Antagonistic activities of Klp10A and Orbit regulate spindle length, bipolarity and function in vivo

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Summary

The metaphase-spindle steady-state length occurs as spindle microtubules ‘flux’, incorporating new subunits at their plus-ends, while simultaneously losing subunits from their minus ends. Orbit/Mast/CLASP is required for tubulin subunit addition at kinetochores, and several kinesins regulate spindle morphology and/or flux by serving as microtubule depolymerases. Here, we use RNA interference in S2 cells to examine the relationship between Orbit and the four predicted kinesin-type depolymerases encoded by the Drosophila genome (Klp10A, Klp59C, Klp59D and Klp67A). Single depletion of Orbit results in monopolar spindles, mitotic arrest and a subsequent increase in apoptotic cells. These phenotypes are rescued by co-depleting Klp10A but none of the other three depolymerases. Spindle bipolarity is restored by preventing the spindle collapse seen in cells that lack Orbit, leading to functional spindles that are similar to controls in shape and length. We conclude that Klp10A exclusively antagonises Orbit in the regulation of bipolar spindle formation and maintenance.

Key words: Kinesin, Mitosis, Microtubule, Catastrophe factor, Flux, CLASP

Introduction

In mitotic Drosophila cells the replicated centrosomes usually separate from one another around the intact prophase nuclear envelope. As this envelope becomes fenestrated at prometaphase onset, dynamic astral microtubules (MTs) nucleated from each of the opposing centrosomes invade the nuclear space where their plus-ends ultimately become attached to the sister kinetochores of each chromosome. Formation of these kinetochore MTs (kMTs) facilitates bipolar spindle formation and chromosome movement. After chromosome congression the spindle reaches a steady-state length, probably through the ‘flux’ of kMTs, whereby the incorporation of new tubulin subunits at the kinetochore is balanced by a simultaneous and equal loss at the spindle poles (Rogers et al., 2005). This state is maintained until the spindle-assembly checkpoint is satisfied and the cell enters into anaphase.

Drosophila Orbit/Mast (CLASP in vertebrates, hereafter referred to as Orbit) is a highly conserved microtubule-associated protein that stabilises microtubules (Inoue et al., 2000; Lemos et al., 2000; Akhmanova et al., 2001; Maiato et al., 2002; Maiato et al., 2003; Maiato et al., 2005). Mutations in orbit or RNA interference (RNAi) knockdown do not affect prophase centrosome separation but cause spindle collapse at flux-like velocities only after kMT formation during prometaphase (Maiato et al., 2002; Maiato et al., 2005). The kinetochores in such cells fail to remain associated with MT plus-ends and the chromosomes become buried in the centre of a monoaster causing a mitotic arrest (Maiato et al., 2002). The recent finding that Orbit is required for the incorporation of tubulin subunits at the kinetochore for MT elongation and flux (Maiato et al., 2005) might therefore offer an explanation for the spindle collapse seen in Orbit-deficient cells.

The Drosophila genome encodes two families of putative kinesin-like protein (Klp) microtubule depolymerises, kinesin-8 and kinesin-13 (Lawrence et al., 2004), and members of each family have been implicated in regulating spindle morphology and/or flux. For example, depletion of Klp67A (kinesin-8) prevents proper centrosome separation and results in abnormally long, bipolar – yet monoastral – spindles (Goshima and Vale, 2003; Gandhi et al., 2004). The kinesin-13 family includes Klp10A, Klp59C and Klp59D. Both Klp10A and Klp59C have MT depolymerising activity in vitro and are required for proper chromosome movement in embryos. Klp10A localises transiently to centromeres but is spindle-pole- and/or centrosome-associated throughout mitosis where it is proposed to drive MT flux. The loss of Klp10A function causes a spindle collapse phenotype similar to that reported in Orbit-lacking cells. However, cells that lack functional Klp10A ultimately form bipolar spindles and enter anaphase (Goshima and Vale, 2003; Rogers et al., 2004).

Here, we investigate the interplay between Orbit and each of the four Drosophila Klp-type microtubule depolymerases using RNAi in S2 cells. We find that knockdown of Orbit alone results in monopolar spindles, mitotic arrest and subsequent apoptosis. Co-depletion with Klp10A was exclusively able to rescue spindle bipolarity allowing anaphase onset and cell-cycle progression, thereby diminishing the degree of apoptosis. We conclude that Orbit and Klp10A are an antagonistic MT-stabilising and -depolymerising pair that regulates spindle formation and morphology prior to entry into anaphase.
Results
Knockdown of Klp10A but not of the other kinesin-type depolymerases moderates the amount of apoptosis in Orbit-deficient cells
To determine which, if any, of the kinesin-8 and/or kinesin-13 family members might counteract the activity of Orbit, we performed double RNAi depletion experiments for orbit combined with each of the four identified Drosophila Klp depolymerases in S2 cells. The effects of downregulation were initially assessed by flow cytometry 72 hours after treatment (Fig. 1). Depletion of Orbit alone (Fig. 1A) resulted in a significant increase in apoptotic cell debris as well as the proportion of 4N (G2-M) cells relative to 2N (G1), as expected from a mitotic delay followed by cell death. Simultaneous RNAi for orbit with either Klp59C, Klp59D or Klp67A showed no rescue of the proportions of cells of different ploidies in comparison to orbit knockdown alone, and the dual depletion with Klp67A dramatically increased the amount of cell death (supplementary material Fig. S1). By contrast, the single knockdown of Klp10A did not increase the number of apoptotic cells (Fig. 1B). Following double orbit and Klp10A RNAi (Fig. 1C), the proportion of 4N cells remained largely unchanged compared with the Orbit single downregulation, whereas that of 2N cells increased. This was associated with a marked diminution of apoptotic cells. Quantification of cells stained with Trypan Blue revealed that knockdown of orbit caused an approximately threefold increase in the number of apoptotic cells relative to controls (26.7% vs 8.7%). Co-depletion of Orbit and Klp10A reduced the proportion of dead cells to 14%. Together, these data suggest that the apoptosis associated with orbit RNAi can be selectively rescued by also depleting Klp10A but not other kinesin-type MT depolymerases.

Bipolar and bi-centrosomal spindles form in cells depleted simultaneously but not individually of Orbit and Klp10A
Previous independent observations have revealed that Orbit and Klp10A regulate spindle microtubule dynamics. We therefore reasoned that the partial rescue seen in the FACS profiles of double depleted cells probably occurred during mitosis and examined spindle morphology in fixed cell populations. In cells treated with orbit double-stranded RNA (dsRNA) alone, the mitotic index rose from 4.4% to 14.5%. Almost all of the spindles in these cells were abnormal (93.4%; n=137). Of these, 69% were monopolar with both centrosomes (as revealed by γ-tubulin staining) contacting one another at the centre of a large monoastral (Fig. 2B). Many of the remaining abnormal spindles were bipolar with a centrosome and aster capping each end, but were consistently shorter (6.46±0.13 μm; range 4.35-9.78 μm; n=100) than equally staged controls (7.60±0.16 μm; range, 4.93-10.78 μm; n=100; Fig. 2B,E).

Downregulation of Klp10A often led to monoastral but bipolar spindles upon which the majority of the chromosomes were equatorially positioned (Fig. 3C,C’). These spindles tended to be abnormally dense and significantly longer than their control counterparts (average length 11.14±0.22 μm; range 7.04-15.81 μm; n=100; compare Fig. 2A,A’ with C,C’). The mitotic index of Klp10A RNAi treated cells rose to 7.0%. This slight increase together with the detection of anaphase cells (data not shown) suggests that Klp10A depletion delays but does not prevent anaphase onset. DOUBLE RNAi of orbit and Klp10A resulted in a mitotic index of 7.2%, a twofold decrease compared with orbit knockdown alone. Likewise, this decreased the number of abnormal mitotic cells from the 93.4% seen after orbit RNAi to 55.1% and the proportion of bipolar spindles rose from 31% to 62%. The average pole to pole distance of spindles in the double-RNAi-treated cells was 7.59±0.18 μm (range 5.40-12.03 μm; n=100), a value intermediate between the length of bipolar spindles following the individual knockdown experiments and one
Spindle bipolarity and mitotic progression are rescued in double-depleted cells by preventing prometaphase spindle collapse

Single depletions of either Orbit or Klp10A have been shown to cause spindle collapse shortly after prometaphase onset leading, at least transiently, to the formation of monopolar spindles. This raises the possibility that the bipolar spindles seen in double-RNAi cells could arise either through the prevention of the collapse or through a re-separation of the centrosomes after it. To differentiate between these mechanisms, GFP-α-tubulin-expressing S2 cells were followed by time-lapse imaging after downregulating Orbit and Klp10A individually or together.

As represented in Fig. 3A, the centrosomes in control cells were independent during prophase and either continued to separate or maintained relative separation during spindle formation (0-400 seconds; see also Fig. 4C and supplementary material Movie 1). Following chromosome alignment, as revealed by a non-fluorescent equatorial ‘shadow’, the spindle shortened slightly and assumed a length of about 7 μm (1480 seconds). Approximately 15 minutes later this cell entered anaphase (2180 seconds). The duration of prometaphase in control cells, i.e. the time from the initiation of spindle formation to anaphase onset, was 30±4±2.7 minutes (n=19).

In all of the orbit-RNAi-treated cells filmed (n=13), the centrosomes were separated from one another during promphase. In 85% (11 of 13) of these cells the centrosomes began to collapse towards one another to form a monoaster after a nascent bipolar spindle was detected (Fig. 3B; 0-900 seconds, Fig. 4C, supplementary material Movie 2). In several cells transient multipolar spindles formed as bundles of MTs collapse towards one another to form a monoaster after a nascent bipolar spindle was detected (Fig. 3B; 0-900 seconds). None of these 11 cells entered into anaphase during the 200-minute filming period. Furthermore the centrosomes never separated from one another after their initial collapse. In a few (two of 13) instances short, bipolar spindles formed resembling those seen in our fixed-cell studies. Despite the reduced separation between the centrosomes, these cells underwent anaphase and exited mitosis.

We found that the centrosomes also collapsed together during prometaphase to form transient monopolar spindles in 50% of the cells treated with Klp10A dsRNA (n=20) (Fig. 3C;
Klp10A antagonises Orbit in spindle formation

In the cell shown in Fig. 3C, the single monoaster then extended to form a centrosome-free spindle pole distal to the first and generated a monoastral bipolar spindle (Fig. 3C; 1400, 1700s). Such monoastral bipolar spindles required 39.2±3.6 minutes (n=10) to advance from prometaphase into anaphase. It is noteworthy that when Klp10A-depleted cells entered anaphase and cytokinesis they formed atrophied and abnormally long central spindles and intracellular bridges (data not shown).

As illustrated in Fig. 4, in contrast to the spindle collapse we observed in the cells following orbit or Klp10A RNAi alone, in most cells depleted of both proteins the centrosomes remained separated beyond prophase and formed a stable bipolar spindle. We found that 14 of 20 cells maintained or increased their centrosome separation distance after prometaphase onset and formed bipolar and bi-astral spindles. Despite forming bipolar spindles with kinetics similar to controls, double-knockdown cells were often delayed in entering anaphase. Unlike control cells, in which prometaphase lasted about 30 minutes, only six of 14 double-RNAi-treated cells entered anaphase within 45 minutes of spindle formation (Fig. 4A, supplementary material Movie 3) and the remaining eight cells did not enter into anaphase within 65 minutes. Because our FACS data indicated that orbit and Klp10A double-RNAi cells reduced the frequency of apoptosis seen following orbit knockdown alone, we reasoned that although they undergo a prolonged prometaphase, these double-depleted cells were not permanently arrested. We therefore repeated our time-lapse experiments and followed 14 cells for up to 200 minutes, an interval 6.5 times greater than that required for control cells to enter anaphase. Of these 14 cells filmed, three failed to form bipolar spindles and did not advance into anaphase during the course of filming. Of the remaining 11 cells that formed bipolar spindles, two entered anaphase within about 30 minutes of prometaphase onset. Another three cells progressed into anaphase within 45 minutes, whereas the remaining six cells required between 74 and 147 minutes for anaphase onset (Fig. 4B, supplementary material Movie 3). These live cell studies indicate that the simultaneous RNAi of Klp10A and orbit prevents the centrosome-collapse phenotype observed in single knockdown cells. The resulting bipolar spindles are functional enough to promote chromosome congression and anaphase onset, although the timing of this latter event can be substantially delayed.

Fig. 3. Spindle formation in living S2 cells expressing GFP-tubulin after orbit or Klp10A RNAi. Selected frames from time-lapse sequences showing spindle formation and mitotic progression in (A) control, (B) orbit RNAi- and (C) Klp10A RNAi-treated cells. (A) In control cells the two separated centrosomes oppose one another on the nuclear envelope during prophase. At prometaphase onset (0 s) the nuclear envelope becomes fenestrated and astral MTs interact with the kinetochores to form a bipolar spindle (100) that becomes more robust as the chromosomes congress to the equatorially positioned metaphase plate (400), the fluorescence ‘shadow’ at the equator indicates the presence of the chromosomes. The spindle in this cell slightly shortens at metaphase (1480) after assuming a steady state length which it maintains until anaphase onset (2180) (see also supplementary material Movie 1). (B) orbit RNAi does not prevent prophase centrosome separation. As the chromosomes become bi-oriented during prometaphase, the nascent bipolar spindle collapses upon itself (0-630 seconds) to form a monopolar spindle (900). In this cell, transient multi-poles (1260-1770 seconds; arrowheads) form that are probably generated by individual or small clusters of chromosomes as evidenced by the shadow at their equators (see text for details). (C) Knockdown of Klp10A does not perturb the initial separation of the centrosomes at prophase but, as illustrated here, causes their subsequent collapse during prometaphase in 50% of the cells followed by time-lapse microscopy (0-520 s). Unlike Orbit-depleted cells, those lacking Klp10A are able to form stable bipolar spindles (1400-1700 seconds) that are monoastral. These spindles are fully functional and cells can enter into anaphase (3220). Time (in brackets) is in seconds relative to prometaphase onset. Bars, 10 μm (see also supplementary material Movie 2).
The delay in anaphase onset seen in orbit and Klp10A double-RNAi cells correlates with decreased centromeric tension and BubR1 retention

Our fixed and live cell observations indicated that the chromosomes in orbit and Klp10A double-downregulated cells reached an equatorial position with the same frequency as control cells. Since chromosome congression is a hallmark of attachment, we investigated whether the kinetochores are under tension, the absence of which could account for the delayed anaphase entry (Taylor et al., 2004; Tan et al., 2005). We therefore measured the distance between sister centromeres as revealed by CID (centromere identifier), the Drosophila equivalent of modified histone CENP-A (Blower and Karpen, 2001) (Fig. 5A,B). In control prophase cells the average intra-centromeric distance was 0.63±0.01 μm (n=35). Since this period is prior to spindle attachment, this is the relaxed or resting separation distance. After bi-orientation and metaphase alignment the separation was 0.96±0.02 μm (n=81), which we define as the tensed state.

We carried out similar measurements following RNAi against orbit and Klp10A either individually or simultaneously (Fig. 5A,B). In Orbit-depleted cells that formed monopolar spindles the average intra-centromeric distance was 0.72±0.01 μm (n=92), consistent with a lack of bi-orientation. This value increased to 0.94±0.04 μm (n=43) when the chromosomes attached to either transient multipolar or short bipolar spindles. The average centromeric separation distance after Klp10A RNAi varied according to spindle morphology and for monoastral bipolar spindles was 0.87±0.03 μm (n=70). Surprisingly, this distance rose by 73% to 1.20±0.03 μm (n=93) when the chromosomes had congressed upon bipolar spindles that had an aster at each end. A similar, but lesser, trend was observed in cells that were RNAi-treated for both orbit and Klp10A. The average intra-centromeric distance of chromosomes on bipolar, monoastral spindles in these cells was 0.80±0.02 μm (n=50), whereas those on bipolar spindles capped at each pole by a centrosome and aster was 0.86±0.01 μm (n=110). These average values are less than tensed controls and, moreover, the double-knockdown population had a distribution of distances with a greater proportion of the centromeres under less tension than that observed in the controls (Fig. 5B).

We next examined the localisation of the tension-sensitive BubR1 checkpoint protein (Logarinho et al., 2004; Howell et al., 2004) in control and knockdown cells. As shown in Fig. 5C, in control cells BubR1 was detected as a faint signal on the kinetochores of chromosomes that had congressed to the spindle equator. Equatorially positioned chromosomes in Klp10A RNAi cells similarly lacked BubR1, whereas intensely staining punctae could be detected within the same cell on chromosomes adjacent to the spindle poles, which are presumably mono-oriented and not under tension. BubR1 was seen as multiple spots on the chromosomes buried in the monoaasters of Orbit-lacking cells, but like with control metaphase cells, this signal intensity was reduced for equatorially positioned chromosomes on bi- or multi-polar spindles. As illustrated in Fig. 5C, we found that BubR1 staining intensity appeared higher on the kinetochores of congressed chromosomes of orbit and Klp10A double-knockdown cells than in controls and that this signal intensity could vary between adjacent chromosomes. From these data we conclude that the bipolar spindles in cells lacking Orbit and Klp10A interact with the kinetochores to generate tension. The amount of tension is less than that observed in the controls correlating with the retention of the BubR1 checkpoint protein on kinetochores and delayed anaphase entry.
Discussion

Bipolar spindle formation and maintenance occur through the actions of multiple motor and microtubule dynamics-altering proteins (Sharp et al., 2000). Here, we examined the interplay between Orbit, a protein needed for tubulin-dimer incorporation into kinetochore MTs, and each of the four microtubule depolymerising kinesins Klp67A, Klp10A, Klp59C and Klp59D. We found that the co-depletion of Klp10A but not of the other KLP MT depolymerases diminished the number of apoptotic cells and prevented the spindle collapse associated with orbit knockdown. In contrast to individual downregulation of Orbit or Klp10A that resulted in abnormally short and/or monopolar or long spindles, respectively, spindles in double-deficient cells were bipolar and of an average length indistinguishable from controls. These spindles promoted chromosome alignment and anaphase entry, indicating that they were functional. Since both Orbit and Klp10A have been directly implicated in microtubule flux (Rogers et al., 2004; Maiato et al., 2005), these data suggest that this process is not required to determine mitotic spindle morphology or chromosome congression in Drosophila tissue culture cells.

During the course of this work, it was reported that microtubule flux is not essential for bipolar spindle formation and chromosome congression in mitotic vertebrate cells (Ganem et al., 2005). In contrast to the knocked down MT-stabilising and -depolymerising protein pair described here, the experiments by Ganem et al. (Ganem et al., 2005) used the co-depletion of two kinesin-13 depolymerases: Kif2A, the orthologue of Klp10A and Kif2C/MCAK/XKCM1, the vertebrate counterpart of Klp59C. Both Kif2A and Kif2C have overlapping localisations at centromeres and spindle poles.
and/or centrosomes (e.g. Kline-Smith and Walczak, 2002; Gaetz and Kapoor, 2004; Ganem and Compton, 2004) and loss of either leads to a prometaphase spindle collapse (Kline-Smith and Walczak, 2002; Ganem and Compton, 2004). This monopolar spindle phenotype was rescued by simultaneously depleting both of these MT depolymerases (Ganem and Compton, 2004), suggesting they form an antagonistic pair. However, an orthologous antagonism of Klp10A and Klp59C does not exist during mitosis in Drosophila, because loss of Klp59C function in tissue culture cells (Goshima and Vale, 2003) or syncitial embryos (Rogers et al., 2004) does not alter spindle morphology, and dual perturbations result in spindle abnormalities identical to that observed following single Klp10A disruptions (Rogers et al., 2004).

We propose that bipolar spindle formation occurs in at least two phases. During the first, MTs nucleated from the separated centrosomes invade the nuclear volume and make their initial interactions with the kinetochores. Time-lapse analyses indicate that this step is not affected by the depletion of either Orbit or Klp10A (Maiato et al., 2002; Maiato et al., 2005; Goshima and Vale, 2003) (this study) and is thus Orbit- and Klp10A-independent. The second phase, spindle stabilisation, occurs after chromosome bi-orientation and probably results from antagonistic pairs of molecules regulating the dynamics of the plus- and minus-ends of kinetochore MTs. It is at this stage that Orbit and Klp10A become engaged as evidenced by the collapse of the nascent spindle following their individual perturbations (e.g. Maiato et al., 2002; Maiato et al., 2005; Goshima and Vale, 2003) (this study). Although we cannot rule out the possibility that the spindle collapse results from a loss of interpool MT integrity, we feel this is unlikely. First, because the rate at which spindles collapse after orbit RNAi is similar to the rate of MT flux in Drosophila tissue culture cells (compare 0.7 μm/minute with ~0.6 μm/minute for each half spindle) (Maiato et al., 2002; Maiato et al., 2005), consistent with a flux depolymerising shortening kMTs in the absence of new tubulin-polymer growth at the kinetochore. Second, because kMTs that form independently from the centrosome and primary spindle axis (i.e. a mini-spindle) also shrink in the absence of Orbit (Maiato et al., 2005). Since these bundles of kMTs are not present between two half spindles it unlikely that their shortening is the result of intervening interpool MTs. Likewise, it has been previously demonstrated that the spindle collapse associated with the loss of Kif2A is kMT-dependent. Spindle bipolarity was restored in these cells by co-depleting the Nut2 kinetochore protein, thereby preventing kMT formation without affecting any other MT MTs (Ganem and Compton, 2004). In the case of Orbit and Klp10A single-depleted cells, we envisage that the spindle collapse is due to an imbalance of regulatory components following the activation of the flux machinery during this second phase. Collapse could result, for example, as Klp10A depolymerises the non-polymerising kMTs that result from orbit downregulation. Conversely, because flux-generated tension has been proposed to promote tubulin subunit incorporation at kinetochores (Maddox et al., 2003), depletion of Klp10A could also affect polymerisation of kMTs, which – in the presence of other active phase two depolymerises – would cause spindle collapse. In the absence of Orbit and Klp10A the flux machinery would not become engaged and spindle length would be determined by other antagonistic molecular pairs.

Our data in flies, in concert with that found in vertebrates, indicate that although microtubule flux is a characteristic of many animal cells it is not essential for pre-anaphase chromosome movements or spindle formation. Nevertheless, the plasticity it imparts is probably advantageous for spindle and kinetochore interactions, for example by promoting kMT polymerisation (Maddox et al., 2003) or by generating tension for satisfying the spindle checkpoint. We found that cells simultaneously depleted of Klp10A and Orbit tended to spend variable but extremely prolonged periods of time in mitosis before entering anaphase. Since both our fixed and live cell studies did not reveal an increase in non-equatorially positioned chromosomes compared with controls, we believe that the prometaphase arrest was not due to activation of the checkpoint through unattached kinetochores. Although never fully relaxed, the centromeres of bi-oriented chromosomes in double-knockdown cells tended to be under diminished tension relative to controls. This corresponded to the retention of BubR1 at kinetochores. Cells depleted only of Klp10A also spent more time in prometaphase than their control counterparts (Goshima and Vale, 2003) (this study), although this duration was substantially less than that observed for orbit and Klp10A double-RNAi cells. Despite decreased intra-centromeric tension in Klp10A downregulated cells, we did not observe BubR1 on the kinetochores of congressed chromosomes. One explanation for this is that, even without flux, spindle MTs can still produce tension by transducing cortical forces. Here, the long spindles that form in the absence of Klp10A would position the asters in direct contact with the cortex where their component MTs would make increased numbers of contacts with cortical motor proteins such as cytoplasmic dynein. Just as cortex-based forces by this motor act along astral MTs for spindle positioning (Dujardin and Vallee, 2002), astral pulling could generate tension across the centromeres and kinetochores of congressed chromosomes. If true, centromeric tension should correlate with the presence or absence of asters. We found this to be the case, and sister centromeres were separated to a greater extent when they were on bi-astral spindles than when attached to bipolar spindles with a single aster (Fig. 5B). Moreover, in those Klp10A-depleted cells displaying bipolar spindles capped at each end by an overgrown aster, the average intra-centromeric distance was greater than that seen in the controls.

Together, our observations indicate that Orbit and Klp10A are an antagonistic molecular pair, consistent with their previous individual implicated roles in MT flux, a process we show here to be dispensable for bipolar spindle formation and chromosome congression. Our data further suggest that astral-mediated pulling forces are involved in checkpoint satisfaction. To our knowledge, the role that these forces may serve in the checkpoint has not been previously reported and thus merits further investigation.

Materials and Methods
Cell culture
Drosophila S2 cells were cultured in Schneider insect medium supplemented with 10% foetal calf serum (FCS) and penicillin-streptomycin. S2 cells expressing GFP-α-tubulin (Goshima and Vale, 2003) were grown in the same medium supplemented with 100 μg/ml hygromycin B.

RNAi-mediated protein depletion
The following double-stranded RNAs (dsRNAs) were used: Orbit (against basepairs 1685-2193, using primers 5’-CCCCGTCTGGCCGAACACCTGGAAACC’-3’ and 5’-
ACGTGCAAGCCCGCACCCTGTAGAGT-3′, corresponding to the MT-binding domain), Klp95C, Klp59D (see Rogers et al., 2004), Klp10A (against the first 880 bp of a Klp10A open reading frame using the primers 5′-ATGGACATGATTACGGTG-3′ and 5′-CATCGATCCTCTTGCGAG-3′, which terminates before the predicted motor domain sequence) and Klp67A (against baspair 1050-1851, using the primers 5′-GAAGACAAGATGTCCTCAAGT-3′ and 5′-GCTGTCGACCAAGCCCCAG-3′, corresponding to a region beyond that predicted to encode the motor domain). For each reaction 20 μg of dsRNA was transfected into S2 tissue culture cells with Transfast (Promega) and observed 72 hours later. For control reactions either dsRNA against GFP (primers 5′-CTTACGGGCCTACCCC-3′ and 5′-TGTCGGGCACAC-3′) or Transfast alone was used and gave similar results.

**FACs analysis, Trypan Blue staining and western blots**

The cell-cycle profiles of S2 cell samples were analysed using a Becton Dickinson FACS Calibur (BD Biosciences, San Jose, CA, USA). S2 cells were plated on coverslips and were sectioned each cell of interest both before and during the course of filming to allow the detection of ectopic centrosomes and removed those cells from the data set. z-sectioning was further necessary to follow MT behaviour as we chose not to artificially flatten our cells with reagents such as concanavalin A, which might influence spindle formation. Cells grown on coverslips were mounted in a POC-R chamber (Zeiss) and maintained in supplemented Schneider insect media at 25°C by using a Tempcontrol 37-2, stage- and objective-lens-heating system (Zeiss). Cells were imaged with a Perkin Elmer Ultraview RSIII spinning disk confocal head mounted on a Zeiss Axiovert200 microscope with a 100× (N.A. 1.4) lens and a 2×2 bin. At 20- to 30-second intervals z-series consisting of no more than seven 1-μm steps were acquired.

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