New insights into the functions of the two mitotic kinases, NIMA and CDK1, through the cell cycle

DISSERTATION

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By

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Abstract

In Aspergillus nidulans, the Never in Mitosis A (NIMA) kinase plays an essential role along with the Cdk1 kinase in initiating mitosis. NIMA is the founding member of a family of NIMA related kinases (Neks) that are conserved in all eukaryotic cells. Therefore, to better understand the conserved aspects of mitotic regulation, it is of fundamental importance to study the mechanism of NIMA function through the cell cycle. nimA is an essential gene in A. nidulans, hence the non-essential S. cerevisiae nimA ortholog, KIN3, was used by our collaborators to perform a synthetic lethal screen to identify novel genetic interactors of KIN3 (SL Anglin et al. unpublished). Since KIN3 does not have an essential mitotic role, we hypothesized that extending this genetic analysis to A. nidulans might help us examine potential non-mitotic roles of nimA. Consistent with this expectation, we identified a conserved genetic interaction between nimA and the trafficking pathway involved in the turn-over of cell membrane proteins, the ESCRT (Endosomal Sorting Complex Required for Transport) pathway. Analysis of cells that lack ESCRT function in combination with having partial NIMA function revealed that NIMA has a role in regulating cell tip growth. From parallel microscopy experiments we found that in addition to mitotic nuclear structures, NIMA locates to the tips of growing hyphal cells. Ectopically
expressed NIMA also locates to the plus ends of interphase microtubules (Mts) in a manner dependent on the well-studied plus end binding protein, Eb1. Perturbation of NIMA function modifies Eb1 behavior, Mt dynamics and polarized cell growth. Our results contend that NIMA has a cytoplasmic function in cell tip growth during interphase involving the regulation of microtubule dynamics in concert with the ESCRT pathway.

Using our genetic approach, we also identified a conserved genetic interaction of nimA with the Set1 methyl transferase complex. Unlike the genetic interaction between NIMA and the ESCRT pathway, we find that the methylation of the histone H3K4 by the Set1 complex is essential in cells that have partial function of either the NIMA or Cdk1 mitotic kinases. We find that the function of the Set1 complex is required along with Cdk1 activation by Cdc25 phosphatase to regulate the successful transition from G2 into mitosis. The Set1 complex is also required for normal mitotic progression, since the absence of Set1 in combination with partially impaired NIMA function causes mitotic defects monitored by the spindle assembly checkpoint.

We detected that in contrast to cells with partially active Cdk1 that exhibit a G2 delay (but complete the subsequent mitosis successfully), cells with partial NIMA activity exhibit mitotic defects. A systematic exploration of these mitotic defects allowed us to identify multiple functions for NIMA in specific aspects of mitosis post G2-M transition, that correlate with the dynamic mitotic localization of NIMA. Thus our data support functions for NIMA both in the cytoplasm to
regulate microtubule dynamics and cell growth during interphase and also in the nucleus during mitosis. We suggest NIMA might help integrate these two key aspects of cell growth and development.
Dedication

Dedicated to Appa and Amma
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I am very fortunate to have trained under Dr. Stephen A. Osmani’s mentorship and will always be indebted to him for taking me on as his student. I find myself at a loss for words to express my gratitude for all the effort he has put in to guide and train me. Steve’s clarity of logical thinking, attention to detail and eloquence are just few of the qualities that I have tried to imbibe over the years. I could not have asked for anything more in my mentor and he is in every way the ideal role model for me.

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scientist but also mature as an individual and a citizen, which I hope would enable me to make a meaningful difference in people’s lives in the future.
Vita

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Govindaraghavan, M., Varadaraj, A., Hashmi, S.H., De Souza, C.P.C., and
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**Fields of Study**

Major Field: Molecular, Cellular and Developmental Biology
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List of commonly used abbreviations

APC/C - Anaphase Promoting Complex/ Cyclosome
DAPI - 4', 6-diamidino-2-phenylindole
ESCRT - Endosomal Sorting Complex Required for Transport
H3K4 - Histone 3 Lysine 4
H3S10 - Histone 3 Serine 10
+TIPs - Plus tip binding proteins
Mt - Microtubule
MTOC - Microtubule Organizing Center
NE - Nuclear Envelope
Nek - NIMA related kinase
NPC - Nuclear Pore Complex
SAC - Spindle Assembly Checkpoint
SIME - Spindle Independent Mitotic Exit
SIN - Septation Initiation Network
ts - temperature sensitive
Wt - Wildtype
Chapter 1: Introduction

1.1 The filamentous fungus, Aspergillus nidulans, a model organism for cell biology research

1.1.1 Growth and development of A. nidulans

Research using A. nidulans has been at the forefront of cell cycle research since the identification of cell cycle specific mutations by N. Ronald Morris several decades ago (Pontecorvo, Roper et al. 1953, Morris 1975). A. nidulans is a filamentous fungus in the phylum Ascomycota. The vegetative life cycle of A. nidulans begins as a dormant spore (conidium) which responds to environmental cues and undergoes an initial period of isotropic growth following which an extreme form of polarized growth is initiated (Figure 1.1, a-d). The growth of this swollen spore is tightly coupled with nuclear divisions; such that a multinucleated germling is formed (Figure 1.1, e-h). As shown in Figure 1.1, each successive mitosis in the multinucleated cell is preceded by a period of growth, indicating that regulatory mechanisms function to ensure that mitosis is triggered only after the cell has grown enough to support the doubling of nuclei. It was suggested early on that mechanisms monitoring the ratio of nuclear to cytoplasmic volume might regulate this coordination between cell growth and mitosis (1978) (Smith and Berry). The nuclei in A. nidulans hyphal cells undergo parasynchronous
mitosis, which is a term used to describe a wave of mitosis that is initiated at one end of cell compartment and spreads along the nuclei in the cell towards the other end (Rosenberger and Kessel 1967, Clutterbuck 1970). After three mitotic divisions, septation, a process akin to cytokinesis, separates the cell into two asymmetric compartments (Figure 1.1, i) (Harris 2001). Hereafter, septation is triggered after every round of nuclear division (Fiddy and Trinci 1976). Septation gives rise to two cell compartments, one of which is initially devoid of an actively growing cell tip, called a basal cell (Figure 1.1, i, k). The nuclei in a basal cell are arrested in G1 (marked in red in Figure 1.1) until a new subapical growing tip emerges, which acts as a signal to promote the entry of these nuclei into the cell cycle to undergo parasympathetic mitoses (Figure 1.1, j) (Nayak, Edgerton-Morgan et al. 2010, Edgerton-Morgan and Oakley 2012).

Thus, the vegetative growth of A. nidulans forms a network of interconnected cells called the mycelium. This process occurs through a regulated and complex interplay of nuclear division, cell growth and septation (Fiddy and Trinci 1976, Bergen and Morris 1983, Wolkow, Harris et al. 1996). As can be imagined, the misregulation of one of these processes or the lack of crosstalk between them can result in unwanted consequences. For instance, it would be detrimental to have a rate of nuclear division that cannot be supported by the rate of cell growth. Likewise, septation timing and spacing have to be regulated to allow maximum growth and thus a competitive advantage to the
organism. However, very little is understood about the regulatory network that integrates cell cycle progression with septation and cell growth.

After a period of mycelial growth, the growth of aerial branches (which is triggered by exposure to an air surface) is seen which then develop into the organs that form asexual spores called conidiophores (Adams, Wieser et al. 1998). The development of conidiophores is a fascinating process (Timberlake 1990). It begins with the differentiation of a section of a hyphal cell to give rise to what is called the foot cell (Figure 1.2), which may have multiple nuclei. An aerial conidiophore stalk extends from the foot cell. After a period of aerial growth, the tip of the conidiophore stalk swells forming the conidiophore vesicle. Now, the conidiophore vesicle undergoes a dramatic transformation with synchronized division of nuclei coupled with the formation of about 60 buds called metulae having a single nucleus (Mims, Richardson et al. 1988). The metulae are elongated and they in turn undergo two rounds of budding to produce a tier of about 120 uninucleated cells called phialides. Subsequently, each phialide undergoes hundreds of mitotic divisions to give rise to a string of uninucleated conidial spores. It is important to note that the phialide undergoes asymmetric divisions and behaves like stem cells with the phialide nucleus undergoing multiple mitotic divisions while the daughter nuclei segregated into the conidia are removed from the cell cycle and enter G0. Therefore in the string of conidia, the oldest conidia can be found farthest from the conidiophore stalk. In this manner, each foot cell is able to generate, on the order of thousands of conidia.
Under the right nutritive conditions the germination of the conidia occurs to complete the asexual life cycle.

*A. nidulans* is homothallic and undergoes sexual development through either self or cross fertilization. The meiotic spores are contained in a spore bearing organelle called the ascus, which is a distinguishing feature of the Ascomycota (Adams, Wieser et al. 1998). The ascus contains 8 ascospores which are produced by a meiotic cell division followed by a mitotic cell division. Hundreds of asci are enveloped by a fruiting body called the cleiostothecium. A specialized kind of cell termed Hülle cells surround the cleiostothecia and possibly act as nurse cells to provide nutrition and support to the growing fruiting body. Like the conidia formed during asexual development, ascospores are dormant and can enter the vegetative phase of development under appropriate nutritional environments. The availability of the sexual cycle in *A. nidulans* offers enormous opportunities for studies using classical and molecular genetics. Early on, the mapping of new genes to linkage groups was made possible by the ability to outcross and self-cross *A. nidulans* strains carrying mutations that disrupt different cellular processes, and this continues to be an advantage today during the identification of genes by forward genetic screens. Moreover, in the molecular genetic era, the generation of strains suitable for microscopic and biochemical analysis according to experimental needs is largely facilitated by the heterothallic nature of *A. nidulans*. 
In addition, two *A. nidulans* hyphal cells carrying nuclei of two different genotypes can fuse to give a heterokaryon, an aspect of the life cycle termed the parasexual cycle (Pontecorvo 1953). Though heterokaryons carry nuclei of two different genotypes in the same cytoplasm, the conidia formed during asexual reproduction are uninucleate and will carry a nucleus of one or the other genotype. [As a side note, this aspect of *A. nidulans* development has been exploited for performing and analyzing essential gene deletions, by a technique called the heterokaryon rescue. Further details are in Chapter 2, Section 2.2.3 (Osmani, Oakley et al. 2006)]] On the other hand, *A. nidulans* haploid cells in which all nuclei are of the same genotype are called homokaryons. The nuclei within homokaryons or heterokaryons can fuse to give diploid nuclei. When *A. nidulans* diploid strains undergo asexual reproduction, they produce conidia that carry diploid nuclei. Thus even though *A. nidulans* is normally haploid, formation of stable diploids that complement each other for the lack of two different nutritional markers can be induced in a laboratory setting by providing the appropriate selection (see Section 2.2.5 for details). Likewise, the haploidization of diploid *A. nidulans* cells can be triggered to recover two haploid strains of parental genotype.

1.1.2 *A. nidulans* as a model organism

*A. nidulans* is a powerful model organism for cell biology research (Osmani and Goldman 2008). The ability to use different experimental
approaches (see below) enables one to address fundamental questions in cell biology - identify novel proteins, study previously uncharacterized functions for proteins and the mechanistic details of cellular processes. Importantly, decades of research using *A. nidulans* has shown that it shares many conserved regulatory mechanisms of cellular processes with higher eukaryotes (as will be described in later sections of this Chapter). Therefore, studies performed using the genetically tractable *A. nidulans* can be invaluable to further research in higher eukaryotes that may not be as amenable to definitive genetic studies.

Studying filamentous fungal biology is also important from a medical standpoint, given the threat posed by fungal diseases (for instance caused by *A. fumigatus*) in immune-compromised individuals and the ability of certain fungi to produce powerful carcinogenic mycotoxins (example, *A. flavus*). Some species of the *Aspergillus* genus, like *A. niger* and *A. oryzae*, are important in industrial production of enzymes and utilized in oriental food processes. Being a non-pathogenic fungus in the same genus as these medically and industrially important *Aspergilli*, *A. nidulans* is the model system for gaining an in-depth understanding of fungal biology which can have far reaching applications (Osmani and Goldman 2008).

*A. nidulans* has been an historically important model system to carry out forward genetic screens, which has given rise to a repertoire of mutants defective in cell cycle, nuclear distribution and septation (Morris 1975). The development of *A. nidulans* as one of the first genetic model systems was greatly facilitated by
the fact that the organism can undergo self-fertilization (hence termed homothallic) as well as cross-fertilization. Technical advantages include its short life cycle which makes it easy to propagate using defined minimal media. Now with the advent of live cell microscopy, the short cell cycle of about 75 minutes (at 37°C) enables the study of multiple consecutive complete cell cycles. The asexual and sexual development of *A. nidulans*, which involves the generation of multiple different cell types as described above, offers the opportunity to examine the function of a gene in several different cell types. For instance, a gene that is required for cytokinesis may not be essential for the growth of the multinucleated hypha, since septation is not required for mycelial growth; however, it might be expected to be essential for conidiation.

*A. nidulans* has a fully sequenced and annotated genome (Wortman, Gilsenan et al. 2009). The availability of protein and genome analysis tools as well as the genome sequences of multiple species of Ascomycota has made reverse genetic and comparative genomics approaches routine. In addition, this has also aided the identification of new genes and new functions of genes through forward genetics screens using mutagenesis and multi copy number plasmids and subsequent complementation of the mutant phenotype (De Souza, Hashmi et al. 2006, Ukil, Varadaraj et al. 2008). The development of molecular genetics techniques in *A. nidulans* revolutionized research using this organism, enabling the control of expression of a gene using regulatable promoters, and the deletion and tagging of endogenous gene open reading frames (ORFs) (Waring,
May et al. 1989, Yang, Ukil et al. 2004). The fusion of proteins of interest with fluorophores enables detection of localization through live cell imaging and fusion of the protein with S-peptide allows biochemical studies through affinity purification followed by mass spectrometry analysis (Liu, Osmani et al.).

Importantly, standard techniques for gene tagging involve the expression of the protein from its endogenous promoter. The multinucleated feature of the fungus has been used to develop the heterokaryon rescue technique that enables the examination of the phenotype of essential genes (Osmani, Oakley et al. 2006). In addition, the ability to generate diploid strains as well as convert them into haploids allows one to conduct genetic complementation experiments and test for the dominance of a mutant allele (Todd, Davis et al. 2007). The use of fusion PCR to generate gene deletion and gene tagging constructs and the use of strains deleted for An-ku70 (that increases the chances of homologous recombination in the cell) has recently accelerated the pace of genetic manipulation in A. nidulans (Yang, Ukil et al. 2004, Szewczyk, Nayak et al. 2006).

### 1.2 Principles of cell cycle regulation

Cell division is a fundamental process of cell biology. Decades of research has revealed the principles behind the regulation of this highly coordinated process as well as the genetic regulatory networks that implement this regulation. The cell cycle is composed of successive phases of growth and division. The
growth phase can be further divided into G1, and G2 phases interspersed by the S phase. Cell division comprises of the division of nuclei during mitosis which is followed by cytokinesis, in the case of single celled organisms, giving rise to two daughter cells (Alberts, Wilson et al. 2008). In \textit{A. nidulans} vegetative hyphal cells, septation a process similar to cytokinesis gives rise to cell compartments that have multiple nuclei (Figure 1).

During S phase, DNA is duplicated as is the microtubule organizing center (MTOC). The duplicated MTOCs form the mitotic spindle, which is a microtubule (Mt) based mechanical structure required for the separation of the duplicated DNA during mitosis. In fungi, the MTOC is a structure associated with nuclear envelope called the spindle pole body (SPB), while higher eukaryotes nucleate Mts from the cytoplasm-localized centrosomes. In mitotic prophase, the chromatin condenses, and a spindle starts to form. During metaphase, the Mts of the bipolar spindle attach to proteinaceous structures on chromosomes, called kinetochores. When all kinetochores have been attached to the spindle in a bipolar fashion, anaphase is triggered resulting in the segregation of duplicated chromosomes.

Forward genetic screens in \textit{S. cerevisiae} and \textit{S. pombe} gave early insights into the identity of genes that regulate the cell cycle. A screen for cell cycle mutants was conducted contemporary with these studies in \textit{A. nidulans}, which led to the independent identification of several conserved cell cycle regulators in this organism (see Section 1.4 for details). In addition, egg extracts
from the model amphibian, *Xenopus laevis* served as an excellent system to undertake biochemical studies. Genetic and biochemical studies in these different model systems converged to identify several master regulators of mitosis regulation. Chief among them is the Cdk1-cyclin kinase. Formation of a complex with cyclin B contributes to the activation of the kinase activity of Cdk1 during mitosis. The abundance of cyclin B is regulated in such a manner that it peaks at the G2-M transition so that the spike in Cdk1 activity can promote entry into mitosis and its degradation is required for mitotic exit.

The processes of DNA replication, growth, mitotic entry and mitotic exit need to happen in the right order in a step wise manner. Therefore, mechanisms need to be in place to ensure that mitosis is not initiated before DNA is fully replicated, or that anaphase is not triggered before all the kinetochores are attached to the spindle stably in the correct orientation. One way to ensure this is to have a subsequent process be dependent on a former process, for instance mitotic entry could be biochemically dependent on finishing DNA replication. However, the discovery of checkpoint proteins showed that an alternate mechanism is at work to ensure the correct order of events in the cell cycle. Checkpoints are regulatory mechanisms that detect the occurrence of errors in completing a phase of the cell cycle and then cause an arrest of the cell cycle so that the errors can be corrected before progressing ahead. Proteins that function as part of checkpoints are sensitive to the fidelity of the processes required to complete each phase of the cell cycle. For instance, checkpoint mediated
mechanisms ensure that mitosis is initiated only after S phase is completed and DNA is replicated. Also, when DNA is damaged by intracellular or extracellular agents, this is sensed by the proteins of the DNA damage checkpoint, which impose an inhibition on proteins that promote mitosis initiation. Thus entry into mitosis is halted until the damaged DNA is repaired. Consistent with this idea then, when cells in which the genes encoding DNA damage checkpoint proteins are mutated experience DNA damage, then they unable to detect the damaged DNA and therefore commit to a catastrophic mitosis. Therefore, checkpoint proteins are central to cell cycle regulation. As another example, the spindle assembly checkpoint (SAC) ensures that there is accurate attachment between chromatin and spindle microtubules before anaphase is triggered. The components of SAC inhibit the activity of the anaphase promoting complex/cyclosome (APC/C) until all the kinetochores are accurately attached in a bipolar fashion to either spindle poles. Once bipolar attachments are established and SAC is fulfilled, the inhibition of APC/C is lifted. APC/C now promotes the ubiquitination and degradation of securin, an inhibitor of the protease called separase, thereby relieving separase of negative inhibition. Finally, the separase mediates the degradation of cohesin proteins (that hold the sister chromatids together), thus effectively promoting the segregation of the sister chromatids to either spindle pole, and the onset of anaphase.

A recent study in *S. cerevisiae* examining the mitotic exit event involving the release of Cdc14 phosphatase from the nucleolus suggests an additional
layer of complexity to cell cycle regulation (Lu and Cross 2010). Evidence suggests that some mitotic events – for instance, in this case, the release of Cdc14 from the nucleolus – are intrinsically oscillatory. This means that they can happen in independent cycles, but are entrained to a master oscillator, the oscillation of Cdk1 activity, in such a way that these events happen once per cell cycle in a specific order. When Cdk1-cyclin B function is held at a constant level (without oscillations), then the occurrence of these sub aspects of mitosis is not prevented, but remarkably, the frequency of the oscillations is altered, resulting in an altered order of mitotic events. Therefore, it has been proposed that the oscillation of the master regulator locks the oscillations of other mitotic events in phase so that they happen in the correct order (Morgan 2010).

In addition, another important aspect of mitosis initiation is the biphasic manner of activation of Cdk1-cyclin B complexes (Morgan 2007). Cdk1-cyclin B complex formation activates Cdk1 activity, however, the accumulation of cyclin B happens gradually during G2. In contrast, Cdk1 activity increases from negligible to the maximum in a short period of time during mitotic initiation. How this is achieved is a fascinating aspect of regulation. Cdk1 is inhibited by phosphorylation at tyrosine 15 in the kinase enzymatic active site during G2 by the Wee1 kinase. This inhibition is reversed by the activation of the Cdc25 phosphatase during mitotic activation. When cyclin B levels reach a threshold, a small amount of Cdk1 activity is promoted. The resulting Cdk1 function activates its activating Cdc25 phosphatase while simultaneously inactivating the Wee1
kinase by phosphorylation. Thus, the establishment of positive and negative feedback loops results in a switch-like increase in Cdk1 activity.

1.3 Mitosis in *Aspergillus nidulans*

In *A. nidulans* vegetative hyphal cells, nuclei undergo parasynchronous mitoses in a common cytoplasm. The microtubule organizing center (MTOC) called the spindle pole body (SPB) is embedded within the nuclear envelope of each nucleus. During interphase, the SPB nucleates cytoplasmic microtubules, whereas in mitosis, spindle microtubules are nucleated on the nuclear side of the SPB. In higher eukaryotes like human cells, the breakdown of the nuclear envelope at mitosis allows the interaction of mitotic spindle Mts nucleated by the cytoplasmic centrosomes with the nuclear chromatin. Therefore, the mitosis in these organisms is called open mitosis (De Souza and Osmani 2007). In contrast, in lower eukaryotes like *S. cerevisiae*, nuclei undergo closed mitosis, wherein the nuclear envelope is maintained in an interphase configuration during mitosis. Mitosis in *A. nidulans* is unique in that the nuclear envelope remains intact, while around half of the nuclear pore complex (NPC) proteins disperse abolishing active nuclear transport (De Souza, Osmani et al. 2004, Osmani, Davies et al. 2006). Since one of the first mitotic events in higher eukaryotes during nuclear envelope breakdown (NEBD) is the disassembly of the NPCs, the behavior of NPC proteins in *A. nidulans* represents an intermediate between the
open and closed forms of mitosis (De Souza and Osmani 2007). Therefore, mitosis in *A. nidulans* is referred to as being semi-open.

Another aspect of *A. nidulans* mitosis that is more similar to higher eukaryotes than to lower eukaryotes is the mitotic segregation of the nucleolus. In *S. cerevisiae*, nucleolar proteins do not disassemble from chromatin but segregate along with the chromatin. In contrast, during open mitosis in higher eukaryotes, the nucleolus undergoes sequential disassembly and then reassembly in daughter nuclei. Similarly, the nucleolus of *A. nidulans* is expelled from the segregating chromatin during anaphase/telophase, and then undergoes step-wise disassembly and reassembly into the daughter nuclei (Ukil, De Souza et al. 2009). In addition, we also find the evolution of a unique nuclear envelope segregation pattern in *A. nidulans* that was possibly necessitated by the occurrence of multiple semi-open mitoses in a common cytoplasm. During nuclear division in *A. nidulans*, the nuclear envelope restricts at two points (instead of just one) giving rise to two membrane compartments enveloping the segregating daughter nuclei on both sides and a transient membrane bound compartment in the middle containing the nucleolus. Almost immediately afterwards, the nucleolus disassembles and reassembles into the daughter nuclei. Moreover, the process of expulsion of the nucleolus, and subsequent disassembly-reassembly can occur in the absence of a spindle, which alludes to an overarching theme in mitosis that sub aspects of mitosis can occur potentially
independently of each other, but in the context of the cell cycle are regulated to occur in the correct order (Lu and Cross 2010).

Interestingly, the examination of mitosis in the absence of the spindles revealed another important phenomenon which has broad implications for mitosis regulation. In *A. nidulans*, when microtubules are depolymerized in the presence of the microtubule poison, benomyl, mitosis is initiated, but the absence of a functional spindle causes a mitotic arrest dependent on the spindle assembly checkpoint (SAC). Upon prolonged treatment of cells with benomyl, however, nuclei exit mitosis in a regulated manner by inactivating the SAC and degrading cyclin B. This process has been termed spindle independent mitotic exit (SIME) (De Souza, Hashmi et al. 2011). Further study of this process would help in the understanding the mechanisms by which SAC is inactivated in cells, a process which is not very well understood currently. Microtubule poisons are often used in cancer therapeutics and therefore it is important also from a medical perspective to understand the different mechanisms employed by eukaryotic cells in response to microtubule depolymerization.

### 1.4 Regulation of mitosis by the NIMA and CDK1 kinases

A forward genetic screen aimed at identifying molecular regulators of the cell cycle was carried out in *A. nidulans* (Morris 1975). Mutations in the gene that is the focus of my dissertation research, *nimA*, was identified as part of this screen. Temperature sensitive mutations in *nimA* result in a G2 arrest. *nimA* was
cloned by complementation of the ts phenotype of nimA5 mutant allele and was shown to encode a protein kinase (Osmani, May et al. 1987, Osmani, Pu et al. 1988, Osmani, McGuire et al. 1991, Lu, Osmani et al. 1993). The expression and kinase activity of NIMA is cell cycle regulated, peaking at the G2-M transition and decreasing as the cells transition through mitosis (Osmani, May et al. 1987, Osmani, McGuire et al. 1991). Therefore, early evidence helped in the recognition of NIMA as a regulator of mitotic initiation. Moreover, nimA remains the only kinase that is required for mitotic initiation, other than the Cdk1 kinase. NIMA is degraded through mitosis and its degradation is essential for mitotic exit, a behavior that is in essence similar to cyclin B (Pu and Osmani 1995). Therefore NIMA in A. nidulans is a master regulator of mitosis on par with Cdk1-cyclin B.

NIMA is a phosphoprotein (Lu, Osmani et al. 1993). In cells carrying a temperature sensitive mutant of the Cdk1 activating Cdc25 phosphatase, nimT23, at the restrictive temperature, NIMA has basal kinase activity (Ye, Xu et al. 1995). This NIMA activation is mediated by autophosphorylation and possibly by an unidentified kinase (Osmani and Ye 1996). Upon Cdk1 activation, NIMA is hyperphosphorylated potentially directly by Cdk1, which correlates with a further spike in kinase activity. These data are consistent with a part of NIMA function being downstream of Cdk1 activity. However, nimA5 cells that are inhibited for mitotic entry have high Cdk1 activity, showing that Cdk1 activity is not sufficient to trigger mitotic entry. Moreover, when the inhibition on Cdk1 by the Wee1 kinase is undermined by mutating the phosphorylation residue on Cdk1 that is
targeted by Wee1, the resulting Cdk1\textsuperscript{AF} does not promote mitosis, showing that activation of Cdk1 is not sufficient for mitosis. In contrast, the analogous mutation in \textit{S. pombe} results in lethality due to premature mitosis (Osmani and Ye 1997). Importantly, in \textit{A. nidulans}, Cdk1\textsuperscript{AF} in combination with mutants that elevate NIMA activity (Ye, Fincher et al. 1998) enter pre-mature mitosis, showing that mitotic initiation in cells with high Cdk1 activity is dependent on NIMA activity.

These extensive studies have led to a model whereby NIMA and Cdk1 function in parallel to activate mitosis, and help activate each other in order to trigger mitotic entry. How does NIMA promote Cdk1/cyclin B activation?

A genetic screen for extragenic mutations that suppress the temperature sensitivity of \textit{nimA1} (another \textit{nimA} ts allele) identified mutations in two genes encoding proteins of the nuclear pore complex (NPC), suggesting that NIMA can perhaps regulate NPC proteins during mitosis allowing mitotic regulators including Cdk1-cyclin B access to their substrates specifically during mitosis (Wu, Osmani et al. 1998, De Souza, Horn et al. 2003). Indeed, expression of NIMA can promote mitotic dispersal of NPCs even out of cell cycle phase (De Souza, Osmani et al. 2004). This striking observation offers a mechanism of NIMA function in regulating nuclear permeability during mitosis. Recent evidence has shown that cyclin B is localized to the nucleus and the spindle pole body during interphase and disperses during mitosis (Shen \textit{et al.} unpublished). Therefore, the opening of the NPCs possibly allows the substrates of Cdk1/cyclin B that are excluded from the nucleus in interphase access to the nucleoplasm and thus

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facilitates contact with mitotic regulators. In addition, NIMA overexpression can promote chromatin condensation (De Souza, Osmani et al. 2000). The modification of the histone H3S10 is a universal mark of mitotic condensed chromatin. Interestingly expression of NIMA can promote the phosphorylation of H3 even out of cell cycle phase, showing that NIMA function is sufficient to affect this modification. In higher eukaryotes, H3S10 phosphorylation is mediated by another mitotic kinase, the Aurora kinase. Expression of the Aurora kinase can promote H3S10 phosphorylation in A. nidulans in a manner dependent on NIMA function (De Souza et al. unpublished).

The mitotic kinases in A. nidulans, as in other systems studied, are a target of inactivation in response to DNA damage and replication stresses. The inhibitory phosphorylation of Cdk1 that is regulated by the Wee1 kinase and Cdc25 phosphatase is wired to the DNA damage checkpoint (Ye, Fincher et al. 1997). Thereby mitotic entry is prevented by retaining this inhibitory modification of Cdk1 during DNA damage. In A. nidulans, the expression of the Cdk1\textsuperscript{AF} mutant that cannot be inhibited by Wee1 is not lethal (since, as explained earlier, lack of NIMA function in these strains prevents mitotic entry). However, in the presence of DNA damage, expression of Cdk1\textsuperscript{AF} causes premature mitotic entry and is lethal, showing that the DNA damage checkpoint over mitosis is via the phosphorylation of Cdk1 at this site. Yet another checkpoint over mitosis functions to monitor replication stress. Interestingly, there seem to be two parallel checkpoint mechanisms in A. nidulans to prevent mitotic entry in the presence of
replication stress (Ye, Fincher et al. 1996). One is mediated by the inactivation of Cdk1 by the tyrosine 15 phosphorylation of Cdk1. The other is mediated by NIMA inactivation. BIME, a component of the anaphase promoting complex/cyclosome (APC/C), is required to inhibit NIMA activation under replication stress. The absence of BIME function in combination with the expression of the Cdk1\textsuperscript{AF} mutant can cause the entry of cells into a catastrophic mitosis with unreplicated DNA (Ye, Fincher et al. 1998).

1.5 The family of NIMA-related kinases (Neks) – NIMA orthologs in other organisms

The discovery of NIMA in \textit{A. nidulans} led to the discovery of NIMA related kinases in other organisms, and the family of Neks was found to be conserved from lower eukaryotes all the way up to humans and also plants. In fact, the expression of the \textit{Neurospora crassa nimA} ortholog, \textit{nim1} is able to rescue the temperature sensitivity of the \textit{nimA1} allele in \textit{A. nidulans} (Pu, Xu et al. 1995), demonstrating functional interchangeability between these two model organisms and underlining the conserved nature of NIMA function. Moreover, overexpression of NIMA can cause mitotic events including chromatin condensation not only in \textit{A. nidulans} but also strikingly, in fission yeast, \textit{Xenopus} and human cells (O’Connell, Norbury et al. 1994, Lu and Hunter 1995). This result strongly contends that the mechanisms of NIMA function are conserved in all organisms. However, there are some differences in the requirement of \textit{nimA}
for entry into mitosis. Even though deletion of the *S. cerevisiae* ortholog, *KIN3*, or the *S. pombe* ortholog, *fin1*, result in a delay at G2-M, they are not essential for entering mitosis as *nimA* is in *A. nidulans* [Sarah Lea Anglin *et al.*, unpublished, (Barton, Davies et al. 1992, Schweitzer and Philippsen 1992, Krien, Bugg et al. 1998)].

Moreover, recent evidence from *S. cerevisiae* indicates that *KIN3* is important for a functional G2-M checkpoint over mitosis upon DNA damage (Moura, Castilhos et al. 2010). Some interesting insights have come from the comparative study of the budding yeast *KIN3* and the closest human ortholog to NIMA, Nek2 (Chen, Riley et al. 2002). Though the deletion of *KIN3* is non-essential, generation of a mutant version of *KIN3* which has the same point mutation as *nimA7* results in temperature sensitivity (Chen, Riley et al. 2002). It has been proposed that this gain of function phenotype potentially results from the mutated *KIN3* being able to bind substrates but being unable to release them, therefore being more deleterious than the absence of *KIN3*. Remarkably though, this *ts* phenotype of the *KIN3* mutant can be suppressed by the expression of human NIMA ortholog, Nek2. As further evidence of the functional conservation between the two orthologs, *KIN3* is able to phosphorylate a Nek2 substrate, the human kinetochore protein, Hec1 *in vitro*. The catalytically dead mutant version of *KIN3* retains the ability to bind human Hec1 but is unable to phosphorylate it, indicating the dominant negative nature of the *KIN3* mutation.
Likewise, there is substantial intersection between the functions of fin1 in S. pombe and its A. nidulans counterpart (O'Connell, Krien et al. 2003). For instance, fin1 can promote chromatin condensation upon overexpression in the absence of Cdc2 activity (Krien, Bugg et al. 1998). Fin1 activity is cell cycle regulated and intriguingly shows two peaks of activity – one, corresponding to G2-M and the other at septation (Grallert, Connolly et al. 2012). As might be expected from these regulated peaks in Fin1 activity, Fin1 has been demonstrated to have roles in G2-M transition and as part of the septation initiation network. Fin1 localizes to SPB from G2 through to mitotic exit (Krien, West et al. 2002). The components of the septation initiation network (SIN) are operative only on one of the SPBs in S. pombe, and Fin1 location correlates with, and its activity is required, to maintain the complex asymmetric regulation of the SIN at the two separated SPBs (Grallert, Krapp et al. 2004). Fin1 is required to locate the Polo kinase to the SPB (Grallert and Hagan 2002), though this function is not conserved in A. nidulans (Bachewich, Masker et al. 2005).

Importantly, a role for Fin1 as part of the regulatory cascade that mediates entry into mitosis has been postulated based on very recent evidence in S. pombe (Grallert, Chan et al. 2013). In S. pombe, the checkpoint allowing adequate growth before triggering mitosis is mediated by the activity of the pom1 kinase that localizes to cell tips and inhibits the activation of Cdk1 kinase, preventing mitotic entry. Growth of the cell physically distances pom1 kinase from the Cdk1 regulatory circuit (that is localized to nodes in the middle of the
cell) effectively relieving this inhibition on Cdk1 activity (Tatebe, Nakano et al. 2008, Moseley, Mayeux et al. 2009). It has been shown that Fin1 activity regulates this activation of Cdk1 potentially through an effect on Wee1 (Grallert, Connolly et al. 2012). Furthermore, recent evidence delineates a role for Fin1 in phosphorylating a SPB component, Cdc12 that in effect results in the detachment of the PP1 phosphatase (a negative regulator of Cdk1 activity and thus of mitotic entry) from the SPB. This correlates with the recruitment of Polo kinase to the SPB (Grallert, Chan et al. 2013, Grallert, Patel et al. 2013). The recruitment of Polo kinase to the SPB by Fin1 plays a major role in establishing the positive feedback that causes a surge in Cdk1 kinase function resulting in a switch like activation and initiation of mitosis. Therefore, Fin1 plays a central role in the G2-M transition in S. pombe through at least two mechanisms – mitigating the inhibition of Cdk1 activity by the pom1 kinase and in detaching PP1 from the SPB and thus establishing a functioning positive feedback of Polo kinase activity to cause a switch like increase in Cdk1 activity.

There are 11 NIMA related kinases in humans, named Neks 1-11 (Fry, O'Regan et al. 2012). They share a conserved domain structure with NIMA, consisting of an N-terminal kinase domain and a C-terminal regulatory domain, which contains coiled coil domains (Wei, Ngo et al. 2011). Moreover, like NIMA, Nek2 and Nek6 were also shown to prefer a hydrophobic reside (F/L) at the -3 position to the site of phosphorylation indicating that there are likely many conserved substrates between NIMA and the NIMA related kinases in higher
eukaryotes (Pu, Xu et al. 1995, Lizcano, Deak et al. 2002, Alexander, Lim et al. 2011). Though it is difficult to conclude from studies done so far whether or not human Neks are essential for mitotic entry, they are certainly required for mitotic events that happen at G2-M transition. Neks may be activated either by autophosphorylation (Rellos, Ivins et al. 2007) or by another activating kinase (for instance, Plk1 in the case of Nek9 or Nek9 in the case of Nek6/7) (Belham, Roig et al. 2003, Bertran, Sdelci et al. 2011).

One of the most well studied aspects of Nek function is their contribution to the disjunction of duplicated centrosomes during the G2-M transition. Interestingly, studies have defined two parallel and redundant pathways involving multiple members of the Nek family. As a side note, this potentially indicates that the functions of NIMA have expanded in humans and been distributed among the multiple members of the Nek family to confer further fine tuning of cell cycle regulation. One pathway of centrosome disjunction involves the removal of proteins like C-Nap1, rootletin and β-catenin that link the mother centrosome with the daughter centrosome. The mitotic specific phosphorylation of the linker proteins mediated by Nek2, which is the closest human ortholog of NIMA, facilitates centrosome disjunction (Fry, Mayor et al. 1998, Bahe, Stierhof et al. 2005, Bahmanyar, Kaplan et al. 2008). In addition, the upstream regulatory cascade of Nek2 regulation has been shown to involve phosphorylation and activation of Nek2 by the Mst2 kinase of the Hippo pathway (that functions in a plethora of cellular functions including organ size and cell cycle regulation) and
the activation of Mst2 kinase by the Polo like kinase1 (Plk1) (Mardin, Lange et al. 2010, Mardin, Agircan et al. 2011). Interestingly, the most upstream regulator known in the second arm of centrosome disjunction regulation which operates redundantly to the Nek2 pathway described above also is Plk1. Plk1 activates the Nek9 kinase which in turn activates Nek6 and Nek7 ultimately resulting in the phosphorylation of the BIMC kinesin, Eg5 (Bertran, Sdelci et al. 2011). Activated Eg5 kinesin is thought to mediate lateral interaction between spindle microtubules thus helping generate the mechanical force for the separation of the centrosomes. In addition to centrosome separation, Neks are also implicated in the formation of a stable bipolar spindle. For instance, in the absence of adequate amounts of Nek6 or Nek7 kinases, spindles have reduced density (O'Regan and Fry 2009). Consistent with these observations, Nek9 has been shown to promote the recruitment of gamma tubulin to mitotic centrosomes (Kim, Lee et al. 2007, Sdelci, Schutz et al. 2012).

Nuclear pore complex (NPC) disassembly is one of the early mitotic events that was first shown to be under the regulation of NIMA in A. nidulans (De Souza, Osmani et al. 2004). Subsequently, it has been shown that this function is at least partially conserved in the mammalian Nek6 and Nek7 kinases. The depletion of Nek6 and Nek7 impairs mitotic NPC disassembly, and as in A. nidulans, these kinases are able to phosphorylate a NPC protein, Nup98 in vitro [Shen et al, unpublished, (Laurell, Beck et al. 2011)]. In addition, it has been
proposed that Nek9 has a function in initiating nuclear envelope breakdown by interacting with the dynein associated protein, BICD2 (Holland, Milne et al. 2002).

Another cellular process that multiple Neks are involved in is ciliogenesis (Quarmby and Mahjoub 2005). Cilia are protrusions from the cell surface that are involved in movement of the organism or the extracellular space (motile cilia) or in sensing the environment and signaling to the cell (primary cilia). Intriguingly, organisms that have cilia show an expansion of genes encoding NIMA family members. (Parker, Bradley et al. 2007). Initial insights into the involvement of Neks in cilary regulation came from studies in Chlamydomonas, in which one of the 10 Neks, Fa2, is required for the environment-induced severing of flagella (Mahjoub, Montpetit et al. 2002). In addition, mouse embryonic fibroblasts isolated from Nek7 knock-out mice are defective in the formation of primary cilia (Fry, O'Regan et al. 2012). It has also been suggested that Nek2 might function in coordinating cilia resorption with mitotic entry based on studies using human cell lines (Spalluto, Wilson et al. 2012). Since primary cilia have important roles in signaling, misregulation of cilia can have pleiotropic effects causing diseases called ciliopathies. One of the common features of ciliopathies is the occurrence of poly cystic kidney disease. Underlining the importance of ciliary regulation by Neks, mutations in Nek1 and Nek8 are widespread in human ciliopathy patients and were found to be causative in mouse models of polycystic kidney disease (Quarmby and Mahjoub 2005, Fry, O'Regan et al. 2012).
1.6 Polarized cell growth in *A. nidulans*

All growth in *Aspergillus nidulans* happens at cell tips via targeted exocytosis of secretory vesicles. Fusion of these vesicles at the cell apex, results in the addition of membrane and delivery of membrane proteins (such as cell end markers, see below). Just posterior to the zone of exocytosis at the cell apex is the collar of endocytic patches marking the endocytic zone (Taheri-Talesh, Horio et al. 2008). Excess membrane and proteins that need to be present only at the cell tip are thought to be internalized through endocytosis. Thus, the positioning of the two zones of exocytosis and endocytosis is important for polarized growth. However, little is known about the regulation of these processes.

Microtubules (Mts) are important for long distance transport in *A. nidulans*. Absence of Mts often results in the alteration of cell growth direction, but does not completely inhibit growth itself (Horio and Oakley 2005). The localization of tropomyosin cables focused at the cell tip and extending along the hypha indicates that actin plays a role in cell growth (Taheri-Talesh, Horio et al. 2008). Depolymerization of actin stops polarized growth and results in swelling of the cell tip, suggesting that non-polarized growth can still take place under these conditions. Therefore, it has been proposed that secretory vesicles are transported to the vicinity of the cell tip in a Mt dependent manner and thereafter move to the cell apex on actin cables. The cluster of electron dense material at the cell apex, consisting of secretory vesicles is called the Spitzenkörper (Harris,
Read et al. 2005). It is thought that the location of the Spitzenkörper is critical for maintaining the direction of polarized growth.

It has been recognized that the localization of 'landmark' or 'cell end marker' proteins contributes to defining the site of polarized growth (Chang and Peter 2003). In *S. pombe*, the protein complex formed by the cell end marker proteins functions to organize the actin cytoskeleton by the recruitment of the formin For3, thereby establishing the polarity axis. Landmark proteins, Tea1, Mod5 and Tea2, identified in *S. pombe* are conserved in *A. nidulans* (Fischer, Zekert et al. 2008). The absence of either of these proteins results in meandering hyphae potentially caused by the dislocation of the Spitzenkörper from the cell apex also seen in these mutants (Takeshita, Higashitsuji et al. 2008, Higashitsuji, Herrero et al. 2009). Therefore, these proteins have been proposed to function in determining growth directionality in *A. nidulans*. The landmark protein, Mod5, is prenylated and its localization in *A. nidulans* is consistent with an association with the membrane (Takeshita, Higashitsuji et al. 2008). Therefore it is important to understand the mechanism by which Mod5 is located to the membrane. Increasing evidence suggest that lipid rafts that are rich in sterols (called sterol rich domains, SRDs) play an important role in determining the polarity of cells. Studies in *A. nidulans* show that disrupting the function of SRDs results in the mislocalization of cell end marker proteins, Tea1 and Mod5 (Takeshita, Diallinas et al. 2012), showing that the membrane composition contributes to establishment of polarity axis. The Cdc42 cascade is an essential
regulator of polarized growth in budding and fission yeast (Fischer, Zekert et al. 2008). However, the Cdc42 ortholog is not essential in *A. nidulans* (Virag, Lee et al. 2007). Furthermore, *A. nidulans* possesses another Rho GTPase, a Rac1 homolog, although the relationship between the two Rho GTPases in regulating cell growth is not well understood in *A. nidulans*. Therefore, it is likely that there are unique mechanisms in place in filamentous fungi that confer their characteristically extreme form of polarized growth.

### 1.7 Overview of my dissertation research

The budding yeast ortholog of *nimA*, *KIN3* is a non-essential gene. A synthetic lethal screen was carried out using a *KIN3* deleted strain (SL Anglin et al., unpublished). This forward genetic screen led to the identification of a number of genes involved in diverse cellular processes. *nimA* is an essential gene in *A. nidulans* and therefore, a similar synthetic genetic screen would be technically difficult to perform in this organism currently. So the goal of my project was to gain further insights into the function of the *nimA* gene using the information garnered from the *S. cerevisiae* screen. Therefore my project started with identifying the synthetic genetic interactions of *nimA* that are conserved between *A. nidulans* and *S. cerevisiae*. These studies revealed the presence of three previously unknown conserved synthetic lethal interactions of *nimA*. The study of the genetic interaction between two of the genes, *An-vps23* and *An-vps25*, converged with the study of NIMA localization, to support the current
model that NIMA has previously unrecognized cytoplasmic functions during interphase. The description of these studies is presented in Chapter 3. The study of the genetic interaction between the third gene from the screen, \textit{An-swd1} and \textit{nimA}, revealed to us the essential function of the Set1 complex mediated histone H3 methylation in G2-M transition and mitotic progression as well as the relationship of the Set1 complex with Cdk1 function. These results are presented in Chapter 4. During the course of these studies, we observed an interesting phenomenon wherein partial inactivation of NIMA, instead of causing an expected G2-M delay, was seen to cause mitotic defects. We undertook investigations to try and define the mitotic defects in these cells and these results are presented in Chapter 5. Therefore, taken together, my results presented here have given us novel insights into the mitotic and non-mitotic functions of the NIMA kinase and further opened up exciting avenues of NIMA function to be explored in the future.
Figure 1.1 A schematic representation of *A. nidulans* vegetative growth. *A. nidulans* conidia (a) undergo isotropic growth (b) followed by mitosis (c). Germination marks the switch to polarized growth (d) and after a period of growth (e), the cell undergoes the second mitosis (f). This process is repeated (g-h) and cells with 8 nuclei typically form the first septum (i). Nuclei marked in red are in G1 arrest and do not undergo mitosis. Once a new cell tip starts to grow, the nuclei enter cell cycle (j). After the first septum is formed, septation typically takes place after every round of mitosis in apical cell (i-k). The nuclei in the basal compartment are arrested in G1 until the branch forms (k-l). Nuclei in the same compartment (indicated in one color) undergo parasymporhous mitosis.
Figure 1.1

- a \(\downarrow\) Isotropic growth
- b \(\downarrow\) Mitosis
- c \(\downarrow\) Germination
- d \(\downarrow\) Polarized growth
- e \(\downarrow\) Mitosis
- f \(\downarrow\) Polarized growth
- g \(\downarrow\) Mitosis
- h \(\downarrow\) Septation
- i \(\downarrow\) Emergence of a second growing tip
- j \(\downarrow\) Septation
- k \(\downarrow\) Branch formation
**Figure 1.2:** Conidiophore formation in *A. nidulans*: The differentiation of the foot cell marks the beginning of the asexual development leading to stalk and vesicle formation. The vesicle gives rise to metulae, phialides and conidia to form the mature conidiophore. See text for details. This figure is adapted from (Todd, Davis et al. 2007).
Chapter 2: Materials and Methods

2.1 Retrieval and analysis of genome and proteins sequences

The genome or protein sequences were obtained from the Aspergillus Genome Database (http://aspgd.org/) or the Aspergillus Comparative Database at the Broad Institute (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html). Sequences were viewed and mapped for open reading frames on Gene Runner Version 3.05 (Hastings Software Inc.). BLAST searches for protein and gene orthologs were performed at http://aspgd.org/ or at http://blast.ncbi.nlm.nih.gov/Blast.cgi. CustalW alignment was done using the Biology Workbench (http://seqtool.sdsc.edu/). Generation of sequence contigs after sequencing was done using MegAlign Version 5.00 (DNASTAR Inc.).

2.2 Generation of recombinant A. nidulans strains

2.2.1 Generation of constructs for gene deletion, gene tagging with fluorophores and site directed mutagenesis

Constructs for gene deletion or endogenous tagging were generated by fusion PCR (Yang, Ukil et al. 2004, Szewczyk, Nayak et al. 2006). The primers were designed on Primer Design software, Version 2.01 (Scientific and
Educational Software). The constructs were made using nutritional markers, *An-pyrG* or *An-pyroA*. Mutations were introduced within the histone H3 gene by site directed mutagenesis using primers carrying a single mismatched base. The histone gene was amplified using this primer carrying the appropriate mutation and then fusion PCR was performed to generate a construct that had the mutated H3 ORF fused with a nutritional marker (Yang, Ukil et al. 2004, Szewczyk, Nayak et al. 2006). The mutation was confirmed by sequencing before transformation.

### 2.2.2 Transformation of *A. nidulans*

*A. nidulans* transformation was carried out using published protocols (Szewczyk, Nayak et al. 2006). 1X10⁹ conidia of the recipient strain were inoculated in complete medium and allowed to break dormancy. At this time point, the conidia were transferred to media containing cell wall digesting enzymes. The digestion of the cell wall resulted in the formation of protoplasts with large vacuoles. This process was monitored under the microscope. After 2-2.5 hours of treatment with enzyme, the protoplasts were harvested, washed and suspended in an isotonic solution. The transformation of the fusion construct into the protoplasts was done as described in (Szewczyk, Nayak et al. 2006). The transformants were grown on appropriate media to select for the nutritional marker present in the construct. If deletion of a gene was being performed, the transformants were analyzed using the heterokaryon rescue technique as
described in Section 2.2.3. Once the transformant colonies are detected on selective media, the conidia from at least 4 transformant colonies were isolated using an inoculation loop and streaked on selective media to single colonies three times and then spot inoculated on selective media. The integration of the construct at the correct location in the genome was checked by performing PCR using primers that anneal outside the construct using the genomic DNA extracted from the different transformant colonies as the template.

The protocol for extraction of genomic DNA is as follows. The conidia from the transformant colonies were isolated and inoculated in 30 mL selective liquid media in petridishes. After 24 hours of growth at 32°C, a fine mycelial mat is formed in the media, which was harvested by vacuum filtration of the culture through Miracloth (Calbiochem) and washed with Stop Buffer (9gm/L NaCl, 65mg/L NaN3, 20mL 0.5M EDTA [pH 8.0], and 2.1g/L NaF). Thereafter the mycelia was frozen in liquid nitrogen and dried in a lyophilizer (Savant) overnight. The dried mycelia was ground and genomic DNA was extracted using the Wizard Plus Miniprep DNA Purification System (Promega Corporation) as described previously in (Osmani, Davies et al. 2006).

2.2.3 Analysis of gene deletions by the heterokaryon rescue technique

The deletion of genes required for normal growth results in the formation of heterokaryons in A. nidulans, which were analyzed by the heterokaryon rescue technique as published in (Osmani, Oakley et al. 2006). Conidia from at least 16
individual transformant colonies were streaked on selective and non-selective media and incubated for 24-36 hours at 32°C. If the gene deleted is non-essential, then growth is observed on both selective and non-selective media. If the gene deleted is essential, then growth is observed on non-selective media but will be restricted on the selective media by the absence of the essential gene. The phenotype of essential gene deletions can be studied by growing the conidia isolated from heterokaryons on selective media. Since An-pyrG is one of the tightest nutritional markers in regular use for genetic manipulation of A. nidulans, this marker was used to select for gene deletions.

In cases where the above analysis indicated that the gene deletion is essential, the strain was propagated as heterokaryons by the procedures as described below. The mycelial mat of the deletion transformant was carefully removed from the edge of the colony from the original transformation plates and placed on the center of plates that are selective for the nutritional marker. After 3-4 days the heterokaryon can be seen to be growing radially from the mycelial mat. For genomic DNA extraction, the mycelial mat from the heterokaryon colony was isolated, cut into several pieces and inoculated into petridishes containing selective liquid media. Mycelia was harvested and genome DNA extraction was done as described in Section 2.2.2. The presence of the two nuclei of different genotypes in the heterokaryons was confirmed by performing PCR using primers that anneal outside the gene deletion construct. For long term storage, the mycelial mat of the growing edge of the heterokaryon colony and the solid media
underneath was cut into small square cubes and placed into eppendorfs. These were frozen at -80°C and can be revived by placing on petridishes containing selective media again.

2.2.4 Generation of strains by meiotic crossing

To generate recombinant strains using meiotic crossing, parental strains that complement each other for two nutritional markers were chosen. These strains were spot inoculated on complete media in petridishes and allowed to grow until the radially growing colonies were close together. At this point, mycelial mat at the intersection of the two colonies consisting of both parental strains was removed and implanted into petridishes containing media that is selective for both the parental strains. This forces sexual reproduction between the two strains which results in the formation of cleiostothecia after about 2-3 weeks at 32°C. The cleiostothecia were cleaned from the surrounding cells under a dissecting microscope (Bausch and Lomb) and crushed in an eppendorf containing 500µl of 0.2% Tween 20. The ascospores thus released, were plated on complete media and individual colonies were spot inoculated on media lacking one of the several nutritional supplements to work out the genotype of the strain (Pontecorvo, Roper et al. 1953).
2.2.5 Generation of diploids

Diploid generation was performed following the general protocol as published in (Todd, Davis et al. 2007). It is recommended though not required that the two parental strains used for diploid generation are mutant for the production of Wt conidial pigment (green) such that their mutations complement each other. This aids the easy identification of diploids which would form green conidia. 3 mL of complete agar media was placed at the bottom of a sterile capped glass test tube and was allowed to set. Thereafter, 2 mL of sterile water was added to the top. Conidia from each of the parent strains were isolated using an inoculation loop and floated on the surface of the water. These tubes were incubated at 32°C undisturbed for 24-36 hours and the formation of the mycelial mat on the surface of the water was monitored. This mat was removed using forceps and washed in water to remove all nutrients, teased apart into small pieces and placed on petridishes containing minimal media lacking all supplements except those required by both parental strains. These plates were incubated for several days until the appearance of heterokaryons that form by the fusion of hyphae of the two different genotypes was detected. The conidia from these heterokaryons were isolated and added to agar containing liquid selective media at 50°C in a wide range of dilutions. The selective media was poured into petridishes and the plates were again allowed to incubate for several days. The selection imposed at this step allows the growth of only diploids.
2.2.6 Storage of haploid and diploid strains

The strain to be processed for long term storage was spot inoculated on appropriate media. After 2 days of growth at 32°C, the plates were incubated at room temperature (23°C) for another 3-4 days. This allows the conidia to set down a thicker cell wall, which helps towards long term viable dormancy. The mature conidia were then harvested into 1mL of sterile 7.5% milk (7.5g of Carnation Nonfat Dry Milk in 100mLs of dH₂O and autoclaved for 20 minutes) using a glass spreader and transferred into pre-cooled screw top vials containing baked silica. The silica was vortexed to allow mixing of the spores and silica and the vials were transferred back on ice and allowed to cool for another hour. After this incubation, the vials were left at room temperature to allow the silica beads to dry completely and stored at room temperature. The strains can be revived by inoculating a few silica beads onto a petridish with complete solid media.

2.3 Examination of synthetic lethality of gene deletions with nimA7 or nimT23

For comparison of colony growth between different strains, the strains to be compared were allowed to grow from silica beads to form fresh colonies at 32°C. One loopful of conidiospores were harvested from each strain using an inoculation loop and then transferred into 500µl of 0.2% Tween 20. The concentration of spores in this suspension was calculated using a hemocytometer. Then the suspension was diluted if necessary and roughly 100
spores of each strain were spread on the appropriate plates using a glass spreader. The plates were incubated at temperatures as per the nature of the experiment.

2.4 Epifluorescence microscopic analysis of fixed cells

2.4.1 Fixation and DAPI/ Calcofluor staining

One loopful of conidiospores harvested from strains to be analyzed using an inoculation loop was transferred into 500µl of 0.2% Tween 20. The concentration of the spores in this suspension was calculated using a hemocytometer. No. 11/2 coverslips (Corning Labware and Equipment) were sterilized using ethanol and then placed in 100x15 mm petri dishes (Fisher Scientific). 300µl of appropriate liquid media was placed on these coverslips. Then conidia at a final concentration of $1 \times 10^6$ spores/mL were inoculated into the media. The cells were allowed to grow at the appropriate temperature for the required amount of time. Thereafter, the cells were fixed by draining the media and inverting the coverslips on to 200µl of DAPI fix (4% gluteraldehyde, 0.02% NP40, 50mM PO4, pH 6.5). After 20 mins of fixation, the coverslips were washed in water three times and then inverted on 10µl of DAPI solution (made in the ratio of 1:4 = 1.5µg/ml DAPI: Citifluor mounting solution) placed on glass microscopy slides (Corning). If Calcofluor staining was being done, after fixation, the coverslips were inverted on Calcofluor solution (0.7 µg/ml Calcofluor, 5mM KOH) for 15 mins and then washed once before being mounted on DAPI/Citifluor
solution. The cells were allowed to be stained for 15 minutes in the dark and the coverslips were sealed. The cells were viewed using an E800 microscope (Nikon, Inc.) with DAPI, FITC and Texas Red filters (Omega Optical, Inc.). An UltraPix digital camera (Life Science Resources, Ltd) was used to capture images.

2.4.2 Fixation of cells carrying GFP and/or RFP tagged markers

For fixation of cells carrying GFP/RFP markers, the same procedure as described in Section 2.4.1 was used. Importantly, the immunofluorescence fix (6% paraformaldehyde, 0.1% glutaraldehyde, 5% DMSO, volume made up to 25.5 ml with PHEM (45mM PIPES, 45mM HEPES, 10mM EGTA, 5mM MgCl$_2$, [pH 6.9])) was used instead of the DAPI fix.

2.4.3 Quantitation of cell length and number of nuclei per cell length

The images captured using the epifluorescence microscope as described in Section 2.4.1 were analyzed using the image analysis software, ImageJ, version 1.46m (http://rsbweb.nih.gov/ij/). The cell length was measured using the line tool and number of nuclei in each cell was noted manually. The ratio of number of nuclei per cell length was calculated for each cell and then the average of this parameter was computed.
2.5 Confocal microscopy of live cells

2.5.1 Growth of cells and drug treatments

Conidia were inoculated in 3 mL of liquid minimal media without riboflavin in 35 mm glass bottom petri dishes (MatTek Cultureware). Cells were observed using a 60X 1.49 TIRF objective lens on a Nikon Eclipse TE 2000-U (Nikon, Inc.) microscope along with an UltraView ERS spinning disk confocal system (Perkin Elmer). Images were captured using a Hamamatsu ORCA-AG camera and 488 and 568 nm argon ion lasers. For treatment of cells with benomyl, 2 mL of media was carefully removed from the dish. Liquid media containing 2.4µg/ml of benomyl was added and mixed gently. 2 mL of media was again removed from the top and replaced with 2 mL benomyl containing media. Imaging was started immediately afterwards. For treatment of cells with latrunculin B, 1.5 mL was removed from the dish and discarded, leaving 1.5 mL behind. From the remainder of the media, 500µl was removed and latrunculin was added to a concentration of 40µg/ml. This media was mixed thoroughly and added back to the dish. The media was gently mixed to get even concentration of latrunculin B.

2.5.2 Growth of cells using agar-anchor method

In order to observe long cells growing synchronously and radially from a single point in a manner similar to growth of cultures in flasks, we developed the agar-anchor method. Conidia isolated from the required strain were transferred into 50µL of 0.2% Tween 20 to give a concentrated spore suspension. 3µL of this
spore suspension was placed a little offset from the center of the imaging dishes described above and allowed to dry completely. Then about 50 µL of molten agar containing complete media was placed on the spores. The agar was allowed to solidify and then liquid media was gently added to the dish. The dish was incubated at the appropriate temperature and imaged using live cell microscopy. This method is also applicable for treatment of cells with drugs or induction of expression from a regulatable promoter.

2.5.3 Temperature controlled experiments

Conidia were inoculated in 2mL liquid media without riboflavin in Bioptechs Delta T™ Dishes (Bioptechs Inc.). The cells were grown at the required temperature in humidity chambers to prevent evaporation of the media. Before imaging, the objective and the stage were heated using equipment from Bioptechs Inc. Initially a dish containing water was used to calibrate the temperature. Thereafter, the dish with the cells adhered to the bottom was transferred to the temperature controlled setup on the microscope. The sample was allowed to equilibrate for about 10 mins and imaging was started thereafter.

2.6 Analysis of protein levels by Western Blotting

Strains were grown for protein preparation in shaking liquid flask cultures. The harvested mycelium was freeze dried, as described previously, and ground with a mortar and pestle. Protein extraction was done using 6M urea sample
buffer. The extracted protein was run on 8-10% SDS-polyacrylamide gels using the rainbow recombinant proteins (GE Healthcare) as molecular weight markers. Proteins were transferred to a nitrocellulose membrane using a gel transfer apparatus (Bio-Rad Laboratories, Inc.) at 180mA. The membrane was treated in 5% milk (5g of Carnation Nonfat Dry Milk in 100 mL of TBS buffer – 2mM Tris base, 5M NaCl at pH 7.5) followed by incubation in primary antibody in 5% milk. The membrane was washed 3 times in TBST buffer (TBS buffer with 0.5% Tween 20) and then incubated in the secondary antibody in 5% milk. The membrane was washed three times in TBST before protein detection using the enhanced chemiluminescence reagent kit (Thermo Fisher Scientific Inc.). The primary antibodies used were anti-NIMA (1:2000) and anti-tubulin (Sigma B512 – 1:10,000). ECL peroxidase-labeled secondary antibodies used were anti-rabbit (1:5000) and anti-mouse (1:5000) (GE Healthcare).

2.7 Titration of protein levels using the regulatable alcA promoter

Strains carrying an ectopic gene under the alcA promoter can be grown on media containing different carbon sources to provide conditions of gene repression, non-induction-non-repression (derepression) and induction. In liquid flask cultures containing yeast extract-lactose media, alcA expression was induced by the addition of 100mM threonine. For live cell imaging, cells carrying alcA regulated gene constructs were grown in minimal media containing glucose (which represses the expression of alcA). Thereafter media exchange was done
to switch the cells into minimal media containing either glycerol (460µL/mL, for derepression) or 1% ethanol (for induction). Cells were also germinated in glycerol or ethanol to examine the effect of continuous expression off of alcA on the cell growth.

2.8 Analysis of live cell microscopy movies

2.8.1 Pixel intensity profiles

The line tool in ImageJ was used to make the ROI along which the pixel intensity was to be plotted. The pixel intensity plot was generated using the ‘plot profile’ function under the ‘Analyze’ tab. The values that were generated by this function were used to create a customized graph in Microsoft Excel with pixel intensity % on the y axis and the cell length in µm on the x axis.

2.8.2 Kymographs

The line tool was used to make line along which the movement was to be tracked. The width of the line can be adjusted to incorporate a wider area into the quantitation. The kymograph plugin was used to generate the kymograph.

2.8.3 Calculation of rate of movement of dynamic proteins

The movement of the protein is represented as slanting lines on the kymographs. The slope of the line was used to compute the rate of movement. The line tool was used to draw a line exactly on the tracked path of the particle.
The angle of this line was measured using the Measure tool under ‘Analyze’ tab. The tangent of this angle was calculated in Microsoft Excel after conversion of the units of the angle from degrees to radians. Thereafter, the reciprocal of this value was generated, which represents the slope of the line in the units of pixel per frame. Pixel was converted to units of µm (4.4 pixels = 1µm) and the time delay between individual frames in the movie was used to convert the units from per frame to per second or per minute.

2.8.4 Quantitation of signal intensity

Mean pixel intensity within a specified ROI was measured using the Measure function in ImageJ. The value for mean background fluorescence using the same ROI was measured from a region outside the cell and subtracted from the values obtained inside the cell. The graphs were plotted according the requirement using Microsoft Excel.

2.9 Strains

The *A. nidulans* strains used in the studies described in this dissertation are listed in Table 2.1.
### Table 2.1: List of *A. nidulans* strains used in this study

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>KF45</td>
<td>nimA-GFP::pyrG4; NDC80-cR::pyroAAF; argB2; nirA14; sE15; wA3</td>
</tr>
<tr>
<td>CDS683</td>
<td>alcA::NIMA-GFP::pyr4+ (pyrG89); Nup49-CR::pyroAAf; chaA1 (pyroA4? nirA14?)</td>
</tr>
<tr>
<td>CDS131</td>
<td>alcA::ΔNNimA-GFP::pyr4 (pyrG89); pyr4+; pyroA4; wA</td>
</tr>
<tr>
<td>R153</td>
<td>pyroA4</td>
</tr>
<tr>
<td>MG394</td>
<td>alcA::NIMA-GFP::pyr4+ (pyrG89); tub-GFP; Sad1-CR::pyroA4+ (pyroA4?); pabaA1; wA</td>
</tr>
<tr>
<td>MG385</td>
<td>alcA::NIMA-GFP::pyr4+ (pyrG89); Eb1-CR::pyroAF (pyroA4?); ΔnKu::argB/argB+; sE15; nirA14?</td>
</tr>
<tr>
<td>MG409</td>
<td>alcA::NIMA-GFP::pyr4+ (pyrG89); argB2; sE15; wA3</td>
</tr>
<tr>
<td>MG410</td>
<td>alcA::NIMA-GFP::pyr4+; pyrG89; ΔAn-Eb1::pyrG4+ (pyrG89); argB2; sE15; wA3</td>
</tr>
<tr>
<td>MG397</td>
<td>ndc80-CR::pyroA4+::alcA::ΔNNimA-GFP::pyr4+; NIMA-GFP; pyrG89; wA3</td>
</tr>
<tr>
<td>MG395</td>
<td>tub-GFP; Eb1-CR::pyroA4+ (pyroA4?); argB2; sE15; nirA14?</td>
</tr>
<tr>
<td>MG44</td>
<td>nimA7; ΔKu70::argB (argB2); riboA1; pyroA4; pyrG89; nicB8/A2+; wA3</td>
</tr>
<tr>
<td>MG8</td>
<td>ΔAn-zuo1::pyrGAf (pyrG89); ΔAn Ku70::argB (argB/B2); pyroA4; argB2; sE15; wA3</td>
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Table 2.1: List of A. nidulans strains used in this study. Question marks indicate that the nutritional marker may be present but has not been confirmed.
Chapter 3: A cytoplasmic function for the mitotic NIMA kinase in regulating microtubule dynamics and cell growth in concert with the ESCRT pathway

3.1 Introduction

3.1.1 Apical dominance

The filamentous growth of *A. nidulans* gives rise to basal cell compartments, marked by septal boundaries and actively growing apical cells as described in Section 1.1.1. Growth of de novo cell tips can occur from basal cell compartments. However, the initiation of new cell tips is suppressed near the vicinity of a growing cell tip, underlining a general rule that there is only one growth site per cell compartment (Figure 3.1A). The phenomenon by which the emergence of secondary polarity axes is suppressed at the cell apex is called apical dominance. Maintaining a single dominant cell tip is likely significant to maintain the growth advantage of the organism and probably contributes to the fast growth rates of hyphal cells. It has been suggested that the generation of reactive oxygen species (ROS) at the cell tip might contribute to the maintenance of a single dominant cell tip (Semighini and Harris 2008). Although apical dominance is a recognized feature of filamentous growth, the regulation of this phenomenon is not understood.
Studies from our lab (described here) and other labs have shown that microtubules (Mt) depolymerization can have a noticeable effect on cell growth in *Aspergillus nidulans* (Horio and Oakley 2005). Upon Mt depolymerization, the first observable phenotype is a change in the growth direction and often the emergence of another growing cell tip (Horio and Oakley 2005, Taheri-Talesh, Horio et al. 2008). It has been proposed that in the absence of Mts, the Spitzenkörper, the cluster of secretory vesicles at the cell apex (Section 1.60, is no longer maintained at the cell apex but can drift, thereby leading to changes in growth. Our data, as will be described in this chapter, now show that the continued absence of polymerized Mts can result in a dramatic breakdown of apical dominance completely changing cell tip morphology.

### 3.1.2 Microtubule Plus end binding proteins

Plus end binding proteins dynamically interact with the growing end of the microtubules (Akhmanova and Steinmetz 2008). The tracking ability of these proteins may be due to a specific affinity for the growing end of Mt lattice or due to an interaction with plus end directed motors (Galjart 2010). This growing family of + tip interacting proteins (or +TIPs) possesses the ability to regulate the dynamicity of Mts by influencing the polymerization rate, catastrophe or bundling (Akhmanova and Steinmetz 2008). Plus end binding proteins also interact with proteins on the plasma membrane or intracellular organelles and thus contribute to cell migration, organelle dynamics and chromosome segregation (Tamura and
Draviam 2012). Therefore, the biology of plus end binding proteins has gained enormous interest over the last decade.

One of the most well studied plus end tracking proteins is the Eb1 family of proteins. Interestingly the localization of several proteins to the Mt plus end is dependent on Eb1 (Vaughan 2005). Eb1, on the other hand, locates the Mt plus ends autonomously at least in vitro (Jiang and Akhmanova 2011). Eb1 has a calponin (CH) homology domain in its N terminal which mediates Mt binding. The C terminal domain of Eb1 consists of two distinct regions that seem to mediate interaction with two different classes of plus end binding proteins. The extreme C terminus has the conserved EEY/F motif that mediates interactions with the first class of plus end tracking proteins which have the cytoskeleton associated protein Gly-rich (CAP-Gly) domain at their N terminus (Honnappa, Okhrimenko et al. 2006). The C terminus of Eb1 also comprises of an end binding homology (EBH) domain that mediates dimerization thereby forming a hydrophobic pocket, which interacts with the second kind of plus end binding proteins that possess the consensus motif, (S/T)x(I/L)P or the SXIP motif (Honnappa, Gouveia et al. 2009).

3.1.3 The Endosomal Sorting Complex Required for Transport (ESCRT) Pathway

When proteins on the plasma membrane are internalized by endocytosis, they can either be recycled back to the plasma membrane or be degraded
Internalization and subsequent degradation of membrane proteins like receptors is an important regulatory mechanism to downregulate the function of plasma membrane localized proteins. In addition, this pathway also in effect removes excess membrane thus maintaining the composition of the plasma membrane. After endocytosis, the target proteins that need to be degraded are present on the membrane of the endosome. To facilitate their degradation in the vacuole, they are internalized into the lumen of endosomes (Figure 3.1B). Thus, several vesicles carrying target proteins to be degraded are formed within the endosome, and this organelle is called the multivesicular body (MVB) or the late endosome. When the MVB fuses with the vacuole, these intraluminal vesicles are delivered into the lumen of the vacuole resulting in the proteolysis of the target protein.

A complex pathway consisting of multiple distinct protein complexes mediates the invagination of the early endosome membrane and the scission of the membrane neck, thereby catalyzing the formation of the intraluminal vesicles inside the MVB (Williams and Urbe 2007). This pathway is called the Endosomal Sorting Complex required for Transport or the ESCRT pathway. The genes encoding ESCRT pathway components were discovered from forward genetic screens in S. cerevisiae aimed at identifying genes involved in intracellular vesicular trafficking. The absence of ESCRT function in S. cerevisiae results in the formation of the characteristic class E compartment (Raymond, Howald-Stevenson et al. 1992, Henne, Buchkovitch et al. 2011). The class E
compartment is a prevacuolar organelle that contains mis-sorted vacuolar and Golgi proteins (Coonrod and Stevens 2010). ESCRT pathway components are highly conserved in all eukaryotes studied. Over the last decade, research on the ESCRT pathway has expanded considerably, especially with the identification of mammalian orthologs and biochemical and structural characterization of the protein complexes (Henne, Buchkovich et al. 2011). These studies have helped elucidate the function of the 5 protein complexes involved in this pathway – ESCRT 0, ESCRT I, ESCRT II, ESCRT III and vps4. The S. cerevisiae proteins that function as part of each of these complexes are indicated in Figure 3.1C. The target protein that is to be degraded gets mono-ubiquitylated, and this modification serves as a signal to sort the protein into the ESCRT pathway. The VPS27 subunit of ESCRT 0 directly binds to the ubiquitin moiety as well as phosphotidylinositol 3-phosphate (PIP3) thus recruiting the first ESCRT complex to the surface of the endosome. Thereafter, the proteins of ESCRT II are recruited to the membrane through a direct interaction between HSE1 and VPS23. VPS23 in ESCRT I also interacts with the ubiquitylated target protein. Subsequently, the ESCRT II complex is recruited to the membrane through protein and lipid based interactions with the target protein and PIP3. Finally the ESCRT III complex is recruited followed by the VPS4 AAA-ATPase and its associated proteins. The membrane binding of the ESCRT III complex proteins induces oligomerization of the SNF7 subunit which is proposed to contribute to membrane curvature and invagination (Henne, Buchkovich et al. 2011). Finally
the ATPase activity of the \textit{VPS4} results in the dissociation of the ESCRT proteins from the membrane.

Though the \textit{VPS} proteins were identified as being required for MVB generation, they have since been found to be responsible for the scission of the membrane neck during viral budding and cytokinesis (Wollert, Yang et al. 2009). Moreover, specific subunits of the ESCRT machinery have been shown to be involved in pH signaling in \textit{S. cerevisiae}, \textit{Candida albicans} and \textit{A. nidulans} (Xu, Smith et al. 2004, Hayashi, Fukuzawa et al. 2005). Interestingly, recent evidence from \textit{A. nidulans} indicates that some ESCRT pathway components might be localized to the plasma membrane during their function in pH signaling and that this function might be independent of their function at the endosome (Galindo, Calcagno-Pizarelli et al. 2012). In mammalian cells and \textit{Drosophila}, depletion of ESCRT pathway function causes a block in the degradation pathway of growth factor receptors and thus causes continued signaling promoting cell proliferation (Gilbert and Moberg 2006). Therefore, the function of the ESCRT proteins is important not only for the correct sorting of membrane proteins but also for the regulation of signaling.
3.2 Results

3.2.1 Cytoplasmic localization of NIMA during interphase

3.2.1.1 The mitotic NIMA kinase locates to growing cell tips

To enable detection of endogenous NIMA in live cells, strains carrying replacement of NIMA with NIMA tagged with GFP were generated and observed using spinning disc live cell confocal microscopy. In addition to confirming the previously defined mitotic locations of ectopically expressed NIMA (De Souza, Osmani et al. 2000), these studies revealed an additional unexpected location for NIMA at cell tips during interphase. As shown in Figure 3.2A, a low amount of the NIMA protein could be detected, often in a dome shaped location, at the growing cell tip. Quantitation using pixel intensity profiles (Figure 3.2 B-D) shows a peak in NIMA signal intensity juxtaposed with the growing tip. Intracellular transport towards the cell tip in *A. nidulans* is mediated by both microtubules (Mts) and actin based systems (Fischer, Zekert et al. 2008). Therefore we sought to determine whether Mts and actin were necessary for locating NIMA to the cell tip. We find that NIMA is able to locate to both cell tips and newly emerging branches in the presence of benomyl, a microtubule poison, indicating that NIMA locates to cell tips in a largely microtubule independent manner at least in the short term (data not shown). Interestingly, we find that depolymerization of actin results in an increase of NIMA signal in 54% of the cell tips (Figure 3.2E) studied. Quantitation of this signal along the cell tip shows that there is a one and a half fold increase in NIMA signal at the cell tip as well as accumulation of NIMA away
from the tip resulting in a broader pixel intensity profile extending back from the cell tip compared to untreated cells (Figure 3.2F).

3.2.1.2 Overexpression of NIMA in growing hyphae promotes the growth of multiple cell tips at the cell apex

The interphase cytoplasmic location described above suggests that NIMA might have a role in regulating cell tip growth. To test this hypothesis, WT cells or cells carrying either NIMA-GFP or a truncated version of NIMA comprising only the C terminal regulatory domain (NIMA-RegD-GFP), under the regulatable promoter alcA, were grown to the hyphal stage under repressive conditions in liquid flask cultures (Waring, May et al. 1989). The expression of the NIMA constructs was then induced by the addition of threonine (a robust inducer of alcA expression). In WT cells, before or after addition of threonine, and before induction of alcA in cells carrying the two different versions of NIMA, cell tips exhibited normal uniform morphology (Figure 3.3). Strikingly, the induction of NIMA-GFP promoted a dramatic modification of cell growth which created multiple branching events revealing a breakdown of apical dominance (Figure 3.3C, green arrow). Some NIMA-GFP expressing cells also showed uniform swelling (Figure 3.3C, blue arrow) or recurrent swelling events (data not shown). These phenotypes, as quantitated in Figure 3.3D suggest that excess NIMA promotes swelling and branching of apical cell tips with high penetrance. Importantly, cells expressing NIMA-RegD-GFP that lacks the N terminal kinase
domain under similar conditions resembled WT cells (Figure 3.3B, D), suggesting that the kinase domain of NIMA is required to promote misregulation of cell growth. Likewise, the expression of NIMA-GFP results in more severe defects in colony growth and conidiation compared to the expression of NIMA-RegD-GFP that lacks the kinase domain (Figure 3.4B). Collectively the data indicate that, in addition to its well established roles in mitotic regulation, NIMA likely plays roles during interphase involving the regulation of normal cell tip growth.

3.2.1.3 Expression of ectopic NIMA results in reduced polymerized tubulin, potentially leading to the establishment of extraneous growth axes at the cell tip

As introduced earlier, depolymerization of microtubules (Mts) in A. nidulans can affect cell growth (Horio and Oakley 2005). Therefore we thought it was possible that exposure of cells to benomyl, under similar growth conditions employed in the alcA NIMA induction experiments above, might lead to the kind of dramatic cell tip morphology defects that we observed in the case of NIMA overexpression. To test this idea, WT cells were grown in a manner similar to the experiment in Figure 3.3, and then subjected to treatment with benomyl. After 1.5 hours of exposure to benomyl, we find that virtually every cell tip displays multiple branching events (Figure 3.5A). This led us to hypothesize that the effect of NIMA on cell tip growth might be via a modification of the microtubule network. To test this hypothesis directly, we generated strains carrying alcA-NIMA-GFP
that also express tub-GFP (Ovechkina, Maddox et al. 2003) to visualize the effects of NIMA on Mts. These cells were grown for 24 hours under repressive conditions and then exchanged into media to promote expression driven by the alcA promoter (Figure 3.5B). Upon the induction of NIMA expression, cell tip swelling and the growth of multiple cell tips at the cell apex, were observed by live cell microscopy (Figure 3.5B; cell 1 and 2), that largely phenocopied the effects seen previously upon NIMA expression in liquid flask cultures. In addition, we determined that the expression of NIMA is accompanied by an obvious modification of the microtubule network resulting in barely detectable microtubule tracks (Figure 3.5B, cell 1). Entry into mitosis following NIMA expression was also seen as expected, as detected by nuclear NIMA accumulation and spindle formation (indicated by arrows). Though microtubule tracks ceased to be prominent upon NIMA induction, we noticed comet like structures in the cytoplasm (Figure 3.5B, arrowheads). To further define these comets, we germinated cells carrying alcA-NIMA-GFP and tub-GFP under repressing and non-repressing, non-inducing (derepression) conditions (Figure 3.5C, D). As expected, we find WT-like microtubule tracks when the expression of alcA-NIMA-GFP is repressed (Figure 3.5C), while microtubule tracks were far less obvious under derepression conditions (Figure 3.5D). Interestingly, we also observed comet like structures when NIMA-GFP expression was constitutively derepressed (Figure 3.5D, arrowheads). Using time lapse imaging with shorter delays, we determined that these comets were highly dynamic (see below). The
nature of movement of these comets suggested that these were likely not segments of microtubules and further analysis of this is presented below. Taken together, these results indicate that the expression of NIMA results in a sustained effect on the interphase cytoplasmic microtubule network such that there is reduced polymerized tubulin. Therefore, it is possible that the depolymerization of Mts upon induction of NIMA contributes to the generation of multiple growth axes at the cell apex.

3.2.1.4 Ectopic NIMA-GFP displays a microtubule dependent localization to dynamic cytoplasmic comets

Since the overexpression of NIMA-GFP results in reduced polymerized tubulin as well as breakdown of apical dominance, we sought to determine the localization of ectopic NIMA-GFP. To this end, we observed cells expressing ectopic NIMA-GFP from a regulatable alcA promoter under derepression conditions which results in a 10 fold increase in NIMA protein compared to endogenous levels (as determined microscopically). We find that NIMA-GFP locates to dynamic cytoplasmic comets (Figure 3.6A, Figure 3.5D), which exhibit an average rate of movement of approximately 9 +/- 6 µm/min (n=104). This rate is similar to the published average polymerization rate of Mts in Aspergillus nidulans (Xiang, Han et al. 2000, Efimov, Zhang et al. 2006), suggesting that the NIMA comets might be associated with the polymerizing end of the Mts. In the presence of the microtubule depolymerizing drug benomyl, the NIMA comets
immediately disperse (Figure 3.6C), consistent with the hypothesis that the comets are dependent upon polymerizing Mts for their formation. Immediate dispersal of NIMA comets in benomyl is also remarkably similar to the behavior of the Mt plus end binding protein Eb1 in *A. nidulans* (data not shown). Also, if NIMA comets disappeared when Mts are depolymerized with benomyl, we would expect the same result when cytoplasmic Mts are depolymerized in mitosis. And consistently, our preliminary data shows that during mitosis, NIMA is seen to locate to the nucleus. Thereafter at the end of mitosis, NIMA comets reappear (Figure 3.7). We note that before mitotic entry, NIMA signal is seen to be dispersed for a reasonable period of time (at least 27 minutes in the cell shown in Figure 3.7). This may suggest that in these cells, there is a time delay between the depolymerization of cytoplasmic microtubules and the appearance of NIMA at the nucleus, which in the case of Wt cells would happen almost simultaneously. Alternatively, this may indicate that the localization of NIMA to the comets is cell cycle regulated in such a way that it does not locate to the comets at G2 but does so immediately after mitosis in G1. Examination of alcA-NIMA-GFP expressing cells through a complete cell cycle with respect to a mitotic marker in the future should help us distinguish between these two possibilities. However, these data say that the localization of NIMA to the cytoplasmic comets is not seen during mitosis when cytoplasmic Mts are expected to be depolymerized.

NIMA-GFP comets exhibit bidirectional movement near the cell tip (82% cells, Figure 3.6A). The montage in Figure 3.6A shows an example of a cell in
which we were able to track the movement of NIMA-GFP comets towards and away from the tip. The kymograph generated using the ROI indicated in Figure 3.6B shows the bidirectional movements of NIMA comets near the cell tip. 54% of these cells also displayed swollen tip morphology (Figure 3.6A) or swelling along the germ tube (data not shown), indicating that germination of cells under conditions that derepresses alcA-NIMA-GFP expression affects normal cell growth.

3.2.1.5 Ectopic NIMA-GFP colocalizes with the microtubule plus end binding protein Eb1 and locates to the polymerizing end of Mts in an Eb1 dependent manner

The rate of movement of the microtubule dependent alcA-NIMA-GFP comets was similar to the rate of microtubule polymerization indicating NIMA might be locating to the plus ends of Mts. To test this we generated strains carrying alcA-NIMA-GFP in the background of a well-studied Mt plus end binding protein, Eb1, tagged with chRFP. In A. nidulans, Eb1-CR appears as dynamic comets in the cytoplasm, representing the plus ends of the Mts (Figure 3.8, Figure 3.10C and (Egan, Tan et al. 2012)). Upon induction of NIMA-GFP, we were able to observe colocalization between the NIMA comets and Eb1-CR (Figure 3.8A, arrows, 3.8C). In support of the idea that NIMA and Eb1 might function together at the plus ends of Mts, we find that the behavior of Eb1 is modified in alcA-NIMA induced cells. In cells repressed for alcA-NIMA-GFP
expression, near the vicinity of the cell tip, the vast majority of Eb1 comets move unidirectionally towards the cell tip (Figure 3.8E). However, in cells carrying alcA-NIMA-GFP, under derepression conditions, Eb1 exhibits movement both towards and away from the tip (Figure 3.8E). Often we were able to track the movement of Eb1 comets moving around cell tips (Figure 3.8D, arrows), very similar to the movement of NIMA-GFP under similar induction conditions (Figure 3.6A, B), suggesting strongly that the presence of ectopic NIMA alters Eb1 behavior and perhaps regulates its function. However, the average rate of Eb1 does not change significantly (p>0.05) in repressed (7.3um/min) vs. derepressed conditions (8.5 um/min). Also the rate of Eb1-CR movement under derepressed conditions is similar to the rate of NIMA-GFP under the same conditions, which is consistent with both of them locating to the Mt plus ends.

We noticed that the number of cells that showed comets was reduced and the signal of NIMA at the comets was weaker in the presence of Eb1-CR. To confirm this observation, we co-cultured cells of two different genotypes both carrying alcA-NIMA-GFP. One strain also had tagged nup49-CR (a nuclear pore complex protein) in the background while the other strain was double tagged for Eb1-CR. Images of randomized fields of the co-culture were captured. If the comet location of NIMA was unaffected by the presence of Eb1-CR then, among the population of cells that show alcA-NIMA-GFP comets, we would expect to see roughly equal percentage of cells carrying either nup49-CR (~50%) or Eb1-CR (~50%). When we quantitate the number of cells that showed alcA-NIMA-
GFP comets in the co-culture, we find that 85% of the cells with alcA-NIMA-GFP comets were double tagged for nup49-CR, while the rest 15% of the cells with alcA-NIMA-GFP comets were double tagged for Eb1. This suggests that it is more difficult to detect NIMA at the comets when Eb1 is also tagged in the background of that strain. Eb1 is known to act as an interaction hub at the Mt plus end since it interacts with and is responsible for recruiting multiple Mt plus end binding proteins (Jiang and Akhmanova 2011). We hypothesized that the recruitment of NIMA to the plus end of the Mts might be hindered when the C terminus of Eb1 is tagged with CR and therefore, perhaps less accessible for binding. To directly ask whether Eb1 is required for the localization of NIMA to Mt plus ends, we generated strains expressing ectopic NIMA in WT cells and Eb1-null cells generated by endogenous replacement of the Eb1 ORF with a nutritional marker. Strikingly, virtually no cells (0.01%; n= 204) show NIMA comets when Eb1 is deleted, compared to 65% (n=400) of alcA-NIMA expressing cells that show distinct NIMA comets under derepression conditions (Figure 3.8B).

Eb1 interacts with several plus end binding proteins, and it has been determined that the largest class of Eb1 binding proteins carry a small consensus motif, SXIP, amidst a region rich in basic residues, as introduced earlier (Honnappa, Gouveia et al. 2009, Kumar and Wittmann 2012). We determined that the *Aspergillus nidulans* NIMA sequence has a SKIP sequence surrounded by multiple basic residues that is potential binding site for Eb1. This sequence is
well conserved as shown by the alignment of this domain in NIMA orthologs in Figure 3.8F. It is worth pointing out that the similarity between these NIMA orthologs is confined to the N terminal kinase domain, the island of SXIP motif and a small stretch of residues at the extreme C terminal (data not shown). The fact that this motif is so well conserved while the neighboring sequences are not, suggests that this motif very likely plays a significant function, perhaps by promoting interaction with Eb1.

3.2.1.6 The C-terminal regulatory domain of NIMA exhibits dynamic cytoplasmic movements

Our lab has shown previously that when an ectopic copy of the C-terminal regulatory domain of NIMA (NIMA-RegD-GFP) that lacks the kinase domain is expressed, it displays localization at the nuclear periphery at mitotic onset similar to the full length version but acts in a dominant negative fashion to delay the onset of partial NPC disassembly (De Souza, Osmani et al. 2004). This observation, in addition to other lines of evidence, has previously indicated that NIMA promotes partial nuclear pore complex disassembly during the G2-M transition. Therefore we considered the possibility that examination of the locations of the C-terminal domain of NIMA tagged with GFP might reveal previously unidentified cytoplasmic locations and functions for NIMA. To this end, we expressed the C-terminal regulatory domain of NIMA that lacks the N-terminal kinase domain from the inducible alcA promoter C-terminally tagged with GFP
under non-inducing, non-repressing conditions. We find that the NIMA-RegD-GFP locates to dynamic foci in the cytoplasm, similar to the location of ectopic NIMA-GFP (Figure 3.9A). NIMA-RegD-GFP foci move a bit slower than NIMA-GFP, though; at an average rate of 5.8 µm/min +/- 2 µm/min (n=135) (see below for discussion). We also detected NIMA-RegD-GFP locating to more stationary structures (white arrowhead and septum, yellow arrow), which will be discussed in Section 3.2.1.7. Kymographs were generated from different parts of the cell - near the tip, the middle of the cell and near the septum. These show that the predominant movement of NIMA-RegD-GFP at either end of the cell is towards the cell tip or towards the septum. In the middle of the cell, NIMA-RegD-GFP is seen to move in both directions (Figure 3.9A). However, we do see a fraction of cells (~33%) that exhibit an occasional instance of NIMA-RegD-GFP moving away from the cell tip. The level of expression of NIMA-RegD-GFP was variable and in cells with higher levels of expression there was an increase in the number and size of immotile foci. As a result, the detection of the dynamic cytoplasmic foci of NIMA-RegD-GFP appears sensitive to the expression level of NIMA-RegD-GFP (data not shown). The dynamic location of NIMA-RegD-GFP suggests a possible role for microtubules in their movement. Treatment of cells expressing NIMA-RegD-GFP with benomyl abolishes all movement as shown by kymographs generated from the movies (Figure 3.9B). We conclude that the cytoplasmic movement exhibited by NIMA-RegD-GFP is dependent on microtubules.
3.2.1.7 The mobile foci of NIMA-RegD-GFP originate and depart from the spindle pole body region

In addition to the dynamic cytoplasmic foci, NIMA-RegD-GFP also located to cytoplasmic foci that were far less dynamic (Figure 3.9A-B, arrow). We hypothesized that some such foci might represent the spindle pole body (SPB). Indeed, we find that the less dynamic NIMA-RegD-GFP foci localize with the kinetochore/SPB marker, Ndc80 (Yang, Ukil et al. 2004) (Figure 3.9C). Kinetochores are known to cluster at the SPB and therefore during interphase, a single focus of Ndc80 is expected. However, we observed that upon expression of NIMA-RegD-GFP, some kinetochores seem to be separated from the prominent Ndc80 focus, such that only the main Ndc80 focus, which most likely corresponds to the SPB, colocalizes with NIMA-RegD-GFP (Figure 3.9C, arrow). As seen from the kymograph, NIMA-RegD-GFP foci originate from the SPB region and move away from the SPB in both directions (Figure 3.9D, yellow arrows). Since ectopic NIMA-GFP associates with the plus ends of Mts, we think that the movement of NIMA-RegD-GFP could also represent Mt plus ends. If so, then this would indicate that the regulatory domain of NIMA has the information to locate the full length protein to the Mt plus ends.

However, the behavior of NIMA-RegD-GFP is different from ectopic NIMA-GFP in its location to the SPB. Evidence from studies in S. cerevisiae and our lab suggests that plus end binding proteins like Eb1 might first associate with the SPB and then associate with polymerizing Mts (Cuschieri, Miller et al. 2006). We
think it is possible NIMA may also behave similarly. Then in the absence of the kinase domain, NIMA-RegD-GFP acting in a dominant negative manner may associate with the SPB for a much longer time than NIMA-GFP and therefore exhibit a constant SPB signal. Another difference between the location of the ectopic truncated NIMA protein and full length NIMA is the movement of NIMA-RegD-GFP towards the SPB. This movement is not typical of most microtubule plus end binding proteins, which usually associate with the growing end of the Mts. This could be explained if NIMA-RegD-GFP bound to the ends of Mts more stably than NIMA-GFP, due to its dominant negative nature. Then we may expect to see it associating with not only polymerizing Mts but also Mts undergoing catastrophe. In other words, the movement of NIMA-RegD-GFP foci towards the SPB may be reflective of the continued association of this truncated version of NIMA with the Mt end that is depolymerizing. Since NIMA-RegD-GFP has been seen to act in a dominant negative manner, we may also expect to see an alteration of the Mt growth rate in these cells. In agreement with this, the rate of NIMA-RegD-GFP foci movement is 5.8 µm/min +/- 2 µm/min. If the movement of NIMA-RegD-GFP foci was reflective of the Mt growth rate, this would indicate a negative effect of the expression of the regulatory domain of NIMA on Mt dynamics.
Partial inhibition of NIMA function results in increased stability of Mts and changes in the dynamics of the plus end binding protein, An-Eb1

Since increase in NIMA leads to reduced polymerized tubulin, we sought to determine whether inhibition of NIMA would have the opposite effect. To test this hypothesis, we generated WT and nimA7 strains carrying tub-GFP and Eb1-CR, to enable us to visualize microtubule (Mt) tracks and the growing end of Mts simultaneously. These cells were grown at 35°C, a semi permissive temperature for nimA7 (that results in partial NIMA function), and then observed using live cell time lapse microscopy with acquisitions less than one second apart. In WT cells, microtubule tracks with Eb1 at the plus end can be readily observed growing towards the tip as denoted by the kymograph in Figure 3.10A. In the vicinity of the cell tip Eb1 moves predominantly unidirectionally towards the tip with an average rate of 15.7 +/- 4.7 µm/min (n=63, 6 cells) at this temperature. The distribution of the rate values is indicated by the histogram in Figure 3.12A. Interestingly, in 66% of nimA7 cells at this temperature, we detected instances of Eb1 moving away from the tip (Figure 3.10B). Importantly, these nimA7 cells also exhibit cell tip swelling. When the movement of Eb1 comets is tracked in cells of this 66% of the nimA7 population, 48% of these movements (n=56 tracked movements) were directed away from the tip. The average rate of Eb1 movement in cells with partial NIMA function is 25.6 +/- 24 µm/min, which is significantly higher than the average rate of Eb1 in WT cells at this temperature (p<0.0005). However, there is some variability in this distribution as indicated by the
histogram in Figure 3.12B. Among the population of nimA7 cells which have more WT-like tip morphology, Eb1 was found to move unidirectionally towards the tip. Also, the rate of Eb1 movement in nimA7 cells that have normal tip morphology is 15 +/- 5 µm/min (n=28 tracked movements), which is similar to the rate of Eb1 in WT cells. These data indicate that the misregulation of cell growth and modification of Eb1 behavior in nimA7 cells are intimately linked.

In addition to the alteration of Eb1 behavior, nimA7 cells also show modified microtubule dynamics. In contrast to WT cells, where Mts grow towards the tip and then undergo catastrophe to retract away from the tip (Figure 3.10A, C), the Mts in nimA7 cells can grow towards and then around the cell tip as well as show instances of bending and pushing against the cell tip wall (Figure 3.10D, Figure 3.11). Thus, our data indicate that under conditions where NIMA function is reduced, the dynamics of the plus end binding protein Eb1 is altered as is the dynamics of Mts (Figure 3.10E). In contrast to ectopic NIMA expression that leads to reduced polymerized tubulin, these data suggest that partial NIMA inhibition has the opposite effect on Mts, by promoting microtubule stability.

3.2.2 Genetic evidence towards a role for NIMA in cell growth

3.2.2.1 Synthetic lethal screen using the deletion of KIN3, the nimA ortholog in Saccharomyces cerevisiae.

Our studies detailed above suggest that NIMA has a previously unrealized interphase role in regulating cell growth. Since nimA7 cells at the semi
permissive temperature exhibit defects in cell growth and regulation of microtubule dynamics (Figure 3.10-3.12), we were interested to use cells with partial NIMA function to identify novel genes that function together with NIMA in regulating cell growth. Unlike nimA, the S. cerevisiae orthologue, KIN3 is a non-essential gene. Therefore, a KIN3 deleted strain was used to perform a genetic screen to identify non-essential gene deletions that are synthetically lethal with the absence of KIN3. Eighty-three genes were identified as potentially being synthetic lethal, or synthetic sick, with ΔKIN3 in the screen. Of these, eleven genes were confirmed by tetrad analysis as having synthetic genetic interactions with the KIN3 deletion (Table 3.1) (SL Anglin, unpublished).

3.2.2.2 Identification of conserved genes that are essential in cells having partial NIMA function

As a first step towards testing whether the genetic interactions identified in S. cerevisiae were conserved in A. nidulans, we identified the A. nidulans orthologues of the S. cerevisiae genes that are synthetic lethal with KIN3 using pBLAST searches at the Aspergillus Genome Database (www.aspgd.org) and the Aspergillus Comparative Database of the Broad Institute (Table 3.1). Next, we deleted each of these genes in a WT strain and in a strain carrying the nimA7 temperature sensitive mutation. The gene deletion construct in each case was made using fusion PCR and was transformed using the nutritional marker pyrG<sup>AF</sup> to delete the coding sequence of each target gene (Yang, Ukil et al. 2004,
Szewczyk, Nayak et al. 2006). The replacement of the endogenous gene was verified by performing diagnostic PCR using primers targeting outside the gene deletion constructs. The phenotype of the gene deletions in NIMA\(^+\) backgrounds is indicated in Table 3.1.

Genetic interaction between a gene deletion and \textit{nimA7} was identified by comparing the growth of the double mutants to the single mutants at the semi permissive temperature for the \textit{nimA7} temperature sensitive allele. This analysis revealed three synthetic genetic interactions of \textit{nimA} (Table 3.1, Figure 3.13) that are shared by \textit{\Delta KIN3} in \textit{S. cerevisiae}. Our analysis of the genetic interaction between \textit{nimA7} and \textit{\Delta An-vps23} and \textit{\Delta An-vps25} is presented here. The analysis of the genetic interaction between \textit{nimA7} and \textit{\Delta An-swd1} will be described in Chapter 4.

\textbf{3.2.2.3 Deletion of An-vps23 or An-vps25 results in the formation of heterokaryons indicating they are essential for normal growth}

In \textit{A. nidulans}, during heterokaryon rescue (Osmani, Oakley et al. 2006), deletion of essential genes (or genes required for normal growth) leads to the formation of heterokaryons which carry both WT nuclei lacking the nutritional marker of the deletion cassette as well as nuclei lacking the essential gene but which carry the nutritional \textit{pyrG}\(^{AF}\) marker gene. The presence of two genetically different nuclei in the heterokaryon is confirmed using diagnostic PCR. During asexual spore development, heterokaryons form uninucleate spores which can
carry either WT nuclei or nuclei with the target gene deleted. The phenotypes caused by the lack of the essential gene can be studied by germinating the conidia from the heterokaryon on selective media for pyrG\(^+\). Deletion of An-vps23 or An-vps25 results in the formation of heterokaryons (Figure 3.14A, B). When conidia from \(\Delta\)An-vps23 or \(\Delta\)An-vps25 heterokaryons are spread on pyrG selective plates to allow single colony formation, small aconidial colonies are formed (Figure 3.15), indicating that An-vps23 and An-vps25 are not absolutely essential although the deletion alleles can only be effectively propagated through heterokaryons.

The colonies formed by An-vps23 or An-vps25 deleted spores often give rise to one or more spontaneously occurring better growing sectors (Figure 3.15, arrowheads). Figure 3.15C shows a magnified image of two \(\Delta\)An-vps23 colonies after 72 hours and 96 hours growth at 35°C. One colony does not show the generation of a faster growing sector and grows slowly and conidiates poorly, while the other displays the emergence of faster growing sectors that are distinct in appearance and show better conidiation (arrowhead). The data indicate that the growth defects caused by deletion of An-vps23 or An-vps25 exert a selection pressure for spontaneous suppressor mutations that partially rescue the growth phenotype of the null alleles. Consistent with this possibility, we see that the generation of the faster growing sectors depended on some degree of growth of the mutant colonies. While our study was in progress similar suppressor mutations were independently identified by Herb Arst’s group and were defined
to be present in at least two cation tolerance genes, one of which is the transcription factor \textit{sltA} (Calcagno-Pizarelli, Hervas-Aguilar et al. 2011). By propagating the \(\Delta \text{An-vps23}\) and \(\Delta \text{An-vps25}\) in heterokaryons we ensured we did not select for suppressor mutations in our strains.

\textbf{3.2.2.4 An-vps23 and An-vps25 are essential in cells with partial NIMA function}

To test for synthetic genetic interaction between \(\text{An-vps23}\) or \(\text{An-vps25}\) and \(\text{nima}\), the colony growth of conidia isolated from the double mutants \(\Delta \text{An-vps23} + \text{nima7}\) and \(\Delta \text{An-vps25} + \text{nima7}\) was compared with the growth of the single mutants \(\Delta \text{An-vps23}\) or \(\Delta \text{An-vps25}\) or \(\text{nima7}\). At the semi-permissive temperature of 35°C, strains carrying the \text{nima7} allele are able to form restricted colonies (Figure 3.15). At 35°C, conidia from strains deleted for \(\text{An-vps23}\) or \(\text{An-vps25}\) also form small aconidial colonies (note at 32°C these mutants display cold sensitivity and grow poorly) and eventually generate some suppressor fans as described above. In contrast, conidia from double mutant strains barely form visible colonies even after 96 hours of incubation at 35°C (Figure 3.15). This growth assay indicates that when \text{nima} is partially inactivated, \(\text{An-vps23}\) and \(\text{An-vps25}\) become essential for growth.

It is possible that this synergistic growth defect in the double mutants is due to a general defect in the G2-M transition. To test this hypothesis, we sought to determine whether the deletion of \(\text{An-vps23}\) or \(\text{An-vps25}\) in combination with
partial inactivation of the mitotic Cdc25 phosphatase (*nimT* in *A. nidulans* (O’Connell, Osmani et al. 1992)) would cause similar growth defects. Partial function of Cdc25 phosphatase, an activator of Cdk1 kinase, results in a G2-M delay. We generated strains deleted for *An-vps23* or *An-vps25* that also carry a temperature sensitive mutant allele of cdc25, *nimT23*. Comparison of colony growth of the double mutant with the single mutants shows that there is no synergistic growth defect at the semi-permissive temperature for *nimT23*, 37.5°C (Figure 3.14). This result indicates that the synthetic genetic interaction between *nimA7* and ∆*An-vps23* or ∆*An-vps25* is likely not due to general G2-M defects but is specific to a partial loss of *nimA* function.

### 3.2.2.5 Deletion of *An-vps23* or *An-vps25* modifies the terminal phenotype of cells lacking *nimA* function

Our data indicates that partial loss of *nimA* function is poorly tolerated when combined with the absence of *An-vps23* and *An-vps25*. We therefore determined if the terminal phenotype of the double mutants at the fully restrictive temperature would be more severe than the terminal phenotypes of either single mutant. To test this we germinated conidia from WT, ∆*An-vps23*, ∆*An-vps25*, *nimA7* and ∆*An-vps23+nimA7* or ∆*An-vps25+nimA7* at the restrictive temperature for *nimA7* (42°C). The cells were then fixed and stained with DAPI to visualize nuclei and analyzed by microscopy. WT cells grown at 42°C exhibited normal growth morphology generating multinucleated cells as shown by the
representative image in Figure 3.16B and the illustration in Figure 3.16A. At the fully restrictive temperature, \textit{nimA7} cells are defective in initiating mitosis and arrest with a single nucleus unlike the multinucleated WT hypha at the same time point. \textit{nimA7} cells can however undergo short term growth to the point that can be supported by a single nucleus (Figure 3.16B, C). Unlike WT cells, \textit{An-vps23} deleted cells are wider such that nuclei can be found adjacent to each other across the width of the germtube (Figure 3.16B). \textit{An-vps23} deleted cells also noticeably show closely spaced septa and branches (unlike WT cells) as identified by brightfield images and indicated by yellow arrows in Figure 3.16B. These dramatic growth defects indicate that \textit{An-vps23} is required for normal cell growth morphology. Consistent with our expectation, we find that the double mutant $\Delta \textit{An-vps23}+\textit{nimA7}$, exhibits severe growth defects when compared to either single mutant (Figure 3.16C). Moreover, the double mutants also show novel cell growth defects not seen in either single mutant (Figure 3.16B, D). These phenotypes include swelling at the cell tip (Figure 3.16B, red arrow), swollen conidia indicating a defect in polarized germination (Figure 3.16B, green arrow), impairment of growth (Figure 3.16B, blue arrow), and cell lysis (Figure 3.16B, purple arrow). Remarkably the \textit{An-vps25} deleted cells and $\Delta \textit{An-vps25}+\textit{nimA7}$ double mutants show very similar cell growth defects as their \textit{An-vps23} counterparts (Figure 3.17), indicating a common basis for the synthetic genetic interaction between \textit{An-vps23} and \textit{An-vps25}, with \textit{nimA7}. 
3.2.2.6 *nimA* functions in concert with *An-vps23* to maintain apical dominance at the cell tip

To gain insights into the basis for the synthetic genetic interaction between *An-vps23* and *nimA*, we sought to determine the cellular phenotype of the double mutants in comparison with the single mutants at the semi permissive temperature. WT strains, Δ*An-vps23* or *nimA7* single mutant strains and double mutants were grown at the semi permissive temperature for 11 hours, fixed and stained with DAPI to visualize nuclei. At the permissive temperature for *nimA7*, 32°C, almost all WT and *nimA7* cells have normal tip morphology (Figure 3.18A, B). However, cells with partial *nimA7* function (*nimA7* cells at 35°C) exhibit a minor fraction of cells which show a breakdown of apical dominance (with two cell tips at the apex instead of one) compared to the WT cells at 35°C (Figure 3.18B). Among Δ*An-vps23* cells, a fraction of cells exhibit defects in maintaining apical dominance, a phenotype that is independent of temperature (Figure 3.18B). Interestingly, in the double mutants, there is a larger population of cells that exhibit defects in maintaining a single growing tip that is greater than the sum of the corresponding populations in the single mutants (Figure 3.18A, B). Importantly, this phenotype is only seen at 35°C, showing that the manifestation of the phenotype is dependent on the partial inactivation of *nimA*. In addition to an increase in the percentage of double mutants displaying a breakdown of apical dominance, double mutant cells also show more severe forms of the phenotype (Figure 3.18C). For instance, around 10% of double mutant cells have
three growing tips at the cell apex and around 12% have as many as four tips at the cell apex compared to a far smaller percentage of cells showing either phenotype in the single mutants.

3.2.2.7 The function of multiple ESCRT pathway components is required when nimA is partially inactivated

The synthetic genetic interaction between \( An-vps23 \) and \( An-vps25 \) with \( nimA7 \) could be specific to the function of these genes or could be due a general role for the ESCRT pathway in cells with partial nimA function. To address this, we generated strains deleted for \( An-vps28 \) (component of ESCRT I complex), \( An-vps24 \) (component of ESCRT II complex), \( An-vps36 \) (component of ESCRT III complex) and \( An-vps4 \) (an AAA-ATPase protein which interacts with ESCRT III complex proteins) in WT or \( nimA7 \) strain backgrounds. The deletion of each of the \( An-vps \) genes in combination with partial nimA function resulted in synthetic growth defects to at least the same extent as \( \Delta An-vps23+nimA7 \) or \( \Delta An-vps25+nimA7 \) (Figure 3.19). This indicates that the function of the ESCRT pathway becomes essential when nimA is partially inactivated.

3.2.2.8 Deletion of An-vps4 causes mitotic defects

During our analysis of the double mutants lacking partial NIMA function in combination with genes encoding multiple ESCRT component subunits, we noticed that the poor colony growth of the single mutants of \( \Delta An-vps28, \Delta An-vps24 \),
vps36 and $\Delta An$-vps24 exerts a selection pressure giving rise to spontaneous suppressor colonies as seen for $\Delta An$-vps23 and $\Delta An$-vps25 (Figure 3.19 and data not shown). But the deletion of $\Delta An$-vps4 did not have the same effect even after prolonged incubation. $\Delta An$-vps4 colonies also grew much further compared to the colony size of other vps deletions at a time point before the formation of suppressor colonies is induced. However, An-vps4 exhibited the strongest genetic interaction with nimA among all the vps genes tested. Therefore we were curious to see the morphological phenotypes of the An-vps4+nimA7 in comparison with the single mutants at the semi permissive temperature. We observed fixed and DAPI stained cells grown at this temperature by epifluorescence microscopy. Figure 3.20A shows that after 11 hours, Wt cells have formed long multinucleated cells, whereas cells with partial NIMA function have fewer nuclei and exhibit abnormal nuclear morphology due to mitotic defects (described in Chapters 4 and 5). Interestingly, absence of An-vps4 results in abnormal nuclear DAPI staining; with condensed chromatin (yellow arrowheads) and unevenly distributed chromatin compared to WT nuclei that are evenly stained (red arrows) (Figure 3.20A). The double mutants also show nuclear morphology defects as shown in Figure 3.20A. The DAPI staining abnormalities in the double mutant did not look obviously different from $\Delta An$-vps4. We did find apical dominance defects in these cells similar to their vps23 counterparts. Quantitation of these defects in terms of the percentage of cells that exhibit it (Figure 3.20B) as well as the severity of the defect (Figure 3.20C),
shows that there is a marginal increase in maintaining apical dominance in the
double mutants. Therefore, we strongly suspect that the mitotic defects that
potentially underlie the abnormalities in nuclear segregation in the double
mutants may be responsible for the strong genetic interaction between \textit{nimA} and
\textit{An-vps4}. Consistent with our results, the mammalian VPS4 has been proposed
to have mitotic functions at the centrosome (Morita, Colf et al. 2010). Since VPS4
is an enzyme, it likely could have a regulatory role in mitosis and hence, the
genetic interaction between NIMA and VPS4 is significant and warrants further
study (see also Chapter 6).

3.2.2.9 \textbf{VPS23 localizes to dynamic cytoplasmic foci}

The components of the ESCRT pathway are expected to be in contact
with the endosomal membrane during the formation of multivesicular body
(MVB). To test if this was true in \textit{A. nidulans}, we C-terminally tagged \textit{An-vps23}
and replaced the \textit{An-vps23} gene with the tagged version by homologous
recombination. The tagging of \textit{An-vps23} is functional since strains carrying
VPS23-GFP as the only copy of \textit{An-vps23} can be maintained as haploid strains
and do not exhibit any growth defects characteristic of \textit{An-vps23} deletion. Using
confocal microscopy, we find that VPS23-GFP locates to cytoplasmic foci (Figure
3.21A). When VPS23-GFP was followed through time using delays of less than a
second apart, we can detect the fast movement of these VPS23-GFP foci at an
average rate of about 147.5 +/- 2.45 µm/min at 23°C, which is similar to the
published rate of the early endosomal marker RabA in *A. nidulans* [150µm/min at 25°C, (Abenza, Pantazopoulou et al. 2009)]. The kymograph generated from a VPS23-GFP movie shows that the movement is bidirectional and that there are some VPS23 foci that are more stationary (Figure 3.21B). Late endosomes are more stationary in *A. nidulans* compared to early endosomes, therefore the pattern of VPS23 movement indicates it locates to both organelles (Abenza, Pantazopoulou et al. 2009). The average rate of movement in either direction is similar. The lipid binding dye, FM4-64 upon contact with cell membranes gets internalized and stains endosomal foci (Penalva 2005). We find upon staining VPS23-GFP cells with FM4-64 that VPS23-GFP foci co-locate with FM4-64 foci (data not shown). Therefore, we conclude that VPS23 associates with endosomes in *A. nidulans*. Independently, another recent study that examined the localization of VPS23 in the context of pH signaling also find that it locates to early and late endosomes in *A. nidulans* (Galindo, Calcagno-Pizarelli et al. 2012). Given the synthetic genetic interaction between An-vps23 and nimA7 and the role of NIMA in Mt dynamics, we were interested in determining whether the location of VPS23-GFP is altered in cells with partial NIMA function. To this end, we generated strains that carried VPS23-GFP and the nimA7 temperature sensitive allele. We germinated cells carrying VPS23-GFP that are either NIMA+ or nimA7 at the elevated temperature of 35°C and captured images of live cells at the same temperature using a heated stage and objective, and comparable image acquisition parameters. Kymographs were generated from these movies.
and rates were calculated as described in Chapter 2. As shown in Figure 3.20D, our preliminary results show that there is a minor increase in the average rate of VPS23 movement in cells with partial NIMA function vs. the WT. However, we do not find any major changes in VPS23 movement from the movies and kymographs. Therefore, the data suggest that the VPS23 localization and possibly function is not drastically altered when NIMA function is insufficient.

3.2.2.10 The mitotic and cell tip locations of NIMA are unaffected in the absence of ESCRT pathway function

In the view of the established role of the ESCRT pathway in the turn-over of proteins and the genetic interaction with nimA, we wanted to determine whether the intracellular localization of NIMA is affected in the absence of ESCRT pathway function. We deleted An-vps23 in strains carrying NIMA-GFP and generated heterokaryons. We observed NIMA location in Wt and \( \Delta An-vps23 \) cells at the temperature at which the genetic interaction was observed, 35°C. There was no obvious difference in the localization of NIMA to the cell tip between Wt cells and \( \Delta An-vps23 \) cells (Figure 3.22A). Roughly the same proportion of cells show distinct NIMA dome location at the cell tip in Wt cells (28%, n=117) compared to \( \Delta An-vps23 \) cells (32%, n=129). When NIMA-GFP is followed through mitosis in Wt cells, it is seen to locate to the SPBs, nucleus, spindle and then to the SPBs in a temporally resolved manner (see chapter 5 for details). We see that this pattern of NIMA’s mitotic location can be seen in the
absence of the ESCRT pathway at 35°C or at RT (22°C) (Figure 3.22B, C). The largely typical mitotic location of NIMA at the lower temperature says that the cold sensitivity of An-vps23 deletion mutants is likely not due to a defect in mitosis (Figure 3.15). Our analysis of fixed and DAPI stained cells lacking An-vps23 and An-vps25 showed that the nuclear morphology is normal and that mitosis is possibly not affected in these mutants (Figures 3.16 and 3.17). These data from live cell imaging of NIMA-GFP in ∆An-vps23 (Figure 3.22) further confirm that there are no dramatic problems in progressing through mitosis in vps-null mutants.

3.2.2.11 The function of the ESCRT pathway is required for normal location of NIMA to forming septa

Septation is a process analogous to cytokinesis that results in the partitioning of the A. nidulans hyphal cell into cell compartments. Septation involves the formation and the contraction of the actomyosin ring, the invagination of plasma membrane and the subsequent deposition of cell wall material (Harris 2001). The septal structure does not completely seal off cell compartments on either side, instead it has a central pore that allows cytoplasmic movement across the septum between cell compartments ((Kaminskyj 2000), Shen et al., unpublished data). The regulation of septation and the mechanics of septum formation are poorly understood in A. nidulans and nothing is known about how septal pores are formed. Recent evidence from the lab has shown
that NIMA locates to the forming as well as mature septa in *A. nidulans* indicating a potential role for NIMA in septum formation and the permeability of formed septa (unpublished data). In Wt cells, NIMA-GFP locates to the septation site early and forms a ring that colocalizes with the actino-myosin ring that subsequently contracts to form a disc of NIMA signal that corresponds with septa as identified by brightfield images. NIMA continues to be detectable at formed septa during interphase (Shen et al. unpublished). An example of normal NIMA location in ∆An-vps23 cells during septation is shown in Figure 3.23A, which essentially mirrors the localization of NIMA at the septum in Wt cells. (The foci of NIMA signal seen in the cytoplasm in Figure 3.23A most likely represent the location of NIMA to SPBs of G1 nuclei.) In contrast, as shown by the examples in Figure 3.23B, in 62.5% (n=8) of ∆An-vps23 cells, NIMA is seen to locate to the site of septation but the signal is weak and its normal dynamics seems impeded. We also were able to find examples where NIMA formed a ring as is typical of Wt cells; however the rings were not seen to contract even after prolonged imaging (Figure 3.23C). These data suggest that in the absence of ESCRT complex function, septum formation is defective possibly at the step of action-myosin ring contraction. This is consistent with studies implicating some subunits of the ESCRT pathway (including the *An-vps23* ortholog) in cytokinesis in mammalian cells and highlights the fact that septation possibly shares many conserved aspects with cytokinesis (Chen, Hehnly et al. 2012). It is important to point out that from our preliminary analysis, we see that this defect in NIMA location to
forming septa is potentially specific to the lower temperature, 22°C, and that 100% (n=10) of the septation events in ∆An-vps23 grown and imaged at 35°C showed normal NIMA dynamics. This suggests that the defect in the location of NIMA to forming septa in ∆An-vps23 cells contributes to the cold sensitivity of this deletion mutant (Figure 3.15). However, we see a negligible effect on the signal of NIMA at formed septa in the absence of An-vps23 at RT (5%, n=57) or at 35°C (1.7%, n=59).

3.3 Discussion

3.3.1 Microtubules, apical dominance and NIMA

As our data shows, long term treatment with Mt depolymerizing drugs leads to dramatic modifications at the cell tip with the emergence of multiple growing tips. The localization of the Spitzenkörper, the cluster of secretory vesicles at the cell apex, is important for determining the growth direction of the hyphal cell. It has been proposed that microtubules may contribute to hyphal cell growth by ensuring that the Spitzenkörper is located correctly. Therefore, it may be expected that depolymerization of Mts might allow the Spitzenkörper to drift and therefore allow a second growing tip to emerge near the dominant tip (Horio and Oakley 2005). However, since the effects we see are quite extreme and result in production of multiple tips, and even branching of the extraneous tips, we think it is unlikely that this dramatic phenotype may be caused just by drifting of the Spitzenkörper. Rather we think that these data potentially reveal an
inhibitory role that Mts may play (directly or indirectly) on the formation of new branches near the tip. For instance, transport of proteins that inhibit the formation of new branches is likely to be Mt dependent. Abolishing the location of such proteins might allow growth from ectopic sites. In fact, it has been shown that in *A. nidulans*, the prenylated cell end marker protein, TeaR (the ortholog of *S. pombe* Mod5), which recruits other cell end marker proteins to the cell apex is present over a wider area on the cell tip (than TeaA, see below) and sometimes in multiple foci along the cell apex (Takeshita, Higashitsuji et al. 2008). However, TeaA (*S. pombe* Tea1), which is recruited by TeaR, colocalizes with only one of the TeaR spots. This suggests that there are other spots on the cell membrane which are potentially ‘primed’ for cell tip generation, but perhaps are inhibited by a mechanism requiring microtubules. Under such a scenario, the depolymerization of Mts would be expected to relieve the suppression on these additional growth sites.

In *S. pombe*, there is further evidence for the existence of a mechanism involving the inhibition of secondary polarity axes by polymerized microtubules. Wild type *S. pombe* cells are rod shaped and grow in a linear fashion at the two opposite cell ends in a regulated manner through the cell cycle. The treatment of *S. pombe* cells with Mt depolymerizing drugs or the mutation of tubulin genes can result in the emergence of an additional cell growth tip leading to T-shaped cells or the formation of multiple branches (Sawin and Nurse 1998, Castagnetti, Novak et al. 2007). This remarkable similarity in phenotypes between *S. pombe* and *A.*
*nidulans*, suggests that there is a conserved mechanism involving the suppression of the growth of secondary cell tips by Mts which is relieved upon drug induced depolymerization of Mts. Therefore, further exploration of the benomyl-induced growth of these extraneous tips is likely to help understand not just filamentous fungal biology but also potentially uncover a conserved regulatory aspect of cell growth. Furthermore, we suppose that the growth of multiple branches near the tip might be part of an adaptive response in the absence of the rapid hyphal extension that is made possible by microtubules. Our observation that overexpression of NIMA phenocopies depolymerization of Mts with benomyl strongly suggested that in cells expressing extra NIMA, there might be less polymerized tubulin. Indeed, this is what we find. Although there are few Mt tracks under these conditions (as detected by tub-GFP), the NIMA and Eb1 comets seen under similar conditions disperse upon treatment with benomyl (data not shown), indicating that their movement is still dependent on microtubules. In this regard, we note that the phenotype of *A. nidulans* cells deleted for the plus end binding protein, AlpA (Dis1/XMAP215), is very similar to NIMA overexpressing cells in that there are very few visible Mt tracks (Enke, Zekert et al. 2007). AlpA is not essential, indicating that fewer Mts or less bundled Mts than that seen in WT cells are sufficient to support cell growth, consistent with our data. Dis1/XMAP215 is a well-known processive Mt polymerase (Brouhard, Stear et al. 2008). Therefore, there is an exciting possibility that NIMA might be causing the depolymerization of cytoplasmic Mt
architecture via regulation of AlpA (Dis1/XMAP215) and/or other Mt plus end binding proteins.

Some indications linking NIMA-related kinases with Mt regulation has surfaced from other model systems. For instance, mammalian Nek6 and Nek7 can phosphorylate tubulin in vitro, suggesting that the regulation of Mts by NIMA related kinases may be a conserved function and could be through direct modification of tubulin subunits (O'Regan and Fry 2009, Fry, O'Regan et al. 2012). Many Mt plus end binding proteins have been described to bind to tubulin dimers and influence their function (Akhmanova and Steinmetz 2008). In that respect, NIMA is certainly at the right location to carry out such a function in A. nidulans. In fact, a potential function for the NIMA family of kinases at the plus ends of Mts was predicted by a recent review (Fry, O'Regan et al. 2012). Our data provides the first evidence to support the model proposed in the review that NIMA and NIMA related kinases might have an interphase function involving the regulation of the plus end binding proteins. Also, consistent with our results being applicable for Neks in higher eukaryotes, a recent study proposes a role for Nek7 in regulating Mt dynamics in HeLa cells and mouse embryonic fibroblasts (Cohen, Aizer et al. 2013).

One interphase function that several human Neks are known to be involved with is the regulation of cilia (Quarmby and Mahjoub 2005). The growth of cilia is polarized and therefore, might share some conserved mechanisms similar to filamentous fungal growth. The core of the cilia is made up of a bundle
of microtubules called the axoneme. The growth as well as movement of the cilia depends on microtubule function. So it might be expected that plus end binding proteins would be important for ciliary function. In agreement, Eb1 is found at the tips of cilia, a region where the antergrade transport towards the tip and retrograde transport away from the tip are thought to be coordinated (Emmer, Maric et al. 2010). It has been suggested that Neks might mediate cilia regulation by affecting the centrosome at the base of the cilia. However, another possibility, supported our data, is that the Neks might regulate microtubules in the axoneme directly.

3.3.2 NIMA and Eb1

Our results indicate that NIMA locates to the Mt plus ends in an Eb1 dependent manner. Eb1 is a focal point for the plus end binding protein interactome. Eb1 interacts with two classes of proteins, as introduced in Section 2.1.4. The largest group of Eb1-interacting proteins possesses the SXIP domain (Kumar and Wittmann 2012). The family of SXIP containing Eb1 binding site proteins is growing fast and our data adds A. nidulans NIMA to this list of proteins. We find that this motif is conserved in the Ascomycota, but we do not find it in NIMA orthologs of the model yeasts or in humans. This is likely due to the fact that SXIP is a short linear motif (SLiM) and the evolution of SLiM proteins have been proposed to be convergent, i.e. unrelated proteins may have evolved to contain this motif at different points in evolution (Neduva and Russell 2005).
Therefore, motifs that are evolved in a convergent manner often are not very highly conserved between orthologs (Neduva and Russell 2005). However, the mechanism of Mt dynamics regulation involving NIMA and Eb1 is very likely to be conserved between organisms. As another example of this phenomenon, the budding yeast Aurora kinase (Ipl1) has been shown to contain an SXIP motif and to physically interact with the budding Eb1 ortholog, Bim1 during mitosis (Zimniak, Stengl et al. 2009). However, the A. nidulans ortholog of the Aurora kinase (AN5815) or mammalian Aurora kinases do not possess a corresponding SXIP sequence. The existence the SXIP domain was discovered using a bioinformatics search program designed to identify SliMs (http://dilimot.russelllab.org/) (Neduva and Russell 2006). Using this program, we find that the SXIP domain sequence is readily detected as the common protein motif between the protein sequences of A. nidulans NIMA, budding yeast Ipl1, and mammalian +TIP Clip-170.

The binding between SxIP domain containing proteins and Eb1 is aided by the presence of positively charged amino acids, especially arginine residues that interact electrostatically with the negatively charged C terminus of Eb1. Also, as has been demonstrated in the case of CLASP2, a human +TIP, the arginine residues near SxIP motif are engaged in salt bridge interactions with glutamate residues in the Eb1 C terminus (Kumar and Wittmann 2012). Furthermore, these interactions are targets for regulation, since phosphorylation by CDK1 and GSK3 at residues nearby disrupts this interaction (Kumar, Chimenti et al. 2012).
Likewise, many other Eb1 binding proteins, like CLIP170, are also known to be regulated by phosphorylation (Tamura and Draviam 2012). We note that NIMA also carries two arginine residues one amino acid away from the SxIP domain, which very likely reinforces the interaction with Eb1 and excitingly, there are multiple consensus CDK1 phosphorylation ‘SP’ sites on either side of the SxIP domain (Figure 3.8, data not shown). So these features of NIMA SxIP domain are consistent with the function of NIMA at the plus ends of the microtubules being regulated by phosphorylation potentially by CDK1 (also see Chapter 6, Discussion).

In cells expressing ectopic NIMA, we find that the NIMA comets and Eb1 comets move at a rate similar to Mt polymerization. However, the direction of movement of Eb1 is altered to give many instances of movement away from the tip which is rarely observed in Wt cells. Therefore, along with decreasing the amount of polymerized tubulin, ectopic NIMA expression also affects the movement of Eb1. As mentioned earlier, we know that the Eb1 movement in these cells is dependent on Mts since the addition of benomyl results in the dispersal of Eb1 from comets. Therefore, although the rate of Mt growth is unaltered in cells expressing additional NIMA, the nature of Mt tracks is affected. When NIMA function is reduced on the other hand, the rates of Eb1 are altered such that there is a proportion of Eb1 comets that move faster than what is typical for Wt cells. In addition, when interphase Mts were observed by tub-GFP, we were able to follow the Mt tracks continuing to grow even after reaching the
tip resulting in loops of Mts near the cell apex, suggesting that Mts are possibly more stable under these conditions. Consistent with microtubules growing around the tip, we also see instances of Eb1 moving away from the tip. One reason we see Eb1 movement around the cell tip when NIMA is both increased and decreased could be that some of these movements might be due to the tip swelling that is observed in cells with either increased or decreased NIMA function. We have seen that when swelling of the tip is induced by an independent method, by depolymerization of actin, in Wt cells then Eb1 can be seen to move around the cell tip (data not shown). However, the fact that NIMA locates to the plus ends of the microtubules when expressed ectopically indicates that there is a direct effect of NIMA on the regulation of Mt plus end dynamics.

Elegant studies have shown that artificially changing the shape of the S. pombe cell by mechanical forces can promote the contact of Mts with sites on the plasma membrane other than the growing cell tip (Minc, Bratman et al. 2009). This can in turn result in the delivery of polarity factors in a manner dependent on the Eb1 ortholog, Mal3 and induce the formation a new growth site in cells that are deleted for Tea1 (Minc, Bratman et al. 2009). Therefore, we think it is possible that when NIMA function is reduced, the continued growth of Mts after reaching the tip might result in the Mts contacting a site somewhat removed from the growing cell tip leading to formation of another tip.
3.3.3 The interphase locations of the NIMA kinase

Our data shows that the endogenous NIMA protein locates to growing cell tips. When expressed under the control of a regulatable promoter such that NIMA levels were ~10 fold higher than the endogenous level (as determined microscopically), NIMA could be seen to locate at microtubule plus ends in an Eb1 dependent manner. The reason endogenous NIMA is not detectable at Mt plus ends is likely due to its low abundance. In support of this argument, studies involving the localization of plus end binding proteins in mammalian cells have typically involved ectopic expression of the protein (Kumar and Wittmann 2012). Furthermore it has been reported that several +TIPs including Eb1 are virtually undetectable at the Mt growing ends at their endogenous levels (Vaughan 2005, Kumar and Wittmann 2012).

Multiple examples of +TIPs have been reported in the literature that locate to an intracellular organelle or structure, like the plasma membrane (CLASPs, ACF7) and ER membrane (STIM1) (Kumar and Wittmann 2012). It has been proposed in these cases that the presence of these proteins at that intracellular site facilitates ‘search and capture’ of Mts. For instance, in the case of CLASPs, they are seen to locate to the leading edge of migrating cells, in addition to being on the plus ends of Mts, where they are thought to bind to Mts to facilitate the movement of the cell in that direction (Goodson and Folker 2006, Lansbergen, Grigoriev et al. 2006). Likewise, STIM1, an resident ER membrane protein also locates to the plus ends of Mts in an Eb1 dependent manner, and thus mediates
the interaction between Mts and the ER membrane and allows the extension of ER tubules (Grigoriev, Gouveia et al. 2008).

On similar lines, we think it is possible that the localization of NIMA to the cell apex in long hyphal cells, as well as to the plus ends of the Mts, helps ensure that the growing end of the microtubules are captured at the cell tip (Figure 3.24). This would enhance the efficiency of the deposition of cell end marker proteins and secretary vesicles and enable the continuous membrane extension at the cell apex contributing to normal polarized growth. Moreover, catastrophe events of Mts have been observed to be correlated with contact of the plus ends with the cell tip (Brunner and Nurse 2000), therefore contact of the Mts with the cell membrane facilitated by NIMA might contribute to triggering catastrophe. When NIMA function is compromised, we might expect to see that the Mts do not make sufficient contact with the cell membrane and therefore continue to grow (Figure 3.11C).

Another important aspect about NIMA localization to the cell tips is that it is visible in long hyphal cells (Figure 3.2A) but not obvious in germlings (data not shown). This relates to an observation made previously that an obvious Spitzenkörper is present in longer hyphal cells but not in small germlings (Taheri-Talesh, Horio et al. 2008). Therefore there are fundamental differences between the mechanisms of growth of germlings vs. long hyphal cells. It is possible that the localization and function of NIMA at the cell tip is part of a mechanism that mediates a switch to faster growth rates in hyphal cells.
3.3.4 NIMA, the ESCRT pathway and cell growth

Our data shows that the growth of cells with reduced NIMA function is dependent on the function of the ESCRT pathway. Mutant cells that lack ESCRT pathway function and have reduced NIMA function show defects in maintaining a single growing tip that is both more penetrant and more severe than either single mutant. Moreover, cell growth is severely inhibited when cells lack NIMA as well as vps23, showing that the growth of the uninucleated cells that lack NIMA is almost completely dependent on the function of ESCRT pathway. The localization of NIMA to the cell tip in the absence of the ESCRT pathway and the location of VPS23 in the absence of partial NIMA function is largely normal. These data together suggest that the ESCRT pathway and NIMA function in parallel pathways to regulate cell growth, specifically in the maintenance of apical dominance. How might this be explained?

In *A. nidulans*, the growth of the cell tip is regulated by a delicate balance of exocytosis and endocytosis. The presence of sterol rich domains (SRD) and cell end marker proteins at the cell apex is proposed to regulate the targeted delivery of secretory vesicles (Takeshita, Higashitsuji et al. 2008, Takeshita and Fischer 2011, Takeshita, Diallinas et al. 2012). However the requirement and sufficiency conditions for the generation of a de novo tip growth are poorly understood. As explained in Section 3.3.3, in cells with reduced NIMA function it is possible that mis-regulation of Mt dynamics and the reduced efficiency by which Mts are captured at the cell apex causes mislocalization of polarity factors.
and in turn promotes extraneous cell tip growth. It is important to note however, that though targeted exocytosis might be defective in cells with partial NIMA function, the contribution of endocytosis towards maintaining the cell tip membrane composition would be expected to be unaffected, and therefore the maintenance of apical dominance may be expected to not be drastically affected under these conditions.

The main phenotype of the ESCRT pathway mutants in budding yeast is the presence of the Class E compartment, which contains mis-sorted proteins that should be delivered to the vacuole (Russell, Shideler et al. 2012). In mammalian cells, depletion of ESCRT pathway components also results in an enlarged endosome with abnormal morphology (Doyotte, Russell et al. 2005). Many studies show that the depletion of ESCRT function results in the accumulation of target proteins that were meant for degradation in an intracellular compartment. Interestingly, though, some studies have shown that in the absence of ESCRT complex function, the target proteins can be recycled back to the plasma membrane in S. cerevisiae as well as in fibroblasts (Babst, Odorizzi et al. 2000, Luo and Chang 2000, Bugnicourt, Froissard et al. 2004). This effect is likely to be protein specific because not all ESCRT target proteins accumulate on the plasma membrane in ESCRT pathway mutants. Mammalian cells have a mechanism whereby ubiquitylated proteins that are set to enter the ESCRT pathway can be ‘rescued’ by deubiquinating enzymes and thus recycled back to the plasma membrane (MacGurn, Hsu et al. 2012). It is possible that, when the
ESCRT pathway is blocked due to the absence of one of its components, some of the target proteins are similarly rescued and erroneously sent back to the plasma membrane. If so, we might expect that in the absence of the ESCRT pathway, the removal of certain cell end marker proteins, as well as excess membrane from the cell tip, might be less efficient.

We speculate that the enhanced apical dominance defects in the double mutants could result from the reduced efficiency in targeted secretion of cell end markers at the cell apex (because of reduced NIMA) combined with potential defects in maintaining normal membrane composition at the cell apex in the absence of the ESCRT pathway. We hypothesize that NIMA and the ESCRT pathway might thus function in parallel pathways to regulate polarized growth (Appendix A, Figure A.1). The proposed model can be tested by examining the distribution of sterol rich domains (SRDs) at the cell tip and location of proteins that have been proposed to play a role in tip determination such as TeaA and proteins regulating polymerized actin dynamics. Such analysis would not only help us test the above model but also help delineate the requirement and sufficiency conditions for de novo cell tip formation thus advancing our understanding of polarized growth.
Figure 3.1: Schematic representation of *A. nidulans* cell growth and the ESCRT pathway (A) A schematic representation of Wt cells which have one dominant cell tip per growth compartment. In apical dominance mutants, this regulation is impaired. The nuclei in the same color undergo parasympathetic mitoses. The cell tip region within the dotted rectangle is magnified in (B), which shows the predicted function of the ESCRT pathway in *A. nidulans* based on studies in other model systems. Red bars represent plasma membrane localized proteins that are targeted by secretary vesicles and are internalized when they encounter the endocytic collar (C) The *S. cerevisiae* ESCRT protein complexes involved in the membrane invagination and scission events during MVB biogenesis.
Figure 3.1
Figure 3.2 The mitotic NIMA kinase locates to growing cell tips. (A) NIMA-GFP, expressed from the endogenous promoter (KF45), exhibits a dome-shaped localization at the tips of growing hyphal cells. (B) For each of the time points in (A), the pixel intensity along the dotted yellow line indicated in (A) is plotted as a percentage of the maximum value along the line. The increase in NIMA-GFP signal at the cell tip is shown by percentage pixel intensity profiles from 10 cells, where the pixel profile of each cell is indicated in a different color (C) and by the average intensity profile (D). (E) Depolymerization of actin by latrunculin B leads to an increase NIMA signal at the cell tip (KF45), as also quantified in (F). Bars, 5µm.
Figure 3.2
Figure 3.3: Induction of ectopic NIMA-GFP in hyphal cells results in defects in maintaining a single growing cell tip. (A) Hyphal cells carrying alcA-NIMA-GFP (CDS683) or alcA-NIMA-RegD-GFP (CDS131) were grown under repressive conditions and then exchanged into media promoting the expression of the respective NIMA constructs. Representative images of cells after 6 hours of alcA induction are shown. Breakdown of apical dominance in NIMA-GFP expressing cells is indicated by green arrow; example of cell tip swelling is indicated by blue arrow. Quantitation of tip growth defects is shown in (B). Bar, 5µm. (PI – Pre-induction, I – alcA expression induced using threonine for 2, 4 or 6 hours as indicated.)
Figure 3.3
Figure 3.4: Colony growth of strains expressing ectopic NIMA constructs. (A) Growth of strains carrying different NIMA constructs as indicated under conditions of repression (R), non-repression and non-induction (depression – DR) and expression (E). (B) Growth of strains carrying different NIMA constructs upon induction with threonine compared to uninduced control. Wt=R153, alcA-NIMA-RegD-GFP=CDS131, alcA-NIMA-GFP=CDS683.
Figure 3.5: Induction of alcA-NIMA-GFP results in reduced polymerized tubulin. (A) Depolymerization of Mts using benomyl in WT hyphal cells (R153) results in the emergence of multiple growing tips near the cell apex. (B) Long hyphal cells carrying tub-GFP and alcA-NIMA-GFP (MG394) grown under repressive conditions were exchanged into media that would promote the expression of NIMA-GFP. Cell tip swelling (cell 1 and 2) and breakdown of apical dominance (cell 2) characteristic of NIMA induction is seen. Mitotic nuclei showing nuclear NIMA and spindle formation are marked by yellow and red arrows respectively. NIMA-GFP comets are indicated by arrowheads. (C) Cells germinated under conditions that repress the expression of alcA-NIMA-GFP (MG394) show normal WT microtubule network. (D) When alcA-NIMA-GFP (MG394) expression is derepressed, Mt tracks are not detectable and dynamic alcA-NIMA-GFP comets are seen (arrowheads). Bars, 5μm.
Figure 3.5
Figure 3.6: Ectopically induced NIMA-GFP displays a distinctive microtubule dependent localization. (A) NIMA-GFP (CDS683) forms dynamic cytoplasmic comets (yellow arrowhead). (B) NIMA-GFP (CDS683) comets often exhibit bi-directional movement near the cell tip as shown by the kymograph. (C) Upon addition of the microtubule poison, benomyl, NIMA-GFP (CDS683) immediately disperses throughout the cytoplasm. Bars, 5µm.
Figure 3.6
Figure 3.7: The cytoplasmic comet localization of ectopic NIMA-GFP is not seen during mitosis. Maximum intensity projection images of a cell expressing alcA-NIMA-GFP (CDS683) grown under derepression conditions (glycerol) followed through mitosis. The bright field image shown is of a single Z section. Bar, 5μm.
Figure 3.8: Ectopically induced full-length NIMA locates to the plus ends of microtubules in an EB1 dependent manner. (A) NIMA-GFP comets locate to Mts plus ends defined by their colocalizaton with EB1-CR (arrows, strain: MG385). (B) The location of NIMA-GFP to the Mt plus ends is abolished in strains deleted for EB1. Strains: Wt = MG409, ΔEb1 = MG410 (C) NIMA-GFP comets colocalize with EB1 (MG385) through time. (D) Under the induction conditions where NIMA-GFP comets move around the tip (Figure 4A), EB1 comets are seen to exhibit similar behavior (arrowheads, MG385). (E) Alignment of the SXIP containing domain of NIMA orthologs across fungal species as highlighted in yellow reveals a conserved consensus site for Eb1 binding. Bars, 5μm.
Figure 3.8
**Figure 3.9:** Ectopically expressed C-terminal regulatory domain of NIMA displays microtubule dependent cytoplasmic movement. (A) ROIs near the tip, middle of cell and near the septum (as indicated) were used to generate the kymographs. Arrowhead indicates spindle pole body (SPB) location. Strain=CDS131. (B) Kymographs generated from cells expressing NIMA-RegD-GFP (CDS131) before and after addition of benomyl. Arrowhead indicates SPB location (C) Some NIMA-RegD-GFP colocalizes with ndc-80-CR at the SPB (white arrow, CDS703). (D) Kymograph shows NIMA-RegD-GFP foci arriving (red arrow) and departing (yellow arrow) from the SPB (CDS703). Bar, 5µm.
Figure 3.9
**Figure 3.10:** Partial inhibition of NIMA results in alteration of microtubule dynamics and Eb1 behavior. (A) In WT cells (MG395), Eb1-CR typically moves unidirectionally towards the cell tip as illustrated by the kymograph generated using the indicated ROI. (B) When NIMA function is partially impaired (MG397), the movement of Eb1-CR is altered to give instances of Eb1 comets moving away from the tip. (D) Microtubule tracks in cells with partial NIMA function bend and push against the cell membrane, a phenotype not typical of WT (C) cells. (E) Model illustrating the effect of partial inhibition of NIMA on MT dynamics. Bars, 5µm.
Figure 3.10
**Figure 3.11:** Partial inhibition of NIMA function results in an alteration of microtubule (Mt) dynamics. A representative *nimA7* cell shows Eb1 moving away from the tip (red arrows). The Mt corresponding to this Eb1 comet can also be visualized to be growing around the tip (yellow arrows) forming a loop of Mt especially at the time points of 4.05s and 7.29s (MG397). Bar, 5µm.
Figure 3.11

nimA7 (35°C)
Figure 3.12: The rate of movement of Eb1 is altered in cells with partial NIMA function. (A) Histogram showing the distribution of the rates of movement of Eb1 in WT cells (MG395) at 35°C. (B) Histogram showing the distribution of the rates of movement of Eb1 in nimA7 cells (MG397) at the semi permissive temperature of 35°C.
Table 3.1: *A. nidulans* orthologues of genes that are synthetically lethal with \( \Delta KIN3 \) in *S. cerevisiae*.

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<tr>
<th><em>S. cerevisiae</em> Gene Name</th>
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<th>Deletion phenotypeb</th>
<th>E-valuec</th>
<th>Function in <em>S. cerevisiae</em></th>
<th>Genetically interacts with nimA7 in <em>A. nidulansd</em></th>
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<td>Zuo1</td>
<td>AN7143.3</td>
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<tr>
<td>Ssz1</td>
<td>AN4616.3</td>
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<td>Hsp70 protein that interacts with Zuo1 to a form a ribosome-associated complex</td>
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<td>Mph1</td>
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<td>Viable</td>
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<td>Member of DEAH family of helicases; functions in an error-free DNA damage bypass pathway</td>
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<td>Bud14</td>
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<td>7.0e-44</td>
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(Continued)
### Table 3.1: Continued

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<td>Hsl7</td>
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<td>Vps25</td>
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<td>2.7e+00</td>
<td>Component of the ESCRT-II complex; involved in ubiquitin-dependent sorting of proteins into endosome</td>
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**Table 3.1:** *A. nidulans* orthologues of genes that are synthetically lethal with Δ*KIN3* in *S. cerevisiae.* <sup>a</sup>Designation given by the *A. nidulans* Genome database.
Refers to the growth phenotype of the deletion strains on complete media (Figures 3.12, 3.14, and 4.1). Values obtained on performing BLAST using the *S. cerevisiae* gene to the *A. nidulans* Genome database at AspGD. This study. Refers to genes that have been previously studied in *A. nidulans* using the name given in brackets (Oka, Maruyama et al. 2004, Higashitsuji, Herrero et al. 2009, Bauer, Graessle et al. 2010, Calcagno-Pizarelli, Hervas-Aguilar et al. 2011).
Figure 3.13: nimA7 does not exhibit genetic interaction with 7 out of the 10 A. nidulans orthologs of S. cerevisiae genes that interact with KIN3 (See also Table 1). The images show colonies grown from spores of indicated genotypes for 96 hours at 35°C, the semi permissive temperature of nimA7. Strains in the order left to right, top to bottom: R153, MG44, MG8, MG61, MG19, MG67, MG10, MG62, MG12, MG63, MG22, MG68, MG14 and MG64.
Figure 3.14: Deletion of *An-vps23* or *An-vps25* is not synthetic lethal with reduced Cdk1 function. (A) Deletion of *An-vps23* leads to the formation of heterokaryons, analyzed here by the heterokaryon rescue technique. Growth of conidia isolated from WT (R153), *pyrG* (SO451) and ∆*An-vps23* (MGH21) heterokaryons on non-selective and selective media shows that the deletion of *An-vps23* severely impairs growth. (B) Diagnostic PCR confirms the presence of WT and *An-vps*-deleted nuclei in the heterokaryons. (C and D) Comparison of colony growth at 37°C, a semi permissive temperature for *nimT23*, indicates no synthetic growth defect in the double mutants of *nimT23* and ∆*An-vps23* (C) and ∆*An-vps25* (D) compared to either single mutants. Strains: Wt=R153, *nimT23*=MG99, ∆*An-vps23*=MGH21, ∆*An-vps25*=MGH26, *nimT23 +∆An-vps23*=MGH31, *nimT23 +∆An-vps25*=MGH39.
Figure 3.14
**Figure 3.15:** Functions of *An-vps23* and *An-vps25* become essential upon partial inhibition of NIMA function. (A and B) At the semi restrictive temperature for *nimA7*, absence of *An-vp23* or *An-vps25* is poorly tolerated resulting in tiny colonies even after 96 hours of incubation. Black arrowheads mark ∆*An-vps23* and ∆*An-vps25* colonies that exhibit the presence of suppressor mutations at 96 hours but not at 72 hours. (C) The inset in (A) has been magnified to show the emergence of colonies carrying suppressor mutations from one colony and not from the adjacent one. Strains: Wt=R153, *nimA7*=MG44, ∆*An-vps23*=MGH21, ∆*An-vps25*=MGH26, *nimA7* + ∆*An-vps23*=MGH19, *nimA7* + ∆*An-vps25*=MGH14.
Figure 3.15

A

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B

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C

ΔAn-vps23

72 hrs
96 hrs

Figure 3.15
**Figure 3.16:** Deletion of *An-vps23* modifies the terminal phenotype of cells lacking NIMA function. (A) A schematic representation of the representative cell morphological phenotypes of WT, *nimA7*, ∆*An-vps23* and *nimA7+∆An-vps23*. The red dotted rectangle marks the region of the cell in each strain that is depicted in (B). In (B), closely spaced septa (as identified by brightfield images, not shown) in ∆*An-vps23* are marked by yellow arrows. The double mutants show a synergistic growth defect (blue arrow) as quantified in (C) and display novel phenotypes not seen in either single mutant such as defects in germ tube emergence (green arrowhead), tip swelling (red arrowhead), and cell lysis (purple arrowhead) as categorized in (D). The p values indicated in (C) show that growth differences between the double mutant and the single mutants after growth for 15 hours at the indicated temperatures are highly statistically significant. Bar, 5 µm.

Figure 3.16
Figure 3.17: Deletion of An-vps25 modifies the terminal phenotype of cells lacking NIMA function. (A) Representative cells of indicated genotype stained with DAPI. Septa were identified by bright field (not shown) and indicated by yellow arrows. The double mutants show a synergistic growth defect (blue arrow) as quantified in (C) and display novel phenotypes not seen in either single mutant such as defects in germ tube emergence (green arrowhead), tip swelling (red arrowhead), and cell lysis (purple arrowhead) as categorized in (D). The p values indicated in (C) show that growth differences between the double mutant and the single mutants after growth for 15 hours at the indicated temperatures are highly statistically significant. Bar, 5 µm. Strains: Wt=R153, nimA7=MG44, ΔAn-vps25=MGH26, nimA7+ΔAn-vps25=MGH14
Figure 3.17
**Figure 3.18:** NIMA functions in concert with *An-vps23* to maintain apical dominance at the cell tip. (A) Representative images of WT, *nimA7*, ∆*An-vps23*, and double mutant cells fixed and DAPI stained after 11 hours of growth at the indicated temperature. Quantitation of tip morphology defects is shown in (B). (C) Quantitation of number of additional tips at the cell apex as a measure of the severity of tip morphology defect in the double mutant. Only strains that show appreciable defects in tip morphology as seen in (B) are represented here.

Figure 3.18
**Figure 3.19:** The ESCRT pathway becomes essential when NIMA function is partially reduced. The images show the colony growth of strains of the indicated genotypes after 96 hours. Strains: Wt=R153, _nimA7_=MG71, \(\triangle An-vps28=MGH53\), _nimA7_+\(\triangle An-vps28=MGH55\), \(\triangle An-vps24=MGH49\), _nimA7_+\(\triangle An-vps24=MGH51\), \(\triangle An-vps36=MGH57\), _nimA7_+\(\triangle An-vps36=MGH59\), \(\triangle An-vps4=MGH45\), _nimA7_+\(\triangle An-vps4=MGH47\).
Figure 3.19

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**Figure 3.20:** Cells lacking An-vps4 in combination with partial NIMA function show enhanced defects in cell growth. (A) WT, nimA7, ∆An-vps4 and the double mutant cells grown at the semi-permissive temperature for nimA7 were fixed and stained with DAPI. ∆An-vps4 cells also show nuclear segregation defects (yellow arrowheads) as compared to WT nuclei (red arrows). Quantitation of tip morphology defects is shown in (B). (C) Quantitation of number of additional tips at the cell apex as a measure of the severity of tip morphology defects in the double mutant. Only strains that show appreciable defects in tip morphology as seen in (B) are represented here. Bar, 5 µm. Strains: Wt=R153, nimA7=MG71, ∆An-vps4=MGH45, nimA7 + ∆An-vps4=MGH47.
Figure 3.20
**Figure 3.21:** Localization of VPS23 is consistent with its predicted role in the ESCRT pathway (A) Still image of a cell expressing VPS23-GFP (MG112) from its endogenous location. Bar, 5µm (B) Kymograph of the movement of VPS23-GFP foci. (C) Kymograph of VPS23-GFP foci in benomyl (D) Comparison of the average rate of VPS23-GFP foci in Wt (MG286) and nimA7 (MG282) at 35°C.
Figure 3.21
**Figure 3.22:** The mitotic and cell tip location of NIMA is unchanged in the absence of ESCRT complex function. (A) NIMA-GFP at the cell tips of WT (KF005) and ΔAn-vps23 (MGH61) cells at 35°C. (B) and (C) NIMA-GFP followed through mitosis in ΔAn-vps23 cells (MGH61) at 35°C (B) and at RT (22°C).
Figure 3.22
**Figure 3.23:** The location of NIMA to forming septa is affected in the absence of An-vps23 function. (A) Example of NIMA location to a forming septum in ΔAn-vps23 cells (MGH61) that is typical of Wt cells at RT (22°C). (B) and (C) Examples of abnormal NIMA location to a forming septum in ΔAn-vps23 cells (MGH61) at RT (22°C).
Figure 3.23
Figure 3.24: A schematic representation of proposed functions for NIMA in the cytoplasm. NIMA indicated in purple is present at the cell tip and at the plus ends of microtubules. Red bars represent cell marker proteins that are located to the cell tip by Mt based transport. The regulation of Mt capture (indicated by double side arrow) and Mt dynamics by NIMA (through Eb1 binding) could contribute to the organization of the cell tip growth apparatus, comprising the zone of exocytosis and endocytic collar and thus regulate polarized cell growth.
Chapter 4: A novel function for the methyltransferase Set1 complex in regulating mitosis together with the mitotic kinases, CDK1 and NIMA

4.1 Introduction

In the eukaryotic nucleus, the DNA needs to be packaged within the spatially constrained nucleus, while being available for gene expression and replication. This requires DNA to be compacted in a highly ordered fashion starting from the basic unit of chromatin structure, the nucleosome. The nucleosome consists of 146 basepairs of DNA wrapped around an octamer made up of histone proteins – H2A, H2B, H3 and H4. When viewed under an electron microscope, this gives an appearance of a beads-on-a-string topology with a thickness of about 10nm (Alberts, Wilson et al. 2008). Further packaging of the 10 nm fiber occurs with the help of the linker histone H1 protein to give the 30 nm fiber. The packaging of DNA has to be regulated through the cell cycle. While interphase chromatin has a relaxed configuration mostly in the forms of 30 nm fibers and is more amenable to gene expression, during mitosis, DNA assumes one of the most condensed forms of chromatin structure, the chromosome. During interphase, different regions of the chromatin may exhibit different levels of condensation. The less condensed form of chromatin that
usually comprises of actively transcribed genes is called euchromatin, whereas more condensed regions of the chromatin that are typically resistant to gene expression are called heterochromatin. One mode of regulating the chromatin landscape is through the post translational modification of histones. The histone proteins consist of a conserved C-terminal globular domain, and an N-terminal tail. The N-terminal tail extends out of the nucleosome and therefore is accessible to proteins in the nucleoplasm. Modifications of residues on the different histone tails have the ability to regulate the recruitment and binding of specific protein complexes including transcription factors and DNA repair factors to specific stretches of the genome. In addition, modifications on adjacent histones can influence each other and work in concert or antagonistically to each other resulting in extensive crosstalk (Suganuma and Workman 2008). This results in further fine tuning of the regulation of histone functions and thereby gives more flexibility to the regulation of DNA accessibility.

One such histone modification takes place on the lysine 4 of the histone H3. The methylation of this histone residue is catalyzed by the Set1 complex, the subunits of which are conserved from yeast to humans (Dehe and Geli 2006). The methyltransferase catalytic activity of the complex is found within the Set1 protein. The Set1 protein has a highly conserved C-terminal SET domain that confers the catalytic activity. The other subunits of the Set1 complex have roles in maintaining complex stability and also in regulating the methylation activity of the complex to give mono-, di-, or tri methylated H3K4 (Dehe, Dichtl et al. 2006).
S. cerevisiae has a single Set1 protein as does A. nidulans. All the Set1 complex subunits found in yeast are conserved through to humans. In addition, Drosophila and humans have some subunits specific to higher eukaryotes not found in S. cerevisiae or A. nidulans (Shilatifard 2012). Also, seven putative H3K4 methyl transferase encoding genes have been identified in Arabidopsis thaliana (Ng, Wang et al. 2007).

The methylation of H3K4 is found most commonly on actively transcribed genes. The occurrences of H3K4 methylation on expressed genes has been spatially resolved revealing that the trimethylated H3K4 is found on the 5’ end of the gene, while dimethylated H3K4 is found in the middle of the gene (Dehe and Geli 2006). In addition, H3K4 also binds to many transcription factors including TFIID and chromatin remodeling enzymes (Sims and Reinberg 2006, Kim and Buratowski 2009). However, the function of H3K4 methylation in the regulation of gene expression is not completely understood. The Set1 complex function is not essential in S. cerevisiae, S. pombe or in A. nidulans (as will be described in this work) (Dehe, Dichtl et al. 2006). Moreover in budding yeast, loss of H3K4 methylation leads to a global defect in RNA Pol II transcription however the degree of change is small, less than two fold (Santos-Rosa, Schneider et al. 2002, Boa, Coert et al. 2003). Also, the inactivation of Set1 in Dictyostelium only changes the expression of 75 genes (Chubb, Bloomfield et al. 2006). Therefore, the Set1 complex seems to be not required for establishing global transcription levels. Interestingly, a recent study implicates the involvement of the Set1
complex not in determining the level of transcription, but the inheritance of transcription states from the mother cell to daughter cells in *Dictyostelium* (Muramoto, Muller et al. 2010, Peterson 2010). Using an innovative system to track the production of RNA in a single cell in real time, it has been shown that gene transcription in *Dictyostelium* happens in pulses (Golding and Cox 2006). Using the same technique, it was found that transcription frequency, defined as the amount of time that a gene is transcribed in an hour is more similar between daughter cells and the mother cell than between unrelated cells in a population (Muramoto, Muller et al. 2010). Interestingly, the mutation of H3K4 to alanine eliminates the memory of transcriptional frequency. The inheritance of active transcriptional states is important during development when gene expression profiles are required to be maintained through generations of cells in the same differentiation program.

In contrast to lower eukaryotes that have a single Set1 protein, Drosophila has 3 and humans have 6 set1 proteins. This suggests that the roles of the Set1 complex have expanded during evolution. Consistent with this prediction, the Drosophila orthologs – Set1, Trx and Trr and the human Set1 orthologs – Set1A, Set1B, Mixed Lineage Leukemia (MLL) 1-4 have been found to function in a non-redundant manner (Shilatifard 2012). Moreover, a function for MLL complexes in the expression of Hox genes, which play important regulatory roles in development, has emerged from studies in mice and flies. Also revealing an essential function for the Set1/MLL complexes in development, the absence of
MLL1 is embryonic lethal in mice (Ansari, Mishra et al. 2009). Importantly, MLL1 draws its name from the fact that the rearrangement of this gene is widely seen in several aggressive human leukemias. A common rearrangement seen at the MLL1 locus is translocation of the N-terminal region of MLL1 to another gene (Ansari, Mishra et al. 2009). The SET domain of MLL1 is present at the extreme C terminus of the protein, therefore, most of the fusion proteins resulting from the above translocation event would be expected to carry the regulatory domains of MLL1 which comprise of protein and DNA interaction domains. Therefore, the identification and characterization of the network of proteins that function along with Set1 complexes might be expected to give us leads into how the chimeric MLL proteins function in cancerous cells. In addition, an involvement of the MLL complexes in regulating the expression of cell cycle regulatory proteins like cyclins and Cdk1 inhibitors has also been shown (Milne, Hughes et al. 2005, Takeda, Chen et al. 2006). Therefore the Set1/MLL complexes are likely to have pleiotropic effects on cellular processes through their functions in gene expression in higher eukaryotes like Drosophila and humans.

Growing evidence indicates that the Set1 complex is involved in a gamut of cellular functions, many of which may be independent of its roles in gene expression. For instance a direct involvement of the H3K4 methylation in DNA repair and replication checkpoints in budding yeast, in a manner that is possibly independent of gene expression, has been proposed (Faucher and Wellinger 2010). For instance, the absence of Set1 complex subunits or H3K4 methylation
makes the deletion of genes encoding proteins of the MRX complex (a conserved protein complex involved in DNA damage repair) more sensitive to DNA damaging agents. Strikingly, the Set1 complex and H3K4 methylation locate to sites of DNA double strand breaks (Faucher and Wellinger 2010). In the absence of methylated H3K4, budding yeast cells show a clear defect in repairing damaged DNA by non-homologous end joining (NHEJ); one of the major DNA repair pathways. Moreover, absence of the Set1 complex also makes cells sensitive to replication stress caused by the drug, hydroxyurea that inhibits DNA replication. Also, studies have indicated that a role for methylated H3K4, potentially in the recruitment of DNA repair factors, is conserved in human cells (Pena, Hom et al. 2008).

Though H3K4 was considered a mark of euchromatin, it is present at heterochromatic centromeric DNA in fission yeast, Drosophila and human cells (Sullivan and Karpen 2004, Cam, Sugiyama et al. 2005). A possible function for this centromeric histone modification is emerging from studies showing that the methylation of H3K4 is essential for the recruitment of centromere specific histone variant, CENPA that is essential for centromere formation, in a human artificial chromosome system (Bergmann, Rodriguez et al. 2011). Since kinetochores, protein complexes that mediate the interaction between chromosomes and the spindle and are hence essential for chromosome segregation, are assembled on centromeres, Set1 mediated H3K4 methylation is likely important for centromere and thus kinetochore formation and normal
mitosis. Moreover, the Set1 complex has been shown to regulate kinetochore function through another mechanism. Remarkably, the *S. cerevisiae* Set1 complex, which was considered to be a histone methyltransferase, also methylates a non-histone protein, Dam1 (Zhang, Lin et al. 2005). Dam1 is an inner kinetochore protein in budding yeast which has a crucial role in mediating the interaction between kinetochore proteins and spindle microtubules during chromosome segregation (Tien, Umbreit et al. 2010). The Set1 complex methylates kinetochore-associated Dam1 and it has been proposed that this modification might regulate Dam1 function during mitosis (see also Discussion) (Latham, Chosed et al. 2011).

Our genetic studies presented in Chapter 3 revealed a conserved genetic interaction between the mitotic NIMA kinase and the Set1 complex. In *S. cerevisiae*, the deletion of KIN3, the ortholog of *nimA* is synthetic lethal with the absence of *swd1*, a subunit that is essential for the integrity of the Set1 complex (See Chapter 3, Table 3.1). As will be described in this Chapter, we find that this genetic interaction is also present in *A. nidulans*, underlining a conserved functional relationship between the Set1 complex and NIMA. Furthermore, our studies reveal a genetic interaction of the Set1 complex with the conserved mitotic Cdk1 kinase as well. Overall, our studies, as described here, highlight the importance of Set1 complex mediated H3K4 methylation in regulating G2-M transition together with Cdk1 mediated phosphorylation as well as in regulating a successful mitosis together with NIMA mediated phosphorylation.
4.2 Results

4.2.1 Genetic interaction between nimA and nimX and the components of the Set1 complex

As described in Section 3.2.2, a synthetic lethal screen conducted in S. cerevisiae uncovered several novel genetic interactions of KIN3, the budding yeast ortholog of nimA. By employing the temperature sensitive mutant allele of nimA, nimA7, it was determined that three of these genetic interactions are conserved in A. nidulans. The analysis of two of the three genes, An-vps23 and An-vps25, was described in the previous chapter. Insights obtained from our analysis of the genetic interaction between nimA and the third gene, An-swd1, will be the focus of this chapter. Deletion of the An-swd1 gene yielded viable strains indicating it is non-essential. However the haploid nulls grow slower and conidiate poorly compared to the Wt (Figure 4.1). We noticed that the conidiation defects in ∆An-swd1 could be at least partially suppressed in media containing sucrose (Figure 4.1), and therefore sucrose was used as the main carbon source to propagate ∆An-swd1 strains more effectively. In addition, ∆An-swd1 confers cold sensitivity as evident from the poor growth at 20°C, even after 7 days of incubation, and an improvement in colony growth with increasing temperature (Figure 4.1).

To test for the synthetic genetic interaction between nimA and An-swd1, we compared the growth of strains carrying both nimA7 and ∆An-swd1 with that of the single mutants and a Wt strain at 35°C, a temperature that is semi
permissive for nimA7. Conidia from Wt, nimA7, ΔAn-swd1, and nimA7 + ΔAn-swd1 strains were isolated from colonies grown at 32°C, and were inoculated on plates and allowed to grow at either 32°C or 35°C. 32°C is more permissive for the growth of nimA7 than 35°C, and nimA7 and Wt colonies grow to similar extents at this temperature (data not shown). While the growth of Wt colonies at 35°C is indistinguishable from their growth at 32°C, the temperature sensitivity of nimA7 strains allows a more restricted colony growth at the higher temperature. ΔAn-swd1 strains grow poorly at 32°C compared to 35°C, as seen in Figure 4.1, and the presence of nimA7 in addition to ΔAn-swd1 has a marginal effect on colony size at 32°C (data not shown). However, in contrast to nimA7 and ΔAn-swd1 colonies, the double mutants form very tiny colonies at 35°C revealing a synergistically enhanced growth defect compared to either single mutant (Figure 4.2A). Therefore, when NIMA is partially inactivated (using the semi permissive temperature of the ts mutant allele, nimA7), the deletion of An-swd1 is highly deleterious to growth.

It is possible that this synergistic growth defect was caused by the G2-M defects expected in cells with partial NIMA function. In order to test this, we employed a mutant allele of the Cdc25 phosphatase, nimT, an activator of the mitotic CDK1 kinase (NIMX in A. nidulans). In cells carrying the temperature sensitive mutant allele of nimT, nimT23, at the semi-permissive temperature of 37.5°C, the function of nimT, and therefore CDK1, is partially reduced. So these cells are expected to be delayed at mitotic entry. Comparing the colony growth at
the semi-permissive temperature for nimT23, we find that the deletion of An-swd1 is synthetic lethal with nimT23, such that nimT23 + ΔAn-swd1 cells do not form any colonies at the semi permissive temperature, instead appear as short stunted cells when viewed under the microscope (Figure 4.2C, inset). In comparison, the single mutants grow to form visible colonies at the same temperature. Taken together, these dramatic growth defects in the double mutants, nimA7 + ΔAn-swd1 and nimT23 + ΔAn-swd1, points to the requirement of the Set1 complex subunit, An-swd1, for survival upon when mitotic kinases are partially functional.

The only known function of An-swd1 is as a part of the Set1 complex and in all systems studied, swd1 is essential for the function of the Set1 complex (Dehe, Dichtl et al. 2006, Shilatifard 2012). However it is possible that An-swd1 plays other unrecognized cellular functions independent of the Set1 complex and that the abrogation of these Set1-independent functions is responsible for the genetic interaction between ΔAn-swd1 and nimA7. Therefore, to determine whether the loss of Set1 complex function in ΔAn-swd1 cells is responsible for the synergistic growth defects in ΔAn-swd1 + nimT23 and ΔAn-swd1 + nimA7, we identified the A. nidulans ortholog of set1 that encodes the catalytic methyltransferase protein of the Set1 complex. set1 was replaced with a nutritional marker by homologous recombination in cells of Wt, nimA7 and nimT23 genotypes. Next, the growth of the double mutants that lack the Set1 protein in combination with nimA7 or nimT23 at the respective semi-permissive
temperatures was assessed. The growth defects of the double mutants, ∆An-set1 + nimT23 and ∆An-set1 + nimA7, at 37.5°C and 35°C respectively, are remarkably similar to the growth defects of their An-swd1 deletion double mutant counterparts (Figure 4.2B, D). These data indicate that the lack of Set1 complex function (either in ∆An-swd1 or ∆An-set1) is lethal when combined with reduced mitotic kinase function.

4.2.2 Cells with partial NIMA and CDK1 function depend on H3K4 methylation for survival

The most conserved substrate of the Set1 complex is the histone H3. In addition to the methylation of H3 at K4, the Set1 complex has been shown to methylate the kinetochore protein, Dam1, in S. cerevisiae (Zhang, Lin et al. 2005). We were interested to determine whether the absence of H3K4 methylation in ∆An-swd1 and ∆An-set1 cells was responsible for the synthetic lethality (Figure 4.2). To address this, we generated cells carrying a mutant version of H3, where the lysine, K4, was mutated to arginine. Since the Set1 complex is a lysine methyltransferase, the histone H3-K4R cannot be methylated even though the Set1 complex is fully functional in these cells. A construct carrying H3-K4R generated by site directed mutagenesis fused to a nutritional marker was transformed into Wt, nimA7 and nimT23 strains, to replace the endogenous histone H3 gene with the mutant version. Strains carrying the mutant H3-K4R as the only copy of histone H3 exhibited growth and conidiation
defects very similar to $\Delta An$-swd1 and $\Delta An$-set1 strains, indicating that the loss of methylation at this histone residue is responsible for the poor growth and conidiation in Set1 complex mutants (Figures 4.3, 4.2). Moreover, in combination with nimA7 or nimT23 at their respective semi restrictive temperatures, non-methylatable H3-K4R causes synthetic lethality compared to the single mutants (Figure 4.3). At 32°C, the temperature that is more permissive for both ts alleles, while the nimA7+H3K4R double mutant grows to a similar extent as H3K4R single mutant, the double mutant nimT23+H3K4R exhibits a growth defect compared to either single mutant (data not shown). The combination of H3-K4R and reduced Cdc25$^{nimT}$ function causes lethal effects that are comparable to the combination of $\Delta An$-swd1 or $\Delta An$-set1 with reduced Cdc25$^{nimT}$ function. However, the combination of reduced NIMA function and H3-K4R is tighter (causes more severe growth defects) compared to the combination of reduced NIMA function with the deletion of $\Delta An$-swd1 or $\Delta An$-set1. This suggests that in the absence of Set1 complex function, H3K4 can perhaps be methylated by another unknown enzyme, however when the K4 residue is mutated and cannot be methylated, it produces a stronger synthetic genetic interaction with partial loss of NIMA function. This genetic interaction between NIMA and the Set1 complex is likely specific to the histone modification involving H3K4 since the deletion of Dot1, the gene encoding the H3K79 methyl transferase does not genetically interact with NIMA (Chapter 3, Table 3.1).
Histone H3 is a substrate of not only the Set1 complex but also the NIMA kinase. NIMA phosphorylates the serine 10 residue of histone H3 (H3S10) \textit{in vitro}, is required for this phosphorylation during mitosis and can promote the phosphorylation of this residue out of cell cycle phase upon overexpression (De Souza, Osmani et al. 2000). Therefore, we hypothesized that the combination of loss of methylation at H3K4 and the loss of phosphorylation at H3S10 might underlie the synthetic genetic interaction between \textit{swd1} and \textit{nimA7}. In addition, since Cdk1 is essential for complete activation of NIMA, it is possible that there is reduced or delayed H3S10 phosphorylation in \textit{nimT23} cells, as in \textit{nimA7} cells, at the respective semi permissive temperature. To test this hypothesis, we generated a mutant version of H3 that had an alanine instead of serine at position 10 (H3-S10A) and also a version of H3 that had both K4 mutated to arginine and S10 mutated to alanine (H3-K4RS10A), by site directed mutagenesis. Constructs carrying H3-S10A or H3-K4RS10A were used to replace the endogenous H3 gene. We find that mutating H3-S10 to a non-phosphorylatable residue does not compromise viability, and H3-S10A strains grow similar to Wt at all temperatures tested (Figure 4.4). If there was a synthetic genetic interaction between H3-K4R and H3-S10A, then it would be expected that having histone H3 that is neither methylatable at K4 nor phosphorylatable at S10 as the only H3 would be lethal. However, we were able to generate haploid strains that carried H3-K4RS10A. We compared the growth of this double mutant strain to either single mutant at different temperatures to test for any enhanced
growth defects in the double mutants. As shown in Figure 4, we find that there is marginal inhibition of colony growth in H3-K4RS10A compared to either single mutant at 32°C. However, the double mutant shows considerable temperature sensitivity at the higher temperature of 42°C. Therefore, the combination of a methylated K4 and phosphorylated S10 in H3 is probably required for a cellular process that is more important for growth at the higher temperatures. But since the H3-K4RS10A does not phenocopy the growth defects shown by ∆An-swd1 + nimA7 and ∆An-swd1 + nimT23 at the semi-permissive temperature we think that the loss of H3S10 phosphorylation is not causative of the genetic interaction between the Set1 complex and the mitotic kinases.

4.2.3 In the absence of sufficient CDK1 activity, Set1 complex function is essential for G2-M transition

To gain further insights into the potential cause for the synthetic lethal interaction between CDK1 and the Set1 complex, we decided to observe microscopically the nuclear morphology of the double mutant, ∆An-swd1 + nimT23, compared to either single mutant and Wt cells. To this end, strains that carried a chromatin marker, histone H1 tagged with ch-RFP as well as tub-GFP, in the background of ∆An-swd1, nimT23, ∆An-swd1 + nimT23 or Wt genotypes were generated. These strains were grown for 7 h at the semi permissive temperature of 37.5°C, the same temperature at which the synthetic lethality was observed, and fixed and analyzed by confocal microscopy (Figure 4.5). We find
that at this time point, the majority of Wt cells have 8-16 nuclei. In contrast, most nimT23 cells have 2-4 nuclei, consistent with the expected delay in mitotic entry at this temperature (Figure 4.5A, B). Deletion of An-swd1 results in a majority of cells having 4-8 nuclei, which would be consistent with there being defects in cell cycle progression and/or growth. Strikingly, however, most double mutant cells had a single nucleus, as indicated by the quantitation in Figure 4.5B. The presence of uninucleated cells may reflect defects in overall cell growth and/or a defect in mitotic entry. To discern between these two possible causes for the increased number for uninucleated cells in the double mutant, we calculated the average cell length for each of the cell types (Figure 4.5C). We found that the average cell length of the single mutant lacking An-swd1 is half as much as Wt cells. nimT23 cells also are similarly inhibited for growth and the growth of the double mutant does not differ significantly from either single mutant (Figure 4.5C). Therefore, a more profound growth defect cannot explain the marked lack of mitotic division in the double mutant.

In A. nidulans, analysis of various cell cycle mutants defective G2-M transition has shown that short term growth continues even when there is a delay in mitotic entry (Osmani and Ye 1996). Therefore in such a situation, fewer nuclei are expected to be present per unit length. We calculated the average number of nuclei in a fixed length of the cell to test whether there was an enhanced G2-M delay in the double mutants, given that they had fewer nuclei but grew to the same extent as the single mutants (Figure 4.5D). In the case of Wt cells, there
are about 4-5 nuclei in a span of 20 μm. Consistent with there being a G2-M delay, there are on average only about 2 nuclei in the same length of a nimT23 cell. An-swd1 null cells show a slight reduction in the number of nuclei per 20μm, showing 3-4 nuclei on average. However, the double mutant had on average only 1 nucleus within a span of 20μm, in strong support of a defect in mitotic entry (Figure 4.5D). These data indicate that there is an enhanced G2-M defect in the double mutant compared to cells with only partial Cdk1 function. In other words, abolishing Set1 complex function makes the ‘never in mitosis’ (nim) phenotype of nimT23 cells tighter at a lower temperature.

4.2.4 Cells lacking Set1 complex function in addition to partial CDK1 function exhibit enhanced delay in entering the first mitosis

Our data so far are consistent with an enhanced G2-M delay in ∆An-swd1 + nimT23 cells at the semi permissive temperature. To further define this defect, we aimed to follow these cells by live cell confocal microscopy at the elevated temperature, 37.5°C. In addition, to identify the earliest defect(s), we were interested in comparing the first mitosis in the two single mutants as well as in the double mutant. We find that in the absence of sufficient Cdk1 function, nimT23 cells still complete mitosis successfully, showing chromatin condensation concomitant with spindle formation and the generation of two daughter nuclei (Figure 4.6B). We also confirmed that mitotic nuclear pore complex disassembly occurs normally in cells with reduced CdcnimT function. As seen in Figure 4.6C,
nimT23 cells at the semi permissive temperature, show the dispersal of the peripheral nuclear pore complex protein, Nup49, upon spindle formation at mitotic entry and its return to the nuclear periphery at the end of mitosis.

The absence of An-swd1 in Wt cells as well as in combination with partial Cdk1 function does not give rise to any catastrophic mitotic defects as followed by chromatin segregation and spindle formation (Figure 4.7 C, D). However, we noticed that the length of cells at the time of entry into the first mitosis was quite different. In the vast proportion of Wt and An-swd1-null cells, the first mitosis is triggered in the swollen conidia before germtube extension. In contrast, in nimT23 cells, 51% of the cells that entered first mitosis had already sent out a germtube (Figure 4.7). Strikingly though, the cell length of the double mutant, ΔAn-swd1 + nimT23, was on average 4 times that of nimT23 cells at the time of mitotic entry (Figure 4.7C). This phenotype is indicative of a defect in entering the first mitosis due to which cells with one nucleus continue to grow resulting in the characteristic ‘nim’ phenotype.

It is important to note that in spite of this delay in triggering mitosis, no double mutant cells were observed to undergo mitotic failure (Figure 4.7B). During our analysis of fixed ΔAn-swd1 + nimT23 cells, we detected the presence of spindles in telophase which is not typical during normal mitosis. In Wt cells, the spindle typically gets disassembled soon after chromatin segregation in anaphase and telophase spindles are not present for very long. Therefore, we looked to see if our live cell imaging revealed a sustained presence of the
spindle. Consistent with our fixed cell imaging, we find that double mutant cells show the obvious presence of the spindle well after the chromatin has been segregated (Figure 4.7B, arrowheads), suggesting a defect in disassembling the mitotic spindle. Upon calculating the time spent in mitosis for the double mutant compared to either single mutant, we find that there is an increase in mitotic delay in the double mutants compared to either single mutant (Figure 4.7D).

Our results described so far are consistent with mitotic initiation being delayed in the double mutants (nimT23+ΔAn-swd1), more so than either single mutant (Appendix A, Figure A.2). If the cells were delayed in the G2 phase of interphase, then we would expect that the cells would enter mitosis immediately once they are downshifted to a lower temperature (and nimT\textsuperscript{cdc25} regains full functionality). To test this prediction, we germinated nimT23 or nimT23+ΔAn-swd1 cells at 37.5°C for 5-6 hours. Then the temperature of the cells was quickly reduced by replacing the media with media at room temperature and immediately followed by live cell microscopy. We find that more than 70% of nimT23+ΔAn-swd1 cells enter mitosis within 30 minutes of being brought down to room temperature (23°C), as represented by the histogram in Figure 4.8B. Similarly, nimT23 cells also exhibited a prompt synchronous entry into mitosis (Figure 4.8A). Interestingly, we note that peak of mitotic entry in the double mutant cells is slightly shifted to the right compared to nimT23 cells. This could be reflective of a tighter G2 arrest or of an arrest at a point in G2 earlier than the arrest point of nimT23, in the double mutants, which takes them longer to overcome. The peak
of mitosis seen in nimT23+ΔAn-swd1 immediately after downshift to the lower
temperature indicates that these cells were in G2 before the downshift and that
the mitotic initiation is likely impaired by defects in G2.

One reason for the prolonged G2 could be the presence of DNA damage
that delays mitotic entry until the DNA is repaired. Therefore, we wondered
whether the reason nimT23+ΔAn-swd1 cells are delayed in G2 might be due to a
defect in DNA repair pathways. To test this hypothesis, we subjected nimT23 and
ΔAn-swd1 cells to DNA damaging agents (DEO and MMS) at the semi
permissive temperature, along with Wt strain as a negative control and a strain
mutant for scaA, a gene known to be required for DNA damage response in A.
nidulans (Figure 4.8C) (Bruschi, de Souza et al. 2001). We find that deletion of
An-swd1 or reducing Cdk1 function makes cells sensitive to DNA damage.
Therefore, we think it is possible that in the double mutants (which lack An-swd1
in addition to having impaired Cdk1 function), the spontaneous occurrences of
DNA damage are not repaired as efficiently and therefore the double mutants
take longer to transition through G2 even in the absence of external DNA
damaging agents.

4.2.5 Partial decrease in NIMA function results in mitotic defects

In line with the analysis of the CDK1- Set1 complex genetic interaction, we
sought to determine the potential cause for the genetic interaction between NIMA
and the Set1 complex by performing microscopic analysis of Wt and nimA7, ΔAn-
**swd1** and **nimA7+ΔAn-sw1** mutants. Strains were generated that carried the chromatin marker histone H1-chRFP, and tub-GFP to monitor microtubule dynamics, in the background of Wt and each of the mutant genotypes. These strains were grown for 7 hours at the semi permissive temperature for **nimA7**, a temperature that reveals the synthetic genetic interaction, and fixed for microscopic analysis. It was expected that the reduction of NIMA, like the reduction of Cdc25, would result in a delay in transitioning into mitosis, resulting in germlings with widely spaced but normal nuclei. Intriguingly, however, we find that **nimA7** cells at the semi permissive temperature display chromatin segregation defects suggesting a failure to complete mitosis successfully (Figure 4.9A). This defect was highly penetrant in **nimA7** cells. We quantitated this defect by counting the number of nuclei per cell in **nimA7** vs Wt cells. As shown in Figure 4.9B, the population of Wt cells is divided between cells that have mainly 8 or 16 nuclei and some nuclei that have 2 or 4 nuclei. This distribution of nuclei number is reflective of consecutive successful rounds of synchronous mitoses that generate 1, 2, 4 and 16 nuclei in Wt cells. In contrast, **nimA7** cells are quite distinctive, having a significant number of cells with odd numbers of nuclei, which is rarely observed in Wt cells (Figure 4.9B). This indicates a failure to double the nuclei during one or multiple mitotic events during the growth of the **nimA7** cells. Moreover, at this time point, no **nimA7** cells are seen to possess 8 or 16 nuclei. We were interested in further exploring this unexpected phenotype in **nimA7** cells to gain insights into the functions of NIMA in mitotic progression and undertook
extensive live cell imaging of these cells at the semi permissive temperature. These results will be discussed in detail in Chapter 5. However, we conclude that reduction of NIMA function causes mitotic defects rather than just delaying mitotic entry as seen with the reduction in Cdk1 activity.

4.2.6 Absence of Set1 complex function in addition to partial NIMA function results in fewer nuclei and reduced growth

On quantitating the distribution of cells with different numbers of nuclei in ∆An-swd1, we find a majority have 4 nuclei compared to the majority of Wt cells that have 8 or 16 nuclei (Figure 4.9B). Importantly, the double mutant (nimA7+△An-swd1) cells have a higher number of uni- and bi-nucleated cells, a lower proportion of 3 and 4 nucleated cells and no cells with 5 or 6 nuclei compared to nimA7 cells (Figure 4.9B, dotted lines). These data are consistent with there being a synergistic growth defect in the double mutants compared to either single mutant. The increase in cells with 1 and 2 nuclei in the double mutants could be either due to a defect in mitotic entry or an indirect effect of growth differences between the strains. To distinguish between these two possibilities, we calculated the average cell length of the Wt strain in comparison to both single mutants and the double mutant (Figure 4.9C). Consistent with our data from growth at 37.5°C, we see that the deletion of An-swd1 gives rise to a growth defect resulting in an average cell length of 50% that of Wt cells. Partial reduction of NIMA function also results in some retardation of growth.
Importantly, the growth of the double mutant is more inhibited than either single mutant (Figure 4.9C). These data show that the reduced number of nuclei in nimA7+An-swd1 is accompanied by a cell growth defect. To further confirm this, we calculated the distance between nuclei as a measure of G2-M transition defect as done in Figure 4.3. Comparison of average internuclear distance shows that there is little difference in this parameter between nimA7 and nimA7+ΔAn-swd1 cells (Figure 4.9D). Moreover, during live cell imaging of each of the single mutants in comparison with the double mutants, we did not see a delay in entry into first mitosis. In the case of nimT23+ΔAn-swd1 cells (Figure 4.7C), the first mitosis happens in long germlings unlike the two single mutants and the Wt, indicating that the nuclear division was delayed while cell growth continued. However, we did not find the nimA7+ΔAn-swd1 cells to be longer than either single mutants and the Wt at the time point before entering the first mitosis (data not shown). Though this is consistent with the absence of a G2-M delay in nimA7+ΔAn-swd1, since we know from cell length quantitation that nimA7+ΔAn-swd1 cells are inhibited for growth (Figure 4.9C), it is difficult to ascertain whether nimA7+An-swd1 cells have an enhanced G2-M defect. However, these data show that a synthetic growth defect and reduced numbers of nuclei (Figure 4.9) underlies the synthetic lethality between absence of Set1 complex function and impaired NIMA function (Figure 2A).
4.2.7 Absence of Set1 complex function prolongs the time spent in mitosis when NIMA is reduced

As described above, cells with reduced NIMA function exhibit mitotic defects. Analysis of mitosis in these cells by live cell imaging showed that they exhibit a delay during mitosis compared to Wt cells at the same temperature (Figure 4.10, see also Chapter 5). One potential cause for the reduced number of nuclei in \( \text{nima}^7+\text{An-swd}1 \) cells could be the presence of enhanced mitotic defects in the double mutant compared to either single mutant, which might in turn result in a greater mitotic delay in the double mutant. To test if this was true, we collected time lapse images of \( \Delta \text{An-swd}1 \) and \( \text{nima}^7+\Delta \text{An-swd}1 \) cells at the semi permissive temperature and compared the time taken to complete mitosis with that of \( \text{nima}^7 \) cells and Wt cells. Using tub-GFP to follow spindle formation, we followed the first mitosis during spore germination to detect potential early defects in mitosis. We find that the time taken for the double mutants to complete the first mitosis is greater than \( \text{nima}^7 \) cells (Figure 4.10A). However, this effect is not synergistic considering there is a marginal increase in the mitotic time in \( \Delta \text{An-swd}1 \) cells as well (Figure 4.10A). Interestingly, when we follow the second mitosis in these cells, we see that the double mutants exhibit a mitotic delay that is more enhanced compared to either single mutant (Figure 4.10B). In addition, we see that there is more variability in the time taken to complete first mitosis in \( \text{nima}^7 \) and the double mutants compared to the other two cell types (Figure 4.10C). This variability in length of mitosis is enhanced in the second mitosis in
the double mutant but not in nimA7 (Figure 4.10D). These data taken together support the idea that mitotic progression is impaired to a greater extent in cells lacking the Set1 complex in addition to having partial NIMA function as compared to either single mutant.

4.2.8 Absence of Set1 complex function makes cells with partial NIMA function more dependent on the spindle assembly checkpoint

The spindle assembly checkpoint (SAC) monitors defects in mitosis including correct kinetochore-spindle attachment to regulate the transition from metaphase into anaphase. Mutants that give rise to mitotic defects are often dependent on the spindle assembly checkpoint for the correction of these errors and thus, survival. Therefore, the presence of mitotic defects in a mutant cell background can be revealed by determining whether elimination of SAC function affects the growth of the mutant strain. Since the nimA7+ΔAn-swd1 double mutant cells exhibit enhanced mitotic delay compared to either single mutant, we hypothesized that they might be dependent on the SAC for survival, more so than either single mutants. To test this, we generated strains that carry the deletion of An-md2A, the A. nidulans ortholog of the SAC gene, mad2, in Wt, nimA7, An-swd1 and nimA7+ΔAn-swd1 backgrounds. We then asked whether the double mutants are more sensitive to the absence of An-md2A when compared to either single mutant. We compared the colony growth of all the different strain genotypes, as indicated in Figure 4.11, at 32°C, a temperature
which is more permissive for nimA7 cells than 35°C (Our live cell imaging indicates that 32°C may not be completely permissive for nimA7 cells, since some mitotic defects can be detected). The ∆An-mad2+ nimA7 and ∆An-mad2+ ∆An-swd1 colonies formed after 96 hours of growth, when compared to the single mutants show that the absence of Mad2 does not affect the growth of either nimA7 or ∆An-swd1 significantly. However, the absence of Mad2 in nimA7+∆An-swd1 double mutants severely inhibits growth (Figure 4.11). This indicates that the growth of nimA7+∆An-swd1 is dependent on SAC function.

This dependence of nimA7+∆An-swd1 double mutants on SAC for colony growth at a temperature where both the single mutants are not influenced by the absence of SAC, points to the presence of mitotic defects in the double mutants not present in either single mutants.

Data presented in sections 4.2.5 and 4.2.6 indicates that the absence of Set1 complex function severely inhibits the ability of cells with reduced Cdc25 (and hence reduced Cdk1) function to initiate mitosis. If G2-M defects are the main cause for the synthetic genetic interaction between Set1 and Cdc25
nimT, then we would not expect the ∆An-swd1+ nimT23 double mutant to be more dependent on SAC function (a checkpoint that detects mitotic errors) compared to either single mutant. Consistent with this expectation, we find that the colony growth of ∆An-swd1+ nimT23+∆An-mad2 cells is no more inhibited for colony growth than the double mutant ∆An-swd1+ nimT23. This lends further support to the idea that mitotic events, at least those monitored by the SAC, are unaffected
in \(\Delta An\text{-}swd1+ nimT23\) and the main cause for the synthetic lethality, as supported by our data, is likely to be enhanced delays in the G2-M transition. These data also act as a control for our observation that \(nimA7+\Delta An\text{-}swd1\) cells are dependent on SAC function for survival.

### 4.2.9 The Set1 complex may act as a negative regulator of septation

To explore further the synthetic genetic interaction between \(An\text{-}swd1\) and \(nimA\), we decided to investigate the effect of the deletion of \(An\text{-}swd1\) in cells that completely lack NIMA function. We reasoned that due to the synthetic genetic interaction of the two genes, deletion of \(An\text{-}swd1\) might modify the terminal phenotype of \(nimA7\) cells at 42°C. At this restrictive temperature, \(nimA7\) cells grow to form germlings, but since mitotic entry is blocked in the absence of NIMA function, they arrest with a single G2 nucleus. Wt, \(nimA7\), \(\Delta An\text{-}swd1\), and \(nimA7+\Delta An\text{-}swd1\) were grown at 42°C for 11 hours and fixed and stained with DAPI to visualize nuclei and calcofluor to reveal septa (Figure 4.12A). We find that as expected, Wt cells grow to form multinucleated septated cells at both 32°C and 42°C. Cells carrying the \(nimA7\) mutation grew similar to Wt cells at 32°C but were unseptated and arrested with a single nucleus at 42°C (Figure 4.11A). \(\Delta An\text{-}swd1\) cells also resembled Wt cells for the most part, except that a small proportion of cells showed closely spaced septa (Figure 4.12, data not shown). Importantly, the double mutants were severely inhibited for growth compared to either single mutant with 20% of cells being defective in establishing
polarized cell growth, leading to an arrest as swollen conidia (Figure 4.12A). Strikingly, the double mutant cells carrying a single nucleus, that are more inhibited for growth than the nimA7, showed occurrences of septation. In *A. nidulans* Wt cells, the first septation event is typically triggered after 3 rounds of mitosis. Therefore, nimA7 cells that are uninucleated show virtually no septa at this temperature. In contrast, 18% of the double mutants had one, two or even three septa, even though they were uninucleate. These data reveal a potential role for the Set1 complex as a negative regulator of septation and suggest that NIMA is not required for septation. On the other hand, analysis of nimT23+ΔAn-swd1 cells at 42°C revealed that these uninucleated cells did not show septation (data not shown) indicating that normal activation of Cdk1 is required for septation.

In some septated uninucleated nimA7+ΔAn-swd1 cells at 42°C, we noticed that the septum was erroneously placed so that it cut the nucleus. In our time lapse imaging of these cells at the semi permissive temperature, we were able to detect septation events (~5%) where the septum was formed such that it passed through the nucleus. One such example is depicted in Figure 4.12B. At 35°C, this nimA7+ΔAn-swd1 cell enters mitosis as indicated by the presence of the mitotic spindle. However, nuclear segregation fails and the nucleus, as marked by the nuclear transport marker NLS-DsRed, remains as a single unit connected by a thin bridge (yellow arrowhead). Interestingly, septation is initiated at this point, resulting in a septum (red arrowhead) that cuts the bridge
connecting the two parts of the nucleus. The identification of such erroneous septation events in nimA7+ΔAn-swd1 cells suggests that the absence of Set1 complex function in combination with reduced NIMA function results in a deregulation of septation. Similar analysis of nimA7 cells in the future will allow us to determine whether the misregulation of septation so as to allow the septum to pass through the nucleus is unique to the nimA7+ΔAn-swd1 cells.

4.2.10 The mitotic locations of NIMA are not affected by the absence of Set1 complex function

NIMA has a dynamic location during mitosis and is predicted to play multiple functions at several different nuclear locations including the SPBs and NPCs. One possible reason for enhanced mitotic defects in nimA7+ΔAn-swd1 double mutants as seen in Figures 4.10 and 4.11, could be that the Set1 complex function is required for NIMA to locate to one or more of its mitotic locations. If this were true, then in the double mutants, a combination of loss of location and reduced function of NIMA might be expected to generate enhanced mitotic defects. To test this hypothesis, we generated strains that are deleted for An-swd1 that also carried the endogenous NIMA fused with GFP as the only copy of the protein. We observed these cells by live imaging at the same temperature that the synthetic genetic interaction was observed, at 35°C. We find that the mitotic locations of NIMA are unaltered in ΔAn-swd1 cells (Figure 4.13A). As seen in Wt cells (data not shown), NIMA locates to spindle pole body (SPB) at
mitotic entry, then transitions to the nuclear periphery, the nucleus and then back
to the separated SPBs at mitotic exit. The NIMA kinase also locates to forming
and formed septa in Wt cells (Shen et al. unpublished). Interestingly, we
observed that 5% of ΔAn-swd1 cells show an obvious defect in NIMA location at
the septum. In the cell in Figure 4.13B, the septum on the left shows normal Wt
like location of NIMA at the mature septum. In contrast, the septum on the right
shows NIMA being offset to one side of the septum (Figure 4.13B, arrowhead).
The corresponding brightfield image for this cell shows the presence of a septum
(data not shown). However, we did not see any defects in NIMA location at
forming septa (n=25). Therefore, these data support a role for the Set1 complex
in accurately locating NIMA to formed septa. Whether this reflects a broader role
for the Set1 complex in locating septal components or is specific to NIMA
regulation is not known at this point.

4.2.11 SWD1 locates to the nucleus and the kinetochore/SPB region during
interphase and disperses during mitosis

Since An-swd1 exhibits synthetic genetic interaction with two mitotic
kinases in A. nidulans, we were interested to determine its interphase and mitotic
locations. An-swd1 was endogenously tagged with GFP. This tagged version is
functional since it does not cause any growth defects seen with the ΔAn-swd1
strain (data not shown). Analysis of SWD1-GFP by live cell confocal microscopy
reveals that it is a nuclear protein during interphase (Figure 4.14A). In addition,
SWD1 also locates as a strong focus at the nuclear periphery. The spindle pole body of *A. nidulans* is embedded in the nuclear envelope and all the kinetochores cluster at the SPB in interphase. Therefore we sought to determine whether SWD1 locates to the SPB/kinetochore region by generating strains that carried a red tagged SPB marker (GCP3) or a kinetochore marker (ndc80) in addition to SWD1-GFP. As seen in Figure 4.14B and C, SWD1 does indeed locate to the kinetochore-spindle pole body region during interphase.

To determine the mitotic location of SWD1 we followed its location in cells that also carried histone H1-chRFP by time lapse microscopy (Figure 4.14A). Entry into mitosis can be followed by condensation of chromatin marked by H1-chRFP. In *A. nidulans*, the nuclear pore complexes partially disassemble during mitosis, thus abolishing active nuclear transport. Therefore proteins that are actively imported into the interphase nucleus disperse into the cytoplasm during mitosis and are reimported once transport is established at mitotic exit. We find that upon entry into mitosis, SWD1 disperses throughout the cytoplasm and is reimported at the end of mitosis (Figure 4.14A).

To observe more closely the dispersal of SWD1 upon mitotic entry, we used NLS-DsRed, a marker protein that is actively transported into the nucleus during interphase, as a marker for mitotic nuclear pore complex disassembly. Interestingly, we find that dispersal of SWD1 does not coincide with NLS-DsRed dispersal; rather SWD1 exhibits a more gradual dispersal from the nucleus. As shown in the montage in Figure 4.15A, at 8’, NLS-DsRed is mostly dispersed
from the nucleus, though a nuclear signal for SWD1 is still detectable. This difference in dispersal time is quantitated in the pixel intensity plots of the indicated ROI passing through the nucleus, which shows that at 8', a peak of only green SWD1 signal is present while the red signal has already reduced significantly (Figure 4.15B). These data indicate that SWD1 is not held by active transport in the nucleus. In the absence of active transport, SWD1 stays in the nucleus for a short while, perhaps through interaction with chromatin or other nuclear proteins. Moreover, since SWD1 is expected to be in complex with other subunits of the Set1 complex to form a huge protein complex, it is possible that this complex formation prevents the immediate dispersal of SWD1 into the cytoplasm upon mitotic entry. We also observed a delay in the reimport of SWD1 into the nucleus at mitotic exit compared to the reimport of NLS-DsRed (Figure 4.15B).

4.2.12 The location of SWD1 is unaltered when the function of NIMA or Cdk1 is reduced

To determine whether the Set1 complex might be under the regulation of NIMA and/or Cdk1, we asked whether the localization of SWD1 is altered in cells with nimT23 at 37.5°C or in nimA7 cells at 35°C. We generated cells that carried SWD1-GFP in the background of either of these two temperature sensitive mutants and observed the location of SWD1 through mitosis using live cell microscopy. As shown in Figure 4.16A, the interphase and mitotic location of

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SWD1 in Wt cells is not changed at the higher temperature of 37.5°C. In cells with reduced Cdc25\textsuperscript{nimT}, and hence reduced Cdk1 function, SWD1 behaved similar to the Wt, with mitotic specific dispersal and reimport after mitotic exit (Figure 4.16B). In cells with partial NIMA function too, we find that SWD1-GFP disperses almost concomitant with NLS-DsRed (Figure 4.16C). At reimport, SWD1 takes longer to accumulate in the nucleus, consistent with what we see in Wt cells (Figure 4.15A). Thus we find that SWD1 undergoes mitotic dispersal in cells with partial NIMA function, in a manner similar to NLS-DsRed. However, the mitotic localization of SWD1-GFP is important in the light of the mitotic defects seen in \textit{nimA7} cells and so these data will be discussed further in Chapter 5.

4.3 Discussion

4.3.1 The Set1 complex and Cdk1

Our data strongly suggests that the absence of the Set1 complex when \textit{nimT} (cdc25), the positive activator of Cdk1 is partially inactive, leads to an enhanced G2-M delay. This suggests that the Set1 complex is a positive regulator of mitotic entry in a manner possibly through a mechanism involving Cdk1 activation (Appendix A, Figure A.2). Alternatively, it is possible that the Set1 complex is involved in a process that is monitored by a G2-M checkpoint. In support of this idea, we find that cells lacking Set1 function are sensitive to DNA damage. A role for the Set1 complex in DNA damage has also been proposed based on studies in other model systems (Corda, Schramke et al. 1999, Faucher
and Wellinger 2010). Moreover, we also find that lowering Cdk1 activity makes cells sensitive to DNA damage. This was surprising because previous studies have shown that the DNA damage checkpoint acts by inhibiting Cdk1 via phosphorylation in *A. nidulans* (Ye, Fincher et al. 1997). Therefore a decrease in \( \text{nimT}^{\text{cdc25}} \) activity which should increase the inhibitory phosphorylation on Cdk1 was not expected to interfere with Cdk1 inactivation. It is known that the inhibitory phosphorylation on Cdk1 is retained in the presence of DNA damage by activation of the Wee1 kinase combined with simultaneously inactivation the Cdc25 phosphatase (den Elzen and O'Connell 2004). Therefore it is possible that in \( \text{nimT23} \) cells at 37.5°C, the sensitivity to DNA damage results from \( \text{nimT}^{\text{cdc25}} \) being inhibited by both the DNA damage checkpoint and the temperature sensitivity, thereby causing a defect in recovering from DNA damage rather than due to a defect in actual DNA repair mechanisms. Therefore, considering these data, we hypothesize that the combination of lack of Set1 complex function and inadequate Cdk1 function causes increased sensitivity to DNA damage due to a combination of inefficient recovery from DNA damage (in the absence of Set1 function) and a deficiency in re-entering mitosis after checkpoint-mediated G2 arrest (due to \( \text{nimT23} \) mutation). Therefore the \( \text{nimT23}^{\Delta \text{An}-\text{swd1}} \) double mutants are perhaps unable to cope with naturally occurring spontaneous DNA damage and thus exhibit lethality even in absence of external DNA damaging agents.
4.3.2 The Set1 complex and NIMA

Our data shows that the Set1 complex has a function in ensuring proper mitotic progression which is uncovered upon inactivation of NIMA. The absence of the Set1 complex in cells with reduced NIMA makes them more sensitive to the absence of the spindle assembly checkpoint (SAC). The reliance of nimA7 ΔAn-swd1 on SAC function is greater than either single mutant, suggesting that compromised kinetochore-Mt attachments in these cells perhaps leads to the requirement of SAC activity for survival. One possibility is that NIMA-mediated phosphorylation and Set1 complex mediated H3K4 methylation are required to jointly regulate the function of the kinetochore. The human ortholog of NIMA, Nek2, phosphorylates Hec1 (the ortholog of A. nidulans Ndc80), a kinetochore protein that is central to normal Mt attachment during mitosis. It has been shown that this phosphorylation by Nek2 is necessary for proper chromosome segregation (Chen, Riley et al. 2002, Wei, Ngo et al. 2011). Our data on cells with reduced NIMA function are also consistent with a function for NIMA in kinetochore segregation as followed by Ndc80 dynamics (Chapter 5, Section 5.2.2). Therefore, NIMA might regulate kinetochore function through phosphorylation of Ndc80 and possibly other target proteins. Importantly, the presence of H3K4 methylation at the heterochromatic centromeres is a conserved histone modification, strongly suggesting that this modification may have a role in regulating centromere function (Sullivan and Karpen 2004, Cam, Sugiyama et al. 2005). In line with this function being conserved in A. nidulans,
our data shows that An-swd1, the subunit of the Set1 complex locates distinctly
to the kinetochore/SPB region. Recent evidence shows that diminished levels of
H3K4 methylation affects the recruitment and deposition of the histone CENPA at
centromeres of human artificial chromosomes providing direct evidence for the
involvement of Set1 mediated methylation in centromere function (Bergmann,
Rodriguez et al. 2011). The centromeres are held together by the protein,
cohesion, until anaphase, the inactivation of the spindle assembly checkpoint
(SAC) triggers cohesion degradation and hence anaphase onset. H3K4
methylation is implicated in preventing premature separation of centromeres
during mitosis (Eot-Houllier, Fulcrand et al. 2008). Studies suggest that
centromeric methylated H3K4 may provide a binding site for the protein,
Shugosin which maintains the cohesion at the centromeres until anaphase,
indicating that H3K4 methylation might have key regulatory roles at the
centromere during mitosis. Since kinetochore formation is intimately linked with,
and is dependent on, centromere integrity, we propose that one cause for the
synthetic lethality and SAC monitored mitotic defects in cells lacking adequate
NIMA and Set1 complex function could be the misregulation of centromere-
kinetochore functions.

It is worth pointing out that a parallel genetic interaction has been
uncovered between another mitotic kinase, the Aurora kinase, and Set1
mediated methylation in budding yeast. In this case, temperature sensitive
mutations in the budding yeast Aurora kinase, Ipl1, can be suppressed by
deletion of Set1 complex subunits (Zhang, Lin et al. 2005). Though this suppression has been proposed to be due to the absence of Set1 complex mediated methylation on Dam1, the inner kinetochore protein, the mutation of H3K4 to a non methylatable residue also suppresses the growth defects of the ts lpl1 allele to a reasonable extent (Zhang, Lin et al. 2005). This points to an unexplored functional relationship between methylation at H3K4 by the Set1 complex and Aurora kinase mediated phosphorylation. The Aurora kinase has a well-documented role in regulating kinetochore-Mt attachment via the phosphorylation of kinetochore proteins (Lampson and Cheeseman 2011). Therefore, these data taken together with our results, indicates that a complex interplay between phosphorylation of kinetochore proteins mediated by NIMA and Aurora kinase and Set1 mediated H3K4 methylation functions to regulate kinetochore function.

Interestingly, the Set1 complex has been implicated in regulating gene expression in the context of mitotic exit, in different model systems. An intriguing observation was made in mammalian cells regarding the presence of MLL complex (one of the human Set1 complex orthologs) during mitosis on genes that are not active during mitosis, but are activated soon after mitosis is completed. Based on these data, it has been suggested that MLL complexes likely act as ‘mitotic bookmarks’ facilitating the return of the mitotic cell to an interphase state by marking the genes that need to be turned on at mitotic exit (Blobel, Kadauke et al. 2009). H3K4 methylation is retained on mitotic chromatin in S. pombe,
*Dictyostelium* and human cells (Noma and Grewal 2002, Muramoto, Muller et al. 2010). In human cells, a mechanism of mitotic repression of genes that are actively transcribed in interphase has been described. During mitosis, a nucleosome that borders the transcription start site, slides upstream such that it now covers the transcription start site preventing the binding of transcription factors and thus turning off gene expression specifically in mitosis (Kelly, Miranda et al. 2010). The histone H3 which is part of this sliding nucleosome is methylated at K4 in mitosis as well as in interphase, supporting the idea that this chromatin mark might guide the return of transcription factors to target-genes at the end of mitosis. In addition, it was shown in budding yeast that H3K4 methylation mediates the inheritance of transcription frequencies (the amount of time a gene is expressed in a fixed amount of time) from the mother cell to daughter cells (Muramoto, Muller et al. 2010). Therefore, another potential explanation for the synergistically enhanced mitotic defects in *nimA7 + ∆An-swd1* could be the requirement of the Set1 complex function to mediate the resetting of gene expression to an interphase like state after mitosis. If this was true then we might expect that the double mutants would do reasonably well in the first mitosis, but subsequent mitoses might be affected by the inability to resume normal gene expression after one round of mitosis. In agreement with this expectation, we find that the time taken by *nimA7 + ∆An-swd1* cells to complete the first mitosis is similar to the single mutants during the first nuclear division; however, an increased mitotic arrest is seen in subsequent mitoses.
In *S. cerevisiae*, the Set1 complex subunit, Spp1, has been shown to be required specifically for trimethylation of H3K4 while being dispensable for dimethylation (Dehe, Dichtl et al. 2006). Therefore, using the deletion of Spp1 ortholog in *A. nidulans* (AN2850), we could potentially address the question of whether the synthetic lethality of cells lacking all H3K4 methylation and partial mitotic kinase function, is due to loss of trimethylation of H3K4 specifically. The H3 methylation found on *Drosophila* and human centromeres is a dimethyl mark, whereas trimethylated H3K4 is typically associated with gene expression (Sullivan and Karpen 2004). If the deletion of An-Spp1 in combination with reduced mitotic kinase function did not phenocopy the lethality of cells lacking the Set1 complex along with reduced mitotic kinase function, it would suggest that the synthetic lethality is likely not due to the loss of H3K4 trimethylation.

4.3.3 The Set1 complex and septation

In *A. nidulans*, nuclear division and septation are jointly regulated such that the first septum is laid down typically after 3 rounds of mitoses (as described in Section 1.1.1). Hence, though cells lacking NIMA function (*nimA7* at 42°C) grow to a size similar to Wt cells that have 4-8 nuclei, the absence of nuclear division prevents septation. Interestingly, we observe that cells lacking NIMA function in combination with the absence of Set1 complex function (∆An-swd1+nimA7 at 42°C) can induce the formation of septa. Since these cells exhibit a growth defect more pronounced than cells lacking NIMA only, and are
uninucleate, this was quite unexpected. These data suggest that the absence of the Set1 complex obviates the need for nuclear division and growth to promote septation, indicating that the Set1 complex possibly acts as a negative regulator of septation. This is also important in the light of the recent localization data from the lab showing NIMA locates to forming and formed septa (see also, Section 3.2.1.19). The fact that we can obtain septa that can be stained by calcofluor and are visible by DIC in the absence of NIMA function suggests that NIMA is not absolutely required for the formation of septa. Nevertheless, it is possible that NIMA is involved in normal septum formation; however the absence of Set1 complex function is able to bypass that requirement resulting in septa that are potentially not completely normal.

One possibility is that the absence of the Set1 complex may affect the levels of proteins that regulate septum formation. Alternatively, the Set1 complex may be directly involved in septum formation possibly by methylating and regulating the function of proteins involved in septum formation. A line of evidence that supports the latter hypothesis is the finding that one of the subunits of the Set1 complex, Spa5 (the ortholog of budding yeast Shg1) locates to septa in the filamentous fungus, *Neurospora crassa* (Lai, Koh et al. 2012). Though we have not seen SWD1 (or SHG1) locate to the septa in *A. nidulans* so far, there is a possibility that this location might be developmentally regulated and that observation of cells at the right developmental stage might reveal a potential septal location of SWD1/SHG1. On the other hand, it is possible that the Set1
complex locates to the septum at levels that are not detectable by microscopy in A. nidulans. It is worth pointing out that orthologs of Spa5/Shg1 have not been identified outside of fungi (Shilatifard 2012). Therefore we speculate that Shg1 could confer a fungal specific role on the Set1 complex in regulating septation.
Figure 4.1: Deletion of An-swd1 confers cold sensitivity. Colony growth of \( \Delta \text{An-swd1} \) (MG41) strains from single conidia at the indicated temperatures on media with or without sucrose, in comparison to Wt (R153) strains.
Figure 4.2: The function of the Set1 complex is essential for cells that have partially functional mitotic kinases, Cdk1 and NIMA. (A) and (B) Colony growth of strains of the indicated genotype at the semi permissive temperature of nimA7 after 96 hours. (C) and (D) Colony growth of strains of the indicated genotype at the semi permissive temperature of nimT23 after 96 hours. The inset shows 10X magnified images of cells on the plate. Strains: Wt=R153, nimA7=MG71, nimT23=MG99, ΔAn-swd1=MG41, ΔAn-swd1+ nimA7=MG65, ΔAn-swd1+ nimT23=MG104; ΔAn-set1=MG177, ΔAn-set1+ nimA7=MG179, ΔAn-set1+ nimT23=MG181.
Figure 4.2
Figure 4.3: Mutation of lysine 4 of histone H3 is lethal when NIMA or Cdk1 function is reduced. Colony growth of the strains of the indicated genotype at the semi permissive temperature for nimA7 or nimT23 as shown after 96 hours.

Strains: Wt=R153, nimA7=MG71, nimT23=MG99, H3K4R=MG316, H3K4R+nimA7=MG318, H3K4R+nimT23=MG267
Figure 4.4: Lack of the methylatable lysine 4 and phosphorylatable serine 10 in histone H3 results in poor growth at elevated temperatures. Colony growth of the strains carrying either Wt H3, H3K4R, HS10A or the double mutant at different temperatures as shown after 96 hours. Wt=R153, H3K4R=MG316, H3S10A=MG327, H3K4R+S10A=MG320
Figure 4.5: The G2-M transition in cells having partial Cdk1 function is dependent on the Set1 complex. (A) Representative images of cells of the indicated genotypes carrying H1-chRFP and tub-GFP grown at the semi permissive temperature for nimT23 (37.5°C) and fixed for microscopic analysis. (B) Categorization of cells of different genotypes based on the number of nuclei. (C) Quantitation of average cell length of cells of each genotype as indicated. (D) Calculation of the number of nuclei in 20µm of cell length in Wt cells compared to the single mutants and the double mutant. Strains: Wt=HA365, nimT23 = HA375, ΔAn-swd1=MG160, nimT23+ΔAn-swd1 = MG161
Figure 4.5
Figure 4.6: Cells with partial Cdk1 activity complete mitosis successfully. Mitosis in cells of Wt (MG300) genotype (A) and carrying nimT23 mutant allele (MG304) (B) was followed by live cell imaging using tub-GFP as a marker for the mitotic spindle and H1-chRFP as a marker for chromatin at 37.5°C, the semi permissive temperature for nimT23. (C) Mitotic nuclear pore complex disassembly in nimT23 cells as followed by the localization of nup49-CR and tub-GFP through time (CDS580).
Figure 4.6
**Figure 4.7:** Cells lacking *An-swd1* in addition to having partial Cdk1 exhibit a pronounced delay in initiating the first mitosis. (A) Mitosis in a representative ∆An-swd1 cell (MG276) resulting in two daughter nuclei. (B) Mitosis in a representative cell lacking *An-swd1* in addition to having partial Cdk1 function (MG302) is successful in generating two daughter nuclei. Arrowhead indicates the spindle that continues to be visible in telophase. (C) Cell length at the time of entry into the first mitosis in the indicated genotypes. (D) Time in mitosis as measured using tub-GFP and H1-chRFP as markers for mitosis. Bars, 5µm.
**Figure 4.7**

**A**

**ΔAn-swd1, 37.5°C**

<table>
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<tr>
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<th>H1-chRFP</th>
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</table>

**B**

**nimT23+ΔAn-swd1, 37.5°C**

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<th>H1-chRFP</th>
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**C**

Cell length at entry into 1st mitosis at 37.5°C (μm)

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<th>ΔAn-swd1</th>
<th>nimT23+ΔAn-swd1</th>
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<td>4</td>
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</table>

**D**

Time in mitosis (mins)

<table>
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<tr>
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<th>nimT23</th>
<th>ΔAn-swd1</th>
<th>nimT23+ΔAn-swd1</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>5</td>
<td>4</td>
<td>10</td>
</tr>
</tbody>
</table>
Figure 4.8: The G2-M arrest in cells lacking An-swd1 in addition to having partial Cdk1 can be quickly reversed by lowering the temperature. (A, B) The time taken for cells of the indicated genotype, germinated at 37.5°C and downshifted to 22°C, to form a spindle (marked by tub-GFP) is represented as a histogram. Strains: nimT23 = MG304, nimT23+ ΔAn-swd1 = MG302. (C) Colony growth of nimT23 or ΔAn-swd1 strains at the semi permissive temperature (37.5°C) after 72 hours in the presence or absence of DNA damaging agents (0.015% DEO, 0.025% MMS). WT=R153, scaA1 = JLA3, nimT23 = SO53, ΔAn-swd1 = MG218.
Figure 4.8

A and B: Histograms showing the distribution of time taken to enter M phase after downshift from 37.5°C for two conditions: nimT23 (n=23) and nimT23+ΔAn-swd1 (n=16).

C: Photographs of cell growth on agar plates with and without MMS and DEO treatments. The plate shows WT and ΔAn-swd1 strains on YAGUU medium.
Figure 4.9: Synthetic growth defects in cells that lack Set1 complex function in addition to partial NIMA function. (A) Representative images of cells of the indicated genotypes carrying H1-chRFP and tub-GFP grown at the semi permissive temperature for nimA7 (35°C) and fixed for microscopic analysis. (B) Categorization of cells of different genotypes based on the number of nuclei. (C) Quantitation of average cell length of cells of each genotype as indicated. (D) Calculation of the number of nuclei in 20µm of cell length. Strains: Wt=HA365, nimA7=MG153, ΔAn-swd1=MG160, nimA7+ΔAn-swd1 = MG159.
Figure 4.9
**Figure 4.10:** The absence of Set1 complex function in combination with partial NIMA function results in enhanced mitotic delays. (A) The time spent in the first mitosis by each of the mutant strains is indicated compared to the Wt strain. (B) The time spent in the second mitosis by each of the mutant strains is indicated compared to the Wt strain. (C and D) Variability in the time spent in mitosis within the cell population in each of the indicated strains is represented by a box and whiskers plot for the first mitosis (C) and subsequent mitosis (D). Spindle formation was used as a measure of determining time in mitosis. Strains used to compute the average time in mitosis: Wt – MG224, *nimA7* – MG190, MG227, and MG229, ΔAn-swd1 – MG244, *nimA7+ΔAn-swd1* – MG213, MG243.
Figure 4.10

A

First mitosis

Time in mitosis (mins)

WT nimA7 △An-swd1 nimA7+△An-swd1

n=13 n=21 n=8 n=21

4 mins

B

2nd mitosis

Time in mitosis (mins)

WT nimA7 △An-swd1 nimA7+△An-swd1

n=2 n=14 n=9 n=37

8 mins

C

First mitosis

Time in mitosis (mins)

WT nimA7 △An-swd1 nimA7+△An-swd1

~3 mins

D

2nd mitosis

Time in mitosis (mins)

WT nimA7 △An-swd1 nimA7+△An-swd1

~9 mins
**Figure 4.11:** Absence of the Set1 complex makes cells with partial NIMA function dependent on the spindle assembly checkpoint (SAC). Colony growth of strains of the indicated genotypes grown at the permissive temperature (32°C) for the *ts* mutant alleles, *nimA7* and *nimT23*, for 96 hours. Strains: Wt = R153, *nimA7* = CDS790, ΔAn-swd1 = MG218, *nimA7*+ΔAn-swd1 = MG215, ΔAn-mad2 = CDS629, ΔAn-swd1+ΔAn-mad2 = MG383, *nimA7*+ΔAn-mad2 = MG381, *nimT23*+ΔAn-mad2 = MG405, *nimA7*+ΔAn-swd1+ΔAn-mad2 = MG384, *nimT23*+ΔAn-swd1 = MG219, *nimT23* = SO53, *nimT23*+ΔAn-swd1+ΔAn-mad2 = MG402.
Figure 4.11
**Figure 4.12**: Lack of the Set1 complex promotes septation in the absence of NIMA function (A) Cells of Wt, \textit{nimA7}, \textit{ΔAn-swd1} and \textit{nimA7+ΔAn-swd1} genotypes were grown at the fully restrictive temperature for \textit{nimA7}, 42°C. DAPI and calcofluor staining reveal nuclei and septa respectively. Septa in the double mutant are indicated by arrows. Strains: Wt=HA365, \textit{nimA7}=MG153, \textit{ΔAn-swd1}=MG160, \textit{nimA7+ΔAn-swd1} = MG159 (B) Live cell imaging of a \textit{nimA7+ΔAn-swd1} cell (MG243) shows that septa can be misplaced resulting in a cutting of nuclei. Open arrowhead indicates the link between the two nuclei. The red arrow indicates the presence of the septum as seen by brightfield microscopy.
Figure 4.12

A

32°C  42°C

WT

nimA7

ΔAn-swd1

nimA7+ ΔAn-swd1

B

nimA7 + ΔAn-swd1, 35°C
Figure 4.13: The septal location, but not the mitotic location, of NIMA is altered in the absence of the Set1 complex function. (A) The dynamic mitotic location of NIMA to the G2 spindle pole body, the nuclear periphery, the nucleus and G1 SPBs is unaffected in An-swd1-deleted cells (MG173). (B) 5% of ∆An-swd1 cells show mislocalization of NIMA at formed septa (arrow) while the rest of the cells show typical Wt-like localization (left septum).
Figure 4.13

A

NIMA-GFP; An-swd1
35°C, Mitosis

B

NIMA-GFP; An-swd1
35°C

Figure 4.13
Figure 4.14: SWD1 locates to the nucleus and the spindle pole body/kinetochore region in interphase. (A) Montage of images showing the location of SWD1-GFP and the chromatin marker, H1-chRFP through mitosis (MG107). (B and C) The SWD1-GFP focus locates at the spindle pole body (B, MG140) and the kinetochore (C, MG146) region. Bars, 5µm.
Figure 4.14
**Figure 4.15:** SWD1-GFP disperses later than NLS-DsRed upon entry into mitosis. (A) Cells carrying SWD1-GFP and NLS-DsRed (MG139) were followed through mitosis. (B) Pixel intensity profiles were plotted across the dotted line indicated in (A) at the different time points as indicated.
Figure 4.15
Figure 4.16: The location of SWD1-GFP is not altered when the function of CDK1 or NIMA is reduced. (A) The localization of SWD1-GFP during Wt mitosis (MG132) at 37.5°C. (B) SWD1-GFP in cells also carrying the nimT23 allele (MG165) followed through mitosis at 37.5°C. (C) Behavior of SWD1-GFP in nimA7 with respect to that of NLS-DsRed at the semi permissive temperature of 35°C (MG411).
Figure 4.16
Chapter 5: The NIMA kinase is required for the successful completion of multiple mitotic events post G2-M transition

5.1 Introduction

5.1.1 NIMA and Cdk1 are both required for mitotic initiation

In *A. nidulans*, in addition to the conserved mitotic kinase Cdk1, the function of NIMA is required for mitotic entry (Osmani and Ye 1996). Cells in which the ts nimA allele is inactivated by incubation at the restrictive temperature arrest with a single G2 nucleus, with duplicated spindle pole bodies (SPB) and cytoplasmic microtubule (Mt) architecture (Osmani, May et al. 1987). When restored to the permissive temperature, these cells immediately enter mitosis, indicating that mitotic initiation is dependent on NIMA being active. Likewise, the inactivation of Cdk1 using a temperature sensitive allele of its activating phosphatase, nimTcdc25, also results in a reversible G2 arrest (Osmani, McGuire et al. 1991, O'Connell, Osmani et al. 1992). In addition, both NIMA and Cdk1 are targets of the checkpoint over mitosis that monitors replication stress. Given the essential roles for both Cdk1 and NIMA in mitotic initiation, the decrease in function of either of these genes would be expected to result in a prolonged G2 phase, which would allow the cell to accumulate enough Cdk1 or NIMA activity to
affect a successful mitosis. In the case of partial inactivation of mitotic Cdk1 (imposed by the incubation of cells carrying a ts allele of nimT at the semi permissive temperature), we see that this prediction is correct (Chapter 4, Figures 4.5, 4.6). There is a delay in G2, but the mitosis that follows is successful and does not have any obvious errors. This suggests that when the activation of Cdk1 is slowed by partial inactivation of its activator, cdc25, mitosis is triggered only after enough Cdk1 activity that is required for completing mitosis is accumulated. In contrast, fixed cell microscopic analysis of cells with reduced NIMA function grown at the semi permissive temperature revealed abnormal chromatin segregation suggesting that mitosis might be triggered erroneously when NIMA function is inadequate (Figure 4.7). We undertook live cell imaging experiments to try and determine the mitotic defects in cells with reduced NIMA function. Insights obtained from these studies will be described in this chapter.

5.1.2 The NIMA kinase and mitotic progression

Since NIMA function is essential for all aspects for mitosis, it has been difficult to assess whether NIMA is required for specific mitotic events subsequent to the start of mitosis. Some insights into potential functions for NIMA during mitotic progression were obtained from the analysis of cells that are mutated for the gene encoding the anaphase promoting complex (APC) subunit, BIME in addition to carrying a nimA5 mutant allele (nimA5+bimE7). While nimA5 cells exhibit a G2 arrest at the restrictive temperature, the absence of bimE...
function gives an arrest in mitosis reflective of the functions of BIME in mitotic
exit. However the absence of bimE function in combination with the nimA5 allele
abrogates the G2-M checkpoint and promotes pre-mature mitotic entry at the
restrictive temperature in the absence of normal NIMA function (Osmani,
O'Donnell et al. 1991). Therefore the nimA5+bimE7 double mutant cells undergo
an aberrant mitosis, and exhibit defects that are most likely due to the absence of
normal NIMA function. Mitotic nimA5+bimE7 cells show abnormal spindle
formation and nuclear envelope invaginations never seen in Wt cells suggesting
a role for NIMA in regulating spindle formation and mitotic NE segregation. Live
cell confocal microscopy of endogenously GFP tagged NIMA revealed that it has
a dynamic location during mitosis (De Souza, Osmani et al. 2000) (Shen et al.
unpublished). NIMA localizes to the spindle pole body at the beginning of mitosis,
then moves around the nuclear periphery and then is seen in the nucleus and on
the spindle in metaphase before moving back to the SPBs at mitotic exit and
remaining at the spindle poles until early G1. This strongly suggests that NIMA
has location-specific functions throughout mitosis. Consistent with this prediction,
our studies using cells with partial NIMA function presented here provide
convincing evidence for NIMA being required to complete multiple distinct mitotic
events successfully.
5.2 Results

Microscopic analysis of fixed cells with partial NIMA function revealed a highly penetrant chromatin mis-segregation phenotype suggesting that there are likely defects in mitotic progression in these cells (Chapter 4, Figure 4.8). To investigate this further, we decided to follow \textit{nimA7} cells through mitosis by live cell spinning disk confocal microscopy, at the same semi permissive temperature at which the fixed cell analysis was performed, in comparison to Wt cells using a heated stage and objective. This enabled us to investigate the potential functions of NIMA during mitosis after mitosis had been initiated. We examined the first mitosis in conidia to identify the earliest defects thus avoiding the possibility of looking at cumulative effects of previous mitotic failures. These studies revealed that around 42% of \textit{nimA7} cells at the semi permissive temperature fail to complete the first mitosis successfully resulting in a single diploid nucleus. The remaining portion of the \textit{nimA7} cell population undergoes nuclear division yielding two nuclei, however our data suggest that the mitosis in these cells is defective (Section 5.2.9). The cells that remain uninucleated after the first mitosis display three temporally separated defects in distinct mitotic processes – an inability to form a bipolar spindle (11% of the total cell population), a defect in chromatin segregation during anaphase (16% of total cell population) and a failure in restricting the nuclear envelope during telophase (15% of total cell population) (Table 5.1).
5.2.1 Normal NIMA function is required for the establishment of a bipolar spindle after the initiation of mitosis

To follow mitotic progression, we generated nimA7 strains that carried tub-GFP to monitor spindle formation and the kinetochore protein, Ndc80, tagged in red to follow chromosome segregation. In A. nidulans, during interphase, the kinetochores are clustered at the spindle pole body (SPB) on the nuclear periphery. In nimA7 cells grown at RT (22°C), kinetochores as marked by Ndc80 are seen in a single focus in interphase at the first time point in Figure 5.1A. Upon spindle formation, the Ndc80 signal spreads along the spindle at metaphase indicating the release of the kinetochores from the SPBs. Subsequently, in anaphase when the kinetochores segregate, the Ndc80 signal is seen to divide into two foci (Figure 5.1A, time point 10'). When NIMA function is partially reduced by growing the same strain at 35°C, we find that 11% of the cells show a clear failure to segregate kinetochores (Figure 5.1B) and form a monopolar spindle. In A. nidulans, spindle microtubules are initially formed from duplicated, unseparated SPBs at mitotic entry. This is followed by the disjunction of the duplicated spindle pole bodies resulting in the bipolarization of the spindle. Therefore, some spindles in nimA7 cells appear to be arrested at the monopolar stage potentially because of an inability to separate the duplicated SPBs at the beginning of mitosis. The spindle continues to stay monopolar through the entire length of mitosis and then mitotic exit occurs as seen by the disassembly of spindle microtubules (Figure 5.1B). We do not see the segregation of the Ndc80
focus into two foci at any time point in such cells. We have also confirmed the monopolarity of the spindle in nimA7 cells using tub-GFP and a SPB marker, Sad1, at the semi permissive temperature (Figure 5.1C). Here again, as exemplified by the cell in Figure 5.1C, the focus representing duplicated SPBs is not seen to separate at any point, revealing that the spindle is monopolar. These data indicate that in nimA7 cells with partial NIMA function, there is a defect in separating the duplicated SPBs to form a bipolar spindle.

The monopolar spindle phenotype seen in nimA7 cells is remarkably similar to the phenotype of cells mutated for the bimC kinesin. The function of BIMC is essential to mediate lateral interactions between microtubules nucleated from unseparated duplicated SPBs in prophase, which then facilitates the separation of the two SPBs away from each other resulting in a bipolar spindle at metaphase. Therefore, we considered the possibility that BIMC might be misregulated in nimA7 cells. To test whether the localization of BIMC is affected in these cells, we generated WT and nimA7 cells that also carried BIMC tagged in green and histone H1 tagged in red. In interphase, BIMC locates in the nucleus (Figure 5.1D). When mitosis is initiated in Wt cells, BIMC relocates to the SPB. At anaphase it locates along the spindle and then goes back to the SPB during anaphase-telophase. Upon following the localization of BIMC in nimA7 cells by live cell imaging, we find that in 14% (n=14) cells followed, BIMC locates to a single spot in the nucleus upon mitotic entry and did not show a transition to the spindle. It is highly likely that these cells have a monopolar spindle due to the
fact that they exit mitosis without attempting to do anaphase (as seen by H1-chRFP). Therefore, our data suggests that BIMC can locate to the SPB in cells that fail mitosis without reaching anaphase. However, we note that the BIMC signal at mitotic SPB of nimA7 cells seems higher compared to Wt cells. In WT cells, there is a possibility that some of the BIMC protein disperses while some of it locates to the SPB. It is possible that in nimA7 cells, the defect in partial NPC disassembly (described in Section 5.2.3) prevents the dispersal of BIMC and therefore all of the protein locates to the SPB. Alternatively, this increase in BIMC signal could be a direct effect of reduced NIMA function. In our analysis, we also noted one instance of mitosis in nimA7 cells where all BIMC signal dispersed from the nucleus, then located to the SPB briefly before finally returning to interphase localization; such behavior of BIMC during mitosis is not typical of Wt cells. Therefore, reduced NIMA function likely affects BIMC function during mitosis and this can be further confirmed by visualization of BIMC with respect to monopolar spindle formation (using tub-GFP) in nimA7 cells.

### 5.2.2 Cells with reduced NIMA function show defects in anaphase completion

During our live cell microscopic analysis of Ndc80-CR and tub-GFP in nimA7 cells, we noticed examples of nimA7 cells where upon spindle formation, the kinetochores initially stayed in a single focus (Figure 5.2A, open arrowheads), but then later show an attempt to segregate giving rise to two foci. At time point
20’, when there are two Ndc80 foci, two tub-GFP foci are also visible, denoting tubulin polymerization from the two separated SPBs (Figure 5.2A, filled arrowheads). This suggests that duplicated SPBs have separated to form a bipolar spindle. Surprisingly though, the segregation of Ndc80 foci is not completed, and the kinetochores collapse back to a single focus at the next time point (Figure 5.2A, open arrowhead), suggesting a defect in completing anaphase. Since the presence of two tubulin foci suggests that the SPBs were separated at time point 20’, we were interested in following the dynamics of SPBs directly by examining the SPB marker protein GCP3. In nimA7 cells that are double tagged for GCP3 and Ndc80, we noted instances where, as shown in Figure 5.2B, the SPBs divided into two distinct foci (blue arrows), indicating successful disjunction of the two SPBs. However, the two SPBs seem to be delayed at this stage for a period of time. Previous studies have shown that in Wt cells, upon SPB disjunction, kinetochores lie between the two separated SPBs and then get segregated into two equal foci that colocalize with either SPBs. This completes the separation of the kinetochores during anaphase (De Souza, Hashmi et al. 2009). In nimA7 cells, on the other hand, when the SPBs separate, the kinetochores unexpectedly are seen to stay at one of the SPBs (white arrowhead). At 3’, the kinetochores display a location more typical of metaphase, lying in between the two SPBs. Subsequently, as seen in time point 4’, the kinetochores appear to segregate to two foci (though possibly unequally), however, this separation is not sustained and Ndc80 collapses back to a single
focus that colocalizes with only one of the SPBs (yellow arrow, time point 5’). Remarkably, the failure to segregate the kinetochores is followed by the separated SPBs fusing back to give a single GCP3 focus (blue arrow at time point 9’). Finally the nucleus exits mitosis with a single GCP3/Ndc80 focus.

Overall, these data reveal that some nimA7 cells have defects in kinetochore segregation. It has been our experience that it is difficult to detect the phenotype wherein, the SPBs separate but fail to remain separated and thus come back together, possibly due to the fact the orientation that the nucleus needs to be in order to aid the visualization of this phenomenon is limited. However, we have successfully confirmed this phenotype with multiple nimA7 strains carrying other marker proteins that locate to the SPB, such as Sad1 and tinA (Figure 4.8C, data not shown). In all the mitoses (n=50) that were followed in nimA7 cells where the dynamics of the SPB could be followed through mitosis, we detected this phenomenon in 12% of the nimA7 mitoses.

To further confirm this anaphase defect, we followed a chromatin marker, histone H1-chRFP with tub-GFP in WT cells compared to nimA7 cells at the same semi permissive temperature. In a typical Wt cell, chromatin condensation occurs along with spindle formation during mitotic entry. During anaphase, spindle elongation separates the chromatin into two and subsequent DNA decondensation occurs during G1 with the formation of two daughter nuclei (Figure 5.2C). In 31% of the nimA7 cells at the same temperature, we find that a bipolar spindle is established (as can be seen by two tubulin foci at the separated
SPBs; Figure 5.2D, arrows) and the spindle elongates, however the spindle collapses before anaphase is accomplished. As shown by H1-chRFP dynamics, chromatin condenses and then tries to segregate (Figure 5.2D, arrowhead), however segregation fails and chromatin collapses back to a single nucleus. This suggests that a proportion of cells with partial NIMA function are defective in a process downstream of SPB separation that is required for successful anaphase/telophase completion.

5.2.3 Cells with reduced NIMA function initiate mitosis but are unable to undergo partial nuclear pore complex disassembly

The nuclear pore complex (NPC) proteins (or Nups) in A. nidulans can be divided into two categories based on their behavior in mitosis – peripheral Nups that disperse during mitosis and core Nups that stay at the nuclear periphery during mitosis. Previous studies have shown that NIMA inactivation prevents initiation of mitosis and therefore prevents NPC disassembly while ectopic expression of NIMA can promote partial nuclear pore complex disassembly, even out of cell cycle phase (De Souza, Osmani et al. 2004). Therefore, the current model for G2-M transition is that NIMA initiates mitosis at least in part by promoting NPC disassembly, which allows tubulin and mitotic regulators to access the nucleoplasm. We hypothesized that the mitotic failure in 42% of the cells with partial NIMA function might be due to a defect in disassembling the NPCs. To test this, we generated strains having WT nimA or the nimA7 mutant
allele in which the location of peripheral Nups can be followed through mitosis by observing Nup49-CR in addition to tub-GFP. Nup49 is a peripheral nucleoporin that locates around the nuclear periphery at NPCs during interphase and then disperses into the cytoplasm at mitosis (De Souza, Osmani et al. 2004). We find that in cells with reduced NIMA function, the majority of Nup49-CR signal continues to remain at the nuclear periphery even as the cell enters mitosis and forms a mitotic spindle (Figure 5.3A). This dramatic defect indicates that in addition to being sufficient to promote NPC disassembly, NIMA function is also required for normal NPC disassembly. This suggests that the failure to disassemble NPCs normally might contribute to the mitotic defects in cells with reduced NIMA function. This defect in Nup49 dispersal suggests that the abrogation of nuclear transport seen upon entry into mitosis in Wt cells may be defective in nimA7 cells. To test this, we generated Wt and nimA7 strains that would enable the visualization of nuclear transport (by following NLS-DsRed, a marker protein that is actively transported into interphase nuclei) along with spindle formation by following GFP-tagged tubA. In Wt cells, during interphase, NLS-DsRed is exclusively nuclear (Figure 5.3D). During mitosis, partial NPC disassembly results in the dispersal of NLS-DsRed until active transport is re-established in daughter nuclei. When NIMA function is reduced by growing nimA7 cells at the semi permissive temperature, we find that NLS-DsRed does not disperse upon mitotic entry, as marked by formation of the mitotic spindle, but continues to remain nuclear (Figure 5.3E). However at anaphase, when the
spindle elongates, NLS-DsRed briefly disperses but is immediately imported back into the nucleus that has not undergone mitotic segregation. Although the cell shown in Figure 5.3E clearly attempts mitosis it undergoes mitotic failure resulting in a single nucleus being generated after mitosis.

During mitotic nuclear pore complex disassembly, the Nup2 nucleoporin displays a unique translocation from the NPCs to chromatin, unlike other peripheral Nups that disperse into the cytoplasm (Osmani, Davies et al. 2006). Studies in the lab have shown that NIMA can promote the transition of Nup2 from the NPCs to chromatin even out of cell cycle phase (Markossian et al. unpublished). However it is not known whether NIMA is required to promote the transition of Nup2 to mitotic chromatin once mitosis has been initiated. Since our data shows that normal function of mitotic NIMA is required for the dispersal of the peripheral nucleoporin, Nup49, we thought it possible that NIMA might also be required to promote the transition of Nup2 to mitotic chromatin. To test this, we generated strains to visualize Nup2 and a chromatin marker in nimA7 mutant strains. In Wt cells, Nup2 undergoes an abrupt transition from the NPCs to chromatin at mitotic entry. Preliminary analysis of nimA7 strains carrying Nup2 at the semi permissive temperature suggests that the transition of Nup2 from the pore to the chromatin is not abrupt, but more gradual in 66% cells (n=6). As shown in Figure 5.3C, Nup2 seems to transition from a distinct ring-like nuclear pore signal around nuclei, to a more central signal that does not show the ring at
the nuclear periphery (Figure 5.3C, arrow). Subsequently we see Nup2 locating to chromatin and in the cell shown in Figure 5.3C, the nucleus fails to divide and Nup2 returns to a ring like nuclear periphery signal after exit from mitosis. These data suggest that complete NIMA function is required to promote a quick transition of Nup2 from the NPCs to the chromatin upon mitotic initiation, which is consistent with the requirement for NIMA in the dispersal of peripheral Nups (Figure 5.3A). Alternatively, it is possible that chromatin condensation is a pre-requisite for Nup2 to translocate to chromatin. In such a scenario, defects in chromatin condensation in nimA7 cells might have an indirect effect on the behavior of Nup2 during mitosis.

One of the major proposed roles of NPC disassembly is to allow the entry of tubulin into the nucleus so that spindle formation can take place. Our data so far suggest that in mitotic nimA7 cells, partial NPC disassembly is defective and so is the abolishment of active transport. However, since spindle-like structures are able to form in these cells we were interested to test whether tubulin is able to enter these nuclei during entry into mitosis. To this end, we observed single confocal sections of the tubulin signal through the middle of nimA7 cells and quantitated the change in tubulin signal inside the nucleus during the G2-M transition in Wt cells and in nimA7 cells (Figure 5.4A). In Wt interphase nuclei, unpolymerized fluorescently-tagged tubulin is excluded from the nucleus revealing a nuclear shadow (Figure 5.4A, arrow), and therefore the % signal intensity of tubulin (with the pixel intensity inside the nucleus at G2-M being
normalized to 100%) within the nucleus at this time point is low (Figure 5.4B). Upon mitotic entry, this mitotic shadow fills in as tubulin equilibrates across the nuclear envelope, as shown by the increase in the tubulin signal. This occurs concurrently with the dispersal of NLS-DsRed from the nucleus (Figure 5.4A). Therefore, in Wt cells, upon mitotic entry, we see a drop in the nuclear NLS signal accompanying the increase in nuclear tubulin signal (Figure 5.4B).

Interestingly, when we perform a similar quantitation for nimA7 cells, we find that tub-GFP increases sharply at G2-M, however the nuclear signal of NLS-DsRed does not decrease precipitously as it does in the Wt cells (Figure 5.4A, C). This shows that in cells with reduced NIMA function, even though NLS-DsRed is unable to escape from the nucleus in mitosis, tubulin is able to access the nucleoplasm.

One possibility is that in the absence of normal NIMA function, the nuclear pores are not being disassembled to the extent that occurs with full NIMA activity. Therefore, tubulin a 50KD protein is able to diffuse through the incompletely opened nuclear pores; however NLS-DsRed which forms a tetrameric complex of higher molecular weight is retained in the nucleus. Therefore the different behaviors of tubulin and NLS-DsRed in mitotic nimA7 cells may just be an effect of their different sizes. To test this hypothesis, we examined the behavior of two other proteins, one that is similar in size to tubulin and the other that is expected to form a large complex and so would have a bigger effective size.

Heterochromatin protein 1, HP1, localizes in the nucleus and at the spindle pole
body during interphase and disperses during mitosis. Consistent with our prediction, HP1 does indeed disperse concurrent with spindle formation as tubulin enters the nucleus (Figure 5.3B). Therefore, although the large NLS-DsRed tetramer complex cannot escape nuclei the smaller HP1 protein can. This indicates that the entry of tubulin into the mitotic nimA7 nucleus is not due to active nuclear transport but is likely due to the opening of the mitotic NPCs by the partially functional NIMA7 just enough so that smaller proteins like tubulin and HP1 can freely diffuse across the nuclear envelope. Secondly, we also followed the location of SWD1, which is expected to be part of the large molecular weight Set1 complex. As shown in the previous chapter, SWD1 behaves very similar to NLS-DsRed in mitotic nimA7 cells, dispersing only briefly at a time point corresponding to spindle elongation (Figure 4.17C). This further confirms that the disparity between the behavior of NLS-DsRed and tubulin is due to their respective sizes. Collectively these data indicate that when NIMA is fully active, then active transport is abolished even for bigger proteins during mitosis. However, when only partial NIMA activity is present, the NPC disassembly is incomplete allowing free diffusion across the nuclear envelope for only smaller proteins and not for the larger ones.

Our data suggests that the defect in NPC disassembly contributes to the mitotic failure seen in roughly half of the cells with insufficient NIMA function. One prediction of this hypothesis would be that if the inability of partial NIMA activity to promote complete disassembly of the mitotic NPC causes mitotic defects then it
might be possible to suppress these defects by forcing the disassembly of the NPCs to mimic Wt NPC behavior. Previous studies in our lab have shown that in the absence of two nucleoporins, *nup37* and *elys*, many of the core Nups also disperse, a phenomenon termed extreme disassembly, which allows the faster release of NLS from the nucleus in mitosis (Liu, De Souza et al. 2009).

Therefore, we reasoned it was possible that in the absence of *nup37* and *elys*, NPC disassembly in *nimA7* cells might be restored to a Wt situation. Therefore we generated strains that lack the two nuclear pore complex proteins *nup37* and *elys*, in addition to being *nimA7* and carrying mitotic marker proteins, NLS-DsRed and tub-GFP. We find, as shown in Figure 5.3F, active transport is still largely functional in mitotic *nimA7Δnup37Δelys* cells. However, as can be seen from this example, the signal of NLS reduces slightly upon mitotic entry (Figure 5.3F, time point 2'). Interestingly, we also find that 80% (n=10) of these cells completed the first mitosis to give two nuclei compared to 58% (n=26) of nuclei which divide to two in *nimA7* cells. These preliminary data suggest that though, as followed by NLS dispersal, the mitotic NPC disassembly in *nimA7Δnup37Δelys* cells does not resemble Wt cells, the slightly increased mitotic porosity of the nuclear envelope may be just enough to rescue the majority of the mitotic failures in *nimA7* cells.
5.2.4 Complete NIMA function is required for successful nuclear envelope segregation and thus nucleolar segregation

In our analysis of nimA7 cells carrying NLS-Dsred and tub-GFP as mitotic markers, we noticed instances where two distinct daughter nuclei are visible at the end of mitosis, however, the nuclei then revert back to become 1 nucleus (Figure 5.5A). In the example shown in Figure 5.5A, the nucleus as marked by NLS-DsRed does not disperse at mitotic entry marked by spindle formation, but disperses momentarily at spindle elongation. Subsequently, two NLS-DsRed importing daughter nuclei can be seen at time point of 14’. However, they fuse to go back to being 1 nucleus at the end of the movie. We wondered whether these nuclei might be defective in nuclear envelope (NE) segregation, which is required for the separation of the two daughter nuclei in telophase. In A. nidulans Wt nuclei, the nuclear envelope undergoes a double pinch giving rise to 3 membrane compartments transiently (Figure 5.5B, arrowheads). The two NE associated compartments at either end encompass the segregated chromatin of the two daughter nuclei, while the middle compartment contains the nucleolus (Ukil, De Souza et al. 2009). This unique segregation pattern in Wt cells is shown in Figure 5.5B, using the nuclear membrane protein, AN0162, as a marker for mitotic NE dynamics (the double pinch is indicated by arrowheads). Interestingly, when we observe cells with reduced NIMA function using the same marker, AN0162, we find that 15% of cells show a defect in executing the NE double pinch (Figure 5.5C). As can be seen at time point of 14’ in the montage, the
nucleus attempts to segregate the NE, however, it fails to do so (Figure 5.5C, arrows). Moreover, we noticed instances where an intra-nuclear ring of the nuclear envelope was visible (time point 16’ in Figure 5.5C; 71%, n=7). We refer to this NE configuration as the ‘theta’ (θ) structure, owing to its resemblance to the said Greek notation. The NE segregation is coupled to nucleolar segregation such that during the double restriction of the NE, the nucleolus contained in the middle compartment of the NE disassembles and subsequently reassembles into the daughter nuclei. Given this unique mitotic segregation pattern of the nucleolus in *A. nidulans*, we considered the possibility that the θ nuclei might be a result of inaccurate NE and nucleolar segregation such that the intranuclear NE ring encompasses the nucleolus. Indeed, when we followed strains that were generated to carry a red tagged nucleolar marker protein, fibrillarin in addition to AN0162-GFP, we find that the nucleolus is within the intra nuclear NE inclusion in every θ nucleus observed (Figure 5.5D, n=5). In addition we also find at least one fibrillarin focus that is separated from the bulk of the nucleolar fibrillarin protein in 42% (n=7) of the cells that undergo mitotic failure, consistent with an inability to maintain the integrity of the nucleolus. Overall our data provide convincing evidence that the successful generation of daughter nuclei via the NE double pinch and nucleolar segregation requires complete NIMA function.
5.2.5 Insufficient NIMA function causes a delay in mitosis

As described in the previous sections, 42% (n=26) of the nimA7 cells are unsuccessful in generating two nuclei in the first mitosis due to either a failure in establishing a bipolar spindle (section 5.2.1) or defects downstream of spindle bipolarization, due to defective anaphase completion (section 5.2.2) or an inability to segregate the nuclear envelope and nucleolus (section 5.2.4). The remaining half of the nimA7 population completes an apparently successful mitosis to give rise to two nuclei (However, as will be described later, the mitosis in these cells is not completely normal). Using time lapse movies of mitoses, we calculated the average time taken by Wt cells and nimA7 cells to complete mitosis. Spindle formation was used as a marker to determine the start of mitosis and disassembly of the spindle was used to define the end of mitosis. The presence of mitotic defects in nimA7 cells suggested that they might be delayed in mitosis, perhaps in an attempt to fix those errors. Consistent with that expectation, we find that reducing NIMA function more than doubles the time spent in mitosis (Figure 5.6A). We also note that there is wide variability in this delay as indicated by the box and whiskers plot (Figure 5.6B). We wondered whether this variability was dependent on whether or not the nuclei complete mitosis successfully. Therefore we plotted the individual instances of mitotic times on the box and whiskers plot, marking the successful mitoses in green and the unsuccessful ones in red. We find that there is no correlation between the time spent in mitosis and whether the nucleus successfully divided into two or
not. In order to determine whether the delay in mitosis seen in cells with partial NIMA function is specific to this temperature sensitive allele of nimA, we followed mitosis in two other temperature sensitive nimA alleles, nimA1 and nimA5, following spindle formation through mitosis. We find that nimA1 and nimA5 cells also spend more than twice as long as a WT cell in mitosis (Figure 5.6C), demonstrating that the reduction of NIMA function, irrespective of the kind of allele used, results in prolonged mitosis.

5.2.6 Cells with partial NIMA function are dependent on the spindle assembly checkpoint for their survival

The spindle assembly checkpoint (SAC) functions to monitor mitotic errors and imposes a delay in mitosis so that these errors can be corrected before anaphase is triggered. Given that lowering NIMA function results in multiple mitotic defects as well as a delay in mitosis, we hypothesized that the successful completion of mitosis in nimA7 cells might be dependent on the SAC. To test this, we generated strains carrying nimA7 that are also deleted for the gene essential for SAC function, mad2. To examine whether the deletion of mad2 affects the growth of nimA7 cells, we compared the colony growth of nimA7+ Δmad2 with the single mutants at the semi permissive temperature, at which the mitotic defects were observed. We find that the double mutants lacking MAD2 in addition to having partial NIMA function are unable to form any visible colonies at this temperature (Figure 5.7A), indicating the absolute requirement of the SAC.
for the survival of nimA7 cells at this temperature. To determine whether the absence of mad2 worsens the mitotic defects of nimA7 cells, we generated strains carrying NLS-DsRed and tub-GFP that are also double mutants, carrying nimA7+Δmad2. When these cells were followed through mitosis, we observed that they show a striking inability to complete mitosis successfully, with more than 90% of the double mutant cells failing to divide their nucleus in the first mitosis (Figure 5.7C). These data show that the occurrences of first mitoses that result in two nuclei in nimA7 cells are completely dependent on the SAC. In these cells, though tubulin equilibrates across the nuclear envelope (NE) and the spindle forms upon mitotic entry, NLS-DsRed does not disperse (Figure 5.7B). Moreover, in the absence of the mitotic delay imposed by the mad2-mediated checkpoint, tubulin exits the nucleus within an average of 5 mins after being equilibrated across NE at mitotic entry, whereas tubulin remains equilibrated across NE for as long as 12 mins on average in nimA7 cells (Figure 5.7D). This indicates that the delay in mitosis in cells with partial NIMA function is mediated by the SAC. However surprisingly, we see that even though tubulin is exported from the nucleus giving rise to the nuclear shadow (Figure 5.7B, yellow arrow), spindle like bundled microtubules continue to extend from the SPB for a period of time (Figure 5.7B). When we quantitate the time period that the mitotic spindle as well as the cytoplasmic bundled microtubules are seen in nimA7+Δmad2, we find that it is comparable to the time that nimA7 cells spend in mitosis (Figure 5.7D). This suggests that even though the absence of mad2 allows tubulin to access the
nucleoplasm for only 5 mins on average, the SPB continues to perhaps remain in a mitotically active state, nucleating bundled microtubules in the cytoplasm which appear similar to spindle microtubules.

5.2.7 Partial NIMA function results in the extension of Mts on the cytoplasmic side of the SPB during metaphase

One of the key events necessary for mitotic entry is the switch in the Mt nucleating activity of the SPB from the cytoplasmic side (during interphase) to the nuclear side (during mitosis). The observation that nimA7 cells lacking mad2 continue to show bundled Mts being nucleated from the cytoplasmic side of the SPB even after mitotic exit is surprising (Figure 5.7B, D). One potential explanation for this phenomenon would be that during mitosis in nimA7 cells, the SPB is mis-regulated such that microtubules are erroneously nucleated on the cytoplasmic side as well as on the nuclear side of the NE. In the absence of mad2, tubulin is exported out of the nucleus within 5 mins of mitotic entry in nimA7 cells, as described previously, resulting in tubulin being present only on the cytoplasmic side of the nucleus. The presence of spindle like Mts extending from the SPB on the cytoplasmic side in these cells suggests firstly, that there is a defect in turning off the mitotic activity of the SPB, and secondly, that the SPB is mis-regulated to allow the nucleation of spindle-like Mts on the cytoplasmic side.
In typical Wt cells, during metaphase, no astral microtubules are visible (Ovechkina, Maddox et al. 2003). Subsequently, in anaphase and telophase, spindle elongation is associated with the appearance of astral microtubules on the cytoplasmic of the SPB. If the SPB was indeed misregulated to allow nucleation of Mts on either side of the NE during mitosis, then we would expect to see some cytoplasmic Mts during metaphase in *nimA7* cells. Consistent with this prediction, we readily find *nimA7* cells in which cytoplasmic Mts are detectable even when a metaphase-like spindle is present (Figure 5.8A, arrows).

We notice that the detection of these cytoplasmic Mts during metaphase is easier in germinated cells that are likely undergoing the second round of mitosis. This is possibly because the larger size of germinated cells helps the detection of the cytoplasmic Mts extending from the SPB compared to the smaller sized conidia. In *nimA7* cells that have two nuclei, we were able to detect instances wherein the microtubules emanating from the two neighboring nuclei seem to interact, which is only possible if there were some microtubules being nucleated on the cytoplasmic side of the nucleus. Figure 5.8B shows a dramatic example of this phenomenon. This interaction between neighboring nuclei is also seen in the second mitosis in Figure 5.10 at 5' (arrowhead). These data strongly suggest that there is a defect in cells with partial NIMA function in regulating the directionality of Mt nucleation from the SPBs during mitosis.

The presence of increased cytoplasmic Mts during metaphase is reminiscent of the phenotype of cells lacking the two hybrid interacting protein of
NIMA, tinA, in combination with a mutation in the anaphase promoting complex/cyclosome, *bimE7*, i.e., ΔtinA+bimE7. While the lack of function of *bimE* results in an extended metaphase delay, in the double mutant that also lacks tinA, dramatic extensions of cytoplasmic Mts are notable in metaphase (Osmani, Davies et al. 2003). This has led to a proposed function for tinA as a negative regulator of astral microtubules. In Wt cells, TINA locates to the SPB specifically at mitosis and stays at the separated SPBs through mitotic exit (Osmani, Davies et al. 2003). We reasoned that the presence of increased cytoplasmic microtubules in *nimA7* cells could be due to absence of TINA at the SPB. Therefore, to test for a defect in TINA localization to mitotic SPB in *nimA7* cells, we generated *nimA7* cells that also carried TINA-GFP and H1chRFP. However, as shown in Figure 5.8C, TINA is found to still locate to mitotic SPBs in *nimA7* cells in the first mitosis. Further confirmatory experiments aimed at following TINA location in the second mitosis of *nimA7* cells and also in combination with a spindle marker would be helpful to establish whether TINA location is affected in cells with partial NIMA function. Our data, so far, suggest that the appearance of Mts extending on the cytoplasmic side of mitotic SPBs in *nimA7* cells is possibly not due to the absence of TINA at the SPB.
5.2.8 The brief dispersal of NLS-DsRed at anaphase in *nimA7* cells does not depend on the formation of a mitotic spindle

As shown in Figure 5.3E, though NLS-DsRed does not disperse at the start of mitosis, it is released from the nucleus briefly when the spindle elongates in anaphase when NIMA is not fully active. One possible explanation for this brief release of NLS-DsRed would be that the mechanical force from spindle elongation leads to some tearing of the NE resulting in the transitory release of actively transported proteins from the nucleus. To test this hypothesis, we germinated *nimA7* cells carrying tub-GFP and NLS-DsRed in benomyl, a microtubule poison. Spindle formation is blocked in benomyl treated cells; however mitotic entry and exit can be followed by examining the entry and exit of free tubulin into and out of the nucleus. In cells that were identified to be in mitosis by the presence of nuclear tubulin, we asked whether these cells showed dispersed NLS-DsRed during mitosis. Interestingly, we find that in the absence of the spindle, NLS-DsRed disperses in all the cells observed (n=16). These data prove that the dispersal of NLS-DsRed in *nimA7* cells is not dependent on spindle elongation. In addition, we find that NLS-DsRed continues to remain dispersed for an average of 42 mins (Figure 5.9A; n=10) in stark contrast to untreated *nimA7* cells where NLS disperses for a brief period of time before getting reimported (Figure 5.3E). A schematic representation of events in *nimA7* cells upon treatment with benomyl in given in Figure 5.9B. After an average of 13 mins after tubulin entry into the nucleus (n=10), NLS is seen to disperse and
remain dispersed for an average of 40 mins. In Wt *A. nidulans* cells, treatment with benomyl results in a similar arrest in mitosis for an average of 40 mins, after which the spindle assembly checkpoint (SAC) is actively turned off and exit from mitosis occurs by a process termed spindle independent mitotic exit (SIME) (De Souza, Hashmi et al. 2011). This strongly suggests that in *nimA7* cells, upon treatment with benomyl the SAC is engaged resulting in the extended mitotic arrest. Moreover, the difference between the time point of tubulin entry into the nucleus and NLS dispersal is variable, and probably depends on the level of NIMA function in that particular cell (Figure 5.9B).

5.2.9 Apparently successful first mitoses in cells with diminished NIMA function are often followed by a defective second mitosis

As referred to previously, the mitoses in *nimA7* cells that generate two daughter nuclei in the first mitosis are not entirely normal. This is because, when these cells with 2 nuclei are followed into their second mitosis, we find that the two nuclei often behave different from each other (71%, n=7). As shown in the example in Figure 5.10, in the second mitosis, both nuclei show spindle formation at 3'. However, one nucleus does not disperse the NLS-DsRed through the length of time that the spindle is visible and does not attempt anaphase, whereas the other nucleus attempts to divide and shows very brief NLS-DsRed dispersal. Since the nucleus that disperses NLS-DsRed does so in the presence of an
actively transporting nucleus, much of the NLS-DsRed is taken up by the actively transporting nucleus. Therefore, the daughter nuclei of the second nucleus have unequal amounts of NLS-DsRed. These daughter nuclei most likely also have unequal amounts of essential nuclear proteins. Overall, this shows that the majority of the first mitoses in nimA7 cells are unsuccessful in generating two equal daughter nuclei. This in turn results in downstream defects in the second mitosis and differences in behavior between the two daughter nuclei in the second mitosis.

5.2.10 The localization of NIMA7-GFP to the nuclear periphery is not sufficient to promote NLS dispersal

As introduced in Section 5.1.2, NIMA dynamically locates to multiple nuclear structures during mitosis including the spindle pole bodies and the nuclear periphery. The location of NIMA to the nuclear pore complexes at the nuclear periphery coincides with the initiation of partial NPC disassembly (Shen et al. unpublished). Therefore, we wanted to test whether loss of localization of NIMA to the nuclear periphery in nimA7 cells is responsible for the inability of these cells to open the pores at mitotic entry. To this end, we tagged the nimA7 gene with GFP. We then followed cells carrying GFP tagged WT NIMA as well as those carrying NIMA7-GFP at the semi permissive temperature, in addition to NLS-DsRed. As shown in Figure 5.11A, in the first mitosis, WT NIMA locates to
the periphery which is coincident with the dispersal of NLS-DsRed. In first mitosis, the location of NIMA is difficult to detect due to the small amounts of protein in the conidia. Therefore, we cannot detect NIMA at every mitotic location that we see it locating to in a longer hyphal cell with more NIMA protein. In Wt cells where NIMA was detectable around the nuclear periphery in mitosis, NLS-DsRed was seen to disperse at the same time. In contrast, in nimA7-GFP cells, the instances where we detected NIMA7 at the periphery were not accompanied by NLS dispersal (Figure 5.11B). This suggests that NIMA7 is capable of locating to the nuclear periphery at mitosis, but that this location is not sufficient to trigger NPC disassembly. It is, although, also possible that NIMA7-GFP does not locate to the nuclear periphery at amounts necessary for NPC disassembly. In the example in Figure 5.11C, NIMA7 also shows localization to the nucleus in addition to the nuclear periphery. This nucleus shows brief incomplete NLS dispersal (possibly at anaphase) before failing nuclear division and fusing back to form a single nucleus. We note that NIMA7 locates to the SPBs at mitotic exit in this cell similar to WT NIMA-GFP shown in Figure 5.11A. Overall, these data show that the defects in mitotic NPC disassembly in nimA7 cells are likely not due to a complete failure in locating the NIMA7 protein to the nuclear periphery.
5.3 Discussion

5.3.1 Partial NIMA function allows entry into mitosis instead of causing a G2 delay

The checkpoints monitoring DNA damage and replication ensure that mitosis is not triggered without completing the repair of the damaged DNA or the replication of the genome, respectively. However, in addition to these two important processes, another prerequisite for successful mitosis is the availability of proteins that are important for mitotic progression. We find that when the function of the NIMA kinase is partially inhibited, nuclear division is initiated with inadequate NIMA function which results in downstream mitotic defects and lethal consequences. In comparison, the partial inactivation of Cdk1 leads to a G2-M delay which allows for enough time to eventually accumulate sufficient Cdk1 activity and therefore the ensuing mitosis is completed successfully. Why does partial inactivation of NIMA allow mitotic entry?

Cells that completely lack NIMA function (for instance nimAts alleles such as nimA5 at the completely restrictive temperature) exhibit full activation of Cdk1 (Osmani, McGuire et al. 1991). On the other hand, NIMA is dependent, in part, on Cdk1 to become completely active. Therefore, even though in cells lacking Cdk1 activity (nimT23 cells at the restrictive temperature), NIMA is phosphorylated and has a basal level of activity, NIMA is subsequently hyper-
phosphorylated and further activated in a Cdk1-dependent manner (Ye, Xu et al. 1995). These data together indicate that at least some part of NIMA function happens downstream of Cdk1 activation. Therefore, we think in cells with partial NIMA function, the low level of NIMA7 activity is sufficient to trigger mitotic entry since in these cells Cdk1 is fully active (Appendix A, Figure A.4). However, since NIMA function is required for specific mitotic events to occur successfully (as our data show), we find defects in mitotic processes in cells in which NIMA function is inadequate.

However, it is important to note that the basal level of NIMA activation happens in a Cdk1-independent manner. This activity correlates with auto-phosphorylation of NIMA and phosphorylation mediated possibly by an unidentified kinase (Ye, Xu et al. 1995). Also significant in this regard is that the basal level of NIMA activation primes it for the Cdk1-dependent hyper-phosphorylation and activation. This is because when NIMA is completely inactivated (nimA5 at the restrictive temperature), it is not hyperphosphorylated even though Cdk1 activity is high in these cells (Ye, Xu et al. 1995). Moreover, cells that carry the Cdk1 hyperactive allele (which cannot be inhibited by wee1 phosphorylation) are not lethal in A. nidulans, however, this allele is lethal in the presence of mutants that increase NIMA activity. These data together, suggest that NIMA plays a partially non-redundant role with Cdk1 to initiate mitosis.
The fact that cells with reduced Cdk1 function do not show mitotic defects might be related to the strategy used to inactivate Cdk1. Partial inactivation of Cdk1 was done by growing cells at the semi permissive temperature of the *ts* allele of the Cdk1 activator, *nimT* (*cdc25*). In this scenario, it may be expected that the accumulation of active Cdk1 will be slower potentially since the rate of generation of these active Cdk1 molecules by the partially active NIMT23 is slower. However, once the inhibitory phosphorylation on Cdk1 is removed, it would be as active as Cdk1 in Wt cells. Therefore, we might expect cells to proceed through mitosis normally, even though the transition into mitosis takes longer. However, since partial inactivation of NIMA was carried out using a *ts* allele of *nimA*, therefore NIMA7 activity would be expected to not be comparable to Wt NIMA. Therefore, following entry into mitosis (possibly by the mechanism described above), cells having NIMA7 would be defective in mitotic processes that require the normal function of NIMA.

5.3.2 Normal NIMA function is essential for the successful completion of multiple mitotic events downstream of G2-M transition

NIMA expression and kinase activity are regulated through the cell cycle such that there is maximum NIMA activity at G2-M transition (Osmani, May et al. 1987). Since NIMA is required for the entry into mitosis, in the absence of NIMA function, no mitotic events can be triggered. Therefore it has been difficult to
ascertain the requirement of NIMA for specific mitotic events from the study of nimA7 cells at the fully restrictive temperature because the G2-M transition is blocked in these cells. In this regard, using the semi permissive temperature of the nimA7 allele has been a powerful approach in teasing out the involvement of NIMA in mitotic progression. The mitotic defects exhibited by cells with partial NIMA function have been analyzed by following the localization of proteins that localize to the nucleus, chromatin, spindle pole body, kinetochore, spindle, nuclear pore complex, nuclear envelope, and the nucleolus. These studies enabled us to categorize the mitotic defects in these cells in a systematic manner, revealing defects in discrete mitotic events including bipolar spindle formation, nuclear pore complex disassembly and nuclear envelope segregation (Figure 5.12). These defects are discussed below:

5.3.2.1 NIMA and mitotic nuclear pore complex disassembly

During mitosis in A. nidulans, the nuclear envelope stays intact, so it was thought that the mitosis was closed like in unicellular fungi such as S. cerevisiae. Forward genetic screens in A. nidulans identified that loss of function mutations in genes encoding two nuclear pore complex (NPC) proteins, sonA and sonB can suppress the temperature sensitivity of the nimA1 mutant allele (Wu, Osmani et al. 1998, De Souza, Horn et al. 2003). The examination of the localization of SONA and SONB through mitosis indicated that some NPC proteins dispersed from the nuclear periphery at mitosis. Subsequently a systematic study of all
NPC proteins in *A. nidulans* showed that a subset of nucleoporins called peripheral Nups, disperse during mitosis, while the core nucleoporins remain at the nuclear periphery. Thus it was demonstrated that *A. nidulans* undergoes a semi-open mitosis which allows the entry of tubulin into the nucleus at mitotic entry. Moreover, it was shown that activation of NIMA can promote nuclear pore complex disassembly out of cell cycle phase (De Souza, Osmani et al. 2004) and SONB is phosphorylated by NIMA *in vitro*. Therefore, the current model proposes that NIMA promotes mitotic initiation, at least in part, by promoting the phosphorylation of peripheral Nups leading to their dispersal from the NPCs, thus changing the transport properties of the nuclear pores specifically during mitosis. However, it was not known whether NIMA function was required for mitotic NPC dispersal once mitosis was initiated. One possibility is that once mitosis is initiated NIMA might work redundantly with other mitotic kinases to regulate NPC disassembly. Consistent with that idea, it was shown that multiple human kinases including NIMA related kinases – Nek6 and Nek7, Cdk1, and Plk1 have the ability to phosphorylate Nup98 nucleoporin *in vitro*, suggesting a role for these kinases in regulating Nup98 dynamics in mitosis (Laurell, Beck et al. 2011). Therefore it becomes important to address the question of which kinase is important and/or required for modifying the permeability of the nucleus in mitosis.

One unifying phenotype we see in all cells with reduced NIMA function is an inability to promote partial nuclear pore complex disassembly in mitosis, as seen by the presence of Nup49 at the nuclear periphery as well as the nuclear
retention of NLS DsRed during mitosis. Therefore our data presented here, provides strong evidence that NIMA is not only sufficient but also required for mitotic disassembly of NPCs. In fact, we find that in cells with reduced NIMA function, the active transport of smaller proteins like tubulin and HP1 is abolished in mitosis. However, larger proteins or proteins that are part of macromolecular complexes like NLS-DsRed and SWD1 are still retained in the nucleus. This indicates that in the absence of normal NIMA function, there is some disassembly of NPCs, but perhaps this is not enough to allow all proteins to diffuse into the cytoplasm (Appendix A, Figure A.5). In fact, we know that even in Wt cells the mitotic disassembly of NPCs is not sufficient to cause the dispersal of certain proteins like proteasome subunits, which form a big macromolecular complex (Yi Xiong and Berl Oakley, unpublished). The proteasome subunits, however, disperse in strains deleted for the NPC proteins, nup37 and elys, in which the majority of the core Nups disperse in mitosis, a phenomenon called extreme disassembly (Liu, De Souza et al. 2009). Our data further shows that the lack of nup37 and elys (that causes extreme disassembly) does not suppress the defect in NPC disassembly in cells with partial NIMA function, indicating that extreme disassembly is contingent upon the presence of normal NIMA function. These data indicate that NIMA function is indispensable for mitotic partial nuclear pore complex disassembly. This is consistent with the in vitro data showing that dominant negative versions of NIMA can inhibit nuclear envelope breakdown (Laurell, Beck et al. 2011) and the depletion of NIMA related kinases, Nek6 and
Nek7 delays the mitotic dispersal of an actively imported nuclear marker protein in HeLa cells. Therefore, NIMA likely has a conserved role in permeabilizing the nuclear envelope at mitosis.

It has been shown that the nuclear pore complex disassembly at mitotic entry and reassembly at mitotic exit happens in a hierarchical and step wise manner (Dultz, Zanin et al. 2008). We think it is possible that in cells with reduced NIMA function, the early steps in NPC dispersal happen, allowing the mitotic diffusion of tubulin and HP1. However, the late disassembling Nups are possibly inhibited from disassembling resulting in the retention of some actively transported proteins. We have shown that Nup49, a peripheral nup, fails to disperse in mitosis without sufficient NIMA activity. Further extending this study and performing a systematic examination of the behavior of all nucleoporins in cells with partial NIMA function has the potential to help us resolve the NPC disassembly process temporally. In addition, further calibration of the NIMA function (reducing it by increasing the temperature of incubation of nimA7 or increasing it by using a temperature lower than 35°C) also could help us further define which steps of NPC disassembly require NIMA function and give insights into potential NIMA substrates at the nuclear periphery.
5.3.2.2 NIMA and the regulation of spindle formation

Our data clearly implicates NIMA in regulating the separation of duplicated spindle pole bodies since reduced NIMA function can lead to monopolar spindle formation. These data are consistent with studies showing that the human orthologs of NIMA function in different pathways to enable segregation of centrosomes. While Nek2 regulates the removal of centrosomal linker proteins through phosphorylation, the Nek9-Nek6/Nek7 phosphorylation cascade regulates the function of the BIMC kinesin, Eg5 during centrosome separation (see Section 1.5 for details) (Fry, O'Regan et al. 2012). Therefore, the role of NIMA in regulating microtubule organizing centers in mitosis is clearly conserved through to higher eukaryotes. Therefore studies in *A. nidulans* could be potentially useful to understand the function of not only NIMA but also of NIMA related kinases in other systems.

In addition to the separation of microtubule organizing centers (MTOCs), another key event that occurs during mitotic initiation is the switch from nucleation of cytoplasmic microtubules (Mts) to functionally and structurally different bundled nuclear spindle microtubules. In organisms in which SPBs are embedded in the nuclear envelope, cytoplasmic and spindle microtubules need to be extended from either side of the nuclear envelope in interphase and mitosis respectively. This calls for a dramatic change in SPB function at mitotic entry. The localization of NIMA at the SPB during mitotic entry as well as mitotic exit...
suggests that it may have functions in switching the nucleation of Mts at the SPB from the cytoplasmic side to the nuclear side at mitotic entry and from the nuclear side to the cytoplasmic side at mitotic exit. Our data is consistent with such a role for NIMA, showing that in cells with partial NIMA function, Mts can be erroneously nucleated on the cytoplasmic side of the nuclear envelope during mitosis. We propose that this misregulation of SPBs results in reduced spindle microtubules on the nuclear side and thereby contributes to defects in anaphase. Exploring this phenotype further would be extremely valuable in understanding the regulation of the SPB during mitosis.

5.3.2.3 NIMA and kinetochore/SPB segregation

In cells with partial NIMA function, defects in anaphase completion can occur after the formation of a bipolar spindle, revealing a requirement for normal NIMA function in the segregation of chromatin successfully. The examination of the behavior of kinetochores in these cells provides further insights into a possible cause for the anaphase failure. We find that kinetochores are able to segregate in nimA7 cells, however, they fail to remain segregated and collapse back together. In Wt cells, duplicated sister chromatids are held together by the cohesion complex until segregation in anaphase. In higher eukaryotes, the segregation of sister chromatids occurs in two steps. In prophase and prometaphase, cohesion is removed from the chromatid arms, resulting in the sister chromatids being held together only at the centromere/kinetochore region.
Subsequently, when anaphase is triggered, the cohesin that is holding the centromeres together is cleaved by separase which allows the movement of the sister chromatids towards the spindle poles. Since in nimA7 cells, we see an initial separation of the kinetochores, we think one reason the kinetochores collapse back together could be the aberrant presence of cohesion between the sister chromatids. Failure to remove the cohesion between the sister chromatids in pro metaphase might result in the failure to segregate the chromatin even though the centromeric/kinetochore region can be separated. Another possibility that might explain the failure to segregate chromatin in nimA7 cells has to do with the topology of DNA during mitosis. Catenation or intertwining of DNA is a natural consequence of DNA replication, and therefore proteins including topoisomerase II function to unravel the chromatin before entry into mitosis. If this process does not occur successfully, we might expect a failure to segregate the sister chromatids because the chromatin is intertwined. In the future, it would be interesting to determine which of these defects contributes to the anaphase defect in nimA7 cells.

Another remarkable phenomenon we detect in cells with partial NIMA function is that subsequent to a failure to divide, the separated SPBs in cells that form a bipolar spindle, can come back to a single focus. This is surprising since we would not expect there to be any link between the SPBs once they are separated. There are a couple of scenarios that might give rise to this observation. One, it is possible that it is more energetically favorable for the
proteinaceous SPBs to minimize the contact with the double lipid bilayer of the nuclear envelope. The second possibility deals with the less understood nature of physically interaction between kinetochores and the spindle pole bodies. In *A. nidulans*, as in other systems with NE embedded MTOCs, the kinetochores are clustered at the interphase SPB. However, the nature of attachment between these two structures is not understood. In mitotic metaphase, microscopic examination of kinetochore proteins in conjunction with spindle pole body proteins shows the presence kinetochore between the two separated SPBs along the spindle (De Souza, Hashmi et al. 2009). This led to the thought that kinetochores are released from the SPBs before segregated in anaphase on spindle Mts. Segregated kinetochores again appear as a focus and locate near the SPBs. Therefore, the connection between the kinetochores and the SPBs appears to be mitotically regulated. It is possible that in nimA7 cells, the connection between the kinetochores and the SPBs is not completely severed, such that, since kinetochore segregation fails and the kinetochores came back together, the existence of a physically link between the SPBs and the kinetochores brings the SPBs together as well. If this was true, that would implicate NIMA in the poorly understood mechanism by which kinetochores are released from the SPB at prophase/metaphase. Interestingly, we have also observed that in interphase cells expressing the dominant negative version of NIMA, the kinetochore protein, Ndc80, can be found in more than one focus, one of which corresponds to the SPB. These data also support the idea that NIMA
likely has a function in regulating the link between SPBs and kinetochores through the cell cycle.

5.3.2.4 NIMA and mitotic nuclear envelope dynamics

The segregation of the nuclear envelope in *A. nidulans* is unique in that it involves two restriction points transiently giving rise to three membrane bound compartments, two of which envelope the separated daughter chromatin and the middle compartment surrounds the nucleolus (Ukil, De Souza et al. 2009). Moreover, nucleolar segregation is intimately linked to NE segregation, such that the NE double pinch is accompanied by the disassembly and reassembly of the nucleolar proteins. How NE segregation and nucleolar segregation are regulated is poorly understood. Interestingly, in cells with partial NIMA, we find instances where NE and nucleolar segregation is attempted, however the membrane fails to restrict and the NE goes back to encompass the single chromatin mass. Though it is not known what triggers the disassembly of the nucleolus, it has been suggested that the mechanical forces exerted by the NE during restriction might contribute to its disassembly. In agreement with this hypothesis, we find that cells with partial NIMA function that fail to complete mitosis either because of a defect in establishing a bipolar spindle or due to anaphase defects do not disassemble the nucleolus (Table 5.1, data not shown). However, nuclei that attempt to restrict the NE (either resulting in the resolution of two daughter nuclei or in a failure to complete NE segregation), exhibit the disassembly and
reassembly of the nucleolus. In addition, studies from the lab have shown that nucleolar disassembly can happen in the absence of spindle formation (in the presence of the microtubule poison, benomyl), leading to the model that nucleolar disassembly-reassembly and spindle formation are independent yet coupled processes (Ukil, De Souza et al. 2009). Our data now complements the previously published study by showing that spindle formation (in nimA7 cells that have a monopolar spindle or that fail anaphase) can occur in the absence of nucleolar disassembly-reassembly. Also, to directly probe the role of NIMA in nucleolar segregation independent on its role in spindle formation, it will be informative to test whether cells with partial NIMA function can disassemble and reassemble their nucleoli in the absence of spindle forces.

A critical aspect of the behavior of the nuclear marker, NLS-DsRed in nimA7 cells is the observation that even though it does not disperse at mitotic entry in cells that form a bipolar spindle and attempt anaphase, as discussed previously, NLS disperses briefly at a time point that coincides with spindle elongation. This suggested that in the absence of NPC disassembly perhaps the tearing of the NE due to spindle forces contributes to the transient release of NLS from the nucleus. However, to our surprise, we find that NLS disperses in cell with partial NIMA function even in the absence of the spindle (in the presence of a microtubule poison, benomyl). This suggests that the dispersal of NLS at anaphase is not just a consequence of mechanical forces on the NE, rather it is likely to be an actively regulated process. This observation is also significant in
view of the fact that nucleolar disassembly and reassembly can also happen in
the absence of spindle microtubules, as mentioned earlier (Ukil, De Souza et al.
2009). Taken together with our data, this suggests that in the absence of a
spindle, mechanisms exist that actively cause the permeabilization of the NE
membrane even in the absence of NPC disassembly. Furthermore, our data
suggests that perhaps the NE is actively remodeled at telophase, that there
might be a regulated transient NE breakdown that occurs in *A. nidulans* in the
absence of the spindle. This is consistent with our data from *nimA7* cells in the
absence of benomyl, that even though there is no obvious change in the
localization of Nup49 in telophase, we see the dispersal of NLS-DsRed,
suggesting that this dispersal may not be dependent on changes in NPC
behavior but rather be dependent on a change in the integrity of the nuclear
envelope, that is perhaps is too transient to be detectable by microscopic studies
done so far. Indeed NE breakdown specifically at anaphase (not at mitotic entry)
has been demonstrated in the fungus, *Schizosaccharomyces japonicas* (Yam,
He et al. 2011). This fungus does not under partial NPC disassembly at
prophase, however, at late anaphase active transport is abolished for a brief
period, very similar to the behavior of NLS in *nimA7* cells. Since in *A. nidulans* Wt
cells, NPC disassembly at mitotic entry abolishes active transport, any additional
permeabilization of NE would not be detected. However, since in *nimA7* cells,
NPC disassembly is inhibited retaining NLS in nucleus, we were able to uncover
this process of transient late dispersal of NLS that might be due loss of NE
integrity. Importantly, another parallel between \textit{nimA7} cells and \textit{S. japonicas} Wt cells is in the dispensability of the spindle for abolishment of active transport at late anaphase, further suggesting that the reason for increased permeabilization of the NE in both cases might be a regulated modification of NE integrity. It is important to also consider at this point that in Drosophila embryos that undergo synchronous mitosis during early development, the nuclear envelope breakdown happens after all kinetochores have successfully attached to the spindle (Paddy, Saumweber et al. 1996).

5.3.3 The effect of the partial inhibition of NIMA on the spindle assembly checkpoint

Though there is variability in the time spent in mitosis among \textit{nimA7} cells, the average time spent in mitosis by cells with partial NIMA function that fail to complete the first mitosis vs. those that complete an apparently successful first mitosis is very similar. This was surprising considering the different fates of either population of cells. In \textit{A. nidulans}, when spindle assembly is blocked using the microtubule depolymerization drug, benomyl, mitosis is extended for an average for 40 mins by the activity of the spindle assembly checkpoint (SAC). Thereafter, the SAC is actively turned off and cells exit mitosis by a process termed spindle independent mitotic exit (SIME) (De Souza, Hashmi et al. 2011). Therefore, defects in establishing a bipolar spindle and/or normal kinetochore-Mt
interactions would be expected to engage the SAC and prolong mitosis by an average of 40 mins. It is surprising therefore, that we do not see an average delay as long as 40 mins in *nimA7* cells which form a monopolar spindle or that fail to segregate chromatin. These data suggested that the SAC function might not be completely normal in *nimA7* cells, and therefore, cells exit mitosis without displaying the mitotic delay characteristic of SAC activity. Mad1 and Mad2, proteins that are essential for SAC function, reside at the NPCs during interphase. At mitosis, they locate to kinetochores and upon the establishment of kinetochore-Mt attachment, disperse briefly before being recruited to the NPCs of daughter nuclei. The mitotic location of Mad1 and Mad2 at the kinetochores is essential for SAC activity. Since the function of NIMA is required for the dispersal of the peripheral Nups, Nup49 and Nup2, we attempted to determine whether the mitotic location of Mad1 was affected in *nimA7* cells. Our data support the idea that the location of mad1 to mitotic kinetochores is largely unaffected in *nimA7* cells (data not shown). However, this does not rule out the possibility that the SAC is not fully active in *nimA7* cells due to a defect in a process other than mad1 localization.

Though we don’t see a difference in the mitotic delay between cells that show successful vs. unsuccessful mitoses, the data is pretty clear in indicating that the occurrences of successful mitoses in *nimA7* is completely dependent on the spindle assembly checkpoint. Moreover, the prolonged permeabilization of the NE to tubulin indicating a mitotic delay in *nimA7* cells is also dependent on
the activity of the SAC. Therefore, the SAC is functional to some extent, even if not completely normally in nimA7 cells.

Another interesting observation relating to the function of NIMA and the activity of the SAC came from the experiment involving the treatment of nimA7 cells with benomyl. We find that in these cells, NLS-DsRed disperses for an average time equivalent to SAC imposed mitotic arrest in Wt cells. This striking result suggests that the SAC can be activated in nimA7 cells when spindle formation is abolished as well. These data suggest that the machinery that is required to impose SAC mediated delay is probably intact in nimA7 cells. However, NIMA is perhaps required for the SAC to detect certain mitotic errors and therefore the SAC might not be completely engaged in nimA7 cells. However, the detection of the absence of a spindle by the SAC is probably not dependent on normal NIMA function, and therefore treatment with benomyl is able to impose a mitotic delay on nimA7 cells. Our data, therefore, shows that NIMA is required for not only for the execution of specific mitotic events but also the detection of errors if they were to occur. This further underlines the importance of normal NIMA kinase function in successful mitotic completion.
<table>
<thead>
<tr>
<th>Phenotype</th>
<th>First Mitosis</th>
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<tr>
<td></td>
<td>1 nucleus to 2 nuclei (58%)</td>
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<tr>
<td></td>
<td>Absence of anaphase (11%)</td>
</tr>
<tr>
<td></td>
<td>Defective anaphase (16%)</td>
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<tr>
<td>Spindle</td>
<td>Bipolar</td>
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<td>Kinetochores/SPB</td>
<td>Segregate</td>
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<tr>
<td>Chromatin</td>
<td>Segregates</td>
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<tr>
<td>Nuclear envelope</td>
<td>Restricts to give two daughter nuclei</td>
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<tr>
<td>Nucleolus</td>
<td>Disassembles and reassembles into daughter nuclei</td>
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**Table 5.1:** Compilation of the analysis of first mitosis in cells with partial NIMA function. Phenotypes of cells with reduced NIMA function are represented in a tabular form. Please refer to Sections 5.2.1-5.2.4 for details. N.O = Not observed.
Figure 5.1: Cells with reduced NIMA function form a monopolar spindle. (A) and (B) nimA7 cells with microtubules marked by tub-GFP and carrying the kinetochore protein, Ndc80, tagged in red (MG190) followed through mitosis at RT (22°C) (A) or at 35°C (B). (C) Images from a movie of nimA7 cells carrying a spindle pole body (SPB) marker and tub-GFP undergoing mitosis (MG372). (D) and (E) WT (MG291) or nimA7 cells (MG290) carrying kinesin BIMC tagged with GFP and histone H1-chRFP followed through mitosis. Bars for A-D are 5µm. Bar for E is 2.5µm.
Figure 5.1
Figure 5.2: Cells with reduced NIMA function fail to segregate the chromatin successfully. (A) A nimA7 cell carrying GFP-tub and the kinetochore marker (MG190), Ndc80-CR followed through mitosis at the indicated temperature. Open arrowheads indicate kinetochore foci. The filled arrowheads point to tub-GFP foci at separated SPBs. (B) Montage of images from a movie in which a nimA7 cell carrying the SPB marker, GCP3 and the kinetochore marker Ndc80 (MG274), attempts to divide the kinetochores. Blue arrows indicate the separated SPBs which later come back together. Yellow arrows show key steps in the missegregation of the kinetochore foci. The white arrowhead points to the retention of the kinetochores at one of the SPBs. (C) and (D) Histone H1-chRFP and tub-GFP were used to mark mitosis in Wt (MG300) (C) and nimA7 (MG298) (D) at 35°C. Arrows in (D) show the separated SPBs, arrowhead points to chromatin that is attempting to segregate. Bars, 5µm.
Figure 5.2
Figure 5.3: Cells with reduced NIMA function have defects in mitotic partial nuclear pore complex (NPC) disassembly. (A) nimA7 cells carrying tub-GFP and the peripheral nucleoporin, nup49, (MG321) undergoing mitosis at the elevated temperature at indicated. (B) Time lapse images showing mitosis in nimA7 cells carrying the heterochromatin binding protein, HP1 and H1-chRFP (MG378). (C) Simultaneous imaging of peripheral nucleoporin, Nup2 and H1-chRFP in nimA7 cells at the indicated temperature (MG257). Arrowhead indicates the localization of nup2 in the nucleus compared to the localization around the nuclear periphery seen in the previous time point (see text for details). (D) and (E) Nuclear transport marked by NLS-DsRed and spindle formation (tub-GFP) followed in Wt cells (D, MG273) and nimA7 cells (E, MG229) undergoing mitosis. (F) Cells deleted for the NPC proteins, nup37 and elys, and also carrying nimA7 followed with time through mitosis (MG360). Bars, 5µm.
Figure 5.3
Figure 5.4: Cells with reduced NIMA function allow tubulin to enter the nucleus.

(A) 1 Z slice images of tub-GFP and NLS-DsRed in Wt and nimA7 cells at 35°C during G2-M transition. The shadow of the nucleus revealed by tubulin-GFP is marked by yellow arrows. In the Wt cell, the shadow of only one of the two nuclei is visible in this Z plane. (B) and (C) The signal intensity of NLS-DsRed or tub-GFP, in the ROI (yellow dotted) as indicated, from single Z sections through the middle of the cell was quantitated and plotted through time during G2-M transition of Wt (A, MG273) or nimA7 (B, MG229) cells.
Figure 5.4

A

WT (35°C)

\[\text{tub-GFP} \quad \text{NLS-DsRed}\]

1'

2'

3'

nimA7 (35°C)

\[\text{tub-GFP} \quad \text{NLS-DsRed}\]

1'

2'

B

WT cells, 35°C

NLS $\rightarrow$ mitosis

Tubulin $\rightarrow$ mitosis

C

nimA7 cells, 35°C

NLS $\rightarrow$ mitosis

Tubulin $\rightarrow$ mitosis

Graphs showing signal intensity in nucleus over time (mins):

- Tubulin
- NLS
Figure 5.5: Cells with reduced NIMA function fail to segregate the nuclear envelope. (A) Mitosis in a nimA7 cell followed by NLS-DsRed and tub-GFP localization (MG229). (B) and (C) The behavior of the nuclear envelope (NE) marker protein, AN0162, and tub-GFP was followed through mitosis in Wt (B, MG224) and nimA7 cells (C, MG227). Arrowheads in (B) point the restriction sites of NE in telophase. Arrows in (C) at 14’ indicate an attempt to segregate the NE. Arrow at 26’ shows the formation of the ‘theta’ structured nucleus (see text for details). (D) Nucleolar marker, fibrillarin tagged in red (MG294), localizes within the NE inclusion marked by AN0162-GFP (arrow). The arrowhead points to a speck of fibrillarin that is separated from the rest of the nucleolus. Bars, 5µm.
Figure 5.5

A. nimA7 at 35°C
   tub-GFP
   NLS-DsRed
   Merge

B. WT at 35°C
   tub-GFP
   AN0162-CR
   Merge

C. nimA7 at 35°C
   tub-GFP
   AN0162-CR
   Merge

D. nimA7 at 35°C
   GFP-AN162
   fib-CR
   Merge
Figure 5.6: Cells with reduced NIMA function show a prolonged mitosis. (A) Quantitation of the average time spent in mitosis by Wt and \( nimA7 \) cells at 35°C. Spindle formation was used as a measure of determining time in mitosis. Strains used to compute the average time in mitosis: Wt – MG224, \( nimA7 \) – MG190, MG227, and MG229. (B) Box and whiskers plot showing the distribution of time in mitosis for Wt and \( nimA7 \) cells. The green squares and red circles were manually marked on the plot secondarily. (C) The quantitation of time in mitosis for Wt and three different alleles of \( nimA \) at their respective semi permissive temperatures, as indicated. Strains used: Wt – MG224, \( nimA1 \) – MG310, \( nimA5 \) – MG308, \( nimA7 \) – MG229.
Figure 5.6

A

Average time in Mitosis (mins)

n=21

WT
nimA7

n=13

B

Time in Mitosis (mins)

WT
nimA7

1 to 2
1 to 1

C

Time in Mitosis (mins)

WT (35°C)
n=19
nimA7 (35°C)
n=12
nimA5 (35°C)
n=30
nimA1 (37.5°C)
n=15
Figure 5.7: Cells with reduced NIMA function depend on the spindle assembly checkpoint (SAC) for survival. (A) Colony growth of strains of the indicated genotypes at 35°C after 72 hours. Strains: Wt – R153, nimA7 – MG298, ΔAn-mad2 – MG362, nimA7+ΔAn-mad2 – MG313. (B) Mitosis as followed using NLS-DsRed and tub-GFP in a cell carrying nimA7 that also lacks mad2 (MG313). The red arrow points to the entry of tubulin into the nucleus. The yellow arrow points to the shadow of the nucleus generated when tubulin is exported out of the nucleus. Bar, 5µm. (C) Quantitation of the number of cells that complete the first mitosis successfully vs. those which do not in the strains of the indicated genotype. Unequal division refers to a mitosis resulting in daughter nuclei of different sizes. (D) Quantitation of the time during mitosis when tubulin is equilibrated vs. the time that tubulin is seen polymerized to form spindle like structures in strains of the indicated genotypes.
**Figure 5.7**
Figure 5.8: Polymerization of microtubules on the cytoplasmic side of spindle pole body (SPB) in mitotic nimA7 cells. (A) tub-GFP marked microtubules extend on the cytoplasmic side during metaphase in the mitotic germling carrying nimA7 (arrows, MG298). Bar, 5µm. (B) Microtubules polymerized on the cytoplasmic side in this nimA7 germling with two nuclei interact during mitosis (arrowhead, MG298). Bar, 5µm. (C) Behavior of the NIMA interacting protein, TINA tagged with GFP during mitosis with respect to chromatin marked by histone H1-chRFP (MG353). Bar, 2.5 µm.
Figure 5.8
Figure 5.9: The transient NLS-DsRed dispersal in nimA7 cells is not due to spindle forces. (A) nimA7 cells germinated in benomyl were followed through mitosis at the indicated temperature using NLS-DsRed and tub-GFP as markers (MG229). The topmost row of images shows 1 Z slice of tub-GFP at two time points as indicated. The arrowhead points to the nuclear shadow that is filled in by tub-GFP at the next time point. The rest of the images are maximum intensity projections. (B) A schematic representation of the effect on benomyl on nimA7 cells. See text for details.
Figure 5.9
Figure 5.10: A successful first mitosis in *nimA7* cells may be followed by a failed attempt at second mitosis. Nuclear transport is marked by NLS-DsRed and spindle formation by tub-GFP (MG229). Arrow at 5’ points to the transient Mt connection between the two nuclei. The arrow at 12’ points to the micronucleus formed after second mitosis. Bars, 5µm.
Figure 5.11: Localization of NIMA7-GFP through mitosis. (A) Localization of NIMA-GFP to nuclear periphery and SPBs during mitosis in Wt cells (KF33). (B) and (C) Localization of NIMA7-GFP followed through mitosis along with NLS-DsRed (MG250). Bars, 5µm.
Figure 5.12: Schematic representation of the mitotic defects seen in cells with partial NIMA function. Comparison of Wt mitosis (A) with the mitotic defects seen in nimA7 cells (B) at the semi permissive temperature.
Chapter 6: Discussion

One of the outstanding questions in cell biology is how cell growth is coordinated with mitosis. The regulation of mitotic initiation in *A. nidulans* has been a topic of extensive studies and these have revealed that DNA damage and replication errors activate checkpoints over mitosis, through the regulation of the kinases that are essential for mitotic entry in *A. nidulans* – Cdk1 and NIMA. In addition, it has been recognized that before commitment to mitosis, mechanisms must ensure that there has been sufficient cell growth to support the doubling of the number of nuclei. This is especially critical in the multinucleated cells of *A. nidulans*. Therefore, *A. nidulans* is a good model system to address this basic question of how cell growth and mitosis might be integrated. One mechanism by which this could be achieved is if proteins that are essential for mitosis were also involved in cell growth. Our results presented in Chapter 3 describe a new role for the mitotic NIMA kinase in cell growth in *A. nidulans*. We find that NIMA locates to the plus ends of Mts as well as to the growing cell tips juxtaposed with the membrane. Perturbation of NIMA function can lead to a dramatic modification of hyphal growth including defects in maintaining a single dominant apical cell tip. Our studies implicating NIMA in maintaining polarized cell growth are consistent with results from *S. cerevisiae* that describe a similar role for Cdk1 in cell growth,
potentially through the regulation of membrane trafficking and the organization of endocytosis and exocytosis zones (McCusker, Denison et al. 2007, McCusker, Royou et al. 2012). Therefore, further study of this interphase function of NIMA can potentially help uncover conserved aspects of growth-mitosis coordination.

The location of NIMA to the growing cell tip in addition to Mt plus ends is significant considering that a growing number of Mt plus end binding proteins (+TIPs) also locate to other intracellular sites. For instance, during mitosis +TIPs like APC and CLASPs locate to kinetochores in addition to the growing end of the Mts (Bahmanyar, Nelson et al. 2009, Maffini, Maia et al. 2009). This facilitates the mitotic process of search and capture of kinetochores by the spindle Mts (Tamura and Draviam 2012). Interestingly, recent studies have demonstrated that some +TIPs also locate to other intracellular sites such as the cell membrane and the endoplasmic reticulum membrane and may play a similar function in Mt capture at these intracellular sites during interphase. For instance, the +TIP, CLASP1 and 2 also locate to the plasma membrane in a manner independent of their Mt plus end location (Lansbergen, Grigoriev et al. 2006). The interaction of cell-membrane-located-CLASP2 with the Mt plus end binding protein, Eb1 has been shown to be important for organizing the interphase array of Mts and in mediating the movement of migrating cells (Mimori-Kiyosue, Grigoriev et al. 2005, Kumar, Chimenti et al. 2012). We hypothesize that the localization of NIMA to the growing cell tip in addition to the Mt plus end might be reflective of a similar role for NIMA in Mt capture at the growing hyphal cell tip. For instance, the
interaction between cell-tip-localized-NIMA and Mt plus end localized Eb1 would ensure that the growing end of the Mts makes end-on contacts with the cell tip. This would ensure that polarity factors and secretory vesicles are targeted accurately to a defined spot at the cell apex. In addition, interaction of the Mt plus ends with the cell membrane has been seen to correlate with the initiation of Mt catastrophe (Brunner and Nurse 2000). Therefore, when there are inefficient end-on interactions between interphase Mt plus ends and the cell tip, we may expect Mts to continue to grow even after reaching the cell tip. Indeed, this is what we find in cells that have reduced NIMA function. In addition our studies show that NIMA likely has a direct role in regulating the dynamics of Mts. We find that an increase in NIMA function can result in reduced polymerized tubulin, and perturbation of NIMA also affects the behavior of the +TIP, Eb1. Interestingly, the deletion of the A. nidulans ortholog of Dis1/XMAP215, the conserved Mt polymerase localized to the polymerizing end of Mts, also results in reduced interphase Mt tracks, a phenotype that is strikingly similar to the ectopic expression of NIMA. This strongly suggests that NIMA likely has a direct effect on Mt dynamics through the regulation of +TIPs such as Eb1 and XMAP215. Therefore our studies are consistent with a dual role for NIMA in regulating cytoplasmic microtubules in interphase – one, in mediating the end-on interaction of the Mt plus ends with the cell apex, and second, in the regulation of the Mt plus end binding interactome and thereby Mt dynamics (Figure 6.1A). We hypothesize that via the regulation of interphase Mts, NIMA ensures directed
membrane trafficking, and in turn the maintenance of apical dominance and regulated polarized growth.

The fact that the location of NIMA to the Mt plus ends is dependent on Eb1 function is significant. In fission yeast, the complex of Tea1/Tea4 is thought to play a central role in determining the cell tip. However, recent evidence implicates Eb1 in cell tip determination independent of Tea1/Tea4. De novo formation of extra cell tips can be induced in fission yeast cells that are deleted for Tea1 function by changing the shape of the cell and forcing microtubules to make contact with sites other than the cell tips. Surprisingly, these extraneous cell tips are formed by a process that is dependent on Eb1 function, suggestive of a novel pathway involving Eb1 that regulates the establishment of polarity axes (Minc, Bratman et al. 2009). Therefore, we think it is possible that an Eb1 dependent pathway involving NIMA might be conserved in A. nidulans in the regulation of new cell tips, as supported by our data.

Our genetic studies uncover additional players in the regulation of cell growth that work in concert with NIMA. Our results suggest that NIMA and the ESCRT pathway possibly function in parallel pathways to maintain apical dominance. The ESCRT pathway has an established function in the turnover of membrane proteins. Moreover, there is evidence to suggest that some membrane proteins might accumulate on the plasma membrane when ESCRT function is blocked (as detailed in Section 3.3.4). Therefore, we hypothesize that
a block in ESCRT functions could result in defects in membrane trafficking and thus affect polarized cell growth. In Wt cells, polarized cell tip growth is made possible by the balance of exocytosis and endocytosis in spatially separated zones, together referred to as the tip growth apparatus (Taheri-Talesh, Horio et al. 2008). We hypothesize that a combination of inefficient targeting of cell end markers (in cells with reduced NIMA function) as well as an inability to maintain normal membrane composition (in the absence of the ESCRT pathway) might cause enhanced defects in the localization of cell tip specific proteins and sterol rich domains and thus cause increased defects in apical dominance. This hypothesis can be tested by the analysis of the tip growth apparatus in cells that lack either adequate NIMA or the ESCRT pathway in comparison with the double mutants.

It is tempting to think that the interphase localization of NIMA might feed into its requirement for mitotic initiation. In other words, NIMA’s involvement in cell growth may potentially allow it to initiate mitosis at the right time, after adequate growth has occurred. How might this happen? Recent studies in *S. pombe* have indicated the existence of a checkpoint that monitors damage to microtubules giving rise to a delay in G2 (Balestra and Jimenez 2008). These studies show that drug induced depolymerization of Mts results in a delay in mitotic entry in Wt cells that is dependent on the inhibition of Cdk1 activity. That this delay is specific to Mt depolymerization was demonstrated using tubulin alleles resistant to the Mt depolymerizing drug. Moreover, evidence also confirms
that this checkpoint is distinct from the mitotic spindle assembly checkpoint and stress induced delay in entering mitosis. Such Mt damage induced delay in mitotic entry is also seen in primary human cell lines, suggesting this might be a conserved mechanism (Uetake and Sluder 2007). In *A. nidulans*, upon prolonged exposure to benomyl (a Mt poison), cells undergo a spindle assembly checkpoint (SAC) mediated arrest in mitosis and then subsequently exit mitosis by inactivating the SAC in a regulated manner in a process termed spindle independent mitotic exit (SIME) (De Souza, Hashmi et al. 2011). It has been noticed that the time spent in interphase after SIME (in benomyl) is longer than the time spent in interphase in Wt cells not treated with benomyl, suggesting that the absence of polymerized Mts might give rise to a transient delay in interphase. However to confirm that this is not due to a failure to complete the previous mitosis (due to the absence of Mts), the length of interphase needs to be monitored in cells treated with Mt damaging drugs, that are synchronized before G2, say in S phase. Given NIMA’s location to Mts plus ends and the cell tip, we hypothesize that NIMA might be able to function as a sensor for Mt integrity and this may influence NIMA’s role in G2-M transition.

Studies from *S. pombe* point to the existence of a potentially unexplored mechanism, by which mitosis and cell growth may be integrated. In *S. pombe*, two independent studies demonstrated that a gradient of the tip localized inhibitor kinase, Pom1, holds the key to regulating cell length with mitosis in *S. pombe* (Martin and Berthelot-Grosjean 2009, Moseley, Mayeux et al. 2009). In small
cells, the gradient of Pom1 kinase extending away from the cell tip overlaps with
the interphase nodes in the middle of the cell, where some mitotic regulatory
proteins are located, and in essence allows the inhibition of Cdk1 by the Wee1
kinase and thus prevents mitotic entry. However as the cell grows, the Pom1
gradient continues to move away from the medial nodes and at the tipping point,
the inhibition of mitotic entry is lifted. It is important to note, however that even
though Pom1 and Wee1 are in the same pathway and both effectively inhibit
Cdk1 and thus mitotic entry, Pom1 deletion does not mimic Wee1 deletion.
Therefore it is possible that there are other mechanisms that operate in parallel
with this Pom1 mediated pathway to link cell growth to mitotic regulation in S.
pombe. Our data suggest that NIMA might be perfectly poised to fulfill such a role
in A. nidulans and potentially other organisms.

NIMA has consensus sites for Cdk1 phosphorylation very close to the
SXIP domain that mediates its interaction with Eb1 (Chapter 3). This suggests
that the location of NIMA to Mt plus ends could be regulated by phosphorylation
through the cell cycle. In fact, it has been shown that phosphorylation of sites
close to the SXIP domain disrupts salt bridge interactions between the +TIP,
CLASP2 and Eb1 and thus regulates the recruitment of CLASP2 to Mt plus ends
(Kumar, Chimenti et al. 2012). We hypothesize that in the absence of Cdk1
activity in interphase, NIMA is recruited to Mts plus ends and the cell membrane
and regulates the interphase Mt dynamics and thus cell growth (Figure 6.1A).
Upon mitotic entry, phosphorylation of NIMA by Cdk1 at consensus sites near the
Eb1 interacting motif disrupts the NIMA-Eb1 interaction and causes the dispersal of NIMA from cytoplasmic Mts. This would potentially promote the disassembly of cytoplasmic Mts and facilitate mitotic specific localization of NIMA (Figure 6.1B). Likewise, at the end of mitosis, decrease in Cdk1 activity allows NIMA to return to its interphase functions. Future experiments can test this hypothesis by mutating the Cdk1 consensus sites that are near the Eb1 binding motif on NIMA as well as by generating a mutant version of NIMA that is deleted for the Eb1-interacting-SKIP domain. This approach may also enable us to specifically disrupt the interphase function of NIMA and to address the requirement of NIMA in polarized growth.

In *A. nidulans*, the cell cycle needs to be coordinated not only with hyphal cell growth as explained previously, but also through development. During asexual development, a part of the hyphal cell differentiates to form a specialized foot cell that gives rise to the aerial conidiophore, or spore forming structure. The conidiophore comprises of four different cell types - the vesicle, metulae, phialides and the conidia (as introduced in Section 1.1.1), and its development involves a complex interplay between mitosis and differentiation. The vesicle is an aerial cell that arises from the foot cell and has multiple nuclei. A layer of metulae forms from the vesicle by budding. The nuclei in the vesicle arrange themselves next to the newly formed metulae and the mitosis of the nuclei within the vesicle occurs in such a way that one of the daughter nuclei moves into the metulae. Subsequently, two phialides bud off from each metula, following which
two rounds of mitosis happen within each metula resulting in a layer of uninucleated phialides. Thereafter the metulae undergo cell cycle arrest. The phialides undergo continuous rounds of mitosis to form a string of uninucleated conidia. The conidia are arrested in G1 and are dormant until appropriate nutritive conditions are obtained. It is important to note that mitosis is regulated differently in each of these cell types and needs to be tightly coordinated with the development of the conidiophore. Therefore, mitotic regulatory proteins, Cdk1 and NIMA, might be expected to play a central role during this process. Consistently, the expression as well as kinase activity of both the mitotic kinases is upregulated during condidiophore formation and is under the regulation of the transcription factor, brlA, which is an important regulator of vesicle formation (Ye, Lee et al. 1999). It has also been recognized that the movement of the nucleus into the metulae and phialides is a microtubule dependent process since it requires the activity of the dynein heavy chain (nudA in A. nidulans) (Osmani 1994). Given our results showing that NIMA has an important role in regulating microtubule dynamics through the cell cycle and its requirement for mitosis, we expect NIMA might have an essential function during conidiophore formation.

In addition, another characteristic feature of conidia generation from phialides is the tight coupling of nuclear division with septation, such that every mitosis is followed by septation. This is in contrast to vegetative hyphal cells where septation is only loosely coupled with nuclear division, such that multiple nuclei are partitioned within cell compartments (Figure 1.1). Therefore, a
dramatic change in the coordination between cell cycle and septation needs to happen during the switch from mycelial growth to conidiophore formation. Recent evidence from the lab shows that NIMA locates to forming as well as formed septa in hyphal cells. However, little is known about the function of NIMA in the regulation of septation. Therefore, it is important to note that my studies presented here have uncovered two key players that function with NIMA in septation regulation. An-vps23 is an essential component of the ESCRT pathway and its deletion results in the abnormal localization of NIMA to forming septa (Chapter 3). Therefore the ESCRT pathway is likely to play important roles in septation in hyphae, analogous to its proposed functions in cytokinesis in higher eukaryotes (Chen, Hehnly et al. 2012). Moreover, haploid strains deleted for An-vps23 (as well as other ESCRT genes) show extremely poor conidiation and so can only be propagated as heterokaryons. These conidiation defects are consistent with a positive role for the ESCRT pathway in septation which becomes critical during the generation of uninucleate conidia.

In addition, we also find that the loss of Set1 complex function promotes pre-mature septation in cells that lack NIMA function (Chapter 4). This was unexpected as these cells show an obvious growth defect and have a single nucleus (septation is promoted in Wt cells only after 3 rounds of mitosis). Moreover, Shg1, a Set1 complex subunit locates to septa in the filamentous fungus, Neurospora crassa (Lai, Koh et al. 2012). Therefore, the Set1 complex likely has conserved functions in septation. Thus my studies offer exciting leads
in the form of a positive regulator of septation (the ESCRT pathway) and a potential negative regulator of septation (the Set1 complex), both of which work together with NIMA. In the future, it would be informative to explore the function of these proteins in relation to NIMA’s requirement during asexual development.

NIMA gets hyperphosphorylated at the G2-M transition in a manner dependent on Cdk1 function, autophosphorylation and possibly an unidentified kinase (Osmani and Ye 1996). This mitotic form of NIMA is essential for promoting mitosis. Our results from studies involving cells with partial NIMA function have allowed us to delineate for the first time the sub aspects of mitosis that NIMA is required for. As summarized in Figure 6.1C, we find NIMA to be essential for almost every step in mitosis – spindle pole body (SPB) disjunction, nuclear pore complex disassembly, kinetochore segregation and nuclear envelope restrictions (Chapter 5). Moreover, the defects that were observed in these mitotic events correlate well with the localization of NIMA to each of these locations. Therefore, our data enables us to relate the location studies of NIMA with its functions. Given the different mitotic events that require NIMA function and NIMA’s dynamic localization, it might be possible to separate these different mitotic functions of NIMA by performing a domain analysis of NIMA. For instance, such a domain deletion analysis has the potential to reveal the domain of NIMA that may be involved in locating it to SPB vs. NPCs. Affinity purifications of these domains and comparing the proteins pulled down could lead us to the proteins that are important for NIMA to locate correctly. Early work on NIMA domains
showed the presence of sequences that are required for NIMA degradation and mitotic exit (Pu and Osmani 1995). Also recent work on a NIMA binding protein, TINA, has shown that NIMA does not locate to the SPBs of G1 nuclei in the absence of TINA ((Osmani, Davies et al. 2003), Shen et al. unpublished). We might expect that the deletion of the NIMA domain that binds TINA might phenocopy this failure to locate to G1 SPBs.

In fact, we suspect that the localization of NIMA to G1 SPBs may be significant for its recruitment to the plus ends of Mts in interphase. Plus end binding proteins like Eb1 and Kar9 have been shown to be loaded on to polymerizing Mt plus ends at the SPB in *S. cerevisiae* (Cuschieri, Miller et al. 2006). Hence G1 SPBs may likewise serve as a loading zone for NIMA as it transitions into its interphase function. In further support of this idea, we find that the regulatory domain of NIMA expressed under non-repressing and non-inducing conditions, which exhibits dominant negative behavior, constitutively localizes to SPBs and to foci that move away and towards the SPB at a rate less than the published Mt growth rate in *A. nidulans* (Sections 3.2.1.6 and 3.2.1.7), consistent with the dominant negative nature of its behavior (Chapter 3).

With regard to the mitotic functions of NIMA, we note that the deletion of the AAA-ATPase, *An-vps4* is synthetic lethal with partial loss of NIMA functions (Chapter 3). Interestingly, absence of *An-vps4* by itself causes chromatin segregation defects suggesting defects in mitotic progression. Emerging evidence from higher eukaryotes indicates that Vps4 might have a mitotic
function distinct from its function in the ESCRT pathway, since Vps4 depleted cells show centrosome amplification and multipolar spindle formation (Morita, Colf et al. 2010). Given our finding that reducing NIMA function causes monopolar spindle formation (a phenotype opposite to the depletion of mammalian Vps4), it is possible that the synthetic lethality between nimA and An-vps4 is due to enhanced defects in regulating the spindle pole body. Therefore, further examination of the mitotic defects in Vps4-null cells and cells that lack Vps4 in addition to adequate NIMA function by live cell imaging, especially with regard to the regulation of the spindle pole body during mitosis has the potential to give novel insights into the conserved interplay between these two enzymes in mitotic regulation.

In addition, it would be of importance to determine the mechanistic basis of the involvement of Set1 complex with NIMA and Cdk1 in mitosis. Our data indicate that the Set1 complex is essential for mitotic entry in cells with reduced Cdc25nimT (and hence Cdk1) function (Chapter 4). One possible mechanism by which the Set1 complex and Cdk1 kinase might function together to initiate mitosis is if the Set1 complex had a role in activating Cdk1. Then we might expect that in the absence of Set1 complex function, cells have partially impaired Cdk1 function. The combination of this defect in Set1-null cells with partially reduced Cdk1 function (due to reduced cdc25nimT function) might underlie the failure to transition from G2 into M correctly in these cells. Cells with partial NIMA initiate mitosis but fail to complete it successfully because of lack of normal NIMA

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function. Therefore, when Set1-null cells that potentially have partially impaired Cdk1 function also carry a mutation that results in partial NIMA function, then mitosis is promoted in these cells in the absence of adequate NIMA as well as Cdk1 (Appendix A, Figure A.3). This could potentially give rise to the enhanced mitotic defects we see in cells that lack Set1 complex function in addition to having reduced NIMA function. In addition we also find that lack of Set1 complex function or reduced cdc25nimT function results in a marked sensitivity to drugs that cause DNA damage. Therefore the delay shown by the cells that lack Set1 complex function in addition to having reduced Cdc25nimT function in initiating mitosis could possibly stem from an inability to recover from naturally occurring DNA damage in the cells in the absence of external DNA damaging agents (see Chapter 4 for details).

Another mechanistic basis for the spindle assembly checkpoint-monitored mitotic defects in cells lacking Set1 complex function in combination with reduced NIMA function is a misregulation of kinetochore function. Defects in kinetochore structure and kinetochore-Mt attachments can lead to the activation of the spindle assembly checkpoint. Studies have shown that methylated H3K4 is important for maintaining centromere identity (and therefore for kinetochore structure) and that the human NIMA ortholog can regulate the kinetochore protein, Hec1 (Ndc80) by phosphorylation (see Chapter 4 for details) (Chen, Riley et al. 2002, Bergmann, Rodriguez et al. 2011). We hypothesize therefore, that the absence of Set1 mediated H3K4 methylation at the centromere may
become essential in mitosis in the absence of adequate NIMA mediated phosphorylation of kinetochore proteins. This can be tested in the future by analyzing the integrity of the kinetochore in H3K4R strains that also lack adequate NIMA function by biochemical and microscopic approaches. Moreover, insights gained from the NIMA domain analysis described above can also be used to potentially define the mitotic function of NIMA, the loss of which causes the synthetic interaction with the Set1 complex. An alternate possibility is that the Set1 complex function is required for regulated gene expression of important regulators of mitosis. To assess that in the context of the cell cycle, global gene expression studies can be undertaken in cells that lack Set1 mediated H3K4 methylation at different stages of the cell cycle.

Overall, my studies reveal a novel cytoplasmic function for NIMA in interphase and a requirement for NIMA to successfully complete multiple mitotic events, while also uncovering new key players that function along with NIMA to fulfill its functions in interphase cell growth (ESCRT pathway), mitosis (Set1 complex, An-vps4) and septation (ESCRT and Set1). Furthermore, these findings also raise exciting questions to be explored in the future as well as allow us to propose a testable model for integrating NIMA’s interphase and mitotic functions (Figure 6.1).
Figure 6.1: A model for integrating the interphase and mitotic functions of NIMA.

(A) In interphase, NIMA locates to the plus ends of Mts and regulates Mt dynamics. In addition, interaction between cell tip localized NIMA and Eb1 (indicated by arrow) ensures the correct targeting of Mt plus ends to the cell tip.

(B) Just before mitotic entry, phosphorylation of NIMA by Cdk1 causes the dissociation of NIMA from Eb1, which potentially allows the mitotic disassembly of cytoplasmic Mts, while simultaneously promoting the mitotic specific localization and functions of NIMA.
Figure 6.2: Schematic representation of the localization and functions of NIMA through mitosis. NIMA is indicated in green at its different mitotic locations. The proposed function for NIMA at each step is labeled in blue.
References


"CLASP1 and CLASP2 bind to EB1 and regulate microtubule plus-end dynamics at the cell cortex." J Cell Biol 168(1): 141-153.


Figure A.1: NIMA and the ESCRT pathway work in concert to maintain apical dominance and thus polarized growth. See Section 3.3.4 for details.
Figure A.2: The Set1 complex functions in the G2-M transition. (A) In Wt cells, Cdk1 function is predicted to increase slowly and then undergo a biphasic mode of activation, as shown by the blue trace, to promote mitotic entry. (B) As in Wt cells, cells lacking Set1 complex function are also predicted to show normal biphasic activation of Cdk1. (C) Cells that have partially reduced function of nimT23 would be expected to take longer to attain the threshold level of Cdk1 function that would allow its biphasic activation and therefore exhibit a delay in G2 compared to Wt cells (dotted line). (D) Our results (detailed in Chapter 4) indicate that when the Set1 complex function is absent in addition to having partially active Cdk1, then there is a pronounced G2 delay suggesting that the activation of Cdk1 may take a lot longer (as depicted in the graph, red dotted arrow) than nimT23 cells. However we find that when mitosis is eventually triggered, it is completed successfully, suggesting that the subsequent biphasic activation of Cdk1 occurs normally. See Section 4.3.1 for details.
Figure A.2

A

WT

CDK1 Activity

G2

M

Time

B

ΔSet1 complex

CDK1 Activity

G2

M

Time

C

nimT23

CDK1 Activity

G2

Time

D

nimT23 + ΔSet1 complex

CDK1 Activity

G2

M

Time

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Figure A.3: The Set1 complex and NIMA regulate mitotic progression. Our results (as described in Chapter 4) show that cells that lack the Set1 complex function in combination with partial NIMA function exhibit enhanced defects in mitotic progression. One potential explanation for the above result is that the Set1 complex might be playing novel mitotic roles together with NIMA in ensuring successful mitotic completion (black arrows). Alternatively, in the light of our analysis of the genetic interaction between the Set1 complex and Cdk1 which indicates that the Set1 complex has functions in promoting G2-M transition (black dotted arrow), it is possible that the abrogation of the function of the Set1 complex in G2-M transition might in combination with partial NIMA function cause more pronounced mitotic defects. See Chapter 6 for details.
Figure A.4: Partial NIMA function allows entry into mitosis but causes mitotic defects. (A) In Wt cells, Cdk1 function is predicted to increase slowly and then undergo a biphasic mode of activation (blue trace). Based on previous studies, NIMA activity (green trace) would be expected to increase to a basal level and then NIMA gets further activated in a Cdk1 dependent manner (Ye, Xu et al. 1995). Activation of both NIMA and Cdk1 is known to be required for mitotic entry. (B) Previous studies have shown that when NIMA function is inhibited using a temperature sensitive nimA allele at the restrictive temperature, then mitotic entry is blocked even though Cdk1 is fully active (Osmani, McGuire et al. 1991). This result is represented in graphical format in (B). (C) We hypothesize that in nimA7 cells at the semi permissive temperature, the partial function of NIMA (green trace) might allow mitotic entry owing to the fully functional Cdk1 (blue trace). However, since NIMA function is inadequate, mitotic errors ensue. See Section 5.3.1 for details.
Figure A.4

A: WT, M → NIMA
CDK1
G2
Time

B: Restrictive temperature
M
NIMA
G2
Time

C: Semi permissive temperature
M
NIMA
G2
Time

Mitotic defects
Figure A.5: NIMA is required for normal mitotic nuclear pore complex disassembly. This figure shows the cross-section of a nuclear pore complex. (A and C) In Wt and nimA7 cells in G2 at the semi permissive temperature, the nuclear pore channel is expected to be lined by peripheral nuclear pore complex proteins (red wavy lines) that mediate active transport. (B) In Wt cells, the peripheral nuclear pore proteins are known to disperse into the cytoplasm, resulting in the abolishment of active transport and the free diffusion of molecules in and out of the nucleus. This is represented schematically in (B). (D) Our results (described in Chapter 5) indicate that in cells with partial NIMA function, the majority of the peripheral nuclear pore complex protein Nup49 remains at the nuclear periphery and the mitotic nuclear pores are opened just enough to allow small proteins, like dimeric tubulin-GFP (~130kD) and HP1 (~50kD) to diffuse in and out of the nucleus but not larger proteins like the NLS-DsRed tetrameric complex (predicted to be several hundred kDs). These data indicate that cells with partial NIMA function do not disassemble all peripheral nuclear pore complex proteins during mitosis and this hypothesis is represented schematically in (D). See Section 5.3.2.1 for details.
Figure A.5