Abstract

Title of Thesis: THE ROLE OF AURORA KINASE IN THE DEVELOPING MALE GAMETOPHYTE OF *MARSILEA VESTITA*

Alisha Barnes, Master of Science, 2018

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The development of the male gametophyte of *Marsilea vestita* occurs via an ordered sequence of cellular events, which result in the formation of 32 motile sperm. The blepharoplast is a subcellular structure that arises *de novo* at ~3.5 hours following microspore hydration and functions as a microtubule organizing center during the final mitotic division. I investigated the roles of aurora kinases in the formation and maturation of the blepharoplast. Three unique aurora kinase isoforms were found in the transcriptome of the male gametophyte, each with a unique N-terminal sequence and expression pattern. RNAi knockdowns of each isoform resulted in different stages of developmental arrest, and the absence of blepharoplasts, thereby suggesting that each isoform has a unique function. Centrin phosphomimics acted to stabilize centrin, enabling centrin aggregates to form. My results suggest that each isoform of aurora kinase plays an important role in male gametophyte development in *Marsilea*.

THE ROLE OF AURORA KINASE IN THE DEVELOPING MALE GAMETOPHYTE OF *MARSILEA VESTITA*

by

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Dedication

This dissertation is dedicated to my family whose help and support was crucial in this process.

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CstF	Cleavage stimulation factor
CPSF	Cleavage and polyadenylation specificity factor
PAP	Polyadenylate polymerase
snRNP	Small nuclear ribonucleoproteins
sIF6	Eukaryotic translation initiation factor 6
PCK	Protein kinase c
RNAi	RNA interference
dsRNA	Double stranded RNA
siRNA	small interfering RNA
ssRNA	single stranded RNA
RISC	RNA-induced silencing complex
MTOC	Microtubule organizing center
PCM	Pericentriolar material
bb	Basal bodies
DAPI	4',6-diamidino-2-phenylindole
mvAK	*Marsilea vestita* aurora kinase

Chapter 1: Introduction

The spatial and temporal expression of certain genes and their protein products is critical for regulating the cellular activities responsible for transforming a single cell into a differentiated multicellular organism. All multicellular organisms that reproduce sexually proceed through similar steps, including the growth of the embryo and subsequent juvenile stages, the formation of sex organs and the process of reduction division in certain cells, and the syngamy of the sex cells to form the zygotes giving rise to the next generation Plants are unique due to the alternation of generations that creates two distinct multicellular stages in their life cycle (Hofmeister, 1859). In animals, when the diploid germ-line cells undergo meiosis, the resulting haploid cells are directly transformed into haploid gametes, and thus, do not develop an intervening multicellular haploid organism. The alternation of generations in plants begins with two single cell gametes, a sperm and an egg, that are fertilized to form the zygote that develops via mitosis into a diploid sporophyte. The diploid sporophyte produces haploid spores through meiosis. These spores then develop into multicellular haploid gametophytes that produce the male and female gametes via mitosis. The fusion of these gametes completes the life cycle. While all plants exhibit alternation of generations, the sporophyte and gametophytes can vary drastically in different species. Major determinants in this variation are in the modes of gamete dispersion and the adaptations to the environments in which the plants live (Bateman and Dimichele, 1994; Bell and Hemsley, 2000).

The development of a single-celled zygote into a differentiated sporophyte is dictated by the genome of the organism, which executes the genomic instructions via a multifaceted array of gene regulations. This complex regulation allows for spatial and

temporal regulation throughout the organism and ultimately allows for the cells with identical DNA content to differentiate into the different cell types expressed in the plant. Each cell type is differentiated through the expression of different genes. The expressed genes result in altered patterns of transcription and translation, and often regulate the production of intercellular signaling molecules including hormones and ion gradients. These molecules spread through the developing plant ultimately establishing both the root and shoot apical meristems. These meristems establish the primary axis of elongation growth, which will further dictate the positioning and subsequent development of lateral organs. These broad patterns of organismal development are ultimately controlled by the regulation of gene expression in the dividing meristematic cells, which are also influenced by the interactions with differentiated cells surrounding the meristems (Hay and Tsiantis, 2010; Huijser and Schmid, 2011).

Gene expression is controlled before, during, and after the synthesis of the protein for which the gene codes. Initially, the production of the mRNA, the template for the protein, is controlled through the regulation of initiation through the binding of transcription factors, either promoters or repressors of transcription. The access to the DNA is controlled by the histones around which the DNA is wrapped. If the DNA is tightly wrapped around the histones due to the interaction between the histone tails and DNA, the transcription proteins are not able to access the DNA, preventing transcription. Once the pre-mRNA is created, the amount of translation of the mRNA can be controlled through the stability, distribution, and splicing of mRNA, and the initiation of translation at the ribosome. Finally, the protein is regulated through post-translational regulation, including methylation and phosphorylation.

Gene Regulation

Gene regulation allows for differential gene expression in cells. This process allows for the expression of different genes in different cell types and, in addition, allows for the regulation of gene expression at different stages of development at the cellular level. This is essential because different distinct processes occur at different stages of development, and almost all cells within an individual organism have identical genetic material. Differential regulation allows for all cells in an organism to have one set of genes in all the cells, but each cell can potentially be unique due to the regulation of its gene expression. The regulation of gene expression also allows for resource conservation by conserving metabolites and energy.

Transcriptional Gene Regulation

The many mechanisms used to regulate gene expression during transcription and translation include both post-transcriptional and post-translational regulation. Transcriptional regulation allows for the production of mRNA intermittently depending on cellular signals. As the first step of regulation, this is the most important and common point of regulation for most genes. Translation of mRNA is regulated primarily during the initiation of translation at the ribosome (Gilbert, 2006; Jacob, 1961). The mRNA then can be modified or stored for later use depending on the regulators present.

Regulation at the initiation of transcription allows for the production of mRNA and for the subsequent protein to be regulated by the environment both inside and outside of the cell. Genes can be regulated temporally by controlling the number of mRNA

transcripts that are made at a given time. For example, Enhancers are 50- 200 base pair regions found upstream of the promoter, and they serve as a binding site, or a control element, for several different transcription factors. The assembly of the transcription complex occurs at the promoter, and ultimately controls the initiation. Different transcription factors can bind far upstream from the promoter. The bound DNA then can loop towards the promoter so the bound transcriptional factor can interact as the moderator, another component of the transcriptional complex. The moderator initially serves as a hub for the binding of transcription factors, which is in turn bound to the proteins at the promoter sites. Using this system, many different transcription factors, each with different environmental inputs and purposes, can interact with the complex at the promoter and influence the initiation of transcription (Gagniuc, 2013; Lodish, *et al*., 2000).

Transcriptional activators bind upstream of the gene of interest and interact with additional transcriptional factors to promote the transcription of the gene. These activators facilitate the binding of RNA polymerase and other transcription factors that affect rates and extents of transcription. The gene remains active until the activator is either removed or replaced, this usually occurs when environmental factors change. Transcriptional repressors act similarly, except instead of activating transcription, they prevent the initiation of transcription. Interestingly, the same protein can act as both a repressor and activator. If the protein binds partially over the promoter, the assembly of the transcription initiation complex, including RNA polymerase and other transcription factors essential to initiation, will be inhibited and transcription will not occur. The same protein can then bind further upstream, leaving the promoter accessible, and aid in the

recruitment of transcription factors and initiation. Activators affect initiation in many different ways. They not only can attract transcription factor, but also determine the position and create modifications to transcription factors and initiation complex. All of these pathways culminate in the initiation of RNA transcription using the RNA polymerase in response to the needs of the cell (Busby, 1999; Ptashne, 1997).

Chromatin is packaged into a highly organized and condensed structure. This allows for a large amount of DNA to be stored within the small volume of the nucleus and remain accessible to regulatory proteins. The basic unit of this packaging is the nucleosome. The nucleosome is made up of DNA wound around an eight-histone protein core called an octamer. The nucleosomes then continue along the DNA, much like bobbins or beads connected by linking DNA. The histones have tails that hold onto the DNA that is wrapped around the core. The nucleosomes are then folded repeatedly to condense the chromatin, while allowing access to the DNA inside. The access of the DNA plays a major role in regulating RNA transcription. If the DNA is not easily accessible by the various proteins needed for transcription this can serve as a repressor to RNA transcription without needing to have the protein bound directly to the DNA (Kornberg, 1974).

To make the DNA accessible for transcription, post-translational modifications are made to lysine in the histone N-terminal end of the histone tail. Acetylation of histones causes the interaction of the histone core and DNA to decrease due to the addition of the negative charged acetyl group from the histone acetyltransferase. This allows the DNA to be more open and accessible to transcription factors. This action, however, is reversible through the use of histone deacetylases, which remove the added

acetyl groups. When the acetyl groups are removed, the DNA once again becomes tightly associated with the histone, primarily due to the increased positive charge of the histones (Eberharter, 2002; Imhof, 1997).

Histone methylation is another method used to regulate gene expression. The effect of methylation depends on the particular amino acid residue in the particular histone that are methylated. For example, when histone 3 is methylated at lysine 9, the chromatin condenses, and subsequently, the DNA is more tightly bound. This can culminate to form heterochromatin, which contains silenced genes. Conversely, if histone 3 is methylated at arginine 2, it will act as an activator. Chromatin is bound less tightly by the histones, which allows for easier access of the transcription factors. In addition to the physical changes to the nucleosome, the pattern of covalent modifications can also act as a positive signal for regulatory proteins (Zhang, 2001).

The transcriptional regulation in *M. vestita* occurs before the desiccation of the sporocarp. A large number of pre-mRNAs are formed and stored before desiccation occurs, along with some proteins. The chromatin is silenced during desiccation, and remains so during development. Once the microspore is hydrated, a cascade of events is set in motion that controls the programmed development of the male gametophyte. Spermidine is a major regulator for development, as its controls many different developmental processes. The stored and masked pre-mRNAs are masked are unmasked, processed and trafficked to the cytoplasm in a sequential manner that controls each step in gametophyte development (Wolniak *et al.*, 2011, 2015).

Post-transcriptional Gene Regulation

Gene expression is further regulated at the mRNA level through post-transcriptional regulation that control the stability and the distribution of mRNA. Because mRNA stability depends on the presence of the 5' cap and poly-adenosine tail, these sites are the primary targets for regulation. The 5' cap consists of a modified guanine that is added immediately after the first nucleotide emerges from the RNA polymerase II complex. Three enzymes work in sequence to attach the cap: A phosphatase removes the phosphate from the 5' nucleotide; a guanylyltransferase then adds a guanosine monophosphate in 5' to 5' direction; and finally, a methyltransferase adds a methyl group to the guanosine. The cap also performs several critical roles in the life of the transcript, including distinguishing the transcript from other RNA molecules in the cell. Only transcripts from RNA polymerase II are capped during transcription. The cap also acts as a regulator of nuclear transport and ensures the integrity of the transcript by preventing degradation by exonucleases (Visa *et al*., 1996). Once the properly capped mRNA exits the nucleus, the 5' cap interacts with eIF4 translation initiation factors. Other components of the translation machinery, most importantly the ribosomal subunits, recognize these newly bound initiation factors, which enables translation to proceed (Mangus, 2003; von der Haar, 2004). Transcription is terminated when a signal sequence is transcribed in the 3' untranslated region of the mRNA. Two protein complexes, cleavage stimulation factor (CstF) and cleavage and polyadenylation specificity factor (CPSF), recognize the signal sequence on the pre-mRNA and initiates the polyadenylation of the 3' end, which creates the poly(A)tail. The binding of CstF and

CPSF to the 3' untranslated region recruits other required proteins to assemble at the 3' end of the transcript. The newly synthesized pre-mRNA is cleaved, and polyadenylate polymerase (PAP) adds approximately 200 adenosine nucleotides 5'- 3' to the new 3' end of the pre-mRNA with the aid of polyadenylate binding protein 2. This binding protein increases the binding affinity of PAP to the mRNA strand and does not require a template like many other polymerases do (Humphreys, 2005; Revel 1978).

The transcribed RNA sequence contains many more nucleotides than are needed to translate the finished protein. The pre-mRNA is therefore edited to only include the exons. This is achieved through splicing which is carried out concurrently with transcription. Some mature mRNAs retain introns. This is due to alternate splicing where different combinations of multiple introns and exons as a single unit, creating several different transcripts in addition to the originally conventionally spliced product. This allows for multiple proteins to be made from the same gene, each having a unique function. This enables the cell to further conserve resources (Clancy, 2008; Roy, 2006).

Splicing is a series of snRNP- pre-mRNA interactions that involves a series of base-pairings where the subsequent step disrupts the previous base pairing, allowing a new pairing to be made by forcing a rearrangement of the pre-mRNA. Each step in splicing the pre-RNA uses a unique set of proteins, and these proteins are exchanged freely throughout the entirety of the splicing. A spliceosome comprised of five small nuclear ribonucleoproteins (snRNP) controls pre-mRNA splicing. Once the snRNP has completed its role in the spliceosome, it falls away from the complex. It can take as many as 200 individual proteins to complete one step. This complexity helps to ensure accuracy by preventing indiscriminant bonding and reactions, since checks are made at

every rearrangement to ensure correct splicing. The dynamic nature of this complex also allows for flexibility, which enables alternate splicing (Staley, 1998).

A complex series of reactions and rearrangements results in the removal of the intron, or multiple introns, a process that is strongly conserved in eukaryotes.

All introns contain a 5' donor site, a branch site close to the 3' end, and a 3' acceptor site, each has a consensus sequence which is recognized by the specific snRNA contained in the appropriate snRNP. To initiate splicing, the 5' and 3' ends of the intron must be brought together. Two snRNPs initially are bound to the donor and acceptor sites on either end of the intron. Two more snRNPs enter and bring the 5' and 3' ends together, forming an open loop. Using RNA-RNA binding, rearrangements are made in the spliceosome. With the addition of additional snRNPs, these rearrangements result in the formation of the active site of the spliceosome. The first cleavage is made on the 5' end of the intron. This newly created free end of the intron is brought to the 3' end of the intron and is held by a snRNP to create a lariat. Additional rearrangements are made resulting in the cleavage of the 5' end of the intron. The dangling exon terminus which was created is held by the spliceosome to increase the efficiency of splicing. Another cut is made at the 3' splice site releasing the lariat, which causes the spliced intron to be released from the mRNA strand. The two free ends of the exons remaining are brought together and ligated. This mature transcript exits the nucleus and moves into the cytosol (Burge, 1996; Nilsen, 1996; Staley, 1998).

Translational Gene Regulation

Once the transcript exits the nucleus, the mRNA is translated either by ribosomes on the rough endoplasmic reticulum or by free ribosomes in the cytoplasm. The first step of translation is the recognition of the initiation codon by the 40S ribosomal subunit. Then the 60S ribosomal subunit binds to the 40s subunit to form the assembled 80S ribosome (Klinge *et al.*, 2011; Rabl *et al.,* 2011). The ribosome complex then travels the length of the mRNA sequence converting the codons found in the mRNA to a sequence of amino acids constituting the polypeptide. The codons of the mRNA sequence are paired with the matching amino acids, which are delivered to the ribosome by corresponding aminoacyl-tRNAs. The ribosome catalyzes the formation of peptide bonds to link the delivered amino acids, resulting in the emergence of the newly formed polypeptide from the 80S ribosome. As the polypeptide emerges, it begins to fold into its final 3-D conformation. Once the termination sequence is reached on the mRNA, the completed functional protein is released and the two subunits fall off the mRNA (Wilson, 2012). These subunits will be recycled to translate other mRNA sequences.

Translation initiation is regulated through the ability to form the complete 80S ribosome. Normally the 60S subunit is bound by eukaryotic translation initiation factor 6 (eIF6). When eIF6 is bound to the ribosomal subunit, the 60S subunit is not able to bind the 40S subunit unless the eIF6 is phosphorylated, which is done by a protein kinase c (PCK) bound to the 40S subunit by the RACK1, a receptor for activated kinase C 1. Once eIF6 is phosphorylated, it is released from the ribosomal subunit and the 2 subunits are able to form the 80s ribosome (Ceci *et al.*, 2003; Gartmann *et al.*, 2010; Miluzio *et al.*, 2009). RACK1 is a signaling protein that can recruit a variety of proteins including PCK

in response to various signaling molecules (Adams *et al.*, 2011). The total amount of protein translated in the cell at a given time can be regulated through this pathway.

Translation can also be regulated by RNA interference (RNAi), which results in the targeted degradation of mRNA transcripts. By degrading specific transcripts, genes can be suppressed after transcription has occurred. RNAi is initiated by the cleavage of double stranded RNA (dsRNA) by dicer, which produces small interfering RNAs (siRNA). These siRNA are then unwound and separated to produce single stranded RNAs (ssRNA), including both a passenger strand and guide strand (Bagasra *et al.*, 2004; Fire, 1999). The passenger strand is degraded while the guide strand is incorporated into the RNA-induced silencing complex (RISC). RISC then finds and binds to the mRNA sequences complementary to the guide sequence. The catalytic portion of RISC called argonaute 2 cleaves the mRNA. The resulting fragments are degraded preventing translation (Hutvagner, 2008).

Post-translational Gene Regulation

After the protein is produced, kinases and phosphatases work together to regulate the protein's function in response to the needs of the cell. Kinases respond to environmental and endogenous signals by catalyzing phosphorylation of the protein. This flexible mechanism can be used to activate, deactivate, or alter the protein's function through conformational changes in the protein's 3-D structure that can be reversed by phosphatases (Cohen, 2000; Day *et al.*, 2016).

Glycosylation, which occurs in the endoplasmic reticulum and Golgi body, is another form of post translational regulation. Glycosylation covalently attaches

carbohydrates to specific residues of proteins and influences folding of certain proteins and stabilization of others. In the endoplasmic reticulum, glycosylation serves as a marker for proper folding to ensure that only properly folded proteins are trafficked to the golgi body. The carbohydrates attached to soluble proteins can be bound by receptors in the trans-golgi network to aid in proper delivery of the protein (Dalziel *et al.*, 2014).

Another form of post-translational modification, methylation, is a reversible process mediated by methyltransferases that results the addition of a methyl group at specific amino acids in the protein. It has a wide variety of effects depending on the protein and the amino acid that is being methylated and an individual amino acid can be methylated several times. The degree of methylation has a direct effect on protein-protein interactions, trafficking, and signaling (Biggar and Li, 2015; McBride and Silver, 2001). Methylation of non-histone proteins is commonly found in cell signaling transductor mediated by signaling pathways including MAPK, WNT, BMP, Hippo and JAK-STAT pathways. This is achieved through crosstalk with other post-transcriptional modifications to regulate transcription, translation, chromatin remodeling and many other cellular processes (Biggar and Li, 2015).

Although most forms of post-translational modification are reversible, proteolysis is an example of an irreversible form of post translational regulation. Proteolysis breaks down peptide bonds by using proteases. Another pathway to degrade proteins is through ubiquitination. When proteins are tagged covalently with ubiquitin molecules, they are targeted to the proteasome. The proteasome is an ATP-dependent protease complex that breaks down proteins and releases the previously bound ubiquitin molecules (Ciechanover, 2005; Glickman, 2002). Some proteins even need to be further processed

to remove unneeded portions leaving the rest of the protein intact. This occurs with the removal of N-terminal targeting signals that tag proteins to be trafficked to specific organelles (Creighton, 1993). The vast and varied array of gene regulation methods creates an exquisitely sensitive and varied web of interconnected processes that controls all the functions of the cell in response to the needs of the cell.

The study of gene regulation requires a high degree of precision and accuracy because the expression of individual genes requires the interplay of many physiological and environmental interactions. These interactions form a large signaling network with very complex connectivity. An additional layer of complexity is introduced because many genes are temporally and spatially controlled. Therefore, to study gene interactions, a synchronous population of cells is needed that will remove the differences in cell cycle stages and the degree of differentiation in a population of cells. To study individual genes, each gene and its connected genes needs to be isolated; otherwise, there is no way to know, with certainty, if the effects observed can reasonably be attributed to the gene of interest.

Microtubule Organizing Center

One of the critical regulation points in development is the regulation of the proteins involved in mitosis. To direct this process, a large diverse complex of proteins works together to ensure the proper partitioning of the DNA. These proteins ensure the precise and accurate division of the cell contents and DNA. A major regulator and organizer of division is the microtubule organizing center (MTOC), which is a recruiter and organizer of many mitotic proteins, and also serves an anchor for the mitotic spindle.

The MTOC serves as the nucleation point for the microtubules and, in metazoans, is a centrosome, a membrane-less organelle that contains two centrioles surrounded by pericentriolar material (PCM). Plant cells have a centrosome that includes the equivalent of the PCM, but no centrioles. The PCM is a mix of structural and regulatory proteins that nucleate microtubules (Salisbury *et al.*, 2004). As the cell exits interphase into prophase, different proteins are recruited to the centrosome. Some of these proteins play structural roles to increase the size of the centrosome, while others interact with proteins in the centrosome to alter their activity. The final product is a bipolar spindle that arches across the cell and is anchored by centrioles inside a large complex centrosome at each end. The chromosomes attach to the spindle, which aligns and guides the chromosomes to each pole. This ensures equal separation of the chromatids resulting in a complete set of chromosomes in each daughter cell (Mitchison and Salmon, 2001).

Centrin

Centrin, an acidic calcium-binding phosphoprotein, is a crucial centrosomal component that is found in all eukaryotic cells and is both an essential structural component of the PCM (Salisbury, 2004) and crucial for the function of the centrosome (Salisbury, 2007; Hartman and Fedorov, 2002). It is involved in the centrosome reproduction cycle and the coordination of nuclear and cytoplasmic division (Lutz *et al*., 2001; Paoletti *et al.*, 1996). Centrin contains four EF-hand domains, helix-loop-helix calcium-binding structures; these domains are highly conserved in the centrins from all species studied to date (Weber *et al.*, 1994; Veeraraghavan *et al.*, 2002; Craig *et al.*, 2006; Thompson *et al.*, 2006).

Centrin has been shown to localize to MTOCs including centrosomes, yeast spindle pole bodies and basal bodies (bb). In *Chlamydomonas reinhardtii,* centrin forms the Ca^{2+}-modulated contractile fibers that are necessary for the proper segregation of the flagellar apparatus during cell division. Furthermore, centrin isoforms have also been detected within the precursors of centrioles and bb, indicating that they play a possible role in these structures (Laoukill *et al.*, 2000). Centrin plays a critical role in the correct segregation of the centrioles during cell division. When centrin is phosphorylated during prophase, it aids in the separation of the pair of centrioles preceding centrosome duplication (Lutz *et al*., 2001).

Phosphorylation of centrin is also involved in several centrosome functions, including the duplication, maturation, and separation of the centrioles, microtubule nucleation, and the specification of cleavage furrow formation (Lutz *et al*., 2001). In laser ablation studies of centrioles, centrin has been implicated in controlling the switch between the semi-conservative and *de novo* pathways for centriole assembly (Marshall *et al.*, 2001; Khodjakov *et al.*, 2002; Marshall, 2002; La Terra *et al.*, 2005). When centrin is knocked down, it causes a loss of centrioles indicating its essential involvement in centriole duplication. Centrin has been shown to form complexes with Sfi1p to create a network of calcium sensitive fibers within the PCM of the centrosome. This allows elastic connections between different elements of the centrosome, allowing for morphological changes in the centrosome (Salisbury, 2004).

Aurora kinases

Aurora kinases are serine/threonine kinases found in all eukaryotes (Brown *et al.*, 2004) that serve as key regulators of cellular division, by ensuring the accurate progression through mitosis (Nigg, 2001; Taylor and Peters, 2008). Three isoforms of aurora kinases are found in vertebrates: aurora A, B, and C. All three are unique in their localization and protein interactions. Aurora A localizes to the centrosome and helps to recruit PCM components (Carmena and Earnshaw, 2003); it is bound by Cep192 and targeted, along with Plk1, in a pericentrin-dependent manner to the centrosome. Cep192 then helps to promote the activation of both kinases (Joukov *et al.*, 2014). The recruitment of aurora A to the centrosome is critical for mitosis and is highly conserved (Kress *et al.*, 2013). It regulates centrosome maturation, spindle assembly, and mitotic entry (Barr and Gergley, 2007; Marumoto *et al.*, 2002; Macurek *et al.*, 2008; Seki *et al.*, 2008). It also has in integral role in the centrosome's structure and duplication in addition to establishing the bipolar spindle assembly (Dutertre *et al.*, 2002). The localization of aurora A is cell cycle dependent. It only localizes to centrosomes that have duplicated, from the end of S phase to the beginning of the G1 phase of the next cell cycle (Dutertre *et al.*, 2002). Kinase activity is not necessary for the centrosomal localization. Kinase dead mutants still locate to the centrosome (Giet and Prigent, 1998). Aurora A is also necessary for the formation of a bipolar spindle and the proper alignment of chromosomes (Glover *et al*., 1995; Hégarat *et al.*, 2011). There is also some evidence that aurora A plays a role in the targeting and anchoring of γ-tubulin in the PCM. This is achieved through the binding of aurora A to centrosomin which in turn

binds γ-tubulin at microtubule-nucleating sites (Terada *et al.*, 2003). The kinase activity serves to promote the regulation of the function and assembly of centrosomal proteins through phosphorylation (Giet *et al*., 2002).

Aurora A stabilizes centrin through the phosphorylation of serine 170. The stabilization then allows centrin to be colocalized to the centrosome with aurora A. During prophase compared to interphase cells, there is a large increase in centrin at the spindle poles that coincides with an increase in aurora A localization at the poles (Fig 1-1a and b). This is not simply a colocalization, but the apparent formation of a physical complex linking aurora A and centrin. Phosphorylated centrin is more stable than unphosphorylated centrin because it cannot interact with the APC/C protein degradation complex. When phospho-centrin is over expressed, it accumulates at the centrosome and remains there. When non-phosphorylatable centrin is expressed, there is no localization at the centrosome, the results where the same when a truncated mutant was expressed. This indicates that aurora A is transiently phosphorylating centrin during G_2 and metaphase, and then centrin is dephosphorylated at the metaphase-anaphase transition point. This dephosphorylation leads to the disassociation of centrin from the PCM, thus allowing for the separation of the centrioles (Lukasiewicz *et al.*, 2011).

Aurora B is a member of the chromosomal passenger complex (Fig 1-1c), which aids in the proper alignment of the chromosomes on the spindle from prophase to metaphase, and regulates the assembly of the kinetochore (Kunitoku *et al.*, 2003). It has a role in sister chromatid cohesion, spindle disassembly, cytokinesis, and regulation of the synaptonemal complex during meiosis (Hochegger *et al.*, 2013).

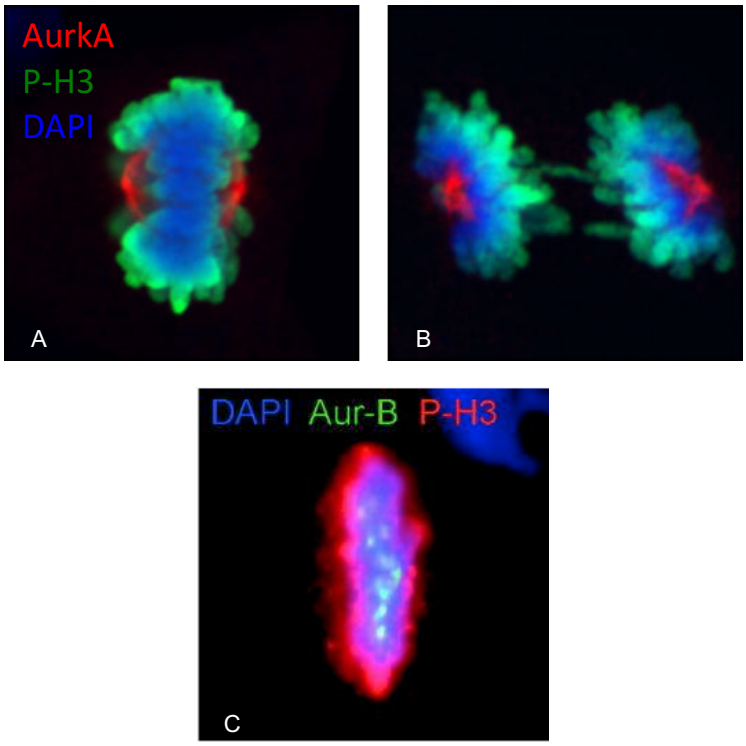


Figure 1-1. Localization of Aurora A and B during mitosis.
Aurora A localizes to the centrosome throughout mitosis **(A,B)**. In contrast, Aurora B localizes to the kinetochores of the centrosomes as a chromosomal passenger protein **(C)**. (*A, B- Cell Signaling Technology; C- Hauf, et al., 2003)*

Early in mitosis, aurora B appears as punctate staining on the centromeres, and regulates the formation and cohesion of the kinetochores (Kaitna *et al.*, 2002; Murata-Hori *et al.,* 2002). During anaphase, it detaches and remains at the metaphase plate to regulate cytokinesis (Ruchaud *et al.,* 2007; Vader *et al.*, 2006). Phosphorylation of kinetochore proteins ensures the correct attachment to the spindle in response to tension and attachment states of the kinetochore (Welburn *et al.*, 2010; Chan *et al.*, 2012). Aurora B has in integral role in the spindle assembly checkpoint in that its error correcting role ensures the proper separation of chromatids. It also has been proposed that aurora B could play a direct role in the spindle assembly checkpoint (Nezi and Musacchio, 2009). In some organisms, a single aurora performs the same functions as aurora A and B (Abe *et al.*, 2010). Aurora A and B have been shown to have a combined role in the depolymerization of the spindle in anaphase and the correct segregation of the chromosomes (Hégarat *et al.*, 2011).

Aurora C is found only in mammalian testes (Carmena and Earnshaw, 2003), is a chromosomal passenger protein, and functions in chromosome alignment and segregation, microtubule stabilization, and spindle assembly. Many of its functions overlap with those of aurora B. However, aurora C is unique in that it is critical in the formation of gametes during meiosis (Avo Santos *et al.*, 2011).

Three aurora isoforms are also found in plants. In *Arabidopsis thaliana* aurora 1 and 2 have very similar roles as aurora A and B, respectively, ensuring the correct assembly of the spindle and cytokinesis (Demidov *et al.*, 2005; Kawabe *et al.* 2005). Aurora 1 and 2 locate to the nuclear membrane during interphase and then relocate to preprophase band, phragmoplast, and the nascent cell plate during mitosis (Fig 1-2a)

(Kawabe *et al.*, 2005). During cell division, they associate with the spindle poles and then dissociate and relocalize to the midzone (Fig 1-2b-e). Finally, they concentrate at the midline of the phragmoplast along the cell plate (Fig 1-2f) (Demidov *et al.*, 2005). Live cell imaging has shown that auroras regulate the attachment of microtubules to the kinetochore during the alignment of the chromosomes. They are also critical in cohesion dissociation during chromosome segregation. These studies were performed by using the drug hesperadin, which is an aurora kinase inhibitor that targets aurora B in human cells, and atAK3 in *Arabadopsis* (Kurihara *ae al.*, 2007, 2008; Aliagas-Martin *et al.*, 2009).

Aurora 3 localizes to *A. thaliana* centromeres early in mitosis and then becomes ubiquitously distributed in the chromosomes after metaphase, whereas aurora B, in vertebrates, remains at the metaphase plate to regulate cytokinesis (Fig 1-2g-l) (Schumacher *et al.*, 1998; Adams *et al*., 2000; Kaitna *et al.*, 2000). As with aurora B, aurora 3 is involved in the alignment of the chromosomes on the plant spindle and plays a role during cytokinesis (Demidov *et al.*, 2005; Kawabe *et al.* 2005). When looked at more closely, the localization of plant and animal auroras are similar.

Plants lack a centrosome with centrioles as a MTOC, but instead have MTOCs in the nuclear membrane during interphase (Staiger and Lloyd, 1991). The evidence available shows that AtAUR 1 and 2 colocalizing with γ-Tubulin and acting at the MTOCs of *Arabidopsis* (Drykova *et al.*, 2003). AtAur3 plays a major role in the phosphorylation of serine 10 of histone H3 (Kurihara *et al.*, 2007). The phosphorylation of H3 is thought to be one of the critical events at the onset of mitosis. This phosphorylation occurs early in G2 and coincides with chromatid condensation, whereas aurora A and B serve this function in animal cells (Crosio *et al.*, 2002).

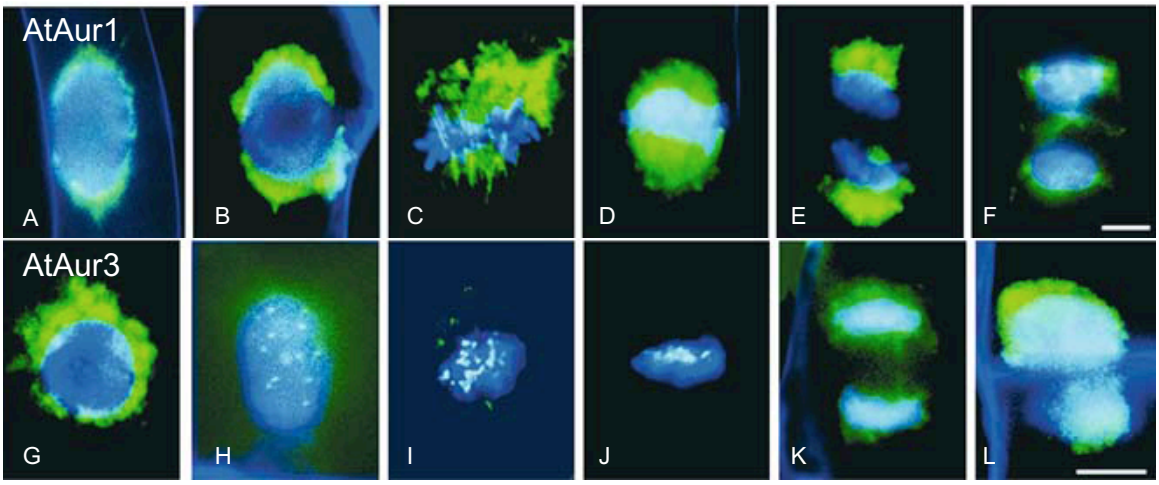


Figure 1-2. Localization of Aurora kinase in *Arabidopsis thaliana.*
During mitosis, AtAur1 localizes to the spindle poles (**A-E)** and finally relocalizes to the midzone **(F)**. AtAur1 and 2 show very similar localizations. AtAur3 colocalizes with the condensed chromosomes **(H,I)**, finally aligning at the metaphase plate **(J).** AtAur3 then is distributed along the length of the chromosomes at anaphase **(K).** During interphase, both 1 and 3 are localized to the cytoplasm and nuclear membrane **(A,G)**. Bar = 10μm. (*Kawabe, et al., 2005*

Plant and non-plant aurora kinases all share a high degree of amino acid sequence similarity, over approximately 60%, particularly in the kinase domains (Carmena and Earnshaw, 2003; Demidov *et al.*, 2005). The unique 5' amino ends that can vary greatly in length and composition allows for the different localizations and functions of the different isoforms. The conserved catalytic domain contains an activation loop and a destruction loop that are both very highly conserved among different species (Carmena and Earnshaw, 2003; Demidov *et al.*, 2005; Kawabe *et al.* 2005). Auroras are activated by autophosphorylation of a threonine residue within the activation loop of the catalytic domain (Walter *et al.*, 2000).

Marsilea vestita as an experimental system

Marsilea vestita is a small heterosporous fern that grows in vernal ponds and other semi-aquatic habitats. The sporophyte stage of *M. vestita* consist of a horizontal rhizome bearing leaves resembling four-leaf clovers (Fig 1-3a). This stage forms reproductive structures called sporocarps that are modified leaves. Each sporocarp (Fig 1- 3b) contains clustered sporangia, containing the male microspores and female megaspores. A desiccated sporocarp resembles a small kidney bean (Fig 1-3b). The sporocarps remain dormant indefinitely while stored in dry conditions. After rehydration, the sporocarp opens to release the mega- and microspores into the water (Fig 1-3c, d). The microspores proceed to develop into male gametophytes forming motile spermatozoids, and the megaspores develop into female gametophytes producing solitary eggs (Laetsch, 1967).

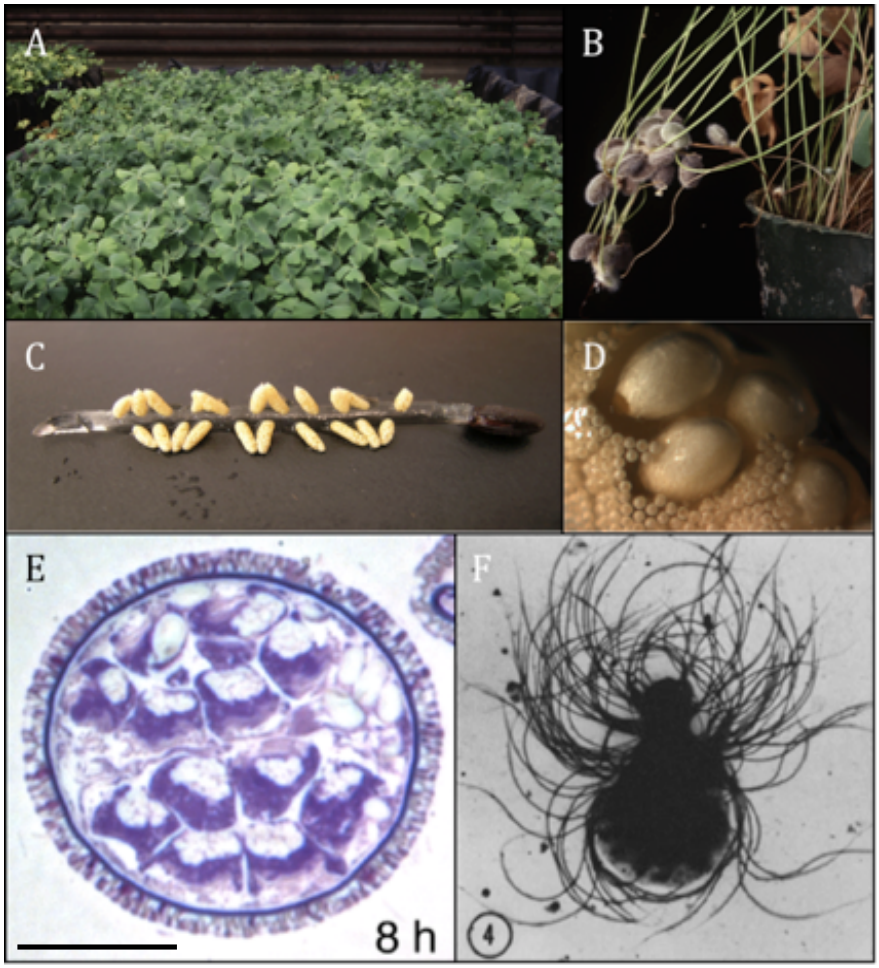


Figure 1-3. The life cycle of *Marsilea vestita*.
The mature sporophyte superficially resembles a four-leaf clover (**A**). The desiccated sporocarp holds and protects the mega and microspores (**B**). When the sporocarp is hydrated, yellow sori emerge, containing the mega and microspores (**C**). The mega and microspores are released. The small microspores surround the larger megaspores (**D**). After 8 hours of development 32 spermatogenous cells are surrounded by sterile jacket cells (**E**). After 11 hours, fully matured spermatozoids are released (**F**). Bar = 25µm. (*C,D and E courtesy of C. van der Weele; F, Mizukami & Gall, 1966*

The male gametophyte of *Marsilea vestita* provides a unique opportunity to study gene regulation in a synchronous cell population. This gametophyte is easy to work with and has a short synchronous development time, thus eliminating many problems that complicate experimental investigations of other systems. *M. vestita* is grown in artificial ponds in the greenhouse complex at the University of Maryland. After rehydrating the sporocarps, the development of the microspores into male gametophytes occurs quite quickly as it takes 11 h at 20°C to complete the maturation process, resulting in 32 helical motile spermatozoids (Sharp, 1914). The timing of the divisions and ultimately the total development time is directly dependent on temperature. The rate of divisions remains synchronous throughout the microspore for the entirety of development (Helper, 1976), which allows for the ability to know the precise timing of different events during male gametophyte development. The microspores can be used directly or fixed after harvesting at a known time point to study specific aspects of development.

One useful feature of microspore hydration for experimental manipulation is that water is taken up through the porous membranes of the microspore. Thus, various molecules and drugs added to the water can be taken up by the microspore, which is especially useful in RNAi experiments (Klink and Wolniak, 2000 and 2001). RNAi allows for the targeted destruction, referred to as knockdown, of specific mRNA sequences during development. RNAi knockdown experiments can cause the appearance of altered phenocopies of the treated cells as compared to untreated cells. The knockdown experiments have the potential to disclose the timing of the mRNA action during male gametophyte development.

Moreover, a range of phenocopies can be seen by using different concentrations of dsRNA. A high concentration will result in a complete knockdown of gene expression, and the phenocopy will reflect the point at which that protein was first critical, perhaps at the first or second division. A lower concentration of dsRNA will result in less severe phenocopies, which may allow for the identification of several times of gene action during male gametophyte development (Klink and Wolniak, 2001).

RNAi treatments can be specific an mRNA of interest (Klink and Wolniak, 2001; Tsai and Wolniak, 2001; Tsai *et al*., 2004). A series of western blot assays were performed to determine if the treatment was specific to the targeted mRNA or if it had a global effect on other mRNAs. When samples were treated with centrin dsRNA, reduced amounts of centrin protein was seen on blots, but the levels of other proteins such as β-tubulin and P28 remained constant (Fig 1-4; Klink and Wolniak, 2001). When dsRNA encoding a fragment of the HIV genome was added, no effect was seen in the abundance of centrin or P28. These studies show the specificity and efficiency of RNAi that can be achieved with *M. vestita* (Klink and Wolniak, 2001).

Another unusual feature of the development of *Marsilea* male gametophytes is that the male gametophyte is a transcriptionally quiescent organism. Instead of relying on the transcription of new mRNA, mRNA that was stored during microspore unmasked during development to control the entirety of development, in addition to some stored proteins (Hart and Wolniak, 1998; Hart and Wolniak, 1999; Klink and Wolniak, 2001). Thus, tight temporal and spatial control of the processing and translation of masked transcripts enables the rapid changes occurring during male gametophyte development (Gross and Cousineau, 1963). Many of the stored pre-RNAs retain introns, and the final

splicing of these transcripts after hydration is essential for the translation of these transcripts (Wolniak *et al.*, 2015). In addition, the expression of a specific transcript can be altered by shortening or lengthening its poly(A) tail, affecting transcript stability (Wolniak *et.* al, 2011). Masked mRNAs are aggregated in nuclear speckles that are asymmetrically distributed only among the spermatogenous cells (Boothby and Wolniak, 2011). Specific mRNAs are unmasked at specific times thereby allowing for translation and the accumulation of these proteins in specific cells of the gametophyte and at the appropriate stage of development. In addition, further translational and post-translational regulation of these proteins control the spatial and temporal development of the male gametophytes. (Wolniak *et al.*, 2011).

Spermatogenesis in *Marsilea vestita*

Spermatogenesis in the microspore is a well-defined synchronous process that is activated by the addition of water to the dry sporocarp (Fig 1-3e) (Hepler, 1976; Wolniak *et al.,* 2000). During 9 sequential divisions, each male gametophyte developing from a microspore produces 1 prothallial cell, 6 sterile jacket cells, and 32 chemotactic coiled spermatozoids that develop approximately 144 cilia (Fig 1-4f). This process is linked intrinsically to temperature; at 20°C, the motile sperm will emerge in 11 h. The size and position of each cell within the spore wall determines its fate, because there is no movement of the cells relative to each other. The microspore initially consists of a single cell with a large nucleus that is surrounded by plastids that will serve as a source of sugar for the developing cells (Fig 1-5a) (Hepler, 1976). Upon hydration, the organelles are rearranged with the nucleus and plastids being located at opposite poles of the microspore

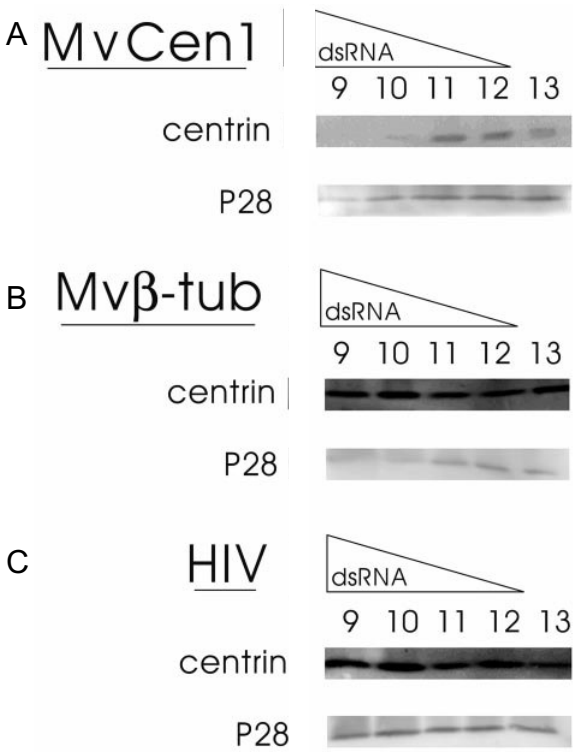


Figure 1-4. RNAi effects are specific to the protein of interest and dependent on concentration.
As the concentration of dsRNA is decreased, more protein is detected on immunoblots. When centrin dsRNA is added, only centrin protein levels are effected **(A)**. When β-tubulin dsRNA is added, neither centrin or p28 levels are effected **(B)**. HIV dsRNA shows no effect on either protein **(C)** *(Klink and Wolniak, 2001)*.

(Fig 1-5b) (Rice and Laetsch, 1967). The first asymmetric division occurs by 45 min forming a germ cell and a prothallial cell, the latter of which does not divide any further (Fig 1-5c). Approximately 2 h after initiation, the germ cell divides symmetrically creating 2 antheridial initials which divide 3 more times producing 6 sterile jacket cells and 2 primary spermatogenous cells (Fig 1-5d-g). Each spermatogenous cell then divides 4 more times to produce 32 spermatids by the end of the division phase (Fig 1-5h-k). Each spermatogenous cell differentiates to form a motile spermatid consisting of an elongated nucleus coiled around a multilayered structure (Sharp, 1914; Hepler, 1976; Myles and Hepler, 1977). The multilayered structure is a complex cytoskeletal structure that forms at the anterior end of the spermatid and extends to elongate and coil the cell. Basal bodies which are formed from a centrosome-like body called the blepharoplast, align along the multilayered structure and serve as templates for ciliogenesis (Fig 1-6) (Myles, 1975; Myles and Hepler 1977 and 1982).

Blepharoplast formation

Blepharoplasts arise synchronously in all the spermatogenous cells due to the components being equally divided in previous divisions (Tsai and Wolniak, 2001). The blepharoplast arises *de novo* approximately 4 h after initiation in each spermatogenous cell (Fig 1-8a). It serves as a MTOC and approximately 144 bb will eventually arise from it. The blepharoplast first forms as an amorphous mass during telophase of the 7^{th} division, approximately 3.5 h, and then it disappears after that division. It reappears during telophase of the 8^{th} division, again as an amorphous mass located on the distal side of the nucleus (Fig 1-7a). During this division, it splits in half, and each half then migrates to each daughter cell, ensuring that each daughter cell receives one

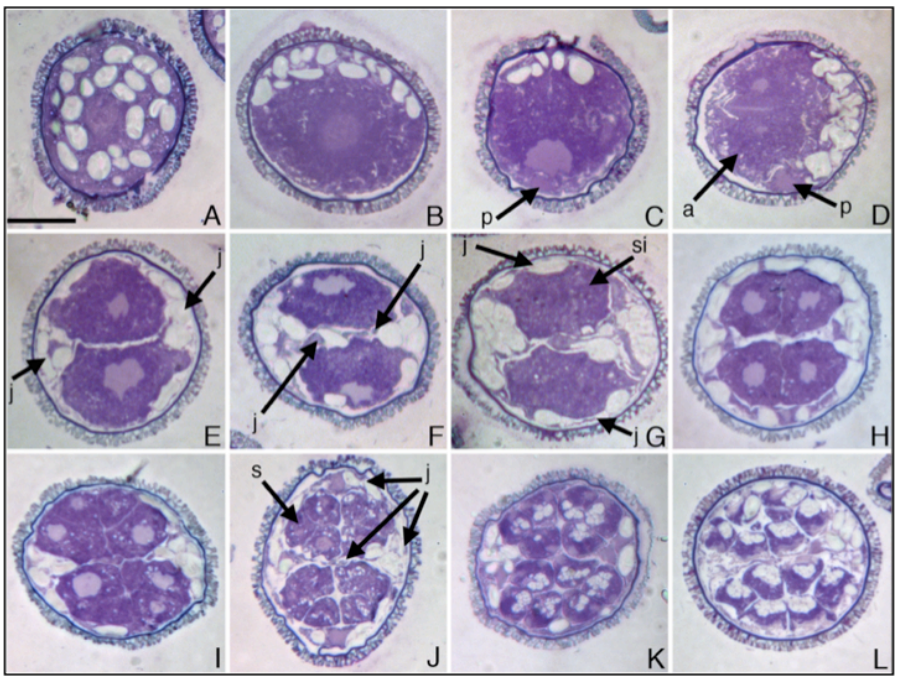


Figure 1-5: The development of the microspore of *Marsilea vestita* into 32 motile spermatids.
When the dry microspore (**A**) is hydrated, there is a rearrangement of the plastids (**B**). The first division creates a prothallial (p) cell and germ cell (**C**). The germ cell continues to divide, creating 2 antheridial (a) initials (**D**). The sterile jacket (j) cells undergo several rounds of division (**E-G**). The spermatogenous cells divide to finally create 32 spermatogenous cells (**H-J).** The spermatogenous cells undergo further differentiation to form the spermatids (**K, L**). Bar = 25 μm. (*Courtesy of C. van der Weele*)

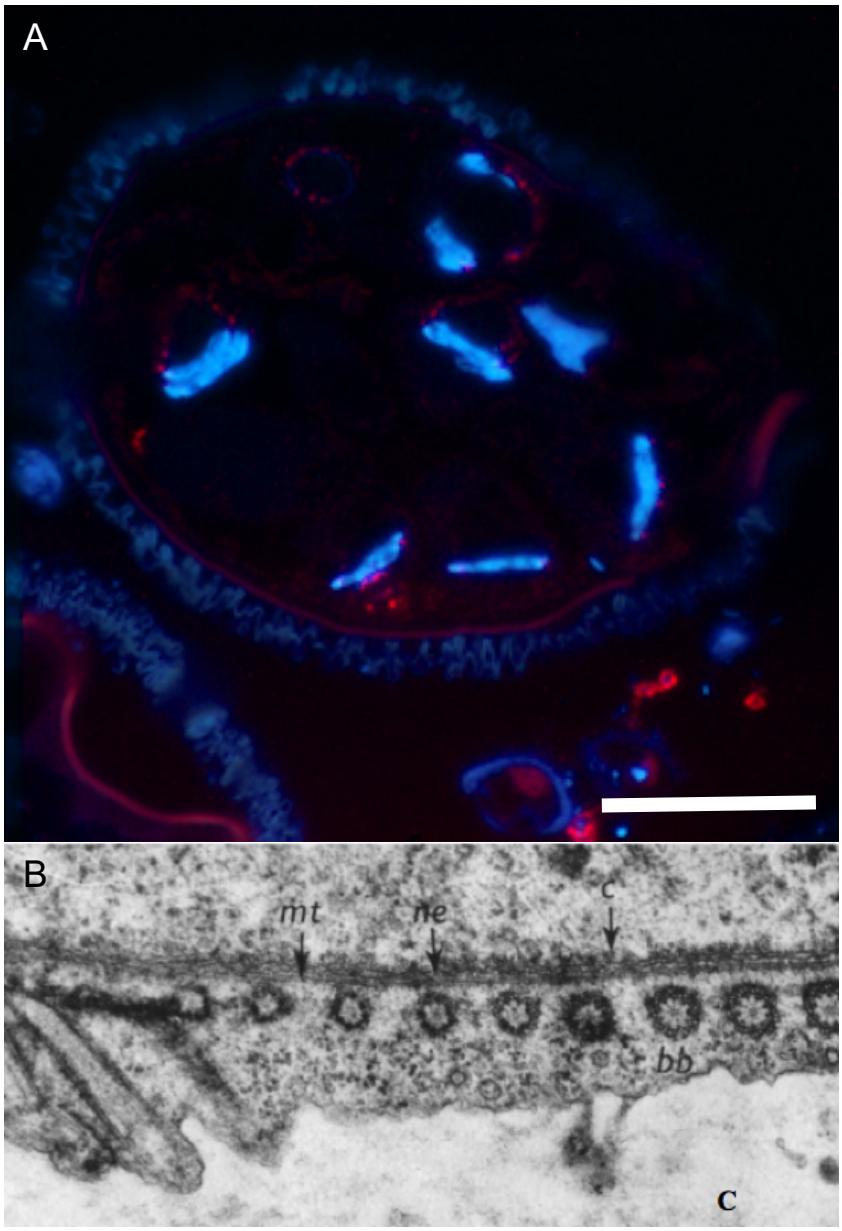


Figure 1-6: The basal bodies aligning along the microtubule ribbon and coiled nucleus.
Immunofluorescent labeling of centrin, red, in an 8 hour microspore. The DNA is labeled with DAPI (**A**). Centrin is localized to the bb. The bb are aligned along the microtubule ribbon (mt) and nuclear envelop (ne) (**B**). Bar = 25µm. *(A courtesy of E.Tomei; B Myles and Hepler, 1977)*

blepharoplast (Hepler, 1976). As with most *de novo* instances of centriole/centrosome formation, the earlier stages of its formation remain unclear (Gall, 2004). The amorphous mass starts to become structured with the formation of two parallel triple-layered plaques that are assembled within a sphere. The distal layers of these plaques become thicker and lengthen as material condenses on them, forming the two hemispherical structures. The growing blepharoplasts will then become spherical in shape. (Fig 1-7b-f). They separate during prophase of the 9th division and begin to move away from the nucleus and each other (Fig 1-7g-h). The blepharoplast then serves as a MTOC for the 9th division, and begins to mature during metaphase and anaphase (Hepler, 1976; Hoffman and Vaughn, 1994 and 1995). The blepharoplast begins as a solid sphere with many interpenetrating channels (Fig 1-8c1, b). The channels swell and shorten, forming procentrioles that are radially aligned around the sphere (Fig 1-8c2, b). At this point, 9-fold symmetry can be seen in each forming procentriole, but it is not as ordered as that symmetry seen in centrioles. The procentrioles continue to enlarge, finally breaking apart into individual bb that migrate to the elongated nucleus and become linearly aligned to then serve as the template for ciliogenesis (Fig 1-6b, 1-8c3 and b4) (Hepler, 1976; Mizukami and Gall, 1966). The composition of the blepharoplast remains largely unknown. Centrin has been shown to be an essential component of the blepharoplast (Klink and Wolniak, 2001). Tubulin is absent in the blepharoplast until just before the formation of bb, and its distribution predicts the locations of the developing bb (Pennell *et al.*, 1986 and 1988).

The rapid ordered and synchronous development of motile sperm provides a unique opportunity to study the *de novo* formation of bb. Normally, plants do not have centrioles or spindle pole bodies, but instead the nuclear envelope serves as the MTOC

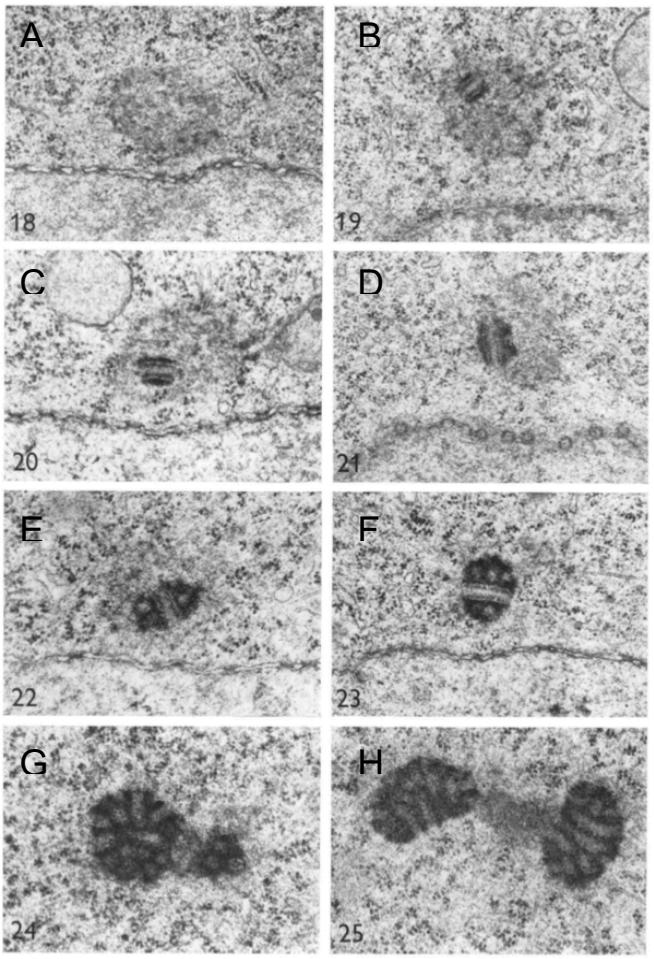


Figure 1-7: The formation of the blepharoplast during the 8th division.
The blepharoplast begins as an amorphous mass (**A**). Two plaques form and thicken as more material is amassed (**B-F**). The blepharoplast becomes spherical (**G**), separates and **b**egins to migrate away from each other (**H**). (*Hepler, 1976*)

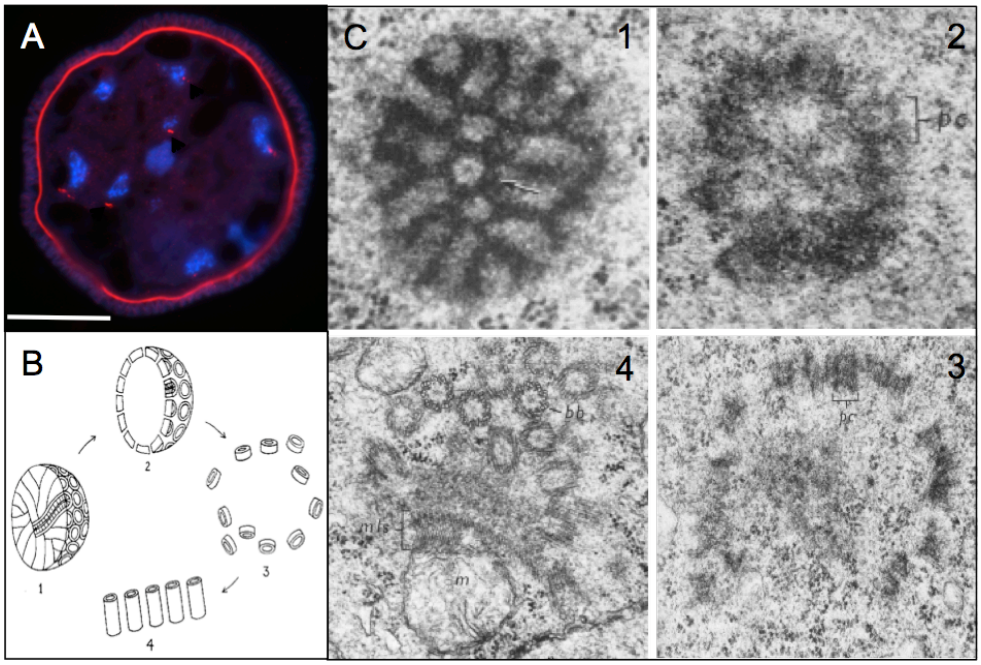


Figure 1-8. The blepharoplast arises *de novo* during telophase of the 7th division, serving as a MTOC and is the source of basal bodies.
Centrin, red, is labeled in a 4 hour microspore. The centrin labeling is localized to the blepharoplasts. DNA is labeled with DAPI (**A**). The blepharoplast undergoes maturation, resulting in individual bb: 1- blepharoplast, 2- radially aligned procentrioles, 3- individual bb, 4- alignment of bb (**B,C**). Bar = 25µm *(B Mizukami & Gall, 1966; C Hepler, 1976*)

that is always present in the cell. *M. vestita* breaks this mold with the *de novo* formation of the centrin-rich blepharoplast and the subsequent formation of bb (Klink and Wolniak, 2001). Centrin is a critical structural component of the blepharoplast, which is unable to form without centrin protein in the cell: there is a small amount of centrin protein present at the onset of development was apparently stored in the desiccated microspore. This centrin appears to be essential for the mitotic divisions that occur early in gametophyte development (Heart and Wolniak, 1998, 1999; Klink and Wolniak, 2001, 2003). At approximately 4 h of development, centrin levels increases drastically due to the translation of stored centrin mRNA and is localized almost exclusively to the blepharoplast and bb (Hart and Wolniak, 1999; Klink and Wolniak, 2001 and 2003). During cell cycle arrest, centrin translation still occurs, but the blepharoplast and bb are not formed, indicating that new centrin translation is not sufficient for the *de novo* formation of bb (Tsai and Wolniak, 2001).

Blepharoplast formation apparently requires a variety of different components. The blepharoplast and centrosomes both serve as MTOCs, while they do have many differences. Due to this overlap in function, the proteins involved in both structures are likely similar. Some proteins like tubulins and centrin serve a structural role, but these alone are not sufficient for *de novo* formation. A variety of additional proteins, including regulatory and signaling proteins, are needed to compose and aggregate the blepharoplast components to create the functioning blepharoplast (Tsai and Wolniak, 2001).

The animal centrosome

Unlike the blepharoplast, the centrosome serves as a MTOC throughout the life of the cell. It is thought that the proteins that make up the blepharoplast would be similar to the composition of the pericentriolar material (PCM) do the fact that both serve as MTOC for cellular division which is a conserved process in most cell types. The centrosome is a membrane-less organelle found in metazoans that serves as the MTOC. It is formed when proteins are recruited to the centrioles to form the PCM (Fig 1-9a, b) (Mahen and Venkitaraman, 2012; Mennella *et al*., 2013; Palazzo *et al*., 2000). The centrosome serves as the main microtubule nucleating center, the anchor of the two poles during cell division, and the integration hub for many signaling pathways (Brittle and Ohkura, 2005; Nigg and Stearns, 2011; Pannu *et al*., 2012). The lack of a membrane allows for rapid, direct interactions with cellular components and for the rapid cycling of proteins between the centrosome and cytoplasm (Schatten and Sun, 2010).

The centrosome consists of 2 centrioles surrounded by a layered PCM that provides the scaffold to support the nucleation of microtubules by γ-tubulin ring complexes (γTuRC) (Fig 9c). The layers that form the PCM have different components and functions (Mennella *et al*., 2013). The components of the PCM can be divided into two major classes, γTuRC and a lattice-like network of filaments made of coiled-coil proteins (Anderson *et al*., 2003; Salisbury, 2007). Regulatory proteins are also incorporated into the PCM to control the functioning of the other PCM components. The lattice-like network is closely associated with the centrioles and promotes the recruitment of PCM material, controlling the size and composition of the centrosome. The PCM is not static; instead there is a dynamic exchange of proteins between the PCM and the

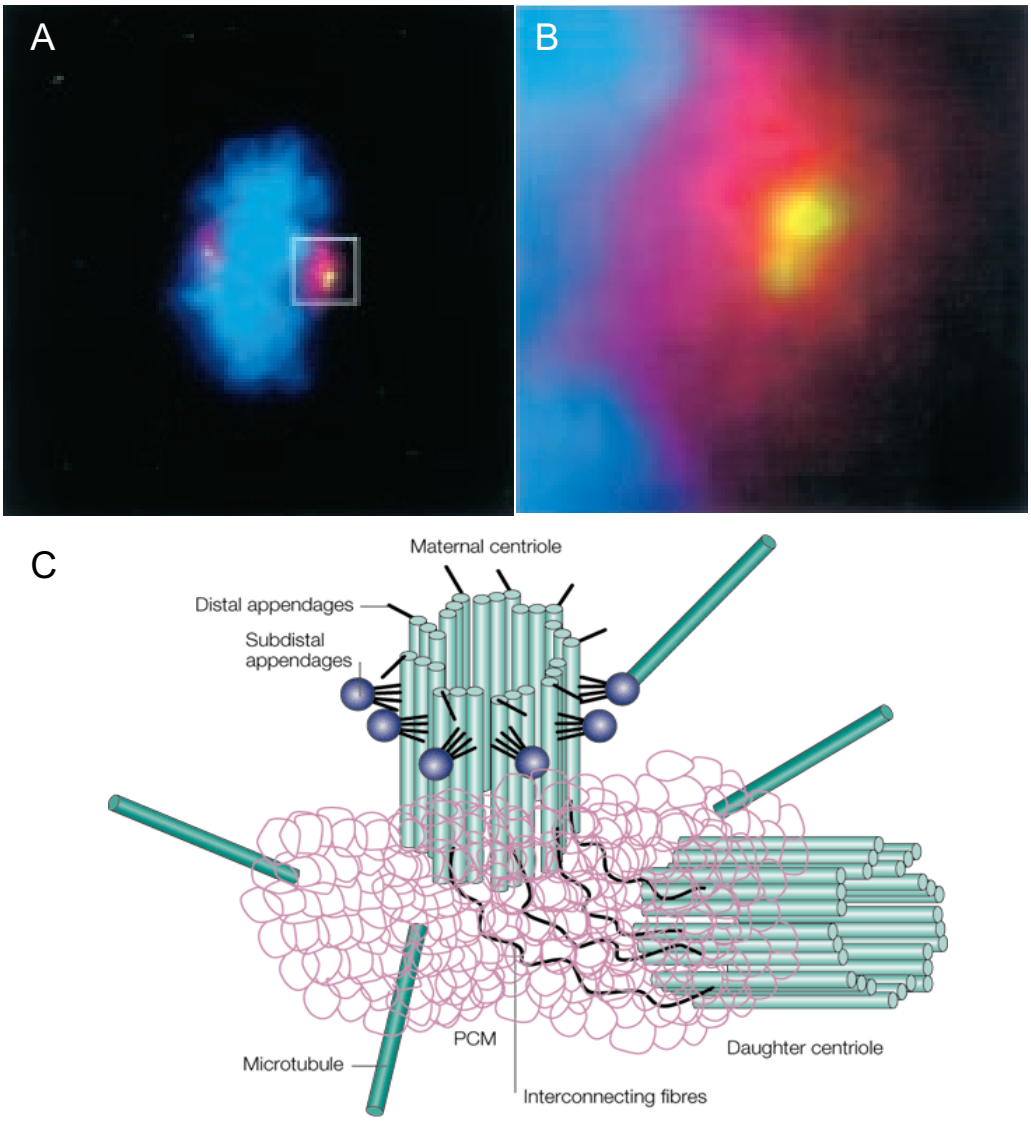


Figure 1-9: The structure of the centrosome.
The centrosome is membrane less organelle (**A**,**B**). The PCM is labeled pink and the DNA in blue. It consists of 2 centrioles surrounded by PCM which nucleates and anchors microtubules (**C**). *(A,B Dutertre, et al 2002; C Doxsey, 2001)*

cytosol allowing for rapid adaption to meet the needs of the cell (Conduit *et al.,2014;* Mennella *et al*., 2013). The make-up and size of the PCM varies depending on stage of the cell cycle. The PCM is an anchoring site of γTuRC during interphase and metaphase. Just before and during mitosis, many additional anchoring sites are needed to establish the bipolar microtubule spindle, drastically enlarging the PCM (Palazzo *et al*., 2000). The additional regulatory proteins and γTuRC needed in the PCM to establish the bipolar spindle are supported by an expand lattice-like structure. Two critical proteins in this expansion are centrin and Sfi1p, which together form an elastic matrix that allows the PCM to change shape and size. Sfi1p has many internal repeats that bind several centrin molecules at once, this complex interacts with γTuRC to form a unit of duplication, which is then repeated throughout the structure (Kilmartin, 2003; Salisbury, 2007). The γTuRC provides the link between the centrosome and microtubules, and serves as the source of cell cycle regulation of the centrosome to nucleate microtubules (Stearns and Kirschner, 1994).

The growth of the centrosome coincides with the progression of the centrosome cycle. The centrosome cycle progresses concurrently with the cell cycle, ensuring that duplicated centrosomes are present to serve as spindle poles during mitosis. At the conclusion of mitosis, each of the daughter cells receives one centrosome (Lukasiewicz and Lingle, 2009). The centrioles duplicate during each cellular division cycle in either a semi-conservative or *de* novo pathway which are both fundamentally similar and have interchangeable features. The *de novo* pathway is thought to be inhibited by the presence of at least one centriole in the cell, which serves as a pattern for the formation of the new centriole (Khodjakov *et al*., 2002). In the *de novo* pathway, the new centriole first

appears as an amorphous structure and then begins to take on the morphology of a centriole before mitosis occurs (Khodjakov *et al*., 2002; Salisbury, 2007). The components of the new centrosome are actively recruited to the new centrioles in a microtubule dependent fashion by PCM, forming the centrosome (Blagden and Glover, 2003; Bornens, M. 2002; Dammerman and Merdes, 2002).

Normally, centriole (and thus centrosome) formation is semi-conservative though instances of *de novo* centrosome formation has been observed or induced in specialized cell types. This can be replicated by destroying the centrosome using laser microsurgery. When the centrosome begins to form, a loose cloud of electron dense material, including γ-Tubulin structural proteins, begins to coalesce. The initial cloud is serves as an organizing center for microtubules while the loose cloud continues to condense and become better structured and the centrioles appear (Khodjakov et al., 2002).

Aurora Kinases in *Marsilea vestita*

The blepharoplast acts as a MTOC for the final spermatogenous division in the male gametophyte of *M. vestita*. Several key centrosomal proteins are critical for blepharoplast formation and function which retain their functionality seen in the centrosome. These include centrin and several tubulins, which are structural components in the PCM, blepharoplast and bb (Pennell *et al.*, 1987). The blepharoplast forms *de novo* just before the final division takes place. Most cell types do not have an organized MTOC during interphase (Pickett-Heaps, 1968), but it forms as a loose aggregate (as seen ultrastructurally) in early prophase. In most animal cells, the MTOC is a centrosome (Boveri, 1887), which is discrete and readily recognizable for the entirety of the cell cycle. MTOCs normally are patterned from the mother cell, resulting in duplicate

MTOCs in the daughter cells. I wanted to investigate the role of aurora kinases and the *de novo* formation of the blepharoplast. Centrin is phosphorylated by aurora A, which stabilizes centrin in centrosomes causing the aggregation and parenting the diffusion of centrin at the centrosome. I hypothesize that the phosphorylation of centrin will stabilize centrin initializing the *de novo* formation of the blepharoplast. This is in contrast to centrin being added to a preexisting existing body, such the centrosome and centrioles. By looking at the centrin-aurora interaction before, during, and after the formation of the blepharoplast, it allows me to examine of the centrin-aurora interaction in isolation from the foundational proteins which the PCM based on.

To characterize aurora kinases in *M. vestita*, the central conserved portion of aurora kinase of *Arabidopsis thaliana* will be used to identify the different isoforms present in a *Marsilea* transcriptome. Using RNAi, the role of each isoform will be studied by targeting the unique 5' portion of the transcript, and the combined effects of the isoforms will be examined by targeting the central conserved portions of the transcripts. Examinations of microspore cell morphologies and the distributions of centrin, aurora kinase, and transiently translated proteins will be achieved through immunocytochemistry of thin sections of developing male gametophytes. The expression profile of each isoform will be deduced using RT-PCR for mRNA, and western blotting for proteins. To investigate the relationship between centrin phosphorylation and blepharoplast formation, transiently translated phosphomimics will be engineered to mimic constitutively phosphorylated and unphosphorylated centrin in addition to transiently translated wild type centrin.

Chapter 2: Methods

Plant material

Marsilea vestita plants were grown in potting soil in 12-inch plastic pots submerged in artificial ponds located in the University of Maryland greenhouse complex. The ponds were drained gradually over the course of 3 months in order to induce the formation and maturation of sporocarps. Naturally desiccated sporocarps were harvested and stored at room temperature in the lab, where they remained viable for at least 20 years. Some sporocarps were nicked and placed in aerated water in order to generate new plants for growing in the artificial ponds. The remaining sporocarps were stored in ambient conditions for future experiments (Klink and Wolniak, 2001).

Microspore culture and fixation

Mechanical isolation of the microspores from the sporocarps was accomplished by grinding dehydrated sporocarps in three one-second bursts in a coffee grinder (Hepler, 1976) and then sifting the debris through a series of 425 and 212 um wire sieves. The isolated microspores were then suspended in commercial spring water (4 mg microspores /ml water), and aerated thoroughly with an Orbitron agitator for 1 h. The initial microspore suspension was transferred to a flask with 9 ml of spring water and further incubated in a shaking water bath for the desired length of incubation. The temperature was maintained at 20°C for the entire process (Hepler, 1976; Hart and Wolniak, 1998; Klink and Wolniak, 2001; Tsai and Wolniak, 2001; Wolniak *et al.*, 2015).

Developed microspores were filtered and collected under vacuum through a polyester filter over a Buchner funnel. The spore walls were then disrupted by striking a hammer on a stainless-steel pestle (Hepler, 1976), which was on placed top of the filter

and a 60μm brass foil spacer with the sample in a stainless-steel mortar. Samples were washed from the mortar by using 15 mL of 4% PFA, then incubated at 4°C for 2h. Thirty-five ml of PBS were then added to each sample, and the samples were incubated at 4°C overnight. Dehydration of the microspores was performed with a graded ethanol series (10, 25, 50, 75, and 100% by volume), followed by 4 consecutive rinses (1 h each) in 100% ethanol. The microspores were embedded by infiltrating them in a series of ethanol-methacrylate concentrations (3:1, 1:1, 1:3 v/v) and by then polymerizing the methacrylate with UV light (5 h at 4°C). Semi-thin sections (1-2 μm) were made then transferred to drops of water on a clean glass slide. The sections were adhered to the slide by drying the slide at 40° on a slide warmer (Klink and Wolniak, 2001).

Immunocytochemistry

Indirect immunofluorescence was performed to localize proteins in the gametophytes. Semi-thin sections were etched in acetone for 15 min, blocked with super block (Hardin and Wolniak, 1998) for 45 min and incubated with antibody. The primary antibodies used were anti-centrin monoclonal 20H5 (Millipore 04-1624) (1:200 dilution) for 1 h at room temperature), anti-mCherry antibody (1:500 dilution) for 1 h at 37°C) (Abcam, ab125096), and anti-phospho-aurora-A, -B, -C (1:50 dilution) for 2 h at 37°C) (Cell Signaling, 2914). Sections were then incubated with Alexa Fluor 594 and Alexa Fluor 488 secondary antibodies (Molecular Probes), respectively for 1 h at room temperature. These sections were simultaneously treated with 0.1μg/ml 4',6-diamidino-2-phenylindole (DAPI). Incident light fluorescence was performed using standard fluorescein (ex 475 nm, em 530 nm), texas red (ex 559 nm, em 630 nm) and UV (ex 358 nm, em 461 nm)

filter sets. Phase contrast images were also taken to examine cellular morphology and to identify the stage of rapid development in the gametophytes. Approximately 100-200 microspores were observed in each sample, and representative images were taken to ensure accurate assessments of the treatments.

Protein isolation

Eight mg of microspores in 10 ml of water were allowed to develop at 20°C in a shaking water bath until they reached the desired stage. The microspores were then centrifuged at 13,200 rpm in an Eppendorf 5415D centrifuge for 5 min to sediment them. The microspores were homogenized using a cold glass dounce tissue grinder on ice to avoid protein degradation. The homogenate was centrifuged, and the supernatant was then collected. An equal volume of sample buffer (62.5mM Tris base pH 6.8, 2% SDS, .02% bromophenol blue, 25% urea, 10% glycerol and 5% 2-mercaptoethanol) was added to the sample, and boiled for 5 min (Klink and Wolniak, 2001). The amounts of protein in the samples were quantified using the Bradford assay (Bradford, 1976).

Western Blot

Western blot analyses were performed in order to visualize proteins. Samples were separated using SDS-PAGE gels and electrotransfered to a PVDF membrane using transfer buffer with and without SDS (Laemmli, 1970). The membranes were blocked at room temperature using nonfat dry milk and potato starch to reduce background signal. The blots were then probed using anti-α tubulin (1:200 dilution) for 1 h at room

temperature and anti- phospho-aurora A, B, C (1:50 dilution) for 2 h at 37°C, after which. Alkaline phosphatase-conjugated 2° antibodies (1:200 dilution) (New England Biolabs) were added to each membrane and incubated for 1 h at room temperature. The membranes were incubated in alkaline phosphatase buffer followed by electrofluorination (Amersham), and imaged with a phosphor imager using the blue laser (Storm 860, Molecular Dynamics). The apparent molecular weight was determined using a prestained protein ladder which was run with the samples on the SDS gels. Each blot was run 4 times, with 4 separate incubations and protein isolations. The results were similar for all of the blots.

Poly(A)+ RNA isolation

After microspores developed for various times (see above), they were sedimented by centrifugation, and as much water as possible was decanted from each sample. The mRNA of each sample was isolated using a magnetic poly(A) RNA isolation kit according to New England Bio Lab's protocol. The isolated RNA was aliquoted into 6 µl volumes and stored at -80°C after being snap frozen using liquid nitrogen.

Identifying aurora kinase isoforms in the M. vestita transcriptome

Three distinct aurora kinase isoforms were identified in the *M. vestita* transcriptome by using Tblastn (https://blast.ncbi.nlm.nih.gov) to screen against the conserved central catalytic domain of the aurora kinases *of Arabidopsis thaliana*. These isoforms, designated as mvAUR1, 2, and 3, were determined to be aurora kinase isoforms

due to a 90% identity between the conserved regions of the identified isoforms and known aurora kinase sequences.

Phylogenetic tree generation

Phylogenetic trees were generated using Clustal Omega (EMBL-EBI). The protein sequences of mvAUR1, 2, and 3 were aligned with atAUR1 and 2 to form a neighbor joining tree visualized as a cladogram. The distance and comparative position between each sequence indicated the evolutionary relationship between the sequences.

Reverse transcription and cDNA amplification

Reverse transcription PCR was performed using isolated poly(A)+ RNA with Avian Myeloblastosis Virus Reverse Transcriptase according to the New England Bio Labs protocol. One nano gram of the resulting cDNA was amplified using *Taq* polymerase for a total of 30 cycles using the primers outlined in appendix 2. The products were run on 1.5% TAE agarose gels, and visualized with EtBr using UV light.

Characterizing aurora kinase mRNA abundance patterns

The relative abundance of mRNA transcripts during development was determined by analyzing RT-PCR products for each isoform at different time points in development. Primers were designed using the unique 5' ends of each isoform (see appendix 2). All of the reactions for each isoform were set up identically, with the exception of the poly(A)+ RNA for each time point. The poly(A)+ RNA from each time point for all of the reactions was isolated at the same time. Equal volumes of each RT-PCR product were

run on a 1.5% TAE (tris base, acetic acid, and EDTA) agarose gel, and visualized using ethidium bromide. A rough quantification of the bands for each transcript was preformed using ImageJ (NIH), by calculating the percent intensity relative to the highest intensity band for each transcript. The reactions were repeated 4 times, each with a new RNA isolation. All of the RT reactions for each isoform were similar in each replicate.

RNA interference (RNAi)

Single stranded RNA was created from a DNA template with a T7 or SP6 promoter by T7 and SP6 RNA polymerases (see appendix 2). Reactions using the RNA polymerases were incubated at 37°C for 2 h. The single stranded RNA was quantified by using a Nano Drop spectrophotometer to ensure equal concentrations of the T7 and SP6 RNA. The ssRNA was annealed starting at 85°C and lowered to 20°C. The dsRNA was quantified using a Nano Drop spectrophotometer, and then visualized using agarose gel electrophoresis to assess its quality (Deeb *et* al., 2010; Klink and Wolniak, 2001; Wolniak *et al.*, 2015). The microspores were initially hydrated in 1ml of water containing 25-100 μg of dsRNA. An untreated sample was included with every experiment as a developmental control. The microspores were then incubated and fixed using the same protocol as used with the control microspores. (Deeb *et al.*, 2010; Heart and Wolniak, 1999). Double stranded RNA was created using a portion of the HIV genome to serve as a negative control: primers- F: CTCCCATCAGCGGACAAATTA; R: ACCTACCAAGCCTCCTACTATC. Approximately 100-200 microspores were observed in each sample, and representative images were taken to ensure accurate assessments of the treatments. The experiment was repeated 3 times with similar results.

Treatment of microspores with the aurora kinase inhibitor hesperadin

Four milligrams of microspores were treated with 0, 1, 5, and 10 μM hesperadin at the onset of hydration. 10 μM hesperadin was also added to one sample at 3 hours after hydration. As a negative control, 10 μM hesperadin was heated to 100°C and added to another sample at the onset of hydration with spring water. All of the samples were cultured for 8 h at 20°C in a shaking water bath, then fixed according to the procedures described above. Approximately 100-200 microspores were observed in each sample, and representative images were taken to ensure accurate assessments of the treatments. The experiment was repeated 3 times with similar results.

Transformation

All transformations were performed using DH5α-competent *E. coli* cells following the protocol from Life Technologies. After the frozen cells were thawed on ice, 25 ng of the vector plasmid was added to the cells. The cells were then incubated on ice for 30 min, transferred to a 42°C water bath for 90 s, and placed immediately on ice for 2 min. Two hundred μl of warmed LB broth was added to the cells and incubated at 37°C with shaking at 125 rpm for 45 min. The cells were plated on LB plates containing 1 mg/ml of ampicillin and 20 mg/ml of x-gal. The plates were then incubated over night at 37°C. Liquid broth was inoculated using white colonies representing successful transformants. and incubated overnight at 37°C. Plasmid was isolated the following day using a Qiagen miniprep.

Transient translation of proteins

To make the mCherry vector, mCherry DNA sequence and PBSK plasmid were cleaved using a double digest with XhoI and HindIII for 1 h at 37°C, followed by heat inactivation with a 20-min incubation at 65°C. The vector and insert were ligated using a sticky-end ligase, following the New England Bio Lab protocol. DH5α-competent cells were then transformed using the final ligated plasmid containing mCherry. The successful transformants were selected and cultured in order to increase cell density. This allowed for a sufficient amount of plasmid to be isolated using a Qiagen miniprep.

The transcript to be expressed was isolated by RT-PCR using primers with EcoR1 and SpeI cleavage sites at the 5' and 3' ends respectively. The PCR product and PBSK-mCherry plasmid were double digested with EcoR1 and SpeI for 1 h at 37°C. The reactions were heat inactivated with a 20-min incubation at 65°C, and ligated using a sticky-end ligase. The ligation was then inserted into DH5α-competent cells, as described above, and plated on LB ampicillin plates with x-gal. The plasmid with the transcript was isolated by using a Qiagen miniprep.

To pull out the mCherry-tagged transcript, primers were designed to add the T7 promoter sequence and Kozak sequence to the 5' of the PCR product during amplification. The ssRNA construct was created using the T7 polymerase as above. The ssRNA was quantified then snap frozen and stored at -70°C.

One hundred μg of the mCherry construct ssRNA was added to 1 ml of RNase-free water and added to 4 mg of microspores. The microspores were developed and fixed as described above. Approximately 100-200 microspores were observed in each sample,

and representative images were taken to ensure accurate assessments of the treatments. The experiment was repeated 3 times with similar results.

Chapter 3: Results

The male gametophyte of *Marsilea vestita* has a well defined and ordered progression of cellular development that concludes with the emergence of motile spermatozoids. An essential step in this progression is the final spermatogenous division, resulting in 32 spermatogenous cells that develop into mature spermatozoids. The blepharoplast forms *de novo* just before this final division, and thus, it serves as a microtubule organizing center to ensure the proper distribution of DNA. After this division occurs, the blepharoplast matures into a hollow sphere of procentrioles, which breaks apart to form individual bb that serve as the anchors of the cilia forming later.

Centrin is a major structural component of the blepharoplast. Centrin must be stabilized so that it coalesces to form the structural component of the blepharoplast. In other systems, aurora kinases have been shown to phosphorylate centrin, causing the stabilization of the protein. Aurora kinases have been found in both metazoan and plant cells. In all systems, they primarily act during cellular division, helping to ensure the proper distribution of genetic material by acting at the spindle poles and the kinetochores. Most organisms have multiple isoforms of aurora kinase, each having a unique function and localization during cellular replication. Three isoforms of aurora kinase (mvAK1,2,3) were identified in *M. vestita* from the transcriptome using BLAST alignment (Fig 3-1). All mvAks identified have the characteristic attributes of all aurora kinases found to date. Each isoform had a variable 5' end, with no similarities to the other isoforms (Fig 3-1a). This difference allows each isoform to interact with a specific set of substrates. The remaining sequence had approximately 90% identity among the

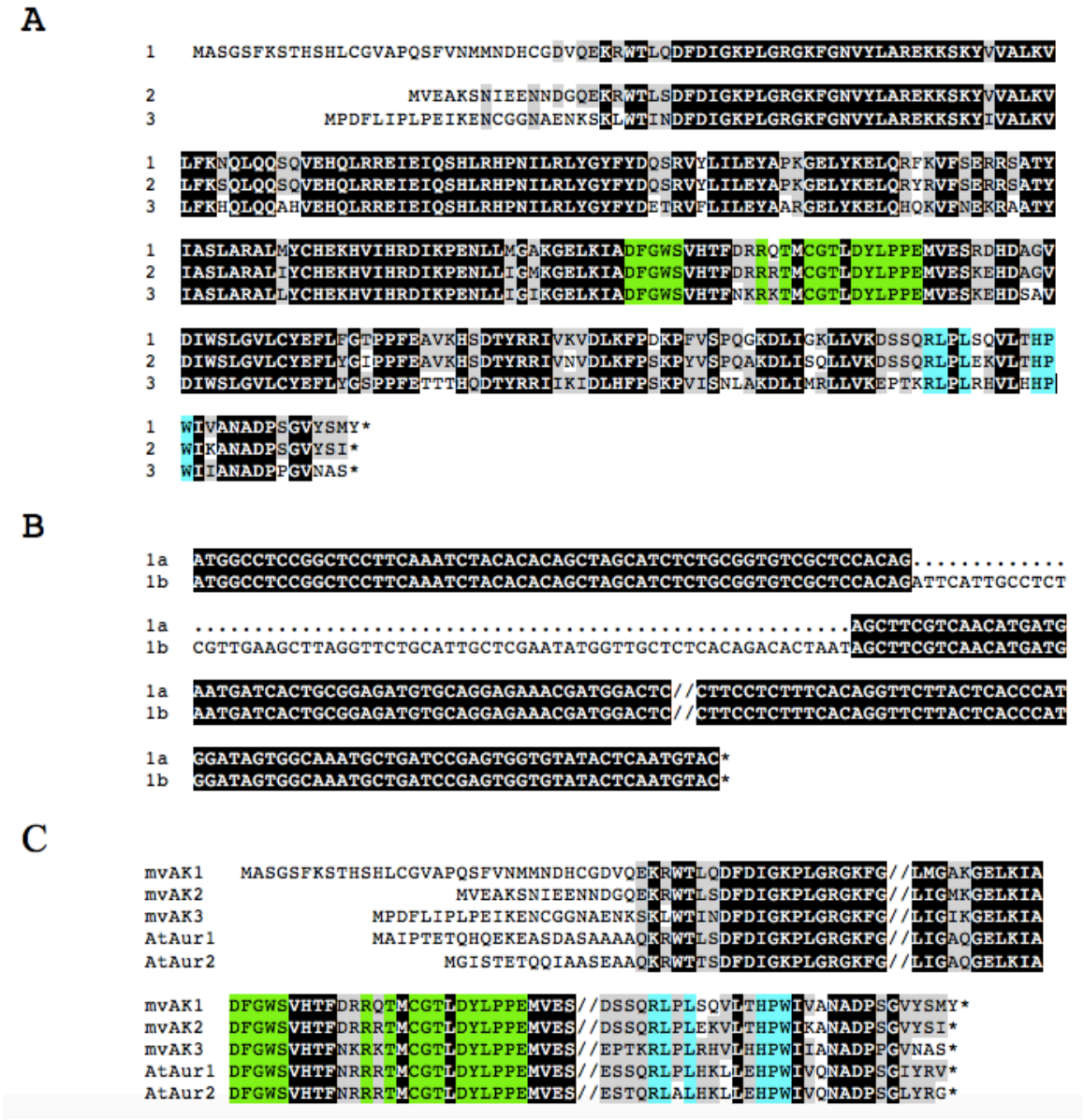


Figure 3-1: Sequences of Aurora isoforms in *Marsilea vestita.*
Three isoforms of aurora kinase were identified in *M. vestita* using RNAseq **(A)**. A unique N-terminal end and a conserved central portion is seen. This is consistent with other aurora kinases. The conserved middle portion has a high homology to same portion in AtAur1,2. The activation, **green**, and destruction loops, **blue**, are also conserved (**A,C**). The unique N-terminal ends of the 3 isoforms differ in length and sequence **(A)**. **Black** shaded residues are identical and **grey** similar. A 68 base splice variant of mvAK1 was found, 1b. The variation is found in the 5' end. When spliced, the sequences have 100% identity **(B)**. The mvAks are very similar in sequence and architecture to the two aurora kinases found in *Arabidopsis thaliana* **(C)**. *AtAur1, NP195009.1*; *AtAur2, NP180159.2*

isoforms. The conserved catalytic domain contains an activation loop and a destruction loop, both of which are highly conserved in all aurora kinases studied so far. The two loops activate and deactivate the kinase domain (Fig 3-1a). A 68-base splice variant was found of mvAK1, 1a and 1b (Fig 3-1b). When spliced, there is 100% identity. In addition to being conserved in the mvAks, the conserved region and architecture was the same as seen in aurora kinases of *Arabidopsis thaliana* (Fig 3-1c). The unique N-terminal ends, which are characteristic of other aurora kinases, indicates that each isoform has a unique function and substrate interaction.

In other systems, the evolutionary relationships among isoforms have revealed a strong correlation with the functions of those isoforms. In general, proteins that are closely related tend to have similar functions, whereas those that diverge tend to have different functions. This pattern is very evident when the divergence occurred early in evolutionary history, resulting in proteins with very different functions. To determine the evolutionary relationships among the 3 isoforms and *A. thaliana* aurora kinases, a neighbor-joining phylogenetic tree was created. mvAK2 and both *Arabidopsis* kinases share a recent common ancestor. That ancestor diverged from a common ancestor of all the aurora kinases studied (Fig 3-2). The close relationship between mvAK2 and *Arabidopsis* aurora kinases argues that they are likely to have similar functions. The early divergence of mvAK1 and 3 from the others suggests the possibility that they carry out different functions. The 3 isoforms found in *Marsilea vestita* do diverge early from their common ancestor, making it likely that these isoforms have different functions.

Dry microspores of *M. vestita* contain a large amount of stored mRNA. This is the only mRNA that is utilized during development, because this system has been shown

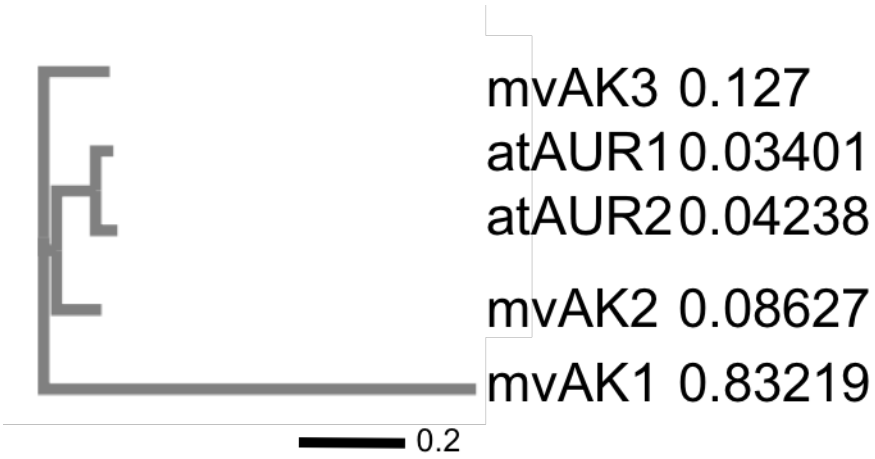


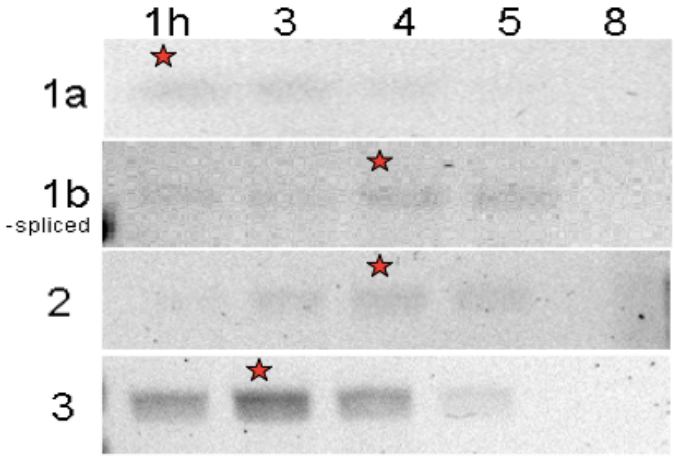
Figure 3-2: Evolutionary relationships of aurora kinase isoforms.
This phylogenetic tree indicates the relationships between the isoforms from *A. thaliana* using a neighbor joining tree. mvAK2 is closely related to both *A. thaliana* kinases. There is a common ancestor of the *Arabidopsis* and mnAK1. mvAK2 and 3 separated from mv1 early in the differentiation. The branch lengths correspond to the relative number of amino acid substitution. Created using Clustal Omega.

to be transcriptionally quiescent (Wolniak *et al.,* 2011). This mRNA is made available for translation at specific times during development when the protein product is required for an important process.

Therefore, one must study the relative levels of mRNA and protein that are present before, during, and after blepharoplast development to begin to understand the timing of aurora kinase activity. Starting in the first hour following spore hydration, the initial microspore cell undergoes a series of organized cell divisions. The blepharoplast is being formed and maturing into bb from 3-5 hours. The division phase ends at 8 h, which is followed by the differentiation phase involving spermatozoid maturation

To study the availability of each isoform transcript during development, RT-PCR was performed using poly(A) RNA isolated from microspores developed at 20°C for 1, 3, 4, 5, and 8 h. Equal amounts of RNA were added to each RT-PCR reaction, and equal volumes of cDNA were loaded on the gel. A different pattern of availability was seen for each isoform. To characterize both variants of mvAK1, the splice site located in the 5' end was utilized. Variant 1a was present and peaked at 1 h, and then it decreased at later times. In contrast, variant 1b was seen at low levels at 1 h, increased to its peak at 4 h, and then decreased at later times (Fig 3-3a, b). mvAK2 also peaked at 4 h, but levels stayed relatively stable until 5 h, after which they decreased during the remaining time. When compared to the other isoforms, mvAK3 had the most intense bands indicating the highest transcript availability. mvAK3 sharply peaked at 3 h, and then steadily decreased during subsequent development. None of the isoforms were visible in the gels at 8 h (Fig 3-3a).

A



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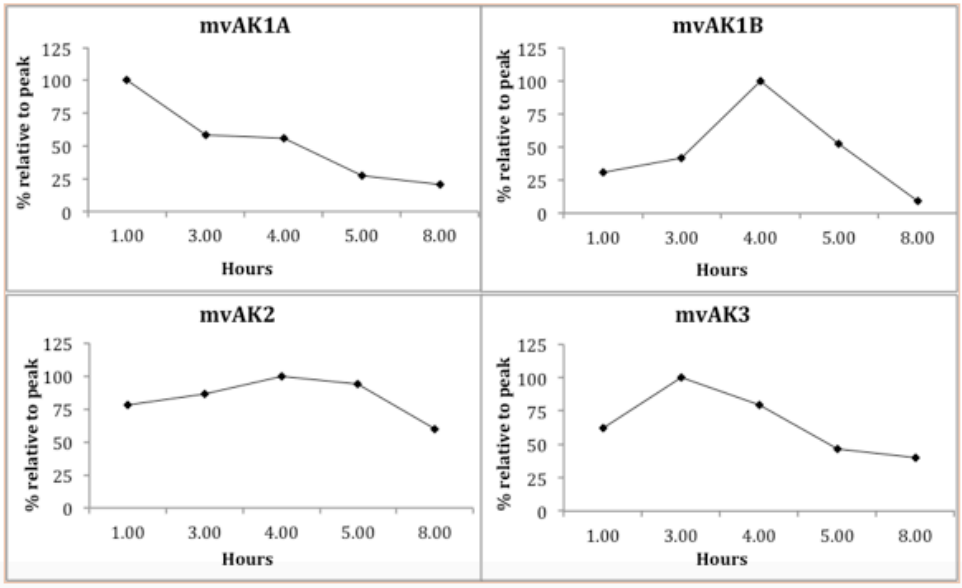


Figure 3-3: Relative abundance of Aurora Kinase transcripts over time.
Primers were made to the unique portion of each AK isoform. RT-PCR and amplification were performed using 1, 3, 4, 5, and 8 h poly(A) RNA. The abundance pattern is different for each isoform **(A)**. Stars indicate the highest intensity band. When a rough quantification of the bands was done using ImageJ, the patterns of expression became very apparent **(B)** mvAk1a peaked at 1 h, while the other isoforms peaked later in development. mvAK1b and 2 peaked at 4 h, and mvAK3 peaked at 3 h. The graphs were generated by calculating the percent intensity at each time relative to the highest intensity value.

ImageJ was then used to calculate the percentages of the peak expression in each gel. The quantification showed the same peaks as was visually identified in the gels, and this analysis confirmed that all the graphs had unique profiles (Fig 3-3b). The relative changes over time were easily discernable. mvAK2 levels showed more stability than the other isoforms. The other isoforms have more variable expression profiles. These unique profiles indicate that each isoform was active at different stages of gametophyte development, which supports the argument that each isoform may have a unique function and perhaps even a unique substrate. It is particularly intriguing that isoforms 1a and 1b, which are splice variants presumably sharing several common exons, exhibited such different profiles.

Western blots were used to examine at the abundance of aurora kinase protein at 1, 3, 4, 5, and 8 h. Because all aurora kinases have the same conserved kinase domain, a human anti-phospho aurora kinase antibody was used to detect the protein. This antibody recognized all the aurora kinase isoforms found in the microspores. The gels were run in the presence or absence of SDS (Fig 3-4). Without SDS, three bands were clearly seen at approximately 50, 40, and 38 kDa the 50 kDa bands were much stronger than the 40 and 38 kDa bands. All isoforms had the greatest intensity at 3 h, and are not seen at 8 h. When SDS was present, 1 distinct band was seen, peaking between 3 and 4 hours (Fig 3-4).

The blepharoplast arises *de novo* during development at approximately 4 h, during the 8^{th} division, which correlates with the expression profiles of aurora kinase observed in Fig 3-4. The expression of centrin was examined because it is a structural protein found in microspores were fixed at 3.5 and 4 h, in order to visualize the cell events occurring

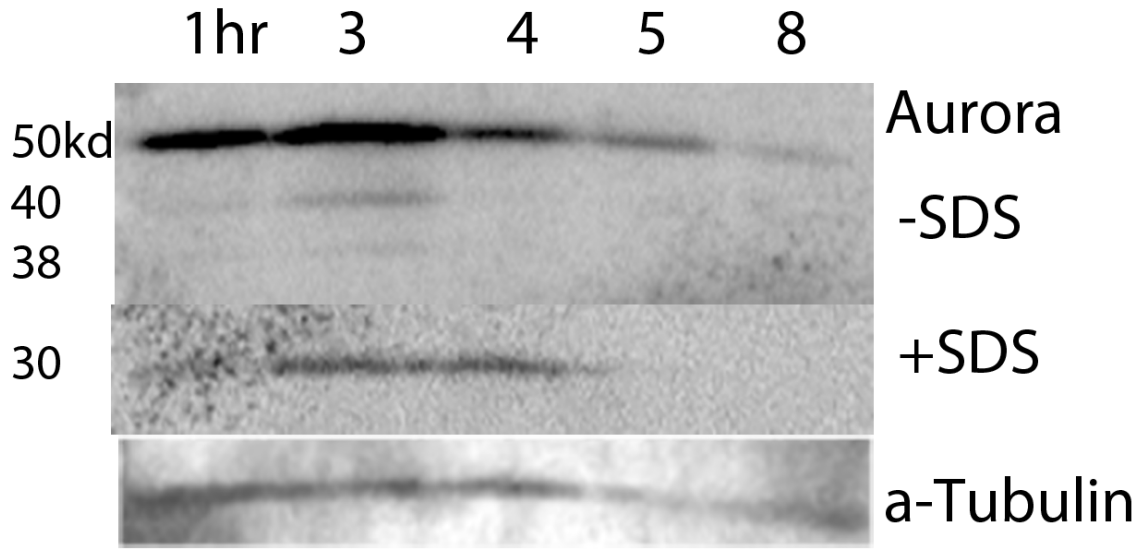


Figure 3-4. Aurora kinase abundance.
Protein was extracted from gametophytes 1,3,4,5 and 8 h after microspore hydration. Western blot analysis was performed to detect phospho- aurora isoforms. The antibody recognized the conserved kinase portion of all three aurora protein isoforms. Bands are seen at 50, 40, and 38 kDa when SDS is not present in the running buffer. All isoforms peak at 3 h, and the 50 kDa product is more abundant than the others. A single band is seen at 30 kDa when the running buffer contains SDS. Alpha tubulin serves as a loading control.

the blepharoplast, and because it is a known substrate of aurora kinase. Microspores were hydrated and then harvested during the 7th and 8th divisions. The sectioned microspores were stained with centrin (red) antibody and DAPI (blue) in order to visualize centrin expression during blepharoplast development at this time in gametophyte development (Fig 3-5). The progression of development can be followed by the number of spermatogenous cells and the shape of the nuclei. A division occurs every 30 min at 20°C, which means that the 6th, 7th, 8th, and 9th divisions occur at approximately 3.0, 3.5, 4.0, and 4.5 h, respectively after the hydration of the microspores. In particular, the nuclei a large and rounded at 6 h (Fig 3-5a). With each successive division, the nuclei become smaller as the number of spermatogenous cells increase (Fig 3-5c and d). After the final 9th division, at 4.5 h, 32 spermatogenous cells are present with small round nuclei. The small rounded nuclei then begin to elongate and spiral, which can be seen in some of the spermatogenous cells (Fig 3-5d).

Just after the 7th division, only a little diffuse centrin staining was seen in the spermatogenous cells (Fig 3-5a). A large increase of centrin was seen in these cells between the 7th and 8th divisions. Some of the staining showed that the protein was beginning to coalesce, but much of it was still diffuse throughout the spermatogenous cells (Fig 3-5b). Just before the 8th division, centrin appeared to be coalescing into amorphous clouds (Fig 3-5c). The blepharoplasts with concentrated centrin staining were clearly seen after the completion of the 8th division (Fig 3-5d). This pattern of centrin staining was correlated with the timing of the *de novo* formation of the blepharoplast between the 7th and 8th divisions. The distribution of centrin was initially diffuse in the cells, and then it coalesces into distinct foci in the blepharoplast.

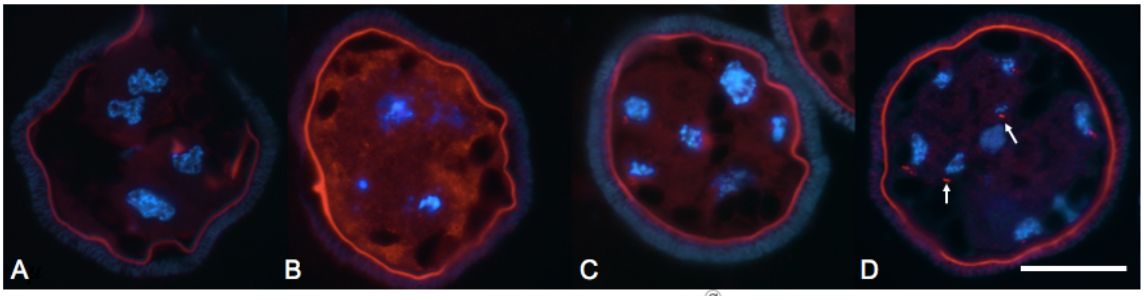


Figure 3-5: The formation and maturation of the blepharoplast visualized with anti-centrin antibody.
Dry microspores were placed into water, and the gametophytes were fixed 3.5-4 h later. After the completion of the 7th division **(A)** centrin (red) was present, but not abundant. Before the 8th division **(B),** centrin is diffusely distributed throughout the spermatogenous cells. During the 8th division, the centrin began to coalesce in distinct locations **(C)**, finally resulting in distinct foci of centrin located adjacent to nuclei **(D)**. Blepharoplasts are indicated by arrows. Sections labeled with DAPI and anti-centrin antibody detected with AlexaFluor594. Bar = 25µm

Aurora kinase has been shown to phosphorylate centrin (Lukasiewicz *et al*., 2011). This phosphorylation helps to stabilize centrin, so that it can remain localized in a microtubule-organizing center. In order to investigate the potential co-localization of centrin and aurora kinase during blepharoplast development, the distribution of aurora kinase and centrin were studied in developing gametophytes using the pan-aurora and centrin antibodies, respectively. The gametophytes were fixed at 3, 3.5, 4, and 4.5 h, and then stained with both centrin (red) and aurora kinase (green) antibodies (Fig 3-6). At 3 h, centrin staining was very low, whereas aurora kinase staining was very apparent and dispersed throughout the spermatogenous cells (Fig 3-6a, e). As the centrin staining began to coalesce into punctate spots, aurora kinase staining remained distributed throughout the spermatogenous cells (Fig 3-6b, f). When the blepharoplasts could be visualized as well defined spots of centrin staining, more aurora kinase staining was seen throughout the spermatogenous the cells (Fig 3-6c, g). Finally, when the bb were formed from the blepharoplast, centrin and aurora kinase co-stained the bb (Fig 3-6d, h).

The overall pattern of aurora kinase staining was that its levels increased during blepharoplast formation, and then it co-localized with centrin at the bb. These observations support the prediction that aurora kinase acts to help stabilize centrin, thus allowing centrin to remain localized in the blepharoplast and the bb in developing gametophytes.

RNAi can be used to cause the elimination of specific mRNAs during organismal development. Thus, it creates a null phenocopy having reduced or no synthesis of the translated protein. By comparing gametophyte development in RNAi-treated vs. control microspores, it was possible to obtain significant insights into the timing and function of

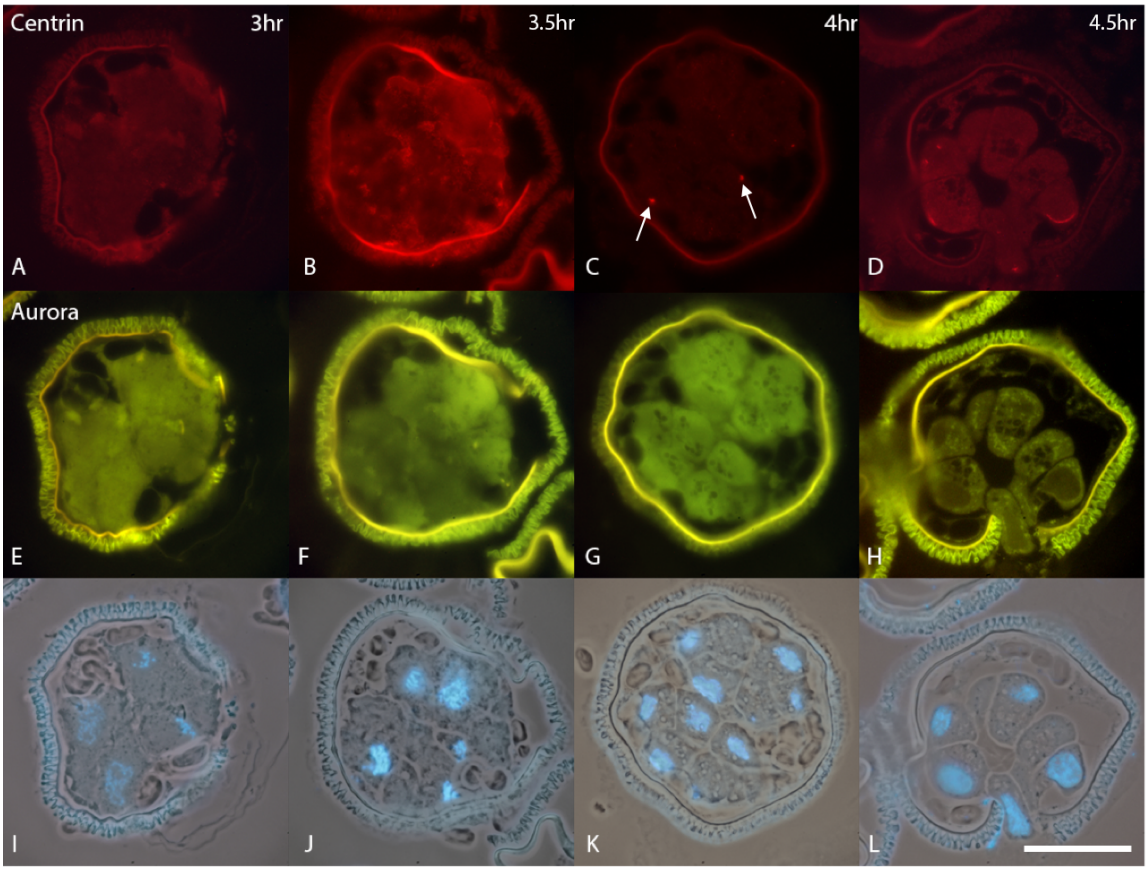


Figure 3-6. Aurora kinase localization coincides with centrin localization.
Dry microspores were placed in water, and the gametophytes were fixed at 3, 3.5, 4, and 4.5 h. Centrin (red) levels were low at 3 h **(A)**, and aurora kinase (green) was detectable in the cytoplasm of the spermatogenous cells **(E)**. After the 7th division (3.5 h) **(I,J)**, centrin was present at high levels, but it was diffusely distributed throughout the cytoplasm. Aurora kinase remained diffuse in the cytoplasm after this division **(F)**. Blepharoplasts, as indicated by arrows, were seen at 4 h **(C)**, just before the 8th and final division. Aurora kinase levels rose just before the 8th division, but the protein remained diffuse in the cytoplasm **(G)**. After the 8th division **(K,L)**, the bb were visualized as distinct foci, and were spread apart from each other**(D)**.Aurora protein was loosely distributed as punctate aggregates in the cytoplasm of the spermatogenous cells **(H)**. The development of the 4.5 h sample seems to be delayed **(L)** compared to the 4 hour sample. The progression of the blepharoplast maturation has continued normally, though. Sections labeled with anti-centrin antibody detected with AlexaFluor594, and anti-aurora kinase antibody detected with AlexaFluor488 and DAPI. Bar = 25µm.

the mRNA transcripts of different aurora kinases and their protein products. All aurora kinase isoforms in developing gametophytes were knocked down simultaneously using double stranded RNA targeting the conserved 5' region. 100, 75, 50, or 25μg of dsRNA was added to the microspores at the onset of hydration, and the developing gametophytes were fixed after 6 h. The different amounts of dsRNA had various cellular effects depending on their concentration. The 100μg dsRNA treatment blocked the rearrangement of the plastids, which is the initial process occurring in hydrated control microspores prior to the first cell division. Centrin staining was diffuse throughout the cytoplasm. In microspores treated with lower concentrations of dsRNA, the gametophytes were observed to undergo more divisions, but centrin staining remained diffuse. In the microspores treated with 50μg dsRNA, the gametophytes progressed through the 7^{th} division, but there was still no evident aggregation of centrin. In the microspores treated with 25μg dsRNA, the gametophytes exhibited a distinct aggregation of centrin at the blepharoplast, but divisions had halted before the 8^{th} division (Fig 3-7).

Hesperadin, a pan aurora inhibitor, was added to the microspores at various concentrations and incubated for 8 h. Hesperadin acts at the ATP binding site in the catalytic pocket of aurora kinase. This site is conserved in all isoforms of the protein. Hesperadin treatments were used to verify that total knockdowns observed with RNAi are attributable to their effects on aurora kinase protein. In these experiments, heat-inactivated hesperadin was used as a negative control. Normal divisions and centrin aggregation were observed in developing gametophytes following the addition of 10 μM heat-inactivated hesperadin to hydrating microspores (Fig 3-8b). Higher concentrations of hesperadin resulted in progressively earlier developmental arrests in the gametophytes

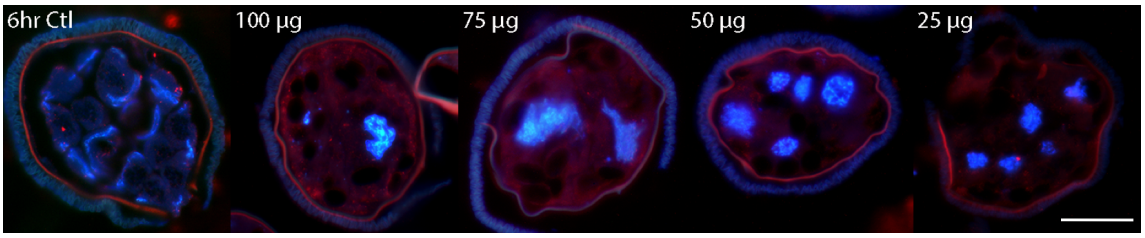


Figure 3-7. Aurora kinase is essential in the formation and maturation of the blepharoplast into basal bodies.
Varying concentrations of dsRNA, targeting the conserved 5' region of mRNA, were added at 0 h to hydrating microspores. The gametophytes were fixed at 6 h. The control shows elongated nuclei, and punctate staining of the bb. The lower concentrations of dsRNA allowed more divisions in the developing gametophytes. Centrin staining, while punctate at times, remained diffuse throughout the cytoplasm in the 100 and 50µg treatments. In the 25µm treatment, a blepharoplast is seen, but the final division has not been completed. Sections labeled with anti-centrin antibody detected with AlexaFluor594 and DAPI. Bar = 25µm.

(Fig 3-8d, e, f, j, k). Only the initial division producing the prothallial cell was completed in the microspore exposed to the highest dose of 10 μM hesperadin (Fig 3-8k). The aggregation of centrin staining was also less evident, mimicking what was seen in the pan Aurora knockdowns (Fig 3-8c, g, h). Centrin aggregation was only seen in the 1 μM treatment (Fig 3-8c). When 10μM hesperadin was added at 3 h, the cell divisions were not affected, but centrin did not coalesce into the blepharoplasts (Fig 3-8i, l). This result suggests that aurora kinase is not needed for the 7th division to occur in untreated cells, but that it is necessary for the centrin aggregation in the blepharoplast.

Each isoform of aurora kinase has a unique N-terminal end, which suggests a unique function for each isoform, as is seen in other organisms. Each isoform was individually knocked down using dsRNA targeted to its unique 5' end. Double stranded RNA was introduced to the microspores at the onset of hydration, and the gametophytes were fixed at 8 h. All treatments caused significant reductions in centrin aggregation. The Ak1a knockdown completed the 7th division, but the nuclei were large and elongated (Fig 3-9g). Centrin was present in the cytoplasm as diffuse aggregates, but there was much less aggregation than what was observed in untreated gametophytes (Fig 3-9b). Ak1b knockdowns successfully progressed through the 6th division, but centrin did not form large aggregates (Fig 3-9h). Centrin was distributed throughout the cytoplasm as small aggregates, as was also seen in the Ak1a knockdowns (Fig 3-9c). Ak2 knockdown resulted in little centrin aggregation, as most of the protein was distributed throughout the cytoplasm (Fig 3-9d). Development was arrested before the 8th division, and the nuclei did not appear different from those in untreated gametophytes (Fig 3-9i). Development was arrested after the 2nd division in Ak3 knockdowns, and the nuclei exhibited a very

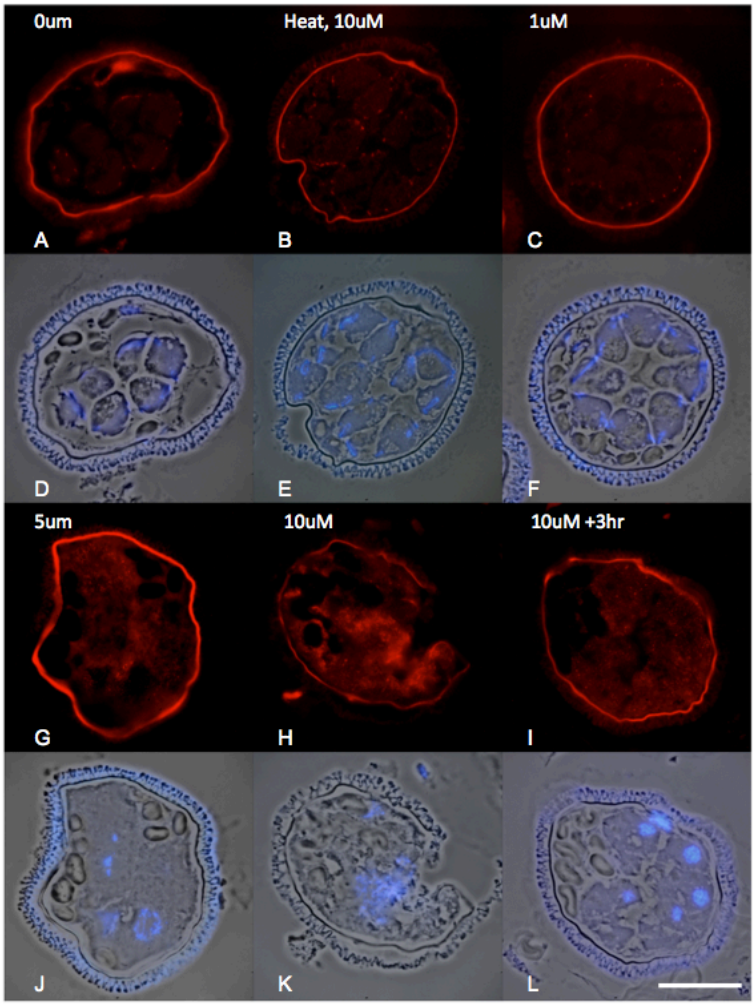


Figure 3-**8**. **The aurora kinase inhibitor, hesperadin, prevents the aggregation of centrin protein.** Microspores were treated with various concentrations of hesperadin at 0 and 3 h of gametophyte development, then incubated for 8 h. Centrin (red) aggregation was blocked and no blepharoplasts formed in all inhibitor-treated gametophytes **(C,G-I)**. Basal bodies became less apparent at higher hesperadin concentrations. In addition, cell divisions are slowed or arrested by the 5 and 10µM treatments **(J, K)**. When the inhibitor (10µM) was added at 3 h into gametophyte development, there was little effect on the progression of cell divisions, but centrin failed to aggregate into blepharoplasts **(I, L)**. The gametophytes exposed to heat-treated inhibitor **(B, E)** progressed as far as the untreated control gametophytes **(A, D)**. Sections labeled with DAPI and anti-centrin antibody detected with AlexaFluor594 secondary. Bar = 25µm.

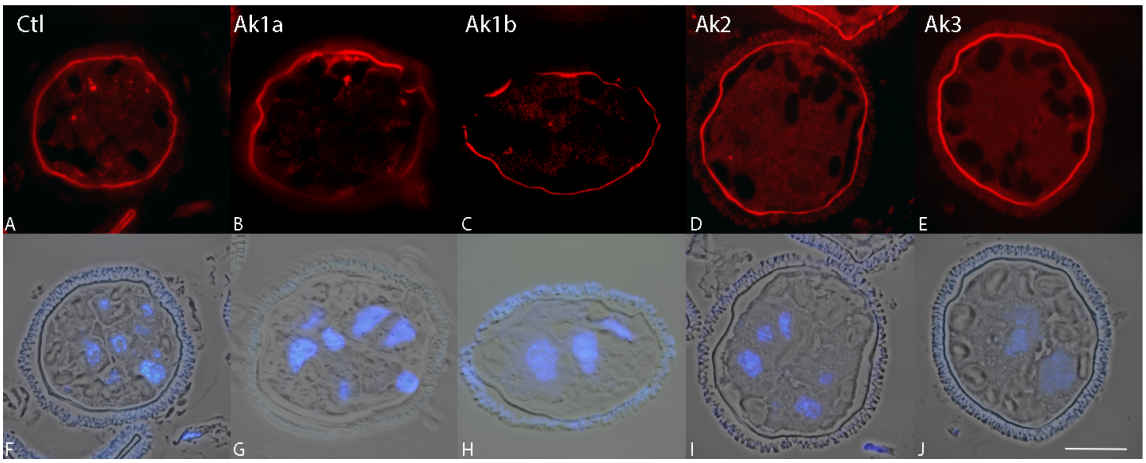


Figure 3-9. RNAi knockdowns of aurora kinase isoforms.
Dry microspores were placed in water with 50µg dsRNA made to target the 5'unique portions of the transcript of each aurora kinase. Gametophytes were fixed at 5 hours after spore hydration. All RNAi-treated gametophytes showed defects in nuclear shapes (blue) and centrin aggregation (red). The control \completed the 8th division, and the blepharoplasts were seen to begin breaking into individual bb **(A, F)**. The Ak1a knockdown almost reached the 8th division, but the nuclei were elongated **(G),** and centrin showed a diffuse pattern of punctate aggregation in the cytoplasm **(B)**. Ak1b knockdowns progressed through the 6th division **(H)**, and exhibited the diffuse pattern of punctate centrin aggregation also seen in Ak1a knockdown **(C)**. The Ak2 knockdown resulted in cell divisions that were halted after the 7th division **(I)**. The centrin staining is very diffuse in the cytoplasm with little obvious aggregation **(D)**. The Ak3 knockdown caused early arrest after the 2nd division and very little aggregation of centrin protein. Centrin was barely detectable **(E, J)**. Sections labeled with DAPI and anti-centrin antibody detected with AlexaFluor594 secondary. Bar = 25µm.

irregular morphology (Fig 3-9e). Very little centrin was seen, but what was present was punctate (Fig 9j). In other systems, centrin is reported to be phosphorylated by aurora kinase, which causes centrin to become stably localized in microtubule-organizing centers (Lukasiewicz *et al*., 2011).

Phospho-mimics with a mCherry tag were created to characterize centrin localization in the developing gametophyte after the addition of ssRNA constructs. The amino acid serine-172, which is phosphorylated by aurora kinase (Lukasiewicz *et al*., 2011), was modified to show how aurora kinase affects centrin localization. Serine-172 is located just after the EF-hand domain near the C-terminal end of centrin. This amino acid was altered so that it mimics non-phosphorylatable state (via a serine-to-alanine replacement) or a constitutive phosphorylated state (via serine-to-aspartic acid replacement) state of the protein. Specifically, if the hydroxyl R-group in serine is replaced with the methyl R-group in alanine, then it mimics a non-phosphorylatable state. Similarly, the negative charge of phosphoserine can be mimicked with aspartate, which has a terminal carboxyl group acting chemically like a phosphate group. Transient translation of unaltered centrin results in an overall increase of centrin concentration with abundant aggregations in the cytoplasm as the gametophytes undergo typical sequence of developmental events (Fig 3-10b). The unphosphorylated phosphomimic, S172A, exhibited very limited aggregation of centrin (Fig 3-10c). The final division was completed in these gametophytes, but their nuclei were not elongated like the nuclei of untreated gametophytes. The phosphorylated phosphomimic, S172D, resulted in more extensive centrin aggregation, although it was somewhat less that what was observed in untreated gametophytes (Fig 3-10d), and the nuclei were very large and malformed.

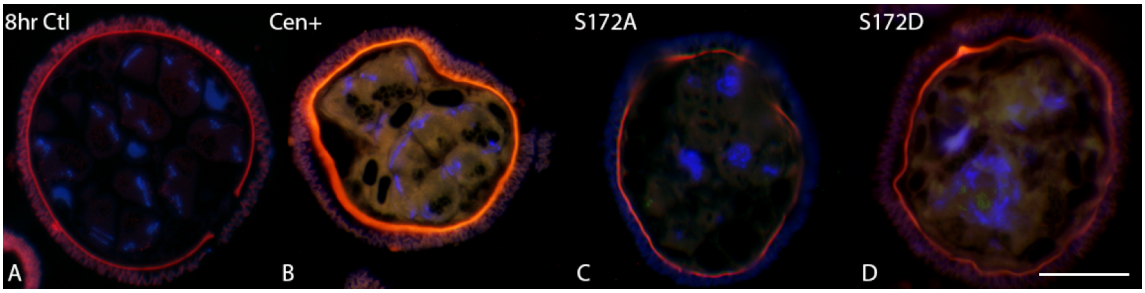


Figure 3-10. Phosphorylation of Centrin is necessary for blepharoplast formation. Dry microspores were placed in water with 100µg of ssRNA for each wild-type and phosphomimic constructs of centrin for 8 h. The 8-h control underwent the final 8th division, and the bb spread along the dorsal side of the elongated nucleus **(A)**. When wild-type centrin is transiently translated **(B),** the gametophytes exhibited high levels of centrin accumulation in numerous aggregations. When S172 was changed to an alanine, mimicking non-phosphorylatable state **(C),** the centrin exhibited relatively few aggregations, and development arrested before the 8th division. When S172 was changed to aspartate, mimicking a phosphorylated protein **(D)**, the gametophytes exhibited numerous centrin aggregations, plus an increased amount of diffuse protein in the cytoplasm. The nuclei were very large and malformed. Centrin, red; mCherry-centrin, green; DAPI, Blue. Bar = 25µm.

Chapter 4: Discussion and Conclusions

The proper segregation of replicated chromosomal DNA in the cell is essential to the survival of any organism. In the male gametophyte of *M. vestita*, blepharoplast separation in the 8th division is critical for the final division to ensure that each spermatid receives a complete set of chromosomes to ensure successful reproduction and thus the survival of the next generation. This process requires the activation of a complex network of structural and regulatory proteins. The hub of this network is the mitotic spindle that is anchored at either pole by the centrosomes that contain centrioles in animal cells (Mitchison and Salmon, 2001). The spindle acts to align the chromosomes properly at the metaphase plate and then serves to move each set of chromatids toward opposite poles at which will form the new daughter cells.

A large and diverse array of proteins is required to complete the duplication, separation, and movement of the chromosomes. Many different structural proteins are needed to build the mitotic spindle, including tubulin that polymerizes to form the microtubules (MT's) arching between the two poles. In addition, γ-tubulin, centrin and Sfi1p are a few of the proteins that form the structure of the centrosome's pericentriolar material (PCM) that nucleates and anchors the MT's (Salisbury, 2004). Motor proteins are also involved in the formation of the spindle throughout mitosis, the separation of the chromatids, and cytokinesis. The entire process of mitosis and cytokinesis is regulated by complex gene regulatory networks acting in response to different signal transduction networks. Tight regulation of gene expression ensures that the proper proteins are present at the correct time and in the right position to carry the complex molecular and cellular events associated with cell division.

Aurora kinase expression in the developing male gametophytes of *M. vestita*

Three aurora isoforms were found in *M. vestita*, each with a unique 3' and a conserved catalytic domain, which is similar to the structure found in aurora kinases in other organisms. One of the isoforms designated as mvAK1 was found to have a splice variant with a unique mRNA expression pattern during gametophyte development. When the three isoforms were aligned with atAUR1 and 2 from *A. thaliana*, mvAK2 was more closely related to the *A. thaliana* isoforms. Isoforms mvAK1 and 3 diverged earlier from the common ancestor of mvAK2 and the *A. thaliana* isoforms. This earlier divergence suggests that the different *M. vestita* isoforms may have different roles during cell division and gametophyte development.

Each of the three isoforms has a unique mRNA abundance pattern throughout the first eight hours of gametophyte development. Isoform mvAK1a exhibited a peak mRNA level at 1h, and then steadily decreases over the next 8 h. Isoforms 1b, 2, and 3 have peak mRNA levels at 3 h, just before the 7^{th} division, (mvAK3) and 4 h, the 8^{th} division (mvAK1b, 2). These patterns also coincided with the early stages of formation and the assembly, of the blepharoplast at 3 and 4h, respectively. Western blots of protein samples treated with SDS confirmed the presence of aurora kinase proteins at 3 and 4 h. One band coincided with the predicted average isoform size of 34 kDa. The SDS gels showed two other bands of higher molecular weights, which may possibly be attributed to the proteins retaining their tertiary structure in the presence of SDS.

These results suggest that the unique N-terminal region may allow each aurora kinase isoform to have a distinct function in *Marsilea*, which is also seen in aurora

kinases in other organisms. Each unique sequence forms a distinct N-terminal structure that is able to interact with a distinct substrate. The results discussed on the following section support the hypothesis that aurora kinase may be necessary for the formation of the blepharoplast, and its maturation into the multilayered structure (MLS).

Interaction of aurora kinase and centrin in the formation of the blepharoplast

The role of aurora kinases in the *de novo* formation of the blepharoplast was studied by looking at the interaction of centrin with aurora kinases in developing male gametophytes of *M. vestita*. After the 7th division in male gametophyte development, an increase in diffuse centrin staining is observed in the cytoplasm. An increase in aurora kinase levels coincides with the coalescence of centrin in the blepharoplasts during the 8th division. Both aurora kinase RNAi and the pan-aurora inhibitor hesperadin prevent the formation of the blepharoplast and the progression of development past the 7th division, which argues that the interaction of centrin and aurora kinase is crucial for the formation of blepharoplasts and the subsequent formation of bb.

Each aurora kinase isoform has a unique 5' terminal sequence, suggesting that each isoform has a unique role during development. Each isoform knockdown resulted in a unique morphology after a 5 h incubation following the completion of all cell divisions. RNAi knockdowns were used to identify the specific divisions that required the mRNA transcripts of each isoform in order to continue gametophyte development, which provided important clues about the function of each isoform. The RNAi of isoforms mvAK1a and 2 resulted in some centrin aggregation and prevented the 8th division, indicating a possible role in the final stabilization and subsequent aggregation of

centrin to form the blepharoplast. The isoform mvAK1b knockdown caused development to halt at the 6th division, but diffuse centrin was still observed in the cytoplasm, which is similar to its staining patterns in untreated gametophytes after the 7th division. The knockdown of mvAK3 halted development after the 2nd division. This isoform is apparently essential in the initial processes of development, and may be interacting with a substrate other than centrin because centrin was not present during early development. This evidence supports the interpretation that each isoform has a unique function during gametophyte development

Centrin phospho-mimics were made to specifically look at the role of centrin aggregation due to phosphorylation by aurora kinase. The non-phosphorylatable phospho-mimic resulted in very few centrin aggregates. The reverse was seen with the continuously phosphorylated phospho-mimic which resulted in an abundance of aggregates and interactions. These observations suggest that the phosphorylation of centrin by aurora kinase is critical for centrin aggregation and the initial steps in blepharoplast formation.

Conclusion and future prospects

Each of the 3 isoforms of aurora kinase active in the male gametophyte of *M. vestita* plays a unique and essential role during gametophyte development, but the gametophyte cannot complete its development without the actions of all 3 isoforms. The evidence presented in this thesis suggests that the isoforms of mvAK1a and 2 may be responsible for phosphorylating centrin as the critical step in the *de novo* formation of the blepharoplast to serve as a MTOC for the final spermatogenous division and subsequent

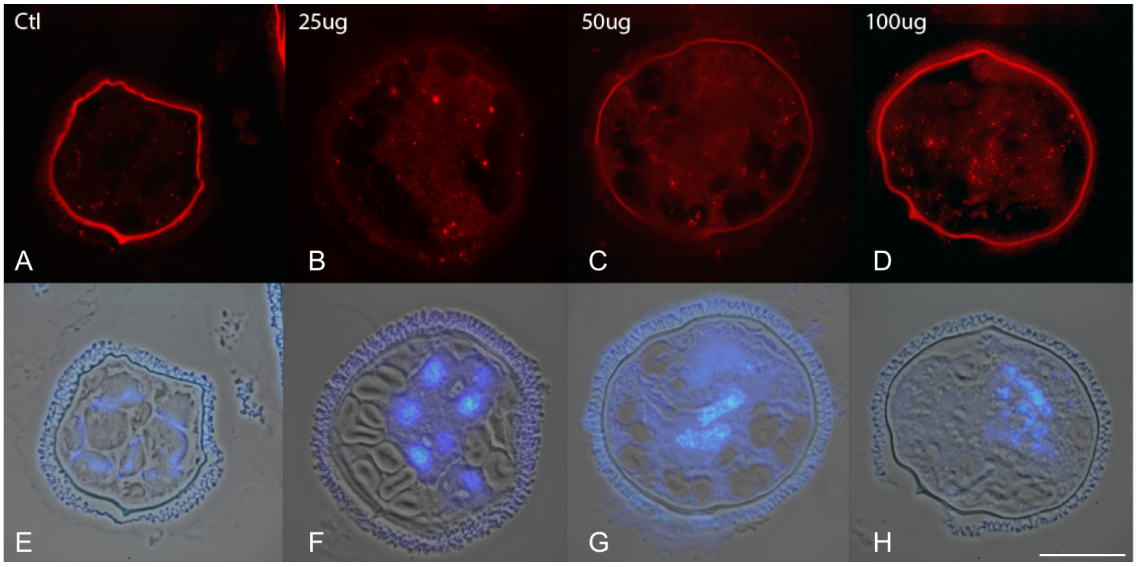
basal body formation. It is likely that aurora kinases may also play a similar role in other organisms that have a *de novo* pathway for centrosome formation.

The mechanism of blepharoplast formation remains largely unknown. One productive area of future research is to attempt to identify additional proteins which form the blepharoplast. The function of homologous proteins in other systems could provide a clue into their role in the *de novo* formation of the blepharoplast. It is informative to search for the candidate blepharoplast proteins among those proteins that are already known to be present in the PCM of centrosomes. A second question is the substrate of each isoform. The sensitivity of different stages of gametophyte development to RNAi knockdowns suggests that centrin is not the only phosphorylation target in developing gametophytes. Other substrates if identified could provide more insight into the mechanisms of aurora kinase activity in gametophyte development.

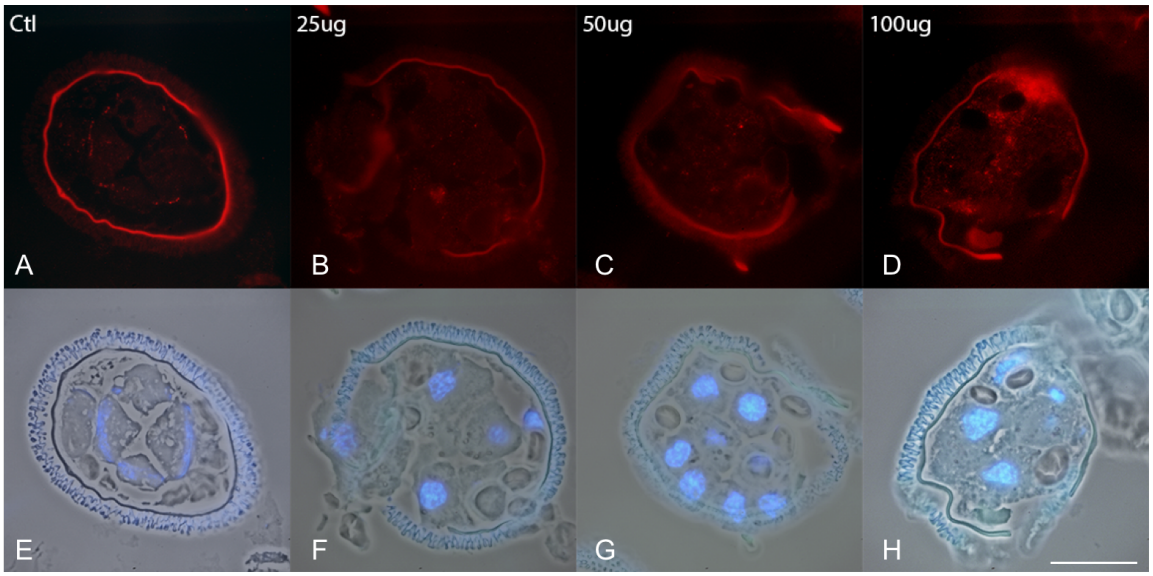
Appendix 1: Additional RNAi knockdown experiments

It is reasonable to suspect that the components of the centrosomes in animal cells are likely to be similar to components of the blepharoplast of *Marsilea vestita* because both function as MTOCs. This is consistent with the observation that centrin and aurora kinase interact in the formation of the blepharoplast. To further expand on this idea, proteins that are involved with the centrosome form and function were knocked down with RNAi using dsRNA complementary to common portions of each transcript of interest. The morphology of sectioned gametophytes was then used to determine the effect of each knockdown on gametophyte development. These experiments serve as a starting point to further understand the *de novo* formation of the centrosome and the blepharoplast in plant cells.

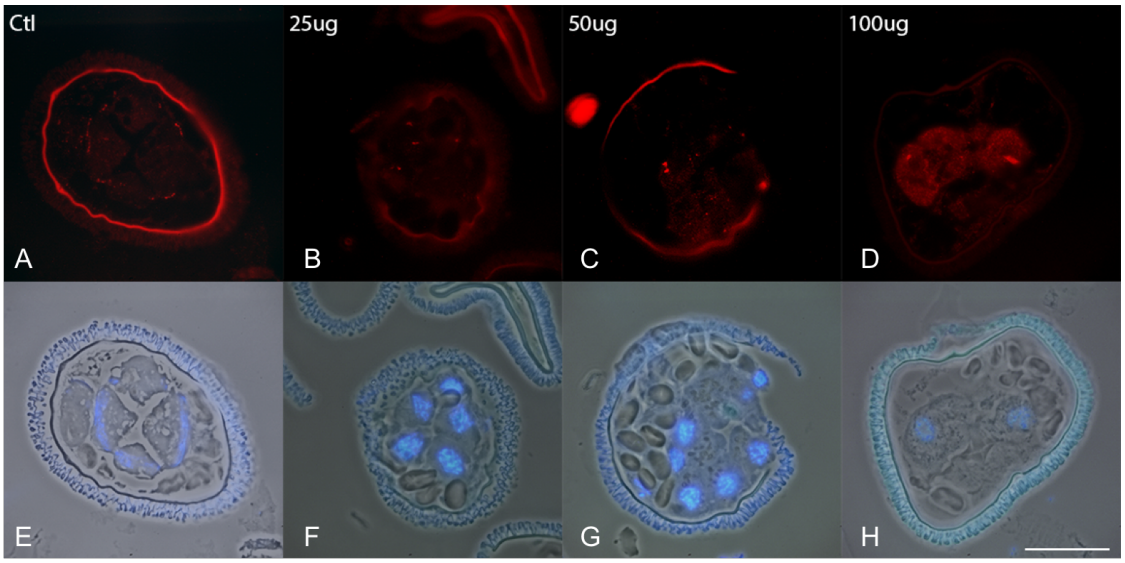
Appendix fig.	Transcript	Putative function
1.	Nedd1	Interacts with γ-tubulin during mitosis
2.	GCP2	γ-tubulin complex protein 2
3.	dynactin	Aids in bidirectional movement of organelles and vesicles and in spindle assembly
4.	Cep78	Regulator of centrosomal function, and required for ciliogenesis.
5.	GCP5	γ-tubulin complex protein 5
6.	centriolin	Involved with centrosome maturation and cytokinesis
7.	Tub γ-1 Chain	PCM component required for MT nucleation, centrosome duplication and spindle formation
8.	GCP3	γ-tubulin complex protein 4
9.	Sfi1	Forms elastic connections with centrin in the PCM
10.	Cep120	Coiled-coil protein which couples the nucleus and centrosome
11.	Cep170	Localized to sub-distal appendages of mature centrioles
12.	GCP4	γ-tubulin complex protein 4
13.	cntrobin	Required for centriole duplication and cytokinesis
14.	Cep41	Required during ciliogenesis



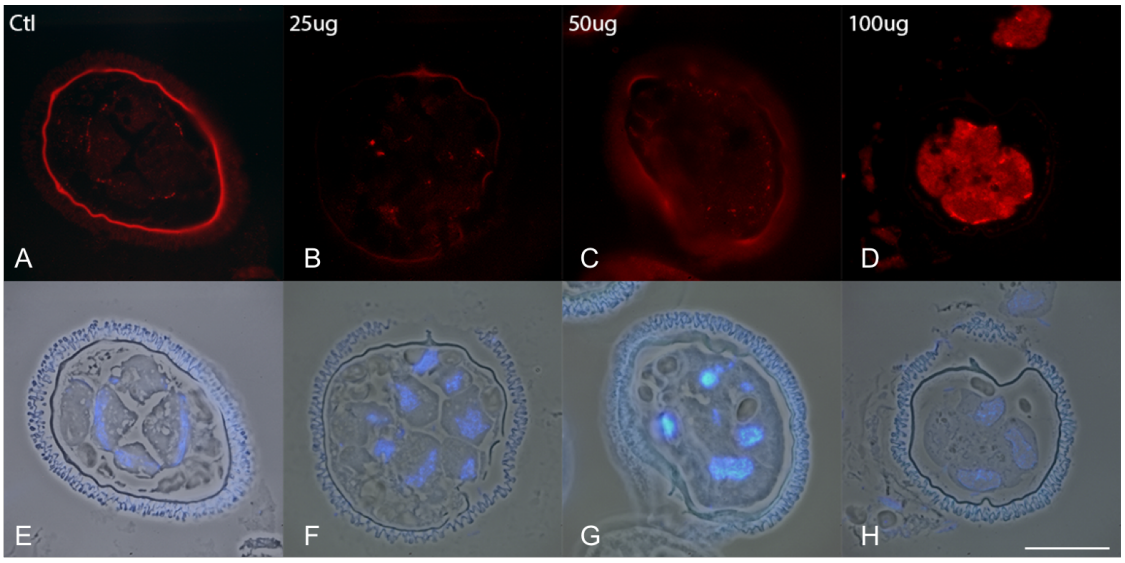
Appendix Fig. 1-1. Knockdown of Nedd1
Dry microspores were placed in water with varying concentrations of dsRNA complementary to a unique portion of the Nedd1 transcript. The control sample had completed all the spermatogenous divisions, and the nucleus has elongated and spiraled (**E**). Centrin has aggregated and localized to the traditional location of the bb. The aggregates were distributed along the side of the nucleus, seen as arcs (**A**). The addition of the dsRNA caused development to terminate prematurely in all of the treated samples. The 25µg sample completed the final spermatogenous division, but the nuclei remain round and have not elongated (**F**). When the concentration of dsRNA is increased, cellular divisions are stopped increasingly earlier. The 50µg sample stopped dividing after two antheridial cells were formed (**G**). The nuclei had an irregular longer shape. The plastids of the 100µg sample had rearranged and one very large nucleus was present. The nucleus is much larger than what is traditionally seen at this stage in development (**H**). Centrin aggregation was present in all of the treated samples. The distribution of the aggregates was no longer restricted to the nuclei. In all the treated samples, a high density of punctate centrin aggregates were distributed throughout the cytoplasm (**B-D**). Some aggregates seen in the 25µg sample were larger than those seen in the control (**B**). The 100µg sample had areas where the aggregates were more condensed than the diffuse aggregates seen in the rest of the cytoplasm (**D**). Sections labeled with DAPI and anti Centrin antibody detected with AlexaFluor594 secondary. Bar = 25µm.



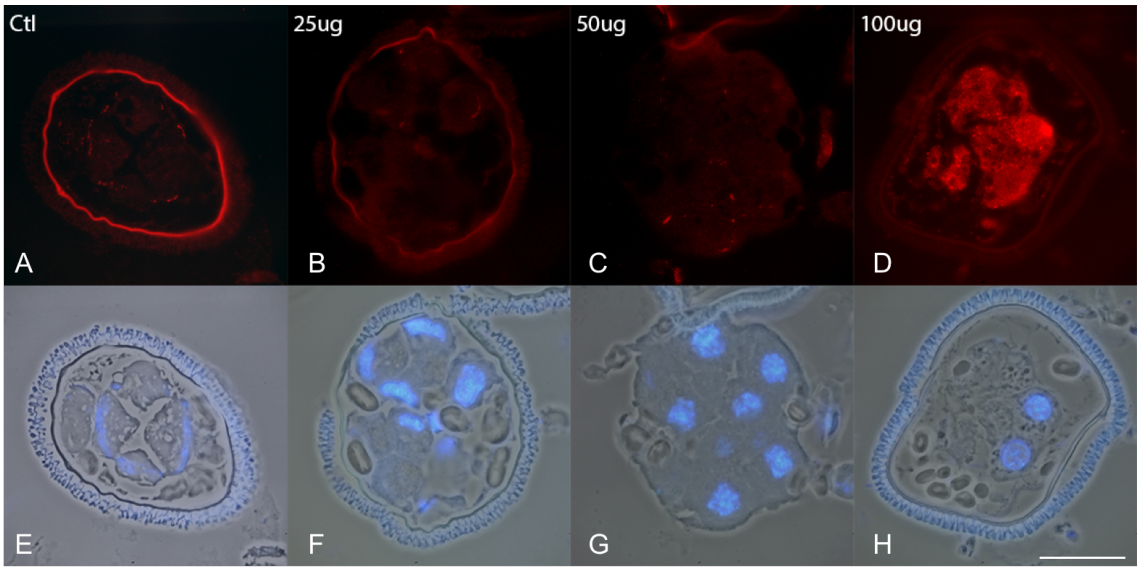
Appendix Fig. 1-2. Knockdown of GCP2
Dry microspores were placed in water with varying concentrations of dsRNA complementary to a unique portion of the GCP2 transcript. The control sample had completed all the spermatogenous divisions, and the nucleus has elongated and spiraled (**E**). Centrin has aggregated and localized to the traditional location of the bb. The aggregates were distributed along the side of the nucleus, seen as arcs (**A**). The addition of the dsRNA caused development to terminate prematurely in all of the treated samples. The 25 and 50µg samples completed the final spermatogenous division (**F**, **G**) while the 100µg sample stopped after the 7th division (**H**). Some aggregation of centrin is seen in the treated samples, diffuse centrin staining is also seen in the cytoplasm of the spermatogenous cells (**B-D**). The 25µg sample has a loose aggregation of centrin located near the nucleus (**B**). The centrin aggregates in the 50µg sample are small and punctate. The aggregates have a range of sizes, most are at the lower end of the range (**C**). The 100µg sample has high levels of centrin staining. Most of the staining is diffuse in the cytoplasm, but at a high density (**D**). Some of the higher density regions are located near the nuclei. Sections labeled with DAPI and anti Centrin antibody detected with AlexaFluor594 secondary. Bar = 25µm.



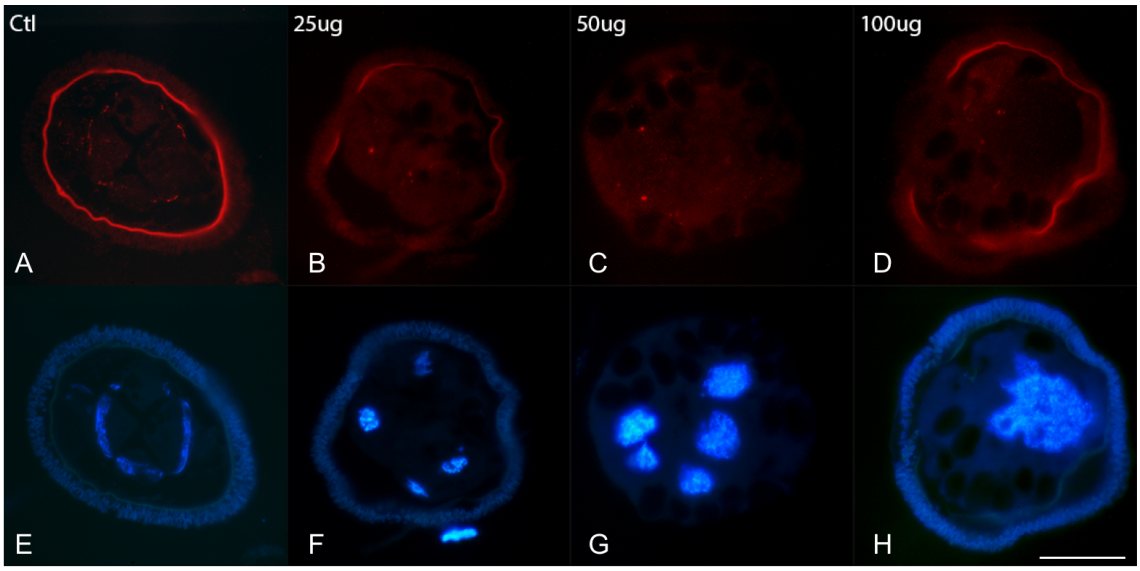
Appendix Fig. 1-3. Knockdown of dynactin
Dry microspores were placed in water with varying concentrations of dsRNA complementary to a unique portion of the dynactin transcript. The control sample had completed all the spermatogenous divisions, and the nucleus has elongated and spiraled (**E**). Centrin has aggregated and localized to the traditional location of the bb. The aggregates were distributed along the side of the nucleus, seen as arcs (**A**). The addition of the dsRNA caused development to terminate prematurely in all of the treated samples. All of the treated samples completed the final spermatogenous division, but did not progress to differentiation. The nuclei were relatively small and rounded (**F-H**). The aggregates were distributed throughout the cytoplasm, and have a range of sizes (**B-D**). Larger and brighter aggregates were also present in all of the treated samples. The 25µg sample was sample had the smallest of the brighter aggregates, with the size increasing as the dsRNA concentration increased (**B-H**). Sections labeled with DAPI and anti Centrin antibody detected with AlexaFluor594 secondary. Bar = 25µm.



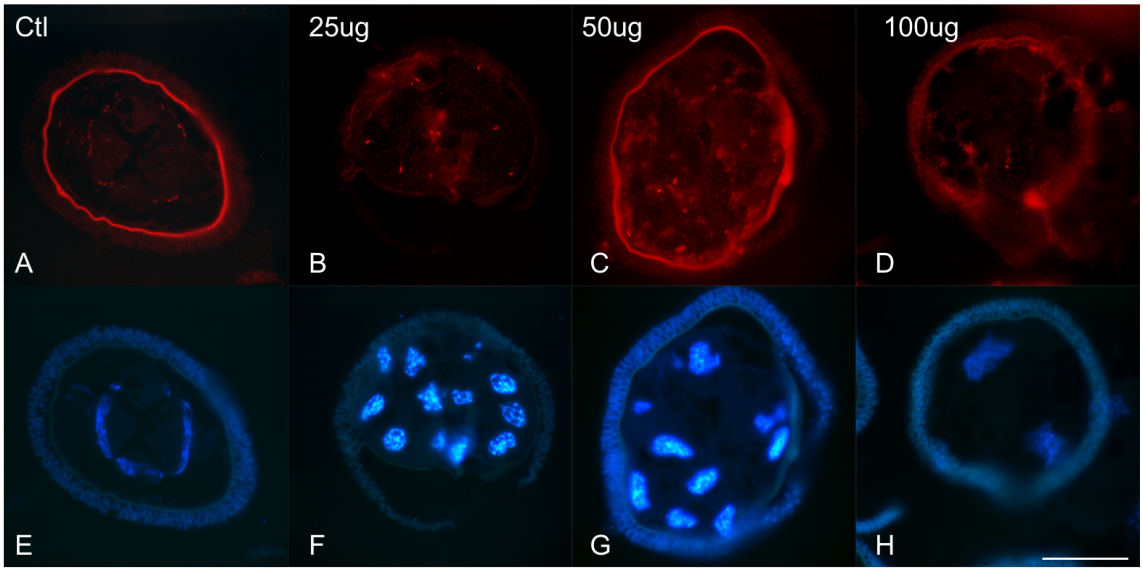
Appendix Fig. 1-4. Knockdown of Cep78
Dry microspores were placed in water with varying concentrations of dsRNA complementary to a unique portion of the Cep78 transcript. The control sample had completed all the spermatogenous divisions, and the nucleus has elongated and spiraled (**E**). Centrin has aggregated and localized to the traditional location of the bb. The aggregates were distributed along the side of the nucleus, seen as arcs (**A**). The addition of the dsRNA caused development to terminate prematurely in all of the treated samples. The 25µg sample completed the last spermatogenous division, but the nuclei were not elongated. The nuclei were small with an irregular shape (**F**). The 50µg sample stopped dividing after the completion of the 8th division. The nuclei had an irregular shape and does not have a consistent size throughout the spermatogenous cells in microspore (**G**). The 100µg sample arrested at the completion of the 7th division. The nuclei have elongated compared to the uniform round nuclei which were normally seen in untreated cells after this division (**H**). Low levels of diffuse centrin staining was seen in the 25 and 50µg. Individual centrin aggregates located near nuclei were seen in the treated samples (**B**, **C**). These aggregates were not distributed along the nuclei as was seen in the control (**A**). Centrin aggregates were seen in the 100µg sample, but very high levels of diffuse staining was present throughout the cytoplasm (**D**). Sections labeled with DAPI and anti Centrin antibody detected with AlexaFluor594 secondary. Bar = 25µm.



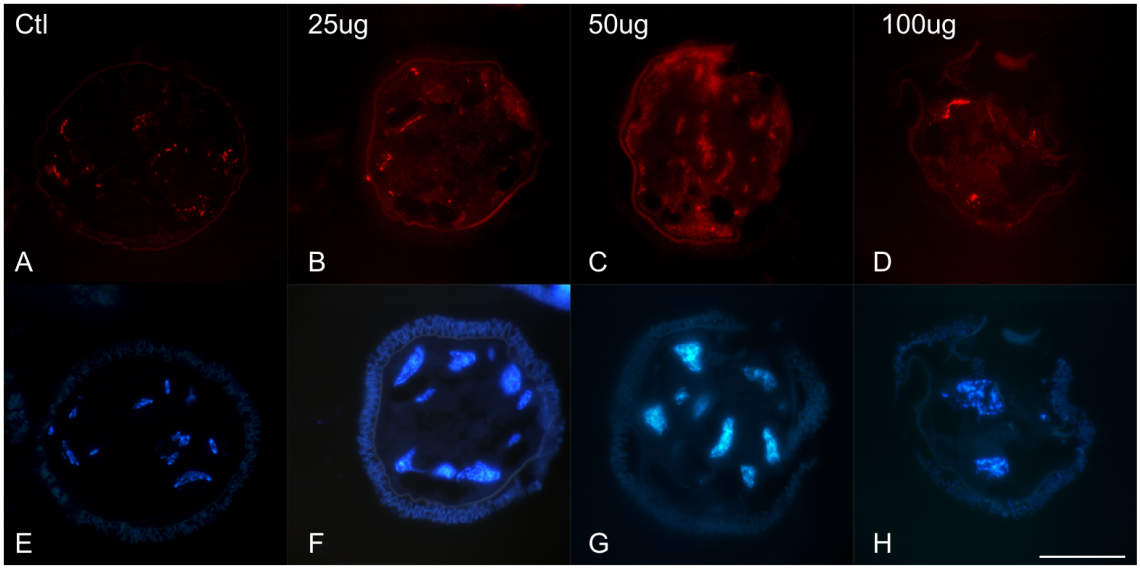
Appendix Fig. 1-5. Knockdown of GCP5
Dry microspores were placed in water with varying concentrations of dsRNA complementary to a unique portion of the GCP5 transcript. The control sample had completed all the spermatogenous divisions, and the nucleus has elongated and spiraled (**E**). Centrin has aggregated and localized to the traditional location of the bb. The aggregates were distributed along the side of the nucleus, seen as arcs (**A**). The addition of the dsRNA caused development to terminate prematurely in all of the treated samples. All the treated samples completed the final spermatogenous division. Some of the nuclei in the 25µg sample had elongated (**F**). The elongated nuclei did not elongate to the same extent seen in the control and have irregularities in the shape and size of the nuclei (**F**). The nuclei in the 50 and 100µg samples had the morphology as nuclei in untreated microspores after the final spermatogenous division (**G**, **H**). Aggregation of centrin were seen in all the treated samples. The aggregates seen in the 25 and 50µg samples localized to the nuclei. In the 25µg sample, the distribution of the aggregates along the edge of the nuclei was similar to the control (**B**). Some aggregates in the 50µg sample were localized near the nuclei, but not all. The aggregates were larger than those seen in the control (**C**). The level of diffuse centrin staining in the cytoplasm of the 100µg sample was higher than was seen in the other treated samples. Aggregates were also present with a diverse range of sizes (**D**). Sections labeled with DAPI and anti Centrin antibody detected with AlexaFluor594 secondary. Bar = 25µm.



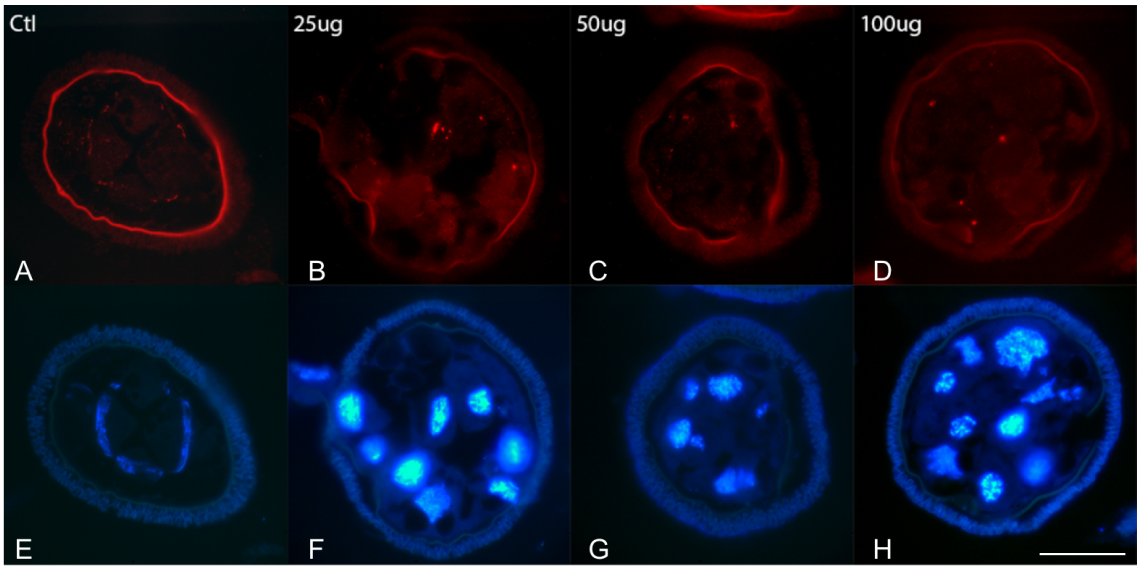
Appendix Fig. 1-6. Knockdown of centriolin
Dry microspores were placed in water with varying concentrations of dsRNA complementary to a unique portion of the centrolin transcript. The control sample had completed all the spermatogenous divisions, and the nucleus has elongated and spiraled (**E**). Centrin has aggregated and localized to the traditional location of the bb. The aggregates were distributed along the side of the nucleus, seen as arcs (**A**). The addition of the dsRNA caused development to terminate prematurely in all of the treated samples. The 25µg sample stopped at the 8th division, the nuclei were small and not uniformly round (**F**). The 50µg sample stopped dividing after the 7th division (**G**). The nuclei were much larger than those seen in untreated microspores after this division. The 100µg sample did not undergo any divisions, but there was rearrangement of the plastids due to rehydration of the microspore (**H**). The nucleus was large with an irregular shape. Centrin aggregates were present in all of the samples with very little diffuse staining in the cytoplasm (**B-D**). All of the aggregates were of a similar size. Sections labeled with DAPI and anti Centrin antibody detected with AlexaFluor594 secondary. Bar = 25µm.



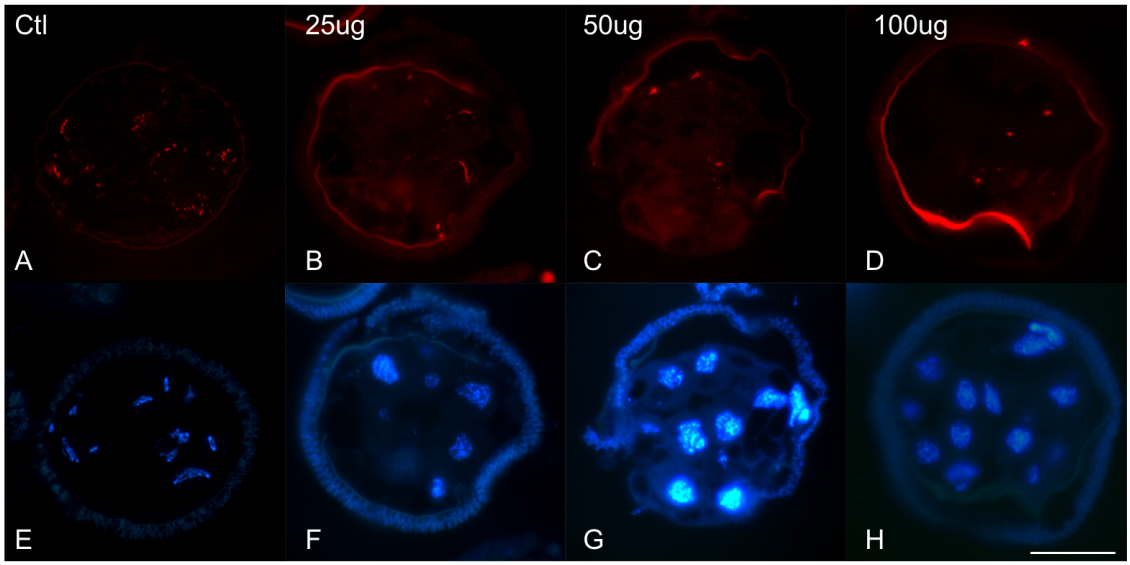
Appendix Fig. 1-7. Knockdown of Tub γ-1 Chain
Dry microspores were placed in water with varying concentrations of dsRNA complementary to a unique portion of the Tub γ-1 Chain transcript. The control sample had completed all the spermatogenous divisions, and the nucleus has elongated and spiraled (**E**). Centrin has aggregated and localized to the traditional location of the bb. The aggregates were distributed along the side of the nucleus, seen as arcs (**A**). The addition of the dsRNA caused development to terminate prematurely in all of the treated samples. The 25 and 50µg samples completed the final spermatogenous division (**F**, **G**). Neither sample had elongated nuclei. The nuclei of the 25ug sample had a similar size as untreated samples after the final division, but had some distortion of the usual rounded (**F**). The nuclei in the 50µg sample had an irregular elongated shape (**G**). The 100µg sample has stopped dividing with 2 antheridial cells formed (**H**). The nuclei also had an irregular shape. Aggregation of centrin was seen in all of the treated samples to varying degrees. The 25µg sample had aggregates localized to the nucleus. Very little diffuse centrin staining was seen in the cytoplasm (**B**). The aggregates seen in the 50µg sample were not localized to the nuclei, and there was a large amount of diffuse centrin staining in the cytoplasm (**C**). Less diffuse centrin was seen in the 100µg sample and there were some small aggregates present which were not localized to the nuclei (**D**). Sections labeled with DAPI and anti Centrin antibody detected with AlexaFluor594 secondary. Bar = 25µm.



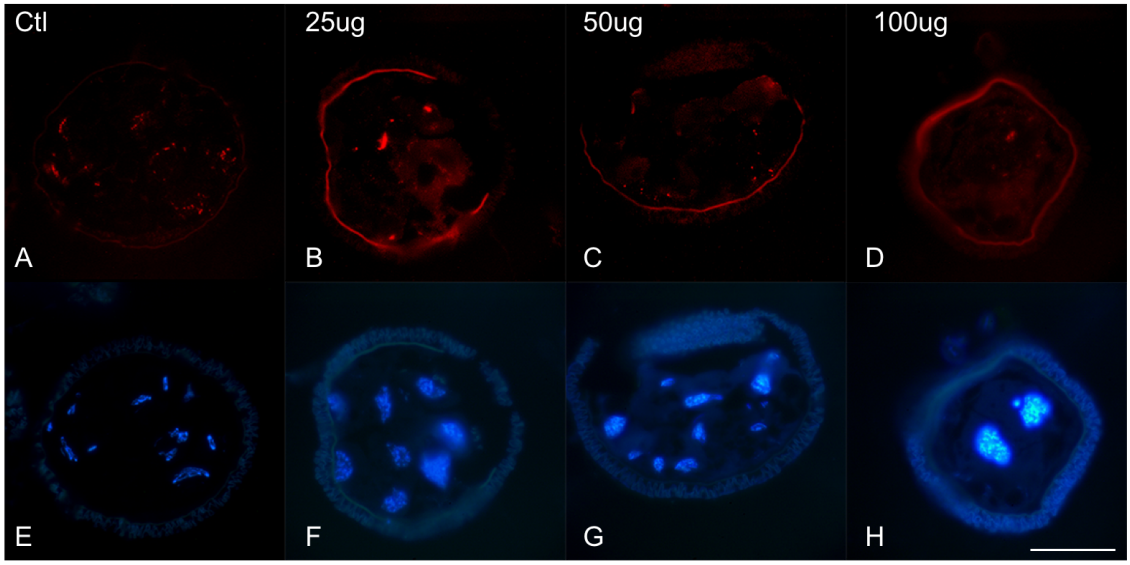
Appendix Fig. 1-8. Knockdown of GCP3
Dry microspores were placed in water with varying concentrations of dsRNA complementary to a unique portion of the GCP3 transcript. The control sample had completed all the spermatogenous divisions, and the nucleus has elongated and spiraled (**E**). Centrin has aggregated and localized to the traditional location of the bb. The aggregates were distributed along the side of the nucleus, seen as arcs (**A**). The addition of the dsRNA caused development to terminate prematurely in all of the treated samples. The 25 and 50µg samples had completed the final spermatogenous division. The nuclei in both sample had begun to elongate, but they have not completed this step (**F**, **G**). The 100µg sample stopped dividing after the 2 antheridial cells formed (**H**). The nuclei are not the uniform size and shape seen in the controls at this stage. Centrin staining was seen in all treated samples. All treated samples had aggregates and diffuse staining in the cytoplasm. The aggregates in all the samples were localized to the nuclei (**B- D**). The 25µg sample had punctate foci distributed similarly to centrin after nuclear elongation (**B**). Large loose aggregates were seen in the 50µg sample (**C**). The 100µg sample had large dense aggregations (**D**). The aggregates are larger than those seen in the 25µg sample. Sections labeled with DAPI and anti Centrin antibody detected with AlexaFluor594 secondary. Bar = 25µm.



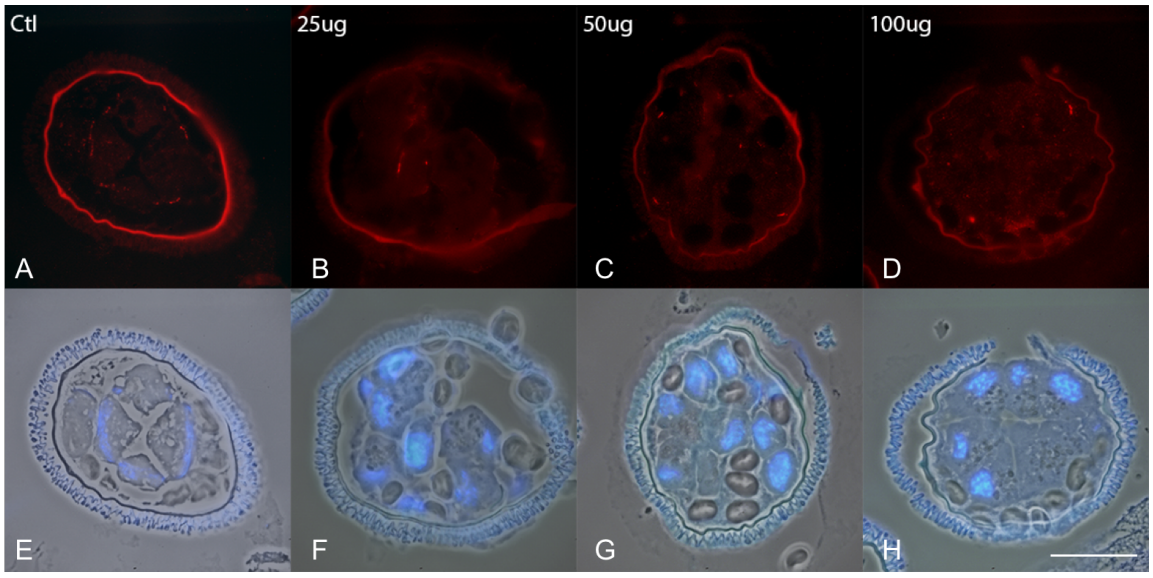
Appendix Fig. 1-9. Knockdown of Sfi1
Dry microspores were placed in water with varying concentrations of dsRNA complementary to a unique portion of the Sfi1p transcript. The control sample had completed all the spermatogenous divisions, and the nucleus has elongated and spiraled (**E**). Centrin has aggregated and localized to the traditional location of the bb. The aggregates were distributed along the side of the nucleus, seen as arcs (**A**). The addition of the dsRNA caused development to terminate prematurely in all of the treated samples. All of the samples completed the final spermatogenous division. None of the samples had elongated nuclei (**F- H**). Many nuclei in each treated sample have the same morphology as control samples after the final spermatogenous division. There are also nuclei in each sample which had an altered morphology and are no long uniformly round (**F- H**). Diffuse cytoplasmic centrin staining was seen in the cytoplasm of each treated sample. Centrin aggregation was seen in each treated sample, localized near the nuclei (**B-D**). The aggregates seen in the 25 and 50µg samples were small and clustered together (**B**, **C**). The aggregates in the 100µg sample were large single clusters, with one cluster per cell (**D**). Sections labeled with DAPI and anti Centrin antibody detected with AlexaFluor594 secondary. Bar = 25µm.



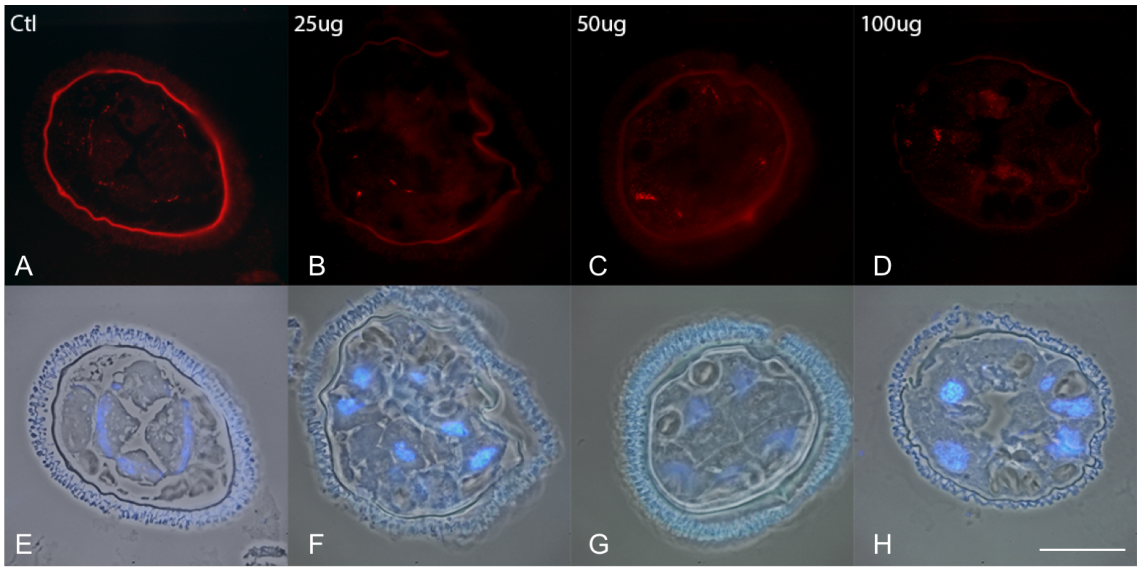
Appendix Fig. 1-10. Knockdown of Cep120
Dry microspores were placed in water with varying concentrations of dsRNA complementary to a unique portion of the Cep120 transcript. The control sample had completed all the spermatogenous divisions, and the nucleus has elongated and spiraled (**E**). Centrin has aggregated and localized to the traditional location of the bb. The aggregates were distributed along the side of the nucleus, seen as arcs (**A**). The addition of the dsRNA caused development to terminate prematurely in all of the treated samples. All of the samples completed the final spermatogenous division. The nuclei in all samples are of a similar size to those seen in control samples after the final spermatogenous division was completed (**F-H**). Centrin aggregation was seen in all treated samples. The aggregates in the 25µg sample were small and punctate, with many distributed in lines along the nuclei (**B**). This was similar to the morphology seen in the control (**A**). The aggregation in the 50µg sample was punctate in some areas and moderately clustered in others (**C**). The centrin aggregates seen in the 100µg sample were single defined aggregates, with one per cell (**D**). Sections labeled with DAPI and anti Centrin antibody detected with AlexaFluor594 secondary. Bar = 25µm.



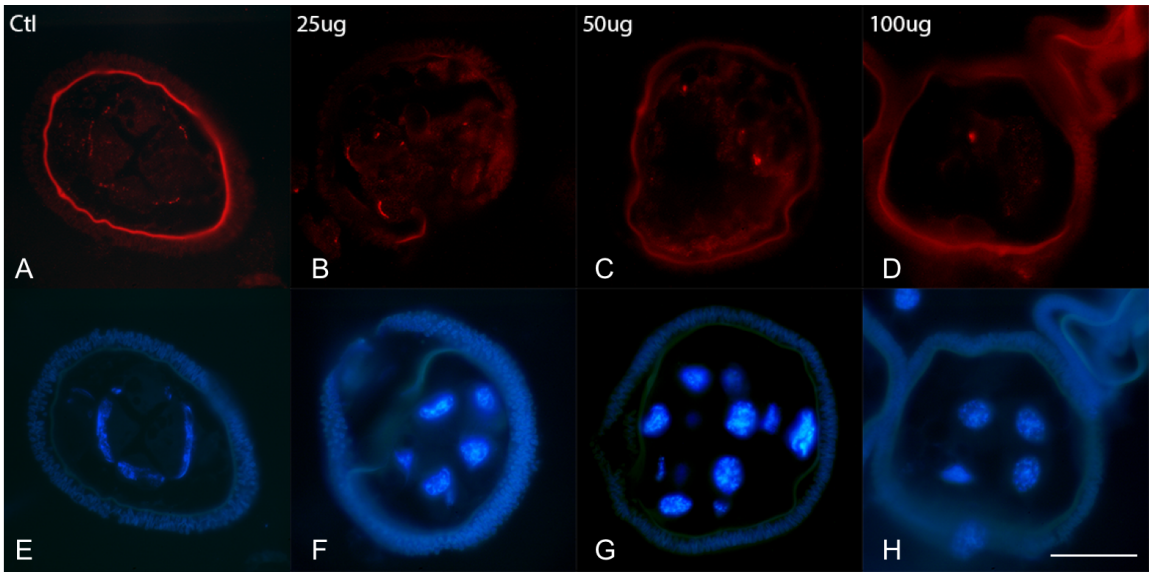
Appendix Fig. 1-11. Knockdown of Cep170
Dry microspores were placed in water with varying concentrations of dsRNA complementary to a unique portion of the Cep170 transcript. The control sample had completed all the spermatogenous divisions, and the nucleus has elongated and spiraled (**E**). Centrin has aggregated and localized to the traditional location of the bb. The aggregates were distributed along the side of the nucleus, seen as arcs (**A**). The addition of the dsRNA caused development to terminate prematurely in all of the treated samples. The 25 and 50µg samples completed the final spermatogenous division (**F**, **G**). The nuclei in both samples were not uniformly round or elongated. The nuclei were irregularly shaped with a range sizes. The 100µg sample stopped dividing after the 2 antheridial cells formed (**H**). Diffuse centrin staining was present in the cytoplasm of all treated samples (**B-D**). Large and small punctate aggregates of centrin localized to the nucleus were seen in the 25µg sample (**B**). The 50µg sample had small aggregates localized to nuclei (**C**). The 100µg sample had aggregates which were irregularly distributed among the spermatogenous cells. The aggregates had a range of sizes. Sections labeled with DAPI and anti Centrin antibody detected with AlexaFluor594 secondary. Bar = 25µm.



Appendix Fig. 1-12. Knockdown of GCP4
Dry microspores were placed in water with varying concentrations of dsRNA complementary to a unique portion of the GCP4 transcript. The control sample had completed all the spermatogenous divisions, and the nucleus has elongated and spiraled (**E**). Centrin has aggregated and localized to the traditional location of the bb. The aggregates were distributed along the side of the nucleus, seen as arcs (**A**). The addition of the dsRNA caused development to terminate prematurely in all of the treated samples. All of the treated samples had completed the final spermatogenous division, but did not continue to differentiation (**F-H**). The nuclei were a similar size and shape as the nuclei after the final spermatogenous division in control microspores. Aggregation of centrin and diffuse cytoplasmic staining was seen in all the treated samples. The morphology of the aggregates differed between the 3 concentrations. The 25µg sample aggregation was similar to the linear aggregation and morphology in the control (**A**,**B**). The 50µg sample had small aggregates with nuclear localization (**C**). The 100µg sample had a large number of aggregates with a range of sizes. Some of the aggregates were nuclear localized (**D**). Sections labeled with DAPI and anti Centrin antibody detected with AlexaFluor594 secondary. Bar = 25µm.



Appendix Fig. 1-13. Knockdown of centrobin
Dry microspores were placed in water with varying concentrations of dsRNA complementary to a unique portion of the centrobin transcript. The control sample had completed all the spermatogenous divisions, and the nucleus has elongated and spiraled (**E**). Centrin has aggregated and localized to the traditional location of the bb. The aggregates were distributed along the side of the nucleus, seen as arcs (**A**). The addition of the dsRNA caused development to terminate prematurely in all of the treated samples. All of the treated samples completed the final spermatogenous division, but did not progress to differentiation (**F-H**). The nuclei in of all the samples were no longer uniformly round. Aggregation of centrin was seen in all the treated samples. The 25µg sample's dense aggregates (**B**) were larger than those seen in the control, but were also not dispersed as seen in the control (**A**). Aggregates in the 50µg sample consist of small clustered punctate foci which have not further condensed into larger distinct aggregates (**C**). The aggregates in the 100µg sampleµ were loosely associated masses of punctate foci (**D**). Diffuse centrin staining was seen in the cytoplasm of all of the treated samples. Sections labeled with DAPI and anti Centrin antibody detected with AlexaFluor594 secondary. Bar = 25µm.



Appendix Fig. 1-14. Knockdown of Cep41
Dry microspores were placed in water with varying concentrations of dsRNA complementary to a unique portion of the Cep41 transcript. The control sample had completed all the spermatogenous divisions, and the nucleus has elongated and spiraled (**E**). Centrin has aggregated and localized to the traditional location of the bb. The aggregates were distributed along the side of the nucleus, seen as arcs (**A**). The addition of the dsRNA caused development to terminate prematurely in all of the treated samples. The 25 and 50µg samples completed the finial spermatogenous division, but have not proceeded into differentiation (**F**, **G**). The 100µg sample completed the 7th division (**H**). Diffuse centrin background staining was present in all of the treated samples (**B-D**). Small aggregates were seen in the 25µg sample which are distributed along the edges of nuclei, similar to the control (**A**,**B**). Large distinct aggregates were present in the 50 and 100µg samples. The aggregates were localized to the nuclei (**C**, **D**). Sections labeled with DAPI and anti Centrin antibody detected with AlexaFluor594 secondary. Bar = 25µm.

Appendix 2: *Marsilea vestita* aurora kinase primer sequences

mvAK1

ATGGCCTCCGGCTCCTTCAAATCTACACACAGCTAGCATCTCTGCGGTGTCGCTCCACA
G**ATTCATTGC**CTCTCGTTGAAGCTTAGGTTCTGCA**TTGCTCGAATATGGTTGCTCTCAC**
AGACACTAATTAGAGCTTCGTCAACATGATGAATGATCACTGCGGAGATGTGCAGGAGA
AACGATGGACTCTACAGGACTTTGATATCGGCAAACCTCTAGGAAGAGGAAAATTCGGC
AATGTATACCTCGCCAGAGAAAAGAAAAGTAAGTACGTTGTAGCTCTTAAGGTACTCTT
TAAGAGCCAGCTTCAACAGTCTCAGGTCGAACATCAACTGCGGCGAGAAATTGAAATCC
AAAGTCATCTCAGGCACCCTAACATTCTCAGACTTTATGGATACTTCTATGATCAGAGC
CGTGTATACCTCATACTGGAATATGCTCCAAAGGGAGAATTGTACAAAGAGCTACAGAG
ATTTAAGGTTTTTAGTGAAAGGAGATCTGCCACGTATATTGCCTCCCTAGCACGTGCAT
TGATGTACTGCCACGAGAAACATGTTATTCATCGTGACATAAAACCTGAGAACTTGCTG
ATGGGAGCCAAGGGAGAGCTGAAGATAGCTGACTTTGGATGGTCAGTCCATACATTTGA
CAGAAGACAAACAATGTGTGGAACATTGGACTATCTTCCTCCAGAGATGGTTGAGAGTC
GTGATCATGATGCTGGTGTTGATATTTGGAGCTTGGGAGTTCTATGCTATGAGTTTCTG
TTTGGAACACCTCCATTTGAGGCAGTGAAACACTCAGATACCTATAGAAGGATTGTCAA
AGTTGATCTGAAGTTTCCAGATAAACCCTTTGTTTCCCCACAGGGAAAGGATCTTATTG
GCAAGCTTCTTGTAAAGGATTCTTCACAAAGGCTTCCTCTTTCACAGGTTCTTACTCAC
CCATGGATAGTGGCAAATGCTGATCCGAGTGGTGTATACTCAATGTACTAG

Bold- retained intron
Grey- conserved central kinase domain

Aurora 1 Frw- SP6
GATTTAGGTGACACTATAGATGGCCTCCGGCTCC
Aurora 1a Rev- T7 133bp
TAATACGACTCACTATAGGGTGCAGAACCTAAGCTTCAACGAGAG
Aurora 1b Rev- T7 108bp
TAATACGACTCACTATAGGGCGCTCCACAGAGCTTCGTC

These primers were used to make dsRNA for RNAi. The primers for rt-PCR amplified the same regions, without the addition of SP6 of T7 sequences to the primers.

Aurora Frw- SP6 352bp
GATTTAGGTGACACTATAGCTCAGGCACCCTAACATTCTC
Aurora Rev- T7
TAATACGACTCACTATAGGGGATCACGACTCTCAACCATCTC

These primers were used to make dsRNA for RNAi knockdown of all aurora isoforms.

mvAK2

ATGGTGGAAGCCAAGTCAAATATCGAAGAAAATAATGATGGACAGGAGAAAAGGTGGAC
ATTAAGCGATTTCGACATTGGAAAGCCTCTAGGAAGAGGCAAATTCGGGAATGTTTACC
TTGCCAGGGAGAAGAAGAGTAAGTATGTTGTAGCGTTGAAGGTTTTATTCAAAAACCAG
CTCCAACAGTCTCAAGTGGAGCACCAATTGCGAAGAGAAATCGAAATTCAAAGCCATCT
TCGACATCCTAACATCCTCAGACTCTATGGATACTTCTATGATCAGAGTCGAGTGTATT
TGATTCTTGAATATGCTCCTAAGGGTGAACTCTACAAAGAACTCCAGCGTTACAGAGTC
TTCAGTGAGAGAAGATCAGCCACGTACATAGCATCTCTTGCAAGAGCTTTGATTTATTG
CCATGAGAAGCATGTCATTCACAGGGACATCAAACCTGAGAATCTCCTAATAGGAATGA
AGGGCGAACTTAAAATAGCAGATTTTGGATGGTCTGTACATACATTTGACCGAAGGCGT
ACTATGTGTGGTACACTTGATTATCTGCCTCCTGAGATGGTTGAAAGCAAAGAACATGA
TGCTGGAGTGGACATTTGGAGTCTGGGAGTTCTATGTTACGAGTTTTTATATGGAATTC
CTCCATTTGAAGCTGTTAAACATTCAGATACTTATAGACGGATTGTTAATGTTGATTTG
AAGTTTCCAAGCAAGCCTTACGTATCTCCACAGGCAAAGGATAG

Grey- conserved central kinase domain

Aurora 2 Frw2 SP6 89bp

GATTTAGGTGACACTATAGATGGTGGAAGCCAAGT

Aurora 2 Rev2 T7

TAATACGACTCACTATAGGGCTCCTGTCCATCATTATTTTCTT

These primers were used to make dsRNA for RNAi. The primers for rt-PCR amplified the same regions, without the addition of SP6 of T7 sequences to the primers.

mvAK3

ATGCGGATTTTTTGATTCCTCTGCCCGAGATCAAAGAGAACTGTGGAGGGAATGCTGAG
AACAAATCTAAGCTCTGGACAATCAACGACTTCGACATCGGAAAACCTCTTGGCAGAGG
AAAATTTGGAAATGTCTACCTCGCCAGAGAGAAGAAGAGCAAATATATAGTAGCACTGA
AGGTGCTATTCAAGCATCAGCTGCAACAGGCACATGTTGAACATCAACTGCGAAGAGAA
ATTGAAATACAGAGCCATCTCAGACATCCCAACATTTTACGTCTCTATGGATACTTTTA
TGACGAGACTCGAGTGTTTCTGATTCTGGAGTATGCAGCAAGAGGGGAACTTTATAAAG
AACTCCAGCACCAAAAAGTTTTCAATGAAAAGAGAGCTGCAACTTACATAGCCTCGTTA
GCTCGAGCTCTTTTATACTGTCATGAGAAGCATGTGATACATAGGGATATCAAGCCGGA
AAATTTATTGATTGGAATAAAGGGAGAGCTTAAAATTGCTGATTTTGGGTGGTCTGTCC
ATACATTCAACAAGAGAAAAACAATGTGCGGGACACTCGATTATCTCCCTCCTGAAATG
GTTGAAAGTAAGGAGCACGATTCAGCTGTAGACATCTGGAGTTTAGGGGTTCTCTGCTA
TGAGTTTCTATATGGATCACCACCTTTTGAAACAACTACTCATCAAGATACTTATCGAC
GAATCATCAAGATCGACCTCCATTTCCCTTCAAAACCTGTGATTTCCAATCTAGCCAAG
GATCTGATTATGAGGCTGTTGGTAAAAGAACCTACCAAAAGGCTTCCATTAAGACATGT
TCTTCATCATCCTTGGATTATTGCGAATGCAGATCCTCCTGGTGTCAATGCTTCACTAG

Grey- conserved central kinase domain

Aurora 3 Frw2 SP6 105bp

GATTTAGGTGACACTATAGGCGGATTTTTTGATTCCTCTGC

Aurora 3 Rev2 T7

TAATACGACTCACTATAGGGAGATTTGTTCTCAGCATTCCCTC

These primers were used to make dsRNA for RNAi. The primers for rt-PCR amplified the same regions, without the addition of SP6 of T7 sequences to the primers.

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