Functions of Gamma-tubulin in the Spindle Assembly Checkpoint and APC/C Regulation in *Aspergillus nidulans*

**DISSERTATION**

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By

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Abstract

Studies from our lab demonstrated that a cold-sensitive γ-tubulin mutant allele, *mipAD159*, causes defects in the coordination of late mitotic events at restrictive temperatures with observed phenotypes such as abnormal chromosome segregation and inhibition of anaphase A (Prigozhina et al., 2004). These abnormalities are not a result of defects in microtubule nucleation since γ-tubulin localized normally to the spindle pole body (SPB), microtubules were abundant and mitotic spindle formation and elongation appeared to be normal (Prigozhina et al., 2004). Nayak et al. (2010) further examined γ-tubulin’s role in mitotic regulation and determined that, at restrictive temperatures, *mipAD159* caused a failure of accumulation of cyclin B, cyclin dependent kinase 1 (Cdk1) and the phosphatase, Ancdc14, in a subset of nuclei. These nuclei were removed from the cell cycle while other nuclei in the same cell accumulated these proteins and cycled normally. Extensive analysis revealed that this failure of accumulation was due to a nuclear autonomous failure of inactivation of the anaphase promoting complex/cyclosome (APC/C) sometime between late mitosis and S phase.

The two projects I have focused on are directed toward further elucidating γ-tubulin’s role in cell cycle regulation. Many mitotic regulatory proteins are known to localize to the SPB, or the centrosome, its functional equivalent in higher organisms, in
mitosis. Therefore, γ-tubulin might be interacting with such proteins and such interactions might be altered in strains carrying mipAD159. I decided to focus on the spindle assembly checkpoint (SAC) proteins Mad2, Mps1, Bub3, BubR1 and Cdc20. I identified the *A. nidulans* homolog of each, created fluorescent protein fusions, and observed them *in vivo* by spinning disk confocal microscopy. I found that these proteins are physically separate from each other in interphase, keeping the SAC inactive until mitosis, when they are all at the SPB/kinetochore region. Additionally, I found that Mps1 and BubR1 fail to accumulate in a subset of *mipAD159* nuclei, and such nuclei are unable to establish a proper SAC.

In other organisms, APC/C binds to two activators. APC/C<sup>Cdh1</sup> is known to be active in G<sub>1</sub>, preventing premature accumulation of S-phase cyclins, and it has to be inactivated for the cell to proceed into S phase. APC/C<sup>Cdc20</sup> is active in mitosis and is required for anaphase and mitotic exit. One unanswered question is which form of the APC/C is constitutively active in *mipAD159* nuclei. Since cyclin B is both an S-phase and mitotic cyclin in *A. nidulans*, either failure of inactivation of APC/C<sup>Cdh1</sup> at G<sub>1</sub>/S or failure of inactivation of APC/C<sup>Cdc20</sup> at the end of mitosis would lead to continuous destruction of cyclin B. I identified the *A. nidulans* homolog of Cdh1 (designated CdhA) created a GFP fusion, deleted the gene, and analyzed both the fusion and the deletion by time-lapse microscopy. Here, I report my data showing that γ-tubulin has a critical role in inactivating APC/C<sup>Cdh1</sup> at the G<sub>1</sub>/S boundary (Edgerton-Morgan and Oakley, 2012).
Dedication

This document is dedicated to my family.
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Chapter 1: Introduction

1.1. Microtubules and Polar Microtubule Organizing Centers (PMTOCs)

Microtubules are hollow tubes, 25 nm in diameter, formed by the lateral and longitudinal association of α-tubulin-β-tubulin (α/β) heterodimers [reviewed in (Job et al., 2003; Wade, 2009)]. The α/β heterodimers assemble in a head-to-tail fashion (Nogales et al., 1998) in a GTP-dependent manner, forming what is termed a protofilament (Fig. 1.1A). Thirteen protofilaments form a microtubule. Each protofilament has the same polarity (α-tubulin on the minus end, the slow growing end, and β-tubulin on the plus end, the more dynamic end) and the lateral interactions between protofilaments are generally α-α and β-β (Nogales et al., 1999). This polarity of microtubules provides directionality for microtubule motors, which are responsible for carrying intracellular cargo [reviewed in (Wade, 2009)]. For example, most kinesins are plus end-directed motors, generally transporting cargo from the center of the cell to the periphery (Vale et al., 1985), whereas dyneins are minus end-directed motors (Paschal and Vallee, 1987).

Microtubules are essential in all eukaryotes and are important for maintenance of cell morphology, cell motility, vesicle transport, organelle positioning, exocytosis, and endocytosis. Significantly, they are the main components of mitotic and meiotic spindle apparatuses and are required for chromosomal segregation. Having such a range of functions requires that microtubule arrays must be very dynamic, capable of rapid
reorganization. This is indeed the case as microtubules themselves are highly dynamic, switching between growth (microtubule assembly) and shrinkage (microtubule disassembly) [reviewed in (Wade, 2009); Fig. 1.1B].

In many types of cells, microtubules are nucleated by polar microtubule organizing centers (PMTOCs). PMTOCs, which include centrosomes in animal cells and spindle pole bodies in fungal cells, is a collective term for a variety of functionally similar, but structurally different organelles that serve the same purpose, to nucleate and organize microtubules [reviewed in (Cuschieri et al., 2007)]. PMTOCs nucleate microtubule assembly such that the minus ends are at the PMTOC and the plus ends are distal. All PMTOCs rely on γ-tubulin for microtubule nucleation. Loss of γ-tubulin results in aberrant mitotic spindles and microtubule curvature in a variety of eukaryotes (Horio et al., 1991; Marschall et al., 1996; Oakley et al., 1990; Sobel and Snyder, 1995; Stearns et al., 1991). Accumulating evidence suggests PMTOCs have additional roles other than microtubule nucleation. Many proteins localize to these organelles that are involved in cell cycle regulation, checkpoints, spindle positioning, mitotic exit, and cytokinesis [reviewed in (Cuschieri et al., 2007)].

PMTOCs, like chromosomes, duplicate once per cell cycle and form the poles of the mitotic spindle. Misregulation of PMTOC duplication can lead to multipolar or monopolar mitotic spindles that can lead to improper segregation of chromosomes, which is typical in cancer and tumor cells (Brinkley and Goepfert, 1998; Kasbek et al., 2007; Pihan et al., 2001).
1.1.1. **Centrosomes**

Centrosomes were discovered in the late 1800’s by Theodor Boveri (Wheatley, 1982), who observed a small region of phase-dense material surrounded by a larger region of less phase density (Wilson, 1911). This material focused the ends of thin cytoplasmic fibers in interphase cells and bipolar spindles in mitotic cells. The densely stained structures Boveri observed are now known as centrioles. Each centrosome has two centrioles lying at right angles to one another. These are surrounded by the pericentriolar material (PCM), and the thin filaments that Boveri observed are microtubules (Fig. 1.2, A and C). The PCM is the site of microtubule nucleation. It consists of a meshwork of fibers and proteins, a large percentage of which are coiled-coiled proteins, which act as a scaffold for microtubule nucleating proteins (Dictenberg et al., 1998; Moritz et al., 1995). In most cells, the centrosomes acts as the main site of microtubule nucleation such that microtubules are polarized with the minus ends anchored at the centrosome and the plus ends distal.

Centrosomes are known to have important roles in the cell cycle. Many regulators of cellular processes localize to the centrosomes, including those involved in cell cycle progression, checkpoint control, spindle function, and ubiquitin-mediated destruction. In cells that have centrosomes, the centrosomes have a dominant role in spindle formation. If there are more than two centrosomes in a cell, which is often the case in cancer cells, this often leads to multipolar spindles in mitosis. Multipolar spindles can lead to chromosome mis-segregation and aneuploidy (Doxsey, 2001; Holland and Cleveland, 2009). In centrosome-ablated mammalian cells, no astral microtubules are
produced, mitotic spindles are formed but are mis-positioned (Heald et al., 1997; Khodjakov et al., 2000; Khodjakov and Rieder, 2001) and many cells fail cytokinesis (Khodjakov and Rieder, 2001). Interestingly, in African green monkey kidney cells (CVG-2 and BSC-1 cells) and rat kangaroo kidney epithelial cells (PtKG-23) with removed centrosomes, G1-S progression is blocked (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001).

1.1.2. Spindle Pole Bodies (SPBs)

The fungal equivalent of the centrosome is the spindle pole body (SPB). Even though it is quite different structurally from the centrosome (Fig. 1.2), its function as a microtubule organizing center is the same (Francis and Davis, 2000; Schiebel, 2000). As with the centrosome, nucleation of microtubules by the SPB establishes their polarity with minus ends at the SPB and plus ends extending into the cytoplasm or nucleoplasm. In *Saccharomyces cerevisiae*, the SPB is embedded in the nuclear envelope throughout the cell cycle and is the sole site of microtubule organization as it nucleates both nuclear and cytoplasmic microtubules. It is cylindrical and consists of three disks or plaques [(Byers and Goetsch, 1974; Byers and Goetsch, 1975; Jaspersen and Winey, 2004; Moens and Rapport, 1971; Robinow and Marak, 1966); Fig. 1.3]. The outer plaque faces the cytoplasm and associates with cytoplasmic microtubules through the binding of γ-tubulin complexes to Spc72, whereas the inner plaque faces the nucleoplasm and associates with nuclear microtubules through the binding of γ-tubulin complexes to Spc110. A third plaque, the central plaque, spans the nuclear membrane with one side associated with a region of the nuclear envelope termed the half-bridge. Additional cryo-
EM and electron tomography studies revealed there are two additional layers between the outer and central plaque, termed intermediate layers 1 and 2 (IL1 and IL2) (Bullitt et al., 1997; O'Toole et al., 1999).

The structure of the SPB in _Aspergillus nidulans_, our model organism, has been studied in less detail; however, it is known that the SPB is embedded in the nuclear envelope with its inner surface contacting the nucleoplasm and its outer face contacting the cytoplasm (Jung et al., 1998; Oakley and Morris, 1983; Fig. 1.4). In _A. nidulans_, the SPB nucleates the assembly of most microtubules [reviewed in (Oakley, 2004)], but there are small amounts of γ-tubulin complex proteins at septa (Xiong and Oakley, 2009) and a small number of microtubules appear to be nucleated at the septum (Konzack et al., 2005). In interphase, there is an abundance of cytoplasmic microtubules extending from the SPB (Jung et al., 1998), but microtubules are absent from the nucleus and tubulin is excluded from nuclei (Ovechkina et al., 2003). As hyphae approach mitosis, cytoplasmic microtubules disassemble (Szewczyk and Oakley, 2011), tubulin rapidly enters the nucleus (Ovechkina et al., 2003), and nuclear microtubules assemble from the duplicated SPBs, forming the mitotic spindle. During anaphase, astral microtubules grow from the cytoplasmic side of the SPB as the spindle elongates (Oakley, 2004). The spindle disassembles at telophase, and the astral microtubules grow to form the interphase cytoplasmic microtubule array (Szewczyk and Oakley, 2011).

As with centrosomes, the SPB and its associated microtubules have important roles in the cell cycle with a dominant role in spindle formation leading to chromosomal segregation (Masuda et al., 2006). Proteins involved in spindle positioning (Segal and
Bloom, 2001), mitotic exit (Bardin et al., 2000; Molk et al., 2004), cytokinesis (Krapp et al., 2001; Tomlin et al., 2002), and cell cycle checkpoints (Castillo et al., 2002; De Souza et al., 2009; Howell et al., 2000) also localize to the SPB.

1.2. γ-Tubulin

1.2.1. Identification

γ-tubulin was discovered by C. Elizabeth and Berl Oakley through a screen for extragenic suppressors of a heat-sensitive β-tubulin mutation, benA33, in *A. nidulans*. In this screen, most of the suppressors were found to have mutations in *tubA*, the *A. nidulans* α-tubulin gene (Oakley et al., 1987a). However, three suppressors were found to be closely linked to the *riboB* gene, suggesting all three were within the same gene, designated *mipA* [microtubule interacting protein; (Weil et al., 1986)]. *mipA* was cloned by chromosome walking from the previously cloned *riboB* gene (Oakley and Oakley, 1989; Oakley et al., 1987b). Sequence analysis revealed that *mipA* encoded a novel member of the tubulin superfamily having approximately 30% sequence identity with α- and β-tubulins from a wide range of species (Oakley and Oakley, 1989), which is roughly the same sequence identity shared by α-tubulin and β-tubulin (Little and Seehaus, 1988). Following the discovery of γ-tubulin in *A. nidulans*, it has been found to be conserved in all eukaryotes. Most γ-tubulin homologs share more than 60% amino acid identity with each other (Oakley and Akkari, 1999) and are functionally conserved as well. In fact, human γ-tubulin was nearly fully functional in *Schizosaccharomyces pombe* cells lacking endogenous γ-tubulin in that it restored viability, supported a near normal cell replication rate, localized to SPBs, and nucleated microtubules (Horio and Oakley, 1994).
Arabidopsis γ-tubulin also functioned in such S. pombe cells supporting growth and replication, localizing to SPBs, and nucleating microtubules; however, there was little growth at low temperatures, slower than normal growth at high temperatures and the distribution of microtubules and cell morphology were abnormal (Horio and Oakley, 2003).

1.2.2. Localization

The initial localization of γ-tubulin in A. nidulans (the first in any organism) revealed that it localized to SPBs (Oakley et al., 1990). Further analysis determined that approximately 32% of the γ-tubulin existed in a soluble form in the cytoplasm (Akashi et al., 1997). Following its discovery and initial localization studies, γ-tubulin has been found to localize to PMTOCs in many other organisms (Horio et al., 1991; Joshi et al., 1992; Spang et al., 1996; Stearns et al., 1991; Zheng et al., 1991). In addition, γ-tubulin was also found in the cytoplasm as part of the large γ-tubulin ring complex (γ-TuRC) (Moritz et al., 1998; Oegema et al., 1999; Stearns and Kirschner, 1994; Zheng et al., 1995) and as part of the γ-tubulin small complex (γ-TuSC) (Knop et al., 1997; Knop and Schiebel, 1997; Oegema et al., 1999). In some cell types, γ-tubulin also localized to the mitotic spindle (Lajoie-Mazenc et al., 1994; Zheng et al., 1991) and to midbodies (Julian et al., 1993).

1.2.3. Function as a Microtubule Nucleator

Oakley et al. (1990) were the first to demonstrate that γ-tubulin had a role in microtubule nucleation. Disruption of the gene encoding γ-tubulin, mipA, in A. nidulans was lethal; however, use of the hetrokaryon rescue technique (Osmani et al., 2006b;
Osmani et al., 1988) allowed the authors to visualize the effects of the disruption in germinating spores produced by the heterokaryon. The absence of functional γ-tubulin abolished mitotic spindles, and decreased both the length and number of cytoplasmic microtubules, ultimately leading to a failure of mitosis and inhibition of nuclear migration (Oakley et al., 1990).

One caveat with this initial gene disruption was that the observed phenotypes might have been secondary consequences of the loss of γ-tubulin. When using the heterokaryon rescue technique, conidia from a gene disruption heterokaryon are incubated in media selective for the conidia carrying the disruption. Germlings carrying the disruption are identified by presence of a germ tube (conidia carrying parental nuclei will barely germinate). However, the first cell cycle normally occurs before germination, at a time at which parental and disruptant conidia cannot be distinguished (Martin et al., 1997). To overcome this and examine the effect of the loss of γ-tubulin in the first cell cycle, Oakley and colleagues created γ-tubulin disruption heterokaryons in strains carrying the temperature sensitive mutations nimA5 [nimA encodes the NIMA kinase that is required for the G2 to M transition (Osmani et al., 1987)] or nimT23 [nimT encodes a homolog of cdc25 phosphatase, which is required for the initiation of mitosis (O'Connell et al., 1992)]. Each allele blocks the cell cycle in G2 at 42°C, thus synchronizing the nuclei. The block is rapidly reversible by shifting to a permissive temperature of 32°C or 25°C, allowing the cell cycle to resume (Martin et al., 1997; Oakley and Morris, 1983; Osmani et al., 1991). Using this method, it was confirmed that γ-tubulin was required for mitotic spindle formation. Cytoplasmic microtubules did disassemble in the γ-tubulin
disruptants in mitosis following release from the block and eventually reassembled after nuclei failed mitosis and re-entered interphase; however, such cytoplasmic microtubules had abnormal curvature, and often long single microtubules or short microtubule bundles were observed (Martin et al., 1997). This indicates that γ-tubulin is not required for cytoplasmic microtubule nucleation, but it might be involved in the formation of proper cytoplasmic microtubule arrays. γ-tubulin is also essential for the assembly of mitotic spindle microtubules in *S. pombe* (Horio et al., 1991; Masuda and Shibata, 1996), *S. cerevisiae* (Marschall et al., 1996), *Drosophila melanogaster* (Moritz et al., 1998), *Xenopus laevis* (Félix et al., 1994), human cystic fibrosis pancreatic cancer (CF-PAC) cells (Joshi et al., 1992), and neurons (Ahmad et al., 1994). Thus, its function in microtubule nucleation is evolutionarily conserved.

1.2.4. Function in the γ-Tubulin Ring Complex

γ-tubulin forms complexes with several other proteins. The majority of these complexes are large ring structures (Moritz et al., 1998; Murphy et al., 2001; Oegema et al., 1999; Stearns and Kirschner, 1994; Zheng et al., 1995). These ring complexes, designated γ-tubulin ring complexes (γ-TuRCs), consist of γ-tubulin and at least five γ-tubulin complex proteins (GCPs; see Table 1.1 for orthologs). γ-tubulin is also part of a smaller complex, designated the γ-tubulin small complex (γ-TuSC). It should be noted that only homologs of γ-TuSC components (γ-tubulin, GCP2 and GCP3), and not the γ-TuRC specific components (GCP4-6), have been found in *S. cerevisiae* (Geissler et al., 1996; Knop et al., 1997; Vinh et al., 2002). γ-TuSCs are capable of nucleating microtubules, but are much less efficient than γ-TuRCs (Oegema et al., 1999). The
structure of the \textit{S. cerevisiae} $\gamma$-TuSC has been solved (Kollman et al., 2008), and it is a V-shaped structure with GCP2 and GCP3 as the arms of the “V” and $\gamma$-tubulin residing at each tip (Fig. 1.5). This structure also revealed that the two $\gamma$-tubulins in the $\gamma$-TuSC are held apart from each other in a confirmation that is incompatible with both lateral and longitudinal microtubule lattice-like interactions (Kollman et al., 2008), explaining why the $\gamma$-TuSC is not an efficient nucleator. Each of the $\gamma$-TuSC components is essential for viability and mitotic spindle assembly and function (Geissler et al., 1996; Knop et al., 1997; Oakley et al., 1990; Paluh et al., 2000; Spang et al., 1996; Vardy and Toda, 2000; Xiong and Oakley, 2009), whereas the $\gamma$-TuRC components GCP4-6 are not essential (Anders et al., 2006; Venkatram et al., 2004; Vogt et al., 2006; Xiong and Oakley, 2009).

Previous models suggest that $\gamma$-TuRCs are assembled from $\gamma$-TuSCs in the cytoplasm and are recruited to the centrosome, where they serve as the major microtubule nucleators [(Moritz et al., 1998); reviewed in (Wiese and Zheng, 1999; Wiese and Zheng, 2006)]. Recent evidence, however, shows that $\gamma$-tubulin directly binds to the C-terminal domain of human GCP4, and, by inference, to the other GCPs based on sequence conservation in regions of all GCPs and structural similarities to GCP2 and GCP3 (Guillet et al., 2011). This suggests a new model of $\gamma$-TuRC assembly in which GCP4-6 are directly incorporated into the ring structure rather than forming a scaffolding cap [reviewed in (Kollman et al., 2011)]. In various organisms, including \textit{A. nidulans}, when GCP4, GCP5, or GCP6 are absent, the $\gamma$-TuSC components still localize to PMTOCs (Anders et al., 2006; Venkatram et al., 2004; Vérollet et al., 2006; Xiong and Oakley, 2009).
1.2.5. Function as a Cell Cycle Regulator

Numerous studies indicate that γ-tubulin complexes function in cell cycle regulation [reviewed in (Cuschieri et al., 2007)]. Importantly, γ-tubulin complexes play a role in the spindle assembly checkpoint (SAC). A subset of human γ-tubulin mutants, when expressed in *S. pombe*, led to mitotic progression in the presence of abnormal spindles (Hendrickson et al., 2001). This was surprising because the prediction is that the presence of abnormal spindles would activate the SAC, arresting these cells in metaphase. Instead, these cells proceeded through anaphase, followed by abnormal cytokinesis (Hendrickson et al., 2001). In *D. melanogaster* and human embryonic kidney (HEK293) cells, γ-tubulin interacts physically and perhaps functionally with the SAC proteins Cdc20 and BubR1 (Müller et al., 2006).

It is also well established that PMTOCs, such as centrosomes and SPBs, have a role in regulating the G1/S transition [(Gromley et al., 2003; Hinchcliffe et al., 2001; Matsumoto and Maller, 2004; Mikule et al., 2007); reviewed in (Doxsey et al., 2005a; Doxsey et al., 2005b; Sluder, 2005)], but there is less information on whether γ-tubulin complex proteins, themselves, play a role in this regulation. It has been demonstrated that Alp4, the *S. pombe* homolog of the γ-TuSC component GCP2, is required in G1 (Vardy and Toda, 2000).

1.3. Cell Cycle Regulation

1.3.1. The Anaphase Promoting Complex/Cyclosome (APC/C)

The anaphase promoting complex or cyclosome (APC/C) is a crucial cell cycle regulator conserved in all eukaryotes. It targets certain substrates for destruction by
adding polyubiquitin chains, which are recognized by the proteasome. The oscillation of cell cycle regulators, such as cyclin-dependent kinases (Cdks), is responsible for proper timing of the phases of the cell cycle. Precise timing of destruction of such regulators is essential for proper DNA replication and chromosomal segregation.

There are two known activators of the APC/C, which aid in target specificity [reviewed in (Acquaviva and Pines, 2006; Pines, 2011; Thornton and Toczyski, 2006; van Leuken et al., 2008); Fig. 1.6]. Cdc20 activates the APC/C (APC/C\textsuperscript{Cdc20}) in mitosis and is required for the metaphase to anaphase transition. APC/C\textsuperscript{Cdc20} targets securin for destruction, which, in turn, frees separase to cleave cohesion, allowing anaphase and chromosome separation to occur [reviewed in (Uhlmann, 2004)]. APC/C\textsuperscript{Cdc20} is also required for mitotic exit through targeting cyclin B for destruction. Cdh1 activates the APC/C (APC/C\textsuperscript{Cdh1}) in G\textsubscript{1}. APC/C\textsuperscript{Cdh1} prevents premature accumulation of S-phase cyclins, and it must be inactivated for the cell cycle to proceed into S [reviewed in (Li and Zhang, 2009)]. Typically, APC/C\textsuperscript{Cdc20} recognizes substrates containing a destruction box motif (minimally the amino acid sequence R-X-X-L), whereas APC/C\textsuperscript{Cdh1} recognizes both the destruction box and another motif, the KEN box (K-E-N) (Zur and Brandeis, 2002). In \textit{S. cerevisiae}, it has been determined that phosphorylation of the APC/C by Cdk1 results in the binding of Cdc20 to the APC/C in mitosis. Mutating the Cdk-dependent phosphorylation sites of the APC/C allows Cdh1 to bind in mitosis and makes Cdh1 essential and Cdc20 non-essential (Cross, 2003) (in wild-type strains, Cdh1 is not essential and Cdc20 is essential). APC/C activity in mitosis is regulated by the spindle assembly checkpoint.
1.3.2. The Spindle Assembly Checkpoint (SAC)

The spindle assembly checkpoint (SAC), as its name suggests, ensures that anaphase does not begin before the mitotic spindle is correctly assembled. Unattached kinetochores (KTs) generate a “wait anaphase” signal monitored by the SAC and the SAC is not satisfied until all KTs are attached in a bipolar fashion to spindle microtubules [reviewed in (Musacchio and Hardwick, 2002; Musacchio and Salmon, 2007; Taylor et al., 2004)]. Once the SAC is satisfied, securin and cyclin B are ubiquitinated by the APC/C, targeting them for destruction by the proteasome [reviewed in (Peters, 2006)].

All SAC proteins concentrate at KTs during prometaphase and are recruited in a hierarchical fashion [(Johnson et al., 2004; Vigneron et al., 2004; Wong and Fang, 2006); reviewed in (Musacchio and Salmon, 2007)]. Once the SAC is fulfilled, the SAC proteins are diminished at KTs (Chen et al., 1998; Chen et al., 1996; Li and Benezra, 1996; Taylor et al., 1998). Mutations or conditions that prevent binding of “core” SAC components (Mad1, Mad2, Bub1, BubR1/Mad3, Bub3, Mps1, Aurora B, and Cdc20) to KTs generally inhibit SAC activity (Ditchfield et al., 2003; Meraldi et al., 2004; Vigneron et al., 2004; Zhao and Chen, 2006).

1.3.3. The Mitotic Checkpoint Complex (MCC)

The mitotic checkpoint complex (MCC) is required for the SAC in that it binds the APC/C. The MCC-bound APC/C is unable to target cyclin B and securin for destruction, thereby inhibiting anaphase and mitotic exit. The MCC was first identified in S. cerevisiae in that Mad3 (the S. cerevisiae ortholog of H. sapiens BubR1), Bub3, Cdc20, and Mad2 were found to physically interact with each other (Hardwick et al., 2003).
This complex accumulated in mitosis, when it seemed to associate with the APC/C, rendering it inactive (Fraschini et al., 2001; Hardwick et al., 2000; Poddar et al., 2005). Within the *S. cerevisiae* MCC, Mad2 and Mad3 bind Cdc20 directly (Hardwick et al., 2000; Hwang et al., 1998). The Mad2-Cdc20 interaction requires Mad1, and the Mad3-Cdc20 interaction requires Mad2 and Bub3 (Fraschini et al., 2001; Hardwick et al., 2000; Hwang et al., 1998). A MCC was also identified through biochemical methods in HeLa cells (Sudakin et al., 2001). This complex of proteins, which included Mad2, Cdc20, Bub3, and BubR1, was found to inhibit activity of mitotic APC/C. In fact, this group estimated that the inhibitory activity of the MCC was more than 3000-fold greater than that of recombinant Mad2 (Sudakin et al., 2001). The authors went on to calculate that the stoichiometry of the MCC components was 1.4 BubR1:1 Bub3:1 Cdc20:1.2 Mad2; therefore, it is unlikely that Mad2 oligomers, which form readily in bacteria (Fang et al., 1998), are present in the MCC of HeLa cells (Sudakin et al., 2001). Importantly, even though 90% of Mad2 from HeLa cell extracts is not associated with the MCC or APC/C, these fractions are unable to inhibit the APC/C (Sudakin et al., 2001).

Components of the MCC are conserved from yeast to vertebrates, suggesting an evolutionarily conserved mechanism of inhibiting the APC/C until all chromosomes are aligned properly in mitosis. All MCC components cycle on and off KTIs rapidly, suggesting that the MCC is created dynamically at or near KTIs (Howell et al., 2000; Howell et al., 2004; Kallio et al., 2002; Shah et al., 2004).

Other proteins have been found to have a role in the SAC that are not part of the MCC. These included Mad1, Bub1, and Mps1, among others. Even though such proteins
are not part of the MCC, they do provide a link between the MCC and KTs in that some are required for KT localization of MCC components. In *X. laevis*, Mad2 requires Mad1 for KT localization (Chen et al., 1998), Bub3 and Mad2 require Bub1 (Sharp-Baker and Chen, 2001), and Mad2 requires Mps1 (Abrieu et al., 2001).

How exactly the MCC, and thereby the SAC, inactivates the APC/C is still incompletely understood. The current model is that Mad1-Mad2 heterodimers bind at unattached KTs, resulting in a conformational change in Mad2 to a “closed” state (C-Mad2). Mad1-C-Mad2 now is a receptor for cytoplasmic Mad2, which is in an “open” conformation (O-Mad2). Formation of the O-Mad2-C-Mad2 dimer releases O-Mad2 in an intermediate form (I-Mad2) that is primed for binding to Cdc20. This binding to Cdc20 converts I-Mad2 to C-Mad2, inhibiting Cdc20 binding to the APC/C [(De Antoni et al., 2005; Mariani et al., 2012); reviewed in (Kim and Yu, 2011; Musacchio and Salmon, 2007; Pines, 2011); Fig. 1.7]. It has been proposed that when Cdc20 is part of the MCC, it is in an orientation that eliminates the substrate recognition site between Cdc20 and the APC/C (Herzog et al., 2009). The key to this MCC-APC/C inhibition may lie with BubR1/Mad3. BubR1-Cdc20 is a more effective inhibitor than Mad2-Cdc20 (Fang, 2002; Tang et al., 2001), and studies in *S. cerevisiae* have suggested that Mad3 acts as a pseudosubstrate inhibitor of the APC/C (Burton and Solomon, 2007; King et al., 2007).
1.4. *Aspergillus nidulans* as a Model System

1.4.1. General Description of the Genus

The genus *Aspergillus* is comprised of filamentous fungi and is classified under the phylum Ascomycota. All *Aspergillus* species are highly aerobic and most rely on carbon-rich sources for growth such as the soil, fruits, vegetables, and trees. Members of this genus are important commercially, industrially, and medically. From a commercial standpoint, *A. niger* is a major source of citric acid, among other things, while *A. oryzae* is used in the fermentation processes that produce sake and soy sauce. *Aspergillus* species also produce certain natural products that are medically useful such as the antifungal agent, echinocandin [reviewed in (Emri et al., 2013)] (although the compounds used medically, such as anidulafungin are chemically modified derivatives of echinocandin) and the anti-cholesterol agent, lovastatin (Moore et al., 1985). Medically, certain *Aspergillus* species can be harmful not only to humans, but to animals as well. Aspergillosis is the term for the class of diseases caused by *Aspergillus*, and the main contributor to these diseases is *A. fumigatus*. Aspergillosis typically occurs in those who have a severely compromised immune system and affects the lungs and sometimes surrounding tissue and can also infect the brain. Aspergillosis has also been reported in birds, especially those in captivity (Beernaert et al., 2010). Another species, *A. flavus*, is also harmful to humans, especially children, as it produces aflatoxin, a highly carcinogenic substance (Groopman et al., 2008).
1.4.2. Asexual Life Cycle

_Aspergillus_ species start their asexual life cycle as ungerminated conidia, which are uninucleate and haploid. Under appropriate growth conditions, conidia produce a germ tube and undergo polarized growth forming a germling. After about three rounds of mitotic division, a septum forms (Harris et al., 1994), dividing the germling into an apical cell, also called a tip cell, and a subapical cell. Both apical and subapical cells are multinucleate, but only the nuclei in apical cells actively proceed through the cell cycle. Such nuclei go through the cell cycle in synchrony. Once septa are formed, the germling is now called a hypha [reviewed in (Lengeler et al., 2000; Timberlake and Marshall, 1988)]. Multiple rounds of division and hyphal extension eventually lead to the formation of a mycelium consisting of numerous branched, filamentous, and multinucleate cells. In liquid culture, this vegetative growth results in non-conidiating mycelia (Varanasi et al., 2004). However, on solid medium, conidiophores will develop in several steps. First, certain hyphal segments differentiate into conidiophore-support cells termed foot cells [reviewed in (Timberlake and Marshall, 1988)]. Short, aerial hyphae, termed stalks, are produced from foot cells (Varanasi et al., 2004). The tips of stalks then swell to form apical vesicles, which bud to produce uninucleate metulae, which then divide, producing phialides. Repeated nuclear divisions of phialides lead to a chain of conidia with the oldest conidium being the most terminal and therefore the first to be dispersed into the air [(Varanasi et al., 2004); reviewed in (Lengeler et al., 2000; Timberlake and Marshall, 1988)]. A diagram of vegetative growth and asexual reproduction is given in Fig. 1.8.
1.4.3. Sexual Life Cycle

About one-third of *Aspergillus* species are known to have a sexual life cycle, with the majority of these species being homothallic, meaning they are self-fertile [reviewed in (Geiser, 2009; Lee et al., 2010)]. Sexual ascospores can be produced by homokaryons (self mating) or by heterokaryons (mating between different strains). In either case, specialized fruiting bodies called cleistothecia form on the surface of the mycelium. In the cleistothecia, mating nuclei undergo fusion, termed karyogamy, and, subsequently, meiosis [reviewed in (Timberlake and Marshall, 1988)]. Following meiosis, a round of mitosis gives rise to eight ascospores inside sac-like structures called asci. The nuclei in the ascospores then undergo a second round of mitosis resulting in the mature ascospores being binucleate [reviewed in (Geiser, 2009; Timberlake and Marshall, 1988)]. When mature, the ascus wall dissolves, releasing the ascospores which remain inside of the cleistothecium [reviewed in (Geiser, 2009)]. Each cleistothecium can contain tens of thousands of ascospores [reviewed in (Lee et al., 2010)], which can be released upon perforation of the cleistothecium. A diagram of sexual reproduction is given in Fig. 1.9.

1.4.4. *A. nidulans* as a Model System for Genetic and Cell Cycle Research

The ability of *A. nidulans* to undergo both asexual and sexual reproduction is one reason why it serves as a great genetic model system. Asexual conidia are produced rapidly and are uninucleate. When conidia germinate, the nucleus divides with the daughter nuclei entering the germ tube. Therefore, it is easy to determine through light or fluorescence microscopy whether a mutation affects nuclear division or nuclear movement. Because there are no mating types, any two strains can, in principle, be
mated. The use of spore color markers and nutritional markers can allow easy identification of hybrid cleistothecia (see also Section 2.8).

*A. nidulans* is normally haploid, but heterokaryons and asexual diploids can be generated for complementation analysis of mutations. A mutation can be mapped to its linkage group (chromosome) by formation of an asexual diploid with a strain with a genetic marker on each chromosome and subsequent haploidization. The mutation will segregate opposite a particular chromosomal marker. Once the chromosome has been determined, a mutation can be mapped to its locus through sexual crosses to strains with several markers on the chromosome.

The genomic sequence of *A. nidulans* has been published (Galagan et al., 2005), making it easier to identify genes involved in such processes as mitosis, meiosis, septation, conidiation, metabolite production, cell polarity, and the cell cycle. There are eight chromosomes and the size of the genome is 30.5 megabases. Annotation has identified 10,678 open reading frames, 1096 of which have been verified (meaning there is experimental evidence for the existence of a gene product) according to the *Aspergillus* Genome Database (http://www.aspergillusgenome.org/cache/A_nidulans_FGSC_A4_genomeSnapshot.html). Even though it has a relatively small genome and is a simple eukaryote, *A. nidulans* has proven itself to be a useful model organism for cellular processes that occur not only in other fungi, but in higher eukaryotes as well. It has been used with great success to study cell cycle control (De Souza et al., 2009; Edgerton-Morgan and Oakley, 2012; Efimov and Morris, 1998; Nayak et al., 2010; Osmani et al., 1994; Prigozhina et al.,
2004), the cytoskeleton (Horio and Oakley, 2005; Jung et al., 1998; Oakley et al., 1990; Szewczyk and Oakley, 2011; Taheri-Talesh et al., 2008; Taheri-Talesh et al., 2012; Zhang et al., 2011), cytokinesis (Kim et al., 2006; Kim et al., 2009b; Si et al., 2010), primary metabolism [reviewed in (Davis and Hynes, 1989; Felenbok, 1991)], secondary metabolism [reviewed in (Bayram and Braus, 2012; Sanchez et al., 2012)] and developmental regulation [reviewed in (Geiser, 2009; Lengeler et al., 2000; Timberlake and Marshall, 1988)]. It should also be noted that *A. nidulans* is non-pathogenic, unlike some closely-related *Aspergillus* species. Therefore, *A. nidulans* is easier to work with and its similarity to potentially pathogenic species [the amino acid identity of predicted protein coding sequences between *A. nidulans* and *A. fumigatus* is about 66% (Galagan et al., 2005)] suggests that many findings made with *A. nidulans* will apply to pathogenic species of *Aspergillus*.

Another advantage of using *A. nidulans* is that it is transformable. Transforming DNA on plasmids (Ballance et al., 1983; Johnstone et al., 1985; Morris and Enos, 1992), cosmids (Yelton et al., 1985), and linear molecules of DNA (Miller et al., 1985) [now produced easily by fusion PCR (Nayak et al., 2006; Szewczyk et al., 2006; Yang et al., 2004)] can integrate into the genome by homologous recombination. Such transformations with DNA can result in gene disruption or replacement, fusion of a tag to the N-terminus or C-terminus of a protein, or replacement of an endogenous promoter with a regulatable promoter.
1.4.5. Cell Cycle Stages

*A. nidulans* has cell cycle stages typical for eukaryotes, but the cell cycle is very short. Bergen and Morris (1983) determined that at 32°C, an entire cell cycle is 100 minutes with G1 being 15 minutes; S, 40 minutes; G2, 40 minutes; and mitosis only 5 minutes. I have further determined that at 25°C, the temperature at which most of my imaging was performed, the cell cycle time is 199 ± 49 minutes with mitosis lasting approximately 10 minutes (Edgerton-Morgan and Oakley, 2012).

1.4.6. Efficiency of Gene Targeting

Linear DNA can integrate into the genome by either of two ways, by homologous recombination which results in an integration at the desired genomic locus, or by non-homologous end joining which results in a random integration in the genome (Fig. 1.10). Tania Nayak, a former graduate student in the lab, sought to increase the frequency of homologous recombination by deleting the *A. nidulans* homolog of the human KU70 gene, designated *nkuA*. KU70 and KU80 are genes required for non-homologous end joining DNA repair, and deletion of homologs of these in *Neurospora crassa* increased the frequency of correct gene replacement (Ninomiya et al., 2004). Deletion of *nkuA* in *A. nidulans* also dramatically improved the frequency of single, correct integrations to about 90% (Nayak et al., 2006).

1.5. The *A. nidulans* γ-Tubulin Mutation, *mipAD159*

1.5.1. Creation by Alanine-scanning Mutagenesis

Charged to alanine mutations are often useful for determining what region(s) of a protein are important for its function(s), and what those functions are, and they also aid in
determining regions of protein-protein interactions. For these reasons Jung et al. (2001) created a series of charged-to-alanine mutations of the *A. nidulans* γ-tubulin gene, *mipA*. Charged amino acids tend to lie on the outside of proteins in regions that are hydrophilic. Substituting such amino acids with alanine, which is neutrally charged and non-polar, often disrupts protein-protein interactions but without causing large alterations of protein structure. Clusters of charged amino acids [two or more in a stretch of five (Wertman et al., 1992)] were mutated to alanine. All together, 41 mutant alleles of *mipA* were created, eight of which proved to be conditionally lethal (Jung et al., 2001). The conditionally lethal alleles were also determined to be recessive.

*mipAD159*, an allele that has D159 and R160 both mutated to alanine, was found to be cold-sensitive and is essentially completely blocked for growth at 20°C (Fig. 1.11). D159 and R160 are located on helix H4 (Fig. 1.12). Germlings grown at a permissive temperature of 37°C had normal cytoplasmic microtubules and spindles; however, most germlings grown at a restrictive temperature of 20°C had a single, obviously polyploid nucleus. Robust mitotic spindles were seen and some germlings had bundled or curved cytoplasmic microtubules (Jung et al., 2001). It is worth noting that the γ-tubulin encoded by *mipAD159* localized normally to the SPB (Jung et al., 2001).

1.5.2. Failure of Coordination of Late Mitotic Events

To determine if the *mipAD159* defects observed by Jung et al (2001) were primarily due to the mutation itself or due to the secondary effects caused by failed mitoses, Prigozhina et al. (2004) set out to determine the precise effects of *mipAD159* on the progression of mitosis. In summary, they found that *mipAD159* caused a failure of
coordination of late mitotic events as observed phenotypes included inhibition of anaphase A (the movement of chromosomes to the poles), abnormal chromosome segregation including nondisjunction, nuclei re-entering interphase without entering anaphase or completing mitosis, and abnormal nuclear movement after mitosis (Prigozhina et al., 2004). Chromosomal condensation, mitotic spindle formation, and spindle elongation in anaphase B were apparently normal [the morphology of mitosis in wild-type A. nidulans is described in (Jung et al., 1998)]. In the presence of benomyl, a microtubule-depolymerizing agent, and consequently an activator of the SAC, the mipA\textsuperscript{D159} strain was blocked in mitosis, but it exited mitosis sooner than the mipA\textsuperscript{+} control strain, indicating that mipA\textsuperscript{D159} causes a premature mitotic exit, and that this does not require microtubules or mitotic spindle formation (Prigozhina et al., 2004).

1.5.3. Failure of Inactivation of the APC/C

To further elucidate the role of γ-tubulin in mitotic regulation in A. nidulans, our lab set out to determine the localization of mitotic regulatory proteins in mipA\textsuperscript{+} and mipA\textsuperscript{D159} strains. Interestingly, we found that three proteins, cyclin B, Cdk1, and Ancdc14, failed to accumulate in a subset of mipA\textsuperscript{D159} nuclei in hyphae grown at a restrictive temperature of 25°C (Nayak et al., 2010). We designated these nuclei cyclin B negative (CB\textsuperscript{−}). Such nuclei were taken out of the cell cycle while other nuclei in the same cell continued to cycle. The percentage of non-cycling nuclei increased over time without an obvious pattern as to whether daughter nuclei would or would not fail to accumulate cyclin B in the subsequent S/G\textsubscript{2} phase (Fig. 1.13). Extensive experimental analyses revealed this failure of cyclin B accumulation was not simply due to abnormal
mitoses (50% of obviously aneuploid nuclei accumulated cyclin B), or to a defect in nuclear transport [dsRed fused to a nuclear localization sequence (Suelmann et al., 1997) accumulated in all mipAD159 nuclei, nuclear pore assembly and disassembly was normal in CB− nuclei, and blockage of nuclear export did not rescue the CB− phenotype] (Nayak et al., 2010). Instead, the failure of cyclin B accumulation was found to be due to a nuclear autonomous failure of APC/C inactivation sometime between late mitosis and S phase. The evidence is as follows. (Nayak et al., 2010) created a version of cyclin B-GFP that lacked the N-terminal region that contained its destruction box (Δdb-cyclin B-GFP) and it was put under control of the regulatable alcA promoter [alcA(p); (Waring et al., 1989)]. A strain was created that carried alcA(p)-Δdb-cyclin B-GFP integrated at the wA locus and mipAD159. The strain was grown at a permissive temperature in a medium that repressed expression of alcA(p), and then shifted to a restrictive temperature of 25°C, and the medium was changed to inducing media. Δdb-cyclin B-GFP accumulated in all nuclei. Controls gave expected results. CB− nuclei were rare in mipA+ controls, and CB− nuclei were as abundant in a strain carrying mipAD159 and alcA(p)-cyclin B-GFP [i.e. cyclin B under the control of alcA(p) with an intact destruction box. This served as a control for any effects of expression from the alcA promoter]. In a parallel experiment a strain was created that carried mipAD159 and expressed cyclin B-mCherry and alcA(p)-Δdb-cyclin B-GFP. It was taken through the same temperature shift and induction procedure. Δdb-cyclin B-GFP accumulated in all nuclei, but in a subset of these GFP positive nuclei, cyclin B-mCherry did not accumulate [(Nayak et al., 2010); Fig. 1.14]. Removal of the destruction box, which is targeted by the APC/C, thus, reversed the
cyclin B accumulation defect caused by \textit{mipAD159}. These results indicate that the defect is due to constitutive APC/C activity and, by inference, that \(\gamma\)-tubulin has a previously uncharacterized role in inactivating the APC/C.

1.6. Aims of Dissertation Research

One goal of my research was to obtain information as to the mechanism by which \(\gamma\)-tubulin regulates APC/C activity. As described in 1.5.3, Nayak et al. (2010) discovered that \(\gamma\)-tubulin has a role in inactivating the APC/C sometime between late mitosis and S. \textit{mipAD159} nuclei with a constitutively active APC/C fail to accumulate cyclin B, Cdk1, and the Ancdc14 phosphatase in interphase and are taken out of the cell cycle while other nuclei in the same cell continue to cycle. What is unknown is whether the constitutively active form of the APC/C is APC/C\(^{Cdh1}\) or APC/C\(^{Cdc20}\). Failure of inactivation of either form could lead to continuous destruction of cyclin B, which is required for S phase as well as mitosis. (Osmani et al., 1994). Therefore, I wanted to determine if \textit{mipAD159} caused a failure of inactivation of APC/C\(^{Cdh1}\) at the G\(_1\)/S boundary or a failure of inactivation of APC/C\(^{Cdc20}\) in late mitosis and, consequently, whether \(\gamma\)-tubulin plays a role in the regulation of APC/C\(^{Cdc20}\) or APC/C\(^{Cdh1}\). In Chapter 4, I discuss my findings, clearly demonstrating that \(\gamma\)-tubulin has a role in inactivating APC/C\(^{Cdh1}\).

The data of Nayak et al. (2010) raise the question of why the APC/C in CB\(^{−}\) nuclei is not inactivated by the SAC in mitosis. If, for example, \(\gamma\)-tubulin acts as a regulator of the SAC, and this function is compromised in strains carrying \textit{mipAD159}, then nuclei might enter anaphase prematurely before all KTs are properly attached to spindle microtubules, possibly leading to aneuploidy or polyploidy. In a potentially
related finding, Prigozhina et al. (2004) found that nuclei were blocked in mitosis by
treatment with the antimicrotubule agent benomyl (which, because it inhibits spindle
formation, activates the SAC), and nuclei exited mitosis significantly sooner in a
$mipAD159$ strain than in a $mipA^+$ control strain. What is not known is if the SAC is
rendered inactive due to a failure or mislocalization of SAC proteins. I therefore decided
to study the localization of SAC proteins in both $mipA^+$ and $mipAD159$ strains. In
addition, tagging and performing live imaging on SAC proteins, will, at a minimum, add
to the general knowledge of cell cycle regulation in $A. nidulans$. If the localization of one
or more SAC proteins is altered in $mipAD159$ strains, it may provide additional insights
into $\gamma$-tubulin functions. The localization of Mad2, Mps1, Bub3, BubR1, and Cdc20 is
discussed in Chapter 3 as well as the absence of Mps1 and BubR1 in a subset of
$mipAD159$ nuclei.

In Chapter 5, I give my concluding remarks and overall discussion. In this
chapter, I connect the findings of my two projects together and propose that the
continuous activation of APC/C$^{Cdh1}$ causes destruction of Mps1 and thereby failure of
BubR1 accumulation, resulting in an inactive SAC in CB$^-$ nuclei.
Panel A shows a microtubule consisting of 13 protofilaments. Each protofilament consists of α/β heterodimers assembled in a head-to-tail fashion. The α/β heterodimers can bind to both ends of the microtubule, but one end grows faster than the other. The end with β-tubulins is the fast-growing end, or plus end, while the end with α-tubulins is the slow-growing end, or minus end. Lateral interactions between α-α and β-β exist except at the seam. A 13-protofilament microtubule is a 3-start helix, meaning each turn of the helix spans three tubulin monomers. Modified from Amos and Schlieper (2005).

Panel B depicts the dynamics of a microtubule, switching between growth (microtubule assembly/polymerization) and shrinkage (microtubule disassembly/depolymerization). Modified from Kinoshita et al. (2002).
Centrosomes and spindle pole bodies (SPBs) are both PMTOCs, but are quite different in structure. A and C. Cartoon representations of the structures of a centrosome (A) and an *S. cerevisiae* SPB (C) [Adapted from Crasta and Surana (2006)]. B. Electron

Figure 1.2: Composition of the centrosome and *S. cerevisiae* spindle pole body

Centrosomes and spindle pole bodies (SPBs) are both PMTOCs, but are quite different in structure. A and C. Cartoon representations of the structures of a centrosome (A) and an *S. cerevisiae* SPB (C) [Adapted from Crasta and Surana (2006)]. B. Electron
Figure 1.2 continued

micrograph of a vertebrate centrosome. PCM is the pericentriolar material; tMTs indicates triplet microtubules that form the centrioles; PC is a procentriole, a centriole precursor. D. Electron micrograph of a SPB in *S. cerevisiae*. Arrowheads indicate the nuclear envelope into which the SPB is embedded; N is the nucleoplasmic side; C is the cytoplasmic side; nMTs are microtubules extending into the nucleus; cMT is a cytoplasmic microtubule; HB is the half-bridge; S is a satellite, which is a SPB precursor. B and D are adapted from Jaspersen and Winey (2004).
The different plaques and layers are indicated on the left-hand side. Tub4 (γ-tubulin) nucleates both cytoplasmic (cMTs) and nuclear microtubules (nMTs) and is attached to both the outer and inner plaques through its interaction with Spc97/Spc98 and their interactions with Spc72 (on the outer plaque) and Spc110 (on the inner plaque). Figure modified from Jaspersen and Winey (2004).
A freeze substitution electron micrograph of a mitotic spindle in *A. nidulans*. The SPB is indicated by the arrow. Microtubules extend from the SPB into the nucleoplasm and the cytoplasm. Microtubules contact chromosomes at KTs, indicated by arrowheads. Scale bar is 1 μm. The original micrograph was courtesy of Dr. I. Brent Heath and was published in Jung et al. (1998).
Panel a depicts the structure of the *S. cerevisiae* γ-TuSC bound to its anchor on the inner SPB plaque, Spc110, as determined by cryo-electron microscopy with a resolution of 8 Å. The γ-TuSC consists of a Spc97 (GCP2)-Spc98 (GCP3) dimer with Tub4 (γ-tubulin) at each tip. Panel b shows how the γ-TuSC oligomer spontaneously assembles into a ring-like structure. The Tub4 molecules are held apart from each other in a configuration that is incompatible with the microtubule lattice. Figure modified from Kollman et al. (2011).
Components of the γ-TuSC (γ-tubulin, GCP2, and GCP3 orthologs) are present in all eukaryotes and are essential for viability and normal spindle microtubule assembly. γ-TuSCs are part of the larger γ-TuRC, which consist of at least two other GCPs. GCP4 and GCP5 orthologs are present in most eukaryotes, and GCP6 orthologs are present in animals, fungi, and fission yeast. S. cerevisiae does not have orthologs of GCP4-6.
Cdh1 binds and activates the APC/C in G1 targeting S-phase cyclins for destruction by the proteasome (orange triangles). This prevents cyclins required for S phase from accumulating prematurely. APC/C$^{Cdh1}$ must be inactivated for cells to enter S, during which S-phase cyclins will accumulate. Cdc20 binds and activates the APC/C in mitosis. APC/C$^{Cdc20}$ targets securin for destruction by the proteasome, freeing separase to cleave cohesin, allowing the separation of chromosomes. APC/C$^{Cdc20}$ also targets cyclin B for destruction, and this destruction is essential for mitotic exit.
Unattached KTs generate a signal to inhibit the APC/C so anaphase does not happen prematurely. Unattached KTs recruit Mps1, and thereby Mad1 and BubR1 to promote assembly of the MCC. Mad2 in the open conformation (O-Mad2) binds to Mad1-closed Mad2 (C-Mad2) dimers at the KT. This catalyzes O-Mad2 to come off in an intermediate form (I), which is in a state to bind Cdc20. When I-Mad2 binds Cdc20, it undergoes a conformational change into C-Mad2. C-Mad2-Cdc20 promotes further interactions with BubR1 and Bub3, forming the MCC. The MCC then binds to and inhibits APC/C\textsuperscript{Cdc20} by blocking substrate recognition. Modified from Kim and Yu (2011).

Figure 1.7: A current model as to how the MCC inhibits the APC/C
As described in the text, *Aspergillus* starts its life cycle as conidia. Conidia germinate and undergo polarized growth, forming germlings. Germlings eventually generate septa, dividing them into subapical and apical cells. At this point, germlings are now hyphae. Hyphae continue to undergo apical growth as they go through multiple rounds of mitotic division, eventually forming a mycelium of multiple hyphae. Foot cells of hyphae produce stalks, which swell to form conidiophore vesicles. Vesicles then produce metulae, which further divide into phialides. Phialides then produce chains of conidia, and the structure is now a mature conidiophore. Conidia are then subject to air dispersal, which can, in the right environmental conditions, start the cycle over again. The figure is modified from Varanasi et al. (2004).

Figure 1.8: Vegetative growth and asexual reproduction in *Aspergillus*
Aspergillus hyphae can either self-cross or outcross with other hyphae. Cleistothecia, the fruiting bodies, form on the surface of the mycelium. Nuclear fusion, followed by meiosis and two rounds of mitosis results in eight ascospores formed in each ascus. One cleistothecium can contain tens of thousands of ascospores, which, when released, undergo germination under the right nutritional conditions. Modified from Lee et al. (2010).
A. One way in which a linear DNA molecule can integrate into the genome is through homologous recombination. This results in an integration at the desired, targeted locus.

B. Another way in which linear DNA can integrate into the genome is through non-homologous end joining (NHEJ). NHEJ is facilitated by KU70 and KU80, two DNA repair proteins, which results in integration of the linear DNA molecule in a random location in the genome. Both the deletion of the *A. nidulans* homolog of KU70, *nkuA*, and the double deletion of *nkuA* and *nkuB*, the *A. nidulans* homolog of KU80, improved the frequency of correct gene targeting events from ≤ 40% to ~ 90%.

Figure 1.10: Homologous recombination versus non-homologous end joining DNA repair.
Multiple \textit{mipA} mutants were stabbed onto complete medium (YAG) and incubated at temperatures ranging from 20-42°C to check if they were cold- or heat-sensitive. \textit{mipAD159} is indicated by the arrowheads. It grows similarly to WT at 42°C, grows slightly slower than WT at 37°C, is noticeably growth-inhibited at 30 and 25°C and is essentially dead at 20°C. Modified from Jung et al. (2001).
Figure 1.12: *mipAD159* maps to helix H4 on the outside of structural models of γ-tubulin
Figure 1.12 continued

A. Structure of γ-tubulin showing sites of conditional lethal alleles. This γ-tubulin structure was predicted from the electron crystallographic structures of mammalian α- and β-tubulins. *mipAD159* is circled in pink. Modified from Jung et al. (2001). B. *mipAD159* (shown in green) mapped onto the structure of human γ-tubulin as determined by x-ray crystallography at 3.0Å resolution (Aldaz et al., 2005). This figure shows four γ-tubulin molecules and how they are predicted to interact in a ring-like structure of the γ-TuRC. This interaction model was kindly provided by the lab of David Agard, and the position of *A. nidulans* *mipAD159* was mapped using PyMOL (Delano, 2002). C. Same structure as B. but as a view from the top of the γ-TuRC. *mipAD159* clearly maps to the outside face of γ-tubulin, and is likely not involved in lateral interactions between γ-tubulin molecules (this figure and unpublished data from América Hervás-Aguilar and Berl Oakley).
A. In a $mipA^+$ strain, cyclin B-GFP begins to accumulate in S phase (not shown) and stays through G2. After division, every nucleus in the pictured tip cell accumulates cyclin B-GFP in the next S and G2 phases. B. In a $mipAD159$ strain, both nuclei, which are in the same tip cell, have cyclin B. After division, which in this case appears to have been normal since there was no evidence for non-disjunction or other abnormality in the video.

Figure 1.13 Failure of accumulation of cyclin B in $mipAD159$ nuclei
Figure 1.13 continued

from which this series of images was taken, one nucleus fails to accumulate cyclin B (arrow in right merge panel).
A linear DNA construct was generated to put a version of cyclin B-GFP that lacked its destruction box under the control of the alcA promoter [\textit{alcA}(p)-Δdb-cyclin B-GFP]. The construct was integrated by transformation into the \textit{wA} locus of a strain carrying a wild-type cyclin B fused to mCherry. The resulting transformant strain was then crossed to a \textit{mipAD159} strain. A verified strain carrying \textit{mipAD159}, cyclin B-mCherry, and \textit{alcA}(p)-Δdb-cyclinB-GFP was grown at a permissive temperature of 37°C in \textit{alcA}(p)-repressing media for 12 hours. The repressing medium was then washed out and replaced with \textit{alcA}(p)-inducing medium and the culture was shifted to a restrictive temperature of 25°C. Tip cells were imaged 1-3 hrs. after the shift to allow the accumulation of dbΔ-cyclin B-

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**Figure 1.14:** CB\textsuperscript{−} nuclei are caused by constitutive APC/C activity

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continued
Figure 1.14 continued

GFP. Pictured here are four nuclei in the same tip cell. dbΔ-cyclin B-GFP accumulates in all four, but two do not accumulate cyclin B-mCherry (arrows), indicating that removal of the destruction box, which the APC/C recognizes to target cyclin B for destruction by the proteasome, rescues the cyclin B accumulation defect of *mipAD159*. The figure is from Nayak et al. (2010).
Chapter 2: Materials and Methods

2.1. Strains and Media

A list of strains used in this study and their genotypes is given in Table 2.1. YAG [5 g/liter yeast extract, 20 g/liter D-glucose, 15 g/liter agar, supplemented with 400 μl/liter of a trace element solution (Vishniac and Santer, 1957)] was used as a solid complete medium. Yeast extract does not provide enough pyrimidines to supplement the pyrG89 mutation nor enough riboflavin to fully supplement the riboB2 mutation, so uridine, uracil and riboflavin were added to YAG if needed (see Table 2.2 for concentrations). Liquid minimal medium (MM) consisted of 6 g/liter NaNO₃, 0.52 g/liter KCl, 0.52 g/liter MgSO₄·7H₂O, 1.52 g/liter KH₂PO₄, 10 g/liter D-glucose, 400 μl/liter of trace element solution (Vishniac and Santer, 1957) and appropriate nutrients needed by each strain for growth (a list of nutrients and their concentrations to fully supplement nutritional markers is given in Table 2.2). pH was adjusted to 6.0-6.5. The same recipe was used for solid MM with the addition of 15 g/liter of agar.

2.2. Identifying A. nidulans Gene Homologs

The A. nidulans genome has been sequenced and was first publically released in 2003 by the Broad Institute [www.broadinstitute.org/; (Galagan et al., 2005)]. Several annotations later (Arnaud et al., 2010; Wortman et al., 2009), the genome sequence is
now also available and searchable through AspGD (www.aspgd.org) and the Central
Aspergillus Data Repository (CADRE; www.cadregenomes.org.uk/index.html). These
websites were used for BLAST searches and sequence downloads.

2.3. PCR for Gene Targeting

Linear constructs for transformation of *A. nidulans* were generated by fusion PCR
(Nayak et al., 2006; Szewczyk et al., 2006; Yang et al., 2004; Yu et al., 2004; Zarrin et
al., 2005).

2.3.1. C-terminal Tagging

The procedure I followed for generating a fusion PCR construct in which a
fluorescent protein sequence is fused to the C-terminus of a gene is given in Szewczyk et
al. (2006). I will summarize the procedure here. Six primers were designed for each
construct. Four were used to amplify two regions of flanking genomic DNA:
approximately 1000 base pairs (bp) 5’ to the stop codon of the gene and approximately
1000 bp 3’ from the stop codon, and two were used for the fusion PCR amplification
(Fig. 2.1). Primers P1, P2, P5, and P6 were designed to have a $T_m$ of about 60°C. The
formula used to calculate $T_m$ was \[ [(C+G) \times 4] + [(A+T) \times 2]. \] P3 and P4 were longer
primers as they both have tails to allow fusion of the flanks to the fluorescent protein-
selectable marker cassette. The tail of P3 was identical to the 5’ end of the fluorescent
protein cassette [the 5’ end of all fluorescent proteins used for C-terminal fusions
included a flexible glycine-alanine (GA) linker, which encodes 5 repeats of the amino
acids GA (Yang et al., 2004)], while the tail of P4 was identical to the 3’ end of the
cassette (the region containing the selectable marker). The tail sequences also had a $T_m$
around 60°C. It was critical to make sure that the reading frame of the gene was maintained when designing P3. P3 did not contain the stop codon of the gene being tagged. The fluorescent protein-selectable marker cassette was amplified from a plasmid. Fusion PCR of the three DNA fragments was done with nested primers P2 and P5. We have found that using nested primers increases the specificity of amplification, decreasing the possibility of obtaining extra bands in the fusion PCR product (Szewczyk et al., 2006).

2.3.2. N-terminal Tagging

In my projects, N-terminal fluorescent protein tagging was only required in the case of *cdc20*. A schematic for generating a five-piece fusion PCR product for N-terminal tagging is given in Wong et al. (2008), and a schematic of my similar fusion PCR strategy for N-terminally tagging *cdc20* with GFP is given in Fig. 2.2. In summary, three fragments were amplified from *A. nidulans* genomic DNA: 1) approximately 1000 bp of a region of the gene’s 5’ untranslated region (5’UTR), 2) approximately 500 bp of the gene’s promoter region, and 3) approximately 1000 bp of the gene’s coding sequence. The fluorescent protein was amplified such that it included an ATG at its N-terminus and a flexible glycine-alanine (GA) linker at its C-terminus. The selectable marker was amplified from a plasmid and inserted between the 5’ flank fragment and the promoter.

2.3.3. Gene Deletions

Gene deletions were done by replacing the gene with a selectable marker as described in Szewczyk et al. (2006). To create a transforming fragment, I used a fusion PCR strategy similar to that used to create constructs for C-terminal tagging. Two flanks
were amplified from *A. nidulans* genomic DNA. Primers P1 and P3 amplified approximately 1000 bp of the 5’UTR directly upstream of the gene’s start codon and primers P4 and P6 amplified approximately 1000 bp of the gene’s 3’ untranslated region (3’ UTR) directly downstream of the gene’s stop codon (Fig. 2.3). P3’s tail was identical in sequence to the 5’ end of the selectable marker cassette, and P4’s tail was identical in sequence to the 3’ end of the cassette. The two flanks were mixed with the selectable marker cassette, already amplified from a plasmid, and fusion PCR was carried out with nested primers P2 and P5.

### 2.3.4. Cloning of Cassettes that Consist of a Fluorescent Protein and a Nutritional Marker from *A. fumigatus*

Selectable markers from *A. fumigatus* were cloned into plasmids, transformed into *E. coli*, isolated, and then purified to serve as long-term cassette sources such that they would not have to be repeatedly amplified from *A. fumigatus* genomic DNA. Similarly, cassettes carrying fluorescent protein sequences along with selectable markers from *A. fumigatus* were created by fusion PCR and cloned into plasmids so that they would not have to be constructed repeatedly. If a fluorescent protein is already in a DNA fragment with a selectable marker, this reduces the number of fragments that must be fused whenever one is generating a gene targeting construct. Plasmids carrying *A. fumigatus* riboB (*AfriboB*) and pyroA (*AfpyroA*) were constructed by Tania Nayak, a former graduate student, before I joined the lab. Plasmids carrying GFP and *A. fumigatus* pyrG (*AfpyrG*) as well as mRFP and *AfpyrG* were kindly provided by the laboratory of Dr. Stephen Osmani. The GFP variant our lab uses is a plant-adapted GFP (Fernández-
Ábalos et al., 1998). Yi Xiong, another former graduate student in our lab, constructed a plasmid carrying mCherry (Shaner et al., 2004) and AfpyrG (see Table 2.3 for a list of plasmids commonly used in our lab).

Since I was planning to make a lot of fluorescent protein-tagged strains, I thought it would be worthwhile to subclone any cassettes carrying fluorescent protein sequences and A. fumigatus selectable markers that our lab did not already have. Fluorescent protein sequences as well as A. fumigatus nutritional markers were amplified from digested bacterial plasmids with primers that had tails that would allow for fusion PCR between the two individual pieces. Fusion PCR was carried out following the protocol described in 2.3.7 and products were purified using the QIAQuick PCR Purification Kit (Qiagen) and analyzed by agarose gel electrophoresis. In some cases, I had to gel purify the PCR product to ensure that only the band of the right size would be subcloned (see 2.3.8). PCR products were subcloned into the pCR II-Blunt-TOPO vector using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) following the manufacturer’s instructions. The resulting vector was transformed into chemically competent One Shot TOP10 E. coli cells (Invitrogen) according to the manufacturer’s instructions. 50 μg/ml kanamycin was used as a selective antibiotic for transformants. Resulting bacterial colonies were inoculated in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0; LB) plus 50 μg/ml kanamycin. Plasmid DNA was isolated using Qiagen’s QIAprep Spin Miniprep Kit and tested for the correct insert and its orientation by restriction digests and PCR. Using this TOPO PCR cloning kit, I cloned a cassette carrying mCherry and AfriboB. I also used a variation of this approach to remove a part of an adjacent upstream
gene’s coding sequence that was present in the *AfpyrG* cassette we had used previously and create a more compact GFP-*AfpyrG* cassette. The 3’ UTR of the adjacent upstream gene remained, however, to act as a processing and termination sequence for mCherry upon integration into the *A. nidulans* genome.

I was also responsible for creating cassettes carrying the fluorescent tag t-Sapphire (Zapata-Hommer and Griesbeck, 2003) and *A. fumigatus* selectable markers and testing this tag in *A. nidulans*. Using fusion PCR and the TOPO cloning method described above, I successfully subcloned t-Sapphire-*AfpyrG*, t-Sapphire-*AfriboB*, and t-Sapphire-*AfpyroA* cassettes. These cassettes were subsequently amplified and fused to histone H1 and transformed into *A. nidulans*. The resulting transformants exhibited fluorescence, albeit fairly weak, when excited with a 403 nm laser but showed some bleed-through in the GFP channel since it emits fluorescence at 511 nm (GFP emits at 509 nm). This bleed-through signal was eliminated by adjusting the minimum intensity cutoff of the GFP channel in Volocity software (Perkin-Elmer).

### 2.3.5. PCR Parameters for Amplifying Genomic DNA Flanks

To amplify each flanking genomic fragment, I mixed the following according to previously published protocols from our lab (Nayak et al., 2006; Oakley et al., 2012; Szewczyk et al., 2006): about 100 ng of *A. nidulans* genomic DNA, each primer to a final concentration of 300 nM, 0.5 μl (1.25 U) of AccuPrime *Pfx* DNA polymerase (Invitrogen), 5 μl of 10X AccuPrime *Pfx* Reaction mix (the 10X mix consists of 3 mM dNTPs, 10 mM MgSO4, and thermostable AccuPrime™ protein), and double-distilled water to a final volume of 50 μl. The reactions were set up on ice in 200 μl thin-wall
PCR tubes, mixed, and spun down before being placed in the thermocycler. Genomic flanks were amplified using the parameters listed in Table 2.4. The annealing temperature was 5°C below the primer T_m, and the extension time was one minute per kilobase pair (kb) of the expected amplified fragment. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), eluted in 50 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and analyzed by agarose gel electrophoresis to verify that the product was a single band of the expected size.

2.3.6. PCR Parameters for Amplifying Cassette

Cassettes were amplified from purified plasmid DNA. The plasmids were cut first to decrease amplification of plasmid DNA beyond the desired fragment and the restriction enzyme(s) were inactivated before PCR. PCR was carried out using the same protocol and thermocycler conditions as that used for amplifying genomic flanks (Table 2.4). The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen), eluted in 50 μl TE buffer, and analyzed by agarose gel electrophoresis.

2.3.7. PCR Parameters for Fusion PCR

Fusion PCR was set up using 0.5μl of each template DNA fragment and 300 nM final concentration of each nested primer. The remainder of the components varied depending on the DNA polymerase used. When I used AccuPrime Taq High Fidelity (Invitrogen), I added 0.2 μl (2.5U) of polymerase and 5 μl of 10X AccuPrime PCR Buffer I (the 10X buffer consists of 600 mM Tris-SO₄ (pH 8.9), 180 mM (NH₄)₂SO₄, 20 mM MgSO₄, 2 mM dGTP, 2 mM dATP, 2 mM dTTP, 2 mM dCTP, thermostable AccuPrime™ protein, 10% glycerol). When I used KOD Hot Start (Novagen), I added
1 μl (1U) of polymerase and 25 μl of a pre-made 2X Master Mix (the 2X mix consists of 2X buffer, 0.4 mM each dNTP, 3 mM MgSO4 and double-distilled water). Phusion Hot Start II (Finnzymes; ThermoFisher Scientific) is another DNA polymerase I used for fusion PCR, and when I did, I used 0.5 μl (1U) of polymerase, 1 μl of 10 mM dNTPs, and 10 μl of 5X Phusion HF buffer (the 5X buffer contains 7.5 mM MgCl2 and other unspecified components). Double-distilled water was then added to each reaction to a final volume of 50 μl for all polymerases. PCR conditions varied by polymerase and are given in Tables 2.5-2.7). PCR products were purified using the QIAQuick PCR Purification Kit (Qiagen), eluted in 50 μl TE, and analyzed by agarose gel electrophoresis.

2.3.8. Band Purification

In rare cases, the fusion PCR produced a mixture of amplification products of different sizes. If this occurred, the correct band was cut out and purified using Qiagen’s QIAQuick Gel Extraction Kit.

2.4. Transformation of A. nidulans

All transformations I performed used nkuAΔ strains and basically followed our published transformation protocol manuscripts (Oakley et al., 2012; Szewczyk et al., 2006), on both of which I was an author.

2.4.1. Strain Inoculation and Growth

1 x 10^8 spores of the strain to be transformed were inoculated into 20 ml YG (plus riboflavin, uridine, and uracil, if required for the strain) in a clean, sterile 50 ml Erlenmeyer flask. Spores were taken from a previously prepared spore suspension in
saline/tween (8.5 g NaCl, 1 ml Tween 80, 1 L double distilled water; S/T), spun down in a microcentrifuge tube, and washed in double-distilled water at least twice to remove any residual S/T before inoculating. The culture was incubated at 30°C in a gyratory shaking water bath at 120 rpm for 13-15 hours.

2.4.2. Protoplasting Procedure

A 2X protoplasting solution was made by dissolving 1280 mg VinoFlow FCE (Novo Nordisk) in 10 ml KCl, citric acid solution (8.2 g KCl, 2.1 g citric acid monohydrate, double-distilled water to 100 ml, pH adjusted to 5.8 with 1.1 M KOH). Once dissolved, the solution was filtered through a sterile 0.22 μm Milllex GV filter unit (Millipore). 8 ml of this solution was added to 8 ml YG [5 g/liter yeast extract, 20 g/liter D-glucose, supplemented with 400 μl/liter of a trace element solution (Vishniac and Santer, 1957) and riboflavin, uracil, and uridine, if needed] in a fresh, sterile 50 ml Erlenmeyer flask to give a 1X protoplasting solution. Hyphae from the inoculated culture were harvested by filtration through sterilized Miracloth (Calbiochem), washed with YG, and then added to the flask containing the protoplasting solution. The culture was then incubated at 30°C in a gyratory shaking water bath at 110 rpm. At 15-30 min intervals, a sterile transfer pipet was use to break up hyphal clumps and ensure that all hyphae had access to the protoplasting enzyme by pulling the protoplasting solution into the pipet and expelling it rapidly. The number of “squirts” was at least one per ml of culture volume and were vigorous enough to break up clumps, but not vigorous enough to cause a lot of foaming. Samples of the culture were examined under the microscope every 15-30
minutes to monitor protoplast formation. Protoplasting usually took between one and two hours.

2.4.3. Protoplast Purification

Protoplasts were purified by layering the protoplasting mixture onto a sterile 1.2 M sucrose cushion (41.08 g sucrose in double-distilled water to a final volume of 100 ml) in a 50 ml sterile disposable centrifuge tube (Sarstedt). The tube was then centrifuged at 2800 x g for 10 minutes at 4°C in a swinging bucket rotor and was allowed to coast down without braking. Protoplasts layered at the interface of the protoplasting solution and the top of the sucrose cushion. The protoplast layer was collected and transferred to a 15 ml sterile disposable centrifuge tube (Sarstedt) where it was mixed with an equal or greater volume of 0.6 M KCl (4.47 g KCl in double-distilled water to a final volume of 100 ml) and centrifuged at 2400 x g for 10 minutes at 25°C. The supernatant was carefully decanted and the protoplast pellet was resuspended in 2 ml of 0.6 M KCl. The protoplast suspension was then split into two sterile 1.5 ml microcentrifuge tubes. Protoplasts were washed three times by centrifugation at 2400 x g for 3 minutes, removal of the supernatant with a micropipettor, and resuspension in 1 ml 0.6 M KCl. After the third wash, each pellet was resuspended in 0.5 ml 0.6 M KCl, 50 mM CaCl₂ and then combined into one microcentrifuge tube before centrifugation at 2400 x g for 3 minutes. After the spin, the supernatant was removed, and the pellet was resuspended in a volume of 0.6 M KCl, 50 mM CaCl₂ (4.47 g KCl, 0.74 g CaCl₂·2H₂O, double-distilled water to a final volume of 100 ml), typically 40 μl for each transformation, and aliquoted into separate tubes for each transformation.
2.4.4. Transformation of Protoplasts

Typically, 4 μl of purified fusion PCR product was added to 40 μl of protoplasts. Tubes were vortexed 6-7 times in one-second bursts at maximum speed on a Vortex Genie. PEG solution [4.47 g KCl, 50 mM CaCl₂·2H₂O, 25 g polyethylene glycol (MW 3350), 0.802 ml 1 M Tris-HCl, 0.196 ml 1 M Tris base, double-distilled water to a final volume of 100 ml] was filtered through a syringe-driven 0.22 μm Millex GV filter, and 20 μl was added to the protoplasts. The protoplast suspension was vortexed with 6-7 one second bursts at maximum speed and incubated in an ice water bath for 25 minutes. Next, 400 μl of freshly filtered PEG solution was added and each transformation reaction mixed thoroughly with a 1000 μl micropipettor before incubation at room temperature for 30 minutes. 100 μl of each transformation mixture was then plated on selective media plates containing 0.6 M KCl and incubated at 37°C.

2.5. Isolation of A. nidulans Genomic DNA

Genomic DNA was isolated by one of three methods, all of which follow previously published protocols (Edgerton-Morgan and Oakley, 2012; Hervás-Aguilar et al., 2007; Lee and Taylor, 1990). The method I helped develop was a very quick, crude method in which a toothpick of spores was taken from a single colony and suspended in 50 μl TE buffer in a 1.5 ml screw cap microcentrifuge tube. 50 μl of 0.45-0.55 mm glass beads were added and the tube vortexed for two minutes at maximum speed. This method does not produce a large amount of genomic DNA, nor is it purified, but it is adequate for diagnostic PCR. I have used this method successfully even with strains that
do not conidiate well as well as heterokaryons since it does not require a large amount of spores.

2.6. Confirmation of Correct Gene Targeting

Even though the use of an nkuAΔ strain greatly improves the frequency of correct gene targeting, we still needed to verify, in our transformants, that a single copy of the transforming sequence had integrated at the correct locus and that there were no additional insertions of the transforming DNA elsewhere in the genome. For this reason, I always tested at least five different transformants by diagnostic PCR and Southern hybridization, and for growth at various temperatures. In addition, I examined fluorescence localization pattern(s) and signal intensity (if a fluorescently tagged gene).

2.6.1. Diagnostic PCR

As mentioned in Section 2.5, I isolated A. nidulans genomic DNA by one of three ways (Edgerton-Morgan and Oakley, 2012; Hervás-Aguilar et al., 2007; Lee and Taylor, 1990). Any of these methods gives enough DNA at adequate purity for diagnostic PCR. Primers for diagnostic PCR usually consisted of a pair of primers outside of the targeted region (primers P1 and P6 used for the initial amplification of A. nidulans flanking regions can be used since they are not part of the transforming fragment but are in the genome outside of the targeted region) as well as a pair consisting of one outside primer and one internal primer (usually inside the selectable marker), especially when outside primers would give a similar size PCR product whether the gene was correctly targeted or not (Fig. 2.4). PCR was performed using each primer at a final concentration of 300 nM, 0.5 μl of genomic DNA, and either Platinum Taq (Invitrogen) plus 10 μl of 10X PCR
buffer, 1.5 μl 50 mM MgCl₂, and 1 μl of 10 mM dNTPs; or AccuPrime Taq Hi Fidelity DNA polymerase (Invitrogen) plus 10 μl of 10X AccuPrime PCR Buffer II (the 10X buffer consists of 600 mM Tris-SO₄ (pH 8.9), 180 mM (NH₄)₂SO₄, 20 mM MgSO₄, 2 mM dGTP, 2 mM dATP, 2 mM dTTP, 2 mM dCTP, thermostable AccuPrime™ protein, 10% glycerol). In each case, double-distilled water was added to each reaction to a total volume of 50 μl. The PCR parameters for Platinum Taq were the same as for Pfx (Table 2.4), and the parameters for diagnostic PCR with AccuPrime Taq Hi Fidelity (generally used only for large diagnostic amplifications and if amplifications with other enzymes failed) are listed in Table 2.5. One Taq Hot Start Quick-Load DNA polymerase (New England Biolabs) was used for diagnostic PCR of DNA isolated from spores using the method published in Edgerton-Morgan and Oakley (2012). In this case, 2 μl of the solution of vortexed spores, TE buffer, and glass beads was immediately taken and added to 18 μl TE. 2 μl of this dilution was immediately added to the PCR reaction tube which included 12.5 μl One Taq 2X Master Mix (the 2X mix consists of 40 mM Tris-HCl, 44 mM KCl, 44 mM NH₄Cl, 3.6 mM MgCl₂, 10% glycerol, 0.1% Tween 20, 0.12% IGE Palmer CA-630, 0.4 mM dNTPs, 50 units/ml OneTaq DNA Polymerase), each primer to a final concentration of 300 nM and double-distilled water to a final volume of 25 μl. The PCR program for One Taq is given in Table 2.8.

2.6.2. Growth on Plates

Transformants that were verified to have a correct insertion were further tested for growth on complete and minimal media at various temperatures from 20°C-42°C. This tests whether all transformants are phenotypically the same and this can, in turn, indicate
whether any defect in growth or conidiation is due to the insertion itself or to a random mutation or integration. In the case of gene tagging, if the transformed strain grows as well as a wild-type control at all temperatures tested, we deduce that the gene fusion is fully functional.

2.6.3. Fluorescence Localization Patterns

Transformants carrying a fluorescent gene fusion were checked under the microscope for their fluorescence localization pattern. Several transformants were examined in case one or more might have a mutation in the fluorescent protein sequence that causes reduced or absent fluorescence. One also becomes more confident that each transformant is correct if various transformants give the same localization pattern.

2.6.4. Southern Hybridizations

For Southern hybridizations, *A. nidulans* genomic DNA was isolated using one of two published procedures [(Lee and Taylor, 1990); (Oakley et al., 1987b)]. Genomic DNA was then digested with an appropriate restriction enzyme. The hybridization was carried out using a dried gel method (Oakley et al., 1987b). Radioactively labeled full-length transforming DNA fragments were used as probes and were labeled using the Prime-It II Random Primer Labeling Kit (Agilent Technologies) and purified using spin-column chromatography through Sephadex G-50 (Maniatis et al., 1982).

2.7. Microscopy

For imaging, spores were inoculated in liquid minimal medium plus appropriate nutritional supplements in four- or eight- chamber cover glasses (Lab-Tek; Thermo Fisher Scientific). I used three systems for imaging. Two were inverted Olympus IX71
microscopes equipped with Prior shutters, filter wheels, and Z-axis drives with mercury light sources. One was equipped with an ORCA ER camera (Hamamatsu Photonics) and the other with an ORCA ERAG camera (Hamamatsu Photonics) and an environmental chamber to maintain stable temperatures. With both of these imaging systems I used GFP/DsRed2X2M-B dual-band Sedat filter sets (Semrock) with a 459-481 nm bandpass excitation filter for GFP, a 546-566 nm excitation filter for mCherry and mRFP, a dual reflection band dichroic (457-480 nm and 542-565 nm reflection bands, 500-529 and 584-679 nm transmission bands), a 499-529 nm emission filter for GFP, and a 580-654 nm emission filter for mCherry/mRFP. Images were acquired with a 60x 1.42 NA planapochromatic objective (Olympus) using Slidebook (Intelligent Imaging Innovations) or Volocity software (PerkinElmer). The third system was an UltraView VoX spinning disk confocal system (PerkinElmer) mounted on an Olympus IX71 inverted microscope. It was equipped with a controlled temperature chamber and a software-controlled piezoelectric stage for rapid Z-axis movement. Images were collected using a 60x 1.42 NA panapochromatic objective (Olympus) (in some cases with a 1.6x Optovar) and an ORCA ERAG camera (Hamamatsu Photonics). Solid state 405-, 488-, and 561 nm lasers were used for excitation. Fluorochrome-specific emission filters were used to prevent emission bleed through between fluorochromes. This system was controlled by Volocity software (PerkinElmer). All three systems were calibrated with a stage micrometer.

For time-lapse multiple channel imaging of live cells, Z-series stacks were collected at each time point specified, and maximum intensity projections from all time points were combined to generate movies.
2.8. Crossing of Strains

*mip4*D159 strains and strains expressing multiple tagged proteins or carrying various nutritional markers were generated through crosses. I first stabbed the two strains close to each other (approximately 5mm apart) on complete media that would support growth of each of the two strains. The plate was incubated at 37°C for two days. By this time, hyphae from each strain would be growing radially and towards each other in the small space between the two stabs. Small pieces of agar containing the hyphae from both strains were cut out and transferred to MM supplemented with nutrients for which both strains are deficient. These plates were taped with masking tape to create slightly anaerobic conditions and incubated at 37°C for at least two weeks until mature cleistothecia were formed. Individual cleistothecia (at least eight) were then picked with sterile toothpicks and rolled on solid medium to remove excess adhering conidia. Rolled cleistothecia were placed in 1 ml S/T in either a sterile 1 dram vial or 1.5 ml microcentrifuge tube and crushed (to release ascospores). In instances in which the parents in the cross carry complementary nutritional markers, ascospores from each suspension were spread on media that would allow growth of spores from hybrid cleistothecia but not selfed cleistothecia. In this case, growth of the ascospores into visible colonies or hyphal masses indicates that the cleistothecium is hybrid. If the parents of the cross carry different conidial color markers, ascospores from hybrid cleistothecia grow into colonies of each color of the parent strains, whereas if the cleistothecium is not hybrid, the colonies will all be the same color. This test can be used to identify hybrid cleistothecia when the parents do not carry complementary nutritional
markers or as an additional verification that cleistothecia are hybrid if the parents do carry complementary nutritional markers. Once hybrid cleistothecia were identified, dilutions of these ascospore suspensions were plated and the resulting progeny tested for the desired alleles.

2.9. Storage of Strains

Strains were stored by harvesting their conidia in approximately 1.5 ml of sterile 7.5 % non-fat milk solution and dividing the suspension into two sterile, dry one-dram vials three quarters full of sterile silica gel (grade 40, mesh size 6-12; Thermo Fisher Scientific). Approximately 750 μl of the suspension was added to each silica vial. Conidia adsorbed onto silica gel can remain viable for many years and they can be revived easily by sprinkling a few granules of the silica gel with adsorbed conidia onto complete agar media.
<table>
<thead>
<tr>
<th>Strain number</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGSC4</td>
<td>Glasgow wild-type</td>
</tr>
<tr>
<td>LO1124</td>
<td>pyrA4; yA2; mipAD159</td>
</tr>
<tr>
<td>LO1316</td>
<td>wA3; pyrG::pyrA; md2A-GFP-AFpyrG; pyrA4</td>
</tr>
<tr>
<td>LO1362 (TN02A7)</td>
<td>pyrG89; pyrA4; nkuA::argB; riboB2</td>
</tr>
<tr>
<td>LO1390</td>
<td>pyrG89?; md2A-GFP-AFpyrG; hhoA-mRFP-AFpyrG; pabaA1; yA2</td>
</tr>
<tr>
<td>LO1438</td>
<td>pyrG89; nimE-GFP-AFpyrG; hhoA-mRFP-AFpyrG pyrA4; nkuA::argB; riboB2; yA2</td>
</tr>
<tr>
<td>LO1439</td>
<td>pyrG89; nimE-GFP-AFpyrG; hhoA-mRFP-AFpyrG; pyrA4; nkuA::argB; mipAD159</td>
</tr>
<tr>
<td>LO1479</td>
<td>wA3; pyrG89?; mpsA-GFP-AFpyrG; hhoA-mRFP-AFpyrG; pabaA1</td>
</tr>
<tr>
<td>LO1501</td>
<td>pyrG89; pabaA1; pyrA4; argB2?; nkuA::argB; riboB2; fwA1</td>
</tr>
<tr>
<td>LO1516</td>
<td>pyrG89; pyrA4; nkuA::argB; riboB2; hhoA-mRFP-AFrioB</td>
</tr>
<tr>
<td>LO1533</td>
<td>pyrG89?; md2A-GFP-AFpyrG; hhoA-mRFP-AFpyrG; pabaA1; mipAD159; yA2</td>
</tr>
<tr>
<td>LO1711</td>
<td>pyrG89; mipA-mCherry-AFpyrG; pabaA1; pyrA4; hhoA-GFP-AFpyroA; nkuA::argB; riboB2; fwA1</td>
</tr>
<tr>
<td>LO1801/1802</td>
<td>pyrG89; cdhA-GFP-AFpyrG; pabaA1; pyrA4; argB2?; nkuA::argB; riboB2; fwA1</td>
</tr>
<tr>
<td>LO1803/1804</td>
<td>pyrG89; cdhA::AFpyrG; pabaA1; pyrA4; argB2?; nkuA::argB; riboB2; fwA1</td>
</tr>
<tr>
<td>LO1805/1806</td>
<td>pyrG89; cdhA-GFP-AFpyrG; pyrA4; nkuA::argB; riboB2?; hhoA-mRFP-AFrioB</td>
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<tr>
<td>LO2019</td>
<td>pyrG89; cdhA::AFpyrG; pyrA4; nkuA::argB; riboB2; hhoA-mRFP-AFrioB</td>
</tr>
<tr>
<td>LO2073</td>
<td>pyrG89; nimE-GFP-AFpyrG; pyrA4; hhoA-mCherry-AFpyroA; nkuA::argB; riboB2</td>
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<tr>
<td>LO2415</td>
<td>pyrG89; cdhA::AFpyrG; pyrA4; nkuA::argB; riboB2</td>
</tr>
<tr>
<td>LO2585</td>
<td>pyrG89; sldB-GFP-AFpyrG; pyrA4; hhoA-mCherry-pyroA; nkuA::argB; riboB2?; mpid159; fwA1</td>
</tr>
<tr>
<td>LO2834</td>
<td>wA3; pyrG89; hhoA-t-Sapphire-AFpyrG; sepK-GFP-AFpyrG; pabaA1?; pyrA4; ndc80-mCherry-AFpyroA; nkuA::argB; nirA14?; sE15?; riboB2; fwA1?; chaA1?</td>
</tr>
<tr>
<td>LO2869</td>
<td>pyrG89; nimE-GFP-AFpyrG; cdhA::AFpyrG; pabaA1; pyrA4; nkuA::argB; riboB2; fwA1</td>
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<tr>
<td>LO3105</td>
<td>wA3?; pyrG89; mpsA-GFP-AFpyrG; hhoA-t-Sapphire-AFpyrG; pabaA1; pyrA4; ndc80-mCherry-AFpyroA; nkuA::argB; nirA14?; sE15?; riboB2?; fwA1?; chaA1</td>
</tr>
</tbody>
</table>

Table 2.1: List of strains used in this study
Table 2.1 continued

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genetic modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>LO3317</td>
<td>pyrG89; nimE-GFP-AFpyrG; pabaA1; pyroA4; riboB2; hhoA-mRFP-AFriboB</td>
</tr>
<tr>
<td>LO3406</td>
<td>pyrG89; sldB-GFP-AFpyrG; pyroA4; hhoA-mCherry-AFpyroA; nkuA::argB</td>
</tr>
<tr>
<td>LO4223</td>
<td>pyrG89; mpsA-GFP-AFpyrG; hhoA-mRFP-AFpyrG; pabaA1; pyroA4; nkuAΔ; riboB2; mipAD159</td>
</tr>
<tr>
<td>LO4580</td>
<td>pyrG89; sldA-GFP-AFpyrG; pyroA4; nkuA::argB; riboB2; hhoA-mRFP-AFriboB</td>
</tr>
<tr>
<td>LO4676</td>
<td>pyrG89; sldA-GFP-AFpyrG; pabaA1; pyroA4; nkuAΔ; riboB2; hhoA-mRFP-AFriboB; mipAD159; fwA1</td>
</tr>
<tr>
<td>LO5242</td>
<td>pyrG89; sldA-GFP-AFpyrG; pabaA1; pyroA4; hhoA-t-Sapphire-AFpyroA; nkuAΔ; riboB2; nimX-mCherry-AFriboB; mipAD159</td>
</tr>
<tr>
<td>LO5442-5444</td>
<td>pyrG89; nimE-GFP-AFpyrG; cdhA::AFpyrG; pabaA1; pyroA4; nkuAΔ; riboB2; hhoA-mRFP-AFriboB; mipAD159; fwA1</td>
</tr>
<tr>
<td>LO5538/5539</td>
<td>pyrG89; pyroA4; AFXpyroA-GFP-cdc20; nkuA::argB; riboB2; hhoA-mRFP-AFriboB</td>
</tr>
<tr>
<td>LO5570</td>
<td>pyrG89; cdhA::AFpyrG; pabaA1; pyroA4; nkuAΔ; riboB2; hhoA-mRFP-AFriboB; mipAD159; fwA1</td>
</tr>
<tr>
<td>LO5571</td>
<td>pyrG89; cdhA::AFpyrG; pabaA1; pyroA4; nkuAΔ; riboB2; mipAD159; fwA1</td>
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<tr>
<td>LO5572</td>
<td>pyrG89; nimE-GFP-AFpyrG; cdhA::AFpyrG; pabaA1; pyroA4; nkuAΔ; riboB2; mipAD159; fwA1</td>
</tr>
<tr>
<td>LO5573</td>
<td>pyrG89; nimE-GFP-AFpyrG; cdhA::AFpyrG; pabaA1; pyroA4; nkuAΔ; riboB2; mipAD159; fwA1</td>
</tr>
<tr>
<td>LO5690</td>
<td>pyroA4; AFXpyroA-GFP-cdc20; riboB2; hhoA-mRFP-AFriboB; mipAD159</td>
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<tr>
<td>LO5851</td>
<td>wA3?; pyrG89; mpsA-GFP-AFpyrG; pabaA1; pyroA4; nirA14; se15?; riboB2; mipA-mCherry-AFriboB; fwA1; chaA1</td>
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<tr>
<td>LO5908</td>
<td>pyrG89; sldA-GFP-AFpyrG; pyroA4; ndc80-mCherry-AFpyroA; riboB2; fwA1</td>
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<tr>
<td>LO6256</td>
<td>pyrG89; cdhA-GFP-AFpyrG; nup49-mCherry-AFpyrG; pabaA1; pyroA4; argB2; nirA14; riboB2; nimX-mCherry-AFriboB; mipAD159; fwA1; chaA1</td>
</tr>
<tr>
<td>LO6518</td>
<td>pyrG89; nup49-mCherry-AFpyrG; pabaA1; pyroA4; AFXpyroA-GFP-Ancdc20; riboB2; fwA1; chaA1</td>
</tr>
<tr>
<td>R153</td>
<td>wA3; pyroA4</td>
</tr>
</tbody>
</table>

continued
Table 2.1 continued

Each strain created is given a unique strain number. Genotypes of these strains are given in the right column. Question marks indicate alleles that were present in one of the parents of a cross but have not been tested in the progeny. All strains also carry veA1.
<table>
<thead>
<tr>
<th>Mutant allele</th>
<th>Supplemented by</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pyrG89</em></td>
<td>1 mg/ml uracil, 2.442 mg/ml uridine</td>
</tr>
<tr>
<td><em>riboB2</em></td>
<td>2.5 μg/ml riboflavin</td>
</tr>
<tr>
<td><em>pyroA4</em></td>
<td>0.5 μg/ml pyridoxine</td>
</tr>
<tr>
<td><em>pabaA1</em></td>
<td>1 μg/ml para-aminobenzoic acid</td>
</tr>
</tbody>
</table>

Table 2.2: Nutritional supplements for mutant alleles

These supplements need to be added to MM if the strain contains any of these mutant alleles. The nutrients in YAG fully supplement *pyroA4* and *pabaA1* but not *pyrG89* or *riboB2*. 
In the example shown, the *hhoA* gene (which encodes histone H1) is fused to

fluorescent protein sequence

Figure 2.1: Schematic for C-terminally tagging a protein with a fluorescent protein sequence
Figure 2.1 continued

monomeric red fluorescent protein (mRFP) sequence. Panel (a) depicts how two flanking sequences are amplified from genomic DNA: 1) ~1000 bp of sequence upstream of the stop codon of *hhoA* is amplified using primers P1 and P3, and 2) ~1000 bp of sequence of downstream of the stop codon of *hhoA* (3’ UTR) is amplified using primers P4 and P6. Primers P3 and P4 are designed with tails consisting of sequences that overlap with the mRFP-*AfpyrG* cassette [P3 with a tail corresponding to the GA linker sequence (Yang et al., 2004) at the 5’ end of mRFP and P4 with a tail corresponding to the opposite end of the end of the cassette]. Panel (b) depicts the fusion PCR reaction in which the pre-amplified mRFP-*AfpyrG* cassette and the two flanking gene regions amplified in panel (a) are mixed together and amplified with nested primers P2 and P5. Panel (c) shows how, upon transformation, integration of this fragment by homologous recombination results in a *hhoA*-GFP fusion gene under control of the endogenous *hhoA* promoter. Modified from (Szewczyk et al., 2006).
The strategy for fusing GFP to the N-terminus of \textit{cdc20} was similar to that of Wong et al. (2008), keeping the fusion gene under the control of the normal \textit{cdc20} promoter. Three fragments from the region of the \textit{cdc20} gene were amplified from \textit{A. nidulans} genomic
Figure 2.2 continued

DNA: 1) the 5’ UTR from -1500 to -521 relative to the start codon of *cdc20* using primers P1 and P3, 2) the *cdc20* promoter region (-520 to -1) using primers P6 and P7, and 3) the *cdc20* coding sequence from +1 to +1025 using primers P10 and P12. *AfpyroA* was amplified from plasmid pTN1 (Nayak et al., 2006) with primers P4 and P5. GFP was amplified from plasmid pFN03 using primers P8 and P9 such that its C-terminus encoded the GA linker (Yang et al., 2004). Primers P3, P5, P7, P9, and P10 are designed with tails of overlapping sequence with adjacent fragments (P3 with a tail corresponding to the 3’ end of the *AfpyroA* cassette, P5 with the 5’ end of the *cdc20* promoter, P7 with the 5’ end of GFP, P9 and P10 with the GA linker sequence), to allow fusion of all of these fragments in a single PCR reaction. The fusion PCR reaction used nested primers P2 and P11. Integration of this fragment by homologous recombination at the genomic *cdc20* locus results in a GFP-*cdc20* fusion gene under control of the endogenous *cdc20* promoter.
Two flanking regions of the gene to be replaced are amplified from genomic DNA. P1 and P3 amplify ~1000 bp upstream of the target gene’s start codon (5’ UTR). P4 and P6 amplify ~1000 bp downstream of the target gene’s stop codon (3’ UTR). P3 and P4 have tails with identical sequences to the ends of the selectable marker cassette (in this case AfpyrG), which is amplified from a plasmid. The two genomic flanks are mixed with the selectable marker and fused together with nested primers P2 and P5. Upon transformation, integration of this fragment by homologous recombination replaces the target gene with AfpyrG.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Carries</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAH1</td>
<td>GFP-\textit{AfpyrA}</td>
<td>\textit{AfpyrA} has a 5’ pyrGF3 primer tail and a 3’ pyrGR primer tail</td>
</tr>
<tr>
<td>pFN03</td>
<td>GFP-\textit{AfpyrG}</td>
<td>Contains part (221 bp) of the coding sequence of the adjacent gene as part of \textit{AfpyrG}'s 5’ UTR; Yang et al. (2004)</td>
</tr>
<tr>
<td>pHE4</td>
<td>t-Sapphire-\textit{AfpyrG}</td>
<td></td>
</tr>
<tr>
<td>pHE5</td>
<td>t-Sapphire-\textit{AfriboB}</td>
<td></td>
</tr>
<tr>
<td>pHE6</td>
<td>t-Sapphire-\textit{AfpyrA}</td>
<td></td>
</tr>
<tr>
<td>pHE10</td>
<td>GFP-\textit{AfpyrG}</td>
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</tr>
<tr>
<td>pHE11</td>
<td>mCherry-\textit{AfriboB}</td>
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<tr>
<td>pHL84</td>
<td>GFP-\textit{AfpyrA}</td>
<td>Contains 305 bp of \textit{AfpyrG}'s 5’ UTR (which includes part of the coding sequence of its adjacent upstream gene) as a tail; Liu et al. (2009)</td>
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<tr>
<td>pHL85</td>
<td>mCherry-\textit{AfpyrA}</td>
<td>\textit{AfpyrA} has 84 bp of \textit{AfpyrG}'s 5’ UTR as a tail; Liu et al. (2009)</td>
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<tr>
<td>pLO87</td>
<td>\textit{AfriboB}</td>
<td>\textit{AfriboB} has a 5’ pyrGF2 primer tail and a 3’ pyrGR primer tail</td>
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<tr>
<td>pLO88</td>
<td>\textit{AfriboB}</td>
<td>\textit{AfriboB} has a 5’ pyrGF3 primer tail and a 3’ pyrGR primer tail</td>
</tr>
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<td>pTN1</td>
<td>\textit{AfpyrA}</td>
<td>Nayak et al. (2006)</td>
</tr>
<tr>
<td>pTN2</td>
<td>\textit{AfriboB}</td>
<td>Nayak et al. (2006)</td>
</tr>
<tr>
<td>pYX1</td>
<td>mCherry-\textit{AfpyrG}</td>
<td></td>
</tr>
<tr>
<td>pXDRFP4</td>
<td>mRFP-\textit{AfpyrG}</td>
<td>Contains part (221 bp) of the coding sequence of the adjacent gene as part of \textit{AfpyrG}'s 5’ UTR; Szewczyk et al. (2006)</td>
</tr>
</tbody>
</table>

Table 2.3: Common plasmids our lab uses containing various fluorescent proteins and selectable markers from \textit{A. fumigatus}

Cassettes containing the fluorescent proteins and markers are amplified from digested plasmid DNA to be used in fusion PCR reactions. Certain selectable markers have continued
various tails corresponding to regions of *AfpyrG*. This allows additional fusion PCR products using AfriboB or AfpyroA as a selectable marker to be made quickly using the genomic DNA flanks already made for fusion to *AfpyrG*. 
<table>
<thead>
<tr>
<th>Cycle number</th>
<th>Denature</th>
<th>Ramp</th>
<th>Anneal</th>
<th>Ramp</th>
<th>Extend</th>
<th>Ramp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C 2 min.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-31</td>
<td>94°C 20 sec.</td>
<td>To 70°C at max rate; 70°C 1 sec.; ramp to annealing temperature at 0.1°C/sec.</td>
<td>55°C(^a) 30 sec.</td>
<td>To 68°C at 0.2°C/sec.</td>
<td>68°C 1 min.(^b)</td>
<td>At max rate to 94°C</td>
</tr>
</tbody>
</table>

**Final Extension**

68°C 5 min.

**Table 2.4: PCR parameters for amplifying genomic DNA flanks and cassettes with AccuPrime *Pfx* and for diagnostic PCR with Platinum *Taq***

Ramping rates (also known as slopes) are important since primers will anneal and extend while the thermocycler is ramping down to and up from the annealing temperature. Since thermocyclers have different maximal ramping rates, programming specific ramp rates allows essentially identical results to be obtained with different thermocyclers. \(^a\)The annealing temperature is 5°C below the primer T\(_m\). In this example, the primer T\(_m\) is 60°C. \(^b\)The extension time depends on the size of the expected continued
product and is 1 min./kb. Flanks are usually approximately 1 kb long. Modified from Szewczyk et al. (2006) and Oakley et al. (2012). This same protocol is used for diagnostic PCR with Platinum Taq.
<table>
<thead>
<tr>
<th>Cycle number</th>
<th>Denature</th>
<th>Ramp</th>
<th>Anneal</th>
<th>Ramp</th>
<th>Extend</th>
<th>Ramp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C 2 min.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-11</td>
<td>94°C 20 sec.</td>
<td>To 70°C at max rate; 70°C 1 sec.; ramp to annealing temperature at 0.1°C/sec</td>
<td>55°C&lt;sup&gt;a&lt;/sup&gt; 30 sec.</td>
<td>To 68°C at 0.2°C/sec.</td>
<td>68°C 5 min.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>At max rate to 94°C</td>
</tr>
<tr>
<td>12-16</td>
<td>94°C 20 sec.</td>
<td>To 70°C at max rate; 70°C 1 sec.; ramp to annealing temperature at 0.1°C/sec</td>
<td>55°C&lt;sup&gt;a&lt;/sup&gt; 30 sec.</td>
<td>To 68°C at 0.2°C/sec.</td>
<td>68°C 5 min.</td>
<td>At max rate to 94°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+ 5 sec. each additional cycle&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>17-26</td>
<td>94°C 20 sec.</td>
<td>To 70°C at max rate; 70°C 1 sec.; ramp to annealing temperature at 0.1°C/sec</td>
<td>55°C&lt;sup&gt;a&lt;/sup&gt; 30 sec.</td>
<td>To 68°C at 0.2°C/sec.</td>
<td>68°C 5 min.</td>
<td>At max rate to 94°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+ 20 sec. each additional cycle&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.5: PCR parameters using AccuPrime Taq Hi Fidelity
Table 2.5 continued

<table>
<thead>
<tr>
<th>Final Extension</th>
<th>68°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>last</td>
</tr>
<tr>
<td></td>
<td>extension</td>
</tr>
<tr>
<td></td>
<td>time + 1</td>
</tr>
<tr>
<td></td>
<td>min.</td>
</tr>
</tbody>
</table>

This protocol was used for fusion PCR to create long transforming fragments. With AccuPrime Taq Hi Fidelity it is important to increase the extension time in cycles 12-26 since the polymerase loses activity. aThe annealing temperature is 5°C below the primer Tm. In this example, the primer Tm is 60°C. bThe extension time depends on the size of the expected product and is 1 min./kb. Fusion PCR products are typically 4-5 kb but can be larger depending on the construct design. cIncrease the extension time for each subsequent cycle by 5 seconds. In this example, cycle 12’s extension time is 5 minutes and cycle 16’s is 5 minutes 20 seconds. dIncrease the extension time for each subsequent cycle by 20 seconds. In this example, cycle 17’s extension time is 5 minutes 20 seconds and cycle 26’s is 8 minutes 20 seconds. Modified from Szewczyk et al. (2006).
<table>
<thead>
<tr>
<th>Cycle number</th>
<th>Denature</th>
<th>Ramp</th>
<th>Anneal</th>
<th>Ramp</th>
<th>Extend</th>
<th>Ramp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C 2 min.</td>
<td></td>
<td>62°C&lt;sup&gt;a&lt;/sup&gt; 10 sec.</td>
<td>To 68°C at 0.2°C/sec.</td>
<td>68°C 2 min. 30 sec.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>At max rate to 94°C</td>
</tr>
<tr>
<td>2-26</td>
<td>94°C 10 sec.</td>
<td>To 75°C at max rate; 75°C 1 sec.; ramp to annealing temperature at 0.1°C/sec.</td>
<td>62°C&lt;sup&gt;a&lt;/sup&gt; 10 sec.</td>
<td>To 68°C at 0.2°C/sec.</td>
<td>68°C 2 min. 30 sec.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>At max rate to 94°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>68°C last extension time + 1 min.</td>
</tr>
</tbody>
</table>

Table 2.6: Fusion PCR parameters using KOD Hot Start

KOD is more processive than AccuPrime *Taq* Hi Fidelity, allowing a shorter extension time. It is also more thermo-stable and extension times do not need to be increased in later rounds. <sup>a</sup>The annealing temperature is 2°C above the primer T<sub>m</sub>. In this example, the primer T<sub>m</sub> is 60°C. <sup>b</sup>The extension time depends on the size of the expected product and is 30 sec./kb. Modified from Oakley et al. (2012).
<table>
<thead>
<tr>
<th>Cycle number</th>
<th>Denature</th>
<th>Ramp</th>
<th>Anneal</th>
<th>Ramp</th>
<th>Extend</th>
<th>Ramp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98°C 30 sec.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-26</td>
<td>98°C 10 sec.</td>
<td>To 75°C at max rate; 75°C 1 sec.; ramp to annealing temperature at 0.1°C/sec.</td>
<td>63°C\textsuperscript{a} 10 sec.</td>
<td>To 72°C at 0.2°C/sec.</td>
<td>72°C 2 min. 30 sec.\textsuperscript{b}</td>
<td>At max rate to 94°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>72°C 5 min.</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.7: Fusion PCR parameters using Phusion Hot Start II

Like KOD Hot Start, Phusion Hot Start II is a very rapid, processive, thermo-stable DNA polymerase. \textsuperscript{a}The annealing temperature is 3°C above the primer T\textsubscript{m}. In this example, the primer T\textsubscript{m} is 60°C. \textsuperscript{b}The extension time depends on the size of the expected product and is 30 sec./kb. Modified from (Oakley et al., 2012).
Genomic DNA is isolated from putative transformants as described in the text.

Diagnostic PCR is then performed using primers outside of the targeted region (P1 and P6). In cases in which the selectable marker and target gene are about the same size, it is necessary to also perform diagnostic PCR with an internal primer inside the selectable marker. Only a correct transformant in which the target gene has been replaced by a selectable marker will amplify with the internal primer. The gel depiction on the right shows an example of expected diagnostic PCR results if this is the case. Lane 1 is

Figure 2.4: Diagnostic PCR to verify correct transformants

continued
Figure 2.4 continued

bacteriophage λ digested with HindIII and serves as a size marker. Lane 2 shows a hypothetical result of a PCR reaction done with outside primers P1 (green arrow) and P6 (gray arrow) and genomic DNA isolated from a putative transformant carrying a gene replacement. In this example, the gene that is replaced is the same size as the selectable marker it is being replaced with. Therefore, P1 and P6 will amplify the same size product with genomic DNA of a WT control (lane 2) as with a correct transformant. Lane 3 shows a hypothetical PCR product with P1 and an internal reverse primer located inside the selectable marker (blue arrow). This amplification will only occur with genomic DNA isolated from correct transformants and not with WT genomic DNA or with genomic DNA isolated from incorrect transformants (lane 4).
<table>
<thead>
<tr>
<th>Cycle number</th>
<th>Denature</th>
<th>Ramp</th>
<th>Anneal</th>
<th>Ramp</th>
<th>Extend</th>
<th>Ramp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C 30 sec.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-31</td>
<td>94°C 20 sec.</td>
<td>To 70°C at max rate; 70°C 1 sec.; ramp to annealing temperature at 0.1°C/sec.</td>
<td>55°C&lt;sup&gt;a&lt;/sup&gt; 30 sec.</td>
<td>To 68°C at 0.2°C/sec.</td>
<td>68°C 5 min.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>At max rate to 94°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>55°C&lt;sup&gt;a&lt;/sup&gt; 30 sec.</td>
<td>To 68°C at 0.2°C/sec.</td>
<td>68°C 5 min.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>At max rate to 94°C</td>
</tr>
<tr>
<td>Final Extension</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>68°C 5 min.</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.8: Verification PCR parameters using OneTaq

<sup>a</sup>The annealing temperature is 5°C below the primer Tm. In this example, the primer Tm is 60°C. <sup>b</sup>The extension time depends on the expected size of the PCR product and is 1 min./kb. Modified from Edgerton-Morgan and Oakley (2012).
Chapter 3: Characterization of the Spindle Assembly Checkpoint in *A. nidulans*

3.1. Introduction

The spindle assembly checkpoint (SAC) has a well-established role in inhibiting anaphase until all kinetochores (KTs) are attached in a bipolar manner to spindle microtubules. How exactly it accomplishes this is not fully understood. It has been demonstrated biochemically that a group of SAC proteins, including Mad2, Cdc20, Bub3, and BubR1, form a mitotic checkpoint complex (MCC) at KTs (Hardwick et al., 2000; Musacchio and Salmon, 2007; Sudakin et al., 2001) activating the SAC and keeping the anaphase promoting complex/cyclosome (APC/C) inactive until the checkpoint is satisfied. Only then will the MCC dissociate from KTs, thereby inactivating the SAC and activating the APC/C. But what keeps the MCC from forming prematurely? It is known that MCC components bind in a temporal order based on biochemical experiments and immunofluorescence (Fraschini et al., 2001; Hardwick et al., 2000; Kulukian et al., 2009; Musacchio and Salmon, 2007; Vigneron et al., 2004), but there has been surprisingly little live imaging of the MCC components, so it is unclear when and how they come to form a complex at KTs. In addition, there is a scarcity of information on the assembly and disassembly of the MCC in filamentous fungi.
In addition, I have recently found that γ-tubulin has an important role in inactivating APC/C^{Cdh1} at the G1/S boundary [(Edgerton-Morgan and Oakley, 2012); see also Chapter 4]. When strains carrying a recessive, cold-sensitive γ-tubulin mutant allele, mip4D159, are grown at a restrictive temperature, a subset of nuclei within a common cytoplasm fail to accumulate cyclin B, cyclin-dependent kinase 1 (Cdk1), and the \textit{A. nidulans} cdc14 phosphatase (Ancdc14) due to a nuclear autonomous failure of inactivation of APC/C^{CdHA} (CdHA is the \textit{A. nidulans} Cdh1 ortholog) [(Edgerton-Morgan and Oakley, 2012; Nayak et al., 2010); see also Chapter 4]. The percentage of such nuclei increases over time, and these nuclei are permanently taken out of the cell cycle. The SAC is apparently not activated in such nuclei in mitosis because the nuclei are not arrested in mitosis. Note, however, that these nuclei can be “reset” (i.e APC/C^{CdHA} can be inactivated) by long-term treatment with the antimicrotubule drug benomyl, which activates the SAC (Hoyt et al., 1991; Li and Murray, 1991). I was, therefore, curious if this failure of inactivation of APC/C^{CdHA} was due to a failure of accumulation or mis-localization of one or more SAC proteins.

There were two goals underlying the work presented in this chapter. The first was to create fluorescent fusion proteins of SAC components and perform time-lapse imaging of them in wild-type cells to determine their behavior through the cell cycle, and, in particular, to observe the assembly and disassembly of the MCC. The second was to determine if the behavior of the SAC components are altered in an informative way by \textit{mip4D159}.
3.2. Results

3.2.1. Identification of SAC Gene Homologs in A. nidulans

As mentioned in Chapter 2, the *A. nidulans* genome has been sequenced (Galagan et al., 2005; Wortman et al., 2009) and is generally well annotated. The *A. nidulans* genome sequence and gene annotation is available through the *Aspergillus* Genome Database (AspGD; [www.aspgd.org](http://www.aspgd.org)), the *Aspergillus* Comparative Sequencing Project, Broad Institute of Harvard and MIT ([www.broadinstitute.org/](http://www.broadinstitute.org/)), and the Central *Aspergillus* Data Repository (CADRE; [www.cadregenomes.org.uk/index.html](http://www.cadregenomes.org.uk/index.html)). I ran BLASTP searches using the published amino acid sequences of known SAC components from other organisms and was able to identify *A. nidulans* genes that encoded SAC component homologs. The lower the E-value between the two amino acid sequences, the greater the possibility that these two sequences are indeed homologs. The results for the SAC components I chose to examine (Mad2, Mps1, Bub3, BubR1, and Cdc20) are given in Table 3.1. A BLASTP search with *S. cerevisiae* Mad2 revealed the predicted protein product of AN2511 to be a strong homolog (E-value of 2e^{-55}). Orthologs from *S. pombe* and *H. sapiens* showed similar identity to the predicted protein product of AN2511.

AN2927 was identified as a homolog of Mps1 in that low E-values were obtained in comparing its sequence to the Mps1 sequences from *S. cerevisiae*, *S. pombe*, and *H. sapiens* (E-values ranged from 6e^{-59} to 4e^{-80}). The predicted protein product of AN2439 was found to be most similar to the Bub3 protein sequence of *H. sapiens* (E-value of 3e^{-71}). The predicted product of AN3946 showed slightly less homology to BubR1 proteins of other organisms (E-values ranged from 2e^{-21} to 3e^{-42}), but, as will be discussed in later
sections, *S. cerevisiae* and *S. pombe* do not have a true BubR1 homolog. Their ortholog, Mad3p, has KEN boxes in its N-terminal domain but lacks a C-terminal kinase domain. Interestingly, in common with higher organisms, the *A. nidulans* BubR1 homolog has N-terminal domain KEN boxes as well as a C-terminal kinase domain. A BLASTP search with *S. cerevisiae* Cdc20 revealed that the predicted protein product of AN0814 is a strong homolog (E-value of 4e⁻⁸⁵). Cdc20 orthologs from *S. pombe* and *H. sapiens* showed even stronger identity to the predicted protein product of AN0814 (E-values of 8e⁻¹³⁰ and 4e⁻⁹⁰, respectively). Based on the available genomic sequence information, I was able to design primers for tagging and deleting these homologs. All fluorescent protein fusions were under the control of their endogenous promoters and were functional (Fig. 3.1, Fig. 3.8).

### 3.2.2. Localization of Kinetochores (KTs) with Respect to the SPB During the Cell Cycle

In *A. nidulans*, the SPB and KTs are in proximity throughout interphase (De Souza et al., 2009; Yang et al., 2004). Only during mitosis, from prometaphase through early anaphase, are the two physically separated enough to be distinguished. This period is very short in *A. nidulans* because mitosis lasts only about 10 minutes at 25°C. I created a strain (LO2834) that carried histone H1-t-Sapphire, Nud1 (a SPB marker) fused to GFP, and Ndc80 (a KT marker) fused to mCherry and imaged it by time-lapse confocal microscopy (Fig. 3.2). Since the SAC proteins are expected to localize to the SPB or KTs in mitosis, these images serve as a reference for the localization pattern of these structures during mitosis. In instances in which the SPB and KTs are too close to distinguish as separate structures I will refer to the structures as the SPB/KT complex.

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3.2.3. Localization of Mad2

Mad2 is the only MCC component that has been localized well in *A. nidulans* (De Souza et al., 2009). To verify the localization of Mad2, Tania Nayak, a former graduate student in the lab, created a strain that carried Mad2-GFP and histone H1-mRFP (LO1390). Dr. Nayak performed preliminary localization analysis during the cell cycle, and I further analyzed it through mitosis by time-lapse spinning disk confocal microscopy. As previously reported (De Souza et al., 2009), we found Mad2 [encoded by *md2A*; (Prigozhina et al., 2004)] localized predominantly to the nuclear periphery in interphase nuclei (Fig. 3.3, A-C) and was also found dispersed in the cytoplasm. Upon entry into mitosis, Mad2 translocated from the nuclear periphery, concentrating at a region that, based on morphology, appears to be the SPB/KT complex [Fig. 3.3, D-F; (De Souza et al., 2009; Szewczyk and Oakley, 2011)]. Its signal then appeared to spread through this region from metaphase to anaphase (Fig. 3.3, G-L). De Souza and colleagues were able to determine that Mad2 concentrated around KTs in early mitosis (De Souza et al., 2009). From anaphase through telophase, Mad2 faintly localized to a region between separating daughter nuclei (Fig. 3.3, M-O) and between segregated KTs (De Souza et al., 2009). It returned first to the nucleoplasm (Fig. 3.3, P-R) and eventually to the nuclear periphery in early G1. *A. nidulans* undergoes a semi-open mitosis in which nuclear pore complexes (NPCs) partially disassemble, meaning peripheral nucleoporins (Nups) disperse while core Nups remain (De Souza et al., 2004; De Souza and Osmani, 2007; Osmani et al., 2006a). Removal of peripheral Nups in mitosis results in open NPC central channels, making the nuclear envelope permeable, now allowing passive diffusion
into and out of the nucleus. De Souza et al. (2009) determined that Mad2 returned to the nuclear periphery at the same time as peripheral Nups re-localized to the nuclear periphery and nuclear transport was re-established.

3.2.4. **Localization of Mps1**

To determine the localization of Mps1 through the cell cycle, I created a strain that carried Mps1-GFP and histone H1-mRFP (LO1479) and imaged it using time-lapse microscopy at 25°C. Through most of the cell cycle, Mps1 (encoded by a gene that I now designate *mpsA*) localized to a dot in each nucleus, which I have demonstrated to be the SPB (Fig. 3.4; see next paragraph). We know that the cell cycle duration in *A. nidulans* at 25°C is 199 ± 49 min. with G₁ lasting about 20% of the cell cycle, or approximately 39 minutes [(Edgerton-Morgan and Oakley, 2012); see also Chapter 1 and Chapter 4]. Long-term time-lapse imaging of 132 nuclei revealed that Mps1-GFP was not visible for 28 ± 12 min (mean ± standard deviation) after mitosis, which would correspond to it being undetectable for most of G₁ (Fig. 3.4, A-C). It is, however, possible that the fusion protein is being made during this time, but at levels we cannot detect microscopically, or it is simply not concentrated at the SPB. Throughout the rest of interphase, Mps1-GFP fluorescence at the dot increased in intensity (Fig. 3.4, D-L).

To verify that Mps1 localized to the SPB in S and G₂, which would be in agreement with its localization to the centrosome or SPB in other organisms (Fischer et al., 2004; Fisk and Winey, 2001; Kasbek et al., 2007; Winey et al., 1991), I created a strain (LO3105) that carried Mps1-GFP, histone H1-t-Sapphire, and Ndc80-mCherry. Mps1-GFP localized immediately adjacent to Ndc80 suggesting that it localized to the
SPB, but, perhaps, not to KTs, in interphase (Fig. 3.4, M-P). Further imaging of a strain that carried Mps1-GFP and γ-tubulin-mCherry (LO5851) verified that Mps1 localized to the SPB in interphase as the two protein fusions colocalized (Fig. 3.4, Q-T).

Upon closer examination of mitosis, I was able to determine that Mps1’s localization changed at mitotic entry [as judged by the beginning of chromosomal condensation; (Fig. 3.5, D-F)]. It moved from a single structure (the SPB) to several dots associated with the chromatin, which I deduce to be KTs since the localization pattern is consistent with it being at KTs [SPBs are not seen as more than two dots during mitosis in *A. nidulans* (Fig. 3.2)], and that is where it localizes in mitotic cells of other organisms (Abrieu et al., 2001; Liu et al., 2003; Stucke et al., 2002; Winey et al., 1991). The Mps1-GFP signal was completely gone prior to anaphase (Fig. 3.5, G-I).

### 3.2.5. Localization of Bub3

The *A. nidulans* Bub3 gene, *sldB*, was originally identified in a synthetic lethality screen for mutations that inhibit growth in the absence of dynein (Efimov and Morris, 1998). I created a strain that carried Bub3-GFP and histone H1-mCherry (LO3406) to determine the localization of Bub3 through the cell cycle. In interphase, I found Bub3 was abundant in the nucleoplasm (Fig. 3.6, A-C) as is the case for Bub3 homologs in other organisms (Campbell and Hardwick, 2003; Kadura et al., 2005; Taylor et al., 1998), but it was excluded from the nucleolus (arrows; Fig. 3.6). As nuclei entered mitosis, the intensity of Bub3 decreased in the nucleoplasm (Fig. 3.6, D-F). This corresponds to the time that NPCs partially disassemble (De Souza et al., 2004; De Souza and Osmani, 2007; Osmani et al., 2006a). However, as the nucleoplasmic signal decreased, Bub3
remained localized to dots in the nucleoplasm that likely correspond to KTs (Fig. 3.6, G-I). Bub3 was only at KTs for a short period of time before the signal was lost prior to anaphase (Fig. 3.6, J-L). It reappeared in the nucleoplasm in early G1 (Fig. 3.6, P-R).

3.2.6. Localization of BubR1

The *A. nidulans* BubR1 gene, *sldA*, was identified in the same synthetic lethality screen that identified *sldB* (Efimov and Morris, 1998). The protein encoded by *sldA* was originally identified as a homolog of Bub1 since it has a C-terminal kinase domain that distinguishes it from its ortholog Mad3p in *S. cerevisiae* and *S. pombe*. However, upon closer examination of its amino acid sequence, I determined that it is actually a homolog of BubR1 as it has two KEN boxes in its N-terminal domain as well as a C-terminal kinase domain (Fig. 3.7). To determine the localization of BubR1 through the cell cycle, I created a strain that carried BubR1-GFP and histone H1-mRFP (LO4580). In interphase, BubR1 was not detectable (Fig. 3.8, A-C). At the onset of mitosis, though, it quickly appeared at the SPB/KT complex (Fig. 3.8, D-F). It then was apparent briefly at dots in the nucleoplasm that appeared to be KTs (Fig. 3.8, G-I) before disappearing prior to anaphase (Fig. 3.8, J-L). By creating a strain carrying BubR1-GFP and Ndc80-mCherry (LO5908) and analyzing it by confocal microscopy, I determined that BubR1-GFP colocalized with Ndc80-mCherry verifying that the nucleoplasmic dots are, indeed, KTs (Fig. 3.9). This KT localization is similar as to what has been reported in *H. sapiens* (Taylor et al., 2001), PtK2 cells (Howell et al., 2004), and *D. melanogaster* (Buffin et al., 2005). BubR1 is probably present in the cytoplasm in interphase but not at levels that I
can detect above background levels. This would also be similar to *H. sapiens* (Burum-Auensen et al., 2007; Taylor et al., 1998) and *D. melanogaster* (Buffin et al., 2005).

### 3.2.7. Localization of Cdc20

To observe the localization pattern of Cdc20 through the cell cycle, I first fused GFP to the 3’ end of its coding sequence keeping expression of the fusion product under control of the endogenous *cdc20* promoter. Viable transformants were obtained and verified to be C-terminally tagged by diagnostic PCR; however, no GFP signal was detected in 10 different, verified transformants. Since no signal was detected in so many C-terminally tagged transformants, I decided to try an N-terminal GFP fusion instead. Again, this fusion was kept under the control of the endogenous *cdc20* promoter (see Chapter 2). Viable transformants were obtained and verified by diagnostic PCR to have a correct integration. Transformants in this case did produce a faint, but detectable, GFP signal. The N-terminal fusion was functional, supporting normal growth over a range of temperatures (20°C-42°C; Fig.3.10) and it was the only copy of the *cdc20* gene in the genome.

I created strains LO5538 and LO5539 that carried GFP-Cdc20 and histone H1-mRFP, to determine the localization of Cdc20 throughout the cell cycle. These strains were imaged using spinning disk confocal microscopy by myself and by Dr. Berl Oakley. In interphase, GFP-Cdc20 levels in the nucleus were significantly lower than in the cytoplasm (Fig. 3.11, A-C; Fig. 3.12, A-C). At mitotic entry, as judged by the beginning of histone H1-mRFP condensation (and based on NPC breakdown revealed by the dispersal of Nup49-mCherry), GFP-Cdc20 entered the nucleus (Fig. 3.12, D-I), briefly
concentrating in a small area that, on the basis of morphology, is likely to be the SPB or very early forming spindle (Szewczyk and Oakley, 2011) (Fig. 3.12, M-O). It then spread through the nucleoplasm, accumulating to higher levels than in the cytoplasm (Fig. 3.11, D-F). It occupied a larger volume than the condensed chromosomes but was not excluded from chromatin. In anaphase and telophase, the concentration of GFP-Cdc20 dropped such that its concentration in the nucleus was similar to its concentration in the cytoplasm (Fig. 3.11, G-I, top nucleus). We noted some variation in the timing of this concentration drop. Sometimes it occurred just before anaphase and sometimes it occurred later such that there was a visible concentration of Cdc20 between separating chromosome masses (Fig. 3.11, G-I, bottom nucleus). After the drop, Cdc20 remained at the same concentration as the surrounding cytoplasm until mitotic exit, when it was rapidly depleted from the nucleoplasm (Fig. 3.11, J-L). Interestingly, the pattern was different from that reported for animal cells, where it is enriched at centrosomes, which are functionally similar to SPBs, through mitosis (Kallio et al., 2002; Kim et al., 2009a; Li et al., 2010; Raff et al., 2002). We only detected an enrichment of GFP-Cdc20 in prophase when SPBs and KT are very close together and the spindle is beginning to form, and it is difficult to determine if the localization is at the SPB, spindle, or KT, or all three. There was clearly no enrichment at SPBs at later stages of mitosis. We can conclude from these data that the location of Cdc20 is regulated during the cell cycle in A. nidulans, and we further hypothesize that the location of Cdc20 is a key regulator of APC/C activity. Previous localization data in A. nidulans revealed that the APC/C is located in the nucleoplasm in interphase and mitosis (Nayak et al., 2010). Since Cdc20 is
depleted from the nucleoplasm until mitotic entry, the entry of Cdc20 into the nucleoplasm must allow an increase in the formation of APC/C<sup>Cdc20</sup>.

3.2.8. The SAC is Spatially Regulated in A. nidulans

Based on my microscopy data, I determined that Mad2, Mps1, Bub3, BubR1, and Cdc20 are all at different locations during interphase. Mad2 localized to the nuclear periphery; Mps1 was at the SPB from late G<sub>1</sub> through G<sub>2</sub>; Bub3 was in the nucleoplasm; and BubR1 was presumably in the cytoplasm along with Cdc20. Only at mitotic onset, did all of these proteins localize to the SPB/KT region. Thus, they are physically separate from each other and, therefore, blocked from forming a complete, functional MCC complex until it is time for the SAC to become active in mitosis (Fig. 3.13). *A. nidulans* undergoes a semi-open mitosis in which peripheral Nups disassemble from the nuclear envelope at mitotic entry, but core Nups remain (De Souza et al., 2004; De Souza and Osmani, 2007; Osmani et al., 2006a). I hypothesize that partial disassembly of NPCs allows (1) Mad2 to dissociate from the nuclear periphery and enter the nucleus, concentrating at the SPB/KT complex [based on my data and localization data reported in De Souza et al. (2009)], (2) BubR1 and Cdc20 to enter the nucleus from the cytoplasm with BubR1 concentrating at the SPB/KT complex and Cdc20 concentrating first at the SPB/KT/forming spindle region before spreading through the nucleoplasm, and (3) Bub3 to leave the nucleoplasm except for the portion that remains at KTs. Mps1 translocates from the SPB to KTs at this stage. These results are in contrast to HeLa cells in which a MCC was found to exist in interphase (Sudakin et al., 2001). In *S. cerevisiae*, a MCC can form in the absence of functional KTs (Fraschini et al., 2001), also suggesting a KT-
independent formation of the MCC. Since Mad2, Cdc20, and presumably BubR1 are all in the cytoplasm in interphase, I cannot rule out the possibility in \textit{A. nidulans} that a KT-independent formation of a pre-made Mad2-BubR1-Cdc20 subcomplex forms in the cytoplasm in interphase as has been reported in mammalian cells (Meraldi et al., 2004).

3.2.9. \textit{mipAD159 Causes a Failure of Accumulation of Mps1 and BubR1}

At a restrictive temperature \textit{mipAD159} causes a failure of inactivation of APC/C\textsuperscript{CdhA} sometime between late mitosis and S phase, leading to failure of cyclin B accumulation and nuclei being permanently taken out of the cell cycle (Edgerton-Morgan and Oakley, 2012; Nayak et al., 2010). One might expect, however, that when such nuclei entered mitosis, the SAC would be activated and this would, in turn, inactivate APC/C\textsuperscript{CdhA}. This is clearly not the case as once nuclei became unable to accumulate cyclin B (CB\textsuperscript{−}), they remained CB\textsuperscript{−} (Nayak et al., 2010). One possible explanation is that the constitutively active APC/C causes mislocalization or destruction of one or more SAC components so a functional SAC cannot be established.

\textit{A. nidulans} is coenocytic with nuclei in each tip cell actively progressing through the cell cycle in a synchronous fashion. Mitotic regulatory proteins, such as cyclin B, accumulate and are destroyed at essentially the same time in all nuclei within a tip cell. However, in strains carrying \textit{mipAD159}, incubated at a restrictive temperature of 25°C, cyclin B, Cdk1 and Ancdc14 fail to accumulate in a subset of tip cell nuclei due to constitutive APC/C\textsuperscript{CdhA} activity, while others in the same cell accumulate them normally (Edgerton-Morgan and Oakley, 2012; Nayak et al., 2010). To determine if a constitutively active APC/C\textsuperscript{CdhA} causes a failure of accumulation or mislocalization of
Mad2, Mps1, and Bub3, I examined the localization of each in strains carrying $mipAD159$. Strains were incubated at 25°C for 21 hours, a time by which CB$^-$ nuclei, and therefore nuclei with a constitutively active APC/C$^{\text{CdhA}}$, would have accumulated (Edgerton-Morgan and Oakley, 2012; Nayak et al., 2010). Z-series stacks of random fields were captured for a one hour period and tip cell nuclei scored for the presence or absence of the SAC protein. As a control, I found that $mipAD159$ caused a failure of cyclin B accumulation in 45.2 ± 13% of tip cell nuclei (mean ± standard deviation of three experiments) compared to 1.5 ± 1.6% in two $mipA^+$ strains (six experiments) (Fig. 3.14). This percentage of CB$^-$ nuclei was similar to that reported previously (Nayak et al., 2010). Since Mad2 and Bub3 are associated with nuclei in interphase, I examined if they were absent from a similar percentage of tip cell nuclei as cyclin B. Note that I only scored tip cells in which at least one nucleus had accumulated Mad2-GFP or Bub3-GFP to make sure I was only counting cells that were actively going through the cell cycle. Mad2 localized to the nuclear periphery of almost all $mipAD159$ nuclei. Only 3.6 ± 0.7% (three experiments) of tip cell nuclei were negative for Mad2 compared to 0.3 ± 0.5% (three experiments) in a $mipA^+$ strain. Likewise, Bub3 accumulated to all $mipAD159$ nuclei as only 0.9 ± 0.4% (three experiments) of tip cell nuclei failed to accumulate Bub3 compared to 0.3 ± 0.5% in a $mipA^+$ strain (three experiments). Therefore, a constitutively active APC/C does not cause a gross mislocalization or destruction of Mad2 or Bub3.

Mps1, in contrast to Mad2 and Bub3, is not visible throughout interphase. As discussed in 3.2.4, Mps1-GFP is undetectable for most of $G_1$. I performed the same type of experiment as I did for cyclin B, Mad2, and Bub3, making sure to only score tip cells
in which at least one nucleus had accumulated Mps1-GFP to ensure that I was not counting nuclei as negative when, in reality, they were in an early stage of the cell cycle at which Mps1 had not accumulated. In a \textit{mipAD159} strain, Mps1 failed to accumulate to the SPB in 23.1 ± 10.3% of nuclei (mean ± standard deviation of three experiments) compared to 1.9 ± 1.7% (three experiments) in a \textit{mipA⁺} strain. \textit{mipAD159}, thus, causes a nuclear autonomous failure of accumulation of Mps1 at the SPB at restrictive temperatures. The percentage of nuclei that were Mps1⁻ was lower than the percentage of nuclei that were CB⁻ in separate experiments under similar conditions, but within one standard deviation. \textit{mipAD159} nuclei with a constitutive APC/C activity are taken out of the cell cycle and remain in a G₁-like state (Edgerton-Morgan and Oakley, 2012; Nayak et al., 2010). Mps1 does not accumulate to detectable levels in G₁ and the failure to accumulate Mps1 could be downstream of the failure to accumulate cyclin B and, thus, exit G₁. However, \textit{A. nidulans} Mps1 has a canonical destruction box (RXXLXXXXN) that can make it a target for either APC/C\textsuperscript{Cdc20} or APC/C\textsuperscript{Cdh1} (Bashir and Pagano, 2004; Pfleger and Kirschner, 2000; Tian et al., 2012; Visintin et al., 1997; Zur and Brandeis, 2002). I believe a more likely explanation for the Mps1⁻ nuclei is that constitutive APC/C\textsuperscript{CdhA} activity leads to continuous destruction of Mps1 by the proteasome. In either case, the absence of Mps1 from nuclei would prevent the establishment of a functional SAC to inactivate the constitutively active APC/C\textsuperscript{CdhA} when such nuclei enter mitosis.

To determine if \textit{mipAD159} caused a mislocalization, or failure of accumulation, of Cdc20 or BubR1 I collected time-lapse images of strains going through mitosis that
carried *mipAD159*, histone H1-mRFP and either GFP-Cdc20 (LO5690) or BubR1-GFP (LO4676). Strains were incubated at a restrictive temperature of 25°C for 17-24 hours before imaging. I chose this range of incubation times because *mipAD159* nuclei with a constitutively active APC/C have accumulated by this time (Nayak et al., 2010). Cdc20 accumulated in virtually all mitotic nuclei as only 1.4 % failed to accumulate it ($n = 71$). *mipAD159*, thus, does not appear to affect Cdc20 accumulation.

With BubR1, however, a subset of mitotic nuclei failed to accumulate the protein (Fig. 3.15). To determine if the nuclei in which BubR1 failed to accumulate were nuclei with a constitutively active APC/C$^{CdhA}$, I created a *mipAD159* strain carrying BubR1-GFP, Histone H1-t-Sapphire, and Cdk1-mCherry (LO5242). Cdk1 forms a complex with cyclin B (Nurse, 1990) and CB$^-$ nuclei also fail to accumulate Cdk1 (Nayak et al., 2010). In our hands, Cdk1-mCherry is brighter and photobleaches less than cyclin B-mCherry and is, therefore, more amenable to long-term imaging. Cdk1-mCherry localizes to the nucleoplasm and SPB only in S and G2 and only in nuclei with an inactive APC/C$^{CdhA}$ (Edgerton-Morgan and Oakley, 2012; Nayak et al., 2010). I found that, indeed, Cdk1$^-$ nuclei (and therefore CB$^-$ nuclei) failed to accumulate BubR1 at mitotic entry (Fig. 3.16, asterisks).

3.3. Discussion

This is the first report of the localization of Mps1, Bub3, BubR1, and Cdc20 in any filamentous fungus. More importantly, given the importance of SAC proteins, there
is surprisingly little in vivo imaging data in other organisms on these proteins during the entire cell cycle or even through mitosis.

With respect to fungi, the localization of these SAC components is valuable information. *A. nidulans* undergoes a semi-open mitosis in which the nuclear envelope remains intact, but the NPCs partially disassemble upon entry into mitosis. Peripheral Nups, including Nup49, disperse throughout the cell in mitosis while core Nups remain associated with the nuclear envelope (De Souza et al., 2004; De Souza and Osmani, 2007; Osmani et al., 2006a). The localization patterns I have seen suggest that dissociation of peripheral Nups from NPCs at mitotic entry is a key regulatory event in the formation of a functional MCC. In interphase, NPCs act as a physical barrier preventing the interaction of MCC components and, thus, activation of the SAC. At mitotic onset, partial disassembly of NPCs removes the physical barrier and allows MCC formation. These data support the notion that NPCs play a key cell-cycle regulatory role in *A. nidulans* (De Souza et al., 2009; De Souza et al., 2011; De Souza et al., 2004; De Souza and Osmani, 2007; Osmani et al., 2006a).

In addition, these data show for the first time that APC/C activity in *A. nidulans* is likely regulated in part by the localization of Cdc20. Cdc20 is depleted from interphase nuclei and enters at mitotic onset, where it accumulates to a higher level than in the cytoplasm. BimA, the *A. nidulans* homolog of the APC/C subunit APC3, is concentrated in the nucleus throughout interphase and additionally localizes to the spindle in anaphase (Nayak et al., 2010). Partial disassembly of NPCs at mitotic onset likely allows Cdc20 to accumulate in the nucleoplasm, permitting Cdc20 to interact with
the APC/C, resulting in APC/C^{Cdc20} activity. Since Cdc20 leaves the nucleoplasm at mitotic exit, whereas the APC/C remains there (Nayak et al., 2010), physical separation of the two proteins is a probable mechanism by which APC/C^{Cdc20} is inactivated at mitotic exit. It has been suggested in other organisms that targeting of Cdc20 for destruction by APC/C^{Cdh1} is necessary for mitotic exit, but this is not the case in *A. nidulans* as strains are viable and mitosis is completed successfully in the absence of Cdh1 (see Chapter 4).

My data also address why the SAC does not inactivate the constitutively active APC/C in CB^−, *mipA*^{D159} nuclei when they enter mitosis. Mps1 fails to accumulate in a subset of nuclei and, while we have not demonstrated directly that these are CB^− nuclei, our data suggest that they are. If so, the absence of Mps1 would prevent the establishment of a functional SAC. In vertebrates, Mps1 is required for KT localization of Mad1, Mad2, as well as two other SAC components, Plk1 and CENP-E (Abrieu et al., 2001; Liu et al., 2003; Martin-Lluesma et al., 2002; Vigneron et al., 2004; Wong and Fang, 2005). It is likely that Mps1’s role in this recruitment is regulatory rather than structural since FRAP experiments have shown that Mps1 exchanges completely with KTs with a half-life of only 10 seconds (Howell et al., 2004). In interphase, Mad2 localized to the nuclear periphery of virtually all *mipA*^{D159} nuclei; however, it still remains to be determined if Mad2 fails to translocate to the SPB/KT complex at mitotic entry in Mps1^− nuclei.
BubR1 also fails to accumulate at KTs in CB⁻ nuclei. In *Xenopus* egg extracts, Mps1 is required for BubR1 to localize to KTs (Vigneron et al., 2004; Wong and Fang, 2006). If this is the case in *A. nidulans*, Mps1⁻ nuclei would not be expected to accumulate BubR1 at KTs in mitosis. Failure of accumulation of BubR1 could also be due, in principle, to a failure of inactivation of APC/C<sup>CdhA</sup> since BubR1 contains two KEN boxes, with the most N-terminal one acting as a destruction signal for APC/C<sup>CdhA</sup> as has been determined in *S. cerevisiae* (King et al., 2007). BubR1 is present in the cytoplasm of hyphae with CB⁻ nuclei, because when the CB⁺ nuclei in the same cells enter mitosis, BubR1 localizes at KTs in those nuclei. If the absence of BubR1 at KTs of CB⁻ nuclei were due to constitutive APC/C<sup>CdhA</sup> activity, it would have to be destroyed after mitotic onset. However, my data on CdhA reveal that in all nuclei, whether CB⁺, CB⁻, *mipa*⁺, or *mipAD159*, CdhA disappears from the SPB and nucleoplasm in mitosis (Edgerton-Morgan and Oakley, 2012). Therefore, APC/C<sup>CdhA</sup> is probably not active in the nucleus at this stage. So, rather than BubR1 failing to accumulate due to a constitutively active APC/C<sup>CdhA</sup>, the absence of BubR1 is more likely due to the absence of Mps1. This remains to be tested experimentally, but, in any case, the absence of both Mps1 and BubR1 would clearly prevent activation of the SAC in mitosis.
Table 3.1: Identification of *A. nidulans* homologs of known SAC components

<table>
<thead>
<tr>
<th>Protein</th>
<th><em>A. nidulans</em> gene designation</th>
<th>Gene ID (AspGD)</th>
<th>E-value to <em>S. cerevisiae</em></th>
<th>E-value to <em>S. pombe</em></th>
<th>E-value to <em>H. sapiens</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mad2</td>
<td><em>md2A</em></td>
<td>AN2511</td>
<td>2e⁻⁵⁵</td>
<td>8e⁻⁶⁵</td>
<td>3e⁻⁵¹</td>
</tr>
<tr>
<td>Mps1</td>
<td><em>mpsA</em></td>
<td>AN2927</td>
<td>5e⁻⁷⁵</td>
<td>4e⁻⁸⁰</td>
<td>6e⁻⁵⁹</td>
</tr>
<tr>
<td>Bub3</td>
<td><em>sldB</em></td>
<td>AN2439</td>
<td>9e⁻³⁴</td>
<td>2e⁻⁴⁸</td>
<td>3e⁻⁷¹</td>
</tr>
<tr>
<td>BubR1</td>
<td><em>sldA</em></td>
<td>AN3946</td>
<td>8e⁻³⁴</td>
<td>3e⁻⁴²</td>
<td>2e⁻²¹</td>
</tr>
<tr>
<td>Cdc20</td>
<td><em>cdc20</em></td>
<td>AN0814</td>
<td>4e⁻⁸⁵</td>
<td>8e⁻¹³⁰</td>
<td>4e⁻⁹⁰</td>
</tr>
</tbody>
</table>

*A. nidulans* homologs of the SAC genes Mad2, Mps1, Bub3, BubR1, and Cdc20 were identified by performing a BLASTP search with SAC proteins of other organisms against the *Aspergillus* genome database (AspGD). The *A. nidulans* gene designations for Mad2, Bub3, and BubR1 have been previously published. It should be noted that *sldA* was originally identified as a homolog of Bub1 since it contained a C-terminal kinase domain that distinguishes it from Mad3p orthologs. However, closer examination of the amino acid sequence revealed the protein product of *sldA* encodes a homolog of BubR1. BubR1 homologs have two KEN boxes in addition to a C-terminal kinase domain. Gene IDs are according to AspGD ([www.aspgd.org](http://www.aspgd.org)).
Figure 3.1: C-terminal GFP fusions of Mad2, Mps1, Bub3, and BubR1 are functional

continued
Figure 3.1 continued

Strains carrying SAC-GFP fusions were stabbed onto a complete medium (YAG) and incubated at the temperatures shown. The times of incubation at each temperature were different to partially compensate for different growth rates at the different temperatures. All C-terminal gene fusions grew at rates comparable to a WT strain (FGSC4) at all temperatures tested. Since deletions or mutations of SAC genes are known to lead to hypersensitivity to the antimicrotubule drug, benomyl, (Efimov and Morris, 1998; Hoyt et al., 1991; Li and Murray, 1991; Prigozhina et al., 2004), I stabbed the same GFP-tagged strains on YAG containing 0.2, 0.4, or 0.6 μg/ml benomyl. There is a hint of increased benomyl sensitivity at high temperatures, indicating that the GFP-fusion proteins might be very slightly impaired for function. As shown by the WT control, *A. nidulans* is less sensitive to benomyl at high temperatures than at low temperatures as has been previously reported (Jung et al., 2001).
Figure 3.2: Localization of SPBs and KTs in interphase and during mitosis
Figure 3.2 continued

In interphase (A-D), the SPB (revealed by Nud1-GFP) and KT's (revealed by Ndc80-mCherry) are in proximity and appear to localize to overlapping dots in the nucleus (chromatin is revealed by Histone H1-t-Sapphire). Upon entry into mitosis, the SPB's become visible as two dots as the poles begin to separate. KT's are visible as an entity between the two SPB's (E-L). In anaphase (M-P), the SPB's are at opposite end of the nucleus and KT's are faintly visible as dots in between. As anaphase proceeds, the KT's are rapidly pulled to the SPB's (Q-X). Images are 3D projections of Z-series stacks of strain LO2834 collected at 30 second intervals.
Cytoplasmic levels of Mad2-GFP are barely visible in this figure because intensity levels were chosen to be optimal to show the nuclear periphery localization. Mad2-GFP is at the nuclear periphery in interphase (A-C) and translocates to the SPB/KT complex early at mitotic onset.

Figure 3.3: Mad2 translocates from the nuclear periphery to the SPB/KT complex at mitotic onset.

continued
in mitosis when chromosomal condensation is first becoming visible (D-F). Its signal then seems to spread through a region of the nucleus (G-L), which De Souza et al. (2009) determined to be a region around KTs. In late anaphase/telophase (M-O), Mad2-GFP remains faintly visible in a region in between separating chromatin masses. The M-O localization pattern was noted in De Souza et al. (2009), which was distinct from KTs as KTs had already segregated to the SPBs by this stage. After mitosis is complete, Mad2-GFP starts to return to the nucleoplasm of daughter nuclei (P-R) and eventually returns to the nuclear periphery (De Souza et al., 2009). All images are 3D projections of Z-series stacks collected at 30 second intervals. This data set is from strain LO1390 incubated at 30°C for 17.5 hours and then shifted to 25°C for three hours before imaging. Imaging was carried out at 25°C.
Figure 3.4: Mps1-GFP localization throughout the cell cycle

A-L. Z-stack projection images from a time lapse data set collected at 10 min intervals at continued
Figure 3.4 continued

25°C (strain LO1479). Mps1 is not visible through most of G1 (A-C), but it becomes faintly visible 28 ± 12 min. after mitosis (arrows; D-F). The mean cell cycle time is 199 ± 49 minutes at 25°C (Edgerton-Morgan and Oakley, 2012), indicating Mps1-GFP becomes visible approximately 14% through the cell cycle, corresponding to late G1. Its signal increases in intensity through S (G-I) and G2 (J-L). M-P. Mps1 localizes immediately adjacent to Ndc80 when it is apparent in interphase, suggesting it localizes to the SPB at this stage. Q-T. Mps1 is verified to localize to the SPB in interphase. Mps1-GFP colocalizes with γ-tubulin-mCherry. An intensity plot of both channels (green is GFP, and red is mCherry) is shown in the right column, which also verifies the colocalization pattern. The intensity plot was made using Volocity software using one optical section in the Z-series stack to minimize signal from out of focus fluorescence. A-P are 3D projection images from a single through-focus Z-series stack of strain LO3105 while Q-T are projection images from a single Z-series stack of strain LO5851.
In G₂, Mps1-GFP is at the SPB (A-C). At mitotic entry (D-F), as judged by the beginning of chromosomal condensation, Mps1-GFP translocates form the SPB to KTs. At the 540 sec time point (G-I), still prior to anaphase, the signal is completely gone. This nucleus divides at the 690 sec. time point (J-L) and Mps1-GFP is still not present.

Images are 3D projections from a time lapse data set collected at 30 sec intervals at 25°C.
In interphase Bub3-GFP is present in the nucleoplasm (A-C). However, it is excluded from the nucleolus (arrows). Upon mitotic entry as judged by histone H1-mRFP condensation, Bub3-GFP disperses from the nucleoplasm, but remains concentrated in one area of the nucleus (D-F). Thirty seconds later (G-I), Bub3-GFP is clearly gone from the nucleoplasm except from KTs. Prior to anaphase, its signal is completely gone (J-L), and it does not return during the remainder of mitosis (M-O shows anaphase). Bub3-GFP continued
Figure 3.6 continued

returns to the nucleoplasm of daughter nuclei in G1 (P-R). Images are 3D projections of Z-series stacks collected at 30 second intervals. With this data set, the strain (LO3406) was incubated at 30°C for 20.5 hours and then shifted to 25°C three hours before imaging. Imaging was done at 25°C.
Alignment of the amino acid sequences encoding Mad3/BubR1 in various organisms reveals that the *A. nidulans sldA* gene product (A.n.) is a homolog of BubR1 and not Bub1 as previously published. Like *S. cerevisiae* Mad3p (S.c.), *S. pombe* Mad3

Figure 3.7: Clustal WS alignment of the N-terminus of BubR1 orthologs
(S.p.), and *H. sapiens* BubR1 (H.s.), *sldA* encodes a protein with two KEN box motifs in its N-terminus. The blue regions indicate areas of identity among all four protein sequences with KEN boxes outlined in red.
In interphase (A-C), BubR1-GFP is not detectable. Its levels in the cytoplasm are barely above background fluorescence levels. At mitotic onset, as judged by histone H1-mRFP condensation (D-F), BubR1-GFP is concentrated at one region in the nucleoplasm, which is likely the SPB/KT complex. It is then visible as dots in the nucleoplasm, which are presumably KTs (G-I), before disappearing by anaphase (J-L). The signal does not return during the rest of mitosis (M-O shows late telophase/early G1). Images are 3D projections of Z-series stacks collected at 45 sec intervals at 25°C.

Figure 3.8: BubR1-GFP localization at mitotic onset
A-C. Two mitotic nuclei in the same apical cell. BubR1-GFP colocalizes with KTs (Ndc80-mCherry) upon mitotic entry. Images are projection images of a single Z-series stack of strain LO5908 incubated and imaged at 30°C. D and E are intensity traces of the GFP (green) and mCherry (red) signals across the lines in the Merge panel. D is a trace of the top nucleus (light purple line) and E is a trace of the bottom nucleus (turquoise line). The traces were created by Volocity software and were made using one optical section in the Z-series stack to minimize signal from out of focus fluorescence. The intensity traces further verify that BubR1 colocalizes with Ndc80.

Figure 3.9: BubR1 localizes to KTs at mitotic entry
GFP-Cdc20 fusions support growth at all temperatures tested. Two GFP-Cdc20 strains (LO5538 and LO5539) as well as the parental strain that was used for transformation (LO1516) and a WT control (FGSC4) were stabbed onto complete media and incubated at the temperatures shown. The times of incubation at each temperature were different to partially compensate for different growth rates at the different temperatures. The GFP-Cdc20 fusion is functional, but the strains that carry it show slightly reduced growth at 30, 37, and 42°C relative to controls.

Figure 3.10: The N-terminal fusion of GFP to Cdc20 is functional
Images are Z-stack projections of strain LO5538 captured using a spinning disk confocal continued
microscope at 30°C using one minute intervals and were taken from the same data set. Intensity traces of histone H1-mRFP and GFP-Cdc20 over the path of the white line shown in C, F, I, and L are in the right-most column. The traces were created by Volocity software and were made using one optical section in the Z-series stack to minimize signal from out of focus fluorescence. A-C. Two nuclei in an interphase cell. GFP-Cdc20 levels are low in the nucleoplasm and are higher in the cytoplasm. D-L. Another hypha from the same data set that is going through mitosis. In prophase (D-F), GFP-Cdc20 levels increase in the nucleoplasm and it occupies a larger volume than the chromatin. One minute later (G-I), the top nucleus is in telophase and GFP-Cdc20 is present at approximately the same level as in the surrounding cytoplasm. GFP-Cdc20 is concentrated in the bottom nucleus, which is in late anaphase, relative to the cytoplasm. At the 5 minute time point (J-L), in early G1, GFP-Cdc20 levels in the daughter nuclei have decreased.
All images are projections of Z-series stacks captured at one minute intervals at 25°C.

Figure 3.12: Movement of GFP-Cdc20 into nuclei at mitotic onset
with a spinning disk confocal microscope. A-I are strain LO5538. At the 0 min. time
point (A-C), GFP-Cdc20 is at lower levels in the nucleoplasm than in the cytoplasm. The
bottom two nuclei at this time point are just beginning to enter mitosis as judged by the
amount of chromosomal condensation. Two minutes later (D-F), the top nucleus is
entering mitosis and the other nuclei are in early prophase. GFP-Cdc20 is increasing in
the nucleoplasm at this point, and by 4 min. (G-I) all nuclei are in prophase and GFP-
Cdc20 levels in the nucleoplasm are higher than in the cytoplasm. Panels J-U are from a
separate data set using strain LO6518. The nuclear pore complex (NPC) is revealed by
nup49-mCherry fluorescence. The 0 min. time point (J-L) is late $G_2$ in which GFP-
Cdc20 is clearly lower in the nucleoplasm than in the surrounding cytoplasm. At the 2
min. time point (M-O), the NPC is disassembling and GFP-Cdc20 has entered the
nucleoplasm, concentrating at the SPB, KTs, and forming spindle complex (arrows). By
the 3 min. time point (P-R), the NPC has completely disassembled and GFP-Cdc20 has
spread throughout the nucleoplasm. At the 14 min. time point (S-U), the NPC has
reassembled around daughter nuclei, and GFP-Cdc20 is again reduced in the
nucleoplasm.
In interphase, Mad2 (light blue) is present at the nuclear periphery, Bub3 (green) is in the nucleoplasm, and BubR1 (orange) is presumably in the cytoplasm. Cdc20 is at low levels in the cytoplasm. Mps1 (dark blue) is not detectable until late G1 when it becomes visible at the SPB and it remains visible there through G2. At mitotic onset, the nuclear continued

Figure 3.13: Schematic of SAC activation in *A. nidulans*
Figure 3.13 continued

envelope partially breaks down, allowing Mad2 to translocate to the SPB/KT complex and BubR1 and Cdc20 to enter the nucleus where they first concentrate at the SPB/KT complex. Mps1 is at KTs, and the majority of Bub3 leaves the nucleoplasm, but some is left at KTs. Thus, these SAC components are all physically separate from each other in interphase and only come together at the SPB/KT complex at the onset of mitosis in *A. nidulans*. This ensures the SAC will not be activated prematurely.
Each strain was incubated for 21 hours at 25°C before imaging. Z-stacks of random fields were taken at 25°C over a one hour period and tip cell nuclei were scored for the presence or absence of each SAC-tagged protein. Cyclin B-GFP strains were continued
used as controls for the percentage of \textit{mip4D159} nuclei that have a constitutively active APC/C. \textit{mip4D159} causes a large increase in tip cell nuclei that are negative for Mps1-GFP and in nuclei that are negative for cyclin B-GFP. Values are means and error bars are standard deviation over three experiments for each strain except for CycB-GFP, HH1-mRFP, \textit{mip4A^+}, which was six experiments with two strains.
Three *mip*AD159 nuclei entering mitosis. All three nuclei are in the same cell. In interphase (0 sec.), none of the nuclei have accumulated BubR1-GFP. However, 180 sec. later, the nucleus on the left is entering mitosis, based on the condensation of histone H1-mRFP, and has accumulated BubR1-GFP. As a wave of mitosis proceeds through the hypha (360 sec.), both the left and right nuclei (asterisks) accumulate BubR1-GFP, whereas the middle nucleus (circle) does not. Although the rest of mitosis and mitotic exit is not shown, the middle nucleus never accumulates BubR1-GFP. Images are Z-stack projections of strain LO4676 captured at three minute intervals at 25°C.

Figure 3.15: BubR1 fails to accumulate in a subset of mitotic *mip*AD159 nuclei.
Images are 3D projections of Z-series stacks of strain LO5242 incubated at a restrictive temperature of 25°C and captured at two minute intervals. All three nuclei, revealed by histone H1-t-Sapphire, are in the same apical cell. At the 0 min. time point, the two left nuclei are positive for Cdk1-mCherry (Cdk1⁺; arrows), which is visible in the nucleoplasm and at the SPB in S through G₂ and exits the nucleus during mitosis.
Figure 3.16 continued

(Edgerton-Morgan and Oakley, 2012; Nayak et al., 2010). The right nucleus (asterisk) is negative for Cdk1-mCherry (Cdk1\(^{-}\)), and therefore has a constitutively active APC/C.

During mitosis, (8 min. and 16 min. time points), the two Cdk1\(^{+}\) nuclei accumulate BubR1-GFP, whereas the Cdk1\(^{-}\) nucleus never accumulates BubR1-GFP. Therefore, the \textit{mipAD159} nuclei that fail to accumulate BubR1 are ones that have a constitutively active APC/C.
Chapter 4: \(\gamma\)-Tubulin Plays a Key Role in Inactivating APC/C^{Cdhl}

4.1. Introduction

As mentioned previously, our lab recently discovered that the \(\gamma\)-tubulin mutation, \textit{mipAD159}, causes a failure of inactivation of the APC/C in interphase, sometime between late mitosis and S. In \textit{mipAD159} cells grown at a restrictive temperature of \(25^\circ\text{C}\), a subset of nuclei failed to accumulate cyclin B, Cdk1, and the \textit{A. nidulans} \textit{cdc14} phosphatase, Ancdc14. Extensive analysis revealed that such nuclei were the consequence of a constitutively active APC/C, resulting in these nuclei being taken out of the cell cycle while other nuclei in the same cell continued to cycle [(Nayak et al., 2010); see also Chapter 1]. This phenomenon was nuclear autonomous and the percentage of such nuclei increased over time. The constitutively active APC/C was not due to a defect or failure of nuclear import or export and was microtubule independent. These data revealed an unexpected, but important role for \(\gamma\)-tubulin in inactivating the APC/C sometime after mitotic exit.

The APC/C is known to bind to two activators in other organisms, Cdc20 and Cdh1 [reviewed in (Acquaviva and Pines, 2006; McLean et al., 2011; Pines, 2011; Thornton and Toczyski, 2006; van Leuken et al., 2008)]. Cdc20 activates the APC/C in mitosis. APC/C^{Cdc20} targets securin for destruction, which allows separase to cleave
cohesin and chromosome separation to occur in anaphase. APC/C\textsuperscript{Cdc20} is also required for mitotic exit through targeting cyclin B for destruction. Cdh1, on the other hand, activates the APC/C in G\textsubscript{1}. APC/C\textsuperscript{Cdhl} prevents premature accumulation of S-phase cyclins and has to be inactivated before the cell cycle can proceed to S.

The results from Nayak et al. (2010) suggest two alternative models for the constitutive activation of the APC/C in \textit{mipA\textsubscript{D159}} nuclei. First, APC/C\textsuperscript{Cdc20} may not be inactivated at the end of mitosis, leading to the destruction of cyclin B and other APC/C\textsuperscript{Cdc20} substrates through interphase. Second, APC/C\textsuperscript{Cdc20} may be inactivated properly, but APC/C\textsuperscript{Cdhl} may not be inactivated at the G\textsubscript{1}/S boundary, leading to the destruction of cyclin B and other APC/C\textsuperscript{Cdhl} substrates and consequent failure of nuclei to enter S phase. Either model would result in affected nuclei being taken out of the cell cycle as is the case with \textit{mipA\textsubscript{D159}}. To determine which, if either of these models is correct and, thereby, take a step toward elucidating the function of \(\gamma\)-tubulin in APC/C regulation, I first needed to obtain basic information on the localization and functions of the \textit{A. nidulans} orthologs of Cdhl and Cdc20.

4.2. Results

4.2.1. Identification of \textit{cdc20} and \textit{cdh1} Orthologs in \textit{A. nidulans}

The identification of the \textit{A. nidulans} Cdc20 homolog is covered in Chapter 3, and it is encoded by AN0814. I will refer to the gene as \textit{cdc20} and its product Cdc20. Similarly, a BLASTP search with \textit{S. cerevisiae} Cdhl (P53197.1 in the NCBI protein database) revealed the predicted protein product of AN2965 to be a strong homolog (E value 9e\textsuperscript{-141}). Cdhl orthologs from mammals showed even stronger identity with the
predicted protein product of AN2965 (E value of $5 \times 10^{-162}$ when using the *H. sapiens* Cdh1 protein sequence Q9UM11.2). The AN2965 gene will be referred to as *cdhA* and its protein product as CdhA. Cdc20 and Cdh1 are closely related proteins, so there was some possibility of cross-identification (meaning AN2965 was actually the *cdc20* ortholog and AN0814 was the *cdh1* ortholog). However, my localization and functional analyses described in Chapter 3 and in detail below indicate that my initial ascriptions are correct.

### 4.2.2. *cdc20* is Essential But *cdhA* is Not

To determine if *cdc20* and *cdhA* are essential genes in *A. nidulans*, I attempted to delete each of them by replacing them with selectable markers from *A. fumigatus*. When I attempted to replace *cdhA* with the *A. fumigatus* pyrG gene (*AfpyrG*) in the recipient strain LO1501, I obtained numerous transformants. These were verified by diagnostic PCR and Southern hybridizations to contain the *cdhA::AfpyrG* gene replacement (Fig. 4.1, C-D). Thus, *cdhA* is not essential in *A. nidulans*. *cdhA*Δ strains grew at all temperatures tested, from 20-42°C, but did show a slight reduction in growth at 42°C (Fig. 4.2).

When I attempted to replace *cdc20* with the *A. fumigatus* riboB gene (*AfriboB*) in the recipient strain LO1711, I obtained relatively few transformants. The small number of transformants suggested that *Ancdc20* might be essential, meaning the correct transformants were dying rather than forming colonies. To determine if *cdc20* is indeed essential, I used the heterokaryon rescue technique (Jung et al., 2001; Martin et al., 2003) (Jung et al., 2001; Martin et al., 2003; Oakley et al., 1990; Osmani et al., 2006b; Osmani...
et al., 1988). When essential genes are deleted in *A. nidulans*, heterokaryons carrying nuclei that do not carry the deletion (untransformed, parental nuclei) as well as nuclei that do carry the deletion often form. Hyphae will grow because since they are multinucleate, the lethal gene replacement will be complemented by the parental, *cdc20*<sup>+</sup> nuclei. For example, in the case of *cdc20::AfriboB*, if *cdc20* is essential, heterokaryon hyphae, which carry both *cdc20::AfriboB* (*cdc20Δ*) nuclei and untransformed (*cdc20<sup>+</sup>*) nuclei will grow on media without riboflavin. *Aspergillus* conidia, however, are uninucleate and conidia from the heterokaryon colonies would either carry *cdc20*<sup>+</sup>, which would need riboflavin to grow, or *cdc20Δ*, which if *cdc20* is essential, would not support growth. In summary, if *cdc20* is essential, transformant hyphae will grow on media lacking riboflavin, but the conidia from these colonies will not grow on the same media lacking riboflavin. I tested six transformant colonies and found all six had hyphae that would grow on media lacking riboflavin, but the conidia would not. These heterokaryons were verified by Southern hybridizations (Fig. 4.1, A-B) and also by diagnostic PCR with multiple primer pairs. I repeated these experiments, transforming the same gene replacement construct into two other strains, LO2073 and TN02A7, and obtained similar results. Therefore, *cdc20* is essential in *A. nidulans*. This was expected because Cdc20 is also essential in mouse (Li et al., 2007) and in yeast (Hartwell et al., 1973; Kim et al., 2010). Since *cdc20* is essential, I chose to focus on CdhA.

### 4.2.3. Localization of CdHA-GFP

To determine the localization of CdHA throughout the cell cycle, I fused GFP in frame with the 3’ end of the *cdhA* gene. This fusion was the only copy of *cdhA* in the
genome and was determined to be fully functional as it supported normal growth at all temperatures tested from 20-42°C (Fig. 4.3). To correlate CdhA-GFP localization with cell cycle stages, I created strains LO1805 & LO1806 that carried both CdhA-GFP and histone H1-mRFP. Long-term live time lapse imaging at 25°C revealed that CdhA-GFP localized to the nucleoplasm but was also concentrated at the SPB in G2 (Fig. 4.4A, A-C; 4.4B). Imaging at shorter time intervals, I determined that CdhA-GFP disappeared from the SPB 4 ± 2 minutes (mean ± standard deviation) before chromosomal condensation (n = 26; Fig. 4.5 A-C). I was also able to determine the localization of CdhA-GFP during mitosis. It began to be visible at SPBs in anaphase and was also apparent at the spindle region in late anaphase/telophase (Fig. 4.4A, D-F; Fig. 4.5 D-I). CdhA-GFP remained visible at the SPB for 39 ± 12 minutes after mitosis (n = 79; Fig. 4.4A, G-I). I determined from the same data sets that the length of the cell cycle is 199 ± 49 minutes (n = 67) at 25°C. Therefore, CdhA is visible at the SPB after mitosis for about 20% of the cell cycle. Bergen and Morris (1983) calculated that G1 of A. nidulans lasts approximately 15% of the cell cycle at 32°C. The disappearance of CdhA-GFP from the SPB, therefore, corresponds reasonably well to the expected timing of the G1/S transition. When CdhA-GFP disappeared from the SPB, it still remained faintly visible in the nucleoplasm throughout the rest of interphase (Fig. 4.4A, J-L). It reappeared at the SPB 48 ± 16 minutes before mitosis (n = 36; Fig. 4.4A, M-O). If one takes mitotic exit as one’s starting point, CdhA reappeared 74.2% through the cell cycle. Bergen and Morris (1983) calculated G1 and S to occupy approximately 55% of the cell cycle at 32°C, so CdhA-GFP reappears at the SPB in G2. Since this localization pattern of CdhA is very
different from that of the APC/C (Nayak et al., 2010), it indicates that CdhA has its own localization determinants and its localization is not dependent on the APC/C.

4.2.4. APC/C-CdhA Targets Cyclin B for Destruction in G1

As mentioned previously, it is known that in many organisms APC/C-Cdh1 prevents S-phase cyclins from accumulating in G1, preventing the initiation of S [reviewed in (Li and Zhang, 2009)]. In *A. nidulans*, cyclin B is an S-phase cyclin as well as a mitotic cyclin (Osmani et al., 1994). It accumulates in the nucleoplasm and at the SPB during S phase and stays there in G2 until it is destroyed during mitosis (De Souza et al., 2009; Nayak et al., 2010). Therefore, the fraction of tip cells (the only cells actively passing through the cell cycle) in which cyclin B is absent reflects the length of G1 (Nayak et al., 2010). If APC/C-CdhA targets cyclin B for destruction in G1, then deletion of *cdhA* would allow cyclin B to accumulate earlier, and the percentage of tip cells with nuclei lacking cyclin B would be reduced.

To test this hypothesis, I grew *cdhA*+ and *cdhA*Δ strains expressing histone H1-mRFP (to serve as a nuclei marker) and cyclin B-GFP for 21 hours at 25°C. I then captured Z-series stacks of random fields during a one hour period and scored tip cells for the presence or absence of cyclin B-GFP in their nuclei (Fig. 4.6). Two *cdhA*+ strains, LO1438 and LO3317, gave essentially identical data. The mean percentage of tip cells with nuclei that did not contain visible cyclin B-GFP was 39.9 ± 5.5% (mean ± standard deviation of six experiments, three for each strain). These data are virtually identical to that published by Nayak et al. (2010). In comparison, 12.8 ± 3.5% of hyphal tips in a *cdhA*Δ strain (LO2869) had nuclei without visible cyclin B-GFP (mean ± standard
deviation of three experiments). This difference is highly significant (p = 0.00008, unpaired Student’s t-test). Thus, deletion of cdhA shortens the period of time in which cyclin B is absent from nuclei and, therefore, it is safe to conclude that APC/C^{CdhA} normally targets cyclin B for destruction in G_1. Given that mitosis at 25°C occupies approximately 5% of the cell cycle (unpublished data from our lab), cyclin B is only undetectable in 7-8% of interphase tip cell nuclei of cdhAΔ strains. It may be accumulating in this short period of time but below the level we can detect. I can conclude that CdhA plays a key role in preventing the premature accumulation of cyclin B in G_1. APC/C^{CdhA}, therefore, targets cyclin B for destruction in G_1, preventing the onset of S, similarly to APC/C^{Cdh1} targeting S-phase cyclins for destruction in G_1 in other organisms.

In addition, I wanted to determine if mipAD159 altered the timing of cyclin B-GFP accumulation in cdhA^+ and cdhAΔ tip cell nuclei. As with the mipA^+ strains just described, I grew strains at 25°C, a restrictive temperature for mipAD159, for 21 hours and captured Z-series stacks of random fields over a one hour period and scored tip cell nuclei for the presence or absence of cyclin B-GFP (Fig. 4.6). Since mipAD159 causes a constitutively active APC/C in a subset of nuclei in the same tip cell, nuclei with a constitutively active APC/C will not accumulate cyclin B while others in the same cell will. For this reason, I scored tip cells as positive if any nuclei in the cell contained cyclin B-GFP. I then subtracted this percentage from 100% to get the percentage of tip cells that were in G_1. In a cdhA^+, mipAD159 strain, LO1439, 39.3 ± 11.4% (mean ± standard deviation of three experiments) of hyphal tip cells were in G_1. This was
essentially identical to the $cdhA^+, mipA^+$ strains. In a $cdhA\Delta, mipAD159$ strain, LO5442, 9 ± 3.6% (mean ± standard deviation of three experiments) of tip cells were in G1, similar to the $mipA^+, cdhA\Delta$ strain. Thus, $mipAD159$ does not appear to affect the timing of accumulation of cyclin B; it just prevents some nuclei from accumulating it at all.

I also wished to determine if the entire cell cycle duration was shortened in $cdhA\Delta$ strains. Through time-lapse imaging of the $cdhA\Delta$ strain, LO2019, the cell cycle timing was determined to be 178 ± 39 minutes (Fig. 4.7). As reported in section 4.2.3, the cell cycle timing of a $cdhA^+$ strain, LO1806, was determined to be 199 ± 49 minutes. Despite overlapping standard deviations, the difference in cell cycle lengths between $cdhA\Delta$ and $cdhA^+$ strains (21 minutes) is statistically significant ($p = 0.005$, unpaired Student’s $t$-test). This is consistent with the idea that deletion of $cdhA$ shortens G1 and allows premature entry into S as expected based on data from other organisms [(Bashir et al., 2004; Blanco et al., 2000; Kitamura et al., 1998; Schwab et al., 1997; Sigl et al., 2009; Sudo et al., 2001; Visintin et al., 1997; Wäsch and Cross, 2002; Wei et al., 2004); reviewed in (Li and Zhang, 2009; Pines, 2011; Wäsch et al., 2010)].

4.2.5. $CdhA$ is Required to Block Subapical Nuclei in G1

In $A. nidulans$ only tip cells are actively going through the cell cycle whereas subapical cells are blocked in G1 and therefore do not accumulate cyclin B (Nayak et al., 2010). Subapical cells re-enter the cell cycle and resume interphase once side branches emerge from them. Since very little is known about the G1 blockage mechanism in subapical nuclei, and given the fact that CdhA is required to target cyclin B for destruction in G1 to prevent the initiation of S-phase in tip cells, I wished to determine if
CdhA is required to prevent the accumulation of cyclin B in subapical cells. I examined subapical cells in both a cdhAΔ and a cdhA⁺ strain (both expressing cyclin B-GFP and histone H1-mRFP) by taking Z-series stacks of random fields of each strain. Surprisingly, in a cdhAΔ strain, LO2869, I found that subapical cells did accumulate cyclin B-GFP (Fig. 4.8). It was detectable in 98 ± 2.4% (mean ± standard deviation of three experiments) of subapical cells (n = 80 individual subapical cells). In a cdhA⁺ strain, LO3317, cyclin B-GFP was present only in nuclei of 2.9 ± 2.6% (mean ± standard deviation of three experiments) of subapical cells (n = 90 individual subapical cells). Thus, this reveals that CdhA is required for the prevention of cyclin B accumulation in subapical cells. CdhA is also not required for cyclin B to accumulate at SPBs as its localization there was not affected in the cdhAΔ strain (Fig. 4.8, arrowheads). I did note through time lapse imaging that even though nuclei in subapical cdhAΔ cells accumulate cyclin B-GFP, they do not go through mitosis. This indicates that there must be at least one mechanism in addition to the absence of cyclin B accumulation that blocks the cell cycle in subapical nuclei. In cdhAΔ hyphae, as in cdhA⁺ hyphae, when a side branch forms the cell cycle resumes and the cell becomes mitotically active.

4.2.6. mipAD159 Causes a Failure of Inactivation of APC/C<sup>CdhA</sup>

I next wished to test the hypothesis that mipAD159 causes a failure of inactivation of the APC/C at restrictive temperatures due to a nuclear autonomous failure of inactivation of APC/C<sup>CdhA</sup>, which results in destruction of cyclin B throughout the cell cycle. If this hypothesis is correct, deletion of cdhA should prevent constitutive APC/C<sup>CdhA</sup> activity and therefore would reverse the constitutively active APC/C caused
by \textit{mipAD159}. Cyclin B, as well as other APC/C\textsuperscript{CdhA} targets would then be able to accumulate in all tip cell nuclei in \textit{mipAD159} strains grown at restrictive temperatures.

To test this hypothesis, I used strains expressing cyclin B-GFP and histone H1-mRFP and also carrying \textit{mipA}\textsuperscript{+} or \textit{mipAD159} and \textit{cdhA}\textsuperscript{+} or \textit{cdhA}\textDelta. I grew strains at 25°C, a restrictive temperature for \textit{mipAD159}, for 21 hours and collected Z-series stacks of random fields for a one hour period. I then scored tip cells in which at least one nucleus contained visible cyclin B-GFP (Fig. 4.9). These cells are in S or G\textsubscript{2} and are still actively going through the cell cycle. As expected, in \textit{mipA}\textsuperscript{+} strains LO1438 and LO3317, only 1.5 ± 1.6\% of nuclei (mean ± standard deviation of six experiments, three for each strain) were cyclin B negative (CB\textsuperscript{−}). CB\textsuperscript{−} indicates nuclei that fail to accumulate cyclin B when other nuclei in the same cell accumulate it normally. In a \textit{cdhA}\textsuperscript{+}, \textit{mipAD159} strain, LO1439, 45.2 ± 13.0\% of nuclei were CB\textsuperscript{−} (mean ± standard deviation of three experiments). These values are in agreement with the data presented by Nayak et al. (2010), and they confirm that \textit{mipAD159} causes constitutive APC/C activity at restrictive temperatures resulting in the continuous destruction of cyclin B. In \textit{cdhA}\textDelta, \textit{mipAD159} strains, however, only 0.8 ± 0.7\% of nuclei were CB\textsuperscript{−} [three strains were used (LO5542, LO5543, and LO5544), values are mean ± standard deviation of nine experiments, three for each strain]. Thus, deletion of \textit{cdhA} reduces the numbers of CB\textsuperscript{−} nuclei to wild-type levels in \textit{mipAD159} strains at 25°C. \textit{mipAD159}, therefore, causes a failure of inactivation of APC/C\textsuperscript{CdhA} at restrictive temperatures.
4.2.7. *mipAD159 Causes a Failure of Dissociation of CdhA from the SPB at G1/S*

In *mipA*+ cells, as mentioned previously, CdhA is present at the SPB early in G1, and it is also apparent faintly in the nucleoplasm. CdhA disappears from the SPB at the time of the G1/S transition, when APC/C^{CdhA} is now inactive, allowing the accumulation of cyclin B. Therefore, CdhA is at the SPB at times when APC/C^{CdhA} is predicted to be active. In fact, it has been suggested that Cdh1 is activated at the centrosome in *Drosophila* (Raff et al., 2002). If the SPB localization of CdhA is important for its activity, *mipAD159* might alter its function by altering its localization.

To investigate this hypothesis, I created a *mipAD159* strain that expressed CdhA-GFP, Cdk1-mCherry, and nup49-mCherry (LO6256). Cdk1-mCherry is used as a marker for nuclei with a constitutive APC/C. It has essentially the same localization pattern as cyclin B (Nayak et al., 2010) as they bind together (Nurse, 1990); therefore, if cyclin B is continuously destroyed, Cdk1 fails to accumulate as well (Nayak et al., 2010). Even though Cdk1 and nup49 are both fused with mCherry, they have distinct localization patterns-Cdk1 to the nucleoplasm and SPB in S through G2 until mitosis (Nayak et al., 2010) and nup49 to the nuclear periphery throughout the cell cycle until mitotic entry (De Souza et al., 2009; Osmani et al., 2006a). Nup49-mCherry, therefore, can serve as a nuclear marker in this case. Time-lapse imaging of this *mipAD159* strain at 25°C revealed that in Cdk1 negative (Cdk1−) nuclei, CdhA-GFP remained at the SPB and in the nucleoplasm throughout the cell cycle. As in *mipA*+ strains, CdhA-GFP was brightest and at the SPB in early G1 immediately after mitosis. It was also visible in the nucleoplasm. However, in S phase when Cdk1-mCherry started to accumulate in nuclei
that had normal APC/C activity, at a time in \textit{mipA}^+ strains when CdhA-GFP would only be present in the nucleoplasm, Cdk1^- nuclei retained CdhA-GFP at the SPB, and it was at a higher concentration in the nucleoplasm than in Cdk1^+ nuclei (Fig. 4.10A). These data reveal that there is a correlation between CdhA dissociation from the SPB and the inactivation of APC/C^{CdhA}. I hypothesize that \textit{mipA}D159 causes a failure of dissociation of CdhA from the SPB at the G1/S boundary, which, in turn, results in constitutive activation of APC/C^{CdhA}.

Interestingly, in mitosis, as judged by the dispersal of Nup49-mCherry, CdhA-GFP disappeared from the SPB and nucleoplasm of Cdk1^- nuclei in \textit{mipA}D159 cells as in Cdk1^+ nuclei in the same cells and as in all nuclei in wild type cells (Fig. 4.10B). Once nuclei become CB^- in \textit{mipA}D159 strains, they remain negative in subsequent cell cycles (Nayak et al., 2010). Thus, even though CdhA disappears from such nuclei, they will remain CB^- in the next cell cycle. This suggests that such nuclei are somehow “marked” so that when they exit mitosis, they once again accumulate CdhA but fail to have it dissociate from the SPB at G1/S. It is possible that a stable, nuclear autonomous change occurs to, or at, the SPB that prevents the destruction of CdhA at the G1/S boundary.

4.2.8. \textit{cdhA}Δ Restores Cyclin B Accumulation to All \textit{mipA}D159 Nuclei, But Does Not Rescue the Cold-sensitivity of this Allele

Since deletion of \textit{cdhA} decreases the percentage of CB^- nuclei to wild-type levels in strains carrying \textit{mipA}D159, I wondered whether \textit{cdhA}Δ would rescue the growth of
mipAD159 strains at restrictive (20°C and 25°C) or sub-permissive (30°C) temperatures. I stabbed a variety of strains carrying mipA+/mipAD159 and cdhA+/cdhAΔ on complete media plates (YAG) and incubated them at 20°, 25°, 30°, 37°, and 42°C. I found that the deletion of cdhA does not suppress the growth defect of mipAD159 at restrictive or sub-permissive temperatures (Fig. 4.11, arrows). Therefore, the cold-sensitivity of mipAD159 strains is not just due to a constitutive activity of APC/C^{CdhA}.

4.3. Discussion

Previous data from our lab revealed that γ-tubulin has a key role in inactivating the APC/C in interphase, specifically between late mitosis and S (Nayak et al., 2010). The data presented here clarify γ-tubulin’s function in that they demonstrate that γ-tubulin has a significant role in inactivating APC/C^{CdhA} at the G1/S boundary.

The key finding in my experiments was that in mipAD159 strains grown at a restrictive temperature, deletion of cdhA reduces the number of CB⁻ nuclei to the very low number seen in mipA⁺ strains (45.2% CB⁻ nuclei for mipAD159 versus 0.8% for mipA⁺). These data reveal that CdhA is required for the constitutive activation of the APC/C that is caused by mipAD159. My data also indicate that, as in other organisms, inactivation of APC/C^{CdhA} is required for the G1 to S transition. It follows that, at restrictive temperatures, mipAD159 causes a nuclear autonomous failure of inactivation of APC/C^{CdhA}, resulting in constitutive destruction of cyclin B and other APC/C^{CdhA} substrates. From these data we can conclude that γ-tubulin has an important role in inactivating APC/C^{CdhA} and, thus, in regulating the G1 to S transition.

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The constitutive activation of APC/C\(_{\text{CdhA}}\) correlates with a failure of CdhA to dissociate from the SPB. Also, since the \(\gamma\)-tubulin encoded by the \textit{mipAD159} allele localizes normally to the SPB (Jung et al., 2001), and given the fact that \textit{mipAD159} nuclei with a constitutively active APC/C retain CdhA at the SPB, it is likely that \(\gamma\)-tubulin’s function in inactivating APC/C\(_{\text{CdhA}}\) occurs at the SPB and my data are consistent with the possibility that the localization of CdhA at the SPB is necessary for APC/C\(_{\text{CdhA}}\) activity. CdhA is at the SPB from late mitosis until the G\(_1/S\) boundary, when APC/C\(_{\text{CdhA}}\) is predicted to be active. When APC/C\(_{\text{CdhA}}\) is inactivated at G\(_1/S\), CdhA leaves the SPB. Interestingly, CdhA re-localizes to the SPB in G\(_2\), a time in which it is not predicted to be active. However, at this time period, cyclin B and Cdk1 have accumulated, and they are known to inactivate Cdh1 by phosphorylating it (Zachariae et al., 1998).

Although Cdh1 complexes with the APC/C in other organisms, the two have different localization patterns in \textit{A. nidulans}. The APC/C is predominantly concentrated in the nucleus throughout the cell cycle as judged by the localization of BimA, the \textit{A. nidulans} homolog of the APC/C component APC3 (Nayak et al., 2010). Therefore, CdhA has its own localization determinants and does not depend on the APC/C for its localization to the SPB. The association of CdhA with the APC/C must also be transient. If it were a stable association, then both would show similar localization patterns at least from late mitosis to the end of G\(_1\).

There is accumulating evidence that PMTOCs, such as the SPB and centrosome, and PMTOC proteins, such as \(\gamma\)-tubulin complex proteins, are not simply microtubule
organizing centers but also have a role in cell cycle regulation (Cuschieri et al., 2007; Maniotis and Schliwa, 1991; Nayak et al., 2010), in particular at the G1/S transition (Doxsey et al., 2005b; Ferguson and Maller, 2010; Matsumoto and Maller, 2004; Mikule et al., 2007; Rieder et al., 2001; Sluder, 2005; Vardy and Toda, 2000). However, the mechanism(s) by which PMTOCs regulate this transition are not completely understood. For example, PMTOCs could be involved in a signal transduction pathway that senses cell cycle events and inactivates the APC/C at G1/S. Raff et al. (2002) have suggested that Cdh1 is activated at centrosomes, and that active Cdh1 diffuses from the centrosome, leading to the destruction of cyclin B. Therefore, γ-tubulin may have a role in both activating and inactivating Cdh1 at PMTOCs.

The fact that mipAD159 is a recessive allele indicates that it is a loss-of-function allele (Jung et al., 2001), although the loss could be partial and, if γ-tubulin has more than one function, some functions might be intact. If CdhA bound directly to γ-tubulin, a loss-of-function γ-tubulin allele would be predicted to cause a loss of CdhA at the SPB rather than it being retained there inappropriately in CB⁻ nuclei. It is, therefore, unlikely that the failure of CdhA to leave the SPB reflects an alteration of binding of γ-tubulin to CdhA. An explanation more consistent with my data is that mipAD159 is defective in activation of an inhibitor required for destruction or dissociation of CdhA from the SPB.
A. Genomic DNA was isolated from putative $cdc20\Delta$ transformant heterokaryons and digested with EcoRV. The $cdc20::AfriboB$ fusion PCR product was radioactively labeled and used as a probe. Arrows show predicted EcoRV sites for the $cdc20$ deletion and the wild-type control. B. With DNA from two putative transformants (Δ1 and Δ2) the probe hybridized to bands of the size expected for $cdc20\Delta$ (1.7 kb, 4.0 kb) and wild-type $cdc20$ (2.2 kb, 5.2 kb). These data verify that these transformants are heterokaryons and therefore indicate that $cdc20$ is essential. FGSC4 DNA was used as wild-type control. $\lambda$ is radioactively labeled bacteriophage $\lambda$ DNA that has been digested with HindIII. C.

Figure 4.1: Southern hybridizations verify that $cdc20$ is essential and $cdhA$ is not
Genomic DNA was isolated from putative cdhAΔ strains and digested with PstI. The cdhA::AfpyrG fusion PCR product was radioactively labeled and used as a probe. Arrows show predicted PstI sites for the cdhA deletion and the wild-type control. D. The probe hybridized to bands of the size expected for cdhAΔ alone (1.9 kb and 4.7 kb versus the wild-type band of 6.7 kb). This verifies that cdhA is not essential and cdhAΔ strains are viable. LO1804 was a putative cdhAΔ transformant, whereas LO2869 and LO2415 were progeny of crosses predicted to carry cdhAΔ. The extra bands in the LO2869 lane are due to the AfpyrG portion of the probe hybridizing to cyclin B-GFP-AfpyrG (5.3 kb, 12.6 kb), which this strain also carries. Radioactively labeled λ DNA digested with HindIII was not used in this gel; however, a picture of the gel was taken before the addition of probe so that the band sizes could be determined. These are marked (in kb) to the left of the gel. R153 DNA was used as a wild-type control.
Strains containing cdhAΔ as well as a control strain were stabbed onto complete media and incubated at the temperatures shown. Strain numbers are shown in the bottom right panel. The times of incubation were different at the different temperatures to partially compensate for different growth rates at the different temperatures. cdhAΔ strains are slightly restricted for growth relative to WT at 42°C. LO1803 and LO1804 carry cdhAΔ and are two of the original transformants of the cdhA::AfpyrG construct, which was put into a fawn-colored strain. LO2019 carries cdhAΔ and histone H1-mRFP. LO2869, which grew slightly worse at low and high temperatures, carries cdhAΔ, histone H1-mRFP, and cyclin B-GFP. The WT control is FGSC4, which is Glasgow wild-type.

Figure 4.2: cdhAΔ strains are viable
Transformants and control strains were stabbed onto complete media and incubated at the temperatures shown. The times of incubation were different to partially compensate for different growth rates at the different temperatures. At each temperature tested, strains carrying CdhA-GFP grew similarly to WT (FGSC4 is Glasgow wild-type). The top row contains two of the original transformants of CdhA-GFP, in which the fusion construct was put into a fawn-colored strain.

Figure 4.3: CdhA-GFP fusions appear to be fully functional
Figure 4.4: Localization of CdhA-GFP throughout the cell cycle
A. Live time-lapse imaging reveals that CdhA-GFP is present in the nucleoplasm and at the SPB in G₂ (A-C). 20 minutes later in late anaphase/telophase, it is present at SPBs and at the spindle region between the separating chromatin masses (D-F). The bottom two nuclei in D-F are actually at the M to G₁ transition, and CdhA-GFP is present brightly on SPBs but not evident in the nucleoplasm. By 30 minutes, in early G₁, CdhA-GFP is still at SPBs but is also faintly visible in the nucleoplasm (G-I). The SPB signal is lost by G₁/S such that in S phase (80 min. time point, J-L), CdhA-GFP is only faintly visible in the nucleoplasm. In G₂ (150 min.), it reappears at SPBs (M-O). Images in A. are Z-stack projections from a time-lapse data set collected at 10 min intervals. The strain is LO1806 and was incubated and imaged at 25°C. B. CdhA-GFP co-localizes with Cdk1-mCherry at SPBs in G₂ (arrows). Cdk1 has previously been determined to co-localize with cyclin B at SPBs in S and G₂ (Nayak et al., 2010). Therefore, we can say with confidence that the foci to which CdhA localizes in G₂ nuclei is the SPB.
Figure 4.5: Localization of CdhA-GFP in mitosis

continued
Figure 4.5 continued

In metaphase (0 min.), CdhA-GFP is barely detectable (A-C). One minute later in anaphase (D-F), it is visible at three of four SPBs (arrows), and a small amount is present between separating chromatin masses (arrowheads in D and F). This spindle-like localization was not seen in many nuclei, possibly because this localization is extremely brief. In telophase (2 min. time point, G-I), CdhA-GFP is present at the SPBs of each daughter nucleus. Images are Z-stack projections from a time-lapse data set of strain LO1806 collected at one minute intervals. LO1806 was incubated and imaged at 25°C.
Strains were incubated for 21 hours at 25°C, a restrictive temperature for \textit{mipA}D159. Z-series stacks of random fields were captured during a one hour period and tip cells were scored for the presence or absence of cyclin B-GFP in nuclei. Comparing a \textit{cdhA}+, \textit{mipA}+ strain with a \textit{cdhA}Δ, \textit{mipA}+ strain, fewer tip cells lack cyclin B in the \textit{cdhA}Δ strain. The period of the cell cycle in which cyclin B is absent is, thus, shorter in \textit{cdhA}Δ strains. This difference in cyclin B accumulation is highly significant (*** = p value of 0.00008). \textit{mipAD159} does not alter the timing of cyclin B accumulation in \textit{cdhA}+ or \textit{cdhA}Δ strains as the percentage of tip cells with nuclei with visible cyclin B-GFP in the \textit{cdhA}+,

![Figure 4.6: Deletion of \textit{cdhA} shortens the period of time in which cyclin B is absent in the cell cycle](image)
Figure 4.6 continued

*mipAD159* strain was identical to the *cdhA+*, *mipA+* strains. Likewise the *cdhAΔ*,
*mipAD159* strain had a similar percentage of tip cells with nuclei that were cyclin B-GFP
persistent to the *cdhAΔ, mipA+* strain. *mipAD159* tip cells do contain some nuclei that do
not accumulate cyclin B due to a constitutively active APC/C. I therefore scored
*mipAD159* tip cells as cyclin B positive if any nuclei in the cell contained cyclin B-GFP.

Values are means and error bars are standard deviations. *cdhA+, mipA+* data are from six
experiments with two different strains. Data from the *cdhAΔ, mipA+; cdhA+, mipAD159;*
and *cdhAΔ, mipAD159* strains are from three experiments each.
Cell cycle duration was calculated using time-lapse images collected at 10 minute intervals of both a cdhA\(^+\) and a cdhA\(\Delta\) strain. Cell cycle duration was the amount of time from the end of one mitosis to the end of the next mitosis. A cdhA\(^+\) strain had a cell cycle time of 199 minutes versus a cell cycle time of 178 minutes in a cdhA\(\Delta\) strain. This difference in cell cycle timing is statistically significant (** = p value of 0.005) and is consistent with the deletion of cdhA shortening or eliminating G\(_1\). Values are means and error bars are standard deviations.

Figure 4.7: Deletion of cdhA results in a shorter cell cycle duration
A. Z-stack projection images showing differences in cyclin B accumulation in subapical cells. Cyclin B-GFP does not accumulate in nuclei of subapical cells in a cdhA\(^+\) strain, continued

Figure 4.8: Subapical cdhA\(\Delta\) nuclei are able to accumulate cyclin B.

A. Z-stack projection images showing differences in cyclin B accumulation in subapical cells. Cyclin B-GFP does not accumulate in nuclei of subapical cells in a cdhA\(^+\) strain, continued
Figure 4.8 continued

but it does accumulate in such nuclei in a \textit{cdhA} \textit{Δ} strain. In fact, it accumulates in the nucleoplasm and at SPBs (arrowheads) indicating that CdhA is not required for cyclin B’s accumulation to the SPB. Arrows in the bright field images (which are taken from a single plane of the Z-stack collection) indicate septa, showing the boundaries of each cell and revealing that what are pictured are indeed subapical cells. B. Quantification of the percentage of subapical cells that have nuclei that have accumulated cyclin B-GFP. Both strains were incubated at 25°C for 21-21.5 hours. Z-stacks of random fields were then taken for a one hour period and subapical nuclei were scored for the presence or absence of cyclin B-GFP. Values are means and error bars are standard deviations of three experiments for each strain.
A. *cdhA*, *mipAD159* tip cell contains two nuclei with a constitutively active APC.

Figure 4.9: Deletion of *cdhA* restores cyclin B accumulation in *mipAD159* nuclei

A. *cdhA*, *mipAD159* tip cell contains two nuclei with a constitutively active APC.

continued
Figure 4.9 continued

Such CB' nuclei (arrows) do not accumulate cyclin B. The histone H1-mRFP panel of this strain shows three nuclei are actually present in this cell, even though they are different in size due to unequal segregation of chromosomes. This is a common phenotype of $mipAD159$ strains (Nayak et al., 2010; Prigozhina et al., 2004). A $cdhA\Delta$, $mipAD159$ strain, however, has tip cells in which all nuclei accumulate cyclin B-GFP. Fluorescence images are projections of Z-series stacks of strains grown at 25°C, a restrictive temperature for $mipAD159$. Bright field images are from single planes of the Z-stacks. Septa (arrowheads) reveal that each cell is a tip cell. B. Quantification of CB' nuclei. All strains were grown at 25°C for 21 hours before Z-series stacks of random fields were collected over a one hour period at 25°C. Nuclei in tip cells were scored for the presence or absence of cyclin B-GFP. Values are means and error bars are standard deviations. $cdhA^+, mipA^+$ data are from six experiments with two different strains; $cdhA^+, mipAD159$ from three experiments; $cdhA\Delta$, $mipAD159$ from nine experiments with three different strains.
Figure 4.10: CdhA stays in the nucleoplasm and at the SPB in mipAD159, Cdk1 negative nuclei

A. A projection image of a single time point Z-series stack shows a tip cell containing
three nuclei. The periphery of each nucleus is shown by the localization of nup49-mCherry. The nucleus at the top is positive for Cdk1-mCherry (visible in the nucleoplasm and at the SPB), but has little or no CdhA-GFP signal indicating that the cell is in S or early G2. The bottom two nuclei are negative for Cdk1-mCherry (designated Cdk1\(^-\); the red fluorescence is only from the nuclear periphery localization of nup49-mCherry). However, in these two nuclei, CdhA-GFP is present both at the SPB (arrows) and in the nucleoplasm. B. Time-lapse imaging reveals that CdhA leaves the nucleus and SPB during mitosis in CB\(^-\) nuclei. At the 0 min time point, a Cdk1\(^-\) nucleus (note only the nuclear periphery localization of nup49-mCherry) has CdhA-GFP at the SPB (arrows) and in the nucleoplasm. Although they are not shown, there are other nuclei in this tip cell that have accumulated Cdk1-mCherry, indicating the cell is in S or G2. 20 minutes later, the nucleus pictured has entered mitosis (note the dispersal of nup49-mCherry) and there is no detectable CdhA-GFP signal. In \textit{mipA}\(^+\) nuclei and in \textit{mipAD159} Cdk1\(^+\) nuclei, this is a point in the cell cycle in which CdhA-GFP would not be present. Therefore, CdhA is dispersing or is being destroyed in mitosis as would normally occur. In the next interphase, after a failed division, CdhA-GFP returns to the SPB (arrows) and nucleoplasm. As reported in Nayak et al. (2010) CB\(^-\) (and therefore Cdk1\(^-\)) nuclei are permanently taken out of the cell cycle, and they remain CB\(^-\) while other nuclei in the same cell go through multiple cell cycles. Such nuclei are kept in G1 and, therefore,
Figure 4.10 continued

accumulate CdhA but do not progress into S. Images here are Z-stack projection images from a time lapse data set collected at 10 minute intervals.
Figure 4.11: Deletion of cdhA does not rescue the cold-sensitivity of
mipAΔD159 strains at restrictive (20°C and 25°C) or sub-permissive (30°C) temperatures.

<table>
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<th>Strain #</th>
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<td>mRFP</td>
<td>Δ</td>
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Figure 4.11 continued

A variety of strains carrying cdhA+ or cdhAΔ and mipA+ or mipAD159 alleles were stabbed on complete media at the temperatures indicated to the left. The strains were stabbed onto complete media and incubated for various times to partially compensate for differences in growth rates at the different temperatures. The table gives the relevant genotype of each strain pictured. GFP refers to the strain having cyclin B fused to GFP, mRFP refers to the strain having histone H1 fused to mRFP, Δ indicates the strain carries cdhAΔ, D159 designates the strain carries the mipAD159 allele, and WT means the strain does not carry a GFP fusion or mRFP fusion, and has a wild-type copy of cdhA or mipA. The strains indicated by arrows, do not carry any fluorescent protein fusions and therefore can be compared without concern for possible effects of such fusions. FGSC4 (black arrow) is cdhA+, mipA+; LO1124 (red arrow) is cdhA+, mipAD159; and LO5571 (blue arrow) is cdhAΔ, mipAD159. cdhAΔ, mipAD159 strains were just as cold-sensitive as the cdhA+, mipAD159 control strain, LO1124, at 20°, 25°, and 30°C. The cold-sensitivity of mipAD159 strains is, therefore, not just due to constitutive APC/C^{CdhA} activity.

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I have focused on two main projects in my dissertation work, both of which have provided further insight into γ-tubulin’s role(s) in cell cycle regulation. By using molecular and cellular biology techniques, genetics, and time-lapse fluorescence microscopy, I was able to determine that γ-tubulin has a critical role in inactivating APC/C^Cdhl. Failure of inactivation of APC/C^Cdhl results in the destruction of APC/C^Cdhl substrates, which include proteins involved in the SAC. Destruction of SAC proteins by a constitutively active APC/C^Cdhl thereby prevents the establishment of a proper SAC in mitosis.

To establish these roles, I first had to fluorescently tag a number of cell cycle regulatory proteins and determine their localization patterns in a wild-type γ-tubulin (mipA^+) background. It is well-established that SAC proteins localize to KTs in early mitosis in other organisms with Mad2, Bub3, BubR1 and Cdc20 forming the mitotic checkpoint complex (MCC) and that MCC components bind hierarchically based on biochemical data and immunofluorescence (Fraschini et al., 2001; Hardwick et al., 2000; Kulukian et al., 2009; Musacchio and Salmon, 2007; Vigneron et al., 2004). Surprisingly, however, given the extreme importance of these proteins in mitotic regulation, there has been very little live imaging of these proteins during mitosis or through the cell cycle. My data provide the first localization reports of the SAC proteins
Mps1, Bub3, BubR1, and Cdc20 in filamentous fungi. Importantly, I found that there is spatial regulation of the SAC in *A. nidulans*. In interphase Mad2 and Bub3 are in the nucleus, but show different localization patterns. Both are physically separated from the other MCC components BubR1 and Cdc20, which are in the cytoplasm. The MCC, thus, cannot form until partial disassembly of the nuclear pore complex (NPC) at mitotic onset allows the components to come into physical contact. Likewise, Mps1 is physically separated from BubR1, Cdc20 and, probably, Mad2 in interphase and cannot interact with them until NPC disassembly at mitotic onset. The locations of the SAC components, thus, keep the SAC inactive until mitosis. These data point out the importance of the nuclear envelope in mitotic regulation in *A. nidulans*. Regulation of the assembly and disassembly of NPCs in *A. nidulans* has been studied extensively by the laboratory of Stephen Osmani, and they have found that NIMA kinase activity as well as Cdk1 activity is essential for mitotic entry (De Souza and Osmani, 2007; De Souza et al., 2000; Osmani et al., 1991). NIMA kinase activity is required for the dispersal of peripheral nucleoporins (Nups) during mitosis (De Souza et al., 2004). Inducing NIMA in cells blocked in S phase led to dispersal of the peripheral Nup, Nup98, and dsRed fused to a nuclear localization sequence, and depolymerized tubulin were able to enter nuclei (De Souza et al., 2004). All of these events are mitosis specific; therefore, NIMA expression is sufficient to lead to changes in the NPC composition and thereby allow nuclear transport as if the nuclei were in mitosis, not in interphase. Although I have not done the experiment, I hypothesize that if I constructed a strain that put NIMA under the control of the regulatable promoter *alcA*, grew the strain in non-inducing medium, arrested the cells
in S phase through the addition of hydroxyurea, and then switched the medium to inducing medium but still including hydroxyurea (De Souza et al., 2004) that, due to the activation of nuclear transport, I would see BubR1 and Cdc20 enter the nucleoplasm while Bub3 exits it, thus activating the SAC even though the nuclei are in S.

The SAC is a key cell cycle regulatory mechanism in eukaryotes. Many of its components are evolutionarily conserved from yeast to humans, whereas others like BubR1 and Mad3 are functional orthologs, or at least share key functions, but have quite different amino acid sequences. BubR1 has a C-terminal kinase domain that Mad3 lacks, suggesting BubR1 has an additional function (Harris et al., 2005; Mao et al., 2003) and/or it has evolved such that this kinase domain is important in higher eukaryotes for its function in the SAC (Mao et al., 2005; Weaver et al., 2003). Although it has not been thoroughly characterized functionally, all indications are that the A. nidulans protein encoded by sldA is a BubR1 homolog in that it is has N-terminal KEN boxes and the C-terminal kinase domain. BubR1 homologs, thus, do not appear to be restricted to higher organisms and these data raise the possibility that the A. nidulans SAC shares common features with SACs of higher organisms that are not shared by yeasts.

Although partial disassembly and assembly of NPCs in mitosis is not universal among fungi, nuclear envelope breakdown and reassembly are common features of mitosis in higher eukaryotes. Such organisms undergo an open mitosis in which the nuclear envelope is completely disassembled at mitotic entry and is not reassembled until after DNA segregation [reviewed in (Antonin et al., 2008; Wente and Rout, 2010)]. In mitosis of mammalian cells, several Nups are found at KT's where they have been
demonstrated to have mitotic roles [(Loïodice et al., 2004; Orjalo et al., 2006; Rasala et al., 2006); reviewed in (Chatel and Fahrenkrog, 2011)]. As in *A. nidulans*, mammalian Mad1 and Mad2 localize to NPCs in interphase and depletion of the NPC component, Tpr, in HeLa cells resulted in the loss of these proteins from the nuclear envelope (Lee et al., 2008). In mitosis, depletion of Tpr led to the dissociation of Mad2 from both Mad1 and from Cdc20, leading to premature activation of the APC/C (Lee et al., 2008). A study in *D. melanogaster* provided an alternative model (Lince-Faria et al., 2009). This study found that depletion of Megator, the *D. melanogaster* ortholog of Tpr, did not result in a loss of Mad1 from KTs in mitosis; however there was the same loss of Mad2 as in HeLa cells as well as an additional loss of Mps1. Mps1 kinase activity is required for the KT localization of Mad2 in HeLa cells (Tighe et al., 2008) and in *D. melanogaster* (Lince-Faria et al., 2009). Therefore, at least in *D. melanogaster*, Tpr affects mitotic Mad2 localization and thereby SAC activity not through Mad1 but through Mps1. Importantly, in living kangaroo rat kidney (PtK2) cells, *H. sapiens* BubR1 did not enter the nucleus and localize to KTs until after nuclear envelope breakdown (Howell et al., 2004). Taken together, these studies provide evidence that disassembly of the nuclear envelope in organisms that undergo an open mitosis provides regulation of mitotic events, including activation of the SAC. Likewise, the reassembly of the nuclear envelope in telophase/G1 re-establishes the physical barrier between the nucleoplasm and cytoplasm as well as active transport. As in *A. nidulans*, *X. laevis* Bub3 (Campbell and Hardwick, 2003) and *H. sapiens* Bub3 expressed in baby hamster kidney (BHK) cells (Taylor et al., 1998) or in PtK2 cells (Howell et al., 2004) is restricted to the nucleoplasm.
In addition, also as in *A. nidulans*, *D. melanogaster* BubR1 (Buffin et al., 2005) and *H. sapiens* BubR1 expressed in BHK cells (Taylor et al., 1998) as well as BubR1 in various normal human tissues (Burum-Auensen et al., 2007) is restricted to the cytoplasm in interphase. Taken together, this suggests that SAC regulation in animals and *A. nidulans* are similar.

My SAC protein localization data also indicate that APC/C activity in *A. nidulans* is regulated, at least in part, by the location of Cdc20. As shown in Fig. 3.12, Cdc20 enters the nucleus at the time NPCs are partially dispersing. Only during this time, when APC/C<sup>Cdc20</sup> is predicted to be active (Uhlmann, 2004), is Cdc20 at a higher level in the nucleoplasm than in the cytoplasm and present at the same location as the APC/C (Nayak et al., 2010). While the APC/C stays in the nucleoplasm at mitotic exit (Nayak et al., 2010), Cdc20 leaves the nucleoplasm. It follows that physical separation of Cdc20 from the APC/C is a probable mechanism of APC/C<sup>Cdc20</sup> inactivation at mitotic exit. Although the mechanism of APC/C<sup>Cdc20</sup> inactivation in other organisms is debated, one current model is that it is inactivated through APC/C<sup>Cdh1</sup> targeting Cdc20 for destruction in late mitosis (Pesin and Orr-Weaver, 2008; Prinz et al., 1998; Robbins and Cross, 2010; Shirayama et al., 1998; Weinstein, 1997). If such destruction occurs in *A. nidulans*, it is not essential for viability or mitotic exit as *cdhAΔ* strains are viable and mitosis is completed successfully as it is in a number of other organisms from yeast to mammalian cells that lack Cdh1 (Blanco et al., 2000; Floyd et al., 2008; Schwab et al., 1997; Sigl et al., 2009; Sigrist and Lehner, 1997; Visintin et al., 1997).
As for Cdh1 orthologs in other organisms, CdhA targets S-phase cyclins for destruction in G1. Cyclin B, which accumulates in the nucleoplasm and at the SPB in S through G2 and is destroyed in mitosis (De Souza et al., 2009; Nayak et al., 2010), is both an S-phase and a mitotic cyclin in A. nidulans (Osmani et al., 1994). Deletion of cdhA resulted in an earlier accumulation of cyclin B and shortened the total length of the cell cycle. In a cdhAΔ. mipAΔ strain, I found cyclin B-GFP was present in 87.2% of tip cell nuclei. Given that mitosis occupies approximately 5% of the cell cycle at this temperature (25°C), only 7-8% of cdhAΔ interphase tip cell nuclei did not have detectable cyclin B. Since cyclin B-GFP must take some time to accumulate to detectable levels, it is likely that it is being expressed prematurely right after mitosis in strains carrying cdhAΔ. In other organisms, not only does depletion or deletion of Cdh1 orthologs lead to premature accumulation of S-phase cyclins, but it also results in premature DNA replication (Sigl et al., 2009), decreased number of functional pre-replication complexes (Wäsch and Cross, 2002; Wäsch et al., 2010), and an activation of p53 suggestive of a DNA damage response (Sigl et al., 2009). Taken together, these data reveal that Cdh1 orthologs in various organisms are crucial for proper G1 regulation.

As discussed in Chapters 1 and 4, data from our lab revealed that a γ-tubulin mutation, mipAD159, caused a nuclear autonomous failure of inactivation of the APC/C sometime between late mitosis and S at restrictive temperatures (Nayak et al., 2010). I was able to determine that APC/C^{CdhA} is the constitutively active form of the APC/C in strains carrying mipAD159, leading to the continuous destruction of cyclin B. Deletion of cdhA reduced the number of CB^- nuclei in strains carrying mipAD159 to the very low
levels seen in \textit{mipA}^{+} strains. Therefore, \textit{mipAD159} causes a failure of inactivation of APC/C^{CdhA} in interphase, resulting in constitutive failure of cyclin B accumulation and, by inference, failure of accumulation of other APC/C^{CdhA} substrates.

My data are the first report of Cdh1 localization in filamentous fungi, and its localization was altered in a very informative way in a strain carrying \textit{mipAD159}. In \textit{mipA}^{+} strains, CdhA had a complex localization pattern. It localized to the SPB and nucleoplasm in G\textsubscript{1} and then disappeared from the SPB at a time that I determined to be the G\textsubscript{1}/S boundary based on my cell cycle timing data as well as that of Bergen and Morris (1983). The presence of CdhA at the SPB in G\textsubscript{1} corresponds with the period during which APC/C^{CdhA} is predicted to be active. APC/C^{CdhA} must be inactivated at the G\textsubscript{1}/S boundary for the cell cycle to proceed, and I found that the G\textsubscript{1}/S transition coincided with the disappearance of CdhA from the SPB. This suggested that the SPB localization of CdhA might be important for APC/C^{CdhA} activity. By creating a strain that carried CdhA-GFP, Cdk1-mCherry, nup49-mCherry, and \textit{mipAD159}, I found that in nuclei with a constitutively active APC/C (and are therefore Cdk1\textsuperscript{+}), CdhA remained at the SPB throughout interphase. These data are consistent with the possibility that \textit{mipAD159} causes a constitutively active APC/C^{CdhA} by altering CdhA localization at the SPB.

Surprisingly, CdhA disappeared from the SPB in CB\textsuperscript{−} nuclei when mitosis occurred in other nuclei in the same cell. Once nuclei become CB\textsuperscript{−} they remain CB\textsuperscript{−} (Nayak et al., 2010), and an obvious possibility was that CdhA would remain at the SPB during mitosis. This was not the case. Instead, in mitosis, CdhA localized to the SPB in CB\textsuperscript{−} nuclei and
remained at the SPB through interphase. This suggests that a stable, nuclear autonomous change occurs to the SPB that prevents the dissociation of CdhA from the SPB and/or its destruction at the G1/S boundary.

Interestingly, in mipA+ strains, CdhA localized to the SPB in G2 before disappearing in mitosis. This is a time period in which APC/C^CdhA is predicted to be inactive, so why would it be at the SPB at this stage? In G2, Cdk1 has accumulated, and it is known to inactivate Cdh1 by phosphorylating it (Zachariae et al., 1998). It has been suggested from data using DT40 chicken cells and human cells that APC/C^Cdh1 is activated in G2 in response to DNA damage (Bassermann et al., 2008; Mocciaro et al., 2010; Sudo et al., 2001). So, perhaps, CdhA is present at the SPB, but in an inactive state, unless the DNA damage response is activated.

Not only does my data further elucidate γ-tubulin’s role in inactivating the APC/C, but it also reveals that it has an indirect role in the SAC. Mps1, which localized to the SPB in late G1 through G2 in mipA+ strains, failed to localize there in 23.1 % of mipAD159 tip cell nuclei that were in S or G2. As mentioned in Chapter 3, A. nidulans Mps1 contains a canonical destruction box, which could be a target for the APC/C. In fact, human Mps1 has been shown to have a destruction box, and it is targeted by both Cdc20 and Cdh1 (Cui et al., 2010; Liu et al., 2013). If a constitutively active APC/C^CdhA is continuously targeting Mps1 for destruction, affected mipAD159 nuclei would never accumulate Mps1. In addition to its role in the SAC, Mps1 is also known to be required for SPB/centrosome duplication, at least in S. cerevisiae, mouse, and human cells (Fisk et al., 2003; Fisk and Winey, 2001; Winey et al., 1991). In A. nidulans, SPB duplication
occurs approximately half way through interphase (Oakley and Morris, 1983), which corresponds to S phase. Therefore, I propose that failure of inactivation of APC/C^{CdhA} leads to continuous destruction of Mps1 and nuclei remain in G1 with unduplicated SPBs. When such nuclei enter mitosis, the absence of Mps1 prevents the formation of a functional MCC, which renders the SAC inactive.

BubR1, another SAC protein and a component of the MCC, also fails to localize to mipA^D159 nuclei that have a constitutively active APC/C^{CdhA}. This was determined directly by live imaging of a strain carrying BubR1-GFP, Cdk1-mCherry, Histone H1-t-Sapphire, and mipA^D159 (Fig. 3.16). This was somewhat surprising because CdhA dissociates from the SPB in mitosis even in CB^1 nuclei and, therefore, might not be active. It is possible that APC/C^{CdhA} is active even though it is not at the SPB. However, it has been demonstrated in Xenopus egg extracts that KT localization of BubR1 is dependent on Mps1 (Vigneron et al., 2004; Wong and Fang, 2006), and it is possible that the absence of Mps1 prevents the accumulation of BubR1 at SPBs. In any case, the absence of both Mps1 and BubR1 would clearly prevent SAC activation. Cdc20, on the other hand, does not depend on BubR1 or Mps1 as it still localizes to the SPB/KT/early forming spindle in mitosis in virtually all mipA^D159 nuclei examined thus far. This is in contrast to a report in Drosophila which found that BubR1 is required for Cdc20 to localize to KT{s} (Li et al., 2010) and in Xenopus which found that Mps1 is required for KT localization of Cdc20 (Vigneron et al., 2004).

Taken together, my work clearly demonstrates that γ-tubulin has an important role in inactivating APC/C^{CdhA} and, thus, in regulating the G1/S transition. My data, thus,
adds to the accumulating evidence that γ-tubulin, and, therefore, PMTOCs, have functions in cell cycle regulation (Cheng et al., 2008; Gromley et al., 2003; Hinchcliffe et al., 2001; Maniotis and Schliwa, 1991; Matsumoto and Maller, 2004; Mikule et al., 2007; Nayak et al., 2010; Srsen et al., 2006; Srsen and Merdes, 2006; Vardy and Toda, 2000). Although the mechanisms may differ, the fact that PMTOCs are involved in G₁/S regulation in phylogenetically distant organisms from yeast to fungi to humans suggests this regulation is of fundamental importance. For example, mammalian cells have a G₁ checkpoint regulated by an undefined sensor at the centrosome; a signal transduction pathway involving the tumor suppressor p53; an activator of p53, p38; and the Cdk inhibitor p21 as a receiver of that signal (Mikule et al., 2007). Cells depleted of centrosomal proteins are arrested in G₁, and p53, p38, and p21 are required for this arrest (Mikule et al., 2007; Srsen et al., 2006). CB⁻, *mipAD159* nuclei are also arrested in G₁; however, BLASTP searches of the *A. nidulans* genome did not reveal any clear homologs of p53, p38, or p21. There could still be some sort of sensor at the SPB that activates a signal transduction pathway with the end result of inactivation of APC/C<sub>CdhA</sub> and transition into S phase. *mipAD159* strains could have a defective SPB sensor or a misregulated signal transduction pathway leading to a constitutively active APC/C<sub>CdhA</sub> and G₁ arrest.

I have not determined the exact mechanism by which γ-tubulin inactivates APC/C<sub>CdhA</sub>, but my data suggest a model in which CdhA must dissociate from the SPB at G₁/S for nuclei to proceed into S. The failure of dissociation of CdhA from the SPB in *mipAD159* nuclei is not due to an increased binding affinity of γ-tubulin for CdhA since
mipAD159 is a recessive mutation (Jung et al., 2001). As mentioned in Chapter 1, mipAD159 maps to the outside face of γ-tubulin, in a position that does not interact with α-tubulin, adjacent γ-tubulin molecules, or GCPs. However, it is in a position that is available to bind with other proteins (Fig. 1.12, B-C). Therefore, it is more likely that γ-tubulin interacts, directly or indirectly, with a protein or protein complex required for destruction of CdhA or dislocation of CdhA from the SPB. For example, γ-tubulin could interact with an inhibitor of APC/C\(^{\text{CdhA}}\) such as Mad2L2 (Pfleger et al., 2001), Bmh1, Bmh2 (Dial et al., 2007; Martinez et al., 2006), Emi1 (Miller et al., 2006), or Acm1 (Martinez et al., 2006). The failure of inactivation of APC/C\(^{\text{CdhA}}\) caused by mipAD159 and consequent failure of accumulation of cyclin B appears to be a random process (Nayak et al., 2010); hence, the interaction between γ-tubulin and an APC/C\(^{\text{CdhA}}\) inhibitor may be compromised in mipAD159 in that the interaction is rendered inefficient, leading to a stochastic loss of APC/C\(^{\text{CdhA}}\) inactivation.
References


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