Klp67A destabilises pre-anaphase microtubules but subsequently is required to stabilise the central spindle

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Summary

Klp67A is a member of the Kip3 subfamily of microtubule destabilising kinesins, the loss of which results in abnormally long and stable pre-anaphase microtubules. Here we examine its role during cytokinesis in *Drosophila* primary spermatocytes that require the coordinated interaction of an interior and peripheral set of central spindle microtubules. In mutants anaphase B spindles elongated with normal kinetics but bent towards the cortex. Both peripheral and interior spindle microtubules then formed diminished bundles of abnormally positioned central spindle microtubules associated with the pavarotti-KLP and KLP3A motor proteins. The minus ends of these were poorly aligned as revealed by Asp protein localisation. Furrows always initiated at the sites of central spindle bundles but could be unilateral or nonequatorially positioned. Ectopic furrows were stimulated by the interior central spindle and formed only after this structure buckled and contacted the cortex. Furrows often halted and regressed as they could not be sustained by the central spindles that became increasing unstable over time and often completely degraded. Consistent with this, actin and anillin failed to form homogenous bands. Thus, the Klp67A microtubule catastrophe factor is required for cytokinesis by regulating both the formation and stability of the central spindle.

Supplementary material available online at
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Key words: Kinesin-like protein, Kip3 subfamily, Cytokinesis, *Drosophila*, Meiosis

Introduction

Cytokinesis in animal cells is effected through the constriction of an actomyosin contractile ring that forms at a pre-defined location between the segregating chromosomes. Experiments from several systems indicate that the central spindle is a universal controller of this process (Gatti et al., 2000). This structure forms as the previously fusiform and bipolar spindle remodels itself during late anaphase; it is comprised of overlapping and interdigitating antiparallel microtubules (MTs), as well as a growing list of accessory proteins that associate with either actin or microtubules including polymerisation regulatory factors, molecular motors, chromosomal passengers, kinases and proteins involved in membrane insertion (e.g. Mastronarde et al., 1993; Giansanti et al., 2004; Skop et al., 2004). Recently, we showed that the central spindle in *Drosophila* primary spermatocytes consists of two biochemically and initially spatially unique microtubule populations: dynamic peripheral astral MTs that probe the cytoplasm until contacting the cortex where they are responsible for cleavage plane determination and a set of interior microtubules found within the persisting nuclear or spindle envelope. Preferential destabilisation of interior central spindle microtubules through mutation of the microtubule associated protein (MAP) Orbit/Mast/Clasp resulted in cleavage furrows that initiated but ultimately regressed, suggesting that these stable MTs are needed for furrow propagation to the midbody stage (Inoue et al., 2004). Thus, the central spindle presents a unique structural problem, as its component MTs must be dynamic and yet still remain stable.

Our knowledge of the molecules responsible for regulating central spindle dynamism remains poor. Indeed, much of our understanding of the molecules that regulate microtubule stability comes from in vitro experiments performed in pre-anaphase *Xenopus* egg extracts. There it has been shown that physiological MT dynamics can be recapitulated by the antagonism of a single stabilising MAP, XMAP215/chTog/Dis1 and a member of the Kin I or kinesin-13 subfamily of kinesins, XKCM1/MCAK (Kinoshita et al., 2001; Lawrence et al., 2004). Members belonging to this latter group are unique in that their motor domain is internally positioned and lacks in vitro motility. Instead, Kin I kinesins nonspecifically bind to both MT ends and induce depolymerisation or catastrophe (Desai et al., 1999; Moores et al., 2003). Subsequent in vivo experiments have shown that disruption of the balance between these stabilising or catastrophe-inducing components dramatically affects spindle morphology. For example,

depletion of Kin I depolymerases in vertebrate somatic cells (Kline-Smith and Walczak, 2002), as well as in *Drosophila* embryos and cultured cells (Goshima and Vale, 2003; Rogers et al., 2004), leads to a common phenotype with spindles often collapsing and their component MTs becoming abnormally long and stable. These spindle defects activate the spindle assembly checkpoint and prevent mitotic exit.

Members of the Kip3/Kinesin-8 subfamily (Lawrence et al., 2004) of kinesins have been classified as catastrophe factors on the basis of observations that their mutation or RNAi depletion leads to long microtubules that are highly resistant to depolymerising drugs. Several studies implicate these kinesins in regulating post-anaphase spindle dynamics and cytokinesis. Budding yeast mutants that lack the founding member of this subfamily, *kip3*, have abnormally long spindles following anaphase onset that continue to elongate such that they curve around the cell cortex. These overgrown spindles then require increased amounts of time to disassemble (Cottingham and Hoyt, 1997; Straight et al., 1998). Similarly, deletion of the fission yeast *kip3* orthologues *klp5/6* leads to chromosome congression and segregation defects on spindles that are almost twice as long as those in wild-type cells (Garcia et al., 2002a; Garcia et al., 2002b; West et al., 2002). Despite this morphological abnormality, the rate of anaphase B spindle elongation appeared unaltered in these mutants (West et al., 2002). Klp5/6 were also isolated in a screen for interactors with the guanine nucleotide exchange factor Scd1 (Li and Chang, 2003), which is part of the Ras1 pathway. Double mutants of *scd1* with either *klp5* or *klp6* prevented actin and the anillin orthologue Mid1 from forming their characteristic and well-defined equatorial bands leading to cytokinesis failure. Interestingly, both of these kinesins also bound directly to the septin-recruiting small GTPase Cdc42 (Li and Chang, 2003).

The *Drosophila* orthologue of Kip3 is Klp67A (West et al., 2001; Savoian et al., 2004). Time-lapse analyses have shown that this catastrophe factor is needed for proper chromosome congression and segregation during male meiosis I (Savoian et al., 2004). In fixed preparations of mutant primary spermatocytes the microtubules appeared overgrown and the spindles were poorly organised before anaphase onset, after which time defined central spindles were conspicuously absent and cytokinesis failed (Gandhi et al., 2004; Savoian et al., 2004). The MT aberrations were not unique to meiosis as mutant syncitial embryos also formed elongated metaphase spindles that subsequently failed to reorganise into central spindles (Gandhi et al., 2004). However, a true cytokinesis does not occur in the early stages of *Drosophila* embryogenesis (Foe and Alberts, 1983) and thus the effects of Klp67A depletion on cleavage are unclear.

We therefore chose to re-examine the role of Klp67A in central spindle morphogenesis and cytokinesis by examining both living and fixed preparations of mutant *Drosophila* primary spermatocytes. Here we show that cytokinesis fails in approximately 44% of *klp67A* hypomorphic mutant cells. The spindle elongated during anaphase B but the centrosomes failed to separate and the resulting central spindle was distorted and contained decreased numbers of microtubule bundles. We found that furrow formation was initiated by peripheral central spindle microtubules as in wild-type cells but furrows could not be sustained as central spindles were not robust and their component MTs degraded over time. Moreover, furrows formed at ectopic sites when distorted bundles of interior central spindle microtubules contacted the cell cortex. These data are the first live cell characterisations of a catastrophe factor mutant during animal cell cytokinesis. They reveal that in the absence of a Kip3 subfamily MT catastrophe factor, the central spindle becomes distorted and unstable. They further provide direct evidence that the interior central spindle microtubules like their peripheral counterparts are sufficient to induce cleavage when proximal to the cortex.

Materials and Methods

Flies and husbandry

Two different *klp67A* mutant alleles were employed in this study: $klp67A^{42705}$ and $klp67A^{EP(3)3516}$ as well as the deficiency chromosome *Df(3L)29A6, kni[r1-1]p[p]*. These mutants are severe hypomorphs and the Klp67A protein is undetectable in testes extracts subjected to western blotting. The different allelic combinations yield similar phenotypes (Savoian et al., 2004) and thus are referred to as *klp67A* mutants in the text. The following alleles were also used: $polo^{1}/polo^{10}$ (Donaldson et al., 2001), $klp3A^{e4}$ (Williams et al., 1995) and *GFP-pavarotti*, which is driven by the ubquitin promoter and located on chromosome II (Minestrini et al., 2003). All flies were reared according to standard procedures and maintained at 25°C.

Immunofluorescence microscopy

Testes were prepared for staining using standard methanol and acetone fixation unless otherwise indicated (Cenci et al., 1994). To label microtubules primary antibodies against β-tubulin (Boehringer Mannheim) were diluted 1:200 or tyrosinated α-tubulin (Harlan Sera-Lab) 1:20. Centrioles were stained with the HsCen1p antibody at a concentration of 1:400. Antibodies to Pav-KLP and Klp3A were used at 1:100 and 1:500, respectively. Asp antibodies were diluted 1:50 and those against anillin at 1:1000. Klp67A staining was performed with the antibody described by Savoian et al. (Savoian et al., 2004) and used at a concentration of 1:50. Protein visualisation utilised commercially available secondary antibodies. For actin localisation cells were fixed with 3.7% formaldehyde according to the protocol of Gunsalus et al. (Gunsalus et al., 1995) and incubated with rhodamine-labelled phalloidin.

Images were acquired with a Leica TCS 4D laser-scanning microscope (Leica Lasertechnik) or with a Nikon Microphot microscope fitted with an MRC1024 scanning confocal head (Biorad), both using a 63× (N.A. 1.4) objective lens. The figures shown are the maximum intensity projections of optical sections acquired at 0.2-0.5 μm steps.

Live cell imaging and analysis

Multi-dimensional imaging was performed according to the method of Inoue et al. (Inoue et al., 2004). Briefly, testes were dissected under Voltalef 10s oil onto clean No. 1½ thickness coverslips that were attached to a metal slide lacking the central portion. Specimens were imaged on a Zeiss Axiovert 200 (Carl Zeiss Microimaging) microscope with a 100× (N.A. 1.4) Differential Interference Contrast (DIC) lens and condenser (N.A. 0.55). At each 1 minute time interval, six near simultaneous DIC and EGFP optical sections of a 1 μm step size were acquired through appropriate filter wheels and an EGFP/DsRed filtercube. Images were captured with a CoolSnap HQ camera (Roper Scientific) and stored on a PC. Image acquisition and subsequent analysis were performed using Metamorph (Universal Imaging). The fluorescence images shown are the maximum intensity projection of all six sections, and the corresponding DIC image is the single centre-most section taken from the z-stack.

Table 1. Quantitation of meiotic defects in onion stage spermatids

Genotype	*n*	% Spermatids per class (%variation in nuclear size)			
		<1 nucleus/cell*	1 nuclei/cell	2 nuclei/cell	>4 nuclei/cell
Oregon R	1636	0	100 (0)	<1 (<1)	0
klp67A/klp67A	2445	19 (48)	36 (22)	44 (36)	1 (60)
klp67A/Df	2127	24 (50)	28 (24)	47 (39)	1 (68)

n, number of onion stage spermatids.
Df, deficiency chromosome *Df(3L)29A6, kni[r1-1]p[p]*.
*, nucleus significantly smaller than in the wild type.

To determine anaphase B spindle elongation velocity we selected cells in which the spindle envelope and therefore the spindle remained nearly co-planar throughout the duration of analysis. At each time point we superimposed the maximum intensity projection of the six β-tubulin-EGFP sections onto the centre-most 1-2 DIC sections and measured the distance between the two opposing centrosomes along a seven segment line drawn through the spindle long axis as demarcated by the midline of the spindle envelope (see Fig. S1 in supplementary material) using Metamorph. This multi-segmented line was necessary as spindles in *klp67A* mutants became bent and twisted and the movement of the centrosomes did not reflect the extent to which the spindle envelope and thus the spindle extended. The distances were then imported into Excel (Microsoft) and plotted. The average maximal spindle elongation velocity represents the slope of the line that best fits the eight or more contiguous and steepest points of the graph.

Results

Drosophila Klp67A is central spindle component required for cytokinesis

Recently we showed that Klp67A is a Kip3 subfamily depolymerase that is a transient component of pre-anaphase kinetochores and is required for proper chromosome congression and accurate anaphase segregation in *Drosophila* primary spermatocytes (Savoian et al., 2004). This earlier study had noted that following anaphase onset, Klp67A redistributed from the kinetochores and a diffuse cloud in a region of low microtubule density at the cell's equator to a tight band on the mid-portion of the central spindle, suggesting a role in cytokinesis (Fig. S2 in supplementary material). In agreement with this, loss of Klp67A leads to aberrant spindle morphology and cleavage defects during meiosis (Gandhi et al., 2004; Savoian et al., 2004). However, the mechanism by which cleavage fails remains to be fully determined.

It is possible to quantitate the frequency of cytokinetic failure during *Drosophila* male meiosis by examining the post-meiotic products in mutant spermatocytes. We have previously shown that extracts of testes from homozygous or hemizygous $klp67A^{42705}$ or $klp67A^{EP(3)3516}$ mutants had virtually undetectable levels of Klp67A protein, indicating that these mutations are severe hypomorphs (Savoian et al., 2004). Such mutants show a dramatic increase in the occurrence of bi-nucleate cells (compare 44% vs <1% in the wild-type; Table 1). Intriguingly, we also observed cytoplast-like structures that contained a Nebenkern, an aggregate of mitochondria that also segregates during meiosis, but no obvious nucleus (see below).

Given the central spindle's fundamental role in both initiating and maintaining the cleavage furrow we next examined the morphology of this structure in *klp67A* mutants. In wild-type cells, the late anaphase/early telophase spindle remodels itself into a compact and polarised series of bundles found at the cell equator (Fig. 1A; inset). However, as illustrated in Fig. 1A, the central spindle could be highly distorted and greatly diminished in *klp67A* mutants. MTs of an apparently increased length were found between the daughter nuclei and these were bundled and organised to varying

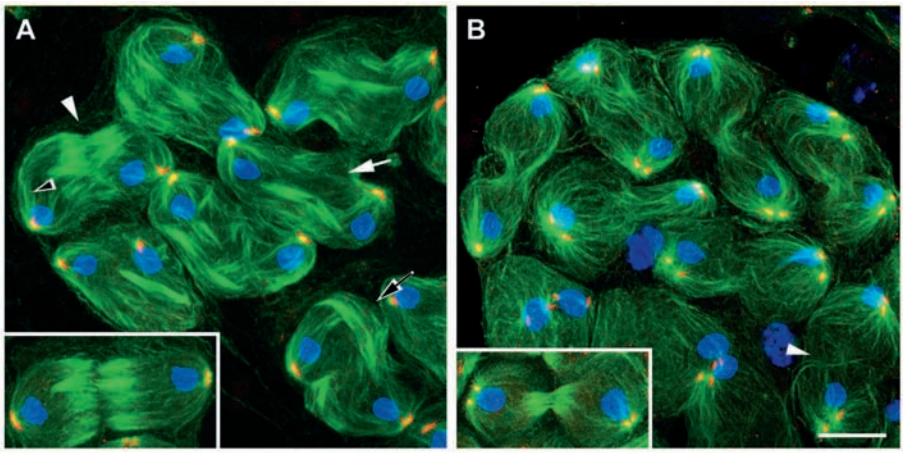

Fig. 1. *klp67A* mutant primary spermatocytes lack robust central spindles and midbodies. (A) Late anaphase cells isolated from *klp67A* mutants or wild-type (inset) testes. Microtubules are in green, DNA in blue and the centriole marker Centrin in red. During late anaphase control cells form a symmetrical central spindle that spans the cell's equator. In *klp67A* mutants the central spindles are diminished and asymmetrical with microtubule bundles positioned on a single side of the cell (white arrowhead) or concentrating in the interior but lacking at the periphery (white arrow). Some centrosome-associated MTs can be abnormally long and run along the periphery (black arrowhead). In other cells, the spindles become bent or S-shaped (black arrow). (B) In control cells constriction of the contractile ring results in the formation of a dense midbody connecting the two daughter cells (inset). This structure was rarely observed in the mutant cells, which although partially constricted lacked discreet microtubule bundles at this later stage. Note how most cells homogenously contain individual, disorganised microtubules but that in some instances few microtubules are visible at the equator (arrowhead). Bar, 10 μm.

Table 2. Frequency of spermatocytes with central spindle abnormalities during meiosis I

	Late anaphase				Late telophase			
	Spindle midzone				Spindle midzone			
Genotype	*n*	% wild-type	% mis-formed*	% absent	*n*	% wild-type	% mis-formed*	% absent
Oregon R	591	99	1	0	428	100	<1	0
klp67A/klp67A	704	61	22	17	521	60	23	17
klp67A/Df	503	56	24	20	413	55	24	21

n, number of onion stage spermatids.
Df, deficiency chromosome *Df(3L)29A6, kni[r1-1]p[p]*.
*, see Fig. 1 for examples.

degrees (see also Table 2). MT distribution was asymmetrical and the bundles could be concentrated on only a single side of the cell (white arrowhead) or in the cell interior but absent near the equator at the periphery (white arrow). A few microtubule bundles extended from the centrosomes (revealed by Centrin staining) and along the cell's edge into the equator (black arrowhead). The spindles in many mutant cells took on an S-shaped conformation as if they were overgrown and then elongated and distorted during Anaphase B (black arrow).

After the central spindle forms, it becomes progressively constricted by the contractile ring until only a thin midbody remains connecting the two telophasic daughter cells together (Fig. 1B; inset). This structure, which is a hallmark of successful cleavage and seen in virtually all wild-type cells (>99%), was noticeably absent or aberrant in *klp67A* mutants (Table 2). Although similarly staged mutant cells appeared at least partly constricted, MT bundles were difficult to discern among large numbers of disorganised spindle and cytoplasmic microtubules (Fig. 1B). The bundles that were present were randomly positioned between the karyomeres, and both the peripheral

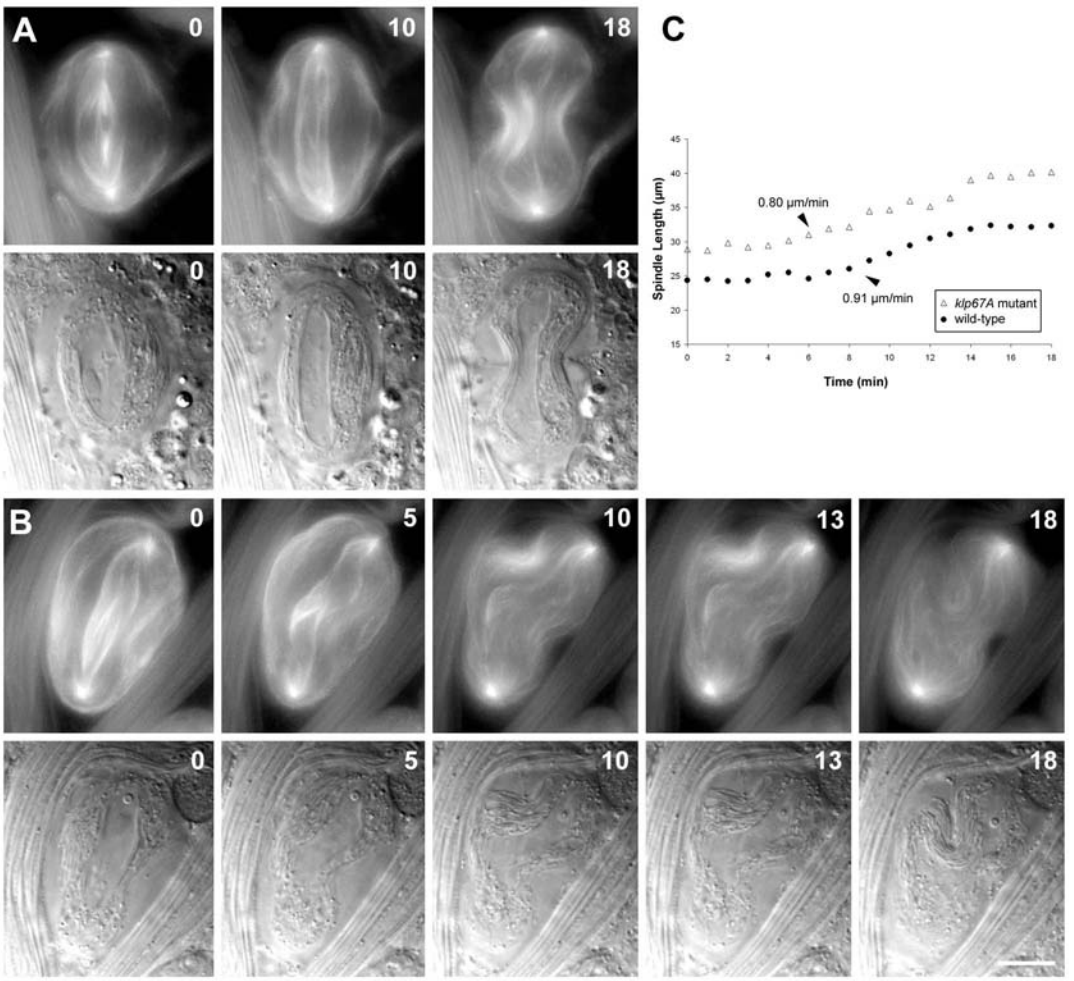

Fig. 2. Anaphase B kinetics are similar between *klp67A* mutants and wild-type cells. Selected frames from time-lapse series comparing anaphase B in a wild-type (A) and *klp67A* mutant primary spermatocyte (B) expressing β-tubulin-EGFP. The maximum intensity projection of the six optical sections and the corresponding single, centre-most DIC image for each time point is shown here and a similar format is used for subsequent figures. (A) In wild-type cells anaphase B occurs after the dyads disjoin (0 minutes). Once initiated, the spindle continually elongates, moving the centrosomes in opposite directions until a new steady-state length is reached (10-18 minutes) (B). (B) Before anaphase onset (0 minutes) *klp67A* mutants formed dense spindles surrounded by long astral microtubules. Unlike in control cells, the centrosomes in the mutants did not undergo significant separation during anaphase B. As the spindle elongated (5-13 minutes) it buckled out of this focal plane before also assuming a new steady-state length (18 minutes). Note how the mutant lacks a discreet central spindle. See main text for details. (C) Kinetic plot of the elongating spindles depicted in (A) and (B). In both cases there is a lag before anaphase B initiates, after which time the spindles extend at the indicated velocities. See Materials and Methods for details of measurements. Bar, 10 µm. Times are in minutes relative to anaphase onset.

cytoplasmic as well as the interior central spindle MTs appeared to be equally affected. In more extreme examples, MTs were found radiating from opposing asters but almost totally lacking in the equatorial regions (arrowhead). These data indicate that in the absence of Klp67A, the late anaphase spindle becomes distorted, central spindles are diminished and cytokinesis fails.

Loss of Klp67A prevents anaphase B centrosome separation but not spindle elongation

Our fixed cell analysis suggested that the spindles in mutants became progressively elongated and distorted following the transition into anaphase. To investigate how the *klp67A* mutation affected anaphase B spindle elongation we employed multidimensional imaging of living wild-type and *klp67A* mutants expressing β-tubulin-EGFP. This allowed us to follow changes in the MT cytoskeleton and membranous components of the cell in a near-simultaneous manner.

The post-metaphase spindle's length could be determined by measuring the distance between the opposing centrosomes along a midline drawn within the persisting spindle envelope (see Materials and Methods and Fig. S1 in supplementary material). After dyad disjunction at anaphase onset in wild-type cells (Fig. 2A; 0 minutes) the spindle began to show its first signs of elongating. While the spindles in some cells showed immediate extension and ostensible centrosome separation, those in other cells could require several minutes before elongation became apparent. Anaphase B onset did not require the central spindle, as the release of its component interior central spindle MTs from the poles occurred on average 9 minutes after chromosome disjunction. Once anaphase B initiated it continued for about 11 minutes (n=5) until a new steady-state spindle length was reached (Fig. 2A, 10-18 minutes; Fig. 2C). This correlated with the released MTs consolidating at the cell's equator to form the central spindle. The average maximal spindle elongation velocity during anaphase B in wild-type cells was about 1.0 μm/minute (n=5) (see Movie 1 in supplementary material).

We next carried out a similar kinetic analysis in ten mutant cells. As expected, the metaphase spindle length in Klp67A-depleted cells tended to be longer than those in the wild-type. We found that after anaphase B initiated, it proceeded for about 12 minutes, a time virtually indistinguishable from that seen in the controls. However, unlike in wild-type cells, the centrosomes capping the *klp67A* mutant spindles did not always separate and in many instances appeared fixed in position. Despite this, the spindles as delineated by their spindle envelopes still elongated but became distorted and buckled towards the cortex (Fig. 2B, 5-18 minutes; Movie 2 in supplementary material). Surprisingly, the kinetics of spindle elongation were similar between controls and mutants (Fig. 2C) and the average maximal rate of spindle elongation in the latter was 0.8 μm/minute (n=10). Therefore, the loss of *klp67A* does not dramatically affect the rate of anaphase B. However, the centrosomes were less mobile leading to the bent and distorted spindles seen in fixed preparations.

Microtubule minus ends are improperly positioned in *klp67A* mutants

Central spindle formation requires that the overlapping plus ends of microtubules are consolidated into bundles and that their minus ends are released from the centrosomes and spindle poles. Molecules at the respective plus and minus ends are both directly involved in these processes and serve as polarity markers for the MTs comprising the central spindle. To investigate the organisation of MT plus ends in this structure, we examined the localisation of two plus end directed motors required for proper central spindle formation. The first of these kinesin-like proteins, pavarotti (Adams et al., 1998; Somma et al., 2002), is known to form a complex with a Rho-family GAP, termed centralspindlin, and is thought to bundle microtubules and deliver a stimulatory signal for cytokinesis (Mishima et al., 2002; Somers and Saint, 2003). In wild-type control cells, pavarotti (Pav-KLP) assumed a well-defined band-like distribution at the central spindle's midpoint, or midzone (Fig.

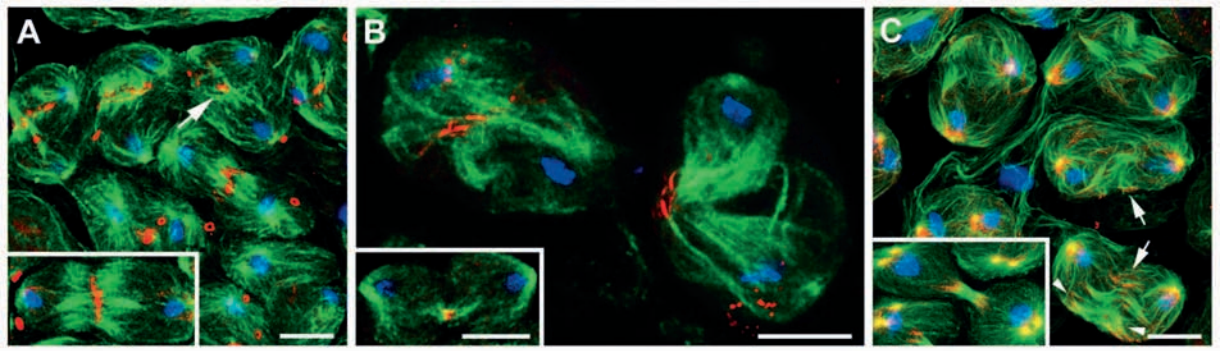

Fig. 3. *klp67A* mutants mislocalise the central spindle-dependent proteins Pav-KLP, KLP3A and Asp. Microtubules are in green and DNA is shown in blue. (A) In control cells (inset) pavarotti-kinesin like protein (Pav-KLP; red) assumes a band-like distribution on the overlapping and interdigitating plus ends of central spindle microtubules. Loss of *klp67A* prevents the formation of this homogeneous band, but Pav-KLP still associates with the variable numbers of central spindle microtubule bundles that form, which in severe cases is very few, where it appears as aggregates (arrow). (B) Like Pav-KLP, KLP3A (red) localises to the midzone of wild-type central spindles and midbodies (inset). In *klp67A* mutants the motor protein is detected as ragged streaks that correspond to the poorly formed central spindle microtubule bundles. (C) Asp (red) associates with microtubule minus ends. In wild-type cells (inset) Asp localises to the centrosomes as well as the ends of the microtubules that have been released from the centrosomes and spindle poles to form the central spindle. Similarly, in mutants, Asp associates with the centrosomes and at the termini of the central spindle microtubules (arrowheads). As indicated by the arrows, irregularly spaced Asp signals are found near the equators of *klp67A* mutant cells, indicative of variable microtubule lengths or positions. Bars, 10 μm.

3A; inset; see also Fig. S2 in supplementary material). Klp67A function was not required for the association of Pav-KLP with central spindle microtubules. In some mutant cells Pav-KLP concentrated at the equator as a poorly formed or atrophied ring, whereas in other cases was seen only as a few foci corresponding to the asymmetrically positioned MT bundles (Fig. 3A; arrow). The second motor protein we immunolocalised was the chromokinesin KLP3A, which although not needed for cleavage in mitotic systems, is crucial for the formation of proper central spindles during *Drosophila* male meiosis (Williams et al., 1995; Giansanti et al., 2004). KLP3A showed a similar localisation pattern to Pav-KLP in *klp67A* mutant cells, whereas in wild-type cells it concentrated into a well-defined centrally positioned band on central spindles and midbodies (Fig. 3B; inset); in the mutants it appeared as a variable number of centrally positioned streaks on the bundles of central spindle MTs (Fig. 3B). Thus the kinesin-like proteins required for MT plus end organisation and bundling are present on the midzones of the diminished central spindles observed in *klp67A* mutants but they do not form homogeneous bands.

The microtubules that are released from the centrosomes and spindle poles can be recognised by their association with the abnormal spindle protein (Asp). This MT minus end binding protein is important for the formation and maintenance of the central spindle (Wakefield et al., 2001; Riparbelli et al., 2002). Following anaphase onset in wild-type primary spermatocytes, Asp decorated the centrosomes and spindle poles as well as the minus ends of the microtubules that translocated to the spindle equator to form the central spindle and resultant midbody (Fig. 3C; inset). Asp consistently associated with the termini of central spindle structures in *klp67A* mutants (Fig. 3C, arrowheads). However, the protein was also seen in asymmetrical punctae or as irregularly spaced streaks in the mid-portion of the cell (Fig. 3C, arrows). From this we conclude that the central spindle MTs found in *klp67A* mutants show considerable variation in length or positioning.

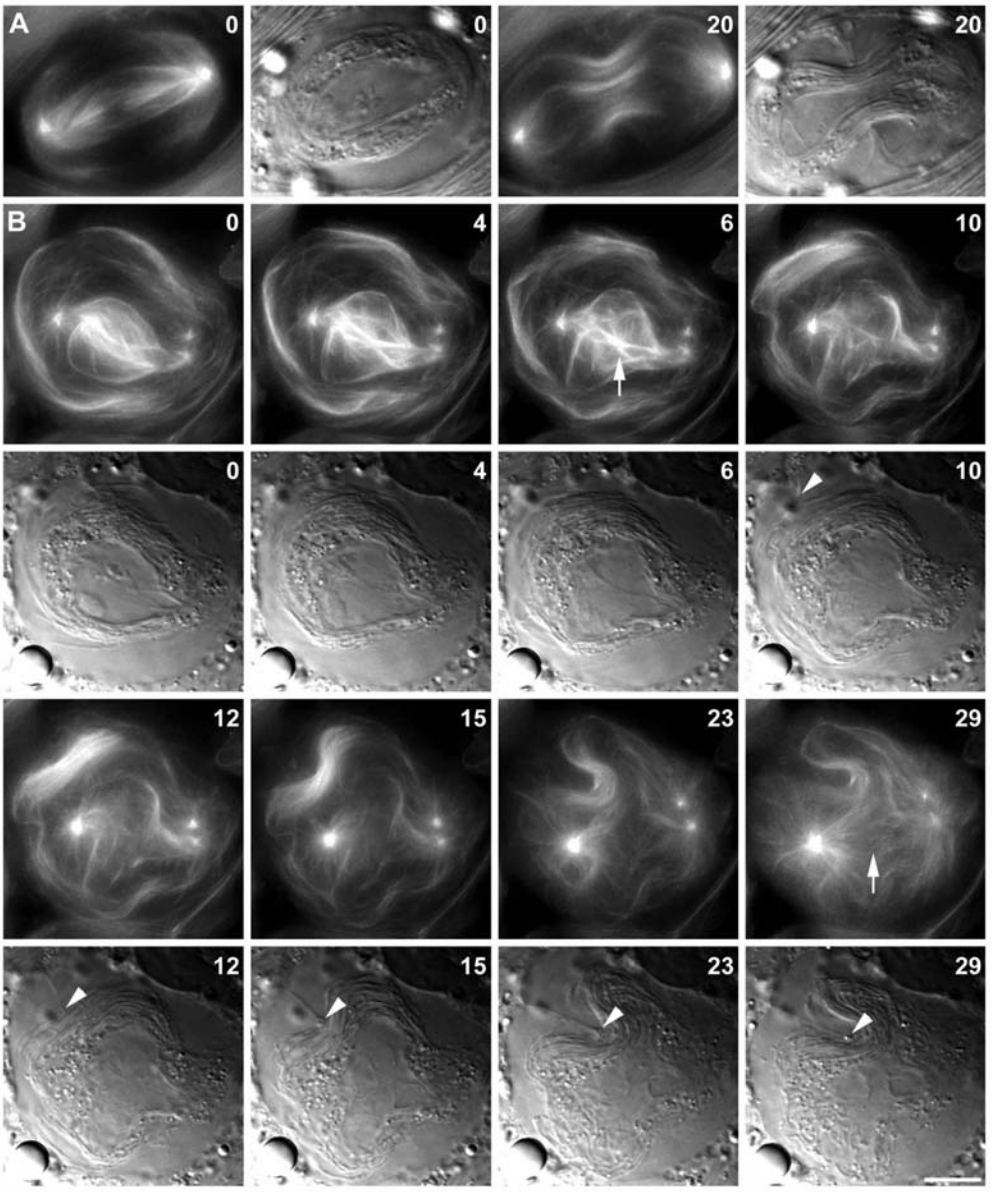

Fig. 4. *klp67A* mutants form diminished and unstable central spindles. Selected frames from time-lapse sequences showing central spindle formation in wild-type (A) and (B) *klp67A* mutant cells. At anaphase onset in wild-type cells (A; 0 minutes) the spindle is linear and has well defined asters that cap each spindle pole. As anaphase ensues the spindle remodels itself into an equatorially positioned central spindle composed of bundles of cytoplasmic, peripheral astral microtubules and interior microtubules within the spindle envelope. This structure initiates and promotes the advancement of the cleavage furrow (20 minutes) before being compacted into a midbody. (B) Central spindle formation is aberrant in *klp67A* mutants. Before anaphase onset (0 minutes) the cell contained increased numbers of abnormally long astral microtubules that encircled the robust spindle. As the cell advances into anaphase these peripheral astral microtubules collect in the upper portion of the cell, where they initiate a unilateral cleavage furrow that advances but fails to fully cleave the cell (10-29 minutes; arrowheads). Concomitantly, the interior central spindle fails to form ostensible bundles as seen in the wild-type and rapidly degrades (compare arrows at 6 and 29 minutes). The peripheral microtubules are also unstable and as a result this cell lacks a recognisable central spindle. See main text for details. Time is in minutes relative to anaphase onset. Bar, 10 μm.

Live-cell imaging reveals that central spindles become unstable in *klp67A* mutants

To gain further insights into how the depletion of Klp67A leads to aberrant central spindles and defective cytokinesis, we re-examined the dynamics of central spindle formation and furrow ingression in living primary spermatocytes. Consistent with the report by Inoue et al. (Inoue et al., 2004), we found that following anaphase onset in wild-type cells (Fig. 4A; 0 minutes), a population of peripheral astral MTs became bundled at the future cleavage site. These bundles remained in contact with the cortex as the furrow ingressed. Shortly thereafter, a central spindle formed that was composed of both the peripheral astral MTs and interior MTs found within the spindle envelope. This structure then became further compacted and consolidated as the cleavage furrow advanced (Fig. 4A; 20 minutes) until a midbody formed between the two daughter cells (see Movie 3 in supplementary material).

Central spindle morphogenesis was dramatically altered in *klp67A* mutants. As with our fixed preparations we found that before anaphase, the spindles in living *klp67A* mutants were abnormally dense and appeared to have increased numbers of long MTs both inside the spindle envelope and in the cytoplasm. At anaphase onset in the cell shown in Fig. 4B (0 minutes), cortically proximal astral microtubules were symmetrically positioned along the cell periphery, whereas those MTs inside the spindle envelope appeared both as thick bundles and as disorganised masses. As anaphase ensued, the astral MTs redistributed and concentrated on the upper portion of the cell at a site displaced from the equator of the cell. This largest and predominant peripheral bundle ultimately directed the formation of a unilateral furrow (10 minutes; arrowhead) that ingressed but halted (12-23 minutes; arrowheads), resulting in cleavage failure (29 minutes; arrowhead). In most mutant cells interior spindle MTs were released from the spindle poles in a timely fashion. However, in a few cases, the bulk of the MTs were shed from the poles while a few MTs or bundles remained. Once released the microtubules translocated towards the equator but only formed feeble overlapping bundles that became less distinct and ultimately degraded (Fig. 4B; compare 6 minutes and 29 minutes; arrows; Movie 4 in supplementary material) (see also Figs 5 and 6). The interior central spindles were unstable in *klp67A* mutants and, unlike control cells in which this structure remained robust throughout cleavage (15/15), interior central spindles were maintained in only 14/31 (45%) of mutant cells. Furthermore, in stark contrast to the 87% (n=15) of wild-type cells whose cleavage furrows went to completion during filming, *klp67A* mutants formed daughter cells in only 48% of our experiments (n=31). We did not observe any defects in the translocation of peripheral spindle MTs. However, this microtubule population was also destabilised in the mutants and completely degraded in 15/31 cells (48%). These observations reveal that the lack of discreet central spindles noted in *klp67A* mutants results from a global loss of microtubule stability following the metaphase-to-anaphase transition.

klp67A mutants reveal that both peripheral and interior central spindle microtubules can induce furrowing

We found that cleavage furrows always initiated in *klp67A* mutants. They could be either unilateral, as in the above example (7/31 cells), or symmetrical, but also fail to progress and form a midbody (9/31). This failure correlated with the lack of an interior central spindle. These data reveal that the aberrant spindles formed in *klp67A* mutants are competent to initiate cleavage. However, the interior central spindles are unstable and the furrows often regress before cytokinesis is completed.

We also found that of the 31 *klp67A* mutant cells filmed, 19% formed furrows at multiple locations on one or both sides of the cell. Such supernumerary furrows initiated on average 8 minutes (n=6) after the predicted, primary cleavage had begun. These additional ectopic furrows could form in cells with unilateral (Fig. 5) as well as symmetrical primary furrows (Fig. 6), indicating that their initiation was not due to 'fragmention' of the contractile ring. As illustrated in Fig. 5 (see also supplementary material, Movie 5), once initiated, ectopic (arrowheads) and primary furrows (arrows) often advanced

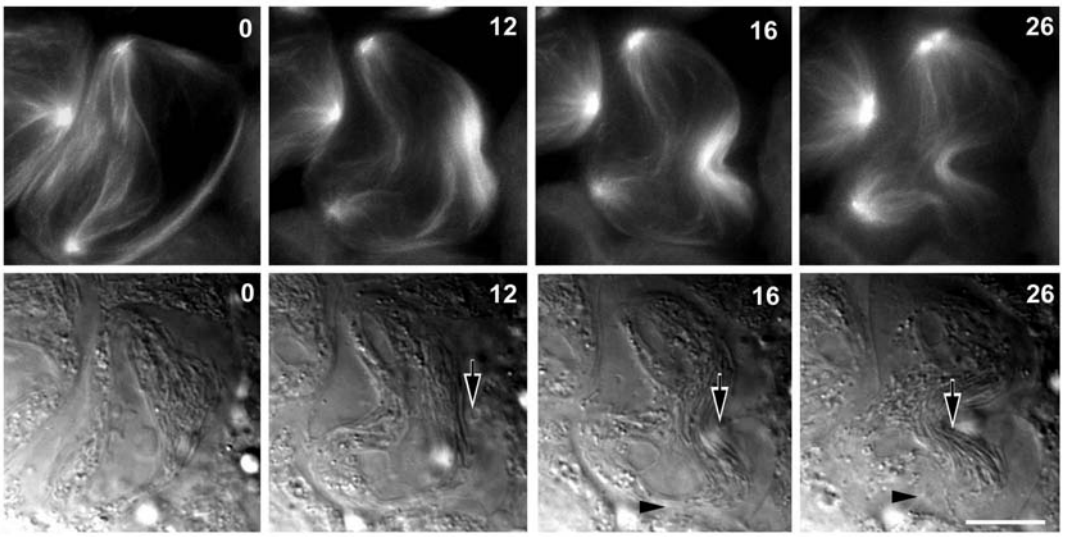

Fig. 5. Ectopic furrows in *klp67A* mutants generate cytoplasts. Selected frames from a time-lapse sequence of a *klp67A* mutant during cytokinesis. At anaphase onset (0 minutes) the spindle was abnormally long and surrounded by a symmetrical cage of astral microtubules. These translocate during spindle elongation and coalesce at the right hand side and lower portion of the cell, where they initiate (12 minutes) the primary (arrows) and ectopic (16 minutes; arrowheads) cleavage furrows, respectively. Ingression of the two unilateral furrows leads to the formation of an anuclear cytoplast (26 minutes). Time is in minutes after anaphase onset. Bar, 10 μm.

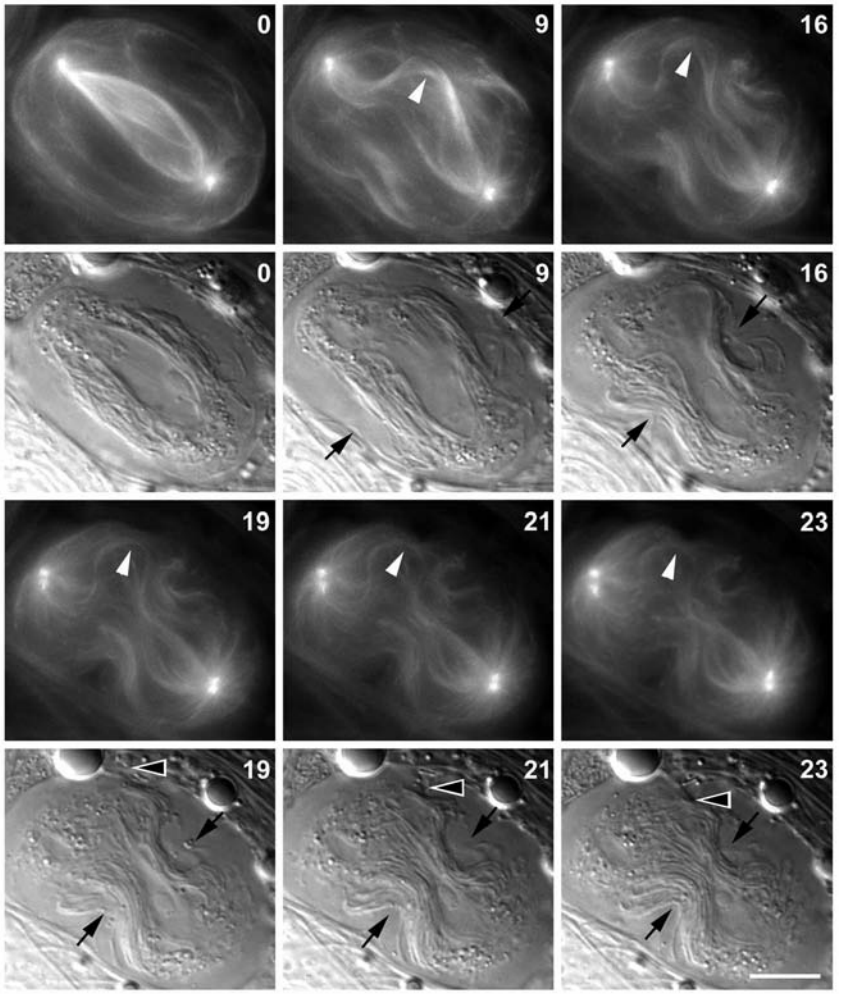

Fig. 6. Interior central spindles can initiate cleavage furrows in primary spermatocytes. Time-lapse sequence of a *klp67A* mutant during cytokinesis. 0 minutes: Image of the cell at anaphase onset. Note the characteristic rings of astral microtubules encircling the dense spindle. After dyad disjunction the interior central spindle (9 minutes; white arrowhead) undergoes anaphase B elongation causing it to buckle outwards. During this time the peripheral astral microtubules concentrate and contact the cell boundary at the equator where they initiate a primary furrow (arrows). The interior central spindle further protrudes and approaches the cell cortex in a region of diminished peripheral microtubule density (9-16 minutes; white arrowheads). As the primary furrow continues to advance (16-23 minutes; arrows), an ectopic furrow (19 minutes; black arrowhead) initiates where the interior central spindle apposes the cell membrane (white arrowhead). This ectopic furrow then ingresses towards the cell centre (21-23 minutes; black arrowheads). Time is in minutes relative to anaphase onset. Bar, 10 μm.

towards a common point (16-21 minutes; arrowheads), resulting in the formation of cytoplasts (26 minutes). Thus, ingression of ectopic cleavage furrows accounts for the origins of the anucleate cells seen in onion stage spermatids.

Surprisingly, the additional furrows could form at areas with greatly diminished numbers of astral MTs (Fig. 6). Here, the spindle was surrounded by a ring of astral MTs at anaphase onset (0 minutes) that shortly thereafter began to concentrate at the equator where they bundled and then initiated primary furrow ingression (9 minutes; arrows). Concomitant with the bundling of the peripheral astral MTs, the interior central spindle (white arrowhead) began to form and buckled outwards towards the cortex. These bundles of interior central spindle MTs were in close proximity to the cortex as the equatorially positioned furrow advanced (16 minutes). During this time the interior central spindle began to diminish and became more disorganised, but the remaining bundles approached to within a few micrometers of the cell boundary (19 minutes; white arrowhead), where despite a very low density of peripheral astral MTs, a furrow then initiated and ingressed (19-23 minutes; black arrowheads; Movie 6 in supplementary material).

The timing of the appearance of the ectopic furrows further suggested that the interior central spindle MTs were responsible for their formation. In *klp67A* mutants the primary furrow began ingressing on average 2 minutes after the peripheral MTs contacted and bundled at the cortex (n=27), a duration identical to that seen in the 15 wild-type cells analysed. Similarly, ectopic furrows initiated approximately 4 minutes after interior central spindle MT bundles approached the cortex (n=6). This observation, taken with the consistent delay in ectopic furrow ingression relative to primary sites, indicates that the few astral MTs that were present at the nonequatorial locations did not induce cleavage. Furthermore, these data reveal that the interior central MTs are both capable and sufficient to induce furrows when proximal to the cell periphery.

Loss of Klp67A disrupts actin and anillin recruitment to the cleavage site

Fission yeast double mutants lacking either *klp5* or *klp6* and *scd1* undergo a defective cytokinesis. This is due at least in part to a failure to efficiently recruit actin and Mid1 to the cell equator (Li and Chang, 2003). We therefore wondered whether depletion of Klp67A would affect the localisation of the fly counterparts of these proteins in primary spermatocytes. To this end we examined the distribution of actin, the principle structural component of the contractile ring (Satterwhite and Pollard, 1992) and the PH domain containing anillin protein, the *Drosophila* orthologue of Mid1 (Field and Alberts, 1995; Bahler et al., 1998). In wild-type cells the contractile ring is a continuous actin belt around the cell's equator (Fig. 7A; inset). Actin was organised to varying degrees in *klp67A* mutants (Fig. 7A). In some cells it appeared as a slightly distorted or diminished ring, but still encircled the cell. In other instances, actin staining was discontinuous and asymmetrically concentrated in a crescent that corresponded to the bundled central spindle MTs. In those cells where the central spindles had almost completely degraded, actin could only be detected as a few streaks or cortically positioned foci.

We next investigated the distribution of anillin. This is one

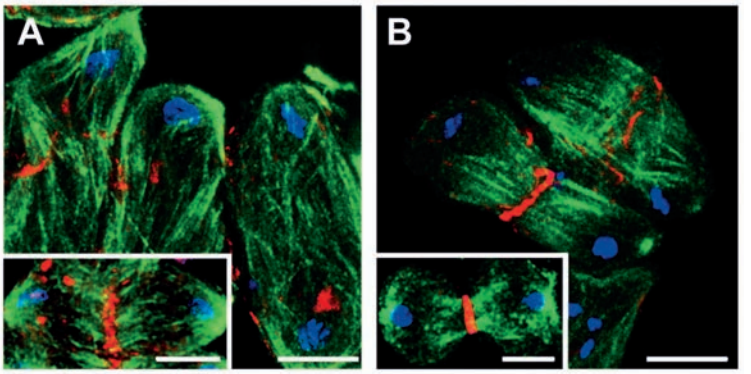

Fig. 7. *klp67A* mutants fail to form continuous actin and anillin rings. Wild-type (insets) and *klp67A* mutants were fixed and stained for actin (A; red) or anillin (B; red). Microtubules are shown in green and DNA in blue. In contrast to wild-type cells, which form continuous rings of actin at their equators, actin staining is often disorganised or in streaks in the mutants and in some instances is found primarily as cortically associated foci. Similarly, anillin staining is aberrant in mutants (B) and is patchy or discontinuous. The extent to which either actin or anillin form homogenous bands correlates with the integrity of the central spindle, with the proteins being more symmetrically distributed when robust microtubule bundles are present. Bars, 10 μm.

of the first proteins to localise to the cleavage plane and concentrates there during late anaphase in wild-type cells (Fig. 7B; inset). It retains this distribution in some mutants (e.g. *klp3A*) in which the central spindle is disrupted (Giansanti et al., 1999). However, as shown in Fig. 7B, we found that in *klp67A* mutants, anillin organisation at the equator correlated with the degree of central spindle integrity. In the presence of well defined central spindles, anillin was focused into its characteristic ring-shape at the midzone. In those cells with poorly organised central spindles this distribution became ragged and heterogeneous, and in those instances where no central spindles could be identified, anillin did not appear to concentrate above background levels. From these observations we conclude that Klp67A function is required for those aspects of central spindle organisation that organise both actin and anillin into ring-like structures in *Drosophila* primary spermatocytes.

Discussion

klp67A mutants form diminished central spindles

We and others have previously shown that before anaphase, depletion of Klp67A, a *Drosophila* member of the Kip3 subfamily of microtubule destabilising proteins, leads to the formation of abnormally robust spindles comprised of increased numbers of long and stable MTs (Goshima and Vale, 2003; Gandhi et al., 2004; Savoian et al., 2004). We have now extended these analyses and characterised the behaviour of MTs in anaphase and cytokinesis in both living and fixed *Drosophila* primary spermatocytes isolated from *klp67A* mutants. Our data indicate that in the absence of *klp67A* the spindle still undergoes anaphase B. However, elongation leads to spindles that are bent and distorted. Likewise, in budding yeast *kip3* mutants the spindles become overgrown (Straight et al., 1998), and fission yeast *klp5/klp6* mutants also form spindles that double in length (West et al., 2002). In surprising contrast to these yeast mutants whose spindles require increased amounts of time to disassemble, the central spindles in *klp67A* mutant primary spermatocytes appeared to contain diminished numbers of MTs and they degraded before cytokinesis could be completed.

klp67A mutant spindles do not linearly elongate during anaphase B

Studies in *Drosophila* embryos indicate that anaphase B results from two complementary mechanisms, a dynein-mediated cortical pulling force on the asters and a pushing force from within the spindle as the tetrameric Bim C subfamily kinesin Klp61F slides antiparallel MTs in opposite directions (Sharp et al., 1999a; Sharp et al., 1999b; Sharp et al., 2000). Several of our observations suggest that similar mechanisms exist for anaphase B in primary spermatocytes. First, spindle elongation and centrosome movement appear to be simultaneous yet independent events as *klp67A* mutants undertook the former with kinetics similar to that observed in the wild-type, but did not undergo the latter. Second, as predicted from models of anaphase B based on the sliding of antiparallel MTs, spindle elongation initiated in wild-type spermatocytes when interior MTs from each half spindle appeared to overlap at the equator. Third, anaphase B continued while interior microtubules remained associated with each spindle pole, but terminated following MT release and translocation. These observations suggest that the mechanisms of spindle elongation are conserved between mitotic and meiotic cells.

Spindle elongation was not perturbed in the absence of Klp67A and occurred with normal kinetics. This is also seen in fission yeast lacking Klp5/6 (West et al., 2002). Unlike yeast, however, the centrosomes in mutant primary spermatocytes were less mobile, leading to the buckling and bending of spindles. Previous fixed cell studies of *klp67A* mutants suggested a similar movement defect during prophase, when the centrosomes could be delayed in associating with the nuclear envelope and separating from one another for bipolar spindle formation (Gandhi et al., 2004) (our unpublished observations). Thus, Klp67A is involved in proper centrosome movement throughout male meiosis I. Klp67A probably serves an indirect role in this process, possibly affecting movement through the alteration of astral MT dynamics and their subsequent interactions with motor molecules such as cytoplasmic dynein and Klp61F.

Klp67A is needed for forming and stabilising the central spindle

We attribute the diminished central spindles in *klp67A* mutants primarily to a loss of microtubule stability. In contrast to the MTs in *klp67A*-deficient primary spermatocytes in prometaphase and metaphase, which were abnormally long and stable (Gandhi et al., 2004; Savoian et al., 2004) (this report), we consistently found that following anaphase onset the microtubules both inside and outside of the spindle envelope underwent degradation. As a result, the interior central spindles and the bundles of peripheral astral MTs became more

disorganised and rapidly disintegrated, leading to cleavage failure. We did not detect a significant difference in the frequency with which either interior or peripheral microtubules destabilised (compare 45% and 52%, respectively). It is important to note, however, that the peripheral MT bundles appeared to take slightly longer to degrade. It is unclear if this reflects differences in the composition of these MT populations, or if it is the direct result of the number of MTs found in each bundle type. Nevertheless, these observations indicate that Klp67A function is required for stabilising both interior and peripheral microtubules after the metaphase-to-anaphase transition.

How does the loss of a catastrophe factor lead to unstable microtubules? The mutant phenotype we have observed in spermatocytes in this and our earlier study (Savoian et al., 2004) suggests that the activity of Klp67A changes in the late stages of meiosis: it destabilises MTs in prometaphase, metaphase and early anaphase, but is required for their stabilisation in late anaphase, telophase and cytokinesis. We envisage at least two ways in which this might be brought about. In the first, we hypothesise that a potential active role for Klp67A as a MT destabilising kinesin-like motor before anaphase might then be replaced by a more passive role as a cap to protect microtubule plus ends against other catastrophe factors in late anaphase. The second also proposes loss of Klp67A function in late division, but supposes that microtubule instability might be a natural default mechanism should the central spindle not be correctly assembled. Downregulation of the motor function of kinesin-like proteins at the metaphase-anaphase transition is not without precedent. It has recently been shown that the activity of MCAK, a Kin I MT depolymerase that localises to the central spindle (Walczak et al., 1996; Maney et al., 1998; Desai et al., 1999) is regulated by aurora B kinase. Phosphorylation of MCAK inhibits its depolymerase activity and also regulates the association of the motor protein with the kinetochore (Andrews et al., 2004; Ohi et al., 2004; Lan et al., 2004). It is therefore possible that Klp67A's depolymerase activity might also be phospho-regulated. Sequence analyses did not identify any CDK1 phosphorylation consensus sequences in Klp67A, but there are multiple putative phosphorylation sites for both polo and uurora B kinases (our unpublished analysis). Each of these kinases localise to the central spindle in a pattern identical to that seen for Klp67A and both are needed for central spindle formation (Carmena et al., 1998; Herrmann et al., 1998; Schumacher et al., 1998; Bonacorrsi et al., 2000; Giet and Glover, 2001). It will therefore be of future interest to determine if the properties of Klp67A are changed following phosphorylation by either of these enzymes.

We also observed an apparent failure to release some microtubules from the spindle poles in *klp67A* mutants. In wild-type cells the Asp protein-associated minus ends of the MTs within the spindle envelope are released from the poles, allowing their translocation towards the equator and 'compaction' to form the interior central spindle, a structure required for cleavage furrow propagation (Inoue et al., 2004). Although a comparison between the sizes of the spindle poles/centrosomes did not indicate defects during microtubule release, in living mutant cells we sometimes noted microtubules or MT bundles that remained associated with the spindle poles within the spindle envelope. Whether this was a true failure to release MTs was unclear as the distorted spindle shape and microtubule degradation in *klp67A* mutants made any analysis of fluorescence intensity along the spindle length impractical in live cells. It is interesting that the peripheral MTs did not appear to be affected and consistently underwent translocation through the cytoplasm. One explanation for the putative ectopic polar MTs and bundles in *klp67A* mutants is that they are simply the overgrown remnants of a nonreleased population of interior spindle MTs. Such a population exists in wild-type control cells where some short microtubules remain associated with the spindle poles during late anaphase/telophase (our unpublished observations). Thus, the presence of these MTs may simply be emphasised by the *klp67A* mutation that makes them longer or initially hyper-stable. Alternatively, the depletion of Klp67A may prevent microtubule release. When interior microtubules are destabilised by mutations in the *orbit/mast* gene that encodes a MT plus end stabilising protein (Maiato et al., 2002), they become severely hampered in their release from the spindle poles (Inoue et al., 2004). This raises the possibility that stable MT plus ends are required for microtubule release and interior central spindle formation, although any mechanism that underlies this is unclear.

The interior central spindle is able to stimulate cleavage furrows

It has recently been shown in cultured mammalian cells (Canman et al., 2003) and in cultured *Drosophila* somatic cells and primary spermatocytes (Inoue et al., 2004) that peripheral astral MTs contact the cell cortex at the future cleavage site and signal cytokinesis. Live cell studies using Pav-KLP tagged with GFP reveal that *Drosophila* embryos (Minestrini et al., 2003) as well as primary spermatocytes (our unpublished observations) have populations of Pav-KLP both at the periphery and confined within the spindle envelope. Thus, a probable candidate for the signal transmitted by either interior or peripheral MTs is the evolutionarily conserved central spindlin complex composed of pavarotti-KLP and the Rho family GTPase activating protein RacGap50C in flies (Somers and Saint 2003), and their respective counterparts Zen-4 and Cyk-4 in *Caenorhabditis elegans* (Mishima et al., 2002) and MKLP1 and MgcRacGAP in mammals (Hirose et al., 2001; Mishima et al., 2002).

However, cytokinesis can still occur in the absence of astral microtubules. Asterless systems can be generated by mutation – for example *asterless* (Bonaccorsi et al., 1998; Giansanti et al., 2001) and *centrosomin* (Megraw et al., 2001) or through microsurgery (Alsop and Zhang, 2003). Our observations of ectopic furrows formed in *klp67A* mutants help to reconcile these seemingly contradictory data. We found that although cleavage furrows formed preferentially where peripheral central spindle MTs contacted the cortex, they were also able to initiate in regions lacking peripheral astral microtubules, but only when the interior central spindles became closely apposed to the cortex. Consistent with this finding, analyses of fixed preparations of aster-lacking spermatocytes revealed central spindle-like microtubule bundles adjacent to the cell cortex at the cleavage site (Bonacorssi et al., 1998; Alsop and Zhang, 2003). Together, these observations indicate that the interior central spindle is able to transmit a signal to stimulate the

formation of the cleavage furrow, but that in wild-type *Drosophila* primary spermatocytes it is positioned too far from the cortex to be effective.

Our observations of the ability of the interior central spindle to stimulate furrowing sheds further light on the role of the (interior) central spindle in furrow maintenance. Previously it was reported that asters/peripheral spindle MTs could generate furrows but that these were unstable in the absence of a central spindle-like structure and quickly regressed (Powers et al., 1998; Raich et al., 1998; Savoian et al., 1999; Inoue et al., 2004). This could be explained if the population of centralspindlin and other putative interacting partners found with it on the peripheral central spindle require a critical mass of similar molecules provided by the interior central spindle to reinforce the formation of a contractile super-complex in order to advance to the midbody stage.

In conclusion, the data we present here provides the first characterisation of the role of a catastrophe factor during cytokinesis. It reveals that the Klp67A protein is a microtubule depolymerase during prometaphase and metaphase when it facilitates chromosome congression and regulates spindle morphology, but then changes function during anaphase. In the absence of Klp67A, initially abnormally long peripheral MTs induce asymmetric furrowing. Such furrows cannot be maintained however, as following anaphase entry Klp67A is needed for stabilising central spindle MTs. The motor protein is also needed for centrosome movement during anaphase B, and in its absence, spindles buckle towards the cortex as they elongate. Cleavage furrows initiate at these ectopic sites indicating that both interior and peripheral MTs are able to initiate furrowing. It will be of considerable future interest to understand how the Klp67A protein mediates each of these roles and how the transition between them is regulated.

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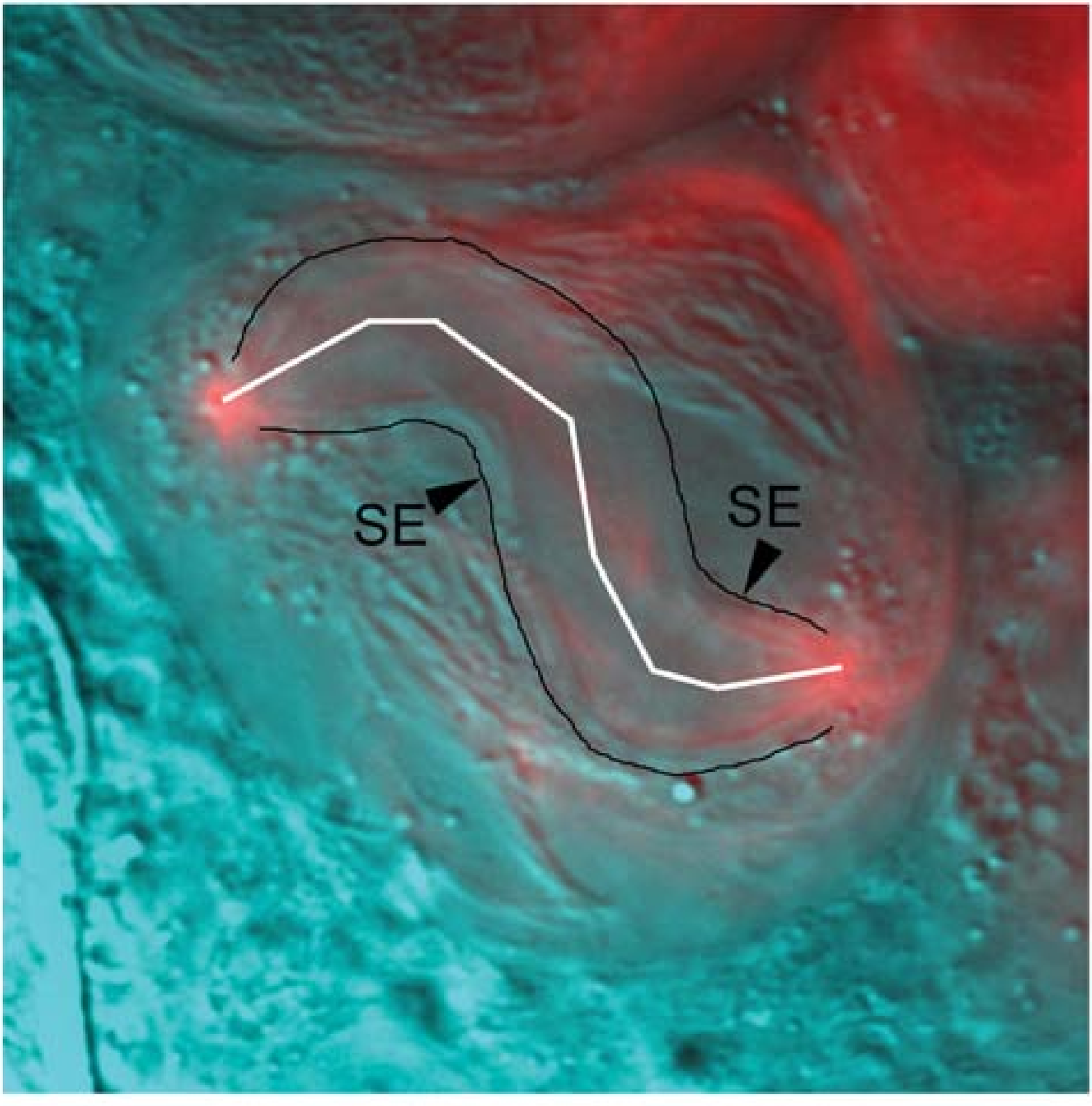
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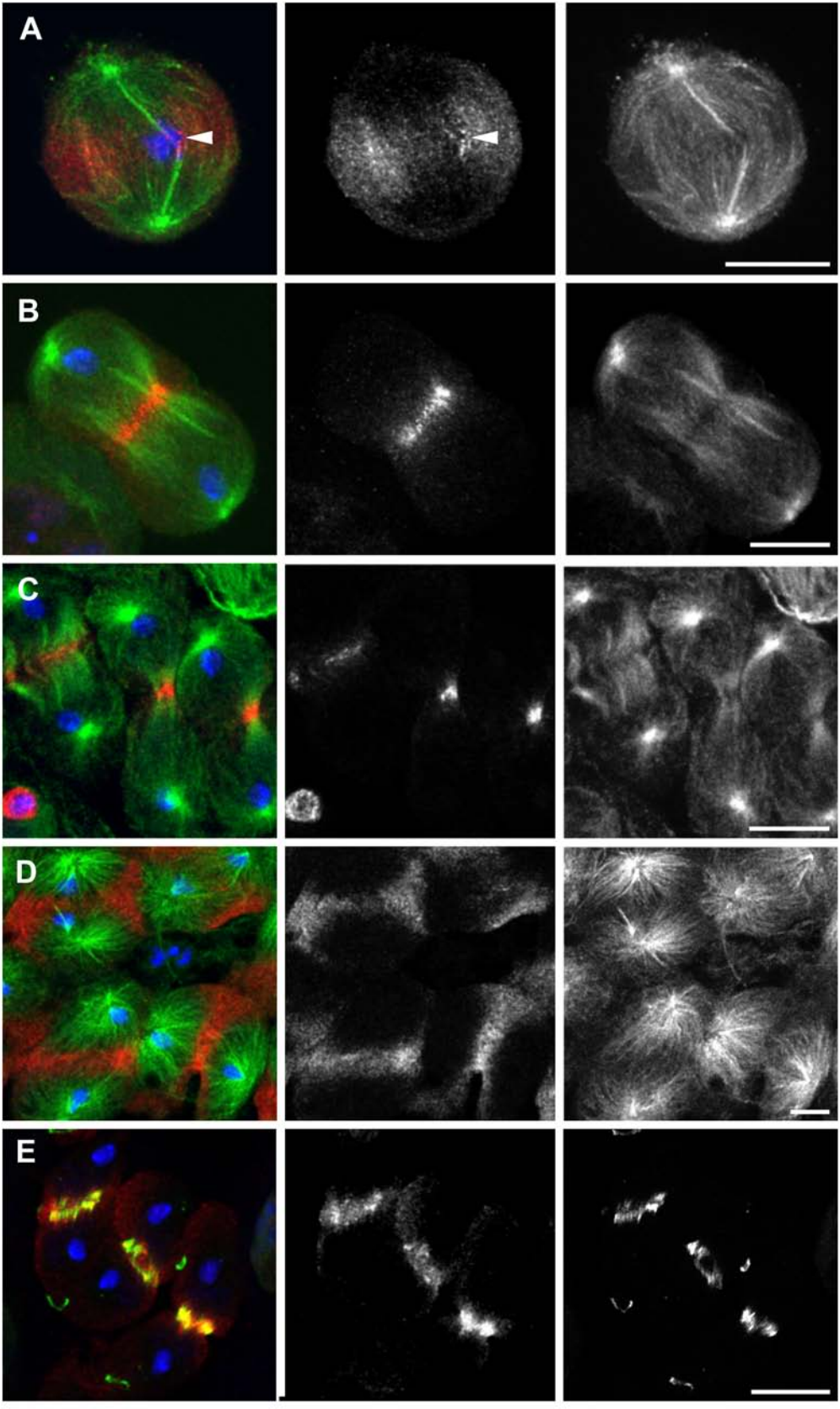
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