The Kinesinlike Protein Subito Contributes to Central Spindle Assembly and Organization of the Meiotic Spindle in *Drosophila* Oocytes□D

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In the oocytes of many species, bipolar spindles form in the absence of centrosomes. *Drosophila melanogaster* oocyte chromosomes have a major role in nucleating microtubules, which precedes the bundling and assembly of these microtubules into a bipolar spindle. Here we present evidence that a region similar to the anaphase central spindle functions to organize acentrosomal spindles. *Subito* mutants are characterized by the formation of tripolar or monopolar spindles and nondisjunction of homologous chromosomes at meiosis I. *Subito* encodes a kinesinlike protein and associates with the meiotic central spindle, consistent with its classification in the Kinesin 6/MKLP1 family. This class of proteins is known to be required for cytokinesis, but our results suggest a new function in spindle formation. The meiotic central spindle appears during prometaphase and includes passenger complex proteins such as AurB and Incenp. Unlike mitotic cells, the passenger proteins do not associate with centromeres before anaphase. In the absence of Subito, central spindle formation is defective and AurB and Incenp fail to properly localize. We propose that Subito is required for establishing and/or maintaining the central spindle in *Drosophila* oocytes, and this substitutes for the role of centrosomes in organizing the bipolar spindle.

INTRODUCTION

In the oocytes of many animals, bipolar spindles form in the absence of centrosomes (Compton, 2000; Karsenti and Vernos, 2001). In the acentrosomal pathway for spindle formation, the chromosomes trigger spindle formation by capturing free microtubules that are present in the cytoplasm (Theurkauf and Hawley, 1992; McKim and Hawley, 1995). These microtubules are then bundled and sorted to generate two poles in a process that involves a variety of motor protein–microtubule interactions (Matthies *et al.,* 1996; Walczak *et al.,* 1998). Plus-end-directed motors of the BimC class such as Eg5 are proposed to generate bundles of antiparallel microtubules, an activity that could be important for promoting the formation of bipolar instead of monopolar spindles (Karsenti and Vernos, 2001). Minus-end-directed motors such as kinesins in the C-terminal motor class or dynein have been proposed to bundle parallel microtubules and taper them into defined poles (Matthies *et al.,* 1996; Walczak *et al.,* 1998).

Although the activities of a variety of motors has been studied in such systems as *Xenopus* extracts, the formation of acentrosomal spindles in vivo is still poorly understood. Although *Drosophila* female meiosis is an excellent system to study acentrosomal spindle formation, the only motor protein with a role in spindle assembly that has been exten-

sively studied is NCD, a C-terminal motor kinesin. Consistent with a role in focusing the poles, *ncd* mutant spindles are frequently multipolar or apolar (Hatsumi and Endow, 1992; Matthies *et al.,* 1996). Nonmotor proteins have also been shown to make important contributions to Drosophila acentrosomal spindle organization. For example, spindle pole-associated proteins TACC and MSPS have a role in bipolar spindle pole formation (Cullen and Ohkura, 2001). The AXS protein is present within a structure ensheathing the meiotic spindle and has a role in meiotic spindle assembly (Kramer and Hawley, 2003). These studies suggest there are important proteins or structures that modulate the interaction of motors and microtubules in acentrosomal spindle assembly.

Previous studies in *Drosophila* oocytes have suggested that the process of acentrosomal spindle formation is initiated by the capture of free microtubules by the chromosomes followed by bundling and sorting of microtubules by minus-end-directed motors and the accumulation of certain proteins at the spindle poles (Matthies *et al.,* 1996; Cullen and Ohkura, 2001). In this article we have built on this model by investigating the role of the *subito* (*sub*) gene in acentrosomal spindle formation. *sub* encodes a kinesinlike protein whose sequence is most similar to the MKLP1 (mitotic kinesin like protein 1) family (Giunta *et al.,* 2002) (now Kinesin 6, Dagenbach and Endow, 2004). Mutants in *sub* have a phenotype consistent with a role in organizing the bipolar spindle. Female meiosis in *sub* mutants is characterized by the formation of monopolar and tripolar spindles and the nondisjunction of homologous chromosomes during the first meiotic division (Giunta *et al.,* 2002).

Here we show that SUB protein is bound to the meiotic central spindle, a pattern that is consistent with its assignment to the MKLP1 class of proteins. SUB and the central
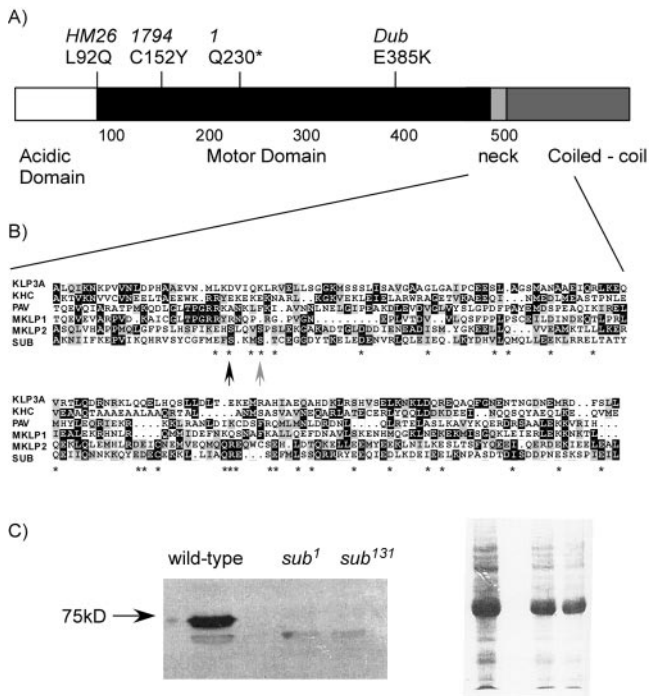
Figure 1. Subito (SUB) is a kinesinlike protein in the MKLP1 family. (A) Schematic of SUB protein organization showing the amino acid changes in the known mutants. sub^{131} deletes most of the subcoding region (Giunta *et al.,* 2002) and sub^1 is a nonsense mutation. (B) Sequence alignment of the neck-linker region in the *Drosophila* and human MKLP1 group along with two other *Drosophila* proteins, conventional kinesin heavy chain and KLP3A. The black arrow indicates the Polo kinase site identified by Neef *et al.* (2003), the gray arrow indicates a second conserved serine, and the stars denote identical or similar amino acids unique to MKLP2 and SUB. (C) Western blot of ovary protein using anti-SUB antibodies. A smaller nonspecific band and a Ponceau S-stained membrane serve as loading controls.

spindle appear at the earliest stages of prometaphase, before bipolar spindle formation. Indeed, SUB is required for assembly of the central spindle. We suggest that the precocious assembly of the central spindle has a primary role in organizing the meiotic spindle in the absence of centrosomes.

MATERIALS AND METHODS

Genetics and Sequencing of sub alleles

The isolation and genetic analysis of most *sub* alleles has been described previously (Giunta *et al.,* 2002), including two alleles of *sub* that were identified in a screen for female sterile mutations (Schupbach and Wieschaus, 1989) and one dominant allele (Moore *et al.,* 1994). sub^{1794} is a fertile hypomorph, whereas sub^{131}, sub^{202}, sub^1, and sub^{HM26} are female sterile alleles. As described previously (Giunta *et al.,* 2002), sub^{131} and sub^{202} were generated by excision of a P-element and delete most of the *sub* coding region. Sequencing demonstrated that sub^1 creates a stop codon and sub^{HM26} causes a missense mutation (Figure 1). Sequencing of these *sub* mutations was performed by PCR amplification from *sub* mutant homozygotes followed by blunt-end cloning into the pT7Blue vector (Perfectly Blunt cloning system, Novagen, Madison, WI). At least two mutant DNA clones and another from a strain of the same genetic background were sequenced at the University of Medicine and Dentistry of New Jersey sequencing facility. Sequences were analyzed using the Wisconsin Package Version 10.0 (Genetics Computer Group).

ncd^1 is a deletion of the coding region and is fertile (Yamamoto *et al.,* 1989), $tacc^{stella592}$ is a female sterile mutant (Lee *et al.,* 2001), and $polo^1$ is a hypomorphic female sterile mutant (Riparbelli *et al.,* 2000). γ-$Tub37C^1$ and γ-$Tub37C^3$ are female sterile alleles in one of the two *Drosophila* γ-tubulin genes (Tavosa-

nis *et al.,* 1997) and α-$Tub67C^1$ and α-$Tub67C^2$ are females sterile alleles of a specialized α-tubulin gene (Matthews *et al.,* 1993).

Germ-line Clone Analysis of Incenp

Drosophila Incenp mutations cause early larval lethality (M. Carmena, personal communication). To study the role of *Incenp* during female meiosis, we generated mutant germline clones in P{hsFLP}12/+; P{FRT(w^{hs})}G13 Incenp^{EP2340}/P{FRT(w^{hs})}G13 P{ovo^{D1-18}}2R females. These females were generated by crossing P{hsFLP}12; P{FRT(w^{hs})}G13 Incenp^{EP2340}/CyO females to +/Y; P{FRT(w^{hs})}G13 P{ovo^{D1-18}}2R/CyO males for 2 d ,followed by heat shock for 1 h at 37°C on days 3 and 4. The P{hsFLP}12/+; P{FRT(w^{hs})}G13 Incenp^{EP2340}/P{FRT(w^{hs})}G13 P{ovo^{D1-18}}2R females were expected to lack mature oocytes because of the dominant *ovo^{D1}* mutation. If a mitotic recombination event occurred, germline cells homozygous for *incenp^{EP2340}* would be produced. However, in no cases were oocytes observed from ~50 heat shocked females. In contrast, most of the 23 females in a control experiment without the *Incenp^{EP2340}* mutation produced mature oocytes. These results suggest that *Incenp* is required for early stages of oocyte development.

Generation of the SUB Antibody

A carboxy-terminal fragment of *sub* encoding amino acids 498–628 was cloned from a cDNA (Stapleton *et al.,* 2002) as an *Eco*RI-*Xho*I fragment into pET30A (Novagen) and expressed in *E. coli* BL(21)DE3. This fragment contains the poorly conserved C-terminal domain of *sub* that follows the motor domain. The fusion proteins were purified using His-binding Ni^{2+} binding resin columns (Novagen) under denaturing conditions. After electroelution, the proteins were concentrated, dialyzed into 50 mM HEPES pH 7.5 and used to raise antibodies in rats (Covance, Denver, PA).

Western Blotting and Immunofluorescence Microscopy

Total ovary protein was isolated by dissecting whole ovaries from yeasted females in phosphate-buffered saline and then grinding and boiling them in SDS gel loading buffer. Protein from ~2–3 ovaries was loaded per lane. The rat anti-SUB primary antibody was used at 1:5000 and the secondary anti-rat HRP antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was used at 1:5000. The secondary was detected using ECL reagents (Amersham, Piscataway, NJ).

For immunofluorescence, stage 14 oocytes were collected from 3- to 7-d-old yeast-fed females and fixed as described previously and mounted in Vectashield (Vector Laboratories, Burlingame, CA; Theurkauf and Hawley, 1992; McKim *et al.,* 1993). Anaphase oocytes were collected by allowing females to lay eggs on grape juice agar plates for 20 min after a short precollection period. The embryos were dechorionated in 50% bleach for 2 min and then fixed in cold heptane/methanol (Rothwell and Sullivan, 2000). Oocytes were stained for DNA with Hoescht and for microtubules with anti-tubulin monoclonal antibody DM1A (at 1:50) directly conjugated to FITC (Sigma, St. Louis, MO). When examining *sub* mutant oocytes, heterozygotes for protein null alleles, either sub^1/sub^{131} or sub^1/sub^{202}, were often used. Heterozygotes were used to eliminate potential genetic background effects but the same results were observed in sub^1, sub^{131}, or sub^{202} homozygotes. The rat anti-SUB antibody was used at 1:75 combined with either a Cy3 or Cy5 anti-rat secondary antibody adsorbed against a range of mammalian serum proteins including mouse and rabbit (Jackson Laboratories, 112-165-167 and 112-175-167). Additional primary antibodies were TACC (1:75), AurB (1:250), INCENP (1:250), RACGAP50C (1:100), POLO (1:15), and MEI-S332 (1:1000) with Cy3-conjugated secondary antibodies (Jackson Laboratories, West Grove, PA). NCD was observed using a GFP fusion protein (Endow and Komma, 1997). Images were collected on a Leica TCS SP confocal microscope (Deerfield, IL) with a 63×, NA 1.3 lens. Images are shown as maximum projections of complete image stacks followed by cropping in Adobe Photoshop (San Jose, CA).

RESULTS

SUB Is a Kinesinlike Protein with Similarities to the MKLP1 Family

We previously suggested that SUB (Giunta *et al.,* 2002) and Pavarotti (PAV; Adams *et al.,* 1998) are two *Drosophila* kinesinlike proteins in the MKLP1 (or Kinesin 6) family (Nislow *et al.,* 1992). Although this family was originally defined by MKLP1, PAV, and their orthologues (Dagenbach and Endow, 2004), sequence and functional studies suggest it could also include paralogs such as MKLP2 (formerly RabK6; Neef *et al.,* 2003). In addition, SUB (referred to as DmKlp54E) has been placed on a branch close to the MKLP1 group in a phylogenetic tree derived from the alignment of kinesin motor domain sequences (Dagenbach and Endow, 2004), and there are several conserved amino acids in the neck-

linker region of all four proteins that are not present in other kinesins (Figure 1). PAV and MKLP1 have the highest level of amino acid identity or similarity and are probably orthologues. Similarly, there are several identical or similar amino acids in the neck-linker regions of SUB and MKLP2 that are not found in other kinesins. This includes the serine residue in MKLP2 that is phosphorylated by Polo kinase (Neef *et al.*, 2003) and the corresponding acidic residue at −2 that is often found at Polo kinase sites. Furthermore, although all four of these MKLP1 homologues have nonconserved N-terminal domains of ~100 amino acids, this domain is basic in PAV and MKLP1 but acidic in SUB and MKLP2. These sequence comparisons and the functional studies described here and in Neef *et al.* (2003) raise the possibility that SUB is the *Drosophila* ortholog of MKLP2.

SUB Localizes to the Central Spindle during Female Meiosis I

Antibodies were raised against the poorly conserved C-terminus of the SUB protein that follows the motor domain (Figure 1). These antibodies recognize an ~75-kDa band on a Western blot that is absent in homozygotes of the null alleles *sub1* and *sub131* (Figure 1C). To examine the localization of SUB during meiosis, we stained mature *Drosophila* oocytes (stage 14), which in wild type, are arrested at metaphase I.

Our identification of prometaphase and metaphase spindles was based on previous studies of fixed (Theurkauf and Hawley, 1992) and living oocytes (Matthies *et al.*, 1996; Skold *et al.*, 2005). There is no congression to a metaphase plate in *Drosophila* oocytes. Instead, the chromosomes come together and condense into a ball to form the karyosome much earlier in oogenesis. After nuclear envelope breakdown (NEB), the chromosomes in the karyosome initiate spindle formation. The initial phases of spindle assembly are characterized by disorganized arrays of microtubules emanating from the karyosome. Once a bipolar spindle forms, it remains stable until the onset of anaphase when the oocyte is activated by passage through the oviduct. As in previous studies (Theurkauf and Hawley, 1992; McKim *et al.*, 1993), staining for tubulin in wild-type oocytes revealed that metaphase I spindles have a prominent band of microtubules that run pole-to-pole and do not terminate at the chromosomes. The bright staining in the middle of this region probably represents the antiparallel overlap of microtubules. We will refer to this region as the meiotic metaphase central spindle (MMCS) in order to distinguish it from the central spindle or midzone present in anaphase of mitotic cells.

Staining of wild-type oocytes with SUB antibodies revealed that SUB protein was found exclusively in the MMCS (Figure 2, A and B). Although SUB always colocalized with tubulin staining, it was also closely associated with the chromosomes. In many spindles, 3D reconstruction revealed that SUB staining appeared more concentrated on one side or in some cases in two clusters on either side of the karyosome. Whether this pattern reflects intrinsic features of the spindle, such as asymmetry within the karyosome, or stochastic properties of central spindle formation, remains to be determined. SUB staining was not detected in the null alleles *sub1* and *sub131* at prometaphase (see below) or metaphase (Figure 2, C and D), confirming the specificity of the antibody.

Because genetic and cytogenetic studies (Giunta *et al.*, 2002) have suggested that SUB has an important role in spindle assembly, we investigated when SUB first appears on the meiotic spindle. SUB appeared from the earliest time points after NEB, even on early prometaphase spindles that

were characterized by a disorganized array of microtubules (Figure 2A). SUB staining at this stage colocalized with microtubules in the region that will become the MMCS. Thus, SUB localizes to the central spindle before bipolar spindle formation.

Stage 14 oocytes arrest at meiotic metaphase I; therefore, we used two methods to observe anaphase I spindles. First, we looked at *mei-218* mutants in which the metaphase arrest is bypassed (McKim *et al.*, 1993). Second, we collected embryos after short periods of egg laying, which allows for the isolation of oocytes undergoing the early meiotic divisions. In both of these experiments, SUB remained in the spindle midzone as the chromosomes moved toward the poles (unpublished data and Figure 2E, respectively).

SUB Is a Mitotic Protein

Because *sub* mutants are viable, it is possible that *sub* has a meiosis-specific function and is only required for the unique situation of assembling acentrosomal spindles during female meiosis. There is, however, genetic evidence that *sub* is expressed in mitotically dividing cells (Moore *et al.*, 1994; Giunta *et al.*, 2002). Consistent with these genetic observations, we observed SUB staining in mitotically dividing cells of the embryo (Supplementary Figure 1). Similar to the metaphase oocytes, SUB protein was observed at the middle of the spindle. SUB protein has also been observed at metaphase of larval neuroblast cells (B. Redding and K. McKim, unpublished results). Thus, SUB protein may be a component of most or all metaphase spindles in *Drosophila.* Although SUB has an important function in pronuclear fusion, some *sub* mutant embryos commence but never complete the early embryonic divisions (Giunta *et al.*, 2002). Analysis of these embryos shows evidence of spindle assembly defects and aneuploidy, indicating that *sub* also has a role in mitotic spindle function (Supplementary Figure 1).

SUB Is Required for Central Spindle Formation

We previously reported that *sub* null mutant females develop monopolar and tripolar spindles in a majority of meiosis I figures (see Table 4 in Giunta *et al.*, 2002) and are sterile because of a requirement during early embryogenesis. In addition, *sub* hypomorphic mutants exhibit a high frequency of homologous chromosome nondisjunction at meiosis I (Giunta *et al.*, 2002). Considered along with the SUB staining pattern, these results suggest that the MMCS may have an important role for in bipolar spindle formation and chromosome segregation. For this study, we reexamined the effects on central spindle formation with a new *sub* mutant data set. Similar to our previous report, 14/17 *sub1/sub131* mutant oocytes had abnormal spindle organization compared with only 4/36 in wild-type oocytes. Among the 14 abnormal *sub1/sub131* spindles, 3 were monopolar, 9 were tripolar, and 2 had other problems such as fraying of the microtubules. Thus, *sub* mutant spindles have a defect in maintaining or establishing bipolarity (Figure 2C), although in a minority of cases, relatively normal bipolar spindles were observed (Figure 2D). Notwithstanding these defects, the ability to taper microtubules into poles is not usually affected in *sub* mutants.

Given the localization of SUB, we examined the MMCS in *sub* mutants. Strikingly, all *sub* mutant spindles, even those that were bipolar, lacked metaphase central spindle tubulin staining (Figure 2, C and D, Supplementary Figure 2). Although the amount of central spindle microtubules was variable in wild type, the prominent bundles of central spindle microtubules often observed in wild-type oocytes were never observed in *sub* mutant oocytes. Instead, there

were often small gaps of microtubules staining in the middle of the spindle or only limited evidence of antiparallel microtubule overlap. Additional evidence that the central spindle fails to form in *sub* mutants is that midzone proteins such as Incenp and AurB did not localize to this region in *sub* mutants (see below). These results suggest that the MMCS is normally organized or maintained by SUB.

SUB Localization Does Not Require Bipolar Spindle Formation

To address whether SUB localization was dependent on bipolar spindle formation, SUB localization was examined in mutants with disrupted spindle organization, including *ncd, tacc, α -tub67C*, and *γ -tub37C* (Figure 3). Like *sub, ncd* encodes a kinesin required for bipolar spindle formation (Hatsumi and Endow, 1992; Matthies *et al.,* 1996). Double mutants with the hypomorph *sub^{1794}* have a meiotic phenotype similar to that of the single mutants (Giunta *et al.,* 2002). *tacc* has an important role in meiotic bipolar spindle formation with some similar phenotypes to *sub* mutants (Cullen and Ohkura, 2001). *γ-Tub37C* is one of two *Drosophila* γ-tubulin isoforms and has previously been shown to have a role in female meiotic spindle formation (Tavosanis *et al.,* 1997). Mutants of the female-specific isoform *α-Tub67C* are sterile, although defects in spindle formation have not previously been shown (Matthews *et al.,* 1993). Despite severe defects in bipolar spindle formation, these mutants exhibited SUB staining in association with the central spindle. In addition, we have documented meiotic spindle defects in *α -tub67C* mutant females for the first time. These results suggest that the MMCS and SUB staining are not dependent on bipolar spindle formation. Instead, SUB most likely localizes and functions before the formation of a bipolar spindle. SUB may simply localize to any region of the spindle containing antiparallel microtubules.

Polo Localization during Female Meiosis

One candidate for regulating SUB localization is Polo kinase because, as described above, the SUB ortholog MKLP2 is phosphorylated by Polo in human cells. Observing the effects of *polo* null mutants is problematic because the homozygotes are lethal. We were, however, able to examine females with a viable but female sterile allele heterozygous to a null allele and found that SUB staining was normal (Figure 3). Indeed, these mutants did not have gross defects in meiotic spindle formation, except for a possible reduction in kinetochore microtubules. The absence of an effect on SUB staining in *polo* mutants is consistent with the results that in HeLa-S3 cells, MKLP2 is a target for Polo phosphorylation but this is not required for localization (Neef *et al.,* 2003).

Because MKLP2 is required for the localization of Polo kinase to the midzone in HeLa-S3 cells (Neef *et al.,* 2003), we examined Polo localization during *Drosophila* meiosis. In addition, *Drosophila* Polo has been shown to have a localization pattern similar in mitotic cells to proteins such as AurB and Incenp (Logarinho and Sunkel, 1998), which, as described below, localize to the MMCS in *Drosophila* oocytes and depend on SUB activity. Polo antibody staining in wild-type metaphase *Drosophila* oocytes appeared weakly in the MMCS and was not visible in all images. In contrast, it was stronger in foci that colocalized with the DNA (Figure 4A). These Polo foci were probably the kinetochores, consistent with previous studies of larval neuroblasts. In these mitotically dividing cells, Polo was localized to kinetochores during metaphase, and midzone staining was strong only during anaphase (Logarinho and Sunkel, 1998). In *sub* mutant oocytes, the foci of Polo staining were still observed, which is consistent with the absence of SUB protein at kinetochores

(Figure 4B). However, the level of Polo staining was variable and may be dependent on spindle structure, because in some *sub* mutant oocytes, particularly those with the most disorganized spindles, Polo staining was weak (Figure 4C).

Midzone Proteins Depend on Subito

Our results suggest that the MMCS has an important function in organizing the meiotic spindle. This led us to examine what other proteins are located in this region of the meiotic spindle and could contribute to microtubule assembly. Several proteins have been localized to the spindle midzone at anaphase of mitotic cells including the passenger proteins AurB/Ial and Incenp. In mitotic cells, the localization pattern of the passenger proteins depends on the mitotic stage. They localize to the centromeres during metaphase and then move to the spindle midzone at anaphase (Adams *et al.,* 2001; Giet and Glover, 2001). We examined the metaphase I localization pattern of AurB and Incenp by antibody staining to determine if the MMCS also contains these proteins and whether they show an early (mitotic metaphase) or late (mitotic anaphase) staining pattern. We also examined RacGap50C, another mitotic midzone component that forms a complex with the SUB paralog PAV (Somers and Saint, 2003).

For Incenp (Figure 4D), AurB (Figure 4E), and RacGap50C (Figure 4G), we found staining similar or identical to SUB. For example, Incenp perfectly colocalized with SUB in the MMCS (Figure 4D), including early prometaphase staining before a bipolar spindle had formed. To determine the relationship of these proteins to the centromeres, we stained with an antibody to MEI-S332 (Moore *et al.,* 1998). SUB and MEI-S332 always occupied distinct regions around the karyosome in both disorganized prometaphase and bipolar metaphase spindles (Figure 4, I and J). By extension, because the midzone proteins and SUB always colocalize, Incenp and AurB do not localize to the centromere regions during meiotic metaphase I. Indeed, the centromeres and MMCS appear to be properly organized and oriented before a bipolar spindle forms.

The localization of these proteins to the MMCS was dependent on *sub* activity. In *sub* mutants, AurB accumulated in a region surrounding the karyosome but not in the region where the MMCS would have been (Figure 4F). Almost identical localization defects were observed with RacGap50C (Figure 4H) and Incenp (unpublished data). This pattern appears to be more extensive than just centromere staining, at least when compared with the MEI-S332 staining and Polo staining described above. Instead, these proteins concentrated in the region where the microtubules are in close proximity to the karyosome. This could occur if, in the absence of a central spindle in *sub* mutants, these proteins concentrate near the plus-ends of microtubules. A similar effect of midzone disruption on AurB and MKLP1 staining has been observed in HeLa cells (Kurasawa *et al.,* 2004). It was suggested that localization toward the plus ends was an intermediate stage in development of the midzone. It is possible these proteins have an affinity for the plus ends of microtubules or other motors, such as the *Drosophila* MKLP1 ortholog Pavarotti, that actively transport them there (Matuliene and Kuriyama, 2002). Unfortunately, we have been unable to test the role of the passenger proteins in acentrosomal spindle formation because *Incenp* appears to be required during early stages of oogenesis (see *Materials and Methods*).

Two Pathways for Meiotic Spindle Formation

Although SUB has an important role in spindle formation, most *sub* mutant spindles retain the ability for form poles.
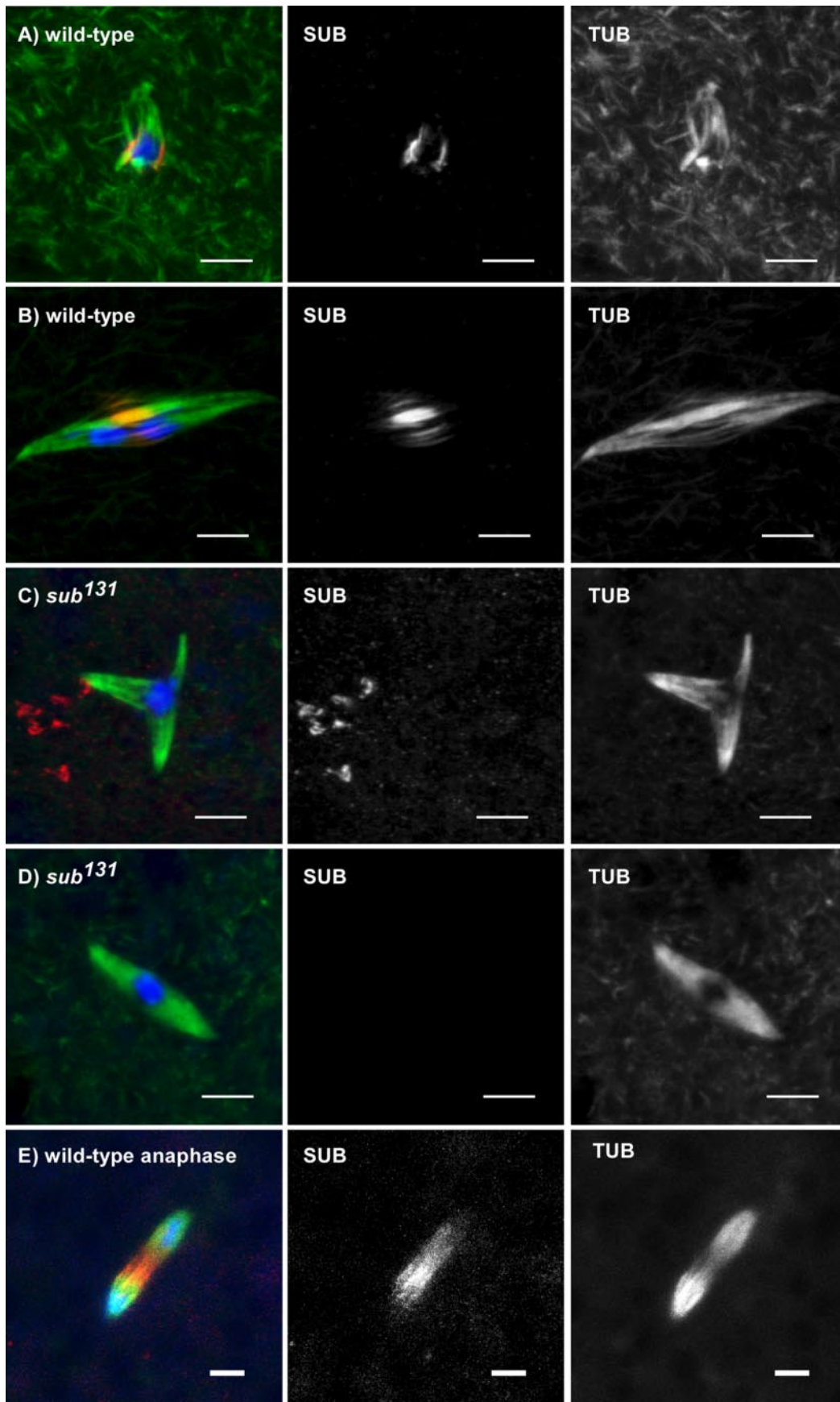
Figure 2. SUB localizes to the meiotic metaphase central spindle in wild-type female meiosis I. SUB staining is in red, tubulin in green, and DNA in blue in addition to gray scale images of the separate SUB and tubulin channels. Before NEB, SUB is excluded from the nucleus
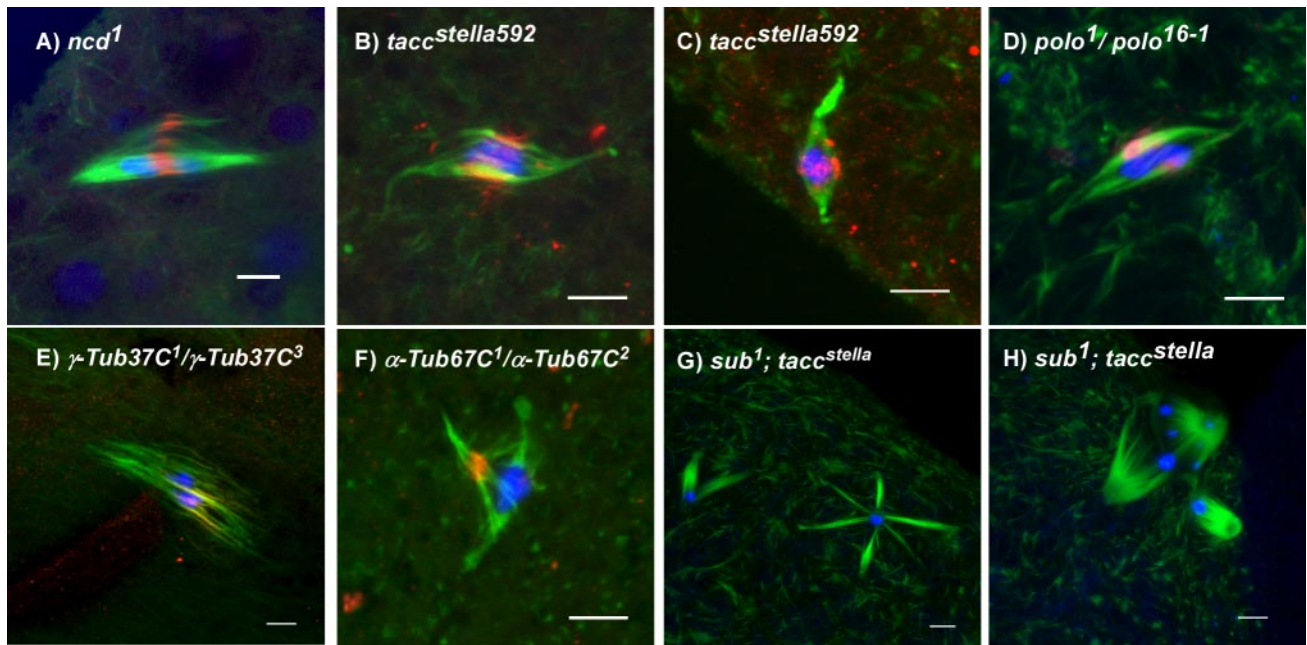
Figure 3. SUB staining in spindle mutants. SUB is still found in the central spindle in a variety of mutants with disorganized meiotic spindles, such as (A) ncd^1, (B and C) $tacc^{stella592}$, (D) $polo^{16-1}/polo^1$, (E) $\gamma\text{-}Tub37C^1/\gamma\text{-}Tub37C^3$ and (F) $\alpha\text{-}Tub67C^1/\alpha\text{-}Tub67C^2$. (G and H) Synergistic effects on spindle formation in *sub; tacc* double mutant oocytes. SUB staining is in red, tubulin in green, and DNA in blue. Scale bars, 5 μm.

Therefore, SUB-independent activities must be functioning to organize spindle poles. TACC and MSPS may have a role in this function because these proteins localize to meiotic spindle poles. Previous genetic and cytological studies have shown that *tacc* and *msps* have an important role in meiotic bipolar spindle formation (Cullen and Ohkura, 2001), and the mutants have been reported to have phenotypes similar to *sub* mutants. To investigate the relationship between TACC and SUB, we examined TACC localization in *sub* mutants and examined the phenotype of double mutant combinations. As reported previously, TACC localized to the acentrosomal spindle poles in wild-type oocytes (Figure 4K). In *sub* mutants, however, the spindle pole staining was weaker and in some cases accumulated near the chromosomes (Figure 4L). Thus, it is possible that the phenotype of SUB mutants may be related to defects in TACC localization.

To test if SUB and TACC function in distinct spindle forming activities or have similar functions during spindle

assembly, we constructed *sub; tacc* double mutants. The sub^1; $tacc^{stella592}$ double mutant had severe spindle formation defects, more dramatic than either single mutant (compare Figure 2C and Figure 3B with Figure 3, G and H). Unlike the single mutants, there were often multiple bundles of microtubules, some associating with chromosomes. Although microtubules were still associating with the chromosomes, most bundles of microtubules were randomly organized. Similar defects were also observed in sub^{1794}/sub^1; $tacc^{stella592}$ but not sub^{1794}/sub^{1794}; $tacc^{stella592}$ females, demonstrating this phenotype was dependent on severe loss of *sub* function. The double mutants appear to retain the ability to assemble kinetochore microtubules. This phenotype could be explained by a combination of *tacc* and *sub* mutant defects: a failure to stabilize the spindle poles (*tacc*) and a failure to organize the microtubules around the chromosomes (*sub*). These results suggest that *sub* and *tacc* contribute to different pathways that function to organize the microtubules of acentrosomal spindles. This is consistent with the observation that SUB and TACC associate with distinct structures or populations of microtubules.

DISCUSSION

Evidence that Subito Belongs to the MKLP1 Family of Kinesinlike Proteins

Meiotic spindle microtubules in most oocytes must be organized without centrosomes to organize the poles. Although the chromosomes play a critical role in spindle formation by capturing free microtubules (replacing the nucleation step of centrosomes), it is not clear what organizes the bundling and elongation of microtubules into a bipolar spindle. Our results suggest that the kinesinlike protein Subito has an important role in *Drosophila* acentrosomal spindle formation, possibly by organizing the prominent central spindle that assembles at meiotic prometaphase. Interestingly, SUB has

Figure 2 (cont). (unpublished data). (A) Prometaphase oocyte: SUB localizes to the presumptive central spindle, even though a mature bipolar spindle has not yet formed. (B) Metaphase I oocyte: A bipolar spindle has formed and SUB staining is associated with the central spindle, which is typically visible as bright bundles of pole-to-pole microtubules. The chromosomes are under tension because they are being pulled toward to the poles but homologues are still connected by chiasmata (visible as thinner DNA staining between the two main masses). (C and D) SUB staining is absent in *sub* null mutant metaphase I oocyte spindles. Although most *sub* mutants spindles have polarity defects (C), some have relatively normal structure (D) (see text). In some cases, nonspecific signals are observed. In either case, however, the bright microtubule staining in the central spindle is greatly reduced in *sub* mutants. (E) SUB localization during meiotic anaphase I. SUB staining remains in the region between the chromosomes as they move toward the poles. Scale bars, 5 μm.
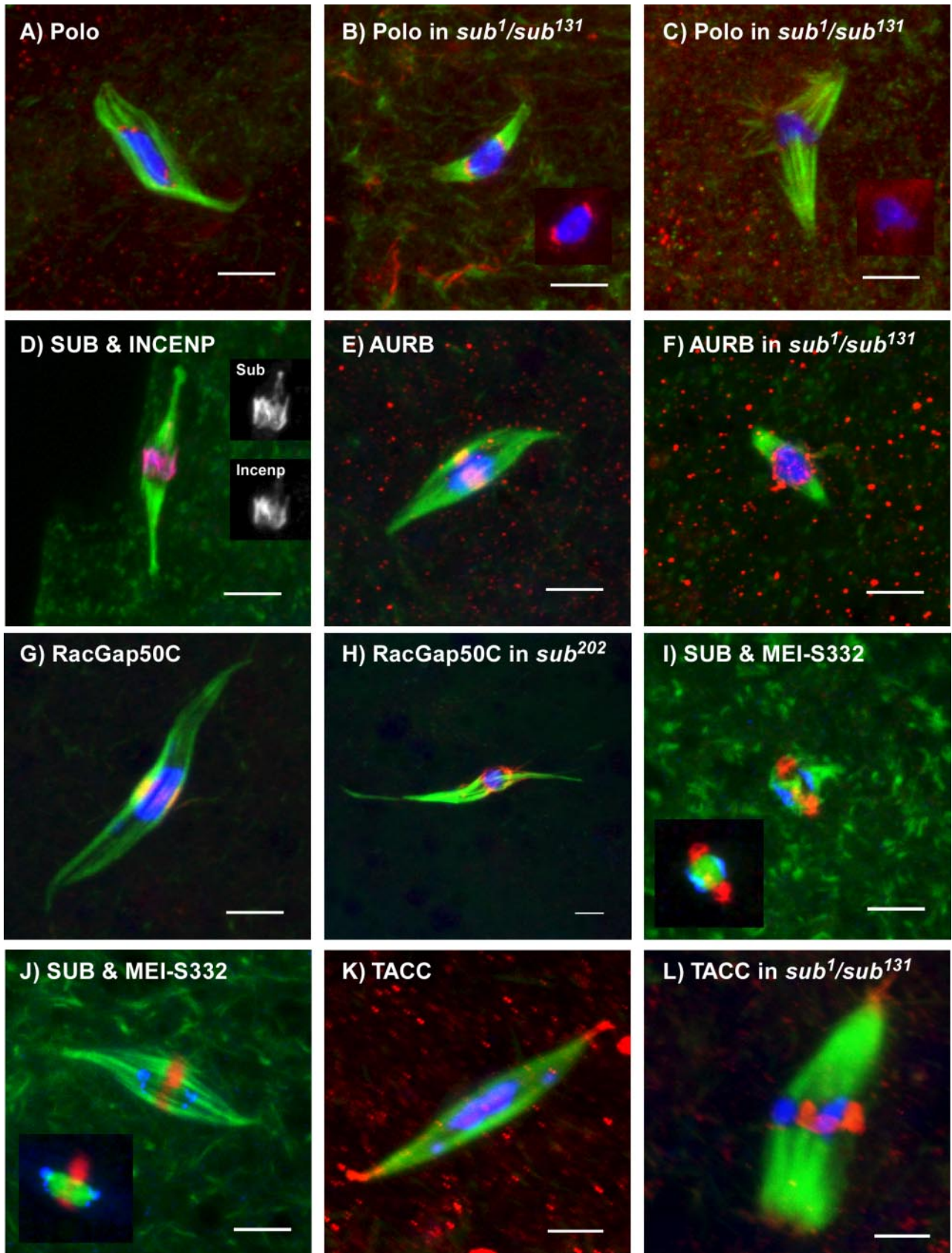
Figure 4. The meiotic metaphase central spindle contains proteins typically found on mitotic midzones. In these wild-type (A, D, E, G, I, J, and K) or *sub* protein null mutant (B, C, F, E,and L) oocytes, microtubules are shown in green and the DNA is i nblue, except in D, I, and J, where blue is another protein. In some cases, images of the less frequentbipolar *sub* mutant spindles were selected to more clearly show the abnormal

several characteristics similar to MKLP2: SUB localizes to a region of antiparallel microtubules, in this case the meiotic metaphase central spindle; it is required for central spindle formation; it is required for the localization of other central spindle proteins, and it has nonmotor domain sequence similarity including amino acids that could be phosphorylated by Polo kinase. Similarly, a phylogenetic tree made from the alignment of kinesin motor domain sequences has SUB in a cluster close to the MKLP1 group (Dagenbach and Endow, 2004). As described below, we suggest these features allow SUB to contribute to the organizing of *Drosophila* acentrosomal spindles by establishing or maintaining the central spindle at prometaphase and metaphase.

Mammalian MKLP1 (Matuliene and Kuriyama, 2002), *Drosophila* PAV (Adams *et al.,* 1998), and the *C. elegans* ortholog ZEN-4 (Raich *et al.,* 1998; Mishima *et al.,* 2002) have been found in the spindle midzone during anaphase and have an important function in cytokinesis. The midzone has been implicated in establishing the placement of the cytoplasmic furrow, although there are exceptions (D'Avino *et al.,* 2005). Furthermore, MKLP1 was found to bundle microtubules and to promote anti-parallel sliding in vitro (Nislow *et al.,* 1992). This is consistent with its localization in the spindle midzone, where microtubules overlap in antiparallel orientation. In addition, from the direction of the antiparallel sliding of microtubules it was concluded that MKLP1 is a plus-end-directed motor. Although less is known about MKLP2, it is also required for the spindle midzone and cytokinesis, and like other MKLP1 family members, the protein accumulates at the midzone (Hill *et al.,* 2000; Fontijn *et al.,* 2001; Neef *et al.,* 2003). Our characterization of SUB suggests that organisms with two MKLP1-like proteins are not restricted to vertebrates.

Two observations suggest that the MMCS is mostly or entirely absent in *sub* mutants. First, *sub* mutant spindles lack the prominent band of antiparallel microtubules arising from the overlap of pole to pole spindle fibers. Second, proteins that normally associate with this region, such as Incenp, AurB, and RacGap50C, are absent in *sub* mutant oocytes. Nonetheless, it is difficult to rule out if other proteins are able to promote formation of a thin and fragile central spindle in *sub* mutants. A candidate with this function could be PAV but, because of its lethal phenotype and because *pav* mutant germlines do not make oocytes (Minestrini *et al.,* 2002), we could not determine if PAV contributes to the meiotic spindle assembly. However, the severe defect in meiotic central spindle formation in *sub* mutants

suggests that PAV cannot compensate in a significant way for the absence of SUB.

Passenger Proteins Appear at the Midzone during Female Meiotic Metaphase I

Our studies suggest a new role for the central spindle in bipolar spindle formation and chromosome segregation. The localization pattern of central spindle components, such as members of the passenger protein complex AurB and Incenp, is consistent with the idea that a central spindle is forming precociously in oocytes. Although it is typical in *Drosophila* and human mitotic cells for AurB and Incenp to initially associate with centromeres and then move to the midzone at anaphase (Adams *et al.,* 2001; Giet and Glover, 2001; Gruneberg *et al.,* 2004), in *Drosophila* oocytes, these proteins appear on the central spindle much earlier in prometaphase. Furthermore, the meiotic division of *Drosophila* oocytes appears to skip the stage in mitotic cells (metaphase) where passenger proteins associate with centromeres. We have not observed SUB, AurB, or Incenp at the centromeres during female meiosis; they appear to be associated only with the nonkinetochore microtubules. This appears to be specific only to a subset of midzone proteins. Polo exhibited kinetochore staining typical of mitotic metaphase at meiotic metaphase I. In addition, KLP3A, a kinesinlike protein that associates with the anaphase midzone in mitotic cells, has been reported to stain along the length of female meiotic spindles and only moves to the midzone at anaphase (Williams *et al.,* 1997).

AurB and Incenp localization to the oocyte MMCS depends on SUB. Similarly, Incenp and AurB midzone localization depends on MKLP2 in mammalian mitotic cells, and MKLP2 may even have a direct interaction with AurB (Gruneberg *et al.,* 2004). An important aspect of SUB function could be to recruit proteins like AurB in order to stimulate chromosome–microtubule interactions (Gassmann *et al.,* 2004; Sampath *et al.,* 2004). Consistent with this model, phosphorylation of the microtubule-destabilizing kinesin MCAK by AurB stimulates chromatin induced spindle assembly in *Xenopus* extracts (Ohi *et al.,* 2004). We have not, however, been able to determine the role, if any, of the passenger proteins in meiotic spindle formation.

Antiparallel Microtubules Organize the Meiotic Acentrosomal Spindle

Previous models for acentrosomal spindle formation suggested that the process was initiated by the capture of free microtubules by the chromosomes followed by bundling and sorting of microtubules by minus-end-directed motors to form the poles (Matthies *et al.,* 1996; Skold *et al.,* 2005). However, these models lack a mechanism to ensure that the kinetochore microtubules are oriented toward only one of two poles. For example, how are the two half spindles oriented relative to each other and what limits the spindle to have only two poles? On the basis of the localization pattern of SUB and the phenotype of *sub* mutants, we present a model for acentrosomal spindle formation in *Drosophila* oocytes that addresses these questions (Figure 5). We propose that a structure composed of antiparallel microtubules is organized during prometaphase. The axis of the spindle is defined by this structure, the MMCS, which provides the scaffold on which to build a bipolar spindle during prometaphase and metaphase. Proteins that localize to the spindle poles have a separate function in spindle pole formation and the functions of the central spindle or spindle-poles are partially redundant for maintaining spindle integrity and establishing poles. As the *sub; tacc* double mutant phenotype

Figure 4 (cont). localization of a midzone protein and that this was due to the absence of SUB rather than spindle structure. (A) POLO (red) localizes to spots on the chromosomes, which are most likely the kinetochores. (B) Polo staining can still be observed in some *sub* mutant oocytes. (C) In other *sub* mutant oocytes with more disorganized spindles, however, Polo staining was reduced. The insets in B and C have the microtubule staining removed in order to see Polo staining clearly. (D) Incenp (red) colocalizes with SUB (blue) in the central spindle. The inset images show the separate channels for SUB and Incenp. (E) AurB (red) localizes to the meiotic central spindle. (G) Similar results were obtained using antibodies against RacGap50C. (F and H) In the absence of SUB, midzone proteins (AurB and RacGap50C are shown) accumulate around the karyosome instead of the MMCS. (I and J) MEI-S332 (blue) is a marker for the meiotic centromeres and this staining does not overlap with SUB (red). The inset shows SUB and MEI-S332 with DNA in green. (K) TACC (red) normally localizes to the spindle poles. (L) In *sub* mutant oocytes, there is less staining at the poles and in some cases TACC it is observed near the center of the spindle. Scale bars, 5 μm.

A) Microtubule capture, establish midzone
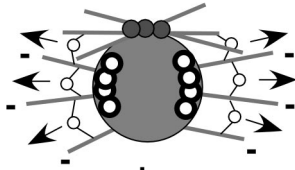
B) Bundle parallel MTs, establish poles

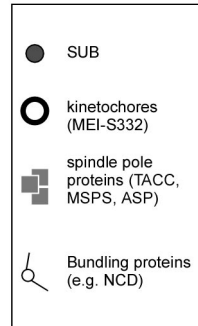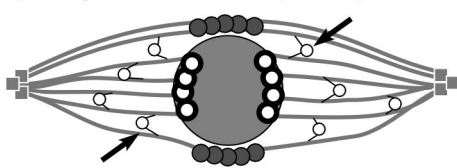C) Elongation of MTs to separate poles

Figure 5. Model for acentrosomal spindle formation in *Drosophila* oocytes. (A) The chromosomes enter prometaphase clustered together in a ball, the karyosome, and capture microtubules that are not organized into a bipolar array. At this time, SUB protein accumulates on microtubules adjacent to the karyosome. (B) Motor proteins, possibly involving minus-end-directed motor proteins such as NCD, bundle parallel microtubules, and taper them into defined poles. In parallel with this process, the spindle is stabilized by proteins that accumulate at the poles. Proteins that localize to the female meiotic spindle poles include Asp (Riparbelli *et al.,* 2002), MSPS and TACC (Cullen and Ohkura, 2001). (C) The direction of elongation/bundling/sliding is dictated by the metaphase central spindle. Critical to the model is that the orientation of the kinetochore microtubules is established through interactions (via crosslinking) with the central spindle. Examples of these interactions are shown with by the arrows. The "backbone" structure provided by the central spindle defines the long axis of the spindle, ensuring that the two poles form on opposite sides of the chromosomes and prevents additional poles from forming. In fixed images, the SUB-staining region appears localized to one side of the karyosome, but in other images there are two or more clusters of SUB staining. Elongation of the spindle could involve the capture of additional microtubules to lengthen the spindle. Thus, spindle elongation could occur via microtubule capture and bundling with or without motor activity, microtubule sliding or growth at the plus ends.

demonstrates, in the absence of these structures, the spindle loses all organization.

SUB and the MMCS could be required at several points in spindles assembly. The MMCS may have a role in the transition from prometaphase, with its disorganized microtubules around the karyosome, to metaphase with a bipolar spindle. The interaction of kinetochore microtubules with pole-to-pole microtubules of the MMCS via parallel microtubule bundling could determine the formation and relative orientation of only two poles (Figure 5). In addition, SUB probably has a role in maintaining spindle bipolarity. By maintaining the MMCS, SUB could attenuate the activity that is active to establish poles during prometaphase but

must be inactive during metaphase. Repeated attempts at new spindle pole formation could generate extra poles in *sub* mutants. Indeed, we observed what appears to be newly formed short half spindles and the ectopic appearance of TACC in the middle of the spindle of *sub* mutants, suggesting that de novo pole formation can occur at metaphase. The presence of monopolar spindles could occur if the MMCS has a role in maintaining half spindles, resulting in the collapse of half spindles at metaphase in *sub* mutants. This dynamic portrayal of the meiotic spindle in *sub* mutants is consistent with real time observations in *ncd* mutants (Matthies *et al.,* 1996). Although wild-type spindles appear to be stable structures over long periods of time, *ncd* mutant spindles are dynamic structures, where bipolar spindles will form only to lose their organization to become apolar, monopolar, or even completely disassemble and then reform again.

The role for the MMCS described above in coordinating spindle pole formation can explain the *sub* genetic and cytological mutant phenotypes. However, we have not ruled out other roles for SUB in chromosomes segregation. An alternative is that SUB contributes to a balance of forces between pushing apart or pulling together the spindle poles (Sharp *et al.,* 1999). In *sub* mutants, this could lead to a defect in spindle pole positioning. Two observations argue against this hypothesis. First, the *sub* mutant phenotype is not alleviated by defects in *ncd* (Giunta *et al.,* 2002), in contrast to Klp61F (Wilson *et al.,* 2004), which has this role in mitotic cells. Second, this function does not easily explain why *sub* mutants often have multiple poles, whereas the length of the half spindles are not dramatically shorter than wild-type. We also cannot rule out a role for SUB in facilitating interactions between the chromosomes and the microtubules. This could have a role in aligning the homolog pairs at metaphase I, similar to what has been proposed for the chromokinesin NOD (Zhang *et al.,* 1990; Theurkauf and Hawley, 1992). Although in *nod* mutants, the nondisjunction phenotype is not associated with defects in spindle organization.

We thus favor a model in which SUB directly contributes to bipolar spindle formation by organizing and/or stabilizing the MMCS. An important implication of this model is that, to compensate for the absence of centrosomes, the oocyte has modified the regulation of the central spindle so that it appears earlier in order to direct spindle formation. This is a novel function for the central spindle and contrasts with the suggestion for mitotic cells that the midzone accumulation of Incenp and AurB needs to be inhibited until anaphase (Pereira and Schiebel, 2003; Mishima *et al.,* 2004). An important question that we are currently investigating is what controls SUB localization. One possibility is that the concentration of a factor that promotes microtubule assembly, such as ran-GTP (Kahana and Cleveland, 1999), is greatest in one region of the karyosome. Given the SUB/MKLP2 conservation of sequence and function, it will be interesting to determine if the central spindle has an important role in organizing the acentrosomal spindles of oocytes in mammals and other animals or in plants. Furthermore, as described here for embryos and will be described elsewhere for other mitotic cells (B. Redding and K. McKim, unpublished results), SUB also has a role in spindle assembly of mitotic cells. This is consistent with the hypothesis that acentrosomal spindle assembly occurs through the modification of functions already present in mitotic cells.

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