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Eg5 Restricts Anaphase B Spindle Elongation in Mammalian Cells

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Abstract

During anaphase, overlapping antiparallel microtubules in the spindle interzone elongate and contribute to chromosome segregation. Kinesin-5 family members are required for spindle elongation in some cells, but in other cases they restrict elongation acting like a brake. To determine how kinesin-5 contributes to spindle elongation in mammalian cells, we treated LLC-Pk1 epithelial cells with small molecule inhibitors of the mammalian kinesin-5, Eg5, at anaphase onset and measured the rate and extent of spindle pole separation using multidimensional tracking of centrosomes in cells expressing GFP- γ -tubulin. Centrosome separation was biphasic, with an initial fast phase followed by a slower phase. Treatment with the small molecule inhibitor, STLC, which weakens the interaction of Eg5 with microtubules, resulted in an increase in the rate of centrosome separation. Conversely, treatment with FCPT, which induces a rigor-like interaction of Eg5 with microtubules, reduced the rate of spindle elongation. In control cells, GFP-Eg5 was localized to spindle microtubules and accumulated in the interzone as anaphase progressed. Spindle fluorescence of GFP-Eg5 was decreased following treatment with STLC and increased in cells treated with FCPT. In anaphase cells, cortical dynein increases and rocking motion of spindle poles was detected consistent with the possibility that dynein mediates spindle elongation. In summary, our results demonstrate that Eg5 is not required for spindle elongation, and in fact, restricts the rate of spindle elongation in mammalian cells.

Keywords

Eg5; anaphase; mitosis

Introduction

One of the first steps of spindle assembly is the separation of the duplicated centrosomes. In most cell types, centrosome separation requires the homotetrameric kinesin-5, which crosslinks and slides antiparallel microtubules extending from each centrosome, generating an outward-directed force [Sawin et al., 1992; Kashina et al., 1996; Sharp et al., 1999;

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Kapitein et al., 2005]. Inhibition of Eg5, by either mutation or small molecule inhibitors, attenuates centrosome separation resulting in the formation of a monopolar spindle [Mayer et al., 1999; Ferenz et al., 2010]. Eg5 is detected in the spindle midzone, where antiparallel microtubules are located [Sharp et al., 1999], and is enriched at spindle poles, where microtubules are oriented in a parallel fashion [Sawin et al., 1992; Blangy et al., 1995; Uteng et al., 2008; Gable et al., 2012], suggesting that Eg5 may also contribute to interactions between parallel microtubules [Ma et al., 2010].

During anaphase, kinetochore microtubules that link each sister chromatid to a spindle pole shorten while over-lapping antiparallel microtubules in the spindle interzone lengthen and slide relative to each other to push the spindle poles apart [McIntosh et al., 2002; Roostalu et al., 2010]. In budding yeast, two kinesin-5 family members, Cin8 and Kip1, contribute to spindle elongation, resulting in an ~fivefold increase in spindle length [Saunders et al., 1997; Straight et al., 1998]. Similarly, in drosophila embryos, the kinesin-5, KLP-61, drives microtubule–microtubule sliding to push spindle poles apart. In these cells, spindle elongation is coupled to a suppression of microtubule disassembly at spindle poles [Brust-Mascher and Scholey, 2002; Civelekoglu-Scholey and Scholey, 2010]. In contrast, kinesin-5 is not required for spindle elongation in other cell types. For example, in *C. elegans*, the sole kinesin-5, BMK-1, is nonessential and mutations in BMK-1 result in faster spindle elongation [Saunders et al., 2007] and in *Dictyostelium discoideum*, deletion of kinesin-5 results in faster and more extensive spindle elongation [Tikhonenko et al., 2008]. In both of these cases, cortical dynein pulling on astral microtubules is thought to power spindle elongation, while kinesin-5 acts to restrict sliding.

In mammalian cells, microsurgery and laser ablation of specific subsets of microtubules demonstrate that astral microtubule-dependent pulling contributes to spindle pole separation and that forces that oppose spindle elongation originate from the midzone [Kronebusch and Borisy, 1982; Aist et al., 1993]. These pulling forces are thought to be generated as astral microtubules interact with the minus-end directed motor, dynein, which dynamically associates with the cell cortex by binding to the tripartite complex LGN, Ga α , and NuMA [Du and Macara, 2004; Zheng et al., 2013]. Cortical dynein is negatively regulated by astral microtubules and polo kinase (Plk1) [Kiyomitsu and Cheeseman, 2012] and is enriched during anaphase, consistent with a role for dynein in anaphase spindle elongation [Collins et al., 2012]. Interestingly, recent experiments using cell-free extracts of drosophila embryos showed that astral microtubules and the spindle interzone both contribute to nuclear separation, even in the absence of a cell cortex, suggesting that dynein in the cytoplasm may also contribute to force production [Kimura and Kimura, 2011; Telley et al., 2012].

Surprisingly, the contribution of Eg5 to spindle elongation in mammalian cells is poorly understood, likely due to the fact that inhibition of Eg5 during early mitosis blocks spindle formation, thus precluding analysis of its role during anaphase. To overcome this limitation and examine the contribution of Eg5 to spindle elongation, we added small molecule inhibitors of Eg5 to cells at anaphase onset and subsequently measured spindle elongation. Our results show that Eg5 is not required to drive spindle elongation, but rather restricts the rate of pole–pole separation.

Results and Discussion

Centrosome Tracking Reveals Two Phases of Spindle Elongation in LLC-Pk1 Cells

To investigate spindle elongation, we used LLC-Pk1 epithelial cells that remain flat during mitosis. Time-lapse imaging of cells expressing GFP- α -tubulin showed that following anaphase onset, kinetochore fibers shorten, bundles of microtubules form in the midzone region between the two sets of segregating chromosomes, and astral microtubules elongate to the cell cortex (Fig. 1A) [Rusan and Wadsworth, 2005]. In these cells, movement of chromosomes toward spindle poles (anaphase A) and spindle elongation (anaphase B) occur simultaneously. To quantify spindle elongation, centrosome position over time was measured using multidimensional tracking in cells expressing GFP- γ -tubulin [Danowski et al., 2001; Magidson et al., 2011]. Initial experiments were performed using manual two-dimensional (2D) tracking of centrosome position (X , Y versus time); in additional experiments, centrosome position was measured using an automated three-dimensional (3D) tracking algorithm (X , Y , Z versus time; see Methods).

Centrosome tracking during anaphase in untreated LLC-Pk1 cells, using either 2D or 3D tracking, showed that centrosome separation occurs in two distinct phases, an initial rapid phase, followed by a slower phase (Fig. 1B, 2D tracking; Fig. 1C, 3D tracking). To determine the rate of centrosome separation, we used images collected at 30 s intervals so that pole separation was completed without photo-bleaching of the γ -tubulin fluorescence (Fig. 1D). Two-dimensional tracking data revealed a rate of $0.034 \pm 0.008 \mu\text{m/s}$ ($n=12$) for the first phase, which was typically completed within a few minutes. The rate of the slow phase was highly variable and the data could not be fit by a straight line; the variation in rate may be due to back and forth motion of the centrosome later in anaphase (see below) [Collins et al., 2012]. As described previously, we confirmed that the motion of the two centrosomes was not always coordinated [Waters, 1993]. As seen in the plots of centrosome coordinates, in some cells one centrosome remained nearly stationary, while the other moved extensively; in other cases, both centrosomes were motile and the motion of each could be similar or distinct (Supporting information Fig. S1).

STLC Reduces Eg5 Binding to Spindle Microtubules

To determine the contribution of Eg5 to spindle elongation, we used S-trityl-L-cysteine, STLC, a small molecule inhibitor that weakens the affinity of the motor for the microtubule [Skoufias et al., 2006]. Eg5 tetramers crosslink and slide antiparallel microtubules and thus contribute to centrosome separation during spindle formation [Sharp et al., 1999; Kapitein et al., 2005; Ferenz et al., 2010]. However, the role of Eg5 during anaphase spindle elongation is unclear. Although Eg5 can be detected to a limited extent within the spindle midzone, where antiparallel microtubules are located, Eg5 is enriched at spindle poles of metaphase and anaphase spindles [Mastroratte et al., 1993; Sharp et al., 1999; Gable et al., 2012; Uteng et al., 2008]. The poleward enrichment of Eg5 may function to crosslink parallel microtubules in the half-spindle [Ma et al., 2011] and/or remove unengaged motors from the spindle midzone [Uteng et al., 2008; Gable et al., 2012]. Using cells expressing mCherry α -tubulin and Eg5 with a localization and affinity purification tag (hereafter referred to as GFP-Eg5) [Gable et al., 2012], we previously showed that GFP-Eg5 is only weakly detected

in the midzone region of early anaphase cells and increased as anaphase progressed. Measurement of the ratio of Eg5 to microtubule fluorescence in the midzone as cells progressed through anaphase confirmed that Eg5 gradually accumulates on midzone microtubules (ratio of Eg5/microtubules increased 2.5-fold; Methods). Following treatment of cells with STLC, spindle fluorescence of GFP-Eg5 decreased and the increase in Eg5 fluorescence in the interzone was not detected (Fig. 2).

Inhibition of Eg5 with STLC at Anaphase Onset Increases the Rate of Spindle Elongation

Because addition of STLC to pre-mitotic cells prevents spindle assembly, we added STLC (10 μ M) to the culture medium following spindle assembly—at anaphase onset—and subsequently acquired images of GFP- γ -tubulin over time (Fig. 1D). Surprisingly, we found that addition of STLC resulted in a statistically significant increase in the rate of the fast phase of centrosome separation measured using 2D tracking (from 0.034 to 0.047 μ m/s; $P=0.009$; Table I). The total extent of spindle elongation (including both the fast and slow phases; see Methods) was significantly increased in STLC-treated cells (8.6 ± 2.7 μ m and 11.6 ± 3.7 μ m, control and STLC, respectively; $P=0.042$; Table I).

Because motion of the centrosome in the Z -axis could potentially lead to an underestimation of centrosome motion, we also used an automated 3D tracking algorithm to determine centrosome position over time (Table I; Methods). Consistent with this possibility, 3D tracking revealed a rate of centrosome separation in control cells that was faster than that measured by 2D tracking (0.034 μ m/s versus 0.049 μ m/s; $P=0.0069$). Importantly, the rate of the fast, initial phase of centrosome separation in the presence of STLC as revealed by 3D tracking was significantly increased (0.049 μ m/s to 0.064 μ m/s; $P=0.025$; Table I). The total extent of spindle elongation increased from 8.73 to 11.56 μ m; $P=0.0004$; Table I). These results demonstrate that Eg5 is not required for pole–pole separation, but rather restricts the rate and extent of elongation.

We next asked whether altered microtubule organization in the midzone could explain the increased rate of centrosome separation observed in STLC-treated cells. Imaging of GFP- α -tubulin in both control and STLC treated cells, revealed multiple bundles of microtubules spanning the region between the segregating chromosomes (Fig. 1E); no qualitative differences were discerned between control and STLC-treated cells. To estimate the extent of microtubule overlap within the midzone, we tracked microtubule plus-ends in this region in cells expressing EB1-GFP [Piehl and Cassimeris, 2003]. The extent of overlap in the central region of the anaphase spindle was similar for both control and STLC treated cells (Fig. 1F and 1G). Together, these observations indicate that the organization of midzone microtubules was not detectably altered following treatment with STLC and suggest that Eg5, which crosslinks antiparallel microtubules, restricts the rate of centrosome separation in LLC-Pk1 cells. Further, these data indicate that other, non-Eg5-mediated forces maintain the overall organization of midzone microtubules [Kurasawa et al., 2004; Zhu et al., 2006; Hu et al., 2011].

Reduction of Spindle Elongation in FCPT- Treated Cells

Our above results are consistent with a model in which Eg5 tetramers interact with midzone microtubules and act as a frictional brake to reduce the rate of spindle elongation. To test this further, we used 2-[1-(4-fluorophenyl)cyclopropyl]- 4-(pyridin-4-yl)thiazole, FCPT, an Eg5 inhibitor that induces a rigor-like binding of the motor to the microtubule [Groen et al., 2008]. Addition of FCPT (10 μ M) at anaphase onset to GFP- γ -tubulin expressing cells (Fig. 1D) reduced the initial rapid rate of spindle elongation, as measured by 2D and 3D centrosome tracking (2D, 0.034 to 0.023; $P=0.018$; 3D, 0.049 to 0.032; $P=0.038$; Table I). The overall extent of spindle elongation, in FCPT-treated cells was reduced, but this difference was not statistically significantly for 2D or 3D tracking (Table I).

The distribution of microtubules in FCPT treated cells was examined using cells expressing GFP- α -tubulin. Following treatment with FCPT, bundles of microtubules were detected in the midzone region (Fig. 2). In addition, kinetochore fiber microtubules were present in late anaphase (compare Fig. 2, Control, 6 min, with FCPT, 7 min), indicating that FCPT treatment slowed the disassembly of kinetochore microtubules. With time, however, cytokinesis and kinetochore fiber disassembly were observed in these cells (not shown). Quantification of GFP-Eg5 fluorescence levels on spindle microtubules in FCPT-treated cells further showed an approximately threefold increase. These data demonstrate that inhibiting microtubule-release by Eg5 motors prevents spindle elongation and kinetochore fiber disassembly.

Cortical Pulling Forces Contribute to Spindle Elongation

The observation that Eg5 restricts spindle elongation in these cells is consistent with a model in which astral microtubule-mediated pulling forces drive spindle elongation. In support of this possibility, we recently showed that in LLC-Pk1 cells in which the spindle is asymmetrically positioned at anaphase onset, dynein and astral microtubule-dependent differential spindle pole motion is required to generate equal sized daughter cells [Collins et al., 2012]. Further, recent work demonstrates that increasing or decreasing the level of cortical dynein results in a corresponding increase or decrease in the extent of spindle elongation [Kotak et al., 2013].

In *C. elegans* embryos, cortical force generators result in rocking of the spindle pole, toward and away from the cell cortex [Grill et al., 2003]. In LLC-Pk1 cells, expressing GFP- γ -tubulin, we observed motion of the centrosome toward and away from the lateral cortex during late anaphase. Most cells showed this rocking motion, although the behavior of each centrosome could vary. Kymographs of centrosome motion in anaphase cells showed that in some cases the motion was oscillatory (Fig. 3D). Immunofluorescence staining confirms that the dynein binding protein p150 dynactin localizes to the polar and lateral cell cortex where it could participate in spindle elongation [Collins et al., 2012] (Fig. 3E). The rocking behavior of the centrosome is not consistent with outward pushing from the spindle midzone, but supports the view that forces acting along the cell cortex pull on astral microtubules and contribute to spindle elongation [Hara and Kimura, 2009].

To determine how cortical pulling forces and midzone motors cooperate to achieve spindle elongation, cells expressing GFP- γ -tubulin were treated, at anaphase onset, with an inhibitor of Polo kinase (BI2536) to increase the level of cortical dynein [Collins et al., 2012; Kiyomitsu and Cheeseman, 2012]. In these cells, the centrosome became less focused, was uncoupled from the spindle and moved toward the cell cortex consistent with enhanced pulling by cortical dynein (Fig. 3A). The rate of centrosome motion in cells treated with BI2536 was $0.042 \pm 0.013 \mu\text{m/s}$, $n=10$, which is not statistically significantly different from controls, however, the disruption of the centrosome made accurate tracking difficult.

Because the centrosome was detached from the spindle in BI2536 treated cells, spindle elongation was measured in cells expressing GFP- α -tubulin. The results show that spindle elongation was statistically significantly inhibited in these cells, consistent with previous studies [Brennan et al., 2007] (Fig. 3B and 3C) This result suggests that Eg5 in the midzone does not actively elongate the spindle when the centrosome and astral microtubules are uncoupled from the spindle. A caveat of this experiment is that the microtubule crosslinking protein PRC1 is phosphorylated by Plk1 in anaphase [Neef et al., 2007]. Members of the Ase1/PRC1 family are required for midzone microtubule crosslinking and elongation in diverse organisms [Fu et al., 2009]. Thus, the alteration of midzone microtubule organization and elongation in BI2536-treated cells may result from regulation of multiple spindle targets.

SUMMARY

Our results demonstrate that inhibition of Eg5 with STLC increases the rate and extent of spindle elongation in mammalian cells, supporting the view that this kinesin normally functions to restrict elongation by acting as a frictional brake. These results are consistent with the braking function of kinesin-5 in *C. elegans* and *Dictyostelium* and are distinct from the requirement for kinesin-5 to drive spindle elongation in other organisms [Saunders et al., 1995; Sharp et al., 2000; Roostalu et al., 2010]. Our data are consistent with earlier studies showing that the midzone restricts elongation in Ptk1 cells [Kronebusch and Borisy, 1982], and further identify Eg5 as one of the molecules that contributes to this restriction.

To perform these experiments, it was necessary to add the inhibitor at the onset of anaphase. Some of the cell-to-cell variation in our results could result from the difficulty in adding the drug at precisely the onset of anaphase for each cell. In addition, the inhibitors need to enter the cell and bind to their target; the time required for this could vary for different inhibitors. Despite these experimental challenges, the data demonstrate that the rate and extent of anaphase spindle elongation is altered following inhibition of Eg5, which is consistent with work in other systems using genetic approaches to eliminate the activity of kinesin-5 family members [Saunders et al., 2007; Tikhonenko et al., 2008].

Our results emphasize the need for regulation of kinesin-5 activity throughout mitosis. For example, in budding yeast the kinesin-5, Cin8, is regulated by the chromosome passenger complex, by Cdk1-dependent phosphorylation of the motor head, and by the microtubule crosslinking protein Ase1 all of which conspire to affect proper spindle elongation during anaphase [Roostalu et al., 2010; Avunie-Masala et al., 2011; Rozelle et al., 2011]. Defects in

any of these regulatory mechanisms lead to defects in chromosome segregation and mitotic exit. In mammalian cells, the microtubule-associated protein TPX2, which interacts with Eg5, reduces the rate of Eg5-dependent microtubule-microtubule sliding *in vitro* and disrupts spindle formation *in vivo* [Eckerdt et al., 2008; Ma et al., 2011]. Although the contribution of TPX2 to anaphase B spindle elongation has not been determined, one possibility is that TPX2 contributes to the braking function of Eg5 during late anaphase. Our results support the possibility that that force production by Eg5 is differentially regulated throughout mitosis [Gable et al., 2012].

In *C. elegans*, spindle elongation is regulated by a balance of cortically generated pulling forces that are opposed by kinesin-5 motors in the spindle interzone [Saunders et al., 2007]. Interestingly, in cells in which an upstream regulator of cortical dynein, GPR1/2, and the kinesin-5, BMK1, are simultaneously inhibited, spindle elongation is restored to near wild-type values. These data demonstrate that spindle elongation can proceed when both the pulling and braking forces are reduced or eliminated [Saunders et al., 2007], raising the question of where the elongation-driving forces come from in the absence of both kinesin-5 and dynein. One possibility is that microtubule polymerization contributes to spindle elongation. In support of this, inhibition of KIF4, which normally limits plus-end microtubule growth, results in extensive elongation of the spindle midzone in HeLa cells [Hu et al., 2011]. Thus, the combination of microtubule polymerization and a balance of motor dependent forces likely cooperate to drive spindle elongation.

Materials and Methods

Materials

All materials for cell culture were obtained from Sigma- Aldrich with the exception of Opti-MEM (Invitrogen) and FBS (Atlanta Biologicals). All other chemical reagents unless specified were obtained from Sigma-Aldrich.

Methods

Cell Culture, Inhibitors, and Live Cell Imaging—Parental LLC-Pk1 pig kidney epithelial cells and LLC-Pk1 cells expressing GFP- α -tubulin, GFP- γ -tubulin, EB1-GFP and LAP-Eg5/mCherry Tubulin were grown in a 1:1 mixture of F-10 medium and Opti-MEM (Invitrogen) as described previously [Rusan et al., 2001; Piehl and Cassimeris, 2003; Gable et al., 2012]. Centrosome tracking was performed in a clonal line of GFP- γ -tubulin cells (clone 3). Cells were plated 2–3 days prior to use in experiments. To treat live cells with inhibitors, cells were plated in MatTek dishes (MatTek Corporation, Ashland, MA) containing 1ml of tissue culture medium lacking phenol red and sodium bicarbonate, and buffered with 5mM HEPES, pH 7.3. To add inhibitors at anaphase onset, cells were monitored using phase contrast optics. As soon as the chromosomes began to separate, the lid of the dish was removed and one ml of medium containing 2 $\times$ the desired final concentration of inhibitor was added, and the lid was replaced and imaging resumed. For some experiments, coverslips were mounted in Rose chambers with a circular 25 mm glass coverslip serving as a lid. To add inhibitors to cells in Rose chambers, half of the volume of medium in the chamber was removed and replaced with the same volume of medium

containing 2× the desired final concentration of inhibitor. All inhibitors were made in DMSO, stored at −20°C, and diluted into tissue culture medium the day of the experiment.

Immunofluorescence—Cells were rinsed in 37°C phosphate buffered saline lacking calcium and magnesium (PBS^{−/−}) and fixed in −20°C Methanol for 10 min, rehydrated in PBS^{−/−}, and fixed in 3.2% paraformaldehyde, 0.1% glutaraldehyde, and 0.05% Triton in PBS^{−/−} for 10 min and rehydrated in PBS^{−/−} containing 0.1% Tween-20 and 0.02% sodium azide (PBS-Tw-Az) for 5 min at room temperature. Primary antibody staining was performed with anti-p150 at 1:100 (BD Transduction Laboratories, Lexington, KY) for 1 h at room temperature. Secondary antibody staining was performed with Cy3-labeled anti-mouse antibodies at 1:400 (Jackson ImmunoResearch Laboratories, West Grove, PA) for 45 min at 37°C or 1 h at room temperature. Coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and sealed with nail polish.

Image Acquisition and Analysis—We acquired images using two different spinning disc confocal microscopes. A Nikon Eclipse TE300 microscope equipped with a CSU 10 spinning disk confocal scan head (PerkinElmer, Wellesley, MA) and an Orca ER cooled CCD camera (Hamamatsu, Bridgewater, NJ) or a Nikon TiE microscope with a CSU-X1 Yokogawa spinning disk confocal scan head (PerkinElmer, Wellesley, MA), and an Andor iXon+ EMCCD camera (Andor, Belfast, Northern Ireland). Both microscope systems were controlled by MetaMorph software (Molecular Devices, Sunnyvale, CA). A 100X 1.4 NA objective was used. For live cell imaging, exposures were adjusted without saturating the camera's pixels; typical exposures were 50–1000 ms. Typically we imaged a subset of image planes in Z, to include only the centrosome. For 2D tracking, a maximum intensity projection was made. Cells were maintained at ~37°C during the experiment, but the heater was turned off while adding inhibitors. BI2536, STLC, and FCPT were used at a final concentration of 10 μM.

Two-dimensional tracking was performed manually using measure tools in Metamorph (Molecular Devices, Sunnyvale, CA) software or Image J (National Institutes of Health, Bethesda, MD) linked to an Excel (Microsoft, Redmond, WA) data sheet. For cells expressing GFP-γ-tubulin, the center of the centrosome was used; for cells expressing GFP-α-tubulin, the end of the spindle was used. For automated 3D tracking, a Metamorph plug-in for Multidimensional motion analysis was used. Prior to tracking, the threshold function was adjusted so as to include the centrosomes and exclude nonspecific fluorescence. The simple threshold segmentation method was used with the *XY* and *Z*-diameter and local intensity above background was altered manually for each movie sequence.

For the “adaptive” kymograph shown in Figure 3, the *X*- and *Y*-axis movements of the centrosome were segregated by selecting a region of interest along the *X*-axis. As the centrosome moved along the *Y*-axis the region of interest was manually moved to keep the centrosome within the box. Similarly, the *Y*-axis movements were tracked by manually moving the region of interest along the *X*-axis.

The extent of spindle elongation was determined as the difference between the spindle length, measured as the distance between centrosomes in 2D or 3D, just prior to anaphase

onset and the spindle length following spindle elongation. For spindle length measured using cells expressing GFP- α -tubulin, the length of the spindle was estimated from one end of the spindle to the other.

To measure the extent of microtubule overlap in the spindle midzone, cells expressing EB1-GFP were imaged at 2 s intervals to generate a movie of microtubule growth events. EB1-GFP comets at microtubule ends in the overlap region were tracked with MTrackJ in Image J. Only microtubule ends that could be followed for sufficient time to generate a track were included in the analysis.

To measure the ratio of Eg5 to tubulin, images of cells expressing mCherry tubulin and GFP-Eg5 were collected and each channel was background subtracted. Fluorescence was measured in the midzone at various times during anaphase and the ratio of Eg5/tubulin determined. The average change in the ratio was determined from the beginning to the end of anaphase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Inhibition of Eg5 results in an increased rate of spindle elongation

(A) Fluorescence images from a time-lapse sequence of an LLC-Pk1 cell expressing GFP- α -tubulin. Time in min:s; 0:00=anaphase. Arrowheads mark the approximate location of the chromosomes. (B, C) Two phases of spindle elongation in LLC-Pk1 cells. Plot of pole-pole distance versus time in cells expressing GFP- γ -tubulin. (B) Data collected by 2D manual tracking; (C) data collected by 3D automated tracking. Triangle, square and diamonds represent control, STLC and FCPT treated cells. (D) Images from time-lapse sequences of cells expressing GFP- γ -tubulin; top, STLC treated; middle, control; bottom, FCPT treated. STLC and FCPT added at anaphase onset. (E) Midzone microtubule organization in cells expressing GFP- α -tubulin; upper – control; lower – STLC treated. (F) Cells expressing GFP-EB1, bottom panels show tracks of growing microtubule plus-ends that overlap in the mid-region of the spindle; control (left) and STLC (right) treated cells. (G). Quantification of overlap length. Marker bars=10 μ m.

Fig. 2. Distribution of Eg5 during spindle elongation

Fluorescence images from time-lapse sequences of cells expressing Eg5-LAP and mCherry tubulin. Time in min:s. Contrast and brightness adjusted in the inset in 18:00 min panel of FCPT treated cell to show Eg5 on interzonal microtubules. Bar=10 μ m.

Fig. 3. Spindle elongation is sensitive to inhibition of Polo kinase1

(A) Images of LLC-Pk1 cells expressing GFP- γ -tubulin and treated with BI2536. Centrosomes (arrowheads) dissociate from spindle and GFP- γ -tubulin becomes diffuse. (B) Cells expressing GFP- α -tubulin and treated with BI2536 at anaphase onset. Note the microtubule bundles in the midzone. (C) graph of spindle elongation in control (triangles) and BI2536-treated cell (squares); cells expressing GFP- α -tubulin used for this experiment. (D) Images and kymographs from time-lapse sequence of cells expressing GFP- γ -tubulin showing oscillations of the centrosome. Images acquired at 3 s intervals. (E) LLC-Pk1 cell stained for p150 subunit of dynactin; arrowheads mark dynactin at cortex. Marker bars=10 μ m.

Table I**Anaphase Centrosome Motion**

<i>2D tracking</i>			
	Control	STLC	FCTP
Rate ($\mu\text{m/s}$) Fast phase	0.034 $\pm$ 0.008	0.047 $\pm$ 0.013	0.023 $\pm$ 0.011
Extent	8.6 $\pm$ 2.7, n=12	11.6 $\pm$ 3.7, n=10	7.5 $\pm$ 3.0, n=8
<i>3D tracking</i>			
Rate ($\mu\text{m/s}$) Fast phase	0.049 $\pm$ 0.017	0.064 $\pm$ 0.015	0.032 $\pm$ 0.011
Extent	8.73 $\pm$ 2.4, n=23	11.56 $\pm$ 2.7, n=9	7.67 $\pm$ 2.2, n=5

The sample numbers (*n*) for rate measurements are the same as the corresponding extent measurements.

Rate measurement for the fast phase of spindle elongation; extent measurement determined from spindle length at end of anaphase minus spindle length at anaphase onset.

Two-dimensional rates: STLC and FCTP each significantly different from control, $P = 0.01$; 2-D extent: STLC significantly different from control, $P < 0.05$; FCPT not different from control.

Three-dimensional rates: STLC and FCTP each significantly different from control, $P < 0.05$; 3-D extent: STLC significantly different from control, $P < 0.001$; FCPT not different from control.