REGULATION OF SISTER CENTROMERE FUSION AND MEIOTIC SPINDLE ASSEMBLY IN *DROSOPHILA* OOCYTES

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ABSTRACT OF THE DISSERTATION

REGULATION OF SISTER CENTROMERE FUSION AND MEIOTIC SPINDLE ASSEMBLY IN *DROSOPHILA* OOCYTES

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Accurate meiotic chromosome segregation prevents aneuploidy that can cause developmental problems and lead to infertility. The majority of errors causing aneuploidy in gametes are maternal in origin due to several innate differences in female meiosis. In contrast to mitosis and male meiosis, bi-polar spindles in female meiosis are assembled in the absence of centrosomes. Instead, the chromosomes direct bi-polar spindle assembly. Abnormalities in spindle formation or structure can cause non-disjunction of the chromosomes. To understand the mechanisms of proper chromosome segregation in female meiosis, I focused on sister centromere cohesion and spindle assembly in *Drosophila* oocytes. One important mechanism for proper chromosome segregation in meiosis is that the sister chromatids co-orient via sister centromere fusion, resulting in microtubule attachment to a shared spindle pole. This phenomenon is unique to meiosis I, and is well-studied in budding and fission yeast, it remains unclear how it is regulated in other organisms.

Previously our lab has identified two proteins that are required for sister centromere fusion. I characterized these two genes and surprisingly found they regulate sister centromere fusion by two different mechanisms. SPC105R, a kinetochore protein, regulates centromere fusion by recruiting proteins that protect cohesins. Protein phosphatase 1 (PP1), on the other hand, regulates this phenomenon through antagonizing proteins that stabilize microtubule

attachment. The latter result suggests that stable microtubule attachment in metaphase I is the prerequisite to release sister centromere fusion without cohesin removal before metaphase II.

The chromosomal passenger complex (CPC) has four subunits (INCENP, Aurora B, Borealin and Survivin/Deterin) and it is essential for spindle assembly in *Drosophila* oocytes. I made several separation-of-function mutations within the *Incenp* subunit of the CPC and found that interactions with Heterochromatin Protein 1 (HP1) are key to direct CPC-dependent spindle assembly in oocytes. HP1 firstly recruits the CPC to chromosomes through interacting with Borealin, and this interaction is also required for building the central spindle, the central overlap region of the microtubules. Additionally, I found evidence that HP1 interacts directly with INCENP within the central spindle in oocytes to promote homologous chromosome bi-orientation. These results give rise to a model where HP1 could be a novel CPC targeting subunit and reveals a mechanism for how the CPC mediates chromosome-mediated spindle assembly.

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Chapter 1

Introduction

Aneuploidy and human fertility

Sexual reproduction is completed when two gametes fuse, form a zygote, and develop into an individual. Gametes are generated through a type of specialized cell division, called meiosis, which yields a haploid cell with a single copy of genetic information. Several different types of errors can happen in meiosis, including duplication, insertion or deletion of a region of a chromosome, translocation of chromosome arms which lead to the unbalanced gene expression, or the most common error, chromosome mis-segregation. Aneuploidy, the incorrect number of the chromosomes, is the result of chromosome mis-segregation in meiosis, and it is the leading cause for genetic problems in human reproduction. While an estimated 10-30% of fertilized human eggs are aneuploid [1] and account for a third of the miscarriages [2], some aneuploid eggs are viable and associated with severe developmental diseases. Some notable examples are trisomy 21 (Down's syndrome), XXY sex chromosome trisomy (Klinefelter's syndrome) and X sex chromosome monosomy (Turner syndrome).

Errors resulting in aneuploidy are mostly maternal-origin: for example, almost 100% of documented trisomy 16 was due to maternal-origin errors and happened during meiosis I [1, 3]. Trisomy 21 occurs 88% of the time due to maternal-origin errors, in contrast to 8% from paternal-origin errors and 4% from mitotic errors during development [1]. Additionally, when analyzing the gametes, around 10-30% of oocytes are aneuploid compared to 2% in sperm [1], and the incidence of aneuploidy in oocytes increases to 35% in older women [4]. This strong correlation of maternal-origin error in aneuploid gametes begs the question: why are oocytes so susceptible to chromosome segregation errors?

One leading hypothesis of this cause is that the maternal age effect, where the cohesion that holds the sister chromatids together is lost/weakened with the women age [5-7]. However, this hypothesis can only explain the elevated aneuploidy rate in aged oocytes, but not the generally high maternal-origin errors. For instance, altered recombination frequency and recombination site have been observed in the case of trisomy 21 and 16 [8]. Thus, some other mechanism(s) that is inherent to female meiosis might be mis-regulated. Understanding the basis of meiosis, and the innate differences in this process between the two sexes, is key to understanding the healthy gamete, especially oocytes. In this chapter, I will introduce meiosis, important details of its molecular regulation, and the unique features of female meiosis.

Meiosis

Meiosis is a type of cell division that generates haploid gametes. It includes two rounds of cell division where the homologous chromosomes segregate in meiosis I and the sister chromatids segregate in meiosis II. Meiosis II, also called equational division, is very similar to mitosis in which centromere cohesion exists between sister chromatids. During metaphase/anaphase transition in meiosis II, this centromere cohesion is removed so that the sister chromatids can segregate. In contrast, meiosis I is a unique process. In early prophase I, the homologous chromosomes undergo DNA double strand breaks (DSBs) to initiate homologous recombination. This process exchanges the genetic code between the homologs, resulting in genetic diversity of the gametes. Once recombination is finished, the homologs are connected by the crossovers, or chiasmata, as a bivalent and held by chromosome arm cohesion. During the metaphase/anaphase transition in meiosis I, the arm cohesion is removed to resolve the crossover, and the homologs can therefore segregate into two haploid daughter cells. Meiosis I is also called reductional division because the homologs separate and reduce the ploidy of the daughter cells by half.

Meiosis I is different from meiosis II and mitosis. Several distinct hallmarks in meiosis I have evolved to regulate the homologous chromosome segregation. These meiosis I hallmarks include crossover formation, synaptonemal complex formation, sister centromere fusion and stepwise cohesion removal. In this dissertation, I will focus on the latter three features that are related to my research.

The formation of synaptonemal complex (SC)

The SC is a ladder like structure that exists on the chromosome axis to facilitate homolog pairing, regulate the formation of crossovers and connect the chromosome pairs [9]. This ladder like structure comprises lateral elements, transverse elements, and central elements. A lot of the proteins in the SC have been identified: the transverse element is Zip1 in yeast and SYCP1 in mice; the central element is SUMO/Smt3 in yeast and SYCE1 and TEX12 in mice; whereas, the lateral element is Red1 in yeast and SYCP2 and SYCP3 in mice [10]. In *Drosophila*, C(3)G is the transverse element and Cona is the central element [10], whereas the lateral elements have not been defined. A recent study has shown that the SC depends on the meiosis-specific cohesins, SUNN, SOLO, C(2)M, SA and SMC1/3, and cohesion regulator, ORD and Nipped-B [11-14]. Additionally, C(2)M and SA localized on the chromosome axis and showed similar properties in their protein dynamics and crossover regulation, suggesting the possibility that they can be part of lateral elements in the SC [11, 13].

The SC initiates assembling in the early zygotene stage, and it starts from the centromeres and few foci on the chromosome arms and fully assembles in the pachytene stage along the chromosome axis [13]. While most of this structure disassembles by late prophase via the phosphorylation by Aurora B kinase or Polo Kinases [15, 16], small amounts of SC at the centromere persist beyond prophase I, possibly until anaphase I in mice [17-19]. These groups of SC have been shown to promote the nonexchange chromosome orient [20]; however,

understanding of how chromosomes orient is still very limited. Although the presence of the centromeric SC might possibly regulate another meiosis-specific phenomenon, sister kinetochore co-orientation, the biological significance and regulation of this centromeric SC remains to be investigated.

Kinetochore geometry

In metaphase I, meiotic cells employ another unique mechanism to ensure that sister chromatids segregate to the same pole. This mechanism changes the sister kinetochore geometry via sister kinetochore fusion in order to facilitate the microtubule attachment from the same pole [21, 22]. A model comparing this process to mitosis is shown in Figure 1. This phenomenon is well-studied in *Saccharomyces cerevisiae*, where a group of specialized proteins, called monopolin, interacts with the kinetochore protein DSN1 and forms a V-shaped structure to pull the sister kinetochores together [23, 24]. While monopolins are not conserved and other specialized proteins have not been identified yet in other eukaryotic cells, we know that cohesin proteins are involved in this process. For example, fission yeast, plant and mouse meiotic cohesin protein, Rec8, is required for this fusion process [25-27]. Also, cohesin protectors, Moa1 and Meikin, are required for this meiotic specific process [25-29]. This unique centromeric cohesion has also been seen in *Drosophila*. The centromere cohesion is enriched on the centromere in early meiosis [13], and this enrichment has not been reported in mitotic cells. These findings have led to more questions: is this centromere cohesion in *Drosophila* sufficient to establish co-orientation? Whether there are any specialized meiotic proteins like centromeric SC responsible for this process in higher eukaryotic organisms remains an unsolved question.

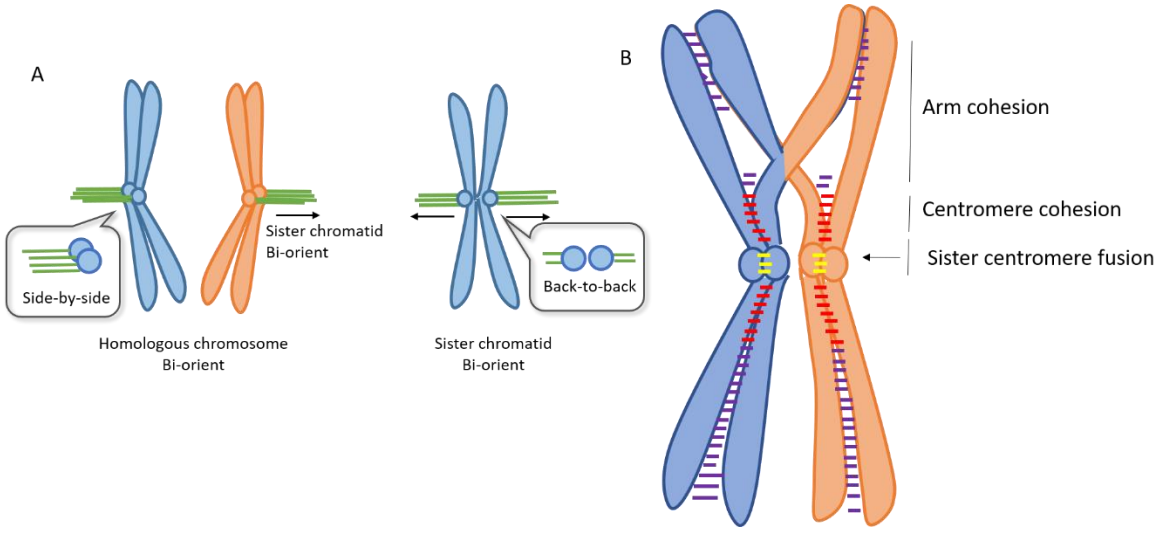

Figure 1. Sister centromere geometry.

(A) When homologs bi-orient and sister chromatids need to co-orient in meiosis I (left), the sister centromeres orient side-by-side to facilitate microtubules attach from the same pole. When sister chromatids bi-orient in meiosis I or in mitosis (right), the centromere orientation turns back-to-back. (B) The cohesions, which mark as dash lines between the sister chromatids, can be classified into three groups based on the chromatid regions: arm cohesion, centromere cohesion and sister centromere fusion. While the first two groups are known to be held by cohesin proteins, whether sister centromere fusion depends on cohesin proteins in *Drosophila* oocytes remains to be tested.

Stepwise cohesin removal

One mechanism important for correct segregation of homologous chromosomes, but not sister chromatids in meiosis I, is the regulation of cohesion removal. Cohesion is established during S-phase before meiosis starts. During the metaphase/anaphase I transition, arm cohesion is removed to release the chiasmata that hold the homologs; whereas, centromere cohesion remains to hold sister chromatids until metaphase/ anaphase II onset. In mitosis, two pathways for cohesion removal exist. One is a prophase pathway, which releases arm cohesion via Wapl; the other functions during the metaphase/ anaphase transition, which releases centromeric cohesion via Separase. Because arm cohesion needs to be remove in meiosis I, one would imagine the mitotic prophase mechanism could be utilized during meiosis. However, Wapl mainly regulates cohesin to change metaphase chromatin morphology [30, 31] and as of writing, only one paper has shown its impact on homolog segregation [32]. Wapl's meiotic role remains to be clarified, leaving Separase as the prime pathway to remove cohesin [33, 34].

In this model of Separase as the primary negative regulator of cohesion, to achieve stepwise cohesin removal, centromere cohesion needs to be protected from removal in metaphase I. Sgo1 has been identified as necessary for this meiotic process [35, 36]. Sgo1 localizes to the centromere by recognizing phosphorylated histone H2A, and recruits protein phosphatase PP2A [37-39]. This PP2A recruitment further counteracts cohesins' phosphorylation by the kinase(s), which is required for Separase removal. However, contradictory results have been shown in many systems. Both in *Drosophila* and mouse oocytes, a Separase- uncleavable Rec8 or the knockdown of Separase only delays but does not abrogate meiosis I [40, 41]. The conventional mitotic cohesin phosphorylation kinase, Polo kinase, has yet been shown to be required for meiosis I chromosome segregation [42]; instead, casein kinase (CK1) and CDC7 kinase have essential roles [43]. However, related literature of cohesin phosphorylation by these two protein kinases all used budding yeast, whether the results are the budding yeast-specific phenomenon needs to be tested. Additionally, the APC/C, the Separase regulator, does not seem to be essential for the meiosis I in

Xenopus oocytes [44, 45]. All these observations challenge the conventional regulation of Separase in the meiotic cohesin removal process, leaving the regulatory mechanisms a mystery. As mentioned previously, sister centromere fusion for co-orientation in most eukaryotic cells depends on cohesin, though its regulation is still unclear. This type of cohesion needs to be maintained during metaphase I and released before metaphase II. Whether this cohesion is also protected by Sgo from Separase or there are other meiosis-specific mechanisms involved, and how it is removed before metaphase II are intriguing questions. One of my research focuses is to characterize this sister centromere fusion in *Drosophila* oocytes. Our lab identified two genes required for maintaining sister centromere fusion, and surprisingly I found that they regulate two independent mechanisms: one is involved in cohesin protection and the other regulates the stability of kinetochore-microtubule attachment. I will address on this topic in chapter two.

Uniqueness of oocytes

Although male and female gametes follow the same principle for meiotic chromosome segregation, there are several differences. These differences might be crucial to understanding how maternal-origin errors arise in gametes. The unique features of oocyte meiosis need to be researched in depth.

Meiotic Arrest

Female meiosis in metazoans involves two cell cycle arrests during development while male meiosis proceeds without interruption. The first arrest happens in prophase after the oocytes have gone through DNA repair and recombination, and the second arrest happens either in metaphase I (mostly in invertebrates including *Drosophila*) or metaphase II (in most vertebrates including mice and humans) [46]. After resuming from prophase arrest, oocytes undergo several

significant processes including nuclear envelope breakdown, meiotic spindle assembly, and rearrangement of the cortical cytoskeleton. This process is called meiotic maturation. This meiotic maturation is for oocytes to prepare for fertilization. During meiotic maturation, the oocytes undergo a second arrest. Hypothetically this arrest is to prevent inappropriate DNA replication because knocking out of c-mos, which is required for oocyte arrest, causes parthenogenetic development of unfertilized mouse oocytes [47, 48]. Resuming from the second arrest is called activation, and this process is regulated through hormone or fertilization signaling [46]. The period of prophase arrest lasts for hours to years depending on organism. In this case, cohesin complexes, known for holding the sister chromatids together, are vulnerable during these arrests and their degradation can result in aneuploid gametes because of premature loss of cohesion.

Acentrosomal spindle assembly

Female gametes are known for their lack of the typical centrosomes. In *Drosophila*, before germ cells enter meiosis, a germ cell divides four times to make 16 cell cysts and one of them will become oocyte. During this duplication the centrosomes are also doubling and after entering meiosis, these centrosomes migrate to the oocyte. In late prophase, the clustered centrosomes are gradually eliminated to ensure the parental centrosomes will be the sole source [49]. This gradual elimination process depends on Polo kinase. Polo Kinase is required to maintain the pericentriolar matrix in prophase in order to keep centrosomes from elimination; however, as Polo Kinase expression declines in meiosis, centrosomes are eventually lost by the end of meiosis [49].

Centrosome elimination accompanies a challenge for the oocytes where the spindle needs to assemble in a non-canonical way. In mitosis, centrosomes function as a microtubule-organizing centers (MTOC) by recruiting γ-tubulin ring complex (γ-tuRC) to nucleate the microtubules. The

microtubules grow toward the kinetochores in a search-and-capture mechanism [50]. Because centrosomes are eliminated in early prophase, oocytes have evolved other mechanisms to build the spindle. In mouse oocytes, after nuclear envelope breakdown, dozens of small microtubule asters called acentriolar MTOCs nucleate microtubules in the cytoplasm and then cluster around the chromosome to assort into a bipolar spindle [51]. In *Xenopus*, *Drosophila* and even human oocytes, the chromosome serve as a MTOC to nucleate and organize the spindle formation [52-55]. These observations demonstrate the importance of the chromosome in directing the spindle assembly in oocytes.

The molecular mechanisms controlling meiotic spindle assembly are relatively well characterized in *Xenopus* egg extracts. Both DNA-coated beads and sperm nuclei can induce spindle assembly in *Xenopus* egg extracts [56, 57], and the process depends on two pathways. First, spindle assembly depends upon the conversion of Ran into RanGTP based on a chromosome-based RCC [58, 59]. Mechanistically, RanGTP can release spindle assembly factors from inhibitors called importins. Based on the gradient of RanGTP from the chromosome, the spindle can be assembled around the chromosome. Second, the Chromosomal Passenger Complex (CPC) is essential for spindle assembly and it is partially regulated through inhibiting the microtubule-severing proteins, MCAK and Op18 [56, 60]. In *Drosophila* oocytes the Ran pathway is not essential for spindle assembly because inhibiting RanGTP only delays meiosis I spindle formation but does not abolish it [61]. Although spindle assembly requires the CPC's activity in *Drosophila* oocytes [62], the mechanism is not the same as in *Xenopus*: co-depleting MCAK and Aurora B, the catalytic subunit of the CPC, did not restore the spindle phenotype suggesting there are other targets for the CPC [63].

The CPC is composed of four subunits, including Survivin, Borealin, a scaffold protein, INCENP and a kinase, Aurora B. Survivin and Borealin interact with the N terminus of INCENP forming the tripartite complex; meanwhile, the C terminus of INCENP contains a domain, the INbox, which interacts with Aurora B (Figure 2). In mitosis, Survivin and Borealin are recruited

by two phosphorylated histone markers, H3T3 and H2AT120, which distribute in the centromere region. The centromere-recruited CPC corrects the errors of kinetochore-microtubule attachments by destabilizing it during metaphase. After entering anaphase, the CPC relocates onto the spindle midzone, also called central spindle, to regulate cytokinesis. Although the CPC plays an important role in meiotic spindle assembly, the understanding of its meiotic function remains limited.

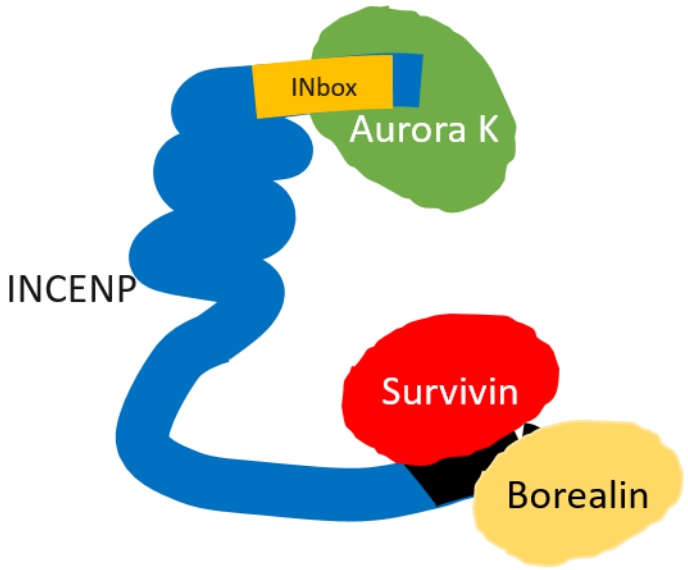

Figure 2: The structure of the chromosomal passenger complex (CPC).

The CPC is a four-member protein complex. The scaffold protein, INCENP, interacts two targeting subunits, Survivin and Borealin, through its N terminus. The C terminus of INCENP contains a conserve domain, INbox, which recruits the other CPC subunit, Aurora B Kinase.

In mouse, acentriolar MTOCs consist of pericentriolar material (PCM) containing γ-tubulin, pericentrin and other spindle assembly proteins including TPX2 and TACC3. Studies have shown that TPX2, as a Ran pathway target, can nucleate MTs and also control MTOC integrity by phosphorylating TACC3 via interaction with Aurora A Kinase [64]. Although the Ran pathway has impacts on MTOC integrity and MT nucleation, dysregulated Ran-GTP levels only impair meiosis II spindle assembly but not meiosis I [65]. These results suggest the CPC or other novel pathway might contribute to the process.

In addition to the regulation of spindle nucleation or organization, spindle assembly factors also play a critical role in assisting spindle formation in the oocytes. In *Caenorhabditis elegans*, the meiotic spindle can be observed before nuclear envelope breakdown. The bundled microtubules form a cage in the nucleus, and this formation depends on several microtubule associated proteins, including KLP-18/kinesin-12 and MESP-1 for assorting MTs [66] and ASPM-1 [67] and MCAK/kinesin-5 [68, 69] for regulating microtubule length . Similarly, spindle assembly in *Xenopus* egg extracts shows that spindle formation is also inhibited by microtubule severing proteins MCAK and Op18 [56, 60]. In *Drosophila* oocytes, Subito/Kinesin-6 is required for central spindle formation and it genetically interacts with the CPC [62, 70, 71]. Acentrosomal spindle assembly is not as efficient as the centrosome-mediated pathway. It takes 3-5 hours in mouse oocytes to assemble spindles [51, 72, 73] while it only takes 30 minutes in mitotic cells [74]. The timing of spindle formation potentially correlates with the size of oocytes and may be due to dilution of these spindle assembly factors [75]. More importantly, the length of spindle formation process has been associated with spindle instability and incorrect kinetochore-microtubule attachments, potentially contributing to the high error-rate of female gametes [52].

To understand how the CPC regulates the spindle assembly in oocytes, the other project in this dissertation is to study the separation-of-function mutation of the CPC. I identified HP1 (heterochromatin protein 1) is the key regulator to recruit the CPC to chromatin and assist the CPC to initiate the spindle assembly. Moreover, I found that the CPC in oocyte regulates the

homologous chromosome bi-orientation from the central spindle than from the conventional pathway, the centromere. The detailed mechanism will be addressed in chapter three.

Large size of the oocytes

The dimorphism of gamete size, also called anisogamy, predominantly exists in eukaryotes, especially in mammals. Anisogamy often describes a larger quantity of physically smaller male gametes verses fewer, but larger, female gametes. Indeed, oocytes are known to have an enormous cytoplasmic volume compared to other cell types. The size of *D. melanogaster* oocytes is approximately 0.2 mm x 0.5 mm, the diameter of a mouse oocyte is approximately 80 µm, approximately 1 mm in *Xenopus* and about 120 µm in humans, compared to 25 µm on average in somatic cells. The large cytoplasmic volume of oocytes might be due to their role in providing mRNA and proteins for zygote development. The great cytoplasmic volume of oocytes has been reported to effect meiosis. Altering the cytoplasmic volume of mouse oocytes causes the timing of acentrosomal spindle assembly and the strength of spindle checkpoint to change proportionally [75]. When the oocyte cytoplasm size increases, it causes a larger spindle to form, a longer time to bi-orient chromosomes, but it hastens the transition to anaphase and decreases the stringency of spindle assembly checkpoint [75]. These observations provide insight into the error-prone nature of oocytes, regardless of the age-effect and highlights the uniqueness of this special cell type.

Ovary model system in *Drosophila melanogaster*

Female *Drosophila melanogaster* have two ovaries, and each ovary contains 15 to 20 ovarioles (Figure 3A). Each ovariole is a string of temporally-ordered oocytes. The anterior section includes the earliest stages in the germarium and the posterior section contains the latest stage of oocytes. The development of the oocytes can be classified into 14 different stages based on the nurse cells, oocyte volume, and follicle cell morphology [76]. Pre-meiotic mitosis takes

place in the tip of the germarium (region 1) and generates 16-cell cysts. The cysts enter meiotic prophase in germarium region 2A which lasts until stage 12. Meiotic maturation occurs in the end of stage 12 when the nuclear envelope breaks down. The oocyte enters prometaphase in stage 13 and reaches the second arrest in metaphase I at stage 14. The oocyte resumes meiosis once it passes through the oviduct where it could get fertilized. Meiosis can be completed within 20 minutes even without fertilization.

Meiotic spindle assembly in *D. melanogaster* beings after nuclear envelope break down in stage 12 oocytes. Due to the lack of centrosomes in oocytes, the microtubules cluster and nucleate around the chromosomes instead and then assort into a bi-polar spindle. The microtubules of the spindle can be classified into two groups: one connects to the kinetochore, called K-fibers; the other group forms antiparallel microtubules overlapping with each other, called the central spindle (Figure 3B).

D. melanogaster has been a great genetic model organism over a century. Modern technologies have been applied to *Drosophila* and have been a great boon to research. This includes the full genome sequence, an open resource of FlyBase, cytological and imaging techniques [77], and programmable gene expression. In my research, Gal4-UAS system was used to control gene expression including expressing shRNA to knockdown gene expression or express transgenes [78] (Figure 3C). Several oocyte-specific promoters express the GAL4 transcription factor, which can then be used to express an shRNA or transgenes which were under control of a UAS promoter (Figure 3D). This well-established system was commonly used in both of my projects.

Overall, the focus of my dissertation is to understand the molecular mechanisms of two fundamental meiotic processes through *D. melanogaster*: one applies to both sexes, where the sister centromere fuses to ensure the sister chromatids can be segregated to the same pole; the other is to focus on a female-specific process, where the spindle assembles without the presence

of centrosomes. I provide strong evidence and novel insight in both projects in hope to bring the reproduction field a better understanding of meiosis, especially in oocytes.

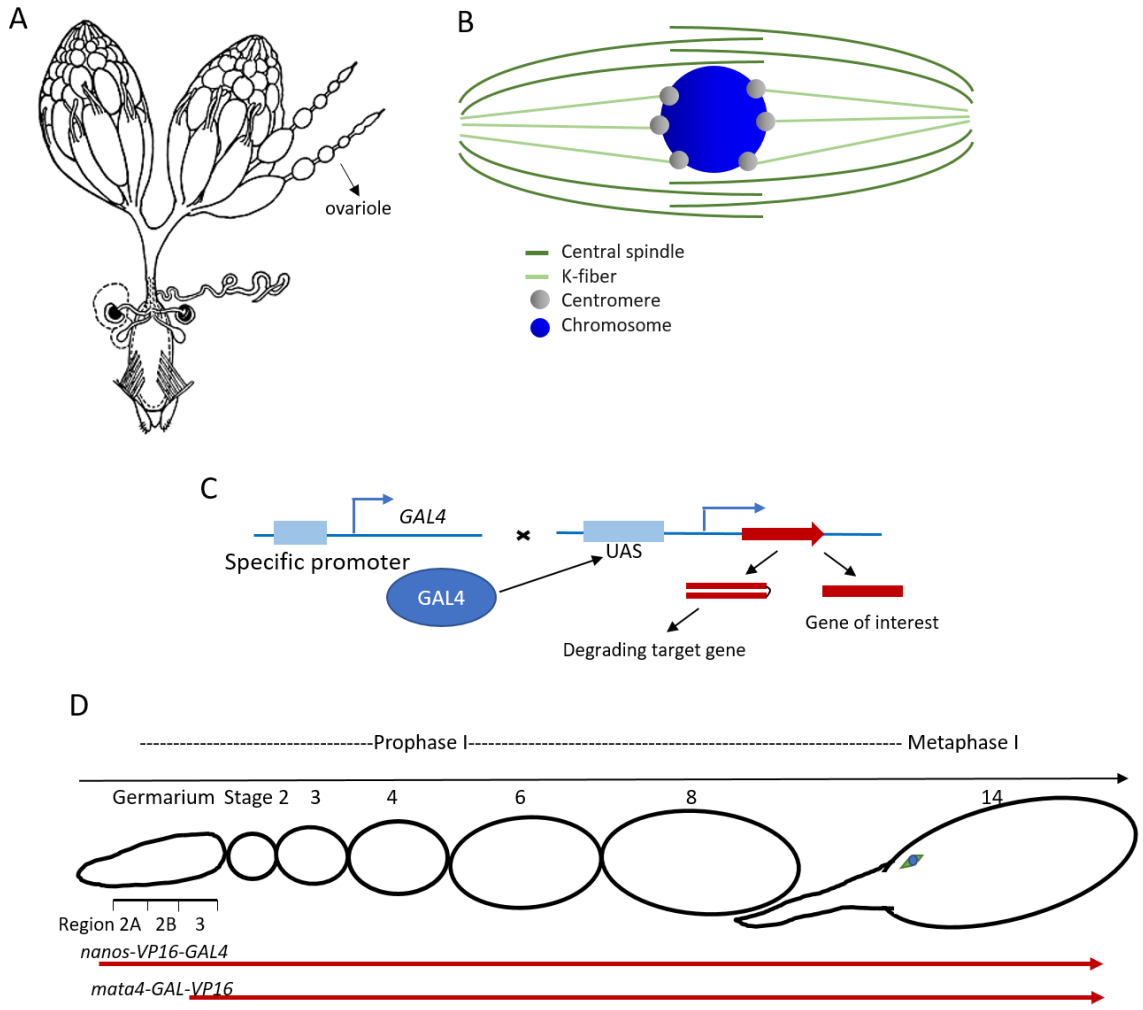

Figure 3. **Schematic of *Drosophila melanogaster* ovaries, the spindle and Gal-UAS system**

(A) Graph of *D. melanogaster* ovaries which are consist of ovarioles. Figure adapted from Miller 1950 [79]. (B) Graph of *D. melanogaster* metaphase I spindle. (C) The Gal-UAS system that we used for expressing shRNA and transgenes in *Drosophila* oocytes. (D) The graph for the two tissue-specific promoters I used in the dissertation. The graph shows the expression of these two genes.

Chapter 2

Sister centromere fusion during meiosis I depends on maintaining cohesins and destabilizing microtubule attachments

I. Preface

This chapter was published online in PLoS Genetics, May, 2019. My contributions to this paper was writing the paper and all the experiments including revisiting the results of *Pp1-87B* RNAi oocyte phenotype quantification, binuclein 2 and Taxol experiments.

II. Abstract

Sister centromere fusion is a process unique to meiosis that promotes co-orientation of the sister kinetochores, ensuring they attach to microtubules from the same pole during metaphase I. We have found that the kinetochore protein SPC105R/KNL1 and Protein Phosphatase 1 (PP1-87B) regulate sister centromere fusion in *Drosophila* oocytes. The analysis of these two proteins, however, has shown that two independent mechanisms maintain sister centromere fusion. Maintenance of sister centromere fusion by SPC105R depends on Separase, suggesting cohesin proteins must be maintained at the core centromeres. In contrast, maintenance of sister centromere fusion by PP1-87B does not depend on either Separase or Wapl. Instead, PP1-87B maintains sister centromeres fusion by regulating microtubule dynamics. We demonstrate that this regulation is through antagonizing Polo kinase and BubR1, two proteins known to promote stability of kinetochore-microtubule (KT-MT) attachments, suggesting that PP1-87B maintains sister centromere fusion by inhibiting stable KT-MT attachments. Surprisingly, C(3)G, the

transverse element of the synaptonemal complex (SC), is also required for centromere separation in *Pp1-87B* RNAi oocytes. This is evidence for a functional role of centromeric SC in the meiotic divisions, that might involve regulating microtubule dynamics. Together, we propose two mechanisms maintain co-orientation in *Drosophila* oocytes: one involves SPC105R to protect cohesins at sister centromeres and another involves PP1-87B to regulate spindle forces at end-on attachments.

III. Introduction

The necessity of sister kinetochores to co-orient toward the same pole for co-segregation at anaphase I differentiates the first meiotic division from the second division. A meiosis-specific mechanism exists that ensures sister chromatid co-segregation by rearranging sister kinetochores, aligning them next to each other and facilitating microtubule attachments to the same pole [21, 22]. We refer to this process as co-orientation, in contrast to mono-orientation, when homologous kinetochores orient to the same pole. Given the importance of co-orientation in meiosis the mechanism underlying this process is still poorly understood, maybe because many of the essential proteins are not conserved across phyla.

Most studies of co-orientation have focused on how fusion of the centromeres and kinetochores is established. In budding yeast, centromere fusion occurs independently of cohesins: Spo13 and the Polo kinase homolog Cdc5 recruit a meiosis-specific protein complex, monopolin (Csm1, Lrs4, Mam1, CK1) to the kinetochore [80-82]. Lrs4 and Csm1 form a V-shaped structure that interacts with the N-terminal domain of Dsn1 in the Mis12 complex to fuse sister kinetochores [23, 24]. While the monopolin complex is not widely conserved, cohesin-independent mechanisms may exist in other organisms. A bridge between the kinetochore proteins MIS12 and NDC80 is required for co-orientation in maize [83]. In contrast, cohesins are required for co-orientation in several organisms. The meiosis-specific cohesin Rec8 is

indispensable for sister centromere fusion in fission yeast [27] and *Arabidopsis* [25, 84]. Cohesin is localized to the core-centromere in fission yeast [85] and mice [26]. In *Drosophila melanogaster* oocytes, we and others have shown that cohesins (SMC1/SMC3/SOLO/SUNN) establish cohesion in meiotic S-phase and show an enrichment that colocalizes with centromere protein CID/CENP-A [11, 86-88]. Like fission yeast and mouse, *Drosophila* may require high concentrations of cohesins to fuse sister centromeres together for co-orientation during meiosis.

In mice, a novel kinetochore protein, Meikin, recruits Plk1 to protect Rec8 at centromeres [26]. Although poorly conserved, Meikin is proposed to be a functional homolog of Spo13 in budding yeast and Moa1 in fission yeast. They all contain Polo-box domains that recruit Polo kinase to centromeres [26]. Loss of Polo in both fission yeast (Plo1) and mice results in kinetochore separation [26, 28], suggesting a conserved role for Polo in co-orientation. In fission yeast, Moa1-Plo1 phosphorylates Spc7 (KNL1) to recruit Bub1 and Sgo1 for the protection of centromere cohesion in meiosis I [28, 89]. These results suggest the mechanism for maintaining sister centromere fusion involves kinetochore proteins recruiting proteins that protect cohesion. However, how centromere cohesion is established prior to metaphase I, and how sister centromere fusion is released during meiosis II, still needs to be investigated.

We previously found that depletion of the kinetochore protein SPC105R (KNL1) in *Drosophila* oocytes results in separated centromeres at metaphase I, suggesting a defect in sister centromere fusion [90]. Thus, *Drosophila* SPC105R and fission yeast Spc7 may have conserved functions in co-orientation [28]. We have identified a second *Drosophila* protein required for sister-centromere fusion, Protein Phosphatase 1 isoform 87B (PP1-87B). However, sister centromere separation in SPC105R and PP1-87B depleted *Drosophila* oocytes occurs by different mechanisms, the former is Separase dependent and the latter is Separase independent. Based on these results, we propose a model for the establishment, protection and release of co-orientation. Sister centromere fusion necessary for co-orientation is established through cohesins that are protected by SPC105R. Subsequently, PP1-87B maintains co-orientation in a cohesin-

independent manner by antagonizing stable kinetochore-microtubule (KT-MT) interactions. The implication is that the release of co-orientation during meiosis II is cohesin-independent and MT dependent. We also found a surprising interaction between PP1-87B and C(3)G, the transverse element of the synaptonemal complex (SC), in regulating sister centromere separation. Overall, our results suggest a new mechanism where KT-MT interactions and centromeric SC regulate sister kinetochore co-orientation during female meiosis.

IV. Results

PP1-87B is required for chromosome organization and sister centromere fusion in meiosis I

Drosophila has three homologs of the alpha type of mammalian Protein Phosphatase1 (PP1α/γ) genes, *Pp1-87B, Pp1-96A* and *Pp1-13C* [91]. We focused our studies on the *Pp1-87B* isoform because it is the only essential gene, is highly expressed during oogenesis, and contributes ~80% of PP1 activity during development [91, 92]. As *Pp1-87B* mutations are lethal, tissue-specific expression of an shRNA targeting *Pp1-87B* was used to define its role in oocytes (see Methods) [93]. The ubiquitous expression of an shRNA for PP1-87B using *tubP-GAL4-LL7* resulted in lethality, suggesting the protein had been depleted. When PP1-87B was depleted in oocytes using *mata4-GAL-VP16* (to be referred to as *Pp1-87B* RNAi oocytes), we observed two phenotypes. The first was disorganization of the metaphase I chromosomes. In wild-type *Drosophila* oocytes, meiosis arrests at metaphase I with the chromosomes clustered into a single chromatin mass at the center of the spindle **(**Figure 4A). In 62% of *Pp1-87B* RNAi oocytes, the single chromosome mass was separated into multiple groups of chromosomes (Figure 4A, B). The second phenotype observed in *Pp1-87B* RNAi oocytes was precocious separation of sister centromeres, as determined by counting the number of centromere protein CENP-C or CID (CENP-A) foci (see Methods) [94]. In wild-type oocytes, we observed an average of 7.3

centromere foci, consistent with the eight expected from four bivalent chromosomes at metaphase I (Figure 4A, C). However, in *Pp1-87B* RNAi oocytes we observed a significantly higher number of foci (mean=12.7). This suggests a defect in sister centromere fusion results in their premature separation during metaphase I.

To determine whether the separated chromosome mass and centromere separation phenotypes in *Pp1-87B* RNAi oocytes is caused by a general loss of cohesion, we used heterochromatic FISH probes directed to the pericentromeric regions of each autosome to mark the homologs. In wild type, two FISH foci are typically observed per homologous chromosome pair in metaphase I because of cohesion between sister chromatids (Figure 4D). To determine if pericentromeric cohesion in *Pp1-87B* RNAi oocytes was affected, we analyzed the number of heterochromatin FISH signals from the dodeca satellite, the most punctate and therefore quantifiable heterochromatic FISH probes. In *ord* mutant oocytes that lack all cohesion, the oocytes had a significantly higher number of dodeca foci (mean = 4.8) compared to wild type (mean = 2.7, Figure 4E). In contrast, the average number of dodeca foci in *Pp1-87B* RNAi oocytes was not significantly higher than wild type (Figure 4E; mean = 3.0), suggesting that pericentromeric cohesion is intact in *Pp1-87B* RNAi oocytes. Secondly, we used these FISH probes to test if there were loss of arm cohesion, defined as when the homologs separate and are observed as two FISH foci in separate chromosome masses. We observed that while 62% of *Pp1-87B* RNAi oocytes (n = 50) had a separated chromosome mass, only 8.5% of the homologs had separated (n =130). These results suggest that arm cohesion is usually retained when PP1-87B is depleted. Hence, the separated chromosome mass phenotype in *Pp1-87B* RNAi oocytes is due to intact bivalents failing to organize correctly at the center of the spindle.

Based on these FISH results, PP1-87B is only required for maintaining sister centromere cohesion but is dispensable for cohesion of the pericentromeric regions and the chromosome arms in oocytes. To refer to this specific type of cohesion, we will use the term sister centromere

fusion. We also observed two defects associated with the defect in sister centromere fusion and a lack of co-orientation in *Pp1-87B* RNAi oocytes. First, the FISH experiments can detect errors in homologs bi-orientation, defined as when pairs of homologous centromeres are separated towards opposite poles (Figure 4D). In *Pp1-87B* RNAi oocytes, 5.3% of the homologs were mono-oriented, defined as when pairs of homologous centromeres are have moved towards the same pole (n=130 vs. n_{wt}=111, p=0.016). These results support the conclusion that the sister centromere fusion defect in *Pp1-87B* RNAi oocytes causes problem for homologous chromosomes to bi-orient.

Second, when the sister centromeres that precociously separate dring meiosis I in mouse and yeast, chiasmata can still direct bi-orientation of these homologs, suppressing the consequences of co-orientation defects [26, 27, 29]. Therefore, we used a crossover defective mutant, *mei*-P22 [95], to generate univalents, and knocked down *Pp1-87B* in these oocytes. If the precocious sister centromere separation causes a co-orientation defect, we would expect the univalents in *mei-P22, Pp1-87B* RNAi oocytes can become bi-oriented. Indeed, we observed that 20% of *mei-P22, Pp1-87B* RNAi oocytes had sister chromatids bi-oriented (n = 15, Figure 4F). These results suggest that PP1-87B is required for sister centromere fusion to facilitate co-orientation in metaphase I of oocytes.

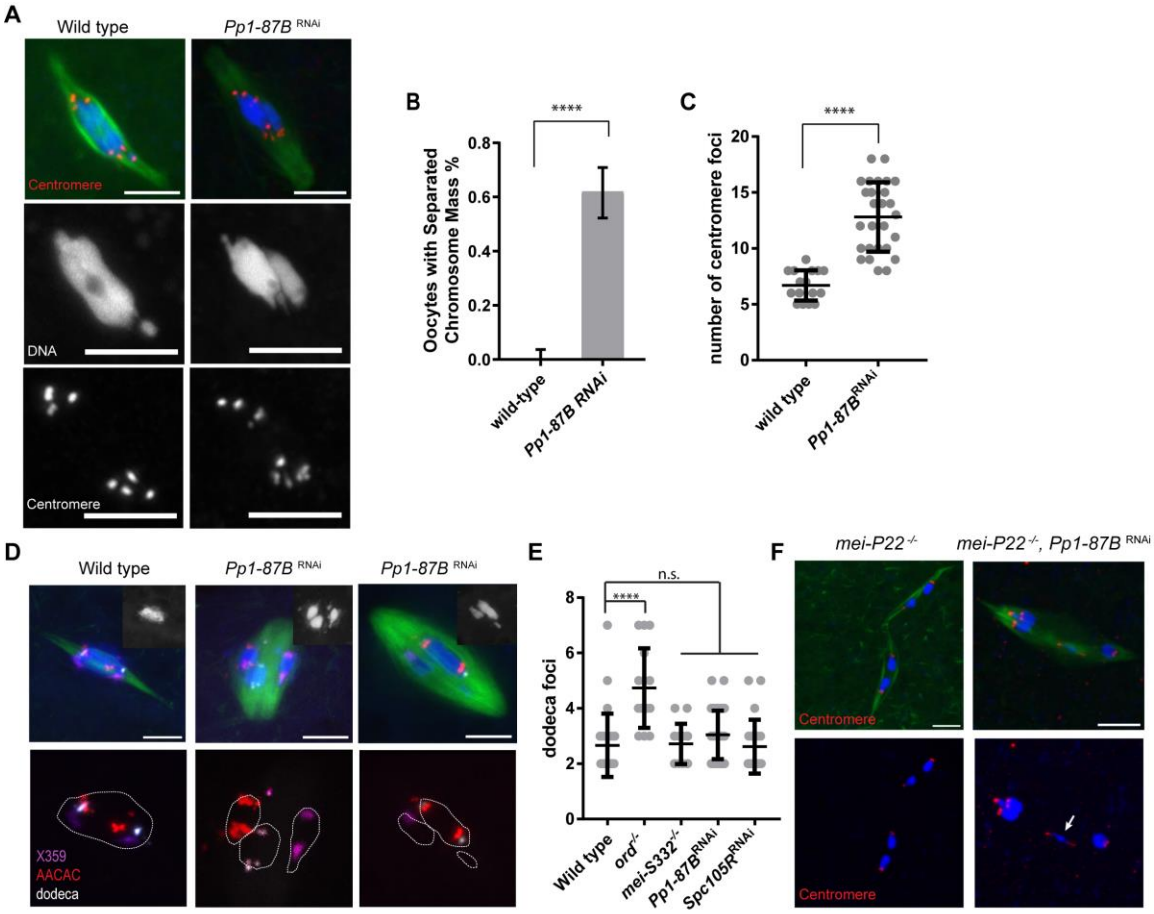

Figure 4. *Pp1-87B* RNAi oocytes have defects in chromosome mass organization and sister centromere fusion.

(A) *Pp1-87B* RNAi oocytes show separated chromosome mass and sister centromere (red) separation in metaphase I with tubulin in green and DNA in blue. DNA and centromeres are shown in separate channels. In wild-type, the fourth chromosomes sometimes appear as a dot separated from the chromosome mass. Scale bars indicate 5 µm. (B) Quantification of the separated chromosome mass phenotype in wild-type (n=20) and *Pp1-87B* RNAi oocytes (n=50). ****= $p < 0.0001$. Error bars indicate 95% confidence interval. (C) Quantification of centromere foci. Error bar shows standard deviation. Number of oocytes: wild type n=16 and *Pp1-87B* RNAi n=27. ****= $p < 0.0001$, (D) Chromosome mass separation defect in *Pp1-87B* RNAi oocytes. DNA channel is shown in the inset. FISH probes for the X (359 bp repeat, purple), 2nd (AACAC, red) and 3rd chromosome (dodeca, white) were used to detect pericentromeric heterochromatin. The chromosome mass is outlined in white. Two examples are shown of *Pp1-87B* RNAi oocytes with homologs bi-oriented and not bi-oriented. Scale bars are 5 µm. (E) Quantification of dodeca foci to detect precocious separation of pericentromeric heterochromatin. Number of oocytes: wild-type (n=27), *ord* (n=15), *mei-S332* (n=14), *Pp1-87B* RNAi (n=50) and *Spc105R* RNAi (n=21). ****= $p < 0.0001$. (F) Recombination defective mutant *mei-P22* displayed homologous chromosome separation indicting precocious anaphase I in oocytes normally arrested in metaphase I. Knocking down *Pp1-87B* in a *mei-P22* mutant background resulted in sister chromatid bi-orientation in meiosis I (arrow). The bioriented univalent can be identified because

it has not segregated to a pole like the other univalents. In addition, it is the only chromosome where the centromeres are oriented towards opposite poles. And given that there are no chiasmata, the only linkage between these two centromeres can be pericentromeric cohesion of sister chromatids.

Co-orientation in *Drosophila* oocytes requires both cohesin-dependent and cohesin-independent pathways

Both cohesin-dependent and -independent mechanisms of sister centromere fusion have been described. Therefore, we investigated whether loss of sister centromere fusion depends on cohesin release. In addition to PP1-87B, the kinetochore protein SPC105R was also tested because it is the only other protein known to be required for sister centromere fusion in *Drosophila* oocytes [96]. To investigate if cohesin is involved in sister centromere fusion, we tested if sister centromere separation in *Pp1-87B*- and *Spc105R*- RNAi oocytes depends on known cohesin removal mechanisms by depleting two negative-regulators of cohesin, Wings Apart-like (*wapl*) and Separase (*sse*). If losing a factor required for cohesin removal rescued the sister centromere separation in *Pp1-87B* or *Spc105R* RNAi oocytes, it would suggest the *Drosophila* sister centromere fusion depends on cohesin.

Upon co-expression of *wapl* shRNA with either *Pp1-87B* or *Spc105R* shRNA, the centromeres remained separated (Figure 5A, B). While WAPL could be required for cohesion release at anaphase I, these results suggest WAPL is not required for the meiosis I sister centromere separation caused by depletion of PP1-87B or SPC105R. However, the centromeres in *wapl, Pp1-87B* RNAi oocytes became thread-like instead of punctate (Figure 5A), leading to additional centromere foci when quantified (Figure 5B). The thread-like centromere phenotype suggests that chromosome structure is affected in *wapl, Pp1-87B* RNAi oocytes, consistent with previous studies that concluded WAPL was involved in regulating chromosome structure [97, 98].

The separated centromere phenotype was rescued in *sse, Spc105R* RNAi oocytes (Figure 5A, C; mean = 8.1), suggesting that centromere separation in *Spc105R* RNAi oocytes depends on the loss of cohesins. This is a surprising result because it suggests that Separase is active during meiotic metaphase I [99]. If Separase is active, these results could be explained if SPC105R recruits proteins that protect cohesins from Separase. To test the hypothesis that SPC105R protects cohesins from Separase, we examined the localization of MEI-S332/SGO, which is required to maintain cohesion during meiosis in several organisms [100]. *Drosophila* orthologue MEI-S332 localizes to centromere and peri-centromeric regions in wild-type meiosis I oocytes, as shown by colocalization and substantial non-overlap distribution with the core centromere (Figure 6). While present during meiosis I and useful as a marker for cohesion protection, MEI-S332 only shows defects during meiosis II [36, 101], possibly due to redundancy with another factor during meiosis I [102, 103]. Consistent with our hypothesis, MEI-S332 localization was almost abolished in *Spc105R* RNAi oocytes (Figure 7A, B). While we cannot rule out non-cohesive functions for Separase, the most likely interpretation is that SPC105R is required to recruit proteins that protect cohesins from Separase.

On the other hand, different from the result of *sse, Spc105R* RNAi, the separated centromere phenotype was not rescued in *sse, Pp1-87B* RNAi oocytes (Figure 5A, C; mean= 13.4). Consistent with cohesin-independence of these phenotypes, the localization of MEI-S332 in *Pp1-87B* RNAi oocytes was not reduced, and in fact, the volume was increased relative to wild-type (Figure 7A,B). Aurora B is required for MEI-S332 localization [104], and although the mechanism is not well understood in *Drosophila,* our results suggest MEI-S332 localization is promoted by Aurora B but constrained by PP1-87B. These results indicate that sister centromere fusion in *Drosophila* oocytes is regulated through two different mechanisms: the SPC105R pathway that is sensitive to Separase, and the PP1-87B pathway that is Separase independent.

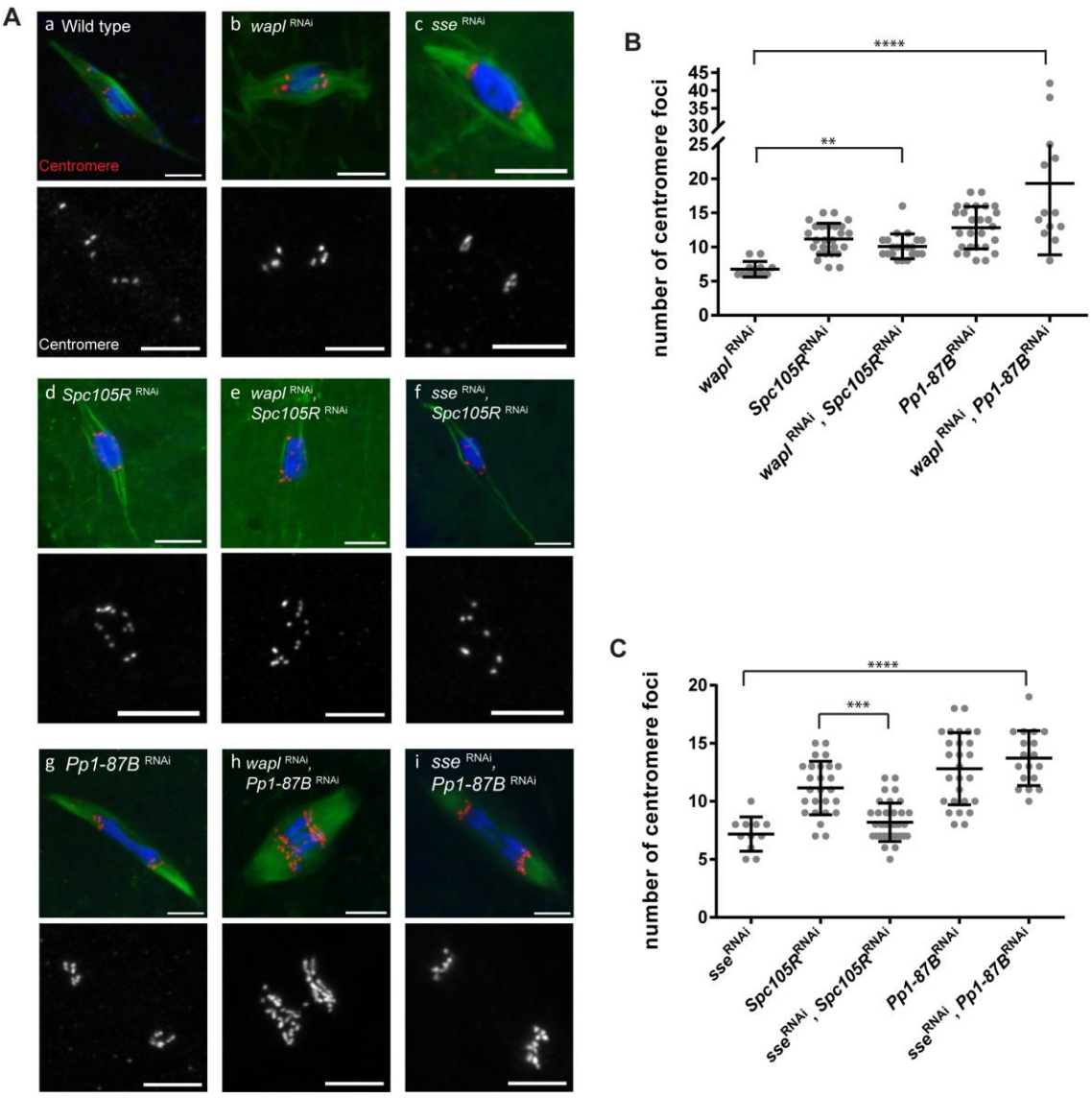

Figure 5. Sister centromere fusion defect rescued by loss of Separase in *Spc105R* RNAi but not *Pp1-87B* RNAi oocytes.

(A) Confocal images showing the centromeres (red) in wild-type, *sse* RNAi, and *wapl* RNAi in combination with *Pp1-87B* RNAi or *Spc105R* RNAi. Centromeres are shown in separate channel. Scale bars are 5 µm. (B, C) Dot plots summarize the quantification of centromere foci number in (A). Error bars indicate standard deviation, **= p <0.01, ***= p <0.001 and ****= p <0.0001. Number of oocytes are *wapl* RNAi (12), *Spc105R* RNAi (26), *wapl* RNAi + *Spc105R* RNAi (21), *Pp1-87B* RNAi (27), *wapl* RNAi + *Pp1-87B* RNAi (13), *sse* RNAi (11), *sse* RNAi + *Spc105R* RNAi (36), *sse* RNAi + *Pp1-87B* RNAi (18).

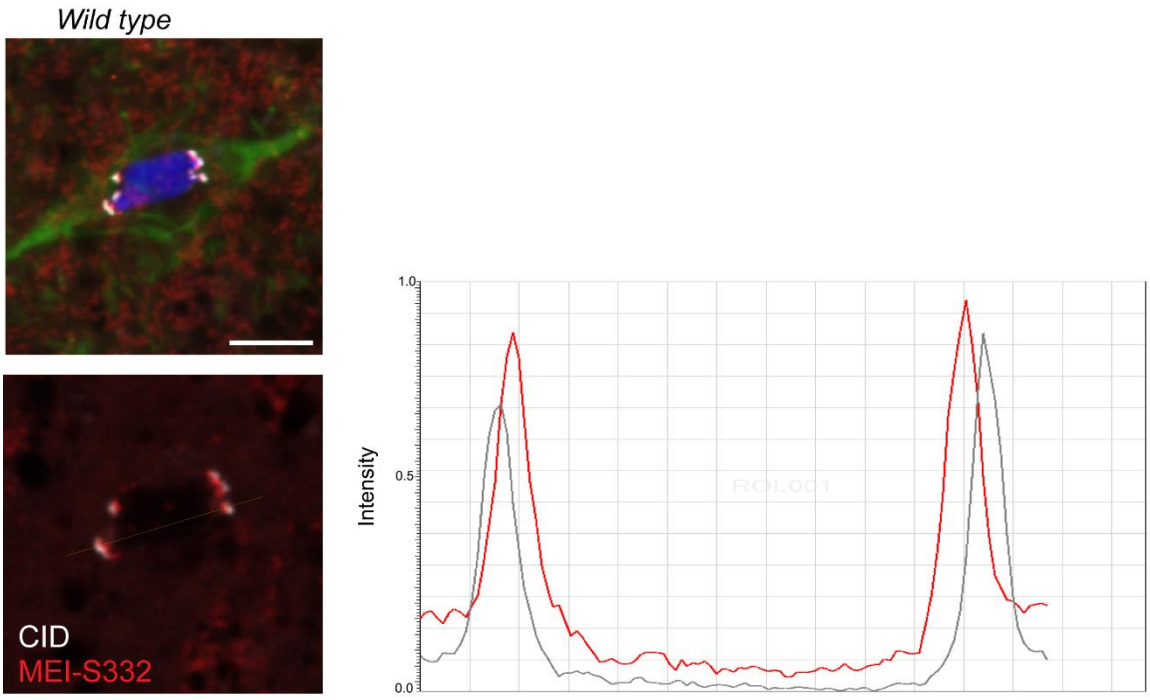

Figure 6: **MEI-S332 localization does not co-localize with centromere**.

Representitive picture of wild type oocytes staining MEI-S332 (red) and CID (white) is shown and measured the intensity of flourensent. MEI-S332 localizes to both the pericentromeric and centromeric regions. Scale bar is 5 µm.

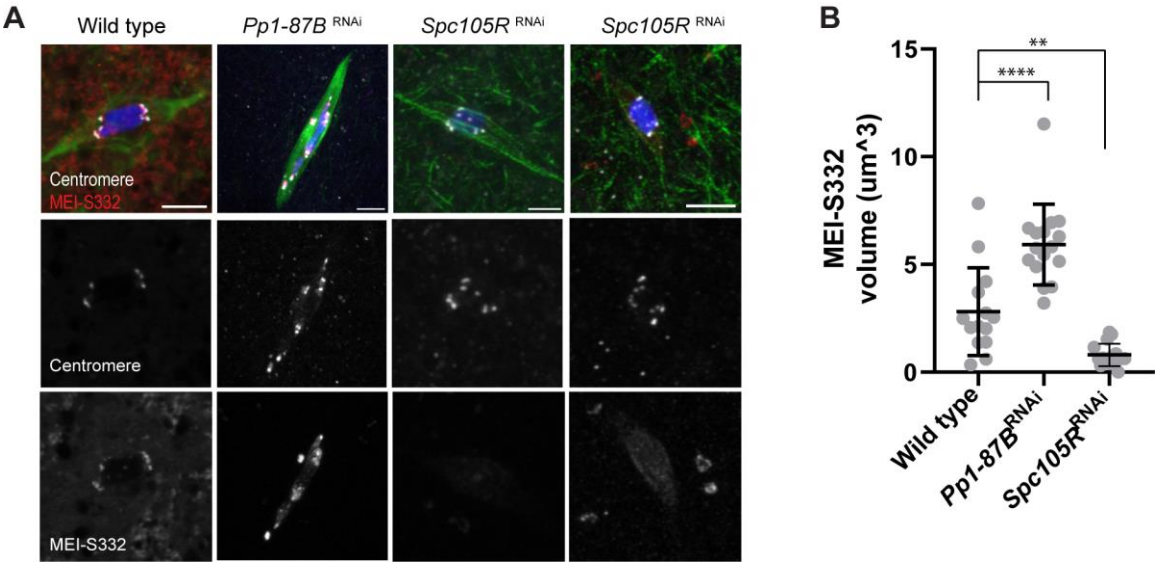

Figure 7: MEI-S332 localizes to centromeres and heterochromatin.

(A) MEI-S332 has enhanced recruitment to the pericentromeric regions in *Pp1-87B* RNAi oocytes and is decreased in *Spc105R* RNAi oocytes. Two images of *Spc105R* RNAi oocytes show MEI-S332 localization either abolished or greatly reduced. Confocal images are shown with centromeres (white), MEI-S332 (red), tubulin (green) and DNA (blue). Scale bar indicates as 5 µm. (B) Quantification of MEI-S332 volume. The number of oocytes for measuring are wild type (14), *Pp1-87B* RNAi oocytes (16) and *Spc105R* RNAi oocytes (16). Error bars indicate standard deviation, **= p <0.01 and ****= p <0.0001.

Separase-independent loss of sister centromere fusion depends on microtubule dynamics

Because the *Pp1-87B* RNAi phenotype was not suppressed by loss of Separase, we investigated cohesin-independent mechanisms for how PP1-87B regulates sister centromere fusion. A critical initial observation was that the spindle volume of *Pp1-87B* RNAi oocytes was larger than wild type (Figure 8A). In addition, PP1-87B was found to localize to the oocyte meiotic spindle (Figure 9). Based on these observations, we tested the hypothesis that PP1-87B regulates microtubules dynamics by co-depleting proteins known to regulate MT dynamics and KT attachments.

Aurora B kinase activity is required for spindle assembly in *Drosophila* oocytes [62] and can be antagonized by PP1 in other systems [105]. Furthermore, they have opposite phenotypes: both the chromosome mass and sister centromeres precociously separate in *Pp1-87B* RNAi oocytes but remain together in Aurora B-depleted oocytes [62]. Therefore, we tested whether Aurora B is required for both the chromosome mass and centromere separation phenotypes of *Pp1-87B* RNAi oocytes. Treatment of metaphase I oocytes (i.e. those that have assembled a spindle) with the Aurora B inhibitor, Binucleine 2 (BN2) [106], caused loss of the spindle (65%, n=29, Figure 8B and C), consistent with previous findings that Aurora B is required for spindle assembly [62]. Interestingly, *Pp1-87B* RNAi oocytes showed partial resistance to BN2 treatment; only 9% had lost the spindle and 50% of oocytes had residual MT around the chromosome mass (n= 22, Figure 8B and C). Regardless of these residual MTs, the increased number of centromere foci in *Pp1-87B* RNAi oocytes (mean = 13.0) was rescued by BN2 treatment to a level (Figure 8D and E, mean = 7.4) similar to wild-type controls (Figure 8D and E, mean = 7.7). Similarly, the increased frequency of chromosome mass separation in *Pp1-87B* RNAi oocytes was rescued by BN2 treatment (Figure 8F and G). In contrast, centromere separation was not rescued by BN2 treatment of *Spc105R* RNAi oocytes (Figure 8B and C, mean = 11.3). These results are concordant with the effects of *sse* RNAi on the *Spc105R* and *Pp1-87B* RNAi phenotypes and support the conclusion that the maintenance of centromere fusion may occur by at least 2 mechanisms.

Suppression of *Pp1-87B* RNAi oocyte phenotypes by BN2 treatment could have been due to loss of Aurora B activity, or loss of the spindle microtubules. To distinguish between these two possibilities, we treated *Pp1-87B* RNAi oocytes with Paclitaxel to stabilize the spindle prior to BN2 treatment of the oocytes. Although these oocytes successfully formed spindles, 18% showed chromosome mass separation, a significant decrease compared to the Paclitaxel and solvent-treated RNAi control oocytes and similar to the results from BN2 treatment of *Pp1-87B* RNAi oocytes (Figure 8F and G). This rescue of chromosome mass separation demonstrates that PP1-

87B antagonizes Aurora B in regulating chromosome organization. On the contrary, the sister centromeres remained separated in these oocytes (Figure 8D and E, mean = 11.1), suggesting that stabilizing microtubule dynamics in *Pp1-87B* RNAi oocytes can override any effect of inhibiting Aurora B on sister centromere fusion. Based on these observations, we propose that PP1-87B regulates sister centromere separation by regulating microtubules dynamics. However, we cannot rule out the possibility that Aurora B is also required for centromere separation independently of the microtubules.

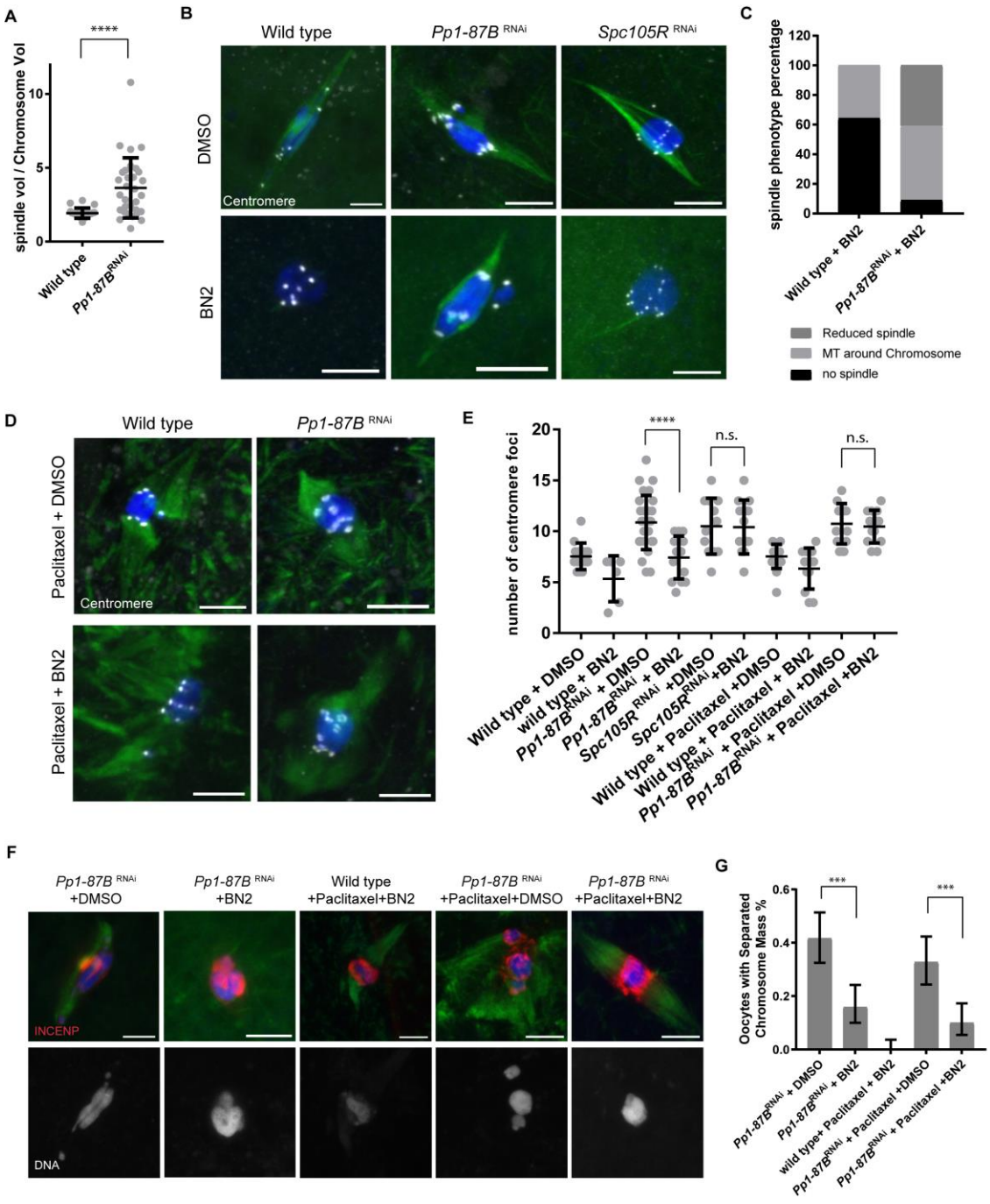

Figure 8: PP1-87B regulation of sister centromere fusion depends on microtubules.

(A) Graph showing the spindle volume relative to the chromosome mass volume. The chromosome mass volumes remain constant while *Pp1-87B* RNAi oocytes (n=31) had increased spindle volume compared to wild type (22) oocytes. ****= p <0.0001 (B) Wild-type, *Pp1-87B* RNAi and *Spc105R* RNAi oocytes treated with 50uM BN2 or the solvent for one hour. All images are shown with DNA (blue), tubulin (green) and centromeres (white), and the scale bars are 5 µm. (C) Quantification of spindle phenotype in wild-type (n=28) and *Pp1-87B* RNAi (n=22) oocytes after one hour of BN2 treatment. (D) Wild type and *Pp1-87B* RNAi oocytes treated with Paclitaxel for 10 minutes followed by either BN2 or DMSO for one hour.

Centromeres are marked in white. Scale bars are 5 µm. (E) Quantification of centromere foci in indicated genotypes of oocytes (n= 15, 6, 29, 14, 12, 12, 17, 12, 15 and 13 in the order of the graph). Error bars show standard deviation and ****=p<0.0001. (F) Chromosome mass organization in *Pp1-87B* RNAi oocytes treated for 10 minutes in Paclitaxel followed by BN2 or DMSO for 30 minutes. INCENP localization is shown in red, DNA in blue and tubulin in green. The single channel of DNA is also shown. Scale bar =5 µm. (G) Quantification from the same experiment in (F). Error bars indicate 95% confidence intervals and ***=p<0.001. The numbers of oocytes were 143,151, 21, 27, 111 in order of the graph.

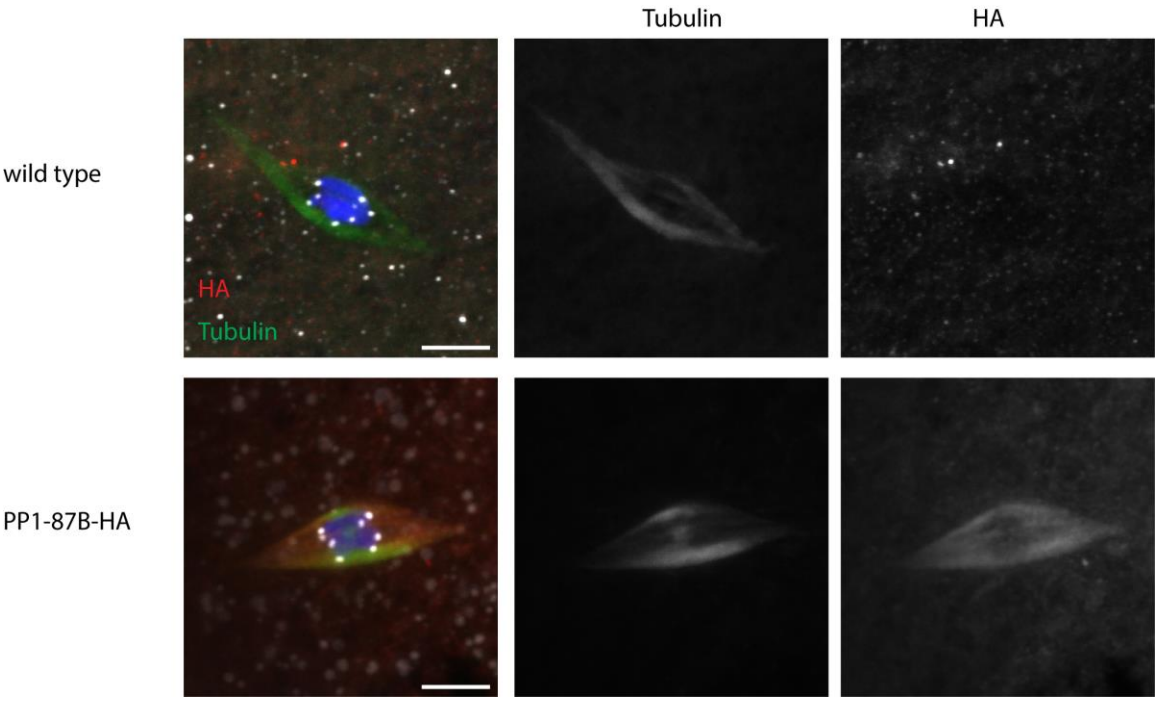

Figure 9: Localization of PP1-87B to meiotic spindle.

An epitope-tagged version of PP1-87B was expressed from a UASP transgene using *mata4-GAL-VP16.* HA-PP1-87B is in red, tubulin in green and DNA in blue and scale bars are 5 µm.

Kinetochore-microtubule interactions regulate chromosome mass organization and sister centromere fusion

The meiotic spindle consists of overlapping microtubules, only a portion of which make contact with the kinetochores. To understand which set of microtubules affect PP1-dependent centromere separation and chromosome mass disorganization, we used knockdowns of kinetochore proteins to specifically abrogate one class of microtubule contracts with the chromosomes. In *Drosophila* oocytes, SPC105R is required for lateral attachments and the localization of NDC80 whereas NDC80 is required for end-on attachments [90]. Thus, we co-depleted PP1-87B and SPC105R (no MT attachments) or NDC80 (lateral MT attachments only) and examined the chromosomes and centromeres. We found that loss of SPC105R, but not NDC80, suppressed the separated chromosome mass phenotype of *Pp1-87B* RNAi oocytes (Figure 10A and C), suggesting that the separated chromosome mass phenotype in *Pp1-87B* RNAi oocytes depends on lateral KT-MT interactions. The sister centromeres are already separated in *Spc105R* RNAi oocytes, and co-depletion of both *Pp1-87B* and *Spc105R* did not enhance the effects of either single knockdowns (Figure 10A, B). In contrast, the centromere separation phenotype was rescued in *Ndc80, Pp1-87B* double RNAi oocytes (mean = 9.0, Figure 10A, B) but not chromosome mass disorganization. We conclude that PP1-87B affects chromosome mass organization through regulating lateral KT-MT attachments and sister centromere fusion through end-on attachments.

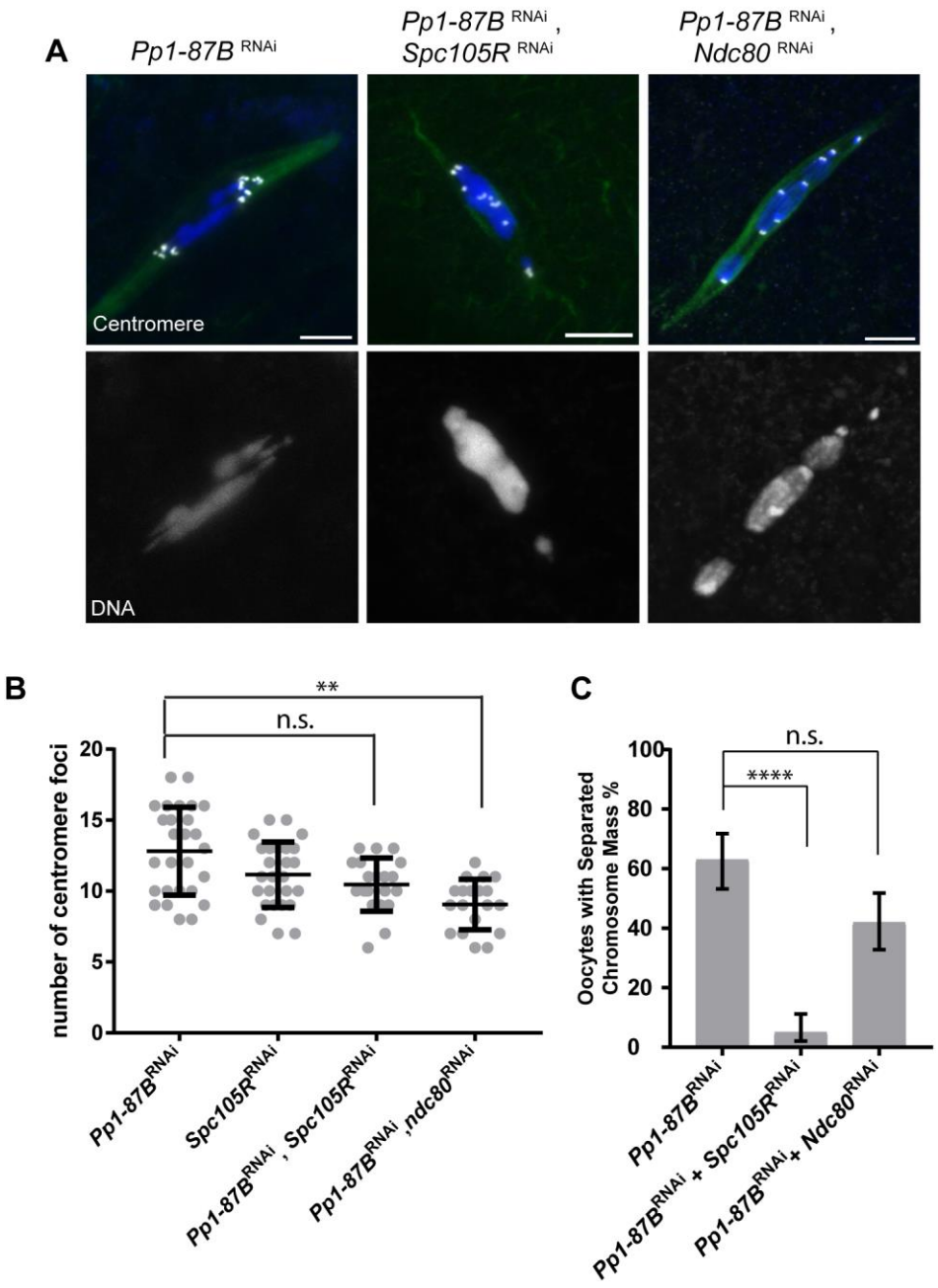

Figure 10. PP1-87B regulates chromosome alignment through lateral attachment and co-orientation via end-on attachment

(A) Confocal images of *Pp1-87B* RNAi oocytes when expressing *Spc105R* RNAi or *Ndc80* RNAi. Centromeres are in white, DNA is in blue and tubulin is in green. Single channel image is selected to show DNA in the merge images. Error bars = 5µm. (B) Dot plot shows the number of centromere foci in each genotype. Oocytes numbers are 27, 26, 20 and 19 in order of the graph. Error bars show standard deviation. **=p<0.01. (C) Quantification of oocytes with a separated chromosome mass. Error bars indicate 95% confidence interval. Numbers of oocytes are 29, 20, and 19 in order of the graph. ****=p<0.0001.

PP1-87B antagonizes Polo and BubR1 in regulating sister centromere fusion

To identify proteins that function with PP1-87B in regulating end-on KT-MT attachments, we depleted proteins with meiotic functions that are involved in regulating microtubule attachments. Polo kinase localizes to centromeres in *Drosophila* metaphase I oocytes [107] (Figure 11), and in other organisms has been reported to stabilize KT-MT attachments [108-111]. Unlike Polo in mice [26], *Drosophila polo* RNAi oocytes do not show precocious sister centromere separation at metaphase I [71]. We depleted *polo* with RNAi in either *Spc105R* or *Pp1-87B* RNAi oocytes. Interestingly, we found that centromere separation in both mutant oocytes were rescued by *polo* RNAi (Figure 12A and B, mean = 6.6 and mean = 6.9). These results indicate that Polo negatively regulates both the separase-dependent (through SPC105R) and the microtubule attachment dependent pathways (through PP1-87B) for sister centromere fusion in *Drosophila*.

Two proteins, BubR1 and MPS1, function along with Polo to regulate KT-MT attachments in several organisms [108, 109, 112, 113]. We predicted that depletion of either one could have a similar effect on the *Pp1-87B* oocyte phenotype as *pol*o RNAi. Centromere separation in *Pp1-87B* RNAi oocytes was suppressed by simultaneous knockdown of *BubR1* (Figure 12A, C; mean = 8.5) but not *mps1* (Figure 12A, C; mean = 10.7). A caveat to this negative result is that, based on the non-disjunction rate, MPS1 is only partially depleted in these females (NDJ = 11%, n = 961, compared to a strong *mps1* loss of function mutant, NDJ = 20.2%, n = 231 [114]). Regardless, these results suggest that PP1-87B promotes sister centromere fusion by antagonizing the activities of Polo and BubR1. In contrast, the frequency of oocytes with a separated chromosome mass phenotype remained similar to *Pp1-87B* RNAi oocytes when PP1-87B were co-depleted with BubR1 (Figure 12D), consistent with the results with NDC80. This

result confirms that the separated chromosome mass phenotype in *Pp1-87B* RNAi oocytes depends on lateral KT-MT interactions.

We propose that PP1-87B destabilizes end-on microtubule attachments by antagonizing Polo and BubR1 activities. In support of this conclusion, the increased spindle volume observed in of *Pp1-87B* RNAi oocytes was suppressed by co-depletion of *polo* or *BubR1* (Figure 12E). In summary, several experiments, including drug treatment (Paclitaxel+BN2), depletion of genes that affect KT-MT attachments, and measurements of spindle volume, support the conclusion that PP1-87B regulates KT-MT attachments, and these activities then affect sister-centromere separation and chromosome mass organization.

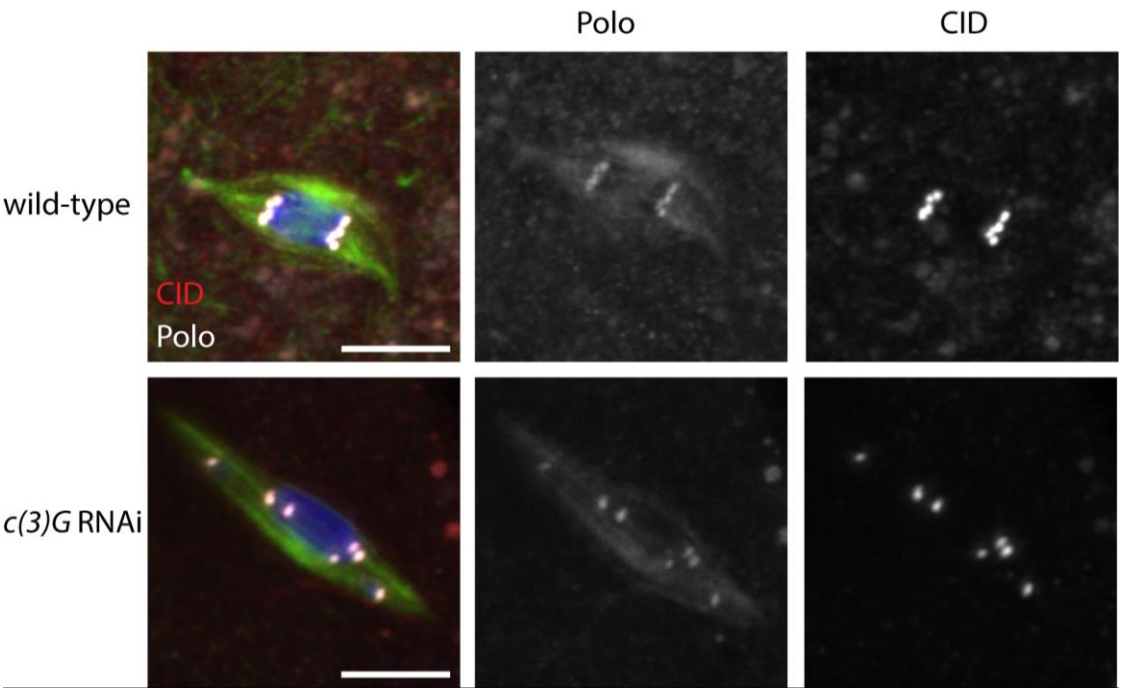

Figure 11: Polo localization does not change in *c(3)G* RNAi oocytes but decreases in *Spc105R* RNAi oocytes.

Wild-type, *c(3)G* RNAi, and *Spc105R* RNAi oocytes with DNA in blue, tubulin in green, Polo in red and CID in white. Single channels are shown in white. All images are maximum projections and scale bars are 5 µm.

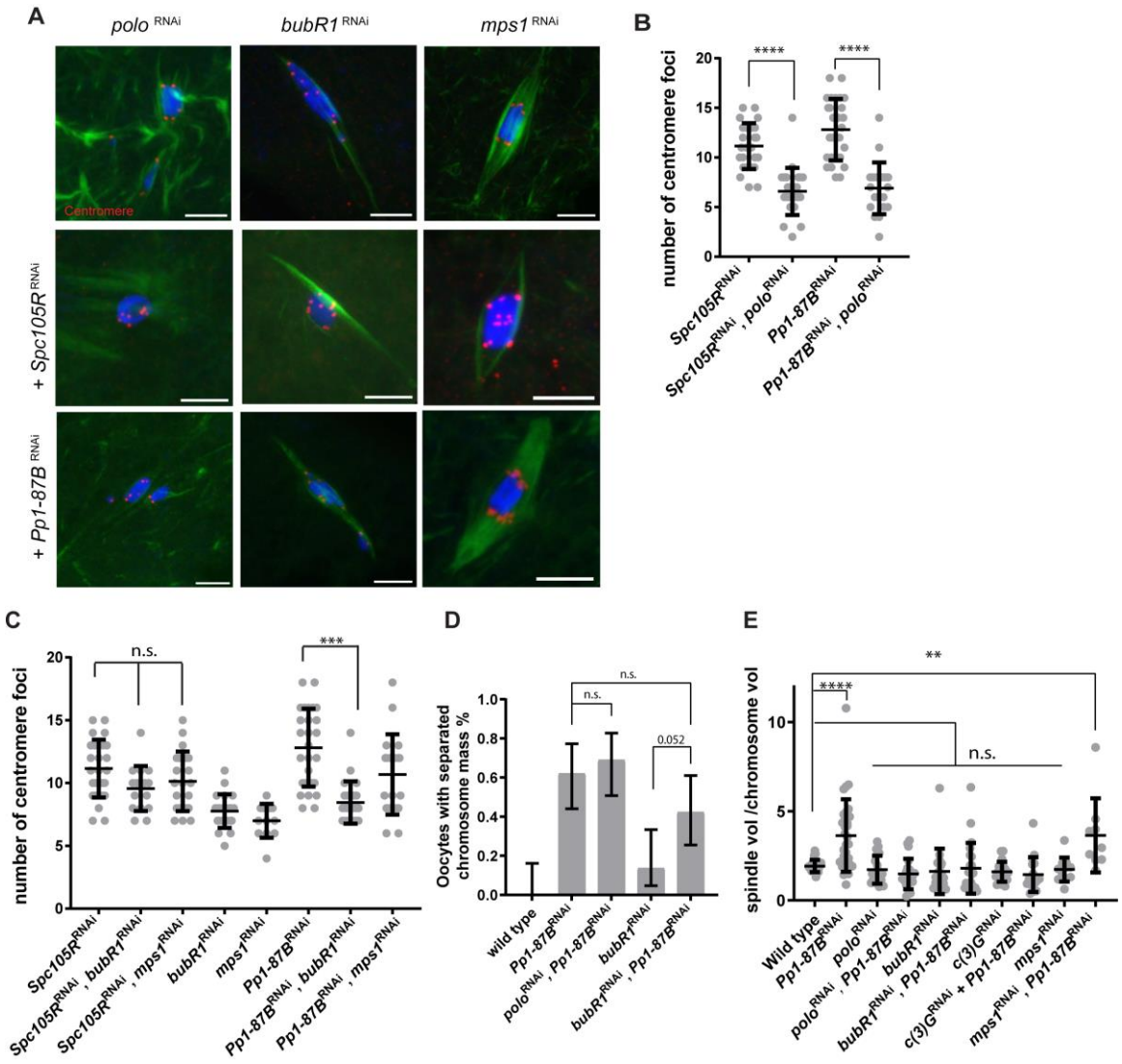

Figure 12: Polo and BubR1 antagonize PP1-87B effects on KT-MT interactions

(A) Confocal images showing *polo, BubR1* or *mps1* RNAi expressed along with *Spc105R* RNAi or *Pp1-87B* RNAi in oocytes. DNA is in blue, tubulin is in green and centromeres are in red. Scale bars = 5µm. (B) Dot plot showing the number of centromeres foci in (A). Oocytes numbers are 26, 24, 27, and 20 in order of the graph. Error bars show standard deviation and ****=p<0.0001. (C) Dot plot showing the number of centromere foci in (A). Oocytes numbers are 26, 16, 21, 22, 12, 27, 22 and 19 in order of the graph. Error bars show standard deviation and ***=p<0.001. (D) Graph showing the percentage of a separated chromosome mass in oocytes depleted for a variety of kinases in the presence or absence of *Pp1-87B* RNAi. Error bars indicate standard deviation. Numbers of oocytes of each genotype are 20, 31, 21, 19, 22 in order of the graph. (E) Dot plot showing the spindle volume relative to the chromosome mass volume. Number of oocytes are: 22, 31, 20, 21, 19, 22, 12, 9 in order of the graph. Error bars show standard deviation **= p<0.01 and ****=p<0.0001.

The transverse element of the synaptonemal complex, C(3)G, is required for release of sister centromere fusion

As described above, simultaneous loss of co-orientation and chiasmata can result in bi-orientation of univalent at meiosis I. We observed this phenomenon with simultaneous depletion of *PP1-87B* and *mei-P22*. The same experiment was done with *c(3)G*, which encodes a transverse element of the synaptonemal complex (SC) [115], because it is also required for crossing over [116]. Compared to *mei-P22*, however, we got surprisingly different results. First, *c(3)G* mutant females that were depleted of *Pp1-87B* failed to produce mature oocytes. We currently do not know why loss of *c(3)G* and prophase depletion of PP1-87B causes a failure in oocyte development, but it suggests C(3)G has a function in mid-oogenesis after its role in crossing over.

To examine the interaction between C(3)G and PP1-87B, *c(3)G* RNAi was used. To test the efficiency of the *c(3)G* RNAi, *nanos-VP16-GAL4* was used to express the shRNA during early prophase, the frequency of X chromosome non-disjunction (NDJ) was similar to that observed in *c(3)G* null alleles (31%, n = 1647) [116]. In addition, C(3)G localization was absent in the germarium (Figure 13). These results suggest that this shRNA knockdown recapitulates the null mutant phenotype. For the double depletion we used *mata4-VP16-GAL* that induced shRNA expression later in oogenesis than *nanos-VP16-GAL4*. This was necessary because early expression of *Pp1-87B* shRNA results in a failure to produce oocytes. When using *mata4-VP16-GAL* to express shRNA, C(3)G was present in pachytene, crossing over was not affected (NDJ= 0%, n = 427), but C(3)G was missing from mid-late prophase (Figure 13, Figure 14A). These results indicate C(3)G is dynamic throughout prophase, and allows us to test if there is a late prophase-metaphase interaction between C(3)G and PP1-87B. Interestingly, RNAi of *c(3)G* rescued the sister centromere separation phenotype in *Pp1-87B*, but not *Spc105R* RNAi oocytes (Figure 14A and B). These results suggest that PP1-87B antagonizes centromeric C(3)G, after most of the SC has been disassembled, to maintain sister centromere fusion at metaphase I. As

with other proteins that regulate end-on attachments, the *Pp1-87B* RNAi increased spindle volume phenotype was rescued to wild type levels by co-depletion of *c(3)G* (Figure 14C).

It is noteworthy that C(3)G is enriched at the centromere regions [13, 117] in pachytene, although its function there is not known. In this location, and because C(3)G has a Polo-binding box, it is possible that C(3)G recruits Polo to the centromere region to regulate microtubule dynamics. However, when examining the localization of Polo in *c(3)G* RNAi oocytes, we did not observe any changes in protein localization compared to wild-type (Figure 11). Whether C(3)G plays a role in regulating microtubule dynamics through Polo or other independent function to regulate sister centromere fusion needs further investigation.

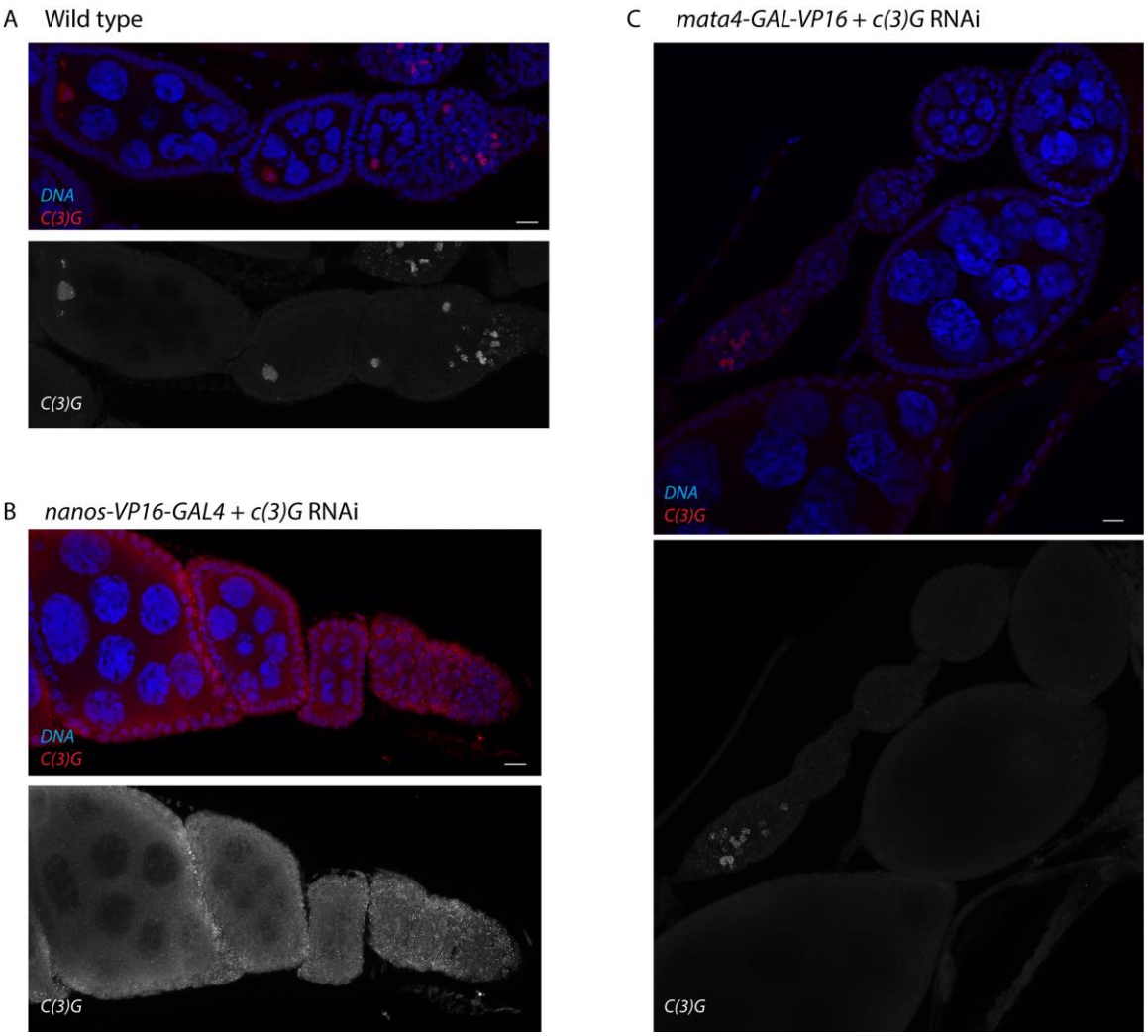

Figure 13: C(3)G is knockdown by shRNA expressed in the germline.

(A) C(3)G (red) forms thread-like structure in the germarium (early prophase), and retains them in oocytes of stages 2-5 of the vitellarium (late prophase). (B) When *nanos-VP16-GAL4* expressed *c(3)G* shRNA in early prophase, C(3)G expression was abolished. (C) When *mata4-GAL-VP16* expressed *c(3)G* shRNA in late prophase, C(3)G localization was present in germarium early pachytene, but absent in the stages 2-5 of the vitellarium. Scale bars are 10 μm.

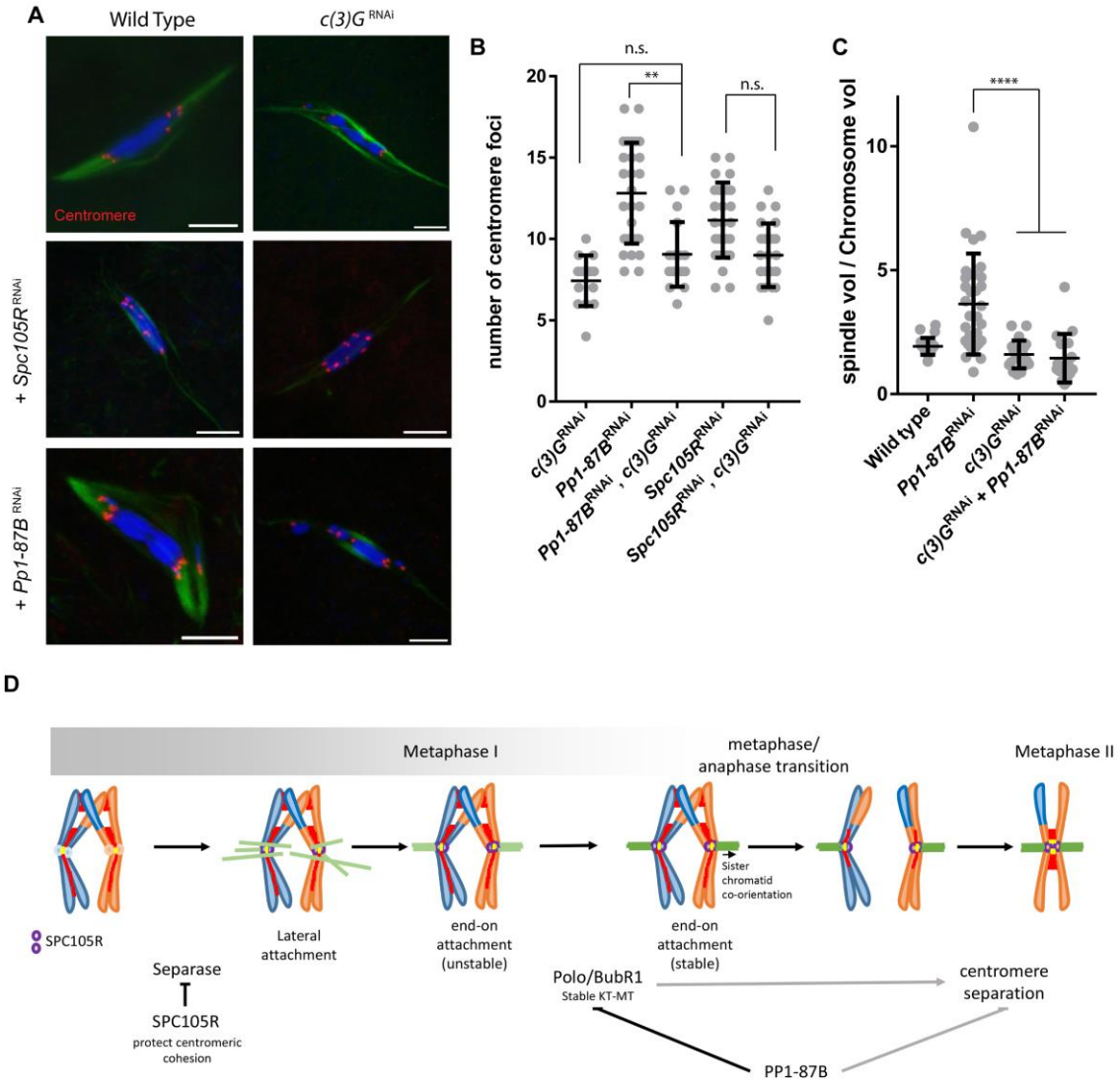

Figure 14. PP1-87B antagonizes C(3)G to regulate sister centromere fusion.

(A) Confocal images of oocytes expressing c*(3)G* RNAi in combination with *Pp1-87B* or *Spc105R* RNAi. The centromeres are shown in red in the merged images. Scale bar= 5 µm. (B) Dot plot showing the number of centromere foci in (A). Number of oocytes of each genotype are 14, 27, 18, 26 and 23 in order of the graph. Error bars indicates standard deviation. **=p<0.01. (C) Graph showing the ratio of the spindle volume to the chromosome mass volume. Number of oocytes are: 22, 31, 20 and 17. Error bars show standard deviation, ****=p<0.0001. (D) Model for regulation of co-orientation in *Drosophila* oocytes.

V. Discussion

The fusion of sister centromeres is important for co-orientation in meiosis I, ensuring that sister kinetochores attach to microtubules from the same pole. Release of this attachment must occur early in meiosis II. Based on our results, we propose that the regulation of sister centromere fusion that ensures its release in meiosis II occurs through at least two mechanisms (Figure 14D). First, *Drosophila* centromere fusion depends on loading cohesins at the centromeres that is protected by a kinetochore protein, SPC105R. Second, sister centromere fusion is released in a Separase-independent manner that depends on KT-MT interactions and is inhibited by PP1-87B.

Sister centromere fusion depends on kinetochore protein SPC105R to protect cohesion from Separase

Assembly of meiosis-specific cohesins at the centromeres probably establishes sister centromere fusion [21]. Indeed, the meiosis-specific cohesin complex SMC1/SMC3/SOLO/SUNN is enriched at *Drosophila* meiotic centromeres and could to have this function [11, 86-88]. Guo et al found that separase is required for progression through both meiotic divisions in oocytes [40]. We found that depleting Separase in metaphase I *Drosophila* oocytes rescued the precocious centromere separation phenotype caused by loss of SPC105R. Although we found no role for WAPL in centromere fusion, we did not rule out other functions in meiosis, especially in anaphase I given that Guo et al. found that oocytes depleted for Separase were delayed in anaphase I but eventually progressed to meiosis II.

SPC105R may protect centromere cohesion by recruiting cohesin protection proteins such as MEI-S332/SGO that subsequently recruit PP2A. The fact that *mei-S332* mutants do not display defects in meiosis I [36, 101] could be due to redundancy with another *Drosophila* PP2A recruiter, Dalmatian [102, 103]. The previous finding that *Drosophila Spc105R* mutants enhance defects in Separase function suggest SPC105R may have a cohesion protection function in other

cell types [118]. However, Separase activation usually coincides with the entry into anaphase when the APC degrades an inhibitor of Separase, Securin [99]. One explanation is that Separase has a novel cohesin-independent function in regulating co-orientation through SPC105R, such as structural or regulatory function within the kinetochore or spindle [119], or that loss of SPC105R activates Separase. We favor, however, the explanation that Separase is active prior to anaphase I and cohesion is maintained only by PP2A activity in metaphase I arrested oocytes. This model can explain why knockout of SPC105R in male meiosis does not show a loss of centromere fusion [120]. In male meiosis where there is no cell cycle arrest, Separase may not be active until anaphase, which would make a protective role for SPC105R difficult to observe.

The transition of sister centromeres from co-orientation to bi-orientation depends on kinetochore-microtubule interactions

Aurora B inhibitor BN2 was applied to mature *Pp1-87B* RNAi oocytes, which were in prometaphase I or metaphase I and therefore, after the spindle had formed and the sister centromeres had separated. Because this treatment caused the sister centromeres to come back together, sister centromere separation in *Pp1-87B* RNAi oocytes appears to be reversible. In contrast, treatment of mature oocytes with BN2 did not reverse centromere separation in *Spc105R* RNAi. This reversible phenotype of *Pp1-87B* RNAi oocytes is consistent with a mechanism that involves the reorganization of centromere and kinetochore geometry, and the nonreversible phenotype of *Spc105R* RNAi with a mechanism that involves the degradation of cohesins. Furthermore, the results from destabilizing microtubule attachments with BN2 treatment suggested that centromere separation in PP1-87B-depleted oocytes depends on KT-MT interactions. In support of this conclusion, we found that PP1-87B affects several spindle-based parameters: it localizes to the meiotic spindle, its knockdown caused an increase in spindle volume, and centromere separation in PP1-87B-deplated oocytes depended on NDC80, Polo and

BubR1. These results suggest that stable end-on attachments are required for release of sister centromere fusion. Similar conclusions have been made in *Drosophila* male meiosis. Sister centromere separation in meiosis II does not depend on Separase [121] but does depend on KT-MT interactions [120, 122]. These findings are not limited to *Drosophila*. Classic micro-manipulation experiments in grasshopper cells demonstrated that the switch in meiosis II to separated sister kinetochores requires attachment to the spindle [123]. Based on all these results, we propose that sister centromeres normally separate early in meiosis II by a process that is Separase-independent but microtubule-dependent (Figure 14D).

Interestingly, univalents in meiosis I can bi-orient in co-orientation-defective mutants that lack crossovers [26]. We observed a similar phenomenon in *Pp1-87B*; *mei-P22* meiosis I oocytes. However, the frequency of univalent bi-orientation was low, raising the question of how meiosis II univalents preferentially achieve bi-orientation. The low frequency of univalent bi-orientation in meiosis I could be due to differences in how each division begins. Meiosis I begins with the centromeres clustered in a chromocenter and rapidly develops a robust central spindle, both of which may bias the sister centromeres to make attachments to the same pole, even in a PP1-87B knockdown oocyte (Figure 15).

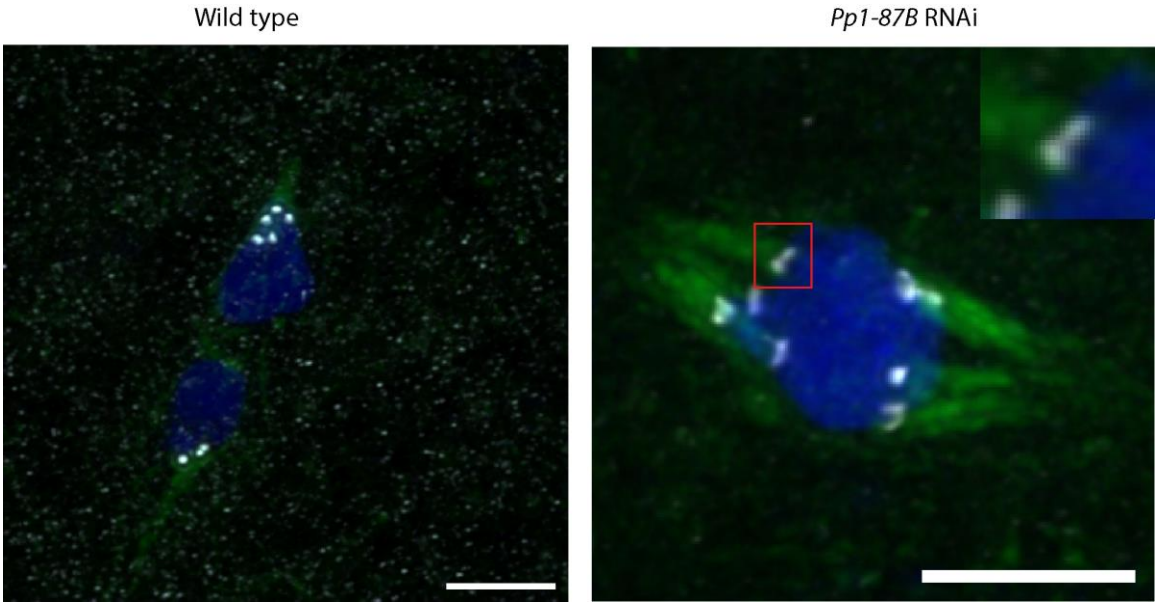

Figure 15: Kinetochore-microtubule attachments in *Pp1-87B* RNAi oocytes.

To observe whether the microtubule attachments in *Pp1-87B* RNAi oocytes are merotelic or syntelic in metaphase I, we used cold treatment to remove the unstable attachments. All females were cold treated for 2 hours before fixation. Presumably because depletion of PP1-87B stabilizes microtubule attachments, the *Pp1-87B* RNAi oocytes show a partial resistance to cold-treatment compared to wild-type. The images were taken and processed through deconvolution. All images are maximum projections and scale bars are 5 µm.

The mechanism regulated by PP1-87B that regulates KT-MT interactions and maintains sister centromere fusion is not known and may be a function utilized in mitotic cells. For example, PP1 has a role in regulating microtubule dynamic in *Xenopus* extracts [124]. In HeLa cells depleted of SDS22, a regulatory subunit of PP1, sister kinetochore distances increase [125], similar to the defect we described here. In budding yeast, suppressing premature formation of stable kinetochore-microtubule attachments is necessary for co-orientation [126]. The mechanism may be related to the role of PP1 in negatively regulating condensin functions that affect chromosome structure [127, 128]. A negative effect on condensin activity, which is known to shape mitotic chromosomes [129, 130], could also explain the chromosome mass separation phenotype of *Pp1-87B* RNAi oocytes.

Our observations are strikingly similar to the phenomenon of cohesin fatigue, where sister chromatids separate in metaphase arrested mitotic cells. Identical to the effect of PP1-87B on centromere separation, cohesion fatigue occurs in a Separase-independent but microtubule-dependent manner [131, 132], however, the mechanism is unknown [133]. Oocytes with a prolonged arrest points, such as metaphase I in *Drosophila*, might prevent cohesion fatigue by concentrating meiotic cohesins at the centromeres and destabilizing KT attachments to reduce MT forces. In *Drosophila* oocytes, the microtubule catastrophe protein Sentin destabilizes end-on KT-MT attachments after the spindle is well established [96]. In fact, active destabilization of kinetochore attachments may be a common feature of oocyte meiosis. Mammalian oocytes also have an extended period of dynamic KT-MT interactions [134], lasting 6-8 hours in mice and up to 16 hours in human [52, 135]. All of these results are in line with our conclusion that oocytes require PP1-87B to prevent premature stable KT-MT attachments and avoiding cohesion fatigue.

On the role of C(3)G and Polo kinase in cohesion and co-orientation

Depletion of Ç(3)G suppresses the *Pp1-87B* centromere fusion defect. This result suggests that centromeric SC has a role in negatively regulating sister centromere co-orientation. While the bulk of SC disassembles in late prophase [16, 19], centromeric SC proteins persist beyond pachytene in *Drosophila* and until at least metaphase I in budding yeast and mouse [17, 19, 20, 117, 136]. It has also been shown that SC proteins interact with the NDC80 complex in two yeast two hybrid experiments [137, 138]. These studies have concluded that centromeric SC is required for bi-orientation of homologs and monopolar attachment. Because both Polo Kinase and C(3)G negatively regulate co-orientation, we hypothesize that C(3)G could be required for Polo Kinase activity, but not localization, at the centromere. Thus, centromeric SC components might be an important mediator of co-orientation.

Co-orientation in yeast and mice depends on Polo kinase, which is recruited by Spo13, Moa1 or Meikin [139]. This is opposite of the known mitotic role of Polo in phosphorylating cohesin subunits and facilitating their removal from binding sister chromatids [100, 140, 141]. In yeast meiosis, however, the phosphorylation of cohesin subunits may depend on two different kinases, Casein kinase I and CDC7 [43, 142, 143]. Which kinase(s) are required in animals to phosphorylate meiotic cohesins for their removal remains unknown. We have shown that Polo is required for loss of centromeric cohesion, which to our knowledge is the first evidence of its kind in animal meiotic cells.

Unlike mice and yeast, depletion of Polo kinase from *Drosophila* metaphase I oocytes does not cause sister centromere separation [71]. One reason for this difference in Polo function could be that it is required at multiple stages of meiosis and its phenotype may depend on when it is absent. Loss of Polo or BubR1 during early *Drosophila* prophase (pachytene) oocytes leads to loss of SC and cohesion defects [144, 145]. Our experiments depleted Polo after cohesion was established. Alternatively, the function of Polo in co-orientation may not be conserved. Importantly, two features of centromere fusion and co-orientation that are conserved are maintenance depending on SPC105R and separation depending on stabilization of KT-MT attachments. Like SPC105R in *Drosophila,* budding yeast KNL1 is required for meiotic sister centromere fusion and co-orientation and is a target of Polo [28]. The differences between *Drosophila* and mouse or yeast can be explained if SPC105R does not require Polo in order to protect cohesion at the centromeres for co-orientation.

While all previous studies of co-orientation have focused on the establishment of centromere fusion, our results identified several key regulators and provide insights into how sister centromere fusion is maintained in meiosis I and released for meiosis II. In contrast to release of cohesion in most regions of the chromosomes, we propose a Separase-independent mechanism that requires stable kinetochore-microtubule attachments promotes centromere separation early in meiosis II. While it is well known that regulating microtubule attachments is important for bi-

orientation, our results are an example of another reason why KT-MT attachments must be properly regulated, to safely navigate the transitions through the two divisions of meiosis.

VI. Methods

Drosophila genetics

Drosophila were crossed and maintained on standard media at 25°C. Fly stocks were obtained from the Bloomington Stock Center or the Transgenic RNAi Project at Harvard Medical School [TRiP, Boston, MA, USA, flyrnai.org, TRiP, Boston, MA, USA, flyrnai.org, 93]. Information on genetic loci can be obtained from FlyBase [flybase.org, flybase.org, 146].

RNAi in oocytes: expression and quantification

Most *Drosophila* lines expressing a short hairpin RNA were designed and made by the Transgenic RNAi Project, Harvard (TRiP) (Table 1). To deplete target mRNA, a cross was performed to generate females carrying both the *UAS:shRNA* and a *GAL4-VP16* transgene. The shRNA can be induced ubiquitous expression by crossing to *tubP-GAL4-LL7* and testing lethality [147], or *mata4-GAL-VP16* and *osk-GAL4-VP16* for oocyte-specific expression [148]. In this paper, *mata4-GAL-VP16* was primarily used for inducing expression of the *UAS:shRNA* after early pachytene but throughout most stages of oocyte development in the *Drosophila* ovary. This allows for 3-5 days of continuous expression to knockdown the mRNA levels. In some cases, we used the *oskar -GAL4-VP16* transgene [149, 150], which causes a similar knockdown and phenotype in PP1-87B as *mata4-GAL-VP16.* Double RNAi crosses were set up based on the available RNAi lines (Table 2).

For measuring the mRNA knockdown level, total RNA was extracted from late-stage oocytes using TRIzol® Reagent (Life Technologies) and reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The qPCR was performed

on a StepOnePlus™ (Life Technologies) real-time PCR system using TaqMan® Gene Expression Assays (Life Technologies), Dm02152292_g1 for *Pp1-87B* and Dm02134593_g1 for the control *RpII140*. Oocyte-specific shRNA expression of *HMS00409* using *mata4-GAL-VP16* resulted in sterility and knockdown of the oocyte mRNA to 35% as measured by RT-qPCR; the same phenotype has been seen when using *osk-GAL4-VP16*, where the mRNA knockdown is also to 35%. For SPC105R, expressing shRNA GL00392 using *osk-GAL-VP16* knocked down the mRNA to 10%.

Generation of Wapl shRNA lines in *Drosophila*

To generate a *wapl* shRNA line, we followed the protocol in Harvard TRiP center (http://fgr.hms.harvard.edu/trip-plasmid-vector-sets) and targeted *wapl* sequence (5'-gaggaggaggatcaacagcaa -3') for mRNA knockdown. This 21-nucleotide sequence was cloned into pVALIUM22 and the whole construct was injected into *Drosophila* embryos (*y sc v; attP40*). The mRNA is knocked down to 4% when using *mata4-GAL-VP16* to express the shRNA in oocytes.

Antibodies and immunofluorescent microscopy

Mature (stage 12-14) oocytes were collected from 100 to 200, 3-4-day old yeast-fed non-virgin females. The procedure is described as in [77]. Oocytes were stained for DNA with Hoechst 33342 (10 μg/ml) and for MTs with mouse anti-α tubulin monoclonal antibody DM1A (1:50), directly conjugated to FITC (Sigma, St. Louis). Additional primary antibodies used were rat anti-Subito antibody [107], rat anti-INCENP [151], guinea pig anti-MEI-S332 [152], rabbit anti-CENP-C [153], rabbit anti-Deterin [154], rabbit anti-Spc105R [155], mouse anti-Polo [156] and rabbit anti-CID (Active Motif). These primary antibodies were combined with either a Cy3,

Alex 594 or Cy5 secondary antibody pre-absorbed against a range of mammalian serum proteins (Jackson Immunoresearch, West Grove, PA). FISH probes corresponding to the X359 repeat labeled with Alexa 594, AACAC repeat labeled with Cy3 and the dodeca repeat labeled with Cy5 were obtained from IDT. Oocytes were mounted in SlowFade Gold (Invitrogen). Images were collected on a Leica TCS 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).

Image analysis

All the CENP-C foci, CID foci, chromosome mass volume, spindle volume and MEI-S332 volume were measured using Imaris image analysis software (Bitplane). For determining centromere foci, an automated spots detection function in Imaris was used. A spot whose XY diameter is 0.20 um, Z diameter is 1.00 um and is in touch with DNA will be counted as a centromere. For the volume measurement, images were resampled first to become isovoxel data. Then the surface detection function was used for defining different objects and measuring their volume. Spindle volume is measured and normalized by the chromosome mass volume to compensate for effects of chromatin volume on microtubule recruitment, although chromosome mass volume in each genotype did not differ significantly.

Binuclein 2 treatment assay

To inhibit Aurora B, oocytes were incubated with either 0.1% DMSO or 50 μM BN2 in 0.1% DMSO for 60 minutes prior to fixation in Robb's media. To stabilize MTs, oocytes were incubated with either 0.1% DMSO or 10 μM Paclitaxel (Sigma) in 0.1% DMSO for 10 minutes, followed by 50 μM BN2 plus 10 μM Paclitaxel in 0.1% DMSO for 60 minutes.

Quantification and statistical analysis

Statistical tests were performed using GraphPad Prism software. All the numbers of the centromere foci or spindle/chromosome mass volume or MEI-S332 volume were pooled together and ran one-way ANOVA followed by post hoc pairwise Tukey's multiple comparison test. Details of statistical evaluations and the numbers of samples are provided in the figure legends.

Table 1: Genotypes of *Gal4* and shRNA lines used for RNAi experiments

RNAi strains		
D. melanogaster: Gal4 driver of *αTub84B*: *y[1] w[*]; P{w[+mC]=tubP-GAL4}cLL7/TM3, Sb[1] Ser[1]*	Bloomington Drosophila Stock Center	BDSC: 5138;
D. melanogaster: Gal4 driver of *αTub67C*: *w[*]; P{w[+mC]=matalpha4-GAL-VP16}V37*	Bloomington Drosophila Stock Center	BDSC:7063;
D. melanogaster: Gal4 driver of *osk*: *w[1118]; P{w[+mC]=osk-GAL4::VP16}A11/CyO*	Bloomington Drosophila Stock Center	BDSC:44241;
D. melanogaster: shRNA of *Pp1-87B*: *y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00409}attP2*	Bloomington Drosophila Stock Center	BDSC:32414; FlyBase: FBgn0004103
D. melanogaster: shRNA of *Spc105R: y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00392}attP2*	Bloomington Drosophila Stock Center	BDSC:35466; FlyBase: FBgn0037025
D. melanogaster: shRNA of *Spc105R: y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01548}attP40/CyO*	Bloomington Drosophila Stock Center	BDSC:36660; FlyBase: FBgn0037025
D. melanogaster: shRNA of *sse: Sse$^{dsRNA.147.UASp}$*	Guo et al., 2016	FlyBase: FBal0319202
D. melanogaster: shRNA of *wapl:* (5'-gaggaggaggatcaacagcaa -3') was created using pVALIUM22	This paper	
D. melanogaster: shRNA of *polo: y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00512}attP40*	Bloomington Drosophila Stock Center	BDSC:36093; FlyBase: FBgn0003124
D. melanogaster: shRNA of *BubR1: y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GLV21065}attP2*	Bloomington Drosophila Stock Center	BDSC:35700; FlyBase: FBgn0263855
D. melanogaster: shRNA of *mps1: y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00184}attP2*	Bloomington Drosophila Stock Center	BDSC:35283; FlyBase: FBgn0000063
D. melanogaster: shRNA of *Ndc80: y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00625}attP40*	Bloomington Drosophila Stock Center	BDSC:37482; FlyBase: FBgn0030500
D. melanogaster: shRNA of *c(3)G: y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ30046}attP40*	Bloomington Drosophila Stock Center	BDSC:62969; FlyBase: FBgn0000246

Table 2: Transgenes used for Double RNAi

Driver (GAL)	shRNA line 1	shRNA line 2
mata4-GAL-VP16	*wapl*	*PP1-87B (HMS00409) or SPC105R (GL00392)*
mata4-GAL-VP16	*sse*$^{dsRNA.147.UASp}$	*PP1-87B (HMS00409) or SPC105R (GL00392)*
mata4-GAL-VP16	*c(3)G (HMJ30046)*	*PP1-87B (HMS00409) or SPC105R (GL00392)*
mata4-GAL-VP16	*Polo (GL00512)*	*PP1-87B (HMS00409) or SPC105R (GL00392)*
osk-GAL4-VP16	*mps1 (GL00184)*	*PP1-87B (HMS00409) or SPC105R (GL00392)*
osk-GAL4-VP16	*bubR1 (GLV21065)*	*PP1-87B (HMS00409) or SPC105R (GL00392)*
mata4-GAL-VP16	*Ndc80 (GL00625)*	*PP1-87B (HMS00409) or SPC105R (GL00392)*
osk-GAL4-VP16	*SPC105R (GL00392)*	*PP1-87B (HMS00409)*

Chapter 3

HP1 assists the Chromosomal Passenger Complex to promote spindle assembly and chromosome bi-orientation in *Drosophila* oocytes

I. Preface

This chapter will be submitted soon. My contribution to this project is writing the paper, revisiting Haspin, Bub1 results and all the experiments except for the live image of oocytes.

II. Abstract

Oocytes assemble bipolar spindles in the absence of centrosomes, and it has been observed that the chromosomes in oocytes direct the process instead. The Chromosomal passenger complex (CPC) has been shown to be required for spindle formation and orchestrate chromosomes biorientation in oocytes; however, the mechanism of spindle assembly and the chromosomal feature that recruits the CPC is not known. Here, we found that heterochromatin protein 1 (HP1) has an essential role in both spindle assembly and chromosome biorientation in *Drosophila* oocytes. An interaction between HP1 and Borealin is crucial for the initial recruitment of the CPC to the chromosomes. This recruitment is sufficient to build kinetochores and form the kinetochore microtubules. We also found that HP1 relocates from the chromosomes onto the spindle microtubules similar to the CPC. Although the localization of the CPC and HP1 on the spindle do not perfectly overlap, the microtubule dependency of their spindle relocation and their similar chromosome localization when microtubules are diminished suggests a CPC-HP1 complex might form before relocating onto the spindle. Additionally, we have shown that the CPC regulates homologous chromosome bi-orientation through the central spindle rather than

the centromeres. A functional central spindle relies on both on CPC localization to the spindle and the HP1-INCENP interaction. Together, we demonstrate that, in oocytes, HP1 plays an important role in regulating the CPC chromosome localization and its central spindle function to further affects both spindle assembly and homologs biorientation.

III. Introduction

Accurate chromosome segregation in cell division is achieved through a functional bipolar spindle which is composed of an array of microtubules (MTs) and associated proteins. In mitosis and male meiosis, two centrosomes serve as microtubule organizing centers (MTOC) to nucleate the MTs. The MTs then grow toward the chromosomes and construct into a bipolar spindle. In contrast, the spindle in female meiosis is assembled in a non-canonical pathway. During prophase of meiosis, oocytes start a process to eliminate the centrosomes [49]. Without centrosomes, MTs cluster around the chromosomes after nuclear envelope breakdown and then assort into the bipolar spindle. Although this process has been observed in the oocytes of many organisms [157], the regulation of this chromosome directed spindle assembly is not well understood.

Several known pathways have been discovered that nucleate MTs and promote spindle assembly in an acentrosomal system. Amongst these, the Ran pathway and the chromosomal passenger complex (CPC) have been addressed the most [158-161]. The Ran pathway is known to form a gradient of its active form, RanGTP, centered at the chromosomes that releases spindle assembly factors from the inhibitor importins [58, 59]. While this pathway is well described in many organisms, experiments that inhibit RanGTP have shown only a delay in meiosis I spindle assembly but does not abolish it in mouse and *Drosophila* oocytes [61, 65], suggesting it is dispensable. On the other hand, the CPC, composed by Aurora Kinase, the scaffold subunit INCENP and two targeting subunits Borealin and Survivin, is essential in spindle formation in the

oocytes of *Xenopus* and *Drosophila* [56, 60, 62, 162, 163]. The CPC is required for promoting spindle assembly independent of the Ran pathway when sperm nuclei were added to a *Xenopus* egg extract [56, 60], and it is partially through suppressing microtubule depolymerase kinesin 13/MCAK and Op18/Stathmin [56, 60]. However, knocking down MCAK in *Drosophila* did not rescue the loss of spindle in the CPC-depleted oocytes [62], suggesting the CPC promotes spindle assembly through other targets. Additionally, the CPC in *Drosophila* oocytes also regulates kinetochore assembly [90] and homolog bi-orientation [62], indicating the multi-function of the CPC in acentrosomal system. Interestingly, the CPC in *Drosophila* oocytes is the sole pathway to that promotes acentrosomal spindle assembly. This may be less apparent in other organisms due to the redundancy of multiple Aurora Kinases [135]; however, this unique requirement makes the *Drosophila* oocyte an ideal model to study how the CPC regulates acentrosomal spindle assembly and its function.

In mitosis, the CPC is recruited to centromeres by interactions between Survivin and Borealin and phosphorylated H3T3 and H2AT120 histone markers. This localization is required for the CPC to destabilize the mis-attachment of MTs for error correction. After the metaphase-anaphase transition, the CPC relocates onto the spindle midzone for cytokinesis [164]. In *Drosophila* oocytes, the CPC has been only observed on the central spindle in metaphase I but not the centromeres [62]. Subito, the *Drosophla* Kinesin-6 and MKLP2 orthologue, localizes to the central spindle and is known to be required for organizing the central spindle and homologous chromosome bi-orientation in *Drosophila* oocytes [107]. Similar to mitotic cell where Subito has a direct interaction with INCENP to recruit the CPC to the spindle midzone, in oocytes they have a genetic interaction and mutual dependence of their localization [62, 70, 71]. These results suggest that the CPC may promote spindle assembly, in part, by activating Subito activity in the central spindle. Additionally, we have also found that the kinetochores are not required to assemble the central spindle [90], showing the kinetochore dependent (K-fiber) and the central spindle dependent MTs could be regulated independently. However, these observations have led

to some intriguing questions: how does the CPC orchestrate spindle assembly if it only localizes on the central spindle? How does the CPC build the kinetochores and K-fibers? How the CPC regulates homolog biorientation in oocytes? And most importantly, how do the chromosomes direct oocyte spindle assembly?

To further understand of how the CPC regulates these functions in oocytes, we made several separate of function mutations of the CPC. We discovered an interaction between the CPC and a heterochromatin protein, HP1. Unlike mitosis where the CPC is known to be recruited to centromeres through recruitment of Survivin by H3T3ph and Borealin by H2AT120ph, we provide evidence that the interaction between the CPC and HP1 is of the most important for oocyte spindle assembly. We found the CPC is recruited to the chromosome by an HP1-Borealin interaction, and then the CPC assembles K-fibers and central spindle independently. K-fiber formation is highly correlated with the presence of kinetochores and the CPC's chromosomal localization. Both the HP1-Borealin interaction and the INCENP SAH domain are essential for building the central spindle, although the latter plays a role in drawing the CPC away to the chromosome rather than assembly. Finally, we showed that CPC on the central spindle plays a major role in regulating the oocyte homologs bi-orientation at the centromeres. Our research sheds light on the mechanism for the important role of the CPC in acentrosomal spindle assembly and homolog bi-orientation in the oocytes.

IV. Results

The CPC localizes in both centromere and central spindle in early meiosis

The CPC plays an essential role in spindle assembly and its activity is crucial for building the kinetochores (KT) and chromosome bi-orientation in meiosis I [62]. However, unlike mitosis where the CPC is known to localize in the centromeres/inter-kinetochore during metaphase to regulate chromosome bi-orientation, the previous research in *Drosophila* oocytes has only

observed that CPC localized to the central spindle [62, 107]. Because the CPC has a crucial mitotic function at the centromeres, it is possible the CPC localizes to multiple locations in addition to the central spindle. To exclude the possibility of the fixation process that might affect the result of immunofluorescent staining, we live imaged the oocytes that carry two transgenes: Aurora B:RFP and Cenp-C:GFP, to examine whether the CPC localizes in the centromeres in the oocytes. The result showed that, in addition to the central spindle, Aurora B:RFP colocalized with Cenp-C:GFP although its intensity is lower than one on the central spindle (Figure 16A), indicating the CPC in meiosis indeed localizes both at the centromere and the central spindle.

Expressing CPC in both centromere and central spindle is not sufficient to assemble wild type spindle

Because the CPC is required for microtubules at both the kinetochores/ centromeres and the central spindle, we predicted that expressing two populations of the CPC that independently localize would reconstitute the wild type spindle. To test the hypothesis, we fused either a centromere protein or a central spindle protein to the *INbox* domain of *Incenp*, the C terminal 644-754 amino acids that recruits and activates Aurora B. We predicted that each fusion would localize and activate Aurora B at these two sites. Previous studies have shown that the CPC is required for KT assembly. For example, when oocytes were depleted of Aurora B or INCENP by tissue-specific expression of shRNAs (referred to as *aurB* RNAi or *Incenp* RNAi oocytes), they failed to localize SPC105R or NDC80 to the centromere regions [62]. In contrast, we found that there was a low level of MIS12 localization in INCENP- or Aurora B-depleted oocytes (Figure 17). Based on these observations, we chose to fuse *mis12* to the *INbox* for centromere targeting. Additionally, we fused the *INbox* with two spindle proteins, Fascetto (*feo*, the *Drosophila* PRC1 homolog) and Subito (*sub*, the *Drosophila* MKLP2 homolog) for central spindle targeting.

Expressing the *INbox* by itself had a dominant effect because it lacks of focus to cluster and activate Aurora B Kinase, similar to a previous report in mammalian cells [165]. Its expression in *Drosophila* oocytes showed a diminished spindle and caused sterility. *mis12:INbox*, *feo:INbox* and *sub:INbox* all showed strong localization patterns when expressed in otherwise wild type oocytes (Figure 16B). When targeting the CPC to the centromere by expressing *mis12:INbox* in *Incenp* RNAi oocytes, only the microtubules (MTs) closed to the kinetochores (K-fiber) formed while the central spindle failed to form (Figure 16C). On the other hand, when expressed either *feo:INbox* or *sub:INbox* in *Incenp* RNAi oocytes, none of the oocytes displayed a spindle around the chromosomes. However, the *sub:INbox* oocytes had MT bundles in the ooplasm (Figure 16C). This result suggests that the interaction between the CPC and central spindle proteins, especially Subito, can promote MT bundling but is not sufficient to promote spindle assembly around the chromosomes. Because most of the *sub:Inbox, Incenp* RNAi oocytes showed the absence of the SPC105R localization (75%, n=12, Figure 16C), one possibility is that the central spindle targeting of the CPC lacked the interaction with the chromosomes necessary to assemble spindle. To test this, we co-expressed *mis12:INbox* and *sub:INbox* in *Incenp* RNAi oocytes to determine if CPC independently targeted to the chromosomes and the central spindle would result in spindle assembly. Interestingly, only the K-fibers formed in these oocytes and the oocyte showed stronger MT bundling in K-fibers without bundling in the ooplasm (Figure 16D), suggesting the SUB:INbox cannot initiate the spindle assembly but can help bundle once initiated. This result indicates that given the two populations of the CPC is not sufficient to form the wild type spindle.

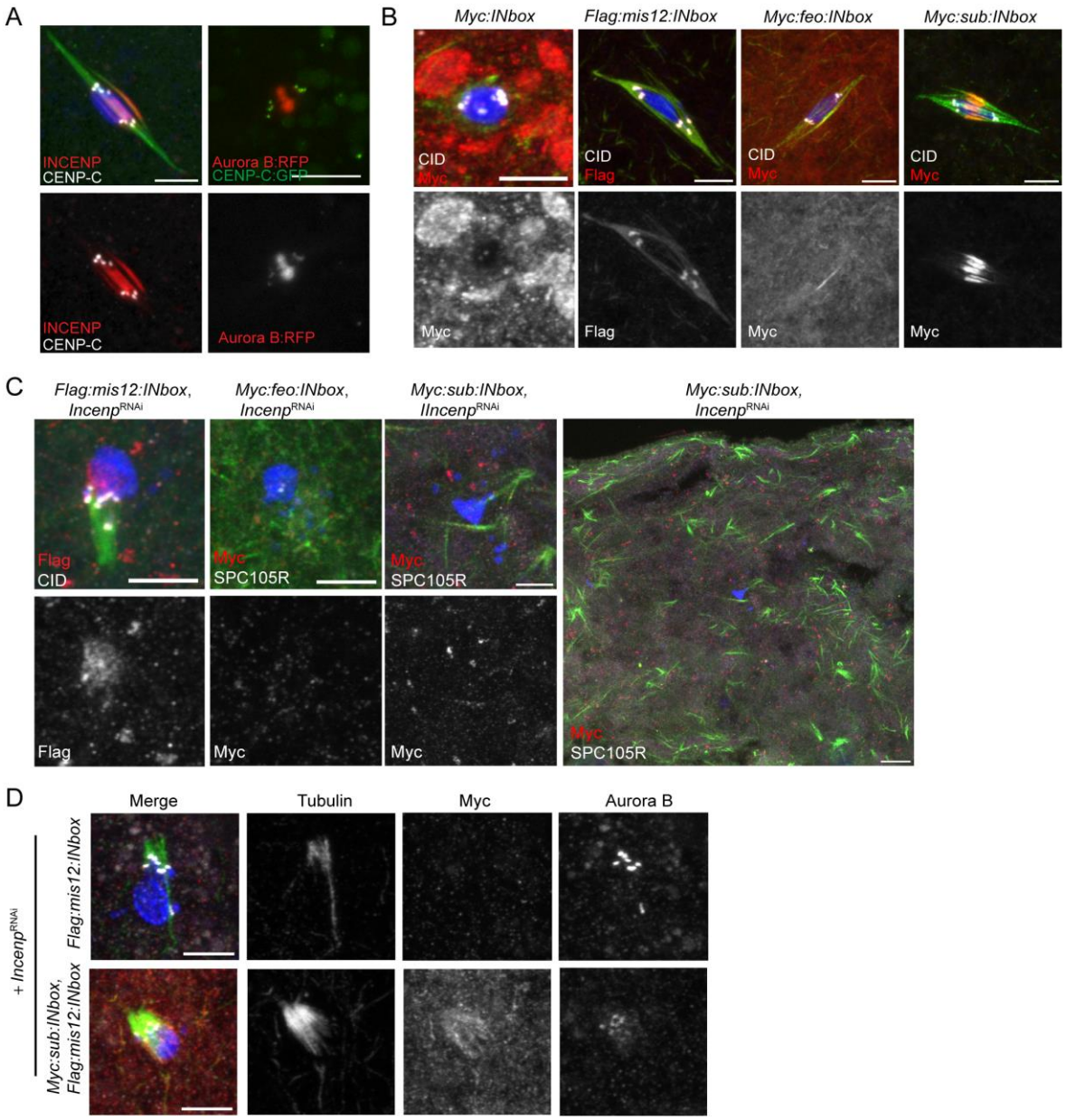

Figure 16: Expressing CPC at both centromere and the central spindle only assemble kinetochore-dependent microtubules.

(A) CPC can be observed on the central spindle in fixed immunostaining process. and can only be observed colocalizing with CENP-C when live imaged wild type oocytes. DNA is in blue and tubulin is in green. Scale bar = 5μm. (B) *Myc:INbox*, *Flag:mis12:INbox*, *Myc:feo:INbox* and *Myc:sub:INbox* were expressed in the wild type oocytes respectively. The separated channels showed the transgene localization. Centromere protein, CID, is shown in white, DNA is in blue and tubulin is in green. Scale bar = 5μm. (C) *Flag:mis12:INbox*, *Myc:feo:INbox* and *Myc:sub:INbox* were expressed in *Incenp* RNAi oocytes. The zoomed out picture of *Myc:sub:INbox* showed microtubule bundled in the ooplasm instead of around chromosomes. Transgenes are in red, CID or kinetochore protein, SPC105R, are in white. DNA is in blue and tubulin is in green. Scale bars represent 5μm in the left three pictures and 10 μm in the zoomed

out picture. (D) *Flag:mis12:INbox* either expressed alone or co-expressed with *Myc:sub:INbox* in *Incenp* RNAi oocytes. Merged pictures showed DNA (blue), tubulin (green), Myc (red) and Aurora B (white). Scale bars indicate 5µm.

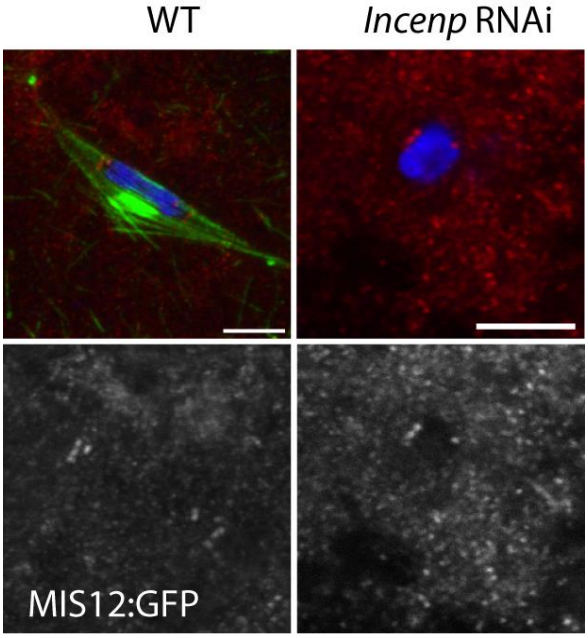

Figure 17: MIS12 localizes independent to the CPC.

MIS12:GFP can be observed in both wild type oocytes and *Incenp* RNAi oocytes. Scale bar represents 5 µm. MIS12-GFP is in red, tubulin is in green and DNA is in blue.

Establishing an RNAi resistant system to study CPC function

Co-expressing the two CPC populations was not sufficient to reconstitute chromosome-based spindle assembly, suggesting that the chromosome and spindle-associated mechanisms may be linked. To investigate how the CPC regulates various meiotic activities, especially spindle assembly, we examined the separate functions of the CPC by targeting it to specific locations or blocking specific interactions. We engineered an RNAi resistant transgene with mismatches (amino acids 437-441, Figure 18A) in the region of *Incenp* targeted by shRNA GL00279. This version of *Incenp* was fused to a 6Xmyc-tag at its N terminus to generate *Incenp*^{myc} (Figure 18A). Expressing *Incenp*^{myc} in an *Incenp* RNAi background rescued spindle assembly in oocytes,

including kinetochore and central spindle localization, but also showed several defects. The homologous chromosomes frequently failed to bi-orient, fertility was reduced and the transgene protein spread along the spindle instead of concentrating in the central spindle (Figure 17B). The same phenotype was also observed in Flag-INCENP and HA-INCENP fusion and *Incenp*^{myc} in previous publication (Figure 19, [62]). These results suggest that an epitope tag in the N terminus of INCENP might interfere with its function. To solve this problem, we removed the myc-tag from *Incenp*^{myc} to generate *Incenp*^{mycless}. Expressing *Incenp*^{mycless} in *Incenp* RNAi oocytes displayed wild type spindle and localization, and restored fertility to wild type levels (Figure 17B). Therefore, we used *Incenp*^{mycless} to construct all the separation of function *Incenp* transgenes.

The N-terminal region of INCENP (defined as amino acids 1-46), has been proposed to have a role in targeting, in part by interacting with the centromere targeting proteins, Survivin and Borealin [166]. To validate substituting this CEN domain can still perform CPC's kinase activity, we replaced this domain with MIS12 (*mis12:Incenp*) and deleted the region necessary for Survivin and Borealin interaction (deletion of amino acids 22-30, *Incenp*$^{\Delta CEN}$) (Figure 17A). When *mis12:Incenp* was expressed in *Incenp* RNAi oocytes (n=36), the oocytes displayed the same phenotype as *Flag:mis12:INbox* where only K-fibers formed (n=25, Figure 17C). When the CEN domain was removed from *Incenp* and expressed in an *Incenp* RNAi background, 83% of the oocytes (n=35) lacked spindles. Surprisingly, 82% of these oocytes (n=22) had SPC105R localization. This result suggests that the interaction between Borealin and Survivin is essential for spindle assembly but not for kinetochore assembly.

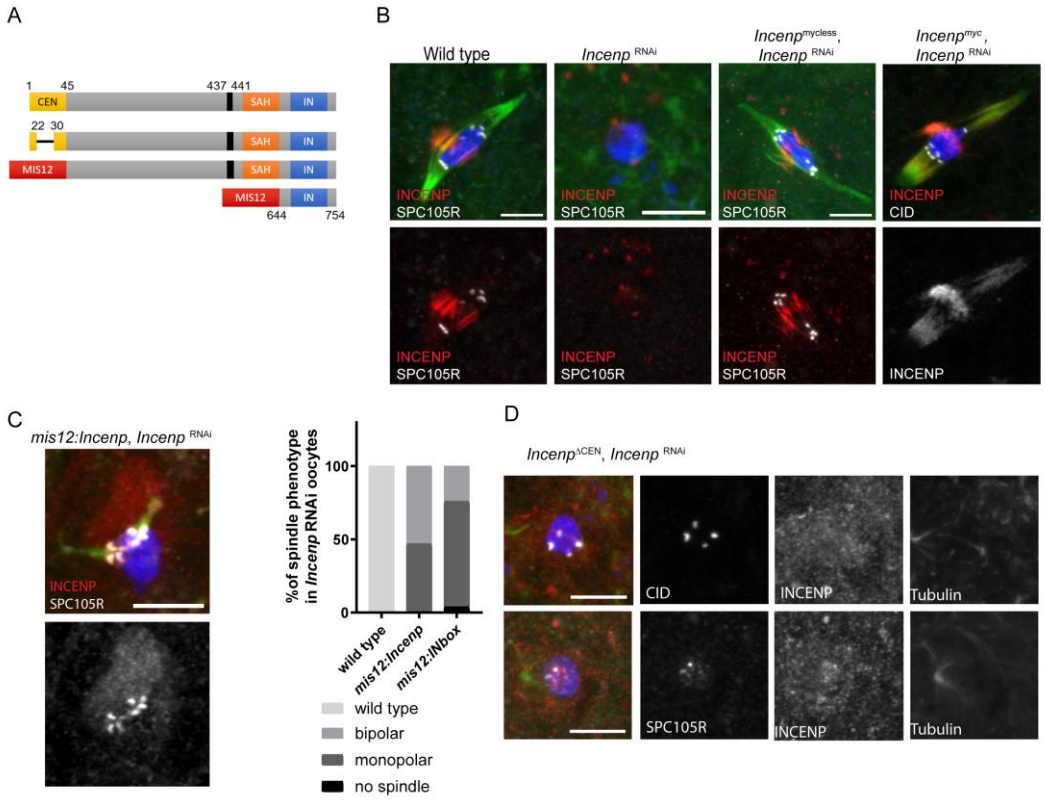

Figure 18: RNAi-resistant INCENP restored CPC function in *Incenp* RNAi oocytes.

(A) Diagram of each modified *Drosophila* INCENP constructs which are all based on INCENP^{mycless} . CEN, centromere-targeting domain; black bar, RNAi resistant mismatch; SAH, single alpha helix; IN, INbox and MIS12, the full-length *Drosophila* MIS12. (B) INCENP^{myc} and INCENP^{mycless} reconstituted the wild type spindle in the *Incenp* RNAi oocytes. However, INCENP^{myc} displayed several defects including mis-localization of INCENP on the spindle and homologous chromosome bi-orientation which can be observed by the uneven distribution of centromere foci. (C) An *Incenp* RNAi oocyte expressing *mis12:Incenp* only has K-fiber assembled. A quantification of spindle phenotype is shown in the graph: the K-fiber can be subgrouped into bipolar or monopolar. Numbers of oocytes are: 23, 36 and 25. (D) Expressing *Incenp*$^{\Delta 22\text{-}33}$ did not support spindle assembly *Incenp* RNAi oocytes; however occasionally, kinetochores can be observed as shown in the lower panel. All the pictures show DNA in blue, INCENP in red and Tubulin in green. White dots are either centromere protein, CID, or kinetochore protein, SPC105R. Scale bars are 5 μm.

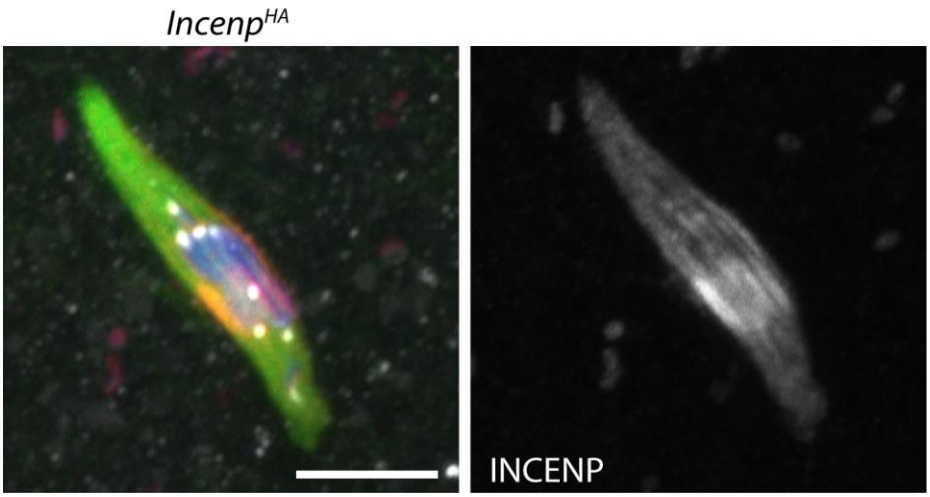

Figure 19: INCENP^{HA} localizes throughout the spindle.

The construct is expressed in the RNAi oocytes. INCENP is in red, CID is in white, tubulin is in green and DNA is in blue. Scale bar is 5 µm.

Borealin but not Survivin plays a key role in meiotic spindle assembly

In mitosis, Survivin and Borealin are the two centromere targeting subunits of the CPC [164]. When the CEN domain was deleted (*Incenp*$^{\Delta CEN}$), the spindle was abolished, suggesting the interaction with Survivin and/or Borealin is crucial for spindle assembly in oocytes. A prior study in mitotic cells has shown that substituting the N-terminal domain of INCENP with Survivin can target the CPC to the centromere and restore CPC function in the absence of Borealin [167]. To test whether Survivin and/or Borealin plays the critical function in oocyte spindle assembly, we replaced N-terminal domain with Survivin (Deterin in *Drosophila*) or Borealin INCENP (referred as *Det:Incenp* and *borr:Incenp*). Interestingly, when expressing these transgenes in *Incenp* RNAi oocytes, only *borr:Incenp*, but not *Det:Incenp*, displayed wild type spindle assembly and proper localization (Figure 20A). Det:INCENP only promoted K-fiber assembly and its localization was a haze in the pericentromeric regions (Figure 20A). Borealin localization could not be detected in *Det:Incenp*, *Incenp* RNAi oocytes (Figure 21), suggesting

this spindle phenotype is independent of Borealin. We then asked if the wild type spindle in Borr:INCENP depends on Survivin. To test this, we expressed *borr:Incenp* in *Survivin* RNAi oocytes. In contrast to *Survivin* RNAi oocytes where 44% of the oocytes did not assemble spindle and the rest only showed MT clustering around chromosome (n=18), all the *borr:Incenp, Survivin* RNAi oocytes displayed a wild type spindle (n=24, Figure 20B and C). Consistent with this observation, Survivin did not localize in *borr:Incenp*, *Incenp* RNAi oocytes (Figure 20D). These results showed that Borealin plays a more important role than Survivin in oocyte spindle assembly.

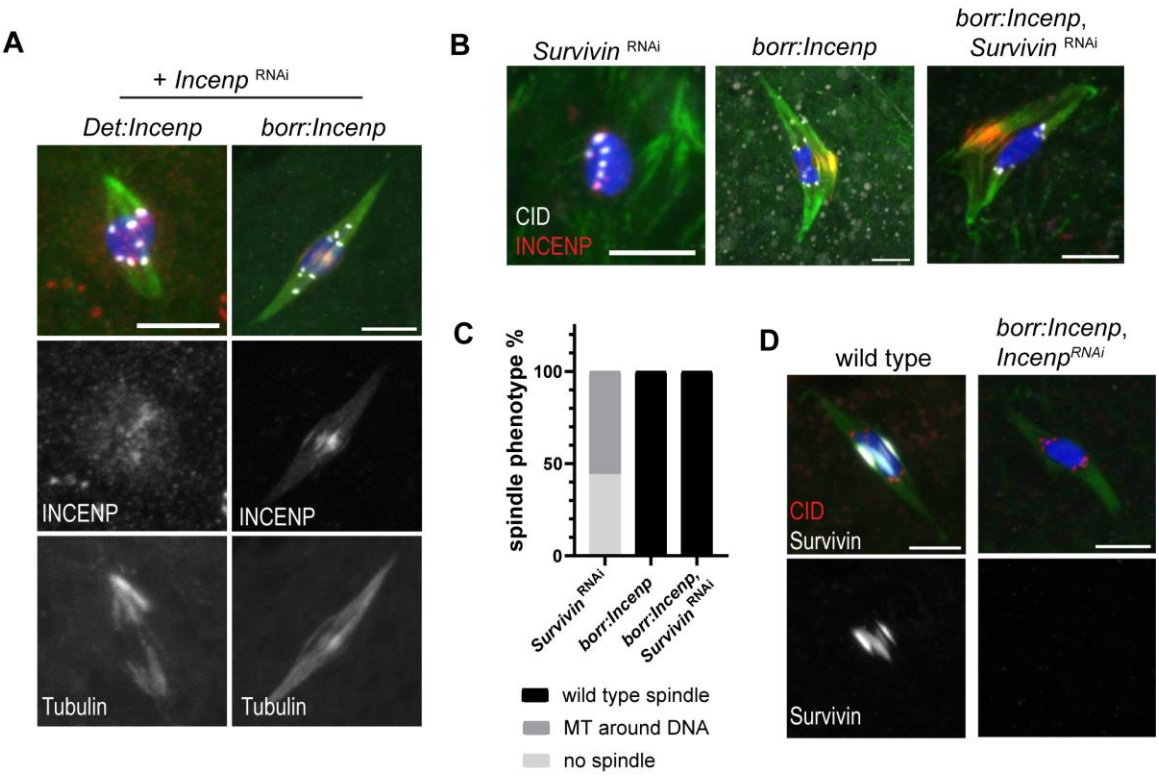

Figure 20: Borealin plays a more essential role than Survivin in assembling meiotic spindle, especially the central spindle.

(A) Borr:INCENP restored the wild type spindle in *Incenp* RNAi oocytes while Det:INCENP only assembled K-fiber. Separated channels showed the localization of the transgenes and spindle. INCENP is in red, DNA is in blue, tubulin is in green and CID is in white. Scale bars are 5μm. (B) Borr:INCENP forms the wild type spindle in *Incenp* RNAi oocytes independent to Survivin. Knocking down Survivin in the oocytes displayed the same phenotype as in *Incenp* RNAi and *aurB* RNAi. INCENP is in red, DNA is in blue, tubulin is in green and CID is in white.

Scale bars are 5µm. (C) A quantification of spindle phenotype in (B) is shown. Oocyte numbers are 18 ,23 and 24. (D) Examining Survivin localization. Survivin localizes on the central spindle in the wild type oocytes whereas in *borr:Incenp, Incenp* RNAi oocytes, Survivin localization disappeared. CID is in red, Survivin is in white, DNA is in blue and tubulin is in green. Scale bars are 5µm.

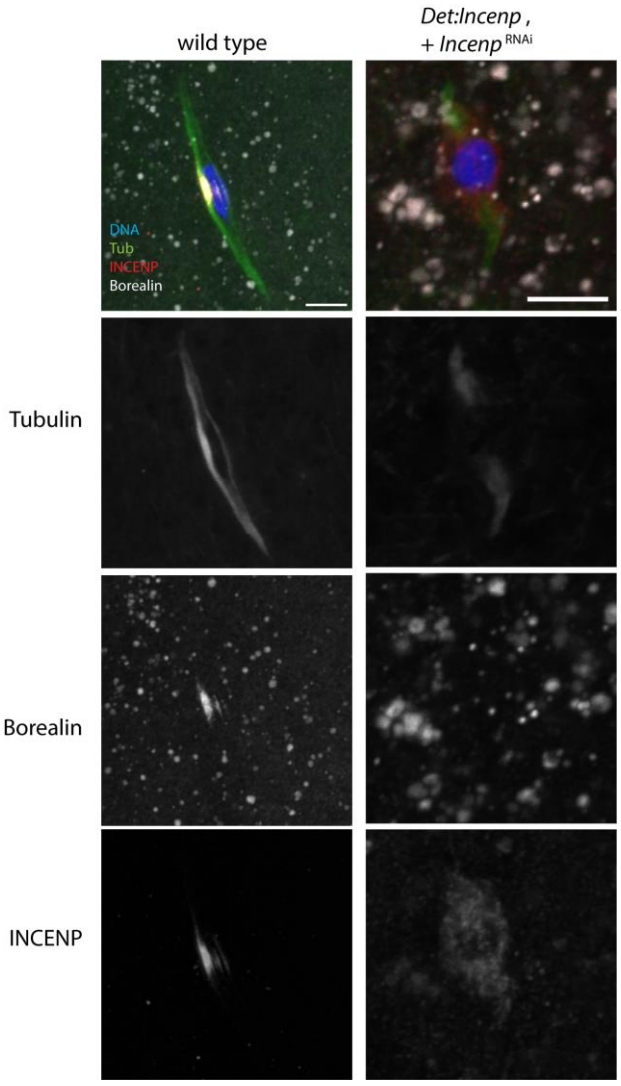

Figure 21: Borealin localization is not detectable in *Det:Incenp, Incenp* RNAi oocytes.

Borealin is in white, INCENP is in red, tubulin is in green and DNA is in blue. Scale bar is 5 µm.

HP1 recruits the CPC to the chromosome through the C terminus of Borealin and this interaction is important for spindle assembly

Survivin and Borealin are known to be recruited by the histone marks: H3T3ph and H2AT120ph respectively [168, 169]. These two histones are phosphorylated by Haspin and Bub1 kinases. When we knocked down these two kinases in oocytes by RNAi, spindle formation and CPC localization were similar to wild type (Figure 22A). Consistent with this, the K-fibers observed in *Det:Incenp, Incenp RNAi* oocytes did not depend on Haspin (Figure 23). These results suggest that the CPC is recruited to the chromosome through a pathway that does not depend on H3T3ph or H2AT120ph. Previous studies have shown that the C terminus of Borealin contains a HP1 interaction site (PxVxL) that is essential for centromere targeting while the N terminus interacts with Survivin and INCENP [170]. Additionally, we found that INCENP, Suvivin and Borealin colocalize with HP1 and H3K9me3, the histone marker that recruits HP1, in *aurB* RNAi oocytes (Figure 22B and C). These observations suggest HP1 could recruit the tripartite of the CPC through Borealin, which might subsequently initiate all the CPC's meiotic functions. To test whether this interaction is essential for spindle assembly in the oocytes, we deleted the HP1 interaction site by removing the C terminus of Borealin from *borr:Incenp* and observed the effect on spindle assembly (Figure 22D). Although 80% of the oocytes still had SPC105R localization, spindle assembly was severely impaired and only 26% of $borr^{\Delta C}$*:Incenp, Incenp* RNAi oocytes had assembled K-fibers (n=15, Figure 22E and F). Because an HP1 interaction site exists both in both Borealin and INCENP, HP1 might recruit INCENP directly, resulting in partial assembly of the spindle in $borr^{\Delta C}$*:Incenp* oocytes. To test whether these two HP1 sites are redundant, we deleted the HP1 site in *Incenp* ($Incenp^{\Delta HP1}$) and both HP1 sites in *borr:Incenp* ($borr^{\Delta C}$*:*$Incenp^{\Delta HP1}$). Interestingly, when expressing $Incenp^{\Delta HP1}$ in *Incenp* RNAi oocytes, spindle assembly was similar to wild type although INCENP displayed irregular ring shaped localization on the spindle (38%, n=13, Figure 22E and G). However, when expressing

borr$^{\Delta C}$*:Incenp*$^{\Delta HP1}$ in *Incenp* RNAi oocytes, although 80% of the oocytes had SPC105R localization (n=15), the spindle was abolished in nearly all the oocytes (n=36, Figure 22E and F). These results suggest that HP1 recruits the CPC, primarily through Borealin, to the chromosome for spindle assembly.

Our results suggest that the interaction between the CPC and HP1 in the heterochromatic regions of the chromosomes is essential for spindle assembly. Therefore, we wondered if the requirement for Borealin in this interaction could be bypassed. HP1 was fused with the N-terminus-deleted *Incenp* (*HP1:Incenp*) and spindle formation was examined. Despite observing that HP1:INCENP localized to the heterochromatin regions and 78% of the oocytes had SPC105R localization (n=23), 53% of the oocytes did not have spindle assembly and the rest only had K-fiber formation (n=49, Figure 22E). HP1-INCENP had similar KT assembly rates as INCENP$^{\Delta CEN}$ and Borr$^{\Delta C}$:INCENP$^{\Delta HP1}$, indicating KT assembly might depend on a low and /or unlocalized CPC activity. Furthermore, targeting the CPC to the heterochromatin regions without the Borealin interaction is not sufficient to build the spindle, confirming the importance of the HP1-Borealin interaction in spindle assembly. Together, all these results suggest that Borealin, Survivin and INCENP form a complex first and then are recruited to heterochromatin region through HP1-Borealin interaction.

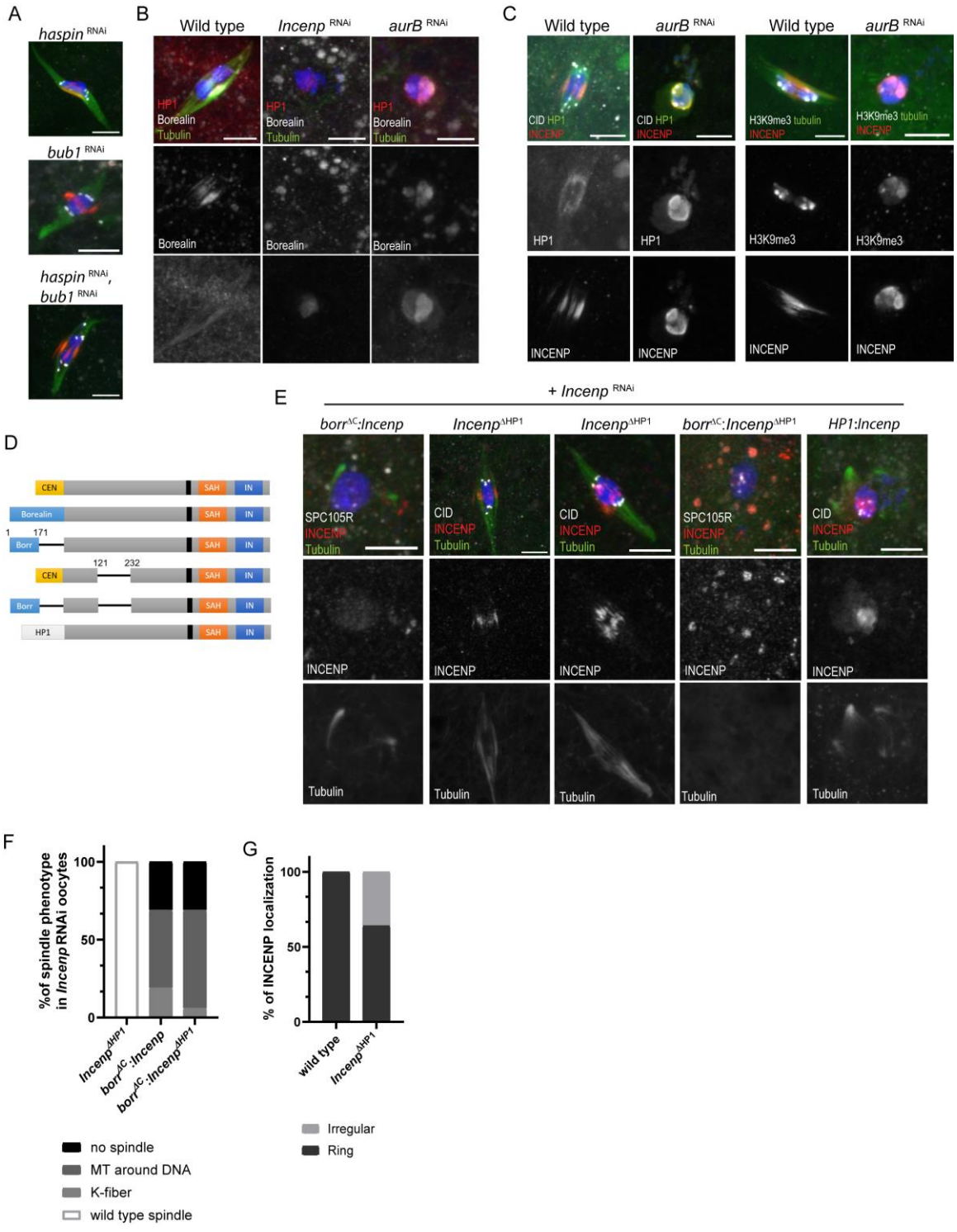

Figure 22: HP1-Borealin interaction is critical for the CPC to assemble the spindle.

(A) Knocking down both Bub1 and Haspin did not affect spindle assembly. INCENP is in red, DNA is in blue, CID is in white and tubulin is in green. Scale bars is 5 μm. (B) Borealin localization in wild type, *Incenp* RNAi and *aurB* RNAi oocytes. Borealin interacts with INCENP and Survivin before recruited by HP1. Borealin is in white, HP1 is in red, tubulin is in green and

DNA is in blue. Scale bars are 5µm. (C) HP1 localization and H3K9me3 distribution in wild type and *aurB* RNAi oocytes. HP1 is in green, INCENP is in red, DNA is in blue and CID is in white. Scale bars represent 5 µm. (D) Diagram of several *Incenp* constructs. HP1 interaction sites (PxVxL) fall in the C terminus of Borealin and INCENP between 121-232 amino acids. Full length *Drosophila* HP1 fused to INCENP by substituting CEN domain. Black bars represent *Incenp* RNAi resistant mismatch. (E) Expressing different *Incenp* transgenes in *Incenp* RNAi oocytes, which includes $borr^{\Delta C}$*:Incenp*, $Incenp^{\Delta HP1}$, $borr^{\Delta C}$*:*$Incenp^{\Delta HP1}$ and *HP1:Incenp*. The quantification of the spindle phenotype is shown in (F). $INCENP^{\Delta HP1}$ displayed irregular localization on the spindle, and the quantification is shown in (G). All the pictures show either CID or SPC105R is shown in white, INCENP is in red, DNA is in blue and tubulin is in green. Scale bars indicates 5µm. (F) Quantification of spindle phenotype in several HP1-interaction-deletion mutants. The numbers of oocytes: 17, 15 and 16 in the order of the graph. (G) Quantification of INCENP's spindle localization in wild type and $Incenp^{\Delta HP1}$, *Incenp* RNAi oocytes. Oocytes numbers are 19 and 17.

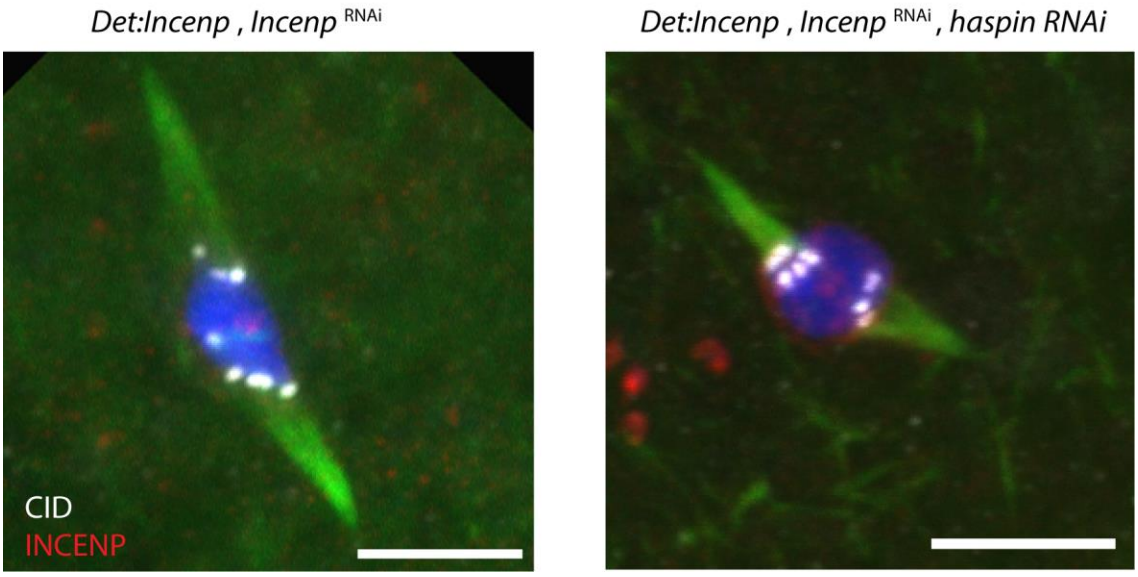

Figure 23: Depletion of Haspin does not affect Det:INCENP assemble K-fiber.

The transgene localized around the DNA, and the spindle phenotypes look alike even when oocytes knockdown Haspin. CID is in white, INCENP is in red, tubulin is in green and DNA is in blue. Scale bar is 5 µm.

Ejection of HP1 and the CPC from the chromosomes depends on microtubules

To confirm if HP1 plays a critical role in recruiting the CPC, we asked if depletion of HP1 has defects in CPC localization and spindle assembly. We first examined the oocytes with *mata-pGAL4* expressed *Su(var)205* shRNA (GL00531). This RNAi line of *Su(var)205* causes a mild knockdown (40% of mRNA remains). Despite this mild knock down *Su(var)205* which caused 21% X-chromosome nondisjunction (n=68), the oocytes displayed wild type spindle with wild type HP1 localization (Figure 24).

In mitosis, it has been shown that phosphorylation of H3S10ph by Aurora B can interfere with the interaction between H3K9me3 and HP1 [171]. This interference could therefore be the mechanism that promotes the transfer of HP1 and the CPC from the chromosome to the spindle. In support with this hypothesis, we observed that HP1 localized on the spindle in wild type oocytes while the H3S10ph signal was all over the chromosomes (Figure 22C and Figure 25A). To test the hypothesis that Aurora B activity could release HP1 and the CPC from the chromosomes, we tested if inhibiting Aurora B activity could cause retention of HP1 on the chromosomes with H3K9me3. Similar to the results in *aurB* RNAi oocytes, when oocytes were treated with the Aurora B inhibitor BN2, the spindle was diminished and the CPC localized on the chromosomes overlapping with HP1 and H3K9me3 (Figure 25B). To test if HP1 and CPC localization depends on MT, we examined oocytes treated with colchicine. When the oocyte spindle was greatly reduced following colchicine treatment, both HP1 and the CPC retreated to the chromosomes and colocalized with H3K9me3 (Figure 25C). These results suggest that the ejection of HP1 and the CPC from the chromosomes depends on the microtubules.

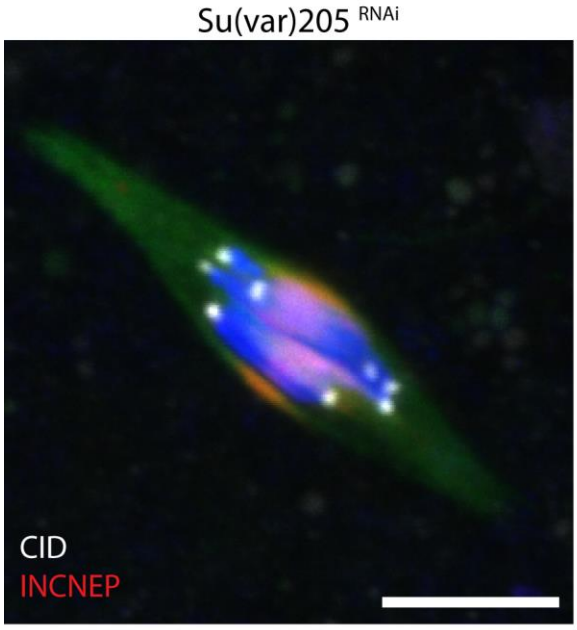

Figure 24: Knocking down Su(var)205 did not affect spindle assembly and CPC localization in oocytes.

Using shRNA GL00531 to knockdown Su(var)205, HP1α, did not cause phenotypical changes in stage 14 oocytes. CID is in white, INCENP is in red, tubulin is in green and DNA is in blue. Scale bar is 5 μm.

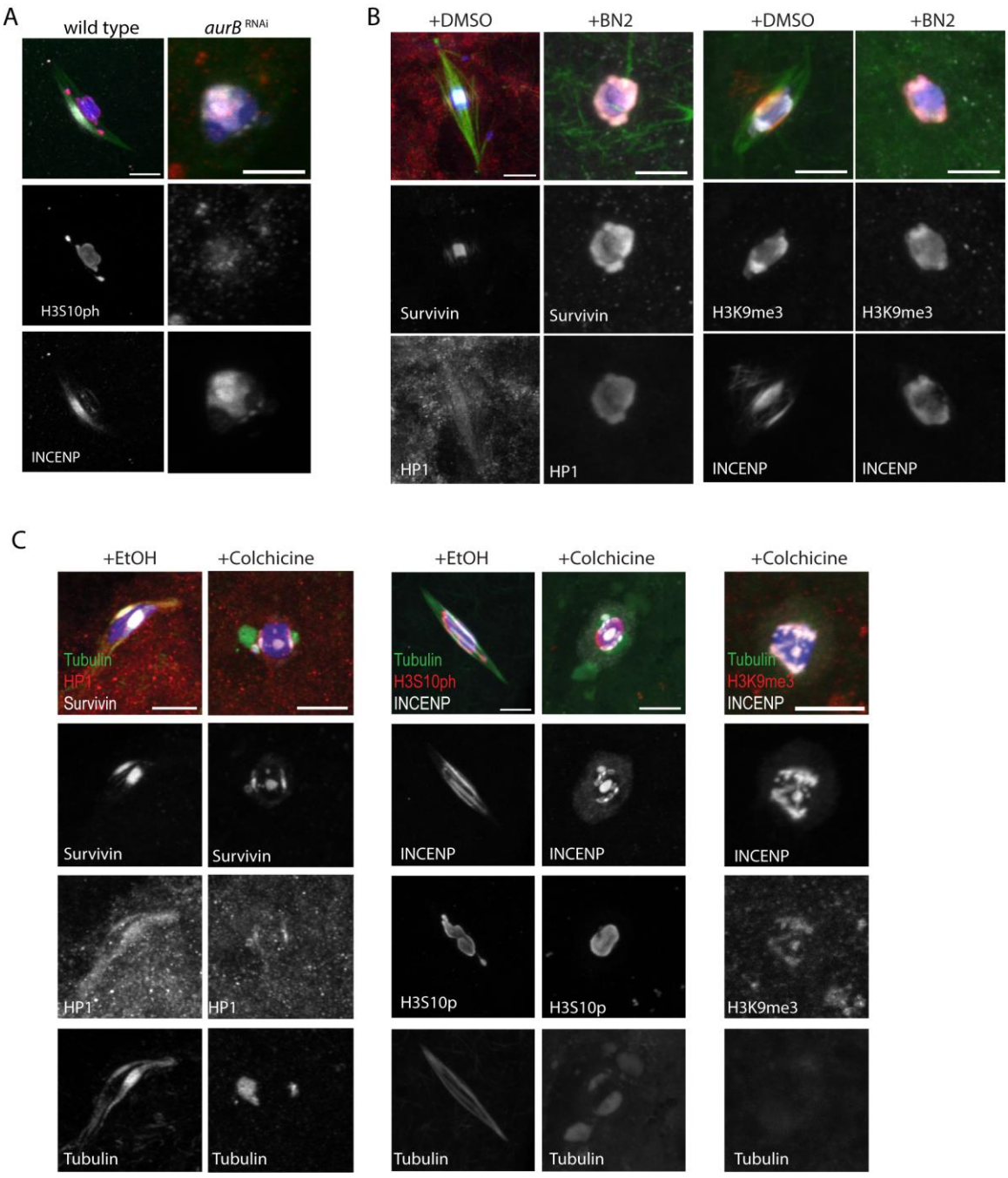

Figure 25: The relocation of HP1 and the CPC onto the spindle depends on microtubules.

(A) H3S10ph distribution in the wild type oocytes and *aurB* RNAi oocytes. INCENP is in white, H3S10ph is in red. (B) Both HP1 and the CPC retreated back to the region of H3K9me3 from the spindle after the wild type oocytes were treated with BN2 to inhibit Aurora B kinase activity. Left two panels: Survivin is in white, HP1 is in red; right two panels: H3K9me3 is in white and INCENP is in red. (C) Both HP1 and the CPC retreated to the region of H3K9me3 when the spindle was diminished in colchicine treatment. DNA was shown in blue and tubulin was shown in green in all the pictures. Scale bars represent as 5 μm in all the pictures.

INCENP SAH domain is required for central spindle assembly

Because the CPC and HP1 relocation depends on MT, we asked how the CPC interacts with MTs and whether the interaction affects spindle assembly and HP1 localization. INCENP is known to interact with MTs through its single-α-helix (SAH) domain [172], whereas another study has suggested that a conserved domain within the INCENP N-terminal region is required for spindle transfer (STD) (Figure 26A, [173]). To test if either of these MT interaction domains are important for CPC to relocate and assemble the meiotic spindle, we made transgenes with each site deleted. When expressing *Incenp*$^{\Delta STD}$ in *Incenp* RNAi oocytes, we observed wild type spindle assembly and normal CPC, HP1 and Subito localization (Figure 26B and E); however, these oocytes displayed reduced fertility and homolog bi-orientation defects. In contrast, when we expressed *Incenp*$^{\Delta SAH}$ in the RNAi background, the oocytes only had K-fiber formation without central spindle assembly and HP1 remained on the chromosomes (Figure 26C and D). While we observed the CPC primarily localized to the chromosomes (Figure 26C), occasionally we could detect the CPC colocalizing with Subito as foci in the ooplasm even though there were not any noticeable central spindle assembled (Figure 26E). These observations suggest that INCENP$^{\Delta SAH}$ can interact with Subito; however, this interaction is not sufficient to assemble the central spindle. Furthermore, the INCENP SAH domain is required for relocating the CPC and HP1 and building the central spindle.

Central spindle assembly involves both INCENP^{SAH}-MT and HP1-Borealin interactions

Subito is known to be essential for central spindle assembly in *Drosophila* oocytes [107], and genetically interacts with the CPC [70, 71]. One Subito allele, Sub$^{\Delta N}$, has been shown to have dominant effects that include an ectopic spindle phenotype, in which the spindle forms without chromosomes [174]. This ectopic spindle is bipolar with Subito and the CPC located in the center in what resembles a central spindle (Figure 26Fa). In addition, the formation of this ectopic

spindle depends on the CPC activity (Figure 26Fb). Hence, this allele provides a genetic tool to examine the CPC interactions required for central spindle formation.

We firstly examined whether INCENP SAH domain is required for ectopic spindle assembly, we expressed *Incenp*$^{\Delta SAH}$ in *Incenp* RNAi and Sub$^{\Delta N}$ oocytes. Interestingly, the ectopic spindle phenotype was suppressed when INCENP$^{\Delta SAH}$ was expressed and only a single bipolar spindle formed around the chromosomes (Figure 26Fc). This result is in line with Figure 26E, suggesting that Subito can interact with INCENP$^{\Delta SAH}$ and the INCENP SAH domain might play a role in drawing the CPC away from the chromosome through interaction with MTs. This data further implicates that the CPC builds the central spindle after interacting with MTs.

Next, we asked what other interaction within the CPC is required for the ectopic spindle assembly. We found HP1 colocalized with Sub$^{\Delta N}$ and the chromosomes in Sub$^{\Delta N}$ oocytes (Figure 27). To test whether the interaction of HP1 with the CPC is critical for this process, we want to examine the series of HP1-deleted transgenes. For this, we firstly examined if Borr:INCENP could promote ectopic spindle assembly. Similar to the result from expression in the *Incenp* RNAi oocytes, Borr:INCENP could replace wild-type INCENP protein and support ectopic spindle formation (Figure 26Fd). We then examined if ectopic spindles formed in HP1-deleted transgenes. When expressing *Incenp*$^{\Delta HP1}$ in *Incenp* RNAi; Sub$^{\Delta N}$ oocytes, ectopic spindle assembly was observed (Figure 26Fe). In contrast, when expressing *borr*$^{\Delta C}$*:Incenp* or *borr*$^{\Delta C}$*: Incenp*$^{\Delta HP1}$ in *Incenp* RNAi; Sub$^{\Delta N}$ oocytes, the ectopic spindles disappeared (Figure 26Ff and g). Most of these oocytes only had MTs cluster around the chromosome. These results demonstrate the importance of the HP1-CPC interaction, especially HP1-Borealin, even in ectopic spindle assembly that occurs in the absence of chromosomes.

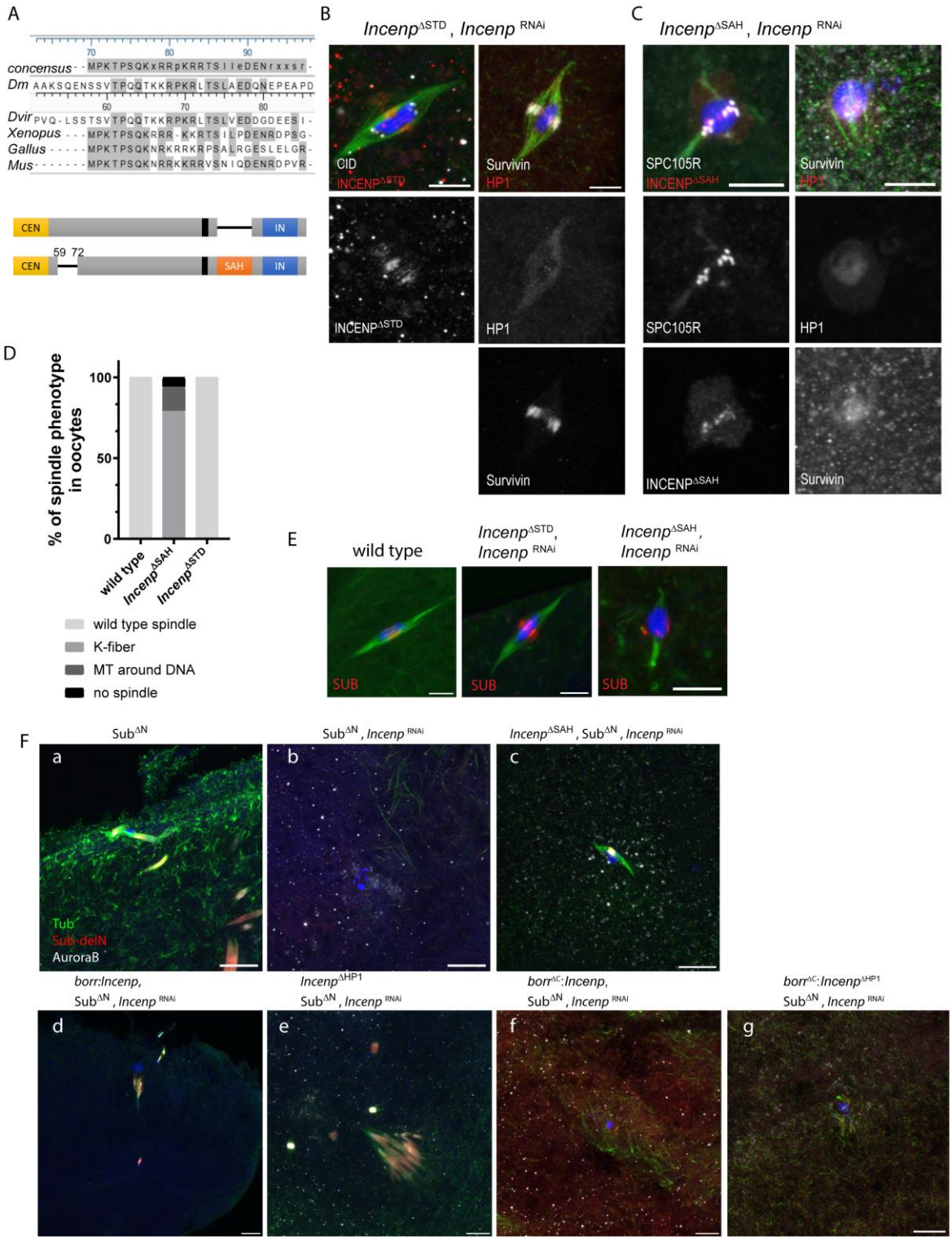

Figure 26: INCENP SAH domain is required for the central spindle assembly.

(A) Partial INCENP sequence alignment of *Drosophila melanogaster*, *D. virilis*, *Xenopus*, *Gallus*, *Mus*. A conserved region is shown between amino acid 59 to 72 in *D. melasnogaster*. A diagram shows two INCENP deletions that might have spindle interaction. (B) Expressing

Incenp$^{\Delta STD}$ in *Incenp* RNAi oocytes displayed wild type spindle. Tubulin is in green and DNA is in blue. Scale bars are 5µm. (C) Expressing *Incenp*$^{\Delta SAH}$ in *Incenp* RNAi oocytes only promotes K-fiber formation. Tubulin is in green and DNA is in blue. Scale bars are 5 µm. (D) Spindle phenotypes in INCENP$^{\Delta STD}$ and INCENP$^{\Delta SAH}$ are quantified. Oocyte numbers are 19, 72 and 20. (E) Subito localization in wild type, INCENP$^{\Delta STD}$ and INCENP$^{\Delta SAH}$ oocytes. Subito is in red, tubulin is in green and DNA is in blue. Scale bars are 5 µm. (F) Examination of ectopic spindle formation in a. Sub$^{\Delta N}$, b. Sub$^{\Delta N}$, *Incenp* RNAi, c. Sub$^{\Delta N}$, *Incenp* RNAi, *Incenp*$^{\Delta SAH}$, d. Sub$^{\Delta N}$, *Incenp* RNAi, *borr:Incenp*, e. Sub$^{\Delta N}$, *Incenp* RNAi, *Incenp*$^{\Delta HP1}$, f. Sub$^{\Delta N}$, *Incenp* RNAi, *borr*$^{\Delta C}$*:Incenp*, and g Sub$^{\Delta N}$, *Incenp* RNAi, *borr*$^{\Delta C}$*:Incenp*$^{\Delta HP1}$. Tubulin is in green, Aurora B is in white, and Sub$^{\Delta N}$ is in red. Scale bars are 10µm.

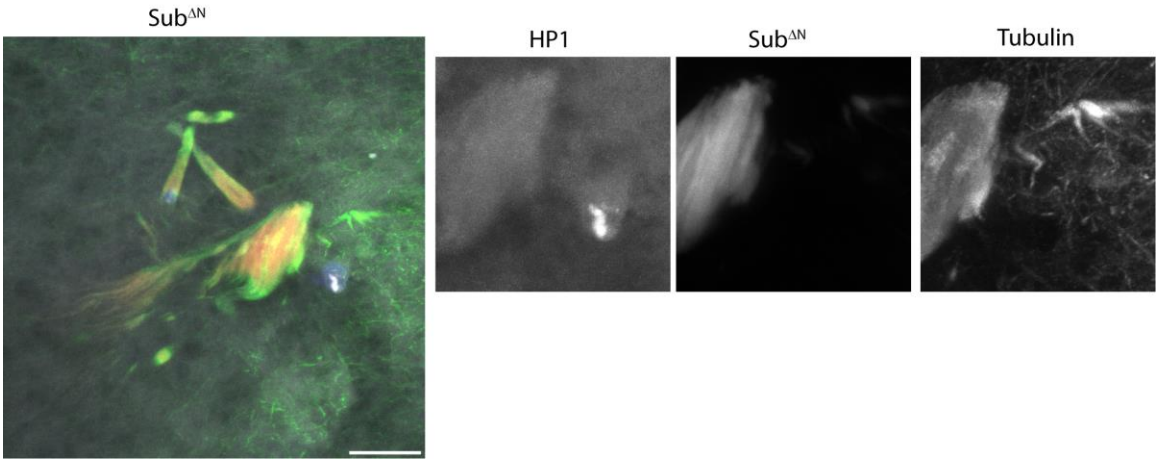

Figure 27: HP1 localization overlapped with DNA and Sub$^{\Delta N}$ in the Sub$^{\Delta N}$ oocytes.

HP1 is in white, Sub$^{\Delta N}$ is in red, tubulin is in red and DNA is in blue. Scale bar is 10 µm.

Homolog bi-orientation is regulated through central spindle and proper spindle localization of the CPC and HP1

In mitotic cells, the CPC is known to correct error attachment and achieve homolog bi-orientation by destabilizing the KT-MT attachments [164]. Similarly, the CPC in oocytes also

regulates homolog bi-orientation based on the bi-orientation defects in two hypomorphic alleles of the CPC (*Incenp*^{QA26} and *ial*^{1689}) [62]; however, while the majority of the CPC localizes on the central spindle in oocytes, the regulation remains unknown. To test if the CPC at the centromere has a similar role in regulating homolog bi-orientation by destabilizing the KT-MT attachments, we examined wild type oocytes overexpressing the CPC at the centromere (MIS12:INbox). MIS12:INbox displayed functional kinase activity by having a strong phospho-INCENP signal at the centromeres (Figure 28A). We expected that overexpressing CPC at the centromere would hyper-phosphorylate substrates that lead to unstable attachments and bi-orientation defects. However, when we tested these oocytes with colchicine to destabilize MTs, the results between the wild type and MIS12:INbox oocytes were comparable; the spindle was diminished to the same extent in both genotypes (Figure 28B and C). Furthermore, when bi-orientation was examined using FISH probes, centromere-targeting MIS12:INbox oocytes were similar to wild type (5%, n=37) (Figure 28D and E). We then asked whether the CPC regulates homologs bi-orientation from its location in the central spindle. Surprisingly, central-spindle-targeted CPC (SUB:INbox and Feo:INbox). displayed bi-orientation defects (28% in SUB:INbox oocytes, n=50 and 23% in Feo:INbox oocytes, n=30) (Figure 28D and E). These results suggest the CPC regulates homolog bi-orientation from the central spindle rather than centromere in the oocytes.

The importance of the central spindle for homologs bi-orientation can also be observed in several INCENP transgenes. All the tagged-INCENPs regardless of the charge neutrality and INCENP$^{\Delta HP1}$ had wild type spindle morphology but had defects in fertility and failed to limit CPC localization to the central spindle (Figure 17B, Figure 22E and G). Further examination by FISH showed these oocytes had a homolog bi-orientation defect. When we examined the HP1 localization in INCENP$^{\Delta HP1}$, we found even it localized on the spindle but did not overlap with the CPC (Figure 28F). This result suggests the spindle localization of HP1 is important for regulating homolog bi-orientation. Together, these results show that after HP1 and the CPC relocate onto the spindle, they are both critical for the homologs biorientation.

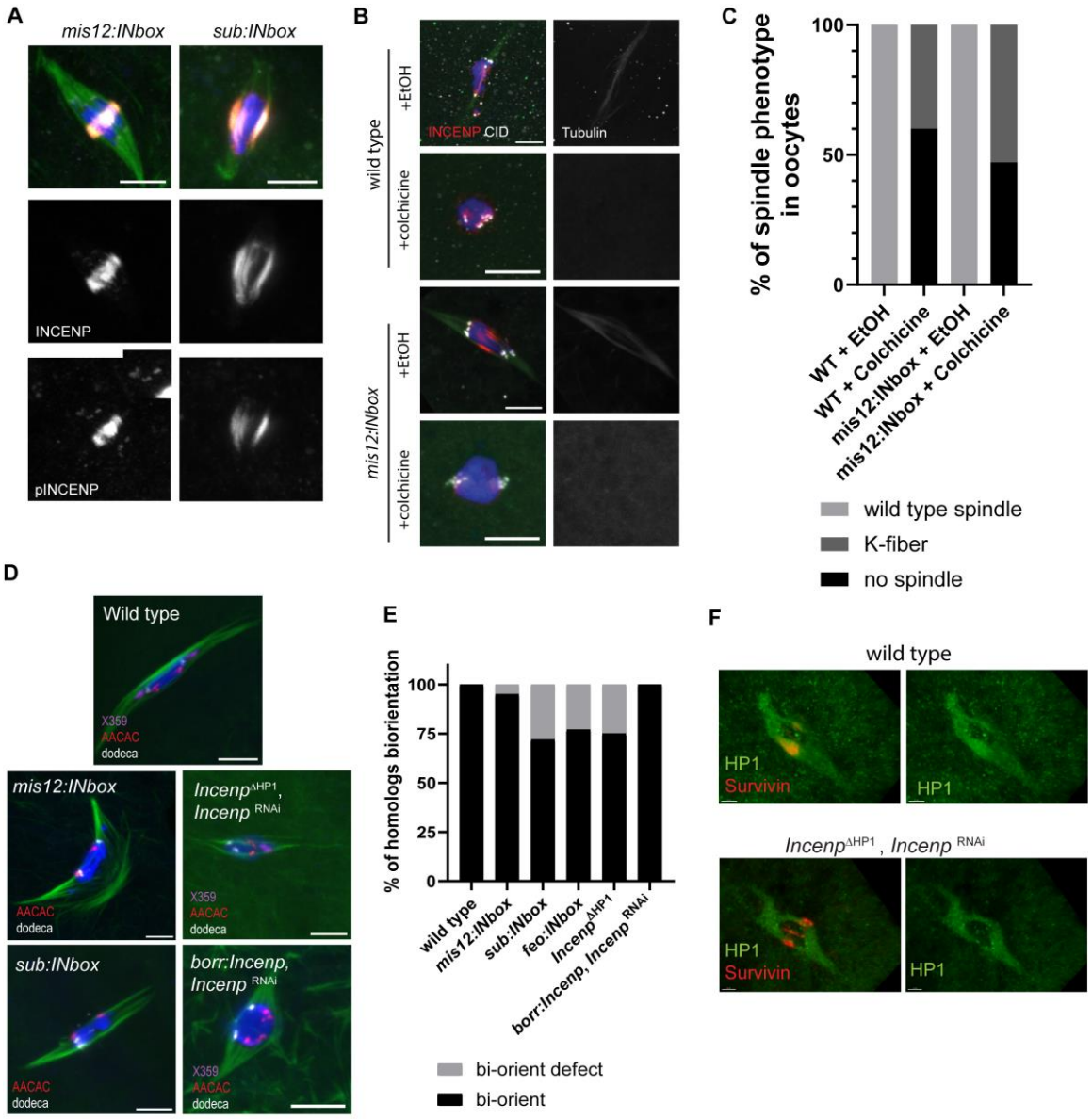

Figure 28: Homolog biorientation depends on the proper localization of the CPC and HP1 on the spindle.

(A) Localization of phosphorylated INCENP in MIS12:INbox and SUB:INbox oocytes. The Inlet shows the signal of pINCENP at the kinetochore. INCENP is in red and pINCENP is in white. Scale bars represent 5µm. (B) Wild type oocytes and MIS12:INbox oocytes treated with colchicine for 30 minutes. The spindle is diminished in the similar fashion. INCENP is in red and CID is in white. Scale bars are 5µm. Quantification is shown in (C), and the oocytes numbers are: 5,10,7 and 17 in the order of the graph. (D) Oocytes with different constructs were tested for homolog bi-orientation by using FISH assay. FISH probes against X (359 bp repeat, purple), 2nd (AACAC, red) and 3rd chromosome (dodeca, white) are used for detecting pericentromeric heterochromatin in homologs and further distinguish whether the homologs bi-orient properly or not. (E) Bi-orientation defect rates are quantified. Homolog numbers are 50, 37, 50, 30, 63 and 27 in the order of the graph. (F) HP1 and Survivin localization in wild type and *Incenp$^{\Delta HP1}$*, *Incenp*

RNAi oocytes. HP1 is in green, Survivin is in red and overlapping region is in yellow. Scale bars are 2 μm.

V. Discussion

Our research demonstrates an outline of chromosome-directed spindle assembly pathway regulated through the CPC and HP1, which previously had been suggested to be a novel partner of the CPC [175, 176]. We found that after nuclear envelope breakdown, INCENP along with Borealin and Survivin form a tripartite complex and can be recruited to the heterochromatin regions marked by H3K9me3 through a Borealin-HP1 interaction and independent of Aurora B. We propose that, once the tripartite structure locates onto the chromosomes, it recruits Aurora B and promotes several activities, including phosphorylation of H3S10, assembly of the kinetochore and the formation of K-fibers. Similar to research in mitosis [171], we propose that in oocytes, H3S10 phosphorylation weakens the interaction between Borealin-bound HP1 and H3K9me3. Meanwhile, the INCENP SAH domain provides the strong force for the CPC to associate with MTs [177], which is essential for releasing the CPC from chromatin and building the central spindle. When the CPC-HP1 complex move onto the MTs, the complex interacts with central spindle nucleating proteins, such as Subito, to form the central spindle (Figure 29).

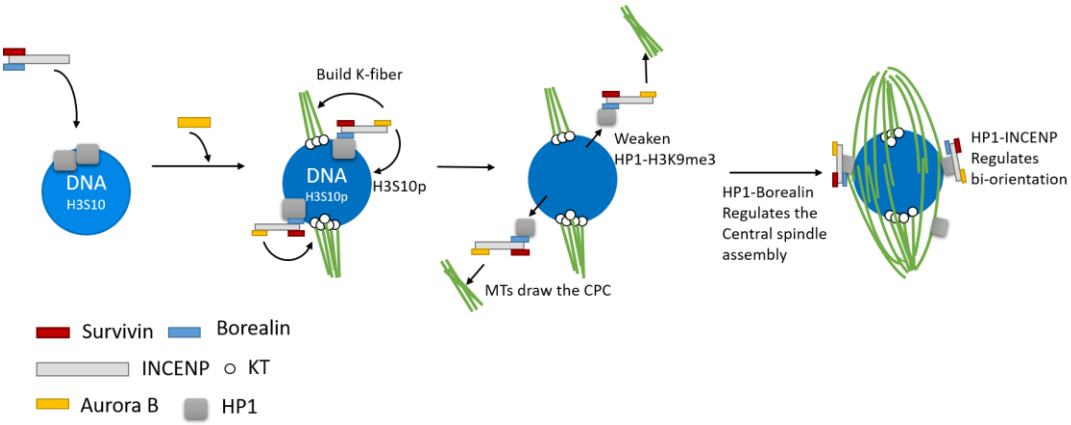

Figure 29. Model of spindle assembly in oocytes.

CPC-dependent spindle assembly pathway in oocytes

Our findings suggest that the meiotic spindle assembly depends on two independent pathways: the K-fibers and the central spindle. K-fiber formation has been observed in mitotic cell; however, this pathway is not predominant in the presence of centrosomes [178-180]. While the regulation of K-fiber assembly mostly focuses on Ran pathway [181-183], the mechanism is still very unclear. We found that in *Drosophila* oocytes, the growth of K-fibers depends on the CPC, especially through Survivin and Borealin. Our observations have shown that Survivin-INCENP and Borealin-INCENP can promote KT and K-fiber assembly without the tripartite complex forming. These results suggest that both Survivin and Borealin can still be recruited to heterochromatin through INCENP HP1 domain. However, when forcing the CPC to heterochromatin regions (HP1:INCENP) by substituting the CEN domain with HP1, KTs can form but K-fiber assembly is not efficient. This result firstly in line with a study in *Xenopus* egg extracts suggesting the KTs can assemble independently to the CPC's centromere localization [184]. Furthermore, it indicates that in order to build K-fibers, Survivin and Borealin need to be recruited to the centromeres to cluster the CPC and function more efficiently. Interestingly, the conventional pathways of recruiting Survivin and Borealin to the centromere via Haspin and

Bub1 does not seem to be essential in *Drosophila* oocytes (Figure 22A). One possibility is that the trace remaining proteins in these RNAi experiments was sufficient to direct Survivin and Borealin to the centromeres. Another possibility is that in oocytes the regulation of recruiting CPC to the centromeres might be different from mitotic cells. Indeed, Haspin has been shown to recruit the CPC to the pole in mouse oocytes [185]; however, an alternative pathway to recruit the CPC to the centromeres has not been described.

A recent study in *Drosophila* oocytes has revealed a new pathway to promote spindle assembly, where Augmin recruits Dgp71WD/Nedd1, a recruiter of r-tubulin complex. Subito also plays a role in recruiting Dgp71WD through its N-terminal region to nucleate MTs [186]. Both of these pathways can regulate the localization of Dgp71WD and construct the spindle. Indeed, Subito has been shown to have the ability to induce spindle assembly in meiosis: a misregulated Subito allele, $Sub^{\Delta N}$, can promote ectopic spindle formation without the chromosomes [174]. Here in this study, we show that this ectopic spindle assembly is CPC-dependent, suggesting Subito functions downstream of the CPC to assemble MTs. Interestingly, when Subito is deleted, the mutant spindle appears to contain more than just K-fibers [107], suggesting there are additional CPC substrates on the spindle. Hence, it is possible that the Augmin pathway could also be regulated by the CPC by nucleating from the existing K-fibers. This hypothesis can be supported by the findings that Augmin interacts with NDC80 to promote K-fiber formation in *Drosophila* mitotic cells [187], and no stable spindle is formed when co-depleted SPC105R and Subito in *Drosophila* oocytes [90]. Together, these results demonstrate there are two pathways for assembling the spindle, one from the centromeres to assemble K-fiber and the other from Subito to build the central spindle.

The spindle is restricted to assemble around the chromosome and the knowledge of this regulation is still limited [186, 188, 189], especially in the acentrosomal system. The RanGTP pathway could provide an ideal explanation, where RanGTP forms a gradient centered from the chromosome [56]; however, the RanGTP pathway may not be essential for spindle assembly in

Drosophila oocytes. Hence, how the CPC restricts meiotic spindle assemble around the chromosome remains unclear. The misregulated Subito, $Sub^{\Delta N}$, displayed the CPC-dependent ectopic spindle, suggesting the N terminus of Subito is the key to restrict the spindle forms around chromosome [174], either through regulating its own activity by an auto-inhibitory mechanism [70, 190] or through chromosome interactions. In this paper, we found that HP1 is associated with Subito in the $Sub^{\Delta N}$ oocytes (Figure 27), suggesting HP1 might assist the ectopic spindle formation along with the CPC. This result explains why the ectopic spindle assembly always coincide with nuclear envelope breakdown possibly because the chromosomes licenses it through eliciting HP1.

The meiotic role of HP1

HP1 has been discovered to interact with the CPC directly in *Drosophila* [191] and to assist the CPC localization and its function [192]. Early research in HeLa cells found that HP1 assists the CPC in releasing from the heterochromatin regions to the central spindle [173]. A recent study in HeLa cells, on the other hand, demonstrated that HP1 targets the CPC to the heterochromatin regions for activation before mitotic entry and allowing the CPC to be redirected to the kinetochore [176]. These results indicate a variety of HP1 and the CPC localizations and functions. Our work is in accordance with these findings, we found that an HP1-Borealin interaction plays a major role in localizing the CPC, including recruiting the CPC to the chromatin and then releasing the CPC to relocate onto the spindle. In addition to these observations, we discovered HP1's novel function in regulating homolog bi-orientation in oocytes. Oocytes expressing $Incenp^{\Delta HP1}$ had defects in bi-orientation and the localization of HP1 and INCENP on the spindle, supporting a spindle-associated function for HP1. Based on the observation that $INCENP^{\Delta HP1}$ had irregular localization (Figure 22E and G), HP1 might be required for establishing or maintaining the central spindle structure. In addition, in $Incenp^{\Delta HP1}$oocytes, the localization of INCENP and HP1 were not overlapped (Figure 28F) even

though the HP1 binding site in Borealin was still intact, suggesting their localizations are not dependent to each other. These results indicate that INCENP- HP1 interaction becomes critical once the CPC and HP1 moves onto the spindle.

So how does HP1 regulate these meiotic processes with the CPC? HP1 is known to form dimers through its chromo shadow domain (CSD) [193]. At the same time, the CSD can interact with proteins containing a conserved binding site (PXVXL) [194]. This interaction might possibly bring the candidate proteins together, such as INCENP and the Aurora B phosphorylation substrates. MKLP2 (Subito homolog) has been shown to be required for the CPC's translocation from the centromeres to the central spindle in mitotic cells, and this process depends on a direct interaction between INCENP and MKLP2 [195-197]. While Subito is also required for CPC's translocation to central spindle in mitotic cell [197], a direct interaction in *Drosophila* oocytes remains elucidated. Interestingly, Subito has a conserved HP1 binding site (Figure 30) and a point mutation in this HP1 interaction domain causes female sterility [107, 174]. This strongly implies Subito interacts with HP1 and, therefore, could be regulated by the CPC from this interaction. If HP1 plays a role in regulating CPC's substrate phosphorylation, then there may be other CPC's substrates on the spindle because HP1 localizes along the length of the spindle. Thus, investigating possible CPC substrates through the CSD conserved binding site might be an alternative way to understand its regulation. In addition to the CSD, the chromo domain (CD) of HP1 is known to interact with H3K9me3; however, HP1 has been reported to participate in DNA repair process in a CD-independent regulation [198]. Therefore, whether HP1's spindle regulation is CD-independent, or the CD has novel interactions with spindle proteins needs further investigation. Lastly, a histone lysine methyltransferase, Su(var)3-9, is known to regulate H3K9me3 in *Drosophila* [199]. It would be interesting to knock out Su(var)3-9 to test whether recruiting HP1 onto the chromosome is essential for spindle assembly. Alternatively, *Drosophila* has five HP1 paralogs and their localization patterns differ from each other. In mitotic cells, HP1a/Su(var)205 localizes to the heterochromatin, HP1c localizes to

euchromatin, and HP1b localizes to both heterochromatin and euchromatin [200-204]. HP1d/Rhino is expressed only in the ovary and HP1e is only in testis [201, 205]. Because all the subtypes of mammalian HP1s have been shown to interact with INCENP [175] and their localization varies spatiotemporally (HP1α localizes at centromere in metaphase whereas HP1β localizes in interphase) [203], testing the different *Drosophila* paralogs for their effect on CPC's meiotic localization and function will provide a more detailed analysis of the regulation of meiosis and the MTs recruitment site(s) on the chromatin.

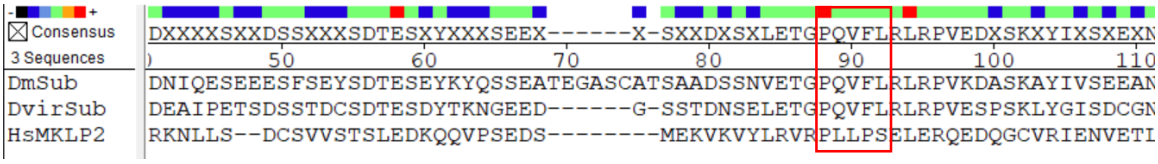

Figure 30. The HP1 interaction site in Subito.

CPC's regulation of Homolog Bi-orientation

Bi-orientation of meiotic chromosomes has been suggested to be regulated through the central spindle [70]. Our results are consistent with this finding by showing the misregulated CPC on the central spindle, but not the kinetochores, disrupts bi-orientation. Although we observed a strong pINCENP signal at the centromeres in metaphase I oocytes (Figure 28A), the centromere-targeting of the CPC in meiosis did not cause KT-MT destabilization as predicted from the literature (Figure 28B and 7C, [164]). This could be explained by the recruitment of phosphatases such as PP1 or PP2A to the centromeres [134, 206, 207], thus the high levels of the CPC at the centromere did not show more sensitive to the MT destabilizing drug.

A functional central spindle for homolog bi-orientation relies on the intricate regulation involving the N-terminus INCENP. The first evidence comes from deleting the HP1 interaction site (121-232 amino acid) of INCENP. INCENP$^{\Delta HP1}$ in oocytes displayed irregular CPC central

spindle localization and loss of HP1 colocalization with the CPC (Figure 25E and Figure 28F), indicating the structure of the central spindle might be compromised, therefore leading to a homolog bi-orientation defect. Additional evidence comes from the mislocalization of epitope-tagged INCENP proteins (Figure 18B, Figure 19 and [62]). These N-terminal tagged INCENPs have defects in homologs bi-orientation and fertility. Previous findings have shown that the positive charges of Borealin balanced the conserved negative charges in the N-terminus of INCENP and this affects CPC localization [166]. However, changing from negatively charged tags (Myc- tag and Flag- tag) to more a neutral charged tag (HA) displayed the same results (Figure 19), suggesting the charge of the N- terminus tag is not the cause for the phenotype, but it is the tag itself. In addition, the conserved spindle transfer domain in INCENP also affects the homolog bi-orientation. INCENP$^{\Delta STD}$ has been shown to be required for the CPC's central spindle relocation in mitosis [173]; however, INCENP$^{\Delta STD}$ oocytes did not display obvious defect in Subito and INCENP localization but had defect in homolog bi-orientation. Whether deleting STD affects the tripartite interaction because the STD is adjacent to the CEN domain, or STD has a novel interaction that is crucial for regulating homolog bi-orientation is an interesting question. Together, these results show that the N-terminus INCENP not only affects the CPC and HP1 localization but also plays an important role for a functional central spindle.

Our results provide a mechanism for how the CPC promotes acentrosomal spindle assembly and bi-orientation. The chromosome-based recruitment and regulation of the CPC in oocytes is based mechanism that depends on HP1. We propose that HP1 in oocytes functions as an important targeting protein to regulate its activity spatiotemporally in order to assemble microtubules around the chromosomes, assemble a central spindle ensuring bipolarity, and promoting the bi-orientation of homologs.

VI. Method and materials

Generation of RNAi resistant INCENP

Incenp cDNA (RE52507) from *Drosophila* Genomic Resource Center was cloned into pENTR (Invitrogene, Carlsbad, CA). In order to express this Incenp construct in *Incenp* RNAi oocytes, we created 8 mismatches by Change-it Site-directed Mutagenesis kit (Affymetrix) at the site that is complementary to *Incenp* shRNA (GL00279) to prevent from silencing. The primers for the site-directed mutagenesis are: 5'-ATGAGCTTTTCAACCCACTcCTgCAGtcgCCcGTcAAgATGCGCGTGGAGGCGTTCGA -3' and 5'-TCGAACGCCTCCACGCGCATCTTGACGGGCGACTGCAGGAGTGGGTTGAAAAGCTCATG -3'. RNAi resistant INCENP constructs including Myc-INCENP, HA-INCENP and Flag-INCENP were further generated by using the LR Clonase reaction (Gateway systems, Invitrogen) to the pPMW, pPHW or pPFW vector that carries UASp-promoter. INCENP^{mycless} was generated by removing the Myc-tag in Myc-INCENP using Gibson Assembly kit (New England Biolabs). INbox constructs were generated by taking the last 101 amino acids (655-755) of INCENP including INbox and TSS activation site. Fusion proteins of INCENP were created by using MIS12 cDNA (RE19545), Survivin cDNA (LP03704), Su(var)205 cDNA (LD10408) and Borealin cDNA (LD36125) the constructs were injected into *Drosophila* embryos through Model System Injections (Durham, NC).

Drosophila genetics and RNAi

Flies were crossed and maintained on the standard media at 25℃. All loci information was obtained from Flybase. Flies stocks were obtained from the Bloomington Stock Center or the Transgenic RNAi Project at Harvard Medical School (TRiP, Boston, USA), including *aurB*

(GL00202), *Incenp* (GL00279), *Su(var)205* (GL00531) and *Bub1* (GL00151). $Sub^{\Delta N}$ allele was from [174].

All the short hairpins for RNA silencing and transgenes were carried UAS promoter for UAS/GAL4 binary expression system [78]. All these short hairpin RNA lines and transgenes using in this paper were expressed by *mata4-GAL-VP16*, which induces expression after early pachytene throughout most stages of oocyte development in *Drosophila*.

For quantifying the knockdown of these RNAi lines, total RNA was extracted from late-stage oocytes using TRIzol® Reagent (Life Technologies) and reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The qPCR was performed on a StepOnePlus™ (Life Technologies) real-time PCR system using TaqMan® Gene Expression Assays (Life Technologies). Dm03420510_g1 for *haspin*, Dm0103608_g1 for *Su(var)205*, Dm02141491_g1 for *Deterin* and Dm02134593_g1 for the control *RpII140*.

Generation of Survivn and Haspin shRNA lines in *Drosophila*

To generate a Survivin and Haspin shRNA line, a targeted Survivin sequence (5'-CGGGAGAATGAGAAGCGTCTA -3') and a targeted Haspin sequence (5'-GGAAGACAGTAGAGACAAATG- 3') were cloned into pVALIUM22 respectively, based on the protocol described in Harvard TRiP center. The construct was injected into Drosophila embryos (y sc v; attP40). The mRNA was further measured to confirm the mRNA depletion to 5% for Survivin and 15% for Haspin when using *mata4-GAL-VP16* to express the shRNA in the germline.

Antibodies and immunofluorescence microscopy

Mature (stage 12-14) oocytes were collected from 100-200, 3-4 day old yeast-fed non-virgin females. The protocol is described in [77]. Hoechst 33342 (10ug/ml, Invitrogen) was used for staining DNA and mouse anti-a tubulin monoclonal antibody DM1A(1:50) conjugated with FITC (Sigma, St. Louis) was used for staining MTs. Primary antibodies used in this paper were rabbit anti-CID (1:1000, Active motif), Guinea pig anti-MEI-S332 (1:300, [152]), rabbit anti-SPC105R (1:4000, [155]), rabbit anti-CENP-C (1:5000, [153]), mouse anti-Myc (1:50, 9E10, Roche, Indianapolis), mouse anti-Flag (1:500, Thermo Fisher), rat anti-INCENP (1:400, [151]), rabbit anti-Aurora B (1:1000, [208]), rabbit anti-Survivin (1:1000, [154]), rabbit anti-Borealin (1:100, [209]), mouse anti-HP1 (1:50, C1A9, Developmental Hybridoma Bank), rabbit anti-H3K9me3 [1:1000, Active motif], rabbit anti-H3S10ph (1:1000, Active motif), rat anti-Subito (1:75, [107]), rat anti-a-tubulin (Clone YOL 1/34, Millipore) and rabbit anti-pINCENP (1:1000, [210]). The secondary antibodies including Cy3 and AlexFluor647 (Jackson Immunoresearch West Grove, PA) or AlexFluor488 (Molecular Probes) were used in accordance with the subjected primary antibodies. FISH probes were designed against X-chromosome (359 repeats), 2nd chromosome (AACAC satellite) and 3rd chromosome (dodeca satellite) obtained by Integrated DNA Technologies. Oocytes were mounted in SlowFade Gold (Invitrogen). Images were collected on a Leica TCS SP8 confocal microscope with a 63x, NA 1.4 lens, and shown as maximum projections of complete image stacks. Images were then cropped in Adobe Photoshop (PS6).

Drug treatment assay

To inhibit Aurora B Kinase activity, oocytes were treated by either 0.1% DMSO or 50uL BN2 in 0.1% DMSO in 60 minutes before fixation in Robb's media. To test MTs stability, oocytes were incubated in 250 uM colchicine in 0.5% ethanol or just 0.5% ethanol as control for 15 or 30 minutes before fixation.

Chapter 4

Discussion

Accurately executing chromosome segregation in meiosis depends on a combination of sophisticated regulations, and many of them remain unclear. My research addressed two fundamental questions of meiosis: the regulation of sister centromere fusion in metaphase I and the formation of the acentrosomal spindle in *Drosophila* oocytes.

In chapter two, we found that sister centromere fusion was regulated by Separase, suggesting the fusion in *Drosophila* depends on cohesin proteins that are similar to the discoveries in other organisms. We also discovered that, in addition to cohesion, the stability of kinetochore-microtubule attachments is a key to regulate the fusion and release of sister centromeres from metaphase I to metaphase II. In the second project we discovered that HP1 plays a critical role in assisting the CPC to promote the chromosome-mediated spindle assembly and homologous chromosome bi-orientation. We propose that HP1 acts as a novel subunit for the CPC to target to the chromatin and the spindle in the oocytes.

The key regulation revealed by both projects happens in the centromere/kinetochore regions. SPC105R/KNL is the scaffold kinetochore protein. It is required in *Drosophila* oocytes for assembling the outer kinetochore, lateral MT attachments, regulating sister centromere fusion and importantly, providing a platform for the regulatory proteins including Bub1, BubR1 and PP1 at the centromeres. PP1-87B in mitosis is recruited to the KNL/SPC105R N terminal RISF motif to regulate microtubule attachments against Aurora B in mitosis [207]. Although we showed PP1-87B and SPC105R function in two different pathways to regulate sister centromere fusion in meiosis, it is still possible that PP1-87B is recruited to the kinetochore via SPC105R then onto the spindle. This recruitment might not only antagonize Polo but also antagonize the CPC in the

regulation of stable MT attachments. This conclusion arises from our observation that overexpressing the CPC in the centromere (MIS12:INbox) did not cause MTs more sensitive to colchicine and the sister centromere fusion defect in *Pp1-87B* RNAi oocytes can be suppressed after BN2 treatment, indicating the possible phosphatase antagonism happens in the centromere. Additionally, SPC105R plays a role in protecting cohesin from Separase to regulate sister centromere fusion. SPC105R domain analysis will provide insights into the regulation of cohesion protection, possibly the regulation of Separase irrespective of its role as a cysteine protease or a novel function in centromere, and the other functions we mentioned above.

The conventional pathways to recruit the CPC to the centromere, on the other hand, do not seem to be essential in *Drosophila* oocytes. Several observations have laid out the different requirements of the CPC for kinetochore assembly and the K-fiber formation. First, low and/or unlocalized CPC activity is sufficient to assemble the kinetochore. Similar to the conclusion in *Xenopus* egg extract [184], several transgenes including INCENP$^{\Delta CEN}$ still had higher percentage of oocytes assemble the kinetochore than *Incenp* RNAi oocytes suggesting that kinetochore assembly does not depend on the CPC centromere localization. Another possibility is these transgenes were overexpressed and the trace amount of the CPC activity was enough to assemble the kinetochore but not K-fibers. This hypothesis can be supported by the observations that about thirty percent of *Incenp* RNAi oocytes assemble kinetochore (n=32) and thirty percent of wild type oocytes had kinetochores after the BN2 treatment (Arunica Das's unpublished data); however, how the CPC regulates the kinetochore assembly needs to be tested.

Secondly, the role of Survivin and Borealin for the CPC localization/activity is critical for K-fiber formation regardless of HP1 localization because the HP1:INCENP did not assemble K-fiber efficiently. What controls the localization of the CPC at the centromere/ kinetochore in *Drosophila* oocytes remains an interesting question. Recent studies have revealed several pathways to recruit the CPC to the centromere: in budding yeast, an inner centromere protein

complex (COMA) can recruit the CPC to centromere independent to Bub1 and Haspin [211]; in *Xenopus* egg extract, both CENP-C and CENP-T can localize Bub1 [212]. Although *Drosophila* is lacking most of the centromere proteins in the CCAN complex [213], this study opens up a possibility that the CPC localize by other unknown pathways. Alternatively, Bub1 and BubR1 might play a redundant role to recruit the CPC, therefore, the spindle assembled regularly when co-depleting Haspin and Bub1 in the oocytes. Testing double and triple mutants might give some insights of the CPC's centromere recruitment pathway.

In chapter two, we observed that the separated chromosome mass phenotype in *Pp1-87B* RNAi oocytes was suppressed by *Spc105R* RNAi and BN2 treatment, suggesting that lateral attachments and Aurora B regulate chromosome movement against PP1. Interestingly, this phenotype is very similar to the previous report where Cmet and Cana, the two CENP-E orthologs, direct chromosome movement and homolog bi-orientation through lateral attachment in *Drosophila* oocytes [90]. These results suggest a hypothesis that Aurora B- PP1 antagonism could regulate these processes through CENP-E. Indeed, a report in a human cell line has shown that PP1 can bind to CENP-E and reverse Aurora B phosphorylation at T422 [214]. Interestingly, I found this phosphorylation site only present in Cmet (T395) but not in Cana (Figure 31). According to a previous report, Cana only affects chromosome alignment but not homolog bi-orientation [90]. Although the sample number of Cana mutant in the paper was low and the result needs to be repeated, it indicates that there might be a differential regulation between two CENP-E homologs in *Drosophila*.

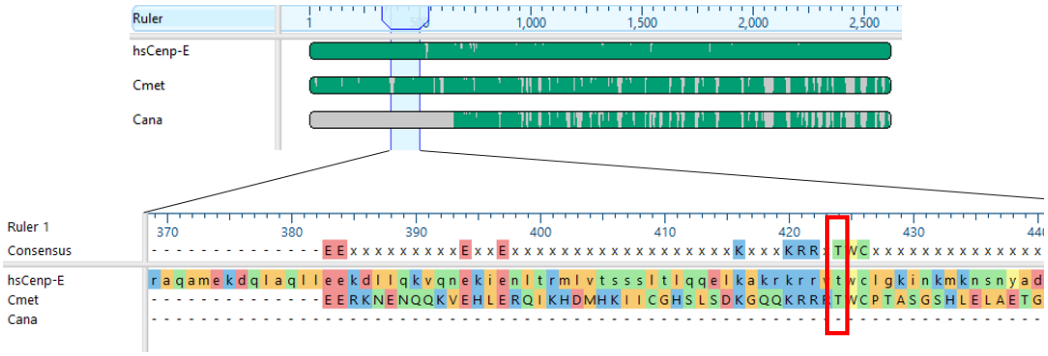

Figure 31. Alignment of Cenp-E and the conserved site that interacts with both PP1 and Aurora B

The regulation of homolog bi-orientation depends on the functional central spindle that includes proper localizations of the CPC and HP1 (Figure 28). An *Incenp* hypomorphic allele (*Incenp*^{Q26A}) has homolog bi-orientation defects even though the protein localizations on the central spindle was normal [162]. This allele has a point mutation in the INbox, that might weaken the Aurora B interaction. The compromised CPC function could result in dysfunctional central spindle, which agreed with the published result that the MT intensity on the central spindle was declined [162]. There are two other hypomorphic alleles: *Incenp*^{P746L}, whose mutation is closed to Aurora B activation TSS motif, and an Aurora B hypomorphic allele (*ial*^{1689}), whose mutation is at a conserved proline in the N terminus [162, 215]. They showed either female sterility or have defects in homolog bi-orientation, suggesting their role in forming functional central spindle. However, examining the CPC's localization or spindle phenotype in oocytes remains to be done.

We showed that when the HP1 interaction site within Borealin and INCENP was deleted, spindle assembly and homolog bi-orientation were affected. We have not been able to knockdown HP1 effectively. Because HP1 plays several important roles in different stages of oocyte development, a pleiotropic mutant phenotype is expected if knocking it down successfully. Therefore, through abolishing the interaction site within Borealin and INCENP, the results

differentiate this specific function of the HP1. Another option to examine HP1's separated function is a temporal controllable or inducible HP1 CSD or $HP1^{I165E}$ transgene [216]. This transgene is expected to have dominant negative phenotype similar to the previous results [217] because it can bind to the substrates but fail to localize or dimerize, and provide a good knockdown in specific timing. In addition, based on HP1's spindle localization, it is possible the HP1 interacts with multiple proteins including INCENP and Subito on the spindle. These interactions might be a new way to cluster the CPC's substrates and regulate their protein activities. Identifying these possible targets through mutating their HP1 interaction sites could be an avenue for understanding the CPC regulation.

Oocytes are known to have higher aneuploidy rate comparing to sperms. It has been reported that the separated sister centromeres is commonly seen in the aged oocytes, suggesting aged oocytes have a higher chances to loss centromeric cohesion loss which leads to aneuploidy [5-7]. In addition to the age effect, the development of the oocytes also makes oocytes more susceptible to error. The lack of centrosomes makes oocytes develop other mechanisms to assemble the spindle; however, it takes a longer time to assemble the spindle. This long process is accompanied by spindle instability and kinetochore-microtubule mis-attachment, both creating a susceptibility for errors to occur in oocytes [52]. Researchers have observed that human oocytes resemble to fly oocytes in which they both lacks MTOCs and chromosome, instead, directs their meiotic spindle assembly [52], suggesting that *D. melanogaster* is an ideal model organism to address on the topic in the acentrosomal spindle assembly. My research used *D. melanogaster* oocytes to understand both the oocyte spindle assembly and the regulation of sister centromere fusion in meiosis I. Despite that there are still many unanswered questions, my work provides the basis for new models describing these important meiotic mechanisms that leads to a better understanding of female meiosis.

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