Kinesin 6 family member Subito participates in mitotic spindle assembly and interacts with mitotic regulators

Jeff M. Cesario, Janet K. Jang, Bethany Redding, Nishit Shah, Taslima Rahman and Kim S. McKim*

Waksman Institute and Department of Genetics, Rutgers, The State University of New Jersey, Piscataway, NJ 08854-8020, USA

*Author for correspondence (e-mail: mckim@rci.rutgers.edu)

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Summary

Drosophila **Subito is a kinesin 6 family member and ortholog of mitotic kinesin-like protein (MKLP2) in mammalian cells. Based on the previously established requirement for Subito in meiotic spindle formation and for MKLP2 in cytokinesis, we investigated the function of Subito in mitosis. During metaphase, Subito localized to microtubules at the center of the mitotic spindle, probably interpolar microtubules that originate at the poles and overlap in antiparallel orientation. Consistent with this localization pattern, *subito* mutants improperly assembled microtubules at metaphase, causing activation of the spindle assembly checkpoint and lagging chromosomes at anaphase. These results are the first demonstration of a kinesin 6 family member with a function in mitotic spindle assembly, possibly involving the interpolar microtubules. However, the role of Subito during mitotic anaphase resembles other kinesin 6 family members. Subito localizes to the spindle midzone at anaphase and is required for the localization of Polo, Incenp and Aurora B. Genetic evidence suggested that the effects of *subito* mutants are attenuated as a result of redundant mechanisms for spindle assembly and cytokinesis. For example, *subito* double mutants with *ncd*, *polo*, *Aurora B* or *Incenp* mutations were synthetic lethal with severe defects in microtubule organization.**

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Key words: Mitosis, Microtubule, *Drosophila*, Central spindle, Kinesin-like protein, Checkpoint

Introduction

In most animal cells the role of centrosomes in mitotic spindle formation has been described by the 'search and capture' hypothesis (Kirschner and Mitchison, 1986). Characterized by 'outside-in' spindle formation, the centrosomes nucleate and organize bundles of astral microtubules that search the cytoplasm for kinetochores. Chance contacts between centrosomal microtubules and a kinetochore results in transient binding that remains unstable until the two paired kinetochores of each replicated chromosome achieve bipolar attachment (Nicklas, 1997). At this stage, each chromatid is pulled in opposite directions while sister-chromatid cohesion resists this force, resulting in tension on the kinetochores and stabilization of the chromosomes at the metaphase plate. This model explains both chromosome congression to the metaphase plate and spindle assembly itself. If spindle assembly is defective, a checkpoint is activated, which delays the progression of mitosis into anaphase. Factors that signal the spindle assembly checkpoint include reduced microtubule occupancy of the kinetochores or the absence of tension on the kinetochores (Logarinho et al., 2004).

In contrast to this conventional model, there are situations where spindle formation occurs in the absence of centrosomes, such as in the oocytes of many animals and some plant cells (for reviews, see Compton, 2000; McKim and Hawley, 1995) (Wadsworth and Khodjakov, 2004). These examples indicate the presence of centrosome-independent spindle assembly pathways in cells lacking centrosomes. Similar pathways may also be found in cells with centrosomes (e.g. Maiato et al., 2004; Wadsworth and Khodjakov, 2004), however, owing to redundancy, the role of centrosome-independent spindle assembly pathways in cells with centrosomes has been difficult to study. A defect in the centrosome-independent spindle assembly pathway may go unnoticed because of the ability of the centrosomes to form bipolar spindles, even in the absence of chromosomes (e.g. Brunet et al., 1998; Bucciarelli et al., 2003; Faruki et al., 2002; Walczak et al., 1998).

The *Drosophila* oocyte, which lacks centrosomes, is a genetically tractable system for the identification of genes required for acentrosomal spindle assembly. Using this system, we identified Subito as a kinesin-like protein required for meiotic spindle assembly in *Drosophila* females (Giunta et al., 2002). *Subito* (*sub*) mutants exhibit high levels of meiotic nondisjunction, which is associated with the formation of monopolar and tripolar spindles in metaphase I oocytes. Immunolocalization studies have shown that Subito is present on microtubules before bipolar spindle formation and associates with interpolar microtubules at metaphase (Jang et al., 2005). Interpolar microtubules do not terminate at the kinetochores, but instead originate at both poles and overlap in the middle of the spindle in antiparallel orientation (Mastronarde et al., 1993). During anaphase, these microtubules form the midzone, which has an important role in cytokinesis (e.g. Giansanti et al., 1998; Neef et al., 2006). Our results suggest that interpolar microtubules have an important role in organizing acentrosomal spindle formation in *Drosophila* oocytes.

These localization studies are consistent with the conclusion

from sequence analysis showing that Subito is a member of the kinesin 6 group of kinesin-like proteins (Miki et al., 2005). Kinesin 6 proteins are believed to bundle antiparallel microtubules (Nislow et al., 1992) and the family includes mitotic kinesin-like proteins 1 (MKLP1) and 2 (MKLP2). Relative to other kinesin-like proteins, members of the kinesin 6 family have a unique insertion of approximately 65 amino acids in loop L6 of the motor domain. In addition, kinesin 6 proteins fall into two subgroups, the KIF20-MKLP2-Subito group, present in a wide range of animals, fungi and slime molds, and the KIF23-MKLP1-Pavarotti group present only in animals (Miki et al., 2005). Loss of MKLP1 or MKLP2 results in a disorganized midzone at anaphase and subsequent cytokinesis defects (Neef et al., 2003).

Since Subito is required for acentrosomal spindle formation in oocytes, we investigated whether it is also required for spindle formation in mitotic cells. *Sub* null mutants are viable, demonstrating that it does not have an essential role in spindle formation during mitosis (Giunta et al., 2002). Cytological and genetic analysis of *sub* single mutants and double mutants with *BubR1*, *polo*, *Aurora B* and *Incenp* indicate that Subito has a role during spindle assembly in metaphase. This function has not previously been attributed to a kinesin 6 family member. These results also demonstrate that Subito is required for organizing the microtubules in the midzone during anaphase, a function consistent with studies of other kinesin 6 family members including MKLP1 (Matuliene and Kuriyama, 2002) and MKLP2 (Fontijn et al., 2001; Neef et al., 2003) in humans, ZEN-4 in *Caenorhabditis elegans* (Raich et al., 1998) and Pavarotti (Adams et al., 1998) in *Drosophila*, which are all required for cytokinesis.

Results

Subito localizes to interpolar metaphase microtubules

To determine whether Subito is present on mitotic spindles, we compared Subito and Tubulin staining in mitotically dividing cells of the larval brain. Subito was most concentrated with tubulin fibers at the center of the metaphase spindle. These are probably interpolar microtubules, which run from opposite poles to the middle of the spindle where they overlap in antiparallel orientation (Compton, 2000; Mastronarde et al., 1993). Subito appeared to be concentrated in foci on interpolar fibers or extending for short distances along the microtubule (Fig. 1A). As the chromosomes moved to the spindle poles during anaphase, Subito remained in the center of the spindle, in the region destined to become the midzone (Fig. 1B,C). No Subito staining was observed in mutant larval neuroblasts homozygous for the null alleles sub^{1} or sub^{131}. Furthermore, when microtubules were depolymerized following colchicine treatment, specific Subito staining was lost. In particular, the foci of staining were not observed, although the colchicine-treated cells did have considerable delocalized Subito staining. These results suggest that Subito localization is microtubule dependent.

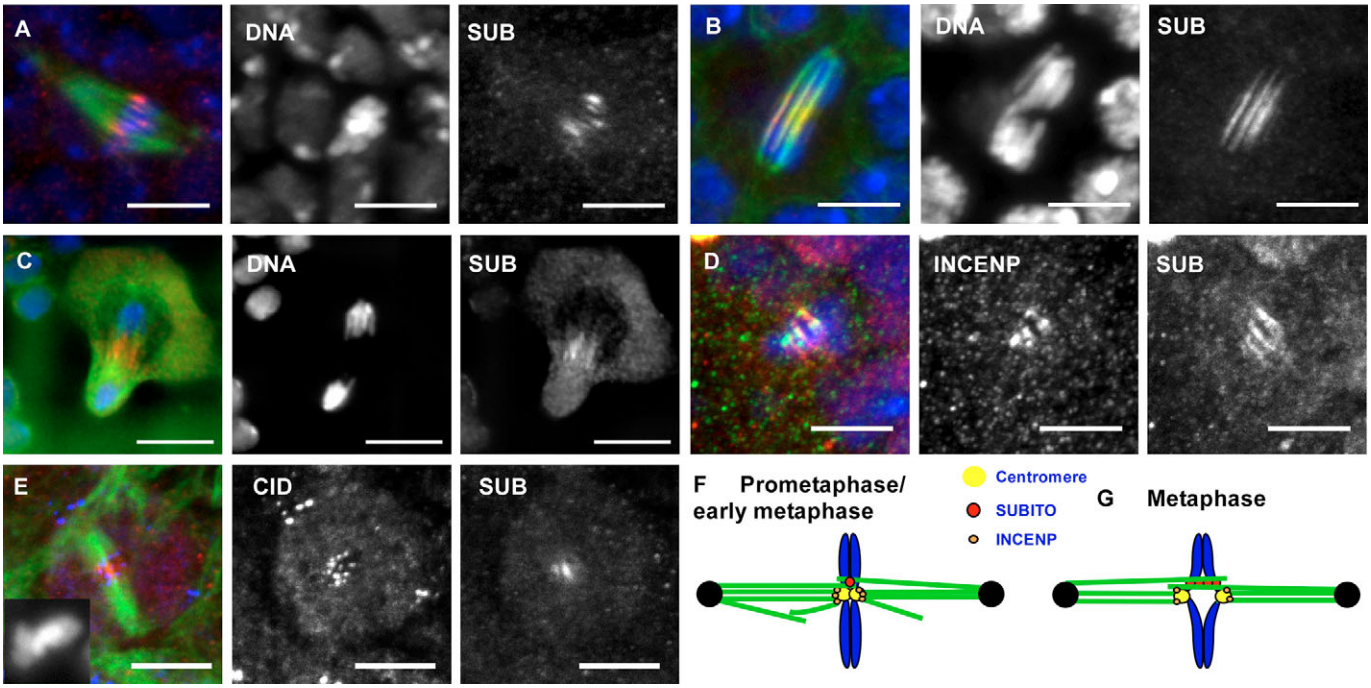

Fig. 1. Subito localization in wild-type brains. (A) A whole-mount metaphase cell with the chromosomes aligned in the middle of the spindle. The DNA (blue) is condensed and Subito (red) is present on the microtubules (green). (B) A whole-mount early anaphase cell with Subito on the spindle between the separating chromosomes. (C) A squashed late anaphase cell with Subito on the midzone microtubules and beginning to concentrate on a smaller portion of the spindle than in early anaphase. (D) A whole-mount metaphase cell showing the overlap of Subito (red) and Incenp (green). In some cases, Subito appears to be spreading out along the interpolar microtubules whereas in other cases, it appears in foci. (E) A whole-mount metaphase cell showing Subito (red) present n the microtubules between paired CID foci (blue). The greyscale inset shows DNA staining. Bars, 5 μm. (F,G) Model for the localization pattern of Subito at metaphase. Subito interacts with antiparallel microtubules, which only exist between centromeres. Just before anaphase, the centromeres are pulled in opposite directions, increasing the length of antiparallel microtubules between them.

Subito colocalizes at centromeres with Polo, Incenp and MEI-S332 during metaphase

Although Subito appeared to colocalize with microtubules, the concentration of staining near the chromosomes raised the possibility that Subito was associating with kinetochores. To test this possibility, we stained whole mount larval brains with both Subito and proteins known to associate with centromeres or kinetochores at metaphase. For example, the passenger protein Incenp localizes to centromeres at metaphase (Adams et al., 2001b). We found that the signals from Subito and Incenp overlapped (Fig. 1D), suggesting that Subito was present near the centromeres. In some cases, the Subito signal appeared to spread out over a short distance along the microtubules. Nonetheless, at least part of the Subito signal usually overlapped with Incenp. Similar results were obtained using antibodies to Aurora B, Polo and MEI-S332 (data not shown). Like Incenp, all of these proteins have been shown to localize to centromeres or kinetochores at metaphase (Adams et al., 2001b; Giet and Glover, 2001; Logarinho and Sunkel, 1998; Moore et al., 1998; Moutinho-Santos et al., 1999). To directly compare Subito localization to the centromeres, larval brains were stained for CID, a centromere-specific histone H3 (Blower and Karpen, 2001; Henikoff et al., 2000) and Subito. In some cells, there were 16 disordered foci of CID staining, suggesting that the sister centromeres had not yet aligned with the poles. In these nuclei, Subito colocalized with the CID staining. In other cells, the CID foci were neatly arranged in two rows, suggesting that the sister centromeres had aligned with the poles (Fig. 1E). The DNA staining suggested they were still in metaphase, although it is possible they were at the earliest stages of anaphase. In these cases, Subito was spread out along microtubules between pairs of CID foci.

To explain the colocalization of Subito with both microtubules and centromeres, we suggest that the antiparallel overlap of interpolar microtubules may preferentially interact with centromeres during metaphase (Fig. 1F,G). Early in metaphase the centromeres have not separated and thus there are only short tracks of antiparallel microtubles. By contrast, once the centromeres are under tension and pulled towards opposite poles, the distance between them increases and the length of antiparallel overlap increases. This is reflected in the more elongated regions of Subito staining. At present, however, we have not determined the cause of the centromere association; whether it is Subito or the antiparallel microtubules independently of Subito that are attracted to the centromeres. Alternatively, we have not ruled out the possibility that Subito interacts with the plus ends of the microtubules that approach the kinetochores and then move to interpolar microtubules when the centromeres separate.

Subito is required for the localization of mitotic kinases but not other midzone proteins

The Subito localization pattern, particularly to the midzone at anaphase, resembles several other proteins, including Polo (Logarinho and Sunkel, 1998), the passenger proteins Incenp and Aurora B (Adams et al., 2001b; Giet and Glover, 2001) and proteins required for cytokinesis such as Pavarotti (Adams et al., 1998) and Fascetto (Verni et al., 2004). To investigate the relationship between Subito and these proteins, we examined whether their localization depended on *sub* in mitotic larval brain cells. As described above, Subito colocalized near the centromeres with proteins such as Polo, Incenp and Aurora B during metaphase. Polo and Subito continued to colocalize at the midzone during anaphase (Fig. 2A). Although Polo staining at the centromeres was not affected in *sub* mutants during metaphase, it was strongly reduced at the spindle

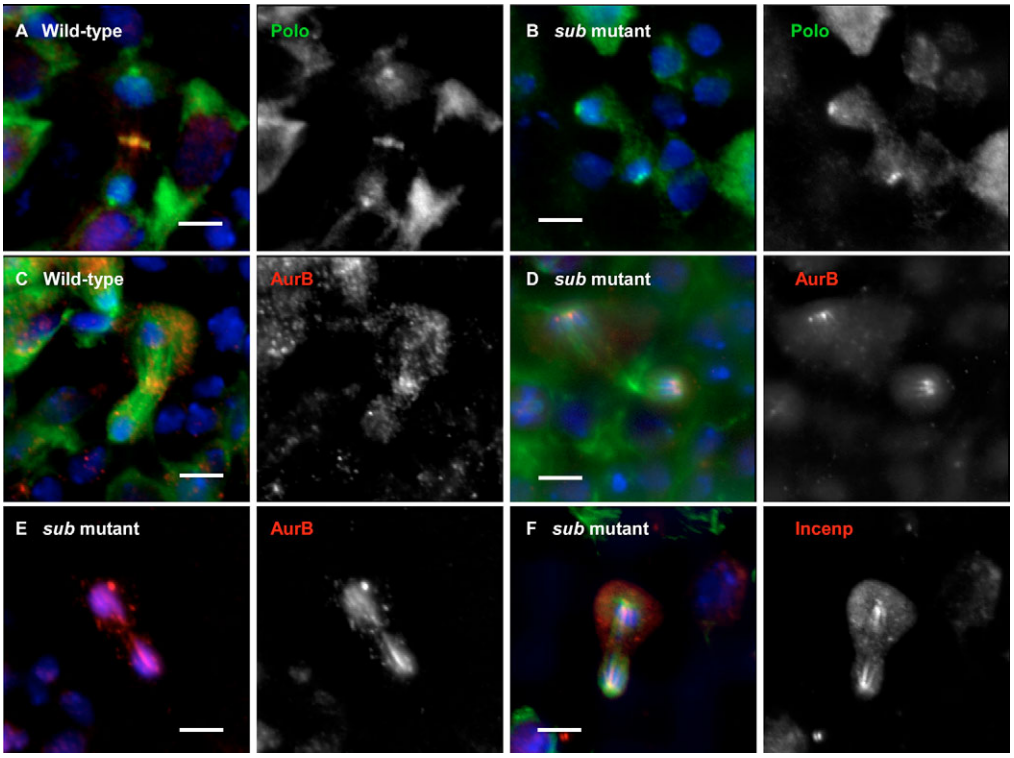

Fig. 2. Subito is required during anaphase for the localization of Polo and Aurora B but not Fascetto or Pavarotti. All images are stained for DNA (blue) and, except for A and B, tubulin (green). Polo (green) colocalizes with Subito (red) at the midzone in wild-type anaphase (A) but not in sub^{1}/sub^{131} mutants (B). (C) Aurora B (red) is localized on the chromosomes during metaphase (not shown) and during anaphase it moves off of the chromosomes and localizes on the spindle midzone. (D) In sub^{1}/sub^{131} mutant anaphase, Aurora B protein is still present on the anaphase chromosomes but not at the midzone. (E,F) Although usually more concentrated near the chromosomes in *sub* mutant anaphases, Aurora B and Incenp (red) often appeared to have spread out along the microtubules; they were never concentrated in the midzone. In panel E, there was no tubulin staining. Bars, 5 μm.

midzone during anaphase (Fig. 2B). Some early anaphase cells had faint Polo staining in the midzone, but by late anaphase it was no longer detectable.

A similar set of observations was made with Aurora B and Incenp. Aurora B was located at the centromeres during metaphase in wild-type cells but moved to the spindle midzone during anaphase (Fig. 2C). Unlike metaphase, where Subito staining often occupied a larger domain than Polo, Aurora B or Incenp, the anaphase localization patterns of these proteins were more closely matched. In *sub* mutant cells, Aurora B was at the centromeres during metaphase but failed to move to the midzone during anaphase (n=45, Fig. 2D). Fig. 2D also shows that in *sub* mutant cells, the midzone microtubules were usually disorganized. Similarly, Incenp failed to move to the spindle midzone in *sub* mutant anaphases (n>40). The abnormal staining of Aurora B and Incenp was not simply a retention of centromere localization at anaphase. Particularly in the early stages of anaphase, the Aurora B and Incenp staining in *sub* mutants often appeared to spread out along the microtubules (Fig. 2E,F). The brightest staining was still near the centromeres, as if the passenger proteins were unsuccessfully attempting to move to the midzone. In no case, however, did the protein concentrate in the midzone.

Pavarotti and Subito are both members of the kinesin 6 family. Pavarotti is required for cytokinesis and becomes enriched at the spindle midzone during late anaphase and telophase (Adams et al., 1998) (Fig. 3A). We found that Pavarotti could still localize to late anaphase and telophase spindles in *sub* mutant brains although the intensity of staining was often reduced and less restricted within the midzone compared to the wild type (Fig. 3B,C). These results show that Subito is required for the normal localization pattern of Pavarotti. Subito might have a direct role mediating Pavarotti localization via protein-protein interactions (see Discussion). Alternatively, because the level of Pavarotti staining might be related to the severity of the microtubule phenotype, the localization defects could be a secondary consequence of the disorganized midzone in *sub* mutants. For example, Pavarotti might bind to the antiparallel microtubules that depend on Subito.

Fascetto is the *Drosophila* Prc1 homolog and is enriched in the spindle midzone starting at anaphase and has an important role in cytokinesis (Verni et al., 2004). Staining of *sub* mutant brains showed that Fascetto localized strongly to the spindle midzone at anaphase and telophase (Fig. 3E,F), suggesting that Fascetto localization is independent of Subito. Because telophase looked normal in *sub* mutants, it is possible that spindle organization improved over time, ultimately promoting cytokinesis. In addition, the control experiments showed that the enrichment of Subito early in anaphase occurred before the appearance of Fascetto, which is consistent with the observation that Fascetto does not localize to the midzone until late anaphase (Verni et al., 2004). In HeLa cells depleted of MKLP2 by RNAi, PRC1 midzone staining still occurs in (Neef et al., 2003).

The effect of *sub* mutants on mitotic progression

When sub^{131}/CyO males were crossed with sub^{1}/CyO females, the expected number of sub^{131}/sub^{1} progeny were produced (Table 1), indicating that *sub* is not required for viability. However, *sub* mutant homozygotes exhibited morphological defects such as interruptions in the abdominal cuticle pattern and clipped wing tips (data not shown). To investigate other non-essential functions of *sub* in mitotically dividing cells, we compared cell-cycle progression in wild-type and *sub* mutant larval brains by measuring the mitotic index.

Nuclei in mitosis were identified by staining for histone H3 phosphorylated on Ser10 (Gurley et al., 1978). The mitotic index in *sub* mutant larvae was approximately twice that of the wild type (Fig. 4), suggesting there was a delay in the progression of *sub* mutant cells through mitosis. To test if the elevated mitotic index in *sub* mutants was caused by activation of the spindle assembly checkpoint, a sub^{131} $BubR1^{k03113}$ double mutant was generated. BubR1 is required for the spindle assembly checkpoint (Basu et al., 1999). Therefore, if the spindle checkpoint was activated in *sub* mutants, we anticipated a reduced mitotic index in the sub^{131} $BubR1^{k03113}$ double mutant relative to the *sub* single mutant. In fact, the sub^{131} $BubR1^{k03113}$ double mutant had a lower mitotic index (0.65) than either the *sub* single mutant (2.66) or the wild type

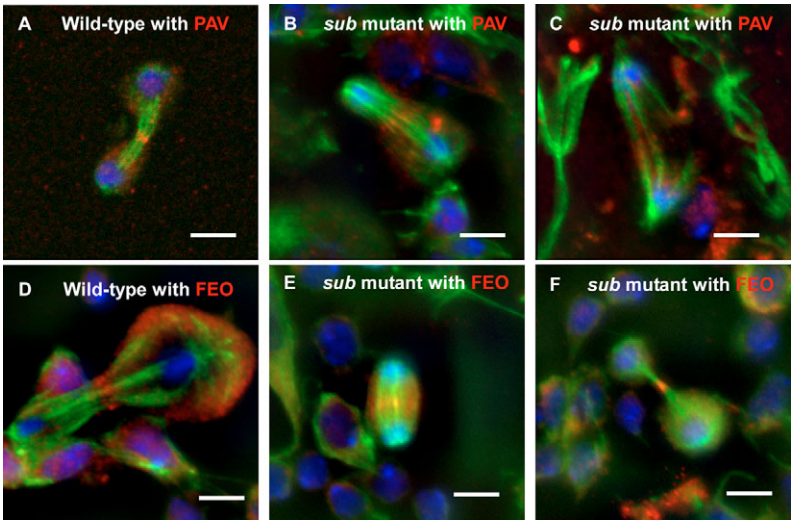

Fig. 3. (A) Pavarotti (red) is located in the midzone of wild-type late anaphase. (B,C) Pavarotti shows variable midzone staining in sub^{1}/sub^{131} mutants, which may be related to the degree of microtubule disorganization. (D) Fascetto (FEO, red) localization to the midzone in the wild type. (E,F) Fascetto staining was strong during anaphase and particularly at telophase. Bars, 5 μm.

Table 1. Viability of *sub* double mutants

Cross	*sub/CyO**	*sub/sub; +/+ or sub/sub; Incenp/+*	*sub/sub; polo/+*	Total
sub^{131}/CyO, Cy × sub^{1}/CyO, Cy	644	302 (313)†		946
sub^{1}/CyO; $polo^{16\text{-}1}$/TM3, Sb × sub^{131}/CyO; +/+	1655	308	6 (308)†	1969
$Incenp^{3747}$ sub /CyO × sub/CyO‡	1028	0 (514)†		1028
Df(2L)Exel7049 sub^{1}/CyO × sub^{131}/CyO	527	0 (263)†		527

The number of progeny scored from the indicated crosses is shown. *Depending on the cross, this could include *sub/CyO; +/+* and either *sub/CyO;* $polo^{16\text{-}1}$*/+* or *sub* $Incenp^{3747}$*/CyO* or *Df(2L)Exel7049* sub^{1} */CyO*. †The expected number of progeny if there was no synthetic lethality is shown in parentheses. ‡Combined data of $Incenp^{3747}$ sub^{1}/CyO × sub^{131}/CyO and $Incenp^{3747}$ sub^{131}/CyO × sub^{1}/CyO.

(1.32, Fig. 4). The low mitotic index in the double mutant was consistent with our control data and previous observations that the *BubR1* mutant has a lower mitotic index than the wild type (Basu et al., 1999). Thus, we conclude that *BubR1* is required for the increased mitotic index in *sub* mutants and that *sub* mutants have a defect during prometaphase or metaphase that activates the spindle assembly checkpoint. If spindle assembly errors do occur in a sub^{131} mutant and are not corrected in a $BubR1^{k03113}$ mutant background, a synergistic effect on mitosis or development would be expected. This was observed; although *BubR1* mutants are lethal, two synergistic phenotypes were observed in the double mutants. First, sub^{131} $BubR1^{k03113}$ double mutant third instar larvae were slow growing and they appeared 24-48 hours later than the *BubR1* single mutant larvae. Second, the brains of the double mutant larvae were small and contained fewer cells than the single mutants. It is likely that these phenotypes resulted from the failure to repair spindle assembly errors that arise during metaphase in *sub* mutants. We were unable to characterize the cytological phenotype of the double mutant because there were so few mitotic cells.

A low mitotic index in *sub ncd* and *ncd mutants*

We previously reported that mutations in *ncd*, which encode a kinesin 14 or C-terminal motor kinesin-like protein, and *sub* genetically interact (Giunta et al., 2002). The double homozygous mutant $sub^{1}/sub^{131};ncd^{1}/ncd^{1}$ is lethal at the third larval instar stage, whereas the sub^{1}, sub^{131} and ncd^{1} single mutant homozygotes are viable. We examined *sub;ncd* double mutants to investigate whether synergistic effects on spindle assembly were the cause of the double mutant lethality. Unlike *sub* single mutants, which exhibited an elevated mitotic index, the $sub^{1}/sub^{131};ncd^{1}/ncd^{1}$ brains had a mitotic index similar to that of the wild type (Fig. 4). Since the mitotic index of ncd^{1} brains was also similar to the wild type, these results suggest that ncd^{1} suppresses the mitotic delay phenotype of *sub* mutants.

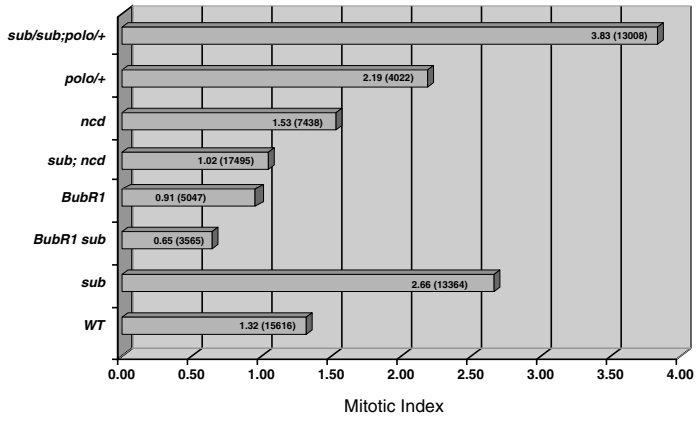

Fig. 4. Mitotic index in wild-type and mutant brains. The mitotic index was defined as the percentage of cells in mitosis. The number on each bar is the mitotic index and the total number of cells counted is shown in parentheses.

The *ncd* mutant brains consistently had a lower metaphase to anaphase ratio, and this was also observed in the *sub;ncd* double mutant (Table 2). A more rapid progression through metaphase than observed in wild-type or *sub* mutants could cause the lower mitotic index and lethality because of segregation errors in the double mutant. If the high mitotic index in the *sub* single mutant was due to activation of the spindle assembly checkpoint, it was possible that an inability to activate this spindle assembly checkpoint caused the *sub;ncd* double mutant lethal phenotype. Currently, however, cytological studies have not confirmed this hypothesis nor revealed insights into the cause of the *sub;ncd* synthetic lethal phenotype (see supplementary material Fig. S1, Table S1).

Spindle assembly and chromosome segregation defects in *sub* mutant mitotic cells

Mitotic spindle assembly was assayed in squashed or whole-mount larval brains from wild-type and *sub* mutants stained for DNA and tubulin. Although most *sub* mutant metaphase spindles were bipolar, we identified an increased frequency of spindle assembly defects. These included frayed microtubules, unequal distribution of microtubules in the two half spindles and disorganized or absent interpolar microtubules (Fig. 5B-D). The frayed spindles and unequal distribution of microtubules in the half spindles could be a secondary consequence of a defect in organizing interpolar spindle fibers, a role Subito has been shown to have in meiosis (Jang et al., 2005). The effect on interpolar microtubules was not fully penetrant, however, because *sub* mutant spindles with interpolar microtubules were observed. Unlike oocytes, therefore, it appears that interpolar microtubules can form in the absence of *sub* in mitotic cells, although perhaps less efficiently or less stably.

To compare the wild type and mutants, the metaphase spindles were classified as ‘disorganized’ if they had any of the features described above. In sub^{131} mutant brains, 43.4% of the metaphase spindles were classified as disorganized compared to 10.9% in wild-type brains (Table 2). An even greater frequency of metaphase spindle defects was observed in sub^{1}/sub^{131} brains. A second *sub* mutant phenotype was observed during anaphase. Although the chromosomes usually

Table 2. Characterization of mitotic figures in wild-type and *sub* mutant brains

Genotype	Anaphase	Metaphase	M/A*	Disorganized metaphase (%)	Lagging chromosomes at anaphase (%)
Wild type	140	468	3.3	10.9	9.3
sub^{131}	81	380	4.7	43.4	51.8
sub^{1} / sub^{131}	203	687	3.4	70.0	46.3
ncd / ncd^{1}	212	315	1.5	18.1	55.2
sub^{1} ncd^{1}	88	146	1.7	65.7	43.2
sub^{131}; ncd^{1}	275	454	1.7	61.9	51.6
sub^{1} / sub^{131} ncd^{1}	292	459	1.6	65.1	54.8
$Incenp^{3747}$ sub^{1} / + sub^{131}	231	599	2.6	71.9	75.6

*M/A, metaphase to anaphase ratio.

moved uniformly to the poles during wild-type anaphase (Fig. 5E), lagging chromosomes were frequently observed in *sub* mutant anaphases (Fig. 5F). The frequency of these abnormal anaphases was 9.3% in wild-type brains but rose to 46.3-51.8% in *sub* mutant brains (Table 2). Like the disorganized microtubules described above, the presence of lagging chromosomes is consistent with defects in spindle organization at metaphase.

sub genetically interacts with regulators of mitosis

Since Subito, Polo and the passenger proteins colocalize and because physical interactions have been detected between their human homologs (Gruneberg et al., 2004; Neef et al., 2003), experiments were performed to determine genetic interactions between mutations in *polo, Incenp* or *Aurora B* and *sub*. The interaction with *polo* was tested using the $polo^{16\text{-}1}$ allele that causes recessive early larval lethality (Lukinova et al., 1999). When sub^{1}/CyO;$polo^{16\text{-}1}$/TM3, Sb and sub^{131}/CyO;+/+ flies were crossed, few sub^{1}/sub^{131};$polo^{16\text{-}1}$/+ progeny were recovered and the rare survivors were sick and small with abdominal patterning defects (Table 1). Thus, heterozygosity for $polo^{16\text{-}1}$ increased the severity of the *sub* homozygous phenotype, resulting in late larval developmental arrest. Synthetic lethality was also observed in *sub* homozygotes that were simultaneously heterozygous for the strong hypomorph $polo^{S025604}$ (or $polo^{9}$) (Donaldson et al., 2001).

The interaction with *Incenp* was tested using the EMS-induced allele $Incenp^{3747}$, which causes embryonic lethality (Chang et al., 2006) whereas *Aurora B* was tested using the deletion *Df(2L)Exel7049*. When the appropriate heterozygotes were crossed together, we did not recover any $Incenp^{3747}sub^{1}$/+sub^{131} or *Df(2L)Exel7049* sub^{1}/+sub^{131} adults (Table 1). Since the synthetic lethality was observed in a genotype where one copy of the gene encoding Aurora B was deleted, the genetic interaction with the passenger protein mutants probably resulted from reduced levels of gene product rather than allele-specific interactions. Not all mitotic regulators caused lethality in *sub* homozygotes. The double mutant sub^{1}/sub^{131};$aur^{87Ac\text{-}3}$/+, which carried a null allele of the *Drosophila* Aurora A homolog, was viable. Therefore, the reduced dosage of Polo or the passenger proteins Incenp and Aurora B caused lethality in the absence of *sub*.

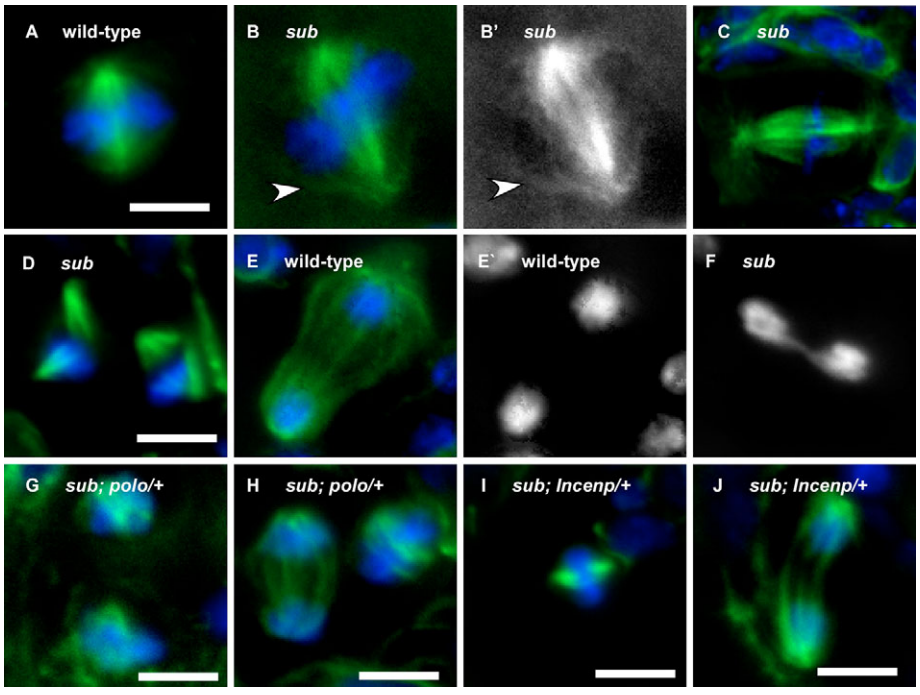

Fig. 5. A variety of mitotic spindle defects and lagging chromosomes in sub^{1}/sub^{131} mutants stained for DNA (blue) and microtubules (green). Compared with (A) wild-type metaphase, *sub* mutant metaphase spindles are bipolar but exhibit several defects including (B) frayed microtubules (B', tubulin channel shown with increased levels), (C) asymmetric half spindles and (D) poorly oriented half spindles or absent interpolar microtubules. Although wild-type anaphase (E) (E', DNA channel only) involves the uniform segregation of chromosomes to the poles, in *sub* mutant anaphases there was an increased frequency of lagging chromosomes (F). A synergistic effect on spindle assembly was observed during (G) metaphase and (H) anaphase in sub^{1}/sub^{131}; $polo^{16\text{-}1}$/+ mutants or metaphase (I) and (J) anaphase spindles in sub^{1}/sub^{131};*Incenp*/+ mutants. Bars, 5 μm.

To examine the lethal interactions involving *sub* more closely, we looked for evidence of spindle assembly defects. Severe defects in spindle organization were observed in the brains of $sub^{1}/sub^{131};polo^{16\text{-}1}/+$ mutant larvae (Fig. 5J,K). The spindles were unusually short. For example, the metaphase spindles were not much longer than the thickness of the chromosomes. Furthermore, $sub^{1}/sub^{131};polo^{16\text{-}1}/+$ larvae showed a higher mitotic index than either sub^{1}/sub^{131} or $polo^{16\text{-}1}/+$ mutants and was nearly four times that of the wild type (Fig. 4), consistent with a defect in spindle assembly. Similar results were found in $Incenp^{3747}sub^{1}/+sub^{131}$ mutant brains. Short and disorganized metaphase spindles were common (Fig. 5L, Table 2), consistent with a spindle assembly defect. Furthermore, the double mutant anaphase spindles usually lacked an organized midzone (Fig. 5M). Although Subito and the passenger proteins always colocalized, the effects of the double mutant on midzone organization were more severe than those observed with the *sub* single mutant, suggesting that Incenp may have a separate role to *sub* in spindle assembly and stabilization of midzone microtubules during anaphase.

Subito and Polo physically interact

Consistent with our observations that Subito and Polo colocalize, Neef et al. (Neef et al., 2003) have shown that the mammalian homologs of Polo and Subito, Plk1 and MKLP2, physically interact. To examine whether Subito and Polo physically interact, we performed co-immunoprecipitation experiments. Lysates were prepared from embryos or oocytes expressing a *sub* transgene fused in frame to three copies of the HA epitope tag. As shown in Fig. 6, we were able to precipitate Subito efficiently. In addition, Polo was detected in the anti-HA immunoprecipitate from the $Subito^{HA}$ lysates. Therefore, in both oocytes and embryos, Subito and Polo may exist in a complex.

The consequences of the spindle abnormalities in *sub* mutants

A defect in spindle assembly would be expected to result in hyperploidy or hypoploidy and a failure in cytokinesis would be indicated by the presence of polyploid cells. Aberrant chromosome segregation was detected by counting chromosome numbers in larval brain cells treated with colchicine to arrest them at metaphase. Consistent with the observed increase in spindle assembly defects, *sub* mutants had an increase in all aneuploid types (Table 3, Fig. 7A,B). Although *sub* mutants fail to localize Polo, Aurora B, and Incenp to the midzone at anaphase, *sub* mutants had only a small increase in polyploidy. This was not surprising, because *sub* mutants lack some of the characteristics of *Drosophila* mutants with cytokinesis defects (see Discussion).

To investigate the nature of synthetic lethality with *Incenp*, the same experiment was performed on double mutant larvae. $Incenp^{3747}sub^{1}/+$ sub^{131} double mutant larvae exhibited a high frequency of polyploid cells (Table 3, Fig. 7C), suggesting a severe defect in cytokinesis. These results suggest that Subito has a redundant function in cytokinesis. Although s*ub* single mutants did not have a severe defect in cytokinesis, reducing the dosage of Incenp caused a severe defect in cytokinesis. These results suggest that Incenp may have a function in promoting cytokinesis that is independent of Subito.

Discussion

Subito has an important role in assembling the acentrosomal female meiotic spindle in *Drosophila* (Giunta et al., 2002; Jang et al., 2005). The results presented here from the analysis of *sub* are the first to demonstrate a function for a kinesin 6 family member in mitotic spindle assembly.

Relationship of Subito to the kinesin 6 family

Subito is one of the two *Drosophila* kinesin 6 family members and probably the ortholog of MKLP2 (Dagenbach and Endow, 2004; Jang et al., 2005; Miki et al., 2005). In support of this classification, there are striking similarities between Subito and MKLP2. Both are required for localization of the passenger proteins to the midzone during anaphase (this report) (Gruneberg et al., 2004). In addition, both Subito and MKLP2 interact with Polo kinase (or Plk1 in human) and are required for its localization to the midzone during anaphase (this report) (Neef et al., 2003). Plk1 phosphorylates MKLP2 at Ser528 and this phosphorylation promotes Plk1 binding to MKLP2. Plk1 phosphorylation negatively regulates MKLP2 microtubule bundling activity in vitro but is not required for the localization of MKLP2 to the midzone.

Despite belonging to the same family, the two kinesin 6 family members probably have unique functions. The distinct phenotypes of *sub* and *pav* mutants indicate they have non-

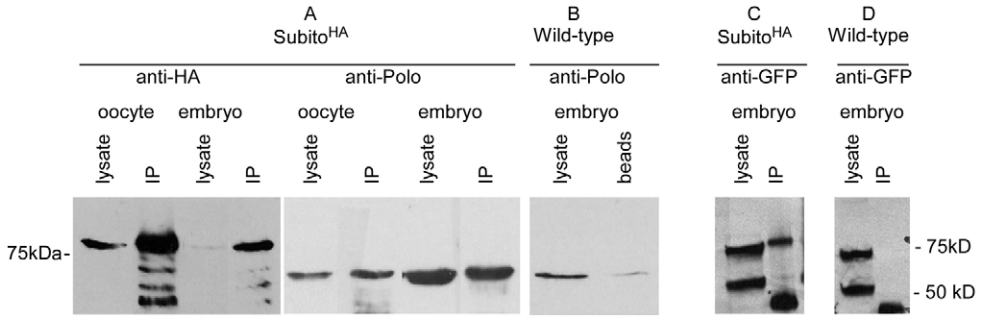

Fig. 6. Subito and Polo co-immunoprecipitate. (A) HA-tagged Subito was immunoprecipitated from mixed stage 0- to 2-hour-old embryos and metaphase-arrested oocytes and then used for western blotting. (B) A control experiment was performed on tissue that did not express the HA-tagged Subito. (C) This experiment was repeated using GFP-tagged Polo (Moutinho-Santos et al., 1999). HA-tagged Subito was immunoprecipitated from mixed stage 0- to 2-hour-old embryos. (D) In the control, using embryos not expressing HA-tagged Subito, GFP-Polo was not precipitated. The lower band is a nonspecific protein in the lysate.

Table 3. Frequency of aneuploidy in *subito* and *Incenp* mutants

Genotype	2*n*	2*n*–1	2*n*+1	2*n*–2	2*n*+2	2*n*–3	2*n*+3	Polyploid	% Hypoploid and hyperploid	% Polyploid	Total cells
Wild type	217	9	3	2	1	0	0	0	6.5	0.0	232
sub^{1} / sub^{131}	172	10	16	0	5	0	2	11	15.3	5.1	216
$Incenp^{3747}$ sub^{1} / + sub^{131}	51	5	7	0	3	0	4	75	13.1	51.7	145
$Incenp^{3747}$ / +	178	5	0	0	0	0	1	8	3.1	4.2	192

overlapping functions (see also Jang et al., 2005). Similarly, and despite having similar localization patterns, MKLP2 and MKLP1 have nonredundant functions in cytokinesis (Fontijn et al., 2001; Neef et al., 2003). MKLP2, but not MKLP1, has been shown to physically interact with Aurora B and Incenp (Gruneberg et al., 2004). However, it has also been suggested that the MKLP2-dependent localization of Aurora B to the midzone is required for it to phosphorylate MKLP1 (Neef et al., 2006). The importance of this phosphorylation on MKLP2 localization is unclear and our results are consistent with this indirect relationship between Subito and Pavarotti.

It is possible that all members of the kinesin 6 group interact with antiparallel microtubules (Nislow et al., 1992). Our immunolocalization data is consistent with this because Subito is found on interpolar microtubules, which are characterized by an overlap of antiparallel microtubules in the midzone at mitotic anaphase in embryos (Jang et al., 2005), brains and testis (J.K.J. and K.S.M., unpublished results). However, the localization of Subito to metaphase interpolar microtubules in the vicinity of the centromeres was a surprising finding. Although it is likely that Subito also associates with antiparallel microtubules at metaphase, we cannot rule out the possibility that Subito interacts with the plus ends of the microtubules that interact with the kinetochores. Surprisingly, a specific localization pattern of other kinesin 6 family members to metaphase microtubules has not been observed (Adams et al., 1998; Minestrini et al., 2003; Somers and Saint, 2003) (K.S.M., unpublished results). This is not due to the absence of the appropriate substrate, since metaphase interpolar microtubules are present in most spindles (Compton, 2000; Mastronarde et al., 1993). Either Subito is regulated differently than MKLP2, with an associated additional function in spindle assembly, or the localization pattern of MKLP2 at metaphase has not been informative with respect to its function.

Evidence of a redundant role for Subito during cytokinesis

Since Subito is required to localize Polo, Aurora B and Incenp to the spindle midzone at anaphase, it is surprising that *sub* mutants are viable. Loss of MKLP2 causes cytokinesis defects (Neef et al., 2003). *Drosophila* mutants with strong defects in cytokinesis fall into the categories of male sterile (Giansanti et al., 2001), embryonic lethal (e.g. *pav* mutants) (Adams et al., 1998) or pupal lethal (e.g. Castrillon and Wasserman, 1994; Gunsalus et al., 1995; Verni et al., 2004). In fact, *Incenp* and *polo* mutants have embryonic lethal phenotypes that may be caused by a failure of cytokinesis (Carmena et al., 1998; Chang et al., 2006). Unlike the loss of *Incenp*, *Aurora B* or *Polo*, *sub* mutants do not have any of these phenotypes and appear to complete cytokinesis most of the time in larval brains. In addition, because *sub* mutant males are fertile, and most mutants with strong defects in cytokinesis during spermatogenesis are male sterile (Giansanti et al., 2001), Subito does not appear to be essential for cytokinesis in the testis. A cytokinesis phenotype was also not evident in cultured *Drosophila* cells depleted of Subito by RNAi (C. Wu and K.S.M., unpublished results) (Echard et al., 2004; Eggert et al., 2004; Goshima and Vale, 2003). These same studies did identify cytokinesis defects when *Polo, Aurora B* and *Incenp* were depleted. Thus, it seems likely that in some cell types, such as larval brains, the presence of Subito and the localization of the passenger proteins are not required for cytokinesis to occur.

A close examination of *sub* mutants, however, revealed that anaphase did not proceed normally. In addition to the failure

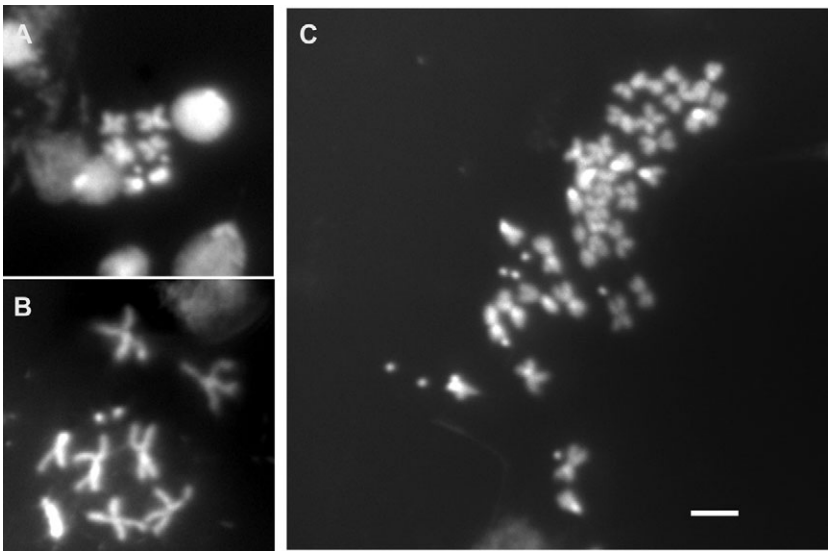

Fig. 7. Metaphase spreads in colchicine-treated swollen cells. (A) A normal karyotype in a wild-type cell. (B) A hyperploid cell in a sub^{1}/sub^{131} mutant brain. (C) A polyploid cell in a $Incenp^{3747}$ sub^{1} /+ sub^{131} brain. Bar, 5 μm.

to accumulate Polo, Aurora B and Incenp in the midzone, the absence of Subito resulted in disorganized midzone microtubules at anaphase and a small increase in the frequency of polyploid cells. When the dosage of *Incenp* was reduced in *sub* mutants, the frequency of polyploidy was markedly increased. Therefore, Subito appears to have a similar function to MKLP2 in promoting cytokinesis, although there may be functional redundancy. Since the ability to complete cytokinesis in *sub* mutants depends on *Incenp* and *Aurora B* dosage, it is possible that unlocalized Incenp or Aurora B may promote cytokinesis. However, our observation that Incenp and Aurora B have a limited ability to spread along anaphase microtubules in the absence of Subito suggests an alternative; enough passenger protein activity may be present to promote cytokinesis. This model can account for the sensitivity of *sub* mutants to *Incenp* or *Aurora B* dosage because high levels of these proteins may be needed to promote cytokinesis if not concentrated in the midzone. It is also possible that anaphase may last longer and/or the microtubule organization improves with time in *sub* mutants. This would account for the relatively normal Feo localization and high success completing cytokinesis in *sub* mutants.

Subito interacts with Ncd, Polo and the passenger proteins during spindle assembly

Several lines of evidence suggest that Subito has a role in mitotic spindle assembly. First, Subito initially localizes to interpolar microtubules at metaphase. Second, abnormally formed metaphase spindles were found in *sub* mutants more frequently than in the wild type. Third, *sub* mutant brains have an elevated mitotic index. Although the magnitude of the increase in *sub* mutants was lower than reported in some other mutants with spindle assembly defects (e.g. Basto et al., 2000; Donaldson et al., 2001), these mutants are lethal. Consistent with the conclusion that *sub* mutants have a defect in spindle assembly, the elevated mitotic index was dependent on *BubR1*, suggesting that the spindle assembly checkpoint is activated in the absence of Subito. Fourth, *sub* mutations exhibit synthetic lethality in combination with *polo*, *Incenp* and *Aurora B* mutations, and the cytological phenotype includes defects in spindle assembly and increased mitotic index (see below). Finally, RNAi of *sub* in *Drosophila* S2 cells results in frequent mitotic spindle abnormalities (C. Wu and K.S.M., unpublished results) (Kiger et al., 2003). These observations all point to a role for Subito in spindle assembly.

The defects associated with *sub* mutants are less severe in mitotic cells than during female meiosis, possibly because of redundant spindle assembly pathways in mitosis. The double mutant studies suggest that the defects in spindle assembly or chromosome alignment in *sub* mutants are compensated for in two ways. First, the activation of the spindle assembly checkpoint allows defects in microtubule organization to be corrected. Second, the presence of redundant spindle assembly pathways allows microtubules to be assembled in the absence of *sub*. Our double mutant studies support both of these mechanisms.

The phenotype of the *sub;polo*$^{16\text{-}1}$/+ double mutant was consistent with a redundant role for Subito in spindle assembly. Compared with the single mutants, the double mutants exhibited grossly abnormal metaphase and anaphase spindles. Similar to our results with *sub*, a role for Polo in spindle assembly was previously shown through the analysis of *polo* hypomorphs that had an elevated mitotic index in larval brains, indicating that the spindle assembly checkpoint was activated (Donaldson et al., 2001). During metaphase, Polo localizes to the centromeres where it has a role in spindle formation but during anaphase it localizes to the spindle midzone where it has a role in cytokinesis (Carmena et al., 1998; Logarinho and Sunkel, 1998; Moutinho-Santos et al., 1999). The very high mitotic index in the double mutants, however, suggests a more severe defect in spindle assembly than either single mutant. We suggest that the abnormal spindle phenotype in *sub/sub;polo/+* mutants arise from a combination of defects in two partially redundant spindle assembly pathways: improper assembly of kinetochore microtubules in *polo/+* mutants and a reduction in assembling interpolar microtubules in *sub* mutants. Although *polo* mutants are recessive lethal, there is other evidence for dominant phenotypes, such as an elevated mitotic index in *polo*$^{16\text{-}1}$/+ brains (see also Clarke et al., 2005).

The combination of these two spindle assembly defects in *polo/+;sub/sub* mutants might result in the severe spindle assembly phenotype and lethality in the double mutant. Similar conclusions apply for the interactions between *sub* and *Incenp* or *Aurora B*. Like *Polo,* the passenger proteins have an important role in spindle assembly (Adams et al., 2001a; Vernos, 2004). Indeed, the effects of all three mutants are strikingly similar, suggesting that Subito, Polo and the passenger proteins have important interactions during metaphase and anaphase. Interestingly, there is evidence of a direct interaction between Plk and Incenp in mammalian cells (Goto et al., 2006).

The role of Subito in mitotic spindle function

Like its kinesin 6 homolog MKLP1 (Nislow et al., 1992), Subito is probably a plus-end-directed motor that crosslinks and slides interpolar antiparallel microtubules. Our results suggest that this activity is important from metaphase through anaphase. Interestingly, the metaphase and anaphase interpolar microtubules have functional differences. Metaphase interpolar microtubules are observed in the absence of Subito whereas their anaphase counterparts depend on Subito. Another important difference is that Polo and the passenger proteins only localize to anaphase interpolar microtubules in the midzone. We have previously suggested that the precocious appearance of anaphase-like interpolar microtubules is an important feature of acentrosomal meiotic spindle assembly in *Drosophila* oocytes (Jang et al., 2005). The passenger proteins Aurora B and Incenp localize to the interpolar microtubules at metaphase of meiosis I, rather than the centromeres, which is typical during mitotic metaphase. Therefore, the regulation of the passenger protein localization pattern is modified in oocytes to bypass the centromere localization that is characteristic of mitotic metaphase, resulting in precocious localization to interpolar microtubules (Jang et al., 2005).

Despite these differences, the same biochemical activities of Subito could be used to organize both centrosomal mitotic and female acentrosomal meiotic spindles. In mitotic cells, kinetochores can initiate microtubule fiber formation, but these fibers are not directed toward either spindle pole (Khodjakov et al., 2003; Maiato et al., 2004). Failure to organize these fibers could result in disorganized and frayed spindles, as we have observed in *sub* mutants. A function for Subito and

interpolar microtubules could be to properly orient undirected kinetochore fibers. Interpolar microtubules could interact with and direct the organization of kinetochore microtubules via motors that bundle parallel microtubules. We have proposed this mechanism for organizing a bipolar spindle in the acentrosomal meiosis of *Drosophila* oocytes (Jang et al., 2005). With motor-driven sliding of antiparallel microtubules, this is an example of a centrosome-independent model for the spindle assembly pathway. This is consistent with previous conclusions that centrosome-independent mechanisms for spindle assembly are active in mitotic cells (Maiato et al., 2004; Wadsworth and Khodjakov, 2004). Indeed, since bipolar spindles can form in the absence of centrosomes in neuroblasts and ganglion mother cells (Bonaccorsi et al., 2000; Megraw et al., 2001), it appears that centrosome-independent mechanisms for spindle assembly are active in the mitotic cells we have analyzed.

Another possibility is that Subito functions as part of the centrosomal assembly pathway. For example, an array of interpolar microtubules could help channel centrosome microtubules towards the kinetochores. This activity could reduce the element of chance associated with making contacts between centrosome microtubules and kinetochores. It has also been proposed that centrosomal microtubules may capture the minus ends of kinetochore microtubules (Khodjakov et al., 2003; Maiato et al., 2004). An involvement of Subito in this process would be surprising, however, because the ability to bundle microtubules in parallel has not been described for a kinesin 6 family member. Nonetheless, if Subito was involved in the interactions of centrosomal and kinetochore microtubules, subsequent plus-end-directed movement would explain why Subito localization overlaps with centromeres. Whether or not these models are correct, the redundant nature of spindle assembly and function may explain why a role for kinesin 6 motor proteins in spindle assembly has not been described previously.

Materials and Methods

Genetic stocks and identifying mutant larvae

All fly cultures and crosses were raised at 25°C. Third instar larva homozygous for second and third chromosome mutants were identified using stocks containing the mutant heterozygous to the translocation *T(2;3)B3,CyO:TM6B, Tb*. The dominant *Tubby* (*Tb*) marker was used to select the homozygous larvae. The *sub* mutants used in this study were the protein null alleles, sub^{1} and sub^{131} (Giunta et al., 2002; Jang et al., 2005; Schupbach and Wieschaus, 1989), examined as either trans-heterozygotes or as homozygotes. In addition, mutant alleles $Incenp^{3747}$ (Chang et al., 2006), $polo^{16\text{-}1}$ (Lukinova et al., 1999), $aur^{87Ac\text{-}3}$ (Glover et al., 1995), ncd^{1} (Yamamoto et al., 1989) and $BubR1^{k03113}$ (Basu et al., 1999) were used in this study. *Df(2L)Exel7049* (Parks et al., 2004), which deletes 32B1;32C1, was used because it is a deletion of the *Aurora B* locus.

Dissection and fixation of larvae

Larval brain tissue was prepared by both squashing and whole-mount methods. In preparation for squashing, the larvae were dissected in saline and the brains were fixed in 3.7% formaldehyde in 1× PBS for 30 minutes. For the purpose of depolymerizing microtubules and activating the spindle assembly checkpoint, the brains were incubated for 1 hour in 5×10^{-5} M colchicine. For karyotype analysis, the brains were incubated for 1.5 hours in 5×10^{-5} M colchicine followed by hypotonic swelling in 0.5% sodium citrate. The brains were then transferred to 45% acetic acid for 3 minutes (except for karyotype analysis) before transferring to ~8 μl of 60% acetic acid on a siliconized coverslip where they were firmly squashed between the coverslip and slide. The slides were briefly frozen in liquid nitrogen and the coverslips were flicked off. The slides containing the tissue were placed in ethanol at –20°C (chilled on dry ice) for 10 minutes, then transferred to a slide chamber containing 0.1% Triton X-100 in PBS for 10 minutes. Rubber cement was used to form wells on the slides and two 5-minute washes were done in PBS. The tissue was blocked with 1% BSA in PBS for 45 minutes. Primary antibodies were diluted in 1% BSA in PBS and then 250 μl was added to each slide and incubated overnight at 4°C in humid chambers. The next day, two washes in PBS and one wash in 1% BSA in PBS were performed for 5 minutes each. The secondary antibodies were then added and the slides were again incubated overnight at 4°C. The next day, two more 5-minute washes in PBS were done. The DNA was stained with 0.2 μl/ml of a 10 mg/ml Hoechst 33258 solution in 1% BSA in PBS for 7 minutes and the slides were washed with 1% BSA in PBS for 5 minutes. The tissue was mounted in Vectashield (Vector).

For the preparation of whole-mount brain tissue, third instar larvae were dissected in PBS and fixed in 3.7% formaldehyde for 20 minutes. They were then washed in PBS for 20 minutes, PBS with 0.3% Tween 20 for 10 minutes and then PBS with 10% normal goat serum (NGS) for 40 minutes. Primary antibodies were incubated with the brains in PBS-0.1% Tween 20 (PBST). The brains were then washed four times in PBST and then incubated with secondary antibodies in PBST/10% NGS. During the following four washes in PBST, the DNA was stained with Hoechst.

The rat anti-Subito antibody was used at 1:200 combined with a Cy3 anti-rat secondary antibody (1:200, Jackson Labs). Additional primary antibodies were mouse anti-α-tubulin (1:50, clone DM1A, Sigma) directly conjugated to FITC, rat anti-α-tubulin (1:75, Clone YOL 1/34, Chemicon), rabbit anti-CID (1:100, Abcam), rabbit anti-Aurora B (1:500), rabbit anti-Feo (1:100) (Verni et al., 2004), rabbit anti-Incenp (1:250) (Adams et al., 2001b), mouse anti-Polo (1:15) (Llamazares et al., 1991), rabbit anti-Pavarotti (1:750) (Adams et al., 1998), guinea pig anti-MEI-S332 (1:4000) (Moore et al., 1998) and Phospho-Histone H3 (Ser-10) (1:1000, Upstate) with Cy3- or FITC-conjugated secondary antibodies (1:200-1:500, Jackson Labs).

Image capture and analysis

Images were collected on two systems: a Zeiss Axioplan II fluorescent microscope using a 63× NA 1.4 lens and software from Vaytec to collect and process *Z*-stacks or a Leica TCS SP confocal microscope with a 63× NA 1.3 lens. Images are shown as maximum projections of image stacks. The mitotic index was measured as the number of mitotic cells/total cells counted from randomly selected fields. The mitotic cells were identified by Phospho-Histone H3 (Ser10) staining and approximately ten photographs of randomly selected fields were analyzed from each slide.

Co-immunoprecipitation experiments

A transgene was constructed by fusing the *sub* coding region to three copies of the HA epitope tag and sub-cloning this into the pUASP vector. This was expressed in oocytes and early embryos using the *nosGAL4:: VP16* driver (Van Doren et al., 1998). This combination rescued the meiotic and maternal effect embryonic lethal phenotypes of *sub* mutants (data not shown).

Lysates from embryos and oocytes were prepared in IP buffer [50 mM Tris-HCl pH 8.0, 400 mM NaCl, 0.5% (v/v) NP-40, 0.1% (w/v) deoxycholate and protease inhibitors]. The cleared extract was incubated with the 3F10 anti-HA antibody covalently linked to beads (Roche) overnight at 4°C and then the beads were washed twice with IP buffer. The lysate and beads were mixed with loading buffer for SDS-PAGE and the proteins transferred to PVDF membranes for immunoblotting. The western blots were performed using rat anti-Subito at 1:2000 (Jang et al., 2005), rat anti-HA (clone 3F10, Roche) at 1:5000, mouse anti-Polo (MA294) (Llamazares et al., 1991) at 1:80, mouse anti-GFP (clone JL.8, Clontech) at 1:2000 and detected with HRP-conjugated secondary antibodies (Jackson Labs) at 1:5000 and ECL reagents (GE Healthcare).

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References

Adams, R. R., Tavares, A. A., Salzberg, A., Bellen, H. J. and Glover, D. M. (1998). *pavarotti* encodes a kinesin-like protein required to organize the central spindle and contractile ring for cytokinesis. *Genes Dev.* **12**, 1483-1494.

Adams, R. R., Carmena, M. and Earnshaw, W. C. (2001a). Chromosomal passengers and the (aurora) ABCs of mitosis. *Trends Cell Biol.* **11**, 49-54.

Adams, R. R., Maiato, H., Earnshaw, W. C. and Carmena, M. (2001b). Essential roles of Drosophila inner centromere protein (INCENP) and aurora B in histone H3 phosphorylation, metaphase chromosome alignment, kinetochore disjunction, and chromosome segregation. *J. Cell Biol.* **153**, 865-880.

Basto, R., Gomes, R. and Karess, R. E. (2000). Rough deal and Zw10 are required for the metaphase checkpoint in Drosophila. *Nat. Cell Biol.* **2**, 939-943.

Basu, J., Bousbaa, H., Logarinho, E., Li, Z., Williams, B. C., Lopes, C., Sunkel, C. E. and Goldberg, M. L. (1999). Mutations in the essential spindle checkpoint gene bub1 cause chromosome missegregation and fail to block apoptosis in Drosophila. *J. Cell Biol.* **146**, 13-28.

Blower, M. D. and Karpen, G. H. (2001). The role of Drosophila CID in kinetochore formation, cell-cycle progression and heterochromatin interactions. *Nat. Cell Biol.* **3**, 730-739.
Bonaccorsi, S., Giansanti, M. G. and Gatti, M. (2000). Spindle assembly in Drosophila neuroblasts and ganglion mother cells. *Nat. Cell Biol.* **2**, 54-56.
Brunet, S., Polanski, Z., Verlhac, M. H., Kubiak, J. Z. and Maro, B. (1998). Bipolar meiotic spindle formation without chromatin. *Curr. Biol.* **8**, 1231-1234.
Bucciarelli, E., Giansanti, M. G., Bonaccorsi, S. and Gatti, M. (2003). Spindle assembly and cytokinesis in the absence of chromosomes during Drosophila male meiosis. *J. Cell Biol.* **160**, 993-999.
Carmena, M., Riparbelli, M. G., Minestrini, G., Tavares, A. M., Adams, R., Callaini, G. and Glover, D. M. (1998). Drosophila polo kinase is required for cytokinesis. *J. Cell Biol.* **143**, 659-671.
Castrillon, D. H. and Wasserman, S. A. (1994). Diaphanous is required for cytokinesis in Drosophila and shares domains of similarity with the products of the limb deformity gene. *Development* **120**, 3367-3377.
Chang, C., Goulding, S., Adams, R. R., Earnshaw, W. C. and Carmena, M. (2006). INCENP is required for cytokinesis and asymmetric cell division during development of the nervouse system. *J. Cell. Sci.* **119**, 1144-1153.
Clarke, A. S., Tang, T. T., Ooi, D. L. and Orr-Weaver, T. L. (2005). POLO kinase regulates the Drosophila centromere cohesion protein MEI-S332. *Dev. Cell* **8**, 53-64.
Compton, D. A. (2000). Spindle assembly in animal cells. *Annu. Rev. Biochem.* **69**, 95-114.
Dagenbach, E. M. and Endow, S. A. (2004). A new kinesin tree. *J. Cell Sci.* **117**, 3-7.
Donaldson, M. M., Tavares, A. A., Ohkura, H., Deak, P. and Glover, D. M. (2001). Metaphase arrest with centromere separation in polo mutants of Drosophila. *J. Cell Biol.* **153**, 663-676.
Echard, A., Hickson, G. R., Foley, E. and O'Farrell, P. H. (2004). Terminal cytokinesis events uncovered after an RNAi screen. *Curr. Biol.* **14**, 1685-1693.
Eggert, U. S., Kiger, A. A., Richter, C., Perlman, Z. E., Perrimon, N., Mitchison, T. J. and Field, C. M. (2004). Parallel chemical genetic and genome-wide RNAi screens identify cytokinesis inhibitors and targets. *PLoS Biol.* **2**, e379.
Faruki, S., Cole, R. W. and Rieder, C. L. (2002). Separating centrosomes interact in the absence of associated chromosomes during mitosis in cultured vertebrate cells. *Cell Motil. Cytoskeleton* **52**, 107-121.
Fontijn, R. D., Goud, B., Echard, A., Jollivet, F., van Marle, J., Pannekoek, H. and Horrevoets, A. J. (2001). The human kinesin-like protein RB6K is under tight cell cycle control and is essential for cytokinesis. *Mol. Cell. Biol.* **21**, 2944-2955.
Giansanti, M. G., Bonaccorsi, S., Williams, B., Williams, E. V., Santolamazza, C., Goldberg, M. L. and Gatti, M. (1998). Cooperative interactions between the central spindle and the contractile ring during Drosophila cytokinesis. *Genes Dev.* **12**, 396-410.
Giansanti, M. G., Bonaccorsi, S., Bucciarelli, E. and Gatti, M. (2001). Drosophila male meiosis as a model system for the study of cytokinesis in animal cells. *Cell Struct. Funct.* **26**, 609-617.
Giet, R. and Glover, D. M. (2001). Drosophila aurora B kinase is required for histone H3 phosphorylation and condensin recruitment during chromosome condensation and to organize the central spindle during cytokinesis. *J. Cell Biol.* **152**, 669-682.
Giunta, K. L., Jang, J. K., Manheim, E. M., Subramanian, G. and McKim, K. S. (2002). *subito* encodes a kinesin-like protein required for meiotic spindle pole formation in *Drosophila melanogaster*. *Genetics* **160**, 1489-1501.
Glover, D. M., Leibowitz, M. H., McLean, D. A. and Parry, H. (1995). Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles. *Cell* **81**, 95-105.
Goshima, G. and Vale, R. D. (2003). The roles of microtubule-based motor proteins in mitosis: comprehensive RNAi analysis in the Drosophila S2 cell line. *J. Cell Biol.* **162**, 1003-1016.
Goto, H., Kiyono, T., Tomono, Y., Kawajiri, A., Urano, T., Furukawa, K., Nigg, E. A. and Inagaki, M. (2006). Complex formation of Plk1 and INCENP required for metaphase-anaphase transition. *Nat. Cell Biol.* **8**, 180-187.
Gruneberg, U., Neef, R., Honda, R., Nigg, E. A. and Barr, F. A. (2004). Relocation of Aurora B from centromeres to the central spindle at the metaphase to anaphase transition requires MKlp2. *J. Cell Biol.* **166**, 167-172.
Gunsalus, K. C., Bonaccorsi, S., Williams, E., Verni, F., Gatti, M. and Goldberg, M. L. (1995). Mutations in twinstar, a Drosophila gene encoding a cofilin/ADF homologue, result in defects in centrosome migration and cytokinesis. *J. Cell Biol.* **131**, 1243-1259.
Gurley, L. R., D'Anna, J. A., Barham, S. S., Deaven, L. L. and Tobey, R. A. (1978). Histone phosphorylation and chromatin structure during mitosis in Chinese hamster cells. *Eur. J. Biochem.* **84**, 1-15.
Henikoff, S., Ahmad, K., Platero, J. S. and van Steensel, B. (2000). Heterochromatic deposition of centromeric histone H3-like proteins. *Proc. Natl. Acad. Sci. USA* **97**, 716-721.
Jang, J. K., Rahman, T. and McKim, K. S. (2005). The kinesin-like protein Subito contributes to central spindle assembly and organization of the meiotic spindle in Drosophila oocytes. *Mol. Biol. Cell* **16**, 4684-4694.
Khodjakov, A., Copenagle, L., Gordon, M. B., Compton, D. A. and Kapoor, T. M. (2003). Minus-end capture of preformed kinetochore fibers contributes to spindle morphogenesis. *J. Cell Biol.* **160**, 671-683.
Kiger, A. A., Baum, B., Jones, S., Jones, M. R., Coulson, A., Echeverri, C. and Perrimon, N. (2003). A functional genomic analysis of cell morphology using RNA interference. *J. Biol.* **2**, 27.
Kirschner, M. and Mitchison, T. (1986). Beyond self-assembly: from microtubules to morphogenesis. *Cell* **45**, 329-342.
Llamazares, S., Moreira, A., Tavares, A., Girdham, C., Spruce, B. A., Gonzalez, C., Karess, R. E., Glover, D. M. and Sunkel, C. E. (1991). *polo* encodes a protein kinase homolog required for mitosis in *Drosophila*. *Genes Dev.* **5**, 2153-2165.
Logarinho, E. and Sunkel, C. E. (1998). The Drosophila POLO kinase localises to multiple compartments of the mitotic apparatus and is required for the phosphorylation of MPM2 reactive epitopes. *J. Cell Sci.* **111**, 2897-2909.
Logarinho, E., Bousbaa, H., Dias, J. M., Lopes, C., Amorim, I., Antunes-Martins, A. and Sunkel, C. E. (2004). Different spindle checkpoint proteins monitor microtubule attachment and tension at kinetochores in Drosophila cells. *J. Cell Sci.* **117**, 1757-1771.
Lukinova, N. I., Roussakova, V. V. and Fortini, M. E. (1999). Genetic characterization of cytological region 77A-D harboring the presenilin gene of Drosophila melanogaster. *Genetics* **153**, 1789-1797.
Maiato, H., Rieder, C. L. and Khodjakov, A. (2004). Kinetochore-driven formation of kinetochore fibers contributes to spindle assembly during animal mitosis. *J. Cell Biol.* **167**, 831-840.
Mastronarde, D. N., McDonald, K. L., Ding, R. and McIntosh, J. R. (1993). Interpolar spindle microtubules in PTK cells. *J. Cell Biol.* **123**, 1475-1489.
Matuliene, J. and Kuriyama, R. (2002). Kinesin-like protein CHO1 is required for the formation of midbody matrix and the completion of cytokinesis in mammalian cells. *Mol. Biol. Cell* **13**, 1832-1845.
McKim, K. S. and Hawley, R. S. (1995). Chromosomal control of meiotic cell division. *Science* **270**, 1595-1601.
Megraw, T. L., Kao, L. R. and Kaufman, T. C. (2001). Zygotic development without functional mitotic centrosomes. *Curr. Biol.* **11**, 116-120.
Miki, H., Okada, Y. and Hirokawa, N. (2005). Analysis of the kinesin superfamily: insights into structure and function. *Trends Cell Biol.* **15**, 467-476.
Minestrini, G., Harley, A. S. and Glover, D. M. (2003). Localization of Pavarotti-KLP in living Drosophila embryos suggests roles in reorganizing the cortical cytoskeleton during the mitotic cycle. *Mol. Biol. Cell* **14**, 4028-4038.
Moore, D. P., Page, A. W., Tang, T. T., Kerrebrock, A. W. and Orr-Weaver, T. L. (1998). The cohesion protein MEI-S332 localizes to condensed meiotic and mitotic centromeres until sister chromatids separate. *J. Cell Biol.* **140**, 1003-1012.
Moutinho-Santos, T., Sampaio, P., Amorim, I., Costa, M. and Sunkel, C. E. (1999). In vivo localisation of the mitotic POLO kinase shows a highly dynamic association with the mitotic apparatus during early embryogenesis in Drosophila. *Biol. Cell* **91**, 585-596.
Neef, R., Preisinger, C., Sutcliffe, J., Kopajtich, R., Nigg, E. A., Mayer, T. U. and Barr, F. A. (2003). Phosphorylation of mitotic kinesin-like protein 2 by polo-like kinase 1 is required for cytokinesis. *J. Cell Biol.* **162**, 863-875.
Neef, R., Klein, U. R., Kopajtich, R. and Barr, F. A. (2006). Cooperation between mitotic kinesins controls the late stages of cytokinesis. *Curr. Biol.* **16**, 301-307.
Nicklas, R. B. (1997). How cells get the right chromosomes. *Science* **275**, 632-637.
Nislow, C., Lombillo, V. A., Kuriyama, R. and McIntosh, J. R. (1992). A plus-end-directed motor enzyme that moves antiparallel microtubules in vitro localizes to the interzone of mitotic spindles. *Nature* **359**, 543-547.
Parks, A. L., Cook, K. R., Belvin, M., Dompe, N. A., Fawcett, R., Huppert, K., Tan, L. R., Winter, C. G., Bogart, K. P., Deal, J. E. et al. (2004). Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. *Nat. Genet.* **36**, 288-292.
Raich, W. B., Moran, A. N., Rothman, J. H. and Hardin, J. (1998). Cytokinesis and midzone microtubule organization in Caenorhabditis elegans require the kinesin-like protein ZEN-4. *Mol. Biol. Cell* **9**, 2037-2049.
Schupbach, T. and Wieschaus, E. (1989). Female sterile mutations on the second chromosome of *Drosophila melanogaster*. I. Maternal effect mutations. *Genetics* **121**, 101-117.
Somers, W. G. and Saint, R. (2003). A RhoGEF and Rho family GTPase-activating protein complex links the contractile ring to cortical microtubules at the onset of cytokinesis. *Dev. Cell* **4**, 29-39.
Van Doren, M., Williamson, A. L. and Lehmann, R. (1998). Regulation of zygotic gene expression in Drosophila primordial germ cells. *Curr. Biol.* **8**, 243-246.
Verni, F., Somma, M. P., Gunsalus, K. C., Bonaccorsi, S., Belloni, G., Goldberg, M. L. and Gatti, M. (2004). Feo, the Drosophila homolog of PRC1, is required for central-spindle formation and cytokinesis. *Curr. Biol.* **14**, 1569-1575.
Vernos, I. (2004). The chromosomal passenger complex takes center stage during mitosis. *Dev. Cell* **7**, 145-146.
Wadsworth, P. and Khodjakov, A. (2004). E pluribus unum: towards a universal mechanism for spindle assembly. *Trends Cell Biol.* **14**, 413-419.
Walczak, C. E., Vernos, I., Mitchison, T. J., Karsenti, E. and Heald, R. (1998). A model for the proposed roles of different microtubule-based motor proteins in establishing spindle bipolarity. *Curr. Biol.* **8**, 903-913.
Yamamoto, A. H., Komma, D. J., Shaffer, C. D., Pirrotta, V. and Endow, S. A. (1989). The claret locus in Drosophila encodes products required for eye color and for meiotic chromosome segregation. *EMBO J.* **8**, 3543-3552.

Table S1. Frequency of aneuploidy in *subito* and *Incenp* mutants

Genotype	2*n*	2*n*–1	2*n*+1	2*n*–2	2*n*+2	2*n*–3	2*n*+3	Polyploid	% Hypoploid and Hyperploid	% Polyploid	Total cells
Wild type	217	9	3	2	1	0	0	0	6.5	0.0	232
sub^{1}/sub^{131}	172	10	16	0	5	0	2	11	15.3	5.1	216
ncd^{1}	192	4	1	0	0	0	1	15	2.8	7.0	213
$sub^{1};ncd^{1}$	185	6	3	1	2	0	0	6	5.9	3.0	203
$sub^{131};ncd^{1}$	68	1	6	3	5	0	2	5	18.9	5.6	90

The *sub;ncd* double mutant lethality was not associated with the expected increased frequency of aneuploidy anaphase.
We have not checked for other possible phenotypes, such as an increase in cell death, which may be associated with the synthetic lethal phenotype.