Study towards understanding the mitotic role of the Aurora protein kinase in *Aspergillus nidulans* 

A Senior Honors Thesis

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by

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ABSTRACT

Mitosis is the process by which cells segregate their DNA equally during cell division and growth. Because uncontrolled mitosis can lead to cancer, mitotic regulators like the Aurora protein kinase in *Aspergillus nidulans* are very important. Kinases are enzymes which are capable of activating or inactivating proteins by phosphorylating them. It had already been demonstrated that another mitotic regulator, the NIMA protein kinase, was essential. To determine whether or not *Aurora* was required for mitosis and growth, I deleted the Aurora gene from the *A. nidulans* genome via homologous recombination of a deletion construct. Transformed strains survived only by forming heterokaryons which contained 2 genetically distinct nuclei. This is typical after deletion of genes that are essential for survival. In addition, I endogenously tagged the Aurora gene with Green Fluorescent Protein (GFP). This allows for visualization via confocal microscopy of temporal and spatial location of Aurora during the cell cycle. I found that *Aurora*-GFP had a mitotic specific location on mitotic spindles. The techniques used for these experiments include Polymerase Chain Reaction, western blots, gel electrophoresis, and growth tests on various types of media. Because the NIMA and Aurora kinases are both implicated in mitotic initiation, it is hypothesized that they work together to achieve this event. In addition to this, both NIMA and Aurora have homologous genes in the human genome, therefore my studies might shed light upon the origins and treatments of cancer in humans.
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DEDICATION

This work is dedicated
to my parents, Tony and Souhad,
and to my sister, Lori,
and to all of my extended family
and friends.
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CHAPTER I
INTRODUCTION

1.1 Problem Statement

Cancer, one of the leading causes of death today, is a result of uncontrolled cell division, and this debilitating disease still does not have a cure. In a healthy cell, there exist numerous mitotic regulators that constantly monitor the cells rate of division. These become faulty in cancer cells, and uncontrolled division ensues. Enzymes that phosphorylate proteins, called kinases, play key roles in regulating mitosis. This phosphorylation often activates or inactivates the target protein. In the nucleus, the DNA is wrapped around an octomer of histone proteins, forming nucleosomes (Strachan and Read 2010). In many organisms including the model genetic organism *Aspergillus nidulans*, the phosphorylation of histone protein H3 in the nucleus, around which DNA is wound, has been linked to the initiation of mitosis. Two protein kinases, NIMA and Aurora have been implicated in mitotic phosphorylation of Histone H3 on the Serine 10 residue in *A. nidulans*. By studying these kinases with the ultimate goal of discovering how they work together to bring about H3 phosphorylation and mitosis, a great deal can be learned about mitotic regulation. Because both NIMA-like kinases and Aurora kinases are present in higher eukaryotes like humans, the advancements in the study of these kinases in *A. nidulans* may be applicable to human cancer.

It has been demonstrated that NIMA is essential for mitosis to occur, but whether Aurora kinase has essential roles is not known (De Souza, *et. al.* 2000). Determining if a mitotic regulator is essential can tell a great deal about its mechanism and function in the cell cycle. The aim of this project is to determine the essentiality of the Aurora kinase in *A. nidulans* so that the
Aurora kinases in higher eukaryotes can also be understood in greater detail. I want to answer two questions: where does *Aurora* locate during the cell cycle, and is it necessary for cell division and growth?

### 1.2 Biology of *Aspergillus nidulans*

The filamentous fungus *Aspergillus nidulans* is a very powerful model organism. A non-pathogenic species, its genome of 31Mb has been entirely sequenced along with thousands of mapped genes (Galagan J. E. *et al.* 2005). Nuclei divide several times without cytokinesis and up to eight nuclei share a common cytoplasm until a septum forms. Branching can occur in any direction, including vertically. It displays a rapid growth rate at about 0.5 mm per hour at 37°C and the cell cycle is normally synchronous within individual multinucleated cells. Conidia (asexual spores) color pigmentation is another benefit that allows sporulation to be studied easier. Methods have been established for the transformation of *A. nidulans* strains with DNA. Typically *A. nidulans* grows as a haploid but can be induced to become diploid or form a heterokaryon, in which two distinct nuclei exist in the common cytoplasm. *A. nidulans* can undergo sexual or asexual reproduction.

Of relevance to this study is the asexual mode of reproduction in which internal and external events induce spore formation on multinucleated structures termed conidiophores. This structure can release spores which then can germinate into colonies elsewhere. During spore germination it will undergo polarized growth and give rise to a tube-like structure with multiple nuclei in common cytoplasm, called hyphae. Branching from the tube occurs to produce a rounded colony. Aerial hyphae give rise to conidiophores. During this development, conidiophores form, which again produce uninucleate spores to complete the asexual life cycle.
The fungus can also switch to sexual reproduction when the center of the colony begins to produce fruiting bodies called cleistothecia. These contain meiotic ascospores after diploid nuclei had undergone meiosis. Studying *Aspergillus nidulans* not only sheds light upon human genetics because of conservation of many genes from *A. nidulans* to higher eukaryotes, but can also be extrapolated as a safe way to learn more about pathogenic fungi that cause aspergillosis (Adams, *et. al.*1998).

![Aspergillus nidulans mycelium and conidiophores (Mims).](image)

1.3 *The Cell Cycle*

The eukaryotic cell cycle consists of interphase and mitosis. Interphase is comprised of gap stage $G_1$, followed by DNA replication in the $S$ phase, then growth in $G_2$. Mitosis then occurs between the two growth phases. Cells typically double in size during interphase as they prepare to divide. When a cell like a fibroblast is not dividing, since fibroblasts only divide when there is damage to a tissue, a cell is said to be in $G_0$. Mitosis is also divided into stages, in the order prophase, metaphase, anaphase, and telophase, followed by cytokinesis when the cell...
divides. In some organisms such as *A. nidulans*, mitosis only takes up about 5% of the entire cell cycle.

In order for a cell to proceed through phases of the cell cycle, it must pass through various checkpoints along the way to ensure that the cell is replicating DNA and growing normally and will be able to produce two viable daughter cells. Checkpoints ensure that one cell cycle phase is complete before the next one can begin. Cancer or other major health problems such as birth defects can ensue if a cell is allowed to grow and divide without being controlled by the checkpoints properly. For example, in mammals a mutation in the p53 gene has been linked to many cancers; this gene typically regulates a cell's proceeding from G1 to S (Cooper, 2000).

Figure 2-Eukaryotic cell cycle shown with each phase size relative to the time spent in each stage. Example checkpoints are illustrated where they stop the cycle if DNA is damaged or chromosomes are misaligned. In normal cells the cycle does not proceed until the error is fixed. (modified from Phases of the Eukaryotic Cell)
1.4 The Aurora Protein Kinase

The Aurora Protein Kinase has not yet been studied extensively in *Aspergillus nidulans*, but it has demonstrated vital roles in other eukaryotes like yeast, nematodes and mammals. In yeast and nematodes, the lpl/Aurora kinase and Glc7/PP1 phosphatase are responsible for correct phosphorylation of histone H3 at ser10 and subsequent chromosome condensation. It was demonstrated that in yeast, Ipl1 kinase is an upstream mitotic H3 kinase because its peak expression corresponds to that of H3 phosphorylation (Hsu *et. al.* 2000). Over-expression of Ipl/Aurora kinase is evident in many types of cancers (De Souza, *et. al.* 2000).

In mammals, there exists three forms of Aurora kinase termed Aurora-A, Aurora-B and Aurora-C. Aurora A function is required for centrosome maturation, separation, bipolar spindle assembly and controls activation of cyclin-B/Cdk-1 and thus mitotic initiation. Aurora-B is linked to many mitotic specific events such as H3 Ser 10 phosphorylation, chromosome condensation, sister chromatid cohesion and mitotic spindle assembly. Aurora C has been less studied but plays an important role in male fertility and spermatogenesis in mice (Vader, *et. al.* 2008).

In *A. nidulans*, the *Aurora* gene measures 3.4 kb. As seen in Figure 3, *Aurora* is highly conserved from *A. nidulans* to yeast and humans. Studying this kinase in the powerful model organism *A. nidulans* can help us understand its role and study its evolution to homologues found in higher eukaryotes. Using BLAST sequence homology searches we identified (Figure 3) the only Aurora kinase gene encoded in the genome of A. nidulans (An5815.3).
Consensus key (see documentation for details)
* - single, fully conserved residue
: - conservation of strong groups
. - conservation of weak groups
- no consensus

CLUSTAL W (1.81) multiple sequence alignment

Figure 3-Conservation of *Aurora* in mammals, *A. nidulans* and *S. cerevisiae*
1.5 The NIMA Protein Kinase

The NIMA kinase has been demonstrated to have a role as an essential mitotic regulator and its activation has been shown to be required for the mitotic specific phosphorylation of histone H3 at Ser-10. If H3 mitotic phosphorylation is blocked, the chromosomes cannot condense and the cell cannot enter mitosis. Similarly, if H3 is not dephosphorylated, the chromosomes abnormally remain condensed in mitosis. NIMA is able to phosphorylate histone H3 in vitro, which strongly suggests that it is the phosphorylators in vivo as well, but this is still undetermined. NIMA temperature sensitive mutants arrest in G2 with uncondensed chromatin. NIMA levels peak as the cell enters mitosis. NIMA exhibits a chromatin-like localization during H3 phosphorylation, then at mitosis it was seen to localize at the spindle. These findings suggest that NIMA is necessary and sufficient to allow for H3 Ser 10 phosphorylation and subsequent entry into mitosis (De Souza, et. al. 2000). However, in other systems it has been proposed that the mitotic histone H3 kinase is Aurora suggesting that in A. nidulans NIMA might activate Aurora which then phosphorylates the H3 S10 site (Hsu et.al. 2000). Alternatively in A. nidulans NIMA could function as the H3 S10 kinase with no role for Aurora.
CHAPTER II
MATERIALS AND METHODS

2.1 Oligonucleotide Primer Selection and Polymerase Chain Reaction (PCR)

When choosing oligonucleotide primers to order, they were chosen with sequences complimentary to the ends of the desired segments of DNA so that they would hybridize to the template during PCR. Primers with more guanine-cytosine pairs are favorable because of the three hydrogen bonds that occur between this nucleotide base pair, versus the two hydrogen bonds that keep adenine and thymine together. This allows then for a somewhat stronger binding between primer and template. An optimal ratio is 50-60 % GC pairs. This was done using MS-DOS primer software. The primer sequences were then sent to Integrated DNA Technologies where they were custom-made.

To amplify the desired region, PCR was conducted using as template *A. nidulans* genomic DNA or plasmids containing the appropriate cassettes according to Roche Expand Long Template PCR System. PCR involves denaturation of the double-stranded DNA into single strands at ~ 94 °C. Next, the temperature is decreased to 56-60 °C, allowing the primers to anneal to their complimentary sequences, which are at the ends of the desired amplified segment. Next, the temperature is increase to ~ 68 ° C for the polymerase to extend the primers as dNTP’s are added and DNA is synthesized from 5’ to 3’ direction. The entire cycle is then repeated after the temperature is raised to ~ 94 °C again. Over time, the desire segment is amplified exponentially (Polymerase Chain Reaction). Temperatures, number of cycles and other conditions were optimized according to the specific reaction. In this project PCR was done using a 9700 Thermal Cycler (Perkin Elmer) or a Gradient Thermal Cycler (Eppendorf).
2.2 Agarose Gel Electrophoresis

PCR products were run on an agarose gel for separation and visualization. Because of the phosphate backbone, the DNA is negatively charged. Therefore the DNA is loaded into wells at the negative pole of the gel tank, and migrates towards the positive end. Larger fragments migrate at a slower rate through the gel. Ethidium bromide is used as a fluorescent intercalator of DNA to allow for visualization of the bands in UV light (Strachan and Read, 2010). In this project, most PCR samples were run on 0.8 % agarose gels. For gel purification procedures, 1 % low melt agarose gels were used.

2.3 Aspergillus nidulans Selective Media

Vitamins: 1 mL/L each: p-aminobenzoic acid (PABA), nicotinic acid, choline, D-biotin, pyridoxine HCl, riboflavin HCl

YG: 5 g/L yeast extract, 10 g/L dextrose, 1 mL/L trace elements, 1 mL/L vitamins, 1 M MgSO₄

YGUU: YG with 1.2 g/L uridine and 1.1 g/L uracil

YAG: 5 g/L yeast extract, 10 g/L dextrose, 1 mL/L trace elements, 1 mL/L vitamins, 1 M MgSO₄, 15 g agar/L.

YAGUU: YAG with 1.2 g/L uridine and 1.1 g/L uracil

MAG: 5 g/L malt extract, 2 g/L bacto peptone, 10 g/L dextrose, 1 mL/L trace elements, 1 mL/L vitamins.

MAGUU: YAG with 1.2 g/L uridine and 1.1 g/L uracil

50 X Minimal Media (MM) Urea: 15 g urea, 13 g KCl, 12.5 g MgSO₄, 500 mL water, 1 mL/L trace elements.
**Note:** transformed strains were positive for the nutritional marker *pyrG*, which allows for growth in conditions lacking UU. Untransformed strains remained *pyrG89* mutants lacking the *pyrG* nutritional marker that needed UU to grow. *pyrG* was amplified from the species *A. fumigates*, allowing for transformation into *A. nidulans* but not at the site where it would be found in *A. nidulans*, i.e. transformation occurs and only strains that are deleted for *Aurora* will take up the *pyrG*, and the untransformed strains will remain *pyrG89* mutants and selected against on YAG media (Yang et al. 2004) while *pyrG*\(^+\) transformants are selected for.

### 2.4 *Aspergillus nidulans* Transformation

**Spore Preparation:** Fresh conidia were prepared for transformation by pouring overlays and incubating the plates at 32 °C for 30-42 hours. The spores were then harvested in sterile 0.2 % tween 80. Debris was separated from the mix by differential centrifugation, and the spores were isolated, washed, and quantitated using a hemocytometer.

**Preparation of Transformation Solutions:**

**Solution 1**- 0.8 M ammonium sulfate, 100mM citric acid, [pH 6] with KOH autoclaved and stored at 4 °C.

**Solution 2**- 10 g/L yeast extract, 20 g/L sucrose, and 20 mM magnesium sulfate autoclaved and stored at 4 °C.

**Solution 3**- 0.4 M ammonium sulfate, 1 % sucrose, 50 mM citrate, [pH 6] with KOH. Autoclaved at 10 psi for 15 minutes and stored at 4°C.

**Solution 4**- 25 % PEG 8000, 100 mM calcium chloride, 0.6 M potassium chloride, and 10 mM tris-Cl [pH 7.5] heated to dissolve PEG and filter sterilized; aliquot in 15 mL tubes and stored at -20 °C.
**Solution 5**- 0.6 M potassium chloride, 50 mM calcium chloride, 10 mM MOPS, [pH 6] with KOH, filter-sterilized and stored at 4 °C.

**Generating Protoplasts**: In order for DNA mediated transformation to occur, the cell wall must be degraded and the fungus made into a protoplast so that the cell can uptake foreign DNA. To do this, 1 x 10⁹ spores are inoculated in YGUU and placed in a 32 °C air shaker. The spores were shaken at 250 rpm until they start to germinate, which is after 5.5-6 hours. The mix is then centrifuged and the cells are isolated then resuspended in lytic solution. Lytic solution is composed of 25 mL solution 1, 25 mL solution 2, 100 mgs BSA, 500 mgs vinoflow FCE, and 50 mgs driselase all mixed at room temperature, spun in a bench top centrifuge, and the supernatant, filter-sterilized into a sterile flask. Spores were added and the flask placed at 32 °C for 2-2.5 hours. The sample was pipetted up and down every 30 minutes to prevent clumping of germinating spores. The process is complete when, under the microscope, the majority of cells have formed protoplasts which display a distinctive vacuole by phase contrast microscopy. The sample is centrifuged and the pellet is washed in solution 3 and resuspended in solution 5, and kept on ice overnight.

**Transformation**: 100 µL of protoplast and 4 µg of DNA are added to eppendorf tubes and kept on ice for 10 min. Solution 4 at room temperature is added to the mix and after 20 min the samples are ready to plate. Typically aliquotes of 10 µL, 50 µL, 100 µL, and 500 µL are plated and the DNA/Protoplast/Solution 4 mix is added to top agar of YAG + 1 M sucrose at 47 °C, and poured onto YAG + 1 M sucrose plates. Subsequent growth on YAG will select for transformed \([\text{pyrG}^+]\) strains because unlike the untransformed
pyrG89 mutants lacking pyrG nutritional marker, the transformed strains do not need UU supplemented in the media.

2.5 Heterokaryon Test

An essential gene is required for the growth of an organism. To see whether or not a gene is essential in Aspergillus nidulans, a heterokaryon test can be conducted. This is typically done following the deletion of the essential gene by transformation. The transformed protoplasts will typically have the target gene, gene X, deleted and in its place a nutritional marker like pyrG that will allow for growth on media without uridine and uracil (UU) supplements, in effect allowing for the selection of transformed strains. If the deleted gene is essential, then the organism will rescue viability via selection for heterokaryons with two distinct nuclei in the common cytoplasm. This allows for both the nutritional marker and the essential gene to be expressed in the organism and growth on selective media lacking UU. Following the transformation then, there exists two types of cells, wild-type [pyrG\(^{-}\)/gene X\(^{+}\)] (white nuclei in Figure 4) and the transformed strain [pyrG\(^{+}\)/gene X\(^{-}\)] (black nuclei in Figure 4).

The critical aspect of the test occurs following sporulation of the fungus, during which conidia (uninucleate spores) are generated. When these spores are streaked onto pyrG\(^{+}\) selective media like YAG, essentiality of gene X can be determined: if it is essential, no growth is seen because the black nuclei lack the essential gene, and the white nuclei, although possessing the essential gene, lack the nutritional marker that would be necessary for them to survive on the YAG pyrG\(^{+}\) selective media. On non-selective media like YAGUU however, growth will be observed, since the white nuclei have gene X and nutrition is supplied in the media (Osmani et al. 2006).
2.6 Green Fluorescent Protein

Shimomura *et.al.* first isolated Green Fluorescent Protein (GFP) in the 1960’s from jellyfish.

When illuminated with 488 nm light, this protein emits light at ~ 508 nm wavelength, which corresponds to that of green light (Tsien, 1998). At ~ 0.7 kb, this gene can be inserted in-frame into a genome at the 3’ end of a target gene before the stop codon (Yang, *et. al.* 2004). This

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Figure 4-Steps a-e illustrate the heterokaryon test in *Aspergillus nidulans*. Osmani, et al., Nature Protocols, 2006.
allows for the target gene expression to be visualized by GFP and thus its localization
determined during the cell cycle. This process is also known as “tagging” the gene. GFP can be
fused to the *pyrG* nutritional marker, to allow for selection of the GFP-pyrG construct tagging
the target gene on YAG selective media.

2.7 Confocal Microscopy

A gene tagged with GFP can be visualized in live cells time via confocal microscopy. In
the described experiments, imaging was done with an Orca-ER camera (Hamamatsu) configured
on a TE2000-U inverted microscope (Nikon) linked to an Ultraview controlled ERS spinning
disk confocal system (Perkin Elmer). Z-stack series of images were collected in real time and the
image produced had high contrast and little glare (Dunn, *et. al*. 2008).

For live cell imaging, spores were inoculated in a minimal media (MM) glucose urea
with 1 ml/L vitamins without riboflavin, 6.4 g/L arginine 1.2 ml 1 M PO₄ [pH 6.5] and 500 µL
sodium thiosulfate/100 mL. Following autoclaving, stock 1 g/5 ml sodium thiosulfate/5 ml H₂O
was used after filter sterilization.

2.8 Western Blot Analysis

Also known as immunoblotting, western blotting involves the identification of target
proteins via anti-body binding to a specific protein. For protein preparation, mycelia were
harvested, lyophilized, and proteins extracted with 2x SDS-PAGE sample-buffer containing urea
(0.625 M TRIS [pH 6.8], β-mercaptoethanol, glycerol, water, bromophenol blue and sodium
dodecyl sulfate (SDS)), which denatures the protein, breaking all non-covalent bonds and leaving
the protein anionic. Next, for this project, the sample was run on 10 % polyacrylamide gel
electrophoresis (SDS-PAGE) at 30 mAmperes for 4 hours to allow the proteins to migrate to the 
positive end and be separated according to size. Then the proteins are transferred onto a 
nitrocellulose sheet (transferred at 100 mAmps). Non-specific binding sites were then blocked by 
immersion in 5% non-fat dry milk powdered in PBS-T. Washing and binding with primary anti-
body anti-mouse GFP (1:5000) and secondary Horseradish Peroxidase (HRP) labeled antibody 
from mice was followed by enhanced chemiluminescence (ECL) exposure (using a kit and 
according to the manufacturer's instructions) on film for 20 minutes (Strachan and Read 2010).
3.2 GFP-Tagging of Endogenous *Aurora*

We expected that defining the localization of *Aurora* will shed light upon the function of this protein kinase and help define its potential role in mitotic regulation in *A. nidulans*.

A. Generation of *Aurora*-GFP::pyrG construct

The 3’ end and 3’ UTR of *Aurora* were amplified via PCR and fused to form a construct with Green Fluorescent Protein (GFP) and pyrG. Again the nutritional marker was added in order to allow for selection on YAG plates on which untransformed pyrG89 strains cannot grow. By homologous recombination, the *Aurora*-GFP::pyrG construct was inserted into the *A. nidulans* genome, placing GFP in-frame with the C-terminus of endogenous *Aurora*. The generated CDS 799 *Aurora*-GFP was confirmed using diagnostic PCR and western blot before it was used for confocal live cell spinning disc microscopy.

![Diagram of GFP tagging](image)

Figure 10- Endogenous tagging of the *Aspergillus nidulans* Aurora with GFP
Figure 11- Gels show PCR amplified GFP::pyrG and 3’ end segments in comparison to λHindIII and pGEM markers. See Figure 6 for 3’UTR on gel.

Figure 12- To ensure GFP tagging did not cause functional defects, growth of transformed strains was tested (bottom 2 rows) against controls SO451 (1) strain CDS459 (2) and CDS 759 (3).

B. The Aurora kinase locates at the mitotic spindle

GFP tagging allowed for Aurora visualization via time lapse confocal microscopy of live cells in *A. nidulans*. Aurora is only detectable in ~5% of cells at a given time, where it displays a mitotic spindle-like localization as depicted in Figure 13. At 0.0 min and 8.0 min the signal seems to be degraded or dispersed, suggesting that Aurora-GFP expression is limited to mitosis, which is only ~5% of the cell cycle. The signal starts off as a dot, most likely at the spindle pole bodies, and then extends, reminiscent of spindle elongation during mitosis (Figure 13). Here a germling with two nuclei that enter mitosis can be seen as the endogenously GFP-tagged Aurora
locates on the spindles during mitosis and stretches as the spindle does during anaphase of mitosis. Western blot analysis was conducted, using an anti-GFP antibody indicating the expressed Aurora-GFP has the expected band size corresponding to the expected molecular weight. The recipient parent SO451 strain serves as negative control (Figure 14). Diagnostic PCR demonstrates the presence of the corrected targeted Aurora-GFP construct (Figure 15).

Figure 13- Aurora-GFP was only detectable during mitosis when it displayed a spindle-like localization.
Figure 14- Western Blot

Figure 15- Diagnostic PCR
3.1 Deletion of *Aurora*

Aurora deletion will allow for the determination of the essentiality of the gene for *A. nidulans* growth. Whether or not *Aurora* is essential can help determine if there is a specific role for this kinase as a mitotic regulator and give us the potential to determine if this kinase is required for H3 Ser10 phosphorylation.

A. Generation of the deletion construct Δ*Aurora::pyrG*

The 5’ and 3’ untranslated regions (UTR) on either side of the *Aurora* gene were amplified using PCR and gene specific primers as described in section 2.1. Samples were run on a 0.8 % agarose gel and displayed the correct predicted band sizes of 0.95 and 1.0 kb, respectively. These were combined with a previously amplified *pyrG* nutritional marker in a fusion PCR reaction to generate the deletion construct. This entails the amplified fragments being fused to one another because of hybridization between complimentary extended tails of sequence on each fragment (Yang *et al.* 2004) For example, the extended flank of the lagging strand primer for the 5’ UTR piece will be complimentary to the leading strand primer of *pyrG* allowing the two segments to fuse.

Next the fused deletion construct was transformed, via the naturally occurring process of homologous recombination—directed via the homology of the 5’ and 3’ UTR segments with the corresponding regions in the genome—into a *pyrG89* and Δ*Ku70* mutant of *A. nidulans*. *pyrG89* is a mutant in which the organism must have UU supplemented in the media in order for it to grow. Transforming on a Δ*Ku70* background prevents non-homologous recombination end-joining of DNA. Transformed strains are now Δ*Aurora::pyrG*, with *Aurora* deleted and in its place *pyrG* (see Figure 5).
Figure 5-Aurora deletion construct generation. The 5’ and 3’ UTR of the Aurora gene were amplified and fused at either side of the pyrG nutritional marker. This deletion cassette was then introduced into the *A. nidulans* genome after transformation via homologous recombination to delete *Aurora*.

Figure 6- Ethidium bromide stained gels show PCR amplified 5’UTR and 3’ UTR segments in comparison to λHindIII and pGEM markers as well as the fusion PCR final product.
B. Heterokaryon Test and Microscopic Examination of Phenotype

The heterokaryon rescue technique was utilized to determine the essentiality of Aurora and examine the phenotype caused by the *Aurora* null allele. *Aurora* deleted transformants were streaked on YAG and YAGUU plates at 32 °C and incubated for 48 hours. Transformants 1-4 were able to grow on YAGUU but not YAG, suggesting they had formed heterokaryons; formation of a heterokaryon is typically seen when an essential gene is deleted. SO451 [pyrG⁻], which cannot grow on YAG plates, and strain R153 [pyrG⁺] which can, are controls. Spores from a typical heterokaryon caused by deletion of *Aurora* were streaked on YAG and microscopically examined to determine the terminal phenotype caused by deletion of *Aurora*. The results showed that growth was limited such that deletion of *Aurora* generated only small germ tubes (Figure 8), demonstrating that *Aurora* is essential for growth in *A. nidulans*.

C. Diagnostic PCR analysis proves *Aurora* is essential

The results described above suggest that *Aurora* is an essential gene for growth in *A. nidulans*. Once it was deleted, the organism attempted to save itself by forming a heterokaryon, and no growth was seen on the YAG plate, which affirms that although the uninucleate spores from the transformed strains are capable of growing on YAG, (*pyrG⁺*) they lack *Aurora*, and cannot grow without this essential gene. To confirm that the putative heterokaryons contained the deletion construct as well as the wild type allele, following inoculation of candidate heterokaryons in YG, diagnostic PCR of DNA prepared from the heterokaryons (1-4) and a wild type control (SO451) was conducted to confirm the presence of both a wild type *Aurora* allele and a deleted *Aurora* allele in the heterokaryons (Figure 9). These results demonstrate that *Aurora* is an essential gene in *A. nidulans*. 
Figure 7-Heterokaryon Test Plates

Any growth is limited to small germ tubules, the rest remained as ungerminated spores.

Figure 8- Delta Aurora::pyrG

Figure 9-Diagnostic PCR
3.3 NIMA Over-expression in the *Aurora* null strain

It had been suggested that the Aurora kinase is the phosphorylator of histone H3 at Ser-10 (Hsu, *et. al.* 2000), but NIMA can phosphorylate H3 *in vitro* (De Souza, *et. al.* 2000). It was of interest then to study whether induction of NIMA can lead to this phosphorylation in the absence of *Aurora*. If this is the case, it is likely that NIMA actually phosphorylates histone H3 *in vivo*, and that it can do so in the absence of Aurora kinase. This can be tested by over-expressing NIMA-GFP in place of *Aurora* to see if NIMA is sufficient for histone H3 Ser10 phosphorylation, growth and cell division in *A. nidulans*.

A. Generation of Construct

To generate the construct (Figure 16) four-way PCR fusion was used. First, a cassette was generated in which an alcA promoter was fused to nimA-GFP. The promoter allows for inducible expression of exogenous NIMA. This alcA::nimA-GFP was then fused to the 5’ UTR of *Aurora* (gel band size depicted in Figure 17). Next, *pyrG* nutritional marker was fused to the 3’ UTR of *Aurora* (Figure 18). The four-way fusion PCR was then conducted to give the final construct of approximately 7.2 kb (Figure 19). Although the fusion PCR worked, other bands were also present necessitating gel purification of the appropriate band. Following gel purification on 1% low melt agarose gel and DNA extraction using the QIAquick Gel Extraction Kit Protocol (QIAquick Spin Handbook, 23) much DNA was lost, so further amplification of the piece will be needed in order to transform *A. nidulans*. 
Figure 16- Generation of alcA::nimA-GFP construct

Figure 17- Displayed in lane 2 is the fusion product of 5’ UTR and alcA::nimA-GFP segments.
B. Discussion and Future Directions

In conclusion, my results demonstrate that Aurora is essential for growth in *A. nidulans*, and it appears to locate on the mitotic spindle, suggesting its function is during mitosis. Whether the Aurora kinase or NIMA kinase (or even a different kinase downstream) is the actual phosphorylator of H3 at Ser-10 is still unknown, as some evidence exists supporting either kinase as the possible candidate for H3 phosphorylation.
Tests similar to those applied to the *Aurora* deletion project can be conducted on the alcA::nimA-GFP construct once enough is amplified for a transformation. Confocal microscopy can be done to see where the over-expressed NIMA locates in live cells. The inducible promoter can also be utilized so that *Aurora* localization can be visualized before and after NIMA activation, thus testing the hypothesis that NIMA plays a role in transporting *Aurora* into the nucleus so that it can phosphorylate histone H3.

More testing can also be conducted on the Δ*Aurora::pyrG* strains, for example conducting a 4',6-diamidino-2-phenylindole (DAPI) stain to determine if lack of growth of the heterokaryons on YAG was due to a failure in mitosis. DAPI intercalates with DNA to allow visualization of nuclei; if *Aurora* is essential for cell division, a germ tube will be evident with only one fluorescing nucleus, whereas normally *A. nidulans* would undergo polarized growth to produce many nuclei in the germ tube.

Also, an anti-body for histone H3 phosphorylation can be applied to determine if H3 is phosphorylated at Ser10 in *Aurora* null strains. The ultimate goal is to learn exactly how the interplay between NIMA and *Aurora* is linked to phosphorylation of histone H3 at Ser10, which is implicated in chromosome condensation and mitotic initiation. Demonstrating that *Aurora* plays an essential role at the mitotic spindle brings us a step closer to reaching this goal.


Mitotic phosphorylation of histone h3 is governed by ipl/aurora kinase and glc7/pp1 phosphatase in budding yeast and nematodes. *Cell, 102*, 279-291.


Phases of the Eukaryotic Cell.


Polymerase Chain Reaction. Dolan DNA Learning Center.

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