Regulation of asymmetric spindle positioning in the early *C. elegans* embryo

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ABSTRACT

Erin Kate McCarthy: Regulation of asymmetric spindle positioning in the early *C. elegans* embryo
(Under the direction of Dr. Bob Goldstein)

Asymmetric cell division is necessary for proper development in many organisms, and results in daughter cells of unequal size or cell fate, or both. The first mitotic division of the *C. elegans* embryo is asymmetric, due to movement of the mitotic spindle to an asymmetric position. While this cell division is a well-studied model of asymmetric spindle positioning, the mechanisms that regulate this event are not completely understood. In order to better understand asymmetric spindle positioning, I have studied microtubule dynamics and the timing of spindle movement during mitosis in the one-cell stage *C. elegans* embryo.

The mitotic spindle of the one-cell stage division shifts towards the posterior cortex of the embryo due to an increase in microtubule pulling forces originating from one side (Grill et al., 2001; Labbe et al., 2004). It is not clear, however, how microtubules contribute to generating the pulling forces required to segregate chromosomes during anaphase in *C. elegans*. To test this, I monitored the dynamics of kinetochore microtubules through the use of photobleaching and high resolution confocal microscopy. Combined with previous data from other labs, my results suggest that the forces that segregate chromosomes are provided by astral microtubules in early *C. elegans* embryos, and not by kinetochore microtubules.

It is not clear in any developmental system how asymmetric spindle positioning is timed. I found that the mitotic spindle begins to shift at a precise time in the early *C. elegans* embryo, soon after chromosomes have completed congression to the metaphase plate. This observation suggested an interesting hypothesis—that machinery timing mitotic progression might serve a dual function, also timing asymmetric spindle movement until the appropriate time. Upon manipulation of the cell cycle machinery, my results suggest that components of the spindle checkpoint pathway serve a novel role as a timer for asymmetric spindle positioning in the one-cell *C. elegans* embryo. This additional role for the spindle checkpoint pathway may ensure that chromosomes attach to the spindle before the spindle shifts to an asymmetric position. This work demonstrates a fundamental new link connecting cell and developmental biology, between mitotic regulation and asymmetric cell division.

To my cheering section, my family.

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It is difficult to imagine the number of people who have helped, encouraged, and loved me to get me where I am today—academically and personally. To save myself, I will gradually build up enough courage to think, write, edit, and read about my gratitude to those more personal to me. In a grand finale of gratitude, I may end up in a puddle on the floor.

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Where would I be without my advisor, Dr. Bob Goldstein? Bob has been a fantastic mentor on so many levels, and his courage, creativity, and persistence are inspiring to me. As a scientist, Bob has such a unique way of looking at science problems. His creativity in approaching experiments is well-known, and I hope some has spread into my own approach. His persistence is key to his success. There have been many times when I thought it was time to "cut the cord" on an experiment, yet Bob pushed me to think of a better way to perform the experiment. Sure enough, he was often

right. Finally, Bob's courage in science is admirable. There have been many times when Bob approaches a field, question, or experiment that others have deemed a bit risky. Add together Bob's creativity, persistence, and courage, and you have the success that Bob has worked hard to attain. I will always look up to Bob as a fantastic scientist.

As an advisor and friend, Bob is wonderful. Bob "It's your career!" Goldstein allows each of us in the lab to approach a project that we are interested in personally, and gives each of us the freedom to direct our projects how we think is best. With this independence are challenges that each of us, especially graduate students, must face. Although there were times when I would be frustrated with the (lack of) progress in my project, I am proud to know that I have played a major role in determining how and why my project later became successful. Bob's approach to advising also extends to career (the "big C") guidance—his willingness and enthusiasm to allow each of us to explore different career paths makes it easy to be honest about our personal goals. Bob has been more than generous with the lab, and I thank him for the many lab lunches, dinners, and parties he hosted. Bob has also been generous in sharing his fantastic family; Jenny, Connor, and Duncan have made me smile and laugh too many times to count. Bob's approach in running his lab has set the scientifically interactive, close-knit, and fun tone in the lab—I thank Bob and look up to him for this feat. And finally, Bob's friendship is something for which I am so grateful. I hope that the trust, honesty, generosity, and humor he extended to me were reciprocated properly and thanked along the way.

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My brother, Brian, may not understand or enjoy the science that I am so passionate about, but that has never stopped him from listening to my concerns and providing his calm, reasonable, big-brother advice. Brian's humor has always made me laugh, and his warmth reminds me so much of Mom it amazes me. Brian is one of my closest friends, and we have helped each other through so much "life" that I cannot imagine what I would do without him. I also want to thank Brian for bringing Heather into our lives. Heather is an incredibly sweet and supportive person—she is always enthusiastic to hear about worms and my project, and her enthusiasm always rejuvenates my excitement for science.

There is absolutely no way I can thank my parents properly in words. Mom and Dad raised Brian and I in an environment that valued hard work, education, humor, and above all, love for one another. This stable foundation has been a blessing, and something that I look up to when thinking about my own future. My mom always

encouraged us to do our best, and was always proud of any achievement—this gave me the self-confidence to set goals I otherwise may not have made. Mom's optimism, strength, and deep care for her family is a major influence in how I live my own life. Although my time with Mom was cut short in 2002, my memories of her laugh, smile, and hug are constantly with me. My dad is the reason I am in science. Although Dad has no higher education in science, he has a natural-born curiosity in understanding how things work. Dad encouraged my own curiosity, and is so proud of any achievement. I'm thankful for his dedication to family, and his support of anything I want to accomplish. I'm also grateful for Dad's sense of humor – one may thank or scorn him for passing that on to Brian and I!

Finally, I am so thankful for my wonderful husband John. In addition to being my best friend, cheerleader, therapist, and CPA, he has truly been a partner in everything. Graduate school is filled with many up and down emotions, and it is so wonderful to have someone like John along for that ride. John would help push me along when I would be frustrated, whiney (I'll admit it), or down about my project. When my nerves would kick in for a committee meeting, a poster presentation, a talk, or any moment my simmering anxiety boiled over, John would be there to calm me and to convince me that I'm the best one to do what I'm doing. As John begins his graduate program this fall, I hope I can provide the same support and stability that he provided me. His humor, honesty, openness, and relaxed demeanor have made me a better person, and I thank and love him so much.

PREFACE

When I started graduate school in August 2002, I was infatuated with biology, and naïve. I thought science was just dandy and graduate school would be hard work, but fun. I had no clue how to weather the ups and downs of failed experiments, stalled projects, incomplete ideas, and an unreliable self-confidence as a scientist. Although I am still quite naïve and my enthusiasm for biology sometimes gets in the way of reason, I now truly appreciate what "research" is to me.

Even before I joined the Goldstein lab, I had always been interested in mitosis, spindles, and microscopy. When Bob introduced me to the work in his lab, I was immediately charmed by the *C. elegans* embryo and asymmetric spindle positioning in the one-cell stage. I began my rotation by looking at proteins that regulate this movement of the spindle, using a technique that a former post-doc in the lab, Dr. Jean-Claude Labbe had developed. I counted the residence time of microtubules reaching the cortex at the time of spindle displacement, with the goal of finding differences in microtubule residence times in different genetic backgrounds. Starting this project was laborious, so Bob and I worked with Dr. Yoni Fridman, a then-graduate student in the Computer Science Department, to develop a program that could perform these counts of microtubule residence time. At the time, I was distracted by so many interesting questions about spindle positioning; thus, this program is still being tested and improved.

I spent several months optimizing my techniques for visualizing microtubules during spindle positioning, and reading the literature. I wondered if the astral microtubules that pulled the spindle towards the posterior cortex were undergoing poleward flux. Although there was/is no precedent for astral microtubules undergoing poleward flux, I was enthusiastic about the idea of finding something unexpected. In order to eventually test this, I first optimized the microscopy setup for photobleaching spindle microtubules which I knew would be easier to image and photobleach than astral microtubules. Although I wasn't able to photobleach astral microtubules, I did uncover results that eventually and indirectly led me to the project that consumed much time, energy, and passion (I'll get to this later).

When I photobleached the spindle microtubules, I was struck at how "cleanly" the photobleached marks remained on microtubules. When Bob and I analyzed the photobleached images and considered all of the literature on the one-cell stage division in worms, we concluded that these microtubules were as dynamic as "sticks" (Bob's description!). My results on spindle microtubule dynamics were included as part of Jean-Claude's 2004 Journal of Cell Biology paper "The forces that position a mitotic spindle asymmetrically are tethered until after the time of spindle assembly" (Labbe et al., 2004). My role as second author and collaborator on this paper was educational in terms of understanding the hard work and patience that is needed for manuscript preparation, submission, and all things editorial. This work is included in Chapter II, as excerpts of the paper that are relevant to my contribution.

After publication of Jean-Claude's paper, I had squeezed a tiny space for myself in the published world of spindle positioning. I realized from the role I played in writing

my results for this paper and discussing my opinions on our conclusions and statements, that I enjoyed writing. I enjoyed the literature search, the thinking, the organizing, the interpreting, the debating, and the editing that went into a paper. Bob recognized my enthusiasm for the process, and gave me two fantastic opportunities. With Bob, I wrote a Dispatch for Current Biology on meiotic spindle positioning (McCarthy and Goldstein, 2005). As meiotic spindle positioning is a very different process from mitotic spindle positioning, I dedicated a few weeks to review the literature, come up with an interesting approach to the Dispatch (a collaborative effort in a lab meeting), and write. I am including this Dispatch, which introduces a new hypothesis to the field of meiotic spindle positioning, as Chapter IV. This Dispatch allowed me to consider the diversity of mechanisms that could position a spindle, and broadened my thinking about the one-cell stage embryo. In addition, the process of writing this Dispatch has inspired me to think about a career in scientific writing. Shortly after writing this Dispatch, Bob then offered me the opportunity to write a review on the field of spindle positioning for Current Opinions in Cell Biology. This review is a thorough introduction to this fascinating biological event, and I have included this as Chapter I (McCarthy and Goldstein, 2006). Throughout this introductory chapter, I will provide updates on spindle positioning since publication of the review.

During the editorial process for Jean-Claude Labbe's paper, I had a chance to look at comments from anonymous reviewers. One of the reviewers suggested we look at several wild-type embryos to make sure that one of our representations of spindle position timing was accurate. When Bob and I started looking through many movies of the one-cell stage division that I had put together, we realized that not only was our

representation of spindle position timing accurate, but the event's timing was more precise than we or others had ever reported. We noted that the chromosomes would line up at the metaphase plate upon attachment to the spindle, and precisely after lining up, the entire spindle would move to its asymmetric location. I can remember where we were sitting when we were discussing how this event could be timed, consistently from embryo to embryo. I remember that my mind was racing a mile a minute, and I left the lab that night absolutely loving science.

I spent a lot of time in the next few years trying to show that the mechanism that regulates spindle position timing is tied up with cell cycle machinery, and specifically the spindle checkpoint. I had dribs and drabs of data that together didn't make up a whole story, until Bob and I had lunch with Dr. Todd Stukenberg. Todd suggested a very experimental, non-commercial chemotherapy drug that his collaborators had used to manipulate the mitotic machinery (Potapova et al., 2006). Seven months went by and we jumped through several hoops, and I finally had the CDK inhibitor drug, flavopiridol (to Bob this is always flavo-flavo-piridol). The results from these drug treatments and all follow-up experiments began to pour in. Around this same time, Dr. Andy Golden very generously shared several checkpoint mutants that exposed an additional aspect to this story than just RNAi knockdowns of the checkpoint proteins. From all of these experiments, I was able to show that spindle position timing was indeed regulated by the mitotic machinery, and the spindle checkpoint itself. This story has shown that a well-studied cell biological checkpoint was co-opted to regulate spindle positioning, a developmentally significant event. Although the spindle positioning field is quite competitive and saturated with very intelligent scientists, this story brings in a new

perspective and set of questions about spindle positioning. As I write this, Bob and I are in the process of submitting this manuscript and I am enthusiastic about finding a nice home for this story. The most updated version of this manuscript is included as Chapter III.

As I explained above, most of my Chapters are parts of or entire manuscripts that I have written throughout my wonderful time in the Goldstein lab. Chapter I is an introduction to the field of asymmetric spindle positioning, published in *Current Opinions in Cell Biology* (McCarthy and Goldstein, 2006), including updates of recent findings in the field. Chapter II is my contribution to Jean-Claude Labbe's manuscript on the forces that position the spindle, which was published in *Journal of Cell Biology* (Labbe et al., 2004). This Chapter will have relevant sections from that paper's introduction, results, and discussion. Chapter III is my most updated version of the manuscript on spindle position timing. Although the manuscript has not yet been accepted to a journal, I feel confident that we will find the right journal with the right readership. Chapter IV is the Dispatch article that I wrote in *Current Biology*, on meiotic spindle positioning (McCarthy and Goldstein, 2005); although it is quite different from my project, it is an article that I had a fantastic time writing. Finally, Chapter V is a discussion on how I think my research has contributed to the field of asymmetric spindle positioning, as well as perspectives I have on the field.

As I reflect on the course my project followed, I've realized that research requires more out of me than I had originally thought. I'm proud of the spindle position timing story that I have begun to sort out, and I'm excited to see how the field will respond to this story. Two key events – lunch with Todd when he informed us of flavopiridol, and

Andy's generosity in sharing unpublished strains – were monumental to the progress and direction of my project. This has reinforced my enthusiasm for friendly scientific interaction. Finally, although I hopefully have many years of science ahead of me, I feel as if graduate school has transformed, matured, and energized me both as a scientist and person.

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APC	Anaphase promoting complex
CDK	Cyclin-dependent kinase
C. elegans	*Caenorhabditis elegans*
c-LβL	*clasto*-lactacystin β-lactone
dsRNA	double-stranded RNA
FRAP	Fluorescence recovery after photobleaching
gf	gain-of-function
GFP	Green fluorescent protein
MTOC	Microtubule organizing center
PNEBD	Pronuclear envelope breakdown
RNAi	RNA interference
WT	wild-type

CHAPTER I

ASYMMETRIC SPINDLE POSITIONING

When a spindle is positioned asymmetrically in a dividing cell, the resulting daughter cells are unequal in size. Asymmetric spindle positioning is driven by regulated forces that can pull or push a spindle. The physical and molecular mechanisms that can position spindles asymmetrically have been studied in several systems, and some themes have begun to emerge from recent research. Recent work in budding yeast has presented a model for how cytoskeletal motors and cortical capture molecules can function in orienting and positioning a spindle. The temporal regulation of microtubule-based pulling forces that move a spindle has been examined in one animal system. Although the spindle positioning force generators have not been identified in most animal systems, the forces have been found to be regulated by both PAR polarity proteins and G-protein signaling pathways in more than one animal system.

Introduction

When a mitotic spindle is positioned asymmetrically within a cell, cell division results in daughter cells that are unequal in size. Such asymmetry in spindle position occurs commonly, for example in budding yeast mitotic divisions and in countless developmental cell divisions. In animal development, asymmetric divisions like these

often have an additional, important role in unequally partitioning cell-fate determinants. Asymmetry in size of cells alone is likely to be important to partition such determinants precisely (Whittaker, 1980) and to allow large stem cells to divide repeatedly without becoming depleted of cytoplasm (Watt and Hogan, 2000). Asymmetric spindle positioning was first seen over a century ago (Figure I.1), yet the mechanisms involved are only now beginning to be elucidated (Lillie, 1901). Here, we discuss the physical forces that asymmetrically position spindles, the molecular machinery that may generate and regulate these forces, and the checkpoints that can monitor spindle position in some systems. We will highlight some recent findings that have shed light on the molecular mechanisms of asymmetric spindle positioning.

Forces that asymmetrically position a spindle

In a symmetrically dividing cell, passive mechanisms locate the spindle at the center of the cell (Wilson, 1925). Certain cell shapes alone can dictate asymmetric division planes (Harris and Gewalt, 1989; Rappaport and Rappaport, 1994), but in most cases of asymmetric division, it is likely that forces are actively exerted on a spindle from one or more specialized sites on the cell cortex. To assess the regional sources of these pulling and pushing forces, researchers have cut spindles in half, or eliminated one side of a spindle, and followed the subsequent movement of the experimentally isolated spindle parts (Aist and Berns, 1981; Labbe et al., 2004; Tolic-Norrelykke et al., 2004). Such experiments have been performed to date on only one type of asymmetrically dividing cell, the relatively large (50 μm long) one-cell stage *C. elegans* embryo (Figure I.2) (Grill et al., 2001; Labbe et al., 2004). Here, experiments have demonstrated that

microtubule pulling forces are pervasive throughout the cell cortex, and that these pulling forces are stronger on one side of the cell — at the posterior cortex — causing the spindle to shift from the center of the embryo towards the posterior. These posterior pulling forces are generated early in mitosis, even before the spindle is completely assembled. Spindles are not shifted this early in the cell cycle, however, as astral microtubules tether the spindle to the anterior cortex until near the time that spindle assembly is completed (Labbe et al., 2004). Determining whether pulling forces dominate similarly in other asymmetrically dividing cells awaits experiments in other systems.

Force-generating mechanisms

Molecular motors

Early studies in *Chaetopterus* oocytes demonstrated the presence of a unique attachment site to which the spindle migrates when experimentally pulled away from the cortex (Lutz et al., 1988), and morphologically unique sites in the cortex of certain sea urchin embryonic cells toward which spindles move (Dan, 1979). Whether movement in either case is driven by molecular motors or by other mechanisms, such as microtubules depolymerizing (Lombillo et al., 1995) while maintaining continuous attachment to a cortical site, is not clear. These studies have been influential, however, in suggesting that regions of the cortex may be specialized for spindle attachment and spindle pulling.

Molecular motors that walk along microtubules or actin filaments can contribute to spindle positioning directly, by generating a pulling or pushing force, or indirectly, by transporting cargo proteins that contribute to spindle positioning. Budding yeast is one of the best-studied cases of motors functioning directly to position a spindle (Huisman and

Segal, 2005; Pearson and Bloom, 2004). Spindle orientation is initially dependent on myosin, which functions through interactions with the plus-end microtubule binding proteins Kar9 and Bim1 to move microtubules along actin cables to the bud tip. Later, during anaphase, spindle positioning is dependent on dynein, which binds microtubule plus ends and guides the spindle through the neck and into the daughter cell (Figure I.3).

The posterior cell of the two-cell stage *C. elegans* embryo may undergo spindle rotation through attachment of microtubules to a cortical capture site enriched in actin, dynein and components of the dynactin complex, which are recruited to the cell division remnant of the previous one-cell stage division (Hyman, 1989; Skop and White, 1998; Waddle et al., 1994). The use of conditional dynein mutants suggests that dynein is essential for spindle positioning in this cell, but is dispensable for spindle positioning at the one-cell stage (Schmidt et al., 2005). These results might not completely rule out a role for dynein in positioning the spindle in the one-cell stage embryo, as whether dynein was completely nonfunctional was not clear. These disrupted dynein motors might still function in walking along a microtubule more slowly than normal and might inefficiently release upon reaching the minus end of a microtubule, since the authors observed a decrease in the rate of spindle positioning and an enrichment of dynein near centrosomes (Schmidt et al., 2005).

Meiotic divisions in animal eggs are extreme forms of asymmetric division, producing tiny polar bodies and large egg cells. Recent work on *C. elegans* and *Xenopus* meiotic division has identified motors required to position spindles: a microtubule-based motor in *C. elegans*, and an actin-based motor in *Xenopus* (Weber et al., 2004; Yang et al., 2005). Yang and colleagues found a role for a kinesin motor in *C. elegans* meiotic

spindle positioning. Meiotic spindles in *C. elegans* have defects in translocation to the cortex in oocytes lacking a kinesin-I homolog or its associated light chains, or a putative cargo protein that has been shown to interact with both the kinesin light chains and a heterochromatin binding protein in a yeast two-hybrid screen (Li et al., 2004). Meiotic spindles in *C. elegans* oocytes lack centrosomes and astral microtubules, and the potential link between kinesin-I and the meiotic spindle suggests a model in which the kinesin-I motor activity might directly translocate the spindle to the cortex. *Xenopus* meiotic spindles are positioned adjacent to the cortex by the interaction of microtubules and F-actin (Gard et al., 1995). Recent work has found that an unconventional myosin, Myo10, interacts directly with microtubules (Weber et al., 2004). Disruption of Myo10 function results in defects in nuclear positioning, an event that normally requires microtubules (Gard et al., 1995; Weber et al., 2004), and in spindle structure and rotation, which normally requires actin filaments (Weber et al., 2004). These results suggest a role for Myo10 in linking the actin and microtubule networks for their function in nuclear and spindle positioning.

Other motors function indirectly in spindle positioning, by transporting other motors or non-motor proteins that affect spindle movement. Recent studies in budding yeast have shown that Kip2 kinesin plays roles in transporting dynein and Bik1, a CLIP-170-related microtubule-stabilizing protein, to the plus ends of astral microtubules, from which dynein is presumably delivered to the cortex (Carvalho et al., 2004; Sheeman et al., 2003). In asymmetrically dividing *Drosophila* neuroblasts, dynein has been shown to be required for apical localization of inscuteable mRNA, and thus of Insc protein, which plays a role in both spindle orientation and segregation of cell fate determinants (Hughes

et al., 2004; Wodarz, 2005). Localization of basally localized proteins required for asymmetric division in this system requires the actin-based motors myosin VI and myosin II (Barros et al., 2003; Petritsch et al., 2003).

Cortical capture of microtubules

A cortical capture mechanism can perform several jobs during cell division: microtubules that interact with the cortical capture site can orient the spindle along a specific axis, continued interaction with the site can maintain proper orientation, and cortical proteins that depolymerize microtubules can function in generating pulling forces to move a spindle to an asymmetric position. Cortical capture mechanisms may use microtubule motors, instead of microtubule depolymerizing proteins, to 'reel in' a spindle. Examples of this type of cortical capture include the *Chaetopterus* oocyte and the two-cell stage *C. elegans* embryo, as mentioned above.

In budding yeast, astral microtubules are captured at the bud tip cortex and function in positioning the spindle along the mother–bud axis (Pearson and Bloom, 2004). Several recent studies have focused on understanding cortical capture in budding yeast and identifying proteins that may regulate microtubule dynamics at the capture site. Kar9 functions in linking microtubule plus ends, via Bim1, to Myo2, which guides microtubules along actin cables towards the bud tip. Live-cell imaging experiments in budding yeast have distinguished the roles of Kar9 and actin-associated Bud6 in microtubule cortical capture: Kar9 functions in delivery of microtubules along actin cables into the bud, while Bud6 functions in securing microtubule capture at the bud tip (Huisman et al., 2004). It has also been shown that subunits of the type I phosphatase

complex act via Bud14 to regulate the interaction of microtubules at the bud cortex, thus maintaining spindle position within the bud neck (Knaus et al., 2005). Other recent work has shown how an asymmetry in spindle pole bodies may contribute to cortical capture: the cyclin-dependent kinase Cdc28 and cyclin B Clb4 are localized to the bud-ward spindle pole body (SPB), and are translocated to the plus ends of astral microtubules in a manner dependent on Kar9 (Maekawa and Schiebel, 2004; Maekawa et al., 2003). This complex regulates the interaction of microtubules with the bud cortex, although how Cdc28–Clb4 modifies cortically bound microtubules remains unknown. These findings are in contrast to a previous model, in which the Cdc28–Clb4 complex is associated with the mother cell SPB, where it prevents Kar9 binding (Liakopoulos et al., 2003).

Microtubule dynamics and length

Some asymmetric cell divisions may depend directly on microtubule dynamics to position a spindle. It is possible, for example, that by locally regulating the stability of microtubules, the duration of their interaction with microtubule motors can be controlled. It is also possible that locally stable microtubules that reach the cortex but do not interact with motors can occlude movement of a spindle. By imaging microtubules at the cortex in early *C. elegans* embryos, Labbe et al. (2003) found that microtubules reaching the anterior cortex are more stable than those reaching the posterior cortex. Whether this difference is required to move the spindle asymmetrically is not yet known. Recent work in *C. elegans* has also examined the effects of specific mutations in tubulin isoforms on spindle positioning — certain dominant mutations of these tubulin isoforms affect microtubule dynamics (Wright and Hunter, 2003) as well as spindle positioning events

(Ellis et al., 2004; Lu and Mains, 2005; Phillips et al., 2004) — but how altered microtubule dynamics affect spindle positioning is unclear.

Microtubule length must also be regulated to correctly position a spindle, and this is especially apparent during the meiosis-to-mitosis transition in animal development. The *C. elegans* homologs of the microtubule-severing protein katanin are required to keep microtubules short during meiosis but must be downregulated in mitosis to allow the growth of a larger spindle that fills the one-cell-stage embryo (Clandinin and Mains, 1993; Kurz et al., 2002; Mains et al., 1990; Srayko et al., 2000). Early *C. elegans* embryos lacking MBK-2, a member of the Dyrk family of protein kinases, have short microtubules and defects in spindle positioning (Pang et al., 2004). This phenotype is rescued by knockdown of katanin, suggesting that MBK-2 protein normally functions to downregulate katanin, thereby controlling spindle size during mitosis (Pang et al., 2004). Other proteins that affect microtubule length independently of the katanin pathway include the Doublecortin-related kinase ZYG-8 (Gonczy et al., 2001) and the TAC-1/ZYG-9 complex, members of the TACC family and the associated XMAP215 family, respectively (Bellanger and Gonczy, 2003; Le Bot et al., 2003; Srayko et al., 2003).

Spindle positioning can occur by means of asymmetries in microtubule aster size, of which the most studied example is in *Drosophila* neuroblasts. In these cells, the spindle is shifted basally, where the centrosome and associated microtubules are small compared to the apical centrosome and its microtubules (Figures I.2, I.3) (Kaltschmidt et al., 2000). In an extreme case of spindle pole asymmetry, one-cell-stage embryos of the freshwater oligochaete Tubifex divide asymmetrically with only one spindle pole

containing the microtubule-nucleating protein gamma-tubulin and astral microtubules (Ishii and Shimizu, 1995).

2007 Update: Force generating mechanisms in C. elegans embryos

Microtubule-based motors are one way in which forces can be generated, and several advances in understanding the role of motors in spindle positioning have been made in *C. elegans* embryos. After using lasers to ablate centrosomes during anaphase, and tracking speed and direction of microtubule fragments, it was determined that the number of force-generators is higher in the posterior half of the embryo (Grill et al., 2003). In addition, by using computer modeling combined with experimental data, Pecreaux and colleagues showed that partial depletion of cytoplasmic dynein and a G-protein regulator causes a complete loss of anaphase spindle oscillations despite the remaining presence of the proteins (2006). Anaphase spindle oscillations, then, require a minimum threshold of force generators (Pecreaux et al., 2006).

Regulation of microtubule dynamics have also been proposed to generate force. Recent images of the cortex in early *C. elegans* embryos suggest that microtubules contact the cortex very briefly, and these short residence times are uniform across the embryo's cortex (Kozlowski et al., 2007). Computer simulations of this low cortical residence time of microtubules suggest that these microtubule dynamics are sufficient for asymmetric spindle positioning (Kozlowski et al., 2007). The cortical residence time of microtubules in this study, however, are quite different from those measured earlier in flattened embryos (Labbe et al., 2003).

Through the use of transgenic *C. elegans* strains expressing EB1:GFP to visualize the plus ends of growing microtubules, there is now a systematic description of microtubule dynamics in the early embryo, as well as further characterization of factors known to effect microtubule-dependent processes (Srayko et al., 2005). Finally, a study of LET-711 in embryos demonstrates that a reduction of LET-711 leads to longer, more stable microtubules in the early embryos, as well as larger centrosomes (DeBella et al., 2006). LET-711 is proposed to affect centrosomes-associated proteins, such as ZYG-9, to indirectly function in regulating microtubule dynamics (DeBella et al., 2006).

Regulators of force-generating mechanisms

Polarity establishment

C. elegans has been a well-studied model for polarity establishment (Figure I.3). Recent research has aimed at understanding how polarity-establishing proteins function in controlling asymmetric spindle positioning. PAR proteins are essential for downstream events that may affect spindle positioning; these downstream events include the regulation of microtubule stability at the cortex (Labbe et al., 2003), the generation of pulling forces (Grill et al., 2001; Labbe et al., 2004), and the asymmetric localization of other proteins required for spindle positioning, such as LET-99 and GPR-1/2 (Colombo et al., 2003; Gotta et al., 2003; Srinivasan et al., 2003; Tsou et al., 2002). *Drosophila* neuroblasts also localize a PAR protein complex to the apical cortex of the dividing cell (Wodarz, 2005). It is important, then, to determine if similar polarity-establishing mechanisms are used in other asymmetrically dividing cell types. Recent work in mouse oocytes has revealed the localization of homologs of PAR6 and PAR3 to a cortical actin

cap near the meiotic spindle (Duncan et al., 2005; Vinot et al., 2004). Polarity establishment in animal cells may not always be regulated by the PAR proteins, however. HAM-1, for example, is localized asymmetrically and is required for asymmetric division in *C. elegans* neuroblasts (Frank et al., 2005).

2007 Update: Polarity in mouse ooctyes

The asymmetric division of mouse oocytes has provided an up and coming model for polarity and asymmetric cell division, in which both actin and microtubules play important roles. Recent work has shown that CDC42 plays a role in affecting both the actin and microtubule cytoskeletons during meiotic division in mouse oocytes (Na and Zernicka-Goetz, 2006). Specifically, actin and formin-2 are required for the process of spindle migration during meiosis (Dumont et al., 2007). Ran-GTPase and Rac have been implicated in asymmetric spindle positioning and polarity establishment in mouse oocytes. Ran-GTPase, presumably through its association with chromatin, functions to establish a cortical actin cap, an indication of polarity within dividing oocytes (Deng et al., 2007). And, Rac likely plays a role in meiotic spindle anchoring to the cortex once it has positioned itself there (Halet and Carroll, 2007). These data and others support a model in which the mouse meiotic spindle induces the actin cap required for polarity establishment, and this polarized region of cortex further maintains spindle position through its role in anchoring.

G-protein signaling

G-protein signaling is a major regulator of asymmetric spindle positioning in several systems including *C. elegans* and *Drosophila*. G-protein signaling acts downstream of the PAR proteins, affecting spindle orientation without affecting the localization of cell fate determinants (Gotta and Ahringer, 2001). Spindle positioning is affected through the non-receptor-dependent Gα/Gβγ complex when a regulator of this pathway induces the exchange of GDP for GTP on Gα, followed by the separation of Gβγ from Gα (Gotta and Ahringer, 2001). Either of these subunits, or both, may promote downstream signaling. Recent work in *C. elegans* embryos has revealed that RIC-8 acts as a guanine nucleotide exchange factor, stimulating GTP binding to and activation of a Gα protein to induce pulling forces (Afshar et al., 2004; Couwenbergs et al., 2004; Hess et al., 2004), in addition to being required for the cortical localization of a second Gα protein (Afshar et al., 2005). Another regulator of G-protein signaling, RGS-7, functions in stimulating the hydrolysis of GTP-Gα to GDP-Gα, modulating those forces (Hess et al., 2004). While the PAR proteins are required for generating an asymmetry in pulling forces (Grill et al., 2001; Labbe et al., 2004), G-protein signaling is required for generating strong pulling forces on both sides of the cell (Colombo et al., 2003; Gotta et al., 2003), indicating that PAR proteins differentially regulate forces that are strictly dependent on G protein signaling.

In *Drosophila* as in *C. elegans*, G-protein signaling functions in regulating the spindle orientation downstream of cell fate determinant segregation (Izumi et al., 2004; Wodarz, 2005). Neuroblasts lacking a functional Gβγ complex cannot correctly orient spindles (Izumi et al., 2004). The activity of this G-protein signaling pathway is

regulated by Pins (Partner of Inscuteable) and Loco (Locomotion defect), which localize apically along with Gα (Yu et al., 2005). Pins and Loco function synergistically as guanine nucleotide dissociation inhibitors to facilitate the generation of free Gβγ (Schaefer et al., 2000; Yu et al., 2005), while Loco may have an additional function as a GTPase-activating protein regulating the equilibrium of GDP-Gα and GTP-Gα (Yu et al., 2005). Recently, studies in *Drosophila* neuroblasts and sensory organ precursor cells demonstrated a role for Ric-8 in spindle positioning, in which Ric-8 regulated the cortical localization and activity of Gα and Gβγ subunits (David et al., 2005; Hampoelz et al., 2005; Wang et al., 2005).

How G-protein signaling causes an asymmetry in microtubule pulling forces is unknown in asymmetrically dividing cells. Recent work in mammalian cells has, however, suggested a model. Mammalian Pins, called LGN, links cortical Gα to NuMA, a microtubule binding protein (Du and Macara, 2004). When either Gα or YFP:LGN is overexpressed, spindles in these cells have pronounced oscillations that are NuMA-dependent (Du and Macara, 2004), suggesting that these proteins regulate spindle positioning forces. It will be interesting to see if similar mechanisms are used in asymmetrically dividing cells, such as in *C. elegans* and *Drosophila*, where the LGN homologs GPR-1/2 and Pins become localized asymmetrically (Colombo et al., 2003; Gotta et al., 2003; Wodarz, 2005).

2007 Update: G-protein signaling

Work from Du and Macara paved the way towards a better understanding of the link between G-protein signaling and spindle positioning (2004). In three separate

studies of *Drosophila* neuroblasts, the microtubule binding protein Mud is shown to be a NuMA ortholog (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). Similar to NuMA, Mud binds directly to a G-protein signaling component, Pins, at its cortical crescent in the dividing neuroblast, and can also bind centrosomes and microtubules (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). Although it still unknown mechanistically how this link provides a layer of regulation in spindle positioning, this function of NuMA/Mud may be conserved.

Similarly, the role of G-protein signaling in regulating spindle positioning is also conserved. Recent work in sea urchin has identified the role of AGS/Pins G-protein signaling activator in asymmetric cell divisions (Voronina and Wessel, 2006). AGS functions in generating the asymmetric micromere cell divisions of 16-cell stage embryos (Voronina and Wessel, 2006).

2007 Update: Role of centrosomes in asymmetric spindle positioning

Two interesting studies in *Drosophila* have revealed an unusual centrosome cycle, and its effect on asymmetric spindle positioning in dividing larval neuroblasts (Rebollo et al., 2007; Rusan and Peifer, 2007). In these asymmetrically dividing cells, only one, apical, mature centrosome maintains MTOC function during the early stages of the cell cycle, while the other organizes an aster only after moving to the opposite, basal side of the neuroblast. While Rebollo and colleagues suggest that spindle positioning is determined by the position of the apical centrosome (2007), Rusan and Peifer suggest that this original position of the apical centrosome functions in coarsely aligning the spindle,

followed by later spindle-cortical interactions that function to ensure proper alignment (2007).

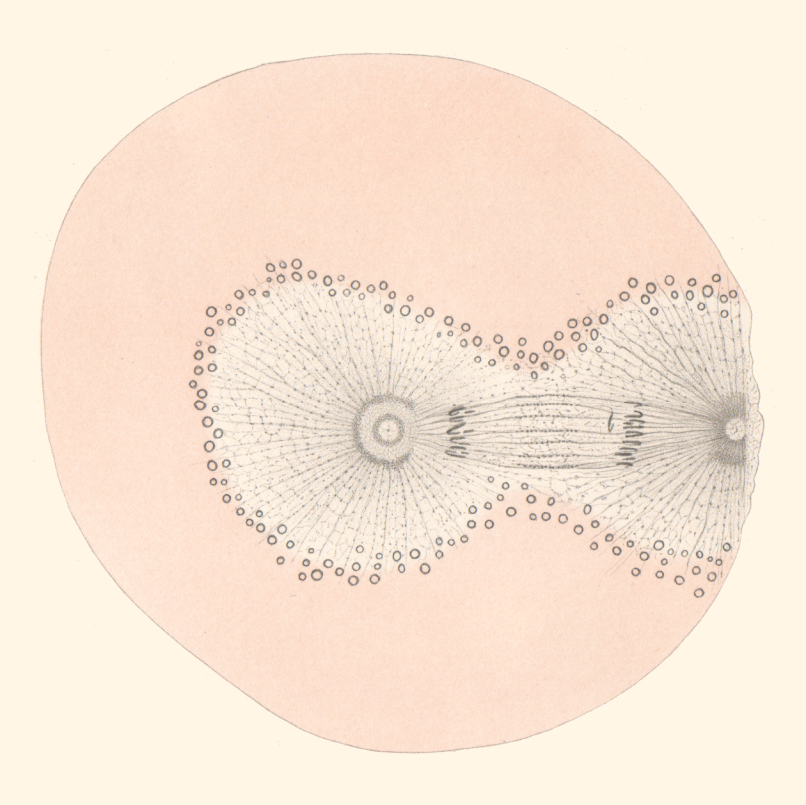
Monitoring asymmetric spindle positioning

In budding yeast, spindle positioning is monitored, ensuring accurate chromosome segregation. The budding yeast spindle position checkpoint delays activation of the mitotic exit network (MEN) in cells with mispositioned spindles by activating the Bub2–Bfa1 complex (Pearson and Bloom, 2004). Activation of this signaling pathway is triggered by changes in MEN protein dynamics at spindle poles upon penetration of the daughter-bound spindle pole into the bud (Molk et al., 2004). Recent work by two groups describes how Kin4 kinase acts as part of this monitor by inhibiting MEN signaling in cells with mispositioned spindles (D'Aquino et al., 2005; Pereira and Schiebel, 2005). The MEN signaling pathway ultimately triggers anaphase onset by regulating Cdc14 release from the nucleolus. Prior to this, a small wave of Cdc14 release occurs via the FEAR network (Cdc-fourteen early anaphase release), which triggers early anaphase events. The FEAR network has recently been demonstrated to play a role, via Cdc14, in ensuring proper nuclear position during anaphase (Ross and Cohen-Fix, 2004). Fission yeast cells, although they divide symmetrically, monitor spindle positioning by a checkpoint that also regulates anaphase onset timing (Gachet et al., 2001; Oliferenko and Balasubramanian, 2002; Tournier et al., 2004). Whether or not spindle position is monitored in animal cells or in other organisms is not yet clear.

Conclusions

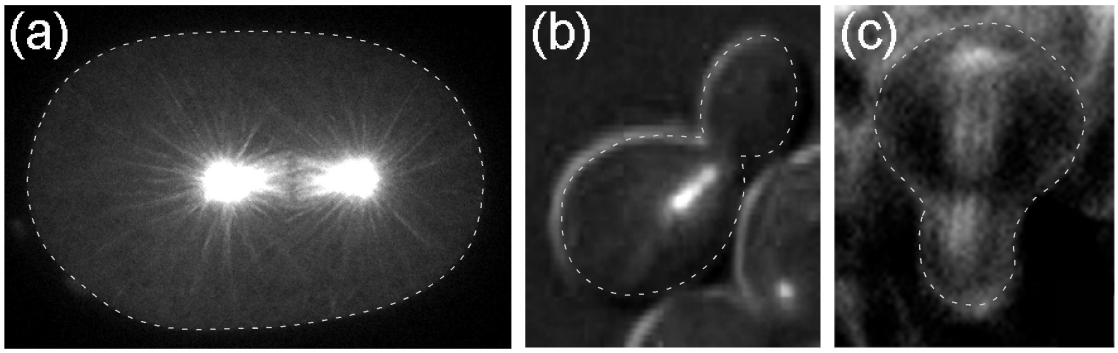
The movement of a spindle to an eccentric location is a complex process requiring motor activities that act at specific times in a cell. The examples cited here provide glimpses of the mechanisms by which this occurs. It will be interesting to determine to what extent these mechanisms function similarly in other systems. In addition, it will be interesting to see how the mechanisms that control positioning in asymmetric divisions are similar or different to those that function in symmetrically dividing cells.

Figure I.1. Asymmetric spindle positioning observed in 1901.



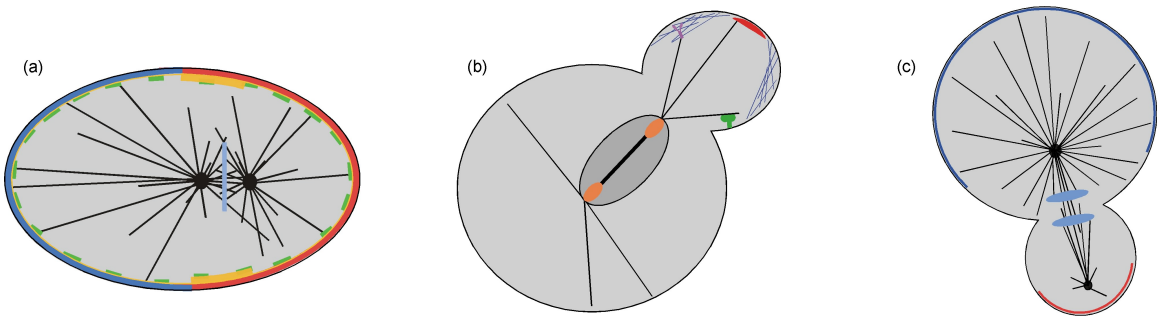
Asymmetric spindle positioning in a mussel, a drawing from a 1901 publication based on staining of embryos with textile dyes used at the time by cytologists (Lillie, 1901). Several theories of how spindles are positioned asymmetrically already existed by this time. Reprinted with permission.

Figure I.2. Images of examples of asymmetric spindle positioning.



The mitotic spindle is positioned asymmetrically **(a)** closer to the posterior region of the *C. elegans* embryo, **(b)** at the bud neck in budding yeast cells and **(c)** along an apical–basal axis in *Drosophila* neuroblasts. Live-cell imaging of cells expressing tubulin:GFP or tau:GFP have allowed the analysis of changes in spindle position, orientation, structure, size and dynamics. Budding yeast image provided by J Molk and K Bloom. *Drosophila* image provided by A Brand.

Figure I.3. Models of asymmetric spindle positioning.



The mitotic spindle is positioned asymmetrically in these cell divisions. **(a)** In the *C. elegans* one-cell stage embryo, the mitotic spindle is positioned close to the posterior cortex. This is dependent on the presence of cortical proteins (PAR-3 is blue, PAR-2 is red, LET-99 is orange) that regulate force generators, which might be dynein patches localized at the cortex (green). **(b)** In budding yeast, the spindle is oriented when myosin (purple) binds plus ends of microtubules to direct them to cortical proteins at the bud tip cortex (red), which may provide a pulling force. Dynein (green) positions the spindle into the bud neck during anaphase through interactions of astral microtubules at the cortex. **(c)** In *Drosophila* neuroblasts, the spindle is asymmetric during anaphase when the apical microtubules are able to grow longer than basal microtubules. Cortical complexes that are required for spindle orientation and cell fate determination include the PAR/aPKC and Pins/Gα complexes, which are localized apically (dark blue), and Miranda, Prospero and Numb, which are localized basally (red).

CHAPTER II.

KINETOCHORE MICROTUBULES DO NOT UNDERGO SIGNIFICANT POLEWARD FLUX DURING ANAPHASE IN *C. ELEGANS*

Introduction

The first division of the early *C. elegans* embryo gives rise to two daughters of different size and molecular composition; e.g., only the posterior daughter inherits germline determinants such as P granules and the protein PIE-1 (Pellettieri and Seydoux, 2002). This asymmetry in cell size results from the position of the first mitotic spindle, which forms at the center but moves to the posterior of the one-cell embryo before cytokinesis (Albertson, 1984). Posterior spindle displacement is dependent on the PAR proteins, which are required to establish and maintain polarity in the embryo (Kemphues et al., 1988), and heterotrimeric G-protein signaling, which acts downstream of the PAR proteins (Gotta and Ahringer, 2001). Spindle-cutting experiments demonstrated that PAR proteins and G proteins function to generate an imbalance in pulling forces that act on each side of the spindle during anaphase, creating a stronger pulling force toward the posterior of the embryo and possibly regulating posterior spindle displacement (Grill et al., 2001; Grill et al., 2003). These experiments also demonstrated that spindle microtubules function to limit the rate of spindle pole separation during anaphase (Grill et

al., 2001), possibly because the antiparallel sliding of polar microtubules in the spindle occurs at a limiting rate.

Although little is known about how the mitotic spindle is positioned before asymmetric cell divisions, there has been intensive study on the generation of forces that drive movements of spindle components during normal mitotic divisions. Such work is informative, and also provides model approaches, for studying how spindles are positioned asymmetrically. Segregation of chromosomes, for example, occurs through a fine regulation of microtubule-dependent forces that act on centrosomes and sister chromatids through the mitotic phase of the cell cycle (for review see Cleveland et al., 2003). These forces have been defined as anaphase A and B forces.

Anaphase A forces function to shorten the distance between each sister chromatid and its respective spindle pole. In *Drosophila* embryos and *Xenopus laevis* extract spindles, these forces are mediated, at least in part, by a complex regulation of microtubule dynamics: the kinetochore microtubules, which directly mediate the connection between chromosomes and the spindle pole, generally undergo a process termed poleward flux, a microtubule behavior in which the kinetochore-bound plus end of microtubules undergoes polymerization, whereas the spindle pole-associated minus end is concomitantly depolymerized (Brust-Mascher and Scholey, 2002; Desai et al., 1998; Maddox et al., 2002; Mitchison, 1989). At metaphase, the rates of microtubule polymerization and depolymerization are equal, and individual tubulin dimers translocate along microtubules in a plus-to-minus end direction, leaving kinetochore microtubules at a roughly constant length (Maddox et al., 2003; Mitchison, 1989). At anaphase onset, the microtubule plus ends switch from polymerization to depolymerization, whereas the

minus ends continue to depolymerize, resulting in movement of the chromosomes toward the spindle pole (Desai et al., 1998; Maddox et al., 2003).

Anaphase B forces are responsible for the increase in distance between the two spindle poles, which generally occurs at anaphase onset. This increase can occur through the generation of pushing forces by motors on overlapping, antiparallel spindle microtubules (Aist and Berns, 1981; Inoue et al., 1998). Pole–pole separation can also be mediated by astral microtubules, which extend from the centrosomes and make contact with the cell cortex. For instance, cortically bound, minus end–directed motor proteins, such as dynein, could mediate such a function (Inoue et al., 1998). Astral microtubules are also required to position the spindle in the center of dividing cells (O'Connell and Wang, 2000). In symmetrically dividing cells, the forces acting on astral microtubules are likely equal on each side of the spindle and remain equal during both spindle positioning and spindle pole separation.

Both anaphase A and anaphase B forces are temporally regulated by the cell cycle machinery. This level of regulation is mediated, in part, by components of the spindle checkpoint and ensures that segregation does not initiate before all chromosomes make kinetochore–microtubule attachments and align at the metaphase plate (for review see Cleveland et al., 2003). Interestingly, during prometaphase and metaphase, poleward microtubule flux as well as microtubule plus end dynamics generate forces, as evidenced by tension at the kinetochore (Pearson et al., 2001). These forces contribute to chromosome congression and are at dynamic equilibrium when sister chromatids are aligned at the metaphase plate, indicating that some forces are active before cells enter anaphase (Mitchison and Salmon, 1992; Waters et al., 1996). The absence of

chromosome segregation during this time might be mediated by cohesins, which link sister chromatids together and are degraded at the metaphase–anaphase transition (Nasmyth, 2002).

We show that photobleaching segments of microtubules in early *C. elegans* embryos during anaphase revealed that spindle microtubules are not undergoing significant poleward flux. Together with the known absence of anaphase A, these data suggest that forces from outside the spindle are the major components contributing to chromosome separation during anaphase. We propose that the forces acting on microtubules to asymmetrically position the mitotic spindle are modulated throughout the cell cycle and that these same forces are used to drive chromosome segregation at anaphase.

Results

Asymmetric spindle positioning begins in metaphase

In the one-cell stage *C. elegans* embryo, the spindle forms at the center of the cell and moves toward the posterior before cytokinesis. Previous experiments assessing the forces acting on the spindle were performed at anaphase B (Grill et al., 2001; Grill et al., 2003). As a baseline for further studies, we first determined the stage of the cell cycle during which posterior spindle displacement occurs by imaging embryos expressing both gamma-tubulin and histone H2B fused to GFP (Oegema et al., 2001), which allowed us to simultaneously monitor the behavior of centrosomes and chromosomes, respectively. After the spindle arrived at the center of the embryo, both the centrosomes and chromosomes began to move posterior of the center 60.9 ± 20.8 s before anaphase (n =

7), near the time when sister chromatids were first aligned at the metaphase plate (Fig. II.1). Chromosome separation occurred after the spindle began moving toward the posterior in the cell, and the posterior spindle pole continued to move posteriorly after entry into anaphase. Therefore, the mitotic spindle begins to move to an asymmetric position during metaphase, before anaphase onset, which is consistent with observations made previously (Oegema et al., 2001). This result suggests that spindle positioning is unlikely to be regulated by anaphase entry.

Spindle-positioning forces also drive sister chromatid segregation

In vertebrate and *Drosophila* spindles, microtubule poleward flux is a significant component of chromosome segregation (Desai et al., 1998; Maddox et al., 2002; Maddox et al., 2003). One striking observation made previously in *C. elegans* embryos is that the mitotic spindle does not undergo anaphase A during chromosome segregation (Oegema et al., 2001; Fig. II.2, A and E). Furthermore, despite an asymmetry in pulling forces on each side of the spindle, we have found that chromosome segregation and centrosome separation in each spindle half appear symmetric (Fig. II.2A), suggesting that the forces within the mitotic spindle itself may also be symmetric. Together with the finding that the spindle midzone limits the rate of anaphase pole separation (Grill et al., 2001), these observations suggested that the forces responsible for mediating pulling on the asters during posterior spindle displacement may also drive the segregation of chromosomes at anaphase B. However, poleward flux has been shown to generate tension at the kinetochore of mitotic spindles in *Xenopus* extracts, through constant microtubule plus end net polymerization and minus end depolymerization (Desai et al., 1998; Maddox et

al., 2003). Therefore, one possibility remained that spindle microtubules might be under tension during chromosome segregation through poleward flux, despite the apparent absence of anaphase A.

To test this possibility, we used an approach that relies on the photobleaching of microtubule-associated fluorophores and quantification of FRAP in living specimens. Such an approach has proven successful in the past to study a broad variety of cellular events, including the dynamics of spindle microtubules (Salmon et al., 1984; Saxton et al., 1984; Zhai et al., 1995). We photobleached a small region of the central spindle in embryos expressing a gene encoding ß-tubulin fused to GFP at either prometaphase or at anaphase onset and quantified FRAP in this region (see Appendix A: Materials and Methods for Chapter II.). Spindle microtubules photobleached at the time of prometaphase showed a fast fluorescence recovery time (average t1/2 = 10.6 s), suggesting a rapid turnover of tubulin subunits in the microtubule polymer during this stage of the cell cycle (Fig. II.2, B and D). This fast recovery precluded detecting if the photobleached region moved during metaphase, thus preventing analysis of microtubule dynamic properties, such as poleward flux, because no mark could be followed on the microtubule lattice. However, microtubules photobleached at anaphase onset showed a slower rate of fluorescence recovery (average t1/2 = 17.7 s), indicating a slower turnover of tubulin subunits within microtubules at this stage (Fig. II.2, C and D). We were able to monitor the movement of the photobleached region during the course of anaphase and found that the photobleached region on spindle microtubules remained at a constant distance from the spindle pole as it followed the spindle pole (Fig. II.2E). This finding demonstrates that, during anaphase, spindle microtubules are not undergoing significant

poleward flux in *C. elegans* embryos. The fact that a majority of spindle microtubules are mediating kinetochore attachments in *C. elegans* (O'Toole et al., 2003) suggests that a significant number of the bleached microtubules are attached to kinetochores. The finding that kinetochore microtubules do not undergo significant flux implies that kinetochore microtubules do not contribute dynamic forces during anaphase. Together with the findings that the spindle midzone limits the rate of anaphase pole separation (Grill et al., 2001) and the absence of anaphase A (Oegema et al., 2001), these results suggest that in *C. elegans* spindle microtubules are relatively static in anaphase. We conclude that the forces that drive pole and chromosome separation are provided by astral microtubules.

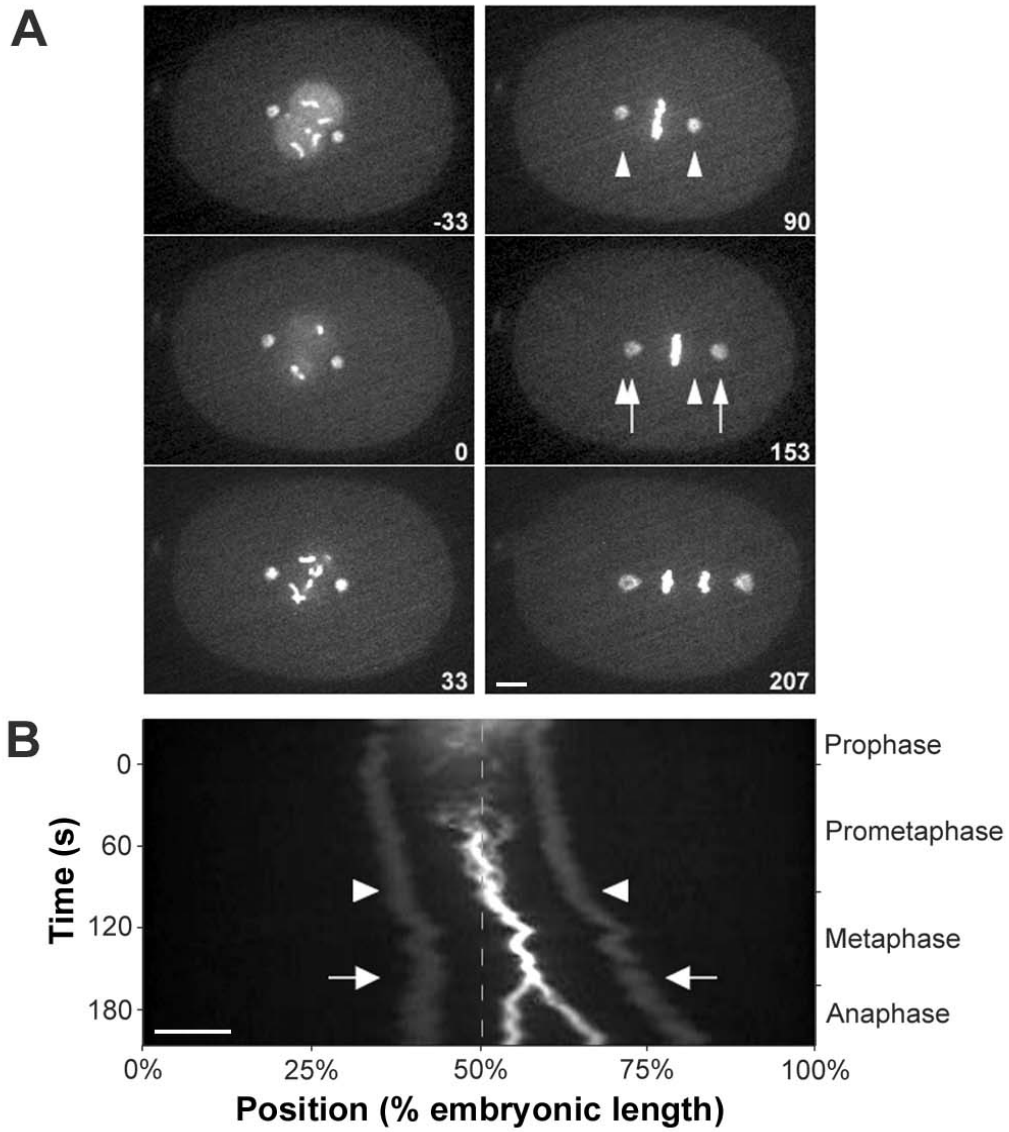
Discussion

We have observed that spindle microtubules do not undergo significant poleward flux during mitotic anaphase. This result, together with the observation that chromosome segregation occurs without anaphase A (Oegema et al., 2001), suggests that spindle microtubule dynamics are regulated differently in *C. elegans* zygotes compared with mammalian and *Drosophila* cells, which have been shown to undergo poleward flux (Brust-Mascher and Scholey, 2002; Desai et al., 1998; Maddox et al., 2002). Because the spindle midzone was shown to limit the rate of anaphase pole separation (Grill et al., 2001), this further suggests that the forces responsible for mediating pulling on each aster, and for positioning the spindle, are also involved in segregating chromosomes in the *C. elegans* zygote. Poleward flux and microtubule plus end dynamics have been proposed to be responsible for generating tension at the kinetochores (Maddox et al.,

2003; Mitchison and Salmon, 1992; Waters et al., 1996), and an asymmetry in astral microtubule flux during late prophase and prometaphase could potentially account for the early asymmetry in pulling forces that we observed in early *C. elegans* embryos.

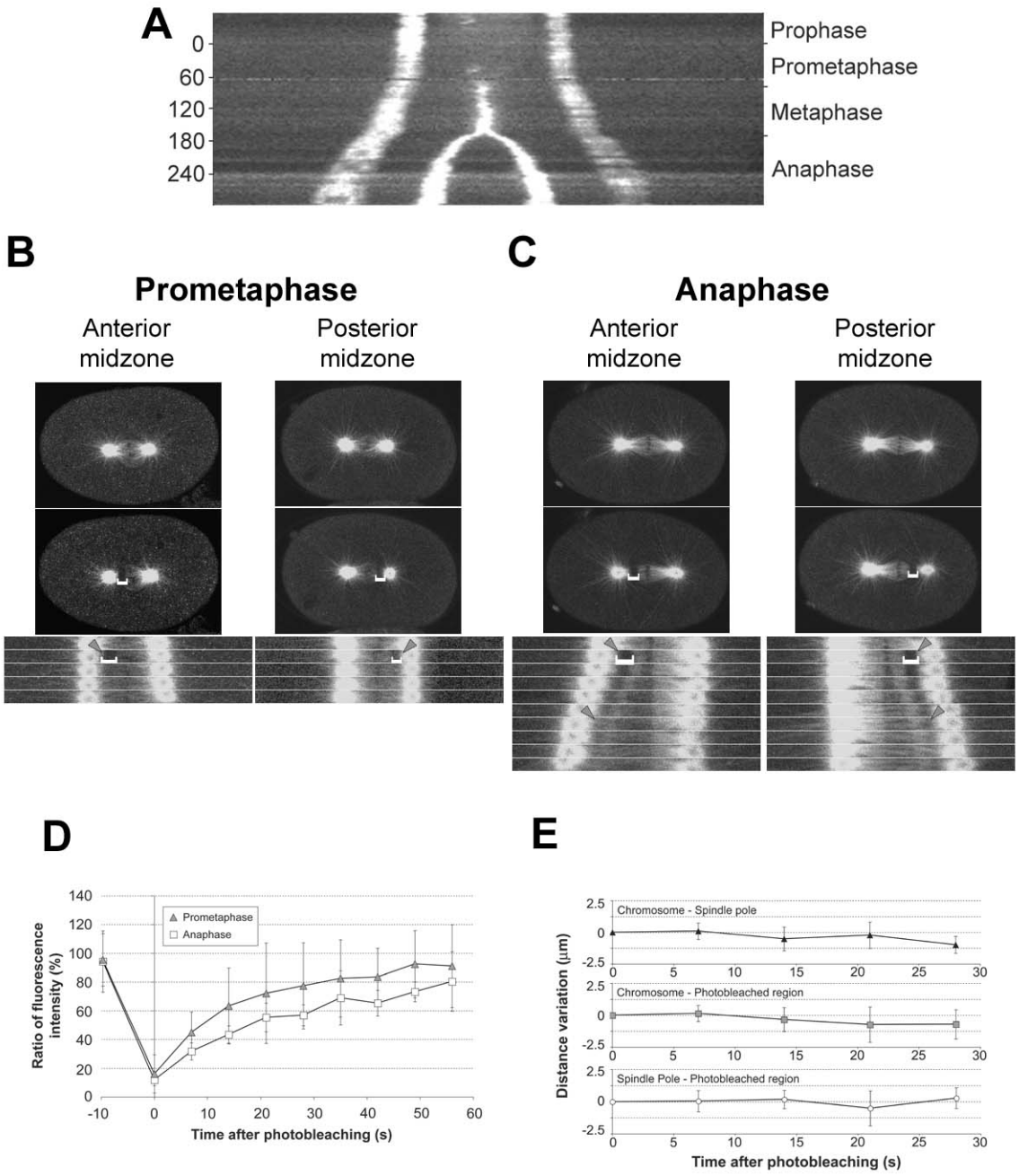
We are currently unable to image individual astral microtubules long enough *in vivo*, by conventional confocal microscopy, to determine whether or not they undergo flux. It also remains to be established whether or not the absence of significant poleward flux in spindle microtubules that we measured is specific to *C. elegans* zygotes or is a more general property of asymmetrically dividing cells. In this sense, it is interesting to note that spindle microtubules do not undergo poleward flux in *S. cerevisiae*, which also divides asymmetrically (Maddox et al., 2000).

Figure II.1. Posterior spindle displacement begins at metaphase.



(A) Time-lapse images of an early *C. elegans* embryo expressing both gamma-tubulin and histone H2B fused to GFP. (B) Kymograph analysis of spindle behavior from these time-lapse images. In both panels, arrowheads point to centrosomes at early metaphase and arrows point to the centrosomes at late metaphase, before anaphase onset. Displacement of the spindle toward the posterior can be observed during metaphase. Displacement began during early metaphase or at the end of prometaphase in all embryos examined in this way (n = 8). Bars, 5 µm.

Figure II.2. Chromosome segregation occurs without anaphase A and significant poleward flux.



(A) Spindle-centered kymograph of an embryo expressing both gamma-tubulin and histone H2B fused to GFP. This kymograph was produced similarly to the one in Fig. II.1B, except that posterior movement of the spindle was eliminated: the spindle in each frame of time-lapse images was rotationally aligned and recentered on the midpoint between the centrosomes to allow the observation of symmetries in the spindle. Frames were acquired at 7-s intervals. (B and C) Time-lapse images of embryos expressing ß-tubulin::GFP in which a short region of anterior (left) or posterior (right) spindle microtubules were photobleached during prometaphase (B) or anaphase (C) onset. The bottom panels follow FRAP as well as movement of the photobleached region (indicated by gray arrowheads). Frames were acquired at 7-s intervals. For photobleaches of the spindle during prometaphase, kymographs were aligned to the location of chromosomes in the center of the spindle. For photobleaches of the anterior or posterior half of the spindle at anaphase onset, kymographs were aligned to the center of the posterior or anterior centrosome, respectively. (D) Quantification of FRAP during prometaphase (gray triangles) and anaphase (open squares). To correct for fluorophore bleaching and embryo to embryo variations, fluorescence intensity in the photobleached region is expressed as a ratio of bleached over unbleached midzone microtubules in the same embryo. FRAP occurs faster during prometaphase (t1/2 = 10.6 s; polynomial equation: y = –3E – 07 x4 + 4E – 05x3 – 0.0021x2 + 0.0559x + 0.1571; R2 = 0.997) compared with anaphase (t1/2 = 17.7 s; polynomial equation: y = 4E – 08x4 + 2E – 06x3 – 0.0005x2 + 0.0303x + 0.1223; R2 = 0.989). Time points were acquired at 7-s intervals. Error bars represent SD over six embryos. (E) Quantification of the distance variation between chromosomes to photobleached region (black triangles), chromosomes and spindle poles

(gray squares), and spindle pole to photobleached region (open circles) during anaphase. The distance remains constant between these three positions throughout anaphase. Time points were acquired at 7-s intervals. Error bars represent SD over six embryos.

CHAPTER III.

A CELL CYCLE TIMER FOR ASYMMETRIC CELL DIVISION

Asymmetric cell division is an important process for animal development. In many cells that divide asymmetrically, the mitotic spindle shifts to an asymmetric position, resulting in daughter cells of different sizes (Betschinger and Knoblich, 2004; McCarthy and Goldstein, 2006). Regulating the timing of such spindle shifts may be critical, since moving the spindle before it fully assembles could lead to aneuploidy. Little is known about how spindle shifts are timed in asymmetric cell divisions. Here we show that components of the spindle assembly checkpoint pathway serve a novel role as a timer for asymmetric spindle positioning in the one-cell *C. elegans* embryo. We found that the mitotic spindle begins to shift at a precise time in the one-cell stage, soon after chromosomes have completed congression to the metaphase plate. Reducing the function of spindle checkpoint pathway components caused a delay in spindle positioning. Conversely, premature inactivation of cyclin-dependent kinase (CDK) caused the mitotic spindle to shift prematurely, often before chromosome congression was completed. Furthermore, we found that the timing of the spindle shift depends on spindle checkpoint proteins, and that the pathway timing the spindle shift is wired somewhat differently than the checkpoint pathway timing anaphase entry. Based on our results, we conclude that the spindle shift waits briefly for inactivation of CDK activity by the anaphase promoting

complex, by an unexplored mechanism. This additional role for the spindle checkpoint pathway may ensure that chromosomes attach to the mitotic spindle before the spindle shifts to an asymmetric position. This work demonstrates a fundamental new link between cell and developmental biology, between cell cycle checkpoint regulation and asymmetric cell division.

Introduction

The mitotic spindle of the one-cell stage *C. elegans* embryo is moved to an asymmetric position by an inequality in net pulling forces on the two sides of the spindle (Grill et al., 2001; Grill et al., 2003; Labbe et al., 2004). We have found previously that before the spindle begins to move asymmetrically, pulling forces on one side of the spindle are balanced by a microtubule-based tether on the other side. This tether is released near the time that the spindle begins to shift (Labbe et al., 2004). This suggested to us the possibility that the timing of spindle movement might be carefully regulated in asymmetric cell divisions, perhaps by a cell cycle checkpoint mechanism. Early-stage animal embryos lack many cell cycle checkpoints (Hartwell and Weinert, 1989), but some exceptions have been found in which checkpoints can monitor early embryonic cell cycles (Brauchle et al., 2003; Encalada et al., 2005; Holway et al., 2006). Because chromosomes begin shifting asymmetrically in metaphase or anaphase in asymmetric cell divisions of diverse animal systems (Ishii and Shimizu, 1995; Kaltschmidt et al., 2000; Labbe et al., 2004; Oegema et al., 2001; Ren and Weisblat, 2006; Roegiers and Jan, 2004; Shimizu, 1996; Zhang and Weisblat, 2005), we hypothesized that the spindle checkpoint pathway might function as a timer for such asymmetric movements.

Results and Conclusions

We examined the precise timing of spindle positioning in one-cell stage *C. elegans* embryos by tracking movements of all of the chromosomes and both centrosomes, using multiple-plane imaging of histone H2B:GFP and gamma-tubulin:GFP (Oegema et al., 2001)(Figure III.1A). We quantitatively analyzed the degree of chromosome congression and the position of the spindle throughout mitosis. We found that the spindle began to shift toward the posterior cortex soon after chromosome congression was completed (Figure III.1B,C,D; Figure III.2). Metaphase, defined here as the time from completion of chromosome congression to the beginning of anaphase chromosome separation, lasted an average of 66.9 ± 8.8 seconds. The spindle began to shift early in metaphase, starting an average of 10.8 ± 11.3 seconds after we observed the completion of congression. This timing and level of precision suggested further testing of whether mitotic progression pathways time the spindle shift.

Mitotic progression depends in part on the degradation of proteins by the proteasome at the transition from metaphase to anaphase (Gutierrez and Ronai, 2006). We disrupted the proteasome to determine if the spindle shift is timed by proteasome activity. We used two treatments—the pharmacological inhibitor clasto-lactacystin β-lactone (c-LβL) for rapid proteasome disruption, and *rpt-6*(RNAi) for specific targeting of a proteasome component. RPT-6 is a component of the 19S proteasome subunit, and its disruption has been shown to delay mitotic timing in the early embryo without disrupting the earlier process of meiosis (Gonczy et al., 2000). Treatment with c-LβL after laser-permeabilization of the eggshell did not have an apparent effect on spindle

morphology (Figure III.3B). Both treatments caused a delay of anaphase onset, as expected. Strikingly, we found that these treatments also delayed asymmetric spindle positioning (Figure III.3), demonstrating that proteasome function is required for timely spindle positioning.

The proteasome has a large number of targets, a subset of which are targeted for degradation by the anaphase promoting complex (APC), a multi-subunit E3 ubiquitin ligase (Gutierrez and Ronai, 2006). The APC is activated by spindle checkpoint signaling once all chromosomes are attached to the mitotic spindle and aligned at the metaphase plate (May and Hardwick, 2006). We targeted *C. elegans* homologs of two key functional components of the APC for disruption; these components have been implicated in human cancer, perhaps because of their roles in orderly mitotic progression and prevention of aneuploidy (Wang et al., 2003; Zachariae and Nasmyth, 1996). Because the APC is required for progression through meiosis in *C. elegans* (Davis et al., 2002; Dong et al., 2007; Golden et al., 2000), we used two methods that can allow meiotic progression and then disrupt mitosis. First, we used a fast-acting temperature-sensitive allele of *mat-3*, the *C. elegans* homolog of APC8/CDC23 (Golden et al., 2000). We shifted *mat-3*(or180ts) embryos to the restrictive temperature after meiosis, but just prior to mitosis, and we found that this delayed both anaphase onset and asymmetric spindle positioning (Figure III.3C). Second, we used carefully timed dsRNA injections to attempt partial depletion of MAT-1, the *C. elegans* homolog of APC3/CDC27. At 3-6 hours after injection of *mat-1* dsRNA, embryos progressed through meiosis successfully, but in mitosis anaphase onset was delayed, and we found that asymmetric spindle

positioning was delayed as well (Figure III.3C). We conclude that APC function is required for timely spindle displacement.

A key activator of the APC is Cdc20/Fizzy, which is bound to and inhibited by checkpoint proteins until kinetochore attachment to the spindle is complete. Upon checkpoint inactivation, Cdc20/Fizzy is able to bind to and activate the APC (May and Hardwick, 2006). Similar to APC components, the *C. elegans* Cdc20/Fizzy protein FZY-1 is required before mitosis for meiotic progression. To disrupt FZY-1 function in mitosis, we attempted partial depletion of FZY-1 by timed dsRNA injections. At 10 hours post-injection, *fzy-1*(RNAi) delayed anaphase onset, and we found that it also delayed asymmetric spindle positioning (Figure III.3C). Together, our results suggest that FZY-1, the APC and proteasome activity are required for timely spindle positioning.

If mitotic progression determines the onset of spindle positioning, the converse effect on timing from our previous experiments should be possible: premature inactivation of an APC target should result in premature asymmetric spindle positioning. Cyclin B is a target of the APC, and degradation of cyclin B leads to inactivation of CDK (Pines, 2006). To test whether CDK inactivation temporally regulates the spindle shift, we used a highly specific pharmacological inactivator of CDK, the anticancer drug flavopiridol (Potapova et al., 2006; Sedlacek, 2001), since loss of maternal CDK in *C. elegans* results in meiotic defects before first mitosis (Boxem et al., 1999). We first tested whether flavopiridol can inactivate CDK in *C. elegans* embryos, applying the drug to one-cell stage embryos at the beginning of mitosis, prior to pronuclear envelope breakdown. A cyclin-CDK complex promotes entry into mitosis; thus, inhibition of CDK activity at this early stage should block mitotic entry (Pines, 2006). We found that upon

treating *C. elegans* embryos with flavopiridol prior to mitosis, pronuclear envelope breakdown failed to occur, and most microtubules were found unassociated with centrosomes (Figure III.4A), both suggesting that flavopiridol successfully blocked progression into mitosis. Based on this and further results below, we conclude that flavopiridol is likely to be an effective inhibitor of CDK activity in *C. elegans* embryos.

To test whether CDK inactivation functions as a timer for spindle positioning, we treated embryos with flavopiridol later, shortly after pronuclear envelope breakdown (PNEBD). Flavopiridol treatment at this stage of mitosis did not appear to disrupt microtubules or the mitotic spindle (Figure III.4A), and it did succeed in causing premature anaphase onset. Anaphase bridges formed in some embryos, although most embryos succeeded in separating chromosomes (10/14 cases). This result suggests that CDK inactivation promotes chromosome separation in *C. elegans* by functioning upstream of separase activity, as can occur in certain other systems (Stemmann et al., 2006). We found that flavopiridol treatment caused the spindle to shift prematurely (Figure III.4B). This premature shift may have a developmental consequence, as we found that chromosome congression was often not complete as the spindle began to shift and in some cases, chromosomes never completed congression (3/11 cases, compared to 0/21 cases of wild-type) (Figure III.4C,D).

Although our data suggest that CDK inactivation times the spindle shift, the APC targets other proteins for degradation in addition to cyclin B (Pines, 2006). To determine whether the proteasome and APC time asymmetric spindle positioning primarily through CDK inactivation, we determined whether flavopiridol treatment could rescue most of the delay caused by disrupting proteasome or APC activity. First, we used c-LβL to disrupt

proteasome function in one-cell stage embryos as before and then added flavopiridol after PNEBD. Flavopiridol rescued most of the c-LβL-induced anaphase delay. We found that flavopiridol also rescued most of the spindle shift delay (Figure III.5A). Second, we treated *mat-1*(RNAi) embryos with flavopiridol and found that the delay in both anaphase onset and spindle positioning was completely rescued (Figure III.5A). We conclude that the APC and the proteasome function as a timer for both anaphase and spindle positioning in the one-cell *C. elegans* embryo primarily through their roles in inactivating CDK. Taken together, our results suggest that the time at which the mitotic spindle shifts to an asymmetric position in this system is regulated by the well-known pathway that determines when anaphase will occur (Figure III.5B).

Does the spindle checkpoint directly regulate spindle positioning? Spindle checkpoint components normally function by keeping Cdc20/Fizzy inactive until spindle assembly is completed (May and Hardwick, 2006). Spindle checkpoint components in *C. elegans* regulate anaphase timing when the spindle is damaged (Encalada et al., 2005). In the absence of spindle damage, RNAi depletion of checkpoint components does not affect the timing of anaphase (Encalada et al., 2005) or spindle positioning (data not shown). However, we found, using strong loss-of-function mutants of checkpoint components (Stein et al., 2007), that MDF-2/Mad2 and MDF-3/Mad3 do regulate the timing of spindle positioning (Figure III.3C). A number of aspects of this finding were surprising: The timing of only spindle positioning and not anaphase was affected in these experiments, the timing of spindle positioning was delayed rather than shortened, and this delay appeared to be at least partially CDK-independent (Figure III.3C, Figure III.6). Our results suggest that these checkpoint components regulate the timing of spindle

positioning, but by an unexpected mechanism. MDF-2/Mad2 and MDF-3/Mad3 likely function here by keeping FZY-1/Cdc20 inactive as in other systems, as we found that a gain-of-function allele of *fzy-1* (Stein et al., 2007) produced the same results as loss of function of *mdf-2* or *mdf-3* (Figure III.3C, Figure III.6). Our finding that both unregulated FZY-1 activity and loss of function of *fzy-1* can delay the spindle shift suggest an unexpected dual function for FZY-1 in timing the spindle shift, potentially delaying the shift when unregulated and promoting it when activated at the appropriate time. The results suggest that an additional layer of regulation may exist -- an MDF-2, MDF-3, and FZY-1-dependent pathway for regulation of spindle positioning that is at least partially APC- and CDK-independent (Figure III.5B). Experiments in budding yeast cells have shown previously that Cdc20/Fizzy can function independently of the APC, although the mechanism by which it does so is not clear (Clarke et al., 2003). Given these results and our finding that CDK inactivation functions as a timer for spindle positioning, we conclude that the spindle checkpoint pathway does play a role in timing spindle positioning, but that the pathway timing the spindle shift in *C. elegans* is wired somewhat differently than the pathway timing anaphase entry.

Regulation of the timing of spindle positioning may play an important role in development. In the absence of the new role we have identified for the spindle checkpoint pathway, the spindle might shift before one or more chromosomes are attached. As the spindle moves, so will nearby cytoplasm (Kozlowski et al., 2007). However, a countercurrent flow of cytoplasm displaced by movement of the spindle might sometimes push unattached chromosomes away from the spindle, and prevent proper chromosome segregation. We propose that the regulation of spindle positioning

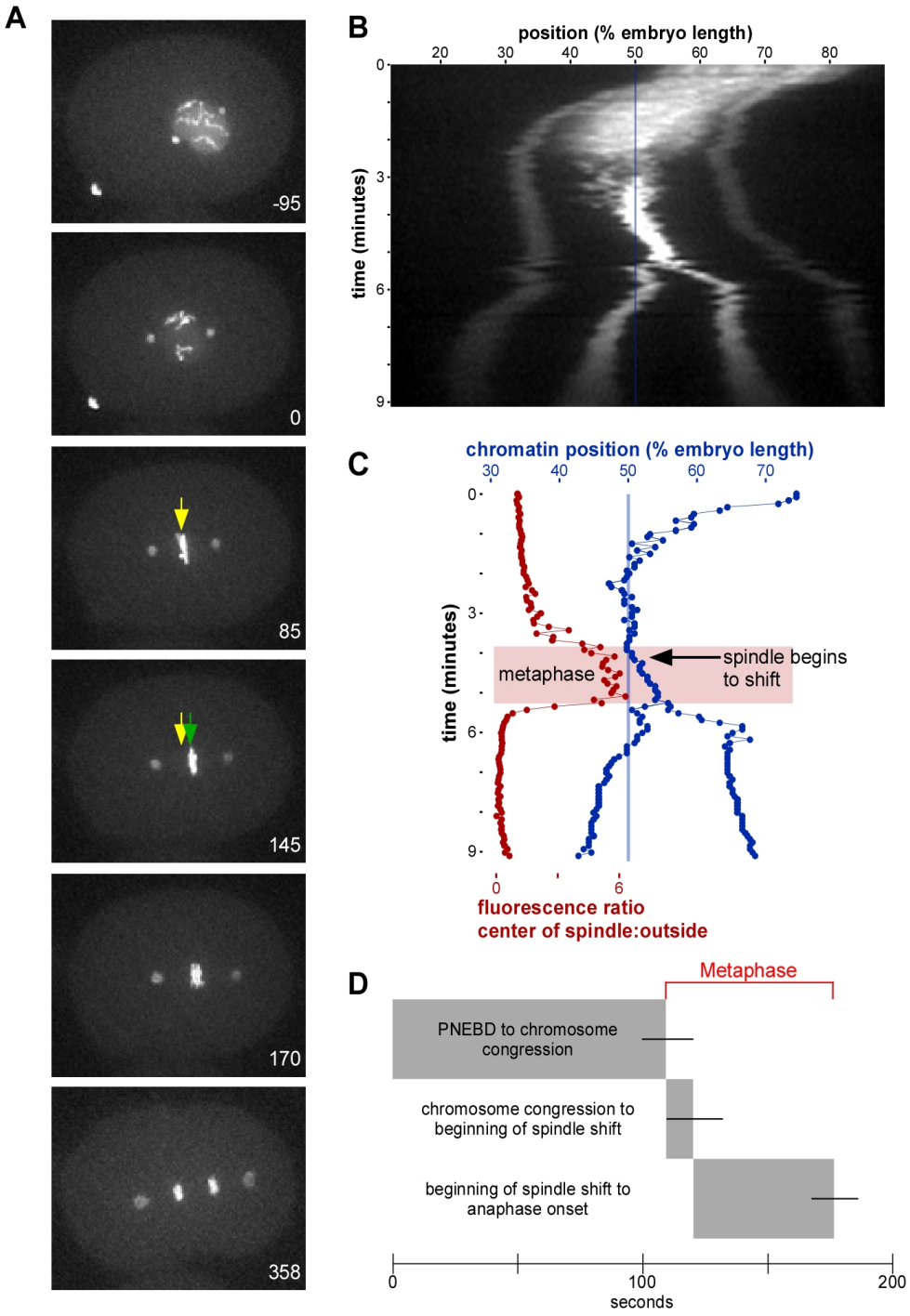
by the spindle checkpoint pathway might ensure that the spindle is fully assembled before it moves to an asymmetric position, ensuring that all chromosomes move together.

How might CDK inactivation impinge on the mechanism of asymmetric spindle positioning? The mechanism by which mitotic spindles are positioned asymmetrically has been a topic of intense recent interest. In a number of animal systems, a critical set of asymmetrically localized molecules has been identified, including Cdc42, PAR proteins, Gα subunits and their regulators (Bellaiche and Gotta, 2005; Cowan and Hyman, 2004). Gα may link to the mitotic spindle through the microtubule binding protein Numa (Du and Macara, 2004). The forces that act on mitotic spindles as they move away from the center of a cell have been characterized (Bellaiche and Gotta, 2005; Cowan and Hyman, 2004). Still, a complete mechanism has yet to be described: How Numa and a set of asymmetric molecules interact to result in asymmetric forces remains a fascinating and incompletely understood issue. It will be interesting to learn if any of the critical, asymmetrically localized proteins in *C. elegans*, or the as-yet unidentified motor(s) that move the spindle asymmetrically, are regulated by CDK-dependent phosphorylation. Separase, an APC-regulated protease involved in separating chromosomes, may have multiple targets including some not involved in chromosome separation (Gutierrez and Ronai, 2006), for example in disengaging duplicated centrioles (Tsou and Stearns, 2006). It is conceivable that separase targets might include one or more proteins involved in positioning the mitotic spindle.

Our finding that the spindle checkpoint pathway times spindle positioning forges a new link between a cell biological process and development. Chromosomes are first positioned asymmetrically soon after chromosome congression -- during metaphase or

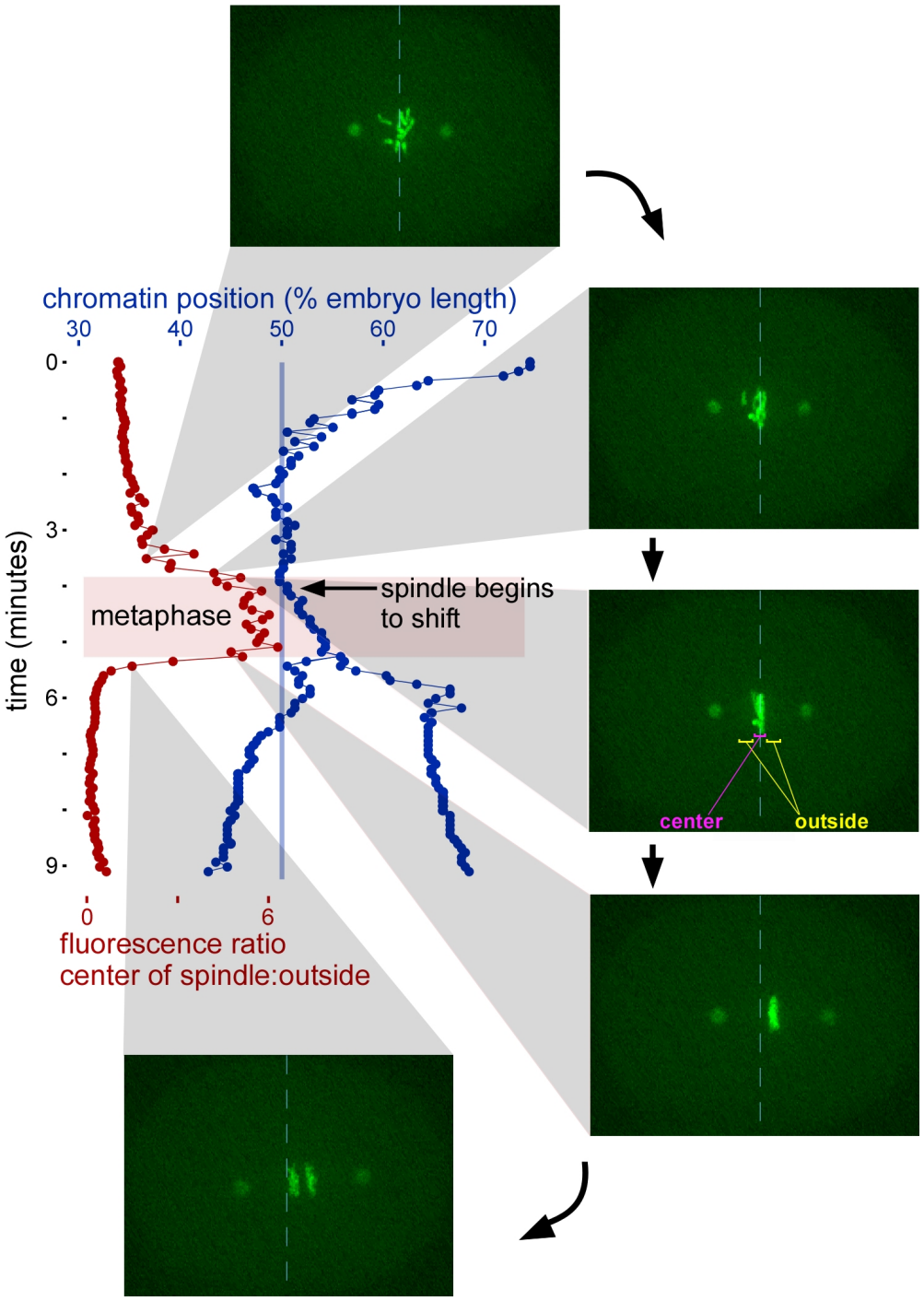
anaphase -- in several other models of asymmetric cell division, including neuroblast divisions in *Drosophila* (Kaltschmidt et al., 2000) and leech (Zhang and Weisblat, 2005), sensory organ precursor divisions in *Drosophila* (Roegiers and Jan, 2004), as well as early embryonic cell divisions in leech (Ren and Weisblat, 2006) and *Tubifex* (Ishii and Shimizu, 1995; Shimizu, 1996). It is possible therefore that mitotic progression is a widespread temporal regulator of asymmetric spindle positioning.

Figure III.1. The mitotic spindle begins to shift soon after the completion of chromosome congression.



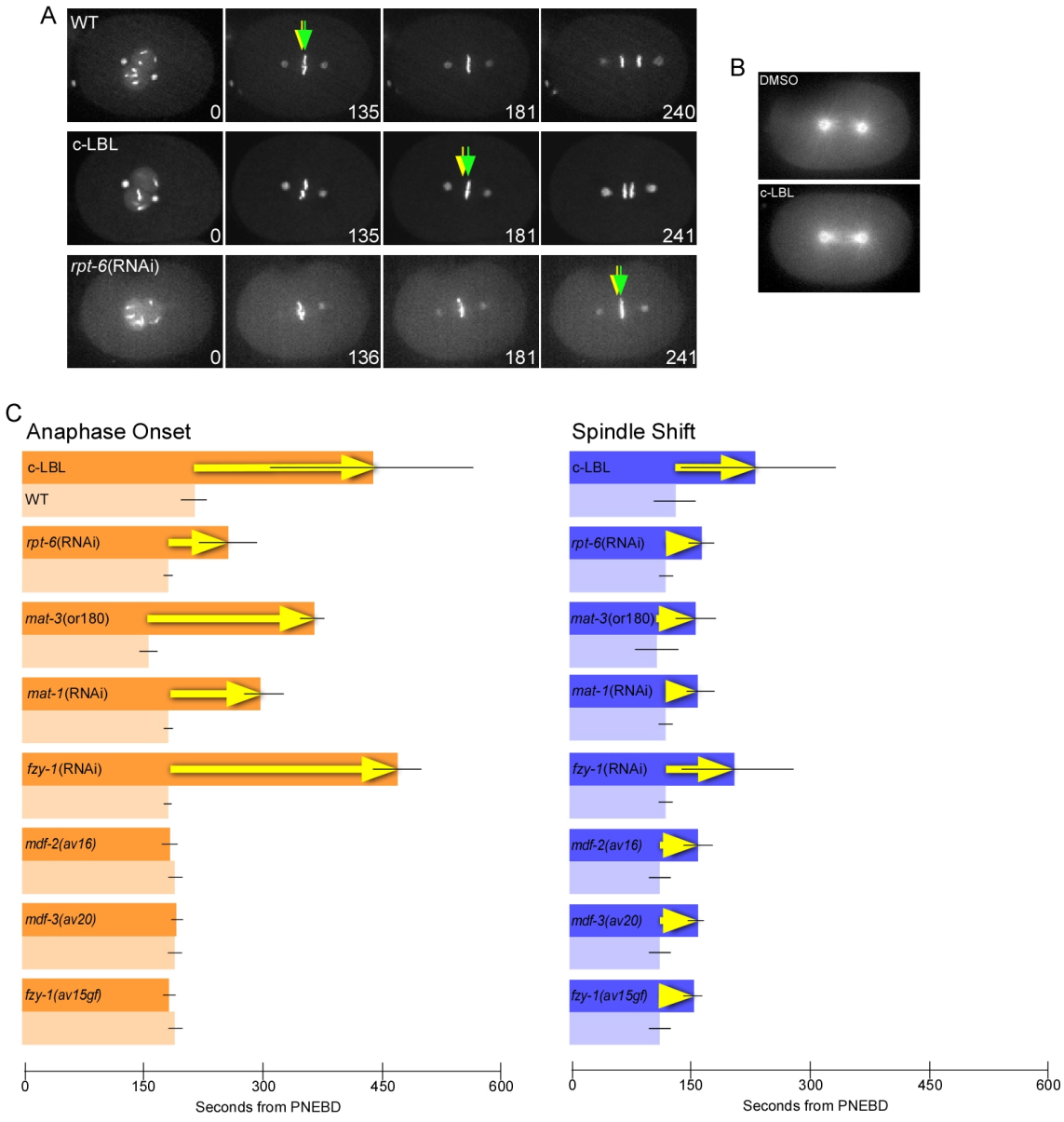
A) Wild-type one-cell stage *C. elegans* embryo expressing histone H2B:GFP and gamma-tubulin:GFP, with seconds before or after pronuclear envelope breakdown (PNEBD) indicated. Chromosomes complete congression to the metaphase plate at 50% embryo length (yellow arrow) before the spindle shifts (green arrow). B) Kymograph analysis of the embryo in A, with the blue line indicating 50% embryo length, and the y-axis representing time after pronuclear meeting near the posterior cortex. C) Quantitative analysis of the embryo in A and B. Chromatin position is indicated in blue, and the degree of compactness of the chromatin, measured as a ratio of fluorescence intensities from the center of the spindle to directly outside this region, is indicated in red. Metaphase is indicated in pink. D) Quantitative analysis of spindle positioning, chromosome congression, and anaphase onset times from 21 z-projected embryos. Error bars represent the 95% confidence intervals for significance.

Figure III.2. Images of specific stages of the embryo from Fig III.1A,B,C, expressing histone H2B:GFP and gamma-tubulin:GFP.



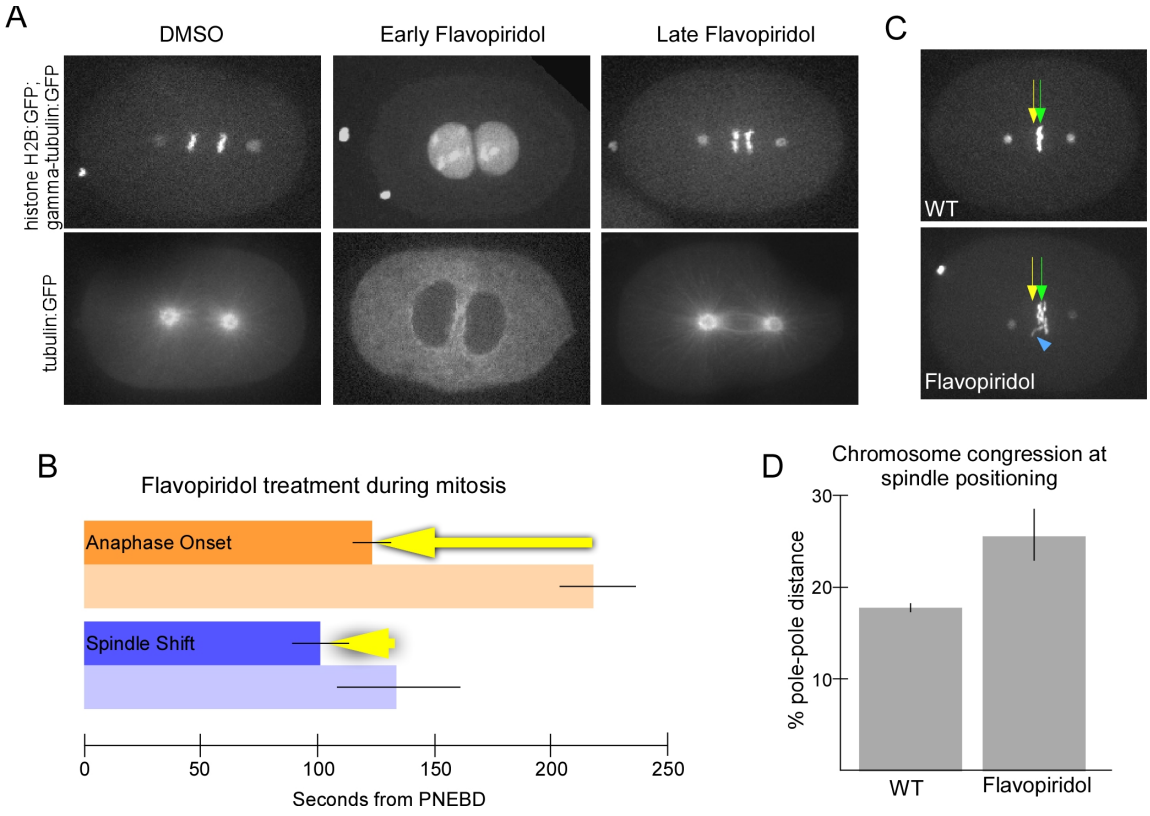
Annotated on the images are 50% embryo length (dotted line) and areas where fluorescence intensity was measured, in the center of the spindle and directly outside (indicated on center right image). As the ratio reaches its maximum at metaphase, both the position on the graph and the image of the embryo show the beginning of movement of the spindle with a compact metaphase plate.

Figure III.3. Proteasome function, the APC, and FZY-1 are required for timely spindle positioning.



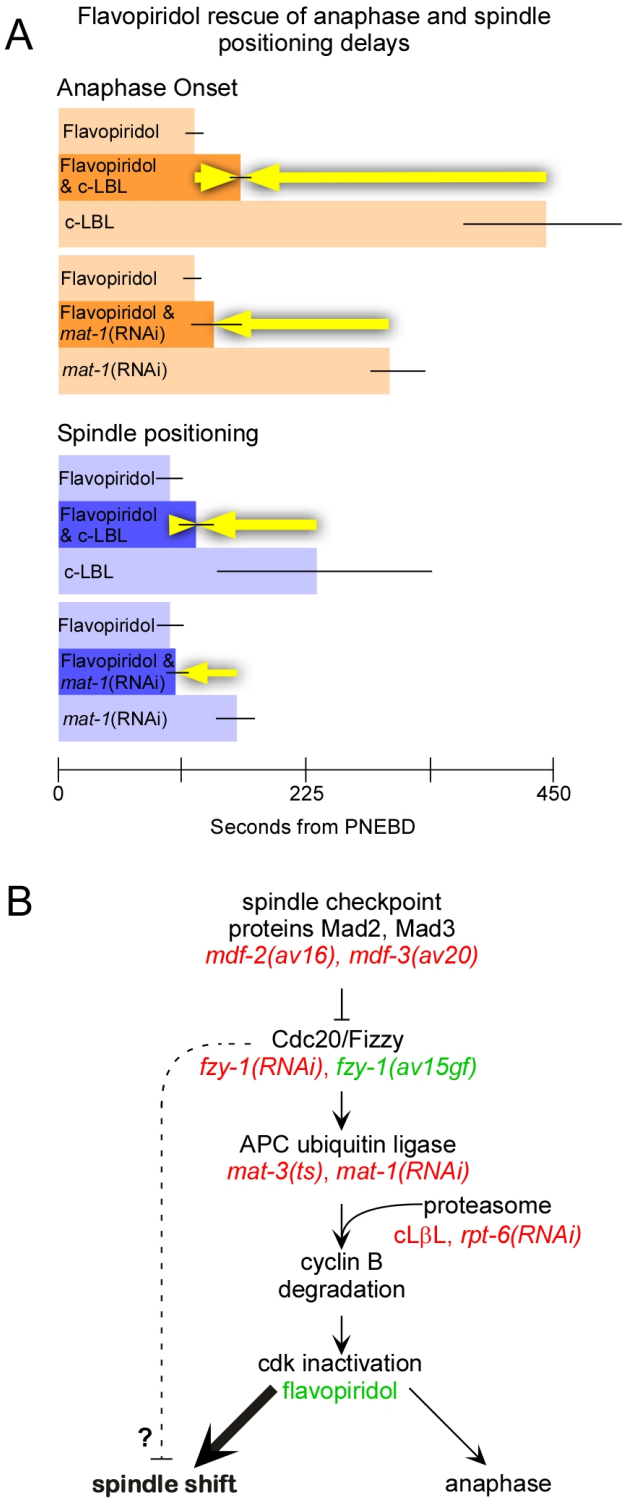
A) Time-lapse images of embryos monitored for spindle positioning and anaphase onset. Time is indicated as seconds after pronuclear envelope breakdown (PNEBD), and the beginning of the spindle shift is indicated with a green arrow (50% embryo length is yellow arrow). B) Images of embryos expressing tubulin:GFP in either control DMSO or c-LβL treatment show that the spindle appears normal after drug treatment. C) Quantitative analysis of the time between PNEBD and either anaphase onset (left) or the spindle shift (right). Wild-type controls are shown in a lighter shade than the experimental treatments, and yellow arrows denote a statistically significant difference in values and direction of change in timing. Wild-type controls include the following: laser-permeabilized embryos in DMSO (for comparison to c-LβL); embryos in which the temperature was raised to 25°C as in temperature-shift experiments (for comparison to *mat-3(or180)*); embryos that were grown at 24°C (for comparison to checkpoint alleles); and embryos that were raised and imaged at 20°C (for comparison to all other backgrounds in Figure 2). Error bars represent the 95% confidence intervals for significance. For statistical values, see Appendix B: Materials and Methods for Chapter III.

Figure III.4. Spindle positioning is timed by Cdk inactivation.



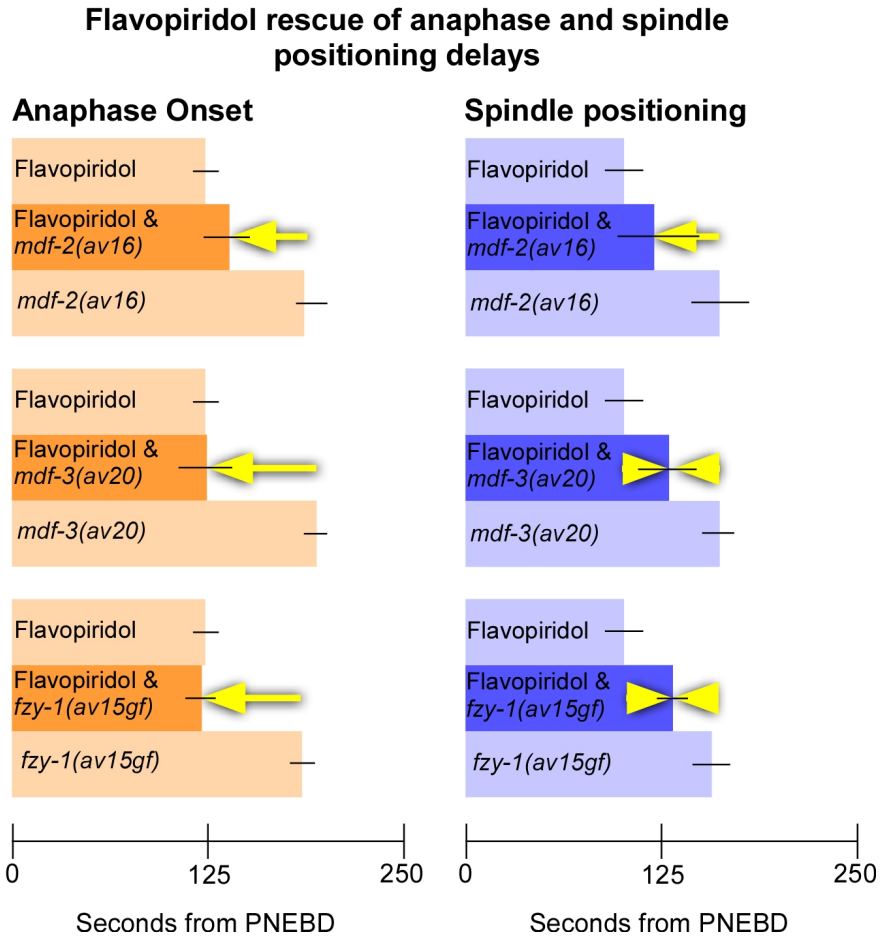
A) Embryos expressing histone H2B:GFP and gamma-tubulin:GFP, or expressing alpha-tubulin:GFP, were laser-permeabilized. Embryos permeabilized in DMSO proceeded through mitosis similar to wild-type, and had normal spindle morphology. When embryos were treated with flavopiridol prior to mitosis, they did not undergo PNEBD (11/12 H2B:GFP- and gamma-tubulin:GFP-labeled embryos), nor did they maintain centrosome-nucleated microtubules (8/9 tubulin:GFP-labeled embryos). Embryos treated with flavopiridol later in mitosis proceeded through mitosis, and had normal spindle morphology (8/8 tubulin:GFP-labeled embryos). B) After flavopiridol treatment during mitosis, anaphase onset and spindle positioning occurred earlier than in wild-type (lighter shaded bars, laser-permeabilized embryos in DMSO). Error bars represent the 95% confidence intervals for significance, and yellow arrows denote statistical significance and direction of change in timing. C) Z-projection images of wild-type and flavopiridol-treated embryos. At this time point, each spindle was positioned at 53% embryo length (green arrows; yellow arrows indicate 50% embryo length). In flavopiridol-treated embryos, chromosomes are often not aligned at this point, compared to wild-type. In some cases, significant regions of chromosomes are not aligned at the metaphase plate (blue arrowhead). D) Measurement of chromosome congression at the time of spindle positioning. After flavopiridol treatment, chromosomes are not aligned on the metaphase plate as tightly as in WT. For statistical values, see Appendix B: Materials and Methods for Chapter III.

Figure III.5. The proteasome and APC function in spindle positioning primarily through Cdk inactivation.



A) Flavopiridol treatment rescued most of the delay of anaphase onset and spindle positioning induced by the proteasome inhibitor c-LβL. Flavopiridol treatment completely rescued the delay induced by *mat-1*(RNAi), for both anaphase onset and spindle positioning. Error bars represent the 95% confidence interval for significance, and yellow arrows denote statistical significance. For statistical values, see Appendix B: Materials and Methods for Chapter III. B) Model for regulation of spindle positioning by spindle checkpoint pathway components. Treatments used in our experiments are shown in red (loss of function) or green (gain of function).

Figure III.6. The spindle checkpoint regulates spindle positioning through a partially CDK-independent mechanism.



Flavopiridol rescued the delay in spindle positioning in checkpoint alleles (*mdf-2(av16)* p=0.014, n=9; *mfd-3(av20)* p=2.9x10^{-3},n=10; *fzy-1(av15gf)* p=4.9x10^{-3}, n=10), and shortened the time to anaphase onset (*mdf-2(av16)* p=2.9x10^{-5}; *mfd-3(av20)* p=1.9x10^{-8}; *fzy-1(av15gf)* p=1.2x10^{-9}). In these mutants flavopiridol did not rescue spindle positioning timing as effectively as it rescued anaphase timing. The time to anaphase onset for each mutant is not statistically distinguishable from flavopiridol treatment alone (*mdf-2(av16)* p=0.056; *mfd-3(av20)* p=0.91; *fzy-1(av15gf)* p=0.73). Spindle positioning was incompletely rescued in two of these flavopiridol-treated mutants, compared to drug treatment alone (*mdf-2(av16)* p=0.14; *mfd-3(av20)* p=0.011; *fzy-1(av15gf)* p=1.1x10^{-3}). As might be expected if flavopiridol rescued the anaphase delay more effectively than the spindle shift delay, in the flavopiridol-treated checkpoint alleles, the spindle shift often occurred at or after anaphase onset (*mdf-2(av16)* 4/9 cases; *mfd-3(av20)* 7/10 cases; *fzy-1(av15gf)* 7/10 cases), which was never observed in wild-type (0/29 cases). Error bars represent the 95% confidence intervals for significance, and yellow arrows denote statistical significance.

CHAPTER IV

ASYMMETRIC DIVISION: A KINESIN FOR SPINDLE POSITIONING

The meiotic spindles of animal eggs move to extremely asymmetric positions, close to the cell cortex. A recent paper has identified a motor complex that may move the meiotic spindle toward the cortex in *Caenorhabditis elegans* eggs.

Some of the most extreme cases of asymmetric cell division are the meiotic divisions of maturing oocytes. Each meiotic division results in the partitioning of chromosomes between the oocyte and a polar body. These two cells must differ in size drastically to provide the maturing oocyte with a substantial amount of cytoplasm to support development. How oocytes position meiotic spindles is largely an open question. Additionally, it is not well understood how an oocyte regulates meiotic events in the same cytoplasm that will later sustain mitotic events, as these events may rely on very different mechanisms.

One of the hurdles in understanding meiotic divisions is the surprising variety of strategies that appear to be used in different systems. Oocytes of the worm *Chaetopterus* have spindles that, when pulled away from the cortex, will return to the original cortical site (Lutz et al., 1988). Such experiments have suggested that there is a site in the cortex that can pull on astral microtubules of the meiotic spindle. Astral microtubules function

in similar movements during meiosis in certain other systems, such as fission yeast (Ding et al., 1998; Svoboda et al., 1995).

In many other systems, including the nematode *Caenorhabditis elegans*, the fruitfly *Drosophila* and mice (Albertson and Thomson, 1993; Gueth-Hallonet et al., 1993; Theurkauf and Hawley, 1992), meiotic spindles lack centrosomes and astral microtubules. Even in these anastral systems, studies indicate that a diversity of mechanisms are used. For example, meiotic spindle positioning in mice depends on an actin-based mechanism, while *C. elegans* meiotic spindles can move normally even when actin filaments are depolymerized (Verlhac et al., 2000; Yang et al., 2003).

C. elegans meiotic spindles provide us with a fascinating model in which to study how a spindle with minimal tools can position itself near the cortex. If there are no astral microtubules that can be used to pull the spindle to the cortex, and actin filaments do not play an active role, what mechanisms remain? A recent paper by Yang et al. (2005) has provided some initial clues. These authors have identified players required to translocate the *C. elegans* meiotic spindle to the cortex. From this, we can begin to build models for how a meiotic spindle can be positioned without the use of astral microtubules or actin filaments.

Yang et al. (2005) speculated that kinesin motors might function to translocate the meiotic spindle to the cortex and began an RNA interference (RNAi) screen of the *C. elegans* kinesin homologs, using live imaging to monitor meiotic spindle translocation inside living worms. During both meiosis I and II in wild-type oocytes, the spindle is generally translocated to the cortex with its long axis parallel to the cortex, followed by spindle rotation and spindle shortening at the cortex (Figure IV.1).

Yang et al. (2005) found that, in oocytes depleted of the kinesin-I homolog UNC-116, meiotic spindles remain stationary when wild-type spindles would normally translocate, and polar bodies often fail to form. RNAi downregulation of two kinesin light-chain homologs, KLC-1 and KLC-2, produced a similar result. Although the spindle did not move at the correct time in these backgrounds, it did move to the cortex later, at the time when wild-type meiotic spindles would normally undergo spindle rotation and shortening, suggesting that a partially redundant mechanism exists for spindle positioning.

As more than 5000 *C. elegans* protein–protein interactions have been identified by two-hybrid screens and by other methods (Li et al., 2004), checking for interaction partners has become a routine step for *C. elegans* researchers who develop an interest in new proteins. Yang et al. (2005) showed that both of the kinesin light chains, KLC-1 and KLC-2, can bind a protein that, by RNAi experiments, is also required for spindle translocation. This protein, which they call KCA-1, for kinesin cargo adaptor, appears to be a novel and nematode-specific kinesin cargo protein. KCA-1 can also bind a heterochromatin protein (Li et al., 2004), suggesting a possible direct link between the kinesin motor complex and the meiotic chromatin.

How might kinesin-I function to move the meiotic spindle? Yang et al. (2005) have proposed a model in which KCA-1 serves as a cargo adaptor to bridge the meiotic chromosomes and UNC-116. They propose that UNC-116 walks along cytoplasmic microtubules toward the cortex, carrying along KCA-1 and the spindle. Although KCA-1 has been shown also to bind a heterochromatin protein, whether the heterochromatin protein is required for spindle translocation has not been reported. One alternative to this

model is that kinesin-I might act more indirectly, for example to set up a microtubule architecture required for spindle movement, or to carry other motors to the spindle or the cortex.

Earlier studies by Yang and colleagues (2003) demonstrated a role for another protein in this process. A putative katanin-like microtubule severing protein, MEI-1, also functions in translocation of the meiotic spindle to the cortex. The microtubule severing activity of MEI-1 keeps microtubules short during meiosis. Later, during mitosis, when the mitotic spindle must be much larger, MEI-1 is degraded (Clandinin and Mains, 1993; Clark-Maguire and Mains, 1994; Kurz et al., 2002). MEI-1 protein is enriched at spindles in *C. elegans* oocytes (Clark-Maguire and Mains, 1994), and oocytes depleted of MEI-1 have defects in spindle translocation, such as delayed movement to the cortex (Yang et al., 2003). From these findings, it has been hypothesized that MEI-1 functions to keep meiotic spindles both small and close to the cortex (Yang et al., 2003).

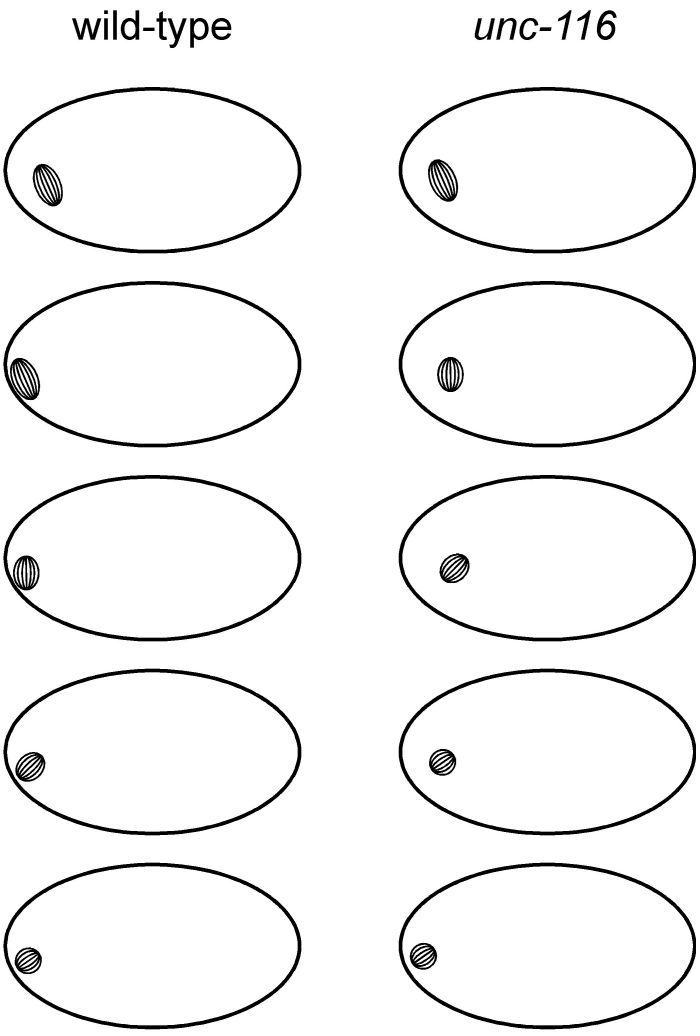
Given the roles of both MEI-1 and the UNC-116 complex, it is interesting to speculate how these proteins may function together in translocating the meiotic spindle to the correct location at the cortex. As kinesin-I is typically a plus-end-directed motor, the model proposed by Yang et al. (2005) of kinesin-I-dependent translocation would require that many microtubules near the meiotic spindle are oriented with their plus ends at the cortex, something that has not yet been examined.

One interesting possibility is that the microtubule severing activity of MEI-1 may produce a directional bias in microtubule orientation that a plus-end motor could exploit for spindle translocation — a bias in which most microtubules near the spindle have their plus ends at the cell cortex. Depending on the balance of plus end- and minus end-

stabilizing proteins near microtubules, it is conceivable that severed microtubules could undergo catastrophe at newly created plus ends and might be stable at newly created minus ends. This would leave intact primarily the microtubules with their plus ends near the cortex (Figure IV.2), a bias that could result in a plus end directed motor moving toward the cell cortex.

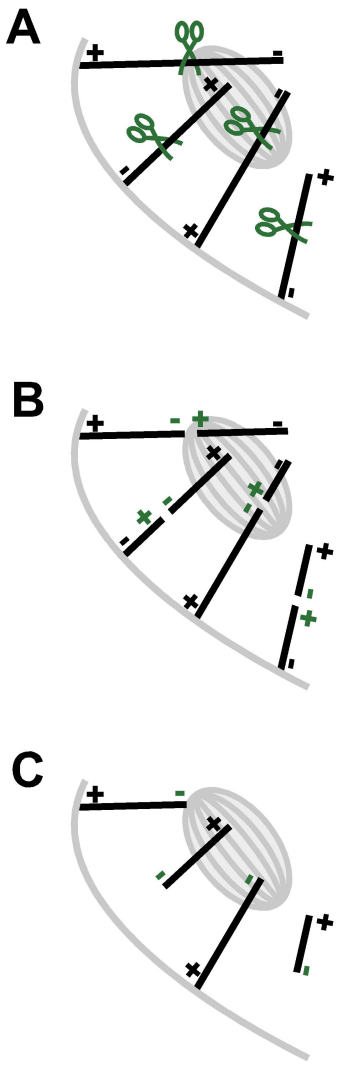
Although little is yet known about the molecular mechanisms of meiotic spindle positioning, it is clear that various systems employ strikingly different mechanisms. By using a genetically tractable organism in which these events also can be well visualized, Yang and colleagues (2005; 2003) have created a new model for how a spindle can be positioned. Whether similar strategies are used in other systems to move mitotic or meiotic spindles will be an interesting question for future work.

Figure IV.1. *C. elegans* meiosis.



In wild-type *C. elegans* oocytes, the meiotic spindle translocates to the cortex prior to spindle rotation and shortening (left). In oocytes lacking UNC-116 (right), the meiotic spindle does not translocate to the cortex until after spindle rotation and shortening begin.

Figure IV.2. Microtubule severing for spindle translocation.



(A) The *C. elegans* katanin homolog MEI-1 (represented by green scissors), may function in severing cytoplasmic microtubules near the meiotic spindle. (B) Severing generates new plus and minus ends (marked in green). (C) It is plausible that the newly created plus and minus ends may behave differently. In the scenario drawn, newly created plus ends undergo catastrophe, and new minus ends are stable. This would leave only plus ends contacting the oocyte's cortex.

CHAPTER V.

DISCUSSION AND PERSPECTIVE

Asymmetric cell division is an important feature of development in many organisms. The asymmetric cell division of the early *C. elegans* embryo results from movement of the mitotic spindle to an asymmetric location. My investigation of the early *C. elegans* embryo has focused on two main topics surrounding asymmetric spindle positioning—the molecular mechanisms required to move the spindle, and the regulation of when the spindle is asymmetrically positioned. In this Chapter, I'd like to discuss my results and contributions to the field, additional tools that I have helped to build in the Goldstein Lab, as well as my perspective on important questions still open in the field.

Summary and Conclusions

In my attempts at photobleaching and imaging different populations of microtubules in the early embryo, I found that spindle microtubules were very cleanly photobleached. As my first significant result in my investigation on microtubule dynamics, I focused my thinking on how different dynamics of microtubules could contribute to anaphase chromosome segregation. I knew that kinetochore microtubules were not shortening during anaphase (Oegema et al., 2001), but I also knew that poleward flux could still function in generating a tension at kinetochores (Waters et al.,

1996). Once I analyzed the movement of the photobleached regions throughout different stages of mitosis, I was able to conclude that kinetochore microtubules were not undergoing poleward flux during anaphase. So, these microtubules were not shortening or undergoing poleward flux. With this lack of dynamics, combined with the previous finding that midzone microtubules were not contributing to, but were limiting, spindle pole separation during anaphase (Grill et al., 2001), I concluded that anaphase pulling forces were provided by the astral microtubules, the same microtubules that pull the spindle to its asymmetric location.

Although my results may not have opened many new avenues of research in the field, it was good to establish a photobleaching protocol in an organism like *C. elegans* embryos. Because of the size and characteristics of the one-cell stage embryo as a "cell," many straight-forward, beautiful experiments that are easily done in flat, tissue-culture cells are often not done or possible in this system. I was at the threshold of what could be seen and photobleached in order to try this simple great experiment, and it worked and contributed a little bit more to our understanding of the one-cell stage embryo. To see these results get published in collaboration with Jean-Claude Labbe (Labbe et al., 2004) was purely icing on the cake.

It was serendipitous that I then started studying the timing of spindle positioning. As mentioned in the Preface, the question of how spindle positioning is timed arose while Bob and I were addressing a reviewer's comment on Jean-Claude's paper. It was (and still is) striking for me to think that nobody had reported and followed up on how precise the timing for spindle positioning was in the early embryo. I manipulated the mitotic machinery at several different timpoints, from before mitotic entry to the final

inactivation of CDK, and monitored how the timing of spindle positioning changed in response. I found a mostly consistent story in which CDK inactivation serves to time spindle positioning, although some results are harder to interpret. The fact that the checkpoint alleles from the Golden lab (Stein et al., 2007) delayed spindle position timing, but not that of anaphase onset, was surprising. By using two conditions to manipulate Cdc20/FZY-1, I was able to show that the pathway for spindle position timing diverges, by distinguishing CDK-dependent and –independent pathways. Within both branches of the pathway, however, I have concluded that mitotic progression, and specifically the mitotic spindle checkpoint, is playing a role in timing spindle positioning. By finding a link between this precise timing and mitotic progression, I feel as if I have set some groundwork for thinking about spindle positioning from this aspect.

In showing a cell biological checkpoint's role in a process that is a hallmark of early embryogenesis in *C. elegans*, it is interesting to speculate on the biological significance of regulating the timing of spindle movement. Although it is only speculation at this point, I think this link ensures that chromosomes are properly aligned and attached to the mitotic spindle before moving to one side of the embryo. Chromosomes could potentially be lost in the cytoplasm if the spindle is pulled to its asymmetric location without proper assembly of the metaphase spindle. I look forward to seeing if and how this speculation is turned into experiments.

Tool building

During my time in the Goldstein Lab, my research followed several paths. Some, such as the ones in this dissertation, provided results that allowed immediate

contributions to understanding asymmetric spindle positioning. Other paths were not as immediately rewarding, but do help provide an indirect contribution to the lab and field. I have helped to build several tools that I hope to see in use at some point.

In trying to understand the molecular mechanisms involved in moving the spindle, my original focus was on the dynamics of astral microtubules, which are known to transduce the pulling force that moves the spindle (Grill et al., 2001; Labbe et al., 2004). Previous work in the lab (Labbe et al., 2003) using CIMS (Cortical Imaging of Microtubule Stability) suggested that I could look at residence time of microtubules at the cortex in many different genetic backgrounds that were previously untested, and have a better understanding of the role of cortical proteins on microtubule dynamics at the cortex. The first, giant step in the project was to collaborate with Dr. Yoni Fridman to develop a program that automates and annotates the counts of microtubule plus-ends at the cortex. After many test trials and improvements to the program, we found that this program is limited by the image quality, which is limited by the transgenic tubulin:GFP strains. I am confident that better strains of labeled tubulin will allow this program to accurately perform its analysis of microtubule residence time. Although I will not personally see this program to its success, I hope that my initial questions and framework will aide future lab members.

Another tool that may be used in understanding asymmetric spindle positioning that I have created is an mCherry:tubulin construct currently being transformed into worms that will hopefully allow better visualization of microtubules. Expression of mCherry:tubulin in embryos will likely result in less background fluorescence, which is an obstacle in trying to acquire high-resolution images in the yolky light-scattering

embryo. Once embryos expressing this construct are imaged, many questions can be approached more easily. As mentioned above, the CIMS program will likely function better on its automated measurements of microtubule residence time with improved imaging. In addition, better imaging will hopefully create better opportunities to photobleach additional populations of microtubules in the early embryo.

Remaining questions in asymmetric spindle positioning

One of my goals was to understand the dynamics of astral microtubules while they were pulling the spindle towards the posterior cortex. I had tried to photobleach astral microtubules during spindle positioning to determine if there is a novel, poleward flux mechanism that could generate the pulling force. If astral microtubules could undergo depolymerization at the minus end, potentially in combination with depolymerization at the plus end, the mechanism pulling the spindle could by analogy look similar to how kinetochore microtubules function in rapid anaphase poleward-movements of chromatids in certain organisms (Brust-Mascher and Scholey, 2002; Desai et al., 1998; Maddox et al., 2002; Maddox et al., 2003; Mitchison, 1989). This could open up a new line of questions on this analogy, and help the field understand how the spindle is moved. This experiment was quite risky; there was no precedent for non-spindle microtubules undergoing flux. In addition, in the case that I did not find poleward flux on astral microtubules, it would not cross anyone's radar as a surprising result. However, although there was plenty of evidence that the spindle moved by microtubule-dependent pulling forces, nobody had yet shown how this pulling force was generated. Similar to the CIMS project above, my attempts at photobleaching astral

microtubules were limited by the quality of existing tubulin:GFP strains. I will always be enthusiastic about this experiment, and I hope that future lab members may be able to answer this question. This project, although exciting in its potential, taught me about the need to balance risky experiments with experiments more sure to result in an interesting result.

An additional question that has only recently appeared is a study by another group that finds drastically different numbers for microtubule residence time at the cortex (Kozlowski et al., 2007), compared to Jean-Claude's numbers (Labbe et al., 2003). This group also finds that through computer simulations of the one-cell stage *C. elegans* embryo, these shortened, uniform residence times are conducive to spindle movement to the posterior of the embryo. Whether the numbers generated by the Goldstein lab or their lab are more representative of proper development remains unanswered, as the fluorescent labels of tubulin and the embryo mounting techniques are different.

Although time will give me great perspective on my graduate work, I currently feel that my biggest contribution to the field is my story on the timing of spindle positioning. The big impenetrable "black box" in the field is the identification of the mechanism that is pulling the spindle. My hope is that my conclusions on the role of mitotic progression on the timing of spindle positioning may lead someone's search down a path that will identify the exact mechanism. Perhaps the role of CDK in timing the event may suggest downstream effectors of CDK (of which there are many) that may more directly affect spindle positioning. This is, of course, a pipe dream.

The asymmetric spindle positioning field has made great progress since I started graduate school, and there are still so many great questions left unanswered. I think that

the current focus on the link between G-protein signaling and microtubules will help build a mechanism that will most likely, but not definitely, involves microtubule motors. I hope that the minutiae many people are studying will eventually add up to be a very significant story and model of asymmetric cell division, and I hope that the computer modelers and simulators will provide biologists with testable hypotheses that can also lead to understanding.

I am enthusiastic about following the research on asymmetric spindle positioning, and to see the historical context of all of the work in which I participated, witnessed, and admired.

APPENDIX A:

MATERIALS AND METHODS FOR CHAPTER II.

Strains

All strains were maintained as described by Brenner (1974) and were grown at 20 degrees C. The strains and alleles used were TH32: *unc119*(ed3) III; ruIs32[*unc-119(+) pie-1::GFP::histoneH2B*]; ddIs6 [*unc-119(+) pie-1::GFP::tbg-1*] (a gift from K. Oegema and T. Hyman, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany), AZ244: *unc-119(ed3)* III; ruIs57[*unc-119(+) pie-1::GFP::tubulin*] (Praitis et al., 2001).

Fluorescence imaging

To image embryos expressing the genes encoding both tubulin and histone H2B fused to GFP, embryos were mounted on agar pads and time-lapse images were acquired using a CSU10 Yokogawa spinning-disk confocal system (Perkin-Elmer) mounted on an inverted microscope (model Eclipse TE300; Nikon). The embryos were illuminated at 488 nm with an air-cooled Ar/Kr laser (Melles Griot). Digital images were acquired by a 16-bit cooled CCD camera (model Orca ER; Hamamatsu) and the acquisition system was controlled by MetaMorph software (Universal Imaging Corp.). Fluorescence images were acquired with 650 ms exposure at 3-s intervals using a 100x Plan Apochromat NA1.4 objective and 2 x 2 binning in the camera. Images were analyzed using MetaMorph software and Microsoft Excel, and processed with Adobe Photoshop.

To image microtubules, embryos expressing the gene encoding β-tubulin fused to GFP were mounted on agar pads and imaged using a 63x NA1.4 Plan-Apochromat DIC objective lens on a laser scanning confocal microscope (model LSM 510; Carl Zeiss MicroImaging, Inc.). Images were acquired using an optical slice of ~2.0µm. A selected region of interest was photobleached using 50–150 iterations of 100% 488 nm laser power. Fluorescence intensity and distances of photobleached regions were measured using Metamorph software and analyzed using Microsoft Excel as described previously (Maddox et al., 1999).

APPENDIX B:

MATERIALS AND METHODS FOR CHAPTER III.

C. elegans strains

Published strains used in this study include the following: TH32: *unc119(ed3)* III; ruIs32[*unc-119(+) pie-1::GFP::histoneH2B*]; ddIs6 [*unc-119(+) pie-1::GFP::tbg-1*], AZ212: *unc119(ed3)* III; ruIs32[*unc-119(+) pie-1::GFP::histoneH2B*]; ddIs6, and OD3: (ltIs24[pAZ132; *pie-1::GFP::tba-2* + *unc-119(+)*], a gift from Paul Maddox), cultured at 20°C. For imaging of the checkpoint and APC alleles, strains of *mdf-2(av16)*, *mdf-3(av20)*, *fzy-1(av15)*, and *mat-3(or180)* (gifts from Andy Golden) were crossed into TH32 or AZ212. Checkpoint alleles were cultured at 24°C, and *mat-3(or180)* was cultured at 15°C and moved to 25°C one minute prior to experiments and recorded at 25°C.

RNA interference

mat-1 and *fzy-1* functions were disrupted by injecting dsRNA as described previously (Fire et al., 1998), and imaging embryos at multiple time points after injection to identify a time when embryos reached first mitosis without meiotic defects, but had a delay in anaphase timing. *rpt-6* function was disrupted by feeding bacteria expressing dsRNA as described previously (Kamath et al., 2001).

Drug treatments

Embryos were treated with the following drugs: 20uM c-LβL (Calbiochem) for all experiments, 200uM flavopiridol (NCI) for experiments prior to the entry into mitosis, and 400uM flavopiridol for experiments during mitosis. As each drug was stored in DMSO, controls were carried out in egg buffer and the appropriate amount of DMSO for each drug. To permeabilize embryos for drug treatment, embryos were mounted in the drug on poly-L-lysine coated and washed coverslips, with clay feet used as spacers, coated in small pieces of charcoal, and sealed with valap (equal parts petroleum jelly, lanolin, and paraffin). Charcoal pieces attached to the eggshell were targeted with a 2-mW pulsed laser (model VSL-337; Laser Science Inc.) containing Coumarin 440 dye in a lasing chamber (Photonic Instruments), to produce small holes in the eggshell. To treat embryos with flavopiridol during mitosis, slides were mounted in egg buffer and sealed on only two sides. Shortly after PNEBD, the drug was added to an unsealed side, while egg buffer was wicked from the other side. For the experiment in which flavopiridol was used to rescue the effects of c-LβL, embryos were permeabilized in c-LβL. During mitosis, a combination of both drugs was washed into the chamber.

Imaging and Analysis

Embryos (other than drug-treated embryos) were mounted as described previously (Labbe et al., 2004). Time-lapse images were acquired using a CSU10 Yokogawa spinning-disk confocal system (McBain) mounted on an inverted microscope (Eclipse TE2000; Nikon). The embryos were illuminated at 488 nm with a 50mW air-cooled Argon laser (Laser Physics). Digital images were acquired by a 16-bit cooled CCD

camera (Orca ER; Hamamatsu) and the acquisition system was controlled by MetaMorph software (Universal Imaging Corp.). For quantifying the duration of events in mitosis, images were acquired with 650 ms exposure at 3 second intervals. Images for multiplane z-series were acquired at 5 second intervals with 400ms exposure time, in 5 steps of 1.25µm each. All images were acquired using 100x Plan Apochromat VC NA1.4 or 60x Plan Apochromat NA1.4 objectives, and 2 x 2 binning in the camera. Images were analyzed using MetaMorph software and Microsoft Excel, and processed in Adobe Photoshop (Adobe Systems).

To quantitatively assess the degree of chromosome congression as the spindle shift began, we measured fluorescence intensity, using MetaMorph, from histone H2B:GFP; gamma-tubulin:GFP embryos along the length of a rectangular box running from the anterior to the posterior end of the embryo through the width of the chromatin in the plane of view, and through a projection of the entire spindle in all of the z-planes recorded. Fluorescence intensities were exported to Microsoft Excel, and further analysis was carried out in Microsoft Excel. Chromatin position was identified as the peak position of a 13 pixel-wide running average of fluorescence intensity values (or 5-pixel wide for one timepoint at anaphase to better resolve anaphase separation of chromatin), and two peaks were found similarly after anaphase. The pixel size used was 0.14µm. The degree of compactness of the chromatin before, during and after metaphase is reported as the fluorescence signal ratio at the center of the spindle:outside (Figure III.1C), obtained by collecting average pixel value along a 13-pixel-wide region at the center of the chromatin position (defined here as the peak value a 31-pixel wide running average) and average pixel value for two 16-pixel wide regions on either side of the

center region, subtracting from each the background level of fluorescence, defined as the minimum pixel intensity value of a 158-pixel wide region in the center of the embryo. The most relevant regions are indicated on the center right panel of Figure III.2. These region widths were selected to ensure that values produced were sensitive to individual chromosomes out of the metaphase plates observed in several recordings. To quantify the progress of chromosome congression as in Figure III.4, the width of the area in which chromosomes reside in the spindle was calculated as a percentage of the spindle pole-pole distance.

To analyze the timing of PNEBD in embryos, we measured the fluorescence intensity of the histone H2B:GFP signal within a 20x20 pixel square positioned in an area of the pronucleus free of a chromosome. PNEBD was defined as the time when the fluorescence intensity dropped to 50% the initial measurement (subtracting a 20x20 pixel square of background within the embryo). Chromosome congression (Figure III.1) was defined as the time when the chromosome mass resided within 15% of the distance between spindle poles. The beginning of the spindle shift was defined as the time when the chromosomes moved to 52% embryo length and did not return past this mark. Anaphase onset was defined as the time when the single chromosome mass first became resolveable as two masses.

Kymographs (Figure III.1) were created using Metamorph software, using an 80-pixel tall line that spanned the embryo's length. Using only frames after PNEBD, the kymograph was created using average intensities at each time frame.

Statistics

We used two-tailed t-test p-values to determine significance in all experiments. For experiments represented in Figure III.3, the p-values and n-values are the following: For treatments in which the proteasome is disrupted, anaphase onset was delayed in both *rpt-6*(RNAi) (p=3.7x10-6, n=16; compared to wild-type embryos grown at 20°C, n=28) and c-LβL treated embryos (p=5.1x10-4, n=9; compared to DMSO controls, n=12). Spindle positioning was also delayed in both *rpt-6*(RNAi) (p=3.2x10-6) and c-LβL treated embryos (p=0.03). Disruption of the APC delayed both anaphase onset timing (*mat-3(or180)* p=1.3x10-15, n=11; compared to wild-type embryos quickly shifted to 25°C, n=11)(*mat-1*(RNAi) p=7.5x10-14, n=20; compared to wild-type embryos grown at 20°C, n=28) and spindle position timing (*mat-3(or180)* p=0.01; *mat-1*(RNAi) p=5.5x10-5). RNAi targeting *fzy-1* delayed both anaphase onset (p=1.6x10-26, n=12; compared to wild-type embryos grown at 20°C, n=28) and spindle positioning (p=6.2x10-4). For experiments in which checkpoint alleles were used, the time of anaphase onset was not altered, but the time spindle positioning was delayed (*mdf-2(av16)* $p=2.4x10^{-4}$, n=10; *mfd-3(av20)* $p=5.5x10^{-6}$,n=14; *fzy-1(av15gf)* $p=9.2x10^{-5}$, n=12), compared to wild-type embryos that were grown at 24°C (n=15).

For experiments represented in Figure III.4, the p-values and n-values are the following: After flavopiridol treatment during mitosis (n=15), anaphase onset (p=3.0x10-11) and spindle positioning (p=0.02) occurred earlier than in wild-type (DMSO, n=12). For chromosome congression measurement at the time of spindle positioning, metaphase plates in flavopiridol-treated embryos (n=7) were not as compact as WT embryos (n=21) (p=7.0x10-3).

For experiments represented in Figure III.5, the p-values and n-values are the following: Flavopiridol treatment rescued most of the delay of anaphase onset ($p=1.3x10-5$, n=14) and spindle positioning ($p=9.6x10-3$) induced by the proteasome inhibitor c-LβL (n=9). The delay was not completely rescued compared to flavopiridol treatment alone ($p=4.2x10-7$ for anaphase; p=0.023 for the spindle shift; n=15). In addition, flavopiridol treatment rescued the delay induced by *mat-1*(RNAi) (n=20), for both anaphase onset ($p=1.5x10-7$, n=7) and spindle positioning ($p=1.3x10-3$). The rescue timing was not statistically different from flavopiridol treatment alone (p=0.078 for anaphase onset; p=0.60 for spindle positioning; n=15).

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