REGULATION OF MEIOTIC CENTRAL SPINDLE FUNCTION, HOMOLOG BI-ORIENTATION AND CO-ORIENTATION IN *DROSOPHILA* OOCYTES

By

ARUNIKA DAS

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ABSTRACT OF THE DISSERTATION:

Regulation of meiotic central spindle function, homolog bi-orientation and co-orientation in *Drosophila* oocytes

By ARUNIKA DAS

Dissertation director:
Kim S. McKim

In the oocytes of many animals including humans, spindles are assembled in the absence of centrosomes. It is poorly understood what organizes the bipolar spindle in this system and directs attachment of chromosomes to opposing poles, a process known as bi-orientation. Previously it was shown that the kinesin 6 motor protein, Subito, is important for meiotic central spindle assembly and localization of the chromosomal passenger complex (CPC). I analyzed the role of different domains of Subito, to understand what restricts the activity of the motor to the chromatin, in meiosis. I found that the N-terminus of Subito, is required for localization of the kinesin to microtubules possibly in conjunction with the C-terminus. I also identified domains of Subito N-terminus that are required for mitosis, but may be dispensable for meiosis.

I also analyzed mutants obtained from a synthetic lethal screen with Subito and found that the centralspindlin complex, is required for both homolog bi-orientation and Subito localization in meiosis. Surprisingly, I also found that downstream targets of this complex like Rho1 and Sticky (Citron kinase), are important for bi-orientation. In addition, another target from the screen, Polo kinase, which is a kinetochore protein is required for maintaining karyosome structure. Altogether, these results have given rise to

a model where late cytokinesis proteins are involved in regulating central spindle assembly and directing bi-orientation possibly by modulating error correction by the kinase, Aurora B.

It is unclear how the CPC function is regulated to establish tension between homologs and correct errors. Phosphatases have been shown to be important for a similar function in mitosis. However the role of phosphatases in meiosis was unknown. I have found that *Drosophila* PP1 is required for maintaining karyosome structure, peri-centromeric cohesion and co-orientation at metaphase I, in an Aurora B dependent manner. Kinetochore assembly by Aurora B, is also opposed by PP1. These results taken together provide insight on how Aurora B activity is balanced in meiosis, and emphasize the important role played by PP1 in oocytes.

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DEDICATION

This dissertation is dedicated to my grandparents, Umarani Das, Lala Himanshu Das, Shanti Ghosh and Mikku Ghosh. They never understood what I was doing but was immensely supportive and enthusiastic about my work. They are not here to see me complete this today, but I think they would be happy and proud about this. I would also like to dedicate this work to the plethora of fruit flies that I sacrificed to understand science. Your contribution will not be forgotten.

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CHAPTER 1: INTRODUCTION

I. Preface:

Parts of this chapter will be incorporated into a review to be submitted soon.

Aneuploidy and its effects

Meiosis is a specialized cell division that generates haploid gametes (e.g. egg and sperm) from a diploid cell after two rounds of division. This is essential for sexually reproducing organisms and is thought to generate diversity required for long term survival of the species The first division meiosis I (MI) segregates homologous chromosomes reducing ploidy, whereas meiosis II (MII) separates sister chromatids and is analogous to mitotic divisions. Diversity in the germline is achieved by recombination (exchange of genetic material) and chromosome segregation. Also, there are sex-specific differences in gamete formation. In males, sperm is continually made, however, in human females all oocytes are generated during fetal development where they enter prophase. After homologous chromosomes synapse and recombine, oocytes enter meiotic arrest. The timing of this arrest differs depending on the organism. In human females, resumption of meiosis occurs years later before the oocyte is ovulated in preparation for fertilization. Furthermore, out of the four products of female meiosis only one is designated as the oocyte and the rest form polar bodies and are degraded [1, 2].

Mis-segregation of chromosomes is a rare event and results in aneuploidy, as coined by Gunnar Tackholm in1922 [3]. “Ploidy” refers to the total chromosome content of a cell. Aneuploidy is any chromosome number that deviates from a multiple of the

haploid set [4].This may be gains or losses in the number of chromosomes, non-balanced rearrangements including deletions, amplifications and translocations. Aneuploidy during meiosis is more frequent and is usually constitutional or present in the whole organism. It is reported that 35% of spontaneous abortions, 4% of stillbirths and 25% of fertilized zygotes contain incorrect number of chromosomes [5-8]. This is a leading cause of miscarriages, infertility and birth defects like Down's syndrome or Turner's syndrome. There are many consequences to both somatic and germline aneuploidies (reviewed in [9]). Errors in meiosis are termed non-disjunction. MI errors include failure to form a chiasmata due to improper recombination, failure to release arm cohesion leading to random segregation of homologues or premature separation of sister chromatids. Most of these errors occur in MI and are maternal in origin and these processes will be investigated closely in the course of my study. Understanding the basic mechanism of chromosome segregation will provide further insights as to how they are generated in the first place and allow us to deal with such abnormalities.

The focus of my study is to understand the mechanism of chromosome segregation in the female germline. Humans show a high incidence of aneuploidy during meiosis and the origins of this aneuploidy seems to be primarily maternal. For e.g. 100% of documented trisomy 16 is due to errors in the first maternal division, MI [2, 8]. Also, the levels of aneuploidy increase dramatically to almost 35% in older women [10]. Since more women are now conceiving in their later years, this is a problem that needs better understanding.

In order to address these questions, the molecular mechanisms of meiosis has been studied in model organisms. In fungi all the products of meiosis are packaged

together and are hence easy to study [11]. Recent advances in genetic techniques have enabled intensive studies in *Arabidopsis*, *C.elegans*, *Drosophila* and mice as well [12-15]. Using these genetically tractable systems we have been able to dissect some of the differences between meiosis and mitosis and more importantly between female and male chromosome segregation. Although the field of female meiosis has been established, there are still a wide range of unique processes that govern the formation of the oocyte that we do not understand.

Chromosome segregation: The main requirements

Faithful chromosome segregation has basic requirements in all types of cells. 1) Forces need to be generated for chromosome movement. This is provided by a bipolar structure built from microtubules composed of tubulin dimers and associated motor proteins. It has been estimated that a single microtubule can generate ~65pN of force upon the chromosomes [16]. Microtubules are dynamic and can be depolymerized in a polarized fashion because of the presence of "plus" ends near the chromatin and "minus" ends at the poles; this mediates movement of the chromosomes towards the spindle poles. 2) Chromosomes require coupling to the microtubules by a structure that can bind to both chromatin and microtubules and is flexible enough to resist pulling forces until correct attachments are established. A proteinaceous structure called the kinetochore is responsible for this function, the regulation of which is complex, as it is the primary site of error correction [17]. 3) The chromosomes themselves need to be held together prior to segregation so that they do not prematurely separate. This is called cohesion and is achieved by a complex called the cohesin complex [18]. For accurate segregation this complex requires release just after the chromosomes are bi-oriented during metaphase-

anaphase transition so that the spindle forces can pull/push chromosomes apart. This process is modified in meiosis and has specialized functions (see below). 4) Lastly cytokinesis or division of the membrane has to occur to generate daughter cells. Most or all of these functions is under strict regulation by a complex called the chromosomal passenger complex that is required for almost all aspects of segregation in both mitosis and meiosis [19, 20].

Chromosome segregation in mitosis

The chromosomal passenger hypothesis proposes that there is one complex that coordinates chromosome segregation and cytokinesis [21]. This idea was further solidified when the inner centromere protein (INCENP) was shown to be in a complex with the kinase Aurora B and both were required for cell division [22, 23]. Additional members of the complex were then identified as being Survivin (Deterin) and Borealin (Dasra) to form a functional complex [24-26].

Activation of Aurora B: The complex is activated by a multistep process where Aurora B initially binds the IN box on Incenp [22] and this enables phosphorylation of the C-terminal TSS motif on INCENP and autophosphorylation of T232 in the T-loop of Aurora B [27, 28]. Both these events are required for achieving full activity of the complex and are thought to occur in trans [29]. This activity can also be increased by binding to microtubules and chromatin [30, 31] and is important for the spatial restriction of its phosphorylation gradient.

Localization of CPC to centromeres and relocation to spindle midzone: In early mitosis the CPC is enriched at inner centromeres and is recruited specifically by two independent chromatin modifications [32]: phosphorylation of H3T3 by Haspin kinase and H2AT120

(H2AS121 in humans and fission yeast) by Bub1 kinase [33-35]. The N-terminus of INCENP forms a three helix bundle with Survivin and Borealin and this comprises the localization module of the CPC [36-38]. Survivin binds H3T3 through the BIR domain and Borealin is recruited to Shugoshin proteins (Sgo1/Sgo2/MEI-S332) which bind H2AT120 and are interdependent on the CPC for localization [35, 39]. Overlap between these marks enriches the CPC at the inner centromeres (Figure 1A). This localization is also under the control of extensive feedback loops [40, 41] . During metaphase-anaphase transition the CPC relocates to the spindle midzone and directs cytokinesis and abscission (reviewed in [26, 42]). This relocation is dependent on removal of inhibitory phosphorylation by Cdk1 which in turn promotes the interaction of the CPC with MKlp2 [43-45] or Bim1 (EB1) in budding yeast [46]. The spindle midzone is stabilized by the action of centralspindlin complex (MgcRacGAP/ RacGAP50C and MKlp1/Pavarotti/ZEN4), protein regulator of cytokinesis 1 (PRC1) and the kinesin Kif4. Aurora B promotes centralspindlin clustering and microtubule binding to stabilize the midzone structure [47]. Although less studied, Aurora B is also thought to regulate cytokinesis directly through the protein RhoA (Rho1), and abscission checkpoint through the ESCRT complex [26].

Regulation of kinetochore-microtubule interactions and tension sensing by Aurora B phosphorylation gradients: Faithful chromosome segregation requires bi-orientation of the sister kinetochores through the bipolar attachment (amphitelic attachment) to opposite spindle poles. However, errors in attachment like syntelic and merotelic attachments can lead to aneuploidy and must be corrected before cytokinesis can occur. Hence, a crucial function of Aurora B is to destabilize incorrect kinetochore attachments by sensing a lack

of tension generated between the two sister kinetochores [48]. The kinetochore has a core KMN network comprising of the KNL1, Mis12 and Ndc80 complexes all of which are substrates of Aurora B [49-52]. Amphitelic attachments are under tension and hence the kinetochores are further apart from the destabilizing phosphorylation of Aurora B at the inner centromere. Merotelic and syntelic attachments are not under tension closer to Aurora B localized site. Hence they can be destabilized due to reduction of the affinity for microtubules by phosphorylation of kinetochore components by Aurora B [50, 53-59]. This is known as the spatial separation model where a gradient of Aurora B activity is generated to regulate attachments [53, 60]. Furthermore, KNL1 associated pool of a phosphatase PP1, counteracts Aurora B activity and ensures stabilization upon bi-orientation (Figure 1B) [61, 62]. However this model has been recently challenged by the finding that Aurora B activity required for actual destabilization resides outside of its primary localization at the inner centromeres [63, 64].

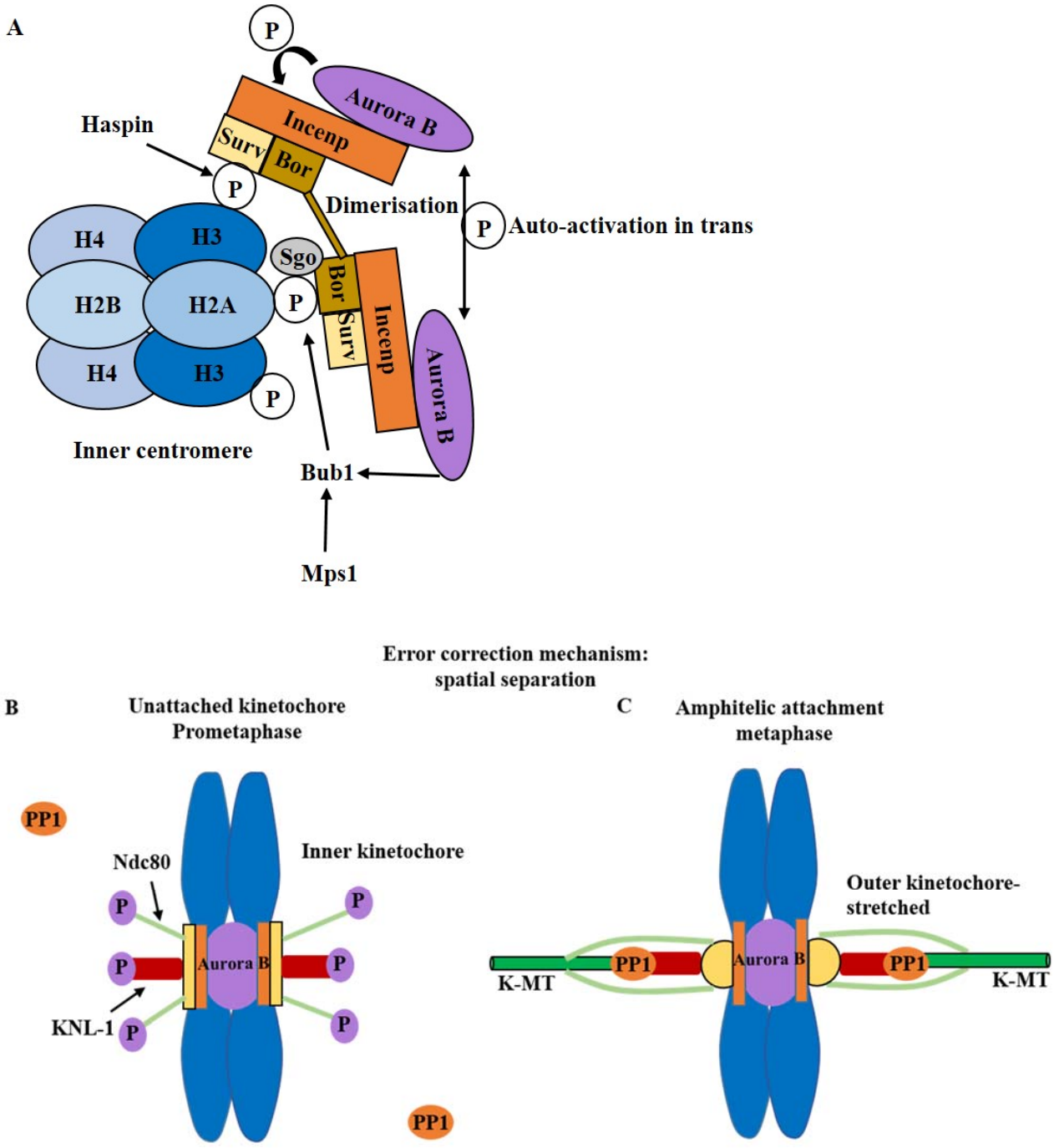


Figure 1: CPC localization and regulation in mitosis

A. Cartoon depiction of the two histone marks necessary for CPC localization to inner centromeres in mitosis. B. Aurora B phosphorylates KMN network components reducing their affinity for microtubules and hence destabilizing attachments. C. once the kinetochores have made end-on attachments, tension separates them spatially from Aurora B phosphorylation and PP1 dephosphorylates kinetochore to stabilize these attachments.

Regulation of Aurora B activity by phosphatases: Activity of Aurora B is counteracted by mainly two phosphatases. This is important for maintaining the correct phosphorylation balance of substrates in the cell. This is achieved by various methods. The T-loop of Aurora B is dephosphorylated by PP1/PP2A to decrease its activity, or its localization is affected by dephosphorylation of H3T3 [65, 66]. Dephosphorylation of Aurora B substrates like KNL1, Ndc80 is also crucial for mediating correct attachments. In fact the spindle assembly checkpoint is silenced primarily by the activity of PP1 but is also modulated by a negative feedback signal from the dephosphorylation of kinetochore proteins by PP2A. The specificity of these phosphatase activities are conferred by regulatory subunits, which direct differential substrate recognition, like Sds22, Repo-Man, CENP-E and kinesin 8 for PP1 and B56 or EB1 for PP2A [67-74].

Chromosome segregation in meiosis

Although the mitotic chromosome segregation field has advanced over the years and the mechanism for the various intricate steps are well elucidated, meiosis still has a long way to go in terms of understanding the mechanisms that modify the mitotic machinery to segregate the chromosomes and reduce ploidy. As in mitosis the basic requirements for segregation are similar except they have some specific modifications in the first division.

Pulling chromosomes apart: acentrosomal spindle assembly

In somatic cells and spermatocytes, microtubules are nucleated from centrosomes or the major microtubule organizing center (MTOC). These microtubules then grow inward towards the chromatin to make contact at kinetochores in a search and capture mechanism [75, 76]. During female meiosis in many species including humans, flies and nematodes, chromosome segregation is preceded by centrosome elimination to avoid

duplication of centrosomes after fertilization. The normal number is reconstituted from the male gamete following zygote formation. However this means that oocyte meiotic spindles are formed in the absence of centrosomes, also called acentrosomal spindle assembly. The mechanisms of this acentrosomal system is beginning to be outlined in various systems. It was shown in frog egg extracts that beads coated with DNA could support formation of bipolar spindles [77]. These studies also elucidated the role of a small GTPase Ran in chromatin associated spindle assembly through the formation of a gradient that recruits spindle assembly factors required for nucleation and elongation of microtubules [78].

However, in vivo, this pathway seems to be redundant with others. In fact in mouse, frog and *Drosophila* oocytes, inhibiting Ran perturbs or delays meiotic spindle assembly but does not prevent chromosome mediated spindle assembly [79, 80]. This indicates that Ran independent pathways must be present in the oocyte. Further studies in *Drosophila* and *Xenopus* oocytes have shown that the chromosomal passenger complex (CPC) is important for spindle assembly [81-83]. The mechanism by which it initiates spindle assembly and its downstream targets are not well understood, but in *Xenopus* extracts it was seen to be partially by inhibiting microtubule depolymerizer kinesin 13, MCAK (mitotic centromere associated kinase) and Oncoprotein 18 (Op18/Stathmin) [31, 81]. However, in *Drosophila* oocytes, loss of spindle microtubules in the absence of Aurora B does not seem to depend on the kinesin 13, KLP10A [83, 84]. Instead it may depend on the concurrent regulation of multiple microtubule depolymerizers and stabilizers.

Localization of the CPC in meiosis: Although the localization of the CPC during prometaphase is restricted to the inner centromere in most mitotic cells, in meiosis I CPC localization is different depending on the organism. In *C. elegans*, the chromosomes are holocentric and the structure of the bivalent is different than monocentric organisms. The site of the crossover differentiates the bivalent into two distinct domains with a short and the long arm [85]. The CPC localizes along the axis of the short arm called the mid-bivalent region in a ring around the chromosomes. This localization is important for regulating both chromosome congression and preferential removal of cohesion from the mid-bivalent region [86-88]. In *Drosophila* oocytes, which are monocentric, the chromosomes are compacted to form a sphere called the karyosome. The centromeres are located at the edges of this karyosome with the homolog arms presumably towards the center. There is a central overlap region of microtubules, surrounding the center of the karyosome, where the CPC localizes in a ring [83] and sometimes to the centromeres at a low level (S.J. Radford personal comm.).

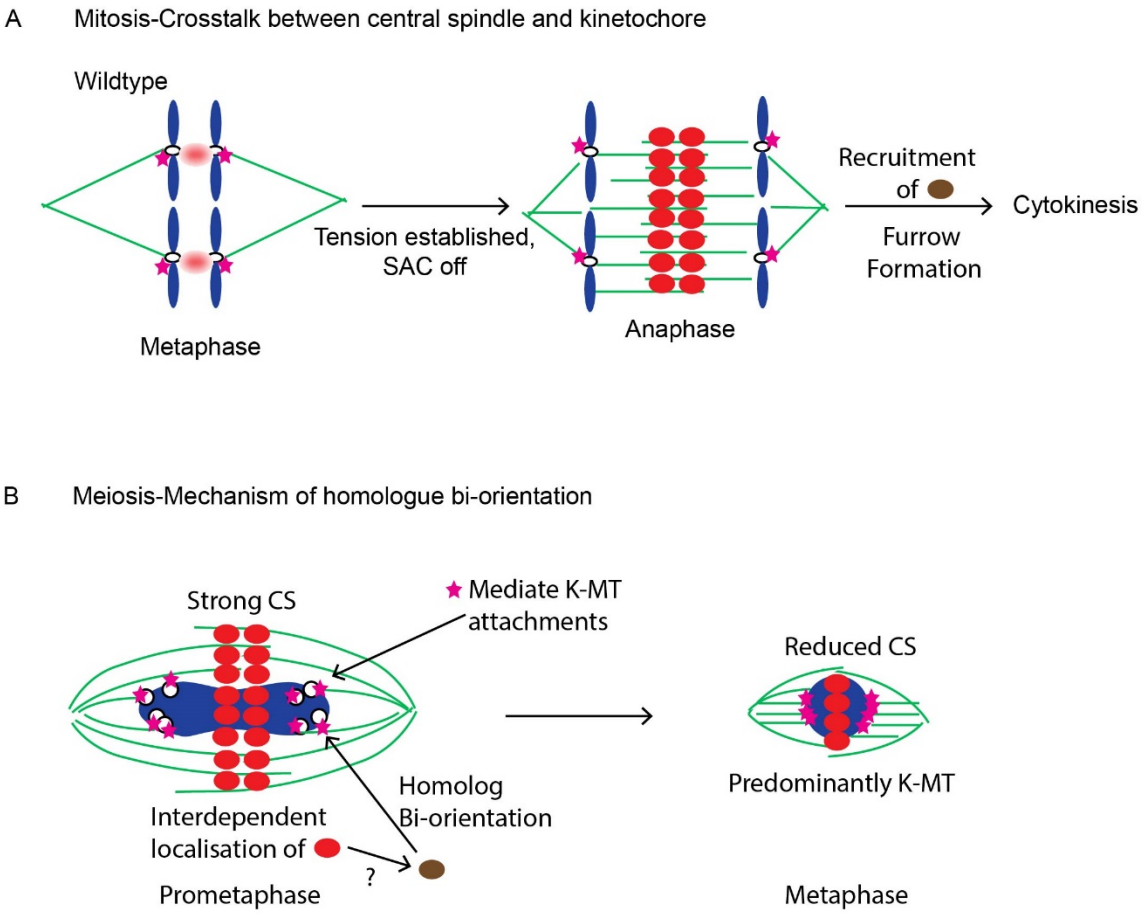
In mice the localization of Aurora kinases is complicated by the presence of the meiosis specific Aurora kinase C. This kinase localizes to the centromeres and along the chromosome arms (called the interchromatid axis, ICA) at MI whereas Aurora B localizes to the spindle [89, 90]. However the ICA localization may be the site of active Aurora kinase instead of the kinetochore population. This ICA localization of Aurora C is also dependent on H3T3 phosphorylation by Haspin kinase [90]. My studies have shown that Haspin is not required for CPC localization in *Drosophila*; it is possible that multiple redundant pathways are responsible for localization of the CPC in this system. However, the mid-bivalent ring seen in C. elegans, the central spindle ring in *Drosophila* and the

ICA localization of Aurora C in mice may be analogous to each other, indicating that although there may be inherent differences in the sites of localization (chromatin versus microtubules), there is still an overall pattern that is conserved in most organisms regardless of centromere or bivalent structure.

Taken together these results evoke some additional questions. Is the centromere localization of Aurora B required for its function? There is some evidence from studies in yeast mitosis that this centromere population may be dispensable for chromosome segregation but it may not be applicable to higher organisms. Additionally work from De Luca et al 2011, suggests that a pool of active Aurora B at unattached kinetochores may be sufficient for error correction in mitosis which is not its primary centromeric localization site. Conversely, in meiosis it is possible that a chromosome arm (worm and mouse) or microtubule (*Drosophila*), activated population of Aurora B is responsible for its functions and somehow in oocytes the kinetochore population may be diminished in importance. However this model remains to be tested.

Central spindle organization and function: In mouse oocytes, there are non-centrosomal MTOCs which recruit microtubule asters and then are clustered to form spindle poles [79, 91]. Establishment of bipolarity however requires the formation of a central microtubule array and is regulated by Hepatoma Up-Regulated Protein (HURP) in a Ran and kinesin 5 dependent manner [92, 93].This is similar to *Drosophila*, where the meiotic spindle appears to be made up of primarily two kinds of interdependent microtubules: the kinetochore and the central spindle. An outstanding question in the field, that my studies have addressed, is how the meiotic central spindle is regulated and what its functions are.

This central microtubule overlap region is thought to be essential for establishing bipolarity and homolog bi-orientation. The CPC possibly recruits other proteins important for bundling, like the kinesin 6, Subito (MKlp2 homolog). Subito mutants are viable but sterile due to a failure to complete meiosis which can be attributed to the failure in pro-nuclear fusion [45, 94-96]. This central region known as the meiotic metaphase central spindle is analogous to the spindle midzone that forms during anaphase/telophase in mitosis [45]. The functions of this overlap region, although well studied in mitosis, are not well known in meiosis. An obvious question that arises is what cytokinesis proteins are regulating at metaphase I where no cellular division occurs. To answer this question, a part of this study focuses on identifying proteins that are involved in regulation of this structure through genetic interactions with Subito. Since this central overlap region may have mechanistic similarities with the mitotic midzone, albeit with a different function, we have also investigated the role of certain key cytokinesis mediators in meiosis. This study has brought to light novel functions of proteins previously thought to be active late in the cell cycle, and increased the repertoire of factors required not only for organizing the central spindle but also for homologous chromosome bi-orientation (Figure 2) (Das et al 2015, accepted).



Key:

Central spindle proteins	Late anaphase/ telophase proteins	Kinetochore/ centromere proteins
Incenp Aurora B Survivin Tumbleweed Pavarotti Subito	Rho1 Sticky RhoGEF2	Polo Spc105R BubR1

Figure 2: Overview of the role of CPC and central spindle proteins in regulating cytokinesis in mitosis and bi-orientation and polarity in meiosis.

Congression of bivalents and establishing microtubule-chromatin attachments: In the absence of centrosomes, the microtubules are thought to first establish lateral attachments to the chromosomes and achieve congression by sliding along these lateral bundles during early prometaphase [86, 97] [98]. This is interesting as chromatin movements in C. elegans and mouse oocytes do not require kinetochores [99, 100]. In C. elegans oocytes, chromosome congression is mediated by the chromokinesin KLP19 [86] which localizes in a ring to the mid-bivalent region in a CPC dependent manner and mediates lateral sliding of chromosomes. It still remains to be tested whether these movements involve attachments to the kinetochores which encapsulate the bivalent as the chromosomes are holocentric.

Surprisingly, due to the lack of any kinetochore fibers in prometaphase in moose oocytes, congression was proposed to be kinetochore independent. The chromosomes in this case congress to the equator, leaving a ring in the center, in close proximity to the spindle microtubules forming what is known as the “prometaphase belt”. This congression is also mediated mainly through lateral attachments as stable end on attachments form later in prometaphase [97, 101]. The prime candidate for lateral movement of chromosomes in the absence of end-on kinetochore attachment, is via perhaps a chromokinesin similar to C. elegans. Although the plus end directed force generator Kid, is dispensable for this process in mice, it is required for polar ejection forces in *Xenopus* egg extracts [102, 103]. The kinesin motor CENP-E, required for kinetochore related lateral movements [104], is a less likely candidate in mouse oocytes

since the speed of such movements in oocytes is slower than that measured for CENP-E directed ones [104, 105].

However in *Drosophila*, The central spindle is thought to be analogous to the "prometaphase belt" which then promotes stable end-on attachments late in prometaphase. In fact in the absence of the CPC, the chromosomes fail to execute any movements and the centromeres remain clustered [83]. Kinetochore attachments have also been shown to be important for congression and segregation in *Drosophila*. Minus end directed movements of the centromere are opposed by kinesin motor CENP-E to achieve congression through Ndc80 mediated lateral attachments. In *Drosophila* both lateral attachments via Ndc80 and end-on attachments through SPC105R are important for homolog bi-orientation [98]. All of these data support a universal mechanism of chromosome congression early in prometaphase through lateral attachments which may be kinetochore dependent or chromokinesin mediated, which are converted to stable attachments later in the spindle elongation phase. It should also be noted that even though end-on kinetochore attachments were absent in mice in prometaphase, the lateral interactions may still require the kinetochore as in other organisms.

Homologous chromosome bi-orientation: Establishment of bi-orientation in meiosis requires a unique geometry where the two sister kinetochores must be aligned side by side to attach to microtubules from the same pole (termed mono- or co- orientation; [106]) resulting in bivalents attached to opposite poles. Given the unique geometry of meiosis I kinetochores, there still remains the question of how tension is sensed. In mitosis the spatial separation model explains how syntelic or merotelic attachments are destabilized by Aurora B in the absence of tension [17, 60]. A similar model has been

proposed to be active in meiosis except, its role is to destabilize attachments made to co-oriented sister kinetochores. Studies in fission yeast have shown that Ark1 (Aurora homolog) can promote both bi-orientation of bivalents and of separated sister chromatids giving rise to the idea that the mechanism which underpins kinetochore attachment is conserved from mitosis to meiosis [107-109]. This is supported by findings in budding yeast which suggest that Ipl1 can ensure bi-orientation of sister kinetochores if the bivalent structure is lost and mitotic like chromosomes are created [107, 110].This is corroborated by evidence from *Drosophila* oocytes where hypomorphic mutants in Incenp and Aurora B show bi-orientation defects [83].

Mouse oocytes contain both Aurora B and C, which are 75% identical in the kinase domain, leading to various overlapping functions. Functional compensation between Aurora B and C in mouse oocytes has made separation of function difficult. But studies using an Aurora C gatekeeper mutant (L93A), which specifically inhibits the C isoform, and dominant negative AURKC mutants, has revealed elevated levels of misalignment and incorrect kinetochore attachments in keeping with results from other systems. Interestingly this study also suggests that the ICA localization of Aurora C is more important for attachment regulation [89, 111]. Another study showed that Aurora B/C activity is required for destabilizing kinetochore-microtubule attachment. Due to the close proximity of the CPC complex to the attachment site, stabilization is not achieved until the counteracting phosphatase PP2A, is recruited to the kinetochores in a Cdk1 dependent manner [101]. Hence Aurora activity seems to be more important for preventing premature stabilization of attachments rather than error correction, in a Cdk1

dependent manner in contrast to mitosis [112] (Davydenko 2013, Kabeche and Compton 2013).

Stepwise regulation of cohesion-part I, co-orientation: During MI there are two unique processes that ensure accurate segregation of homologs rather than sisters: recombination during prophase that along with arm cohesion generates chiasmata holding the homologs together until anaphase I; and co-orientation at metaphase I which is required to fuse sister centromeres such that they co-segregate to the same pole of the cell.

Sister kinetochores in budding yeast are held together by the monopolin complex comprised of Mam1, Casein kinase Hrr25, Csm1 and Lrs4 [113-115]. This complex is regulated by Cdc5 (Plk1 homolog) and Dbf4 dependent kinase [116-119]. Monopolin is recruited by the kinetochore component Mis13 and is regulated temporally by kinetochore microtubule attachments [120, 121]. However in fission yeast the monopolin homolog is required to prevent merotelic attachments in mitosis [1]. Instead a meiosis specific cohesin Rec8, along with the protein Moa1 is responsible for fusing sisters by localizing to core centromeres [122, 123]. Additionally another study identified Aurora B as a Rec8/Moa1 independent regulator of co-orientation. Co-orientation defects seen in the absence of Aurora B, was attributed to the stabilization of merotelic attachments which pulled the sisters apart at anaphase I [107]. It should be noted that in meiosis, Sgo1 is present only at peri-centromeric regions and not at the core centromeric region [124].

In mice, a non-conserved protein, MEIKIN holds the sister kinetochores together in a Rec8 and Plk1 dependent manner [125]. Surprisingly, in plants, co-orientation depends on linkages made by the kinetochore component Mis-12 [126]. In *Drosophila*, although homologs of MEIKIN or monopolin have yet to be found, co-segregation also

seems to be dependent on a core component of the KMN network, SPC105R (KNL-1 homolog) [98]. Additionally, my studies have shown that the phosphatase PP1, is required to maintain co-orientation geometry in *Drosophila* possibly by counteracting the effects of Aurora B on a co-orienting protein complex. My study also brings to light an interesting nuance in phenotype, depending on whether we affect establishment or maintenance of co-orientation. In any of these cases, the general mechanism of function of these complexes is thought to be a molecular clamp for multiple microtubule attachment sites in two sister kinetochores (Figure 3). This linkage gives a special geometry to the meiotic kinetochores and helps in reductional division. However from my study and those conducted in fission yeast [107], Aurora B seems to be required to also maintain co-orientation possibly by preventing merotelic attachments to a pair of co-oriented sister kinetochores. This function may be opposed by PP1, which is known in mitosis to stabilize correct microtubule attachments [62]. This brings up the question again of how these molecules can distinguish between tension generated from a merotelic attachment as opposed to that generated from an amphitelic attachment. It is possible however that merotelic attached sisters are closer to the central spindle ring of CPC and are hence destabilized. But this is a model that would require further study.

Another aspect that requires further study is whether co-orientation is cohesin mediated. Even though it seems to be cohesin independent in budding yeast, there is evidence in both *Drosophila* and mice oocytes that it may be dependent on cohesin subunits. It will be interesting to investigate how these subunits interact with the kinetochore proteins and whether there are direct linkages between the kinetochore structure and the cohesin rings or if these are independent pathways.

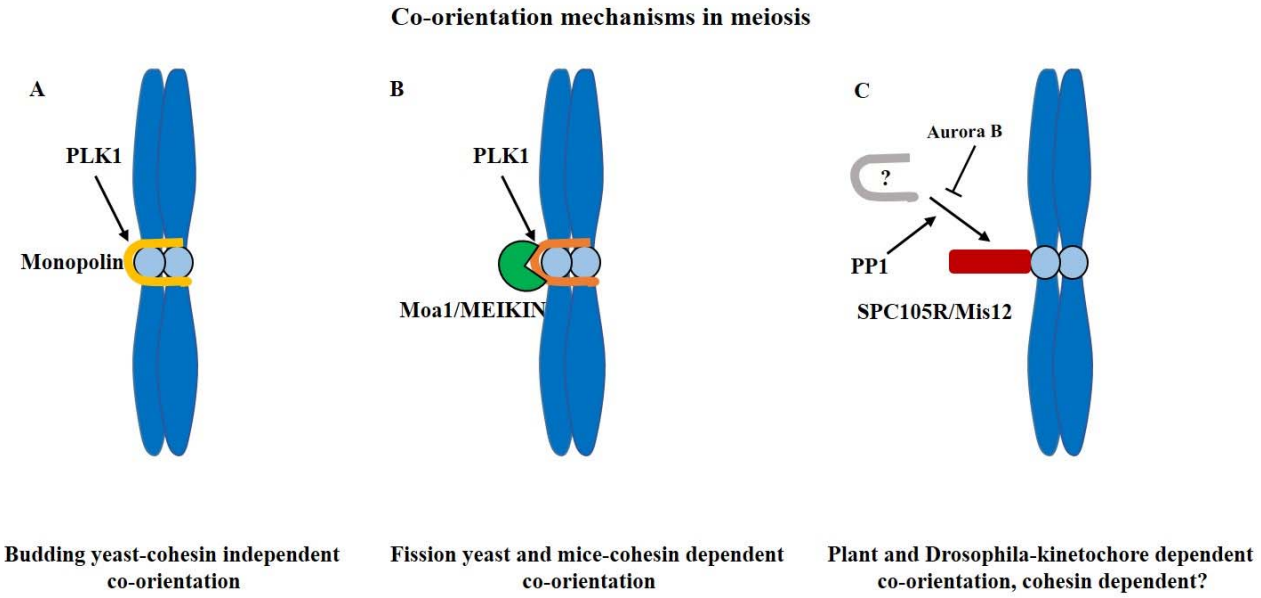


Figure 3: Co-orientation mechanisms in meiosis.

Details are in text.

Stepwise regulation of cohesion-part II, protection of peri-centromeric cohesion: A primary difference between mitosis and meiosis, is that, cohesion is needed to be established and maintained at both core centromeres and peri-centromeric regions as opposed to only peri-centromeric regions in mitosis. In mitosis the canonical cohesin complex generates peri-centromeric cohesion which is maintained until anaphase, when the sisters separate and peri-centromeric cohesion is released by Separase mediated cleavage of a Kleisin subunit [18]. Dissolution of arm cohesion occurs in mitotic prophase by Plk1 and Aurora B [127] and peri-centromeric cohesion is protected until anaphase by Sgo1 whose localization depends on PP2A, Aurora B and Bub1 [128, 129].

In meiosis, peri-centromeric cohesion has to be protected locally until anaphase of meiosis II. In budding yeast, Ipl1 is required for this function by recruiting PP2A/Rts1 which presumably blocks Separase mediated cleavage of Rec8 [108]. In *Drosophila* male meiosis, Aurora B is required to localize MEI-S332/Sgo1 to the chromosomes for protection of meiosis specific cohesin complexes at the peri-centromere. PP2A also inhibits Sororin removal from the peri-centromeric regions hence protecting it from removal [130]. There is also evidence that implicate LAB-1 and PP1 in counteracting Aurora B mediated removal of cohesion along the long arm [131, 132]. In *Drosophila*, my studies have shown that PP1 may be involved in a similar mechanism to protect peri-centromeric cohesion at meiosis I.

Drosophila as a model organism: Drosophila females typically contain a pair of ovaries with 10-20 ovarioles each. The ovarioles contain oocytes in various stages of oogenesis in a developmental timeline with a total of 14 stages [133]. The anterior tip contains 16 cell cystocytes where prophase occurs and there is a prolonged arrest. Meiotic maturation

is initiated in stage12-13 when the nuclear envelope breaks down and the oocyte resumes meiosis but arrests a second time in metaphase I [134]. The data collected in my study predominantly focuses on events that occur in prometaphase until metaphase arrest, which is when chromosomes are bi-oriented on a bipolar spindle. Since conventional RNAi methods are not functional in the germline, modified short hairpin RNA (shRNA) has been used extensively in my study to knockdown mRNA expression of essential genes in order to study their functions. I have primarily addressed two main questions in my work:

1) Does the early formation of the spindle midzone at metaphase I have effects on homolog bi-orientation and what proteins are involved?
2) Are phosphatases important for meiosis I and how do they regulate CPC activity to achieve specialized chromatin functions required in meiosis?

CHAPTER 2: SPINDLE ASSEMBLY AND CHROMOSOME SEGREGATION REQUIRES CENTRAL SPINDLE PROTEINS IN *DROSOPHILA* OOCYTES

I. Preface:

This chapter was published early online, as presented here, in Genetics, November, 2015 with the title "Spindle assembly and chromosome segregation requires central spindle proteins in *Drosophila* oocytes". My contributions to the paper were: writing of the paper and all of the experiments except conducting the screens, non-disjunction analyses and the western for *Polo* RNAi quantification.

II. Abstract

Oocytes segregate chromosomes in the absence of centrosomes. In this situation, the chromosomes direct spindle assembly. It is still unclear in this system, what factors are required for homologous chromosome bi-orientation and spindle assembly. The *Drosophila* kinesin-6 protein Subito, though non-essential for mitotic spindle assembly, is required to organize a bipolar meiotic spindle and chromosome bi-orientation in oocytes. Along with the chromosomal passenger complex (CPC), Subito is an important part of the metaphase I central spindle. In this study we have conducted genetic screens to identify genes that interact with *subito* or *Incenp*. In addition, the meiotic mutant phenotype for some of the genes identified in these screens were characterized. We show for the first time, in part through the use of a heat shock inducible system, that the Centralspindlin component RacGAP50C and downstream regulators of cytokinesis Rho1, Sticky and RhoGEF2, are required for homologous chromosome bi-orientation in

metaphase I oocytes. This suggests a novel function for proteins normally involved in mitotic cell division, in the regulation of microtubule-chromosome interactions. We also show that the kinetochore protein, Polo kinase, is required for maintaining chromosome alignment and spindle organization in metaphase I oocytes. In combination our results support a model where the meiotic central spindle and associated proteins are essential for acentrosomal chromosome segregation.

III. Introduction

Chromosomes are segregated during cell division by the spindle, a bipolar array of microtubules. In somatic cells, spindle assembly is guided by the presence of centrosomes at the poles. In this conventional spindle assembly model, the kinetochores attach to microtubules from opposing centrosomes and tension is established. This satisfies the spindle assembly checkpoint, which then allows the cell to proceed to anaphase [135]. Cell division is completed by recruiting proteins to a midzone of antiparallel microtubules that forms between the segregated chromosomes, signaling furrow formation [136, 137]. However, spindle morphogenesis in oocytes of many animals occurs in the absence of centrosomes. This may contribute to the high rates of segregation errors that are maternal in origin and are a leading cause of miscarriages, birth defects and infertility [138]. How a robust spindle assembles without guidance from the centrosomes is not well understood. While it is clear that the chromosomes can recruit microtubules and drive spindle assembly [30, 139], how a bipolar spindle is organized and chromosomes make the correct attachments to microtubules is not understood.

The *Drosophila* oocyte provides a genetically tractable system for the identification of genes involved in acentrosomal spindle assembly. Substantial evidence

in *Drosophila* suggests that the chromosomes direct microtubule assembly, subsequent elongation of the spindle and establishment of spindle bipolarity [75, 140, 141]. We have also shown that the kinesin-6 protein Subito, a homolog of human MKLP2 with a role in cytokinesis [142], is also essential for organizing the meiotic spindle and the bi-orientation of homologous chromosomes [45, 83, 96]. Subito colocalizes with members of the chromosomal passenger complex (CPC), which is composed of the scaffolding subunit INCENP, the kinase Aurora B and the targeting subunits Survivin (Deterin) and Borealin (Dasra) [25]. The CPC has a critical role in assembling the acentrosomal spindle and segregating chromosomes [82, 83]. In addition, with Subito, the CPC localize to the equatorial region of the meiotic metaphase I spindle and are mutually dependent for their localization [45, 83]. This equatorial region is composed of anti-parallel microtubules and is a structure that includes a plethora of proteins [45]. Assembling a central microtubule array may be a conserved mechanism to organize a bipolar spindle in the absence of centrosomes [139].

The meiotic central spindle, while assembling during metaphase, has several features and proteins associated with the midzone present during anaphase in mitosis. Indeed, Subito is required for the localization of the CPC to the midzone at anaphase [95]. The mitotic spindle midzone proteins function in anaphase and telophase to direct abscission, furrow formation and cytokinesis [136, 143]. The role of these proteins in the *Drosophila* acentrosomal meiotic spindle assembly pathway is unclear, however, since there is no cytokinetic function required at metaphase I and *Drosophila* does not extrude polar bodies [144]. It is possible these proteins are loaded in the central spindle at metaphase for a function later in meiotic anaphase, as has been proposed for Centrosomin

[145]. Alternatively, these central spindle proteins could be adapted for a new role, like Subito, in spindle assembly and/or bi-orientation of homologous chromosomes.

To identify and study the function of meiotic central spindle proteins, we carried out screens for genes that interact with *subito* (*sub*) and the CPC component *Incenp.* The first was an enhancer screen for mutations that are synthetically lethal with *sub*. Second, since synthetic lethality is a mitotic phenotype, a screen was performed for enhancement of the meiotic nondisjunction phenotype caused by a transgene overexpressing an epitope-tagged *Incenp* [83]. In these screens we identified new mutations in CPC components (*Incenp, aurB*, *borr*), the Centralspindlin gene *tumbleweed* (*tum*) and the transcription factor *snail*. Mutations in at least 16 additional loci were also identified and we directly tested candidate mitotic central spindle proteins for functions in meiosis. Several proteins were found to be required for microtubule organization and homologous chromosome bi-orientation during metaphase of meiosis I, including proteins in the Rho-GTP signaling pathway required for cytokinesis such as TUM (RacGAP50C), Rho1, Sticky (Citron kinase homolog) and RhoGEF2. Not all mitotic midzone proteins are required for the meiotic central spindle, however, demonstrating meiosis-specific features of this structure. For example, Polo kinase may only be required for kinetochore function while the RhoGEF Pebble was not required for meiosis. In summary, this is the first documentation that proteins known to be required for anaphase/telophase and cytokinesis in mitotic cells are also essential in meiotic acentrosomal spindle assembly and chromosome bi-orientation.

IV. Materials and methods:

Deficiency and mutagenesis screens for synthetic lethality

To test synthetic lethality of 3rd chromosome mutations and deficiencies, *cn sub bw/ CyO; e/ TM3, Sb* females were crossed to *Df/ TM3, Sb* females. The *cn sub bw/ +; Df/TM3* males were then crossed to *sub bw/ CyO* or *cn sub/ CyO* females to generate *cn sub bw/ sub bw; Df/+* progeny. The frequency of these progeny was compared to *cn sub bw/ sub bw; TM3/+* siblings to measure the synthetic lethal phenotype as a percent of relative survival.

The mutagenesis screen was performed for the second chromosome using Ethyl Methane Sulfonate (EMS). *y/y^{+}Y; sub^{131} bw sp/ SM6* males were exposed to 2.5 mM EMS in 1% sucrose overnight. About 25 mutagenised males were mated to 50 *al dp b pr Sp bw/ SM6* virgin females. Single *sub^{131} bw sp */ SM6* (asterisk denotes random mutations) males were mated with virgin *cn sub^{1} bw/SM6* females and the progeny were scored for the absence of brown eyed flies, which indicates a synthetic lethal interaction between the heterozygous EMS induced mutation and the *sub* mutant. Initially, 51 synthetic lethal lines were isolated. Each line was retested twice by crossing *sub^{131} bw sp */SM6a* sibling male progeny to *cn sub^{1}bw/SM6a* females and examining again for brown eyed progeny. Eventually 19 lines carrying a synthetic lethal mutation (*sub^{131} bw sp */SM6a*) were established and used for complementation testing and mapping.

Genetics, mapping and complementation testing

To generate recombinant chromosomes for mapping or to remove the *sub^{131}* allele, we mated *sub^{131} bw sp */SM6a* males to *al dp b pr cn c px sp/CyO* virgin females, collected *sub^{131} bw sp */al d b pr cn c px sp* virgin females and mated them to *al dp b pr*

Bl cn c px sp/CyO males (Fig S2B). Recombinants that were al^- and c^+ were collected and, because these recombinants likely carried the *sub* mutant allele, tested for synthetic lethality. In contrast, recombinants that retained the *c* mutation likely did not carry the *sub* mutant allele. These were used to evaluate whether the synthetic lethal mutation had a recessive lethal phenotype.

For establishing complementation groups, sub^{131} *bw sp */SM6a* flies were crossed to *c px sp */CyO* flies. A failure to complement was established by the absence of straight winged (Cy+) progeny with a total of at least a hundred flies being scored. For some mutations we used deficiency mapping. Three deficiencies, *Df(2L)r10, Df(2L)osp29* and *Df(2L)Sco[rv14],* failed to complement *22.64* and *27.18*. The allele of *snail* used for complementation was sna^1. One deficiency, *Df(2R)Exel7128* failed to complement *15.173* and *16.135*. The alleles of *tum* used for complementation were tum^{AR2} and tum^{DH15}.

X-chromosome non-disjunction was measured by crossing females to *y Hw w* /B^SY males. The Y chromosome carries a dominant *Bar* allele such that XX and XY progeny are phenotypically distinguishable from exceptional XXY and XO progeny that received two nor no X-chromosomes from their female parent. Nullo-X and triplo-X progeny are inviable, which is compensated in nondisjunction calculations by doubling the number of XXY and XO progeny.

Generating germline clones by FLP-FRT

Males of the genotype *w/Y;* ovo^D *FRT40A/CyO* were mated to *y w hsFLP70; Sco/CyO* virgins and *y w hsFLP70;*ovo^D *FRT40A/CyO* males were selected from the progeny. These were mated to either *22.64 pr FRT40A*/*CyO* (or *27.89*) virgins for the

experiment or *b pr FRT40A/CyO* virgins for the control [146]. Third instar larval progeny from these crosses were heat shocked at 37C for one hour on the 4th day. Female progeny of the genotype *y w hsFLP; ovo^{D} FRT40A/ 22.64 FRT40A* and *y w hsFLP; ovo^{D} FRT40A/ b pr FRT40A* were yeasted for 3-4 days and stage 14 oocytes were collected and analyzed.

Sequencing

DNA was extracted from a single fly [147]and amplified using standard polymerase chain reaction. The gene of interest was amplified using specific primer sets spanning the length of the gene. This DNA was then sent for sequencing to Genewiz Inc. Since the stocks were balanced, the resulting sequence was analysed using Align-X (Invitrogen) and Snapgene software for the presence of heterozygous SNPs indicating possible EMS induced mutations.

Expression of RNAi in oocytes and quantification

Expression of short hairpin RNA lines designed and made by the Transgenic RNAi Project, Harvard (TRiP) was induced by crossing each RNAi line to either *P{w^{+mC}=tubP-GAL4}LL7* for ubiquitous expression or *P{w^{+mC}=matalpha4-GAL-VP16}V37* for germ line specific and oocyte expression (referred to as "drivers"). The latter is expressed throughout oogenesis starting late in the germarium [83]. For expression of *tum* RNAi we used *P{GAL4-Hsp70.PB}89-2-1*. In this method, 2 day old adult females were yeasted for 2 days with males and then heat shocked for 2 hours at 37˚C. They were allowed to recover for 3 ½ hours and then oocytes were collected and fixed. At this time point the oocytes that were~ stages 10-11 at the time of heat shock are being laid as mature oocytes. Later time points did not yield sufficient quantities of

oocytes in the *tum* RNAi as oogenesis had arrested by then. *tum* RNAi females were sterile for 72 hours after heat shock whereas wild type regained fertility soon after heat shock.

For reverse transcriptase quantitative PCR (RT-qPCR), total RNA was extracted from late-stage oocytes using TRIzol® Reagent (Life Technologies). cDNA was consequently prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The qPCR was performed in either a StepOnePlus™ (Life Technologies) or Eco™ (Illumina) real-time PCR system using TaqMan® Gene Expression Assays (Life Technologies, Dm01823196_g1 (*polo*), Dm01794608_m1 (*Rho1*), Dm018202757_g1 (*sticky*), Dm01794707_m1, (*RhoGEF2*), Dm01822327_g1 (*pebble*).

Antibodies and immunofluorescent microscopy

Stage 14 oocytes were collected from 50 to 200, 3 to 4 day old yeast fed non-virgin females by physical disruption in a common household blender in modified Robb's media [140, 148]. The oocytes were either fixed in and 100 mM cacodylate/8% formaldehyde fixative for 8 min or 5% formaldehyde/heptane fixative for 2.5 min and then their chorion and vitelline membranes were removed by rolling the oocytes between the frosted part of a slide and a coverslip [148]. For FISH, oocytes were prepared as described (Radford et al 2012). Oocytes and embryos were stained for DNA with Hoechst 33342 (10μg/ml) and for microtubules with mouse anti-α tubulin monoclonal antibody DM1A (1:50), directly conjugated to FITC (Sigma, St. Louis) or rat anti-α tubulin monoclonal antibody (1:75) (Millipore). Additional primary antibodies were rat anti-Subito antibody (used at 1:75) [45], rat anti-INCENP (1:400) [83], rabbit anti-TUM

(1:50) [149], rabbit anti-SPC105R (1:4000) [150], rabbit anti-Sticky (1:50) [151] and mouse monoclonal anti-Rho1 (P1D9, 1:50) [152]. These primary antibodies were combined with either a Cy3 or Cy5 secondary antibody pre-absorbed against a range of mammalian serum proteins (Jackson Immunoresearch, West Grove, PA). FISH probes used were to the AACAC repeat (2nd chromosome) and dodeca repeat (third chromosome). Oocytes were mounted in SlowFade gold (Invitrogen). Images were collected on a Leica TCS SP5 or SP8 confocal microscope with a 63x, NA 1.4 lens. Images are shown as maximum projections of complete image stacks followed by merging of individual channels and cropping in Adobe Photoshop (PS6).

V. Results

sub mutants interact with multiple third chromosome loci including *Deterin* (Survivin) and *pavarotti* (MKLP1)

Null mutants of *sub* are viable but female sterile [96]. CPC members INCENP and Aurora B are mislocalised in the larval neuroblasts of *sub* mutants, which may be the reason why a reduction of INCENP or Aurora B dosage by 50% causes *sub* homozygotes to die [95]. This observation suggests that the *sub* mutant is a sensitized genetic background to perform forward genetic screens to identify mitotic proteins with possible functions in meiosis similar to the CPC or Subito. Thus, we performed screens for mutations that show a dominant lethal interaction with *sub*, also known as synthetic lethality (Figure 4 and Figure 5). The advantage of these screens is that we can recover mutations in essential genes and identify genes encoding central spindle proteins even if there is no direct physical interaction.

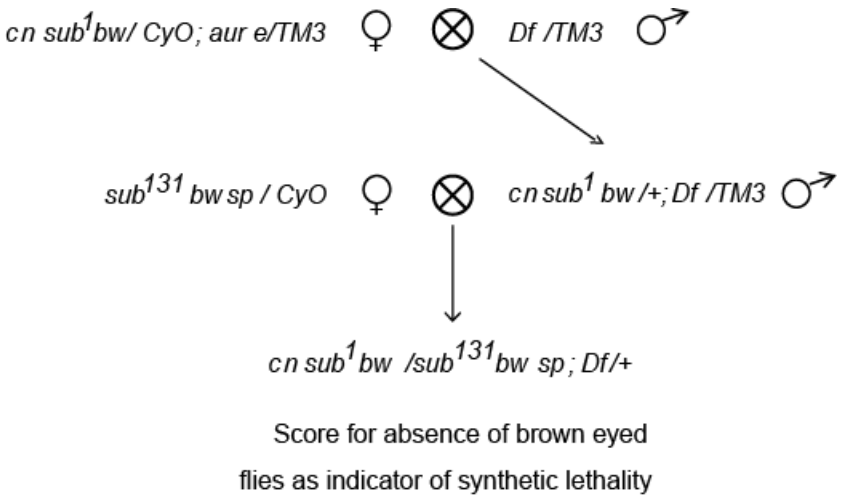


Figure 4: Synthetic lethal deficiency screen on the third chromosome.

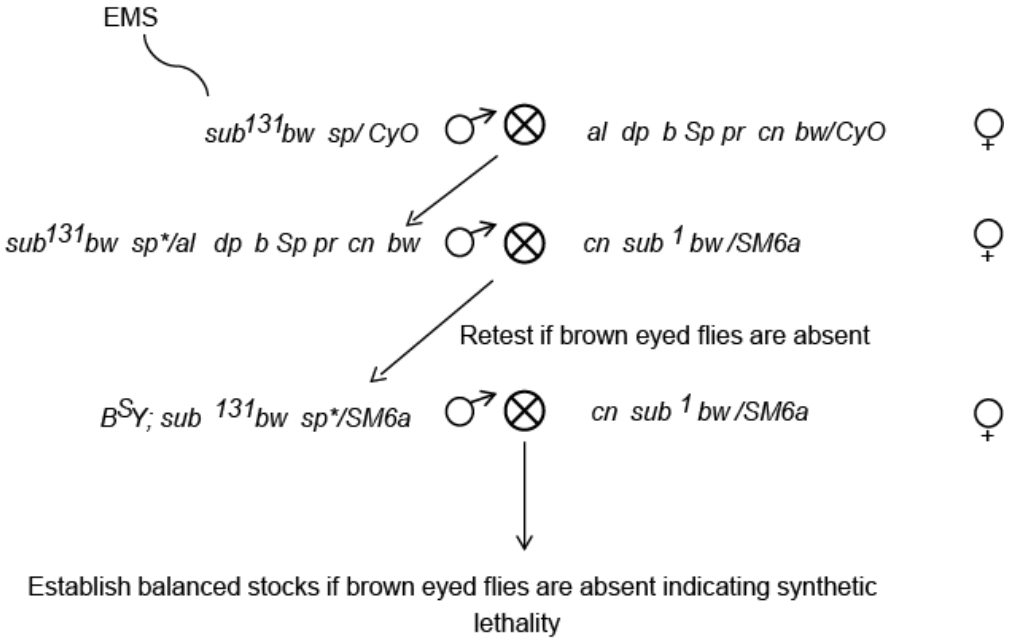


Figure 5: Synthetic lethal screen on the second chromosome.

Crossing scheme for isolating heterozygous mutations that induce synthetic lethality in a *sub*^{131}*/sub*^{1} mutant. An asterisk indicates the EMS-treated chromosome. Synthetic lethality is assessed by the absence of straight winged, brown eyed flies in the second step and these mutations are further retested and balanced.

On the third chromosome we screened 81 deficiencies obtained from Bloomington Stock Center for synthetic lethality, covering approximately 75% of the chromosome. Synthetic lethality was calculated as a ratio of sub^{1}/sub^{131};*Df*/+ to sub^{1}/sub^{131};+/+ progeny. Seven deficiencies, *Df(3L)ZN47, Df(3R)23D1, Df(3R)DG2, Df(3L)rdgC-co2, Df(3L)GN24, Df(3R)Exel9014* and *Df(3L)ri-XT1*, were identified that displayed synthetic lethal interaction with *sub* at viability rates between 0-10% (Table 1). Three additional deficiencies, *Df(3R)Antp17, Df(3L)emc-E12, Df(3R)BSC43*, exhibited a milder synthetic lethal interaction, with a viability rate between 10-30% (Table 1).

Table 1: Deficiencies that are synthetic lethal with *sub* and/or dominantly enhance *Incenp*^{myc}

Deficiency	Cytology	Viability [1]	Total [1]	% X-Non disjunction [2]	Total[2]	Candidate genes
sub				20.3	1438	
+				1.3	158	
Df(3L)emc-E12	61A;61D3	30.1	272	22.6	257	*fwd*
Df(3L)ED4177	61C1;61E2	67.0	309	2.6	1440	*fwd*
Df(3L)GN24	63F6-63F7;64C8-64C9	0	118	1.2	1027	*pavarotti*
Df(3L)Exel9000	64A10;64A12	30.1	359	5.3	219	*pavarotti*
Df(3L)ZN47	64C4-64C6;65D2	0	98	2.1	391	*Mad2, RCC1*
Df(3L)rdgC-co2	77A1;77D1	7.3	191			*polo*
Df(3L)ri-XT1	77E2-77E4;78A2-78A4	6.1	70	12.2	460	*Spc105R, pitsire*
Df(3L)BSC452	77E1;77F1	39	163	13.1	565	
Df(3L)BSC449	77F2;78C2	122	180	4.8	565	
Df(3R)Antp17	84A1-84A5;84D9	16.7	28			

Df(3R)DG2	89E1-89F4;91B1-91B2	0	36	0.0	297	*Deterin*
Df(3R)ED5780	89E11;90C1			8.7	577	
Df(3R)BSC43	92F7;93B6	10.1	89	0.0	439	
Df(3R)23D1	94A3-94A4;94D1-94D4	0	175	6.1	457	
Df(3R)Exel6191	94A6;94B2	113.3	224	3.8	311	
Df(3R)Exel6273	94B2;94B11	112.3	155	4.5	532	
Df(3R)ED6091	94B5;94C4	158.1	191	0.0	156	
Df(3R)Exel6192	94B11;94D3	111.1	133	6.0	807	?
Df(3R)Exel9013	95B1;95B5	132.8	288	8.4	1197	?
Df(3R)Exel9014	95B1;95D1	0	49	11.1	2234	?
Df(3R)Exel6196	95C12;95D8	140.6	77	6.5	2043	?

[1] % viability was calculated from the ratio of $sub^{131}/sub^{1};Df/+::sub^{131}/sub^{1};+/+$ flies obtained (Fig S2).
[2] X-chromosome non-disjunction was measured by crossing females to $y\ Hw\ w\ /B^{S}Y$ males (Materials and Methods).

For each of the seven deficiencies with the strongest synthetic lethal phenotype, we looked at sets of overlapping deficiencies and specific mutations to identify candidate genes. *Df(3R)DG2* uncovers the gene *Deterin* (also known as *survivin*), which we expect to be synthetic lethal with *sub* similar to the other members of the CPC. A null allele of *Deterin* was tested and also exhibited a synthetic lethal interaction (4% $sub^{1}/sub^{131};Det^{e01527}/+$ progeny, n=184) . Deficiency *Df(3L)rdgC-co2* uncovers *polo*, which we expected to be synthetic lethal based on previous results [95]. Within *Df(3L)GN24* we tested 6 smaller deficiencies and found synthetic lethality with *Df(3L)Exel9000*. Within this deficiency is *pavarotti*, which encodes the *Drosophila* homologue of MKLP1 that localizes to the central spindle in both mitosis and meiosis similar to Subito [45, 153, 154]. A null allele of *pavarotti* also was synthetic lethal with *sub* (0% $sub^{1}/sub^{131}; pav^{B200}/+$ progeny, n=69).

Two of the deficiencies identified as synthetic lethal with *sub, Df(3R)Exel9014* and *Df(3L)ri-XT1*, disrupt the kinetochore protein encoding gene *Spc105R* (Table 1). *Df(3R)Exel9014* does not delete *Spc105R* but the chromosome carries a second mutation that is a null allele, $Spc105R^{1}$ [155]. One of two smaller deficiencies within *Df(3L)ri-XT1*, *Df(3L)BSC452,* also deletes *Spc105R* and had a synthetic lethal phenotype. We directly tested synthetic lethality with a $Spc105R^{1}$ chromosome that lacked *Df(3R)Exel9014*. $Spc105R^{1}$ on its own was not synthetic lethal with *sub* (n=253). We also tested two additional kinetochore mutants, but neither *mis12* (n=337) nor *spc25* (n=131) were synthetic lethal with *sub*. These results suggest that there is no synthetic

lethal interaction between *sub* and kinetochore mutants. *Df(3R)Exel9014* and *Df(3L)ri-XT1* must interact with *sub* because of loci other than *Spc105R*.

For two of the deficiencies, *Df(3L)ZN47* and *Df(3R)23D1,* we did not identify a smaller interacting region. It is possible that the interaction lies in a gene disrupted only by the larger deficiency. Alternatively, the genetic interaction may involve haploinsufficiency for more than one gene within the larger deficiency. There are also possibly more complex interactions of positive and negative regulators. In this case, a smaller deficiency could have less severe synthetic lethal phenotype than a point mutant. This was observed with deletions of *pav*. While a *pav* mutation and *Df(3L)GN24* had severe synthetic lethal phenotypes, the smaller deficiency *Df(3L)Exel9000* had a relatively mild synthetic lethal phenotype.

Overall, in addition to confirming genetic interactions between *sub* and *polo, pav* and *Det,* the third chromosome deficiency screen for synthetic lethality identified at least seven additional interacting loci.

Mutagenesis screen for synthetic lethal mutants on the second chromosome reveals new alleles of CPC genes and centralspindlin component *Tumbleweed*

A mutagenesis screen of the second chromosome was done to identify genes that genetically interact with *sub.* We screened 5314 second chromosomes mutagenized with EMS and isolated 19 lines with a synthetic lethal phenotype (Materials and Methods). (Figure 5). We expected to obtain alleles of the CPC since three of its members, *Incenp*, *aurB* and *borr*, are on the second chromosome. Complementation testing with deficiencies uncovering these genes and existing mutants revealed three alleles of *Incenp,* two of *aurB* and one of *borr* (Table 2). Most of these mutations were also homozygous

lethal. However, *Incenp*$^{18.197}$ is a hypomorphic allele that causes recessive sterility and not lethality. The rest of the mutations were put into 11 complementation groups. There are 2 groups with 2 alleles each (*22.64, 27.18* and *15.173, 16.135*) and 9 that are represented by one allele each (Table 2).

Some synthetic lethal mutations that complemented all CPC mutants were genetically mapped (Table 2). We picked two types of recombinants, those that also retained the *sub* mutation so that the synthetic lethal mutation could be mapped, and those that did not have the *sub* mutation, to determine if the mutation had a recessive phenotype, such as lethality or sterility. A detailed example of this approach is described in Appendix I for the synthetic lethal mutations *27.89*.

Table 2: Mutations obtained from EMS screen of the second chromosome

Complementation groups	**Mutant localization**	**Allele**	**Phenotype[1]**	**Mutation**
Incenp	43A2-43A3	22.68	Lethal	Q611-Stop
		47.125	Lethal	ND
		18.197	♀ sterile	P746L
aurB	32B2	35.33	Lethal	L166F
		49.149	Lethal	Q95-Stop
borr		44.356	Lethal	Lost
snail	35D2	22.64	Lethal	ND
		27.18	Lethal	Q275-Stop
tumbleweed	50C6	15.173	Lethal	P463L
		16.135	Lethal	ND
6	31B1-32D1	27.89	Lethal	
7	34D1-43E16	27.88	viable	
8	?	48.116	Lethal	
9	25A2 – 34D1	44.13	Lethal	
10	?	46.10	Lethal	
11	?	47.90	?	
12	?	47.134	?	lost
13	?	49.178	?	lost
14	?	10.33	?	

[1] Based on phenotype of recombinant chromosome lacking the *subito* mutation.
? = not determined.

Mutation *27.89* was mapped between *dp* and *b* on chromosome 2R. Using SNP mapping, the synthetic lethal mutation was mapped to a 300kb region (see Appendix I). Surprisingly, it is possible that *27.89* is homozygous lethal but viable when heterozygous to a deficiency (see Appendix I), although we have not excluded a second lethal mutation on the *27.89* chromosome. To examine if *27.89* has a germline phenotype, we generated germline clones to collect *27.89* homozygous oocytes to determine if there was an effect on meiosis. In fact, homozygous *27.89* germline clones failed to develop into mature oocytes. This inability to generate mature germline clones is a phenotype shared by other mutations isolated in the screen such as *Incenp*, *aurB*, and *tumbleweed*, which are involved in the early mitotic cell divisions that occur pre-oogenesis. This indicates that *27.89* may play a role in cell division.

Mutation *22.64* was mapped to the interval between *b* and *pr* and, based on complementation to deficiencies, we found that *22.64* and *27.18* failed to complement existing alleles of *snail,* which encodes a zinc finger containing transcriptional repressor [156, 157]. This was a surprising finding because *snail* has not previously been shown to regulate spindle assembly. An analysis of mature oocytes using germline clones has revealed that *snail* mutants do not grossly affect meiotic spindle assembly (Figure 6). Further work is necessary to address why *snail* mutations enhance the *sub* mutant phenotype and if *snail* has a role in meiotic or mitotic spindle function. Interestingly, a *Drosophila* paralog of Snail, Worniu, has been shown to regulate cell cycle progression in neuroblasts [158].

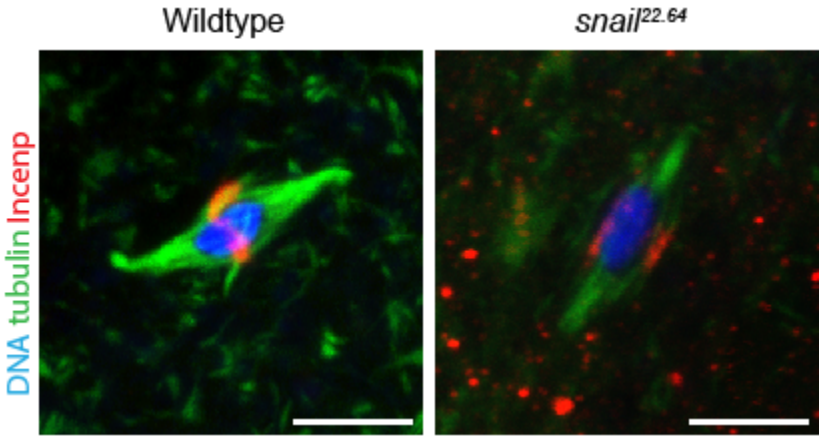


Figure 6: *snail* is not required for spindle assembly in meiotic metaphase I

snail$^{22.64}$ mutant oocytes, (generated using germ line clones) [146] shows no effect on meiotic spindle assembly or central spindle localization. Wild-type or mutant oocytes were stained for DNA (blue), tubulin (green) and Incenp (red). Incenp (Inner centromere Protein) is a member of the CPC which localizes to the central spindle if formed correctly as shown here. Scale bars represent 5 μm.

Both *15.173* and *16.135* genetically mapped to a region on chromosome 2R between *cn* and *c* and failed to complement a deficiency in this region, *Df(2R)Exel7128.* Based on this mapping, we found that both mutations failed to complement existing alleles of *tum,* which encodes the *Drosophila* homolog of RacGAP50C [159]. RacGAP50C is a Centralspindlin component that, as described earlier, also includes Pavarotti. Thus, all known members of two complexes, the CPC and Centralspindlin, genetically interact with *sub*. This is consistent with previous observations that Subito, Incenp, and RacGAP50C colocalize at the central spindle during mitosis [95] and meiosis [45]. Below are the results from analyzing the meiotic phenotype of oocytes depleted for RacGAP50C.

Mutations that enhance the dominant meiotic chromosome segregation phenotype of an *Incenp* allele

While the synthetic lethal screens revealed genes that interact with *sub*, these genes may not function in meiosis. In order to test interacting genes for a function in meiosis, we determined if they enhanced the nondisjunction phenotype of a transgene expressing the CPC member *Incenp* tagged with the *myc* epitope at its N-terminus (*UASP:Incenp^{myc}*). Females expressing *UASP:Incenp^{myc}* with *nos-GAL4:VP16* in addition to the endogenous alleles show approximately 1% X-chromosome non-disjunction. Females also heterozygous for a null allele of *sub* show approximately 20% X-chromosome non-disjunction [83] (Table 1). It is not known if the phenotype arises from the N-terminal tag or overexpression of Incenp. We used *UASP:Incenp^{myc}* to screen for mutations that dominantly enhance the nondisjunction phenotype, similar to *sub*.

We tested deficiencies that showed a synthetic lethal interaction with *sub* (Table 1). Using a cutoff for enhancement of 4% increase over the control, ten deficiencies showed an increase in nondisjunction ranging from 5-19% over control levels (Table 3). This assay appears to be more sensitive than the synthetic lethal phenotype for detecting interactions. For example, the strong nondisjunction phenotype of *Df(3L)emc-E12* contrasts with the mild synthetic lethal phenotype. Similarly, while *Df(3R)BSC452* had a milder synthetic lethal phenotype than the larger *Df(3R)ri-XT1,* it had a similar nondisjunction phenotype with *UASP:Incenp^{myc}.* Taking into account that some of these deficiencies overlap, these experiments identified at least six loci that genetically interact with *UASP:Incenp^{myc}*. These results suggest that some of the deficiencies identified as synthetic lethal also have at least one gene required for meiotic chromosome segregation.

Table 3: Frequency of mono-orientation in oocyte knockouts of central spindle proteins

GENOTYPE	AACAC % mono-orientation (N)[1]	DODECA % mono-orientation (N)[2]	P-value[3] (AACAC)	P-value[3] (DODECA)	Total
Wild type	4 (2)	0	NA	NA	45
Wild type (HS)[4]	5.5 (1)	5.5 (1)	NA	NA	18
tum HMS01417 (HS)[4]	50 (10)	45 (9)	0.004	0.009	20
Rho1 HMS00375	35 (9)	15 (4)	0.001	0.019	26
sticky GL00312	27 (6)	18 (4)	0.013	0.015	22
RhoGEF2 HMS01118	20 (5)	13 (3)	0.045	0.039	24
pbl GL01092	0	0	n.s.	n.s.	15
polo GL00512	61.9 (13)	47.6 (10)	0.009	0.01	21

[1]Percentage of total oocytes with 2nd chromosome AACAC probe mono-oriented.
[2]Percentage of total oocytes with 3rd chromosome Dodeca probe mono-oriented.
[3]Fisher's exact test was used to calculate the P-values compared to wild-type. n.s. = not significant.
[4]HS: These values were obtained from independent experiments with the heat shock driver.

In addition, we tested several candidate genes for enhancement of *UASP:Incenp^{myc}* (Table 4). A mutation in *non-claret disjunctional* (*ncd*), which encodes a kinesin-14 motor protein, was notable because it enhanced as strongly as *sub.* The groups of genes that most consistently enhanced *UASP:Incenp^{myc}* were Cyclin B and its regulators. Also relevant to the current study is the finding that mutants in cytokinesis genes such as *four wheel drive* (*fwd*), which encodes phosphatidylinositol (PI) 4-kinase III β [160] and *twinstar,* which encodes cofilin [161], enhanced *UASP:Incenp^{myc}*. Some mutants had surprisingly weak enhancement phenotypes, such as *pav, Df(3L)Exel9000*

that deletes *pav* and *tum,* which are strongly synthetic lethal. Other notable mutations that did not interact with *UASP:Incenp^{myc}* were in central spindle component gene *feo* (encodes PRC1) and checkpoint genes *BubR1* and *zw10.* These results suggest the enhancement of *UASP:Incenp^{myc}* depends on a specific defect. Indeed, there was evidence for allele specific interactions, with mutations in genes such as *fzy*, which encodes a Cdc20 homolog*, ord,* which encodes a nonconserved cohesion protein*, spc25,* which encodes a kinetochore protein, and *Incenp*. Furthermore, a *fwd* mutant enhanced *UASP:Incenp^{myc}* while a deficiency, *Df(3L)ED4177,* had a weaker phenotype. These results suggest that specific types of alleles may cause enhancement of *UASP:Incenp^{myc}*. It is possible that all the genes that interact with *UASP:Incenp^{myc}* affect the localization or regulation of *sub* (see Discussion).

Table 4: Genetic interactions with *UASP:Incenp^{myc}*

Type/ Homolog	Mutant	Nondisjunction progeny	Total progeny	Nondisjuction (%)
Cell Cycle				
CDC2/CDK1	*Cdc2^{E1-24}*	28	1347	4.2
	Cdc2^{B47}	22	1316	3.3
CKS30A	*Cks30A^{RA74}*	56	777	14.4
	Cks30A^{KO}	57	1333	8.2
Cdc20	*cort*	42	1626	5.2
Cyclin B	*CycB^{2}*	66	569	23.2
Cyclin B3	*CycB3^{L6540}*	18	1120	3.2
	CycB3^{2}	23	1367	3.4
CDC20	*fzy^{1}*	61	2044	6.0
	fzy^{6}	84	915	18.4
	fzy^{7}	61	1045	11.7
WEE1	*myt^{R3}*	18	741	4.9
Cdc25	*twe^{1}*	55	1130	9.7
	twe^{1}	32	960	6.7
WEE1	*wee^{DS1}*	14	988	2.8
	wee^{ES1}	10	874	2.3
Kinetochore				
CENP-C	*Cenp-C^{IR35}*	78	3319	4.7

NSL1	*Kmn1*^{G0237}	14	1361	2.1
NUF2	*Nuf2*^{EY18592}	23	2667	1.7
SPC25	*Spc25*$^{A34-1}$	60	1350	8.9
KNL1	*Spc105R*^{1}	20	2030	2.0
Motor protein/spindle				
MAST	*chb*^{4}	30	1169	5.1
CENP-E	*cmet*^{04431}	14	1257	2.2
Dynein	*dhc*$^{4-19}$	104	2359	8.8
	dhc$^{6-10}$	64	1702	7.5
PRC1	*feo*^{EA86}	4	633	1.3
NCD	*ncd*^{1}	209	1287	32.5
RAN	*ran*^{G0075}	10	441	4.5
Sentin	*ssp2*^{136}	56	2563	4.4
	ssp2^{32}	31	2692	2.3
Cohesion				
ORD	*ord*^{10}	20	2010	2.0
	ord^{5}	8	846	1.9
	ord^{3397}	132	3507	7.5
Securin	*pim*^{IL}	14	965	2.9
SMC1	*SMC1*^{exc46}	28	893	6.3
Separase	*sse*	9	431	4.2
Three Rows	*thr*^{3}	33	1318	5.0

Checkpoint				
MPS1	*ald^{c3}*	47	1393	6.8
	ald^{B4-6}	32	1976	3.2
BUBR1	*BubR1^{k03113}*	5	980	1.0
ZW10	*zw10^{1}*	13	2671	1.0
CPC/ POLO				
Aurora B	*aurB$^{2A43.1}$*	26	2139	2.4
	aurB$^{49-149}$	74	1904	7.8
	aurB$^{35.33}$	13	2273	1.1
	aurB^{1689}	9	2397	0.8
Aurora A	*aurA^{87Ac-3}*	7	951	1.5
Survivin	*Det^{e01527}*	8	436	3.7
INCENP	*Incenp$^{18.197}$*	52	2955	3.5
	Incenp$^{22.68}$	111	1602	13.9
	Incenp^{QA26}	49	2555	3.8
	Incenp^{3747}	86	2978	5.8
POLO	*polo$^{16-1}$*	13	2644	1.0
	polo$^{16-1}$	1	537	0.4
Cytokinesis				
ASP	*asp^{1}*	7	1227	1.1
Four wheel drive	*fwd^{2}*	53	855	12.4
Pavarotti/MLP1	*pav^{B200}*	24	1260	3.8

Subito/MKLP2	*sub*^{1}	144	1179	24.4
	sub^{1} *(18^{o})*	55	1573	7.0
Twinstar/ Cofilin	*tsr*^{1}	25	840	5.8
RacGAP50C	*tum*^{AR2}	10	2111	0.9
	tum^{DH15}	40	2710	2.9

Polo kinase is required for karyosome maintenance and homologous chromosome bi-orientation at metaphase I

In the previous sections, we identified genes that genetically interact with *sub* and *Incenp.* To determine if any are required during meiosis I for chromosome segregation, we examined oocytes lacking some of these proteins for meiotic defects. Loss of these genes might be expected to have a phenotype similar to *sub* mutants, with defects in spindle bipolarity and homolog bi-orientation.

Mutants of *polo* mutants are synthetic lethal with *sub* [95]. Since *polo* mutants are recessive lethal, we used *polo* RNAi (TRiP GL00014 and GL00512) to test the function of Polo in acentrosomal spindle assembly and chromosome segregation. Expression of both shRNA lines using ubiquitous *P{tubP-GAL4}LL7* resulted in lethality, suggesting the protein had been knocked down by the shRNA. Oocyte specific shRNA expression was achieved using *matalpha4-GAL4-VP16* and this resulted in sterility and knockdown of the mRNA as measured by qRT-PCR (Figure 7 and Table 5).

In wild-type oocytes, the chromosomes cluster together in a spherical mass referred to as the karyosome in the center of a spindle with well-defined poles and a

central spindle containing Subito and the CPC (Figure 8A, G). In *polo* GL00014 RNAi oocytes, there were defects in both chromosome and spindle organization. There were multiple karyosome masses (2-5) in most oocytes (Figure 8B) (69%, N=31). In addition, there were defects in spindle microtubules that we have classified into three types. First, 55% of the oocytes had disorganized spindles, with characteristics like frayed microtubules, untapered spindle poles and displaced karyosomes (Figure 8B). Second, 39% of the spindles appeared "hollow", composed primarily of central spindle microtubules and few or no kinetochore microtubules, those microtubules ending at the chromosomes (Figure 8C). Third, 16% of the oocytes had mono- or tripolar spindles (Figure 8D). Localization of central spindle proteins INCENP and Subito was not affected (Figure 8H), suggesting Polo is not required for central spindle assembly. Similar observations were made when the other shRNA, GL00512, was expressed (Figure 8I). The multiple karyosome phenotype (78%, n=14) and spindle defects (Table 5) were observed at similar frequencies with the two shRNAs.

Table 5: Spindle phenotypes and knockdown of shRNA lines used in this study

GENOTYPE	**Knockdown of mRNA (%)** [1]	***tubP-GAL4*** [2]	***matalpha4-GAL-VP16*** [2]	**% abnormal spindles** [3] **(N)**	**P-value** [4]
Wild type	NA	Viable	Fertile	0 (30)	-
polo GL00014	36	lethal	sterile	70 (31)	0.001
polo GL00512	9	lethal	sterile	100 (9)	0.001
Rho1 HMS00375	28	lethal	sterile	40 (43)	0.0001
sticky GL00312	35	lethal	sterile	30 (38)	0.0016
RhoGEF2 HMS01118	11	lethal	sterile	6 (18)	0.375
pbl GL01092	6	lethal	sterile	10 (21)	0.165

[1] Effect on mRNA expression evaluated by qRT-PCR (see Materials and Methods)
[2] Phenotype when crossed to the indicated GAL4 expressing line.
[3] Abnormal spindles were scored as any spindles that have frayed microtubules, not tapered poles or disorganized central spindle.
[4] Fisher's exact test was used to calculate the P-values compared to wild-type

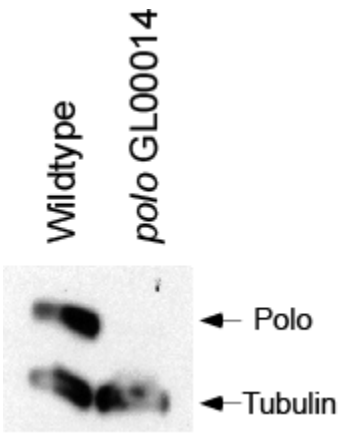


Figure 7: Quantification of *polo* RNAi

Western showing that the *polo* GL00014 hairpin does indeed knockdown POLO protein in the ovaries. POLO was detected using mouse monoclonal MA294 [162] and Tubulin was used as a loading control.

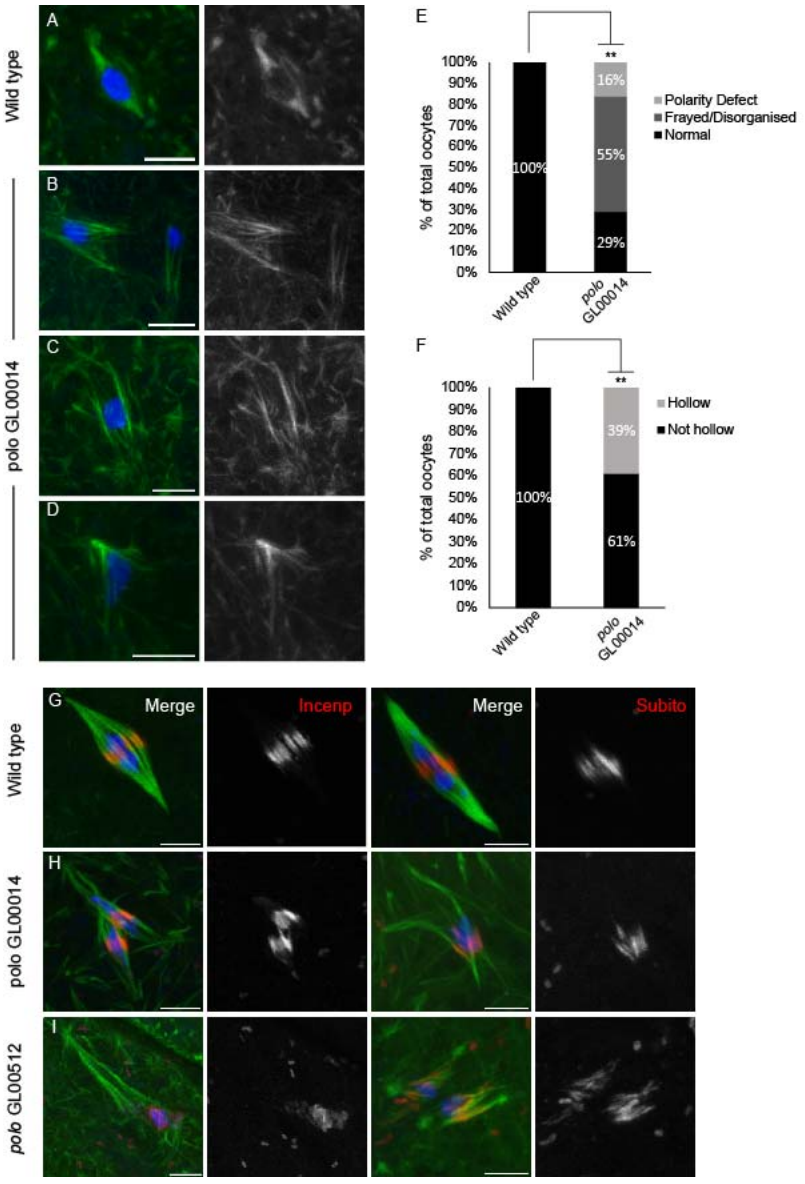


Figure 8: Polo is required for karyosome and spindle organization at meiotic metaphase I.

DNA is in blue, INCENP or Subito is in red and tubulin is in green. (A) A wild-type bipolar spindle and (B, C, D) *polo* RNAi oocytes showing monopolar, frayed/disorganized and hollow spindles respectively. (E,F) Spindle defects in *polo* RNAi (n=33) oocytes compared to wild type (n = 13). Percentage of oocytes with disorganized (E) or hollow (F) spindles are graphed separately. Asterisk denotes significantly higher spindle defects (for E, P = 0.001, for F, P = 0.009). (G) Wild-type bipolar spindle showing either INCENP or Subito staining at meiotic central spindle. (H,I) *polo* GL00014 or GL00512 RNAi oocytes showing INCENP and Subito localization. Scale bars represent 5 μm.

Polo accumulates at the kinetochores during meiotic metaphase of *Drosophila* oocytes [45]. Therefore, we examined the centromeres and kinetochores directly in Polo knockout oocytes. At metaphase in wild-type oocytes, the centromeres are attached to microtubules and oriented towards the two poles while the central spindle forms between them with proteins like Subito and INCENP localized in a ring around the karyosome. The kinetochore protein SPC105R localized normally in GL00014 oocytes (Figure 9A), suggesting Polo is not required for kinetochore assembly. With an average of 6.5 SPC105R foci per oocyte compared to 6.7 in wild type, these results also show that Polo is not required for cohesion at the centromeres at metaphase I (Figure 9B), in contrast to a recent report in mouse [125].

In wild-type oocytes, each pair of homologous centromeres orients towards opposite poles (known as bi-orientation). To test if *polo* knockdown oocytes have bi-orientation defects we performed FISH on *polo* RNAi oocytes with probes to the 2nd (AACAC) and 3rd (Dodeca) chromosome heterochromatin. Wild-type oocytes normally shows the second and third chromosome signals oriented towards opposite poles (Figure 9C, Table 3). In *polo* knockdown oocytes, the 2nd and 3rd chromosomes were frequently mono-oriented compared to wild type (Figure 9D-F, Table 3). Due to the separated karyosome phenotype, in some cases these defects were observed in oocytes where the 2nd and 3rd chromosomes were in different masses with their own spindles. Importantly, in most cases where the karyosomes had separated, the homologous chromosome pairs were in the same mass, indicating that the cohesion holding the bivalents together had not been released. These results show that Polo is required for microtubule attachment,

chromosome bi-orientation and karyosome structure, but is not required for central spindle function.

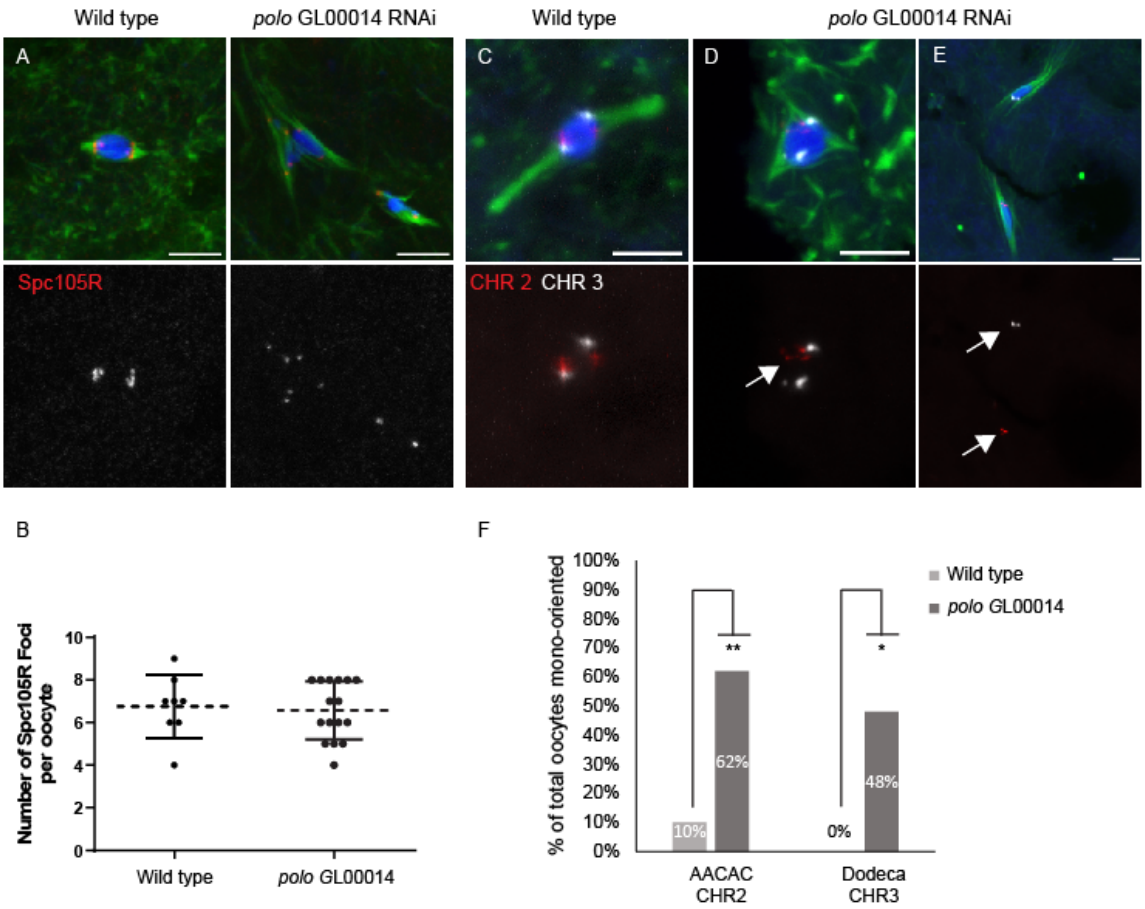


Figure 9: Polo is required for bi-orientation but not kinetochore protein localization.

A) Wild-type and *polo* RNAi oocytes were stained with SPC105R antibody to examine localization of kinetochore components. SPC105R is in red, DNA in blue and tubulin in green while the single channel shows SPC105R in white. (B) Graph showing the number of SPC105R foci in wild-type and *polo* GL00014 RNAi oocytes is not significantly different. (C-E) Probes to the AACAC repeat on the 2nd chromosome (red) and the Dodeca satellite on the 3rd chromosome (white) were used to assess bi-orientation. (C) In wild-type oocytes the 2nd and 3rd chromosome bi-orient towards the two poles within a single karyosome. (D,E) *polo* RNAi oocytes showing mono-orientation (arrows) without and with a karyosome defect, respectively. Scale bars represent 5 µm. (F) Summary of orientation defects in wild type and *polo* GL00014 RNAi oocytes. Asterisk shows significantly higher mono-orientation compared to wild type. P-values in Table 3.

Centralspindlin is required for meiotic spindle organization and homologous chromosome bi-orientation

We identified the Centralspindlin components *pav* and *tum* as synthetic lethal mutations with *sub*. The role of the Centralspindlin proteins in mitotic spindle midzone formation and stabilization leading to cytokinesis is well documented [163-166]. Their contribution to acentrosomal spindle assembly, however, has not been characterized. To test the role of the Centralspindlin complex in oocyte meiotic spindle assembly, we expressed short hairpin RNA (shRNA) against both *tum* and *pav* (HMS01417 and HMJ02232, respectively) [167] with *GAL4::VP16-nos.UTR*, which expresses GAL4 with the germline specific promoter from the *nanos* gene [168]. Both lines failed to generate mature oocytes, probably due to cytokinesis defects in the mitotic germline divisions, which would also preclude using the FLP-FRT system to generate germline clones. To circumvent this problem, we expressed each shRNA with *matalpha4-GAL-VP16*, which expresses throughout most of meiotic prophase but, importantly, after premeiotic S-phase [83]. However, these two shRNAs expressed with *matalpha4-GAL-VP16* also produced very few mature oocytes, indicating a role for these proteins in oogenesis which prevented analysis of their meiotic function.

Because of the requirement for *tum* and *pav* in oogenesis, we developed an alternative method to knock down gene expression in oocytes. We chose to focus on *tum* with the goal of knocking down expression after its requirement in oogenesis, but prior to spindle assembly in mature oocytes. To achieve this, a heat shock inducible driver (*P{GAL4-Hsp70.PB}89-2-1*) was used to express *tum* shRNA (Figure 11A). The *Drosophila* oocyte undergoes 14 developmental stages to form a mature oocyte [169].

Therefore, application of heat shock to a female will result in induction of the RNAi in all stages present at the time. At 5 hours after induction of *tum* shRNA by heat shock, the adult females produced inviable embryos, suggesting they had stage 14 oocytes depleted of TUM. This was confirmed using an antibody to TUM, which showed an absence of TUM protein on the spindle in a majority of the heat shock treated oocytes (Figure 10). At times greater than 5 hours after heat shock, in which stage 14 oocytes would have been at stage 10 or earlier at the time of heat shock, stage 14 oocytes were not produced. These results suggest that oocytes depleted of TUM at stage 10 or earlier fail to develop. With the 5 hour time point, however, we could investigate *tum* knockdown oocytes for defects in acentrosomal meiotic spindle assembly and chromosome segregation.

Similar to wild-type, in heat shocked wild-type oocytes or *tum* shRNA oocytes that were not heat shocked, the chromosomes were clustered with their centromeres oriented towards the two poles while the central spindle proteins like Subito and Incenp localize in a ring around the karyosome (Figure 11B). In oocytes depleted of *tum* by heat shock induced RNAi, Subito was mislocalized over the entire spindle (65%, n=20, $p<<0.05$) instead of its normal restriction to the central spindle in wild type (n=14) (Figure 11C). Since TUM localization is abnormal in *sub* mutants [45], these results indicate that Subito and TUM are interdependent for their localization during meiosis. TUM depleted spindles also had frayed microtubules or polarity defects (70%, n=20, $p<<0.05$) as compared to wild type (14%, n=14) (Figure 11D, E). These oocytes frequently had grossly elongated or broken karyosomes (Figure 11F) (47%, n=45, $p<0.0004$) compared to wild type oocytes (9%, n=33).

Defects in spindle assembly can lead to mono-orientation, where homologous centromeres are oriented towards the same pole. To test if *tum* knock down oocytes had bi-orientation defects, we performed fluorescent in situ hybridization (FISH) with probes to the heterochromatic regions of the 2nd (AACAC repeat) and 3rd (Dodeca satellite repeat) chromosomes. We found that in *tum* knockdown oocytes, 50% of oocytes had AACAC mono-oriented ($n=20$, $p<0.05$) and 45% of oocytes had Dodeca mono-oriented ($n=20$, $p<0.05$) as compared to 5.5% in wild type ($n=18$) (Figure 11F, G, Table 3). These results show that TUM is required for meiotic spindle assembly and chromosome bi-orientation.

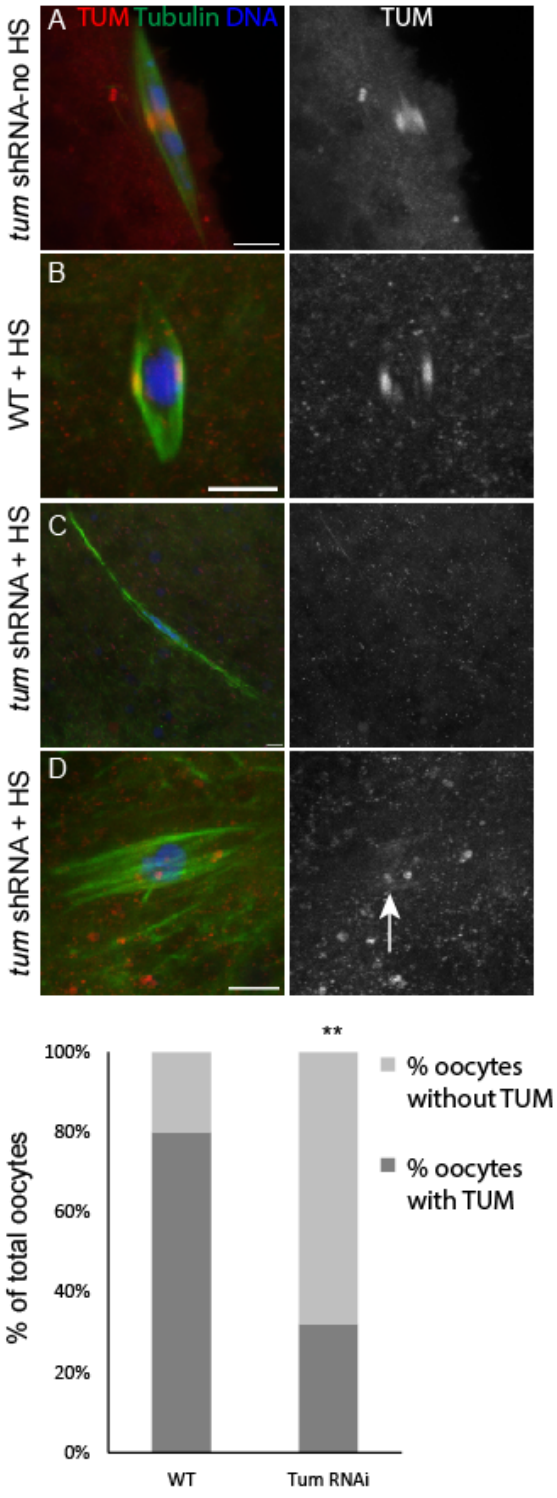
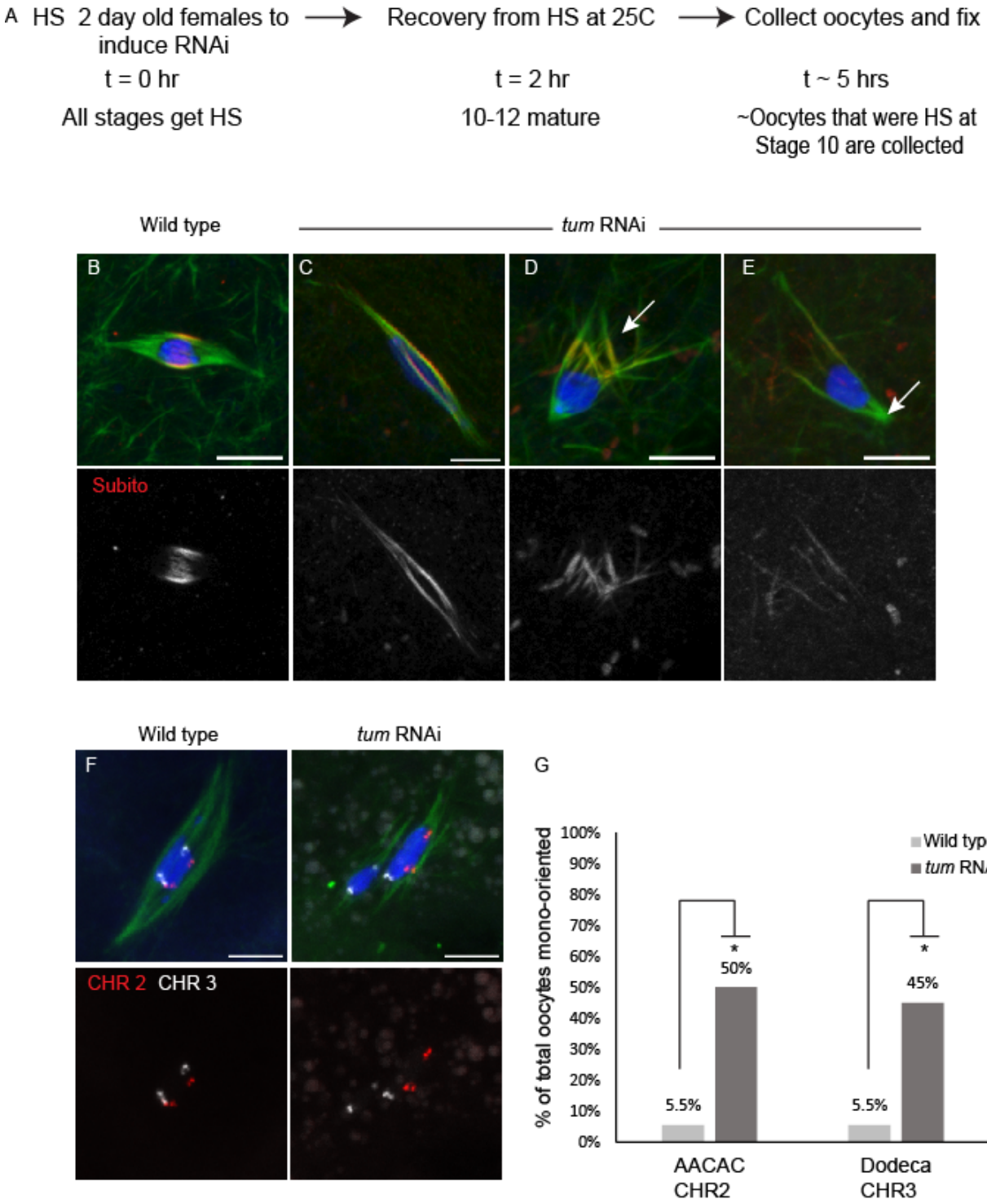


Figure 10: TUM knock down by heat shock induced RNAi.

Oocytes are shown with TUM (red) to assess the level of knockdown following heat shock. Tubulin is in green and DNA is in blue. A-B) Both *tum* shRNA without heat shock and wild-type females with heat shock show TUM staining at the central spindle in almost all oocytes. C-D) TUM localization is greatly reduced or eliminated in oocytes. Faint staining (arrow) in some oocytes can be attributed to the non-uniformity of the heat shocked oocytes in adult females E) Quantification of TUM localization. The WT category includes both heat shocked and non-heat shocked oocytes; 80% of these oocytes had TUM localization to the spindle (n=24). In contrast, only 32% of *tum* RNAi oocytes had TUM localization, which is significantly lower than the controls, and was usually more diffuse and fainter than wild-type (n=28, Fisher's exact p-value =0.0012). The scale bars are 5 µm.

Figure 11: TUM is required for proper localization of Subito to the central spindle and chromosome segregation during meiosis I.

(A) Protocol used to induce RNAi expression late in oogenesis to bypass early requirement of the TUM protein in oocyte development. The heat shock treatment caused some mild karyosome defects in these controls. However, these were occasionally observed in wild type and the mutant defects described below were qualitatively different because they involved spindle organization defects not observed in the controls. (B-E) Wild type and *tum* RNAi females were heat shocked and examined for central spindle components. DNA is shown in blue, tubulin in green and Subito in red in merged images. B) Subito localizes to the central spindle region in wild type. (C-E) *tum* RNAi oocytes, showing diffuse Subito staining all along the length of the spindle (C), frayed spindles in (D) and monopolar spindles in (E). (F) Wild type and *tum* RNAi oocytes showing FISH probes AACAC (chromosome 2) in red and Dodeca (chromosome 3) in white. (G) Summary of mono-orientation frequency in *tum* RNAi oocytes compared to wild-type. Asterisk indicates significantly different values. Scale bars represent 5 μm. P-values are calculated by Fisher's exact test (Table 3).


A HS 2 day old females to induce RNAi
t = 0 hr
All stages get HS
Recovery from HS at 25C
t = 2 hr
10-12 mature
Collect oocytes and fix
t ~ 5 hrs
~Oocytes that were HS at Stage 10 are collected
Wild type
tum RNAi
B
C
D
E
Subito
Wild type
tum RNAi
F
CHR 2 CHR 3
G
% of total oocytes mono-oriented
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Wild type
tum RNAi
*
50%
45%
5.5%
5.5%
AACAC CHR2
Dodeca CHR3

Meiotic function of Centralspindlin may depend on Rho1 activation

Since the above results show the Centralspindlin complex is required for meiotic chromosome segregation, we investigated the role of the proteins activated by this complex. Pebble, a Rho Guanine Exchange Factor (GEF, ECT2 homologue), associates with the Centralspindlin complex during mitotic anaphase and together they regulate the GTPase Rho1 (RhoA) and its downstream effectors such as Citron kinase (encoded by *sticky*) [170-172]. There is also a second GEF, RhoGEF2, that may play a role in the germline [173]. Rho1 and Sticky (citron kinase homolog) are recruited by Centralspindlin to the spindle midzone during mitosis [151, 174, 175]. We failed to detect localization of Rho1 to the meiotic spindle using available antibodies. However, these negative results could be explained by localization to membranes or the actin cytoskeleton, or fixation conditions, which we have found some antibodies are very sensitive to in *Drosophila* oocytes [148]. In contrast, we did detect Sticky on oocyte meiotic spindles (Figure 12).

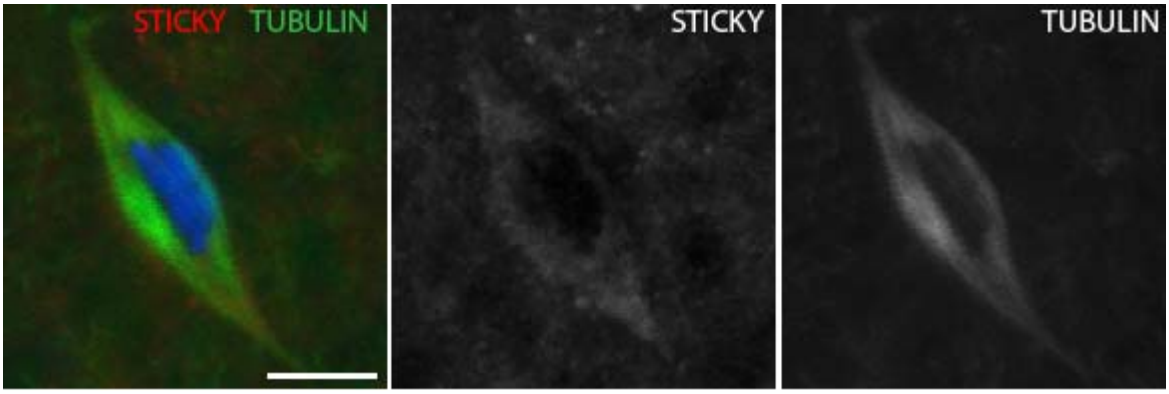


Figure 12: Sticky localizes to the meiotic spindle.

Wild-type oocytes were stained with rabbit anti-Sticky antibody shown in red in merge and white in single channel. Tubulin is shown in green in the merge and white in single channel and DNA is in blue. The scale bar is 5 μm. We also tested a, but were unable to detect any localization.

To examine their roles in spindle microtubule organization and homologous chromosome bi-orientation in oocytes, *matalpha4-GAL-VP16}V37* was used to express shRNAs against *Rho1*, *sticky*, *RhoGEF2* and *pebble* (HMS00375, GL00312, HMS01118 and GL01092 respectively). Expression of each shRNA with *P{tubP-GAL4}LL7* caused lethality, suggesting the proteins were indeed knocked down. Consistent with this, all four shRNAs caused significant knockdowns when evaluated using qRT-PCR of oocytes.

We used antibodies against Subito and INCENP as markers for the integrity of the meiotic central spindle. Wild-type metaphase spindles have a well-defined band of Subito and INCENP and a bipolar spindle (n=30) (Figure 13A, F). However *Rho1* RNAi oocytes showed a significantly higher level of abnormal spindle microtubule organization (40%, $p<<0.05$) accompanied by aberrant central spindle protein localization (Figure 13B, F, Table 5). *Sticky* RNAi oocytes also showed significant microtubule disorganization (30%, $p<<0.05$) and Subito and INCENP mis-localization compared to wild-type control oocytes (Figure 13C, F, Table S2). *RhoGEF2* and *pbl* RNAi oocytes did not show any significant defects in either spindle formation or Subito or INCENP localization (Figure 13, E, F, Table 5). These results indicate that some mitotic cytokinesis proteins regulate acentrosomal spindle assembly and central spindle integrity in meiosis.

In order to test whether *Rho1*, *sticky*, *RhoGEF2* and *pebble* RNAi oocytes show bi-orientation defects, we performed FISH on knockdown oocytes. *Rho1*, *sticky* and *RhoGEF2* showed significantly higher frequency of oocytes with mono-orientation defects compared to wild-type oocytes (Figure 14A-D, F). In contrast, *pbl* RNAi oocytes showed no AACAC or Dodeca mono-orientation defects (n=15) (Figure 14E, F, Table 3).

These results indicate that Rho1, Sticky and RhoGEF2, but not Pebble, are required for the kinetochores to make correct attachments to microtubules that result in bi-orientation.

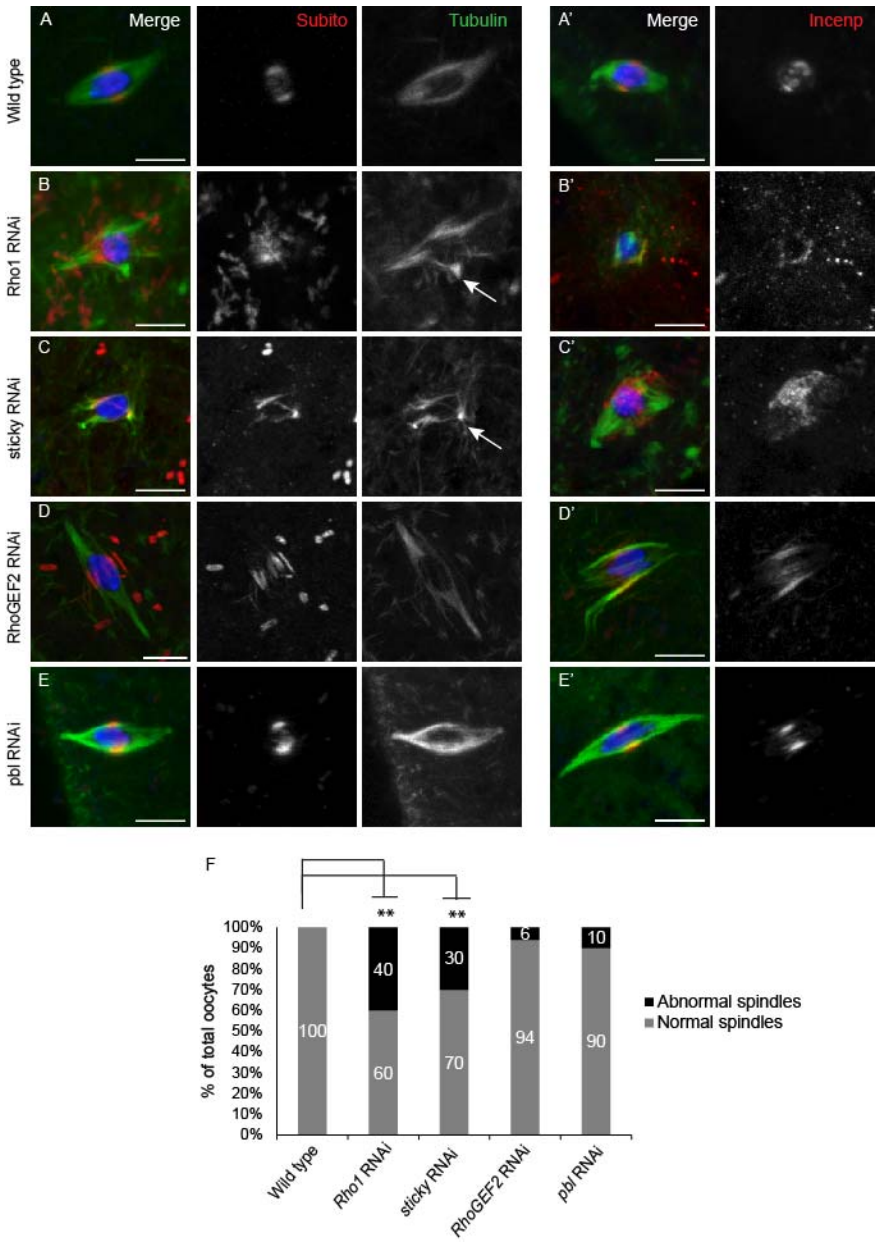


Figure 13: Mitotic midzone proteins affect microtubule organization and central spindle protein localization in meiotic metaphase I.

Oocytes were stained with DNA (blue), Tubulin (green) and Subito or INCENP (red). (A,A') Wild-type oocytes localize Subito or INCENP to the central region of a bipolar metaphase spindle. (B,B') *Rho1* and (C,C') *sticky* RNAi oocytes show disorganized microtubules (marked with arrows) and aberrant Subito or INCENP localization. (D,D') *RhoGEF2* and (E,E') *pbl* RNAi oocytes resemble wild type in both microtubule organization and Subito localization. (F) Graph summarizing the spindle defects in wild type and RNAi oocytes. Significantly different P-values are indicated by asterisks. Scale bars represent 5 μm.

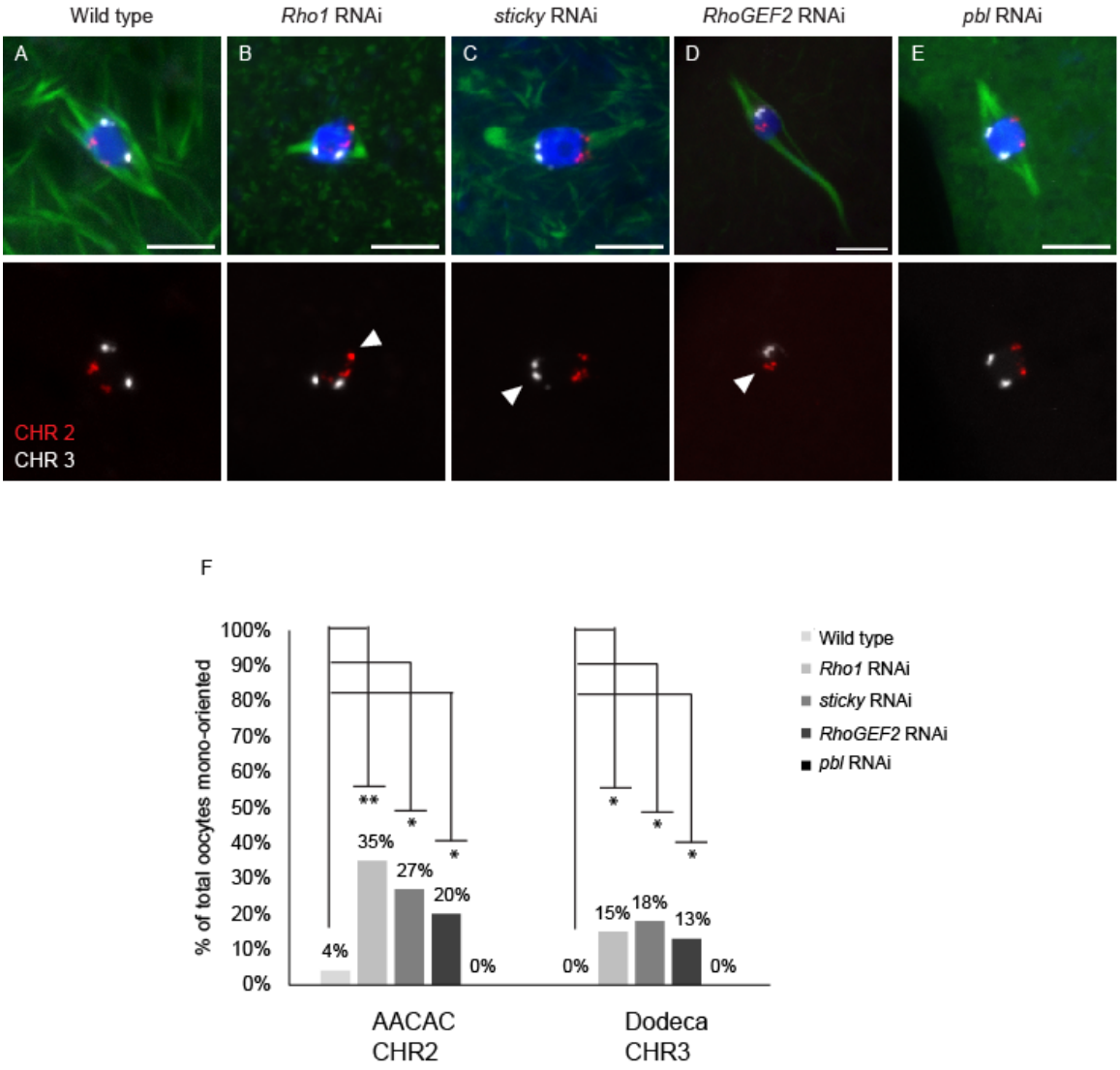


Figure 14: Homologous chromosome bi-orientation is affected by *Rho1*, *sticky* and *RhoGEF2* but not by *pbl* RNAi.

(A-E) Top panels show merged images with FISH probes AACAC (chromosome 2) in red and Dodeca (chromosome 3) in white. DNA is in blue and tubulin is in green. (A) The probes in wild type are bi-oriented towards the two poles. (B,C,D) *Rho1*, *sticky* and *RhoGEF2* RNAi oocytes show one or both probes mono-oriented. (E) *pbl* RNAi oocyte with no orientation defects. Bottom panels show only the probes, with mono-orientation marked by arrowheads. Scale bars represent 5 µm. (F) Summary of orientation defects. Significantly higher mono-orientation defects in mutants are indicated by asterisks, P-values are indicated in Table 3.

VI. Discussion

While the microtubules of the acentrosomal spindle may be nucleated from cytoplasmic MTOCs [91] or from the chromatin itself [77], additional factors are required to organize them and segregate chromosomes. One such factor is the kinesin-6 motor protein Subito, which functions in cytokinesis during mitotic anaphase but during acentrosomal meiosis it is required to organize a bipolar spindle [96]. Similarly, another prominent central spindle component is the CPC, which is also required for acentrosomal spindle assembly [82, 83]. Based on these and other studies, we and others have suggested that, in the absence of centrosomes, the central spindle has a critical role in organizing the microtubules and chromosome alignment [39, 45, 83, 139]. Thus, we have initiated the first comprehensive study of central spindle protein function in acentrosomal spindle assembly and chromosome segregation.

In addition, cytological analysis of mitotic cells has shown that Subito is required to localize the CPC to the midzone during cytokinesis [95], consistent with the studies of its human homolog, MKLP2 [44]. This function only becomes essential when the dosage of the CPC is reduced. We have used this observation to identify genes that interact genetically with *sub*, with the expectation that we might find other genes that function in meiotic spindle assembly like the CPC and Subito. We identified proteins associated with the mitotic central spindle or midzone, such as all CPC and Centralspindlin components. Furthermore, we confirmed that several mitotic central spindle genes have a role in meiotic acentrosomal spindle assembly. These are functions during metaphase I, rather than anaphase and cytokinesis as in mitotic cells. Finally, this study has identified

at least 16 novel loci that interact with *sub* (synthetic lethal) and at least six novel loci on the third chromosome that interact meiotically with *Incenp*.

Polo may only function at the kinetochore during female metaphase I

We had previously found that *polo* mutations cause synthetic lethality and there is a direct interaction between Polo and Subito [95]. Therefore, we determined if Polo has a meiotic central spindle function. Previous work in *Drosophila* has shown that Polo inhibition by Matrimony is important for the maintaining prophase arrest [176, 177], but its role in meiosis I has not been characterized. Polo has diverse roles in mitosis ranging from centrosome maturation, spindle assembly, kinetochore attachment the SAC response, and cytokinesis [178, 179]. Correlating with these diverse functions, Polo localizes to the centrosomes and centromeres at metaphase, and the midzone at anaphase. Meiotic metaphase is different, however, because Polo retains its localization to the centromeres [45], unlike meiotic central spindle proteins like Subito and the CPC. In analyzing oocytes lacking Polo, we observed two prominent phenotypes. First, the chromosomes were disorganized, resulting in the failure to maintain a single karyosome. Second, these oocytes form aberrant spindles that appear to be composed mostly of central spindle. The spindles often appear "hollow", which can reflect loss of kinetochore but not central spindle microtubules [98]. These results are consistent with a role for Polo in stabilizing microtubule – kinetochore attachments [180-183] but with no function in the central spindle. These results also show that, while the meiotic metaphase central spindle contains many proteins found in the anaphase midzone, it also has important differences. Indeed, it remains to be determine if Polo relocalizes to the midzone at anaphase I.

Mitotic spindle midzone proteins regulate acentrosomal spindle function.

From our genetic screens, we identified mutations in all the components of two essential mitotic central spindle components: the CPC and Centralspindlin. Our analysis of TUM shows that Centralspindlin also plays an important role in organizing the acentrosomal spindle and localizing Subito. It is possible that since Centralspindlin colocalizes with Subito in meiosis, it is involved in stabilizing the interpolar microtubules in the central spindle. TUM localization is in turn dependent on Subito, demonstrating the underlying interdependence of the meiotic central spindle proteins [45].

In its cytokinesis role, Centralspindlin signals to the actomyosin complex via the RhoA pathway. Pebble, the *Drosophila* homolog of the guanine exchange factor (GEF) ECT2, is critical for cytokinesis [166, 171, 184], interacts with RacGAP50C [170, 172] and activates RhoA. Indeed, we found that Centralspindlin downstream effectors Rho1 (RhoA) and Sticky (Citron kinase) are required for accurate meiotic chromosome segregation. Loss of these proteins resulted in spindle assembly and centromere bi-orientation defects. This is the first report that the contractile ring proteins have been shown to be involved in meiotic chromosome segregation. Given these results, however, it was surprising that Pebble was not found to be critical for meiosis. *Drosophila*, however, has RhoGEF2 that is also a GEF and is required to regulate actin organization and contractility in the embryo [173].

A hierarchy of central spindle assembly and function

None of the knockdowns we have studied have the same phenotype as a *sub* mutant with spindle bipolarity defects. Similarly, while we identified several interesting genes that interact with *Incenp,* most did not interact as strongly as *sub* mutants. We

suggest these data reflects a direct physical interaction between Subito and the N-terminus of INCENP, as recently described for MKLP2 [185]. That we observed consistent genetic interactions between *Incenp* and Cyclin B and some of its regulators, which are also known to regulate Subito/ Mklp2 localization [43, 185] is consistent with a specific direct interaction between Subito and Incenp. A surprisingly strong interaction was also observed between *Incenp* and *ncd* mutants, suggesting the NCD motor has an important role in central spindle assembly. Indeed, we previously observed an allele specific genetic interaction between *ncd* and *sub* [96]. These results are striking because *ncd* mutants do not have cytokinesis defects, suggesting that NCD may have a specific function in the central spindle of acentrosomal meiosis.

Based on the lack of mutants with phenotypes similar to *sub,* we suggest the integrity of the meiotic central spindle and spindle bipolarity may depend only on the activity of Subito to bundle antiparallel microtubules. Our results also show, however, that contractile ring proteins are required in meiosis to maintain the organization of microtubules and promote homolog bi-orientation. One interpretation of these data is that the actin cytoskeleton is required for the organization or function of the meiotic central spindle microtubules. While the actin cytoskeleton is required to position the meiotic spindle in some systems [186-188], it could also affect functioning of the spindle itself. Indeed, the formin mDIA3 has been shown to be involved in recruiting Aurora B for error correction [189]. RhoA has been shown to regulate microtubule stability, possibly through its downstream effectors mDia or Tau [190-192]. In the future, it will be important to directly perturb the actin cytoskeleton and examine chromosome alignment and segregation.

An alternative is that the contractile ring proteins directly regulate microtubule organization. Interestingly, RhoGEF2 has been found to associate with microtubule plus ends in a process that depends on EB1 [193]. Citron kinase (*sticky*), rather than functioning simply as a downstream effector of RhoA, directly interacts with Pavarotti and another Kinesin, Nebbish (Klp38B) and is required for RhoA and Pavarotti localization and midzone formation [174, 175]. In the future, it will be important to determine if the meiotic function of Citron kinase depends on interactions with actomyosin components, or only with the microtubules.

Our results are the first to implicate proteins required during mitosis for midzone function and cytokinesis in meiotic chromosome segregation. In cytokinesis, a precise position of a division plane must be established [136]. This activity may also be important for the acentrosomal spindle; a precise division plane may be established during metaphase I in order to sort each pair of homologous chromosomes. This process could result in the two kinetochores of each bivalent interacting with the microtubules from opposite poles. Activities such as those promoted by the Centralspindlin complex may fine tune the central spindle structure to create a precise division plane. Further studies will be required, however, to determine if the meiotic spindle depends on interactions with the actin cytoskeleton for chromosome segregation, or these proteins exert their effects only through central spindle microtubules at meiosis I.

CHAPTER 3: LOCALIZATION AND ACTIVITY OF THE KINESIN-6 SUBITO IS REGULATED BY THE N-TERMINUS

I. Preface:

This chapter will be submitted soon, as an individual paper. Some data presented here has been previously reported in Jeffry Cesario's dissertation, 2010. My contribution to this paper is: writing of the entire paper, organization, analysis and compilation of the data into figures, and experiments on the serine substitution mutants, the deletion mutants of the C-terminus and 42-76 deletion of the N-terminus. Some of the genetic assays and scoring for non-disjunction was done by Anna Maria Hinman.

I. Abstract

The kinesin 6 Subito, MKLP-1 homolog, is required for establishing *Drosophila* oocyte spindle bipolarity and chromosome biorientation. We have previously proposed that the N-terminus negatively regulates Subito activity in oocytes, restricting it to the anti-parallel central spindle microtubules associated with the chromatin. In this study we show that the N-terminus has antagonistic regulators in the first and second halves that restrict its functions to the meiotic central spindle. We also show that this negative regulation may be partly due to the phosphorylation of two serine residues in the N-terminus. We have further identified conserved regions of the N-terminus required for localization of the protein. Surprisingly, this analysis has revealed separation of function mutants, identifying domains of the N-terminus required for mitotic function, but not meiotic. This mechanism may be applicable to other kinesin 6 members and extend to other families.

II. Introduction:

Accurate segregation of chromosomes in most animal cells is directed by spindle assembly and subsequent cytokinesis. While spindle assembly is guided by the presence of centrosomes, cytokinesis is orchestrated by a plethora of proteins which localize to the anaphase central spindle after faithful segregation has occurred (reviewed in [143]). The kinesin 6 family of proteins is essential for formation of the mitotic spindle midzone and promoting cytokinesis [194, 195]. This interaction however has to be restricted spatiotemporally and that requires additional regulation of the protein's localization and activity. In mitosis, the localization and function of the kinesin 6, MKlp2, is dependent on and regulated by Aurora B and the chromosomal passenger complex [44, 196]. In fact, MKlp2 is required for relocation of the CPC to the spindle midzone and this interaction is negatively regulated by Cdk1 [43]. However although a lot is known about the function and regulation of MKlp2 in mitosis, its requirements in meiosis is not well known.

The function of the kinesin 6 motors is important in both mammalian cell culture and invertebrates [44, 194, 197, 198]. Furthermore, the functions of these proteins may be differentially regulated in different cell types. Indeed, Subito, the *Drosophila* MKlp2 homolog is non-essential for mitosis but is required to build a bipolar spindle and bi-orient chromosomes in meiosis [45]. Meiotic spindle assembly and chromosome segregation is inherently different than mitosis, in that, there is no centrosome in *Drosophila* oocytes [199]. Spindle assembly may be initiated by chromosome bound signal, from the CPC [82, 83].

The formation of a meiotic metaphase central spindle In *Drosophila*, similar to the mitotic anaphase spindle midzone may compensate for lack of centrosomes [45].

Subito and Pavarotti, the MKlp1 homolog, and other cytokinesis factors are known to localize to this structure at meiotic metaphase [95]. It is unclear however, how the localization and activity of these motors are regulated to function in meiosis. In fact, the specific domains required for regulation of function is not well studied. It is known that in oogenesis, the central stalk region of Pavarotti (Mklp1) is required for localization to ring canals and that this domain is also important for binding to spindle midzone microtubules [200, 201]. For MKlp2, the C-terminal domain has been shown to be important for binding Mad2, regulated by the mitotic checkpoint [202]. In fact the C-terminus also regulates interaction with microtubules in a Cdk1 dependent manner. This same phosphoregulation may also be important for interactions between the C and N-termini of MKlp2 forming a folded state [185]. This sort of auto inhibitory mechanisms have been shown to be important for regulation of kinesin activity (reviewed in [203]). Similar domain analysis is lacking in *Drosophila*, for Subito. Earlier, we had shown that an N-terminal deletion of Subito causes ectopic spindles to appear in the ooplasm unrelated to chromosome signals [94]. These results support the hypothesis that kinesin 6 is negatively regulated, through its N-terminal domain to direct oocyte spindle assembly in the vicinity of the chromatin.

Our study investigates the contribution of the N and C-terminus of Subito, to the regulation of motor localization and activity. We have found that the first 21 amino acids in the N-terminus is important for localization of the motor to the meiotic spindle. Surprisingly deleting the first 41 amino acids restores localization indicating that there are antagonistic regulators in the N-terminus possibly a positive one in the first half and a negative one in the second. We have further identified two conserved serine residues in

the N-terminus which may be involved in the negative regulation of Subito, indicated by the increase in ectopic bundling in the ooplasm shown by the non-phosphorylatable mutant protein similar to the N-terminal deletion. This phosphoregulation however may be essential for structures important for embryogenesis, since these sites are non-essential for meiotic function but are required for mitotic divisions. Interestingly we have been able to identify other conserved regions within the N-terminus that also show similar separation of function. In fact while deletion of the entire first 41 amino acids is required for both mitotic and meiotic function, the conserved region 24-33 is also dispensable for meiosis in keeping with the fact that the conserved residue Ser24 is not required for meiosis. We have also shown that deleting the entire coiled coil domain in the C-terminus also hampers localization indicating additional levels of regulatory input from other parts of the kinesin.

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 [168]. 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 deletions 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 non-virgin females by physical disruption in a common household blender [148] [140].The oocytes were fixed in modified and 100 mM cacodylate/8% formaldehyde fixative for 8 min and then their chorion and vitelline membranes were removed by rolling the oocytes between the frosted part of a slide and a coverslip. For immunofluorescence rolled oocytes were extracted in PBS/1% Triton-X-100 for 1-2 hours and blocked in PBS/0.1% Tween-20/0.5% BSA (PTB) for an hour and then antibodies were added. For FISH, rolled oocytes were stepped into 20%, 40% and 50% formamide solutions followed by 5 hour incubation in 50% formamide at 37^{o}C. Oocytes were incubated with the FISH probes at 91 ^{o}C for 3 minutes and then put into the 37 ^{o}C water bath overnight. Oocytes

were stepped out of formamide and blocked in PTB for 4 hours before addition of antibodies [83].

Embryos were collected in cages with grape juice plates containing yeast. 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 [204]. Embryos were then rehydrated using PBS and blocked for an hour in PTB before addition of antibodies.

Oocytes and embryos were stained for DNA with Hoescht 33342 (10µg/ml) and for microtubules with mouse anti-α tubulin monoclonal antibody DM1A (1:50), directly conjugated to FITC (Sigma, St. Louis) or rat anti-α tubulin monoclonal antibody (1:75) (Millipore). The primary antibodies were rat anti-SUB antibody (used at 1:75) [45], rat anti-HA (Roche, clone 3F10) (1:25), and rat anti-INCENP (1:500) [205]. These primary antibodies were combined with either a Cy3 or Cy5 secondary antibody preabsorbed against a range of mammalian serum proteins (Jackson Immunoresearch, West Grove, PA). FISH probes used were to the AACAC repeat (2nd chromosome) and dodeca repeat (third chromosome). Oocytes were mounted in SlowFade gold (Invitrogen). Images were collected on a Leica TCS SP2 or SP5 confocal microscope with a 63x, NA 1.3 or 1.4 lens respectively. Images are shown as maximum projections of complete image stacks followed by merging of individual channels and cropping in Adobe Photoshop (PS3).

IV. Results:

subito null mutants have tripolar or monopolar spindles during meiosis and are sterile due to a defect in pro-nuclear fusion [45]. We have previously shown that the N-

terminus is important for restricting the activity of the kinesin to the chromosomes [94]. Deletion of the entire N-terminus of Subito (SUB), epitope tagged with HA, results in ectopic bundles of microtubules in the ooplasm away from the chromosomes even in the presence of endogenous Subito but is not as severe as the GFP tagged SUB with the N-terminus deleted [94](Figure 15A). This phenotype was not observed in full length Subito tagged with HA confirming that the N-terminus is involved in negatively regulating Subito's activity. Within the N-terminus there are several regions conserved in other insects (Figure 15A, B).To determine whether these conserved regions contribute to the regulation of Subito in meiosis, we created a series of deletions and substitutions in the N-terminus (Figure 15C). These mutant transgenic proteins were made by fusing the coding region of the mutant variants to three copies of the HA epitope tag at the N-terminus. The transgenes were expressed in the germline using a binary system combining *UASp-GAL4* system adapted from yeast [168]. For all the experiments described below, the *UASp:sub* transgenes were expressed using *P{GAL4::VP16-nos.UTR}MVD1*, which has *GAL4* fused to the *nanos* promoter and induces the expression of *UASp* containing transgenes in the female germline.

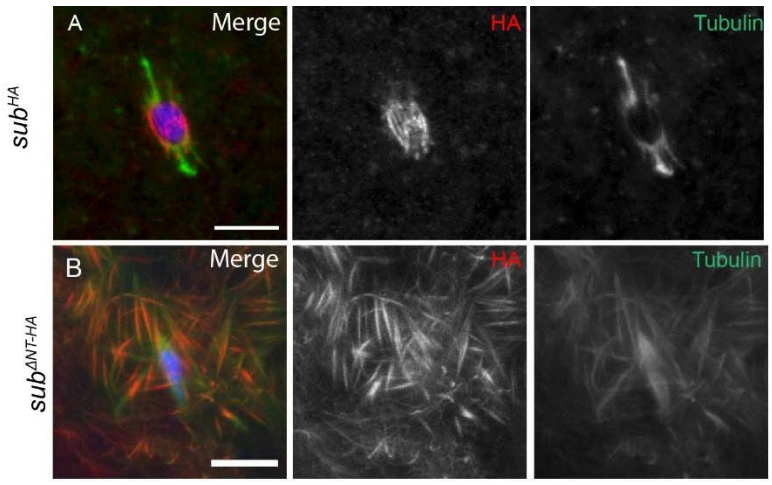
Two large deletions were created which split the N-terminus in half and together span the entire N-terminus ($sub^{\Delta(1-41)}$ *and* $sub^{\Delta(42-76)}$) (Figure 15C). A series of smaller deletions were also created, eliminating coding regions within the N-terminus that are highly conserved in other *Drosophila* species ($sub^{\Delta(1-21)}$ and $sub^{\Delta(24-33)}$) keeping the motor domain and the C-terminus intact. In addition, two conserved serine residues have been shown to be phosphorylated in *Drosophila* Kc167 cell line [206]. Transgenes were engineered that substituted these conserved serines with alanines at amino acid position

16 and 24 to test for possible regulation by kinases (*sub*^{S16A}, *sub*^{S24A}, and *sub*^{S16AS24A}). Additionally, we also created two deletions in the conserved regions of the C-terminus that are described later. For all the transgenes constructed, fertility and X-chromosome non-disjunction rates were tested in the presence of endogenous SUB and found to have no dominant effects (Table 6).

Table 6**:** Fertility and non-disjunction phenotypes in N-terminus mutants in the wild type background

Transgene[1]	Progeny/female parent	% Non-Disjunction	Total flies
sub^{HA}	76	0	1514
$sub^{HA\text{-}Myc}$	41	0	3146
$sub^{\Delta NT}$	43	0.23	869
$sub^{\Delta(1\text{-}21)}$	47	0	1889
$sub^{\Delta(24\text{-}33)}$	54	0.08	2581
$sub^{\Delta(1\text{-}41)}$			
$sub^{\Delta(42\text{-}76)}$	36	0.52	4251
$sub^{S16AS24A}$	38	0	1219
$sub^{\Delta CT1}$	40	0	1632
$sub^{\Delta CT2}$	32	0.26	1531

[1]Each transgene was expressed in the presence of endogenous SUB protein and resultant progeny was tested for fertility and X-chromosome non-disjunction.



C Alignment of SUB N-terminus in various insects showing deletions and Ser16 and Ser24

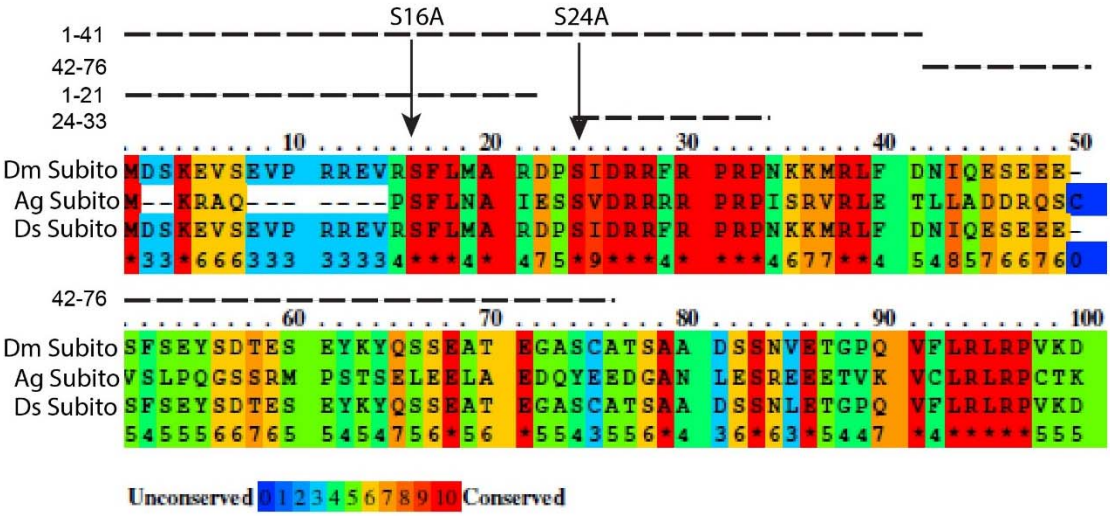


Figure 15**:** Ectopic bundling by *sub*$^{\Delta NT\text{-}HA}$ and schematic of all deletions in N-terminus

A. Wild type oocytes expressing *sub*$^{\Delta NT\text{-}HA}$, stained with DNA (blue), HA (red) and Tubulin (green) and single channels are shown in white. Ectopic bundling of microtubules can be seen in the ooplasm with SUB$^{\Delta NT\text{-}HA}$ localized to them. B. Alignment of Subito N-terminus in 3 species using the online PRALINE alignment program for *Drosophila melanogaster*, *Anopheles gambiae* and *Drosophila simulans*, showing the conserved domains in red. Different deletions made are also indicated above the sequence and the two conserved serine sites that may be involved in phospho-regulation of the protein.

Amino acids 1-21 is required for localization of Subito and is antagonized by second half of the N-terminus

To pinpoint the regulatory region in the N-terminus, we looked at localization and ectopic bundling activity of the deletion mutants. Of the two larger deletions, $SUB^{\Delta(1-41)}$ is able to localize similar to wild type in the oocyte (n=11) and does not show ectopic spindles unlike the deletion of the entire N-terminus (Figure 16B). This deletion is also able to rescue spindle defects of a $sub^{131/1}$ mutant oocyte similar to WT (Figure 16A, C). This suggests that either the deletion of the second non-conserved half of the N-terminus is important for regulation, or there are additional factors within the first 41 amino acids that may be responsible for negative regulation.

Surprisingly the second half of the N-terminus $SUB^{\Delta(42-76)}$ though non-conserved had dominant effects on the spindle morphology. It has significantly higher abnormal spindles including fraying and stunted poles as compared to the wild type SUB^{HA} transgene (55%, n=31) but does not show any ectopic bundling phenotype (Figure 16D). However, this deletion mutant does rescue central spindle defects in a $sub^{131/1}$ null background (Figure 16E).This shows that while deleting the second half can interfere with the function of the wild type protein complex, it cannot promote microtubule bundling away from the chromosomes.

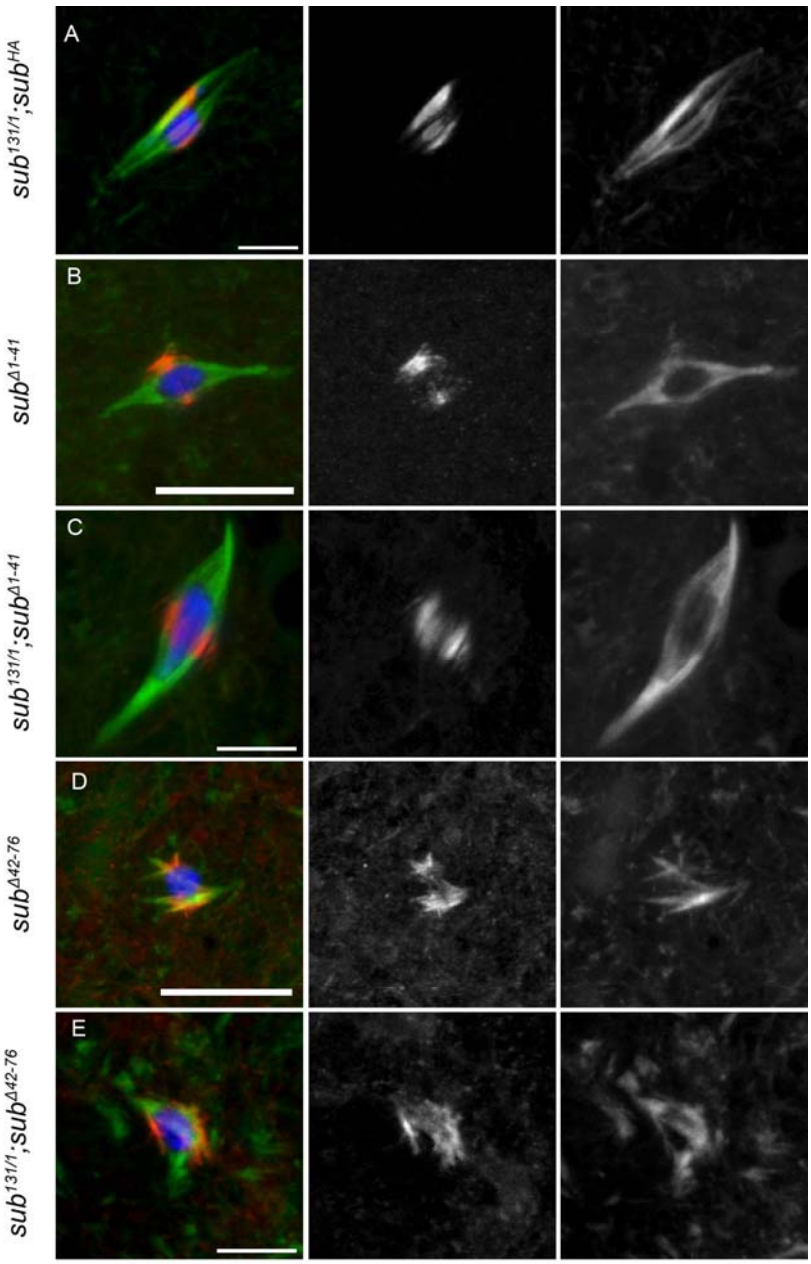


Figure 16**:** Localization and effects of full length and mutant SUB transgenes, in wild type or *sub*$^{131/1}$ null background.

For all panels oocytes have been stained with DNA (blue), HA (red), tubulin (green) and single channels for HA and tubulin are shown in white. Scale bars are 5µm. A, C, E. Localization of full length SUB$^{\Delta NT\text{-}HA}$, SUB$^{\Delta(1\text{-}41)}$ and SUB$^{\Delta(42\text{-}76)}$ in wild type background. Spindles are normal in A and C but are abnormal in E. B, D, F. Localization of indicated transgenes in *sub*$^{131/1}$ null background. All 3 transgene constructs are able to rescue spindle formation and localize similar to wild type.

To test if the conserved regions within the first 41 amino acids are responsible for negative regulation, we tested smaller deletions in the N-terminus. There are two domains well conserved within the first 41 amino acids each with a conserved serine that may be important for regulation (Figure 17B). Surprisingly while $SUB^{\Delta(24-33)}$ localizes normally to the central spindle and does not show any dominant effects (n=25), $SUB^{\Delta(1-21)}$ fails to localize even though it expresses in the ovaries (n=9) (Figure 17A-D). This is an interesting result since the larger deletion mutant $SUB^{\Delta(1-41)}$ localizes normally. This suggests that there are antagonistic regulatory elements in the N-terminus required for localization of the protein. There must be a positive regulatory element in the first 21 amino acids that may be negatively influenced by the next 20 amino acids such that deletion of the entire 41 amino acids, restores localization. Additionally this is the first evidence that the kinesin 6 N-terminus can promote direct microtubule binding apart from the C-terminus, as opposed to simply being a regulator.

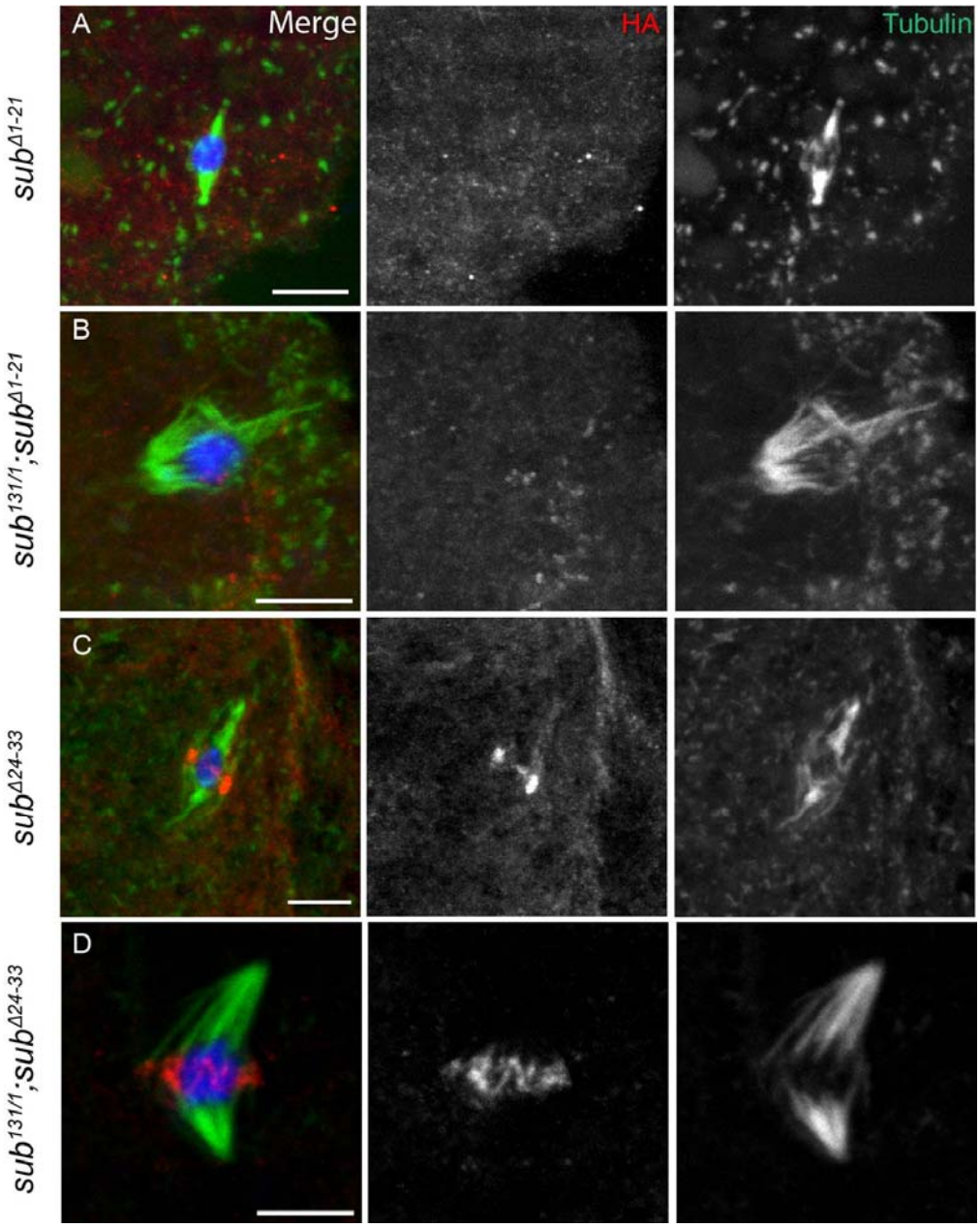


Figure 17**:** Amino acids 1-21 is required for localization of Subito at metaphase I.

Oocytes are stained with DNA (blue), HA (red), tubulin (green). A,C. Localization of $SUB^{\Delta(1-21)}$ and $SUB^{\Delta(24-33)}$ in wild type background. $SUB^{\Delta(1-21)}$ does not localize to the central spindle but $SUB^{\Delta(24-33)}$ shows normal localization. B,D. Localization of indicated transgenes in *sub*$^{131/1}$ null background, showing that $SUB^{\Delta(1-21)}$ is unable to rescue defects possibly due to lack of localization, however, $SUB^{\Delta(24-33)}$ is able to rescue spindle formation and localizes normally.

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

Since we have established that there must be two antagonistic signals in the N-terminus, it is possible that these are mediated by phosphorylation of the two domains. To determine if phosphorylation of conserved serines 16 and 24, has a role in regulating Subito, constructs were created that substituted these serines to alanines individually, sub^{S16A} and sub^{S24A}, and in tandem, $sub^{S16AS24A}$. If these serines are important for antagonistic regulation we may expect the double phosphorylation mutant to have a phenotype similar to that of $sub^{\Delta(1-41)}$. Formation of ectopic spindle assembly was not observed in sub^{S16A} or sub^{S24A} oocytes and both SUB^{S16A} and SUB^{S24A} localized normally to the central spindle, indicating that these serines may not be important for localization of the protein. However in contrast to $sub^{\Delta(1-41)}$, expression of $sub^{S16AS24A}$ in wild type oocytes, resulted in high levels of abnormal spindles (52%, n=42) (Figure 18A). The localization of this mutant protein in the absence of endogenous SUB, is abnormal and not restricted to the ring shape characteristic of the wild type protein suggesting that although these sites are not required for localization they are required for function (45%, n=11) (Figure 18B). Upon examination of the ooplasm, bundles of microtubules were observed similar, but reduced, compared to that seen in the $sub^{\Delta NT\text{-}HA}$ mutant oocytes (Figure 18C, D). This indicates that these conserved serines are indeed important for regulating both localization and activity of the protein in meiosis (Figure 18C). Both serines are necessary to restrict the motor activity of the kinesin but there may be more than one negative regulatory input accounting for the severity of the $sub^{\Delta NT\text{-}HA}$ mutant

phenotype. Overall, there appears to be complex regulators present in the N-terminus for both localization and possibly function.

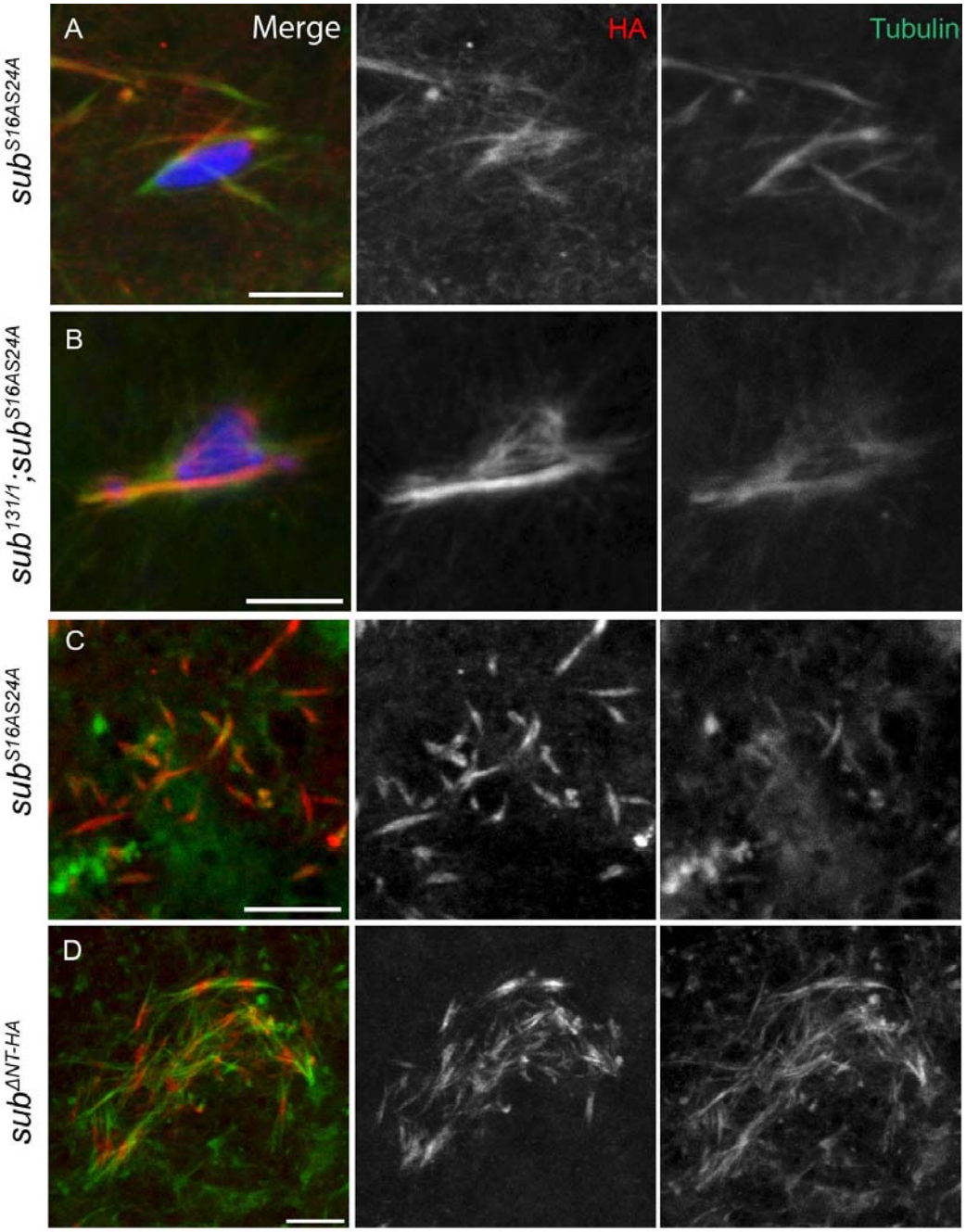


Figure 18**:** SUB^{S16AS24A} shows ectopic microtubule bundling although lesser than SUB$^{\Delta NT\text{-}HA}$

Oocytes are stained with DNA (blue), HA (red), tubulin (green). A, B. SUB^{S16AS24A} localizes to the spindle normally in wild type background but is diffusely localized all over the spindle in *sub$^{131/1}$* null mutant background. C, D. Ectopic microtubule bundling in SUB^{S16AS24A} expressing oocytes as compared to SUB$^{\Delta NT\text{-}HA}$.

The second half of the N-terminus has dominant effects on microtubule organization and is required for meiotic function

Since SUB is crucial for meiosis but is non-essential for mitosis, its activity may be differentially regulated in the two processes. To test if the N-terminus is responsible for this difference, we tested the role of the various mutant transgenes in the absence of endogenous SUB. For this purpose, we used available null and hypomorphic alleles of *subito*. To test for embryonic defects we used a null mutant $sub^{131/1}$ which is sterile due to pro-nuclear fusion defects. To test for chromosome segregation errors in meiosis, we expressed the deletion mutants in a background of a hypomorphic allele sub^{1794}, which as a transheterozygote with the null allele sub^{131} yields 41% X-chromosome non-disjunction (Table 8). These assays will allow us to assess the roles of the different transgenes and determine if some domains are important for one or the other process.

$SUB^{\Delta(1-41)}$ is unable to rescue sterility of $sub^{131/1}$ null mutant indicating that this domain is important for mitotic functions of SUB in the germline (Table 7). Interestingly $SUB^{\Delta(1-41)}$ only partially rescues non-disjunction of a sub^{1794}/sub^{131} trans-heterozygote (Table 8) indicating that this deletion is also required for meiotic functions.

The deletion of the second half of the N-terminus $SUB^{\Delta(42-76)}$, though non-conserved, failed to rescue sterility of a $sub^{131/1}$ null mutant and non-disjunction of sub^{1794}/sub^{131} suggesting that it is also required for both mitotic and meiotic functions of the kinesin (Table 7and Table 8). From these results we can conclude that the first two domains are both important for the activity of the kinesin.

Interestingly, $SUB^{\Delta(24-33)}$ rescues non-disjunction of *sub*^{1794}/*sub*^{131} to wild type levels indicating this sub-domain is not required for meiosis (Table 8). This may also suggest that the conserved serines may have a differential role in mitosis versus meiosis. Indeed, although $SUB^{S16AS24A}$ was able to rescue non-disjunction of *sub*^{1794}/*sub*^{131} it did not rescue sterility of the *sub*$^{131/1}$ mutant (Table 7 and Table 8). This indicates that while the conserved domain 24-33 and perhaps the phosphorylation sites are not important for meiosis, they are required for mitotic divisions. Hence we have determined that although the entire first 76 amino acids are required for mitosis, certain domains are dispensable for meiosis, like 24-33, and possibly the two phosphorylation sites, in the presence of SUB^{1794}. This indicates that kinesin 6 activity is indeed differentially regulated between meiosis and mitosis by regulating parts of the N-terminus.

C-terminus of Subito does not affect spindle organization but regulates localization of the protein to the central spindle.

The C-terminus of MKlp2, is known to be regulated by Cdk1 phosphorylation and mediates binding to microtubules and interaction with various cell cycle regulators [185]. This was supported by our previous study where the C-terminus of SUB was capable of binding to microtubules [94]. In this study, we have shown that the N-terminal 21 amino acids are also important for microtubule binding, however this could be due to formation of dimeric structures through interaction with the C-terminus. There is a coiled coil domain in the C-terminus which may be important for various functions including microtubule and protein interactions [207]. Additionally, a genome wide mass spectrometry analysis in *Drosophila* embryos has shown that the C-terminus of Subito is

also phosphorylated [208]. The phosphorylation of the C-terminus could regulate microtubule interactions, similar to the phosphorylation of the neck region.

Since a complete deletion of the C-terminus failed to express in the ovaries [94], we constructed two smaller deletions to investigate the role of the C-terminus. The first deleted the last 22 amino acids, $sub^{\Delta CT1}$, and the second deleted the last 43 amino acids, $sub^{\Delta CT2}$. Both deletion constructs span the amino acid region predicted to be phosphorylated by mass spectrometry but the second deletion, $sub^{\Delta CT2}$, also deletes the coiled coil domain completely. Genetic analysis revealed normal levels of X chromosome non-disjunction for both mutants (Table 6). The mutant protein $SUB^{\Delta CT1}$ localizes to the central spindle and spindle assembly and karyosome organization was not affected (Figure 19C). However $SUB^{\Delta CT2}$ did not localize very well (Figure 19D). This suggests that the C-terminus might regulate recruitment of the protein complex. This also suggests a role for the coiled coil domain in regulating interaction with the microtubules or perhaps other protein complexes, like the CPC, that might activate the protein. However, expression of $sub^{\Delta CT1}$ or $sub^{\Delta CT2}$ did not rescue sterility of the $sub^{131/1}$ null mutant (Table 7).

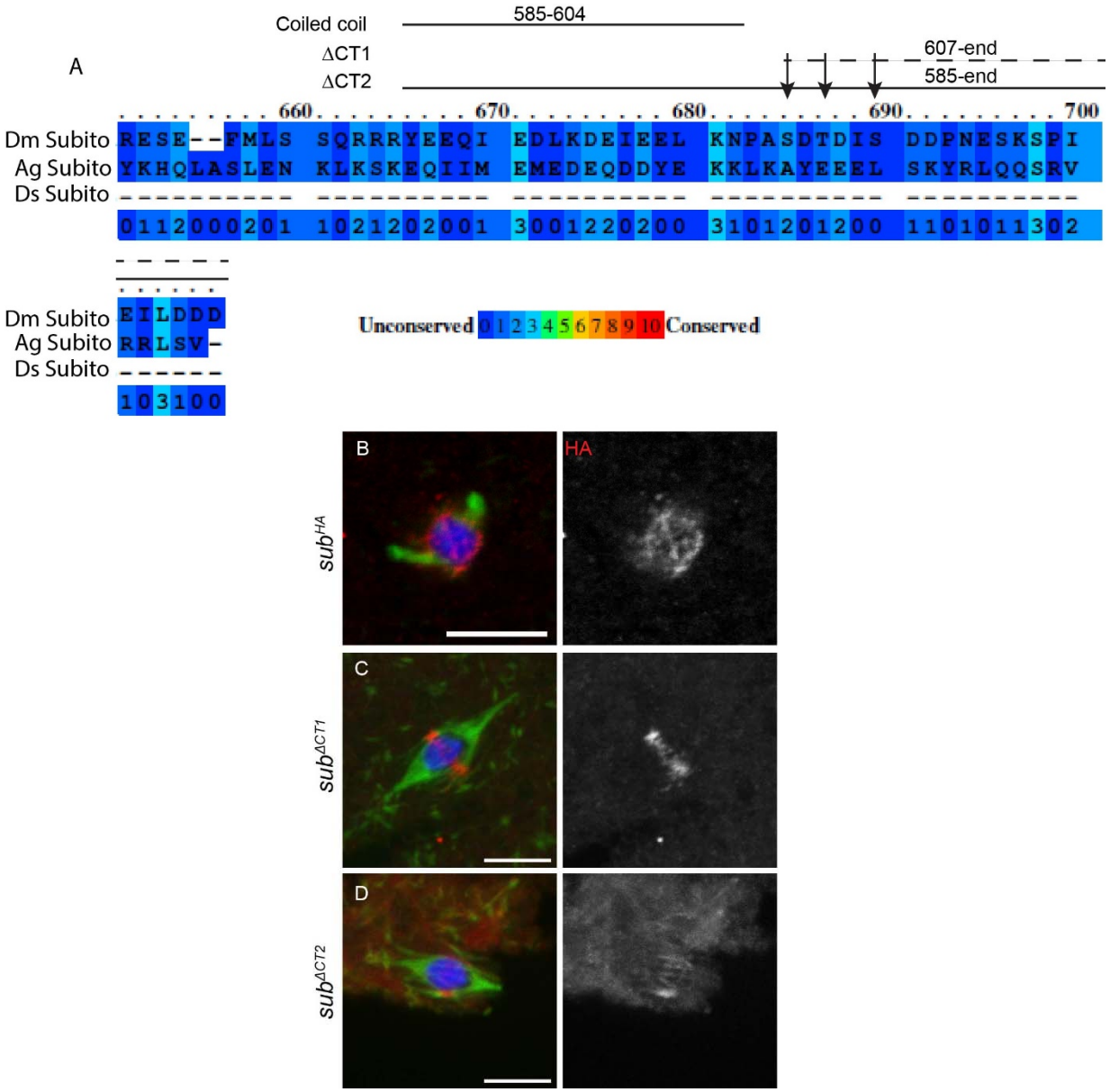


Figure 19**:** C-terminus of Subito is required for localization of the motor

A. Alignment of the C-terminus showing the deleted regions and the conserved coiled coil. B-D. Localization of indicated transgenes in a wild type background.

V. Discussion:

The *Drosophila* homolog of MKlp2, Subito has been previously shown to be required for bundling central spindle microtubules in meiosis even though its role in mitosis is non-essential. The N-terminus of Subito has been shown to be one of the factors which regulate its bundling activity. The object of this study was to further characterize the N-terminus to pinpoint domains of regulatory functions and elucidate which domains are important for meiosis versus mitosis. Additionally we have also looked at the role of C-terminus in microtubule binding in more detail in this study.

Localization and function of Subito is regulated by both the N and C-terminal domains:

We have shown in agreement with previous results that the N-terminus deletion of Subito causes ectopic microtubule bundling in the ooplasm away from the chromatin. Upon further examination of individual domains we found that region 1-21 is important for localization to the meiotic central spindle. This is surprising since in both Subito and the vertebrate homolog Mklp2, the C-terminus is thought to be important for microtubule binding [94, 185]. Curiously, a larger deletion, 1-41, restores microtubule binding indicating that this deletion eliminates a negative regulation of localization.

Regulation of kinesin function traditionally may occur through two ways: autoinhibition involving a folded state where the C-terminal tail inhibits the motor, and another model where the kinesin is activated through clustering and dimerization and remains inactive in the monomeric state [209-211]. For MKlp2 a higher-order clustered structure has been shown to be important for microtubule binding and function which is

negatively regulated by CDK1 [185]. In *Drosophila* oocytes it is possible that the C-terminus is required for dimerization of the protein which activates it. $sub^{\Delta 1\text{-}21}$ mutant may abolish this interaction perhaps through a regulatory element in the domain 22-41. Deletion of this entire domain may restore localization by allowing the C-terminal domain to interact with the motor domain, hence dimerizing and activating the protein. In fact this is supported by the fact that the C-terminus expressed by itself has been previously shown to bind microtubules and the C-terminus deletion mutants, especially the coiled-coil mutant are unable to localize properly or rescue function of the protein.

Different domains of the N-terminus is required for meiosis and mitosis

Here, we show that there is a distinct separation of function in the N-terminus between meiosis and mitosis. All of the N-terminal mutants failed to rescue sterility of a Subito null mutant indicating a failure to rescue pro-nuclear fusion defects in the zygote. However, the mutants $sub^{\Delta(24\text{-}33)}$ and $sub^{S16AS24A}$ were able to rescue meiotic defects of Subito. This indicates two things: Phosphoregulation of this kinesin may be more important for mitosis, and that these sites or the conserved domain are not important for meiotic function. This may be attributed to difference in the structures of the anti-parallel microtubules in mitosis versus meiosis. At meiotic metaphase I, even though there is a central spindle highly analogous to the mitotic spindle midzone [45], regulation by kinases and CDK1 may be inhibited. Alternatively, considering that sub^{1794} is a point mutant in the motor domain, and the two serines are in the N-terminal region, a resulting complex between SUB^{1794} and $SUB^{S16AS24A}$ may provide enough functionality, to rescue meiosis.

Also there is evidence that there are diffusible factors which activate the protein, close to the chromatin, as opposed to the rest of the cytoplasm in the oocyte. Since, the putative dimerization/microtubule binding regulatory domain 1-21 is present in $SUB^{\Delta 24-33}$ mutant protein, it may still be able to form higher order structures required to activate the protein in the absence of regulatory phosphorylations. However in mitosis these sites may be inhibitory to the function of the protein and may preclude formation of a functional midzone. This is interesting because it may indicate that in meiosis there is a mechanism which bypasses negative regulation by CDK1. Indeed, in mitosis, reduction of Cdk1 allows MKlp2 to associate with the CPC and relocate to spindle midzone. This regulation must needs be modified in meiosis, as the central spindle structure forms at metaphase I, where the CPC, Subito and Cyclin B, all localize to it concurrently. This further supports a model where Subito is regulated predominantly by domains involved in higher order structure formation in meiosis, whereas in mitosis, both clustering and phoshoregulation may be required. Future research efforts will be required however to confirm the presence of the higher order structures in oocytes and also to test if mutating CDK1 sites indeed have no effect in meiosis.

In summary we have shown that the kinesin 6 Subito is differentially regulated in meiosis as opposed to mitosis. This may represent a conserved mechanism for other organisms as well given that structures analogous to the central spindle has been seen in mammalian and worm oocytes [86, 97]. We have also shown that N-terminus of kinesin 6 is also important for its localization to the microtubules, along with the C-terminus, despite the fact that microtubule binding is thought to reside in the C-terminus.

Table 7**:** Rescue of a *sub*$^{131/1}$ null mutant with N-terminus mutant transgenes

Transgene[1]	Progeny/female parent	% Non-Disjunction	Total flies
sub^{HA}	42.5	0.24	850
sub$^{HA\text{-}Myc}$	38	0	1524
sub$^{\Delta NT}$	0	sterile	0
sub$^{\Delta(1\text{-}21)}$	0	sterile	0
sub$^{\Delta(24\text{-}33)}$	0	sterile	0
sub$^{\Delta(1\text{-}41)}$	4	semi-sterile	447
sub$^{\Delta(42\text{-}76)}$	12	semi-sterile	12
sub^{S16AS24A}	0	sterile	0
sub$^{\Delta CT1}$	0	sterile	0
sub$^{\Delta CT2}$	0	sterile	0

[1]Each transgene is expressed in a *sub*$^{131/1}$ null mutant lacking endogenous protein. Resultant progeny were tested for fertility and X-chromosome non-disjunction.

Table 8: Rescue of non-disjunction in a sub^{1794}/sub^{131} hypomorph by mutant transgenes

Transgene	Progeny/female parent	%Non-disjunction	Total Flies
sub^{1794}/sub^{131}	2	41%	591
sub^{HA}	19	0.24%	2464
$sub^{\Delta(24-33)}$	12	1%	3054
$sub^{\Delta(1-41)}$	17	29%	1846
$sub^{\Delta(42-76)}$	6	21%	1441
$sub^{S16AS24A}$	12	1.20%	1829

CHAPTER 4: AURORA B ACTIVITY IS OPPOSED BY PP1-87B IN FEMALE MEIOTIC METAPHASE I

I. Preface:

This chapter will be submitted as an individual manuscript soon. My contribution to this paper includes: writing of the paper and all the experiments, except the standardization of Binucleine 2 treatment on wild type oocytes.

II. Abstract:

In female meiosis of most animals including *Drosophila*, a bipolar spindle assembles without the guidance of centrosomes. In *Drosophila* oocytes, knockdown of the master cell cycle regulator, Aurora B kinase, results in a failure to assemble spindle microtubules and kinetochores. How the activity of this kinase is regulated was unknown but phosphatases have been known to oppose Aurora B function in mitosis. We have examined the role of the serine/threonine protein phosphatase 1 (PP1) in female meiotic chromosome segregation and spindle assembly. In PP1 depleted oocytes, we observed disorganized spindle microtubules and the karyosome, a structure into which all the chromosomes are compacted, is dispersed into several masses with loss of sister-centromere co-orientation. To determine if PP1 antagonizes Aurora B, we used an inhibitor Binucleine 2. If Binucleine 2 is added after spindle assembly is complete, the microtubules disassemble and kinetochore proteins do not localize, indicating that sustained Aurora B activity is important in meiosis I. This indicates that the spindle assembly factors require continual phosphorylation for accurate segregation to occur due to either the presence of multiple phosphatases or protein exchange. The karyosome

defect and loss of co-orientation in oocytes lacking PP1, is rescued by addition of the Aurora B inhibitor. However, the complete loss of the meiotic spindle caused by the Aurora B inhibitor is not restored by loss of PP1. Thus, other phosphatases may negatively regulate spindle assembly and/or Aurora B-dependent phosphorylation is required to maintain incorporation of spindle associated proteins throughout meiosis. Furthermore, the co-orientation defect is dependent on the presence of microtubules, but the karyosome defect is directly dependent on Aurora B. Loss of kinetochore protein SPC105R at centromeres, upon adding the inhibitor of Aurora B, is also rescued by a concomitant loss of PP1. These results together suggest that PP1 antagonizes Aurora B for maintaining co-orientation, karyosome integrity and kinetochore protein localization.

III. Introduction:

In females of many species, the meiotic spindle assembles in the absence of centrosomes [212] and is directed by chromosome based cues. These are provided largely by two pathways depending on the organism: the chromosomal passenger complex (CPC) and RanGTP [80, 82, 83, 213, 214]. Aurora B is the catalytic subunit of the CPC and regulates mitotic spindle assembly and cytokinesis (reviewed in [25]). Regulation of Aurora B activity in mitosis is thought to be dependent on a spatial gradient seen to be present at spindle midzone and inner centromeres for regulation of chromosome segregation and kinetochore-microtubule attachments [53, 54, 56]. In *Drosophila*, the CPC is required for spindle assembly, homologous chromosome bi-orientation and kinetochore localization in female meiosis [82, 83, 98]. However all or most of these experiments, were performed with either tissue specific knockdowns or hypomorphic mutants. These studies therefore, do not address whether continuous Aurora B activity is

required to maintain spindle microtubules or kinetochore localization status. They also do not address regulation of Aurora B gradients which may also be present in oocytes.

It is known that Aurora B regulation of kinetochore assembly is counteracted by a phosphatase PP1 [61, 215]. Additionally dephosphorylation of Aurora B targets at the kinetochore by an associated population of PP1 is thought to silence the spindle assembly checkpoint and induce anaphase by stabilizing kinetochore-microtubule attachments [62, 74, 216, 217] . In accordance with this, mutations in PP1 rescue conditional mutants of Aurora B in both yeast and worms [218, 219]. Aurora B localization and function is also regulated by targeting of PP1 to the chromatin by Repo Man and Sds22 which dephosphorylates histone 3 at Thr3 [68, 220, 221].

In meiotic systems however the role of reversible phosphorylation is not well understood. Previous studies in *C. elegans* oocytes have shown that PP1 is required to oppose Aurora B's phosphorylation of cohesin components in meiosis I [131]. In mouse oocytes, inhibition or depletion of PP1 induces GVBD and meiotic resumption [222, 223]. The functions of Aurora B in an acentrosomal system goes beyond regulating the kinetochore-microtubule interface and is more holistic in nature [83, 98]. It is thus plausible that phosphatases have as yet unidentified roles that are modified in the acentrosomal system. In addition, although the antagonism between Aurora B and PP1 has been studied in *Xenopus* extracts on reconstituted kinetochores [215], the difference between establishment and maintenance of these functions have been largely unknown in in vivo systems. Here we use the Aurora B specific inhibitor, Binucleine 2, [224, 225] and a tissue specific RNAi to the PP1α isoform in *Drosophila* oocytes to evaluate the importance of phosphatases in meiosis and the underlying antagonism between these

proteins. We show that PP1 opposes Aurora B in regulating karyosome structure and maintaining the unique centromere geometry required for faithful segregation. Meiosis I is unique because sister chromatids co-orient while separating homologs. This is achieved by first, selectively removing arm cohesion, and second establishing cohesion to fuse sister centromeres called co-orientation [1, 226]. We have shown a direct role of PP1 in maintenance of both peri-centromeric cohesion and co-orientation in meiosis I. Also in accordance with an earlier study [215], we show that kinetochore assembly is regulated by Aurora B/PP1 antagonism.

IV. Materials and Methods:

Expression of RNAi in oocytes and quantification

Expression of short hairpin RNA lines designed and made by the Transgenic RNAi Project, Harvard (TRiP) was induced by crossing each RNAi line to either *P{w^{+mC}=tubP-GAL4}LL7* for ubiquitous expression or *P{w^{+mC}=matalpha4-GAL-VP16}V37* for germ line specific and oocyte expression. The latter is expressed throughout oogenesis starting late in the germarium [83]. For reverse transcriptase quantitative PCR (RT-qPCR), total RNA was extracted from late-stage oocytes using TRIzol® Reagent (Life Technologies). cDNA was consequently prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The qPCR was performed in either a StepOnePlus™ (Life Technologies) or Eco™ (Illumina) real-time PCR system using TaqMan® Gene Expression Assays (Life Technologies,Dm ##).

Antibodies and immunofluorescent microscopy

Stage 14 oocytes were collected from 50 to 200, 3 to 4 day old yeast fed non-virgin females by physical disruption in a common household blender [140, 148]. The

oocytes were either fixed in modified Robb's media and 100 mM cacodylate/8% formaldehyde fixative for 8 min or 5% formaldehyde/heptane fixative for 2.5 min and then their chorion and vitelline membranes were removed by rolling the oocytes between the frosted part of a slide and a coverslip. For FISH, oocytes were prepared as described (Radford et al 2012). Oocytes and embryos were stained for DNA with Hoechst 33342 (10μg/ml) and for microtubules with mouse anti-α tubulin monoclonal antibody DM1A (1:50), directly conjugated to FITC (Sigma, St. Louis). Additional primary antibodies were rat anti-Subito antibody (used at 1:75) [45], rat anti-INCENP (1:400) [83] rabbit anti-SPC105R (1:4000) [150], rabbit anti-CENP-C (1:5000) (gift from Christian Lehner), rabbit anti-Deterin (?). These primary antibodies were combined with either a Cy3 or Cy5 secondary antibody pre-absorbed against a range of mammalian serum proteins (Jackson Immunoresearch, West Grove, PA). FISH probes used were to the AACAC repeat (2nd chromosome) and Dodeca repeat (third chromosome). Oocytes were mounted in SlowFade gold (Invitrogen). Images were collected on a Leica TCS SP5 or SP8 confocal microscope with a 63x, NA 1.4 lens. Images are shown as maximum projections of complete image stacks followed by merging of individual channels and cropping in Adobe Photoshop (PS6). CENP-C foci and FISH foci were counted on Imaris v6? (Bitplane) and graphs were plotted using Graphpad Prism software.

Drug treatments

For drug treatments, oocytes were treated with 0.001% DMSO or 25 uM BN2 for 25 minutes prior to fixation in Robb's media. For Taxol experiments, oocytes were treated

with 10uM taxol or DMSO for 10 minutes and then 25uM BN2 or additional DMSO was added for 25 minutes and then fixed.

V. Results:

PP1-87B is required for maintaining meiotic chromosome structure and microtubule organization

Drosophila has four genes that encode for the catalytic subunit of PP1. *Pp1-87B*, *Pp1-96A* and *Pp1-13C* are homologous to mammalian PP1α/γ isoforms [227]. Pp1-87B is essential and contributes to ~80% activity during development while the other isoforms are non-essential. [228, 229]. Hence we focused our studies on *Pp1-87B* isoform. As *Pp1-87B* mutants are lethal, we used *Pp1-87B* RNAi (Transgenic RNAi project, TRiP *HMS00409*) [167] to test the function of PP1-87B in oocytes. Expression of the shRNA line using ubiquitous *P{tubP-GAL4}LL7* resulted in lethality, suggesting the protein had been knocked down by the shRNA. Oocyte specific shRNA expression of *HMS00409* was achieved using *matalpha4-GAL4-VP16* and this resulted in sterility and knockdown of the mRNA to 35% as measured by qRT-PCR in oocytes.

Pp1-87B knockdown oocytes had significant spindle organization defects, like unfocused poles and extensive fraying (79%, n=43, p=0.0001, Figure 20Figure 20B, D) compared to wild type controls (Figure 20A). We also saw chromosome organization defects as compared to wild type. In wild type the chromosomes in *Drosophila* oocytes are clustered to form a sphere known as the karyosome (Figure 20A). This karyosome was separated into multiple masses in 42% of oocytes (n=43, p=0.0001, Figure 20B, C) as compared to 0% in wild type (n=27, Figure 20A). To test if the karyosome separation was due to premature anaphase, we also used Subito (a kinesin 6 motor protein) and

Survivin (a component of the CPC) antibodies, both of which bind to central spindle microtubules and are usually in a ring around the karyosome at metaphase (Figure 20A). In *Pp1-87B* knockdowns, although the localization pattern was not wild type, yet both Subito and Deterin were associated with the karyosome indicative of metaphase localization pattern (Figure 20 B).

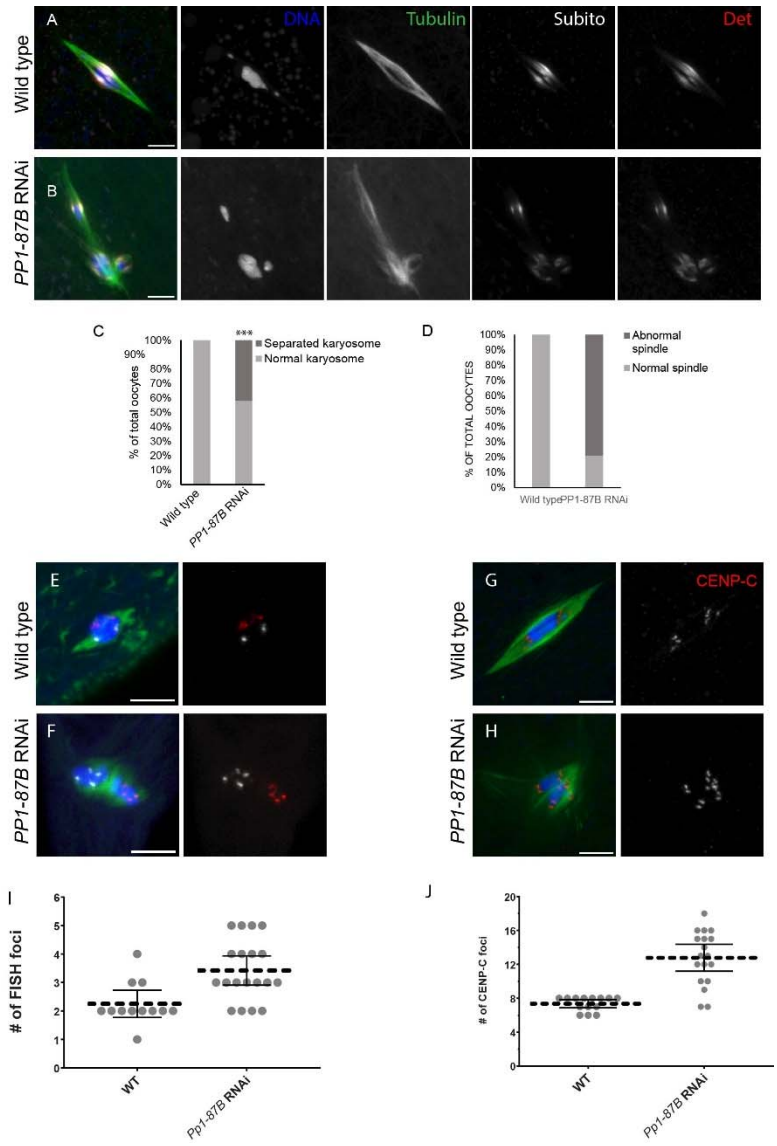


Figure 20: PP1-87B is required for maintaining chromosome structure and preventing centromere and pericentromeric separation.

All images show DNA in blue and Tubulin in green A-B. Subito is shown in white, Deterin in red in merged images. All single channels are in white. *Pp1-87B* knockdown oocytes show separated karyosome (arrow in B) and disorganized spindles. C-D. Quantification of separated karyosome and spindle defects; P-values are calculated using Fisher's exact test. E-F. Wild type and *Pp1-87B* knockdown oocytes stained with CHR 2 probe (red) and CHR 3 probe (white). G-H. CENP-C is shown in red in merge and white in single channel. *Pp1-87B* has higher number of CENP-C foci compared to Wildtype. I-J. Quantification of number of foci for CHR 3 probe and CENP-C. Error bars are indicate mean with 95% CI.

PP1-87B is required for pericentromeric cohesion and sister centromere co-orientation but not arm cohesion.

To confirm that *Pp1-87B* does not induce premature anaphase we used fluorescently tagged probes to the AACAC repeat on the 2nd and the Dodeca satellite on the 3rd chromosome. In wild type normally we observe two foci per homologous chromosome, oriented towards opposite poles (Figure 20E). All the FISH foci are present in one karyosome mass at metaphase. At anaphase, in wild type, we would expect to see two karyosome masses with the homologs separated resulting in one FISH focus per homologous chromosome per karyosome mass. In *Pp1-87B* knockdown oocytes although 70% had separated karyosome (n=20) only 21% of those had separated homologs, indicating that *Pp1-87B* knockdown oocytes usually do not lose arm cohesion. Interestingly, we observed more than two foci per homolog in *Pp1-87B* knockdown oocytes suggesting separation of sister chromatids (Figure 20F, I). Since the AACAC repeat yields diffuse foci not easily quantifiable, we used the Dodeca satellite which is punctate to quantify this phenotype. The average number of Dodeca foci is significantly higher than wild type (3.4 versus 2.2, p= 0.0025, Mann Whitney U test, Figure 20I) suggesting that PP1 may be involved in the regulation of sister chromatid cohesion in oocytes.

In meiosis, there is specialized cohesion at the centromeres not required at mitosis. This additional cohesion ensures co-orientation of homologous sister chromatids at metaphase I and may or may not require the cohesin complex depending on the organism [106, 123, 125]. We used CENP-C antibody to test if co-orientation is also

affected in PP1 knockdown oocytes. As *Drosophila* has 4 chromosomes, after replication, at metaphase I we expect 8 foci to be visible. Due to clustering of centromeres sometimes we observe a lower number of foci but the average number of CENP-C foci in wild type oocytes is close to 8 (Figure 20J). Loss of co-orientation would result in an average number of CENP-C foci higher than 8. Indeed, in *Pp1-87B* knockdown oocytes we see significantly higher average number of CENP-C foci (12.7, $p<0.0001$, Mann Whitney U test, Figure 20J) compared to wild type oocytes (7.3) indicating a loss in co-orientation. Thus we can conclude that Pp1-87B may be required for both sister chromatid cohesion and co-orientation but is dispensable for arm cohesion.

Sustained Aurora B activity is required to maintain the meiotic spindle and kinetochore localization

If the defects observed in the phosphatase *Pp1-87B* knockdowns are due to constant unregulated activity of Aurora B kinase then inhibition of the kinase should rescue the defects. We developed a system to temporally inhibit activity of Aurora B, in order to address whether the defects in *Pp1-87B* RNAi are dependent on Aurora B and also test the reverse. We first tested whether constant Aurora B activity is required in *Drosophila* oocytes for maintaining spindle microtubules. We treated mature stage 14 oocytes with a *Drosophila* isoform specific inhibitor of Aurora B, Binucleine 2 (BN2) [224, 225, 230]. This allows us to inhibit Aurora B activity after spindle assembly is complete. If Aurora B activity is not required after the spindle is assembled we would expect that the mature oocytes would be resistant to drug treatment. On the other hand if Aurora B is required to continually phosphorylate its targets, we would expect phenotypes similar to that of the RNAi knockdown. We observed that there were very

few or no microtubules left after treatment with 25μM BN2 for 25 minutes, in 58% of oocytes (n=41) as compared to 3% in the solvent treated control (n=35) (Figure 21, A-C). We also treated oocytes with 25μM BN2 for 10 minutes and an hour. All oocytes had a spindle after a 10 minute treatment and there was no significant increase in number of oocytes without a spindle after one hour of treatment and therefore for further experiments we treated oocytes for 25 minutes. From the data we conclude that factors required for maintaining spindle microtubules need constant Aurora B activity.

We know that Incenp localizes in a distinctive ring on the central spindle microtubules at meiotic metaphase I ([83] and Figure 21A). This localization pattern is lost in the BN2 treated oocytes; instead, Incenp localization is dispersed all over the chromatin in 100% of treated cells (n=17) regardless of their microtubule status (Figure 21B). This is characteristic of a loss of Aurora B activity as shown in Aurora B knockdown oocytes and DMel2 cells [20, 83, 220]. Hence we conclude that Aurora B is not only required for initiation, but is also essential for maintenance of the meiotic spindle.

Studies with in vitro egg extracts have shown that continuous Aurora B activity is required to maintain Ndc80 protein at centromeres and this is opposed by PP1 [215]. Conversely, another study found that kinetochore proteins could be reconstituted and assembled in vitro regardless of their phosphorylation states [50]. The difference between these studies may be related to the differential regulation of establishment of the KMN network as opposed to maintenance of its function and microtubule attachment. Previous work from our lab has shown that Aurora B is indeed required to establish kinetochore network [98]. Hence we tested if continuous Aurora B activity was required in oocytes to maintain kinetochore localization. Indeed, treatment with BN2 resulted in loss of

kinetochore protein SPC105R (KNL-1 homolog) localization in 70% of oocytes (n=10, Figure 21E) as compared to 0% in solvent treated control oocytes (n=9, Figure 21D). Since SPC105R is also required for localization of NDC80, Nuf2 and Nsl1 in *Drosophila* oocytes [98], we conclude that in female meiosis, Aurora B activity is required for maintaining kinetochore assembly in oocytes. This requirement of constant Aurora B activity in oocytes may be due to the presence of active phosphatases such as PP1 with similar targets since protein turnover generally takes longer than the time in which we observe phenotypes due to inhibition.

Having established a system to study the antagonism between Aurora B kinase and Pp1-87B, we next tested if the defects seen in *Pp1-87B* RNAi oocytes could be rescued by inhibition of Aurora B and vice versa. First, we tested if the loss of kinetochore localization in BN2 treated oocytes could be rescued by a concomitant loss of *Pp1-87B*. We treated *Pp1-87B* RNAi oocytes with BN2 and then examined them for SPC105R localization. Both the solvent and the BN2 treated *Pp1-87B* RNAi oocytes had SPC105R localization in 100% of oocytes (n=10 each, Figure 21F, G). We conclude that *Pp1-87B* opposes Aurora B in oocytes for kinetochore localization.

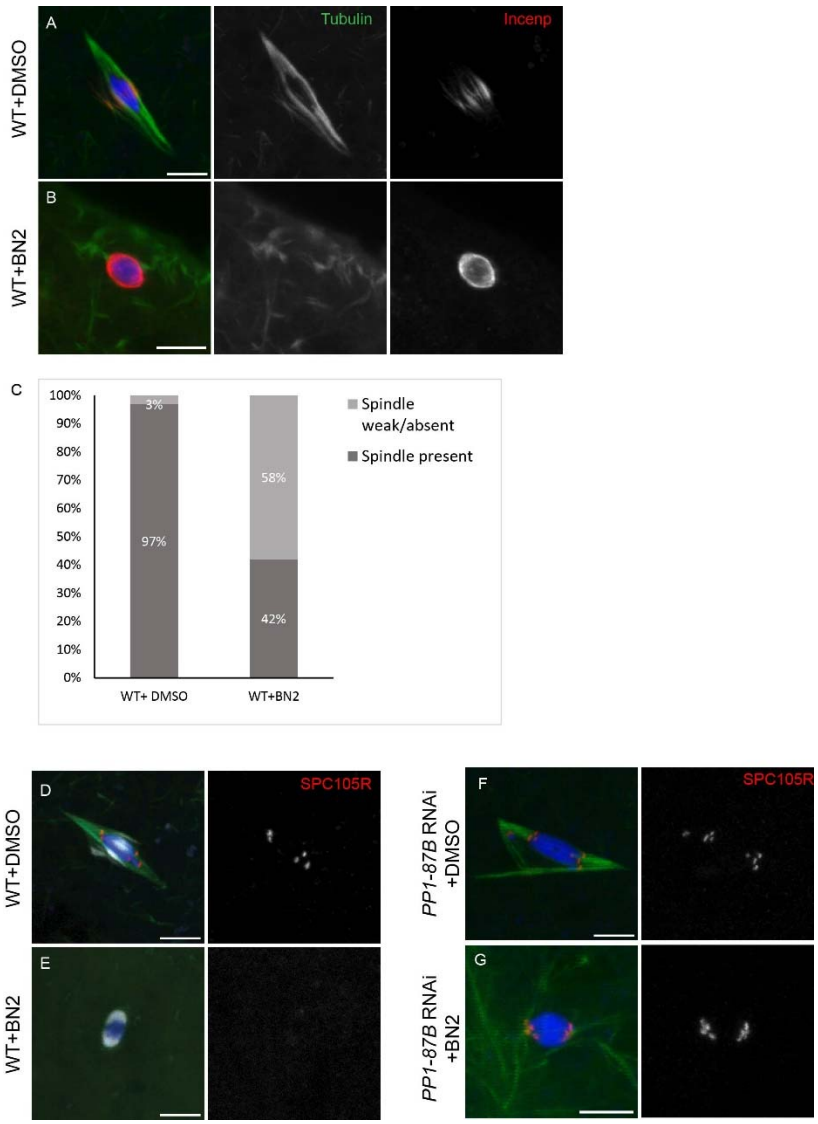


Figure 21: Constant Aurora B activity is required or maintaining spindle microtubules and SPC105R localization in oocytes

A-B. Wild type stage 14 oocytes were treated with either 0.001% DMSO control or 25uM BN2 and stained with antibodies against Incenp (red), Tubulin (green) and Hoechst for the DNA. Tubulin and Incenp are shown separately in white channels. C. Quantification of number of oocytes containing spindles. Significance tests were performed using Fisher's exact test. D-E. Wild type DMSO or BN2 treated oocytes stained with Tubulin (green) and SPC105R (red) and Incenp (white) antibody. The red and green channels are shown separately in white. F-G. Kinetochore localization is restored when PP1 is knocked down prior to BN2 treatment. Scale bars represent 5 μm.

Aurora B opposes PP1-87B in maintaining karyosome structure

Next, *Pp1-87B* RNAi oocytes were treated with BN2 as described earlier and spindle disintegration and karyosome separation was measured. There is a loss of spindle microtubules in the absence of continuous Aurora B activity (Figure 21). If PP1 acts on Aurora B substrates then in the absence of PP1 we may expect that the spindle microtubules are retained. However, similar to the wild type oocytes treated with BN2, *Pp1-87B* RNAi oocytes treated with BN2, also have fewer or no spindle microtubules in a significant number of oocytes (68%, n=144) as compared to the *Pp1-87B* RNAi solvent treated controls (100%, n=114, Figure 22A, B). This suggests that either PP1 is not the only phosphatase that is acting on Aurora B substrates or that the substrates are turned over. The separated karyosome defect in *Pp1-87B* RNAi solvent treated controls oocytes (51%, n=143) was rescued by addition of BN2 (20%, n=151) (Figure 22A, B and G). Thus we can conclude that in the absence of Pp1-87B, the chromosome structure defects we see are dependent on Aurora B.

The loss of spindle microtubules in BN2 treated *Pp1-87B* RNAi oocytes presents a further question of whether the karyosome separation is dependent on the presence of microtubules. To test whether the defects in *Pp1-87B* RNAi are dependent on microtubules, we first tested whether the spindle microtubules in BN2 treated oocytes could be retained by stabilization with Taxol treatment prior to inhibition of Aurora B. Wild type oocytes were treated with 10uM Taxol for 10 minutes and then 25uM BN2 was added for 25 minutes. 100% of the Taxol and BN2 treated controls had spindle microtubules (n=21, Figure 22 C, F), compared to wild type oocytes treated with only

BN2 (Figure 21E). However, even in wild type oocytes, treatment with Taxol had some other effects on the spindle that we investigated prior to usage in *Pp1-87B* RNAi oocytes. Incenp was still mis-localized all over the chromatin, in wild type oocytes treated with both Taxol and BN2, and the central spindle microtubules were indiscernible (Figure 22C, E). To test whether the central spindle microtubules were indeed absent, we used the central spindle marker Subito in the wild type oocytes treated with Taxol alone. As compared to wild type solvent treated controls, oocytes treated with Taxol alone, still retain Subito but the localization is sometimes abnormal, extending to the rest of the microtubules (Figure 23). This indicates that although the Taxol treatment is retaining microtubules they may not be completely wild type. As expected, in oocytes treated with Taxol and BN2 combined, Subito localizes diffusely to the parallel microtubules. In wild type oocytes treated with BN2 alone, Subito does not localize (n=6). However, as majority of the spindle microtubules are unaffected, we can still use this treatment protocol to ask whether spindle microtubules affect the phenotypes seen in *Pp1-87B* RNAi.

We first treated *Pp1-87B* RNAi oocytes with Taxol only to confirm that this treatment did not significantly change the phenotypes seen in solvent treated *Pp1-87B* oocytes. As with wild type, 100% of the *Pp1-87B* RNAi oocytes treated with Taxol prior to BN2, retained spindle microtubules (n=102). We then measured separated karyosome in the *Pp1-87B* RNAi treated with only Taxol (52%, n=27) and found it to be similar to the *Pp1-87B* RNAi oocytes treated with DMSO (Figure 22). Next we measured whether addition of BN2 to *Pp1-87B* RNAi oocytes treated with Taxol still rescues the karyosome separation. We only observed 18% separated karyosome in the Taxol and BN2 treated

oocytes, which is a significant decrease compared to Taxol only and DMSO treated *Pp1-87B* RNAi oocytes ($p<0.0001$). This suggests that the karyosome separation is dependent on Aurora B activity and not indirectly on the presence of microtubules.

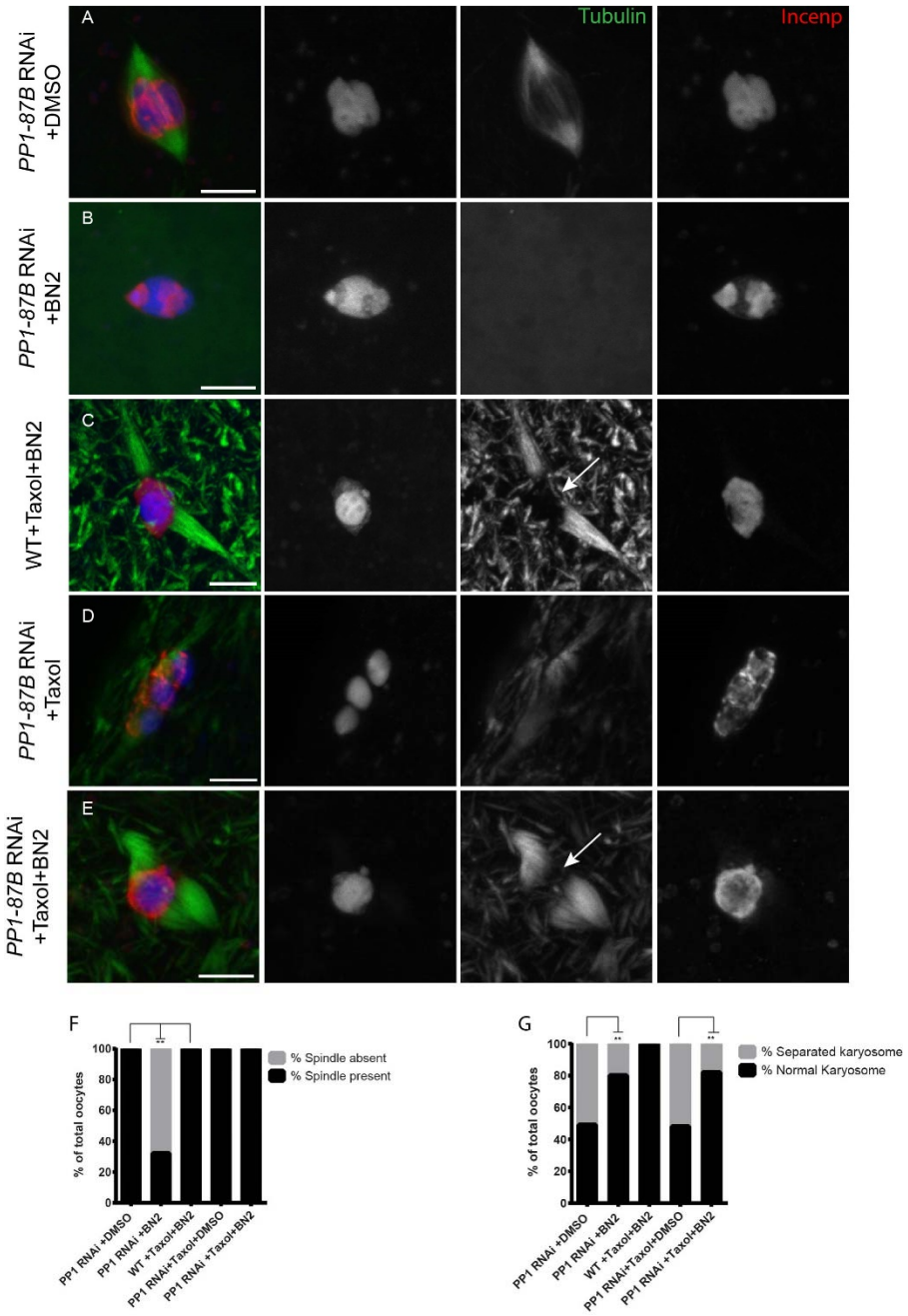


Figure 22: Karyosome defect in *Pp1-87B* knockdowns depend on Aurora B activity

All merged images show DNA (blue), Tubulin (green) and Incenp (red) with single channels in white. A-B. *Pp1-87B* RNAi oocytes treated with DMSO or BN2 showing rescue of karyosome defect. Karyosome in A is separated in Z-direction. BN2 treated PP1 RNAi oocytes lose spindle microtubules as well. C-E. Wild type oocytes treated with Taxol prior to BN2 retain spindle microtubules. *Pp1-87B* RNAi oocytes still show rescue of karyosome defect even upon the restoration of spindle microtubules. Arrows indicate where central spindle microtubules are normally. F-G. Quantification of separated karyosome and loss of spindle microtubules.

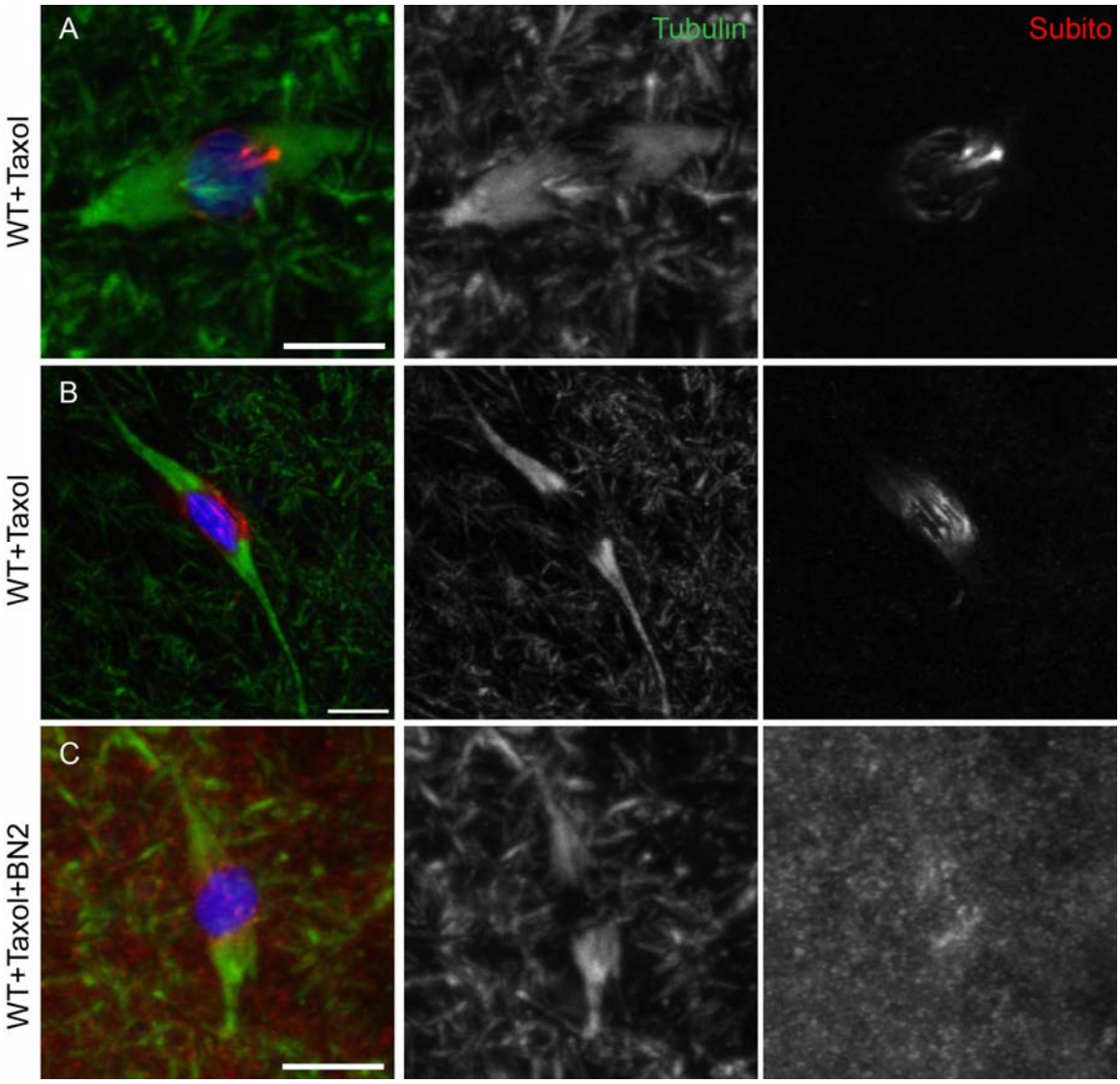


Figure 23: Subito still localizes to the central spindle microtubules in Taxol treated wild type oocytes.

All merged images show DNA (blue), Tubulin (green) and Subito (red) with single channels in white. A-B. Wild type oocytes treated with Taxol only, showing faint and diffuse Subito in A, but wild type localization in B. C. Wild type oocytes treated with Taxol and BN2 combined, showing Subito localizing faintly to parallel microtubules, instead of the central spindle.

Centromere separation in *Pp1-87B* RNAi is dependent on continuous Aurora B activity and may regulate the kinetochore protein SPC105R

Next we tested whether the centromere separation in *Pp1-87B* RNAi was also dependent on Aurora B activity. Wild type solvent treated controls and BN2 treated oocytes both showed an average number of CENP-C foci similar to untreated wild type oocytes (7.7 and 6.9 respectively) as quantified earlier (Figure 24A, B). *Pp1-87B* RNAi solvent treated controls also had similar number of average CENP-C foci (13) compared to the untreated *Pp1-87B* RNAi oocytes (Figure 24C). This number was significantly higher than wild type as expected, but surprisingly, this defect was also rescued by BN2 treatment (average number of CENP-C=8, Figure 24D, I), indicating that co-orientation is dependent on Aurora B (Figure 24I). We also tested if this phenotype is dependent on the presence of microtubules using the Taxol treatments described before. However, unlike the karyosome phenotype, the co-orientation defect persisted in *Pp1-87B* RNAi oocytes after combined treatment with Taxol and BN2 (average number of CENP-C=12), as compared to Taxol treatment alone on *Pp1-87B* RNAi oocytes (average number of CENP-C=12) (Figure 24E, F). This suggests that the centromere separation phenotype may be dependent on the presence of microtubules indicating that it might be dependent on a microtubule bound protein or tension or forces generated by microtubules.

It is well established in mitotic cells that PP1 is targeted to the centromere by the kinetochore protein KNL-1 [61, 216]. Interestingly In *Drosophila* oocytes, the KNL-1 homolog, SPC105R is not only dependent on Aurora B for localization ([98] and Figure 24E) but also SPC105R RNAi (TRiP GL00392) oocytes show a co-orientation defect similar to PP1 knockdowns (average number of CENP-C=11), ([98] and Figure 24G).

However this co-orientation defect in SPC105R RNAi oocytes was not attenuated by inhibition of Aurora B by BN2 (average number of CENP-C=10, Figure 24H, I); this is expected if SPC105R is indeed a target of the kinase. This also demonstrates an instance where centromere separation is observed despite the absence of microtubules, indicating that microtubule forces or tension or even associated proteins are not always required for this separation. Alternatively establishment of co-orientation could be solely dependent on SPC105R, in which case, oocytes lacking this protein will have never fused their kinetochores initially.

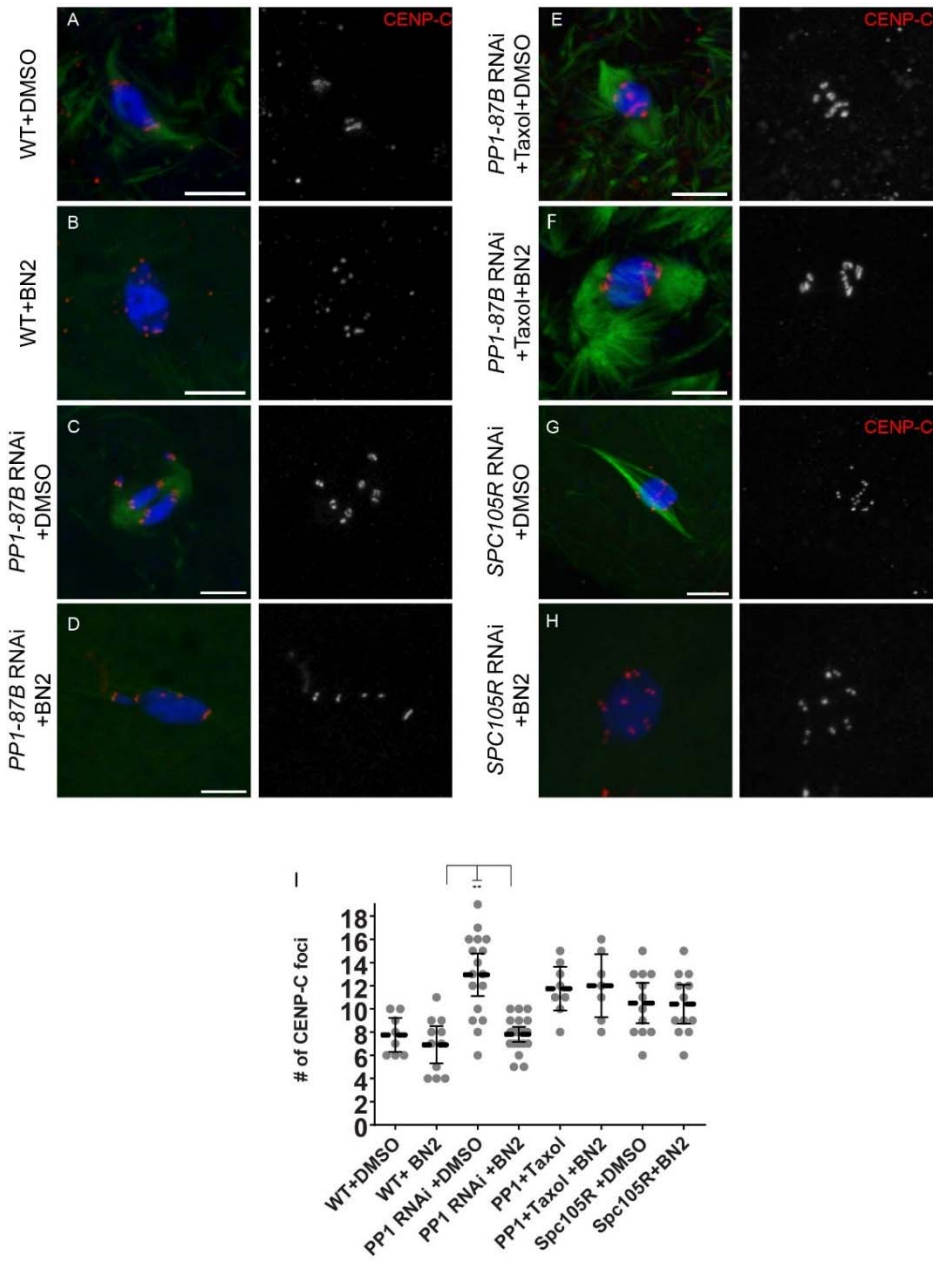


Figure 24: Effects of inhibition of Aurora B activity on centromere separation and kinetochore localization

A-D. Wild type and *Pp1-87B* oocytes treated with either DMSO or BN2 showing inhibition of Aurora B is rescues centromere separation defect in *Pp1-87B* RNAi oocytes. E-F. Rescue of centromere separation is dependent on presence of microtubules as seen in *Pp1-87B* oocytes treated with taxol only, and taxol and BN2 combined. G-H. Centromere separation in Spc105R RNAi oocytes is not dependent on constant Aurora B activity. I. Quantification of CENP-C foci in all genotypes.

V. Discussion

The chromosomal passenger complex (CPC) is crucial for assembling spindle microtubules in the absence of centrosomes [83]. However, the role of this kinase in maintenance of spindle assembly is largely unknown since the oocyte is one of the only cell types which requires this unique activity. In this study, we have shown that continuous Aurora B activity is required to maintain a bipolar spindle in the *Drosophila* oocyte. This aspect of Aurora B function was not examined in previous studies of Aurora B inhibition in D-Mel2 cells or larval neuroblasts [225] and possibly represents a specialized and crucial function for the kinase in female meiosis. It is to be noted that in mouse oocytes, inhibition of Aurora B does not show a similar spindle disintegration phenotype (Schindler, personal comm.). This can be explained by compensatory activity from Aurora C which is not present in *Drosophila* [89]. We also show that inhibition of Aurora B at prometaphase leads to disassembly of kinetochore components consistent with earlier results in frog egg extracts [215]. This data together suggest that not only is the CPC important for initiating spindle and kinetochore assembly, its sustained activity is required to prevent rapid disassembly.

The specific roles of PP1 in meiosis I with respect to spindle and kinetochore assembly was unknown. This is largely due to the fact that PP1 is involved in a wide range of biological processes making it hard to pinpoint specific functions. However we have attempted to investigate its role in the female germline using tissue specific knockdowns. We have shown that PP1 is required to maintain proper chromosome structure during meiotic metaphase I. This is in agreement with previous studies showing that PP1 regulates chromatin condensation through an as yet unidentified factor, in

conjunction with the condensin complex [68, 71]. In fact, in the *Drosophila* oocyte there are two levels of chromatin architecture that is observed: one which condenses the homologs and the second which forms the aggregated karyosome structure. PP1 is definitely required for the second and could be required for the first function as well. Interestingly, this role in regulating chromatin architecture in meiosis is opposed by continuous Aurora B activity. Moving forward it would be pertinent to study targets of PP1 and Aurora B in meiosis, which are involved in regulating chromosome structure and their interactions with the condensins.

Although the role of PP1 in cohesion has been investigated earlier, these specific functions were not previously reported. In *C. elegans*, it was shown that the protein LAB-1 was required to protect cohesion from removal through recruitment of PP1. There is further evidence in *C. elegans* that removal of cohesin is dependent on Aurora B and that the antagonism by PP1 may be a conserved mechanism even in monocentric organisms. In this work we have shown that PP1 is required to maintain cohesion at the centromeres and peri-centromeric regions. Curiously, although inhibition of Aurora B post spindle assembly does not seem to cause centromere separation on its own, it does rescue sister centromeres that are already separated, as seen in PP1 knockdown oocytes. This may suggest a role for Aurora B in removing cohesion at these regions. Additionally this phenotype is dependent on perhaps a microtubule bound component. We know that SPC105R also shows centromere separation similar to PP1 but does not have chromatin defects [98]. It is possible that an SPC105R associated pool of PP1 is responsible for regulating a protein keeping sister centromeres fused. In keeping with this hypothesis, centromere separation in SPC105R knockdowns is not dependent on Aurora B. This may

predict a working model (Figure 25) where sister centromere cohesion is established by an as yet unidentified protein, recruited possibly by SPC105R and then maintained at the centromere by the opposing activities of PP1 and Aurora B.

Overall in this study we have ventured to dissect some of the many functions that PP1 possibly has in meiosis. This is a significant advance in the field since regulation of meiosis by kinases is well understood but the role of phosphatases was vastly understudied. Although the antagonism of PP1 and Aurora B has also been observed in mitosis, meiotic functions of these kinases are modified and hence required further study which we have been able to achieve with our system. This system can also distinguish between establishment and maintenance of the different factors required for the events leading to accurate chromosome segregation and provides a platform to study the interactions between other kinases and phosphatases in a temporal manner.

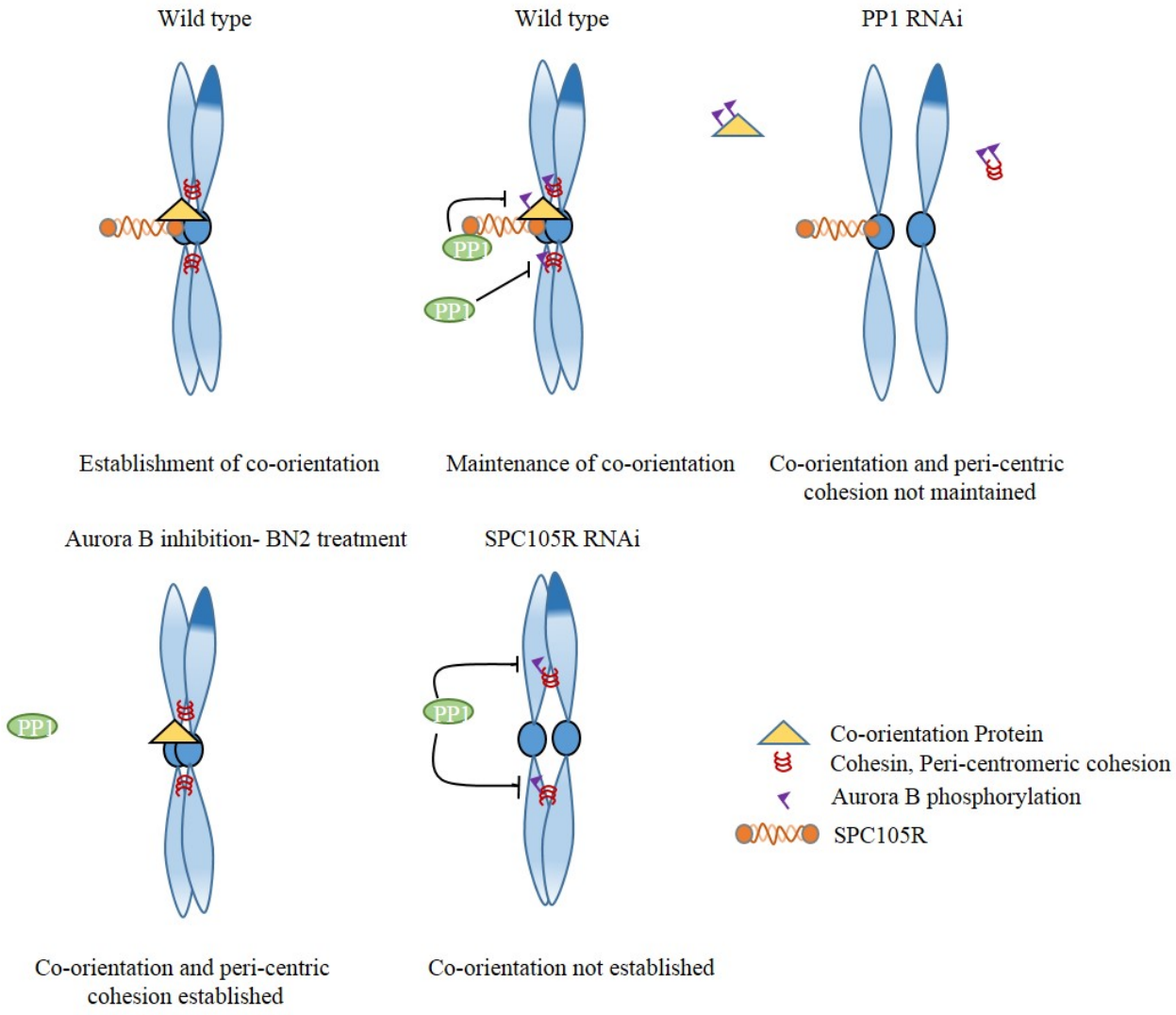


Figure 25: Possible mechanism of Co-orientation in meiotic metaphase I

CHAPTER 5: TOUSLED LIKE KINASE IS REQUIRED FOR HOMOLOGOUS CHROMOSOME BI-ORIENTATION IN *DROSOPHILA* OOCYTES

I. Introduction:

The gene Tousled in *Arabidopsis thaliana* encodes a protein kinase which is required for flower development leading to the concept that this protein regulates plant organ patterning [231, 232]. Dominant negative mutants in *Drosophila* or mouse cells led to chromosomal abnormalities and problems in mitosis [233]. In *C. elegans,* it was shown that the Tlk is required for chromosome condensation and cytokinesis [234]. Interestingly the cytokinesis defect was found to be due to a reduced localization of Aurora B (AIR-2), a chromosomal passenger complex (CPC) member, to the spindle midzone at anaphase. Furthermore, Tlk was also found to be phosphorylated at S364 by AIR-2 and this was required for its proper localization. Curiously AIR-2 was conversely activated by phosphorylated Tlk. This shows that there is an additional substrate activator of Aurora B other than INCENP [235]. However there was no evidence in worms for a direct role of this kinase in phosphorylating H3S10 unlike human cell lines. Previously Tlk was shown to be primarily an S-phase kinase and these studies demonstrate that this kinase also has mitotic roles [236].

In *Drosophila*, the CPC localizes to a ring shaped structure around the chromatin at meiotic metaphase I, in oocytes, which is analogous to the spindle midzone at anaphase of mitosis [83]. Factors important for this localization pattern is not very well understood. In this study we have shown that *Drosophila Tlk* is not required for formation of the

central spindle or localization of the CPC and other central spindle components such as Subito (MKLP-2 homolog) [96].

There are a few physiological substrates of TLK namely the antisilencing factor Asf1, which is required for deposition of H3 and H4 histones during DNA replication and repair, the histone H3 and the Aurora kinase B [237-239]. TLK has been shown to be inactivated in response to IR treatment or genotoxins in an ATM/Chk2 dependent manner. It has also shown to be an interacting partner of the Rad1-Rad9-Hus1 (9-1-1) complex to repair double strand breaks (DSBs) [240]. In *Drosophila* embryos, Tlk has been shown to be involved in chromosome segregation by regulating mars [241]; overexpression of Tlk or Asf1 causes a block in endoreplication in salivary glands [242]. In meiosis, DSB formation is tightly regulated and undergoes repair in a homology directed manner [11, 243]. Hus-1 is thought to be important for this process in early *Drosophila* prophase [244]. In this study we have shown that Tlk is required for oogenesis and possible for repair of DSBs in both oocytes and nurse cells.

II. Results:

TLK is not required for CPC localization at meiotic metaphase I

To test whether Tlk is required for localization of Aurora B to the central spindle we used a short hairpin RNA (HMS) against *Drosophila Tlk* to knockdown the expression of the mRNA in the female germline. We used two different promoters *P{w*$^{+mC}$*=matalpha4-GAL-VP16}V37* and *P{GAL4::VP16-nos.UTR}MVD1*, to express the UASp driven shRNA against *Tlk*. With the *nanos* promoter, the hairpin failed to produce mature oocytes and hence precluded the study of spindle assembly and CPC localization. However this indicated an early requirement of *Tlk* in the germline which we also

investigated (see below). But using a post-meiotic S-phase promoter to express the shRNA generated mature oocytes that were sterile, indicating the protein had indeed been depleted. However in these TLK RNAi oocytes we saw no defects in spindle organization (n=16) or CPC localization. The scaffolding member INCENP localized to the center of the spindle similar to wildtype, in the knockdown oocytes (Figure 26A, n=6). We also examined localization of the central spindle component Subito and it also was not affected (Figure 26A, n=3). These results taken together indicate that in *Drosophila* oocytes, *Tlk* is not required for CPC or Subito localization and spindle organization. We also did not see any obvious chromosome defects such as decondensed chromatin or separated chromosomes, also indicating that *Tlk* may have no role in chromosome organization at least at metaphase I (Figure 26, n=16).

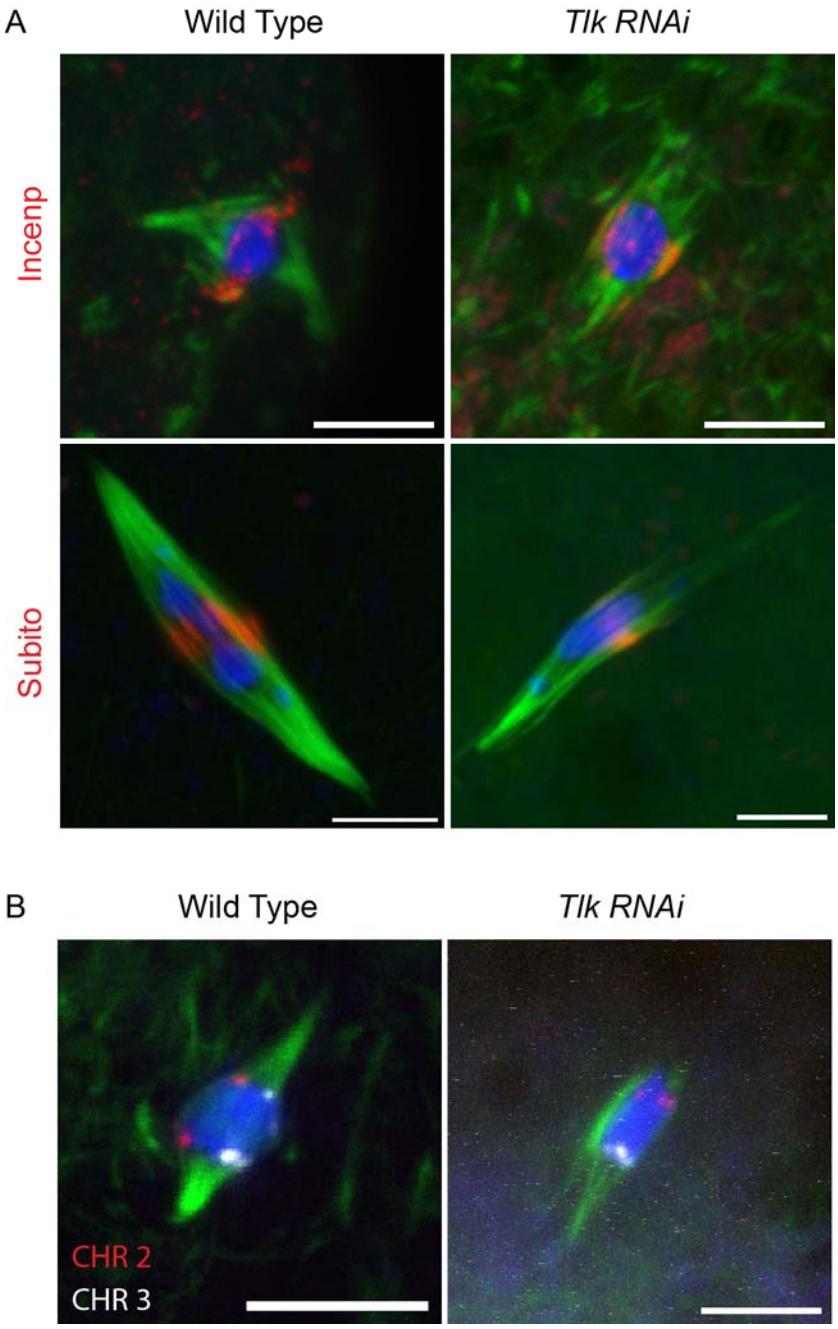


Figure 26: Tlk is required for bi-orientation but not for CPC localization, spindle organization or karyosome structure.

A. Wild type and Tlk RNAi oocytes (expressed using matα) stained with DNA (blue), Tubulin (green) and INCENP or Subito (red). B. Wild type and Tlk RNAi oocytes showing bi-orientation using CHR2 probe (AACAC in red) and CHR3 probe (Dodeca in white). Scale bars are 5µm.

TLK is required for homologous chromosome bi-orientation at metaphase I

To investigate whether *Tlk* affects activity of Aurora B similar to *C. elegans*, we tested for bi-orientation defects in *Tlk* RNAi oocytes using fluorescent in situ hybridization. In wild type, probes to the second and third homologs bi-orient towards each pole. Our preliminary data indicate that *Tlk* RNAi oocytes show a bi-orientation defect where either the second (AACAC probe) and/or the third chromosome (Dodeca probe) are mono-oriented in 100% of *Tlk* RNAi oocytes (Figure 26B, n=3). Considering that wild type orientation defects are negligible in *Drosophila*, despite the low numbers, TLK RNAi orientation defect may be significant.

TLK is required for DSB repair and completing oogenesis in prophase

Expression of *Tlk* RNAi in S-phase had severe defects with the *nanos* promoter. Although we saw evidence of a germline, by the presence of the synaptonemal complex element c(3)G, there were no stages observed beyond that of the germarium. To test if *Tlk* was required for DSB repair we used an antibody against phosphorylated H2AV which marks unrepaired breaks. These are usually observed as foci in zygotene and are repaired in pachytene as oocyte designation occurs. In *Tlk* RNAi, expressed in the germarium with *nanos*, we saw diffuse localization of yH2AV in region 2A surprisingly, where normally, we observe punctate foci (Figure 27A, C). This pattern of yH2AV staining is similar to that seen in *tefu* (ATM) mutants [245]. Surprisingly when we expressed the RNAi with the *bam* promoter, we obtained similar results (Figure 27B). *bam* only expresses in the third mitotic germline division, the 8 cell cyst. This indicates that *Tlk* is required early for the regulation of germline events such as DSB repair. The unrepaired breaks may also fail to satisfy the pachytene checkpoint as we saw two

oocytes in region 3, indicating a delay in oocyte selection, when expressing the RNAi with either *nanos* or *bam* (Figure 27D-F). However, the DSB repair defect seems more prominent in the *Tlk* RNAi expressed with *bam* (Figure 27E).

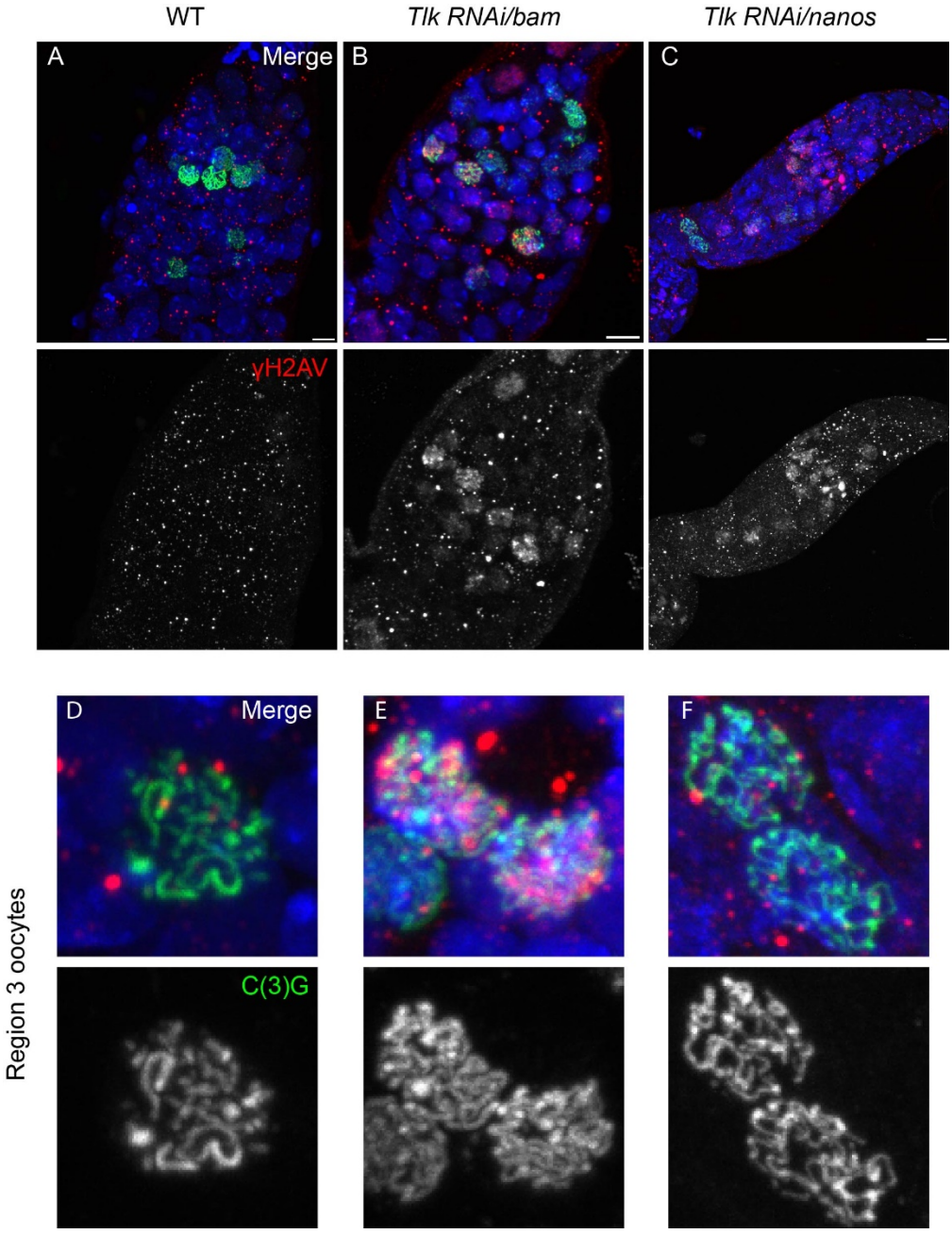


Figure 27**:** Tlk is required to repair DSB and may fail to satisfy the pachytene checkpoint.

The germarium is stained with DNA (blue), C(3)G (green) and γH2AV (red). Single channels are in white. Scale bars are 5µm. A-C. Wild type and *Tlk* RNAi germaria showing diffuse staining of γH2AV in region 2A and 3 (B,C). D-F. Region 3 of the germaria showing 2 oocytes in *Tlk* RNAi. Diffuse γH2AV staining is more prominent in *Tlk* RNAi/*bam* promoter.

III. Discussion:

In this study we have shown a direct role of TLK in bi-orientation at meiosis I, in the formation of an oocyte and in DSB repair in early prophase. In *Drosophila* ovaries Tlk has been shown to be important in the follicle cells for the regulation of the cytoskeleton [246]. This could be due to its role in activating Aurora B which is required for cytoskeletal rearrangements. It has also been shown to be important for border cell migration in the *Drosophila* ovary through regulation of the Jak/STAT pathway [247]. These results taken together with ours establish an essential role of Tlk in female germline development and chromosome segregation.

Although we do not see any defects in Aurora B localization at metaphase I, it is possible that the bi-orientation defects arise from decreased Aurora B activity if indeed Tlk is a substrate activator of Aurora B as reported in *C. elegans* [235, 248]. Additionally we occasionally observe Aurora B localization at the centromeres and this population may be selectively regulated by Tlk to regulate correct attachments of the homologs. In fact in *C. elegans*, tlk localization does not mimic that of the passenger complex indicating that this regulation may be indirect [235].

In early prophase it is obvious that Tlk can generate germline cells by the presence of synaptonemal complex but these cysts are unable to mature. This may be due to DSB repair checkpoints which are unsatisfied in the absence of TLK. This supports a role for Tlk in homologous recombination as opposed to somatic DSB repair pathways. It remains to be investigated which complex is being affected during repair. There is evidence that the DSB induced checkpoint complex, 9-1-1, is important in *Drosophila* germarium for DSB repair [244]. Since Tlk phosphorylates Rad9 [249], also a complex

member, these would be prime candidates for downstream targets in *Drosophila* meiosis. In the future we would like to investigate these interactions further especially Tlk's regulation by ATM/CHK1 or ATR/CHK2.

CONCLUSION

My research focus was directed towards understanding the mechanism of spindle assembly and homolog bi-orientation in the first meiotic division in *Drosophila* oocytes. Specifically, my research involved studying the formation and regulation of the central spindle at metaphase I and the regulation of the proteins that localize to it.

The chromosomal passenger complex has been shown previously to be required for spindle assembly, kinetochore localization and all chromosome movements. However, how this complex regulates these crucial functions remains unclear. In fact, many downstream targets of this complex in meiosis is not known. We do know that the CPC localizes predominantly to the central spindle at metaphase I and so does various other cytokinesis proteins like Subito. Subito mutants are viable but sterile, and they demonstrate reduced dosage dependent lethality with alleles of the CPC. In a synthetic lethal screen with Subito we recovered mutations in 14 complementation groups on the second chromosome and additional ones on the third chromosome, including components of the CPC and centralspindlin complex. We found that the centralspindlin complex member TUM (RacGAP50C) is required for both Subito localization and homolog bi-orientation. This demonstrated for the first time that cytokinesis proteins have alternate specialized functions associated with homolog bi-orientation in meiosis I. There is evidence that this complex may be a direct substrate of Aurora B, and the functions of that conserved phosphorylation site in meiosis remains to be investigated. TUM is a GTPase activating protein and in the future it will be important to investigate whether this activity is required for its meiotic function. This central spindle structure may in fact be

analogous to the mouse meiotic prometaphase belt and thus be a conserved mechanism for segregating chromosomes in acentrosomal systems [97].

Interestingly, I also found that mitotic downstream targets of TUM, like Rho1 GTPase also regulate bi-orientation. Since we were unable to localize Rho1 to the meiotic spindle; it is possible that there is a very low level of protein present undetectable by immunofluorescence. Alternatively, Rho1 present at the cortex may be directing homolog bi-orientation. The mechanism of this, if true, is unclear but will be prudent to study in the future. This will also assess the direct role of the actin cytoskeleton in spindle assembly and chromosome segregation. Another aspect that remains a question in the field is the interactions between the different central spindle proteins. Although our screens have shown genetic interactions between most of the components, and we know that they co-localize at the central spindle in meiosis, we do not know whether these proteins physically interact and the stoichiometry of that interaction. Structural studies to identify the domains and regulatory sites important for meiosis would be the next crucial step in *Drosophila*. Specifically, PRC1 homolog Fascetto (FEO), will be interesting to study. PRC1 is a crucial component of the mitotic spindle midzone and could be very important for regulating the meiotic central spindle structure as well. In the future, analysis of *feo* knockdown oocytes may serve to lend insight into the mechanism of establishment of bipolarity in acentrosomal spindles. It is also plausible to expect that given the evidence in *Drosophila,* these proteins may have similar roles in other organisms as well and indeed the formin family of proteins has been found to be important for kinetochore attachments [189].

Some of my work has tried to focus on identifying regulatory domains in the protein Subito by deletion analysis. This work has identified complex positive and negative regulators in the N-terminus of the kinesin, which may be conserved in vertebrate Mklp2. We have identified domains that are specifically required for mitosis but dispensable for meiosis. This work can be extended to the other members of the central spindle proteins and will doubtless contribute to understanding the relationship between structure and function of these proteins. In fact previously it was thought that only the C-terminus contributes to microtubule binding but our studies have shown that the N-terminus may also be important for binding and since this region is conserved, it may be a mechanism seen in other organisms as well.

However all these studies may still not answer a predominant question in the field of oocyte meiosis, which is how homolog bi-orientation and error correction is mechanistically achieved? Aurora B is required to phosphorylate kinetochore proteins in mitosis, in order to reduce their affinity for microtubule binding, in case of incorrect attachments. The spatial gradient of Aurora B is the current model for sensing tension in mitosis [250]. However in meiosis, where Aurora B is not present at the kinetochores, or is only transiently present at the actual attachment site, how tension is sensed is still unclear. Further studies into regulation of Aurora B with respect to its chromatin associated functions will hopefully address some of these questions. My work has looked at the counteracting effect of the phosphatase PP1, in meiosis. I have found that PP1 has a broad range of functions, most of which are dependent on counteracting Aurora B. Since PP1 also has a plethora of substrates, it is difficult to pinpoint mechanism due to pleiotropic effects. Nevertheless, I have found that co-orientation and chromatin structure

are both regulated by an antagonistic phosphorylation balance between Aurora B and PP1. It remains to be investigated whether co-orientation is cohesin mediated or through a cohesin-independent meiosis specific complex. There is evidence for both mechanisms in yeast and other systems. The role of specific kinetochore proteins in this process along with other kinases like Polo are also speculative at best and require careful study.

Overall, my dissertation has looked at genetic interactions between central spindle proteins to dissect their functions in meiosis I, attempted to understand structural regulation of an important regulator Subito and lastly looked at the holistic regulation of the centrally important complex, CPC by phosphatases. My work represents significant contribution to the field of meiotic spindle and homolog bi-orientation. Furthermore, studies from PP1, have elucidated some interesting thoughts about how co-orientation, a meiosis specific phenomenon, may be regulated. Since this field is still young, these studies will help to understand the causes of errors in meiosis I better.

APPENDIX I

27.89 is located between the visible recessive markers dumpy and black

Recombination mapping was done by isolating recombinants between the *27.89* *sub*^{131} chromosome and a chromosome that contained eight 2nd chromosome recessive visible phenotype markers: *aristaless* (*al*), *dumpy* (*dp*), *black* (*b*), *purple* (*pr*), *cinnabar* (*cn*), *curved* (*c*), *plexus* (*px*), *and speck* (*sp*) (Figure 28). Flies that have had a crossover between the two chromosomes were identified by crossing to another chromosome with all of the markers. Crossovers were then tested to see if the *27.89* mutation remained on the recombinant chromosome by crossing to the *sub*^{1} allele and checking for synthetic lethality. Using the knowledge of which crossovers retained *27.89* one could deduce whether the mutation is to the left or right of each marker.

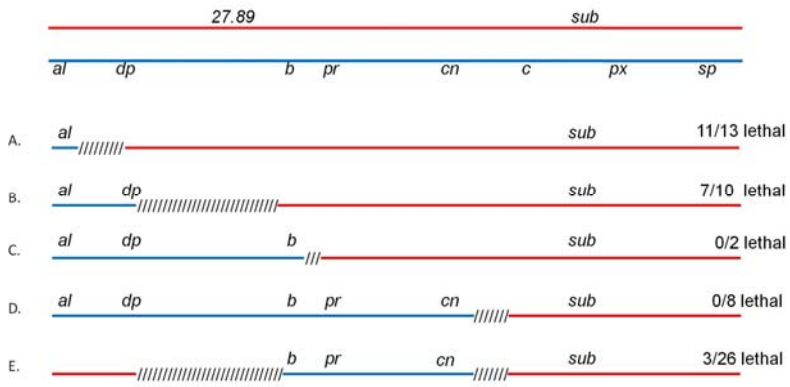


Figure 28: Genetic map showing the recombinants used for the mapping of *27.89*.

The red lines represent the original mutagenized *27.89* *sub*^{131} chromosome. The blue lines represent a chromosome with several recessive visible markers. The slash marks represent the possible area of crossing over for each recombinant. 13 of these crossed over between *al* and *dp*, and 11 of the 13 were synthetic lethal when crossed back to *sub*^{1}. There were 10 crossovers in between *dp* and *b*, of which 7 were synthetic lethal and thus had retained *27.89*. Two events crossed over between *b* and *pr* and neither of them retained *27.89*. Likewise, of the 8 that crossed over between *cn* and *c*, none displayed synthetic lethality. Finally, of the 26 double crossovers that crossed over once between *dp* and *b* and then again between *cn* and *c*, 3 had retained *27.89* and showed synthetic lethality.

For the mapping of *27.89*. 59 recombinants were isolated. Nearly all of the recombinants that crossed over to the left of *dp* (the *al* recombinants) contained *27.89*. Most critically, the recombinants that crossed over in between *dp* and *b* (both the *al dp* recombinants as well as the double crossover *b pr cn* recombinants) showed a mixture of having or lacking *27.89*. These data suggest that 27.89 is likely located in between *dp* and *b.*

Mapping 27.89 to a 303 kilobase region using Single Nucleotide Polymorphisms

To map *27.89* at higher resolution, we used single nucleotide polymorphisms (SNPs) between *dp* and *b* [251, 252]. We isolated recombinants between the *27.89* chromosome and a chromosome of a different background so that there would be a large number of SNPs between the chromosomes. The other chromosome was marked with a Minos element (*Mi[GFP]*) inserted just to the left of *subito* (*sub*). Each individual recombinant was tested for synthetic lethality and the location of the crossover relative to the SNP was determined by PCR followed by a restriction enzyme digest or sequencing of the amplified DNA. For this SNP mapping scheme, a total of 594 recombinants that were al^{+} dp^{+} and GFP^{-} were collected from *al dp 27.89/Mi[GFP]* females. These were selected to isolate recombinants between *dumpy* and the Minos element while ensuring sub^{131} remained on the chromosome.

The SNP marker 939 was used to map the recombinants because is located just to the left of *black* and it was used to discard recombinants that occurred between *black* and *Mi[GFP]*. Similarly, the SNP 865 was used between it was located between *dumpy* and

939. The finding that 65 out of 66 recombinants that crossed over to the right of the SNP 939 were not synthetically lethal (i.e. they did not contain *27.89*), while all 45 of the recombinants that crossed over to the left of SNP 865 were synthetic lethal (i.e. they all contained *27.89*), is consistent with the previous mapping that *27.89* is between *dp* and *b* (Figure 29A). More importantly, of the 28 recombinants between 865 and 939, 11 were synthetic lethal when crossed *sub*^{1} and 17 were not. This mixture of recombinant types indicates that *27.89* is located between SNPs 865 and 939.

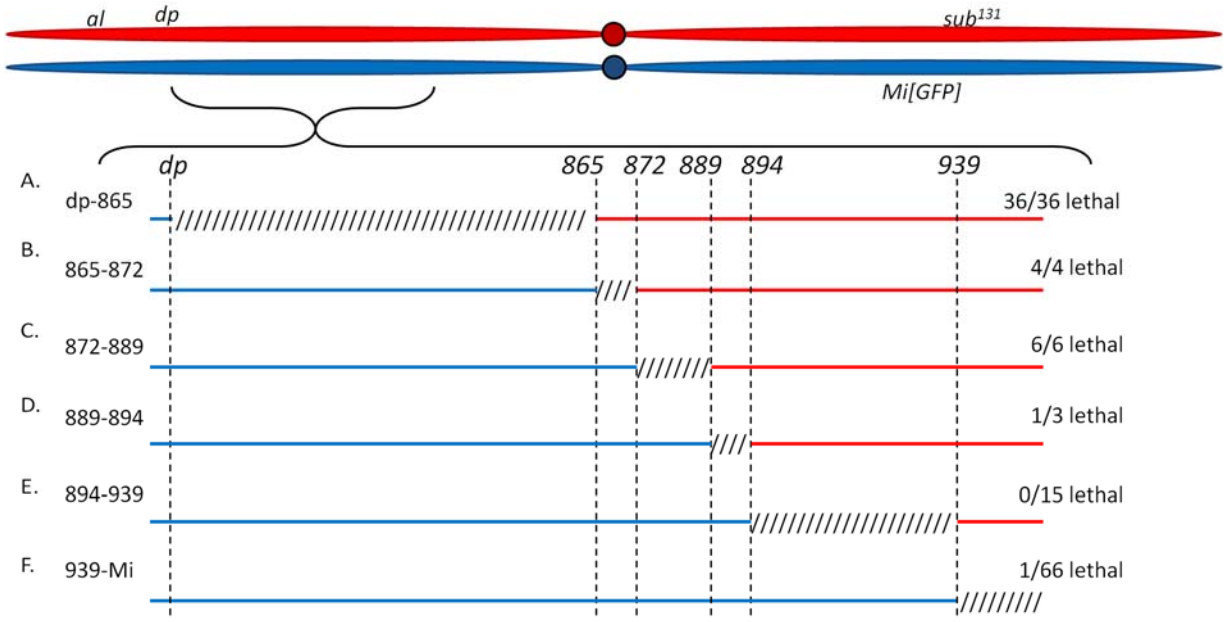


Figure 29: Schematic diagram of the recombinants used for Single Nucleotide Polymorphism marker mapping of *27.89*.

Red lines represent the *27.89* mutant chromosome with the *al* and *dp* markers, *al dp 27.89 sub*^{131}. Blue lines represent a chromosome with many differing SNPs as well as a *Mi[GFP]* insertion just to the left of *subito*. The slash marks represent the possible area of crossing over for each recombinant. The locations of the individual SNP markers are indicated by the vertical dashed lines.

The 28 recombinants between 865 and 939 were tested with additional SNPs in the region 872, 889, and 894. 15 of the 28 recombinants crossed over between 894 and

939, all of which did not have *27.89*, implying that *27.89* is located to the left or very close to the right of 894 (Figure 29B). 4 of the 28 recombinants crossed over in between 865 and 872, and all of these crossovers contained the *27.89* mutation suggesting that *27.89* is most likely located to the right or close to the left of 872 (Figure 29E). The 9 remaining recombinants crossed over between 872 and 894, 7 of which retained *27.89* and 2 of which did not. The SNP 889 further divided these 9, into 6 crossovers between 872 and 889, all of which had *27.89*, and 3 crossovers between 889 and 894, of which one contained *27.89* and 2 did not (Figure 29C and D). These data indicate that *27.89* is located between 889 and 894, and likely closer to 889. This is a region of approximately 300 kb.

27.89 exhibits homozygous lethality, yet complements all deficiencies within the region between SNPs 872 and 894

The original *27.89* chromosome contained two mutations, *27.89* and sub^{131}. The sub^{131} allele was removed by isolating recombinants of the *27.89* sub^{131} chromosome as discussed above. By picking cn^{+} c^{-} recombinants (*curved* (*c*) is located a short distance to the left of *sub*) a stock was generated that carried only *27.89*. The recombinant *27.89* cn^{+} c^{-} chromosome was homozygous lethal. This could mean that *27.89* is a homozygous lethal mutation. Another possibility, however, was that there was another EMS induced lethal mutation elsewhere on the chromosome. To check if *27.89* is homozygous lethal or there is another EMS induced lethal on the chromosome, recombinants *al dp 27.89* sub^{131}, *27.89 b pr cn* sub^{131} and *27.89 c* were crossed to each, resulting in much of the original mutagenized chromosome remaining heterozygous. Even after removing much

of the mutagenized chromosome we still failed to observe *27.89* homozygotes. Thus, these results support the conclusion *27.89* is homozygous lethal.

We also attempted to map *27.89* using chromosomal deletions. Using the SNP mapping data, we crossed *27.89* to all deficiencies spanning the distance between SNPs 872 and 894 (Figure 30). None of these deficiencies failed to complement *27.89* for lethality. To determine if the problem lies with the deficiencies, we acquired known homozygous lethal mutations in genes that the deficiencies are supposed to delete. Complementation tests were done between these mutations and their corresponding deficiencies, and it was determined that all of the deficiencies in the region that had complemented *27.89* failed to complement other known lethal mutations. Therefore, it is possible that *27.89* both fails to generate homozygotes yet is viable when heterozygous to a deficiency. There are currently two explanations for this result, either *27.89* is a recessive hypermorph, that is viable over a deficiency, or the region between *dp* and *b* where *27.89* itself is located, contains a second site lethal mutation.

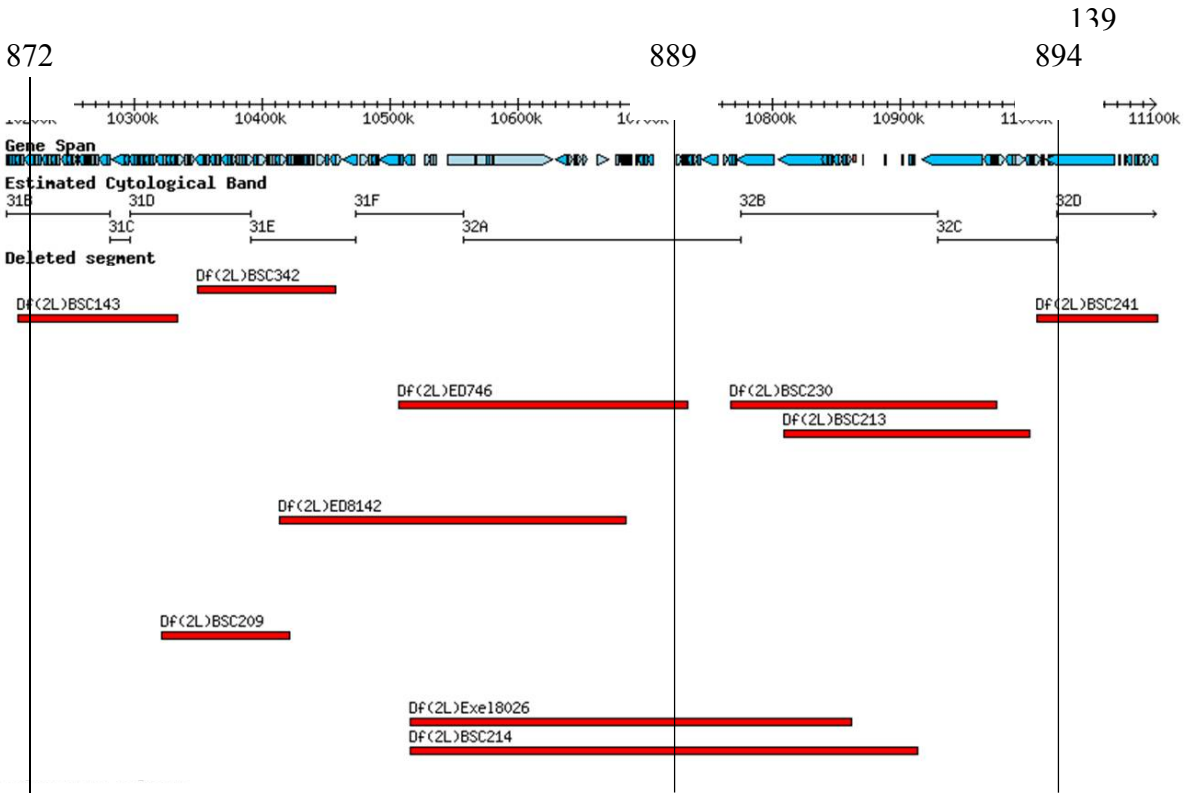


Figure 30: Schematic diagram of the chromosomal deletions used to deficiency map *27.89*.

The red lines represent chromosomal deficiencies. The key SNP markers with which *27.89* was mapped are labeled at the top and delineated by the vertical black lines. Figure adapted from Flybase **[253]**.

APPENDIX II: CONDENSIN COMPLEX IS NOT REQUIRED FOR LOCALIZATION OF CPC IN MEIOSIS I OR CHROMATIN ORGANIZATION

The condensin complex is a highly conserved ring shaped pentameric structure with two SMC (structural maintenance of chromosome) family proteins, SMC2 and SMC4, and three additional subunits. There are two complexes in higher organisms, depending on the auxiliary subunits. Condensin I has CAP-H, CAP-D2 and CAP-G. Condensin II has CAP-H2, CAP-D3 and CAP-G2 [254]. *Drosophila* has both complexes although CAP-G2 has not been identified yet. In this study I investigated the role of condensins in regulating CPC function. It is known that the CPC is required for recruiting condensins to the chromosomes [255]. However, not much is known about the reverse regulation. There is some evidence that relocation of the CPC from the chromatin to the central spindle microtubules may be dependent on SMC2 mediated chromatin changes, regulated by PP1 [68].

To test if this was true in *Drosophila* oocytes, I used shRNA to knockdown expression of the two SMC subunits, SMC-2 and Gluon (SMC-4) and Barren (Cap-H). In wild type oocytes Incenp localizes to the central spindle (Figure 31A). I stained for Incenp in these knockdown oocytes and found that SMC-2 did not mislocalize Incenp (Figure 31B). However, Gluon knockdown oocytes showed diffuse localization of Incenp in 25% of oocytes (Figure 31D) but are normal in the rest (Figure 1C). Also in 25% of oocytes, the karyosome was separated into two pieces whereas in wild type oocytes it is usually in one mass at metaphase (Figure 31D). The rest of the oocytes were normal as shown (Figure 31C). Barren also showed diffuse Incenp in 27% of oocytes (n=11, Figure

31 E). It will be interesting to investigate the role of condensin in chromatin organization and CPC function.

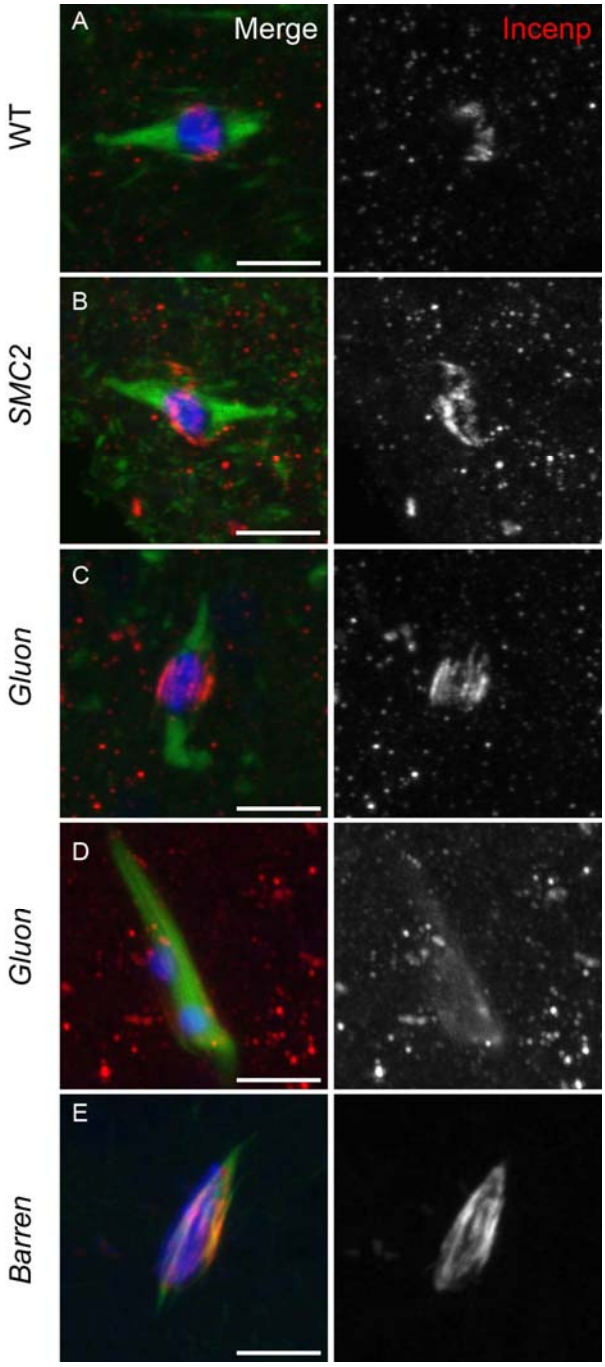


Figure 31: Condensins do not affect CPC localization but may be required for karyosome structure.

All the panels show oocytes stained with DNA (blue), Incenp (red in merge or white in single channel) and tubulin (green). B-E. Indicated condensin subunits, knocked down in oocytes using shRNA.

APPENDIX III: CENTRAL SPINDLE MICROTUBULES MAY BE IMPORTANT FOR CHROMOSOME MOVEMENTS IN *PP1-87B* KNOCKDOWN OOCYTES

To test if the karyosome separation seen in *Pp1-87B* knockdown oocytes, is due to the presence of microtubule directed forces, we used colchicine treatment to remove most of the microtubules. 150μM of colchicine, added for 30 minutes, was sufficient to remove almost all of the microtubules except those attached end-on to kinetochores. We observed that in wild type oocytes treated with either solvent only or colchicine, there was no karyosome defect (n=10 and 15 respectively Figure 32A, B). In *Pp1-87B* knockdown oocytes treated with solvent only, 62.5% were separated as expected (n=8, Figure 32C). However, in *Pp1-87B* knockdown oocytes treated with colchicine we see only 37.5% separated karyosome (n=22, Figure 32D). This could indicate that while the karyosome separation is not dependent on microtubules if Aurora B is inhibited, in the presence of active Aurora B, the lateral attachments may be important for chromatin movements in a *Pp1-87B* knockdown background.

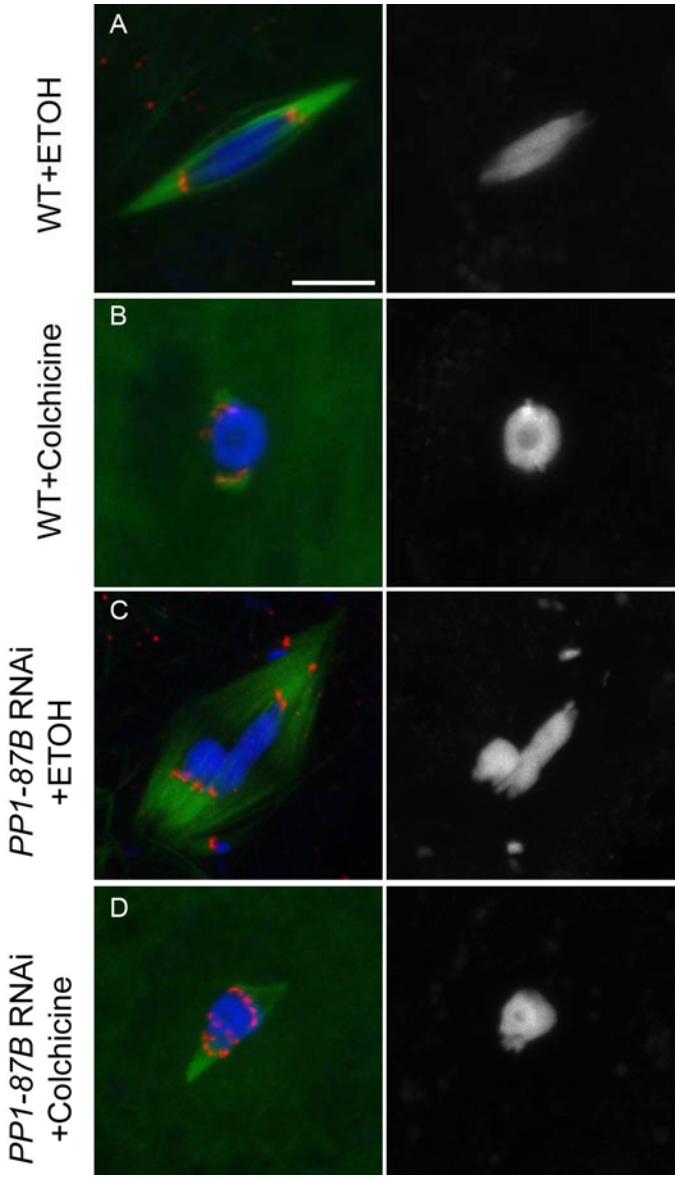


Figure 32: Colchicine treatment of *Pp1-87B* knockdown oocytes reveals that karyosome defect may depend on central spindle

Wild type and *Pp1-87B* knockdown oocytes showing CENP-C (red), DNA (blue) and tubulin (green). A,C. Solvent treated WT or Pp1 RNAi oocytes. B,D. Colchicine treated WT and Pp1 RNAi oocytes.

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