The Chromosomal Passenger Complex Is Required for Meiotic Acentrosomal Spindle Assembly and Chromosome Biorientation

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ABSTRACT During meiosis in the females of many species, spindle assembly occurs in the absence of the microtubule-organizing centers called centrosomes. In the absence of centrosomes, the nature of the chromosome-based signal that recruits microtubules to promote spindle assembly as well as how spindle bipolarity is established and the chromosomes orient correctly toward the poles is not known. To address these questions, we focused on the chromosomal passenger complex (CPC). We have found that the CPC localizes in a ring around the meiotic chromosomes that is aligned with the axis of the spindle at all stages. Using new methods that dramatically increase the effectiveness of RNA interference in the germline, we show that the CPC interacts with *Drosophila* oocyte chromosomes and is required for the assembly of spindle microtubules. Furthermore, chromosome biorientation and the localization of the central spindle kinesin-6 protein Subito, which is required for spindle bipolarity, depend on the CPC components Aurora B and Incenp. Based on these data we propose that the ring of CPC around the chromosomes regulates multiple aspects of meiotic cell division including spindle assembly, the establishment of bipolarity, the recruitment of important spindle organization factors, and the biorientation of homologous chromosomes.

DURING cell division, chromosomes interact with a bipolar array of microtubules and associated proteins that constitute the spindle. These interactions serve to physically separate chromosomes along the spindle axis toward the spindle poles, resulting in the partitioning of chromosomes into daughter cells. During mitotic cell division in many cell types, two centrosomes are the predominant sites of microtubule organization and define bipolarity during spindle assembly. In contrast, meiotic spindle assembly in the females of many species proceeds without centrosomes (Szollosi *et al.* 1972; Theurkauf *et al.* 1992; Albertson and Thomson 1993). Instead, microtubules accumulate around the chromosomes, and spindle poles are organized and extended outward in the absence of any obvious cues that establish bipolarity.

The chromosomes, therefore, replace the centrosomes in two distinct processes, often grouped together under the

term “spindle assembly”: they recruit or nucleate microtubules and direct the organization of a bipolar spindle. In *Xenopus laevis* egg extracts lacking centrosomes, chromatin-induced spindle assembly is dependent on RanGTP (Carazo-Salas *et al.* 1999) and the chromosome passenger complex (CPC) (Sampath *et al.* 2004). The CPC is composed of Incenp, Aurora B kinase, Deterin (also known as Survivin), and Borealin and has a diverse range of functions in chromosome–microtubule interactions, sister-chromatid cohesion, cytokinesis, and others (Ruchaud *et al.* 2007). The relative contribution of RanGTP and the CPC to acentrosomal spindle assembly *in vivo*, however, is less well understood. Indeed, while a gradient of RanGTP is thought to be required for spindle assembly in some cell types, there is mounting evidence that this may not be true for meiotic acentrosomal spindles. For example, a RanGTP gradient is required for spindle assembly around chromatin-coated beads in *Xenopus* extracts, but not sperm nuclei (Maresca *et al.* 2009). Furthermore, in mouse (Dumont *et al.* 2007) and *Drosophila melanogaster* (Cesario and McKim 2011) oocytes, RanGTP may be dispensable for meiosis I spindle assembly. On the other hand, chromosome alignment and segregation are defective after knockdown of the CPC in

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both mouse and *Caenorhabditis elegans* oocytes, but spindle assembly has not been closely examined (Schumacher *et al.* 1998; Speliotes *et al.* 2000; Kaitna *et al.* 2002; Rogers *et al.* 2002; Shuda *et al.* 2009; Sharif *et al.* 2010).

Characterizing the role of the CPC in *Drosophila* oocytes has been difficult due to its essential role in the mitotic divisions that precede meiosis. In *Drosophila* oocytes with reduced CPC function, the initiation of meiotic spindle assembly is delayed (Colombie *et al.* 2008), suggesting that the CPC may play a role in spindle assembly *in vivo*. However, a definitive demonstration of the role of the CPC in acentrosomal spindle assembly awaited generating oocytes lacking proteins like Incenp or Aurora B. Using new RNA interference (RNAi)-based methods (Ni *et al.* 2011), we have been able to knock out CPC activity in the oocyte and define its role in acentrosomal spindle assembly. Using these methods, we demonstrate that the CPC is required for several aspects of acentrosomal meiotic spindle assembly, including the recruitment of microtubules, organization of a bipolar spindle, and homologous chromosome biorientation. We propose a mechanism for these functions based on the localization pattern of CPC proteins and the effects of depleting them in spindle assembly.

Materials and Methods

Drosophila stocks and genetics

Flies were reared on standard media at 25°. Genetic loci not described in the text are described on FlyBase (flybase.org; Tweedie *et al.* 2009). To generate the *Incenp^{myc}* transgene, the entire *Incenp* coding region was amplified by PCR from the cDNA clone RE52507 (*Drosophila* Genomics Resource Center, Bloomington, IN) and cloned into pENTR4 (Invitrogen, Carlsbad, CA). It was then fused at its N terminus to six copies of the myc epitope tag in the vector pPMW (*Drosophila* Genomics Resource Center) using a Clonase (Invitrogen) reaction to make *pP{UASP: Incenp^{myc}}*. This was injected into embryos to make transgenics by Model System Genomics (Duke University, Durham, NC). This construct was expressed in oocytes using the *nanos-GAL4:VP16* driver (Rorth 1998).

The *ial¹⁶⁸⁹* allele was identified from a collection of EMS-mutagenized second chromosome fly stocks (Koundakjian *et al.* 2004) by screening for elevated levels of X-chromosome nondisjunction. Recombination mapping with visible markers and single-nucleotide polymorphisms (FlySNP Project, Berger *et al.* 2001) narrowed the candidate region to 32A5–32C1. Although the *ial¹⁶⁸⁹* allele is homozygous viable, two overlapping deficiencies [*Df(2L)Exel8026* and *Df(2L)Exel7049*] in this region were inviable in combination with the *ial¹⁶⁸⁹* allele, suggesting that this mutant is a hypomorph. The region of overlap contains the *ial* gene: sequencing revealed a missense mutation (C82T) that results in an amino acid substitution (P28S). Both the X-chromosome segregation defect and the inviability over deficiency were rescued by expression of an *ial* transgene (data not shown),

confirming that these defects are a result of the mutation in *ial*.

Antibodies, immunofluorescence, and microscopy

Stage 14 oocytes were prepared as described (Mckim *et al.* 2009). Briefly, 100–300 nonvirgin females were fattened on yeast for 3–5 days and then pulsed in a blender to disrupt abdomens. Late-stage oocytes were separated from bulk fly tissues and then fixed in an 8% formaldehyde/100 mM cacodylate solution. Chorion and vitelline membranes were removed by rolling oocytes between the frosted part of a glass slide and a coverslip. For standard immunofluorescence, rolled oocytes were extracted in PBS/1% Triton-X-100 for 1½ to 2 hr and blocked in PBS/0.1% Tween 20/0.5% BSA for 1 hr, and then antibodies were added. For FISH, rolled oocytes were stepped into 20, 40, and 50% formamide solutions, followed by 1–5 hr in 50% formamide at 37°. FISH probes were added, and oocytes were incubated at 91° for 3 min, followed by incubation overnight at 37°. Oocytes were stepped out of formamide solution and blocked for 4 hr in 10% normal goat serum, and then antibodies were added.

Oocytes were stained for DNA with Hoechst 33342 (10 µg/mL) and for microtubules with mouse anti- α -tubulin conjugated to FITC (1:50 dilution or 1:30 for FISH experiments, clone DM1A) (Sigma, St. Louis). We raised an antibody against Incenp by expressing the C-terminal 297 amino acids (starting at an internal *Bgl*II site) in *Escherichia coli* and injecting gel-purified protein into rats (Covance, Princeton, NJ) (Wu *et al.* 2008). This antibody was used at 1:400. Additional primary antibodies included rat anti-SUB (1:75) (Jang *et al.* 2005), rabbit anti-CID (1:500) (Henikoff *et al.* 2000), chicken anti-CID (1:100) (Blower and Karpen 2001), guinea pig anti-MEI-S332 (1:300) (Moore *et al.* 1998), and mouse anti-myc (1:20, Roche, Indianapolis). All primary antibodies were combined with either Cy3- or Cy5-conjugated secondary antibodies pre-absorbed against a range of mammalian serum proteins including mouse and rat (Jackson ImmunoResearch, West Grove, PA). FISH probes used were to the 359-bp repeat (X chromosome), AACAC repeat (second chromosome), dodeca repeat (third chromosome), and the 1.686-gm/cm³ repeat (second and third chromosomes) as described (Dernburg *et al.* 1996). Oocytes were mounted in SlowFade Gold (Invitrogen), and images were collected on a Leica TCS SP2 or SP5 confocal microscope with a $\times 63$, N.A. 1.3 or 1.4 lens, respectively. Images are shown as maximum projections of complete image stacks followed by merging of individual channels and cropping in Adobe Photoshop.

Oocytes were cold-treated by placing females in an Eppendorf tube on ice for 40 min to 2.5 hr prior to preparation. All preparation steps prior to fixation were performed at 4°. When on ice, the females are immobile, but when returned to room temperature after 2 hr of cold treatment, the females immediately resumed activity. Treated and recovered females were mated and tested for fertility

and nondisjunction, which was not different from untreated wild-type flies (data not shown).

Results

Bipolarity is established in prometaphase with a ring of central spindle proteins

Spindle assembly in *Drosophila* oocytes begins during prometaphase with microtubules accumulating around a condensed mass of chromosomes, termed the karyosome, followed by the organization and extension of two spindle poles (Theurkauf and Hawley 1992; Matthies *et al.* 1996). Several proteins localize to the central spindle at metaphase I, including the kinesin-6 Subito and Incenp, a component of the CPC (Jang *et al.* 2005). Previously, Colombié *et al.* (2008) observed a delay in spindle assembly in a hypomorphic *Incenp* mutant, which led to the hypothesis that the CPC plays an important role in the chromosome-driven spindle assembly of *Drosophila* oocytes. This hypothesis makes two predictions: first, the CPC should be associated with the meiotic spindle at all times, from the earliest stages of spindle assembly, which we test in this section; and second, the CPC should be required for spindle assembly, testing of which will be described in a later section.

We have previously observed that both Subito and Incenp localize to the central spindle at metaphase I (Jang *et al.* 2005). To test the prediction that the CPC should be associated with the meiotic spindle at all times, we examined Subito and Incenp localization in oocytes that were collected under conditions that promote the isolation of all stages of spindle assembly from prometaphase to metaphase arrest (Gilliland *et al.* 2009). In this large collection ($n > 100$), there were no oocytes with tubulin, but without Subito or Incenp. Furthermore, Incenp localization is absent prior to nuclear envelope breakdown (NEB) (data not shown). Therefore, we suggest that spindle assembly begins early in prometaphase with the simultaneous accumulation of central spindle proteins and tubulin at the karyosome. This conclusion assumes that at least some of the >100 oocytes that we imaged were in prometaphase. Based on several criteria, including those established by Gilliland *et al.* (2009), we believe that this is true. First, we used well-fed mated females in which oocytes are proceeding continuously through development. Nuclear envelope breakdown occurs between stages 12 and 13 of oogenesis. In well-fed mated females, stage 13 lasts <1 hr and stage 14 lasts ~ 2 hr, which is then followed by ovulation, activation, progression past metaphase I, and egg laying (King 1970). Since prometaphase lasts at least 20 min (Matthies *et al.* 1996; Gilliland *et al.* 2009), a conservative estimate is that, in a collection from well-fed females, $\sim 10\%$ (or ≥ 10 in our collection of >100 oocytes) of oocytes should be in prometaphase. Second, we often observed oocytes that do not have the “lemon” configuration of the karyosome indicative of metaphase I arrest as described by Gilliland *et al.* (2009), suggesting that these oocytes are in prometaphase. Finally,

our results are consistent with live imaging studies in which both tubulin and Subito accumulate simultaneously around the karyosome following nuclear envelope breakdown (S. Takeo and R. S. Hawley, personal communication). While we cannot rule out the possibility that there is a very brief stage in prometaphase during which Subito and Incenp are not present, our results suggest that the central spindle proteins accumulate very early during spindle assembly either concurrently with or soon after microtubules begin to assemble around the karyosome.

During our previous experiments on Subito and Incenp localization at metaphase I, we concluded that both proteins localize to two main bands on either side of the karyosome (Jang *et al.* 2005). Using imaging techniques with improved sensitivity, we observed that the Subito and Incenp signals between the two main bands (Figure 1, A and B) localized to a ring around the karyosome. Some spindles tend to show a more uniform ring of Subito or Incenp (Figure 1, C and D; “Rotation”), while other spindles tend to show the more discontinuous ring. These results hint that the ring of central spindle proteins may change shape during the course of spindle assembly, perhaps beginning as a continuous ring and becoming enriched at the prominent central spindle microtubules later on. Importantly, however, the ring is always present and always observed perpendicular to the axis of the spindle, that is, with the spindle axis running through the ring. This orientation relative to the spindle axis is suggestive of a role for the ring in the establishment or maintenance of spindle bipolarity. Taken together, our results suggest that the central spindle proteins accumulate early in spindle assembly in a ring around the karyosome, the orientation of which correlates with the bipolarity of the spindle.

Central spindle protein Subito depends on microtubules while Incenp interacts with noncentromeric chromatin

The location of the ring of central spindle proteins at prometaphase suggests that it interacts with either microtubules or chromosomes. During mitosis, the CPC localizes to centromeres at metaphase and midzone microtubules at anaphase (Adams *et al.* 2001; Cesario *et al.* 2006; Chang *et al.* 2006; Ruchaud *et al.* 2007). Subito does not colocalize with the centromere protein MEI-S332 at metaphase of meiosis I in oocytes (Jang *et al.* 2005), and since Subito and Incenp colocalize (Jang *et al.* 2005), it seemed likely that the CPC also would not localize to meiosis I centromeres in oocytes. Indeed, we found that Incenp does not colocalize in oocytes with either MEI-S332 or CID, a centromere-specific histone H3 (Figure 1, E and F), although it does in mitotic metaphase of larval neuroblasts and in male meiotic metaphase I (Cesario *et al.* 2006; Resnick *et al.* 2006). Similar results in meiosis I of oocytes have been found with two different Incenp antibodies, two Aurora B antibodies, RFP-tagged Aurora B and GFP-tagged Deterin (Adams *et al.* 2001; Giet and Glover 2001; Jang *et al.* 2005; Colombié *et al.* 2008; data not shown). In addition, Incenp did not

Figure 1 Central spindle proteins form a ring in prometaphase I and metaphase I oocytes. Tubulin is in green, DNA in blue, and Subito or Incenp in red, except in insets in E and F in which CID and MEI-S332 are green, respectively. (A–D) Prometaphase I oocytes showing a ring of Subito or Incenp around the karyosome. The insets show only the karyosome and Incenp or Subito. The insets in A and B show the karyosome rotated $\sim 90^\circ$ in the Z direction. The rotation panels for C and D show the images rotated $\sim 90^\circ$ in the Z direction. Arrows point to the spindle poles. (E) An oocyte showing Incenp primarily localizing to the center of the karyosome. The inset shows that CID and Incenp do not colocalize. (F) An oocyte showing Incenp localizing to the central spindle. The inset shows that MEI-S332 and Incenp do not colocalize.

colocalize with centromere probes in the experiments described below. These results suggest that the CPC is not at centromeres during meiosis I in oocytes.

If the ring of central spindle proteins is not at centromeres during metaphase of meiosis in oocytes, it may be either at another chromosomal location or, similar to the localization of the CPC at anaphase of mitosis, on microtubules. By treating oocytes with colchicine, which depolymerizes microtubules, we previously showed that Subito localization depends on microtubules (Jang *et al.* 2005). Microtubules are also sensitive to cold (Salmon and Begg 1980; Rieder 1981), which is more easily applied and reversed than colchicine treatment, so we exposed oocytes to cold temperatures to determine if Incenp localization depends on microtubules. Cold treatment caused the loss of most microtubules and, consistent with previous results, the complete loss of Subito from the spindle in 4/4 oocytes (Figure 2A). In most cases, two small bundles of microtubules remained visible on each side of the karyosome, which could be kinetochore microtubules since these can be resistant to cold treatment (Salmon and Begg 1980; Rieder 1981). Microtubules and Subito localization returned within 1 hr at room temperature (Figure 2B). In contrast, Incenp was resistant to cold treatment: Incenp localized to a ring around the karyosome in nine of nine cold-treated oocytes (Figure 2C). These results suggest that Incenp can interact with the chromatin independently of microtubules.

To further investigate if Incenp can interact with the chromosomes, *nod* mutants were examined. In *nod* mutants, univalent achiasmate chromosomes are frequently separated from the main mass of chromosomes in the karyosome

(Theurkauf and Hawley 1992). In the oocyte shown in Figure 2D, Incenp colocalized with the achiasmate fourth chromosomes that have moved precociously toward the spindle poles. Overall, we interpret these data to indicate that Incenp binds to noncentromeric chromatin on each chromosome.

Spindle assembly depends on the CPC

The results presented thus far show that the CPC is at the right place at the right time to play a role in the establishment of meiotic spindle bipolarity. Previous investigations into the role of the CPC during meiosis *in vivo* have been complicated by the essential role of the CPC in mitotic cell division. Null mutants in genes encoding members of the CPC in *Drosophila* are inviable (Chang *et al.* 2006; S. J. Shah and K. S. McKim, unpublished data), and null mutant oocytes made by mitotic recombination do not complete oogenesis (Jang *et al.* 2005). Recent developments in RNAi technology by the Transgenic RNAi Project (TRiP) (Harvard Medical School, Cambridge, MA) have made it possible to knock down gene expression in the *Drosophila* female germline (Ni *et al.* 2011), and we took advantage of this new tool to test for a role of the CPC during meiotic spindle assembly.

We obtained transgenic lines from TRiP that express short hairpin microRNAs specific to *Incenp* (GL00279) and to *ial* (GL00202), which encodes Aurora B, under the control of the Gal4/UAS system (Brand and Perrimon 1993). The choice of female germline-specific Gal4 driver was critical for these experiments, and we tested two that are commonly used. The first, *nanos-GAL4:VP16*, drives expression of UASP transgenes beginning early in the germarium,

Figure 2 Incenp interacts with the chromosomes while Subito depends on microtubules. (A) An oocyte after a 2-hr cold treatment showing an absence of Subito localization. The cold treatment depolymerizes most microtubules with the possible exception of some kinetochore microtubules. (B) An oocyte after a 2-hr cold treatment followed by recovery at room temperature for 1 hr. Tubulin and Subito localization are present at normal levels. (C) An oocyte after a 2-hr cold treatment showing Incenp localization around the karyosome. (D) Incenp staining in a *nod* mutant oocyte. Arrows indicate Incenp associating with the univalent achiasmate fourth chromosomes. Bars, 5 μ m.

which contains premeiotic and early meiotic prophase cells (Supporting Information, Figure S1) (Rorth 1998). Expression of either *Incenp* or *ial* RNAi with *nanos-GAL4:VP16* produced ovarian cysts that did not develop past the germarium (data not shown). In contrast, expression of *Incenp* or *ial* RNAi using the *mata4-GAL-VP16* driver, which expresses at high levels just after oocytes exit the germarium (Figure S1) (Sugimura and Lilly 2006), allowed the completion of oogenesis (data not shown). Therefore, we were able to bypass the requirement for the CPC in early oogenesis and examine the role of this important complex during meiotic spindle assembly by driving RNAi expression with the *mata4-GAL-VP16* driver.

Oocytes in which we used the *mata4-GAL-VP16* driver to express either the *Incenp* or *ial* RNAi constructs will herein be referred to as CPC RNAi oocytes. Under these conditions, Incenp and Aurora B protein expression, respectively, were almost undetectable, confirming the effectiveness of RNAi knockdown (Figure S2). In wild-type oocytes, a bipolar spindle forms with Incenp and Subito localizing to the central spindle (Figure 3, A and B). In CPC RNAi oocytes, we observed a complete lack of microtubules surrounding the karyosome (Figure 3, C–F). This phenotype was completely penetrant ($n = 42$), making it unlikely that spindles form but then disassemble in the absence of the CPC. The results were identical with either RNAi construct, suggesting that this is specific to the CPC and is not an off-target effect. The complete absence of organized microtubules around the karyosome suggests that the CPC is required to recruit microtubules for acen-trosomal spindle assembly.

Spindle assembly does not depend on downregulation of a microtubule-depolymerizing motor

In *Xenopus* egg extracts, the lack of microtubule accumulation around chromatin-coated beads in the absence of CPC activity is dependent on the presence of MCAK, a microtubule-depolymerizing kinesin-13 family member (Sampath *et al.* 2004). This suggests that the CPC may act to promote meiotic spindle assembly through the downregulation of microtubule-depolymerizing proteins. In fact, the CPC is

known to phosphorylate members of the kinesin-13 family *in vitro*, resulting in a reduction in their activity (Andrews *et al.* 2004; Lan *et al.* 2004; Ohi *et al.* 2004; Knowlton *et al.* 2009).

To test whether a similar mechanism is active in *Drosophila* oocytes, we examined meiotic spindle assembly in oocytes expressing short hairpin microRNAs specific to *ial* and to *Klp10A* (HMS00920), which encodes one of three *Drosophila* kinesin-13 proteins. In the accompanying article in this issue by Radford *et al.*, we have shown that *Klp10A* is an essential gene. Furthermore, in *Drosophila* oocytes lacking KLP10A, the length of both cytoplasmic and spindle microtubules is dramatically increased, suggesting that KLP10A regulates microtubule length through depolymerization. KLP10A is the strongest candidate for a kinesin-13 motor that would be negatively regulated by the CPC because preliminary experiments with the two other *Drosophila* kinesin-13 proteins, KLP59C and KLP59D, have failed to yield evidence that they regulate microtubule length in oocytes (S. J. Radford and K. S. McKim, unpublished results). *Klp10A* RNAi resulted in almost complete knockdown of KLP10A expression with phenotypes indistinguishable from that of a null mutation (Figure 4, A and B, and accompanying article in this issue by Radford *et al.* 2012). In *ial Klp10A* double RNAi oocytes, we observed the same complete absence of spindle microtubules as with *ial* single RNAi, whereas the cytoplasmic microtubules resembled *Klp10A* single RNAi (Figure 4, C and D). This demonstrates that the lack of spindle microtubules in the absence of the CPC is not due to hyperactive KLP10A activity.

CPC activity is required for correct localization of Incenp and Subito

As an alternative to the downregulation of depolymerizing enzymes, the CPC may promote meiotic spindle assembly through the recruitment of spindle assembly factors to the chromosomes. Consistent with protein blotting results (Figure S2), we did not detect Incenp localization in *Incenp* RNAi oocytes (Figure 3C); however, Incenp did localize to the karyosome in *ial* RNAi oocytes (Figure 3E). The

Figure 3 Spindle assembly failure and central spindle protein mislocalization in the absence of the CPC. Tubulin is in green, DNA in blue, and Incenp or Subito in red. (A and B) Wild-type oocytes showing a bipolar spindle and Incenp (A) or Subito (B) localization to the central spindle. (C and D) *Incenp* RNAi oocytes lack both microtubule accumulation around the karyosome ($n = 12$) and Incenp ($n = 3$) (C) or Subito ($n = 4$) (D) localization. (E) *ial* RNAi oocytes lack microtubule accumulation around the karyosome ($n = 30$), but show Incenp localization that is enriched on certain regions of the karyosome ($n = 5$). (F) *ial* RNAi oocytes lack Subito localization ($n = 4$). (G) Incenp^{myc} localized (detected with a Myc antibody) to the microtubules throughout the spindle in 7/16 oocytes. (H) Example of relatively normal Incenp localization in an Incenp^{myc} oocyte. Even when concentrated in the central spindle, Incenp^{myc} is often found throughout the spindle as well. In G and H, Incenp^{myc} was expressed in an Incenp⁺ background. Panels are accompanied by the Incenp (A', C', E', G', and H') or Subito (B', D', and F') localization pictured alone. Bars, 5 μ m.

localization of Incenp in the absence of microtubules confirms the results with cold treatment, showing that Incenp can interact directly with the chromosomes, although this is insufficient to promote spindle assembly in the absence of Aurora B. Interestingly, instead of being restricted to a ring as observed in wild type (Figure 3A), the localization of Incenp in *ial* RNAi oocytes was disorganized (Figure 3E). Its distribution was not uniform, suggesting that Incenp still showed some chromatin specificity in *ial* RNAi oocytes, but the absence of a ring structure suggests that the kinase activity of the CPC may play a role in organizing the karyosome, shaping the ring, or both. Alternatively, the

mere presence of Aurora B protein may be required to restrict Incenp to the ring localization pattern.

There was also a lack of Subito localization in both *Incenp* and *ial* RNAi oocytes (Figure 3, D and F) although Subito protein expression was normal (Figure S2). Because Subito localization depends on microtubules (Figure 2A) (Jang *et al.* 2005), the absence of Subito localization in CPC RNAi oocytes may result from the lack of microtubule accumulation rather than from a direct interaction between Subito and the CPC. Nonetheless, these results indicate that Subito, an important factor required for spindle bipolarity, is not recruited in the absence of CPC activity. Furthermore, in

Figure 4 The absence of a spindle in a CPC knockdown does not depend on *Klp10A*. (A and B) *Klp10A* RNAi oocytes showing overgrowth of cytoplasmic and spindle microtubules. (C and D) *ial Klp10A* double RNAi oocytes showing overgrowth of cytoplasmic microtubules but a lack of spindle microtubule accumulation around the karyosome. Bars, 5 μ m.

ial RNAi where *Incenp* is present on the chromosomes, *Subito* is still absent, indicating that the basis for *Subito* localization is not simply an interaction with *Incenp*.

CPC is required for centromere separation and biorientation

Because the CPC regulates spindle assembly, we investigated its role in chromosome segregation. To generate gametes with the correct number of chromosomes, the two homologous chromosomes must make connections with microtubules oriented toward opposite spindle poles, known as biorientation. To determine if homologous chromosome biorientation depends on central spindle proteins, we performed FISH on CPC knockdown and *subito* (*sub*) mutant oocytes using probes to the highly repetitive heterochromatic sequences present on the X, second, and third chromosomes. We performed FISH on two CPC hypomorphs and the more severe CPC RNAi knockouts. The hypomorphs include *Incenp*^{QA26} (Resnick *et al.* 2006; Colombie *et al.* 2008) and an allele of *ial* that we identified, *ial*¹⁶⁸⁹, from a collection of EMS-mutagenized second chromosome stocks (Koundakjian *et al.* 2004) as a mutant that exhibits elevated X-chromosome nondisjunction (see *Materials and Methods* and Table 1).

Prior to spindle assembly in *Drosophila* oocytes, homologous centromeres are paired (Dernburg *et al.* 1996). Once NEB occurs and the spindle assembles, the homologous centromeres separate toward opposite spindle poles. We propose that this happens rapidly because the vast majority of wild-type oocytes that we examined had bi-oriented centromeres (Table 2; Figure 5, A, B, and D). Because this collection includes oocytes from prometaphase through to the metaphase arrest, these results suggest that centromere biorientation occurs early in spindle assembly. Indeed, three of the four wild-type oocytes with mono-oriented second chro-

Table 1 Chromosome segregation in *Incenp*^{myc}

Genotype	Total	Nondisjunction (%)
<i>ial</i> ¹⁶⁸⁹ /+	973	0.4
<i>ial</i> ¹⁶⁸⁹	773	5.8
<i>Bwingsy</i> /+; <i>ial</i> ¹⁶⁸⁹ /+	833	0.0
<i>Bwingsy</i> /+; <i>ial</i> ¹⁶⁸⁹	791	26.0
<i>Incenp</i> ^{myc} /+	8008	1.4
<i>Incenp</i> ^{myc} /+; <i>sub</i> ¹ /+	3864	20.1
<i>Incenp</i> ^{myc} /+; <i>Incenp</i> −/+	3246	10.4
<i>FM7</i> /+; <i>Incenp</i> ^{myc} /	3011	23.9

Females carrying *ial*¹⁶⁸⁹ were crossed to *C(1:Y)1, y v f B; y⁺; C(4)RM, ci ey^R* males. In *ial*¹⁶⁸⁹ homozygotes, fourth-chromosome nondisjunction was not detected. The *Incenp* transgene (*P{UASP:Incenp*^{myc}) was expressed using the *nanos-GAL4:VP16* driver and the indicated females were crossed to *y w/B^SY* males.

mosome centromeres had disorganized spindles, which may have been in the early stages of spindle assembly (Figure 5C).

In both *Incenp*^{QA26} and *ial*¹⁶⁸⁹ mutant oocytes, we frequently observed mono-orientation of homologous chromosomes (Table 2; Figure 5, E and F). Resnick *et al.* (2009) showed that there were chromosome orientation errors in an *Incenp*^{QA26} mutant that was also heterozygous for an *ial* deficiency. Our results show that orientation errors are also observed in the *Incenp*^{QA26} single mutant. Although these mutants have orientation problems, there is evidence of chromosome movement. Oriented correctly or not, the centromeres are usually at the edge of the karyosome closest to one pole. Two distinct FISH signals are often visible, as opposed to the one when the centromeres are paired prior to NEB. Therefore, we refer to the independent movement of the centromeres after NEB as “separation.” In both *Incenp* and *ial* RNAi oocytes, on the other hand, we usually observed only one focus for each pair of centromeres (Table 3; Figure 5, G and H). The failure to see distinct foci for each centromere suggests that the centromeres have failed to separate following NEB. These results suggest that the CPC is required for two steps during chromosome biorientation in oocytes: the separation of centromeres and the proper orientation of those centromeres toward opposite spindle poles for chromosome segregation. Interestingly, the frequency of mono-orientation in *Incenp*^{QA26} mutants was substantially >50%, which could be explained if this mutant, like the CPC RNAi oocytes, has a mild defect in homolog separation in addition to the defect in biorientation.

These results show that the CPC is critical for centromere separation and biorientation, but do not directly test the importance of the central spindle ring localization pattern. To examine if the localization of the CPC to the central spindle is important, we examined oocytes expressing a transgene under the control of the Gal4/UAS system that encodes full-length *Incenp* with a myc-tag at the N terminus (*Incenp*^{myc}). Expression of this transgene in the female germline using the *nanos-GAL4:VP16* driver results in abnormal chromosome segregation, including missegregation of both chiasmate and achiasmate X chromosomes (Table 1). This dominant phenotype may result from either the overexpression

Table 2 Mono-oriented centromeres in wild-type and mutant oocytes

	Bipolar spindles	Frequency of mono-oriented centromeres			Combined mono-oriented (%)
		X	Second chromosome	Third chromosome	
Wild-type	69/75 (92%)	0/24 (0%)	4/46 (9%)	0/44 (0%)	3.5
<i>sub¹/sub¹³¹</i>	25/48 (52%)	8/17 (47%)	22/39 (56%)	10/25 (40%)	49.4
<i>Incenp^{Q426}</i>	7/9 (78%)	7/9 (78%)	ND	6/9 (67%)	72.2
<i>ial^{I689}</i>	16/16 (100%)	3/10 (30%)	ND	0/16 (0%)	^a
<i>ncd</i>	15/18 (83%)	ND	8/27 (30%)	7/28 (25%)	27.3

ND, not determined.

^a Not applicable because *ial^{I689}* mutants affect only the X chromosome.

of *Incenp* (Figure S2) or the addition of the N-terminal tag because the N terminus contains sequences important for *Incenp* localization (Ainsztein *et al.* 1998; Mackay *et al.* 1998; Klein *et al.* 2006). In fact, myc-tagged *Incenp* localized throughout the spindle in 7/16 oocytes (Figure 3G) while in 9/16 oocytes the protein was concentrated at the central spindle like wild-type *Incenp* (Figure 3H). Even when *Incenp* was concentrated at the center, however, it was disorganized. There was also a correlation between localization pattern and spindle length. The spindles with mis-localized *Incenp* were usually longer than spindles where *Incenp* was concentrated in the center. Interestingly, a bipolar spindle formed in 14/16 *Incenp^{myc}* mutant oocytes. The correlation between *Incenp* mis-localization and chromosome missegregation, however, suggests that the ring localization of the CPC promotes proper chromosome biorientation, leading to proper chromosome segregation during female meiosis.

Subito is required for centromere biorientation

Since the CPC is required for *Subito* localization, we examined *sub* mutants to provide insights into how the CPC regulates homolog orientation. As in wild type, the homologous centromeres are tightly paired prior to NEB (Figure 5I). Similar to hypomorphic CPC mutants, we frequently observed mono-orientation of homologous centromeres in *sub* mutants (Table 2, Figure 5, J and K). Although chromosome biorientation is defective, there was no failure to separate and the centromeres always were oriented toward one of the poles present in the spindle, indicating that movement toward a pole does not depend on *Subito*. Indeed, the frequency of mono-orientation was close to 50%, suggesting that chromosomes orient randomly in *sub* mutant oocytes.

In summary, these results confirm that the two steps of chromosome biorientation—centromere separation and orientation—both depend on the CPC but are genetically separable. The role of the CPC in biorientation may be through regulating the activity of proteins like *Subito*. Unlike *sub* mutants, however, the CPC hypomorphs usually generated bipolar spindles (Table 2) and localized *Subito* normally (Colombie *et al.* 2008). Therefore, simply localizing *Subito* correctly is not sufficient. The level of CPC ac-

tivity needed for biorientation may be greater than that needed to form a bipolar spindle.

Because of the frequent appearance of monopolar and tripolar spindles in *sub* mutant oocytes, we were able to examine the relationship between chromosome orientation and spindle poles using *sub* mutants. In 49 *sub* mutant oocytes with the centromeres of the three major chromosomes marked, we observed 103 poles in a mix of monopolar, bipolar, and tripolar spindles. All of the poles were associated with at least one centromere (Figure 5, J and K), suggesting that spindle pole formation may be established or stabilized by the attachment to at least one centromere.

Non-claret disjunctional is required for chromosome biorientation

The result that homologs fail to bi-orient properly in *sub* mutants suggests that the central spindle microtubules play a role in homolog biorientation, but how they interact with the chromosomes is not known. The fact that the centromeres appear to move toward the poles in *sub* mutants suggests that there is a population of *Subito*-independent microtubules that connect to the chromosomes, driving poleward centromere movement. We hypothesize that bundling between these chromosome-associated microtubules and the central spindle microtubules that depend on *Subito* provides a mechanism for proper homolog biorientation. One candidate for this activity is the kinesin-14 non-claret disjunctional (NCD), which is known to bundle microtubules (McDonald *et al.* 1990). Mutations in *ncd* cause elevated homolog nondisjunction during female meiosis and genetically interact with *sub* mutants (Giunta *et al.* 2002). In addition, *ncd* mutants display fraying of female meiosis I spindles, which is consistent with a role in bundling microtubules (Hatsumi and Endow 1992; Matthies *et al.* 1996). We performed FISH on *ncd* mutant oocytes and found an elevated frequency of mono-oriented homologous centromeres (Table 2, Figure 5L), suggesting that NCD is indeed required for homolog biorientation. Consistent with previous results (Hatsumi and Endow 1992; Matthies *et al.* 1996), bipolar spindles were more common in *ncd* (83.3%) than in *sub* mutants (52.1%). This is consistent with the primary defect in *ncd* mutants being a bundling defect as opposed to *sub* mutants where the primary defect is maintaining bipolarity.

Figure 5 Chromosome orientation defects in the absence of central spindle proteins. In all panels, tubulin is in green and insets show only the FISH signals. In D–I, the DNA is in blue. For all other panels, DNA was imaged but is not shown for clarity. (A and B) Wild-type oocytes showing biorientation of the second chromosome. The centromeres do not colocalize with Incenp, which is at the central spindle. (C) Early prometaphase wild-type oocyte showing a monopolar spindle with the second chromosome centromeres still paired while the third chromosome centromeres are separated and interacting with microtubules as if moving toward what will be opposite poles. (D) A wild-type oocyte showing biorientation of the centromeres of both the X and third chromosomes. (E) An *Incenp*^{QA26} mutant oocyte in which both the X and third chromosomes are mono-oriented. (F) An *ial*¹⁶⁸⁹ mutant oocyte in which the X chromosome is mono-oriented, but the third chromosome is bi-oriented. (G and H) *Incenp* and *ial* RNAi oocytes, respectively, showing a lack of microtubule accumulation around the karyosome and a failure of homologous centromeres to separate. (I) A *sub* mutant oocyte prior to nuclear envelope breakdown. The X chromosome centromeres are paired. The inset shows a FISH probe that detects both the second and third chromosome centromeres. Since there are only two discrete signals, the autosomal centromeres are likely also paired. (J) A *sub* mutant oocyte in which both the second and third chromosomes are mono-oriented on a monopolar spindle. (K) A *sub* mutant oocyte in which at least one centromere is oriented toward each of the three poles present in a tripolar spindle. (L) An *ncd* mutant oocyte showing biorientation of the second but mono-orientation of the third chromosome centromeres. The three dots indicate that one pair of sister centromeres has separated. One of the FISH signals is on a part of the spindle that has frayed, a common defect in *ncd* mutant spindles. Bars, 5 μ m.

These results are consistent with our hypothesis that NCD bundles chromosomal and central spindle microtubules to promote homologous centromere bi-orientation.

Discussion

Previous work using *Xenopus* egg extracts demonstrated that both RanGTP and the CPC are required for chromatin-induced spindle assembly (Sampath *et al.* 2004). In contrast, RanGTP appears not to be required for acentrosomal spindle assembly in *Drosophila* (Cesario and McKim 2011) and mouse oocytes (Dumont *et al.* 2007). We have shown that the CPC is essential for the accumulation of microtubules around the chromosomes in *Drosophila* oocytes, suggesting that *in vivo* the CPC is the critical factor for regulating acentrosomal spindle assembly. A model is presented for acen-

trosomal spindle assembly with implications for how the CPC simultaneously promotes bipolarity and homolog bi-orientation (Figure 6).

CPC promotes spindle assembly and establishes the spindle axis

Our results support a model in which the primary step in the establishment of meiotic spindle bipolarity is the accumulation

Table 3 Centromere separation in wild-type and CPC RNAi oocytes

	Frequency of X-chromosome centromere separation	Frequency of third-chromosome centromere separation
Wild type	24/24 (100%)	44/44 (100%)
<i>Incenp</i> RNAi	1/5 (20%)	1/5 (20%)
<i>ial</i> RNAi	0/7 (0%)	4/18 (22%)

Figure 6 Model for the relationship between the central spindle, spindle bipolarity, and centromere orientation during acentrosomal spindle assembly. (A) Early in prometaphase, the CPC (red circles) interacts with the chromosomes (blue circles). The CPC is recruited by interacting with either the chromosomes or cooperatively with the chromosomes and microtubules (Tseng *et al.* 2010). (B) A complex of Subito (orange circles) and the CPC interacts with antiparallel microtubules (green lines). These antiparallel bundles may predict the eventual bipolarity of the spindle and may contribute to the orientation of homologous centromeres

(white circles). (C) A stable metaphase spindle forms through the tapering of microtubules to form two poles. Subito and the CPC remain at the central spindle, perhaps stabilizing it to maintain spindle bipolarity. The chromosomes may achieve end-on contact with microtubules that connect to the poles. Alternatively, lateral interactions between the chromosomes and microtubules may predominate. (D) Late prometaphase or metaphase spindle. Chromosome-associated microtubules may be bundled with central spindle microtubules by a cross-linking motor like NCD (black) to promote biorientation. (E) A pathway for biorientation.

of the CPC in a ring encircling the chromosomes (Figure 6A). The enrichment of CPC proteins in a ring around the karyosome may provide the increased local concentration of Aurora B that has been postulated to be necessary to activate the Aurora B kinase for chromosome-based spindle assembly in *Xenopus* egg extracts (Kelly *et al.* 2007; Maresca *et al.* 2009; Tseng *et al.* 2010). We propose that the CPC has two critical functions in *Drosophila* oocytes: it promotes microtubule accumulation near the chromosomes and also constrains microtubule growth into two poles by establishing the spindle axis (Figure 6, B and C). This replaces two functions of the centrosomes: recruitment of microtubules and organizing a bipolar spindle. Previous studies have suggested that the CPC promotes spindle assembly by suppressing the microtubule-depolymerizing activity of a kinesin-13 protein near the chromosomes (Sampath *et al.* 2004). In contrast, we have shown that downregulating KLP10A, a *Drosophila* kinesin-13 protein known to regulate spindle length (accompanying article in this issue by Radford *et al.*), is not a sufficient explanation for the activity of the CPC. While we cannot rule out a role for the CPC in regulating the two additional kinesin-13s encoded by the *Drosophila* genome KLP59C (Rogers *et al.* 2004) and KLP59D (Rath *et al.* 2009) during acentrosomal spindle assembly, evidence summarized below suggests that the CPC positively regulates spindle assembly factors.

For the second function—constraining microtubule assembly toward two poles—a simple model is suggested by the shape of the ring: the ring may act like a tube that restricts microtubules to assemble in only two directions. Additionally, the CPC ring establishes the location for recruitment of other spindle assembly factors that regulate bipolarity, including Subito. A direct physical interaction between Subito and Incenp would be consistent with results showing that the mammalian Subito ortholog MKLP2 physically interacts with Aurora B and Incenp (Gruneberg *et al.*

2004). This must depend on Aurora B activity since we did not observe Subito localization in *ial* RNAi oocytes even though Incenp was associated with the chromatin. We suggest that the CPC interacts with chromosomes in a ring, promotes microtubule accumulation, and recruits proteins like Subito to these microtubules, which results in the establishment or stabilization of antiparallel microtubules, spindle bipolarity, and the formation of two poles (Figure 6, C and D).

Subito and the CPC appear to have a mutual dependency. We previously reported that the meiotic central spindle localization of the CPC depended on Subito (Jang *et al.* 2005). To explain these results, we suggest that the CPC is first recruited to the chromosomes and then moves to the central spindle microtubules. In the absence of Subito and the central spindle microtubules, the interaction of Incenp with the chromosomes persists and the CPC does not move to the microtubules. While interacting with the chromosomes the CPC can apparently promote spindle assembly, but not biorientation.

What controls the localization of the CPC ring and how it gets targeted to the region between bi-oriented centromeres remains to be uncovered. In the absence of Aurora B, the localization pattern of Incenp within the karyosome is disorganized, suggesting that the kinase activity of the CPC may play a role in shaping the ring, but underlying features of the chromosomes may also be important. It is intriguing that the passenger proteins are not detected in the centromere regions as they are in mitotic and centrosomal meiotic cells. Our results are consistent with data from *C. elegans* oocytes (Kaitna *et al.* 2002; Rogers *et al.* 2002; Monen *et al.* 2005) and mouse oocytes (Shuda *et al.* 2009; Sharif *et al.* 2010), showing that the CPC interacts with noncentromeric chromatin at metaphase of meiosis I. In *C. elegans*, the CPC forms a ring at the center of each bivalent that colocalizes with cohesion proteins distal to chiasmata

(Rogers *et al.* 2002; Wignall and Villeneuve 2009). The *C. elegans* CPC ring is a complex structure that, as in *Drosophila*, contains motor proteins (Klp-19) (Powers *et al.* 2004) and is required for segregation of homologs at meiosis I (Wignall and Villeneuve 2009; Dumont *et al.* 2010). The importance of noncentromeric CPC in a variety of organisms suggests that the unique demands of acentrosomal meiosis have resulted in a meiosis-specific CPC/central spindle localization pattern with a conserved role in spindle assembly and chromosome segregation. Finding out the identity or structural features of the chromosome locations to which the CPC ring localizes will be critical to understanding how the chromosomes organize acentrosomal spindles.

Homologous chromosome biorientation at prometaphase depends on the CPC and central spindle microtubules

Centromeres are paired in *Drosophila* oocytes prior to NEB (Dernburg *et al.* 1996). Based on our examination of oocytes depleted of the CPC and spindle assembly motors Subito and NCD, we propose the following pathway leading to homolog biorientation (Figure 6E). First, the CPC binds in a ring to the chromosomes and recruits spindle assembly factors such as Subito. This stage is defined by the observation that the CPC can bind chromosomes independently of microtubules and, in its absence, the microtubules and Subito fail to accumulate around the chromosomes. Second, microtubules with attachments to the chromosomes provide a poleward force on the centromeres. This stage is defined by the observation that, in the absence of the CPC, and consequently in the absence of microtubules, the homologous centromeres fail to separate. Third, the homologs bi-orient through interactions with the central spindle microtubules. This stage is defined by the observation that, in *sub* mutants, the central spindle is absent, but microtubules with attachments to the chromosomes still form and the homologous centromeres separate but fail to bi-orient.

The nature of the microtubule attachments to the chromosomes that lead to centromere separation is not known. Some previous studies have suggested that chromosome alignment depends on lateral interactions during acentrosomal meiosis (Brunet *et al.* 1999; Schuh and Ellenberg 2007; Wignall and Villeneuve 2009; Walczak *et al.* 2010). However, an alternative model incorporates an important role for kinetochore microtubules (Dumont *et al.* 2010). Kinetochore microtubules in oocytes have been inferred by Hughes *et al.* (2011) and could be the cold-resistant karyosome-associated microtubules that we have observed (Salmon and Begg 1980; Rieder 1981). Whether the microtubules connect to the chromosomes through traditional end-on kinetochore attachments or lateral attachments, we propose that these microtubules are bundled with central spindle microtubules to achieve biorientation. Interactions between central spindle microtubules and the microtubules with attachments to the chromosomes could be mediated by the kinesin-5 KLP61F (van den Wildenberg *et al.* 2008;

Brust-Mascher *et al.* 2009) or by the kinesin-14 NCD (McDonald *et al.* 1990). Indeed, we have shown here that NCD is required for homolog biorientation. The frayed spindles that are typical of *ncd* mutants (Hatsumi and Endow 1992; Matthies *et al.* 1996; Jang *et al.* 2005) could be explained by the loss of bundling between chromosome and central spindle microtubules.

A possible mechanism for how the CPC ring may facilitate biorientation at meiosis is suggested by two recent studies in mammalian mitotic and meiotic cells (Kitajima *et al.* 2011; Magidson *et al.* 2011). In both systems, prometaphase chromosomes move toward the outside edges of the developing spindle and then congress via lateral interactions to a ring around the central part of the spindle. This “prometaphase belt” facilitates and enhances the rate of biorientation by bringing kinetochores into the vicinity of a high density of microtubules, which leads to stable kinetochore–microtubule attachments. We propose that the ring of CPC protein promotes a prometaphase belt-like organization to enhance the interaction of centromeres with a high density of microtubules in *Drosophila* oocytes.

Summary

Chromosome-based spindle assembly is a well-described phenomenon, but the responsible chromatin-based factors in intact oocytes have not been previously identified. Our data suggest that the CPC interacts with noncentromeric chromatin and not only promotes the accumulation of microtubules around the chromosomes, but also regulates multiple aspects of spindle function, including the establishment of bipolarity and biorientation of homologs. Indeed, the localization to a central spindle ring and not centromeres may be critical for these functions. At this location, the CPC could regulate several different types of target protein that organize microtubules. One type is represented by Subito, which is required for spindle bipolarity, perhaps through the stabilization of antiparallel microtubules in the central spindle (Jang *et al.* 2005). Another type of target protein may function to promote microtubule attachment to the chromosomes. Indeed, these results provide the starting point for investigating what controls the localization of the CPC and what are its critical targets during acentrosomal meiosis.

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Note added in proof: See S. J. Radford *et al.* (pp. 431–440) in this issue, for a related work.

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Supporting Information

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The Chromosomal Passenger Complex Is Required for Meiotic Acentrosomal Spindle Assembly and Chromosome Biorientation

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File S1

Supporting Materials and Methods

GAL4 Driver Expression in Drosophila Ovaries

Females carrying the driver and *UASp-lacZ* transgene (gift from P. Rørth, Singapore) were dissected in PBS and ovarioles were separated with a tungsten needle, maintaining connections to ovaries. Ovaries were fixed and stained for *lacZ* expression using the β -Gal Staining Kit from Invitrogen. After staining, ovaries were transferred to 60% glycerol/PBS for 30 min then mounted on a slide and ovarioles were further teased apart using a tungsten needle. Images were collected using a PixeLINK® CCD camera mounted on a dissecting microscope.

Protein Blotting

Protein samples were prepared by collecting stage 14 oocytes by the same method used for immunostaining, but instead of fixation, oocytes were weighed and SDS gel loading buffer was added to obtain a final concentration of 1 mg oocytes/8 μ L total volume. The mixture was boiled for five minutes and 8 μ L was loaded per lane (20 μ L was loaded for *ial* RNAi and *Incenp* RNAi samples) on an SDS-PAGE gel. Primary antibodies used were rat anti-*Incenp* (1:10,000), rabbit anti-Aurora B (1:5000) (GIET and GLOVER 2001), rat anti-Subito (1:4000) (JANG *et al.* 2005), rabbit anti-KLP10A (1:10,000,000) (ROGERS *et al.* 2004) and rat anti- α tubulin (1:4000, clone YOL 1/34, Millipore, Billerica, MA, USA). Secondary antibodies used were goat anti-rabbit-HRP and goat anti-rat-HRP (both 1:5000, Jackson ImmunoResearch), detected using ECL Plus (Amersham, Piscataway, NJ, USA).

Figure S1 The expression pattern of two GAL4 drivers in the *Drosophila* female ovary. A single ovariole is pictured for each genotype with the anterior tip, where the germarium is located, positioned to the left. Dark staining shows expression of the *UASp-lacZ* transgene under the control of either *P{GAL4::VP16-nos.UTR}CG6325^{MVD1}* or *P{mata4-GAL-VP16}V37*. The earliest *lacZ* expression is indicated by an arrow. The *nanos-GAL4:VP16* driver begins to express early in oogenesis (in the germarium) while the *mata4-GAL-VP16* driver doesn't express until later (beginning in the vitellarium.)

Figure S2 Expression of central spindle proteins in CPC RNAi or sub mutants examined by Western blot. Protein samples from mature oocytes were probed on four separate blots with anti-Incenp, anti-Aurora B, anti-Subito, and anti-tubulin as a loading control. Incenp expression is practically undetectable in the *Incenp* RNAi sample, Aurora B expression is practically undetectable in the *ial* RNAi sample, and Subito expression is undetectable in the *sub* mutant. Aurora B expression is also decreased in the *Incenp* RNAi sample and increased in the *Incenp*^{myc} sample, suggesting that Aurora B is stabilized by the presence of Incenp. Approximately 2.5-fold more material was loaded of the *ial* RNAi and *Incenp* RNAi samples relative to the other samples, except in the anti-Subito blot in which all samples were loaded evenly.