# ON THE CONTROL OF MICROTUBULE REORGANIZATION IN CAENORHABDITIS ELEGANS OOCYTES PRIOR TO FERTILIZATION

By

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To my loving family;
my amazing mom,
my three sisters,
and my future husband Brandon

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CHAPTER I

# INTRODUCTION

Reproduction is essential for the survival of a species. Sexual reproduction requires fertilization, the union of two parental germ cells, a sperm and egg, to form a complete genetic organism. In order for successful reproduction to take place, a multitude of complex and precise signaling processes are required both before and after fertilization. Prior to fertilization, both sex cells must undergo a process called, meiosis, to ensure that they are fully competent to generate a new being. Studying the mechanisms of meiosis has been an interesting and important field for scientists because of the drastic effect that meiotic errors can have on a species. Through understanding how meiosis occurs, treatments can be developed to prevent the incidence of miscarriage and birth defects, such as Trisomy 21. I have always been extremely fascinated by the complex process of meiosis and how an individual can arise from two cells despite the numerous errors that may be encountered. My thesis work has focused on the effect of hormonal signaling on the microtubule cytoskeleton during meiosis in the oocyte and how this signaling may be important for the proper segregation of chromosomes and preparing the oocyte for fertilization.

## Microtubule Cytoskeleton

The microtubule cytoskeleton plays many critical roles in the cell. It is involved in cell shape, cell movement, positioning membrane-enclosed organelles, RNA

localization, protein trafficking, cell polarity, cell division, and chromosome separation (reviewed by Kirschner, 1987; Avila, 1992; Lasko, 1995; Apodaca, 2001; Kline-Smith and Walczak, 2004). Even though microtubules are mainly localized in the cytoplasm of the cell, they also are found in motile projections, such as cilia and flagella, on the cell surface (reviewed by Huitorel, 1988). The microtubule cytoskeleton is a highly dynamic structure that is constantly changing in stability and growth. Because of the microtubule's dynamic nature and its diverse functions in the cell, complex regulation of its assembly and behavior is required.

***Microtubule Structure and Dynamic Nature***

Microtubules are long, hollow cylinders composed of alternating protein subunits of tubulin. There are two types of subunits, α- and β-tubulin, that assemble as dimers in a head-to-tail fashion to form a microtubule (Fig. 1). The microtubule has an intrinsically polar composition because, when assembled, the α-tubulin subunit is exposed at one end and the β-tubulin is exposed at the other. These two ends are called the minus, or slow growing end, and the plus, or fast growing end, respectively. The α-tubulin subunit is tightly bound to a molecule of GTP and cannot be hydrolyzed or exchanged. In contrast, the β-tubulin subunit can freely exchange GTP with GDP, a process that can greatly affect microtubule dynamics (reviewed by Caplow, 1992; Desai and Mitchison, 1997; Wade and Hyman, 1997).

At the plus end, when β-tubulin remains bound to GTP, a GTP cap is formed and promotes microtubule growth (Fig. 1). Once GTP is hydrolyzed to GDP, faster than the addition of tubulin subunits, the plus end loses the GTP cap and the microtubule begins to

shrink. This process is called depolymerization or "catastrophe" (Fig. 1). Catastrophe can be "rescued", to promote microtubule growth or polymerization, by adding additional GTP bound tubulin subunits to the plus end to form a new GTP cap (Fig. 1). This process of growing and shrinking is an intrinsic property of microtubules and is known as dynamic instability (reviewed by Mitchison and Kirschner, 1984; Carlier, 1992; Cassimeris, 1993; Desai and Mitchison, 1997; Wade and Hyman, 1997). Dynamic instability was initially observed by Mitchison and Kirschner using an *in vitro* polymerization assay that allowed them to visualize and measure the growth of individual fluorescent microtubules (Mitchison and Kirschner, 1984). Another behavior intrinsic to microtubules is treadmilling, which occurs when tubulin subunits are added to the plus end while simultaneously losing tubulin subunits from the minus end (reviewed by Waterman-Storer and Salmon, 1997). In animal cells, dynamic instability is more likely to occur than treadmilling because microtubules typically have one end, the minus end, attached to a microtubule organizing center (MTOC), or centrosome (reviewed by Waterman-Storer and Salmon, 1997).

The centrosome is composed of a protein complex called the γ-tubulin ring complex, where γ-tubulin is a type of tubulin that is involved in the nucleation of microtubule growth (Fig. 1), and two perpendicular centrioles, which are responsible for organizing the pericentriolar material of the centrosome (reviewed by Schatten, 1994; Pereira and Schiebel, 1997; Schnackenberg and Palazzo, 1999; Schiebel, 2000). Microtubules are nucleated from the centrosome, and the plus ends grow in an astral-like conformation towards the cell periphery (Fig. 1) (Rieder, 1990). The centrosome is not only involved in nucleating microtubules, but is responsible for organizing the

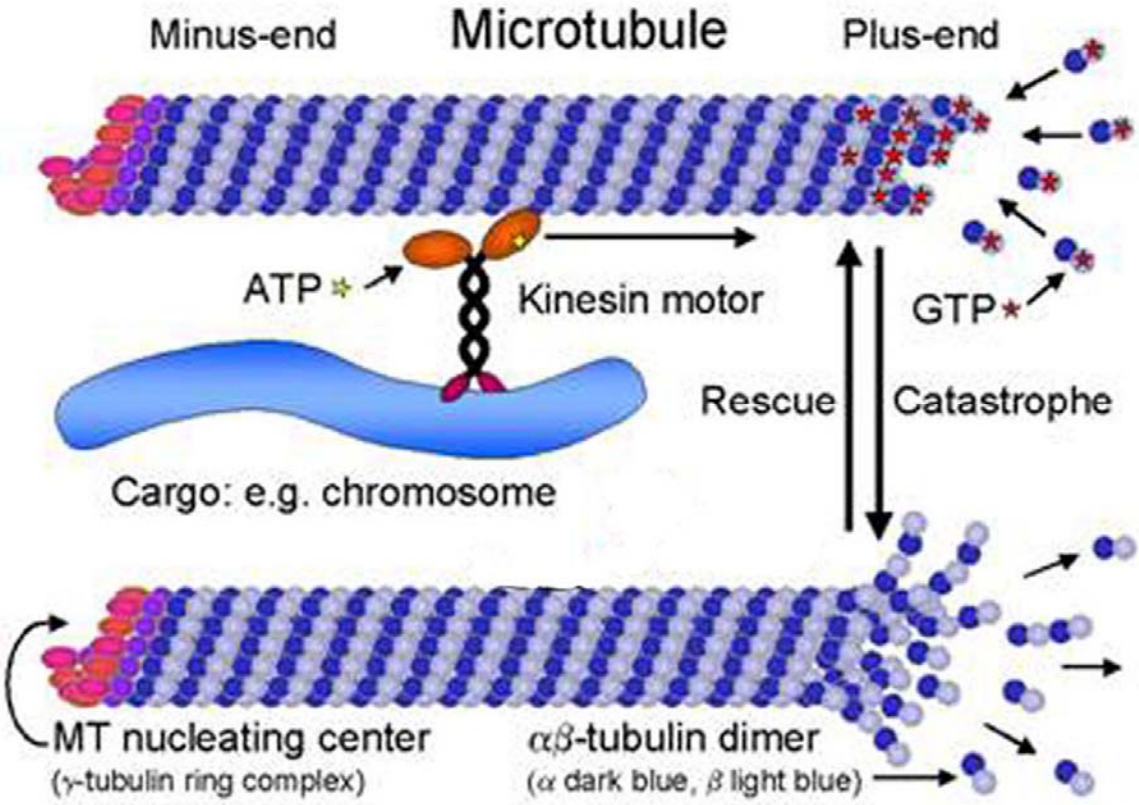



**Figure. 1. The dynamic nature of the microtubule.** A microtubule is a dynamic structure composed of α- and β-tubulin heterodimers with a slow growing minus-end and a fast growing plus-end. The minus-end is typically attached to a centrosome where it is nucleated by the γ-tubulin ring complex. The ability of the β-tubulin subunit to exchange bound GTP with GDP can affect the stability and dynamic nature of the microtubule. When the plus-end is capped by GTP, microtubule growth is promoted. When this GTP cap is lost, the microtubule undergoes catastrophe and depolymerizes. By restoring the GTP cap, growth can be rescued. One function of the microtubule is to interact with proteins such as the motor, kinesin, to help transport different cargoes (e.g. chromosomes) within the cell. Kinesin is a plus-end directed motor and uses ATP hydrolysis to fuel its movement. Microtubule interaction with kinesins may also affect microtubule dynamics, such as promoting depolymerization.

microtubule array and coordinating cell division. Before the cell enters mitosis, the division process in a eukaryotic cell, the centrosome duplicates (reviewed by Hinchcliffe and Sluder, 2001). After NEBD, the daughter centrosomes separate to form the two poles of the spindle and astral microtubules assemble to assist in separating chromosomes during mitosis (Rieder, 1990; Schatten, 1994). Despite the importance of the centrosome, it is not sufficient to regulate the dynamics of growing microtubule plus ends. Therefore, other factors are needed to regulate the microtubule cytoskeleton.

***Regulation of Microtubule Dynamics***

Microtubule dynamics can be altered in a myriad of ways in the cell. The ability to vary the dynamic stability of microtubules is especially important for cell division, which endures several cytoskeletal changes (reviewed by Joshi, 1998). During interphase of mitosis, there are a few, long microtubules emanating from the centrosomes. At the start of prophase, this array is abruptly converted into a large number of short, dynamic microtubules that begin to form the mitotic spindle by growing from a centrosome and then rapidly shrinking back until they capture a chromosome. Once a microtubule encounters a chromosome, it is selectively stabilized against shrinkage. Further, laser ablation of the centrosome demonstrates that microtubule dynamics, microtubule associated proteins, and chromatin are sufficient to drive the assembly of the bipolar mitotic spindle (Khodjakov et al., 2000). The regulation of microtubule dynamics is also essential in post-mitotic neurons for establishing cell polarity, axon outgrowth, cell signaling, adhesion, and the organization of cellular organelles (reviewed by Joshi, 1998). Neurons sends out two types of processes, dendrites and axons, both of which lack local

machinery for the synthesis of proteins and require a unique mechanism for the transport and assembly of proteins to the site of new growth. Dendrites are shorter than axons and contain microtubules that lie parallel to each other with mixed polarity. In contrast, axons contain short overlapping microtubules that are oriented in the same direction, with their plus-ends distal to the cell body of the neuron. Microtubule dynamics are regulated in the axon so that microtubules growing in the wrong direction undergo catastrophe and those microtubules growing in the correct direction are stabilized against disassembly. Interestingly, there are conflicting views on how microtubules assemble to promote axonal growth. One view supports that tubulin subunits in the cell body are transported along stable microtubules in the axon to the growing tip where they are assembled into microtubules (Bamburg et al., 1986). A contrasting view holds that microtubules are assembled in the cell body before being transported into the axon (Lasek, 1982). Currently, fluorescent tubulin injections have supported the former view and have indicated a role for microtubule associated proteins in tubulin transport (Miller and Joshi, 1996). Overall, different cell types have devised several mechanisms in order to manipulate the microtubule cytoskeleton including the utilization of a variety of proteins and posttranslational modifications. In addition to inherent factors that affect microtubule dynamics in cells, pharmacological drugs have been widely applied to study the properties and functions of microtubules.

*Microtubule Interacting Proteins*

Many kinds of proteins interact with microtubules directly and are essential for regulating microtubule stability and contributing to their function. One type are

microtubule-associated proteins (MAPs), which can either stabilize or destabilize a microtubule by binding to the side or top of the filament (reviewed by Avila et al., 1994; Hirokawa, 1994; Maccioni and Cambiazo, 1995; Drewes et al., 1998). One example of a MAP is the evolutionarily conserved protein, XMAP215. Originally discovered in *Xenopus* egg extracts, XMAP215 was found to stimulate the polymerization rate at the plus ends of microtubules (Gard and Kirschner, 1987). XMAP215 binds along the sides of the microtubule to stabilize free microtubule ends and prevent depolymerization (reviewed by Kinoshita et al., 2002). In addition, XMAP215 has been shown to have the opposite effect on microtubule dynamics by destabilizing microtubules, suggesting that XMAP215 has multiple activities (Shirasu-Hiza et al., 2003).

Another example of a conserved MAP is the plus-end binding protein, EB1 (reviewed by Tirnauer and Bierer, 2000). The first functional studies of EB1 have come from the yeast homologue, *BIM1*, and have shown that *BIM1* promotes microtubule polymerization by increasing the frequency of rescue (Schwartz et al., 1997; Tirnauer et al., 1999). In other organisms such as *Drosophila* and *Xenopus,* the ability of EB1 to promote microtubule growth indicates that it may have a crucial role in mitosis by assisting in the assembly of the spindle or the interaction of spindle microtubules with the cell cortex (Rogers et al., 2002; Tirnauer et al., 2002; Tirnauer et al., 2004). Studies in humans have additionally shown that EB1 may help target the adenomatous polyposis coli (APC) protein to the ends of microtubules in order to facilitate the interaction of the APC with the cell membrane (Morrison et al., 1998).

A second type of microtubule interacting protein are microtubule motors, which can aid in transporting molecules along the microtubule or in separating chromosomes

(reviewed by Hunter and Wordeman, 2000; Vale and Milligan, 2000; Wittmann et al., 2001). There are two categories of conserved microtubule motor proteins, dyneins and kinesins. Dyneins are minus-end directed motors composed of two to three heavy chains, which act as the motor, and a variable number of light chains (Porter and Johnson, 1989). Cytoplasmic dynein is localized within a large protein complex and associates with another complex, dynactin, in order to transport cargo (Porter and Johnson, 1989). Dynein is very important for regulating the length of microtubules in the meiotic and mitotic spindles, and together with dynactin, helps to target depolymerizing activities to the spindle poles (Mountain and Compton, 2000; Gaetz and Kapoor, 2004). Dynein has also recently been found to affect microtubule dynamics directly by acing as a potent microtubule nucleator in order to organize microtubules into radial arrays (Malikov et al., 2004). In addition, it has been proposed that dynein orients and sorts microtubules according to their polarity and this activity is needed for both centriolar and acentriolar spindle formation (Gibbons, 1996; Heald et al., 1996). Studies in *Xenopus* egg extracts have shown that bipolar spindles can assemble around DNA-coated beads, and that dynein is required for the formation of the spindle-pole through the translocation of microtubules (Heald et al., 1996).

Kinesin is the second type of motor protein and was discovered after experiments using axoplasm from squid giant axons revealed a novel force-generating protein that induced the movement of microtubules on glass, latex beads on microtubules, and axoplasmic organelles on microtubules (Vale et al., 1985). Kinesin motor proteins move along the microtubule, usually towards the plus end, while carrying many different kinds of cargo, such as organelles, vesicles, chromosomes, and cytoskeletal elements (Fig. 1)

(Vale et al., 1985; reviewed by Mountain and Compton, 2000; Hirokawa and Takemura, 2004). Kinesins are composed of two heavy and two light chains, two globular head domains, and a coiled-coiled domain involved in dimerization (Amos and Cross, 1997). Kinesin binds to microtubules through one of its head domains and uses ATP hydrolysis to fuel its movement (Fig. 1) (Amos and Cross, 1997). In addition to transporting molecules, kinesins have been implicated in having roles in mitosis and meiosis. In particular, the conserved BimC family of kinesins are essential for spindle formation in all eukaryotes and are important for driving chromosome segregation (reviewed by Kashina et al., 1997; Mountain and Compton, 2000; Kwok et al., 2004). Kinesin function is not limited to its motor activity. One family of kinesins, Kin I, have been found to affect microtubule dynamics independent of motility (reviewed by Desai et al., 1999; McNally, 1999; Walczak, 2000). One example, XKCM1, a non-conventional kinesin-related protein, was isolated from *Xenopus* eggs and was found to have a novel role in promoting microtubule depolymerization during mitotic spindle assembly (Walczak et al., 1996). XKCM1 binds to both ends of microtubules and induces a destabilizing conformational change which results in protofilament peeling (Desai et al., 1999). Upon depolymerization, tubulin heterodimers have been found to form an ATP-dependent complex with XKCM1, however, further work will be needed to determine whether these complexes have a role on microtubule dynamics (Desai et al., 1999).

As alluded to above, protein modifiers of microtubule stability and dynamics are not limited to binding along the microtubule. Proteins can also bind to free subunits of tubulin and affect their incorporation into filaments. Op18/stathmin, in particular, promotes microtubule destabilization by diminishing the availability of free tubulin for

polymerization (reviewed by McNally, 1999; Walczak, 2000; Rubin and Atweh, 2004). Op18/stathmin was first purified from *Xenopus* egg extracts and was proposed to promote microtubule destabilization by increasing the rate of catastrophe (Belmont et al., 1990). Two distinct mechanisms for the destabilization of microtubules by Op18/stathmin were later characterized. The N-terminal region of the protein bound to the ends of polymerized microtubules to promote catastrophe (Belmont and Mitchison, 1996; Howell et al., 1999). In contrast, the C-terminal region prevents tubulin polymerization by binding directly to tubulin heterodimers (Jourdain et al., 1997; Howell et al., 1999). During mitosis, regulation of Op18/stathmin through phosphorylation is very important for its function in spindle formation (Feuerstein and Cooper, 1983). Phosphorylation of op18/stathmin switches off its depolymerizing activity at the onset of mitosis to allow for microtubule polymerization and the assembly of the spindle. Dephosphorylation of Op18/stathmin, prior to mitotic exit, reactivates its depolymerizing activity (Brattsand et al., 1994; Marklund et al., 1996). Thus, the regulation of tubulin subunits in addition to the microtubule filament is important for the control of microtubule dynamics.

*Posttranslational Modifications*

The αβ-tubulin heterodimer is subject to several reversible posttranslational modifications that affect both the function and dynamics of microtubules (reviewed by Barra et al., 1988; Westermann and Weber, 2003). The extensive array of modifications includes acetylation/deacetylation, tyrosination/detyrosination, polyglutamylation, polyglycylation, palmitoylation, and phosphorylation, and they occur at the carboxy-terminus of the tubulin tail, with the exception of acetylation. Acetylation occurs on the

α-tubulin subunit and is mostly associated with stable microtubule populations (L'Hernault and Rosenbaum, 1985; Cambray-Deakin and Burgoyne, 1987). Some studies have proposed that tubulin acetylation may play a role in the differentiation of microtubule structure and function (Piperno and Fuller, 1985). Although it is still unclear whether acetylation directly affects microtubule dynamics, inhibitor studies of the tubulin deacetylase, HDAC6, have shown an increase in stability in normally dynamic microtubule populations (Matsuyama et al., 2002). When HDAC6 was inhibited in fibroblast cells, cell migration was repressed suggesting that microtubule acetylation may function in cell motility (Hubbert et al., 2002; Palazzo et al., 2003).

Tyrosination/detyrosination occurs on the α-tubulin subunit in an ATP-dependent cycle and is catalyzed by tubulin tyrosine ligase (TTL) (Ersfeld et al., 1993). While TTL prefers to tyrosinate non-assembled tubulin, tubulin tyrosine carboxypeptidase (TTCP) prefers to detyrosinate assembled microtubules (Webster et al., 1987). Studies have linked tyrosinated tubulin with dynamic microtubule populations, and detyrosinated tubulin with stable microtubule populations (Kreis, 1987). Detyrosinated microtubules have been proposed to interact with intermediate filaments, and this interaction is mediated by kinesin (Liao and Gundersen, 1998).

Polyglutamylation can occur on either α- or β-tubulin. The main function of polyglutamylation is influencing the interaction between microtubules and associated proteins. MAPs, such as kinesin, have been shown to have a high affinity for polyglutamylated microtubules (Larcher et al., 1996). Additionally, centriole tubulin is highly polyglutamylated indicating a function in centriolar maturation, stability, and recruiting proteins to anchor centriole microtubules (Bobinnec et al., 1998; Bornens,

2002). Polyglycylation is another posttranslational modification that can occur on either α- or β-tubulin, and it is the prominent modification in axonemal and sperm tubulin suggesting a role in axon organization and cell motility. Genetic studies in *Tetrahymena* have revealed that mutations in β-tubulin polyglycylation sites cause a failure in cytokinesis, and that this defect is due to incomplete severing of microtubules (Xia et al., 2000; Thazhath et al., 2002). This result suggests that polyglycylation may have a role in assigning microtubules for severing.

The less understood modifications, palmitoylation and phosphorylation, are not widely observed and their functions are not fully clarified. Studies in budding yeast have discovered that reducing palmitoylation on α-tubulin leads to defects in astral microtubule positioning suggesting it is needed to establish microtubule interactions with the cell cortex (Caron et al., 2001). Phosphorylation is a minor modification to β-tubulin and may be involved in regulating MAP interactions and neuronal differentiation, however, its precise function remains unknown (Gard and Kirschner, 1985).

*Microtubule-Specific Drugs*

Since the microtubule cytoskeleton is crucial for the survival of eukaryotic cells, the production of cytoskeletal toxins have naturally evolved from plants, fungi, and sponges as a mechanism for these organisms to ward off potential predators. These toxins function by either binding to the microtubule filament or tubulin subunits and either stabilize or destabilize the microtubule (reviewed by Wilson et al., 1999). Since microtubules are among the most strategic, subcellular targets of anticancer chemotherapeutics due to their involvement in the cell cycle, understanding the

mechanism of microtubule toxins is critical for the treatment of cancer (reviewed by Jordan et al., 1998).

There are four widely used microtubule-specific drugs: colchicine, vinblastine, nocodazole, and taxol. Colchicine, vinblastine, and nocodazole all function to destabilize microtubules by binding to tubulin subunits and preventing microtubule polymerization. Colchicine is isolated from a meadow saffron plant, and as one the first anti-mitotic agents studied, it was found to alter the tubulin heterodimer structure upon binding (Jordan et al., 1998). Vinblastine is isolated from a periwinkle plant, and prevents microtubule polymerization by binding to the β-tubulin subunit and physically blocking heterodimer attachment (Jordan et al., 1998). Interestingly, in low levels, vinblastine can bind and stabilize the ends of microtubules by suppressing dynamic instability and treadmilling without the occurrence of depolymerization (Wilson et al., 1999). Nocodazole, a derivative of benzimidazole, is also commonly used to destabilize microtubules, and studies have proposed that nocodazole increases GTPase activity by binding to tubulin dimers resulting in the increase of GTP hydrolysis (Vasquez et al., 1997).

Taxol is extracted from the bark of a yew tree and has a profound impact on microtubule dynamics and stability (Manfredi and Horwitz, 1984). Taxol functions differently from the other three toxins in that it binds and stabilizes microtubules causing a net increase in tubulin polymerization (Manfredi and Horwitz, 1984). Since microtubule disassembly is critical for mitotic spindle function and taxol renders microtubules dysfunctional by preventing depolymerization, taxol is used to kill dividing cells for the treatment of cancer (Rowinsky et al., 1990). Overall, experimental use of

microtubule drugs can be very useful in studying microtubule dynamics and the cell cycle.

**Female Meiosis**

Meiosis is an essential process for sexually reproducing organisms. During female meiosis, a diploid (2N) oocyte must undergo DNA replication followed by two consecutive rounds of cell divisions (Meiosis I and Meiosis II) in order to produce a haploid (N) gamete (Fig. 2). Haploid gametes must be generated with the correct number of chromosomes in order to produce a diploid zygote upon fertilization. Similar to mitosis, DNA replication occurs during S phase, and each round of cell division is divided into four stages, prophase, metaphase, anaphase, and telophase. During prophase, the replicated chromosomes condense and the nuclear envelope breaks down (NEBD) to allow the spindle microtubules to attach to the kinetochores of the chromosomes. The chromosomes are aligned on the equator of the spindle at metaphase, separated to opposite poles during anaphase, and cell division is completed during telophase.

The first cell division of meiosis, however, differs from mitosis in two ways. First, prophase of meiosis I is divided into five sequential stages: leptotene, zygotene, pachytene, diplotene, and diakinesis. Duplicated homologous chromosomes, each containing two sister chromatids, condense and pair during leptotene. A synaptonemal complex, or a physical protein link, forms between the two sets of sister chromatids during zygotene and promotes genetic recombination, or the exchange of genetic information important for genetic diversity. Pachytene begins when synapsis is

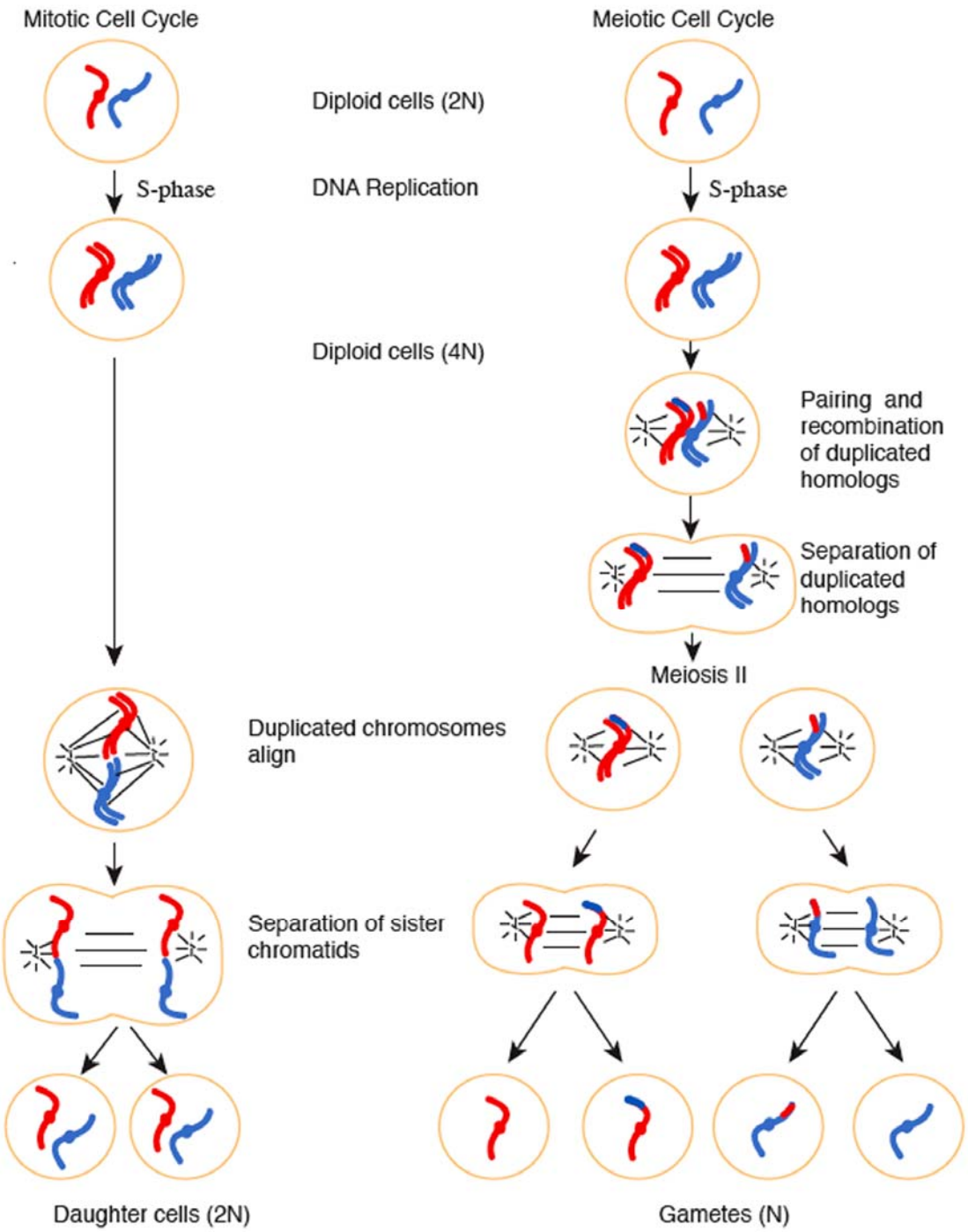



**Figure 2. Comparison of mitosis and meiosis.** During mitosis duplicated chromosomes align and sister chromatids are separated generating two genetically identical diploid cells. In contrast, during meiosis I duplicated homologous chromosomes align and homologs are separated. Meiosis II is similar to mitosis in that sister chromatids are separated, however, four haploid gametes instead of two are generated. Figure adapted from Alberts et al., 2002.

complete, and diplotene occurs when desynapsis begins. At this stage, connections called chiasmata are visible and indicate a crossover event had taken place. Diplotene ends with diakinesis, the transition stage to metaphase. The second difference during meiosis occurs during meiotic anaphase I when homologous chromosomes are separated instead of sister chromatids to generate two diploid cells. The second meiotic cell division occurs similar to mitosis, except that four genetically distinct haploid gametes are produced, instead of two identical diploid cells (Fig. 2).

***Oocyte Meiotic Maturation***

Depending on the species, oocytes arrest at one or two places during meiosis to allow for oocyte growth and the accumulation of nutrients. The release from meiotic arrest and the physiological changes in the oocyte before fertilization is termed oocyte meiotic maturation and is required for preparing the oocyte for successful fertilization (Masui and Clarke, 1979). The hallmarks of meiotic maturation include the transition between diakinesis of prophase I to metaphase I, NEBD, the rearrangement of the cortical cytoskeleton, and meiotic spindle assembly (reviewed by Schmitt and Nebreda, 2002). These features of oocyte meiotic maturation are regulated by the surrounding somatic cells and encompass changes in both the nucleus and the cytoplasm (Buccione et al., 1990; Carabatsos et al., 2000; Eppig, 2001). Nuclear maturation is the process of releasing the meiotic arrest at prophase I and driving the progression of meiosis to metaphase II (Eppig et al., 1994). Nuclear maturation includes changes in chromatin organization, nuclear membrane dynamics, and nucleolar function and stability (Van Blerkom, 1991). Cytoplasmic maturation is the process that prepares the oocyte for

activation, formation of pronuclei, and preimplantation (Eppig et al., 1994). Cytoplasmic maturation is associated with the redistribution of organelles, activation of signaling for ovulation, and alterations in the cytoskeleton (Van Blerkom, 1991; Yamamoto et al., 2006). Experiments isolating immature mouse oocytes from follicles demonstrated that these oocytes arrested at metaphase I and were deficient in maternal factors required for embryonic development, suggesting that both nuclear and cytoplasmic maturation is required for the competency of an oocyte (Eppig et al., 1994). Since oocyte meiotic maturation must be coordinated with other aspects of oogenesis, including oocyte growth, meiotic chromosome congression, and ovulation, the regulation of intracellular signals governing these processes is required for meiotic progression (reviewed by Masui, 2001; Yamamoto et al., 2006).

*Regulation of Meiotic Maturation*

The signals and timing of meiotic divisions vary greatly among species. In mammals, oocytes are held in arrest during meiotic prophase I by surrounding somatic follicle cells until a surge of luteinizing hormone (LH) from the pituitary gland stimulates the resumption of meiosis (reviewed by Channing et al., 1978; Mehlmann, 2005). In contrast, meiotic arrest in species such as nematodes, sponges, and mollusks, is regulated by signals from the sperm (reviewed by Masui and Clarke, 1979; Miller et al., 2001). Despite these different mechanisms in signaling, the molecular pathways are strikingly similar and conserved among organisms. For example, studies of meiotic maturation in amphibian oocytes led to the discovery of Maturation Promoting Factor (MPF) (Masui and Markert, 1971; reviewed by Masui, 2001; Tunquist and Maller, 2003). This

regulator of meiotic progression was later discovered to be composed of a cyclin-dependent protein kinase, Cdk1, catalytic subunit and a cyclin B regulatory subunit (Dunphy et al., 1988; Gautier et al., 1988; Lohka et al., 1988; Gautier et al., 1990). Further studies have revealed that cyclin-dependent protein kinases are universal regulators of meiotic and mitotic cell cycle progression in eukaryotes (reviewed by Nurse, 1990; Murray, 2004).

Several other signaling pathways, that include components such as cyclic adenosine monophosphate (cAMP) and mitogen-activated protein kinase (MAPK), are also conserved during meiotic maturation but have different roles depending on the species. For instance, in animals such as frogs, fish, and mice, the cAMP-dependent protein kinase (PKA) is a potent inhibitor of meiotic maturation in oocytes (Maller, 1985; Eppig, 1989). Therefore, decreasing cAMP levels or direct inhibition of PKA, is sufficient to induce meiotic maturation in these organisms. In animals such as pigs, sheep, and rabbits, however, meiotic maturation occurs with an increase in cAMP levels (reviewed by Schmitt and Nebreda, 2002). Similarly, the role and timing of MAPK activation also varies during meiotic maturation. In mammals, even though MAPK is activated during meiotic maturation, it is not required for NEBD to occur. *Xenopus* can also undergo NEBD in the absence of MAPK, but kinetic delays in maturation occur (Maller et al., 2001). In contrast, MAPK activation in starfish oocytes is not related to meiotic maturation (reviewed by Schmitt and Nebreda, 2002).

As mentioned above, communication between the somatic cells and oocyte is crucial for the regulation of meiosis. Mammalian oocytes are held in meiotic arrest by an unknown signal from the surrounding somatic follicle cells and the orphan $G\alpha_s$-linked

receptor, GPR3, in the oocyte (Mehlmann et al., 2004). Given that the isolation of an oocyte from its surrounding somatic cells causes spontaneous maturation, studies have further shown that the interactions between the soma and germline are mediated by gap-junctions and paracrine factors (reviewed by Eppig, 1991; Webb et al., 2002). In support of this, follicle cells have also been suggested to transfer cAMP to oocytes via gap junctions to maintain arrest (Anderson and Albertini, 1976; reviewed by Wickramasinghe and Albertini, 1993; Webb et al., 2002). Other studies show that LH stimulation increases cAMP levels within the granulosa cell compartment, concomitantly decreasing cAMP levels within the oocyte, thereby promoting meiotic resumption and cumulus expansion (Downs and Hunzicker-Dunn, 1995; Tsafriri et al., 1996). These conflicting results indicate that soma-germline interactions may play a crucial role in both the positive and negative regulation of meiotic maturation (reviewed by Voronina and Wessel, 2003).

***Meiotic Spindle Assembly***

The viability of an embryo after fertilization depends critically on the faithful segregation of the genetic material during meiosis. Non-disjunction occurs when chromosomes fail to properly segregate during either of the meiotic divisions (Fig. 3A). This defect can give rise to aneuploid embryos, containing too many or too few chromosomes, typically with dire consequences owing to the deleterious effects of abnormal gene dosage. In humans, non-disjunction during female meiosis I represents the leading cause of miscarriage and congenital birth defects, such as Trisomy 21 or Down syndrome (Hassold and Hunt, 2001). A “two-hit” model has been proposed to

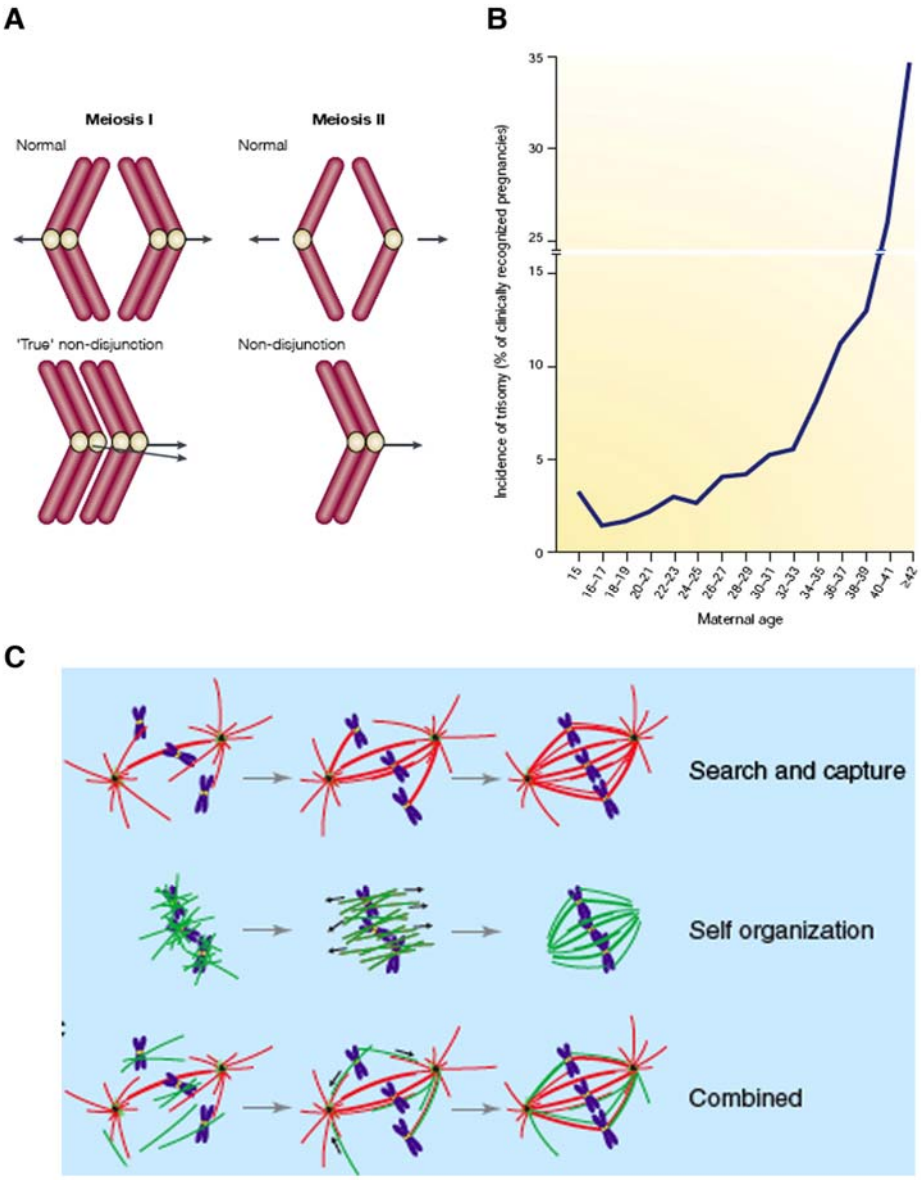



**Figure 3. Non-disjunction and meiotic spindle assembly.** (A) Diagram comparing chromosome segregation during meiosis I and II. Top panel represents normal chromosomes segregating to opposite poles. Bottom panel represents non-disjunction with chromosomes segregating to the same pole (adapted from Hassold and Hunt, 2001). (B) Graph depicting the relationship between non-disjunction and maternal age (adapted from Hassold and Hunt, 2001). (C) Models of spindle assembly. In the absence of a centrosome, microtubules self organize randomly around chromatin in a bipolar array by motor proteins (middle panel). In the presence of a centrosome, microtubules randomly search and capture chromatin from the centrosome (top panel) or both centrosome (red) and chromosome (green) nucleated microtubules are captured to generate a spindle (bottom panel) (adapted by Gadde and Heald, 2004).

explain the causes for non-disjunction during meiosis (Lamb et al., 1997). The first hit refers to the risk involved in crossovers during fetal development (Lamb and Hassold, 2004), and the second hit occurs decades later when the oocyte resumes meiosis. Previous studies in humans and mice have also suggested that disturbances in the hormonal regulation of folliculogenesis are associated with chromosome congression failure during meiotic spindle assembly, and may represent a significant risk factor for non-disjunction errors (Hodges et al., 2002). This link between hormonal signaling and meiotic errors may help in understanding why the incidence of non-disjunction dramatically increases as human females age (Fig. 3B).

The meiotic spindles of most animal oocytes are distinctive from mitotic spindles of somatic cells in terms of their mechanism of assembly, their function, and their modes of regulation. The female meiotic spindles of many species are both acentriolar and anastral (Szollosi et al., 1972; Albertson and Thomson, 1993; Schatten, 1994; Matthies et al., 1996). Instead of relying on centrosomes for the assembly of a bipolar spindle (Fig. 3C, top and bottom panels), the meiotic chromatin functions to nucleate microtubules, which self-organize through incompletely understood mechanisms that involve sorting by microtubule motors and microtubule dynamics (Fig. 3C, middle panel) (Heald et al., 1996; Matthies et al., 1996; Walczak et al., 1998; Skold et al., 2005). Biochemical studies in *Xenopus* egg extracts, which provide a model for assembly of the meiosis II spindle, indicate that DNA-coated beads can nucleate the self-organization of microtubules into bipolar spindles (Heald et al., 1996). Cultured mammalian cells might utilize analogous mechanisms in parallel with centrosome-dependent mechanisms as bipolar spindles can assemble after laser ablation of centrosomes (Khodjakov et al.,

2000). Chromatin-based microtubule assembly pathways employ the Ran GTPase, which has been shown to nucleate microtubules (Carazo-Salas et al., 1999; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999). Mechanisms utilizing the Aurora B kinase chromosomal passenger complex have also been revealed to stabilize microtubules associated with chromatin (Sampath et al., 2004).

Assembly of a bipolar meiotic spindle through chromatin-dependent mechanisms requires that the cytoplasmic microtubules gain access to the nuclear environment, which occurs after NEBD during meiotic maturation. At present, it is not understood how signals that regulate oocyte growth and meiotic progression might influence meiotic spindle assembly and chromosome segregation. If genes or processes are conserved between mitosis and meiosis, then examination of regulators involved in mitotic progression and spindle assembly may help to elucidate the mechanisms of regulation during meiosis. For instance, Cdk1 and cyclin B are essential for mitotic spindle morphogenesis in addition to regulating cell cycle progression. Cdk1 has been shown to up-regulate microtubule dynamics when added in cell-free *Xenopus* extracts (Verde et al., 1990; Verde et al., 1992), and Cdk1 inactivation is necessary for proper anaphase spindle dynamics and cytokinesis (Wheatley et al., 1997). Large amounts of Cdk1 induce the depolymerization of interphase microtubules when injected into mammalian cells (Lamb et al., 1990) and the destabilization of microtubule arrays when added on lysed mammalian cells (Lieuvin et al., 1994). Among Cdk1 substrates are a number of microtubule effectors (Ubersax et al., 2003) such as MAP4, which has been shown to be phosphorylated by Cdk1 *in vivo* (Ookata et al., 1997). Furthermore, MAP4 interacts with cyclin B, and this interaction could target Cdk1 to microtubules (Ookata et al., 1995;

Charrasse et al., 2000). Other substrates of Cdk1 are microtubule motors, such as the kinesin-related proteins Eg5, Kid, or MKLP1, and have implications in different steps of mitosis (Blangy et al., 1995; Sawin and Mitchison, 1995; Ohsugi et al., 2003; Mishima et al., 2004). Phosphorylation by Cdk1 has been shown to regulate the localization of these motors in the mitotic spindle (Blangy et al., 1995; Ohsugi et al., 2003; Mishima et al., 2004). Likewise, Op18/stathmin, a protein that sequesters tubulin dimers and destabilizes microtubules during interphase, is inactivated through phosphorylation by Cdk1 at the onset of mitosis (Cassimeris, 2002). Overall, these studies indicate that a protein involved in mitotic progression also has a function in mitotic spindle assembly and regulating microtubule dynamics. Therefore, signaling pathways that function in meiotic progression could have a dual function in meiotic spindle assembly, however, further studies will be needed to determine whether this overlap exists.

### *C. elegans* as a Genetic Model for Studying Meiosis

The nematode, *Caenorhabditis elegans*, is an excellent system to study meiosis. *C. elegans* is one of the only genetic model organisms in which the events of oocyte development, meiotic maturation, fertilization, and ovulation can be directly observed in a live, intact animal (Ward and Carrel, 1979; McCarter et al., 1999; reviewed by Hubbard and Greenstein, 2000). Since *C. elegans* are transparent, several microscopy and imaging techniques are very useful in order to visualize *in vivo* real-time dynamics of cytoskeletal elements and proteins. In addition to these techniques, several tools, such as RNAi, and the availability of mutants provide a powerful means to characterize phenotypes and gene function (reviewed by Maine, 2001). Further, *C. elegans* is one of the few systems in

which the meiotic maturation signal has been identified (Miller et al., 2001), and recent studies have begun to uncover the receptor signaling pathways involved in the regulation of this process (Miller et al., 2003; Miller et al., 2004; Corrigan et al., 2005; Govindan et al., 2006). The coupling of convenient tools and techniques with the natural properties of *C. elegans* makes this organism an attractive model for the study of meiosis and meiotic maturation.

### *C. elegans Reproductive Development*

*C. elegans* has two natural sexes, hermaphrodites and males. Hermaphrodites are self-fertile and contain both sperm and oocytes. Males contain only sperm and must mate with hermaphrodites to produce progeny. Hermaphrodite reproduction is limited by the availability of sperm because a limited amount of sperm is produced early in gametogenesis before switching exclusively to oogenesis as an adult (Kimble, 1988). Since hermaphrodites produce only a fixed number of sperm, meiotic maturation rates are initially high for the first two days of adulthood but decline as sperm are used for fertilization (McCarter et al., 1999; Kosinski et al., 2005). After a hermaphrodite utilizes all of its sperm for fertilization, the animal remains sterile unless it mates with a male. In male/female nematode species (e.g., *C. remanei*), oocytes arrest at diakinesis until the female is inseminated during mating. Likewise, in sex-determination mutants of *C. elegans*, which fully feminize the hermaphrodite gonad so that no sperm is produced (e.g. *fog-2* or *fog-3*), oocytes also arrest until sperm are supplied by males. Therefore, the maintenance of oogenesis is required even in the absence of sperm to ensure the continual production of oocytes for reproduction.

*Oogenesis*

During early embryogenesis, the hermaphrodite gonad begins to develop upon hatching and contains four precursor cells (Z1, Z2, Z3, and Z4) that divide to create the primordium of somatic and germ line cells (Fig. 4A) (Kimble and Hirsh, 1979). During post-embryonic development, the Z1 and Z4 cells give rise to the somatic gonad including the distal tip cells (DTCs), sheath cells, spermathecae, and the uterus (Fig. 4A). The Z2 and Z3 cells give rise to the germline (Fig. 4A). Development of both the germline and the soma are coordinated by intracellular signaling, and by functioning together, they maintain the hermaphrodite reproductive tract.

Oocytes grow and develop in two symmetrical U-shaped gonad arms that each end proximally at a spermatheca, a specialized storage compartment for sperm (Fig. 4B). Only the most proximal oocyte undergoes meiotic maturation before it is ovulated into the spermatheca and fertilized. The two spermathecae share a common, centralized uterus that collects newly fertilized embryos. The embryos exit the animal through an opening in the middle of the uterus, called the vulva. The distal end of each gonad arm contains a DTC followed by mitotically proliferating germ cells. As the germ cells migrate towards the proximal arm, they enter meiosis, a decision regulated by the Notch-family receptor, GLP-1, and initiate meiotic recombination in a region called the transition zone (Crittenden et al., 1994). The DTC also regulates germ cells and their transition to meiosis by sending out tentacle-like processes along the distal gonad to promote germ line mitosis (and/or inhibiting meiosis) and control gonadal outgrowth (Hall et al., 1999).

As germline nuclei transit through the loop region, where the gonad tube bends

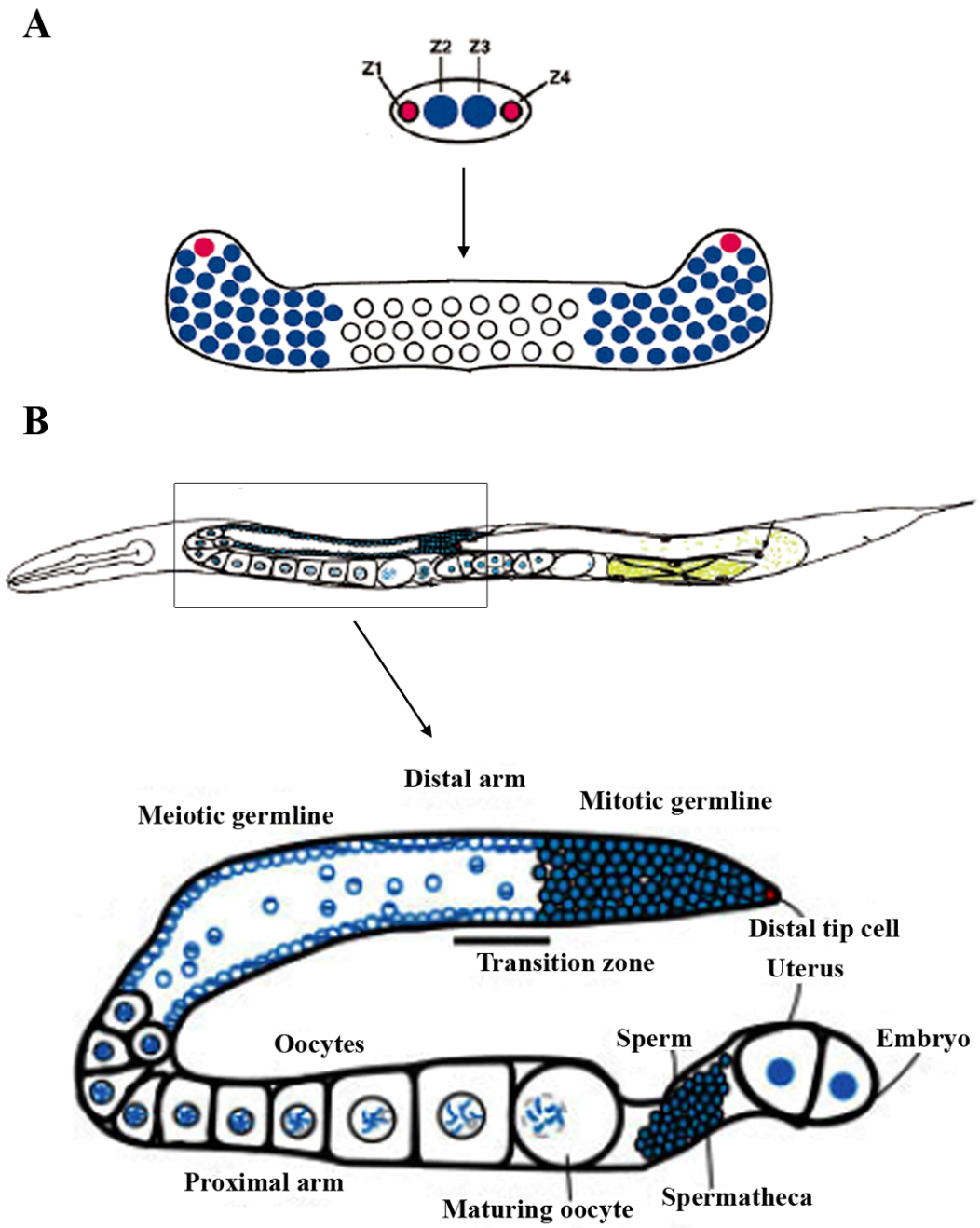



**Figure 4. Oogenesis in the *C. elegans* gonad.** (A) Overview of hermaphrodite gonadal development. The L1 larval stage contains four precursor cells: Z1, Z2, Z3, and Z4. Z1 and Z4 (depicted in red) give rise to the somatic gonad and the DTCs. Z2 and Z3 (depicted in blue) give rise to the germline (adapted from Hubbard and Greenstein, 2000). (B) Cartoon of the hermaphrodite gonad. Upper image illustrates the two U-shaped arms of the gonad. The right arm is illustrating sheath cells surrounding the proximal oocytes. The left arm is enlarged in the bottom panel with the distal gonad defined by the DTC and the proximal gonad defined by the sperm. Only the most proximal oocyte matures in an assembly-line fashion.

ventrally, they exit pachytene, become more fully enclosed by plasmamembrane, and form a queue in the proximal gonad arm. This progression beyond the pachytene stage of meiosis I, requires the Ras/MAPK signaling pathway (Church et al., 1995). Mutations in *let-60* (RAS), *mpk-1/sur-1* (MAP kinase), and *mek-2* (MAP kinase kinase) block meiotic exit from pachytene and result in gonad disorganization (Church et al., 1995). Cell ablation studies have further suggested that cells in the sheath/spermathecal lineages promote pachytene exit (McCarter et al., 1997). Once germ cells enter the diplotene stage, their chromosomes begin to condense and individual oocytes begin to grow and form. During diakinesis of meiotic prophase I, six discrete highly condensed bivalents, corresponding to the homologous chromosome pairs, can be visualized. In the absence of sperm, oocytes will arrest in diakinesis.

*Oocyte Meiotic Maturation*

*C. elegans* oocytes remain arrested in diakinesis of prophase I in the absence of sperm. When sperm are present, the most proximal (-1) oocyte resumes meiosis and undergoes meiotic maturation (see Fig. 5) (McCarter et al., 1999). During meiotic maturation, *C. elegans* oocytes are subjected to both cytoplasmic and nuclear changes including NEBD, the rearrangement of the actin cortical cytoskeleton, and meiotic spindle assembly. Similar to mammals, ovulated oocytes that have not completed meiotic maturation are not competent for fertilization and embryogenesis (Rose et al., 1997; Bui and Sternberg, 2002; Yamamoto et al., 2006). Given the importance of meiotic maturation for fertilization, it is crucial to investigate the specific changes that occur in the oocyte during this process. Recently, it has been discovered in *C. elegans*

that the oocyte microtubule cytoskeleton reorganizes prior to fertilization (Harris et al., 2006). Given that microtubules have been shown in other systems to be involved in redistributing organelles and proteins in the cytoplasm during maturation (Van Blerkom, 1991; Theurkauf et al., 1992; Sun and Schatten, 2006) and are essential after NEBD for meiotic spindle assembly and function, this finding has important implications for the involvement of microtubules in both nuclear and cytoplasmic maturation.

Once the proximal oocyte is ovulated into the spermatheca, the second oocyte (-2) becomes the most proximal oocyte and prepares for maturation. Subsequent oocytes will continue to mature in an assembly-line fashion as long as sperm are present (McCarter et al., 1999). Since wild-type meiotic maturation is spatially restricted to the –1 oocyte, an unknown inhibitory mechanism must prevent distal oocytes from maturing out of order (McCarter et al., 1999). Since oocytes mature with clockwork regularity, the events of oocyte maturation, ovulation, and fertilization have been carefully recorded (McCarter et al., 1999). First, the oocyte nucleolus disappears 70 minutes prior to fertilization. NEBD then occurs in the most proximal oocyte 6 minutes prior to fertilization, which is followed by the rounding of the oocyte through cortical rearrangement of the actin cytoskeleton (3 minutes) and ovulation into the spermatheca (0.7 minutes). Finally, the oocyte is fertilized within the spermatheca before being pushed into the uterus approximately 3 min after fertilization. When sperm are abundant, this process occurs approximately every 23 minutes for each gonad arm. When sperm are absent, this rate dramatically decreases to approximately 1/40th of the wild type rate (0.1 vs. 2.5 maturations per gonad arm per hour) (McCarter et al., 1999).

In *C. elegans*, specialized contractile myoepithelial cells of the somatic gonad, the

gonadal sheath cells, are closely apposed to oocytes and are required to maintain both the structure and integrity of the gonad (McCarter et al., 1997; Rose et al., 1997) (see Fig. 4B). Laser ablation studies indicate that the somatic sheath influences events of hermaphrodite germline development including germline proliferation, male germ cell fate, exit from the pachytene, and ovulation of the oocyte (McCarter et al., 1997). To drive ovulation, the sheath cells contract such that the most proximal oocyte is pushed/pulled into the spermatheca allowing fertilization to occur (Rose et al., 1997; Hall et al., 1999). In addition to their contractile role, signaling pathways in the sheath cells have been shown to both inhibit and promote oocyte meiotic maturation (Miller et al., 2003; Govindan et al., 2006).

The *C. elegans* Major Sperm Protein (MSP) has been identified as the hormonal signal for meiotic maturation and sheath contraction (Miller et al., 2001). Interestingly, MSP is also the key cytoskeletal element required for the actin-independent amoeboid locomotion of nematode spermatozoa (Italiano et al., 1996; Bottino et al., 2002). Since sperm are contained in the spermatheca and cannot interact with oocytes until they are ovulated, non-motile spermatids and motile spermatozoa have been proposed to bud novel vesicles in order to deliver the MSP signal to distant oocytes (Kosinski et al., 2005). This method of MSP release creates a spatial gradient across the gonad arm with the most proximal oocytes receiving a higher concentration of MSP compared to the more distal oocytes (Fig. 5) (Kosinski et al., 2005). MSP is also temporally graded in hermaphrodites because as sperm are consumed in fertilization, the total amount of MSP decreases (Kosinski et al., 2005).

Meiotic maturation and ovulation in *C. elegans* are coupled to sperm availability

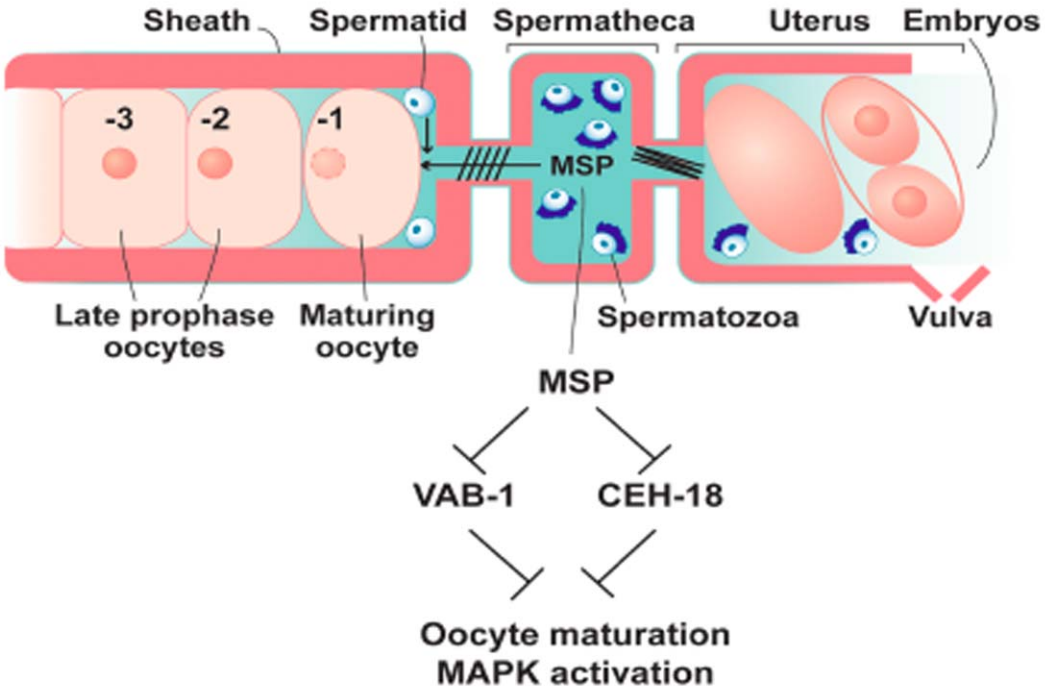



**Figure 5. Model for MSP signaling.** Diagram of the hermaphrodite reproductive tract. Oocytes undergo meiotic maturation in an assembly-line fashion in response to the extracellular MSP signal (depicted in light blue) from sperm, which exhibits a graded distribution from the proximal to distal gonad. Two parallel pathways, defined by VAB-1 and CEH-18, act to inhibit oocyte meiotic maturation and gonadal sheath cell contraction. In the presence of sperm, MSP removes this negative regulation to allow meiotic maturation to occur. MSP has been shown to directly bind the VAB-1 Eph receptor on oocyte surfaces. Receptors regulating the CEH-18 pathway remain to be identified. In all depictions and photomicrographs of the gonad, proximal is at the right, and distal is at the left. The –1 through –3 oocytes are indicated.

through a complex regulatory network involving germline and somatic controls. MSP signaling occurs through two parallel genetic pathways (Fig. 5) (Greenstein et al., 1994; Rose et al., 1997; Miller et al., 2003; Corrigan et al., 2005). One arm of the pathway is defined by VAB-1, an MSP-binding Eph receptor protein-tyrosine kinase expressed in oocytes and sheath cells (Kuwabara, 2003; Miller et al., 2003). The other pathway defined by *ceh-18*, encodes a POU-homeoprotein expressed in gonadal sheath cells but not oocytes. Additional MSP receptors likely exist since a receptor for the *ceh-18* pathway has not been identified and animals in which *vab-1* and *ceh-18* are eliminated show oocyte meiotic maturation rates above female levels and respond to MSP (Miller et al., 2003). Together, the *ceh-18* and *vab-1* parallel pathways negatively regulate oocyte meiotic maturation, MAPK activation in the proximal oocytes, and gonadal sheath cell contractions in the absence of sperm (Miller et al., 2003; Corrigan et al., 2005). Additionally, these pathways constitute a sperm-sensing checkpoint to conserve metabolically costly oocytes when sperm are unavailable for fertilization.

Once oocytes receive the MSP signal, one or more signal transduction pathways are likely activated within the oocyte triggering MAPK activation and oocyte maturation in the proximal oocyte. Several downstream components have been uncovered to function in the VAB-1 pathway. VAB-1 has been found to regulate the signaling properties of two Ca2+ channels that are encoded by NMR-1, the N-methyl D-aspartate type glutamate receptor subunit, and ITR-1, the inositol 1,4,5-triphosphate receptor (Corrigan et al., 2005). In the absence of MSP, Ephrin/VAB-1 signaling functions upstream of ITR-1 to inhibit meiotic resumption, while in a separate pathway, NMR-1 prevents the signaling of UNC-43 Ca2+/calmodulin-dependent protein kinase II

(CaMKII). MSP binding to VAB-1 stimulates NMR-1-dependent UNC-43 activation, and UNC-43 acts redundantly in oocytes to promote oocyte maturation and MAPK activation. These results support a model in which VAB-1 switches from a negative regulator into a redundant positive regulator of oocyte maturation upon binding to MSP. In addition, four conserved proteins have been recently uncovered in a genome-wide RNAi screen and shown to function either downstream or in parallel to VAB-1. These genes include a disabled protein (DAB-1), a vav family GEF (VAV-1), a protein kinase C (PKC-1), and a STAM homolog (PQN-19) (Govindan et al., 2006).

Several positive effectors of oocyte meiotic maturation have been identified in *C. elegans*. The polo-like kinase homolog PLK-1 is required for the completion of meiosis and to promote NEBD (Chase et al., 2000). Despite that *plk-1* RNAi oocytes can be fertilized, the resulting embryos are unable to separate their meiotic chromosomes or form and extrude polar bodies (Chase et al., 2000). The cyclin-dependent kinase cdc2 homologue, NCC-1, has a similar phenotype to PLK-1 and is needed for the completion of M phase in meiosis (Boxem et al., 1999). The TIS-11 zinc finger domain–containing proteins, OMA-1 and OMA-2, are redundantly required for oocyte meiotic maturation and ovulation (Detwiler et al., 2001). In *oma-1;oma-2* double mutant hermaphrodites MAPK activation is not sustained and NEBD does not occur. The precise biochemical functions of OMA-1 and OMA-2 are unknown, however, they might act as regulators of translation or proteolysis (Detwiler et al., 2001; DeRenzo et al., 2003; Lin, 2003). OMA-1 and OMA-2 may function upstream of additional regulators of meiotic maturation including the two conserved cell cycle regulators, the MYT1–related kinase, WEE-1.3 and the cyclin-dependent kinase homolog, CDK-1 (Detwiler et al., 2001). Depletion of

WEE-1.3 in hermaphrodites causes precocious oocyte maturation *in vivo* (Burrows et al., 2006). Although these oocytes are ovulated, they are fertilization incompetent perhaps due to other defects such as chromosome coalescence, aberrant meiotic spindle organization, and the early expression of a meiosis II post-fertilization marker (Burrows et al., 2006). Furthermore, co-depletion studies of CDK-1 and WEE-1.3 demonstrate that WEE-1.3 is dispensable in the absence of CDK-1, suggesting that CDK-1 is a major target of WEE-1.3 in *C. elegans* oocytes. Another gene that may act either upstream or in parallel to *oma-1* and *oma-2* is *cgh-1*, a DEAD-box helicase (Navarro et al., 2001; Navarro and Blackwell, 2005). Mutations in *cgh-1* lead to ectopic MAPK activation, suggesting *cgh-1* functions to inhibit MAPK (I. Yamamoto and D. Greenstein unpublished results). Even though some genes involved in regulating meiotic maturation have been identified in the oocyte, several other pathways and their components remain to be determined.

Somatic control of meiotic maturation, likely through the *ceh-18* pathway, involves antagonistic $G\alpha_{o/i}$ and $G\alpha_s$ signaling pathways that define negatively- and positively-acting inputs, respectively (Fig. 6) (Govindan et al., 2006). $G\alpha_s$ signaling is necessary and sufficient to trigger oocyte MAPK activation and meiotic maturation, in part, through antagonizing inhibitory sheath/oocyte gap-junctional communication. While these studies provide initial insights into the complex signaling pathways regulating meiotic maturation in response to MSP, they raise new questions about how the *vab-1* and *ceh-18* pathways control nuclear and cytoplasmic events during maturation, as well as the division of labor among the pathways.

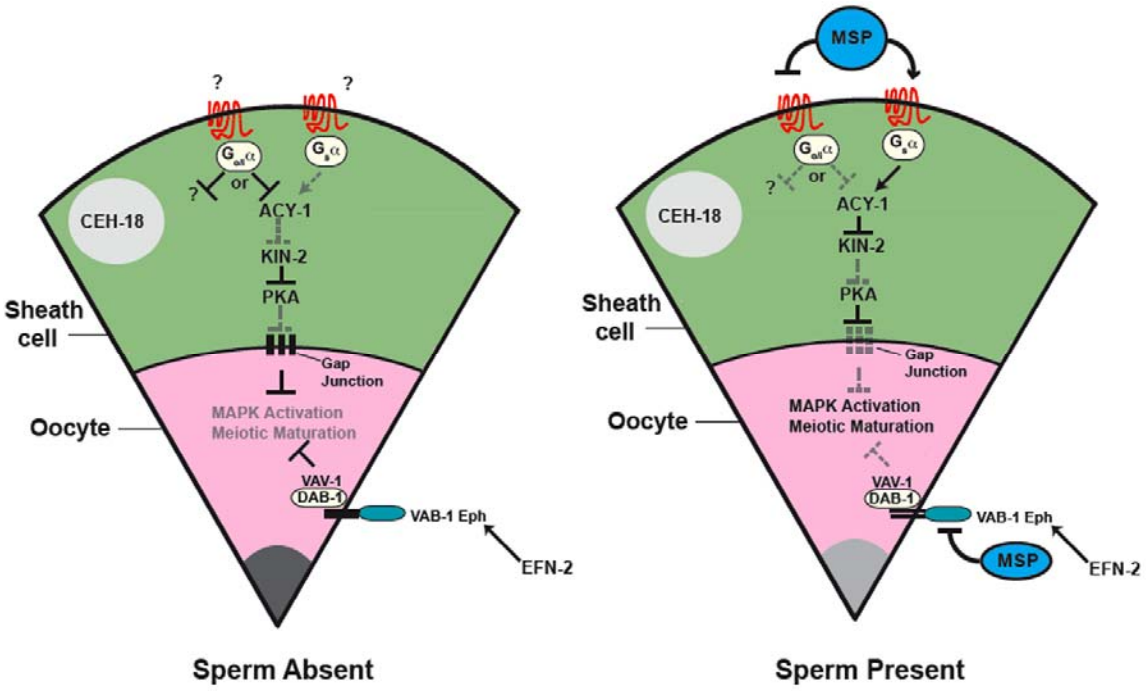



**Figure 6. A model for the parallel control of meiotic maturation in *C. elegans* by antagonistic G protein signaling from the soma and oocyte.** The germline and somatic meiotic maturation control network is depicted in either the absence (left panel) or presence (right panel) of MSP. $G\alpha_{o/i}$ negatively regulates meiotic maturation and oocyte MAPK activation and antagonizes a $G\alpha_s$ pathway that promotes maturation. The $G\alpha_s$ pathway is drawn showing the involvement of the regulatory subunit (KIN-2) of cyclic-AMP-dependent protein kinase A (PKA) and adenylyl cyclase (ACY). The $G\alpha_s$ pathway is proposed to directly destabilize the inhibitory sheath/oocyte gap junctions, but a parallel function is also possible (adapted from Govindan et al., 2006).

*Meiotic Spindle Assembly*

The *C. elegans* anastral, barrel-shaped meiotic I spindle forms in the most proximal oocyte after NEBD by nucleating around meiotic chromatin (Albertson and Thomson, 1993; Howe et al., 2001; Yang et al., 2003). The maternal centrioles are lost during oogenesis, likely disappearing before the gonad loop region (see Fig. 4) via an unknown mechanism (Wolf et al., 1978; O'Connell et al., 2001). Therefore, the meiotic spindle must assemble without MTOC serving as centrosomes and instead rely on microtubule motors and dynamics to drive the organization of microtubules around chromatin.

The assembly of the *C. elegans* meiotic spindle depends on the function of several genes. First, meiotic spindle assembly requires the two genes, *mei-1*and *mei-2*, which respectively encode the p60 and p80 subunits of katanin, a dimeric microtubule severing AAA-ATPase first purified from sea urchin eggs (Mains et al., 1990; Clandinin and Mains, 1993; McNally and Vale, 1993; Clark-Maguire and Mains, 1994; Clark-Maguire and Mains, 1994; Srayko et al., 2000). Antibodies to MEI-1 and MEI-2 demonstrate that these proteins localize to the polar ends of meiotic spindle microtubules and meiotic chromatin, and co-expression of MEI-1 and MEI-2 in HeLa cells results in the disassembly of microtubules (Srayko et al., 2000). These data support that MEI-1/MEI-2 microtubule-severing activity is required for meiotic spindle organization. MEI-1/MEI-2 katanin activity is restricted to meiosis by ubiquitin-mediated protein degradation promoted by the CUL-3 E3 ubiquitin ligase complex during cleavage stages (Kurz et al., 2002; Pintard et al., 2003). MEI-1 protein disappears in the period between meiosis II exit and the formation of the first mitotic spindle (Clark-Maguire and Mains, 1994).

MEI-1/MEI-2 katanin ubiquitin-mediated degradation must be precisely coordinated with the exit from meiosis because the inappropriate inclusion of MEI-1/MEI-2 katanin into the mitotic spindle causes malorientation in cell division planes owing to excessive shortening of astral microtubule arrays (Clark-Maguire and Mains, 1994; Vasquez et al., 1994; Kinoshita et al., 2001). In addition to regulating microtubule length, *mei-1* and *mei-2* are also subsequently required for the normal segregation of meiotic chromosomes and the translocation meiotic spindle to the cortex while maintaining the stability of this interaction (Srayko et al., 2000; Yang et al., 2003).

Another important regulator of the meiotic spindle is ZYG-9. ZYG-9 is the *C. elegans* homologue of the *Xenopus* XMAP215, a microtubule-binding protein that promotes rapid microtubule polymerization as well as antagonizes the activity of the conserved microtubule-depolymerizing protein, XKCM1/MCAK (Vasquez et al., 1994; Kinoshita et al., 2001). The *C. elegans* ZYG-9 protein is concentrated on the meiotic spindle during maturation (Matthews et al., 1998). The *zyg-9* gene is required for meiotic spindle assembly and for the formation of the exceptionally long microtubules in the mitotic spindle apparatus of early embryos (Albertson, 1984; Kemphues et al., 1986). During meiosis, *zyg-9* mutant embryos exhibit disorganized spindles and numerous cytoplasmic clusters of short microtubules (Kemphues et al., 1986). The exact mechanism of ZYG-9 function is unknown, however, it has been proposed that it influences microtubule length by affecting microtubule stability at the spindle poles by antagonizing other centrosomal factors that normally facilitate microtubule disassembly at the minus ends (Matthews et al., 1998). Several studies have shown that, in the absence of centrosomes, spindle microtubules form and elongate in the region

surrounding chromatin and are subsequently reorganized into a bipolar array with microtubule minus ends focused at the spindle poles (reviewed by Hyman and Karsenti, 1996). ZYG-9 is not required for the nucleation of microtubules in the meiotic spindle, since disorganized arrays of microtubules are detected around meiotic chromosomes in strong *zyg-9* alleles (Mains et al., 1990). ZYG-9 may organize the meiotic spindle through its recruitment to the region surrounding the meiotic chromosomes, where it, along with other proteins, promotes the elongation and rearrangement of microtubules into an ordered bipolar array (Matthews et al., 1998).

Microtubule motors are also critical for the formation of the meiotic spindle. RNAi of either of the kinesin genes, *klp-15* or *klp-16,* results in the failure of meiotic spindle assembly, and consequently leads to defects in meiotic chromosome segregation (Robin et al., 2005). KLP-15 is a minus-end directed motor and is believed to aid in spindle assembly by helping to move microtubules (Robin et al., 2005). Dynein also plays a role in meiotic spindle formation as shown by dynein heavy chain, *dhc-1*, mutants, which exhibit defects in centrosome separation and spindle assembly (Cockell et al., 2004). Dynein anchored at the cell cortex has been implicated in governing centrosome and spindle positioning by reeling in the plus ends of microtubules (reviewed by Dujardin and Vallee, 2002; Gonczy, 2002; Xiang, 2003). Additionally, dynein anchored at the nuclear periphery is thought to generate tension on the nuclear envelope to help trigger NEBD (Beaudouin et al., 2002; Salina et al., 2002). Overall, the identification of genes involved in organizing the meiotic spindle is important to understand spindle assembly, however, elucidating how hormonal signaling affects these genes is required to fully comprehend the regulation of meiotic spindle assembly.

CHAPTER II

# IDENTIFICATION OF MICROTUBULE REORGANIZATION IN *C. ELEGANS OOCYTES*

## Introduction

Meiosis generates haploid gametes, which combine through fertilization to generate a diploid zygote (reviewed by Page and Hawley, 2004). The viability of the resulting embryo, and the whole species for that matter, depend critically on the faithful segregation of the genetic material during meiosis. Non-disjunction during either of the meiotic divisions gives rise to aneuploid embryos, containing too many or too few chromosomes. In humans, non-disjunction during female meiosis I represents the leading cause of miscarriage and congenital birth defects, such as Trisomy 21 or Down syndrome (Hassold and Hunt, 2001). Previous studies in humans and mice suggest that disturbances in the hormonal regulation of folliculogenesis are associated with chromosome congression failure during meiotic spindle assembly, and may represent a significant risk factor for non-disjunction errors (Hodges et al., 2002). At present, it is not understood how signals that regulate oocyte growth and meiotic progression might influence meiotic spindle assembly and chromosome segregation.

Assembly of a bipolar meiotic spindle requires that the cytoplasmic microtubules gain access to the nuclear environment, which occurs upon NEBD during the meiotic maturation process and defines the transition between diakinesis and metaphase of meiosis I. While there have been many biochemical and cell biological investigations of meiotic spindle assembly, few studies have examined microtubule organization before

cytoplasmic microtubules become associated with chromatin upon NEBD. For example, early studies in sea urchin oocytes have revealed that cortical microtubules undergo a dramatic structural reorganization from an extensive basket-like array to a diminishing population of shorter microtubules during meiotic maturation (Boyle and Ernst, 1989). Despite this observation of oocyte microtubule rearrangement, a functional significance has not been elucidated in this organism.

In addition to studies in sea urchin, several changes in microtubule organization in *Drosophila* oocytes have been classified. During oogenesis, microtubule organization in *Drosophila* oocytes change in density by decreasing at the posterior pole and increasing at the anterior cortex (Theurkauf et al., 1992). Through the use of microtubule inhibitors, this structural asymmetry has been deemed necessary for the localization of determinants required for anterior-posterior polarity in the embryo. During meiotic maturation in *Drosophila*, microtubules reorganize again from a subcortical population to short randomly oriented filaments in the cytoplasm (Theurkauf et al., 1992). The subcortical array of microtubules is associated and required for cytoplasmic streaming, the mixing of ooplasm with incoming cytoplasm from the nurse cells (Gutzeit, 1986). When microtubules reorganize into short random filaments, cytoplasmic streaming stops and meiotic maturation is allowed to occur (Theurkauf et al., 1992). The observation that vesicles are often intimately associated with subcortical microtubules raises the possibility that vesicle translocation away from the plasma membrane is dependent upon microtubules originating at the cortex (Theurkauf et al., 1992). Despite the identification and hypothesized function of these cytoskeletal rearrangements, the signaling mechanisms that bring about these major cytoplasmic changes and their potential

biological significance remain unclear.

The nematode *C. elegans* is an ideal system for studying the effects of hormonal signaling on oocyte microtubules during the meiotic maturation process. These animals are transparent so meiotic maturation can be directly observed (Ward and Carrel, 1979; McCarter et al., 1999), and recent studies have begun to elucidate the key intercellular signaling pathways that regulate this process (reviewed by Yamamoto et al., 2006). In *C. elegans*, sperm trigger oocyte meiotic maturation using the major sperm protein (MSP) as a signaling molecule (Miller et al., 2001; Miller et al., 2003; Kosinski et al., 2005; Govindan et al., 2006). Since hermaphrodites produce only a fixed number of sperm, meiotic maturation rates are initially high, but decline as sperm are consumed by fertilization and levels of the MSP signal decline (McCarter et al., 1999; Kosinski et al., 2005). In sex-determination mutants of *C. elegans* that feminize the gonad (e.g., *fog-2* or *fog-3*) such that no sperm are produced, oocytes arrest at the diakinesis stage until sperm are supplied by mating.

Here we investigate whether meiotic signals affect the organization of the microtubule cytoskeleton in *C. elegans* oocytes by analyzing microtubules in both the absence of sperm (females) and in the presence of sperm (hermaphrodites or mated females). We describe how the presence of sperm influences the localization of oocyte microtubules prior to NEBD in *C. elegans*.

## Materials and Methods

### *Nematode Strains, Nomenclature, and Phenotypic Analysis*

Standard techniques were used for nematode culture at 20°C, except where indicated otherwise. The *C. elegans var. Bristol* strain, N2, was used as the wild-type hermaphrodite strain. We used *fog-2(q71)V* (Schedl and Kimble, 1988), which feminizes the germ line, to generate *XX* animals that do not produce sperm (females), a situation we refer to as a "minus sperm" condition to simplify the flow of information. Similarly, the "plus sperm" condition refers to experiments using either mated females or hermaphrodites as indicated. Key strains and alleles used were: *LGI: lon-2(e678), LGX: tth-1(gk43)*. The rearrangement used was: *szT1(I, X)*. Transgenes used were: AZ244 *pie-1::gfp::tubulin* (Praitis et al., 2001). MSP injections (200 nM MSP-142) and analysis of oocyte MAPK activation were described in Miller et al. (2001).

### *Fluorescence Microscopy*

*Preparation of Dissected Gonads and Antibody Staining*

Gonads were dissected, fixed, and stained for immunofluorescence microscopy as described (Rose et al., 1997). GFP fluorescence was analyzed in whole-mount using 0.1% tricaine/0.01% tetramisole treatment for 30 minutes as an anesthetic (McCarter et al., 1999). Antibodies used were: YL1/2 rat monoclonal anti-α-tubulin (Kilmartin et al., 1982) (Accurate Chemical and Scientific Corporation); mouse monoclonal anti-actin (MP Biomedicals); anti MAPK-YT (Sigma); and Cy2- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories).

Wide-field fluorescence microscopy employed Zeiss Axioskop or Axioplan microscopes using 40X and 63X (NA1.4) objective lenses. Images were acquired with an ORCA ER (Hamamatsu) charge-coupled device camera using OpenLab (Improvision) or MetaMorph (Universal Imaging) acquisition software. Pixel intensities were measured in arbitrary fluorescent units. All exposures were within the dynamic range of the detector. DNA was detected with DAPI.

*Laser-Scanning Confocal Microscopy*

Confocal images were acquired on a Zeiss LSM510 microscope with a pinhole of 1.0 Airy Units and 63X (NA 1.4) objective lens. Gain and offset were set so that all data were within the dynamic range of the PMT. Band pass filters were used to optically isolate the Cy2 and Cy3 fluorophores, and no cross-talk was observed.

***Quantitation of Cortical Microtubule Enrichment***

To calculate the cortical microtubule enrichment factor (CMEF), z-stack images of dissected gonads labeled with anti-tubulin and anti-actin antibodies were obtained on the confocal microscope and analyzed using MetaMorph (Universal Imaging) software. Cross-sectional images through medial focal planes were analyzed to avoid the overlying gonadal sheath cells. For each oocyte analyzed, ten line scans, each 10 pixels thick, were drawn from either the proximal to distal cortex or from the dorsal to ventral cortex, avoiding the nucleus. For each pixel, the corresponding intensities for tubulin and actin were then exported into an Excel (Microsoft) spreadsheet. The cortex was defined operationally, using an observer blind method, as the region of the micrograph in which

the actin intensity was greater than one standard deviation above its mean. Use of line scans, instead of regions of interest, reduced the potential for sampling bias, however, the use of a mathematical definition of the cortex tended to reduce the magnitude of the increase in cortical enrichment in females.

The CMEF was defined as the ratio of the average microtubule intensity at the cortex to the average microtubule intensity in the cytoplasm. Use of a ratio enabled comparisons between separate experiments that may have had varying overall staining levels. Because we measured pixel intensities, we could exclude the possibility that our results were due to oversampling cortical regions of adjacent oocytes relative to the cytoplasm. In time-course analyses, CMEF values among oocytes –1 to –5 within each day examined, and among days examined within each oocyte, were tested using Fisher's protected least significant difference (*lsd*) method at $p$=0.01.

## Results

### *Sperm Dependence of Microtubule Reorganization*

During female meiosis, dynamic microtubules assemble around chromatin instead of relying upon centrosomal MTOCs (Varmark, 2004). Meiotic spindle assembly commences when the cytoplasmic microtubules gain access to the chromatin upon NEBD during the oocyte meiotic maturation process. To investigate the origins of this specialized microtubule behavior during *C. elegans* oocyte meiotic maturation, we examined the organization of the dynamic microtubule cytoskeleton in oocytes prior to the onset of NEBD in the absence and presence of the MSP meiotic maturation signal.

Since tyrosinated α-tubulin is associated with newly formed microtubule populations (Westermann and Weber, 2003), we used YL1/2 monoclonal antibodies to stain tyrosinated-α-tubulin in dissected and fixed gonads from unmated and mated *fog-2(q71)* females and analyzed the results using laser-scanning confocal microscopy (Fig. 7A,B). In unmated females, in which sperm are absent, microtubules are highly enriched at the proximal and distal cortical edges of oocytes, but are sparsely dispersed throughout the cytoplasm (Fig. 7A). By contrast, in mated females, in which sperm are present, microtubules in proximal oocytes (–1 to –4) are evenly dispersed throughout the oocyte cytoplasm in a dense net-like array (Fig. 7B). Strikingly, the cortical enrichment of microtubules observed in the absence of sperm (Fig. 7A) is not observed in presence of sperm (Fig. 7B). This result suggests that the presence of sperm in the reproductive tract influences the organization of the oocyte microtubule cytoskeleton. Moreover, these data indicate that the sperm-dependent change in microtubule organization occurs prior to both NEBD, which is spatially restricted to the most proximal (–1) oocyte, and to fertilization, which occurs as the oocyte enters the spermatheca during ovulation (Fig. 5).

To determine whether the detection of tyrosinated α-tubulin in dissected and fixed gonads reflects the localization of bulk microtubules in living animals, we compared oocyte microtubules in unmated and mated *fog-2(q71)* females containing a β-tubulin-GFP fusion using confocal microscopy (Fig. 7C,D). Using this method, we observed a similar difference between the microtubule organization of unmated and mated females, with oocytes exhibiting an enrichment of microtubules at the proximal and distal cell cortex in the absence of sperm (Fig. 7C).

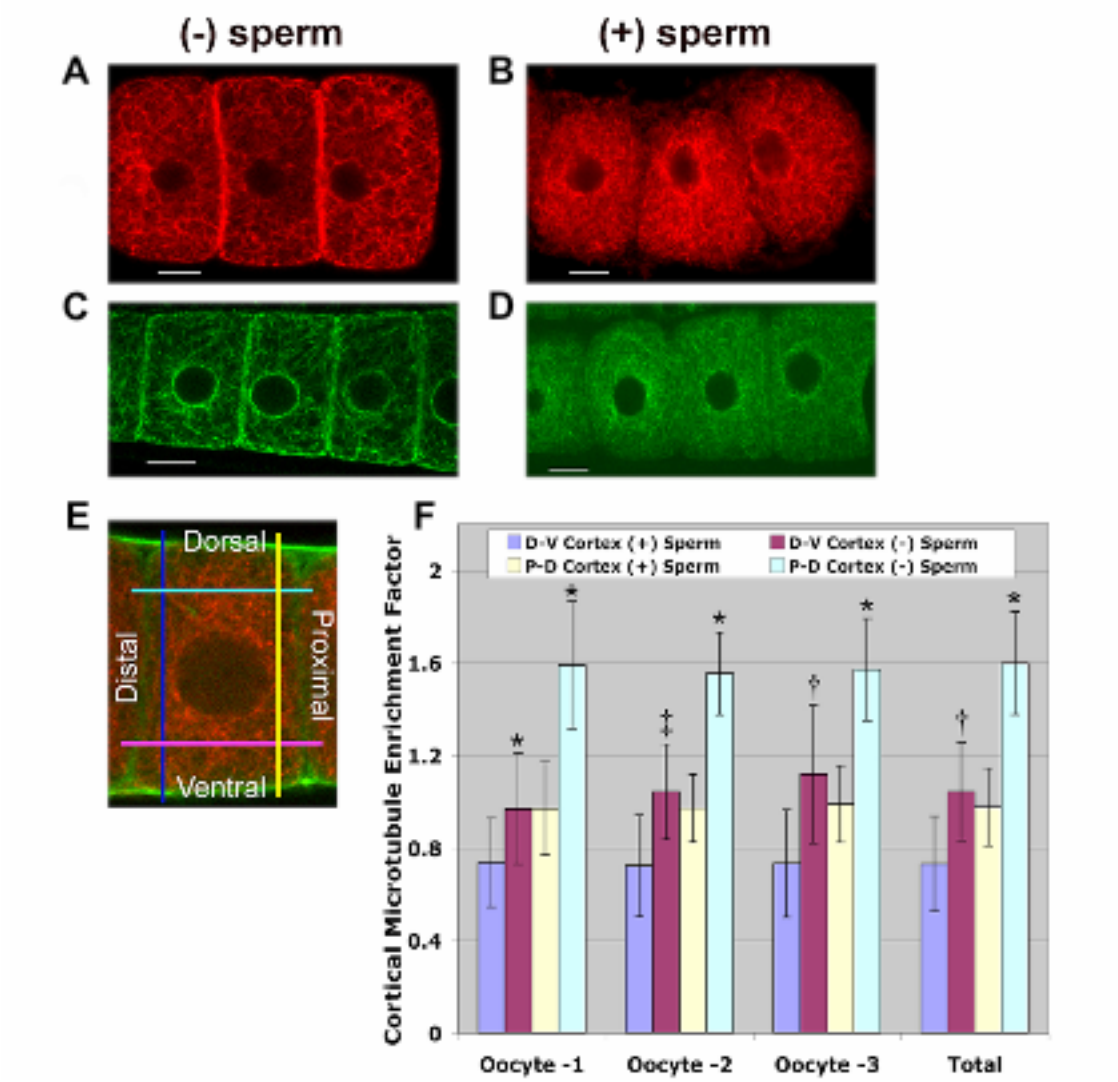



**Figure 7. Organization of microtubules in *C. elegans* oocytes.** (A-D) Single confocal images of the oocyte microtubule cytoskeleton in dissected and fixed gonads (A and B) or living animals (C and D) showing the localization of α-tubulin (A and B) or β-tubulin::GFP (C and D). (A and C) In unmated *fog-2(q71)* females, microtubules are cortically enriched between oocytes. (B and D) Microtubules are evenly dispersed throughout the cytoplasm in the most proximal oocytes in mated *fog-2(q71)* females. The perinuclear accumulation of microtubules observed in panel (C) and in subsequent figures is variable. (E) Single confocal image of a proximal oocyte in a dissected gonad stained for α-tubulin (red) and actin (green), illustrating the strategy for quantifying microtubule enrichment along the proximal-distal and dorsal-ventral axes with representative line scans drawn using Metamorph software. (F) Comparison of CMEFs from unmated and mated females at the proximal-distal and dorsal-ventral cortices of the -1 through -3 oocytes (in this and subsequent bar graphs the –1 oocyte is on the left). *t*-test between mated and unmated females, **p*<0.0001, †*p*<0.01, or ‡*p*<0.05, error bars represent s.d. Scale bars: 10 μm.

### *Measurement of Cortical Microtubule Enrichment*

To quantify this sperm-dependent difference in microtubule organization, we measured the average pixel intensity of cortical and cytoplasmic microtubules of the three most proximal oocytes in unmated (n=25) and mated (n=34) females, and calculated a cortical microtubule-enrichment factor (CMEF), expressed as the ratio of cortical to cytoplasmic intensities (Fig. 7E,F; see Materials and methods). In the absence of sperm, we observed a significant ($p$<0.0001) 1.5-fold enrichment of microtubules at the proximal and distal cortices (CMEF=1.51 ± 0.20). By contrast, microtubules were uniformly distributed throughout the cytoplasm of oocytes and were not enriched at the proximal and distal cortices of the cell in the presence of sperm (CMEF=1.02 ± 0.20) (Fig. 7F). In the absence of sperm, microtubule enrichment is limited to the proximal and distal cortical edges between oocytes as demonstrated by the lack of comparable microtubule enrichment at the dorsal and ventral cortical edges of oocytes in females (CMEF=1.04 ± 0.21, n=30) (Fig. 7F). In addition, microtubules were not highly enriched at the most proximal edge of the –1 oocyte, closest to the spermatheca, in females (CMEF=1.12 ± 0.32, n=10) compared to the distal edge adjacent to the –2 oocyte (CMEF=1.67 ± 0.29, n=10). These results define a sperm-dependent process, we term "oocyte microtubule reorganization," referring to the global transition between cortically enriched microtubules, as observed in unmated female oocytes, and uniformly distributed microtubules, as observed in oocytes from mated females.

### *Spatial and Temporal Distribution of Cortical Microtubule Enrichment in Hermaphrodites*

*C. elegans* hermaphrodites produce a fixed amount of sperm. Consequently,

extracellular levels of MSP in the reproductive tract and meiotic maturation rates progressively decline when sperm become depleted as hermaphrodites age (Kosinski et al., 2005). To determine whether microtubule reorganization is influenced by the decline in sperm numbers that occur as hermaphrodites age, we performed a time-course analysis of microtubule organization in proximal oocytes (Fig. 8A-C). We found that CMEF values along the proximal-distal axis progressively increase over the first five days of adulthood from 0.95 ± 0.08 at day 1 (n=76) to 1.40 ± 0.04 at day 5 (n=62) (Fig. 8B), approaching the CMEF levels seen in unmated females (Fig. 7F). To distinguish between aging and the presence of sperm as factors promoting microtubule reorganization in hermaphrodites, we mated 4-day-old hermaphrodites and analyzed their microtubules on day 5. These mated old hermaphrodites displayed reorganized microtubules characteristic of young hermaphrodites (CMEF=1.03 ± 0.23, n=73), suggesting that the presence of sperm, not age, is the key factor controlling microtubule organization in oocytes (Fig. 8B).

Since the CMEF measurements in this time-course analysis represent quantitative parameters describing oocyte microtubule organization, we applied the least significant difference (*lsd*) approach for performing pairwise comparisons to statistically analyze the CMEF values (see Materials and Methods). This analysis indicates that microtubule reorganization is not an all-or-none phenomenon in that there is a temporally graded response with intermediate CMEF levels observed in oocytes from 2-3-day-old hermaphrodites (Fig. 8A,B). If MSP signaling were directly responsible for microtubule reorganization, we would expect to observe a spatially graded response of oocyte microtubules because sperm in the spermatheca generate an extracellular MSP gradient

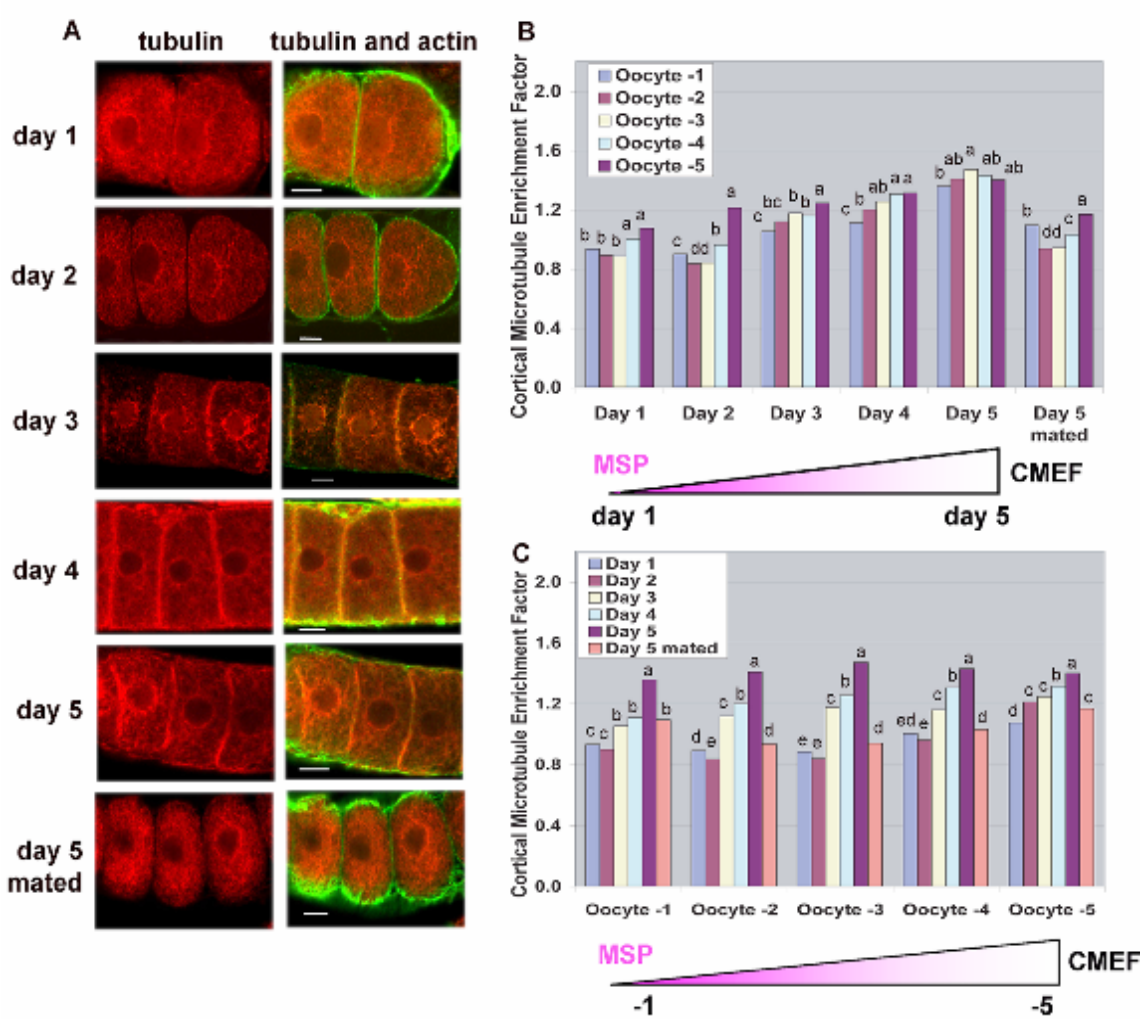

A
tubulin
tubulin and actin
day 1
day 2
day 3
day 4
day 5
day 5 mated
B
Cortical Microtubule Enrichment Factor
Oocyte -1
Oocyte -2
Oocyte -3
Oocyte -4
Oocyte -5
Day 1
Day 2
Day 3
Day 4
Day 5
Day 5 mated
MSP
CMEF
day 1
day 5
C
Cortical Microtubule Enrichment Factor
Day 1
Day 2
Day 3
Day 4
Day 5
Day 5 mated
Oocyte -1
Oocyte -2
Oocyte -3
Oocyte -4
Oocyte -5
MSP
CMEF
-1
-5

**Figure 8. Oocyte microtubule time course in hermaphrodites.** (A) Single confocal images of hermaphrodite oocytes labeled with tubulin (red) and actin (green) during the time course. Day-4 hermaphrodites were mated with males and analyzed on day 5 (bottom panel). (B) Comparison of cortical microtubule enrichment over time (between 13-17 gonad arms were analyzed per time point). When MSP is present during the first few days of adulthood, the CMEF is low. As hermaphrodites age and MSP is depleted, the CMEF increases. The wedge depicting the MSP gradient is an estimate for illustrative purposes–see Kosinski et al. (2005) for measurements. The *lsd* method was used to compare the means of different oocytes at each time. Means with the same letter designation are not significantly different from one another within each individual day grouping (e.g., a=a, b=bc, and bc=c); whereas means with different letter designations are significantly different ($p<0.01$; eg., b ≠ c, but bc=c and bc=b). Distal oocytes exhibit higher CMEF values than proximal oocytes. (C) Comparison of cortical microtubule enrichment by oocyte. The *lsd* method was used to compare means at different time points for each individual oocyte. For each oocyte, CMEF values are lower earlier in adulthood when extracellular MSP levels are higher. Scale bars: 10 μm.

such that proximal oocytes are exposed to greater MSP levels than distal oocytes (Kosinski et al., 2005). To test this possibility, we analyzed CMEF values among oocytes within a time point using *lsd* (Fig. 8C). Significantly lower CMEF values were observed in proximal (–1, –2, –3) than in distal (–4, –5) oocytes, supporting the idea that microtubule reorganization is spatially graded over the proximal gonad arm (Fig. 8B,C).

***MSP is Sufficient for Oocyte Microtubule Reorganization***

These time-course data are consistent with the hypothesis that MSP may be sufficient to signal microtubule reorganization. To test this possibility directly, we injected 200 nM purified MSP or a control buffer into the uterus of unmated females. After various recovery times (30, 60, or 120 min), we quantified oocyte microtubule organization (Fig. 9A,B). We also verified that the purified MSP injections were sufficient to activate MAPK, stimulate meiotic maturation, and sheath cell contractions (Fig. 9C, and data not shown). Females injected with MSP exhibited a decreased average CMEF value of 1.23 ± 0.30 (n=15) by 30 min following MSP injection compared to buffer injected controls, which displayed an average CMEF value of 1.61 ± 0.42 (n=60). Oocytes located more distal from the uterine injection site (e.g., oocytes –2 and –3), tended to exhibit higher CMEF values than the most proximal oocyte. These results provide evidence that the MSP signal is sufficient to reorganize microtubules in proximal oocytes. In addition to the spatial distribution of cortical enrichment, allowing a longer time of recovery after MSP injection caused the CMEF of the most proximal oocyte to increase (Fig. 9B). These results further support a temporal distribution in that as the MSP signal diminishes, oocyte microtubules become cortically enriched.

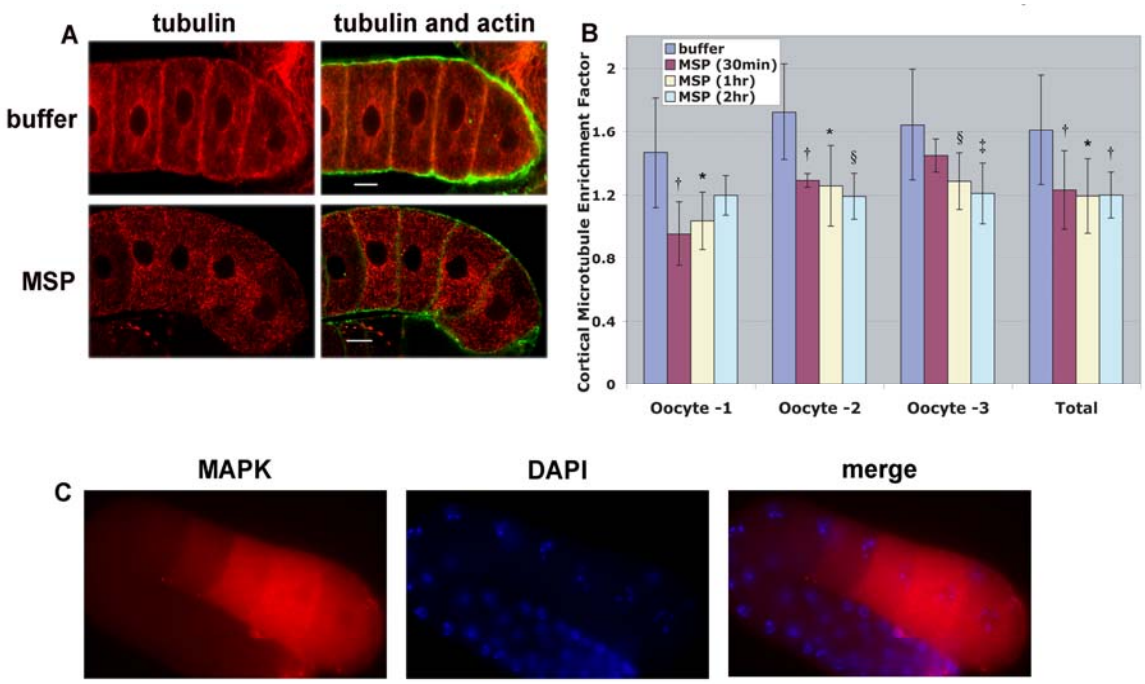



**Figure 9. MSP is sufficient to reorganize oocyte microtubules.** (A) Effect of MSP on oocyte microtubule reorganization. Females were injected with either 200 μM purified MSP or a control buffer. Single confocal images of oocytes are labeled with tubulin (red) and actin (green). Microtubules appear cortically enriched in buffer-injected females and reorganized in MSP-injected females. (B) Quantification of oocyte microtubule reorganization in MSP-injected females, which were allowed to recover for 30 min, 1 hour, or 2 hours after injection (*t*-test between buffer injected females and each MSP injection condition, **p*<0.0001, §*p*<0.001, †*p*<0.01, ‡*p*<0.05, error bars represent s.d.). (C) Verification that MSP injections are sufficient to activate MAPK in female oocytes as initially described by Miller et al. (2001). Fluorescence images of an MSP injected female labeled with both MAPK (red) and DAPI (blue). Scale bars: 10 μm.

## Discussion

### *MSP Signaling Reorganizes Oocyte Microtubules*

In addition to nuclear changes presaging the final steps of meiotic chromosome segregation, the meiotic maturation process encompasses cytoplasmic alterations, collectively referred to as "cytoplasmic maturation". Cytoplasmic maturation includes the reorganization of cytoplasmic organelles, cytoskeletal remodeling, activation of signaling for ovulation, and cellular events important for fertilization and polarity establishment (reviewed by Voronina and Wessel, 2003; Greenstein and Lee, 2006; Yamamoto et al., 2006). The full extent of the cytoplasmic meiotic maturation program and its regulation is considerably less well understood than the control of meiotic cell cycle progression.

We have investigated changes in the oocyte microtubule cytoskeleton during early steps of the meiotic maturation process in *C. elegans*. We show that the sperm-derived meiotic maturation signal, MSP, triggers cytoplasmic microtubule reorganization in the oocyte prior to NEBD in a temporal and spatial manner. When MSP is absent, as in females or older hermaphrodites, microtubules are enriched at the proximal and distal cortices of oocytes. In mated females or younger hermaphrodites, microtubules are dispersed evenly in a net-like fashion throughout the cytoplasm of proximal oocytes. We used a quantitative assay for oocyte microtubule reorganization to show that purified MSP is sufficient to direct this cytoskeletal remodeling.

Interestingly, we observed an asymmetry of cortical microtubule accumulation in the -1 oocyte in the absence of sperm in that microtubules were enriched at the distal, but not the proximal cortex. While the significance of this finding is unclear, we

speculate that this asymmetry may be part of the mechanism by which this oocyte "knows" it is in the most proximal position and thus competent to undergo meiotic maturation upon receiving the MSP signal. Overall, these studies reveal that a remarkable change in the microtubule cytoskeleton occurs in response to the MSP meiotic maturation hormone and that these changes occur prior to both NEBD and fertilization.

### *Are Other Populations of Microtubules Affected by MSP?*

Even though our studies primarily used tyrosinated α-tubulin antibodies, which are associated with dynamic, newly formed microtubule populations (Westermann and Weber, 2003), tubulin is subject to several other posttranslational modifications that affect both the function and dynamics of microtubules (reviewed by Barra et al., 1988; Westermann and Weber, 2003). Future experiments may involve examining the localization of microtubule populations with other posttranslational modifications, such as acetylation and polyglutamylation. It remains unclear whether acetylated or stable populations of microtubules exist in oocytes and undergo reorganization in response to MSP. The localization of polyglutamylated tubulin would also be interesting to examine is because of its proposed interaction with associated proteins. For example, MAPs, such as kinesin, have been shown to have a high affinity for polyglutamylated microtubules (Larcher et al., 1996). If the localization of polyglutamylated tubulin changed in the presence of sperm, this may indicate that MAPs are also being translocated to a different location in the oocyte. Inhibitors of posttranslational modifications may also help in determining their effect on microtubules. Studies have shown that inhibiting the tubulin

deacetylase, HDAC6, may help to examine the effect of destabilizing microtubules that were previously stabilized by acetylation (Matsuyama et al., 2002). There are several deacetylases in *C. elegans* that could be analyzed for an effect on microtubules. In particular, RNAi of *hda-1* has been shown to affect gonadogenesis and causes large oocytes to grow in the gonad (Dufourcq et al., 2002).

### *Microtubule Reorganization is Distinct from the Cortical Actin Rearrangement of the Most Proximal Oocyte*

The microtubule changes we describe here, which we term "oocyte microtubule reorganization," are distinct from "oocyte cortical rearrangement," or the rounding-up of the oocyte just prior to ovulation, described by McCarter and Schedl (1999), in at least two significant ways. First, oocyte microtubule reorganization occurs prior to the onset of NEBD, whereas oocyte cortical rearrangement occurs after the onset of NEBD (McCarter et al., 1999). Second, oocyte cortical rearrangement is spatially restricted to the maturing –1 oocyte and occurs in the time window between the onset of NEBD and ovulation (3-5 min prior to ovulation). By contrast, proximal oocytes that receive the MSP signal undergo microtubule reorganization. The possibility that oocyte cortical rearrangement (McCarter et al., 1999) represents an actin-based process is consistent with static images of phalloidin- or anti-actin-stained fixed gonads and embryos (Strome, 1986), but real-time analysis will be needed to address this possibility fully. MSP signaling thus induces an early reorganization of the microtubule cytoskeleton, which might be followed by later changes in the actin cytoskeleton.

To test whether cortical actin found between oocytes plays a direct role in microtubule reorganization we examined oocyte microtubules in *tth-1(gk43)* mutants.

*tth-1* encodes tetraThymosinβ, which is expressed between oocytes and is crucial for meiotic maturation by regulating actin polymerization and organization (Van Troys et al., 2004). It has been reported that *tth-1(gk43)* mutants do not contain actin filaments between oocytes and oocytes become deformed when ovulated into the spermatheca and uterus (Van Troys et al., 2004). Therefore, *tth-1* has been proposed to be required for oocyte rigidity and sheath contractions. Since *tth-1* mutant hermaphrodites do not contain cortical actin between oocytes, we examined whether microtubule reorganization was affected. Even though the gonad arms in these mutants were small, containing one or two oocytes, microtubules were still dispersed throughout the cytoplasm (data not shown). We cannot rule out the possibility that actin still may interact with microtubules at the cortex in oocytes since we did not examine feminized mutants of *tth-1*. Further work will be needed to determine whether actin anchors and regulates cortical microtubules in *C. elegans* oocytes.

CHAPTER III

# MICROTUBULE DYNAMICS IN *C. ELEGANS* OOCYTES

## Introduction

The meiotic spindles of most animal oocytes are distinctive from mitotic spindles of somatic cells in terms of their mechanism of assembly, their function, and their modes of regulation. The female meiotic spindles of many species are both acentriolar and anastral (Szollosi et al., 1972; Albertson and Thomson, 1993; Schatten, 1994; Matthies et al., 1996). Instead of relying on centrosomes, which are eliminated during oogenesis in many species, the meiotic chromatin directs the assembly of a bipolar spindle both by nucleating microtubules and organizing preexisting microtubules, which then self-organize through incompletely understood mechanisms involving microtubule motors and dynamics (Heald et al., 1996; Matthies et al., 1996; Walczak et al., 1998; Skold et al., 2005). Biochemical studies in *Xenopus* egg extracts, which provide a model for assembly of the meiosis II spindle, indicate that DNA-coated beads can drive the self-organization of microtubules into bipolar spindles (Heald et al., 1996). Cultured mammalian cells might utilize analogous mechanisms in parallel with centrosome-dependent mechanisms as bipolar spindles can assemble after laser ablation of centrosomes (Khodjakov et al., 2000). Chromatin-based microtubule assembly pathways employ Ran-GTP-dependent mechanisms (Carazo-Salas et al., 1999; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999), as well as mechanisms utilizing the Aurora B kinase chromosomal passenger complex (Sampath et al., 2004).

While many studies have addressed the chromatin-based mechanism of female meiotic spindle assembly, it is less clear how signaling influences microtubule localization and dynamics prior to NEBD in the absence of centrosomes. In other systems, microtubule end binding proteins have proven useful to study microtubule dynamics (Schroer, 2001; Akhmanova and Hoogenraad, 2005). Recently, a stable transgenic strain expressing the fluorescent marker EBP-2::GFP, an EB1 human homolog that decorates the growing plus ends of microtubules, has been developed and analyzed in *C. elegans* embryos where in the past dense microtubule arrays in the embryo has hindered the direct visualization of individual microtubules *in vivo* (Srayko et al., 2005). This study has utilized EBP-2::GFP hermaphrodites to study the growth rate, nucleation rate, and distribution of astral and spindle microtubules during mitosis. They have found that astral microtubules grow from centrosomes at an extraordinary speed of 0.7 μm/s and are limited by the availability of free tubulin and driven by the ZYG-9-TAC-1 complex (Srayko et al., 2005). In our study, we investigate the localization and dynamics of growing microtubule plus ends in *C. elegans* oocytes prior to fertilization using EBP-2::GFP hermaphrodites.

One useful tool for characterizing microtubule stability in *C. elegans* is fluorescence recovery after photobleaching (FRAP). Experiments with FRAP started 30 years ago to visualize the lateral mobility and dynamics of fluorescent proteins in living cells. Its popularity increased when non-invasive fluorescent tagging became possible with GFP. Many researchers use GFP to study the localization of fusion proteins in fixed or living cells, but the same fluorescent proteins can also be used to study protein mobility in living cells. FRAP has the potential to study cytoskeletal protein dynamics,

activity, and stability within a single living cell. These measurements can be made with most standard confocal laser-scanning microscopes equipped with photobleaching protocols (reviewed by Reits and Neefjes, 2001). One study in *C. elegans* has employed FRAP analysis to characterize *tac-1*, which has been discovered to be essential for pronuclear migration and spindle elongation in one-cell-stage embryos (Bellanger and Gonczy, 2003). TAC-1 is present in the cytoplasm and is enriched at centrosomes in a cell cycle-dependent manner, and its centrosomal localization is independent of microtubules but requires the activity of γ-tubulin and the Aurora-A kinase AIR-1. The utilization of an *in vivo* FRAP-based assay in embryos expressing GFP-TAC-1, established that centrosomal TAC-1 exchanges rapidly with the cytoplasmic pool and that inactivation of *tac-1* results in defective microtubule assembly (Bellanger and Gonczy, 2003). This study suggests that FRAP analysis is helpful in describing protein stability and dynamics in *C. elegans*. Our study uses FRAP analysis to examine tubulin exchange rates in oocytes of tubulin::GFP mated and unmated females.

## Materials and Methods

### *Nematode Strains, Nomenclature, and Phenotypic Analysis*

Standard techniques were used for nematode culture at 20°C, except where indicated otherwise. The *C. elegans var. Bristol* strain, N2, was used as the wild-type hermaphrodite strain. We used *fog-2(q71)V* (Schedl and Kimble, 1988), which feminizes the germ line, to generate *XX* animals that do not produce sperm (females), a situation we refer to as a "minus sperm" condition to simplify the flow of information. Similarly, the "plus sperm" condition refers to experiments using either mated females or

hermaphrodites as indicated. Key strains and alleles used are described in WormBase (http://www.wormbase.org), and transgenes used were: TH66 *pie-1::ebp-2::gfp* (Srayko et al., 2005); and AZ244 *pie-1::gfp::tubulin* (Praitis et al., 2001).

### *Fluorescence Microscopy*

#### *Preparation of Dissected Gonads and Antibody Staining*

Gonads were dissected, fixed, and stained for immunofluorescence microscopy as described (Rose et al., 1997). For nocodazole experiments, gonads were dissected and soaked in a 10 μg/mL solution of nocodazole for 30 minutes. GFP fluorescence was analyzed in whole-mount using 0.1% tricaine/0.01% tetramisole treatment for 30 minutes as an anesthetic (McCarter et al., 1999) or in unfixed dissected gonads, which were mounted on agar pads using double-sided tape as a spacer to prevent the cover glass from smashing the gonads. Antibodies used were: anti-CeGrip-1 (kindly provided by Karen Oegema and Tony Hyman).

#### *Laser-Scanning Confocal Microscopy*

Confocal images were acquired on a Zeiss LSM510 microscope with a pinhole of 1.0 Airy Units and 63X (NA 1.4) objective lens. Gain and offset were set so that all data were within the dynamic range of the PMT. Band pass filters were used to optically isolate the Cy2 and Cy3 fluorophores, and no cross-talk was observed.

*Spinning Disk Confocal Microscopy*

Spinning disk confocal microscopy used a Perkin Elmer Ultraview LCI system on a Zeiss 200M inverted microscope with a 63X (1.4 NA) objective lens. Images were acquired using an ORCA ER (Hamamatsu Photonics) camera and Ultraview (Perkin Elmer) software. The thickness of the sample and the small size of the EBP-2::GFP foci, coupled with the inherent photosensitivity of the meiotic maturation process (McCarter et al., 1999), represented technical difficulties. We were able to increase the signal-to-noise by utilizing a preparation in which the worm was decapitated to allow buffer exchange with the pseudocoelom or the gonad arm outside the carcass. Movies were obtained over one minute in a single confocal plane (~0.4 μm thick) with a gain of 255, 1 X 1 binning, and an exposure of 250 ms. Images were acquired as a stream with no recovery time between images. The density of growing plus ends was measured in different regions of the oocyte by counting the number of brightly labeled EBP-2::GFP foci in a 36.4 $\mu m^3$ region using Metamorph software. The directional movement of growing plus ends that remained in the focal plane for 3-5 consecutive frames was analyzed for n=200 EBP::GFP foci in each region of the oocyte using LSM Examiner (Zeiss) software. To calculate the rate of movement, we measured the distance a plus end traveled over 5 frames (~1.5 sec) using Metamorph. Recordings and measurements were made in the –1 and –2 oocytes prior to NEBD and similar results were obtained.

*Fluorescence Recovery After Photobleaching (FRAP)*

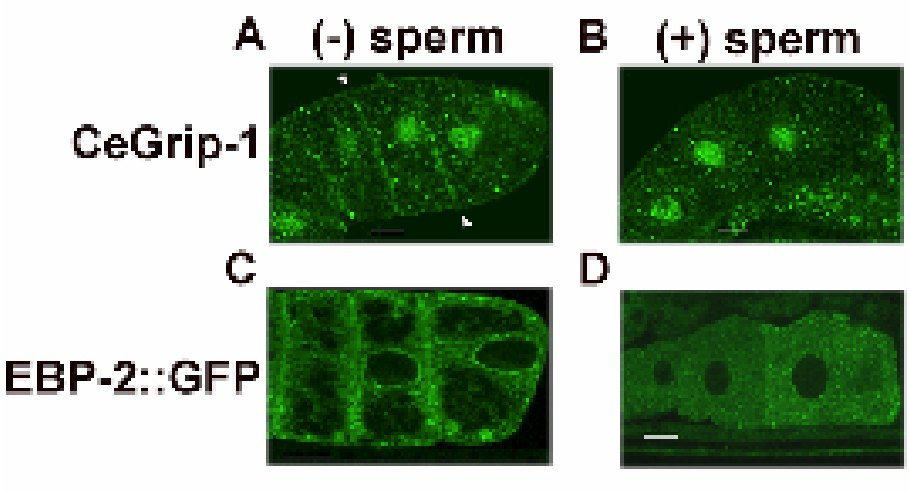
FRAP was conducted on the LSM510 confocal microscope on a single confocal slice. A box of 77 X 24 pixels (73.92 $\mu m^2$) was bleached with 100% laser power after

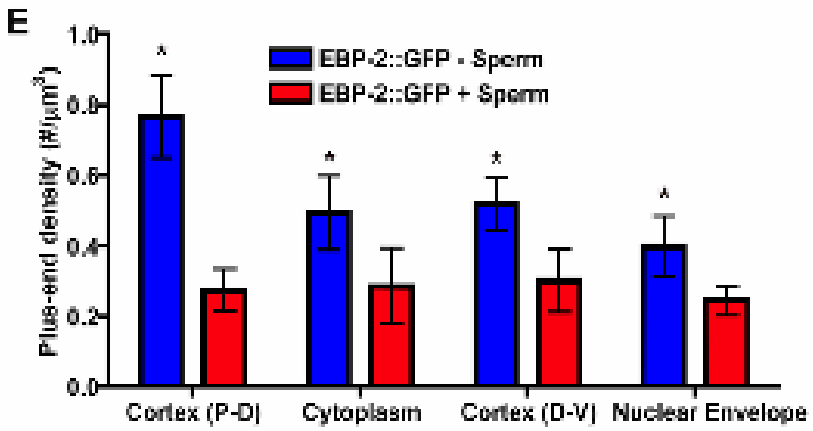
three pre-bleach images and fluorescence recovery was measured every 1.57 seconds over 77 images. Fluorescence recovery was observed in the cytoplasm and at the cortex of anesthetized *pie-1::gfp::tubulin* mated and unmated females (15 laser-pulse iterations per bleach) and *pie-1::gfp* hermaphrodites and females (100 laser-pulse iterations per bleach). More laser-pulse iterations were needed to bleach GFP fluorescence in the *pie-1::gfp* animals due to the greater GFP signal in oocytes. Fluorescence intensities within the bleached area of the cell were normalized to the intensities of the whole cell after correcting for the background fluorescence. FRAP experiments and measurements were made in the –1 and –2 oocytes prior to NEBD and similar results were obtained.

## Results

### *MSP Signaling Affects the Localization of Microtubule Plus and Minus Ends*

During oogenesis, centrioles and centrosomes are lost in many organisms; and in *C. elegans*, they disappear prior to the late stages of oocyte differentiation. Therefore, the organization of microtubule plus and minus ends in oocytes is not specifically known. To further analyze the organization of microtubules in *C. elegans* oocytes, we examined the localization of CeGrip-1, a component of the γ-tubulin ring complex, which nucleates and anchors the minus ends of microtubules (Hannak et al., 2002), and thus approximates the localization of microtubule minus ends. In the absence of sperm, we observed an enrichment of CeGrip-1 at the cortex (Fig. 10A, arrowheads), which is not seen in the presence of sperm (Fig. 10B). Rather, CeGrip-1 is diffusely distributed throughout the cytoplasm in the presence of sperm (Fig. 10B; the nuclear staining appears to represent a


A
(-) sperm
B
(+) sperm
CeGrip-1
C
D
EBP-2::GFP


E
EBP-2::GFP - Sperm
EBP-2::GFP + Sperm
Plus-end density (#/μm²)
1.0
0.8
0.6
0.4
0.2
0.0
Cortex (P-D)
Cytoplasm
Cortex (D-V)
Nuclear Envelope

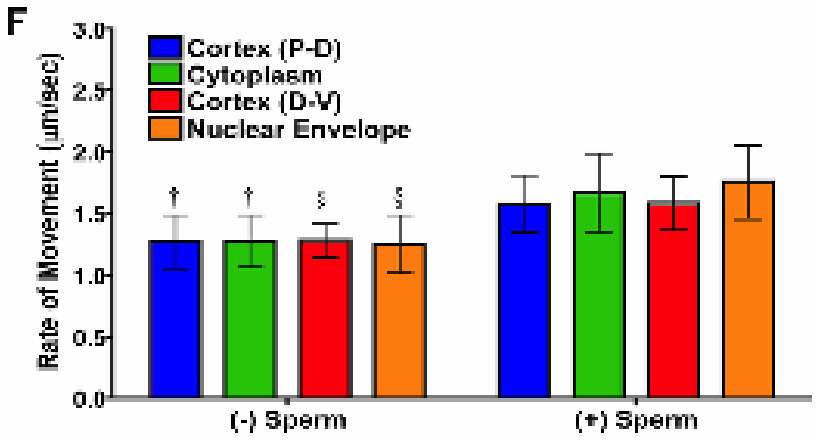

F
Cortex (P-D)
Cytoplasm
Cortex (D-V)
Nuclear Envelope
Rate of Movement (μm/sec)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
(-) Sperm
(+) Sperm

**Figure 10. MSP affects the localization and dynamics of microtubules.** (A and B) Single confocal images of a fixed unmated (A) and mated (B) *fog-2(q71)* female labeled with CeGrip-1 antibodies. CeGrip-1 is dispersed in a punctate fashion throughout the cytoplasm in the presence of sperm (B), but there is cortical enrichment (arrowheads) in the absence of sperm (A). The nuclear staining is likely due to cross-reactivity of the antibody. (C and D) Single confocal images of a live unmated female (C) and hermaphrodite (D) expressing EBP-2::GFP. EBP-2::GFP is enriched at the cortex in unmated females (C), but is dispersed in a punctate fashion throughout the cytoplasm in hermaphrodites (D). (E) Density of growing microtubule plus ends at different regions of the oocyte in females and hermaphrodites (*t*-test between females and hermaphrodites, **p*<0.0001, error bars represent s.d.). (F) Rate of plus-end movement in females and hermaphrodites at different regions of the oocyte (*t*-test between female and hermaphrodite, §*p*<0.001, †*p*<0.01, error bars represent s.d.). Scale bars: 10 μm.

non-specific cross-reaction). Overall, this data suggests that microtubule nucleation at the oocyte cortex, as defined by minus end localization, is increased in the absence of sperm.

In addition to minus end distribution, we investigated the localization of growing plus ends using an EBP-2::GFP fusion construct (Srayko et al., 2005). Single confocal images reveal that growing microtubule plus ends are enriched at the cortex in the absence of sperm (Fig. 10C). By contrast, growing plus ends are distributed throughout the cytoplasm in the presence of sperm (Fig. 10D). This result mimics the distribution of CeGrip-1, suggesting that there is an increase in both microtubule nucleation and growth at the cortex of oocytes in the absence of sperm.

***MSP Signaling Affects the Dynamics of Microtubule Plus Ends***

To visualize the dynamic nature of microtubules in oocytes, we used spinning disk confocal microscopy to image the behavior of EBP-2::GFP, which marks growing microtubule plus ends (Srayko et al., 2005), in oocytes of living females and hermaphrodites. We visualized growing plus ends in one-minute stream acquisitions by tracking the movement of EBP-2::GFP foci that remained in the focal plane for 3-5 consecutive frames. We observed that growing plus ends appeared to move randomly throughout the oocyte cytoplasm in the presence of sperm (Fig. 11B). In the absence of sperm, however, we observed a directional movement bias of growing microtubule plus ends at the oocyte cortex, in which they appeared to travel up and down along the proximal-distal cortex, or back and forth along the dorsal-ventral cortex (Fig. 11A). Interestingly, in the cytoplasm of female oocytes, the plus ends appeared to move along

tracks or bundles extending from the nucleus to the cortex (Fig. 11A, white arrows). Growing microtubule plus ends that contacted the nuclear envelope appeared to move more rapidly, similar to observations made in embryos by Srayko et al. (2005), who suggested this behavior is due to dynein motors localized at the cytoplasmic face of the nuclear envelope. We also noticed that the dissection protocol increased the rate of spontaneous maturation of the –1 oocyte, and that growing microtubule plus ends appeared to move randomly (Fig. 11A, asterisk) in these spontaneously maturing oocytes.

In order to quantify sperm-dependent differences in microtubule behavior, we examined the density, direction, and rate of movement of growing plus ends at multiple regions of the oocyte in the absence and presence of sperm, at the proximal-distal cortex, the dorsal-ventral cortex, the cytoplasm, and around the nuclear envelope. To compare the density of growing plus ends in EBP-2::GFP females and hermaphrodites, we measured the number of actively growing plus ends in a 36.4 $\mu m^3$ volume at various regions of the oocyte, focusing on strong staining foci within the focal plane. We observed that oocytes contained a higher density of growing plus ends in the absence of sperm at each region of the oocyte (Fig. 10E). In addition, the proximal and distal cortex exhibited the largest difference in plus-end density. In the absence of sperm, the number of growing plus ends at the proximal and distal cortex was 0.77 ± 0.02 plus ends/$\mu m^3$ (n=30), whereas, there was a significant decrease (almost three-fold, $p<0.0001$) in growing plus ends at the cortex in the presence of sperm (0.28 ± 0.01 plus ends/$\mu m^3$, n=30) (Fig. 10E).

***Directionality and Rate of Movement of Growing Plus Ends***

To assess the trajectories of growing plus ends in a quantitative manner, we tracked the movement of EBP-2::GFP foci that remained in the plane of focus over five consecutive frames (~1.5 sec) for each region of the oocyte. We defined the distal to proximal axis as 0° and divided the oocyte medial plane into eight categories (A-H), each spanning a 45°-interval (Fig. 11C). Growing plus ends were assigned to a category based on the direction vector of their movement. In the absence of sperm, growing plus ends displayed a directional movement bias at the proximal-distal and dorsal-ventral cortex of the oocyte (Fig. 11A,D,E and Fig. 12B). At the proximal and distal cortex in females, plus-end movement predominately fell into categories B, C, F, and G, corresponding to movement up and down along the cortex between oocytes (Fig. 11A,D and Fig. 12B). Likewise, at the dorsal or ventral cortex of the oocyte in females, moving plus-ends predominately fell into categories A, D, E, and H, corresponding to plus-end movement along the dorsal and ventral cortex of the oocyte (Fig. 11A,E and Fig. 12B). By contrast, in the presence of sperm growing plus ends exhibited uniformly distributed movement in each region of the oocyte (Fig. 11B,D,E and Fig. 12A). These data support our initial visual observations and suggest that MSP signaling affects both the spatial organization of microtubules and their directionality of movement.

To assess the rate of movement of growing microtubule plus ends at different regions of oocytes, we measured the distance EBP-2::GFP foci traversed over five consecutive frames (~1.5 sec) in females and hermaphrodites (Fig. 10F). The rate of movement of EBP-2::GFP foci reflects microtubule growth as well as the physical translation of microtubules via motor action (Srayko et al., 2005). We observed that the

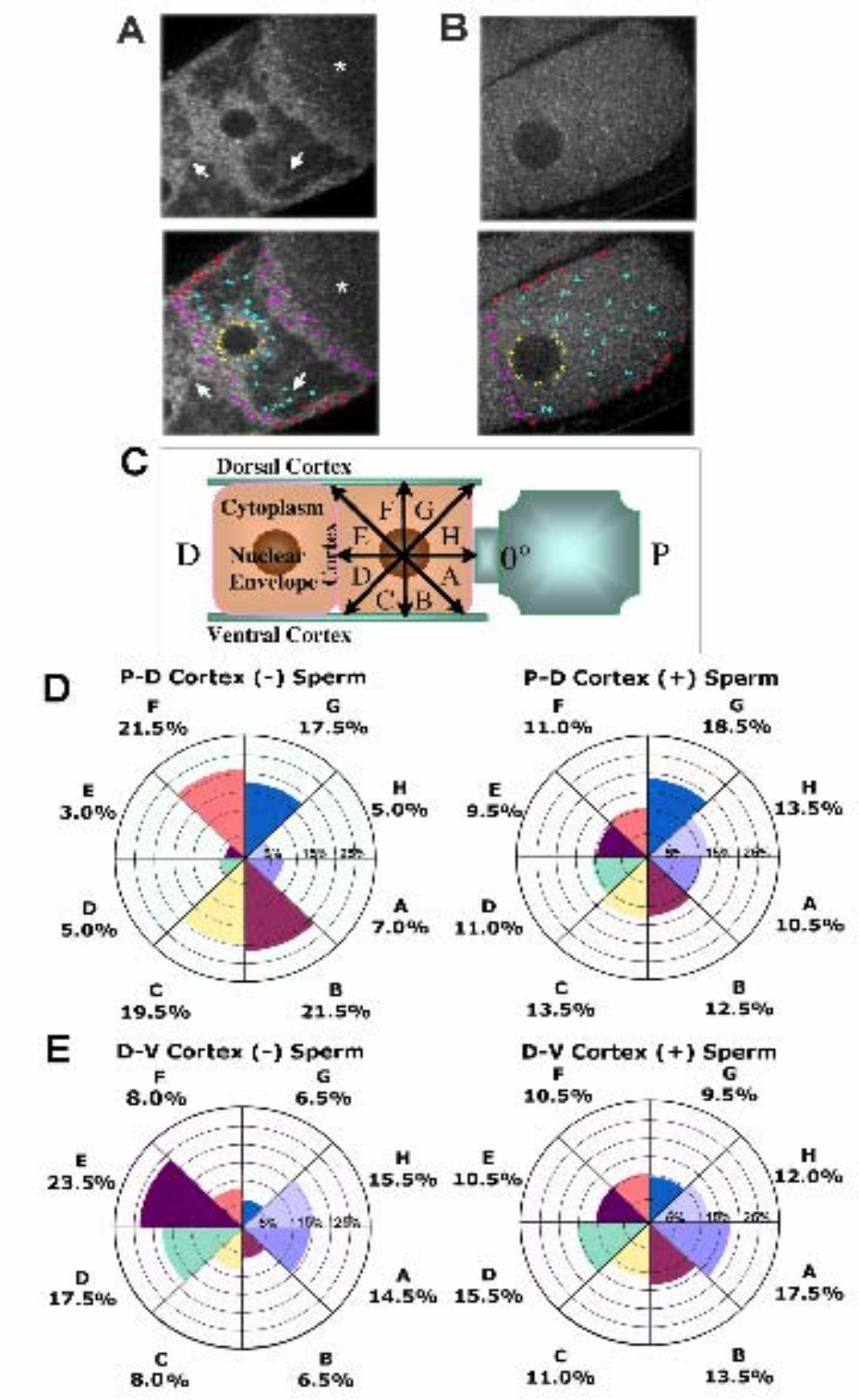

A
B
C
Dorsal Cortex
Cytoplasm
Nuclear Envelope
Cortex
D
P
0°
F
G
H
A
B
C
Ventral Cortex
D
P-D Cortex (-) Sperm
F 21.5%
G 17.5%
H 5.0%
A 7.0%
B 21.5%
C 19.5%
D 5.0%
E 3.0%
P-D Cortex (+) Sperm
F 11.0%
G 18.5%
H 13.5%
A 10.5%
B 12.5%
C 13.5%
D 11.0%
E 9.5%
E
D-V Cortex (-) Sperm
F 8.0%
G 6.5%
H 15.5%
A 14.5%
B 6.5%
C 8.0%
D 17.5%
E 23.5%
D-V Cortex (+) Sperm
F 10.5%
G 9.5%
H 12.0%
A 17.5%
B 13.5%
C 11.0%
D 15.5%
E 10.5%

**Figure 11. Analysis of growing plus-end movement in oocytes.** (A and B) Upper panels show spinning-disk confocal images of an EBP-2::GFP unmated female (A) and hermaphrodite (B). Lower images are overlaid with multicolored arrows that correspond to the direction of plus-end movement at different regions of the oocyte (red=D-V cortex, purple=P-D cortex, blue=cytoplasm, and yellow=nuclear envelope). (A) The white arrows indicate tracks within the unmated female oocyte that growing plus ends predominately move along. The white asterisk indicates an oocyte that has spontaneously matured in the absence of sperm. (C) Diagram showing the strategy for categorizing the directional movement of growing plus ends. In a clockwise manner from the proximal edge of the oocyte, categories were assigned in 45° intervals (labels from A-H), corresponding to the direction vector of plus-end movement. (D and E) Circular diagrams demonstrating the percentage of plus ends per 45° category at the P-D cortex (D) or the D-V cortex (E) of females and hermaphrodites, as indicated. The chi-square test was used to test the null hypothesis that microtubule plus ends exhibit uniform directional movement. The null hypothesis was rejected in females at the P-D and D-V cortices ($p<0.001$). Scale bars: 10 μm.

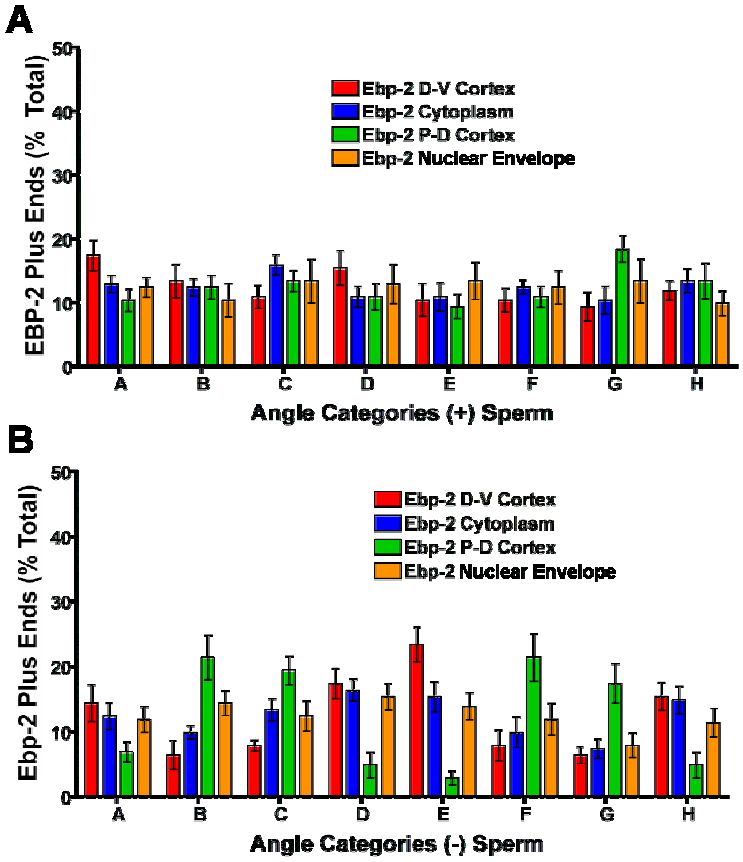



**Figure 12. Direction of growing plus-ends at different regions of the oocyte.** Percentages of growing plus ends (EBP-2::GFP foci) in the corresponding angle categories at different regions of the oocyte in hermaphrodites (A) or females (B). Error bars represent s.d..

rates of movement of growing plus ends were largely position independent in the absence and presence of sperm, with the exception of some plus ends that contacted the nuclear envelope (Fig. 10F and data not shown). We observed a significant ($p$<0.001) difference in the rate of plus-end movement between females and hermaphrodites (Fig. 10F). In females, plus ends move at a slightly slower rate (1.27 ± 0.01 μm/sec, n=400; Fig. 10F) compared to hermaphrodites (1.65 ± 0.08 μm/sec, n=400; Fig. 10F). The slower movement rate observed in females might be reflective of the overall increase in the density of growing plus ends observed in the absence of sperm (Fig. 10E).

***FRAP Analysis of Oocyte Microtubules***

The data presented above suggest that MSP affects microtubule organization and dynamics in oocytes. To determine whether the exchange rate of free tubulin into microtubules is also affected by the presence of sperm, we conducted FRAP experiments in unmated and mated female oocytes expressing β-tubulin::GFP (Fig. 13A,C). We bleached a 74-$\mu m^2$ area within several regions of the oocyte and allowed recovery to occur over 2 minutes. We normalized the recovered-fluorescence intensity values to the whole-cell and background fluorescence intensities and calculated the recovery halftimes at each region (Table 1). Examination of the β-tubulin::GFP FRAP curves at the proximal and distal cortices indicated a significantly slower recovery in the absence of sperm (Fig. 13A), with a recovery halftime at the proximal-distal cortex of 14.06 ± 3.90 sec (n=19) in females ($p$<0.001; Table 1) compared to 9.55 ± 3.97 sec (n=20) in mated females ($p$<0.001; Table 1). FRAP curves at the dorsal and ventral top of the oocyte also demonstrated a significantly slower recovery in the absence of sperm (Fig. 13C), with a

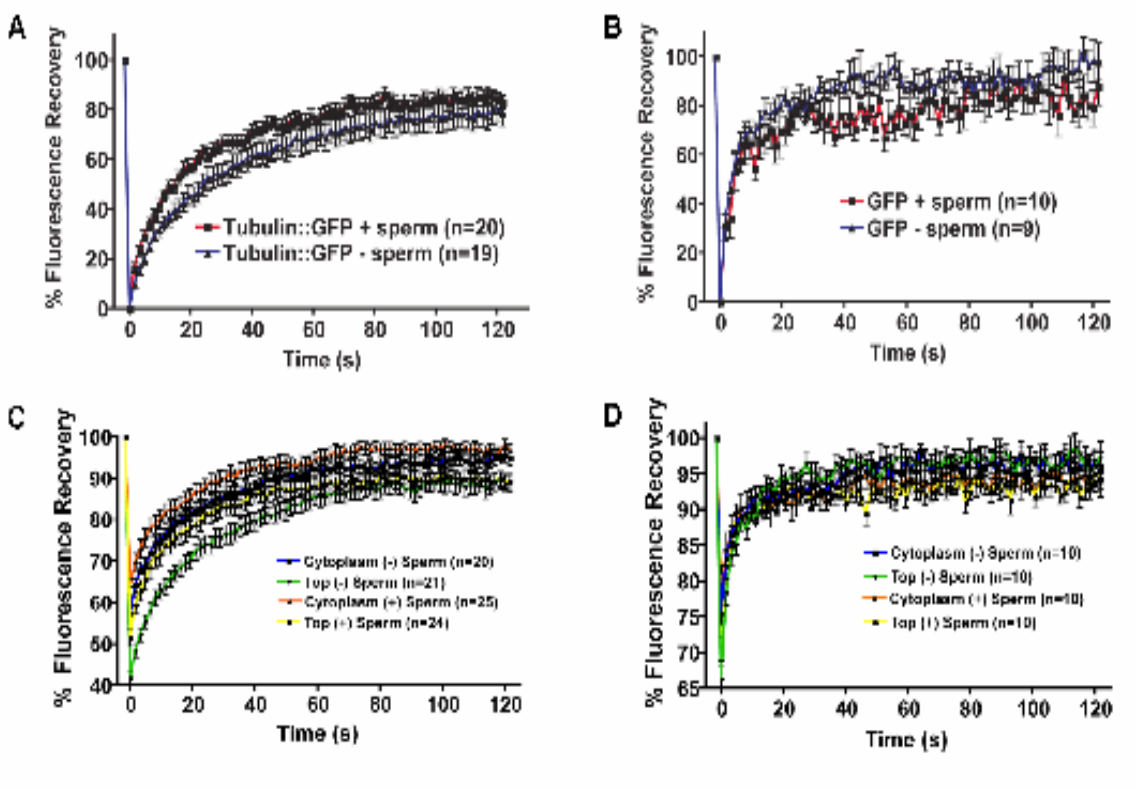



**Figure 13. FRAP analysis in *C. elegans* oocytes.** (A and C) FRAP curves of tubulin::GFP in unmated and mated females. (A) The fluorescence-recovery curves at the proximal and distal cortex do not overlap at initial times, suggesting that tubulin::GFP is incorporated from free pools slightly faster in the presence of sperm. (C) The fluorescence recovery curves at the dorsal-ventral top in the presence and absence of sperm do not overlap at initial times, indicating a slower exchange of tubulin in the absence of sperm. The recovery curves for the cytoplasm overlap indicating there is no difference in diffusion rate in the presence or absence of sperm. (B and D) FRAP curves of germline-expressed GFP in females and hermaphrodites. The fluorescence-recovery curves tightly overlap at initial times, suggesting that there is no difference in the diffusion rate of free GFP in the absence and presence of sperm at all regions. Table 1 reports the halftimes for the FRAP analysis.

**Table 1**

FRAP Halftimes (s)

| Genotype | Sperm | Cytoplasm (n) | P-D Cortex (n) | D-V Cortex (n) |
|---|---|---|---|---|
| GFP[a] | + | 3.57 ± 1.78 (n=10) | 3.43 ± 1.62 (n=10) | 2.89 ± 0.92 (n=10) |
| GFP | – | 3.28 ± 1.63 (n=9) | 3.81 ± 1.11 (n=9) | 2.92 ± 0.66 (n=10) |
| Tubulin::GFP[b] | + | 9.78 ± 4.94 (n=23) | 9.55 ± 3.97 (n=20) | 8.29 ± 4.77 (n=22) |
| Tubulin::GFP | – | 12.02 ± 5.44 (n=18) | 14.06 ± 3.90 (n=19)[c] | 13.74 ± 4.45 (n=19)[c] |

[a] Hermaphrodites were analyzed for the sperm (+) condition.
[b] Mated females were analyzed for the sperm (+) condition.
[c] *t*-test between the presence and absence of sperm is significant ($p < 0.001$)

recovery halftime of 13.74 ± 4.45 sec (n=19) in females compared to 8.29 ± 4.77 sec (n=22) in mated females ($p<0.001$; Table 1). The recovery curves of the cytoplasm in the presence and absence of sperm overlapped and were not significantly different (Fig. 13C), indicating that there is no difference in the tubulin exchange rate in the cytoplasm. These data suggest that free tubulin may be incorporated into microtubules more slowly at a population level in the absence of sperm.

The increased number of growing microtubule plus ends in females (Fig. 10E) may compete for free tubulin, thereby producing the longer halftime. As a control, we performed FRAP analysis on oocytes expressing cytoplasmic GFP in the germ line in the absence and presence of sperm (Fig. 13B,D). The initial fluorescence recovery curves tightly overlapped when we compared data from the proximal and distal cortex of females and hermaphrodites (Fig. 13B) along with other regions of the oocyte (Fig. 13D). Accordingly, the halftimes at the cortex did not differ markedly (Table 1; $p>0.1$). These data indicate that the presence of sperm does not affect the diffusion rate of cytoplasmic GFP. Taken together, these analyses of microtubule dynamics suggest that changes in the cortical density of growing microtubule plus ends may represent a major factor in MSP-dependent microtubule reorganization.

***Depolymerization of Oocyte Microtubules with Nocodazole***

The presence of MSP affects microtubule dynamics by altering the density, direction, rate of movement, and tubulin exchange of microtubules. These experiments, however, do not reveal any information on whether microtubule polymerization is required for microtubule reorganization. Several studies in *C. elegans* have used

nocodazole, by soaking or injections, to examine its effects in either embryos (Matthews et al., 1998; Hannak et al., 2001), the germline (Kitagawa and Rose, 1999), or in oocytes (T. Doniach, Worm Breeders Gazette, 1988). Experiments using dissected hermaphrodite gonads soaked in a solution of nocodazole observed that the oocyte cytoplasm resembled a cottage cheese mixture, but did not examine microtubule localization (T. Doniach, Worm Breeders Gazette, 1988). Using a similar soaking technique with dissected gonads in nocodazole, we examined the organization of oocyte microtubules in the absence and presence of sperm. In unmated females soaked in nocodazole for 30 min (n=22), we observed either oocytes with no change in cortical enrichment of microtubules (Fig. 14A) or oocytes devoid of microtubules except for tubulin labeled nuclei (Fig. 14B, same phenotype in the presence or absence of sperm).

When we examined microtubules treated with nocodazole in the presence of sperm (n=26), we observed that 85% of gonads contained cortically enriched microtubules (Fig. 14C, arrows). The other 15% of gonads contained oocytes that either only had tubulin labeled nuclei (Fig. 14B) or still had rearranged microtubules (data not shown). This result is surprising because it suggests that inhibition of new tubulin polymerization prevents microtubule reorganization in response to MSP. We cannot tell from these experiments whether the major effect of nocodazole is to 1) inhibit new polymerization in the cytoplasm and through an indirect mechanism inhibit cortical microtubule breakdown; or 2) paradoxically induce new tubulin polymerization at the cortex. Live cell imaging will be required to differentiate between these two possibilities. As a control, we tested whether this induced cortical enrichment was due to nocodazole or MSP being washed out of the dissected gonads during soaking. We analyzed oocyte

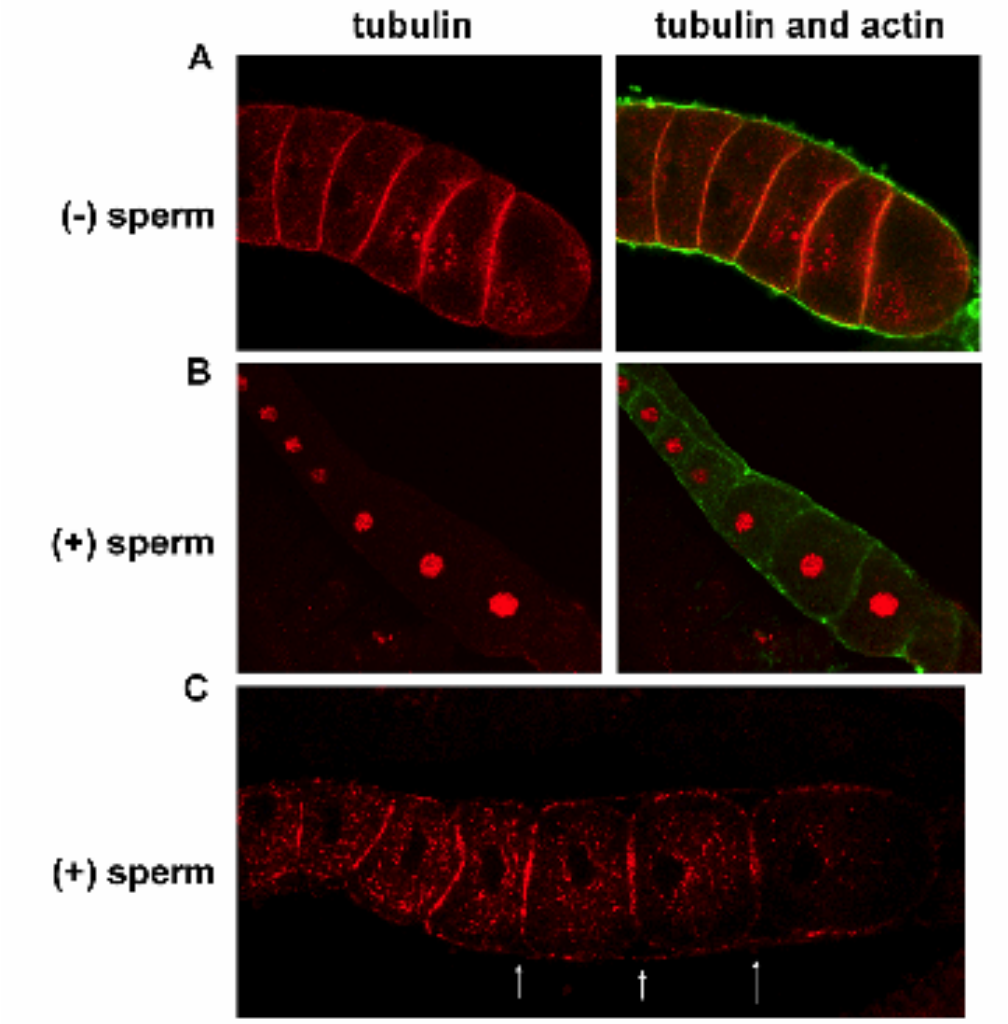



**Figure 14. Microtubule reorganization involves polymerization from the cortex.** (A) Single confocal image of oocytes in the absence of sperm labeled with tubulin (red) and actin (green) after soaking in a 10 μg/mL solution of nocodazole for 30 minutes. Microtubules are still cortically enriched between oocytes. In the most proximal oocytes, blebs of tubulin are observed within the nucleus. (B) Single confocal image of oocytes in the presence of sperm treated as described in (A). Microtubules are no longer in the cytoplasm, but have localized in the nucleus. This staining pattern has also been observed in the absence of sperm (data not shown). (C) Single confocal image of oocytes in the presence of sperm labeled with tubulin (red) after nocodazole treatment as described in (A). Microtubules are cortically enriched (arrows) and not rearranged throughout the cytoplasm as seen in oocytes not treated with nocodazole (see Fig. 7B).

microtubules after immersing dissected N2 hermaphrodites for 30 minutes in buffer without nocodazole, and we observed that the microtubules in all gonads contained reorganized microtubules (n=9; data not shown). This result reaffirms that nocodazole may have caused this effect, however, the control experiment was not done in the presence of DMSO, the solvent for nocodazole, therefore we cannot rule out that the effect on microtubules was due to DMSO and future experiments should address this issue. Overall these data suggest that oocyte microtubule reorganization may require microtubule polymerization.

The small percentage of gonads in both the presence and absence of sperm that contained tubulin stained nuclei and no cytoplasmic or cortical microtubules may indicate that excessive exposure of nocodazole causes tubulin to be internalized by the nucleus. In some gonads, blebs of tubulin are visible within the nucleus (Fig. 14A, look at nuclei in proximal oocytes). This observation is also interesting because CeGrip-1 antibodies labeled the nucleus, which we had supposed was cross-reactive staining. In the absence of centrosomes, perhaps components of the γ-tubulin ring complex are localized in the nucleus to help nucleate microtubules around chromatin after NEBD. This nuclear localization of tubulin is similar to the change in microtubule localization in the most proximal oocyte during meiotic maturation, and indicates that depolymerization of microtubules is perhaps important for this process. Overall, these data indicate that microtubule reorganization in the presence of sperm may require new polymerization of microtubules. In addition, the maintenance of cortical microtubule enrichment after nocodazole treatment in unmated females may indicate that cortical microtubules are relatively stable over the 30 minute nocodazole treatment period.

## Discussion

### *MSP Changes the Dynamics of Oocyte Microtubules*

Our studies reveal that an extraordinary change in the microtubule cytoskeleton occurs in response to the MSP meiotic maturation hormone and that these changes occur prior to both NEBD and fertilization. Using spinning disk confocal microscopy to assess oocyte microtubules in living animals, we have found that the presence of MSP affects the localization and dynamics of microtubules. We have examined microtubule polarity by observing the localization of microtubule plus and minus ends. Using EBP-2::GFP hermaphrodites and unmated females, we have revealed that the presence of MSP affects the dynamics of microtubules through changes in plus end density, directionality, and rate of growth. In addition, FRAP analysis has suggested that the rate of free tubulin incorporation into microtubules may be slightly accelerated in the presence of sperm.

Examining the localization of microtubule minus and plus ends has implied that an increase in the nucleation and growth of microtubules occurs at the cortex in the absence of sperm. Our nocodazole experiments also suggest that microtubule reorganization in the presence of sperm requires new polymerization since microtubules in hermaphrodite oocytes become or stay cortically enriched after treatment with the tubulin sequestering agent. These two results raise some interesting questions about the origin of microtubule growth in the presence and absence of sperm. If microtubules are polymerizing from the cortex in hermaphrodites, why are the plus and minus ends redistributed into the cytoplasm? One aspect we have not tested that may help to answer this question is whether the localization of minus ends change in response to nocodazole

treatment. If minus ends redistribute to the cortex, then that would help explain why we see cortically enriched microtubules after nocodazole treatment. If minus ends remain distributed in the cytoplasm, then perhaps either tubulin subunits accumulate at the cortex or the cortex stabilizes nucleating microtubules to make them more resistant to nocodazole treatment. Since we have not analyzed dynamic minus ends to determine their rate of movement and stability, it would be interesting to examine minus end dynamics to fully understand how microtubules are moving through the oocyte. The fact that we cannot determine the movement or length of an entire microtubule is one caveat to our experiments. Microtubules could be anchored to the cortex by their minus ends with their plus ends growing into the cytoplasm, they could be treadmilling throughout the cytoplasm, or a combination of both. We do not know whether short microtubules populate the cortex in the absence of sperm or long microtubules grow from the cortex in the presence of sperm. This type of information in combination with changes in the directionality of growth would help determine how microtubules are reorganizing in the presence of MSP.

It had been previously reported that microtubules were not required for the anterior/posterior (A/P) polarity of the embryo, such as the asymmetric distribution of P-granules, after treatment with microtubule depolymerizing drugs (Strome and Wood, 1983). Instead, embryos treated with the microfilament inhibitors cytochalasins D and B demonstrated that P granule segregation was dependent on actin (Strome and Wood, 1983). To determine whether microtubules were involved before fertilization in establishing A/P polarity, T. Doniach applied nocodazole to oocytes while they were still in the ovary. She reported that the oocytes were fertilized normally and still observed

signs of A/P polarity, but no polar body formation, pronuclear migration, spindle formation, or cleavage occurred. Although she did not examine P-granule distribution or intact microtubules, it was interesting that she noted that nocodazole gave the oocyte cytoplasm a 'cottage cheesy' texture since EM images of female oocytes appeared to have a similar appearance. In addition, it would be fascinating to measure meiotic maturation rates in hermaphrodites after nocodazole injection to see if they are reduced. P granule distribution may also be worthwhile to investigate in both the presence and absence of sperm and treatment with nocodazole to see if microtubules are involved.

Our dynamic studies propose that the increase in microtubule plus end density may affect other aspects of microtubules such as rate of movement and tubulin exchange rate. We suggest that an increase of plus ends at the cortex would result in slower growing microtubules due to limiting space. However, our nocodazole studies may indicate that microtubules are stabilized by the cortex because tubulin sequestration did not have an immediate affect on cortical microtubules in the absence of sperm. This suggests that stabilization in addition to microtubule density may affect cortical microtubules. Since we used tyrosinated-tubulin antibodies that only detects dynamic, newly formed microtubules, we cannot determine the effect of nocodazole on stable microtubule populations. It is possible that our nocodazole experiment only prevents new polymerization from occurring and does not depolymerize stable microtubule populations in the oocyte. By analyzing stable microtubule populations, as defined by acetylation for example, would we be able to determine if microtubule stability is increased at the cortex and whether nocodazole affects these microtubule populations. In addition, proteins or other cytoskeletal elements, such as actin, at the cortex may help to

stabilize microtubules, and by using mutants or RNAi in combination with nocodazole we could determine the effect of these cortical interactors on microtubule stability. In order to confirm that microtubule polymerization occurs from the cortex to promote microtubule reorganization, we could examine the nucleation of microtubules in oocytes after nocodazole treatment. This experiment could help establish where microtubules are being nucleated from, what direction they are growing, and how quickly they are polymerizing.

We hypothesize that our FRAP analysis helps to determine microtubule stability through measuring the exchange rate of free tubulin into microtubules. *In vitro* studies have demonstrated that microtubule polymerization rates are directly dependent on free-tubulin concentration (Mitchison and Kirschner, 1984; Walker et al., 1988). Indeed, microtubules appear to grow faster in the presence of MSP and result in a more rapid bleach recovery indicating a high concentration of free tubulin. In addition, given that there are more microtubules at the cortex, it may take longer for tubulin to incorporate into microtubules if the same or a smaller amount of free tubulin is available. Since our FRAP analysis shows only a slight effect in recovery halftime, this raises the possibility that the pool of free tubulin is the same in the presence and absence of sperm and that polymerization rates depend on the number of microtubules. The amount of free tubulin may also be less in the absence of sperm, and we could test this possibility by using FRAP analysis on the meiotic spindle at different days of adulthood. If the pool of free tubulin is less when there is a decreased level of MSP, such as in a 3-day-old mated female or hermaphrodite, then there should be a slower recovery rate of tubulin in the meiotic spindle compared to that of 1-day-old mated females or hermaphrodites with a

higher level of MSP. This result may also suggest that microtubules are more stable when MSP levels are low. Given that we cannot distinguish between free tubulin exchange and microtubules growing into the bleached plane, our FRAP data in concert with our nocodazole experiments may suggest that microtubules are more stable at the cortex because of slower movement and polymerization. Our FRAP data is also supported by our nocodazole experiment because if the polymerization of new microtubules is important for reorganization, then the oocyte would need to increase the rate of free tubulin incorporation into microtubules for this change in polymerization to occur. Further experiments utilizing speckle microscopy with fluorescently labeled tubulin may help to determine microtubule stability by watching the direction and incorporation of labeled free tubulin into microtubules (Waterman-Storer and Salmon, 1998; Waterman-Storer and Salmon, 1999).

### *Does MSP Help Promote Meiotic Spindle Assembly through Changes in the Localization and Dynamics of Oocyte Microtubules?*

Since MSP affects the localization and dynamics of oocyte microtubules, the question remaining to be answered is why would the oocyte need to make these changes. One possibility is that alterations in microtubule dynamics may promote search and capture of meiotic chromatin after NEBD (Fig. 19A). Thus, MSP may "prime" microtubule dynamics for meiotic spindle assembly early in the meiotic maturation process, so that the spindle can form rapidly upon NEBD. Since cortical microtubule enrichment, as assessed by CMEF measurements, depends on oocyte position and sperm availability (Fig. 8B,C), we asked whether the meiotic spindle assembles more rapidly at the young-adult stage (day 1) when sperm are abundant compared to later in adulthood

(day 3) when sperm are limiting. We made tubulin::GFP movies using spinning disk confocal microscopy of day 1 (n=5) and day 3 (n=3) adults. The time from NEBD to assembly of a bipolar meiotic spindle was approximately the same in both cases (9.86 ± 3.48 vs. 11.0 ± 2.73 min, respectively). Thus, sperm availability affects the frequency of meiotic maturation, but apparently not the timing of spindle assembly once maturation occurs. It remains a formal possibility, however, that oocyte microtubule reorganization may have a redundant role in influencing the timing of meiotic spindle assembly.

When we examined tubulin::GFP animals at day 1 and day 3 of adulthood, we noticed that the timing of ovulation was slightly affected. Day 1 animal oocytes ovulated about 10 minutes after the appearance of a spindle, and day 3 animal oocytes ovulated about 15 minutes after the appearance of a spindle (data not shown). In the majority of day 3 animals, ovulation did not even occur, and in some oocytes, NEBD did not occur or the nuclear envelope reformed over time if ovulation did not occur shortly after NEBD. Since ovulation normally occurs 5 minutes following NEBD, our results indicate that there is some photodamage taking place and perhaps the older oocytes are more sensitive to laser treatment. It is possible that ovulation may have been delayed in the older gonads due to defects in meiotic spindle assembly. Even though we can visually detect the presence of the meiotic spindle in the tubulin::GFP animals using the spinning disc confocal, we cannot determine whether the spindle is correctly assembled. If there is a cell cycle checkpoint for meiotic spindle assembly during meiosis I, our result may indicate that microtubule reorganization may additionally affect either microtubule stability or correctly localizing proteins to the meiotic spindle to ensure proper assembly.

CHAPTER IV

# GENETIC REGULATION OF OOCYTE MICOTUBULE REORGANIZATION

## Introduction

Great strides have been made in understanding the control of cell-cycle progression during the meiotic maturation process, culminating in the discovery of MPF (Cdk1/cyclin B) (Masui, 2001). MAPK cascades also play an important role in controlling meiotic progression (Fan and Sun, 2004). By contrast, comparatively less information is available about the intercellular signaling pathways that regulate meiotic resumption. Unifying conclusions from studies in vertebrate and invertebrate systems are that soma-germline interactions play a crucial role and that regulation involves both positively- and negatively-acting pathways (Voronina and Wessel, 2003). As meiotic maturation signals have been characterized in several invertebrate systems, studies in these organisms may offer both comparative and mechanistic insights.

In *C. elegans*, meiotic maturation and ovulation are coupled to sperm availability through a complex regulatory network involving germline and somatic controls (Miller et al., 2003; Corrigan et al., 2005; Govindan et al., 2006). Parallel genetic pathways defined by *vab-1*, which encodes an MSP-binding ephrin receptor, and *ceh-18*, which encodes a POU-homeoprotein expressed in gonadal sheath cells but not oocytes, together compose an MSP-sensing control mechanism that regulates meiotic maturation (Fig. 5; Miller et

al., 2003; Govindan et al., 2006). Somatic control of meiotic maturation, likely through the *ceh-18* pathway, involves antagonistic $G\alpha_{o/i}$ and $G\alpha_s$ signaling pathways that define negatively- and positively-acting inputs, respectively (Govindan et al., 2006). $G\alpha_s$ signaling is necessary and sufficient to trigger oocyte MAPK activation and meiotic maturation, in part, through antagonizing inhibitory sheath/oocyte gap-junctional communication. This finding, together with the results from mammalian systems (Jamnongjit and Hammes, 2005; Mehlmann, 2005), suggests that the involvement of the $G\alpha_s$ pathway may be an ancestral feature of meiotic maturation signaling. While these studies provided initial insights into the complex signaling pathways regulating meiotic maturation in response to MSP in *C. elegans*, they raise new questions about how the *vab-1* and *ceh-18* pathways control nuclear and cytoplasmic events during maturation, as well as the division of labor among the pathways.

Using laser-scanning and spinning-disk confocal microscopy, we found that cortical microtubule localization and plus-end dynamics are altered in the presence of the MSP signal. By investigating the known genes involved in MSP signaling, we have elucidated several regulators of microtubule reorganization. The VAB-1 Eph/MSP receptor pathway has no apparent role in regulating the distribution of oocyte microtubules. By contrast, the somatic *ceh-18* pathway involving antagonistic $G\alpha_{o/i}$ and $G\alpha_s$ signaling influences the microtubule cytoskeleton of the oocyte. In addition, we uncovered several genes in the oocyte that also function to regulate microtubule organization. Similar to that reported for meiotic maturation, we propose that microtubule reorganization is regulated through complex cell autonomous and non-autonomous signaling pathways.

## Materials and Methods

*Nematode Strains and Phenotypic Analysis*

Standard techniques were used for nematode culture at 20°C, except where indicated otherwise. The *C. elegans var. Bristol* strain, N2, was used as the wild-type hermaphrodite strain. We used *fog-2(q71)* and *fog-3(q443)* mutations, which feminize the germ line, to generate *XX* animals that do not produce sperm (females), a situation we refer to as a "minus sperm" condition to simplify the flow of information. Similarly, the "plus sperm" condition refers to experiments using either mated females or hermaphrodites as indicated. In all tested cases, mutations in *fog-2* and *fog-3* gave identical results and could be used interchangeably, however, for technical reasons most experiments were performed using *fog-2(q71)*, unless genetic markers on *LGI* were utilized. Key strains and alleles used are described in WormBase (http://www.wormbase.org), in Govindan et al. (2006), or as follows:

*LGI*: *gsa-1(ce81)*, *gsa-1(ce94)*, *goa-1(sa734)*, *rrf-1(pk1417)*, *fog-3(q443)*

*LGII*: *vab-1(dx31)*, *ptc-1(ok122)*

*LGIII*: *mpk-1(ga111*ts*)*, *cgh-1(ok492)*, *acy-1(ce2gf)*

*LGIV*: *unc-24(e138)*, *oma-1(zu40te33)*, *fem-3(e1990)*

*LGV*: *oma-2(te51)*, *emo-1(oz1)*, *fog-2(q71)*

*LGX*: *ceh-18(mg57)*, *kin-2(ce179)*

Rearrangements used were *hT2(qIs48)(I, III)*, *mIn1II*, *DnT1(IV, V)*.

RNA interference employed a modification of the method of (Kamath and

Ahringer, 2003), which was performed as described (Govindan et al., 2006). Analysis of oocyte MAPK activation was described in Miller et al. (2001). To reduce the extent of MAPK activation, *fog-2(q71)* females were microinjected with the MEK1/2 inhibitor, U0126 (100 μM in egg salts; Sigma). cAMP-soaking experiments utilized an 8 mg/ml dibutyryl cyclic AMP (Sigma) in M9 buffer.

### *Fluorescence Microscopy*

#### *Preparation of dissected gonads and antibody staining*

Gonads were dissected, fixed, and stained for immunofluorescence microscopy as described (Rose et al., 1997). Antibodies used were: YL1/2 rat monoclonal anti-α-tubulin (Kilmartin et al., 1982) (Accurate Chemical and Scientific Corporation); mouse monoclonal anti-actin (MP Biomedicals); anti-MAPK-YT (Sigma); and Cy2- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories).

Wide-field fluorescence microscopy employed Zeiss Axioskop or Axioplan microscopes using 40X and 63X (NA1.4) objective lenses. Images were acquired with an ORCA ER (Hamamatsu) charge-coupled device camera using OpenLab (Improvision) or MetaMorph (Universal Imaging) acquisition software. Pixel intensities were measured in arbitrary fluorescent units. All exposures were within the dynamic range of the detector. DNA was detected with DAPI.

#### *Laser-scanning confocal microscopy*

Confocal images were acquired on a Zeiss LSM510 microscope with a pinhole of

1.0 Airy Units and 63X (NA 1.4) objective lens. Gain and offset were set so that all data were within the dynamic range of the PMT. Band pass filters were used to optically isolate the Cy2 and Cy3 fluorophores, and no cross-talk was observed.

## Results

### *ceh-18, but not vab-1, Affects Microtubule Reorganization*

MSP promotes oocyte meiotic maturation by antagonizing two parallel negative regulatory circuits: an oocyte VAB-1, Eph/MSP receptor, pathway and a somatic gonadal sheath cell pathway defined by the POU-homeoprotein, CEH-18 (Fig. 5, Miller et al., 2003). To determine whether either of these pathways affect MSP-dependent oocyte microtubule reorganization, we examined oocytes from unmated and mated *vab-1(null)* and *ceh-18(null)* mutant females (Fig. 15). *vab-1(dx31)* null mutants did not have an effect on microtubule reorganization either in the absence (CMEF=1.91 ± 0.20, n=15; Fig. 15B,E) or presence (CMEF=0.97 ± 0.18, n=20; Fig. 15A,E) of sperm. By contrast, *ceh-18(mg57)* null mutant females did not exhibit cortical microtubule enrichment in the absence of sperm (CMEF=1.03 ± 0.12, n=18) (Fig. 15D,E), but contained evenly dispersed microtubules throughout the cytoplasm as in mated females (CMEF=0.92 ± 0.14, n=14) (Fig. 15C,E). Likewise, oocytes in *vab-1(dx31);ceh-18(mg57)* mutant females exhibited reorganized microtubules (CMEF=0.99 ± 0.12, n=18) (Fig. 15E). These results indicate that the *ceh-18* pathway inhibits microtubule reorganization in the absence of MSP, thereby suggesting a role for the gonadal sheath cells in regulating the organization of oocyte microtubules.

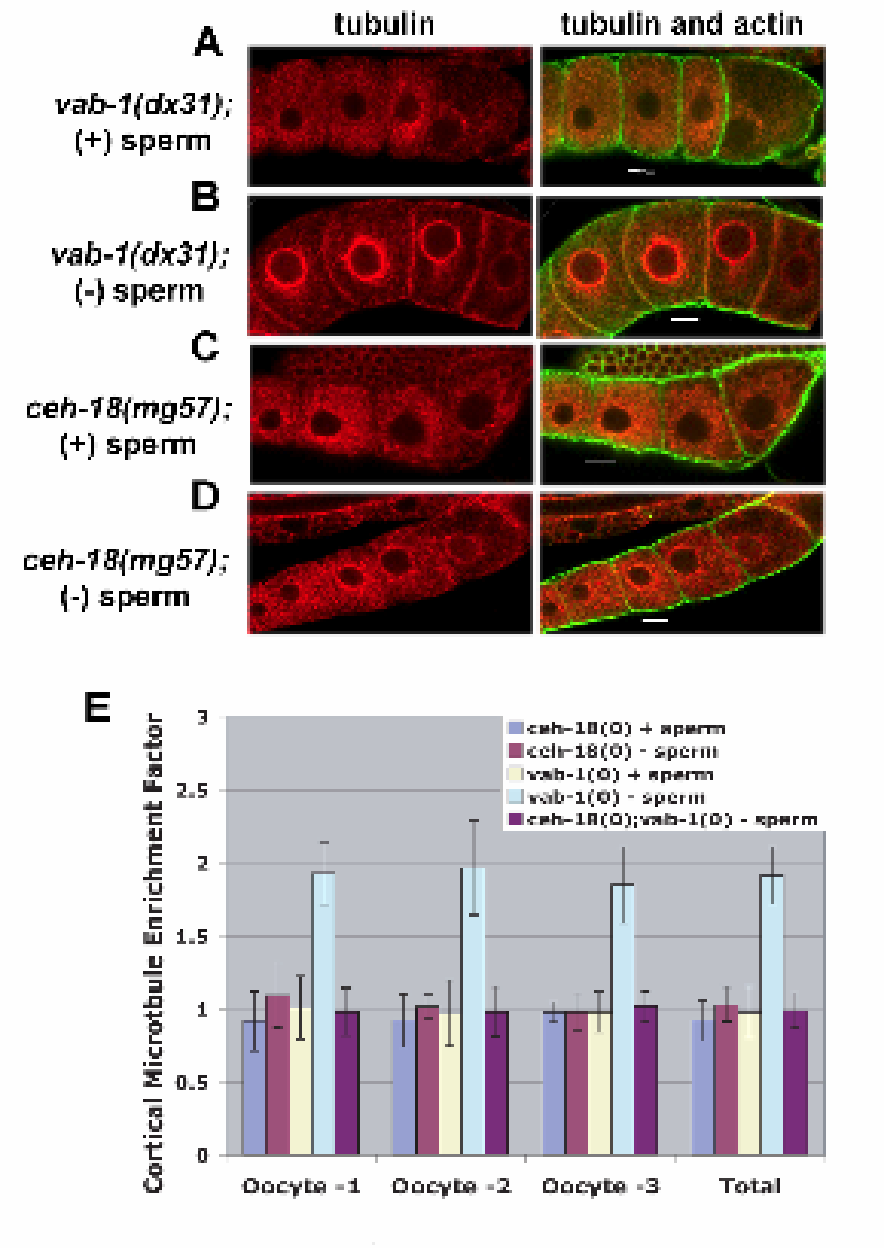



**Figure 15. MSP signaling genes regulate oocyte microtubule reorganization.** (A and B) Single confocal images of *vab-1(dx31)* mated (A) and unmated (B) females labeled with tubulin (red) and actin (green). Null mutations in *vab-1* have no effect on microtubule organization. (C and D) Single confocal images of *ceh-18(mg57)* mated (C) and unmated (D) females. Unmated females (D) do not exhibit cortical microtubule enrichment. (E) CMEF measurements in *ceh-18* and *vab-1* mutants in the absence and presence of sperm (error bars represent s.d.). Scale bars: 10 μm.

### *$G\alpha_s$ Signaling Promotes Oocyte Microtubule Reorganization*

A recent study suggested that the gonadal sheath cells regulate meiotic maturation via antagonistic $G\alpha_s$ and $G\alpha_{o/i}$ signaling pathways, which function in parallel to the VAB-1 Eph/MSP receptor pathway (Govindan et al., 2006) (Fig. 6). In this study, $G\alpha_s$ signaling was shown to be necessary to promote meiotic maturation and MAPK activation in oocytes in the presence of MSP (Govindan et al., 2006) (Fig. 18A). In addition, $G\alpha_s$ signaling was shown to be sufficient for these responses in the absence of MSP. When we examined hermaphrodites treated with *gsa-1* RNAi, which encodes $G\alpha_s$, with DIC, we observed that the first maturing oocyte of the gonad arm either did not undergo maturation, or had defective ovulation by being severed in the spermatheca or slipping back into the gonad arm after being fertilized in the spermatheca (data not shown). These defects resulted in oocytes stacking in the gonad arm (data not shown). To test whether *gsa-1* affects microtubule reorganization, we analyzed microtubules following *gsa-1* RNAi in a hermaphrodite background (Fig. 16B). We observed that oocyte microtubules were cortically enriched at the proximal and distal edges following *gsa-1* RNAi in the presence of sperm (CMEF=1.73 ± 0.30, n=54) (Fig. 16B,K) compared to the empty vector RNAi control (CMEF=0.80 ± 0.19, n=33) (Fig. 16A,K), indicating that *gsa-1* is required for MSP-dependent microtubule reorganization.

To determine whether *gsa-1* functions in the soma or the germ line to promote microtubule reorganization, we conducted RNAi analysis in an *rrf-1(null)* mutant background. *rrf-1* encodes an RNA-dependent RNA polymerase (RdP) that is required for the RNAi response in many somatic cells (Sijen et al., 2001), but is dispensable for

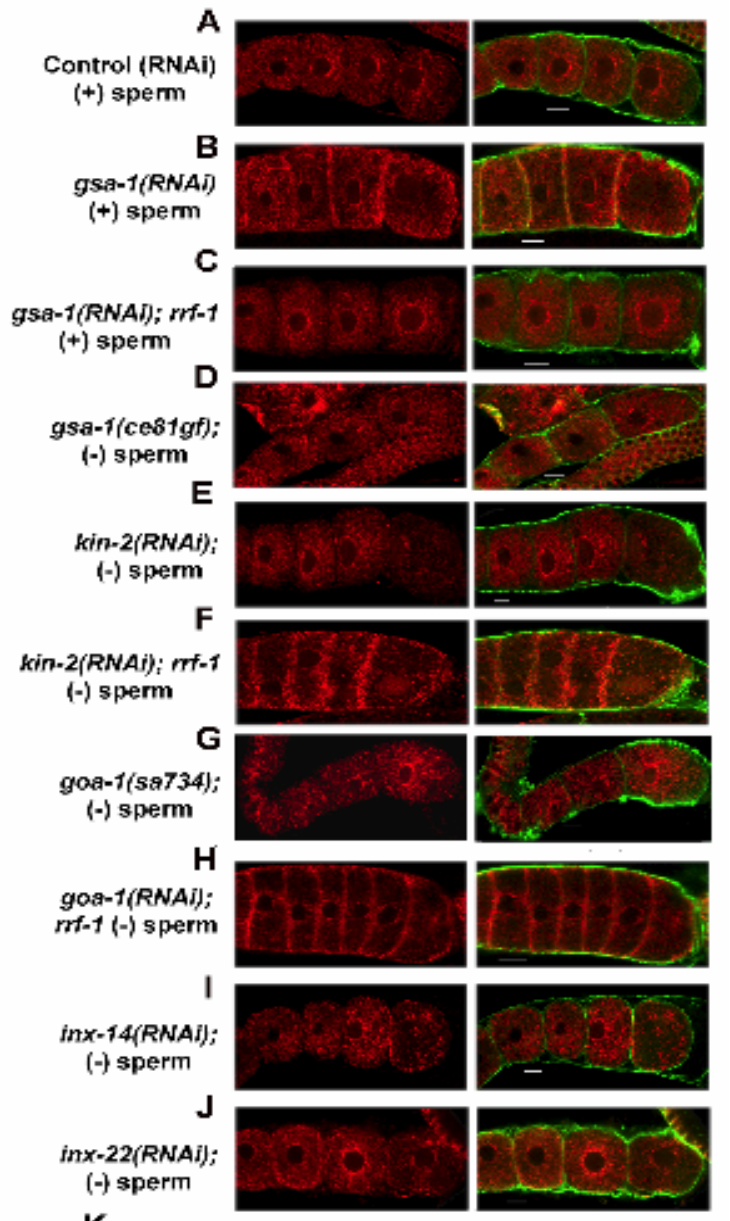
germline RNAi, which employs the EGO-1 RdP (Smardon et al., 2000). Thus, an RNAi response in an *rrf-1(null)* background is indicative of a germline function, whereas an absence of a response suggests sufficiency of gene function in the soma. We observed that microtubules were not enriched at the proximal-distal cortex of oocytes following *gsa-1(RNAi)* in an *rrf-1(null)* mutant background (CMEF=0.68 ± 0.16, n=27; Fig. 16C,K), suggesting that $G\alpha_s$ signaling in the soma is sufficient to promote microtubule reorganization in oocytes in the presence of MSP.

To test whether *gsa-1* activity is sufficient to promote microtubule reorganization in the absence of MSP, we examined two gain-of-function (gf) *gsa-1* alleles, *ce94*gf and *ce81*gf, which are predicted to stabilize the GTP-bound form of $G\alpha_s$ through G45R and R182C substitutions, respectively (Schade et al., 2005). In *gsa-1(ce81*gf*)* and *gsa-1(ce94*gf*)* females, we observed that oocyte microtubules were dispersed evenly throughout the cytoplasm (CMEF=0.88 ± 0.16, n=17; Fig. 16D,K; and data not shown), like in mated females. In canonical $G\alpha_s$ signaling, activated $G\alpha_s$ stimulates adenylyl cyclase resulting in production of cAMP, which binds the regulatory subunit of cAMP-dependent PKA thereby releasing the active catalytic subunit (Cabrera-Vera et al., 2003). Thus, we tested the involvement of *kin-2*, which encodes the regulatory subunit of cAMP-activated protein kinase, in regulating oocyte microtubule reorganization. We examined oocyte microtubules in *kin-2(ce179);fog-3(q443)* females or following *kin-2(RNAi)* in a *fog-2(q71)* female background (Fig. 16E,K, and data not shown). In both cases, we observed that oocyte microtubules were reorganized despite the absence of MSP (CMEF=0.85 ± 0.22, n=15, and CMEF=1.04 ± 0.18, n=39, respectively).

To determine whether *kin-2* functions in the germ line or soma to prevent

microtubule reorganization in the absence of MSP, we tested the effect of *kin-2(RNAi)* in an *rrf-1(pk1417)fog-3(q443)* female background. We observed cortically enriched microtubules (CMEF=1.76 ± 0.39, n=30) (Fig. 16F,K), indicating that *kin-2* function is sufficient in the soma to prevent oocyte microtubule reorganization when MSP is absent. This observation is consistent with the finding that *kin-2* function is sufficient in the soma to prevent meiotic maturation and MAPK in the absence of MSP (Govindan et al., 2006) (Fig. 18B). The contribution of both $G\alpha_s$ and KIN-2 to microtubule reorganization in oocytes suggests that cAMP may also be involved in this regulation. To test this hypothesis, we soaked unmated *fog-2(q71)* females in an 8 mg/ml solution of dibutyryl-cAMP for two hours. Although the effects were weak, variable, and required high levels of dibutyryl-cAMP, they were quantifiable in that the dibutyryl-cAMP-soaked females exhibited decreased CMEF values (CMEF=1.24 ± 0.21, n=33; Fig. 18H) compared to the buffer control (CMEF=1.61 ± 0.34, n=60; $p<0.001$; data not shown).

In addition to analyzing *kin-2* and cAMP, we examined whether adenylyl cyclase had an affect on microtubule reorganization. *C. elegans* has four adenylyl cyclase genes, however, we could not test all four genes because *acy-4* was not found in the RNAi library. We did conduct RNAi on *acy-1*, *acy-2,* and *acy-3* alone and in combination in the presence and absence of sperm, but did not notice any affect on microtubules (data not shown). We cannot rule out the possibility that these genes function alone or redundantly in microtubule reorganization since RNAi is not always efficient, especially when mixing bacterial clones, and when we cannot test the involvement of *acy-4*. Only one adenylyl cyclase mutant was available, so based on weak meiotic maturation results from Govindan et al. (2006), we tested the feminized gain-of-function *acy-1(ce2gf)*




| Label | RNAi or Mutant | Sperm | Label | RNAi or Mutant | Sperm |
|---|---|---|---|---|---|
| a | Control | + | f | *kin-2(RNAi);rrf-1;fog-3* | - |
| b | *gsa-1(RNAi)* | + | g | *kin-2(ce179);fog-3* | - |
| c | *gsa-1(RNAi);rrf-1* | + | h | *goa-1(sa734);fog-3* | - |
| d | *gsa-1(ce81gf);fog-3* | - | i | *inx-22(RNAi);fog-2* | - |
| e | *kin-2(RNAi);fog-2* | - | j | *inx-14(RNAi);fog-2* | - |

**Figure 16. RNAi screen for regulators of microtubule reorganization.** (A-K) Single confocal images of dissected gonads from mutants or RNAi-treated animals labeled as indicated. (A) Hermaphrodites treated with empty-vector control RNAi. (B) Hermaphrodites treated with *gsa-1* RNAi. (C) *rrf-1(pk1417)* hermaphrodites treated with *gsa-1* RNAi. (D) *gsa-1(ce81*gf*)fog-3(q443)* unmated females. (E) Unmated *fog-2* females treated with *kin-2* RNAi. (F) Unmated *fog-3(q443)rrf-1(pk1417)* females treated with *kin-2* RNAi. (G) *goa-1(sa734)fog-3(q443)* unmated females. (H) *fog-3(q443)rrf-1(pk1417)* unmated females treated with *goa-1* RNAi. (I) Unmated *fog-2* females treated with *inx-14* RNAi. (J) Unmated *fog-2* females treated with *inx-22* RNAi. (K) CMEF measurements for the indicated genotypes (between 6-18 gonad arms were analyzed; error bars represent s.d.). Scale bars: 10 μm.

mutant for an effect on microtubules. *acy-1* has been reported to function downstream of *gsa-1,* however, the gain-of-function mutant females did not have reorganized microtubules (data not shown). Since we did not discover which of the four adenylyl cyclase genes are involved in $G\alpha_s$ signaling, additional experiments are needed to determine whether one or multiple adenylyl cyclase genes regulate microtubule reorganization. In general, we have uncovered that $G\alpha_s$ signaling in the soma, likely in the gonadal sheath cells, is necessary and sufficient to promote oocyte microtubule reorganization.

### *$G\alpha_{o/i}$ Signaling Inhibits Oocyte Microtubule Reorganization in the Absence of Sperm*

Recently, Govindan et al. (2006) provided evidence that *goa-1*, which encodes a $G\alpha_{o/i}$ protein, functions in the sheath cell control of meiotic maturation and MAPK activation in oocytes. Specifically, *goa-1* was shown to negatively regulate meiotic maturation and MAPK activation when MSP was absent, and *gsa-1* was shown to be epistatic to *goa-1*. To determine whether *goa-1* is a regulator of oocyte microtubule reorganization, we examined *goa-1(sa734)fog-3(q443)* females and observed evenly dispersed microtubules (CMEF=0.81 ± 0.26, n=24) (Fig. 16G,K). By contrast, cortically enriched microtubules were observed following *goa-1(RNAi)* in an *rrf-1(null)* female background (Fig. 16H). These data suggest that *goa-1* is required in the soma to prevent oocyte microtubule reorganization in the absence of the MSP signal.

### *Regulation of Oocyte Microtubule Reorganization by $G\alpha_s$, $G\alpha_{o/I}$, and Gap Junctions*

To investigate the relationship between *goa-1* and *gsa-1* in the regulation of

oocyte microtubule reorganization, we performed *gsa-1(RNAi)* on *goa-1(sa734)* hermaphrodites and examined oocyte microtubules. Microtubules were cortically enriched at the proximal and distal cortical edges of oocytes consistent with the finding that *gsa-1* is required for oocyte microtubule reorganization in a hermaphrodite background (Fig. 17J). When we conducted *gsa-1(RNAi)* in a *goa-1(sa734)fog-3(q443)* female background, however, oocyte microtubules were reorganized (Fig. 17A). It has been recently reported that *gsa-1(RNAi)* in a *goa-1(sa734)* female background results in low meiotic maturation rates and an absence of detectable MAPK activation in oocytes (Govindan et al., 2006), a result we confirmed (data not shown). Thus, microtubule reorganization might represent a separable element of the meiotic maturation process requiring different thresholds of signaling. Together, these data suggest the involvement of antagonistic $G\alpha_s$ and $G\alpha_{o/i}$ signaling in sheath cells in the regulation of microtubule reorganization in oocytes.

$G\alpha_s$ signaling appears to promote meiotic maturation in part by affecting the synthesis or stability of sheath/oocyte gap junctions (Govindan et al., 2006). The existence of gap-junctional communication between sheath cells and oocytes provides a potential mechanism for coordinating their behaviors (Hall et al., 1999). To determine whether gap-junctional communication between sheath cells and oocytes regulates oocyte microtubule reorganization, we used RNAi to inactivate *inx-14* and *inx-22*, which likely encode innexin components of the sheath/oocyte gap junctions (Govindan et al., 2006). When we conducted *inx-14(RNAi)* or *inx-22(RNAi)* in a *fog-2(q71)* female background, we observed oocytes that contained evenly dispersed microtubules (CMEF=0.95 ± 0.23, (n=15) and CMEF=1.05 ± 0.27, (n=18), respectively) (Fig. 16I-K). These data suggest

that gap-junctional communication between sheath cells and oocytes prevents microtubule reorganization in the absence of MSP.

### *Germline Regulators of Oocyte Microtubule Reorganization*

To identify oocyte regulators of microtubule reorganization, we conducted a candidate-gene screen, focusing on genes implicated in meiotic maturation control or microtubule regulation (summarized in Table 2; Fig. 17). MSP-dependent MAPK activation and meiotic maturation require the downstream action of OMA-1 and OMA-2, two TIS-11 zinc-finger proteins expressed in the germ line (Detwiler et al., 2001). When we examined microtubules in *oma-1(zu405te33);oma-2(te51)* double mutant hermaphrodites, microtubules were enriched at the proximal and distal cortex (Table 2; Fig. 17B). This observation indicates that OMA-1 and OMA-2 may function to promote microtubule reorganization directly, or indirectly, as a consequence of their requirement for promoting meiotic maturation. Analysis of the three negative regulators of meiotic maturation and MAPK activation in oocytes, *emo-1(oz1)* (Iwasaki et al., 1996), *ptc-1(ok122)* (Govindan et al., 2006), and *cgh-1(ok492)* (I. Y., and D. G., unpublished results), indicated that mutants in these genes exhibit reorganized oocyte microtubules in a female genetic background (Fig. 17C-E).

Recently, Burrows et al. (2006) demonstrated that the Myt1 homolog WEE-1.3 is required for the normal timing of NEBD during the meiotic maturation process in hermaphrodites and for proper meiotic spindle assembly. Consistent with the proposal that *wee-1.3* regulates oocyte microtubules (Burrows et al., 2006), we found that *wee-1.3(RNAi)* in a *fog-2(q71)* female background causes oocyte microtubule reorganization

**TABLE 2**

Screen for regulators of microtubule reorganization

**I. Genes that promote microtubule reorganization in the presence of sperm**

| Gene | Description |
|---|---|
| gsa-1[c] | Heterotrimeric Gsα protein subunit |
| oma-1/oma-2[b] | Redundant TIS11 zinc finger proteins |

**II. Genes that prevent microtubule reorganization in the absence of sperm**

| Gene | Description |
|---|---|
| *kin-2*[c] | cAMP-dependent protein kinase (regulatory subunit) |
| *cgh-1*[c] | RNA DEAD-box helicase |
| *ceh-18*[b] | POU-class homeobox transcription factor |
| *wee-1.3* | Cyclin-dependent kinase WEE1 |
| *emo-1*[b] | Sec61p ortholog |
| *ptc-1*[b] | Patched receptor |
| *gpb-1*[a] | Heterotrimeric $G_{\beta}$ protein subunit |
| *goa-1* [c] | Heterotrimeric $G_{o/i}\alpha$ protein subunit |
| *inx-14*[a] | Gap junction protein (innexin family) |
| *inx-22*[a] | Gap junction protein (innexin family) |

**III. Cytoskeletal regulators that had no effect on microtubule reorganization in the presence or absence of sperm**

| Gene[a] | Description | Gene[a] | Description |
|---|---|---|---|
| *cls-2* | Microtubule tip-binding protein | *mei-1* | Catalytic subunit of katanin |
| *dhc-1* | Dynein heavy chain | *mei-2* | Targeting subunit of katanin |
| *dnc-1* | Dynactin | *ndc-80* | HEC/Ndc80p protein |
| *gip-1* | Gamma-tubulin-binding protein | *nmy-2* | Nonmuscle myosin II |
| *khc-1* | Kinesin heavy chain | *ptl-1* | Microtubule binding protein |
| *klc-1* | Kinesin light chain | *tac-1* | Acidic coiled-coil protein |
| *klp-7* | Kinesin-like motor protein | *tba-2* | Alpha tubulin |
| *klp-13* | Kinesin-like protein | *tba-9* | Alpha tubulin |
| *klp-15* | C-terminal motor kinesin | *zen-4* | Kinesin-like protein |
| *klp-16* | Kinesin motor protein | *zyg-9* | Microtubule-associated protein |
| *klp-18* | Kinesin-like protein | C55A6.2 | Tubulin-tyrosine-like ligase |
| *mbk-2* | Yak1-related kinase | F16D3.4 | Tubulin-specific chaperone D |

[a] Used RNAi for phenotypic analysis
[b] Used mutants for phenotypic analysis
[c] Used both RNAi and mutants for phenotypic analysis

17F). Burrows et al. (2006) reported that *wee-1.3(RNAi)* in a female background is conditions *wee-1.3(RNAi)* was sufficient to cause NEBD as evident by endomitotic oocytes, oocytes that undergo meiotic maturation but remain trapped in the gonad arm resulting in multiple rounds of DNA replication (Fig. 18C). This result indicates that the microtubule effect could be downstream of *cdk-1* activation. While *cdk-1(RNAi)* in a hermaphrodite background did not block oocyte microtubule reorganization (data not shown), it may be difficult to completely deplete CDK-1 from the germline using RNAi, as discussed previously (Boxem et al., 1999; Burrows et al., 2006).

To begin to address the mechanism of microtubule reorganization, we used RNAi to test whether known microtubule regulators, such as kinesin and dynein motors and microtubule-associated proteins (Table 2, section III), function to promote oocyte microtubule reorganization when sperm are present, or, alternatively, whether they are needed for blocking reorganization in female backgrounds. We found that none of the tested microtubule regulators (Table 2, section III) affected oocyte microtubule reorganization, indicating that further work will be needed to determine the mechanism at the level of individual microtubules.

***Oocyte Microtubule Reorganization does not Require High Levels of MAPK Activation***

MSP signaling triggers MAPK activation in oocytes (Miller et al., 2001). Because there is a general correlation between MAPK activation and oocyte microtubule reorganization: negative regulators of MAPK activation (e.g. *kin-2*, *inx-14*, *inx-22*, *ptc-1*, *cgh-1*, *goa-1*, *gpb-1*, *ceh-18)* block microtubule rearrangement in females; whereas positive regulators (e.g. *oma-1/2* and *gsa-1*) are required in hermaphrodites, we addressed

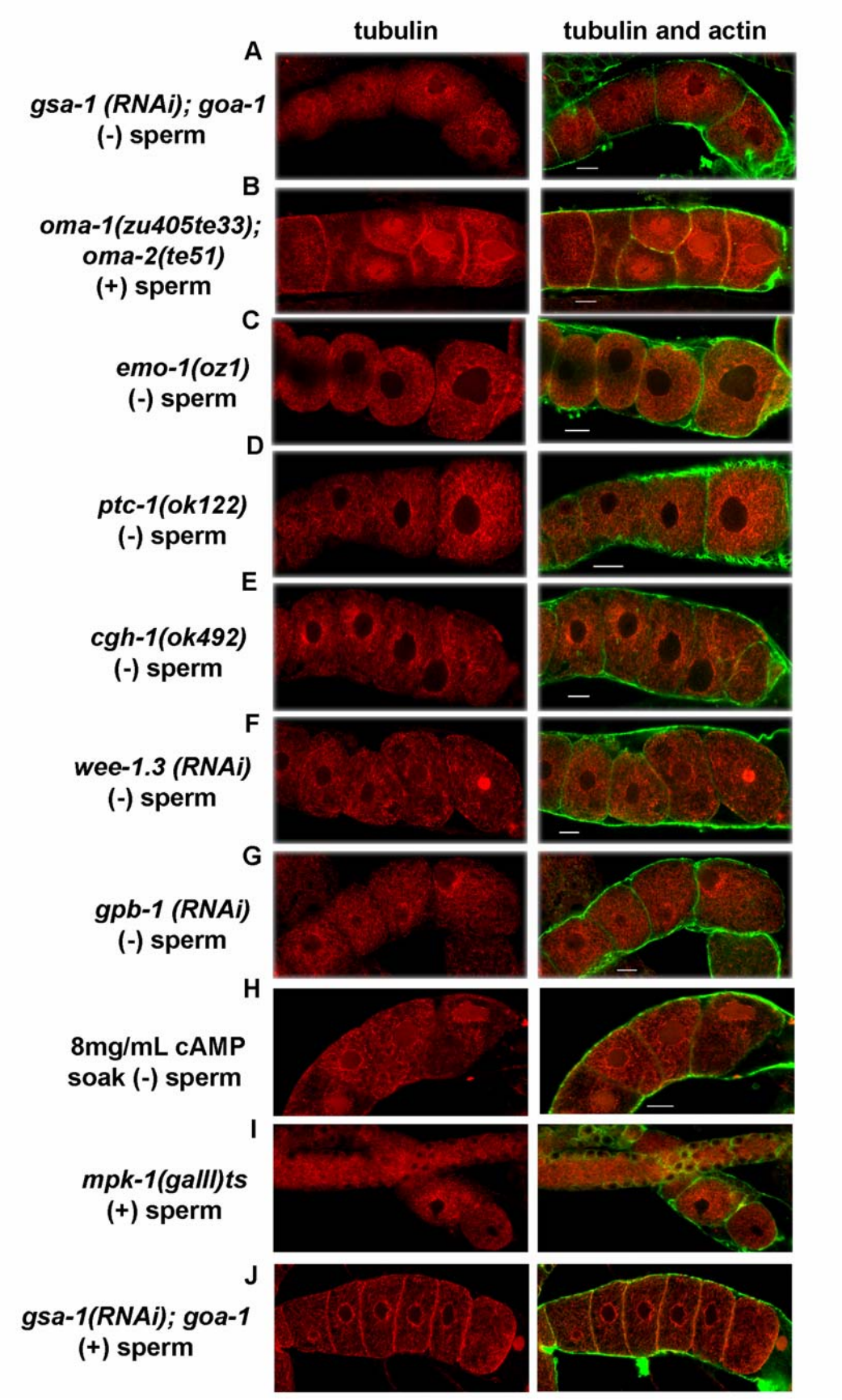

tubulin
tubulin and actin
A
gsa-1 (RNAi); goa-1
(-) sperm
B
oma-1(zu405te33);
oma-2(te51)
(+) sperm
C
emo-1(oz1)
(-) sperm
D
ptc-1(ok122)
(-) sperm
E
cgh-1(ok492)
(-) sperm
F
wee-1.3 (RNAi)
(-) sperm
G
gpb-1 (RNAi)
(-) sperm
H
8mg/mL cAMP
soak (-) sperm
I
mpk-1(galll)ts
(+) sperm
J
gsa-1(RNAi); goa-1
(+) sperm

**Figure 17. Additional genetic mutations or RNAi treatments that affect microtubule reorganization.** (A-K) Single confocal images of animals labeled with tubulin (red) and actin (green). (A) *goa-1(sa734)fog-3(q443)* unmated females treated with *gsa-1* RNAi do not exhibit cortically enriched microtubules. (B) *oma-1(zu405te33);oma-2(te51)* hermaphrodites contain oocytes with cortically enriched microtubules. (C-E) *emo-1(oz1)* (C), *ptc-1(ok122)* (D), and *cgh-1(ok492)* (E) unmated females contain reorganized microtubules. (F and G) Unmated *fog-2(q71)* females treated with either *wee-1.3* RNAi (F) or *gpb-1* RNAi (G) contain reorganized microtubules. (H) Unmated *fog-2(q71)* females soaked in a 8 mg/mL solution of cAMP for 2 hours contain reorganized microtubules. (I) 2 day adult *mpk-1(ga111)ts* hermaphrodite shifted to 25°C at 1 day of adulthood. Microtubules are reorganized in the few remaining oocytes of the disorganized gonad. (J) *gsa-1* RNAi in *goa-1(sa734)* hermaphrodites contain oocytes with cortically enriched microtubules. Scale bars: 10 μm.

the requirement for *mpk-1* MAPK. Null mutations in *mpk-1* MAPK cause a sterile phenotype in which germ cells fail to progress through pachytene thereby disrupting oogenesis at an early stage (Church et al., 1995). Therefore, we tested the involvement of *mpk-1* MAPK using three methods that reduce but not eliminate *mpk-1* activity: 1) the temperature-sensitive (ts) *mpk-1(ga111*ts*)* allele; 2) *mpk-1(RNAi)*; and 3) the chemical inhibitor U0126. One-day-old adult hermaphrodites were shifted to the restrictive temperature (25°C) for 12-16 hrs and their oocyte microtubules were analyzed by confocal microscopy. All gonad arms examined containing oocytes exhibited microtubule reorganization for both *mpk-1(ga111*ts*)* (n=10; Fig. 17I) and *mpk-1(RNAi)* (n=8).

We also injected the gonads of young adult hermaphrodites with a 100 μM solution of the MEK1/2 inhibitor, U0126, as an additional approach for inhibiting MAPK activation. When we assessed the efficacy of the U0126 injections by staining dissected gonads with antibodies to the diphosphorylated, activated form of MPK-1 MAPK (MAPK-YT), we found that only 50% of the gonads exhibited observable MAPK-YT staining (n=40) (Fig. 18D). In contrast, staining of U0126-injected gonads with anti-α-tubulin antibodies indicated that microtubules were reorganized in all proximal oocytes. Together these results suggest that MAPK activation may not be essential for MSP-dependent microtubule reorganization. This result is consistent with the observation, described above, that oocyte microtubules are reorganized following *gsa-1(RNAi)* in a *goa-1(sa734)fog-3(q443)* female background, despite the lack of observable MAPK activation in oocytes. This also applies to *wee-1.3* RNAi that contain reorganized microtubules in a female background, but also does not contain MAPK activation (Fig.

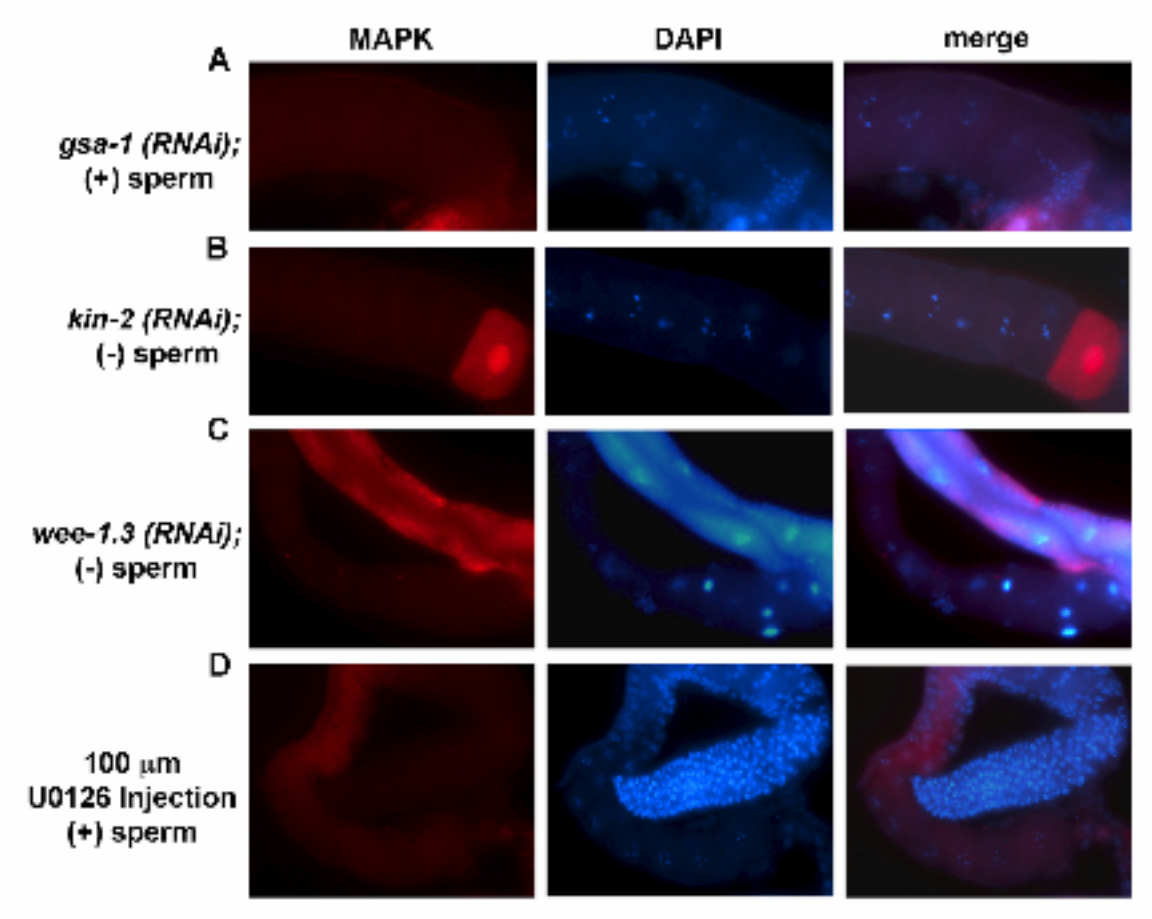



**Figure 18. Oocyte microtubule reorganization does not require high levels of MAPK activation.** (A-D) Fluorescent images of gonads labeled with MAPK antibodies (red) and the DNA stain, DAPI (blue). (A) *gsa-1* RNAi in hermaphrodites do not contain oocytes with MAPK activation. (B) *kin-2* RNAi in unmated females contain oocytes with activated MAPK. (C) *wee-1.3* RNAi in unmated females do not have activated MAPK and contain endomitotic oocytes in the proximal gonad arm as shown by DAPI. (D) 50% of hermaphrodites injected with a 100 μm solution of the MEK1/2 inhibitor, U0126, no longer have activated MAPK in proximal oocytes. Activated MAPK is still visible in the distal gonad.

18C). The interpretation of these results has the major caveat that we cannot exclude the possibility that residual levels of activated MAPK may be available for promoting oocyte microtubule reorganization.

## Discussion

### *The Regulation of Microtubule Reorganization*

Microtubules play critical roles during oogenesis by controlling cell shape, protein trafficking, RNA localization, and cell polarity. Despite these essential functions, it is incompletely understood how meiotic or follicular signals regulate microtubule organization and function in oocytes. We used quantitative confocal microscopy and genetics to analyze the regulation of oocyte microtubule reorganization. Our analyses defined two groups of regulators: the first group promotes microtubule reorganization in the presence of MSP; and the second group prevents reorganization in the absence of MSP (Table 2). A null mutation in the POU-homeobox gene *ceh-18* leads to microtubule reorganization in a female genetic background in which MSP is absent. This finding, taken together with the observation that *ceh-18* is expressed in gonadal sheath cells but not oocytes, and is required for proper sheath cell differentiation and function (Greenstein et al., 1994; Rose et al., 1997), suggests a role for the gonadal sheath cells in controlling the microtubule cytoskeleton of the oocyte. Consistent with this idea, *goa-1*, which encodes a $G\alpha_{o/i}$ protein shown to inhibit meiotic maturation through its action in the soma (Govindan et al., 2006), is a negative regulator of oocyte microtubule reorganization. Further, we used the *rrf-1* genetic background, in which somatic cells are less sensitive to

RNAi, to provide evidence that *goa-1* functions in the soma to prevent oocyte microtubule reorganization in the absence of sperm.

To explore how sheath cell signaling might influence the localization and dynamics of microtubules within oocytes, we tested several known germline-expressed microtubule regulators (e.g., microtubule-associated proteins and motors) for their potential involvement using RNAi, but none of the tested microtubule regulators had an effect. While it will take further work to uncover the mechanism by which the somatic gonad controls microtubule localization and dynamics in oocytes, signaling through sheath/oocyte gap junctions is likely to play an important role, as *inx-14(RNAi)* or *inx-22(RNAi)* cause microtubule reorganization in female genetic backgrounds. These findings mesh with prior TEM studies that indicated that sheath/oocyte gap junctions were rare or absent in *ceh-18* mutants (Rose et al., 1997; Hall et al., 1999). These observations raise the possibility that small molecules or ions transferred through these junctions might critically influence microtubule localization and dynamics.

MSP signaling stimulates the basal contraction of the proximal sheath cells (McCarter et al., 1999; Miller et al., 2001). Several observations allow us to discount the possibility that the absence or presence of external mechanical pressure from sheath cell contractions represents a major factor regulating MSP-dependent oocyte microtubule reorganization. For example, *oma-1(zu40te33);oma-2(te51)* mutant hermaphrodites exhibit substantial sheath cell contractions (5.6 ± 3.6 contraction/min; n=14; I.Y., D.G., unpublished results), but lack oocyte microtubule reorganization, whereas *ceh-18(mg57)* females exhibit low basal sheath cell contraction rates, but have reorganized oocyte microtubules.

***Does Microtubule Reorganization Regulate the Localization of Proteins Required for Meiotic Maturation?***

To address the potential function of oocyte microtubule reorganization, we attempted to determine the consequence of blocking the process. We found two situations in which oocyte microtubule reorganization was disrupted despite the presence of MSP: 1) *oma-1(zu40te33);oma-2(te51)* hermaphrodites; and 2) *gsa-1(RNAi)* hermaphrodites. Since meiotic maturation, ovulation, and fertilization do not occur in the *oma-1;oma-2* double mutant (Detwiler et al., 2001), we were unable to discern whether there is a detrimental consequence for the embryo. In a similar vein, meiotic maturation only occurs at a low rate following *gsa-1(RNAi)* (Govindan et al., 2006). The few fertilization events we observed following *gsa-1(RNAi)*, typically one per gonad arm, resulted in viable hermaphrodite offspring, perhaps suggesting a non-essential or modulatory role for oocyte microtubule reorganization. Nonetheless, several considerations affect the interpretation of this result. First, for technical reasons, we could not address whether microtubule reorganization can occur in the few maturing oocytes observed following *gsa-1(RNAi)*, similar to that seen in spontaneous meiotic maturation events in a female background. Second, we were unable to examine *gsa-1(null)* hermaphrodites owing to the fact that *gsa-1* is an essential gene (Korswagen et al., 1997), and thus the caveat that RNAi was used in this experiment should be considered. Mosaic analysis could be performed in the *gsa-1(null)* mutant to examine whether loss of *gsa-1* in the soma is sufficient to regulate microtubule reorganization and confirm our RNAi results. Genetic or experimental (i.e. RNAi) perturbations that specifically affect microtubule reorganization, but not other components of the meiotic maturation process,

will be needed to address the question of biological significance more fully.

While it is formally possible that MSP-dependent oocyte microtubule reorganization is non-essential, our bias is that this dramatic cytoskeletal transformation has functional importance for the worm. In our first proposed model, we suggested that microtubule reorganization might facilitate the assembly of the meiotic spindle (Fig. 19A). In a second, but not mutually exclusive model, cortical microtubule enrichment in the absence of MSP might facilitate trafficking of proteins needed to maintain meiotic diapause (Fig. 19B). For instance, meiotic arrest depends on the assembly of sheath/oocyte gap junctions and the function of the VAB-1 MSP/Eph receptor (Miller et al., 2003; Corrigan et al., 2005; Govindan et al., 2006). These regulators are likely to depend on microtubules for trafficking to the cortex (reviewed by Musch, 2004). By contrast, removal or depletion of negative regulators of meiotic maturation from the cortex upon microtubule reorganization might aid in flipping an "all or none" switch that drives the oocyte to meiotically mature once it reaches the most proximal position.

Our work here defines two classes of microtubule arrangements in oocytes: one associated with diapause; and the other associated with meiotic maturation. Both modes appear to represent active states with several genes required for their maintenance, suggesting complex regulation. We anticipate that the underlying mechanisms controlling cytoplasmic microtubule organization in this system may be relevant to the oocytes or epithelial cells of other organisms in which most microtubules are non-centrosomal.

CHAPTER V

# GENERAL DISCUSSION AND FUTURE DIRECTIONS

## Summary

Investigating the molecular and physical changes that occur in the oocyte during meiotic maturation is very important for understanding the meiotic defects that affect an organism's ability to reproduce. Previously in *C. elegans*, it was discovered that sperm release the cytoskeletal protein, MSP, which functions as the hormonal signal for meiotic maturation and ovulation (Miller et al., 2001). This finding in combination with the recent genome-wide RNAi screen for negative regulators of meiotic maturation (Govindan et al., 2006) make *C. elegans* a very powerful genetic tool for studying the control of meiotic maturation. *C. elegans* is also the only model organism for which the events of oocyte development, meiotic maturation, and ovulation are directly observable in the intact animal, therefore, the use of microscopy and other live cell imaging techniques is highly advantageous to study these processes. The observation that MSP stimulates meiotic spindle assembly in the absence of centrosomes resulted in the intriguing question as to whether MSP affected microtubules in oocytes. We have generated evidence that oocyte microtubules respond to MSP through changes in their localization and dynamics before NEBD and fertilization. We have also supported that these changes in microtubule organization are regulated by a complex signaling network involving antagonistic $G\alpha_{o/i}$ and $G\alpha_s$ pathways in the somatic cells of the gonad and

corresponding gap-junctional communication with oocytes.

**Genetic Regulation of Microtubule Reorganization**

To identify genes that may regulate microtubule reorganization in the presence and absence of MSP, we conducted a candidate screen, using RNAi or mutants, examining genes involved in meiotic maturation and microtubule related processes. Our analyses defined two groups of regulators of microtubule reorganization. The first group promotes microtubule reorganization in the presence of MSP, and the second group prevents reorganization in the absence of MSP (Table 2). Despite testing several known germline-expressed microtubule regulators (e.g. microtubule-associated proteins and motors) for their potential involvement in microtubule reorganization using RNAi and mutants, none of the tested regulators had an effect on oocyte microtubule organization. Several reasons may account for this result. First, for the majority of genes, RNAi was the sole technique used. Since we did not monitor protein depletion after RNAi treatment, we do not know the efficacy of RNAi. Sometimes the RNAi clones lose the plasmid for the gene of interest resulting in a negative result. RNAi is also not 100% effective and even if the bacterial clone is functional, there may be variability in the severity of phenotypes among the fed population. In addition, the clone in the RNAi library may be incorrect. Without sequencing the clone, the mistake will go undetected and the true phenotype for the gene of interest would not be observed.

Another possible reason for why we did not identify any microtubule regulators is because of genetic and functional redundancy. Perhaps other genes compensated for the loss of function of the gene of interest resulting in no obvious phenotype. For example, *oma-1* and *oma-2* are genes that function redundantly to regulate meiotic maturation and

microtubule reorganization. As a result, microtubules are only cortically enriched when both of these genes are non-functional. Multiple parallel pathways, similar to the *ceh-18* and *vab-1* pathways identified for the control meiotic maturation, may also be involved in microtubule organization. Therefore, components in all pathways would need to be removed before a full effect is realized. Multiple pathways are already evident for the control of microtubule reorganization including the two antagonistic $G\alpha_s$ and $G\alpha_{o/i}$ pathways in the sheath cells and pathways in the oocyte involving genes such as *oma-1* and *oma-2*.

We tried to test whether several genes were redundant by combining equal concentrations of the RNAi bacteria. For instance, we tested different combinations of three of the adenylyl cyclase genes (*acy-1, acy-2,* and *acy-3)*, and we tested both proposed catalytic subunits of PKA (*kin-1* and F47F2.1). Despite the negative results when combining these RNAi genes, we cannot rule out the possibility that the combination RNAi may not have been effective and that these genes still function redundantly. The two interacting genes, *mei-1* and *mei-2*, which respectively encode the p60 and p80 subunits of katanin, when expressed together result in the disassembly of microtubules (Srayko et al., 2000). RNAi of either *mei-1* or *mei-2* did not result in any phenotype on oocyte microtubule reorganization, but we did not examine the effect of RNAi on both genes. These two genes may function redundantly to regulate microtubule length in oocytes prior to NEBD. Another candidate pair of genes that may function redundantly in microtubule reorganization that we did not detect in our screen are *dhc-1* and *dnc-1*, which encode dynein and dynactin respectively. Dynein associates with dynactin in order to transport cargo (Porter and Johnson, 1989). Dynein is very important

for regulating the length of microtubules in the meiotic and mitotic spindles, and together with dynactin, helps to target depolymerizing activities to the spindle poles (Mountain and Compton, 2000; Gaetz and Kapoor, 2004). This indicates that dynein and dynactin may function together for different functions, but both complexes may not be required and one may be sufficient in regulating microtubules. We also used RNAi for either *dhc-1* or *dnc-1*, but not both genes simultaneously. In a female background, no phenotype was observed for *dnc-1* RNAi; however, we did observe a slight effect with *dhc-1* RNAi. We noticed that 31% of the gonads treated with *dhc-1* RNAi contained rearranged microtubules in a female background (n=16). This result was not prominent enough to classify *dhc-1* as a regulator of microtubule reorganization. It is possible that this percentage could increase if we eliminated both dynein and dynactin. Interestingly, a similar effect was noticed for *dnc-1* RNAi in a hermaphrodite background. 30% of animals with *dnc-1* RNAi had a mild cortical enrichment of microtubules (n=10). Since we only used RNAi to examine the phenotypes *dhc-1* and *dnc-1*, it is possible that their role in microtubule reorganization was overlooked. Until we can test mutants in these genes, our results cannot confirm that dynein or dynactin are major players in microtubule reorganization.

*C. elegans* also contain many kinesin like proteins any of which can function redundantly. We have tested *klp-7*, *klp-9* (a.k.a. *zen-4*), *klp-13, klp-15*, *klc-16, klc-18, khc-1,* and *klc-1*. Only RNAi of *khc-1*, *klp-13*, and *klp-16* had a slight affect on microtubule organization in oocytes. About 14% of oocytes in *klp-13* RNAi hermaphrodites (n=14) and about 38% of oocytes in *khc-1* hermaphrodites (n=8) contained microtubules that appeared cortically enriched. In a female background, about

25% of gonads with *klp-16* RNAi exhibited reorganized microtubules (n=4). Both *klc-1* and *khc-1* may be needed or multiple kinesin like proteins may function redundantly in oocytes to regulate microtubule organization. Several other genes also had a minor effect on microtubule organization. In a female background, the genes F36H12.8, encoding a possible tau tubulin kinase (25%, n=4), *cyb-1,* encoding a cyclin B protein (40%, n=5), *cyb*-3, encoding another cyclin B protein (63%, n=8), *cap-1*, encoding an f-actin capping protein (50%, n=4), and *let-99,* encoding a protein containing a DEP domain (40%, n=10), resulted in gonads with reorganized microtubules. In a hermaphrodite background, RNAi of *cdc-25.1*, a gene encoding a cdc25 phosphatase (50%, n=12), and *elp-1*, a gene encoding a microtubule associated protein (38%, n=8), resulted in cortically enriched oocyte microtubules. As mentioned above, the exclusive use of RNAi and the low number of affected gonads do not enable us to confirm that these genes are involved in microtubule reorganization.

Another problem we encountered when performing RNAi in hermaphrodites and females that may affect our identifying additional regulators of meiotic maturation is examining genes that perturb the organization of the gonad. RNAi of several genes that may be involved in cytoskeletal processes cause disorganized gonads without any observable oocytes and make examining microtubule organization impossible. These genes include the Ran GTPase, *ran-1*, *klp-15*, a non-muscle myosin, *nmy-2*, a GAP/Rho, *cyk-4*, the two polarity genes, *par-2* and *par-5*, and an actin gene, *act-4*. These cytoskeletal related genes could be required early in oogenesis, which would make it difficult to study their role in the mature oocyte and would require applying RNAi at a later stage of *C. elegans* development. Finally, we did not exhaust the list of possible

genes that may be involved in regulating microtubule reorganization. A recent RNAi screen of 40 genes involved in microtubule processes contained several genes that we did not test including genes involved in tubulin assembly (e.g. F53F4.3), microtubule dynamics (e.g. *mel-26*), spindle assembly (e.g. *tpxl-1*), spindle position (*zyg-8*), and nuclear envelope structure (*npg-9*) (Srayko et al., 2005). Further examination of additional microtubule related genes using RNAi and mutants may eventually help to uncover key regulators of microtubule reorganization.

***Future Characterization of Microtubule Reorganization***

We have characterized several aspects to microtubule reorganization, but several questions remain to be addressed. As mentioned above, microtubule regulators and other components of the G protein and oocyte pathways are unknown. An additional RNAi and mutant screen would be beneficial to try to identify these key players in microtubule reorganization. Some candidates include the genes reported in Srayko et al. (2006) and the remaining kinesin genes. Since there may be functional redundancy, microtubule organization can be examined with either combination RNAi or RNAi in mutant backgrounds. For example, a *dhc-1* mutant, such as the temperature sensitive EU828 strain, could be treated with *dnc-1* RNAi and tested for microtubule defects in the oocyte. Additionally, G protein coupled receptors could be examined since one has not been recognized for either of the $G\alpha_s$ and $G\alpha_{o/I}$ pathways. Any remaining genes from the genome-wide RNAi screen for negative regulators of meiotic maturation that have not been tested for an affect on microtubule reorganization are also plausible candidates to test. Identification of new genes involved in both meiotic maturation and microtubule

organization may help to further characterize and establish the relationship between regulators of microtubule reorganization in the oocyte including *oma-1/oma-2*, *ptc-1*, *emo-1*, *cgh-1*, and *wee-1.3*.

The involvement of *wee-1.3* in microtubule reorganization raises the possibility that cyclin B and CDC25 is involved, and the slight RNAi effect on microtubules supports this assumption. The involvement of *wee-1.3* also implies that microtubule reorganization may be downstream of *cdk-1* activation. As previously mentioned, RNAi of *cdk-1* in the presence or absence of sperm did not have any effect on oocyte microtubule reorganization (data not shown), however, it may be difficult to completely deplete CDK-1 from the germline using RNAi. It is very interesting to consider that *wee-1.3* and *cdk-1* may have additional functions to their roles in meiotic progression especially since Cdk1 has been implicated in mitotic spindle assembly by phosphorylating a number of microtubule- or tubulin-binding proteins. A recent report has even shown that Cdk1 phosphorylates β-tubulin both *in vitro* and *in vivo* and suggests that tubulin phosphorylation by Cdk1 could be involved in the regulation of microtubule dynamics during mitosis (Fourest-Lieuvin et al., 2006). Future experiments include investigating microtubule organization in various *cdk-1*, *cyb-3*, and *cdc-25.1* mutants to determine whether these genes are involved in regulating microtubule reorganization in addition to *wee-1.3*.

**The Biological Significance of Microtubule Reorganization**

We have characterized a significant change in microtubule organization and dynamics in response to hormonal signaling during meiotic maturation. Even though we

have determined several genetic regulators, the biological significance of microtubule reorganization in oocytes remains elusive. We propose several possibilities and future experiments for identifying the function of microtubule reorganization including a role in meiotic maturation, meiotic spindle assembly, protein distribution, and patterning the shape and Cytoplasmic components of the oocyte.

### *The Effect of Microtubule Reorganization on Meiotic Maturation*

MSP has been shown to be sufficient to promote meiotic maturation (Miller et al., 2001). We have found that MSP is also sufficient to reorganize microtubule in oocytes prior to NEBD. This correlation leads to the hypothesis that microtubule reorganization promotes meiotic maturation. Since *vab-1* and *ceh-18* are two pathways both required for meiotic maturation, our analysis of these mutants have determined that only the *ceh-18* pathway is required for microtubule reorganization. Meiotic maturation rates are still elevated in the *vab-1* null mutant female, which contain oocytes with cortically enriched microtubules (Miller et al., 2003). This suggests that microtubule reorganization is not required for meiotic maturation. The observation that unmated females mature their most proximal oocyte at low rates also makes this suggestion. However, we observed an asymmetry of cortical microtubule enrichment in the -1 oocyte in the absence of sperm in that microtubules were enriched at the distal, but not the proximal cortex. Since females have low rates of maturation, we speculate that this asymmetry may be part of the mechanism by which this oocyte "knows" it is in the most proximal position and thus competent to undergo meiotic maturation. This observation may help to support that microtubule reorganization is sufficient to promote meiotic maturation. Indeed, *gsa-1*

RNAi treated and *oma-1/oma-2* mutant hermaphrodites contain cortically enriched microtubules but have low rates of meiotic maturation (Detwiler et al., 2001, Govindan et al., 2006). Additionally, RNAi of *kin-2* and *ceh-18* null mutant females have reorganized microtubules and elevated rates of meiotic maturation (Miller et al., 2003, Govindan et al., 2006). Despite these observations, we cannot assume that microtubule reorganization causes meiotic maturation. Additional experiments that affect only the organization of microtubules and not other aspects of meiotic maturation would establish the relationship between microtubule organization and meiotic maturation.

***Microtubule Reorganization in Facilitating Meiotic Spindle Assembly***

Oocyte microtubules modify their localization and dynamics in response to MSP. Since meiotic spindle assembly in the absence of centrosomes relies on microtubule dynamics and motors, one of our hypotheses predicts that the change induced by MSP promotes the search and capture of meiotic chromatin after NEBD (Fig. 19A). Thus, MSP may help prepare microtubules for meiotic spindle assembly early in the maturation process, so that the spindle can form rapidly upon NEBD. Cortically enriched microtubules may then help to prevent the assembly of a meiotic spindle until their localization and dynamics are altered by MSP. The low meiotic maturation rates in females (McCarter et al., 1999), however, make it extremely difficult to analyze meiotic spindle assembly in the absence of sperm. Uncovering how different levels of MSP signaling affects meiotic spindle assembly will provide the best method to test this model. As mentioned earlier, sperm availability affects the frequency of meiotic maturation, but not the timing of spindle assembly once maturation occurs. It remains a formal

possibility, however, that oocyte microtubule reorganization may either have a redundant role in influencing the timing of meiotic spindle assembly, affect the stability of meiotic spindle microtubules, or in correctly localizing proteins to the meiotic spindle, or chromatin, to ensure proper assembly. Further experiments will be needed to address the role of microtubule reorganization in meiotic spindle assembly.

***Redistribution of Proteins in Response to Microtubule Reorganization***

Similar to the microtubule-dependent localization of morphogenetic determinants in *Drosophila* oocytes (Theurkauf et al., 1992), oocyte microtubule reorganization in *C. elegans* may also be important for redistributing proteins throughout the oocyte. As previously mentioned, regulators of meiotic maturation, such as *vab-1*, are likely to depend on microtubules for trafficking to the cortex (reviewed by Musch, 2004). Therefore, cortical microtubule enrichment in the absence of MSP might facilitate or prevent the trafficking of proteins needed to maintain meiotic diapause (Fig. 19B). In support of this hypothesis, DAB-1, a negative regulator of meiotic maturation, has been shown to change its cytoplasmic localization in the oocyte in unmated females to being cortically enriched between oocytes in the presence of sperm (Govindan et al., 2006). If this localization pattern is dependent on microtubules and is required for meiotic diapause, then cortical microtubule enrichment would help prevent meiotic maturation by not being able to physically traffic *dab-1* to the cortex. By contrast, removal or depletion of negative regulators of meiotic maturation from the cortex upon microtubule reorganization might aid in flipping an "all or none" switch that drives the oocyte to meiotically mature once it reaches the most proximal position. For example, *goa-1*

localization has been recently shown to be cortically enriched in the oocyte when sperm is absent and diffuse throughout the cytoplasm when sperm is present (Govindan et al., 2006). Again, if microtubule reorganization is required for this redistribution, then cortically enriched microtubules may help to keep *goa-1* at the cortex and prevent meiotic maturation. Determination of the dependence of these localization patterns on microtubules would help to support this model.

Microtubule reorganization in response to MSP signaling might also function in refashioning the cortex or plasma membrane domains for the later developmental events of fertilization or embryogenesis. At anaphase I, the DYRK-family kinase MBK-2 translocates from the cell cortex to intracellular puncta in a trafficking process that depends on CDK-1 and the anaphase-promoting complex (McNally and McNally, 2005; Stitzel et al., 2006). While prior studies have not addressed whether microtubules play a key role in promoting MBK-2 redistribution, it is interesting that microtubules in the embryo are themselves a downstream target of MBK-2. MBK-2 phosphorylates the MEI-1 subunit of katanin, a dimeric microtubule-severing AAA-ATPase (Srayko et al., 2000), which marks it for ubiquitin-mediated degradation by a CUL-3/MEL-26 E3 ubiquitin ligase (Furukawa et al., 2003; Pintard et al., 2003). MEI-1 is required for assembly of anastral barrel-shaped female meiotic spindles (Clark-Maguire and Mains, 1994), and the inappropriate inclusion of MEI-1 into mitotic spindles is lethal because excessive shortening of astral microtubule arrays disrupts spindle positioning and the asymmetric partitioning of cell-fate determinants (Clandinin and Mains, 1993). The pioneering work of Mains and colleagues (Mains et al., 1990) brought attention to the fact that meiotic and mitotic spindles form in a common cytoplasm, yet each utilizes

different assembly mechanisms and mediates distinct functions, implying uniquely tailored regulatory modes.

In addition to proteins involved in maturation, microtubule reorganization may also redistribute proteins involved in meiotic spindle assembly. Interestingly, MEI-2 localizes MEI-1 to the spindle during meiotic maturation (Clark-Maguire and Mains, 1994), but the mechanism of this localization is unclear. It may be possible that reorganized microtubules traffic these proteins to the meiotic spindle, however, reports that show that MEI-1 and MEI-2 interact differently with the two redundant α- and β-tubulin isotypes indicate that these interactions affect severing but not localization (Lu et al., 2004; Lu and Mains, 2005). Other proteins, such as ZYG-9, and motors, such as dynein and kinesin, may also require microtubules to transport them to the meiotic spindle. Examination of their localization in the presence and absence of MSP may help to determine whether they redistribute their position in the oocyte, and blocking reorganization, with drugs or mutants, could establish if microtubules are involved.

### *Oocyte Shape Change and Organelle Repatterning in the Presence Of MSP*

Microtubule reorganization may play an essential role in regulating oocyte shape and the redistribution of organelles. In the absence of sperm, *C. elegans* oocytes are rectangular in shape and stack in the gonad arm. Electron micrographs of female oocytes reveal a different cytoplasmic environment compared to hermaphrodites in that the organelles are clustered and highly organized (Mary Kosinski, Kent McDonald, David Greenstein, unpublished results). When sperm is present, oocytes become slightly rounded, less compacted in the gonad arm, and organelles are dispersed in the cytoplasm.

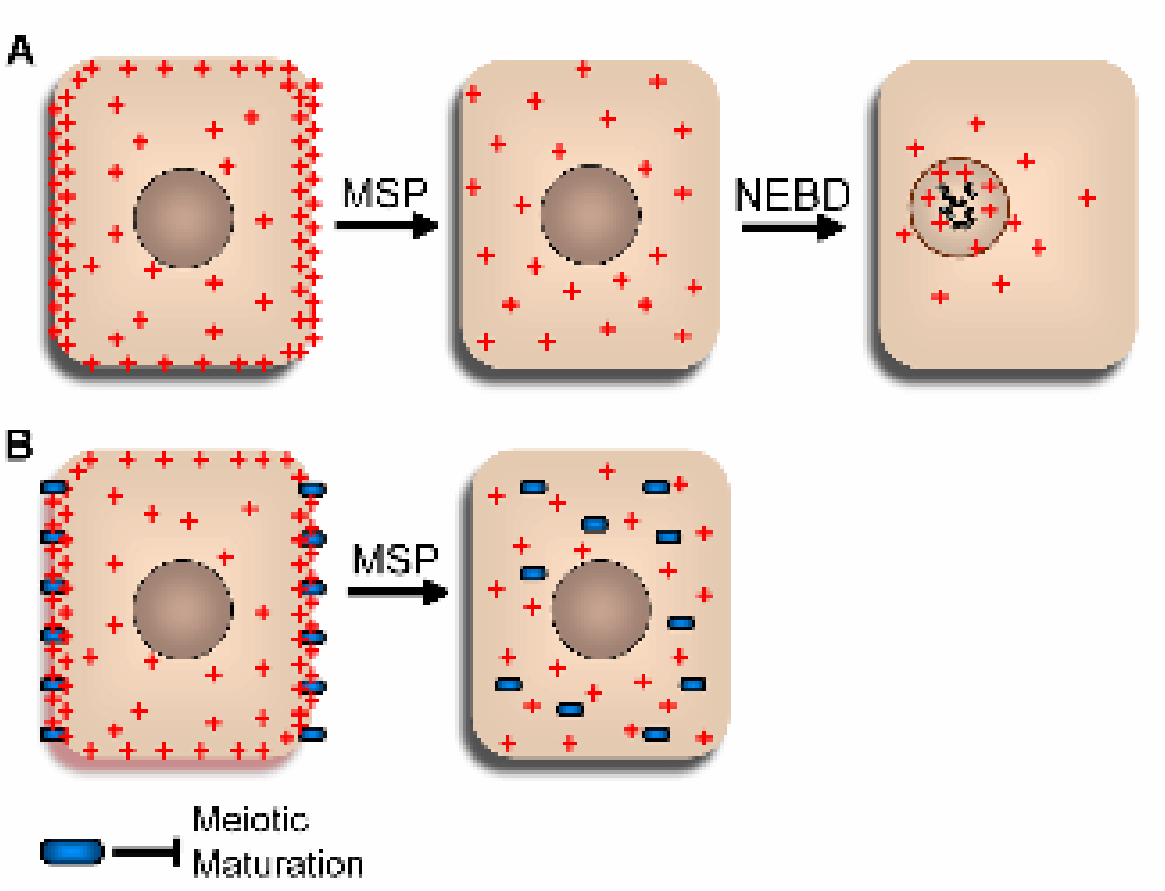



**Figure 19. Models for the biological significance of MSP-dependent oocyte microtubule reorganization.** (A) Microtubule reorganization prepares the oocyte for meiotic spindle assembly upon NEBD. Changes in microtubule localization and dynamics (indicated by plus signs) in the presence of MSP facilitate the search and capture of chromatin by microtubules. (B) Microtubule reorganization repatterns the oocyte cortex to facilitate the irreversible cell-cycle transition of meiotic maturation. Cortical reorganization may affect the trafficking of membrane proteins that inhibit meiotic maturation (depicted in blue).

If microtubules could be induced to reorganize in the absence of sperm without spontaneously maturing, then the question could be addressed whether cortical enrichment was required for the rectangular stacked oocytes. Positioning of organelles may also play a factor in cell shape. In mammalian oocytes, microtubule reorganization is necessary for positioning mitochondria in a perinuclear pattern during cytoplasmic and nuclear maturation (Van Blerkom, 1991). Perhaps similar to mammalian systems, microtubules may assist in organelle positioning by keeping organelles in the absence of sperm tightly compacted in oocytes. Further studies are needed to link microtubules with cell shape and the relocation of organelles in the oocyte. Possible experiments include labeling organelles in oocytes with specific antibodies and determining their localization in the presence or absence of sperm.

***Uncovering the Biological Significance of Microtubule Reorganization***

We have proposed several models for the biological significance of microtubule reorganization, and only through further experiments would we be able to address these models. To address whether microtubule reorganization facilitates the meiotic maturation process, meiotic maturation, the redistribution of proteins, or repatterning of organelles several experiments could be conducted to analyze which biological process or combinations of processes are involved. First, an extensive analysis using pharmacological drugs would be very informative. The application of nocodazole in hermaphrodites would be very helpful in determining whether cortical enrichment of microtubules affect meiotic maturation. Since microtubules are cortically enriched in hermaphrodite oocytes after nocodazole treatment, if meiotic maturation rates decrease

after nocodazole injection into hermaphrodite gonads, this would indicate that either cortical enrichment helps prevent meiotic maturation or new microtubule nucleation at non-cortical sites is necessary for meiotic maturation. Comparing labeled proteins or organelles in unmated and mated females would determine whether MSP affects their localization, and comparing the localization of proteins and organelles before and after nocodazole treatment in hermaphrodites could determine whether their localization is reversibly dependent on microtubules. Oocyte shape could also be examined after nocodazole treatment in hermaphrodites by measuring the volume, height, and width of these oocytes and comparing these measurements to that of female oocytes. This would reveal whether driving cortical enrichment and/or new microtubule polymerization at non-cortical sites causes the oocyte to change its shape. Finally, if meiotic maturation occurs in nocodazole treated hermaphrodites, meiotic spindle assembly could be examined. However, if there is an effect on meiotic spindle assembly, it would be difficult to determine if this effect was due to the nocodazole treatment or the change in oocyte microtubule localization.

In addition to nocodazole, we could test the effects of the microtubule stabilizing drug, taxol, on oocytes in the presence and absence of sperm. If we injected taxol into the gonads of females and then mated them with males, we could ascertain whether stabilizing microtubules prevents microtubule reorganization in the presence of MSP. If this result is the case, then we could examine meiotic maturation rates to determine whether microtubule reorganization promotes meiotic maturation. Additionally, we could test whether oocyte shape was affected after taxol treatment in the presence of sperm. The rectangular oocyte shape in the absence of sperm, however, may be a

consequence of too many oocytes stacking in the gonad arm. If taxol or nocodazole negatively affects meiotic maturation rates, then it may be difficult to determine whether shape change is a direct result of microtubule organization or oocytes stacking because they are not maturing. Examining meiotic spindle assembly after treatment with taxol would be very interesting especially if microtubules do not reorganize in the presence of sperm. If maturation does occur, we could examine any progeny or lack of progeny for defects. If a meiotic spindle even assembles, we could also measure how long spindle assembly occurs after NEBD. However, it may be hard to determine whether the affect on spindle assembly after taxol treatment was due to microtubule reorganization or whether another process that taxol may have affected. Tubulin RNAi (e.g. *tba-2*) in either hermaphrodites or females can be used in addition to nocodazole and taxol. Since *tba-2* RNAi caused a dramatic decrease of microtubules in oocytes (data not shown), the processes of meiotic maturation and protein localization could be tested for their dependence on microtubules.

We can further test the affect of microtubule reorganization on meiotic spindle assembly by examining whether non-disjunction occurs in mutants or animals treated with RNAi for genes that regulate microtubule reorganization. For instance, we can test whether the gain-of-function *gsa-1* mutant females, which contain reorganized microtubules, have defects in meiotic spindle assembly by mating them with *tra-2* males and counting the number of male progeny. *tra-2* males have an XX genotype, like a female, instead of the normal XO genotype for males. Since *tra-2* males are XX, then mating with females should only produce female progeny. However, if non-disjunction occurs during meiosis in the female, then some of the progeny will be male. Therefore,

the percentage of male progeny would be a good indicator of whether non-disjunction is occurring. In addition to testing for non-disjunction with mutants and RNAi, we can also conduct a hermaphrodite time course, similar to that described for microtubule organization, and mate with the *tra-2* males at different days. This time course would be extended for a longer period of time, to about 10 or 15 days, to simulate the aging effect in human females. If we mated 15-day-old hermaphrodites with *tra-2* males, we can identify whether non-disjunction occurs and if there is a higher percentage of males compared to a 5-day-old adult. This experiment would not only provide support for our hypothesis that microtubule reorganization affects meiotic spindle assembly, but it would also provide a model to study the effect of non-disjunction in aging human females.

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