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Cytoskeleton, Polarized Growth, and the Cell Cycle in Aspergillus nidulans

Reinhard Fischer, Norio Takeshita, and John Doonan

CONTENTS

15.1 Introduction

Microtubules (MTs), filamentous actin (F-actin), and their associated motor proteins, kinesin, dynein, and myosin, play important roles in all eukaryotes providing cells with a dynamic structural framework called the cytoskeleton. The cytoskeleton plays crucial roles in many processes that require reorganization of the

cytoplasm, such as growth, nuclear division, and cell division. In this chapter, we review the organization of the cytoskeleton in filamentous fungi, its role in polarized growth, mitosis and cell division. We focus on *Aspergillus nidulans* because work on this species has provided major insights in this area, most of which pertains to eukaryotes generally, but other fungal systems are mentioned and compared throughout the chapter.

Genetic, biochemical, and cell biological approaches in *A. nidulans* and other fungi have provided many important insights into MT functions over the years and continue to lead to new views of many MT-related processes. For example, there is increasing evidence that MT cables, as visualized by immunostaining or GFP-tubulin fusion proteins, consist of several MTs and their dynamics differ in fast-growing hyphal tips as compared with young germlings. Whereas the spindle-pole bodies were considered as the only, or the main, MT organizing centers (MTOCs) in filamentous fungi, additional MTOCs lying outside the nuclei are contributing to the generation of the complex MT array. In addition to new insights into the MT network and its dynamics, the roles of several kinesins have been elucidated recently and their interplay with dynein investigated. Furthermore, it has become clear that MT functions are interwoven with those of the actin cytoskeleton and that three main structures are required at the tip for polarized growth, the Spitzenkörper (vesicle supply centre), the polarisome, and cell end (tip) markers at the cortex.

Another important function for MTs is in mitosis. The spindle derives its structure from a highly organized set of MTs that is generated between two MTOCs, the spindle-pole bodies, which are embedded in the nuclear envelope. At the beginning of mitosis, the spindle-pole bodies are activated by a regulatory kinase network that allows the innermost face of the spindle pole body to act as a MTOC and kinesin proteins to provide the motive power to drive pole separation, producing a bipolar scaffold on which the chromosomes are separated.

For the purpose of the studies described here, hyphal growth starts with the germination of a conidiospore, a uninucleate haploid asexual spore produced at conidiophores. This dormant cell has a thick, resistant and highly pigmented wall that is hydrophobic and adapted to spreading across liquid surfaces. On landing in a suitable environment, the cells take up water, grow, and enter their first cell cycle (see also Fig. 15.4). The first hyphal nuclear division cycle takes about 75–120 minutes, depending on growth conditions. The nuclear division cycle can be considered as four sequential phases, Gap1 (G1), S-phase when the genomic DNA is replicated, Gap2 (G2) and mitosis when the replicated DNA is separated on the mitotic spindle. The dormant spore is arrested in G1 with a highly condensed nucleus and no detectable MTs or actin filaments, but after 4–5 hours on a suitable media, will swell to several times its original size and enter S-phase, replicating its DNA. During this time, cytoskeletal elements appear and actin accumulates at the incipient point of growth. Mitosis is estimated to last 5 minutes at 37°C; G2, 30 minutes; S-phase, 25 minutes; and G1, 15 minutes (Bergen and Morris, 1983; Bergen et al., 1984). Under different growth conditions the duration of G1 and G2 phases vary but the length of mitosis and S-phase remain constant (Bergen and Morris, 1983). In this chapter, we focus mostly on mitosis and controls associated with entry into mitosis. Mitosis is a critical part of the cell cycle and involves the dramatic and highly coordinated rearrangement and separation of nuclear components. Sister chromatid separation on a spindle of MTs, the central and essential feature of nuclear division, has many common features across all eukaryotes and studies in *A. nidulans* have revealed many useful insights into the underlying mechanisms.

15.2 The Microtubule Cytoskeleton

Microtubules are hollow tubes composed of 13 protofilaments, each of which is made up with the heterodimer αβ-tubulin, as the building block. MTs have an inherent instability but under suitable conditions can continuously elongate at their plus end, where αβ-tubulin dimers are added. One parameter that determines the elongation rate is the concentration of tubulin dimers in the cell. Both tubulin subunits contain a bound GTP. The nucleotide-binding pocket on α-tubulin is located at the interface between the $\widetilde{\alpha}$ and β-tubulin subunits and thus, this GTP is rather stable. On the other hand, GTP in the β-tubulin subunit is exposed and easily undergoes hydrolysis. Once β-tubulin contains GDP, further assembly is blocked and the MT is prone to catastrophic disassembly (Nogales and Wang, 2006).

15.2.1 Fungicide-Resistance Genes Identify Tubulins in *Aspergillus nidulans*

Mutations in the $\widetilde{\alpha}$ and β-tubulins were amongst the first cell cycle mutations to be characterized at the molecular level in *A. nidulans*. Screens for fungicide resistant mutants (Davidse and Flach, 1977, 1978) produced a number of strains resistant to growth on benomyl, an antimicrotubule drug. Strains resistant and sensitive to benomyl were shown to produce altered tubulin proteins (Gambino et al., 1984) that respectively either increased or decreased the stability of MTs. In both cases the cells arrested in mitosis, demonstrating that MTs were essential for mitosis. While the mitotic block caused by fragile MTs was not surprising, the mitotic block in *benA33* strains where MTs are unusually stable (Jung et al., 1998; Oakley and Morris, 1981) indicated the importance of MT turnover for mitotic progression. Strains with hyperstable MTs arrest with persistent spindles. The mitotic spindle, therefore, was shown to be a highly dynamic structure, not just a passive scaffold on which the chromosomes were separated. Subsequent work has shown that the organization of the spindle is actively monitored by checkpoint mechanisms that are intimately involved in regulating all stages of mitosis.

The *ben*A gene encodes two of the three β-tubulin isotypes (Sheir-Neiss et al., 1976; Sheir-Neiss et al., 1978). The other β-tubulin gene (*tubC*) plays a specialized but nonessential role in conidiation (May et al., 1985; Weatherbee et al., 1985). *A. nidulans* has two $\widetilde{\alpha}$-tubulin genes, *tubA* and *tubB*. Mutations in *tubA* were identified as suppressors of *benA*-mediated benomyl resistance (Oakley et al., 1987). Molecular disruption of the *tubA* gene leads to a mitotic block in vegetative cells (Doshi et al., 1991), while disruption of the other $\widetilde{\alpha}$ tubulin gene, *tubB*, leads to a block in meiosis (Kirk and Morris, 1991). *tubA* encodes the major vegetative α-tubulin protein while *tubB* is highly expressed during sexual development, so the most likely reason for the differences in phenotype is differential expression, rather than any major functional difference (Kirk and Morris, 1993).

Suppressor analysis of the *benA33* mutation uncovered a new member of the tubulin superfamily, *mipA* or γ-tubulin (Weil et al., 1986), which has a crucial role in MT organization and mitosis. Biochemical analysis of MTs, from *A. nidulans* (Weatherbee and Morris, 1984) as well as a variety of other sources, established long ago that the basic backbone consisted of equimolar amounts of α- and β-tubulin molecules, but failed to detect this novel and crucial member of the family. Until the recent advent of highly sensitive mass-spectroscopy-based methods of protein identification, biochemical approaches were simply not sensitive enough to routinely identify unsuspected minor components in such preparations and so important regulatory proteins such as γ-tubulin were not found. γ-tubulin, clearly related to both α-tubulin and β-tubulin, was sufficiently distinct from both to define a completely new class of tubulin (Oakley and Oakley, 1989) that has since been shown to be crucially important for MT organizing centers (MTOCs) in other eukaryotes (Horio et al., 1991; Joshi et al., 1992; Liang et al., 1996; Martin et al., 1997; Stearns et al., 1991). γ-tubulin is located at the spindle poles, where it is necessary for normal MT assembly during both interphase and mitosis (Oakley et al., 1990). Its SPB location, the phenotype of cells lacking it, and the genetic evidence that *mip*A interacts with β- and not α-tubulin led to a now widely accepted model whereby γ-tubulin determines both the location and polarity of MT initiation (Oakley, 1992). γ-tubulin forms the basis of a high molecular weight complex known as the γ-tubulin ring complex (γTuRC) that provides a template for MT assembly. Some ring complexes are embedded in structures such as the spindle pole body, but others are more dispersed (see later).

γ-tubulin may also have a checkpoint function. Cells with a mutant allele of γ-tubulin were originally reported to have a similar mitotic index to that of freely cycling wild type cells, suggesting that they cannot monitor successful completion of mitosis. *mip*AD159, another allele, allows spindles to form, but anaphase A is delayed, and late mitotic events are defective (Prigozhina et al., 2004). However, careful reexamination of γ-tubulin deletion strains indicate that nuclei arrest with condensed chromatin for about one cell cycle (Martin et al., 1997). Although spindle assembly is completely abrogated, other aspects of mitotic entry, such as SPB phosphorylation and chromatin condensation occur normally. Interestingly, the authors report that γ-tubulin is not required for cytoplasmic MT assembly, although these are abnormal.

Coordination of the complex series of events that occur during mitosis involves checkpoint controls that monitor spindle function. Thus, defects in the spindle may lead to prolonged chromatin condensation because the checkpoint pathway can sense that mitosis is incomplete and prevents a return to interphase. Mutation screens based on this logic identified the BUB genes, originally in the budding yeast, that are

required to monitor MT function (Hoyt et al., 1991; Li and Murray, 1991). These mutants, which are supersensitive to antiMT drugs, fail to arrest if progress through mitosis is delayed. Ascertaining that mitosis has been correctly and completely executed is, therefore, a critical checkpoint in the cell cycle. Under normal circumstances antiMT drugs block the cell cycle at M, probably because a crucial checkpoint that monitors the completion of mitotic events has not been satisfied. Similar genes have been found in *A. nidulans*, as the result of a screen for synthetic lethal mutants aimed at understanding the function of cytoplasmic dynein (Efimov and Morris, 1998). Cytoplasmic dynein is a MT motor protein involved in vesicle transport, mitosis, nuclear migration, and spindle orientation, and dynein mutations impair nuclear migration. Synthetic lethal mutations that significantly reduced growth in the absence of dynein mapped to nine different genes. Mutations in *sldA* and *sldB* also confer hypersensitivity to the MT-destabilizing drug benomyl and are in genes homologous to the checkpoint genes *BUB1* and *BUB3*. *sld*A and B mutations are also synthetically lethal when combined with mutations in the *bimC* kinesin (see later).

15.2.2 Organization of the Interphase Microtubule Cytoskeleton

Microtubules are visible in fixed cells by immunolocalization light microscopy (Bourett et al., 1998; Czymmek et al., 1996; Fischer and Timberlake, 1995) or by electron microscopy (Jung et al., 1998) but these methods do not reveal the highly dynamic MT behaviors that occur in living cells. *In vitro* studies, using mammalian brain tubulin, have shown that MT behavior is complex and their organization can be modified by several mechanisms. These mechanisms include treadmilling, where subunits tend to fall off the minus end and are added to the plus end. However, many MTs have their minus ends capped by virtue of having them embedded in a MTOC, in which case a process called dynamic instability may be more important. Dynamic instability describes the process by which a plus end can alternate between growth and disassembly (Nogales and Wang, 2006). MTs can also interact with each other due to the action of MT-associated proteins that can either crosslink different MTs, or facilitate sliding of one MT relative to another (MacRae, 1992).

Direct observation of MT behavior became possible after the discovery of the green fluorescent protein, which was fused to tubulin and expressed in cells. In *S. cerevisiae* interphase cells, short MTs are attached to nuclei and their growth toward the cortex and subsequent shrinkage causes short-distance movement of the nuclei. The situation changes once the yeast cell enters the division cycle. The nuclear spindle pole body divides, and as the two daughter organelles move to opposite sides of the nucleus, they nucleate the spindle MTs. The spindle- pole bodies span the nuclear envelope and, from their cytoplasmic faces, also nucleate cytoplasmic MTs that in turn mediate MT-cortex interactions (Hoepfner et al., 2000). In *S. pombe,* interphase cells contain several cytoplasmic MTs, which span the entire cell. Because they serve as tracks to deliver so called cell-end markers, these MTs determine growth directionality in this yeast (Tran et al., 2001). Q1 Q1

In filamentous fungi, GFP-tagged MTs were first studied in *A. nidulans* in X. Xiang's laboratory (Bethesda, USA). MTs are quite inflexible structures and their orientation probably mainly depends on the shape of the cell. Hence, the bundles of MTs are mostly aligned parallel to the growth axis and their number ranges from 3 to 6. *A. nidulans* MTs extend with a speed of about 14 μm per min, reach the cortex, pause for some time and undergo a catastrophic event. Subsequently, MTs shrink with a speed of about 30 μm per min and they may either depolymerize all the way to the MTOC, or else rescue occurs and they may recommence elongation (Han et al., 2001). Slightly different values were recently obtained in the group of B. Heath (Sampson and Heath, 2005). They also observed that short MT fragments were able to slide toward the hyphal tip. In *N. crassa,* the MT network was first visualized by N. Reads group in Edinburgh (Scotland) and has been analyzed recently in more detail (Freitag et al., 2004; Mouriño-Pérez et al., 2006). From observations of the MT cytoskeleton, it is obvious that the organization is quite different in these two filamentous fungi. In *N. crassa* the MT cytoskeleton is far more complex than in *A. nidulans* and the number of nuclei in one compartment is much higher in *N. crassa* than in *A. nidulans* (Freitag et al., 2004; Suelmann et al., 1997). Another big difference is the regulation of mitosis. Whereas nuclear division is synchronized in *A. nidulans,* it is not, in *N. crassa* (Freitag et al., 2004; Suelmann et al., 1997), a difference that probably contributed to the differential use of these two models in the genetic dissection of cellular processes. Q1

Real-time studies of MT organization and dynamics by immunofluorescence are impossible, but fluorescently labeled tubulin has recently allowed observations in living cells (Czymmek et al., 2005; Ding et al., 1998; Fischer and Timberlake, 1995; Freitag et al., 2004; Han et al., 2001). The filamentous structures observed using both immunostaining and GFP-labeled tubulin, consist of several individual MTs with mixed orientation. There is increasing evidence for this organization coming from studies with *S. pombe* where it was recently shown that the orientation of neighboring MTs can be opposite within the one bundle. Moreover, a kinesin-like motor protein in combination with dynein is required for sliding of individual MTs within a bundle and for maintenance of MT polarity (Carazo-Salas et al., 2005). This suggests a mechanism whereby MT bundles can quickly increase or decrease in length. In *A. nidulans,* Konzack et al. reported that fluorescence intensity of a MT varies dynamically and that the regions with low intensity can recover brightness after some time. Similarly, after localized bleaching of a given MT, brightness returns quickly (Veith et al., 2005), indicating active turnover of tubulin subunits within the bundle. In addition, thin MT filaments occasionally detach from a MT for some time before they merge again to form a thick MT (Veith and Fischer, unpublished results). These observations are in agreement with a model that MT filaments consist of a bundle and individual MTs within a bundle undergo individual behavior and dynamics.

15.3 Origin of Microtubules

Microtubules cannot efficiently assemble *de novo* in a eukaryotic cell and require MT organizing centers (MTOC), of which γ-tubulin is a characteristic and necessary component (see earlier). In higher eukaryotes γ-tubulin forms a 2.2 MDa ring complex, the γTuRC, consisting of 12–14 (different numbers exist in the literature) γ-tubulin subunits associated with other proteins, (Aldaz et al., 2005). It has been known for a long time that fungal spindle-pole bodies (SPB) are very active MTOCs (Jaspersen and Winey, 2004). The SPB is embedded into the nuclear envelope, divides prior to mitosis and, by definition, localizes at the poles of the mitotic spindle. SPBs consist, in *S. cerevisiae,* of an inner and an outer plaque and they are able to polymerize MTs on both sides of the nuclear envelope. The outer MTs formed during mitosis are called astral MTs, but the interphase SPBs are also active MTOCs both in *S. cerevisiae* and in filamentous fungi (Heath, 1981). The protein composition of the *S. cerevisiae* SPB has been defined by John Kilmartin's lab (Adams and Kilmartin, 1999) using Mass Spec-based identification of peptide fragments from highly purified SPBs. The availability of complete annotated genome sequences from several species, combined with the increasing sensitivity of MS peptide identification, now makes it possible to undertake similar experiments in filamentous fungi.

It seems that the SPBs are the only places from which the yeast *S. cerevisiae* polymerizes MTs (see accompanying movies in Hoepfner et al., 2000). However, cytoplasmic MTs only have a minor and nonessential role in *S. cerevisiae*, that of positioning of the nucleus prior to mitosis (Maekawa and Schiebel, 2004). The cytoplasmic MT array is not very pronounced and usually limited to a few MTs growing out of the SPB into the cytoplasm. In contrast, filamentous fungi employ MTs for their fast, polarized growth during interphase (Horio and Oakley, 2005; Riquelme et al., 2003). Nevertheless, it was assumed for a long time that SPBs are the only place for MT initiation (Czymmek et al., 2005; Oakley, 2004; Sampson and Heath, 2005). This assumption was based on the finding that the intracellular αβ-tubulin pool is used for the assembly of spindle MTs as well as for cytoplasmic MTs. Indeed, cytoplasmic MTs are generally disassembled prior to mitosis and regenerate thereafter (Ovechkina et al., 2003; Sampson and Heath, 2005). In order to determine the origin of new MTs, regrowth of MTs was observed in *S. pombe* after depolymerization of MTs by drugs (Mata and Nurse, 1997). These studies revealed that MTs are generated not only from the SPB but also from other MTOCs around the nucleus and in the cytoplasm. During cell division an equatorial MTOC becomes very important (EMTOC) (Hagan, 1998; Sawin et al., 2004; Venkatram et al., 2005). The origin of MTs from the cell centre leads to an orientation with their plus ends toward the growing ends.

Recently, another tool was used to determine the origin of MTs. Using MT plus-end localizing proteins, such as homologues of the mammalian EB1, MT initiation was analyzed in the plant pathogenic basidiomycete *Ustilago maydis.* It was found that MT nucleation occurs at three places, at dispersed

cytoplasmic sites, at a polar MTOC and at the SPB (Straube et al., 2003). Whether MTOCs exist near the apical dome of other tip growing cells is a matter of debate as MTOCs have been observed within the apical dome of plant cells such as moss protonemata (Doonan et al., 1985).

In filamentous fungi, our knowledge of MT organization is restricted to a few species, such as the chytridiomycete *Allomyces macrogynus,* the basidiomycete *U. maydis,* and the ascomycete, *A. nidulans,* which is one of the best-studied examples. Whereas Sampson and Heath (2005) reported that MTs emanate only from SPBs, Konzack et al. (2005) demonstrated that additional MTOCs also exist. This discrepancy may be due to different methods used. In the first study, the authors observed that GFP labeled MTs and the location of nuclei was determined by the absence of cytoplasmic fluorescence. The authors of the second study used simultaneous labeling of nuclei with a red fluorescent protein and GFP labeled tubulin. In addition, a plus-end tracking protein, KipA, was used to determine the origin of MTs. MTOCs were found at the SPBs but also in the cytoplasm and at septa of *A. nidulans* (Fig. 15.1). This model recently received further support from the characterization of a novel MTOC-associated protein,

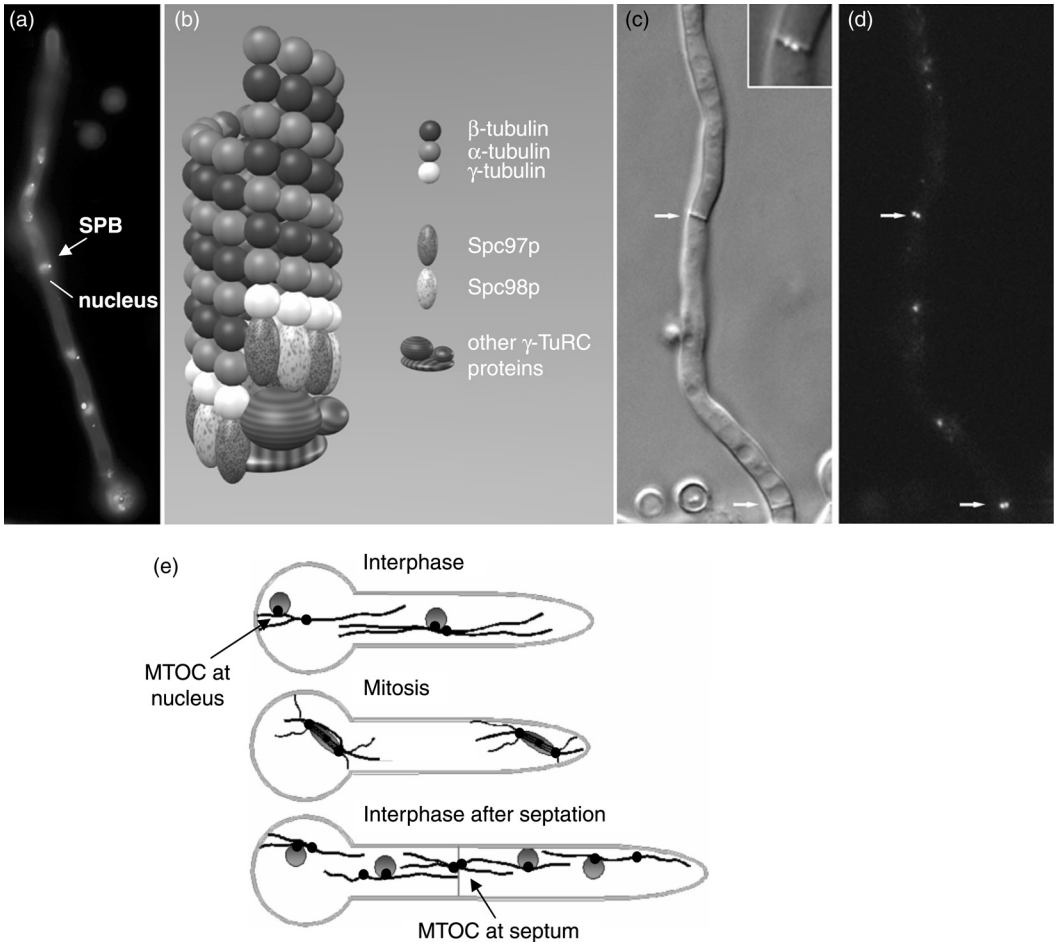

FIGURE 15.1 MTOCs in *A. nidulans.* (a) Hypha with DAPI-stained nuclei and GFP-labeled spindle-pole body (SPB) associated ApsB. Nuclei are evenly spaced and at each nucleus a SPB is visible. (b) Scheme of an MTOC with γ-tubulin and other proteins described in *S. cerevisiae*. (Adapted from Oakley, *Trends Cell Biol*, 10, 2000; Pereira and Schiebel, *J Cell Sci*, 110, 1997.) (c, d) MTOCs visualized by GFP-ApsB fusion, at septa. Left, phase contrast; right, same hypha under fluorescent conditions. Inset in (c) enlargement of the septum and overlay of phase contrast and fluorescent image. (e) MTOCs are found at the nuclei, in the cytoplasm and at septa. (From Konzack et al., *Mol Biol Cell*, 16, 2005. With permission.)

ApsB (Veith et al., 2005). Here, the authors demonstrated that MTOCs at septa are important for the production of the interphase cytoplasmic MT array (Fig. 15.1). These findings are in agreement with the results obtained in *S. pombe* and *U. maydis*.

It is still an open question whether there are MTOCs at hyphal tips of filamentous fungi. Whereas γ-tubulin can be visualized at tips of *A. macrogynus* hyphae and thus MTs polymerize from the tip to the back (McDaniel and Roberson, 1998), γ-tubulin has not yet been detected at the tip in *A. nidulans,* but using the kinesin motor KipA, Konzack et al. (2005) found MTs can also polymerize from the tip. However, it has to be considered that a MT occasionally might not depolymerize upon contact with the cortex but could bend along the cortex toward the rear of the hypha. If this MT would continue growth, it could explain the observed comets from the tip to the back of the hypha. In *N. crassa* the situation appears to be far more complicated because of the higher number of MTs and nuclei (Freitag et al., 2004; Mouriño-Pérez et al., 2006) and detailed studies of MT origin have yet to be performed.

15.3.1 The Microtubule Plus End

It is well accepted that the plus end consists of a large protein complex that is involved in the regulation of MT dynamics as well as in the regulation of interactions with cortical actin, membrane proteins, or proteins associated with the kinetochore of chromosomes (Akhmanova and Hoogenraad, 2005; Hestermann et al., 2002; Schuyler and Pellman, 2001b). Given the diversity of interacting partners, it is obvious that the protein composition of the plus end complex may vary depending on the function of the MT and is likely to be a highly controlled and organized structure. There are three different ways that proteins can reach the MT plus end and remain associated with it while the MT is growing (Al-Bassam et al., 2006; Howard and Hyman, 2003).

In fungi, the MT plus ends have been best characterized in *S. cerevisiae* and S. *pombe*. MT-cortex interactions play important roles for the positioning of the mitotic spindle and nuclear migration in *S. cerevisiae* (Schuyler and Pellman, 2001a). Dynein is a prominent example of a MT plus-end associated protein (Fig. 15.2) that localizes to the MT tip and hitchhikes with the growing filament to the cell periphery. Once at the cortex, dynein is activated and pulls the attached MT toward the cortex. This leads to translocation of the nucleus (Maekawa et al., 2003; Maekawa and Schiebel, 2004; Schuyler and Pellman, 2001a; Sheeman et al., 2003). The kinesin motor protein, Kip2, appears to be responsible for the plus end localization of several proteins, for example, the CLIP170-like protein Bik1 (Carvalho et al., 2004). As in *S. cerevisiae*, the CLIP170-like protein of *S. pombe*, Tip1, also localized to MT plus ends. The motor responsible for this localization is Tea2 (Busch et al., 2004). However, MTs are not so important for polarized growth in yeasts in comparison to filamentous fungi. However, only some components that localize at MT plus ends have been found in filamentous fungi, but amongst these are subunits of the dynein motor complex and recently the Stu2 (Alp14)-homologue AlpA (Enke et al., 2007; Zhang et al., 2002). Interestingly, conventional kinesin, KinA, is required for dynein MT tip localization (Zhang et al., 2003) (Fig. 15.2). The CLIP170-like protein, ClipA, in *A. nidulans* also accumulated at MT plus ends and its localization is also dependent on the Tea2/Kip2 homologue KipA (Efimov et al., 2006).

The role that plus-end localized proteins play for polarized growth remains an open question. As mentioned earlier, MT-cortical interactions are necessary for dynein-dependent nuclear positioning prior to mitosis in *S. cerevisiae* (Carminati and Stearns, 1997). In *A. nidulans* dynein is also required for nuclear positioning and migration and recently Veith et al. showed that the interaction of MT-plus ends with the cortex contribute to the dynamics of mitotic spindles (Veith et al., 2005; Xiang et al., 1994; Xiang and Fischer, 2004). Whether interphase nuclei are moved as a result of similar MT-cortex interactions is not yet clear.

Whereas the MT-plus end protein complex is widely accepted as having a role in force generation required to translocate organelles, a role in polarized growth is less obvious. Some new ideas came from observations of MTs in growing tips of *A. nidulans*. Konzack et al. (2005) described how MTs merge into one point in the apex. Given that vesicles constantly travel toward the vesicle supply centre, the position of MT ends determines the vesicle supply centre location. In the *kipA* (*tea2/kip2*) mutant where MTs did

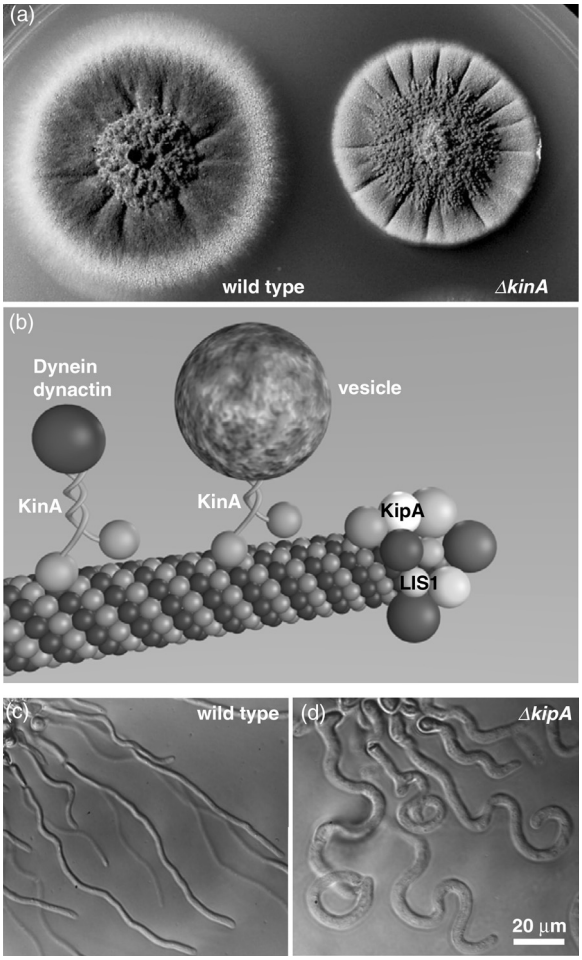

FIGURE 15.2 The role of conventional KinA and Kip2 family kinesin KipA. (a) Comparison of a wild type with a conventional kinesin deletion mutant (from Requena et al., *Mol Microbiol,* 42, 2001)). (b) Scheme of a MT with the MT plus-end complex. This protein complex consists of several proteins, e.g., KipA or LIS1. Conventional kinesin transports vesicles and components of the plus-end complex, for instance, dynein (from Zhang et al., *Mol Biol Cell,* 14, 2003). A direct interaction between KinA and dynein or dynactin has not yet been verified. Modified after (from Hestermann et al., *J Muscle Res Cell Motil,* 23, 2002). (c, d) When KipA, which is suggested to be involved in the delivery of cell end markers, is missing, hyphae loose directionality. (From Konzack, *Mol Biol Cell*, 16, 2005. With permission.)

not merge into a single point, the hyphae grew in meandering curves rather than straight lines. This was explained by the lack of cell-end markers, which mediate cortical contact, and are normally transported by KipA (see later). There is good evidence for such a situation in *S. pombe,* where it was shown that the cortex protein Tea1 is transported by Tea2 (Browning et al., 2003; Martin and Chang, 2003; Sawin and Snaith, 2004) (Fig. 15.3). If either of the two genes is deleted, *S. pombe* cells appear curved or T-shaped (Browning et al., 2000; Snell and Nurse, 1994). Hence, Tea1 and other proteins were named cell polarity determinants or cell end marker proteins. However, to prove such a model in *A. nidulans*, cargoes of KipA have to be identified and characterized. Another crucial piece in the puzzle is the identification of cortex proteins. Whereas cortical contacts of MTs involved in nuclear migration require the cortical protein, ApsA, in *A. nidulans* (Num1 in *S. cerevisiae*) (Veith et al., 2005) this interaction appears not to be necessary for polarized growth (unpublished results). In *S. pombe*, the Mod5 protein acts as a membrane anchor for the polarized growth machinery (Snaith and Sawin, 2003). However, in filamentous fungi, a protein with significant sequence similarity has not yet been identified.

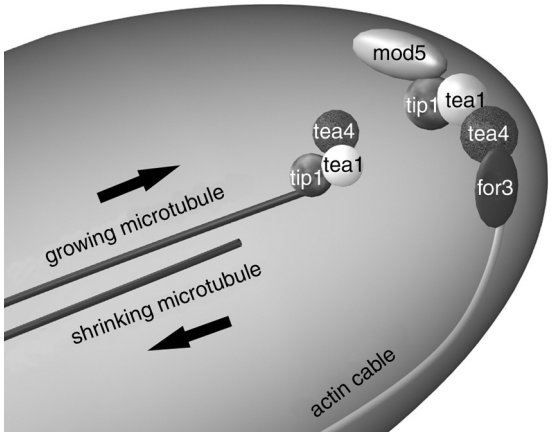

FIGURE 15.3 Model of polarized growth in S. pombe. (Reprinted from Martin and Chang, *Curr Biol,* 16, 2003. With permission from Elsevier.)

15.3.2 The MT Lattice

MT function and dynamics are not only determined by the plus and minus ends, but also by the filament lattice, which in higher eukaryotes can be decorated with a number of different microtubule-associated proteins (MAPs), which in turn may control the activity of associated motor proteins (Baas et al., 1994; Baas and Qiang, 2005; Cassimeris and Spittle, 2001). Despite the abundance of those proteins in higher eukaryotes, it is not clear yet whether proteins like Tau exist in filamentous fungi. A very profitable approach involves the direct isolation of MAPs, based on their ability to bind to and copurify with MTs. Many of the classical mammalian MAPs were found due to their copurification with brain tubulin, which can be easily induced to assemble into MTs *in vitro*. Tubulin from other species does not so readily self-assemble but taxol can promote *in vitro* MT assembly, and has extended this approach to plants where MAPs are not readily identified due to structural divergence (Korolev et al., 2005). However, filamentous fungi are quite closely related to yeast and many other MAPs such as katanin and spastin that lead to MT severing can be recognized from conserved sequences in the *A. nidulans* and *Aspergillus. fumigatus* genomes (Konzack, unpublished results). Experimental data for the role of this class of MT-associated proteins are not yet available for filamentous fungi. Q2

15.3.3 MT-Dependent Motor Proteins

Microtubules and their dynamics are, in principle, able to create force and transport proteins, such as Tea1 in *S. pombe* (see earlier). However, at least two classes of motor proteins have evolved that mediate fast MT-dependent movement within the cell. These are the minus-end directed dynein and the plus-end directed kinesins (Fig. 15.2), although some kinesins can also move in a minus-end mode. Both motor classes are characterized by a motor domain in which ATP is hydrolyzed (Hirokawa, 1998). The location of the motor domain within the protein can be N- or C-terminal or even the middle region.

The mechanism by which chemical energy is converted into conformational changes and force generation is best understood in conventional kinesin. Interested readers should refer to several recent reviews (Adio et al., 2006; Schliwa and Woehlke, 2003; Woehlke and Schliwa, 2000; Yildiz and Selvin, 2005).

Whereas all fungi employ a single dynein for their transport processes, their genomes usually contain several kinesin-encoding genes. For instance, *A. nidulans* harbors 11 and *N. crassa* 10, different kinesins (Fuchs and Westermann, 2005; Rischitor et al., 2004). BimC was the first kinesin discovered in *A. nidulans* and defines the entire class of BimC-like kinesins (Enos and Morris, 1990). The gene was discovered in a screen for temperature-sensitive *A. nidulans* mutants with defects in mitosis (*bim* = block in mitosis).

BimC has a C-terminal motor, which forms a tetramer with two motor domains opposite to each other. Because every head domain can bind to a MT, this arrangement allows crosslinking of adjacent MTs and BimC provides the motive force to build the spindle. One of the BimC's essential functions is to separate the SPBs. Shortly after their activation as intranuclear MTOCs, BimC is required to move the SPBs to opposite sides of the nucleus as this step fails in a temperature-sensitive mutant, *bim*C3 (Enos and Morris, 1990). BimC is not required for spindle assembly *per se* as MT elongation is not impaired in *bim*C3 and a "unipolar" spindle is formed where MTs running from the spindle pole bodies do not overlap. BimC defects seem to lead to a mild checkpoint induction since *bim*C3 mutants only arrest transiently in mitosis with a temporally elevated mitotic index. The mutant cells do exit mitosis after some time, suggesting that the checkpoint becomes attenuated. Whereas BimC was discovered in a genetic screen (Morris, 1976), four other kinesins were characterized using reverse genetic approaches (Konzack et al., 2005; Prigozhina et al., 2001; Requena et al., 2001; Rischitor et al., 2004).

A second motor with functions in mitosis is the C-terminal kinesin-like protein KlpA with similarity to *S. cerevisiae* Kar3 (Prigozhina et al., 2001). The gene was isolated through a PCR approach and characterized subsequently. Deletion of *klpA* alone did not produce any severe phenotype but suppressed a *bimC* mutation (O'Connell et al., 1993) suggesting that these two motors act in opposing directions during the establishment of the spindle.

Another kinesin with a function in mitosis is the Kip3 family member, KipB, with a motor domain that is localized closer to the N-terminus. Gene deletion did not cause any defect in hyphal extension or organelle movement, but chromosome segregation was defective (Rischitor et al., 2004). This was surprising, because a similar motor in *S. cerevisiae*, Kip3, is involved in nuclear migration (Miller et al., 1998). However, the *A. nidulans* KipB results are in good agreement with results for the homologous proteins in *S. pombe*, Klp5 and Klp6 (West et al., 2002).

Two motors with N-terminal motor domains and pronounced roles in polarized growth are conventional kinesin, KinA, and the CENP-E family kinesin KipA. Deletion of *kinA* resulted in slower hyphal **Q3** growth, which is similar to effects in other fungi (Lehmler et al., 1997; Requena et al., 2001; Seiler et al., 1997; Wu et al., 1998b) (Fig. 15.2). It is generally accepted that this motor transports vesicles toward the extending tip and provides cell wall components to the growing tip (Seiler et al., 1999). In addition, KinA appears to be involved in other cellular processes related to polarized growth, namely mitochondrial and nuclear distribution. Whereas nuclear distribution was affected in *N. crassa* and *A. nidulans*, mitochondrial distribution was changed in *N. haematococca* (Wu et al., 1998b). This may be due to the fact that **Q4** mitochondrial movement depends on the actin cytoskeleton in *A. nidulans* (Suelmann and Fischer, 2000) and on the MT cytoskeleton in *N. crassa* (Fuchs et al., 2002; Fuchs and Westermann, 2005). Whether mitochondrial distribution is also altered in *N. crassa* conventional kinesin mutants has not yet been studied. The mechanism by which conventional kinesin may contribute to mitochondrial or nuclear distribution is not yet clear, but the effects may be indirect. KinA is required for transportation of dynein subunits to the plus end of MTs (Zhang et al., 2003) and dynein is a crucial motor for nuclear migration. Exclusion of dynein from the MT plus ends in cells lacking KinA could cause the observed nuclear clustering (Xiang et al., 1994). In addition, it has to be considered that conventional kinesin may well be involved in delivering other components of the MT plus end complex. Lack of KinA could thus influence the dynamics of MTs as well as their cortical interaction.

KipA of *A. nidulans* is similar to Tea2 in *S. pombe* and is characterized by an N-terminal motor domain (Konzack et al., 2005). Accumulation at the plus ends is dependent on an intrinsic motor activity because mutant proteins, in which a crucial residue for ATP hydrolysis was replaced, lost the ability to accumulate at MT tips, but decorated them evenly. These findings were in agreement with studies of Tea2 in *S. pombe* (Browning et al., 2003). Gene deletion caused a surprising phenotype in *A. nidulans* (Fig. 15.2). Delta *kipA* strains grew as well as wild-type strains but hyphal morphology and MT behavior was changed. In wild type, MTs form a focus at the apex, but are dispersed in the mutant. This suggested that the MT foci were important for controlling the direction of growth. MT foci would direct and deliver the vesicles accurately to one place, and the Spitzenkörper and hyphae would grow straight. If the MTs could not merge into one point, vesicle delivery would be less accurate with the result that new growth occurs in arbitrarily directions and leads to a twisted morphology. The KipA protein might transport proteins that are necessary for temporal anchorage of MT at the cortex at a specific point. An example for such proteins

in fission yeast are Tea1 and Tip1 (Browning et al., 2003; Busch et al., 2004). However, MT fixation at the cortex through Tea1 was not shown. Tea1 may be evolutionarily conserved among fungi, because a similar protein has been localized to the growing hyphal tip in *A. nidulans* (Takeshita, Konzack and Fischer, unpublished results).

Deletion of any kinesin motor (besides *bimC*) does not cause severe phenotypes. Interestingly, even a strain in which KinA, KipA, and KipB were deleted, was still viable although hyphal growth and development were quite severely affected (Konzack et al., 2005). This shows that kinesins can substitute for each other to some extent, and this was recently confirmed in the case of the Unc-104 homologues, Nkin-2 and Nkin-3, from *N. crassa*. Whereas Nkin-2 associates with mitochondria and connects mitochondria with MTs, Nkin-3 was found in the cytoplasm. Surprisingly, after depletion of Nkin-2, Nkin-3 was upregulated and also bound to mitochondria and MTs (Fuchs and Westermann, 2005). Homologues of these two motors also exist in *A. nidulans* and are currently being investigated (Zekert and Fischer, unpublished results). UncA plays an important role in hyphal tip extension, whereas UncB is likely to play a role in the nucleus and at septa (N. Zekert and R. Fischer, unpublished data).

As mentioned earlier, fungi usually contain only a single dynein protein, although in some basidiomycetes the heavy chain is encoded by two genes (Eshel et al., 1993; Martin et al., 2004; Straube et al., 2001; Xiang et al., 1994; Yamamoto and Hiraoka, 2003). Dynein has a crucial role in nuclear migration but is also implicated in vesicle transport (Seiler et al., 1999). Because dynein moves toward the MT minus end, it is difficult to imagine that it is directly involved in polarized growth, given that MTs are mainly oriented with their plus-ends to the membrane. Indeed, deletion of dynein does not cause an immediate block of hyphal extension and the impact on colony growth could partly be due to the lack of nuclei and other organelles that are translocated with the help of dynein (Xiang et al., 1994).

Besides the concerted action of the cytoskeleton and associated motor proteins to translocate organelles, cytoplasmic streaming has to be considered as another mechanism to push forward the cytoplasm and organelles. Mouriño et al. showed recently in *N. crassa* that the MT array was able to advance as a unit, as the hypha elongates. The basis for this bulk flow has not yet been defined (Mouriño-Pérez et al., 2006).

If MTs play a role in vesicle delivery to the growing hyphal tip, the question remains as to how the sites for cell extension are marked. First insights into this process came from studies in *S. pombe* with the definition of cell-end markers.

15.3.4 Cell-End Markers at the Cortex

As described earlier, one of the first proteins (Tea1) to label the growing end of a yeast cell was discovered in *S. pombe* during a genetic screen for polarity mutants (Mata and Nurse, 1997). It was shown recently that the main membrane anchor, which recruits proteins such as Tea1, is Mod5 (Browning et al., 2003; Snaith and Sawin, 2003). This protein is posttranslationally modified with a prenyl residue, conferring membrane association. The anchored Mod5 then recruits the formin protein, For3 (Bretscher, 2005; Martin et al., 2005; Martin and Chang, 2006) (Fig. 15.3). For 3 initiates the growth of actin filaments away from the growing tip that can be used as tracks for directing the vesicles necessary for cell extension.

Given that the growth machinery is largely conserved in filamentous fungi, and although a crucial component, Mod5, has not yet been identified in filamentous fungi, and it has to be considered that a **Q2** similar protein exists, the question remains as to what targets Mod5 or analogous proteins to the membrane near the hyphal tip rather than along the length of the cell. This points to a key function of the membrane itself, perhaps involving sterol-rich lipid rafts that may cause asymmetric distribution of membrane-associated proteins (Grossmann et al., 2006; Hancock, 2006). There is recent evidence that these membrane domains play a role in polarized growth of filamentous fungi (Martin and Konopka, 2004) and the laboratory of S. Harris showed that a ceramide synthase is important for hyphal morphogenesis (Li et al., 2006).

Because the installation of the growth machinery at a specific place determines growth directionality, one would expect that external signals influence the architecture of proteins. Indeed, recently a kinase with such a potential was described in *A. nidulans* (Li et al., 2006). The ATM kinase has a well-characterized role in DNA damage response (see section titled "Genome Surveillance") but Li et al. (2006) found that deletion also affects the establishment of polarized growth. The reason appeared to lie

in a disorganization of MTs in the apex similar to the defect in the kinesin mutant Δ*kipA* (Konzack et al., 2005). Whereas MTs form a focus within the apical dome of wild-type hyphae, they are dispersed in the *atmA* and the *kipA* mutants. In both cases the authors argue that MT-cortex interaction might be affected, but it is unclear if this is due to a direct effect of ATM kinase at the tip or an indirect effect through the DNA damage checkpoint pathway.

Two further candidates for regulation of tip growth are Pod6 and Cot1, described from *N. crassa*. Both proteins are distributed evenly along the hypha and their role in tip growth remains to be explained (Seiler et al., 2006). The BimG phosphatase, better known for its role in mitosis, is also found in the apical dome (see section titled "Anaphase") where it might control vescile recycling.

15.4 Cell-Cycle Controls

Cell growth and division require careful coordination of many processes to ensure that two healthy and viable daughter cells are formed. Visually, this is most dramatically illustrated by mitosis when many subcellular structures undergo dramatic and extensive reorganization (Fig. 15.4): cytoplasmic MTs begin to disassemble and disappear, being replaced by the mitotic spindle as the spindle pole is activated. Nuclear structure changes too, with the chromosomes becoming condensed in preparation for separation. In some groups, but not in the filamentous fungi, the nuclear envelope breaks down and the nucleolus also disperses coincident with cession of ribosome biosynthesis and reduced protein production. The consequences of mitosis are profound, leading to complete and irreversible separation of the genome into two daughter nuclei. Premature entry into mitosis is prevented by a system of "checkpoints" that ensures that the prospective mother cell is capable of producing two viable daughters—that DNA replication is complete, and the cell is big enough, to name but two important attributes that must be satisfied. Therefore, the decision to enter mitosis is one of the critical transitions in the cell cycle and is under strict control by a network of regulatory pathways known as checkpoints, because, if taken at the wrong time, this would be effective suicide. These checkpoint pathways link mitosis with other cell cycle events, such as

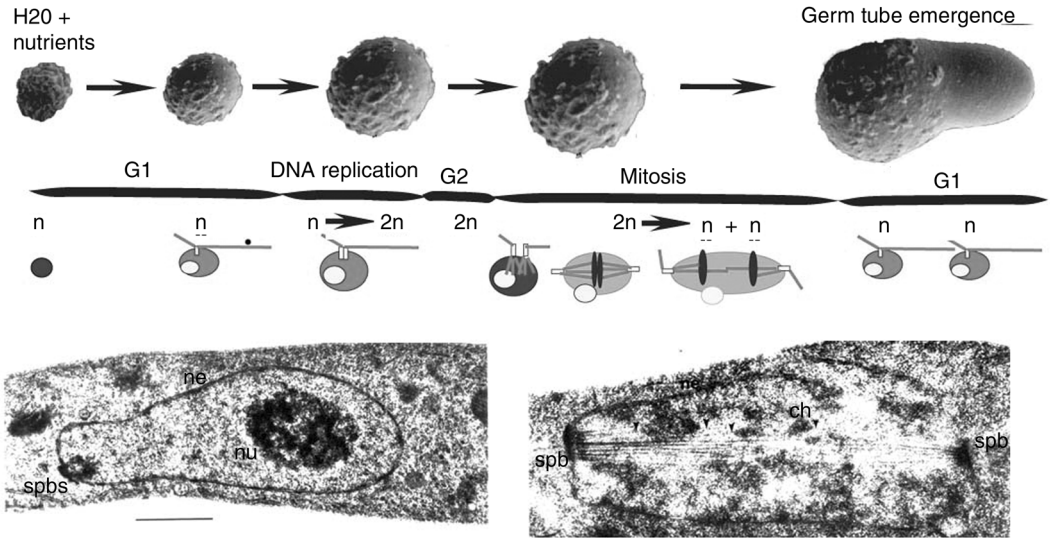

FIGURE 15.4 Hyphal germination and nuclear division cycle in *A. nidulans*. *Note*: Scanning electron micrographs of germinating spores are arranged above the corresponding cell-cycle phases (G1, DNA replication, G2 and Mitosis) and a schematic of the nuclear division cycle showing MTs (green) SPB (small empty rectangles), and DNA (blue where the state of DNA condensation is indicated by the intensity of blue). The lower images show transmission electromnmicrographs of interphase and mitotic nuclei (from Kerry O'Donnell, *J Cell Sci,* 99, 1991; modified from Doonan, *J Cell Sci*, 103, 1992) and various features are marked as follows: spb, spindle-pole body; ne, nuclear envelope; nu, nucleolus; ch, chromosome, arrowheads indicate bundles of spindle microtubules. **Q12**

FIGURE 15.5 Regulatory network controlling the mitosis in *A. nidulans*. *Note*: Arrows represent positive interactions or regulation; bars indicate negative regulation. Solid lines indicate genetic or protein-protein interaction evidence for the regulation. The nuclear schematic is as described in Figure 15.4 while cell cycle events are indicated inside the rectangles.

septation, DNA replication, and cell growth. Checkpoint pathways have been genetically dissected in several model organisms, including *A. nidulans*. Some of the important features of this network in *A. nidulans* are shown in Figure 15.5. **Q5**

15.4.1 Genetic Analysis of the Nuclear Division Cycle

Chromatin condensation during mitosis is particularly marked in *A. nidulans* hyphae and this provided the basis for a cytological screen for cell cycle mutations. These screens directly identified mutations with altered cell-cycle dynamics and have implicated at least thirty genes in various aspects of DNA replication and nuclear division. These include temperature-sensitive lethal mutant collections (Morris, 1976; Orr and Rosenberger, 1976a,b), aneuploid generating mutants (Upshall and Mortimore, 1984), and mutations sensitive to DNA damage (Käfer, 1986; Oza and Käfer, 1990; Shanfield and Käfer, 1969). Although not all of the corresponding genes have been isolated, those that have been cloned provided profound insights into the general mechanisms that regulate mitosis in eukaryotes.

Two of the key regulators, NimA kinase and $BimE^{APC/C}$ were identified using mutants from Ron Morris' Lab. Morris (1976) screened for mutants that were conditionally defective in nuclear division but could continue limited growth at restrictive temperature. Such hyphae contained fewer nuclei per unit length, suggesting that nuclear division was specifically impaired. The mutants were further classified on the basis of chromatin configuration (condensed and, therefore, mitotic, or noncondensed and, therefore, interphase) and presence or absence of spindles at restrictive temperature. Those mutants with interphase-like nuclei were given the acronym *nim*, *n*ever *i*n *m*itosis; those with condensed nuclei were called *bim* for *b*locked *i*n *m*itosis. To establish where the interphase mutations had their point of action relative to S-phase, the *nim* mutants were tested using a reciprocal shift method where the arrest point of a mutant is determined relative to the reversible S-phase arrest induced by hydroxyurea (HU), an inhibitor of DNA synthesis (Bergen et al., 1984). Surprisingly, no strains were identified as blocking in G1 although several mutations produced nuclei that could not be scored due to abnormal nuclei. In addition, three mutations

(in the *nimL*, *nimM* and *nimN* genes) were irreversible conditional lethals whose position of blockage could not, therefore, be determined. However, the latter three are all supersensitive to low doses of HU, implying that they may be involved in DNA metabolism (Doonan, unpublished data). Five other mutations, *sodB1, nimC3, nimG10, nimK14* and *nimQ20*, conditionally block in S-phase. Mutations in at least six genes conditionally prevent the transition from G2 to M. These include *nimA*, *nimB*, *nimE*, *nimT*, *HfaB,* and *HfaF* and, together with the genes required for mitotic progression, these genes have provided some unique insights into eukaryotic mitotic control.

15.4.2 Regulation of the G2/M Transition

A phosphorylation cascade, cumulating in the activation of the NimX cyclin-dependent protein kinase (cdk), plays a key role in regulating mitotic entry. This heterodimeric protein kinase is composed of two subunits, a catalytic kinase subunit encoded by *cdc2* gene in *S. pombe* (the *nimX* gene in *A. nidulans*) and a regulatory cyclin subunit encoded by cdc13 in *S. pombe* (Moreno et al., 1989) (*nimE* in *A. nidulans*). The kinase is kept in an inactive state by phosphorylation on tyr15 (Fleig and Gould, 1991). Activation as a mitosis-specific histone H1 kinase occurs by dephosphorylation, which is undertaken by a tyrosine-specific protein phosphatase (reviewed by Fleig and Gould, 1991), encoded by NimT in *A. nidulans* (Osmani et al., 1991b). In all of these features, the $NimX^{cdc2}$ kinase conforms to the typical eukaryotic mitotic cdk kinases. Mutations in the genes that encode the regulatory subunits of this kinase conditionally block the cell cycle in G2 (O'Connell et al., 1992). Mutant *nimT23* strains, therefore, arrest in G2 with phosphorylated and inactive p34cdc2 kinase.

Another distinct type of mitotic kinase, NimA, was discovered in *A. nidulans* and its analysis has revealed a novel parallel control pathway that acts both alongside and on the p34 kinase as a positive regulator of mitosis (Osmani et al., 1991b). At restrictive temperature, mutations in the *nimA* gene block in G2 (Bergen et al., 1984) and return of cells to the permissive temperature results in a synchronous entry into mitosis within a few minutes (Oakley and Morris, 1983). Activation of NimA and $NimX^{CDC2}$ kinases are, in part, independent of each other: NimA is fully active when $NimX^{CDC2}$ is inactivated by mutations in the *nimT* gene and $NimX^{CDK1}$ activity is very high in *nimA5* mutations (Osmani et al., 1991a). Expression of NimA in *A. nidulans* and other cell types can induce aspects of mitotic progression, especially chromatin condensation, and this does not require Cdc2 kinase activity (Lu and Hunter, 1995; O'Connell et al., 1994).

The parallel behavior of these two kinases seemed to be confirmed when NimX p34cdc2 kinase activity was examined in a *nimA5* mutant background. Despite the *nimA5* mutant being blocked in interphase at restrictive temperature, it had a fully activated p34 H1 kinase (Osmani et al., 1991b). Moreover, the NimX kinase was dephosphorylated on tyrosine 14, one of the final steps in its activation. Therefore, an active p34 kinase is insufficient to allow *A. nidulans* cells to enter mitosis. NimA kinase is fully active as a kinase in the *nimT23* mutant, which fails to dephosphorylate and activate $NimX^{CDC2}$.

The underlying mechanism by which mitotic entry is dependent on both NimA and $NimX^{CDC2}$ kinases involves the NimA-dependent nuclear import of $NimE^{cyclinB}$ (Wu et al., 1998a), and possibly other cell-cycle regulators (De Souza et al., 2000). Although NimX is dephosphorylated in *nim*A5 mutants, $NimE^{cyclinB}$ and $NimX^{CDC2}$ are retained in the cytoplasm. NimA, therefore, acts upstream of $NimX^{CDC2}$ by controlling the subcellular localization of the *nim*E encoded cyclin regulatory subunit and the cyclin-dependent kinase, $NimX^{CDC2}$.

To control the timed nuclear uptake of $NimE^{cyclinB}$ $NimX^{CDC2}$, NimA could either module nuclear pore function in a cell-cycle dependent manner or it could directly modify the CDK, but the available evidence supports the former mechanism. NimA interacts genetically with *sonA*, a homologue of the yeast nucleocytoplasmic transporter GLE2/RAE1, and interacts with SonB, a FG-repeat nucleoporin (De Souza et al., 2003). The *sonA1* mutation suppresses defective nuclear division and $NimE^{cyclinB}$ localization in *nimA1* cells without markedly increasing $NimX^{CDC2}$ or NimA activity, as measured in cell homogenates. NimA activation leads to partial disassembly of nuclear pores not only at mitosis, but also when ectopically expressed in S-phase cells. These results indicate that NimA promotes the nuclear localization of the $NimX^{CDC2}$/ $NimE^{cyclinB}$ complex, by modulating nuclear pore stability. The dual action of the two kinases may have evolved as a mechanism to facilitate the entry of cytoplasmic proteins into the nucleus in the

absence of mitotic nuclear envelope disassembly (De Souza et al., 2004). In organisms where the nuclear envelope does break down at mitosis, the other roles of NimA-related proteins may appear more prominent.

NimA also has additional roles and targets in *A. nidulans*. A major effect of ectopic NimA expression is chromosome condensation, due to the phosphorylation of histones by NimA. Prior to mitosis, NimA is uniformly distributed within the cell but, as the cell enters mitosis, it accumulates in regions of the nucleus that are undergoing chromatin condensation precisely coinciding with the phosphorylation of histone H3 on serine-10 (De Souza et al., 2000). Histone H3 is a substrate of NimA *in vitro* and its phosphorylation on serine-10 is crucial to condensation of the nucleosomes in many species. NimA is also localized to the spindle and spindle-pole bodies during later stages of mitosis, suggesting that other targets may be located on the spindle poles or microtubules. TinA, a protein that locates to the mitotic spindle poles and influences astral microtubules, interacts in yeast 2H with NimA, and deletion of *tinA* is synthetically lethal with *bimE*$^{APC/C}$ mutations (Osmani et al., 2003). TinA might provide a structural connection between NimA and other cell-cycle regulators that show spindle localization during part or all of mitosis. These include components of the anaphase-promoting complex (Mirabito and Morris, 1993) and BimGPP1 (Fox et al., 2002), which also genetically interact with *nimA* to control entry into mitosis. In fission yeast, Fin1 (the NimA homolog) promotes the association of polo kinase with the spindle pole body (Grallert and Hagan, 2002), although this does not appear to be the case in *A. nidulans* as POLO is found at the poles throughout the cell cycle (Bachewich et al., 2005). In animals, NimA-related kinases promote the assembly of the centrosome, an analogous structure to the spindle pole body. These data suggest an evolutionarily conserved role for NimA kinases in spindle-pole function (Hayward and Fry, 2006).

NimA activity is tightly controlled at a number of levels, leading to an increase in kinase activity during mitosis of approximately twenty-fold of that observed in S-phase (Osmani et al., 1991a). First, accumulation of *nimA* transcript increases to a maximum at mitosis and falls precipitously as the cells return to interphase (Osmani et al., 1987). This is an important regulatory mechanism as inappropriate production of *nimA* mRNA (from an inducible promoter) can drive cells into premature mitotic-like state, even in the presence of a HU block (Osmani et al., 1988). Moreover, overexpression of NimA prolongs mitosis leading to the formation of elongated spindles and condensed fragmented chromatin. This suggested that NIMA must be destroyed in order for cells to exit mitosis.

The accumulation of high levels of mutant NimA5 protein in double-mutant *nimA5 bimE7* due to a compromised APC function might explain the rather odd mitotic phenotype of these strains. After a short delay, the double mutant strain enters mitosis with aberrant spindles and nuclear membranes whereas a single *nimA5* mutant does not (Osmani et al., 1988). NimA protein is relatively stable in cells that have been arrested in mitosis and a destruction motif probably resides in the C-terminus of the protein as deletion leads to protein accumulation and delayed mitotic exit (Pu and Osmani, 1995b). NimA levels during interphase also respond to checkpoint mechanisms that monitor DNA replication (Ye et al., 1996). If progression through S-phase is delayed, the checkpoints normally act to delay entry into mitosis and this involves delaying the activation of NimX^{CDC2} and NimA kinases. Under such circumstances, NimX^{CDC2} is kept inactive via phosphorylation of tyrosine 14. Indeed, cells unable to phosphorylate tyrosine 14 on NimX^{CDC2}, either due to mutation of NimX (*nim*X^{cdcAF}), or to deletion of the wee1 kinase that carries out the phosphorylation, are viable but sensitive to S-phase delay and will enter mitosis with partially replicated DNA, and die. This premature mitotic entry is dependent on NimA accumulation, so tyrosine phosphorylation of NimX^{CDC2} plays a key role in controlling the timing of NimA protein accumulation, at least when DNA replication is defective. APC^{BimE} also plays an important role in controlling NimA activity during interphase—if DNA replication is completely blocked, double *bimE7nimX*^{cdcAF} mutants enter mitosis even at very high levels of DNA synthesis inhibitors, and this is associated with accumulation of NimA. This is consistent with other data that suggests the APC is activated in S or G2 to prevent premature activation of mitotic regulators (Lies et al., 1998; Ye et al., 1998). Another regulatory mechanism involves protein phosphorylation. NimA is a phosphoprotein (Lu et al., 1993) that becomes hyperphosphorylated during G2 (Ye et al., 1995). The final activation of hyperphosphorylated NimA depends on fully activated NimX^{CDC2} and is important for the coordination of early mitotic events. Several of the temperature-sensitive *nim*A alleles produce proteins that accumulate normally at the correct

time, but fail to undergo this final NimX-dependent activation step (Pu and Osmani, 1995a). Phosphorylated NimA may be a substrate for Pin1, a peptidyl-prolyl cis/trans isomerases (PPI) that might affect its activity by altering the conformation of the protein. In human cells, a NimA protein kinase interacts with, and is negatively regulated by, Pin1 (Lu et al., 1996), which is required for normal progression through mitosis. In *A. nidulans*, PinA (a pin1-like protein) interacts genetically with *nimA5* (Joseph et al., 2004): overexpression of *pinA* reduces the severity of the *nimA* phenotype while reduction in PinA levels increases the severity. Pin1 may be dependent on cdc2 phosphorylation as its preferred targets are proline residues proximal to phosphoS/phosphoT, the product of Cdc2 phosphorylation, and it has been suggested that it amplifies the effect of phosphorylation by inducing a really major change in protein shape on proteins that have been earmarked by the kinase. Consistent with the idea that PinA may have additional substrates, reducing PinA has diverse effects on the *A. nidulans* cell cycle (Joseph et al., 2004).

15.4.3 Involvement of Calcium in the G2/M Transition

Calcium has been widely implicated in cell-cycle transitions in plants and animals. The gene for calmodulin, one of the major internal cellular receptors for calcium, has been cloned and sequenced from *A. nidulans* (Rasmussen et al., 1990). Using site-specific gene replacement to place the calmodulin gene under the control of the inducible *alc*A promoter indicates that calmodulin is required for G2/M progression (Lu et al., 1992). A multifunction calcium calmodulin-dependent protein kinase (CaMK) may have an overlapping role as strains with reduced CaMK activity also seem to be impaired in G2/M progression (Dayton and Means, 1996). Two additional CaMKs have been characterized and play roles in spore germination and cell-cycle progression (Joseph and Means, 2000).

15.4.4 APC and the Metaphase-Anaphase Transition

The anaphase-promoting complex (APC), or cyclosome, is an ubiquitin ligase that assembles polyubiqitin chains on to its substrates to direct them for degradation by the 20S proteosome (Gutierrez and Ronai, 2006). The APC is required for mitotic progression, particularly for the metaphase to anaphase transition and for mitotic exit. Mutants that lack APC function tend to arrest in mitosis and in *A. nidulans,* produce the typical *bim* phenotype. Genetic and biochemical analyses of the APC in diverse organisms have revealed a huge complex involving at least a dozen subunits, and two of the defining proteins were discovered as *bim* mutations in *A. nidulans*. The *bimE7* mutation causes cells to block in metaphase at restrictive temperature, and is also known as APC1 (Zacharie et al., 1996). Loss of *bimE* function overrides a variety of interphase blocks, driving cells into premature mitosis (Osmani et al., 1988), but this requires functional $NimX^{CDC2}$ (James et al., 1995). The *bimA* gene encodes the APC3 subunit, a TPR protein (O'Donnell et al., 1991). Mutations in *bimA* also arrest in mitosis and override interphase arrests in a similar way to *bimE* mutations, but the phenotype is generally much weaker (Mirabito and Morris, 1993; Ye and Osmani, 1997).

Work in yeast suggests that the only essential function of APC is the ubiquitylation of securin, the separase chaperone, and cyclins. This has lead to the suggestion that APC and CDK-cyclins have coevolved (Thornton and Toczyski, 2003). In *A. nidulans*, the metaphase arrest caused by loss of APC function can be overridden by mutations in at least three other genes that affect either DNA replication or chromosome structure. Reductional divisions occur in *nimO18bimE7* (James et al., 1999) and *nimQ20bimE7* (Ye and Osmani, 1997) double mutants. NimO is structurally similar to the regulatory subunit of the cdc7 kinase, Dbf4, which phosphorylates the MCM proteins of the prereplicative complex thereby facilitating DNA replication. The cell achieves precise control over DNA synthesis, ensuring that one complete round of genome replication occurs in each cell cycle, by the binding of the prereplicative complex to discrete regions of the chromosome known as origins of replication. NimQ is homologous to one of the MCM proteins. Both *nimO* and *nimQ* are, therefore, required for DNA replication and mutants grow at restrictive temperature arrest with unreplicated DNA.

The other mutations that can bypass the *bimE7* metaphase arrest lie in the *nimU* gene (Pitt et al., 2004), which encodes a Pot1-like protein. Pot1 proteins form part of the shelterin complex, which acts to protect telomeric DNA from degradation and recombination, in part by regulating telomerase activity

(Price, 2006). The *nimU23* mutation was originally identified as having a decreased index of interphase nuclei and was, therefore, classified as being a *nim* "never-in-mitosis" mutant (Morris, 1976). Reciprocal shift experiments with HU indicated that the arrest point for *nimU24* was in G2 (Bergen et al., 1984) but the logic of these experiments depend on there being a discrete arrest point for a given mutation: mutants that are "leaky" or do not arrest cell cycle progression can be misclassified. Loss of *nimU* function reduces the mitotic index because cells progress through mitosis too fast. *nimU* mutants show a number of defects consistent with this, including premature spindle elongation and early mitotic exit (Pitt et al., 2004) and this produces elongated large aberrant nuclei that continue to cycle but spend a reduced time in mitosis. Double *nimU24bimE7* mutants enter mitosis with nearly normal *bimE7* dynamics and a significant percent of cells progress through into anaphase. This indicates that NimU is required for spindle checkpoint control at the metaphase-anaphase transition and this is independent of APC function. The spindle checkpoint can be triggered by perturbing microtubule stability, either with drugs or by mutation, and results in prolonged mitosis due to inhibition of mitotic exit (Oakley and Morris, 1981). However, mitotic exit remains APC-dependent as double mutants remain blocked in mitosis with two masses of chromatin. NimU mutants may also fail to activate PP1 at metaphase as these strains also have reduced protein phosphatase activity (unpublished results). It also remains to be determined if timing of sister chromatid separation in *nimU* mutants is dependent on the APC and this should now be testable since centromeres can now be marked with GFP (Yang et al., 2004). The mechanism by which a telomere component affects the mitotic checkpoints, therefore, remains unclear.

The $BimE^{APC}$ complex also regulates the SPB's activity as a cytoplasmic MTOC. Loss of *bimE* function leads to cells containing metaphase spindles with short or no astral MTs, indicating that APC function is required for the activation of the cytoplasmic face of the SPB as an active MTOC at the end of metaphase. This requirement for APC acts through the TinA protein (Osmani et al., 2003). TinA is a coiled-coil-containing protein that was isolated as interacting with NimA in a yeast-two-hybrid screen. TinA localizes to the SPB at G2/M in a NimA-dependent manner and may act to suppress MT nucleation from the cytoplasmic face of the SPB during early stages of mitosis.

15.4.5 Anaphase

When all the chromosomes have attached correctly, via their kinetochores, to the spindle, the APC is activated and the cell enters anaphase. APC activation leads to the degradation of securin, a protein that inhibits the protease, separase. When separase is released from inhibition it causes the release of sister chromatid cohesion by cleaving cohesin. In *A. nidulans*, separase function is encoded by *bimB* (May et al., 1992), and one of the four subunits of cohesin is encoded by the *sudA3* gene (Holt and May, 1996). *Sud*A was isolated as a cold sensitive suppressor of *bimD* mutants. BimD is structurally related to SPo76 of Sordia, Pds5p of budding yeast, and As3 from humans, which are required for sister chromatin cohesion, DNA damage response, and normal cell-cycle progression (van Heemst et al., 2001). In yeast, Pds5p and a cohesin subunit, Scc1, are mutually required for each other's recruitment to the chromosomes during G1 and cleavage of cohesin releases Pds5 at the metaphase-anaphase transition. In *A. nidulans*, as in vertebrates, BimD dissociates from the chromatin in prophase rather than the metaphase-anaphase transition, which may reflect differences in condensed chromatin structure between yeast and other eukaryotes (van Heemst et al., 2001). BimD also affects the rate of cellcycle progression, as mutants seem to progress faster through the cell cycle and cellular morphogenesis (van Heemst et al., 2001) while overexpression blocks the cell in G1 (Denison et al., 1993). The human ortholog, As3, acts as a tumor suppressor and it has been suggested that BimD plays a second role in modulating cell-cycle progression rates under unfavorable conditions (van Heemst et al., 2001).

Protein dephosphorylation plays a key role in anaphase. During mitotic entry many proteins become phosphorylated and, both to progress through mitosis and return to interphase, one might suppose that they need to be dephosphorylated. Using an antibody, MPM2, that recognizes phosphoproteins (Engle et al., 1988), a mutation was identified with temperature-sensitive arrest in anaphase. A mutation in the *bimG* gene, which encodes a type1 protein phosphatase (PP1), led to the formation of large nuclei, which failed to complete anaphase (Doonan and Morris, 1989), and also caused reduced PP1 activity (Doonan et al., 1991). At the level of gene expression, the *bimG11* allele leads to a temperature-sensitive

splicing event due to a mutation in an intron. An interfering truncated protein is produced (Hughes et al., 1996), which is recessive to the wild-type protein. Consistent with this, the *bimG11* allele is suppressed by cold sensitive mutations in the *sugB* gene, which encodes a splicing factor (Assinder et al., unpublished). *bimG11* is complemented by the structurally similar mammalian phosphatase (Doonan et al., 1991) but a related PP1 from Arabidopsis is only able to support hyphal growth (Arundhati et al., 1995). Interestingly, the major difference between the mammalian and plant PP1 is a C-terminal region where a functionally important regulatory site for cdc2 phosphorylation has been identified in the yeast homolog, dis2 (Yamano et al., 1994). However, mutation of the presumptive phosphorylation site in *bimG* did not seriously impair its function (Fox, unpublished). BimG-GFP fusions locate to several subcellular compartments (Fig. 15.6), including the cytoplasm, the spindle pole body, the nucleolus, the spindle, and the septation site (Fox et al., 2002), reflecting the multiple functions of BimG and the pleiotropic nature of the mutation. Notably, BimG-GFP association with the spindle pole is transiently reduced during early mitosis, at a time when MPM-2 phosphoprotein staining is increased. Since MPM-2 staining intensity is dramatically enhanced by the *bim*G11 mutation, it seems likely that BimG is largely responsible for limiting phosphorylation of nuclear structures in G2. BimG associates with the nucleolus until quite late in mitosis and time-lapse observations suggest that nucleolar BimG-GFP is segregated into

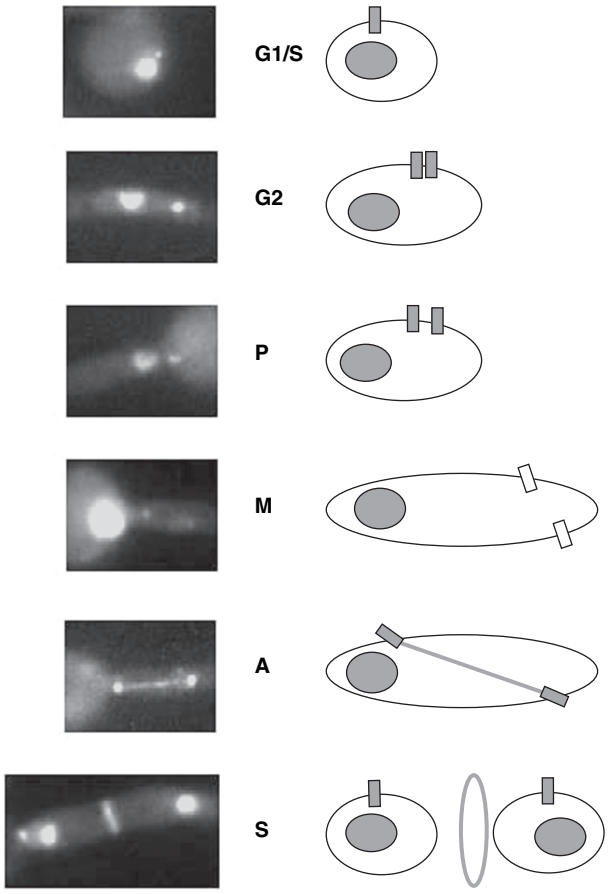

FIGURE 15.6 BimG distribution during mitosis. *Note*: Left panels show the distribution of BIMG-GFP fusion protein in a *nimA5* mutant. The top panel represents time zero (nimA5 block point) and subsequent panels show representative stages of mitosis. The right panels show schematic representations of nuclei showing BIMG distribution in green. Large green oval, nucleolus; small circles, SPB; bar, telophase spindle with BimG; ring, incipent septum. The stages of the cell cycle are given as follows: G1/S, G2, P (prophase), M (midphase), A (anaphase/telophase) and S (septation). **Q13**

the daughter nuclei during late anaphase/telophase by streaming along the spindle. BimG is necessary for nucleolar segregation as mutants have a highly MPM-2-stained nucleolus, whose persistence is associated with spindle defects.

As the daughter nuclei separate during telophase, at some point, the nuclear envelope surrounding the two daughters must break into two and provide each daughter with its own envelope. In higher eukaryotes, this occurs by complete disassembly of the nuclear envelope and reassembly but *A. nidulans* retains a nuclear envelope throughout mitosis. NimA inactivation may play a central role in daughter nucleus separation and envelope segregation (Davies et al., 2004). TinC preferentially interacts with inactive NimA, locates to membranous structures, and is required for the final stages of nuclear separation. Expression of an N-terminal deletion derivative of TinC leads to uncoupling of DNA separation and nuclear envelope separation. TinC belongs to a fungal-specific group of proteins that includes the HetC gene of *N. crassa*, a multiallelic gene that defines hetrokaryon incompatibility classes in that fungus (Sarkar et al., 2002). **Q6**

15.4.6 Mitotic Exit and the Septation Initiation Network

Cell division is completed with the insertion of a septum (cytokinesis or septation) between two recently divided nuclei (see review by Harris, 2001). In germinating conida, the initial cell delayed until after the third mitosis (Fiddy and Trinci, 1976; Momany and Taylor, 2000), so that the typical hyphal segment is multinucleate. Septation is not absolutely essential for the hyphal stage of growth as mutants that conditionally lack septa can produce microcolonies, but these fail to undergo any sexual or asexual development (Morris, 1976).

Septation involves the specialization of a site on the cell periphery during mitosis and the subsequent recruitment of actin filaments to form a contractile ring, similar to that observed at the division site in animals. The ring of actin filaments then contracts toward the centre of the cell, presumably pulling the cell membrane along. New cell-wall material is secreted to produce the septum. A number of mutants that have conditionally defective septation have been isolated (Morris, 1976). These have been classified into two distinct groups, called early and late. Late mutants, including *sepA, D, G*, and *H* undergo continuous nuclear division and hyphal growth at restrictive temperature but fail to form septa, while early mutants, such as *sepB, E, I,* and *J,* undergo only three nuclear divisions before arresting as aseptate cells. The late mutants probably represent genes involved in the process of septation itself, while the early genes may be involved in the coordination of mitosis and septation.

The *sepH* gene (Bruno et al., 2001), a member of the late group, is essential for the initiation of the actin ring and specially affects localization of the septin protein AspB (Momany and Hamer, 1997; Westfall and Momany, 2002), and SepA, a formin-related protein (Harris et al., 1997), which also locates to the septation site (Sharpless and Harris, 2002). SepH belongs to the family of protein kinases required for mitotic exit as defined by cdc7 from *S. pombe* and to CDC15 from *S. cerevisiae*. The yeast proteins are required for cytokinesis and for B-cyclin degradation at the end of mitosis. SepH is not required for hyphal growth and in the early stages of colony formation; the growth rate of *sepH* mutants is actually slightly higher than wild type. SepH function depends on normal cell-cycle progression: *sepH1* mutants grown at restrictive temperature will form septa on return to permissive temperatures but not if the cells have been treated with drugs that perturb cell-cycle progression.

T. Wolkow used temperature-sensitive mutants to show that septation could occur after a single mitosis, provided the cell was large enough. Indeed, downshift of *nimA5* cells back to permissive temperature is a useful means of obtaining a population of cells undergoing synchronized cytokinesis. The position of the mitotic nucleus seems to be critical in determining the position of the septum as *nim* mutants, grown at semipermissive temperature, form a septum at the site of the first nuclear division, provided the cell is large enough. Nud mutations that affect the migration and positioning of nuclei also affect the position of septa, which tend to be positioned close to the clusters of poorly separated nuclei. Wolkow et al. also proposed a model whereby septation is inhibited by the proximity of the hyphal tip leading to an asymmetric cell division. Completion of mitosis is important for cytokinesis as most *nim* and *bim* mutations conditionally block septation. One exception to this general rule is *hfaB* mutants,

which display a conditional "cut" phenotype, whereby a septum is laid down through a nondivided nucleus (Hughes et al., 2000).

The DNA damage-checkpoint pathway inhibits septation, acting through phosphorylation of $NimX^{CDC2}$ on tyr15 (Harris and Kraus, 1998). *sepB3* mutants are conditionally defective for septation and define one of the early sep genes (Harris and Hamer, 1995). SepB is required for efficient chromosome segregation as mutants accumulate defects that eventually arrest growth and prevent septation. DNA damage induces phosphorylation on tyr15 (Ye et al., 1997) and if this is prevented either due to mutation of the tyrosine kinase, AnkA (the wee1 orthologue), or by mutation of tyr15 in $NimX^{CDC2}$ then the cells will undergo septation despite having suffered DNA damage (De Souza et al., 1999; Kraus and Harris, 2001). Thus, *sepB3 nimX*^{cdc2AF} double mutants contain a NimX protein that cannot be phosphorylated by the tyrosine kinase AnkA, but they form septa normally. This demonstrates that the DNA damage-checkpoint pathway affects septation indirectly, through regulation of $NimX^{CDC2}$. A second indication of the close involvement of NimX in septation comes from the phenotype of a suppressor of *nimX* mutants, *snxB1* (McGuire et al., 2000), which can lead to hyperseptation.

The positioning of septa may also be influenced by the energy balance or nutrient status of the cell. As the carbon source has been reported to affect the size of the tip cell formed after cell division (Muller et al., 2000). The AnkA-wee1 kinase is implicated in delaying septation under high carbon growth conditions as conditional mutations in AnkA septate at a given size regardless of carbon source (Kraus and Harris, 2001) and cell-size control in response to nutrient status may act through AnkA. Q7

The APC has also been implicated in septation as a conditional mutation in sepI actually defines a new allele of the *bimA* gene, *bimA10* (Wolkow et al., 2000). The *bimA10* allele causes a splicing defect that leads to the production of an aberrant protein with an altered C-terminus and this leads to the accumulation of replication errors that trigger the DNA damage checkpoint. *bimA10* double mutants with DNA damage-checkpoint mutants such as *uvsB110* or *uvsD153* partially suppress the failure to septate normally, indicating that the primary effect of the *bimA10* allele is in DNA replication and its effects on septation are secondary through activation of the DNA-damage pathway.

The spindle-pole body seems to play an important role in septum positioning since many proteins found there seem to affect septation. SnaD, a protein found at the spindle-pole body (Liu and Morris, 2000), affects the timing of mitosis and septation. Mutations in *snaD* suppress the growth defects of *nudA* mutations, not by affecting nuclear migration directly but by delaying the timing of mitosis and septation. This has the result that tip cells have a better chance of containing a nucleus and thereby retaining viability. Conversely, the *snaD* mutants have a defect in conidiation where the timing of septation is critical for cellular development. BimG PP1, another spindle-pole component, seems to play a role in septation. GFP studies indicate that BimG locates to the site of septation just after mitosis and follows the contractile ring as it divides the cell (Fox et al., 2002). Temperature-shift experiments support the idea that BimG plays a direct role in septation, but it is difficult to exclude potential indirect effects caused by perturbance of mitosis.

15.4.7 Genome Surveillance

Activation of the DNA-damage repair pathway can slow or arrest the cell cycle at several points, including G1/S, S-phase, and G2/M. The G2/M transition and septation are clearly very sensitive to genome damage, perhaps because these stages involve irreversible structural rearrangement of the cell that leads to separation of the genetic material. There is the possibility of repair by somatic recombination between two recently replicated DNA while they remain in the same nucleus, but after mitosis such recombinational repair is less likely and almost impossible after septation. It is easy to image, therefore, that there has been a strong selective advantage for delaying both mitosis and septation in the event of DNA damage.

Consistent with this idea, there is a conserved signal transduction pathway that detects and responds to DNA damage. In *A. nidulans*, the uvsB and uvsD genes encode crucial components and are structurally related to the ATM/ATR kinases (De Souza et al., 1999; Hofmann and Harris, 2000). The UvsB kinase is related to Rad3 while UvsD is similar to Rad26, both PI-3 related kinases that modulate cell-cycle progression by phosphorylating several other proteins, including downstream kinases such as Rad53/Chs2, and ultimately leads to inhibitory phosphorylation on tyr14 of $NIMX^{cdc2}$, thereby delaying or

blocking mitosis and septation (Fig. 15.6). The NIMX^{cdc2AF} mutation cannot be so inhibited and, therefore, does not respond to DNA damage by inducing cell-cycle arrest. Loss of these checkpoint functions can have downstream effects on the DNA-replication checkpoint and allow rereplication of a genome that has not completed mitosis successfully. This leads to endoreduplication and increases in ploidy.

Genetic dissection of this pathway has revealed several additional components (reviewed by (Goldman and Käfer, 2004)) and these are gradually being placed into what might be better described as a genome-monitoring network. Thus, uvsB acts both in the DNA replication and the intra-S-phase checkpoints (Fagundes et al., 2004) and affects both mitosis and septation (Hofmann and Harris, 2000) but other components seem to be specific for particular branches of the network. The *musN* gene functions downstream of uvsB, probably in the septation-specific branch, since mutations in *musN* suppress only the septation checkpoint in *uvsB* mutant backgrounds (Hofmann and Harris, 2000). NpkA, a cdc2-like kinase, was isolated as a gene that is transcriptionally upregulated in response to camptothecin, a drug that inhibits type I topoisomerase and induces replication-mediated DNA double strand breaks (Fagundes et al., 2004). Deletion of *npkA* partially suppresses the intra S-phase checkpoint defect of *uvsB* mutants but not the DNA-replication checkpoint. Consistent with this, deletion of *npkA* is additive with *ankA^{wee1}* mutations, but its interaction with *bimE* mutants suggests that there is additional functional redundancy to be uncovered.

The *sldL* gene encodes a Rad50 homologue that interacts genetically with *bimE$^{APC/C}$* mutants in response to DNA damage (Malavazi et al., 2005). Rad50 is a large coiled-coil protein related to the SMC protein family and is believed to bind to double stranded breaks in damaged DNA, effectively bridging the gap and allowing DNA repair enzymes to bind. Using the ScaA protein as bait in a yeast-two-hybrid screen, Semihini et al. (2003) isolated the Mre nuclease, one of these repair enzymes (Semighini et al., 2003). **Q8**

A. nidulans, in common with many fungi, can exist as haploid or diploid strains and occasionally interconvert between the two. This interconversion is the basis of the parasexual cycle that has been so useful for classical genetic analysis and mapping of mutations. Diploid strains arise very rarely, probably as a result of accidental nuclear fusion, but can be recognized and isolated from suitably marked hetrokaryotic strains. The reverse process, haploidization, can be induced by transient growth on antimicrotubule drugs **Q6** that lead to chromosome loss. Although the process of ploidy control remains poorly understood, it is amenable to genetic and molecular dissection. Targeted disruption of the *chpA* (cysteine- and histidine-rich-domain-[CHORD]-containing protein A) gene in haploid *A. nidulans* strains gives rise to *chpA* knockout haploids, which are morphology normal, and heterozygous diploids, which develop abnormal condiophores. However, *chpA* knockout diploids were impossible to isolate and attempts to disrupt the remaining *chpA* gene in heterozygous diploids lead to unstable aneuploids suggesting that ChpA is required for mitosis in diploid cells (Sadanandom et al., 2004). The molecular mechanism is unclear but related proteins in plants and animals interact with SGT1, a multifunctional protein associated with protein turnover.

15.5 Hyphal Morphogenesis and the Cell Cycle

bimG11 mutants also affect hyphal morphogenesis. At restrictive temperature, germinating spores fail to switch from isotropic to polar growth with the result that *bimG11* spores swell to great size. GFP fusions indicate a direct role for BimG at the hyphal tip since the protein forms a collar around the base of the hyphal dome (Fox et al., 2002). *bimG11* mutant strains have reduced levels of chitin in their cell wall (Borgia, 1992), and it was proposed that the phosphatase affected the pathway for chitin biosynthesis. However, it seems unlikely that the cell-wall defect is the sole basis of the phenotype as the cell swelling phenotype is much more extreme in germinating spores than in hyphae, which stop growing rather than swelling when shifted to restrictive temperature. Thus the roles of BimG seem to change after germ tube emergence: before emergence, the primary role is to define a point of polarized growth and a minor role in growth itself but after emergence the primary role is to maintain growth and the polarity maintenance role is less important.

The mechanism by which BimG establishes polar growth is uncertain, but seems to involve NimX^{CDC2}. *nimX^{cdcAF}* partially suppresses the spore germination defect of the *bimG11* mutant as double mutant cells

germinate at restrictive temperature (Fox, Ph.D. thesis). The hyphae produced under these circumstances are unusually wide and have reduced cell extension rates, supporting the idea that BimG has an additional role in controlling growth, perhaps in vesicle recycling in the growing tip. PP1 is known to affect vesicle membrane recycling in other organisms (Peters et al., 1999) and the location of the BimG halo approximately coincides with sites of endocytosis. Alternatively, BimG may affect actin function or organization since actin is intimately associated in a highly dynamic manner with both the tip and the septum.

Although $NimX^{CDC2}$ is not required for hyphal outgrowth (*nimX* mutants germinate normally at restrictive temperature (James et al., 1995), regulation of either $NimX^{CDC2}$ or NimA activity might be required to couple spore germination with the nuclear division cycle. APC activity is essential for hyphal outgrowth as *bimE7* and *bimA* mutants have delayed and reduced hyphal outgrowth under nonpermissive conditions. This requirement for APC depends on *nimX* as mutations that reduce $NimX^{CDC2}$ activity suppress the polarization defect (James et al., 1995). NimA may also play a role as overexpression inhibits outgrowth so that under high nutrient conditions emergence is delayed until after the first mitosis, but under low nutrient conditions emergence takes place after the first S-phase. Completion of S-phase is essential for emergence under both conditions as mutations and drugs that lead to S-phase arrest tend to block emergence. The only exception to this general rule so far is *nimX3*, which blocks the nuclear cycle in late G1 but allows emergence before DNA replication is complete (Harris, 1999).

Germ tube emergence is also under developmental control. During spore formation and maturation, the nucleus becomes highly condensed and the cells enter a deeply dormant state that is dependent on WetA, a protein required to prevent precocious germination of nascent spores while they are still attached to the parent colony (Clutterbuck, 1969). However, nothing is known about the cell cycle stage-dependency of germination in *wetA* mutant spores.

15.6 Branching and Cell Cycle Control

Superficially similar to germ tube emergence, branching is also important for fungal growth and morphogenesis, and seems likely to be coupled to cell-cycle progression. A ts^- mutation in the *ahbA* gene leads to reduced branching and abnormal development associated with reduced nuclear number. When the gene was cloned, the *ahbA1* mutation was found to be an allele of *nimX* (Lin and Momany, 2004). These data support the longstanding idea that the number of nuclei in a hyphal segment influences branching frequency (Dynesen and Nielsen, 2003) but the signals coupling nuclear number to branching are poorly understood. *AhbB1*, another gene isolated by Lin and Momany (2004), may provide a clue. Mutation of this gene also reduces branching, and it encodes a cytochrome P-450, possibly involved in steroid and fatty acid metabolism. Other lipid-diriviaized compounds may also act as signals (Cheng et al., 2001). Sphingolipids are major components of the plasma membrane and their metabolism can generate potent signaling molecules. Mutations in the *aur1* gene, which encodes inositol phosphorylceramide synthase, blocks in G1, perturbs the actin cytoskeleton and inhibits polarized cell growth. However, this mutation leads to changes in multiple lipid-derived signaling compounds, reducing sphingolipids and increasing levels of ceramide. To deconvolve this complexity, Cheng et al., looked at *lcbA* mutants defective in serine palmitoyltransferase (SPT), the first enzyme in the sphingolipid biosynthesis pathway, and at the effect of myriomycin, a specific inhibitor of SPT. Reducing SPT activity led to a defect in actin-dependent hyphal morphogenesis without affecting the cell cycle. Thus, it seems likely that lipid-derived signals could be involved in the coordination of hyphal growth and branch formation with cell-cycle progression.

15.6.1 Developmental Regulators Impose New Discipline on the Cell Cycle

Coupling of the nuclear division cycle with cell growth and cytokinesis during hyphal growth differs dramatically from that observed during later stages of development. During both the asexual and sexual cycles, cell sizes and shapes and nuclear number per cell become more or less strictly defined and are often crucial for the identity and function of particular cell types (reviewed by Fischer and Kües, 2006). **Q9** For example, the fertile hyphae within fruiting bodies are binucleate and, during asexual spore formation,

cell and nuclear division become coupled so that each spore receives only one nucleus. Genes that control morphogenesis affect how cell and nuclear division are coupled. Thus the condiospores, end products of the *brl*A-controlled asexual reproductive pathway, contain a single nucleus per cell whereas the aerial hyphae from which they are derived contain many nuclei per cell. One effect of *brlA*, therefore, is to couple cell division more tightly with nuclear division than normally occurs in hyphal growth. A *brlA*-dependent increase in $NIMX^{CDC2}$ activity combined with the requirement for correct regulation of $NimX^{CDC2}$ activity suggests that there is a direct developmental regulation of the cell cycle, acting through BrlA (Ye et al., 1999). Ectopic expression of the *brlA* gene in hyphae leads to one-to-one coupling between nuclear and cell division in hyphae leading to ectopic spore formation at the hyphal tips (Adams et al., 1988) as well as increased levels of $NimX^{CDC2}$ (Ye et al., 1999). Not only are $NimX^{CDC2}$ levels increased during sporulation, but regulation of its activity via inhibitory phosphorylation on tyr15 is also crucial as $nimX^{cdc2af}$ strains conidiate poorly with morphologically abnormal conidiophores. Tyr15 dephosphorylation had previously been found essential for conidiation: *nimT23* mutants could be partially complemented by an extra copy of the *nimE* gene, which allowed hyphal growth but development was impaired (O'Connell et al., 1992). This suggests that extra *nimE* (cyclin B) may increase the amount of pre-MPF (tyrosine phosphorylated cyclinB/cdc2) available for *nimT23*-mediated activation and, eventually, the mutant phosphatase activates enough kinase to allow entry into mitosis. The filamentous hyphal cells apparently can cope with this rather sloppy control of mitotic entry but the cells that comprise the reproductive structures cannot, and development is impaired. The transcriptional regulation of $NimX^{CDC2}$ could be quite direct as BrlA is a transcription factor with two TFIIIA-type zinc fingers and the *nimX* upstream regulatory region has seven potential BrlA- binding motifs. BrlA also modulates cyclin expression: *nimE* transcript size is altered by expression of *brlA*.

Other sporulation-specific cell cycle controls may also operate to add additional layers of regulation. For example, an additional cyclin, *pclA*, is induced during sporulation in a *brlA*-dependent manner (Schier et al., 2001) and this physically interacts with $NimX^{CDC2}$ (Schier and Fischer, 2002). PclA is related to pho85 cyclins in yeast but there is no evidence that PclA interacts with PhoA, one of the two *A. nidulans* orthologs of the yeast pho85 cyclin-dependent kinase. Deletion of the *pclA* gene severely reduced sporulation, indicating an important requirement for this cyclin during asexual development while mutation of *phoA* promotes the sexual development pathway. It is possible that these genes affect the developmental decisions made in response to environmental conditions (Bussink and Osmani, 1998).

The septation initiation network (SIN), the regulatory pathway that activates the contractile actin ring during septation, also seems to be more stringently controlled during conidiation. The *mobA* gene, a homolog of the yeast SIN gene, *mboA*, is not required for colony formation, but mobA mutants fail to conidate. A screen for mutations that bypass the requirement for mobA has identified a number of genes that also bypass the requirement for SEPH kinase (Kim et al., 2006).

Analysis of the KfsA function supports the idea that septation is differentially regulated during development. The *kfsA (kinase for septation*) gene was discovered in a reverse-genetic approach and is involved in the regulation of septation in the conidiophore, (Takeshita et al., 2007). The protein displayed some similarity to Kin4 of *S. cerevisiae*. Kin4 appears to monitor spindle misalignments and delays septation until nuclei are correctly distributed (D'Aquino et al., 2005; Pereira and Schiebel, 2005). In *A. nidulans,* KfsA localized to septa after the actin ring disappeared and neither deletion nor overexpression affected overall growth or the visual appearance of the colony. However, the number of conidiophores with ectopic septa in the stalk was increased and binucleate metulae were produced when KfsA levels were perturbed. This suggests similar roles for KfsA in *A. nidulans* and Kin4 in *S. cerevisiae*, but KfsA's role in *Aspergillus* is only really critical during development.

The enhanced coordination between cytokinesis and nuclear division may involve other components of the *brlA* regulatory network. Absence of *abaA* function leads to incomplete separation of spores but cell growth and nuclear division continue (Sewall et al., 1990) while ectopic expression of *abaA* induces aberrant compartmentalization of the hyphae (Mirabito et al., 1989). Regulation of *nimX* may also be important for the suppression of septation during certain stages of condiophore development as a suppressor of *nimX* leads to ectopic septa in the stalk (McGuire et al., 2000) as also occurs in strains with the activated $nimX^{cdc2AF}$ allele (Ye et al., 1999). The molecular characterization of the *nimX* suppressor genes should provide insight into the interplay between the cell cycle and developmental regulation of growth.

15.7 The Actin Cytoskeleton

15.7.1 Organization of the Actin Cytoskeleton

Immunostaining of actin or visualization with phalloidin derivatives revealed a spot-like distribution of the protein along the cortex in many fungi with a high concentration at the tip. In germinating spores, these actin spots are initially distributed evenly around the swelling spore but gradually accumulate at the site of hyphal emergence. In comparison, in *Ashbya gossypii,* actin cables are frequently seen (Schmitz et al., 2006). Meanwhile actin has been fused to GFP, which allows *in vivo* studies of the dynamics of actin (S. Osmani, personal communication). Furthermore, Penalva et al. fused an actin-binding protein with GFP, producing a useful tool to study actin localization and behavior in living *A. nidulans* cells (M. Peñalva, Madrid, personal communication). The important role that actin plays in polarized growth becomes obvious when depolymerizing agents, such as latrunculin B, or cytochalasin, are added to growing hyphae. Sampson et al. showed that addition of latrunculin B causes a rapid cessation in hyphal extension (Sampson and Heath, 2005). Likewise, deletion of the myosin gene, *myoA*, is lethal (McGoldrick et al., 1995). There are two likely contributions of the actin cytoskeleton to polarized growth. On the one hand, the actin-myosin cytoskeleton is used for vesicle transportation and secretion and thus the delivery of cell-wall components. On the other hand, cortical proteins are brought into place by this system in *S. cerevisiae* and guarantee proper attachment of MTs to the cortex (Schuyler and Pellman, 2001b). Because MT attachment sites required for polarized growth seem to be very defined in the apical dome (see later), it is conceivable that the actin cytoskeleton plays a role at this point as well. However, further experiments are required to unravel the exact mechanisms.

Another aspect of polarized growth that we should consider is the existence of a Ca^{2+} gradient along the hypha, with a high concentration having been demonstrated at the tip of *Phyllosticta ampelicida* and *N. crassa* (Shaw et al., 2001; Silverman-Gavrila and Lew, 2003). In the absence of the Ca^{2+} concentration gradient, hyphal polarity is affected (Schmid and Harold, 1988). Although this phenomenon has been known for a long time, a direct link to the growth machinery described earlier has not emerged yet. One explanation for the role of Ca^{2+} ions is the stimulation of vesicle fusion with the membrane. The Ca^{2+} concentration appears to be regulated through a stretch-activated phospholipase C at the tip, which catalyzes the formation of inositol (1,4,5) triphosphate (IP_3) and in turn causes the release of Ca^{2+} from special vesicles (Silverman-Gavrila and Lew, 2002).

15.7.2 The Polarisome

A protein complex involved in the organization of the actin cytoskeleton is localized at the incipient bud of *S. cerevisiae* and was named the polarisome. This structure is involved in the organization of the actin cytoskeleton and its appearance resembles the Spitzenkörper in filamentous fungi (Sagot et al., 2002). There is evidence that this protein complex also exists in filamentous fungi as a separate structure to the Spitzenkörper (Harris and Momany, 2004). The existence of polarisome components in filamentous fungi was shown first in *A. nidulans*. Sharpless, and Harris demonstrated that SepA—an orthologue of Bni1, a key component of the yeast polarisome—colocalizes with the Spitzenkörper (Sharpless and Harris, 2002). Similarly, in *A. gossypii*, a filamentous fungus very closely related to *S. cerevisiae* (Wendland and Walther, 2005), a homologue of the *S. cerevisiae* polarisome protein Spa2 was analyzed (Knechtle et al., 2003) and recently also the Bni1 orthologue, AgBni1 (Schmitz et al., 2006). Whereas Spa2 is not essential in *A. gossypii*, it is necessary for fast polarized growth, and deletion of *Agbni1* caused loss of polarization and swelling of the cells to a potato-like appearance. A Spa2 orthologue has been characterized in *Candida albicans* as well and its role studied during filamentous growth (Zheng et al., 2003). The protein persistently localized at hyphal tips and deletion caused defects in polarity establishment. Recently, Crampin *et al*. suggested that the polarisome and the Spitzenkörper are distinct structures that coexist in hyphae (Crampin et al., 2005; Sagot et al., 2002). Similar results for Spa2 (SpaA) were obtained in *A. nidulans*, suggesting that a polarisome or the existence of polarisome components at the growing hyphal tip could be a general theme for filamentous fungi (Virag and Harris, 2006b). According to this model,

filamentous fungal cells employ both the MT and the actin cytoskeleton, while the Spitzenkörper acts as a vesicle supply centre and the polarisome functions in actin organization.

The growth machinery discussed so far describes how fungi could extend their hyphae, but this picture does not yet allow any adaptation of the process to external (e.g., nutrient gradients) or internal signals (e.g., the stage of the cell cycle or life cycle). Little is known so far about the transduction of such signals into, for example, changes of growth direction, although several regulatory proteins have been described, which influence polarized growth, probably through an interaction with the actin cytoskeleton. The principle of this possible regulation is well studied in *S. cerevisiae* (Tcheperegine et al., 2005) and some of the components appear to be conserved in filamentous fungi. Among those are members of the Rho and the Rac family, small GTPases that act as molecular switches (Boyce et al., 2001, 2003, 2005; Guest et al., 2004; Momany, 2005; Virag and Harris, 2006a). However, a detailed analysis of their exact role in polarized growth in filamentous fungi remains to be done. Hyphae respond to environmental stimuli by altering their growth rate, diameter, and branching patterns. Genes such as *phoA* and *phoB, which encode cyclin-dependent protein kinases,* may play an important role in coupling growth to nutrient status (Dou et al., 2003) as available nutrients strongly affected the phenotype of knockouts.

15.7.3 Actin-Dependent Motor Proteins

The function of the actin cytoskeleton depends on the activity of actin-dependent motor proteins, the myosins. Myosins serve a broad range of cellular functions and are grouped into 18 different classes. In *A. nidulans,* a class-I myosin that is required for protein secretion and polarized growth and has an essential role (McGoldrick et al., 1995) and localizes to the growing hyphal tip (Yamashita et al., 2000). **Q10**

Given that myosin motors are involved in vesicle transportation toward the cell cortex and vesicle fusion with the cell membrane, it is very interesting that *A. nidulans* employs a myosin-derived motor domain for the transportation of class-V and class-VI chitin synthases, where the motor domain is directly fused to the enzyme (Horiuchi et al., 1999; Takeshita et al., 2005; Takeshita et al., 2006).

Myosin motor proteins of other classes have been described. For example, in *S. cerevisiae*, a class-V myosin motor is involved in peroxisome's and other organelles' inheritance (Bretscher, 2003a; Fagarasanu et al., 2006). A second class-V myosin is required for RNA transportation (Bretscher, 2003b). **Q2**

15.8 Genes Required for the Establishment of Polarity

So far we have discussed polar growth in the sense of maintaining polarized extension by recruiting the cellular growth machinery for cell-wall assembly at the tip of an existing hypha. As mentioned earlier, the question how polarity is initially established starting from round spores remains largely unanswered. Hyphal emergence must involve localized cell modification such that one area of the wall becomes differentially susceptible to incorporation of new cell wall material. The polarized cytoskeleton described earlier is necessary to control this, the actin cytoskeleton being required per se, and the MTs fine-tuning the direction of growth. The ability to modulate cell-wall rigidity is also required as mutants lacking fibrillar components of the wall grow in a spherical manner. The secretion apparatus is also essential for hyphal outgrowth. Whittaker et al. (1999) showed that the sodVIC gene encodes an α-COPI related protein that is essential for polarized outgrowth (Whittaker et al., 1999). In mutants that lack SodVIC function, nuclear division occurs to produce swollen deformed cells without obvious tips. COPI proteins are important for vesicle formation and recycling. GFP-fusions with SodVIC localize to the Golgi (Assinder et al unpublished), consistent with a role in secretion.

In ways that are not yet clear, polarity is under the control of the cell cycle regulatory network (see section titled "Hyphal Morphogenesis and the Cell Cycle"). Although this control could be indirect and through diverse pathways, it is now clear that cdc2-related proteins interact with, and control, a large number of proteins in other organisms (Ubersax et al., 2003). Consistent with this, cdc2 proteins in other organisms are located not only in the nucleus but also in the cytoplasm where they associate with the microtubules (Maekawa and Schiebel, 2004) and regulate MAP function.

In addition to cell cycle-related controls on spore germination, a number of additional functions have been described that directly affect polarization of the spores. Three temperature-sensitive mutations in the *swoC, swoD,* and *swoF genes*, cause spores to swell at restrictive temperature and prevent the production of a germ tube (Momany et al., 1999). The SwoC protein displayed homology to rRNA pseudouridine synthases of yeast and its role in polarized growth remained obscure. SwoF on the other hand had high identity to N-myristoyl transferases and it was speculated that a polarity determinant could be the substrate for the myristolyation (Shaw et al., 2002). This posttranslational protein modification is found in proteins that switch between membrane-bound and cytoplasmic states (e.g., G-protein α-subunits), and could be important for the localization of cell-end marker proteins as discussed earlier (Bathnagar and Gordon, 1997). Therefore, the identification of prenylated or myristoylated proteins appears to be of prime importance for understanding polarity establishment in filamentous fungi.

15.9 Conclusions

The last few years have provided many new insights into the role of and interplay between actin microfilaments, MTs, and the nuclear division cycle in polarized growth of fungi. Actin plays a major role in cell growth and septation, the microtubules have a central role during mitosis, where they form the structure of the spindle, and through checkpoints mechanisms interact with the regulatory kinases that drive the cell cycle and cell growth. The circuits that connect the cell-cycle regulators to cellular morphogenesis are yielding to genetic and cell biological dissection and it will be very interesting to understand how these become modified to produce the more complex cell shapes that arise during the life cycle. During interphase in hyphae, the main function of microtubules is to deliver and direct vesicles and cell-end markers to the single point of growth at the apex of the cell, but during asexual development, growth first becomes dispersed over a large part of the vescile surface and then becomes restricted again but to multiple points as the metulae are initiated. This function needs special attention, since only one putative cell-end marker protein has been identified so far in *A. nidulans*. If homologues of *S. pombe* cell-end markers exist in filamentous fungi, many questions remain: what is their biochemical function? Which downstream events do they trigger to allow straight hyphal growth? And with which upstream regulatory circuits are they integrated to permit the formation of different cell types? The latter question is particularly interesting since the developmental and morphological complexity of different cell types in *Aspergillus* suggest that the regulatory circuitry will be more complex than in single-celled fungi. The publication of several fungal genome sequences (Galagan et al. 2005) along with their innate genetic tractability and continuous improvement of molecular and microscopy techniques promise a fruitful future for cytoskeletal and cell-cycle research in fungi.

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