Chromosome-directed oocyte spindle assembly depends HP1 and the Chromosomal Passenger Complex

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Abstract

The chromosomes in the oocytes of many animals appear to promote bipolar spindle assembly. In *Drosophila* oocytes, spindle assembly requires the chromosomal 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 HP1 is crucial for the initial recruitment of the CPC to the chromosomes and is sufficient to build kinetochores and recruit spindle microtubules. We also found that HP1 moves from the chromosomes to the spindle microtubules along with the CPC, and based on this, propose a mechanism for how the CPC moves from the chromosomes to the microtubules. 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 is achieved by bi-orientation of homologs on a meiotic spindle prior to anaphase. Bi-orientation is the result of two simultaneous processes: the assembly of microtubules into a bipolar spindle and the correct attachment of some of these microtubules to the kinetochores. In mitosis and male meiosis, the bipolarity of the spindle is defined by centrosomes at each pole. These serve as microtubule organizing centers (MTOC), nucleating microtubules that grow towards the chromosomes and make contact with kinetochores (Cheeseman, 2014; Heald and Khodjakov, 2015; Nicklas, 1997; Watanabe, 2012). In many species, the female meiotic spindle assembles without centrosomes. In these oocytes, 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 (Schuh and Ellenberg, 2007). In contrast, the chromosomes themselves serve as major sites of microtubule nucleation in *Drosophila* (Matthies et al., 1996; Theurkauf and Hawley, 1992) and human (Holubcová et al., 2015) oocytes.

These observations of chromosome-directed spindle assembly suggest the 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; Moutinho-Pereira et al., 2013; Mullen et al., 2019; Radford et al., 2017). The Ran pathway is activated by chromosome localized RCC1. The chromatin factor associated with the CPC is not known, but it is required for promoting spindle assembly when sperm nuclei are added to *Xenopus* egg extracts (Kelly et al., 2007; Sampath et al., 2004). The CPC comprises Aurora B kinase, the scaffold subunit INCENP, and the two targeting subunits Borealin and Survivin (Deterin in *Drosophila*). In *Drosophila*, the depletion of Aurora B or INCENP causes a

complete failure in oocyte spindle assembly (Colombié et al., 2008; Radford et al., 2015; Radford et al., 2012). The results in *Xenopus* and *Drosophila* suggest that the chromosomes carry signals that can recruit and/or restrict the activity of the CPC.

The CPC displays a dynamic localization pattern during cell division that contributes to its known functions. During mitosis, including *Drosophila* cells, the CPC localizes to the centromeres during metaphase, where it is required for correcting kinetochore-microtubule (KT-MT) attachments and checkpoint regulation (Carmena et al., 2012b; Krenn and Musacchio, 2015; Trivedi and Stukenberg, 2016). It then relocates onto the microtubules to form the spindle midzone required at anaphase for cytokinesis (Adams et al., 2001; Carmena et al., 2012a; 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). Another critical component of the prometaphase meiotic central spindle is Subito, the *Drosophila* kinesin-6 and MKLP2 orthologue, in the (Giunta et al., 2002). Subito is required for organizing the central spindle and homologous chromosome bi-orientation in *Drosophila* oocytes (Jang et al., 2005). Given that Subito and INCENP genetically interact and are mutually dependent for their localization (Das et al., 2018; Das et al., 2016; Radford et al., 2012), a model for spindle assembly in Drosophila oocytes is that the CPC activates multiple spindle organizing proteins, including Subito. The goal this study was to determine how the chromosomes are involved in this process.

How the CPC is recruited and promotes chromosome-directed kinetochore and spindle assembly in oocytes 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, we provide evidence that Haspin and Bub1 are not required for spindle assembly in meiosis. Instead, an interaction between Borealin and Heterochromatin protein 1 (HP1) recruits the CPC to the chromosomes and this interaction is sufficient for kinetochore, K-fiber, and central spindle assembly. We also found that HP1 moves onto the spindle concomitantly with the CPC. We propose that once on the central spindle, the CPC and HP1 play a major role in promoting the bi-orientation of homologous chromosomes in oocytes. Thus, our research has revealed a mechanism for how the chromosomes recruit the CPC for spindle assembly, and then how movement of the CPC is associated with homolog bi-orientation in oocytes.

Results

Using RNAi resistant transgenes to study factors that regulate CPC localization

The CPC is required for the initiation of spindle assembly by recruiting kinetochore proteins to the centromeres and recruiting microtubules around the chromosomes (Radford et al., 2015; Radford et al., 2012). In mitosis, the CPC has a dynamic localization pattern that influences its functions (Adams et al., 2001; Cesario et al., 2006; Giet and Glover, 2001). While the CPC localizes to the centromeres in mitotic cells prior to anaphase, it localizes predominantly to the central spindle in prometaphase I *Drosophila* oocytes. We do not observe CPC centromere localization in fixed images (Figure 1A), although it is under certain conditions. For example, centromere localization has been observed in live imaging (Costa and Ohkura, 2019). Furthermore, when we treated wild-type oocytes with colchicine to destabilize microtubules, we found that the CPC localized to the centromeres and chromosomes (Figure 1A). Thus, under certain conditions, the CPC does localize to the centromeres. Because the CPC localizes primarily to the spindle microtubules, and occasionally to centromeres, the relationship between CPC localization and functions in chromatin-directed spindle assembly is not clear.

To address 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 (see Methods). *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* (Figure 1B). Expressing the RNAi-resistant transgene ($Incenp^{WT\text{-}R}$) rescued the defects in *Incenp* RNAi oocytes, including spindle and kinetochore assembly, homolog bi-orientation, and fertility,

restoring them to wild-type levels (Figure 1C, Table 1). Having successfully established this RNAi-resistant system, we utilized this RNAi-resistant *Incenp*$^{WT\text{-}R}$ backbone to construct several separation-of-function *Incenp* mutants.

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

Borealin and Survivin (known as Deterin in *Drosophila*) target the CPC to the centromeres in mitotic cells (Carmena et al., 2012b). We have so far not been successful at generating an effective *borr* shRNA for oocyte RNAi. However, *Deterin* RNAi oocytes had the same phenotype as *Incenp* or *aurB* RNAi oocytes (Figure 1C and D) (Radford et al., 2012). This result suggests that Deterin plays an important role in targeting INCENP and Aurora B to the chromosomes in *Drosophila* oocytes. Borealin and Survivin interact with the N-terminal domain of INCENP, which is thought to be required for centromere localization of the CPC (Jeyaprakash et al., 2007; Klein et al., 2006). Therefore, to test the function of the CPC at the centromere, we deleted conserved amino acids 22-30 in INCENP corresponding to the centromere targeting domain described in chicken INCENP (Ainsztein et al., 1998) (referred to as *Incenp*$^{\Delta CEN}$, Figure 1B, Figure S 1) and is predicted to be required for the interaction with Borealin and Deterin. INCENP$^{\Delta CEN}$ expressed in *Incenp* RNAi oocytes did not localize, recruit Deterin, or promote spindle assembly (Figure 1E). However, 59% of the *Incenp*$^{\Delta CEN}$, *Incenp* RNAi oocytes displayed SPC105R localization, compared to 22% in *Incenp* RNAi oocytes, 7% in *aurB* RNAi oocytes and 25% in *Deterin* RNAi oocytes (Figure 1F). These results suggest that the N-terminal domain of INCENP recruits Borealin and Deterin and is required for spindle assembly. These results also suggest that kinetochores assembly can occur without localization of the CPC to the centromeres and may require less Aurora B activity than spindle assembly.

To directly test whether centromeric CPC can promote spindle assembly and regulate homolog bi-orientation in oocytes, we targeted the CPC to the centromeric regions. We found that kinetochore protein MIS12 localized in either INCENP- or Aurora B-depleted oocytes (Figure 2A). To target the CPC to the centromeres, the entire N-terminus of INCENP (amino acids 1-46, the "BS" domain) was replaced with MIS12 (*mis12:Incenp*) (Figure 2B). Surprisingly, when *mis12:Incenp* was expressed in wild-type oocytes, we did not observe centromere localization (Figure 2C). However, the females were sterile due to the failure to complete the two meiotic divisions and initiate the mitotic divisions (Figure S 2, Table 1). This phenotype demonstrated that the transgene was expressed and toxic to the embryo. When expressing *mis12:Incenp* in *Incenp* RNAi oocytes, however, the fusion protein localized in and around the centromeres and kinetochores and K-fiber formation was observed (Figure 2C and D). These results suggest that targeting INCENP to the centromere regions could promote K-fiber formation. However, because we did not observe *mis12:Incenp* localizing to the centromere in the presence of endogenous INCENP, it was possible that regions outside of the BS domain of INCENP negatively regulate centromere localization.

To avoid the possibility that regions outside the BS domain regulate CPC localization, we fused MIS12 to the INbox domain of INCENP (amino acids 644-754 of the C-terminal domain), which recruits and activates Aurora B (Figure 2B) (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 (Figure 2E) and sterility (Table 1). This observation suggests that unlocalized INbox may act like a competitive inhibitor of INCENP by generating non-productive binding interactions with Aurora B. A dominant-negative effect was also observed when expressing the INbox in mammalian cells, similar to an Aurora B kinase-

dead mutant (Gohard et al., 2014). In contrast, when expressing *mis12:INbox* in wild-type oocytes, we observed MIS12:INbox localization at the centromeres (Figure 2E). We also observed phospho-INCENP, which is a marker of Aurora B activity (Salimian et al., 2011), at the centromeres as well as the central spindle (Figure 2F). Similar to *mis12:Incenp*, when *mis12:INbox* was expressed in *Incenp* RNAi oocytes, SPC105R was recruited, but only K-fibers formed (Figure 2C, D and G). These results demonstrate that centromere-targeted CPC is sufficient to build kinetochores and K-fibers, but not the central spindle. Furthermore, regions outside the BS domain of INCENP negatively regulate centromere localization.

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

Because kinetochore-directed CPC promotes only K-fiber assembly, it is possible that oocyte spindle assembly depends on microtubule-associated CPC. 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 the CPC to these two sites is independent or whether one site might depend on the other. In addition to the centromere-targeting of the CPC described above, the *INbox* was fused with two microtubule-associated proteins, Fascetto (*feo*, the *Drosophila* PRC1 homolog) and Subito. These two fusions, *feo:INbox* and *sub:INbox*, resulted in robust INCENP localization to the central spindle when expressed in wild-type oocytes (Figure 2E and Figure S 3). When expressed in *Incenp* RNAi oocytes, neither *feo:INbox* nor *sub:INbox* oocytes assembled a spindle around the chromosomes (Figure 2G and Figure S 3), though *sub:INbox* oocytes promoted microtubule bundles in the cytoplasm. This result suggests that the interaction between the CPC and central spindle proteins is not sufficient to promote spindle assembly around the chromosomes. Fusing Subito to the INbox promoted microtubule bundling, but not in

the specifically important location around the chromosomes. Additionally, most SPC105R localization was absent in *sub:INbox, Incenp* RNAi oocytes (25% had localization, n= 12, Figure 2G) similar to *Incenp* RNAi (Figure 1F). One possible explanation for these observations is that the central spindle targeting of the CPC lacked the interaction with the chromosomes necessary for spindle and kinetochore assembly.

The problem with the *feo:INbox* and *sub:INbox* experiment could have been the absence of chromosome-directed CPC to recruit microtubules and nucleate central spindle assembly. Therefore, we tested whether the CPC independently targeted to the chromosomes and the central spindle would promote spindle assembly by co-expressing *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 (Figure 2H and Figure S 3). We did observe enhanced microtubule bundling involving the K-fibers, indicating that SUB:INbox was active and could contribute to microtubule bundling. These results indicate that independently targeting two populations of the CPC is not sufficient to assemble a bipolar spindle. Furthermore, central spindle assembly requires more than just bundled antiparallel microtubules; a link to the chromosomes is required.

Borealin, but not Deterin, recruits the CPC to the meiotic chromosomes

The phenotype of *Incenp*$^{\Delta CEN}$ and *Deterin* RNAi oocytes suggests that Borealin and Deterin are critical for chromosome-directed spindle assembly in oocytes (Figure 1D 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*, Figure 3A). In *Det:Incenp* oocytes, INCENP localized to the pericentromeric regions and we observed the assembly of kinetochore and K-fibers, but not the central spindle (Figure 3B). Borealin localization could not be detected

in *Det:Incenp*, *Incenp* RNAi oocytes (Figure S4), suggesting this spindle phenotype is independent of Borealin. In contrast, *borr:Incenp* rescued all spindle assembly, including the central spindle, in *Incenp* RNAi oocytes (Figure 3B). These observations suggest that, while Deterin can promote K-fiber formation, Borealin is critical and sufficient for all aspects of CPC-mediated spindle assembly in *Drosophila* oocytes.

Two observations suggest that the *borr:Incenp* fusion promotes spindle assembly independently of Deterin. First, when *borr:Incenp* was expressed in *Deterin* RNAi oocytes (*borr:Incenp*, *Deterin* RNAi oocytes), a normal bipolar spindle formed (Figure 3C and 3D). In contrast, 44% of *Deterin* RNAi oocytes did not assemble a spindle, and the rest only showed non-specific microtubule clustering around the chromosomes (Figure 3C and 3D). Thus, the *borr:Incenp* fusion was able to rescue the loss of Deterin. Second, Deterin did not localize to the bipolar spindles formed in *borr:Incenp*, *Incenp* RNAi oocytes (Figure 3E). These results show that Borealin plays a direct role in recruiting the CPC for oocyte spindle assembly; whereas, Deterin is required for the INCENP-Borealin interaction.

HP1 recruits the CPC to the chromosomes through the C-terminus of Borealin to promote spindle assembly

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 and CPC localization were normal in *Haspin,* or *Bub1* RNAi oocytes (Figure 4A). Spindle assembly was also normal in *haspin, Bub1* double RNAi oocytes, suggesting Haspin and BUB1 are not required for spindle assembly. Therefore, we investigated other mechanisms for Borealin-mediated CPC recruitment to the chromosomes.

Borealin contains multiple protein interaction domains that interact with INCENP/Deterin or microtubules in the N-terminus and an HP1 interaction site (PxVxL) in the C-terminus (Liu et al., 2014; Trivedi et al., 2019b). HP1 is a good candidate for targeting the CPC to the chromatin and, indeed, has been proposed localize to the CPC by interacting with Borealin and INCENP (Ainsztein et al., 1998; Higgins and Prendergast, 2016). Thus, HP1 could be involved in chromosome-directed spindle assembly by interacting with Borealin in oocytes. In support of this hypothesis, we found that in *aurB* RNAi oocytes, INCENP, Deterin, and Borealin colocalize with HP1 and H3K9me3, the histone marker that recruits HP1 (Figure 4B and 4C). To test the hypothesis that HP1 recruits the CPC to chromatin in oocytes, we deleted the HP1 interacting-domain of Borealin from *borr:Incenp* (referred as $borr^{\Delta C}$*:Incenp*) (Figure 4D). 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 (Figure 4E and 4F). Additionally, 26% of oocytes displayed normal SPC105R localization (Figure 4G), suggesting that K-fiber formation was associated with higher levels of SPC105R. These results suggest an interaction between Borealin and HP1 is important for oocyte spindle assembly.

Because spindle assembly was not completely eliminated in $borr^{\Delta C}$*:Incenp* oocytes, we considered the possibility that HP1 could interact with another CPC member. In fact, a second HP1 interaction consensus site exists in INCENP (van der Horst and Lens, 2014) and this was also deleted ($Incenp^{\Delta HP1}$). While spindle assembly in $borr^{\Delta C}$*:Incenp, Incenp* RNAi was severely affected, spindle assembly in $Incenp^{\Delta HP1}$, *Incenp* RNAi oocytes was similar to wild type, except that in some oocytes INCENP displayed irregular and disorganized central spindle localization (36%, Figure 4E, 4F and 4H). These data suggest that the Borealin-HP1 interaction is critical for oocyte spindle assembly, while the INCENP-HP1 interaction has a minor role. To test for

additive effects, a mutant with both HP1 sites deleted was generated (*borr*$^{\Delta C}$*:Incenp*$^{\Delta HP1}$). Indeed, 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 the oocytes and we measured a small decrease in K-fiber formation ($p = 0.08$, Figure 4E and F). These results suggest that HP1 recruits the CPC to the chromosomes for spindle assembly primarily through Borealin, with possibly a minor contribution from INCENP.

These results suggest that an important role of Borealin is to interact with HP1 for recruitment of the CPC. To test whether this is the sole function of Borealin in oocytes, the BS domain of *Incenp* was replaced with HP1 (*HP1:Incenp*) (Figure 4D). HP1:INCENP localized to part of the chromatin, probably the heterochromatin regions, but 53% of the oocytes failed at spindle assembly, and the rest only had K-fiber formation associated with SPC105R localization (Figure 4E and 4G). These results showed that targeting the CPC to the heterochromatin regions without Borealin is not sufficient for bipolar spindle assembly. These results suggest that Borealin, Deterin and INCENP form a complex that is recruited to heterochromatin regions through a HP1-Borealin interaction. Rather than Borealin being simply an adapter for CPC localization, the HP1-Borealin interaction, or the interaction of Borealin with another protein, is essential for oocyte spindle assembly.

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

The HP1-Borealin interaction can explain how the chromosomes recruit the CPC and initiate spindle assembly. Next, we investigated the mechanism that promotes the transfer of the CPC from the chromosomes to the central spindle. First, we tested the hypothesis that an ejection mechanism transfers the CPC from the chromosomes to the microtubules. Phosphorylation of H3S10 by Aurora B can interfere with the interaction between H3K9me3 and HP1 (Duan et al.,

2008). In wild-type *Drosophila* oocytes, we observed that HP1 and H2S10ph localized to different structures: HP1 to the spindle and H3S10ph to the chromatin (Figure 4C). Thus, a possible mechanism to promote the transfer of the CPC from the chromosomes to the central spindle is that Aurora B phosphorylation of H3S10 releases HP1 and the associated Borealin from the chromosomes. To test this hypothesis, we determined if inhibiting Aurora B activity would cause retention of HP1 on the chromosomes. Similar to the results in *aurB* RNAi oocytes , when oocytes were treated with the Aurora B inhibitor Binucleine 2 (BN2), the spindle was diminished. In addition, INCENP localized on the chromosomes overlapping with HP1, H3K9me3 and H3S10ph (Figure 5A). Because the microtubules are lost upon BN2 treatment, we tested the role of microtubules in CPC and HP1 transfer by treating oocytes with colchicine. When the oocyte spindle was greatly reduced following colchicine treatment, both HP1 and the CPC retreated to the chromosomes and colocalized with H3K9me3 (Figure 5B). These results are consistent with the model that the ejection of HP1 and the CPC from the chromosomes depends on Aurora B activity and the microtubules. It is possible that only the microtubules matter because Aurora B activity is not able to eject HP1 from the chromosomes when microtubules are absent. Thus, we have not shown it Aurora B has a role when microtubules are present.

To test the hypothesis that spindle transfer depends on an interaction between the CPC and the spindle microtubules, we considered CPC components with a microtubule binding domain. Indeed, INCENP has two known microtubule interaction domains: its single-α-helix (SAH) domain (Samejima et al., 2015) and a conserved domain within the N-terminal region (the spindle transfer domain, STD) (Ainsztein et al., 1998) (Figure S 1). To test if either of these microtubule interaction domains is important for the CPC to relocate and assemble the meiotic

spindle, we generated deletions in each site (Figure 5C). In both *Incenp*$^{\Delta STD}$, *Incenp* RNAi and *Incenp*$^{\Delta SAH}$, *Incenp* RNAi oocytes, we observed wild-type spindle assembly and normal CPC localization, suggesting these microtubule binding domains are not required for oocyte meiotic spindle assembly (Figure 5D). However, these oocytes displayed either reduced fertility or sterility (Table 1), suggesting that these two domains of INCENP may have an important role in embryonic mitosis. Whether these two domains are redundant or there are other pathways for the CPC to interact with microtubules remains to be investigated.

An interaction between Subito and the CPC promotes central spindle assembly

We have thus far provided evidence that the CPC localizes to the chromosomes by interacting with HP1, and then Aurora B activity results in ejection of the CPC to the microtubules where it promotes spindle formation. In *Drosophila*, Subito is required to organize the central spindle, which includes recruiting the CPC (Jang et al., 2005). It also genetically interacts with the CPC (Das et al., 2018; Das et al., 2016). However, the CPC can localize to the spindle microtubules in the absence of Subito (Das et al., 2018; Jang et al., 2005), suggesting the ejection of the CPC from the chromosomes may be sufficient for its spindle transfer and Subito is not required. A role for Subito in assisting the transfer of the CPC onto the spindle could be difficult to observe if it is redundant with the ejection mechanism. To determine if Subito can recruit the CPC to the spindle microtubules, and thus have a role in the transfer mechanism, we investigated the role of Subito in a system where the chromosomes were removed from the spindle.

In the *sub* allele, *sub*$^{\Delta N}$, inhibitory N-terminus sequences have been deleted, resulting in an ectopic spindle phenotype where bipolar spindles assemble without chromosomes (Jang et al., 2007; Rome and Ohkura, 2018). These ectopic spindles contain Subito with colocalizing CPC, suggesting the Subito and the CPC interact on these spindles (Figure 6A). Thus, the *sub*$^{\Delta N}$ allele

provides a genetic tool to examine the recruitment of the CPC to the central spindle in the absence of the chromosomes. No spindle assembly of any kind was observed in *Incenp* RNAi; $sub^{\Delta N}$ oocytes, showing that ectopic spindle assembly depends on the CPC (Figure 6B). This result suggests that $SUB^{\Delta N}$ recruits the CPC onto the spindle microtubules independent of chromosomes. Thus, Subito may be capable of facilitating spindle transfer of the CPC.

Despite the absence of chromosomes, HP1 localized to the spindles in $sub^{\Delta N}$ oocytes (Figure S 5), suggesting HP1 can be recruited to the microtubules in the absence of chromosomes. To test whether the interaction between HP1 and the CPC is critical for the ectopic spindle phenotype, we examined our series of HP1 interaction-defective *Incenp* transgenes, expressed in *Incenp* RNAi, $sub^{\Delta N}$ oocytes. Ectopic spindle assembly was observed in *borr:Incenp* or $Incenp^{\Delta HP1}$ oocytes (Figure S 5B, C and Figure 6C). This was consistent with the finding that these transgenes rescued spindle assembly in *Incenp* RNAi oocytes. In contrast, ectopic spindles were not observed in $borr^{\Delta C}$*:Incenp* or $borr^{\Delta C}$*:* $Incenp^{\Delta HP1}$ oocytes (Figure S 5D, E and Figure 6C). These results suggest the HP1-Borealin interaction is critical for spindle assembly even in the absence of chromosomes.

Based on the results that the ectopic spindles are driven by a CPC-Subito interaction, and the finding that ectopic spindles are absent in $borr^{\Delta C}$*:Incenp, Incenp* RNAi; $sub^{\Delta N}$ oocytes, the HP1-Borealin interaction could be important for the CPC to interact with Subito. 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). Similar to a *sub* null mutant, Subito failed to localize to the spindle in sub^{HM26} oocytes, the spindle displayed a tripolar phenotype and the CPC localized throughout the spindle (Figure 6D).

These results suggest that an HP1 interaction is required for Subito localization and function. Thus, these experiments suggest that central spindle formation depends on interactions between HP1, Subito and Borealin.

Homolog 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 mutants of CPC components, *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 is most prominent on the central spindle, we envision that Aurora B activity could regulate bi-orientation while located on the microtubules rather than the chromosomes. To examine the role of the CPC in regulating homolog bi-orientation, we used fluorescence in situ hybridization (FISH) with probes targeting the pericentromeric regions each of chromosomes X, 2 and 3. To compare the role of CPC at the centromeres and central spindle, we examined *Incenp* mutants where the CPC was inappropriately localized to either the centromeres or the central spindle.

We first examined another sterile hypomorphic *Incenp* allele, *Incenp*$^{18.197}$, which was discovered based on genetic interactions with *subito* (Das et al., 2016). Using FISH, we determined that *Incenp*$^{18.197}$ mutant oocytes have homolog bi-orientation defects (11%, n = 42). Interestingly, the spindle was moderately diminished and the majority of INCENP was retained on the chromosomes in *Incenp*$^{18.197}$ mutant oocytes (Figure 7A), suggesting this mutant has a defect in CPC spindle transfer rather than chromosome localization. Second, *Incenp* transgenes with a 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

were 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 and elevated in meiotic nondisjunction in *Incenp* RNAi oocytes (Table 1), and failure to concentrate the CPC to the central spindle (Figure 7A). When the epitope tag was removed to 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 wild-type levels. These results confirmed that the N-terminal epitope tags in INCENP interfered with the CPC's central spindle localization and function. Additionally, these results along with the $Incenp^{18.197}$ mutation, suggest that CPC localization at the central spindle is important for homolog bi-orientation.

To directly test which population of the CPC regulates homolog bi-orientation, we used INCENP fusions to target overexpression of the CPC to specific sites. We predicted that mislocalization or overexpression of the CPC would disrupt bi-orientation. Although forcing CPC localization to the centromere has been shown to cause bi-orientation defects in mitotic cells (Liu et al., 2009), the frequency of bi-orientation in *mis12:INbox* oocytes (in the presence of wild-type INCENP) was not significantly elevated compared to wild-type (Figure 7B and 7C). We also tested whether centromere-targeting CPC 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. The results of colchicine treated wild-type 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 (Figure 7D and 7E). Thus, overexpression of Aurora B activity at the centromeres did not cause bi-orientation defects. In contrast, central-spindle-targeted CPC (*sub:INbox* or *feo:INbox*) caused significantly more bi-orientation defects than in wild type

(Figure 7B and 7C). These results suggest that the CPC regulates homolog 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 homolog bi-orientation defects (Figure 7C), despite having apparently wild-type spindle assembly and CPC localization (Figure 5D). $Incenp^{\Delta HP1}$, *Incenp* RNAi oocytes had wild-type spindle morphology, but had defects in fertility and CPC localization to the central spindle (Figure 4E and 4H). Examination by FISH showed these oocytes had a homolog bi-orientation defect (Figure 7B and 7C). Interestingly, we found HP1 localization on the spindle in $Incenp^{\Delta HP1}$, *Incenp* RNAi oocytes, but unlike wild type, HP1 was not enriched in the overlap with the CPC (Figure 7F). These results are consistent with the model that spindle localization of the CPC and interaction with HP1 is important for regulating homolog bi-orientation. To test HP1 directly, we examined oocytes depleted of HP1 by expressing shRNA *GL00531*. These oocytes displayed wild-type spindles with normal CPC localization (Figure 7G), which could be explained by the relatively mild knockdown of *HP1* (48% of mRNA remains). However, expression of *GL00531* in oocytes caused elevated X-chromosome non-disjunction (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 homolog bi-orientation.

Discussion

Drosophila oocytes generate spindles without centrioles or predefined spindle poles, like in many other organisms. Whether the microtubules assemble around MTOCs as in the mouse (Schuh and Ellenberg, 2007) or more closely around the chromosomes in *Drosophila* and humans (Hadders et al., 2020; Hengeveld et al., 2017), the 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 well known. Our previous studies have shown that the CPC is required for spindle assembly in *Drosophila* oocytes (Radford et al., 2012). Interestingly, the known pathways for recruiting Survivin and Borealin to the centromere via Haspin and Bub1 do not seem to be essential for kinetochore assembly in *Drosophila* oocytes. Instead, we found that Borealin targets the rest of the CPC to chromatin, most likely by interacting with chromatin component HP1, and this interaction is crucial for acentrosomal spindle assembly. Furthermore, HP1 interacts with INCENP to regulate homolog bi-orientation on the spindle.

CPC-dependent, chromosome-directed spindle assembly in oocytes depends on an interaction with HP1

We propose a model that not only explains how the chromosomes recruit spindle assembly factors, but also how the CPC itself moves from the chromosomes to the spindle. This model depends on an interaction between HP1 and a tripartite complex of the CPC comprised of INCENP, Borealin and Survivin/Deterin (Jeyaprakash et al., 2007). After nuclear envelope breakdown, the tripartite complex is recruited to the heterochromatin regions marked by 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 by HP1. Once the CPC localizes to the chromosomes, Aurora B activity results in phosphorylation of

several targets, such as INCENP and H3S10, and assembly of the kinetochore. Spindle assembly likely involves suppressing microtubule depolymerases, such as kinesin 13/MCAK and Op18/Stathmin (Kelly et al., 2007; Sampath et al., 2004). Spindle assembly also involves 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).

Research in HeLa cells first found that HP1 interacts with the CPC (Ainsztein et al., 1998). HP1 promotes CPC localization and may concentrate and enhance Aurora B phosphorylation of targets in the vicinity of the heterochromatin including the kinetochores (Abe et al., 2016; Ruppert et al., 2018). Furthermore, it has been shown previously that an HP1-Borealin interaction can bring the CPC to the centromere regions (Liu et al., 2014). HP1 has also been shown to interact directly with the CPC in *Drosophila* (Alekseyenko et al., 2014). Our evidence suggests that an HP1-Borealin interaction plays an important role in microtubule-independent localization of the CPC to the chromatin.

An HP1 interaction can also explain how the CPC is released from the chromatin to relocate onto the spindle. Similar to research in mitotic cells (Duan et al., 2008), we propose that H3S10 phosphorylation in oocytes weakens the interaction between Borealin-bound HP1 and H3K9me3, facilitating the ejection of the CPC-HP1 complex from the chromosomes. Meanwhile, multiple mechanisms, possibly mediated by at least four protein domains within the CPC (two in INCENP, STD and SAH, and one each in Subito and Borealin) can promote interactions with the microtubules that may be essential for releasing the CPC from chromatin and building the spindle (Trivedi et al., 2019b; van der Horst et al., 2015). When the CPC-HP1 complex moves onto the microtubules, interactions with central spindle nucleating proteins, such as Subito, form the central spindle. This model is attractive for several reasons. First, it identifies

the chromatin-associated molecules that recruit the CPC. Second, it provides a mechanism for transferring the CPC from the chromatin to the microtubules. Indeed, it has been proposed previously that HP1 has role in the transfer of the CPC from the heterochromatin to the spindle (Ainsztein et al., 1998). Third, and most important, it explains how spindle assembly is restricted to the chromosomes in an acentrosomal system (Ohkura, 2015; Reschen et al., 2012; Rome and Ohkura, 2018); it is based on an HP1-Borealin interaction. We and others have suggested that restricting spindle assembly proximal to the chromosomes involves inhibition of spindle assembly factors in the cytoplasm (Beaven et al., 2017; Das et al., 2018; Rome and Ohkura, 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). We propose that the release of the CPC from the chromosomes locally overrides this inhibition and activates spindle assembly factors.

Non-kinetochore microtubules require spindle-associated CPC

In mutants where the CPC was restricted to the chromosomes, but not necessarily the centromeres, kinetochore assembly and K-fiber formation were observed. Our results are similar to studies in *Xenopus*, where centromeric CPC localization is not required for kinetochore assembly (Haase et al., 2017). Correct spatial regulation and specific interactions with inner kinetochore or centromere proteins are required for the CPC to promote outer kinetochore assembly (Bonner et al., 2019), which ensures kinetochore assembly at the correct location and time. Some of our mutants (*HP1:Incenp* and *Incenp*$^{\Delta CEN}$), were able to support limited kinetochore assembly but less K-fibers, suggesting that low levels of CPC are sufficient for kinetochore assembly, but higher levels and/or specific localization are required for spindle assembly. We suggest that, in order to build K-fibers, Borealin needs to be recruited to the centromeric regions to cluster the CPC and function more efficiently. A notable difference

compared to *Xenopus* (Bonner et al., 2019), however, is that in the *Drosophila* oocytes the SAH domain is not required for kinetochore assembly.

In the mutants where the CPC was restricted to the chromosomes, only kinetochore assembly and K-fiber formation were observed. The absence of non-kinetochore microtubules in these mutants suggests that non-kinetochore microtubules require that the CPC leaves the chromosomes and that non-kinetochore microtubules require microtubule associated CPC. This principle is also observed in *sub* mutants, where the central spindle is absent but robust bundles of non-kinetochore microtubules form (Jang et al., 2005). These observations suggest the CPC promotes assembly and bundling of microtubules, independent of Subito. When the CPC is ejected from the chromosomes, it probably activates the Augmin pathway that has been shown to increase the amount of spindle microtubules in *Drosophila* oocytes (Rome and Ohkura, 2018).

Regulation of Homolog 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). However, our analysis of multiple *Incenp* mutants suggests that chromosomal localization of the CPC does not promote these functions. For example, we expected that overexpressing CPC at the centromere would hyper-phosphorylate substrates that lead to unstable attachments and bi-orientation defects. Despite observing pINCENP at the centromeres in *Mis12:Inbox* metaphase I oocytes, the centromere-targeting of the CPC in meiosis did not cause KT-MT destabilization or affect homolog bi-orientation, as might be predicted if centromere-bound CPC can promote destabilization of microtubule attachments.

Mutants with defects specific to spindle localization had the most severe bi-orientation defects. Therefore, the key to homolog bi-orientation may be getting the CPC off the chromosomes and onto the microtubules. Based on the results of several experiments, we propose that homolog bi-orientation of meiotic chromosomes depends on interactions between the CPC and microtubules of the central spindle. First, in previous publication, we showed that defects in central spindle associated proteins results in bi-orientation defects (Das et al., 2016). Second, forcing localization of CPC to the central spindle, but not the kinetochores, disrupts bi-orientation. Third, deleting the HP1 interaction site (121-232 amino acid) of INCENP caused disorganized CPC central spindle localization and loss of HP1 enrichment with the CPC. These results suggest that the structure of the central spindle might be compromised, leading to a homolog bi-orientation defect. Fourth, an N-terminal epitope-tag on INCENP caused the CPC to spread out along the spindle, causing defects in homolog bi-orientation and fertility (Radford et al., 2012). These effects are enhanced by a reduction in Subito activity (Das et al., 2016; Radford et al., 2012), consistent with a function for the CPC in the central spindle. Finally, the conserved spindle transfer domain in INCENP (Ainsztein et al., 1998) is required for homolog bi-orientation. INCENP$^{\Delta STD}$ oocytes did not display obvious mislocalization of Subito and INCENP but had defective homolog bi-orientation. Together, these results show that the CPC and HP1 have an important function within the central spindle that is important for the bi-orientation of homologous centromeres towards opposite poles.

Our data suggests that the INCENP-HP1 interaction is important for bi-orientation once the CPC and HP1 moves onto the spindle. The study of achiasmate chromosome segregation in *Drosophila* has also implicated HP1 (Giauque and Bickel, 2016) or heterochromatin (Karpen et al., 1996) in promoting accurate meiosis I chromosome segregation. HP1 forms dimers through

its chromo shadow domain (Cowieson et al., 2000) while it also interacts with proteins containing a conserved binding site (PXVXL) (Thiru et al., 2004). This suggests HP1 could be involved in a complex pattern of interactions that bring important proteins together, such as the CPC and Aurora B phosphorylation substrates such as Subito, which also has a conserved HP1 binding site that is required for its meiotic functions (Jang et al., 2007; Jang et al., 2005). How this milieu of proteins promotes bi-orientation is still a mystery. The central spindle is a complex structure, containing several proteins including Borealin that have a microtubule binding domain that may allow the CPC to sense its environment (Trivedi et al., 2019b). Several 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 bi-orientation of bivalents.

During meiosis I, the pairs of centromeres within a bivalent bi-orient at a much greater distance than the sister centromeres in mitosis or meiosis II. Therefore, it is plausible that the bi-orientation mechanism has adapted to this difference. We propose that the meiotic central spindle combines two properties required for oocytes to bi-orient their homologous chromosomes during prometaphase I. The first is a mechanism to move and separate the kinetochores of bivalents. This may be analogous to the activity of "bridging fibers" which can separate pairs of sister kinetochores in mitosis (Simunic and Tolic, 2016; Vukusic et al., 2017). In *C. elegans* meiosis, the central spindle separates homologs for chromosome segregation by microtubule pushing (Laband et al., 2017). The second is a mechanism for microtubule bound CPC to be involved in error-correction, which has been observed in several contexts (Funabiki, 2019; Pamula et al., 2019; Trivedi et al., 2019b). This combination of activities would allow the central spindle to direct reductional chromosome segregation at anaphase I.

Methods and materials

Generation of RNAi resistant INCENP

To engineer RNAi resistant transgenes, we obtained *Incenp* cDNA (RE52507) from *Drosophila* Genomic Resource Center and cloned it into the pENTR vector (Invitrogene, Carlsbad, CA). We used the Change-it Site-directed Mutagenesis kit (Affymetrix) to introduce 8 silent mutations in the region corresponding to amino acids 437-441, which is complementary to *Incenp* shRNA (GL00279). (Figure 1B and Figure S 1). The primers for the site-directed mutagenesis are: 5'-ATGAGCTTTTCAACCCACTCCTGCAGTCGCCCGTCAAGATGCGCGTGGAGGCGTTCG A -3' and 5'-TCGAACGCCTCCACGCGCATCTTGACGGGCGACTGCAGGAGTGGGTTGAAAAGCTC ATG -3'. RNAi resistant INCENP constructs including $Incenp^{myc}$, $Incenp^{HA}$ and $Incenp^{Flag}$ were further generated by using the LR Clonase reaction (Gateway systems, Invitrogen) to the pPMW, pPHW or pPFW vectors that carry UASp-promoter. Expressing $Incenp^{myc}$ in an *Incenp* RNAi background rescued spindle assembly in oocytes, as well as kinetochore assembly and spindle localization, but also showed several defects. The homologous chromosomes frequently failed to bi-orient, fertility was reduced and the transgene protein spread along the spindle instead of concentrating in the central spindle. 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. To solve this problem, we removed the myc-tag from $Incenp^{myc}$ to generate $Incenp^{WT\text{-}R}$ by using a Gibson Assembly kit (New England Biolabs). Expressing $Incenp^{WT\text{-}R}$ in *Incenp* RNAi oocytes displayed wild-type spindle and localization, and restored fertility to wild-type levels. We used a plasmid carrying $Incenp^{WT\text{-}R}$ as the backbone in 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 the 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 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* embryos through Model System Injections (Durham, NC).

Drosophila genetics and generation of shRNA transgenics

Flies were crossed and maintained on the standard media at 25 ℃. All loci information was obtained from Flybase. Fly stocks were obtained from the Bloomington Stock Center or the Transgenic RNAi Project at Harvard Medical School (TRiP, Boston, USA), including *aurB* (GL00202), *Incenp* (GL00279), *Su(var)205* (GL00531) and *Bub1* (GL00151), except the $sub^{\Delta N}$ allele, which we generated (Jang et al., 2007) and *mis12::EGFP* (Głuszek et al., 2015). To generate a *Deterin* and *haspin* shRNA line, a targeted *Deterin* sequence (5'- CGGGAGAATGAGAAGCGTCTA -3') and a targeted *haspin* sequence (5'- GGAAGACAGTAGAGACAAATG- 3') were cloned into pVALIUM22 respectively, based on the protocol described in Harvard TRiP center. The construct was injected into *Drosophila* embryos (*y sc v; attP40*). All the short hairpins for RNA silencing and transgenes carried the UAS promoter for UAS/GAL4 binary expression system (Rorth et al., 1998). All these short hairpin RNA lines and transgenes used in this paper were expressed by *mata4-GAL-VP16*, which induces expression after early pachytene throughout most stages of oocyte development in *Drosophila* (Sugimura and Lilly, 2006).

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

(Life Technologies). Dm03420510_g1 for *haspin*, Dm02141491_g1 for *Deterin*, Dm01804657_g1 for *Bub1*, Dm0103608_g1 for *Su(var)205* and Dm02134593_g1 for the control *RpII140*. The knockdown of the respective mRNAs in these oocytes was reduced to 15% in *haspin* RNAi oocytes, 5% in *Deterin* RNAi oocytes, 2% in *Bub1* RNAi oocytes and 48% in *Su(var)205* RNAi oocytes.

Antibodies and immunofluorescence microscopy

Mature (stage 12-14) oocytes were collected from 100-200, 3-4 day old yeast-fed non-virgin females. The immunofluorescence preparation protocol is described in (Radford and McKim, 2016). Hoechst 33342 (10 μg/ml, Invitrogen) was used for staining DNA and mouse anti-a tubulin monoclonal antibody DM1A(1:50) conjugated with FITC (Sigma, St. Louis) was used for staining microtubules. Primary antibodies used in this paper were rabbit anti-CID (1:1000, Active motif), Guinea pig anti-MEI-S332 (1:300, (Moore et al., 1998)), rabbit anti-SPC105R (1:4000, (Schittenhelm et al., 2007)), rabbit anti-CENP-C (1:5000, (Heeger et al., 2005)), mouse anti-Myc (1:50, 9E10, Roche, Indianapolis), mouse anti-Flag (1:500, Thermo Fisher), rat anti-INCENP (1:400, (Wu et al., 2008)), rabbit anti-Aurora B (1:1000, (Giet and Glover, 2001)), rabbit anti-Survivin (1:1000, (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 (1:1000, Active motif), rabbit anti-H3S10ph (1:1000, Active motif), rat anti-Subito (1:75, (Jang et al., 2005)), rat anti-a-tubulin (Clone YOL 1/34, Millipore) and rabbit anti-pINCENP (1:1000, (Salimian et al., 2011)). The secondary antibodies including Cy3 and AlexFluor647 (Jackson Immunoresearch West Grove, PA) or AlexFluor488 (Molecular Probes) were used in accordance with the subjected primary antibodies. FISH probes were designed against X-chromosome (359 repeats), 2nd chromosome (AACAC satellite) and 3rd chromosome (dodeca satellite) obtained by Integrated DNA Technologies. Oocytes were mounted in SlowFade Gold (Invitrogen). Images were collected on a Leica TCS SP8 confocal microscope with a 63x, NA 1.4 lens and shown as maximum projections of complete image stacks. Images were then cropped in Adobe Photoshop (PS6).

Drug treatment assay

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

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Table 1: Summary of transgene fertility

Genotype	**Fertility / nondisjunction [1]**	
	in wild-type oocytes	**in *Incenp* RNAi oocytes**
Myc:Incenp	+++/ 1.4% NDJ (n=862)	+/ 13.7% NDJ (n=95)
HA:Incenp	+++/ 0% (n=112)	+/ 0% (n=78)
Flag:Incenp	++/ 0% (n=48)	+/ 7.2% (n=83)
Incenp^{WT-R}	+++/ 0% (n=267)	+++/ 2.0% (n=461)
Incenp$^{\Delta CEN}$	+/ 0% (n=35)	sterile
Incenp$^{\Delta STD}$	Sterile	++/0% (n=323)
Incenp$^{\Delta SAH}$	+/ 0% (n=68)	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
Wild type	+++/ (n=240)	Sterile

[1] 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 and sterile = no progeny. Nondisjunction = 2XNDJ/total progeny.

Figure legends

Figure 1**: INCENP localization and spindle assembly depends on the N-terminal Borealin/Deterin binding domain.**

(A) INCENP localization in oocytes after colchicine treatment for one hour to destabilize the microtubules. INCENP is in red, CID/CENP-A is in white, tubulin is in green and DNA is in blue. Scale bars in all images are 5 µm. (B) A schematic of *Drosophila* INCENP, showing the location of the centromere-targeting N-terminal region (BS), single alpha helix (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 $Incenp^{WT\text{-}R}$. 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 $Incenp^{WT\text{-}R}$ RNAi-resistant transgene. CID or 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 SPC105R in red. (F) Quantitation of SPC105R localization in RNAi oocytes (n=7, 14, 32, 12 and 22 oocytes). Error bars indicate 95% confidence intervals and ** = p-value < 0.001 run by Fisher's exact test.

Figure 2**: Independent localization of the CPC to the centromere and the central spindle assembles only kinetochore-dependent microtubules.**

(A) MIS12 localization in wild type and *Incenp* RNAi oocytes (arrows). MIS12 is in red, tubulin is in green, DNA is in blue and the scale bar represents 5 µm. (B) A schematic of the CPC constructs designed to target the CPC to the centromeres. (C) Expressing *mis12:Incenp* in wild-type and *Incenp* RNAi oocytes. (D) Quantitation of the spindle phenotype of MIS12 fusions expressed in *Incenp* RNAi oocytes (n=23, 36 and 25 oocytes). (E) *Myc:INbox*, *Flag:mis12:INbox* and *Myc:sub:INbox* expressed in wild-type oocytes. (F) Detection of the Aurora B substrate, phosphorylated INCENP (pINCENP). The arrow and inset shows the pINCENP signal at the centromeres when the INbox is targeted by MIS12. INCENP is in red and pINCENP is in white. (G) *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 represent 5 µm in the left three panels and 10 µm in the low magnification image. (H) *Flag:mis12:INbox* expressed alone or co-expressed with *Myc:sub:INbox* in *Incenp* RNAi oocytes. Merged images showed DNA (blue), tubulin (green), Myc (red) and Aurora B (white). Scale bars indicate 5 µm.

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 to wild-type *Incenp*. (B) The spindle phenotypes of *borr:Incenp* and *Det:Incenp* in *Incenp* RNAi oocytes. Separate channels show the localization of INCENP and the microtubules. 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. Scale bars in all images are 5 µm. (C,D) The effect of Deterin depletion in *borr:Incenp*, *Incenp* RNAi oocytes (n=18 ,23 and 24). (E) Deterin localization in wild-type (n=4) and *borr:Incenp*, *Incenp* RNAi oocytes (n=7). CID is in red, Deterin is in white, DNA is in blue and tubulin is in green.

Figure 4**: HP1-Borealin interaction is critical for CPC-dependent meiotic spindle assembly.**

(A) Knock down of *Bub1* and *Haspin* by RNAi in oocytes. INCENP is in red, tubulin is in green, CID is in white and DNA is blue. Scale bars in all images are 5 µm. (B) Borealin and HP1 localization in wild type, *Incenp* RNAi and *aurB* RNAi oocytes. Borealin is in white, HP1 is in red, tubulin is in green and DNA is in blue. (C) HP1 and H3K9me3 localization in wild-type and *aurB* RNAi oocytes. Arrow shows HP1 localization in wild-type oocytes on the spindle. HP1 is in green, INCENP is in red, DNA is in blue and CID is in white. (D) A schematic of *Incenp* mutant constructs affecting HP1 interactions. The HP1 binding sites (PxVxL) 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 CEN domain. (E) Expressing *Incenp* transgenes shown in (D) in *Incenp* RNAi oocytes, including $borr^{\Delta C}$*:Incenp*, $Incenp^{\Delta HP1}$, $borr^{\Delta C}$*:*$Incenp^{\Delta HP1}$ and *HP1:Incenp*. Two different images of $Incenp^{\Delta HP1}$ are shown to compare ring-shaped and irregular (see arrow) localization of the CPC. The images show CID in white, INCENP in red, DNA in blue and tubulin in green. (F) Quantitation of spindle phenotype in the HP1-interaction-defective mutants shown in (E) (n= 17, 26, 31 and 49 oocytes, in the order as shown in the graph). (G) SPC105R localization was quantified in the different HP1-interaction-defective mutant oocytes (n= 7, 32, 9, 15, 14, 33). Bars indicates 95% confidence intervals and ** = p-value < 0.01 in Fisher's exact test. (H) Quantitation of INCENP spindle localization in wild-type (n=19) and $Incenp^{\Delta HP1}$, *Incenp* RNAi oocytes (n=17). **** = p-value < 0.0001.

Figure 5**: The dissociation of HP1 and the CPC from the chromosomes depends depends on microtubules.**

(A) Wild-type oocytes were treated with DMSO (left panel) or BN2 (right panel) for one hour to inhibit Aurora B kinase activity. In red are heterochromatic marks HP1 or H3K9me3 and the Aurora B target phosphorylated H3S10 (H3S10ph). In white are the CPC components INCENP or Deterin. In all the images, scale bars represent 5 µm. (B) Wild-type oocytes were treated with ethanol or Colchicine for one hour to depolymerize microtubules and analyzed for the same markers as in (A). DNA shown in blue and tubulin shown in green in all pictures. (C) A schematic showing two INCENP deletions removing regions that promote microtubule interactions. (D) Bipolar spindle assembly when $Incenp^{\Delta STD}$ or $Incenp^{\Delta SAH}$ were expressed in *Incenp* RNAi. CID is in white, INCENP is in red, Tubulin is in green, DNA is in blue.

Figure 6**: Analysis of CPC interactions with Subito and microtubules required for central spindle assembly.**

Examination of ectopic spindle assembly in (A) $sub^{\Delta N}$ and (B) $sub^{\Delta N}$, *Incenp* RNAi oocytes. The inset in A is magnified in the single channel images. The boxed region in B contains the chromosomes. Tubulin is in green, Aurora B is in white, and $Sub^{\Delta N}$ is in red. Scale bars are 10µm. (C) Quantitation of ectopic spindle phenotype (see also Figure S 5) (n= 73, 30, 97, 51, 192 and 37 oocytes). **** = *p*-value < 0.0001 in Fisher's exact test. (D) Tripolar spindle and mislocalization of the CPC phenotype in sub^{HM26}/sub^{131} oocytes, with tubulin (green), Deterin (white) or HP1, and Subito (red) localization. Scale bar is 5 µm.

Figure 7: **Disruption of homolog bi-orientation by disruptions of central spindle CPC.**

(A) Disorganized or mislocalized CPC in the *Incenp* hypomorphic allele $Incenp^{18.197}$ and the transgene *Myc:Incenp*. Subito localization was not affected. The CPC is in red, Subito or CID are in white, tubulin is in green and DNA is in blue. Scale bars in all images are 5 µm. (B) *Incenp* mutants examined for homolog bi-orientation using FISH with probes against pericentromeric heterochromatin on the X (359 bp repeat, yellow), 2nd (AACAC, red) and 3rd (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*-value < 0.05 ****= *p*-value <0.0001 in Fisher's exact test. (D) Wild type oocytes and *mis12:INbox* oocytes treated with colchicine for 30 minutes. INCENP is in red and CID is in white. Scale bars are 5 µm. (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 wild-type and $Incenp^{\Delta HP1}$, *Incenp* RNAi oocytes. HP1 is in green, Deterin is in red and overlapping region is in yellow. (G) Spindle in *Su(var)205* RNAi oocytes. CID is in white, INCENP is in red, tubulin is in green and DNA is in blue.

Figure S 1**: Alignment and domain analysis of *Drosophila*.**

The sequence alignment compares *D. melanogaster* INCENP to *D. virilis* and *X. laevis*. The Borealin/ Deterin binding domain is from amino acids 1-46 (yellow). The *CEN* and *STD* deletion mutations are marked 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 blue.

Figure S 2**: Expression of *mis12:INbox* in the wild-type oocytes disrupts meiotic progression.**

Fertilized 0-2hr old *Drosophila* embryos were fixed and stained for INCENP (red), tubulin (green) and DNA (blue). The scale bar is 5 µm.

Figure S 3: **Expression of *Myc:feo:INbox* in wild-type and *mis12:INbox*, *Incenp*^{RNAi} oocytes.**

(A) *Myc:feo:INbox* localizes to the central spindle in the wild-type oocytes. (B) Co-expression 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. The scale bar is 5 µm.

Figure S 4**: Borealin localization is not detectable in *Det:Incenp, Incenp* RNAi oocytes.**

Metaphase I oocytes from wild-type 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 is 5 µm.

Figure S 5: **HP1 localization overlaps with the ectopic microtubules and SUB$^{\Delta N}$ in *sub$^{\Delta N}$* oocytes.**

(A) Ectopic spindle assembly in a *sub$^{\Delta N}$* oocytes. Single channels show a higher magnification of the boxed area in the merged image. HP1 is in white, SUB$^{\Delta N}$ is in red, tubulin is in green and DNA is in blue. Scale bar is 10 µm. Ectopic spindles in (B) *Incenp$^{\Delta HP1}$* and (C) *borr:Incenp* oocytes, but not in (D) *borr$^{\Delta C}$:Incenp* or *borr$^{\Delta C}$:Incenp$^{\Delta HP1}$* oocytes. The insets show magnifications of the boxed regions. Tubulin is in green, Aurora B is in white, and Sub$^{\Delta N}$ is in red. Scale bars are 10µm.

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Figure 1

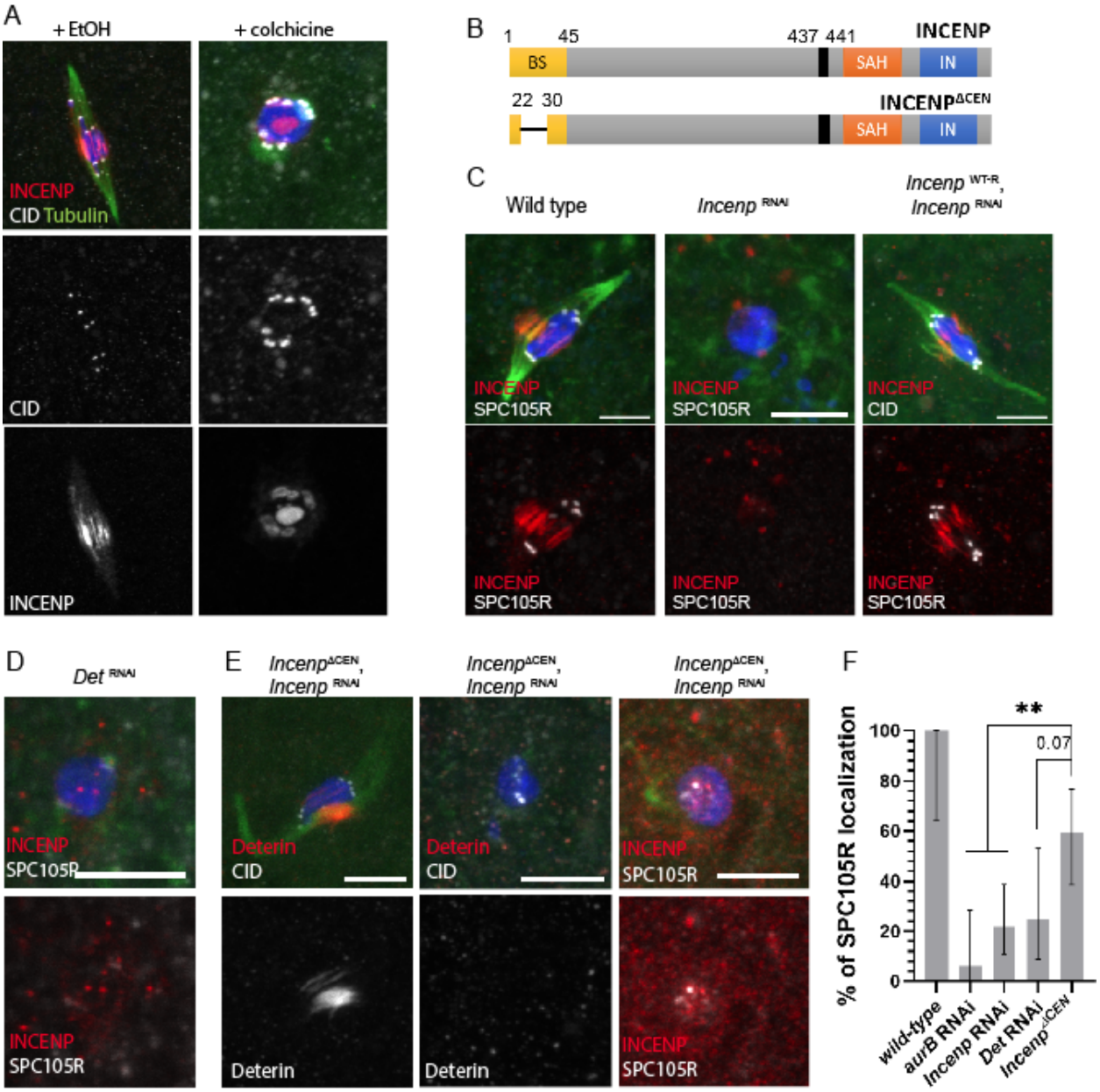

Figure 2

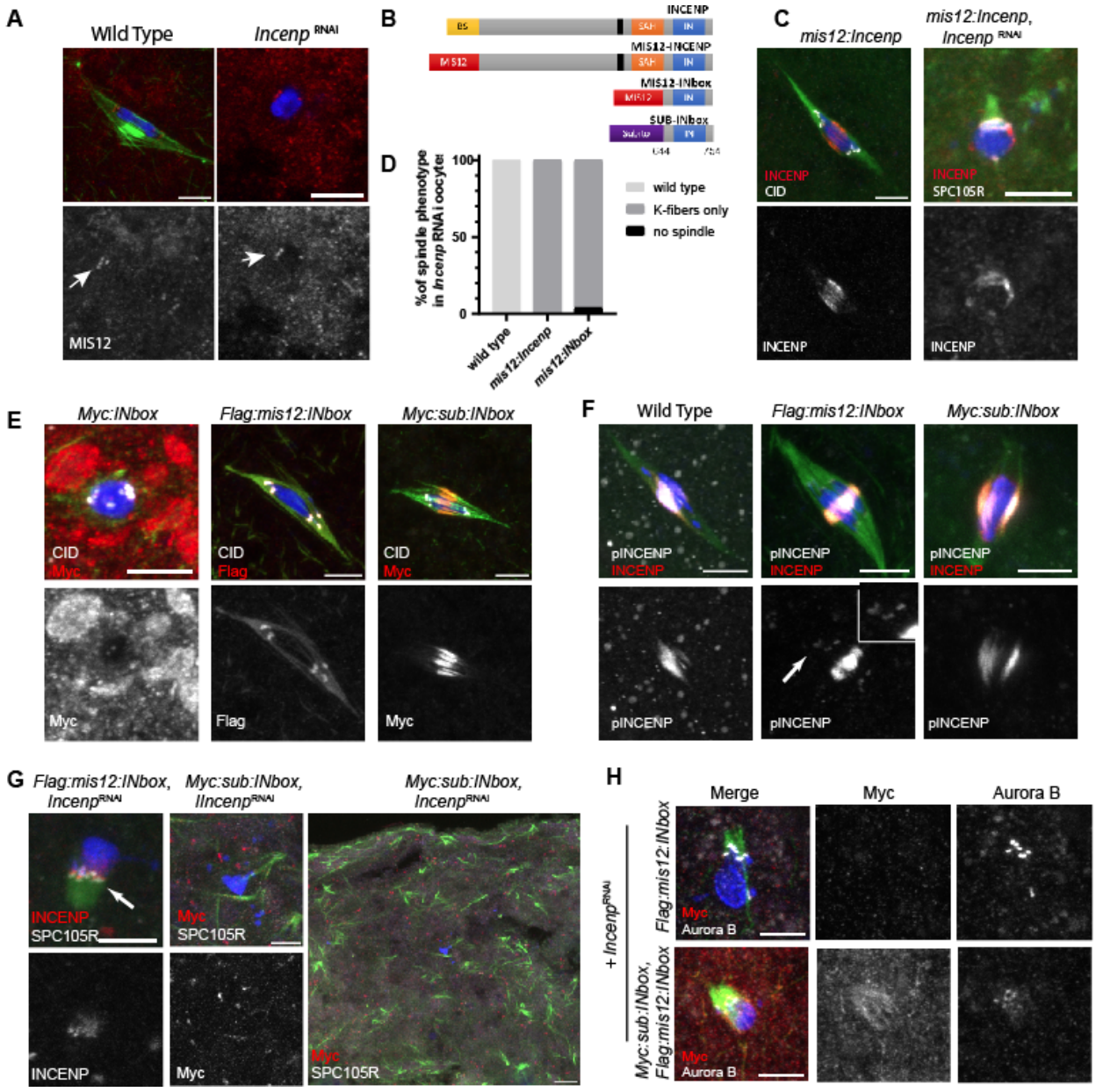

Figure 3

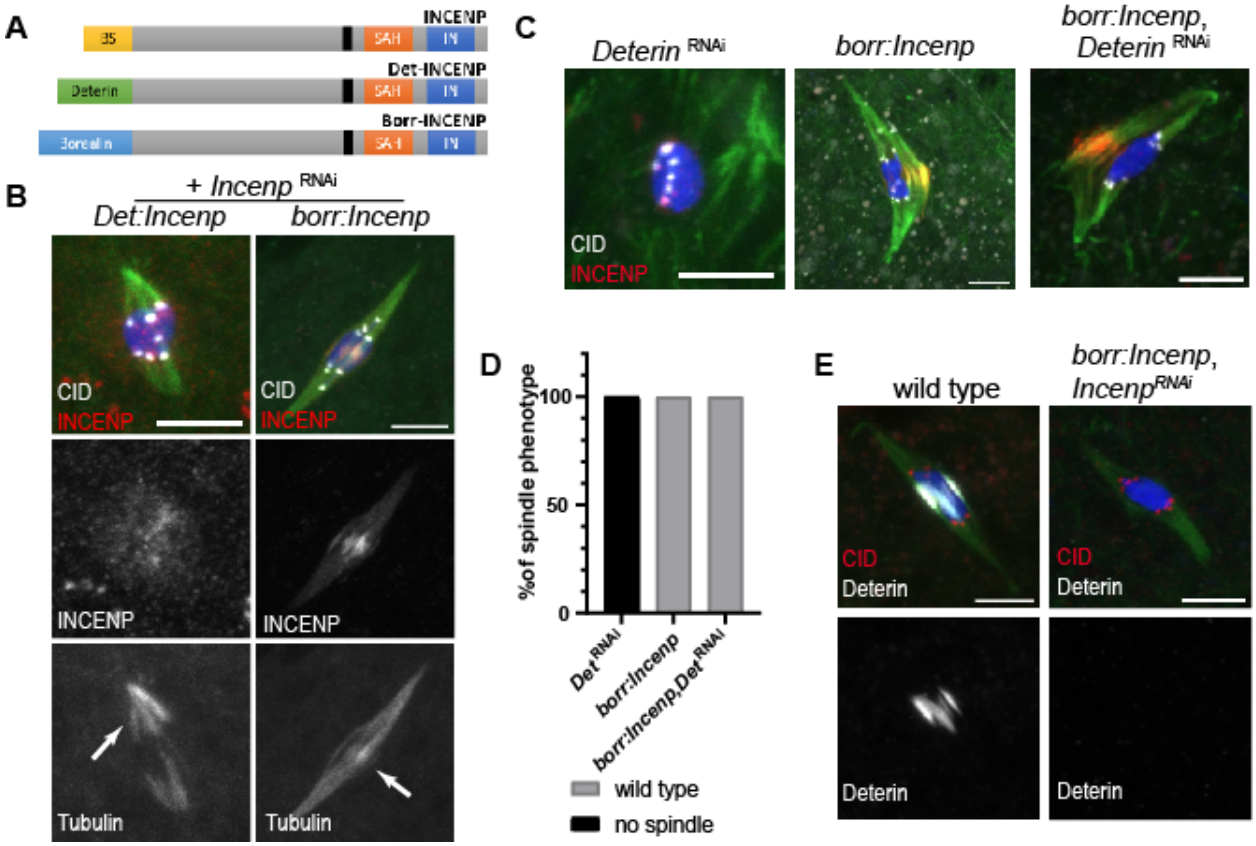

Figure 4

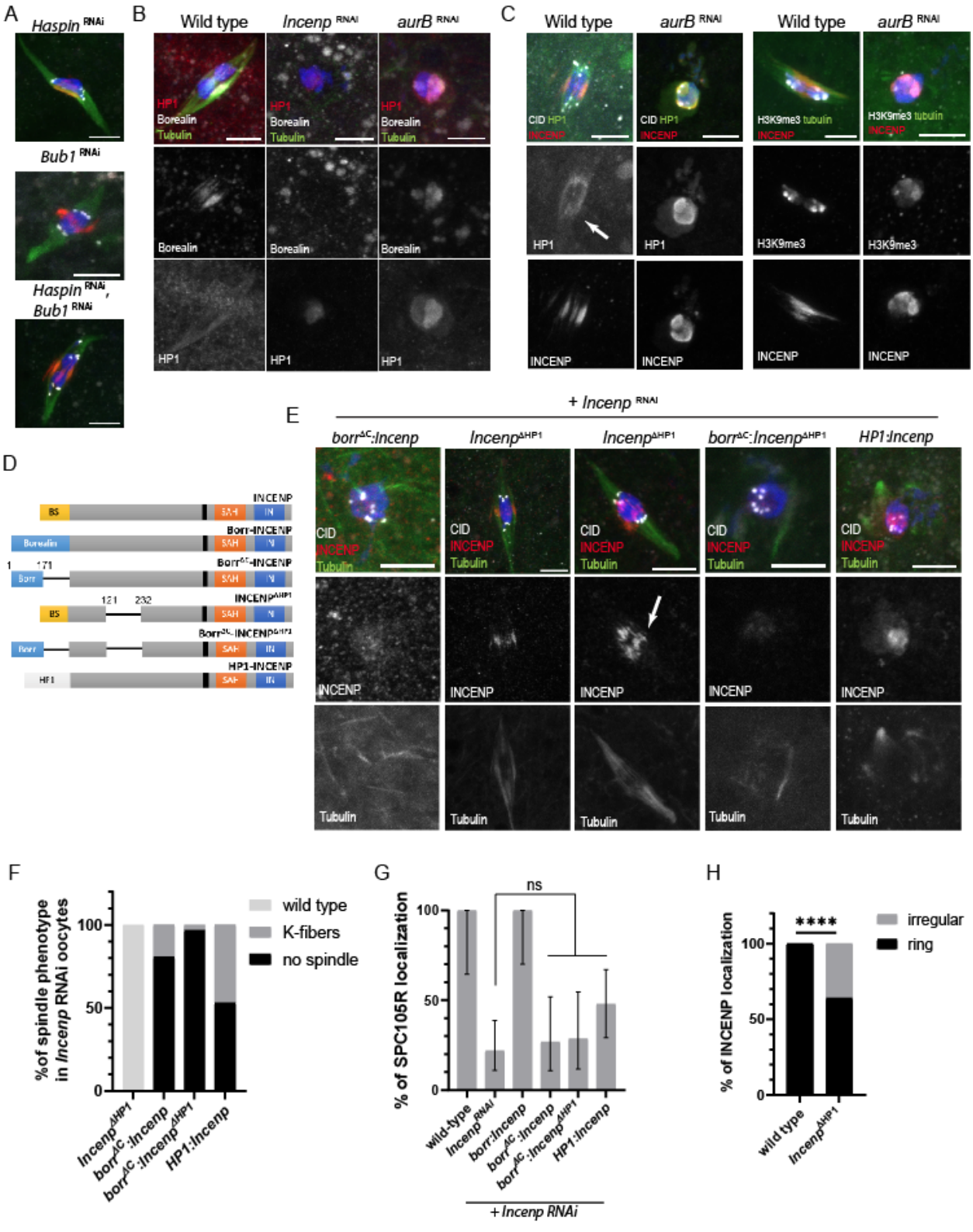

Figure 5

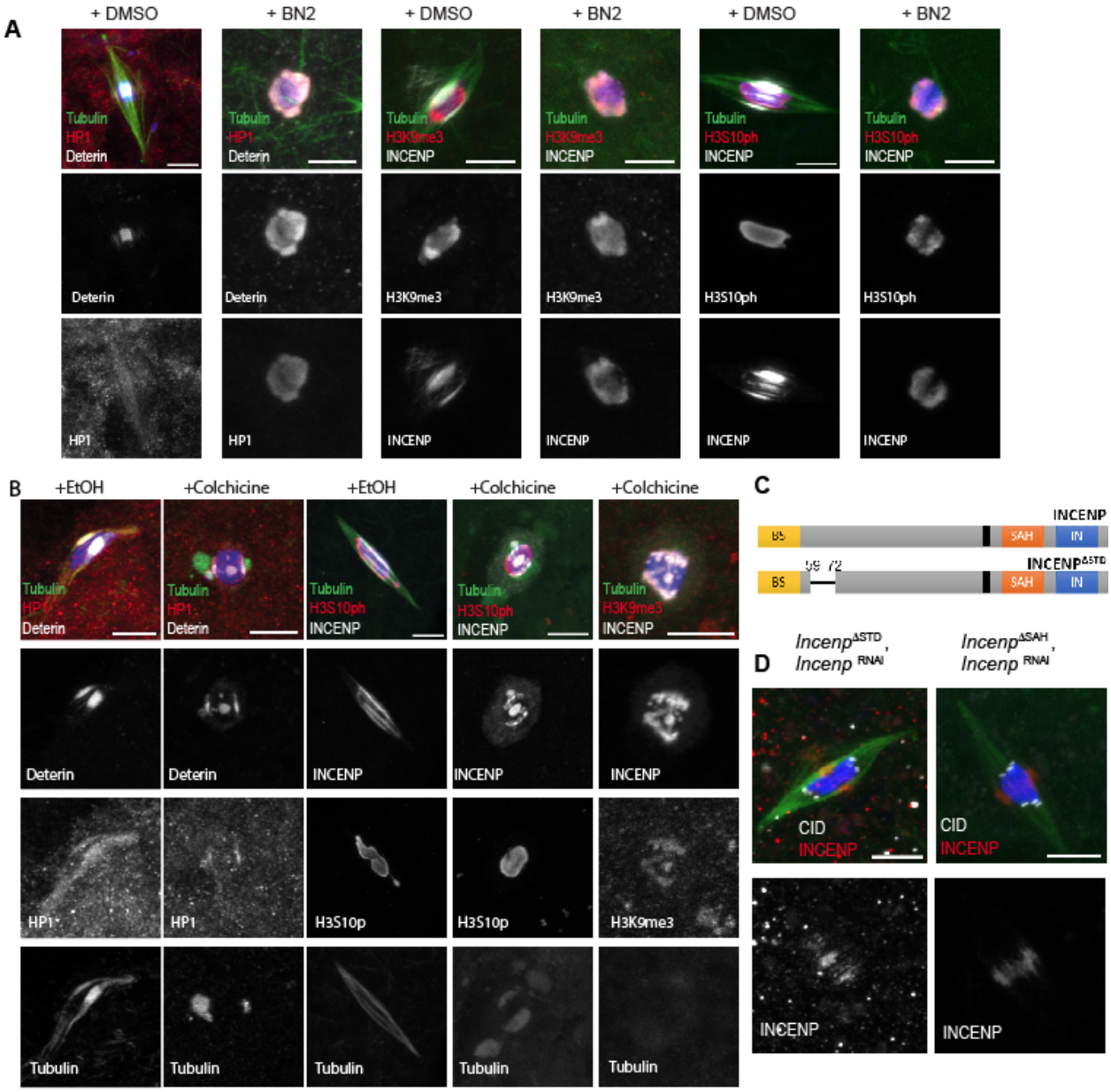

Figure 6

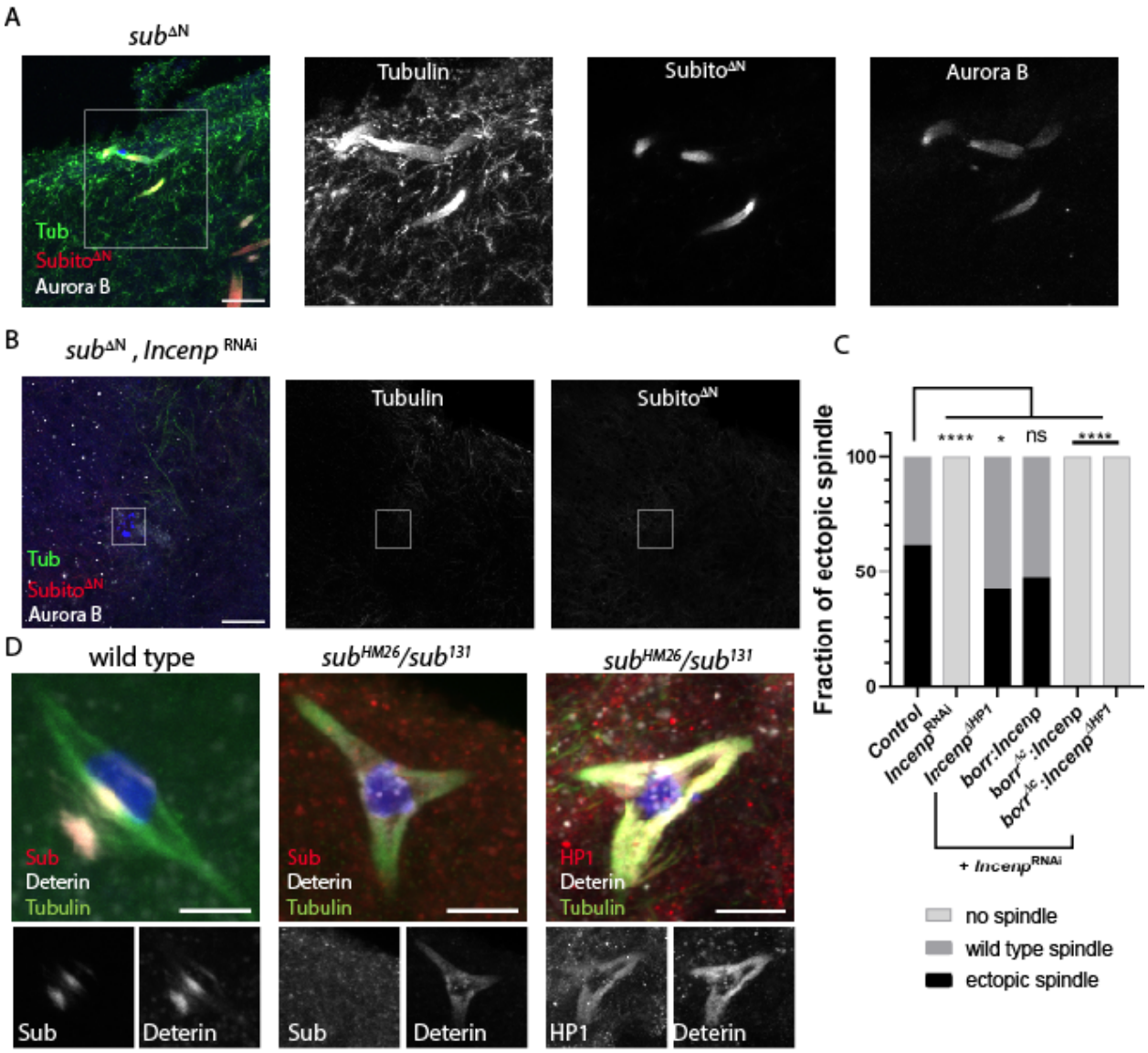

Figure 7