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Inhibition of Polo kinase by BI2536 affects centriole separation during *Drosophila* male meiosis

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Pharmacological inhibition of *Drosophila* Polo kinase with BI2536 has allowed us to re-examine the requirements for Polo during *Drosophila* male gametogenesis. BI2536-treated spermatocytes persisted in a pro-metaphase state without dividing and had condensed chromosomes that did not separate. Centrosomes failed to recruit γ-tubulin and centrosomin (Cnn) and were not associated with microtubule arrays that were abnormal and did not form proper bipolar spindles. Centrioles, which usually separate during the anaphase of the first meiosis, remained held together in a V-shaped configuration suggesting that Polo kinase regulates the proteolysis that breaks centriole linkage to ensure their disengagement. Despite these defects spermatid differentiation proceeds, leading to axoneme formation.

Introduction

Proper chromosome segregation during cell division is crucial to ensure that each daughter receives the full chromosome complement, thus avoiding aneuploidy that represents a hallmark of cell transformation. The proper execution of this process relies on the correct assembly and functioning of the bipolar spindle, which is largely dependent upon the microtubule-nucleating activity of the duplicated centrosome. The Polo family of serine–threonine kinases plays crucial roles in regulating centrosome function and other aspects of mitotic progression. The founder member of the Polo family was originally discovered in *Drosophila*.[1] Flies homozygous for the *polo*[1] mutant exhibit broad and disorganized spindle poles in both syncytial embryos and larval neuroblasts.[1,2] These phenotypes are associated with defects in centrosome organization and migration and a failure of several centrosomal proteins to be recruited to the spindle poles in *polo* mutant *Drosophila* embryos.[3,4] *polo*[1] mutant males exhibit chromosome non-disjunction and cytokinesis defects in male meiosis.[1,5,6] There are also defects in the organization of the female meiotic spindle.[7]

Of the multiple Polo-like kinases (Plks) in vertebrates, Plk1 has multiple roles in mitosis that best match those of Polo in *Drosophila*.[8-10] Interfering with enzyme activity has identified multiple roles for this kinase during cell cycle progression.[11-17] Plk1 is highly expressed in proliferating cells, and its upregulation in human tumors can have prognostic value. This association of Plk1 with a broad range of human tumors has highlighted the kinase as an attractive target for cancer drug development.[18] Depletion of Plk1 by antisense oligonucleotides and small interfering RNA inhibits cell proliferation and decreases viability in cancer cells both in vivo and in vitro.[11,13,19] Among chemical inhibitors targeting Plk1, the dihydropteridinone derivative BI2536, an ATP-competitive kinase inhibitor, is the most extensively investigated. BI2536 treatment results in the mitotic phenotypes that characterize Polo inhibition.[16,20] In cancer cell lines, inhibition of Plk1 activity by BI2536 perturbs spindle pole assembly, leading to mitotic arrest and apoptosis.[21]

Previous studies of the effects of *polo* mutants on male meiosis have been restricted to weak hypomorphic alleles, in which males survive long enough to attempt meiosis.[1,5,6] Here we have overcome this limitation by studying the pharmacological inhibition of Polo kinase in these cells. This has also let us examine the consequences of BI2536 treatment upon both early and late events in meiosis, because *Drosophila* spermatocytes only become transiently delayed by the spindle assembly checkpoint.[22] Because mature spermatocytes are about 25–30 times larger than somatic cells and have larger spindles and very long centrioles, they offer a particularly suitable system for the analysis of cytological consequences of factors affecting cell division. We find that in addition to the typical defects already described to result from reduction of *polo* function, such as failure of centrosome maturation, spindle assembly, and cytokinesis, BI2536-treated spermatocytes show defects in the separation of chromatids and centrioles.

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Results

Centrioles of primary spermatocytes

Our previous studies of the requirements for Polo in male meiosis utilized the hypomorphic mutant allele *polo*.[1,5] We have been unable to study the effects of strong hypomorphic mutants upon meiosis, as these result in larval lethality.[4] We first chose to examine the consequences of treating spermatocytes with BI2536 and following the effects upon centrioles monitored by expression of GFP-tagged Unc. The centrioles of primary *Drosophila* spermatocytes appear as small spots of Unc-GFP as they duplicate at the onset of the first meiotic prophase and gradually elongate during prophase (**Fig. 1A**). Thus, each primary spermatocyte has 2 pairs of connected centrioles that adopt a V-shaped configuration. At this stage, the distal ends of the centrioles push out the cell membrane to form short cilium-like projections (**Fig. 1B**). Unc-GFP is associated with this centriole–cilium complex from prophase I onwards. The labeling was localized in 3 distinct domains: the distal half region of the centriole, the whole cilium, and an intermediate dot-like zone (**Fig. 1A**). Centriole pairs were associated with small astral arrays of microtubules (**Fig. 1A**, inset). We could not find any notable consequences of treating primary spermatocytes with BI2536: they showed separated pairs of V-shaped centrioles (**Fig. 1A**) associated with small asters (**Fig. 1A**, inset); Unc-GFP showed a similar distribution on the centriole and cilium as seen in control spermatocytes; and the cilium-like projection had a similar organization (**Fig. 1B**).

Centriole separation

The centriole pairs moved apart during prometaphase in control primary spermatocytes, where they could be observed at the foci of 2 large asters (**Fig. 2A**) that organize the nascent metaphase spindle. During metaphase of the first meiosis, the centrioles within each pair maintain a V-shaped configuration with their proximal ends in close juxta-position (**Fig. 2A**). The centrioles disorient during the metaphase–anaphase transition of the first meiosis (**Fig. 2A**) and move apart. Thus, each daughter telophase nucleus in primary spermatocytes is associated with 2 widely separated centrioles (**Fig. 2A**). The centrosomal material splits into 2 aggregates and associates with each centriole to form new centrosomes, as it does during the embryonic divisions of the early embryo.[23] Thus, 2 small asters can be observed at each opposite pole of the telophase spindles. Since centriole duplication does not occur during the second meiosis of *Drosophila* male germ cells, the spindle poles of the secondary spermatocytes contain only one centriole (**Fig. 2A**).

Testes from mid-pupae mostly consist of dividing primary and secondary spermatocytes and post-meiotic spermatids. Thus, the incubation in BI2536 would give different phenotypes. Although we were unable to distinguish between the first and the second meiosis in the absence of distinct meiotic spindles, prophase, prometaphase, and post-prometaphase stages were inferred by the chromosome clustering that proceeds in the absence of functional spindles. The spermatid stage was identified by the formation of distinct onion nebenkerns and by the elongation of the axoneme.

BI2536-treated spermatocytes appeared normal during the first meiotic prophase, and chromatin condensed in 3 discrete masses as in control prometaphase cells (**Fig. 2B**). However, in contrast to untreated spermatocytes, asters of microtubules did not associate with centriole pairs in BI2536-treated spermatocytes (**Fig. 2B**). The chromatin compacted further to a single mass, as observed in metaphase or later stages of meiosis in control spermatocytes, but meiotic spindles did not form, and microtubules concentrated at one side of the cells in unfocused arrays that did not contain either γ-tubulin or Cnn. Closer examination of microtubules in 10-nm optical sections revealed that they appeared to concentrate and bend around one side of the spermatocyte nucleus. Two pairs of V-shaped centrioles were maintained in the post-prophase BI2536-treated spermatocytes randomly positioned with respect to the mass of microtubules.

To test if the centriole separation failure observed after treatment with BI2536 might be an indirect consequence of defects in cytokinesis we analyzed spermatocytes treated with latrunculin to inhibit cleavage furrow assembly. The resulting failure of cell division resulted in spermatids with supernumerary nuclei, but the process of centriole separation occurred as usual in control cells (**Fig. S1**). Therefore, the paired centrioles found in BI2536-treated spermatids are not simply the consequence of failed meiotic divisions.

To determine the extent to which microtubule arrays could form independently of centrioles, we examined spermatocytes of *Sas-4* mutants that lack centrioles during gametogenesis.[24] We found that these mutant spermatocytes assembled anastral bipolar spindles that partially supported chromosome movements (**Fig. 2C**). However, following BI2536 treatment of such mutant spermatocytes, microtubules never became organized into bipolar arrays but adopted an arrangement similar to that in BI-2536-treated wild-type cells (**Fig. 2C**). Thus Polo kinase is required to organize microtubules into bipolar arrays in the absence of functional centrosomes. This is likely to reflect the microtubule-organizing ability of chromosomes and kinetochores and is explored in detail elsewhere.[25]

Centrosome maturation

The failure of centrioles to associate with cytoplasmic microtubules in BI2536-treated spermatocytes suggested that inhibition of Polo kinase by BI2536 affects centrosome maturation as it does in mitotic cells. Since the nucleation of microtubules requires protein complexes containing γ-tubulin that are present at the centrosome in prophase and are enriched at the onset of cell division, we examined the distribution of this crucial protein in spermatocytes treated with BI2536. γ-tubulin was present in control cells at the proximal ends of the centrioles during prophase of the first meiosis (**Fig. 3A**), and then, as cells enter the first prometaphase, the amount of γ-tubulin between sister centrioles dramatically increased (**Fig. 3A**). γ-tubulin persisted around the proximal ends of newly separated centrioles after anaphase I and was diffusely present along the centriole wall during spermatid differentiation (**Fig. 3A**). BI2536 treatment did not affect the association of γ-tubulin with the centriole in

primary spermatocytes (**Fig. 3A**), but it showed no significant increase upon completion of prophase (**Fig. 3A**). This strongly suggested a failure of the centrosomes to undergo maturation in the absence of Polo kinase function.

Since the recruitment of γ-tubulin and Centrosomin (Cnn) are mutually dependent in *Drosophila*[26] and dependent upon Polo kianse in cultured cells[27] we asked whether Cnn recruitment was also affected by BI2536-treatment of spermatocytes. We found that Cnn was present during the first prophase in both control and BI2536-treated testes (**Fig. 3B**). At the onset of meiosis in control cells, there was a significant enrichment of Cnn at the centrosome, and it was maintained at a high cencentration around the proximal ends of centrioles as they separated in anaphase I (**Fig. 3B**). Cnn was still present at the proximal ends of the centrioles of young control spermatids, but it gradually decreased as the sperm axoneme underwent elongation (**Fig. 3B**). By contrast, Cnn levels were reduced following BI2536 treatment (**Fig. 3B**) and were barely detectable during spermatid elongation (**Fig. 3B**).

Polo is also required for the activation of *Abnormal spindle (Asp)*, a crucial factor for the centrosome nucleation of microtubules at their minus ends.[28] We therefore asked whether BI2536 treatment would affect the distribution of the *Asp* protein. Whereas in control spermatocytes, Asp was localized in the peri-centrosomal region (**Fig. 3C**), it was not in BI2536-treated spermatocytes (**Fig. 3C**).

Otherwise, there appeared to be no effects of Polo inhibition upon the presence in centrioles of the Plk4-partner protein, Asterless, and Ana 1 (**Fig. S2A and B**), proteins required for centriole assembly.[29,30] Accordingly, there was no effect of BI2536-= treatment upon centriole numbers, indicating that inhibition of Polo kinase did not affect centriole duplication.

Axoneme elongation

At the end of the second meiotic division in control testes, each spermatid inherits one centriole (**Fig. 4A**), from which extends a cilium-like projection (**Fig. 4B**). As spermatids differentiate and the axoneme begins to elongate, the distal dot of Unc-GFP appears to move progressively toward a more distal position (**Fig. 4A**).[31] Although the spermatids in BI2536-treated testes had a single large nucleus, an unusual microtubule network, and 2 pairs of orthogonal centrioles, they still attempted to differentiate (**Fig. 4A**). EM analysis revealed that chromatin of BI2536-treated spermatids underwent further condensation, and mitochondria became organized in onion-like structures, and axonemes began to grow from each centriole. The centrioles in these cells had short cilium-like projections and were disposed in a V-shaped arrangement with their proximal ends close to each other (**Fig. 4B**). This suggests that centrioles remained engaged. Immunofluoresecnce microscopy indicated that the axonemes nucleated by each of the co-joined, paired centrioles elongated, and that the distal puncta of the Unc-GFP dot was displaced further distally as in control cells (**Fig. 4A**). These events could take place without disassembly of the arrays of cytoplamic microtubules in these cells. Moreover, spermatid differentiation and axoneme elongation also proceeded after latrunculin treatment as they did in control and BI2536-treated spermatids (**Fig. S1**).

The axonemes of the elongating spermatids in BI2536-treated cells showed small differences with those of controls. Whereas axonemes of early control spermatids had the characteristic 9+2

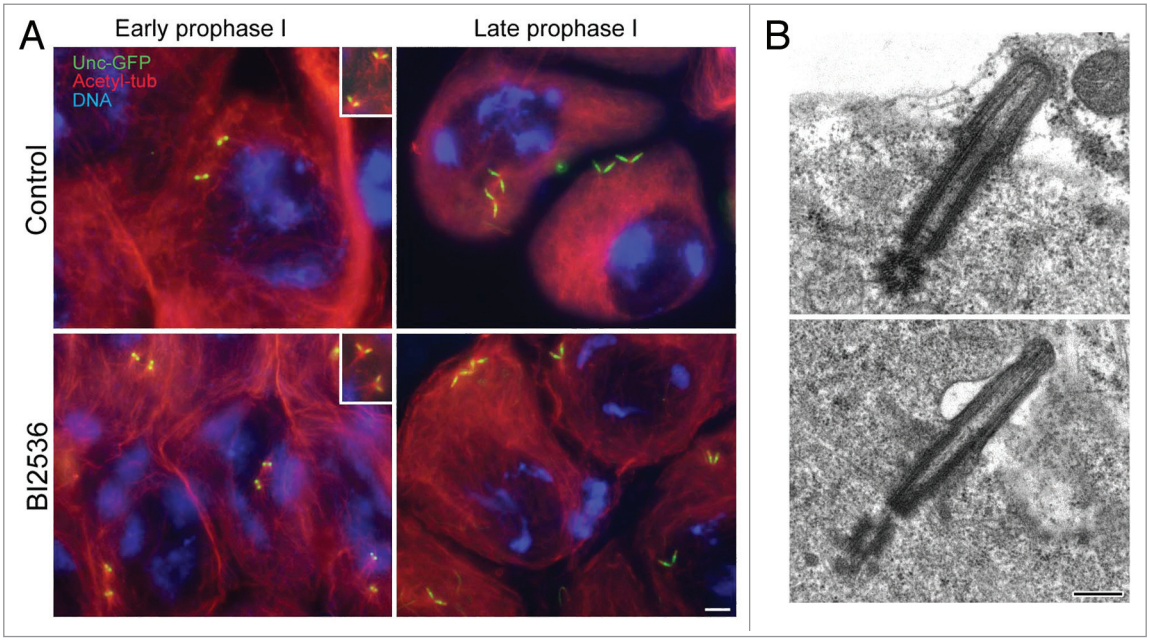

Figure 1. Prophase of the first meiosis is unaffected by BI2536. (**A**) Primary spermatocytes expressing Unc-GFP (green) were stained for acetylated tubulin (red) and DNA (blue). Both control and BI2536-treated spermatocytes have 2 pairs of orthogonal centrioles that are associated to small asters of microtubules (insets). (**B**) Electron micrographs do not reveal significant differences between centriole/cilium complexes in control and treated mature primary spermatocytes. Scale bar = 2.5 μm in (**A**); 250 nm in (**B**).

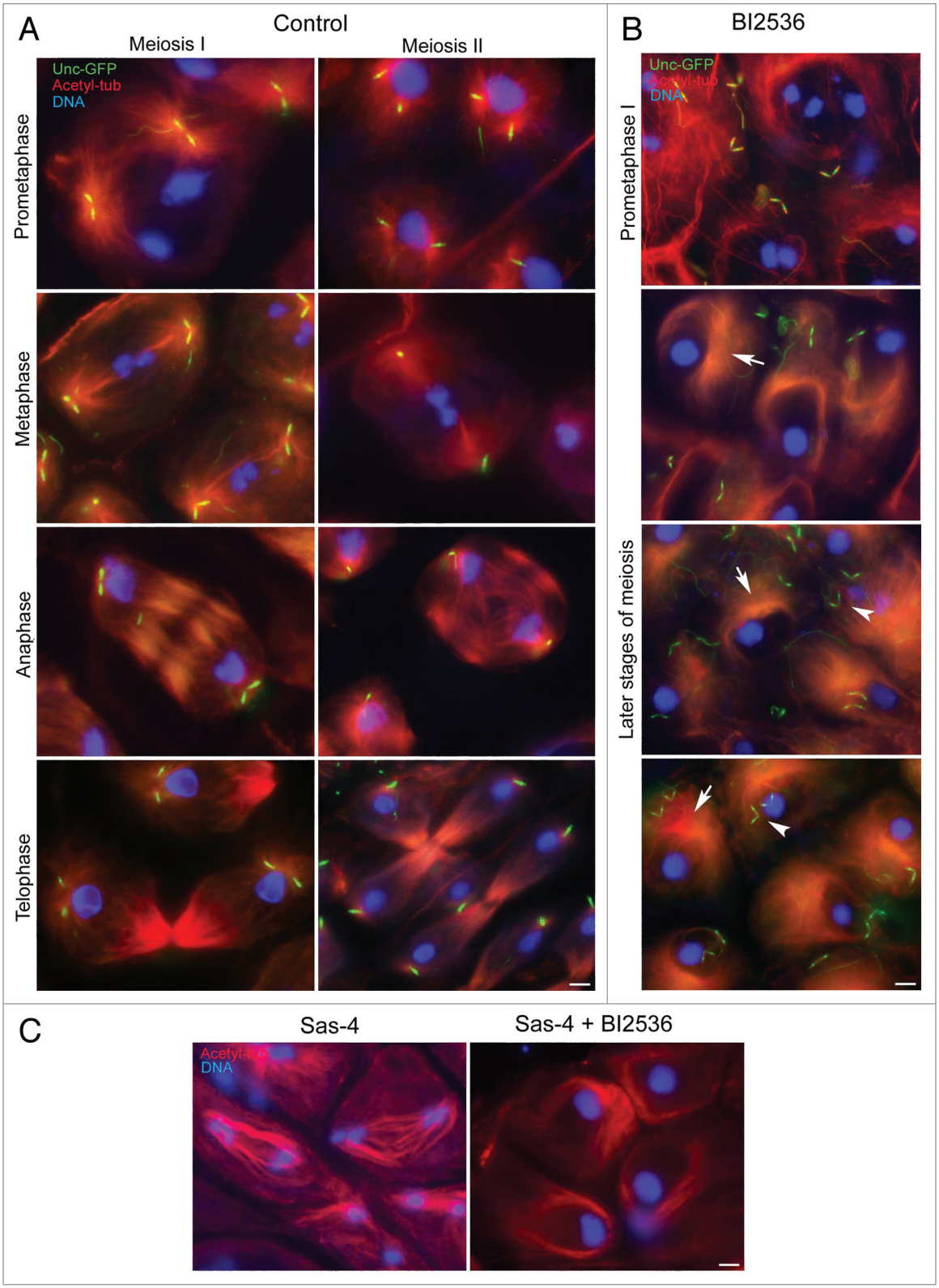

Figure 2. BI2536 affects centriole dynamics and spindle organization during meiosis. Post-prophase control (**A**) and BI2536-treated (**B**) spermatocytes expressing Unc-GFP (green) were stained for acetylated tubulin (red) and DNA (blue). The spindle poles of control primary spermatocytes (**A**) are organized by centrosomes containing 2 close centrioles that separate at anaphase and telophase; thus, the spindle poles contain only one centriole during the second meiosis. BI2536-treated spermatocytes (**B**) lack spindle poles both during prometaphase and later meiotic stages; the microtubules are disposed in asymmetric arrays (arrows) that resemble mono-polar spindles, but the centrioles do not associate with microtubules and are engaged in a V-shaped configuration (arrowheads). (**C**) Sas-4 spermatocytes assemble bipolar anastral spindles, but BI2536 induces the formation of monopolar-like structures. Scale bars = 2.5 μm.

microtubule architecture (**Fig. 4C**), BI2536-treated spermatids displayed axonemes with the usual 9+2 microtubule pattern and also axonemes showing structural defects: 9+0, 9+1, 9+3 patterns or with missing peripheral tubules (**Fig. 4C**). This suggests that Polo kinase participates in promoting the correct elongation of axonemal microtubules.

Discussion

Our findings support previous studies suggesting that *Polo* is required for the organization of the meiotic apparatus and for both chromosome segregation and cytokinesis in *Drosophila* males.[1,5-7] The meiotic defects we find after treatment with BI2536 differ from those described for *polo*[1] mutants in a manner that reflects the weak nature of this hypomorphic allele in contrast to the strong inhibition of Polo kinase activity by BI2536. The absence of a robust spindle assembly checkpoint arrest in spermatocytes enables the formation of late mitotic bipolar spindles that lack a distinct midzone and fail to properly execute cytokinesis in the *polo*[1] mutant.[5] Spindle organization defects are also seen in BI2536-treated spermatocytes, but only asymmetrically positioned monopolar-like spindles were found. The lack of typical pericentrosomal proteins, including γ-tubulin, Cnn, and Asp, in association with these cytoplasmic microtubules and the failure of these molecules to be recruited to centrioles, indicates that centrosome maturation is perturbed. This accords with previous findings following Polo depletion in P-element insertion mutants, RNAi, or treatment of cultured *Drosophila* cells with BI2536.[4,27,32]

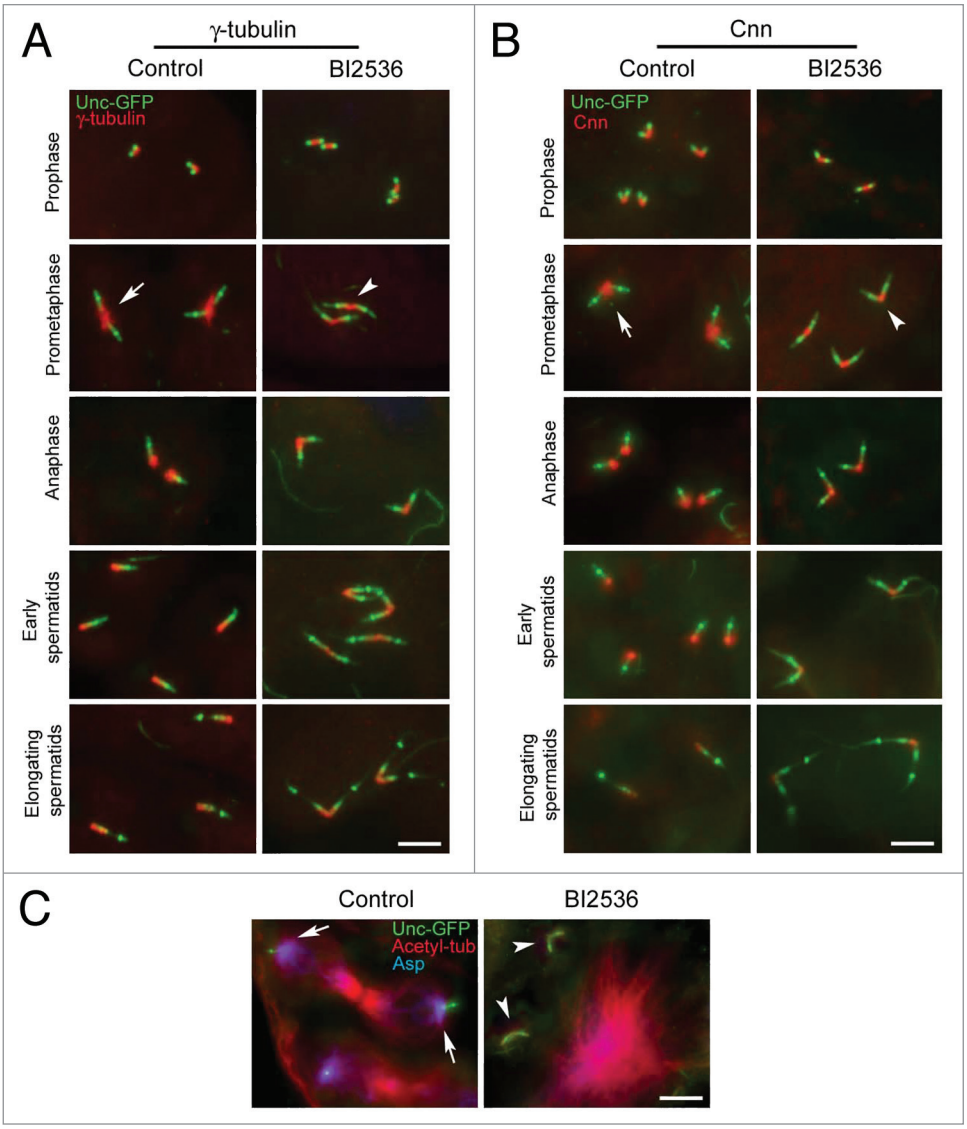

Figure 3. The recruitment of centrosomal proteins is impaired by BI2536. Control and BI2536 treated male germ cells expressing Unc-GFP (green) were counterstained for γ-tubulin (red; **A**) or Cnn (red; **B**). The accumulation of γ-tubulin and Cnn at the centrioles increases in control cells during the meiotic divisions (arrows), but does not augment after BI2536 incubation (arrowheads). (**C**) Localization of Asp protein (blue) in control and treated spermatocytes expressing Unc-GFP (green) and counterstained for acetylated tubulin (red). Asp is mainly found at the centrosomes in control cells (arrow) but does not associate with centrioles after drug treatment (arrowhead). Scale bars = 2.5 μm.

We also find that BI2536 treatment resulted in a failure of microtubules to contact chromosomes. This is likely to reflect some deficiency in the acentrosomal spindle formation mechanism that requires microtubule nucleation at the chromosomes.[33,34] This failure of microtubule–kinetochore interactions that we find after BI2536 treatment appears to reflect a requirement for Polo kinase to recruit Augmin to the kinetochores of primary spermatocytes (Savoian and Glover, unpublished data). It points to a key role for Polo kinase in acentrosomal spindle formation in spermatocytes.

A striking finding of our study is that Polo kinase is required for centriole disengagement. This process normally takes place during the metaphase/anaphase transition and in *Drosophila* spermatogenesis is essential to ensure that each spermatid has a single centriole to nucleate formation of the sperm axoneme. We observe that both spermatocytes and early spermatids have V-shaped configurations of paired centrioles following BI2536 treatment, indicating failure of centriole disengagement. This contrasts to the successful separation of centrioles in the first meiosis of *polo*[1] mutants, in whom the defects in cytokinesis lead

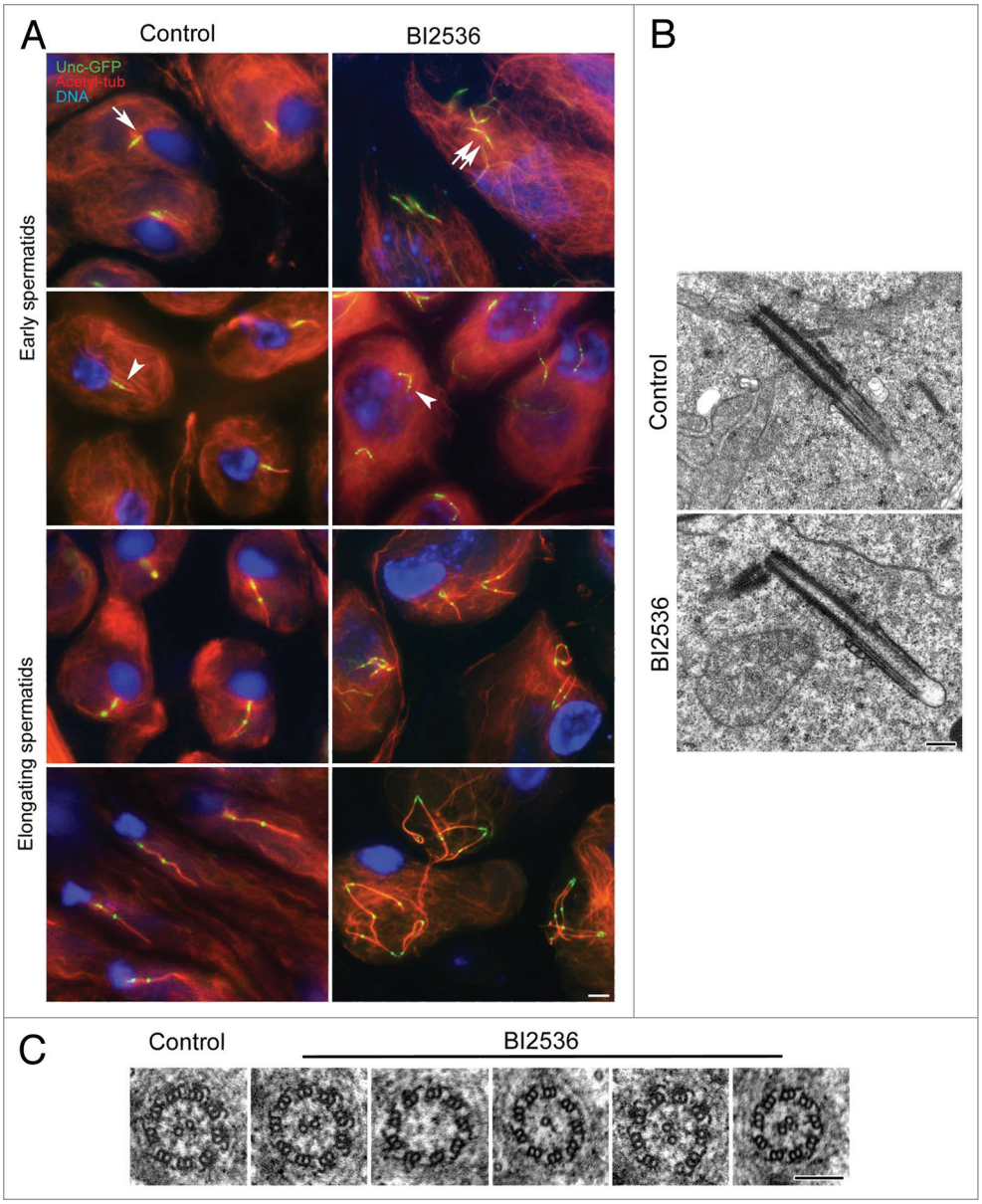

Figure 4. Spermatid differentiation proceeds in the presence of BI2536. (**A**) Control and BI2536-treated spermatids expressing Unc-GFP (green) were counterstained for acetylated-tubulin (red) and DNA (blue). Control spermatids inherit only one centriole at the end of meiosis (arrow), whereas BI2536-treated spermatids inherit 2 pairs of orthogonal centrioles (double arrow). In both control and treated spermatocytes the beginning of axoneme formation is marked by the detachment of the distal Unc-GFP dots (arrowheads) that gradually move away from the centriole. (**B**) The centrioles have a distal cilium-like structure in both control and treated spermatids; treated spermatids display pairs of engaged centrioles. (**C**) Control spermatids have 9+2 axonemes, whereas treated spermatids display the conventional model together with abnormal axonemes. Scale bar = 2.5 μm in (**A**); 250 nm in (**B**); 100 nm in (**C**).

to elongating spermatids with 2 or 4 widely separated axonemes,[5] again most likely reflecting the weak nature of the *polo*[1] allele compared with the effective inhibition of Polo kinase by BI2536. The effect of the drug on centriole dynamics was dose-dependent. No effects were seen at 10 nM concentration, whereas at a concentration of 100 nM we find that approximately 20–30% of the centrioles were engaged in elongating spermatids. Increasing the concentration of BI2536 at 500 nM, we find about 50–60% of engaged spermatid centrioles. The most severe phenotype with 100% of engaged centrioles was found at a final concentration of 1 μM.

Centriole disengagement in vertebrate cells requires the proteolytic activity of separase,[35] which releases engaged centrioles from a "glue" protein. Since separase cleaves cohesin components to allow sister chromatid separation at mitosis,[36,37] cohesin itself may represent the ideal substrate for separase at the centrioles also. Cohesin was, indeed, found at the centrosome in cultured human cells,[38-40] and depletion of cohesin causes premature separation of paired centrioles.[41] Separase and meiosis-specific proteins have been implicated in centriole dynamics during *C. elegans* spermatogenesis.[42] This suggests that cohesin may represent a part of the link that provides connection between the proximal ends of the centrioles.[43] Moreover, the sSgo1 splice variant of Shugoshin 1 (Sgo1) that protects cohesin from separase cleavage at the chromosomes[44,45] has been also found at the centrosomes,[46] where it has been proposed to play a role in preventing cohesin degradation.[47] Consistently, depletion of human sSgo1 through RNAi results in the premature separation of paired centrioles in cultured cells.[47] Reduced Sgo1 amounts in murine embryonic fibroblasts and in adult bone marrow cells leads to multiple spindle poles.[48,49]

A short-lived pool of cohesin is transiently associated in *Drosophila* to centrosomes during metaphase and anaphase,[60] and the product of *MEI-S332*, the *Drosophila* ortholog of the vertebrate cohesin protector Sgo1, seems to be localized at the spindle poles in male meiosis I cells.[51] Polo localizes to centrosomes from metaphase I to metaphase II of *Drosophila* male meiosis,[6] and the release of MEI-S332 in anaphase of the second meiosis and in anaphase of mitosis depends on the Polo kinase activity.[52] Thus the finding of conserved proteins at the centrosome suggests that a mechanism of centriole disengagement like that used in vertebrate cells may also function in *Drosophila*. However, recent experiments in *Drosophila* have argued against a role for cohesin

in maintaining centriole cohesion at least in syncytial embryos.[53] This is consistent with the observation that, although sister chromatid separation is blocked in *Drosophila* separase mutant embryos, centrosomes duplicate correctly.[54] Furthermore, the absence of the *Drosophila* counterpart of pericentrin, proposed as a separase substrate required for centriole cohesion in vertebrate cells, does not prevent the centrosome duplication cycle in flies.[55-57] However, the role played by the separase substrate, cohesin, in centriole cohesion has been controversial. One report indicated that expression of non-cleavable cohesin in HeLa cells did not not prevent disengagement,[58] whereas another using purified mammalian centrioles suggested centriole engagement did depend upon cohesin integrity.[43] Recent observations in early *C. elegans* embryos point to multiple mechanisms contributing to centriole separation, with the removal of pericentriolar material by microtubule-based forces playing a central role.[59] The separase activity becomes critical when the action of the cytoskeleton is reduced. Thus the conflicting roles of cohesin in centriole engagement may be in part explained by the different developmental stages of the systems examined.[59] Plk1 promotes, indeed, a separase-independent removal of the centriolar "glue" at prophase,[58] whereas in anaphase it stimulates a separase-dependent cleavage of centrosomal cohesin,[43] likely by turning cohesin a better substrate for proteolysis after helping to remove sSgo1. This process is reminiscent of the roles of these proteins in removing cohesin from sister chromatids. Plk1 regulates the dissociation of the bulk of cohesin from the chromosome arms in early mitosis,[60] whereas the remaining centromeric cohesin was removed at anaphase by the proteolytic activity of separase[61] after phosphorylation of Sgo1 by Plk1.[62]

Although we cannot categorically rule out the involvement of multiple redundant mechanisms, one of which requires separase, the simplest explanation is that centriole disengagement is regulated through alternative proteins in *Drosophila*, although still under the control of Polo kinase. It has been demonstrated that centrioles can be connected in *Drosophila* spermatocytes through a link that has been postulated to depend upon the *Sas-6–Ana2* complex and that may play a role in centriole engagement.[63] Future work will be required to definitively determine whether this is the case.

Materials and Methods

Drosophila strains

The stocks containing the Unc-GFP transgene and the Ana1-GFP were described previously.[29,64] Flies expressing Asl-Tomato (RFP) were kindly provided by Timothy Megraw. Flies were raised on standard *Drosophila* medium at 24 °C.

Antibodies and reagents

Rabbit anti-centrosomin (Cnn; 1:400) and anti-abnormal spindle (Asp; 1:50) have been previously described.[65,66] Mouse anti-γ-tubulin-GTU88 (1:100) and mouse anti-acetylated tubulin (1:100) were from Sigma-Aldrich. Alexa Fluor 488-, 555-, and 647 secondary antibodies (1:800) were purchased from Invitrogen. BI2536 was purchased by Selleck. Dimethyl sulfoxide (DMSO), Latrunculin B, and Shields and Sang M3 Insect Medium were purchased from Sigma-Aldrich. BI2536 and Latruncilin B were dissolved in DMSO at stock concentration of 1000 μM and stored frozen at 20 °C. The stock solution was diluted to the desired concentration in culture medium prior to incubation with testes.

Culture and drug treatment experiments

Testes were dissected from pupae between 5–7 d in M3 medium. To inhibit Polo kinase, testes were incubated 24 h in M3 medium containing 10 nM, 100 nM, 500 nM, or 1 μM BI2536 for 24 h into a sterile 24-well plate at 24 °C.

To assess the effect of microfilament depolymerization on centriole dynamics the dissected testes were cultured at 24 °C for 24 h in M3 medium containing 0.5 μM Latrunculin B. Incubation of testes in M3 medium containing DMSO but lacking drugs had no effect on centrosome dynamics or on the organization of the cytoskeleton.

Indirect immunofluorescence staining

After incubation, the testes were washed in M3 medium for 10 min and then in phosphate-buffered saline (PBS) for 5 min. Then testes were placed in a small drop of 5% glycerol in PBS on a glass slide and squashed under a small cover glass and frozen in liquid nitrogen. After removal of the coverslip, the samples adhering to the slides were immersed in cold methanol for 10 min. For localization of centrosomal components, testes were washed for 15 min in PBS and incubated for 1 h in PBS containing 0.1% bovine serum albumin (PBS-BSA) to block non-specific staining. Testes were incubated overnight at 4 °C with the specific antisera against Cnn and Asp. For localization of γ-tubulin, testes were incubated in the GTU88 antibody. To visualize microtubules, testes were incubated with anti-acetylated tubulin antibody for 4–5 h at room temperature. After washing in PBS–BSA the samples were incubated for 1 h at room temperature with the appropriate secondary antibodies. In all cases, DNA was visualized with incubation of 3–4 min in Hoechst. Testes were mounted in small drops of 90% glycerol in PBS. Images were taken by using an Axio Imager Z1 (Carl Zeiss) microscope equipped with an AxioCam HR cooled charge-coupled camera (Carl Zeiss). Grayscale digital images were collected separately and then pseudocolored and merged using Adobe Photoshop 7.0 software (Adobe Systems).

Transmission electron microscopy

Testes incubated in M3 medium in the presence or absence of drugs were washed twice in the free-drug medium, then 5 min with PBS, and fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) overnight at 4 °C. Samples were carefully rinsed in PBS and subsequently post-fixed in 1% osmium tetroxide in PBS for 1 h at 4 °C. After rinsing in PBS, the material was dehydrated through a graded series of ethanol, infiltrated with a mixture of Epon–Araldite resin and polymerized at 60 °C for 48 h. Serial ultrathin sections (65–75 nm) were cut with a Reichert ultramicrotome equipped with a diamond knife, collected with formvar-coated copper grids, and stained with uranyl acetate and lead citrate. TEM preparations were observed with a FEI Tecnai G_2 Spirit transmission electron microscope operating at an accelerating voltage of 100 kV and equipped with a Morada CCD camera (Olympus).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/29083

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