Dual roles of Incenp crucial to the assembly of the acentrosomal metaphase spindle in female meiosis

Citation for published version:
Colombie, N, Cullen, F, Brittle, A, Jang, J, Earnshaw, B, Carmena, M, McKim, K & Ohkura, H 2008, 'Dual roles of Incenp crucial to the assembly of the acentrosomal metaphase spindle in female meiosis' Development, vol 135, no. 19, pp. 3239-3246. DOI: 10.1242/dev.022624

Digital Object Identifier (DOI):
10.1242/dev.022624

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Development

Published in final edited form as:
Development. 2008 October ; 135(19): 3239–3246. doi:10.1242/dev.022624.

Dual roles of Incenp crucial to the assembly of the acentrosomal metaphase spindle in female meiosis

Nathalie Colombié1), **C. Fiona Cullen**1), **Amy L. Brittle**1), **Janet K. Jang**3), **William C. Earnshaw**2), **Mar Carmena**2), **Kim McKim**3), and **Hiroyuki Ohkura**1),4)

1)Ohkura group, The Wellcome Trust Centre for Cell Biology, The University of Edinburgh, EH9 3JR, Edinburgh, UK

2)Earnshaw group, The Wellcome Trust Centre for Cell Biology, The University of Edinburgh, EH9 3JR, Edinburgh, UK

3)Waksman Institute and Department of Genetics, Rutgers, the State University of New Jersey, Piscataway, New Jersey 08854-8020, USA

SUMMARY

Spindle formation in female meiosis differs from mitosis in many animals, as it takes place independently from centrosomes, and the molecular requirements of this pathway remain to be understood. Here we report two crucial roles of Incenp, an essential subunit of the chromosomal passenger complex (the Aurora B complex), in centrosome-independent spindle formation in *Drosophila* female meiosis. Firstly, the initial assembly of spindle microtubules is drastically delayed in an *incenp* mutant. This clearly demonstrates, for the first time, a crucial role for Incenp in chromosome-driven spindle microtubule assembly in living oocytes. Additionally, Incenp is necessary to stabilise the equatorial region of the metaphase I spindle, in contrast to mitosis, where the equivalent function becomes prominent after anaphase onset. Our analysis suggests that Subito, a kinesin-6 protein, cooperates with Incenp for this latter function, but not microtubule assembly. We propose that the two functions of Incenp are part of the mechanisms that compensate for the lack of centrosomes during meiotic spindle formation.

Keywords

Drosophila; Aurora; kinase; microtubule; meiosis

INTRODUCTION

Cell division is developmentally controlled depending on specific cell requirements. Meiosis is a specialised cell division that produces sperm and oocytes. It differs from mitosis in many aspects including cell cycle control, chromosome dynamics and spindle morphogenesis. One of the main differences in spindle morphogenesis is the contribution of centrosomes. Unlike mitosis where centrosomes play a central role in spindle assembly, a bipolar spindle forms without centrosomes during female meiosis in many animals including humans and *Drosophila* (McKim and Hawley, 1995; Waters and Salmon, 1997).

The crucial, but unanswered, question is what are the differences in the molecular requirements and regulation of spindle formation between female meiosis and mitosis. Although a bipolar spindle can still form without centrosomes in mitosis if they are

---

[4)] Corresponding author.

artificially eliminated (Khodjakov et al., 2000; Basto et al., 2006), the acentrosomal spindle in female meiosis is much more robust and is likely to possess mechanisms that compensate for the lack of centrosomes.

In the absence of centrosomes, chromosomes play a central role in spindle microtubule assembly. *In vitro* studies using *Xenopus* extracts have revealed central roles of the Ran-importin system in chromosome-mediated spindle microtubule assembly (Gruss et al., 2001; Wiese et al., 2001), whereas recent studies in living mouse oocytes suggest the existence of a Ran-independent pathway (Dumont et al., 2007; Schuh and Ellenberg, 2007). *In vitro* studies found a Ran-independent involvement of the chromosomal passenger complex in spindle microtubule assembly (Sampath et al., 2004; Kelly et al., 2007), but *in vivo* studies have not so far indicated such a role (Adams et al., 2001; Giet and Glover, 2001; Gassmann et al., 2004; Andrews et al., 2004; Lan et al., 2004; Resnick et al., 2006). Therefore, a crucial question remains: whether and how much the chromosomal passenger complex actually contributes to spindle microtubule assembly in living oocytes.

Furthermore, spindle bipolarity needs to be established and maintained without centrosomes in female meiosis. DNA-coated beads in *Xenopus* extracts can organise a bipolar spindle without centrosomes or kinetochores, indicating that spindle bipolarity is generated by the self-organisation of microtubules into anti-parallel arrays (Heald et al., 1996). Genetic studies in *Drosophila* revealed that Subito, the MKLP-2 homologue (kinesin-6), localises to the equatorial region of the metaphase I spindle, and is required for its organisation and bipolarity (Giunta et al., 2002; Jang et al., 2005; Jang et al., 2007). The equatorial region in the metaphase I spindle accumulates proteins that normally localise to the central spindle of mitotic anaphase/telophase, including the chromosomal passenger complex (Jang et al 2005). Therefore, it has been proposed that the equatorial region of the meiotic metaphase I spindle is actually equivalent to the central spindle in mitotic anaphase/telophase, and this structure (sometimes referred to as the meiotic metaphase central spindle) is crucial to establish spindle bipolarity in the absence of centrosomes (Jang et al., 2005).

Despite these biochemical and genetic studies, our knowledge of spindle formation in female meiosis is still limited at the molecular level. In this study we identify an *incenp* mutant which disrupts the bipolarity of the metaphase I spindle in *Drosophila* female meiosis. Incenp is an essential subunit of the chromosomal passenger complex containing Aurora B kinase. The chromosomal passenger complex plays multiple roles in mitosis and meiosis (Vagnarelli and Earnshaw, 2004; Ruchaud et al., 2007), but no role has been reported in spindle morphogenesis in female meiosis.

Here we show that Incenp has two roles in the acentrosomal spindle formation of *Drosophila* female meiosis. First, live imaging analysis showed that the initial assembly of spindle microtubules is drastically delayed in the *incenp* mutant. This is the first and definitive *in vivo* demonstration of a crucial role for a subunit of the chromosomal passenger complex in the centrosome-independent spindle microtubule assembly. Furthermore, we found that Incenp is required for the stability of the spindle equatorial region in meiotic metaphase I to prevent formation of ectopic poles. This is consistent with the precocious localisation of Incenp to the spindle equatorial region at metaphase. These two functions of Incenp may be part of the mechanisms that compensate for the lack of centrosomes in female meiosis.

MATERIALS and METHODS

Genetic, molecular, immunological techniques

Standard techniques of fly manipulation were followed (Ashburner et al., 2005). All stocks were grown at 25°C in standard cornmeal media. w^{1118} was used as wild type. Details of mutations and chromosome aberrations can be found in Lindsley and Zimm (1992) or at Fly Base (http://flybase.org; Drysdale et al., 2005). For live-imaging, UASp -GFP-α-tubulin and GAL4 under the maternal *nanos* promotor on the third chromosome were used.

Standard DNA manipulation and immunological techniques were used throughout (Sambrook et al., 1989; Harlow and Lane, 1988). The primary antibodies used in this study include antibodies against α-tubulin (DM1A; Sigma), γ-tubulin (GLU-88; Sigma), D-TACC (Gergely et al., 2000; Cullen and Ohkura, 2001), Cyclin B (Whitfield et al., 1990), Subito (Jang et al., 2005), Incenp (C. Wu and K. McKim, unpublished) and Aurora B (Adams et al., 2001). For immunoblots, peroxidase-conjugated antibodies (Jackson Lab) were used as secondary antibodies for western blot and detected by ECL kit (Pharmacia).

Molecular identification of the gene mutated in QA26

For the molecular identification of the female sterile mutations on the QA26 chromosome, it was first mapped by recombination with chromosomes carrying visible markers. The region was further narrowed by complementation testing using deficiencies in the area. The female sterile mutation in QA26 is located between the proximal breakpoints of *Df(2R)Drl*^{rv30} and *Df(2R)ED1715*. A tight linkage of the female sterile mutation with spindle defects in female meiosis was confirmed by cytological analysis of the mutant chromosome over small deficiencies. To test whether the female sterile mutation is in the *incenp* locus, a lethal *incenp*^{3747} mutation (Chang et al., 2006) was used for complementation. For further confirmation, the *incenp* coding region were amplified by PCR from the QA26 genomic DNA and sequenced using BigDye (Applied Biosystems).

Cytological analysis

Immunostaining was carried out, as previously described, for D-TACC/Cyclin B (Tavosanis et al., 1997; Cullen and Ohkura, 2001) or Incenp/Aurora B (Theurkauf and Hawley, 1992; Jang et al., 2005) with α-tubulin in non-activated oocytes, and for D-TACC with α-tubulin in activated oocytes (Cullen et al., 2005). Secondary antibodies conjugated with Cy3, Cy5 or Alexa488 (Jackson Lab or Molecular Probes) were used at 1/250-1/1000 dilution. DNA was counterstained with Hoescht, DAPI (0.4μg/ml; Sigma) or propidium iodide (2μg/μl, Sigma). The images were taken using a Plan-Apochromat lens (63X, 1.4NA; Zeiss) attached to an Axiovert 200M (Zeiss) with a confocal scan head (LSM510meta; Zeiss). Confocal images were presented as a maximum intensity projection of the Z-stacks. All digital images were imported to Photoshop/ImageReady (Adobe), and the brightness and contrast were uniformly adjusted for the whole field without changing features of the images. Statistical significance was calculated with Student t test.

Live-imaging of meiosis I spindle was carried out as described (Matthies et al., 1996; Endow and Komma, 1997) except maternally expressed GFP-α-tubulin was used (GAL4-nosUTR combined with UASp-GFP-α-tubulin). Briefly, oocytes were dissected from matured adult females in halocarbon oil (700) and observed using the confocal microscope described above. Typically, a series of Z-sections (separated by 1 μm) which cover the entire spindle was taken every 20-35 seconds.

RESULTS

Identification of an *incenp* mutant defective in the metaphase I spindle in female meiosis

To understand how the acentrosomal spindle is formed in female meiosis, we have cytologically screened collections of female sterile mutants for spindle defects in metaphase I arrested oocytes. From the collection made by Schupbach and Wieschaus (1989), we found that a mutant, QA26, showed an abnormal spindle morphology. It was originally classified as showing “no visible sign of development” (Schupbach and Wieschaus, 1989) along with *γ-tubulin37C* (*TW1*), *subito* and *cks30A* (*rem*) mutants which were later found to have spindle defects at metaphase I (Tavosanis et al., 1997; Giunta et al., 2002; Pearson et al., 2005).

By recombination and deficiency mapping, we localised the mutation to genomic region 43A1-43A4 (see Materials and Methods). This region contains 8 genes, including *incenp* which is known to regulate mitosis. The mutant chromosome did not complement a lethal *incenp* allele and has a point mutation which alters a conserved residue within the IN box, the domain responsible for interaction of Incenp with Aurora B (Adams et al., 2000). This demonstrates that the mutation is an allele of *incenp*. A parallel study (Resnick et al., 2006) has already reported QA26 as an *incenp* allele and described its defects in male meiosis.

In this report we focused on the study of spindle defects observed in non-activated mutant oocytes, although we also observed abnormal meiotic progression in at least some of the activated mutant oocytes (Fig. S1 in the supplementary material). As *incenp* is an essential gene, the phenotype we observed may not represent the full range of Incenp function. Nevertheless, this hypomorphic allele allows us to uncover the role of Incenp in female meiosis.

The *incenp* mutant forms ectopic spindle poles in female meiosis

Incenp is an essential subunit of the chromosomal passenger complex containing Aurora B kinase. The complex accumulates at centromeres during early mitosis, and then translocates to the spindle equatorial region/ central spindle after initiation of anaphase (Adams et al., 2001). In contrast to mitosis, a previous report (Jang et al., 2005) showed that, in female meiosis, Aurora B and Incenp accumulate on the spindle equatorial region in metaphase. We found that the *incenp*^{QA26} mutation disrupts the morphology of the metaphase I arrested spindle in female meiosis.

To determine the role of Incenp in meiotic spindle formation, we examined the morphology of metaphase I arrested spindles in mature non-activated oocytes by immunostaining. The oocytes were fixed and stained for α-tubulin, the pole protein D-TACC and DNA. In wild type, the metaphase I arrested spindle is bipolar with tapered poles (Fig. 1A). Bivalent chromosomes are aligned at the spindle equator with the achiasmatic small 4th chromosomes usually located symmetrically closer to the poles. In the *incenp* mutants, spindle organisation was disrupted in over 50% of oocytes (n=69; Fig. 1D). Although the abnormality varies from oocyte to oocyte, typical defects include the formation of one or more ectopic spindle poles usually around the equator or next to the main poles (Fig. 1B,C). These poles typically have the pole protein D-TACC correctly localised. These results showed that Incenp is required for the proper organisation of the metaphase I arrested spindle in *Drosophila* female meiosis. Chromosome alignment or location was affected to a lesser extent (32% abnormal compared to 15% in wild type). However, it is difficult to conclude which process is primarily defective, spindle formation or chromosome alignment (or both), as these two processes are inter-dependent during acentrosomal spindle assembly.

Instability of spindle bipolarity during metaphase I arrest in the *incenp* mutant

To further understand the spindle abnormalities in the *incenp* mutant, we examined the metaphase I arrested spindle by live-imaging analysis. We first analysed spindle dynamics in a wild-type background using maternally driven GFP-α-tubulin (Materials and Methods). Oocytes were dissected in halocarbon oil and examined under a confocal microscope. In wild type, a metaphase I arrested spindle maintained its overall shape and bipolarity over time (Fig. 2A and Movie 1 in the supplementary material), consistent with previous reports using a Ncd-GFP transgene or injection of fluorescently-labelled tubulin (Matthies et al., 1996; Endow and Komma, 1997).

For live-imaging observation of mutant spindles, we introduced GFP-α-tubulin transgenes into the *incenp* mutant by successive genetic crosses. Consistent with our immunostaining results, among the 47 metaphase I arrested spindles we observed, about 40% (19) showed an abnormal morphology, typically exhibiting ectopic or split poles, at the beginning of the observation. Half of these abnormal spindles (9) became bipolar during our observation that typically lasted for 20 to 40 minutes, while some of the others (6) changed their morphology but stayed abnormal. On the other hand, most of the initially bipolar spindles (20) remained bipolar during the observation, however others (8) lost their bipolarity through the appearance of ectopic poles in most cases or, less often, splitting of poles. Ectopic poles usually grew from the spindle equatorial region, and spindle bipolarity was restored by disassembling the ectopic pole or merging it with one of the main poles (Fig. 2B and Movie 2 in the supplementary material, which shows the clearest example). In total, more than a third of all the observed spindles (17) showed at least one inter-conversion of their morphology between bipolar and abnormal during our observation. Therefore, Incenp is required for the stability of spindle bipolarity during metaphase I arrest.

To understand the requirement for Incenp during spindle formation in female meiosis, we followed spindle formation from beginning of nuclear envelope breakdown. Prophase oocytes expressing GFP-α-tubulin were selected for study and their progression was followed over time. In wild type (Fig. 3A and Movie 3 in the supplementary material), GFP-α-tubulin was excluded from the prophase nucleus, until just before nuclear envelope breakdown, when it entered the nucleus. After nuclear envelope breakdown, the GFP-α-tubulin diffused into the cytoplasm. After a short gap, microtubules assembled around the cluster of meiotic chromosomes (called the karyosome), which can be recognised as a dark spherical shape (that excludes the GFP signal). Multiple transitory poles were formed during very early stages of spindle assembly, but one axis quickly became dominant. Once one axis was established, it was maintained without forming other poles. Then the poles were focused and the spindle elongated before arresting in metaphase. These observations were in agreement with previous reports using Ncd-GFP or injection of fluorescently labelled tubulin (Matthies et al., 1996; Endow and Komma, 1997).

Similarly to wild type, in the *incenp* mutant, spindle microtubules were assembled around the chromosomes after nuclear envelope breakdown, and multiple transitory poles appeared at the beginning of spindle formation. However, unlike wild type, even after one spindle axis became dominant, other poles continued to be formed. These ectopic poles eventually fused with the original poles during spindle formation. In the time sequence shown in Fig. 3B (and Movie 4 in the supplementary material), soon after microtubules were assembled around the chromosomes, multiple poles were temporarily formed. Eventually two dominant poles were established. Then a third pole (arrow in Fig. 3B) appeared near the spindle equatorial region and merged with one of the main poles to re-establish bipolarity. In total, 6 out of 14 *incenp* oocytes observed by us showed abnormalities during the formation of the meiotic spindle, while all the 10 wild-type oocytes observed behaved normally. In summary, time-lapse observation of meiotic spindle formation revealed that the *incenp* mutant exhibits instability of spindle bipolarity, particularly near the spindle equatorial region, before and after the metaphase I arrest.

The spindle equatorial region is partially defective in the *incenp* mutant

The instability of the spindle equatorial region in the *incenp* mutant may be caused by a failure to recruit other proteins to this region and/or by defective organisation of this region. First we examined the localisation of Incenp and Aurora B in *incenp* mutant oocytes. We found that the mutant Incenp protein localised to the spindle equatorial region (Fig. 4B) as does the wild-type Incenp protein (Fig. 4A). Aurora B was also accumulated in the equatorial region, although we are uncertain about the level of the accumulation relative to wild type due to a high background (Fig. 4B). Next we examined the effect of the *incenp* mutation on Cyclin B which also localises to the spindle equatorial region in wild-type female meiosis (Pearson et al., 2005). Immunostaining indicated that Cyclin B was still localised to the spindle equatorial region in the *incenp* mutant (Fig. 4C).

To quantify the integrity of the spindle equatorial region in the *incenp* mutant, we compared the relative microtubule density of this spindle region in wild-type and mutant oocytes expressing GFP-α-tubulin. We measured the intensity of GFP-α-tubulin along the spindle axis (Fig. 4E). In wild type, the spindle equatorial region gave an average of 40% higher GFP-tubulin signal than the pole regions, probably representing the overlapping anti-parallel microtubule array in the equatorial region (Fig. 4D,F). In the *incenp* mutant, in contrast, the GFP-tubulin signal at the spindle equatorial region was significantly reduced ($p<0.01$) to a level comparable with that of pole regions (Fig. 4D,F). This result showed that the spindle equatorial region is structurally as well as functionally defective in the *incenp* mutant.

Our analysis demonstrated that Incenp is required for the stability and organisation of the spindle equatorial region in prometaphase and metaphase in female meiosis. This is consistent with a previous report showing that Incenp precociously localises to the spindle equatorial region in prometaphase and metaphase in female meiosis (Jang et al., 2005), which is in contrast to mitosis or male meiosis when it is localised to centromeres prior to anaphase (Adams et al., 2001; Resnick et al., 2006).

The *incenp* mutation delays spindle microtubule assembly in female meiosis

In addition to defects in spindle bipolarity, we also noticed that the initiation of spindle microtubule assembly was considerably delayed in the *incenp* mutant. For quantification, we measured the time between nuclear envelope breakdown and the first appearance of microtubules around the chromosomes. This process took an average of 354 seconds (~6 minutes) in wild-type oocytes, while it was delayed three-fold to 1,128 seconds (~19 minutes) in the *incenp* mutant (Fig. 5A). The difference is statistically significant ($p<0.001$).

To confirm that this delay was not due to reduced levels of tubulin, we examined the amounts of α- and γ-tubulin in ovaries. Immunoblots showed that the levels of the tubulins were not significantly affected by the *incenp* mutation (Fig. 5C). On the other hand, the length of the metaphase I spindle in the *incenp* mutant was not significantly different from that in the wild-type oocytes (Fig. 5B). Therefore, the spindle length appears to be determined by mechanisms that do not crucially depend on Incenp activity.

In conclusion, these results showed a crucial role of Incenp in the initial assembly of microtubules around chromosomes. Although this function was previously indicated by a study using *Xenopus* extract (Sampath et al., 2004), this is the first *in vivo* evidence to demonstrate the involvement of Incenp, or any subunits of the chromosomal passenger complex, in the assembly of spindle microtubules.

A *subito* null mutation induces instability of the central spindle but does not delay microtubule assembly

Subito, a kinesin-6 protein, was previously shown to localise to the equatorial region of the meiotic metaphase I spindle and is required for the localisation of the chromosomal passenger complex and all known proteins recruited to the equatorial region (Jang et al., 2005). Consistent with this, immunostaining indicated that the *incenp* and *subito* mutants show similar phenotypes.

To further explore the relationship between Incenp and Subito, we first examined whether Subito localisation is affected by the *incenp* mutation. Metaphase I arrested spindles were immunostained for α-tubulin and Subito. We found that the Subito protein still localised to the spindle equatorial region in the *incenp* mutant (Fig. 6A), contrasting with Incenp delocalisation in a *subito* mutant (Jang et al., 2005). This shows that the defects observed in the *incenp* mutant are not due to Subito delocalisation.

Next, we examined spindle organisation in a *subito* null mutant by live-imaging analysis. We introduced GFP-α-tubulin and GAL4 transgenes into the *subito* mutant, and dissected oocytes were observed under a confocal microscope. Live-imaging of metaphase I arrested oocytes revealed spindle instability. During our observations, an ectopic pole formed around the equatorial region in about half of the spindles (11/18) that were initially bipolar. In most cases, the bipolarity was restored as this ectopic pole merged with one of the main poles (Fig. 6B and Movie 5 in the supplementary material). Additionally, among the spindles that exhibited ectopic poles at the beginning of our observation, about a half (9/16) became bipolar before the end.

Consistent with a requirement of Subito for Incenp localisation, the instability of spindle bipolarity is shared by both *incenp* and *subito* mutants. In addition, both mutants exhibited low microtubule density at the spindle equatorial region (this study; Jang et al., 2005). However, there are some notable differences between the *subito* and *incenp* mutants. Firstly, in the *subito* mutant, splitting of the poles was not observed as it was in the *incenp* mutant. Secondly, the *subito* mutant showed much more frequent and dynamic inter-conversions of spindle morphology between bipolar and tripolar. These differences may be due to the stronger nature of the *subito* mutation and/or to other spindle equatorial region proteins affected by the *subito* mutation.

To assess whether Subito functions in the assembly of spindle microtubules, we also measured the time from nuclear envelope breakdown to the first appearance of spindle microtubules in the *subito* mutant. Unlike the *incenp* mutant which takes three times longer than the wild type to begin spindle microtubule assembly, the *subito* mutant did not show a significant delay (354 seconds in wild type vs 408 seconds in *subito*; $p=0.45$). These results clearly showed that this function of Incenp is independent of Subito.

DISCUSSION

From a screen of female sterile mutants for spindle defects in female meiosis, we identified a mutant of Incenp, an essential subunit of the chromosomal passenger complex containing Aurora B kinase. Live-imaging analysis of the mutant revealed roles of Incenp in two critical steps of the acentrosomal spindle formation in female meiosis. The first is to assemble spindle microtubules around chromosomes, and the second is to stabilise the spindle equatorial region to maintain spindle bipolarity. These two functions are separable and differentially regulated in terms of their requirement for Subito, a kinesin-like protein.

The function of Incenp in spindle microtubule assembly in female meiosis

In the absence of centrosomes, which are the major sites of microtubule nucleation in mitosis, chromosomes appear to play a crucial role in the assembly of spindle microtubules. In recent years, the molecular basis of this activity of chromosomes has been under intense investigation. Beads coated with phage DNA can assemble microtubules in *Xenopus* extract without centrosomes or kinetochores (Heald et al., 1996). Mainly using the *Xenopus in vitro* system, a great deal of evidence has been accumulated to support the hypothesis that Ran activated by a chromosome-associated factor, Rcc1, plays a central role in the assembly of spindle microtubules in the absence of centrosomes (Carazo-Salas et al., 1999; Kalab et al., 2002; Goodman and Zheng, 2006). Despite this compelling evidence obtained in *in vitro* studies, the extent of Ran involvement in the process is more ambiguous *in vivo*. Recent studies in mouse oocytes suggest the existence of a Ran-independent spindle assembly pathway in female meiosis (Dumont et al., 2007; Schuh and Ellenberg, 2007).

Candidates responsible for this Ran-independent spindle assembly pathway include the chromosomal passenger complex, evidence for which again comes from an *in vitro* study using the *Xenopus* system (Sampath et al., 2004). In this system, depletion of Incenp or other subunits of the chromosomal passenger complex prevents spindle microtubule assembly (Sampath et al., 2004), and Aurora B can be activated by chromosomes independently from Ran (Kelly et al., 2007). Consistent with this, Aurora B can phosphorylate and inhibit the microtubule destabilising proteins, Kinesin-13 and Op18 (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004; Zhang et al., 2007; Gadea and Ruderman, 2006). In contrast to this *in vitro* evidence, inhibition of the chromosomal passenger complex (or inhibition of Kinesin-13 phosphorylation) produces only a limited defect in microtubule assembly in mitosis *in vivo* (Adams et al., 2001; Giet and Glover, 2001; Gassmann et al., 2004; Andrews et al., 2004; Lan et al., 2004). We found that our *incenp* mutant takes three times longer to initiate spindle microtubule assembly after nuclear envelope breakdown in oocytes. Our results thus provide the first and definitive *in vivo* demonstration that a subunit of the chromosomal passenger complex is required for efficient assembly of spindle microtubules in female meiosis.

The role of Incenp in the equatorial region of the meiotic metaphase spindle

Evidence from the *Xenopus in vitro* system indicated that bipolar spindles can be formed without centrosomes or kinetochores, suggesting that microtubules can self-organise into a bipolar spindle (Heald et al., 1996). A likely candidate for the basis of spindle bipolarity is anti-parallel bundling of spindle microtubules at the spindle equatorial region.

The *incenp* mutant reduces microtubule density in the spindle equatorial region relative to the polar regions, which suggests that the overlap and/or bundling of anti-parallel microtubules is compromised. A bipolar spindle can assemble, but tends to lose its bipolarity by forming ectopic poles around the spindle equatorial region. Eventually, the bipolarity is restored by the merging of poles. The origin of the ectopic poles is unclear, as single microtubules cannot be resolved in our live-imaging analysis in oocytes. The microtubules forming the ectopic poles may originally be derived from spindle microtubules, may grow from chromosomes, or may be spontaneously nucleated in the cytoplasm. In wild type, these microtubules are likely to be quickly bundled and aligned with existing spindle microtubules. However, in the *incenp* mutant, they can temporarily retain an independent orientation from existing spindle microtubules possibly due to compromised anti-parallel bundling in the spindle equatorial region.

Incenp and Aurora B kinase localise to the equatorial region of the meiotic metaphase I spindle (Jang et al., 2005). Consistent with this, we have shown that Incenp function is essential for the organisation of the spindle equatorial region in meiotic metaphase I, and for the establishment and maintenance of spindle bipolarity. Our results reinforce the hypothesis that anti-parallel microtubule bundling in the spindle equatorial region plays a central role in establishing bipolarity of meiotic metaphase I spindles in order to compensate for the lack of centrosomal activity.

Independent regulation of the two Incenp functions

Our study uncovered two functions of Incenp for acentrosomal spindle formation in oocytes. Both functions are likely to be mediated by Aurora B, although we cannot exclude the possibility that Incenp has roles independent of Aurora B.

Subito is a kinesin-like protein that plays a crucial role in the assembly of the spindle equatorial region. It is required for the localisation of other proteins to this region including the chromosomal passenger complex (Jang et al., 2005). Consistent with this, our immunostaining and live-imaging showed that the *subito* mutant and the *incenp* mutant give similar defects in spindle bipolarity. The stronger phenotype in the *subito* mutant is likely to be due to other proteins affected by the *subito* mutation, or to the hypomorphic nature of the *incenp* mutation.

Crucially, we found that the assembly of spindle microtubules is not delayed in the *subito* mutant, while it is greatly delayed in the *incenp* mutant. This indicates that the early function of Incenp in spindle microtubule assembly is independent of Subito. This function may be mediated through phosphorylation and inhibition of microtubule depolymerising proteins by Aurora B. In conclusion, the two functions of Incenp in spindle microtubule assembly and stabilisation of spindle bipolarity are differentially regulated in female meiosis.

The chromosomal passenger complex in centrosome dependent and independent spindle formation

In the light of our findings in female meiosis, the question is whether the chromosomal passenger complex plays similar roles in centrosome-dependent spindle formation in mitosis or male meiosis.

It has been proposed that Aurora B activity is involved in the regulation of microtubule dynamics at kinetochores upon improper microtubule attachment (Lampson et al., 2004; Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004). However, there is little evidence to support the possibility that the chromosomal passenger complex is required for general microtubule assembly in mitosis. Although the centrosome is the major microtubule nucleation site in mitotic cells, the activity of chromosomes to stabilise microtubules is thought to be important for the efficient capture of kinetochores by spindle microtubules (Wollman et al., 2005). Furthermore, when centrosomes are eliminated in mitosis, spindle microtubules are still assembled around chromosomes (Khodjakov et al., 2000; Basto et al., 2006). Ran-GTP is proposed to be responsible for these activities (Caudron et al., 2005), but involvement of Aurora B should be considered.

Does the chromosomal passenger complex play a role in spindle morphogenesis prior to anaphase in centrosome-dependent spindle formation? In *Drosophila*, before anaphase, the chromosomal passenger complex localises to centromeres in male meiosis but to the spindle equatorial region in female meiosis. The same hypomorphic mutation disrupts chromosome alignment in male meiosis but spindle bipolarity in female meiosis, without strong effects on the other functions (Resnick et al., 2006; this study).

Although the pre-anaphase function of the chromosomal passenger complex at the spindle equatorial region has not attracted much attention in the past, there is some evidence to support a role for the complex in the spindle equatorial region prior to anaphase in mitosis. In some vertebrate cell lines, Incenp was observed to localise to the spindle equatorial region during late metaphase (Earnshaw et al., 1991). RNAi of the chromosomal passenger complex in *Drosophila* S2 cells induces defects in spindle bipolarity (Goshima et al., 2007). RNAi of Borealin in mammalian cells disrupts spindle bipolarity as ectopic poles split off from the bipolar spindle after establishment of metaphase but prior to anaphase in mitosis

(Gassmann et al., 2004). Therefore, it is likely that the chromosomal passenger complex functions in the spindle equatorial region prior to anaphase both in mitosis/male meiosis and female meiosis. However, in female meiosis, this function becomes crucial due to the absence of centrosomes.

In summary, our study in female meiosis has revealed roles of Incenp in two vital steps of acentrosomal spindle formation: the assembly of spindle microtubules and the formation of a robust spindle equatorial region. So far, most studies of the chromosomal passenger complex have focused on centromeric functions in prometaphase and the central spindle/cytokinesis function in telophase in mitosis. Further studies will be required to establish to what extent the chromosomal passenger complex contributes to bipolar spindle assembly in mitosis and how different the regulation of the complex is between mitosis and acentrosomal meiosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Will Whitfield for kindly providing Cyclin B antibody, Terry Orr-Weaver for sharing information and Sandrin Ruchaud for critical reading of the manuscript. We also thank Bloomington stock centre for providing fly stocks. This work is supported by The Wellcome Trust (051091, 081849) and NIH (R01GM067142).

REFERENCES

Adams RR, Wheatley SP, Gouldsworthy AM, Kandels-Lewis SE, Carmena M, Smythe C, Gerloff DL, Earnshaw WC. INCENP binds the Aurora-related kinase AIRK2 and is required to target it to chromosomes, the central spindle and cleavage furrow. Curr. Biol. 2000; 10:1075–1078. [PubMed: 10996078]

Adams RR, Maiato H, Earnshaw WC, Carmena M. 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. 2001; 153:865–880. [PubMed: 11352945]

Andrews PD, Ovechkina Y, Morrice N, Wagenbach M, Duncan K, Wordeman L, Swedlow JR. Aurora B regulates MCAK at the mitotic centromere. De.v Cell. 2004; 6:253–268. [PubMed: 14960279]

Ashburner, M.; Golic, KG.; Hawley, RS. *Drosophila*: a Laboratory Handbook. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2005.

Basto R, Lau J, Vinogradova T, Gardiol A, Woods CG, Khodjakov A, Raff JW. Flies without centrioles. Cell. 2006; 125:1375–1386. [PubMed: 16814722]

Carazo-Salas RE, Guarguaglini G, Gruss OJ, Segref A, Karsenti E, Mattaj IW. Generation of GTP-bound Ran by RCC1 is required for chromatin-induced mitotic spindle formation. Nature. 1999; 400:178–181. [PubMed: 10408446]

Caudron M, Bunt G, Bastiaens P, Karsenti E. Spatial coordination of spindle assembly by chromosome-mediated signaling gradients. Science. 2005; 309:1373–1376. [PubMed: 16123300]

Chang CJ, Goulding S, Adams RR, Earnshaw WC, Carmena M. *Drosophila* Incenp is required for cytokinesis and asymmetric cell division during development of the nervous system. J. Cell Sci. 2006; 119:1144–1153. [PubMed: 16507586]

Cullen CF, Ohkura H. Msps protein is localized to acentrosomal poles to ensure bipolarity of *Drosophila* meiotic spindles. Nat. Cell Biol. 2001; 3:637–642. [PubMed: 11433295]

Cullen CF, Brittle AL, Ito T, Ohkura H. The conserved kinase NHK-1 is essential for mitotic progression and unifying acentrosomal meiotic spindles in *Drosophila melanogaster*. J. Cell Biol. 2005; 171:593–602. [PubMed: 16301329]

Drysdale RA, Crosby MA, The FlyBase Consortium. FlyBase: genes and gene models. Nuc. Acids Res. 2005; 33:D390–D395. [PubMed: 15608223]

Dumont J, Petri S, Pellegrin F, Terret ME, Bohnsack MT, Rassinier P, Georget V, Kalab P, Gruss OJ, Verlhac MH. A centriole- and RanGTP-independent spindle assembly pathway in meiosis I of vertebrate oocytes. J. Cell Biol. 2007; 176:295–305. [PubMed: 17261848]

Earnshaw WC, Cooke CA. Analysis of the distribution of the INCENPs throughout mitosis reveals the existence of a pathway of structural changes in the chromosomes during metaphase and early events in cleavage furrow formation. J. Cell Sci. 1991; 98:443–461. [PubMed: 1860899]

Endow SA, Komma DJ. Spindle dynamics during meiosis in *Drosophila* oocytes. J. Cell Biol. 1997; 137:1321–1336. [PubMed: 9182665]

Gadea BB, Ruderman JV. Aurora B is required for mitotic chromatin-induced phosphorylation of Op18/Stathmin. Proc. Natl. Acad. Sci. USA. 2006; 103:4493–4498. [PubMed: 16537398]

Gassmann R, Carvalho A, Henzing AJ, Ruchaud S, Hudson DF, Honda R, Nigg EA, Gerloff DL, Earnshaw WC. Borealin: a novel chromosomal passenger required for stability of the bipolar mitotic spindle. J. Cell Biol. 2004; 166:179–191. [PubMed: 15249581]

Gergely F, Kidd D, Jeffers K, Wakefield JG, Raff JW. D-TACC: a novel centrosomal protein required for normal spindle function in the early *Drosophila* embryo. EMBO J. 2000; 19:241–252. [PubMed: 10637228]

Giet R, Glover DM. *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. 2001; 152:669–682. [PubMed: 11266459]

Giunta KL, Jang JK, Manheim EA, Subramanian G, McKim KS. *subito* encodes a kinesin-like protein required for meiotic spindle pole formation in *Drosophila melanogaster*. Genetics. 2002; 160:1489–1501. [PubMed: 11973304]

Goodman B, Zheng Y. Mitotic spindle morphogenesis: Ran on the microtubule cytoskeleton and beyond. Biochem. Soc. Trans. 2006; 34:716–721. [PubMed: 17052181]

Goshima G, Wollman R, Goodwin SS, Zhang N, Scholey JM, Vale RD, Stuurman N. Genes required for mitotic spindle assembly in *Drosophila* S2 cells. Science. 2007; 316:417–421. [PubMed: 17412918]

Gruss OJ, Carazo-Salas RE, Schatz CA, Guarguaglini G, Kast J, Wilm M, Le Bot N, Vernos I, Karsenti E, Mattaj IW. Ran induces spindle assembly by reversing the inhibitory effect of importin alpha on TPX2 activity. Cell. 2001; 104:83–93. [PubMed: 11163242]

Harlow, E.; Lane, D. Antibodies: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1988. New York

Heald R, Tournebize R, Blank T, Sandaltzopoulos R, Becker P, Hyman A, Karsenti E. Self-organization of microtubules into bipolar spindles around artificial chromosomes in *Xenopus* egg extracts. Nature. 1996; 382:420–425. [PubMed: 8684481]

Jang JK, Rahman T, McKim KS. The kinesinlike protein Subito contributes to central spindle assembly and organization of the meiotic spindle in *Drosophila* oocytes. Mol. Biol. Cell. 2005; 16:4684–4694. [PubMed: 16055508]

Jang JK, Rahman T, Kober VS, Cesario J, McKim KS. Misregulation of the Kinesin-like protein Subito induces meiotic spindle formation in the absence of chromosomes and centrosomes. Genetics. 2007; 177:267–280. [PubMed: 17660552]

Kalab P, Weis K, Heald R. Visualization of a Ran-GTP gradient in interphase and mitotic *Xenopus* egg extracts. Science. 2002; 295:2452–2456. [PubMed: 11923538]

Kelly AE, Sampath SC, Maniar TA, Woo EM, Chait BT, Funabiki H. Chromosomal enrichment and activation of the aurora B pathway are coupled to spatially regulate spindle assembly. Dev. Cell. 2007; 12:31–43. [PubMed: 17199039]

Khodjakov ARW, Cole RW, Oakley BR, Rieder CL. Centrosome-independent mitotic spindle formation in vertebrates. Curr Biol. 2000; 10:59–67. [PubMed: 10662665]

Lampson MA, Renduchitala K, Khodjakov A, Kapoor TM. Correcting improper chromosome-spindle attachments during cell division. Nat. Cell Biol. 2004; 6:232–237. [PubMed: 14767480]

Lan W, Zhang X, Kline-Smith SL, Rosasco SE, Barrett-Wilt GA, Shabanowitz J, Hunt DF, Walczak CE, Stukenberg PT. Aurora B phosphorylates centromeric MCAK and regulates its localization and microtubule depolymerization activity. Curr. Biol. 2004; 14:273–286. [PubMed: 14972678]

Lindsley, DL.; Zimm, GG. The genome of *Drosophila malanogaster*. New York: Academic Press; 1992.

Matthies HJ, McDonald HB, Goldstein LS, Theurkauf WE. Anastral meiotic spindle morphogenesis: role of the non-claret disjunctional kinesin-like protein. J. Cell Biol. 1996; 134:455–464. [PubMed: 8707829]

McKim KS, Hawley RS. Chromosomal control of meiotic cell division. Science. 1995; 270:1595–1601. [PubMed: 7502068]

Ohi R, Sapra T, Howard J, Mitchison TJ. Differentiation of cytoplasmic and meiotic spindle assembly MCAK functions by Aurora B-dependent phosphorylation. Mol. Biol. Cell. 2004; 15:2895–2906. [PubMed: 15064354]

Pearson NJ, Cullen CF, Dzhindzhev NS, Ohkura H. A pre-anaphase role for a Cks/Suc1 in acentrosomal spindle formation of *Drosophila* female meiosis. EMBO Rep. 2005; 6:1058–1063. [PubMed: 16170306]

Resnick TD, Satinover DL, MacIsaac F, Stukenberg PT, Earnshaw WC, Orr-Weaver TL, Carmena M. INCENP and Aurora B promote meiotic sister chromatid cohesion through localization of the Shugoshin MEI-S332 in *Drosophila*. Dev. Cell. 2006; 11:57–68. [PubMed: 16824953]

Ruchaud S, Carmena M, Earnshaw WC. Chromosomal passengers: conducting cell division. Nat. Rev. Mol. Cell. Biol. 2007; 8:798–812. [PubMed: 17848966]

Sambrook, J.; Fritsch, EF.; Maniatis, T. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989. New York

Sampath SC, Ohi R, Leismann O, Salic A, Pozniakovski A, Funabiki H. The chromosomal passenger complex is required for chromatin-induced microtubule stabilization and spindle assembly. Cell. 2004; 118:187–202. [PubMed: 15260989]

Schuh M, Ellenberg J. Self-organization of MTOCs replaces centrosome function during acentrosomal spindle assembly in live mouse oocytes. Cell. 2007; 130:484–498. [PubMed: 17693257]

Schupbach T, Wieschaus E. Female sterile mutations on the second chromosome of *Drosophila melanogaster*. I. Maternal effect mutations. Genetics. 1989; 121:101–117. [PubMed: 2492966]

Tavosanis G, Llamazares S, Goulielmos G, Gonzalez C. Essential role for γ-tubulin in the acentriolar female meiotic spindle of *Drosophila*. EMBO J. 1997; 16:1809–1819. [PubMed: 9155007]

Theurkauf WE, Hawley RS. Meiotic spindle assembly in *Drosophila* females: behavior of nonexchange chromosomes and the effects of mutations in the nod kinesin-like protein. J. Cell Biol. 1992; 116:1167–1180. [PubMed: 1740471]

Vagnarelli P, Earnshaw WC. Chromosomal passengers: the four-dimensional regulation of mitotic events. Chromosoma. 2004; 113:211–222. [PubMed: 15351889]

Waters JC, Salmon ED. Pathways of spindle assembly. Curr. Opin. Cell Biol. 1997; 9:37–43. [PubMed: 9013671]

Whitfield WG, Gonzalez C, Maldonado-Codina G, Glover DM. The A- and B-type cyclins of *Drosophila* are accumulated and destroyed in temporally distinct events that define separable phases of the G2-M transition. EMBO J. 1990; 9:2563–2572. [PubMed: 2142452]

Wiese C, Wilde A, Moore MS, Adam SA, Merdes A, Zheng Y. Role of importin-beta in coupling Ran to downstream targets in microtubule assembly. Science. 2001; 291:653–656. [PubMed: 11229403]

Wollman R, Cytrynbaum EN, Jones JT, Meyer T, Scholey JM, Mogilner A. Efficient chromosome capture requires a bias in the ‘search-and-capture’ process during mitotic-spindle assembly. Curr. Biol. 2005; 15:828–832. [PubMed: 15886100]

Zhang X, Lan W, Ems-McClung SC, Stukenberg PT, Walczak CE. Aurora B phosphorylates multiple sites on mitotic centromere-associated kinesin to spatially and temporally regulate its function. Mol. Biol. Cell. 2007; 18:3264–3276. [PubMed: 17567953]

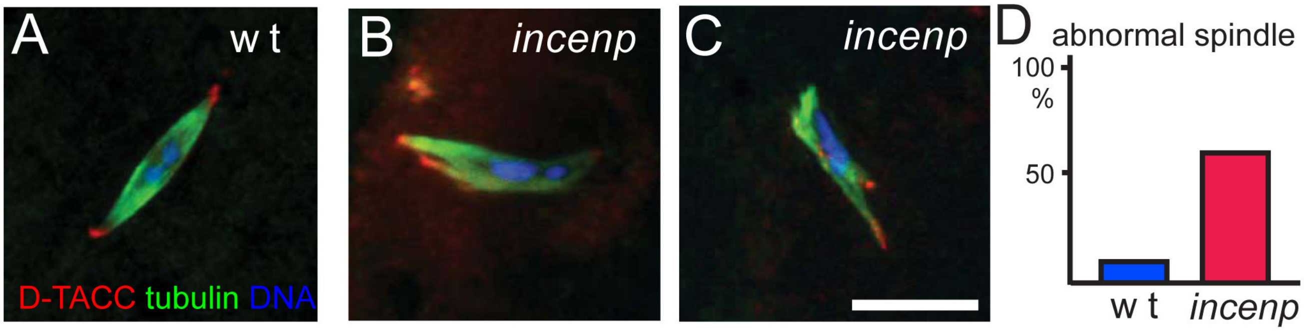

Figure 1. Ectopic poles in the equatorial region of meiotic metaphase spindle in the *incenp* mutant

Metaphase I arrested oocytes from wild type (A) and the *incenp*^{QA26} mutant (B,C) were immunostained for DNA, tubulin and the pole protein D-TACC. Ectopic poles, which often accumulate D-TACC, were formed in the *incenp* mutant. (D) Frequencies of abnormal morphology of meiotic spindles in wild type and the *incenp* mutant. More than 30 spindles were examined. The difference is significant (p<0.001).

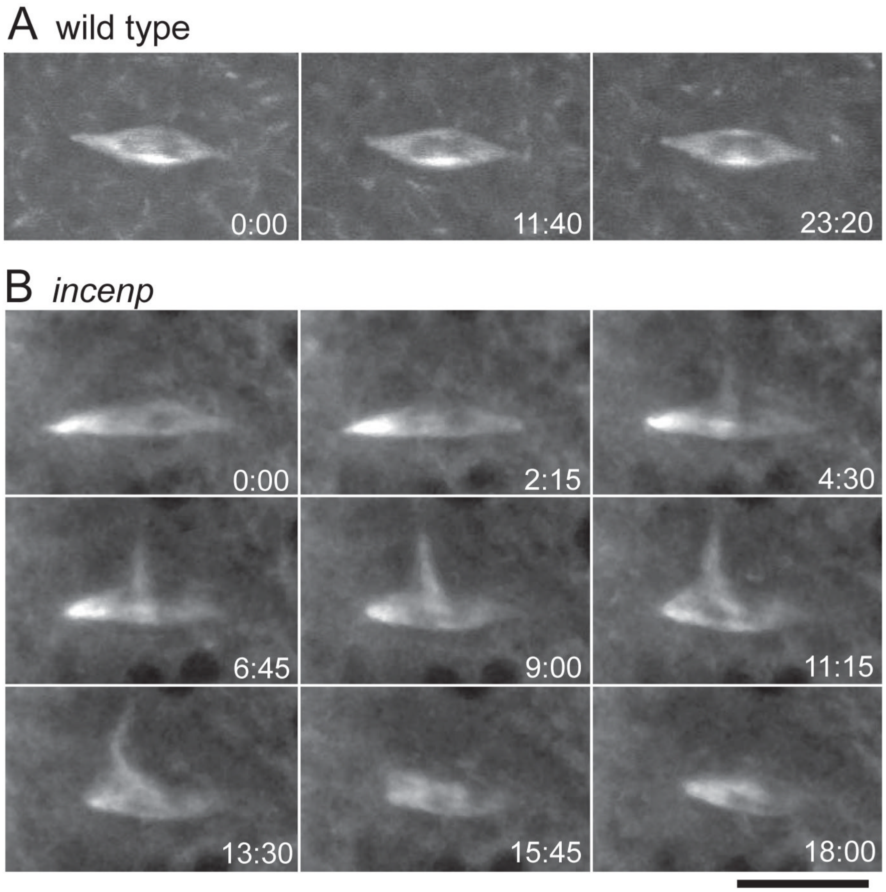

Figure 2. Instability of the metaphase I spindle equatorial region in the *incenp* mutant

A time lapse sequence of a metaphase I arrested spindle in wild type (A) and the *incenp*^{QA26} mutant (B). Oocytes expressing GFP-α-tubulin were dissected and observed under a confocal microscope. The numbers represent min:sec after the arbitrary point. Bar=10μm.

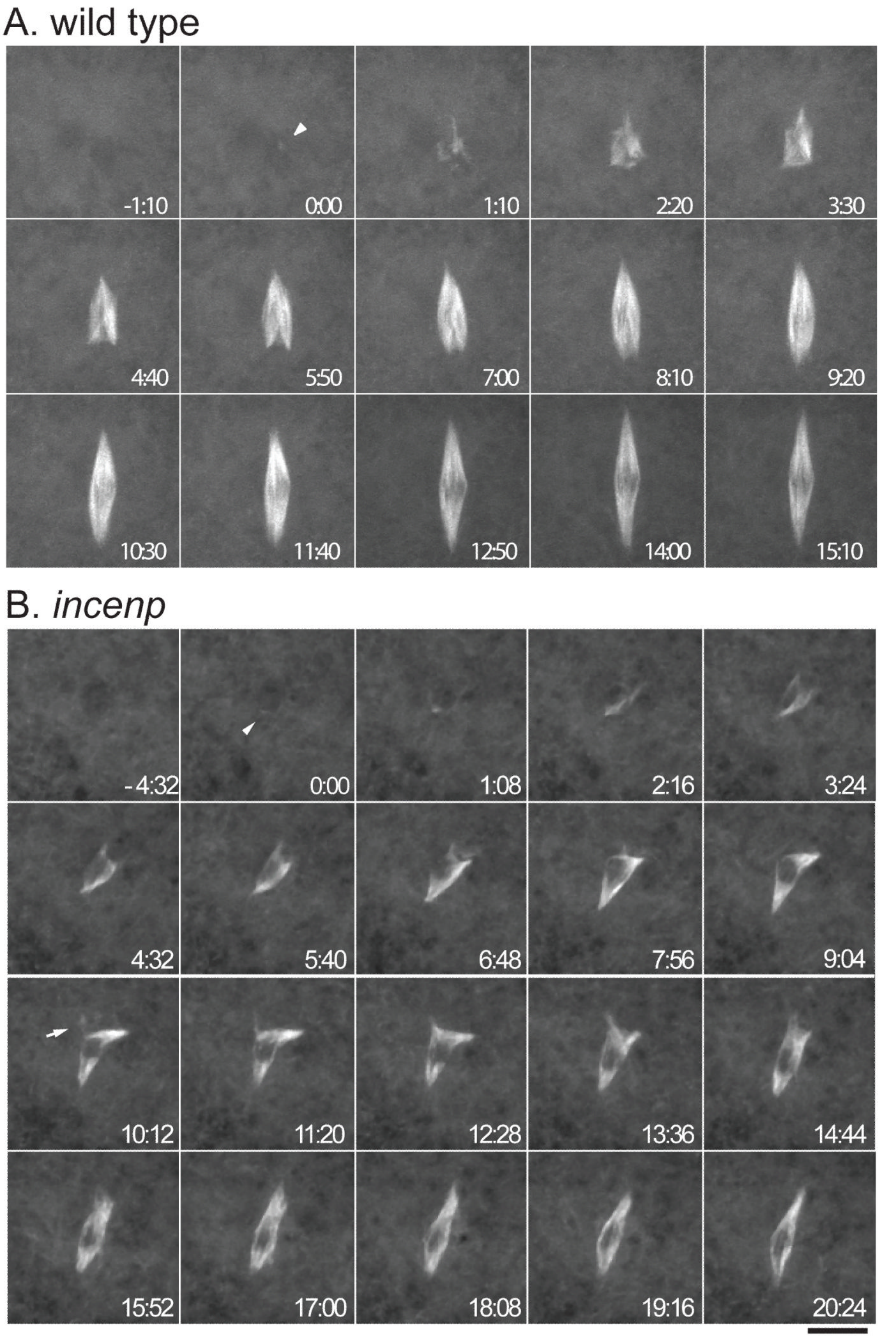

Figure 3. Transitory spindle poles during spindle formation in the *incenp* mutant

A time lapse sequence of spindle formation after nuclear envelope breakdown to metaphase I in wild type (A) and the *incenp*^{QA26} mutant (B). Time zero indicated the first appearance of spindle microtubules (arrowheads) around the chromosomes. At 10:12 in the mutant, an ectopic pole (arrow) was formed around the spindle equatorial region and eventually merged with one of the poles. Bar=10μm.

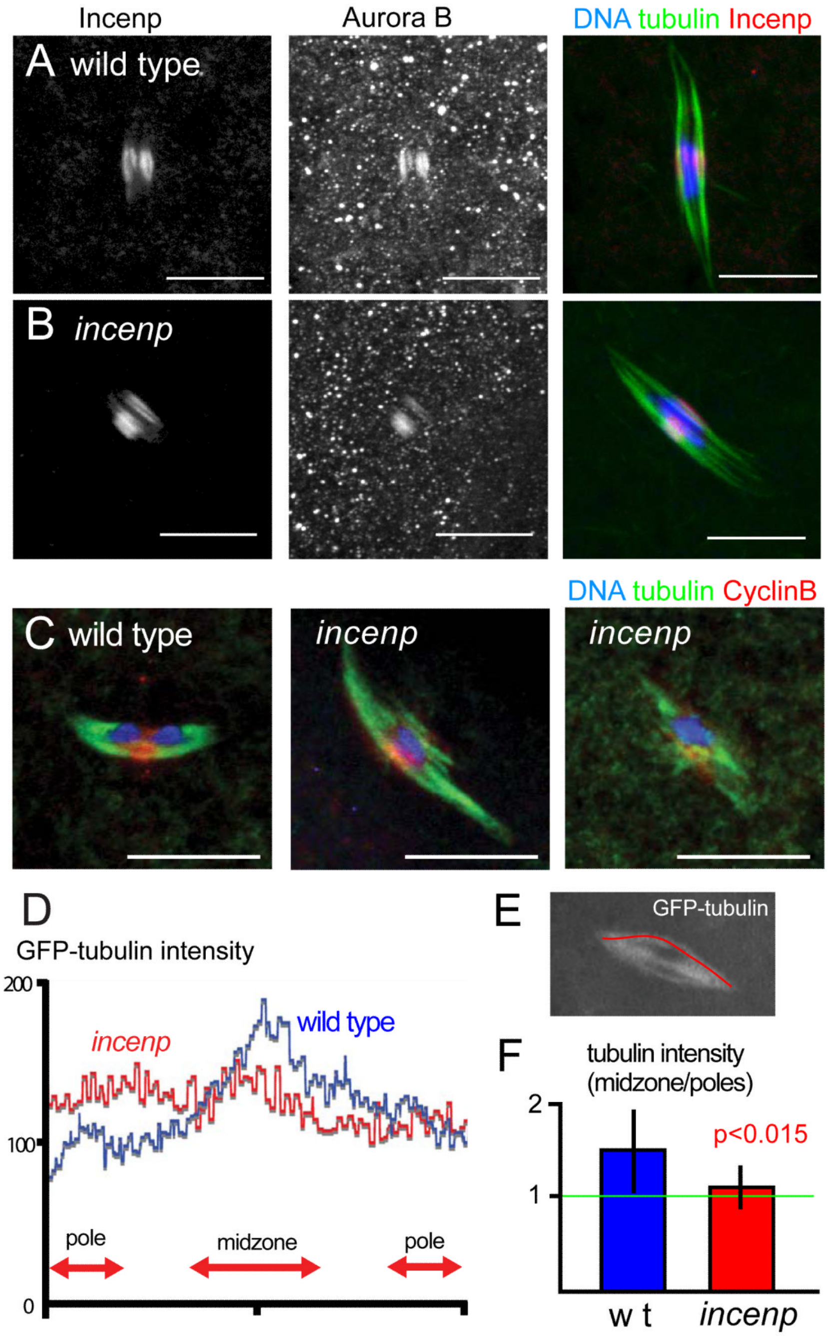

Figure 4. The spindle equatorial region is partially defective in the *incenp* mutant

(A, B) Immuno-localisation of Incenp and Aurora B localises to the equatorial region of meiotic metaphase I spindle in wild type and the *incenp*^{QA26} mutant. Bar=10μm. (C) Immuno-localisation of Cyclin B to the equatorial region of meiotic metaphase I spindle in wild type and the *incenp*^{QA26} mutant. (D,E) Examples of GFP signal intensity plots along the spindles from wild-type and *incenp* oocytes expressing GFP-tubulin, as marked with the red line in (E). (F) The relative intensities of the spindle equatorial region (maximum intensity within the central 4μm) over pole regions (average maximum intensity of 2μm from each pole) are shown as the mean values (bars; 1.4 in wild type and 1.1 in *incenp*) and standard deviations (lines) for multiple wild-type and *incenp* mutant spindles. The difference is significant ($p<0.015$; $n=15$).

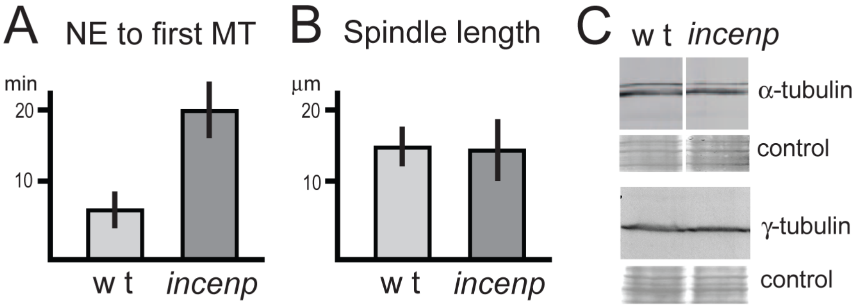

Figure 5. The *incenp* mutation delays spindle microtubule assembly in female meiosis

(A) Time taken from nuclear envelope break down to the first appearance of spindle microtubules around the chromosomes in wild type and *incenp*^{QA26} mutant oocytes expressing GFP-α-tubulin. The mean values (bars) are shown with standard deviations (lines). The difference is significant ($p<0.001$; $n\geq8$). (B) The length of metaphase I arrested spindles in wild type and the *incenp*^{QA26} mutant. (C) The relative amounts of α-and γ-tubulins in ovaries from wild type and the *incenp*^{QA26} mutant were examined by immunoblots. Blotted membranes are stained with MemCode for loading and transfer controls.

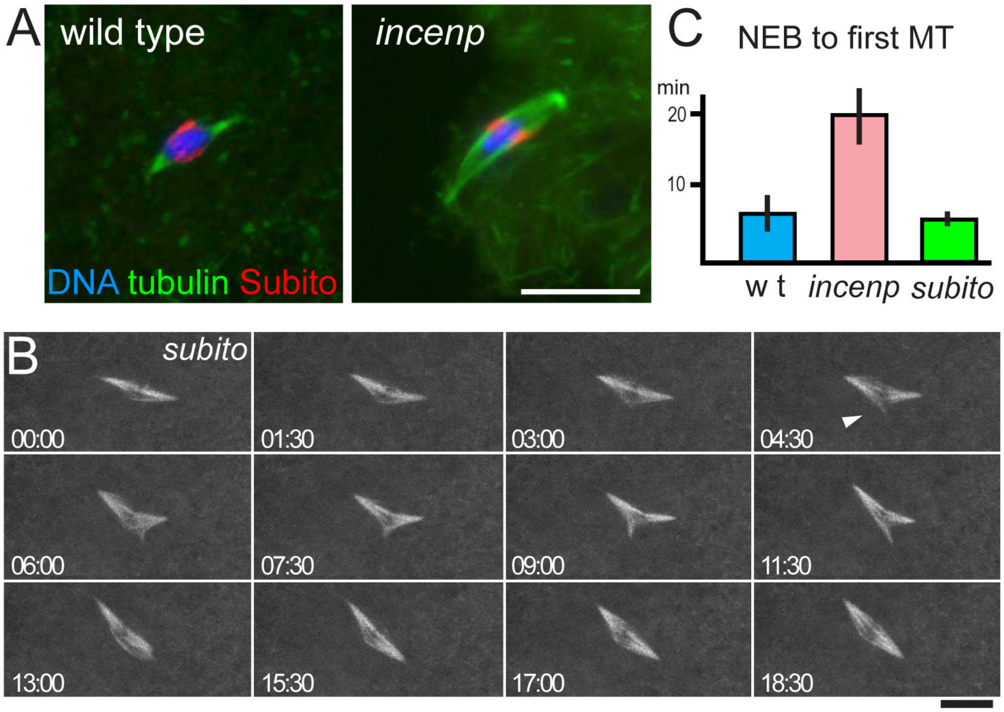

Figure 6. Subito is required for stability of the spindle equatorial region but not spindle microtubule assembly in female meiosis

(A) Subito localises to equatorial regions in wild type and the *incenp*^{QA26} mutant. (B) Instability of the spindle equatorial region in the *subito*^{1} mutant. An example of metaphase I arrested *subito* oocytes expressing GFP-α-tubulin. An ectopic pole (arrowhead) was formed at the spindle equatorial region and merged with one of the main poles. (C) Time taken from nuclear envelope breakdown to the first appearance of spindle microtubules. The *subito* mutant did not show a significant difference to wild type (p=0.45; n=6). Bar=10μm.