Analysis of the initiation of acentrosomal spindle assembly in *Drosophila melanogaster*

By

JEFFRY CESARIO

A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

And

The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Microbiology and Molecular Genetics

Written under the direction of

Kim S. McKim, Ph.D.

and approved by

_____

_____

_____

_____

_____

New Brunswick, New Jersey

October 2010

Abstract of the Dissertation

Analysis of the initiation of acentrosomal spindle assembly in *Drosophila melanogaster*

By JEFFRY CESARIO

Dissertation Director:

Kim S. McKim

In many species, meiotic spindles assemble microtubules in the absence of centrosomes. An acentrosomal mechanism has been proposed where the chromosomes initiate spindle assembly by nucleating and capturing microtubules. While preliminary evidence has supported this model in *Drosophila*, very little is actually known about how a bipolar spindle is initiated and maintained in the absence of centrosomes.

RanGTP is a small GTPase which has been shown to be important for chromosome-dependent spindle assembly in *Xenopus* extracts. I investigated the effect of manipulating the Ran pathway on spindle assembly in *Drosophila* oocytes and embryos. Surprisingly, I found that RanGTP is not essential for the formation of the acentrosomal spindle near the chromosomes. However, I discovered that RanGTP is essential for completing chromosome-independent microtubule assembly, such as pro-nuclear fusion. Thus, RanGTP may be required for microtubule assembly when chromosomes are absent, but redundant with the chromosomal passenger complex when chromosomes are present.

Another contributor to acentrosomal spindle assembly is Subito, a member of the kinesin 6 superfamily. Subito binds and bundles microtubules at the central spindle in both centrosomal and acentrosomal systems. We have previously shown that expression of Subito with an N-terminus deletion results in the formation of chromosome-independent spindles, or ectopic spindles, in the *Drosophila* oocyte. By continuing the mutational analysis within the N-terminus, I have further characterized its regulatory role. We have shown that two conserved serines negatively regulate the motor activity of Subito, while other domains within the N-terminus are essential for positive regulation. So while originally believed to be simply a negative regulator, the N-terminus is actually a complex region of negative and positive regulators.

My studies have helped to clarify the requirements for the initiation of acentrosomal spindle assembly in *Drosophila*. RanGTP is essential for spindle assembly in a chromosome independent manner, suggesting that chromosomal passenger complex is necessary for chromosome dependent microtubule assembly. Furthermore, we suggest a model of auto-inhibition for Subito with the N-terminus interacting with the C-terminus, preventing the binding of microtubules. Together, these results have provided new insights into acentrosomal spindle assembly.

Acknowledgements

First off, I would like to thank my advisor, Dr. Kim McKim. I will be the first to admit that working with me can sometimes be a challenge. This fact only exemplifies how patient and caring Kim is as a teacher and mentor. He never tired or became frustrated with the countless questions I asked him. He even maintained his cool during numerous political conversations, which cannot be easy given our opposing views. I can honestly say that if one day I woke up with half the intellect and a quarter of the patience of Dr. Kim McKim, I will consider my life quite a success. Thank you again, Kim, for your mentoring and friendship through the years.

Next, I would like to thank my parents, Frank and Carol Cesario. Without their support, I would not be the person I am today. My mom has taught me by example what it means to be a caring person. Without question, I always had a lunch packed and waiting for me to take to work in the morning. Little gifts of kindness, like a nicely prepared lunch, often go without any sign of gratitude. So let me say now, I am eternally grateful for every act of kindness you have ever shown me. My dad has instilled the values that have made me the man I am today. He taught me that life is not easy, and the only way to get through it is with hard work. I hope my hard work and determination have made my dad proud. I also want to thank my dad for his gift of cynicism. Cynicism is a trait of every great scientist, and you, dad, have certainly helped me on my scientific path. Thank you and I love you both.

My older sister and brother, Amy and Frank Cesario, you are amazing role models. I want to thank you both for your guidance throughout the years. Though often it goes unsaid, I hope both of you know that I love you. To John Elter and Maureen Cesario, I couldn't ask for better additions to our family. I want to thank you for always being supportive.

I would also like to thank my girlfriend, Jenna Bono. I have learned so much from you in such a short amount of time. I hope this is just the beginning of our journey together. I love you so much. To my friends, thank you for always being there for me. I have known Joseph Elsabee and Thomas Glenn since high school, and I couldn't ask for better friends. Nate Ivanick, though distance has separated us, I still treasure our monthly conversations. I also want to thank Kent, Helen, and Weston Tarrach. Your friendship has been invaluable to me.

I also want to thank all my co-workers in the McKim lab. Dr. Sonam Mehrotra was my original mentor. I want to thank you for all the knowledge you imparted to me. Janet is an incredibly kind person, though I think it is best that we keep our mutual projects to a minimum. Janet, you have become a real friend through the years. Who could ask for a better person to work with than Shree? You keep lab incredibly interesting, and I fear my next place of employment will be quite boring without you. Finally, I would like to thank Dr. Sarah Radford. Though we did not always see eye-to-eye, I think we have become good friends through the years. Don't worry, I won't tell anyone.

Dedication

To my family and friends for their constant support while continuing my education.

Table of Contents

List of Tables

List of Figures

Introduction

The goal in both mitosis and meiosis is to ensure even chromosome segregation. This is accomplished via a complex apparatus composed of microtubules, kinesins, and other microtubule-associated proteins, which is called the spindle. Errors in assembling the spindle can result in aneuploidy, cell death, genome instability, cancer, and birth defects. Mitosis is when a parental cell divides, creating two diploid daughter cells (Figure 1A). Meiosis is the process of dividing one parental cell and creating four haploid cells (Figure 1B). This is accomplished via two divisions: a reductional division, where the homologous chromosomes are separated, and an equational division, where sister chromatids are separated. While the overall outcomes of mitosis and meiosis are different, many of the factors that initiate and regulate these divisions remain the same. This introduction will review the main spindle assembly contributors and regulators while discussing the nuances of both processes.

Spindle assembly in mitosis and meiosis

Mitotic divisions occur with centrosomes as the driving force behind assembling a bipolar spindle. Centrosomes, which are composed of two centrioles, function by migrating to opposite poles early in the cellular division so they can nucleate and assemble tubulin to form microtubules. Microtubules have a net polarity, with the negative end towards the poles, and the positive end closer to the chromosomes. As microtubules are nucleated at the centrosomes, the plus end grows, searching the cytoplasm for kinetochores. This is an extremely dynamic process as microtubules grow and shrink. Chance contacts between kinetochores and microtubules are transient until

both kinetochores have stably attached microtubules. An arrest at metaphase, initiated by the metaphase checkpoint, halts the onset of anaphase and the separation of the sister

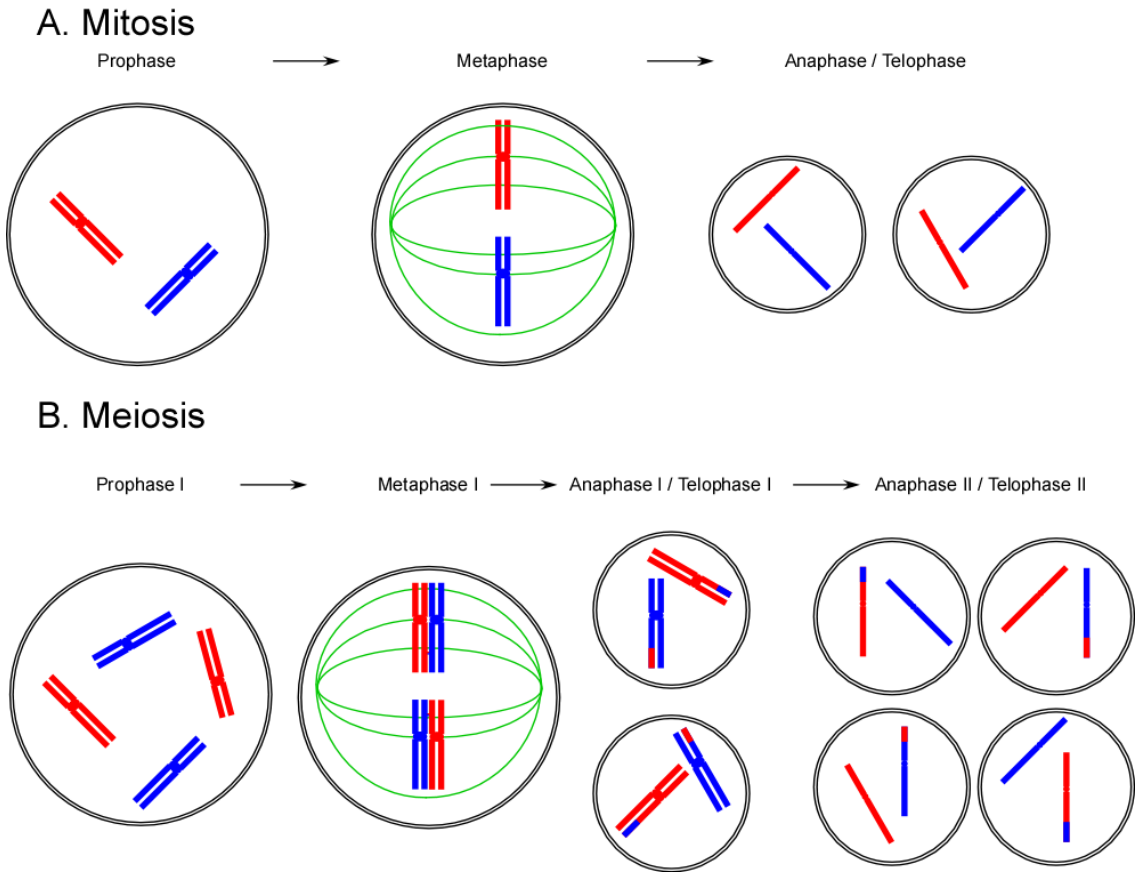

Figure 1 – The difference between mitosis and meiosis. Mitosis is the process of a parental cell dividing into two diploid cells (A). For this to occur, a duplicated chromosome (red and blue), consisting of two sister chromatids, must align at the central spindle during metaphase. Kinetochore microtubules attach directly to the kinetochore from the centrosomes. Interpolar microtubules do not interact with the kinetochores; instead, the microtubules are stabilized at the central spindle by kinesins capable of bundling anti-parallel microtubules. This matrix of microtubules searches the cytoplasm until all the kinetochores are properly attached, satisfying the checkpoint. As anaphase commences, sister chromatids segregate towards opposite poles during anaphase. Meiosis is the process of a diploid cell dividing into four haploid cells (B). During prophase, homologous chromosomes are physically linked by a recombination event. During anaphase, the recombination event is resolved resulting in a crossover. The homologous chromosomes then segregate towards opposite poles during the first meiotic division. The second meiotic division occurs without replication and is more mitotic in nature, since the sister chromatids segregate during this division. The second division results in four haploid daughter cells.

chromatids until all kinetochores have captured microtubules (Blower and Karpen 2001). Lack of tension along the microtubules can also initiate the metaphase arrest, as tension signals proper microtubule attachment to the spindle (Logarinho, Bousbaa et al. 2004). An example of lack of tension is when one kinetochore binds microtubules from both spindle poles, known as a merotelic attachment. When this improper kinetochore microtubule interaction is not corrected, it can result in abnormal chromosomal segregation (Parry, Hickson et al. 2003). As attachments form and stabilize properly, the tension between both poles congresses the chromosomes at the metaphase plate. Once the checkpoint is satisfied, anaphase begins with kinetochore microtubules shrinking and non-kinetochore microtubules elongating. This dramatic change in microtubule stability coupled with the cleavage of cohesin results in sister chromatids being pulled towards opposite poles. The mitotic division finishes with telophase and cytokinesis, as all components of the cell divide evenly into two daughter cells and the nuclear envelope reassembles.

Mitotic and meiotic divisions share many of the same components; hence, features of one division are often applicable to the other. However, there are important differences between female meiosis and mitosis. First, homologous chromosomes orient during the first meiotic division via a linkage termed the chiasma (von Wettstein 1984). The chiasmata physically link homologous chromosomes, holding them together while the bipolar spindle is pulling the chromosomes towards the poles. Chromosomal segregation does not occur until the metaphase checkpoint is satisfied and the chiasmata are resolved. Second, the second meiotic division resembles a mitotic division and initiates without a growth phase. As a consequence, sister chromatids are separated,

resulting in four haploid daughter cells. Third, unlike mitosis in most animals, meiosis is acentrosomal; meaning spindle assembly occurs without the guidance of the centrosomes at the poles. In this situation, the chromosomes play an important role in spindle assembly. In *Drosophila* oocytes, for example, nuclear envelope breakdown (NEB) is followed by the accumulation of microtubules around the chromosomes (Theurkauf and Hawley 1992; Matthies, McDonald et al. 1996). The subsequent bundling and tapering of these microtubules by motor proteins results in a bipolar spindle. These studies suggest that *Drosophila* oocyte chromosomes carry a signal that promotes spindle assembly when released upon NEB. Recent analysis of the kinesin-6 family member Subito confirms this suggestion. MKLP2 is the human homolog of Subito and is thought to be a plus end-directed motor that slides anti-parallel microtubules (Nislow, Lombillo et al. 1992). In *Drosophila* oocytes, a mutation causing the deregulation of *subito* is only capable of inducing chromosome independent spindle assembly after nuclear envelope breakdown (Jang, Rahman et al. 2007). While the components of this signal remain unclear, two mechanisms have been proposed to promote spindle assembly in the absence of centrosomes (Karsenti and Vernos 2001).

The role of the chromosomal passenger complex in acentrosomal spindle assembly

The chromosomal passenger complex (CPC) is a complex of proteins including: Incenp, Survivin, Borealin, and Aurora B, also known as Ial in *Drosophila*. The CPC functions to coordinate a wide range of activities, including chromosome-microtubule interactions, sister chromatid cohesion, and cytokinesis. It controls these activities during both mitosis and meiosis.

During mitotic prophase, the CPC localizes to the chromosome arms where it maintains chromosome structure and organization (Giet and Glover 2001). It then concentrates at the kinetochores during pro-metaphase where it functions to bi-orient the chromosomes (Becker, Stolz et al.). Aurora B is capable of releasing microtubules from the kinetochores (Tanaka, Rachidi et al. 2002). This release is essential to fixing aberrant microtubule to kinetochore attachments. The CPC has also been proposed to initiate centrosomal spindle assembly by inactivating proteins like the microtubule-depolymerizing motor MCAK (Sampath, Ohi et al. 2004). Disrupting the CPC has also been shown to upset spindle bipolarity. Together, these results suggest that the CPC functions to initiate and regulate mitotic spindle assembly (Adams, Maiato et al. 2001). Once the spindle checkpoint has been satisfied, anaphase commences with the CPC transferring to the central spindle (Earnshaw and Cooke 1991). This becomes the site of the cleavage furrow, allowing cytokinesis to successfully complete (Eckley, Ainsztein et al. 1997).

Studying the CPC in meiosis is difficult since null mutations of *Incenp*, *ial*, *survivin*, and *borealin* are all embryonic lethal. However, important differences between mitosis and meiosis have been observed for the CPC. The most intriguing difference is the change in localization. The CPC does not remain bound to the chromosomes at metaphase, like in mitosis. Instead, the CPC accumulates at the central spindle (Jang, Rahman et al. 2005). At anaphase I, the CPC transfers to the kinetochores of the segregating chromosomes and remains bound through telophase I (Resnick, Satinover et al. 2006). Since the CPC localizes to the microtubules and not the chromosomes during meiotic metaphase, the CPC may be initiating acentrosomal spindle assembly. More

recent studies have supported this hypothesis. In *Xenopus* extracts, the CPC is essential for initiating acentrosomal spindle assembly (Maresca, Groen et al. 2009). Analysis of a hypomorphic mutation in *Incenp* has led to the suggestion that this pathway is important for spindle assembly in *Drosophila* oocytes as well (Colombie, Cullen et al. 2008; Resnick, Dej et al. 2009). Nonetheless, more evidence is needed to decipher the varying roles of the CPC during meiosis, including the initiation of spindle assembly.

RanGTP: A small GTPase that assembles large acentrosomal spindles

Ran is a member of the Ras family of small GTP-binding proteins. It was originally discovered for its role in shuttling proteins with nuclear localization sequences into the nucleus (Moore and Blobel 1993). Ran cycles from an active state, RanGTP, to an inactive state, RanGDP (Figure 2). This cycle is complemented by two accessory proteins, RCC1 and RanGAP. RCC1 is a chromatin bound nucleotide exchange factor, which functions to replace the GDP nucleotide with a GTP nucleotide. RanGAP, also known as Ran GTPase activating protein, is found in the cytoplasm where it hydrolyzes RanGTP to RanGDP (Bischoff, Klebe et al. 1994). During G_2 phase, the nuclear envelope creates a barrier where active RanGTP can only be found within the nucleus, since RCC1 is chromatin bound. The Importin complex, which consists of Importin α and Importin β, is capable of binding proteins with nuclear localization sequences (NLS) and transporting them into the nucleus. Once inside the nucleus, RanGTP binds Importin β, releasing Importin α and NLS containing protein (Moroianu 1997). This pathway has been shown to be essential for DNA replication, RNA processing, and gene expression.

It was subsequently discovered that Ran has a role in spindle assembly by releasing spindle assembly factors near the chromosomes (Kalab and Heald 2008). In *Xenopus*, the addition of RanGTP to RCC1-depleted eggs is sufficient to induce self-organization of microtubule asters (Ohba, Nakamura et al. 1999). This result suggests that RanGTP is a major contributor to the initiation of spindle assembly. The production of RanGTP near chromatin and conversion to RanGDP in the cytoplasm can lead to the formation of a gradient of active Ran. A high or specific concentration of RanGTP may be the signal which triggers chromosome-mediated spindle assembly (Caudron, Bunt et al. 2005), though more work is necessary to confirm its role in initiating acentrosomal spindle assembly.

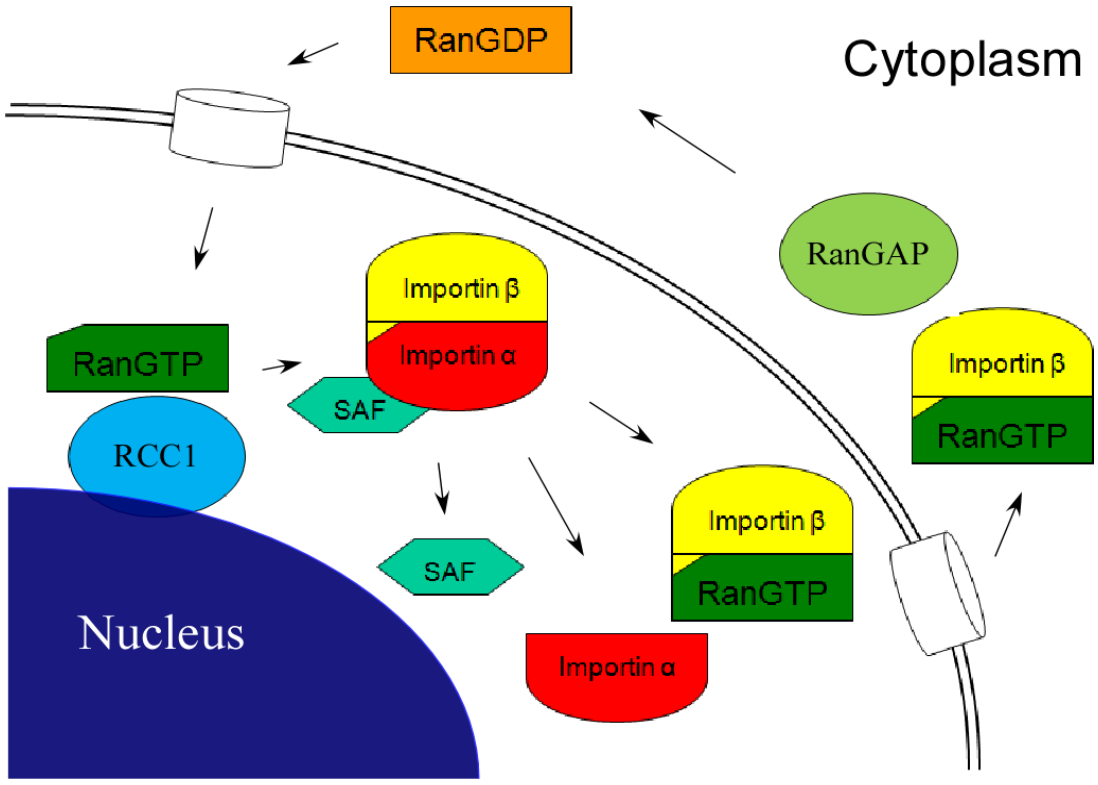

Figure 2 – The cycling of Ran between the cytoplasm and nucleus. RanGTP functions to shuttle proteins containing nuclear localization sequences into the nucleus. Ran exists in two states: inactive or RanGDP (orange) and active or RanGTP (green). RanGAP hydrolyzes RanGTP in the cytoplasm, converting it to RanGDP. RCC1 exchanges a GDP nucleotide for GTP, converting RanGDP to RanGTP. RanGTP in the nucleus interacts with the Importin complex. Importin complex consists of Importin α (red), Importin β (yellow), and a spindle assembly factor (cyan). RanGTP binds to Importin β, freeing Importin α and the spindle assembly factor from the complex. The spindle assembly factor is then active. The RanGTP/Importin β complex returns to the cytoplasm, where RanGAP hydrolyzes RanGTP. This releases Ran from Importin β and allows the cycle to repeat itself.

Kinesins: A motor for every job

While the CPC and Ran remain the primary candidates for initiating acentrosomal spindle assembly, the spindle itself could not be built without kinesins and other microtubule-associated proteins. The kinesins are a large family of motor proteins that promote unidirectional movement of a cargo along microtubules. Several *Drosophila* kinesin proteins have been shown to play important roles in spindle assembly (Goshima and Vale 2003). Since kinesins are capable of performing such a broad range of tasks, a standard nomenclature was recently adopted based on phylogenetic analysis (Lawrence, Dawe et al. 2004). This analysis recognized fourteen kinesin families. For example, a member of the kinesin-4 family interacts with microtubules while attached to chromosomes as their cargo (Mazumdar and Misteli 2005). Another three kinesin families can bundle and slide parallel or anti-parallel microtubules. The first is the kinesin-14 family that includes minus end-directed motors such as NCD in *Drosophila*. NCD and the minus end-directed motor dynein have been proposed to bundle and taper microtubules to establish mitotic (Walczak, Vernos et al. 1998; Goshima, Wollman et al. 2005) and meiotic (Matthies, McDonald et al. 1996; Endow and Komma 1997; Skold, Komma et al. 2005) spindle poles in the absence of centrosomes. The second is the kinesin-5 family, including Klp61F in *Drosophila*, which are plus end-directed motors that function to maintain bipolar spindle assembly and elongation at anaphase. The activity of these proteins may antagonize the forces of the kinesin-14 family during spindle assembly (Kwon, Morales-Mulia et al. 2004; Tao, Mogilner et al. 2006). The third is the kinesin-6 family that includes Subito and Pavarotti in *Drosophila*. As shown for human MKLP1, kinesin-6 proteins are thought to be plus end-directed motors that

slide anti-parallel microtubules (Nislow, Lombillo et al. 1992). Examination of these proteins in human cells (Neef, Preisinger et al. 2003), *Caenorhabditis elegans* (Raich, Moran et al. 1998), and *Drosophila* (Adams, Tavares et al. 1998; Cesario, Jang et al. 2006) has shown they are usually associated with interpolar microtubules in the middle region of the spindle and are important for cytokinesis.

Kinesins are usually capable of binding a cargo. Originally discovered for their essential role in transporting vesicle and organelles throughout the cytoplasm, kinesins have since been recognized to bind a wide variety of proteins that are essential to spindle assembly, such as checkpoint proteins, microtubules, and microtubule stabilizing proteins (Moore 2001). Careful coordination of spindle assembly factors ensures proper spindle assembly. Therefore, the activation of a particular kinesin must be temporally and spatially correct.

Mechanisms for kinesin regulation

Regulation of kinesins occurs by a variety of mechanisms, including protein synthesis and degradation, protein-protein interactions, post-translational modifications, and auto-inhibition. The synthesis and degradation of proteins is a hallmark of the cell cycle. As a cell enters M phase, spindle assembly factors, including kinesins, are translated. This results in a pool of newly synthesized proteins that are inherently active. The kinesins can then bind microtubules, as they assist in assembling the spindle. As the cell exits mitosis, selective degradation of these proteins occurs via ubiquitylation (Manchado, Eguren et al. 2010). In this regulatory mechanism, kinesins, such as Pavarotti, are synthesized when needed and degraded when their function is completed.

Protein-protein interactions can regulate kinesins is a variety of ways. The sequester-and-release mechanism of regulation occurs when regulatory proteins bind a spindle assembly factor, inhibiting the factor and blocking its role in spindle assembly. Under the right conditions, the spindle assembly factor releases from the inhibitory complex, activating the factor to assist during spindle assembly. This regulatory mechanism cycles as the cell returns to the initial condition and, once again, the inhibitory complex binds the spindle assembly factor. An example of the sequestering/releasing mechanism of regulation is the small GTPase, Ran, and Importin complex, mentioned previously in this introduction.

Another form of protein-protein interaction has been shown to alter the motor activity of the kinesin. Plus-end tracking proteins, such as EB1, are involved in a wide range of spindle assembly activities, including the regulation of microtubule dynamics (Coquelle, Vitre et al. 2009). EB1 is essential for proper sister chromatid segregation. EB1 has also been shown to interact with the minus end-directed motor, NCD. This interaction causes a shift in localization of NCD to growing end of microtubules (Goshima, Nedelec et al. 2005). This provides an interesting example of when protein-protein interactions can reverse the unidirectionality of a kinesin.

Protein-protein interactions can also result in post-translational modifications to the kinesin. An interaction between MKLP2, the human homolog of Subito, and Polo-like kinase 1 (Plk1) negatively regulates the bundling activity of the kinesin. This interaction occurs via a serine in the linker domain of Subito. When this serine is phosphorylated by Plk1, it prevents microtubule bundling *in vitro* (Neef, Preisinger et al. 2003). This example of protein-protein interaction demonstrates regulation by protein

modification. This regulation has been shown in other kinesin families, including kinesin-5 (Ferenz, Gable et al. 2010).

Protein modification can also result in structural changes to kinesin, which may affect its activation. Auto-inhibition is a common regulatory mechanism used in many biological processes (Pufall and Graves 2002). This form of regulation also occurs in kinesin and myosin families (Lee, Shin et al. 2004) (Liu, Taylor et al. 2006). For example, a physical interaction between the motor and tail domains of Kinesin-1 blocks the motor, resulting in a functionally inactive kinesin (Cai, Hoppe et al. 2007). This conformation is thought to occur in the absence of a cargo. Upon release from the tail domain, the motor is active and free to interact with microtubules. The release of auto-inhibition is poorly understood, but by temporally and spatially suppressing itself, the kinesin prevents futile ATP hydrolysis and conserves the energy of the cell. Together, these mechanisms show that the tight regulation of kinesins is necessary for proper spindle assembly.

The matchmakers of our genetic building blocks

Though kinesins play a major role in spindle assembly, proper condensation and orientation of the chromosomes is also necessary for the process to occur error free. Sister chromatid cohesion during mitosis is vital to ensuring proper orientation and tension during metaphase. Cohesin is a complex of four proteins, Smc1, Smc3, Mcd1/Scc1 (known as Rad21 in *Drosophila*), and Scc3 (Nasmyth 2001). This complex forms a ring with a thirty-five nanometer hole: wide enough to fit both sister chromatids (Gruber, Haering et al. 2003). In most animals, cohesin binds well to heterochromatin (centromeric) and transcribed regions (Misulovin, Schwartz et al. 2008). The cohesin

bound to the centromere regions promotes tension by resisting the outward pull of microtubules (Figure 3A). Subsequently, when the metaphase checkpoint is satisfied, the anaphase promoting complex activates Separase; a protease which specifically cleaves Securin, disabling the cohesin complex. The premature release of cohesin results in aneuploidy (Hodges, Revenkova et al. 2005).

Since the first meiotic division separates homologous, rather than the sister chromatids, the regulation of cohesin is accordingly different. During prophase of meiosis, chromosomes condense, pair, and a proteinaceous structure called the synaptonemal complex forms along the length of homologous chromosomes. The synaptonemal complex functions to synapse the two chromosomes, in preparation for a recombination event (Figure 3B). A chiasma forms between two non-sister chromatids, physically linking the two chromosomes. After this event, the synaptonemal complex disassembles, as cohesin continues to bind sister chromatids. Meiotic chromosome separation presents a major difference compared to mitosis: the degradation of cohesin must occur in two steps. The two steps of cohesin degradation allow for the resolution of the chiasmata during the first meiotic division, while maintaining enough cohesin to maintain co-orientation of the sister chromatids during the second meiotic division. Shugoshin acts to protect the cohesin at the centromeric regions by inhibiting the degradation of Scc1 (Resnick, Satinover et al. 2006). Shogoshin protects Scc1 by recruiting PP2A, a phosphatase that dephosphorylates Scc1 and prevents cleaving. This protection leaves active cohesin at the heterochromatin, which is sufficient to retain sister chromatid cohesion during the second meiotic division. Since the co-orientation of sister

chromatids is necessary for proper segregation both mitotically and meiotically, the regulation of the cohesin complex must be tightly regulated.

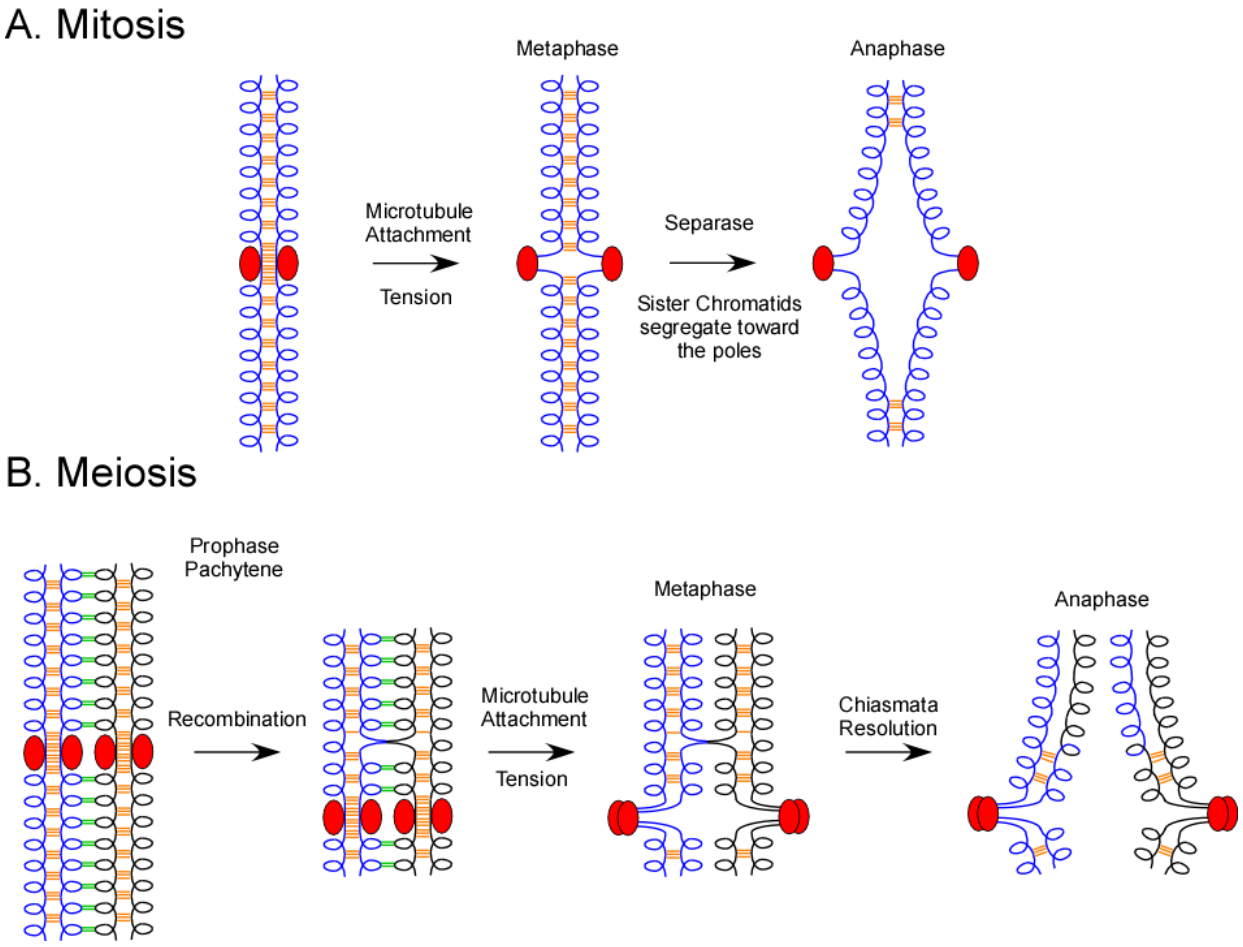

Figure 3 – Differences in sister chromatid cohesion and chromosome alignment between mitosis and meiosis. In mitosis, cohesin (orange) is maintained along the length of the sister chromatids (blue) during pro-metaphase (A). Microtubules search the cytoplasm, randomly attaching to the kinetochores (red). The sister kinetochores are pulled towards opposite poles as cohesin holds the sister chromatids together, creating tension. This tension is a sensor for adequate microtubule attachment to the kinetochores and helps to satisfy the metaphase checkpoint. Once the checkpoint is satisfied, separase is activated, which cleaves cohesin, allowing for segregation of the sister chromatids towards opposite poles. In contrast to mitosis, the synaptonemal complex synapses the parental (blue) and maternal (black) chromosomes during prophase of meiosis I (B). Once the homologous chromosomes have fully synapsed, recombination occurs, forming chiasmata, which physically link the two chromosomes and allow for proper alignment. After recombination, the synaptonemal complex is disassembled, and cohesin is maintained between sister chromatids. Similarly to mitosis, microtubules attach to the kinetochores and tension is established, allowing the checkpoint to be satisfied. At this point the chiasmata are resolved, resulting in a crossover,

and anaphase begins with sister chromatid cohesion maintained at the centromeres. The remaining cohesin ensures that alignment occurs properly during the second meiotic division.

To divide, or not to divide, that is the question

Mitotic and meiotic cell cycles are regulated by a group of proteins called cyclins. Each cyclin pairs with and activates a cyclin-dependent kinase (Cdk). For example, the transition from G_1 to S phase is controlled by cyclin A bound to Cdk2 (Kaldis and Aleem 2005). Cyclin B bound to Cdk1 is known as the maturation-promoting complex and is responsible for the transition from G_2 to M phase (Smith, Jaspersen et al. 2008). During S phase, the concentration of cyclin B gradually increases, as it continually shuttles between the nucleus and cytoplasm. Over time, cyclin B concentrations will build up in the cytoplasm, eventually localizing to the centrosomes prior to mitotic entry (Lindqvist, Rodriguez-Bravo et al. 2009). Cyclin B then interacts with Cdk1, forming the maturation-promoting complex. Active maturation-promoting complex signals the onset of mitosis by initiating nuclear envelope breakdown, centrosome maturation, spindle assembly, and chromatin condensation. However, the mere binding of Cyclin B to Cdk1 is not sufficient to activate the maturation-promoting complex. Before mitosis, two kinases, known in *Drosophila* as Wee1 and Myt1, phosphorylate Cdk1, keeping the maturation-promoting complex in an inactive state. As the cell nears mitotic entry, two phosphatases, known as String and Twine in *Drosophila*, dephosphorylate the complex (Lindqvist, Rodriguez-Bravo et al. 2009). The maturation-promoting complex then phosphorylates these phosphatases, resulting in increased activation of the complex. At the same time, the maturation-promoting complex phosphorylates Wee1 and Myt1, leading to their inactivation. This step results in both a positive and negative feedback loop. Both feedback loops ensures complexes that are committed to an active state.

Once the maturation-promoting complex is active, the cell commits to a mitotic division. As the bipolar spindle forms, the spindle assembly checkpoint, or metaphase checkpoint, ensures that anaphase does not initiate until kinetochores are properly attached to microtubules and tension is retained. This checkpoint is maintained by a complex of Mad2, Mad3/BubR1, and Bub3 (Figure 4A). In a metaphase arrested cell, Mad2 protein interacts with Mad1 near the kinetochore, which causes a conformational change in Mad2 (Vogt, Kirsch-Volders et al. 2008). Mad2 is then released and able to bind BubR1 and Cdc20, also known as Fzy in *Drosophila*. Fzy is replaced by Cortex during female meiosis and Fzr2 during male meiosis as the activator of the anaphase promoting complex (Pesin and Orr-Weaver 2008). The binding of Mad and BubR1 to Fzy inhibits Fzy, and prevents it from activating the anaphase promoting complex. When the spindle assembly checkpoint is satisfied, Mad2 will remain bound to Mad1 (Figure 4B). Mad2 is no longer free to bind BubR1 and inhibit Fzy. As a result, Fzy binds the anaphase promoting complex, forming an active complex. This leads to the degradation of the maturation-promoting complex. The increase in active anaphase promoting complex and decrease in maturation-promoting complex triggers the degradation of cohesin and the onset of anaphase. This tightly regulated system ensures mitotic and meiotic divisions occur properly and in a coordinated manner.

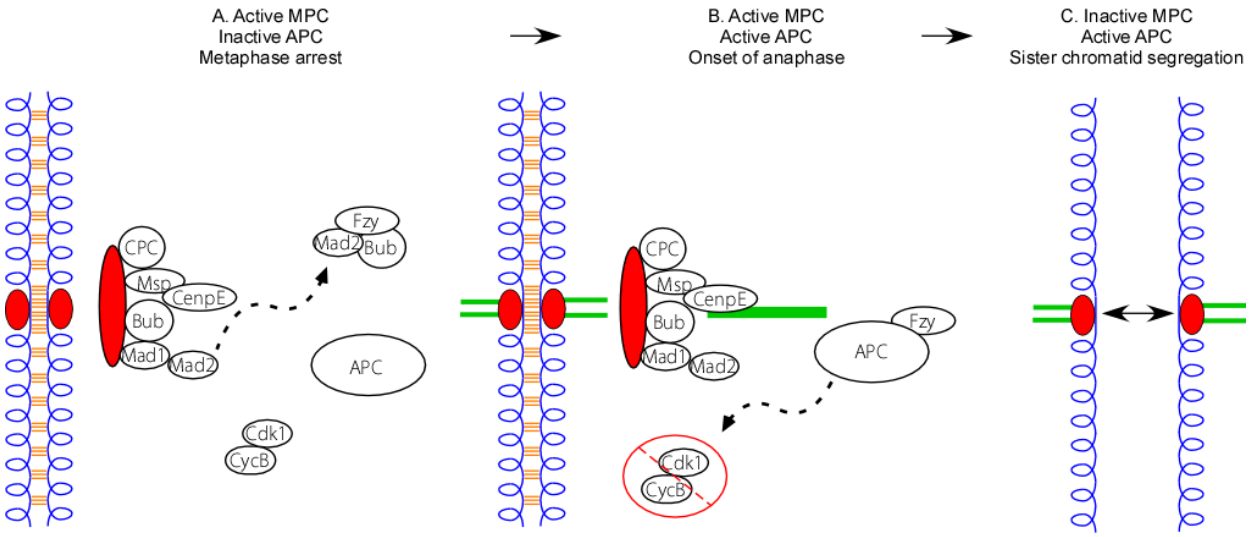

Figure 4 – **The progression from metaphase to anaphase.** Sister chromatids (blue) are held together by cohesin (orange). A complex of proteins form on the kinetochore (red) early in the mitotic division. These proteins include checkpoint proteins, like Mad2 and BubR1, chromosomal passenger complex proteins (CPC), like Incenp and Aurora B, and microtubule attachment proteins, like Mini-spindles (Msps) and CenpE. When the kinetochore is not properly attached to a microtubule or tension is not maintained (A), Mad2 is released from the kinetochore. It then interacts with BubR1 and Fzy, also known as Cdc20. This interaction prevents the interaction of Fzy with the anaphase-promoting complex (APC). At the same time, Cyclin B (CycB) remains bound to Cdk1 maintaining the metaphase checkpoint. When microtubules are bound by the kinetochore and tension is maintained, Mad2 remains bound to the kinetochore (B). This allows Fzy to interact and activate the APC. Active APC has many downstream effects including the degradation of maturation-promoting complex, and the activation of Separase. Active Separase cleaves cohesin, allowing for sister chromatid segregation (C).

Conclusion:

Work in *Xenopus* extracts has shown that, in the absence of chromosomes, RanGTP is capable of initiating spindle assembly. Since *ran* is such a highly conserved gene, this observation led many to believe that RanGTP is responsible for the initiation of acentrosomal spindle assembly. More recent work in *Xenopus* has shown that the CPC must be present for chromosome independent spindle assembly to occur (Dumont, Petri et al. 2007; Maresca, Groen et al. 2009). This finding puts into question whether RanGTP is required for initiating acentrosomal spindle assembly. Nonetheless,

observations from *Drosophila* female meiosis suggests that spindle assembly initiation is dependent upon nuclear envelope breakdown and the subsequent release of factors from the nucleus (Jang, Rahman et al. 2007). We have undertaken an analysis of meiosis in *Drosophila* oocytes and mitosis in embryos and larvae in order to assess the role of the Ran pathway in spindle assembly.

Auto-inhibition is a common regulatory mechanism used in many biological processes (Pufall and Graves 2002). Kinesin-1 perfectly demonstrates how the structural conformation can alter the motor activity (Cai, Hoppe et al. 2007). Subito has demonstrated similar properties of auto-inhibition. The N-terminus of Subito has been shown to negatively regulate the motor activity, as deleting this domain creates a constitutively active kinesin (Jang, Rahman et al. 2007). We hypothesize this negative regulation is the result of auto-inhibition. We sought to further our understanding of auto-inhibition in *Drosophila* oocytes by narrowing the region of amino acids within the N-terminus responsible for negative regulation. Furthermore, by performing FRET experiments, we have undertaken a structural analysis of Subito, to determine if the conformation of Subito allows for auto-inhibition.

Chapter 1: Kinesin 6 family member Subito participates in mitotic spindle assembly and interacts with mitotic regulators.

I. Preface

This chapter was published, as presented here, in the Journal of Cell Science, November 2006. My contributions to the project and paper included: cytological analysis of wild-type and mutant neuroblasts, the mitotic index analysis, chromosomal analysis, and reviewing of the paper.

II. Abstract:

Drosophila Subito is a kinesin 6 family member and ortholog of mitotic kinesin-like protein (MKLP2) in mammalian cells. Based on the previously established requirement for Subito in meiotic spindle formation and for MKLP2 in cytokinesis, we investigated the function of Subito in mitosis. During metaphase, Subito localized to microtubules at the center of the mitotic spindle, probably interpolar microtubules that originate at the poles and overlap in anti-parallel orientation. Consistent with this localization pattern, *subito* mutants improperly assembled microtubules at metaphase, causing activation of the spindle assembly checkpoint and lagging chromosomes at anaphase. These results are the first demonstration of a kinesin 6 family member with a function in mitotic spindle assembly, possibly involving the interpolar microtubules. However, the role of Subito during mitotic anaphase resembles other kinesin 6 family members. Subito localizes to the spindle midzone at anaphase and is required for the localization of Polo, Incenp and Aurora B. Genetic evidence suggested that the effects of

subito mutants are attenuated as a result of redundant mechanisms for spindle assembly and cytokinesis. For example, *subito* double mutants with *ncd*, *polo*, *Aurora B* or *Incenp* mutations were synthetic lethal with severe defects in microtubule organization.

III. Introduction:

The *Drosophila* oocyte, which lacks centrosomes, is a genetically tractable system for the identification of genes required for acentrosomal spindle assembly. Using this system, we identified Subito as a kinesin-like protein required for meiotic spindle assembly in *Drosophila* females (Giunta, Jang et al. 2002). s*ubito* (*sub*) mutants exhibit high levels of meiotic non-disjunction, which is associated with the formation of monopolar and tripolar spindles in metaphase I oocytes. Immunolocalization studies have shown that Subito is present on microtubules before bipolar spindle formation and associates with interpolar microtubules at metaphase (Jang, Rahman et al. 2005). Interpolar microtubules do not terminate at the kinetochores, but instead originate at both poles and overlap in the middle of the spindle in anti-parallel orientation (Mastronarde et al., 1993). During anaphase, these microtubules form the midzone, which has an important role in cytokinesis (Giansanti, Bonaccorsi et al. 1998; Neef, Klein et al. 2006). Our results suggest that interpolar microtubules have an important role in organizing acentrosomal spindle formation in *Drosophila* oocytes.

These localization studies are consistent with the conclusion from sequence analysis showing that Subito is a member of the kinesin 6 group of kinesin-like proteins (Miki, Okada et al. 2005). Kinesin 6 proteins are believed to bundle anti-parallel microtubules (Nislow, Lombillo et al. 1992) and the family includes mitotic kinesin-like

proteins 1 (MKLP1) and 2 (MKLP2). Relative to other kinesin-like proteins, members of the kinesin 6 family have a unique insertion of approximately 65 amino acids in loop L6 of the motor domain. In addition, kinesin 6 proteins fall into two subgroups, the KIF20-MKLP2-Subito group, present in a wide range of animals, fungi and slime molds, and the KIF23-MKLP1-Pavarotti group present only in animals (Miki, Okada et al. 2005). Loss of MKLP1 or MKLP2 results in a disorganized midzone at anaphase and subsequent cytokinesis defects (Neef, Preisinger et al. 2003).

Since Subito is required for acentrosomal spindle formation in oocytes, we investigated whether it is also required for spindle formation in mitotic cells. *sub* null mutants are viable, demonstrating that it does not have an essential role in spindle formation during mitosis (Giunta, Jang et al. 2002). Cytological and genetic analysis of *sub* single mutants and double mutants with *BubR1, polo, Aurora B* and *Incenp* indicate that Subito has a role during spindle assembly in metaphase. This function has not previously been attributed to a kinesin 6 family member. These results also demonstrate that Subito is required for organizing the microtubules in the midzone during anaphase, a function consistent with studies of other kinesin 6 family members including MKLP1 (Matuliene and Kuriyama 2002) and MKLP2 (Fontijn, Goud et al. 2001; Neef, Preisinger et al. 2003) in humans, ZEN-4 in *Caenorhabditis elegans* (Raich, Moran et al. 1998) and Pavarotti (Adams, Tavares et al. 1998) in *Drosophila*, which are all required for cytokinesis.

IV. Materials and Methods:

Genetic stocks and identifying mutant larvae

All fly cultures and crosses were raised at 25°C. Third instar larva homozygous for second and third chromosome mutants were identified using stocks containing the mutant heterozygous to the translocation *T(2;3)B3,CyO:TM6B, Tb*. The dominant *Tubby* (*Tb)* marker was used to select the homozygous larvae. The *sub* mutants used in this study were the protein null alleles, sub^{1} and sub^{131} (Schupbach and Wieschaus 1989; Giunta, Jang et al. 2002; Jang, Rahman et al. 2005) examined as either transheterozygotes or as homozygotes. In addition, mutant alleles $Incenp^{3747}$ (Chang, Goulding et al. 2006), $polo^{16\text{-}1}$ (Lukinova, Roussakova et al. 1999), $aur^{87Ac\text{-}3}$ (Glover, Leibowitz et al. 1995), ncd^{1} (Yamamoto, Komma et al. 1989), and $BubR1^{k03113}$ (Basu, Bousbaa et al. 1999) were used in this study. *Df(2L)Exel7049* (Parks, Cook et al. 2004), which deletes 32B1;32C1, was used because it is a deletion of the *Aurora B* locus.

Dissection and fixation of larvae

Larval brain tissue was prepared by both squashing and whole-mount methods. In preparation for squashing, the larvae were dissected in saline and the brains were fixed in 3.7% formaldehyde in 1x PBS for 30 minutes. For the purpose of depolymerizing microtubules and activating the spindle assembly checkpoint, the brains were incubated for 1 hour in $5x10^{-5}$ M colchicine. For karyotype analysis, the brains were incubated for 1.5 hours in $5x10^{-5}$ M colchicine followed by hypotonic swelling in 0.5% sodium citrate. The brains were then transferred to 45% acetic acid for 3 minutes (except for karyotype

analysis) before transferring to ~8 µl of 60% acetic acid on a siliconized coverslip where they were firmly squashed between the coverslip and slide. The slides were briefly frozen in liquid nitrogen and the coverslips were flicked off. The slides containing the tissue were placed in ethanol at –20°C (chilled on dry ice) for 10 minutes, then transferred to a slide chamber containing 0.1% Triton X-100 in PBS for 10 minutes. Rubber cement was used to form wells on the slides and two 5-minute washes were done in PBS. The tissue was blocked with 1% BSA in PBS for 45 minutes. Primary antibodies were diluted in 1% BSA in PBS and then 250 µl was added to each slide and incubated overnight at 4°C in humid chambers. The next day, two washes in PBS and one wash in 1% BSA in PBS were performed for 5 minutes each. The secondary antibodies were then added and the slides were again incubated overnight at 4°C. The next day, two more 5-minute washes in PBS were done. The DNA was stained with 0.2 µl/ml of a 10 mg/ml Hoechst 33258 solution in 1% BSA in PBS for 7 minutes and the slides were washed with 1% BSA in PBS for 5 minutes. The tissue was mounted in Vectashield (Vector).

For the preparation of whole-mount brain tissue, third instar larvae were dissected in PBS and fixed in 3.7% formaldehyde for 20 minutes. They were then washed in PBS for 20 minutes, PBS with 0.3% Tween 20 for 10 minutes and then PBS with 10% normal goat serum (NGS) for 40 minutes. Primary antibodies were incubated with the brains in PBS-0.1% Tween 20 (PBST). The brains were then washed four times in PBST and then incubated with secondary antibodies in PBST/10% NGS. During the following four washes in PBST, the DNA was stained with Hoechst.

The rat anti-Subito antibody was used at 1:200 combined with a Cy3 anti-rat secondary antibody (1:200, Jackson Labs). Additional primary antibodies were mouse anti-α-tubulin (1:50, clone DM1A, Sigma) directly conjugated to FITC, rat anti-α-tubulin (1:75, Clone YOL 1/34, Chemicon), rabbit anti-CID (1:100, Abcam), rabbit anti-Aurora B (1:500), rabbit anti-Feo (1:100) (Verni, Somma et al. 2004), rabbit anti-Incenp (1:250) (Adams, Maiato et al. 2001), mouse anti-Polo (1:15) (Llamazares, Moreira et al. 1991), rabbit anti-Pavarotti (1:750) (Adams, Tavares et al. 1998), guinea pig anti-MEI-S332 (1:4000) (Moore, Page et al. 1998) and Phospho-Histone H3 (Ser-10) (1:1000, Upstate) with Cy3- or FITC-conjugated secondary antibodies (1:200-1:500, Jackson Labs).

Image capture and analysis

Images were collected on two systems: a Zeiss Axioplan II fluorescent microscope using a 63x NA 1.4 lens and software from Vaytec to collect and process *Z*-stacks or a Leica TCS SP confocal microscope with a 63x NA 1.3 lens. Images are shown as maximum projections of image stacks. The mitotic index was measured as the number of mitotic cells/total cells counted from randomly selected fields. The mitotic cells were identified by Phospho-Histone H3 (Ser10) staining and approximately ten photographs of randomly selected fields were analyzed from each slide.

Co-immunoprecipitation experiments

A transgene was constructed by fusing the *sub* coding region to three copies of the HA epitope tag and sub-cloning this into the pUASP vector. This was expressed in oocytes and early embryos using the *nosGAL4:: VP16* driver (Van Doren, Williamson et

al. 1998). This combination rescued the meiotic and maternal effect embryonic lethal phenotypes of *sub* mutants (data not shown).

Lysates from embryos and oocytes were prepared in IP buffer [50 mM Tris-HCl pH 8.0, 400 mM NaCl, 0.5% (v/v) NP-40, 0.1% (w/v) deoxycholate and protease inhibitors]. The cleared extract was incubated with the 3F10 anti-HA antibody covalently linked to beads (Roche) overnight at 4°C and then the beads were washed twice with IP buffer. The lysate and beads were mixed with loading buffer for SDS-PAGE and the proteins transferred to PVDF membranes for immunoblotting. The western blots were performed using rat anti-Subito at 1:2000 (Jang et al., 2005), rat anti-HA (clone 3F10, Roche) at 1:5000, mouse anti-Polo (MA294) (Llamazares, Moreira et al. 1991) at 1:80, mouse anti-GFP (clone JL.8, Clontech) at 1:2000 and detected with HRP-conjugated secondary antibodies (Jackson Labs) at 1:5000 and ECL reagents (GE Healthcare).

V. Results:

Subito localizes to interpolar metaphase microtubules

To determine whether Subito is present on mitotic spindles, we compared Subito and Tubulin staining in mitotically dividing cells of the larval brain. Subito was most concentrated with tubulin fibers at the center of the metaphase spindle. These are probably interpolar microtubules, which run from opposite poles to the middle of the spindle where they overlap in anti-parallel orientation (Mastronarde, McDonald et al. 1993; Compton 2000). Subito appeared to be concentrated in foci on interpolar fibers or extending for short distances along the microtubule (Figure 5A). As the chromosomes moved to the spindle poles during anaphase, Subito remained in the center of the spindle, in the region destined to become the midzone (Figure 5B,C). No Subito staining was observed in mutant larval neuroblasts homozygous for the null alleles sub^{1} or sub^{131}. Furthermore, when microtubules were depolymerized following colchicine treatment, specific Subito staining was lost. In particular, the foci of staining were not observed, although the colchicine-treated cells did have considerable delocalized Subito staining. These results suggest that Subito localization is microtubule dependent.

Subito colocalizes at centromeres with Polo, Incenp and MEI-S332 during metaphase

Although Subito appeared to colocalize with microtubules, the concentration of staining near the chromosomes raised the possibility that Subito was associating with kinetochores. To test this possibility, we stained whole mount larval brains with both

Subito and proteins known to associate with centromeres or kinetochores at metaphase. For example, the passenger protein

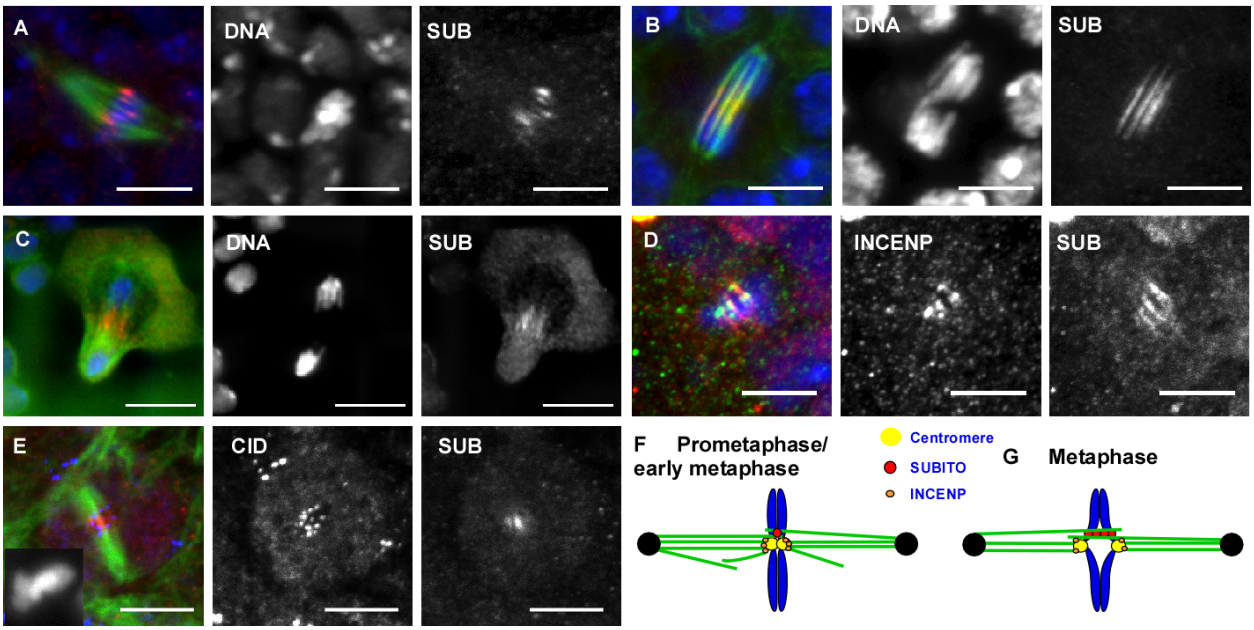

Figure 5 – Subito localization in wild-type brains. A) A whole-mount metaphase cell with the chromosomes aligned in the middle of the spindle. The DNA (blue) is condensed and Subito (red) is present on the microtubules (green). B) A whole-mount early anaphase cell with Subito on the spindle between the separating chromosomes. C) A squashed late anaphase cell with Subito on the midzone microtubules and beginning to concentrate on a smaller portion of the spindle than in early anaphase. D) A whole-mount metaphase cell showing the overlap of Subito (Red) and Incenp (green). In some cases, Subito appears to be spreading out along the interpolar microtubules whereas in other cases, it appears as foci. E) A whole-mount metaphase cell showing Subito (Red) present on the microtubules between paired CID foci (blue). The grey-scale inset shows DNA staining. The scale bar is 5 µm. F, G) Model for the localization pattern of Subito at metaphase. Subito interacts with anti-parallel microtubules, which only exist between centromeres. Just prior to anaphase, the centromeres are pulled in opposite directions, increased the length of anti-parallel microtubules beween them.

Incenp localizes to centromeres at metaphase (Adams, Maiato et al. 2001). We found that the signals from Subito and Incenp overlapped (Figure 5D), suggesting that Subito was present near the centromeres. In some cases, the Subito signal appeared to spread out over a short distance along the microtubules. Nonetheless, at least part of the Subito signal usually overlapped with Incenp. Similar results were obtained using antibodies to

Aurora B, Polo and MEI-S332 (data not shown). Like Incenp, all of these proteins have been shown to localize to centromeres or kinetochores at metaphase (Logarinho and Sunkel 1998; Moore, Page et al. 1998; Moutinho-Santos, Sampaio et al. 1999; Adams, Maiato et al. 2001; Giet and Glover 2001). To directly compare Subito localization to the centromeres, larval brains were stained for CID, a centromere-specific histone H3 (Henikoff, Ahmad et al. 2000; Blower and Karpen 2001) and Subito. In some cells, there were 16 disordered foci of CID staining, suggesting that the sister centromeres had not yet aligned with the poles. In these nuclei, Subito colocalized with the CID staining. In other cells, the CID foci were neatly arranged in two rows, suggesting that the sister centromeres had aligned with the poles (Figure 5E). The DNA staining suggested they were still in metaphase, although it is possible they were at the earliest stages of anaphase. In these cases, Subito was spread out along microtubules between pairs of CID foci.

To explain the colocalization of Subito with both microtubules and centromeres, we suggest that the anti-parallel overlap of interpolar microtubules may preferentially interact with centromeres during metaphase (Figure 5F,G). Early in metaphase the centromeres have not separated and thus there are only short tracks of anti-parallel microtubles. By contrast, once the centromeres are under tension and pulled towards opposite poles, the distance between them increases and the length of anti-parallel overlap increases. This is reflected in the more elongated regions of Subito staining. At present, however, we have not determined the cause of the centromere association; whether it is Subito or the anti-parallel microtubules independently of Subito that are attracted to the centromeres. Alternatively, we have not ruled out the possibility that Subito interacts

with the plus ends of the microtubules that approach the kinetochores and then move to interpolar microtubules when the centromeres separate.

Fascetto is the *Drosophila* Prc1 homolog and is enriched in the spindle midzone starting at anaphase and has an important role in cytokinesis (Verni, Somma et al. 2004). Staining of *sub* mutant brains showed that Fascetto localized strongly to the spindle midzone at anaphase and telophase (Figure 6E,F), suggesting that Fascetto localization is independent of Subito. Because telophase looked normal in *sub* mutants, it is possible that spindle organization improved over time, ultimately promoting cytokinesis. In addition, the control experiments showed that the enrichment of Subito early in anaphase occurred before the appearance of Fascetto, which is consistent with the observation that Fascetto does not localize to the midzone until late anaphase (Verni, Somma et al. 2004). In HeLa cells depleted of MKLP2 by RNAi, PRC1 midzone staining still occurs in (Neef, Preisinger et al. 2003).

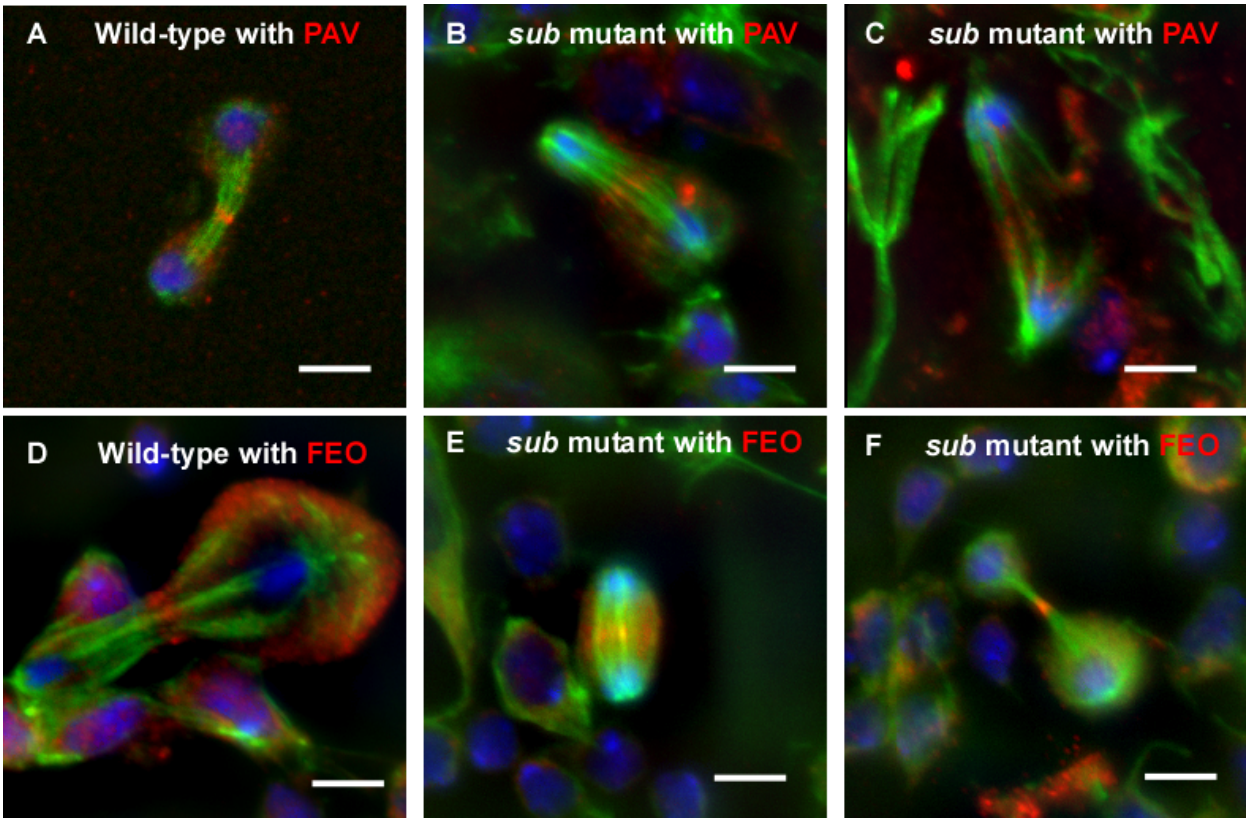

Figure 6 – A) Pavarotti (red) is located in the midzone of wild-type late anaphase. B, C) Pavarotti shows variable midzone staining in sub^{1}/sub^{131} mutants which may be related to the degree of microtubule disorganization. D) Fascetto (red) localization to the midzone in wild-type. E, F) Fascetto localization was strong during anaphase and particularly at telophase. The scale bar is 5 μm.

The effect of *sub* mutants on mitotic progression

When sub^{131}/CyO males were crossed with sub^{1}/CyO females, the expected number of sub^{131}/sub^{1} progeny were produced (Table 1), indicating that *sub* is not required for viability. However, *sub* mutant homozygotes exhibited morphological defects such as interruptions in the abdominal cuticle pattern and clipped wing tips (data not shown). To investigate other non-essential functions of *sub* in mitotically dividing

cells, we compared cell-cycle progression in wild-type and *sub* mutant larval brains by measuring the mitotic index.

Nuclei in mitosis were identified by staining for histone H3 phosphorylated on Ser10 (Gurley, D'Anna et al. 1978). The mitotic index in *sub* mutant larvae was approximately twice that of the wild type (Figure 7), suggesting there was a delay in the progression of *sub* mutant cells through mitosis. To test if the elevated mitotic index in *sub* mutants was caused by activation of the spindle assembly checkpoint, a sub^{131} $BubR1^{k03113}$ double mutant was generated. BubR1 is required for the spindle assembly checkpoint (Basu, Bousbaa et al. 1999). Therefore, if the spindle checkpoint was activated in *sub* mutants, we anticipated a reduced mitotic index in the sub^{131} $BubR1^{k03113}$ double mutant relative to the *sub* single mutant. In fact, the sub^{131} $BubR1^{k03113}$ double mutant had a lower mitotic index (0.65) than either the *sub* single mutant (2.66) or the wild type (1.32, Figure 7). The low mitotic index in the double mutant was consistent with our control data and previous observations that the *BubR1* mutant has a lower mitotic index than the wild type (Basu et al., 1999). Thus, we conclude that *BubR1* is required for the increased mitotic index in *sub* mutants and that *sub* mutants have a defect during prometaphase or metaphase that activates the spindle assembly checkpoint. If spindle assembly errors do occur in a sub^{131} mutant and are not corrected in a $BubR1^{k03113}$ mutant background, a synergistic effect on mitosis or

Table 1

Viability of *sub* double mutants

Cross	*sub/CyO* [a]	*sub/sub; +/+* or *sub/sub; Incenp/+*	*sub/sub; polo/+*	Total
sub^{131}/CyO, Cy ⊗ sub^{1}/CyO, Cy	644	302 (313) [b]		946
sub^{1}/CyO; $polo^{16\text{-}1}$/TM3, Sb ⊗ sub^{131}/CyO; +/+	1655	308	6 (308) [b]	1969
$Incenp^{3747}$ sub /CyO ⊗ sub/CyO [c]	1028	0 (514) [b]		1028
Df(2L)Exel7049 sub^{1} /CyO ⊗ sub^{131} /CyO	527	0 (263) [b]		527

The number of progeny scored from the indicated crosses is shown.

[a] Depending on the cross, this could include *sub/CyO; +/+* and either *sub/CyO;* $polo^{16\text{-}1}$/+ or *sub* $Incenp^{3747}$/CyO or Df(2L)Exel7049 sub^{1} /CyO.

[b] The expected number of progeny if there was no synthetic lethality is shown in parenthesis.

development would be expected. This was observed; although *BubR1* mutants are lethal, two synergistic phenotypes were observed in the double mutants. First, sub^{131} $BubR1^{k03113}$ double mutant third instar larvae were slow growing and they appeared 24-48 hours later than the *BubR1* single mutant larvae. Second, the brains of the double mutant larvae were small and contained fewer cells than the single mutants. It is likely that these phenotypes resulted from the failure to repair spindle assembly errors that arise during metaphase in *sub* mutants. We were unable to characterize the cytological phenotype of the double mutant because there were so few mitotic cells.

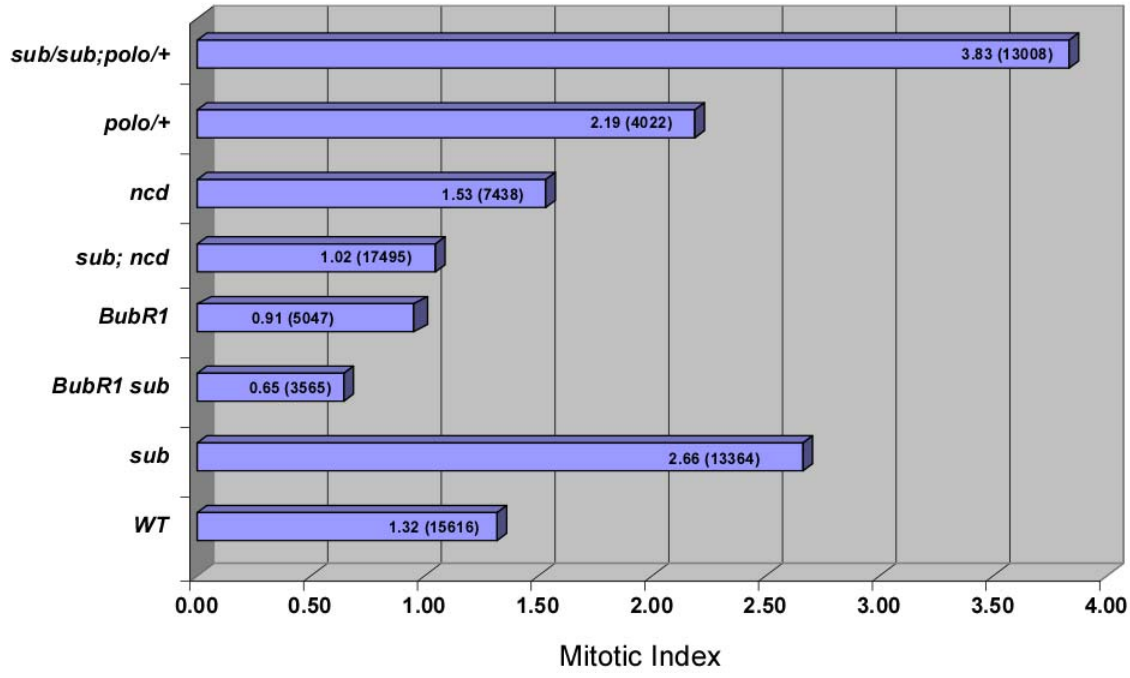

Figure 7 – Mitotic index in wild-type and mutant brains. The mitotic index was defined as the percentage of cells in mitosis. The number on each bar is the mitotic index and the total number of cells counted is shown in parentheses.

A low mitotic index in *sub; ncd* and *ncd mutants*

We previously reported that mutations in *ncd*, which encode a kinesin 14 or C-terminal motor kinesin-like protein, and *sub* genetically interact (Giunta, Jang et al. 2002). The double homozygous mutant $sub^{1}/sub^{131};ncd^{1}/ncd^{1}$ is lethal at the third larval instar stage, whereas the sub^{1}, sub^{131} and ncd^{1} single mutant homozygotes are viable. We examined *sub;ncd* double mutants to investigate whether synergistic effects on spindle assembly were the cause of the double mutant lethality. Unlike *sub* single mutants, which exhibited an elevated mitotic index, the $sub^{1}/sub^{131};ncd^{1}/ncd^{1}$ brains had a mitotic index similar to that of the wild type (Figure 7). Since the mitotic index of ncd^{1} brains was also similar to the wild type, these results suggest that ncd^{1} suppresses the mitotic delay phenotype of *sub* mutants.

The *ncd* mutant brains consistently had a lower metaphase to anaphase ratio, and this was also observed in the *sub; ncd* double mutant (Table 2). A more rapid progression through metaphase than observed in wild-type or *sub* mutants could cause the lower mitotic index and lethality because of segregation errors in the double mutant. If the high mitotic index in the *sub* single mutant was due to activation of the spindle assembly checkpoint, it was possible that an inability to activate this spindle assembly checkpoint caused the *sub;ncd* double mutant lethal phenotype. Currently, however, cytological studies have not confirmed this hypothesis nor revealed insights into the cause of the *sub;ncd* synthetic lethal phenotype (data not shown).

Table 2

Characterization of mitotic figures in wild-type and *sub* mutant brains

Genotype	Anaphase	Metaphase	M/A[a]	Disorganized metaphase (%)	Lagging chromosomes at Anaphase (%)
Wildtype	140	468	3.3	10.9	9.3
sub^{131}	81	380	4.7	43.4	51.8
sub^{1} / sub^{131}	203	687	3.4	70.0	46.3
ncd / ncd^{1}	212	315	1.5	18.1	55.2
sub^{1} ncd^{1}	88	146	1.7	65.7	43.2
sub^{131} ; ncd^{1}	275	454	1.7	61.9	51.6
sub^{1} / sub^{131} ncd^{1}	292	459	1.6	65.1	54.8
$Incenp^{3747}$ sub^{1} / + sub^{131}	231	599	2.6	71.9	75.6

[a] M/A = metaphase to anaphase ratio

Spindle assembly and chromosome segregation defects in *sub* mutant mitotic cells

Mitotic spindle assembly was assayed in squashed or whole-mount larval brains from wild-type and *sub* mutants stained for DNA and tubulin. Although most *sub* mutant metaphase spindles were bipolar, we identified an increased frequency of spindle assembly defects. These included frayed microtubules, unequal distribution of microtubules in the two half spindles and disorganized or absent interpolar microtubules (Figure 8B-D). The frayed spindles and unequal distribution of microtubules in the half spindles could be a secondary consequence of a defect in organizing interpolar spindle fibers, a role Subito has been shown to have in meiosis (Jang, Rahman et al. 2005). The effect on interpolar microtubules was not fully penetrant, however, because *sub* mutant spindles with interpolar microtubules were observed. Unlike oocytes, therefore, it appears that interpolar microtubules can form in the absence of *sub* in mitotic cells, although perhaps less efficiently or less stably.

To compare the wild type and mutants, the metaphase spindles were classified as `disorganized' if they had any of the features described above. In sub^{131} mutant brains, 43.4% of the metaphase spindles were classified as disorganized compared to 10.9% in wild-type brains (Table 2). An even greater frequency of metaphase spindle defects was observed in sub^{1}/sub^{131} brains. A second *sub* mutant phenotype was observed during anaphase. Although the chromosomes usually moved uniformly to the poles during wild-type anaphase (Figure 8E), lagging chromosomes were frequently observed in *sub* mutant anaphases (Figure 8F). The frequency of these abnormal anaphases was 9.3% in wild-

type brains but rose to 46.3-51.8% in *sub* mutant brains (Table 2). Like the disorganized microtubules described above, the presence of lagging chromosomes is consistent with defects in spindle organization at metaphase.

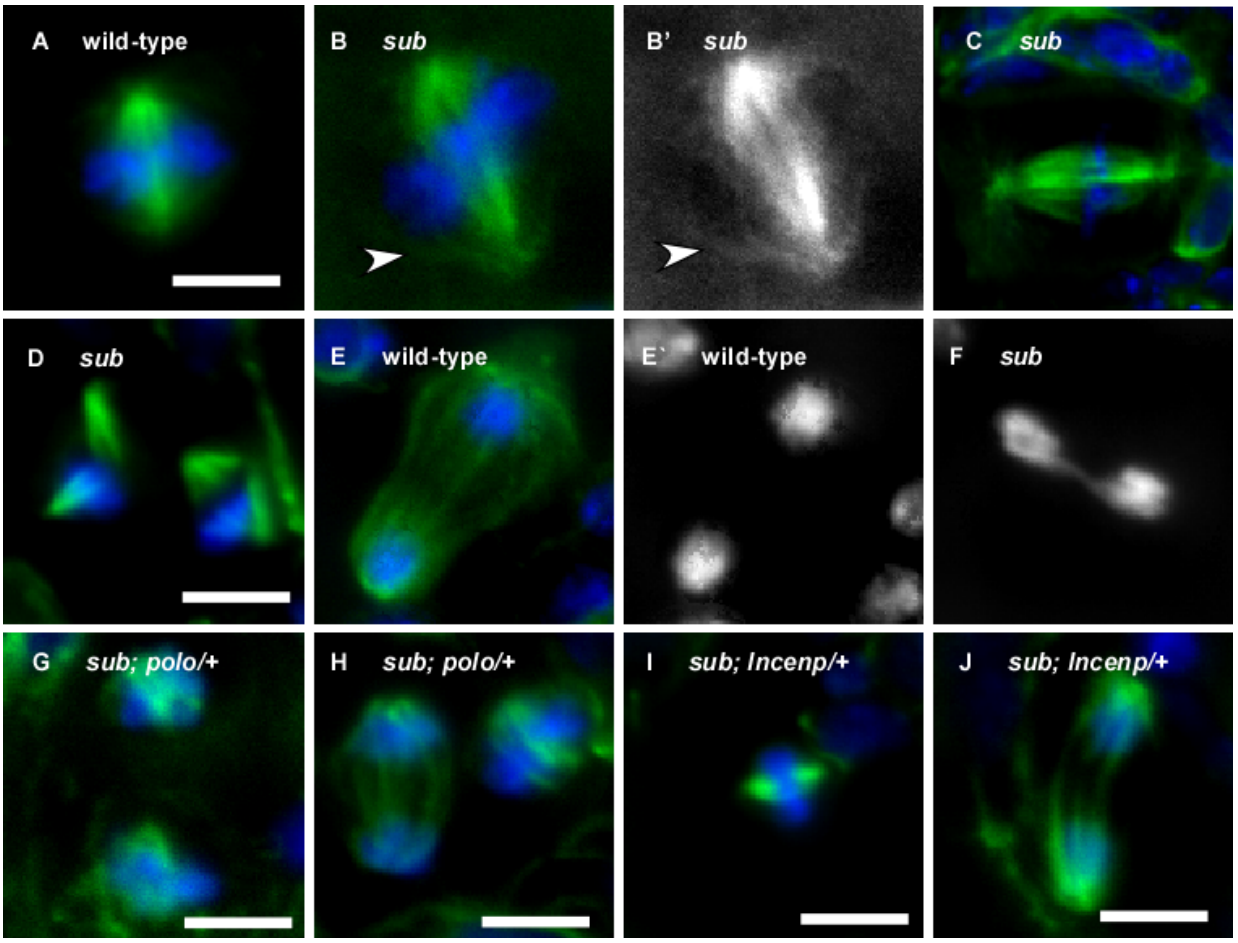

Figure 8 – A variety of mitotic spindle defects and lagging chromosomes in sub^{1}/sub^{131} mutants stained for DNA (blue) and microtubules (green). Compared to (A) wild-type metaphase, *sub* mutant metaphase spindles are bipolar but exhibit several defects including (B) frayed microtubules (B', Tubulin channel shown with increased levels), (C) asymmetric half spindles and (D) poorly oriented half spindles or absent interpolar microtubules. While wild-type anaphase (E, E' DNA channel only) involves the uniform segregation of chromosomes to the poles, in (F) *sub* mutant anaphases there was an increased frequency of lagging chromosomes. A synergistic effect on spindle assembly was observed during (G) metaphase and (H) anaphase in sub^{1}/sub^{131}; $polo^{16\text{-}1}$/+ mutants or metaphase (I) and (J) anaphase spindles in sub^{1}/sub^{131}; *Incenp*/+ mutants. Scale bar is 5 µm.

sub genetically interacts with regulators of mitosis

Since Subito, Polo and the passenger proteins colocalize and because physical interactions have been detected between their human homologs (Gruneberg et al., 2004; Neef et al., 2003), experiments were performed to determine genetic interactions between mutations in *polo, Incenp* or *Aurora B* and *sub*. The interaction with *polo* was tested using the $polo^{16\text{-}1}$ allele that causes recessive early larval lethality (Lukinova et al., 1999). When $sub^{1}/CyO;polo^{16\text{-}1}/TM3, Sb$ and $sub^{131}/CyO;+/+$ flies were crossed, few $sub^{1}/sub^{131};polo^{16\text{-}1}/+$ progeny were recovered and the rare survivors were sick and small with abdominal patterning defects (Table 1). Thus, heterozygosity for $polo^{16\text{-}1}$ increased the severity of the *sub* homozygous phenotype, resulting in late larval developmental arrest. Synthetic lethality was also observed in *sub* homozygotes that were simultaneously heterozygous for the strong hypomorph $polo^{S025604}$ (or $polo^{9}$) (Donaldson et al., 2001).

The interaction with *Incenp* was tested using the EMS-induced allele $Incenp^{3747}$, which causes embryonic lethality (Chang, Goulding et al. 2006) whereas *Aurora B* was tested using the deletion *Df(2L)Exel7049*. When the appropriate heterozygotes were crossed together, we did not recover any $Incenp^{3747}sub^{1}/+sub^{131}$ or *Df(2L)Exel7049* $sub^{1}/+sub^{131}$ adults (Table 1). Since the synthetic lethality was observed in a genotype where one copy of the gene encoding Aurora B was deleted, the genetic interaction with the passenger protein mutants probably resulted from reduced levels of gene product rather than allele-specific interactions. Not all mitotic regulators caused lethality in *sub* homozygotes. The double mutant $sub^{1}/sub^{131};aur^{87Ac\text{-}3}/+$, which carried a null allele of the *Drosophila* Aurora A homolog, was viable. Therefore, the reduced dosage of Polo or the passenger proteins Incenp and Aurora B caused lethality in the absence of *sub*.

To examine the lethal interactions involving *sub* more closely, we looked for evidence of spindle assembly defects. Severe defects in spindle organization were observed in the brains of $sub^{1}/sub^{131};polo^{16\text{-}1}/+$ mutant larvae (Figure 8J,K). The spindles were unusually short. For example, the metaphase spindles were not much longer than the thickness of the chromosomes. Furthermore, $sub^{1}/sub^{131};polo^{16\text{-}1}/+$ larvae showed a higher mitotic index than either sub^{1}/sub^{131} or $polo^{16\text{-}1}/+$ mutants and was nearly four times that of the wild type (Figure 7), consistent with a defect in spindle assembly. Similar results were found in $Incenp^{3747}sub^{1}/+sub^{131}$ mutant brains. Short and disorganized metaphase spindles were common (Figure 8L, Table 2), consistent with a spindle assembly defect. Furthermore, the double mutant anaphase spindles usually lacked an organized midzone (Figure 8M). Although Subito and the passenger proteins always colocalized, the effects of the double mutant on midzone organization were more severe than those observed with the *sub* single mutant, suggesting that Incenp may have a separate role to *sub* in spindle assembly and stabilization of midzone microtubules during anaphase.

Subito and Polo physically interact

Consistent with our observations that Subito and Polo colocalize, Neef et al. (Neef, Preisinger et al. 2003) have shown that the mammalian homologs of Polo and Subito, Plk1 and MKLP2, physically interact. To examine whether Subito and Polo physically interact, we performed co-immunoprecipitation experiments. Lysates were prepared from embryos or oocytes expressing a *sub* transgene fused in frame to three copies of the HA epitope tag. As shown in Figure 9, we were able to precipitate Subito efficiently. In addition, Polo was detected in the anti-HA immunoprecipitate from the

Subito^{HA} lysates. Therefore, in both oocytes and embryos, Subito and Polo may exist in a complex.

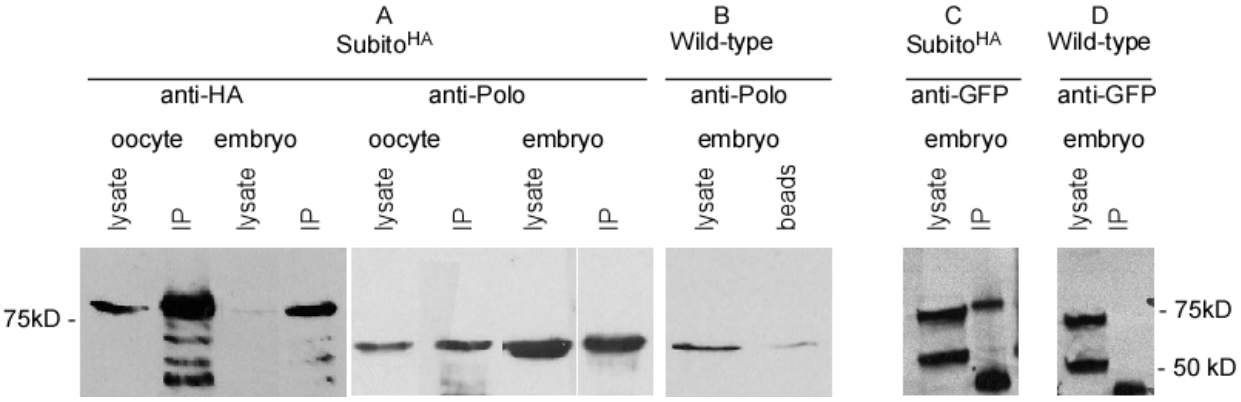

Figure 9 – Subito and Polo co-immunoprecipitate. A) HA – tagged Subito was immunoprecipitated from mixed stage 0—2 hour old embryos and metaphase arrested oocytes and then Western blotted. B) A control experiment was performed on tissue which did not express the HA – tagged Subito. C) This experiment was repeated using GFP – tagged Polo (Moutinho-Santos et al., 1999) HA – tagged Subito was immunoprecipitated from mixed stage 0—2 hour old embryos. D) In the control, using embryos not expressing HA – tagged Subito, GFP – Polo was not precipitated. The lower band is a nonspecific protein in the lysate.

The consequences of the spindle abnormalities in *sub* mutants

A defect in spindle assembly would be expected to result in hyperploidy or hypoploidy and a failure in cytokinesis would be indicated by the presence of polyploid cells. Aberrant chromosome segregation was detected by counting chromosome numbers in larval brain cells treated with colchicine to arrest them at metaphase. Consistent with the observed increase in spindle assembly defects, *sub* mutants had an increase in all aneuploid types (Figure 10A,B). Although *sub* mutants fail to localize Polo, Aurora B, and Incenp to the midzone at anaphase, *sub* mutants had only a small increase in

polyploidy. This was not surprising, because *sub* mutants lack some of the characteristics of *Drosophila* mutants with cytokinesis defects (see Discussion).

To investigate the nature of synthetic lethality with *Incenp*, the same experiment was performed on double mutant larvae. $Incenp^{3747}sub^{1}/+\ sub^{131}$ double mutant larvae exhibited a high frequency of polyploid cells (Figure 10C), suggesting a severe defect in cytokinesis. These results suggest that Subito has a redundant function in cytokinesis. Although s*ub* single mutants did not have a severe defect in cytokinesis, reducing the dosage of Incenp caused a severe defect in cytokinesis. These results suggest that Incenp may have a function in promoting cytokinesis that is independent of Subito.

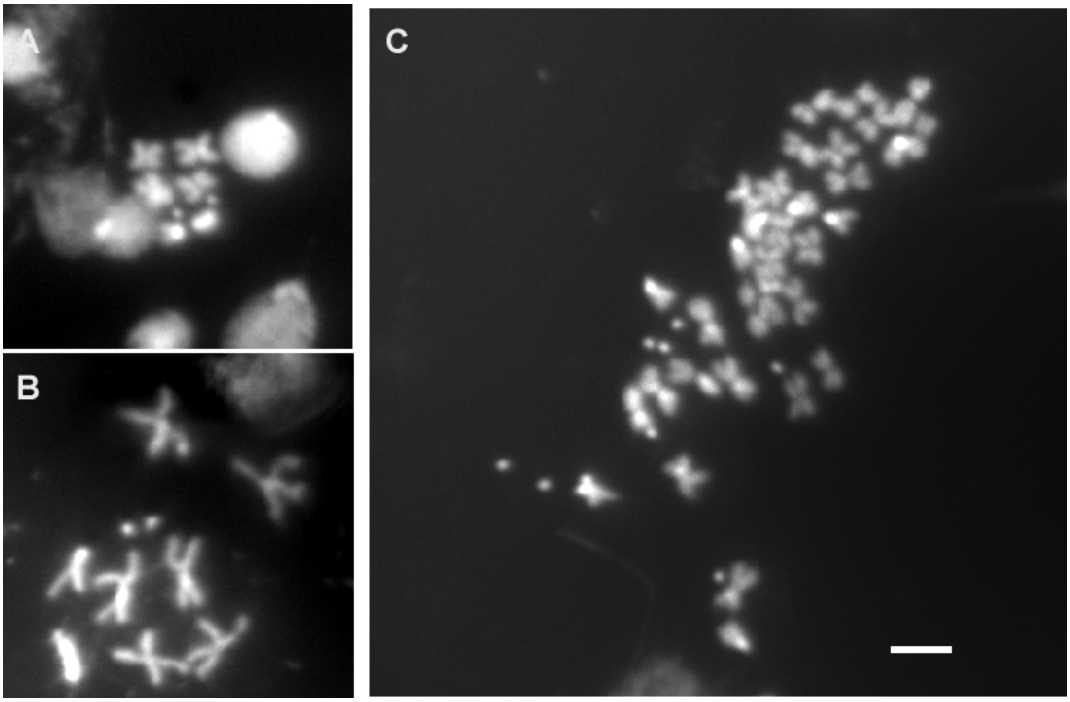

Figure 10 – Metaphase spreads in colchicine treated swollen cells. A) A normal karyotype in a wild-type cell. B) A hyperploid cell in a sub^{1}/sub^{131} mutant brain. C) A polyploid cell in a $Incenp^{3747}$ sub^{1} /+ sub^{131} brain. The scale bar is 5 μm and is the same for all three panels.

VI. Discussion:

Subito has an important role in assembling the acentrosomal female meiotic spindle in *Drosophila* (Giunta, Jang et al. 2002; Jang, Rahman et al. 2005). The results presented here from the analysis of *sub* are the first to demonstrate a function for a kinesin 6 family member in mitotic spindle assembly.

Relationship of Subito to the kinesin 6 family

Subito is one of the two *Drosophila* kinesin 6 family members and probably the ortholog of MKLP2 (Dagenbach and Endow 2004; Jang, Rahman et al. 2005; Miki, Okada et al. 2005). In support of this classification, there are striking similarities between Subito and MKLP2. Both are required for localization of the passenger proteins to the

midzone during anaphase (this report) (Gruneberg, Neef et al. 2004). In addition, both Subito and MKLP2 interact with Polo kinase (or Plk1 in human) and are required for its localization to the midzone during anaphase (this report) (Neef, Preisinger et al. 2003). Plk1 phosphorylates MKLP2 at Ser528 and this phosphorylation promotes Plk1 binding to MKLP2. Plk1 phosphorylation negatively regulates MKLP2 microtubule bundling activity in vitro but is not required for the localization of MKLP2 to the midzone.

Despite belonging to the same family, the two kinesin 6 family members probably have unique functions. The distinct phenotypes of *sub* and *pav* mutants indicate they have non-overlapping functions (see also (Jang, Rahman et al. 2005)). Similarly, and despite having similar localization patterns, MKLP2 and MKLP1 have nonredundant functions in cytokinesis (Fontijn, Goud et al. 2001; Neef, Preisinger et al. 2003). MKLP2, but not MKLP1, has been shown to physically interact with Aurora B and Incenp (Gruneberg, Neef et al. 2004). However, it has also been suggested that the MKLP2-dependent localization of Aurora B to the midzone is required for it to phosphorylate MKLP1 (Neef, Klein et al. 2006). The importance of this phosphorylation on MKLP2 localization is unclear and our results are consistent with this indirect relationship between Subito and Pavarotti.

It is possible that all members of the kinesin 6 group interact with anti-parallel microtubules (Nislow, Lombillo et al. 1992). Our immunolocalization data is consistent with this because Subito is found on interpolar microtubules, which are characterized by an overlap of anti-parallel microtubules in the midzone at mitotic anaphase in embryos (Jang, Rahman et al. 2005), brains and testis (J.K.J. and K.S.M., unpublished results).

However, the localization of Subito to metaphase interpolar microtubules in the vicinity of the centromeres was a surprising finding. Although it is likely that Subito also associates with anti-parallel microtubules at metaphase, we cannot rule out the possibility that Subito interacts with the plus ends of the microtubules that interact with the kinetochores. Surprisingly, a specific localization pattern of other kinesin 6 family members to metaphase microtubules has not been observed (Adams, Tavares et al. 1998; Minestrini, Harley et al. 2003; Somers and Saint 2003)(K.S.M., unpublished results). This is not due to the absence of the appropriate substrate, since metaphase interpolar microtubules are present in most spindles (Mastronarde, McDonald et al. 1993; Compton 2000). Either Subito is regulated differently than MKLP2, with an associated additional function in spindle assembly, or the localization pattern of MKLP2 at metaphase has not been informative with respect to its function.

Evidence of a redundant role for Subito during cytokinesis

Since Subito is required to localize Polo, Aurora B and Incenp to the spindle midzone at anaphase, it is surprising that *sub* mutants are viable. Loss of MKLP2 causes cytokinesis defects (Neef, Preisinger et al. 2003). *Drosophila* mutants with strong defects in cytokinesis fall into the categories of male sterile (Giansanti, Gatti et al. 2001), embryonic lethal (e.g. *pav* mutants) (Adams, Tavares et al. 1998) or pupal lethal (e.g. (Castrillon and Wasserman 1994; Gunsalus, Bonaccorsi et al. 1995; Verni, Somma et al. 2004)). In fact, *Incenp* and *polo* mutants have embryonic lethal phenotypes that may be caused by a failure of cytokinesis (Carmena, Riparbelli et al. 1998; Chang, Goulding et al. 2006). Unlike the loss of *Incenp, Aurora B* or *Polo, sub* mutants do not have any of

these phenotypes and appear to complete cytokinesis most of the time in larval brains. In addition, because *sub* mutant males are fertile, and most mutants with strong defects in cytokinesis during spermatogenesis are male sterile (Giansanti, Gatti et al. 2001), Subito does not appear to be essential for cytokinesis in the testis. A cytokinesis phenotype was also not evident in cultured *Drosophila* cells depleted of Subito by RNAi (C. Wu and K.S.M., unpublished results) (Goshima and Vale 2003; Echard, Hickson et al. 2004; Eggert, Kiger et al. 2004). These same studies did identify cytokinesis defects when *Polo, Aurora B* and *Incenp* were depleted. Thus, it seems likely that in some cell types, such as larval brains, the presence of Subito and the localization of the passenger proteins are not required for cytokinesis to occur.

A close examination of *sub* mutants, however, revealed that anaphase did not proceed normally. In addition to the failure to accumulate Polo, Aurora B and Incenp in the midzone, the absence of Subito resulted in disorganized midzone microtubules at anaphase and a small increase in the frequency of polyploid cells. When the dosage of *Incenp* was reduced in *sub* mutants, the frequency of polyploidy was markedly increased. Therefore, Subito appears to have a similar function to MKLP2 in promoting cytokinesis, although there may be functional redundancy. Since the ability to complete cytokinesis in *sub* mutants depends on *Incenp* and *Aurora B* dosage, it is possible that unlocalized Incenp or Aurora B may promote cytokinesis. However, our observation that Incenp and Aurora B have a limited ability to spread along anaphase microtubules in the absence of Subito suggests an alternative; enough passenger protein activity may be present to promote cytokinesis. This model can account for the sensitivity of *sub* mutants to *Incenp* or *Aurora B* dosage because high levels of these proteins may be needed to promote

cytokinesis if not concentrated in the midzone. It is also possible that anaphase may last longer and/or the microtubule organization improves with time in *sub* mutants. This would account for the relatively normal Feo localization and high success completing cytokinesis in *sub* mutants.

Subito interacts with Ncd, Polo and the passenger proteins during spindle assembly

Several lines of evidence suggest that Subito has a role in mitotic spindle assembly. First, Subito initially localizes to interpolar microtubules at metaphase. Second, abnormally formed metaphase spindles were found in *sub* mutants more frequently than in the wild type. Third, *sub* mutant brains have an elevated mitotic index. Although the magnitude of the increase in *sub* mutants was lower than reported in some other mutants with spindle assembly defects (e.g. (Basto, Gomes et al. 2000; Donaldson, Tavares et al. 2001)), these mutants are lethal. Consistent with the conclusion that *sub* mutants have a defect in spindle assembly, the elevated mitotic index was dependent on *BubR1*, suggesting that the spindle assembly checkpoint is activated in the absence of Subito. Fourth, *sub* mutations exhibit synthetic lethality in combination with *polo, Incenp* and *Aurora B* mutations, and the cytological phenotype includes defects in spindle assembly and increased mitotic index (see below). Finally, RNAi of *sub* in *Drosophila* S2 cells results in frequent mitotic spindle abnormalities (C. Wu and K.S.M., unpublished results) (Kiger, Baum et al. 2003). These observations all point to a role for Subito in spindle assembly.

The defects associated with *sub* mutants are less severe in mitotic cells than during female meiosis, possibly because of redundant spindle assembly pathways in mitosis. The double mutant studies suggest that the defects in spindle assembly or chromosome alignment in *sub* mutants are compensated for in two ways. First, the activation of the spindle assembly checkpoint allows defects in microtubule organization to be corrected. Second, the presence of redundant spindle assembly pathways allows microtubules to be assembled in the absence of *sub*. Our double mutant studies support both of these mechanisms.

The phenotype of the *sub;polo*$^{16\text{-}1}$/+ double mutant was consistent with a redundant role for Subito in spindle assembly. Compared with the single mutants, the double mutants exhibited grossly abnormal metaphase and anaphase spindles. Similar to our results with *sub*, a role for Polo in spindle assembly was previously shown through the analysis of *polo* hypomorphs that had an elevated mitotic index in larval brains, indicating that the spindle assembly checkpoint was activated (Donaldson, Tavares et al. 2001). During metaphase, Polo localizes to the centromeres where it has a role in spindle formation but during anaphase it localizes to the spindle midzone where it has a role in cytokinesis (Carmena, Riparbelli et al. 1998; Logarinho and Sunkel 1998; Moutinho-Santos, Sampaio et al. 1999). The very high mitotic index in the double mutants, however, suggests a more severe defect in spindle assembly than either single mutant. We suggest that the abnormal spindle phenotype in *sub/sub;polo/+* mutants arise from a combination of defects in two partially redundant spindle assembly pathways: improper assembly of kinetochore microtubules in *polo/+* mutants and a reduction in assembling interpolar microtubules in *sub* mutants. Although *polo* mutants are recessive lethal, there

is other evidence for dominant phenotypes, such as an elevated mitotic index in $polo^{16\text{-}1}$/+ brains (see also (Clarke, Tang et al. 2005)).

The combination of these two spindle assembly defects in *polo/+;sub/sub* mutants might result in the severe spindle assembly phenotype and lethality in the double mutant. Similar conclusions apply for the interactions between *sub* and *Incenp* or *Aurora B*. Like *Polo,* the passenger proteins have an important role in spindle assembly (Adams, Maiato et al. 2001; Vernos 2004). Indeed, the effects of all three mutants are strikingly similar, suggesting that Subito, Polo and the passenger proteins have important interactions during metaphase and anaphase. There is evidence of a direct interaction between Plk and Incenp in mammalian cells (Goto, Kiyono et al. 2006).

The role of Subito in mitotic spindle function

Like its kinesin 6 homolog MKLP1 (Nislow, Lombillo et al. 1992), Subito is probably a plus-end-directed motor that crosslinks and slides interpolar anti-parallel microtubules. Our results suggest that this activity is important from metaphase through anaphase. Interestingly, the metaphase and anaphase interpolar microtubules have functional differences. Metaphase interpolar microtubules are observed in the absence of Subito whereas their anaphase counterparts depend on Subito. Another important difference is that Polo and the passenger proteins only localize to anaphase interpolar microtubules in the midzone. We have previously suggested that the precocious appearance of anaphase-like interpolar microtubules is an important feature of

acentrosomal meiotic spindle assembly in *Drosophila* oocytes (Jang, Rahman et al. 2005). The passenger proteins Aurora B and Incenp localize to the interpolar microtubules at metaphase of meiosis I, rather than the centromeres, which is typical during mitotic metaphase. Therefore, the regulation of the passenger protein localization pattern is modified in oocytes to bypass the centromere localization that is characteristic of mitotic metaphase, resulting in precocious localization to interpolar microtubules (Jang, Rahman et al. 2005).

Despite these differences, the same biochemical activities of Subito could be used to organize both centrosomal mitotic and female acentrosomal meiotic spindles. In mitotic cells, kinetochores can initiate microtubule fiber formation, but these fibers are not directed toward either spindle pole (Khodjakov, Copenagle et al. 2003; Maiato, Rieder et al. 2004). Failure to organize these fibers could result in disorganized and frayed spindles, as we have observed in *sub* mutants. A function for Subito and interpolar microtubules could be to properly orient undirected kinetochore fibers. Interpolar microtubules could interact with and direct the organization of kinetochore microtubules via motors that bundle parallel microtubules. We have proposed this mechanism for organizing a bipolar spindle in the acentrosomal meiosis of *Drosophila* oocytes (Jang et al., 2005). With motor-driven sliding of anti-parallel microtubules, this is an example of a centrosome-independent model for the spindle assembly pathway. This is consistent with previous conclusions that centrosome-independent mechanisms for spindle assembly are active in mitotic cells (Maiato, Rieder et al. 2004; Wadsworth and Khodjakov 2004). Indeed, since bipolar spindles can form in the absence of centrosomes in neuroblasts and ganglion mother cells (Bonaccorsi, Giansanti et al. 2000; Megraw, Kao et al. 2001), it

appears that centrosome-independent mechanisms for spindle assembly are active in the mitotic cells we have analyzed.

Another possibility is that Subito functions as part of the centrosomal assembly pathway. For example, an array of interpolar microtubules could help channel centrosome microtubules towards the kinetochores. This activity could reduce the element of chance associated with making contacts between centrosome microtubules and kinetochores. It has also been proposed that centrosomal microtubules may capture the minus ends of kinetochore microtubules (Khodjakov, Copenagle et al. 2003; Maiato, Rieder et al. 2004). An involvement of Subito in this process would be surprising, however, because the ability to bundle microtubules in parallel has not been described for a kinesin 6 family member. Nonetheless, if Subito was involved in the interactions of centrosomal and kinetochore microtubules, subsequent plus-end-directed movement would explain why Subito localization overlaps with centromeres. Whether or not these models are correct, the redundant nature of spindle assembly and function may explain why a role for kinesin 6 motor proteins in spindle assembly has not been described previously.

Chapter 2: RanGTP has a non-essential role in acentrosomal spindle assembly in *Drosophila* oocytes

I. Preface

This chapter was submitted, as presented here, to the Journal of Cell Biology, August 2010. My contributions to the project and paper included: all genetic and cytological analysis, and the writing and reviewing of the paper.

II. Abstract

RanGTP has been shown to be important for chromosome-dependent spindle assembly in *Xenopus* extracts. Since *Drosophila* female meiosis is acentrosomal, we investigated the effect of manipulating the Ran pathway on spindle assembly in *Drosophila* oocytes and embryos. RCC1, a guanine exchange factor responsible for converting Ran to its active RanGTP form, is an important component of this pathway and is present on oocyte chromosomes. Hence, RanGTP is expected to be in the vicinity of the chromosomes after NEB. To investigate the role of RanGTP, we generated females expressing dominant negative GDP-locked (ran^{GDP}) or GTP-locked (ran^{GTP}) forms of *ran*. Females expressing these mutants were sterile, but this was not due to defects in meiosis. Expression of ran^{GDP} in oocytes did not block spindle assembly, although the tapering of microtubules at the poles and localization of TACC and the HURP homolog, Mars, was abnormal. Furthermore, expression of ran^{GTP} did not promote ectopic spindle assembly. Thus, RanGTP may not be essential or sufficient for the formation of the acentrosomal spindle around the chromosomes. In contrast,

expression of *ran*^{GDP} blocked pronuclear fusion, which depends on microtubules nucleated from the sperm aster. Similarly, expression of *ran*^{GDP} suppressed the chromosome-independent spindle assembly phenotype caused by a mutation which deletes the non-motor N-terminus domain of the Kinesin-6 *subito*. Thus, RanGTP may be required for microtubule assembly that is not directly nucleated by the chromosomes. In promoting spindle assembly around chromosomes, RanGTP may be redundant with other factors. Indeed, expression of *ran*^{GDP} in a *sub* mutant background caused a block in oogenesis, a more severe phenotype than in either single mutant. Subito interacts with the chromosome passenger complex (CPC) that has also been implicated in chromosome-mediated spindle assembly. Therefore, RanGTP may be redundant with the CPC when chromosomes are present.

III. Introduction

The proper separation of homologous chromosomes during female meiosis is dependent upon formation of microtubules into a bipolar spindle and the attachment of these microtubules to the chromosomes. Broadly speaking, there are two types of spindle, those with and those without centrosomes. Centrosomes, which are typically found in animal mitotic cells and the meiotic cells of spermatogenesis, are microtubule organizing centers that direct the formation of the spindle into a bipolar structure. Microtubules that grow from these microtubule organizing centers are capable of attaching to the kinetochores or overlapping with microtubules from the opposite pole. The kinetochore microtubules mediate the proper orientation and separation of the homologs to opposite poles, resulting in two daughter cells with equal chromosome numbers.

In *Drosophila* oocytes, as in many oocytes, meiosis is acentrosomal. Spindle assembly occurs without the guidance of the microtubule organizing centers at the poles. In this situation, the chromosomes play an important role in spindle assembly. Nuclear envelope breakdown (NEB) is followed by the accumulation of microtubules around the chromosomes (Theurkauf and Hawley 1992; Matthies, McDonald et al. 1996). The subsequent bundling and tapering of these microtubules by motor proteins results in a bipolar spindle. These studies suggest that *Drosophila* oocyte chromosomes carry a signal which promotes spindle assembly when released upon NEB. It is unclear, however, what are the components of this signal.

At least two mechanisms have been proposed to promote spindle assembly in the absence of centrosomes (Karsenti and Vernos 2001). First, the chromosome passenger complex (CPC) can promote spindle assembly by inactivating proteins like the microtubule-depolymerizing motor MCAK (Sampath, Ohi et al. 2004). Recent studies have suggested that this pathway plays a role in acentrosomal spindle assembly (Maresca, Groen et al. 2009). Analysis of a hypomorphic mutation in *Incenp* has led to the suggestion that this pathway is important for spindle assembly in *Drosophila* oocytes (Colombie, Cullen et al. 2008; Resnick, Dej et al. 2009).

Second, RanGTP can dissociate spindle assembly factors from the repressive Importin complex, thereby promoting spindle assembly (Kalab and Heald 2008). Ran is a member of the Ras family of small GTP-binding proteins. The conversion of RanGDP to RanGTP is facilitated by the chromatin bound guanine nucleotide exchange factor RCC1 (Bischoff and Ponstingl 1991). Conversely, the conversion of RanGTP to RanGDP is facilitated by the cytoplasmic GTPase-activating protein RanGAP (Bischoff,

Klebe et al. 1994). The production of RanGTP near chromatin and conversion to RanGDP in the cytoplasm can lead to the formation of a gradient of active Ran that is highest near the spindle. A high or specific concentration of RanGTP may be the signal which triggers chromosome-mediated spindle assembly (Caudron, Bunt et al. 2005).

The role of RanGTP in chromosome-mediated spindle assembly has been most clearly shown by its activity in *Xenopus* egg extracts which lack centrosomes. Chromatin-mediated microtubule assembly depends on the presence of RanGTP in *Xenopus* extracts (Carazo-Salas, Guarguaglini et al. 1999). Similarly, depletion of RCC1 results in a failure to form microtubule asters. Addition of RanGTP to these RCC1-depleted eggs is sufficient to induce self-organization of microtubule asters (Ohba, Nakamura et al. 1999). Disruption of RanGTP levels also affects mitotic spindle assembly in mammalian cells (Kalab, Pralle et al. 2006; Clarke and Zhang 2008) *Drosophila* (Silverman-Gavrila and Wilde 2006) and *C. elegans* (Askjaer, Galy et al. 2002; Bamba, Bobinnec et al. 2002). We have investigated if the Ran pathway has a role in the assembly of meiotic spindles in *Drosophila* oocytes. The Ran pathway is essential in embryos and larvae, but we found evidence that, while it is active in promoting microtubule assembly in oocytes, there is at least one additional pathway which can promote recruitment of microtubules around the chromosomes.

IV. Materials & Methods

Generation and analysis of transgenic lines:

Full-length and substitution derivatives of *ran* were amplified by PCR. The clones were verified by sequencing and then the fragments were cloned into the pENTR4 vector

(Gateway). The fragment was then recombined using Clonase (Invitrogen) into the pPHW vector which encodes three copies of the HA epitope at the N-terminus of the coding region in a pUASP backbone (Rorth 1998). Amino acid substitutions were made by modifying the wild-type *ran* clone in pENTR4 using the Change IT mutagenesis kit (USB) and the appropriate primers. For the ran^{GDP} transgene, a substitution of a threonine to an asparagine at amino acid twenty-four was accomplished using the following primers: GATGGCGGCACTGGCAAGAACACCTTTGTCAAGCGGCAC for the forward primer and GTGCCGCTTGACAAAGGTCTTGCCAGTGCCGCCATC for the reverse primer. For the ran^{GTP} transgene, a substitution of a glutamine to a leucine at amino acid sixty-nine was accomplished using the following primers: CGCCGAACTTCTCCAGGCCAGCGGTATCCC for the forward primer and GGGATACCGCTGCGGTGGAGAACTTCGGCG for the reverse primer.

For ubiquitous expression in somatic tissues, males carrying a *ran* transgene, *P{UASP::ran}*, were crossed to females carrying a GAL4 transgene with a tubulin promoter (*P{tubP-GAL4}*) (Lee and Luo 1999). A cross with the driver heterozygous to a balancer that provides a Tubby phenotype visible in larvae, *P{tubP-GAL4 }* / *T(2;3)B3, CyO: TM6B, Tb*, results in two genotypes: *P{UASP::ran}* / *P{tubP -GAL4 }* and *P{UASP::ran}* / *T(2;3)B3, CyO: TM6B, Tb*. The percent survival was calculated as (Tb^+ flies) / (Total flies). For expression in the germline and early embryo, males carrying a *ran* transgene were crossed to females carrying a GAL4 transgene with a *nanos* promoter, *P{GAL4::VP16-nos.UTR}MVD1* (Van Doren, Williamson et al. 1998). To measure fertility and chromosome segregation during meiosis, females carrying a transgene and

the *nanos* driver were crossed to *y w/B^{s}Y* males. The non-disjunction frequency was calculated as 2(B^S ♀+ B^+♂) / [B^+ ♀+ B^S ♂+ 2(B^S ♀+ B^+♂)].

Antibodies and immunofluorescent microscopy

Mature (stage 14) oocytes were collected from 50-200 yeast fed females that were aged 3-4 days by physical disruption in a common household blender (Theurkauf and Hawley 1992; McKim, Joyce et al. 2009). The oocytes were fixed in modified Robb's media and cacodylate/formaldehyde fixative for 8 min and then their outer membranes were removed by rolling the oocytes between the frosted part of a slide and a coverslip.

Embryos were collected by placing females and males in cages with grape juice plates for two hours to enrich for those undergoing the syncytial divisions. Embryos were removed from the grape juice plates with water and placed in 50% bleach for 90 seconds to remove the chorion. They were then thoroughly washed with water to remove all traces of bleach. The embryos were fixed using a heptane / methanol fixation (Rothwell and Sullivan 2000).

For squashing neuroblasts, the third instar larval brains were dissected in saline and the brains were fixed in 3.7% formaldehyde in 1x PBS for 30 minutes. The brains were then transferred to 45% acetic acid for 3 minutes before transferring to ~8 µl of 60% acetic acid on a siliconized coverslip where they were firmly squashed between the coverslip and slide. The slides were briefly frozen in liquid nitrogen and the coverslips were flicked off with a razor blade. The slides were placed in ethanol at –20°C (chilled on dry ice) for 10 minutes, then transferred to a slide chamber containing 0.1% Triton X-100 in PBS for 10 minutes. Rubber cement was used to form wells on the slides and two

5-minute washes were done in PBS. The tissue was blocked with 1% BSA in PBS for 45 minutes.

Oocytes, embryos, and neuroblasts were stained for DNA with Hoechst 33342 at 1:1,000 (10mg/ml solution) and for microtubules with mouse anti-α tubulin monoclonal antibody DM1A (1:50), directly conjugated to FITC (Sigma) or rat anti-α tubulin monoclonal antibody (1:75) (Millipore). The primary antibodies were rat anti-SUB antibody (used at 1:75) (Jang, Rahman et al. 2005), rat anti-HA (Roche, clone 3F10) (1:25), rat anti-INCENP (1:500) (Wu, Singaram et al. 2008), mouse anti-RCC1 (1:20) (Frasch 1991), rabbit anti-RanGAP (1:800) (Kusano, Staber et al. 2001), rabbit anti-Mars (Tan, Lyulcheva et al. 2008) and mouse anti-Lamin Dm_0 (1:800) (Klapper, Exner et al. 1997). These primary antibodies were detected with either a Cy3 or Cy5 secondary antibody preabsorbed against a range of mammalian serum proteins (Jackson Labs) and *Drosophila* embryos. TACC was detected using a GFP fusion protein (Gergely, Kidd et al. 2000). Images were collected on a Leica TCS SP2 confocal microscope with a 63x, NA 1.3 lens. Images are shown as maximum projections of image stacks followed by merging of individual channels and cropping in Adobe Photoshop.

Fluorescent in situ hybridization (FISH)

Stage 14 oocytes were collected as described above and then processed for both immunofluorescence and FISH as described (McKim, Joyce et al. 2009). Oligonucleotide probes for the satellite sequence AACAC, in the second chromosome centric heterochromatin, or Dodeca, in the third chromosome centric heterochromatin, were end-labeled with Cy3-dCTP or Cy5-dCTP (GE Healthcare) by Terminal

Deoxynucleotidyl Transferase (Invitrogen). Oocytes were subsequently stained for microtubules and DNA as described above.

V. Results

Generation of dominant *ran* mutants

Because *ran* is required for mitosis (Silverman-Gavrila and Wilde 2006) and is an essential gene in *Drosophila* (Peter, Schottler et al. 2002), we made nucleotide changes predicted to be dominant mutations of *ran*. Previous studies in a variety of systems have characterized mutations that lock Ran in either the GDP (inactive) or GTP (active) states (Kahana and Cleveland 1999; Trieselmann and Wilde 2002). Since Ran is highly conserved, these same changes can be made in *Drosophila*. Transgenes were made by fusing the coding region of the wild-type *ran* or mutant variants to three copies of the HA epitope tag at the N-terminus. They were also put under the control of the UASP promoter, which allows for inducible germline expression regulated by a second transgene expressing GAL4 (Rorth 1998). The *P{UASP:ran^{GDP}}* construct, hereafter referred to as *ran^{GDP}*, contains *ran* with an amino acid substitution of threonine to asparagine at position 24 (T24N). The *P{UASP:ran^{GTP}}* construct, hereafter referred to as *ran^{GTP}*, contains *ran* with an amino acid substitution of glutamine to leucine at position 69 (Q69L). For each allele, at least three transgenic lines were examined for expression levels and phenotypes. Differences in expression levels between different insertions were assayed by Western blot and found to be minimal. For each experiment, the effects of these mutations were compared to flies expressing a wild-type transgene (*P{UASP:ran^{+}}*), hereafter referred to as *ran^{+}*.

Expression of r*an^{GDP}* has a dominant negative effect

To determine if expression of the mutant forms of *ran* would cause lethality similar to the loss of function mutant, we expressed the transgenes using *P{tubP-GAL4}*, which induces ubiquitous expression of UAS transgenes (Lee and Luo 1999). Expression

of *ran*$^{+}$ had no effect on viability (Table 3). Furthermore, dividing neuroblasts from third instar larvae exhibited properly assembled spindles with no detectable abnormalities during metaphase and anaphase. The chromatids were properly condensed at the metaphase plate and appeared to divide evenly during anaphase. Localization of wild-type Ran was examined using antibodies to the HA epitope tag that was fused at the N-terminus of the *P{UASP:ran*$^{+}$*}* transgenes. Ran staining overlapped with the spindle during metaphase and anaphase. Similar to the localization pattern in embryos (Trieselmann and Wilde 2002), Ran was not detected on the chromatids. On the other hand, ubiquitous expression of *ran*^{GDP} or *ran*^{GTP} resulted in lethality prior to the third instar larval stage of development (Table 3). These results suggest that expression of *ran*^{GDP} or *ran*^{GTP} has a dominant negative effect and disrupts the normal functioning of the Ran pathway.

Table 3

Effect of *ran* mutants on viability

Transgene:	Progeny expressing Ran	Progeny not expressing Ran
ran^{+}	755	326
ran^{GDP}	0	464
ran^{GTP}	0	1405

Each transgene was expressed by crossing to *P{ tubP -GAL4} / TM6, Tb*. Progeny expressing *P{ran}* were Tb^{+}.

For each transgene at least two independent insertions were scored, both of which gave similar results.

Maternal expression of *ran*^{GDP} and *ran*^{GTP} causes sterility

To examine the function of Ran in oogenesis and embryogenesis, the *ran* mutations were expressed using the *P{GAL4:VP16-nos.UTR}MVD1* driver (Van Doren,

Williamson et al. 1998) and then genetic assays were performed to measure the frequency of X-chromosome non-disjunction and fertility. This driver has been used extensively to induce high-level expression of UASP transgenes in mature oocytes (Jang, Rahman et al. 2007). When the wild-type Ran transgene was expressed, we found normal levels of X chromosome non-disjunction and high fertility (Table 4). Therefore, expressing wild-type Ran does not have deleterious effects on meiotic chromosome segregation or embryonic development. Expression of ran^{GDP} in the female germline caused a drastic reduction in fertility, with an average of only 6.8 progeny per female parent compared to 62.1 progeny per female expressing wild-type Ran (Table 4). Expression of ran^{GTP} in the oocyte resulted in complete sterility (Table 4). These effects of ran^{GDP} and ran^{GTP} on fertility indicate that there is an important role for RanGTP in either completing meiosis or in the embryonic divisions. *P{GAL4:VP16-nos.UTR}MVD1* also drives expression in the male germline. Males carrying this driver and ran^{GDP} were sterile, suggesting that RanGTP has an essential role in male meiosis.

Maternal expression of ran^{GDP} and ran^{GTP} blocks embryogenesis

The low fertility of the ran^{GDP} mutant females could have been due to defects in the meiotic divisions or embryogenesis. To examine the embryonic mitotic divisions, we examined embryos from mothers expressing wild type or mutant versions of *ran.* Expression of wild-type *ran* resulted in embryos undergoing normal synchronous divisions, with spindle assembly and chromosome organization characteristic of wild-type embryonic divisions (Figure 11A). Furthermore, wild-type Ran localized to the mitotic spindle. This pattern of localization is similar to that determined in neuroblasts and previously by the injection of fluorescently-labeled Ran protein into embryos (Trieselmann and Wilde 2002). Thus, expression of HA-tagged wild-type *ran* exhibited

no deleterious effects on the mitotic divisions of the embryo and mimics the known localization pattern to spindle microtubules.

Table 4

Fertility and non-disjunction phenotypes by *ran* transgenes

Transgene [a]	Regular Progeny	Non-disjunction Progeny	Progeny / Female Parent (N)	Non-disjunction (%)
ran^{+}	2481	1	62.1 (40)	0.08%
ran^{GDP}	498	2	6.8 (74)	0.80%
ran^{GTP}	0	0	0.0 (80)	-
$sub^{\Delta NT}$	0	0	0.0 (20)	-
$sub^{\Delta NT}$; ran^{+}	0	0	0.0 (20)	-
$sub^{\Delta NT}$; ran^{GDP}	338	3	34.1 (10)	1.7%

a - Each transgene was expressed by crossing to the *P{GAL4::VP16-nos.UTR}MVD1* driver. Expressing females were crossed to *y w/* $B^{S}Y$ males to assay non-disjunction and fertility. N is the number of female parents.

For each transgene at least two independent insertions were scored, both of which gave similar results.

The majority of embryos expressing ran^{GDP} arrested development without any evidence of the embryonic mitotic divisions. These embryos fell into two types. About half of the embryos contained the female and male meiotic products (Figure 11B). Some of these embryos contained up to 16 maternal meiotic products. In these cases, Ran^{GDP} protein was closely associated with the chromosomes, as would be expected if it were bound to RCC1. The remaining half of the embryos had no visible nuclei. These results

suggest that meiosis could be completed in the *ran*^{GDP} embryos but the female and male pronuclei do not fuse and the three remaining female meiotic products fail aggregate into a polar body. Consistent with this conclusion, we have observed normal meiosis II spindles in *ran*^{GDP} embryos (data not shown). Expression of *ran*^{GDP} in embryos might disrupt the assembly of the microtubules network nucleated by the sperm centrosome that brings together the female and male pronuclei. It is unclear why, in half the embryos, we did not observe any nuclei. This could be a downstream consequence of a failed attempt at pronuclear fusion although we cannot rule out problems in completing meiosis.

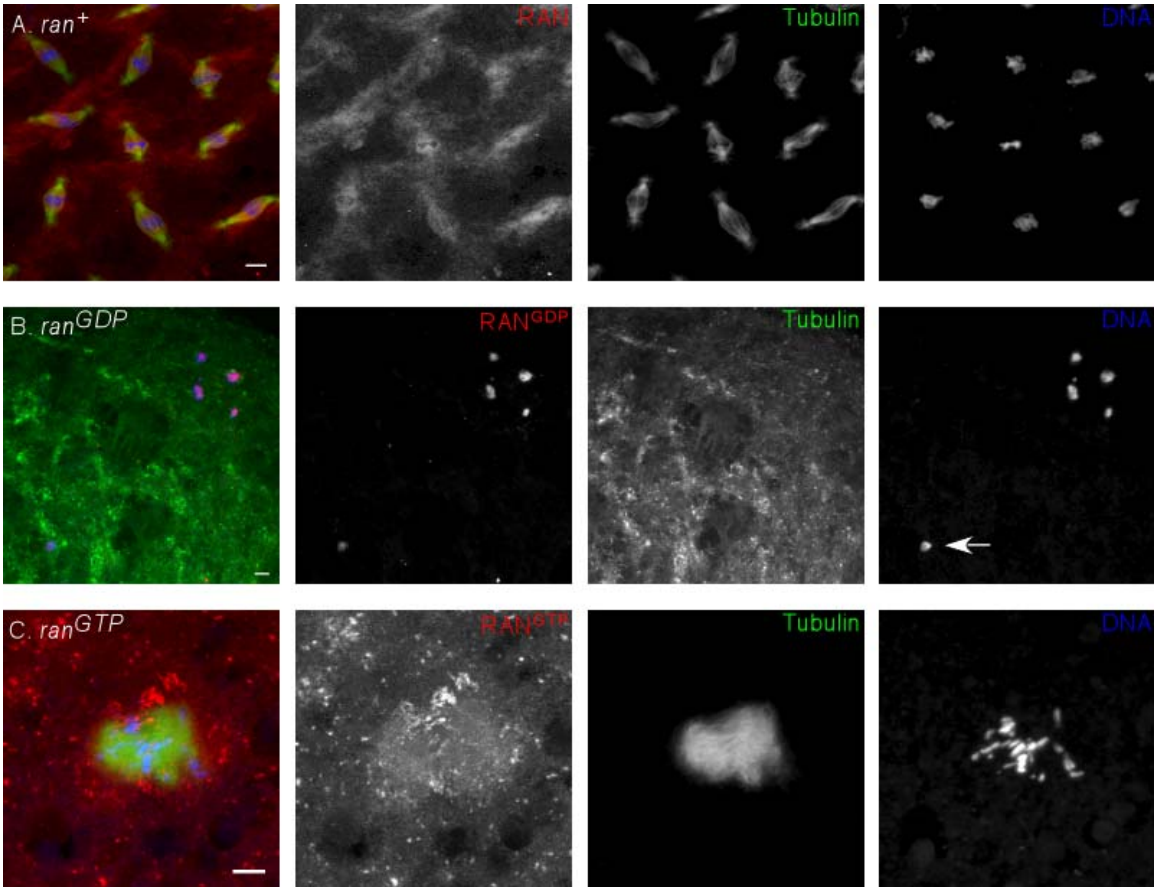

Figure 11 –Ran is required to initiate embryonic development. Spindles form normally when wild-type Ran is expressed in embryos (A) whereas embryogenesis is blocked when Ran^{GDP} (B) or Ran^{GTP} (C) are expressed. The male pronucleus is shown by an arrow. The cluster of four DNA masses are the female pronuclei, three of which normally fuse into a polar body. Transgenes under the control of the UASP promoter were expressed in embryos using the *P{GAL4::VP-nos.UTR}MVD1* driver. DNA is in blue, Ran tagged with the HA epitope is in red, Tubulin is in green and the scale bars are 10 μm.

Similar to ran^{GDP} embryos, maternal expression of ran^{GTP} led to a failure to initiate embryonic development. In most of the mutant embryos, a cluster of DNA and microtubules was observed in the center of the embryo and there was no evidence of any mitotic divisions (Figure 11C). This phenotype was distinctly different that the two types of embryos observed in ran^{GDP} embryos and may be due to a defect shortly after pronuclear fusion (see Discussion).

Upstream (RCC1) and downstream (RanGAP) components of the Ran pathway are present in the *Drosophila* oocyte

The above results show that the sterility in the *ran* mutants can be explained by post-meiotic functions, but did not rule out defects in assembly of the meiotic spindle. To examine if defects in meiosis contributed to the sterility, we examined oocytes expressing the wild-type and mutant *ran* transgenes. Following NEB in *Drosophila* oocytes, microtubules accumulate around the chromosomes, which are bundled tightly together into a karyosome (Theurkauf and Hawley 1992; Matthies, McDonald et al. 1996). This is followed by the extension of poles and lengthening of the spindle. In addition, our previous work has shown that the central spindle is important for organizing bipolarity (Jang, Rahman et al. 2005), which can be detected by staining for Subito, a Kinesin 6 that localizes to the anti-parallel microtubules of the central spindle.

Given the large size of the oocyte relative to the spindle, we examined if two components which contribute to a RanGTP gradient, RCC1 on the chromatin and RanGAP in the cytoplasm, were present. We stained mature oocytes with an antibody raised against RCC1 (Frasch 1991) and found that it localized tightly around the outside of the karyosome (Figure 12A). In contrast, RanGAP was localized to globular structures throughout the ooplasm of mature oocytes (Figure 12B and 2C). The localization pattern of RanGAP does not correlate with any known structure in the *Drosophila* oocyte. These results show that two proteins, RCC1 and RanGAP, are located in discrete locations within the oocyte during assembly of the meiotic acentrosomal spindle and could be in a position to regulate RanGTP.

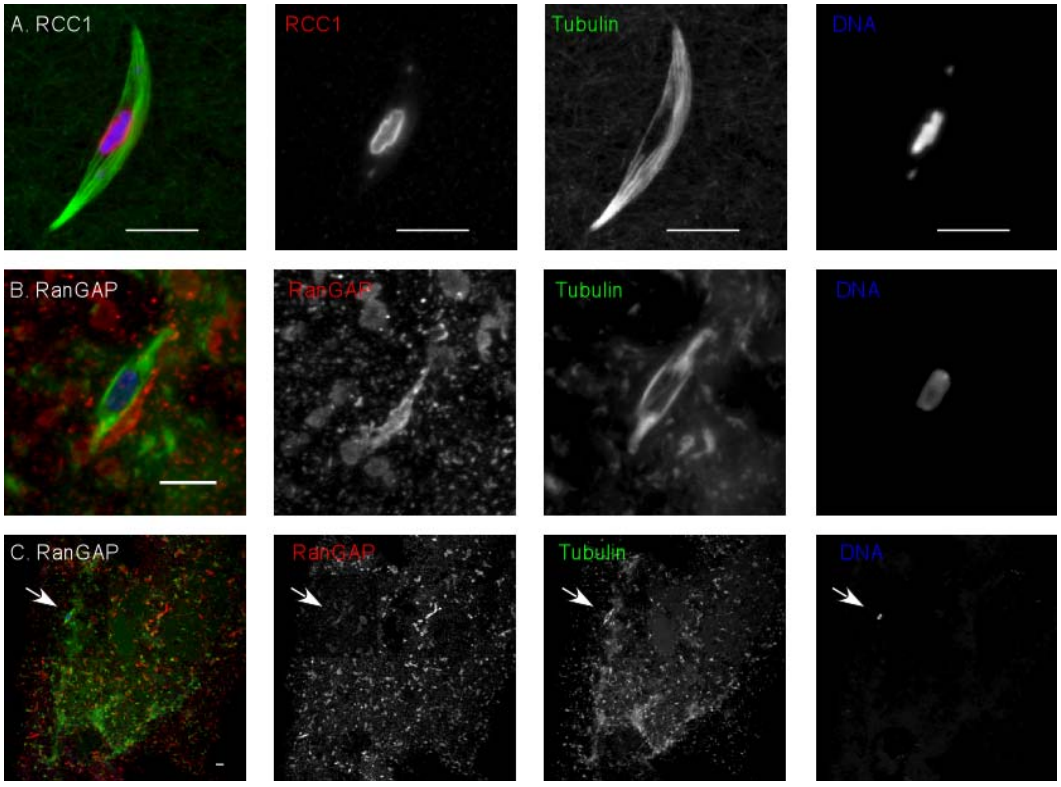

Figure 12 - Localization of RCC1 and RanGAP in stage 14 oocytes. (A) Wild-type oocyte stained with RCC1 antibody (red). (B) Wild-type oocyte stained with RanGAP antibody (red). (C) A low magnification view of the same oocyte in (B) showing the sporadic localization of RanGAP clusters throughout the ooplasm. The arrow points to the karyosome. DNA is in blue, Tubulin is in green and the scale bars are 10 μm.

Expression of Ran^{GDP} effects spindle pole organization in *Drosophila* oocytes

To examine the effect of Ran on meiotic spindle assembly, wild-type and mutant *UASP:ran* transgenes were expressed using the *P{GAL4:VP16-nos.UTR}MVD1* driver. Immunofluorescence of mature oocytes revealed that wild-type Ran localized mostly to an area surrounding of the metaphase I spindle (Figure 13A). Interestingly, this localization pattern showed almost no overlap with the spindle microtubules, unlike the pattern observed in mitotically dividing neuroblasts and embryos. Additional accumulations of Ran were found near the clusters of RanGAP that form throughout the cytoplasm (Figure 14A) but there was generally no overlap.

In the oocytes expressing wild-type Ran, spindle and karyosome morphology were normal (Figure 13A and Table 5). A low frequency of abnormal spindles is expected since some of the oocytes are in early prometaphase when the spindle is first assembling. In addition, Kinesin-6 Subito had a normal localization pattern (data not shown). Therefore, expression of the HA-tagged wild-type Ran does not grossly affect spindle bipolarity or morphology.

Table 5

Characterization of meiotic figures in wild-type and mutant *ran* oocytes

Transgene:	Oocytes	Abnormal Spindle		Abnormal Karyosome	
ran^{+}	39	4	10%	0	0%
ran^{GDP}	16	8	50%	8	50%
ran^{GTP}	18	11	61%	4	22%
mars	11	9	82%	0	0

a - Each transgene was expressed by crossing to the *P{GAL4::VP16-nos.UTR}MVD1* driver.

For each transgene at least two independent insertions were scored, both of which gave similar results.

The form of Ran locked in the inactive GDP state had a different localization pattern than wild-type Ran in mature oocytes. Ran^{GDP} accumulated closely around the chromosomes (Figure 13B), rather than around the outside of the spindle as with wild-type Ran. This localization is similar to the localization of RCC1 (Figure 14B), consistent with the Ran^{GDP} protein binding to RCC1 but not being converted into the GTP form. Oocytes expressing *ran*^{GDP} did not appear to have a problem initiating the assembly of microtubules around the chromosomes or building a bipolar spindle. In addition, among the few progeny from *ran*^{GDP} expressing mothers, X-chromosome non-disjunction was not elevated. However, the *ran*^{GDP} expressing oocytes had an increased

frequency of abnormal spindle and karyosome organization (Table 5). The microtubules were often not tapered at the spindle poles (Figure 13B). Furthermore, the chromosomes were frequently disorganized and failed to condense into a single round or oval karyosome. These results suggest that the Ran pathway has a role in organizing the meiosis I spindle, but may not be essential for promoting chromosome based microtubule assembly.

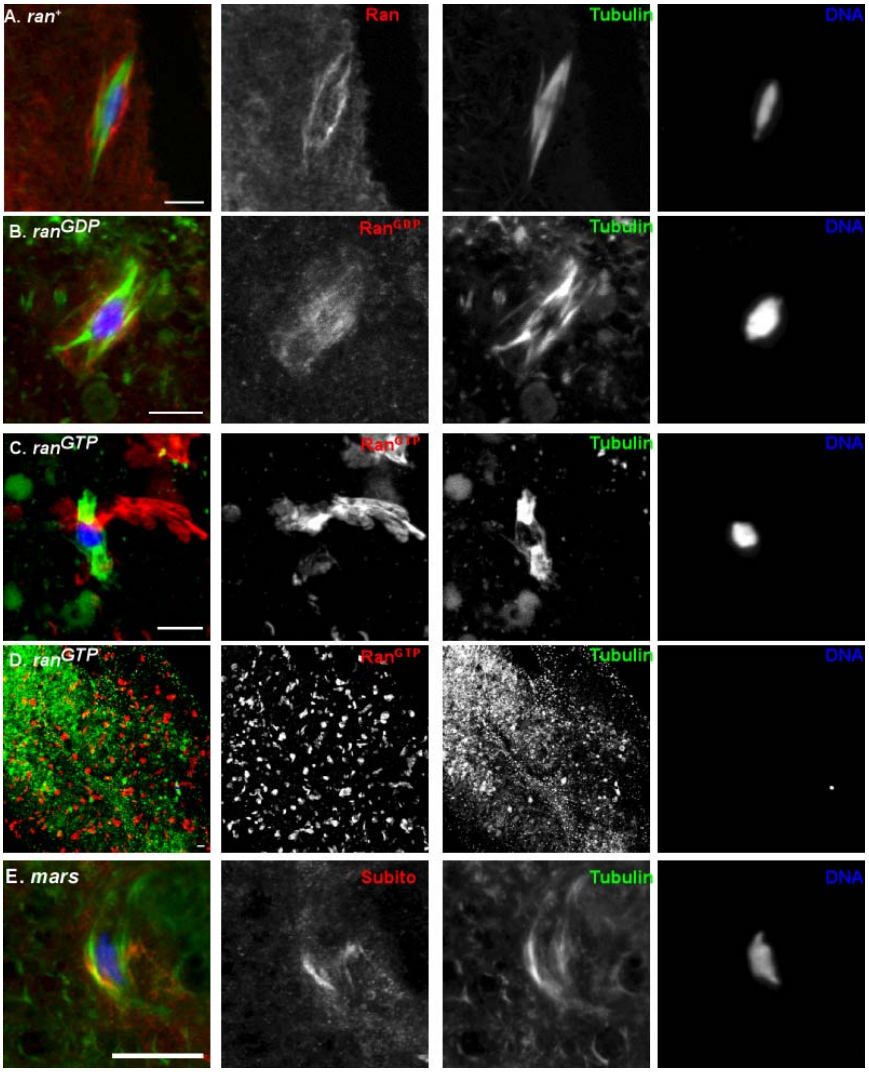

Figure 13 – Effect of Ran on spindle morphology in mature (stage 14) oocytes. The transgenes in these experiments and all subsequent figures were expressed using the *P{GAL4::VP-nos.UTR}MVD1* driver. DNA is in blue, Ran proteins (A-D) or Subito (E) are in red and Tubulin is in green. Ran was detected using an antibody to the HA tag fused to either wild-type *ran* (A), ran^{GDP} (B) or ran^{GTP} (C and D). The images in A – C represent high magnification images centered on the karyosome. The image in D is of the same oocyte as in C but lower magnification to show the localization of mutant Ran^{GTP} throughout the oocyte. In *mars* mutant oocytes (E), the microtubules often fail to be properly tapered at the spindle poles. The scale bars are 10 μm.

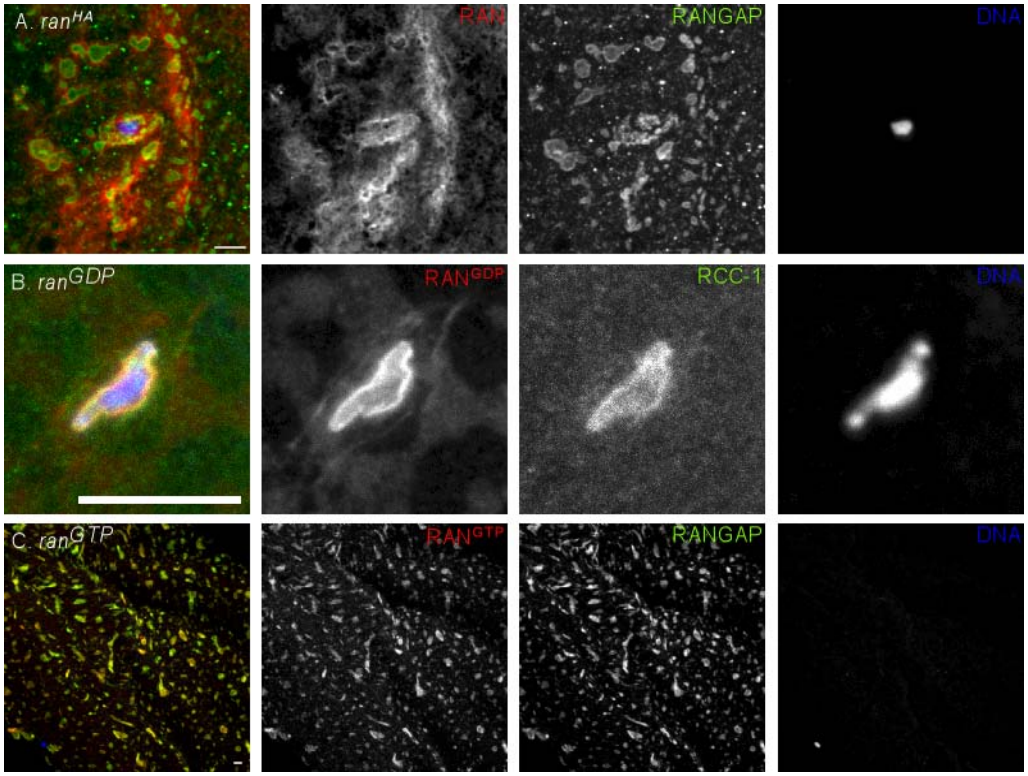

Figure 14 – Co-localization of Ran^{GDP} with RCC-1 and Ran^{GTP} with RanGAP. Ran was detected using an antibody to the HA tag fused to either wild-type *ran* (A), *ran*^{GDP} (B) or *ran*^{GTP} (C). Ran proteins are in red, RCC-1 or RanGAP are in green and DNA is in blue. The Ran protein staining was detected using an antibody to the HA tag. The scale bars are 10 μm.

Since RanGTP promotes spindle assembly through the release of spindle assembly factors, we tested if *ran*^{GDP} mutant oocytes showed evidence of down regulating proteins known to be activated by the Ran pathway. Mars is the *Drosophila* homolog of HURP, a spindle assembly factor regulated by RanGTP (Wilde 2006). Mars has been shown to have a role in the attachment of the centrosome to the mitotic spindle during *Drosophila* embryogenesis (Tan, Lyulcheva et al. 2008; Yang and Fan 2008; Zhang, Breuer et al. 2009). Like *ran*^{GDP} mutants, mature oocytes homozygous for a *mars* mutation were able to assemble a bipolar spindle but failed to properly taper the microtubules at the poles (Figure 13E). To test if these similarities were the result of *ran*^{GDP} mutant oocytes failing to activate Mars, we stained *ran*^{GDP} mutant oocytes with an

antibody against Mars (Tan, Lyulcheva et al. 2008). In wild-type or *ran*$^{+}$ expressing oocytes, Mars colocalized with tubulin except at the spindle poles and the central spindle (Figure 15A). Approximately 50% of oocytes expressing *ran*^{GDP} failed to localize Mars to the meiotic spindle (Figure 15B – 15D), which was significantly different compared to *ran*$^{+}$ oocytes ($z = 3.581$ at 99% confidence interval). Therefore, a Mars localization defect may explain the spindle tapering defect observed in *ran*^{GDP} mutant oocytes. These results are consistent with the conclusion that the RanGTP pathway is not essential for the initiation of acentrosomal spindle assembly in *Drosophila* oocytes, but may have a role in tapering the poles.

Another spindle assembly factor regulated by the RanGTP pathway is the microtubule associated factor Transforming Acidic Coiled Coil, or TACC. TACC localizes to the poles of the meiotic spindle (Cullen and Ohkura 2001) and mitotic spindle (Giet, McLean et al. 2002) where it contributes to the localization of Msps (Minispindles). To examine the localization of TACC during female meiosis, we expressed a GFP fusion gene under the control of a Ubiquitin promoter (Gergely, Kidd et al. 2000). As expected, TACC localized to the poles of most metaphase I spindles (Figure 15E, 15F). There was some variation in this pattern, with TACC tending to be less focused at the poles of shorter spindles. In contrast, TACC colocalized with most microtubules in all *ran*^{GDP} oocytes (Figure 15G, H). The failure to properly localize both Mars and TACC suggests expression of *ran*^{GDP} blocks the activation of some spindle assembly factors in oocytes, but this does not prevent spindle assembly.

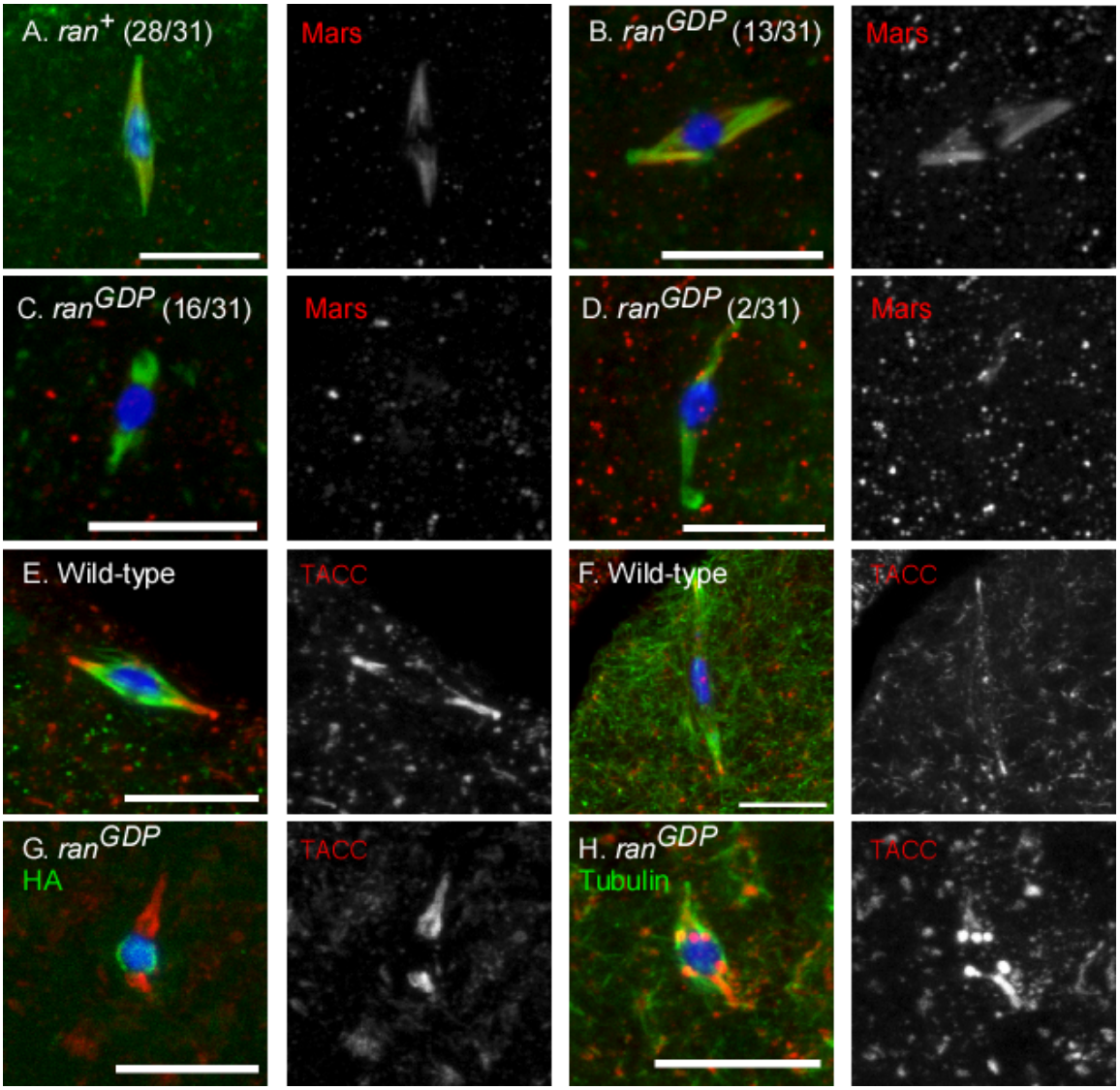

Figure 15 –Localization of Mars and TACC to the meiotic spindle is defective in *ran*^{GDP} mutant oocytes. Red is Mars or TACC-GFP staining, green is Tubulin except in G where it is HA and blue is DNA. In *ran*$^{+}$ oocytes, Mars localizes to most microtubules in the meiotic spindle, with the exception of the poles and the central spindle. The localization of Mars in *ran*^{GDP} mutant oocytes fell into three categories: present (B), completely absent (C) and reduced (D). There was no correlation between the amount of Mars staining and spindle morphology. E) In wild-type, TACC localizes at the poles. F) As the spindle gets longer, TACC staining becomes more concentrated at the poles. G,H) In *ran*^{GDP} oocytes TACC is not restricted to the poles. The scale bars are 10 μm.

Constitutively active RanGTP does not promote spindle assembly

High levels of RanGTP will induce chromatin-independent spindle assembly in *Xenopus* oocytes (Carazo-Salas, Guarguaglini et al. 1999). In contrast, the expression of *ran*^{GTP} did not result in the formation of ectopic spindles in the oocyte, as would be

expected if RanGTP is sufficient to initiate spindle assembly (Figure 13D). Instead, expression of *ran*^{GTP} oocytes caused abnormal spindle assembly reminiscent of *ran*^{GDP} oocytes; they failed to properly taper toward the poles and the karyosome was disorganized (Figure 13C) (Table 5). Despite the similar spindle phenotype, Ran^{GTP} had a localization pattern in mature oocytes that was strikingly different than wild-type or Ran^{GDP}. Ran^{GTP} protein was present in clusters throughout the oocyte (Figure 13C, 3D). Interestingly, Ran^{GTP} and RanGAP colocalized (Figure 14C), suggesting that Ran^{GTP} may be locked in an interaction with RanGAP. Expression of *ran*^{GTP} also changed the localization pattern of other proteins, such as nuclear Lamin (data not shown). These observations suggest that the expression of Ran^{GTP} in the oocyte affects how nuclear envelope proteins interact with the Ran pathway and this may cause a dominant negative phenotype.

Homolog orientation is normal in *ran* mutant oocytes

In both *ran* mutants, the chromosomes were frequently disorganized. To test whether the *ran* mutants affected the orientation of homologous chromosomes at metaphase I, FISH experiments were performed (data not shown). With probes for highly repeated sequences in the centromeric heterochromatin of the second or third chromosomes, biorientation of homologous chromosomes would be observed as the separation of two FISH signals towards opposite poles. As expected, oocytes expressing wild-type *ran* exhibited bioriented centromeres (second chromosome: 10/11 oocytes, third chromosome: 9/9 oocytes). Similarly, the centromeres were bioriented in oocytes expressing *ran*^{GDP} (second chromosome: 13/14 oocytes, third chromosome: 15/15 oocytes) or *ran*^{GTP} (second chromosome: 5/6 oocytes, third chromosome: 6/6 oocytes).

These results suggest that, while the karyosome is disorganized in *ran* mutants, this does not affect chromosome biorientation.

ran mutants genetically interact with _sub_ mutants

The analysis of *ran* mutants suggests that RanGTP is not required to initiate spindle assembly in the oocyte. It is possible that the role of RanGTP in oocyte spindle assembly is hidden by another mechanism that depends on the chromosomes. To address the issue of redundancy, we performed two additional experiments. First, we tested if RanGTP had a role in spindle assembly that did not involve direct interactions with the chromatin. This can be achieved in *Drosophila* oocytes using a mutation that removes the N-terminal domain of *subito* and causes ectopic spindles to form in multiple regions of the oocyte without direct contact with chromosomes (Jang, Rahman et al. 2007). We tested if RanGTP in the cytoplasm stimulates microtubule assembly by constructing a double mutant with the N-terminal deletion mutation, $sub^{\Delta NT}$, and either ran^{GDP} or ran^{GTP}. As observed previously, expression of the construct $P\{UASP:sub^{\Delta NT}\}$ resulted in the formation of ectopic spindles in the oocyte (Table 6, Figure 16). These ectopic spindles tend to be clustered within the oocyte. Typically, two to four clusters of ectopic spindles could be observed in $sub^{\Delta NT}$ mutant oocytes, such as at the posterior tip and a region adjacent to the karyosome (Figure 16A and 6B). Any oocyte containing more than one cluster of spindle formation was considered to have the ectopic spindle phenotype. The frequency of ectopic spindles in $sub^{\Delta NT}$ oocytes expressing ran^{+} was similar to $sub^{\Delta NT}$ alone (96.8% to 97.9% respectively) (Table 6). Strikingly, the dominant negative ran^{GDP} mutation completely suppressed the ectopic spindle phenotype (Figure 16C and 6D, Table 6). The only spindle that formed in ran^{GDP}; $sub^{\Delta NT}$ oocytes was around the karyosome. These results suggest that RanGTP is required for the interaction between

Subito$^{\Delta NT}$ and microtubules that occurs in the absence of the chromosomes. In other words, RanGTP may be required for microtubule assembly that does not depend on direct contacts with the chromosomes. Another surprising finding was that the suppression was reciprocal. The *ran*^{GDP}*; sub*$^{\Delta NT}$ double mutant had increased fertility relative to the two single mutants (Table 4) and this correlated with dramatic improvements in embryonic mitosis (Figure 16E).

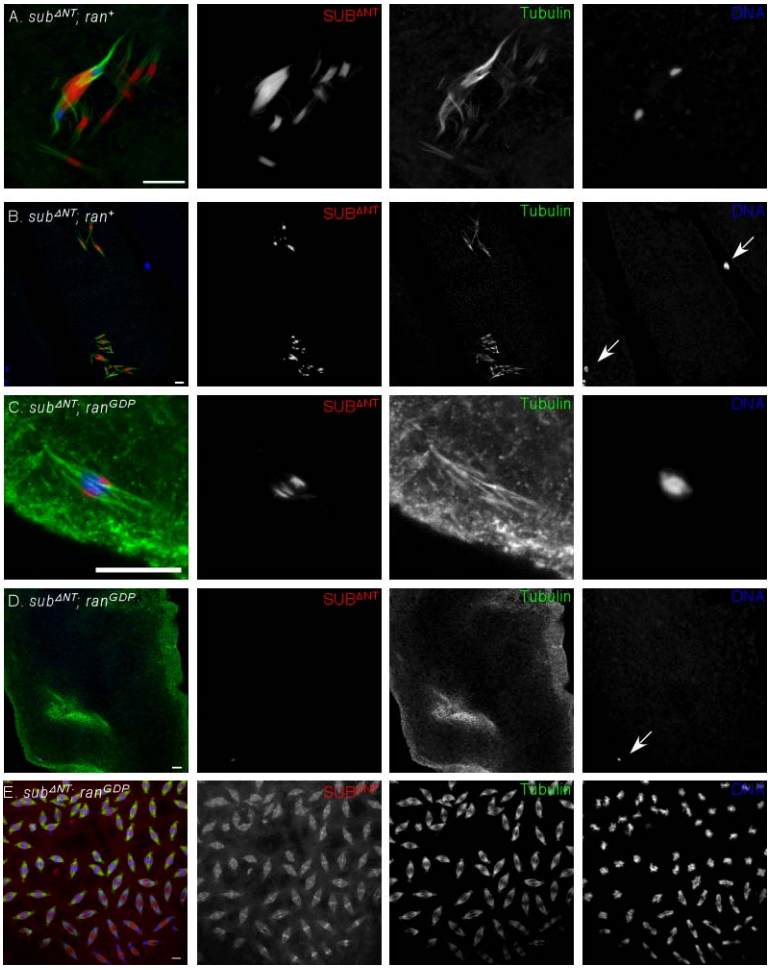

Figure 16 – Ran is required for ectopic spindle formation caused by *sub*$^{\Delta NT}$, a mutation which deletes the N-terminal non-motor domain of Subito. (A,B) Ectopic spindles form in *sub*$^{\Delta NT}$ oocytes expressing wild-type Ran. The yellow dashed circles indicate zones of ectopic spindles in the oocytes. (C,D) Expression of Ran^{GDP} in oocytes suppresses the ectopic spindle phenotype of *sub*$^{\Delta NT}$. B and D are low magnification images of the same oocytes shown in A and C, respectively. Arrows in B and D point to the chromosomes. There are multiple masses of DNA in the *sub*$^{\Delta NT}$ single mutant but only a single karyosome in the double mutant. E) *sub*$^{\Delta NT}$ suppresses the arrest in embryonic development caused by *ran*^{GDP}. In all the images, SUB$^{\Delta NT}$ is a GFP-fusion protein and shown in red, Tubulin is in green, DNA is in blue and the scale bars are 10 μm.

The *ran*^{GTP} mutation also reduced the frequency of ectopic spindle formation in *sub*$^{\Delta NT}$ oocytes (Table 6). If this mutation simply increased the concentration of cytoplasmic RanGTP, we would have predicted a larger number of ectopic spindles.

However, these results are consistent with the other experiments which suggest that *ran*^{GTP} has a negative effect on the Ran pathway and spindle assembly.

Table 6

Characterization of ectopic spindle phenotype in *sub*$^{\Delta NT}$ double mutants

Genotype	Oocytes containing Ectopic Spindles	Total Oocytes:	Ectopic Spindle (%):[a]
sub$^{\Delta NT}$	91	93	98%
sub$^{\Delta NT}$*; ran*$^{+}$	61	63	97%
sub$^{\Delta NT}$*; ran*^{GDP}	0	28	0%
sub$^{\Delta NT}$*; ran*^{GTP}	3	9	33%

Each genotype was expressed by crossing to *P{GAL4::VP16-nos.UTR}MVD1* driver.

[a] - Ectopic spindle (%) is equal to the number of occytes with ectopic spindles divided by the total number of oocytes

The second experiment to address multiple mechanisms for chromosome mediated spindle assembly was to test for redundancy with elements of the chromosome passenger complex (CPC). As discussed in the Introduction, a second pathway for chromosome-mediated spindle assembly requires the chromosome passenger complex (CPC). A test of this hypothesis would involve making a double mutant involving females lacking CPC activity and expressing Ran^{GDP}. This is not possible because mutants lacking CPC activity, such as *Incenp,* are lethal. An alternative is to examine a *sub* mutant that is expressing *ran*^{GDP}. Subito interacts with the CPC and is required for at least some of its spindle assembly functions (Colombie, Cullen et al. 2008). Unlike *sub*^{1} null or *ran*^{GDP} mutants, which produce mature oocytes, the *sub*^{1}; *ran*^{GDP} double mutant had severe defects in oogenesis. All 20 of the ovaries examined were completely devoid of mature (stage 14) oocytes. Only two ovaries were similar in size to wild-type but still

lacked mature oocytes. Another eight ovaries were small, while ten ovaries were missing any evidence of oocyte development. These results show that mutations in both the CPC and Ran pathways have a synergistic effect, suggesting there are two independent pathways that contribute to microtubule dynamics and spindle assembly during oogenesis.

VI. Discussion

The Ran pathway has a variety of targets which affect kinetochores, centrosomes and microtubule associated proteins (Kalab and Heald 2008). RanGTP is potentially an important molecule for spindle assembly in acentrosomal oocytes because it has been identified as a key factor for chromatin-induced spindle formation in *Xenopus* extracts (Carazo-Salas, Guarguaglini et al. 1999; Kalab, Pu et al. 1999; Ohba, Nakamura et al. 1999; Karsenti and Vernos 2001). Therefore, one might predict there is an important role for RanGTP in acentrosomal spindle assembly in *Drosophila* oocytes. Surprisingly, RanGTP may be more important for microtubule assembly in other circumstances, such as when centrosomes are present or when microtubules assemble without direct contact with the chromosomes.

Regulators of RanGTP, RCC1 and RanGAP, during meiosis in *Drosophila* females

Diffusion of RanGTP from its source, the chromatin, into the cytoplasm, where it is converted into RanGDP, can create a gradient which may be important for organizing the spindle (Caudron, Bunt et al. 2005; Kalab, Pralle et al. 2006). The presence of two key regulators in distinct locations of the *Drosophila* oocyte suggests a gradient of RanGTP could be affecting spindle assembly. RCC1, as expected, is localized tightly around the karyosome in mature oocytes. RanGAP localization is more surprising since it is present in many clusters within the oocyte. A candidate protein that may be responsible for generating the concentrations of RanGAP is Ran binding protein 2 (RanBP2), also known as Nup358. This protein is found within the nuclear envelope and binds to RanGAP (Hutten, Flotho et al. 2008). Following NEB, RanGAP in the *Drosophila* oocyte may be anchored to RanBP2-containing cytoplasmic vesicles.

Ran has an unusual localization pattern in oocytes; concentrating around the outside of the oocyte spindle. In contrast, Ran overlaps with the spindle in *Drosophila* mitotic cells (this paper and Trieselmann and Wilde 2002; Silverman-Gavrila and Wilde 2006). We have not determined if these concentrations of Ran are in the GDP or GTP state. However, we can speculate based on the localization patterns of wild-type and mutant proteins. Based on this type of evidence, Trieselmann et al (2002) suggested that the bulk of Ran on the embryonic spindle is in the GTP state. Similarly, the absence of mutant Ran^{GDP} around the outside of the spindle, suggests that the bulk of the Ran localized around the outside of the spindle is in the GTP form. The pattern of mutant Ran^{GTP} staining suggests it enters RanGAP containing vesicles but does not leave because it is not hydrolyzed. Thus, the wild-type Ran that localizes adjacent to the clusters of RanGAP may be the GDP form of the protein that has left RanGAP-containing vesicles.

RanGTP has a nonessential role in acentrosomal spindle assembly

RCC1 and RanGTP are required for chromatin-induced spindle assembly in *Xenopus* extracts (Carazo-Salas, Guarguaglini et al. 1999; Kalab, Pu et al. 1999). In these same extracts, expression of RanT24N blocks spindle assembly (Ohba, Nakamura et al. 1999) and high concentrations of RCC1 or expression of a GTP locked form of Ran leads to spindle formation in the absence of chromosomes and centrosomes (Carazo-Salas, Guarguaglini et al. 1999). Our analysis of RanGTP function in *Drosophila* oocytes is based on these previous studies and assumes that expression of the ran^{GDP} variant RanT24N effectively reduces the concentration of RanGTP. We believe that the ran^{GDP} mutant did have the desired effect of reducing RanGTP production for three reasons. First, Ran^{GDP} localized tightly to the meiotic chromosomes, consistent with the

expectation that this form of Ran remains bound to RCC1 because it has a low rate of GTP exchange. The high affinity of Ran^{GDP} for RCC1 causes a block in the production of RanGTP (Dasso, Seki et al. 1994). Second, the spindle organization defects observed in ran^{GDP} oocytes were similar to defects seen in *mars* mutant oocytes, a protein known to be regulated by the Ran pathway. Third, ran^{GDP} caused dramatic disruptions in chromosome-independent microtubule assembly assays (see below).

Expression of the GDP-locked variant of Ran had relatively mild effects on *Drosophila* oocyte spindle assembly and karyosome organization. Meiosis I spindles were bipolar in ran^{GDP} oocytes. Indeed, reducing the RanGTP concentration in the oocyte was not sufficient to severely effect either meiotic division, since meiosis II spindles (data not shown) and female meiotic products could be seen in the embryos. The most severe defect was that they often had non-tapered poles, possibly due to the abnormal localization of proteins necessary for pole formation such as TACC or Msps (Cullen and Ohkura 2001). Abnormal spindle morphology could be the reason for the disorganized karyosome phenotype. Loss of RanGTP could result in a failure to activate Aurora A, which phosphorylates and activates Tacc (Barros, Kinoshita et al. 2005; Kalab and Heald 2008). In embryos, TACC localization to the centrosomes depends on phosphorylation by Aurora A (Barros, Kinoshita et al. 2005). TACC initially binds all microtubules, but as the spindle matures, TACC is phosphorylated and localizes to the poles. Expression of ran^{GDP} may cause a reduction in Aurora A activity, resulting in the failure to phosphorylate TACC and localize it to the poles. Thus, RanGTP may have a specific role in organizing spindle poles but may not be required for chromosome-promoted spindle assembly.

Expression of the GTP locked mutant, *ran*^{GTP}, gave surprising results because the oocytes showed loss of function spindle phenotypes similar to *ran*^{GDP} mutant oocytes, rather than inducing an uncoupling between spindle assembly and the chromosomes (Carazo-Salas, Guarguaglini et al. 1999). Thus, RanGTP may not be sufficient for initiating spindle assembly in *Drosophila* oocytes. Instead, both the oocyte and embryonic phenotypes of *ran*^{GTP} have links to defects in the organization of membranes or vesicles. For example, expression of the *ran*^{GTP} mutation caused Lamin, RanGAP and RanGTP to colocalize in globular structures located throughout the oocyte. It is unclear what the link is between membranous structures and spindle assembly, although Kramer and Hawley (2003) have proposed that the transmembrane protein Axs is a component of a membranous structure surrounding the meiotic spindle. We suggest that the *ran*^{GTP} mutation causes defects in membranous structures that have a role in spindle organization. Finding that manipulating RanGTP levels with Ran^{GTP} or Ran^{GDP} has similar phenotypes has been observed in other systems. For example, the expression of either form of Ran in mouse oocytes (T24N and Q69L) resulted in similar meiosis II spindle phenotypes (Dumont, Petri et al. 2007). These results suggest that the effects of manipulating RanGTP levels in an intact oocyte are not easily predicted. Other factors such as protein localization may play important roles in regulating the Ran pathway.

Similar to the oocytes, the phenotype of the *ran*^{GTP} mutant embryos may be associated with defects in membrane structure. In these mutants, only a cluster of DNA and microtubules could be observed in the center of the embryo. A strikingly similar phenotype has been observed in dominant negative mutants of *Ketel,* the *Drosophila* homolog of Importin-β (Tirian, Puro et al. 2000; Timinszky, Tirian et al. 2002). In the

Ketel dominant mutants, meiosis I and II occur and the female and male pronuclei come together, but they interact abnormally due to defects in the nuclear envelopes. Subsequently, the first mitotic division fails and the chromosomes disintegrate within a large aggregate of microtubules. Similar to the *Ketel* mutant, ran^{GTP} may cause abnormal interactions among nuclear envelope proteins in the embryo, causing a failure in the first mitotic division.

RanGTP is required for chromosome independent microtubule assembly

Unlike assembly of the meiosis I spindle, expression of ran^{GDP} blocked two other types of microtubule assembly. First, ran^{GDP} mutants had greatly reduced fertility because the embryo mitotic divisions were blocked. In some cases it was clear that this was because expression of Ran^{GDP} prevented fusion of the female and male pronuclei. Several genes with roles in microtubule assembly are also required for pronuclear fusion, including *subito* (Giunta, Jang et al. 2002). This process depends on the assembly of a microtubule array that is nucleated by the centrosome donated by the sperm and acts to draw the female pronucleus towards the male pronucleus. Second, ran^{GDP} suppressed the formation of the ectopic spindles that form in a neomorphic *subito* mutant ($sub^{\Delta NT}$) (Jang, Rahman et al. 2007). The formation of these spindles occurs after nuclear envelope breakdown, consistent with a dependence on release of RanGTP from the nucleus. Both of these examples involve assembly of microtubules without direct interaction with the chromosomes. This finding suggests that the assembly and bundling of microtubules in the oocyte cytoplasm, either as a result of the $sub^{\Delta NT}$ mutant or during the process of pronuclear fusion, depends on RanGTP.

The most surprising result was the mutual suppression shown by the $sub^{\Delta NT}$ and ran^{GDP} mutations. While both mutants decrease fertility, the double mutant is fertile. The simplest explanation is that the loss of spindle assembly factors in ran^{GDP} embryos is balanced by the enhanced spindle assembly activity present in the $sub^{\Delta NT}$ mutant. Expression of ran^{GDP} may suppress the sterility phenotype of $sub^{\Delta NT}$ by abolishing the presence of ectopic spindles, while $sub^{\Delta NT}$ may suppress the reduced fertility phenotype of ran^{GDP} by overcoming the defects in microtubule assembly needed for processes like pro-nuclear fusion. As described in the next section, Ran and Subito may function in two separate microtubule assembly pathways, but their activities may converge on some of the same spindle assembly factors.

Evidence for two spindle assembly pathways in *Drosophila* and vertebrate oocytes

The results of this study suggest that RanGTP is not required, nor sufficient, for acentrosomal spindle assembly in *Drosophila* oocytes. A similar conclusion was drawn from expressing dominant negative form of Ran in mouse oocytes or when RCC1 was depleted from *Xenopus* oocytes (Dumont, Petri et al. 2007). By analyzing mutants similar to the ones we used here, only mild defects in meiosis I spindle assembly were found, such as a delay establishing bipolarity. Dumont et al (2007) reported that the *Xenopus* and mouse meiosis II spindles were more sensitive than meiosis I spindles to disruptions in RanGTP levels. While we did not observe gross defects in meiosis II spindle assembly, we cannot rule out the possibility that an undetected meiosis II defect in *Drosophila* oocytes expressing Ran^{GDP} leads to an irregular number of meiotic products.

The failure to observe evidence supporting a role for RanGTP in acentrosomal spindle assembly may be explained by a predominant chromatin-dependent pathway in oocytes involving the Chromosome Passenger Complex (CPC). The CPC has been proposed to activate chromosome-dependent spindle assembly in *Xenopus* egg extracts (Sampath, Ohi et al. 2004; Maresca, Groen et al. 2009) and *Drosophila* oocytes (Colombie, Cullen et al. 2008). Ironically, our results suggest that, as compared to chromosome-mediated spindle assembly, RanGTP has a greater role in microtubule organization when centrosomes are present or when chromosomes are absent. The ability of the CPC to stimulate spindle assembly depends on direct interactions with the chromosomes and microtubules (Tseng, Tan et al. 2010). In contrast to RanGTP, which is diffusible, the CPC may be more proficient at ensuring that spindles assemble around the chromosomes. RanGTP is critical for pronuclear fusion, where the CPC would not be expected to promote spindle assembly because the microtubules depend on the paternal centrosome.

Chapter 3: The N-terminus of the kinesin-6 family member Subito is a complex domain of positive and negative regulators

I. Abstract:

In many sexually reproducing organisms, bipolar spindles assemble in the absence of centrosomes in the oocytes. Through a poorly understood mechanism in these organisms, the chromosomes have been proposed to initiate spindle assembly by nucleating or capturing microtubules. Subito is a member of the kinesin-6 family that is required for bundling interpolar microtubules located within the central spindle at metaphase I. Through a deletion analysis of Subito, it has been determined that the N-terminus is essential to the negative regulation of Subito in oocytes. By continuing the deletion analysis, we have shown that the N-terminus of Subito is not a simple negative regulator, but has properties of both positive and negative regulators. This kind of complexity has not previously been observed within the N-terminus of kinesins. We propose a model of auto-inhibition for Subito, where the N- and C-terminus interact to prevent interactions with microtubules.

II. Introduction:

The proper separation of homologous chromosomes during female meiosis is dependent upon formation of microtubules into a bipolar spindle around the karyosome. The typical mitotic division contains centrosomes, microtubule organizing centers that direct the formation of the spindle into a bipolar structure. Microtubules emanating from the centrosomes are capable of attaching to the kinetochores or connecting with microtubules emanating from the opposite pole. The kinetochore microtubules mediate

the separation of the homologs to the opposite poles, once proper orientation and connection has been achieved, resulting in two daughter cells.

Unlike most animal mitotic cells, meiosis in many oocytes is acentrosomal. Spindle assembly occurs without the guidance of the centrosomes at the poles. In this situation, the chromosomes play an important role in spindle assembly. Kinesins are a large family of motor proteins that promote unidirectional movement of a cargo along microtubules. Several *Drosophila* kinesins have been shown to play important roles in spindle assembly (GOSHIMA and VALE 2003). For example, the kinesin-4 family members, known as the chromo-kinesins, are able to bind microtubules while attached to chromosomes as their cargo (MAZUMDAR and MISTELI 2005). Three kinesin families can bundle and slide parallel or anti-parallel microtubules. The first is the kinesin-14 family, which includes minus-end directed motors such as NCD in *Drosophila*. NCD and the minus-end directed motor Dynein have been proposed to bundle and taper microtubules to establish mitotic (WALCZAK *et al.* 1998; GOSHIMA *et al.* 2005) and meiotic (MATTHIES *et al.* 1996; ENDOW and KOMMA 1997; SKOLD *et al.* 2005) spindle poles in the absence of centrosomes. The second is the kinesin-5 family, including Klp61F in *Drosophila*, which are plus-end directed motors that function to maintain bipolar spindle assembly and elongation at anaphase. The activity of these proteins may antagonize the forces of the kinesin-14 family during spindle assembly (KWON and SCHOLEY 2004; TAO *et al.* 2006). The third is the kinesin-6 family that includes Subito and Pavarotti in *Drosophila*. As shown for human MKLP1, kinesin-6 proteins are thought to be plus-end directed motors that slide anti-parallel microtubules (NISLOW *et al.* 1992). Examination of these proteins in human cells (NEEF *et al.* 2003),

Caenorhabditis elegans (RAICH *et al.* 1998), and *Drosophila* (ADAMS *et al.* 1998; CESARIO *et al.* 2006) has shown that they are usually associated with interpolar microtubules at the central spindle and are important for cytokinesis. During anaphase, the interpolar microtubules overlap in anti-parallel arrays in the spindle midzone, an area that typically accumulates proteins important for cytokinesis (D'AVINO *et al.* 2005). However, the kinesin-6 protein Subito has been shown to have a role in spindle assembly in *Drosophila*. *Subito* encodes the *Drosophila* homolog of MKLP2 and has an important role in organizing the acentrosomal (JANG *et al.* 2005) and centrosomal spindles (CESARIO *et al.* 2006). The *Drosophila* meiotic spindle develops a prominent bundle of interpolar microtubules during pro-metaphase, referred to as the metaphase I central spindle, which is a critical part of the acentrosomal spindle assembly pathway (JANG *et al.* 2005). In *subito* null mutant oocytes, the central spindle is absent (JANG *et al.* 2005) and there are an abnormal number of spindle poles and high levels of meiotic non-disjunction (GIUNTA *et al.* 2002). We have previously demonstrated that the N-terminus of Subito negatively regulates the motor activity of the kinesin. Expression of a construct of *subito* lacking the N-terminus, $sub^{\Delta NT}$, within the oocyte resulted in ectopic spindles that were not dependent upon chromosomes for initiation of their assembly (Jang, Rahman et al. 2007). This result proved that the N-terminus is necessary to restrict spindle assembly to the karyosome. We sought to resolve the region of the N-terminus essential for the negative regulation of the kinesin and restriction to the karyosome.

Recent research using kinesin-1 has shown that the structure of the kinesin plays a role in negatively regulating its motor activity (Cai, Hoppe et al. 2007). When the tail domain physically interacts with the motor, the kinesin is unable to bind microtubules,

rendering the motor inactive. When the tail is freed from the motor, the kinesin binds microtubules, and travels towards the plus end. Given the similarities in negative regulation, we speculated that the N-terminus of Subito may be functioning to restrict motor activity in a comparable manner.

Our findings prove that the N-terminus of Subito does not function as a simple negative regulator. On the contrary, the N-terminus is a complex region of both negative and positive regulators. This complexity is illustrated by two serines that, via dephosphorylation, act as a switch to activate the binding and bundling of microtubules by the kinesin. Analysis of the conformation of Subito proves that the N and C-termini do not interact when actively binding microtubules in the oocyte. Interestingly, the N- and C-terminus of Subito were tightly associated in *Drosophila* embryos. Since mitotic divisions occur in quick succession during embryogenesis, we propose that only a fraction of Subito is fully activated. Taken together, these results offer a model of auto-inhibition for Subito during acentrosomal spindle assembly.

III. Materials and Methods:

Generation and initial analysis of transgenic lines:

A full-length derivative of *subito* was amplified by PCR. The clone was verified by sequencing and then cloned into pENTR2B vector (Gateway). The fragment was then recombined using Clonase (Invitrogen) into the pPHW vector which encodes three copies of the HA epitope at the N-terminus of the coding region in a pUASP backbone (Rorth 1998). The $sub^{\Delta(1-21)}$ construct was created by cutting the wild-type *subito* pENTR2B construct with BamHI and EcoRI. The resulting 1600 bp fragment was re-cloned back

into pENTR2B. This pENTR2B clone and wild-type *Subito* pENTR2B were both cut with EcoRI resulting in a 3712 bp fragment and a 796 bp fragment respectively. After CIP treatment, these fragments were ligated to each other, resulting in a *subito* clone missing the first 21 amino acid, but maintaining the same open reading frame. The remaining deletion and amino acid substitutions were created using the Change IT mutagenesis kit (USB) and the appropriate primers on the wild-type *subito* clone in pENTR2B.

To measure fertility and chromosome segregation during meiosis, females were crossed to *y w/B^{s}Y* males. The non-disjunction frequency was calculated as 2(B^{S} ♀+ B^{+} ♂) / [B^{+} ♀+ B^{S} ♂+ 2(B^{S} ♀+ B^{+} ♂)]. Ovary protein levels were assayed by Western blot. Whole ovaries were dissected from yeasted females in PBS and then ground and boiled in SDS gel loading buffer. Protein from ~2 to 3 ovaries was loaded per lane. The primary antibody was rat-anti HA "high affinity" (Roche, clone 3F10) used at 1:5000; the secondary HRP-conjugated antibodies (Jackson Labs) were used at 1:5000. The secondary was detected using ECL reagents (Amersham, Piscataway, NJ).

Antibodies and immunofluorescent microscopy

Stage 14 oocytes were collected from 50 to 200 3 to 4 day old yeast fed females by physical disruption in a common household blender (Theurkauf and Hawley 1992; McKim, Joyce et al. 2009). The oocytes were fixed in modified Robb's media and cacodylate/formaldehyde fixative for 8 min and then their outer membranes were removed by rolling the oocytes between the frosted part of a slide and a coverslip. The mass isolation procedure resulted in enrichment for mature stage 14 oocytes.

Embryos were collected in cages with grape juice plates. Two hour collections are suitable for an enrichment of stage four embryos. After washing with water, the embryos were dechorionized using 50% bleach for 90 seconds. They were then thoroughly washed with water to remove all traces of bleach. The embryos were then fixed using a heptane / methanol fixation (Rothwell and Sullivan 2000).

Oocytes and embryos were stained for DNA with Hoescht and for microtubules with mouse anti-α tubulin monoclonal antibody DM1A (1:50), directly conjugated to FITC (Sigma) or rat anti-α tubulin monoclonal antibody (1:75) (Millipore). The primary antibodies were rat anti-SUB antibody (used at 1:75) (Jang, Rahman et al. 2005), rat anti-HA (Roche, clone 3F10) (1:25), and rat anti-INCENP (1:500) (Wu, Singaram et al. 2008). These primary antibodies were combined with either a Cy3 or Cy5 secondary antibody preabsorbed against a range of mammalian serum proteins (Jackson Labs). Images and FRET experiments were collected on a Leica TCS SP2 confocal microscope with a 63x, NA 1.3 lens. Images are shown as maximum projections of image stacks followed by merging of individual channels and cropping in Adobe Photoshop.

IV. Results:

Generation of N-terminus *subito* mutants

To characterize the regulatory region in the N-terminus of Subito, a series of deletion and substitution mutations were created. These transgenes were made by fusing the coding region of the wild-type *Subito* or mutant variants to three copies of the HA epitope tag at the N-terminus. They were also put under the control of the UASP promoter, which allows for germline expression regulated by a second transgene expressing GAL4 (Rorth 1998). For all the experiments described below, the *UASP:sub* transgenes were expressed using the *P{GAL4::VP16-nos.UTR}MVD1* driver, which has *GAL4* fused to the *nanos* promoter and induces the expression of *UAS* containing transgenes in the female germline. Two large deletions were created which split the N-terminus in half and together span the entire N-terminus ($sub^{\Delta(1-41)}$ *and* $sub^{\Delta(42-76)}$) (Figure 17). A series of smaller deletions were also created, eliminating coding regions within the N-terminus that are highly conserved in other *Drosophila* species ($sub^{\Delta(1-21)}$ and $sub^{\Delta(24-33)}$). Finally, substitutions transgenes were engineered that substituted conserved serines with alanines at amino acid position 16 and 24 (sub^{S16A}, sub^{S24A}, *and* $sub^{S16AS24A}$). Both serines have been shown to be phosphorylated in *Drosophila* Kc167 cell line, (Bodenmiller, Malmstrom et al. 2007). For each transgene, at least two insertion lines were examined in case expression levels varied from different insertion sites. In all cases, differences in expression levels as assayed by Western blot were minimal and not the explanation for mutant phenotypes.

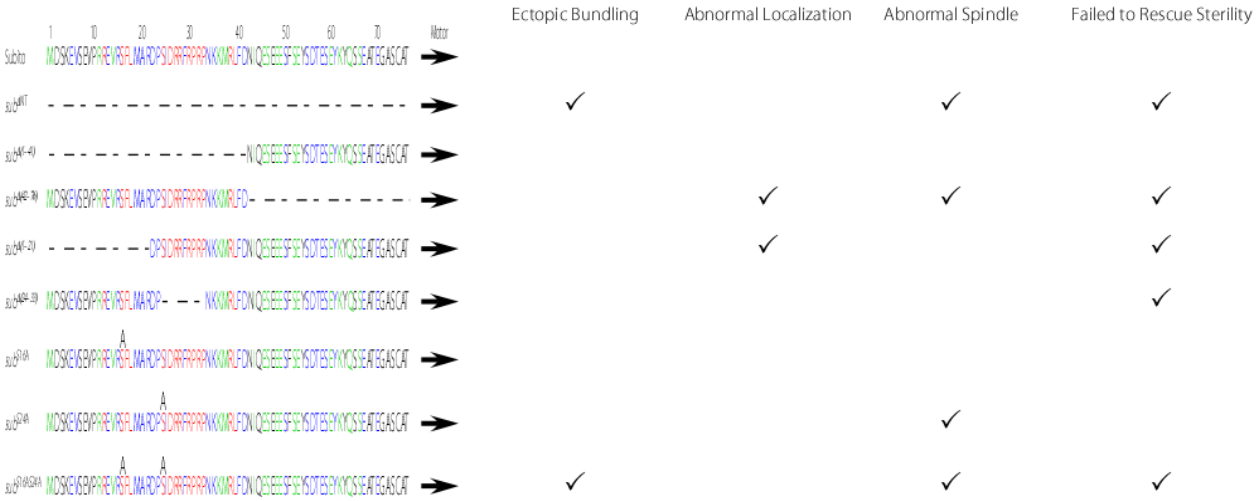

Figure 17 – A graphical representation of the deletion and substitution constructs created within the N-terminus of *subito*. To the right is a table outlining the major genetic and cytological defects observed during the analysis of *subito*; including presence of ectopic bundling, abnormal localization of the mutant protein, abnormal spindle assembly, and failure to rescue the sterility of the *sub* null background.

Deletion of the N-terminus with an HA tag results a less severe chromosome independent spindle assembly phenotype compared to an N-terminus deletion with a GFP tag

The deletion of the entire N-terminus of *subito* resulted in ectopic spindles, when tagged with GFP, SUB$^{\Delta NT}$::GFP (Jang, Rahman et al. 2007). This phenotype was not observed in wild-type *Subito::GFP* oocytes, confirming that the N-terminus negative regulates the motor activity of the kinesin. However, this phenotype was less striking when the mutant protein was HA tagged, SUB$^{\Delta NT}$::HA. The mutant protein was still capable of binding and bundling microtubules in a chromosome independent manner; however, the bundles of microtubules failed to form spindles (Figure 18A). Despite both constructs being expressed with the same driver, *sub$^{\Delta NT}$::GFP* oocytes had a higher level of protein expression compared to *sub$^{\Delta NT}$::HA* oocytes (data not shown). This difference

could be the direct result of the GFP tag providing stability to the protein. If true, the concentration of SUB$^{\Delta NT}$::GFP would increase, which would allow for increased interactions with microtubules. With SUB$^{\Delta NT}$::GFP bound, the resulting microtubule bundles would also become stabilized.

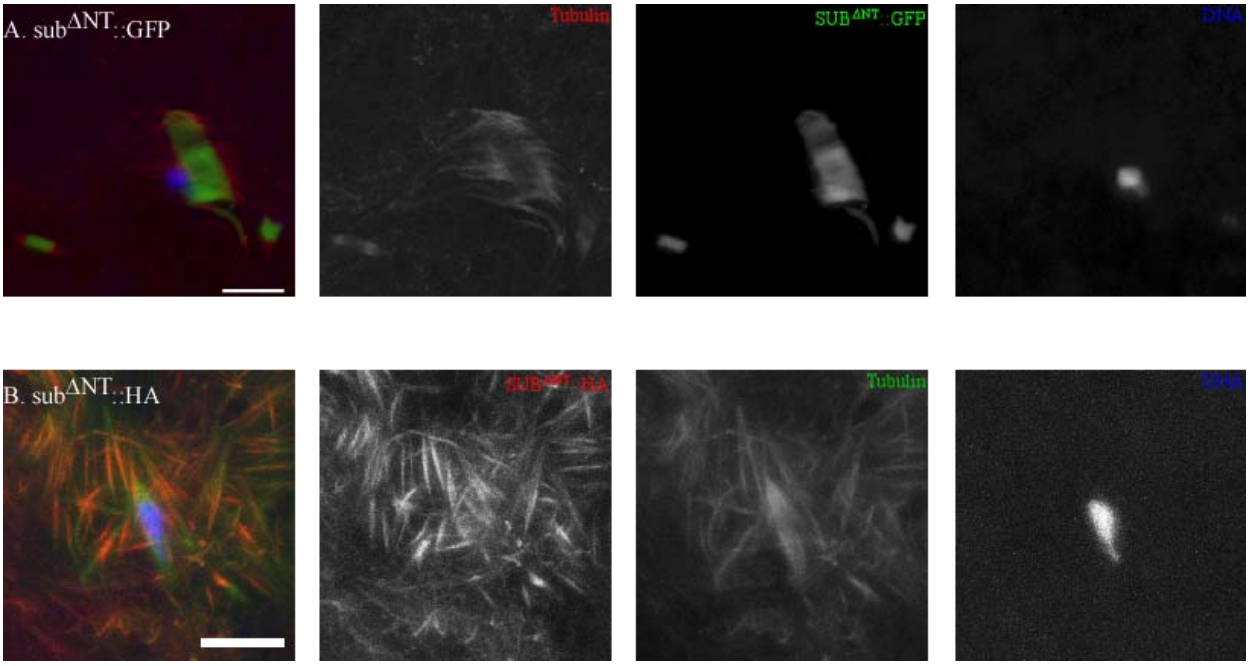

Figure 18 – The chromosome-independent phenotype of the N-terminus deletion mutation of *subito* is more severe when tagged with GFP. While both the HA tagged, *sub$^{\Delta NT}$::HA*, and GFP tagged, *sub$^{\Delta NT}$::GFP*, constructs of the N-terminus deletion of *subito* result in chromosome independent microtubule bundling phenotypes in stage 14 oocytes, *sub$^{\Delta NT}$::HA* mutant oocytes fail to replicate the ectopic spindles seen in *sub$^{\Delta NT}$*::GFP mutant oocytes. (A) Expression of *sub$^{\Delta NT}$::GFP* with a nanos:GAL4 driver results in ectopic spindles. SUB$^{\Delta NT}$::GFP is in red, Tubulin is in green, and DNA is in blue. (B) Expression of *sub$^{\Delta NT}$::HA* with a nanos:GAL4 driver fails to replicate the ectopic spindle phenotype observed in *sub$^{\Delta NT}$::GFP* mutant oocytes. However, chromosome independent bundling of microtubules is observed within these oocytes. SUB$^{\Delta NT}$::HA is in red, Tubulin is in green, and DNA is in blue. The scale bar is 10 um.

As microtubule stabilization continued, INCENP, a member of the chromosomal passenger complex, would sense the stabilized microtubules and initiate spindle assembly through another member of the chromosomal passenger complex, Ial, known as Aurora

B. In *Xenopus* extract, concentration dependent spindle assembly has been demonstrated by clustering INCENP or stabilizing microtubules (Tseng, Tan et al. 2010). We propose that $SUB^{\Delta NT}$::GFP is initiating spindle assembly in a similar manner. By binding and bundling microtubules, the concentration of stabilized microtubules increases, allowing the activation of INCENP. Concentration dependant spindle assembly does not occur with the HA tagged mutant protein, presumably due to a decreased concentration of stabilized microtubules. So while the initial observation that the N-terminus negatively regulates the binding and bundling activity of Subito remains accurate, it is likely that the assembly of chromosome independent spindles is dependent upon the concentration of stabilized microtubules.

Expression of *subito* N-terminus mutants have no dominant effects on fertility or chromosomal segregation

In order to determine if the *subito* mutants disrupted meiotic chromosome segregation, genetic assays were performed by measuring the frequency of X chromosome non-disjunction and fertility. Surprisingly, none of the wild-type or mutant transgenes tested had dominant effects on non-disjunction or fertility (Table 7). *subito* null mutants are sterile, due to a defect during pro-nuclear fusion. However, expression of a wild-type transgene of *Subito* in the female germline is capable of rescuing the sterility defect and producing progeny with low levels of non-disjunction (Table 8). We used this genetic assay to analyze the N-terminus mutant transgenes. Five of the transgenes tested, $sub^{\Delta NT}$, $sub^{\Delta (42\text{-}76)}$, $sub^{\Delta (1\text{-}21)}$, $sub^{\Delta (24\text{-}33)}$, and $sub^{S16AS24}$, failed to rescue the sterility of a *subito* null background (Table 8). A failure to rescue sterility shows that the mutant protein is incapable of performing the wild-type function of the protein. Since the N-terminus mutation tested in this analysis did not affect the motor or C-terminus, it

is likely that these constructs failed to rescue the *sub* null mutant due to a change in the regulation of the kinesin.

Table 7

Fertility & non-disjunction phenotypes by N-terminus mutant transgenes

Transgene:	Total Flies:	Progeny / Female Parent:	Non-Disjunction:
Subito	1514	75.7	0.00%
$Subito^{HA\text{-}Myc}$	3146	41.4	0.00%
$sub^{\Delta NT}$	869	43.5	0.23%
$sub^{\Delta(1\text{-}41)}$			
$sub^{\Delta(42\text{-}76)}$	4251	36.6	0.52%
$sub^{\Delta(1\text{-}21)}$	1889	47.2	0.00%
$sub^{\Delta(24\text{-}33)}$	2581	53.8	0.08%
sub^{S16A}	2946	54.6	0.27%
sub^{S24A}	2189	54.7	0.18%
$sub^{S16AS24A}$	1219	38.1	0.00%

Each transgene was expressed by crossing to the *P{GAL4::VP16-nos.UTR}MVD1* driver. These females were crossed to *y w/* $B^{S}Y$ males to assay non-disjunction and fertility.

Each transgene consists of at least two insertions, both of which gave similar results.

Table 8

Rescue of *sub* null sterility by N-terminus mutant transgenes

Transgene:	Total Flies:	Progeny / Female Parent:	Non-Disjunction:
Subito	850	42.5	0.24%
$Subito^{HA\text{-}Myc}$	1524	38.1	0.00%
$sub^{\Delta NT}$	0	0.0	**Sterile**
$sub^{\Delta(1\text{-}41)}$			
$sub^{\Delta(42\text{-}76)}$	12	0.6	28.57%
$sub^{\Delta(1\text{-}21)}$	0	0.0	**Sterile**
$sub^{\Delta(24\text{-}33)}$	0	0.0	**Sterile**
sub^{S16A}	264	8.8	1.50%
sub^{S24A}	400	10.0	0.00%
$sub^{S16AS24A}$	0	0.0	**Sterile**

Each transgene was expressed by crossing to the *P{GAL4::VP16-nos.UTR}MVD1* driver in a *sub* null background. These females were crossed to *y w/* $B^{S}Y$ males to assay non-disjunction and fertility.

Each transgene consists of at least two insertions, both of which gave similar results.

The second half of the N-terminus retains Subito to the central spindle in the oocyte

An initial analysis of the N-terminus of Subito was done by splitting the N-terminus in halves, in an attempt to replicate the ectopic bundling phenotype seen in $sub^{\Delta NT}$ oocytes. Amino acids one to forty-one are conserved among *Drosophila* species. However, the second half the N-terminus, amino acids forty-two to seventy-six, is mostly non-conserved (Figure 17). To determine if the negative regulatory role of the N-terminus was located within one of these halves, $sub^{\Delta (1\text{-}41)}$ and $sub^{\Delta (42\text{-}76)}$ were created, keeping motor domain and C-terminus fully intact. Upon expression within the germline using Gal4, $SUB^{\Delta (1\text{-}41)}$ failed to form ectopic spindles within the oocyte, suggesting that it had no role in negatively regulating Subito. Additionally, $SUB^{\Delta (1\text{-}41)}$ associated with the central spindle normally (Figure 19B), with no dominant effects on spindle (Figure 20) or karyosome organization. Despite the high conservation of the region, these results prove that the deletion of the first half of the N-terminus did not have any cytological observable effect on the regulation of the kinesin.

An interesting discovery was found when examining the second half deletion. While ectopic spindles were not observed, the spindle associated with the karyosome was grossly abnormal with almost a complete penetrance (Figure 19C and Figure 20). Microtubules formed around the karyosome, but the spindle was short and failed to extend towards the poles. The spindles were quite reminiscent of pro-metaphase spindles observed in wild-type oocytes. These immature spindles are identified shortly after

nuclear envelope breakdown when microtubules just begin to form and bundle around the karyosome (Theurkauf and Hawley 1992; Matthies, McDonald et al. 1996).

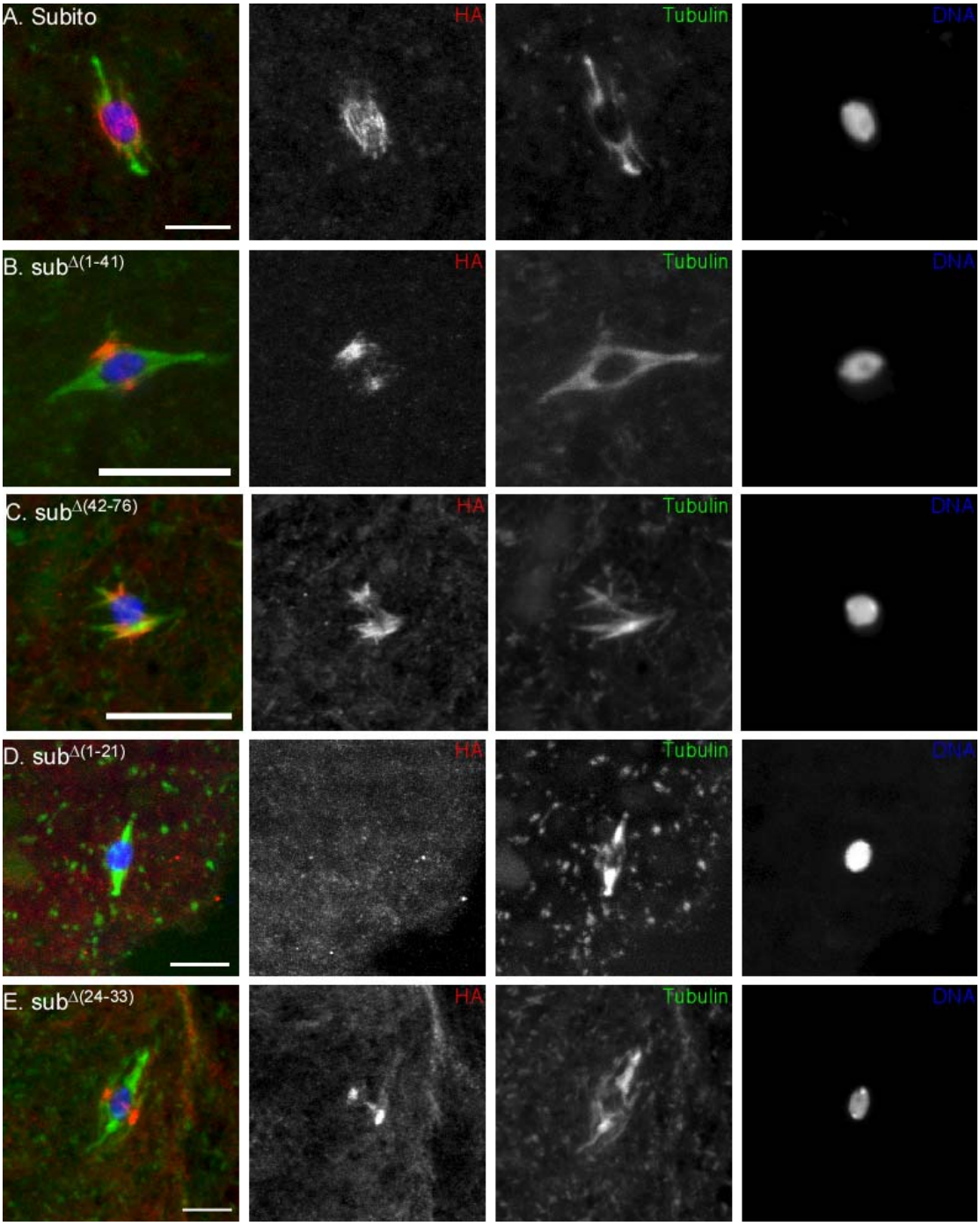

Figure 19 – N-terminus deletion constructs expressed in stage 14 oocytes including, (A) wild-type Subito, (B) sub$^{\Delta(1-41)}$, (C) sub$^{\Delta(42-76)}$, (D) sub$^{\Delta(1-21)}$, and (E) sub$^{\Delta(24-33)}$. subito tagged with HA is in red, Tubulin is in green, and DNA is in blue. The scale bar is 10 um.

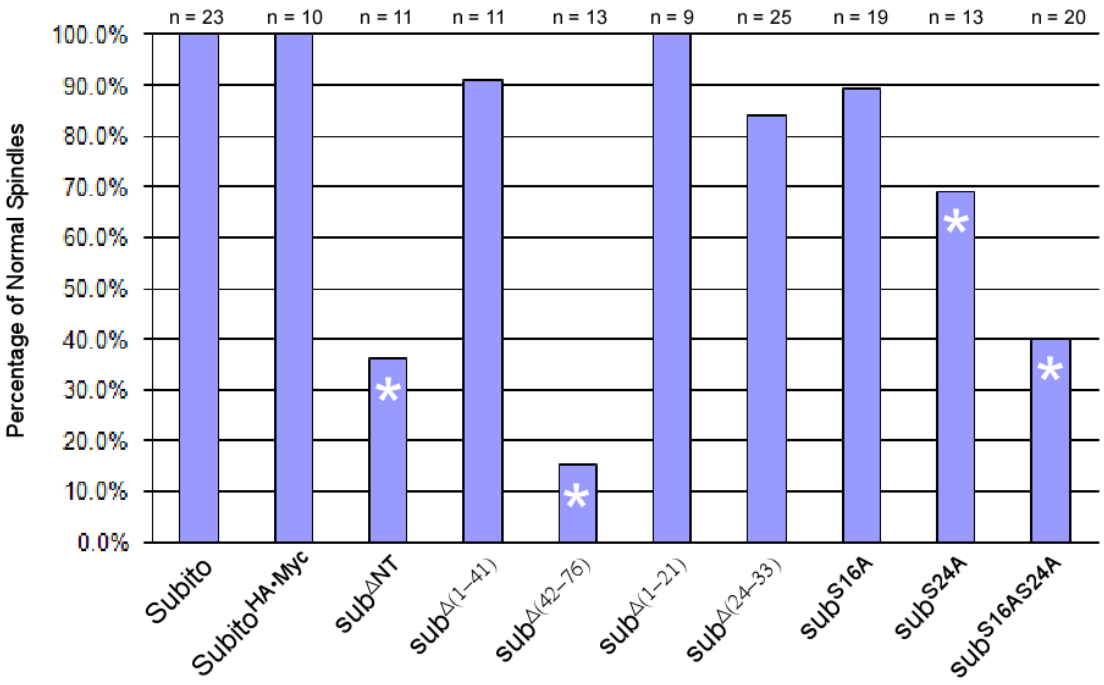

Figure 20 – Graph displaying the percentage of normal spindle morphologies per N-terminus mutant oocytes. Columns with an asterick (*) represent constructs with statistically decreased percentage of cytologically normal spindle assembly. A z-test was performed at 99% confidence to produce these statistics. N-terminus constructs with statistically decreased percentages of normal spindle morphologies were *sub*$^{\Delta NT}$, *sub*$^{\Delta(42-76)}$, *sub*^{S24A}, and *sub*^{S16AS24A}.

The localization of SUB$^{\Delta(42-76)}$ was abnormal as well. The mutant protein was not restricted to the central spindle and stretched along the microtubules towards the negative ends (Figure 19C). The mislocalization of the mutant protein presents two interesting explanations for the spindle assembly defect in *sub*$^{\Delta\,(42-76)}$ oocytes. First, SUB$^{\Delta\,(42-76)}$ is deregulated and allowed to interact with microtubules that are not anti-parallel. Second, SUB$^{\Delta\,(42-76)}$ binds to microtubules normally but is incapable of motoring towards the central spindle. It is important to note that the motor domain is unaffected by the mutation, and the complete deletion of the N-terminus does not affect the localization of the mutant protein to the central spindle, even when assembling ectopic spindles (Figure

19). This suggests that $SUB^{\Delta (42-76)}$ is indeed deregulated and allowed to bind microtubules outside of its normal subset. If true, $SUB^{\Delta (42-76)}$ can bind parallel microtubules, competitively inhibiting the binding of other kinesins which are essential for spindle assembly and achieving pole extension, such as Ncd. These results suggest that the N-terminus restricts microtubule binding by preventing the binding of Subito to parallel microtubules. Furthermore, the binding of an anti-parallel cross linker to parallel microtubules has deleterious effects on spindle assembly.

The first 21 amino acids of Subito function to positively regulate the kinesin

To further study the regulatory role of the N-terminus, the deletion analysis was continued by creating smaller deletions. $sub^{\Delta(1-21)}$ deletes a conserved region of the N-terminus, including a serine, which has been shown to be phosphorylated via mass spectrometry (Bodenmiller, Malmstrom et al. 2007). Expression of $sub^{\Delta (1-21)}$ had no observable effects on spindle assembly or karyosome organization (Figure 20). Quite surprisingly, $SUB^{\Delta (1-21)}$ failed to localize to the central spindle (Figure 19D). This failure to localize suggests $SUB^{\Delta (1-21)}$ cannot bind microtubules, which explains the failure of $SUB^{\Delta (1-21)}$ to rescue sterility of *sub* null mutant (Table 8). Since $sub^{\Delta (1-21)}$ expressed within the ovary, this result confirms that the first twenty-one amino acids of Subito function to positively regulate the motor. While the original hypothesis was that the N-terminus acts to negatively regulate the kinesin, it appears that the N-terminus is more complex, with regions of negative and positive regulation.

$SUB^{\Delta(24-33)}$ can only interact with interpolar microtubules in the presence of wild-type Subito

Continuing the deletion analysis, a second conserved region of N-terminus was deleted, $sub^{\Delta(24-33)}$, which contained a serine shown to be phosphorylated via mass

spectrometry (Bodenmiller, Malmstrom et al. 2007). Expression of *sub*$^{\Delta(24\text{-}33)}$ in a wild-type background had no observable defects in spindle morphology (Figure 20). Furthermore, SUB$^{\Delta(24\text{-}33)}$ localized normally to the central spindle (Figure 19E), suggesting that SUB$^{\Delta(24\text{-}33)}$ had no defects in binding microtubules or localizing to the central spindle. Despite the wild-type appearance, expression of *sub*$^{\Delta(24\text{-}33)}$ was not capable of rescuing the sterility defect of the *sub* null mutant (Table 8). These results suggest that SUB$^{\Delta(24\text{-}33)}$ is dependent upon endogenous Subito to function. This dependence of the mutant protein can be achieved in two ways. Preliminary evidence using yeast two-hybrid suggests that Subito is capable of binding to itself (data not shown). Since Subito is capable of dimerization, SUB$^{\Delta(24\text{-}33)}$ may only bind microtubules when bound to wild-type Subito. When SUB$^{\Delta(24\text{-}33)}$ is dimerized to itself, the resulting complex would be incapable of binding microtubules. A second possibility involves a wild-type complex of Subito initially stabilizing microtubules. Once the stabilization of the microtubules at the central spindle is obtained, the mutant protein is capable of binding microtubules. Both explanations for the mutant phenotype suggest that this region of the N-terminus normally functions to positively regulate the motor activity of the kinesin.

Serines in the N-terminus function to negatively regulate the bundling activity of Subito

Mass spectrometry analysis of Subito suggests that the N-terminus is phosphorylated at two serines, 16 and 24 (Bodenmiller, Malmstrom et al. 2007). To determine if phosphorylation of these serines has a role in regulating Subito, constructs were created that substituted these serines to alanines individually, *sub*^{S16A} and *sub*^{S24A}, and in tandem, *sub*^{S16AS24A}. Formation of ectopic spindle assembly was not observed in

sub^{S16A} or *sub*^{S24A} oocytes. Additionally, SUB^{S16A} and SUB^{S24A} localized normally to the central spindle (Figure 21 B and C). *sub*^{S24A} oocytes had a statistically high level of abnormal spindles (Figure 20). However, neither mutant protein was capable of initiating chromosome independent microtubule binding and bundling as was seen in the *sub*$^{\Delta NT}$ mutant oocytes.

Expression of *sub*^{S16AS24A} in the oocytes, in which both phosphorylation sites are mutated, resulted in high levels of abnormal spindles (Figure 20), despite normal localization to the central spindle (Figure 21D). Upon examination of the ooplasm, bundles of microtubules could be observed (Figure 21E), and SUB^{S16AS24A} was associated with these ectopic microtubule bundles. While the frequency and intensity of these ectopic bundles did not mimic *sub*$^{\Delta NT}$ mutant oocytes (Figure 21E), it is clear that both mutant proteins are capable of binding and bundling microtubules in a chromosome independent manner (Figure 22A-D). This result suggests that phosphorylation of both serines is necessary to inactivate the motor activity of the kinesin. The removal of the phosphorylation results in an active kinesin capable of binding and bundling microtubules. The removal of phosphate groups could be achieved through the phosphotase PP1, which is known to co-localize with the chromosomal passenger complex (Sugiyama, Sugiura et al. 2002). Subito is known to co-localize with components of the chromosomal passenger complex (Jang, Rahman et al. 2005). This localization pattern offers a mechanism for activation for Subito that only occurs near the karyosome. Results from this section confirm that the dephosphorylation of serines in the N-terminus activates Subito. Taken with previous results, this analysis has demonstrated

the complexity of the N-terminus, while providing a model of regulation through the chromosomal passenger complex and PP1.

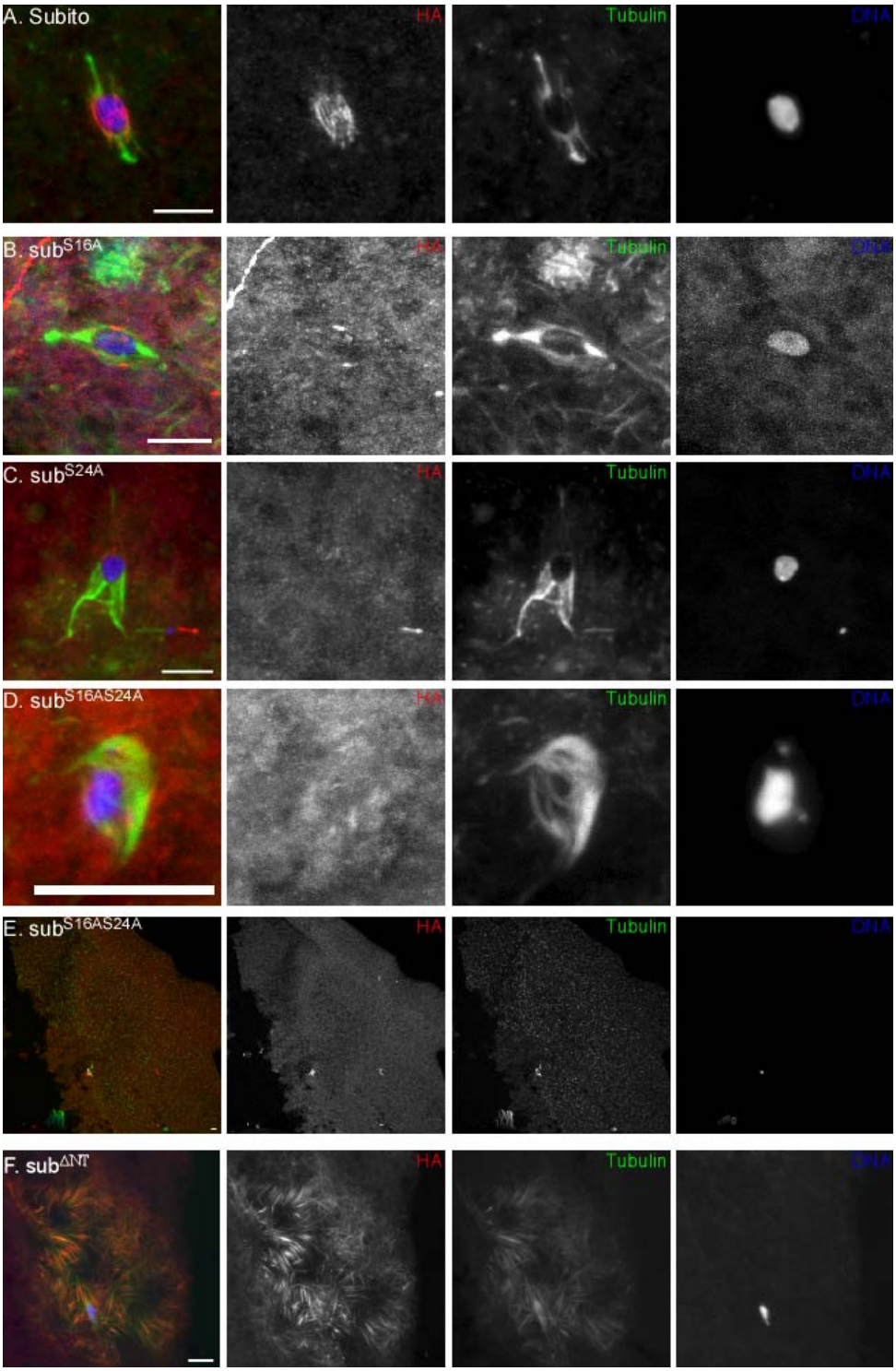

Figure 21 – Serine to alanine substitution constructs in stage 14 oocytes including, (A) wild-type *Subito*, (B) sub^{S16A}, (C) sub^{S24A}, and (D) $sub^{S16AS24A}$. (E) represents a zoomed out view of a $sub^{S16AS24A}$ mutant oocyte so the accumulations of microtubule bundles can be observed. (F) represents a zoomed out view of a $sub^{\Delta NT}$ mutant oocyte so it can be compared to the microtubule bundling seen in a $sub^{S16AS24A}$ mutant oocytes. s*ubito* tagged with HA is in red, Tubulin is in green, and DNA is in blue. The scale bar is 10 um.

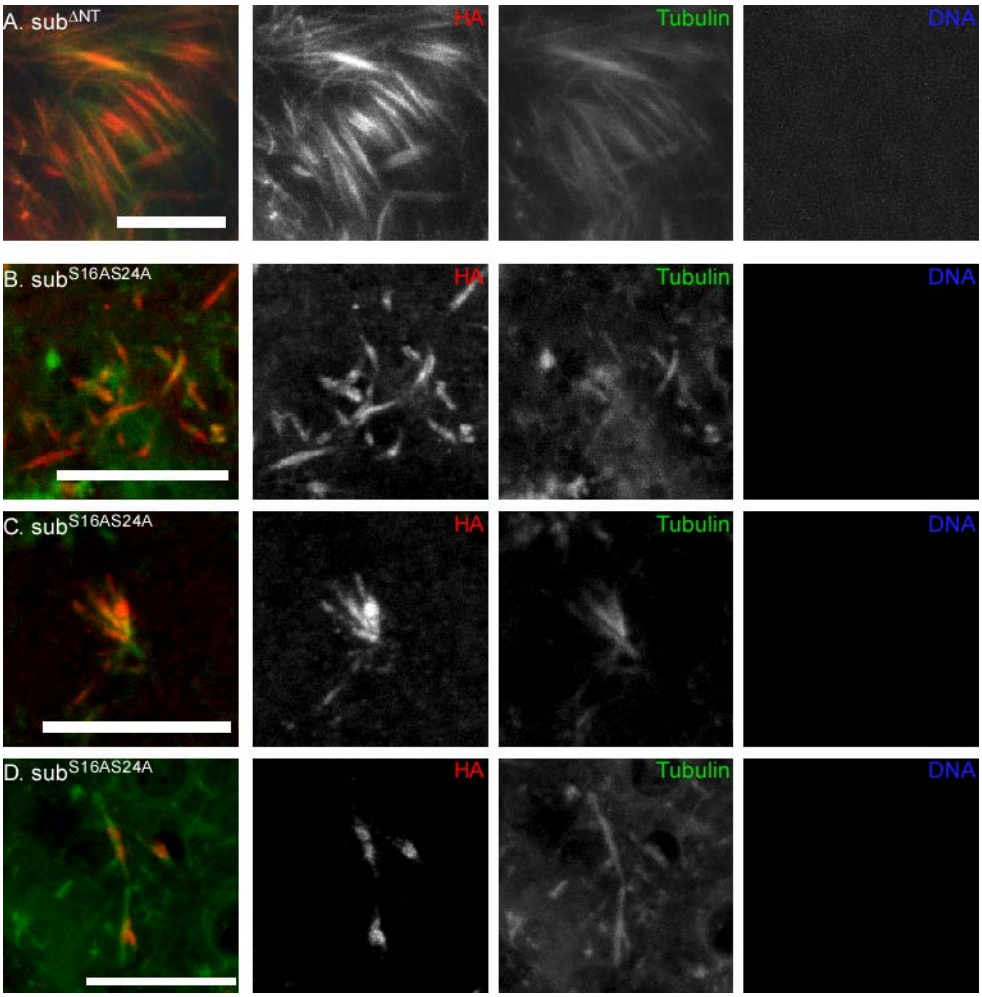

Figure 22 – Ectopic bundling in both $sub^{\Delta NT}$ and $sub^{S16AS24A}$ mutant oocytes. (A) represents a typical $sub^{\Delta NT}$ mutant oocyte with a high level of chromosome independent microtubule bundling. (B-D) represent the chromosome independent microtubule binding seen in $sub^{S16AS24A}$ mutant oocytes, with a lower level of bundling as compared to $sub^{\Delta NT}$ mutant oocytes. The mutant protein is in red, Tubulin is in green, and DNA is in blue. The scale bar is 10 um.

Expression of $Subito^{HA\text{-}Myc}$ has no deleterious effects on meiosis or embryonic development

Recent experiments using FRET have shown that kinesin-1 becomes inactive when the tail and motor physically interact (Cai, Hoppe et al. 2007). Upon separation from the tail, the motor is free to bind microtubules and travel towards the plus end

(Martin, Fathi et al. 2010). Given the importance of the N-terminus in regulating the motor activity of Subito, we speculated that a similar mode of auto-inhibition may be occurring, in which the N-terminus and C-terminus interact and prevent the kinesin from binding microtubules.

To test the conformation of active Subito, a dual tagged construct of *subito* was created with an HA tag on the N-terminus and a Myc tag on the C-terminus, $Subito^{HA\text{-}Myc}$. Expression of $Subito^{HA\text{-}Myc}$ had no dominant effects on X chromosome non-disjunction (Table 7) and was capable of rescuing the sterility of a *sub* null background (Table 8). Cytological experiments in oocytes confirmed that $Subito^{HA\text{-}Myc}$ localized properly to the central spindle during metaphase I (Figure 23A). In embryonic divisions, $Subito^{HA\text{-}Myc}$ bound microtubules during metaphase (Figure 23C), and localized tightly to the central spindle during telophase (Figure 23E). Hence, genetic and cytological experiments proved that $Subito^{HA\text{-}Myc}$ functioned in a wild-type manner, with no abnormal effects on spindle assembly. Control experiments were then performed to confirm that FRET interactions were occurring properly with Cy3/Cy5 donor acceptor pairing. Using Subito and Tubulin as a positive control, Subito a FRET efficiency of 5.51% was obtained (Figure 24). This was statistically higher than the FRET efficiency for the negative control, Subito and DNA, which had a FRET efficiency of 0.02% (Figure 24). These results confirm that oocytes are capable of producing a FRET signal, but only when donor and acceptor are interacting.

Subito has an open confirmation when bound to the central spindle of a meiosis I spindle

Since preliminary results proved that oocytes were an acceptable tissue for performing FRET experiments, we examined $Subito^{HA\text{-}Myc}$ oocytes to determine if the N-

and C-terminus of Subito interact when bound to the central spindle during metaphase I. In oocytes, active Subito$^{HA\text{-}Myc}$ had FRET efficiency of 0.60% (Figure 24). This FRET efficiency was statistically lower than the FRET efficiency for Subito and Tubulin. This result suggests that active Subito has an open confirmation during meiosis, where the N- and C-terminus do not physically interact.

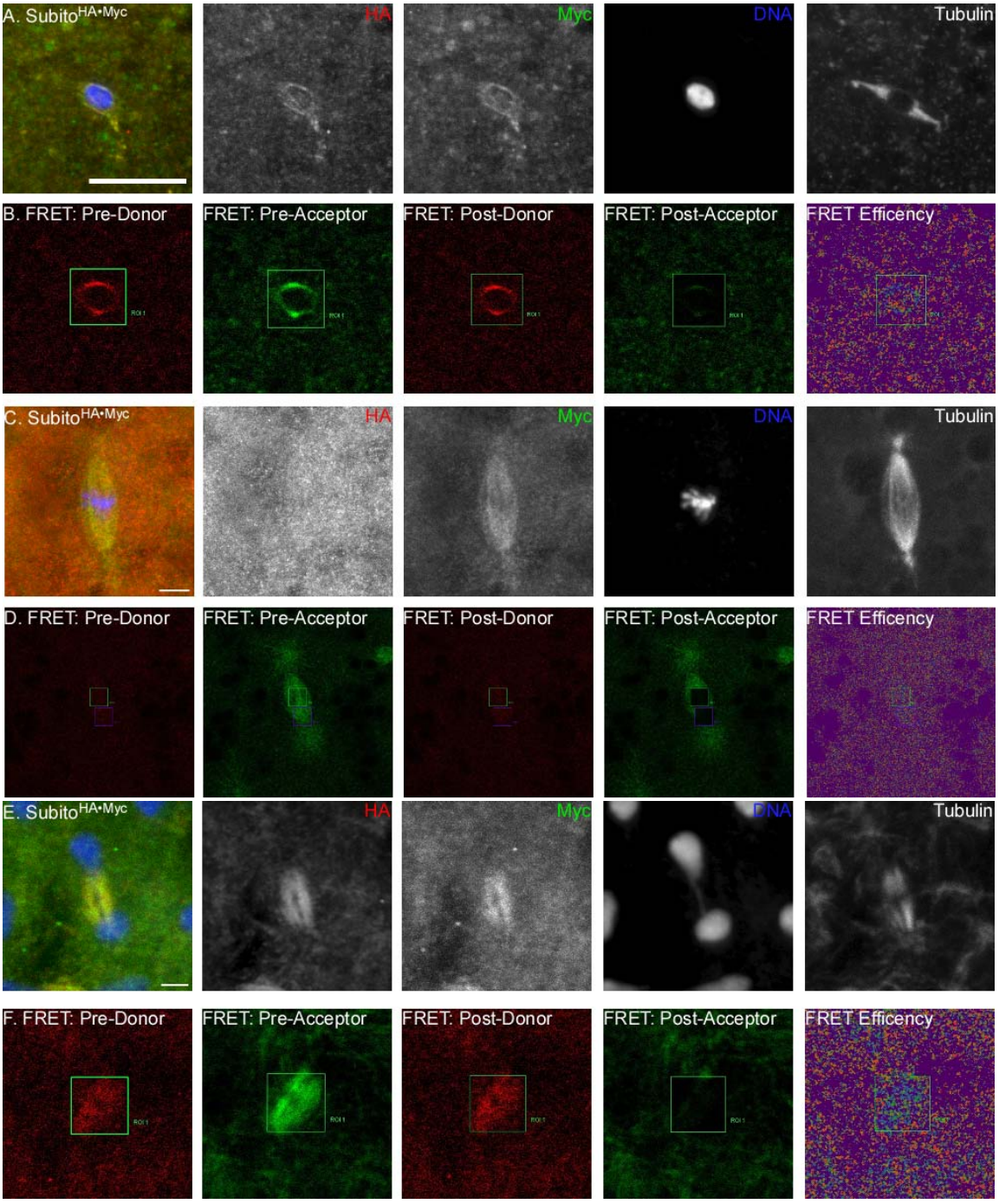

Figure 23 – Subito$^{HA•Myc}$ localizes properly in oocytes (A) and embryos (C and E) and shows no dominate effects on spindle assembly or karyosome organization. (A) shows a stage 14 oocyte, (C) shows a stage 4 embryo at metaphase, and (E) shows a stage 4 embryo at telophase. HA tag is in red, Myc is in green, DNA is in blue, and Tubulin is in gray. An example FRET experiment is shown for oocytes (B), stage 4 embryos at metaphase (D), and stage 4 embryos at telophase (F). The donor represents Cy3 probe and the acceptor represents Cy5 probe. Pre and post represent scans before and after bleaching of the acceptor. FRET efficiency presents that percentage of increased donor intensity after photo bleaching the acceptor. The scale bar is 10 um.

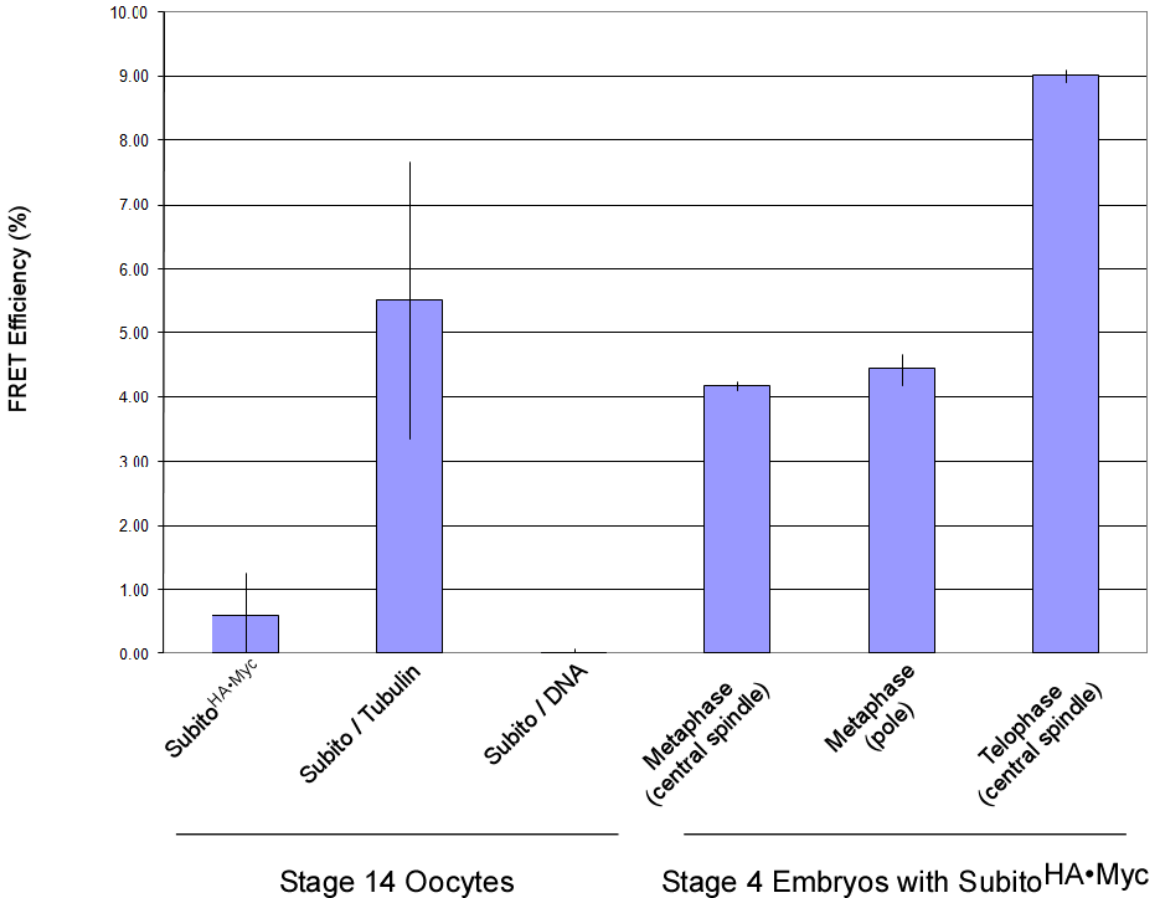

Figure 24 – A graph displaying the FRET efficiencies for *Subito*$^{HA•Myc}$ oocytes and embryos. Error bar present standard deviation for the average FRET efficiency.

Subito has a closed conformation during mitosis

Stage 4 embryos were examined to determine if the confirmation of active Subito has a similar confirmation during mitosis. Subito is known to bind along all microtubules during mitotic metaphase (Figure 23C), but then condenses to the central spindle during anaphase (Figure 23E). Due to this localization pattern, we were able to analyze two populations of Subito: the poles and central spindle. Surprisingly, Subito$^{HA•Myc}$ had a significantly higher FRET efficiency at the poles, 4.44%, and central spindle, 4.19%, when compared to Subito$^{HA•Myc}$ in oocytes, 0.60% (Figure 24). It should be noted that the intensity of N-terminus tag, HA, was decreased when compared to the C-terminus

tag, Myc (Figure 23C). This observation occurred regardless of the fluorescent probe, as swapping of Cy3 with Cy5 at the N-terminus did not result in an increased intensity. Perhaps even more bizarre was the observation that the intensity of the N-terminus probe returned to normal levels at anaphase and telophase (Figure 23E). This result proves that the cytological technique is not the cause of the decreased intensity. Even with a decreased intensity, a high FRET efficiency was obtained during metaphase at both the central spindle and poles. This result proves that the N-terminus and C-terminus of Subito interact while bound to microtubules during mitosis, suggesting a closed confirmation for Subito.

Since the different stages of mitosis are easily visualized during embryogenesis, we were also able to compare Subito$^{HA•Myc}$ at metaphase and telophase. The FRET efficiency at telophase, when Subito condenses tightly to the central spindle, was 9.01% (Figure 24). This value was significantly higher than any other FRET efficiency obtained, and could be the result of the Subito being tightly compacted to the central spindle, forcing the N-terminus into an interaction with the C-terminus of other Subito molecules.

FRET experiments in oocytes have confirmed that active Subito is in an open confirmation during metaphase I. Conversely, FRET experiments in embryos revealed a closed confirmation for Subito during metaphase. These results can be interpreted in two ways. First, Subito has different conformational states during mitosis and meiosis. Second, given the quick succession of mitotic divisions in embryos, it is possible that only a fraction of Subito is fully activated. The exact mechanism of regulation during mitosis remains unclear. Nonetheless, these results prove that Subito has an open

confirmation when active during meiosis. Since the N- and C-terminus have been shown to interact during mitosis, these results suggest a model of auto-inhibition for Subito.

V. Discussion

Subito is a member of the kinesin-6 family capable of binding and bundling microtubules during both mitosis and meiosis. In *Drosophila* oocytes, deletion of the N-terminus results in chromosome-independent spindle assembly (Jang, Rahman et al. 2007). This result confirms that the N-terminus is essential for negative regulation, but also suggests auto-inhibition. Auto-inhibition is a common regulatory mechanism used in many biological processes (Pufall and Graves 2002). This form of regulation also occurs in kinesin and myosin families (Lee, Shin et al. 2004; Liu, Taylor et al. 2006). For example, a physical interaction between the motor and tail domains of Kinesin-1 blocks the motor, resulting in a functionally inactive kinesin (Cai, Hoppe et al. 2007). Upon release from the tail domain, the motor is active and free to interact with microtubules. Since the N-terminus has a role in regulation, we proposed that auto-inhibition may restrict the binding and bundling of microtubules by Subito to the karyosome in *Drosophila* oocytes. The object of this study was to investigate the regulation of Subito by isolating the region of the N-terminus responsible for negative regulation. Furthermore, we wished to determine if auto-inhibition is a regulatory mechanism for Subito, similar to Kinesin-1.

Constructs of *subito* were created with deletions in the N-terminus to isolate the region responsible for this function. Expression of the epitope-tagged transgenes in stage 14 oocytes yielded a wide array of spindle assembly defects and localization

patterns. We found that the N-terminus is complex, containing both negative and positive regulatory domains.

SUB$^{\Delta(42-76)}$ inhibits pole extension during acentrosomal spindle assembly

The kinesin-6 family, as well all other families of kinesins, is grouped based on conversation of the motor domain. The N-terminus is an evolutionary non-conserved domain among most species. However, by comparing the N-terminus to other *Drosophila* species, conservation is observed within the first forty-one amino acids. Surprisingly, a spindle assembly defect was observed by deleting the non-conserved half of the N-terminus, amino acids forty-two to seventy-six. Expression of *sub$^{\Delta(42-76)}$* in stage 14 oocytes resulted in shorter spindles, that appeared blunted and failed to extend to the poles. The localization of the mutant protein was also abnormal. While it localized to the central spindle, SUB$^{\Delta(42-76)}$ also bound to microtubules outside of the central spindle. Since the motor domain is not altered by this mutation, this mislocalization suggests that amino acids forty-two to seventy-six negatively regulate Subito. This region of the N-terminus restricts Subito to the central spindle by preventing interactions with microtubules that are parallel. Expression of *sub$^{\Delta(42-76)}$* results in a high average of abnormal spindles. This spindle assembly defect, coupled with the mislocalization of the mutant protein, suggests that Subito cannot properly bundle parallel microtubules. Bundling of parallel microtubules by Subito results in an inability to extend microtubules towards the poles. Since bundling of parallel microtubules is thought to be done by Ncd, a member of Kinesin-14 family, it is likely that the mutant protein prevents the binding of Ncd (Hatsumi and Endow 1992; Hallen, Liang et al. 2008). Without Ncd at the spindle, parallel microtubules cannot be bundled properly and extended towards the poles. These

results suggest a mechanism of regulation with the N-terminus restricting Subito to the central spindle. Failure to restrict Subito to the central spindle has deleterious effects on spindle assembly.

The first twenty-one amino acids positively regulate the motor activity of Subito

The original goal of our study was to isolate the region of the N-terminus necessary for the negative regulation of Subito. A construct of *subito* with a small deletion in the N-terminus was created, eliminating the first twenty-one amino acids. This region is highly conserved among *Drosophila* species, and includes a serine that is phosphorylated (Bodenmiller, Malmstrom et al. 2007). Upon expression in stage 14 oocytes, $SUB^{\Delta(1-21)}$ failed to localize to the central spindle. Once again, this deletion construct did not alter the motor domain, and should not inhibit microtubule binding. Expression of $sub^{\Delta(1-21)}$ did not affect spindle assembly or chromosome organization. These results suggest that this region of the N-terminus positively regulates Subito. This is an interesting model, since the original observations showed N-terminus functions only to negatively regulate Subito. Nonetheless, the N-terminus is clearly a complex domain, containing both negative and positive regulatory motifs.

Serines sixteen and twenty-four function to negatively regulate the bundling activity of Subito

Mass spectrometry analysis of Subito suggests that the N-terminus is phosphorylated at two serines, 16 and 24 (Bodenmiller, Malmstrom et al. 2007). To determine if phosphorylation of these serines has a role in regulating Subito, constructs of *subito* were created that substituted these serines to alanines individually, sub^{S16A} and sub^{S24A}, and in tandem, $sub^{S1A6S24A}$. Formation of ectopic spindle assembly was not

observed within sub^{S16A} or sub^{S24A} mutant oocytes. However, $sub^{S16AS24A}$ oocytes contained chromosome-independent bundles of microtubules. This phenotype was not as severe as the complete N-terminus deletion. However, it suggests that both serines function in tandem to negatively regulate the protein. It also suggests that, upon dephosphorylation, Subito becomes active. Hence, both states have a role in the regulation of the kinesin. Interestingly, a similar result was obtained in MKLP2, the human homolog of Subito. When a serine in the linker domain was substituted to an alanine, the motor domain was functionally active and capable of bundling microtubules (Neef, Preisinger et al. 2003). Polo- like kinase 1 has been shown to phosphorylate that serine, suggesting that it is responsible for the negative regulation of the MKLP2. We have previously shown that Subito co-localizes with the chromosomal passenger complex during meiosis (Jang, Rahman et al. 2005). Given this localization, we suggest a model of regulation involving the chromosomal passenger and the protein serine/theronine phosphatases type 1, also known as PP1. PP1 has been shown to interact with Aurora B, acting as a negative regulator of kinase activation (Sugiyama, Sugiura et al. 2002). We propose that Aurora B kinase activity phosphorylates most serines in Subito. PP1 then selectively dephosphorylates serines at the central spindle. We believe these serines include serines sixteen and twenty-four. Hence, Aurora B and PP1 work in tandem to fully activate Subito. The analysis of the N-terminus proves that it is a complex domain of positive and negative controls. This complexity is exemplified by the negative regulation of Subito by phosphorylation of the N-terminus. If the chromosomal passenger complex and PP1 function to activate Subito, then it suggests a model where

Subito becomes active near the central spindle. However, a mechanism of auto-inhibition may keep Subito inactive prior to these interactions at the central spindle.

Active Subito has an open confirmation during meiosis I

We examined Subito using FRET to determine the possibility that the N-terminus inhibits the motor domain via an interaction between the N- and C-terminus. To test the conformation of active Subito, a dual tagged transgene of *subito* was created with an HA tag on the N-terminus and a Myc tag on the C-terminus, $Subito^{HA\text{-}Myc}$. Expression of $Subito^{HA\text{-}Myc}$ in the female germline had no dominant effects using both genetic and cytological assays. Expression of $Subito^{HA\text{-}Myc}$ was capable of rescuing the sterility of a *sub* null mutant. Together, these results prove that Subito$^{HA\text{-}Myc}$ functions similarly to the wild-type protein.

Subito$^{HA\text{-}Myc}$ localized normally to the central spindle during metaphase I. FRET analysis showed that the N- and C-terminus do not physically interact on the central spindle. This result suggests that active Subito has an open confirmation, where the N- and C-termini are not interacting. A certain threshold of fluorescent probe was necessary to obtain FRET efficiency. This threshold could not be achieved within the ooplasm, and a FRET efficiency could not be obtained. Hence, inactive Subito could not be analyzed for confirmation of the N- and C-termini. Nonetheless, the low FRET efficiency of Subito$^{HA\text{-}Myc}$ on the central spindle establishes that Subito has an open confirmation when active. However, FRET experiments in embryos reveal a different confirmation for Subito$^{HA\text{-}Myc}$. Since Subito can bind microtubules outside of the central spindle during mitosis in embryos, we analyzed Subito$^{HA\text{-}Myc}$ on both the central spindle and poles. Both locations had high FRET efficiencies. This suggests that active Subito has a closed

confirmation during mitosis, where the N- and C- termini interact. This contradiction to meiosis can be explained by the quick succession of mitotic divisions in embryos.

In oocytes, metaphase I spindles are arrested (McKim, Jang et al. 1993). This allows for a prolonged period of time for Subito to localize to the central spindle and become fully active. However, mitotic divisions during embryogenesis are rapid (Glover 1989); thus, Subito may not have enough time to progress to the central spindle, and become fully active. It is also possible that Subito diffuses through the cytoplasm during mitosis, allowing for random interaction with microtubules. FRAP analysis using Subito::GFP suggests that Subito is more dynamic during mitosis, compared to meiosis. This result suggests that the replacement of Subito from the central spindle occurs at a faster rate in mitosis compared to meiosis. Hence, Subito may remain bound to the central spindle during meiosis, allowing time for the chromosomal passenger and PP1 to fully activate the kinesin. The interaction time during mitosis is decreased, and the chromosomal passenger complex may only activate a small subset of Subito bound to the central spindle. Taken together with the deletion analysis, this paper presents a complex mechanism of regulation for Subito, involving both auto-inhibition and protein interactions with the chromosomal passenger complex and PP1.

Conclusion

My research focus was directed at the understanding of spindle assembly in the absence of centrosomes. Specifically, the emphasis of my research was the initiation of acentrosomal spindle assembly in *Drosophila melanogaster*.

Ran is a small GTPase that has an essential role in transportation of proteins between the cytoplasm and nucleus. It was subsequently shown that RanGTP is important for chromosome-dependent spindle assembly in *Xenopus* extracts. We investigated the effect of manipulating the Ran pathway on spindle assembly in *Drosophila* oocytes and embryos. RCC1, a guanine exchange factor responsible for converting Ran to its active RanGTP form, is an important component of this pathway and is present on oocyte chromosomes. Hence, RanGTP is expected to be in the vicinity of the chromosomes after NEB. To investigate the role of RanGTP, we generated females expressing dominant negative GDP-locked (ran^{GDP}) or GTP-locked (ran^{GTP}) forms of *ran*. Females expressing these mutants were sterile, but this was not due to defects in meiosis. Expression of ran^{GDP} in oocytes did not block spindle assembly, although the tapering of microtubules at the poles and localization of TACC and the HURP homolog, Mars, was abnormal. Furthermore, expression of ran^{GTP} did not promote ectopic spindle assembly. Thus, RanGTP may not be essential or sufficient for the formation of the acentrosomal spindle around the chromosomes. In contrast, expression of ran^{GDP} blocked pronuclear fusion, which depends on microtubules

nucleated from the sperm aster. Similarly, expression of ran^{GDP} suppressed the chromosome-independent spindle assembly phenotype caused by a mutation which deletes the non-motor N-terminus domain of the Kinesin-6 *subito*. Thus, RanGTP may be required for microtubule assembly that is not directly nucleated by the chromosomes. In promoting spindle assembly around chromosomes, RanGTP may be redundant with other factors. Indeed, expression of ran^{GDP} in a *sub* mutant background caused a block in oogenesis, a more severe phenotype than in either single mutant. Subito interacts with the chromosome passenger complex (CPC) that has also been implicated in chromosome-mediated spindle assembly. Therefore, RanGTP may be redundant with the CPC when chromosomes are present.

I further analyzed the initiation of acentrosomal spindle assembly by examining the regulation of Subito in *Drosophila melanogaster*. Subito is a member of the kinesin-6 family that is required for bundling interpolar microtubules located within the central spindle at metaphase I. Through a deletion analysis of Subito, it has been determined that the N-terminus is essential to the negative regulation of Subito in oocytes. By continuing the deletion analysis, we have shown that the N-terminus of Subito is not a simple negative regulator, but has properties of both positive and negative regulators. This kind of complexity has not previously been observed within the N-terminus of kinesins. Furthermore, two serines in the N-terminus are essential for the negative regulation of Subito motor activity. We suggest a model of auto-inhibition for Subito, where the N- and C-terminus interact to prevent interactions with microtubules.

These results have helped to clarify the mechanisms necessary for the initiation of acentrosomal spindle assembly. However, more questions remain. Further analysis of

the chromosomal passenger complex will be necessary to examine the role of the complex in the initiation of acentrosomal spindle assembly. Furthermore, the discovery of spindle assembly factors that are activated by the chromosomal passenger complex will answer how a single complex can initiate the formation of such a complex apparatus. The regulation of Subito appears to be complex as well, with domains within the N-terminus that are essential for the negative and positive regulation of the kinesin. Further characterizing the mechanism of acentrosomal spindle assembly through the chromosomal passenger complex may supply answers to what other factors are responsible for the regulation of spindle assembly factors, including Subito.

Appendix I: Meiotic spindle length is not dependent upon Subito

Klp61F, also known as Eg5, is a kinesin capable of establishing spindle bipolarity in mitosis. It performs this function by binding anti-parallel microtubules, exerting force, and pushing centrosomes towards opposite ends of the cell. Given this role, it is clear that Klp61F is not only essential for establishing the bipolarity of the spindle, but also spindle length. We have previously determined that Subito, a kinesin-6 family member capable of binding anti-parallel microtubules, has a role in maintaining spindle bipolarity during meiosis I in *Drosophila* oocytes. Mitotic divisions in *sub* neuroblasts have defective central spindle. Due to the similarities in function with Klp61F, we hypothesized that Subito has a role in maintaining spindle length during mitosis and meiosis. In addition, we proposed that chromosomal passenger complex and RanGTP are essential for initiating meiotic spindle assembly and maintaining spindle length. There were no statistically differences between wild-type spindle length and the spindle length in *subito* mutants during both meiosis and mitosis. Furthermore, mutants in *Incenp* and *Ran* showed spindles with similar lengths to wild-type in oocytes. These findings prove that, despite its function in stabilizing the central spindle, Subito has no role in establishing or maintaining spindle length.

Kinesins play an essential role in initiating and maintaining spindle assembly. Specifically, Klp61F has been well-documented for its participation in the separation of duplicated centrosomes. Cells lacking Klp61F, via either gene deletion or antibody interference, result in monoastral spindle (Brust-Mascher, Sommi et al. 2009). A member of the kinesin-5 family, Klp61F is a plus ended microtubule kinesin. As the kinesin binds the plus ends of microtubules, it cross-links them with microtubules from the opposite pole. By cross-linking microtubules from opposite poles, Klp61F exerts force upon the centrosomes, pushing them to the opposite ends of the cell (Sharp,

McDonald et al. 1999). This placement of centrosomes establishes bipolarity and, consequently, the length of the spindle (van den Wildenberg, Tao et al. 2008).

We have established that Subito has a very similar role during meiosis and mitosis in *Drosophila*. Subito, a member of the kinesin-6 family, functions by bundling microtubules at the central spindle. Spindles in *sub* oocytes are often monopolar and tripolar, suggesting that Subito is essential for maintaining bipolarity during accentrosomal spindle assembly (Jang, Rahman et al. 2005). *subito* mutants have defective mitotic spindles, with a high incidence of collapsed central spindles (Cesario, Jang et al. 2006). However, centrosomes separate and orient properly. So while Subito and Klp61F have similar functions in ensuring bipolarity, Subito does not function in establishing the mitotic poles.

The activation of Subito is a poorly understood process, but may occur via the RanGTP or chromosomal passenger complex pathway. The role of RanGTP in chromosome-mediated spindle assembly is exemplified by its activity in *Xenopus* egg extracts which lack centrosomes. In these extracts chromatin-mediated microtubule assembly depends on the presence of RanGTP (Carazo-Salas, Guarguaglini et al. 1999). Similarly, depletion of RCC1, an accessory protein which activates Ran, results in a failure to form microtubule asters. Addition of RanGTP to these RCC1-depleted eggs is sufficient to induce self-organization of microtubule asters (Ohba, Nakamura et al. 1999). Disruption of RanGTP levels also affects mitotic spindle assembly in mammalian cells, although it is not as critical when centrosomes are present (Kalab, Pralle et al. 2006; Clarke and Zhang 2008). Similarly, disruption of RanGTP levels in *Drosophila* (Silverman-Gavrila and Wilde 2006) or *C. elegans* (Askjaer, Galy et al. 2002; Bamba,

Bobinnec et al. 2002) embryos hinders spindle assembly. RanGTP has the ability to activate a wide range of spindle assembly factors, including Eg5 and possibly Subito. Eg5 is the *Xenopus* homolog of Klp61F (Wilde, Lizarraga et al. 2001). Hence, during meiosis, RanGTP may function to regulate spindle length.

The chromosomal passenger complex (CPC) is essential for cellular division to occur. The chromosomal passenger complex contains Ial, known as Aurora B in *Drosophila*, Incenp, Survivin, and Borealin. The CPC functions to regulate bipolar attachment. This regulatory role is accomplished by sensing non-attached or merotelically attached kinetochores. Until the cell has properly attached kinetochores, the chromosomal passenger complex prevents the initiation of anaphase. This is thought to occur by Aurora B phosphorylating substrates that prevent continuation of the cell cycle, such as MCAK, a depolymerizing kinesin. Interactions between Aurora B and MKLP2, the human homologue of Subito, have been established, suggesting that Subito is a substrate of the chromosomal passenger complex (Gruneberg, Neef et al. 2004). We sought to examine if Subito, by maintaining the central spindle, sustains spindle length during both meiosis and mitosis. Furthermore, we examined the meiotic spindle length in mutants for two essential spindle assembly factors, RanGTP and Incenp, to determine if acentrosomal spindle length is dependent upon the initiators of spindle assembly.

Subito does not function to regulate spindle length during mitosis or meiosis

Cytological experiments using neuroblasts and oocytes were prepared using antibody specific to microtubules. These images were collected and then measured from pole to pole. Analysis of the *sub* mutant oocytes revealed that spindle lengths were not

significantly shorter than wild-type spindles (Figure 25), with wild-type spindles averaging 12.7 um and *sub* spindles averaging 10.7 um. When neuroblasts were examined, the same proved to be true of mitotic spindles, as wild-type and *sub* spindle lengths were not significantly different (Figure 25). Wild-type spindles averaged 6.2 um from pole to pole, while *sub* spindles averaged 6.0 um. Together, this data suggests that while Subito is necessary for the maintenance of bipolarity, it does not function to establish nor maintain spindle length in the presence or absence of centrosomes.

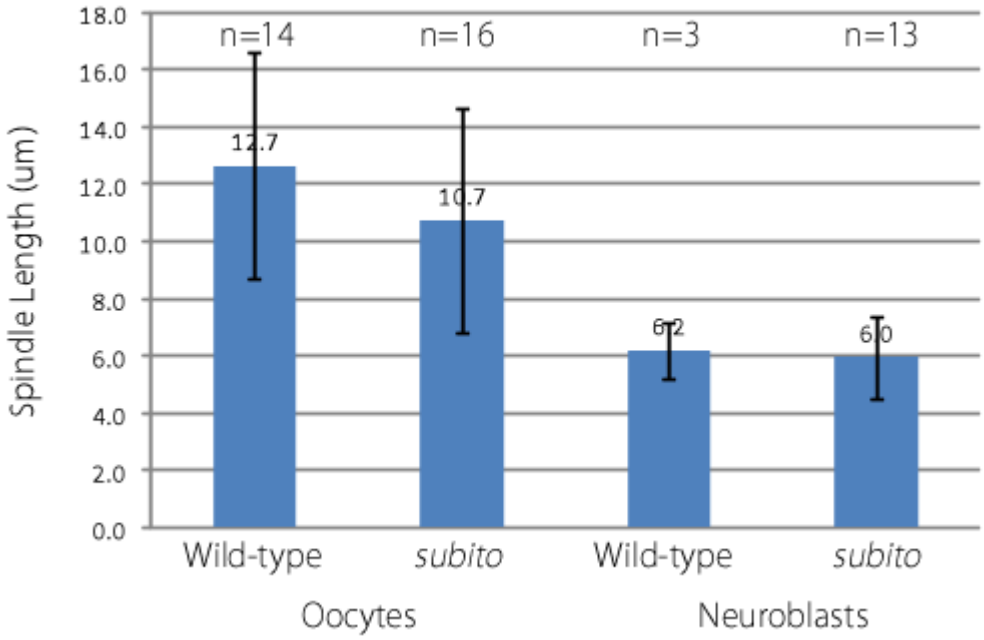

Figure 25 – Spindle length (um) in wild-type and *sub* mutant oocytes and neuroblasts. Error bars are represented by the standard deivation of the average spindle length.

Depletion of RanGTP does not shorten meiotic spindles

A dominant *ran* mutant, ran^{GDP}, was then examined to determine the role of RanGTP in acentrosomal spindle length. This mutant has been to have dominant effects, as it prevents the activation of endogenous RanGDP. Upon expression in oocytes, the length of spindle in ran^{GDP} oocytes were not significantly different than wild-type spindles (Figure 26), 11.4 um compared to 12.7 um respectively. This finding suggests that RanGTP is not essential for the maintenance of spindle length. Expression of a wild-type *Ran* transgene in oocytes resulted in a significantly decreased spindle length, 8.1 um, compared to both wild-type and ran^{GDP} spindles. This is particularly interesting since expression of wild-type Ran has not been shown to have any defects in spindle assembly when analyzed using both genetic and cytological assays (Cesario et al., 2010, under review). However, expression of wild-type Ran could assist in stabilizing microtubule kinetochore interactions. If this is true, it suggests a model of spindle assembly, at least in an acentrosomal system, where spindle length varies over the course of their development. Shortly after nuclear envelope breakdown, microtubules begin to organize around the karyosome. The spindle becomes larger as microtubules extend towards the pole. When the spindle achieves proper kinetochore attachment and tension, the spindle becomes less dynamic and decreases in size. Nonetheless, depletion of RanGTP from oocytes does not negatively affect the length of the spindle.

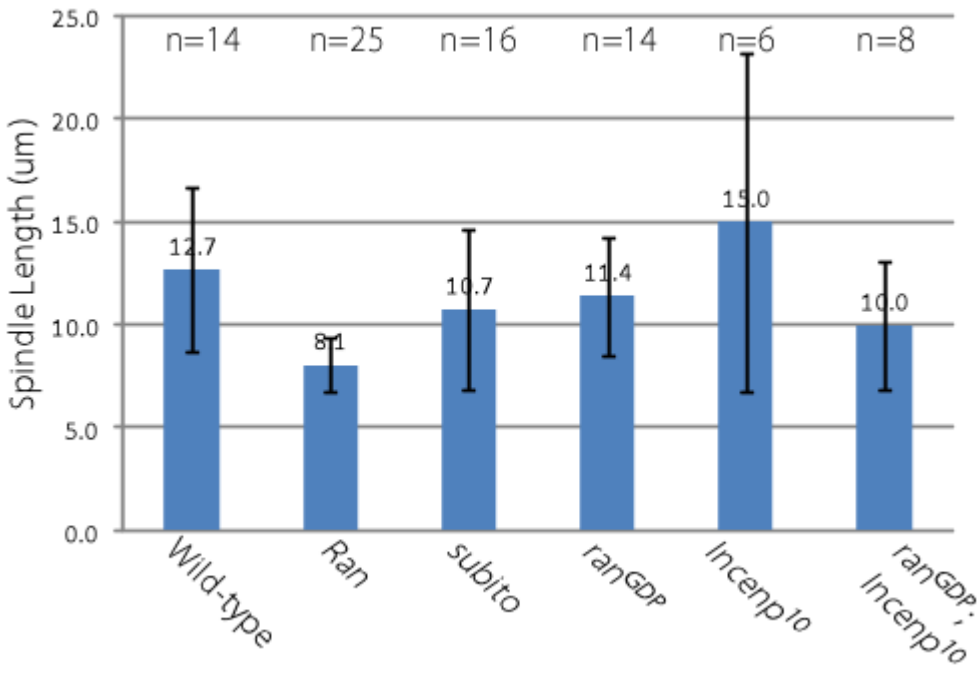

Figure 26 – Spindle length (um) in wild-type and mutant oocytes. Wild-type oocytes include white minus oocytes and oocytes expressing wild-type Ran. Mutant oocytes include *subito* and oocytes expressing a dominate negative ran^{GDP} or $Incenp^{10}$. A double mutant was also examined expressing both ran^{GDP} and $Incenp^{10}$. Error bars are represented by the standard deivation of the average spindle length.

The role of the Chromosomal Passenger Complex in establishing acentrosomal spindle length is unclear

Finally, a dominant mutation of *Incenp*, $Incenp^{10}$, was examined to determine the role of the chromosomal passenger complex in establishing and maintaining acentrosomal spindle length. $Incenp^{10}$ is a construct of wild-type *Incenp* tagged with a Myc tag on the N-terminus. In *Drosophila* as well as other organisms, this has been shown to have a dominant negative effect on spindle assembly and endogenous Incenp localization. Upon expression in oocytes, spindle lengths did not significantly change compared to wild-type spindle length, 15.0 um compared to 12.7 um respectively (Figure 26). Furthermore, oocytes expressing both $Incenp^{10}$ and ran^{GDP} did not significantly decrease spindle length either, 10.0 um. This result suggests that the chromosomal

passenger complex does not control acentrosomal spindle length. However, it is important to remember that the exact nature of the *Incenp*^{10} mutation has not been completely characterized, and endogenous chromosomal passenger complex may still be functioning to establish and maintain spindle length. This is not the case for the *ran*^{GDP} mutation, as it has been shown to directly inhibit endogenous RanGTP production.

Together, these results confirm that, while Subito has a role in maintaining bipolarity, it does not function to maintain pole separation during a mitotic nor meiotic division. It is likely that Klp61F and the chromosomal passenger complex establish and maintain spindle length during both mitosis and meiosis. Since null mutants in both pathways are lethal, it is impossible to directly examine the effect of these proteins on spindle assembly. Klp61F is a known substrate of RanGTP; however, it is possible that the activation of Klp61F is only dependent on RanGTP during mitosis. Additionally, expression of *Incenp*^{10} in oocytes has dominant effects on the localization of Incenp, but the exact nature of the mutation is unknown. While further analysis is needed, it remains likely that the chromosomal passenger complex and Klp61F contribute to the initiation and maintenance of spindle assembly during both mitosis and meiosis.

Appendix II: Deletion of the N-terminus of Subito does not result in ectopic spindle assembly in larval neuroblasts

Expression of a construct of *Subito* lacking the N-terminus in *Drosophila* oocytes results in a large number of bundled microtubules accumulating and forming bipolar spindles. Most of these ectopic spindles were not associated with chromosomes. This result suggests that the N-terminus negatively regulates Subito activity in spindle formation. This meiotic phenotype further suggests that the N-terminus ensures that microtubules are only assembled near chromosomes. Based on this observation, we examined the regulation of Subito during mitosis. We constructed mutant *subito* transgenes that deleted the N-terminus, $sub^{\Delta NT}$, substituted conserved serines in the linker, sub^{SSAA}, hindered the hydrolytic capacity of the motor, sub^{ATP}, and deleted a loop within the motor domain that is specific to the kinesin-6 family, sub^{L6}. By expressing these *subito* constructs with a Tubulin:Gal4 driver, wild-type and mutant neuroblasts were genetically and cytologically examined. Expression of *subito* mutant constructs in a wild-type background did not have a negative effect on spindle assembly or chromosomal organization. $sub^{\Delta NT}$ neuroblasts failed to mimic the ectopic spindle phenotype. The ability to build ectopic spindles during meiosis and not mitosis could be the result of spatial constrictions. The oocyte contains a much higher volume of cytoplasm as compared to the neuroblast. This constriction in space may only allow for spindle assembly to occur near the chromosomes. This reasoning is solidified by the finding that ectopic spindles do form in $sub^{\Delta NT}$ embryos, but also suggests that other factors are probably needed to assist Subito in binding and bundling microtubules. These factors include the maturation-promoting complex, RanGTP, and the chromosomal passenger complex. Together, this result proves the regulation of Subito occurs via a multitude of interactions with other spindle assembly factors.

The kinesins are a large family of motor proteins that promote unidirectional movement of a cargo along microtubules and several *Drosophila* kinesin proteins have been shown to play important roles in spindle assembly (Goshima and Vale 2003). The kinesin-4 family, or chromo-kinesins, are able to interact with microtubules while

attached to chromosomes as their cargo (Mazumdar and Misteli 2005). Another three kinesin families can bundle and slide parallel or anti-parallel microtubules. The first is the kinesin-14 family that includes minus end-directed motors such as NCD in *Drosophila*. NCD and the minus end-directed motor Dynein have been proposed to bundle and taper microtubules to establish mitotic (Walczak, Vernos et al. 1998; Goshima, Nedelec et al. 2005) and meiotic (Matthies, McDonald et al. 1996; Endow and Komma 1997; Skold, Komma et al. 2005) spindle poles in the absence of centrosomes. The second is the kinesin-5 family, including Klp61F in *Drosophila*, which are plus-end directed motors that function to maintain bipolar spindle assembly and elongation at anaphase. The activity of these proteins may antagonize the forces of the kinesin-14 family during spindle assembly (Kwon, Morales-Mulia et al. 2004; Tao, Mogilner et al. 2006). The third is the kinesin-6 family that includes Subito and Pavarotti in *Drosophila*. As shown for human MKLP2, kinesin-6 proteins are thought to be plus-end directed motors that slide along anti-parallel microtubules (Nislow, Lombillo et al. 1992). Examination of these proteins in human cells (Neef, Preisinger et al. 2003), *Caenorhabditis elegans* (Raich, Moran et al. 1998), and *Drosophila* (Cesario, Jang et al. 2006) has shown they are usually associated with interpolar microtubules at the central spindle at metaphase. During anaphase, the interpolar microtubules overlap in anti-parallel arrays at the spindle midzone, an area that typically accumulates proteins important for cytokinesis (D'Avino, Savoian et al. 2005). Unlike the kinesin-5 and -14 proteins, most studies of kinesin-6 proteins have not implicated them in pro-metaphase spindle assembly.

In *Drosophila*, the kinesin-6 protein Subito has been shown to have a role in spindle assembly. *subito* encodes the *Drosophila* homolog of MKLP2 and has an important role in organizing the meiotic acentrosomal (Jang, Rahman et al. 2005) and mitotic spindles (Cesario, Jang et al. 2006). In *Drosophila* oocytes, interpolar microtubules bundle during pro-metaphase, referred to as the metaphase I central spindle, which is a critical part of the acentrosomal spindle assembly pathway (Jang, Rahman et al. 2005). In *subito* null mutant oocytes, the central spindle is absent (Jang, Rahman et al. 2005), resulting in an abnormal number of spindle poles and high levels of meiotic non-disjunction (Giunta, Jang et al. 2002). Thus, Subito, and by inference the central spindle, is required to organize the acentrosomal spindle during *Drosophila* female meiosis. Interestingly, the central spindle forms before the microtubules are organized into a bipolar spindle and may function to direct the kinetochore microtubules toward one of the two poles. During mitotic metaphase, Subito may also organize interpolar microtubules, but the effect of its absence is much more dramatic in meiosis, possibly because Subito activity is more critical in the absence of centrosomes.

Subito first appears on pro-metaphase meiotic spindles, suggesting it functions as the microtubules are recruited to the spindle. Just how the microtubules are recruited to surround the chromosomes is poorly understood. The chromosomes could directly interact with microtubules via chromokinesin molecules (Mazumdar and Misteli 2005). Alternatively, the chromosomes could be the source of a signal, such as RanGTP (Clarke, Tang et al. 2005), which could activate microtubule assembly factors such as motor proteins. In either case, regulating kinesin proteins like Subito could be particularly important when centrosomes are absent and motor proteins may provide most of the

microtubule organizing activity. Previous studies have characterized the role of the N-, motor, and C-terminal coiled coils domains of Subito and found that regulating Subito activity is a critical component of organizing the acentrosomal spindle. Deregulation of Subito leads to the assembly of microtubules into multiple spindles in the absence of chromosomes or centrosomes. Subito appears to be activated by NEB, suggesting there is a diffusible signal that activates Subito and promotes the bundling of microtubules in oocytes. Though, the mechanism for Subito regulation remains unclear during mitosis and meiosis. We sought to further our understanding of the regulation of the motor activity of Subito by continuing the mutational analysis during mitosis.

A genetic analysis was performed to analyze the ability of mutant *subito* protein to function in spindle assembly. Homozygous null mutants of *subito* are viable, but sterile due to a defect in pro-nuclear fusion. In addition, heterozygous null mutants of *Incenp* and *ial*, components of the chromosomal passenger complex, are viable as well. Ial is the *Drosophila* homolog for Aurora B. Combining both mutations results in synthetic lethality (Cesario, Jang et al. 2006). For example, *Incenp sub* / + *sub* is synthetic lethal. We used this genetic interaction to test for mitotic function of wild-type and mutant *subito* constructs. Rescue of synthetic lethality proves that the expression of the *subito* transgene is capable of functioning similarly to the wild-type Subito. Three constructs were examined for this purpose: wild-type *Subito*, sub^{SSAA}, and $sub^{\Delta NT}$. All of these constructs were tagged with GFP. The sub^{SSAA} construct has two serines in the linker domain substituted with alanines. These serines been shown to regulate of the bundling activity of MKLP2, via an interaction between the linker domain and the mitotic polo-like kinase 1 (Neef, Preisinger et al. 2003). Expression within the germline had no

dominant effects on meiotic spindle assembly using both genetic and cytological assays. However, the interaction between Subito and Polo may only occur during mitosis. The *sub*$^{\Delta NT}$ construct deletes the entire N-terminus and has dominant effect on meiotic spindle assembly, including the formation of chromosome-independent spindles (Jang, Rahman et al. 2007).

Rescue of synthetic lethality was initially performed with the wild-type *Subito* construct to ensure that the GFP tagged protein was able to function normally. The experiment was conducted with *subito* homozygous with either *Incenp* or *ial* heterozygous. Expression of a *subito* transgene was performed using the *P{tubP -GAL4}* driver, which is capable of ubiquitously driving expression. The percentage of expected rescued progeny was calculated as the number of rescued progeny divided by the positive control (progeny heterozygous for *subito*), divided by the expected progeny of the rescued progeny. Given complete rescue of synthetic lethality, the percentage of expected rescued progeny would be 100%. As predicted, the wild-type *subito* transgene was capable of rescuing synthetic lethality in both *subito Incenp*/*subito*, 85.9%, and *ial subito* / *subito* background, 166.7% (

Table 9). Additionally, a wild-type construct of *Subito* tagged with HA was tested, showing similarly levels of rescue (

Table 9). In larval neuroblasts, GFP and HA tagged Subito localized to the microtubules during both metaphase (Figure 27A, Figure 28A) and anaphase (Figure 27B, Figure 28B). Both spindle assembly and chromosomal organization appeared to be occurring normally during both stages of mitosis. The combination of both genetic and cytological results proves that GFP and HA tagged transgenes of *Subito* function similarly to endogenous Subito.

Table 9

Rescue of synthetic lethality with *subito* transgenes

Background Phenotype:	Transgene:	% of expected rescued progeny:	Total Flies:
Incenp sub^{131} / sub^{1}	Subito::GFP	85.9%	455
ial sub^{131} / sub^{1}	Subito::GFP	166.7%	253
Incenp sub^{131} / sub^{1}	Subito::HA	105.4%	50
ial sub^{131} / sub^{1}	Subito::HA	253.8%	48
Incenp sub^{131} / sub^{1}	Subito^{SSAA}::GFP	96.2%	486
ial sub^{131} / sub^{1}	Subito^{SSAA}::GFP	114.0%	178
Incenp sub^{131} / sub^{1}	Subito$^{\Delta NT}$::GFP	0.0%	194
ial sub^{131} / sub^{1}	Subito$^{\Delta NT}$::GFP	0.0%	205
ial sub^{131} / sub^{1}	Aurora B::Myc	0.0%	156

Each transgene was expressed by crossing to *P{ tubP -GAL4 } / TM3*. Progeny expressing *P{subito}* are Sb^{+}. Progeny homozygous for the *sub* null mutations have brown eyes.

The rescued progeny contained *sub* null mutation homozygous, *ial* or *Incenp* null mutations heterozygous, the *subito* construct, and the *P{tubP -GAL4}* driver. The positive control for the experiment was progeny containing *subito* construct and driver, but heterozygous for *sub* mutantion. The negative control for the experiment was progeny with *sub* homozygous, *ial* or *Incenp* null mutations heterozygous, the *subito* construct, but lacking the *P{tubP -GAL4}* driver.

Expected progeny was calculated by dividing the total number of rescued progeny by the total number of positive control progeny. That number was then divided by the expected percentage of the rescued progeny.

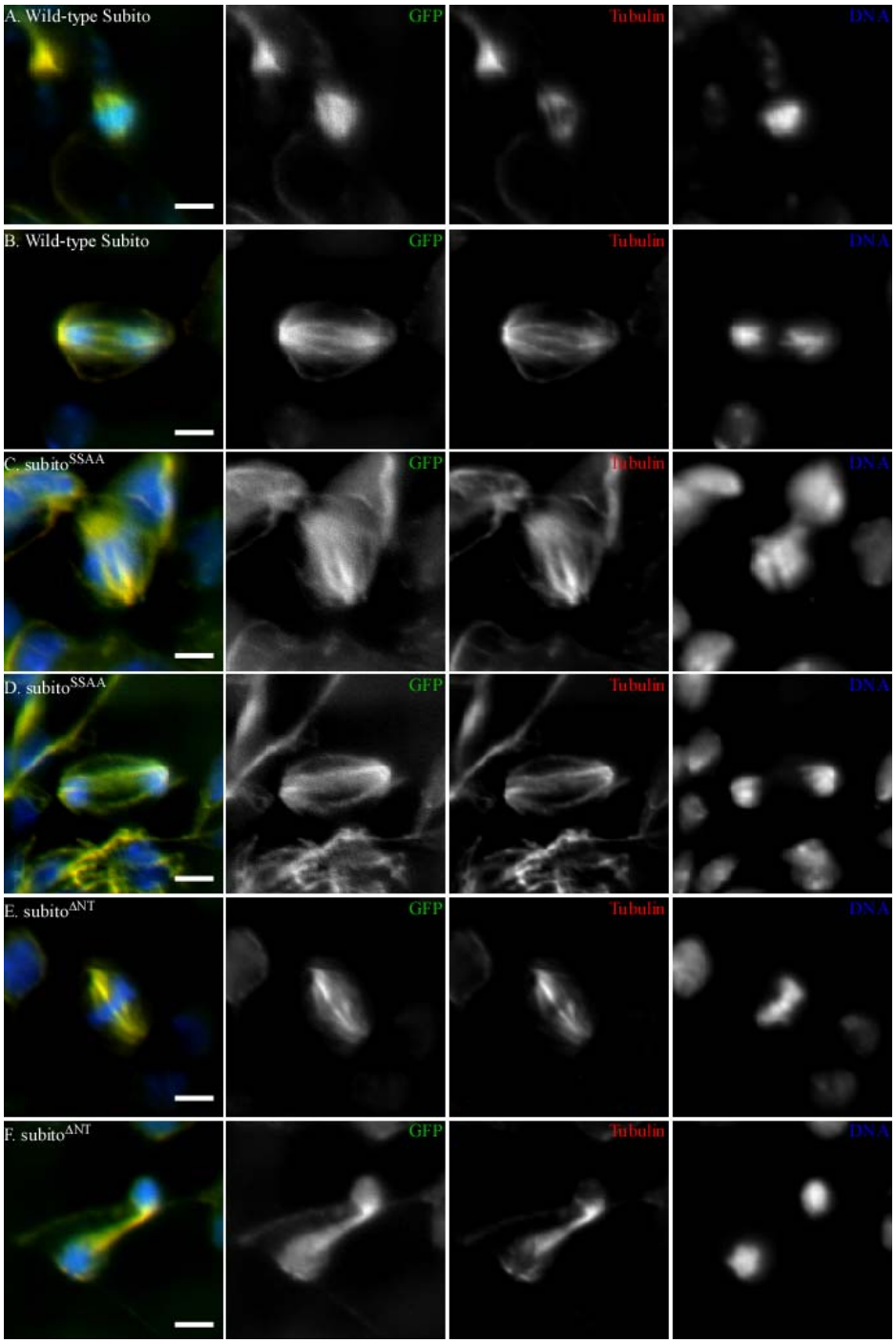

Figure 27 – Cytological analysis of *sub* mutant neuroblasts. For all experiments, *subito::GFP* transgenes were expressed using the *P{tubP -GAL4}* driver. A & B illustrate wild-type *Subito* neuroblasts

at metaphase and anaphase respectively. C & D illustrate *subito*^{SSAA} neuroblasts at metaphase and anaphase respectively. E & F illustrates *subito*$^{\Delta NT}$ neuroblasts at metaphase and anaphase respectively. The wild-type or mutant protein is in green, tubulin is in red, and DNA is in blue.

Since wild-type *Subito* transgenes exhibited normal function, we tested the *sub*^{SSAA} and *sub*$^{\Delta NT}$ constructs using this genetic assay. The expression of *sub*^{SSAA} had no dominant effects on meiotic spindle assembly. However, we proposed that the interaction between Polo and Subito might only occur in a mitotic setting. On the contrary, the expression of the *sub*^{SSAA} was capable of rescuing synthetic lethality for both *Incenp sub* (96.2%) and *ial sub* background (114.0%) (

Table 9). Cytological experiments in neuroblasts showed normal localization of the mutant protein to microtubules at metaphase (Figure 27C) and anaphase (Figure 27D). The analysis of mitotic spindle assembly and chromosomal organization revealed no gross abnormalities (Figure 27C & D). Because the substitution of these serines in the linker domain showed the ability to rescue synthetic lethality and cytological experiments were normal, we can safely assume that these two conserved serines do not function to negatively regulate the motor activity of Subito during mitosis. While this contrasts with results for MKLP2, it is possible that other serines in Subito linker domain interact with Polo and regulate bundling activity in *Drosophila*.

Expression of *sub*$^{\Delta NT}$ in stage 14 oocytes caused chromosome-independent spindle to form throughout the ooplasm (Jang, Rahman et al. 2007), suggesting that the N-terminus functions to negative regulate the motor activity of the kinesin. It is possible

that the N-terminus negative regulates motor activity in a mitotic setting as well. To test this hypothesis, we examined the ability of the mutant protein to rescue synthetic lethality. Expression of $sub^{\Delta NT}$ did not rescue synthetic lethality for either *Incenp sub* (0.0%) or *ial sub*(0.0 %) (Table 9). Coupling this result with the meiotic phenotype of $sub^{\Delta NT}$, suggests that the N-terminus negatively regulates motor activity during mitosis. In neuroblasts, the expression of $sub^{\Delta NT}$ in a wild-type background did not result in formation of ectopic spindles (Figure 27E). This result implies that the right conditions must be satisfied to induce spindle assembly, even when $SUB^{\Delta NT}$ is present. These conditions probably include nuclear envelope breakdown and activation of the maturation-promoting complex, RanGTP, and chromosomal passenger complex. $SUB^{\Delta NT}$ bound microtubules properly at the central spindle and did not appear to have any abnormalities in spindle assembly at either metaphase (Figure 27E) or anaphase (Figure 27F), when expressed in a wild-type background. This is an interesting contradiction, since expression of $sub^{\Delta NT}$ could not rescue synthetic lethality, suggesting it is not capable of functioning in a wild-type manner. It is likely that endogenous Subito is capable of regulating the mutant protein, preventing the formation of additional spindles. However, it is also possible that expression of $sub^{\Delta NT}$ causes mitotic non-disjunction; an observation which would not be visible using a uniform stain for DNA. If true, this suggests that $SUB^{\Delta NT}$ is not properly regulated during mitosis. On the other hand, the ability to build ectopic spindles during meiosis, but not mitotis, could be the result of spatial constrictions. The oocyte contains a higher volume of cytoplasm as compared to the neuroblast. This constriction in space may not allow spindles to form in the vicinity of the chromosomes. This reasoning was supported by the finding that

ectopic spindles do form in embryos expressing the *sub*$^{\Delta NT}$ construct. Embryonic divisions are centrosomal, further proving that centrosomes do not contribute to the inhibition of ectopic spindles. These results suggest that the negative regulation of Subito through the N-terminus is more important to acentrosomal spindle assembly in the oocyte.

Two additional mutant constructs of *subito,* tagged with HA, were also utilized to examine the role of the motor in mitotic spindle assembly. ATP hydrolysis is essential for kinesin movement and has been shown in other kinesins to cause a rigor phenotype where the kinesin can bind to microtubules, but is incapable of movement. The *sub*^{ATP} construct mutates the ATP binding domain of Subito*,* preventing ATP hydrolysis. Expression of *sub*^{ATP} in *Drosophila* oocytes yielded a similar result, in which the mutant protein bound the entire length of the meiotic spindle (Jang, Rahman et al. 2007). This phenotype was mimicked in neuroblast as SUB^{ATP} bound microtubules outside of the central spindle. The inability to localize tightly to the central spindle was observed at both metaphase (Figure 28C) and anaphase (Figure 28D), though no dominant effects on spindle assembly nor chromosomal organization were detected. Nonetheless, this result confirms that ATP hydrolysis is not necessary for microtubule binding, but essential for kinesin movement along the microtubule to the central spindle.

The second motor domain mutant, *sub*^{L6}, deletes the L6 loop within the motor, which is found exclusively within the kinesin-6 family. Very little is known about the function of this unique loop; however, it may function to regulate the motor activity of Subito and Pavarotti, the other member of the kinesin-6 family. Expression of *sub*^{L6} in

neuroblasts had no effects on spindle assembly or chromosome organization. However, similar to sub^{ATP}, the mutant protein failed to properly localize to the central spindle during metaphase (Figure 28E) and anaphase (Figure 28F). This result suggests that the L6 loop is important for the movement of the kinesin to the central spindle. However, it is also possible that simply disrupting the motor can have negative effects on the movement of the kinesin.

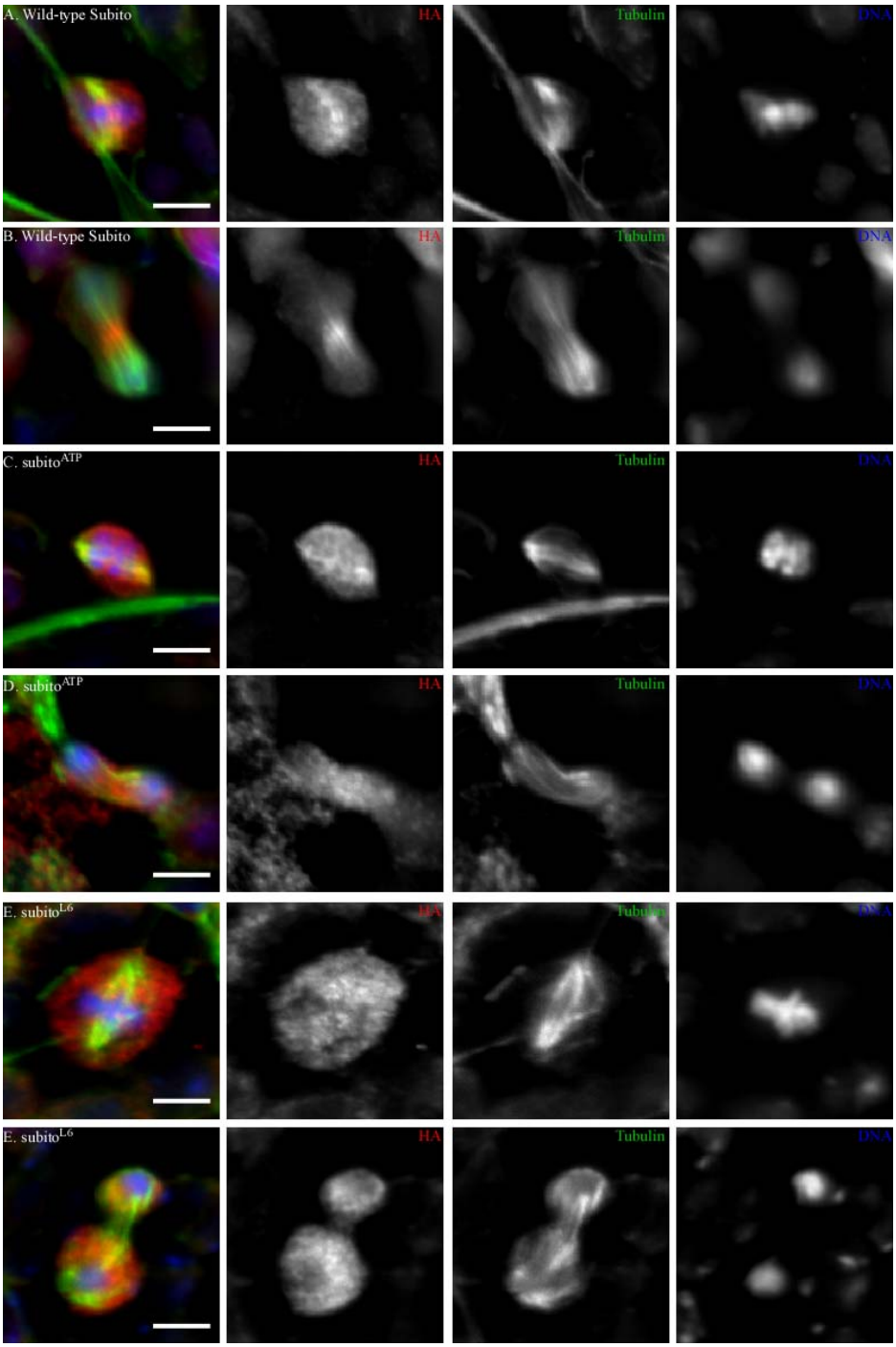

Figure 28 – Cytological analysis of *sub* mutant neuroblasts. For all experiments, *subito::HA t*ransgenes were expressed using the *P{ tubP -GAL4 }* driver. A & B illustrate wild-type *Subito* neuroblasts at metaphase and anaphase respectively. C & D illustrate *subito*^{ATP} neuroblasts at metaphase and anaphase respectively. E & F illustrates *subito*^{L6} neuroblasts at metaphase and anaphase respectively. The wild-type or mutant protein is in red, tubulin is in green, and DNA is in blue.

In conclusion, the regulation of Subito is a complex process. Simply removing the negative regulatory domain found within the N-terminus is insufficient to induce the formation of ectopic spindles during mitosis. Indeed, other factors are needed to assist Subito. These factors probably include nuclear envelope breakdown, maturation-promoting complex, RanGTP, and the chromosomal passenger complex. These findings suggest a model where Subito functions with other spindle assembly factors to assemble a spindle. Phosphorylation of the linker domain does not appear to negatively regulate Subito during mitosis. This is an interesting finding, since previous work demonstrated an interaction with of the linker domain with Polo like kinase 1. However, it is possible that other serines in the linker domain regulate the motor activity of Subito. Analysis of motor mutants during mitosis proves that ATP hydrolysis is essential for kinesin movement along the microtubules, and the L6 loop may function in a similar manner. Taken together, these results provide interesting insights into the regulation of Subito during mitosis. While this research was specific to the regulation of Subito, the mechanism of regulation and motor movement are applicable to a broad range of spindle assembly kinesins.

Appendix III: The C-terminus of Subito is necessary for microtubule interactions during acentrosomal spindle assembly

Subito is a member of the kinesin-6 family, which has been shown to bind and bundle microtubules at the central spindle during both meiosis and mitosis. The proper regulation of Subito occurs through the N-terminus, which has been shown to negative regulate the bundling activity of Subito, restricting acentrosomal spindle assembly to the karyosome. Additionally, serines in the neck domain of Subito have been shown to be important for the regulation of the bundling activity of MKLP2. By substituting these serines to alanines, the mutant protein was capable of bundling microtubules without first being activated by Polo like kinase 1. A genome wide mass spectrometry analysis in *Drosophila* embryos have shown that the C-terminus of Subito is phosphorylated. The phosphorylation of the C-terminus could regulate microtubule interactions with Subito, similarly to the phosphorylation of the neck region. To examine this possibility, two transgenes of *subito* were constructed that deleted that last 22 amino acids, $sub^{\Delta CT1}$, and the last 43 amino acids, $sub^{\Delta CT2}$. Both deletion constructs span the amino acid region predicted to be phosphorylated by mass spectrometry. Western blots established that both transgenes express evenly within the oocyte. Genetic analysis revealed normal levels of X chromosome non-disjunction for both mutants; however, expression of $sub^{\Delta CT1}$ or $sub^{\Delta CT2}$ did not rescue the *sub* null mutant. Cytological analysis of stage 14 oocytes showed a failure to localize to the central spindle for both mutant proteins. Since both transgenes expressed evenly within the ovary, an inability to bind microtubules by both mutant proteins suggests that phosphorylation of the C-terminus activates microtubule binding. However, since both deletions are in the vicinity of a coiled-coil domain, it is possible that this coiled-coil is essential for interactions with microtubules or spindle assembly factors, such as the chromosomal passenger complex.

The kinesins are a large family of motor proteins that promote unidirectional movement of a cargo along microtubules and several *Drosophila* kinesin proteins have been shown to play important roles in spindle assembly (Goshima and Vale 2003). In *Drosophila*, the kinesin-6 protein Subito has been shown to have a role in spindle

assembly. *subito* encodes the *Drosophila* homolog of MKLP2 and has an important role in organizing the meiotic acentrosomal (Jang, Rahman et al. 2005) and mitotic spindles (Cesario, Jang et al. 2006). In *Drosophila* oocytes, interpolar microtubules bundle during prometaphase, referred to as the metaphase I central spindle, which is a critical part of the acentrosomal spindle assembly pathway (Jang, Rahman et al. 2005). In *subito* null mutant oocytes, the central spindle is absent (Jang, Rahman et al. 2005), resulting in an abnormal number of spindle poles and high levels of meiotic non-disjunction (Giunta, Jang et al. 2002). Thus, Subito, and by inference the central spindle, is required to organize the acentrosomal spindle during *Drosophila* female meiosis. The central spindle forms before the microtubules are organized into a bipolar spindle and may function to direct the kinetochore microtubules toward one of the two poles. During mitotic metaphase, Subito may also organize interpolar microtubules but the effect of its absence is much more dramatic in meiosis, possibly because Subito activity is more critical in the absence of centrosomes.

Subito first appears on prometaphase meiotic spindles, suggesting it functions as the microtubules are recruited to the spindle. However, the mechanism that recruits to the chromosomes is poorly understood. The chromosomes could directly interact with microtubules via chromokinesin molecules (Mazumdar and Misteli 2005). Alternatively, the chromosomes could be the source of a signal, such as RanGTP (Clarke, Tang et al. 2005), which could activate microtubule assembly factors such as motor proteins. In either case, regulating kinesin proteins like Subito could be particularly important when centrosomes are absent and motor proteins may provide most of the organizing activity. Previous studies have characterized the role of the N-, motor, and C-terminal coiled coils

domains of Subito and found that regulating Subito activity is a critical component of organizing the acentrosomal spindle. This analysis has revealed that the C-terminal domain may interact independently with microtubules while the motor domain is required for maintaining the interaction with the anti-parallel microtubules (Jang, Rahman et al. 2005). Recent analysis using mass spectrometry showed that the C-terminus is phosphorylated in *Drosophila* embryos (Zhai, Hiesinger et al. 2003). These phosphorylations may regulate Subito interactions with microtubules. To investigate this possibility, two deletions in the C-terminus were created. Previous experiments have shown that the C-terminus is sensitive to deletions, as a complete deletion does not expressed in ovaries. To avoid a failure in expression, the two deletions were kept very small: the first deletion is 21 amino acids, $sub^{\Delta CT1}$, and the second deletion is 41 amino acids, $sub^{\Delta CT1}$ (Figure 29). Both constructs delete the predicted phosphorylation sites.

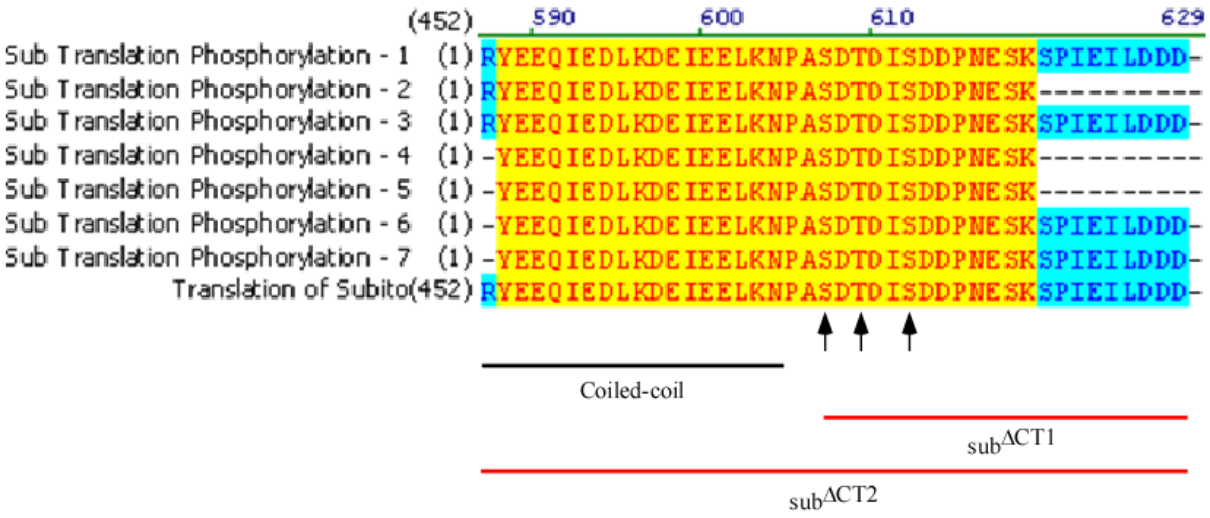

Figure 29 – Phosphorylated peptides of Subito aligned to a translation of wild-type Subito. The arrows point to the serines and threonines predicted to be phosphorylated. A coiled coil is predicted from amino acid 585 to 604. The red lines represent the two deletion of the last 21 amino acids, $sub^{\Delta CT1}$, and the last 41 amino acids, $sub^{\Delta CT2}$.

Transgenes were made by fusing the coding region of the wild-type *Subito* or mutant variants to three copies of the HA epitope tag at the N-terminus. They were also put under the control of the UASP promoter, which allows for germline expression regulated by a second transgene expressing GAL4 (Rorth 1998). For the experiments described below, the *UASP:sub* transgenes were expressed using the *P{GAL4::VP16-nos.UTR}MVD1* driver, which has *GAL4* fused to the *nanos* promoter and induces the expression of *UAS* containing transgenes in the female germline. Western experiments confirm that the both transgenes express evenly within the ovaries (Figure 30).

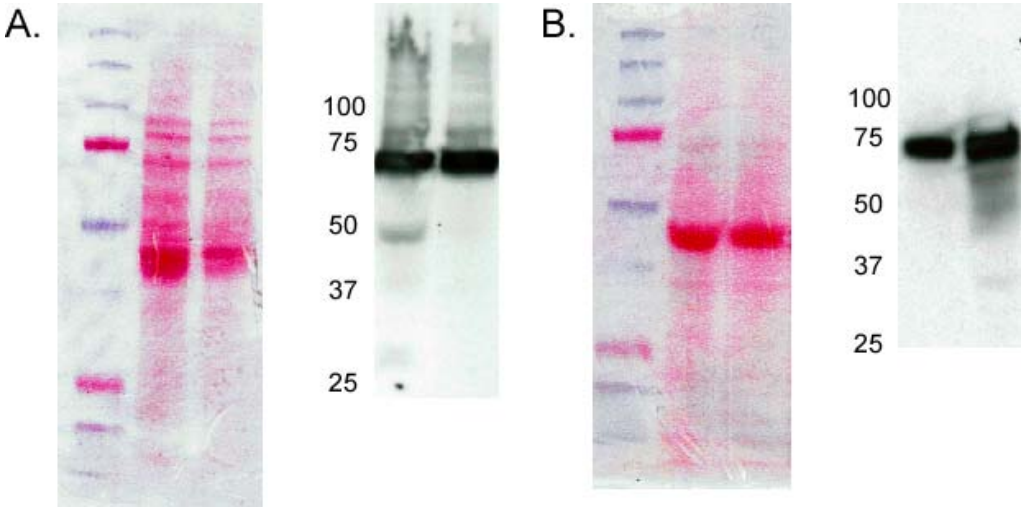

Figure 30 – Western blot of *Drosophila* ovary protein from transgenics expressing subito C-terminal deletions. $sub^{\Delta CT1}$ (A) and $sub^{\Delta CT2}$ (B) transgenes express at similar levels when induced with the *P{GAL4::VP-nos.UTR}MVD1* driver. Proteins were detected using an antibody the HA tag. To left of the blot is the Ponceau Red stained filter to indicate total protein loading.

Genetic assays were performed by measuring the frequency of X chromosome non-disjunction and fertility, in order to determine if the *subito* mutant disrupted meiotic chromosome segregation. Neither mutant transgenes tested had dominant effects on non-

disjunction or fertility (Table 10). Expression of a wild-type transgene of *Subito* in the female germline is capable of rescuing the sterility defect of a *sub* null mutant, producing progeny with low levels of non-disjunction (Jang, Rahman et al. 2007). Using this genetic assay, we analyzed the two C-terminal mutants. Neither construct rescued the *sub* null background (Table 11). A failure to rescue sterility shows that the mutant protein is incapable of performing the wild-type function of the protein. However, cytological analysis was necessary to determine the exact effect of the mutations.

Table 10

Fertility & non-disjunction phenotypes by C-terminus mutant transgenes

Transgene:	Total Flies:	Progeny / Female Parent:	Non-Disjunction:
Subito	1632	40.8	0.00%
$sub^{\Delta CT1}$	1632	40.8	0.00%
$sub^{\Delta CT2}$	1531	31.9	0.26%

Each transgene was expressed by crossing to the *P{GAL4::VP16-nos.UTR}MVD1* driver. These females were crossed to *y w/* $B^{S}Y$ males to assay non-disjunction and fertility.

Each transgene consists of at least two insertions, both of which gave similar results.

Table 11

Rescue of *sub* null sterility by C-terminus mutant transgenes

Transgene:	Total Flies:	Progeny / Female Parent:	Non-Disjunction:
Subito	850	42.5	0.24%
$sub^{\Delta CT1}$	0	0.0	**Sterile**
$sub^{\Delta CT2}$	0	0.0	**Sterile**

Each transgene was expressed by crossing to the *P{GAL4::VP16-nos.UTR}MVD1* driver in a *sub* null background. These females were crossed to *y w/* $B^{S}Y$ males to assay non-disjunction and fertility.

Each transgene consists of at least two insertions, both of which gave similar results.

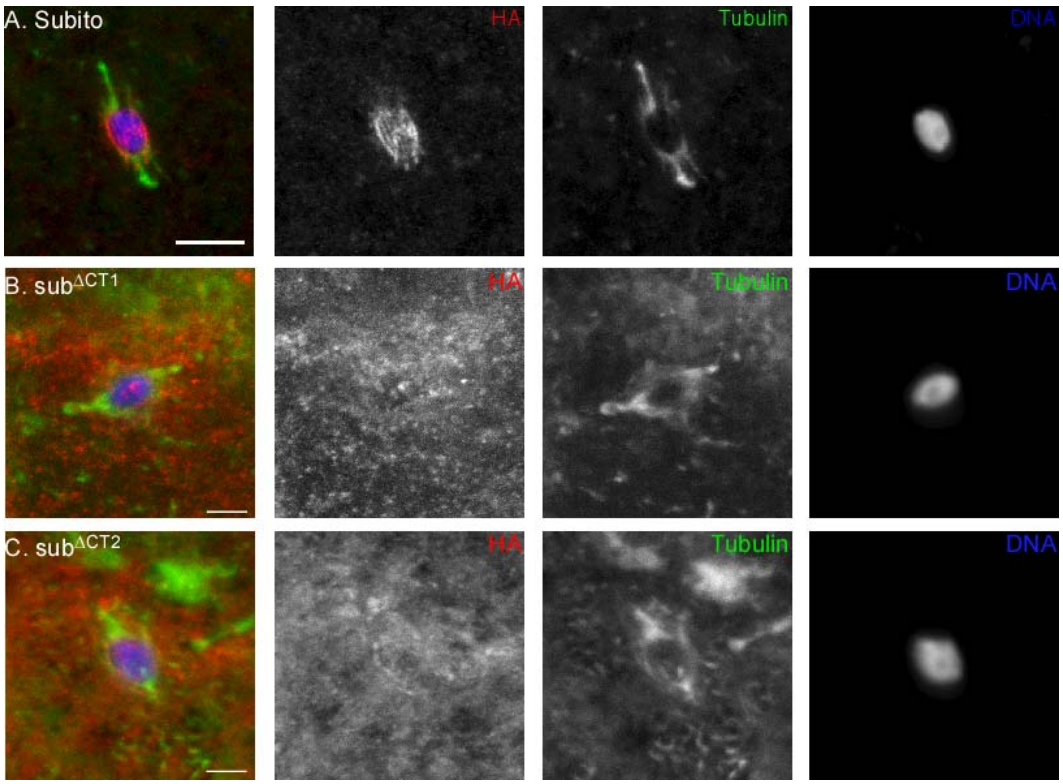

Figure 31 – N-terminus deletion constructs expressed in stage 14 oocytes including, (A) wild-type *Subito,* (B) *sub*$^{\Delta CT1}$, and (C) *sub*$^{\Delta CT2}$. *subito* tagged with HA is in red, Tubulin is in green, and DNA is in blue.

Expression of *sub*$^{\Delta CT1}$ and *sub*$^{\Delta CT2}$ in stage 14 oocytes resulted in wild-type appearance for both spindle assembly and karyosome organization (Figure 31). Interestingly, neither mutant protein was capable of localizing to the central spindle. Since both transgenes expressed in ovaries (Figure 30), this result suggests the phosphorylation event in the C-terminus activates microtubule binding. An inability to activate this activity in both mutant proteins helps to explain the failure to rescue *sub* by both transgenes. It is important to note that that the *sub*$^{\Delta CT1}$ deletions occur in the vicinity of a coiled-coil, while *sub*$^{\Delta CT2}$ completely deletes the coiled-coil (Figure 29). A coiled-

coil is a structural motif that has been implicated in a wide array of functions (Mason and Arndt 2004). For both MKLP2 and Subito, these functions include interactions with microtubules and the chromosomal passenger complex (Gruneberg, Neef et al. 2004) (Jang, Rahman et al. 2005). Preliminary evidence suggests that the C-terminus can dimerize to other Subito molecules (data not shown). Hence, failures to bind microtubules, interact with the chromosomal passenger complex, or dimerize could result in $SUB^{\Delta CT1}$ and $SUB^{\Delta CT2}$ inactivation. Further analysis of the C-terminus would be necessary to determine if phosphorylation of the C-terminus activates Subito or if the coiled-coil domain is essential for microtubule binding.

In conclusion, kinesins are complex machines, with a motor flanked by an N- and C-terminal domain. Due to the presence of coiled-coils, the C-terminus has been linked to the dimerization of Subito, and interactions with microtubules and the chromosomal passenger complex (Echard, Jollivet et al. 1998; Gruneberg, Neef et al. 2004). Our results here suggest that the phosphorylation of the C-terminus activates microtubule binding. However, since both $sub^{\Delta CT1}$ and $sub^{\Delta CT2}$ have deletions near a coiled-coil domain, it is possible that these deletions prevented protein interactions with microtubules or the chromosomal passenger complex. Further analysis will be necessary to isolate the regions of the C-terminus that regulate microtubule binding.

Literature Cited

Adams, R. R., H. Maiato, et al. (2001). "Essential roles of *Drosophila* inner centromere protein (INCENP) and aurora B in histone H3 phosphorylation, metaphase chromosome alignment, kinetochore disjunction, and chromosome segregation." J Cell Biol **153**(4): 865-80.

Adams, R. R., A. A. Tavares, et al. (1998). "*pavarotti* encodes a kinesin-like protein required to organize the central spindle and contractile ring for cytokinesis." Genes Dev **12**(10): 1483-94.

Askjaer, P., V. Galy, et al. (2002). "Ran GTPase cycle and importins alpha and beta are essential for spindle formation and nuclear envelope assembly in living *Caenorhabditis* elegans embryos." Mol Biol Cell **13**(12): 4355-70.

Bamba, C., Y. Bobinnec, et al. (2002). "The GTPase Ran regulates chromosome positioning and nuclear envelope assembly in vivo." Curr Biol **12**(6): 503-7.

Barros, T. P., K. Kinoshita, et al. (2005). "Aurora A activates D-TACC-Msps complexes exclusively at centrosomes to stabilize centrosomal microtubules." J Cell Biol **170**(7): 1039-46.

Basto, R., R. Gomes, et al. (2000). "Rough deal and Zw10 are required for the metaphase checkpoint in *Drosophila*." Nat Cell Biol **2**(12): 939-43.

Basu, J., H. Bousbaa, et al. (1999). "Mutations in the essential spindle checkpoint gene bub1 cause chromosome missegregation and fail to block apoptosis in *Drosophila*." J Cell Biol **146**(1): 13-28.

Becker, M., A. Stolz, et al. "Centromere localization of INCENP-aurora B is sufficient to support spindle checkpoint function." Cell Cycle **9**(7).

Bischoff, F. R., C. Klebe, et al. (1994). "RanGAP1 induces GTPase activity of nuclear Ras-related Ran." Proc Natl Acad Sci U S A **91**(7): 2587-91.

Bischoff, F. R. and H. Ponstingl (1991). "Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1." Nature **354**(6348): 80-2.

Blower, M. D. and G. H. Karpen (2001). "The role of *Drosophila* CID in kinetochore formation, cell-cycle progression and heterochromatin interactions." Nat Cell Biol **3**(8): 730-9.

Bodenmiller, B., J. Malmstrom, et al. (2007). "PhosphoPep--a phosphoproteome resource for systems biology research in *Drosophila* Kc167 cells." Mol Syst Biol **3**: 139.

Bonaccorsi, S., M. G. Giansanti, et al. (2000). "Spindle assembly in *Drosophila* neuroblasts and ganglion mother cells." Nat Cell Biol **2**(1): 54-6.

Brust-Mascher, I., P. Sommi, et al. (2009). "Kinesin-5-dependent poleward flux and spindle length control in *Drosophila* embryo mitosis." Mol Biol Cell **20**(6): 1749-62.

Cai, D., A. D. Hoppe, et al. (2007). "Kinesin-1 structural organization and conformational changes revealed by FRET stoichiometry in live cells." J Cell Biol **176**(1): 51-63.

Carazo-Salas, R. E., G. Guarguaglini, et al. (1999). "Generation of GTP-bound Ran by RCC1 is required for chromatin-induced mitotic spindle formation." Nature **400**(6740): 178-81.

Carmena, M., M. G. Riparbelli, et al. (1998). "*Drosophila* polo kinase is required for cytokinesis." J Cell Biol **143**(3): 659-71.

Castrillon, D. H. and S. A. Wasserman (1994). "Diaphanous is required for cytokinesis in *Drosophila* and shares domains of similarity with the products of the limb deformity gene." Development **120**(12): 3367-77.

Caudron, M., G. Bunt, et al. (2005). "Spatial coordination of spindle assembly by chromosome-mediated signaling gradients." Science **309**(5739): 1373-6.

Cesario, J. M., J. K. Jang, et al. (2006). "Kinesin 6 family member Subito participates in mitotic spindle assembly and interacts with mitotic regulators." J Cell Sci **119**(Pt 22): 4770-80.

Chang, C., S. Goulding, et al. (2006). "INCENP is required for cytokinesis and asymmetric cell division during development of the nervouse system." J. Cell. Sci. **119**: 1144-1153.
Clarke, A. S., T. T. Tang, et al. (2005). "POLO kinase regulates the *Drosophila* centromere cohesion protein MEI-S332." Dev Cell **8**(1): 53-64.
Clarke, P. R. and C. Zhang (2008). "Spatial and temporal coordination of mitosis by Ran GTPase." Nat Rev Mol Cell Biol **9**(6): 464-77.
Colombie, N., C. F. Cullen, et al. (2008). "Dual roles of Incenp crucial to the assembly of the acentrosomal metaphase spindle in female meiosis." Development **135**(19): 3239-46.
Compton, D. A. (2000). "Spindle assembly in animal cells." Annu Rev Biochem **69**: 95-114.
Coquelle, F. M., B. Vitre, et al. (2009). "Structural basis of EB1 effects on microtubule dynamics." Biochem Soc Trans **37**(Pt 5): 997-1001.
Cullen, C. F. and H. Ohkura (2001). "Msps protein is localized to acentrosomal poles to ensure bipolarity of *Drosophila* meiotic spindles." Nature Cell. Biol. **3**: 637-642.
D'Avino, P. P., M. S. Savoian, et al. (2005). "Cleavage furrow formation and ingression during animal cytokinesis: a microtubule legacy." J Cell Sci **118**(Pt 8): 1549-58.
Dagenbach, E. M. and S. A. Endow (2004). "A new kinesin tree." J Cell Sci **117**(Pt 1): 3-7.
Dasso, M., T. Seki, et al. (1994). "A mutant form of the Ran/TC4 protein disrupts nuclear function in *Xenopus* laevis egg extracts by inhibiting the RCC1 protein, a regulator of chromosome condensation." Embo J **13**(23): 5732-44.
Donaldson, M. M., A. A. Tavares, et al. (2001). "Metaphase arrest with centromere separation in polo mutants of *Drosophila*." J Cell Biol **153**(4): 663-76.
Dumont, J., S. Petri, et al. (2007). "A centriole- and RanGTP-independent spindle assembly pathway in meiosis I of vertebrate oocytes." J Cell Biol **176**(3): 295-305.
Earnshaw, W. C. and C. A. Cooke (1991). "Analysis of the distribution of the INCENPs throughout mitosis reveals the existence of a pathway of structural changes in the chromosomes during metaphase and early events in cleavage furrow formation." J Cell Sci **98 (Pt 4)**: 443-61.
Echard, A., G. R. Hickson, et al. (2004). "Terminal cytokinesis events uncovered after an RNAi screen." Curr Biol **14**(18): 1685-93.
Echard, A., F. Jollivet, et al. (1998). "Interaction of a Golgi-associated kinesin-like protein with Rab6." Science **279**(5350): 580-5.
Eckley, D. M., A. M. Ainsztein, et al. (1997). "Chromosomal proteins and cytokinesis: patterns of cleavage furrow formation and inner centromere protein positioning in mitotic heterokaryons and mid-anaphase cells." J Cell Biol **136**(6): 1169-83.
Eggert, U. S., A. A. Kiger, et al. (2004). "Parallel chemical genetic and genome-wide RNAi screens identify cytokinesis inhibitors and targets." PLoS Biol **2**(12): e379.
Endow, S. A. and D. J. Komma (1997). "Spindle dynamics during meiosis in *Drosophila* oocytes." J Cell Biol **137**(6): 1321-36.
Ferenz, N. P., A. Gable, et al. (2010). "Mitotic functions of kinesin-5." Semin Cell Dev Biol **21**(3): 255-9.
Fontijn, R. D., B. Goud, et al. (2001). "The human kinesin-like protein RB6K is under tight cell cycle control and is essential for cytokinesis." Mol Cell Biol **21**(8): 2944-55.
Frasch, M. (1991). "The maternally expressed *Drosophila* gene encoding the chromatin-binding protein BJ1 is a homolog of the vertebrate gene Regulator of Chromatin Condensation, RCC1." Embo J **10**(5): 1225-36.
Gergely, F., D. Kidd, et al. (2000). "D-TACC: a novel centrosomal protein required for normal spindle function in early *Drosophila* embryo." EMBO J. **19**: 241-252.
Giansanti, M. G., S. Bonaccorsi, et al. (1998). "Cooperative interactions between the central spindle and the contractile ring during *Drosophila* cytokinesis." Genes Dev **12**(3): 396-410.

Giansanti, M. G., M. Gatti, et al. (2001). "The role of centrosomes and astral microtubules during asymmetric division of *Drosophila* neuroblasts." Development **128**(7): 1137-45.
Giet, R. and D. M. Glover (2001). "*Drosophila* aurora B kinase is required for histone H3 phosphorylation and condensin recruitment during chromosome condensation and to organize the central spindle during cytokinesis." J Cell Biol **152**(4): 669-82.
Giet, R., D. McLean, et al. (2002). "*Drosophila* Aurora A kinase is required to localize D-TACC to centrosomes and to regulate astral microtubules." J Cell Biol **156**(3): 437-51.
Giunta, K. L., J. K. Jang, et al. (2002). "subito encodes a kinesin-like protein required for meiotic spindle pole formation in *Drosophila* melanogaster." Genetics **160**(4): 1489-501.
Giunta, K. L., J. K. Jang, et al. (2002). "*subito* encodes a kinesin-like protein required for meiotic spindle pole formation in *Drosophila melanogaster*." Genetics **160**: 1489-1501.
Glover, D. M. (1989). "Mitosis in *Drosophila*." J Cell Sci **92 (Pt 2)**: 137-46.
Glover, D. M., M. H. Leibowitz, et al. (1995). "Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles." Cell **81**(1): 95-105.
Goshima, G., F. Nedelec, et al. (2005). "Mechanisms for focusing mitotic spindle poles by minus end-directed motor proteins." J Cell Biol **171**(2): 229-40.
Goshima, G. and R. D. Vale (2003). "The roles of microtubule-based motor proteins in mitosis: comprehensive RNAi analysis in the *Drosophila* S2 cell line." J Cell Biol **162**(6): 1003-16.
Goshima, G., R. Wollman, et al. (2005). "Length control of the metaphase spindle." Curr Biol **15**(22): 1979-88.
Goto, H., T. Kiyono, et al. (2006). "Complex formation of Plk1 and INCENP required for metaphase-anaphase transition." Nat Cell Biol **8**(2): 180-7.
Gruber, S., C. H. Haering, et al. (2003). "Chromosomal cohesin forms a ring." Cell **112**(6): 765-77.
Gruneberg, U., R. Neef, et al. (2004). "Relocation of Aurora B from centromeres to the central spindle at the metaphase to anaphase transition requires MKlp2." J Cell Biol **166**(2): 167-72.
Gunsalus, K. C., S. Bonaccorsi, et al. (1995). "Mutations in twinstar, a *Drosophila* gene encoding a cofilin/ADF homologue, result in defects in centrosome migration and cytokinesis." J Cell Biol **131**(5): 1243-59.
Gurley, L. R., J. A. D'Anna, et al. (1978). "Histone phosphorylation and chromatin structure during mitosis in Chinese hamster cells." Eur J Biochem **84**(1): 1-15.
Hallen, M. A., Z. Y. Liang, et al. (2008). "Ncd motor binding and transport in the spindle." J Cell Sci **121**(Pt 22): 3834-41.
Hatsumi, M. and S. A. Endow (1992). "The *Drosophila* ncd microtubule motor protein is spindle-associated in meiotic and mitotic cells." Journal of Cell Science **103**: 1013-1020.
Henikoff, S., K. Ahmad, et al. (2000). "Heterochromatic deposition of centromeric histone H3-like proteins." Proc Natl Acad Sci U S A **97**(2): 716-21.
Hodges, C. A., E. Revenkova, et al. (2005). "SMC1beta-deficient female mice provide evidence that cohesins are a missing link in age-related nondisjunction." Nat Genet **37**(12): 1351-5.
Hutten, S., A. Flotho, et al. (2008). "The Nup358-RanGAP complex is required for efficient importin alpha/beta-dependent nuclear import." Mol Biol Cell **19**(5): 2300-10.
Jang, J. K., T. Rahman, et al. (2007). "Misregulation of the Kinesin-like Protein Subito Induces Meiotic Spindle Formation in the Absence of Chromosomes and Centrosomes." Genetics **177**(1): 267-80.
Jang, J. K., T. Rahman, et al. (2005). "The kinesin-like protein Subito contributes to central spindle assembly and organization of the meiotic spindle in *Drosophila* oocytes." Mol. Biol. Cell **16**(10): 4684-94.

Jang, J. K., T. Rahman, et al. (2005). "The kinesinlike protein Subito contributes to central spindle assembly and organization of the meiotic spindle in *Drosophila* oocytes." Mol Biol Cell **16**(10): 4684-94.

Kahana, J. A. and D. W. Cleveland (1999). "Beyond nuclear transport. Ran-GTP as a determinant of spindle assembly." J Cell Biol **146**(6): 1205-10.

Kalab, P. and R. Heald (2008). "The RanGTP gradient - a GPS for the mitotic spindle." J Cell Sci **121**(Pt 10): 1577-86.

Kalab, P., A. Pralle, et al. (2006). "Analysis of a RanGTP-regulated gradient in mitotic somatic cells." Nature **440**(7084): 697-701.

Kalab, P., R. T. Pu, et al. (1999). "The ran GTPase regulates mitotic spindle assembly." Curr Biol **9**(9): 481-4.

Kaldis, P. and E. Aleem (2005). "Cell cycle sibling rivalry: Cdc2 vs. Cdk2." Cell Cycle **4**(11): 1491-4.

Karsenti, E. and I. Vernos (2001). "The mitotic spindle: a self-made machine." Science **294**(5542): 543-7.

Khodjakov, A., L. Copenagle, et al. (2003). "Minus-end capture of preformed kinetochore fibers contributes to spindle morphogenesis." J Cell Biol **160**(5): 671-83.

Kiger, A. A., B. Baum, et al. (2003). "A functional genomic analysis of cell morphology using RNA interference." J Biol **2**(4): 27.

Klapper, M., K. Exner, et al. (1997). "Assembly of A- and B-type lamins studied in vivo with the baculovirus system." J Cell Sci **110 (Pt 20)**: 2519-32.

Kramer, J. and R. S. Hawley (2003). "The spindle-associated transmembrane protein Axs identifies a membranous structure ensheathing the meiotic spindle." Nat Cell Biol **5**(3): 261-3.

Kusano, A., C. Staber, et al. (2001). "Nuclear mislocalization of enzymatically active RanGAP causes segregation distortion in *Drosophila*." Dev Cell **1**(3): 351-61.

Kwon, M., S. Morales-Mulia, et al. (2004). "The chromokinesin, KLP3A, dives mitotic spindle pole separation during prometaphase and anaphase and facilitates chromatid motility." Mol Biol Cell **15**(1): 219-33.

Lawrence, C. J., R. K. Dawe, et al. (2004). "A standardized kinesin nomenclature." J Cell Biol **167**(1): 19-22.

Lee, J. R., H. Shin, et al. (2004). "An intramolecular interaction between the FHA domain and a coiled coil negatively regulates the kinesin motor KIF1A." Embo J **23**(7): 1506-15.

Lee, T. and L. Luo (1999). "Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis." Neuron **22**(3): 451-61.

Lindqvist, A., V. Rodriguez-Bravo, et al. (2009). "The decision to enter mitosis: feedback and redundancy in the mitotic entry network." J Cell Biol **185**(2): 193-202.

Liu, J., D. W. Taylor, et al. (2006). "Three-dimensional structure of the myosin V inhibited state by cryoelectron tomography." Nature **442**(7099): 208-11.

Llamazares, S., A. Moreira, et al. (1991). "*polo* encodes a protein kinase homolog required for mitosis in *Drosophila*." Genes & Development **5**: 2153-2165.

Logarinho, E., H. Bousbaa, et al. (2004). "Different spindle checkpoint proteins monitor microtubule attachment and tension at kinetochores in *Drosophila* cells." J Cell Sci **117**(Pt 9): 1757-71.

Logarinho, E. and C. E. Sunkel (1998). "The *Drosophila* POLO kinase localises to multiple compartments of the mitotic apparatus and is required for the phosphorylation of MPM2 reactive epitopes." J Cell Sci **111 (Pt 19)**: 2897-909.

Lukinova, N. I., V. V. Roussakova, et al. (1999). "Genetic characterization of cytological region 77A-D harboring the presenilin gene of *Drosophila* melanogaster." Genetics **153**(4): 1789-97.

Maiato, H., C. L. Rieder, et al. (2004). "Kinetochore-driven formation of kinetochore fibers contributes to spindle assembly during animal mitosis." J Cell Biol **167**(5): 831-40.
Manchado, E., M. Eguren, et al. (2010). "The anaphase-promoting complex/cyclosome (APC/C): cell-cycle-dependent and -independent functions." Biochem Soc Trans **38**(Pt 1): 65-71.
Maresca, T. J., A. C. Groen, et al. (2009). "Spindle assembly in the absence of a RanGTP gradient requires localized CPC activity." Curr Biol **19**(14): 1210-5.
Martin, D. S., R. Fathi, et al. (2010). "FRET measurements of kinesin neck orientation reveal a structural basis for processivity and asymmetry." Proc Natl Acad Sci U S A **107**(12): 5453-8.
Mason, J. M. and K. M. Arndt (2004). "Coiled coil domains: stability, specificity, and biological implications." Chembiochem **5**(2): 170-6.
Mastronarde, D. N., K. L. McDonald, et al. (1993). "Interpolar spindle microtubules in PTK cells." J Cell Biol **123**(6 Pt 1): 1475-89.
Matthies, H. J., H. B. McDonald, et al. (1996). "Anastral meiotic spindle morphogenesis: role of the *non-claret disjunctional* kinesin-like protein." J Cell Biol **134**(2): 455-64.
Matuliene, J. and R. Kuriyama (2002). "Kinesin-like protein CHO1 is required for the formation of midbody matrix and the completion of cytokinesis in mammalian cells." Mol Biol Cell **13**(6): 1832-45.
Mazumdar, M. and T. Misteli (2005). "Chromokinesins: multitalented players in mitosis." Trends Cell Biol **15**(7): 349-55.
McKim, K. S., J. K. Jang, et al. (1993). "Mechanical basis of meiotic metaphase arrest." Nature **362**: 364-366.
McKim, K. S., E. F. Joyce, et al. (2009). "Cytological analysis of meiosis in fixed *Drosophila* ovaries." Methods Mol Biol **558**: 197-216.
Megraw, T. L., L. R. Kao, et al. (2001). "Zygotic development without functional mitotic centrosomes." Curr Biol **11**(2): 116-20.
Miki, H., Y. Okada, et al. (2005). "Analysis of the kinesin superfamily: insights into structure and function." Trends Cell Biol **15**(9): 467-76.
Minestrini, G., A. S. Harley, et al. (2003). "Localization of Pavarotti-KLP in living *Drosophila* embryos suggests roles in reorganizing the cortical cytoskeleton during the mitotic cycle." Mol Biol Cell **14**(10): 4028-38.
Misulovin, Z., Y. B. Schwartz, et al. (2008). "Association of cohesin and Nipped-B with transcriptionally active regions of the *Drosophila* melanogaster genome." Chromosoma **117**(1): 89-102.
Moore, D. P., A. W. Page, et al. (1998). "The cohesion protein MEI-S332 localizes to condensed meiotic and mitotic centromeres until sister chromatids separate." J Cell Biol **140**(5): 1003-12.
Moore, J. D. (2001). "The Ran-GTPase and cell-cycle control." Bioessays **23**(1): 77-85.
Moore, M. S. and G. Blobel (1993). "The GTP-binding protein Ran/TC4 is required for protein import into the nucleus." Nature **365**(6447): 661-3.
Moroianu, J. (1997). "Molecular mechanisms of nuclear protein transport." Crit Rev Eukaryot Gene Expr **7**(1-2): 61-72.
Moutinho-Santos, T., P. Sampaio, et al. (1999). "In vivo localisation of the mitotic POLO kinase shows a highly dynamic association with the mitotic apparatus during early embryogenesis in *Drosophila*." Biol. Cell **91**: 585-596.
Nasmyth, K. (2001). "Disseminating the genome: Joining, resolving, and separating sister chromatids during mitosis and meiosis." Ann. Rev. Genet. **35**: 673-745.
Neef, R., U. R. Klein, et al. (2006). "Cooperation between Mitotic Kinesins Controls the Late Stages of Cytokinesis." Curr Biol **16**(3): 301-7.
Neef, R., C. Preisinger, et al. (2003). "Phosphorylation of mitotic kinesin-like protein 2 by polo-like kinase 1 is required for cytokinesis." J Cell Biol **162**(5): 863-75.

Nislow, C., V. A. Lombillo, et al. (1992). "A plus-end-directed motor enzyme that moves antiparallel microtubules in vitro localizes to the interzone of mitotic spindles." Nature **359**(6395): 543-7.

Ohba, T., M. Nakamura, et al. (1999). "Self-organization of microtubule asters induced in *Xenopus* egg extracts by GTP-bound Ran." Science **284**(5418): 1356-8.

Parks, A. L., K. R. Cook, et al. (2004). "Systematic generation of high-resolution deletion coverage of the *Drosophila* melanogaster genome." Nat Genet **36**(3): 288-92.

Parry, D. H., G. R. Hickson, et al. (2003). "Cyclin B destruction triggers changes in kinetochore behavior essential for successful anaphase." Curr Biol **13**(8): 647-53.

Pesin, J. A. and T. L. Orr-Weaver (2008). "Regulation of APC/C activators in mitosis and meiosis." Annu Rev Cell Dev Biol **24**: 475-99.

Peter, A., P. Schottler, et al. (2002). "Mapping and identification of essential gene functions on the X chromosome of *Drosophila*." EMBO Rep **3**(1): 34-8.

Pufall, M. A. and B. J. Graves (2002). "Autoinhibitory domains: modular effectors of cellular regulation." Annu Rev Cell Dev Biol **18**: 421-62.

Raich, W. B., A. N. Moran, et al. (1998). "Cytokinesis and midzone microtubule organization in *Caenorhabditis* elegans require the kinesin-like protein ZEN-4." Mol Biol Cell **9**(8): 2037-49.

Resnick, T. D., K. J. Dej, et al. (2009). "Mutations in the chromosomal passenger complex and the condensin complex differentially affect synaptonemal complex disassembly and metaphase I configuration in *Drosophila* female meiosis." Genetics **181**(3): 875-87.

Resnick, T. D., D. L. Satinover, et al. (2006). "INCENP and Aurora B promote meiotic sister chromatid cohesion through localization of the Shugoshin MEI-S332 in *Drosophila*." Dev Cell **11**(1): 57-68.

Rorth, P. (1998). "Gal4 in the *Drosophila* female germline." Mech Dev **78**(1-2): 113-8.

Rothwell, W. F. and W. Sullivan (2000). Fluorescent analysis of *Drosophila* embryos. *Drosophila* Protocols. W. Sullivan, M. Ashburner and R. S. Hawley. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press**:** 141-157.

Sampath, S. C., R. Ohi, et al. (2004). "The chromosomal passenger complex is required for chromatin-induced microtubule stabilization and spindle assembly." Cell **118**(2): 187-202.

Schupbach, T. and E. Wieschaus (1989). "Female sterile mutations on the second chromosome of *Drosophila melanogaster*. I. Maternal effect mutations." Genetics **121**: 101-117.

Sharp, D. J., K. L. McDonald, et al. (1999). "The bipolar kinesin, KLP61F, cross-links microtubules within interpolar microtubule bundles of *Drosophila* embryonic mitotic spindles." J Cell Biol **144**(1): 125-38.

Silverman-Gavrila, R. V. and A. Wilde (2006). "Ran is required before metaphase for spindle assembly and chromosome alignment and after metaphase for chromosome segregation and spindle midbody organization." Mol Biol Cell **17**(4): 2069-80.

Skold, H. N., D. J. Komma, et al. (2005). "Assembly pathway of the anastral *Drosophila* oocyte meiosis I spindle." J Cell Sci **118**(Pt 8): 1745-55.

Smith, S. K., S. L. Jaspersen, et al. (2008). "Matrimony ties Polo down: can this kinase get free?" Cell Cycle **7**(6): 698-701.

Somers, W. G. and R. Saint (2003). "A RhoGEF and Rho family GTPase-activating protein complex links the contractile ring to cortical microtubules at the onset of cytokinesis." Dev Cell **4**(1): 29-39.

Sugiyama, K., K. Sugiura, et al. (2002). "Aurora-B associated protein phosphatases as negative regulators of kinase activation." Oncogene **21**(20): 3103-11.

Tan, S., E. Lyulcheva, et al. (2008). "Mars promotes dTACC dephosphorylation on mitotic spindles to ensure spindle stability." J Cell Biol **182**(1): 27-33.

Tanaka, T. U., N. Rachidi, et al. (2002). "Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections." Cell **108**(3): 317-29.

Tao, L., A. Mogilner, et al. (2006). "A homotetrameric kinesin-5, KLP61F, bundles microtubules and antagonizes Ncd in motility assays." Curr Biol **16**(23): 2293-302.

Theurkauf, W. E. and R. S. Hawley (1992). "Meiotic spindle assembly in *Drosophila* females: behavior of nonexchange chromosomes and the effects of mutations in the *nod* kinesin-like protein." Journal of Cell Biology **116**: 1167-1180.

Timinszky, G., L. Tirian, et al. (2002). "The importin-beta P446L dominant-negative mutant protein loses RanGTP binding ability and blocks the formation of intact nuclear envelope." J Cell Sci **115**(Pt 8): 1675-87.

Tirian, L., J. Puro, et al. (2000). "The Ketel(D) dominant-negative mutations identify maternal function of the *Drosophila* importin-beta gene required for cleavage nuclei formation." Genetics **156**(4): 1901-12.

Trieselmann, N. and A. Wilde (2002). "Ran localizes around the microtubule spindle in vivo during mitosis in *Drosophila* embryos." Curr Biol **12**(13): 1124-9.

Tseng, B. S., L. Tan, et al. (2010). "Dual Detection of Chromosomes and Microtubules by the Chromosomal Passenger Complex Drives Spindle Assembly." Dev Cell **18**(6): 903-912.

van den Wildenberg, S. M., L. Tao, et al. (2008). "The homotetrameric kinesin-5 KLP61F preferentially crosslinks microtubules into antiparallel orientations." Curr Biol **18**(23): 1860-4.

Van Doren, M., A. L. Williamson, et al. (1998). "Regulation of zygotic gene expression in *Drosophila* primordial germ cells." Curr Biol **8**(4): 243-6.

Verni, F., M. P. Somma, et al. (2004). "Feo, the *Drosophila* homolog of PRC1, is required for central-spindle formation and cytokinesis." Curr Biol **14**(17): 1569-75.

Vernos, I. (2004). "The chromosomal passenger complex takes center stage during mitosis." Dev Cell **7**(2): 145-6.

Vogt, E., M. Kirsch-Volders, et al. (2008). "Spindle formation, chromosome segregation and the spindle checkpoint in mammalian oocytes and susceptibility to meiotic error." Mutat Res **651**(1-2): 14-29.

von Wettstein, D. (1984). "The synaptonemal complex and genetic segregation." Symp Soc Exp Biol **38**: 195-231.

Wadsworth, P. and A. Khodjakov (2004). "E pluribus unum: towards a universal mechanism for spindle assembly." Trends Cell Biol **14**(8): 413-9.

Walczak, C. E., I. Vernos, et al. (1998). "A model for the proposed roles of different microtubule-based motor proteins in establishing spindle bipolarity." Current Biology **8**: 903-913.

Wilde, A. (2006). ""HURP on" we're off to the kinetochore!" J Cell Biol **173**(6): 829-31.

Wilde, A., S. B. Lizarraga, et al. (2001). "Ran stimulates spindle assembly by altering microtubule dynamics and the balance of motor activities." Nat Cell Biol **3**(3): 221-7.

Wu, C., V. Singaram, et al. (2008). "mei-38 is required for chromosome segregation during meiosis in *Drosophila* females." Genetics **180**(1): 61-72.

Yamamoto, A. H., D. J. Komma, et al. (1989). "The claret locus in *Drosophila* encodes products required for eye color and for meiotic chromosome segregation." EMBO J. **8**(12): 3543--3552.

Yang, C. P. and S. S. Fan (2008). "*Drosophila* mars is required for organizing kinetochore microtubules during mitosis." Exp Cell Res **314**(17): 3209-20.

Zhai, R. G., P. R. Hiesinger, et al. (2003). "Mapping *Drosophila* mutations with molecularly defined P element insertions." Proc Natl Acad Sci U S A **100**(19): 10860-5.

Zhang, G., M. Breuer, et al. (2009). "Mars, a *Drosophila* protein related to vertebrate HURP, is required for the attachment of centrosomes to the mitotic spindle during syncytial nuclear divisions." J Cell Sci **122**(Pt 4): 535-45.

Curriculum Vitae

Jeffry Cesario

Education:

B.S. in Biochemistry **1999 – 2003**

Bucknell University, Lewisburg, PA

Research Commercialization Introductory Course **2010**

National Council of Entrepreneurial Tech Transfer, Washington, DC

Ph.D. in Microbiology and Molecular Genetics **2010**

Rutgers University – Waksman Institute, Piscataway, NJ

Publications:

- Cesario JM, Jang JK, Redding B, Shah N, Rahman T, McKim KS. Kinesin 6 family member Subito participates in mitotic spindle assembly and interacts with mitotic regulators. Journal of Cell Science, 2006
- Jang JK, Rahman T, Kober VS, Cesario JM, McKim KS. Misregulation of the kinesin-like protein Subito induces meiotic spindle formation in the absence of chromosomes and centrosomes. Genetics 2007
- Cesario JM, McKim KS. RanGTP has a non-essential role in acentrosomal spindle assembly. Journal of Cell Science, 2010 (*under review*).
- Cesario JM, McKim KS. The N-terminus of the kinesin-6 family member Subito is a complex domain of positive and negative regulator. (*In preparation*).

Research Experience:

- Undergraduate research with Dr. Marie C. Pizzorno-Simpson
  Bucknell University, PA 2001 – 2003
- Doctoral Research with Dr. Kim McKim
  Rutgers University – Waksman Institute, NJ 2005 – 2010