Protein Phosphatase 2A-B56 maintains the meiotic spindle, kinetochore attachments and cohesion by antagonizing Aurora B in *Drosophila* Oocytes

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Abstract

Meiosis in female oocytes lack centrosomes, the major microtubule-organizing center, which may make them especially vulnerable to aneuploidy. In the acentrosomal oocytes of *Drosophila*, meiotic spindle assembly depends on the chromosomal passenger complex (CPC). Aurora B is the catalytic component of the CPC while the remaining subunits regulate its localization. Using an inhibitor of Aurora B activity, Binucleine 2, we found that continuous Aurora B activity is required to maintain the oocyte spindle during meiosis I. Furthermore, the necessity of a kinase for spindle regulation suggests that spindle dynamics is regulated by phosphatases. Our result have shown that the protein complex Protein Phosphatase 2A (PP2A) opposes CPC activity, probably by dephosphorylating spindle associated proteins such as the Kinesins. PP2A exists in two varieties, B55 and B56. While both antagonize Aurora B, they typically exhibit different localization and function. B55 has only minor roles in meiosis I spindle function. The B56 subunit is encoded by two partially redundant paralogs in the *Drosophila* genome, *wdb* and *wrd*. Knocking down both B56 subunits showed they are critical for multiple functions during meiosis I, including maintaining sister centromere and arm cohesion, end-on microtubule attachments, and the metaphase I arrest in oocytes. We found that WDB recruitment to the centromeres depends on BubR1, MEI-S332, and kinetochore protein SPC105R. However, only SPC105R is required for cohesion maintenance during meiosis I. We propose that SPC105R promotes cohesion maintenance by recruiting two proteins that further recruit PP2A, MEI-S332, and the Soronin homolog Dalmatian.

Introduction

Segregation errors during meiosis I or II results in aneuploidy in the daughter cells and creates serious issues in humans including infertility, spontaneous abortion, and birth defects, such as Down Syndrome, Klinefelter Syndrome, and Turner Syndrome (ORR *et al.* 2015). In females of many species, the meiotic spindle assembles in the absence of centrosomes and is directed largely by the chromosomes. Prior studies have implicated two pathways in acentrosomal spindle assembly: the chromosomal passenger complex (CPC) and RanGTP (CARAZO-SALAS *et al.* 1999; CARAZO-SALAS *et al.* 2001; COLOMBIÉ *et al.* 2008; CESARIO AND MCKIM 2011; RADFORD *et al.* 2012b). In *Drosophila*, the CPC is required for spindle and kinetochore assembly (COLOMBIÉ *et al.* 2008; RADFORD *et al.* 2012b; RADFORD *et al.* 2015). Aurora B is the catalytic component of the CPC while three other subunits, INCENP, Survivin, and Borealin, control the localization of Aurora B (CARMENA *et al.* 2012; VAN DER HORST AND LENS 2014; TRIVEDI AND STUKENBERG 2020). Proposed targets of the CPC include the kinetochore, which is a complex of proteins associated with the centromere, and kinesin family proteins such as Subito and NCD, which contribute to assembly and organization of the meiotic spindle (JANG *et al.* 2005; BEAVEN *et al.* 2017). The CPC may alleviate negative regulation of kinesin proteins such as NCD and Subito (BEAVEN *et al.* 2017; DAS *et al.* 2018; ROME AND OHKURA 2018). It is largely unknown, however, how the formation of the meiotic acentrosomal spindle is regulated, and it is likely that a combination of positive and negative regulatory interactions drives acentrosomal spindle assembly.

While Aurora B is required for initiating spindle assembly, it is not known if Aurora B activity is required to maintain the spindle and localization of kinetochore components in female meiosis. Using an inhibitor of Aurora B activity, Binuclein 2 (BN2) (SMURNYY *et al.* 2010), we

observed the loss of all spindle microtubules in meiosis I oocytes. Thus, continuous Aurora B activity is required to preserve the spindle during meiosis I. These results also suggest that antagonism of Aurora B kinase by a phosphatase could regulate spindle dynamics. Indeed, we found that Protein Phosphatase 2A (PP2A) antagonizes Aurora B in the spindle maintenance function. PP2A is a is highly conserved heterotrimeric serine/threonine phosphatase composed of a scaffolding A subunit, a catalytic C subunit and one of multiple B subunits. In *Drosophila,* the B subunits fall into two groups of paralogs, corresponding to the B55 and B56 types in other organisms. Our results suggest both isoforms of PP2A antagonize the spindle assembly functions of Aurora B.

The two PP2A isoforms have different functions and target proteins. Drosophila PP2A-B55 has several targets many of which are phosphorylated by CDK1 implicated in regulating the progression through G2 and mitosis(RANGONE *et al.* 2011)(VON STETINA *et al.* 2008; WANG *et al.* 2011b; KIM *et al.* 2012). PP2A-B55 is involved regulating the activity of CDK1 and Polo kinases, but the motif recognized by B55 is not known. Besides a function antagonizing Aurora B, we found that in oocytes depleted of Twins*,* the *Drosophila* B55 subunit, meiotic entry and metaphase I proceeded surprisingly normal in our assays. In contrast, when the two *Drosophila* B56 isoforms, WRD and WDB, were depleted, there were dramatic defects in meiosis. These results are consistent with results in *Drosophila* cell lines showing that loss of B56 has more severe defects in the events of metaphase and anaphase than loss of B55 (CHEN *et al.* 2007).

PP2A-B56 is required for at least two functions in mitosis, the stabilization of microtubule attachments to the kinetochores (YOSHIDA *et al.* 2015; TANG *et al.* 2016), and protection of cohesion from cleavage by Separase (GUTIERREZ-CABALLERO *et al.* 2012). These functions of PP2A-B56 have also been reported to be associated with two populations of the

protein, one depends on BubR1, and the other on Shugoshin (KRUSE *et al.* 2013; XU *et al.* 2013). PP2A-B56 has been shown to protect sister chromatid cohesion in *Drosophila* mitosis and in *S. pombe* meiosis (KITAJIMA *et al.* 2006; RIEDEL *et al.* 2006). There is also evidence that PP2A-B56 regulates microtubule attachments in mouse oocyte meiosis I (YOSHIDA *et al.* 2015; TANG *et al.* 2016). However, that there are significant questions regarding how PP2A-B56 activity is regulated during the two meiotic divisions. The first division involves the segregation of homologous chromosomes rather than sister chromatids. Oocytes also have arrest points and then enter anaphase upon external signals. In *Drosophila* females, anaphase occurs upon changes in oocyte environment associated with passage through the oviduct (HEIFETZ *et al.* 2001; HORNER AND WOLFNER 2008). In mouse oocytes, PP2A-B56 regulates microtubule attachments in a way that seems tied to a timer of cell-cycle progression rather than attachment error status (YOSHIDA *et al.* 2015). Because of the two divisions and the differences between mitosis and meiosis I, the roles and regulation of PP2A-B56 in meiosis I may be different (KEATING *et al.* 2020).

Consistent with earlier studies, we found that the PP2A-B56 WRD localizes to meiotic kinetochores and this depends on both BubR1 and MEI-S332 (the *Drosophila* Shugoshin homolog) and is required for sister chromatid cohesion and stabilizing attachments to microtubules. We also found that SCP105R/KNL1, which we previously showed is required for sister centromere cohesion in meiosis (RADFORD *et al.* 2015; WANG *et al.* 2019), is required for PP2A-B56 localization. Surprisingly, MEI-S332 is not required for meiosis I cohesion. We propose that Dalmatian, the *Drosophila* orthologue of Soronin (YAMADA *et al.* 2017), has a cohesion protection function during meiosis I.

Materials and Methods

Tissue specific knockdowns using expression of transgenes and shRNAs

The UAS/Gal4 system was used for tissue-specific expression of transgenes and shRNAs. In most experiments the transgenes and shRNAs, under UAS control, were expressed using *P{w^{+mC}=matalpha4-GAL-VP16}V37* (*mataGAL4*), which induces expression after pre-meiotic DNA replication and early pachytene, and persists throughout most stages of meiotic prophase during oocyte development in *Drosophila* (NI *et al.* 2011). For expression throughout the germline, including the germline mitotic divisions and early meiotic prophase, we used *P{w^{+mC}=GAL4::VP16-nos.UTR}MVD1* (*nos-GAL4-VP16*). For ubiquitous expression, we used *P{tubP-GAL4}LL7*. The RNAi lines used in this study are listed in Table 1. We first selected RNAi lines that caused lethality when under the control of *P{tubP-GAL4}LL7* and sterile under the control of *mataGAL4.* Two RNAi lines, HSM1804 (*mts*) and HMS1921 (PP2A), were not studied in detail because they were fertile with *mataGAL4.*

The *wrd$^{\Delta}$* mutant was generated by FLP-mediated site-specific recombination that removed most of the coding region (MOAZZEN *et al.* 2009). The deletion *Df(3R)189* was made by imprecise excision of a P-element within the *wrd* gene and removes all of *wrd* and a couple genes on each side (VIQUEZ *et al.* 2006). For knockdown of *wrd* and *wdb,* mutations and shRNA were combined to generate *Df(3R)189 mataGAL4/ wrd$^{\Delta}$ HMS01864* females, or two shRNAs were combined to generate *GL00671*/+; *mataGAL4/ HMS01864* females.

Generation of shRNA lines and analysis by RT-PCR

Sequences for shRNAs targeting *wdb* and *dmt* were designed using DSIR (http://biodev.extra.cea.fr/DSIR/DSIR.html) (VERT *et al.* 2006) and the GPP Web Portal

(http://www.broadinstitute.org/rnai/public/seq/search). These were cloned into the pVallium22 vector for expression under control of the UASP promoter.

From 200 *dmt* RNAi/*mat*α females, only 6 total progeny were produced, suggesting the RNAi was effective and that Dmt is essential for fertility. In a cross of *dmt* RNAi to the ubiquitous expressing *TubGal4*, 0 *dmt* RNAi/*TubGal4* progeny were produced among 479 control progeny; consistent with previous data that Dmt is essential for viability.

To measure the amount of mRNA knockdown in oocytes, Taqman qRT-PCR was used. In order to extract RNA from oocytes of interest, female flies were placed in yeasted vials for approximately 3 days. The females were then grinded in a blender containing 1x PBS, and oocytes were filtered through meshes, as described below for cytological analysis of stage 14 oocytes. 50 mg of oocytes were weighed out, and 1mL of TRIzol Reagant was added. RNA extraction was completed, according to manufacturer's instructions (ThermoFisher Scientific). A nanodrop was used to measure the concentration of the RNA, and then 2 µg was used in to prepare cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). After using a nanodrop to determine the concentration of cDNA in the sample, qPCR was performed using a TaqMan Assay (Applied Biosystems) and four replicates per reaction.

Cytology of Stage 14 Oocytes andDrug treatment

To prepare oocytes for cytology, 0-3 day old females were placed in yeasted vials for 1-2 days as described (GILLILAND *et al.* 2009). The females were grinded in a blender with modified Robbs buffer and filtered through a series of meshes to separate the stage 14 oocytes from other body parts. It was at this stage that oocytes were treated with 50 µM BN2 in modified Robbs for one hour. The BN2 was dissolved in DMSO to make a 50mM stock solution and then 1 ul was

added to 999ul of modified Robbs. Controls oocytes were incubated for 1 hr in 1ml of modified Robbs buffer containing 1ul DMSO.

Oocytes were fixed using 37% formaldehyde and heptane and then washed with 1X PBS. Oocyte membranes were mechanically removed by rolling the oocytes between the frosted side of a glass slide and a coverslip. The rolled oocytes were then rinsed into 15mL conical tubes containing PBS/1% Triton X-100 and were rotated for 1.5 to 2 hours. Oocytes were washed in PBS/0.05% Triton X-100 and subsequently transferred to a 1.5 ml Eppendorf tube. Oocytes were then blocked in PTB for 1 hour and then incubated with primary antibodies overnight at 4 °C. The next day, the oocytes were washed 4 times in PTB and the secondary antibody was added. After incubating at room temperature for 4 hours, the oocytes were washed in PTB, stained for DNA with Hoechst33342 (10 µg/ml) and then washed 2X in PTB.

Oocytes were stained for MTs with mouse anti-α tubulin monoclonal antibody DM1A (1:50), directly conjugated to FITC (Sigma, St. Louis). Additional primary antibodies used were rat anti-Subito (JANG *et al.* 2005), rat anti-INCENP (WU *et al.* 2008), rabbit anti-phospho-INCENP (SALIMIAN *et al.* 2011; WANG *et al.* 2011a), guinea pig anti-MEI-S332 (MOORE *et al.* 1998), rabbit anti-CENP-C (HEEGER *et al.* 2005), rabbit anti-Spc105R (SCHITTENHELM *et al.* 2007), rat anti-HA (Roche) and rabbit anti-CID (Active Motif), mouse anti-C(3)G (PAGE AND HAWLEY 2001). These primary antibodies were combined with either a Cy3, Alex 594 or Cy5 secondary antibody pre-absorbed against a range of mammalian serum proteins (Jackson Immunoresearch, West Grove, PA). FISH probes corresponding to the X359 repeat labeled with Alexa 594, AACAC repeat labeled with Cy3 and the dodeca repeat labeled with Cy5 were obtained from IDT.

The oocytes were mounted in Slowfade Gold antifade (ThermoFisher) and imaged using a Leica TCS SP8 confocal microscope with a 63X, N.A. 1.4 lens. Images are shown as maximum projections of complete image stacks followed by merging of individual channels and cropping in Adobe Photoshop (PS6). Centromere foci and spindle intensity were measured using Imaris image analysis software (Bitplane). All data were plotted and statistics calculated using Graphpad Prism software.

Nondisjunction/Fertility Assay

Drosophila crosses were used to determine the rate of nondisjunction and fertility of certain genotypes. In this cross, female virgin flies with a gene of interest were mated to *y w/B*^{s}*Y* males. These males carry a mutant conveying the dominant Bar phenotype on the Y chromosome. Therefore, in normal, healthy, yw/B^{s}Y flies, all the males have Barr eyes, and all the females have wildtype eyes. Nondisjunction of the sex chromosomes during meiosis results in four different outcomes, XXY, OY, XXX, or XO. Gametes with three X chromosomes (XXX) or only one Y chromosome (OY) are inviable, while gametes with two X chromosomes and one Y chromosome (XXY), or only one X chromosome (XO) have unique phenotypes. The nondisjunction rate was calculated by (2*NDJ/(normal progeny + 2NDJ).

Quantification and statistical analysis

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

Results

Sustained Aurora B activity is required to maintain the oocyte meiotic spindle

In the *Drosophila* ovary, the nuclear envelope breaks down and spindle assembly begins in stage 13 oocytes (GILLILAND *et al.* 2009). By stage 14, a bipolar spindle has formed. The chromosomal passenger complex (CPC), through the activity of Aurora B kinase, is required for assembly of a bipolar spindle in *Drosophila* meiosis, based on the observation that no spindle forms when meiosis initiates without CPC activity due to depletion of Aurora B or the targeting subunit INCENP (RADFORD *et al.* 2012b). To test the stability of the meiotic spindle, Aurora B was inhibited after spindle assembly was completed by treating stage 14 oocytes with the drug Binucleine 2 (BN2), which inhibits the kinase activity of Aurora B (EGGERT *et al.* 2004; SMURNYY *et al.* 2010). Spindle assembly was monitored with immunofluorescence for tubulin, kinetochore protein SPC105R/KNL1, central spindle protein Subito/MKLP2 and CPC protein INCENP and Aurora B. In control experiments when wild-type females were treated with DMSO only, 100% of oocytes showed a wildtype, bipolar spindle (Figure 1A). In wild-type oocytes treated with 50 µM BN2 for one hour, the spindle was absent or faint in 85% of oocytes. Only 15% of oocytes had robust bipolar spindles (Figure 1A,B, Figure 2). These results show that maintaining the meiotic spindle depends on Aurora B activity. Because the BN2 treatment occurred after spindles were assembled, and therefore, after the initial phosphorylation of CPC substrates, these results suggest that Aurora B activity is antagonized by phosphatases.

In the absence of Aurora B, INCENP localizes to the meiotic chromosomes (RADFORD *et al.* 2012b). BN2 treatment allowed us to test if kinase activity is required for Aurora B localization. When Aurora B activity was inhibited with BN2, INCENP localized to the chromosomes (Figure 1D). We also observed Aurora B localization to the chromosomes,

showing that upon BN2 treatment, the CPC moves from the microtubules to the chromosomes. Furthermore, inhibition of phosphorylation by BN2 did not prevent Aurora B localization to the chromosomes and/ or its association with INCENP. To directly test Aurora B activity, we examined one of its targets, which is phosphorylation of INCENP (pINCENP) at a conserved serine in the C-terminal domain of INCENP, known as the Inbox (SALIMIAN *et al.* 2011; WANG *et al.* 2011a). Using an antibody for pINCENP, we found that the chromosome localized INCENP in BN2 treated oocytes was not phosphorylated, while solvent treated controls had robust pINCENP in the central spindle (Figure 2). These results show that the Aurora B phosphorylation of INCENP is not required for their interaction or chromosome localization.

The meiotic spindle has two types of microtubules – kinetochore and central spindle (JANG *et al.* 2005; RADFORD *et al.* 2015). Subito, a Kinesin 6 required for central spindle assembly (JANG *et al.* 2005), was lost upon BN2 treatment (Figure 1A), demonstrating Subito localization depends on Aurora B activity and/or microtubules. SPC105R, which is required for kinetochore microtubules (RADFORD *et al.* 2015), was also reduced, although not eliminated (Figure 1B). These results show that maintenance of the two major organizers of oocyte spindle microtubules depends on continuous Aurora B activity. SPC105R localization may require less Aurora B activity than spindle assembly or Subito localization.

PP2A Antagonizes the Spindle Assembly Function of the CPC

To explain why inhibiting CPC activity causes loss of spindle microtubules, we investigated the hypothesis that phosphatases (PP1 or PP2A) antagonize Aurora B targets. To identify the phosphatase responsible for antagonizing Aurora B, shRNA expression under the control of the UAS/Gal4 system was used for germline specific RNAi. Each shRNA was expressed using *mata4-GAL-VP16*, which induces expression after pre-meiotic DNA replication

but throughout most stages of meiotic prophase during oocyte development (RADFORD *et al.* 2012b). This allows for the oocyte to form prior to expression of the shRNA. We will refer to oocytes expressing an shRNA using *mata4-GAL-VP16* as "RNAi oocytes".

PP1-87B is the major PP1 isoform functioning in oocytes (WANG *et al.* 2019). *Pp1-87B* RNAi oocytes were treated with BN2 and spindle disintegration was measured. If PP1-87B opposes the Aurora B spindle assembly function in oocytes, then addition of BN2 after a loss of PP1-87B would rescue loss of spindle microtubules. However, *Pp1-87B* RNAi oocytes treated with BN2 had reduced or no spindle microtubules in a significant number of oocytes when compared to the *Pp1-87B* RNAi solvent treated controls (Figure S 1). In contrast, SPC105R localization and some kinetochore microtubule fibers were retained in *Pp1-87B* RNAi oocytes treated with BN2 (Figure S 1). These observations are consistent with our previous observations that loss of PP1-87B stabilizes kinetochore-microtubule attachments (WANG *et al.* 2019). In summary, PP1 has only a limited role antagonizing the Aurora B spindle assembly function, perhaps only at the kinetochores.

To test if PP2A has a role in spindle assembly, we depleted specific subunits by RNAi. PP2A exists in multiple complexes, although all the complexes contain the same A (PP2A-29B) and C (MTS = Microtubule star) subunits, the latter being the catalytic protein of the complex. Depletion of the A-subunit (*Pp2A-29B*, GLC01651) or C-subunit (*mts*, HMS04478) of all PP2A isoforms resulted in no mature stage 14 oocytes, indicating that PP2A activity is required for oocyte development. However, expression of one shRNA for *mts* (*HMJ22483*) produced stage 14 oocytes, probably due to partial depletion of MTS protein in *HMJ22483* RNAi oocytes (Table 1). When $mts^{HMJ22483}$ RNAi oocytes were treated with BN2, the spindle was retained in 100% of oocytes (Figure 2C,G). Furthermore, SPC105R and Subito localizations were retained (Figure

1C). These results suggest that PP2A antagonizes the role of the CPC in maintaining kinetochores and spindle assembly.

Both PP2A Complexes antagonize the Role of the CPC in spindle maintenance

PP2A exists in at least two complexes, differing in their B subunits Twins (TWS= B55) and Widerborst (WDB = B56) (CHEN *et al.* 2007). In order to determine which PP2A complex opposes the CPC, BN2 treatment was done in the presence of *wdb* or *tws* RNAi. An shRNA line for *tws* (*GL00670*) caused complete sterility when expressed using *mataGal4* and reduced the mRNA to 0% of wild-type levels (Table 1). In *tws* RNAi oocytes treated with BN2, oocyte spindle assembly was restored in ~80% of oocytes (Figure 2C, H). Two shRNA lines were used for *wdb*: one generated by TRiP (*HMS01864*) and one generated in our laboratory (*A5*). Both shRNAs efficiently knocked down *wdb* RNA (Table 1) and the oocytes had similar phenotypes. In both *wdb HMS* and *wdb A5* RNAi oocytes treated with BN2, the spindle was present in ~80% of oocytes (Figure 2C, D). Depletion of WDB also restored localization of spindle associated proteins and INCENP and phospho-INCENP were retained at the central spindle (Figure 2D). These results suggest that both the PP2A complexes, with WDB or TWS, antagonize Aurora B in the process of spindle assembly and maintenance.

Dominant negative alleles of PP2A suppress specific RNAi oocyte phenotypes

A dominant negative allele of *wrd* was made by deleting the first 93 amino acids of *wrd* (HANNUS *et al.* 2002). The mutant form of the protein localized similar to wild-type and expression of the mutant form in the germline did not have deleterious effects on fertility. The mutant did suppress the sterility of three RNAi lines, *Spc105R, AurB* and *CenpC,* but not *BubR1, sub* or *Incenp* RNAi oocytes (Table 2). These results are consistent with the evidence above that *PP2A-B56* activity has a negative effect on kinetochore assembly.

Kinesin 13 Klp10A is a CPC and PP2A Target

The maintenance of the meiotic spindle could depend on Aurora B inhibiting depolymerizing factors or promoting spindle assembly factors. Thus, PP2A could promote the removal of tubulin subunits from the spindle, while Aurora B could promote addition of tubulin subunits to the spindle. Therefore, we tested the hypothesis that PP2A promotes spindle dynamics. However, in *mts* RNAi oocytes, we found tubulin turnover to be unchanged from wild-type (Figure S 2). Thus, PP2A is not required for the turnover of microtubule subunits within the spindle.

Previous work has shown that the kinesin-like protein KLP10A depolymerizes spindle microtubules in oocytes (RADFORD *et al.* 2012a). In the absence of KLP10A, the spindle is very long and disorganized (Figure 3B). Furthermore, KLP10A depletion did not rescue the spindle assembly failure in CPC RNAi oocytes (RADFORD *et al.* 2012a). Thus, the CPC likely promotes initiation of spindle assembly by activating microtubule associated proteins. However, the maintenance of the spindle could depend on inhibiting depolymerizing factors. In fact, the spindle was retained in *Klp10A* RNAi oocytes treated with BN2 (Figure 3C). This suggests that KLP10A is a possible CPC and PP2A target. In contrast to BN2 treatment of wild-type and PP2A RNAi oocytes, the rescue of spindle loss in KLP10A RNAi was variable. In some oocytes, central spindle and kinetochore microtubules appeared to be intact. In other oocytes, the spindle was retained but it appeared, based on Subito or INCENP localization, that the central spindle and/or kinetochore microtubules were absent (Figure 3D). This suggests that the central spindle and kinetochores are more sensitive to loss of Aurora B kinase activity than microtubule bundles in general. While these results suggest KLP10A is inactivated by CPC phosphorylation, and activated when dephosphorylated by PP2A, there is evidence that other factors regulate KLP10A

and spindle length. If PP2A regulates KLP10A, then *PP2A* RNAi oocytes should have long spindles due to inactivation of KLP10A. The fact they do not suggests that other factors must regulate spindle length when PP2A targets are inactivated by Aurora B.

WRD Compensates for Loss of WDB in the germline

Drosophila has two B56-type paralogs, WDB and WRD. WDB and WRD share 68% identity with long stretches of identity. Null mutants of *wrd* are viable and fertile (MOAZZEN *et al.* 2009; HAHN *et al.* 2010). An shRNA targeting *wrd* (*GL00671*) was obtained and found to substantially reduce mRNA levels (Table 1). Consistent with phenotype of the mutants, females expressing *GL00671* in the germline were fertile and nondisjunction was rare (Table 2). In contrast, ubiquitous expression of *wdb* shRNA caused lethality, similar to homozygous null mutations of *wdb*. Thus, WDB appears to be the more important B56 subunit. However, other aspects of the *wdb* RNAi phenotype are milder than expected. Maternal expression of either shRNA line caused reduced fertility but the females were not sterile and meiotic nondisjunction was low (Table 2). The spindle structure of *wdb* RNAi oocytes was similar to wild-type; they were bipolar, and assembled a robust central spindle (Figure 4A). Furthermore, the kinetochores appeared to be making contact with microtubules and orienting towards a pole, suggesting they were making end-on MT-KT attachments. Similar observations were made with *tws* RNAi oocytes. These data suggest that TWS and WDB are not essential for bipolar spindle assembly.

To explain the relatively weak maternal phenotypes of *wrd* RNAi oocytes, we tested the possibility that WRD partially compensates for loss of WDB. To test for redundancy, we generated oocytes lacking both WDB and WRD. Two genotypes were generated to reduce expression of both genes: females expressing shRNA to both *wdb* and *wrd* (wrd^{GL}; wdb^{HMS}), and females expressing *wdb* shRNA and hemizygous for a *wrd* mutation; (wrd^{-} ; wdb^{HMS}). When

using *mata4-GAL-VP16* for expression of the shRNA, the double knockdown females were completely sterile, unlike the single RNAi females. In addition, when using *nos-GAL4-VP16,* which promotes expression of the shRNA in premeiotic germ cells, no oocytes were produced, also unlike the single RNAi females. These results demonstrate that WRD and WDB are partially redundant in the germline and expression of WRD is sufficient for fertility. In contrast, expression of WRD is not sufficient for viability, WDB is required.

PP2A Protects Sister Centromere and Arm Cohesion and promotes end-on microtubule attachments

Drosophila oocytes arrest in metaphase I in their developmental stage 14, and do not proceed into anaphase I and meiosis II until activated by passage through the oviduct (HEIFETZ *et al.* 2001). Stage 14 oocytes can also be induced to proceed past metaphase I by incubation in certain buffers (ENDOW AND KOMMA 1997; PAGE AND ORR-WEAVER 1997). Our methods use a modified Robbs' buffer to avoid activation (THEURKAUF AND HAWLEY 1992). Indeed, most wild-type and *wdb* RNAi oocytes were arrested in metaphase I (Figure 4A, B). Furthermore, even after control oocytes were incubated for one hour in modified Robbs, metaphase I arrest was usually maintained (Figure 4B, C). We were surprised, therefore, to find that the one-hour incubation in modified Robbs buffer with BN2 treatment induced precocious anaphase in *wdb* RNAi oocytes (Figure 2I). To investigate if this was an effect of the BN2 treatment, *wdb HMS* and *A5* RNAi oocytes were incubated in modified Robbs buffer for one hour without BN2. In these conditions, precocious separation of homologous chromosomes was observed in 50% of oocytes (Figure 4B, C). These phenotypes were present in *wdb* RNAi oocytes but not in *tws* RNAi oocytes. These data suggest that 1 hour incubation in modified Robbs buffer induces *wrd*

RNAi oocytes to lose their arm cohesion and precociously enter anaphase 1. This result can be explained if PP2A protects cohesion on the chromosome arms.

To determine if cohesion of the centromeres was affected, we counted the number of CID or CENP-C foci in the oocytes of each genotype. Each centromere detected by immunofluorescence during wild-type meiosis I contains two sister centromeres fused in a process that requires cohesion (WANG *et al.* 2019). Thus, in wild-type oocytes we observed approximately eight centromere foci, as expected from four bivalents at metaphase I. In *wdb* RNAi oocytes, there was an increase in the number of oocytes with greater than 8 centromere foci, indicating that the sister chromatids were separating prematurely (Figure 4D). In fact, 45% of *wdb* RNAi (*HMS* and A5) oocytes had >8 centromere foci, compared to 14% in wildtype (Figure 4D). This suggests that the sister centromeres are precociously separating, possibly due to loss of cohesion.

The sister centromere separation and precious anaphase phenotypes of *wdb* RNAi oocytes were relatively mild comnpared to other cohesion-defective mutants (GYURICZA *et al.* 2016; WANG *et al.* 2019). However, the severity of the spindle phenotypes was dramatically increased in both wrd^{GL}; wdb^{HMS} and wrd^{-} ; wdb^{HMS} oocytes. For example, the precocious anaphase phenotype of chromosomes moving towards the poles was observed without a one-hour incubation in Robbs buffer. Similarly, the number of centromere foci was increased in wrd^{GL}; wdb^{HMS} and wrd^{-} ; wdb^{HMS} oocytes (Figure 5B, C). These results can be explained if PP2A is required for the protection of cohesion during meiosis I. We also observed that wrd^{GL}; wdb^{HMS} or wrd^{-} ; wdb^{HMS} oocytes failed to form end on attachments of the microtubules to the kinetochores. Instead, lateral microtubule attachments were frequently observed (Figure 5,

Figure S 3). Interestingly, chromosomes can still move towards the poles in the absence of end-on attachments.

We previously found that two mechanisms can cause precocious sister centromere separation: loss of cohesion or inappropriate stabilization of kinetochore-microtubule (KT-MT) attachments (WANG *et al.* 2019). The inappropriate stabilization of KT-MT attachments depends on NDC80 and end on KT-MT attachments. To test the role of KT-MT attachments in the wrd^{GL}; wdb^{HMS} or wrd^{-} ; wdb^{HMS} phenotype, we examined wrd^{-} ; wdb^{HMS}; *Ndc80* RNAi oocytes. In *Ndc80* RNAi oocytes, end-on microtubule attachments are absent. The wrd^{-} ; wdb^{HMS}; *Ndc80* RNAi oocytes oocytes exhibited both precocious anaphase and centromere separation, suggesting stabilization of end-on KT-MT attachments was not required for the wrd^{-} ; wdb^{HMS} phenotype. Importantly, we previously showed that end-on attachments were capable of separating sister centromeres in the presence of intact cohesins (WANG *et al.* 2019). Therefore, observing centromere and homolog separation in the absence of end-on attachments is consistent with loss of cohesion in wrd^{-} ; wdb^{HMS} oocytes. Indeed, *Ndc80* RNAi may have little effect on wrd^{-} ; wdb^{HMS} oocytes because PP2A-B56 is required for stabilizing the end-on attachments facilitated by NDC80.

The role of PP2A in bi-orientation

To examine the effects of *PP2A* depletion on chromosome alignment and bi-orientation, we used fluorescent in situ hybridization (FISH). FISH probes were used that detected the pericentromeric regions of all three major chromosomes: the AACAC repeat (2nd chromosome), the dodeca repeat (third chromosome) and the 359 repeat (X chromosome). In wild-type oocytes, correct bi-orientation is indicated by each pair of homologous centromeres separating towards opposite poles (Figure 6A). A low frequency of bi-orientation defects was observed in *tws* and

mts RNAi oocytes (Figure 6B-C, G). These results suggest the TWS/ B55 subunit is not required for making the correct microtubule attachments during prometaphase I. Furthermore, these results suggest that the failure to antagonize Aurora B activity, resulting in spindles that are resistant to BN2 activity, does not have deleterious effects on homolog bi-orientation.

The low levels of bi-orientation defects in the *A5* and *HMS* RNAi oocytes was also low, which could have been due to redundancy with *wrd.* Indeed, oocytes depleted for both *wrd* and *wdb* had a much higher frequency of bi-orientation defects (Figure 6E-G). Furthermore, we confirmed the conclusion, based on chromosome movement towards the poles, that many stage 14 oocytes had entered a precocious anaphase. In wild-type oocytes, pairs of homologous centromeres are separated but remain connected by chiasma and therefore within the same chromatin mass (Figure 6A). In oocytes depleted for both *wrd* and *wdb*, pairs of homologous centromeres had separated towards opposite poles (Figure 6E), indicating precocious loss of cohesion on the chromosome arms.

FISH was used to test if the *A5* and *HMS* RNAi oocytes incubated for one hour had a high frequency of precocious anaphase I. That is, in many of the *A5* and *HMS* RNAi oocytes incubated for one hour, the chromosomes had separated into at least two masses that had moved towards a pole. In these oocytes, the two probe signals for each homolog were usually in different chromatin masses, showing that arm cohesion had been released, allowing the chromosomes to move towards the poles. This was associated with a low frequency of bi-orientation defects (Figure 6D).

Spc105R Is Required for PP2A Localization

Our results show PP2A-B56 (WRD and WDB) has three roles in female meiosis, to antagonize Aurora B kinase in spindle assembly, protect cohesion during meiosis I, and promote

end-on microtubule attachments. Because the function of kinases and phosphatases often depends on their localization, we examined the localization of WDB using either antibodies (SATHYANARAYANAN *et al.* 2004; PINTO AND ORR-WEAVER 2017) or an HA-tagged transgene (HANNUS *et al.* 2002). In wild-type oocytes, we found that WDB localizes prominently to the centromere regions (Figure 7A). In many oocytes, it was also possible to observe weaker localization to the chromosome arms (Figure 7B). Surprisingly, WDB protein was still detected at the centromeres in *wdb* RNAi oocytes (Figure 7E, Figure S 4C). Thus, the RNAi experiments reduced but did not eliminate WDB expression.

The strongest WDB accumulation colocalizes with markers like CID/CENP-A or CENP-C, suggesting it is enriched in the centromere/ kinetochore regions. Therefore, we tested if WDB localization depends on SPC105R, which is a kinetochore protein previously shown to be required for sister centromere cohesion in meiosis I (RADFORD *et al.* 2015; WANG *et al.* 2019). *Spc105R* RNAi oocytes not only display a separated centromere phenotype (WANG *et al.* 2019), they also lacked WDB localization (Figure 7C). These results show that Spc105R is required for the recruitment of WDB. Cohesion itself, however, is not required for WDB localization as shown by the presence of WDB at the centromeres in *sunn* mutant oocytes (Figure 7D). SUNN is a stromalin-related protein required for sister chromatid cohesion in meiosis (KRISHNAN *et al.* 2014).

Evidence for redundant mechanisms to recruit PP2A

In human cell lines, PP2A is recruited to the centromere regions by BubR1 (KRUSE *et al.* 2013; XU *et al.* 2013) and Shugoshin (KITAJIMA *et al.* 2006; RIEDEL *et al.* 2006; TANG *et al.* 2006). Consistent with these prior studies, in *BubR1* RNAi oocytes WDB was absent from the centromeres (Figure S 4F). This was a surprising result, however, because we have previously

shown that *BubR1* RNAi oocytes do not have a cohesion defect nor do they have a precocious anaphase phenotype (WANG *et al.* 2019). Similarly, WDB localization was essentially eliminated in *mei-S332* mutant oocytes, and also reduced in *mei-S332/+* heterozygotes (Figure 7F, Figure S 4D,E). *Drosophila mei-S332* mutants, the first Shugoshin to be discovered, are viable, suggesting MEI-S332 is not required for cohesion in mitosis. Furthermore, chromosome segregation errors in *mei-S332* mutants primarily involve sister chromatids during meiosis II (KERREBROCK *et al.* 1995; TANG *et al.* 1998). Indeed, while MEI-S332 localizes to the centromere regions during meiosis I, the sister centromeres remain fused in *mei-S332* mutant oocytes (see below) (KERREBROCK *et al.* 1995; MOORE *et al.* 1998). Therefore, while both BubR1 and MEI-S332 are required to recruit WDB to the meiotic centromeres, unlike oocytes lacking PP2A, the absence of BubR1 or MEI-S332 does not cause a meiosis I cohesion defect.

Dalmatian May Protect Cohesion During Meiosis I

To explain the lack of a meiosis I defect in *mei-S332* mutants, we tested the hypothesis that another protein could recruit PP2A to the centromeres. A candidate for this is Dalmatian (DMT), which has homology with Soronin but has also been proposed to recruit PP2A in *Drosophila* mitotic cells (YAMADA *et al.* 2017). Consistent with this hypothesis, DMT colocalizes with MEI-S332 at the centromeres in metaphase I oocytes (Figure 8A). Mutations of *dmt* cause lethality (SALZBERG *et al.* 1994) and germ line clones fail to generate oocytes (Methods). To test the function of DMT in meiosis, we created shRNA lines to target *dmt* for tissue-specific RNAi. These shRNA lines caused sterility when expressed in oocytes and lethality when expressed ubiquitously (Table 1), suggesting the RNAi was effective. The strongest of these shRNAs reduced mRNA levels to 5% of wild-type levels. However, in these *dmt* RNAi oocytes, there was no centromere separation defects to indicate a loss of cohesion

(Figure 8C,F). One explanation for the absence of a defect in *dmt* RNAi oocytes could be redundancy with MEI-S332. Therefore, we constructed *mei-S332* mutant females expressing *dmt* RNAi using *matαGal4*. These females, however, also had no centromere separation defects during meiosis I (Figure 8D-F).

In *dmt* RNAi oocytes, DMT localization to centromeres was still observed (Figure 8C) and could explain the lack of a meiosis I phenotype. To investigate the persistence of DMT on the chromosomes in the RNAi oocytes, we determined if DMT is loaded on the chromosomes prior to the domain of shRNA expression by *matαGal4*, and is stable enough to remain on the chromosomes until metaphase I. Indeed, we found that DMT localized to the meiotic chromosomes in the germarium, which contains oocytes early in prophase undergoing pachytene (Figure 8G-I). In contrast, we did not observe localization of WDB at these stages (Figure S 5).

The stable persistence of DMT throughout prophase could explain why *dmt* RNAi oocytes did not exhibit abnormalities in meiosis. Proteins stably maintained should be insensitive to RNAi applied after early prophase/ pachytene, which is what *matαGal4* does. They should be sensitive to RNAi applied during S-phase and early pachytene, which is what *nosGal4* does. Cohesin components are an example of proteins loaded onto the meiotic chromosomes in pre-meiotic S-phase (GYURICZA *et al.* 2016). Consistent with this timing, nondisjunction was observed when *nosGal4* was used to induce expression of shRNAs for cohesins *ord* (32%, n=1270) and *sunn* (15.6%, n=486). In contrast, nondisjunction was not observed with *matαGal4* and the same shRNAs for *ord* (0%, n=1622) or *sunn* (0%, n=547). These results can be explained if cohesins are stably maintained without transcription throughout most of prophase. In contrast, expressing an shRNA to *mei-S332* with either *nosGal4* (40%, n=331) or *matαGal4* (27.8%, n=208) caused high levels of nondisjunction. These results show that *mei-S332* expression is

required late in prophase or during pro-metaphase. To test the hypothesis that MEI-S332 and DMT both recruit PP2A during meiosis I, we expressed *dmt* RNAi using *nosGal4* in *mei-S332* mutant females. In these females, very few oocytes were produced, which can be explained if there is redundancy with DMT and MEI-S33 in the mitotic germline divisions where *nosGal4* also expressed. These data are consistent with the conclusion that *Drosophila* oocytes load cohesins and the protector of cohesin DMT during pre-meiotic S-phase.

Discussion

Oocyte spindle assembly depends on the CPC. The evidence for this has been observed with RNAi oocytes (RADFORD *et al.* 2012b) and, as shown here, by inhibiting Aurora B with Binuclein 2. In both types of experiment, total loss of the meiotic spindle is observed. This study was initiated to identify the phosphatase that antagonizes Aurora B, which we determined to be PP2A. In the process of this analysis, we also investigated the role of PP2A in oocyte meiosis. Depletions of both PP2A subtypes, B55 (e.g. TWS) and B56 (e.g. WDB), suppressed BN2-induced spindle loss. These two complexes are usually targeted to different substrates. These results can be explained if PP2A-B55 depletion reduces the activity of PP2A-B56. This is plausible because B56 activity may depend on Polo kinase, which in turn may be regulated by B55 (RANGONE *et al.* 2011; WANG *et al.* 2011b). Alternatively, PP2A-B55 may directly regulate Aurora B activity via CDK1 (HUMMER AND MAYER 2009; KITAGAWA *et al.* 2014).

Initiation and maintenance of the meiotic spindle

Knockdowns of PP2A components suppressed the BN2-induced loss of the meiotic spindle. The implication is that constant Aurora B activity is required to maintain the spindle, and in its absence, PP2A removes key phosphorylation events on meiotic spindle proteins. This was shown using phosphorylated INCENP, which is a target of Aurora B. Based on indirect evidence, kinetochore protein SPC105R and kinesins KLP10A and Subito also depend on this balance of phosphorylation. In several organisms, Aurora B mediated phosphorylation of DSN1 is required for kinetochore assembly, but Drosophila does not have a DSN1 homolog (PRZEWLOKA *et al.* 2009).

Less well known is the role of PP2A in regulating spindle assembly factors. In Xenopus extracts, the failure of microtubule stabilization in CPC depleted extracts was rescued by loss of

Kinein 13 MCAK, indicating that the CPC negatively regulates spindle depolymerizing factors (SAMPATH *et al.* 2004). However, we have shown that depletion of CPC components by RNAi results in loss of spindle assembly, and this is not suppressed by simultaneous knockdown of Kinesin 13 KLP10A (RADFORD *et al.* 2012a; RADFORD *et al.* 2012b). The different effects of KLP10A knockdown on *aurB* RNAi and BN2 inhibition is most likely is the timing of CPC depletion. In RNAi oocytes, factors required for the initiation of spindle assembly are affected. In contrast, a drug treatment like BN2 affects only maintenance factors, which may include regulation suppressing kinesin depolymerizing activities. A similar finding was made in PP2A promotes the activity of Stathmin by removing inhibitory phosphorylation (TOURNEBIZE *et al.* 1997).

The Drosophila oocyte meiotic spindle is a dynamic structure with constant turnover of tubulin subunits (ENDOW AND KOMMA 1997). However, we did not observe a decrease in tubulin dynamics in PP2A depleted oocytes, suggesting that spindle dynamics are not regulated by PP2A. Instead, we suggest that spindle length is regulated by PP2A-B56. This was most readily observed with the long and disorganized spindles when both B56 subunits were knocked down.

In addition to regulating spindle length, PP2A-B56 probably regulates factors which maintain spindle integrity. Subito localization depends on the CPC and, along with the kinesin NCD, may depend on high levels of Aurora B near the chromosomes to be activated (BEAVEN *et al.* 2017; DAS *et al.* 2018). Interestingly, Subito contains only one predicted PP2A-B56 binding motif (FDNIQESEE) (HERTZ *et al.* 2016), and it is in a region we proposed negatively regulates Subito activity (DAS *et al.* 2018). Overall, it is likely that several spindle assembly factors are

regulated by antagonism between Aurora B and PP2A. This is probably a conserved activity, as PP2A has been proposed to oppose Aurora B activity in the central spindle (BASTOS *et al.* 2014).

Regulation of cohesion and microtubule attachments

In addition to the role of antagonizing Aurora B in spindle maintenance, our analysis of PP2A-B56 mutant phenotypes has revealed its critical roles in regulating key meiosis I events. First, when both B56 subunits, WDB and WRD, were knocked out, a severe loss of cohesion was observed. This is consistent with a reduction in WDB localization in a *mei-S332* (Shugoshin) mutant. Shugoshin can maintain cohesion by recruiting PP2A and removing enabling dephosphorylation of cohesin subunits, preventing Separase from cleaving the Kleisin subunit (GUTIERREZ-CABALLERO *et al.* 2012).

There is a striking difference between the phenotype from loss of PP2A-B56 and MEI-S332, even though the latter is required for its localization. The lack of a meiosis I cohesion defect combined with the absence of a phenotype when kinetochore localization of WDB is reduced can be explained if there is a MEI-S332-indepndent mechanism to recruit PP2A-B56. In fact, the Soronin homolog Dalmatian (DMT), and not MEI-S332, is required for mitotic cohesion (YAMADA *et al.* 2017). Importantly, DMT has sequence features, including the LxxIxE motif, that suggest it recruits PP2A-B56 (HERTZ AND NILSSON 2017). We generated a *dmt* RNAi that efficiently depleted the mRNA. However, no loss of cohesion phenotype was observed. A possible explanation for this phenomenon is that DMT, like the other cohesins required for cohesion, is a stable protein that is only loaded onto chromosomes during pre-meiotic S-phase (GYURICZA *et al.* 2016). Indeed, we observed DMT localization, but not WDB, early in prophase. Cohesion in oocytes, therefore, may be enforced by two redundant mechanisms. One is established during S-phase and ensures protection during the long oocyte prophase, and the

other, based on MEI-S332/SGO, is established late, once the nuclear envelope breaks down and the meiotic divisions begin.

In addition to cohesion loss when both B56 subunits, WDB and WRD, were knocked out, we observed loss of end-on attachments and bi-orientation defects. The bi-orientation defects are likely a consequence of the attachment defects. BubR1 is required for PP2A localization and through this mechanism has been shown to stabilize KT-MT attachments (FOLEY *et al.* 2011; SUIJKERBUIJK *et al.* 2012; KRUSE *et al.* 2013; XU *et al.* 2013). Similarly, in Drosophila oocytes, we have previously shown that BubR1 and Polo stabilize KT-MT attachments (WANG *et al.* 2019). Thus, PP2A activity has two important functions at the centromeres or kinetochores: protecting cohesion and stabilizing KT-MT attachments. The dramatic disorganization of chromosomes and spindle, and precocious entry into anaphase, in B56 depleted oocytes is a consequence of these two defects.

PP2A localization depends on BubR1. However, the PP2A-B56 loss of function phenotypes are much stronger than BUBR1 loss of function. We propose two reasons for this difference. First, there are at least three pools of PP2A-B56. The first is the kinetochore pool, based on the localization of WDB that depends on BUBR1 and MEI-S332. Based on the mild phenotype of BUBR1 RNAi or *mei-S332* mutant oocytes, this pool is not essential for cohesion. Kinetochore localization of WDB is also not essential for spindle instability, because Spc105R RNAi is still sensitive to BN2 (WANG *et al.* 2019). The second pool is the one that regulates spindle integrity and length. This pool may be localized to the spindle and difficult to detect. The third pool is represented by the weak arm staining observed in many of our oocyte images. The weak signal is probably the reason we do not observe it in every oocyte. We do not know if this pool depends on BUBR1 or MEI-S332 (or depends on another factor like Dalmatian). We

propose that the two pools of PP2A that regulate cohesion and KT-MT attachments both depend on SPC105R. It is striking that one protein may provide the spatial separation to allow these two pools of PP2A to function independently.

Table 1: Summary of RNAi lines.

Genotypes	% of *WT* mRNA
Mts; HMJ22483	14
wdb: HMS01864	5
wdb: wdbA5	15
tws: GL00670	0 [a]
wrd: GL00671	5
Spc105R:GL00392	13 [b]
Ndc80 GL00625	6 [b]
BubR1: GL00236	ND
dmt: dmt270	10
dmt: dmt305	7

a = (SAPKOTA *et al.* 2018)

b = (RADFORD *et al.* 2015)

Table 2: Fertility of *PP2A* RNAi females

Genotype	Number of Progeny	Number of Vials	Average Progeny per Vial
mts HMJ22483/ matα	0 [a]	20	0
mts HMS04478/ matα	0 [b]	10	0
wdb HMS/matα	257	10	25.7
wrd GL/matα	1440	20	72
wdb A5/matα	216	33	7
wdb HMS/nos	1044	10	104
wdb A5/nos	533	10	53
wrd-; wdb HMS/matα	4	14	0.3
wrd GL; wdb HMS/ matα	0 [a]	15	0
wrd GL; wdb HMS/nos	0 [b]	15	0
dmt 270/ nos	381	9	42
dmt 305/nos	0 [a]	30	0
dmt 270/ matα	0 [a]	20	0
dmt 305/ matα	0 [a]	20	0

a: Stage 14 oocytes present

b: No Stage 14 oocytes

Figure 1: Constant Aurora B activity is required or maintaining spindle microtubules and SPC105R localization in oocytes. (A) Wild type oocytes treated with either 0.001% DMSO or 50µM BN2. DNA is shown in blue, Subito in red and tubulin in green. All images are maximum projections of Z-stack and scale bars are 5 µm. (B) Localization of kinetochore protein SPC105R (white) was reduced in WT oocytes treated with BN2 (72% faint and reduced localization, n=36) compared to controls (0% faint, n=8). (C) SPC105R localization was retained in *mts*^{HMJ22483} RNAi oocytes treated with BN2 relative to WT oocytes treated with BN2 (16% weak, n=19, P<0.0001, Fisher's exact test). D) Wild type oocytes treated with either 0.001% DMSO or 50µM BN2 immunostained with DNA (blue), INCENP (red), tubulin (green) and Aurora B (white).

Figure 2: PP2A antagonizes Aurora B activity. Oocytes were treated with (A) 0.001% DMSO had pINCENP (n=19/20) while (B) oocytes treated with 50µM BN2 had pINCENP at a reduced frequency (6/15, p=0.007). The BN2 treated oocytes with pINCENP tended to have some residual spindle assembly. Phosphorylated INCENP (pINCENP) is shown in red, INCENP in white, tubulin in green and DNA in blue. (C) Graphs showing the qualitative and quantitative assessment of spindle assembly in WT+DMSO (n=11), WT + BN2 (n=84), *mts* (n=39), *tws* (n=25), *wdb A5* (n=30) and *wdb HMS* (n=50) oocytes treated with BN2. (D) Retention of pINCENP when *wdb* RNAi oocytes were treated with 50µM BN2 (n=15). (E -I) Wild-type and RNAi oocytes treated with 50µM BN2**.** All were stained with Incenp, CID, Tubulin, and Hoechst (DNA). Tubulin staining is shown in the right panel. Scale bars are 5µm.

Figure 3: Aurora B antagonizes KLP10A. (A) Wildtype oocytes treated with BN2. (B) Untreated *klp10A* RNAi oocytes showing characteristic long spindle phenotype. (C) Most wild-

type oocytes treated with BN2 failed to form a spindle (16/20). *Klp10A RNAi* oocytes treated with BN2 mostly developed a long spindle (39 out of 45, $p<0.0001$). (D) In some cases, the spindle in *Klp10A RNAi* oocytes treated with BN2 was detached from the chromosomes, as if the kinetochore attachments were destabilized. The Images are shown with CID/CENP-A in white, Tubulin in green, Subito in red, and DNA in blue. Tubulin channel shown in bottom panel. Scale bars are 5µm.

Figure 4: Spindle assembly in PP2A RNAi oocytes. (A) Wildtype or wdb^{A5} RNAi oocytes with INCENP in red, CID/CENP-A in white, Tubulin in green and DNA in blue. The bottom panel shows CID staining. The Scale bars are 5µm. (B) Percent karyosome phenotype for each genotype. (control n=51; *wdb HMS* n=30; *wdb-A5* n=41; *control 1 hr* n=32. *wdbA5* 1 hr n=35; *wdbHMS* 1 hr n=25). Precocious anaphase is indicated by the separation of chromosomes towards the poles. (C) Wildtype or wdb^{HMS} oocytes incubated in Robbs buffer for 1 hour without BN2. Bottom panel shows the DNA channel. (D) Plot showing the number of centromere foci for each genotype (control n=51; *tws* n=32; *mts* n=50; *wdbA5* n=39; *wdbHMS* n=43).

Figure 5: Precious anaphase in PP2A RNAi oocytes. (A) Metaphase I oocytes in single (*wrd GL* and *wdb HMS*) and double knockdown (w*rd GL*; w*db HMS* and wrd^{Δ} *wrd HMS*) oocytes, with INCENP in red, CID/CENP-A in white, Tubulin in green and DNA in blue. CID and DNA channels are shown below. The number of centromere foci is indicated in the CID channel. Scale bars are 5µm. (B) Number of centromere foci in *wrd GL* (n=13), *wdb HMS* (n=43), *wrd GL*; w*db HMS* (n=33), wrd^{Δ} *wrd HMS* (n=28) and *Ndc80;* wrd^{Δ} *wrd HMS* (n=10) oocytes. (C) Percent karyosome phenotype for each genotype.

Figure 6: Analysis of bi-orientation in PP2A RNAi oocytes. FISH was performed with probes for the three major chromosomes in wild-type (A) and RNAi oocytes of (B) *tws*, (C) *wdbHMS*, (D) *wdb HMS* incubated for 1 hour, (E) *wrd* wrd^{Δ} *wdb HMS,* and (F) *wrd GL wdb HMS*. The paracentric FISH probes were for the X chromosome (359bp repeat, Alexa594, purple), the 2nd chromosome (HET, Cy3, red) and the 3rd chromosome (Dodeca, Cy5, white). An example of one FISH probe is shown in the lower panels (chromosomes 2 or 3). Examples of mono-orientation are shown in D and F. Also shown is tubulin in green, DNA in blue and the scale bars are 5µm. (G) Relative frequency of mono-oriented and bi-oriented centromeres in wild-type (n=117) and RNAi oocytes of *mts* (n=60), *tws* (n=72), *wdb A5* (n=61), *wdbHMS* (n=66), *wrd GL wdb HMS* (n=51F), wrd^{Δ} *wdb HMS* (n=73), *wdb HMS* incubated for 1 hour (D), wrd^{Δ} *wdb HMS* (E), wild-type incubated for 1 hour (n=45), *wdb A5* incubated for 1 hour (n=98) and *wdbHMS* incubated for 1 hour (n=87).

Figure 7: Localization of WDB in metaphase I of meiosis. Stage 14 oocytes showing WDB (red), CID/CENP-C (white), Tubulin (green), DNA (blue). The single channel images show WDB. Images show wild-type control (A,B), RNAi (C,E) or homozygous mutant (D,F) oocytes. WDB was detected using a polyclonal antibody (SATHYANARAYANAN *et al.* 2004; PINTO AND ORR-WEAVER 2017) (A-E) F-H). The WDB channel in panel G is a higher magnification image to show threads of WDB signal, which is believed to be protein on the chromosome arms spanning the distance between the bi-oriented centromeres (arrow). Scale bars are 5µm.

Figure 8: Localization of cohesion protection/ PP2A recruitment proteins in meiosis. (A) Wild-type oocyte showing DMT in red, MEI-S332 in white, tubulin in green and DNA in blue. Also shown are greyscale images of the DMT, MEI-SS332 and tubulin channels. Scale bar is are 5µm. (B,C) Wildtype and *dmt* RNAi oocytes showing with DMT in red, CID in white, tubulin in green and DNA in blue. DMT is shown in the bottom panel and scale bar is 5µm. (D-E) Phenotype of stage 14 *dmt* RNAi and *mei-S332* mutant oocytes, with INCENP (red), CID (white), Tubulin (green) and DNA (blue). (F) Summary of centromere foci in *dmt* RNAi (n=15), *mei-S332* mutant (n=23) or *dmt mei-S332* (n=21) oocytes. There are no significant differences between the three data sets by one-way ANOVA tests. However, *dmt mei-S332* is significantly different than *mei-S332* by a t-test (P=0.013). (G) DMT localization (red) in early prophase/pachytene oocytes. Oocytes are marked by C(3)G (green) along with DNA (blue). (H) DMT localization where the shRNA is expressed at a later stage of prophase than the oocyte in panel using *mataGAL4.* DMT localization is visible in these late stage pachytene oocytes (H) and after pachytene ends (I).

Figure S 1: BN2 treatment in PP1. (A) Wild type (WT) and *Pp1-87B* RNAi oocytes treated with either 0.001% DMSO or 25µM BN2 immunostained with DNA(blue), tubulin (green), pINCENP (red) and INCENP (white). All images are maximum projections of Z-stack and scale bars are 5 µm. (B) Frequency of spindle loss in WT or *Pp1-87B* RNAi oocytes, treated with DMSO or BN2. Error bars show SEM of each category. ***=P<0.001 (Fisher's exact test). (C and D) Kinetochore (SPC105R) localization (red) in *Pp1-87B* RNAi oocytes treated with either DMSO or BN2 (n= 10 and 10). In the merged image, SPC105R is shown in red along with DNA (blue) and tubulin (green).

Figure S 2: FRAP analysis comparing the recovering time of wild-type and *mts* RNAi oocytes expressing *GFPS65C-alpha-Tub84B*.

Figure S 3: Examples end-on attachments in wild-type (A) and lateral attachments (B-D) in *wrd wdb* double knockout oocytes. Stage 14 oocytes are shown with DNA in blue, tubulin in green, CENP-C in white and either WDB (A) or INCENP (B-D) in red, and. All images are maximum projections of Z-stack and scale bars are 5 µm.

Figure S 4: Additional images of WDB localization using an HA-tagged transgene (HANNUS *et al.* 2002). Images show DNA in blue, tubulin in green, HA-WDB in red, and CENP-C in white. Arrow in panel B shows a thread of WDB between the chromosomes. All images are maximum projections of Z-stack and scale bars are 5 µm.

Figure S 5: WDB localization is not observed in meiotic prophase. WDB-HA (red) is not concentrated at the centromeres (white – CENP-C) in early prophase (A – the germarium) and

mid-prophase (B,C – vitellarium oocytes). ORB (green) is a cytoplasmic protein that is enriched in the oocytes (LANTZ *et al.* 1994). Scale bar is 5 µm.

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Figure 1

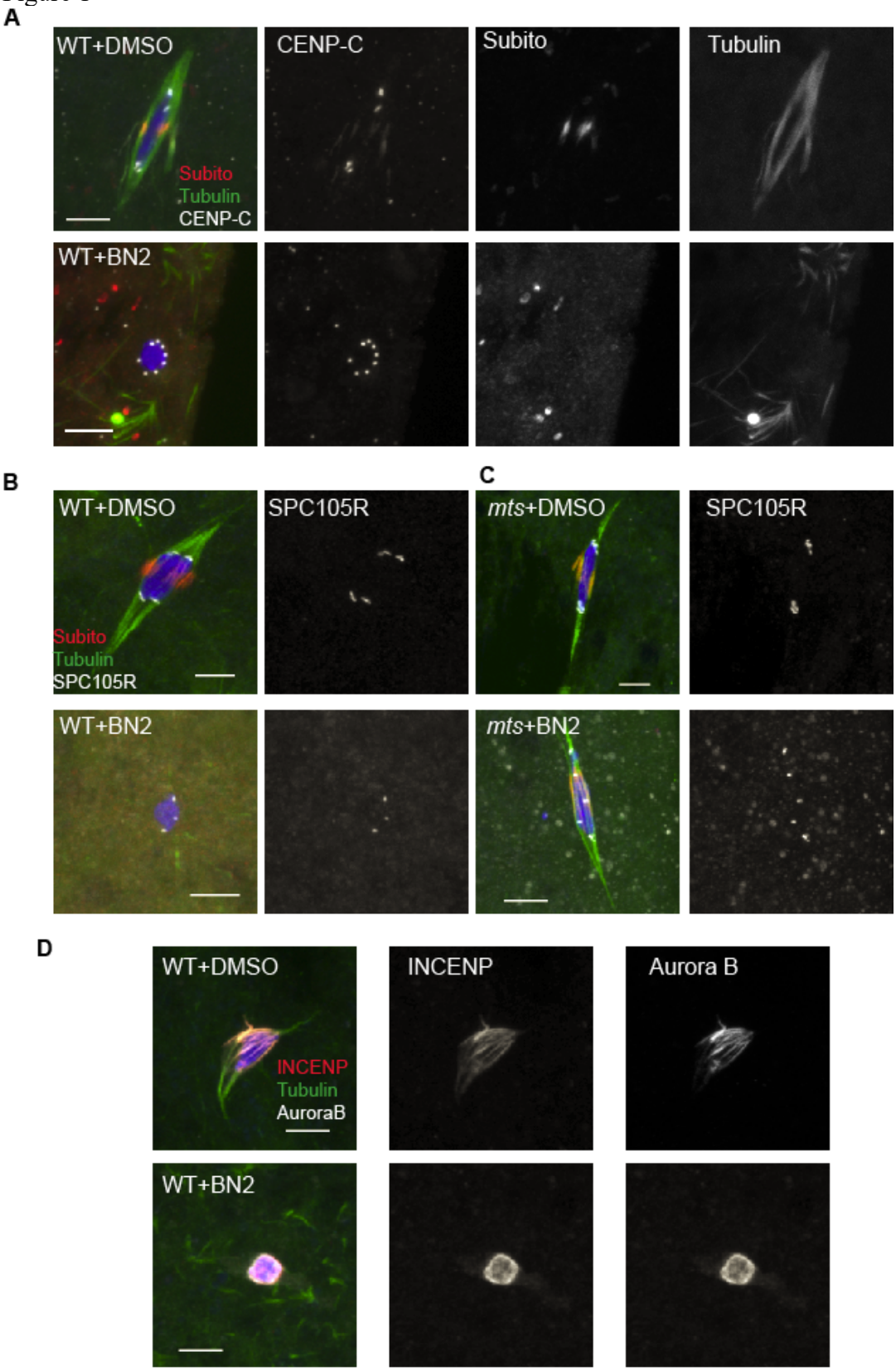

A
WT+DMSO
Subito
Tubulin
CENP-C
CENP-C
Subito
Tubulin
WT+BN2
B
WT+DMSO
Subito
Tubulin
SPC105R
SPC105R
C
mts+DMSO
SPC105R
WT+BN2
mts+BN2
D
WT+DMSO
INCENP
Tubulin
AuroraB
INCENP
Aurora B
WT+BN2

Figure 2

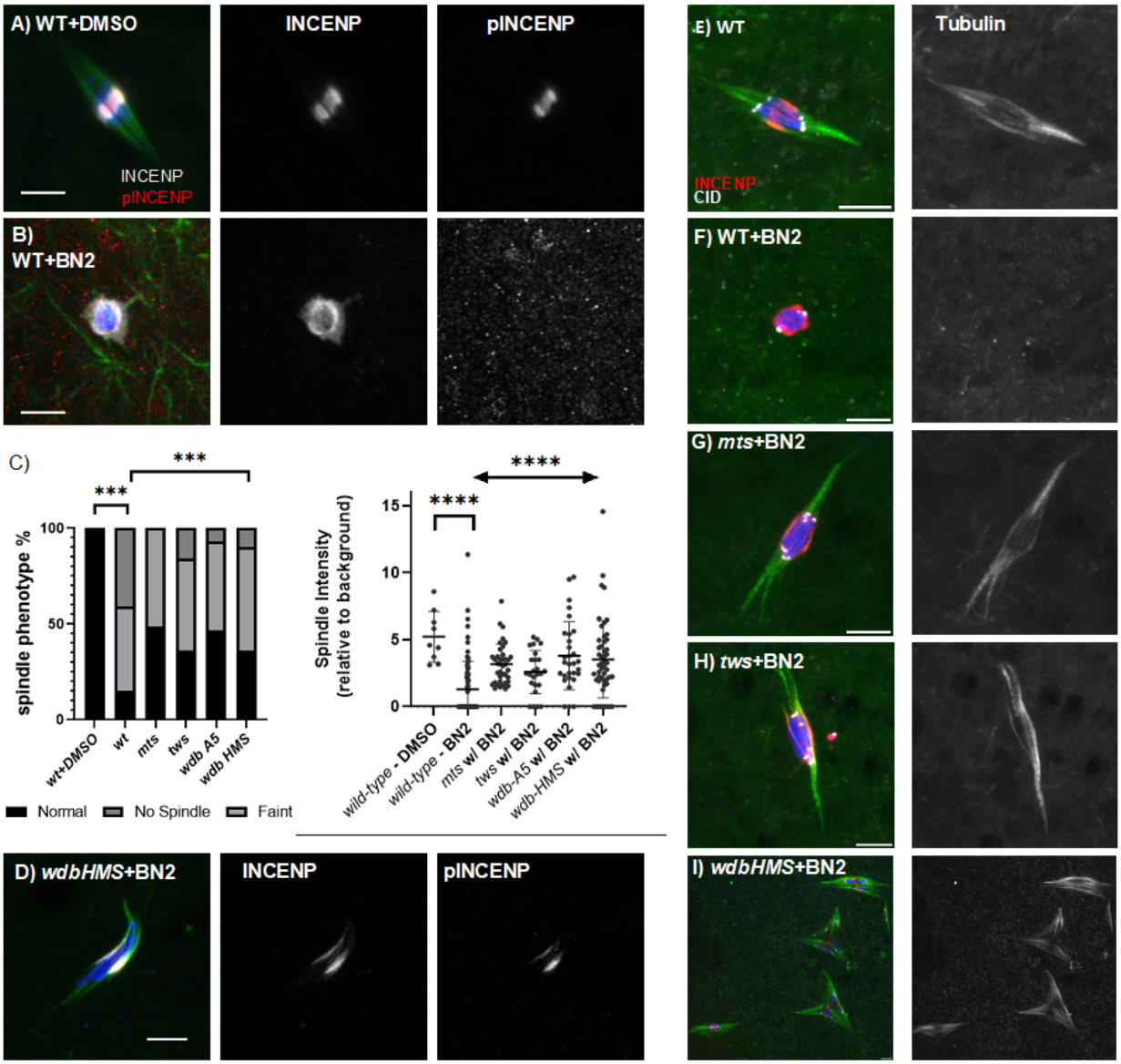

A) WT+DMSO
INCENP
pINCENP
INCENP
pINCENP
B)
WT+BN2
C)
***
***
spindle phenotype %
100
50
0
wt+DMSO
wt
mts
tws
wdb A5
wdb HMS
Normal
No Spindle
Faint
****
****
Spindle Intensity
(relative to background)
15
10
5
0
wild-type - DMSO
wild-type - BN2
mts w/ BN2
tws w/ BN2
wdb-A5 w/ BN2
wdb-HMS w/ BN2
D) wdbHMS+BN2
INCENP
pINCENP
E) WT
INCENP
CID
Tubulin
F) WT+BN2
G) mts+BN2
H) tws+BN2
I) wdbHMS+BN2

Figure 3

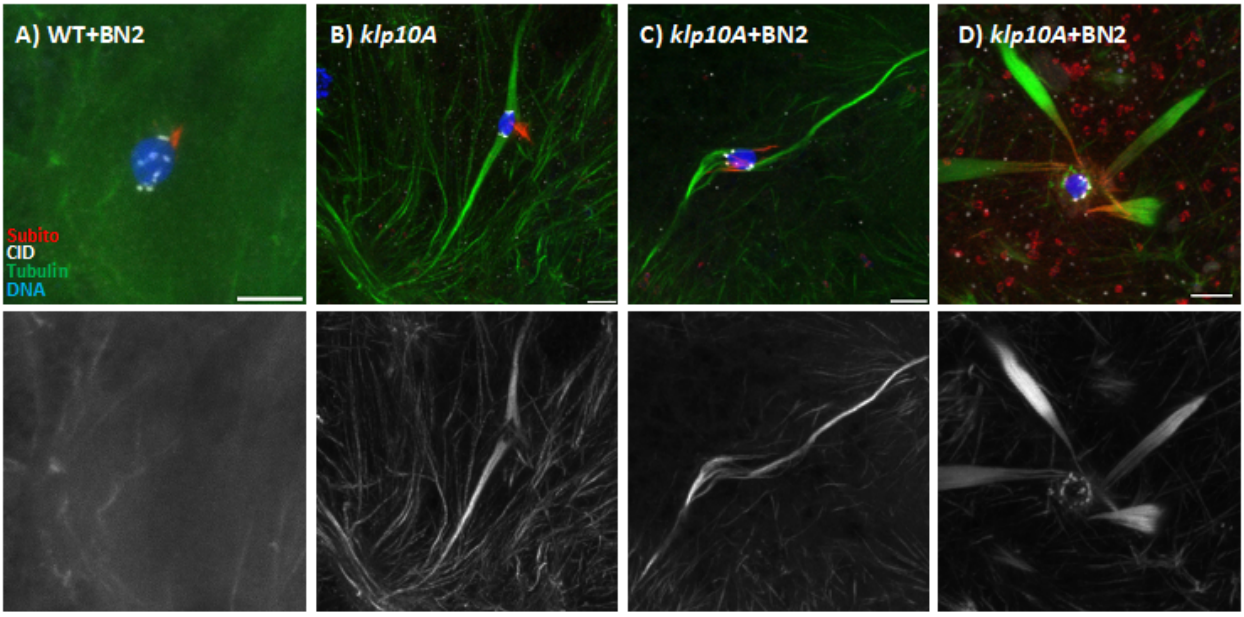

A) WT+BN2
B) klp10A
C) klp10A+BN2
D) klp10A+BN2
Subito
CID
Tubulin
DNA

Figure 4

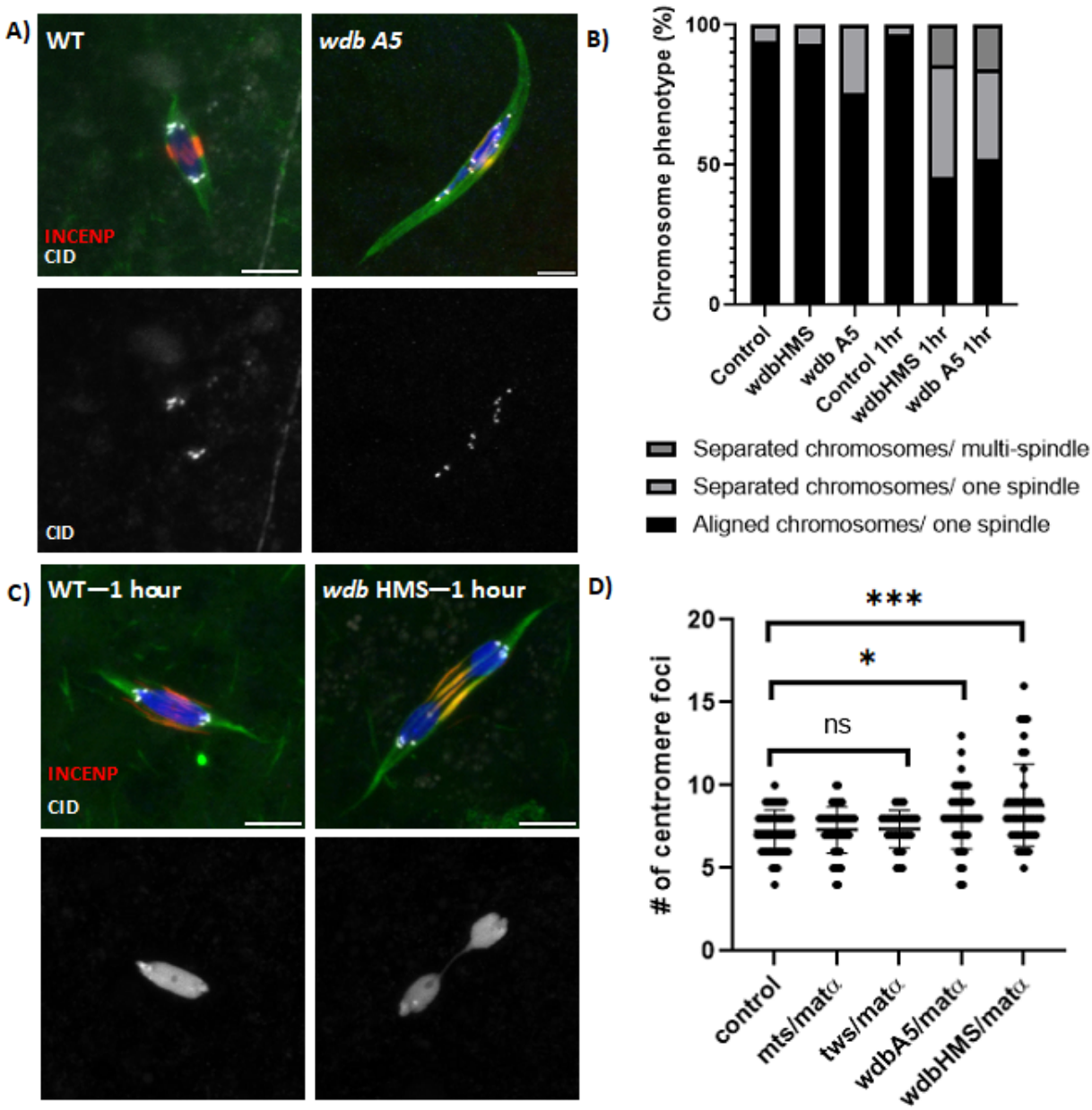

A)
WT
wdb A5
INCENP
CID
CID
B)
Chromosome phenotype (%)
100
50
0
Control
wdbHMS
wdb A5
Control 1hr
wdbHMS 1hr
wdb A5 1hr
Separated chromosomes/ multi-spindle
Separated chromosomes/ one spindle
Aligned chromosomes/ one spindle
C)
WT—1 hour
wdb HMS—1 hour
INCENP
CID
D)
# of centromere foci
20
15
10
5
0
***
*
ns
control
mts/matα
tws/matα
wdbA5/matα
wdbHMS/matα

Figure 5

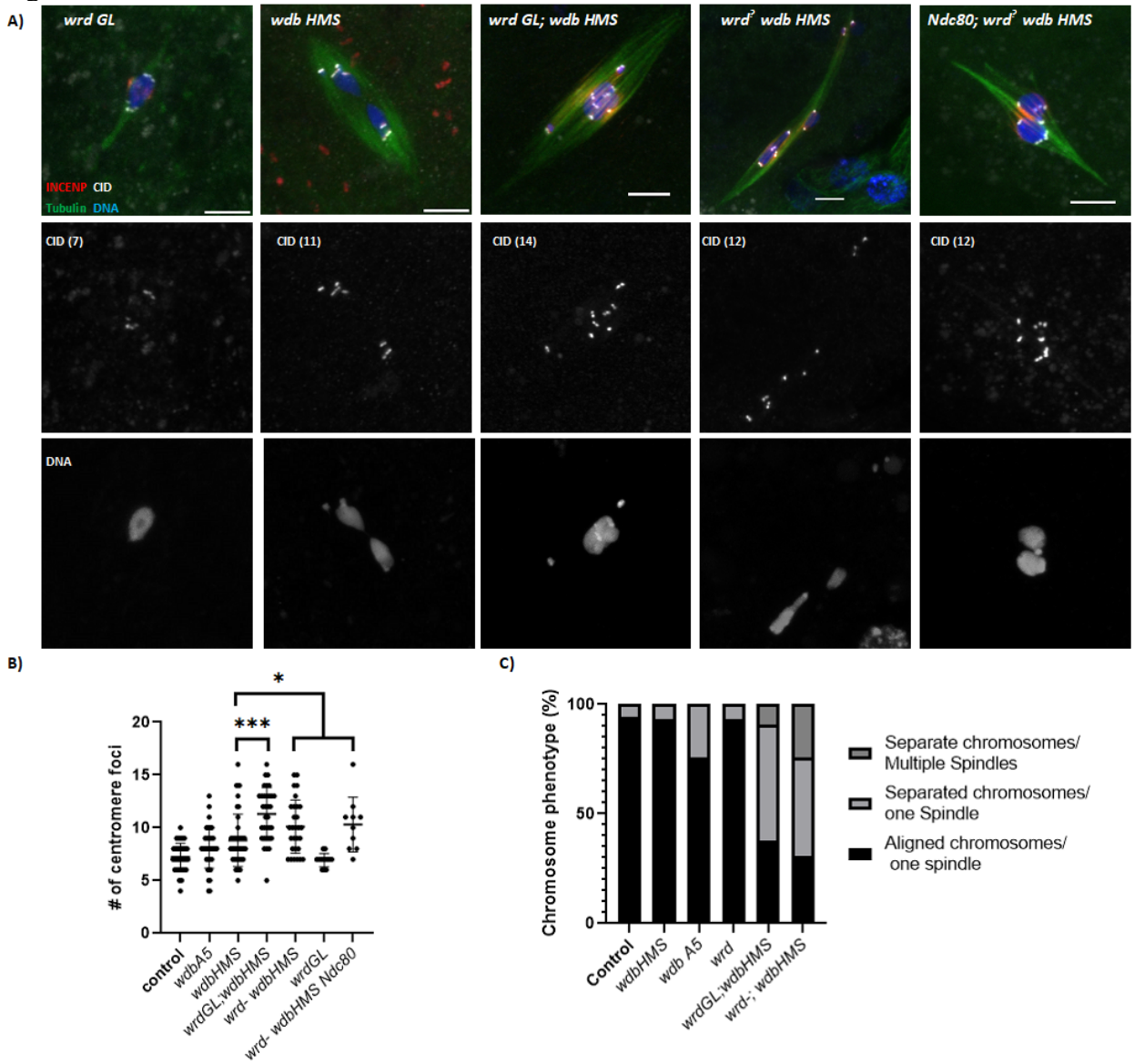

A)
wrd GL
wdb HMS
wrd GL; wdb HMS
wrd² wdb HMS
Ndc80; wrd² wdb HMS
INCENP CID
Tubulin DNA
CID (7)
CID (11)
CID (14)
CID (12)
CID (12)
DNA
B)
# of centromere foci
control
wdbA5
wdbHMS
wrdGL;wdbHMS
wrd- wdbHMS
wrdGL
wrd- wdbHMS Ndc80
C)
Chromosome phenotype (%)
Control
wdbHMS
wdb A5
wrd
wrdGL;wdbHMS
wrd-; wdbHMS
Separate chromosomes/ Multiple Spindles
Separated chromosomes/ one Spindle
Aligned chromosomes/ one spindle

Figure 6

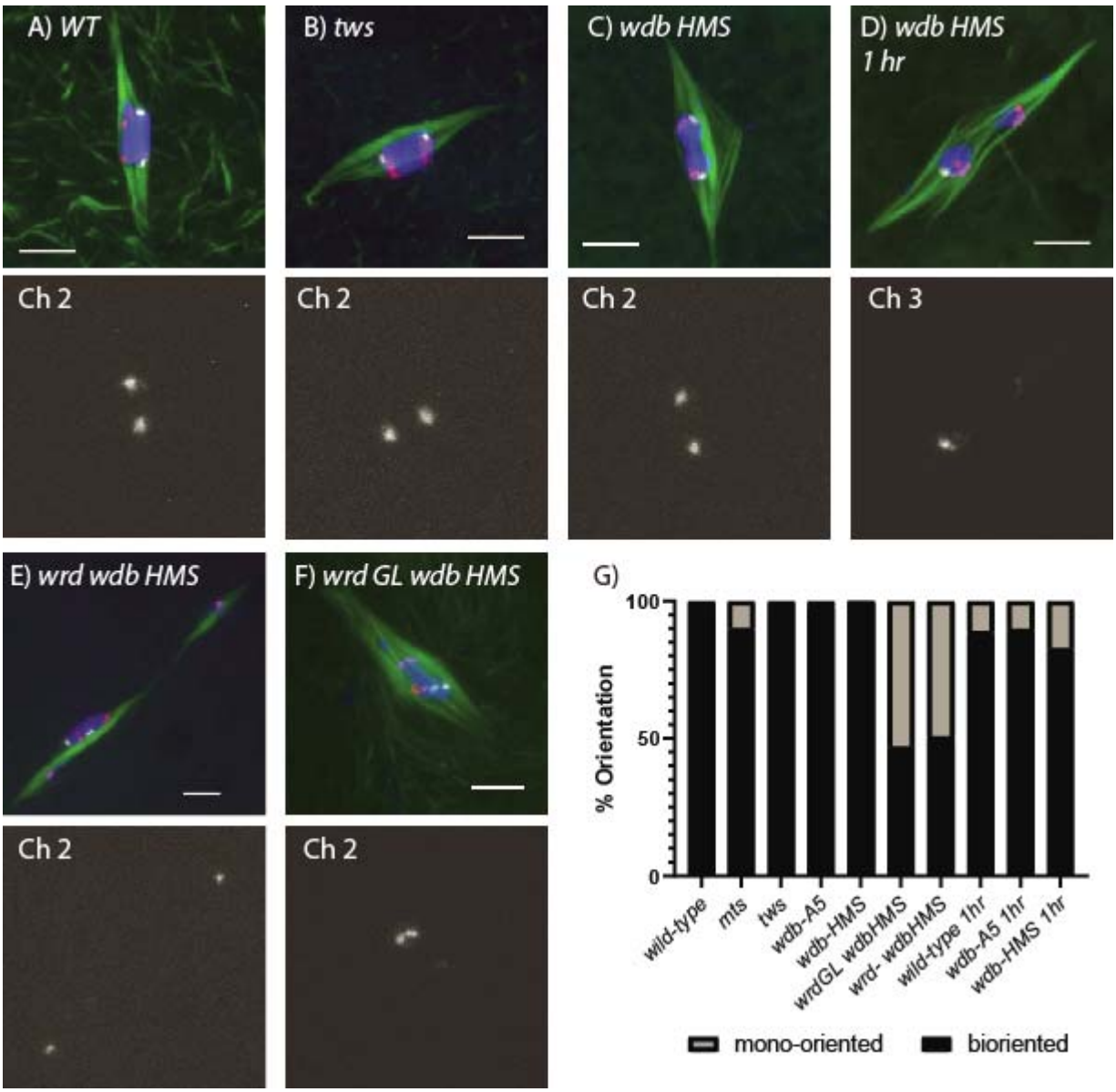

A) WT
B) tws
C) wdb HMS
D) wdb HMS 1 hr
Ch 2
Ch 2
Ch 2
Ch 3
E) wrd wdb HMS
F) wrd GL wdb HMS
Ch 2
Ch 2
G)
% Orientation
100
50
0
wild-type
mts
tws
wdb-A5
wdb-HMS
wrdGL wdbHMS
wrd- wdbHMS
wild-type 1hr
wdb-A5 1hr
wdb-HMS 1hr
mono-oriented
bioriented

Figure 7

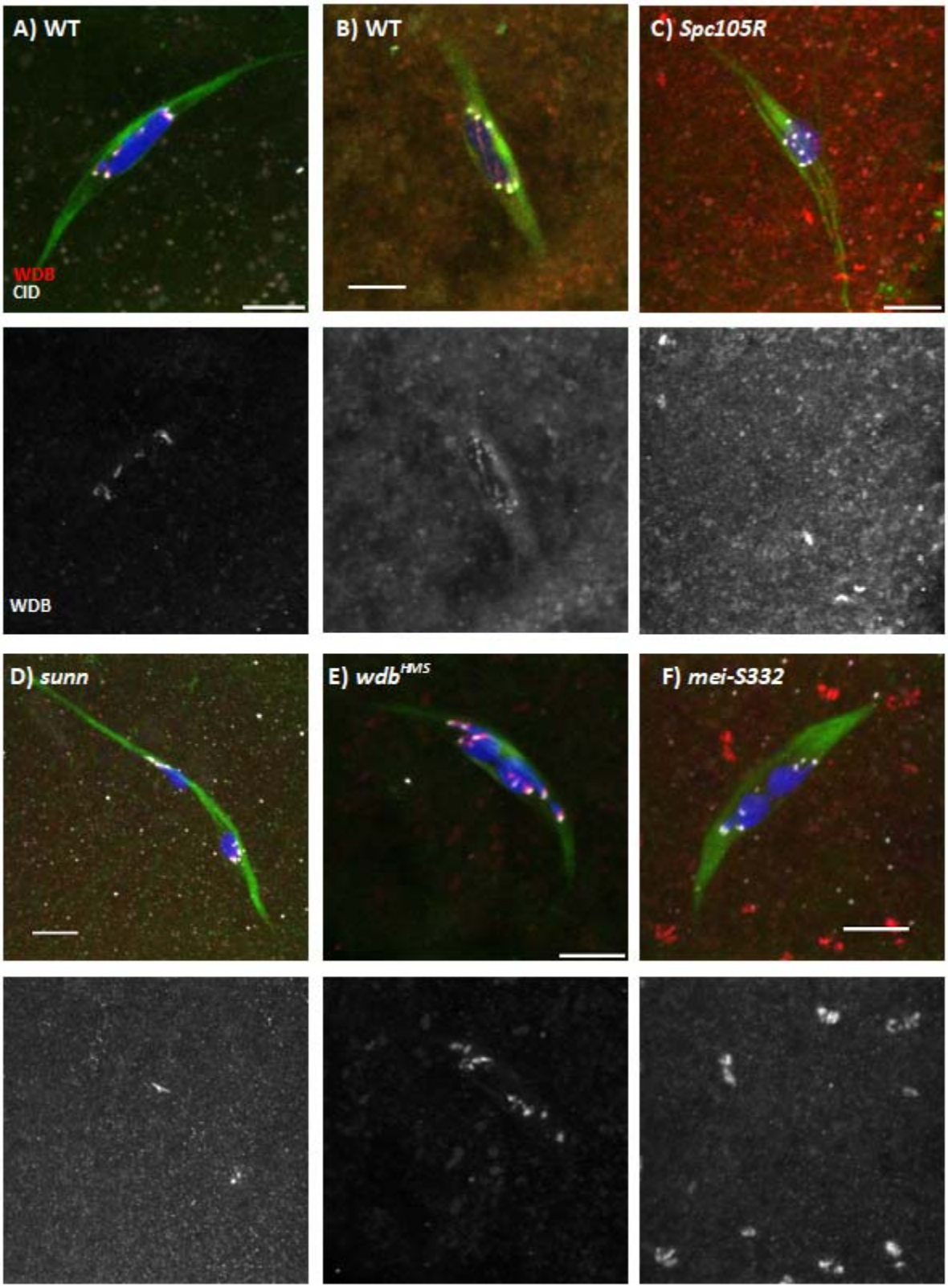
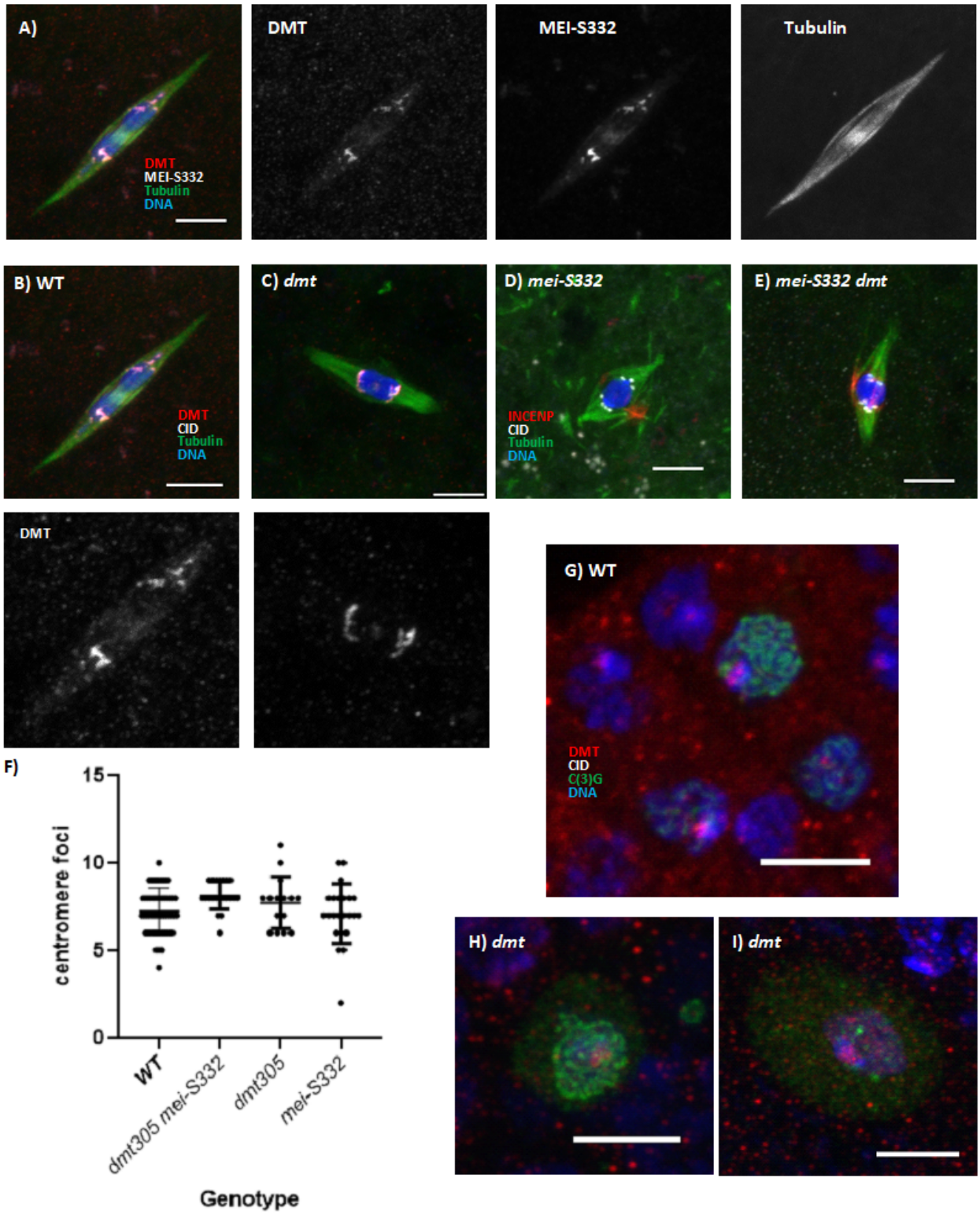


Figure 8


A)
DMT
MEI-S332
Tubulin
DNA
DMT
MEI-S332
Tubulin
B) WT
DMT
CID
Tubulin
DNA
C) dmt
D) mei-S332
INCENP
CID
Tubulin
DNA
E) mei-S332 dmt
DMT
G) WT
DMT
CID
C(3)G
DNA
F)
centromere foci
15
10
5
0
WT
dmt305 mei-S332
dmt305
mei-S332
Genotype
H) dmt
I) dmt