Drosophila Klp67A is required for proper chromosome congression and segregation during meiosis I

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Summary

Drosophila **Klp67A belongs to the Kip3 subfamily of Kinesin-type microtubule catastrophe factors. In primary spermatocytes, loss of *klp67A* leads to defects in karyokinesis and cytokinesis. We show that these cells formed disorganised, bipolar spindles that contained increased numbers of microtubules. The kinetochore fibres were wavy and bent, whereas astral microtubules appeared abnormally robust and formed cortical bundles. Time-lapse studies revealed that during biorientation, the chromosomes in *klp67A* mutant cells continued to reorient for about twice as long as those in control cells. Metaphase plates were poorly defined in the mutants and often formed at non-equatorial positions. Consistent with the above abnormalities in chromosome congression, we found that in wild-type cells Klp67A associated with prometaphase/metaphase kinetochores before redistributing to the central spindle at anaphase onset. Although the timing of this redistribution of kinetochores argues against a role in anaphase chromosome segregation, dyads in the mutants disjoined but exhibited greatly diminished poleward velocities. They travelled on average at approximately 34% of the velocity of their wild-type counterparts and often decondensed at non-polar locations. Hypomorphic mutations of *klp67A* may lead to segregation defects.**

Movies available online

Key words: Kinesin, Karyokinesis, Spindle, Kinetochore, Aster

Introduction

Chromosomes attach to the plus ends of dynamic spindle microtubules (MTs) via their kinetochores (Rieder and Salmon, 1998). In prometaphase I, each chromosome or bivalent is comprised of two homologous dyads. Chromosome biorientation occurs when each of the dyads attaches to one of the opposing spindle poles through bundles of specialised MTs defining the kinetochore fibres (k-fibres). Movement to the metaphase plate, or congression, thus requires that one k-fibre shortens while the other elongates. The k-fibres must then continually shorten to allow anaphase segregation. Although MTs are inherently dynamic polymers, their polymerisation state is further regulated in vivo through the antagonism of stabilising or growth-inducing accessory proteins such as the Dis1/Tog family of microtubule associated proteins (MAPs) and catastrophe factors that promote MT shrinkage (Hunter and Wordeman, 2000; Tournebize et al., 2000; Kinoshita et al., 2001; Ohkura et al., 2001).

Some MT catastrophes are induced by members of the Kinesin superfamily of MT motor proteins. For example, members of the Kin I family, which have their motor domain internally positioned, do not translocate along MTs but concentrate on the ends and promote depolymerisation (Desai et al., 1999; Hunter et al., 2003; Moores et al., 2003). Studies of these proteins both in *Xenopus* extracts and in living mitotic cells indicate that they are required for bipolar spindle formation and maintenance (Walczak et al., 1996; Kline-Smith and Walczak, 2002; Goshima and Vale, 2003). Furthermore, perturbation of centromere-associated Kin I proteins results in disorganised metaphase plates and lagging anaphase chromosomes (Maney et al., 1998; Walczak et al., 2002).

The Kip3 subfamily of Kin N (N-terminal positioned motor domain) Kinesins also promote MT catastrophe. Mutants of the budding yeast founding member *kip3* form abnormally long and stable spindles (Cottingham and Hoyt, 1997; Straight et al., 1998). Similarly, loss of the fission yeast orthologues *klp5/6* also leads to elongated spindles (West et al., 2002). Live cell studies of these mutant cells reveal that prometaphase chromosomes spend prolonged periods at the spindle poles before ultimately congressing. However, stable metaphase plates often fail to form and the chromosomes may continue to oscillate until anaphase onset (Garcia et al., 2002a; Garcia et al., 2002b; West et al., 2002). Furthermore, mini-chromosomes are lost with increased frequency in these mutants indicating segregation defects (Garcia et al., 2002a). Recently, it was reported that perturbation of the *Drosophila* Kip3 member *klp67A* by RNAi in mitotic S2 cells prevented bipolar spindle formation and led to mitotic arrest (Goshima and Vale, 2003). Mutation of the *klp67A* gene also leads to aberrant spindles in *Drosophila* primary spermatocytes and aneuploid cells (Gandhi et al., 2004). However, it is not clear from these studies whether Klp67A behaves in a similar manner to yeast Kip3 family members and thus is a true orthologue.

We have initiated a series of studies to characterise chromosome and spindle behaviour in *Drosophila* primary spermatocytes that have been depleted of the various MT catastrophe factors. Key to our understanding of protein function is the observation of the behaviour of chromosomes in living cells, a criterion that makes primary spermatocytes an excellent system to work with (e.g. Savoian et al., 2000). Here, we characterise karyokinesis in living and fixed *Drosophila* primary spermatocytes that are mutant hypomorphs for the Kip3 family member *klp67A*. Our data indicate that Klp67A transiently associates with pre-anaphase kinetochores before moving to the central spindle. We further confirm that loss of *klp67A* leads to aberrant spindles that contain bent or distorted k-fibres and robust asters. Our live cell observations revealed that loss of *klp67A* affected the ability of chromosomes to stably associate with the spindle during prometaphase and form organised metaphase plates. Anaphase chromosomes disjoined but consistently exhibited greatly diminished segregation velocities and often decondensed before reaching the poles. These observations reveal that Klp67A is a new putative kinetochore component, that when disrupted by hypomorphic mutation, perturbs chromosome congression and severely hampers segregation.

Materials and Methods

Database and sequence analyses

Amino acid sequences corresponding to the different reported motor domains were collected from the Kinesin Homepage (http:/www.proweb.org/kinesin/). The motor sequences were then entered into the NCBI database and BLAST searched against the appropriate genomes to identify short, nearly identical matches.

Fly stocks

Two independent, chromosome III, P-element-generated mutant lines were used in this study, *l(3)S042705*^{S042705} (Deak et al., 1997) and *P{EP}Klp67A*^{EP3516} (Rorth, 1996), hereafter referred to as *klp67A*^{42705} and *klp67A*$^{EP(3)3516}$, respectively. Each of the P-elements maps to the 5′ UTR of the *klp67A* gene with *klp67A*^{EP3516} being located 5 bp upstream of *klp67A*^{42705}. Analyses were performed on hemizygotes using the deficiency chromosome *Df(3L)29A6, kni[r1-1] p[p]/TM6C, Sb, Tb*. As predicted from western blots, a comparison of phenotypes by immunofluorescence and time-lapse microscopy revealed no differences between homozygous, hemizygous and heteroallelic genotype combinations. Oregon R was used as the wild-type control. Flies were maintained at 25°C and reared according to standard procedures.

Antibody production and western blots

klp67A cDNA was isolated from an embryonic library. PCR was used to amplify the portion encoding the non-motor containing C-terminus corresponding to amino acid residues 326-814. The PCR product was fused in frame to a six-Histidine tag during ligation into the pET-23b vector. The recombinant protein was expressed in BL21pLysS cells (Novagen). As the recombinant protein was insoluble, 6 M urea was included in all buffers during purification. The protein was isolated on a Ni-NTA column (Novagen) and subsequently dialysed into PBS. Polyclonal antibodies were generated in a rabbit.

SDS-PAGE gels and western blots were prepared using standard techniques. The affinity purified anti-Klp67A antibody was used at a dilution of 1:500 and recognised a band of the predicted 92 kDa size. Anti-α-tubulin antibodies were used at a dilution of 1:2500 to monitor protein-loading variations. Proteins were imaged using ECL chemiluminescence (Amersham Biosciences).

Immunofluorescence

Testes were isolated from pharate adults in PBS containing 5% glycerol and fixed and processed as previously described (Pisano et al., 1993). Microtubules were labelled with anti-β-tubulin (Boehringer Mannheim) or anti-tyrosinated α-tubulin, YL1/2 (Harlan Sera-Lab) antibodies at dilutions of 1:200 or 1:20 respectively. Centrioles were stained with the HsCen1p polyclonal antibody at a concentration of 1:400. Centromeres were labelled using chicken anti-Cid antibodies at 1:50. For colocalisation of Klp67A with Polo kinase, testes were isolated from GFP-Polo-expressing flies (Moutinho-Santos et al., 1999) and fixed as above. In all cases, Klp67A was labelled using our affinity-purified polyclonal antibody diluted 1:50. For visualisation the appropriate commercially available secondary antibodies were used (Cappel). DNA was stained with either propidium iodide or toto-3-iodide (Sigma).

Fluorescence images were captured using either a Leica TCS 4D laser-scanning microscope (Leica Lasertechnik) or with a Nikon Microphot microscope equipped with an MRC1024 scanning confocal system (Biorad), both using a ×63 (N.A. 1.4) lens. Figures shown are maximum intensity projections generated from optical sections taken at 0.2-0.5 μm *z*-steps. Montages were made using Photoshop (Adobe Systems).

Time-lapse imaging and kinetic analyses

Cultures of primary spermatocytes were prepared according to a reported method (Savoian et al., 2000). Cells were maintained at 24°C and imaged on a Nikon Microphot-FX DIC microscope with a 100× (N.A. 1.25) lens and oil condenser (N.A. 1.4). Specimens were illuminated with filtered 546-nm light. Images were acquired at 4 second intervals using a 2×2 bin with a Spot RT camera (Diagnostic Instruments) running the included software package on a PC. Kinetic analyses were performed as previously described (Savoian et al., 2000). For sequence montages selected frames were imported into and organised in Photoshop. Selected structures were manually pseudo-coloured.

Results

Diminution of *klp67A* leads to meiotic defects

We have started to characterise the roles of putative MT catastrophe factors in regulating spindle architecture and function in *Drosophila*. Two groups of the Kinesin superfamily have been identified with catastrophe-inducing characteristics: the Kin I and Kip3 families. Depending on the method of alignment, Klp67A can be placed into either group (e.g. Lawrence et al., 2002; Dagenbach and Endow, 2004). A BLAST search of the *Drosophila* genome using the motor domains of the Kin I founding members MCAK/XKCM1 indicated that despite some sequence similarity to Klp67A (38% and 40% respectively), other *Drosophila* proteins exist with much higher homology. Furthermore, Klp67A has an N-terminal motor domain (Pereira et al., 1997), in contrast to the internal location that defines the Kin I family. Given these data and the high degree of motor sequence identity between Klp67A and Kip3 (49%) as well as Klp5/6 (51%/43%) we favour placing Klp67A in the Kip3 subfamily of Kinesins.

To examine the function of Klp67A we studied male meiosis in mutants. Our investigations were performed with two independent P-element mediated mutations identified in

separate screens; *klp67A*^{42705} (Deak et al., 1997) and *klp67A*$^{EP(3)3516}$ (Rorth, 1996). Both insertions map within 5 bp of one another in the 5′ UTR of the *klp67A* gene. Protein levels were greatly diminished in these mutants; none could be detected in the testes of hemizygous third instar larvae when placed over the deficiency chromosome *Df(3L)29A6* or in those isolated from homozygous, hemizygous or heteroallelic pharate adults (Fig. 1) using our affinity-purified antibody.

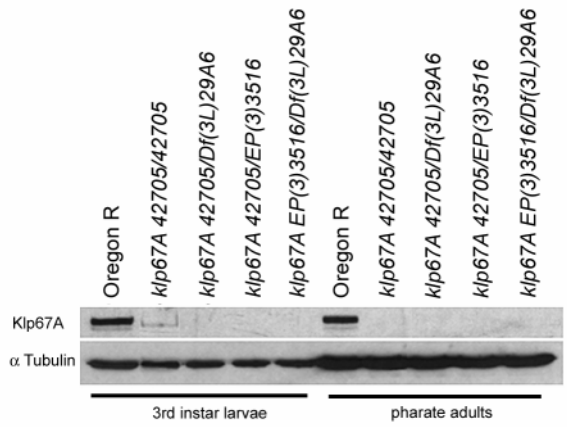

Fig. 1. Testes isolated from P-element-mediated mutants have diminished Klp67A levels. Western blot showing the different allelic combinations used in this study. A band of the expected molecular weight of ~92 kDa was present in Oregon R testes but was greatly diminished in homozygous mutant third instar larvae and not detectable in homozygous pharate adults or hemizygous or heteroallelic individuals at any stage. α-tubulin is shown as a loading control.

klp67A mutants exhibit defects in both karyokinesis and cytokinesis

The phenotypes of *Drosophila* cell division mutants that affect male meiosis can be rapidly assessed by examining the post meiotic onion-stage spermatids. In our experiments, 99% of wild-type spermatids had a single nucleus and phase dense mitochondrial derivative, the Nebenkern (n=1636). In contrast, only 28% of the 2127 spermatids isolated from *klp67A* mutants exhibited this 1:1 ratio. The majority of mutant spermatids (47%) contained two nuclei, which could be of similar or varying sizes (Fig. 2A). The presence of multiple nuclei per cell indicates that *klp67A* mutants undergo a defective cytokinesis. We are investigating the cause of this failure and those results will be described elsewhere. The variation in nuclear size correlates with variable DNA content (Gonzalez et al., 1989) and so suggests errors in chromosome segregation.

To investigate the causes of aneuploidy in *klp67A* mutants we examined meiosis I spindles by indirect immunofluorescence. In wild-type primary spermatocytes, the pre-anaphase spindle has a well-defined fusiform shape that is capped on either end by a centrosome that contains two orthogonally positioned centrioles, from which astral MTs radiate (Fig. 2B). Strikingly, *klp67A* mutants appeared to have an increased number of MTs throughout the cell. We consistently found that *klp67A* mutants formed bipolar spindles with well-separated centrosomes, indicating that Klp67A is not required for initiating or maintaining spindle bipolarity in these cells. However, the spindles were highly abnormal. The astral MTs were long and penetrated deep into the cytoplasm, or formed robust bundles that encircled the spindle (Fig. 2C,D). The k-fibres that link the chromosomes to the spindle were also affected. Rather than being well-defined and straight as in

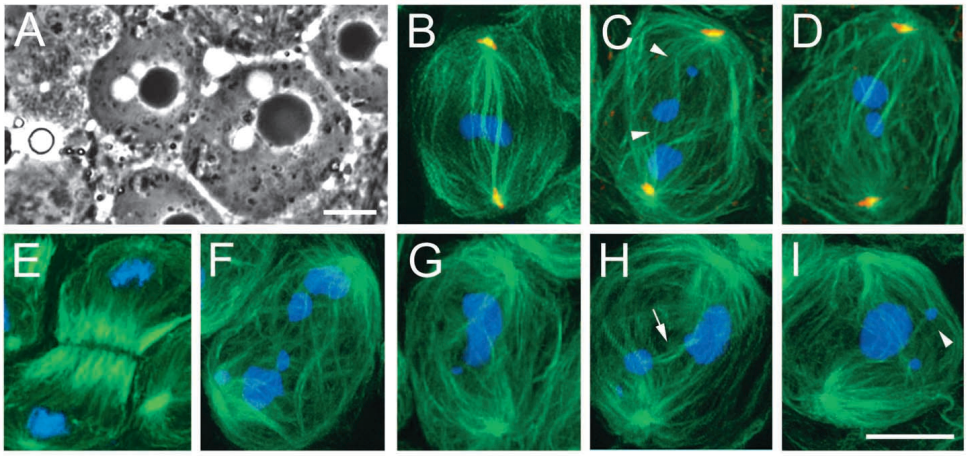

Fig. 2. *klp67A* mutants exhibit karyokinetic and cytokinetic failure during meiosis. (A) Phase-contrast image of onion-stage spermatids taken from a *klp67A* mutant. Unlike wild-type control spermatids which contain a single nucleus and phase dense Nebenkern, those in the mutants may contain two nuclei of equal or varying sizes, indicative of cytokinetic failure and in the latter case, of segregation defects as well. (B-I) A gallery of spindles taken from the wild type and *klp67A* mutant primary spermatocytes; tubulin is shown in green, DNA in blue and the centriole marker, Centrin in red. In *klp67A* mutants the centrosomes separate and form highly aberrant bipolar spindles. Unlike control spindles seen during metaphase (B) and anaphase (E), those in *klp67A* mutants are poorly organised (C,D,F-I). Ectopic MTs are present in the regions of the spindle as well as in the surrounding cytoplasm. The astral MTs are more robust than in wild-type cells and may form bundles that run around the cell periphery (C). The chromosomes sometimes appear entangled in the increased numbers of MTs found in the spindle. Kinetochore fibres in *klp67A* mutants could be hard to discern and often have an elongated and bent morphology (arrowheads in C). In contrast to wild-type anaphase/telophase cells (E) mutants lack a distinct central spindle but are filled with ectopic MTs and abnormal MT structures (F-I). Note how the chromosomes appear to segregate in unequal masses and initiate decondensation at non-polar locations. Some chromosomes may be linked together by non-centrosomal MT bundles (arrow in H) or have MT bundles oriented in opposite directions, suggestive of merotelic malorientation (arrowhead in I). Bars, 10 μm.

control cells, those in the mutants were long and wavy or bent (compare Fig. 2B with 2C, arrowheads). We found such k-fibre defects in 71% (*n*=797) of the mutant spermatocytes examined. By contrast, k-fibre morphology was aberrant in only 1% of wild-type cells (*n*=442). The chromosomes in mutants also appeared to associate with a dense meshwork of ectopic MTs that filled the spindle volume. These sometimes obscured the chromosomes or gave the impression of the chromosomes being entangled (Fig. 2C,D).

To determine if Klp67A was involved in regulating spindle morphology in the later stages of meiosis I, we examined anaphase and telophase cells. Anaphase spindles were also deformed in *klp67A* mutants (compare Fig. 2E to 2F-I), indicating that Klp67A function is needed to regulate spindle morphology throughout karyokinesis. Despite the presence of increased numbers of cytoplasmic MTs in the mutants, the central spindles were abnormal or more usually absent. This may explain the failure of the cells to cleave as revealed in onion-stage spermatids (Fig. 2A). In the mutants, unequally sized chromatin masses corresponding to the decondensing chromosomes could be found at polar proximal positions as expected (Fig. 2F) as well as throughout the spindle. As with those in prometaphase cells, anaphase chromosomes were surrounded by increased numbers of disorganised spindle MTs. Along with these, the chromosomes often associated with ectopic MT bundles that could be subdivided into two types. The first type of MT bundle ran along the length of the chromatin bridging two or more of the DNA masses together (Fig. 2H, arrow). Some of these bundles could be clearly traced to the centrosomes whereas others could not. MT bundles of the second type emanated from a common location on a chromosome and extended in opposite directions (Fig. 2I, arrowhead). This latter observation suggested that some chromosomes in *klp67A* mutants may become merotelically maloriented, with a single chromosome attached to opposite spindle poles. These data indicate that Klp67A is involved in regulating both astral and spindle MT length and/or number throughout meiosis I. They further reveal that aberrant interactions occur between the chromosomes and the spindle that may result in mis-segregation.

klp67A mutants exhibit congression defects

To determine how loss of *klp67A* affects chromosome dynamics, we performed high-resolution, differential interference contrast (DIC), time-lapse imaging of living, wild-type and mutant primary spermatocytes.

We found that the duration of prometaphase I in Oregon R wild-type cells, defined as the first signs of chromosome movement until anaphase onset was 34±2 minutes (*n*=18, range 22-49 minutes). During this time the chromosomes bioriented and traversed the long axis of the spindle. Concurrent with this longitudinal movement the chromosomes underwent dramatic rotations termed reorientations. These movements are proposed to be part of an error correction mechanism to promote proper kinetochore-spindle attachment and biorientation (Nicklas, 1997). For wild-type cells a stable, bi-oriented configuration was rapidly achieved and in the 17 cells observed, the last re-orientation event, i.e., a chromosome rotation of 90° or more along any axis, occurred on average 14±1 minutes after prometaphase onset. Following its final reorientation, a bivalent congressed to a stable position on the spindle where it remained until anaphase onset (see also Movie 1, http://jcs.biologists.org/supplemental/). In 84% of the cells examined (*n*=19) this stable position or metaphase plate corresponded to the spindle equator.

We performed similar analyses on both of the hemizygous P-element strains. The duration of prometaphase in the *klp67A*^{42705}*/Df(3L)29A6* mutant was 30±4 minutes (*n*=18, range 16-53 minutes). The *klp67A*$^{EP(3)3516}$*/Df(3L)29A6* mutant required 34±3 minutes (*n*=14, range 21-56 minutes) to enter anaphase. This indicated that the duration of prometaphase was not significantly different between mutant and wild-type cells.

Prometaphase chromosome behaviour was highly aberrant in both of the hemizygous mutants. The chromosomes exhibited movement in all cells examined indicating that in the absence of Klp67A, kinetochores were able to capture MTs and attach to the spindle. Chromosomes in the mutants appeared far more dynamic than their wild-type counterparts. As illustrated in Fig. 3 (Movie 2, http://jcs.biologists.org/supplemental/), the chromosomes in mutants continued to reorient for much longer than in control cells. The last chromosome reorientations observed in the *klp67A*^{42705}*/Df(3L)29A6* mutants occurred on average 20±4 minutes (*n*=15) after prometaphase onset. The *klp67A*$^{EP(3)3516}$*/Df(3L)29A6* mutants required even more time and their chromosomes underwent their last rotation on average 29±5 minutes (*n*=11) after the first signs of spindle formation. Thus, in contrast to control cells where the chromosomes spent 59% of prometaphase in a stable configuration, chromosomes in the mutants spent on average only ~24% of prometaphase in a non-reorienting state. Furthermore, some chromosomes twisted or rotated during dyad disjunction at anaphase onset.

Unlike in control cells, chromosomes in the mutants formed poorly defined metaphase plates. The chromosomes congressed and found stable positions at the spindle equator in only 56% of the *klp67A*^{42705}*/Df(3L)29A6* mutant cells (*n*=18) and in 41% of the 17 *klp67A*$^{EP(3)3516}$*/Df(3L)29A6* cells examined. Regardless of the position on the spindle, these metaphase plates tended to be staggered with the chromosomes only loosely aligned. The chromosomes often exhibited one or more motions before anaphase onset, behaviour rarely seen in wild-type cells. We conclude that chromosome movements are more dynamic during prometaphase in *klp67A* mutants.

Chromosomes in *klp67A* mutants segregate with greatly diminished velocities

The spindle aberrations observed by immunofluorescence, and the presence of aneuploid spermatids caused us to question what effect loss of *klp67A* had on chromosome segregation. We exploited the fact that *Drosophila* primary spermatocytes have a weak spindle assembly checkpoint (Rebollo and Gonzalez, 2000; Savoian et al., 2000) and were therefore able to perform kinetic analyses of anaphase chromosomes irrespective of any prometaphase abnormalities. In wild-type cells the two dyads comprising each bivalent sometimes behaved differently after entering anaphase. In the example shown in Fig. 4A, one dyad began to travel poleward immediately after disjoining whereas its homologue exhibited a transient, non-motile lag phase. After poleward motion initiated, wild-type dyads consistently moved in a smooth and continuous manner until reaching the

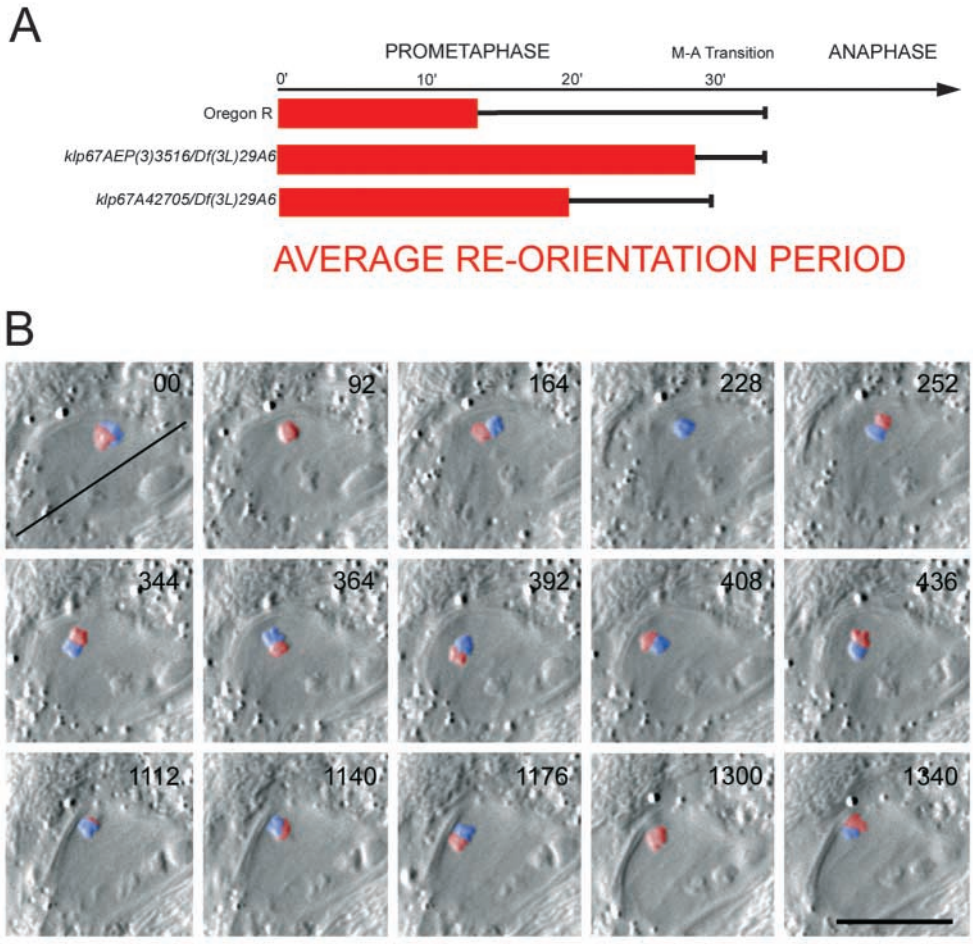

Fig. 3. Chromosomes in *klp67A* mutant primary spermatocytes are abnormally dynamic and undergo exaggerated periods of reorientation during prometaphase. (A) Time-line comparing karyokinetic events in wild-type Oregon R primary spermatocytes and in the two independent P-element-mediated *klp67A* mutant strains examined. The time is in minutes relative to prometaphase onset. Red bars indicate the reorientation period and anaphase onset is indicated by the blunted ends of each line. Although the mutants enter anaphase with similar timing to the controls, their chromosomes reorient for substantially longer. (B) Selected images from a time-lapse sequence during prometaphase in a *klp67A* mutant. The black line (00 timepoint) indicates the long axis of the cell. The two homologous dyads comprising the bivalent of interest are pseudo-coloured red and blue. This chromosome continues to reorient for over 22 minutes before moving out of the plane of focus. During this time it makes a few poleward ingressions but generally remains near the spindle equator. Time in upper right of each panel is in seconds relative to an arbitrary start time. Bar, 10 μm.

poles (Fig. 4B). The chromosomes then began to decondense and reform karyomeres. The average maximal velocity of wild-type anaphase dyads was 3.7±0.2 μm/minute (n=14, range 2.8-4.8 μm/minute).

The distorted morphology of the spindles in *klp67A* mutants made it difficult to monitor the behaviour of both homologous dyads continuously throughout division. In those cases where both dyads could be followed, the pre-anaphase bivalents could be distorted or abnormally resolved, but the homologues remained attached to one another (compare Fig. 4A to 4A′). We did not observe any failures in dyad disjunction at anaphase onset. However, once disjoined, anaphase chromosome movement did not occur in a continuous manner as was observed in the wild type. Dyads could initiate poleward travel and then pause, after which time they might re-initiate movement or in more extreme cases halt and reform karyomeres irrespective of their final spindle position (Fig. 4A′,B′, red dots; see also Movie 3, http://jcs.biologists.org/supplemental/). Poleward chromosome velocity was greatly attenuated in *klp67A* mutants. The average maximal anaphase velocities of the 10 dyads analysed from the $klp67A^{42705}/Df(3L)29A6$ and the 14 dyads in the $klp67A^{EP(3)3516}/Df(3L)29A6$ mutants were 1.5±0.3 μm/minute (range 0.3-3.5 μm/minute) and 1.0±0.3 μm/minute (range 0.3-1.8 μm/minute), respectively. These velocities were on average, approximately 34% of that seen in wild-type cells (3.7±0.2 μm/minute). Furthermore, in a few cells it appeared as though the disjoined dyads did not undergo any significant anaphase A movement. In these cases dyad separation was minimal and resulted in a single restitution nucleus. In the absence of *klp67A*, nuclear reformation occurred at non-polar locations in 72% (n=18) of the $klp67A^{42705}/Df(3L)29A6$ and 89% (n=18) of the $klp67A^{EP(3)3516}/Df(3L)29A6$ cells examined. Non-polar chromosome decondensation initiated in only 5% of the 18 wild-type cells analysed. We therefore conclude that the aneuploidy observed in *klp67A* mutants occurs at least in part as a result of diminished segregation velocities.

The observation that some dyads failed to segregate or initiate movement and then abruptly halted in combination with the immunofluorescence data led us to suspect that in some cases kinetochores were becoming improperly attached to the spindle. The cell shown in Fig. 5 (Movie 4, http://jcs.biologists.org/supplemental/) is an example in which the homologous dyads had resolved into their component chromatids but remained attached to one another at their centromeres. At anaphase onset one of the dyads (red chromatids) travelled towards its predicted, proximal spindle pole. The other dyad was torn apart when one of the chromatids lagged behind its sister before ultimately changing direction and moving to the incorrect pole (blue chromatids; white arrowheads). This observation is in agreement with our immunofluorescence findings (Fig. 2), and indicated that kinetochores may become mis-attached to the spindle in the absence of *klp67A*.

Klp67A associates with pre-anaphase kinetochores and the central spindle

As our data implicated Klp67A in both karyokinesis and cytokinesis, we wished to determine the localisation of the protein throughout meiosis I. To this end we generated and affinity purified a polyclonal antibody raised against the C-terminal, non-motor containing portion of the Klp67A protein and stained wild-type cells (Fig. 6, red channels). In interphase and prophase cells, Klp67A appeared completely confined within the nucleus (Fig. 6A). As the nuclear envelope became

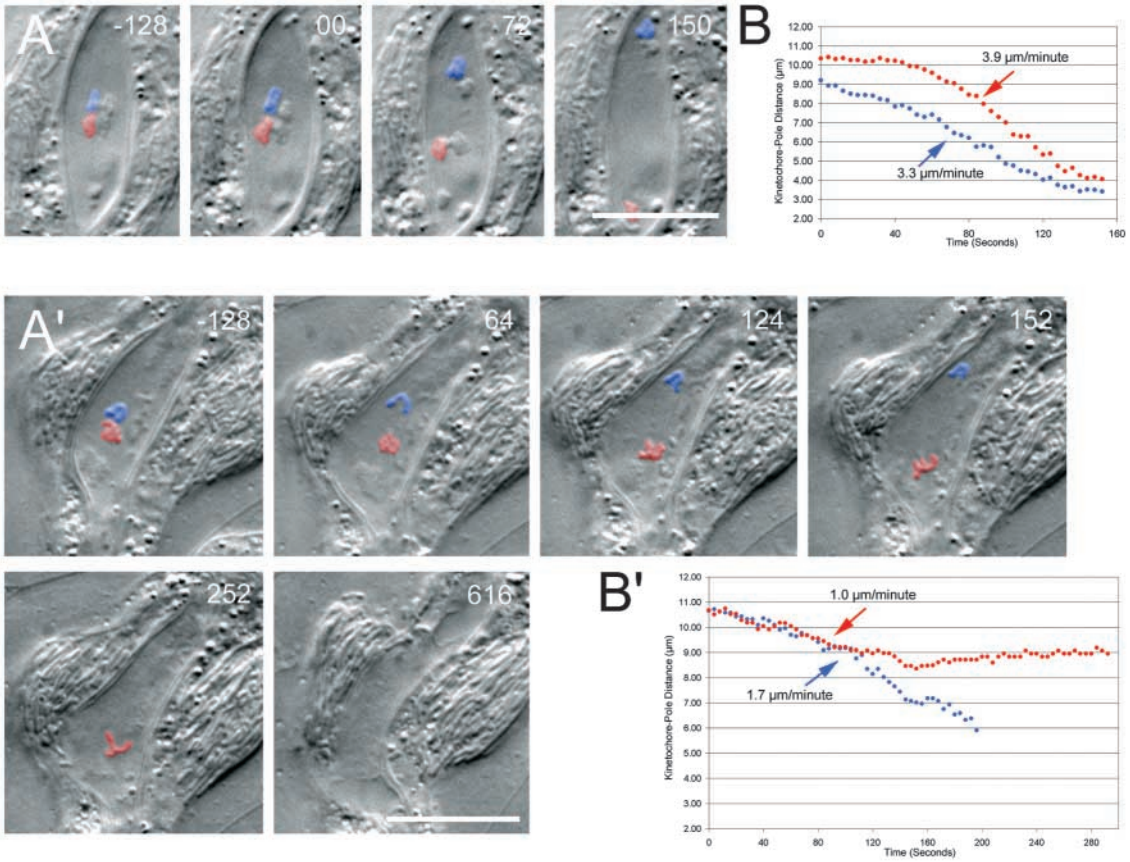

Fig. 4. Dyads in *klp67A* mutants segregate at greatly reduced velocities. (A) Selected images from a time-lapse sequence showing a wild-type cell during anaphase. The homologous dyads have been pseudo-coloured and their anaphase movements plotted (B). The average maximal velocity for each of the dyads of interest is indicated. Once wild-type dyads initiate poleward motion it continues uninterrupted until they reach their poles. (A′) Selected frames from a time-lapse sequence during anaphase in a *klp67A* mutant cell. The chromosomes have been coloured as above. (A′) Prior to anaphase onset the dyads appear 'twisted' relative to one another (–128 second time point). Following disjunction, the dyads in this cell separate and initiate movement to their respective poles. One dyad (blue) travels out of the plane of focus at ~200 seconds. The red dyad begins its poleward migration and then abruptly halts at ~152 seconds and remains stationary relative to the spindle pole, and exhibits no further poleward movement before decondensing. (B′) Kinetic plot and average maximal velocities for the dyads shown in (A′). Time in seconds relative to anaphase onset is given in the right-hand corner. Bars, 10 μm.

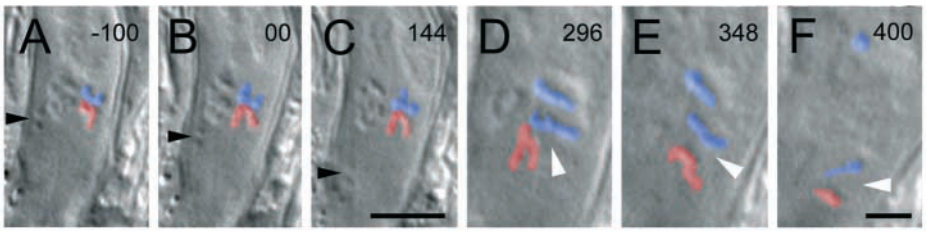

Fig. 5. *klp67A* mutants form unstable metaphase plates and have merotelically maloriented chromosomes. (A-F) Selected frames from a time-lapse sequence showing metaphase (A) and anaphase (B-F) in a *klp67A* mutant primary spermatocyte. Prior to anaphase onset, chromosome IV (black arrowhead) travels away from the metaphase plate before moving out of the plane of focus near the lower pole. The adjacent bivalents are abnormally resolved and their individual chromatids are visible. As anaphase ensues, one bivalent (pseudo-coloured) disjoins. Both (red) chromatids of one dyad remain associated and travel to the lower, proximal pole as predicted. The homologous (blue) dyad separates into its two component chromatids, with one chromatid moving towards the expected upper pole and out of focus (D-F), whereas its sister chromatid (white arrowhead) begins to move towards the upper pole (D) then halts before rapidly changing direction and travelling to the lower, incorrect pole (E,F), resulting in aneuploidy. Time is in seconds relative to anaphase onset. Bar in C, 10 μm; F, 2 μm.

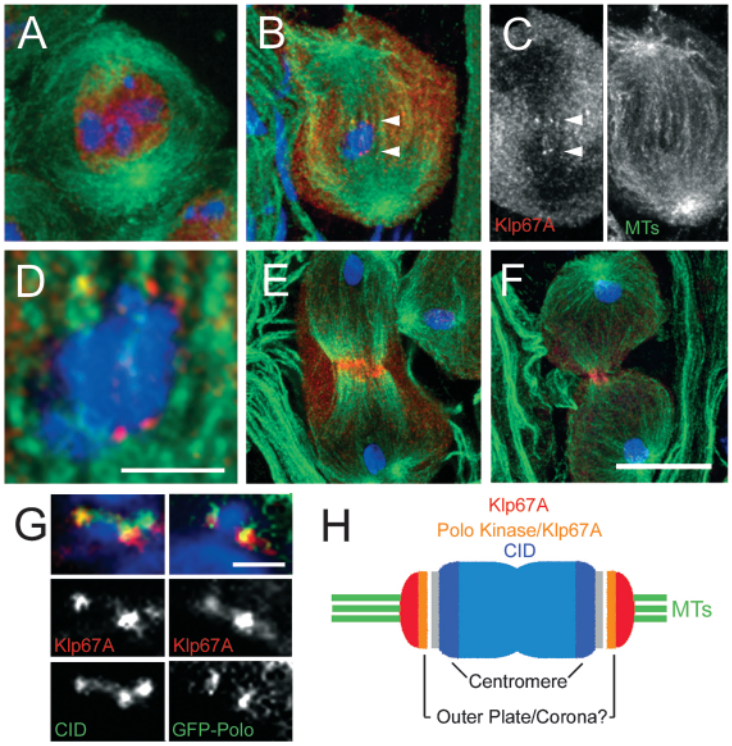

Fig. 6. Klp67A associates with pre-anaphase kinetochores and the central spindle during meiosis I. (A,B and D-F) Microtubules are shown in green, Klp67A in red and DNA in blue. During interphase and prophase (A) Klp67A localises exclusively to the nucleus where it remains until prometaphase. At this time, the protein redistributes to the cytoplasm, but is excluded from regions occupied by spindle MTs (B,C). Klp67A is also detected as pairs of opposing spots on the chromosomes (B,C, arrowheads), that interact with presumptive kinetochore fibres (D). Co-localisation studies (G) using anti-CID antibodies to label centromeres or GFP-Polo-tagged outer kinetochore plates, suggest that Klp67A is a new component of pre-anaphase kinetochores (H). Following anaphase onset Klp67A redistributes to an equatorial band on the central spindle (E), where it remains during cytokinesis. During this time Klp67A is still detected in the cytoplasmic pool, and as telophase initiates, in the reforming nuclei (F). Bar in D, 2 μm; F, 10 μm; G, 1 μm.

fenestrated at prometaphase, the protein was released into the cytoplasm where it was conspicuously absent from the regions occupied by the spindle (Fig. 6B,C). Concomitant with this, Klp67A-positive punctae became visible on the chromosomes (Fig. 6B,C, arrowheads). These spots corresponded to the contact sites between the chromosomes and spindle MTs, raising the possibility that they were centromeres or kinetochores. To test this hypothesis we examined the distribution of Klp67A relative to the centromere protein CID. As shown in Fig. 6G, Klp67A was found external to the centromeres consistent with belonging to the kinetochore. To confirm this, we then looked at the distribution of Klp67A in flies expressing a GFP-tagged form of the outer kinetochore plate protein Polo kinase (Moutinho-Santos et al., 1999; Blower and Karpen, 2001). Interestingly, Klp67A not only co-localised with GFP-Polo, but also extended beyond it (Fig. 6G). This suggests that Klp67A is a kinetochore component that associates with the outer plate and possibly the corona (Fig. 6H). Upon the transition from metaphase to anaphase, the protein was no longer detected at kinetochores but was now manifest as a band located midway along the newly forming central spindle, where it remained as cleavage progressed (Fig. 6E,F). During these later stages Klp67A remained in the cytoplasm in non-microtubule containing regions and also began to accumulate in the reforming nuclei. Together, these observations revealed that Klp67A is a new component of pre-anaphase kinetochores and the central spindle, a localisation pattern consistent with roles in chromosome congression and cytokinesis.

Discussion

The spindle is a dynamic MT machine that is necessary for proper chromosome segregation and the subsequent cleavage of the cell during cytokinesis. In an attempt to define the roles of putative MT catastrophe factors in these processes more clearly, we have examined *Drosophila* primary spermatocytes with a mutation in the Kip3 family member *klp67A*. Consistent with a recent report (Gandhi et al., 2004), we found that loss of *klp67A* leads to aneuploidy and cytokinesis defects. To investigate specifically the cause of the aneuploidy we have examined chromosome behaviour in fixed preparations and by time-lapse microscopy of living cells. Our findings are the first characterisation of karyokinesis in living, meiotic animal cells deficient for a Kip3 family member.

Klp67A regulates spindle morphology

Previous reports have shown that depletion of Kip3 family members leads to MTs with enhanced stability in mitotic budding yeast cells (Cottingham and Hoyt, 1997), in meiotic fission yeast cells (West et al., 2001) and mitotic *Drosophila* cells in culture (Gandhi et al., 2004). In the present study we found that meiotic mutant *klp67A Drosophila* cells formed bipolar spindles that contained long and bent k-fibres with an apparent increase in the number of MTs throughout the cell. In some cases the density of spindle MTs was so great that they appeared to enmesh or otherwise obscure the chromosomes. Likewise, the asters grew to an exaggerated extent and formed robust bundles that could encircle the cell. Similar findings have been made in *klp67A* mutant embryos in which the mitotic spindle assumes an overgrown and elongated, bent shape (Gandhi et al., 2004). Thus, Klp67A is a critical determinant of spindle morphology in both mitosis and meiosis.

Loss of *klp67A* affects chromosome congression

Our time-lapse observations of *klp67A* mutants revealed that

their chromosomes continued to reorient for approximately twice as long as those in wild-type cells. Re-orientations are proposed to occur as kinetochores release and then capture MTs as part of a mechanism to correct erroneous attachments. Micromanipulation experiments in grasshopper spermatocytes indicate that tension placed across the chromosome halts re-orientations. Under wild-type conditions tension should be maximal when a chromosome is amphitelically oriented, i.e., with each kinetochore attached to an opposing spindle pole (Nicklas, 1997). The simplest explanation for the increased reorientations in the mutants is that the chromosomes are not under tension. We were unable to measure the inter-centromere distance between the homologues of bioriented chromosomes to address this directly. However, the k-fibres in mutants were abnormally long and bent, an observation that strongly suggests that the chromosomes are not stretched towards opposing spindle poles. In the metaphase spindles of vertebrate somatic cells (Mitchison et al., 1986; Mitchison and Salmon, 1992) and *Drosophila* embryos (Brust-Mascher and Scholey, 2002), the k-fibre reaches a steady state length as tubulin subunits are added at the kinetochore while being continually lost near the spindle pole by a process termed 'flux'. Our finding that Klp67A localises to kinetochores but is not detectable at spindle poles suggests that these elongated k-fibres result from changes in the dynamics at the kinetochore-proximal, MT plus ends.

Alternatively, the chromosomes may continue to reorient because the kinetochores are transiently losing and then regaining their attachment to the spindle. The fission yeast Kip3 members Klp5/6 have been implicated in making and maintaining stable spindle attachments (Garcia et al., 2002b; West et al., 2002). If the kinetochores in *klp67A* mutants were impaired in attaching to the spindle, the chromosomes should exhibit no motion at prometaphase onset, or attach to move towards the spindle poles and then remain there for prolonged periods of time. We did not observe this. Consistently, the chromosomes moved along the spindle before forming poorly organised metaphase plates. Although these metaphase plates tended to be non-equatorially positioned, they were not directly adjacent to the poles. This observation argues against the *klp67A* mutation hindering spindle attachment. However, the presence of unstable metaphase plates can be interpreted as resulting from the loss and rapid regaining of attachment to the spindle. A more provocative explanation for the moving of chromosomes off the metaphase plates, and their staggered organisation is that loss of *klp67A* leads to an imbalance of forces on homologous kinetochores, possibly by altering the numbers of MTs that constitute the opposing k-fibres (e.g. Hays and Salmon, 1990).

Aberrant chromosome segregation in *klp67A* mutants

Our live cell analyses further revealed that the aneuploidy observed in *klp67A* mutant onion-stage spermatids resulted from motility defects during anaphase. Lagging chromosomes have also been reported in fission yeast mutant for the Kip3 members *klp5/6* (Garcia et al., 2002a; West et al., 2002). We found that dyads in wild-type cells disjoined and then either immediately, or after a short pause, segregated and then decondensed at the poles. Although the dyads always disjoined in mutant cells, karyomeres often formed at non-polar locations. Our kinetic analyses revealed that chromosomes in the mutants travelled at approximately one third of the rate seen in wild-type cells. In addition to this reduced velocity, the chromosomes in the mutants could start moving and then abruptly halt (see Fig. 4A′,B′).

The cause of these aberrant motions is uncertain. In wild-type prometaphase I cells, the chromosomes rapidly travel poleward as the kinetochores first associate with the spindle by a dynein-dependent mechanism (Savoian et al., 2000). Our observations of such 'fast' chromosome movement during spindle attachment in mutants (unpublished observations) indicate that the diminished segregation velocities are not caused by an absence of dynein, which is also involved in anaphase chromosome segregation (Savoian et al., 2000; Sharp et al., 2000). We envisage three, non-mutually exclusive mechanisms to explain the reduced velocities and aberrant segregation behaviour seen in *klp67A* mutants. First, the chromosomes may encounter stearic hindrance. This could occur if for example, the chromosomes were to pass through an area of the spindle that was enriched with ectopic MTs as suggested by the immunofluorescence data. Second, segregation could become hindered if some kinetochores were improperly attached to the spindle, possibly becoming merotelically maloriented (e.g. Fig. 2I, arrowhead). This type of mis-attachment is invisible to the spindle assembly checkpoint and would not trigger a metaphase arrest and prolong prometaphase (Cimini et al., 2001; Cimini et al., 2002). We did note several instances where chromosomes underwent an apparent reorientation during disjunction. Such rotations can only occur if the kinetochore has an attachment to a distal spindle pole or ectopic MT bundle. In the example shown in Fig. 5, no rotation was observed, but the sister chromatids comprising one of the dyads are clearly associated with opposing sources of MT bundles. Furthermore, errors in attachment may occur after anaphase entry as anaphase kinetochores are still able to capture MTs and attach to the spindle (Gorbsky et al., 1998). Under this model, segregation could initiate normally but then become aberrant as a kinetochore encounters and then captures adjacent, ectopic MTs. Finally, in the absence of *klp67A*, the k-fibre itself could become hyper-stable or fail to undergo some other critical anaphase event and so limit the rate of chromosome movement. Our immunolocalisation experiments indicated that the bulk of Klp67A redistributes from kinetochores to the central spindle at anaphase, an observation that suggests that Klp67A may not itself have a direct role in dyad segregation. However, we cannot rule out the possibility that some Klp67A remains on anaphase kinetochores and contributes to anaphase function, but which is undetectable using our current techniques. Regardless of the mechanisms involved, our data clearly reveal that *Drosophila* Klp67A is involved in chromosome congression, accurate chromosome segregation and is a key regulator of spindle morphology throughout meiosis I.

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