New aspects of septin assembly and cell cycle control in multinucleated A. gossypii
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Chapter I

Nuclei in the filamentous ascomycete Ashbya gossypii divide asynchronously and most nuclei have the potential to divide (Alberti-Segui et al., 2001; Gladfelter et al., 2006). Although cytoplasmic extension is restricted to growing tips and emerging branches, the distances between nuclei are uniform along the entire hyphal length. This implies active control of nuclear distribution and division to maintain an ideal nuclear to cytoplasmic ratio, potentially depending on environmental conditions. The question of nuclear distribution has already been addressed earlier (Alberti-Segui et al., 2001). We have investigated how the rate of mitosis is regulated in response to intra- and extracellular signals. Here we show that homologues of S. cerevisiae morphogenesis checkpoint components are involved in starvation response in A. gossypii: Phosphorylation of the cyclin dependent kinase AgCdc28p at tyrosine 18 by the protein kinase AgSwe1p is used to delay mitosis under low-nutrient conditions, leading to an increase in the average distance between nuclei. This effect is markedly reduced in Agswe1Δ or Agcdc28Y18F mutants where the CDK cannot be phosphorylated. Overexpression of AgSWE1 leads to decreased nuclear density even under non-starving conditions. Addition of rapamycin mimics starvation response, suggesting that AgSwe1p may be under control of AgTor1/2p.

In unperturbed budding yeast cells, ScSwe1p is recruited to the septin ring at the mother-bud neck where it is phosphorylated and subsequently degraded. We have speculated that the septins in A. gossypii could serve as spatial markers to locally inactivate AgSwe1p and increase nuclear division rate in areas of growth. Time-lapse analysis has revealed that mitoses in wild type are most common near branching points. Interestingly, AgSep7p-GFP localizes to branching points and septin deletion mutants show random distribution of mitoses. We propose a model in which AgSwe1p may regulate mitosis in response to cell intrinsic morphogenesis cues and external nutrient availability in multinucleated cells.

Chapter II

Septins are evolutionary conserved proteins with essential functions in cytokinesis, and more subtle roles throughout the cell cycle. Much of our knowledge about septins originates from studies with S. cerevisiae, where they form a ring-like protein scaffold at the mother-bud neck. We have asked what functions the septins may hold in an organism that does not complete cytokinesis prior to sporulation. Interestingly, all budding yeast septins are conserved in A. gossypii and one is even duplicated (S. Brachat, personal communication; Dietrich et al., 2004). In vivo studies of AgSep7p-GFP have revealed that septins assemble into discontinuous hyphal rings close to growing tips and sites of branch formation and into asymmetric structures at the base of branching points. Rings are made of filaments which are long and diffuse close to growing tips and short and compact further away from the tip. During septum formation, the septin ring splits into two to form a double ring.

Agcdc3Δ, Agcdc10Δ, and Agcdc12Δ mutants display aberrant morphology and are defective for actin-ring formation, chitin-ring formation, and sporulation. Due to the lack of septa, septin deletion mutants are highly sensitive, and lesion of a single hypha can have catastrophic consequences for a young mycelium. Strains lacking AgCDC11A show morphological defects comparable with other septin deletion mutants, but actin- and chitin-ring formation are not disabled. Deletion of AgCDC11B results in no detectable phenotype under standard laboratory conditions.
General Introduction

Filamentous fungi differ in many striking ways from common single-celled eukaryotes. The mycelium formed by hyphal growth is a dense, complex network of branched filaments, whose size is only limited by external conditions such as availability of resources, environmental stresses, and competing organisms. Mitosis are either synchronized (all nuclei divide simultaneously), or they occur in an asynchronous manner (Nygaard et al., 1960; Clutterbuck, 1970). In either way, nuclear division is not followed by cytokinesis, leading to multinucleated hyphae which share one common cytoplasm. Thus, although by appearance such mycelia certainly do not correspond to the common perception of a cell, a mycelium formed by true hyphal growth has to be considered as one single, multinucleated cell. Filamentous growth enables these organisms to rapidly cover and exploit solid surfaces, and the polarized force generated by apical extension allows them to penetrate tissue which would otherwise not be accessible. However, this growth mode also bears new challenges: How can one single cell have the flexibility to react to conditions, which may greatly differ from one part of the mycelium to the other? How can it prevent local damage from spreading through the entire mycelium? How are hyphal extension and branching regulated to ensure optimized growth and resource exploitation? How does such a cell maintain an optimal protein per cytoplasm ratio, although the cytoplasmic volume may increase with various speed in different places of the mycelium?

We sought to study some of these questions in the filamentous Ascomycete Ashbya gossypii, a pathogen of cotton and citrus fruits and phylogenetically a close relative of S. cerevisiae (Ashby and Nowell, 1926; Prillinger et al., 1997; Wendland et al., 1999; Dietrich et al., 2004). A. gossypii is exclusively found in a filamentous, multinucleated hyphal form. It has a very small haploid genome (9.2 Mb) encoding 4718 genes, which shows a high degree of synteny (conservation of gene order between different species) and homology with the S. cerevisiae genome. Combined with highly efficient homologous recombination allowing PCR-based gene targeting, these features make A. gossypii an attractive system to perform studies of regulation of filamentous growth based on genetic functional analysis (Steiner et al., 1995; Altmann-Johl and Philippsen, 1996; Mohr, PhD Thesis, 1997; Wendland et al., 2000; Wendland and Philippsen, 2000; Dietrich et al., 2004).

The A. gossypii life cycle starts with the only known phase of isotropic growth in wild type: germination of the haploid spore to form a germ bubble. This is followed by apical growth, extending two germ tubes in succession on opposing sites of the germ bubble. More axes of polarity are established with lateral branch formation in young mycelium. Maturation is characterized by apical branching and a dramatic increase of growth speed (up to 200 μm/h at 30°C, (Knechtle et al., 2003)), which enables it to cover an 8 cm-Petri-dish of full medium in about 7 days. Sporulation is thought to be induced by nutrient deprivation, leading to contraction at the septa, cytokinesis and subsequent abscission of sporangia which contain up to 8 haploid spores (Figure Intro.1, adopted from Brachat, PhD thesis, 2003) (Ayad-Durieux et al., 2000; Brachat, PhD thesis, 2003; Knechtle et al., 2003). Hyphae are compartmentalized by septa, which in young parts appear as rings that allow transfer of nuclei (Alberti-Segui et al., 2001) and in older parts may appear as closed discs. Compartments typically contain around eight nuclei. Nuclear pedigree analyses carried out by Peter Philippsen and Amy Gladfelter have shown that neighboring nuclei are in different division cycle stages. Most nuclei have the potential to divide, with the time between two divisions varying greatly from 46 – 250 min (Gladfelter et al., 2006). Despite limitation of growth to tips and branching sites, distances between the nuclei are largely uniform along the entire hyphal length, with an average distance of 4 – 5 μm. This is surprising as one might expect cytoplasmic expansion to cause a decrease of nuclear density in areas of growth. The fact that this is not the case indicates active maintenance of nuclear to cytoplasmic ratio along the hyphae. How may such a regulation be achieved? C. Alberti-Segui has shown that deletion of the microtubule-based dynein motor AgDhc1p leads to clustering of nuclei at the hyphal tips of A. gossypii. Thus, long range nuclear migration has been identified as the key element in regulating nuclear distribution (Alberti-Segui et al., 2001). Besides nuclear distribution, also the division rate of nuclei needs to be controlled in order to
maintain an ideal nuclear to cytoplasmic ratio. This ratio may vary depending on environmental conditions. Mutants where such regulatory mechanisms are affected should display altered nuclear densities under standard growth conditions. Deletion of the kinesin motor AgCin8p leads to a dramatic increase of the distance between two nuclei to about 11 μm (Alberti-Segui, PhD thesis, 2001). However, AgCin8p generates the pushing force of the nuclear spindle and therefore has to be regarded as a requirement for efficient mitosis rather than a regulator. What do we know about regulation of nuclear division in other organisms?

The molecular machinery of eukaryotic cell cycle control is most fully worked out for budding yeast Saccharomyces cerevisiae. Under conditions of limited nutrients, budding yeast cells arrest as small, unbudded cells in early G1 phase (“Gap-1”). Addition of nutrients allows them to begin growing. If they achieve a critical size and no mating pheromone is present, the cells commit to a complete cell cycle. This execution point at late G1 is called START (Pringle and Hartwell, 1981; Tyers et al., 1992). Bud emergence corresponds to the initiation of S phase (Synthesis), during which DNA replication, spindle pole body (SPB, fungal equivalent of the centrosome) separation and nuclear positioning occur. In G2 phase (Gap-2), apical growth of the bud is replaced by isotropic growth, and the SPBs migrate to opposite sides of the nucleus forming a short metaphase spindle which is oriented in parallel to the mother-bud axis (Byers and Goetsch, 1975; Jacobs et al., 1988; Donaldson and Kilmartin, 1996; Carminati and Stearns, 1997; Shaw et al., 1997). During M phase (Mitosis), the spindle extends at least 5-fold, separating the sister chromatids to opposite poles, thus leading to nuclear division (Winey and Byers, 1993; Snyder, 1994). With mitotic exit the anaphase spindle disassembles, and the cell cycle is concluded by cytokinesis and separation (abscission) (Kilmartin and Adams, 1984; Epp and Chant, 1997; Bi et al., 1998; Lippincott and Li, 1998b, 1998a; Vallen et al., 2000).

Lee Hartwell spearheaded cell cycle research in S. cerevisiae with his screen for conditional cell division cycle (cdc) mutants in the 1960s and 1970s (Hartwell, 1971a). Under restrictive conditions, cdc mutants arrest with characteristic cell division morphologies, called terminal phenotypes. His detailed examination of these mutants revealed interdependence of many cell-cycle events but also demonstrated that at least three independent cycles can be distinguished in budding yeast: the cytoplasmic cycle (bud formation), the chromosome cycle (replication) and the centrosome cycle (SPB cycle). Each of these cycles depends on START for initiation and each must be completed for cells to undergo the metaphase to anaphase transition. cdc28 mutants have a terminal phenotype at this principal restriction point of the yeast cell division cycle. This lead to the discovery of cyclin dependent kinases (CDKs), whose changes in activity are responsible for driving key cell cycle transitions (Hartwell et al., 1974; Nasmyth and Reed, 1980). S. cerevisiae possesses at least five CDKs, with Cdc28p being the principal regulator of the cell cycle. Cdc28p activity is regulated by nine cyclins that accumulate, for the most part, at START and at G2/M that are regulated transcriptionally and proteolytically, several protein kinases (e.g. Sic1p, Swe1p) and phosphoprotein phosphatases (e.g. Mih1p), and at least four other binding proteins. Cdc28p cyclins have been classified into two groups: the three G1 cyclins Cln1p, Cln2p, and Cln3p, and the six B-type (mitotic) cyclins Clb1p to Clb6p (Richardson et al., 1989; Surana et al., 1991; Fitch et al., 1992; Richardson et al., 1992; Schwob and Nasmyth, 1993). Regulation of cyclin associated Cdc28p activity is mainly achieved by successive waves of cyclins, provided by a mechanism of transient expression and subsequent degradation. Each wave stimulates degradation of the preceding cyclins and expression of the following cyclins. This allows the Cyclin-CDK complex to drive the cell through the different stages of the cell cycle.

Although bud formation, DNA replication and the centrosome cycle can proceed independently from each other once START has been passed, other events require tight coordination. Surveillance mechanisms termed checkpoints are set at various stages of the cell cycle and allow delayed processes to catch up and re-coordinate upon perturbation (Hartwell and Weinert, 1989). START itself can be regarded as checkpoint, preventing initiation of a cell division cycle under bad nutritional conditions or in the presence of mating partners (Gallego et al., 1997). TOR (target of rapamycin) signaling plays a central role in adjusting cell growth to the nutritional environment (Barbet et al., 1996; Helliwell et al., 1998; Harris and Lawrence, 2003; Jacinto and Hall, 2003; Wullschleger et al., 2006). To prevent the cells from disastrous segregation of incompletely replicated or damaged DNA, the “DNA damage and replication checkpoint” can delay the cell cycle in G1 and prior to anaphase in multiple ways. The “morphogenesis checkpoint” prevents formation of binucleate cells by delaying nuclear division until a bud has been formed (Lew and Reed,
Could such a surveillance mechanism have adapted to regulate the rate of mitosis in A. gossypii? One could say that the morphogenesis checkpoint in S. cerevisiae regulates "nuclear density" by preventing formation of multinucleated cells. It has been argued that not only formation of a proper neck but also cytoplasmic extension of the bud could be a requirement before the cells are released into mitosis (Harvey and Kellogg, 2003). Recent analyses have demonstrated that ScSwe1p is also used to delay mitosis in response to environmental signals such as high osmolarity (Clotet et al., 2006). We sought to investigate whether A. gossypii homologues of morphogenesis checkpoint components (AgSwe1p, AgMih1p, AgHsl1p, AgHsl7p, septins) have evolved to control nuclear division rate in response to hyphal extension and extracellular stimuli such as stress and availability of nutrients (Chapter I). The septins are crucial for the recruitment and inactivation of ScSwe1p in budding yeast. Thus, we further investigated the possibility of morphogenesis checkpoint proteins providing spatial control of mitosis, with the septins functioning as cortical markers to locally trigger mitosis in areas of growth.

The functions of S. cerevisiae septins are far from being restricted to the recruitment of morphogenesis checkpoint components. This conserved family of GTP-binding proteins was discovered through Lee Hartwell's genetic screening for S. cerevisiae mutants defective in cell-cycle progression (Hartwell, 1971b). The original temperature sensitive septin mutants (cdc3, cdc10, cdc11 and cdc12) form multinucleated cellular clusters with elongated buds and thus were assigned essential functions in cytokinesis (Cooper and Kiehart, 1996; Longtine et al., 1996; Field and Kellogg, 1999). Together with a fifth septin, ScSep7p (ScShs1p), the septins form a ring encircling the mother-bud neck, but they also occur at the presumptive bud site, at the bud scar after cytokinesis and at the tip of the shmoo in presence of mating pheromones (Longtine et al., 1996; Mino et al., 1998; Gladfelter et al., 2001b). The septin ring serves several functions (Kinoshita, 2003): Firstly, it is a spatial landmark for bud site selection (Chant et al., 1995; Sanders and Herskowitz, 1996). Further, the septins form a cortical barrier that prevents membrane-associated molecules from diffusing from the bud cortex into the mother cortex (Barral et al., 2000; Takizawa et al., 2000; Fatty et al., 2002). They form a scaffold to recruit molecules for the cytokinesis apparatus, cell-wall synthesis, and for positioning of the mitotic spindle (DeMarini et al., 1997; Kusch et al., 2002). Finally, they recruit the protein kinases ScGin4p, ScKcc4p and ScHsl1p, which belongs to the morphogenesis checkpoint, and one component of the mitosis exit network, ScTem1p (Carroll et al., 1998; Jimenez et al., 1998; Longtine et al., 1998; Barral et al., 1999; Lippincott et al., 2001).

Despite profound morphological differences between budding yeast and A. gossypii, homologues to all five mitotic S. cerevisiae septins have been identified in A. gossypii, with CDC11 being duplicated (S. Brachat, personal communication; Dietrich et al., 2004). What function do the septins have in an organism that does not undergo complete cytokinesis unless for sporulation? Where do they localize in the absence of a bud neck or a division plane? The aim of Chapter II is to shed light on these questions by studying localization of AgSep7p-GFP and by functional analyses of deletion mutants.
Figure Intro.1. *A. gossypii* life cycle (adopted from S. Brachat, Thesis, 2003). *A. gossypii* mycelium grown on solid medium gives rise to needle-shaped spores, recognizable as white fluff in the middle of the colony. Isotropic growth during germination leads to the formation of a germ bubble in the center of the spore. After a permanent switch to polarized, apical growth, two germ-tubes emerge successively on opposing sites of the germ bubble. Nuclear division occurs without subsequent cytokinesis, leading to multinucleated hyphae. New axis of polarity are established by lateral branching in young mycelium. During maturation of the mycelium, the tip extension rate is markedly increased and apical branching (tip splitting) now is the predominant form of branching. Upon growth for an extended time, the sporulation program is initiated, leading to the formation of sporangia and concluding the cycle.
Chapter I

A conserved cell cycle control module leading to CDK tyrosine phosphorylation functions in the \textit{A. gossypii} starvation response

Introduction

Multinucleated cells are common throughout the biosphere. Filamentous, pathogenic fungi, metastasizing tumor cells and the cells of the musculoskeletal and blood systems of mammals are examples of the diverse types of cells which contain many nuclei in one cytoplasm (Mills and Frausto, 1997; Woodhouse \textit{et al.}, 1997; Su \textit{et al.}, 1998; Wakefield \textit{et al.}, 2000; Winding \textit{et al.}, 2000; Atkins \textit{et al.}, 2001). In some cases these cells are produced from specialized cell cycles in which nuclear division occurs without cell division leading to large syncytia. For fungi, multinucleated cells may extend over hundreds of meters so that different regions of a single cell experience dramatically different microenvironments.

Mitosis in multinucleated cells can occur either in a coordinated, synchronous manner where all nuclei divide simultaneously or asynchronously where individual nuclei divide independently in time and space. Both synchronous and asynchronous patterns of mitosis are observed in syncytial cells and the different modes of nuclear division bring different potential advantages to cells (Nygaard \textit{et al.}, 1960; Clutterbuck, 1970; Gladfelter \textit{et al.}, 2006). In synchronous division, nuclei in distant regions of the cell can be coordinated and a cell can globally respond to a signal or stimulus with a limited spatial distribution. In asynchronous division, the cell can restrict mitosis to particular nuclei providing a more local and spatially controlled response to a signal.

Asynchronous or local control of mitosis, which is observed in several filamentous fungi including \textit{Neurospora crassa} and \textit{Ashbya gossypii}, may enable “cells” or mycelia to target both growth and nuclear division into nutrient rich regions while arresting these processes in areas of the mycelium lacking sufficient resources (Minke \textit{et al.}, 1999; Freitag \textit{et al.}, 2004; Gladfelter \textit{et al.}, 2006). Additionally, asynchronous control of mitosis may allow mycelia to link morphogenesis programs, such as branching patterns and septal positioning, to nuclear division. Thus external triggers such as nutrients and/or internal signals involving shape of the mycelium could spatially direct asynchronous mitosis in multinucleated hyphae.

To prevent the formation of aberrant binucleate cells in \textit{Saccharomyces cerevisiae}, it is crucial not to start nuclear division before a proper bud has been formed. Therefore, mitotic entry is tightly coordinated with bud formation by the morphogenesis checkpoint (Figure I.1). Mitosis is promoted by a complex of mitotic cyclin with the cyclin dependant kinase (ScCdc28p). When bud formation is delayed due to defects in the actin cytoskeleton or if the septin scaffold (see Chapter II) is perturbed then the intrinsically unstable Swe1p kinase (Wee1 homologue) is stabilized and translocates to the nucleus where it can inhibit Cdc28/Cib2p, through phosphorylation of the tyrosine 19 residue on Cdc28p (Sia \textit{et al.}, 1996; McMillan \textit{et al.}, 1998; Sia \textit{et al.}, 1998; McMillan \textit{et al.}, 1999a; McMillan \textit{et al.}, 1999b). This provides a G2-delay for cells to recover proper morphogenesis prior to mitosis ensuring aberrant binucleate cells do not form in the absence of budding. The G2-delay is determined by the ratio of active ScSwe1p to the phosphatase ScMih1p (Cdc25 homologue), which can reverse phosphorylation of the CDK (Ciliberto \textit{et al.}, 2003). If proper morphogenesis is not recovered after several hours, ScMih1p activity leads to adaptation and the unbudded cells undergo mitosis and become binucleate (Sia \textit{et al.}, 1996). During unperturbed cell growth, ScSwe1p is targeted for ubiquitin mediated degradation in the proteasome by phosphorylation (Sia \textit{et al.}, 1998). ScSwe1p is sequentially phosphorylated at multiple sites by at least three protein kinases (Sakchaisri \textit{et al.}, 2004; Lee \textit{et al.}, 2005). If proper morphogenesis is underway, ScCla4p (PAK) localizes to the septin cortex at the neck and phosphorylates ScSwe1p during S-Phase, which is only found at low levels during this stage (Mitchell and Sprague, 2001; Sakchaisri \textit{et al.}, 2004). In G2, ScSwe1p becomes abundant and potently inhibits Cib-ScCdc28p. Before the onset of M-phase, a raise in levels of mitotic Cib leads to Cib-CDK dependent phosphorylation of ScSwe1p (Sia \textit{et al.}, 1998; McMillan \textit{et al.}, 2002; Asano \textit{et al.}, 2005). This promotes interaction of
ScSwe1p with the Polo-like kinase ScCdc5p, phosphorylation and subsequent degradation of ScSwe1p (Bartholomew et al., 2001; Park et al., 2003; Sakchaisri et al., 2004; Asano et al., 2005). In addition, data from frog egg extracts raise the possibility of a positive feedback loop, where Clb-CDK could phosphorylate and activate ScMih1p and by that further antagonize ScSwe1p activity (Izumi et al., 1992; Ciliberto et al., 2003). Efficient translocation of ScCdc5p to the neck and tethering of ScSwe1p to the septin filaments both require interaction with ScHsl1p and ScHsl7p, which are recruited by the septins (Barral et al., 1999; Shulewitz et al., 1999; Longtine et al., 2000; McMillan et al., 2002; Sakchaisri et al., 2004; Asano et al., 2005). Thus, in S. cerevisiae, the septin scaffold functions to coordinate bud morphogenesis and nuclear progression by regulating the abundance of ScSwe1p. Additionally, defects in the actin cytoskeleton are thought to trigger a second signaling pathway, operating through a MAPK (Mpk1), which leads to inhibition of ScMih1p (Harrison et al., 2001).

Interestingly, similar cell cycle modules in other model organisms are used by the DNA damage/replication checkpoint (S. pombe, X. laevis) and by the stress response pathway (S. pombe) (Rhind et al., 1997; Rhind and Russell, 1998; Yamada et al., 2004; Petersen and Hagan, 2005). The homologues of all morphogenesis checkpoint factors in S. cerevisiae are present with varying degrees of homology in the genome of the multinucleated, filamentous fungus A. gossypii, which never reproduces by budding and rather is exclusively found in a filamentous, multinucleated hyphal form (Dietrich et al., 2004). Given the absence of budding yet the conservation of all factors involved in the morphogenesis checkpoint, we speculated that this cell cycle module may have co-opted for control of nuclear density in A. gossypii and sought to identify triggers that would lead to a delay in nuclear division. We show here that AgSwe1p regulates nuclear progression in response to external nutrient status through inhibitory phosphorylation of the CDK. In addition, the septin proteins may use this module to link morphogenesis to nuclear cycle in this filamentous fungus by directing where mitosis takes place in the mycelium. We propose that this dual role for Swe1p may be used to facilitate a spatially controlled reaction to limited nutrient availability in the natural world.
Figure I.1. Morphogenesis checkpoint in *S. cerevisiae*. The G2-delay is determined by the ratio of active ScSwe1p to the phosphatase ScMih1p. This ratio is regulated at multiple steps to link proper control of mitotic entry with morphogenetic events. See Chapter I, Introduction for detailed information.
Results

Phosphorylation of Cdc28-Y18 is a candidate for regulation of the nuclear cycle

Phosphorylation of a conserved tyrosine residue of the CDK is a widespread way to delay the cell- or nuclear cycle in response to a variety of intra- or extracellular signals, which differ between different organisms (Rhind et al., 1997; Rhind and Russell, 1998; Yamada et al., 2004; Petersen and Hagan, 2005; Nakashima et al., unpublished). Alignment of the respective sequence among a selection of eukaryotes revealed Tyr18 to be a likely candidate of regulation among a selection of eukaryotes represented the key regulator of the nuclear cycle. To test the essential function of the CDK, AgCdc28p would only possible, if AgCdc28p represented the key regulator of the nuclear cycle. To test the essential function of the A. gossypii CDK, Agc28Δ deletion mutants were created. Heterokaryotic mycelia were able to grow at normal rates under selective conditions, indicating that transformed nuclei were still able to divide. This was in contrast to homokaryotic mycelium grown from spores: growth stopped at the stage of bipolar germlings, with rare attempts of branch formation. Hoechst staining revealed up to 4 nuclei per germling (Figure I.2B top right), dominated by nuclear debris which may be the result of nuclear breakdown (Fig I.2B large picture). Although up to two mitoses could still occur in the deletion mutants, AgCdc28p must play an essential role already early in the development of mycelium.

Minimal AgCdc28-Y18 phosphorylation under ideal growth conditions

We asked which conditions may cause A. gossypii to phosphorylate its CDK and potentially delay the nuclear cycle by using a phospho-specific anti-cdc2 antibody on mycelia and total proteins. Even under ideal growth conditions, phosphorylation of the CDK may be an integral part of nuclear cycle regulation, for instance to maintain asynchrony of mitoses. To test this, phosphorylated CDK was assayed for in mycelia by immunofluorescence to see if a subset of nuclei may be enriched for modified AgCdc28p (Figure I.3A). No phosphorylated protein was detected by this method but potentially this could be due to technical problems resolving the protein with this antibody by immunofluorescence. Thus, whole cell extracts from mycelia were also generated to evaluate if the antibody could recognize the A. gossypii CDK by western blot. Only minimal phosphorylation on AgCdc28p (compared to inducing conditions in later experiments) was apparent in either asynchronously growing mycelia or mycelia that had been artificially synchronized (approximately 70% synchrony) in G2/M with nocodazole and released for progression through mitosis (Figure I.3B). Hence, it can be assumed that CDK phosphorylation only plays a marginal role during normal growth conditions.

AgCdc28p is phosphorylated on tyrosine 18 when hyphae are starved for nutrients

Given the common role of CDK phosphorylation in different checkpoint responses across eukaryotes, we evaluated if AgCdc28p was phosphorylated when mycelia were exposed to potential checkpoint triggers and environmental stresses including Hydroxyurea (to impair DNA replication), nocodazole (to impede spindle assembly), starvation, osmotic shock, and high temperature (42 °C). Latrunculin A could not be used at the required concentration for large volume cultures and therefore was not assayed. No or limited phosphorylation was detected using the anti-phosphoTyr15-cdc2 antibody on whole cell extracts from cultures treated with Hydroxyurea or nocodazole (Figure I.3C). High temperature stress and osmotic shock resulted in moderate phosphorylation on a protein of the predicted size of AgCdc28p. Surprisingly, starvation that was induced by high-density growth (evaluation described in materials and methods) resulted in a level of phosphorylation that was markedly stronger than in any other condition tested (Figure I.3C). This phosphorylated form of AgCdc28p was visible both in whole cell extracts from high-density cultures and in the nuclei of starving intact mycelia visualized by immunofluorescence (Figure I.4A). The high-density/nutrient deprivation-induced phosphorylation was reversed, along with the phenotype associated with starvation (prominent vacuoles) but relatively slowly upon feeding the mycelia with fresh media (Figure I.4B). The phosphorylation was reduced after 3 hours in fresh media and at the minimal levels found in low-density cultures after 5 hours. The response was independent of the type of nutrient that was restricted and was observed when either carbon (AFM with 0.1% glucose), nitrogen (ASD-Asn) or amino acids (ASD with one quarter amino acid concentration) was the limiting resource even when the cultures were grown to low density (Figure I.4C).

To confirm that the phosphorylation observed was as predicted on the tyrosine 18 residue of AgCdc28p, this residue was mutated to phenylalanine which cannot be phosphorylated. The phosphorylated AgCdc28p found in high-
density cultures of the reference strain is not present in lysates generated from Agcdc28Y18F mycelia grown to similar high-density (Figure I.4D). This indicates that the anti-phospho-cdc2 antibody is recognizing the conserved tyrosine phosphorylation on the A. gossypii CDK.

This phosphorylated AgCdc28p observed in densely grown cultures could be formed in response to nutrient deprivation or could be a density-dependent reaction to a quorum-sensing molecule that accumulates at high density. Several approaches were taken to attempt to distinguish between these possibilities. First, it was examined whether cultures grown on plates could release factors that would inhibit growth of new cultures on the same plates. For that, AFM plates were inoculated with A. gossypii wt and grown for ten days. The mycelium was scraped off, leaving behind a mixture of spores and small pieces of mycelium. Half of the plates were soaked with 5 ml deionized water to replace the liquid lost by evaporation. To replace the consumed nutrients, 5 ml of 6xAFM were added to the other half of the plates. If the plates contained quorum from the previous cultures, no growth should be observed on any of the plates. However, two days later, dense growth was observed on the plates treated with 6xAFM, but not on the watered plates. This implies that growth on used plates is not inhibited by quorum factors, but by nutrient deprivation.

In the next approach, cultures were treated with rapamycin which likely inhibits the kinase AgTor1/2p, which has a conserved role in nutrient sensing in a variety of eukaryotes. Inhibition of Tor kinase mimics starvation in many cells and in yeast simulates nitrogen starvation (Martin and Hall, 2005). In A. gossypii mycelium grown at low density in rich nutrient conditions, AgCdc28Y18 phosphorylation clearly accumulated with time in the presence of rapamycin (Figure I.4E). Thus, the appearance of the phosphorylated form does not depend upon high density here but does appear during this mock starvation. Furthermore, when mycelia grown at low density are transferred to MOPS/KCl buffer (osmotically stable but without nutrients) to induce rapid low-density starvation, CDK phosphorylation appears within 30 minutes and accumulates to levels comparable to high-density starvation by 105 minutes (Figure I.4E). Thus, AgCdc28Y18 phosphorylation appears in response to nutrient deprivation rather than other possible signals that may accumulate at high-density growth.

The nuclear cycle is delayed in specific stages of division during starvation

If phosphorylation of the CDK was inhibitory, as would be predicted, then we would expect a delay or inhibition of the nuclear cycle that leads to a change in nuclear density when hyphae are limited for nutrients. In fact the nuclear to cytoplasmic ratio was significantly different between high and low density grown cultures. Nuclei from mycelia grown to low density which presumably were not yet limited for nutrients were an average of 4.6 μm apart whereas in nutrient limited conditions this distance expanded to 10.2 μm (N>500 nuclei) (Figure I.5A). This can only be explained by polarized hyphal growth continuing (presumably until internal energy supplies such as lipid droplets are depleted) while the nuclear cycle is delayed or blocked under starvation. Agcdc28Y18F mycelia which were resistant to CDK phosphorylation showed an intermediate nuclear density in low nutrients of 7.9 μm (N>300 nuclei) suggesting that some but not all of the delay in nuclear division was due tyrosine phosphorylation based inhibition of AgCdc28p (Figure I.5B).

If such a delay was stochastic such that limited nutrients can block nuclei in any stage of the nuclear cycle then high density cultures would be expected to have similar proportions of nuclei in each nuclear cycle stage as found in low-density cultures. If, however, the delay was regulated such that it occurs in a specific window of time, the proportions of nuclei in each stage of nuclear division should vary between the different growth conditions. Under high-density growth conditions, more nuclei accumulated in hyphae with duplicated SPBs (41%) and metaphase spindles (16%) compared to low density cultures (28% duplicated SPBs, 4% metaphase, N=400 nuclei). Thus the deprivation of nutrients leads to accumulation of nuclei in specific stages of division, both just prior to and during metaphase (Figure I.5C).

Homologues of the yeast morphogenesis checkpoint components in A. gossypii

In S. cerevisiae, inhibitory phosphorylation of ScCdc28p at Tyr19 by the kinase ScSwe1p is the principle response to a delay in bud formation (absence of proper septin collar leads to stabilization of ScSwe1p) or perturbation of the actin cytoskeleton. Despite dramatic morphogenetic differences between A. gossypii and budding yeast, homologues of the entire S. cerevisiae morphogenesis checkpoint are present in A. gossypii. Whereas the CDK, the polo kinase Cdc5p and the septins are highly conserved between the two organisms, considerable diversity was observed between the homologues.
of the Swe1 regulators Hs1p and Hs7p and the phosphatase Mih1p (Table 1). Given that AgCdc28p is strongly phosphorylated in starving mycelium which results in a nuclear cycle delay, we asked whether the components of this cell cycle control module have evolved in A. gossypii to link CDK activity to availability of nutrients.

**AgSwe1p is responsible for AgCdc28Y18 phosphorylation**

We generated deletion mutants of the kinase AgSwe1p, its putative inhibitors AgHsl1p, AgHsl7p, the septins, and the phosphatase AgSwe1p to investigate their role in AgCdc28Y18 phosphorylation. As in Agcdc28Y18F mutants, Agswe1Δ mutants failed to accumulate phosphorylated AgCdc28p in high-density conditions in contrast to reference mycelia (Figure I.7A). Additionally, low-density grown Agswe1Δ mycelia that were incubated with rapamycin also did not have detectable AgCdc28p phosphorylation showing that both, the rapamycin and starvation induced modification involved AgSwe1p (Figure I.7A). Agmih1Δ mutants had comparable levels of phosphorylation to reference mycelia (Figure I.7A) suggesting that under these conditions AgMih1p may be down regulated in wild type. Growing Agcdc12Δ and Aghsl1Δ mutants to high density was only partially possible due to the growth defects of these mutants. As a result, only moderate phosphorylation was observed. Interestingly, when AgMih1p was deleted in addition to Agcdc12Δ or Aghsl1Δ, the CDK phosphorylation was markedly increased although these double mutants were afflicted by the same culturing problems as the single mutants (Figure I.7B). This implies that AgMih1p may at least be one phosphatase of the CDK that keeps AgSwe1p dependent phosphorylation in check. This would entail reduced reversibility of AgCdc28p phosphorylation in Agmih1Δ mutants. Experiments were undertaken to see whether Agmih1Δ single mutants or combination mutants with Aghsl1Δ and Aghsl7Δ displayed impaired capability to recover after starvation, upon feeding the mycelia with fresh full medium. No conclusive results were obtained because it was not possible to grow all the strains to the same density and therefore they were starved to a different degree when the recovery experiment was started (data not shown). In none of the cases, however, did the mutant strains display reduced recovery capacity compared to the reference strain. This indicates the existence of additional, AgMih1p independent mechanisms by which the CDK phosphorylation can be reverted.

**Only minimal role of morphogenesis checkpoint homologues in regulating nuclear density under non-starving conditions**

We wanted to know whether the changes in AgCdc28Y18 phosphorylation caused by some of the mutants were also reflected in the average distances between nuclei and the lengths of nuclear cycle stages. Single and combination mutants were evaluated for growth and nuclear density. Under normal growth conditions in rich media all mutant mycelia, with the exception of septin mutants, grew with wild-type like radial growth rates (Figure I.6A). Nuclear density was comparable to the reference (4.6 μm between nuclei) in Aghsl1Δ (3.8 μm), Agswe1Δ (3.8 μm), Agmih1Δ (3.8 μm) and Agcdc12Δ (4.7 μm) mutants (N> 300 nuclei scored for each strain, (Figure I.6B). The nuclear cycle phase proportions were similar to the reference for the different mutant strains (Figure I.6C, nuclear cycle stage scoring based on spindle appearance by anti-tubulin immunofluorescence) however, there was a moderate increase in the percentage of metaphase and anaphase nuclei in Aghsl1Δ (12% vs 7% in wt) and Agcdc12Δ (14%) mutants suggesting there may be some delay late in the nuclear division cycle due to the absence of these proteins.

Based on homologues in other systems, Aghsl1Δmih1Δ double mutants or double deletion strains of AgMIH1 and any of the crucial septins would be predicted to have a synthetic interaction due to the release of inhibition of AgSwe1p in the absence of the phosphatase, AgMih1p, which likely opposes AgSwe1p. In yeast the analogous mutations lead to inviable cells which are blocked at the G2-M transition (McMillan et al., 1999a). Amazingly, there was no additive effect in terms of growth in Aghsl1Δmih1Δ, Agcdc3Δmih1Δ, Agcdc10Δmih1Δ or Agcdc12Δmih1Δ double mutant strains in A. gossypii. Even analyzing nuclear density or nuclear division cycle stages did not reveal any synthetic effect in Aghsl1Δmih1Δ or Agcdc12Δ–mih1Δ (Figure I.6A-C). These data combined suggest that despite the conservation of this cell cycle control module in the genome there is only a minimal “global” role for the septins/AgHsl1p/AgSwe1p, and AgMih1p in A. gossypii for regulating the frequency of nuclear division under standard laboratory (low-density / rich nutrient) growth conditions.

**Starvation induced nuclear cycle delay depends on AgSwe1p**

Strong AgCdc28-Y18 phosphorylation was only observed under starving conditions, except in Agcdc18Y18F and Agswe1Δ mutants. Thus, even if components of this cell cycle control
module have no clear effect on the nuclear cycle under standard laboratory conditions, they may still be involved in regulation of the nuclear cycle delay we had observed under starving conditions. To investigate this possibility, we evaluated average distances of nuclei in deletion mutants grown to high density (starvation). Agcdc12Δ (13.3 μm), Aghsl1Δmih1Δ (14.7 μm) and Agcdc12Δmih1Δ (15.5 μm) mutants all had in average higher nuclear densities than the reference (10.2 μm) (Figure I.8C). One caveat to these experiments however is that the slower growth rate of septin mutants necessitates a longer period of overall growth to achieve high density and thus the growth time is not identical between septin mutant strains and other strains. Agswe1Δ mycelia grown to high density failed to respond as strongly to these conditions as the reference (10.2 μm), but still showed some increase in average nuclear distance (8.0 μm) compared to non-starving conditions (3.8 μm). This was comparable to the 7.9 μm measured in starving Agcdc28Y18F hyphae (Figure I.7C). Agswe1Δ mutant mycelia had fewer nuclei with duplicated SPBs than reference hyphae at high density but retained a similar proportion of mitotic nuclei (Figure I.8A). These results suggest that AgSwe1p induced AgCdc28p phosphorylation is responsible for a delay in nuclear division in G2 and that some other factors are responsible for the increased proportion of metaphase nuclei in high-density growth.

To determine if Swe1p activity was sufficient for AgCdc28p phosphorylation and altered nuclear division even in the absence of high-density starvation, AgSwe1p was overexpressed in mycelium grown in low-density conditions. For this experiment the AgSwe1p promoter was replaced with the S. cerevisiae HIS3 promoter which leads to high, constitutive expression of proteins in A. gossypii (Dominic Höpfner, personal communication). Protein levels were assayed by western blot against a 6HA-epitope tag fused to the C-terminus of AgSwe1p to confirm that the HIS3 promoter leads to overexpression of the AgSwe1p protein (Figure I.8B). Additionally, AgSWE1 expression under its own promoter was evaluated for regulation and to confirm that the HA fusion protein was functional during high and low-density growth. Under control of the native promoter, AgSwe1p-6HA was barely detected in low-density cultures and appeared to migrate slower compared to in high-density growth, where it was more abundant and the bulk of the protein appeared in a faster migrating form that also predominated in the hyphae with AgSwe1p overexpressed. Unlike in reference mycelia, phosphorylated AgCdc28p was readily detected in low-density conditions when AgSwe1p was overexpressed from the ScHIS3 promoter (lanes 2 and 4 compared to 6, Figure I.8B).

To determine if this Y18 phosphorylation of AgCdc28p could alter nuclear cycle progression even in low-density cultures, nuclear density was evaluated in these hyphae overexpressing AgSwe1p. The overexpressed AgSwe1p and presumably the subsequent AgCdc28p phosphorylation led to a 50% decrease in nuclear density with an average distance between nuclei of 8.4 μm compared to 4.6 μm in the reference at low density (Figure I.7C). Additionally, the overexpressed AgSwe1p led to a dramatic delay in the nuclear cycle leading to a population in which almost 60% of nuclei had duplicated SPBs compared to only 28% in reference low density cultures further suggesting that AgSwe1p-induced AgCdc28p phosphorylation acts prior to metaphase (Figure I.8A).

Mitosis are concentrated at sites of branch formation

So far, we have not succeeded to clearly show involvement of the septins or AgHsl1p and AgHsl7p in regulating AgSwe1p activity, mainly due to technical difficulties. Nevertheless, they are the key regulators of ScSwe1p in budding yeast. Are the A. gossypii homologues of these proteins involved in a way of AgSwe1p regulation which is far more subtle than starvation response? One way how the septins could regulate AgSwe1p is by locally inactivating this kinase to provide an increase in the rate of mitosis in regions of growth. To see whether the septins are in the right place for such a regulation, we localized septin proteins in A. gossypii using a GFP-tagged septin, AgSep7p-GFP, and by immunofluorescence using an antibody generated against ScCdc11p. Detailed information on septin localization is found in chapter II (Figure II.2). Anticipatory, septins did actually localize to regions of growth: bar like rings were found near growing tips and close to sites of branch formation, asymmetric structures were found at the bases of branches. In order to have a mitosis promoting effect, the septins would have to bring together AgSwe1p and its inhibitors in these regions. We attempted to evaluate this by localizing AgSwe1p and one of its possible inhibitors, AgHsl7p. AgSwe1p was not visible when tagged either with GFP or epitopes in normally growing mycelia but AgHsl7p was visible in A. gossypii hyphae. AgHsl7-GFP expressed from the endogenous promoter was concentrated in discontinuous rings reminiscent of septin rings (Figure I.9A-D). Co-localization by immunostaining against GFP
and Cdc11p showed that in fact AgHsl7p co-localized with some but not all septin rings and in no case was AgHsl7p observed in the absence of a septin ring (Amy Galdfelter, personal communication). AgHsl7p was not observed in cortical rings in either Agcdc12Δ or Aghs1Δ mutants suggesting that this likely Swe1p regulator is concentrated in specific regions of the mycelium by septins (Figure I.9E, F).

To investigate whether the rate of nuclear division is increased in these regions, we measured the mitotic index in tips, branch points and regions between these growth areas as illustrated in Figure I.10A, using several methods. First we observed mycelia growing on agar expressing GFP-labeled Histone H4 as nuclear marker (AgH4-GFP) using time-lapse microscopy to follow nuclear dynamics and morphogenesis over multiple hours in vivo (Movie 1). Mitoses were frequently observed near newly emerging branches at the junction between the new branch and the “mother” hypha. Example frames from the movie are shown in Figure I.10B, where mitosis occurred at the branching site at 171′, 183′, 201′, and 213′. Mitoses are labeled with a box just prior to division and new daughter nuclei are then outlined with circles. In 84 total mitoses captured by time-lapse, 10 (12%) were at hyphal tips (11% of hyphal length), 43 (51%) were at current or future branching sites (30% of total hyphal length) and 31 (37%) were in the middle of hyphae (in interregion not near tip or branch sites, 59% of total hyphal length). Additionally, to assay mitotic frequency and position using an alternative method carried out by Amy Gladfelter, reference mycelia were grown in liquid medium, then processed for anti-tubulin immunofluorescence to visualize mitotic spindles. 71% of nuclei with mitotic spindles were adjacent to either a tip, main hyphal or branch septin ring and 52% of nuclei with duplicated SPBs were adjacent to septin rings (Figure I.10D, N=230 nuclei).

If septins are required for this spatial pattern of mitotic progression then mycelia lacking septins would be predicted to have a random spatial distribution of mitoses and potentially an altered frequency of mitoses. Amy Gladfelter observed that in mutant mycelia lacking the septin AgCdc12p both mitotic nuclei and nuclei with duplicated SPBs were no longer commonly observed at branch points as seen in the reference strain and instead appeared to be randomly distributed in hyphae. In reference mycelia grown in liquid, 45% of nuclei were near a branch whereas in Agcdc12Δ only 13% were at branches (N>200 nuclei). In contrast, similar proportions of dividing nuclei in Agcdc12Δ mutants (32%) were observed in growing tip regions compared to the reference (30%) suggesting that the septin ring at branch points wields greater influence over the nuclear cycle than the diffuse structures at the tips of hyphae. Thus at least certain septin rings seem to provide spatial directions to the mitotic machinery and potentially help to establish a sub-cellular region that favors nuclear progression.
### Table 1. Homologues of the yeast MC components in *A. gossypii*

<table>
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<td>60 %</td>
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*Domains identified using InterPro from the UniProtKB/Swiss-Prot database*
Figure I.2. Conserved tyrosine phosphorylation of the CDK. (A) Alignment of N-terminal sequences of cyclin dependant kinases among a selection of eukaryotes. The arrow indicates the phosphorylatable, conserved tyrosine residue (Tyr18 in AgCdc28p). (B) Hoechst staining of Agcdc28Δ mutants (AgHPH35), grown for 10 h in liquid AFM at 30 °C.
Figure I.3. Minimal role of AgCdc28-Y18 phosphorylation under ideal growth conditions, but strong phosphorylation during starvation. (A) The reference strain (ΔΔΔ) was grown under low density conditions and stained with Hoechst for DNA visualization (left) and assayed for phosphorylated CDK by immunofluorescence using the anti-phospho-cdc2(Tyr15) antibody (right). Bar, 10 µm. (B) Protein extracts from ΔΔΔ mycelia grown for 11 h under low density conditions then arrested and released from nocodazole (4 h) were assayed for phosphorylated CDK on a Western blot, using the anti-phospho-cdc2 (Tyr15) antibody. A non-specific band from the ponceau red treated membrane was used for the loading control. Extracts from the yeast strain DLY5544 (cdc12-6, PGAL1-SWE1) served as size control (+) for this and all following westerns. (C) ΔΔΔ mycelia were grown for 15 h at 30°C before exposure to checkpoint stimuli or environmental stresses followed by lysis and Western blotting of whole cell extracts using the anti-phospho-cdc2(Tyr15) antibody. When not indicated otherwise, the cultures were grown at low density before applying the various stresses. Nocodazole was used at 15 µg/ml, hydroxyurea at 50 mM. As loading control, a non-specific cross reacting band was used for the blot at the top, non-specific band from the ponceau red treated membrane was used for blot at the bottom.
Figure I.4. Starvation induced AgCdc28-Y18 phosphorylation is reversible, independent of the type of restricted nutrient and not the result of quorum sensing. (A) The reference strain (ΔΔt) was grown for 16 h under high density conditions, stained with Hoechst for DNA visualization (left) and assayed for phosphorylated CDK by immunofluorescence using the anti-phospho-cdc2(Tyr15) antibody (right). Bar, 10 µm. (B) ΔΔt mycelia were grown under high density conditions in 20 ml of AFM until prominent vacuole formation was observed (18 h). The mycelia were washed and resuspended in 200 ml of fresh AFM to recover. Samples were taken after 3 h and 5 h, cells were lysed and the levels of CDK phosphorylation in whole cell extracts were detected by Western blotting using the anti-phospho-cdc2(Tyr15) antibody (left). A non-specific cross-reacting band was used as loading control. The degree of vacuolization was observed by phasecontrast microscopy (right). (C) Low density ΔΔt mycelia were grown in full medium (AFM) for 12 h before shifting them into media limited for different resources: AFM Glc 0.1% (20 times less glucose than standard growth media), ASD(synthetic media without yeast extract), ASD-Asn (synthetic media lacking asparagine), and ASD with 0.25 normal amino acid concentration. Samples were taken 5 h after the media shift and assayed for CDK phosphorylation as described above. (D) ΔΔt and Agcdc28Y18F(AgHPH36) mycelia were grown to the indicated densities, lysed and processed by Western blot using the anti-phospho-cdc2(Tyr15) antibody. A non-specific cross-reacting band was used as loading control. (E) The reference strain was grown for 15 h under low density conditions before adding 200 nm Rapamycin or transferring the cells to MOPS/KCl (40 mM MOPS [pH 7.0], 137 mM KCl) except for the left lane where the culture was grown to high density for a positive control. CDK phosphorylation was detected by Western blotting as described above.
Figure I.5. The nuclear cycle is delayed in specific stages of division during starvation. (A) Reference (ΔΔt) and (B) Agcdc28Y18F (AgHPH36) mycelia grown under high and low density conditions were grown to low and high densities, fixed and stained with Hoechst to visualize nuclei. Bars, 10 µm. (C) ΔΔt mycelia were grown for 16 h at 30°C to high/low density and then fixed and processed for anti-tubulin immunofluorescence to evaluate the percentage of nuclei in different nuclear division cycle stages (Ana: Anaphase, Meta: Metaphase, 2 SPB = 2 spindle pole bodies, 1 SPB = 1 spindle pole body). (D) Example of scoring method used in C (red: nuclei stained with Hoechst, green: anti-tubulin). Bar, 5 µm.
Figure I.6. Minimal role of homologues of the S.c. morphogenesis checkpoint components. (A) Radial growth on solid medium of the reference strain (ΔΔt) and deletion mutants Agmih1Δ (AgHP23), Ag hsl1Δ (hsl1ΔNAT1), Ag hsl1Δmih1Δ (AgHP28), Agcdc12Δ (AgHP15), Agcdc12Δmih1Δ (AgHP27), Agswe1Δ (ASG35) compared to ΔΔt (100 %). A small piece of homokaryotic mycelium (~1 mm³) from each mutant was spotted on full medium plates and grown for 4 days. (B) Nuclei visualized in different strains. Mycelia (same strains as above) were grown overnight at 30°C in selective, liquid medium, fixed and processed for Hoechst dye staining. Numbers indicate average distance between two nuclei, which was determined by dividing variable lengths of hyphae by the number of nuclei observed in such a segment. (C) Percentage of nuclei (N>400) in different nuclear division cycle stages evaluated by Hoechst and anti-tubulin staining (Ana: anaphase; Meta: metaphase; 2 SPB: 2 spindle pole bodies; 1 SPB: 1 spindle pole body).
Figure I.7. AgSwe1p is responsible for AgCdc28Y18 phosphorylation and mitotic inhibition in starving hyphae. (A) ∆Δt (reference), Agswe1Δ (AgHPH24) and Agmih1Δ (AgHPH23) mycelia were grown to high density and whole cell extracts were assayed for CDK phosphorylation as described above (left). ∆Δt and Agswe1Δ (AgHPH24) cells were grown 15 h to low density and then incubated with Rapamycin (200 nM). A non-specific cross-reacting band was used as loading control. (B) Agcdc12Δ (AgHPH15), Agcdc12Δ-Agmih1Δ (AgHPH27), Aghsi1Δ (hs1ΔNAT1) and Aghsi1Δ-Agmih1Δ (AgHPH29) cultures were grown 19 h to high and low density and assayed for CDK phosphorylation. (C) Nuclei of mycelia grown for 16 h at 30 °C visualized by Hoechst staining. Bar, 10 µm.
Figure I.8. AgSwe1p is responsible for AgCdc28Y18 phosphorylation and mitotic inhibition in starving cells. (A) ΔΔt (reference), Agswe1Δ (AgHPH24) and PscHIS3-AgSWE1 (AgHPH37) mycelia were grown at 30°C to high/low density and then fixed and processed for anti-tubulin immunofluorescence to evaluate the percentage of nuclei in different nuclear division cycle stages. (B) Whole cell extracts from PscHIS3-AgSWE1 (AgHPH37, lanes 1-2), PscHIS3-AgSWE1-6HA (AgHPH38, lanes 3-4), AgSWE1-6HA (AgHPH39, lanes 5-6) were generated from cultures grown for 16 h at 30°C to either high or low density. Extracts were then probed by Western blot using either the anti-phospho-cdc2 or anti-HA antibody. A non-specific cross-reacting band was used as loading control. (C) ΔΔt (reference), Agmih1Δ (AgHPH23), Agswe1Δ (AgHPH24), Agcdc28Y18F (AgHPH36), PscHIS3-AgSWE1 (AgHPH37), Aghsl1Δ (hsl1ΔNAT1), Ags1Δmih1Δ (AgHPH28), Agcdc12Δ (AgHPH15), and Agcdc12Δmih1Δ (AgHPH27) mycelia were grown to either high or low density, fixed and nuclei were visualized by Hoechst stainin to calculate the average distance between two nuclei for each strain and condition (N>200). The average distance was determined by dividing variable lengths of hyphae by the number of nuclei observed per measured segment.
Figure I.9. AgHsl7p localizes to septin-dependent cortical rings. (A-D) AgHSL7-GFP (ASG41) mycelia were grown for 12 hours at 30°C and then imaged live. (B) 90° 3D reconstruction of the discontinuous ring shown in (A). (E, F) AbsHsl1Δ, AgHSL7-GFP (AgHPH20) and Agcdc12Δ, AgHSL7-GFP (AgHPH19) were grown for 12 hours at 30°C and then imaged live. Phasecontrast, fluorescence and merged images are ordered from left to right (A) or from top to bottom (C-F). Bars, 10 µm.
Figure I.10. Mitoses are enriched at branching points. (A) Scheme (designed by Peter Philippsen) showing the different regions of a developing mycelium that were considered for determining the spatial mitotic index: tip (T), interregion (I), future branch site (FB) and branch site (B). Tip nuclei were the 2 nuclei closest to the growing tip, branch/future branch nuclei were within 5 µm of a branch site (10 µm zone) and nuclei that were not within the tip or branches were scored as in the inter-region. (B) Myelia expressing GFP-labeled Histone H4 (AgH4-GFP) as nuclear marker were grown at 25°C on diluted medium containing agar and images were collected at 3 min intervals. Hyphal outlines are in red and nuclei are in green, mitoses are labeled with a box just prior to division and new daughter nuclei are outlined with circles. (C) Percentage of mitoses found at sites of branch formation, at tips and in the inter-region of hyphae evaluated by Amy Gladfelter by scoring Hoechst and anti-tubulin stainings (N>200 nuclei). (D) Reference strain mycelia (ΔΔt) were grown overnight for 16 hours at 30°C and processed for anti-tubulin and anti-Cdc11p immunofluorescence (Amy Gladfelter). In overlay, DNA is blue, tubulin is red and septins are green. Bars, 10 µm.
Figure I.11. Localization of mitoses at future branch sites. Mitoses in AgH4-GFP mycelium were monitored by in vivo time-lapse microscopy (Movie 1) as described for Figure I.10. Panels that contain a mitotic event are highlighted by a blue "M". Bars, 10 µm.
Figure I.12. Model of spatial control of mitosis by septins and nutrient availability. (A) Wild-type mycelia grown in the laboratory have mitoses enriched at branch sites near assembled septin rings. (B) As branch sites mature, new septin rings acquire mitosis promoting capacity, leading to mitotic events in interregions. (C) Mitoses are no longer localized preferentially to branch sites in mycelia lacking septins. (D) In low nutrient conditions, nuclei are arrested or delayed with duplicated SPBs and high CDK-Tyr18 phosphorylation levels. (E) Hypothesis for how mitoses may be spatially controlled in response to heterogeneous nutrient supplies in the natural world, where a single mycelium may simultaneously experience “feast and famine” conditions. Local pools of nutrients may trigger clustering of receptors/signaling factors for sensing nutrient status which promote local mitosis through the down-regulation of AgSwe1p, enabling the cell to exploit nutrients without duplicating nuclei distant to nutrient source.
Discussion

In this work, we have sought to identify mechanisms for how nuclear density may be regulated in multinucleated hyphal cells. We have shown that mitosis is limited in response to nutrient deprivation, and that this is achieved through inhibitory AgCdc28p phosphorylation, promoted by the protein kinase AgSwe1p. We have shown further that mitoses are concentrated in the vicinity of branching sites, where they may be promoted by the septins. Here we discuss these data and propose a hypothetical model in which local regulation of AgSwe1p activity may enable the multinucleated mycelium to react to a spatially limited pool of nutrients and restrict mitoses to the area of the nutrient source (Figure I.12).

AgCdc28p is the only cyclin dependent kinase in A. gossypii. Although the CDK is essential for the nuclear division cycle, heterokaryotic deletion mutants are able to grow normally under selective pressure, which is only possible if the resistance marker used for the deletion is spread over the entire mycelium. This indicates that transformed nuclei are able to divide in heterokaryotic mycelium, despite the lack of the gene encoding the crucial kinase. This can either be explained by AgCdc28p being able to travel from wild-type nuclei into transformed nuclei, or by cytoplasmic localization being sufficient to perform the essential functions.

We observed that AgCdc28p is phosphorylated on the Y18 residue in high-density growth or conditions that mimic starvation and this is correlated with an accumulation of nuclei with duplicated SPBs and metaphase spindles and a decreased nuclear density (Figure I.12D). Overexpression of AgSwe1p is sufficient to induce some changes to the nuclear division cycle, the delay in G2, even without nutrient deprivation.

How might AgSwe1p activity be enhanced by nutrient limitation? In budding yeast cells, ScSwe1p participates in the filamentous differentiation response to nutrient limitation through inhibiting CDK/Clb2 complexes which extends G2 and promotes hyperpolarized bud growth (Edgington et al., 1999; La Valle and Wittenberg, 2001). It is not clear how the status of environmental conditions is transmitted to ScSwe1p and its regulators that are partially responsible for sustaining this differentiation program. Both the STE MAPK and the RAS/cAMP pathways have known "anchors" at the plasma membrane sensor level and the nuclear transcriptional response level, however ScSwe1p can act independently of these pathways in filamentation (Ahn et al., 1999). How ScSwe1p senses nutrient status in budding yeast has not been published so far, but there is strong data suggesting that the inactivating kinase of ScSwe1p, ScCdc5p, is under control of TORC1 (Nakashima et al., unpublished). In fission yeast, nutrient limitation signals through a MAP kinase called Spc1 and triggers a SpWee1-mediated G2 delay potentially through the regulation of SpNim1 (Shiozaki and Russell, 1995; Belenguer et al., 1997). A. gossypii is constitutively filamentous and unlike in S. cerevisiae strains, filamentous morphology changes are not observed under nutrient deprivation. Nevertheless, AgSwe1p activity makes dramatic contributions to the nuclear division cycle kinetics in starving A. gossypii mycelium. It was remarkable that inhibition of the Tor1/2p by rapamycin could activate Swe1p similarly to nutrient deprivation suggesting that Swe1p activity in response to starvation may be regulated downstream of this master-regulator of cell growth. This Tor1/2p path and/or a MAPK/RAS/cAMP path could communicate nutrient status and influence AgSwe1p in a variety of ways either by influencing its stability, its localization, its transcription, its intrinsic kinase activity, or affinity for AgCdc28p. Future work will be aimed at understanding how this conserved kinase senses and responds to nutrient fluctuations.

We have studied the distribution of mitoses in fixed and living mycelium. Both methods revealed a preference of mitosis to occur at branching sites. This could simply be due to cytoplasmic increases that trigger a cell-size cue that promotes mitosis. However, this would not explain why nuclei do not have a similarly pronounced preference for division near growing tips, nor would it explain why branching sites already have a mitosis promoting effect even before the branch emerges. It has been shown previously (Knechtle et al., 2003) that polarization sites, such as branching points, do not occur randomly but at preformed landmarks in A. gossypii. It could be the same landmarks that locally promote mitosis to provide nuclei which will be required when the branch emerges. Throughout eukaryotes, septins are among the first proteins that localize to polarization sites where they serve as a scaffold for a vast number of proteins. Recent studies have shown that A. gossypii septins are no exception (Andreas Kaufmann, personal communication). Together with the localization pattern of the septins in A.
gossypii (see Chapter II), this makes them good candidates to establish landmarks for branch formation. In agreement with this view, septin deletion mutants, which are missing organized septin rings, have a random spatial distribution of mitoses. However, septin rings are not only found near existent and future branching sites but also at growing tips. Why do these structures not promote mitosis? Septin proteins have different appearing organizations depending upon their location in hyphae and potentially these morphologically different structures are also functionally distinct. Rings composed of elongated bands form at the tips, more compact rings of short septin bars appear more distant from the growing tip along the main hyphae and asymmetric bar structures are featured at most branchpoints. Conceivably, these different varieties of septin rings could recruit different pools of proteins, attract the same proteins with different affinities, or function to delineate different plasma or organelle membrane domains. Support for functional difference in the structures comes from the observation that only mitosis frequency near branches, not at tips, is diminished in septin deletion mutants. Thus, the rings of the main hyphae near branches and potentially the asymmetric structure that decorates the bases of branches may be directing mitoses in this area. Notably, this influence over the nuclear division cycle is transient and is most pronounced as the branch tip emerges, as seen in Movie 1. As the branch matures, while the ring persists there are fewer mitoses in this area suggesting a decay of the local mitotic inducing potential with time. This coincides with subtle changes in the structure of the asymmetric ring (greater distance between the two halves of the ring, see Chapter II, Figure II.3A) however it is unclear if this change is relevant for mitosis regulation.

How might the septins locally promote mitosis at branching sites? Based on the morphogenesis checkpoint in S. cerevisiae and conservation of these factors, it is easy to hypothesize that septins direct mitosis through the recruitment and local inactivation of Swe1p. Potentially in unbranched areas of mycelia, some nuclei are delayed in division by active AgSwe1p. This delay then is relieved upon septin ring formation at branchpoints which leads to a local depletion/ inhibition of AgSwe1p and then nuclear division at such sites. The observation that deletions of AgSWE1, AgHSL1, AgMIH1, or AgCDC12 similarly shift mitoses away from branches to random positions supports the possibility that the balance of AgSwe1p activity controls the spatial pattern of mitosis. Mycelia lacking Swe1p would no longer have the means to limit mitoses in unbranched areas and Mycelia lacking septins would not be able to promote mitoses at branches, both of which result in random division phenotypes. Furthermore, the localization of AgHsl7p, which functions in S. cerevisiae as an adaptor that helps bring ScSwe1p to the septins where it is targeted for degradation, is suggestive that the links in this regulatory system may be preserved in A. gossypii. Thus, in response to some cell intrinsic patterning signals that direct branching in rich nutrient conditions, there is evidence that the septins may direct mitosis spatially through AgSwe1p activity.

There are some problems, however, in overlaying the details of the yeast checkpoint system onto the A. gossypii spatial control system that functions in response to cell intrinsic cues. AgHsl7p is not observed at all branch septin sites and rather seems to be more common at septin rings on the main hyphae although this could just be due to detection problems. Additionally, the AgHsl7p has a key residue differences in its sequence compared to the ScHsl7p at phenylalanine 242. When ScHSL7F242 is mutated to leucine, the same substitution observed in the A. gossypii sequence (Appendix 3.6, arrow), ScHsl7p no longer can interact with ScHsl1p, and Swe1p is no longer targeted for degradation in yeast cells (Cid et al., 2001b). This raises the question as to whether AgHsl1p and AgHsl7p are able to form a stable complex that could promote AgSwe1p recruitment. More notably, when mycelia are grown in high nutrient conditions, no phosphorylated AgCdc28Y18 is detectable by immunofluorescence and only a faint band is seen on Western blots. This implies that strong Cdc28Y18 phosphorylation does not occur under high nutrient conditions, leaving only little room for changes in phosphorylation levels caused by spatial control of mitosis. It is amazing that this control system is based on very subtle changes of Cdc28Y18 phosphorylation, although AgSwe1p is capable of much stronger phosphorylation of its substrate, as we have seen in starving mycelia. It is conceivable that such a system is fragile and therefore cannot be of great importance for the fitness of A. gossypii mycelium, at least not under laboratory conditions. This is endorsed by none of the analyzed single or double deletion mutants having any noticeable effect on nuclear density or distribution under rich nutrient conditions. With that, A. gossypii is in clear contrast to budding yeast, where the double deletion of the phosphatase ScMih1p and the ScSwe1 inhibitor ScHsl1p in leads to a lethal block at the G2-M transition. However, even if A. gossypii does not depend on spatial control of mitosis, we would expect to see at least some influence on the distribution and/or density of nuclei when this control mechanism is
eliminated. The fact that this is not the case suggests the existence of redundant, yet unidentified mechanisms to regulate nuclear division and distribution. If local control of mitosis is abolished, this could be compensated by other "global" ways to decrease AgSwe1p activity, coupled with more efficient distribution along microtubuli. Another possibility of compensation is offered by controlling where and how often branch formation occurs. Evidence for interdependence of branch formation and mitosis has already been provided in other filamentous fungi (Müller et al., 2000; Westfall and Momany, 2002; Dynesen and Nielsen, 2003). Notably, these mechanisms can be bypassed or overstrained by overexpressing AgSWE1 from to SchHIS3 promoter, which results in decreased nuclear density even under non-starving conditions. AgSwe1p activity could globally be restricted on a transcriptional level in addition to locally controlling protein inhibition/degradation. This transcriptional control would no longer be functional when AgSwe1p is expressed from an exogenous promoter. Alternatively or additionally, the septins may interact more directly with nuclei at branches by capturing the ends of astral microtubules. Such associations have been observed by tubulin immunofluorescence of A. gossypii mycelia (Amy Gladfelter, personal communication) and potentially such physical connections which may generate tension on the astars could be a trigger for mitosis. Swe1p may itself have other targets than AgCdc28 that are relevant for nuclear cycle progression and potentially Swe1p-dependent phosphorylation impacts their activity and in turn progression. Or AgSwe1p may be able to directly inhibit AgCdc28p independent of Y18 phosphorylation and there is actually precedent for this alternative inhibition of the CDK in the morphogenesis checkpoint in budding yeast (McMillan et al., 1999).

Whereas spatially controlled mitoses do not seem to be an important feature for A. gossypii grown in laboratory conditions, this may be different in its natural environment. Filamentous fungi inhabit heterogeneous environments in the natural world. There is spatial and temporal irregularity in many factors such as water, temperature, minerals, pH in addition to carbon and nitrogen sources. A. gossypii is found outside the laboratory as a tropical plant pathogen where it is transmitted by sucking insects and can infect cotton and citrus fruits leading to dry rot (Ashby and Nowell, 1926; Batra, 1973). Given the large potential size of a filamentous fungal mycelium in which many interconnected hyphae share a common cytoplasm, a single mycelium may simultaneously experience "feast and famine" conditions. In many fungi, branching is induced by exposure to pockets of nutrients allowing the mycelium to forage and exploit that presumably limited source. A mycelium would ideally target these resources locally, producing more nuclei to support the exploring hyphae while the rest of the nuclei and mycelium remain quiescent. In a syncytium, however, it is unclear how a mycelium may delineate the active and inactive regions. Our current laboratory culturing conditions of A. gossypii make it difficult to simulate the nutrient heterogeneity such mycelia would encounter in the natural world. However, we have attempted to integrate the data we have generated on Swe1p spatially directing mitosis in the presence of abundant nutrients and Swe1p inhibiting mitosis during starvation into a model. In this model, sudden increases in the pools of nutrients would lead to branch initiation and a promotion of mitoses in the limited area. Interestingly, the Swe1p-dependent response to low nutrients was exacerbated in mycelia lacking septins. Thus, the septins which reside close to and at the branching sites might regulate the Swe1p-dependent response to nutrient status. However, considering the problems we were facing when growing the septin mutants, this data has to be handled with care. The involvement of the TOR complex in starvation triggered phosphorylation of AgCdc28Y18 makes it more likely that this response is spatially controlled by clustering of receptors/signaling factors for sensing nutrient status on the cell surface, which then inactivate a pool of AgSwe1p to generate mitoses locally (Figure I.12E).
Chapter II

Septins – cytokinesis factors in the absence of cell division in A. gossypii

Introduction

A characteristic feature of filamentous fungi is their multifaceted morphology. Some are able to choose between yeast-like growth, pseudohyphal growth or true hyphal growth. Others, like A. gossypii, are restricted to the hyphal form, which is most different from unicellular growth and propagation. It is of high interest to study factors that are involved in morphogenesis of these organisms. A protein family of such morphogenesis factors are the septins. They were discovered in 1970 by Hartwell and colleagues in a screen for ts mutants affecting cell division (cdc mutants). The screen revealed four mutants which prevented cytokinesis at restrictive temperature. The corresponding genes represent the four original septins, ScCDC3, ScCDC10, ScCDC11, and ScCDC12. Despite disrupted cytokinesis, the cells continued budding, DNA synthesis, and nuclear division, which resulted in large multinucleated cells with multiple, elongated buds (Hartwell et al., 1970; Hartwell, 1971a; Hartwell et al., 1974). By analyzing electron micrographs in 1976, Byers and Goetsch discovered ~20 evenly spaced striations of 10-nm filaments around the mother-bud neck in wild-type but not septin-mutant cells. Immunofluorescence studies revealed that the septin proteins colocalize into a ring at the neck (Byers and Goetsch, 1976; Haarer and Pringle, 1987; Kim et al., 1991; Longtine et al., 1996; Field and Kellogg, 1999). The localization of all four septins is disrupted in conditional Sccdc3 and Sccdc12 mutants, indicating interdependence of the septin proteins. Strong support for this finding was provided by biochemical studies: The four original septins co-purified on affinity columns, together with a fifth septin protein, ScSep7p or ScShs1p (Carroll et al., 1998; Frazier et al., 1998). Purified septins from budding yeast, Drosophila, Xenopus, and mammalian cells are able to self associate in vitro to form highly ordered, filamentous structures (Frazier et al., 1998; Kinoshita et al., 2002; Casamayor and Snyder, 2003; Caviston et al., 2003; Dobbelrae et al., 2003; Versele et al., 2004). How the septins interact in vitro to form heteropentamers that assemble into filaments was studied in detail in S. cerevisiae (Versele et al., 2004). Based on these and former studies, the septins are composed of a variable N-terminus with a basic phosphoinositol binding motif, a conserved core comprising a GTP-binding domain, a septin-unique element and a C-terminal extension including a predicted coiled coil (Saraste et al., 2000; Field and Kellogg, 1999; Zhang et al., 1999; Casamayor and Snyder, 2003; Versele et al., 2004).

Micrographs of purified filaments raised the possibility that the septins are organized in parallel to the mother-bud axis. The 10-nm striations seen on electron micrographs may be the result of lateral interaction between the filaments (Frazier et al., 1998; Longtine et al., 1998; Gladfelter et al., 2001b). Mutant strains lacking factors important for septin organization support this view. Instead of continuous rings, the septins form bars oriented along the mother-bud axis in deletion mutants of ScGIN4, ScNAP1 and ScCLA4 (reviewed in Longtine et al., 2000; Gladfelter et al., 2001b).

The septins act as a scaffold, recruiting a plethora of proteins (reviewed by Longtine et al., 1996). These protein complexes are involved in cytokinesis, chitin deposition, cell polarity (reviewed by Faty et al., 2002), spore formation (Fares et al., 1996), in the morphogenesis checkpoint (see Chapter I), spindle alignment checkpoint and bud site selection (Hartwell, 1971a; Byers and Goetsch, 1976; Fares et al., 1996; Longtine et al., 1998; Gladfelter et al., 2001b). In contrast to septin mutants, disruption of one single pathway only leads to a delay in cytokinesis, not complete failure of cell division. Hence, the septins are predicted to act at the most upstream level of cytokinesis.

Cytokinesis is driven through two septin dependent, redundant processes: recruitment and contraction of the actomyosin ring and formation of the septum by vesicle fusion with the plasma membrane (Epp and Chant, 1997; Bi et al., 1998; Lippincott and Li, 1998b, 1998a; Vallen et al., 2000). In contrast to septin mutants, disruption of one single pathway only leads to a delay in cytokinesis, not complete failure of cell division. Hence, the septins are predicted to act at the most upstream level of cytokinesis.

The role of septins in cell polarity is reviewed in detail by Faty et al., 2002. After the apical-isotropic switch in budding yeast, cortical...
components, supposedly of the exocyst and polarisome, are delocalized from the apical pole to the entire plasma membrane of the bud, but not the mother cell. The septin ring at the neck serves as a cortical barrier that prevents membrane diffusion of these factors between the two compartments. This asymmetric distribution is abolished in septin mutants (Barral et al., 2000; Takizawa et al., 2000; Faty et al., 2002).

Some conditional septin mutants do not form buds at their normal axial location. Moreover, the typical localization of some bud-site-selection factors in a double ring at the neck is lost or disturbed in these mutants. This indicates that the septins may serve as anchoring site for such factors in axially budding cells (Flescher et al., 1993; Chant et al., 1995; Cabib et al., 1998).

It seems that one single septin organization should not be sufficient to fulfill such a variety of tasks. Accordingly, the septin cortex undergoes several changes throughout the cell cycle (reviewed by Gladfelter et al., 2001b): The first visible septin structure is a distinct ring which appears ~15 min before bud emergence. After bud emergence, the ring broadens to assume the shape of an hourglass around the mother-bud neck. During cytokinesis, the septin cortex splits into a double ring which eventually disappears. How can the septin cortex undergo so dramatic changes, although some of its functions may require it to be a stable structure? FRAP analysis has revealed that the turnover of septins at the neck undergoes multiple changes during the cell cycle. The predominant, functional conformation is characterized by a low turnover rate (frozen state), during which the septins are phosphorylated. Structural changes require a destabilization of the septin cortex (fluid state) induced by dephosphorylation prior to bud emergence, ring splitting and cell separation (Dobbelaere et al., 2003).

The composition of the septin cortex does not only vary throughout the cell cycle but also along the mother-bud axis. This inherent polarity of septin filaments allows concentration of some proteins primarily to the mother side of the neck, some to the center and others to the bud site (DeMarini et al., 1997; Gladfelter et al., 2001b).

Since their discovery in S. cerevisiae, septin homologues have been found throughout the eukaryotes, with the exception of plants. The variety of different shapes that septins can assume within a single cell is especially apparent in filamentous fungi (reviewed by Gladfelter, 2006). The genome of C. albicans encodes homologues to all S. cerevisiae septins (CaCdc3p, CaCdc10p, CaCdc11p, CaCdc12p, CaSep7p). They form a diffuse band at the base of emerging hyphae, a bright double ring at septation sites, an extended diffuse cap at hyphal tips and elongated filaments stretching around the spherical chlamydospores (Sudbery, 2001; Kaneko et al., 2004; Martin and Konopka, 2004b; Wightman et al., 2004; Martin et al., 2005). Five septins are found in A. nidulans (AnAspAp, AnAspBp, AnAspCp, AnAspDp, AnAspEp). AnAspBp forms single rings at septation sites that eventually split into double rings. Additionally, AnAspBp forms a ring at sites of branch emergence which broadens into a band as the branch grows (Westfall and Momany, 2002). As a effect of maturation double rings reflect hyphal polarity by disassembling the tip proximal ring in C. albicans or the more basal ring in A. nidulans (Warenda and Konopka, 2002; Westfall and Momany, 2002). Bases for the various patterns of septin organization could be different modifications and/or localization of different septin interaction partners.

The septins in both, A. nidulans and C. albicans control aspects of filamentous cell shape. CaCdc3p and CaCdc12p are essential for proliferation in yeast or hyphal forms. Cacdc10Δ and Cacdc11Δ mutants are viable but show aberrant chitin localization and cannot properly maintain hyphal growth direction (Warenda and Konopka, 2002; Martin et al., 2005). Conditional mutants of the essential AnAspBp display diffuse chitin deposition and a hyper-branching phenotype (Westfall and Momany, 2002).

Here, we add data on the function and localization of the septins in a fungus which grows exclusively in the hyphal form. Our studies have lead to the discovery of novel structures and revealed the astonishing fact that none of the analyzed septin genes in A. gossypii is essential, despite significant roles in morphogenesis.
Results

Septins are conserved in two organisms of most diverse morphology

Abscission (complete cytokinesis) is not observed in healthy, multinucleated A. gossypii hyphae, at least not until the sporulation program is initiated (Brachat, PhD thesis, 2003). Does this mean this Ascomycete does not require a full set of septin proteins as we find them in S. cerevisiae? Interestingly, not only were homologues to all budding yeast septins identified in A. gossypii, one of them even revealed two homologues, AgCDC11A (AER445C) and AgCDC11B (AFR436C), as a result of tandem duplication followed by chromosome break (Sophie Brachat, personal communication; Dietrich et al., 2004). Pairwise alignment with the S. cerevisiae homologues was used to identify the domains described by Versele et al. (2004). With the exception of Cdc3p, all mitotic septins and their domain composition were found to be highly conserved between the two organisms (Figure II.1), despite their substantially different life cycles.

Continuous AgSep7p-GFP ring at the neck of budding yeast

To understand where the septins may be required in A. gossypii, we first studied their localization by using the AgSep7p-GFP construct. The GFP fusion was obtained by in vivo recombination in S. cerevisiae (see materials and methods). We used this opportunity to investigate whether AgSep7p was able to interact with the S. cerevisiae septin complex, despite the low conservation (38% identity) between the C terminal extensions (CTEs) of AgSep7p and ScSep7p. Versele et al. (2004) had shown that the CTE of ScSep7p is required for proper interaction with ScCdc11p. Remarkably, a bright, continuous single or double ring structure at the neck was observed which did not look differently from septin formations in budding yeast (Figure II.2A) (Field and Kellogg, 1999; Yuzuyk and Amberg, 2003). This revealed that the AgSEP7 promoter is functional in budding yeast and the protein does properly localize and not disturb the assembly of the S. cerevisiae septins, despite the differences in the CTE.

Intermitted hyphal AgSep7p-GFP rings in A. gossypii

Surprisingly, very different structures were observed when we studied the localization of AgSep7p-GFP in A. gossypii, using strains carrying plasmids or integrated constructs. Most noticeably, ring-like structures were not continuous like in budding yeast, but rather made from 9 – 12 parallel bars (Figure II.2B). These bar rings changed their appearance depending on their position within the mycelium. They were long and diffuse close to the growing tips and got shorter and more compact the further away they were from the tip (Figure II.2C). In older parts of the mycelium we found double rings with various distances between the two rings, probably representing mature septa (Figure II.2B). At low magnification, these double rings and some compact single rings had a continuous appearance. To see whether the bars eventually fuse to form continuous rings, we investigated ambiguous structures at higher magnification and by using 3D reconstruction. Out of 28 single rings, bars were identified in 25 rings. In three cases, evaluation was not possible due to poor picture quality. The resolution of light microscopy was not high enough to resolve the structure of double rings. In some cases they appeared to be made from a large number of short bars, but this remains uncertain.

We had the possibility to study septin structures in giant hyphae of a strain expressing a constitutively active form of AgCdc42p created by (Köhli, Diploma thesis, 2003). We observed unusually long septin filaments at the tip and a double ring like structure composed of bars (Figure II.2D). It is not unlikely, however, that septin structures are influenced by mutations of AgCdc42p (Richman et al., 1999; Cid et al., 2001a; Gladfelter et al., 2001a; Caviston et al., 2003). Thus, we have to be careful when concluding from these “macro structures” to wild-type septin structures.

The bar and gap organization of hyphal rings was confirmed by immunofluorescence using an anti-Cdc11p antibody (Amy Gladfelter and Michael Köhl, personal communication). This made us wonder whether each bar consist of all mitotic septins, leaving real gaps in between, or whether there are various categories of filaments, made from different septin compositions. In the latter case it was conceivable that the septins actually form continuous rings which appear as bar-like when only one septin is labeled (Figure II.2E). To distinguish between the two possibilities, AgSep7p-GFP and AgCdc11A/Bp were co-immunostained using anti-GFP and anti-Cdc11p antibodies. Due to high background and low signal intensity we found only one ring were both stainings were useable. In this ring we could clearly observe co-localization of AgSep7p-GFP bars and AgCdc11A/Bp bars. This puts the first hypothesis (Figure II.2E, left) in favor, but is not sufficient to exclude the second hypothesis.
Variety of septin organizations

Septins in *A. gossypii* were not only found to localize as single and double rings at the hyphal cortex, they also formed a variety of asymmetric structures at the base of branches. Depending on the age/length of the branch, these structures had different appearances (Figure II.3A). Already at the base of branches that were just about to emerge, we observed septin localizations in the angles of the branch with the mother hyphae but not in between (Figure II.3A, first picture). It is not clear, whether this was an imaging artifact or whether the two localizations were actually not connected in most branches. In some cases it looked as if the two deposits were about to join each other to form a ring like organization. With proceeding growth of the branch, a diffuse ring appeared at the growing tip. In longer branches, the septin localization at the base of the branch was usually joined by a ring composed of bars on top of it, with various distances between the two formations. At the base of mature branches we could observe double rings which looked like those seen in other old parts of mycelium, distal from the base. Despite the different appearance of hyphal septin rings and the asymmetric septin organizations at the base of branches, both formations eventually lead to double rings of comparable appearance.

Another phenomenon, small rings or patches with a diameter of 0.5 µm, was only seen in mycelium expressing *AgSEP7-GFP* from plasmid and thus likely was an artifact due to local overexpression. These rings were of remarkable similarity with septin structures found in other organisms under some unnatural conditions (Figure II.3B) (Kinoshita et al., 2002; Kozubowski et al., 2003).

The commonly observed *A. gossypii* septin structures are summarized in Figure II.4A and put in relation to their position within the mycelium in Figure II.4B. Most of the structures seen with *AgSep7p-GFP* were confirmed by anti-Cdc11p immunofluorescence in fixed mycelium (Amy Gladfelter and Michael Köhl, personal communication).

Chronological order of septin localization

By analyzing time-lapse movies of *AgSEP7*-GFP strains we aimed at gaining further insight in the temporal organization of septin structures. Unfortunately, the *AgSep7*-GFP signal was relatively weak for the purpose of time-lapse analysis which – combined with the strong background of *A. gossypii* in the GFP channel – made it difficult to follow the formation of septin structures in detail. In Movie A, we were able to observe the transition from a hyphal single ring to a double ring (Figure II.5A). Remarkably, the single ring was fading around 100 min before the signal reappeared as a double ring. The best result was obtained with a movie of GFP labeled nuclei together with *AgSep7*-GFP (Movie B, Chapter I). Using minimal medium (2xASD) instead of full medium resulted in less background at the cost of reduced growth. Nevertheless we could follow the basic steps of septin deposition during hyphal growth: A broad septin signal appeared at sites of branch formation when the first bulging of a new branch was visible. When the bulge took hyphal shape, the septin deposit was split in two parts, one portion staying at the base of the branch, the other moving together with the growing tip. Eventually, a portion parted from the deposit at the tip to form a hyphal ring, whereas the other part remained at the tip (Figure II.5B). We tried to study these events in detail, by concentrating on two branching sites with acceptable quality. At each time point (Δt = 2 min), we analyzed each focal plane individually (5 planes covering 3 µm). The sketch shown in Figure II.6 was drawn based on these observations. In contrast to long exposure, high magnification picture stacks, signal strength and resolution in the movie were often too low to distinguish single septin bars and only a diffuse glow was visible, which is represented as such in the sketch. Analysis of both, fluorescence and phase contrast planes revealed that fainting of the structure and transition to a double ring occurred at about the same time as septum formation. This lead to a sandwich of two bright septin rings with the septum in between (indicated as red in Figure II.6). More movies will be required in order to confirm this data.

One ring to rule them all

The manifoldness of septin structures – many of them so far not described in wild-type budding yeast – made us wonder whether recruitment of other proteins is limited to only some of these conformations. Amy Gladfelter has observed that *AgHsl7p-GFP* co-localizes with some, but not all septin rings (personal communication). Here we wanted to know which of the septin structures are likely to be involved in the formation of actin rings. For that, we used mild fixation to stain the actin cytoskeleton while preserving the integrity of GFP. Actin rings were limited to mature, contracted septin structures and in no case was an actin ring observed in the absence of a septin ring (Figure II.7A).

Does this imply that the compact septin rings are essential for actin ring formation? To answer this and learn more about septin functions in a filamentous fungus in general, *AgCDC3, AgCDC10,* and *AgCDC12* were deleted in an *AgSEP7*-GFP
background. This allowed us to investigate the importance of single septins for the association into the structures we had observed. Each deletion resulted in delocalization of AgSep7p-GFP to the cytoplasm; no distinct septin structures were visible in these mutants. (Figure II.7B)

We have seen that actin rings are only found at compact septin rings. Further, septins have been found to permanently localize to growing tips. What is the effect of a total loss of septin found to permanently localize to growing tips. Are they important for both, actin ring formation and polarization of actin patches? No actin rings were detectable in the mutants Agcdc3Δ and Agcdc10Δ (Figure II.7C). Though these strains exhibited a certain resistance to the staining procedure, actin patch polarization was still visible. Thus, while septin rings are essential for the formation of actin rings, the septin protein pool found at growing tips does not seem to be important for the polarization of the actin cytoskeleton.

It was shown previously that even in the absence of actin rings, malformed chitin rings, and aberrant septa can still be observed in Agmyo1Δ mutants, permitting low sporulation levels (Helfer, Diploma thesis, 2001). In Agcdc3Δ and Agcdc10Δ mutants neither ring formation, nor septum formation, nor sporulation were observed (Figure II.8A). Agcdc12Δ strains were not stained for actin or chitin, but AgSep7p-GFP localization, septum formation, and sporulation were abolished in this mutant. Thus, the septin structures in A. gossypii are recruiting more components than those required for actin ring formation. Unlike in Agmyo1Δ, there is no alternative way for septum formation that could substitute septin deletions.

When performing microscopy of septin deletion mutants, we could directly observe that damage induced leakage of the cytoplasm was not restricted to one compartment like in the reference strain but extended to the entire young mycelium (data not shown). As a result, liquid cultures of septin null mutants were detectable in the mutants Agcdc3Δ and Agcdc10Δ (Figure II.7C). Though these strains exhibited a certain resistance to the staining procedure, actin patch polarization was still visible. Thus, while septin rings are essential for the formation of actin rings, the septin protein pool found at growing tips does not seem to be important for the polarization of the actin cytoskeleton.

We tried to get further insight in how the septins are required for growth by studying the morphology of small and mature mycelia in septin null mutants. Possible effects on the nuclear cycle, which could also lead to a decrease in radial growth, have already been discussed in Chapter I. Morphological defects in small mycelia grown in liquid (10 – 15 h) were already revealed when looking at the stainings described above. Mutant hyphae exhibit an erratic thickness with characteristic bulges in the area of branch points and especially around the spore (Figures II.7B, 7C, 8A and 8B).

Mature mycelia grown on plates (8 days) revealed invasive growth and a curly phenotype of hyphae, indicating reduced capability of maintaining growth direction. Lateral branching and apical branching occurred in septin null mutants, but the branching pattern appeared random. Long stretches free of branches or only with attempts of branch formation alternated with regions of unusually dense branching. Lateral branching was still ubiquitous close to growing tips, where in reference mycelia of the same age only apical branching was found (Figure II.9B). Apparently, septins in A. gossypii are involved in a variety of morphogenetic processes.

**Cortical barrier?**

In S. cerevisiae, the septins have been shown to form a cortical barrier around the mother bud neck which prevents membrane diffusion between mother and bud and maintains cortical polarity. Thus, components of the exocyst and the polarisome are restrained to the bud (Snyder, 1989; Barral et al., 2000; Takizawa et al., 2000; Faty et al., 2002). We wondered whether the reduced growth rate and erratic thickness of hyphae in A. gossypii septin deletion mutants could be the result of absent diffusion barriers. The septins might be required to efficiently maintain a pool of growth components at the hyphal tips by preventing diffusion towards older parts of the hypha (Figure II.10A). We used Filipin to stain sterols as an indicator of membrane polarity (Wachtler et al., 2003). Polarization of sterols at hyphal tips was clearly visible in reference hyphae and septin null mutants (Figure II.10B). Thus, the septin ring close to hyphal tips in A. gossypii apparently is not required to maintain sterol polarity. However,
it cannot be excluded that they serve as a barrier for certain membrane proteins.

Separating the “twins” (AgCDC11A/B)
The fact that the genome of A. gossypii encodes two homologues to ScCDC11 as a result of tandem duplication followed by chromosome break made AgCDC11A and AgCDC11B highly interesting candidates for functional analysis. Do both homologues occupy distinct roles, or are they redundant? The high degree of conservation between the two genes made it difficult to specifically delete and verify only one homologue at a time. With the exception of CDC11A/B-I, Primers were designed to provide best possible diversity (Figure II.11). Verification PCRs for each strain were done with primer sets for both, AgCDC11A and AgCDC11B. Both ends of each replaced ORF were verified unambiguously, no unspecific binding to the wrong locus was detected (Figure Appendix 1.9B).

In terms of morphology, Agcdc11AΔ isolates exhibited high similarity to other septin null mutants (Agcdc3Δ, Agcdc10Δ, Agcdc12Δ). As in those strains, the actin cytoskeleton was difficult to stain in Agcdc11AΔ mycelium, resulting in cloudy images with actin patches being barely visible. Amazingly, actin rings and chitin rings were both detectable in this strain (Figure II.12A). Fitting to these results, sporulation was not abolished entirely but only reduced to 30%. Whereas deletion of AgCDC11A leads to a mild septin phenotype, we did not observe any mutant phenotype of Agcdc11BΔ null mutants at all. Morphology, actin staining, chitin staining, and sporulation were as in reference mycelium (Figure II.12B). Apparently, the two homologues have diverged enough to be of different importance for the development of A. gossypii mycelium. More experiments are required to determine whether AgCDC11B is expressed under laboratory conditions, whether the two homologues complement each other to exercise functions similar to the other septins, or whether they have partially adopted novel roles, tailored for the requirements in a filamentous fungus in natural environment.
<table>
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<th></th>
<th>Variable N-terminus</th>
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<td>73%</td>
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**Figure II.1. Domain composition of *A. gossypii* and *S. cerevisiae* mitotic septins.** The septins are composed of a variable N-terminus with a tract of basic and hydrophobic residues constituting a phosphoinositide-binding motif, a conserved core comprising a GTP-binding domain, a septin-unique element and a C-terminal extension (CTE) including a predicted coiled coil. The domains were identified by pairwise alignment with the *S. cerevisiae* homologues, using the boundaries defined by Versele et al., 2004.
Figure II.2. AgSEP7-GFP in A. gossypii and S. cerevisiae. (A) S. cerevisiae cells bearing AgSEP7-GFP on the plasmid pagHPH002 were grown in liquid YPD with selection and imaged live. The inlay shows the localization of ScSep7p-GFP as reference (Yuzuk and Amberg, 2003). (B, C) A. gossypii mycelium with integrated AgSEP7-GFP (AgHPH007) was grown for 12 h at 30°C, washed in minimal medium and subjected to in vivo fluorescence microscopy. The inlay in (B) shows a 90° 3D reconstruction of the discontinuous ring. The large picture shows how the septin rings contract the further away they are from the tip. 1: diffuse localization at the tip, 2: intermediate ring, 3: contracted ring, 4: double ring. (C) Diffuse tip localization consists of elongated septin filaments; rings distal from the tip are made from more contracted bars. (D) AgSEP7-GFP in a background of constitutively active AgCdc42⁺p (AgHPH016), grown as described above, 90° 3D reconstruction in the inlay. (E) Schematic representation of the two hypotheses according to which one bar represents one septin (left) or each bar is composed of all the septins (right). Bars, 10 μm.
Figure II.3. Diversity of septin organizations. (A) A. gossypii mycelium with integrated AgSEP7-GFP (AgHPH007, AgHPH008) was grown for 12 h - 15 h at 30°C, washed in minimal medium and imaged live. Pictures show septin structure at branching sites, with young branches at the left and old branches at the right. Outline of the mycelium is indicated by dotted lines. (B) Mycelium bearing AgSEP7-GFP on plasmid (AgHPH006) (left) was grown as above. Small ~0.5 μm rings are only observed in plasmidic strains and have similarity with rings seen in S. cerevisiae cells with overexpressed ScBNI4 (middle) or in mammalian cells with disrupted actin cytoskeleton (right). Bars, (A) 10 μm, (B) 5 μm.
Figure II.4. Schematic overview of septin organizations seen in *A. gossypii*. (A) Early, intermediate and mature structures in hyphae (top) and at branching sites (bottom). (B) Septin structures put in relation with their positions within the mycelium. Schemes are based on microscopic observations of AgSep7-GFP.
Figure II.5. Time-lapse analysis of septins in *A. gossypii*. (A) AgSEP7-GFP mycelia (pagHPH02) were pregrown at 30°C in liquid AFM for 10 h and transferred to a time-lapse slide containing solid minimal medium (2xASD) at 25°C. Every 2 min, a Z-series of 7 frames was taken at 10% intensity and 150 ms exposure (Movie A). The figure was assembled from a selection of frames with a 20 min interval. The arrow indicates a single ring which splits into a double ring. (B) AgSEP7-GFP, AgH4-GFP mycelium (pagHPH09) was pregrown in selective liquid AFM for 14 h at 30°C and transferred to time-lapse slide containing solid 2xASD at 25°C. Every 2 min, a Z-series of 5 frames was taken at 10% intensity and 100 ms exposure (Movie B). The figure shows a selection of pictures with 10 min intervals, on which one can observe the division of the original septin organization at the emerging branch into three distinct parts: a structure staying at the branching site, a hyphal ring and a faint tip localization staying at the growing tip. Bars, 5 μm.
Figure II.6. Scheme of development of septin structures. The drawings are based on individual Z-plane analysis of Movie B. Green: AgSep7p-GFP, red: cell wall and septum. The arrow indicates that the hyphal rings are stationary, whereas the diffuse apical localization moves together with the tip. Maturation of hyphal double rings is not illustrated, but follows a similar pattern as double ring formation at branching sites. Due to technical limitations, bars were not always resolvable in the movie, which is drawn as diffuse signal.
Figure II.7. Actin ring formation depends on septin rings. 

(A) AgSEP7-GFP (green) mycelia (AgHPH05) were grown for 13 h at 30°C, mildly fixed with paraformaldehyde and suspected to Rhodamin-Phalloidin staining (red).

(B) Agcdc3Δ, AgSEP7-GFP (AgHPH10), Agcdc10Δ, AgSEP7-GFP (AgHPH14) and Agcdc12Δ, AgSEP7-GFP (AgHPH15) mycelia were grown for 12 h and monitored by in vivo fluorescence microscopy. 

(C) ΔtΔt (reference), Agcdc3Δ (AgHPH10) and Agcdc10Δ (AgHPH14) mycelia were grown for 12 h, fixed with formaldehyde and stained with Rhodamin-Phalloidin. Bars, 10 μm.
Figure II.8. Septins are required for chitin ring and septum formation. (A) $\Delta \Delta$ (reference), Agcdc3Δ (AgHP10) and Agcdc10Δ (AgHP14) mycelia were grown for 12 h in liquid AFM at 30°C, fixed with ethanol and stained with Calcofluor. (B) Brightfield pictures of an intact (left) and empty (right) Agcdc3Δ mycelium (AgHP10). Bars, 10 μm.
Figure II.9. Morphological defects of mature septin deletion mycelia (A) Radial growth on solid medium of the reference strain (ΔlΔt) and deletion mutants Agcdc3Δ (AgHPH10), Agcdc10Δ (AgHPH14) and Agcdc12Δ (AgHPH15) compared to ΔlΔt (100%) at 30°C and 37°C. A small piece of homokaryotic mycelium (~1 mm³) from each mutant was spotted on full medium plates and grown for 4 days. (B) Micrographs of ΔlΔt, Agcdc3Δ (AgHPH10), Agcdc10Δ (AgHPH14) and Agcdc12Δ (AgHPH15) mycelia grown on full medium plates at 30°C for 8 days, illustrating the disordered branching pattern and wavy phenotype. Bar, 20 μm.
**Figure II.10. Septins are not required for sterol polarity.** (A) Scheme illustrating how the septins might be required for cortical polarity in A. gossypii. Lack of septin barriers would lead to diffusion of membrane associated material away from the tip, resulting in reduced tip extension and swollen hyphae. (B) The reference strain (ΔΔt) and deletion mutants Agcdc3Δ (AgHPH10), Agcdc10Δ (AgHPH14) and Agcdc12Δ (AgHPH15) were grown for 12 h at 30°C in full medium, stained with low concentrations of Filipin (2-4 μg/ml) and monitored after 2 min. Polarization of sterols is not affected in A. gossypii septin mutants. Bar, 10 μm.
Figure II.11. Pairwise alignment of the highly conserved AgCDC11A and AgCDC11B genes. Oligos are designed to bind in places with highest possible diversity. The open reading frames are marked with bold, colored letters (red: AgCDC11A, green: AgCDC11B).
Figure II.12. Deletion of AgCDC11A or AgCDC11B results in a partial or no septin phenotype. ΔΔt (reference), Agcdc11AΔ (AgHPH17) and Agcdc11BΔ (AgHPH18) mycelia were grown for 12 h in liquid AFM at 30°C, fixed with formaldehyde and stained with Alexa-Phalloidin (A) or fixed with ethanol and stained with Calcofluor (B). Agcdc11AΔ mutants display morphological defects similar to the other septin null mutants, but neither actin nor chitin rings are missing. No phenotype of Agcdc11BΔ mutants has been found. Bars, 10 μm.
Discussion

The filamentous ascomycete Ashbya gossypii carries a Saccharomyces-like genome but its morphogenesis differs markedly from budding yeast. Large areas of nutrients are quickly covered by A. gossypii mycelium through permanently polarized hyphal growth and branch formation. Except during germination and in some mutants, isotropic growth is not observed. In stark contrast to budding yeast where nuclear division is obligatory followed by cytokinesis and cell separation (abscission), cytokinesis is not observed during vegetative growth of A. gossypii. It never propagates by yeast-like or pseudohyphal growth like other filamentous fungi. Nonetheless, A. gossypii is not only equipped with homologues to all S. cerevisiae septins – the key players in cytokinesis, one is even duplicated (AgCDC11A, AgCDC11B).

Cytokinesis is likely to be a crucial part of the sporulation program. Thus the A. gossypii septins could be required only late in the life cycle. However, our localization studies with AgSep7-GFP have revealed a complex set of structures that is already established in young mycelium, which makes it unlikely that the septins are only responsible for sporulation. A striking difference between AgSep7-GFP rings seen in A. gossypii and budding yeast is in their structural organization: whereas in S. cerevisiae they are continuous, they are intermittent in A. gossypii, made from parallel bars. How can the very same protein, expressed from the same promoter, lead to such different structures in different organisms? We must keep in mind that we are not looking at all septins at the same time, but only at one single component of the ring, AgSep7-GFP. In budding yeast, the septins form heteropentamers with ScSep7p (ScShs1p) being the most peripheral component of this structure (Versele et al., 2004). Presumably, the role of Sep7 lies in recruiting other proteins to the septin ring, and not in defining the structural organization of the ring itself. To investigate whether and which septins are crucial for the observed structural differences, one could replace different subsets of septins in S. cerevisiae with their A. gossypii homologues and see which combinations lead to bar formation. There is also the possibility that the septin structure is not determined by the septins themselves. Discontinuous rings are known from mating S. cerevisiae cells at the neck of shmooes (Ford and Pringle, 1991; Kim et al., 1991; Longtine et al., 1998; Cid et al., 2001a) or at the mother-bud-neck of budding yeast cells bearing mutations of septin regulators (reviewed in (Gladfelter et al., 2001b). It has further been shown that the septin ring at the neck undergoes changes in protein dynamics to allow structural changes like single ring to double ring transition, and that this is regulated through the phosphorylation state of the septins (Dobbelnaere et al., 2003). In C. albicans, it has been speculated that sumoylation of the septins could be the bases for the different patterns of septin structures (Martin and Konopka, 2004b). Thus, modification of the septins could be a key element in determining their organizational structure. The fact that we see a great variety of different septin organizations in A. gossypii itself also makes it unlikely that having bar-like rings in A. gossypii and continuous rings in S. cerevisiae is solely the results of different septin proteins. The transient state of signal fading seen during single ring to double ring transition in A. gossypii is evidence for profound structural rearrangements which likely comes along with changes in protein dynamics. Mutants of potential septin modification proteins could help to understand how such transitions are regulated and whether different structures are related to different modifications. FRAP analysis could be used to analyze protein dynamics of individual structures.

What is the nature of the septin bars we see in A. gossypii? Do they represent filaments made from individual septins? If so, it was possible that the gaps between the bars are in fact filled by the other septins. All the septins together would then form a continuous ring. Our colocalization analysis of AgCdc11p and AgSep7-GFP and in vitro interaction studies in budding yeast (Versele et al., 2004) rather support the hypothesis that each filament incorporates all the septins, though. There is still the possibility that AgSep7-GFP bars and AgCdc11p bars just appear to colocalize because they are very close together. Ideally, we would tag all the septins and observe whether they together form continuous rings or still bar-like rings.

How does this potential discontinuity of the rings influence the recruitment of other proteins to these structures? AgHsl7-GFP colocalized with some but not all septin rings and actin rings were limited to mature, contracted septin structures. We have shown that even contracted septin rings consist of bars. Thus, the bar-like organization of septin rings in A. gossypii does not prevent recruitment of other proteins or even formation of continuous structures like actin rings. Nevertheless, localization of actin rings or AgHsl7-GFP to the asymmetric septin structures.
at young branching sites or colocalization with young, diffuse hyphal rings has never been observed. Possibly, hyphal septin rings need to mature before they can recruit other proteins. Alternatively, septin structures of different localization and age may recruit different sets of protein.

Interestingly, we have seen that a diffuse portion of AgSep7-GFP always stays at the growing tips. Do these faint, elongated filaments only serve as precursor for hyphal ring formation, or are they also involved in recruiting and/or maintaining actin patches at the tips? Deletion of single septin proteins (Agcdc3Δ, Agcdc10Δ and Agcdc12Δ) leads to complete delocalization of AgSep7-GFP: neither distinct structures nor polarization at the tips is seen in these mutants. Although the actin cytoskeleton is difficult to stain in septin deletion mutants and actin patches are less clearly visible than in the reference strain or even reduced in number, actin patch polarization does not seem to be affected. Even if the septins are not crucial for actin patch polarity, nevertheless maintenance of the permanent localization of septins to the growing tips is already established in young mycelium, which is already established in young mycelium, this clearly indicates significant roles of A. gossypii septins during vegetative growth. We have seen that the lack of septa in septin mutants causes the mycelium to be very susceptible to hyphal damage. Whereas in wild type, damage induced lysis is restricted to the affected hypha, it spreads over the entire mycelium in septin mutants grown in liquid. Considering this, it is surprising that these mutants are able to form large colonies on solid medium at all. One can imagine that even without mechanical stress from shaking, lysis-inducing events should occur at some point during several days of growth. Nonetheless, complete lysis of mature colonies has never been observed. Mere size of the mycelium could prevent leakage of the entire cytoplasm. Especially when immobilized on solid medium, sites of leakage should eventually be clogged by escaping cytoplasm. Even so, the amount of cytoplasm lost during such incidents could be enough to notably reduce growth efficiency.

Reduced radial growth rate is also likely linked to the morphological defects we have observed: A. gossypii septin deletion mutants exhibit erratic thickness of the hyphae, show difficulties in maintaining growth direction and have a disordered branching pattern. Regulation of hyphal thickness may be required for optimal tip extension speed, whereas adjusting direction of growth and the branching pattern should be critical to efficiently grow towards and exploit regions rich in nutrients. How are the septins involved in these processes? It has been shown that the branching pattern of A. gossypii is largely determined by AgRax1p and AgRax2p. AgBud10p is required for stable localization of AgRax2p at growing tips (Boudier, PhD thesis, 2005). Septin mutants in S. cerevisiae display mislocalisation of ScBud3p, ScBud4p and ScBud10p and disruption of the axial budding pattern (Flescher et al., 1993; Chant et al., 1995). It is likely that the homologues of these proteins are similarly localized by the septins in A. gossypii, which would explain why the branching pattern is disturbed in septin mutants. Strikingly, the septins at the branching site do not disappear after branch emergence but rather stay there to form an asymmetric structure of yet unknown function. AgHsl7p has never been found to localize to these places. Does this imply that the septins at the base of branches recruit a different set of proteins than the hyphal rings? Are they required for stabilization of the angle between the branch and the mother hyphae, thus helping to maintain direction of growth? Another unanswered question is proposed by the faint but permanent localization of septins to the growing tips. This localization is not required for actin patch polarity, nevertheless maintenance of the
A very interesting aspect of the septins in *A. gossypii* is the duplication of *CDC11*. Despite 85% identity between the two proteins, deletion of each results in some different phenotypes. Whereas *Agcdc11BΔ* cannot be distinguished from the reference, *Agcdc11AΔ* strains display morphological defects that are typical for septin mutants. Strikingly, actin and chitin rings are still formed in *Agcdc11AΔ* mycelia and sporulation is not completely abolished but occurs at 30% efficiency. This indicates that altered morphology of small mutant mycelium is not the result of absent septa. It would be highly interesting to delete *AgCDC11A* in the *AgSEP7-GFP* background to see which of the septin structures are affected by this deletion. This has the potential to answer many of the above questions by yielding insight into which structures are required for which morphogenetic process. So far we could not resolve the function of *AgCdc11Bp*. The two proteins may be partially redundant, allowing actin and chitin ring formation even if one of them is disrupted. This could be tested by analyzing double deletion mutants. Alternatively, *AgCdc11Bp* could have adopted a function which is of advantage in the natural environment of *A. gossypii*, but has so far escaped our observations under laboratory conditions. We have just begun to study these twins. More detailed examination may reveal the processes in which the two proteins are involved and why it was worthwhile for *A. gossypii* to preserve two copies of *CDC11*.

Thanks to the peculiar morphogenetic processes coming along with filamentous growth, future studies of the septins in *A. gossypii* and other filamentous fungi may draw secrets from these versatile proteins, which uninucleated cells would have kept forever.
A. gossypii methods, media and growth conditions

A. gossypii strains used here are listed in Table 2. To test the influence of high culture density, 10 – 20 ml of full medium were densely inoculated with spores and the cultures were grown until vacuoles were clearly observable by phase contrast microscopy. For all other conditions, 50 – 100 ml of full medium were inoculated at low density, regularly controlled for the absence of visible vacuoles and fresh medium was added 1 – 2 hours before taking samples or exposing the cultures to conditions of potential stress. Nocodazole stocks for the arrest and release experiment were 3 mg/ml in DMSO and used at a final concentration of 15 μg/ml (Sigma). Hydroxyurea was directly added to the cultures to a final concentration of 50 mM. Rapamycin stocks (LC Laboratories, Woburn, MA) were 1 mg/ml in 90 % EtOH, 10% Tween-20 and used at a final concentration of 200 nM (Loewith et al., 2002). To simulate the starvation response, low density cultures were washed twice and resuspended in 40 mM MOPS [pH 7.0], 137 mM KCl. To test the lack of certain nutrients, different media based on Ashbya Synthetic Dextrose (ASD) medium were used: 1.70 g/l YNB w/o amino acids and w/o ammoniumsulfate (Difco), 0.69 g/l CSM-LEU (Bio 101), 0.01 g/l adenine, 1.00 g/l myo-inositol, 20 g/l glucose, 50 mM MOPS [pH 6.5].

Plasmid and strain construction

All DNA manipulations were carried out according to (Sambrook, 2001) with DH5αF’ as host (Hanahan, 1983). Plasmids used here are listed in Table 3. PCR was performed using standard methods with different polymerases from Roche (Basel, Switzerland), Amersham Biosciences (Little Chalfont, Buckinghamshire, United Kingdom), or GenScript (Piscataway, NJ). Oligos were synthesized at MWG (Ebersberg, Germany) or Microsynth (Switzerland) and all restriction enzymes came from New England Biolabs or Roche. Oligonucleotide primers are listed in Table 4. Plasmid isolation from yeast and sequencing were performed as described in (Schmitz et al., 2006).

A. gossypii deletion mutants were generated using the PCR based one-step gene targeting approach with non-homologous selection markers (McElver and Weber, 1992; Baudin et al., 1993; Wach et al., 1994; Wendland et al., 2000). For G418 resistance, the deletion cassettes were amplified off the pGEN3 template using “gene name”-S1/S2 primer pairs. ClonNAT® resistance was obtained using pUC19NATPS as template. N’S1/N’S2 primer pairs were used for expression of the NAT1 resistance module under the control of the promoter and terminator of the targeted gene. NS1/NS2 primer pairs lead to NAT1 expression under the control of the ScPDC1 promoter and terminator. Correct integration was verified by analytical PCR with oligonucleotides “gene name”-(N)G1, -(N)G4, -I1/I2 (genomic) and G2.1, G3, V2PDC1P, V3PDC1T, V2*NAT1, V3*NAT1 (marker). To exclude phenotypes by random mutations, at least two independent transformants were characterized for each deletion. Transformation of multinucleate mycelium leads to heterokaryotic cells, which contain a mixture of transformed and wild-type nuclei, and usually do not display an apparent phenotype. For subsequent analysis, homokaryotic mycelium was obtained by isolating and growing single spores. To evaluate phenotypes of lethal mutants or mutants with sporulation deficiency, spores from heterokaryotic mycelium were germinated and analyzed under selective conditions.

For the creation of the double deletion strains AgHPH29 and AgHPH31, at least three homokaryotic isolates bearing the first deletion that were phenotypically not distinguishable were tested for correct integration of both ends of the deletion cassette and absence of the wild-type gene using verification PCR. One of these isolates was grown and transformed with the deletion cassette for the second gene, using a different selection marker. If no homokaryotic mycelium of the first deletion mutant was available yet (for creation of AgHPH27 and AgHPH28), spores from heterokaryotic mycelium were grown under selection and transformed with the second deletion cassette. In both cases, three primary transformants resulting from the second transformation were isolated. Verification PCR was used to test correct integration of both deletion markers. To test absence of both wild-type genes, homokaryotic mycelium was isolated from the double deletion mutants and verified using PCR.

To construct a strain with integrated histone-H4-GFP (AgHPH004), the plasmid pAG-H4-GFP-
was excised with pUCHPH002 and pUCHPH003. The cassettes were gel purified and separately ligated into the ORF of histone H4 (AgHHF1) with flanking homologies to the AgADE2 locus. H HF1-GFP was under the control of the AgHHF1 promoter and was terminated by ScADH1-T. 5 μg pH PH001 were isolated from ScADH1-T. The plasmid pH PH001 was digested with EcoRI– HindIII, and transformed into the partially deleted AgADE2 locus of the Agade2Δ101 strain (Knechtle, PhD thesis, 2002). Transformants were obtained on minimal medium lacking adenine (ASD–ADE).

A C-terminal fusion of GFP to AgSEP7 was obtained by amplifying pGUG and pGUC with S7-G-S1 and S7-G-S2 or S7-G-S1 and S7-G-CS2, respectively. To generate AgSEP7-GFP-NAT1, the pGUC cassette was first constructed by digesting pGUG with BglII and BamHI and ligating the gel purified 1026 bp fragment into the dephosphorylated pUC19NATPS vector which was linearized with BamHI. The S7-G–S1 primer was designed with homology to the C-terminal region of AgSEP7 before the stop codon. The PCR products were each transformed together with pAG4401, containing AgSEP7, into the yeast strain CEN.PK2 for in vivo recombination. The resulting plasmids pAGPH002 and pAGPH003 were purified and transformed into A. gossypii ∆Δt cells, leading to the strains AgPH05 and AgPH06. For integration into the genome, the two constructs were first subcloned into pUC19 to eliminate ARS activity. pUC19 was opened with SalI and XbaI, AgSEP7-GFP-NAT3 was excised from pAGPH002 with EcoRI and SphI and pAGPH003 was cut with Xhol and SpeI to extract AgSEP7-GFP-NAT1. The fragments were gel purified and separately ligated into the dephosphorylated pUC19 fragment to produce pUCHPH002 and pUCHPH003. The cassettes were excised with EcoRI and transformed into A. gossypii ∆Δt cells, giving rise to AgPH07 and AgPH08.

To generate ASG41 (AgHSL7-GFP-GEN3, constructed by Amy Gladfelter), the oligos Hsl7-S1 and Hsl7-S2 (with homology to the C-terminus of AgHSL7) were used with the template pGUG to generate PCR product which was used to directly transform A. gossypii cells.

To make the nonphosphorylatable Y18F mutation of AgCDC28, the plasmid pAGPH007 was created using in vivo gap repair in S. cerevisiae. First, the plasmid pRS416CDC28GEN3 was created by Amy Gladfelter: AgCDC28 was amplified from genomic DNA using oligos Cdc28alaEcoR1 and Cdc28allBamH1, with homology upstream of Cdc28 including the promoter region and limited downstream sequence. This PCR fragment was purified and digested with EcoRI and BamHI, sites present in the oligos, to clone it into pRS316 that was opened with these same enzymes. The entire CDC28 ORF was sequenced to confirm that PCR did not introduce point mutations. pRS416CDC28GEN3 was cut with Xhol at two sites, one being 23 bp upstream of Y18, the other being located in the pRS416 backbone. The resulting fragments were cotransformed into the yeast strain DHD5 (Arvanitidis and Heinisch, 1994) together with the annealed oligonucleotides CDC28-Y18F-A / CDC28-Y18F-B and CDC28-glue-A / CDC28-glue-B for in vivo recombination. The pair CDC28-Y18F-A / CDC28-Y18F-B overlapped the first Xhol site and contained the Y18F replacement flanked by a total of 15 silent mutations to improve the fidelity of subsequent genomic integration into A. gossypii. The second Xhol site was overlapped by the pair CDC28-glue-A / CDC28-glue-B, which contained no mutations. The gap-repaired plasmid (pAGPH007) was isolated and verified by sequencing of the altered regions. pAGPH007 was digested with EcoRI, BamHI and PstI and transformed into A. gossypii ∆Δt cells, resulting in AgPH36. Correct integration and presence of the altered region was verified by analytical PCR with the oligonucleotides CDC28-IB / CDC28-Y18F-IA (binds to altered sequence, only), G3 / CDC28-G4.2 and CDC28-IB / CDC28-IA-Y18 (binds to wild-type sequence, only). For additional verification, a fragment containing the altered sequence was amplified using CDC28-G1.2 / CDC28-IB and digested with KpnI, which cuts the mutated fragment at two places and the wild-type fragment and one site, only (Appendix 1.8).

The plasmid carrying AgSWE1 (pAGPH004) was generated by amplification of the gene from genomic A. gossypii DNA using the primers SWE1-B1 and SWE1-B2, which contain EcoRI and BamHI restriction sites. The fragments were purified, cut and ligated into pRS416. The resulting plasmid was verified by sequencing.

To C-terminally tag SWE1 with GFP or 13-myc, the pGUC or pAG13-myc cassette was amplified with SWE1-G-S1 and SWE1-G-NS2 or SWE1-MS1 and SWE1-MS2, respectively. The resulting PCR products were cotransformed into the yeast strain DHD5 together with pAGPH004 for in vivo recombination, giving rise to pAGPH005.

**Materials and Methods**
and pAGPH006. Correct fusion was verified by sequencing. Transformation of the plasmid pAGPH006 into A. gossypii ΔΔt cells produced the strain AgPH34. To obtain AgPH32, pAGPH005 was digested with BmgBI and BssHII before transforming it into A. gossypii ΔΔt cells, leading to genomic integration.

For overexpression of AgSWE1, the plasmid pAGPH008 was constructed: the ScHIS3 promoter was amplified off pAGrPXC (kindly provided by Dominic Hœ pfner) using the primer pair MH1 and SWE1-SH2. The GEN3 cassette was amplified using the oligonucleotides MH2 and SWE1-SH2 with pGEN3 as template. MH1 and MH2 contained homology to both, the GEN3 cassette and the ScHIS3 promoter, generating an overlap between the two PCR products. SWE1-SH1 and SWE1-SH2 added 45 bp homology to the very START of SWE1-SH1 and SWE1-SH2 added 45 bp homology to the very START of SWE1, and the Swe1F1 and Swe1F2 oligos, which have 54 bp homology to a region upstream of SWE1, together as template in a subsequent PCR reaction with the primers SWE1-SH1 and SWE1-SH2. The resulting GEN3-ScHIS3 cassette was fused in front of AgSWE1 by in vivo recombination with pAGPH004 in the yeast strain DHD5, leading to pAGPH008. The plasmid was purified and verified by sequencing and transformed into A. gossypii ΔΔt cells to produce AgPH37. To C-terminally tag GEN3-ScHIS3-AgSWE1 with HA, a 980 bp fragment carrying the carboxy end of AgSWE1 fused to 6HA was cut out of pAGSWE1-HA-NAT1 using MscI and ScaI. pAGPH008 was opened with MscI and XbaI. T4 DNA polymerase was used to fill the 5′ protruding end generated by XbaI. The dephosphorylated 8910 bp vector fragment was gel purified and ligated with the 980 bp insert to produce pAGPH009. In frame fusion of the tag was verified by sequencing. Transformation of the plasmid pAGPH009 into A. gossypii ΔΔt cells produced the strain AgPH38. To generate pAGSWE1-HA-NAT1 (done by Amy Gladfelter), the Swe1F1 and Swe1F2 oligos, which have homology to the C-terminus of AgSWE1 and the universal tagging cassette, were used in a PCR reaction with the pN1-6HA (pAGT105-kindly provided by Andreas Kaufman) template and the resulting product was cotransformed into yeast cells with the plasmid pAGPH004. Recombination of the plasmid with the linear PCR fragment resulted in yeast cells resistant to CloNAT. Plasmids were rescued from these resistant yeast strains and correct fusion of 6HA to AgSWE1 was verified by restriction digest.

**Protein extraction and Western blotting**

Cells grown under the desired conditions were collected, washed once with ice-cold PBS containing 1 mM Na2VO4 and 1 mM β-glycerolphosphate and suspended in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% NP-40, 2 mM sodium pyrophosphate, 1 mM Na3VO4, 1 mM β-glycerolphosphate and Roche complete protease inhibitor cocktail). An equal volume of 0.5 mm glass beads was added to this suspension, and cells were broken by vigorous vortexing during 6 x 30 sec intervals in a bead beater at 4 °C. Total cellular proteins were separated by SDS-PAGE (12%) and transferred to nitrocellulose membranes (Amersham Biosciences), using standard conditions. Blocking was performed with 5% milk in TBS-Tween-20 (0.1%). For detection of phosphorylated Cdc28p, rabbit anti-phospho-cdc2(Tyr15) (Cell Signaling, #9111) was used at 1:1000 in TBS, 5% BSA, 0.1% Tween-20, and HRP-anti-rabbit (Cell Signaling, #7074) was used at 1:2000 in TBS, 5% milk, 0.1% Tween-20. As positive control for phosphorylated CDK, total cellular proteins were extracted from the yeast strain DLY5544 (cdc12-6, GAL/SWE1), grown in YP 2% sucrose and induced by adding 2% galactose. For detection of HA tagged proteins, mouse anti-HA was used at 1:1000 in TBS, 5% BSA, 0.1% Tween-20, and HRP-anti-mouse (Jackson Immunoresearch) was used at 1/50 for immunofluorescence. For detection of phosphorylated Cdc28p, rabbit anti-phospho-cdc2(Tyr15) (Cell Signaling, #9111) was used at 1:1000 in TBS, 5% BSA, 0.1% Tween-20, and HRP-anti-mouse (Jackson Immunoresearch) was used at 1:2000 in TBS, 5% milk, 0.1% Tween-20. Detection of labeled proteins was performed by using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). Either non-specific cross reacting bands or bands from ponceau red staining of the membrane were used as loading controls.

**Fluorescence stainings, image acquisition and processing**

DNA stainings (Hoechst 33342) and anti-tubulin immunofluorescence stainings were performed as described previously (Gladfelter et al., 2006). Rabbit anti-ScCdc11p (Santa Cruz Biotechnology) was diluted in PBS and used at a 1/10 dilution. Rabbit anti-phospho-cdc2(Tyr15) was used at 1/50 for immunofluorescence. For in vivo observation of mycelium containing GFP tagged proteins, the cells were grown for 10 - 12 hours in full medium and then washed and resuspended in ASD or MOPS/KCl for observation under the microscope.

Visualization of the actin cytoskeleton by Alexa Fluor Phalloidin 488 was done as described in (Knechtle et al., 2003) with slight modifications: to improve the yield when staining young mycelia, 9 ml of culture were mixed with 1 ml of 37 % formaldehyde and 10 μl of 10 % Triton-X and fixed for 10 min. Mycelia were collected in 15 ml Falcon tubes by 1 min centrifugation in a Heraeus Minifuge at 1000 rpm and resuspended
in 900 μl PBS containing 4% formaldehyde and processed as described previously. For staining of the actin cytoskeleton while preserving the GFP signal mild fixation was used: The mycelia were fixed 20 min in 2–4% p-formaldehyde, washed twice with PBS and resuspended in 100 μl PBS containing 0.6 μM Rhodamine-Phalloidin. After incubating the cells for one hour on ice, they were washed five times with PBS, resuspended in 50–200 μl mounting medium and mounted on slide (Sambrook, 2001; Knechtle, PhD thesis, 2002; Laissue, PhD thesis, 2004, Andreas Kaufmann, personal communication).

Calcofluor white was used to stain chitin rings. 20 μl of a 1 mg/ml Calcofluor solution were added to 100 μl of liquid culture, incubated for 5 min, washed twice with dH₂O and mounted on a glass slide for microscopic inspections.

To visualize the distribution of sterols, a fresh stock of 2 mg/ml Filipin in DMSO was prepared. 2–4 μl of this solution were added to 2 ml liquid culture (2 μg/ml final concentration) and incubated for 2 min. During the incubation time, the mycelia were washed twice with dH₂O, once with AFM and resuspended in 500 μl AFM. The toxic effect of Filipin resulted in rapid death of the mycelia, necessitating immediate microscopic inspection.

The microscopy setup used was the same as described in (Hoepfner et al., 2000; Knechtle et al., 2003; Gladfelter et al., 2006). The following filter sets were used for the different fluorophores: #02 for Hoechst, Calcofluor and Filipin, #10 for Alexa 488, #15 for rhodamine 568 (Carl Zeiss) and #4108 for GFP (Chroma Technology).

Multiple planes (20–50 for still pictures and 3–7 for time-lapse pictures) with a distance between 0.3 and 0.5 μm in the Z-axis were taken. MetaMorph 4.6r9 software (Universal Imaging Corp) was used for “no-neighbors” 2D-deconvolution. The stacks were flattened into a single plane using stack arithmetic “maximize” command. Outlines of the cells were obtained by doing phase-contrast Z-series. The stacks were passed through the Metamorph filter “detect edges: Laplace 2” and flattened into a single plane using the stack arithmetic “sum” command. Fluorescent and phase-contrast images were scaled and overlayed using “color align”.

Time-lapse series were taken as described in (Gladfelter et al., 2006) and assembled into movies using QuickTimePro 6.5. Mitotic events in Movie 1 were labeled manually using Adobe Photoshop CS.
Table 2. *A. gossypii* strains used in this study

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With the exception of plasmidic strains (square brackets), all analyzed mycelia were homokaryotic (all nuclei have same genotype). This was obtained either by sporulating homokaryotic strains or by applying selective pressure during germination.
### Table 3. Plasmids used in this study

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pAG plasmids are based on the pRS416 vector Sikorski and Hieter, 1989.
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**MATERIALS AND METHODS**
Lower case letters are regions of homology to the cassette containing a selectable marker. Underlined letters represent intragenic replacements (alternative codons, point mutations).
I. Verification PCRs

II. Plasmid maps

III. Sequence alignments
Appendix 1.1. Oligos used for verification of deletion strains. (A) Verification of correct integration of the GEN3 deletion cassette. (B) Verification of correct integration of the ClonNAT deletion cassette. (C) Verification of absence of the targeted ORF in homokaryons. PCR products are obtained in wild type and heterokaryons, only.
Appendix 1.2. Verification of Agmih1Δ strains. (A) heterokaryons (B) homokaryons
Appendix 1.3. Verification of AgseptinΔmih1Δ strains. (A) Verification of the heterokaryotic septin knock-outs. (B) Verification of the homokaryotic Agmih1Δ background.
Appendix 1.4. PCR verification of Agcdc12Δ:GEN3 (AgHPH27) homokaryons.

(A) Verification of correct integration of the GEN3 cassette at the AgCDC12 locus and absence of the wt allele  
(B) Verification of the homokaryotic Agmih1Δ background.

The Promega 1 kb DNA ladder was used as marker (lowest three bands: 750, 500 and 250 bp).

[Diagram of PCR results showing different bands for different combinations of samples.]
Appendix 1.5. Verification of AghsllΔmihlΔ strains. (A) For the construction of AgPH28, spores from heterokaryotic AgPH23 (AghmihlΔ::GEN3) were grown under selection and transformed with the AghsllΔ::NAT1 cassette. The AghmihlΔ background was verified by one end only, since both ends were already verified in AgPH23. (B) For AgPH29, spores from homokaryotic AgPH22 (AghmihlΔ::NAT1) were used. Verification of the homokaryons including AghmihlΔ background is shown in Appendix 1.6.
Appendix 1.6. PCR verification of AghlslΔmihlΔ (AgHPH29) homokaryons. (A) Verification of correct integration of the GEN3 cassette at the AgHSL1 locus and absence of the wt allele. (B) Verification of the homokaryotic AgmihlΔ background. The Promega 1 kb DNA ladder was used as marker (lowest three bands: 750, 500 and 250 bp).
Appendix 1.7. Verification of Agsl7Δ::mih1Δ strains. (A) Verification of heterokaryons. Unspecific bands are the result of low annealing temperature. The pattern of true transformants can clearly be distinguished from false positives or the reference strain. (B) Verification of homokaryons. Agmih1Δ background. Spores from homokaryotic AgPHH22 (Agmih1Δ::NAT1) were used for transformation.
AgHPH31 (Ag srew1Δ::GEN3, Agmih1Δ::NAT1)

**A**

1, 3, 5: SWE1-G1 + G2.1 → 721 bp  
2, 4, 6: SWE1-G4 + G3 → 824 bp

**B**

1, 4, 7: MIH1-G1 + V2PDC1P → 506 bp  
2, 5, 8: MIH1-G4 + V3PDC1T → 401 bp  
3, 6, 9: MIH1-G1 + MIH1-I1 → 503 bp (wt)

**C**

Appendix 1.8. Verification of Ag srew1Δmih1Δ strains. (A) heterokaryons, (B) Agmih1Δ background, (C) homokaryons
Appendix 1.9. Verification of Agcdc11AΔ and Agcdc11BΔ strains.
(A) heterokaryons, (B) CDC11A verification oligos G1 and G4 were tested for unspecific binding to the AgCDC11B promotor and terminator region in Agcdc11BΔ strains and vice versa. No crossreaction was detected. (C) Verification of homokaryons.
Appendix 1.10. Verification of Agcdc28Y18F strains. (A) Verification by PCR. CDC28-Y18F-IA binds specifically to the mutated sequence (incl. Phe18), but binding is weak. Bands that were seen by eye on a UV table but were too weak for printing using the gel imager are painted in green. CDC28-G4.2 + G3 was used to confirm correct integration of the GEN3 module. CDC28-IA-Y18 was designed to bind to the wild-type sequence, only (incl. Tyr 18), but results in unspecific, weaker bands in the mutant strains. The pattern is clearly distinguishable between true mutants and false positives or the wild-type allele. A band of correct size (564 pb) is only observed for false positives or the wild-type allele, not for true positives. The lowest band in true positives corresponds to the second lowest (false) band in wild type. (B) A 732 bp fragment including Tyr 18 / Phe 18 was amplified and digested with KpnI, which cuts the mutant fragment three times and the wild-type fragment twice. Additional bands are likely to be the result of the 1.5 kb unspecific PCR product.
Appendix 2.1. pAG-H4-GFP-KanMX6 was constructed by C. Alberti-Segui, but no map or sequence was provided. We reconstructed the map based on information gathered from Alberti-Segui et al., 2001 and Alberti-Segui, Thesis, 2001. pAG4401 is part of the pAG collection created by C. Mohr (C. Mohr, Thesis, 1995; Dietrich et al., 2004). The map is shown to illustrate the construction of pAGPH002 and pAGPH003 (see Appendix 2.2).
Appendix 2.2.
Appendix 2.3. pAGSWE1-HA-NAT1 was created by A. Gladfelter.
Appendix 2.4. pRS416CDC28GEN3 was constructed by A. Gladfelter and is shown here to illustrate the methods used to distinguish mutant from reference strains (see Appendix 1.7).
Appendix 3.1. Alignment of AgCdc28p and ScCdc28p (1/1)
Appendix 3.2. Alignment of AgSwe1p and ScSwe1p (1/2)
Appendix 3.5.  
Alignment of AgHsl1p and ScHsl1p

(1/2)
Appendix 3.6.
Alignment of AgHsl7p and ScHsl7p

(1/2)
Appendix 3.6. Alignment of AgHsl7p and ScHsl7p

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Appendix 3.7.
Alignment of AgCdc3p and ScCdc3p

(1/1)
Appendix 3.8. Alignment of AgCdc10p and ScCdc10p
Appendix 3.9. Alignment of AgCdc11Ap, AgCdc11Bp and ScCdc11p (1/1)
Appendix 3.10.

Alignment of AgCdc12p and ScCdc12p

(1/1)
Appendix 3.11. Alignment of AgSep7p and ScSep7p (1/1)


Boudier, K. (2005). The role of AgRax1p, AgRax2p, AgBud7p and AgBud10p in mycelial development of the filamentous fungus *Ashbya gossypii*. *PhD thesis*, Biozentrum, Universität Basel, Switzerland.

Brachat, S. (2003). Exploring the potential of complete sequence information and synteny: Comparative genome annotation and analysis of *Ashbya gossypii* and *Saccharomyces cerevisiae*. *PhD thesis*, Biozentrum, Universität Basel, Switzerland.


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None of this would have possible without the unwavering support and love of my parents, Madeleine and Fritz Helfer. Especially, I would like to thank my dear Katrin for sweetening my life since the last year of my thesis work, for her unfailing support and patience, for buoying me up in desperate moments, and most of all: for her love.
Curriculum vitae

Hanspeter Helfer

Date of birth: 11.10.1976
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Nationality: Swiss

Education:

Primary school: 1983 – 1989 Primarschule Selzach, Switzerland
Grammar school: 1992 – 1997 Kantonsschule Solothurn, Switzerland
University: 1997 – 2002 Diploma in Biology II (Molecular Biology), Universität Basel, Switzerland
Diploma Thesis: 2000 – 2002 In the lab of Prof. P. Philippsen, Biozentrum, Basel, Switzerland
  “Effects of myosin gene deletions on growth and development of the filamentous fungus Ashbya gossypii”
Ph.D.: 2002 – 2006 In the lab of Prof. P. Philippsen, Biozentrum, Basel, Switzerland
  “New aspects of septin assembly and cell cycle control in multinucleated A. gossypii”

Publications

H Helfer and AS Gladfelter. AgSwe1p regulates mitosis in response to morphogenesis and nutrients in multinucleated A. gossypii cells. MBC, selected for InCytes, Epub ahead of print August 9, 2006.

Meetings and Seminars

**European Conference on Fungal Genetics, Pisa, Italy, April 2002.** Poster presentation: Effects of myosin gene deletions on growth and development of the filamentous fungus *Ashbya gossypii.*

**Swiss Yeast Meeting, Zürich, Switzerland. September 2002.** Poster presentation: Effects of myosin gene deletions on growth and development of the filamentous fungus *Ashbya gossypii.*

**European Conference on Fungal Genetics, Copenhagen, Denmark, April 2004.** Oral presentation: In search of a link between morphogenesis and nuclear division in the filamentous fungus *Ashbya gossypii.*

**Fungal Genetics Conference, Asilomar, CA, USA, March 2005.** Poster presentation: Spatial and nutritional control of mitosis in *Ashyba gossypii.*
Erklärung

Ich erkläre, dass ich die Dissertation

“New aspects of septin assembly and cell cycle control in multinucleated A. gossypii”
nur mit der darin angegebenen Hilfe verfasst und bei keiner anderen Fakultät
ingereicht habe.

Basel, Mai 2006

Hanspeter Helfer