OOCYTE MEIOTIC SPINDLE ASSEMBLY IN *CAENORHABDITIS ELEGANS*

by

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A DISSERTATION

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DISSERTATION ABSTRACT

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Title: Oocyte Meiotic Spindle Assembly in *Caenorhabditis elegans*

As in many organisms, *Caenorhabditis elegans* oocytes assemble bipolar meiotic spindles in the absence of centrosomes. While the assembly of the mitotic spindle in *C. elegans* has been studied in some detail, how the poles assemble in the absence of centrosomes remains poorly understood. In an ongoing screen for temperature-sensitive (TS), embryonic-lethal mutants, we have identified TS mutations in multiple genes required for oocyte meiotic spindle pole assembly. We have so far identified mutations in four genes: *or1178*ts in *mei-1,* which encodes the catalytic domain of the microtubule severing complex katanin; *or447*ts in *klp-18*, which encodes a kinesin 12 family member; *or645*ts in *aspm-1,* which encodes a microtubule scaffolding protein; and *or1092*ts and *or1292*ts in *klp-7*, which encode a kinesin 13/MCAK family member. By using live cell imaging of oocytes from transgenic strains expressing GFP and mCherry fusion to proteins associated with the spindle, we have found and confirmed other findings that *klp-18* promotes spindle bipolarity and that MEI-1 promotes pole assembly both by severing microtubules and by recruiting ASPM-1. More recently, we have found that *klp-7* is required for maintaining bipolarity in the meiotic spindle by preventing the number of poles that can form. In *klp-7(-)* mutants, we observed in addition to extraneous poles an excess accumulation of microtubules during Meiosis I. Futhermore, reducing *klp-7*

function can restore bipolarity in a *klp-18(-)* monopolar spindle mutant background. We also observed that disruption of the kinetochore factor KNL-1 in *klp-7(-)* mutants exacerbates the extra spindle pole phenotype. We suggest that in oocyte meiosis, *klp-7* is required to limit microtubule accumulation and pole assembly and that it may carry out these functions in a kinetochore-dependent manner.

This dissertation includes previously published co-authored material.

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I would also like to express my appreciation to Professor Bruce Bowerman for the valuable mentorship he has given me. Bruce has supported my growth as a scientist by sending me to conferences each year and encouraging me to apply for talks. He taught me how to communicate science by going through my seminar talks with me in great detail; it is during these sessions where I feel I learned to speak like a geneticist! He provided me the opportunity to teach as a full instructor for the Human Genetics course this past Spring, and he was supportive of my decision to participate in the Science Literacy training program. I am also very grateful that he did not simply write my manuscripts for me, but instead extended the effort and time walking me through the process of writing a manuscript until I understood how to write each section. The first manuscript he gave me significant help, but with the second manuscript I was able to write one from to start to finish that Bruce was pretty pleased with—that was a great feeling! I am also thankful for the right amount of support he has given me; he helped

guide my project and provided intellectual insight, but remained hands-off allowing me room to include my own hypotheses and experiments. Getting to pursue my own ideas in addition to Bruce's made my science all the more fun and exciting. All and all I'd say we make a great team!

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This dissertation is dedicated to my parents,
my three siblings, and my grandmother.

Thanks for your love and support.

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CHAPTER I

INTRODUCTION

The research described in this dissertation was performed in the laboratory of Professor Bruce Bowerman, in effort to understand the genetic mechanisms behind early development in the nematode *C. elegans.* The projects described in the following chapters were co-developed and guided by Professor Bowerman with helpful input from fellow lab mates Dr. John Yochem, Dr. Kenji Sugioka, Dr. Chien-Hui Chuang, Josh Lowry, Sara Christensen, Valerie Osterberg, Dr. Sean O'Rourke, and Dr. Danielle Hamill. Chapter I outlines an introduction to meiosis, focusing on oocyte meiotic spindle assembly. Chapter II covers recent work on the mechanisms of meiotic spindle assembly and includes excerpts from material in *Molecular Biology of the Cell* published in 2014. This work contains important contributions from Valerie Osterberg, Sara Christensen, Meredith Price, Chenggang Lu, Kathy Chicas Cruz and Paul Mains. Chapter III includes my latest work on identification of a new gene *klp-7* required for meiotic spindle assembly. This project was co-developed with Bruce Bowerman and is being prepared for submission to *Current Biology*. Finally, Chapter IV briefly outlines future directions I would like to pursue to follow up on the findings presented in Chapter II and III.

Introduction to Oocyte Meiosis

Sexual reproduction requires both males and females to generate gametes through a specialized cell division called meiosis. Meiosis splits an organism's diploid genome into a haploid one creating sperm or egg sex cells, which is of course necessary to provide future offspring with a complete diploid set. During the process of meiosis a

structure called the spindle assembles to organize and segregate chromosomes; this structure is unique in egg meiosis in comparison to other kinds of cell division. My dissertation examines the genetic mechanisms behind spindle assembly in oocyte meiosis.

Meiotic spindle assembly in both male and female meiosis is critical for ensuring that sperm and egg carry a haploid genome so that offspring will be born with precisely two complete sets of chromosomes. Aneuploid zygotes lead to a myriad of complications in a developing fetus or child, and many miscarriages result from aneuploid embryos (Nagaoka et al., 2012). Children born with Trisomy 13 usually do not survive their first year, and those with Trisomy 21 have Down Syndrome. Some intersex conditions result from an abnormal number of sex chromosomes; for instance Turner's Syndrome is caused by a loss of an X chromosome (XO) and Kleinfelter's syndrome results from an extra X chromosome (XXY). All of these conditions result from a failure to align and segregate chromosomes during egg or sperm meiosis. Understanding how the meiotic spindle functions is critical to our understanding of disorders that arise from aneuploid genomes.

Two sequential meiotic cell divisions produce a haploid oocyte from a diploid precursor. Oocyte meiosis in many organisms, including humans, is unique in the way it segregates its chromosomes (Dumont and Desai, 2012). Like sperm meiosis, it occurs twice to produce a haploid oocyte from a diploid one. But unlike sperm meiosis, only one gamete (instead of four) result from one diploid sex cell. During female meiosis I and II, cells undergo a reduction division by which chromosomes are extruded into polar bodies that do not divide further into new cells (**Figure 1.1)**. This process requires a small

bipolar spindles which assembles adjacent to the cell cortex and ultimately extrudes extraneous chromosomes into polar bodies during highly asymmetric cell divisions (Fabritius et al., 2011).

C. elegans provides an excellent model organism with its powerful genetics and transparent anatomy to study the dynamics of acentrosomal spindle assembly (Yamamoto et al., 2006; Muller-Reichert et al., 2010). Upon ovulation, the nuclear envelope breaks down and is fertilized after being pushed into the spermathecum (**Figure 1.1**). Fertilization cues the oocyte to move out of prophase and begin assembling a spindle. At prometaphase, chromatin stimulates microtubules to nucleate and polymerize, organizing into a small compact bipolar spindle that is parallel to the cell cortex. The spindle shortens to form a tight barrel shape and rotates so that the spindle is perpendicular to the cortex. At anaphase the homologous chromosomes segregate and move toward each pole and one set of sister chromatids extrudes into a polar body. The process repeats itself during Meiosis II to separate sister chromatids with the remaining set of chromosomes becoming the haploid oocyte contribution to the zygote (Albertson and Thomson, 1993; McNally et al., 2006).

Generation of Microtubules for Spindle Assembly

In many animals, including nematodes, insects, and vertebrates, oocyte meiotic spindles assemble without the centrosomes that dominate bipolar spindle assembly during mitosis. Centrosomes serve as both the microtubule generators and microtubule organizing centers (MTOC) during mitosis and sperm meiosis. Centrosomes generate the microtubules that "search and capture" chromosomes to orchestrate alignment and

segregation (Kirschner and Mitchison, 1986). Contrary to the centrosome-dependent pathway observed in mitosis and sperm meiosis, *C. elegans* oocytes generate microtubules in an acentrosomal pathway, and instead depend upon motor proteins and other microtubule-associated factors to organize microtubules into two poles (Dumont and Desai, 2012; Meunier and Vernos, 2012).

In systems where centrosomes are missing chromatin appears to stimulate microtubule nucleation, though it is not as efficient as centrosomes are. In an influential experiment to identify mechanisms of acentrosomal assembly, microtubules were observed to organize around chromatin coated beads in *Xenopus* egg extract (Heald et al., 1996). In many organisms this requires the small GTPase Ran, which is higher in concentration near the spindle and helps to stabilize microtubules near chromatin. The *C. elegans* GTPase Ran homolog *ran-1* is not required for meiotic spindle assembly in *C. elegans*, which is surprising given that microtubules immediately assemble around chromatin upon nuclear envelope breakdown. In contrast to nematodes and frog eggs, mouse and *Drosophila* oocytes appear to use other mechanisms to generate microtubules in the absence of centrosomes. Upon NEB in mouse oocytes, many non-centrosomal MTOCs assemble around the chromatin generating aster microtubules that are then organized into bipolar arrays (Dumont and Desai, 2012). This mechanism is similar in *Drosophila* oocytes except that MTOCs are assembled away from the chromatin, and subsequently incorporated into the spindle. Our understanding of acentrosomal spindle assembly mechanisms remains limited, which is why we are using *C. elegans* oocytes as a model system to investigate the genetic requirements for this process.

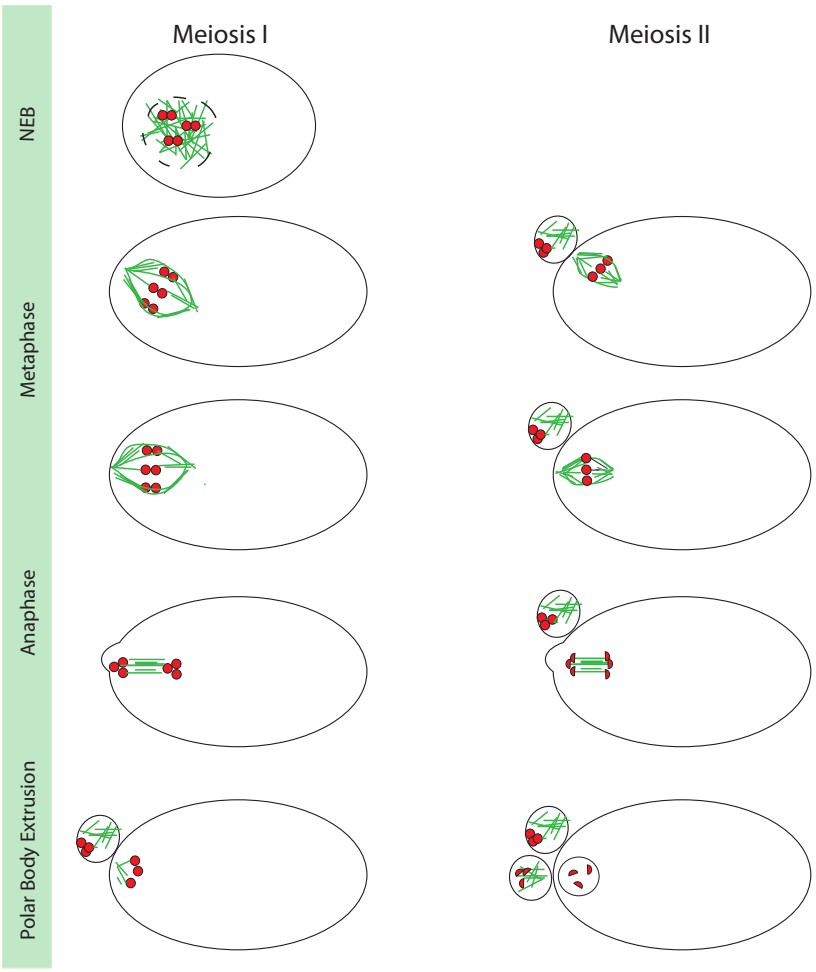

Figure 1.1. Schematic of oocyte meiosis. After nuclear envelope breakdown (NEB), microtubules (green) nucleate around chromosomes (red) and initiate Meiosis I by assembling a meiotic spindle. Chromosomes align at the metaphase plate, the spindle rotates, separates chromosomes during anaphase, and finally extrudes half the chromosomes into a polar body. The process repeats its during Meiosis II. This diagram shows three, instead of six, homologous chromosomes in Meiosis I, and three sister instead of six chromatids in Meiosis II for simplicity.

The Molecular Samurai Katanin: Keeping the Meiotic Spindle in Line

There are two central questions that remain incompletely answered regarding the *C. elegans* oocyte meiotic spindle. The first is how does the meiotic spindle procure enough microtubules without either centrosomes or a Ran GTPase-dependent mechanism, and at the same time remain so small? The second is how does the meiotic spindle organize into a bipolar array without centrosomes to serve as poles? Both of these

questions can be explained in part by discussing Katanin, named after the Japanese samurai sword *katana,* most famously known for its ability to sever microtubules. Katanin appears to be critical both in providing ample microtubule number and in organizing two spindle poles. The *C. elegans* gene *mei-1* encodes the p60 catalytic subunit of katanin (McNally and Vale, 1993; Hartman et al., 1998). The C-terminal region of MEI-1 contains the AAA ATPase microtubule severing activity, while the N-terminal region binds to the p80 subunit MEI-2 (Mains et al., 1990; Clandinin and Mains, 1993; Clark-Maguire and Mains, 1994; Srayko et al., 2000; Yang et al., 2003; McNally et al., 2006; Srayko et al., 2006; McNally and McNally, 2011). Loss of *mei-1* results in meiotic spindles that are dramatically depleted of microtubules, which implicates a role for *mei-1* in generating more nucleation sites by severing microtubules (Srayko et al., 2006). While katanin is likely required to generate microtubules at the spindle, it also appears to be required to limit the spindle's length. Separation-of-function point mutations in *mei-1 (mei-1(c46ct103))* which reduce only microtubule severing cause the spindle to be abnormally long (McNally and McNally, 2011). Furthermore, mutations in both alpha and beta tubulin, which prevent severing of microtubules by MEI-1, was shown to prevent the reduction in oocyte meiotic spindle length that normally occurs during metaphase in wild-type spindles. MEI-1 is necessary for stimulating controlled microtubule production, generating enough microtubules to assemble a spindle, but placing a check on how long the spindles can be.

It is clear that *mei-1* also serves as a central requirement for pole assembly, as loss of *mei-1* produces a disorganized apolar spindle, which suggests that *mei-1* is needed to assemble poles. But how does *mei-1* accomplish such a feat? Microtubule-severing

appears to be only part of the answer. The MT-severing defective allele *mei-1(ct46ct103)* is still capable of assembling bipolar spindles, albeit with poles that are loosely focused early in Meiosis I. Since pole assembly is compromised, but not eliminated, there must be another *mei-1*-mediated mechanism for pole assembly. Another possibility is that ASPM-1, a microtubule scaffolding protein recruited to the spindle poles by MEI-1, is required for pole assembly. Loss of *aspm-1* resembles the *mei-1(ct46ct103)* phenotype in that spindles are long, bipolar, and poles are loosely focused. Chapter II in this dissertation addresses the question of how *mei-1* mediates pole assembly, whether by severing microtubules, by recruiting ASPM-1 to the spindle poles, or both.

Kinesins in Meiotic Spindle Assembly

My work also focuses on two kinesins required for establishment of the meiotic spindle in *C. elegans: klp-18* and *klp-7.* There are other kinesins required for spindle positioning, rotation and chromosome segregation, but these two are required to create the bipolar array. Chapter II focuses on the role of *klp-18* in establishing two spindle poles, and in Chapter III we identify for the first time the requirement of *klp-7* in limiting the number of poles during spindle assembly.

In order to establish two poles with microtubules oriented in anti-parallel directions, oocyte meiotic spindles rely upon either kinesin-12 or kinesin-5 family members to drive poleward movement of microtubules. The mechanisms proposed for meiosis are based on what we have observed in mitosis. Vertebrate kinesin 12 family members form homodimers and may promote mitotic spindle bipolarity by cross-linking anti-parallel microtubules through an interaction with the microtubule-binding protein

TPX-2 (Tanenbaum et al., 2009; Vanneste et al., 2009; Sturgill and Ohi, 2013). In this system, the kinesin 12 family members hKlp2 and Kif15 are partially redundant with kinesin 5 for bipolar mitotic spindle assembly (Tanenbaum et al., 2009; Vanneste et al., 2009). Recent work has shown in the *C. elegans* oocyte that only the kinesin 12 family member KLP-18 drives bipolar movement of microtubules, and that the kinesin-5 family member BMK-1 does not (Bishop et al., 2005; Connolly et al., 2014). RNAi-knockdown of *C. elegans klp-18* results in chromosome mis-alignment and segregation defects during oocyte meiotic cell division, and in assembly of a monopolar meiotic spindle akin to what is observed in oocytes or embryos depleted of kinesin-5 in other systems (Segbert et al., 2003; Wignall and Villeneuve, 2009; Howe and FitzHarris, 2013; Connolly et al., 2014). Chapter II of this dissertation elaborates more specifically on the mechanism *klp-18* plays in assembling a bipolar spindle and how it works with *mei-1* and *aspm-1* to mediate pole assembly.

Recently we discovered a new kinesin to be involved in oocyte meiotic spindle assembly: *klp-7,* which I will discuss in Chapter III. KLP-7 belongs to the MCAK/Kinesin 13 family which is known for its ability to mediate kinetochore-microtubule attachments. During mitosis, the MCAK/kinesin 13 family localizes to kinetochores and poles and functions by depolymerizing microtubules at either end (Ems-McClung and Walczak, 2010). These kinesins do not walk along the microtubules, like many kinesins, but remain stationary, and are thought to destabilize incorrect kMT attachments. Most of the work on these kinesins is on how they prevent aneuploidy in mitosis by maintaining amphitelic attachments, but not on their role in establishing spindle bipolarity. We have observed in *C. elegans* oocytes that *klp-7* is important for

establishing and maintaining spindle bipolarity. Without *klp-7,* meiotic spindles assemble three or sometimes four spindle poles. MCAKs appear to be required for meiotic spindle assembly in other organisms, but to our knowledge our observation of a multi-polar spindle phenotype is completely novel and has not been observed in any other organism. Depletion of MCAK in *Drosophila* or *Xenopus* oocytes results in an extra long meiotic spindle (Mitchison et al., 2005; Radford et al., 2012). And interestingly, depletion of MCAK in mouse oocytes does not affect its ability to correct merotelic attachments, but it does prevent proper chromosome alignment along the metaphase plate (Illingworth et al., 2010; Howe and FitzHarris, 2013).

While defects in oocyte spindle bipolarity with MCAK depletion has not been observed in other organisms, it has been observed during mitosis in human cell lines which suggests a conserved mechanism for MCAK involvement in spindle bipolarity. Studies have suggested kinetochores along with centrosomes contribute forces necessary for establishing and maintaining spindle bipolarity. MCAKs may play an important role in balancing these forces, since they destabilize incorrect kinetochore-microtubule attachments. Proper kinetochore-microtubule attachments is critical for maintaining the opposing tension between sister chromatids, and incorrect attachments may place strain on the spindle structure. One group examined the role MCAK plays in spindle bipolarity (Toso et al., 2009). They used cells with centrosome separation defects where monopolar spindles were present during mitosis, and they found that depleting MCAK restored bipolarity in this system. They concluded that deleting MCAK re-stabilized kinetochore-MT attachments, which increased the opposing tension between sister chromatids, and thereby crudely rescued bipolarity to the otherwise monopolar mitotic spindle. Our work

in Chapter III focuses on the impact *klp-7* has on spindle bipolarity, including its impact in monopolar *klp-18(-)* oocytes. We also examine the relationship between *klp-7* and kinetochore protein *knl-1* to identify whether *klp-7* is regulating pole assembly using a kinetochore-dependent mechanism.

Bridge to Chapter II

In 2010, I began my investigation in oocyte meiotic spindle assembly with a screen for temperature-sensitive embryonic lethal alleles with defects in oocyte meiosis. In Chapter II, I share my findings on three temperature-sensitive alleles that were recently discovered either by myself or others in the lab. These alleles were *mei-1, klp-18* and *aspm-1*. Using spinning disc microscopy to capture meiosis of strains expressing fluorescent meiotic spindle assembly markers, Chapter II investigates the single and double mutant phenotypes for these three genes and how they work together to assemble spindle poles. This work was recently published in *Molecular Biology of the Cell.* I also found another novel allele *klp-7* which is the subject matter of Chapter III, whose role has not been previously identified in meiotic spindle assembly. Using similar techniques outlined in Chapter II, I investigated its role in spindle assembly and its genetic requirements with other spindle assembly factors. The work discussed in Chapter III will be submitted to *The Journal of Cell Biology*.

CHAPTER II

MEI-1-MEDIATED MECHANISM OF POLE ASSEMBLY

This chapter contains important contributions from Valerie Osterberg who constructed a transgenic strain. Sara Christensen, Meredith Price, Chenggang Lu helped identify temperature-sensitive mutant alleles. Kathy Chicas Cruz performed *RNAi* injections in the Lockery lab. Paul Mains provided intellectual and editorial contribution along with useful *C. elegans* strains. Bruce Bowerman helped guide the project and provided me with excellent and instructive mentorship during the process of writing my first manuscript. This chapter includes work published in *Molecular Biology of the Cell.*

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Meiotic Spindle Assembly Genes: *mei-1. aspm-1*, and *klp-18*

While positioning of the *C. elegans* oocyte meiotic spindle has been investigated extensively (Yang et al., 2003; Yang et al., 2005; Ellefson and McNally, 2009; van der Voet et al., 2009; Ellefson and McNally, 2011), less is known about its assembly. Three *C. elegans* genes known to contribute to oocyte meiotic spindle assembly are *aspm-1*, which encodes a protein with a single calponin homology domain and two IQ repeats (van der Voet et al., 2009); *mei-1*, which encodes the catalytic subunit of the microtubule severing complex katanin (Clark-Maguire and Mains, 1994); and *klp-18*, which encodes a kinesin 12 (Segbert et al., 2003).

Both *C. elegans aspm-1* and the *Drosophila* ortholog Asp are essential and required for the proper execution of oocyte meiotic cell divisions (Riparbelli et al., 2002; van der Voet et al., 2009). In *C. elegans* oocytes, ASPM-1 is required for the meiotic spindles to align orthogonally and be in close proximity to the overlying plasma membrane (van der Voet et al., 2009; Ellefson and McNally, 2011). In the mouse, ASPM-1 is expressed specifically in the primary sites of prenatal cortical neurogenesis and is localized to mitotic spindle poles (Bond et al., 2002; Fish et al., 2006). RNA interference (RNAi)-knockdown of mouse ASPM-1 results in abnormal orientation of neural stem cell divisions and an associated loss of cortical neurons (Fish et al., 2006). Moreover, mutations in ASPM-1 are the most common cause of microcephaly in humans (Bond et al., 2002). However, the molecular mechanisms underlying these ASPM-1 requirements remain largely unknown.

RNAi-knockdown of *C. elegans klp-18/*kinesin 12 results in chromosome mis-alignment and segregation defects during oocyte meiotic cell division (Segbert et al., 2003), and in assembly of a monopolar meiotic spindle (Wignall and Villeneuve, 2009). In vertebrates, the kinesin 12 family members hKlp2 and Kif15 are partially redundant with kinesin 5 for bipolar mitotic spindle assembly (Tanenbaum et al., 2009; Vanneste et al., 2009), although *C. elegans klp-18* does not appear to be required for mitotic spindle assembly (Segbert et al., 2003). Vertebrate kinesin 12 family members form homodimers and may promote mitotic spindle bipolarity by cross-linking anti-parallel microtubules through an interaction with the microtubule-binding protein TPX-2 (Tanenbaum et al., 2009; Vanneste et al., 2009; Sturgill and Ohi, 2013).

The *C. elegans* gene *mei-1* encodes the p60 catalytic subunit of the widely

conserved microtubule severing complex called katanin (McNally and Vale, 1993; Hartman et al., 1998), previously shown in *C. elegans* to be required for proper assembly and orientation of oocyte meiotic spindles (Mains et al., 1990; Clandinin and Mains, 1993; Clark-Maguire and Mains, 1994; Srayko et al., 2000; Yang et al., 2003; McNally et al., 2006; Srayko et al., 2006; McNally and McNally, 2011). The C-terminal region of MEI-1 contains the AAA ATPase microtubule severing activity, while the N-terminal region binds to the p80 subunit MEI-2 (Mains et al., 1990; Clandinin and Mains, 1993; Clark-Maguire and Mains, 1994; Srayko et al., 2000; Yang et al., 2003; McNally et al., 2006; Srayko et al., 2006; McNally and McNally, 2011). A partial reduction of *mei-2* and hence microtubule severing was shown to prevent the reduction in oocyte meiotic spindle length that normally occurs during wild-type development (McNally et al., 2006). In addition, MEI-1 is required for the recruitment of ASPM-1 to meiotic spindle poles, and a homozygous viable *mei-1* allele with compromised microtubule severing activity—*ct103*—can still recruit ASPM-1 to meiotic spindle poles (McNally and McNally, 2011; Gomes et al., 2013). This microtubule severing-defective *ct103* allele mediates the assembly of bipolar spindles that, although longer than normal, can still shorten but are often mis-positioned. Another microtubule-severing defective mutant with a similar phenotype was initially described as an allele of *mei-1* but was later reported in a correction to be an allele of *mei-2* (McNally and McNally, 2011; Gomes et al., 2013). Other alleles that more completely reduce *mei-1* function have more severe spindle defects (Mains et al., 1990; Clandinin and Mains, 1993; Clark-Maguire and Mains, 1994; Srayko et al., 2000; Yang et al., 2003; McNally et al., 2006; Srayko et al., 2006; McNally and McNally, 2011). Finally, the N-terminus of MEI-1 also can bind microtubules, and

this may contribute to polar organization (McNally and McNally, 2011). In sum, these results have led to the conclusion that MEI-1 has two distinct functions, microtubule severing and spindle pole organization (McNally and McNally, 2011), although the role of microtubule severing remains unclear.

After isolating conditional (heat-sensitive) alleles of *C. elegans aspm-1, mei-1,* and *klp-18*, we used live cell imaging, with transgenic fluorescent protein fusions, to investigate the requirements for these loci in oocyte meiotic spindle assembly. Our results indicate that KLP-18 promotes spindle bipolarity, while both the MEI-1-dependent recruitment of ASPM-1 to the spindle, and the microtubule-severing activity of MEI-1, contribute to spindle pole assembly.

Results

Temperature-sensitive mutations with abnormal numbers of oocyte pronuclei map to the conserved genes *bmk-1, aspm-1, klp-18,* and *mei-1*

After the completion of oocyte Meiosis I and II in wild-type *C. elegans* zygotes, two spherical and haploid pronuclei appear, one from the egg and one from the sperm (**Figure 2.1A**) (Albertson, 1984; Albertson and Thomson, 1993). To identify essential genes that mediate meiotic spindle assembly, we used Nomarski optics to examine live one-cell stage embryos made by a collection of TS embryonic-lethal mutants at the restrictive temperature (**Materials and methods, Appendix A**). We looked for mutants with either no or more than one oocyte pronucleus, as indicators of defects in chromosome segregation during Meiosis I or II, and hence possibly defects in oocyte

meiotic spindle assembly. Here we report our isolation of four such recessive ts mutants: *or447*ts*, or627*ts*, or645*ts, and *or1178*ts (**Figures 2.1** and **A.1** and **Table 1**).

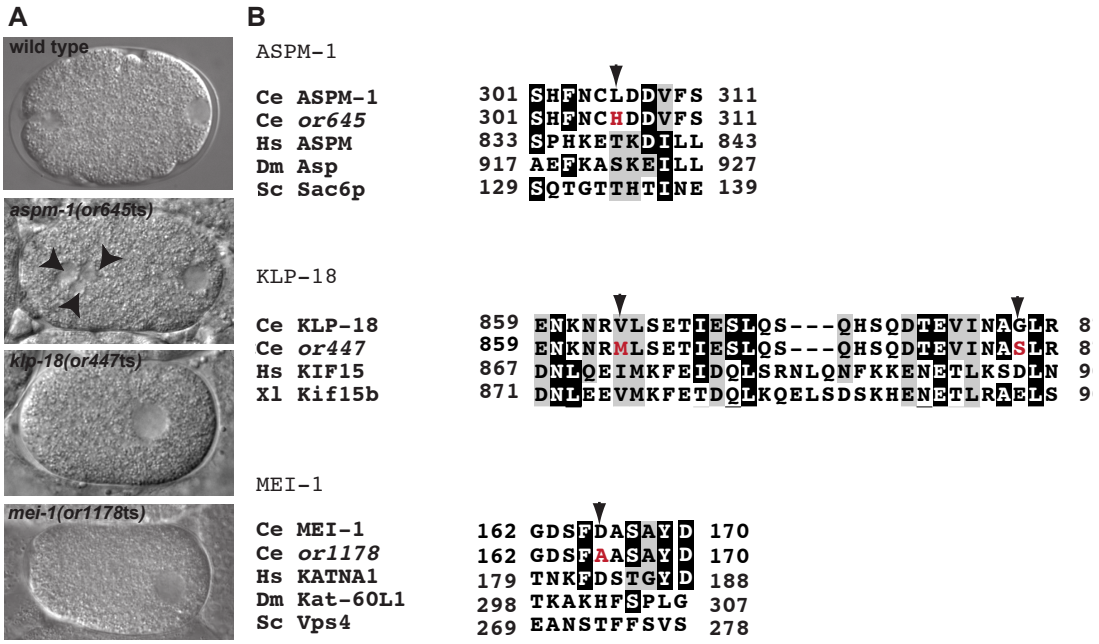


Figure 2.1. The TS mutant alleles of *aspm-1, klp-18, and mei-1* have abnormal numbers of maternal pronuclei and encode mis-sense mutations. (A) Nomarski images of one-cell stage wild-type and mutant embryos. Embryos are positioned with the anterior (maternal) and posterior (paternal) pronuclei to the left and right, respectively; genotypes are indicated. Note the presence of extra maternal pronuclei in *aspm-1(or645*ts*)* mutant embryos (arrowheads), and the absence of maternal pronuclei in *klp-18(or447*ts*)* and *mei-1(or1178*ts*)* mutants. (B) Partial sequence alignments of orthologs from *C. elegans, Homo sapiens (Hs), Xenopus laevis (Xl), Drosophila melanogaster (Dm),* and *Saccharomyces cerevisiae (Sc)* with the wild-type and mutant *Caenorhabditis elegans (Ce)* proteins. Arrowheads indicate altered residues, with wild-type amino acids in black and mutant amino acids in red. Note the two mutations in *klp-18.* The alignment was performed using Boxshade. If the residue is identical to the column consensus there is a black background, if the residue is similar to the column consensus there is a grey background.

After mapping the mutations and performing complementation tests, we sequenced amplified genomic DNA fragments from candidate genes for each mutation to identify the causal mutations (**Figure 2.1B**, **A.1A** and **Materials and methods**,

Appendix A). First, we found that *or627*ts is an allele of *bmk-1*, the *C. elegans* ortholog of kinesin 5/Eg5 (**Figure A.1A** and **Materials and methods, Appendix A**). In vertebrates, this kinesin is required for bipolar mitotic spindle assembly, but *C. elegans bmk-1/*kinesin 5 is not essential, is not required for meiotic spindle assembly, and has only a minor role in mitotic spindle assembly dynamics (Bishop et al., 2005; Saunders et al., 2007). Consistent with these previous studies, we found that *or627*ts is a recessive gain of function *bmk-1* mutation (**Materials and methods**, **Appendix A**). While this mutation results in fully penetrant meiotic spindle defects (**Table 1** and **Figures A.1B** and **A.1C, Appendix A**), we chose to focus further analysis on the three recessive, loss-of-function mutations we isolated in *aspm-1*, *mei-1,* and *klp-18*

Allele	**Homozygote Embryonic Viability (15°C)**	**Homozygote Embryonic Viability (26°C)**	**Heterozygote Embryonic Viability (26°C)**
wild type	99% n=358	97% n=197	----------
aspm-1(or645)	99% n=216	38% n=271	99% n=555
klp-18(or447)	91% n=308	4% n=504	99% n=260
mei-1(or1178)	98% n=127	0% n=262	99% n=256
bmk-1(or627)	79% n=175	1.4% n=561	99% n=403
mei-1(ct46ct103)		65% n=194	
***bmk-1* Deletion Alleles**			
bmk-1(ok391)		99% n=176	
bmk-1(tm969)		99% n=154	
***bmk-1* Complementation Test**			
bmk-1(or627)/bmk-1(ok391)		40% n=341	
bmk-1(or627)/bmk-1(tm969)		27% n=617	
***bmk-1* Recessive-Gain of Function Test**			
bmk-1(or627) + bmk-1(RNAi)		77% n=731	

Table 2.1. Embryonic viability of oocyte meiotic spindle defective mutants. Embryonic viability (percent hatching) was scored for wild type and each TS mutant at permissive (15°C) and restrictive temperature (26°C) temperatures, and *bmk-1* deletion alleles and *mei-1(ct46ct103)* mutants at 26°C (see Materials and methods). Embryonic

viability at the restrictive temperature from heterozygous TS mutants was examined to determine whether the mutations are recessive or dominant.

In genomic DNA from *or645*ts mutants, which failed to complement the deletion allele *aspm-1(ok1208)* for maternal-effect embryonic lethality, we found a single leucine to histidine change at codon 306 in the *aspm-1* open reading frame, 5' of sequences that encode the conserved Calponin homology domain (**Figure 2.1B**). Similarly, *or447*ts failed to complement the deletion allele *klp-18(ok2519)* and contained two mis-sense mutations that both affect the C-terminal coiled-coiled region of KLP-18: a valine to methionine change at codon 854, and a glycine to serine change at codon 876 (**Figure 2.1B**). Finally, *or1178*ts failed to complement the conditional allele *mei-1(or646*ts*)* and resulted in an aspartate to alanine change at codon 166 that affects sequences N-terminal to the conserved AAA ATPase domain (**Figure 2.1B**). The non-conditional deletion alleles *aspm-1(ok1208)* and *klp-18(ok2519)* both result in adult sterility, and studies of these gene requirements during early embryogenesis in *C. elegans* thus far have used RNAi to reduce gene function. To our knowledge, *aspm-1(or645*ts*)* and *klp-18(or447*ts*)* are the first conditional alleles identified for these loci, and *mei-1(or1178*ts*)* is the strongest TS allele yet isolated for this locus (O'Rourke et al., 2011); thus all three should prove useful for further studies of their requirements throughout development (**Table 2.2**).

Allele	**Fertile Adults***	**Larval Lethal**	**Male**	**Sterile**	**n**
aspm-1(or645)		56%		44%	118
klp-18(or447)	67%		4%	29%	117
mei-1(or642)	98%		2%		120
mei-1(or646)	94%		3%	3%	167
mei-1(or1178)	66%			33%	129
mei-2(sb39)	85%			15%	107

*all adults laid dead embryos

Table 2. Post-embryonic phenotypes of oocyte meiotic spindle defective mutants
Larval lethality, sterility, and gender were scored after L1 larvae were matured to adulthood at restrictive temperature (26^{o}C).

To compare oocyte meiotic spindle assembly in live wild-type and mutant embryos, we used spinning disc confocal microscopy and transgenic strains expressing translational fusions of GFP to β-tubulin, and mCherry to Histone2B, to mark microtubules and chromosomes during Meiosis I spindle assembly (**Materials and methods, Appendix A**). We used both our TS mutant alleles and RNAi knockdown, which result in similar phenotypes, to reduce gene functions (**Figures 2.2**, **2.4,** and **A.2, Appendix A**). Because the *klp-18* gene is tightly linked to the mCherry::Histone2B transgene integration site (data not shown), we used RNAi to reduce *klp-18* function in most experiments.

*aspm-1(or645*ts*)* meiotic spindles are bipolar but assemble abnormally and are mis-oriented

Of the three mutants we isolated, we saw the least severe spindle defects in *aspm-1(or645*ts*)* mutants (**Figure 2.2**). In previous studies that used RNAi to reduce its function, *aspm*-1 was shown to act with dynein to rotate the Meiosis I spindle such that it orients orthogonally to the plane of the plasma membrane (Ellefson and McNally, 2009; van der Voet et al., 2009; Wignall and Villeneuve, 2009). We similarly found that bipolar Meiosis I spindles assembled in *aspm-1(or645*ts*)* mutant zygotes and failed to rotate to an orthogonal orientation or to stay in close proximity to the plasma membrane (**Figure**

2.2A), followed in 4/15 cases by failed attempts to extrude chromosomes into a polar body (**Figure 2.2B**).

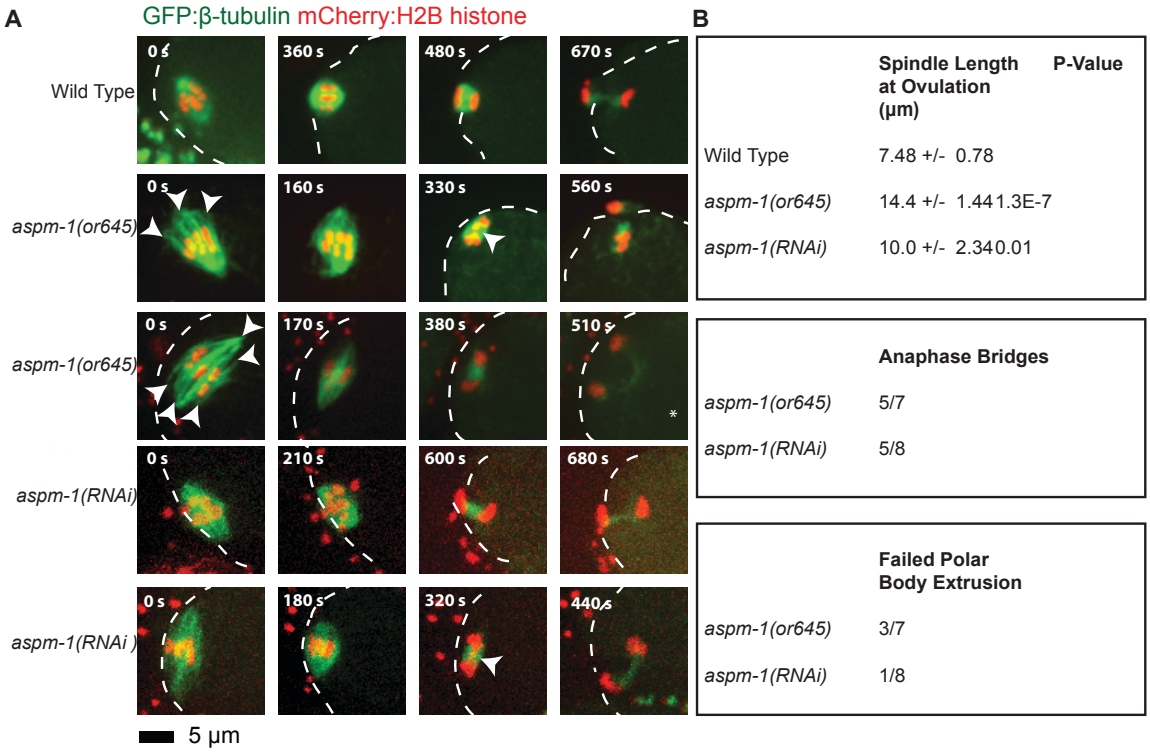


Figure 2.2. *aspm-1(-)* mutants assemble long bipolar oocyte meiotic spindles with unfocused pole ends and aberrantly organized chromosomes. (A) Spinning disc confocal images were recorded over time during Meiosis I in live wild-type (Supplemental Movie 1) and *aspm-1(-)* mutant embryos (Supplemental Movies 2, 3) expressing mCherry:Histone2B and GFP:β-tubulin translational fusions to mark chromosomes and microtubules, respectively. Indicated time points begin at ovulation. A white dashed line marks the edge of the plasma membrane. In the first column, white arrowheads mark unfocused pole ends, and in the third column arrows mark the lagging chromosomes during anaphase, as quantified in B. The Asterisk indicates an embryo in which polar body extrusion failed. Bottom rows are examples of mutant embryos with more focused poles. (B) Quantification of spindle defects in *aspm-1(-)* mutants. Meiotic spindles were measured directly after ovulation from one end of the pole to the other using spinning disc confocal images.

In addition to the previously reported meiotic spindle positioning defects, we also observed earlier defects in spindle morphology in *aspm-1(or645*ts*)* mutants. After oocyte

pronuclear envelope breakdown, ovulation and fertilization, *aspm-1(or645*ts*)* mutants assembled Meiosis I spindles that were large compared to wild type (**Figure 2.3**). Prior to metaphase, the mutant spindles also appeared to have unfocused poles, often with more than a single focus of microtubule ends visible at one or both poles (**Figures 2.2A** and **A.2A**), similar to a previous observation reported for a single fixed embryo (Wignall and Villeneuve, 2009). To further examine the integrity of the oocyte meiotic spindle poles, we reduced *aspm-1* function in a transgenic strain that expresses a GFP fusion to MEI-1, which as shown previously localizes to the two poles in wild-type spindles (McNally et al., 2006). We again observed unfocused but bipolar spindles early in Meiosis I, with more than a single focus of GFP:MEI-1 detected at one or both poles in most mutant embryos (**Figure 2.3**).

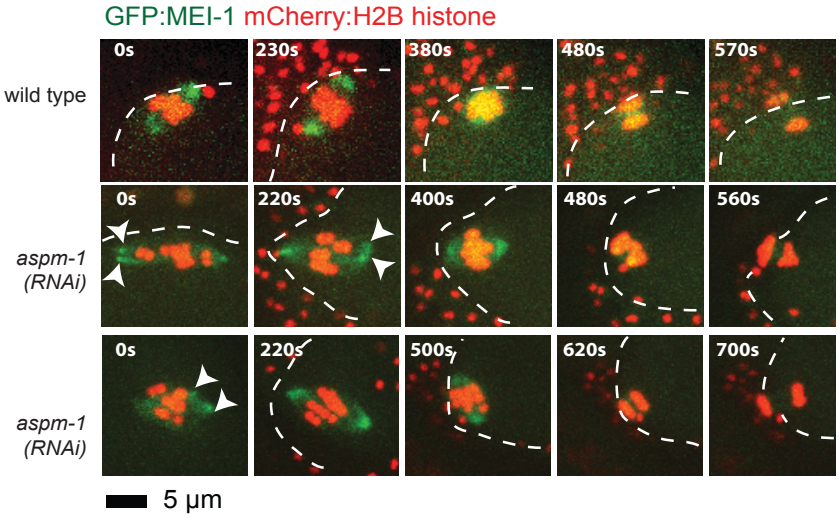


Figure 2.3. *aspm-1(-)* mutants assemble long bipolar oocyte meiotic spindles with unfocused pole ends and aberrantly organized chromosomes. MEI-1 marks unfocused pole ends in *aspm-1(RNAi)*. Spinning disc confocal images taken over time during Meiosis I in live wild-type (Supplemental Movie 4) and *aspm-1(-)* (Supplemental Movie 5) mutant embryos expressing GFP::MEI-1 and mCherry::Histone2B translational fusions to mark spindle poles and chromosomes, respectively. Times indicated begin at ovulation. Scale bar as shown. Arrowheads indicate mutant spindle poles that initially appear fragmented but later coalesce into more focused poles resembling those observed in wild-type embryos

Finally, the meiotic chromosomes in *aspm-1(or645*ts*)* mutants appeared disorganized. During prometaphase of Meiosis I, chromosomes in some cases appeared more dispersed within the spindle relative to wild-type (**Figure 2.2**). Nevertheless, as the meiotic cell cycle progressed the mutant spindles shortened and maintained bipolarity, and chromosomes congressed to a metaphase plate, more nearly resembling wild-type spindle morphology. However, we then observed lagging chromosomes during anaphase and telophase (**Figure 2.2**). Defects in chromosome segregation and failed polar body extrusion presumably account for the penetrant phenotype of multiple maternal pronuclei observed using DIC microscopy, with nuclear membranes assembling around multiple chromosome masses present in oocytes upon the completion of Meiosis I and II (**Figure 2.1** and **Table 2.1**). We conclude that ASPM-1 has important roles in meiotic spindle assembly, positioning and function, but is not required for spindle bipolarity.

Bipolar oocyte meiotic spindles fail to assemble in both *mei-1(-)* and *klp-18(-)* mutants

In contrast to *aspm-1(-)* mutants, *klp-18(-)* and *mei-1(-)* mutants assembled oocyte meiotic spindles that appear to lack bipolarity (**Figure 2.4**). Indeed, both *klp-18(-)* and *mei-1(-)* mutants frequently extruded all oocyte chromosomes into the first polar body, leaving zygotes with no maternal genome contribution (**Table 2.1** and **Figures 2.1A** and **2.4**).

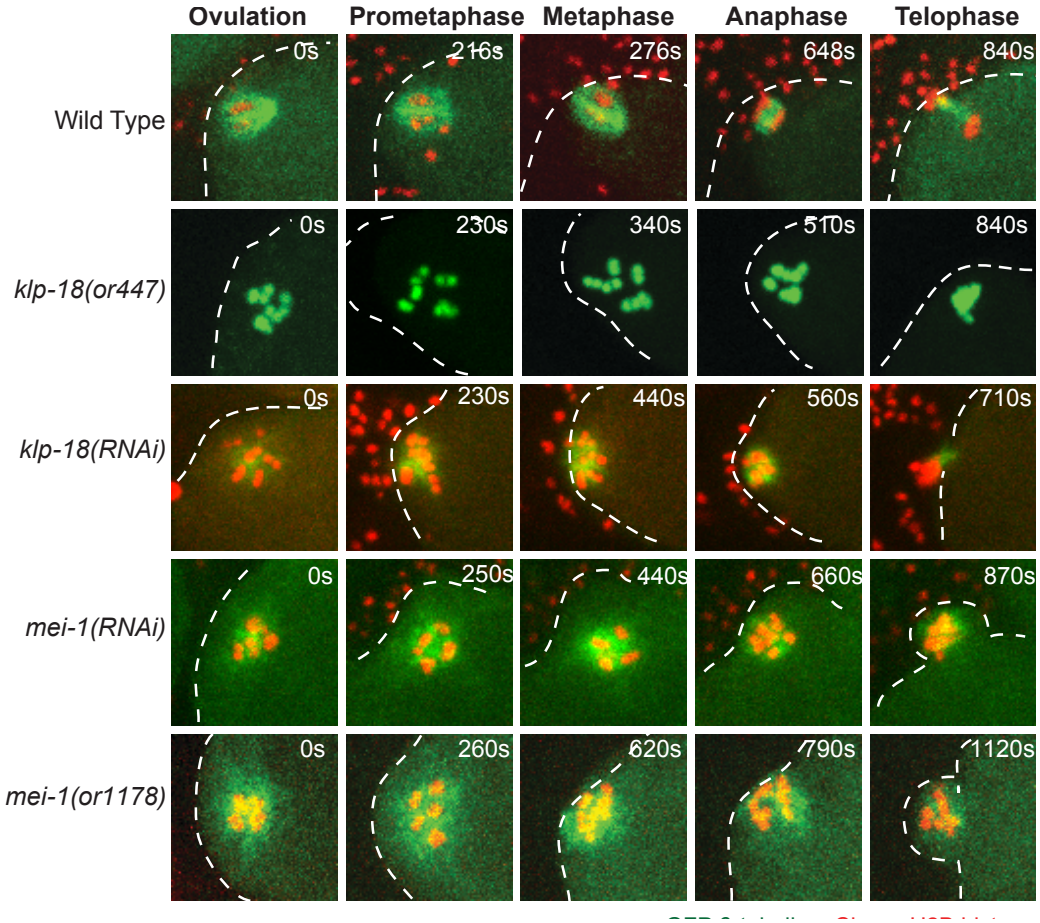


Figure 2.4. Monopolar and apolar oocyte meiotic spindles assemble in *klp-18(-)* and *mei-1(-)* mutants, respectively. Time-lapse spinning disc confocal images from immobilized worms were recorded during Meiosis I in wild-type (Supplemental Movie 1) and mutant zygotes (Supplemental Movies 6, 7, 8, 9) expressing mCherry:Histone2B and GFP:β-tubulin to mark chromosomes and microtubules, respectively, from ovulation to polar body extrusion; but only GFP:Histone2B marks the chromosomes in *klp-18(or447)* oocytes. Anterior is to the left, times indicated are relative to ovulation, and a white dashed line marks the edge of the zygote plasma membrane. In this and subsequent figures, each image shown is a projection of six consecutive frames taken at 1.5 µm intervals in a z-stack for each time point.

While both mutants lacked bipolarity, the meiotic spindle assembly defects we observed in *mei-1(-)* and *klp-18(-)* mutants were distinct. In *mei-1(-)* mutants, the oocyte Meiosis I spindle was highly disorganized: the chromosomes were variably dispersed without ever congressing to a metaphase plate, and microtubules appeared loosely organized. Ultimately, an interspersed assembly of microtubules and chromosomes was

often extruded into a single, abnormally large polar body (**Figure 2.4**). By contrast, during oocyte meiotic spindle assembly in *klp-18(-)* mutants, chromosomes also were initially scattered amongst loosely organized microtubules, but the chromosomes and microtubules progressively congressed into a smaller area, with chromosomes at the periphery of a single bright focus of microtubule density (**Figure 2.4**).

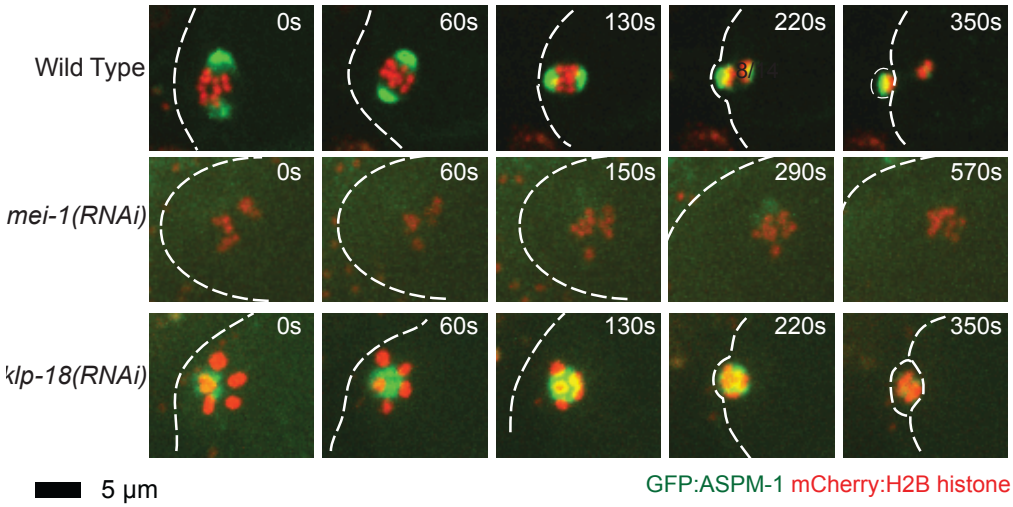


Figure 2.5. Monopolar and apolar oocyte meiotic spindles assemble in *klp-18(-)* and *mei-1(-)* mutants, respectively. ASPM-1 marks a single pole in *klp-18(-)* mutants, and no pole in *mei-1(-)* mutants. Spinning disc confocal images taken over time during Meiosis I in live wild-type (Supplemental Movie 10) and mutant embryos (Supplemental Movies 11, 12) expressing GFP:ASPM-1 and mCherry:Histone2B translational fusions to mark spindle poles and chromosomes, respectively. Times indicated begin at ovulation. Scale bar as shown.

klp-18(-) mutant oocyte meiotic spindles are monopolar, while *mei-1(-)* mutant spindles are apolar

Based on their distinct morphologies, we hypothesized that *mei-1(-)* meiotic spindles are apolar, and that *klp-18(-)* mutants assemble monopolar spindles. To further investigate whether *klp-18(-)* and *mei-1(-)* spindles are monopolar or apolar, we used

ASPM-1 as a spindle pole marker. From prevous studies of fixed embryos, ASPM-1 is known to localize to oocyte meiotic spindle poles (Wignall and Villeneuve, 2009; McNally and McNally, 2011; Gomes et al., 2013). To examine ASPM-1 localization dynamics in live oocytes, we used recombineering to construct a functional N-terminal translational fusion of GFP to ASPM-1 driven by the endogenous promoter (*Materials and methods*). In wild-type transgenic oocytes, GFP:ASPM-1 marked both spindle poles before metaphase and persisted through anaphase (**Figure 2.5**).

We next used transgenic strains expressing the GFP:ASPM-1 fusion to further compare *mei-1(-)* and *klp-18(-)* mutant oocytes (**Figure 2.5**). Consistent with previous studies using fixed embryos (McNally and McNally, 2011; Gomes et al., 2013), GFP:ASPM-1 was nearly absent from the disorganized meiotic spindles observed in *mei-1(-)* mutant oocytes. By contrast, ASPM-1 brightly marked a single focus in *klp-18(-)* mutant spindles, consistent with a previous analysis of fixed specimens (Wignall and Villeneuve, 2009). These results provide the first analysis of *C. elegans* ASPM-1 localization in live embryos and support the conclusions that *mei-1(-)* oocytes lack meiotic spindle polarity, and that *klp-18(-)* mutants assemble monopolar spindles (see Discussion).

mei-1 is required for monopolar spindle assembly in *klp-18(-)* mutants

We next wanted to identify genetic requirements for oocyte meiotic spindle pole assembly, using the monopolar spindles in *klp-18(-)* mutants as a simplified model for wild-type spindle pole assembly. Wild-type mutant embryos with bipolar oocyte meiotic spindles capture and segregate chromosomes into a single metaphase plate that then

transitions during anaphase into two nearly identical masses, sometimes connected by anaphase bridges in mutants. These dynamics made it difficult to directly and quantitatively compare bipolar spindle dynamics to the apolar and monopolar *mei-1(-)* and *klp-18(-)* spindles. We therefore have limited our quantitative comparisons to mutants with apolar or monopolar oocyte meiotic spindles.

To use *klp-18(-)* mutants as a model for analyzing pole assembly, we first developed a simple quantitative measurement to compare differences in spindle size and organization. The measurement entails tracking the two-dimensional area occupied by oocyte chromosomes over time, in projected Z-stacks. We then compared the area at ovulation, immediately after the egg has been fully engulfed by the spermatheca, with the smallest area occupied by the chromosomes during Meiosis I (**Figure 2.6** and **Materials and methods, Appendix A**).

We found that *klp-18(-)* oocyte chromosomes were distributed over a relatively large area at ovulation but then congressed into a much smaller area (**Figure 2.6**). By contrast, chromosomes in *mei-1(-)* oocytes occupied a smaller area initially but generally failed to congress (**Figure 2.6**). We then calculated the ratio of the area occupied by oocyte chromosomes at ovulation over the smallest area occupied during Meiosis I (**Figure 2.6**). In *klp-18(-)* mutants, this "Area Occupied by Chromosomes" (AOC) ratio was on average 3.6, while in *mei-1(-)* mutants the AOC ratio was on average 1.2. As an independent measure of spindle pole assembly, we also compared the smallest areas occupied by chromosomes in *mei-1(-)* and *klp-18(-)* mutants and found that chromosomes came to occupy a smaller area in *klp-18(-)* compared to *mei-1(-)* mutants (**Figure 2.7**). We conclude that oocytes fail both to assemble spindle poles and to organize

chromosomes in *mei-1(-)* mutants, while *klp-18(-)* mutants assemble organized and monopolar oocyte meiotic spindles that appear to capture and bring chromosomes toward the monopole.

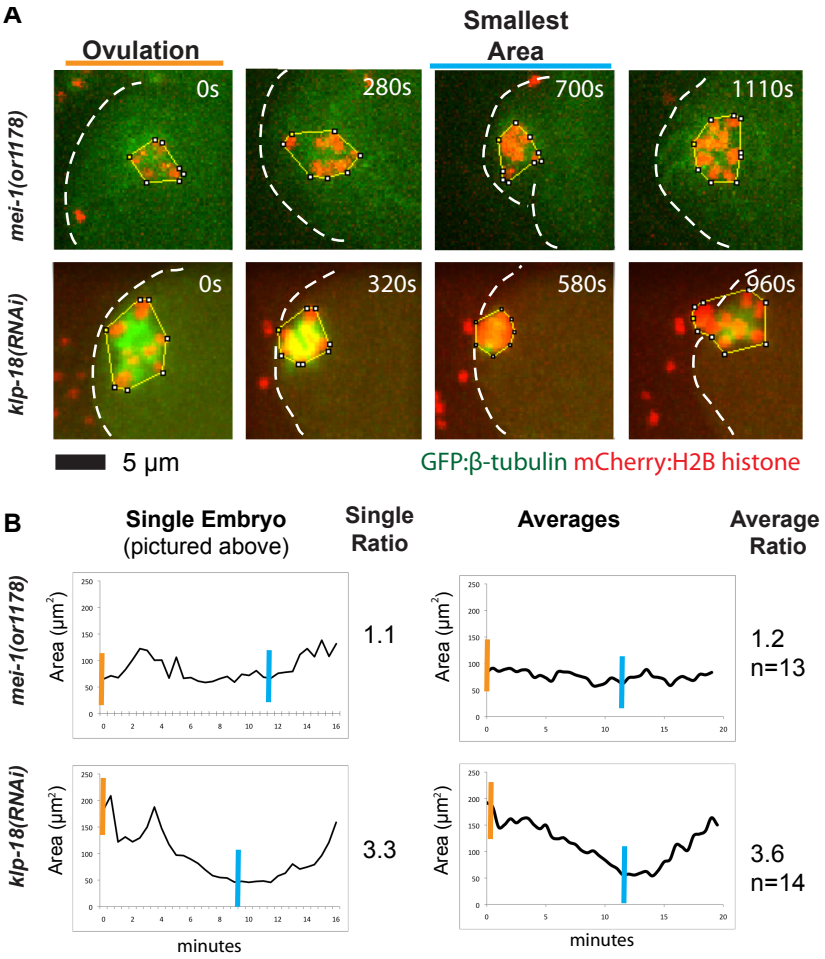


Figure 2.6. Measuring the area occupied by chromosomes over time quantitatively distinguishes oocyte meiotic spindle-defective mutant phenotypes. (A) Spinning disc confocal images were recorded over time during Meiosis I in live wild-type and mutant embryos expressing mCherry:Histone2B and GFP:β-tubulin translational fusions to mark chromosomes and microtubules, respectively. (B) On the left, Image J polygon tool was used to measure the area occupied by chromosomes at each time point for the embryos shown in (A). Examples of how the area was traced are shown in each image (A). On the right, measurements were made beginning at ovulation and taken every 30 seconds, ending when chromosomes were extruded into a polar body. If chromosomes did not extrude into a polar body, the movie was ended after a failed attempt to extrude chromosomes. Averages for the indicated number of embryos are shown. The compaction ratios of the areas occupied at ovulation (orange lines) divided by the smallest area occupied during Meiosis I (blue lines) are shown to the right.

We next asked if the reduction in meiotic spindle size that occurs over time in *klp-18(-)* mutants requires *mei-1* function. We reasoned that a loss of any obvious spindle pole assembly might prevent the assembly of a monopolar spindle in *klp-18(-)* mutants. To this end, we examined oocyte meiotic spindle assembly dynamics in mutants lacking both *mei-1* and *klp-18* function (**Figure 2.7** and **A2**). As expected, we found that spindle assembly dynamics in the double mutants resembled those observed in *mei-1(-)* single mutants: the initially dispersed oocyte chromosomes failed to congress and thus did not occupy a smaller area later in Meiosis I. We conclude that *mei-1* is required for the assembly of monopolar meiotic spindles in *klp-18(-)* mutants, consistent with previous observations (McNally and McNally, 2011).

The microtubule-severing activity of MEI-1 is required for monopolar oocyte meiotic spindle assembly in *klp-18(-)* mutants

The MEI-1 protein could promote oocyte meiotic spindle assembly through two distinct functions: the microtubule severing mediated by its C-terminal AAA ATPase domain, and recruitment of ASPM-1 to the meiotic spindle (McNally and McNally, 2011). While the partially microtubule severing-defective *mei-1(ct46ct103)* allele is homozygous viable (**Table 2.1**) and assembles abnormally long but bipolar Meiosis I spindles (McNally and McNally, 2011), we found that the spindle poles initially appeared loosely organized compared to wild-type (**Figures 2.8** and **2.9**), although the microtubule signal was reduced in these mutants and we have not quantified this defect. The loosely organized poles could result from a role for microtubule severing in pole assembly, or be an indirect consequence of the initially larger size of the mutant spindles relative to wild

Figure 5

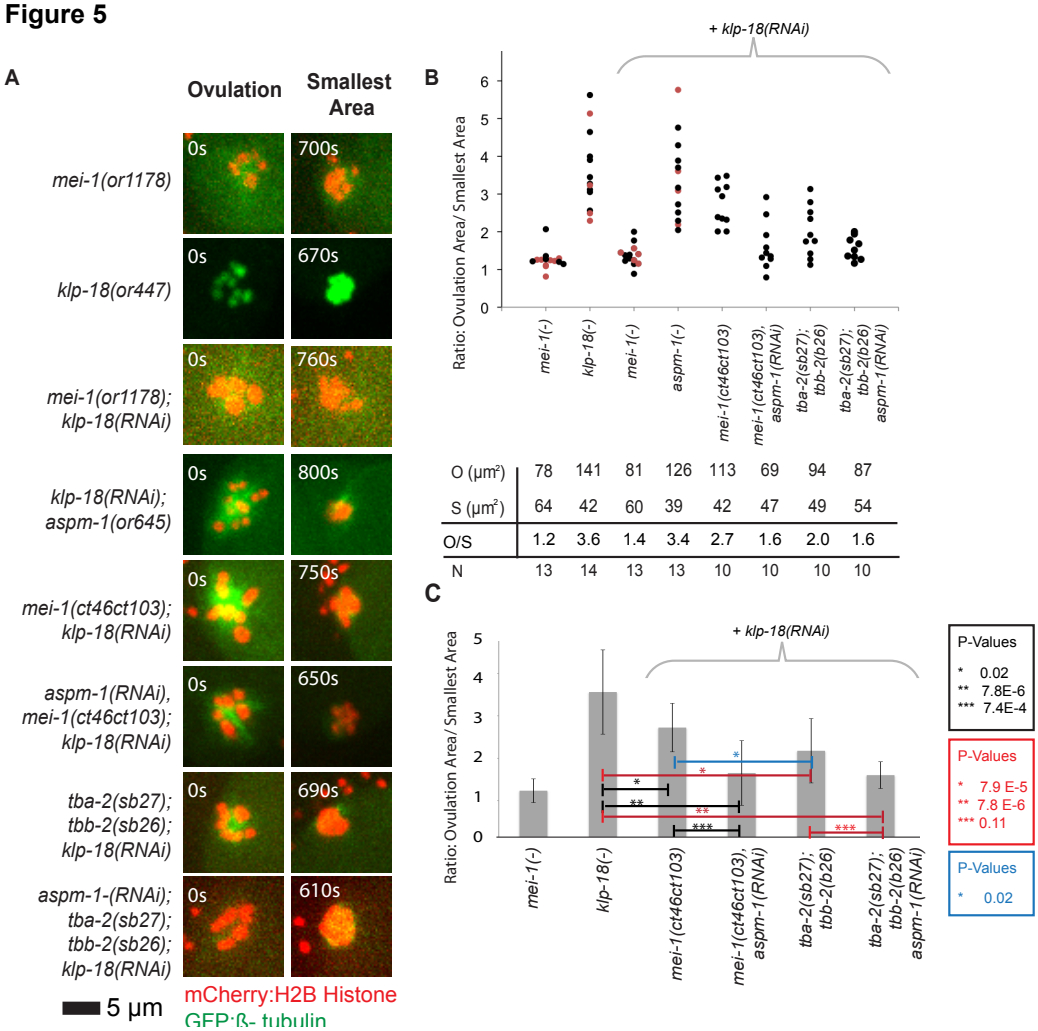


Figure 2.7. Assembly of monopolar oocyte meiotic spindles in *klp-18* mutant requires both the katanin activity of MEI-1 and ASPM-1. (A) Representative spinning disc confocal images were recorded over time during Meiosis I in live mutant embryos expressing mCherry:Histone2B and GFP:β-tubulin translational fusions to mark chromosomes and microtubules, respectively, with the exception of *klp-18(or447*ts*)* oocytes which were expressing GFP::Histone2B only. (B) Scatter plot showing the AoC ratios at ovulation (O) over the smallest (S) areas occupied during Meiosis I. For *mei-1(-)* and *klp-18(-),* measurements were taken using RNAi (black dots) and TS mutations (red dots) to reduce gene function. Average areas for combined genotypes at O, S, O/S and the number of embryos analyzed for each genotype (N) are shown below the graph. (C) Bar graph showing the average O/S ratio occupied during Meiosis I for selected genotypes. P-values from student's test are indicated in black for observations made in the *mei-1(ct46ct103)* background, in red for *tba-2(sb27);tbb-2(sb26)* background, and in blue when comparing these two when *klp-18* is knocked down. P-values under 0.05 indicate a statistically significant difference.

type. To distinguish between these possibilities, we asked if the microtubule severing activity of *mei-1* is required for monopolar spindle assembly in *klp-18(-)* mutants. Intriguingly, the spindle dynamics in *mei-1(ct46ct103); klp-18(-)* double mutants were intermediate compared to those in *mei-1(or1178*ts*)* and *klp-18(-)* single mutants: the double mutant AOC ratio of 2.7 was lower than the 3.6 ratio observed in *klp-18(-)* mutants but more than the 1.4 ratio observed after reducing all *mei-1* function in *klp-18(-)* mutants (**Figure 2.7**). Similarly the smallest area occupied by chromosomes in the double mutant was less than that observed in *mei-1(-)* but more than that observed in *klp-18(-)* single mutants (**Figure 2.7**). We conclude that the microtubule severing activity of *mei-1* plays a role in the assembly of monopolar oocyte meiotic spindles in *klp-18(-)* mutants.

While the *mei-1(ct46ct103)* allele has been shown to have substantially reduced microtubule severing activity (McNally and McNally, 2011), the mutation also could have other effects on MEI-1 function that are responsible for the loss of spindle pole focusing observed in *klp-18(-)* mutants. To independently test whether microtubule severing plays a role in oocyte meiotic spindle pole focusing, we used mutations in the alpha- and beta-tubulin genes *tba-2* and *tbb-2* [*tba-2(sb27)* and *tbb-2(sb26)*] that were isolated as suppressors of a dominant *mei-1* allele with constitutive microtubule severing activity; these tubulin mutants apparently are resistant to microtubule severing (Lu et al., 2004; Lu and Mains, 2005). We found that the AOC ratio, and the smallest area occupied by chromosomes, in *tba-2(sb27); tbb-2(sb26); klp-18(-)* triple mutants, also were intermediate between the values observed for *klp-18(-)* single mutants and for *klp-18(-)* mutants lacking all *mei-1* function (**Figure 2.7**), further supporting a role for microtubule severing in oocyte meiotic spindle pole assembly.

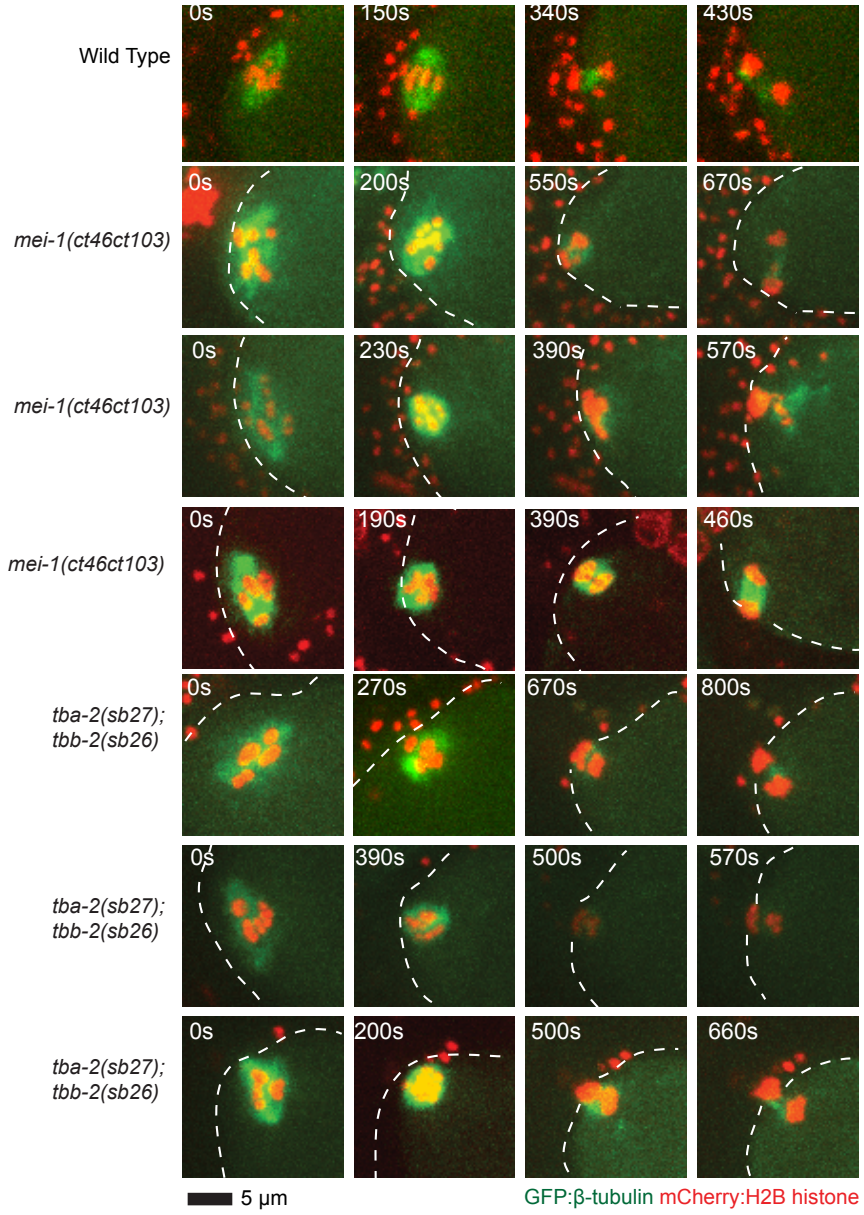


Figure 2.8. Microtubule severing defective *mei-1(ct46ct103)* and *tba-2(sb27);tbb-2(sb26)* mutant oocytes assemble bipolar oocyte meiotic spindles. Spinning disc confocal images were recorded over time during Meiosis I in live wild-type (Supplemental Movie 1) and mutant embryos (Supplemental Movies 19, 20) expressing mCherry:Histone2B and GFP:β-tubulin translational fusions to mark chromosomes and microtubules, respectively. Indicated time points begin at ovulation. A white dashed line marks the edge of the plasma membrane. Scale bar as shown.

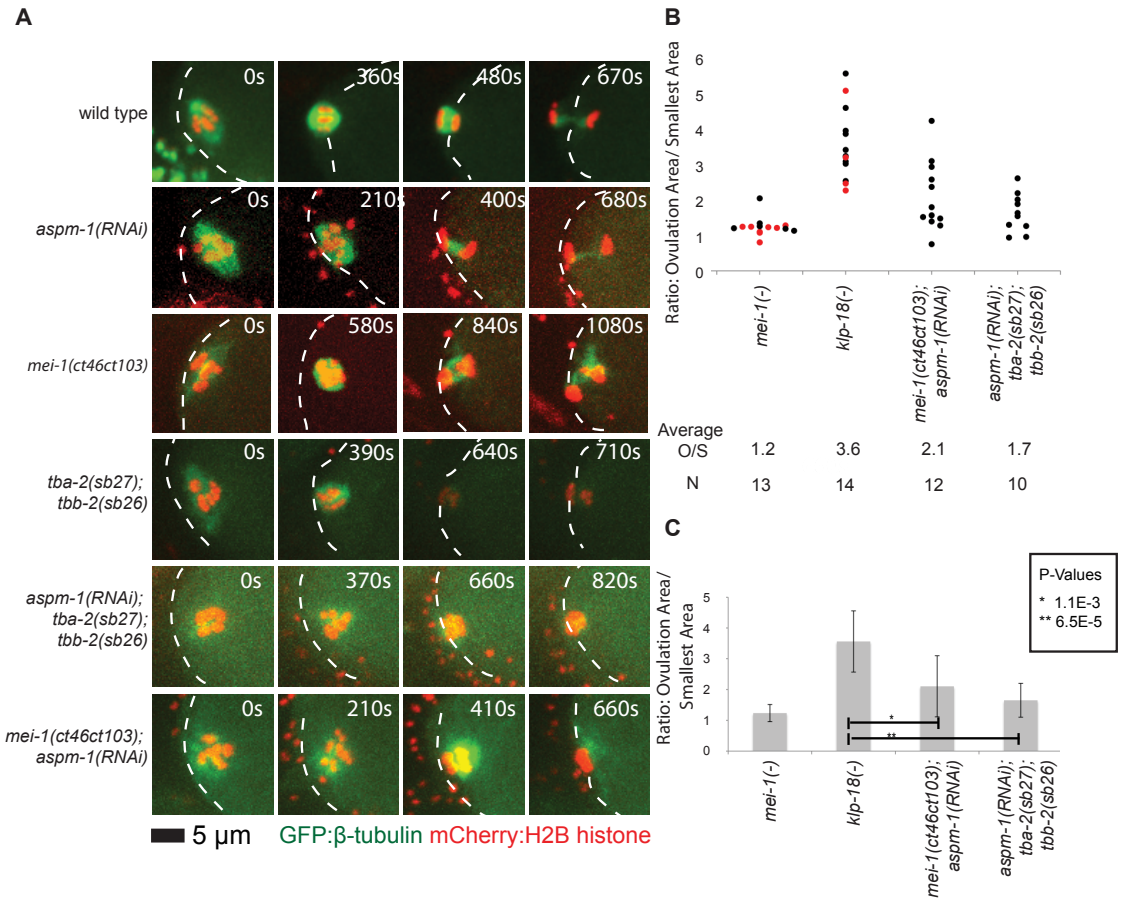


Figure 2.9. Mutants lacking either *mei-1*-mediated microtubule severing or ASPM-1 are bipolar, while mutants lacking both are apolar. (A) Spinning disc confocal images were recorded over time during Meiosis I in live wild-type (Supplemental Movie 1) and mutant embryos (Supplemental Movies 2, 3, 19, 20, 21, 22) expressing mCherry:Histone2B and GFP:β-tubulin translational fusions to mark chromosomes and microtubules, respectively. Indicated time points begin at ovulation. A white dashed line marks the edge of the plasma membrane. Scale bar as shown. (B) For *mei-1(-)* and *klp-18(-),* measurements were taken using RNAi (black dots) and TS mutations (red dots) to reduce gene function. Average ratios for combined genotypes at (O/S) and the number of embryos analyzed for each genotype (N) are shown below the graph. (C) Bar graph showing the average ratio of the areas occupied at ovulation over the smallest area occupied during Meiosis I for selected genotypes. P-values from student's test are indicated; P-values under 0.05 indicate a statistically significant difference.

***aspm-1* is required for monopolar oocyte meiotic spindle assembly in *klp-18(-)* mutants that lack *mei-1* microtubule severing activity**

We next asked if *aspm-1* is required for the compact monopolar appearance of the meiotic spindle in *klp-18(-)* oocytes as they approach completion of Meiosis I. Two observations led us to suspect a role for *aspm-1*. First, as in *mei-1(ct46ct103)* mutants, the meiotic spindle poles in *aspm-1(-)* oocytes initially appeared loosely organized (**Figures 2.2** and **2.8**), consistent with a transient role in focusing spindle poles during prometaphase. Second, the ASPM-1 protein localized to a single monopolar focus in *klp-18(RNAi)* oocytes, but was largely absent from apolar *mei-1(-)* spindles (Figure 3B). We therefore examined spindle assembly in live mutant oocytes lacking both *aspm-1* and *klp-18* function. We found that spindle dynamics in the double mutant were nearly identical to those in *klp-18(-)* single mutants, with AOC ratios of ~3.4 and ~3.6, respectively (**Figure 2.7** and **A2, Appendix A**). However, we then considered the possibility that while a role for *aspm-1* might not be apparent in the *aspm-1(-); klp-18(-)* double mutant, a contribution by *aspm-1* might be detected in the already compromised context of the *mei-1(ct46ct103); klp-18(-)* double mutant. We therefore used RNAi to knock down both *klp-18* and *aspm-1* in *mei-1(ct46ct103)* mutants. We found that reducing both *aspm-1* and microtubule severing activity in a *klp-18(-)* mutant background did result in a more severe loss of spindle pole focusing compared to *mei-1(ct46ct103); klp-18(RNAi)* double mutant, with AOC ratios of 1.6 and 2.8, respectively (Figure 5). Similarly, reducing *aspm-1* function in the *tba-2(sb27); tbb-2(sb26); klp-18(-)* mutant further reduced the AOC ratio compared to *tba-2(sb27); tbb-2(sb26); klp-18(-)*, although the reduction was in this case not statistically significant, due in part to the more severe loss of compaction

observed in the tubulin mutant background relative to the microtubule-severing mutant background (**Figure 2.7**). We conclude that *mei-1* contributes to oocyte meiotic spindle pole focusing both through its microtubule severing activity and through its recruitment of ASPM-1 to the spindle.

We have relied on AOC ratios to quantitatively compare different mutant phenotypes with respect to monopolar spindle assembly, as we believe this measurement best represents the movement of chromosomes into a smaller volume over time. However, this measurement includes the initial area occupied by chromosomes, and one significant difference between *mei-1(-)* and *klp-18(-)* single mutants is that chromosomes initially occupy a significantly larger area in *klp-18(-)* mutants (**Figures 2.6**, **2.7B**). We suspect that this difference may be due to the reduced number of microtubules present in *mei-1(-)* mutants relative to wild-type zygotes (Srayko et al., 2006), although we have not quantified this phenotype in our analysis. Because this difference between *mei-1(-)* and *klp-18(-)* mutants may confound our reliance on AOC ratios, we have also compared the smallest volume occupied by chromosomes during Meiosis I in different mutant backgrounds (Figure 5B). When using this measurement, we also observed a significant difference between *mei-1(-)* and *klp-18(-)* single mutants ($p = 2.9 \times 10^{-5}$), and between *klp-18(-)* and *mei-1(-); klp-18(-)* double mutants ($p = 0.003$). While we did not observe a significant difference when comparing this value between *klp-18(-)* mutants and double and triple mutants specifically lacking microtubule severing activity, we did observe a significant difference when comparing *klp-18(-)* and *klp-18(-); tba-2(sb27); tbb-2(sb26); aspm-1(RNAi)* mutants ($p = 0.02$); this quadruple mutant exhibited the most reduced

AOC ratio of all mutants specifically lacking microtubule severing but not all *mei-1* function (**Figure 2.7**).

As a final test of whether both *aspm-1* and microtubule severing contribute to spindle pole focusing, we used RNAi to reduce *aspm-1* function in *mei-1(ct46ct103)* and in *tba-2(sb27); tbb-2(sb26)* mutants. While *aspm-1(-*) and *mei-1(ct46ct103*) single mutants and the *tba-2(sb27); tbb-2(sb26)* double mutant all assembled abnormal but bipolar oocyte Meiosis I spindles, we found that the *mei-1(ct46ct103); aspm-1(RNAi)* double mutant and the *tba-2(sb27); tbb-2(sb26); aspm-1(RNAi)* triple mutant both were unable to assemble a bipolar spindle and instead assembled disorganized and apparently apolar spindles that resembled those observed in mutants lacking all *mei-1* function (**Figure 2.9**), although the AOC ratios were more variable (**Figure 2.7** and **2.9**). These results support our conclusion that both *aspm-1* and microtubule severing activity contribute to meiotic spindle pole assembly. Finally, the AOC ratio in *klp-18(-)* mutants that lacked both microtubule severing and ASPM-1 was not as low as the AOC ratio in *klp-18(-); mei-1(or1178ts)* double mutants with a more complete loss of mei-1 function. While this difference was not statistically significant, we cannot rule out the possibility that *mei-1* acts through additional factors to promote bipolar oocyte meiotic spindle assembly.

Discussion

By using live cell imaging to quantify microtubule and chromosome dynamics during *C. elegans* oocyte meiotic spindle assembly, we have confirmed previously identified requirements for *aspm-1, mei-1,* and *klp-18*, three essential genes known to be

required for oocyte meiotic spindle positioning or assembly. Our results further indicate that the calponin homology domain protein ASPM-1, in addition to its previously documented role in spindle positioning, also contributes to assembly of the acentrosomal oocyte meiotic spindle poles and is required for chromosome segregation during anaphase. Furthermore, our results provide the first evidence that microtubule severing by MEI-1 also contributes to spindle pole focusing. Finally, our results confirm in live embryos the previous conclusion that *klp-18/*kinesin 12 is not required for spindle pole assembly but instead promotes oocyte meiotic spindle bipolarity.

klp-18 **promotes oocyte meiotic spindle bipolarity**

The conclusion that *klp-18(-)* mutants assemble monopolar oocyte meiotic spindles is based on three observations. First, during Meiosis I in *klp-18(-)* mutants, the chromosomes are initially dispersed but over time the chromosomes move toward each other to ultimately occupy a small volume. The chromosomes surrounded a central core of microtubule density as they congressed, consistent with the capture and movement of chromosomes by microtubules emanating from a monopole. Second, the oocyte meiotic spindle pole marker ASPM-1, found at both poles in wild-type, localizes to a single focus in *klp-18(-)* mutants, and the dispersed chromosomes moved toward this ASPM-1 focus. Third, consistent with our live cell analysis, immunofluorescent images of fixed embryos were consistent with monopolar oocyte meiotic spindles assembling in *klp-18(-)* mutants (Wignall and Villeneuve, 2009).

The human and mouse kinesin 12 family members hKlp2 and Kif15 are partially redundant with the kinesin 5 family member Eg5 in promoting mitotic spindle bipolarity

(Tanenbaum et al., 2009; Vanneste et al., 2009). Chemical inhibition of Eg5 early in mitosis results in a loss of bipolarity, but chemical inhibition beginning at metaphase has no affect (Kapoor et al., 2000). While RNAi-knockdown of either hKlp2 or Kif15 alone does not affect bipolarity, knocking them down after chemical inhibition of Eg5 beginning at metaphase does lead to a loss of mitotic spindle bipolarity (Tanenbaum et al., 2009; Vanneste et al., 2009). In *C. elegans*, neither *klp-18/*kinesin 12 nor *bmk-1/*kinesin 5 are required for mitotic spindle bipolarity (Tanenbaum et al., 2009; Vanneste et al., 2009). Although it remains possible that they are more fully redundant during mitosis than are the vertebrate orthologs, we have not detected any loss of mitotic spindle bipolarity in early embryos lacking both *klp-18* and *bmk-1* function (data not shown).

Both kinesin 5 and 12 family members are thought to mediate bipolar spindle assembly by cross-linking and pushing in opposite directions anti-parallel microtubules to promote separation of the two spindle poles (Tanenbaum et al., 2009; Vanneste et al., 2009; Sturgill and Ohi, 2013). For Eg5 the cross-linking results from the formation of homotetramers of the kinesin heavy chain, with the oppositely projecting motor domain dimers binding anti-parallel microtubules (Weinger et al., 2011). In contrast, kinesin 12 family members form homodimers that can bind one microtubule, with cross-linking possibly achieved by binding Tpx2, which also binds microtubules. In *C. elegans*, RNAi knockdown of Tpx2 compromises mitotic spindle assembly but no requirement for oocyte meiotic spindle assembly was reported (Ozlu et al., 2005). Thus it remains unclear whether and how *klp-18* cross-links microtubules to promote meiotic spindle bipolarity in oocytes.

The temporal pattern of chromosome distribution in *klp-18(-)* mutants is distinct from that observed in *C. elegans* mutants with monopolar mitotic spindles in early embryonic cells (Kitagawa et al., 2009; Song et al., 2011). In these mutants, chromosomes are captured by astral microtubules emanating from a single centrosome and over time congress to form an arc peripheral to the monopole. We conclude that the acentrosomal meiotic spindle monopole in *klp-18(-)* mutants is more compact compared to a centrosomal mitotic monopole, accounting for the movement of oocyte meiotic chromosomes into a much smaller volume.

mei-1 and *aspm-1* promote oocyte meiotic spindle pole assembly

The microtubule severing complex katanin comprises two subunits, p60 and p80**,** encoded in *C. elegans* by the genes *mei-1* and *mei-2* (Clark-Maguire and Mains, 1994; Hartman et al., 1998; Srayko et al., 2000). Inactivation of either gene results in similar meiotic spindle defects and embryonic lethality. Partial loss-of-function mutations have been used to identify requirements for *mei-1* and *mei-2* in oocyte meiotic spindle positioning (Yang et al., 2003; McNally et al., 2006) and for the decrease in spindle length that occurs around metaphase (McNally et al., 2006). More recently, an analysis of a *mei-1* allele, *ct103*, isolated as an intragenic suppressor of a gain of function *mei-1* allele *ct46* (Mains et al., 1990; Clandinin and Mains, 1993), has shown that this homozygous viable katanin-defective *mei-1* allele can recruit ASPM-1 to bipolar meiotic spindles and mediate a partial decrease in spindle length (McNally and McNally, 2011; Gomes et al., 2013). The *mei-1(ct46ct103)* allele was shown to have a substantial reduction in microtubule severing activity compared to wild-type *mei-1*, when expressed

in tissue culture cells and assayed by quantifying the immunofluorescence signal from microtubules (McNally and McNally, 2011; Gomes et al., 2013). These results suggest that the microtubule severing activity of *mei-1* is not required for oocyte meiotic spindle pole assembly. However, by quantifying spindle morphology dynamics over time in *klp-18(-)* mutants, which assemble monopolar spindles, we have found that the microtubule severing activity of katanin does contribute to meiotic spindle pole assembly. This conclusion is based on several observations. First, the partially katanin-defective allele *mei-1(ct46ct103)* assembles bipolar oocyte meiotic spindles, but the poles appear only loosely focused early in Meiosis I. Second, decreasing microtubule-severing activity with *mei-1(ct46ct103)* or increasing resistance to microtubule severing with *tba-2(sb27); tbb-2(sb26)* leads to a partial loss of monopolar spindle assembly in *klp-18(-)* mutants, as judged by the failure of oocyte chromosomes to move into as small of a volume as they do in mutants lacking only *klp-18* function. Third, while reducing *aspm-1* function in *klp-18(-)* mutants does not significantly affect the movement of chromosomes into a smaller volume, reducing *aspm-1* function in mutants that lack both *klp-18* function and have reduced microtubule severing activity further limited the ability of chromosomes to move into a smaller volume. This again supports a role for microtubule severing in pole assembly. Finally, double mutants that lack both *aspm-1* function and microtubule severing fail to assemble bipolar spindles, even though the single mutants assemble bipolar if initially loosely organized spindles. These latter two observations support our conclusion that both ASPM-1 recruitment by MEI-1 and MEI-1 microtubule severing activity play independent roles in oocyte meiotic spindle pole assembly. However, microtubule severing appears to play a more significant role in pole assembly than does

ASPM-1, as reducing ASPM-1 function alone in a *klp-18(-)* background did not significantly alter the movement of chromosomes into a more compact volume. Finally, while *mei-1* is an essential gene, we do not know if microtubule severing and pole assembly are each individually required for viability.

Our analysis does not provide mechanistic insight into how ASPM-1 and microtubule severing each promote pole assembly. Nevertheless, as illustrated in Figure 8, we speculate that severing of microtubules nucleated by chromatin during acentrosomal spindle assembly (Heald et al., 1996; Khodjakov et al., 2000) could liberate microtubules that contribute to spindle assembly (Burbank et al., 2006). Alternatively, it is possible that the *mei-1(ct46ct103)* and *tba-2(sb27); tbb-2(sb26)* mutants influence pole assembly not through microtubule severing itself but perhaps through related interactions with microtubules that do not necessarily lead to severing. ASPM-1, whose polar recruitment depends on MEI-1 (McNally and McNally, 2011), in turn might bring minus ends into close proximity at spindle poles through its interactions with the NuMA-related protein LIN-5 and cytoplasmic dynein (van der Voet et al., 2009; Ellefson and McNally, 2011)**.** Consistent with such a model, a GFP fusion to the *C. elegans* dynein heavy chain (GFP:DHC-1) was largely absent from *mei-1(-)* mutant spindles (**Figure A3, Appendix A**).

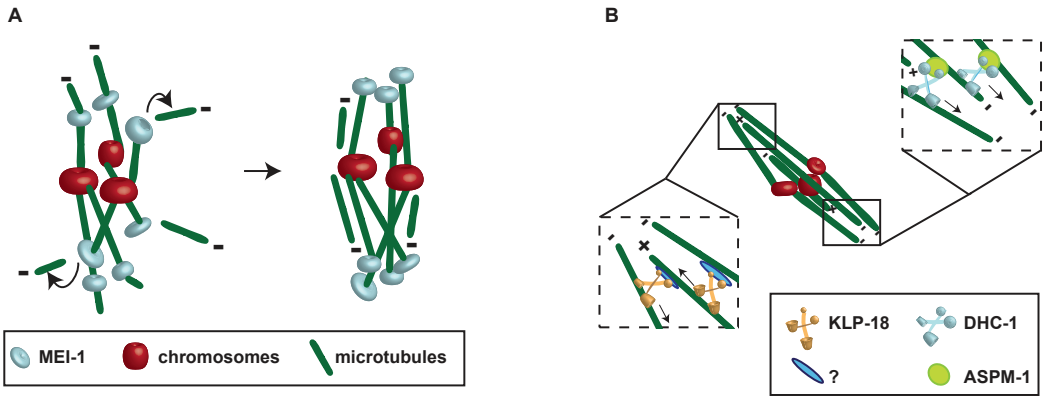


Figure 2.10. Models for how MEI-1 contributes to pole focusing
(A) MEI-1 may mediate spindle pole assembly by severing chromatin-nucleated microtubules. (B) KLP-18 may promote bipolarity by cross-linking anti-parallel microtubules with the help of an unknown factor. ASPM-1 may focus spindle poles by cross-linking the minus-ends of parallel microtubules through a dynein-dependent mechanism.

In sum, our analysis has identified possible roles for two *mei-1* functions—ASPM-1 recruitment and microtubule severing—in the assembly of oocyte meiotic spindle poles. However, we cannot rule out other roles for *mei-1* in oocyte meiotic spindle assembly, involving for example the ability of the MEI-1 N- terminus to bind microtubules (McNally and McNally, 2011).

Quantitative analysis of oocyte meiotic spindle morphology-defective mutants can define spindle assembly pathways

Our understanding of oocyte meiotic spindle assembly has been limited in part by the difficulties of observing oocyte meiotic cell divisions using live-cell imaging methods, and by the very small size of the meiotic spindle. Our use of live imaging of oocyte meiotic spindle assembly in multiple mutant backgrounds, our quantification of spindle morphology dynamics, and our use of the spindle pole marker GFP::ASPM-1,

have together enabled us to identify previously unappreciated roles for genes known to be required for oocyte meiotic spindle assembly.

Our analysis of oocyte meiotic spindle assembly has in addition benefitted from the identification of conditional mutations in essential, maternally expressed genes. Many essential genes that are required for early embryogenesis also have earlier requirements during either gonadogenesis or germline development, with null alleles and RNAi often resulting in sterility and thus precluding or complicating the analysis of requirements during Meiosis I and II. Conditional alleles also facilitate the construction and analysis of mutants lacking the function of multiple genes, without having to rely entirely on the less efficient and less reproducible approach of using RNAi, especially when reducing multiple gene functions. Moreover, conditional mutations can be used to sensitive genetic backgrounds for modifier screens designed to identify additional factors (Dorfman et al., 2009; O'Rourke et al., 2011).

Further investigation of the molecular genetic pathways that control oocyte meiotic spindle assembly will benefit from continued efforts to identify conditional alleles of essential genes required for this process, from the further application of live cell imaging using fluorescent protein fusions to spindle-associated proteins, and from the continued application of quantitative approaches to the analysis of spindle morphology and dynamics.

CHAPTER III

IDENTIFICATION OF THE GENE *KLP-7*, AND ITS MECHANISM OF LIMITING POLE ASSEMBLY

This chapter contains material which is currently in preparation for submission to the journal *Current Biology.* The lab work in this chapter was performed by myself. Together Bruce Bowerman and I posed mechanisms for the role of *klp-7* in meiotic spindle assembly and wrote the manuscript, and we plan to submit these findings Summer 2014.

KLP-7: a Kinesin-13/MCAK Family Member

In this chapter we report our identification of conditional loss of function mutations in the *C. elegans* gene *klp-7* and investigate its role in bipolar oocyte meiotic spindle assembly. The *klp-7* gene encodes a kinesin-13/MCAK family member, a class of kinesins that do not walk along microtubules but rather associate with their minus and plus ends to promote depolymerization (Ems-McClung and Walczak, 2010).

The role of kinesin-13/MCAK family members in oocyte meiotic spindle assembly is not as well understood as it is in centrosome-dependent mitotic spindle assembly. During mitosis, these microtubule depolymerases appear to have multiple roles: two prominent functions are to mediate the correct attachment of paired sister chromatid kinetochores to opposing poles, and to promote microtubule depolymerization and the poleward flux of microtubule subunits during anaphase (Wordeman et al., 2007; Ems-McClung and Walczak, 2010). During mitosis in the early *C. elegans* embryo,

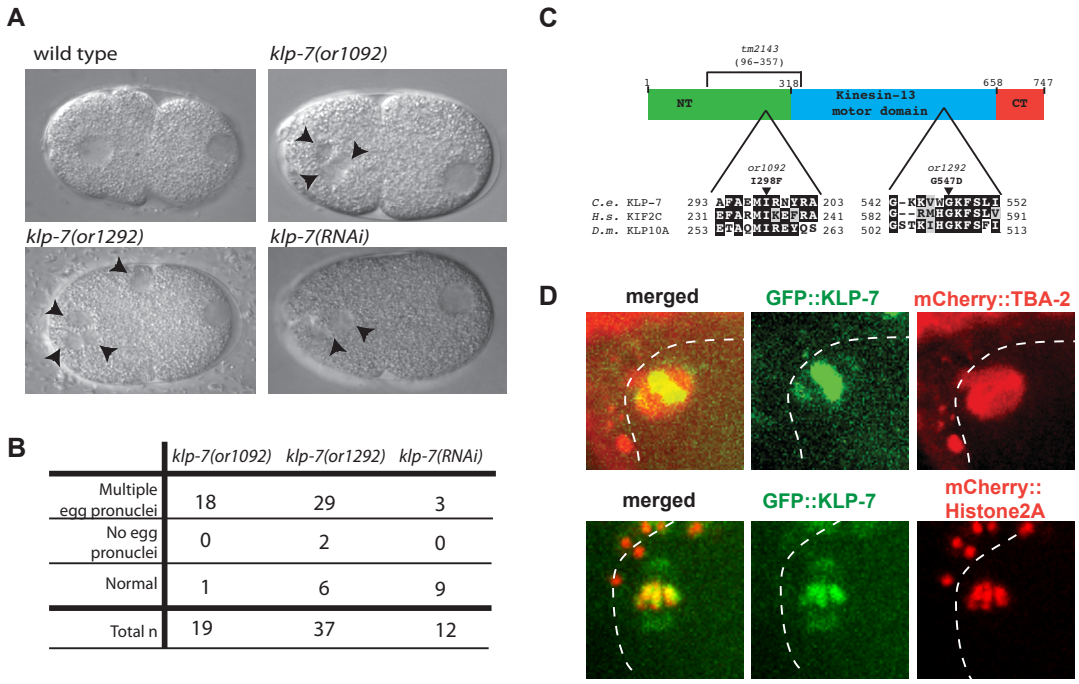
KLP-7/MCAK limits the number of microtubules that grow out from the centrosomes (Srayko et al., 2005).

Requirements for *klp-7* during *C. elegans* oocyte meiotic spindle assembly have not been described, although kinesin-13/MCAK family members have been shown to influence oocyte meiotic spindle length in *Drosophila* and *Xenopus* (Mitchison et al., 2005; Ohi et al., 2007; Zou et al., 2008). Here we investigate for the first time the function of KLP-7, the sole kinesin-13/MCAK family member in *C. elegans*, during acentrosomal oocyte meiotic spindle assembly and present evidence that it functions to limit both microtubule accumulation and meiotic spindle pole number.

Results

Two recessive, temperature-sensitive alleles of *klp-7,* the single *C. elegans* kinesin 13/MCAK family member

To identify essential genes that mediate oocyte meiotic spindle assembly, we used Nomarski optics to examine live one-cell stage embryos produced by a collection of temperature-sensitive, embryonic-lethal mutants at the restrictive temperature. Wild-type zygotes typically possess a single egg pronucleus that appears after the completion of Meiosis I and II, in addition to a single sperm pronucleus (Albertson, 1984; Albertson and Thomson, 1993) (**Figure 3.1A**). We found two recessive mutants called *or1092*ts and *or1292*ts that produced one-cell stage embryos with multiple egg pronuclei (**Table 1, Figure 3.1A**), a mutant phenotype associated with defects in oocyte meiotic spindle function (O'Rourke et al., 2011; Connolly et al., 2014).



B

	klp-7(or1092)	klp-7(or1292)	klp-7(RNAi)
Multiple egg pronuclei	18	29	3
No egg pronuclei	0	2	0
Normal	1	6	9
Total n	19	37	12

Figure 3.1. Identification of temperature-sensitive *klp-7(-)* mutants and localization of KLP-7 to Meiosis I spindles. A. Nomarski images of live one-cell stage wild-type and mutant embryos. Anterior oocyte and posterior sperm pronuclei are to the left and right, respectively. Note the presence of extra oocyte pronuclei in *klp-7(-)* mutants. B. Frequency of abnormal number of oocyte pronuclei in one-cell stage *klp-7(-)* mutants after the completion of Meiosis I and II; wild-type zygotes always have a single oocyte pronucleus. C. Domain diagram and partial sequence alignment of KLP-7 and orthologs. Arrowheads indicate altered residues in TS alleles; the *tm2143* deletion is marked with a bracket. D. Spinning disc confocal images were recorded during Meiosis I in live wild-type embryos expressing mCherry:TBA-2 and GFP:KLP-7, or mCherry:Histone2A and GFP:KLP-7, translational fusions to mark microtubules and KLP-7, or chromosomes and KLP-7, respectively.

To identify the causal mutations in *or1092*ts and *or1292*ts mutants, we used both visible marker genetic mapping and whole genome sequencing to identify candidate mutations in the *C. elegans* gene *klp-7* (**Materials and methods, Appendix 2**), which encodes the only kinesin 13/MCAK family member in *C. elegans* (Schlaitz et al., 2007). In *or1092*ts genomic DNA, we found an isoleucine to a phenylalanine change (ATT to TTT) at codon 298 in the *klp-7* kinesin neck domain; and in *or1292*ts a glycine to an

aspartic acid change (GGC to GAC) at codon 547 in the *klp-7* motor domain (**Figure 3.1B**). Consistent with these two mutations being responsible for the conditional lethality, *or1092*ts and *or1292*ts failed to complement one another, both failed to complement an apparently partial loss-of-function in-frame deletion allele, *klp-7(tm2143)* (**Table 3.1; Materials and methods**, **Appendix 2**), and a GFP fusion to KLP-7 (Schlaitz et al., 2007), which localized to both chromosomes and spindle poles during Meiosis I and II (**Figure 3.1C**), rescued *klp-7(or1092*ts*)* embryonic lethality at the restrictive temperature (**Table 3.1**). We conclude that *or1092*ts and *or1292*ts are alleles of *klp-7.* While genome-wide RNAi screens have reported embryonic lethality and mitotic spindle defects after *klp-7(RNAi)* knockdown (WormBase), defects in oocyte pronuclear number or meiotic spindle function have not been observed previously. However, *klp-7* RNAi knockdown can result in abnormal numbers of oocyte pronuclei (**Figure 3.1, Table 3.1**), further indicating that the two recessive and conditional mutations we have identified reduce *klp-7* function. To our knowledge, *or1092*ts and *or1292*ts are the first temperature-sensitive *klp-7* alleles yet identified, and their mutant phenotypes suggest that *C. elegans klp-7* influences oocyte meiotic spindle function.

klp-7 limits microtubule assembly during oocyte meiotic spindle assembly

To investigate the functional requirements for *klp-7*, we examined oocyte Meiosis I spindle assembly in live *klp-7(-)* mutants using spinning disc confocal microscopy and transgenic strains that express translational fusions of GFP and mCherry to β-tubulin and Histone2B to mark microtubules and chromosomes, respectively (**Materials and methods**, **Appendix 2**). We found that soon after ovulation and throughout Meiosis I in

Allele	Homozygote Embryonic Viability (15°C)	Homozygote Embryonic Viability (26°C)	Heterozygote Embryonic Viability (26°C)
N2	99% n=358	97% n= 197	----------
klp-7(or1092)	90% n=323	0.1% n=1530	99% n=408
klp-7(or1292)	93% n=318	0.8% n=367	97% n=604
klp-7(tm2143)		7% n=136	----------
klp-7(RNAi)	----------	1% n=164	----------

Genotype	Embryonic Viability (26°C)
klp-7(or1092)/klp-7(or1292)	2% n=238
klp-7(or1092)/klp-7(tm2143))	1% n=517
klp-7(or1292)/klp-7(tm2143))	0.5% n=220
***klp-7* Rescue**	
klp-7(or1092) unc-68(e587) + gfp::klp-7	94% n=16
klp-7(or1092) unc-68(e587)	1% n=257

Table 3.1. Embryonic viability of the *klp-7* mutants. Embryonic viability (percent hatching) was scored for wild type and each TS mutant at permissive (15°C) and restrictive temperature (26°C) temperatures. Embryonic viability at the restrictive temperature from heterozygous TS mutants was examined to determine whether the mutations are recessive or dominant. Complementation tests at the restrictive temperature were also performed.

klp-7(-) oocytes, the microtubule signal intensity was greatly increased compared to wild-type oocytes, with the oocyte chromosomes rapidly becoming surrounded by a prominent area of microtubule density (**Figure 3.2A**, **A2.1** and **2.2**). To quantitatively compare microtubule dynamics in *klp-7(-)* and WT oocytes, we tracked the ratio over time of the area occupied by microtubules and the area occupied by chromosomes (MT/C ratio; see **Materials and Methods, Appendix 2**). In wild-type oocytes, this ratio slowly increased to 2.5 at about four minutes post-ovulation, followed by a gradual decrease (**Figure 3.2B**). In contrast, in *klp-7(or1292*ts*)* oocytes, the ratio increased more rapidly and for a substantially longer period of time, reaching a peak value of 4.9 at 8 minutes post-

ovulation. As other kinesin 13/MCAK family members are known to promote microtubule depolymerization (see Introduction), we conclude that reducing the function of *klp-7* stabilizes microtubules and thereby results in their excessive accumulation during oocyte Meiosis I.

klp-7 limits spindle pole number during oocyte meiotic spindle assembly

We further examined microtubule and chromosome dynamics during Meiosis I in live *klp-7(-)* mutants, comparing them not only to wild-type oocytes, but also to other mutants with oocyte meiotic spindle assembly defects (**Figure 3.2A**). During wild-type spindle assembly, the initially scattered chromosomes converged on the metaphase plate and then moved apart in two discrete masses toward the opposing poles during anaphase (**Figure 3.2 A, C**). In contrast, as we and others have shown previously (Connolly et al 2014), *mei-1(-)* oocyte meiotic spindles appeared to be apolar with chromosomes remaining scattered and disorganized throughout meiosis I, while *klp-18(-)* spindles appeared to be monopolar, with chromosomes initially scattered and peripheral to the microtubules but then congressing toward a single centrally located focus of microtubules.

The microtubule and chromosome dynamics in live *klp-7(-)* oocytes were strikingly distinct from those in WT, *mei-1(-)*, and *klp-18(-)* oocytes. In WT oocytes, the chromosomes and microtubules were initially interspersed but then became organized into a bipolar microtubule spindle with chromosomes congressing to the metaphase plate.

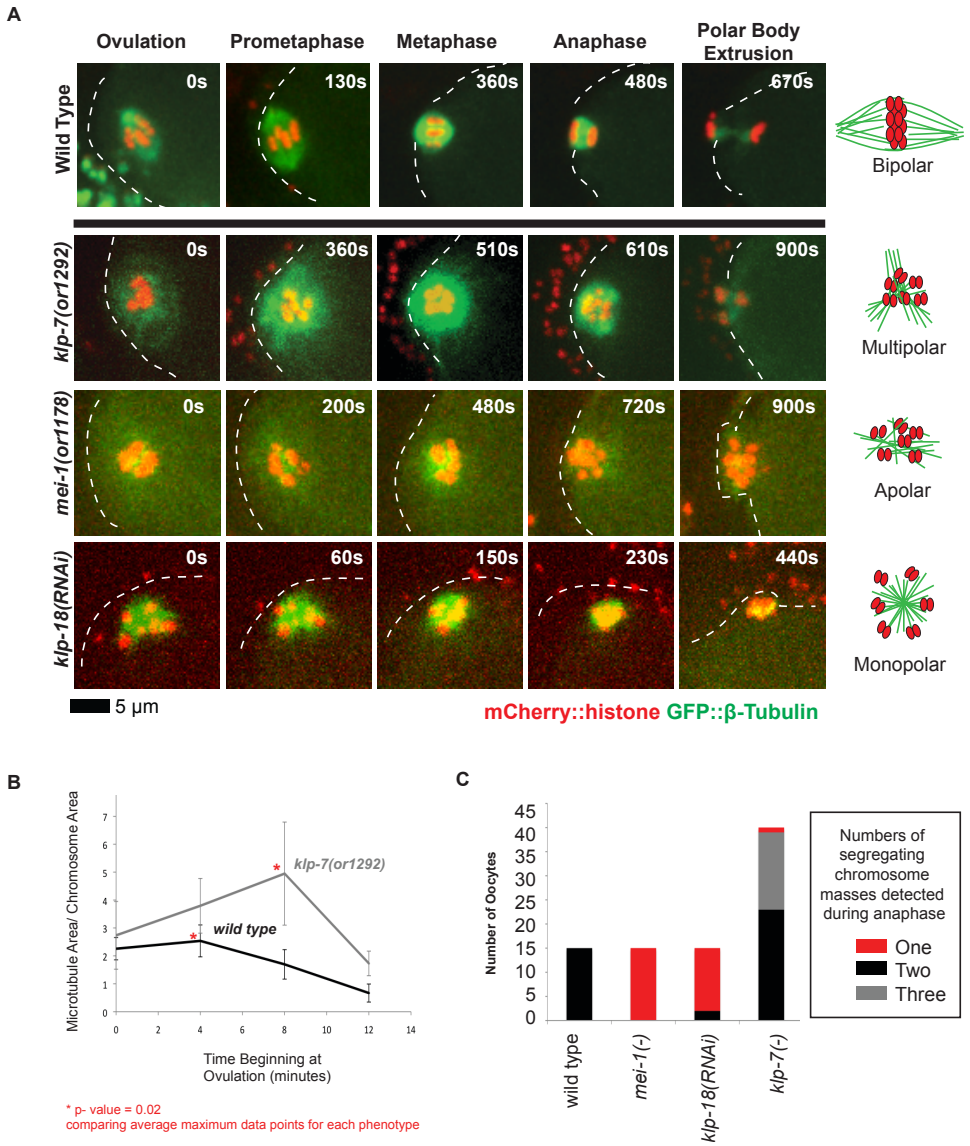


Figure 3.2. *klp-7(-)* mutants accumulate an excess of microtubules and exhibit tripolar chromosome segregation during oocyte Meiosis I. A. Time-lapse spinning disc confocal images during Meiosis I in live wild-type and mutant embryos expressing mCherry:Histone2B and GFP:β-tubulin translational fusions to mark chromosomes and microtubules, respectively. A white dashed line marks the edge of the plasma membrane. Times post-ovulation indicated in all frames. Schematics to the right illustrate our interpretations of the pole phenotypes. B. Microtubule area and chromosome area were measured using Image J beginning at ovulation and at every 4 minutes for a total of four time points. Error bars depict the standard deviation among embryos at each time point. The maximum ratio of the area occupied by microtubules and by chromosomes (MT/C) occurred in *klp-7(-)* at 8 minutes (), compared to the maximum MT/C in wild type at 4 minutes (), with P value for this difference shown below. C. Bar graphs show the number of segregating chromosomes masses detected during anaphase for the indicated genotypes.

In *klp-18(-)* oocytes the chromosomes were located at the periphery of a central core of microtubules from early in Meiosis I, and over time the chromosomes congressed into a single tight aggregate that was often entirely extruded into the first polar body (**Figure 3.2A, C**). In *mei-1(-)* oocytes, chromosomes and microtubules were interspersed throughout meiosis I without any apparent congression but nevertheless were often all extruded into the first polar body (**Figure 3.2A, C**). The *klp-7(-)* oocyte meiotic spindle morphology differed substantially from both *klp-18(-)* and *mei-1(-)* mutants in that microtubules surrounded the chromosomes throughout most of Meiosis I, until later time points when the chromosomes often moved in three directions, segregating into three distinct masses (**Figure 3.2 A, C**). Thus the chromosomes in *klp-7(-)* oocytes appeared to move toward three poles, a novel phenotype that to our knowledge has not been previously observed during oocyte Meiosis I in *C. elegans* or other animals.

To test the hypothesis that *klp-7(-)* oocytes assemble tripolar spindles, we next examined two different pole markers, GFP:ASPM-1 and GFP::MEI-1, which both mark the meiotic spindle poles early in wild type oocyte Meiosis I **(Refs)** (**Figures 3.3A, B**). As Meiosis I proceeded in wild-type oocytes, the two GFP:ASPM-1 foci appeared to merge near the metaphase to anaphase transition, encompassing the segregating chromosomes and ultimately residing mostly between the two chromosome masses by late anaphase. Similarly, the two GFP:MEI-1 foci in wild-type oocytes appeared to merge and surround the chromosomes, but then faded to undetectable levels by late anaphase. Remarkably, in all *klp-7(-)* oocytes examined, we observed more than two foci of both GFP:ASPM-1 and GFP:MEI-1 early in Meiosis I (**Figures 3.3A, B** and **C, A2.3**). As

meiosis continued, the GFP::ASPM-1 and GFP::MEI-1 foci sometimes coalesced into two poles (**Figure 3.3B**) and in all cases converged further as chromosomes segregated into two or three distinct masses. We conclude that *klp-7* acts to prevent the assembly of extra spindle poles during oocyte Meiosis I.

To further test our hypothesis that extra oocyte meiotic spindle poles form in *klp-7(-)* oocytes, we next examined the orientations of paired homologous chromosomes with respect to the wild-type and mutant spindle poles. In bipolar wild-type spindles, homologous chromosomes are oriented with one kinetochore pointing toward each pole, and the midbivalent region parallel to the metaphase axis and thus perpendicular to the axis defined by the two poles (Dumont et al., 2010). The midbivalent region can be identified by a narrow gap between homologous chromosomes (**Figure 3.3D**). In *klp-7(-)* oocytes homologous chromosome pairs often aligned properly along an axis between 2 of 3 poles, and in other cases appeared not to be aligned with respect to any two poles (**Figure 3.3D**). In some oocytes, one homologous pair of chromosomes oriented properly with respect to one pair of poles, while another pair of homologous chromosomes oriented properly with respect to a different pair of poles. These results suggest that the extra poles detected in *klp-7(-)* oocytes are functional with respect to paired homologous chromosome capture and alignment.

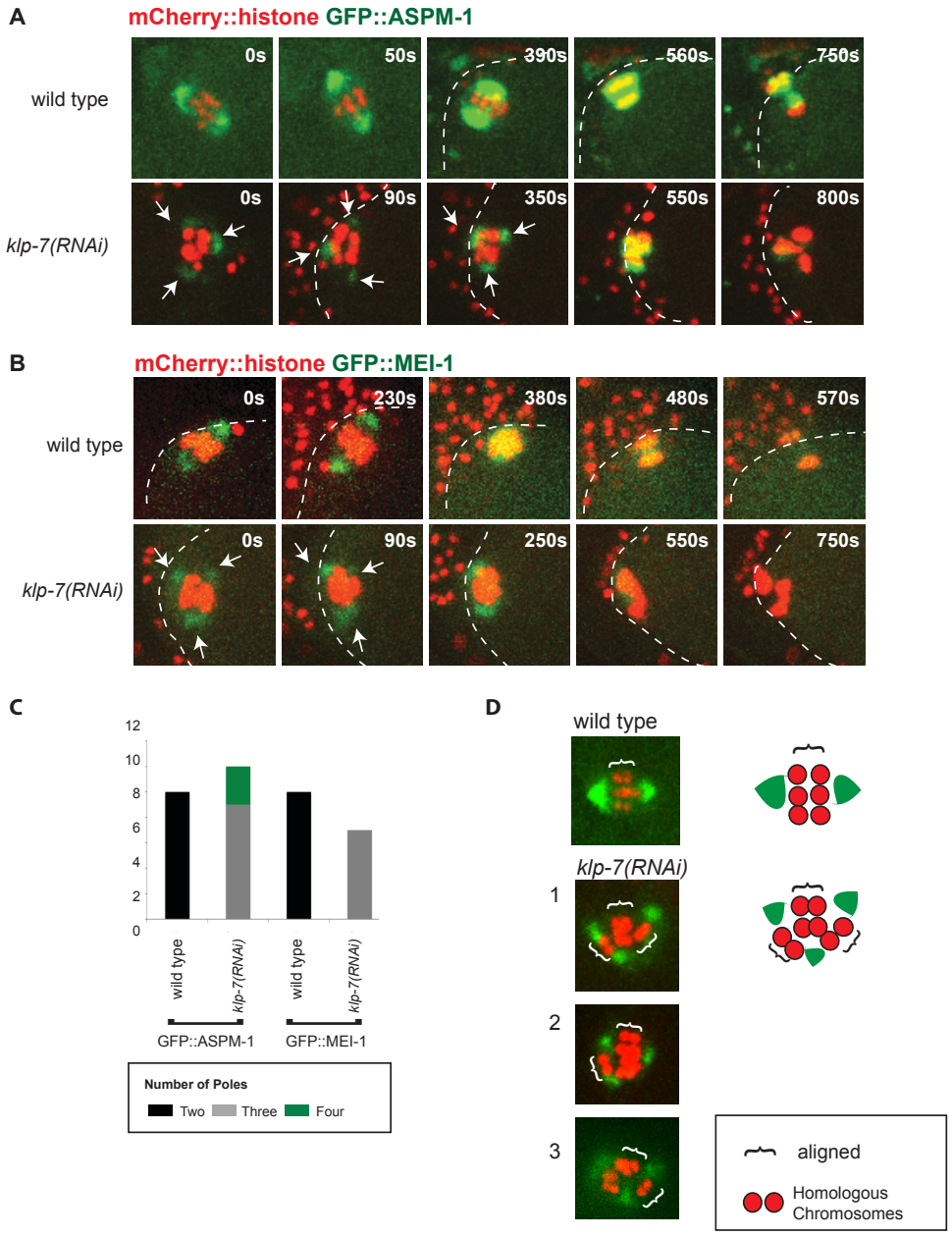


Figure 3.3. *klp-7(-)* mutants assemble extra spindle poles during oocyte Meiosis I. A, B. Time-lapse spinning disc confocal images of Meiosis I in live wild-type and mutant embryos expressing mCherry:Histone2B to mark chromosomes and GFP:ASPM-1 (A) or GFP:MEI-1 (B) translational fusions. A white dashed line marks the edge of the plasma membrane. C. Bar graph indicating the number of spindle poles observed during Meiosis I in wild type and *klp-7(-)* oocytes using both GFP:ASPM-1 and GFP:MEI-1 foci to score pole number. D. Paired homologous chromosome aligned along multiple spindle axes in *klp-7(-)* oocytes during Meiosis I. Examples of homologous chromosome pairs that appear oriented along an inter-polar axis are indicated by brackets. Schematics and legend at right illustrate representative examples.

Loss of *klp-7* restores meiotic spindle bipolarity in *klp-18(-)* mutant oocytes

We next used a genetic approach to further test the idea that *klp-7* prevents the assembly of extra oocyte meiotic spindle poles. As we and others have shown that *klp-18(-)* mutants assemble monopolar oocyte spindles, we wondered whether reducing *klp-7* function in a *klp-18(-)* background might promote the formation of another pole and thereby restore spindle bipolarity. First, we examined *klp-7(-); klp-18(-)* double mutant oocytes from a transgenic strain that expressed GFP:ß-tubulin and mCherry:Histone2B to mark microtubules and chromosomes, respectively. Remarkably, we did not observe any monopolar spindles in these double mutant oocytes; instead bipolarity was restored in nearly all cases, with two discrete masses of chromosomes segregating to opposite poles, and one mass extruded into a polar body (**Figure 3.4A**). In one example, three discrete chromosome masses moved apart late in Meiosis I, indicating the tripolar oocyte meiotic spindles can assemble in the absence of *klp-7,* even in a *klp-18(-)* background.

We also examined *klp-7(-); klp-18(-)* double mutant oocytes from a transgenic strain expressing GFP:ASPM-1 and mCherry:Histone2B to mark oocyte meiotic spindle poles and chromosomes (**Figure 3.4B)**. While GFP:ASPM-1 localized to a single focus in *klp-18(-)* oocytes and to three foci in *klp-7(-)* oocytes, it clearly marked two poles in *klp-7(-); klp-18(-)* double mutant oocyte spindles. While bipolar spindle assembly in the double mutants was delayed, and the poles were not as tightly focused compared to wild-type oocytes, we conclude that reducing *klp-7* function can restore bipolarity in a monopolar spindle mutant background. These results further support our conclusion that

klp-7 opposes the assembly of extra oocyte meiotic spindle poles.

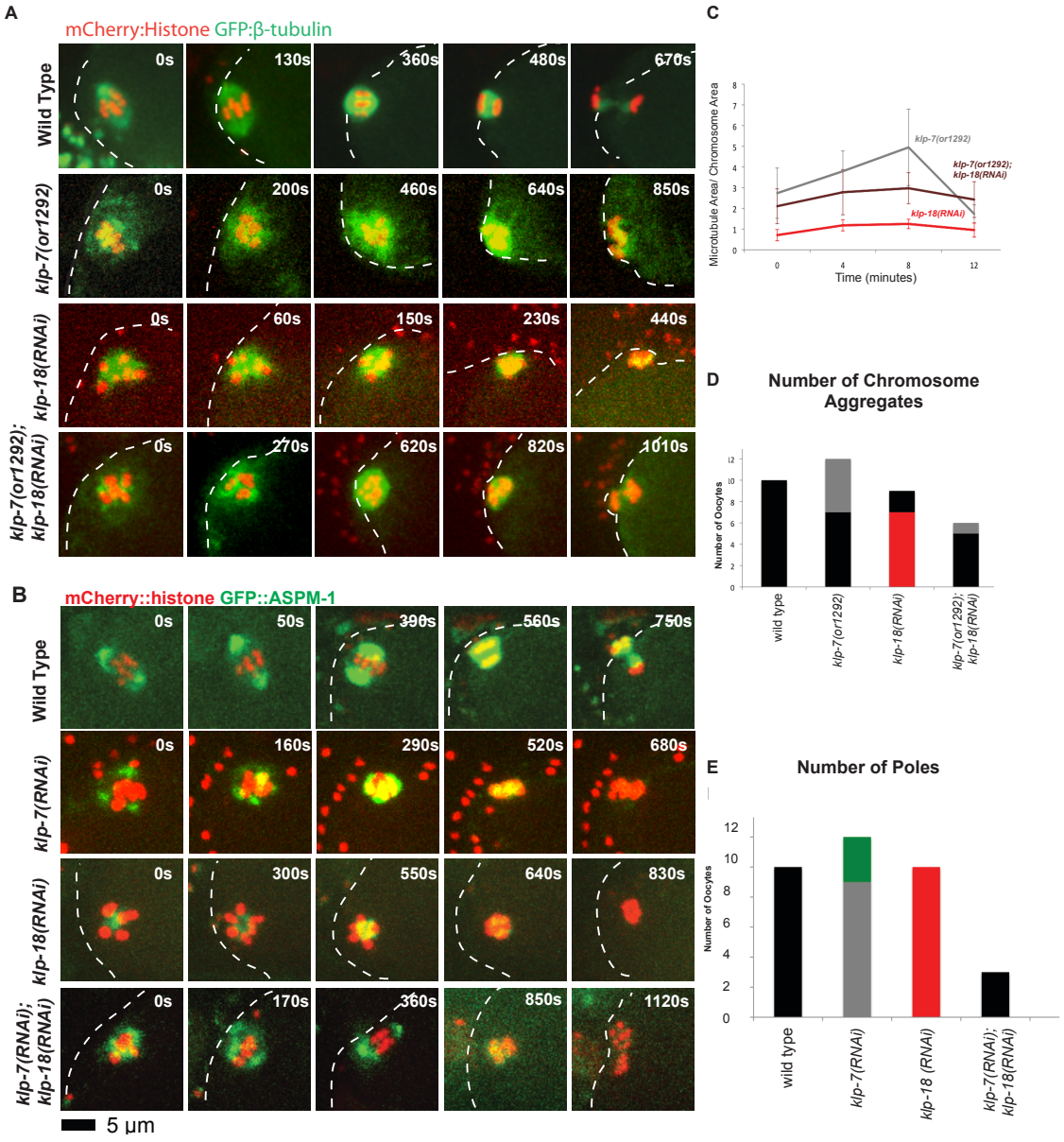


Figure 3.4. Reducing *klp-7* function restores oocyte meiotic spindle bipolarity in *klp-18(-)* mutants. A, B. Time-lapse spinning disc confocal images during Meiosis I of live wild-type and mutant embryos expressing either mCherry:Histone2B and GFP:β-tubulin to mark chromosomes and microtubules, respectively (A), or mCherry:Histone2B and GFP:ASPM-1 to mark chromsomes and spindle poles, respectively (B). A white dashed line marks the edge of the plasma membrane. Time post-ovulation in seconds shown in each frame. C. Microtubule area and chromosome area beginning at ovulation over time for twelve minutes during Meiosis I. Error bars depict the standard deviation among movies at each time point. D. Bar graphs show the number of segregating chromosomes masses detected during anaphase for the indicated genotypes. E. Bar graphs indicate the number of GFP:ASPM-1 foci observed for indicated genotypes.

We next asked whether loss of *klp-7* increased the accumulation of microtubules during Meiosis I in a *klp-18(-)* background, as we observed in *klp-7(-)* single mutants relative to wild-type oocytes. We measured the MT/C ratio over time in *klp-18(-)* oocytes during Meiosis I and found that it increased slowly to a maximum of 1.25. In *klp-7(-); klp-18(-)* double mutants, the MT/C ratio increased slowly to a maximum of 2.97, further supporting our conclusion that reducing *klp-7* function stabilizes microtubules during Meiosis I (**Figure 3.4C**). Furthermore, *klp-7(-);klp-18(-)* double mutants more frequently extruded two rather than one chromosome (**Figure 3.4D**), and prelimary data shows they also assemble two rather than one pole as indicated by GFP:ASPM-1 localization (**Figure 3.4D**). These findings support the idea that *klp-7* is needed to prevent accumulation of microtubules which is ultimately required to limit the number of poles assembled.

Microtubule attachment to kinetochores limits spindle pole assembly

Because an increased accumulation of microtubules correlated with the assembly of extra oocyte meiotic spindle poles in *klp-7(-)* single mutants and *klp-7(-); klp-18(-)* double mutants, we speculated that microtubule attachment to kinetochores might also influence spindle pole assembly and number during oocyte Meiosis I: perhaps more unattached microtubules might also promote the assembly of extra poles. To investigate this possibility, we examined spindle pole assembly in transgenic strains expressing GFP:ASPM-1 to mark spindle poles and mCherry:Histone2B to mark chromosomes, after reducing the function of the core kinetochore component *knl-1* **(Figure 3.5)**. To our surprise, in *knl-1(-)* single mutants we observed multiple foci of GFP:ASPM-1 early in Meiosis I, much as we observed in *klp-7(-)* single mutants. However, the foci in *knl-1(-)*

mutants always converged to form two poles that often appeared fragmented. The chromosomes in *knl-1(-)* single mutants also were disorganized although they ultimately segregated into two sometimes misshapen masses that moved toward opposite poles during anaphase, as reported previously (Dumont and Desai, 2012). Remarkably, in *klp-7(-); knl-1(-)* double mutants oocytes, we never observed three distinct GFP:ASPM-1 foci but rather numerous variably sized foci that sometimes formed a nearly continuous ring around a mass of centrally located chromosomes. Chromosome segregation was variable, sometimes appearing roughly normal and sometimes very abnormal. These results suggest that loss of microtubule attachment to kinetochores, in addition to increased microtubule number, can promote the assembly of extra meiotic spindle poles.

Discussion

We have identified two temperature-sensitive alleles of *klp-7,* the lone kinesin-13/MCAK family member in *C. elegans*. Consistent with the microtubule depolymerase activity documented for other kinesin-13 family members, we detected a substantial increase in the accumulation of microtubules during oocyte Meiosis I spindle assembly. Remarkably, chromosomes in *klp-7(-)* mutant oocytes usually segregate into three discrete masses due to the assembly of functional tripolar meiotic spindles. Our results suggest that both an excess numbers of microtubules, and a loss of microtubule attachment to kinetochores, can promote the assembly of extra oocyte meiotic spindle poles. We speculate that in the absence of centrosomes, extra and unattached microtubules result in the assembly of extra spindle poles mediated by non-limiting

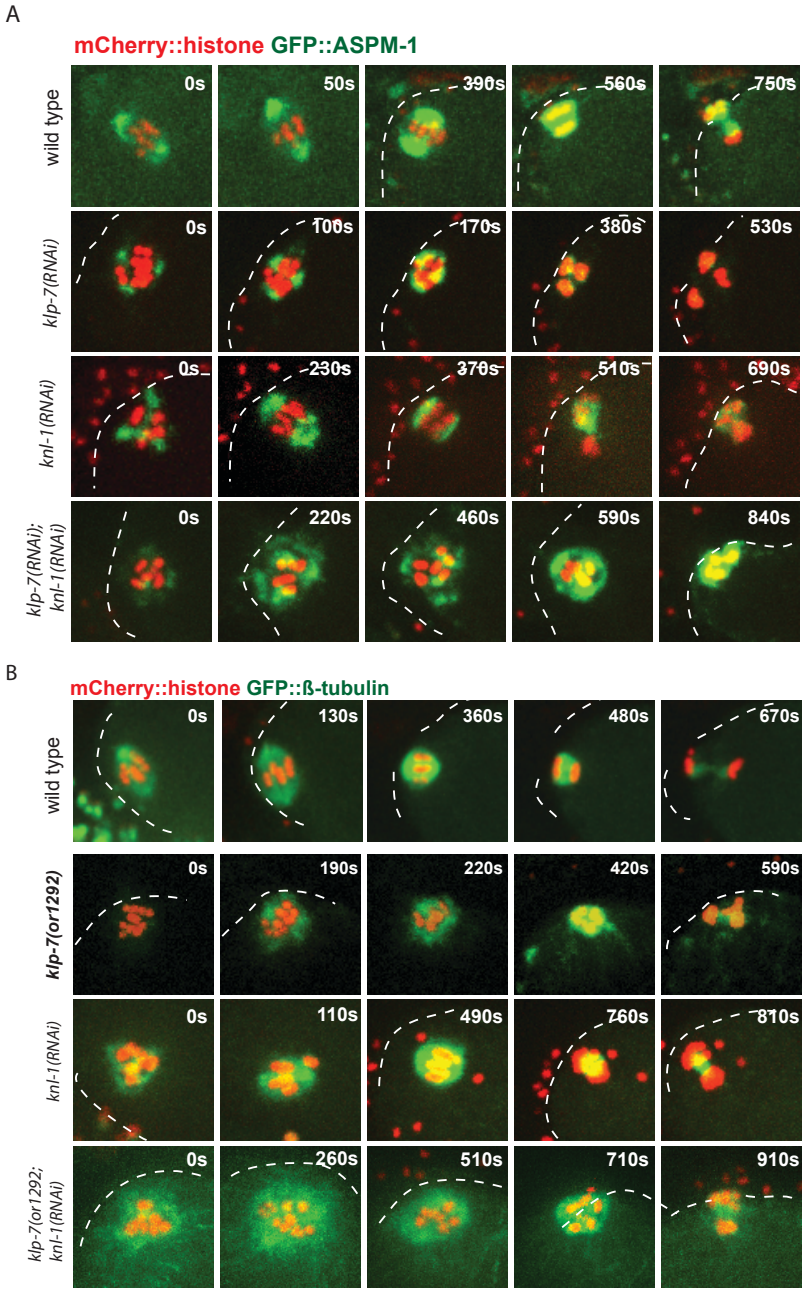


Figure 3.5. Disrupting kinetochore function results in transient extra spindle poles and exacerbates the extra spindle pole phenotype in *klp-7(-)* oocytes during Meiosis I. A, B. Time-lapse spinning disc confocal images during Meiosis I in live wild-type and mutant embryos expressing either mCherry:Histone2B and GFP:ASPM-1 to mark chromosomes and spindle poles, respectively (A), or mCherry:Histone2A and GFP:β-tubulin to mark chromosomes and microtubules, respectively (B). A white dashed line marks the edge of the plasma membrane. Time post-ovulation in seconds shown in each frame. C. Multiple GFP::ASPM-1 foci were observed in *klp-7(RNAi);knl-1(RNAi)* oocytes and were quantitated in the table. The maximum number of foci observed for a given oocyte was recorded.

acentrosomal pole organizing activities. Thus the assembly of bipolar oocyte meiotic spindles appears to depend critically on the regulation of microtubule number and on microtubule attachment to kinetochores.

klp-7 limits microtubule assembly during oocyte meiotic spindle assembly

The excess accumulation of microtubules during Meiosis I in *klp-7(-)* mutants is consistent with the known function of other kinesin-13/MCAK family members that promote the de-polymerization of microtubules from both their minus and plus ends. The *Drosophila* kinesin-13 family member KLP-10A is thought to act at oocyte meiotic spindle poles to depolymerize microtubules and maintain spindle stability, and in *Xenopus* extracts MCAK depletion results in abnormally long meiotic spindles (Mitchison et al., 2005; Ohi et al., 2007; Zou et al., 2008), although these studies did not address whether microtubule levels were affected. However, more recent studies of *klp-7* during early embryonic mitosis in C. elegans have shown that the loss of *klp-7* results in a two-fold increase in the number of microtubules that grow out from centrosomes, perhaps reflecting an increase in the stability of microtubules after their initial nucleation at gamma-tubulin ring complexes (Schlaitz et al., 2007). Our results provide the first evidence that a loss of kinesin-13/MCAK function can result in an increased accumulation of microtubules during the acentrosomal process of oocyte meiotic spindle assembly.

***C. elegans klp-7* limits spindle pole number during oocyte meiotic cell division**

Remarkably, the loss of *C. elegans klp-7* function during oocyte Meiosis I results not only in the excess accumulation of microtubules but also in the assembly of tripolar meiotic spindles. Our conclusion that extra oocyte meiotic spindle poles assemble in the absence of *klp-7* function follows from four observations: (i) In most *klp-7(-)* mutants, chromosomes segregate as the discrete masses; (ii) Two spindle pole markers, GFP:ASPM-1 and GFP:MEI-1, localize to three discrete foci in most *klp-7(-)* oocytes; (iii) homologous pairs of chromosomes often align along more than one of the axes defined by the three spindle poles in *klp-7(-)* oocytes; and (iv) reducing *klp-7* function can rescue oocyte meiotic spindle bipolarity in *klp-18(-)* mutants.

While the loss of kinesin 13/MCAK family members has not been reported previously to result in the assembly of functional tripolar oocyte meiotic spindles, these kinesins have been implicated in the regulation of spindle pole number during meiotic cell division in *Drosophila* and during mitosis in cultured human cells. Expression of a dominant negative allele of the kinesin-13/MCAK family member Klp10A in *Drosophila* oocytes resulted in shorter Meiosis I spindles with loosely organized poles, and in one example an apparent small third pole, based on immunofluorescence imaging of tubulin in a fixed sample. In HeLa cells, depletion of a kinetochore protein caused delays or losses of centrosome separation during mitosis, and the delays were rescued and bipolarity restored by MCAK co-depletion (Toso et al 2009). While we are not aware of any other studies that have documented tripolar segregation of chromosomes in the absence of kinesin 13 function, an influence on spindle pole number is not unique to *C.*

elegans oocyte meiosis and may therefore reflect a conserved property of this microtubule depolymerase family.

One potential mechanism to explain how *klp-7* limits additional pole assembly is illustrated in Figure 3.6. Previous findings have shown that kinesin-13/MCAKs destabilize microtubule-kinetochore attachments (Toso et al 2009). Presumably in *klp-7(-),* there is an increase in stable non-amphitelic attachments, which likely places opposing tension on the spindle poles, causing them to split open and result in additional poles. This model coincides with previous findings where they found that deleting MCAK rescued monopolar spindles (Toso et al 2009), and concluded that the opposing tension between sister chromatids is necessary for maintaining bipolarity in mitotic spindles. This model would therefore suggest that *klp-18(-)* monopolar spindles do not provide the needed opposing tension between sister chromatids, which is why deleting MCAK crudely restores the necessary tension for bipolarity.

Extra microtubules in *klp-7(-)* mutant oocytes correlate with the assembly of extra meiotic spindle poles

In both *klp-7(-)* single and *klp-7(-); klp-18(-)* double mutants, the assembly of spindle poles correlates with an increase in microtubule accumulation during oocyte meiotic spindle assembly. We also have shown that KLP-7 is present at both the spindle poles and in association with chromosomes, presumably at kinetochores, during Meiosis I. We do not know if the assembly of extra microtubules results from loss of *klp-7* function at the spindle poles or at kinetochores. However they might originate, we speculate that in the absence of centrosomes as microtubule organizing centers, extra

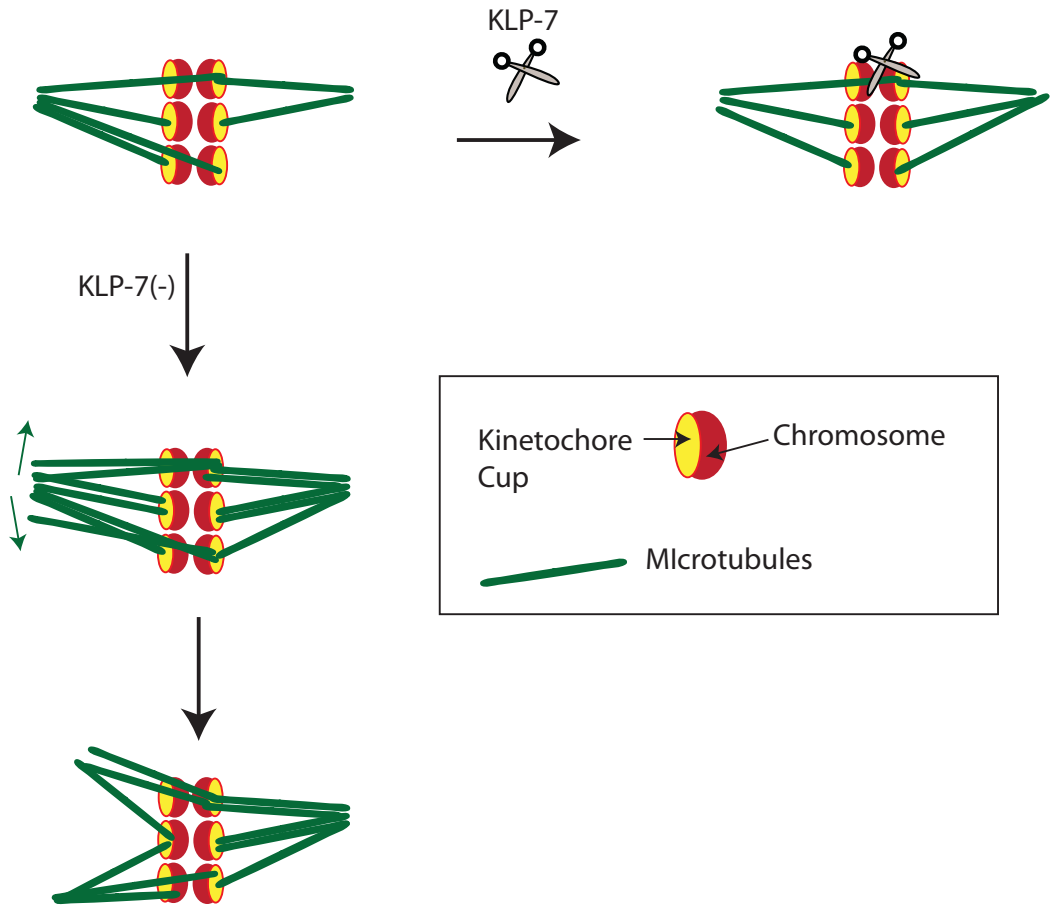


Figure 3.6. Model for Limiting Pole Assembly by *klp-7*. In this model, *klp-7* destabilizes incorrect microtubule (MT) attachments in wild type meiotic spindles. If *klp-7* is absent, incorrect MT attachments are accumulated and stabilized. Incorrect attachments puts unfavorable constraints on the poles, which drives opposing tension between microtubules at spindle poles causing them to split open.

microtubules during oocyte meiotic cell division promote the assembly of extra spindle poles, presumably mediated by non-limiting pole organizing activities that normally assemble bipolar spindles. Similar reasoning may also explain why kinetochore disruption, and hence a loss of microtubule attachment to chromosomes, also resulted in the more transient assembly of extra oocyte meiotic spindle poles, and in an enhanced assembly of extra spindle poles when kinetochores were disrupted in *klp-7(-)* mutants.

These mutant backgrounds may prove useful for further investigation of the requirements for acentrosomal pole assembly during oocyte meiotic cell division in future studies.

Temperature-sensitive mutations as genetic tools for the investigation of essential processes

The conditional *klp-7* alleles we identified are useful tools for investigating the requirements for this kinesin-13 family member. The deletion allele *klp-7(tm2143)* results in an in-frame 261 amino acid deletion that only partially reduces function with little if any loss of meiotic spindle function (Table 1, Materials and methods). Furthermore, genome-wide RNAi screens and previous studies that used RNAi to reduce *klp-7* function revealed requirements during early embryonic mitotic cell division but did not detect requirements during oocyte meiotic cell division. While we have found that RNAi knockdown can be used to investigate *klp-7* requirements during oocyte meiotic cell division, mutant alleles are particularly valuable for analyzing mutant phenotypes that result from reducing the function of multiple genes simultaneously, with RNAi becoming less effective as more genes are targeted. Moreover, RNAi varies in effectiveness depending on the targeted developmental stages and tissues. Thus the strong loss-of-function and conditional alleles we have identified should facilitate studies of *klp-7* throughout embryonic, larval and adult life.

CHAPTER IV

FUTURE DIRECTIONS

We are interested in continuing to characterize and define the genetic networks behind the genes discussed in both Chapter II and III using similar techniques. We would like to make more transgenic fluorescent strains in different colors of these genes to help us gain a better understanding of these genetic pathways that govern spindle assembly in the oocyte. In particular we are interested in understanding the genetic relationship between *mei-1* and *klp-7*, whether *klp-7* is regulated by the Aurora or Polo Kinases as MCAKs are in other organisms, and lastly determine whether the quantity of microtubules used in assembling the spindle affects the number of poles formed.

Genetic relationship between *mei-1* and *klp-7*

We are interested in examining the relationship between *mei-1* and *klp-7* to answer the following questions: is *mei-1* required for the assembly of multiple poles in *klp-7(-)*? And will reducing *mei-1* function increase or decrease microtubule amount in *klp-7(-)*? First, we want to examine whether and how *mei-1* functions to assemble multipolar spindles by looking at both its severing activity and recruitment of ASPM-1 to the spindle. As previously discussed in Chapter II, *mei-1* uses both microtubule- severing in conjunction with ASPM-1 for pole assembly, and we are curious to see if these mechanisms are still in place to assemble multi-polar spindles in *klp-7(-)* oocyte (Connolly et al., 2014). Using the same strains and mechanism discussed in Chapter II, we plan to examine pole assembly in *mei-1(ct46ct103);klp-7(-)* and *aspm-1(-);klp-7(-)* oocytes. Second, we are also interested to learn how *mei-1(-)* may impact microtubule

amount in *klp-7(-)* oocytes. We predict two potential outcomes. First, *mei-1(-)* may increase the amount of microtubules at the spindle. As we observed in *mei-1(ct46ct103)* mutants, meiotic spindles are abnormally long. Potentially knocking down *mei-1* in *klp-7(-)* may lead to an increased amount of long microtubules. Second, while *mei-1(ct46ct103)* has extra long spindles *mei-1(-)* has been shown to have fewer microtubules than wild type (Srayko et al., 2006). As discussion in Chapter I, *mei-1* may generate microtubules by severing them to provide more fragments for microtubules to nucleate from. Therefore reducing *mei-1* function in *klp-7(-)* oocytes may actually diminish the quantity of microtubules observed in *klp-7(-)* oocytes. We are therefore curious to look at both *mei-1(-);klp-7(-)* and *mei-1(ct46ct103); klp-7(-)* oocytes to examine microtubule quantity and morphology.

Regulation of KLP-7 by Aurora and Polo Kinases

The aurora and polo kinases are known to regulate the various roles kinesin-13/MCAK plays during mitotic spindle assembly by phosphorylating various positions along the MCAK protein. We are eager to learn whether the *C. elegans* homologs also mediate KLP-7 activity during oocyte meiotic spindle assembly. In particular, we plan to determine whether *air-1, air2* or *plk-1* are required for MT depolymerization by KLP-7 (Ems-McClung and Walczak, 2010). The *C. elegans* homolog of Aurora A *air-1,* has not been shown to play a role in oocyte meiotic spindle assembly (Bishop et al., 2004). However, *air-1* does appear to nucleates microtubules during mitotic spindle assembly, independent of gamma tubulin (Toya et al., 2011). We are curious to determine whether *air-1* has a similar role in oocytes that can be observed in the context of a *klp-7(-)*

background. Secondly, we want to determine whether the *C. elegans* homolog of Aurora B *air-2* is necessary for MT depolymerization by KLP-7. Unlike *air-1*, *air-2* has been previously identified as an essential player in oocyte meiotic spindle assembly, required for chromosome segregation and polar body extrusion (Bischoff and Plowman, 1999; Bishop et al., 2005). We are curious to determine whether *air-2(-)* impacts MT depolymerization and spindle morphology in *klp-7(-)* oocytes. Lastly, we plan to examine the *C. elegans* homology of the cell cycle regulatory protein Polo-like kinase *plk-1,* which when knocked down in nematodes prevents oocyte nuclear envelope breakdown, unsuccessful chromosome segregation, and a failure to extrude polar bodies (Chase et al., 2000). We are interested to learn whether part of the function of *plk-1* is to regulate MT depolymerizing activity of KLP-7.

The relationship between extra microtubules and extraneous spindle poles

Lastly, we would like to delve deeper into the question raised in Chapter III: does extra microtubules lead to extra spindle poles. We want to see whether preventing microtubule nucleation would inhibit the assembly of extraneous poles in *klp-7(-)* oocytes. We want to see if we can inhibit microtubule nucleation in *klp-7(-)* oocytes by reducing gamma tubulin function, which is known to nucleate microtubules at the centrosomes during mitotic spindle assembly (Bobinnec et al., 2000). Gamma tubulin localizes to the meiotic spindle (data not shown), but a role for gamma tubulin has not been established for *C. elegans* meiotic spindle assembly. We are curious to see whether knocking down gamma tubulin *tbg-1* will decrease the number of microtubules at the meiotic spindle, and if so examine whether that will affect the number of spindle poles

formed in *tbg-1(-); klp-7(-)* double mutant. These results will help us establish whether the meiotic spindle is required to incorporate a precise amount of microtubules into the spindle to maintain bipolarity.

Final remarks

Lastly, we plan to continue screening for and identifying new temperature-sensitive embryonic lethal alleles for genes involved in meiotic spindle assembly. By characterizing genes required for this process, we hope to elaborate fully on the genetic mechanisms in place behind acentrosomal spindle assembly. Hopefully our findings can be used to understand in a broader context the genetic networks that are required for oocyte meiosis in other organisms including female humans.

APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER II: MEI-1-MEDIATED MECHANISM OF POLE ASSEMBLY

Methods

C. elegans strains

N2 Bristol was used as the wild type strain with standard nematode protocols used as described in (Brenner, 1974). The TS alleles were isolated in a screen for TS-embryonic lethal mutant using the methods described in (Kemphues et al., 1988; O'Connell et al., 1998; Encalada et al., 2000). TS mutations were isolated from a *lin-2(e1309)* background and outcrossed at least three times with the N2 strain as described (Encalada et al., 2000). TS strains were maintained at the permissive temperature of 15^{o}C, and L4s were shifted to the restrictive temperature 26^{o}C for 2-5 hours before cutting open young adult worms to isolate mutant embryos for phenotypic analysis, bypassing larval lethality and sterility phenotypes that result from earlier upshifts (Table 2). TS mutations and deletion alleles are listed by chromosomes: *mei-1(or646*ts*)* I; *mei-1(or1178*ts*)* I; *mei-1(ct46ct103)* I; *aspm-1(or645*ts) I; *aspm-1(ok1208)* I; *mei-2(sb39*ts) I; *klp-18(or447*ts) IV; *klp-18(ok2519)* IV; *bmk-1(or627*ts) IV; *bmk-1(tm696)* V; *bmk-1(ok391)* V. Transgenic GFP::β-tubulin and mCherry::Histone2B strains were derived from AZ244 (β-tubulin::GFP) and OD56 (mCherry::Histone2B). A GFP::ASPM-1 construct was made by recombineering using the fosmid WRM0624aG02 as previously described in (O'Rourke et al., 2007). Transgenic strains were generated by microparticle bombardment as previously described (Praitis et al., 2001). The GFP::ASPM-1 fusion was functional in that it rescued embryonic lethality when crossed into the *aspm-*

*1(or645*ts) mutant background (data not shown). Transgenic strains were crossed into a *him-5(e1490)* background, and males from the *him-5* strains were used to introduce the transgenes into TS mutant strains.

Genetic Analysis

Viability counts of embryos (% hatching) were determined by singling at least 7 L4s onto individual plates, growing them at the permissive or restrictive temperature until eggs were laid, and then removing the mother and counting the eggs. We allowed the embryos to develop for 18-24 hours and then counted the number of unhatched embryos. With all four alleles, we found mutant/+ worms produced roughly one-quarter homozygous self-progeny (data not shown), indicating that each recessive mutant phenotype is caused by mutation(s) at single or tightly linked loci. Complementation tests were performed by first generating *him-8* or *him-5* strains that were homozygous for a TS mutant allele; males from these strains were then crossed into heterozygous strains carrying the deletion alleles. Embryonic viability in the broods of F1s were scored at the restrictive temperature (described above). Recessive gain-of-function test of *bmk-1(or627*ts) allele (using RNAi to knock down the mutant gene product) was performed after using hypochlorite to isolate embryos from homozygous mutant worms and leaving them in M9 overnight at 15^{o}C. The synchronized hatched and starved homozygous mutant L1s were then put onto agar plates seeded with *E. coli* expressing double stranded *bmk-1* RNA (Fraser et al., 2000) (see below) and matured to adulthood at 26^{o}C. Young adults were then removed, and the eggs laid were scored for percent hatching as described above.

Positional Cloning of TS Mutant Loci

Using visible markers, we found that *or645*ts resides within a 1.4 cM interval between *unc-29* and *lin-11* on Linkage Group 1 (data not shown). Data from genome-wide RNAi knockdown screens (Wormbase.org) indicate that *aspm-1* is the only gene in this region that results in the production of one-cell zygotes with multiple maternal pronuclei, as observed in *or645*ts mutants. Furthermore, *or645*ts failed to complement the *aspm-1* deletion allele *ok1208* (data not shown). Homozygous *or645*ts genomic DNA was purified using a Qiagen DNeasy Blood and Tissue kit. We amplified overlapping DNA fragments that span the *aspm-1* locus by PCR, and purified the product using QIAquick PCR Purification kit. Overlapping DNA fragments were sequenced by Sequetech.

To identify the causal mutation in *or447*ts mutants, we again used visible markers and mapped the mutation to a 0.11 cM interval between *dpy-20* and *bli-6* on Linkage Group IV). The only *C. elegans* gene within this interval that, when reduced in function using RNAi, results in oocyte meiotic cell division defects is the kinesin 12 family member *klp-18*. Moreover, in genetic crosses *or447*ts failed to complement a non-conditional and likely null *klp-18* deletion allele, *ok2519* (data not shown), consistent with *or447*ts being a *klp-18* allele. From *or447*ts genomic DNA, we PCR amplified and sequenced overlapping DNA fragments that span the *klp-18* locus.

Using a genome-wide SNP mapping and whole genome sequencing approach (Doitsidou et al., 2010), we mapped *or1178*ts to an ~9 Mb region on Linkage Group I. The *mei-1* gene was the most promising candidate in this region, based on both phenotypic similarity to *or1178*ts as reported in earlier studies of *mei-1* mutants (Srayko

et al., 2000), and the results from multiple genome-wide RNAi screens (WormBase.org). *or1178*ts failed to complement the previously identified *mei-1(or646*ts*)* allele, while complementing both *aspm-1(or645*ts*)* and *mei-2(sb39*ts*)* mutants, consistent with *or1178*ts being a *mei-1* allele. From *or1178*ts genomic DNA, we PCR amplified and sequenced overlapping fragments spanning the *mei-1* locus.

We used PCR-targeted SNP mapping to place *or627*ts within a small interval from 6.24 and 6.54 Mb on LG V (data not shown). There were no annotated genes in this region that when knocked down by RNAi resulted in an *or627*ts-like mutant phenotype. We therefore used a genome interval pull-down method, followed by Illumina-based DNA sequencing, to identify any mutations within this interval that might be responsible for the embryonic lethality (O'Rourke et al., 2011). The DNA sequence data revealed a single mis-sense mutation in one annotated gene within this interval (O'Rourke et al., 2011) (Figure S1 B). In vertebrates, this kinesin is required for bipolar mitotic spindle assembly, but surprisingly analysis of RNAi knockdown defects, and results from analysis of two *bmk*-1 deletion alleles, indicate that *C. elegans bmk-1/*kinesin 5 is not essential, is not required for meiotic spindle assembly, and has only a minor role in mitotic spindle assembly dynamics (Bishop et al., 2005; Saunders et al., 2007). We therefore asked whether *or627*ts might be a recessive gain-of-function mutation. Consistent with this possibility, *or627*ts partially failed to complement the *bmk-1* deletions alleles *tm969* and *ok391* (Table 1). More conclusively, when we used RNAi to knock down mutant *bmk-1* function in homozygous *or627*ts worms, embryonic viability at the restrictive temperature was strongly rescued (Table 1).

RNA Interference

RNAi to reduce the function of *mei-1*, *klp-18* or *aspm-1* was performed by three methods: feeding, injection and soaking (Fire et al., 1998). RNAi by feeding was performed by placing L1 larvae synchronized by hypochlorite as described previously (Kamath et al., 2001). For co-depletions, we used the same process, but seeded plates with an equal mixture of the double-strand RNA-expressing *E. coli* strains. We maintained the affected worms at room temperature, and examined their phenotypes in zygotes within whole mounted young adults. TS strains were treated the same way, but maintained at 15^{o}C and shifted to 26^{o}C as L4 larvae. RNAi experiments by injection were done as described previously (Zipperlen et al., 2001) 24 hours before imaging. To create the double stranded RNA, a PCR product was generated from the *aspm-1* and *klp-18* clones available from the Ahringer library (Fraser et al., 2000). RNAi was produced by *in vitro* transcription using Promega RiboMAX kits, and the sample was diluted to 0.8 μg/μL. Finally RNAi by soaking was performed by purifying dsRNA and diluting in a soaking buffer according to Wormbook http://www.wormbook.org. L4 worms were soaked for 24 hours at room temperature and imaged as young adults.

Microscopy

Live imaging of fluorescent fusion proteins during meiosis was accomplished by mounting embryos on 8% agar pads, with 1μL each of 1μm polystyrene beads and M9 on microscope slides covered with a coverslip. Zygotes were analyzed on a Leica DMI 4000B microscope fitted with a Leica 63X/ 1.40-0.60 HCX Plan Apo oil objective lens in

a room maintained at 25^{o}C. Time-lapse videos were obtained with a Hamamatsu EM-CCD digital camera using Volocity software (Perkin Elmer Inc.). Six stacks of 1.5 μm thick were used to collect images every 10 seconds. After recording, the videos were cropped and adjusted for contrast in Image J (http://rsb.info.nih.gov/ij/). Measurements of chromosome compaction were taken using the Image J polygon selection tool to trace around the two-dimensional perimeter of chromosomes to obtain an area. The area of pixels was then multiplied by 0.214 μm^2/pixel, a value specific to our Leica DMI 4000B.

Supplemental Figures:

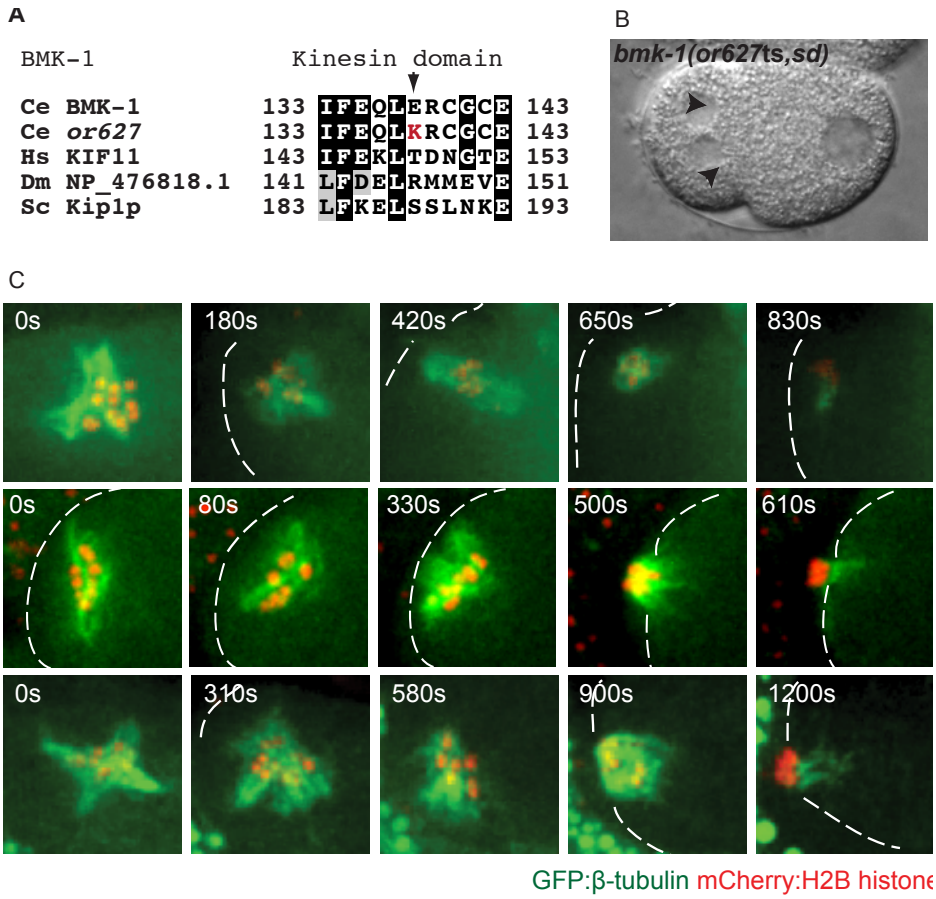


Figure A.1. TS gain-of-function mutant *bmk-1(or627*ts*)* has a defective meiotic spindle. (A) Partial sequence alignments of orthologs from *C. elegans Homo sapiens (Hs), Drosophila melanogaster (Dm),* and *Saccharomyces cerevisiae (Sc)* with the wild-type and mutant *Caenorhabditis elegans (Ce)* BMK-1. Arrowheads indicate altered residues, with wild-type amino acids in black and mutant amino acids in red. **(**B) Nomarski image of one-cell stage mutant embryos. Embryo is positioned with the anterior (maternal) and posterior (paternal) pronuclei to the left and right. Note the presence of extra maternal pronuclei in *bmk-1(or627)* (arrowheads). (C) One cell Time-lapse spinning disc confocal images from immobilized worms were recorded during Meiosis I in wild-type and mutant zygotes expressing mCherry::Histone2B and GFP::β-tubulin to mark chromosomes and microtubules, respectively, from ovulation to polar body extrusion. Anterior is to the left, times indicated are relative to ovulation, and a white dashed line marks the edge of the zygote plasma membrane. In this and subsequent figures, each image shown is a projection of 6 consecutive frames taken at 1.5 µm intervals in a z-stack for each time point. See the text for details.

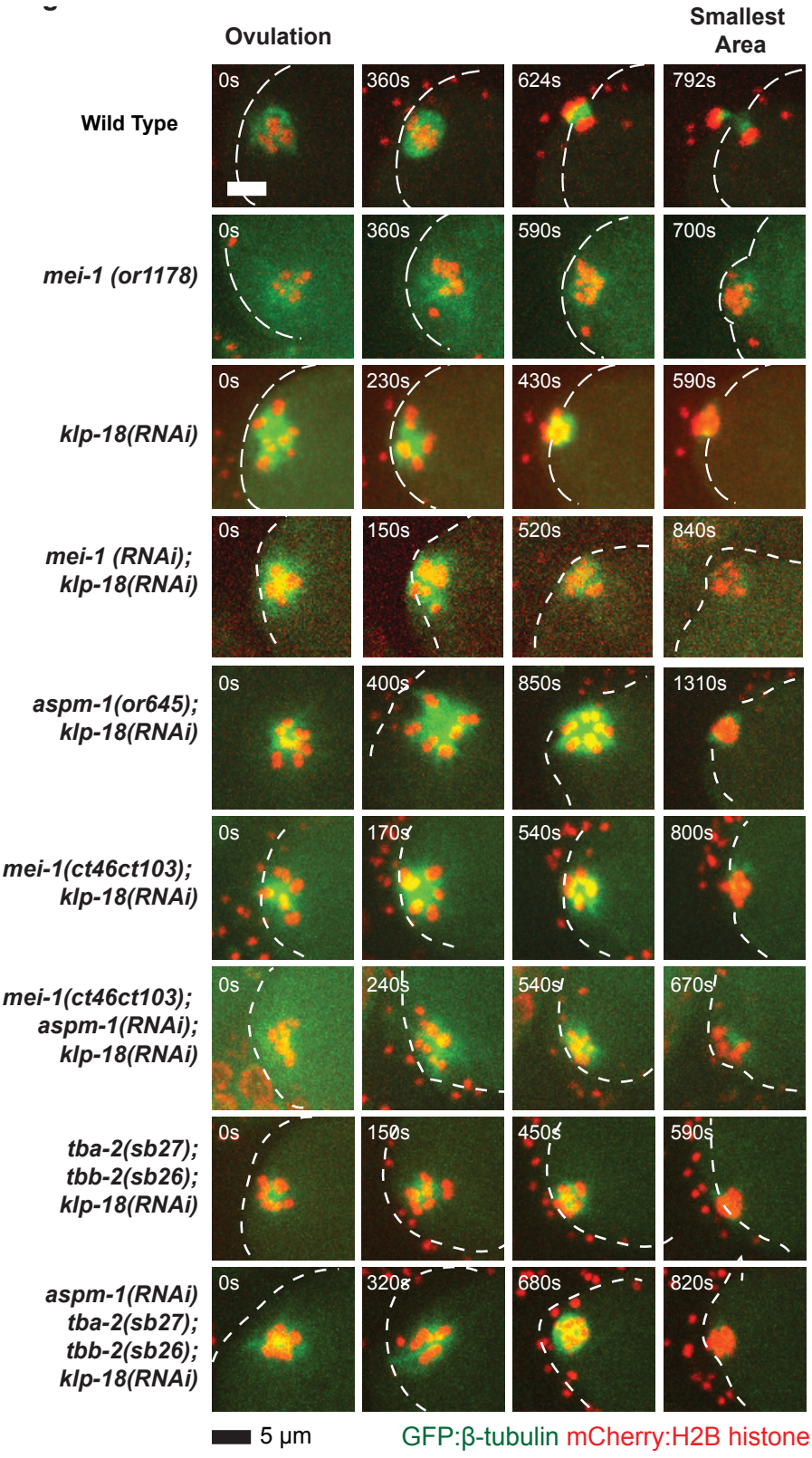

Ovulation
Smallest Area
Wild Type
0s
360s
624s
792s
mei-1 (or1178)
0s
360s
590s
700s
klp-18(RNAi)
0s
230s
430s
590s
mei-1 (RNAi); klp-18(RNAi)
0s
150s
520s
840s
aspm-1(or645); klp-18(RNAi)
0s
400s
850s
1310s
mei-1(ct46ct103); klp-18(RNAi)
0s
170s
540s
800s
mei-1(ct46ct103); aspm-1(RNAi); klp-18(RNAi)
0s
240s
540s
670s
tba-2(sb27); tbb-2(sb26); klp-18(RNAi)
0s
150s
450s
590s
aspm-1(RNAi) tba-2(sb27); tbb-2(sb26); klp-18(RNAi)
0s
320s
680s
820s
5 µm
GFP:β-tubulin mCherry:H2B histone

Figure A.2 (previous page). Assembly of monopolar oocyte meiotic spindles in *klp-18* mutant requires both the microtubule severing activity of MEI-1 and ASPM-1. Spinning disc confocal images were recorded over time during Meiosis I in live mutant embryos expressing mCherry:Histone2B and GFP:β-tubulin translational fusions to mark chromosomes and microtubules, respectively. Indicated time points begin at ovulation. A white dashed line marks the edge of the plasma membrane. The 5 μm bar is drawn to scale.

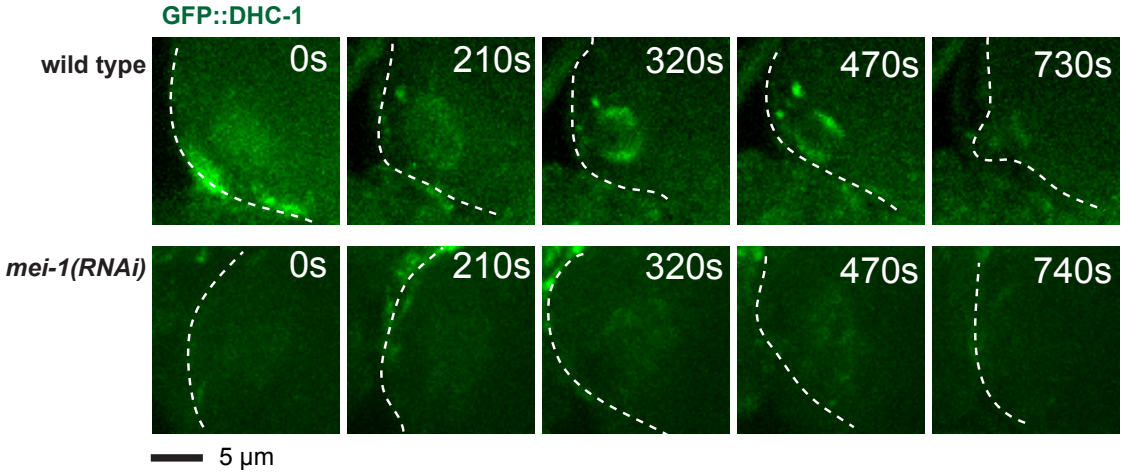


Figure A.3. *mei-1* is required to recruit Dynein to the spindle poles. Spinning disc confocal images were recorded over time during Meiosis I in live mutant embryos expressing GFP:DHC-1 translational fusion to mark dynein (O'Rourke et al., 2007). Indicated time points begin at ovulation. A white dashed line marks the edge of the plasma membrane. The 5 μm bar is drawn to scale.

APPENDIX B

SUPPORTING INFORMATION FOR CHAPTER III:

KLP-7: MECHANISMS OF LIMITING POLE ASSEMBLY

Materials and Methods

C. elegans strains

N2 Bristol strain was used as the wild type strain with standard nematode protocols used as described in (Brenner, 1974). The TS alleles were isolated in a screen for TS-embryonic lethal mutant using the methods described in (Kemphues et al., 1988; O'Connell et al., 1998; Encalada et al., 2000). TS mutations were isolated from a *lin-2(e1309)* background and outcrossed into the N2 strain as described (Encalada et al., 2000). TS strains were maintained at the permissive temperature of 15^{o}C, and L4s were shifted to the restrictive temperature 26^{o}C for 2-5 hours before cutting open young adult worms to isolate mutant embryos for phenotypic analysis, bypassing larval lethality and sterility phenotypes that result from earlier upshifts. TS mutations and deletion alleles are listed by chromosomes: *mei-1(or1178*ts*)* I; *klp-7(or1092*ts) III; *klp-7(or1292*ts) III; *klp-7(tm2143*) III. Transgenic GFP::β-tubulin and mCherry::Histone2B strains were derived from AZ244 (β-tubulin::GFP) and OD56 (mCherry::Histone2B). GFP::ASPM-1 (Connolly et. al, 2013)**.** GFP::KLP-7 TH108 was a gift from the Hyman lab. Transgenic strains were crossed into a *him-5* background, and males from the *him-5* strains were used to cross the transgenes into TS mutant strains.

Mapping

We determined that both *or1092* and *or1292* were located between ~10 and 13 Mb region on Linkage Group III using a genome-wide SNP mapping and whole genome sequencing approach (Doitsidou et al., 2010) (data not shown). We next used visible-marker mapping and found that each mutation resides within a 6.31 cM interval between *unc-69* and *dpy-18* (data not shown) with *or1092*ts mapping approximately to 4.7 cM and *or1292*ts mapping approximately to 4.2 cM. Data from genome-wide RNAi knockdown screens indicated *klp-7* as a potential candidate since embryos were reported as having large polar bodies, however there was no reference to abnormal number of maternal pronuclei (Sonnichsen et al., 2005). We identified the mutations using the whole genome sequencing data, and discovered *or1092* to be an Isoleucine to a Phenylalanine substitution at position 298, and *or1292* to be a Glycine to an Aspartic acid at position 547.

Genetic Analysis

Viability counts of embryos (% hatching) were determined by singling at least 7 L4s onto individual plates, growing them at the permissive or restrictive temperature until broods were produced, and then removing the mother and counting the eggs (see Table 1). We allowed the embryos to develop for 18-24 hours and then counted the number of unhatched embryos that remained. Embryonic viability in the broods of F1s were scored at the restrictive temperature (described above). A test for dominance was also performed by examining F1 heterozygous TS mutant (TS mutant/+) as described above (see Table 1)

RNA Interference

RNAi to reduce the function of *klp-7* was performed by placing L1 larvae synchronized by hypochlorite hatch-off onto 60-mm NGM agar plates with 100 mg/ml ampicillin and 1 mM isopropyl-beta-D-thiogalactopyranoside, and seeded with the double stranded RNA-expressing HT115 E. coli (Fire et al., 1998) (Kamath et al., 2001). For co-depletions, we used the same process, but seeded plates with an equal mixture of the double-strand RNA-expressing *E.coli* strains. We maintained the affected worms at room temperature, and examined their phenotypes in zygotes within whole mounted young adults. TS strains were treated the same way, but maintained at 15^{o}C and shifted to 26^{o}C as L4 larvae.

Microscopy

Live imaging of fluorescent fusion proteins during meiosis was accomplished by mounting embryos on 6% agar pads, with 1μL each of 1μm polystyrene beads and M9 on microscope slides covered with a coverslip. Zygotes were analyzed on a Leica DMI 4000B microscope fitted with a Leica 63X/ 1.40-0.60 HCX Plan Apo oil objective lens in a room maintained at 25^{o}C. Time-lapse videos were obtained with a Hamamatsu EM-CCD digital camera using Volocity software (Perkin Elmer Inc.). Six stacks of 1.5 μm thick were used to collect images every 10 seconds. After recording, the videos were cropped or adjusted for contrast in ImageJ (http://rsb.info.nih.gov/ij/). Measurements of microtubule and chromosome area were taken using the Image J polygon selection tool to trace around the two-dimensional perimeter of either microtubules or chromosomes to obtain an area. The microtubule perimeter was determined by using an Image J program

to generate a threshold. Using the polygon tool we selected for only the oocyte, drawing around the plasma membrane, and set the threshold to “Max Entropy.” The program selects against the background of the oocyte and leaves the pixels colored that should be measured.

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