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ARTICLE

Borealin directs recruitment of the CPC to oocyte chromosomes and movement to the microtubules

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The chromosomes in the oocytes of many animals appear to promote bipolar spindle assembly. In *Drosophila* oocytes, spindle assembly requires the chromosome passenger complex (CPC), which consists of INCENP, Borealin, Survivin, and Aurora B. To determine what recruits the CPC to the chromosomes and its role in spindle assembly, we developed a strategy to manipulate the function and localization of INCENP, which is critical for recruiting the Aurora B kinase. We found that an interaction between Borealin and the chromatin is crucial for the recruitment of the CPC to the chromosomes and is sufficient to build kinetochores and recruit spindle microtubules. HP1 colocalizes with the CPC on the chromosomes and together they move to the spindle microtubules. We propose that the Borealin interaction with HP1 promotes the movement of the CPC from the chromosomes to the microtubules. In addition, within the central spindle, rather than at the centromeres, the CPC and HP1 are required for homologous chromosome bi-orientation.

Introduction

Accurate chromosome segregation during cell division requires bi-orientation of homologous chromosomes in meiosis I and sister chromatids in mitosis or meiosis II. Bi-orientation is the result of two simultaneous processes—the assembly of microtubules into a bipolar spindle and the correct attachment of the kinetochores to microtubules. In mitosis and male meiosis, the bipolarity of the spindle is defined by centrosomes at each pole. These serve as microtubule-organizing centers (MTOCs), nucleating microtubules that grow toward the chromosomes and make contact with kinetochores (Cheeseman, 2014; Heald and Khodjakov, 2015; Nicklas, 1997; Watanabe, 2012). In the oocytes of many species, the female meiotic spindle assembles without centrosomes. Spindle assembly initiates when microtubules cluster around the chromosomes after nuclear envelope breakdown (Dumont and Desai, 2012; Radford et al., 2017). In mouse oocytes, this involves the accumulation of acentriolar MTOCs around the chromosomes (Dumont et al., 2007; Schuh and Ellenberg, 2007). In contrast, chromosomes or chromatin recruit the microtubules in the oocytes of *Drosophila* (Matthies et al., 1996; Theurkauf and Hawley, 1992) and humans (Holubcová et al., 2015).

Chromatin-coated beads in *Xenopus* extracts (Heald et al., 1996; Sampath et al., 2004) and mouse oocytes (Deng et al., 2009), and chromosomes without kinetochores in *Drosophila* oocytes (Radford et al., 2015), build spindles. Similarly, kinetochore-independent chromosome interactions between the chromosomes and the spindle in *Caenorhabditis elegans* oocyte meiosis have been observed (Dumont et al., 2010; Muscat et al., 2015; Wignall and Villeneuve, 2009). These results suggest that oocyte chromatin carries signals that can recruit and organize spindle assembly factors. Potential targets of these signals include the Ran pathway and the chromosomal passenger complex (CPC), both of which have been shown to promote chromosome-directed spindle assembly (Bennabi et al., 2016; Drutovic et al., 2020; Mullen et al., 2019; Radford et al., 2017). The CPC comprises Aurora B kinase, the scaffold subunit INCENP (inner centromere protein), and the two targeting subunits, Borealin and Survivin (Deterin in *Drosophila*). In *Drosophila*, the depletion of Aurora B or INCENP causes a complete failure of meiotic spindle assembly in oocytes (Colombié et al., 2008; Radford et al., 2012). Similarly, the CPC is required for promoting spindle assembly in *C. elegans* oocytes (Dumont et al., 2010) and when sperm nuclei are added to *Xenopus* egg extracts (Kelly et al., 2007; Maresca et al., 2009; Sampath et al., 2004). These results suggest that oocyte chromosomes carry signals that can recruit and activate the activity of the CPC. Indeed, the *Xenopus* studies demonstrate that spindle assembly requires that the CPC interacts with both chromatin and microtubules (Tseng et al., 2010; Wheelock et al., 2017).

The CPC displays a dynamic localization pattern during cell division that contributes to its known functions. During mitosis,

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the CPC localizes to the centromeres during metaphase, where it is required for correcting kinetochore-microtubule (KT-MT) attachments, cohesion regulation, and checkpoint regulation (Carmena et al., 2012a; Krenn and Musacchio, 2015; Trivedi and Stukenberg, 2020). It then relocates onto the microtubules to form the spindle midzone required for anaphase and cytokinesis (Adams et al., 2001; Carmena et al., 2012b; Cesario et al., 2006; Chang et al., 2006). In *Drosophila* prometaphase I oocytes, however, the CPC is most abundant on the central spindle, similar to the anaphase midzone of mitotic cells, and is not usually observed at the centromeres (Jang et al., 2005; Radford et al., 2012). Thus, while the CPC is required for spindle assembly in *Drosophila* oocytes, how the chromosomes are involved in this process is not known.

To test the hypothesis that the chromosomes recruit and activate the CPC to spatially restrict oocyte spindle assembly, we developed an RNAi-resistant expression system to generate separation-of-function mutants of the CPC. The most thoroughly studied pathways for localization of the CPC to chromosomes involves two histone kinases, Haspin and Bub1, which phosphorylate H3T3 and H2AT120 (Hindriksen et al., 2017) and recruit Survivin and Borealin, respectively, to the inner centromeres; however, Haspin and Bub1 are not required for spindle assembly in oocyte meiosis. Instead, an interaction between Borealin and the chromatin recruits the CPC to the oocyte chromosomes to initiate kinetochore and spindle assembly. Furthermore, heterochromatin protein 1 (HP1) may interact with the CPC in multiple phases of spindle assembly. HP1 colocalizes with the CPC on the chromosomes and then moves with the CPC onto the spindle. Thus, our research has revealed a mechanism for how the meiotic chromosomes recruit the CPC for spindle assembly and how the CPC moves to the microtubules. We also propose that within the central spindle, the CPC and HP1 promote the bi-orientation of homologous chromosomes in oocytes.

Results

Using RNAi-resistant transgenes to study factors that regulate CPC localization

The *Drosophila* meiotic spindle is composed of two types of microtubules. Kinetochore microtubules (K-fibers) are defined by those that end at a kinetochore, and the central spindle is defined by microtubules that make antiparallel overlaps in the center of the spindle and contain the kinesin 6 Subito (Jang et al., 2005). The CPC localizes predominantly to the central spindle in prometaphase I *Drosophila* oocytes and can also be observed at centromeres in oocytes treated with colchicine to destabilize microtubules (Fig. 1 A). Thus, the CPC can localize to meiotic chromosomes in addition to spindle microtubules in metaphase oocytes.

To study the relationship between CPC localization patterns and function, we developed a system to target the CPC to distinct chromosomal or spindle locations. Oocyte-specific RNAi was used to knock down INCENP instead of using mutants, because the CPC is essential for viability. *Incenp* or *aurB* RNAi oocytes fail to recruit kinetochore proteins, such as SPC105R or NDC80, to the centromeres, or recruit microtubules around the chromosomes (Radford et al., 2015; Radford et al., 2012). We constructed an *Incenp* transgene to be RNAi resistant with silent mismatches in the region targeted by shRNA *GL00279* (Fig. 1 B and Fig. S1). Expressing the RNAi-resistant transgene ($Incenp^{WT\text{-}R}$) rescued the defects in *Incenp* RNAi oocytes, including spindle and kinetochore assembly, homologue bi-orientation, and fertility, restoring them to WT levels (Fig. 1 C and Table 1). The $Incenp^{WT\text{-}R}$ backbone was then used to construct separation-of-function *Incenp* mutants. These mutants were analyzed in either a WT (i.e., $Incenp^{WT\text{-}R}$ oocytes) or RNAi background (i.e., $Incenp^{WT\text{-}R}$, *Incenp RNAi* oocytes).

Targeting the CPC to centromeres is sufficient, but not required, to promote K-fiber assembly

Borealin and Survivin—known as Deterin in *Drosophila*—target the CPC to the centromeres in mitotic cells (Carmena et al., 2012b; Hindriksen et al., 2017). Borealin and Survivin interact with the N-terminal domain of INCENP, which is required for centromere localization of the CPC (Jeyaprakash et al., 2007; Klein et al., 2006). A *Deterin* shRNA was found to have the same phenotype as *Incenp* or *aurB* RNAi oocytes (Fig. 1 D). This result suggests that Deterin is required to target INCENP and Aurora B to the chromosomes in *Drosophila* oocytes. To test the function of the CPC at the centromere, we deleted conserved amino acids 22–30 in INCENP ($Incenp^{\Delta CEN}$; Fig. 1 B and Fig. S1) that correspond to the centromere-targeting domain described in chicken INCENP and which is predicted to be required for the interaction with Borealin and Deterin (Ainsztein et al., 1998; Jeyaprakash et al., 2007). In $Incenp^{\Delta CEN}$, *Incenp* RNAi oocytes, the $INCENP^{\Delta CEN}$ protein had weak localization to the chromosomes and did not recruit Deterin or promote spindle assembly (Fig. 1 E). Furthermore, $Incenp^{\Delta CEN}$, *Incenp* RNAi oocytes had an intermediate level of SPC105R localization compared with WT or *Incenp*, *aurB* RNAi or *Deterin* RNAi oocytes (Fig. 1, C–F). These results suggest that the N-terminal domain of INCENP recruits Deterin and is required for spindle assembly in *Drosophila* oocytes, although some kinetochore assembly is possible without it.

To directly test whether centromeric CPC can promote spindle assembly and regulate homologue bi-orientation in oocytes, we targeted the CPC to the centromeric regions. Based on a strategy used in HeLa cells, INCENP was fused to the kinetochore protein MIS12 (Liu et al., 2009). MIS12 loads onto centromeres during prophase (Schittenhelm et al., 2007; Venkei et al., 2012), is independent of other kinetochore proteins (Feijão et al., 2013; Przewloka et al., 2007), and localizes to foci on the chromosomes in WT and *Incenp* RNAi oocytes (Fig. S2 A; Głuszek et al., 2015). To target the CPC to the centromeres, the N-terminal amino acids 1–46 of INCENP—the BS (Borealin-Survivin) domain—were replaced with MIS12 (*mis12:Incenp*; Fig. 2 A). Surprisingly, when *mis12:Incenp* was expressed in WT oocytes, we did not observe centromere localization (Fig. 2 B); however, the females were sterile due to the failure to complete the two meiotic divisions and initiate the mitotic divisions (Fig. S2 B and Table 1). This phenotype demonstrated that the transgene was expressed and toxic to the embryo. When expressing *mis12:Incenp* in *Incenp* RNAi oocytes, the fusion protein localized around the centromeres, but only partial spindle

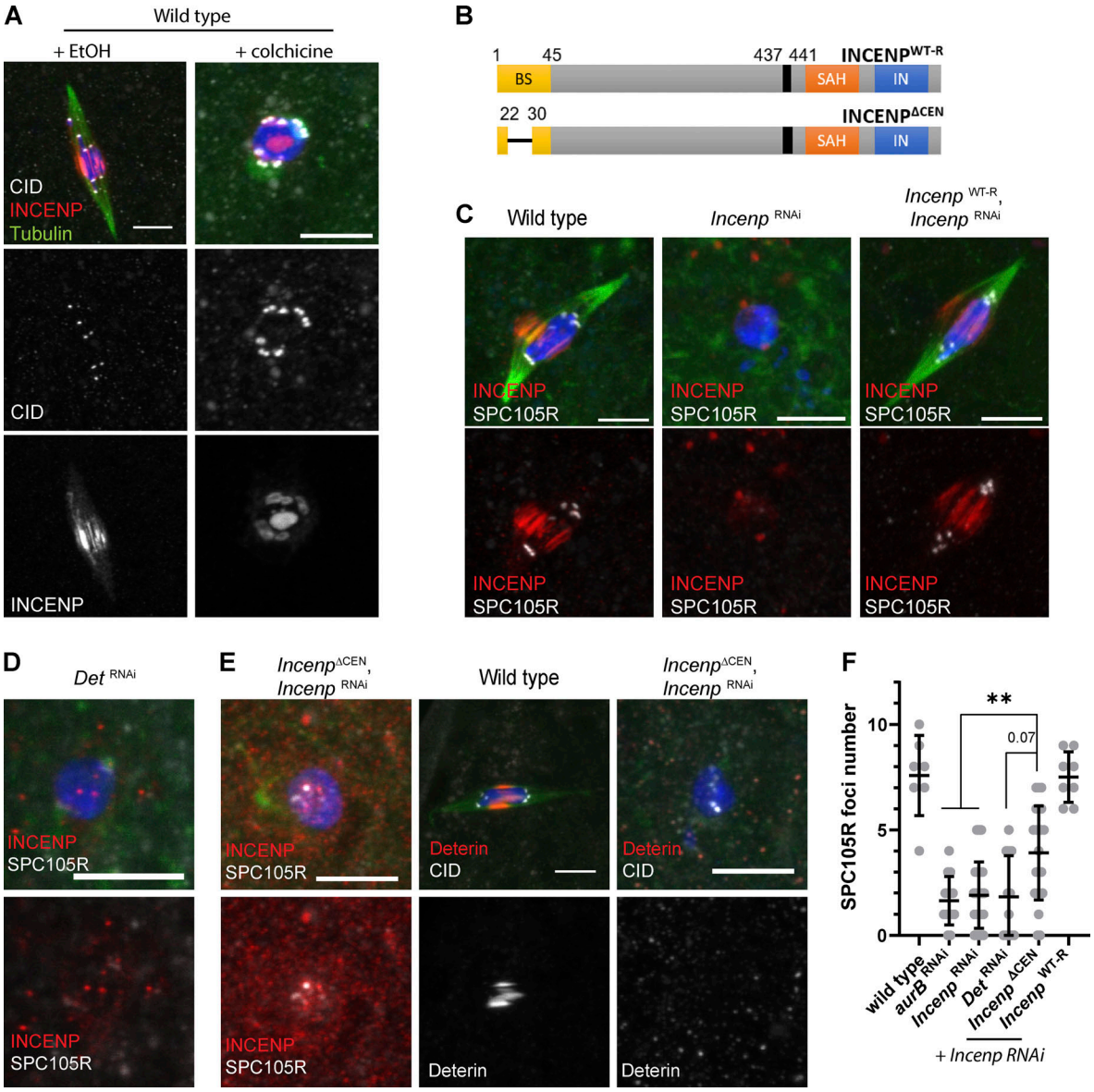

Figure 1. **INCENP localization and spindle assembly depends on the N-terminal Borealin/Deterin binding domain. (A)** INCENP localization in oocytes after a 60-min colchicine treatment to destabilize the microtubules. The sister centromeres appeared to be separating in colchicine-treated oocytes, the mechanism of which is not known. INCENP is in red, CID/CENP-A is in white, tubulin is in green, and DNA is in blue. **(B)** A schematic of *Drosophila* INCENP showing the location of the centromere-targeting BS binding region, SAH, and INbox (IN) domains. The black box (437–441) shows the sequence targeted by the shRNA *GL00279* that were mutated to make RNAi-resistant *Incenp*$^{WT-R}$. The conserved amino acids 22–30 are deleted in *Incenp*$^{\Delta CEN}$. **(C)** INCENP (red) localization in *Incenp* RNAi oocytes and oocytes also expressing the *Incenp*$^{WT-R}$ RNAi-resistant transgene. Kinetochore protein SPC105R is in white. **(D)** Spindle and kinetochore assembly defects in *Deterin* RNAi oocytes. **(E)** Spindle and kinetochore assembly defects in *Incenp*$^{\Delta CEN}$, *Incenp* RNAi oocytes, with Deterin or INCENP in red and SPC105R or CID in white. **(F)** Quantitation of SPC105R localization in RNAi oocytes (n = 7, 14, 32, 12, 22, and 8 oocytes). Error bars indicate 95% confidence intervals; **, P < 0.01 run by Fisher's exact test. Scale bars, 5 µm (all images).

assembly was observed. Kinetochore assembly and K-fiber formation was observed, which was defined as oocytes with robust SPC105R localization and microtubules emanating from the kinetochores (Fig. 2, B and C; and Fig. S2 C). Unlike WT oocytes, however, these spindles were usually short and lacked a central spindle. These results suggest that targeting INCENP to the centromere regions could only promote kinetochore assembly and K-fiber formation.

In the presence of endogenous INCENP, MIS12:INCENP did not localize to the centromeres. To test the possibility that regions outside the BS domain of INCENP negatively regulate centromere localization, we fused MIS12 directly to the INbox domain of INCENP (amino acids 655–755 of the C-terminal domain), which is sufficient to recruit Aurora B (Fig. 2 A; Bishop and Schumacher, 2002). Expressing unfused *INbox* in the presence of endogenous INCENP had a dominant-negative effect on oocyte spindle assembly, causing a diminished spindle (Fig. 2 D) and sterility (Table 1). This observation suggests that unlocalized INbox has a dominant-negative effect. Similar to observations in mammalian cells (Gohard et al., 2014), INbox

Table 1. **Summary of transgene fertility**

Genotype	Fertility/NDJ[a] In WT oocytes	In *Incenp* RNAi oocytes
Myc:Incenp	+++/1.4% NDJ (*n* = 862)	+/11.4% NDJ (*n* = 184)
HA:Incenp	+++/0.5% (*n* = 552)	+/1.3% (*n* = 315)
Flag:Incenp	++/5.2% (*n* = 771)	+/8.1% (*n* = 150)
$Incenp^{WT\text{-}R}$	+++/0% (*n* = 267)	+++/0.9% (*n* = 3,806)
$Incenp^{\Delta CEN}$	+/0% (*n* = 35)	Sterile
$Incenp^{\Delta STD}$	Sterile	++/0% (*n* = 323)
$Incenp^{\Delta SAH}$	+/0% (*n* = 111)	Sterile
Det:Incenp	Sterile	Sterile
Mis12:Incenp	Sterile	Sterile
Mis12:Inbox	Sterile	Sterile
Feo:Inbox	Sterile	Sterile
Sub:Inbox	Sterile	Sterile
Inbox	Sterile	Sterile
$Incenp^{\Delta HP1}$	Sterile	Sterile
HP1:Incenp	++/0% (*n* = 113)	Sterile
Borr:Incenp	+/0% (*n* = 74)	+/0% (*n* = 18)
$Borr^{\Delta c}$*:Incenp*	Sterile	Sterile
$Borr^{\Delta c}$*:Incenp* $^{\Delta HP1}$	Sterile	Sterile
WT	+++/0% (*n* = 240)	Sterile

NDJ = 2XNDJ/total progeny.
[a]Females were crossed to *y Hw w/BSY* males in vials. Fertility is based on the number of progeny per vial: +++ = 20–50 per vial; ++ = 10–20 per vial; and + = 1–10 per vial; sterile = no progeny.

could be acting like a competitive inhibitor of INCENP by generating nonproductive binding interactions with Aurora B. When expressing *mis12:INbox* in WT oocytes, MIS12:INbox was present at the centromeres (Fig. 2 D). MIS12:INbox was also observed on the spindle, although the mechanism and consequences of this are not known. Phospho-INCENP, which is a marker of Aurora B activity (Salimian et al., 2011), was observed at the central spindle and in the vicinity of the centromeres (Fig. 2 E), showing that MIS12:INbox can successfully recruit and activate Aurora B. Similar to *mis12:Incenp*, when *mis12:INbox* was expressed in *Incenp* RNAi oocytes, SPC105R was recruited and K-fibers formed, but there was no central spindle (Fig. 2, C, F, and G; and Fig. S2 C). The centromeres were unable to bi-orient and were often clustered together and oriented toward the same pole of a monopolar spindle. These results demonstrate that centromere-targeted CPC is sufficient to build kinetochores and K-fibers, consistent with findings in *Xenopus* extracts (Bonner et al., 2019), but not the central spindle.

Independent targeting of the CPC to both the centromere and central spindle is not sufficient to assemble a WT spindle

Because centromere-directed Aurora B only promotes K-fiber assembly, it is possible that oocyte spindle assembly depends on microtubule-associated Aurora B. Indeed, our prior studies have suggested the CPC is simultaneously required for kinetochore and central spindle microtubule assembly in oocytes (Radford et al., 2015); therefore, we performed experiments to determine whether the recruitment of Aurora B to these two sites is independent or whether one site might depend on the other. To target Aurora B to the spindle, the *INbox* was fused with two microtubule-associated proteins, Fascetto (*feo*, the *Drosophila* PRC1 homologue) or Subito (Fig. 2 A). These two fusions, *feo:INbox* and *sub:INbox*, resulted in robust INbox localization to the central spindle when expressed in WT oocytes (Fig. 2, D and E; and Fig. S2 D). When expressed in *Incenp* RNAi oocytes, neither *feo:INbox* nor *sub:INbox* oocytes assembled a spindle around the chromosomes (Fig. 2 F and Fig. S2 C). These results suggest that microtubule-associated Aurora B is not sufficient to promote spindle assembly around the chromosomes. Fusing Subito to the INbox promoted microtubule bundles in the cytoplasm, but not in the specifically important location around the chromosomes (Fig. 2 F). Additionally, most SPC105R localization was absent in *sub:INbox, Incenp* RNAi oocytes (Fig. S2 C), similar to *Incenp* RNAi (Fig. 1, C and F). One possible explanation for these observations is that the central spindle targeting of Aurora B lacked the interaction with the chromosomes necessary for spindle and kinetochore assembly.

The problem with the *feo:INbox* and *sub:INbox* experiments could have been the absence of chromosome-associated Aurora B to recruit microtubules and nucleate central spindle assembly;

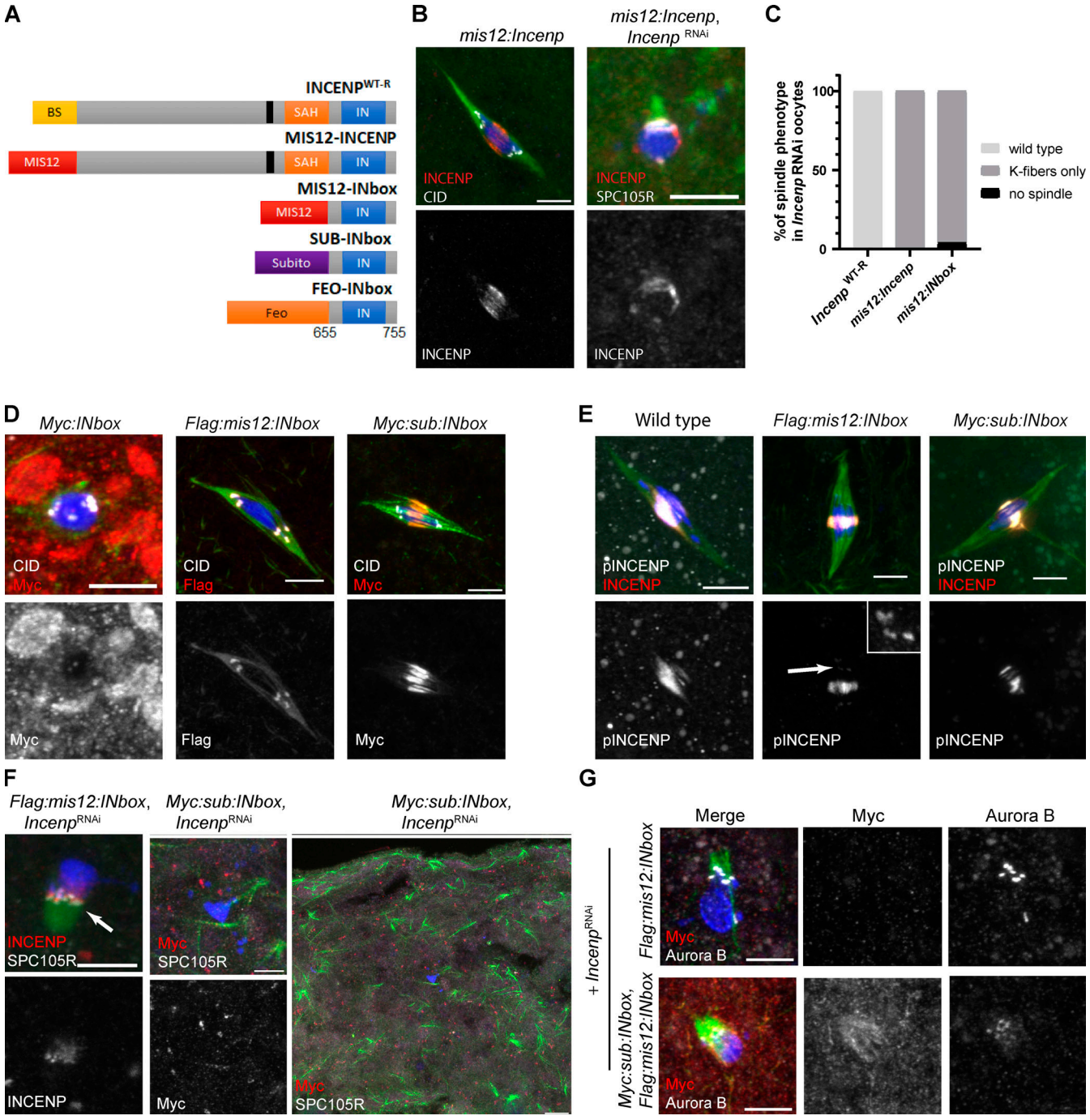

Figure 2. **Independent localization of the CPC to the centromere and the central spindle assembles only kinetochore-dependent microtubules. (A)** A schematic of the CPC constructs designed to target the CPC to the centromeres. **(B)** Expression of *mis12:Incenp* in WT and *Incenp* RNAi oocytes. **(C)** Quantitation of the spindle phenotype of MIS12 fusions expressed in *Incenp* RNAi oocytes (n = 23, 36, and 25 oocytes). **(D)** *Myc:INbox*, *Flag:mis12:INbox*, and *Myc:sub:INbox* expressed in WT oocytes. **(E)** Detection of the Aurora B substrate, phosphorylated INCENP (pINCENP). The arrow and inset (3 µm wide, levels increased) show the pINCENP signal at the centromeres when the INbox is targeted by MIS12. INCENP is in red and pINCENP is in white. **(F)** *Flag:mis12:INbox* and *Myc:sub:INbox* expressed in *Incenp* RNAi oocytes. The arrow points to K-fibers in *Flag:mis12:Inbox* oocytes. The low-magnification image of *Myc:sub:INbox* shows microtubule bundles in the cytoplasm instead of around the chromosomes. Transgene proteins are in red, SPC105R in white, DNA is in blue, and tubulin is in green. Scale bars, 5 µm (left four panels) and 10 µm (low-magnification image). **(G)** *Flag:mis12:INbox* expressed alone or coexpressed with *Myc:sub:INbox* in *Incenp* RNAi oocytes. Merged images showed DNA (blue), tubulin (green), Myc (red), and Aurora B (white). Scale bars, 5 µm.

therefore, to test whether independent targeting of Aurora B to the chromosomes and microtubules would promote spindle assembly, we coexpressed *mis12:INbox* and either *sub:INbox* or *feo:INbox* in *Incenp* RNAi oocytes. Interestingly, only the K-fibers formed in these oocytes, suggesting that *sub:INbox* and *feo:INbox* cannot contribute to spindle assembly, even in the presence of K-fibers (Fig. 2 G and Fig. S2 E). These results indicate that independently targeting two populations of Aurora B is not

sufficient to assemble a bipolar spindle (see also Tseng et al., 2010).

Borealin, but not Deterin, is sufficient for most meiotic spindle assembly

Central spindle assembly may require an interaction between the CPC and the chromosomes before the bundling of antiparallel microtubules. Furthermore, the phenotype of *Incenp*$^{\Delta CEN}$ and *Deterin* RNAi oocytes suggests that Borealin and Deterin are critical for chromosome-directed spindle assembly in oocytes (Fig. 1, D and E). To test whether an interaction of Deterin and/or Borealin with INCENP is sufficient to target the CPC for oocyte spindle assembly, we replaced the BS domain of INCENP with Deterin or Borealin (referred to as *Det:Incenp* and *borr:Incenp*; Fig. 3 A). In *Det:Incenp* oocytes, INCENP localized to the chromatin close to the centromeres and K-fibers were formed; however, DET:INCENP failed to localize to the spindle and no central spindle was observed (Fig. 3 B). Borealin localization could not be detected in *Det:Incenp, Incenp* RNAi oocytes (Fig. S3 A), suggesting that this spindle phenotype is independent of Borealin.

In contrast, *borr:Incenp* rescued spindle assembly, including the central spindle, in *Incenp* RNAi oocytes (Fig. 3 B), although the degree of rescue in some oocytes was variable. In *borr:Incenp, Incenp* RNAi oocytes, 29% had a frayed central spindle, and in 47%, INCENP localized to the microtubules but failed to be concentrated in the central spindle (n = 28; Fig. 3, B and D). A bipolar spindle also formed when *borr:Incenp* was expressed in *Deterin* RNAi oocytes, although similar to *borr:Incenp*, there were some spindle abnormalities (48% frayed spindles and 17% diffuse INCENP localization; Fig. 3, C and D). In contrast, 44% of *Deterin* RNAi oocytes did not assemble a spindle, and the rest only showed nonspecific microtubule clustering around the chromosomes (Fig. 3, C and D). Thus, the *borr:Incenp* fusion promotes spindle assembly independent of Deterin. Deterin localized to the spindle in 64% of *borr:Incenp, Incenp* RNAi oocytes, but only when BORR:INCENP was concentrated in the central spindle (Fig. 3 E). These observations demonstrate that Borealin is sufficient to move the CPC from the chromosomes to the microtubules and promote spindle assembly in *Drosophila* oocytes. An important role for Borealin in CPC-dependent spindle assembly has also been shown in *Xenopus* (Kelly et al., 2007). Deterin, in contrast, promotes the localization of INCENP to the chromatin and the formation of kinetochores and K-fibers, and may have a role stabilizing the interaction of Borealin and INCENP with microtubules. Deterin is not, however, sufficient to promote movement of the CPC to the microtubules.

Recruitment of the CPC to the chromosomes and spindle assembly depends on the C-terminal domain of Borealin

Deterin and Borealin are known to be recruited by the histone markers H3T3ph and H2AT120ph, respectively (Wang et al., 2010; Yamagishi et al., 2010). These two histones are phosphorylated by Haspin and BUB1 kinases, respectively; however, spindle assembly, CPC localization, fertility, and chromosome segregation were normal in *Haspin* or *Bub1* RNAi oocytes or *Haspin, Bub1* double-RNAi oocytes (Fig. S4, A and B). Furthermore, ubiquitous expression of *Haspin* or *Bub1* shRNAs did not cause lethality, and *Haspin*-null mutants are viable and fertile (Fig. S4 B; Fresán et al., 2020). These results suggest that Haspin and BUB1 are not required for the CPC to promote meiotic spindle assembly in oocytes. In addition, *Drosophila* CPC localization does not depend on MEI-S332/SGO (Resnick et al., 2006), which, in vertebrates, has been shown to recruit Borealin (Bonner et al., 2020; Broad et al., 2020; Kawashima et al., 2007). Therefore, we investigated other mechanisms for Borealin-mediated CPC recruitment to the chromosomes.

In addition to recruitment by BUB1 activity, Borealin can be recruited to chromosomes by an interaction between its C-terminal domain and HP1 (Liu et al., 2014) or nucleosomes (Abad et al., 2019). Although the C-terminal domain of Borealin is poorly conserved, there is evidence in support of the hypothesis that the CPC interacts with HP1 during chromosome-directed spindle assembly in oocytes. INCENP and Borealin colocalize with HP1 and H3K9me3, the histone marker that recruits HP1, in *aurB* RNAi oocytes (Fig. 4, A and B). Furthermore, HP1 is present on chromosomes in *Incenp* RNAi oocytes (Fig. 4 A), showing that the CPC is not required for HP1 localization. To test the hypothesis that HP1 recruits the CPC to chromatin in oocytes, we deleted the C-terminal domain of Borealin from *borr:Incenp* (referred as *borr*$^{\Delta C}$*:Incenp*; Fig. 4 C). Spindle assembly was severely impaired in *borr*$^{\Delta C}$*:Incenp, Incenp* RNAi oocytes. Only 19% of oocytes assembled K-fibers, and none of them assembled the central spindle (Fig. 4, D–F). Most of the oocytes that assembled K-fibers (75%) had normal SPC105R localization (Fig. 4 F and Fig. S3 B), suggesting that K-fiber formation was associated with SPC105R localization. Because CPC components and HP1 colocalize when Aurora B activity is absent, we favor the interpretation that an interaction between Borealin and HP1 is required to build both K-fibers and the central spindle in oocytes; however, we cannot rule out a role for an interaction between Borealin and the nucleosomes in recruiting the CPC to the chromosomes.

Two putative HP1 interaction sites exist in INCENP (Ainsztein et al., 1998; van der Horst and Lens, 2014), and these were deleted to make *Incenp*$^{\Delta HP1}$ (Fig. 4 C). While spindle assembly failed in most *borr*$^{\Delta C}$*:Incenp, Incenp* RNAi oocytes, spindle assembly in *Incenp*$^{\Delta HP1}$*, Incenp* RNAi oocytes was similar to WT. In a minority of these oocytes (36%), however, INCENP displayed irregular and disorganized central spindle localization (Fig. 4, D, E, and G). Thus, an INCENP–HP1 interaction may only have a minor role in oocyte spindle assembly. To test for additive effects, a mutant with all HP1 sites deleted was generated (*borr*$^{\Delta C}$*: Incenp*$^{\Delta HP1}$). A more severe spindle assembly defect was observed in *borr*$^{\Delta C}$*:Incenp*$^{\Delta HP1}$*, Incenp* RNAi oocytes. Specifically, the spindle was abolished in nearly all oocytes, and we measured a small decrease in K-fiber formation (P = 0.08; Fig. 4, D and E). These results suggest that the C-terminal domain of Borealin recruits the CPC to the oocyte chromosomes, with a minor contribution from INCENP. To test whether the only function of Borealin in oocytes is to interact with HP1 for recruitment of the CPC, the BS domain of *Incenp* was replaced with HP1 (*HP1:Incenp*; Fig. 4 C). HP1:INCENP localized to part of the chromatin, probably the heterochromatin regions, but 53% of oocytes failed at spindle

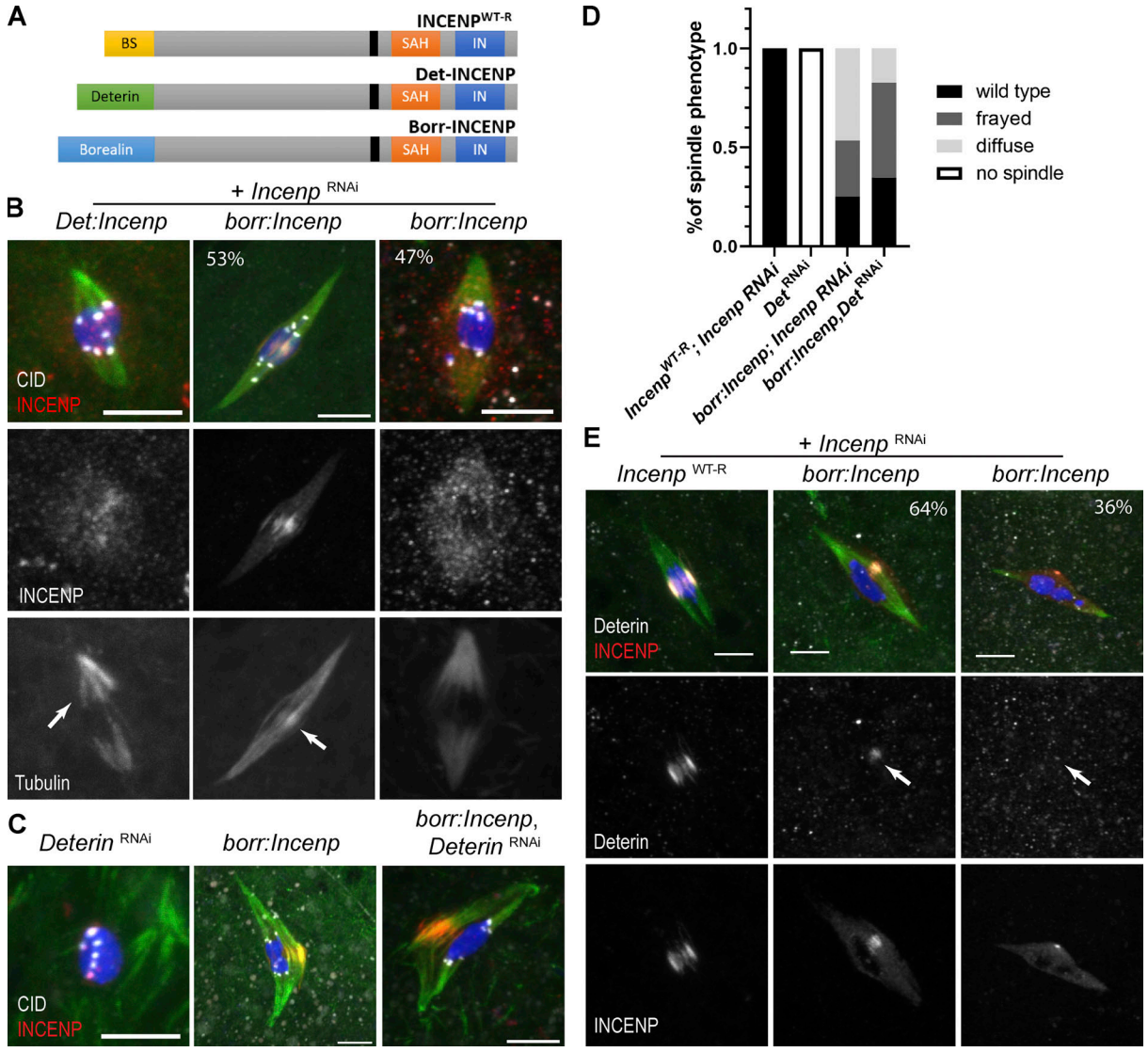

Figure 3. **Borealin is sufficient to recruit the CPC for meiotic spindles assembling. (A)** A schematic of the *Det:Incenp* and *borr:Incenp* fusions compared with WT *Incenp*. **(B)** The spindle phenotypes of *Det:Incenp* (n = 18) and *borr:Incenp* (n = 28) in *Incenp* RNAi oocytes. The separate channels show the localization of INCENP and the microtubules. The second *borr:Incenp* image is an example of a spindle with diffuse INCENP on the spindle. Arrows show K-fibers in *Det:Incenp* oocytes and central spindle fibers in *borr:Incenp* oocytes. INCENP is in red, DNA is in blue, tubulin is in green, and CID is in white. **(C)** Deterin depletion in *borr:Incenp* oocytes has a spindle phenotype similar to *borr:Incenp*. **(D)** Summary of the effect of Deterin depletion in *borr:Incenp* oocytes (n = 23, 18, 28, and 23). **(E)** Deterin localization in WT and *borr:Incenp, Incenp* RNAi oocytes (n = 22). The first *borr:Incenp* image is an example with a defined central spindle and the second has a diffuse spindle and INCENP. INCENP is in red, Deterin is in white, DNA is in blue, and tubulin is in green. Scale bars, 5 µm (all images).

assembly and the rest only had K-fiber formation associated with SPC105R localization (Fig. 4, E, F, and H; and Fig. S3). Thus, targeting the CPC to the heterochromatin regions without Borealin is not sufficient for bipolar spindle assembly. Rather than Borealin being an adapter for CPC localization, an interaction between Borealin and HP1 and/or nucleosomes appears to be essential for the transfer of the CPC to the microtubules and oocyte spindle assembly.

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

To determine if Borealin is sufficient to target the CPC to the chromatin, we examined the behavior of BORR:INCENP fusion proteins when Aurora B activity was inhibited. Similar to the results in *aurB* RNAi oocytes, when WT oocytes were treated with the Aurora B inhibitor binucleine 2 (BN2; Smurnyy et al., 2010), the spindle was drastically diminished and INCENP co-localized with HP1 and H3K9me3 on the chromosomes (Fig. 5 A). The same result was observed with *borr:Incenp, Incenp* RNAi oocytes. The BORR:INCENP fusion colocalized with HP1 on the chromosomes in BN2-treated oocytes (Fig. 5 A). In contrast, in BN2-treated *borr*$^{\Delta C}$*:Incenp, Incenp* RNAi oocytes, the BORR$^{\Delta C}$:INCENP fusion did not localize to the chromosomes. These results suggest that the Borealin C-terminal domain is required to target the CPC to the chromosomes, including sites enriched with HP1.

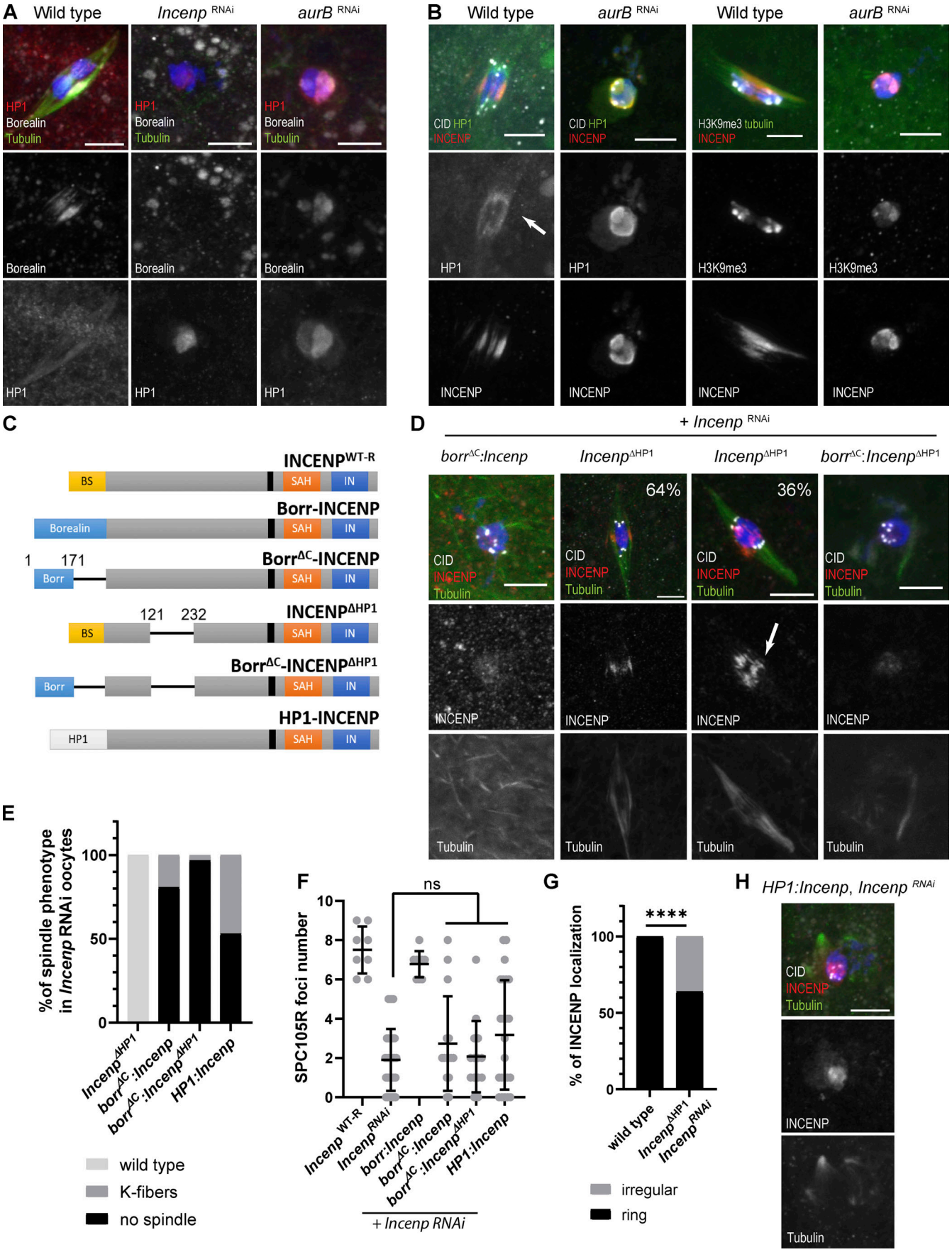

Figure 4. **Chromatin–Borealin interaction is critical for CPC-dependent meiotic spindle assembly. (A)** Borealin and HP1 localization in WT, *Incenp* RNAi, and *aurB* RNAi oocytes. Borealin is in white, HP1 is in red, tubulin is in green, and DNA is in blue. **(B)** HP1 and H3K9me3 localization in WT and *aurB* RNAi oocytes. Arrow shows HP1 localization in WT oocytes on the spindle. HP1 or tubulin is in green, INCENP is in red, DNA is in blue, and CID or H3K9me3 are in white. **(C)** A schematic of *Incenp* mutant constructs affecting HP1 interactions. Proposed HP1 binding sites are located in the C terminus of Borealin and in INCENP between amino acids 121–232. Full-length *Drosophila* HP1 was fused to INCENP by substitution for the BS domain. **(D)** Expression of *Incenp* transgenes shown in C in *Incenp* RNAi oocytes, including $borr^{\Delta C}$:*Incenp*, $Incenp^{\Delta HP1}$ and $borr^{\Delta C}$:$Incenp^{\Delta HP1}$. Two different images of $Incenp^{\Delta HP1}$ are shown to compare ring-shaped and disorganized (see arrow) localization of the CPC. The images show CID in white, INCENP in red, DNA in blue, and tubulin in green. **(E)** Quantitation of spindle phenotype in the HP1 interaction–defective mutants shown in D (n = 17, 26, 31, and 49 oocytes, in the order as shown in the graph). **(F)** SPC105R localization (see Fig. S3) in the HP1 interaction–defective mutant oocytes (n = 8, 32, 9, 15, 14, and 33). Bars indicates 95% confidence intervals; ns, not significant in Fisher's exact test. **(G)** Quantitation of INCENP spindle localization in WT (n = 19) and $Incenp^{\Delta HP1}$, *Incenp* RNAi oocytes (n = 17). ****, P < 0.0001. **(H)** Expressing *HP1:Incenp* (see C) in *Incenp* RNAi oocytes. The images show CID in white, INCENP in red, DNA in blue, and tubulin in green.

In WT oocytes, HP1 is on the spindle, but in the absence of Aurora B activity, is on the chromosomes (Fig. 5 A). To test if Aurora B activity promotes the transfer of HP1 from the chromosomes to the spindle, we used colchicine to depolymerize the microtubules without inhibiting Aurora B activity. Colchicine treatment caused the CPC to retreat to the chromosomes and colocalize with H3K9me3 in WT oocytes (Fig. 5 B). Chromosome-associated INCENP was also observed in colchicine-treated *borr:Incenp*, *Incenp* RNAi, consistent with the conclusion that Borealin promotes CPC localization to the chromosomes. HP1, in contrast, was barely detectable in colchicine-treated WT oocytes (Fig. 5 B), unlike the observation in BN2-treated and *aurB* RNAi oocytes (Fig. 5 A; and Fig. 4, A and B). These results suggest that Aurora B activity negatively regulates HP1 localization. To test this hypothesis, we compared colchicine-treated $Incenp^{\Delta HP1}$, *Incenp* RNAi and $borr^{\Delta C}$:$Incenp^{\Delta HP1}$, *Incenp* RNAi oocytes. Only in the latter, which fails to recruit Aurora B to the chromosomes, did HP1 localize to the chromatin. Because $Incenp^{\Delta HP1}$, *Incenp* RNAi oocytes recruit Aurora B to the chromosomes while $borr^{\Delta C}$:$Incenp^{\Delta HP1}$, *Incenp* RNAi oocytes do not, the most likely explanation is that once HP1 is ejected from the chromosomes, Aurora B activity prevents its return. Thus, ejection of HP1 from the chromosomes depends on Aurora B activity and Borealin. Ejection of the CPC from the chromosomes, however, depends on Aurora B activity, Borealin, and the microtubules.

Recruitment of the CPC to the chromosomes and spindle assembly does not depend on INCENP microtubule-interacting domains or Subito

We have thus far provided evidence that Borealin targets the CPC to the chromosomes, and then Aurora B activity results in spindle assembly and movement of the CPC to the microtubules. Because CPC localization to the chromosomes depends on the presence of microtubules, we examined if the microtubule-binding domains within the CPC promoted spindle localization. INCENP has a single-α-helix (SAH) domain that binds microtubules (Samejima et al., 2015) and a conserved domain within the N-terminal region—the spindle transfer domain (STD)—that is required for transfer to the midbody (Ainsztein et al., 1998), possibly by interacting with the kinesin 6 MKLP2/Subito (Fig. S1; Serena et al., 2020). To test if either of these microtubule interaction domains are important for the CPC to relocate to the meiotic spindle, we generated deletions in each site (Fig. 6 A). In both $Incenp^{\Delta STD}$, *Incenp* RNAi and $Incenp^{\Delta SAH}$, *Incenp* RNAi oocytes, we observed bipolar spindle assembly and normal CPC localization and a central spindle, suggesting that these microtubule-binding domains are not required for oocyte meiotic spindle assembly (Fig. 6 B). However, these females displayed either reduced fertility or sterility (Table 1), suggesting that these two domains of INCENP have an important role in embryonic mitosis. Whether these two domains are redundant in meiosis, or that spindle localization of the CPC depends only on Borealin, remains to be investigated.

In *Drosophila*, kinesin 6 Subito is required to organize the central spindle, which includes recruiting the CPC (Das et al., 2018; Jang et al., 2005; Radford et al., 2012); however, the CPC can localize to the spindle microtubules in the absence of Subito (Fig. 6 C; Das et al., 2018; Jang et al., 2005), suggesting that ejection of the CPC from the chromosomes may be sufficient for spindle transfer and Subito is not required. Interestingly, we found that Subito has a conserved HP1 binding site (amino acid 88–92, PQVFL). To test if the putative Subito HP1 binding site is required to build the central spindle, we examined sub^{HM26}, a *sub* allele that has a point mutation (L92Q) in the HP1 binding site (Jang et al., 2005). $Subito^{HM26}$ failed to localize to the spindle in oocytes, the spindle displayed a tripolar phenotype, and the CPC localized throughout the spindle, all similar to a *sub*-null mutant (Fig. 6 C). Thus, an HP1 interaction may be required for Subito localization, although we have not yet shown a direct interaction between HP1 and Subito.

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

In mitotic cells, the CPC has an important role in error correction by destabilizing incorrect KT-MT attachments at the centromeres (Carmena et al., 2012b; Funabiki, 2019). Two hypomorphic CPC mutants, $Incenp^{QA26}$ and $aurB^{1689}$, could be defective in error correction because they are competent to build a bipolar spindle, but have bi-orientation defects in oocytes (Radford et al., 2012; Resnick et al., 2009). Because the CPC in oocytes is most prominent on the central spindle, Aurora B activity could regulate bi-orientation while located on the microtubules rather than the chromosomes. To compare the role of CPC at the centromeres and central spindle in regulating homologue bi-orientation, we used FISH to examine three sets of *Incenp* mutants where the CPC is inappropriately localized to either the centromeres or the central spindle. FISH probes targeted the pericentromeric regions each of chromosomes X, 2, and 3.

We first examined a sterile hypomorphic *Incenp* allele, $Incenp^{18.197}$, which was discovered based on genetic interactions

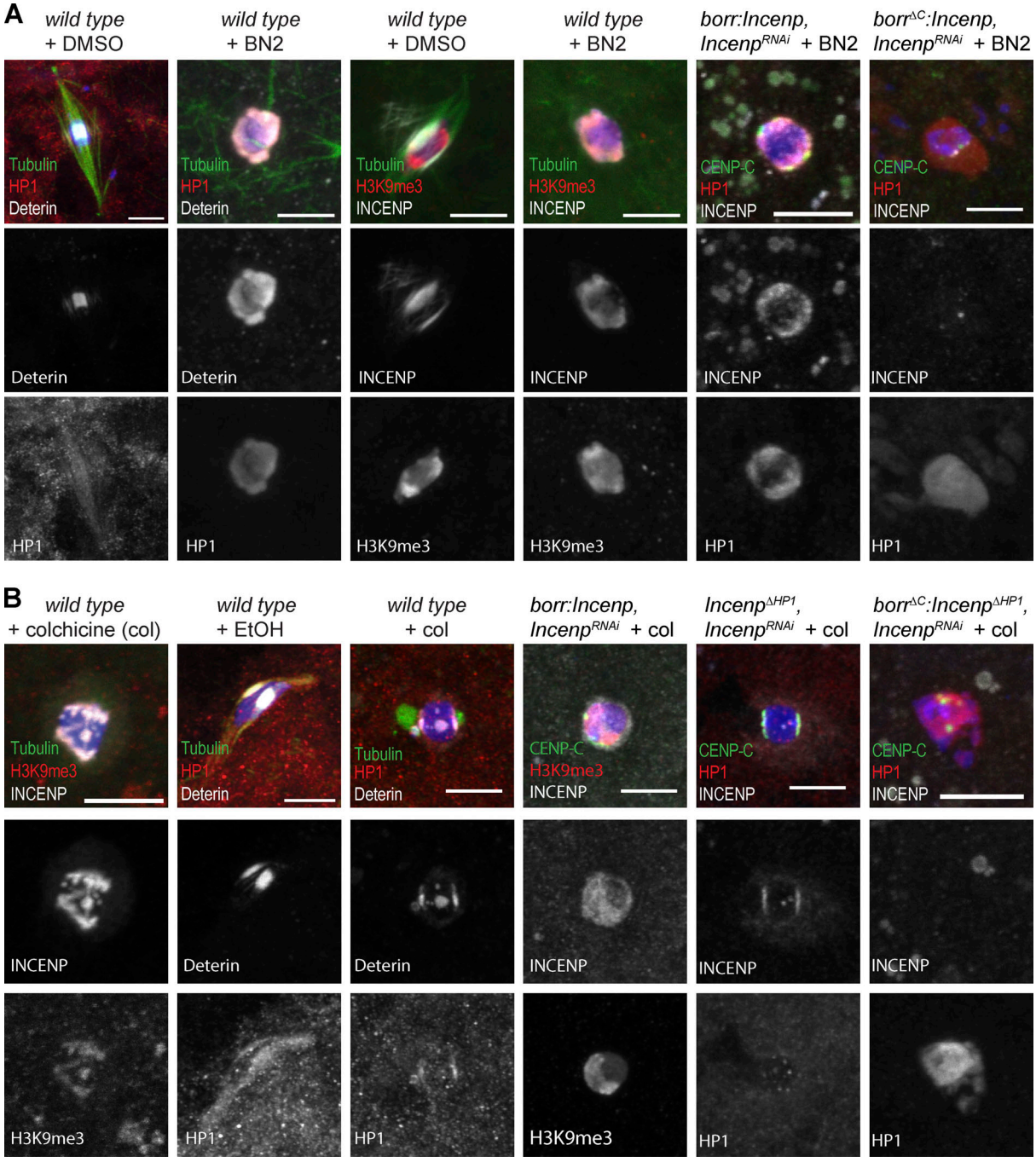

Figure 5. **The dissociation of HP1 and the CPC from the chromosomes depends on Aurora B kinase activity and microtubules. (A)** WT oocytes were treated with DMSO or BN2 for 1 h to inhibit Aurora B kinase activity. In red are heterochromatic marks HP1 or H3K9me3 and in white are the CPC components INCENP or Deterin. Scale bars, 5 µm. **(B)** WT oocytes were treated for 60 min with ethanol or colchicine to depolymerize microtubules, and were analyzed for the same markers as in A. In all images, DNA is in blue and tubulin is in green.

with *subito* (Das et al., 2016). In *Incenp*$^{18.197}$ oocytes, the spindle was moderately diminished and a portion of INCENP was retained on the chromosomes in 67% of oocytes (n = 15; Fig. 7 A), suggesting this mutant has a defect in CPC spindle transfer rather than chromosome localization. Using FISH, we determined that *Incenp*$^{18.197}$ mutant oocytes have homologue bi-orientation defects (11%; n = 42). Second, *Incenp* transgenes with an MYC tag fused to the N terminus have dominant defects in meiosis (Radford et al., 2012). To investigate whether the N-terminal tag was the cause of this defect, a new set of transgenes was constructed using the RNAi-resistant backbone and different tags (MYC, HA, and FLAG). We found that, regardless of the fused tag, all the transgenes had similar phenotypes: reduced fertility, elevated meiotic nondisjunction (NDJ) in *Incenp* RNAi oocytes (Table 1), and failure to restrict the CPC to the central spindle (Fig. 7 A). When the epitope tag was removed to

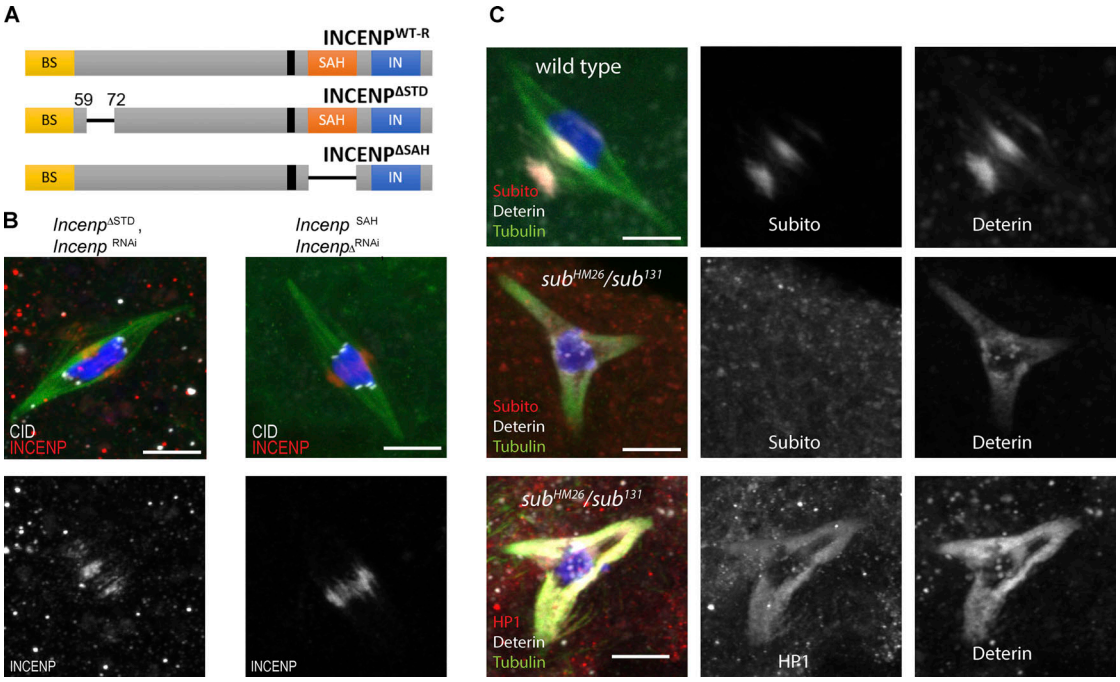

Figure 6. **Analysis of CPC interactions with Subito and microtubules required for central spindle assembly. (A)** A schematic showing two INCENP deletions removing regions that promote microtubule interactions. **(B)** Bipolar spindle assembly when *Incenp*$^{\Delta STD}$ or *Incenp*$^{\Delta SAH}$ were expressed in *Incenp* RNAi oocytes. CID is in white, INCENP is in red, tubulin is in green, and DNA is in blue. **(C)** Tripolar spindle and mislocalization of the CPC phenotype in *sub*^{HM26}/*sub*^{131} oocytes, with tubulin (green), Deterin (white), and HP1 or Subito (red) localization. Scale bars, 5 µm.

generate *Incenp*$^{WT\text{-}R}$ (this transgene was the backbone used to generate the mutants in this study), the defects in *Incenp* RNAi oocytes were fully restored to WT levels. These results confirmed that the N-terminal epitope tags in INCENP interfered with the CPC's central spindle localization and function.

Third, to test which population of the CPC regulates homologue bi-orientation, we used INbox fusions to target overexpression of Aurora B to specific sites. We predicted that overexpression of Aurora B would disrupt bi-orientation by destabilizing KT-MT attachments. Although forcing Aurora B localization to the centromeres has been shown to cause bi-orientation defects in mitotic cells (Liu et al., 2009), the frequency of bi-orientation in oocytes expressing *mis12:INbox* was not significantly elevated compared with controls (Fig. 7, B and C). We also tested whether centromere-targeting Aurora B can destabilize microtubules by treating the oocytes with colchicine. K-fibers are more resistant to colchicine treatment than the central spindle, and the amount of K-fibers after colchicine treatment is a measure of attachment stability (Wang et al., 2019). The results with colchicine-treated WT and *mis12:INbox* oocytes were comparable; the spindle was diminished to the same extent, indicating that the stability of the KT-MTs was similar in each genotype (Fig. 7, D and E). Thus, overexpression of Aurora B activity at the centromeres did not cause bi-orientation defects. In contrast, central spindle–targeted Aurora B (*sub:INbox* or *feo:INbox*) caused significantly more bi-orientation defects than in WT (Fig. 7, B and C). These results suggest that the CPC regulates homologue bi-orientation from within the central spindle rather than at the kinetochores.

If targeting the CPC to the central spindle is required for bi-orientation, *Incenp* mutants with defects interacting with microtubules should have bi-orientation defects. Indeed, *Incenp*$^{\Delta STD}$, *Incenp* RNAi oocytes had homologue bi-orientation defects (Fig. 7 C), despite having apparently WT spindle assembly and CPC localization (Fig. 5 D). *Incenp*$^{\Delta HP1}$, *Incenp* RNAi oocytes had WT spindle morphology, but had defects in fertility and CPC localization to the central spindle (Fig. 4, D and G). Interestingly, we found that HP1 spindle localization in *Incenp*$^{\Delta HP1}$, *Incenp* RNAi oocytes was different from WT. HP1 was not enriched in the overlap with the CPC (Fig. 7 F). Like several of the *Incenp* mutants, *Incenp*$^{\Delta HP1}$ causes a dominant sterile phenotype (Table 1). Therefore, we examined *Incenp*$^{\Delta HP1}$-expressing oocytes by FISH and found that these also had a homologue bi-orientation defect (Fig. 7, B and C). These results are consistent with the model that CPC localization to the spindle and interaction with HP1 is important for regulating homologue bi-orientation.

To test HP1 directly, we examined oocytes depleted of HP1 by expressing shRNA *GL00531*, because null mutations in HP1 [*Su(var)205* in *Drosophila*] cause lethality. These oocytes displayed WT spindles with normal CPC localization (Fig. S4), which could be explained by the relatively mild knockdown of *HP1* (48% of mRNA remains). Expression of *GL00531* in oocytes did cause elevated X chromosome NDJ (8.7%; n = 321). These results support the conclusion that, during prometaphase I, HP1 and the CPC relocate from the chromosomes to the central spindle where they are both critical for homologue bi-orientation.

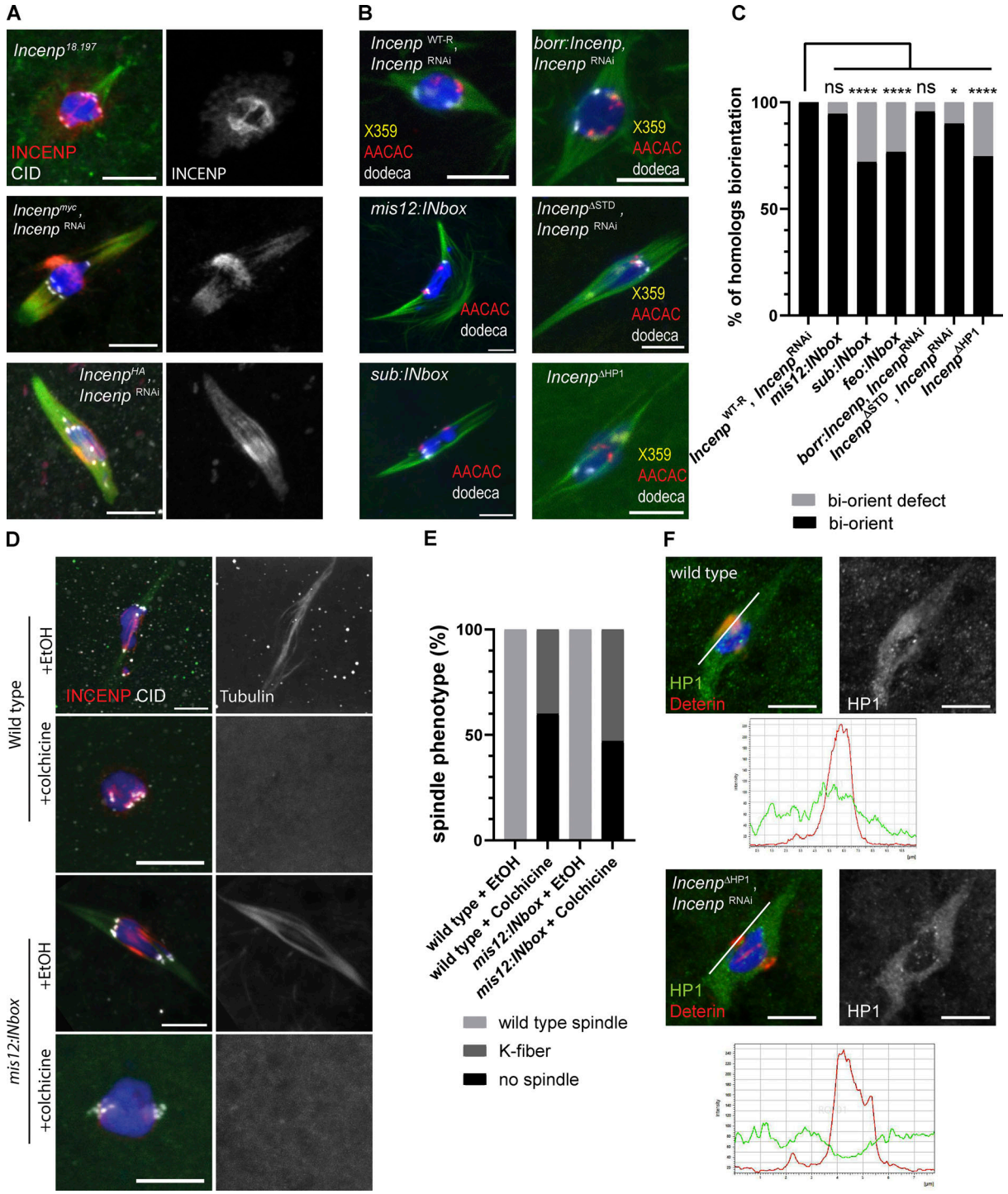

Figure 7. **Disruption of homologue bi-orientation by disruptions of central spindle CPC. (A)** Disorganized or mislocalized CPC caused by the *Incenp* hypomorphic allele *Incenp*$^{18.197}$ or the transgenes *myc:Incenp and HA:Incenp*. The CPC or MYC are in red, CID is in white, tubulin is in green, and DNA is in blue. **(B)** *Incenp* mutants examined for homologue bi-orientation using FISH with probes against pericentromeric heterochromatin on the X (359 bp repeat, yellow), second (AACAC, red), and third (dodeca, white) chromosomes. **(C)** Rates of bi-orientation defects were quantified (n = 57, 37, 50, 30, 69, 60, and 63 in the order of the graph). *, P < 0.05; ****, P < 0.0001 in Fisher's exact test. **(D)** WT oocytes and *mis12:INbox* oocytes treated with colchicine for 30 min. INCENP is in red and CID is in white. **(E)** Quantitation of spindle assembly after colchicine treatment (n = 5, 10, 7, and 17 in the order of the graph). **(F)** Localization of HP1 and Deterin in WT and *Incenp*$^{\Delta HP1}$, *Incenp* RNAi oocytes. HP1 is in green, Deterin is in red, and overlapping region is in yellow.

Discussion

Drosophila oocytes assemble bipolar spindles despite lacking centrioles and predefined spindle poles. Whether the microtubules assemble around MTOCs, as in the mouse (Dumont et al., 2007; Schuh and Ellenberg, 2007), or more closely around the chromosomes in *Drosophila* and humans (Hadders et al., 2020; Hengeveld et al., 2017) as well as *C. elegans* (Gigant et al., 2017), oocyte chromatin appears to play a role in focusing microtubule assembly in the vicinity of, if not contacting, the chromosomes. The chromosome-associated molecules that drive this process, however, are not known. Our previous studies have shown that the CPC is required for spindle assembly in *Drosophila* oocytes (Radford et al., 2012). We found that the known pathways for recruiting Survivin and Borealin to the centromeres via Haspin and Bub1 are not essential for oocyte spindle assembly, although a minor role in spindle assembly has not been ruled out. Borealin, however, has two important functions: targeting the CPC to oocyte chromatin, consistent with studies in *Xenopus* extracts (Kelly et al., 2007), and subsequent movement to the microtubules.

CPC-dependent, chromosome-directed spindle assembly in oocytes depends on a Borealin–chromatin interaction, possibly involving HP1

Although INCENP can recruit HP1 (Kang et al., 2011), several lines of evidence suggest that HP1 recruits the CPC before oocyte spindle assembly. When Aurora B is absent or inhibited in *Drosophila* oocytes, a complex of INCENP, Borealin, and Survivin colocalizes with HP1 on chromosomes (Fig. 5 A). HP1 has also been shown to physically interact with the CPC in *Drosophila* (Alekseyenko et al., 2014). In HeLa cells, HP1 promotes CPC localization to chromatin and precedes H3T3 phosphorylation by Haspin kinase (Ruppert et al., 2018). Also in human cells, an interaction between Borealin and nucleosomes (Abad et al., 2019) or HP1 (Liu et al., 2014) recruits the CPC to the chromosomes. Borealin is also sufficient to recruit the CPC to chromatin in *Xenopus* (Kelly et al., 2007). Thus, evidence from *Drosophila* oocytes and vertebrate cells is consistent and suggests that Borealin interacts with histones and HP1 to recruit the CPC to the chromatin. Aurora B could promote HP1 ejection from the chromosomes by phosphorylating HP1 (Williams et al., 2019) or H3S10 (Duan et al., 2008; Fischle et al., 2005; Hirota et al., 2005), possibly aided by HP1-interacting proteins, like POGZ (Nozawa et al., 2010). Thus, Aurora B activity could promote the transfer of a CPC–HP1 complex from the oocyte chromosomes to the microtubules.

We propose a model that not only explains how the chromosomes recruit spindle assembly factors, but also how the CPC moves from the chromosomes to the spindle (Fig. 8). After nuclear envelope breakdown, a tripartite complex of the CPC composed of INCENP, Borealin, and Survivin/Deterin (Jeyaprakash et al., 2007) is recruited to the chromatin, particularly in regions enriched for H3K9me3 and HP1. This localization is independent of Aurora B activity, suggesting that Borealin, in association with INCENP, is responsible for the recruitment of the CPC to HP1 and chromatin. Once the CPC localizes to the chromosomes, Aurora B activity results in phosphorylation of several targets and assembly of the kinetochores. HP1 is then ejected from the chromatin (Fig. 4 and Fig. 5), which could be a mechanism for how the CPC is released from the chromatin and relocates onto the microtubules.

This model explains how spindle assembly is restricted to the chromosomes in an acentrosomal system (Ohkura, 2015; Reschen et al., 2012; Romé and Ohkura, 2018). It is based on a chromatin/HP1–Borealin interaction. We and others have suggested that restricting spindle assembly proximal to the chromosomes involves the inhibition of spindle assembly factors in the cytoplasm (Beaven et al., 2017; Das et al., 2018; Romé and Ohkura, 2018). Spindle assembly could involve activating spindle-promoting factors, such as kinetochore proteins (Emanuele et al., 2008; Haase et al., 2017) and kinesins that bundle microtubules (Beaven et al., 2017; Das et al., 2018). For example, the kinesin NCD that has been shown to be inhibited by 14–3-3, which is released by Aurora B phosphorylation (Beaven et al., 2017). Spindle assembly may also involve suppressing microtubule depolymerases, such as kinesin 13/MCAK and Op18/Stathmin (Kelly et al., 2007; Sampath et al., 2004). We propose that the release of the CPC from the chromosomes locally activates spindle assembly factors.

Nonkinetochore microtubules require spindle-associated CPC

In mutants where the CPC was targeted to the chromosomes (*mis12:Incenp, Det:Incenp)*, kinetochore assembly and K-fiber formation were observed (Fig. 2 and Fig. 3). In other mutants (*HP1:Incenp* and *Incenp$^{\Delta CEN}$*), only limited kinetochore assembly and K-fibers were observed. Thus, low levels of CPC are sufficient for kinetochore assembly, but higher levels and/or specific localization are required for spindle assembly. Similar conclusions regarding localization and dosage have been made in *Xenopus;* kinetochore assembly can occur without localization of the CPC to the centromeres and may require less Aurora B activity than spindle assembly, but centromeric CPC localization is required for error correction (Haase et al., 2017; Kelly et al., 2007; Tseng et al., 2010; Xu et al., 2009). A notable difference compared with *Xenopus,* however, is that the SAH domain is not required for kinetochore assembly in *Drosophila* oocytes (Bonner et al., 2019; Wheelock et al., 2017).

The absence of nonkinetochore microtubules in the mutants where the CPC was targeted to the chromosomes suggests that spindle assembly requires microtubule-associated CPC. For example, DET:INCENP fusion promotes kinetochore and K-fiber assembly, but not central spindle assembly (Fig. 3). Central spindle assembly depends on the transfer of the CPC from the chromosomes to the microtubules (Fig. 5), and Deterin does not have this activity. The CPC contains multiple spindle-interacting domains, including two in INCENP (STD and SAH; van der Horst et al., 2015). In addition, it has been proposed that an HP1–INCENP interaction in HeLa cells promotes the transfer of the CPC from the heterochromatin to the spindle (Ainsztein et al., 1998). It is possible, however, that Borealin provides this activity in *Drosophila* oocytes. Borealin has a microtubule-binding site (Trivedi et al., 2019b), which could drive spindle transfer, and explain how the BORR:INCENP fusion is sufficient for oocyte spindle assembly but the DET:INCENP fusion is not. Deterin has a role in stabilizing the central spindle (Fig. 3 and Fig. 8).

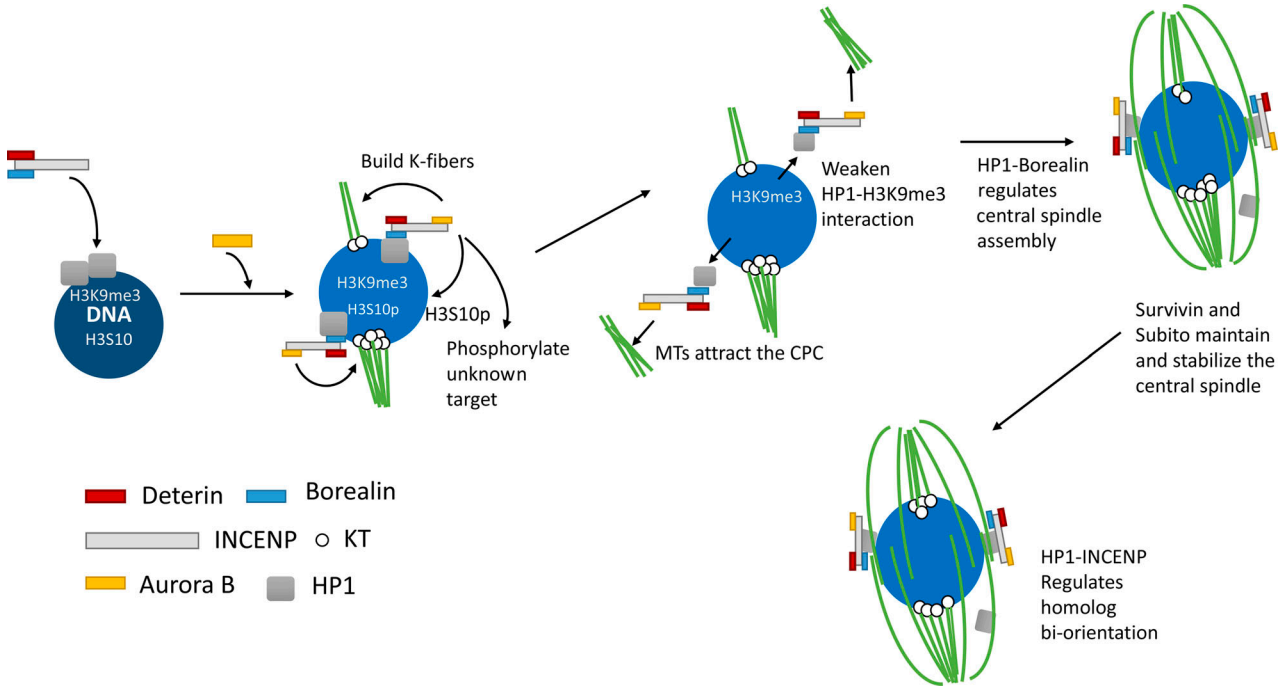

Figure 8. **Model for spindle assembly in *Drosophila* oocytes.** After nuclear envelope breakdown, a complex of INCENP, Borealin, and Deterin/Survivin is recruited to the chromosomes. Localization studies suggest that CPC recruitment is enriched in heterochromatic regions containing H3K9me3 and HP1. Aurora B is recruited, which results in kinetochore assembly, limited microtubule (MT) recruitment in the form of K-fibers, and phosphorylation of other targets, including H3S10 and possibly HP1. Aurora B activity also results in Borealin-dependent ejection of HP1 and the CPC from the chromosomes to the microtubules. Once on the microtubules, the kinesin 6 Subito causes enrichment of the CPC and HP1 in the central spindle. HP1 could be involved in a complex pattern of interactions that bring important spindle proteins together (Eissenberg and Elgin, 2014). For example, Aurora B could be brought together with potential phosphorylation substrate Subito, which has a conserved HP1 binding site that is required for its meiotic functions.

Subito is required to promote the release of CPC from chromatin in *Drosophila* (Cesario et al., 2006) and human (Serena et al., 2020) mitotic cells, but this is not the case in oocytes. In *sub* mutants, the central spindle is absent but robust bundles of CPC-containing nonkinetochore microtubules form (Jang et al., 2005). These observations suggest that the CPC promotes assembly and bundling of nonkinetochore microtubules independent of Subito. When the CPC is ejected from the chromosomes, it may activate the Augmin pathway, which has been shown to increase the amount of spindle microtubules in *Drosophila* oocytes (Romé and Ohkura, 2018). Subito, like its human homologue (Adriaans et al., 2020), is required to transport or recruit the CPC to the central spindle. Preventing Subito from interacting with the CPC could be an important regulatory modification in oocytes to ensure that microtubules do not assemble in the absence of chromosomes (Jang et al., 2007).

Regulation of homologue bi-orientation by the CPC

Several previous studies have suggested that chromosome-localized CPC regulates error correction, bi-orientation, and checkpoint silencing (Andrews et al., 2004; Foley and Kapoor, 2013; Liu et al., 2009; Tanaka et al., 2002), although some of these functions may not require precise centromere localization (Hadders et al., 2020; Hengeveld et al., 2017). Our analysis of multiple *Incenp* mutants suggests that chromosomal localization of the CPC may not promote these functions, which is consistent with work in other systems (Campbell and Desai, 2013; Fink et al., 2017). For example, the centromere targeting of the CPC in meiosis did not cause KT-MT destabilization or affect homologue bi-orientation (Fig. 7), as might be predicted if centromere-bound CPC can promote destabilization of microtubule attachments. Instead, several lines of evidence show that mutants with defects specific to spindle localization had the most severe bi-orientation defects (Fig. 7). For example, forcing localization of CPC to the central spindle, but not the kinetochores, disrupted bi-orientation. In addition, INCENP$^{\Delta STD}$ oocytes had defective homologue bi-orientation, suggesting that the conserved spindle transfer domain in INCENP is required for homologue bi-orientation. These results are consistent with the hypothesis that homologue bi-orientation of meiotic chromosomes depends on interactions between the CPC and microtubules of the central spindle.

An INCENP–HP1 interaction may be important for bi-orientation once the CPC and HP1 move to the spindle. Deleting the HP1 interaction site (121–232 amino acid) of INCENP caused disorganized CPC central spindle localization, loss of HP1 enrichment with the CPC, and bi-orientation defects (Fig. 4 and Fig. 7). HP1 or heterochromatin has also been shown to promote accurate achiasmate chromosome segregation during meiosis I in *Drosophila* oocytes (Giauque and Bickel, 2016; Karpen et al., 1996). HP1 interacts with a variety of proteins through its chromo-shadow domain (Eissenberg and Elgin, 2014) and could be involved in a complex pattern of interactions that bring important spindle proteins together (Fig. 7). The central spindle is a

complex structure, containing several proteins that have microtubule-binding domains, including Borealin and INCENP, that may allow the CPC to simultaneously interact with microtubules and regulate KT-MT attachments (Trivedi et al., 2019b; Wheelock et al., 2017). Several *Drosophila* central spindle components have been suggested to form structures by phase separation (So et al., 2019), including HP1 (Liu et al., 2020) and the CPC (Trivedi et al., 2019a). We suggest the central spindle forms a unique structure that allows for sensing the bi-orientation of bivalents.

During meiosis I, each pair of centromeres within a bivalent has to bi-orient while at a much greater distance apart than the sister centromeres in mitosis or meiosis II. How these centromeres on homologous chromosomes communicate is not known. The meiotic central spindle may provide a direct connection between homologous centromeres by combining two properties. The first is a mechanism to coordinate the movement and separation for each kinetochore of a bivalent. This may be analogous to the activity of bridging fibers, which is a structure that can separate pairs of sister kinetochores in mitosis (Simunić and Tolić, 2016; Vukušić et al., 2017) and use length-dependent forces to align chromosomes (Jagrić et al., 2021). In *C. elegans* meiosis, the central spindle separates homologues for chromosome segregation by microtubule pushing (Laband et al., 2017). The second is a mechanism for microtubule-bound CPC to regulate KT-MT attachments and error correction, which has been observed in several contexts (Fink et al., 2017; Funabiki, 2019; Pamula et al., 2019; Trivedi et al., 2019b). This combination of activities could be how the central spindle facilitates reductional chromosome segregation at anaphase I.

Materials and methods

Generation of RNAi-resistant INCENP

To engineer RNAi-resistant transgenes, we obtained *Incenp* cDNA (RE52507) from the Drosophila Genomic Resource Center and cloned it into the pENTR4 vector (Invitrogen). We used the Change-it Site-directed Mutagenesis kit (Affymetrix) to introduce eight silent mutations in the region corresponding to amino acids 437–441, which is complementary to *Incenp* shRNA (GL00279; Fig. 1 B and Fig. S1). The primers for the site-directed mutagenesis are: 5′-ATGAGCTTTTCAACCCACTCCTGCAGTCGC CCGTCAAGATGCGCGTGGAGGCGTTCGA-3′ and 5′-TCGAAC GCCTCCACGCGCATCTTGACGGGCGACTGCAGGAGTGGGTTG AAAAGCTCATG-3′. An RNAi-resistant *Incenp* coding region was inserted into the pPMW, pPHW, or pPFW vector that carries the UASp promoter using the LR Clonase reaction (Gateway; Invitrogen) to make $Incenp^{myc}$, $Incenp^{HA}$, and $Incenp^{Flag}$. Each construct was then injected in *w* embryos by Model System Injections. Multiple transgenic lines were selected to balance in a *y w* background and crossed to *mata4-GAL-VP16* with/without *Incenp* RNAi for further testing. The transgenic lines on the third chromosome were chosen for generating a recombinant line with *Incenp* RNAi if the phenotype was comparable with the ones on the X or second chromosome. Expressing $Incenp^{myc}$ in an *Incenp* RNAi background rescued spindle assembly and kinetochore assembly in oocytes as well as spindle localization; however, several defects were also observed, such as reduced fertility and elevated X chromosome NDJ, and the transgene protein was mislocalized along the spindle instead of concentrating in the central spindle. The same defects were observed previously with an $Incenp^{myc}$ variant without the silent mutations (Radford et al., 2012). These results suggest that an epitope tag in the N terminus of INCENP might interfere with its function, although the HA tag may have less impact than the other epitopes (Table 1). To solve this problem, the Gibson Assembly kit (New England Biolabs) was used to remove the myc tag from $Incenp^{myc}$ to generate $Incenp^{WT\text{-}R}$. Expressing $Incenp^{WT\text{-}R}$ in *Incenp* RNAi oocytes displayed WT spindle and localization, and restored fertility to WT levels. We used a plasmid carrying $Incenp^{WT\text{-}R}$ as the backbone for Gibson assembly reactions to generate all the *Incenp* mutations and fusions used in this study. For each mutation, at least two transgenic lines were analyzed for their ability to rescue *Incenp* RNAi with shRNA GL00279.

INbox constructs were generated by taking the last 101 amino acids (655–755) of INCENP, including INbox and TSS motif activation site. Fusion proteins of INCENP were created by using *mis12* cDNA (RE19545), *Deterin* cDNA (LP03704), *Su(var)205* cDNA (LD10408), and *Borealin* cDNA (LD36125). The constructs were injected into *Drosophila w* embryos by Model System Injections.

Drosophila genetics and generation of shRNA transgenics

Flies were crossed and maintained on the standard media at 25°C. All loci information was obtained from Flybase. Fly stocks were obtained from the Bloomington Drosophila Stock Center or the Transgenic RNAi Project (TRiP) at Harvard Medical School, including *aurB* (*GL00202*), *Incenp* (*GL00279*), *Haspin* (*GL00176*), *Su(var)205* (*GL00531*), and *Bub1* (*GL00151*), except *mis12::EGFP* (Głuszek et al., 2015) and $Haspin^{l28}$ (Fresán et al., 2020). To generate *Deterin* (*LW501*) and *Haspin* (*HK452*) shRNA lines, a *Deterin* sequence (5′-CGGGAGAATGAGAAGCGTCTA-3′) or a *Haspin* sequence (5′-GGAAGACAGTAGAGACAAATG-3′) was cloned into pVALIUM22 following the protocols described by the Harvard TRiP center. The construct was injected into *Drosophila* embryos (*y sc v; attP40*).

The pVALIUM22 vector carries the UASp promoter, allowing for expression of short hairpins for RNA silencing and transgenes using the UAS/GAL4 binary expression system (Rørth et al., 1998). All shRNA lines and transgenes were expressed by crossing to *mata4-GAL-VP16*, which induces expression after early pachytene and throughout most stages of oocyte development in *Drosophila* (Sugimura and Lilly, 2006).

For quantifying the mRNA knockdown of these shRNAs, total RNA was extracted from oocytes using TRIzol Reagent (Life Technologies) and reverse transcribed into cDNA using the High-Capacity cDNA Reverse-Transcription Kit (Applied Biosystems). Quantitative PCR was performed on a StepOnePlus (Life Technologies) real-time PCR system using TaqMan Gene Expression Assays (Life Technologies); Dm03420510_g1 for *Haspin*, Dm02141491_g1 for *Deterin*, Dm01804657_g1 for *Bub1*, Dm0103608_g1 for *Su(var)205*, and Dm02134593_g1 for the control *RpIII140*. Knockdown of the respective mRNAs in these oocytes was reduced to 15% in *Haspin HK452* RNAi oocytes, 32% in *Haspin GL00176* RNAi oocytes, 5% in *Deterin LW501* RNAi

oocytes, 2% in *Bub1 GL00151* RNAi oocytes, and 48% in *Su(var)205 GL00531* RNAi oocytes. Based on these quantitative RT-PCR results, we chose to use *HK452* for all *Haspin* experiments. To test for effects on mitosis, shRNA lines were tested for lethality when under the control of *P{tubP-GAL4}LL7*, which results in ubiquitous expression.

Antibodies and immunofluorescence microscopy

Stage 14 oocytes were collected from 100–200 3–4-d-old yeast-fed nonvirgin females (Gilliland et al., 2009). The protocol for fixation and immunofluorescence of stage 14 oocytes has been described previously (Radford and McKim, 2016). To observe whether spindle assembly was affected in postmeiotic mitosis, embryos were collected from several hundred yeast-fed females for 2 h. The chorion was removed by treating embryos with 50% bleach for 90 s and then moving the embryos to tubes containing 500 µl heptane and 500 µl methanol and shaking vigorously for 30 s to fix. Rehydrated embryos were processed for immunofluorescence microscopy. Hoechst 33342 (10 µg/ml; Invitrogen) was used for DNA and mouse anti–α-tubulin monoclonal antibody DM1A (1:50) conjugated with FITC (Sigma-Aldrich) was used for microtubules. Primary antibodies used in this paper were rabbit anti-CID (1:1,000; Active Motif), rabbit anti-SPC105R (1:4,000; Schittenhelm et al., 2007), rabbit anti–CENP-C (1:5,000; Heeger et al., 2005), mouse anti-Myc (1:50; 9E10; Roche), mouse anti-Flag (1:500; Thermo Fisher Scientific), rat anti-INCENP (1:400; Wu et al., 2008), rabbit anti–Aurora B (1:1,000; Giet and Glover, 2001), rabbit anti-Survivin (1:1,000; Szafer-Glusman et al., 2011), rabbit anti-Borealin (1:100; Gao et al., 2008), mouse anti-HP1 (1:50; C1A9; Developmental Hybridoma Bank), rabbit anti-H3K9me3 (1:1,000; Active Motif), rat anti-Subito (1:75; Jang et al., 2005), rat anti–α-tubulin (clone YOL 1/34; Millipore), and rabbit anti-pINCENP (1:1,000; Salimian et al., 2011). The secondary antibodies used included Cy3 and Alexa Fluor 647 (Jackson Immunoresearch) or Alexa Fluor 488 (Molecular Probes). FISH probes for the X chromosome (359 repeats), second chromosome (AACAC satellite), and third chromosome (dodeca satellite) were synthesized and conjugated to either Alexa Flour 594, Cy3, or Cy5 by Integrated DNA Technologies (Dernburg et al., 1996; Radford and McKim, 2016). Oocytes were mounted in SlowFade Gold (Invitrogen). Images were collected on a Leica TCS SP8 confocal microscope with a 63×, 1.4 NA lens and shown as maximum projections of complete image stacks. Images were then cropped in Adobe Photoshop.

Drug treatment assays

To inhibit Aurora B kinase activity, oocytes were incubated with 50 µM BN2 in 0.1% DMSO for 60 min before fixation in Robb's media. To depolymerize microtubules, oocytes were incubated in 250 µM colchicine in 0.5% ethanol or only 0.5% ethanol as a control for either 30 or 60 min before fixation, depending on whether we wanted to destabilize spindle microtubules (Fig. 7) or completely remove all spindle microtubules (Fig. 1 and Fig. 5).

X chromosome NDJ assays

To determine whether each *Incenp* mutant transgenes affected meiotic chromosome segregation, we measured fertility and X chromosome NDJ. Transgenic virgin females were generated by crossing *mata4-GAL-VP16* to either the *Incenp* transgene or the *Incenp* transgene with *Incenp* shRNA or other RNAi lines. These transgenic females were crossed to *y Hw w/B^{S}Y* males. The males carry a dominant mutation, *Bar*, on the Y chromosome, which makes chromosome mis-segregation phenotypically distinguishable in the progeny. Crosses were set in vials and fertility was measured based on the progeny number. To compensate for the inviability of nullo-X and triplo-X progeny, the NDJ rate was calculated as 2 × (XXY and XO progeny)/total progeny, where total progeny was 2 × (XXY and XO progeny) + XX and XY progeny.

Image analysis and statistics

To measure kinetochore localization, SPC105R foci were identified based on size and intensity and then counted using Imaris image analysis software (Bitplane) with the parameters used in Wang et al. (2019). To determine whether HP1 and the CPC were colocalized in Fig. 7 F, line scans were drawn from pole to pole across the central spindle. The intensities of HP1 and Deterin were measured using Leica SP8 software. When measuring bi-orientation by FISH, each data point corresponds to one pair of homologous centromeres. Homologous chromosomes were considered bi-oriented if two FISH signals localized at the opposite ends of chromosome mass. Pairs of homologous chromosomes on the same side of the spindle were considered to have a bi-orientation defect. The appropriate statistical tests for each experiment as indicated in the figure legends were performed using Prism software (GraphPad).

Online supplemental material

Fig. S1 shows an alignment and domain analysis of *Drosophila*. Fig. S2 shows that expression of *mis12:INbox* in the WT oocytes disrupts meiotic progression. Fig. S3 shows Borealin localization in *Det:Incenp, Incenp* RNAi oocytes and the localization of the CPC to the chromosomes. Fig. S4 shows the phenotype of *Haspin, Bub1*, and *Su(var)205* (*HP1*) knockdowns.

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Supplemental material

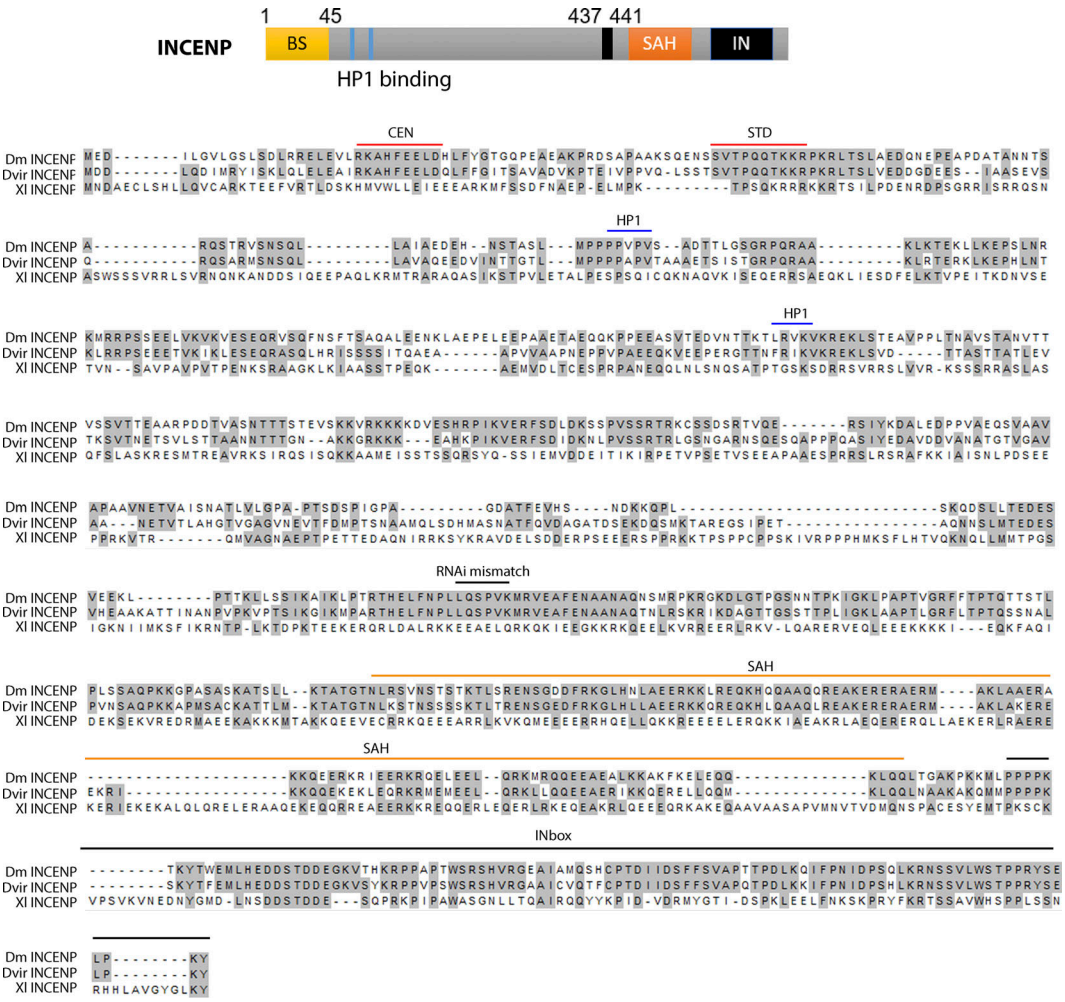


Figure S1. **Alignment and domain analysis of *Drosophila*.** The sequence alignment compares *Drosophila melanogaster* (Dm) INCENP to *Drosophila virilis* (Dvir) and *Xenopus laevis* (Xl). The Borealin/Deterin binding domain is from amino acids 1–46 (yellow). *CEN* and *STD* deletion mutations are marked in red, two potential HP1 interaction sites are marked in blue, the RNAi mismatch region is marked in black, the SAH domain is marked in orange, and the INbox (IN) is in black.

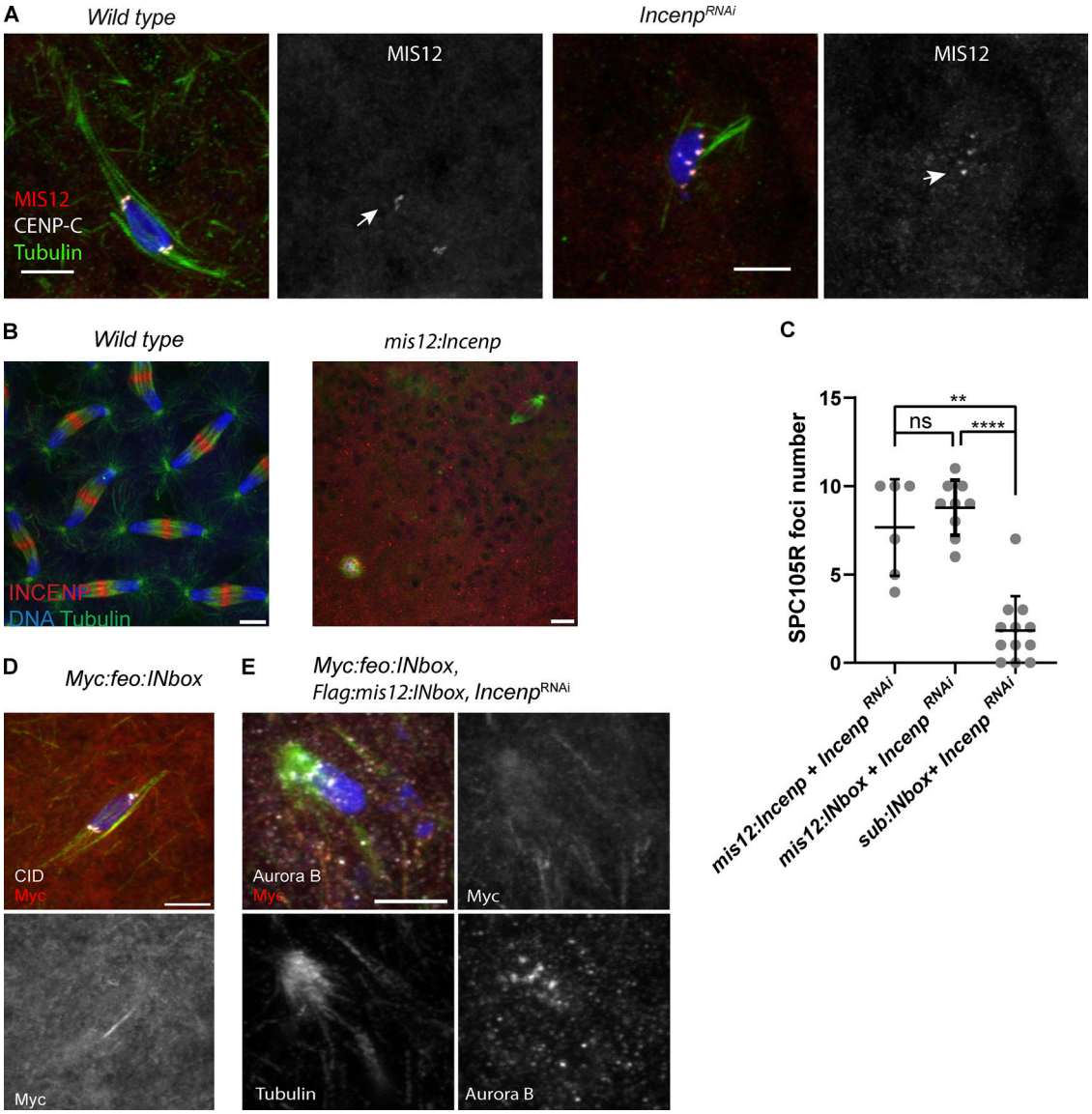


Figure S2. **Expression of *mis12:INbox* in WT oocytes disrupts meiotic progression. (A)** MIS12 localization in WT and *Incenp* RNAi oocytes (arrows). MIS12 is in red, tubulin is in green, DNA is in blue, and CENP-C is in white. Scale bar, 5 µm. **(B)** Fertilized 0-2-h-old *Drosophila* embryos were fixed and stained for INCENP (red), tubulin (green), and DNA (blue). Scale bar, 5 µm. **(C)** Quantitation of SPC105R localization in oocytes with *mis12* fusions (n = 6, 9, and 12 oocytes). Error bars indicate 95% confidence intervals; ****, $P < 0.0001$; **, $P < 0.01$ run by Fisher's exact test. **(D)** Expression of *Myc:feo:INbox* in WT and *mis12:INbox, Incenp*^{RNAi} oocytes. *Myc:feo:INbox* localizes to the central spindle in WT oocytes. **(E)** Coexpression of *FLAG:mis12:INbox* and *Myc:feo:INbox* in *Incenp* RNAi oocytes. In these images, the Myc tag (FEO:INbox) is red, Aurora B or CID is white, tubulin is green, and DNA is blue. Scale bar, 5 µm.

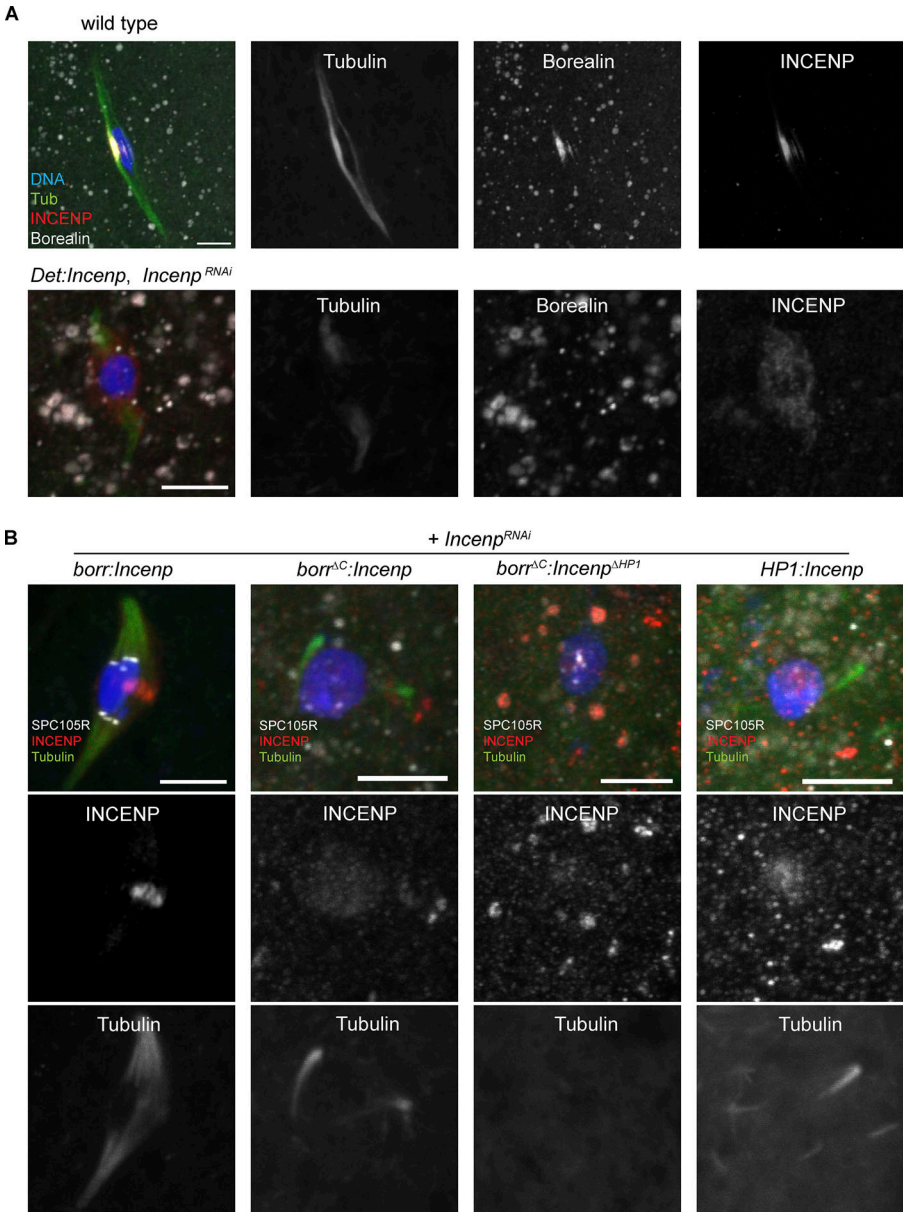
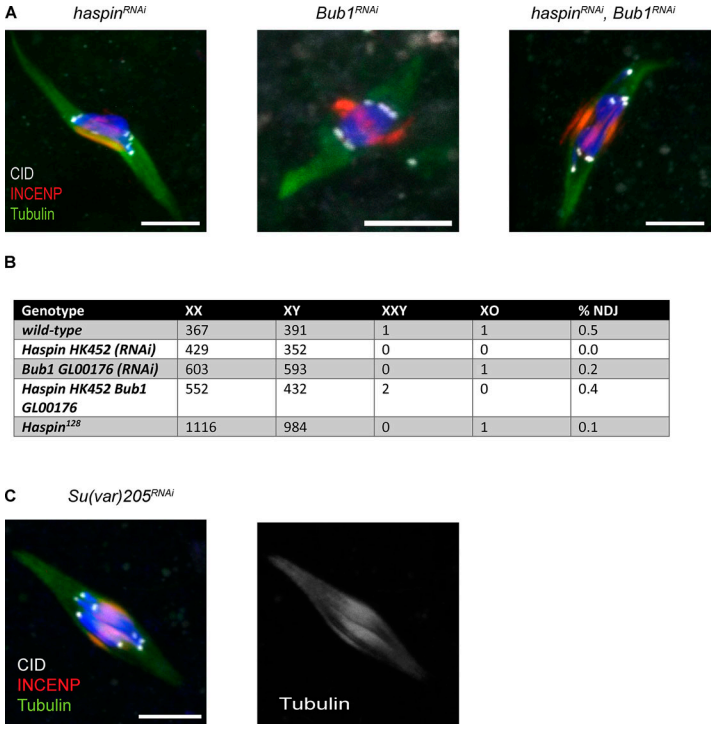


Figure S3. **Borealin localization in *Det:Incenp, Incenp* RNAi oocytes and the localization of the CPC to the chromosomes. (A)** Metaphase I oocytes from WT and *Det:Incenp, Incenp* RNAi females. Borealin is in white, INCENP is in red, tubulin is in green, and DNA is in blue. Scale bar, 5 µm. **(B)** Expression of *Incenp* transgenes shown in Fig. 4 C in *Incenp* RNAi oocytes, including $borr^{\Delta C}$*:Incenp, Incenp*$^{\Delta HP1}$, and $borr^{\Delta C}$*:Incenp*$^{\Delta HP1}$. The images show SPC105R in white, INCENP in red, DNA in blue, and tubulin in green. Scale bar, 5 µm.



Genotype	XX	XY	XXY	XO	% NDJ
wild-type	367	391	1	1	0.5
Haspin HK452 (RNAi)	429	352	0	0	0.0
Bub1 GL00176 (RNAi)	603	593	0	1	0.2
Haspin HK452 Bub1 GL00176	552	432	2	0	0.4
Haspin^{128}	1116	984	0	1	0.1

Figure S4. **Phenotype of *haspin*, *Bub1*, and *Su(var)205* (*HP1*) knockdowns. (A)** Metaphase I oocytes from *haspin HK420 or Bub1 GL00151* single RNAi or *haspin, Bub1* double RNAi females. Centromere protein CID is in white, INCENP is in red, tubulin is in green, and DNA is in blue. **(B)** Fertility and X chromosome NDJ in *haspin* and *Bub1* RNAi and *haspin* mutant females. **(C)** Spindle in oocytes depleted of *Drosophila* HP1 using *Su(var)205 GL00531* RNAi oocytes. CID is in white, INCENP is in red, tubulin is in green, and DNA is in blue. Scale bars, 5 µm (all images).