SYSTEMATIC ANALYSIS OF PHOSPHATASE GENES IN *ASPERGILLUS NIDULANS* AND A ROLE OF FCP1 IN CELL CYCLE REGULATION

DISSERTATION

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ABSTRACT

Many critical steps of cell cycle control had been shown to involve the reversible phosphorylation of proteins, thus regulating their activities and subcellular localizations. While much research has been focused on the functions of various protein kinases concerning cell cycle progression, relatively less is understood how protein phosphatases counteract the activities of those kinases. In Aspergillus nidulans, 28 different genes that encode protein phosphatase catalytic subunits have been identified to be present in its genome by sequence homology search. A systematic deletion analysis of these protein phosphatase catalytic subunit genes was carried out to identify protein phosphatases that are involved in mitotic regulation. Of the 27 different genes that were examined, the deletion of four phosphatase encoding genes proved to be lethal, while the deletion of another four resulted in severe growth defect phenotypes. Of those eight genes, nimT and bimG were confirmed to have essential functions required for mitotic progression as their deletions led to classical G$_2$ and M phase arrest phenotypes. While not as obvious, An-pphA and An-nem1 deleted cells demonstrated mitotic abnormalities as well, with likely defects in mitotic entry (An-pphA) and DNA segregation (An-nem1).
One of the essential phosphatase genes in A. nidulans that was further examined for its potential function in cell cycle regulation was An-fcp1. In addition to being confirmed as a phosphatase with an essential function through the deletion analysis, a conditional mutant allele of An-fcp1 was isolated in a genetic screen showing a strong synthetic interaction with the CDK1F mutation. CDK1F is a hyperactive kinase mutant incapable of inhibitory phosphorylation of the tyrosine (Y) 15, and the synthetic effect of the genetic interaction shown between the loss-of-function phosphatase mutation An-fcp1<sup>TS</sup> and the activated kinase mutation CDK1F suggested an elevated level of phosphorylation on a common substrate. An-fcp1 had been known to target the RNA polymerase II C-terminal domain (RNAPII CTD), and the phosphorylation level of RNAPII CTD was indeed elevated in the An-fcp1<sup>TS</sup>+CDK1F double mutant, confirming that the synthetic interaction of An-fcp1<sup>TS</sup> and CDK1F at least be partially mediated via the abnormal phosphorylation of RNAPII CTD. Cytologically the An-fcp1<sup>TS</sup>+CDK1F double mutant was marked by severe mitotic defects ranging from lagging nuclei to inability of nuclei to exit mitoses. The severity of the mutant phenotype in the An-fcp1<sup>TS</sup>+CDK1F double mutant contrasted from the phenotypes of either the An-fcp1<sup>TS</sup> or CDK1F single mutants, confirming the synthetic nature of their genetic interaction. Also, the distinctively mitotic defects of the An-fcp1<sup>TS</sup>+CDK1F double mutant strongly suggests an important function of FCP1 in regulating mitotic progression beyond its known activity of RNAPII CTD dephosphorylation.
DEDICATION

This work is dedicated to my parents and my brother.
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CHAPTER 1

INTRODUCTION

1.1. PROBLEM STATEMENT

To assure the sustained survival of their cells, eukaryotic organisms have developed a highly conserved control mechanism to maintain and pass down their genetic information in an accurate and faithful manner over the entire processes of cellular growth and division. This coordinated control cycle of cellular growth, replication and segregation of genetic information in eukaryotic cells is referred to as the eukaryotic cell division cycle.

Many critical steps of cell cycle regulation have been shown to be controlled by reversible protein phosphorylation/dephosphorylation. Accordingly, numerous protein kinases have been identified as important regulators of the cell cycle.
progression. In contrast, relatively little is known about the cell cycle functions of the enzymes that counteract the protein kinase activities, the protein phosphatases. Also, while the cyclin dependant kinase CDK1 has been firmly established as the critical factor determining onset of mitosis, studies conducted in different eukaryotic organisms suggest that the inhibitory tyrosine15 phosphorylation of CDK1 may not be the only means of mitotic entry checkpoint control. The overall goal of this work is to examine potential mitotic regulatory functions of all protein phosphatase catalytic subunits identified in A. nidulans, and to identify mutations that demonstrate synthetic interactions with the CDK1F mutation and investigate their potential role in cell cycle.

The specific aims of the studies in this dissertation are: i) to conduct a systematic deletion and localization analysis of all identified protein phosphatase catalytic subunits in A. nidulans, ii) to conduct a synthetic lethality genetic screen with the CDK1F mutation, iii) to identify and characterize mutations obtained through that screen, and iv) to investigate the cell biological nature of the synthetic lethality of these mutations with CDK1F.
1.2. ASPERGILLUS NIDULANS

1.2.1. Classification and general description

*Aspergillus nidulans*, also known as *Emericella nidulans*, is a filamentous fungus extensively described first in the late 19th century by Eidam (Eidam, 1883). The genus *Aspergillus* belongs to the phylum Ascomycota and includes other species of filamentous fungi such as *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus oryzae* and *Aspergillus niger*. Being obligate aerobic organisms, *Aspergilli* are found ubiquitously in virtually all oxygen-rich environments throughout nature. Many species of *Aspergillus* play important roles in areas ranging from medicine to food industry and scientific research. As human pathogens *A. fumigatus* and *A. flavus* can cause a range of fungal infectious diseases collectively termed Aspergillosis. *A. oryzae* has long been used in many East-Asian cuisines for converting plant starch into sugars during the production of various fermented food items, while *A. niger* accounts for most of the industrial citric acid production world wide. In the field of biological sciences, *A. nidulans* is an important organism extensively utilized as an experimental research system, particularly for studying eukaryotic molecular genetics and cell biology. This dissertation describes my research regarding the regulation of the eukaryotic cell division cycle employing *A. nidulans* as a model system.
1.2.2. Life cycle

1.2.2.1. Asexual development

The asexual life cycle starts when a uninucleate haploid cell called a conidiospore initiates polarized cellular growth by germination (Figure 1.2A, B). The process of germination starts with the isotropic growth phase when the conidiospore swells up rapidly and nuclear DNA decondenses, followed by a phase of polarity establishment (PE) and polarity maintenance (PM) as a germ tube is being projected into one particular direction. Continued polarized growth accompanied by several rounds of parasynchronous mitoses results in the formation of a multinucleate germling. Once an 8 to 16 nuclei stage has been reached (through 3 to 4 rounds of mitosis), a septum is formed that physically divides the cytoplasm into different compartments (a process equivalent to cytokinesis). Nuclei within the compartment distal to the growing tip cease to undergo mitosis, while those nuclei proximal to the growing tip will continue to mitose until further septation separates them from the growing compartment. When favorable environmental conditions allow for continued growth, a compartment that initially ceased to engage in mitoses can resume polarized growth and nuclear division by projecting a new branch in an angle roughly perpendicular to the length of the original germling. In a liquid environment *A. nidulans* will continue this growth pattern to form a hyphal network. Once exposed
to air – as in growth on solid matter – specialized cell types called foot cells are formed within hyphae. This cell type differentiation leads to the developmental transition into the formation of the asexual reproductive structures called conidiophores (Figure 1.2C). As a foot cell re-engages in cell division, an aerial hypha will branch off it to give rise to a bulge-shaped vesicle, from which sequentially primary and secondary sterigmata are formed through the process of budding division. At the apex of a conidiophore secondary sterigmata undergo repeated rounds of budding division to produce chains of new conidiospores and thus complete the asexual life cycle. Conidiospores are mononucleate cells that remain metabolically dormant until environmental conditions favor initiation of vegetative, polarized growth. The large number of these conidiospores at the apex of the conidiospores and their pigmentation are what give A. nidulans colonies their distinct color and “powdery” surface appearance. While the color of conidiospores of wild type strains is green, mutations in different genes result in colors of yellow, chartreuse, white and fawn, which provide readily visible markers useful for genetic analyses.

1.2.2.2. Sexual development

The sexual life cycle of A. nidulans can be divided into 3 main steps; heterokaryon formation by hyphal fusion, diploid formation by karyogamy, and
haploidization through meiotic cell division. As a homothallic fungus *A. nidulans* is capable of hyphal fusion (anastomosis) and nuclear fusion (karyogamy) to undergo self-crossing between two organisms of the same strain as well as out-crossing between two organisms of different genetic make-ups. Out-crossing of two strains is induced when two *A. nidulans* strains that have mutually exclusive nutritional requirements grow together in an environment that is lacking in the nutritional requirements of both strains. As meiotic recombination of the auxotrophic markers could produce a progeny strain that is prototrophic – unlike either of the parent strains – regarding the condition of that particular environment, the two parent strains are effectively "forced" to engage in sexual development. Initiation of sexual development is marked by hyphal fusion between the two organisms to form heterokaryons (Figure 1.1). Heterokaryon formation defines a transient developmental state in which two unlike nuclei share a common cytoplasm to overcome their mutually exclusive limitations of nutritional requirement. Dikaryons are formed when septation separates two unlike nuclei into one septal compartment, and subsequent karyogamy between these nuclei results in transient diploid nuclei. Each diploid nucleus undergoes one round of meiosis followed by one round of mitotic cell division, giving rise to eight haploid cells. This process occurs within developmentally specialized cells called asci, and the individual haploid progenies are termed ascospores, with each ascus
containing eight ascospores. Ascospores undergo one more round of mitoses absent of cytokineses, thus a mature ascospore is a binucleate cell. Multiple asci are arranged in a nonlinear fashion within fruitbodies called cleistothecia, where each cleistothecium can contain more than 10,000 ascospores.

1.2.2.3. Parasexual cycle

Although transient in nature, heterokaryons can be maintained under laboratory conditions for research purposes. At a certain frequency, heterokaryons can generate stable diploid nuclei within vegetative hyphae when nuclear fusion occurs without the formation of specialized structures required for sexual development Figure 1.1). Haplodization of these stable diploid cells to reproduce the parental haploid homokaryons can be experimentally induced by treatment with microtubule poisons such as benomyl or nocodazole. This process of haploidization of diploid nuclei in the absence of meiotic cell division is referred to as the parasexual life cycle (Pontecorvo, 1945). Forming a stable diploid organism is a genetic tool useful in determining whether mutations are recessive or dominant, and since chromosome segregation is independent of cross-over during haploidization, the parasexual life cycle has traditionally been used in mapping genes to linkage groups.
1.2.3. A model organism for biological research

*A. nidulans* was described as a potential model organism for cell biological research first by the geneticist Pontecorvo in 1945 (Pontecorvo, 1945). Over sixty years later today, *A. nidulans* has firmly established itself as a powerful experimental research system for various biological fields of study including cell cycle research.

Several aspects of *A. nidulans* as an organism distinguish it as an inviting system for cell cycle research. As a eukaryote it has a rather short cell cycle time, high growth rate, and well developed genetics with a large pool of mutations affecting various aspects of the cell cycle (Bergen and Morris, 1983). Under favorable growth conditions *A. nidulans* completes the cell cycle in about 100 minutes while spending 15 minutes in G\(_1\) phase, 40 minutes in S phase, 40 minutes in G\(_2\) phase and 5 minutes in M phase (Bergen and Morris, 1983). Conidiospores can be maintained for long periods in dormancy without significant loss of viability. Since it is important to accurately determine time points in cell cycle progression experiments, the consistent time frame of conidiospores reengaging in vegetative growth after inoculation into growth media is useful in designing cell cycle staged experiments (Champe and Simon, 1992). The pattern of nuclear division in *A. nidulans* is also an accommodating feature particularly for studying mitosis. In *A. nidulans* nuclear division occurs in a parasynchronous
manner where all mitotic divisions within a single septal compartment occur almost simultaneously. Starting with the nucleus most proximal to the apical tip of each hypha, nuclear division proceeds along the length of the hyphae in a rapid wave (Rosenberger and Kessel, 1967; Rosenberger and Kessel, 1968). Thus, as a spore germinates and engages in polarized growth, by simply counting the number of nuclei present one can easily determine the mitotic history of that cell. *A. nidulans* can grow over the wide range of temperatures from 15°C to 44°C. This facilitates the isolation of conditional mutations based on heat sensitivity and cold sensitivity phenotypes, an important tool for studying the function of essential genes (Doonan, 1992). The ability of *A. nidulans* to form transient heterokaryons and stable diploid cells allows for easy complementation analyses to determine dominance/recessiveness of mutations and investigation of terminal phenotypes of essential gene deletions (Todd et al., 2007; Osmani et al 2006b). The lack of requirements for mating types in *A. nidulans* and the availability of color and auxotrophic mutations as genetic markers makes it feasible to generate desirable strains by forcing meiotic recombination via sexual out-crossing as well. The ability to introduce foreign DNA through targeted integration into the recipient genome via homologous recombination allows for easy complementation of mutant phenotypes. From a cytological standpoint, Robinow and Caten demonstrated in 1969 that *A. nidulans* shows the common hallmark features of eukaryotic mitosis
observed in other organisms. In 1976 Ron Morris chose *A. nidulans* as the model organism in which to conduct a temperature sensitive screen aimed at identifying mutations with defects in cell cycle progression (Morris, 1976). Based on direct observation of nuclear and spindle morphology using techniques established by Robinow and Caten, mutants were classified into three groups according to nuclear morphology, nuclear distribution and the ability to form mitotic spindles (Robinow and Caten, 1969; Morris, 1976). The defining phenotypes of the three classes of mutants identified were 1) defective mitoses 2) lack of septa 3) abnormal nuclear distribution. Mutants with mitotic defects were subdivided into two groups according to their mitotic indices. Cells that could not enter mitosis at restrictive temperatures were termed *nim* (*never in mitosis*) mutants. Cells that were arrested in mitosis were termed *bim* (*blocked in mitosis*) mutants. Cells with nuclear distribution defects were termed *nud* (*nuclear distribution*) mutants (Morris, 1976). The isolation of the nine *bim* mutants in six genes and twenty six *nim* mutants in twenty three genes proved to become the foundation for future studies of mitotic regulation in *A. nidulans* (Osmani and Mirabito, 2004).

Additionally, traditional methods of cell cycle arrest and synchronization utilizing hydroxyurea and anti-tubulin drugs such as nocodazole and benomyl can be readily and effectively applied to *A. nidulans* cultures (Bergen and Morris 1983). Protocols for visualization of cellular contents such as DNA by DAPI and proteins
by immunofluorescence are well established. Vector based expression systems are available for exogenous protein expression. An alcA promoter based system allows for suppression and induction of protein expression by altering the media carbon source (Waring et al., 1989; Doonan et al., 1991). An autonomously replicating plasmid based on the AMA1 sequence allows for high efficiency complementation of recessive mutations and recovery of the complementing DNA (Aleksenko et al., 1996; Aleksenko and Clutterbuck, 1997). The complete sequencing and annotation of the A. nidulans genome with the results compiled as a user friendly database readily available on the world wide web, dramatic improvement of accurate gene targeting frequency by utilizing a Δku70 strain (Nayek et al., 2006), availability of cell cycle specific fluorescent protein fusions as cytological markers further make A. nidulans a powerful model organism for cell cycle research. The recent publications of standardized protocols for generating gene-targeting constructs (Szewczyk et al., 2006) and identification and analysis of essential genes (Osmani et al., 2006b) are examples of additional efforts to make A. nidulans an even more amenable model organism for conducting biological research.
1.3. THE EUKARYOTIC CELL DIVISION CYCLE

1.3.1. Overview

For cells of any living organism, it is imperative for their survival to fully and accurately maintain their genetic information as they grow and faithfully pass it down to the daughter cells as they divide. The cell division cycle (cell cycle in short) is the cyclical process of cells coordinating their growth, replication and segregation of their genetic information so as to duplicate their full complement of cellular contents and divide into viable daughter cells. Generally the eukaryotic cell cycle can be divided into interphase and M-phase. During interphase the cells grow in size, accumulate nutrients and replicate the genome, while M-phase accounts for the period when segregation of the duplicated cell contents – most critically the genetic information – is carried out. Under normal circumstances eukaryotic cells spend most of their time in interphase engaged in cellular growth as M-phase is limited to a short period when separation of the chromosomes (mitosis) is followed by the partitioning of the cytoplasm (cytokinesis) resulting in two daughter cells.
1.3.2. Interphase

Interphase is the period of the cell cycle during which a cell prepares itself for the next division into daughter cells. It is composed of three phases; $G_1$ (gap1), $S$ (DNA synthesis) and $G_2$ (gap2) phase. Upon exiting M-phase of a previous cell cycle by completing cytokinesis, a cell progresses through the $G_1$ phase marked by elevated rates of overall biosynthetic activities leading to the increase of its size and essential cellular contents such as organelles and nutritional factors. Once sufficient growth has been achieved, the $G_1$ phase ends with the cell committing itself into the S phase. If unfavorable environmental conditions do not allow for the $G_1$ phase to be completed, cells can exit the active cell division cycle and enter a suspended state of mitotic dormancy called the $G_0$ phase, only to re-engage in cell division activities when favorable environmental conditions return. The S phase is defined as the cell cycle phase during which the chromosomal DNA is replicated in its entirety. To ensure that chromosomal replication occurs only once during each round of cell division, commencement of DNA replication at chromosomal replication origins is tightly regulated. Different S phase cyclin-dependant kinases (CDKs) and other licensing factors control the formation of the Origin Recognition Complexes (ORCs) and the Minichromosome Maintenance complexes (MCMs) required for initiation of DNA replication. Once synthesis of chromosomal DNA under progress these multiprotein complexes are transformed into post-replication
complexes that do not allow replication origins to re-engage in their activities until the S-phase of the next cell cycle. Pre-replication complexes are reestablished following the inactivation of mitotic CDK/cyclin complexes of the prior cell cycle. Completion of the S phase is followed by the second gap phase, the $G_2$ phase. The main function of the $G_2$ phase is to ensure the readiness of the cell for the following M-phase. Damage to the DNA or incompletion of DNA replication are recognized by various checkpoint protein complexes that retain the major mitotic CDK/cyclin complexes in their inactive form, thus preventing premature onset of mitosis.

1.3.3. M-phase

M-phase is the period of the cell cycle during which the physical partition of the cellular contents is attained resulting in two identical daughter cells. It is comprised of two phases; mitosis and cytokinesis. Mitosis is defined by the segregation of the chromosomal DNA to two qualitatively identical daughter nuclei. As nuclear division occurs, dynamic rearrangement of nucleocytoplasmic macrostructures can be observed via light and electron microscopy. According to these hallmark mitotic events such as chromatin condensation, nuclear envelop breakdown/nuclear pore complex disassembly and mitotic spindle formation, mitosis is further divided into the five different stages of prophase, prometaphase, metaphase, anaphase and telophase. Prophase is distinguished by the
condensation of chromatins to form highly structured chromosomes. As the chromosomal DNA is duplicated during S phase, identical chromatids attached at their centromere region via cohesin protein complexes form a pair of chromatids called sister chromatids to make up a mitotic chromosome. The breakdown of the nuclear envelope marks the transition into the next stage, the prometaphase. With the nuclear envelope as a physical barrier separating the nucleoplasm and the cytoplasm removed, originally cytoplasmic mitotic spindles are able to search and attach to the kinetochores of chromatids via kinetochore microtubules. The prometaphase is complete once every chromosome has the kinetochore microtubules properly attached in a bipolar fashion with the two sister chromatids each having mitotic spindles attached that are projecting from opposite spindle poles. In the following metaphase, chromosomes align forming the metaphase plate in the middle of the cell. This arrangement is maintained by the balance of the forces generated by the mitotic spindles pulling into opposite directions toward the two spindle poles. Only when all chromosomes have completed their bipolar spindle attachment and are properly aligned along the metaphase plate can the cell progress into anaphase. The cytological onset of anaphase is characterized by the separation of the sister chromatids. As a consequence of the activation of the anaphase promoting complex, the cohesin proteins holding the sister chromatids together are degraded and the chromatids start to move toward the opposite
spindle pole bodies. Subsequently the spindle poles move further apart through the relative elongation and sliding of the spindle microtubules. During telophase, the separated chromatids move closer to the poles and nuclear envelopes are reassembled around the chromosomes at each pole, followed by the decondensation of the DNA. The M-phase and thus the entire cell cycle is completed when cleavage of the cytoplasm during cytokinesis separates the newly formed daughter nuclei into two daughter cells.

1.3.4. Cell cycle regulation

A hallmark characteristic of the eukaryotic cell cycle is the strict dependence of cell cycle progression upon the completion of preceding events. In 1989, Leland Hartwell and Ted Weinert articulated this feature of sequential dependence through the concept of “checkpoint” control (Hartwell and Weinert, 1989). They argued that at certain critical transitions, inhibitory control mechanisms stipulate the successful completion of prior events as conditions for the cells to progress into the next stage of the cell cycle, thus functioning as “checkpoints” (Hartwell and Weinert, 1989). Evidence drawn from the vast body of work on cell cycle research conducted over the last two decades overwhelmingly confirm checkpoints as the principle means of cell cycle regulation. As in part described earlier, there are three main checkpoint control steps identified in the eukaryotic cell cycle. At the G₁
to S phase transition, cells will only proceed into S phase when certain growth and environmental requirements are met, while they otherwise exit the cell cycle and enter the stationary G₀ phase. At the G₂ to M phase transition, the DNA replication and DNA damage checkpoints prevent any cells from entering M phase unless DNA replication and DNA damage repair have been completed. During mitosis, the spindle assembly checkpoint prevents any cells from progressing into anaphase unless sister chromatids are aligned properly along the metaphase plate with correct bipolar spindles attached. The main focus of the eukaryotic cell division cycle is the faithful maintenance and transmission of the genetic information over the entire course of cellular growth and division. The instatement of the checkpoint controls at the most critical junctions of the cell cycle illustrate the efficient and highly effective manner in which this demanding task is achieved.

1.4. CDK1 PROTEIN KINASE

1.4.1. Identification of cyclin dependant kinases

By the end of the 1960s, it had been demonstrated that treatment with the steroid hormone progesterone could trigger the onset of meiosis in the eggs of the frog *Rana pipiens* (Masui, 1967). In 1971, Masui and Market further established the fact that injection of the cytoplasm taken from those eggs induced with
progesterone into inactive oocytes led to effective meiotic activation in the recipient oocytes as well. Concluding there being an entity ultimately triggering these effects, they termed this hypothetical factor the Maturation Promoting Factor (MPF). Independent of the efforts to identify the physical nature of the MPF, Hunt and colleagues isolated a protein from the sea urchin oocytes the cellular abundance of which showed a dramatic pattern of fluctuation along the cell cycle. Its expression level continued to rise to reach a pique at mitosis, only to be destroyed right around each cleavage division (Evans et al., 1983). According to the cyclical change of expression level, they proposed to call this protein a cyclin and further suggested a potential role of it in cell cycle oscillation. The critical function of cyclin proteins in cell cycle progression was confirmed when Swenson et al. demonstrated that when injected into Xenopus oocytes, mRNA of clam cyclinA was translated into proteins and drove the cells into meiosis. Efforts to investigate cell cycle regulation by means of classical genetics were led mainly by Leland Hartwell and Paul Nurse. In the 1970s, by conducting separate temperature sensitive mutant screens in the yeast species Saccharomyces cerevisiae (Hartwell) and Schizosaccaromyces pombe (Nurse), both were able to isolate a series of cdc (cell division cycle) mutants with cell cycle defect phenotypes at restrictive temperatures (Hartwell et al., 1974; Nurse et al., 1976). One of the mutations identified in S. pombe was cdc2, and it was later established that Cdc2
is required for both S phase and M phase initiation (Nurse et al., 1976; Nurse and Bisset, 1981). Successful attempts to complement the temperature sensitive phenotype of the cdc2 mutation with the S. pombe as well as the S. cerevisiae DNA libraries led to the cloning of Cdc2 and confirmed it as the functional homologue of CDC28 of S. cerevisiae (Beach et al., 1982). In 1986, Simanis et al. determined the biochemical function of both Cdc2 and CDC28 as protein kinases with their cellular activity reaching the highest level during M phase (Simanis and Nurse, 1986). With a human Cdc2 cDNA cloned by complementing the S. pombe cdc2 mutation, it became also clear that this principle cell cycle regulator was extremely conserved throughout the eukaryotic system ranging from species of yeast to humans (Lee and Nurse, 1987). Meanwhile, by 1988 the MPF had been defined physically when proteins with molecular weights of 45 kDa and 34 kDa were correlated to a purified fraction from Xenopus eggs exhibiting high MPF activity (Lockha, 1988), and the 34kD protein was subsequently identified as a Xenopus homologue of Cdc2 (Gautier et al., 1988). In addition to Cdc2, the genetic screen conducted in Nurse’s group led to the identification other factors crucial for cell cycle regulation. The study of the cdc13 mutation led to the characterization of an M phase specific cyclin partner of Cdc2 (Booher and Beach, 1988; Moreno et al., 1989), while CDC25 was identified as an activator of Cdc2 and the Wee1 protein kinase was shown to carry out an inhibitory function over mitotic onset
(Nurse and Thuriaux, 1980; Russell and Nurse, 1986; Russell and Nurse, 1987; Gould and Nurse, 1989; Moreno et al., 1990). The terminology for Cdc2, CDC28 and their human counterparts were standardized into CDK1 in 1991 when at the Cold Spring Harbor Symposium on the Cell Cycle the term Cyclin Dependant Kinases (CDKs) was decided as the names to be used for kinases which associated with cyclins.

The last two decades of cell cycle research have seen the identification of many additional CDKs and associating cyclins with varying functions in cell cycle regulation. In yeasts CDK1 alone is known to regulate different steps of cell cycle progression by partnering with nine different cyclins. In mammals, eleven CDKs (CDK1-11) have been identified of which five (CDK1, CDK2, CDK3, CDK4 and CDK6) are considered to have bona fide cell cycle functions with CDK2, CDK3, CDK4 and CDK6 regulating interphase progression by forming complexes with different interphase cyclins. Still CDK1 is considered the main determinant of mitotic onset by forming a complex with cyclinB, and recent data suggests that even in mammalian systems CDK1 is the only essential cell cycle CDK and its activity alone might be sufficient to drive the entire cell division cycle (Santamaria et al., 2007).
1.4.2. CDK1 – checkpoint control and regulation by phosphorylation

As mentioned above, in most eukaryotic systems CDK1 is the main factor determining the cell’s entry into mitosis. In late G$_2$ phase, the cell monitors its readiness for mitotic onset via the DNA replication and DNA damage checkpoint mechanisms. When DNA replication is incomplete or damage to the DNA persists, the CDK1/cyclinB complex is retained in its inactive state unable to initiate mitotic entry. The activity of the CDK1/cyclinB complex concerning mitotic entry is dependent on the phosphorylation status at several key amino acid of CDK1. Signals of incomplete DNA replication or DNA damage converge onto the Wee1/Myt1 kinases which in turn phosphorylate CDK1 at its tyrosin15 residue (in higher eukaryotes also the threonine 14 residue). This inhibitory phosphorylation prevents the CDK1/cyclinB complex activation and blocks the cell from entering premature mitosis (Gould and Nurse, 1989; Norbury and Nurse, 1991; Parker and Piwnica-Worms, 1992; Mueller et al., 1995; Rhind and Russell, 1997; Rhind and Russell, 1998). After the genomic conditions for mitotic entry are satisfied – completion of DNA replication and DNA damage repair, the cell is released from this checkpoint arrest as the protein phosphatase CDC25 activates the CDK1/cyclinB complex by dephosphorylating Tyr15 of CDK1 (Russell and Reed, 1989; Gautier et al., 1991). Although the exact mechanism of the inhibitory Tyr15 phosphorylation is not fully understood, several studies have suggested that Tyr15
phosphorylation may interfere with the affinity of CDK1 to peptide/protein substrates and subsequently decrease its catalytic activity (Marcote et al., 1993; Atherton-Fessler et al., 1993; Welburn et al., 2007). While Tyr15 is the target of inhibitory phosphorylation, phosphorylation of the threonine 161 residue is required for the activation of CDK1 during mitosis (Solomon et al., 1992). Thr161 phosphorylation is carried out by the CDK activating kinase (CAK) activity (Fesquet et al., 1993; Solomon et al., 1994), and while it is not understood as essential for the CDK1 function of mitotic initiation, this phosphorylation persists until later stages of mitosis and has been shown to be required for proper mitotic progression (Solomon et al., 1992).

1.4.3. CDK1F mutant allele

Although the phosphorylation status of Tyr15 is critical in determining the activity of CDK1, results from studies of a mutant allele of Cdk1 suggest that it may not be the only control mechanism regulating mitotic entry. When the tyrosine15 amino acid is converted into phenylalanine (Y15F), the mutant CDK1 protein is no longer capable of inhibitory phosphorylation and remains constantly active. In S. pombe, expression of this mutant allele is known to have a lethal effect as the cells continue to enter uncontrolled, premature mitoses. Nevertheless, in higher eukaryotes the equivalent mutations of CDK1F and CDK1AF (threonine14 to
alanine –T14A – in addition to Y15F) result in only partial phenotypes of premature mitosis without the lethal consequences observed in S. pombe. In A. nidulans the CDK1F mutant strains show a hyperbranching phenotype, elevated sensitivity to UV irradiation and intolerance to moderate levels of hydroxyurea, but are viable under normal growth conditions. In human HeLa cells, the expression of a CDK1AF allele caused low levels of premature DNA condensation and reduced G2 phase delay in response to X-irradiation but otherwise failed to induce mitotic events (Jin et al., 1996; DeSouza et al., 1999). The ability of higher eukaryotic cells to tolerate the absence of CDK1 inhibition via Thr14/Tyr15 phosphorylation strongly suggests the existence of other regulatory pathways for mitotic progression not functioning through inhibitory phosphorylation of CDK1. Part of this dissertation will describe a genetic screen conducted with the aim of identifying such new mitotic control genes and the characterization of a mutation isolated through that screen.

1.5. PROTEIN PHOSPHATASES

1.5.1. Protein dephosphorylation – counteracting protein kinases

Phosphorylation of a protein is defined as the enzyme-catalyzed release of the terminal phosphate group of an ATP molecule and its covalent addition to the
hydroxyl group of serine, threonine, or tyrosine side chains of proteins. The effects of protein phosphorylation can manifest itself mainly in two different ways. As one phosphate group carries two negative charges, its addition to a protein can rearrange the electrostatic equilibrium and thus lead to a significant conformational change of the recipient protein. Given that the structure of a protein determines its function, the simple addition of one phosphate group can cause a fundamental change of the biochemical property of the given protein. Alternatively, the phosphate group added can act as a critical part of the local protein structure recognized by the binding motifs of other proteins. The absence or presence of a phosphate group can dramatically alter the binding affinity of two proteins, and in this way protein phosphorylation may play a key function in regulating multiprotein complex formation. The enzymes driving phosphorylation reactions are collectively referred to as protein kinases, and since the detachment of the terminal phosphate group from an ATP molecule involves the release of a large amount of free energy, phosphorylation catalyzed by kinases is essentially considered a unidirectional reaction. Hence the removal of the added phosphate group to reverse a phosphorylation reaction – dephosphorylation – requires a different set of enzymes called protein phosphatases. In many cellular processes reversible phosphorylation/dephosphorylation reactions play important roles by regulating protein activity and subcellular localization. The extent of protein phosphorylation
is well illustrated by the estimation that on average about one-third of all the proteins in a typical eukaryotic cell are thought to be in a phosphorylated state. As the obligate partners of protein kinases counteracting their function in this regulation circuitry, protein phosphatases have been firmly established as key coordinators of diverse biological events. As a comprehensive description of all protein phosphatases would go beyond the scope of this dissertation, this chapter will limit itself to a brief overview of basic protein phosphatase classification and relevant protein phosphatases involved in cell cycle regulation.

1.5.2. Classification of protein phosphatases

Generally three main criteria are used to classify protein phosphatases; sequence homology, structure and catalytic mechanism concerning substrate specificity. According to these features, protein phosphatases are divided into the three main groups of classical serine/threonine phosphatases, protein tyrosine phosphatases and the aspartate based catalysis protein phosphatase (reviewed in Moorhead et al., 2007).

The classical Ser/Thr phosphatases can be further subdivided into the phosphoprotein phosphatases (PPP) family and the protein phosphatase Mg$^{2+}$/Mn$^{2+}$ dependent (PPM) family. The PPP family is made up of classical members such as PP1, PP2A, PP2B and their related phosphatases. PP1, PP2A
and PP2B were initially identified based on their biochemical properties such as substrate specificity, metal ion requirements and reactivity to phosphatase inhibitors. All members of the PPP family share high sequence similarities and have in common a conserved phosphoesterase catalytic motif, although their biological functions range widely from mitotic regulation (PP1), immune response (PP2B), DNA damage response (PP4) and blue-light signaling in plants (PP7). PP2C type protein phosphatases make up the PPM family and is characterized by its remarkable structural similarity to PPPs despite the lack of any sequence similarity.

The protein Tyr phosphatase (PTP) superfamily is distinguished by its CX₅R catalytic signature motif shared by all of its members. It consists of the classical PTPs, the dual specificity phosphatases (DSPs), the CDC25 type phosphatases and the low molecular weight phosphatases (LMWP). The classical PTPs consist both of receptor and non-receptor PTPs with functions related to cell adhesion and cell-cell signaling. While the DSPs belong to the PTP superfamily, they literally can dephosphorylate not only tyrosine residues but show phosphatase activities toward Ser/Thr residues as well. Of the DSPs the function of Cdc14 (mitotic exit) and PTEN (PIP₃ signaling) are relatively well understood while little is known about many other members. The CDC25 type phosphatases are well known for their
essential function in regulating mitotic entry via activation of CDK1. Little is known about the function of the LMWPs.

The Aspartate based catalysis phosphatases are defined by the shared signature catalytic motif of DXDXT/V. Its founding member is the FCP1 phosphatase with an essential function in coordinating the phosphorylation status of the RNA polymerase II largest subunit C-terminal domain and thus regulating the activity of the transcription machinery.

1.5.3. Protein phosphatase holoenzymes – catalytic and regulatory subunits

Recent advances in genomic studies have led to the systematic identification of the full protein phosphatase complements in many organisms. Surprisingly, significantly fewer protein phosphatases seem to counteract the function of many more different protein kinases. In humans, while 518 protein kinases have been identified, only 147 protein phosphatases are known so far. Furthermore, while more than 98% of all active protein phosphorylation is estimated to occur on serine and threonine residues, 107 of the 147 protein phosphatase catalytic subunits are thought to target tyrosine residues. This again is in clear contrast to the human kinome, where 428 kinases are thought to phosphorylate serine and threonine residues with only 90 kinases targeting tyrosine residues. These discrepancies are mainly due to the unique regulation of Ser/Thr phosphatases’ activities. Except for
PP2C type phosphatases, most Ser/Thr phosphatase catalytic subunits associate with a staggering number of different regulatory subunits to form distinct phosphatase holoenzymes. While a large number of protein phosphatase holoenzymes may share the same catalytic subunit, the different make-up of regulatory subunits confers the holoenzymes with their distinct substrate specificities and cellular localizations (Moorhead et al., 2007). Concerning the research of protein phosphatases, the inevitable pleiotropic effects following manipulations of certain protein phosphatase catalytic subunits due to this regulatory mechanism have been a cause of frustration. In light of those difficulties, our expanding knowledge over many phosphatase regulatory subunits involved in phosphatase activity regulation is expected to greatly facilitate our advances in understanding protein phosphatase functions and activities.

1.5.4. Protein phosphatases – cell cycle regulation

In the early days of cell cycle research, several protein phosphatases had been implicated to be deeply involved in cell cycle regulation. This is not surprising when considering the many events of the eukaryotic cell cycle that are under the control of reversible protein phosphorylation. Especially during mitosis, it has been firmly established that the function of protein kinases such as CDK1, Aurora, NIMA and Polo-like kinases are of critical importance for proper mitotic progression. As
enzymes counteracting the effects kinases, protein phosphatases have naturally been considered as logical candidates to have important cell cycle functions. As of now, CDC25 and Cdc14 of the PTP superfamily, and PP1, PP2A of the classical Ser/Thr phosphatase family are the protein phosphatases whose mitotic functions are most studied.

Identified in 1986 for its essential function in mitotic entry (Russell and Nurse, 1986), CDC25 functions to activate CDK1 by removal of the inhibitory Tyr15 phosphorylation of CDK1. The activity and localization of CDC25 itself is regulated by phosphorylation as well. It has been shown that CDC25 is activated by CDK1 mediated phosphorylation, forming a positive feedback loop to facilitate rapid entry into mitosis. Phosphorylation by Polo-like kinases is important in determining the subcellular localization of CDC25.

The DSP family member Cdc14 is known mostly for its role of regulating later stages of mitosis. In the budding yeast S. cerevisiae, Cdc14p remains sequestered in the nucleoli during interphase but is released in anaphase via the FEAR (Cdc fourteen early anaphase release) and MEN (mitotic exit network) signaling cascade. Once released Cdc14p localizes to the spindles and spindle pole body to promote mitotic exit by reversing the phosphorylation of mitotic proteins carried out by CDKs during earlier phases of mitosis. The first evidences of PP1 functioning in mitotic regulation were presented in 1989 as Doonan et al. demonstrated that the
The *bimG* gene of *A. nidulans* is required for the completion of anaphase and encodes a homolog of mammalian PP1. This was followed by studies in the fruit fly *Drosophila melanogaster* and yeast *S. pombe* further confirming the essential function of PP1 in mitotic progression (Axton et al., 1990; Kinoshita et al., 1990). As the M-phase arrest phenotypes of different PP1 mutations in yeasts and other organisms show a high degree of heterogeneity, it is generally believed that PP1 has multiple targets during mitosis, also consistent with its diverse localization to chromatin, nuclear lamin, nucleolus and spindle pole body during different stages of mitosis.

PP2A is another classical Ser/Thr phosphatase that has confirmed mitotic functions. While (along with PP1) the mitotic function of PP2A in *S. pombe* was identified as early as 1990 (Kinoshita et al., 1990), studies in recent years point to a more specific role of PP2A in controlling sister chromatid cohesion by interacting with shugosin proteins at the centromere (Tang et al., 2006).

In 2007, Chen et al. published the findings of their research concerning protein phosphatases that are required for proper cell cycle progression in *D. melanogaster* (Chen et al., 2007). Based on an RNAi-based genome wide knock-down analysis of protein phosphatase genes (including both catalytic and regulatory subunits), the authors recognized 22 of the 117 genes examined to have some role in cell cycle regulation. According to their results, Puckered (JNK
MAP-kinase inhibitory phosphatase) and PP2C are required for proper $G_2$-$M$
progression in addition to CDC25 string, while Pp1-87B downregulation led to
strong mitotic arrest and chromosome congression failure phenotypes.
PP1-Flapwing knockdown resulted in chromosome alignment and segregation
defects, and the down-regulation of several other protein phosphatases including
nonreceptor tyrosine phosphatases, dual-specificity phosphatases and PP2As
were also observed to yield varying degrees of mitotic defects in *D. melanogaster*.
While their research mostly supported prior understandings about mitotic
phosphatase functions, the new findings of additional phosphatase genes may be
involved in regulating mitotic progression which were previously not known
reconfirmed the importance and need for further studies concerning protein
phosphatase functions in cell cycle regulation.
Figure 1.1. Lifecycles of *A. nidulans*. *A. nidulans* is a filamentous fungus of the genus *Aspergillus*. Capable of both asexual as well as a sexual lifecycles, *A. nidulans* primarily remains in a haploid state under normal conditions. Transient diploid nuclei are formed during the sexual life cycle in specialized cells termed asci. Under a laboratory conditions stable diploids can be readily formed and maintained on restrictive growth media.
Figure 1.2. Asexual cycle of *A. nidulans*. The asexual life cycle of *A. nidulans* strats with the uninucleate, haploid conidiospore (A). When nutritional conditions are favorable, the conidiospore initiate polarized growth and nuclear division to produce a multi-nucleate germling. (B). When growth is sustained, specialized cells called foot cells develop within the hyphae. From these cells, the conidiophore develops (C). After the foot cell extends to a certain height, the tip of the foot cell swells up to form a vesicle. Primary and secondary sterigmata develop from the vesicle. Rapid budding division (both mitosis and cytokinesis) at the apex of the secondary sterigmata produce conidia chains. For conidiophores to form in *A. nidulans* an air interface is required. When germinated in liquid culture normally *A. nidulans* fails to conidiate.
CHAPTER 2

MATERIALS AND METHODS

2.1. General DNA preparation and cloning

2.1.1. Plasmid maxiprep and miniprep

Plasmid minipreps started with inoculation of a single bacterial colony from an LB plate into 2 mL 2xTYP (16 g/L yeast extract, 16 g/L tryptone, 5 g/L sodium chloride, 2.5 g/L potassium phosphate, 490 mg/L magnesium sulfate, [pH 7.4]) and cultures were grown overnight at 37°C shaking at 250rpm in an air shaker (Innova). Minipreps were performed using a miniprep plasmid kit following the instructions provided by the manufacturer (Promega).

2.1.2. DNA cloning.

Restriction digests of DNA were carried out using commercially available endonucleases (New England Biolabs and Promega). Digests were performed following the protocols provided by the manufacturers in supplied buffers. Analysis
of restriction digests were by performed with agarose gel electrophoresis. Restriction fragments were isolated by agarose gel purification using a Gel Extraction Kit (Qiagen) or using a DNA Clean-up Kit (Qiagen). Ligation of purified DNA into sequencing vectors was carried out following the protocols provided by the manufacturers.

2.1.3. Polymerase Chain Reaction (PCR)

PCR was performed using a 9700 Thermal Cycler (Perkin Elmer) or a Gradient Thermal Cycler (Eppendorf). PCR cycling conditions varied based on the individual DNA fragments to be amplified. Pfu Turbo Polymerase (Stratagene) was used for some high fidelity PCR reactions. The Expand Long Template PCR Kit (Roche) was used for generating tagging constructs and PCR screening protocols.

2.1.4. Primers

Oligonucleotide primers used for all purposes were designed using Primer Designer Ver. 2.0 software (Scientific and Educational Software). Tables 2.1, 2.2, 2.3 lists the primers used in this work.

2.1.5. DNA sequencing

DNA sequencing was performed by the Plant Microbial Genomics Facility at The Ohio State University (Columbus, OH). Preparation of sequencing samples was performed according to the guidelines listed at:
Analysis of the sequencing results were carried out with Sequencing Analysis software Ver 3.3 (Perkin Elmer Applied Biosystems) or DNASTar Seqman software (DNASTar).

2.1.6. Bacterial strains

DH5αF *Escherichia coli* cells were used for all general cloning and plasmid amplification applications.

2.1.7. Transformation of bacteria

DH5αF *E. coli* competent cells were used for plasmid amplification procedures. Competent cells stored in 100µL aliquots at -80°C were thawed on ice immediately prior to transformation. 20ng of plasmid DNA was added to the cells and gently mixed by pipetting. After addition of DNA, cells were maintained on ice for 20 minutes and then shifted to a 42°C waterbath for 45 seconds. Following this heat shock, cells were placed back on ice for an additional 2 minutes. 900µl of 2xTYP was added to each tube and the cells were incubated for 1 hour in a shaking air incubator at 37°C and 250 rpm, allowing the antibiotics resistance marker to be expressed. Transformed cells were spread on LB agar plates containing appropriate antibiotics in 100µL and 900µL aliquots. The cells were grown for 15 hours at 37°C before colonies were taken.

http://www.biosci.ohio-state.edu/~pmgf/services-dnasequencing.htm
2.1.8. Storage and stock preparation of bacteria

2mL of 2xTYP with right antibiotic selection were inoculated with a single bacterial colony using a sterile wooden applicator. Cultures were grown overnight (16 hours) at 37°C at 250 rpm in a shaking air incubator. Cultured cells were harvested by centrifugation for 2 minutes at 14,000 rpm. The pellet of bacterial cells was resuspended in 1mL of 2xTYP and 20% glycerol in a sterile 1.5mL Eppendorf tube and was stored at -80°C.

2.2. Culture and genetics of A. nidulans

2.2.1. A. nidulans specific media

YG media: (10g/L dextrose, 5g/L yeast extract, 10 mM magnesium sulfate, supplemented with 1µg/mL p-aminobenzoic acid (paba), 0.5µg/mL pyrodoxine HCL (pyro), 2.5µg/mL riboflavin HCL (ribo), 2µg/mL nicotinic acid , 20µg/mL choline, 20ng/mL D-biotin, 0.5g/L D-arginine and 1mL/L trace elements). Strains carrying the pyrG89 auxotrophic mutation in the absence of a complementing pyrG or pyr4 allele in the genetic background were grown in YGUU (YG media supplemented with 1.2 g/L uridine and 1.12 g/L uracil).

YAG media: (YG media with 15g/L agar)

MAG media: (5 g/L malt extract, 20 g/L bacto peptone, 10g/L dextrose, supplemented with 1µg/mL p-aminobenzoic acid (paba), 0.5µg/mL pyrodoxine HCL (pyro), 2.5µg/mL riboflavin HCL (ribo), 2µg/mL nicotinic acid , 20µg/mL choline, 20ng/mL D-biotin, 0.5g/L D-arginine, 1mL/L trace elements and 20g/L
agar). Strains carrying the *pyrG89* auxotrophic mutation in the absence of a complementing *pyrG* or *pyr4* allele in the genetic background were grown on MAGUU (MAG media supplemented with 1.2 g/L uridine and 1.12 g/L uracil).

Minimal Media Urea: (10mM urea, 7mM potassium chloride, 1mM magnesium sulfate, 1mL/L trace elements, and nutritional supplements required for individual strains). Dextrose (10g/L) or glycerol (0.47mL/L) was added prior to autoclaving. Ethanol (1mL/L) was added after autoclaving. After autoclaving add potassium phosphate [pH 6.8] to 12 mM and sodium thiosulfate to 3.2 mM. For solid media 15g/L agar was added prior to autoclaving.

Minimal Media Urea fructose/threonine: (10mM urea, 7mM potassium chloride, 1mM magnesium sulfate, 1mL/L trace elements, and nutritional supplements required for individual strains). Fructose (final concentration 9g/L) and D-threonine (final concentration 40mM) were prepared as sterile stock solutions and added after autoclaving. Potassium phosphate [pH 6.8] (to 12mM) and sodium thiosulfate (to 3.2 mM) were also added after autoclaving. For solid media 15g/L agar was added prior to autoclaving.

Minimal media Low Nitrate: (82mM sodium nitrate, 7mM potassium chloride, 2mM magnesium sulfate, 11mM potassium phosphate monobasic, 111mM dextrose, 1mL/L Clive Roberts Trace Elements, nutritional supplements as required, and 15g/L agar [pH 6.7]).
2.2.2. Preparation of *A. nidulans* conidia stock suspensions

*A. nidulans* conidiospores were inoculated at 1X10^7 spores/mL into 4mL of MAG media (strains carrying the *pyrG89* auxotrophic mutation in the absence of a complementing *pyrG* or *pyr4* allele in the genetic background were supplemented with uracil and uridine) containing only 0.75% agar at 48°C. Inoculated media was vortexed to suspend the cells evenly and poured onto MAG (or MAGUU if nutritional requirement was present) plates. These plates were incubated at 32°C for 40 hours to allow for conidiation of inoculated spores. Newly grown conidiospores were harvested from the surface of the plates in 10mL of 0.2% Tween 20. A sterile glass spreader was used to gently scrap the top of the fungal lawn to release conidia into the Tween 20 solution. Suspended conidia were transferred to sterile 15mL falcon tubes (Corning). The suspensions were centrifuged at 3,800 rpm for 2 minutes to sediment the conidiospores. Hyphal debris was removed by gently resuspending and recovering only the top conidial layer of the pellet. The collected conidia were washed two times in 10mL of 0.2% Tween 20. After the final wash the conidia were resuspended in stock storage solution (8.5mM sodium chloride, 200µM Tween 80). Conidiospore suspensions could be stored for up to a month at 4°C.

2.2.3. Conidiospore Quantitation

Concentration of conidiospores in suspensions were quantitated to allow for accurate inoculation densities for germination in growth media. Conidial suspensions were quantitated by counting 10 µl of a 1 x 10^-3 dilution of conidia in
0.2% Tween 20 using a Bright-Line hemocytometer (Reichert-Jung). Four fields of conidia were counted for each sample and the average value used for quantitation. The number of conidia obtained from this count was multiplied by $1 \times 10^7$ to determine the concentration of the original suspension in spores/mL.

2.2.4. Long term storage and stock preparation of *A. nidulans*

Strains were streaked to isolate single colonies three times. A single colony was replica plated on a selective media plate by a sterile toothpick and incubated at 32°C for 48 hours and then at room temperature for 3 days. 5mL of sterile 7.5% milk (7.5g of Carnation Nonfat Dry Milk in 100mLs dH$_2$O and autoclaved for 20 minutes) was applied onto the surface of the colonies, and mature conidia were harvested by rubbing of the colony surfaces using a sterile glass spreader. A sterile transfer pipette was used to transfer 500µL of the suspended spores into three screw top glass vials containing baked, sterile silica. The silica was vortexed briefly to evenly distribute the spores and returned to ice for 30 minutes. The silica was left at room temperature for 3 days with the vial lid loosened for complete drying. The vials were placed in a room temperature desiccator. Strains were re-grown when needed from silica stocks by placing 5-10 silica pieces onto appropriate solid media plates, and incubating at 32°C for several days in an air incubator.

2.2.5. Strain generation by meiotic crossing

Parental strains which both contained at least one auxotrophic mutation marker which was complemented in the other strain were induced to undergo...
meiosis. The use of parental strains with different color mutations enabled easy, visual confirmation of successful meiotic crossing events.

Parental strains were alternately spot inoculated onto appropriate growth media with ~1 cm between the spots of inoculations. The plates were incubated at 32°C until the boundaries of adjacent colonies abutted each other. A strip of hyphal mat at the interface of the two colonies was recovered with sterilized tweezers. This hyphal mat was sliced and placed onto the surface of a minimal media low nitrate plate. The plate was loosely sealed with tape and incubated at 32°C for 2 weeks for meiosis to occur and cleistothecia to form. Cleistothecia maturation was assessed by size and color (dark pink to black) with a dissecting microscope (Bausch and Lomb). Mature cleistothecia were recovered with a sterilized glass pipette and manipulated across the surface of a 4% water agar plate to remove any debris from the surface of the cleistothecia. Cleaned cleistothecia were crushed in 1mL of 0.2% Tween 20 in a sterile 1.5mL Eppendorf tube to release ascospores. Ascospores were plated and grown on appropriate solid growth media. Individual colonies were replica plated on a range of minimal media plates lacking various supplements to determine the segregation of nutritional markers and identify strains with desired genotypes.

2.2.6. Diploid formation

Conidia from two haploid strains with complementary nutritional and different color mutations were floated on the surface of 2mL of dH₂O itself poured on the top of 2mL of MAGUU media solidified at the bottom of a sterile 10mL flint glass tube
(Fisher Scientific). The spores were germinated overnight at 32°C to allow a mixed mycelial mat to form. Sterile tweezers were used to recover the mixed mycelial mat and slice them into small pieces. These pieces were inoculated onto the surface of minimal media plates restrictive in the nutritional requirements of both strains to induce heterokaryon formation. Spores were harvested from the surface of heterokaryons with 0.2% Tween 20 and added at varying dilutions to limiting minimal media and plated. Plates were incubated at 32°C to allow colonies to grow. Green colonies were selected as diploids as the different color mutations in the parental strains would have to have been complemented in the diploids, thus leading to the green spore.

2.3. General *A. nidulans* techniques

2.3.1. Transformation of *A. nidulans*

1 x 10^9 fresh conidia were inoculated into 50mL YGUU. The cultures were grown at 32°C at 250 rpm in an air shaker for 5.5 hours or until conidia had started to germinate and show a “schmoo” shape. Germlings were harvested by centrifugation in a swinging bucket rotor at 3,800 rpm for 2 minutes and resuspended in a protoplasting mix containing 25mL Solution1 (105.6g/L ammonium sulfate, 19.2g/L citric acid, [pH 6.0]), 25mL Solution2 (10g/L yeast extract, 20g/L sucrose, 1μg/mL acid paba, 0.5μg/mL pyro, 2.5μg/mL ribo, trace elements, 4.92 g/L magnesium sulfate), 100mg bovine serum albumin (BSA), 500mg Vinoflow FCE and 50mg driselase. The resuspended spores were
incubated in a clean sterile flask at 32°C and 170 rpm for 2 hours or until the cell wall was degraded that vacuoles of the protoplasts become visible under examination using phase contrast light microscopy.

Protoplasts were harvested by centrifugation in a swinging bucket rotor for 2 minutes at 3,800 rpm and washed two times in Solution 3 (52.8g/L ammonium sulfate, 10g/L sucrose, 9.6g/L citric acid, [pH 6.0]) and resuspended in 1mL of Solution 5 (44.7g/L potassium chloride, 7.35g/L calcium chloride, 2.09g/L MOPS [pH 6.0]).

Transformation was initiated by mixing 4µg DNA, 100 µl protoplasts, and 50µl of Solution 4 (250g/L PEG 8000, 7.35g/L calcium chloride, 44.7g/L potassium chloride, 10mL 1M Trizma Base [pH 7.5]). The reaction was placed on ice for 20 minutes before adding 1mL of room temperature Solution 4. After 20 more minutes of room temperature incubation, the reactions were plated onto YAG sucrose (5g/L yeast extract, 3.6g/L dextrose, 342.3g/L sucrose, 2.47g/L magnesium sulfate, 1 µg/mL PABA, 500ng/mL pyro, 2.5µg/mL ribo, trace elements, 15g/L agar) in 4mL YAG sucrose overlays (same as above except 8 g/L agar) in 50µL, 100µL, 200µL, 400µL and remainder volumes. The transformation plates were incubated at 32°C for 3 days or until colonies had been formed.
2.3.2. Small scale genomic DNA extraction

A small amount of freeze-dried mycelium was crushed with a sterile toothpick. 150µl of Miniprep Lysis Solution (Promega) was added and vortexed to mix. Contents were left at room temperature for 7 minutes. 150µl Miniprep Neutralization Solution was added and mixed. These samples were centrifuged at 14,000 rpm in a Model 5420 table top refrigerated centrifuge (Eppendorf) at 4°C for 10 minutes to pellet mycelial debris. The supernatant was processed using Miniprep Purification Kit (Promega) following to the manufacturer’s instructions. Genomic DNA was eluted from the column in 50 µl dH₂O.

2.3.3. Large scale genomic DNA extraction

*A. nidulans* conidia were grown in appropriate media overnight in an air incubator at 32°C at 250 rpm. Inoculums were allowed to grow until they reached a packed cell volume of 0.5mL from a 10mL culture sample. Mycelium was harvested by vacuum filtration through Miracloth (Calbiochem). Recovered mycelium was washed with Stop Buffer (9g/L sodium chloride, 65mg/L sodium azide, 20mL 0.5M ethylenediaminetetraacetic acid (EDTA) [pH 8.0], 2.1g/L sodium fluoride). After pressing out excess liquid, the mycelium was removed from the Miracloth, placed in a sterile falcon tube (Corning) and frozen in liquid nitrogen and dried overnight in a lyophilizer (Savant). To extract genomic DNA, 40mg of dried mycelium was thoroughly ground in a 1.5mL Eppendorf microcentrifuge tube. To this ground mycelium was added 250µL 0.5% SDS DNA Extraction Buffer (200mM Trizma Base [pH 8.5], 250mM NaCl, 25mM EDTA, 0.5% SDS), 175µL phenol, and
75µl chloroform. The tubes were rocked for 15 minutes at room temperature and subsequently the mycelial debris was pelleted by centrifugation at 14,000 rpm for 20 minutes. To the recovered supernatant was added 400µl of chloroform and spun at 14,000 rpm for 10 minutes. The supernatant was mixed with an equal volume of 5M lithium chloride, incubated on ice for 10 minutes to precipitate RNA, and centrifuged at 14,000 rpm for 10 minutes. DNA was precipitated from the supernatant using 2'-propanol and resuspended in 50µL TE.

2.3.4. Protein preparation

Conidiospores were inoculated at 1x10⁶ spores/mL into appropriate liquid media. The inside of the culture flasks were coated with GelSlick (Cambrex Bio Science) prior to the preparation of media to prevent mycelium from sticking to the inside surface of the flask. Cultures were germinated overnight at 28°C at 250rpm in a shaking air incubator. The next day the culture growth was monitored by measuring the packed cell volume of the culture. 10mL of culture was removed and centrifuged for 2 minutes at 3,800rpm in a clinical swinging bucket centrifuge (Thermo IEC). Packed cell volumes of ~0.2mL of mycelium was indicative of the culture growing in log phase. Mycelium was harvested by vacuum filtration through Miracloth (Calbiochem). Recovered mycelium was washed with Stop Buffer (9g/L sodium chloride, 65mg/L sodium azide, 20mL 0.5M ethylenediaminetetraacetic acid (EDTA) [pH 8.0], 2.1g/L sodium fluoride). After pressing out excess liquid, the mycelium was removed from the Miracloth, placed in a sterile falcon tube (Corning) and frozen in liquid nitrogen and dried overnight in a lyophilizer (Savant). Dried
samples were weighed and ground into a powder with a pestel and mortar.

Protein was prepared by adding 2.5mL of HK buffer (1µl/mL leupeptin [10 mg/mL in dH₂O], 0.4µl/mL trypsin and chymotrypsin inhibitor [25mg/mL in DMSO], 0.5µl/mL N-tosyl-L-phenylalanine chloromethyl ketone [TPCK] [50mg/mL in DMSO], 5µl/mL aprotinin [1.5mg/mL], 760µg/mL N-p-tosyl-arginine methyl ester hydrochloride [TAME], 6.2mg/mL p-nitrophenyl phosphate [PNPP], 800µg/mL benzamidine, 200µg/mL sodium vanadate, 420µg/mL sodium fluoride, 71µl/mL 1M β-glycerophosphate, 35µl/mL 0.5M Ethylene-glyco-tetra-acetic acid [EGTA] [pH 8.0], 12µl/mL 0.5M ethylene-diamine-tetra-acetic acid [EDTA] [pH 8.0], 30µl/mL 1.0M Trizma Base [pH 7.5], 24µl/mL 10% Nonidet P-40, ammonium sulfate [final concentration 320mM], glycerol [final concentration 20% w/v]) per each 1g of mycelium. Ground mycelium slurries were centrifuged at 20,000rpm at 4°C for 15 minutes in Beckman, Avanti-Je Centrifuge (rotor JA 25.50) to pellet mycelial debris. The supernatant was transferred to fresh 15mL falcon tubes, the protein concentration estimated and frozen in liquid nitrogen. Frozen proteins were stored at -80°C until use. Immediately prior to use protein samples were rapidly thawed in cold water and centrifuged at 14,000 rpm at 4°C.

2.3.5. S-tag purification in A. nidulans

After purification of proteins, 300µl of S-protein Agarose slurry (150ul packed beads volume) [ Novagen # 69704] was added to each 100mg of protein. The solutions were incubated on ice at 4°C for 2 hours while gently rocking. After incubation, the S-protein beads were recovered by centrifugation at 3,800rpm for 2
minutes in a clinical swinging bucket centrifuge (Thermo IEC). The spun-down beads were washed with equal volume of protein wash buffer 5 times (i.e., if total sample volume of S-tag pull down solution was 10ml, it was washed with 10ml of wash buffer. The protein wash buffer was identical to the protein extraction buffer except that NaCl [final concentration 300mM] substituted ammonium sulfate and glycerol was omitted). After washing, the beads were transferred to 1.5mL Eppendorf tubes and washed at least 2 times more, all at 4°C. 4X sample buffer was added and the samples were boiled for 5 minutes. The samples were vortexed and the beads removed from the supernatant by centrifugation for 5 minutes at 14,000rpm. The supernatants were taken and loaded onto 6% polyacrylamide protein gels.

2.3.6. Coomassie staining of protein gels

After gel electrophoresis was complete, polyacrylamide protein gels were fixed in 50% ethanol and 10% acetic acid overnight. The next day, the fixed gels were rinsed 3 times for 5 minutes with dH2O. Residual water was removed as much as possible, and Biorad Bio-Safe Coomassie solution (enough amount of to cover the gel) was added. Staining was continued for 1 hour, and background staining was removed by destaining in water for 30 minutes.

2.3.7. Silver staining of protein gels

After gel electrophoresis was complete, polyacrylamide protein gels were fixed in 50% ethanol and 10% acetic acid for 10 minutes. The fixed gels were washed in
50% ethanol for 15 minutes, and subsequently washed in dH₂O three times for 5 minutes each. After washing, the gels were incubated in 0.02g/100mL of Na₂S₂O₃·5H₂O solution for 1 minute. This was followed by washing in dH₂O for three times 30 seconds each. Then the gels were incubated in a silver nitrate solution (AgNO₃ 2g/L +750μl 37% formaldehyde solution) for 20 minutes. The gels were washed in dH₂O twice for 20 seconds each, and after that the bands were visualized in the developing solution (Na₂CO₃ 6g/100mL, 37% formaldehyde 50μL/100mL, Na₂S₂O₃·5H₂O (0.02g/100mL) 2mL/100mL). When bands became visible the developing reaction was stopped with 50% ethanol and 10% acetic acid.

2.3.8. in vitro λ-phosphatase assay

After purification of proteins via S-tag affinity purification, three tubes with equal amount of proteins were prepared for no phosphatase, phosphatase and phosphatase + phosphatase inhibitors treatment. To initiate the λ-phosphatase assay (New England Biolab), the individual samples were washed in 2X phosphatase buffer (i.e. volume of phosphatase buffer equal to volume of beads) which included MnCl₂ and proper protease inhibitors (prepared according to manufacturers protocol). This step served the purpose of equilibrating the S-protein beads in the phosphatase buffer and removing phosphatase inhibitors present in the protein extraction buffer. After washing, the three samples were treated with either control buffer only or equal volumes of 1x phosphatase mix; no phosphatase, phosphatase (40 units λ-phosphatase), phosphatase (40 units λ-phosphatase) and phosphatase inhibitors (1mM Na Vanadate 50mM Na
Fluoride). The beads were mixed with the solutions and incubated at 30°C for 30 minutes (mixing occasionally). Once the treatment was completed, sample buffer was added to a final concentration of 1X and boiled to stop the reaction. The supernatant was collected after pelleting of the beads by centrifugation at 14,000rpm and analyzed by SDS-PAGE.

2.3.9. Cell cycle arrest by *nimT23* mutation

For G₂ arrest, cultures grown overnight (packed cell volume 0.1-0.2 of 10mL culture spun at full speed for 1 minute) at 42°C were heated in a water bath ready at 55°C. The culture was maintained in the water bath to bring it up to 42°C. Upon reaching that temperature, the cultures were immediately transferred to a 42°C air shaker. The cultures were further incubated for 3 more hours at 42°C. After the 3 hours incubation, 500μL of the culture was set aside for measuring the chromosome mitotic index while the rest was harvested in standard manners. The sample put aside is immediately fixed and DAPI stained. For mitotic arrest, cultures were shifted to 42°C and benomyl (2.4μg/mL) was added to the samples after 2 hours and 45 minutes of incubation at 42°C. After 15 more minutes of further incubation at 42°C, the cultures were cooled rapidly in an ice/water bath until they reached 30°C. The cultures were incubated at 30°C for 40 more minutes before being harvested in standard manners. A 500μL sample of the culture was set aside for measuring the chromosome mitotic index.
2.4. Phosphatase gene deletion in *A. nidulans*

The DNA constructs used for the deletion of *A. nidulans* phosphatase genes were all generated using the 3-way fusion PCR method. The deletion cassette which was common in all deletion constructs was amplified with the primer pair FN02 and LU212 using the pFN03 plasmid as template DNA. The pFN03 plasmid contained an insert which includes a GA5 linker sequence, a GFP coding sequence and an *Af-pyrG* auxotrophic marker sequence. The primers FN02 and LU212 specifically targeted and amplified the *Af-pyrG* auxotrophic marker sequence. The PCR mix was a total volume of 50μL, including 0.75μL Expand Long Template enzyme mix, each dNTPs at 500μM final concentration, 500ng pFN03 plasmid template DNA, 5μL 10X buffer 3, each 300nM (final concentration) of the primers FN02 and LU212. The PCR cycling conditions were as following. 94°C 2 minutes, 25 cycles (94°C 15 seconds, 55°C 30 seconds, 68°C 2 minutes), 68°C 5 minutes. The gene specific primers used to amplify the 5’-flanking as well as the 3’-flanking sequences of all phosphatase genes are listed in Table 2.1. The basic PCR cycling conditions for amplifying these fragments were as following with minor changes introduced for individual genes. 94°C 2 minutes, 25 cycles (94°C 15 seconds, 55°C 30 seconds, 68°C 2 minutes), 68°C 5 minutes. Once all three fragments for fusion PCR were generated, 500ng of each of the three fragments were used a template DNA for fusion PCR reaction. The PCR mix was a total volume of 50μL, including 0.75μL Expand Long Template enzyme mix, each dNTPs at 500μM final concentration, 500ng of each template DNA, 5μL 10X buffer 3, each 300nM (final concentration) of the appropriate primers (for conceptual
layout of fusion PCR see Chapter 3 Introduction). The basic PCR cycling conditions for amplifying these fragments were as following with minor changes introduced for individual constructs. 94°C 2 minutes, 25 cycles (94°C 15 seconds, 55°C 30 seconds, 68°C 6 minutes + 15 seconds increment each cycle), 68°C 5 minutes. Once the deletion constructs were amplified, transformation was carried out according to the standard protocol. The primary transformants were examined for the formation of heterokaryons to determine the essentiality of the deleted genes according to the standard heterokaryon rescue procedure (see Chapter 3 Introduction).

2.5. Endogenous C-terminal protein tagging in A. nidulans.

The DNA constructs used for the endogenous C-terminal protein tagging were all generated using the 3-way fusion PCR method. The Both the GFP tagging cassette as well as the affinity S-tagging cassette were amplified with the primer pair FN01 and FN02 due to the common GA5 linker and Af-pyrG sequence available in the pAO81 (S-tag) and pFN03 (GFP) plasmids. The gene specific primers used to amplify the 5'-flanking as well as the 3'-flanking sequences of all genes whose encoded proteins were tagged are listed in Table 2.2. PCR conditions were similar as in generating the deletion constructs. Once the tagging constructs were amplified, transformation was carried out according to the standard protocol.
2.6. *alcA* promoter insertion in *A. nidulans*

The DNA constructs used for *alcA* promoter insertion were all generated using the 3-way fusion PCR method. The *alcA* promoter cassette which is common in all constructs was amplified using the primer pair SSriboalcA1 and SSriboalcA2. The gene specific primers used to amplify the 5'-flanking as well as the 3'-flanking sequences of all genes where the *alcA* promoter had been inserted are listed in Table 2.3. The fusion PCR procedure was carried out in standard manners. Unlike in other transformation procedures, transformations for *alcA* promoter insertion were performed entirely under inducing conditions for *alcA* promoter driven gene expression. Instead of YAG sucrose, 0.6M KCl was used in Minimal Media Urea fructose/threonine to provide osmotic buffering and induction of the *alcA* promoter.

2.7. Time lapse confocal microscopy

Conidia were inoculated at 1.5X10^5/mL in minimal medium without riboflavin in a microdish and were observed after 18 hours growth at room temperature with the Nikon Eclipse TE300 spinning disc confocal microscope system. When maintenance of particular temperatures was required, the Biotech Delta T4 Culture Dish System was used according to manufacturers protocols.
Table 2.1 Oligonucleotide primers for gene deletion

<table>
<thead>
<tr>
<th>gene</th>
<th>region</th>
<th>primer</th>
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<td>AN0103</td>
<td>5' diag.</td>
<td>SS0103A</td>
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Table 2.2 Oligonucleotide primers for *alcA* promoter insertion constructs

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</tr>
<tr>
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</tr>
<tr>
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<td>3' flank.</td>
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Table 2.3 Oligonucleotide primers for C-terminal S-tag/GFP tagging constructs

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<td><strong>An-fcp1</strong></td>
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3.1. INTRODUCTION

While much of the research concerning cell cycle regulation has been focused on understanding the functions of various mitotic kinases, considerably less has been known about how the activity of these mitotic kinases are countered and balanced by protein phosphatases. This chapter will describe a systematic deletion analysis of protein phosphatase genes in A. nidulans with the aim of identifying protein phosphatase catalytic subunits that are involved in cell cycle regulation. An overview of the experimental procedures extensively used and often referred to in this chapter is provided.
3.1.1. $\Delta nkuA$ gene deletion and transformation characteristics of *A. nidulans*

Introduction of foreign circular or linear DNA into *A. nidulans* results in the integration of the transformed DNA sequence into its genome. Nevertheless, in a wild type strain, targeting specific genes or genome sequences by homologous recombination can be a time consuming and labor intensive task as the general frequency of correct, sequence specific integration of transformed DNA is rather low (Yu et al., 2004). A major breakthrough in overcoming this limitation of *A. nidulans* as a model system was achieved when Berl Oakley and his team generated an *A. nidulans* strain in which the homologue of the Ku70 gene (*nkuA*) was deleted (Nayak et al., 2006). As the Ku70 protein functions in the non-homologous end joining (NHEJ) mediated DNA repair pathway, the deletion of the *nkuA* gene in *A. nidulans* led to a dramatic decrease of heterologous, nonspecific insertion of transformed DNA into the genome. In a $\Delta nkuA$ *A. nidulans* strain, correct integration of gene-targeting DNA constructs was shown to occur in >90% of transformed colonies when the homologous flanking sequences were >0.5KB in size. Although the role of Ku70 function concerning NHEJ-mediated DNA repair had been confirmed in many organisms (Hopfner et al., 2002; Lisby and Rothstein, 2004), *A. nidulans* is likely to retain functionally redundant mechanisms to fulfill this DNA repair function independent of *nkuA* since the gene deletion resulted neither in general growth defects (including meiotic defects) nor
enhanced sensitivity to various DNA damaging agents (Nayak et al., 2006), largely eliminating the concern that \( \Delta nkuA \) be an unsuitable genetic background due to physiologically detrimental effects. Together with the development of fusion PCR methods for efficient generation of gene targeting DNA constructs (Yang et al., 2004; Szewczyk et al., 2006), the establishment of the \( \Delta nkuA \) strain as an efficient gene-targeting system enabled genome-wide, systematic large-scale approaches to target genes of interest in \( A. nidulans \) (Osmani et al., 2006a).

3.1.2. Fusion PCR – a method for generating gene targeting constructs

After the completion of the 13X coverage genome sequencing of \( A. nidulans \), one of the remaining difficulties preventing researchers from taking advantage of this vast information effectively was the lack of methods to easily generate gene-targeting DNA constructs. The fusion PCR-based method of amplifying gene-targeting constructs solved this problem by eliminating the need to perform ligation reactions required in conventional cloning strategies. This method was employed to produce three different types of gene-targeting constructs used to generate \( A. nidulans \) strains described in this chapter and throughout this dissertation. An overview of the three construct types is provided below.
3.1.2.1. Gene deletion constructs

To delete the entire open-reading frame of a given gene, initially three different DNA fragments have to be amplified. The first fragment is a gene deletion cassette consisting of the heterologous (but functionally complementary) *A. fumigatus pyrG* gene (*Af-pyrG*) to minimize the chance of the eventual targeting construct integrating into the native *A. nidulans pyrG* locus upon transformation (Figure 3.1A). In addition, two fragments of the genome sequences immediately upstream and downstream of the gene (>0.5KB in length) to be deleted are PCR amplified as targeting regions (Figure 3.1B, C). In amplifying these two fragments, the 3’-end primer of the upstream sequence is designed to have a 20 bp long 5’ extension that is a reverse complement of the first 20 bp sequence of the *Af-pyrG* cassette (Figure 3.1D). The 5’-end primer of the downstream sequence is designed to have a 20 bp long 5’ extension identical to the last 20 bp sequence of the *Af-pyrG* cassette (Figure 3.1E). A fusion PCR reaction using a mixture of these three fragments in equal molar amounts (estimation deduced by DNA concentration and length of the fragments) as templates and the 5’-end primer of the upstream sequence and the 3’-end primer of the downstream sequence amplifies the complete gene deletion construct with the three initial fragments fused into a single DNA fragment (Figure 3.1F, G). Upon transformation of this construct, transformant colonies are tested for proper replacement by using a
primer set that targets sequences outside of this construct in diagnostic PCR reactions (Figure 3.1H).

3.1.2.2. Endogenous C-terminal GFP-tagging constructs

Generation of endogenous C-terminal tagging constructs by fusion PCR differs from the generation of deletion constructs in that it involves the insertion of a sequence encoding the tag with only minimal removal (the termination codon of the protein to be tagged) of the original genome sequence. Here the procedure for tagging of a green-fluorescent protein moiety is described as an example. In order to insert a GFP tag at the C-terminus of a protein of interest, the two targeting fragments amplified for homologous recombination are the >0.5KB long sequences of the genome encoding the C-terminal region of the gene to be tagged except the termination codon (Figure 3.2B) and the immediate downstream genome sequence following the termination codon of the gene (Figure 3.2C). In amplifying these targeting fragments, the 3’-end primer of the upstream sequence is designed to have a 20 bp long 5’ extension that is a reverse complement of the first 20 bp sequence of the tagging cassette (Figure 3.2D), and the 5’-end primer of the downstream sequence is designed to have a 20 bp long 5’ extension identical to the last 20 bp sequence of the tagging cassette (Figure 3.2E). The tagging cassette itself is made up of the sequences encoding a GA5 (five-time repeat of
glycine-alanine residues) linker immediately followed by a sequence encoding a GFP polypeptide and an Af-pyrG auxotrophic marker (Figure 3.2A). The GA5 linker is a flexible bridge translationally fusing the protein of interest and the GFP polypeptide, minimizing the interference of the functional conformation of the two protein entities (Osmani et al., 2006a). The final fusion PCR reaction is carried out using a mixture of the three fragments as templates and the 5'-end primer of the upstream sequence and the 3'-end primer of the downstream sequence (Figure 3.2F). After transformation using this construct, the proper integration is confirmed using a primer set that targets sequences outside of this construct in diagnostic PCR reactions (Figure 3.2G).

3.1.2.3. Endogenous alcA promoter insertion constructs

To generate an alcA promoter insertion construct, the targeting sequences amplified are the >0.5KB genome region immediately upstream of the start codon of the gene of interest (Figure 3.3B) as well as the first >0.5KB sequence of the gene of interest beginning with the start codon itself (Figure 3.3C). As in the case of the other types of targeting constructs, amplification of these targeting fragments is carried out using a 3'-end primer of the upstream sequence designed to have a 20 bp long 5’ extension that is a reverse complement of the first 20 bp sequence of the tagging cassette (Figure 3.3D) and a 5’-end primer of the
downstream sequence designed to have a 20 bp long 5’ extension identical to the last 20 bp sequence of the tagging cassette (Figure 3.3E). The alcA promoter insertion cassette is made up of a heterologous Aspergillus fumigatus riboB auxotrophic marker followed by the alcA promoter sequence to be inserted (Figure 3.3A). Although in general nutritional markers other than Af-riboB could be used for this procedure as well, in the work described in this dissertation the Af-riboB was chosen to leave the possibility of using the Af-pyrG marker in the future for further manipulation of the strains generated (We thank Dr. Berl Oakley for kindly providing us with the Af-riboB and alcA promoter sequences. A fusion PCR reaction is carried out using a mixture of the three fragments as templates and the 5’-end primer of the upstream sequence and the 3’-end primer of the downstream sequence (Figure 3.3F). As proper insertion of the alcA promoter would affect the expression of the gene it is targeting, any transformation of the alcA promoter insertion construct has to be carried out under a condition where the induction of the alcA promoter is achieved (see 3.1.3). After transformation, diagnostic PCR analysis is performed with primers that target sequences outside of this construct (Figure 3.3G).
3.1.3. Gene expression in *A. nidulans* using the *alcA* promoter

In *A. nidulans*, the targeted regulation of gene expression can be achieved by placing the gene of interest under the control of the promoter of the alcohol dehydrogenase I gene *alcA*. As a part of the alcohol utilization pathway, *alcA* gene expression is either induced or suppressed in response to different carbon sources present in the growth medium. While *alcA* gene expression is highly induced in response to ethanol and threonine via the function of the positive transcriptional regulator AlcR (Kulmburg et al., 1992), the presence of glucose results in the tight suppression of *alcA* mediated by the central catabolite regulator CreA (Bailey and Arst, 1975; Kulmburg et al., 1993). When fructose, lactose or glycerol are added as the carbon source of the medium, transcriptional regulation is neither under inducing or repressing control which leads to a moderate level of *alcA* gene expression (Pateman et al., 1983; Fillinger et al., 1995). Since these regulations of *alcA* gene expression occur at the transcriptional level and are mediated by the *alcA* gene promoter, the expression pattern of a given gene placed immediately downstream of the *alcA* promoter mimics that of the *alcA* gene in response to the different substances mentioned above. The ease with which the expression level of a protein can be controlled makes the *alcA* promoter expression system a useful tool in studying the function of genes carrying out essential cellular activities as the
expression of the essential gene can be turned off or on allowing study of its function without its deletion.

3.1.4. Heterokaryon rescue in *A. nidulans*

Determining whether or not the function of a given gene is essential can become a challenging task if attempts to delete the gene do not produce a clonal population of viable null organisms. Although consistent failure to recover a null organism would argue that the particular gene is essential, such a judgment would be inherently inconclusive as it ultimately depends on inference from negative data. Furthermore, analysis of the terminal deletion phenotype would be impossible due to the absence of a null organism. An alternative approach that had been used to determine the essentiality of genes was to carry out the gene deletion procedure in diploid organisms. The loss of one of the two copies of a given gene would not affect viability, thus enabling the recovery of a viable deletion transformants even if the function of the gene being deleted is essential. Nonetheless, confirmation that the function of the deleted gene is essential would have to be based on the inability to recover viable deletion strains after the diploid organism is induced to segregate into haploid strains, thus leaving the original problem unresolved. In *A. nidulans*, these difficulties can be overcome by utilizing the heterokaryon rescue technique (Osmani et al., 1988; Osmani et al., 2006b). *A. nidulans* are capable of forming
heterokaryons that maintain two types of genetically unlike nuclei within a common cytoplasm. When an essential, hypothetical gene \textit{geneX} is deleted in a \textit{pyrG}^{-} strain via transformation using the \textit{pyrG} auxotrophic marker, the null allele \textit{\Delta geneX} can still be maintained in a viable transformant cell when a heterokaryon is formed which retains the two types of unlike nuclei with the genotypes \textit{\Delta geneX+pyrG}^{+} and \textit{geneX+pyrG}^{-} (Figure 3.4). To confirm the formation of such a heterokaryon, the uninucleate conidiospores (of the primary transformants) into which either one of the two types of nuclei are incorporated can be tested for their ability to grow on two different sets of media which are either restrictive or permissive of the nutritional requirements of a \textit{pyrG}^{-} strain. On the media on which a \textit{pyrG}^{-} strain cannot grow due to the nutritional restriction (usually YAG; yeast extract and glucose based complete rich medium except uridine and uracil, the nutritional requirements of \textit{pyrG}^{-} strains), neither of the \textit{\Delta geneX+pyrG}^{+} nor \textit{geneX+pyrG}^{-} conidiospores will grow since the \textit{\Delta geneX+pyrG}^{+} spore lacks the essential \textit{geneX} function and the \textit{geneX+pyrG}^{-} spore is \textit{pyrG}^{-}. On the media where a \textit{pyrG}^{-} strain can grow (usually YAGUU; YAG media with uridine and uracil added to it), the \textit{geneX+pyrG}^{-} conidiospores will successfully propagate as there are no nutritional restrictions due to the \textit{pyrG}^{-} genotype while the \textit{\Delta geneX+pyrG}^{+} spores still cannot grow due to the lack of the essential \textit{geneX} function. As the two different \textit{\Delta geneX+pyrG}^{+} and \textit{geneX+pyrG}^{-} conidiospores form an undistinguishable
mixture on the surface of a heterokaryon colony, when this mixture is randomly
taken and streaked out on the two different sets of media, the real observation
comes in the form of two differing growth patterns on the two different media sets.
While healthy colony growth (from geneX+pyrG− spores) is observed on the
permissive media set (YAGUU), no colonies are formed on the restrictive media
set (YAG; Figure 3.5B). Since the ΔgeneX+pyrG+ genotype is nevertheless
isolated into individual mitotic progeny conidiospores, the terminal growth and
basic cytological phenotypes of the essential gene deletion can also be analyzed.
On medium restrictive for pyrG− spores, the growth limit of those spores is well
defined and understood as they only swell up while failing to project a germ tube.
By identifying conidiospores exhibiting different terminal growth patterns (the
ΔgeneX+pyrG+ spores) one can analyze the terminal phenotype of the geneX
deletion (Figure 3.5C). The formation of heterokaryons can be further confirmed by
conducting diagnostic PCR analysis. As heterokaryons maintain two different
nuclei within their cells, diagnostic PCR carried out with primers that target
sequences outside of the gene deletion construct will reveal two bands that are of
different sizes, one coming from the wild type and the other the null allele (unless
the deleted gene is of exactly the same size as the Af-pyrG gene deletion cassette;
Figure 3.5C). In contrast to the deletion of an essential gene, if a nonessential
gene is deleted, a clonal population of viable null homokaryon organisms can be
obtained. When the heterokaryon rescue technique is used for the deletion analysis, conidia from the primary transformants will grow on the media sets that are both restrictive and permissive for pyrG- strains (YAG and YAGUU; Figure 3.6B) and diagnostic PCR result will show a single band amplified from the null allele of the deleted gene (Figure 3.6C).

3.2. RESULTS

3.2.1. Identification of 28 different protein phosphatase catalytic subunit genes in the A. nidulans genome by sequence homology search

To identify all protein phosphatase catalytic subunit genes present in the A. nidulans genome, first the protein sequences of all the protein phosphatase catalytic subunit genes in the budding yeast S. cerevisiae were identified in the Saccharomyces Genome Database (SGD; http://www.yeastgenome.org). After the identification of these sequences, each were used for BLAST searches to look for sequence homologues in the Aspergillus Comparative Database (http://www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html) of the Broad Institute. As a result, 28 different hypothetical genes predicted to encode protein phosphatase catalytic subunits were identified (Table 3.1).
3.2.2. Generation of protein phosphatase gene deletion strains

27 of the 28 identified protein phosphatase catalytic subunit encoding genes were deleted. The generation of the gene deletion constructs and confirmation of the gene deletions was carried out in the way described in the Introduction of this chapter (See figure 3.1A, data not shown). As the work covered in this dissertation is limited only to the catalytic subunits of protein phosphatases, from now on the simple term “protein phosphatase(s)” will be used to refer to them unless specifically stated otherwise.

3.2.2.1. Five protein phosphatases have essential functions in A. nidulans

Of the 27 protein phosphatase genes identified in A. nidulans, the deletion of 4 protein phosphatase genes resulted in the consistent generation of heterokaryons (Figure 3.7A), confirming their cellular function to be essential. In addition to the 4 essential protein phosphatase genes, Means and his colleagues had in the past determined the cnaA gene (AN8820 in the A. nidulans genome database; coincidentally the one gene of the 28 that I could not delete for minor technical reasons) as essential (Rasmussen et al., 1994), bringing the total of essential protein phosphatase genes to five. The four essential protein phosphatase genes identified as essential in this dissertation are AN0410 (bimG), AN3941 (nimT), AN6391 (pphA), AN2902 (An-fcp1).
3.2.2.2 Four nonessential protein phosphatases that show significant growth defects upon gene deletion.

Among the nonessential protein phosphatases, gene deletion of AN0164 (An-ppg1), AN1343 (An-nem1), AN3810 (An-ssu72) and AN4419 (An-yvh1) yielded growth defect phenotypes readily distinguishable in the small sizes of growing colonies (Figure 3.13). When the diameter of their colonies were measured over the period of 5 days while growing at room temperature, all four of them showed <35% of the colony sizes of the control wild type strain.

3.2.3. Generation of strains with the protein phosphatase genes under the control of the \textit{alcA} promoter.

As a tool to more easily study the protein phosphatase genes whose deletions proved to be lethal, or result in severe growth defect phenotypes, strains were generated where these genes were placed under the control of the inducible \textit{alcA} promoter (see Figure 3.1C). When the growth characteristics of these strains were compared to the corresponding gene deletion strains, the suppression of the genes on glucose containing medium proved to be effective enough for the growth defects exhibited by the \textit{alcA} regulatable strains to closely mimic that of the gene deletion strains (Figure 3.7B).
3.2.4. Phenotypic analysis of essential protein phosphatase gene deletions

Basic phenotypic analysis of the essential protein phosphatase gene deletions was carried out in two different ways. From the primary transformants, conidiospores were streaked on YAG (rich medium without uridine and uracil) plates and the growth of conidiospores at room temperature was followed for three days (Figure 3.8, 9). Also, the conidiospores taken from the primary transformants were suspended into YG liquid medium and inoculated on cover slips, grown for 18 hours at room temperature, DAPI stained and imaged by fluorescence microscopy.

Deletion of AN0410 (bimG) showed the most severe growth defect phenotype as the \( \Delta \text{bimG} \) spores on YAG medium failed to project germ tubes or engage in polarized growth at all (Figure 3.8, 10A). Previous studies conducted with the \( \text{bimG11} \) conditional mutant allele had revealed that loss of BIMG activity led to a mitotic arrest of the cell. When the nuclei of \( \Delta \text{bimG} \) spores were visualized by DAPI staining, the nuclei were arrested in mitosis with DNA remaining highly condensed, phenotypes consistent with the observations made in \( \text{bimG11} \) mutants. Deletion of \( \text{An-fcp1} \) was slightly less severe as some \( \Delta \text{An-fcp1} \) spores were able to germinate and project a very short germ tubes (Figure 3.8, 11A). The \( \text{nimT} \) gene deletion had a less detrimental effect on initial germination. Up to a certain length polarized germ tube projection continued (Figure 3.9), but DAPI staining revealed the nuclei
of the ∆nimT cells showing a severe G₂ phase arrest phenotype as big, stretched out nuclei unable to enter mitosis with uncondensed DNA were observed (Figure 3.10B). The gene deletion of pphA resulted in the least tight growth defect as ∆pphA spores were able to grow into germlings better than those of the other three essential phosphatase gene deletions (Figure 3.9). When the nuclei were analyzed after DAPI staining, irregular morphology stained DNA was observed, indicative of a potential mitotic defect (Figure 3.11B).

3.2.5. Phenotypic analysis of nonessential protein phosphatase gene deletions

For the analysis of the nonessential phosphatase gene deletions, clonal condiospore stocks were generated for each of the deletion strains. After 6.5 and 9 hours of growth in rich liquid medium at 32°C, the cells were fixed and stained with DAPI to visualize the DNA. Control wild type cells (strain R153) grown under the same conditions reached 4 and 16 nuclei stages respectively after 6.5 and 9 hours growth (Figure 3.13), and the growth and cytological defects exhibited by the phosphatase null mutants were compared to them.

Deletion of An-ppg1 appeared to affect the overall growth rate of the ∆An-ppg1 cells without any pronounced mitotic defects. At both time points of 6.5 and 9 hours of growth, ∆An-ppg1 cells had fewer nuclei as well as much shorter germ tubes compared to the wild type cells indicating an overall slowdown of
growth as a consequence of An-ppg1 deletion (Figure 3.14, 15). Nevertheless, the presence of cells that had already undergone two rounds of mitoses (four nuclei; equivalent to wild type cells) after 6.5 hours suggest that the rate of mitoses may not be affected by the deletion of An-ppg1 as much as overall growth, at least during the early stages of germination. This phenotype of having relatively high number of nuclei in contrast to short germ tubes was also observed in cells of the strain where the endogenous An-ppg1 gene was placed under the control of the alcA promoter. When these cells where grown under the same conditions and thus the expression of An-ppg1 was suppressed (as the same rich liquid medium used to grow the deletion strains was glucose based), cells appeared to have close to the same number of nuclei while significantly shorter germ tubes compared to wild type cells at both after 6.5 and 9 hours of growth (Figure 3.16, 17).

In the An-nem1 null mutants, though the overall growth rate of the cells was also affected, signs of DNA fragmentation were the most significant deletion phenotypes. In some cells grown for 6.5 hours, the actual number of nuclei was higher than in the wild type cells, suggesting an acceleration of cell cycle progression during initial germination (Figure 3.18). The nuclei were small in size and of irregular shapes with some of them appearing to have condensed DNA. A feature more pronounced in the cells that had grown longer (9 hours) was the presence of fiber-like structures positively stained by DAPI that appeared to be
interlinking the nuclei (Figure 3.19). Taken together, these phenotypes suggested defects in mitotic regulation leading to fragmented nuclei that failed to fully separate from each other. Still, this deletion phenotype did not seem to have an accumulative effect on the overall growth rate of the cell. In \textit{alcA-An-nem1} cells with the expression of \textit{An-nem1} suppressed in glucose medium, cells showed phenotypes very similar to the deletion strains while a relatively high growth rate could be seen (Figure 3.20, 21).

Gene deletion of \textit{An-ssu72} appeared to cause the overall slowdown of cellular growth while no obvious mitotic defect phenotypes could be observed. Although the nuclei in the cells grown for 6.5 hours seemed to be slightly larger in size, cells that had grown for 9 hours did not share these nuclear morphological features (Figure 3.22, 23).

The most severe growth defect phenotype among the nonessential phosphatase gene deletion strains was shown by the \textit{\Delta An-yvh1} strain. After 6.5 hours of growth, most of the spores had failed to germinate, and many of those cells had not undergone their first mitosis either (Figure 3.24). Even the cells that had managed to initiate germination were small in size not only in terms of the length of the germ tube but also in the extent of how much the spore had swollen up prior to germination. When \textit{\Delta An-yvh1} cells were grown for 9 hours, the germtubes were still very short in length and many of the nuclei appeared to be
large and stretched out in their length resembling G\textsubscript{2} phase nuclei, indicating potential defects in mitotic progression (Figure 3.25).

3.2.6. Localization of essential and select nonessential protein phosphatases

As a means to further study the function of the essential protein phosphatases, as well as the ones whose deletion strains exhibited growth defect phenotypes, endogenous C-terminal GFP tagging on seven of those proteins was carried out to determine their subcellular localization (BIMG had previously been tagged and its localization was being studied by my colleague Leena Ukil in the Osmani lab). After the recovery of stable transformants, proper insertion of the GFP-tagging constructs was confirmed by diagnostic PCR (data not shown). C-terminal GFP tagging of the PP\textsubscript{HA} phosphatase did not yield functional PP\textsubscript{HA}::GFP chimeric proteins as all the transformants examined turned out to be diploids. Since PP\textsubscript{HA} was shown to be an essential phosphatase, the formation of stable diploid strains confirmed that the PP\textsubscript{HA}::GFP chimera was not functional and thus the transformants were forming diploids to compensate for the essential PP\textsubscript{HA} function. When the localization of the remaining six phosphatases was examined by confocal microscopy, only An-FCP\textsubscript{1}::GFP, An-SSU72::GFP and NIMT::GFP showed concentration of their GFP signals to specific cellular regions. Both An-FCP\textsubscript{1} and An-SSU72 showed distinct nuclear localization (Figure 3.26,
27), consistent with their known functions of targeting the nuclear protein RNAPII. While the localization of NIMT was less clear, concentration of the GFP signal to particular foci within several cells was observed (Figure 3.28).

3.3. DISCUSSION

3.3.1. Total number of protein phosphatase catalytic subunits

A genome wide homology search for protein phosphatase genes revealed the presence of 28 different catalytic domains of protein phosphatases in *A. nidulans*. The relatively small number of phosphatase catalytic subunits was surprising considering that these 28 different proteins were able to counteract the function of all different protein kinases, with a significantly higher number of protein kinases known to exist. As mentioned in the overall introduction (Chapter 1), this probably is due to the unique way the activities of protein phosphatases are regulated. By forming complexes with different kinds of regulatory subunits, a single protein phosphatase catalytic subunit would be able to function in many different phosphatase holoenzymes, making it possible to counter the activities of many different protein kinases.
3.3.2. Essential protein phosphatases

This study showed that the phosphatase genes \textit{bimG}, \textit{nimT}, \textit{An-fcp1} and \textit{pphA} to encode proteins with essential cellular functions. The fact that three of those genes were identified previously in genetic screens aiming for identifying cell cycle mutants (\textit{bimG} and \textit{nimT} through the landmark screen by Ron Morris in 1976 and \textit{An-fcp1} in a screen described in Chapter 4 of this dissertation) reflects the importance of phosphatase mediated protein dephosphorylation in regulating cell cycle progression. The loss-of-function mutant phenotype of \textit{bimG} and \textit{nimT} had been well documented through studies of the conditional mutant alleles \textit{bimG11} and \textit{nimT23}, and the terminal phenotypes displayed by the corresponding deletion strains confirmed their functions to be critical in mitotic regulation. While \textit{An-fcp1} and \textit{pphA} deletions did not yield phenotypes as clearly demonstrative of mitotic defects as \textit{bimG} and \textit{nimT} deletions, the nuclear morphology visualized by staining the DNA with DAPI suggests that there are potentially important functions of these genes in cell cycle regulation as well. The availability of the conditional \textit{An-fcp1}\textit{TS} mutant allele (discussed in Chapters 3, 4 and 5) as well as the strains in which \textit{An-fcp1} and \textit{pphA} are under the inducible promoter \textit{alcA} should prove to be useful in further study of the functions of these genes.
3.3.3. Gene deletion phenotype of four nonessential but important protein phosphatases.

The gene deletion of four protein phosphatases did not cause lethality but had physiological effects detrimental enough to cause strong growth defects. Of those four phosphatase genes, AN-SSU72 is functionally linked to the essential phosphatase An-FCP1 as the physiological target of both phosphatases had been shown to be the C-terminal domain of RNA polymerase II (RNAPII CTD; Krishnamurthy et al., 2004). This may reflect the importance of maintaining a proper phosphorylation status of RNAPII CTD for the cell to engage in proper transcriptional activity, by itself an activity absolutely vital for cellular survival. Unlike An-SSU72, the phosphatase An-NEM1 is linked to An-FCP1 by belonging to the same protein phosphatase family that shares the CPD type catalytic domain. Defined first by the discovery of Fcp1, the CDP family is made up of protein phosphatases that engage in Aspartate based catalysis of target proteins and have in common the signature catalytic motif of DXDXT/V. While only three different CPD phosphatases are present in *A. nidulans*, two of them are either essential (*An-fcp1*) or cause severe growth defects upon deletion (*An-nem1*), indicating the importance of the biological function conserved in this phosphatase family. Within the context of cell cycle regulation, *An-nem1* is of added potential interest as its deletion not only caused an overall decrease of cellular growth but was also
marked by a noticeable DNA fragmentation phenotype, a strong indication that it may play an important role in mitotic regulation.

3.3.4. Suppression of protein phosphatase expression using the alcA promoter

Comparison of the growth patterns of essential and important protein phosphatase gene deletion strains with strains in which the corresponding phosphatase genes were placed under the control of the inducible alcA promoter confirmed the feasibility of utilizing the alcA promoter insertion system as a tool to achieve regulated expression of proteins of interest. When the expression of essential phosphatase genes were suppressed by putting the alcA-promoter driven strains on glucose-based medium, virtually no colony growth was observed, confirming the tightness of this expression system (Figure 3.7B). The induction and suppression of important but nonessential phosphatase genes was similarly effective, although the suppression appeared to be not as tight as the null allele. This may be due to the different threshold concentration levels of different proteins to have a physiological impact as well as other factors influencing the expression and activity of proteins such as their structural stability and half-life.
3.3.5. Localization of essential and select nonessential protein phosphatases.

The attempt to study the localization of protein phosphatases by endogenous GFP tagging proved to be more challenging a task than initially expected. First, since many protein phosphatase catalytic subunits are rather small in size (consistent with their tendency to form complex holoenzymes with other regulatory subunits), the translational fusion with the GFP moiety rendered the original phosphatase protein nonfunctional in the case of PPHA. Also, except for An-FCP1, An-SSU72 and NIMT, all other protein phosphatases proved to have a nondescript localization pattern where the GFP signals were spread over the entire volume of the cells. This may again reflect the pleiotropic manner in which phosphatase catalytic subunits function. If the spatial targeting of the phosphatase activity is achieved by it forming a complex with other factors, even a protein that exhibits ubiquitous presence throughout the cell could deliver localized, specific functions by interacting with different regulatory units. A possible way of overcoming these difficulties may lie in investigating the subcellular localization of the different regulatory subunits of phosphatase holoenzymes. By combining protein affinity purification experiments to identify the proteins that form the phosphatase holoenzyme protein complexes with localization studies of these identified regulatory subunits could provide an effective means to further study the localizations and functions of protein phosphatases.
Figure 3.1 Generation of a gene deletion construct by 3-way fusion PCR.
The *Af-pyrG* gene deletion cassette (A) is fused with fragments of the genome sequences upstream (B) and downstream (C) of the gene to be deleted (*genX*) by fusion PCR, forming a functional deletion construct (fusion PCR reaction with primers G and F). The fusion of the three individual fragments is dependant on the overlapping sequence incorporated in primers (D, E). After transformation, proper replacement of *genX* by *Af-pyrG* is confirmed by diagnostic PCR using a primer set that targets sequences either side of this integrated construct (H).
Figure 3.1
Figure 3.2 Generation of a C-terminal GFP tagging construct by 3-way fusion PCR. The tagging cassette is made up of sequences encoding a flexible GA5 (5X glycine-alanine) linker followed by a sequence encoding a GFP polypeptide and an Af-pyrG auxotrophic marker (A). After individual PCR amplification of fragments A, B and C, due to the overlapping sequences incorporated into primers (D, E), A, B and C fuse into one construct via 3-way fusion PCR (F). Proper integration is confirmed by diagnostic PCR using a primer set that targets sequences either side of this integrated construct (G)
**Figure 3.2**
Figure 3.3 Generation of an \textit{alcA} promoter insertion construct by 3-way fusion PCR. The \textit{alcA} promoter cassette is made up of the \textit{Af-riboB} auxotrophic marker followed by the \textit{alcA} promoter sequence (A). As the \textit{alcA} promoter has to be placed immediately upstream of the gene of interest, the \textit{Af-riboB} marker is positioned upstream of the \textit{alcA} promoter sequence. After individual PCR amplification of fragments A, B and C, due to the overlapping sequences incorporated into primers (D, E), A, B and C fuse into one construct via 3-way fusion PCR (F). Proper integration is confirmed by diagnostic PCR using a primer set that targets sequences either side of this integrated construct (G)
Figure 3.3
Figure 3.4 Heterokaryon formation following the deletion of an essential gene in a pyrG− strain using a pyrG deletion construct. When an essential geneX is deleted, heterokaryon formation enables the pyrG− geneX+ and pyrG+ geneX− nuclei to reciprocally complement each other’s respective lack of pyrG and geneX function (C). When these nuclei are incorporated into uninucleate conidiospores, pyrG− geneX+ spores will grow on neither YAG nor YAGUU plates, but pyrG+ geneX− will grow on YAGUU plates, an easy growth read-out to test if a gene is essential or not.
**Figure 3.5 Expected results when the heterokaryon rescue technique is used to delete an essential gene.** Gene deletion leads to the formation of heterokaryons. When replica plated on YAG and YAGUU media plates, conidiospores from primary transformants in which gene replacement occurred correctly will not grow on YAG plates (B). Heterokaryon formation can be verified by diagnostic PCR. Null and wild type alleles coexisting in the heterokaryotic mycelium give rise to two bands of different sizes after diagnostic PCR amplification using DNA from the primary transformant as template (C). The terminal phenotype of essential gene deletions can be studied by identifying conidiospores on YAG media that carry the null allele of the deleted gene (D).
Figure 3.6 Example of anticipated results when heterokaryon rescue technique is applied to delete a nonessential gene. When a deleted gene is nonessential, homokaryons carrying the null allele of the deleted gene can be isolated from the primary transformants. These conidiospores are capable of growing on both YAG and YAGUU media plates (B). Diagnostic PCR verifies the presence of a single null allele in the transformants (C).
Table 3.1 The genes encoding protein phosphatase catalytic subunits in *A. nidulans*. Genes encoding 28 different catalytic subunits of protein phosphatases were identified. Based on their catalytic domain motifs these phosphatases can be categorized into 9 different groups (see domain). Five have essential functions (labeled in red) and four more cause significant growth defects when deleted (labeled in blue).
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Table 3.1
Figure 3.7 *nimT, An-fcp1, pphA* (AN6391) and *bimG* are essential genes. Using the heterokaryon rescue technique, *nimT, An-fcp1, An-ppg1* (AN6391) and *bimG* were shown to be essential genes. (Concerning the deletion of *nimT*, the size of the deletion cassette was almost the same as the deleted *nimT* gene [second and third lane]. To verify the gene deletion, additional PCR analysis was carried out using a primer targeting a sequence outside of the *pyrG* deletion cassette with a primer targeting a sequence inside of the *pyrG* deletion cassette. Only the gene deletion set produces a band [last lane], confirming the deletion of *nimT* (A). As expected for essential genes, almost complete loss of growth was achieved when the expression of *nimT, An-fcp1, AN6391* and *bimG* was suppressed using an *alcA* promoter (B).
A

ΔnimT

ΔpphA

ΔAn-fcp1

ΔbimG

YAGUU  YAG

B

WT

alcA::An-fcp1  alcA::nimT

alcA::bimG  alcA::AN6391

Figure 3.7

EtOH  glucose
Figure 3.8 Terminal growth characteristics of \textit{bimG} and \textit{An-fcp1} deletion strains. The growth of $\Delta$\textit{bimG} and $\Delta$\textit{An-fcp1} strains tested on YAG plates. The $\Delta$\textit{bimG} cells failed to grow beyond swelling even after three days at room temperature, making them almost indistinguishable from the \textit{pyrG} cells. $\Delta$\textit{An-fcp1} cells grew to project a short germ tube after one day but fail to grow beyond that even after three days further incubation (white arrows).
Figure 3.9 Terminal growth characteristics of \( \Delta \text{nimT} \) and \( \Delta \text{AN6391} (\Delta \text{pphA}) \) deletion strains. The growth of \( \Delta \text{nimT} \) and \( \Delta \text{AN6391} (\Delta \text{pphA}) \) strains tested on YAG plates. The \( \Delta \text{nimT} \) cells grew to considerable length after one day at room temperature, but failed to grow further after three days (black arrows). \( \Delta \text{AN6391} (\Delta \text{pphA}) \) cells grew to project short germ tubes after one day but did not grow much beyond that after three days incubation (white arrows).
Figure 3.10 Terminal phenotype of $\Delta bimG$ and $\Delta nimT$. Deletion of $bimG$ results in polarity defect and a tight mitotic arrest of the nucleus with condensed DNA (A). Deletion of $nimT$ leads to defects of the nuclei to enter mitosis, indicated by the long nuclei with decondensed DNA (B).
Figure 3.11 Terminal phenotype of ΔAn-fcp1 and ΔAN6391 (ΔpphA). Cells deleted of ΔAn-fcp1 show irregular distribution of nuclei along the germ tube and a general inhibition of growth (A). In AN6391(pphA) deleted cells, nuclei are stretched out in their length (white arrows) while DNA appears irregular but decondensed (B).
Figure 3.12 Deletion of four nonessential phosphatase genes results in significant growth defects. Although only five phosphatase genes were determined to be essential, there were four additional genes which when deleted conferred significant growth defects upon cells. The colony diameters of the phosphatase deletion strains were measured over five days at room temperature. The colony sizes of four deletion strains were significantly smaller than the rest (A). The growth defects of those four deletion strains could be partially recapitulated by putting the deleted genes under the control of the inducible alcA promoter and growing them on suppressing glucose medium. alcA::AN0129 is a negative control of suppression of a nonessential phosphatase gene. alcA::nimT is a positive control suppression of essential phosphatase gene (B).
**Figure 3.12**

A: Graph showing colony size (diameter in mm) over growth days (1 to 5) for various strains.

B: Petri dishes with different strains grown in media containing EtOH and glucose.
Figure 3.13 Growth and DNA staining of wild type control strain R153. The wild type strain R153 was grown for 6.5 (A) and 9 hours (B) each, fixed and DNA stained with DAPI. At these time points the cells have predominately 4 nuclei and 16 nuclei respectively.
Figure 3.14 Deletion phenotype of An-ppg1 (AN0164) after 6.5 hours growth.

While rate of mitosis seem to less affected, germination is much slowed down when An-ppg1 is deleted in comparison to wild type cells.
Figure 3.15 Deletion phenotype of An-ppg1 (AN0164) after 9 hours growth.

Overall cellular growth is slowed as both the size of the cells as well as the number of nuclei is less than in the wild type cells. Overall nuclear morphology however appears normal.
Figure 3.16 Phenotype of *alcA-An-ppg1* (AN0164) strains grown in suppressive medium for 6.5 hours. The level of suppression was close to the null allele. Overall growth is slowed as germ tubes were very short, but mitoses seemed to occur properly as some cells exhibited eight nuclei.
Figure 3.17 alcA-An-ppg1 (AN0164) strains grown in suppressive medium for 9 hours. These cells appeared normal as did their nuclear structure but cell growth was reduced compared to the control strains.
Figure 3.18 Deletion phenotype of *An-nem1* (AN1343) after 6.5 hours growth. Growth was slowed in *An-nem1* (AN1343) deletion strains as germ tubes were short. The most notable phenotype was the nuclear structure which appeared small and condensed with no obvious nucleolar structure within nuclei evident.
Figure 3.19 Deletion phenotype of An-nem1 (AN1343) after 9 hours growth.

Although the An-nem1 (AN1343) deletion phenotype of small condensed nuclei persists, the cells could still maintain their growth.
Figure 3.20 Phenotype of *alcA-An-nem1* (AN1343) strain after 6.5 hours growth. Similar to the *An-ppg1* gene, suppression of *alcA-An-nem1* gene expression in YG medium came close to the null allele as cellular growth is effectively slowed and nuclear morphology defects are still observable.
Figure 3.21 Phenotype of \textit{alcA-An-nem1} (AN1343) strain after 9 hours growth. As in the 6 hour growth of this deletion strain, nuclear structure defects persists but the cells could continue to grow.
Figure 3.22 Deletion phenotype of *An-ssu72* (AN3810) after 6.5 hours growth. The deletion of *An-ssu72* resulted in slower cell growth where after 6.5 hours only very short germlings were observed and although nuclear division had taken place, daughter nuclei appeared to remain associated with each other and did not separate normally after mitosis.
Growth and nuclear division continued and the nuclear defects observed at earlier time points were largely alleviated but some cells still revealed evidence of potential defects in progression through mitosis.
Deletion of An-yvh1 results in a very strong growth defect. After 6.5 hours, few spores had swollen up let alone started to germinate.
Figure 3.25 Deletion phenotype of An-\textit{yvh1} (AN4419) after 9 hours growth.

Even after growing for 9 hours \textit{An-\textit{yvh1}} deleted spores had only very short germ tubes. Some of their nuclei seemed large in size and stretched in their length.
Figure 3.26 Localization of An-SSU72 by endogenous C-terminal GFP tagging. An-SSU72 showed a pattern of nuclear localization, consistent with the reported nuclear function of its homologues in other organisms.
Figure 3.27 Localization of An-FCP1 followed by endogenous C-terminal GFP tagging. An-FCP1 showed a clear nuclear localization pattern, as expected of a protein involved in transcription.
Figure 3.28 NIMT localizes to small foci in the cell. The localization of NIMT is not very defined, yet in some cells a concentration of the GFP signal in small foci could be observed.
CHAPTER 4

GENETIC SCREEN FOR IDENTIFICATION OF SYNTHETIC INTERACTIVE MUTATIONS WITH $nimX^{CDK1F}$

4.1. INTRODUCTION

Although inhibitory Tyr15 phosphorylation of CDK1 is a very important control mechanism of determining mitotic entry, the lack of lethality associated with the CDK1F mutation, in which Tyr15 phosphorylation is prevented, in various organisms supports the idea that it be not the only means of regulating onset of mitosis. This chapter describes a synthetic interactive genetic screen conducted with the aim of identifying additional mitotic control genes that do not function through inhibitory Tyr15 phosphorylation of CDK1. A brief introduction is provided to overview the basic scheme of the screen as well as introducing several concepts and methods of genetic manipulation which were employed.
4.1.1. Scheme of the genetic screen

To identify new mitotic control genes with functions independent of Tyr15 phosphorylation of CDK1, a genetic screen was designed to isolate mutations that show synthetic interactions with a mutant allele of \( \text{nimX}^{\text{CDK1}} \) that encodes a kinase incapable of Tyr15 phosphorylation, the \( \text{nimX}^{\text{CDK1F}} \) allele. Because this allele encodes a kinase that is not subject to negative Tyr15 phosphorylation control it is considered an activated allele and is dominant over the wild type allele. To enhance the efficacy of genetic manipulation at certain steps of the screen, the \textit{A. nidulans} strain FRY24 was used in which a \( \text{nimX}^{\text{CDK1F}} \) mutant allele had been inserted at the chromosomal locus of the \( \text{nimX}^{\text{CDK1}} \) to form a tandem repeat of the wild type \( \text{nimX}^{\text{CDK1}} \) and mutant \( \text{nimX}^{\text{CDK1F}} \) alleles, linked together by the auxotrophic marker \textit{pyr4} (Figure 4.1). One of the phenotypes of CDK1F mutants is their sensitivity to moderate levels of hydroxyurea (HU). Due to the inability of the cell to inhibit CDK1 activity through Tyr15 phosphorylation, the DNA replication checkpoint control is compromised in CDK1F mutants. Thus, when DNA replication is slowed down by HU, CDK1F mutants enter lethal premature mitoses and therefore fail to grow properly. The \textit{pyr4} auxotrophic marker linking the two different \( \text{nimX}^{\text{CDK1}} \) alleles is utilized in the screen for its responsive property to the reagent 5’-fluoroorotic acid (5’-FOA). In cells that express a functional \textit{pyr4} gene 5’-FOA is converted into the toxic compound 5’-flourouracil, thus enabling the counterselection for cells that do not have a functional \textit{pyr4} gene.

The screen mainly consisted of two successive selection procedures
following the initial introduction of new mutation via UV irradiation. After random UV mutagenesis, surviving cells were tested for initial temperature sensitivity. Since the $nimX^{CDK1F}$ mutant allele by itself does not cause a temperature sensitive phenotype, strains that showed initial temperature sensitive phenotypes were selected for subsequent eviction of the $nimX^{CDK1F}$ allele. By utilizing the toxicity that HU and 5'-FOA confer upon cells expressing $nimX^{CDK1F}$ and $pyr4$, a double counterselection was carried out for cells having evicted both the $nimX^{CDK1F}$ allele and $pyr4$ gene via homologous recombination between $nimX^{CDK1F}$ and the wild type $nimX^{CDK1}$ alleles (Figure 4.1). After verifying the eviction of $nimX^{CDK1F}$, the mutant strains were analyzed for any change to their initial temperature sensitive phenotypes. The loss of the $nimX^{CDK1F}$ allele had left the strains with only those mutations in their genetic background introduced newly during the UV mutagenesis procedure. A decrease of temperature sensitivity as a consequence of $nimX^{CDK1F}$ eviction would confirm that the newly introduce mutation and the $nimX^{CDK1F}$ mutation are synthetically interacting, in combination causing the temperature sensitivity. Mutations identified as having synthetically lethal genetic interactions with $nimX^{CDK1F}$ were further analyzed genetically to verify whether they are single mutations and their allelic relationship examined. Dominance/recessiveness of the obtained mutations were also determined to select only recessive mutations for easy cloning purposes (Figure 4.1). Single, recessive mutations were consequently cloned by complementation using an AMA1 plasmid-based $A. nidulans$ genomic DNA library.
4.1.2. UV mutagenesis

Exposure of cells to ultraviolet (UV) (wavelength 200-400nm) electromagnetic radiation can cause the formation of various kinds of mutagenic DNA lesions. Although different wavelength components of UV radiation are known to have distinct mutagenic properties, the most common types of mutagenic lesions induced by UV irradiation are cis-syn cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidone photoproducts [(6-4)PPs] (Figure 4.2). CPDs are generated when the C-4 and C-5 atoms of two adjacent pyrimidine bases form a cyclobutane ring, while a (6-4)PP is defined by the covalent linkage between C-4 and C-6 atoms of two adjacent pyrimidines. The exact contributions of these DNA lesions to actual mutageneses are not fully understood, but the high frequency of base transition mutations in dipyrimidine sequences observed in actual UV mutageneses is consistent with the prevalent induction of CPDs and (6-4)PPs upon UV irradiation. Since it is easy to administer, largely non-biased in its mutagenic nature concerning gene functions and generally produces point mutations, UV irradiation is a widely used method for random mutagenesis in various eukaryotic organisms including A. nidulans.

4.1.3. Temperature sensitivity

Organisms that display distinct mutant phenotypes only under certain environmental conditions (nonpermissible or restrictive conditions) while otherwise (under permissible conditions) resembling a wild type are termed conditional mutants. One of the conditional mutant phenotypes most widely used
in cell cycle studies is temperature sensitivity. A temperature sensitive (ts) mutant is defined by having distinct (ranges of) temperatures as permissive and restrictive growth conditions within the temperature range where wild type organisms are normally viable. Ts mutations are generally loss-of-function hypomorphs due to compromised structural stability of the 3-dimensional protein conformation. Being conditional mutants in whom induction of the mutant phenotypes can be easily managed by simply shifting the growth temperatures, ts mutants can especially be useful in studying the function of essential genes. In *A. nidulans*, wild type organisms display robust growth at temperatures from 15°C to 44°C, with 32°C to 39°C considered the temperature range ideal to achieve maximum growth rate. Ts mutants usually show growth defects at temperatures higher than 37°C, while mutant strains displaying growth defects at the temperature of or lower than 32°C are commonly termed *cold sensitive* mutants.

4.1.4. AMA1 plasmid-based *A. nidulans* genomic DNA library

The AMA1 plasmid is an autonomously replicating plasmid first isolated in 1990 by Clutterbuck and his colleagues (Gems et al., 1990). When a plasmid re-isolated from an unstable *A. nidulans* colony resulting from an *A. nidulans* gene bank transformation exhibited extremely high transformation frequencies (~250 times higher than conventional integrative plasmid vectors), the 6.1-kB sequence insert of the plasmid was termed AMA1 (*autonomously* maintained in *Aspergillus*). The AMA1 sequence was later shown to be an inverted duplication of a low-copy
number dispersed genomic repeat (Aleksenko and Clutterbuck, 1996), and when present as an insert confers upon a plasmid properties that make it suitable as an episomal vector system. Besides the high transformation frequency, when transformed into *A. nidulans* the AMA1 plasmids are maintained as extrachromosomal episomes and transformants show high phenotypic stability (i.e., high percentage of spores carry the plasmid). In addition, since one transformant carries only one type of plasmid as a rule, it is easy to rescue them into *E. coli* for further analysis. AMA1 plasmids are maintained at high copy numbers (10-30 copies present per haploid genome) and transcriptional regulations remain mostly intact for genes expressed off the plasmid (Aleksenko et al., 1996; Aleksenko and Clutterbuck, 1997). Taking advantage of these properties, the genomic DNA library of *A. nidulans* cloned into an AMA1 plasmid was utilized with great efficiency for cloning the mutations identified in this genetic screen.

4.2. RESULTS

4.2.1. Determination of the dosage of UV irradiation

It had been known from previous studies conducted in the lab that UV irradiation maximizes the generation of temperature sensitive mutants (at a frequency of <1% of the surviving colonies) when a UV dosage is used that yields a survival rate ranging between 0.1-5%. In order to determine the UV
dosage for mutagenesis that would lead to ~1% spore survival rate upon irradiation, a viability count of the A. nidulans conidiospore stock to be used in the mutagenesis was carried out followed by a UV kill curve analysis. The FRY24 spore stock subsequently used for UV mutagenesis showed 64% viability largely independent of inoculation concentration (Table 4.1A) on MAGUU complete rich growth medium plate. Of different UV radiation dosages tested at different spore inoculation concentrations, 3.15x10⁴ μJ/cm² at 7500 total spores/plate (~5000 viable spores/plate) yielded the desired survival rate (1.08%) and colony number per plate (~50/plate; Table 4.1B).

4.2.2. Initial screening for temperature sensitivity

The screen started with 750,000 spores (500,000 viable) plated on 100 MAGUU complete rich medium plates and subsequently exposed to 3.15x10⁴ μJ/cm² of UV radiation. 5,000 surviving colonies were replica plated onto two identical sets of MAGUU plates. To identify strains that showed temperature sensitivity as a consequence of UV mutagenesis, one set of the replica plated colonies was incubated at 32°C (permissive temperature) and the other set at 42°C (restrictive temperature). When the growth rates at the two different temperatures were compared after 48 hours, 89 of the 5,000 surviving colonies that were tested exhibited scorable degrees of temperature sensitivity at 42°C.
4.2.3. Determination of synthetic lethality

In order to confirm the synthetic lethal genetic interaction between the newly introduced mutation(s) by UV irradiation and the \( \text{nim}X^{CDK1F} \) dominant mutant allele at the restrictive temperature (42°C), all 89 temperature sensitive mutants were inoculated on MAGUU complete rich medium containing 8mM HU and 1% 5'-FOA (Figure 4.1). Colonies capable of growing on this counterselective medium had lost the \( \text{nim}X^{CDK1F} \) allele and thus were evaluated for any change to their initial temperature sensitivity phenotypes by testing their growth at temperatures ranging from 32°C to 42°C. Of the initial 89 mutant strains, 9 strains showed substantial loss of their temperature sensitivity upon eviction of the \( \text{nim}X^{CDK1F} \) allele (Figure 4.3), indicating a synthetic genetic interaction between the newly introduced mutations and the \( \text{nim}X^{CDK1F} \) mutant allele. Although they still demonstrated considerable degrees of growth defects at 42°C compared to wild type strains, at temperatures between 32°C and 42°C the eviction of \( \text{nim}X^{CDK1F} \) in each strain resulted in significant relief from the mutant phenotype shown by the original strains from which they were derived.

4.2.4. Genetic analysis of mutations

The nature of the genetic alterations in the nine mutant strains obtained was further analyzed genetically with the aim of obtaining single, recessive mutations and understanding any potential allelic relationships among them. When each of the haploid mutants was induced to form a diploid with a wild type strain, two of the nine mutants exhibited persistence of their temperature
sensitive mutant phenotypes in the diploid colonies, indicating those mutations to be dominant (Figure 4.4). The remaining seven mutants were backcrossed to a wild type strain to examine the segregation pattern of their mutant phenotypes. Of the seven mutants, only three strains (#13, #25, #66) showed strict segregation of each of their mutant phenotypes as a single complementation group with ~50% of the cross progeny showing the temperature sensitive mutant phenotypes (Figure 4.5). In the other four mutant strains, the mutant phenotypes segregated in a non-mendelian ratio, indicating that the temperature sensitivity was due to multiple mutations incorporated during UV mutagenesis (data not shown). When these three candidate strains were crossed to each other, none of their mutations appeared to be allelic based on the ability to uncover the wild type phenotype in progeny of all three crosses (Figure 4.6).

4.2.5. Cloning of the genes encoding the isolated mutations

The isolation of three different mutant strains was followed by molecular cloning of the mutations by complementation with a non-integrating high-copy-number AMA1-plasmid based A. nidulans genomic DNA library (Liu et al., 2004; Figure 4.7). After transformation of the mutant strains with the genomic DNA library, colonies that showed complementation of their mutant phenotypes (i.e. lost their temperature sensitivity) were recovered and their episomally maintained AMA1 plasmids rescued into E. coli. After amplification and re-isolation of the plasmids from E. coli, the minimum sequences of their inserts capable of complementing the temperature sensitive phenotype of each mutant were
isolated and identified through restriction digest analyses and DNA sequencing. Within these sequences open reading frames predicted by the A. *nidulans* genome database were PCR amplified and re-transformed into the corresponding mutant strains to test their ability to complement the mutant phenotypes. Following this strategy, the mutations of strain #25 and #66 were cloned, while strain #13 was not further investigated for technical reasons.

The DNA inserts of the plasmids capable of complementing the #25 mutation shared a 7.4KB region of the *A. nidulans* genome contig 1.51 scaffold 3. The *Aspergillus* genome database predicted an open reading frame encoding a protein similar to the protein phosphatase FCP1, identified both in yeast (*S. cerevisiae*) and humans. This cloned fragment could have been able to rescue the temperature sensitive phenotype of strain #25 in two different ways. First, if it had been including the wild type copy of the gene mutated in strain #25, the wild type gene simply could be complementing the recessive mutant allele. On the other hand, since the AMA1 plasmid is maintained at high copy numbers within the nucleus, the fragment cloned could have been acting as a high copy number suppressor of the mutation present in strain #25. To distinguish between these two possibilities, a linear DNA fragment of the predicted open reading frame of the gene encoding an FCP1 sequence homologue was PCR amplified and transformed into strain #25. Without a promoter region to drive its own expression, this linear fragment could only complement the mutation if it were the wild type copy of the mutated gene and replaced the mutation via double homologous recombination. Upon transformation it could indeed complement the
mutant phenotype, confirming that the predicted homologue of Fcp1 had been mutated in strain #25 (Figure 4.7).

Inserts of the plasmids complementing mutant #66 comprised of two different groups based on restriction digest pattern and subsequent DNA sequencing. One group shared a region of DNA that included the open reading frame of a hypothetical gene predicted to encode the bifunctional polynucleotide phosphatase/kinase Pnk1p (Figure 4.8B; lanes indicated by red arrows), while another group of inserts had a region in common that was predicted to encode the general transcription factor TFIIB (Figure 4.8B; lanes indicated by yellow arrows). To verify the gene that in fact was mutated in the strain #66, several linear DNA fragments that included different parts of the sequences within the two different inserts were PCR amplified (Figure 4.7C). Of those various fragments, only the one that included the full hypothetical open reading frame of the predicted gene Pnk1 was able to complement the mutant phenotype of strain #66 (Figure 4.7C and 4.7D; arrow and box in green). These results concluded that the gene homologous to Pnk1 was mutated in the strain #66, while TFIIB is probably a high copy number suppressor of the mutation.

4.2.6. Sequencing of mutation of An-Fcp1

As described above, strain #25 has been demonstrated to have a mutation on a hypothetical gene predicted to encode a protein homologous to the protein phosphatase FCP1. A protein BLAST search at NCBI with the predicted An-FCP1 sequence (Aspergillus nidulans FCP1) identified both Fcp1p in S.
cerevisiae (e-value 6e-94) as well as human FCP1 (e-value 1e-37) as its sequence homologues (Figure 4.8). All three homologues share the basic protein structure by having an NLI interacting factor-like phosphatase domain and a BRCT domain, suggesting conserved functions of the proteins. To understand the nature of the mutation introduced in the strain #25 on An-fcp1, the entire open reading frame was PCR amplified from the strain #25 and sequenced. Sequencing results showed that in the #25 mutant a highly conserved Leucine residue close to the critical phosphotransferase motif required for phosphatase catalytic activity was substituted to Alanine. As the mutated Leucine is part of a predicted beta-sheet structure adjacent to the catalytic core of An-FCP1, the Leucine to Alanine substitution was predicted to compromise the protein structure of An-FCP1 and thus lead to the temperature sensitive phenotype at 42°C (Figure 4.9). Although this mutation on An-fcp1 was isolated based on the relative decrease of temperature sensitivity upon the eviction of nimX^{CDK1F}, from a double mutant strain, the mutant by itself had been shown to cause a certain degree of temperature sensitivity as well. Thus, it was decided that this mutation be referred to as An-fcp1^{TS}. 
4.3. DISCUSSION

4.3.1. Frequency of temperature sensitive mutations

A UV dosage resulting in the survival of ~1% of irradiated spores generated temperature sensitive mutants at a frequency of ~1.8% of surviving colonies. Although the viability of spores did not change depending on the inoculation concentration, the survival rate of the spores seemed to be influenced by the inoculation concentration with spores generally showing a higher survival rate at higher inocula (Table 4.1). Aiming for ~50 surviving colonies per plate for technical convenience purposes, UV mutagenesis was carried out with the dosage of $3.15 \times 10^4 \mu$J/cm$^2$ with each 5000 viable spores inoculated on individual plates, repeated on 100 different plates to yield more than 5000 surviving colonies.

4.3.2. Eviction of \textit{nimX}^{\text{CDK1F}} mutant allele

Eviction of the \textit{nimX}^{\text{CDK1F}} allele from the FRY24 strain was efficiently achieved by growing the cells on HU+5′-FOA double selection medium. Although homologous recombination in \textit{cis} between the wild type and mutant alleles of Cdk1 during mitotic cell division occurred only at a low frequency, the tight selection pressure achieved with the HU+5′-FOA double selection medium enabled efficient recovery of progeny colonies that have lost both the \textit{pyr4} and \textit{nimX}^{\text{CDK1F}} alleles.
4.3.3. Frequency of mutations synthetically lethal with \( \text{nim}X^{\text{CDK1F}} \) mutant allele

Of 89 strains showing temperature sensitivity at 42°C, nine strains showed significant recovery from their mutant phenotypes upon eviction of \( \text{nim}X^{\text{CDK1F}} \). As four of the nine strains that lost their temperature sensitivity were later determined to have multiple mutations and two more not further analyzed as they were dominant, finally three mutations were verified as single mutations that show synthetic lethality with \( \text{nim}X^{\text{CDK1F}} \) at 42°C. Nevertheless this screen was determined as not having been saturating in that none of the three mutations isolated were shown to be located on a same gene.

4.3.4. Predicted function of genes identified through the screen

The two mutations that were isolated and cloned through this screen occurred on the \( A. \text{nidulans} \) genes predicted as \( \text{An-fcp1} \) and \( \text{An-pnk1} \). FCP1 had been defined in other eukaryotic systems as a protein phosphatase targeting and dephosphorylating the C-terminal domain of the RNA polymerase II largest subunit. Additional work carried out to understand its function in \( A. \text{nidulans} \) will be addressed in the following chapters. Little is known about the function of Pnk1. Although identified both in \( S. \text{pombe} \) and humans (Jilani et al., 1999; Mejer et al., 2002), the exact function of this protein is still largely unknown. As a protein that has both phosphatase and kinase activity toward DNA polynucleotides, Pnk1p has been suggested to play a role in responding to DNA damage caused by gamma-radiation and camptothecin, and the synthetic interaction of the \( \text{An-pnk1}^{\text{TS}} \) with \( \text{nim}X^{\text{CDK1F}} \) could be mediated through defects in DNA damage control.
Figure 4.1 Overview of the genetic screen. A genetic screen designed to identify mutations that show synthetic lethality with the $nimX^{CDK1F}$ mutation. A strain which has a tandem repeat of the $nimX^{CDK1}$ and $nimX^{CDK1F}$ alleles is utilized for easy determination of synthetic lethality.
Figure 4.2 Structure of the major UV-induced photoproducts in DNA. The molecular structure of cyclobutane pyrimidine dimers and (6-4) photoproducts. [Reproduced with the permission from Cambridge University Press and authors Matsumura and Ananthaswamy; (2002) Short-term and long-term cellular and molecular events following UV irradiation of skin: implications for molecular medicine. Expert Reviews in Molecular Medicine Molecular Medicine. 2 December, Copyright 2002]
Table 4.1 Viability of the FRY24 spore stock and UV irradiation kill curve.

The spore stock of the FRY24 strain used in the screen was measured at 64% (A). The UV dosage of 3.15x10^4 μJ/cm^2 irradiated at 7,500 spores/plate yielded the desired survival rate (1.08%) and colony number per plate (~50).
Figure 4.3 Mutants that show synthetic lethality with \( nimX^{CDK1F} \). Nine mutant strains that exhibit significant loss of temperature sensitivity upon the eviction of the \( nimX^{CDK1F} \) mutant allele. Growth at 32, 37, 40 and 42°C was tested and compared. On each plate colonies on the right-hand side are of the strains before \( nimX^{CDK1F} \) is evicted, and the colonies on the left-hand side are of the strains that have lost \( nimX^{CDK1F} \), indicated at the bottom by either Cdk1F or Cdk1.
**Figure 4.4 Diploid analyses to determine dominance/recessiveness of mutations.** Haploid mutant strains are induced to form diploid organisms with haploid wild type strains. Manifestation of the temperature sensitive mutant phenotype in diploids determines a mutation as dominant. Diploid colonies of mutants #12 and #48 show persistence of temperature sensitivity mutant phenotypes, likely due to a semi-dominant mutation.
Figure 4.5 Genetic confirmation of three mutant strains having each single mutations. Mutant strains #13, #25, #66 were back-crossed to wild type strains and the segregation pattern of their temperature sensitive phenotype was examined. The mutant phenotype segregates as a single complementation group with 50% of the progeny of the crosses showing temperature sensitive phenotypes, verifying that it is caused by a single mutation.
Figure 4.6 Allelic relationship of mutations. Mutant strain #13, #25 and #66 are crossed to each other to determine whether their mutations are located on the same gene. Outsegregation of the wild type phenotype (lack of temperature sensitivity) in all three crosses verifies that the mutations defining these three strains are all located on different genes.
Figure 4.7 Cloning of mutation #25. The mutant strain #25 is transformed with the AMA1-plasmid based genomic DNA library and colonies that display complementation of the temperature sensitive mutant phenotype are recovered (A). Controls are underlined in red (#25-mutant 25 after eviction of $nimX^{CDK1F}$; #25F- mutant 25 before eviction of $nimX^{CDK1F}$; GR5- wild type; FRY24- strain initially used for UV mutagenesis containing a copy of $nimX^{CDK1F}$). The AMA1 plasmids recovered from the colonies that are complemented of the temperature sensitive mutant phenotype are rescued into *E.coli* and their inserts analyzed by restriction digestion with enzymes KpnI and SphI. Blue arrows indicate lane of λ-HindIII DNA size marker (B). Several plasmid inserts appeared to share overlapping regions based on restriction digest pattern (lanes indicated in yellow arrows). A PCR amplified linear DNA fragment of this overlapping genomic DNA sequence (C) was capable of complementing the mutant temperature sensitivity when transformed into the #25 strain, confirming the open reading frame included in that fragment to be the wild type copy of the gene mutated in strain #25.
 Colonies of mutant strain transformed with AMA1 plasmid genomic DNA library and tested for complementation of mutant phenotype

#25 #25F GR5 FRY24

**Figure 4.7**
Figure 4.8 Cloning of mutation #66. Transformation of strain #66 with the AMA1 plasmid based genomic DNA library yielded transformants exhibiting complementation of the temperature sensitive mutant phenotype (A). Inserts isolated from transformants fell into two different groups determined first by restriction digest pattern and subsequent DNA sequencing (B). One group shared a region including a predicted gene encoding the bifunctional polynucleotide phosphatase/kinase Pnk1 (E), while the other included the coding sequence of general transcription factor TFIIB (D). The genome region of the predicted genes are depicted in the A. nidulans database (D, E). Linear DNA fragments of different parts of these two regions (D #1, 2, 3; E #4, 5, 6) are amplified (C #1-6) and tested for capability to complement #66 mutant phenotype. Only the full open reading frame of predicted Pnk1 gene is able to rescue #66 mutant phenotype (#5 in C, E, F indicated in green).
Transformed colonies tested for complementation of mutant phenotype

\#66 \#66F GR5 FRY24

Figure 4.8
Figure 4.8 Sequence alignment of An-FCP1 with Fcp1p of S. cerevisiae. A. *nidulans* FCP1 shows high sequence homology to yeast Fcp1p (e-value 6e-94).
Figure 4.9 Protein structure of An-FCP1 and location of An-fcp1<sup>TS</sup> mutation.

The mutation introduced by UV irradiation caused the amino acid substitution of a Leucine to Alanine. The mutated Leucine is highly conserved and part of a predicted beta-sheet structure adjacent to the phosphotransferase motif required for phosphatase catalytic activity.
5.1. INTRODUCTION

One of the mutant genes isolated through the synthetic lethality screen with CDK1F was determined as an *A. nidulans* sequence homologue of a gene known to encode the protein phosphatase FCP1 in other eukaryotic organisms. FCP1 is a protein phosphatase involved in regulating the phosphorylation status of the RNA polymerase II largest subunit C-terminal domain (RNAPII CTD). As a target of dynamic phosphorylation/dephosphorylation, RNAPII CTD itself is known as an important protein subdomain critical in regulating the assembly and processing of the RNAPII dependent transcription machinery. Here I will provide a brief overview of the functions of the RNAPII CTD and the protein phosphatase FCP1 in determining RNAPII-mediated transcriptional activity. Introduction of
important experimental procedures used extensively in studying the function of An-fcp1 will also be included.

5.1.1. RNA polymerase II largest subunit C-terminal domain

DNA dependent mRNA transcription in eukaryotic cells is catalyzed by the 12-subunit multiprotein enzyme RNA polymerase II (RNAPII). To initiate mRNA transcription of any given gene, RNAPII further forms a multiprotein complex called the preinitiation complex (PIC) along with six general transcription factors (GTFs) designated TFIIA, -B, -D, -E, -F and –H. The assembly of this PIC at a gene promoter region is followed by transcription initiation, promoter clearance and transcription elongation, all of which are carried out in a tightly regulated manner. Of the 12 different subunits of RNAPII, the largest subunit has a distinct carboxyl-terminal domain (CTD) critical in determining the overall activity of the RNAPII transcriptional machinery. In many eukaryotic organisms, the RNAPII CTD is made up of multiple repeats of the conserved heptapeptide sequence Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 (YSPTSPS), functioning as a target of dynamic and reversible protein phosphorylation. These canonical repeats in the RNAPII CTD are essential in those organisms where they are conserved, as the removal of a significant number of them has been shown to have a lethal effect (Nonet et al., 1987; Zehring et al., 1988). In yeast, the very tandem repetition pattern of the heptads is important as well, proven as the insertion of alanine residues between the repeated heptapeptides leads to lethality (Stiller and Cook,
Among the seven residues making up the conserved peptide, the phosphorylation status of the Ser2 and Ser5 residues are considered critical. During the transcription cycle the RNAPII CTD has to be in an unphosphorylated state for the PIC to properly assemble, while transcription initiation is marked by mainly Ser5 phosphorylation of the CTD (Komarnitsky et al., 2000; Schroeder et al., 2000). The transition to transcription elongation coincides with the additional phosphorylation of Ser2 residues (Lee and Greenleaf, 1997; Murray et al., 2001), and moving toward transcription termination Ser2 phosphorylation is believed to dominate the overall phosphorylation pattern of RNAPII CTD. After the RNAPII has completed a transcription cycle, the CTD has to be completely dephosphorylated so that it can be recycled and be incorporated into a new PIC for a new round of mRNA transcription. Within the context of the cell division cycle, Xu et al. showed that RNAPII CTD remains hyperphosphorylated during mitosis (Xu et al., 2003) presumably rendering it unable to form PICs, consistent with the observation that bulk cellular transcription mediated by RNAPII ceases during mitosis. Although the CTD is vital for cell survival and transcription, in vitro studies show that it does not constitute a core part of the RNAPII catalytic activity itself (Zehring, 1988; Tyree et al., 1993), and it is thought mainly to function as a scaffold to which different nuclear factors necessary for proper regulation of mRNA transcription can bind.

The dynamic change of the RNAPII CTD phosphorylation status observed throughout the transcription cycle requires the activities of different protein
kinases and phosphatases. CDK7 of the TFIIH GTF complex has been shown to primarily phosphorylate Ser2 when transcription initiation starts, while CTK1 and CDK9 are known to target Ser5 during transcription elongation. The mitotic kinase CDK1 was confirmed by many groups to phosphorylate RNAPII CTD in vitro, which in combination with the fact that RNAPII CTD is hyperphosphorylated during mitosis suggest a potential function of CDK1 regulating transcription during mitosis and perhaps playing a role in the release of RNAPII from DNA during mitosis.

Among protein phosphatases, FCP1 was the first one identified to target specifically the RNAPII CTD (Archambault et al., 1997), followed by the characterization of SSU72 (Krishnamurty et al., 2004) and SCP1 (Zhang et al., 2006). Sequence analyses show FCP1 and SCP1 to belong together to an evolutionary conserved phosphatase family, while SSU72 has been shown to preferentially catalyze the dephosphorylation of Ser5 of the CTD heptapeptide repeats. Although different kinases and phosphatases have been identified and determined to be involved in RNAPII CTD regulation, much is yet unknown how exactly these different factors coordinate RNAPII CTD phosphorylation and consequently regulate mRNA transcription and how this might be regulated during mitosis when transcription is turned off.
5.1.2. Protein phosphatase FCP1

FCP1 was first identified as a protein with phosphatase activity toward the RNAPII CTD while associating with the general transcription factor TFIIF (TFII★-associating component of CTD phosphatase I; FCP1) both in humans and S. cerevisiae (Archambault et al., 1997). As a founding member of the aspartate-dependent protein phosphatases, the catalytic domain of FCP1 contains a DxDxT/V phosphoesterase motif essential for its phosphatase activity (Kobor et al., 1999). The primary targets of FCP1 dephosphorylation are the Serine2 and Serine5 of the RNAPII CTD heptapeptide repeats, though Serine2 has been suggested to be the preferred target residue in S. pombe (Hausmann and Shuman, 2002; Lin et al., 2002). FCP1 has been shown to be required for RNAPII mediated transcription in general (Kobor et al, 1999), and this requirement most likely stems from the FCP1 function as a phosphatase that reactivates RNAPII after the completion of each transcription cycle by dephosphorylating RNAPII CTD (Cho et al., 1999). Independent of its phosphatase activity, FCP1 has also been reported to facilitate transcription elongation (Friedl et al., 2003). These different activities of FCP1 are known to be regulated by the phosphorylation of FCP1 itself (Palancade et al., 2002; Abbott et al., 2005). Defective FCP1 activity has been linked to certain development disorders as well. In humans, aberrant splicing of the FCP1 mRNA due to a single nucleotide substitution in one of the Fcp1 gene introns has been reported
to cause an autosomal recessive developmental disorder called cataracts facial dysmorphism neuropathy syndrome (CCFDN, Varon et al., 2003).

5.1.3. S-tag protein affinity-purification system

The S-tag protein affinity purification system is based on the unique properties displayed by the cleavage products of the enzyme Ribonuclease A (RNase A). RNase A is an enzyme with the well-known function of catalyzing the hydrolysis of RNA molecules (Richards and Wyckoff, 1971). In the 1950s Richard and his colleagues were able to show that the digestion of RNase A with the protease subtilisin leads to the hydrolysis of a single polypeptide bond within RNase A (Richards and Vithayathil, 1959). The enzymatic cleavage converts RNase A into RNase S in which the two individual polypeptide fragments resulting from this proteolytic reaction, S-peptide and S-protein, remain tightly bound to each other. In 1993, Kim and Raines proposed a protein affinity-purification system utilizing the tight association of S-peptide and S-protein (Kim and Raines, 1993).

Establishing a standardized procedure for the purification of individual proteins or protein complexes from heterogenic biomatters is inherently challenging due to the unique nature in which each protein interacts with its local environment. A commonly employed strategy to overcome these difficulties is to generate fusion proteins in which target proteins are translationally fused with a carrier polypeptide. The affinity of this carrier polypeptide to particular ligands can
be used for the purification of the fusion proteins following a relatively standardized protocol. For this strategy to be effective, the make-up of the fusion protein has to meet certain criteria. First, a carrier peptide ought to be as small as possible to minimize its chance of interfering with the biochemical properties of the target proteins. Second, the binding affinity of the carrier-ligand should be sufficiently high for transient immobilization of the target proteins while separating them from their environs, yet low enough to release them efficiently from the ligands for ultimate purification purposes. Kim and Raines designed a protein purification system utilizing the first 15 amino acid residues of the S-peptide (S15) as a carrier and the S-protein as a ligand (Kim and Raines, 1993). Made up of only 15 amino acids, S15 meets the criterion of being a small polypeptide, while it had also been determined to retain the original interaction properties with the S-protein (Potts et al., 1963). Concerning the binding affinity of the S-peptide versus the S-protein, the original dissociation constant of the RNase S had been shown to be around $10^{-9}$ M. This level of binding affinity proved to be sufficient for tight immobilization of target proteins as well as their effective release off the S-proteins while maintaining the reaction under native conditions. With the S15 polypeptide as an affinity “S-tag” translationally fused to the target proteins and the S-protein fixed on the surface of agarose beads for easy immobilization and purification procedures, this S-tag protein affinity-purification system was extensively used for the purification of the RNAPII in this work and subsequent analyses of the phosphorylation status of its CTD described in this chapter. The
highly optimized experimental conditions of the S-tag purification procedures applied in *A. nidulans* that enabled efficient purification of the proteins of interest were established by my colleague Hui-Lin Liu in the Osmani lab (unpublished data).

5.1.4. Mitotic cell cycle arrest in *A. nidulans* using the *nimT23* mutation and benomyl

In *A. nidulans*, synchronization of a cell population in M-phase is achieved through the sequential activation of G2 and M phase checkpoint control mechanisms utilizing a temperature sensitive mutant allele of a cell cycle regulation gene as well as a chemical reagent that poisons spindle microtubules. *nimT23* is a temperature sensitive mutant allele of *nimT*, the gene encoding the functional orthologue of the phosphatase CDC25. As CDC25 activity is required for mitotic entry due to its essential function of activating CDK1, a cell culture of a *nimT23* mutant strain will arrest in growth at late G\(_2\) phase of the cell cycle when incubated at the restrictive temperature of 42°C. This G\(_2\) phase arrest serves as an initial synchronization step to increase the final mitotic index of the cell population. The cell culture can subsequently be arrested in M phase by shifting it to the permissive temperature of 32°C after adding the microtubule poison benomyl. While the cells are released from the G\(_2\) phase arrest and enter mitoses synchronously due to the shift to a permissive temperature, the presence of benomyl triggers the activation of the spindle assembly checkpoint. As
benomyl depolymerizes microtubules and therefore disables the proper formation of mitotic spindles, APC/C activation is blocked and cells are not able to progress from metaphase to anaphase. Consequently, the entire cell population becomes arrested and highly synchronized in mitosis. This method of experimentally induced mitotic synchronization is widely used when examining the specific mitotic state of various proteins and other subcellular components (Ye et al., 1997).

5.2. RESULTS

5.2.1. The protein sequence of A. nidulans RNA polymerase II CTD is unique from other eukaryotes but conserved within genus Aspergillus

Previously it had been reported that the canonical tandem repeats of the YSPTSPS heptapeptides are highly conserved with relatively few deviations within the heptapeptide sequence throughout the eukaryotic taxa of fungi, green plants and metazoans (Stiller and Hall, 2002). To examine the RNAP II CTD of A. nidulans, the hypothetical gene predicted to encode RNAPII in A. nidulans was identified from the Aspergillus comparative genome database. The locus AN0809.3 on chromosome VIII of A. nidulans was predicted to encode a hypothetical protein made up of 1746 amino acids that is similar to the RNA polymerase II largest subunit of both humans (e=0.0) and S. cerevisiae (e=0.0). Although the e-value over the entirety of the protein effectively defines it as the
RNA polymerase II largest subunit gene, sequence alignment of only the C-terminal domain of A. nidulans RNAPII with the human and S. cerevisiae counterparts clearly reveals a significant degeneration of both the YSPTSPS heptad sequence and the tandem repetition pattern of the heptad itself (Figure 5.1). On the other hand, this apparent degeneration of the canonical heptapeptide repeat of the RNAPII CTD is highly conserved within the genus Aspergillus, demonstrated by the comparison of the CTD sequences of A. nidulans, A. fumigatus, Neosartorya fischeri, A. clavatus, A. niger and A. oryzae (Figure 5.2).

5.2.2. C-terminal S-tagging and deletion analysis of RNA polymerase II largest subunit

To investigate the phosphorylation status of its C-terminal domain in A. nidulans the RNAP II was endogenously tagged on its C-terminus with an S-tag peptide sequence to enable affinity protein purification. A gene replacement construct comprising ~2KB of the final 3’end sequence of the A. nidulans RNAP II gene (excluding the termination codon), the S-tag sequence and the auxotrophic marker pyrG sequence was generated via 3-way fusion PCR and transformed into strains with the desired genetic backgrounds (Figure 5.3). Proper integration of the construct via double homologous recombination was confirmed by diagnostic PCR using a primer set recognizing sequences outside of the transformation construct. Insertion of the S-tag and pyrG sequence in the
transformants results in a ~6.5KB band while the PCR result in an untransformed strain yields a ~4.5KB band. To verify the functionality of the C-terminal S-tagged RNAP II, deletion of the RNAP II was conducted as well to determine the essentiality of the protein. As predicted, RNAP II in *A. nidulans* carries out an essential function as the deletion of the RNAPII encoding gene results consistently in the formation of heterokaryons confirmed both by growth on YAG/YAGUU (restrictive/permisive nutritional conditions respectively) media as well as diagnostic PCR (Figure 5.4). Because RNAPII is an essential gene and the S-Tagged version of this gene did not cause any growth defects I conclude the S-Tagged version is fully functional (Figure 5.5).

5.2.3. S-tag purification of RNA polymerase II largest subunit

Utilizing an S-tag translationally fused to its C-terminus, RNAPII was affinity purified from a whole cell protein extract derived from an exponentially growing culture of a wild type strain. When purified proteins were visualized by Coomassie staining following SDS-PAGE, most clearly two polypeptide species with different gel migration rates where identified (Figure 5.6). While one species formed a single band running at approximately 150KDa, the second species migrated within the range of ~200-250KDa as it resolved into several subspecies including comparatively distinct bands at ~200KDa and ~250KDa with a continuous smear between them. The two different species migrating at ~200-250KDa and ~150KDa were predicted to be the RNAPII largest subunit and the
second largest subunit respectively, with the unique electrophoretic property of the RNAPII largest subunit likely due to the differential phosphorylation status of the RNAPII CTD in the different subspecies.

5.2.4. An-FCP1 is a protein phosphatase targeting RNA polymerase II CTD

To investigate whether An-FCP1 is a protein phosphatase dephosphorylating RNAPII CTD, the phosphorylation status of the RNAPII CTD in the absence of FCP1 activity was examined utilizing the Fcp1<sup>TS</sup> mutant strain. As a conditional mutant allele of the essential gene An-fcp1, An-fcp1<sup>TS</sup> was shown to cause temperature sensitivity at the restrictive temperature of 42°C. RNAPII was S-tag affinity purified from cultures of wild type and An-fcp1<sup>TS</sup> strains grown at 32°C and further incubated for 6 hours either at the permissive temperature of 32°C or the restrictive condition of 42°C. When visualized by silver staining following SDS-PAGE, the migration pattern of RNAPII did not differ between wild type and An-Fcp1<sup>TS</sup> strains at 32°C, showing the separation of two distinct subspecies of the molecular weights of ~200KDa and ~250KDa and a smear in between (Figure 5.7. Once incubated at 42°C, RNAPII isolated from the An-Fcp1<sup>TS</sup> mutant strain only showed a prominent ~250KDa band with the lower sized ~200KDa band and the continuous smear between the two major bands disappearing. On the contrary, RNAPII isolated from the wild type strain failed to show the disappearance of the lower molecular weight subspecies with especially the smear ranging down to ~200KDa clearly persisting (Figure 5.7A).
The exclusive disappearance of the lower molecular weight subspecies of RNAPII in the An-Fcp1<sup>TS</sup> strains at 42°C strongly indicated a loss of unphosphorylated and hypophosphorylated isoforms of RNAPII due to the absence of An-FCP1 protein phosphatase activity thus converting all protein to the fully phosphorylated species. To verify that the varying electrophoretic mobilities of RNAPII subspecies were due to differential phosphorylation of the RNAPII CTD, an in vitro phosphatase assay was conducted with RNAPII isolated from both wild type and An-Fcp1<sup>TS</sup> strains incubated at 42°C after growth at 32°C. Regardless of the different isoform composition within each RNAPII sample, treatment with λ-phosphatase in vitro led invariably to the collapse of the bands into a single, higher gel mobility band roughly of the size of 200KDa. This reaction could be effectively prevented with the addition of phosphatase inhibitors (Sodium Vanadate and Sodium Fluoride) in the reaction cocktail, conclusively showing that the electrophoretic separation of RNAPII subspecies were due to differential phosphorylation of RNAPII CTD (Figure 5.7B).

5.2.5. Phosphorylation of RNA polymerase II CTD during mitosis

Since Xu et al. showed that RNAP II CTD remains hyperphosphorylated in human cells arrested in mitosis by treatment with the microtubule poison nocodazole (Xu et al., 2003), the mitotic phosphorylation status of RNAPII CTD was examined in <i>A.nidulans</i>. Utilizing the microtubule poison benomyl and a strain with the temperature sensitive nimT23 mutant allele (Figure 5.8A), <i>A.
A. nidulans cell cultures were synchronized in late G₂ and M phase (86% synchronization rate based on chromosome mitotic index). RNAPII isolated from neither of these cultures showed a change of their phosphorylation pattern in comparison to RNAPII isolated from an exponentially growing cell culture (Figure 5.8B).

5.3. DISCUSSION

5.3.1. Protein sequence of A. nidulans RNA polymerase II CTD

While the protein sequence of RNAP II CTD in A. nidulans partially retains elements of the Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 (YSPTSPS) heptapeptide sequence as well as its tandem repetition pattern, when compared to yeast species (S. cerevisiae, S. pombe) and humans, the A. nidulans RNAP II CTD shows a significant deviation from those conserved features. Sequence comparison of different Aspergillii shows that this unique RNAP II CTD sequence is nevertheless highly conserved within the genus Aspergillus. Aspergillii are considered evolutionary intermediates between yeasts and higher eukaryotes like mammals. Nevertheless, the RNAP II CTD sequence of Aspergillii deviates from the hallmark features while they are conserved both in yeasts and humans. The fact that this unique RNAPII CTD sequence is highly conserved within the genus Aspergillus challenges the paradigm that Ser2 and Ser5 phosphorylation
within the YSPTSPS heptapeptide sequence be the primary determinant of RNAP II CTD activity may not apply to all eukaryotic systems.

5.3.2. S-tag purification of RNA polymerase II largest subunit

Affinity purification of the RNAP II largest subunit using an endogenous C-terminal S-tag fusion yielded in the isolation of mainly two different proteins. The RNAP II largest subunit resolved into different electrophoretic subspecies likely due to differential phosphorylation on the CTD. Two distinct bands running each at ~200KDa and ~250KDa could be seen as well as a faint, continuous smear between the two bands. Another protein co-purified with the RNAP II largest subunit could be seen as a band running at ~150KDa. Considering the size and the roughly equal intensity of the band compared to the largest subunit, this protein was likely to be the RNAP II second largest subunit, known to form a complex with RNAP II largest subunit in a 1:1 stoichiometric manner. The electrophoretic migration pattern of RNAP II observed was consistent with the expected RNAP II phosphorylation pattern, showing a potential range of no phosphorylation, hypophosphorylation and hyperphosphorylation.

5.3.3. An-FCP1 function as a protein phosphates

Since FCP1 has been shown to be a protein phosphatase targeting and dephosphorylating RNAP II CTD, absence of FCP1 activity was expected to yield
in the accumulation of hyperphosphorylated isoforms of RNAP II. When RNAP II was purified from a cell culture of an \textit{An-fcp1}^{TS} temperature sensitive mutant strain incubated at the restrictive temperature of 42°C, a distinct accumulation of the higher molecular weight (~250KDa) RNAP II isoform was observed. A subsequent phosphatase assay confirmed that the different electrophoretic mobility of RNAP II subspecies was due to differing degrees of protein phosphorylation. RNAPII isolated from both a wild type culture as well as an \textit{An-fcp1}^{TS} mutant incubated at 42°C showed that when treated with a recombinant lambda phosphatase, all bands of RNAPII collapsed into the 200KDa lower molecular weight isoform, determining that the different bands were representing different phosphoisoforms. As the loss of An-FCP1 activity at 42°C was shown to result in the accumulation of hyperphosphorylated, higher molecular weight (~250KDa) RNAP II isoforms, An-FCP1 was conclusively identified as a RNAP II CTD targeting protein phosphatase.

5.3.4.. Phosphorylation of RNA polymerase II CTD during mitosis

A study conducted in human cell lines concluded that RNA polymerase II CTD remains hyperphosphorylated during mitosis (Xu et al., 2003), potentially linking RNA polymerase II activity during mitosis with the mitotic kinase CDK1. Nevertheless, in \textit{A. nidulans} the phosphorylation pattern of RNAP II CTD did not show a quantitative change during mitosis. Although a distinct qualitative change of RNAP II CTD phosphorylation not detectable by quantitative measurements of
phosphorylation may not be entirely precluded, RNAP II isolated from cells arrested and synchronized in either late G\textsubscript{2} and M phase did not show any change in their bulk RNAP II CTD phosphorylation.

5.3.5. Genetic interaction of protein phosphatase \textit{An-fcp1} with mitotic kinase \textit{nimX}\textsuperscript{CDK1}.

The \textit{An-fcp1}\textsuperscript{TS} mutation was initially identified as it caused a synthetic temperature sensitivity phenotype in combination with \textit{nimX}\textsuperscript{CDK1\textsuperscript{F}}, the hyperactive mutant allele of the mitotic kinase \textit{nimX}\textsuperscript{CDK1}. \textit{An-FCP1} had been shown to be a protein phosphatase targeting the RNAPII CTD, and \textit{An-fcp1}\textsuperscript{TS} was confirmed as a conditional loss-of-function mutant allele leading to hyperphosphorylation of RNAPII at the restrictive temperature of 42°C. The characterization of \textit{An-FCP1} function revealed that the synthetic interaction between \textit{An-fcp1}\textsuperscript{TS} and \textit{nimX}\textsuperscript{CDK1\textsuperscript{F}} was a combined effect of an inactivated phosphatase (\textit{An-FCP1}\textsuperscript{TS}) and a hyperactive kinase (\textit{NIMX}\textsuperscript{CDK1\textsuperscript{F}}). The fact that \textit{An-FCP1} is a phosphatase targeting the RNAPII CTD suggested a model where the synthetic interaction between \textit{An-fcp1}\textsuperscript{TS} and \textit{nimX}\textsuperscript{CDK1\textsuperscript{F}} was mediated by the abnormally elevated phosphorylation status of RNAPII CTD, potentially linking RNAPII CTD as a common substrate of \textit{An-FCP1} and \textit{NIMX}\textsuperscript{CDK1}. The next chapter will describe experiments and their results trying to address this possibility.
**Figure 5.1** Amino acid sequence alignment of the RNA polymerase II C-terminal domain of *A. nidulans*, *Homo sapiens* and *Saccharomyces cerevisiae*. While the YSPTSPS heptapeptide sequence and its tandem repetition pattern is highly conserved both in humans (*H. sapiens*) and *S. cerevisiae*, *A. nidulans* CTD exhibits a significant deviation from this feature. Conserved YSPTSPS heptapeptides (ones with no more than one amino acid substitution) are shown in red (and blue when immediately repeated) and in a box when conserved in *A. nidulans*.
A. nidulans  --FGG-FSPAATSPAGYSSPFSANPTSPGYSPTSSYPSPGMGITSPRFMT------SP  1614
A. fumigatus  --FGGGFSPAATSPAGYSSPFSANPTSPGYSPTSSYPSPGMGITSPRFMT------SP  1605
N. fischeri  --FGGGFSPAATSPAGYSSPFSANPTSPGYSPTSSYPSPGMGITSPRFMT------SP  1605
A. clavatus  --FGGGFSPAATSPAGYSSPFSANPTSPGYSPTSSYPSPGMGITSPRFMT------SP  1612
A. niger  --FGGGFSPAATSPAGYSSPFSANPTSPGYSPTSSYPSPGMGITSPRFMT------SP  1605
A. oryzae  --FGGGFSPAATSPAGYSSPFSANPTSPGYSPTSSYPSPGMGITSPRFMT------SP  1606
A. terreus  FGGFSPAATSPAGYSSPFSANPTSPGYSPTSSYPSPGMGITSPRFMT------SP  1605

A. nidulans  GFSPASPSFAPTSPAYGQASPTSPYSPTSPNYSPTSPSFSPASPA  1674
A. fumigatus  GFSPASPSFAPTSPAYGQASPTSPYSPTSPNYSPTSPSFSPASPA  1665
N. fischeri  GFSPASPSFAPTSPAYGQASPTSPYSPTSPNYSPTSPSFSPASPA  1665
A. clavatus  GFSPASPSFAPTSPAYGQASPTSPYSPTSPNYSPTSPSFSPASPA  1672
A. niger  GFSPASPSFAPTSPAYGQASPTSPYSPTSPNYSPTSPSFSPASPA  1665
A. oryzae  GFSPASPSFAPTSPAYGQASPTSPYSPTSPNYSPTSPSFSPASPA  1666
A. terreus  GFSPASPSFAPTSPAYGQASPTSPYSPTSPNYSPTSPSNLSPASPV  1665

A. nidulans  FSPTSPYSPTSPAYGGA-RHLSPTSPTSPQYTSPTSPNFAGSPTS  1733
A. fumigatus  FSPTSPYSPTSPAYGGA-RHLSPTSPTSPQYTSPTSPNFAGSPTS  1725
N. fischeri  FSPTSPYSPTSPAYGGA-RHLSPTSPTSPQYTSPTSPNFAGSPTS  1725
A. clavatus  FSPTSPYSPTSPAYGGA-RHLSPTSPTSPQYTSPTSPNFAGSPTS  1732
A. niger  FSPTSPYSPTSPAYGGA-RHLSPTSPTSPQYTSPTSPNFAGSPTS  1726
A. oryzae  FSPTSPYSPTSPAL-RAGNMSPTSPYQYTSPTSPSNIGGSPTS  1726
A. terreus  FSPTSPYSPTSPAL-RAGNMSPTSPYQYTSPTSPSNIGGSPTS  1724

A. nidulans  PGGPTSPGYSPTSPAFSPTSPQ----------------  1746
A. fumigatus  PGGPTSPGYSPTSPAFSPTSPQ----------------  1751
N. fischeri  PGGPTSPGYSPTSPAFSPTSPQ----------------  1751
A. clavatus  PGGPTSPGYSPTSPAFSPTSPQ----------------  1751
A. niger  PGGPTSPGYSPTSPAFSPTSPQ----------------  1751
A. oryzae  PGGPTSPGYSPTSPAFSPTSPQ----------------  1751
A. terreus  PGG----------------AYSPSPYTPSTSPQ----------------  1743

Figure 5.2 Amino acid sequence alignment of the RNA polymerase II C-terminal domain of *A. nidulans*, *A. fumigatus*, *Neosartorya fischeri*, *A. clavatus*, *A. niger* and *A. oryzae*. Although deviating from the hallmark features of RNAPII CTDs in other eukaryotes, the amino acid sequences of RNAPII CTD of species within the genus Aspergillus is highly conserved.
Figure 5.3 Endogenous C-terminal S-tagging of RNA polymerase II largest subunit. An S-tag gene replacement construct generated by 3-way fusion-PCR (blue arrow) is transformed for endogenous C-terminal tagging of the RNAP II into wild type and An-Fcp1<sup>TS</sup> mutant strains (A). Proper integration of the construct via double homologous recombination was confirmed by diagnostic PCR using a primer set (red arrows) that targets sequences outside of the construct. Insertion of the S-tag and pyrG sequence in the transformants results in a ~6.5KB band (transformed into 1-wild type; 2-An-fcp1<sup>TS</sup> mutant strain), while the PCR result in an untransformed strain yields a ~4.5KB band (3-negative control) (B).
Figure 5.4 Heterokaryon rescue gene deletion analysis of RNA polymerase II largest subunit in *A. nidulans*. (A) All six randomly chosen primary RNAPII gene deletion transformants failed to proliferate on YAG medium when conidiospores of their colonies were streaked out. (B) Formation of heterokarya was confirmed by diagnostic PCR yielding both WT RNAPII band and ∆RNAPII bands (~7KB and ~10KB respectively; lane 1) versus only the WT RNAPII band (2) in the negative control.
Figure 5.5 Endogenous S-tagging of RNAPII CTD does not result in growth defects. RNAPII CTD was endogenously S-tagged in both wild type and An-fcp1TS strains. In Neither of the strains resulted the S-tagging in any growth defects. As RNAPII function is essential, this confirms S-tagged version of RNAPII to be functional (two colonies of the S-tagged An-fcp1TS strain are different transformants).
Figure 5.6 S-tag purification of RNA polymerase II largest subunit. S-tag affinity purified RNAPII was visualized by Coomassie staining following SDS-PAGE (M-size marker; 1-negative control from untagged strain; 2-RNAPII). RNAPII largest subunit separated into subspecies with a size range of ~200-250 KDa and RNAPII second largest subunit (~150KDa) was co-purified (lane 2).
Figure 5.7 An-FCP1 is a protein phosphatase targeting RNAPII CTD. Inactivation of An-FCP1 in Fcp1<sup>TS</sup> strains by incubation at 42°C results in the accumulation of RNAPII subspecies of slower gel mobility (**A**). The differences of electrophoretic mobility shown by different RNAPII isoforms are due to differential phosphorylation. λ-phosphatase treatment of purified RNAPII results in the collapse of all bands into one that migrates at a lower molecular weight range (**B**).
Figure 5.8 RNAPII CTD phosphorylation during mitosis. *A. nidulans* cell cultures can be synchronized and arrested at G$_2$ and M phase of the cell cycle utilizing the *nimT23* mutation as well as the microtubule poison benomyl (A). Cells arrested at G$_2$ and M phase do not exhibit different levels of RNAPII CTD phosphorylation compared to exponentially growing cells (B).
6.1. INTRODUCTION

This chapter will describe the work that was focused on elucidating the molecular and cell biological nature of the synthetic genetic interaction observed between the mutations \( An-fcp1^{TS} \) and \( nimX^{CDK1F} \). To help the understanding and interpretation of the data being presented, a brief introduction is provided about the concept of genetically synthetic interactions as well as the dynamic rearrangement of the nuclear pore complex within the context of cell cycle progression that characterizes the "open" aspects of mitosis in \( A. nidulans \).

6.1.1. Genetically synthetic interactions

The concept of synthetic lethality as one form of genetic interaction was first put forth by Bridges in 1922 and the very term coined by Dobzhansky in
1945 to describe lethal relationship of mutations observed in the fruitfly species *Drosophila melanogaster* and *Drosophila pseudoobscura* (Dobzhansky, 1945). It is a genetic interaction defined by the manifestation of a lethal phenotype due to the combination of two individually viable loss-of-function mutations. More generally, a genetically synthetic interaction determines the relationship of two mutations that in combination cause a more severe mutant phenotype in comparison to the individual single mutations.

While studying the physical interaction partners of a given protein can be useful in deconstructing the immediate linear biochemical pathway in which that protein is involved, this approach is less effective in expanding our understanding into other biological pathways that carry out related or parallel functions. Unlike physical protein-protein interactions, genetic interactions reflect the wider functional relationship of the involved genes and mutations. In particular, determination of synthetically interacting genetic partners of a given mutation is useful as a means to identify genes that provide redundant biochemical activities or regulate functionally parallel cellular pathways. When different cellular pathways provide biological activities that together are required to fulfill an important cellular function, the combination of different mutations in these separate branch pathways can yield a defective mutant phenotype more severe than those of either of the individual mutations. Thus, identifying the synthetic interaction partners of a mutation can reveal the presence or relevance of functionally related, parallel cellular pathways (Figure 6.1).
6.1.2. Partially open mitosis in *A. nidulans*.

In dividing eukaryotic cells, the faithful segregation of genetic information into the two daughter cells is achieved during mitosis of the cell division cycle. While in higher eukaryotes the process of the nuclear division is accompanied by the complete breakdown and reassembly of the nuclear envelope (*open* mitosis), lower eukaryotic organisms have been known to undergo *closed* mitoses during which the nuclear envelope remains intact. An open mitosis of a higher eukaryote is characterized by the disassembly of both the nuclear pore complex as well as the nuclear envelope at the G₂/M transition, thus temporarily eliminating the distinction between the nucleoplasm and cytoplasm during M phase. On the other hand, organisms engaging in closed mitoses still have to establish a mechanism by which the molecular machinery required to initiate mitotic onset can enter the nuclei via the nuclear pore. In organisms like the budding yeast *S. cerevisiae* this is achieved through active transport specific to the mitotic phase of the cell cycle and the nuclear pore complex remains intact throughout mitosis to facilitate this transport. In *A. nidulans*, the rapid nuclear localization of molecules regulating mitotic entry is accomplished by the mitotic-specific rearrangement of the nuclear pore complex that serves as a diffusion barrier between the nucleus and the cytoplasm (Osmani et al., 2006). Upon entry of M phase, while the nuclear envelope remains intact, 14 of the 24 identified proteins making up the *A. nidulans* nuclear pore complex (*Nups*) disperse away from their original location at the NPC only to relocate back to it once mitosis is complete. As the Nups that disperse during mitosis are predicted to occupy the central
channel of the nuclear pore, the mitotic-specific partial disassembly of the *A. nidulans* nuclear pore complex is most likely to leave the central channel of the nuclear pore open, allowing passive diffusion between the nucleoplasm and cytoplasm to occur unhindered while abolishing active nucleocytoplasmic transport. This understanding is consistent with the observations that fluorescent protein constructs directed to either the nucleus or the cytoplasm with nuclear localization or export signals (NLS and NES, respectively) disperse randomly throughout the cell only while mitosis is occurring (De Souza et al., 2004; De Souza and Osmani, 2007).

The concurrence of an open nuclear pore with an intact nuclear envelope during mitosis determines mitosis in *A. nidulans* as partially open, incorporating features of both open and closed mitoses. This unique property of *A. nidulans* mitosis may pose certain questions in examining the regulation of the localization of proteins like transcription factors or RNA polymerase II, proteins that are known to function while associating with chromosomal DNA. As passive diffusion is enabled between the nucleoplasm and cytoplasm during mitosis, a protein functioning while bound to the chromosomal DNA and thus localizing to the nucleus during interphase might either remain nuclear or disperse throughout the cell during mitosis depending on whether its association to chromatin is maintained. If such a protein disperses throughout the cell upon onset of mitosis, it raises the possibility that there be an active process through which it is released from the chromosomal DNA, allowing it to disperse when the nuclear pores are opened. Part of this chapter will describe work carried out to examine
the localization pattern of RNAP II and its potential regulation during cell cycle progression within the context of the partially open mitosis of *A. nidulans*.

6.2. RESULTS

6.2.1. Generation of a strain that has a \( \text{nimX}^{\text{CDK}1F} \) allele (F) as the only copy of the \( \text{nimX} \) gene.

It was described in Chapter 4 how an *An-fcp1\(^{TS}\)* single mutant had been derived from a strain which contained the *An-fcp1\(^{TS}\)* mutation as well as a repeat of both the wild type \( \text{nimX}^{\text{CDK1}} \) (Y) and mutant \( \text{nimX}^{\text{CDK1F}} \) (F) alleles linked by the \( \text{pyr4} \) marker (For the purpose of making it easier to follow the genotypes, limited to this Chapter 6, the wild type \( \text{nimX}^{\text{CDK1}} \) will be refered to as simply “Y”, and the mutant \( \text{nimX}^{\text{CDK1F}} \) allele as “F”). Eviction of the auxotropic marker \( \text{pyr4} \) and the F allele was achieved by double-counterselection on medium containing both 8mM HU and 1% 5’-FOA. Since this eviction process ultimately depended on randomly occurring homologous recombination between the wild type Y and mutant F alleles, a single selection procedure on medium containing only 5’-FOA was predicted to yield F single mutants (without an extra copy of wild type Y) at a frequency determined by the relative location of the tyrosine 15 to phenylalanine (F) mutation on the Y gene. Counter to expectations, attempts to recover a single F mutant in this way failed. When FRY24 (Y+\( \text{pyr4} \)+F ) strain spores were grown on medium containing only 5’-FOA, all outgrowing colonies proved to be of wild
type Y genotypes (data not shown). This likely reflected the advantage of viability the wild type Y bore over the mutant F allele. Although the mutant F allele is dominant over wild type Y in strains that contain both mutant F and wild type Y alleles, to strictly verify the effect of the F mutation, a strain that has a single mutant F allele as the only copy of the gene in its genome was generated by transformation-mediated gene replacement. A gene replacement construct of the mutant F allele was generated incorporating the pyroA auxotrophic marker by 3-way fusion PCR and transformed into a wild type target strain. Along with the F single mutant a nimX\textsuperscript{CDK1AF} (AF) single mutant was also generated to examine the effect of the additional threonine14 to alanine amino acid substitution. All three strains of the Y+F, F and AF genotype displayed severe sensitivity to 8mM HU, confirming aberrant CDK1 activity not controlled by inhibitory Tyr15 phosphorylation. Both of the F and AF single mutants showed slightly lower growth rates compared to the Y+F and wild type strains. The overall lower fitness of the F and AF single mutants could also be observed as they exhibited lower rates of conidiospore formation, indicated by their darker color tone (although both being white strains) and less powdery appearance of the colony surface (data not shown). The Y+F and AF mutants also showed fanning of the colonies (much more severe in the AF mutant), suggesting genomic instability and subsequent separation of the originally clonal colonies into subpopulations of different genotypes. In the Y+F mutant this phenomenon could be attributed to the possibility of homologous recombination between the wild type Y and mutant F alleles, while in the AF mutant it is likely due to the uncontrolled CDK1 activity.
that is perhaps even higher and less coordinated than in the F single mutant leading to defective cell cycle progression and mitoses.

6.2.2. Comparison of the synthetic interaction of $An-fcp1^{TS}$ and F mutations in $An-fcp1^{TS}$+ F versus $An-fcp1^{TS}$+Y+F strains.

To examine whether the presence of a wild type Y allele had an effect on the synthetic interaction of $An-fcp1^{TS}$ and F, temperature sensitivity of the mutant strains, F, Y+F, $An-fcp1^{TS}$, $An-fcp1^{TS}$+F and $An-fcp1^{TS}$+Y+F were compared to a wild type strain at temperatures ranging from 32°C to 42°C. As described previously in chapter 4, the $An-fcp1^{TS}$ mutant by itself showed increasing degrees of temperature sensitivity compared to the wild type strain at temperatures higher than 37°C. Also, the strains F and Y+F did not show any significant temperature sensitivity phenotypes. When strains $An-fcp1^{TS}$, F and $An-fcp1^{TS}$+F were compared, the $An-fcp1^{TS}$ and F strains showed higher growth rates at all the tested temperatures of 32°C to 42°C, most significantly between the temperatures of 32°C to 40°C. When the three strains $An-fcp1^{TS}$, Y+F and $An-fcp1^{TS}$+Y+F were compared, the growth rate differential was most pronounced at the temperatures between 37°C and 40°C, while at 32°C colony growth of the $An-fcp1^{TS}$+Y+F mutant was comparable especially to that of the Y+F mutant. The higher degree of temperature sensitivity shown by the $An-fcp1^{TS}$+F mutant versus the $An-fcp1^{TS}$+Y+F re-confirmed the synthetic interaction of the $An-fcp1^{TS}$ and F mutations and showed that the manifestation of this genetic interaction was mitigated by the presence of the WT allele Y in the $An-fcp1^{TS}$+Y+F mutant.
6.2.3. RNAPII CTD phosphorylation in wild type, An-fcp1\textsuperscript{TS}, F and An-fcp1\textsuperscript{TS}+F strains.

In Chapter 5, An-FCP1 had been shown to be a protein phosphatase targeting the RNAPII CTD. The loss of An-FCP1 phosphatase activity in the An-fcp1\textsuperscript{TS} mutant at the restrictive temperature of 42°C led to the accumulation of hyperphosphorylated RNAPII isoforms. As the temperature sensitivity of the An-fcp1\textsuperscript{TS} mutant due to the inactivation of An-FCP1 at 42°C coincided with the hyperphosphorylation of RNAPII CTD, experiments were conducted to investigate whether the synthetic interaction between the An-fcp1\textsuperscript{TS} and F mutations were mediated by the phosphorylation status of the RNAPII CTD as well. First, RNAPII was S-tag purified from F mutant cultures incubated at either 32°C or 42°C. When visualized by silver staining after SDS-PAGE, RNAPII appears as two distinct bands with the sizes of ~200KDa and ~250KDa and a smear in between these two bands, respectively representing the unphosphorylated, hyperphosphorylated and hypophosphorylated isoforms of RNAPII CTD (Figure 6.5A). This phosphorylation pattern of RNAPII was consistent with that of RNAPII purified from wild type cells (see Figures 5.6, 5.7).

To compare the degree the RNAPII CTD phosphorylation in the An-fcp1\textsuperscript{TS} and An-fcp1\textsuperscript{TS}+F mutant strains, RNAPII was S-tag purified from cell cultures of both strains incubated at the temperatures of 37°C, 38°C, 39°C and 40°C and visualized by Coomassie staining after SDS-PAGE. These temperatures were chosen as the synthetic interaction between the An-fcp1\textsuperscript{TS} and F mutations measured by colony growth rate was most pronounced within that temperature...
range (Figure 6.4). If RNAPII is in fact a common substrate of CDK1 and FCP1 we expected there to be a higher level of phosphorylation in the strain with the activated allele of CDK1 (F) and the FCP1 mutant (An-fcp1TS) when compared to either single mutant strain. In the An-fcp1TS strain, although RNAPII appeared to exist primarily in the hyperphosphorylated state (250KDa) throughout the temperatures of 37°C to 40°C, the amount of RNAPII CTD still remaining as a 200KDa sized unphosphorylated isoform was enough to appear as visible bands at all temperatures and most distinct at 37°C. In comparison, RNAPII samples purified from the An-fcp1TS+F double mutant cells at all temperatures of 37°C to 40°C exhibited even higher degrees of hyperphosphorylated CTD isoform concentration as the 200KDa unphosphorylated isoforms failed to visualize as distinct bands and only appeared as faint smears (Figure 6.5B). Concerning the conditions of the cell cultures, all the ones from which protein samples were prepared in this experiment had initially been grown at 32°C and subsequently shifted to the different temperatures for a 5.5 hour long incubation period. To determine whether these temperature shifts were affecting the phosphorylation status of RNAPII CTD, cell cultures of both the An-fcp1TS and An-fcp1TS+F mutant strains was grown entirely at 37°C. After SDS-PAGE, the S-tag purified RNAPII samples from both cultures were visualized by Coomassie staining. Similar to the previous data set, RNAPII prepared from the An-fcp1TS cells showed accumulation of hyperphosphorylated CTD isoforms (250KDA) yet with a visible amount of unphosphorylated CTD isoforms remaining. On the other hand, RNAPII of the An-fcp1TS+F double mutants almost exclusively existed as
hyperphosphorylated CTD isoforms where other CTD isoforms were hardly detectable (Figure 6.5C).

The phosphorylation status of RNAPII CTD did not differ in the F single mutant compared to wild type strains regardless of different temperatures (Figure 6.5A), while the synthetic interaction of the $An-fcp1^{TS}$ and F mutations was reflected in the increased phosphorylation level of RNAPII CTD in the $An-fcp1^{TS} + F$ double mutant. At the temperature range of 37°C to 40°C, the $An-fcp1^{TS} + F$ double mutant showed an elevated level of RNAPII CTD phosphorylation compared to the $An-fcp1^{TS}$ single mutant (Figure 6.5B, C). As the RNAPII CTD phosphorylation pattern in the F single mutant is certain to remain unchanged (comparable to that of a wild type strain) within that temperature range, the $An-fcp1^{TS} + F$ double mutant would maintain a higher level of RNAPII CTD phosphorylation compared to the F single mutant as well. These results were consistent with the observation that both the $An-fcp1^{TS}$ and F single mutants showed significantly higher growth rates than the $An-fcp1^{TS} + F$ double mutant (Figure 6.4) at the same temperatures. The correlation between temperature sensitivity and level of RNAPII CTD phosphorylation in the $An-fcp1^{TS}$, F and $An-fcp1^{TS} + F$ mutants suggests that the genetic interaction between $An-fcp1^{TS}$ and F may be mediated by the phosphorylation of RNAPII CTD.

6.2.4. Localization of RNAP II during mitosis in A. nidulans.

Although not consistent with the observations made in A. nidulans described in Chapter 5 of this dissertation (Figure 5.8B), in mammalian cells the
RNAPII CTD was reported to become hyperphosphorylated during mitosis (Xu et al., 2003). RNAPII maintained at this CTD phosphorylation status would not be able to stay bound to the chromosomal DNA due to its inability to form transcription pre-initiation complexes once a given transcription cycle was completed. As A. nidulans undergoes a partially open mitosis, any nuclear protein that does not remain associated with nuclear structures, like the chromosomes, would be predicted to disperse throughout the cell once mitosis was initiated. To examine its subcellular localization throughout the cell cycle in A. nidulans, RNAPII was C-terminally tagged with a green-fluorescent protein moiety and followed by time-lapse confocal microscopy. Since RNAPII is an essential protein, the lack any growth defects as a consequence of the endogenous GFP tagging of RNAPII confirmed that RNAPII::GFP was fully functional (data not shown). In exponentially growing cells, RNAPII located exclusively to the nuclei with a partial exclusion most likely from the nucleolus indicated by a darker area within each nucleus. At certain time points, the fluorescence intensity within the nuclei started to dissipate evenly into the entire cell, remained dispersed for 4-5 minutes, and re-concentrated back into the nuclei that in the mean time had doubled in their numbers. The doubling of the number of nuclei and the time frame of the RNAPII staying dispersed most certainly indicated that while RNAPII localized to the nuclei during interphase, it dispersed throughout the cell when mitosis started and relocalized to the nuclei once mitosis was completed. The observation that RNAPII dispersed from the nuclei during mitosis strongly indicates that, as in higher eukaryotes, transcription
if turned off during mitosis in A. nidulans. Also, it suggests an active regulation of RNAPII during mitosis that prevents it from remaining associated to chromosomal DNA, potentially through specific phosphorylation events on its CTD.

6.2.5. Comparison of RNAP II localization during mitosis in WT, An-fcp1<sup>TS</sup>, F and An-fcp1<sup>TS</sup>+F strains.

To determine whether the genetically synthetic interaction of the F and An-fcp1<sup>TS</sup> mutations affected the localization pattern of RNAPII, the endogenously GFP tagged RNAPII was followed both by fixed-cell fluorescence microscopy as well as live-cell time-laps confocal microscopy. The conidiospores of the wild type, An-fcp1<sup>TS</sup>, F and An-fcp1<sup>TS</sup>+F strains were germinated for 8 hours at 37°C, fixed and the frequency at which their RNAPII-GFP signal dispersed was counted. Also, cells of the four strains grown under the same conditions were followed and imaged by time-laps confocal microscopy while the germlings continued to grow at 37°C. Concerning the length of the germlings, number and distribution of the nuclei and frequency of mitotic nuclei (i.e. dispersed RNAPII-GFP signals), the wild type and the F strains did not appear to have a notable difference, consistent with the fact that at 37°C neither of the strains did show any visible growth defects (Table 6.1, Figure 6.7A, B). When the An-fcp1<sup>TS</sup> strain was examined, most notably it showed a higher frequency of dispersed RNAPII-GFP signals measured by the nuclear count in the fixed-cell sample (Table 6.1) while no other significant cytological defects were observed (Figure 6.7C). The most striking
mutant phenotype was displayed by the $An$-fcp1$^{TS}$+F double mutants. Not only did this strain show a higher frequency of dispersed RNAPII-GFP signals when the nuclei of fixed cells where counted (Table 6.1), but many of the cells displayed nuclei that were severely stretched out in their length while the RNAPII-GFP failed to disperse at all, indicative of failure to enter mitosis (Figure 6.7Da). Some of the nuclei had lost their proper distribution along the germ tube as they clumped tightly together (Figure 6.7Db), and cells in which the RNAPII-GFP signal remained dispersed, presumably due to their inability to exit mitoses, were also observed (Figure 6.7Dc). To examine whether the higher frequency of dispersed RNAPII-GFP signals in the $An$-fcp1$^{TS}$+F double mutant (and to a lesser degree in the $An$-fcp1$^{TS}$ strain as well) within a population of cells was due to the lengthening of the period when the RNAPII remains dispersed, the time points of RNAPII-GFP signal dispersal and relocation to the nuclei in representative cells of the wild type, $An$-fcp1$^{TS}$, F and $An$-fcp1$^{TS}$+F cells were measured. Time laps imaging revealed that the RNAPII-GFP signal in the $An$-fcp1$^{TS}$+F double mutants remained dispersed >3 times longer than the wild type and F mutants and almost twice as long as in the $An$-fcp1$^{TS}$ strain (Figure 6.8).

The severity of the cytological defects observed in the $An$-fcp1$^{TS}$+F double mutant cells strongly supported the synthetic interaction of the $An$-fcp1$^{TS}$ and F mutations. While the $An$-fcp1$^{TS}$ and F single mutants showed moderate or no notable defects compared to wild type cells, the $An$-fcp1$^{TS}$+F double mutant exhibited phenotypes that went certainly beyond the simple addition of that of the two single mutant strains.
6.3. DISCUSSION

6.3.1. Mutant strain with a single F allele as the only copy of the \textit{nimX} gene.

As random mitotic recombination between the wild type Y and mutant F alleles in the FRY24 strain did not yield the eviction of the wild type allele, a F single mutant strain was generated by transformation mediated gene replacement. Compared to the Y+F strain (FRY24), colonies of this F single mutant showed lower levels of overall fitness indicated by decreased conidiospore formation and slightly lower growth rate (Figure 6.3). Although the F mutation is dominant over the wild type allele, the presence of the wild type copy in the FRY24 strain would mean that for the mutant F protein to function in this strain, it would have to compete with the wild type Y protein for cyclinB binding partners, activating Thr161 phosphorylation by CAKs (CDK activating kinases) and interaction with target proteins to phosphorylate as well. The lack of the “buffering effect” resulting from this competition with the wild type protein would explain the higher penetrance of the F allele in the F single mutant in comparison to the FRY24 strain with a Y+F genotype.

6.3.2. Effect of the wild type Y allele on the synthetic interaction between \textit{An-fcp1}^{TS} and F.

Consistent with the mitigating effects of the wild type Y allele concerning overall fitness in the Y+F strain (FRY24), the synthetic interaction between \textit{An-fcp1}^{TS} and F was more pronounced in the \textit{An-fcp1}^{TS}+F double mutant that did
not have a wild type Y gene copy. While the growth temperature had to be 37°C or above for the \textit{An-fcp1}^{TS}+Y+F mutant to show the effects of \textit{An-fcp1}^{TS} and F interaction, in the \textit{An-fcp1}^{TS}+F mutant the synthetic interaction was already visible at 32°C as the double mutant exhibited a notably lower growth rate in comparison to the \textit{An-fcp1}^{TS} and F single mutants (Figure 6.4). The enhancement of synthetic interaction between \textit{An-fcp1}^{TS} and F in the absence of the wild type Y allele further confirmed the close genetic relationship between \textit{An-fcp1} and \textit{nimX}^{CDK1}.

6.3.3. RNAPII CTD phosphorylation in wild type, \textit{An-fcp1}^{TS}, F and \textit{An-fcp1}^{TS}+F strains

An-FCP1 was confirmed to be a protein phosphatase targeting the CTD of RNAPII (Figure 5.7), and its inactivation in the \textit{An-fcp1}^{TS} strains at elevated temperatures led to the growth arrest of the cells and accumulation of hyperphosphorylated RNAPII CTD isoforms. The synthetic interaction between \textit{An-fcp1}^{TS} and F represented a combination of an inactivated protein phosphatase (An-FCP1^{TS}) with a hyperactive protein kinase (F) synthetically leading to growth defects which were more severe than when having only the inactivated protein phosphatase (An-FCP1^{TS}) or the hyperactive protein kinase (F) individually. Since the An-FCP1 was confirmed to dephosphorylate RNAPII CTD, and Cdk1 had been shown to phosphorylate RNAPII CTD in vitro in other organisms, we propose a model where An-FCP1 and Y were targeting RNAPII CTD as their substrates and the synthetic interaction between \textit{An-fcp1}^{TS} and
\(nimX^{CDK1F}\) was mediated by the hyperphosphorylation of the target RNAPII CTD due to the inactivation of its protein phosphatase (An-FCP1\(^{TS}\)) and the hyperactivity of its protein kinase (F; Figure 6.9). To test this model, phosphorylation of RNAPII CTD in \(An-fcp1^{TS}\), F and \(An-fcp1^{TS}+F\) strains was examined and compared at temperatures where the synthetic interaction of F and \(An-fcp1^{TS}\) was pronounced. At temperatures of 37°C to 40°C, the \(An-fcp1^{TS}+F\) strain demonstrated extreme accumulations of the hyperphosphorylated CTD isoforms of RNAPII, while in the \(An-fcp1^{TS}\) single mutant a portion of the RNAPII protein pool remained unphosphorylated on its CTD and the F strain maintained an even distribution of unphosphorylated and hyperphosphorylated CTD isoforms even at the higher temperature of 42°C (Figure 6.5A). Although the differential of CTD phosphorylation between the \(An-fcp1^{TS}+F\) double mutant and the F and \(An-fcp1^{TS}\) single mutants was not as pronounced as their overall growth rate differential measured by colony size, the reproducible outcome of these experiments (Figure 6.5B versus 6.5C) suggested that the synthetic interaction of F and \(An-fcp1^{TS}\) was mediated, at least in part, by the hyperphosphorylation of the RNAPII CTD.

6.3.4. Localization of RNAP II during mitosis in \textit{A. nidulans}.

As mitosis in \textit{A. nidulans} had been determined to be partially open, nuclear proteins in \textit{A. nidulans} that do not remain attached to the chromosome during mitosis can disperse into the cytoplasm via passive diffusion once the nuclear pores open up at the beginning of each mitotic phase. In contrast,
proteins such as histones, which remain associated with chromatin during mitosis, are not released from nuclei during mitosis. While the function of RNAPII stipulates that it binds the chromosomal DNA when it is active during interphase, the state of this association and thus the localization of RNAPII during mitosis when cellular transcription is globally shut down remained unclear. To investigate the subcellular localization of RNAPII within the context of cell cycle progression, RNAPII was endogenously tagged with a GFP construct at its C-terminus and followed by time-lapse microscopy (Figure 6.6). The predominant nuclear localization of RNAPII in growing cells confirmed that it remains exclusively in the nucleus during interphase as it is engaged in transcriptional activities. The complete dispersal of RNAPII during mitosis suggests that its association to the DNA may be actively disrupted specific to mitosis, effectively disabling it from continuing transcription. During the transcription cycle, it had been shown that the CTD of RNAPII has to be completely dephosphorylated for the RNAPII to form a pre-initiation complex and re-engage in transcriptional activity (Komarnitsky et al., 2000; Schroeder et al., 2000). In mammalian cells the RNAPII CTD was also reported to be hyperphosphorylated during mitosis (Xu et al., 2003). Although in A. nidulans this mitotic-specific CTD hyperphosphorylation event could not be observed, the fact that RNAPII completely disperses during mitosis suggests that there still may be a mitotic specific phosphorylation pattern of RNAPII CTD in A. nidulans. It may not be distinguishable by measuring bulk phosphorylation of the CTD, as detected by mobility shifts during SDS PAGE, but might still be able to actively dissociate RNAPII from chromatin and thus
contribute to the mitotic specific dispersal. The work described in this dissertation has identified that Y shows a strong genetic interaction with the RNAPII CTD phosphatase gene An-fcp1. Taken together with the fact that CDK1 is a major mitotic protein kinase which is capable of phosphorylating RNAPII CTD in vitro, the CDK1 kinase could be considered a compelling candidate to promote such a mitotic specific phosphorylation event of RNAPII CTD.

6.3.5. RNAP II localization in wild type, An-fcp1TS, F and An-fcp1TS+F strains.

When the localization pattern of RNAPII was examined in the An-fcp1TS, F and An-fcp1TS+F strains at 37°C and compared to wild type cells, the An-fcp1TS+F double mutants revealed by far the most severe cytological defects. Some nuclei became stretched out in their length without the RNAPII-GFP signal dispersing at all during a time period sufficiently long for two rounds of A. nidulans cell cycle to be completed, strongly suggesting their inability to initiate mitosis (Figure 6.7Da). Other nuclei examined appeared to gather into clumps of nuclei indicating defects in proper nuclear distribution (Figure 6.7Db). While in general the time required for the RNAPII-GFP signal to relocate to the nuclei after each round of mitoses was measured to be much longer than in wild type cells (Figure 6.8), in some cases the RNAPII-GFP signal remained dispersed without relocating back to the divided daughter nuclei at all, presumably due to their inability to exit mitoses (Figure 6.7Dc). Overall, the higher frequency of cells observed to show the RNAPII-GFP dispersed when fixed cells where analyzed together with the time-point measurements of live cells undergoing mitosis.
showed that mitoses in the An-fcp1<sup>TS</sup>+F double mutants continued on average as much as 3 times longer than in the wild type and F mutants and twice as long as in the An-fcp1<sup>TS</sup> cells (Table 6.1, Figure 6.8).

The various mitotic defects observed in the An-fcp1<sup>TS</sup>+F double mutants strongly indicates that the synthetic interaction between the An-fcp1<sup>TS</sup> and F mutation may impact the cells in ways that go beyond the immediate disruption of the RNAPII driven transcription cycle. Although RNAPII CTD is so far the only known substrate of FCP1, it cannot be ruled out that FCP1 and CDK1 may share multiple common substrates as well. Since the effects of mutations on An-fcp1 and nimX<sup>CDK1</sup> would become amplified in a pleiotropic manner if they were to share many substrates, this may explain why the difference of RNAPII CTD phosphorylation between F, An-fcp1<sup>TS</sup> and An-fcp1<sup>TS</sup>+F mutants is only moderate compared to the severity of the cytological defects observed in the double mutant. It is also possible CDK1 and FCP1 do not directly share a common substrate but function in different cellular pathways that eventually feed into the same target or targets. The interaction between An-fcp1<sup>TS</sup> and nimX<sup>CDK1F</sup> is genetic in nature, and An-fcp1<sup>TS</sup> and nimX<sup>CDK1F</sup> could still show their synthetically interacting effects if the separate biological pathways in which they function merge onto common cellular targets that provide important activities concerning mitotic regulation. In fact, reports suggesting the involvement of FCP1 in DNA damage responses (Cho et al., 2006) indicate that FCP1 may functionally relate to CDK1 in a manner more directly linked to mitotic regulation. Overall, the severe mutant phenotypes observed in the double mutant compared to that exhibited by the An-
$fcp1^{TS}$ and F single mutants confirms the synthetic nature and high relevance of the genetic interaction between $An-fcp1^{TS}$ and F.
Figure 6.1 Synthetic interactions as genetic relationships. When pathway A and B are contributing together to an important cellular function, a combination of mutations on both α and β can result in a more severe mutant phenotype than those of either α or β individually due to the compensatory nature of the two cellular pathways. Thus, there is a genetically synthetic interaction between the mutations of α and β. As this interaction is genetically defined, there is no requirement for α and β to physically interact.
Figure 6.2 Dynamic rearrangement of the nuclear pore complex during mitosis in *A. nidulans*. 24 proteins (Nups) are identified to comprise the nuclear pore complex (NPC) in *A. nidulans*. During interphase the nuclear pore functions as a conduit of nucleocytoplasmic exchange regulating active transport between the nucleoplasm and the cytoplasm. With onset of mitosis, the NPC undergoes partial disassembly as 14 of the Nups dissociate from the nuclear pore. This partial disassembly of the NPC results in the opening of the central channel and allows random diffusion to occur specifically during mitosis. Nups that disperse are labeled in light blue. (Reproduced with the permission of the authors from Osmani et al., 2006)
Figure 6.2
Figure 6.3 Generation of strains which have only *nimX^{CDK1F}* as a sole copy of *nimX^{CDK1}*. A strain was generated in which the wild type *nimX^{CDK1}* had been replaced with a mutant *nimX^{CDK1F}* allele. The nutritional marker *pyroA* was used to generate a gene-replacement construct (A). Unlike the FRY24 strain (indicated as *nimX^{CDK1}+nimX^{CDK1F}*), the newly generated strain has only the mutant *nimX^{CDK1F}* allele without an additional wild type *nimX^{CDK1}*.

Compared to the FRY24 strain, the *nimX^{CDK1F}* strain showed a slightly slower growth rate on rich growth medium and a lesser degree of conidial spore formation (darker, less powdery colony surface). A strain co-generated in which the wild type *nimX^{CDK1}* gene had been replaced with a *nimX^{CDK1AF}* mutant allele exhibited an even lower growth rate and possible genomic instability indicated by extensive fanning of the colony. All three mutant strain showed equal degrees of sensitivity against 8mM hydroxyurea (B).
Figure 6.3
**Figure 6.4 Synthetic genetic interaction of** \(nimX^{CDK1F}\) **and** \(An-fcp1^{TS}\) **mutations.** The \(An-fcp1^{TS}\) mutation by itself caused gradually increasing degrees of temperature sensitivity compared to the wild type strain (colony indicated as \(nimX^{CDK1}\)) as growth temperatures were elevated from 32°C to 42°C. Nevertheless, within the temperature range of 32°C to 40°C, the \(An-fcp1^{TS}\) and \(nimX^{CDK1F}\) mutants individually showed significantly higher rates of growth in comparison to the \(An-fcp1^{TS}+nimX^{CDK1F}\) double mutant strain (colony \(An-fcp1^{TS}+nimX^{CDK1F}\)), confirming the genetically synthetic interaction of \(An-fcp1^{TS}\) and \(nimX^{CDK1F}\) mutations. The synthetic interaction was less severe in the presence of a wild type copy of the \(nimX^{CDK1}\) (compare colony \(An-fcp1^{TS}+nimX^{CDK1F}\) with \(An-fcp1^{TS}+nimX^{CDK1}+nimX^{CDK1F}\)).
Figure 6.4
Figure 6.5 Phosphorylation of RNAPII C-Terminal Domain in \( \text{nimX}^{\text{CDK1F}} \) and \( \text{An-fcp1}^{\text{TS}+\text{nimX}^{\text{CDK1F}}} \) mutant strains. The phosphorylation pattern of RNAPII CTD in the \( \text{nimX}^{\text{CDK1F}} \) mutant strain resembles that of the wild type strain (see Figure 5.7) independent of growth temperatures (A). Compared to the \( \text{An-fcp1}^{\text{TS}} \) single mutant, the \( \text{An-fcp1}^{\text{TS}+\text{nimX}^{\text{CDK1F}}} \) double mutant exhibits a higher degree of hyperphosphorylated RNAPII accumulation at the temperature ranges of 37°C to 40°C (B). This tendency is more pronounced when the culture for protein samples preparation was grown entirely at 37°C (C).
Figure 6.6 Dynamic localization of RNAPII throughout the cell cycle in *A. nidulans*. RNAPII exhibits exclusively nuclear localization during interphase but disperses completely into the cytoplasm when the nuclei enter mitosis. Once mitosis is complete, RNAPII is re-imported into the nuclei.
Table 6.1 Frequency of RNAPII-GFP dispersal in wild type, \textit{An-fcp}^{TS}, \textit{nimX}^{CDK1} and \textit{An-fcp}^{TS}+\textit{nimX}^{CDK1} strains at 37°C. Cells of the individual strains were grown for eight hours at 37°C and fixed. The number of cells in which the RNAPII-GFP signal appears dispersed was counted as well as the total number of cells. The experiment were repeated three times independently.
Figure 6.7 Localization pattern of RNAPII examined in the wild type, *An-fcp1*<sup>TS</sup>, *nimX<sup>CDK1F</sup>* and *An-fcp1*</sup><sup>TS</sup>+*nimX<sup>CDK1F</sup>* strains at 37°C. *An-fcp1*<sup>TS</sup> (C) and *nimX<sup>CDK1F</sup>* strains (B) did not exhibit structural defect phenotypes compared to wild type cells (A). The most severe cytological defects were revealed by the *An-fcp1*</sup><sup>TS</sup>+*nimX<sup>CDK1F</sup>* double mutants. Some nuclei could be seen stretched out in their length without the RNAPII-GFP signal dispersing at all strongly suggesting their inability to enter mitosis (Figure 6.7Da). Several nuclei appeared to gather into clumps suggesting defects in proper nuclear distribution (Figure 6.7Db). Some germlings exhibited sustained dispersal of the RNAPII-GFP signal and failure to relocate back to the divided daughter nuclei, indicative of defects in mitotic exit (Figure 6.7Dc).
Figure 6.7
Figure 6.8 Time-point measurements of mitotic duration in wild type, \textit{nimX}^{CDK1F}, \textit{An-fcp1}^{TS}, and \textit{An-fcp1}^{TS}+\textit{nimX}^{CDK1F} strains at 37°C. While the wild type and \textit{nimX}^{CDK1F} strains require \(~4\) minutes to complete one round of mitosis, the \textit{An-fcp1}^{TS} mutant requires \(~7.5\) minutes to mitose. The \textit{An-fcp1}^{TS}+\textit{nimX}^{CDK1F} double mutant on average requires more than 13 minutes, to complete one round of mitosis. An alternative explanation would be that while the duration of mitoses remain intact, the dispersal pattern of the RNAP\II is altered in the \textit{An-fcp1}^{TS} and \textit{An-fcp1}^{TS}+\textit{nimX}^{CDK1F} strains where it leaved the nuclei before mitotic onset and came back long after mitoses were completed.
Figure 6.9 Hypothetical phosphorylation pattern of a common substrate shared by a kinase/phosphatase pair. Under normal conditions the countervailing balance between the kinase and phosphatase activities maintains the phosphorylation of the common substrate at a physiologically functional level. When under particular circumstances this balance is disturbed by the elevation of the kinase activity combined with the inactivation of the phosphatase, the common substrate will become hyperphosphorylated and its biological function disrupted.
In living cells, the physiological phosphorylation status of proteins can fluctuate dynamically, balanced by the countervailing activities of protein kinases and protein phosphatases. Within the context of the eukaryotic cell division cycle, many important control measures regarding cell cycle progression had been shown to be implemented at the level of protein phosphorylation. This aspect of the cell division cycle makes proper understanding of the counteractive effects that mitotic protein kinases and phosphatases confer upon their substrates crucial in deciphering the nature of cell cycle regulation. To address this need in a more comprehensive manner, this project aimed to examine potential mitotic functions of all protein phosphatase catalytic subunits in A. nidulans, paralleled with the effort to identify mutations that show genetically synthetic interactions with the hyperactive mitotic kinase mutant nimX^{CDK1F}. Taking advantage of the advances in the field of genomics, a systematic gene targeting strategy was employed to delete all genes encoding protein phosphatase catalytic subunits in
A. *nidulans* and subsequent mitotic defects were identified. Together with this genomic approach for analyzing protein phosphatase functions, a classical forward genetics strategy engaging the function of the mitotic kinase $nimX^{CDK1}$ was set up in the form of a synthetic interaction genetic screen to isolate mutations that combine with $nimX^{CDK1F}$ to synthetically cause increased temperature sensitivity.

The systematic phosphatase gene deletion analysis confirmed the essential function of four protein phosphatases (BIMG, NIMT, An-FCP1 and PPHA), and four other protein phosphatases were shown to have important functions because their gene deletions led to strong growth defects (AN-PPG1, AN-NEM1, AN-SSU72 and AN-YVH1). When the basic gene deletion phenotypes of these eight select protein phosphatases were examined, *bimG* and *nimT* deletions respectively resulted in classical M-phase and late G2 phase arrest phenotypes. This result confirmed previous studies showing that the functions of BIMG and NIMT are required for cell cycle progression, conclusions that were drawn from studies conducted with the conditional mutant alleles *bimG11* and *nimT23*. In addition, in this study, *bimG* was identified as the only protein phosphatase whose function is essential for polarized growth in *A. nidulans*. While not showing classical cell cycle arrest phenotypes, the deletion phenotypes of *An-nem1* and *pphA* strongly suggested important mitotic functions of An-NEM1 and PPHA. $\Delta$*An-nem1* cells showed signs of DNA fragmentation, while $\Delta$*pphA* cells displayed phenotypes strongly indicative of defects in nuclear DNA segregation. Although more subtle, the gene deletion phenotypes of *An-
ppg1, An-ssu72 and An-yvh1 also suggested involvement of their function in cell cycle regulation. ΔAn-ppg1 cells showed a possible de-coupling of polarized growth and mitotic progression, and during early stages of germination ΔAn-ssu72 cells showed signs of defects in nuclear distribution and DNA segregation. The deletion of An-yvh1 not only yielded a particularly strong growth defect phenotype, but many ΔAn-yvh1 cells also displayed nuclei that are stretched in their length resembling defects of mitotic progression.

The severity of terminal growth defect resulting from the deletion of An-fcp1 made it difficult to determine mitotic specific defects in ΔAn-fcp1 cells based on microscopic examination of their nuclear DNA. More insight into the potential function of AN-FCP1 concerning cell cycle regulation came from the genetic interaction the An-fcp1TS mutant allele demonstrated with the mitotic kinase mutation nimXCDK1F. In the genetic screen conducted to identify mutations that synthetically interact with nimXCDK1F, An-fcp1TS was isolated as a mutation that causes temperature sensitivity in combination with nimXCDK1F. An-fcp1TS was shown to be a conditional loss-of-function mutation of An-fcp1, while nimXCDK1F is a hyperactive mitotic kinase mutant incapable of being downregulated by inhibitory Tyr15 phosphorylation, a mechanism critical in determining the catalytic function of CDK1. Normally Tyr15 phosphorylation of CDK1 during interphase keeps the kinase inactive until the CDC25 phosphatase removes this inhibitory phosphate to allow entry into mitosis. The CDK1F mutation prevents Tyr15 phosphorylation leading to hyperactivation of CDK1 activity. The observation that an inactivated phosphatase together with a hyperactive kinase caused severe
growth defects was incorporated into a model designed to explain the synthetic interaction through the potential imbalance of phosphorylation occurring on a protein which is a substrate of both the kinase and the phosphatase (See also Figure 6.9). Since the physiological substrate of AN-FCP1 had been shown to be the C-terminal domain of RNA polymerase II largest subunit (RNAPII CTD), whether the synthetic interaction of \( \text{nimX}^{\text{CDK1F}} \) and \( \text{An-fcp1}^{\text{TS}} \) was being mediated through the phosphorylation of RNAPII CTD functioning as a common substrate was examined. Under conditions where the synthetic interaction of \( \text{nimX}^{\text{CDK1F}} \) and \( \text{An-fcp1}^{\text{TS}} \) was most pronounced, RNAPII CTD exhibited an elevated level of phosphorylation in the \( \text{nimX}^{\text{CDK1F}} + \text{An-fcp1}^{\text{TS}} \) double mutants compared to wild type, \( \text{nimX}^{\text{CDK1F}} \) and \( \text{An-fcp1}^{\text{TS}} \) strains. This result supports the hypothesis that the synthetic interaction of \( \text{nimX}^{\text{CDK1F}} \) and \( \text{An-fcp1}^{\text{TS}} \) was at least in part mediated through the phosphorylation of RNAPII CTD.

The fact that the mitotic kinase CDK1 genetically interacted with an important regulator of transcriptional activity suggested two different possibilities concerning CDK1 function in transcriptional regulation. Considering that CDK1 function is crucial for mitotic progression, one possibility was the regulation of RNAPII mediated transcription specific during mitosis mediated by the kinase activity of CDK1. The unique cellular localization of RNAPII during mitosis was consistent with this possibility. While RNAPII is exclusively nuclear during interphase, at the G2/M transition it was shown to completely disperse throughout the cell only to recover its original localization once mitosis was completed. This pattern suggested that there may be a mitotic specific phosphorylation of RNAPII
CTD carried out by CDK1 to ensure that cellular transcription is shut down during mitosis. Another possibility is that CDK1 may be involved more generally in regulating transcriptional activity through RNAPII CTD phosphorylation. The mitotic status of RNAPII CTD phosphorylation turned out to be no different from that during interphase, at least in the bulk amount, suggesting that indeed the effect of CDK1 on transcription may be of a general nature and independent of mitosis. A more accurate examination of RNAPII CTD phosphorylation during mitosis could be critical in determining which of these possibilities is valid, since there is a possibility that a mitotic specific phosphorylation of RNAPII CTD may not be distinguishable by measuring the bulk level of phosphorylation.

The strong synthetic interaction between \( nimX^{CDK1F} \) and \( An-fcp1^{TS} \) is consistent with the serious cytological abnormalities shown by the \( \text{n}imX^{CDK1F}+An-fcp1^{TS} \) double mutant. Unlike the single mutants, cells of the \( \text{n}imX^{CDK1F}+An-fcp1^{TS} \) double mutant strain display a range of severe mitotic defects including prolonged mitotic duration, defective DNA segregation, uneven distribution of nuclei and inability to exit mitoses. Although FCP1 was demonstrated to dephosphorylate RNAPII CTD, it is unlikely that compromised transcription alone would cause the distinctive mitotic defects observed in the \( \text{n}imX^{CDK1F}+An-fcp1^{TS} \) double mutant strain. In fact, the manifestation of the genetically synthetic interaction between \( \text{n}imX^{CDK1F} \) and \( An-fcp1^{TS} \) in severe mitotic defects strongly suggests an important function of FCP1 in regulating mitotic progression beyond its known activity of RNAPII CTD dephosphorylation. As a kinase/phosphatase pair, \( \text{n}imX^{CDK1F} \) and \( An-fcp1 \) could be sharing substrates other than RNAPII CTD
as well, and the genetic interaction of \( \text{nimX}^{CDK1F} \) and \( \text{An-fcp1}^{TS} \) could be mediated through the phosphorylation of these shared substrates. Alternatively, An-FCP1 may be part of a mitotic regulatory pathway that functions in parallel of the inhibitory Tyr15 phosphorylation of NIMX^{CDK1}.

The systematic analysis of protein phosphatases and the synthetic interaction screen of \( \text{nimX}^{CDK1F} \) described in this dissertation demonstrate the relative advantages of modern genomics and classical genetics, and how in fact they can complement each others’ potential shortcomings. Landmark genetic screens of the past had already identified phosphatase genes that are clearly required for mitotic progression in \( A. \text{nidulans} \) (\( \text{nimT} \) and \( \text{bimG} \)), but it was the genomic gene targeting approach that conclusively determined all protein phosphatases that have essential cellular functions. The fact that only five phosphatase genes out of twenty eight were essential indicates a high degree of functional redundancy within this group of enzymes as well. On the other hand, while the outcome of the An-fcp1 gene deletion analysis was rather limited in furthering our understanding of the specific function of An-fcp1, the synthetic interaction of \( \text{nimX}^{CDK1F} \) and \( \text{An-fcp1}^{TS} \) revealed through a genetic screen convincingly demonstrated the potential relevance of An-fcp1 function in mitotic regulation.

The exact mechanism through which CDK1 and FCP1 coordinate their cellular activities has yet to be completely understood, but this work has shown how a kinase and a phosphatase as a pair may balance each others activity and
in that way regulate and calibrate important cellular activities. Combined with the systematic identification of all protein phosphatases, this concept presents a potent scheme for further studying the functions of kinases and phosphatases in regulating important cellular events. The generation of strains in which endogenous phosphatase genes are under the control of the regulatable \textit{alcA} promoter avails an additional molecular tool that can be readily utilized for this purpose. The ability to group regulatable alleles of protein phosphatases with loss-of-function or hyperactive protein kinase mutants further enhances the feasibility to determine kinases and phosphatases of countervailing functions. In studying the function of \textit{An-fcp1} specifically, the availability of \textit{alcA} promoter driven conditional phosphatase alleles can be used to confirm the specificity of the genetic interaction between \textit{nimX}^{CDK1} and \textit{An-fcp1} as well as potentially the identification of additional substrates that are shared by CDK1 and FCP1. In general, the identification of mitotic kinase/phosphatase pairs that counteract each others activities and the determination of their potential common substrates could provide us with a much better understanding of how mitotic regulation is achieved. Extending the methodologies and concepts developed during this dissertation work can also be further utilized to study numerous other core cellular functions that are regulated by reversible protein phosphorylation.


complements the Aspergillus cell cycle mutation, bimG11. J. Biol. Chem. 266, 18889-18894.


