Characterization of γ-tubulin complex proteins and investigation of the regulation of nuclear proteasome localization in *Aspergillus nidulans*

DISSertation

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Abstract

γ-Tubulin is conserved and essential for microtubule nucleation from lower eukaryotes like unicellular fungi to higher eukaryotes like humans. γ-Tubulin together with other proteins forms complexes and functions in microtubule nucleation and organization. Although these γ-tubulin complex proteins (GCPs) have been identified in several model organisms, the functions of individual GCPs remain unclear and contradictory data from studies using different model organisms have prevented the establishment of a coherent model. Although the existence of GCPs in the filamentous fungus, Aspergillus nidulans, was suggested by early biochemical experiments (Akashi et al., 1997), these proteins remain unidentified and their functions remain undetermined. In my dissertation research, I have identified and deleted each of the genes that encode these proteins, created GFP and mCherry fusions of each of them, and observed them in vivo using spinning disk confocal microscopy. Briefly, I have found that different GCPs play different roles: two are required for the existence of the γ-tubulin complex in cells and are thus essential for formation of the mitotic apparatus and for cell reproduction. Three others are not essential, but play a role in the assembly of large γ-tubulin complexes and have minor functions in the fidelity of chromosomal segregation. We were also able to establish a hierarchy of binding of GCPs to the spindle pole body and, based on these findings, we were able to propose a model for the structure of the γ-tubulin complexes.
To understand the microtubule-nucleation and non-nucleation functions of γ-tubulin, our lab created a series of conditionally lethal γ-tubulin mutants. Extensive analysis of one mutant, *mipAD159*, revealed that the coordination of late mitotic events is disrupted even though mitotic spindles assemble with normal kinetics (Prigozhina et al., 2004). Further investigations revealed that this allele caused a nuclear autonomous failure of inactivation of the anaphase promoting complex/cyclosome (APC/C), which caused constitutive destruction of mitotic regulatory proteins (Nayak et al., 2010). As degradation of proteins targeted by the APC/C is carried out by the proteasome, we were interested in determining if γ-tubulin mutations affect the proteasome. Surprisingly, time-lapse imaging of a γ-tubulin mutant and γ-tubulin deletants as well as deletants of two essential GCPs revealed that the proteasome, which is highly enriched in the nucleus, separates physically from the chromosomes in mitosis. This resulted in the formation of nucleus-like structures containing little or no chromatin but often having a nucleolus. This implies that the proteasome binds to, or itself forms, a nuclear matrix and our high resolution multi-dimensional optical reconstructions of the nuclei support this notion. In an attempt to determine the nature of the nuclear matrix by using a candidate approach, I found that the nuclear localization of the proteasome does not rely on the Mlp1 (the orthologue of Megator) scaffold but does depend on the presence of the *A. nidulans* homolog of *Schizosaccharomyces pombe* Cut8 (An-Cut8). The lack of proteosomes in mitotic nuclei of *An-cut8* deletants, however, does not cause a delay in cyclin B degradation and obvious defects in mitotic progression. My data do not seem to support the hypothesis that Cut8 as an anchor for the nuclear proteasome as has been proposed in
S. pombe, instead, they appear more consistent with the notion that Cut8 is involved in the nuclear transport of the proteasome or proteasomal subunits.
Dedication

This work is dedicated to my family
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Chapter 1: Introduction

1.1 Microtubules and microtubule organizing centers

Microtubules are hollow tubes, about 25 nm in diameter, that are found in all eukaryotic cells. They are involved in many important cellular activities such as formation of the mitotic apparatus, serving as tracks for directional transport of proteins and vesicles, and maintenance of cell shape, polarity and motility. They are composed of heterodimers of α- and β-tubulin that assemble, head to tail, into protofilaments. Thirteen protofilaments form the microtubule lattice in vivo. In the lattice α-tubulins interact laterally with α-tubulins and β-tubulins with β-tubulins except at a seam where α-tubulins interact laterally with β-tubulins. As α- and β-tubulin heterodimers always bind head-to-tail, microtubules are polar polymers. The ends are designated plus and minus and β-tubulin is tip-most at the plus end and α-tubulin at the minus end (Figure 1.1). Both α- and β-tubulin bind GTP, but only the nucleotide bound to β-tubulin is exchangeable as the GTP of α-tubulin is buried in the longitudinal contact of the dimer and is, thus, non-exchangeable. The GTP in β-tubulin can be hydrolyzed to GDP and tubulin dimers in which GTP is bound to β-tubulin are known as GTP-tubulin and those with GDP are known as GDP-tubulin. The vast majority of tubulin dimers in microtubules are known to be GDP-tubulin and, because of the difference in dissociation constants between GDP and GTP, the vast majority of free tubulin dimers are GTP-tubulin. Binding of one GTP-
tubulin dimer during microtubule polymerization appears to be coupled to hydrolysis of the GTP in the β-tubulin of the dimer at the tip of the protofilament to which the dimer binds (Nogales et al., 1998). All the tubulin dimers in the microtubule lattice except at the end are therefore probably GDP-tubulin.

GDP-tubulin dimers have a greater intrinsic curvature than GTP-tubulin dimers. GTP hydrolysis, thus, induces a bend to the protofilament and increases the intrinsic curvature of the lattice. The bend is constrained by intermolecular interactions among dimers, and the energy released from hydrolysis is stored as a structural constraint in the lattice. This structural constraint makes the microtubule unstable and prone to depolymerization, which occurs by loss of lateral contacts between tubulin dimers and protofilament peeling (Howard and Hyman, 2003; Nogales and Wang, 2006). The coexistence of polymerizing and depolymerizing microtubule populations that interconvert between these two states is a general property of microtubules and is termed dynamic instability (Mitchison and Kirschner, 1984). With respect to individual microtubules, a single microtubule can undergo either growing or shortening at both ends and can interconvert frequently between the two states in a stochastic manner. It can also grow at one end while it shortens at the other end (Horio and Hotani, 1986). The transition from the polymerizing state to the depolymerizing state is called catastrophe, and the opposite transition is referred to as rescue (Walker et al., 1988). Microtubules assembled in physiological environments have higher polymerization and catastrophe rates than microtubules assembled from purified tubulin with a similar concentration due to the presence of many microtubule associated proteins that stabilize microtubules and catastrophe factors that destabilize proteins (Cassimeris et al., 1988; Kinoshita et al.,
In many eukaryotic cells, cytoplasmic microtubules originate from microtubule organizing centers (MTOCs) with their minus ends in contact with the MTOC. The major MTOC in fungi is the spindle pole body (SPB) and in animal cells it is the centrosome. SPBs and centrosomes are morphologically distinct, but functionally conserved.

The structure of SPBs in *Saccharomyces cerevisiae* has been well studied by electron microscopy (EM) (Jaspersen and Winey, 2004). The SPB is embedded in the nuclear envelope throughout the cell cycle and appears multi-layered. It consists of three major plaques: an outer plaque facing the cytoplasm, a central plaque that spans the nuclear envelope, and an inner plaque facing the nucleoplasm. Between the outer and central plaque, two additional layers, the first and second intermediate layers (IL1 and IL2) were also observed by cryo-EM and electron tomography methods. An electron-dense region of the nuclear envelope, termed the half-bridge, is associated with one side of the central plaque and new SPB assembly occurs from here (Figure 1.2 A).

The centrosome is composed of two orthogonally arranged centrioles surrounded by an amorphous mass of protein called pericentriolar material (PCM). The two centrioles are cylindrical structures formed mainly by arrays of triplet microtubules organized with nine-fold radial symmetry and they are held together by interconnecting fibers (Doxsey, 2001; Sluder, 2005). One centriole of the mammalian centrosome has distal and subdistal appendages and is called the mother or maternal centriole. The other centriole that lacks these sub-structures is called the daughter centriole. The PCM is composed of a large number of different proteins, some of which are arranged in a three-dimensional lattice structure. Microtubules are nucleated from the PCM (Figure 1.2 B)
(Doxsey, 2001; Sluder, 2005). Both the centrosome and the SPB duplicate once per cell cycle before the entry into mitosis when they initiate bipolar mitotic spindle formation (Jaspersen and Winey, 2004; Azimzadeh and Bornens, 2007).

Although the structure of SPBs in the filamentous fungus, *Aspergillus nidulans* has been studied in less detail, early electron micrographs show that the SPB is also embedded in the nuclear envelope (Oakley and Morris, 1983). In interphase, most, if not all, cytoplasmic microtubules assemble from the SPB while tubulin is excluded from nuclei. As the cell approaches mitosis, the cytoplasmic microtubules disassemble, tubulins rapidly enter mitotic nuclei, and microtubules assemble in the nucleoplasm from the duplicated SPBs to form the mitotic spindle. As the spindle elongates during anaphase, astral microtubules grow from the cytoplasmic side of the SPBs. The spindle disassembles at telophase and the astral microtubules keep growing to form the interphase cytoplasmic microtubule array (Ovechkina et al., 2003).

1.2 **γ-tubulin**

α- and β-tubulin, which share about 40% amino acid identity, are the founding members of the tubulin superfamily of proteins and they were both discovered by biochemical methods. γ-Tubulin, the third member of this superfamily, which shares approximately 30% amino acid identity with α- and β-tubulin, was first discovered as the product of the *mipA* (microtubule-interacting protein) gene of *Aspergillus nidulans* by Oakley and Oakley (1989). The *mipA* locus was identified in a genetic screen for intergenic suppressors of a β-tubulin mutation that confers heat sensitivity and hyperstabilizes microtubules. Since then it has become clear that γ-tubulin is present in all
eukaryotes and is highly conserved (Oakley and Akkari, 1999). For example, the amino acid sequences of human and *Xenopus laevis* γ-tubulin are 98% identical, and *A. nidulans* and human γ-tubulin share 67.4% identity (Zheng et al., 1991; Oakley and Akkari, 1999). γ-Tubulin is functionally conserved, as well, as expression of human γ-tubulin in *S. pombe* cells lacking endogenous γ-tubulin supports viability and near-normal growth (Horio and Oakley, 1994).

Initial studies in *A. nidulans* demonstrated that γ-tubulin localizes at the SPBs (Oakley et al., 1990) and subsequent studies demonstrated that γ-tubulin is located at MTOCs in a variety of organisms. γ-Tubulin is associated with mitotic spindles in many animal cells as well as with midbodies in some types of dividing cells and the phragmoplast in plant cells (Horio et al., 1991; Joshi et al., 1992; Lajoie-Mazenc et al., 1994; Julian et al., 1993; Stearns et al., 1991; Zheng et al., 1991; Oakley and Akkari, 1999; Lambert, 1993). A great deal of γ-tubulin also exists in the cytoplasm as a soluble pool and it associates with the TCP-1 chaperonin complex or with γ-tubulin complex proteins (See 1.3) (Melki et al., 1993; Wiese and Zheng, 1999).

γ-Tubulin is required for MTOCs to nucleate mitotic spindle microtubules in many organisms such as *A. nidulans*, *S. pombe*, *S. cerevisiae*, *D. melanogaster*, *X. laevis* and mammalian cells (Oakley et al., 1990; Martin et al., 1997; Horio et al., 1991; Sunkel et al., 1995; Sobel and Snyder, 1995; Spang et al., 1996; Marschall et al., 1996; Joshi et al., 1992), though alternative, γ-tubulin-independent, pathways for microtubule assembly *in vivo* may exist (Job et al., 2003). Perturbing γ-tubulin function also inhibits cytoplasmic microtubule assembly from MTOCs (Joshi et al., 1992; Ahmad et al., 1994). Evidence that γ-tubulin may be involved in regulating the dynamics of microtubules, in organizing
spindle microtubules and cytoplasmic microtubule arrays, in interactions with motor proteins, and in coordinating mitotic events, has also emerged more recently (Paluh et al., 2000; Prigozhina et al., 2004; Vogel and Snyder, 2000; Jung et al., 2001; Prigozhina et al., 2001; Vogel et al., 2001; Vardy et al., 2002; Sampaio et al., 2001; Hendrickson et al., 2001).

1.3 γ-tubulin complex proteins in other eukaryotes

γ-Tubulin forms complexes with other proteins in almost all organisms examined so far. Stearns and Kirschner (Stearns and Kirschner, 1994) first demonstrated that γ-tubulin is present in a large complex of approximately 25S in the cytoplasm of frog eggs and vertebrate somatic cells. Zheng et al. (1995) purified this complex in an active form and demonstrated that it has a 25 nm open ring structure and contains at least seven different proteins. Similar γ-tubulin ring structures were revealed at the minus end of the microtubule from Drosophila centrosomes by immuno-electron microscopic tomography (Moritz et al., 1995). In Drosophila embryo extracts, γ-tubulin was found in two distinct complexes, the γ-tubulin small complex (γ-TuSC) composed of γ-tubulin, and proteins designated Dgrip84 and Dgrip94, and a larger complex called the γ-tubulin ring complex (γ-TuRC), which is formed of γ-TuSCs and three additional proteins, Dgrip75, 128, and 163 (Moritz et al., 1998; Oegema et al., 1999; Gunawardane et al., 2000). γ-TuRCs are the major γ-tubulin complexes in human cells and its components, named hGCP2-6, have been determined to be homologs of Drosophila Dgrips (Detraves et al., 1997; Fava et al., 1999; Murphy et al., 2001). The two other γ-TuSC components in S. cerevisiae and S. pombe were discovered through genetic screens (Geissler et al., 1996; Knop et al., 1997;
The homologs of other γ-TuRC-components have been found in *S. pombe* but are not present in *S. cerevisiae* (Fujita et al., 2002; Venkatram et al., 2004; Anders et al., 2006). Table 1.1 summarizes γ-tubulin complex components in major model organisms. I will use “GCPs” to refer to γ-tubulin complex proteins. I will reserve the term γ-TuRC for cases in which the complex has been shown by electron microscopy to be a ring complex and the more general term “large γ-TuC” for cases in which a large γ-tubulin complex has been shown to exist but the structure has not been studied by electron microscopy. In most cases the γ-TuRC and the large γ-TuC are likely to be the same.

Functional studies have shown that the γ-TuRC nucleates microtubules much more efficiently than the γ-TuSC *in vitro* and that the γ-TuRC disassembles in 500 mM KCl, releasing γ-TuSCs (Moritz et al., 1998; Oegema et al., 1999). Examination of the *Drosophila* γ-TuRC by electron microscopy led to a lockwasher model in which the ring wall is composed of repeating V-shaped γ-TuSCs with γ-tubulin contacting the minus end of microtubule protofilaments and the other GCPs comprising a cap asymmetrically topping the ring and attaching the γ-TuRC to the centrosome (Figure 1.3) (Moritz et al., 2000). Based on a great deal of data, it has been commonly accepted that in animal cells γ-TuRCs are assembled from γ-TuSCs and other GCPs in the cytoplasm and are recruited to the centrosome where they serve as the major microtubule nucleators (Moritz et al., 1995; Moritz et al., 1998; Wiese and Zheng, 1999; Wiese and Zheng, 2006; Schnackenberg et al., 1998).

The individual γ-TuSC components have been shown to be essential for cell viability and normal mitotic spindle assembly and function in many organisms (Oakley et
al., 1990; Colombie et al., 2006; Knop et al., 1997; Geissler et al., 1996; Spang et al., 1996; Martin et al., 1997; Vardy and Toda, 2000; Barbosa et al., 2000; Knop and Schiebel, 1997; Paluh et al., 2000; Hannak et al., 2002). However, the data from the functional analyses of the large γ-TuC-specific GCPs are contradictory in some cases and show apparent differences among species that have prevented the emergence of a coherent model for GCP4-6 function.

There are apparent differences with respect to the roles of the GCPs in γ-TuC assembly and localization. For example, Zhang et al. (Zhang et al., 2000) found that the *X. laevis* GCP6 homolog, Xgrip210, is required for assembly of the γ-TuRC and for localization of γ-tubulin and Xgrip109, the *Xenopus* GCP3 homolog, to the centrosome. In *D. melanogaster*, on the other hand, depletion of Dgrip163 (GCP6) did not inhibit localization of γ-TuSC components to the centrosome although Dgrip163 depletion did inhibit γ-TuRC formation (Verollet et al., 2006). Although the GCP6 homolog is apparently required for assembly of the γ-TuRC in *X. laevis* and *D. melanogaster* (Verollet et al., 2006; Zhang et al., 2000), this seems not to be the case for the large γ-TuC in *Schizosaccharomyce spombe* (Fujita et al., 2002). Xgrip109 (GCP3) is apparently required for localization of Xgrip210 (GCP6) to the centrosome in *X. laevis* (Zhang et al., 2000), but temperature-sensitive alleles of Alp4 (GCP2) and Alp6 (GCP3) did not affect the localization of Gfh1 (GCP4) or Alp16 (GCP6) to the SPB in *S. pombe* (Venkatram et al., 2004). These differences could reflect differences in GCP6 function among species, but there are also apparently contradictory findings within species. In *D. melanogaster*, for example, large γ-tubulin complexes were found in ovarian extracts of flies with null alleles of Dgrip75 (GCP4) or Drip128 (GCP5) (Vogt et al., 2006), but a subsequent study
found that depletion of Dgrip75 or Dgrip128 caused a strong reduction of γ-TuRCs in a D. melanogaster cell line (Verollet et al., 2006). In S. pombe, as mentioned, a deletion of the GCP6 homolog did not apparently affect the size or assembly of the large γ-TuC (Fujita et al., 2002), but subsequent data suggests that deletion of the GCP6 homolog should, minimally, cause the loss of GCP4 and 5 homologs and, thus, a reduction in the size of the complex (Anders et al., 2006). There is also controversy as to whether the major γ-TuCs in S. pombe are the 22S large complexes or the ~8-9S small complexes (Fujita et al., 2002; Anders et al., 2006; Venkatram et al., 2004). Finally, it should be remembered that the Saccharomyces cerevisiae genome does not contain identifiable homologs of GCP4-6 although it has γ-TuSCs, (also known as the Tub4p complex) (Geissler et al., 1996; Marschall et al., 1996; Knop et al., 1999; Knop and Schiebel, 1997). In spite of the absence of GCP4-6, 22S high-salt-resistant γ-TuCs can be identified in S. cerevisiae extracts under certain buffer conditions (Vinh et al., 2002).

The functions of GCP4-6 in microtubule nucleation and function are also unclear. In S. pombe, neither single deletions or simultaneous triple deletions of Gfh1p (GCP4), Mod21p (GCP5) and Alp16p (GCP6) are lethal nor do they inhibit the formation of mitotic spindles or spindle elongation (Anders et al., 2006; Fujita et al., 2002; Venkatram et al., 2004). In D. melanogaster, Verollet et al. showed that the simultaneous depletion of Dgrip75 (GCP4), Dgrip128 (GCP5), Dgrip163 (GCP6) and another γ-TuC associating protein, Dgp71WD, did not prevent the centrosomal targeting of the γ-TuSC nor did it severely inhibit basic microtubule assembly activities (Verollet et al., 2006). Flies with either mutated Dgrip75 (GCP4) or Dgrip128 (GCP5) are viable but meiosis is defective and a Dgrip75 (GCP4) mutant altered localization of bicoid RNA in oogenesis (Vogt et
al., 2006; Schnorrer et al., 2002).

Since GCP4-6 homologs are found in evolutionarily distant organisms (Wiese and Zheng, 2006), it follows that they must have significant functions, but the nature of those functions is not yet clear. A comprehensive analysis of GCP function in a single organism is warranted to generate consistent data that may be useful to create a coherent model for GCP functions.

1.4  *A. nidulans* γ-tubulin mutant *MipAD159*

γ-Tubulin is well known to play a central role in the nucleation of microtubule assembly in eukaryotes but its localization to the mitotic spindle in mammalian cells has lead to speculation that it may have additional non-nucleation functions (Lajoie-Mazenc et al., 1994). In fact, experimental evidence from *S. pombe, S. cerevisiae, A. nidulans,* and mammalian cells has indicated that γ-tubulin complex proteins may be involved in the regulation of microtubule dynamics, cytoplasmic microtubule organization and mitotic events (Vogel and Snyder, 2000; Vogel et al., 2001; Hendrickson et al., 2001; Jung et al., 2001; Prigozhina et al., 2001; Cuschieri et al., 2007; Vardy et al., 2002; Sampaio et al., 2001; Prigozhina et al., 2004; Fujita et al., 2002; Paluh et al., 2000).

The *A. nidulans* *mipAD159* mutant was created in a clustered charged-to-alanine scanning mutagenesis that aimed to create conditionally lethal alleles of γ-tubulin (Jung et al., 2001). The mutation changes aspartic acid at amino acid (aa) 159 and the arginine at aa 160 to alanine. It does not prevent γ-tubulin from localizing to the SPBs, but causes tight cold sensitivity. At a restrictive temperature of 20°C, *mipAD159* causes spindle
abnormalities including split, multipolar, and bent spindles, and obvious polyploid nuclei are common (Jung et al., 2001). Later data from reciprocal block experiments and live imaging revealed that the primary defects caused by \textit{mipAD159} are not the inhibition of mitotic spindle formation, instead, the coordination of late mitotic events were disrupted, leading to severe mitotic defects such as precocious mitotic exit even in the absence of microtubules (Prigozhina et al., 2004). The spindle defects noted earlier were almost certainly the consequence of failed mitosis rather than the cause. These results indicate that \(\gamma\)-tubulin has a microtubule-independent role in mitotic regulation and led to our examination of the localization of mitotic regulatory proteins in the \textit{mipAD159} mutant in the hope of revealing the nature of the mitotic mis-regulation and clarifying the role of \(\gamma\)-tubulin in mitotic regulation.

Surprisingly, we found that three proteins, cyclin B, Cdk1, and AnCdc14 were absent from a subset of nuclei in hyphae carrying \textit{mipAD159}. The nuclei lacking these proteins were removed from the cell cycle while the other nuclei keep cycling (Nayak et al., 2010). Cyclin B is the only essential cyclin in \textit{A. nidulans}, and it is encoded by the \textit{nimE} gene (Morris, 1975; O'Connell et al., 1992; Osmani et al., 1994). In \textit{A. nidulans}, both Cdk1 and Cyclin B are required for progression through G1 and G2 (Osmani et al., 1994). Early research using frog extracts showed that destruction of Cyclin B is required for the initiation of anaphase (Holloway et al., 1993) and subsequent investigations of the destruction process revealed a ubiquitin ligase complex, the anaphase-promoting complex/cyclosome (APC/C) (King et al., 1995; Sudakin et al., 1995). The APC/C recognizes cyclin B through a special amino acid sequence on cyclin B known as the destruction box or D-box. APC/C activation during mitosis causes polyubiquitination of
cyclin B and polyubiquitinated cyclin B is subsequently degraded by the 26S proteasome (Castro et al., 2005) (Figure 1.4). In the following cell cycle, the APC/C must be inactivated to allow the accumulation of S-phase cyclins [cyclin B in *A. nidulans* since cyclin B and Cdk1 are required for DNA synthesis in *A. nidulans* (Osmani et al., 1994)]. Because cyclin B and AnCdc14 both are substrates of the APC/C and proteasomes, examining the localizations of APC/C and 26S proteasome might shed light on the mechanism by which *mipAD159* causes mis-regulation of the destruction processes.

1.5 Proteasomes

Proteasomes are ATP-dependent proteinase complexes that are ubiquitous in all forms of life. The proteasomes in archaea and bacteria are 20S proteasomes and the 26S proteasome is conserved among eukaryotes. The 26S proteasome rapidly degrades proteins tagged with polyubiquitin chains by ubiquitin ligases. This is used to prevent the accumulation of mis-folded polypeptides and to control diverse cellular events such as cell-cycle progression, DNA repair, apoptosis, signal transduction, transcription, and metabolism. As increased proteasome activity has been observed in tumor cells and decreased proteasome activity has been suggested to be a contributor to aging and neurodegenerative diseases, understanding the regulation of proteasome activity is of clinical importance (Murata et al., 2009).

The 26S proteasome is about 2.5 MDa and is comprised of the 20S core particle (CP) which is approximately 700 kDa in mass and catalyzes proteolysis and the 19S regulatory particle (RP; also called PA700) which is approximately 900 kDa and is involved in binding of ubiquitinated proteins, de-ubiquitination of protein substrates,
unfolding of globular polypeptides and facilitating the entry of polypeptides into the 20S core (Murata et al., 2009). The CP is a cylindrical particle formed of four stacked heteroheptameric rings: two outer α-rings and two β-rings, each composed of seven distinct α- and β-subunits (α_{1-7} and β_{1-7}), respectively. The structure of the 20S proteasome from *S. cerevisiae* is known and the size and shape is similar to the 20S proteasome of the archaebacterium *Thermoplasma acidophilum*, which is about 148 Å in length with a maximum diameter of 113Å (Groll et al., 1997). The RP consists of two subcomplexes: the base subcomplex that is composed of 6 different AAA+ ATPase subunits (RPT) and 3 non-ATPase subunits (RPN) and the lid subcomplex that is comprised of 9 non-ATPase subunits. The RP attaches at either or both ends of the 20S CP to form the 26S proteasome (Figure 1.5). Although vertebrates have two additional subtypes of 20S proteasomes, immunoproteasomes and thymoproteasomes, no subtypes of proteasomes have been found in lower eukaryotes to date (Murata et al., 2009).

The 26S proteasome is assembled in an ordered pathway with the assistance of many chaperones. The assembly begins with the formation of a α-ring and the subsequent incorporation of β-subunits to form a half-proteasome. Two half-proteasomes dimerize and mature into an active 20S proteasome. Findings from yeast suggest that the assembly of the base and the lid of the RP are independent (Isono et al., 2007), but the exact sequence of events is unclear. Whether a lid and a base complete an RP first and then join the CP to form the 26S proteasome or the CP serves as a template for base formation and the subcomplex is joined by a lid to complete a 26S proteasome also remains controversial (Besche et al., 2009).

Early studies using various immunoeytochemical labeling and subcellular
biochemical fractionation methods revealed that the 26S proteasome is present both in the
nucleus and in the cytoplasm in many different cell types, but their relative abundance
within those compartments varies among cell types. Despite some discrepancies in initial
reports, the general pattern of distribution of the proteasomes consists of a uniform
labeling of the cytoplasm with an exclusion from vacuolar structures and a slightly
stronger labeling of the nuclei with an exclusion from nucleoli. Most proteasomes appear
to be diffusely distributed in the cytoplasm but there is some enrichment in the
centrosomes, the cytoskeletal elements, the outer surface of endoplasmic reticulum, and
microsomes (Palmer et al., 1996; Rivett et al., 1992; Wojcik and DeMartino, 2003;
Rivett, 1998). In the nucleus, the proteasomes have been reported to be localized in the
euchromatin regions, the periphery of heterochromatin and around the nucleolus (Rivett
et al., 1992; Wojcik and DeMartino, 2003). Proteasomes have been reported to be loosely
associated with the nuclear matrix in rat hepatocytes but tightly associated in mouse
myoblasts. Upon interferon-γ induction in mammalian cells, a fraction of the nuclear
proteasomes, mainly immunoproteasomes, becomes associated with the PML nuclear
bodies (Palmer et al., 1996; De Conto et al., 2000; Rivett et al., 1992; Wojcik and
DeMartino, 2003; Rivett, 1998).

The intracellular distribution of proteasomes changes in higher eukaryotes during
 cell cycle progression. In immortalized ovarian granulosa cells, the proteasomes
accumulate around the condensing chromosomes in early mitosis, associate mainly with
spindle fibers in late anaphase, and persist in the nuclei in telophase and early interphase
(Amsterdam et al., 1993). In HeLa cells, tight association of the proteasomes with the
spindle poles and the midbodies was also detected (Wojcik et al., 1995). However, a
study of human fibrosarcoma cells using a GFP-tagged immunoproteasome-specific 20S core subunit, LMP2, showed that proteasomes diffuse freely through dividing cells and probably are transported unidirectionally over the nuclear membrane into daughter nuclei after mitosis (Reits et al., 1997). In *S. pombe*, which undergoes a closed mitosis without breakdown of the nuclear envelope, the proteasome localizes predominantly at the inner side of the nuclear periphery both in interphase and throughout mitosis. Intriguingly, the proteasome appears more dispersed in meiosis I during mating and becomes restricted between the separating chromosomes in meiosis II before dispersing and then reappearing around the nuclei of the spores in the ascus (Wilkinson et al., 1998). Early reports described the localization of the proteasome in actively dividing budding yeast cells as being either in the nucleoplasm or associated with the nuclear envelope (Wendler et al., 2004; Russell et al., 1999; Isono et al., 2007; Enenkel et al., 1998; McDonald and Byers, 1997), but a recent study using a strain expressing a GFP-tagged CP subunit, Pre6p, confirmed the nucleoplasmic localization of the proteasome and suggested that the enrichment of the proteasome at the nuclear periphery could be induced by starvation, chemical fixation, and cell wall stresses (Laporte et al., 2008).

Observations on the localization of the proteasome in *S. cerevisiae*, which has a closed mitosis, and in post-mitotic mammalian neuron cells and rat ovarian granulosa cells undergoing apoptosis all suggested that the proteasome or proteasomal subunits is transported through nuclear pores (Wojcik and DeMartino, 2003). However, as the effective diameter of the NPC channel for passive diffusion is up to 9 nm (Gorlich and Kutay, 1999), the nuclear localization of the proteasome must rely on active transport.

Studies in fission yeast using mutants of either lid or base subunits have revealed
that the components of the base, the lid and the CP are transported and assembled in the nucleus independently before these subcomplexes are joined (Isono et al., 2007), but how the subcomplexes or the proteasomal subunits are transported across the nuclear pores remains unclear although several studies indicate that importin α/karyopherin α/Srp1, the classic nuclear localization sequence (cNLS) receptor, could play an important role (Knuehl et al., 1996; Wendler et al., 2004; Lehmann et al., 2002; Wang et al., 1997). Several putative cNLS in proteasomal α subunits function in directing chimeric proteins into the nucleus (Knuehl et al., 1996; Wang et al., 1997) and the precursors of the 20S proteasome accumulate in the cytoplasm in the srp1-49 mutant at restrictive temperatures (Lehmann et al., 2002). However, other studies argued against that the NLS is sufficient to effect the nuclear transport of the proteasome and suggested that phosphorylation could be important in the transport of some proteasomal subunits (Benedict and Clawson, 1996; Rivett, 1998; Mayr et al., 1999).

Whether importin α/karyopherin α/Srp1 is directly involved in nuclear import of proteasome precursors also remains unclear. The srp1-49 mutation prevented the precursors of the 20S proteasome to localize to the nucleus, but the specificity of srp1-49 in effecting the cNLS-dependent transport has been questioned in other studies. First, the srp1-49 mutant exhibits phenotypes quite distinct from the srp1-31 mutant which shows much stronger and clearer defects in NLS binding and protein import (Yano et al., 1994). Second, although the protein degradation defects caused by the srp1-49 mutation can be suppressed by overexpression of either RPN11, a regulatory particle of the 26S proteasome or STS1/DBF8, a protein involving in the ubiquitin/proteasome pathway (see 1.7), the protein degradation defects are srp1-49 allele-specific and were not found in
srp1-31. This suggests a role for Srp1 in regulation of protein degradation separate from its known role as the NLS receptor (Tabb et al., 2000). Although both the srp1-49 and the srp1-33 mutations cause mitotic arrest, whether the arrest is due to protein degradation defects is also controversial because different labs observed opposite extents of mitotic arrest between the two mutants. Some detected more severe mitotic arrest in srp1-49 mutants, and thus interpreted the mitotic arrest to be a consequence of defects in nuclear import of hypothetical cell cycle regulatory proteins, while others observed more severe mitotic arrest in srp1-49 mutants, and interpreted it to be a result from defects in the function related to protein degradation. (Loeb et al., 1995; Tabb et al., 2000).

1.6 Cut8/STS1/DBF8

Cut8 is a nuclear envelope protein in S. pombe. The cut8 gene was named for a mutant in which nuclear division but not cytokinesis is blocked thus causing a cut (Cell Untimely Tom) phenotype (Hirano et al., 1986). The cut8 mutant exhibits chromosome mis-segregation defects and mitotic delay (Tatebe and Yanagida, 2000), and overexpression of Cut1 (separase) suppressed the mutant phenotypes (Samejima and Yanagida, 1994). As the destruction of poly-ubiquitinated mitotic cyclin and Cut2/securin was inhibited in the cut8 mutant and the 26S proteasome was found to be mis-localized from the nucleus to the cytoplasm, the function of Cut8 appeared to be directly linked to the nuclear localization of the 26S proteasome (Tatebe and Yanagida, 2000).

To further characterize the role of Cut8 in regulating proteasome localization, Yanagida and colleagues demonstrated that Cut8 is a short-lived nuclear protein with a half-life of 5 minutes, that is degraded by the 26S proteasome in the nucleus and that the
polyubiquitination of Cut8 is required for its interaction with the proteasome and the nuclear enrichment of the proteasome (Tatebe and Yanagida, 2000). They also found that Rhp6/Ubc2, a ubiquitin-conjugating E2, and the E3 ligases Ubr1 and Rhp18 were responsible for the ubiquitination of Cut8 and, therefore, for the nuclear accumulation of the proteasome. Based on these data, they proposed that Cut8 is a substrate of the proteasome and a dosage-dependent sensor and anchor for the proteasome in the nucleus. In their model, when Cut8 becomes scarce due to degradation by the proteasome, the concentration of proteasomes within the nucleus will be reduced, which will then feed back to allow the accumulation of Cut8 in the nucleus and subsequently more proteasomes to be tethered to the nuclear membrane (Figure 1.6) (Takeda and Yanagida, 2005). This anchoring model is supported by recent photobleaching experiments on the nuclear import of proteasomes, as the rate of proteasome nuclear import is reduced and the rate of proteasome nuclear export is increased in cut8Δ cells compared to wild type cells (Cabrera et al., 2010).

The S. cerevisiae homolog of S. pombe Cut8 protein is STS1/DBF8. The sts1 gene (Sec Twenty-three Suppressor) in S. cerevisiae was identified as an extragenic multicopy suppressor of a sec23 mutant, which is defective in ER-to-Golgi transport and shows ribosomal RNA instability (Liang et al., 1993). The identical gene was independently discovered and named dbf8 (Dumbbell Formers) in a screen for mutants that arrest as large-budded cells at restrictive temperatures (Houman and Holm, 1994). Although the sequence similarity of Cut8 and STS1/DBF8 is weak, they both have a short half-life [only a few minutes (Romero-Perez et al., 2007; Tatebe and Yanagida, 2000)] and dbf8 mutants also exhibit high levels of chromosome nondisjunction and a severe delay in
mitotic progression (Houman and Holm, 1994). Several studies in *S. cerevisiae* also suggest that STS1/DBF8 is involved in regulating the ubiquitin/proteasome pathway. First, overexpression of either RPN11 or STS1 suppressed the protein degradation defects in the *srp1-49* mutant and STS1 interacts with RPN11 and SRP1 (Tabb et al., 2000). Second, *sts1* mutants accumulated high levels of proteasome substrates and overexpression of STS1 suppressed the growth defects of a *rad23Δ, rpn10Δ* double deletion but not the DNA repair defect of *rad23Δ* (Romero-Perez et al., 2007).

In spite of reported functional similarities between Cut8 and STS1/DBF8, there are discrepancies suggesting that they may function through distinct mechanisms. *Sts1/dbf8* is an essential gene in *S. cerevisiae*, whereas *cut8* is dispensable for viability in *S. pombe*. *Cut8Δ* is sensitive to DNA damage and Cut8 is required for DNA damage repair (Takeda and Yanagida, 2005; Kearsey et al., 2007), whereas an *sts* mutant apparently does not display sensitivity to UV light (Romero-Perez et al., 2007). The accumulation of multiubiquitinated proteins is intense in an *sts1* mutant but not in a *cut8* mutant (Romero-Perez et al., 2007; Tatebe and Yanagida, 2000). Moreover, the ubiquitination of Cut8 is required for the interaction between Cut8 and proteasomes in *S. pombe*, whereas unconjugated STS1 was detected with proteasomes purified from *S. cerevisiae* (Romero-Perez et al., 2007; Takeda and Yanagida, 2005).

Besides the unicellular fungi, putative homologs of Cut8 were also found in the filamentous fungus *Neurospora crassa*, in *Drosophila melanogaster* and *D. pseudoobscura*, in the mosquito *Anopheles gambiae*, and in the slime mold *Dictyostelium discoideum*. The *Drosophila* homolog of Cut8 was also required for the nuclear enrichment of the proteasome (Takeda and Yanagida, 2005), suggesting that the
proteasome localization in the nucleus could be due to a conserved mechanism.

1.7 Nuclear division in A. nidulans and other eukaryotes

Mitosis varies among organisms and can be classified into several types with regard to the behavior of the nuclear envelope. Closed mitosis is exemplified by unicellular fungi such as S. cerevisiae and S. pombe, in which the nuclear envelope remains intact, the spindle forms within the nucleus and the segregated chromosomes are encompassed by the nuclear envelope as it splits to form daughter nuclei. Mammalian and higher plant cells engage in open mitosis, in which the nuclear envelope breaks down at mitotic onset, remains disassembled until telophase and then reforms around the segregated chromosomes. This type of mitosis might have evolved because centrosomes are cytoplasmic and the disassembly of the nuclear envelope is required to facilitate the interaction of microtubules with kinetochores. Between the extremes of closed and open mitosis, several other organisms, including Aspergillus nidulans, are known to have variants of semi-open mitosis. For example, in Drosophila early embryos in which nuclei undergo mitosis synchronously in syncytia, the nuclear envelope breaks down only near the centrosome at early mitosis to allow the microtubules to reach the kinetochores within the nucleus and then completely breaks down after the onset of chromosome segregation [Paddy et al., 1996; Kiseleva et al., 2001 and reviewed in (De Souza and Osmani, 2009)]. In the basidiomycete Ustilago maydis, in which SPBs are embedded within the nuclear envelope in interphase, the nuclear envelope ruptures at the tip and remains in the mother cell while the SPBs and chromosomes migrate into the bud cell and form a spindle in the cytoplasm [Theisen et al., 2008 and reviewed in (De Souza and Osmani, 2009)].
*Aspergillus nidulans* engages in a different form of semi-open mitosis in which the nuclear pore complexes (NPCs) partially disassemble during mitosis increasing the permeability of the nuclear envelope and the exchange of molecules between the cytoplasm and nucleoplasm, and, thus, allowing the spindle to form within the nucleus (De Souza et al., 2004). As the complete breakdown of the nuclear envelope in mammalian cells involves a stepwise disassembly of the NPCs, studies of the mechanisms of the partial disassembly of the NPCs in *A. nidulans* and how these mechanisms fit into the regulation of other cell cycle events may help us to understand cell cycle regulation in higher eukaryotes because many of the regulatory mechanisms may be conserved. For example, it has been found that nuclear pore proteins or Nucleoporins (Nups) undergo mitotic-specific phosphorylation in both *A. nidulans* and higher eukaryotes (Glavy et al., 2007; De Souza et al., 2004; Dephoure et al., 2008; Laurell et al., 2011).

NPCs are composed of distinct subcomplexes (Figure 1.7 a). The outer ring of the NPC is formed of Nup84 subcomplexes, the largest subcomplexes, and the inner ring is formed of Nup170 subcomplexes. Together, they form the core scaffold that is anchored by transmembrane Nups at the curved surface of the nuclear envelope. The central channel of the nuclear pore is occupied by Nups containing phenylalanine-glycine (FG) repeats (FG Nups), which create a diffusion barrier. FG Nups are tethered to the core scaffold by linker Nups. At the cytoplasmic side of the NPC, peripheral Nups form filamentous structures (cytoplasmic filaments), while at the nucleoplasmic side of the NPC, peripheral Nups form a basket-like structure (Nuclear basket) (De Souza and Osmani, 2009). In *A. nidulans*, when nuclei enter mitosis, the NPCs undergo partial
disassembly. The peripheral Nups, FG Nups, and linker Nups disperse and this removes the diffusion barrier, but the NPC core scaffold and the transmembrane Nups remain in the nuclear envelope (Figure 1.7 b) (Osmani et al., 2006a; De Souza et al., 2004). The *A. nidulans* Nup84 complex (An-Nup84-120 complex) is more similar to that of higher eukaryotes than *S. cerevisiae* with respect to composition and mitotic behavior. The An-Nup84-120 complex contains two additional Nups, Nup37 and ELYS, that are not found in *S. cerevisiae* and which were thought to be vertebrate specific (Liu et al., 2009). Deletion of both of An-*nup37* and An-*elys* caused the dispersal of the An-Nup84-120 complex from the NPC core scaffold during mitosis but only caused very slight mitotic defects (Liu et al., 2009).

The disassembly of NPCs during open and semi-open mitosis is not just required to eliminate the barrier to allow spindle formation within the nucleus, as some proteins that are released by this process have been found to have roles in regulating spindle assembly, in coordinating spindle assembly with chromosome segregation, and in nuclear reassembly. One example is the relocation of Mlp proteins from the NPC nuclear basket to a mitotic spindle matrix. Mlp proteins tether the spindle assembly checkpoint (SAC) proteins Mad1 and Mad2 to the nuclear basket in *S. cerevisiae*, *A. nidulans* and mammalian cells during interphase (Scott et al., 2005; Lee et al., 2008; De Souza et al., 2009). In both semi-open and open mitosis, Mlp proteins are also required for the proper mitotic localization and the fulfillment of the SAC functions of Mad1 and Mad2 (Figure 1.8) (Lee et al., 2008; De Souza et al., 2009; Lince-Faria et al., 2009).

The mechanism of segregation of the nucleolus at mitosis also varies among eukaryotes. Nucleoli are formed around specific genetic loci called nucleolar organizing
regions (NORs), which are composed of clusters of ribosomal DNA (rDNA) repeats (Boisvert et al., 2007). The nucleolus does not disassemble during closed mitosis but in open mitosis it disassembles and reassembles in parallel with the disassembly and reassembly of the nuclear envelope. *A. nidulans* has an intermediate form of nucleolar segregation, in which the nucleolus remains intact until chromosome segregation occurs. This process removes NORs from the nucleolus, and what is left between the reforming daughter nuclei, termed the nucleolar remnant, undergoes sequential disassembly during the telophase to G1 period. The released nucleolar proteins are imported into daughter nuclei to form new nucleoli in the following interphase (Ukil et al., 2009). It is known that this process can happen in the absence of a functional mitotic spindle (Ukil et al., 2009), but the nature of the driving force and how this process is regulated remains to be determined.

1.8 *A. nidulans* as a model system for genetics and cell biology research

*A. nidulans* belongs to the ascomycota phylum and has been one of the most important model organisms in eukaryotic genetics. It is normally haploid and undergoes asexual development, but it also undergoes sexual development that allows for conventional genetic analysis of meiotic progeny (Pontecorv and Roper, 1952; Todd et al., 2007). Heterokaryons and diploids can form through a parasexual cycle and be used for complementation analysis, dominance tests, and tests for cytoplasmic inheritance (Roper, 1952; Pontecorv and Roper, 1952; Todd et al., 2007). In addition, heterokaryons can also form during DNA-mediated transformation and this process has been successfully exploited to propagate deletants of essential genes (Martin et al., 1997;
Osmani et al., 2006a; Oakley et al., 1990; Osmani et al., 1988). Diploids can haploidize either spontaneously at a low frequency or they can be stimulated to haploidize at higher frequencies by treatment with chemicals interfering with mitotic spindles. In this process, the diploid loses chromosomes randomly until haploidy is reached, which makes it possible to map a mutation to a chromosome through linkage analysis or to assess the haploid lethality of a recessive mutation (Todd et al., 2007).

Many important discoveries, particularly in the areas of cell cycle regulation and cytoskeleton research, have been made using *A. nidulans* as an experimental organism. For example, Dr. N. Ronald Morris and colleagues conducted some of the first genetic studies on the cell cycle and they led to the discovery of genes that affect stages of the mitotic process such as *bim* (blocked in mitosis) and *nim* (never in mitosis) genes, genes that affect septation like the *sep* genes, and genes that affect nuclear distribution like the *nud* genes (Morris, 1975). Moreover, screening suppressors of a *benA* mutant uncovered *mip* genes (Weil et al., 1986) and the *mipA* gene was found to encode a previously undiscovered member of the tubulin superfamily, which was named γ-tubulin (Oakley and Oakley, 1989). Homologs of these genes discovered in studies of *A. nidulans* were subsequently found in other model organisms, resulting in extensive research progress.

Recently a great deal of progress has been made that facilitates studies of *A. nidulans*. The first version of the genome sequence of *A. nidulans* was completed in 2005 (Galagan et al., 2005), allowing many genes to be identified on the basis of homology and, thus, to be cloned easily by PCR. The genome has already been through four major re-annotations, leading to many new findings (Wortman et al., 2009). Gene targeting efficiency was also largely enhanced owing to the development of fusion PCR techniques.
and the construction of strains deficient in nonhomologous end joining. Linear fragments generated through fusion PCR techniques can be directly used for transformation (Nayak et al., 2006; Szewczyk et al., 2006; Yang et al., 2004). It was demonstrated first in *Neurospora* that the efficiency of gene replacement can be largely enhanced by deletions of the homologs of human *KU70* and *KU80* that encode proteins functioning in nonhomologous end-joining of double-stranded DNA (Ninomiya et al., 2004). In *A. nidulans*, transformation using strains carrying a deletion of the *nkuA* gene (the homolog of *ku70* gene) also greatly reduced the frequency of heterologous integration so that approximately 90% of transformants carry a single homologous insertion (Nayak et al., 2006). These improvements in rapid construction of transforming fragments, and in the frequency of correct gene targeting in combination with improvements in transformation (Szewczyk et al., 2006), have made high-throughput studies of genes in *A. nidulans* feasible. For example, our labs have conducted systematic deletions and mitotic localization of the NPC proteins (Osmani et al., 2006) and the analysis of all protein phosphatase genes in *A. nidulans* (Son and Osmani, 2009).

### 1.9 Aims of my dissertation research

One of the aims of my dissertation research is to characterize and analyze the functions of the γ-tubulin complex proteins in *A. nidulans*. Although γ-tubulin was first discovered in *A. nidulans* and evidence that *A. nidulans* γ-tubulin is present in complexes with other proteins emerged more than ten years ago (Akashi et al., 1997), the identities of these γ-tubulin complex proteins remain elusive. The components of the γ-TuSC have been found to be highly conserved among *S. cerevisiae*, *S. pombe* and most higher
eukaryotes, whereas, the other components of the γ-TuRC are absent in *S. cerevisiae*. It will be interesting and worthwhile to determine the composition of γ-tubulin complexes in *A. nidulans*, as information in this regard are lacking for filamentous fungi, many of which are very important medically and commercially. Moreover, a comprehensive analysis of GCPs in a single organism is warranted for generating a coherent model of GCP function, since the data from previous studies of individual GCPs using different organisms are contradictory both among species and within species. The genome sequence of *A. nidulans* is available and I, thus, may find *A. nidulans* homologs of GCPs easily through BLAST searches. The efficient gene targeting system that has been developed for *A. nidulans* should facilitate a systematic deletion and tagging of genes encoding GCP candidates for *in vivo* functional analyses. In addition, the unique heterokaryon rescue technique that has been developed in *A. nidulans* should allow me to examine the phenotypes of lethal gene deletions. I will describe the progress I made on this project in Chapter 3.

The second project of my dissertation research began from analyzing the localization of the proteasome in wild type, *mipAD159*, and *mipAΔ* *A. nidulans* cells. Our studies of *mipAD159* indicated that γ-tubulin plays some role in regulating mitotic events (Prigozhina et al., 2004) and we had hypothesized that some mitotic regulatory proteins might be mis-localized in *mipA* mutants. Further investigation of *mipAD159* mutant revealed that the degradation of cyclin B in a subset of nuclei within a cell was mis-regulated due to a nuclear-autonomous failure of inactivation of the APC/C between late mitosis and S (Nayak et al., 2010). Because cyclin B and several other mitotic regulatory proteins are substrates of the APC/C and their ubiquitinated forms are degraded by the
proteasome, we were interested in determining if localization of the proteasome was altered in mipA mutants. My examination of proteasome localization, however, revealed that, surprisingly, the proteasome seems to be bound to an undefined nuclear structure in A. nidulans, and the regulation of the inheritance of the nuclear structure can be disrupted in aberrant mitosis. In order to characterize the nature of the nuclear structure, I took both a candidate approach and an S-tag affinity purification approach. One candidate, the A. nidulans homolog of S. pombe Cut8, turned out to be interesting. Although my data does not support the notion that Cut8 serves as the anchor for the nuclear proteasome as the Yanagida group proposed for S. pombe Cut8 (Takeda and Yanagida, 2005), it is consistent with the S. pombe data in that A. nidulans Cut8 is important for the nuclear localization of the proteasome. Using cut8 deletants, I was able to observe some interesting mitotic phenomena for the first time. I will describe this project in detail in Chapter 4.
Table 1.1

Summary of γ-tubulin complex components in major model organisms

<table>
<thead>
<tr>
<th>Organism</th>
<th>S. cerevisiae</th>
<th>S. pombe</th>
<th>A. thaliana</th>
<th>D. melanogaster</th>
<th>X. laevis</th>
<th>H. sapiens</th>
<th>A. nidulans</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-tubulin</td>
<td>Tub4p</td>
<td>Tubg1</td>
<td>γ-tubulin</td>
<td>γ-tubulin</td>
<td>γ-tubulin</td>
<td>γ-tubulin</td>
<td>γ-tubulin</td>
</tr>
<tr>
<td>complex</td>
<td>Spc97</td>
<td>Alp4p</td>
<td>(two genes)</td>
<td>(two genes)</td>
<td>Xgrip110</td>
<td>hGCP2</td>
<td>?</td>
</tr>
<tr>
<td>components</td>
<td>Spc98</td>
<td>Alp6p</td>
<td>AtGCP2</td>
<td>Dgrip84</td>
<td>Xgrip109</td>
<td>hGCP3</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Gfh1p</td>
<td>AtGCP3</td>
<td>Dgrip91</td>
<td>Xgrip76</td>
<td>hGCP4</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Mod21p</td>
<td>AtGCP4</td>
<td>Dgrip75</td>
<td>Xgrip133</td>
<td>hGCP5</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Alp16p</td>
<td>?</td>
<td>Dgrip128</td>
<td>Xgrip210</td>
<td>hGCP6</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>NEDD1</td>
<td>Dgrip163</td>
<td>-</td>
<td>NEDD1/GCP-WD</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>Dgrip71WD</td>
<td></td>
<td></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>(Sobel and</td>
<td>(Horio et al., 1991;</td>
<td>(Liu et al., 1993;</td>
<td>(Zheng et al., 1991;</td>
<td>(Stearns et al., 1991;</td>
<td>(Stearns et al., 1991;</td>
<td>Oakley and</td>
</tr>
<tr>
<td></td>
<td>Snyder, 1995;</td>
<td>Fujita et al., 2002; Vardy and Toda, 2000; Venkatram et al., 2004; Anders et al., 2006)</td>
<td>Liu et al., 1994; Murphy et al., 2001; Erhardt et al., 2002)</td>
<td>Wilson et al., 1997; Oegema et al., 1999; Moritz et al., 1998; Gunawardane et al., 2000)</td>
<td>Stearns and Kirschner, 1994; Zhang et al., 2000; Tassin et al., 1998; Martin et al., 1998; Liu and Wiese, 2008)</td>
<td>Stearns et al., 1991; Lajoie-Mazenc et al., 1994; Detraves et al., 1997; Tassin et al., 1998; Fava et al., 1999; Murphy et al., 2001; Luders et al., 2006; Haren et al., 2006)</td>
<td>Oakley, 1989; Akashi et al., 1997</td>
</tr>
</tbody>
</table>

A putative homolog may exist according to literature, but the genes have not been identified and characterized.

-: No putative homolog has been found.

Note: An additional γ-tubulin complex component has been identified in several species: DgpWD in D. melanogaster, X-NEDD1 in X. laevis, and NEDD1 in A. thaliana and human.
Figure 1.1

The microtubule is formed by polymerization of tubulin heterodimers and can undergo depolymerization. Binding of a GTP-tubulin dimer to the tip of a protofilament induces the conversion of the GTP bound β-tubulin at the tip into a GDP bound form and increases the curvature of the protofilament. Lateral interactions between tubulins constrain the protofilaments within the microtubule lattice, keeping them relatively straight and allowing the growth of microtubules. However, the intrinsic curvature of GDP-tubulin creates an internal stress in the microtubule, which drives microtubule depolymerization. Microtubules are dynamic and can interconvert between growing and shrinking states frequently.
Figure 1. 2

Spindle pole bodies, the major fungal MTOCs (A), and the centrosome, the major MTOC in mammalian cells (B), are morphologically distinct, but functionally conserved. [A was adapted from (Crasta and Surana, 2006) and B is from (Doxsey, 2001)].
**Figure 1. 3**  
The lock washer model for microtubule nucleation by the $\gamma$-TuRC (Moritz et al., 2000). $\gamma$-Tubulin, Dgrip84, and Dgrip91 form the $\gamma$-TuSC, 6 of which are arranged in a ring with 25 nm diameter and serve as a template to nucleate a microtubule, the minus end of which interacts with $\gamma$-tubulin molecules. Dgrip75s, Dgrip128, and Dgrip163 bind to the other end of the ring to form the $\gamma$-TuRC.
Figure 1.4
Degradation of cyclin B through the APC/C-proteasome pathway. During mitosis, the APC/C is activated and polyubiquitinates its substrates, including cyclin B. Cyclin B with a polyubiquitin chain is recognized and subsequently destroyed by the 26S proteasome.
Figure 1. 5
Schematic diagram of the 26S proteasome (Murata et al., 2009). The 26S proteasome is composed of a 20S proteasome core particle and one or two 19S regulatory particles. The 20S proteasome core particle is formed of two α-rings that consist of α1-7 subunits and that locate at the top and the bottom and two β-rings that consist of β1-7 subunits that locate in the middle. The 19S regulatory particle is formed of a lid subcomplex that is comprised of 9 RPNs and a base subcomplex that is comprised of 3 RPNs and 6 RPTs.
The anchoring model of Cut8 for the nuclear proteasome in *S. pombe* (Takeda and Yanagida, 2005). Cut8 is enriched at the nuclear membrane where it is polyubiquitinated by Rhp6, Ubr1, and Rhp18. Polyubiquitinated Cut8 anchors the proteasome at the nuclear membrane and is degraded by the proteasome.
Figure 1. 7

Schematic diagrams of the nuclear pore complex. (a) Interphase NPCs are embedded in the nuclear envelope. (b) NPCs partially disassemble during *A. nidulans* mitosis. (De Souza et al., 2009)
Figure 1.8

Location of Mlp proteins, Mad1 and Mad2 in different forms of mitosis (De Souza et al., 2009). In *S. cerevisiae*, which undergoes closed mitosis, Mlp anchors Mad1 and Mad2 at the NPC nuclear basket during mitosis. In *A. nidulans* and human cells which undergo semi-open mitosis and open mitosis individually, Mlp anchors Mad1 and Mad2 at the spindle matrix at late mitosis.
Chapter 2: Materials and Methods

2.1 Strains

*A. nidulans* strains used in my studies are listed in Table 2.1.

2.2 Gene targeting and transformation

Gene targeting was achieved by transforming with linear DNA molecules. Transformation was carried out as described by Szewczyk et al. (2006). Transforming fragments consisted of a cassette flanked by two fragments amplified from *A. nidulans* genomic DNA. The three fragments were fused together by fusion PCR as previously described (Nayak et al., 2006; Szewczyk et al., 2006) (Figure 2.1 A-2.3 A) and the fusion PCR products were used to transform *nkuA*Δ strains which increased the frequency of insertion of the linear molecules into the correct sites through homologous recombination (Figure 2.1 B-2.3 B). To tag a target protein at its C-terminus with a fluorescent protein, two flanking DNA fragments were amplified from genomic DNA. One was from a region of the coding sequence of the target gene extending from about 1000 bp (or, in some cases 500 bp) upstream through the stop codon and the second was a similar sized fragment amplified from the 3’ untranslated region of the target gene. The region of the central cassette fused to the coding sequence of the target gene began with a sequence encoding a 5-glycine-alanine linker (Yang et al., 2004) fused in frame to the sequence encoding the fluorescent protein. Downstream of these, the cassette contained a gene
encoding a selectable marker (Figure 2.1 A).

The α-tubulin encoded by the tubA gene was fused with GFP at its N-terminus and expressed under its native promoter. This GFP-α-tubulin fusion construct consists of a selectable marker (A. fumigatus pyroA) followed by the normal tubA (α-tubulin) promoter, followed by the GFP coding sequence fused in frame to a glycine-alanine linker which was, in turn, fused to the tubA coding sequence. This construct was integrated at the wA locus.

To delete target genes, flanking DNAs were from the 5’ untranslated region and 3’ untranslated region of the target gene. The central cassette was simply the selectable marker gene (Figure 2.2 A). A. fumigatus pyrG (AfpyrG) (Weidner et al., 1998), A. fumigatus riboB (AfriboB) (Nayak et al., 2006), or A. fumigatus pyroA (AfpyroA) (Nayak et al., 2006) were used as selectable markers.

To replace the promoter of a target gene with the alcA promoter, one flanking DNA fragment was amplified from the 5’ untranslated region of the target gene and a second fragment was amplified that contained the coding sequence of the target gene beginning with the start codon. The central cassette contained a selectable marker followed by the alcA promoter sequence (Figure 2.3 A).

2.3 Diagnostic PCR and southern hybridizations

Genomic DNA from transformants was prepared as described (Lee and Taylor, 1990) or by using the Promega Wizard Plus Minipreps DNA Purification system. Correct transformants were initially confirmed through diagnostic PCR using outside primers, for example, the P1 and P2 primers in figures 2.1-2.3. The presence of correct integrations
and the absence of extra integrations of transforming DNAs were also verified by Southern hybridizations in dried agarose gels as described previously (Oakley et al., 1987). The full-length transforming DNA fragments were radioactively labeled and used as probes.

2.4 Microscopy and imaging

Cells were grown in selective media at 25°C and observed using four or eight-chamber Lab-Tek chambered cover glasses (Nalge Nunc International, Naperville, IL). We used four imaging systems. Two were Olympus IX71 inverted microscopes equipped with mercury light sources, and Prior shutters and filter wheels. One was equipped with a Hamamatsu ORCA ER camera, and the other with a Hamamatsu ORCA ERAG camera. We used Semrock GFP/DsRed2X2M-B dual band “Sedat” filter sets (459-481 nm bandpass excitation filter for GFP and a 546-566 nm excitation filter for mCherry and tdTomato), dual reflection band dichroic (457-480 nm and 542-565 nm reflection bands, 500-529 and 584-679 transmission bands) and two separate emission filters (499-529 nm for GFP and 580-654 nm for mCherry and tdTomato). Images were acquired with an Olympus 60X 1.42 N.A. plan apochromatic objective using Slidebook software (Intelligent Imaging Innovations, Denver, CO) or Volocity software (Perkin Elmer) installed on PowerMac computers. Some Z-series stacks were deconvolved using Slidebook software. For time-lapse two-channel imaging of live cells, Z-series stacks were collected at each time point and maximum intensity projections from all time points were combined to generate movies with Slidebook software. The third system was a PerkinElmer UltraviewVoX system mounted on an Olympus IX71 microscope equipped
with a constant temperature chamber. The system used three solid state lasers (405 nm, 488 nm and 561 nm) and was controlled by Volocity software running on a PowerMac computer. A 60X 1.42 plan apochromatic objective was used for image acquisition. The fourth system was an UltraView ERS system mounted on a Nikon Eclipse TE 2000-U microscope equipped with a Hamamatsu ORCA-AG camera and 488nm argon-ion laser and 568 nm krypton-argon-ion laser. The system was controlled by UltraView ERS software running on a Windows PC computer. A 60X 1.49 TIRF objective lens was used for image acquisition.

2.5 Immunoprecipitation and sucrose gradient sedimentation and Western Blotting

Cytoplasmic extracts of \textit{A. nidulans} were prepared by the method of Akashi et al. (1997) with minimal modification. Conidia were inoculated at a concentration of $2 \times 10^6$ per ml in YG medium and incubated for 20 hours at 37ºC, shaking at 140 rpm. The hyphae were then harvested, frozen immediately in liquid nitrogen, and disrupted by cryoimpaction, producing a frozen powder (Smucker and Pfister, 1975). The powder was suspended in extraction buffer [10 mM HEPES, pH7.4, 1.0 mM MgCl$_2$, 0.1 mM CaCl$_2$, 0.1 M KCl, 50 mM Sucrose, 0.5 mM PMSF, supplemented with protease inhibitors (Complete Mini EDTA-free, Roche)]. Extracts were clarified by centrifugation at 10,000 X g for 15 minutes at 4ºC and the supernatants were collected and the protein concentration was determined.

For immunoprecipitation, 4 µl of affinity-purified rabbit polyclonal $\gamma$-tubulin antibody (Oakley et al., 1990) was added to 1-3 ml of cytoplasmic extract and the mixture was incubated with rotation at room temperature for 45 min. Forty µl of protein
G agarose beads (PIERCE) were then added and the mixture was incubated with rotation for an additional hour at room temperature. Beads were washed four times with PBS (58 mM Na, HPO₄, 17 mM NaH₂PO₄, 68 mM NaCl) and boiled in Laemmli sample buffer for Western Blotting.

For sucrose gradient sedimentation, 300 µl of cytoplasmic extract was loaded onto an 11 ml sucrose density gradient [10-40% w/v sucrose in gradient buffer (10 mM HEPES pH 7.4, 1.0 mM MgCl₂, 0.1 mM CaCl₂, 0.1 M KCl)] and centrifuged at 288,000 X g for 12 h at 4ºC in a Beckman SW41Ti rotor. Fractions (500 µl) were carefully taken from the top of the gradient using a cut off micropipette tip. All 23 fractions were analyzed by western blotting. Band intensities were quantified using Image J software (NIH).

Electrophoresis was carried out with a BioRad Mini Protean II apparatus and protein transfer for western blotting was carried out with a Bio Rad Mini Trans-Blot apparatus following the instructions of the manufacturer. The remainder of the western blotting procedure was carried out following the standard protocol provided by the “Antibody Protocol Guide” from Clontech. The anti-GFP primary antibody was from Clontech (Living Colors A.v. monoclonal antibody JL-8) and the secondary antibody was Alexa Fluor 680 goat anti-mouse IgG (H + L) from Invitrogen. The western blots were scanned using an Odyssey Infrared Imaging System from LI-COR Biosciences. The band intensity was analyzed using ImageJ (NIH).
2.6 Proteomics analysis of the proteasome and its associated proteins through affinity purification of S-tagged RPN5

To analyze RPN5-interacting proteins, an affinity tag, the S-peptide, which is residues 1 to 15/20 of RNase A, was fused to the C-terminus of RPN5 using the gene targeting and transformation methods described in 2.2. Purification with the S-peptide is based on the high affinity between the S-peptide and the S-protein (residues 21 to 124 of RNase A). S-protein agarose beads (Novagen) and YX111, a strain containing S-peptide tagged RPN5 (S-tagged RPN5), were used for affinity purification of proteasomes. Affinity purifications were performed according to Liu et al. (2010) with some modifications. Conidia were inoculated at a concentration of 3 X 10^6 per ml in YG medium and incubated for 13 hours at 36ºC, shaking at 200 rpm. The hyphae were then harvested, frozen immediately in liquid nitrogen, and lyophilized overnight. The dried mycelia were ground into powder at room temperature and suspended and, using a Dounce glass homogenizer, mixed thoroughly on ice with extraction buffer (30 mM Tris, pH 7.5, 5 mM EDTA, pH 8.0, 15 mM EGTA, pH 8.0, 60 mM Beta Gly.PO₄, 500 µM NaVO₃, 10 mM NaF, 15 mM pNPP (Sigma), 1 µg/ml pepstatin A (Sigma), 10 µg/ml leupeptin (Sigma), 10 µg/ml trypsin chymoT inhibitor, 10 µg/ml aprotinin (Sigma), 10 µg/ml TPCK (Sigma), 2 mM TAME (Sigma), 5 mM benzamidine (Sigma), 2 mM ATP, 50 µg.ml PMSF, 1 mM DTT, 0.5% NP-40, 300 mM NaCl). Extracts were clarified by centrifugation at 20,000 g for 15 minutes at 4ºC and the supernatants were collected and the protein concentration was determined using the Bradford protein assay (Bradford, 1976). 400 mg of protein extract was incubated with 500 µl S-protein agarose beads (Novagen) for 1.5-2.5 h in a cold room before the beads were collected by centrifugation.
and washed once with the extraction buffer and 5 times with a wash buffer (30 mM Tris, pH 7.5, 5 mM EDTA, pH 8.0, 300 mM NaCl, 1 mM DTT, 50 µg/ml PMSF, 1 mM ATP, 0.2% NP-40). Proteins bound to the S-protein beads were released by boiling the beads in Laemmli sample buffer. A wild-type strain, R153, processed through the same purification procedure was used as a control.

To identify the main proteins purified, a SDS-PAGE gel was run on the protein samples and the gel was stained with Comassie blue to reveal the protein bands. Selected bands were excised and sent to the OSU Campus Chemical Instrument Center (CCIC) for digestion and mass spectrometry analysis. Alternatively, to identify as many purified proteins as possible, the protein sample was run through the stacking gel and just into the separating gel. This process resulted in all purified proteins being compressed into a tight series of bands. The bands were then excised as one and sent to the OSU Campus Chemical Instrument Center (CCIC) for digestion and mass spectrometry analysis. Proteins identified from the R153 sample were interpreted to be non-specific binding proteins and subtracted from the protein data set obtained from YX111.

2.7 Fluorescence recovery after photobleaching (FRAP) analysis of nuclear imports

FRAP was performed using the photokinesis unit on the UltraView ERS confocal system. The whole nucleus was photobleached and the fluorescence recovery was recorded by time-lapse imaging. The images were collected as three focal plane Z-axis stacks with 1 µm spacing and viewed as accumulated signal intensity projections. Three pre-bleach images and 30 post-bleach images were gathered for each nucleus. Data were
processed according to McNally et al. (2008) and Coffman et al. (2009). Only data in which the nucleus was bleached more than 50% of the original signal were used for analysis. In my analysis, the background intensity (I_b) was first subtracted from the intensity within the bleached nuclei (intentional photobleached area) (I_i) at each time point. To correct for unintentional bleaching, a nucleus of another cell outside of the photobleached region was selected and the intensity within this control nuclei (I_u) was measured and used for background-subtraction. The background-subtracted intensity of the photobleached nuclei (I_i-I_b) was then divided by the background-subtracted intensity of the control nuclei [(I_i-I_b)/(I_u-I_b)] at each time point. The average of the (I_i-I_b)/(I_u-I_b) of pre-bleach time points was set to 1 and used to normalize the (I_i-I_b)/(I_u-I_b) of other time points. The normalized values were plotted with time and the moment of bleaching was defined as time zero.
Table 2.1
Strains used in these studies

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<th>Strain</th>
<th>Genotype</th>
</tr>
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<td>LO1952</td>
<td><em>nkuAΔ</em>: argB; <em>fwA1</em>; <em>pyrG89</em>; <em>pabaA1</em>; <em>pyroA4</em>; <em>riboB2</em>; <em>mipA-</em></td>
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<td></td>
<td><em>mCherry</em>- <em>A. fumigatus</em> <em>pyrG</em>; HH1-<em>GFP</em>-<em>A. fumigatus</em> <em>pyro A</em>;</td>
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<td><em>riboB+A.n</em> (pPL5)</td>
</tr>
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<td>LO1948</td>
<td><em>nkuAΔ</em>: argB; <em>pyrG89</em>; <em>pyroA4</em>; <em>riboB2</em>; GcpB-<em>GFP</em>-<em>A. fumigatus</em></td>
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<td></td>
<td><em>pyro</em>; HH1-<em>mCherry</em>-<em>A. fumigatus</em> <em>pyrG</em>; <em>riboB+A.n</em> (pPL5);</td>
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<td>LO1949</td>
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<tr>
<td></td>
<td><em>pyro</em>; HH1-<em>mCherry</em>-<em>A. fumigatus</em> <em>pyrG</em>; <em>riboB+A.n</em> (pPL5);</td>
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<td><em>nkuAΔ</em>: argB; <em>pyrG89</em>; <em>pyroA4</em>; <em>riboB2</em>; GcpD-<em>GFP</em>-<em>A. fumigatus</em></td>
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<tr>
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<td><em>pyro</em>; HH1-<em>mCherry</em>-<em>A. fumigatus</em> <em>pyrG</em>; <em>riboB+A.n</em> (pPL5);</td>
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<tr>
<td></td>
<td><em>pyro</em>; HH1-<em>mCherry</em>-<em>A. fumigatus</em> <em>pyrG</em>; <em>riboB+A.n</em> (pPL5);</td>
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<td><em>mCherry</em>-<em>A. fumigatus</em> <em>pyrG</em>; HH1-<em>GFP</em>-<em>A. fumigatus</em> <em>pyro A</em>;</td>
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<td>GcpDΔ:: <em>A. fumigatus</em> <em>riboB</em></td>
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<td><em>mCherry</em>-<em>A. fumigatus</em> <em>pyrG</em>; HH1-<em>GFP</em>-<em>A. fumigatus</em> <em>pyro A</em>;</td>
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<td>GcpFΔ:: <em>A. fumigatus</em> <em>riboB</em></td>
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<td><em>pyro</em>; HH1-<em>mCherry</em>-<em>A. fumigatus</em> <em>pyrG</em>; GcpFΔ:: <em>A. fumigatus</em> <em>riboB</em></td>
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<td></td>
<td><em>pyro</em>; HH1-<em>mCherry</em>-<em>A. fumigatus</em> <em>pyrG</em>; GcpEΔ:: <em>A. fumigatus</em> <em>riboB</em></td>
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<td>LO1997</td>
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<td><em>pyro</em>; HH1-<em>mCherry</em>-<em>A. fumigatus</em> <em>pyrG</em>; GcpFΔ:: <em>A. fumigatus</em> <em>riboB</em></td>
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(Continued)
Table 2.1: Continued

| LO1999 | *nkuAΔ::argB; pyrG89; pyroA4; riboB2; GcpD-GFP-A.*fumigatus pyro; HH1-mCherry-A.*fumigatus pyrG; GcpEΔ:: A. *fumigatus* riboB |
| LO2000 | *nkuAΔ::argB; pyrG89; pyroA4; riboB2; GcpD-GFP-A.*fumigatus pyro; HH1-mCherry-A.*fumigatus pyrG; GcpFΔ:: A. *fumigatus* riboB |
| LO2002 | *nkuAΔ::argB; pyrG89; pyroA4; riboB2; GcpE-GFP-A.*fumigatus pyro; HH1-mCherry-A.*fumigatus pyrG; GcpDΔ:: A. *fumigatus* riboB |
| LO2004 | *nkuAΔ::argB; pyrG89; pyroA4; riboB2; GcpE-GFP-A.*fumigatus pyro; HH1-mCherry-A.*fumigatus pyrG; GcpFΔ:: A. *fumigatus* riboB |
| LO2006 | *nkuAΔ::argB; pyrG89; pyroA4; riboB2; GcpF-GFP-A.*fumigatus pyro; HH1-mCherry-A.*fumigatus pyrG; GcpDΔ:: A. *fumigatus* riboB |
| LO2008 | *nkuAΔ::argB; pyrG89; pyroA4; riboB2; GcpF-GFP-A.*fumigatus pyro; HH1-mCherry-A.*fumigatus pyrG; GcpEΔ:: A. *fumigatus* riboB |
| LO1930 | *fwA1; nkuAΔ::argB; argB2; riboB2; pyroA4; GcpDΔ:: A. *fumigatus* riboB; GcpEΔ:: A. *fumigatus* pyrG; GcpFΔ:: A. *fumigatus* pyro; pyrG89; paba A1 |
| LO1915 | *nkuAΔ::argB; argB2; riboB2; pyroA4; pyrG89; HH1-mCherry-A.*fumigatus pyrG; wAΔ:: GFP-a-tubulin-A.*fumigatus* pyrA; |
| LO2064 | *nkuAΔ::argB; argB2; riboB2; pyroA4; pyrG89; HH1-mCherry-A.*fumigatus pyrG; wAΔ:: GFP-a-tubulin-A.*fumigatus* pyrA; riboB+A.*n(pPL5) |
| LO1988 | *nkuAΔ::argB; argB2; riboB2; pyroA4; pyrG89; HH1-mCherry-A.*fumigatus pyrG; wAΔ:: GFP-a-tubulin-A.*fumigatus* pyrA; GcpDΔ:: A. *fumigatus* riboB |
| LO2039 | *nkuAΔ::argB; argB2; riboB2; pyroA4; pyrG89; HH1-mCherry-A.*fumigatus pyrG; wAΔ:: GFP-a-tubulin-A.*fumigatus* pyrA; GcpEΔ:: A. *fumigatus* riboB |
| LO1989 | *nkuAΔ::argB; argB2; riboB2; pyroA4; pyrG89; HH1-mCherry-A.*fumigatus pyrG; wAΔ:: GFP-a-tubulin-A.*fumigatus* pyrA; GcpFΔ:: A. *fumigatus* riboB |
| LO2018 | *nkuAΔ::argB; argB2; riboB2; pyroA4; pyrG89; HH1-mCherry-A.*fumigatus pyrG; wAΔ:: GFP-a-tubulin-A.*fumigatus* pyrA; GcpDΔ:: A. *fumigatus* riboB; GcpEΔ:: A. *fumigatus* pyrG; GcpFΔ:: A. *fumigatus* pyroA |
| LO2804 | *nkuAΔ::argB; argB2; riboB2; pyroA4; pyrG89Δ:: pPL6 |
| LO2823 | *GcpDΔ:: A.*fumigatus* riboB; pabaA1; fw |
| LO2824 | *GcpEΔ:: A.*fumigatus* pyroA; pabaA1; fw |

(Continued)
| LO2826 | GcpFΔ::A. fumigatus pyroA; pabaA1; fw |
| LO2828 | diploid +/pabaA1; +/pyrG89; +/wA3; +/fwA1 |
| LO2829 | diploid +/pabaA1; +/pyrG89; GcpEΔΔ::A. fumigatus pyroA; +/wA3; +/fwA1 |
| LO2830 | diploid +/pabaA1; +/pyrG89; GcpFΔΔ::A. fumigatus pyroA; +/wA3; +/fwA1 |
| LO2011 | Rpn5-GFP-A. fumigatus pyrG; HH1-mRFP-A. fumigatus riboB; riboB2; pyroA4; pyrG89 |
| LO2260 | Rpn5-GFP-A. fumigatus pyrG; HH1-mCherry-A. fumigatus pyroA; riboB2; pyroA4; pyrG89 |
| YX039 | GFP-TubA-A. fumigatus pyrG (at wA locus); Rpn5-mCherry-A. fumigatus riboB; HH1-T-sapphire-A. fumigatus pyroA; pabaA1 |
| YX049 | Rpn5-GFP-A. fumigatus pyrG; HH1-T-Sapphire-A.f pyroA; Fib-mCherry-A.f pyrG; riboB2; fw1 |
| YX052 | Rpn5-GFP-A. fumigatus pyrG; HH1-T-Sapphire-A. fumigatus pyroA; FibCherry-A. fumigatus pyrG |
| YX077 | yAΔ::NLS-DsRed; Rpn5-GFP-A. fumigatus pyrG; pyroA; yA1 |
| YX094 | AnAn-cut8-GFP-A. fumigatus pyrG; pyrG89; pyroA4; riboB2; nkuAΔ::argB |
| YX097 | AnAn-cut8Δ::A. fumigatus riboB; pyroA4; pyrG89; riboB2; nkuAΔ::argB |
| YX111 | Rpn5-S tag-A. fumigatus pyrG |
| YX121 | Rpn5-GFP-A. fumigatus pyrG; yAΔ::NLS-DsRed; AnAn-cut8Δ::A.fumigatus riboB; pyroA4 |
| YX144 | proteasomal β1-GFP-A. fumigatus pyrG; Rpn5-mCherry-A. fumigatus riboB; pyroA4; wA3 |
| YX148 | proteasomal α6-GFP-A. fumigatus pyrG; Rpn5-mCherry-A. fumigatus riboB; pyroA4; wA3 |
| YX170 | A. fumigatus riboB-alcAp-An-cut8 (long version of An-cut8); pyrG89; pyroA4; riboB2; nkuAΔ::argB |
| YX172 | A. fumigatus pyrG-alcAp-An-cut8 (long version of An-cut8); pyrG89; pyroA4; riboB2; nkuAΔ::argB |
| YX177 | Cyclin B-GFP-A. fumigatus pyrG; HH1-mCherry-A. fumigatus pyroA; AnAn-cut8Δ::A. fumigatus riboB; pyrG89; riboB2 |
| YX197 | nup37Δ:: A. fumigatus pyrG; elysΔ:: A. fumigatus pyrG; Rpn5-GFP-A. fumigatus pyrG; yAΔ::NLS-DsRed |

(Continued)
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<tr>
<td>YX214</td>
<td>Mlp1-GFP-A. fumigatus pyrG; Rpn5-mCherry-A. fumigatus riboB; argB2Δ::alcAp-ΔnimE(nondegradable cyclin B)-argB</td>
</tr>
<tr>
<td>YX237</td>
<td>Bop1-GFP-A. fumigatus pyrG; yAΔ::NLS-DsRed; An-cut8Δ:: A. fumigatus riboB; pyrG89</td>
</tr>
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</table>
Strategy to tag a target protein with a fluorescent protein. (A) Fusion PCR is used to generate linear fragments for tagging the target protein with a fluorescent protein such as mCherry. Primer P3 contains the reverse complement of the glycine alanine (GA) linker (Continued)
Figure 2.1: Continued

(red) and primer P4 contains a sequence from the selectable marker (pink). Both anneal to the central cassette during fusion PCR. Primer P1 nest and P2 nest are used in fusion PCR rather than P1 and P3 to increase specificity. (B) The linear DNA fragment inserts at the correct chromosomal site through homologous recombination.
Figure 2.2
Strategy to delete a target gene. (A) Fusion PCR is used to generate linear fragments for deleting the target gene with a selectable marker. Primer P3 and primer P4 both contain sequences complimentary to the selectable marker (light pink and dark pink), which anneal with the central cassette during fusion PCR. Primer P1 nest and P2 nest are used in fusion PCR rather than P1 and P2 to increase specificity. (B) The DNA linear fragment inserts at the correct chromosomal site by homologous recombination.
Figure 2.3

Strategy for replacing the promoter of a target gene with the \textit{alcA} promoter. (A) Fusion PCR is used to generate the linear fragments for replacement of the promoter of the target gene with the \textit{alcA} promoter. Primer P3 contains a sequence complimentary to the selectable marker (pink) and primer P4 contains a sequence complimentary to the \textit{alcA} (Continued)
Figure 2.3: Continued

promoter (purple), both of which anneal to the central cassette during fusion PCR. Primer P1 nest and P2 nest are used in fusion PCR rather than P1 and P2 to increase the specificity of amplification. (B) The DNA linear fragment inserts at the correct chromosomal site by homologous recombination.
Chapter 3: *In vivo* analysis of the functions of \(\gamma\)-tubulin complex proteins

3.1 Introduction

In many eukaryotic cells, microtubules are organized by microtubule-organizing centers (MTOCs) such as the spindle pole body (SPB) in fungi and the centrosome in metazoa. \(\gamma\)-tubulin is a highly conserved component of MTOCs, playing an essential role in microtubule nucleation and organization [(Oakley and Oakley, 1989; Oakley et al., 1990; Zheng et al., 1991; Horio et al., 1991; Moritz et al., 1998; Schnackenberg et al., 1998) and additional data reviewed in (Job et al., 2003)]. \(\gamma\)-tubulin forms complexes (\(\gamma\)-tubulin complexes, \(\gamma\)-TuCs) with several proteins [(reviewed in (Wiese and Zheng, 2006)]. The majority of \(\gamma\)-TuCs purified from *Xenopus laevis*, *Drosophila melanogaster* and humans are large (>25S) complexes that appear to be ring structures with a diameter of 25 nm (\(\gamma\)-tubulin ring complexes or \(\gamma\)-TuRCs) (Stearns and Kirschner, 1994; Zheng et al., 1995; Moritz et al., 1998; Oegema et al., 1999; Meads and Schroer, 1995; Murphy et al., 2001). \(\gamma\)-TuRCs consist of \(\gamma\)-tubulin and at least five GCPs (\(\gamma\)-tubulin complex proteins). GCPs are known as Xgrips (*Xenopus* \(\gamma\)-tubulin ring proteins) in *X. laevis*, Dgrips in *D. melanogaster* and hGCPs in humans [recently reviewed in (Wiese and Zheng, 2006; Raynaud-Messina and Merdes, 2007)]. In addition to the five GCPs, an additional protein containing WD repeats (GCP-WD, originally called NEDD1 in humans) has been found to associate with the \(\gamma\)-TuRC in *D. melanogaster* and humans.
but not in *X. laevis* (Gunawardane et al., 2003; Haren et al., 2006; Luders et al., 2006; Liu and Wiese, 2008). We will reserve the term γ-TuRC for cases in which the complex has been shown by electron microscopy to be a ring complex and the more general term “large γ-TuC” for cases in which a large γ-tubulin complex has been shown to exist but the structure has not been studied by electron microscopy. In most cases the γ-TuRC and the large γ-TuC are likely to be the same.

A smaller (~9.8S) complex, the γ-tubulin small complex (γ-TuSC), that contains only γ-tubulin, GCP2 (Dgrip84) and GCP3 (Dgrip91), was discovered in *D. melanogaster* (Moritz et al., 1998; Oegema et al., 1999). It is a component of the γ-TuRC and γ-TuRCs disassemble in 500 mM KCl, releasing γ-TuSCs (Moritz et al., 1998). A great deal of data indicates that γ-TuRCs are assembled from γ-TuSCs and other GCPs in the cytoplasm and are recruited to the centrosome where they serve as the major microtubule nucleators in animal cells (Moritz et al., 1995; Moritz et al., 1998; Schnackenberg et al., 1998; Wiese and Zheng, 1999; Wiese and Zheng, 2006). Based on these data and electron tomographic data, a structural model has been proposed for the γ-TuRC in which 6-7 γ-TuSCs are arranged in a ring and capped by GCPs (Wiese and Zheng, 1999; Moritz et al., 2000; Keating and Borisy, 2000; Wiese and Zheng, 2000).

Each of the γ-TuSC components is essential for viability and for normal mitotic spindle assembly and function. The functions of GCP4-6 are less clear, however. Although a number of functional analyses have been carried out, the data are contradictory in some cases and show apparent differences among and within species that have prevented the emergence of a coherent model for GCP4-6 function (Zhang et al., 2000; Verrollet et al., 2006; Fujita et al., 2002; Venkatram et al., 2004; Vogt et al., 2006;
Anders et al., 2006; Schnorrer et al., 2002; Vogt et al., 2006).

A number of important questions about γ-tubulin complexes remain unanswered. For example, the arrangement of the subunits within the larger γ-tubulin complex is not clear. It is not clear which components of the γ-tubulin complex are involved in the attachment of the complex to MTOCs and, as mentioned, the functions of GCP4-6 are still a matter of controversy.

Many of the previous studies have been carried out in different organisms and usually only one or two GCPs have been studied at a time. To answer some of the extant questions, and, if possible, create a coherent model for GCP function, we felt that it would be useful to carry out a comprehensive analysis of GCP function in a single organism. We chose to carry out our studies in the model filamentous fungus Aspergillus nidulans. This organism has a number of advantages for studying these proteins. First, the sequencing of the genome facilitates identification of genes that encode γ-tubulin complex proteins. Second, a highly efficient gene targeting system has been developed for A. nidulans (Nayak et al., 2006; Szewczyk et al., 2006) that greatly facilitates tagging or deleting γ-tubulin complex genes. Third, the heterokaryon rescue technique that can be used with this organism allows one to examine the phenotypes of recessive lethal mutations or gene deletions in vivo (Osmani et al., 1988; Martin et al., 1997; Oakley et al., 1990; Osmani et al., 2006a) and this should allow us to examine the phenotypes of deletions of GCP2 and GCP3 homologues, which are predicted to be lethal. Finally, there is very little data on γ-tubulin complexes in filamentous fungi and the genus Aspergillus is very important medically and commercially.
3.2 Results

3.2.1 GCP2-6 homologues exist in *A. nidulans*

A BLAST search of the *A. nidulans* genome database ([www.broad.mit.edu/annotation/genome/aspergillus_group](http://www.broad.mit.edu/annotation/genome/aspergillus_group)) with human GCP protein sequences revealed that strong GCP2-6 homologues exist in *A. nidulans*. They contain the conserved grip1 and 2 motifs found in GCPs in other organisms (Figure. 3.1, 3.2) (Gunawardane et al., 2000; Fujita et al., 2002; Murphy et al., 2001) and we designate the genes *gcpB*-gcpF and their protein products GCPB-GCPF (Table 3.1).

To confirm that these proteins are GCP homologues, we tagged the C-terminus of each protein with the green fluorescent protein (GFP) and examined their localizations. To avoid artifactual results due to improper expression levels, we used the fusion PCR procedure of Nayak et al. (Nayak et al., 2006) to produce a single chromosomal copy of the gene of interest, under control of its native promoter, fused in-frame to the GFP coding sequence at its 3’ end. Unless otherwise specified, all of the tagging in this chapter was performed in this way. All strains carrying GCP fusions grew indistinguishably from wild-type (WT) strains at all temperatures tested (20°C, 25°C, 30°C, 37°C and 42°C) (Fig. 3.3). Time-lapse observations revealed that, like γ-tubulin, GCPB-F localize to spindle pole bodies throughout the cell cycle, appearing as one dot at the nuclear periphery during interphase (Fig. 3.4) and two dots during mitosis. Localization of the GCPs to the SPB was confirmed by co-localization with a tdTomato tagged version of the *A. nidulans* homologue of the SPB protein Nud1 (Fig. 3.5). All GCPs localized to septa, but the fluorescence of GCPD-F was barely above background.
levels. These data are consistent with previous findings that the SPB is the major MTOC throughout the cell cycle (Horio and Oakley, 2005; Sampson and Heath, 2005) and with the finding that microtubules may sometimes grow from septa (Konzack et al., 2005). Konzack et al. have suggested that an MTOC might be present at the hyphal apex, based on observations of the movement of a GFP-kinesin-like protein under control of an inducible promoter (Konzack et al., 2005), but other, more direct, studies (Horio and Oakley, 2005; Sampson and Heath, 2005) have not revealed an apical MTOC and we find no localization of GCPs at the apex.

To further verify that GCPB-F are γ-tubulin complex proteins, we asked if the GFP fusions of GCPB-F co-immunoprecipitated with γ-tubulin and found that this was, indeed, the case (Fig. 3.6 A). Based on sequence homology, co-localization and co-immunoprecipitations, we can conclude with confidence that GCPB-F are γ-tubulin complex proteins.

### 3.2.2 γ-TuSC components are essential but gcpD-F are not

To determine if gcpB-F are essential, we deleted each gene by replacing it with the *Aspergillus fumigatus* riboB gene. Transformants carrying a correct replacement with no additional integration of transforming DNA were confirmed by Southern hybridizations (data not shown). Deletions of *mipA*, the gene that encodes γ-tubulin, were lethal as shown previously (Jung et al., 2001; Oakley et al., 1990; Martin et al., 1997) as were deletions of *gcpB* and *gcpC*. The genes that encode components of the γ-TuSC are, thus, all essential. Single, double or triple deletions of *gcpD-F*, however, showed a striking absence of phenotype. They exhibited no obvious change in colony growth rates in
comparison with the parental control (Fig. 3.7) and the colonies were morphologically normal. Conidiation is often a more sensitive indicator of mitotic defects than colonial growth rate, but deletant colonies produced abundant viable conidia.

### 3.2.3 Roles of GCPD-F in the assembly of γ-tubulin complexes

The roles of GCPD-F in the assembly of γ-tubulin complexes are somewhat controversial due to the differences in results obtained in various studies (Zhang et al., 2000; Fujita et al., 2002; Venkatram et al., 2004; Verollet et al., 2006; Vogt et al., 2006; Anders et al., 2006). We used sucrose density centrifugation to determine the size of γ-tubulin complexes in WT hyphae and to determine the effects, if any, of GCPD-F deletions on the sizes of the complexes. In cytoplasmic extracts of WT hyphae, γ-tubulin sedimented in a broad peak between about 7S and 14S and a sharper peak at about 21S (Fig. 3.6 B). *A. nidulans*, thus, has both large and small γ-tubulin complexes, but the large complexes are somewhat smaller than the γ-TuRCs seen in animal cells (Zheng et al., 1995; Wiese and Zheng, 1999; Job et al., 2003). Deletion of any of the non-essential GCPs greatly reduced the large γ-tubulin complex peak and apparently caused a slight reduction of the sedimentation coefficients of both large and small complexes. These data indicate that GCPD-F play an important role in the assembly or stability of large γ-tubulin complexes in *A. nidulans*.

### 3.2.4 GCPs exhibit a hierarchy of localization to the SPB

We were interested in the effects of deletion of GCPs on the localizations of the other GCPs in the hope that this would shed light on which GCPs attach the γ-tubulin complex to the SPB and, perhaps, on the structure of the γ-tubulin complex. *A. nidulans*
has a particular advantage in this regard because it is possible to use the heterokaryon rescue technique to determine the effects of deletion of essential GCPs on the localization of other γ-tubulin complex components. This technique allows deletions or disruptions of essential genes to be maintained in heterokaryons and the phenotypes of the disruptions or deletions determined in germinating conidia (uninucleate spores) produced by the heterokaryons (Osmani et al., 1988; Oakley et al., 1990; Martin et al., 1997; Osmani et al., 2006b).

We created a comprehensive set of deletions in strains carrying tagged GCPs (Table 3.2). Deletions were made by replacing the target genes with the *A. fumigatus* riboB gene. In the case of the essential genes (*mipA, gcpB, gcpC*), deletant nuclei were maintained in heterokaryons. For microscopic observations, conidia from the heterokaryons were incubated in selective media (lacking riboflavin). In the absence of riboflavin, conidia with untransformed nuclei did not germinate and conidia with deletant nuclei germinated but had the phenotype caused by the deletion.

Deletion of any of the genes encoding γ-TuSC components (*mipA, gcpB, gcpC*) resulted in the loss of SPB localization of all γ-tubulin complex components, both the γ-TuSC components and the non-essential GCPs (Figure 3.8 and Table 3.2). The γ-TuSC components are, thus, mutually dependent for SPB localization and the non-essential GCPs are dependent upon the γ-TuSC for SPB localization. Deletion of the non-essential GCPs (GCPD-F), however, did not affect the SPB localization of the γ-TuSC components (Fig. 3.9 and Table 3.2). The γ-TuSC, thus, does not depend on the non-essential GCPs for the SPB localization. We also examined GCPC localization at the septum in a *gcpF* deletion and found that it localized normally (our unpublished data).
Among the non-essential GCPs, we discovered a hierarchy of SPB localization dependence. Deletion of \( gcpF \) eliminated localization of GCPD and E to the SPB, deletion of \( gcpD \) eliminated the localization of GCPE but not GCPF, and deletion of \( gcpE \) did not alter the SPB localization of GCPD or GCPF (Fig. 3.10 and Table 3.2).

### 3.2.5 Effects of \( gcpB-F \) deletions on mitotic spindles and cytoplasmic microtubules

To investigate the roles of GCPs in \( \gamma \)-tubulin-mediated microtubule nucleation, we deleted \( mipA \) and each of the gcp genes individually in a strain (LO1915) carrying a GFP-\( \alpha \)-tubulin fusion and a histone H1-mCherry fusion. Consistent with our finding that \( \gamma \)-TuSC components are mutually dependent for SPB localization, we found that the \( gcpB \) and \( gcpC \) deletions phenocopied the \( mipA \) deletion (Fig. 3.11). Functional mitotic spindles were absent and cytoplasmic microtubules were extensively reduced relative to controls consistent with previous published immunofluorescence data for \( mipA \) deletion germlings (Oakley et al., 1990; Martin et al., 1997). Occasionally, we observed abnormal curved microtubule bundles in these deletants similar to those observed in a previous study of \( mipA \) deletants (Martin et al., 1997). Like the disruption of \( mipA \), deletions of either \( gcpB \) or \( gcpC \) resulted in blockage of nuclear division. Most deletant germlings had one big nucleus after 20 hours incubation at 25°C (Fig. 3.8, 3.11). Occasionally, more than one nucleus was observed in germlings after longer incubation, with one extremely large nucleus and some tiny chromosomal masses scattered along the germlings.

We examined the effects of the deletions on mitosis and microtubule organization by time-lapse microscopy. When germlings carrying any of these three deletions entered mitosis, GFP-\( \alpha \)-tubulin entered the nucleoplasm, but functional spindles did not form.
Surprisingly, the nuclei underwent stretching and moved vigorously at the late stages of the defective mitosis. We sometimes observed formation of thin, nonfunctional spindles, which, judging from fluorescence intensity, contained only one or a few microtubules (Fig. 3.11). Thin, non-functional mitotic spindles were observed occasionally in previous studies of mipA deletants (Oakley et al., 1990; Martin et al., 1997) and nucleation of the assembly of the thin spindles could be due to the presence of small amounts of γ-TuSC components inherited from parental hypha during conidiation (Martin et al., 1997).

GcpD-F deletants in LO1915 grew indistinguishably from the parental strain at all temperatures tested (20°C, 25°C, 30°C, 37°C, and 42°C) (Fig. 3.12). We also tested their sensitivities to the microtubule depolymerizing drug benomyl. In the presence of benomyl concentrations from 0.2 μg/ml to 1.2 μg/ml, none of these deletants showed sensitivities different from the parental strains (Fig. 3.12 and additional unpublished data).

We examined the effects of the gcpD-F deletions on microtubules in vivo and found no obvious defects. Even in the triple deletion strain (gcpDΔ, gcpEΔ, gcpFΔ), cytoplasmic microtubules and mitotic spindles were not visibly different from the wild type (Fig. 3.11). Maximum spindle length at anaphase onset was also similar in the control and triple deletant (WT: 2.67 ± 0.67 μm; triple deletant: 2.65 ± 0.41 μm, n=50 for each, p=0.91). Astral microtubules nucleated from SPBs in the triple deletant were indistinguishable visibly from controls. To determine if there might be a minor delay in the completion of mitosis that might not lead to an obvious change in growth rate, we captured time-lapse images of mitosis at 20 seconds intervals and compared the time of completion of mitosis between parental strains and triple deletion strains. Data were
collected at 25°C ± 1°C. We set the time when the mitotic spindle was first detectable as time zero and when it disappeared completely as the end of mitosis. The difference in the time for mitotic completion between the control LO2064 (361.4 s ± 31.9 s, n=45) and the triple deletion strain LO2018 (367.6 s ± 49.7 s, n=76) was not statistically significant (p=0.7039). However, 10% of triple deletant nuclei spent longer in mitosis than the longest mitosis (520 sec) observed in the control strain. This could reflect a meaningful mitotic delay in a small subset of nuclei.

### 3.2.6 GCPD-F play a nonessential role in chromosome segregation

Although time-lapse microscopy did not reveal a high frequency of chromosomal segregation defects in the triple deletant, we wondered if the gcp deletions might cause subtle defects that might be revealed by a more sensitive assay. We consequently tested the effects of the gcp deletions on chromosome mis-segregation using a chromosome loss assay. In *A. nidulans*, vegetative diploids can be created from haploids carrying spore color mutations. Chromosome mis-segregation in diploid cells will result in aneuploid nuclei which lose extra chromosomes rapidly thus becoming haploid (Kafer, 1977; Clutterbuck, 1992; Rischitor et al., 2004) To test whether deletion of non-essential gcp genes increases the chromosome loss frequency, we created diploid strains that were homozygous for deletions of *gcpD, gcpE* or *gcpF* and which carried the spore color mutations *wA3* and *fwA1*. Chromosome loss resulted in haploid sectors that were fawn, white and dark green (a haploid sector that does not carry a mutation in a spore color marker gene). The control diploid strain contained the same nutritional and color markers but was WT for *gcpD-F*. The difference in the frequency of chromosome loss became
apparent only after the diploid strains were grown 6 days at 37°C. Compared to the control (33 ± 4 sectors per 20 colonies), the homozygous gpcF deletion diploid produced significantly more haploid sectors (93 ± 10 sectors per 20 colonies; p=0.0048 t test). Although the homozygous gcpE deletion diploids also showed a significantly higher frequency of haploid sectors on the 6th day of incubation (48 ± 5 sectors per 20 colonies; p=0.0229), the difference between the homozygous gcpD deletion diploid and the control was not significant (29 ± 5 sectors per 20 colonies; p=0.4726).

The spindle assembly checkpoint monitors spindle defects and inhibits anaphase until the defects are corrected. If GCPD-F play a role in mitosis as our chromosome loss data suggest, deletions of gcpD-F might create defects that are detected by the spindle assembly checkpoint and subsequently corrected. It follows that if the spindle checkpoint were inactivated, enough chromosomal segregation defects might accumulate in gcpD-F deletions to inhibit growth. We consequently created double mutants each carrying a deletion of a non-essential gcp as well as a deletion of md2A, the gene that encodes the A. nidulans homologue of Mad2 which is essential for the functioning of the spindle assembly checkpoint (Prigozhina et al., 2004). Strains carrying deletions of non-essential gcps were indistinguishable in growth rate from a control strain at all temperatures tested (20°C, 25°C, 30°C, 37°C and 42°C) as was a md2A deletion strain. However, gcpFΔ, md2AΔ double deletants were synthetically sick compared to the parental and WT strains at 37°C and 42°C (Fig. 3.13). The gcpDΔ, md2AΔ double deletants and gcpEΔ, md2AΔ double deletants also showed synthetic sickness although somewhat less severe than gcpFΔ, md2AΔ (Fig. 3.14). The stronger synthetic sickness of gcpFΔ and the greater effect of gcpFΔ on chromosome mis-segregation are probably due to the fact that GCPF
is required for localization of GCPD and E to the SPB. In aggregate, these data indicate that GCPD-F, while not essential for mitosis, do play a role in chromosome segregation in mitosis.

3.2.7 The nonessential GCPs are not required for meiosis

Because studies in *D. melanogaster* have shown that *Grip75* and *Grip128* mutant flies are sterile, suggesting that meiosis is more sensitive to the absence of these proteins than mitosis (Vogt et al., 2006), we tested the ability of triple deletion strains to undergo meiosis. We created *gcpD-F* triple deletions in two different strains carrying conidial color mutations, LO2018 carrying *wA3* and LO1930 carrying *fwA1*. Successful mating and gene segregation between the two strains will produce meiotic progeny that are not only white and fawn in color but also the WT dark green color. We found that the triple deletion strains generated cleistothecia with viable ascospores, and that they crossed successfully with each other producing recombinant progeny (Fig. 3.15). We conclude, therefore, that GCPD-F are not essential for meiosis.

3.3 Discussion

3.3.1 GCP4-6 homologues and large γ-TuCs exist in *A. nidulans*

Our data reveal that *A. nidulans* contains homologues of GCP2-6. They localize to SPBs and co-immunoprecipitate with γ-tubulin and, thus, are genuine γ-tubulin complex proteins. Although one can identify putative GCPs on the basis of sequence identity in the genomes of a number of filamentous fungi, this represents the first experimental verification that GCPs are present in filamentous fungi. We have also found that *A. nidulans* possesses distinct large, soluble ~21S γ-tubulin complexes as well as smaller
complexes (Fig. 3.6). This is of interest because there is controversy as to whether large complexes exist in *S. cerevisiae* and *S. pombe* (Vinh et al., 2002; Fujita et al., 2002; Anders et al., 2006; Venkatram et al., 2004). Large γ-TuCs clearly exist in *A. nidulans*, although their sedimentation coefficients are somewhat lower than the γ-TuRCs found in animal cells (Meads and Schroer, 1995; Murphy et al., 2001; Moritz et al., 1998; Oegema et al., 1999; Zheng et al., 1995; Stearns and Kirschner, 1994). This might be because the large γ-TuCs in *A. nidulans* may lack the homolog of the GCP-WD protein. We have found that *A. nidulans* also contains smaller soluble γ-tubulin complexes that distribute over a fairly broad peak (Fig. 3.6). The broad distribution raises the possibility that the small complexes are heterogeneous. Three things are worth noting in this regard. First, this broad distribution has been noted in a previous study and, in high salt, the broad distribution resolved into a much sharper peak that corresponded in size to the 9.8S γ-TuSCs (Akashi et al., 1997). Second, deletion of any of the non-essential GCPs caused a slight apparent reduction in the sedimentation coefficient of the small complexes (Fig. 3.6) and the greatest reduction occurred in the GCPF deletion. Third, GCPF localizes to the SPB in the absence of GCPD or E and GCPD localizes to the SPB in the absence of GCPE, revealing that an intact large γ-tubulin complex is probably not required for GCPF and GCPD binding to the γ-TuSC. These data raise the possibility that the small complexes are not just γ-TuSCs, but perhaps γ-TuSCs bound to one of the non-essential GCPs in a salt-sensitive fashion (such binding could be hierarchical as discussed below).

### 3.3.2 Roles of the GCPs in binding of γ-TuCs to the SPB

Our deletion experiments reveal that the components of the γ-TuSC (γ-tubulin,
GCPB and GCPC) are each essential for the localization of the other γ-TuSC components to the SPB. The structure of the *Saccharomyces cerevisiae* γ-TuSC has been determined (Kollman et al., 2008) and, based on this structure and previous experimental data (Zimmerman et al., 2004; Takahashi et al., 2002; Nguyen et al., 1998), it is not surprising that elimination of GCPB or C eliminates the localization of all γ-TuSC components to the SPB. It is more surprising, however, that γ-tubulin is required for localization of GCPB and C to the SPB because the structure of the γ-TuSC (at least when the γ-TuSC is free in solution) does not predict that γ-tubulin would be involved in binding GCPB and C together or in binding the γ-TuSC to the SPB.[Note, however, that, while it is not emphasized in the manuscripts, previous data from *D. melanogaster* suggest that depletion of individual γ-TuSC components reduces the localization of other γ-TuSC components to the centrosome (Colombie et al., 2006; Raynaud-Messina and Merdes, 2007).] While there are a number of possible explanations for the failure of GCPB and C to localize to the SPB in a γ-tubulin deletant, one speculative, but intriguing, possibility is suggested by the work of Kollman et al. (2008). The structure of the γ-TuSC reveals that in order for γ-TuSCs to participate in microtubule nucleation, a rotation must occur in GCPB/C that moves γ-tubulin into a position to nucleate microtubule assembly. Kollman et al. raise the possibility that binding of the γ-TuSC to the SPB proteins is associated with the rotation in GCPB/C that brings γ-tubulin molecules into proximity. We speculate that lateral interactions of γ-tubulin molecules, in turn, may help to hold the γ-TuSC in a configuration that binds stably to the SPB and nucleates microtubule assembly. The removal of γ-tubulin would not only eliminate microtubule nucleation, but would prevent the locking of GCPB and C in a configuration in which they bind well to the SPB. In this
view, the active microtubule nucleation structure would be formed and stabilized by the cooperative interactions of γ-tubulin, GCPB and C and one or more SPB proteins.

Our data demonstrate for the first time that the γ-TuSC is required for localization of GCPD-F to the SPB. This, coupled with our finding and the findings of others that the γ-TuSC proteins localize to polar MTOCs in the absence of GCP4-6 homologues (Venkatram et al., 2004; Anders et al., 2006; Verollet et al., 2006), demonstrates that GCPD-F are attached to the SPB via the γ-TuSC rather than vice versa. This finding is apparently at odds with the finding in *Schizosaccharomyces pombe* that Gfh1p (GCP4) and Alp16p (GCP6) localize to the SPB in *alp4* (GCP2) and *alp6* (GCP3) mutants (Venkatram et al., 2004). The studies in *S. pombe* were carried out with temperature-sensitive rather than null alleles, however, and it is possible that these alleles retain some ability to bind GCP4-6 homologues at the temperatures at which the experiments were carried out.

We have discovered a hierarchy of localization of GCPD-F to the SPB. GCPF requires only the γ-TuSC proteins for localization to the SPB and this indicates that GCPF binds directly to the γ-TuSC. GCPD requires the γ-TuSC and GCPF for localization but not GCPE. This suggests that GCPD binds to GCPF and does not bind (or binds very loosely) to the γ-TuSC. GCPE requires all the other γ-TuC components for localization, which is consistent with observations on Mod21p (GCP5) in *S. pombe* (Anders et al., 2006). Our results suggest that the γ-TuSC binds to the SPB, GCPF binds to the γ-TuSC, GCPD binds to GCPF and GCPE binds to GCPD. Although our data are generally quite consistent with the findings of Verollet et al. in *D. melanogaster* (Verollet et al., 2006), they differ in this instance in that Verrollet et al. found that RNAi depletion
of Dgrip75 (GCP4) substantially reduced both the total levels of Dgrip128 (GCP5) and Dgrip165 (GCP6) in the cells and at the spindle poles, whereas we found that deletion of GCPD did not affect GCPF localization at the SPB.

3.3.3 Functions of the GCPs in vivo

As expected, we found that the γ-TuSC components are important for mitotic spindle formation and correct organization of cytoplasmic microtubules. In addition, we have found that the γ-TuSC is required for localization of all γ-TuC components to the SPB.

GCPD-F, however, are clearly less important. They are not essential and colony growth is not slowed measurably in their absence. GCPD-F homologues are also inessential in *D. melanogaster* (Verollet et al., 2006; Vogt et al., 2006) and *S. pombe* (Anders et al., 2006; Venkatram et al., 2004; Fujita et al., 2002). They are quite conserved evolutionarily, however, and this implies that they must have functions important enough to confer a selective advantage.

One function is clearly in the assembly or stability of large γ-TuCs. Large γ-TuC peaks were greatly reduced in sucrose gradients of GCPD-F deletants relative to controls. The simplest interpretation of these data is that GCPD-F collectively stabilize the interactions of γ-TuSCs in solution, helping to form large γ-TuCs and that GCPD-F are all required for the stabilization. This is consistent with some (Verollet et al., 2006) but not all (Vogt et al., 2006) data from *D. melanogaster*. However, recent structural studies of the *S. cerevisiae* γ-TuSC have revealed that the γ-TuSC has an intrinsic tendency to associate laterally with other γ-TuSCs and can spontaneously assemble into rings, similar
to *Drosophila* γ-TuRCs, but with poor nucleating activity because the configuration of the two γ-tubulins within each γ-TuSC is incompatible with the microtubule lattice (Kollman et al., 2008; Kollman et al., 2010). The cryo-EM structure of the γ-tubulin oligomer-ring also suggested that the grip2 motif may be involved in γ-tubulin binding (Kollman et al., 2010). GCP2-6 contain the grip 2 motif and all of the *D. melanogaster* Dgrips can interact with the γ-tubulin (Gunawardane et al., 2000). Our data shows that *A. nidulans* γ-tubulin small complexes distribute in a broad range in the sucrose gradient, suggesting that γ-tubulin small complexes are heterogenous. As GCPD-F all have grip 2 motifs and deletion of any of the *gcpD*-F genes caused the peak of the small complexes to shift toward the lower mass, our data supported the suggestions made by Kollman et al. (2010) that GCP4-6 may each bind γ-tubulin and form alternative γ-TuSCs. Such alternative γ-TuSCs could be important for forming and/or stabilizing the γ-tubulin large complexes. Finally, since the deletion of GCPD-F has little effect on growth and does not cause massive mitotic defects or gross alteration of cytoplasmic microtubules or interfere with meiosis, it follows that large γ-TuCs are not particularly important in *A. nidulans*.

Although GCPD-F are not essential, we have found that they do have a role in insuring the fidelity of chromosomal segregation. When they are deleted, the frequency of chromosome mis-segregation increases and, in addition, GCPD-F deletions are synthetically sick with a deletion of the spindle assembly checkpoint gene *md2A*, the *mad2* homologue. Although we cannot rule out the possibility that the synthetic sickness has to do with a role for the GCPs in mitotic regulation, the simplest explanation is that deletion of GCPD-F, singly or in combination, results in an increase in mitotic spindle defects. If the spindle assembly checkpoint mechanism is functional, the defects are
repaired and the number of abnormal mitoses is low enough that growth is normal. When
the spindle assembly checkpoint mechanism is rendered dysfunctional by deletion of
\textit{md2A}, however, the defects are not repaired and the resulting mitotic defects cause a
significant reduction in growth. It is worth noting that loss of function of GCPD-F
homologues also cause mitotic defects in \textit{D. melanogaster} (Verollet et al., 2006).

We suggest the following model for the function of \(\gamma\)-TuC components in \textit{A.
nidulans} and, perhaps, with only small modifications, in other organisms as well. In the
presence of GCP4-6 homologues, each of them binds to one \(\gamma\)-tubulin and, together, they
form a trimeric \(\gamma\)-TuSC in which GCP5 interacts with GCP4 and GCP4 interacts with
GCP6. This trimeric \(\gamma\)-TuSC can promote the assembly of the large complex in the
cytoplasm and in many organisms may be required for its assembly. It may either initiate
or terminate the oligomerization of conventional \(\gamma\)-TuSCs so that one such alternative \(\gamma\-
TuSC and 5 conventional \(\gamma\)-TuSC form a large complex with 13-fold symmetry. In this
large complex, GCP6 will interact with the neighboring conventional \(\gamma\)-TuSC, while
GCP4 and GCP5 will not be able interact with any conventional \(\gamma\)-TuSC but rely on
GCP6 to remain in the large complex. This large \(\gamma\)-TuC binds to the SPB with the \(\gamma\-
TuSC being the site of binding to the SPB. Like the centrosome, the inner face of the \textit{A.
nidulans} SPB is fibrous and this fibrous material could interact laterally with \(\gamma\)-TuSCs.
The large \(\gamma\)-TuC can nucleate microtubule assembly efficiently. In the absence of GCP4-
6 homologues, the large complex does not form in the cytoplasm, or forms inefficiently,
but the \(\gamma\)-TuSCs can still bind to the SPB. The \(\gamma\)-TuSCs nucleate microtubule assembly \textit{in vitro}
very inefficiently (Oegema et al., 1999) and it is likely that, at the SPBs, they
interact with SPB or centrosomal proteins like Spc110 to form \(\gamma\)-TuSC oligomer-rings

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(Kollman et al., 2008; Kollman et al., 2010) that are not 13-fold but can still nucleate microtubules efficiently. The spindles that assemble in the absence of GCP4-6 homologues are functionally deficient, perhaps having fewer microtubules than normal or abnormal microtubule dynamics (Bouissou et al., 2009) but the problems with the spindles are normally corrected if the spindle assembly checkpoint mechanism is in place.
Table 3.1

Identification of GCP2-6 homologs in *A. nidulans* by a BLAST search of the *A. nidulans* genome database.

<table>
<thead>
<tr>
<th>Homolog</th>
<th>Homo sapiens</th>
<th>A. nidulans</th>
<th>E value</th>
<th>A. nidulans gene symbol</th>
<th>Protein product</th>
<th>Predicted mass of protein (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCP2</td>
<td>Q9BSJ2</td>
<td>AN5873.4</td>
<td>ANID_05873.1</td>
<td>0</td>
<td>gcpB</td>
<td>GCPB</td>
</tr>
<tr>
<td>GCP3</td>
<td>Q96CW5</td>
<td>AN4867.4</td>
<td>ANID_04867.1</td>
<td>0</td>
<td>gcpC</td>
<td>GCPC</td>
</tr>
<tr>
<td>GCP4</td>
<td>Q9UGJ1</td>
<td>AN2176.4</td>
<td>ANID_02176.1</td>
<td>1.58E-12</td>
<td>gcpD</td>
<td>GCPD</td>
</tr>
<tr>
<td>GCP5</td>
<td>AAK77662</td>
<td>AN8120.4</td>
<td>ANID_08120.1</td>
<td>4.09E-18</td>
<td>gcpE</td>
<td>GCPE</td>
</tr>
<tr>
<td>GCP6</td>
<td>AAK82968</td>
<td>AN1005.4</td>
<td>ANID_01005.1</td>
<td>5.26E-11</td>
<td>gcpF</td>
<td>GCPF</td>
</tr>
</tbody>
</table>

The designation of human GCP-encoding genes are from NCBI. The Broad Institute designation for *A. nidulans* genes have changed recently. We have, therefore, listed the new Broad Institute designations as well as the older designations that are used by the other *A. nidulans* genome databases, the Central *Aspergillus* Data Repository (CADRE, [http://www.cadre-genomes.org.uk/](http://www.cadre-genomes.org.uk/)) and the *Aspergillus* Genome Database (AspGD,[http://www.aspgd.org/](http://www.aspgd.org/)).
Table 3.2

Effect of deletion of genes encoding γ-TuSC components on SPB localization.

<table>
<thead>
<tr>
<th>Tagged homolog</th>
<th>mipAΔ</th>
<th>gcpBΔ</th>
<th>gcpCΔ</th>
<th>gcpDΔ</th>
<th>gcpEΔ</th>
<th>gcpFΔ</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-tubulin-mCherry</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GCPB-GFP</td>
<td>-</td>
<td>N/A</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GCPC-GFP</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GCPD-GFP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>GCPE-GFP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>GCPF-GFP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>N/A</td>
</tr>
</tbody>
</table>

N/A, not applicable.
Conservation of grip1 motif in *A. nidulans* and human GCPs. Sequence alignments of *A. nidulans* and human GCPs. Sequence alignments were generated using the ‘T-coffee’ algorithm. Standard RasMol colors are used for amino acids and the numbers refer to the amino acid number within each protein.
Figure 3.2 (Continued)
Figure 3.2: Continued

Conservation of grip2 motif in *A. nidulans* and human GCPs. Sequence alignments of *A. nidulans* and human GCPs. Sequence alignments were generated using the ‘T-coffee’ algorithm. Standard RasMol colors are used for amino acids and the numbers refer to the amino acid number within each protein.
Figure 3.3

Growth of strains carrying GCP-GFP fusions. The temperature of incubation is given at the bottom of each plate. A schematic diagram showing the positions of the colonies carrying the various GFP fusions is at the lower right. The GFP fusions do not affect growth rates at any temperature.
Figure 3.4

Localizations of γ-tubulin and putative GCPs to the SPB and septum. All panels except G are projections of Z-series stacks. A-F were taken with widefield microscopes. (A) γ-tubulin mCherry and histone H1-GFP. (B) GCPB-GFP, histone H1-mCherry. (C) GCPC-
Figure 3.4: Continued

GFP, histone H1-mCherry. (D)GCPD-GFP, histone H1-mCherry. (E) GCPE-GFP, histone H1-mCherry. (F) GCPF-GFP, histone H1-mCherry. GCPF fluorescence is very weak and barely brighter than the hyphal background fluorescence. Arrows in each panel indicate the SPB location of γ-tubulin or the relevant GCP. The variation in nuclear size is due to the cell-cycle stage at which the images were captured. The nuclei in C, for example, are in G1 and the nuclei in D are in late G2. (G,H) Images were taken with an UltraviewVox spinning disk confocal microscope and show the same field. (G) Bright field image of a portion of a long germling that has formed a septum (arrow). (H) GCPC-GFP and histone H1-mCherry. In addition to the spindle pole bodies, GCPC localizes in relatively faint dots at the septum (arrow). A-F are the same magnification. scale bar in A:10 µm. G and H are the same magnification. scale bar in G: 10 µm.
Figure 3.5

Co-localization of GCPs with the *A. nidulans* Nud1 homolog. Each row of images shows the same field. Each of the GCPs co-localizes with the *A. nidulans* Nud1 homolog, a spindle pole body marker. All images are the same magnification.
Figure 3. 6

Co-immunoprecipitation of putative \(\gamma\)-tubulin complex proteins with \(\gamma\)-tubulin. (A) and

(Continued)
size distribution of γ-tubulin complexes as determined by sucrose-density centrifugation (B,C). (A) The leftmost lane shows protein size standards. The remaining lanes are from western blots using anti-GFP. For each GCP-GFP fusion strain the left lane shows a mock immunoprecipitation in which the anti-γ-tubulin antibody was omitted. The right lane shows an immunoprecipitation with the anti-γ-tubulin antibody. The anti-GFP recognizes a band of the expected size in each case, indicating that GCPB-GCPF coimmunoprecipitate with γ-tubulin. (B) One set of representative results of western blots, using an anti-γ-tubulin antibody as a probe, of sucrose-density gradients of cytoplasmic extracts of strains wild-type for the GCP-encoding genes (WT) or carrying gcpD, gcpE and gcpF deletions. (C) Plot of the intensity of the γ-tubulin signal for each fraction from the gradients. For each strain, the signal intensity of each fraction is expressed as the percentage of the total γ-tubulin in the sample. In the wild-type strain γ-tubulin is present in a broad peak between approximately 7S and 14S and in a sharper peak at approximately 21S. In the deletion strains, the higher mass peak is greatly diminished and the higher and lower mass peaks are shifted slightly to the left (lower mass).
Figure 3. 7

Growth of strains carrying deletions of gcp genes. The temperature of incubation is at the upper left of each panel and a schematic showing the genotypes of the strains with respect to the gcp genes is at the upper left. The growth of the gcpΔ strains is indistinguishable from the parental strain at all temperatures.
Figure 3. 8

Abolition of γ-tubulin and GCP localization by deletion of *gcpC*. In each panel a

(Continued)
Figure 3.8: Continued

germling carrying a *gcpC* deletion (*gcpCΔ*) is shown along with one or more ungerminated conidia that are wild-type for *gcpC* but carry *riboB2* that prevents growth in unsupplemented media. The swollen conidia that gave rise to each germling are marked with asterisks. (A) *gcpCΔ* in a strain carrying histone H1-GFP and γ-tubulin-mCherry. Two nuclei of unequal sizes are present. γ-tubulin does not show SPB localization in the *gcpCΔ* germling, but does localize to the SPB of the nucleus in the ungerminated *gcpC+* spore (arrow). (B-E) *gcpCΔ* in strains carrying histone H1-mCherry and GCPB-GFP (B), GCPD-GFP (C), GCPE-GFP (D) and GCPF-GFP (E). Each *gcpCΔ* germling has one nucleus, reflecting the fact that nuclear division is inhibited in the *gcpCΔ* strain. Germlings as long as these would normally have many nuclei. In each case, the GCP fails to localize to the SPB in the *gcpCΔ* germlings (i.e. there is no GCP spot associated with the nucleus), but localizes normally in the ungerminated *gcpC+* conidia (arrows). All images are maximum intensity projections of Z-series stacks that include the entire germling so the absence of GCP fluorescence is not due to the SPB being out of the focal plane. Scale bar: 10 μm.
Figure 3.9

The γ-TuSC does not require GCPD-GCPF for localization to the SPB. (A) GCPC-GFP localization in a gcpD deletion strain. GCPC-GFP localizes normally to SPBs (arrows). Identical results were obtained for gcpEΔ (B) and gcpFΔ (C). GCPB and γ-tubulin also localized normally in gcpD-gcpF deletion strains. Scale bar: 10 µm.
Figure 3. 10

Dependency relationships for localization of non-essential GCPs. The *gcpD*, *gcpE* and *gcpF* genes were deleted in strains carrying fusions of GCPD-GCPF with GFP. The GCP-GFP fusion is shown at the top of each panel and the deletion at the left of each pair of panels. SPB localization of the GCP-GFP fusion is indicated with arrows. GCPF localizes to the SPB in *gcpD* and *gcpE* deletants. GCPD localizes to the SPB in the *gcpE* deletion strain but not in the *gcpF* deletion strain. Neither GCPD nor GCPE localize to the SPB in the *gcpF* deletion strain. Scale bar: 10 µm.
Figure 3.11

Effects of *gcp* deletions on microtubules. All panels are maximum intensity projections of Z-series stacks. Microtubules are shown with GFP–α-tubulin and chromatin with histone H1-mCherry. Deletion of *gcpB* or *gcpC* caused a dramatic reduction in cytoplasmic microtubules and a near absence of mitotic spindles. Some nuclei in *gcpB* and *gcpC* deletants had partially condensed nuclei (e.g. *gcpBΔ*, mitotic), perhaps reflecting partial chromatin decondensation during a slow mitotic exit. Thin, apparently non-functional spindles were seen occasionally in *gcpB* and *gcpC* deletants. An example is shown in the *gcpCΔ* mitotic panel (arrow). The interphase cytoplasmic microtubule array and mitotic spindles were apparently normal in a *gcpD, gcpE, gcpF* triple-deletant.

(Continued)
strain. They were also apparently normal in \textit{gcpD}, \textit{gcpE} and \textit{gcpF} single deletions (our unpublished data). Scale bar: 10 µm.
Figure 3. 12
Growth and benomyl sensitivities of strains carrying gcp deletions. The left column of

(Continued)

Figure 3.12: Continued
plates shows growth of the gcp deletants in a strain expressing GFP-α-tubulin and histone H1-mCherry. The gcp deletant strains grow identically to the parental strain at all temperatures. The right column shows the growth of the same strains on the concentrations of benomyl shown. The gcp deletants are indistinguishable from the parental strain in benomyl sensitivity.
Figure 3. 13

Synthetic interactions of *md2A* and *gcpF* deletions. A schematic diagram is shown at the top. Growth was at 37°C. The different colony colors are due to different spore color markers used in the cross to construct the double mutants. The *gcpF* and *md2A* deletant strains grow as well as the wildtype control, but the five double-mutant colonies are smaller and have rougher edges, revealing that the *gcpF* and *md2A* deletions are synthetically sick.
Figure 3. 14

Recombinant progeny in a cross of two \(gcpD-F\) triple deletion strains. Strain LO2018 carrying deletions of \(gcpD-F\) and \(wA3\), which confers white conidia, was crossed to strain LO1930, which also carries deletions of \(gcpD-F\) but carries \(fwA1\) (fawn conidia). Progeny of the cross were spread on the plate shown. The fact that colonies with fawn, white and green colonies are present indicates that the two strains have crossed successfully. In particular, all colonies with green conidia are recombinant.
Figure 3.15

Synthetic sickness of a deletion of the *A. nidulans* MAD2 homolog (*md2Δ*) and deletions of γ-tubulin complex proteins. A schematic diagram of the genotypes of the colonies is shown over each pair of plates and the temperature of incubation is shown at the left. *md2Δ* and *gcpDΔ* and *gcpEΔ* deletions show weak synthetic interactions. Colonies carrying the double mutants are slightly smaller than either parent and have rough edges at 37°C. The *md2Δ*, *gcpFΔ* interaction is strongest.
Chapter 4: Investigation of a proteasome-associated nuclear matrix in 

*Aspergillus nidulans*

4.1 Introduction

\(\gamma\)-Tubulin is conserved in all eukaryotes and its role in nucleating microtubules is well established. Studies in several different organisms have also suggested that \(\gamma\)-tubulin and \(\gamma\)-tubulin complexes are involved in organizing microtubules and regulating microtubule dynamics and mitotic events (Paluh et al., 2000; Vogel et al., 2001; Jung et al., 2001; Hendrickson et al., 2001; Fujita et al., 2002; Vogel and Snyder, 2000; Bouissou et al., 2009; Vardy et al., 2002; Sampaio et al., 2001; Prigozhina et al., 2001; Prigozhina et al., 2004). Our lab has generated a series of *A. nidulans* \(\gamma\)-tubulin mutants and one of them, *mipAD159*, does not inhibit mitotic spindle formation but disrupts the coordination of late mitotic events (Prigozhina et al., 2001; Prigozhina et al., 2004). Our further investigations revealed that *mipAD159* causes a nuclear autonomous failure of inactivation of the APC/C in the G1 phase of the cell cycle, thus leading to a constitutive destruction of cyclin B (Nayak et al., 2010). The APC/C is an ubiquitin ligase that targets proteins for destruction by the proteasome and in order for constitutive activation of the APC/C to cause destruction of cyclin B, there is a strong prediction that proteasomes would be present in the nuclei in which cyclin B is continuously destroyed. Although proteasomes are extremely important, there is little localization data on proteasomes in
filamentous fungi. These factors, in combination, led us to examine proteasome localization in strains carrying \textit{mipA+} and \textit{mipAD159}.

Ubiquitous in eukaryotes, the 26S proteasome is a 2.5 MDa protein complex composed of the 20S core particle (CP) and the 19S regulatory particle (RP) [reviewed in (Murata et al., 2009)]. Its localization has been examined in many model organisms. In mammalian cells, the proteasome is found to diffuse in cytoplasm as well as to associate with the centrosomes, cytoskeletal elements, the outer surface of endoplasmic reticulum, and microsomes (Palmer et al., 1996; Rivett et al., 1992; Wojcik and DeMartino, 2003; Rivett, 1998). The proteasome was also detected in euchromatin regions, the periphery of heterochromatin, around the nucleolus and associated with the nuclear matrix (Palmer et al., 1996; Rivett et al., 1992; De Conto et al., 2000Wojcik and DeMartino, 2003). The intracellular distribution of proteasomes changes during progression through the cell cycle, but the patterns differed depending on the cell lines and visualization techniques that were used. In some cases, the proteasome appeared to be bound to condensing chromosomes, spindle fibers, and midbodies during different stages of mitosis (Amsterdam et al., 1993; Wojcik et al., 1995), whereas, in another case, it appears diffuse freely throughout the whole cells at mitosis (Reits et al., 1997). In \textit{S. pombe}, which undergoes a closed mitosis without breakdown of the nuclear envelope, the proteasome localizes predominantly at the inner side of the nuclear periphery both in interphase and throughout mitosis (Wilkinson et al., 1998). In actively dividing \textit{S. cerevisiae} cells, the proteasome was described as being either in the nucleoplasm or associated with the nuclear periphery in early studies (Wendler et al., 2004; Enenkel et al., 1998; Russell et al., 1999; McDonald and Byers, 1997; Isono et al., 2007). The nucleoplasmic localization
of the proteasome was confirmed by a recent study using a strain expressing a GFP-tagged 20S CP subunit, Pre6p, which also suggested that the enrichment of the proteasome at the nuclear periphery could be induced by starvation, chemical fixation, and cell wall stresses (Laporte et al., 2008).

In *S. pombe*, the accumulation of the proteasome in the nucleus was abolished in *cut8* mutants. Such depletion of the intranuclear proteasome caused defects in clearance of critical mitotic regulatory proteins including Cyclin B and Securin and leads to a severe mitotic delay and a high level of chromosome nondisjunction (Takeda and Yanagida, 2005; Tabb et al., 2000). Whether the nuclear localization of the proteasome is as important in degradation of its mitotic target proteins and timely mitotic progression in eukaryotes with semi-open or open mitosis as in *S. pombe* is unclear.

We have determined the localization pattern of the proteasome in *A. nidulans* by time-lapse live imaging of fluorescent protein fusions of the highly conserved proteasomal subunit RPN5 in *mipA*+, *mipAD159* and *mipA* deletant (*mipAΔ*) strains. We found that there appears to be a proteasome-associated nuclear structure which contains nucleolar proteins and which can physically separate from the chromosomes. We also found that proteasomes fail to localize to the nucleus in the absence of the *A. nidulans* homolog of the *S. pombe* protein Cut8 (An-Cut8) and that the expression level of An-Cut8 correlates with the accumulation level of proteasomes in the nucleus, which suggests that An-Cut8 functions in nuclear transporting of proteasomal subunits. Surprisingly, in absence of intranuclear proteasomes, mitotic progression was not obviously delayed, however, and the
degradation of cyclin B proceeded in a timely fashion. Our data suggest that the nuclear accumulation of the proteasome in a semi-open mitosis system is not imperative for mitotic progression as it is in closed mitosis and the ubiquitination by APC/C may be sufficient to drive the release of the cyclin B from intranuclear structures.

4.2 Results

4.2.1 In vivo localization of the proteasome component, RPN5, through the cell cycle

To reveal the in vivo localization of 26S proteasomes in A. nidulans, we looked for an A. nidulans homolog of the proteasome lid subunit RPN5 by a BLASTP search of the A. nidulans genome database using the human proteasome subunit RPN5/p55 (Swiss-Prot designation Q54UJ0) as a query sequence. We identified a single excellent homolog (AN4775) (e-value: 5.6e-87) and we hereby designate this gene rpn5. Replacing the rpn5 gene with the AfriboB gene, using the fusion PCR and gene targeting system developed recently in A. nidulans (Yang et al., 2004; Nayak et al., 2006; Szewczyk et al., 2006), resulted in lethality, revealing that RPN5 is essential for viability. Multiple genes encode RPN5 homologs in S. pombe and A. thaliana (Yen et al., 2003; Book et al., 2009), but our BLAST and deletion results demonstrate that there is a single RPN5 gene in A. nidulans.

To test if RPN5 is a true subunit of the 26S proteasome, we tagged the C-terminus of endogenous RPN5 with S peptide. Our affinity purification using a strain endogenously expressing RPN5-S-tag (below) pulled down most proteasomal subunits, confirming that RPN5 is a component of the 26S proteasome (Table 4.1).
To determine the localization of RPN5 in living cells, we created GFP and mCherry fusions of RPN5 separately and tested their functionality by growing strains carrying *rpn5-gfp* and *rpn5-mCherry* over a wide range of temperatures (from 20°C to 42°C) (Figure 4.1). Growth was indistinguishable from controls from over the entire range indicating that the fusion proteins are fully functional. Our imaging experiments were carried out at 24°C.

Microscopic analysis of a strain carrying RPN5-GFP along with a histone H1-mRFP fusion revealed that RPN5-GFP is barely visible in the cytoplasm, and highly concentrated in the nucleoplasm of interphase nuclei but excluded from a nucleolus-like region. We confirmed the fact that RPN5 is excluded from nucleoli by creating a strain that carries RPN5-GFP, Histone H1-T-saphire and Fibrillarin-mCherry, a nucleolus marker (Ukil et al., 2009) (Figure 4.2). To confirm that RPN5-GFP and mCherry are reliable reporters of the 26S proteasome in *A. nidulans*, we tagged two other proteasomal subunits, the α6 subunit and the β1 subunit of the 20S core complex and found that they co-localize with RPN5 throughout the cell cycle (Figure 4.3).

As *A. nidulans* undergoes semi-open mitosis during which the nuclear pores partially disassemble and most soluble nuclear proteins and complexes diffuse into the cytoplasm, it was surprising that our time-lapse imaging shows that RPN5 remains in the nucleus through early mitosis (Figure 4.4). During anaphase, RPN5 is located between separating chromosomes (Figure 4.4). To determine if it co-localized with the mitotic spindle in telophase, we created a strain carrying RPN5-mCherry, GFP-α-tubulin and Histone H1-T-Sapphire. We found that RPN5 occupies a larger volume than the spindle in anaphase (Figure 4.5), and, thus, there is no evidence that RPN5 binds to the spindle.
RPN5 quickly disappears from the dividing nucleus in late anaphase/telophase and returns to the daughter nuclei in early G1 (Figure 4.4).

4.2.2 Physical separation of an RPN5-associated nuclear structure from chromatin during aberrant mitosis

Our previous investigation of a recessive γ-tubulin mutant, *mipAD159*, prompted us to examine the localization of 26S proteasome in both a wild type strain and strains carrying *mipAD159*. We noticed that *mipAD159* did alter the localization of RPN5 at restrictive temperatures but in a surprising way. We found that RPN5 sometimes separated physically from the chromosomes in mitosis creating a separate nucleus-like structure containing little or no chromatin (Figure 4.6). Often chromatin appeared to be physically pulled away from RPN5. These results seem to imply that RPN5 binds to a structure that is separable from chromatin and that *mipAD159* causes them to separate in mitosis.

To determine if the physical separation of chromatin from RPN5 is a specific effect of *mipAD159*, we examined RPN5 localization in deletions of the *mipA* gene as well as deletions of *gcpB* and *gcpC* that encode γ-tubulin complex proteins (Xiong and Oakley, 2009). *MipA, gcpB* and *gcpC* are essential genes and disruption or deletion of any of these genes disrupts mitotic spindle formation (Oakley et al., 1990; Martin et al., 1997; Xiong and Oakley, 2009). In order to observe the effects of deletions of these genes on RPN5 distribution, we used the heterokaryon rescue technique (Osmani et al., 1988; Oakley et al., 1990; Martin et al., 1997; Osmani et al., 2006b). We found that deletion of any of these genes resulted in instances of separation of RPN5 from chromatin, resulting
in the strange nucleus-like chromatin deficient structures seen with \textit{mipAD159} (Figure 4.7 and Figure 4.8). As with \textit{mipAD159}, these structures often appeared to result from the chromatin being physically pulled away from the proteasomes. Surprisingly, disruption of these genes in a strain carrying RPN5-GFP, Histone H1-T-sapphire and Fibrillarin-mCherry revealed that these nucleus-like structures often had Fibrillarin-containing structures (putative nucleoli because Fibrillarin is a nucleolar protein) even though chromatin could not be detected (Figure 4.9). Similar structures were also found in wild-type cells after extended treatment with 2.4 µg/ml benomyl (>24 h) (Figure 4.10). Since there should be no pulling of chromatin in cells treated with benomyl, we do not know if the mechanism of formation of chromatin-deficient nuclei is the same in benomyl as in the mutations and deletions of \(\gamma\)-TuSC genes. The data suggest, however, that the chromatin-deficient nuclei might be a consequence of chromosome segregation defects rather than a \(\gamma\)-tubulin-specific defect.

One possible explanation for our data is that RPN5 binds to, or forms, a matrix that can be inherited independently of chromosomes during aberrant mitosis. This motivated us to examine the localization of RPN5 more carefully in \textit{mipA} cells to determine if we could find evidence for a matrix. We obtained high resolution multi-dimensional data sets (Z-series stacks of RPN5-GFP and Histone H1-mCherry collected at a single time point or at intervals over time) using an UltraviewVox spinning disk confocal system. We iteratively deconvolved the data sets to improve resolution and restore residual out of focus signal to its original position in the specimen and we made multi-dimensional optical reconstructions of the nuclei. Non-deconvolved images suggested that chromatin and RPN5 did not overlap completely. RPN5 appeared to occupy portions of the
nucleoplasm distinct from those occupied by Histone H1, but RPN5 did not simply fill the remainder of the nuclear space (Figure 4.4). Deconvolution confirmed this interpretation and added considerably more information. In mitosis, as chromosomes condensed RPN5 did not simply fill the nucleoplasm outside of chromosomes, but appeared to be structured and the RPN5 structures fit into spaces between the chromosomes (Figure 4.11). Chromatin and RPN5 did not overlap at all. In anaphase, RPN5 also did not overlap with chromatin but was found briefly between the separated chromatin masses (Figure 4.11). Again the RPN5 appeared structured (Figure 4.11). Strands of RPN5 were often visible and often the position of the nucleolus was visible as an empty space in the RPN5. In interphase RPN5 also appeared structured and strands of RPN5 were intertwined with regions of chromatin. The separation of chromatin and RPN5 and the structured appearance of RPN5 are consistent with the notion that the proteasome forms or binds to some type of nuclear matrix.

4.2.3 The localization of RPN5 is independent of the spindle matrix protein MLP1

The data on the nuclear matrix or matrices are copious and sometimes contradictory, but the nuclear matrix is clearly of great interest. We were interested in determining the nature of the putative matrix containing RPN5. One candidate for a matrix to which RPN5 might bind has recently been discovered in *A. nidulans* (De Souza et al., 2009). The *A.nidulans* MLP1 homolog acts as a scaffold for the mitotic regulatory proteins Mad1 and Mad2 (De Souza et al., 2009) within mitotic nuclei and they all co-localize between segregating chromosomes at telophase. This led us to hypothesize that RPN5 may bind to the MLP1 scaffold during mitosis. From our time-lapse imaging of a
strain endogenously expressing MLP1-GFP and RPN5-mCherry, we noticed, however, that the RPN5-mCherry signal occupied a larger area than MLP1 and disappeared from nuclei later than the MLP-GFP signal (Figure 4.12). To test our hypothesis more directly, we deleted the mlp1 gene in a strain expressing RPN5-GFP and Histone H1-mCherry. Our time-lapse imaging of mlp1 deletants showed that the deletion of mlp1 does not alter the localization of RPN5 at mitosis or G1 (Figure 4.13). It has been shown that expressing a non-degradable cyclin B could induce a translocation of MLP1 from between separating chromatin masses to around SPBs (De Souza et al., 2009). We found that such an induced translocation of MLP1 led to a complete different distribution pattern of Mlp1 and RPN5 at telophase. The proteasome, thus, does not appear to bind to the MLP1 scaffold (Figure 4.14).

4.2.4 The nuclear localization of the proteasome depends on AnCut8

Another candidate for the matrix, based on data from S. pombe was Cut8. In S. pombe, Cut8 is highly enriched at the nuclear envelope and is required for the nuclear localization of the 26S proteasome (Tatebe and Yanagida, 2000; Takeda and Yanagida, 2005). A homolog of Cut8 was also found in fly and it proved to be indispensable for enrichment of the proteasome at the nuclear periphery in Drosophila S2 cells (Tatebe and Yanagida, 2000; Takeda and Yanagida, 2005). S. pombe Cut8 has been proposed to be a sensor and anchor for the nuclear proteasome in S. pombe (Takeda and Yanagida, 2005), making it a good candidate for a nuclear matrix component. Although proteasomes are not enriched at the nuclear periphery in A. nidulans as in S. pombe, we thought it would be worthwhile to determine if A. nidulans has a Cut8 homolog, and, if so, its localization
pattern and its importance for the localization of the proteasome in the nucleus. We performed a BLASTP search of the predicted proteins of the *A. nidulans* genome with the *S. pombe* Cut8 protein sequence and found a single *A. nidulans* homolog, AN4411, with 34% aa identity (e-value: 4.9e-29). We designated this gene *An-cut8*. We deleted *An-cut8* and found that it does not cause lethality in *A. nidulans* but does cause reduced growth and conidiation at all temperatures tested (20-42°C) (Figure 4.15). The growth reduction was exacerbated at lower temperatures and growth was strongly inhibited at 20°C. To examine the localization of *An-Cut8*, we fused GFP to the 3’ end of the *An-cut8* gene such that the gene was the only copy in the genome and it was under control of its normal, endogenous promoter. Strains carrying *An-Cut8*-GFP grew indistinguishably from the control from 20°C to 42°C (Figure 4.15). *An-Cut8*-GFP thus seems to be fully functional.

Our time-lapse imaging of strains with *An-Cut8*-GFP and other markers revealed two facts. First, *An-Cut8* is concentrated in the nucleoplasm, but is not enriched at the nuclear periphery. Second, *An-Cut8* disappears from the mitotic nucleus upon mitotic onset while the proteasome remains in the mitotic nucleus until late mitosis. Both *An-Cut8* and the proteasome reappear in the daughter nuclei in early G1 (Figure 4.16). These results indicate that, at least during mitosis when the nuclear pore complexes are partially disassembled, the proteasome does not rely on nuclear *An-Cut8* for retention in nucleus.

To test if *An-Cut8* is required for the nuclear localization of the proteasome during interphase, we deleted *An-cut8* in a strain expressing RPN5-GFP and NLS-DsRed (integrated at the *yA* locus). NLS-DsRed localizes to interphase nuclei, exits nuclei rapidly when the nuclear pore complex partially disassembles at mitotic onset and returns
to the nucleoplasm when the nuclear pore complex reassembles at the transition from telophase to G1 (De Souza et al., 2004; Suelmann et al., 1997). It is, thus an excellent marker for nuclei, mitotic entrance and exit and the assembly and disassembly of the nuclear pore complex.

In strong contrast to the control, RPN5-GFP was undetectable in nuclei in An-cut8Δ strains in interphase, instead, it accumulated in the cytoplasm (Figure 4.17). When the cells entered mitosis, the RPN5 distribution did not change although the nuclear pores partially disassembled (Figure 4.18). At the very end of mitosis, however, the concentration of RPN5 within the mitotic nuclei seemed to increase transiently before the two daughter nuclei formed. The β1 subunit of the 20S core complex showed the same nuclear localization pattern in An-cut8 deletants, therefore, we can conclude that An-Cut8 is required for the nuclear localization of the 26 S proteasome in interphase in A. nidulans (Figure 4.17 A). The fact that An-Cut8 is not required for retention of RPN5 in early mitosis but is required for localization of the proteasome in interphase suggests that the function of An-Cut8 in proteasome localization to the nucleus might be in transport of proteasomal subcomplexes or subunits into the nucleus rather than in retention of proteasomes in the nucleoplasm. The fact that proteasomes remain outside of mitotic nuclei in An-cut8 deletants indicates that mitotic nuclear pores may present a physical barrier inhibiting the passage of the proteasome into and out of the nucleus until telophase when a final removal of NE between separating chromatin masses proceed along with reformation of NE around the two chromatin masses.
4.2.5 The integrity of the mitotic nuclear pores is important for the retention of the proteasome in mitotic nuclei.

In *A. nidulans*, it has been shown that simultaneous deletion of two core nups, *nup37* and *elys*, leads to a mitotic specific disassembly of the An-Nup84-120 nuclear pore complex (Liu et al., 2009) and, presumably, reduces the physical barrier of the mitotic nuclear pores. To determine if the integrity of the mitotic nuclear pores is important for the retention of proteasomes in mitotic nuclei, we made a *nup37Δ, elysΔ* double mutant that contains RPN5-GFP and NLS-DsRed and examined the retention time of RPN5 during mitosis. We count the retention time of RPN5 as being from the beginning of mitosis, which is indicated by the disappearance of NLS-DsRed from the nuclei, to the time when RPN5 disappears from the nuclei. Compared with the control, in which RPN5 remained in mitotic nuclei for 6.9 ± 1.0 min (n=23), the *nup37Δ, elysΔ* double mutant exhibited a precocious dispersal of RPN5 with a retention time of only 1.7 ± 0.5 min (n=29).

To exclude the possibility that the precocious dispersal of RPN5 is due to late mitotic defects such as the transient aggregation of NPCs or the absence of the double restriction of the mitotic NE caused by the deletions of *nup37* and *elys* (Liu et al., 2009), we repeated the experiments in the presence of 2.4 μg/ml benomyl, which arrests the cells in a pseudo-metaphase state. Benomyl treatment extended the retention time of RPN5 within mitotic nuclei to 41.4 ± 12.4 min (n=14) while the *nup37Δ, elysΔ* double mutant still exhibited a precocious dispersal of RPN5 with a retention time of 1.3 ± 0.5 min (n=46) (Figure 4.19), revealing that a greater disassembly of the mitotic nuclear pore complexes does lead to the loss of the proteasome from nuclei in early mitosis, and thus,
that the integrity of the mitotic nuclear pores is important to keeping the proteasome within nuclei.

4.2.6 The absence of An-Cut8 and the consequent lack of nuclear proteasomes does not severely delay mitosis.

Data from *S. pombe* have shown that loss of Cut8 and the subsequent mis-localization of the proteasome in *S. pombe* causes a severe mitotic delay because of defects in degradation of mitotic regulatory proteins such as cyclin B (Tatebe and Yanagida, 2000). Loss of STS1/DBF8 in *S. cerevisiae* even leads to lethality, suggesting a critical role for nuclear proteasomes in cell-cycle progression. Although An-Cut8 is not essential in *A. nidulans*, we were interested to find out if, in an Ancut8Δ mutant, cyclin B degradation is inhibited and mitosis is delayed. We used NLS-DsRed as an indicator for mitotic progression and mitotic duration time was counted from the complete disappearance of NLS-DsRed to the reappearance of NLS-DsRed. Surprisingly, mitosis in the An-cut8Δ mutant was not grossly delayed compared to controls [control: 6.7 ± 1.6 min (n=41); An-cut8Δ: 7.5 ± 1.8 min (n=48) (Figure 4.20)], suggesting that the absence of the proteasome in mitotic nuclei does not severely inhibit mitotic progression. As deletions of *nup37* and *elys* cause proteasomes to leave nuclei early in mitosis, our result is consistent with the previous observation that only a minor mitotic delay occurs in the *nup37Δ, elysΔ* double mutant (Liu et al., 2009). We also observed that spindle formation was normal in an An-cut8Δ strain by visualizing α-tubulin with GFP and that disassembly and reassembly of the nucleolus were normal by tracking the nucleolus marker Bop1-GFP.
Because the ectopic expression of a cyclin B allele, in which the destruction box has been deleted, arrests cells at telophase (De Souza et al., 2009; Nayak et al., 2010), our data indicated that cyclin B was destroyed in a timely manner in An-\textit{cut8}\(\Delta\) strains. To verify this, we created an \textit{An-\textit{cut8}\(\Delta\)} strain with cyclin B-GFP and Histone H1-mCherry/RFP and observed its mitoses by time-lapse imaging. The localization pattern of cyclin B-GFP in \textit{An-\textit{cut8}\(\Delta\)} cells was very similar to the localization pattern of cyclin B-GFP in \textit{An-\textit{cut8}}+ cells (De Souza et al., 2009; Nayak et al., 2010). It accumulated in the nucleoplasm and at the spindle pole bodies during S and G2. At mitotic onset, the cyclin B level in the nucleoplasm was reduced and much of cyclin B that remained in the nucleus was concentrated at the SPBs. It also localized to the mitotic spindle until anaphase (Figure 4.21). Surprisingly, cyclin B was almost gone from nuclei by anaphase A in \textit{An-\textit{cut8}\(\Delta\)} cells, almost at the same time as, if not earlier than, in \textit{An-\textit{cut8}}+ cells (Figure 4.21). Destruction box deleted cyclin B, however, remained at the spindle poles for an extended period and caused blockage of nuclei in telophase in both \textit{An-\textit{cut8}}+ cells (De Souza et al., 2009; Nayak et al., 2010) and \textit{An-\textit{cut8}} deletants (Figure 4.22). The difference between destruction box deleted cyclin B and wild-type cyclin B is that the APC/C does not ubiquitinate the destruction box deleted cyclin B. We can, thus, deduce that ubiquitination of cyclin B is required for cyclin B to leave the SPBs and the nucleus. However, since proteosomes are severely depleted from nuclei in \textit{An-\textit{cut8}\(\Delta\)} strains and, yet, cyclin B still disappeared from the nucleus with more or less normal timing, it appears that destruction of cyclin B in the nucleus is not required for the disappearance of cyclin B from the nucleus nor for mitotic exit. We infer that ubiquitination of cyclin B releases it from the SPB and spindle and it then diffuses out of the nucleoplasm into the
cytoplasm where it is destroyed.

4.2.7 The level of proteasomes in nuclei correlates with the nuclear levels of An-Cut8

It has been suggested that *S. pombe* Cut8 is a dosage-dependent, nuclear enrichment factor for the proteasome, as ectopic overproduction of Cut8 led to an accumulation of the proteasome within the nucleus (Takeda and Yanagida, 2005). To test if this is also true in *A. nidulans*, we fused a GFP coding sequence to the single chromosomal copy of An-Cut8 and placed it under the control of the highly regulatable *alcA* promoter (Waring et al., 1989). The strain with *alcA*-An-Cut8-GFP grew like the parent on *alcA*-inducing media and like the *An-cut8Δ* strain on repressing media (Figure 4.23), suggesting that overproduction of An-Cut8 does not cause growth defects. To examine the effect of the overexpression of An-Cut8 on the accumulation of the proteasome within the nucleus, we created two strains having RPN5-mCherry under the control of its own promoter, one of which has An-Cut8-GFP with its native promoter and the other has the *alcA*-An-Cut8-GFP. Grown under inducing conditions, the strain with *alcA*-An-Cut8-GFP contained highly elevated An-Cut8-GFP and the ratio of the signal intensity of RPN5-mCherry in the nucleus to the cytoplasm was significantly higher than that in the control (*control An-cut8-gfp*: 4.7 ± 1.6, *alcA-An-cut8-gfp*: 13.3 ± 5.7, *p* < 0.0001, t test) (Figure 4.23 and 4.25). The correlation between the accumulation level of the proteasome within the nucleus and the expression level of An-Cut8 was further confirmed by our time-lapse imaging of the strain with *alcA*-An-Cut8-GFP. When the strain was grown in repressing medium and An-Cut8-GFP was efficiently repressed, the RPN5-mCherry signal in the
nucleus was barely higher than in the cytoplasm, but soon after An-Cut8-GFP was
induced and appeared in the nucleus, the RPN5-mCherry signal in the nucleus increased
quickly (Figure 4.26).

We performed photobleaching experiments to examine nuclear import rates of An-
Cut8-GFP. We photobleached the GFP signal of the entire nucleus and then measured the
recovery of the GFP signal in the nucleus over time. Our data showed that the half time
for the fluorescence of An-Cut8-GFP to recover in the nucleus is about $8 \pm 3.5$ min
(Figure 4.27).

4.3 Discussion

We examined the localization of *A. nidulans* proteasome by visualizing the lid
subunit, RPN5 and two 20S core subunits, α6 and β1 throughout the cell cycle. Our data
show that the proteasome is localized in the cytoplasm all the time and it is also
substantially enriched in the nucleus most of cell cycle except for a brief period at the end
of mitosis. The nuclear retention and dispersal of the proteasome during mitosis,
however, is unique compared to the known localization patterns of the proteasome in
other model systems. In mammalian cells, proteasomes were reported to be enriched in
the nucleus at interphase and either completely diffused into the cytoplasm (Reits et al.,
1997) or bound to mitotic spindles at mitosis (Amsterdam et al., 1993), while in *S.
cerevisiae* and *S. pombe*, proteasomes remain in the nuclei all the time (Wilkinson et al.,
1998). This discrepancy could be, at least partially, due to *A. nidulans’* semi-open
mitosis, as our data from *nup37Δ, elysΔ* double mutants show that further disassembly of
the mitotic nuclear pore complexes leads to an untimely dispersal of proteasomes from
the nuclei. The normal dispersal of the proteasome at the end of the mitosis is probably due to the final removal of the nuclear envelope between the daughter nuclei.

While observing the behavior of RPN5 in the γ-tubulin mutant mipAD159, we noticed that RPN5, and the proteasome by extension, could separate physically from the chromosomes during mitosis. This resulted in nucleus-like structures containing nucleoli but little or no chromatin. RPN5 also separated from the chromatin in deletions of genes encoding γ-TuSC components and in cells treated with benomyl. We also noticed that the RPN5 in chromatin-depleted nuclei and in wild type nuclei had a structured appearance, suggesting that it was not simply diffusing freely through the nucleoplasm. These data led us to suspect that the proteasome might bind to some form of nuclear matrix. Proteasomes or proteasomal subunits have been detected in the nuclear matrix of mammalian cells at interphase and in the nuclear matrix of goldfish oocytes through biochemical fractionations and immunofluorescence after chemical fixation (Wojcik et al., 1995; De Conto et al., 2000; Tokumoto et al., 2000), but they have not been reported to bind to a nuclear matrix in fungi.

Although our initial reason for examining the proteasome localization was related to the specific phenotype of the mipAD159 allele (Nayak et al., 2010), the separation of the proteasome-associated structure from the chromatin, however, was not specific for γ-tubulin mutants, as we observed the same phenomena in mipAD, gcpBD, and gcpCD strains. The appearance of the similar structures after long-term benomyl treatment suggests that the separation might be a general result of aberrant mitosis. The physical separation of chromatin and proteasomes are consistent with the proteasomes binding to a matrix that separates from the chromatin, but that is not the only possible mechanism for
the separation. For example, if proteasomes are largely excluded from the condensed chromosomes and if the nuclear envelope pinched very close to the chromosomes, nucleoplasm including the proteasomes might be pinched off forming chromatin-depleted nuclei. Still, the separation plus the apparent structure of proteasomal localization suggested it was worth exploring the possibility that the proteasome binds to a nuclear matrix. Because many differences with respect to the composition of the nuclear matrix have been detected between early-stage cancer cells and non-cancerous cells (Coffey, 2002; Zink et al., 2004), further characterization of the nuclear matrix and investigation of such separation mechanism is of great interest and may shed light on our understanding of cancer formation.

The composition of the nuclear matrix has been investigated extensively in mammalian cells, especially in many cancer cell lines, and more than 300 proteins have been reported to be nuclear matrix (NM) components (Mika and Rost, 2005; Albrethsen et al., 2009). However, the fact that *A. nidulans* and other fungi lack homologs of the most frequently reported NM proteins makes the characterization of the nature of the nuclear structure challenging. Intriguingly, the anaphase localization of proteasomes between segregating chromosomes in *A. nidulans* is similar to that observed in immortalized ovarian granulosa cells, but in those cells the localization was interpreted as overlapping with mitotic spindle staining (Amsterdam et al., 1993). We demonstrated that the proteasome occupied a larger volume than the spindle and was not concentrated on the spindle in *A. nidulans*.

The term spindle matrix has been used for the non-microtubule material surrounding mitotic spindles in open mitosis and the molecular components of the spindle
matrix overlap with those of the nuclear matrix to some extent, for example, lamin B and NUMA are both NM proteins and spindle matrix proteins (Dechat et al., 2008; Dionne et al., 1999; Radulescu and Cleveland, 2010; Ma et al., 2009; Tsai et al., 2006). This may be advantageous to the cell, as modification of a structure is likely less energy-consuming than formation of a completely new structure. However, these proteins are not conserved in invertebrates and fungi, questioning the existence of similar structures in lower eukaryotes.

We explored the possibility that the proteasome might bind to two candidate proteins that may be part of a nuclear matrix. Recently, a nuclear protein in *Drosophila*, Megator, the orthologue of the mammalian TPR protein, was reported to form both a nuclear endoskeleton and the spindle matrix (Qi et al., 2004) and its orthologue in *A. nidulans*, MLP1, was also shown to tether Mad1 and Mad2 to a spindle matrix in mitotic nuclei (De Souza et al., 2009). We have found that MLP1 does not anchor the proteasome because it does not co-localize very well with the proteasome in mitotic nuclei and neither its absence nor its redistribution affects the localization of the proteasome.

*S. pombe* Cut8 has been proposed as a sensor and anchor for the nuclear proteasome at the inner side of the nuclear envelope (Takeda and Yanagida, 2005) and we, consequently, have identified and determined the localization pattern of the *A. nidulans* homolog of the Cut8 protein (An-Cut8). We also determined whether An-Cut8 is required for the nuclear localization of the proteasome. We found no evidence of the enrichment of An-Cut8 at the nuclear periphery as is the case with *S. pombe* Cut8. This discrepancy may not be surprising, given that we observed a similar difference in the nuclear localization of the proteasome. Second, we found that An-Cut8 does not remain
in mitotic nuclei, but, instead, it disappears from the nuclei upon mitotic onset. This is likely due to the partial disassembly of nuclear pore complexes at mitosis in *A. nidulans*, which allows An-Cut8 to freely diffuse out of the nucleus. In principle it could be due to the degradation of An-Cut8 in nuclei, but live cell imaging of mitotic cells with An-Cut8-GFP showed that the signal in the cytoplasm increased at the same time that the signal in the nucleus decreased. This argues that An-Cut8 disperses into the cytoplasm upon mitotic entry as expected of nuclear proteins that are not attached to any nuclear structures and which are small enough to diffuse through the partially disassembled NPCs. Third, our functional analysis of An-Cut8 revealed that An-Cut8 is required for the localization of the proteasome in the nucleus in interphase. As active nuclear transport is shut down during mitosis in *A. nidulans* (De Souza et al., 2004; Suelmann et al., 1997), proteasomes in mitotic nuclei are either imported during interphase or recruited during mitosis. As mitotic nuclear pores physically block the passage of proteasomes from the cytoplasm into the nucleoplasm, our data support that proteasomes in mitotic nuclei are imported during interphase.

In the end, our Mlp1 and An-Cut8 data do not address the question of whether the proteasome binds to a nuclear matrix in a meaningful way. They do, however, provide some insights into mitotic regulation and functioning of An-Cut8. Surprisingly, the apparent absence of proteasomes in mitotic nuclei seems not to inhibit mitotic progression in *A. nidulans* and the slow growth of *An-cut8Δ* at 25°C is probably not due to severe mitotic defects, as several important mitotic events such as cyclin B degradation, spindle formation, and nucleolar disassembly and reassembly were hardly distinguishable from the control. However, ectopic expression of the destruction box-
deleted cyclin B did arrest An-cut8Δ cells at telophase and led to the retention of the cyclin B at the SPBs. This result suggests that ubiquitination of cyclin B is required for the release of cyclin B from SPBs but intranuclear proteasomes are not required for removal of cyclin B from the nucleus nor for mitotic progression. We presume that in An-cut8Δ cells the cyclin B, released by ubiquitination, diffuses out of the nucleoplasm and is destroyed by proteasomes in the cytoplasm.

Our data are at odds with the observations of the S. pombe cut8-563 mutant in which the duration of mitotic spindle dynamics is elongated, spindle morphology is abnormal, and degradation of Cdc13 (Cyclin B) and Cut2 (Securin) is inhibited (Tatebe and Yanagida, 2000). This discrepancy is very likely due to the difference in the nuclear transport state between closed mitosis and semi-open mitosis. In S. pombe, as the nuclear diffusion barrier remains intact and nuclear transport is still active during mitosis (Arai et al., 2010; Asakawa et al., 2010), diffusion of ubiquitinated cyclin B out of the nucleus could be inhibited, thus, making nuclear proteasomes necessary for mitotic progression.

A putative ortholog of Cut8 protein, CG5199, was also identified in Drosophila and its requirement for the enrichment of the proteasome at the nuclear periphery at interphase has been established in Drosophila S2 cell lines (Takeda and Yanagida, 2005). Whether the localization of CG5199 changes through the cell cycle and whether the knock-down of CG5199 affects mitosis remains unexplored. Whether these differences between A. nidulans and S. pombe reflect adaptions specific to each mitotic system is of interest and importance to our understanding of higher eukaryotic models with open mitosis.

The level of proteasome accumulation is regulated by the expression level of An-Cut8, but, how An-Cut8 promotes nuclear localization of the proteasome remains
unclear. One possibility is that, like its homolog in *S. pombe*, An-Cut8 serves as a substrate and, thus, an anchor for the nuclear proteasomes. *S. pombe* Cut8 has a very short half-life of only 5 minutes. Although our FRAP experiments did not measure the half-life of An-Cut8 directly, one would expect the half-life of An-Cut8 to be longer than the half-time of the recovery of nuclear An-Cut8, which is about 8 min. It should be pointed out that the model in *S. pombe* is very odd in that it would seem to be very inefficient to have an anchor protein turn over very rapidly. The model proposed by Takeda and Yanagida also fails to explain why, in the absence of Cut8 protein, proteasomes do not just fail to accumulate at the nuclear periphery, instead, they are absent from the whole nucleus. Finally it is worth noting that, given that An-Cut8 exited nuclei upon mitotic onset, An-Cut8 cannot be responsible for the structured distribution of RPN5 we have seen in mitotic nuclei. Since we observed a lower concentration of proteasomes in the nucleoplasm than in the cytoplasm in *An-cut8Δ*, a simpler and more likely interpretation is that An-Cut8 functions in the nuclear import of proteasomal subunits and/or subcomplexes rather than in anchoring them. This might also be true in *S. pombe* where the nuclear periphery localization of proteasomes could be due to the transport functions of Cut8, as there is a coincidence during anaphase of meiosis between a change of the nuclear proteasome localization (Wilkinson et al., 1998) and an inactivation of nuclear transport (Asakawa et al., 2010; Arai et al., 2010).

We have shown that a greater disassembly of the mitotic nuclear pore complexes does lead to the loss of the proteasome in early mitosis. This raised several possibilities with regard to our postulate of a proteasome-associated nuclear matrix. First, the structural appearance of the RPN5 in mitotic nuclei might just be due to the exclusion of
the condensed chromosomes. However, our observations on the separation of the RPN5
from the chromosomes during aberrant mitosis seem to argue against this. Second, it is
likely that the proteasomes bind to the matrix loosely and have a high dissociate constant,
therefore, when the opening of the nuclear pores is big enough for the proteasomes to
pass through, the proteasomes dissociate from the matrix quickly and diffuse into the
cytoplasm where proteasomes are less concentrated. It is also likely that the structured
appearance of RPN5 is because of an exclusion from a nuclear matrix. Further
experiments are warranted to distinguish these possibilities.
Table 4.1
Proteins identified from the S-tag affinity purification of RPN5

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<tr>
<th>Protein ID</th>
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<td>Lid</td>
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<td>Lid</td>
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<td>ANID_04236</td>
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Figure 4.1

Growth tests of strains carrying fluorescent protein fusions of RPN5. Wild-type control: R153; nkuΔ: TNO2A7; RPN5-GFP: YX138; RPN5-mCherry: YX005. Strains were grown in YAG medium supplemented with uridine, uracil, and riboflavin. The temperature of incubation is at the upper left of each panel.
Figure 4.2

Visualization of RPN5 in *A. nidulans*. Localization of the RPN5 subunit of the 19S RP in an interphase cell. RPN5-GFP was used to reveal the localization of the proteasome (green). Nucleoli were shown by Fibrillarin-mCherry (red). Histone H1-T-sapphire was used to reveal chromatin (blue). Scale bar, 5 µm.
Figure 4. 3
Visualization of proteasomes in *A. nidulans*. Co-localization of RPN5 (red) with other proteasomal subunits (green). (Left panels: the α6 subunit of the 20S CP co-localizes with RPN5. Right panels: the β1 subunit of the 20S CP also co-localizes with RPN5). Scale bar, 5 μm.
Figure 4.4

Localization of RPN5 during mitosis. RPN5 (green) remains in nuclei from early mitosis to late anaphase, disperses into the cytoplasm at telophase, and reappears in the daughter nuclei in early G1. Histone H1 was tagged with mCherry to reveal chromatin. Time-points are shown as min:sec. At t=0:00, RPN5 appears partially condensed. Scale bar, 5 µm.
Figure 4.5

Localization of RPN5 during mitosis. RPN5 occupies a larger volume than the mitotic spindle. GFP-α-tubulin and Histone H1-T-Sapphire were used to show microtubules and chromatin. Scale bar, 1 µm.
Physical separation of RPN5 from chromatin during mitosis in *mipAD159* cells. Images were captured less than 2 hours after *mipAD159* cells were shifted from a permissive temperature of 37 °C to a restrictive temperature of 25°C. This ensured that the mitosis (Continued)
observed was the first mitosis after the temperature shift. Three nuclei in one cell entered mitosis. At \( t = 7 \) min, the chromosomes of the nucleus in the middle segregated successfully, the RPN5 localized between the segregating chromosomes as in wild-type cells, and disappeared at \( t = 8 \) min. The nucleus on the top, however, failed to complete chromosome segregation in the time interval shown and its proteasomes separated from its chromatin, resulting in a chromatin-deficient nuclear structure (arrow). Time-points are indicated as min:sec. Scale bar, 10 µm.
Figure 4.7
Physical separation of RPN5 from chromatin during aberrant mitosis in \textit{mipA} deletants.

(Continued)
Figure 4.7: Continued

The *mipA* gene was deleted from a strain expressing RPN5-GFP and Histone H1-mCherry and the *mipA* deletion was maintained through heterokaryons. At t=0 min, the *mipAΔ* cell contains three nucleus-like structures. The top one contains a lot of RPN5 but no detectable chromatin, the middle one contains a lot of RPN5 but little chromatin, and the bottom one contains a seemingly normal amount of RPN5 (arrow head) and chromatin (arrow). From t=3 min, the chromatin (arrow) of the bottom nucleus moved vigorously through the germling, resulting in separation of RPN5 from the chromatin. Consequently, a proteasome-associated nuclear structure with undetectable chromatin (arrowhead) appeared. Time-points are shown as min:sec. Scale bar, 11 µm.
The chromatin-deficient nuclear structure also appeared in \textit{gcpB} and \textit{gcpC} deletants. \textit{GcpB} and \textit{gcpC} were deleted separately and the deletions were maintained in heterokaryons. The proteasome-associated but chromatin-deficient nuclear structures (arrows) were detected in both deletants. Scale bar, 15 µm.
Figure 4. 9

The proteasome-associated but chromatin-deficient nuclear structures often contain putative nucleoli. The *mipA* gene was deleted from a strain expressing RPN5-GFP, Fibrillarin-mCherry, and Histone H1-T-sapphire. The nucleolar protein Fibrillarin but no chromatin was detected in the proteasome-associated nuclear structures (arrow). Scale bar, 9 µm.
Figure 4. 10

Proteasome-associated nuclear structures appeared after long-term benomyl treatment. A strain expressing RPN5-GFP, Fibrillarin-mCherry, and Histone H1-T-sapphire was grown in minimum medium with 2.4 µg/ml benomyl for 28 h. Several proteasome-associated nuclear structures with putative nucleoli but little or no chromatin were generated. Scale bar, 5 µm.
Figure 4.11
Deconvolved images of a mitotic nucleus. Time-lapse images of mitotic nuclei expressing RPN5-GFP and Histone H1-RFP were taken at 30 second interval and deconvolved subsequently. The condensed chromosomes did not overlap with RPN5 which also appeared structured and fit into spaces between the chromosomes. The position of the nucleolus was visible as an empty space in the RPN5 (arrow head) from early anaphase (30 sec time point) to late anaphase A (one minute time point). Time-points are indicated as min:sec. Scale bar, 2 µm.
Figure 4.12

Mlp1 does not overlap with proteasomes completely. From G1 to late G2 (t=0 min), Mlp1 localizes to the nuclear envelope. When nuclei enter mitosis, Mlp1 translocates to the SPBs and spindles (from t=1:30 to t=2:30). A portion of Mlp1 remains between separating chromosomes (De Souza et al., 2009) but RPN5 does not co-localize with Mlp1 completely, instead, it occupies a larger area. In addition, Mlp1 diminishes in nuclei earlier than RPN5 during mitosis. Time-points are shown as min:sec. Scale bar, 3 µm.
Figure 4. 13

Mlp1 is not required for the nuclear localization of proteasome. RPN5-GFP and histone H1-mCherry were localized in an mlp1 deletion strain. RPN5 localizes normally to nuclei in mitosis and early G1. Time-points are shown as min:sec. Scale bar, 5 μm.
Figure 4. 14

Redistribution of Mlp1 does not affect the nuclear localization of proteasomes. The strain shown (YX214) contains *alcA-dbΔ-cyclin B* integrated at the *argB* locus. Induced expression of dbΔ-Cyclin B arrests cells at telophase, which results in retention of Mlp1 in mitotic nuclei. Mlp1 redistributed from the area between separating chromatin masses to the area surrounding SPBs (De Souza et al., 2009). However, RPN5 did not relocate with Mlp1. Time-points are shown as min:sec. Scale bar, 5 µm.
Figure 4.15

Growth of strains expressing An-Cut8-GFP and An-cut8 deletants at different temperatures on fully supplemented YAG medium. The An-Cut8-GFP transformants (YX094, YX095, and YX096) grow similar to the wild type controls at all temperatures. The An-Cut8 deletants (YX097, YX098, and YX099) are restricted for growth at all temperatures but are particularly restricted at low temperatures.
Localization of An-Cut8 and RPN5 from late G2 to early G1. Two nuclei are shown at T=0. The nucleus at the lower left went through the entire mitotic process in the time interval shown and I will describe the behavior of An-Cut8 and RPN5 in this nucleus. In late G2 (T=0) An-Cut8 and RPN5 both localize to the nucleus. Upon mitotic onset (t=1 min), An-Cut8 quickly left the nucleus, leaving only a faint remnant, while RPN5 remained in the nucleus until telophase (t=7 min). In early G1 (t=12 min), An-Cut8 reappeared in daughter nuclei, followed by RPN5 (t=14 min). Time-points are shown as minutes. Scale bar, 10 µm.

<table>
<thead>
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<th>Merge</th>
<th>An-Cut8</th>
<th>RPN5</th>
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<td>14</td>
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</tbody>
</table>
Figure 4. 17

Effects of the *An-cut8* deletion on the localization of proteasome at interphase. In *An-cut8*+ cells, the proteasomes are highly enriched in the nucleoplasm. NLS-DsRed was used to reveal the nucleoplasm. In *An-cut8Δ* cells, both RPN5 and β1 subunits fail to accumulate in the nuclei, instead, they concentrate in the cytoplasm. Scale bar, 5 µm.
Figure 4.18

Effects of the *An-cut8* deletion on the localization of proteasome during mitosis. When the *An-cut8Δ* cell entered mitosis (t=2 min), NLS-DsRed dispersed into the cytoplasm because of the partial disassembly of the nuclear pore complexes. However, RPN5 remained outside of the mitotic nuclei until telophase (t=7 min). Time-points are shown as minutes. Scale bar, 10 µm.
Figure 4. 19

Effects of the An-cut8 deletion on the nuclear retention of RPN5 during mitosis. Timelapse images were collected at 30-second intervals at 24°C. The mitotic onset time was set as when NLS-DsRed completely disappeared from the nucleoplasm. The retention time of RPN5 was determined from the mitotic onset time to when RPN5 completely disappeared from the nucleoplasm. The absence of Nup37 and Elys shortened the RPN5 retention time from 6.9 ± 1.0 min to 1.7 ± 0.5 min. Benomyl at 2.4 μg/ml was used to completely depolymerize microtubules, thus arresting cells at pseudo-metaphase. Although such a mitotic block did extend the RPN5 retention time to 41.4 ± 12.4 min in the wild-type control, it did not prevent the precocious dispersal of RPN5 in the nup37Δ, elysΔ double mutant (1.3 ± 0.5 min).
Figure 4. 20

Effect on mitotic duration of an *An-cut8* deletion. Time-lapse images were collected at 30-second intervals. The mitotic duration time was counted from the time when NLS-DsRed completely dispersed into cytoplasm to the time when NLS-DsRed appeared in the daughter nuclei. The mitotic duration time of *An-cut8Δ* cells (7.5 ± 1.8 min) was slightly, but not significantly, longer than that of the wild-type control (6.7 ± 1.6 min).
Figure 4. 21

(Continued)
Figure 4.21: Continued

Degradation of cyclin B was not delayed in the *An-cut8* deletant. Images were collected at 30-second intervals at 24°C. At early mitosis (t=0:00), cyclin B localized to the SPBs and spindles in both *An-cut8*+ and *An-cut8Δ* cells. It disappeared from the SPBs and spindles as mitosis progressed (t=2:00). A small portion of the cyclin B remained between segregating chromosomes at early anaphase in the control (t=3:30 in the left panels), while this localization was barely detectable in the *An-cut8Δ* cell (t=4:00). Time-points are shown as min:sec. scale bar, 5 µm.
Ectopic expression of the destruction box-deleted cyclin B led to the retention of the cyclin B at the SPBs in $An\text{-}cut8^\Delta$ cells. The $An\text{-}cut8$ deletant strain YX247 that expresses Histone H1-mcherry and has destruction box-deleted Cyclin B-GFP (dbΔ-Cyclin B-GFP) (Continued)
under the control of the *alcA* promoter inserted into the *wA* locus was grown in repressing medium 24 hours and then switched to the inducing medium about 90 min before the time-lapse imaging began. Ectopic expression of dbΔ-Cyclin B-GFP arrested the cell at telophase and prevented the Cyclin B from dissociating from the SPBs (arrowhead). Time-points are shown as minutes. scale bar, 5 µm.
Overexpression of An-Cut8 does not cause growth defects. On inducing minimal medium with 1% ethanol, strains expressing An-Cut8-GFP under the control of *alcA* promoter (YX170 and YX172) grow indistinguishably from the strain expressing An-Cut8-GFP under its native promoter (YX097) and from the parental control (TNO2A7) at all temperatures from 20°C to 42°C.
Overexpression of An-Cut8 increase the amount of the proteasome in the nuclei. A strains expressing An-Cut8-GFP and RPN5-mCherry under their native promoters and a strain expressing RPN5-mCherry under its native promoter and An-Cut8-GFP under the alcA promoter were grown in liquid inducing medium (minimal medium with 1% ethanol) for 26 hours. Images were taken with the same laser strength and exposure time. Scale bar, 8 µm.
Overexpression of An-Cut8 increased the nuclear accumulation level of the proteasome. Strains were grown as described in liquid inducing medium (minimal medium with 1% ethanol) for 26 hours. The ratio of RPN5-mCherry signal intensity in the nuclei to the cytoplasm was used to reveal the effects of An-Cut8 overexpression on nuclear localization of RPN5. The ratios were obtained through dividing the average signal intensity of a randomly selected area in the nucleus by the average signal intensity of three randomly selected areas of the same size in the cytoplasm. The ratio in cells with normal level of An-Cut8-GFP expression is $4.7 \pm 1.6$ (n=18), significantly lower than the ratio in cells with overexpressed An-Cut8-GFP $13.3 \pm 5.7$ (n=23) ($p < 0.0001$, t test).

**Figure 4. 25**

Overexpression of An-Cut8 increased the nuclear accumulation level of the proteasome.
Overexpression of An-Cut8 promoted the accumulation of the proteasome in the nuclei. The strain expressing RPN5-mCherry under its native promoter and An-Cut8-GFP under the *alcA* promoter was grown in repressing medium for 24 hours and then shifted to inducing medium at t=0. Images were collected at 5 min intervals with the same laser strength. The expression level of An-Cut8-GFP increased through time, accompanied with the nuclear accumulation of RPN5-mCherry. Time-points are shown as minutes. Scale bar, 5 µm.
Figure 4.7

Fluorescence recovery of nuclear An-Cut8 after photobleach. To measure nuclear import activity, an entire nucleus was photobleached by laser, and fluorescence recovery in that nucleus was recorded at 1-minute intervals. The data were processed as described in Chapter 2 material and methods and the bleaching moment was defined as time zero. The fit curve was obtained from the average of 17 experiments and from individual experiments separately to obtain standard deviations. The $t_{1/2}$ was calculated by fitting the data to the exponential rise to maximum model [$f = \max-a\exp(-0.6931471806*x/t)$].

$t_{1/2} = 8.07 \pm 3.43$

$n=17$
Chapter 5: Overall Conclusions and Discussions

I have focused on two major projects in my dissertation work. In the first project, I identified the genes of GCP homologs in *A. nidulans* and named them *gcpB-F*. Individual GCP homologs have been studied in several model organisms, but the contradictory data prevented a coherent view on the roles of GCPs. Our data presents the first example of the existence of GCP2-6 homologs in filamentous fungi and we have carried out the most complete functional analysis of γ-tubulin complex proteins in a single organism.

To study the functions of these GCPs, I deleted each of them and observed the effects of the deletions using both live cell imaging and biochemical methods. I found that GCPB and GCPC are essential, and each of them, as well as γ-tubulin, is required for the SPB localization of all other γ-tubulin complex components and for formation of functional mitotic spindles. GCPD-F are not essential, but they are important for the assembly of large γ-tubulin complexes and they play a minor role in ensuring the fidelity of chromosomal segregation. Our data in *A. nidulans* are consistent with observations on GCP homologs in *D. melanogaster* made by Verollet et al. (2006), suggesting that the functions of GCPs are conserved and that lessons learned from *A. nidulans* may apply to GCPs and the γ-tubulin large complexes of other organisms.

We found that the binding of GCPD-F to the SPB is hierarchical with GCPE depending on GCPD and GCPF, GCPD depending on GCPF but not GCPE, and GCPF
only depending on γ-TuSC components but not GCPD and GCPE. Recent cyro-EM analysis of the *S. cerevisiae* γ-TuSC/Tub4 complex has revealed that γ-TuSCs can efficiently assemble into oligomer rings in the presence of the SPB protein Spc110 under physiological conditions (Kollman et al., 2010). These structural data are consistent with our and Verollet’s observations that γ-TuSCs can localize to the SPBs or centrosomes independent of GCP4-6 homologs and they are competent for most microtubule assembly and organization activities.

Kollman’s structural data and Gunawardane’s biochemical data both suggested that the grip 2 motifs of all GCPs may be involved in direct interactions with γ-tubulin (Gunawardane et al., 2000; Kollman et al., 2010). The structure of the human GCP4 homolog has been solved recently and it exhibits a certain similarity to the structures of Spc97 and Spc98 (Guillet et al., 2010). In addition, biochemical experiments reveal that GCP4 binds to γ-tubulin through the grip 2 motif which is conserved among GCP2-6 (Guillet et al., 2010). These data strongly indicate that GCP2-6 all bind to γ-tubulin. Based on our data and these recent findings published subsequently to our J. Cell Science paper (Xiong and Oakley, 2009), we propose a new model for the arrangement of GCPs in the large γ-tubulin complex (Figure 5.1) that differs from the textbook model and the model we previously suggested (Xiong and Oakley, 2009). In this model, we postulate the existence of a trimeric γ-TuSC formed of γ-tubulin and GCP4-6 homologs with each GCP interacting with one molecule of γ-tubulin and GCP4 lying between GCP5 and GCP6. We hypothesize that GCP6 in this trimeric γ-TuSC binds to the conventional γ-TuSC. Bearing in mind that γ-TuSCs have an intrinsic tendency to oligomerize and that γ-TuSCs must exist in equilibrium with γ-TuSC oligomers, when the trimeric γ-TuSC
binds to a γ-TuSC oligomer, it stops addition of γ-TuSCs to the side of the oligomer to which it binds. γ-TuSCs can still be added to the other end of the oligomer but the intrinsic curvature of the γ-TuSC will, as more γ-TuSCs are added bring the other end of the oligomer to the vicinity of the trimeric γ-TuSC where it binds weakly to GCP-5. This binding prevents further growth and strongly inhibits dissociation of γ-TuSCs from the complex and thereby stabilizes the γ-TuRC. In the absence of GCP4, 5 or 6, the ring closure would not occur and the γ-TuRC would not be stable. This would account for the sparsity of full-size γ-tubulin complexes in the absence of GCP4, 5 or 6. Most microtubules are composed of 13 protofilaments in vivo (Tilney et al., 1975), but microtubules assembled in vitro have a variety of protofilament numbers (Pierson et al., 1978; Chrétien and Wade, 1991). The trimeric γ-TuSC plus five conventional γ-TuSCs would result in a large γ-tubulin complex containing 13 γ-tubulin molecules that would nucleate the 13 protofilaments of microtubules, thus establishing correct protofilament number in vivo.

Consistent with this model, we found that the signals of the GFP fusions of GCP4-6 at the SPBs are much weaker than that of GCP2 and 3 and less GCP4-6 were co-immunoprecipitated with γ-tubulin than GCP2 and 3. The large γ-tubulin complex binds to the SPB through the interaction between the conventional γ-TuSC and SPB proteins and the trimeric γ-TuSC relies on binding to the conventional γ-TuSCs for SPB localization. It follows that the positioning of GCP-4-6 in the trimeric γ-TuSC establishes the hierarchy of binding that we have observed. The presence of the alternative γ-TuSC and its ability to bind to the conventional γ-TuSC may increase size heterogeneity of γ-TuSCs and account for the fact that the γ-TuSC peak on sucrose
density gradient is shifted to the lower mass after GCP4-6 was deleted.

Without the trimeric γ-TuSCs, the conventional γ-TuSCs assemble poorly in the cytoplasm, but, with the help of SPB proteins, they may oligomerize to form large complexes at the SPB and nucleate microtubules. The resulting microtubules may have abnormal dynamics because they lack a seam or have abnormal numbers of protofilaments or the assembly of the large complexes at the SPBs may not be efficient enough to produce a fully functional spindle with normal kinetics. In the presence of a functional spindle assembly checkpoint mechanism mitosis is delayed long enough to allow problems to be corrected in the vast majority of cases. We may be able to test this model using biochemical methods. It is known that the dissociation of γ-TuSCs from the γ-TuRC is salt-sensitive. We may separate the γ-TuSCs resulting from a mild salt treatment through various ways such as native gel, gel filtration, or sucrose gradient centrifugation. The components of the separated small complexes can be identified by either Western Blot or mass spectrometry. We expected to see that GCP4-6 show very similar distributions that are different from GCP2 and GCP3. We would also expect to see that co-immunoprecipitations of GCP5 or GCP4 with GCP2 or GCP3 are always accompanied with precipitation of GCP6 after the salt treatment. Cross-linking agents, in combination with mass spectrometry, might also be used in experiments for identifying the direct physical interactions. Furthermore, structural data on large complexes that consist of GCPs with distinguishable tags may help in confirming, rejecting or modifying our model.

In my second project, our initial goal was to determine if the localization of proteasomes was affected by mip4D159 mutation. As little was known about proteasome
localization in filamentous fungi, we first characterized the localization of the 26S proteasome through the cell cycle in wild-type *A. nidulans*. I discovered a retention-and-dispersal localization pattern of the proteasome during mitosis that, to our knowledge, has not been reported in any other organism. In our subsequent analyses of the *mipAD159* allele, we observed that chromosomes can be pulled clearly out of a nuclear structure that is filled with proteasomes, resulting in nuclei that are deficient in chromatin but with abundant proteasomes. Proteasomes also separated from chromosomes in some cases within individual nuclei. I found similar patterns of separations of chromatin from the proteasomes in *mipAΔ, gcpBΔ*, and *gcpCΔ* strains all of which are deficient in mitotic spindle formation but do exhibit nuclear movement. These data suggest that the separation of chromatin and proteasomes could be a general result of aberrant mitosis. I also found similar chromatin deficient nuclei in strains treated with benomyl for a long time. Since nuclear movement is deficient in these strains it is not certain that the chromatin deficient nuclei form by an identical mechanism in benomyl.

We were interested in characterizing the chromatin-deficient nuclei for several reasons. First, our imaging suggested that the proteasomes within the chromatin-deficient nuclei have structure during and after separation instead of simply filling the nucleoplasm. Although these chromatin-deficient nuclei contained nucleoli, thus, resembling the remnant nucleolar structures observed by Ukil et al. (2009), they accumulated as the cell went through more aberrant mitosis rather than disassemble as the remnant nucleolar structure. These chromatin-deficient nuclear structures are larger than the nucleolus and what else resides in these structures besides proteasomes and nucleoli remain unidentified. The apparent structure of the proteasomes suggested that
they might bind to or form some sort of nuclear matrix.

As the biochemical properties of the chromatin-deficient nuclear structures remain unclear, we took a candidate approach in a hope of understanding how the nuclear proteasome localization is regulated. We found that the nuclear localization of proteasomes does not rely on Mlp1 but it requires the *A. nidulans* homolog of Cut8 protein, An-Cut8. Our examination of *An*-cut8 deleterants led to several interesting observations with important implications.

In *An*-cut8Δ cells, the concentration of proteasomes remains higher in the cytoplasm than in the nucleoplasm through most of mitosis. This suggests that the size of the semi-open mitotic nuclear pores is not big enough for large complexes like proteasomes to move across the nuclear envelope freely. We have confirmed this by using a *nup37Δ, elysΔ* double mutant, in which the mitotic nuclear pore complexes were further disassembled. This result reveals that proteasomes are not bound tightly to a stable nuclear matrix. It is possible that the binding is loose (i.e., the dissociation constant is high and the residence time of the proteasome on the matrix is relatively short) allowing proteasomes to diffuse out relatively quickly. Another possibility is that the apparently structured distribution of the proteasome is simply due to exclusion from the chromatin and that the proteasome does not bind to a matrix. This does not appear to be the case because when proteasomes separate from chromatin during aberrant mitosis, it still appears to have structure (Figure 4.6, 4.7 and 5.2). It may be possible to test this by careful imaging using shorter Z-axis steps. A third possibility is that the structured appearance of the proteasomes is not due to the binding of proteasome to a matrix structure, but, rather a partial exclusion from some portions of the nucleoplasm by a
nuclear matrix structure. Recently developed correlative light and electron microscope analysis after live observation (live CLEM) (Haraguchi et al., 2008) may help us to characterize the locations of intranuclear proteasomes in more detail.

Our data revealed that mitosis progressed in a timely fashion in An-cut8 deletants. This was a surprise, given the finding in S. pombe that deletion of cut8 causes a severe mitotic delay (Takeda and Yanagida, 2005; Tabb et al., 2000) and that in the absence of nuclear proteasomes one would expect that the destruction of key mitotic regulatory proteins such as Cyclin B would be inhibited or blocked completely. Cyclin B is normally destroyed by the proteasome after ubiquitination by the APC/C (Glotzer et al., 1991; Hershko et al., 1991). Cyclin B and Cdk1 form a kinase originally called maturation promoting factor (MPF) that drives much of the cell cycle and the general assumption has been that destruction of Cyclin B releases Cdk1 and inactivates MPF. Nayak et al. (2010) found, however, that in mitosis in A. nidulans Cdk1 is released from Cyclin B before Cyclin B is destroyed. Since the separation of Cyclin B and Cdk1 would necessarily inactivate MPF, it follows that the actual inactivation of MPF in mitosis in A. nidulans is due to whatever causes the separation of Cyclin B and Cdk1. The sparse available biochemical data from other systems indicate that the release of Cdk1 from Cyclin B occurs by a mechanism that involves the proteasome but does not require proteolysis (Nishiyama et al., 2000; Chesnel et al., 2006). We further demonstrated that Cyclin B destruction was not inhibited when proteasomes are absent from the nuclei. A possible explanation is that ubiquitination of Cyclin B by the APC/C drives dissociation of Cyclin B from the spindle pole body, the spindle, the Mlp1 matrix and from Cdk1. The APC/C would thus drive anaphase and mitotic exit even in the absence of intranuclear
proteasomes. In An-cut8Δ strains, dissociation of Cyclin B from intranuclear structures would allow it to diffuse into the cytoplasm where it could be destroyed by cytoplasmic proteasomes. If this model has validity, a similar process must occur with Securin, although we have no direct data on it. In S. pombe with closed mitosis, proteins ubiquitinated by the APC/C could not diffuse out of the nucleus and would not be destroyed in a timely fashion. A future experiment to test this will be to observe Cyclin B localization at mitosis when the proteolysis activity of proteasomes is conditionally inhibited. We expect to see that the nuclei are arrested at telophase, but Cyclin B is absent at the SPBs and spindles. We also suspect that ubiquitination of Cyclin B is enough to trigger the dissociation of Cyclin B-Cdk 1 complex and, thus, initiate the mitotic exit in A. nidulans. Since we have consistently observed that Cdk1 exits from mitotic nuclei before Cyclin B in normal mitosis {Nayak et al., 2010, J Cell Biol, 190, 317-30}, we may see that Cyclin B leaves the mitotic nuclei at the same time as Cdk1 in An-cut8Δ. Furthermore, it may be possible to use the FRET technique to monitor the dissociation of Cyclin B-Cdk1 complexes and test whether the dissociation occurs at the SPBs or in the cytoplasm in An-cut8Δ cells.

Our FRAP experiments show that An-Cut8 is transported into the nucleus with a half time of around 8 minutes. Whether the fluorescence recovery is due to replenishment of An-Cut8 proteolyzed in the nucleoplasm or to an exchange of An-Cut8 between the cytoplasm and the nucleoplasm remains undetermined. We are currently inclined to think that An-Cut8 is unlikely to have a half life as short as 8 minutes, because such a short half life might make An-Cut8-GFP difficult to see because GFP would likely need more than 8 minutes to mature such that it is fluorescent. However, a photoswitchable
fluorescent protein fusion of An-Cut8 may be useful in distinguishing between the two possibilities. Our data also reveal that the accumulation of proteasomes correlates with the amount of An-Cut8. As proteasomes concentrate in the cytoplasm rather than in the nucleoplasm of An-cut8Δ, the simplest and most direct interpretation is that An-Cut8 specifically functions in the nuclear transport of proteasomal subunits in A. nidulans. Since Cut8 homologs also exist in other eukaryotes (Takeda and Yanagida, 2005) with more open mitosis, our results might apply to these model organisms as well.
Model for the arrangement of GCPs in the large γ-tubulin complex. The conventional γ-TuSC is composed of two γ-tubulins and one molecule of GCP2 and GCP3. GCP4-6 each binds to one molecule of γ-tubulin individually and form a trimeric γ-TuSC that can bind to the conventional γ-TuSC through GCP6. As the conventional γ-TuSCs also have intrinsic tendency to oligomerize, binding of the trimeric γ-TuSC to the conventional γ-TuSCs increased the heterogeneity of the cytoplasmic pool of γ-TuSCs. A large γ-tubulin complex may be formed of five conventional γ-TuSCs and one alternative γ-TuSC and the large complex bind to the SPB through the interaction between the conventional γ-TuSC and some SPB protein. The trimeric γ-TuSC may locate at one end of the five-
Figure 5.1: Continued

oligomer γ-TuSC ring structure and ensure that the large complex is a single helix with 13 γ-tubulins.
Proteasomes appear structured during aberrant mitosis in *mipAD159* mutants.
Figure 5.2: Continued

RPN5-GFP was used to reveal the proteasome and Histone H1-mCherry to reveal the chromosomes. Proteasomes do not appear as free diffusible to fill up the nucleoplasm or simply excluded from chromosomes and nucleoli, instead, they seem to bind to a separate nuclear structure. Scale bar, 2.8 µm.
References


Asakawa, H., Kojidani, T., Mori, C., Osakada, H., Sato, M., Ding, D. Q., Hiraoka, Y., 164


Coffey, D. S. (2002). Nuclear matrix proteins as proteomic markers of preneoplastic and cancer lesions: commentary re: G. Brunagel et al., nuclear matrix protein alterations


Aspergillus nidulans. Mol Biol Cell 20, 2146-2159.


Nature 356, 80-83.


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system for *Aspergillus nidulans*. Genetics 172, 1557-1566.


Oakley, B. R., Rinehart, J. E., Mitchell, B. L., Oakley, C. E., Carmona, C., Gray, G. L.,


Mol Biol Cell 15, 1374-1386.


Rischitor, P. E., Konzack, S., and Fischer, R. (2004). The Kip3-like kinesin KipB moves along microtubules and determines spindle position during synchronized mitoses in


Roper, J. A. (1952) Production of heterozygous diploids in filamentous fungi. Experientia 15, 14-15


for cell growth and proper microtubule organization in *Saccharomyces cerevisiae*. J Cell Biol 131, 1775-1788.


tubulin is required for the structure and function of the microtubule organizing centre in *Drosophila* neuroblasts. EMBO J 14, 28-36.


Vardy, L., and Toda, T. (2000). The fission yeast gamma-tubulin complex is required in G(1) phase and is a component of the spindle assembly checkpoint. EMBO J 19, 6098-6111.


