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Article

Kif15 Cooperates with Eg5 to Promote Bipolar Spindle Assembly

Marvin E. Tanenbaum,[1] Libor Macůrek,[1] Aniek Janssen,[1] Erica F. Geers,[1] Mónica Alvarez-Fernández,[1] and René H. Medema[1,*]

[1]Department of Medical Oncology and Cancer Genomics Centre, University Medical Center Utrecht, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands

Summary

Background: The formation of a bipolar spindle is an essential step during cell division. Bipolar spindle assembly is driven by the highly conserved microtubule motor Eg5 (kinesin-5), which can slide antiparallel microtubules apart to drive centrosome separation. However, it is currently unclear whether and how additional motors can contribute to centrosome separation and bipolar spindle formation.

Results: We have developed a novel assay to identify motors involved in spindle bipolarity; via this assay, we identify Kif15/Hklp2 (kinesin-12, hereafter referred to as Kif15). Kif15 is not required for spindle bipolarity in cells with full Eg5 activity but becomes essential when Eg5 is partially inhibited. We show that the primary function of Kif15 is to promote spindle elongation and to ensure maintenance of spindle bipolarity. Nonetheless, ectopic expression of Kif15 can fully reconstitute bipolar spindle assembly in the absence of Eg5 activity, demonstrating that Kif15 can replace all essential functions of Eg5 in bipolar spindle assembly. Importantly, this activity of Kif15 depends on its interaction with the microtubule-associated protein TPX2, indicating that a Kif15-TPX2 complex promotes centrosome separation.

Conclusions: These findings show that, similar to Eg5, Kif15 can drive centrosome separation during bipolar spindle assembly. For this activity, Kif15 requires both its motor domain and its interaction with TPX2. Based on these data, we propose that a complex of Kif15 and TPX2 can crosslink and slide two antiparallel microtubules apart, thereby driving centrosome separation.

Introduction

Eg5 (kinesin-5) is a highly conserved plus-end-directed microtubule motor that provides a major force necessary for bipolar spindle assembly. Eg5 forms a homotetramer with two motor domains on each side, allowing it to walk on two microtubules simultaneously [1, 2]. This unique configuration of Eg5 allows it to slide antiparallel microtubules apart, thereby driving centrosome separation during bipolar spindle assembly in vivo [3–5]. In most organisms, Eg5 is absolutely essential for spindle bipolarity; loss of Eg5 results in monopolar spindles [3–5]. Surprisingly, however, bipolar spindle assembly is restored in cells lacking Eg5 activity when either one of the two minus-end-directed motors NCD/HSET or dynein is inhibited [6–9]. Furthermore, Eg5 is not essential for spindle bipolarity in certain systems, such as *C. elegans* embryos [10]. Finally, Eg5 activity does not appear to be required in mammalian cells for the maintenance of a bipolar spindle in metaphase [11]. Together, these results indicate that additional motors might cooperate with Eg5 to promote bipolar spindle assembly.

One motor that has been implicated in spindle bipolarity is the kinesin-12 motor Xklp2: addition of a dominant-negative construct of Xklp2 to *Xenopus* egg extracts resulted in the formation of monopolar spindles [12]. However, subsequent experiments using protein depletion showed that normal bipolar spindles form after removal of >98% of Xklp2 from egg extracts [13], strongly suggesting that Xklp2 is in fact not essential for spindle bipolarity. Similarly, depletion of Xklp2 does not inhibit bipolar spindle assembly around chromatin beads [14], and RNA interference (RNAi)-mediated knockdown of the human homolog of Xklp2, Kif15/Hklp2 (hereafter referred to as Kif15), also does not inhibit bipolar spindle assembly [15]. Together, these results indicate that Kif15/Xklp2 is not essential for bipolar spindle assembly.

To identify additional motors that promote spindle bipolarity, we took advantage of the fact that Eg5 is not required to maintain spindle bipolarity during metaphase in human cells. Via RNAi, we systematically tested all microtubule motors for an essential role in maintaining metaphase spindle bipolarity in the absence of Eg5 activity and identified Kif15 as the major motor involved in this process. Importantly, we found that, although Kif15 is not required for bipolar spindle assembly in normal cells, it becomes essential when Eg5 activity is compromised. Detailed analysis revealed that initial centrosome separation is not affected in cells lacking Kif15 but that subsequent spindle elongation is strongly inhibited, consistent with a role for Kif15 in the later stages of bipolar spindle assembly. Surprisingly, overexpression of Kif15 can drive bipolar spindle assembly when Eg5 is fully inhibited, demonstrating that Kif15 can take over all essential functions of Eg5 and suggesting that Kif15 can directly generate a force that pushes centrosomes apart. This activity of Kif15 depends not only on its motor activity but also on its interaction with the microtubule-associated protein TPX2, indicating that a complex of Kif15 and TPX2 promotes bipolar spindle assembly.

Results

Identification of Motor Proteins Involved in Bipolar Spindle Maintenance

Although Eg5 is essential for bipolar spindle assembly, it does not appear to be required for bipolar spindle maintenance [11]. To confirm this, we left HeLa cells untreated, treated them for 24 hr with the potent Eg5 inhibitor S-trityl-L-cysteine (STLC; [16]), or first blocked them in metaphase with the proteasome inhibitor MG132 and subsequently treated them with STLC (hereafter referred to as MG/STLC treatment). Although 24 hr treatment with STLC completely blocked bipolar spindle assembly, inhibition of Eg5 in metaphase did not result in substantial spindle collapse (Figure 1A), demonstrating that Eg5 is not required for bipolar spindle maintenance. These results suggest that an additional motor might cooperate with Eg5 in bipolar spindle maintenance. To test this hypothesis, we generated a small interfering RNA (siRNA) library

*Correspondence: r.h.medema@umcutrecht.nl

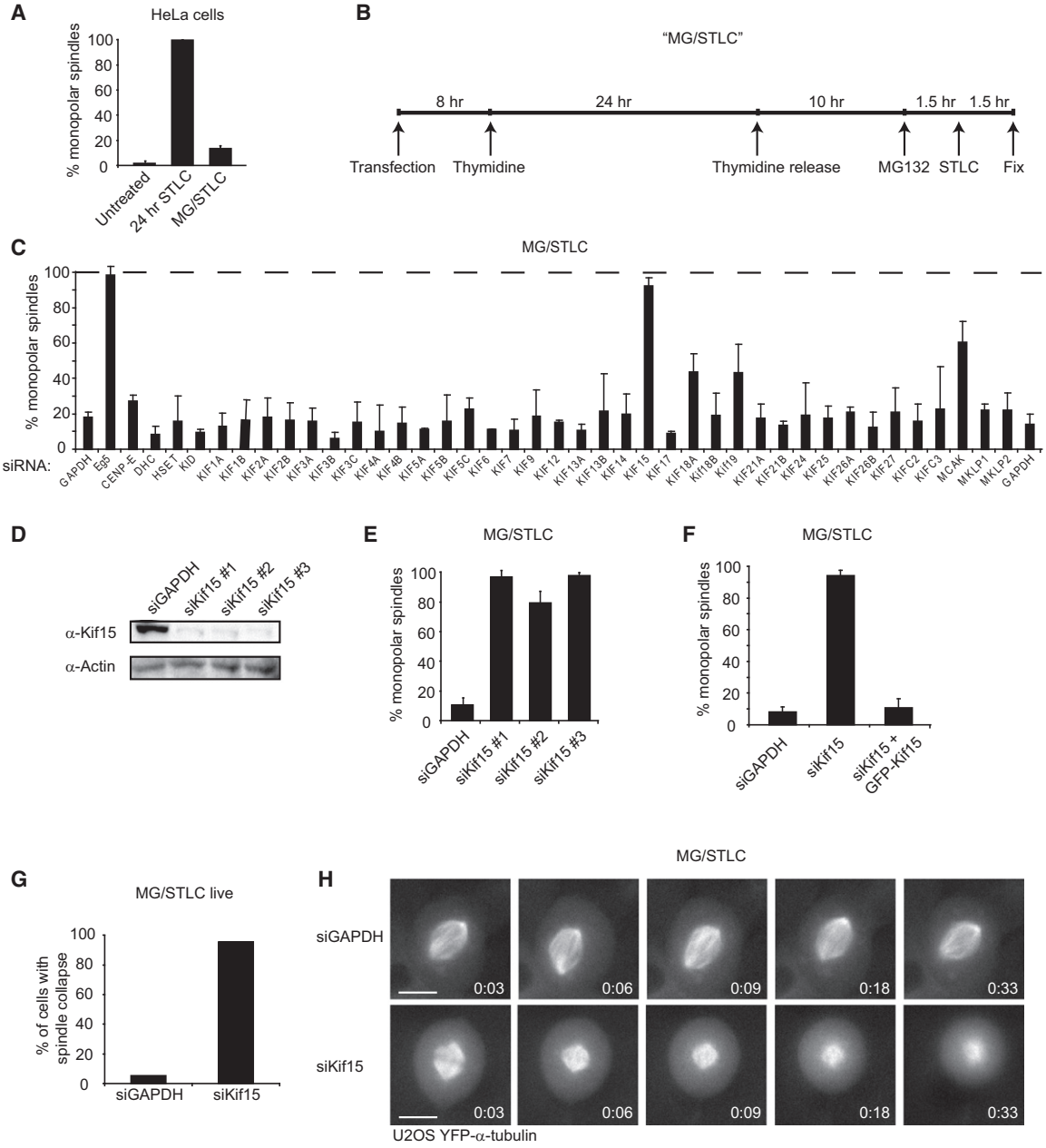

Figure 1. Kif15 Is Involved in Bipolar Spindle Maintenance

(A) HeLa cells were left untreated, treated with 40 μM STLC for 24 hr, or treated with MG132 for 1.5 hr and subsequently with 40 μM STLC for an additional 1.5 hr. Cells were then fixed and stained for α-tubulin and DNA, and the percentage of mitotic cells containing a monopolar spindle was scored (n = 100 per condition).

(B) Experimental setup of the screen performed in (C).

(C) HeLa cells were transfected with siRNAs targeting all human microtubule motors (described in Table S1) and were treated as indicated in (B). The percentage of mitotic cells containing a monopolar spindle was determined (n = 40). Dashed line indicates 100%.

(D) HeLa cells were transfected with the indicated siRNAs, and 48 hr after transfection, cells were harvested. Total Kif15 protein levels were analyzed by western blot.

(E) HeLa cells were transfected and treated as in (C), and the percentage of mitotic cells containing a monopolar spindle was determined (n = 100).

(F) HeLa cells stably expressing GFP-Kif15 under a tetracycline-inducible promoter were transfected with indicated siRNAs. Twenty-four hours after transfection, expression of GFP-Kif15 was induced for 4 hr. Twenty-four hours after GFP-Kif15 induction, MG/STLC treatment was performed, cells were fixed, and the percentage of mitotic cells containing a monopolar spindle was scored.

(G and H) U2OS cells stably expressing YFP-α-tubulin were transfected with indicated siRNAs, and 48 hr after transfection, cells were treated with MG132 for 1.5 hr. Cells were then treated with 40 μM STLC, and images were acquired every 3 min (n = 72 and n = 38 for GAPDH- and Kif15-depleted cells, respectively).

(G) Quantification of all cells.

(H) Representative GAPDH- and Kif15-depleted cells. Scale bars represent 10 μm.

Results in (A), (C), (E), and (F) are averages of three independent experiments. All error bars represent standard deviation (SD).

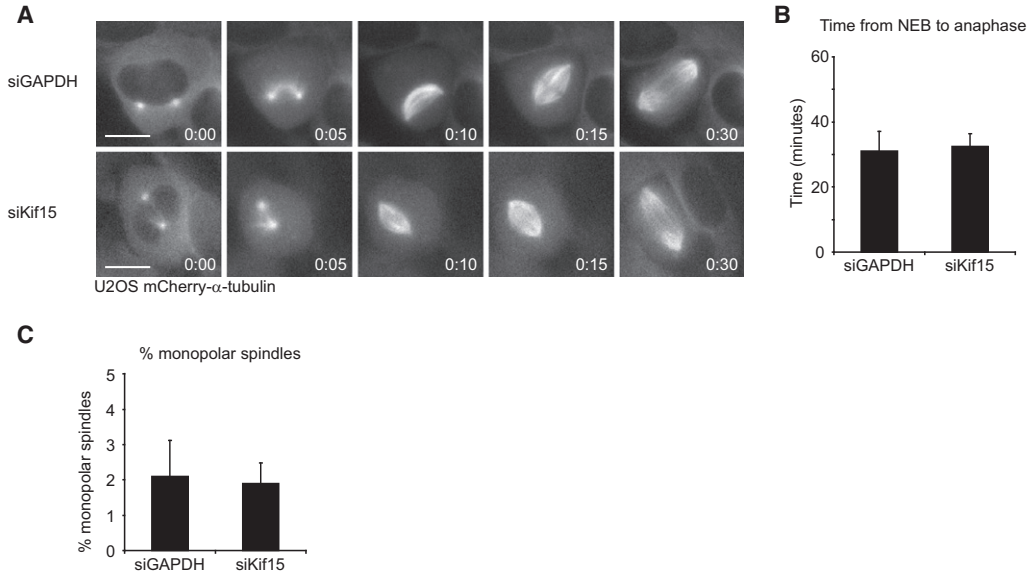

Figure 2. Kif15 Is Not Essential for Bipolar Spindle Assembly in the Presence of Full Eg5 Activity

(A and B) U2OS cells stably expressing mCherry-α-tubulin were transfected with the indicated siRNAs and analyzed by time-lapse microscopy 48 hr after transfection. Images were acquired every 5 min. Scale bars in (A) represent 10 μm.

(B) Time from nuclear envelope breakdown (NEB) to anaphase was determined (n = 50 cells per condition).

(C) U2OS cells were treated as in (A) but were fixed, and the percentage of mitotic cells containing a monopolar spindle was determined (n = 100). Results in (B) and (C) are averages of four independent experiments. All error bars represent SD.

targeting all human microtubule motor proteins and assayed whether any motor was required for bipolar spindle maintenance in the absence of Eg5 activity (Figures 1B and 1C). Indeed, depletion of Kif15 dramatically increased the percentage of monopolar spindles after Eg5 inhibition (Figure 1C). Also, a smaller but reproducible increase in the percentage of monopolar spindles was observed after depletion of Kif18a, Kif19, and MCAK (Figure 1C). Surprisingly, loss of Kif2a and Kif2b, which were previously implicated in spindle bipolarity in mammalian cells [17, 18], did not result in a substantial increase in the percentage of monopolar spindles. However, at least for Kif2a, the siRNA used here reduced Kif2a levels more potently than the siRNA used previously (see Figures S1A–S1D available online), validating our siRNA approach and indicating that Kif2a is not essential for bipolar spindle assembly.

Because the strongest effects were observed after knockdown of Kif15 (>90% of spindles were monopolar), we decided to focus on this motor in more detail. We confirmed the Kif15 knockdown phenotype with two additional siRNAs (Figures 1D and 1E). Furthermore, expression of a siRNA-resistant murine GFP-Kif15 (which localized as endogenous Kif15; see below and Figure S5A) was able to fully restore spindle bipolarity in Kif15-depleted cells after MG/STLC treatment (Figure 1F), even at near endogenous expression levels (Figure S1E), demonstrating that the effects of Kif15 knockdown are indeed specific. Finally, U2OS cells stably expressing YFP-α-tubulin were depleted of Kif15 or GAPDH as a control. Cells were then treated with MG/STLC, and time-lapse analysis was initiated at the time of STLC addition. Whereas only 4% of spindles collapsed within 1 hr of STLC addition in GAPDH-depleted cells, this was the case for 92% of cells after Kif15 depletion (Figures 1G and 1H). Taken together, these results demonstrate that Kif15 and Eg5 act redundantly to control bipolar spindle maintenance.

Kif15 Is Not Essential for Bipolar Spindle Assembly

Because previous studies failed to identify defects in spindle bipolarity after depletion of Kif15/Xklp2 [13–15], we examined bipolar spindle assembly after knockdown of Kif15 in otherwise unperturbed cells by time-lapse microscopy. In control cells, centrosomes separated ~1 hr before nuclear envelope breakdown (NEB), and robust bipolar spindles could be observed within 10 min of NEB (Figure 2A). Anaphase onset was observed 31 ± 6 min after NEB (Figure 2B). Similarly, in Kif15-depleted cells, centrosomes separated normally before NEB and spindle assembly occurred as in control cells (Figure 2A). The time from NEB to anaphase was also unchanged, 32 ± 4 min (Figure 2B). Consistent with this, we did not observe an increase in the percentage of monopolar spindles after depletion of Kif15 (Figure 2C). Taken together, these results demonstrate that Kif15 is not essential for spindle assembly in the presence of full Eg5 activity.

Kif15 Promotes Rapid Elongation of the Spindle

Given that we found that Kif15 is not essential in the presence of full Eg5 activity but is required for bipolar spindle maintenance in the absence of Eg5, it is possible that Kif15 also becomes essential for the assembly of a bipolar spindle when Eg5 activity is reduced. To test this, we partially inhibited Eg5 in U2OS cells with a very low dose of STLC (0.75 μM, in contrast to 40 μM STLC used for full Eg5 inhibition), because at this concentration there was still sufficient Eg5 activity to allow for bipolar spindle assembly in the majority of cells (Figure S2). In control cells treated with a low dose of STLC, spindle poles were often initially close together, consistent with a role for Eg5 in centrosome separation during prophase [9], but the spindle would subsequently "pop" open (i.e., elongate very rapidly) (Figure 3A), and only 8% ± 3% of control cells formed a monopolar spindle at this concentration of STLC (Figure 3D). At higher doses of

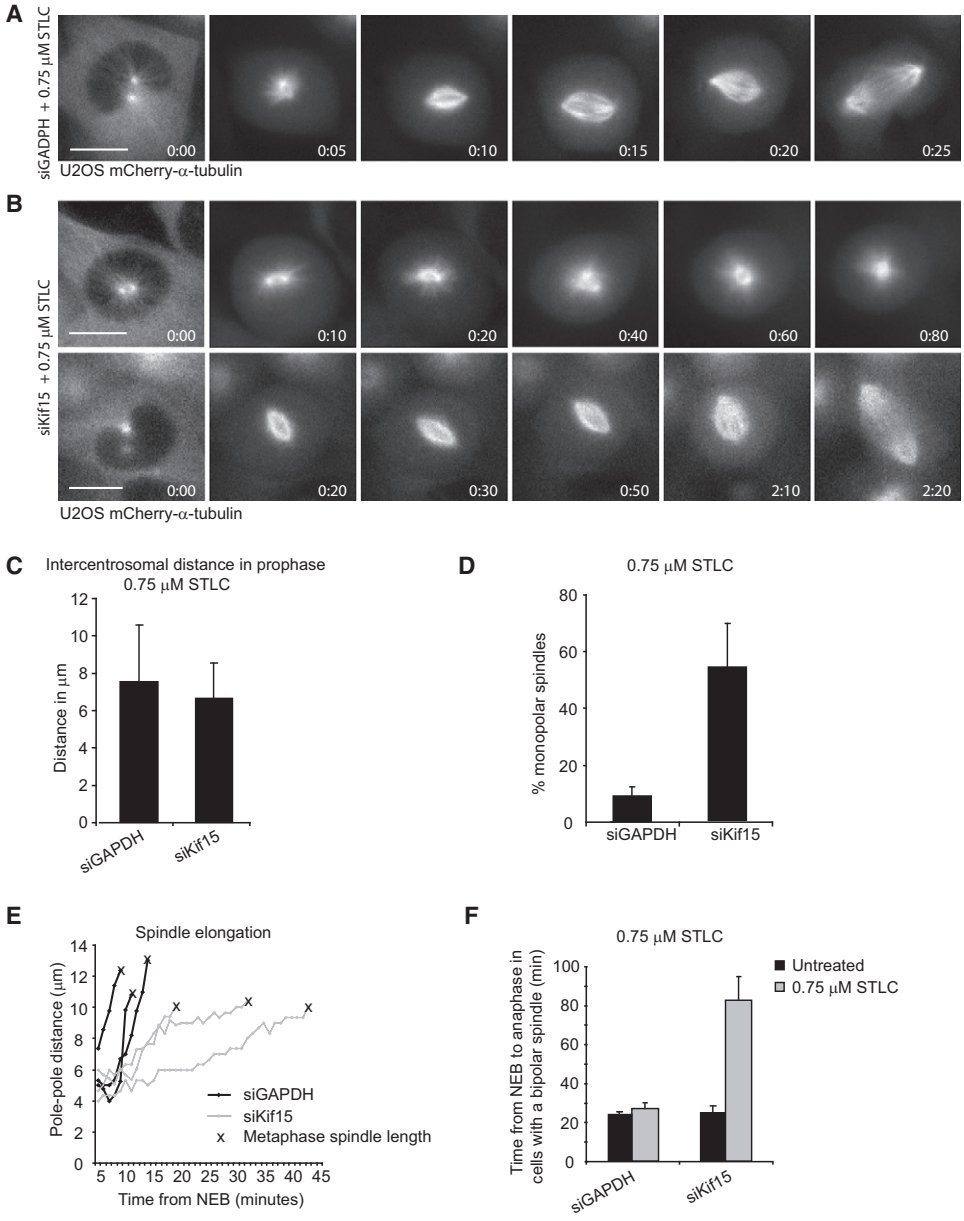

Figure 3. Kif15 Promotes Rapid Spindle Elongation

(A–D) U2OS cells stably expressing mCherry-α-tubulin were transfected with indicated siRNA, and 48 hr after transfection, cells were treated with 0.75 μM STLC and analyzed by time-lapse microscopy (n = 40 cells analyzed per condition).

(A and B) Representative GAPDH- and Kif15-depleted cells are shown. Scale bars represent 10 μm.

(C) Intercentrosomal distance measured at NEB.

(D) Percentage of cells forming a bipolar spindle.

(E) U2OS cells stably expressing GFP-centrin were treated as in (A) but were analyzed by time-lapse analysis at 90 s intervals. The plotted distance between centrosomes of three randomly selected GAPDH-depleted and Kif15-depleted cells was measured over time in three dimensions. Xs mark the time point at which the metaphase spindle length was reached.

(F) U2OS cells stably expressing mCherry-α-tubulin were transfected as in (A) but were either treated with 0.75 μM STLC or left untreated. Time from NEB to anaphase onset was determined in all cells that formed a bipolar spindle (n = 40 cells per condition).

Graphs in (C)–(F) represent averages of three independent experiments. Error bars represent SD.

STLC, a smaller fraction of cells formed a bipolar spindle, but those that did always switched rapidly from an initial monopolar to a bipolar state (data not shown). We never observed cells that contained a spindle of intermediate length for a prolonged period of time, consistent with the notion that the spindle behaves in a bistable manner [9, 19]. In Kif15-depleted cells treated with a low dose of STLC, centrosome separation during prophase was similar to controls cells (Figure 3C). However, the percentage of cells that subsequently formed a monopolar spindle was substantially increased, to 55% ± 15% (Figures 3B and 3D). These results demonstrate that Kif15 indeed acts redundantly with Eg5 to promote bipolar spindle assembly and that this occurs specifically after NEB.

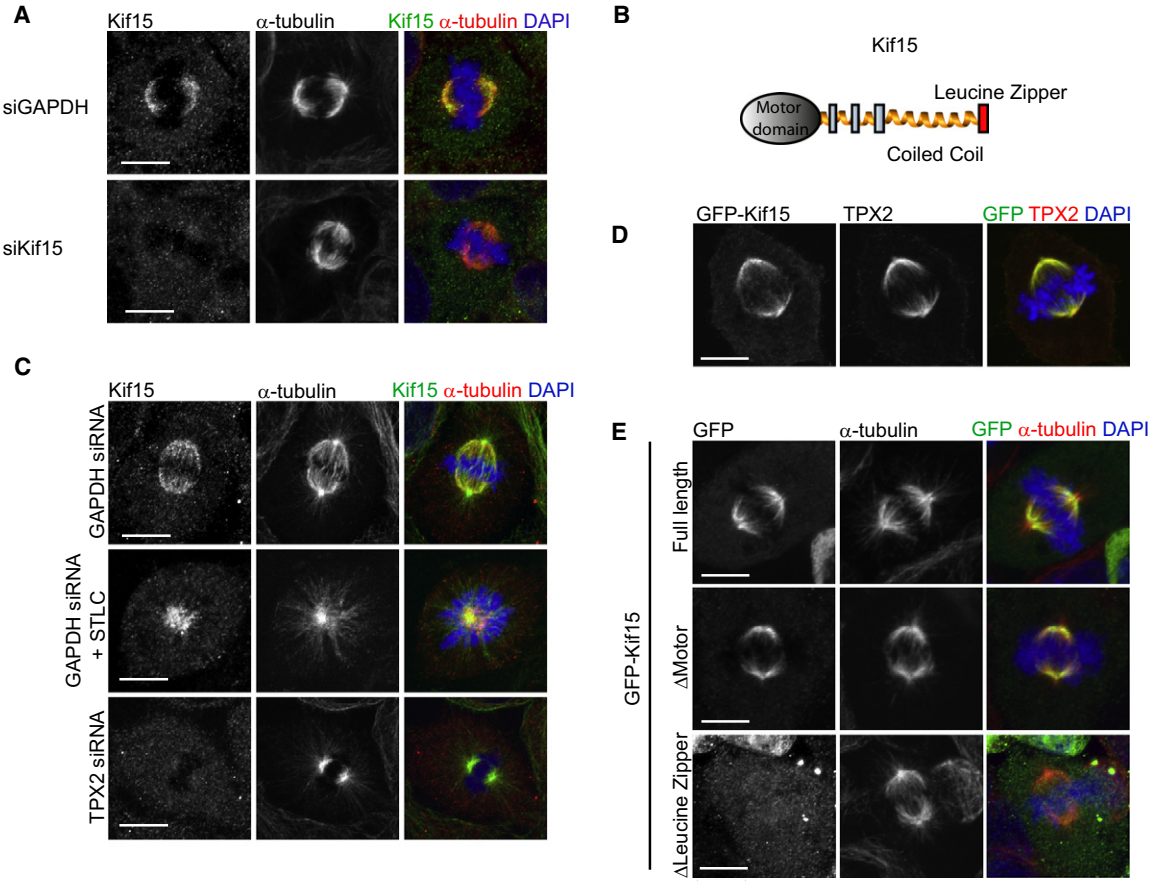

Figure 4. Kif15 Is Targeted to the Spindle through Its C-Terminal Leucine Zipper by TPX2

(A) U2OS cells were transfected with the indicated siRNAs, fixed, and stained for Kif15 and α-tubulin; DAPI was used to visualize DNA. Spindle staining by the Kif15 antibody was specific: no reactivity was observed on the spindle after Kif15 RNAi.

(B) Schematic representation of the Kif15 structure including motor domain, coiled coils, and the leucine zipper. Light blue bars represent interruptions in the coiled coils.

(C) U2OS cells were transfected with the indicated siRNAs, and 36 hr after transfection, cells were fixed and stained for Kif15 and α-tubulin; DAPI was used to visualize DNA. STLC was added during the last 4 hr before fixation.

(D) U2OS cells stably expressing GFP-Kif15 under control of a tetracycline-inducible promoter were induced for 24 hr, and cells were fixed and stained for TPX2; DAPI was used to visualize DNA. GFP-Kif15 was used because of Kif15 and TPX2 antibody incompatibility.

(E) U2OS cells were transiently transfected with the indicated plasmids, fixed, and stained for GFP, α-tubulin, and DAPI 48 hr after transfection.

Scale bars represent 10 μm.

Surprisingly, in Kif15-depleted cells treated with a low dose of STLC, spindles did not rapidly collapse or elongate upon NEB but rather remained at an intermediate length for a prolonged period of time (Figures 3B and 3E; Figure S3A). Although the majority of spindles eventually ended up as monopolar (Figure 3D), the cells that did manage to form a bipolar spindle (mostly cells that had achieved a relatively large intercentrosomal distance in prophase; see Figure S3B) had a final spindle length that was only slightly reduced compared to controls (Figure S3C). Interestingly, in cells that did form a bipolar spindle, chromosome alignment was not completed until spindles had fully elongated (Figure S4A), and anaphase onset was substantially delayed in these cells (Figure 3F). Furthermore, almost all cells (>95%) with spindles of intermediate length exhibited kinetochores positive for the dynactin subunit p150glued (Figure S4B), a marker for unattached kinetochores [20], suggesting that rapid spindle elongation is important for efficient kinetochore-microtubule attachment and chromosome alignment. These results show that Kif15 does not have a major role in initial centrosome separation but rather acts during the later stages of bipolar spindle assembly to promote spindle elongation, consistent with its role in bipolar spindle maintenance in metaphase.

Kif15 Is Targeted to the Spindle through its C-Terminal Interaction with TPX2

To determine the localization of Kif15 during bipolar spindle assembly, we raised a polyclonal antibody against the C terminus of Kif15 that was able to detect endogenous Kif15 by immunofluorescence (Figure 4A). Consistent with previous studies [21, 22], we found that Kif15 localizes all along spindle microtubules, but not on astral microtubules (Figure 4A). Furthermore, Kif15 was not detected along interphase microtubules (data not shown). Similar results were obtained when the localization of GFP-Kif15 was examined (Figure S5A). Together, these results show that Kif15 localizes along microtubules specifically in mitosis.

To better understand how Kif15 could promote bipolar spindle assembly, we first determined how it binds to spindle microtubules. The *Xenopus laevis* homolog of Kif15, Xklp2,

was shown to bind TPX2 with its C-terminal leucine zipper (Figure 4B) and is recruited to the spindle by TPX2 [21]. Indeed, we found that depletion of TPX2 completely prevented Kif15 from binding to the spindle (Figure 4C; see Figures S6A and S6B for TPX2 knockdown). This was likely not due to changes in spindle organization that occurred after TPX2 depletion, because Kif15 localized normally to microtubules of monopolar spindles after STLC treatment (Figure 4C). In contrast, Kif15 depletion did not affect TPX2 recruitment to the spindle (Figure S6C). Furthermore, TPX2 colocalized with GFP-Kif15 all along spindle microtubules (Figure 4D). Interestingly, before NEB, TPX2 did not bind microtubules, because it localized to the nucleus (Figure S5B), likely explaining why Kif15 does not bind microtubules in interphase.

To further confirm that Kif15 is recruited to the spindle through its C-terminal leucine zipper by TPX2, we generated two GFP-Kif15 deletion mutants, the first lacking the entire motor domain and the second lacking the C-terminal leucine zipper. Whereas the motor domain was not required for either TPX2 binding or recruitment to the spindle (Figure 4E; Figure S7), the C-terminal leucine zipper was essential for Kif15 recruitment to the spindle as well as for TPX2 binding (Figure 4E; Figure S7). These results demonstrate that Kif15 is recruited to the spindle through its C-terminal interaction with TPX2 and that the motor domain is not required for recruitment to the spindle.

Kif15 Can Drive Bipolar Spindle Assembly in the Absence of Eg5 Activity

We considered several possible mechanisms by which Kif15 could contribute to spindle bipolarity. First, Kif15 could modulate Eg5 activity, for example by correctly organizing microtubules for Eg5 to slide them apart. Second, Kif15 could statically crosslink antiparallel microtubules within the spindle and thereby prevent spindle collapse by generating friction on inward sliding microtubules. Finally, it is possible that Kif15 could directly generate an outward force by sliding antiparallel microtubules apart. If the last model is correct, overexpression of Kif15 might be able to generate a force sufficient to drive centrosome separation, even in the absence of Eg5 activity. To test this, we generated a U2OS cell line expressing GFP-Kif15 under a tetracycline-inducible promoter. Strikingly, expression of GFP-Kif15 was able to fully restore bipolar spindle assembly after Eg5 siRNA, high-dose (40 μM) STLC treatment, or, to ensure complete inactivation of Eg5, combined Eg5 siRNA and high-dose STLC treatment (Figure 5A). Even more surprisingly, cells expressing GFP-Kif15 that lacked Eg5 activity progressed through mitosis with timing almost identical to untreated control cells (Figure 5B). The only clear difference between untreated control cells and GFP-Kif15-expressing cells that lacked Eg5 activity was that the latter completely failed to separate their centrosomes in prophase (Figure 5C), consistent with the fact that Kif15 does not act before NEB. Quantitative immunofluorescence revealed that 5- to 10-fold overexpression of Kif15 was sufficient to drive bipolar spindle assembly in the absence of Eg5 activity (Figure S8). These results argue against a role for Kif15 in modulating Eg5 function, because Eg5 was completely inhibited in these experiments. Furthermore, it is unlikely that Kif15 prevents spindle collapse solely by generating friction on inward sliding microtubules, because this cannot explain how ectopic expression of Kif15 can push centrosomes apart in cells devoid of Eg5 activity. Taken together, these results strongly support the hypothesis that Kif15 can directly generate a force that pushes centrosomes apart after NEB.

To determine whether Kif15 could fully reconstitute all essential functions of Eg5, we next performed colony formation assays. Cells were either induced to express GFP-Kif15 or were left uninduced and were treated with increasing doses of STLC or, as a control, the microtubule-stabilizing drug paclitaxel. Although both paclitaxel and STLC treatment potently blocked cell proliferation in control cells, expression of GFP-Kif15 was able to completely overcome the inhibition of cell proliferation by STLC, but not by paclitaxel (Figure 5D). Taken together, these results demonstrate that Kif15 can take over all essential functions of Eg5 during spindle assembly and indicate that Kif15 can directly generate a force that is able to push centrosomes apart.

To gain additional insights into the molecular mechanism underlying this function of Kif15, we tested the ability of the Kif15 deletion mutants to drive centrosome separation in the absence of Eg5 activity. Interestingly, neither expression of Kif15-Δleucine zipper, which has a functional motor domain but cannot interact with TPX2, nor expression of Kif15-Δmotor, which localizes normally to the spindle but lacks motor activity, was able to restore bipolar spindle assembly in the absence of Eg5 activity (Figure 5E). Furthermore, although full-length Kif15 was able to drive bipolar spindle assembly in the absence of Eg5 activity, this was no longer the case when TPX2 was depleted (Figure 5E). Taken together, these results show that Kif15 can substitute all essential functions of Eg5 and that this requires both its N-terminal motor domain and its C-terminal interaction with TPX2.

Discussion

Here, we have performed a systematic analysis of all human motor proteins and identified Kif15 as a novel regulator of spindle bipolarity that can drive bipolar spindle assembly in the absence of Eg5. This complete functional overlap was rather unexpected, because it was generally believed that the unique tetrameric conformation of Eg5 provides an irreplaceable centrosome-separating force within the mitotic spindle. Data obtained from the *Xenopus* homolog of Kif15, Xklp2, have demonstrated that this motor forms a dimer [21]. Therefore, it is unlikely that Kif15 can directly crosslink and slide antiparallel microtubules apart.

How, then, does Kif15 contribute to centrosome separation? Here, we show that Kif15 is sufficient to drive centrosome separation and that this requires both the motor domain and the C-terminal interaction with TPX2. It is therefore tempting to speculate that Kif15 is able to slide antiparallel microtubules apart through plus-end-directed motility on one microtubule, while at the same time maintaining a static interaction with a second microtubule through its interaction with TPX2 (Figure 6). Alternatively, TPX2 could crosslink two Kif15 dimers, essentially creating a tetramer that can directly bind two microtubules and slide them apart. Hopefully, future in vitro experiments will be able to address these hypotheses. Intriguingly, in our model, Kif15 and TPX2 mainly act at the spindle equator, where most antiparallel microtubules are present, while both Kif15 and TPX2 localize all along the spindle. Perhaps a specific, active pool of these proteins localizes to the equator. Consistent with this, the sea urchin kinesin-12 motor KRP180 specifically localizes to this region of antiparallel microtubule overlap [23].

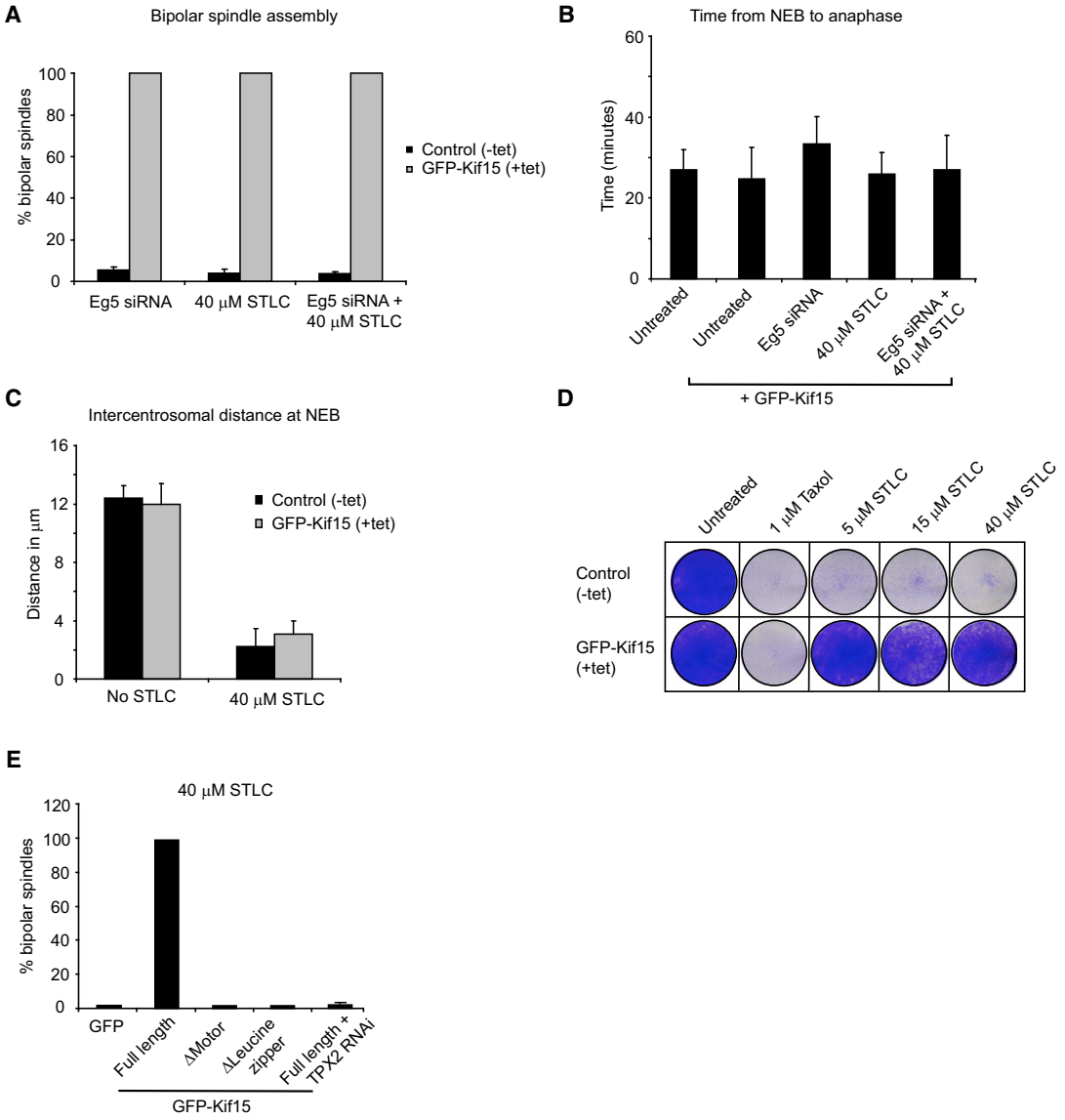

Figure 5. Ectopic Expression of Kif15 Can Drive Bipolar Spindle Assembly in the Absence of Eg5 Activity

(A and B) U2OS cells expressing GFP-Kif15 under a tetracycline-inducible promoter were transfected with Eg5 siRNA where indicated, synchronized through a 24 hr thymidine block, subsequently released for 6 hr, treated with 40 μM STLC where indicated, and analyzed by time-lapse microscopy. GFP-Kif15 expression was induced by addition of tetracycline during the thymidine block. The percentage of bipolar spindles (A) and the time from NEB to anaphase (B) was then determined.

(C) U2OS cells expressing GFP-Kif15 under a tetracycline-inducible promoter were induced to express Kif15 for 24 hr and subsequently treated with STLC for 4 hr. Cells were then fixed and stained for γ-tubulin and GFP, and the intercentrosomal distance immediately before NEB (as determined by high level of chromosome condensation) was measured.

(D) U2OS cells expressing GFP-Kif15 under a tetracycline-inducible promoter were plated in six-well format at a density of 50,000 cells per well, and, where indicated, 1 μg/ml tetracycline was added to induce GFP-Kif15 expression. Twenty-four hours after plating, cells were treated with the indicated drugs. After 5 days, cells were fixed with methanol and stained with crystal violet.

(E) U2OS cells stably expressing mCherry-α-tubulin were transfected with the indicated plasmids and treated as described in (A).

n = 50 cells (A and B), 15 cells (C), or 25 cells (E) were analyzed per condition; data presented are averages of three independent experiments. Error bars represent SD.

Spindles in mammalian cells show clear signs of bistability: they are either monopolar or bipolar with a fixed length. Decreasing the dose of Eg5 activity during bipolar spindle assembly does not result in formation of shorter spindles, but, at a critical low threshold of Eg5 activity, spindles undergo a "switch" and form a monopolar configuration [19]. In contrast, in Kif15-depleted cells, spindles often stay at an intermediate length for a prolonged period of time before turning into either a monopolar or bipolar spindle. Furthermore, we have shown that Kif15-depleted cells are unable to maintain a bipolar spindle in metaphase when Eg5 is inhibited. Interestingly, an independent study by Vanneste et al. in this issue of *Current Biology* also reveals a role for Kif15/Hklp2 in bipolar spindle maintenance [24]. Based on these findings, we propose that initial centrosome separation is mainly dependent on Eg5 but that, once centrosomes have separated, Kif15

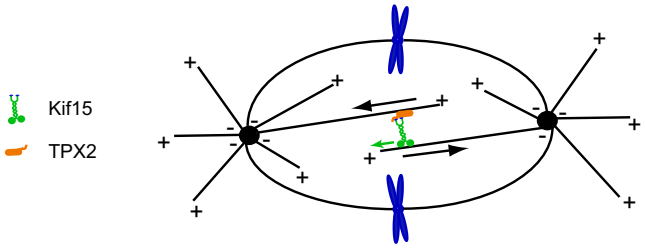

Figure 6. Model of Kif15 and TPX2 Function during Spindle Assembly

Kif15 binds a microtubule with its motor domain and walks toward the plus end of that microtubule. At the same time, Kif15 binds TPX2 on a neighboring, antiparallel microtubule through its C-terminal domain and thereby crosslinks the two microtubules. Now, plus-end-directed motility of Kif15 will result in antiparallel sliding, which will push the centrosomes apart.

contributes to rapid spindle elongation, ensuring a switch-like behavior of the spindle. Consistent with a role for Kif15 during the later stages of bipolar spindle assembly, we found that initial centrosome separation during prophase, which is dependent on Eg5 [9], cannot be reconstituted by Kif15.

Inhibitors of Eg5 have been shown to have potent antitumor activity in preclinical studies and are currently being tested in clinical trials as a potential novel anticancer treatment [25]. Our results could have major implications for inhibition of Eg5 as an anticancer therapy, because they show that Kif15 can take over all essential functions of Eg5. It is therefore possible that tumor cells can acquire resistance to Eg5 inhibitors, simply by upregulating Kif15 protein levels. Combined inhibition of Eg5 and Kif15 might have more potent antitumor activity, and the development of Kif15 inhibitors could therefore be of great value.

Experimental Procedures

Cell Culture, Transfection, and Drug Treatments

U2OS and HeLa cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) with 6% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. siRNA was transfected via reverse transfection with HiPerFect (QIAGEN) according to the manufacturer's guidelines. siRNA information is listed in Table S1. DNA transfections were performed with FuGENE 6 (Roche) according to the manufacturer's guidelines. STLC and paclitaxel (Sigma) were used at the indicated concentrations, MG132 (Sigma) was used at 5 μM, and thymidine (Sigma) was used at 2.5 mM. Tetracycline-inducible expression of GFP-Kif15 was performed as described previously [26].

Plasmid Construction

A murine Kif15 cDNA clone was obtained from Open Biosystems (clone ID 6409082). Using this cDNA as a template, the open reading frame (ORF) of mKif15 was amplified by polymerase chain reaction (PCR) with the primers forward 5′-GATCCCGCGGGCCCCCGGCTGCAAATCTGAG-3′ and reverse 5′-GATCGCGGCCGCTTAGAATTCCTTCTTTTTTTCTTTC-3′ and ligated into the pGEM-T vector. The ORF was subsequently recloned into SacII-NotI sites of a modified version of pcDNA4-TO (Invitrogen) carrying a biotinylation tag and a GFP (referred to as pTON-bEGFP). The Δleucine zipper (amino acids 1–1293) and Δmotor (amino acids 342–1386) were subcloned in the pTON-bEGFP. GFP-TPX2 was as described previously [27].

Immunofluorescence

Cells were grown on 10 mm glass coverslips and fixed either with cold methanol or with 3.7% formaldehyde with 1% Triton X-100 and then postfixed with cold methanol. α-tubulin antibody (Sigma) was used at 1:7500, anti-GFP (custom-made) was used at 1:500, and α-TPX2 (Santa Cruz) was used at 1:500. The polyclonal Kif15 antibody was raised against a GST-tagged protein encompassing amino acids 962–1387 of murine Kif15 and was affinity purified. Primary antibodies were incubated overnight at room temperature, and secondary antibodies (Alexa 488 and 561, Molecular Probes) were incubated for 1 hr at room temperature. DAPI was added before mounting with Vectashield (Vector Laboratories). Images were acquired on a Zeiss LSM 510 META confocal microscope with a Plan Apochromat 63×/NA 1.4 objective. Z planes were acquired with 1 μm intervals. Brightness and contrast were adjusted with Adobe Photoshop 6.0. Images are maximum-intensity projections of all Z planes unless stated otherwise.

Time-Lapse Microscopy

U2OS cells stably expressing mCherry-α-tubulin or YFP-α-tubulin were plated on eight-well glass-bottom dishes (Labtek). Cells were imaged on a Zeiss Axiovert 200M microscope equipped with a Plan Neofluar 40×/NA 1.3 oil-immersion objective in a permanently heated chamber in Leibovitz L-15 CO_2 independent medium. Images were acquired every 3 to 5 min with a Photometrics CoolSNAP HQ charged-coupled device camera (Scientific) and GFP/mCherry filter cube (Chroma Technology Corporation). Z stacks were acquired with 2 μm interval between Z slices. Images were processed and fluorescence intensities were quantified with MetaMorph software (Universal Imaging). For measurements of dynamics of centrosome separation, image stacks of U2OS cells stably expressing GFP-centrin were acquired every 90 s. The distance between centrosomes was then calculated after measuring the X, Y, and Z distances.

RT-PCR

HeLa cells were harvested at 48 hr following siRNA transfection. Total RNA was isolated with the QIAGEN RNeasy Mini Kit according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA with SuperScript II reverse transcriptase (Invitrogen) and oligo(dT) primers. The resultant cDNA was used as a template for PCR amplification with specific primers (sequences detailed in Table S2).

Immunoprecipitation and Western Blotting

For coimmunoprecipitations, 293T cells were cotransfected with indicated Kif15 constructs, GFP-TPX2, and the biotinylase BirA via standard calcium phosphate transfection. Paclitaxel was added 24 hr after transfection to synchronize cells in mitosis. Whole-cell lysates were then prepared, and Kif15 mutants were precipitated with streptavidin magnetic beads (Invitrogen). TPX2 and Kif15 were then detected with α-GFP and α-TPX2 antibodies. For analysis of Kif15 knockdown, whole-cell lysates were prepared 48 hr after siRNA transfection and Kif15 was detected with an affinity-purified Kif15 antibody (1:500 dilution).

Colony Formation

U2OS cells expressing GFP-Kif15 under a tetracycline-inducible promoter were seeded at a density of 50,000 cells per well of a six-well plate, and GFP-Kif15 was induced by addition of 1 μg/ml tetracycline. Twenty-four hours after plating and induction of GFP-Kif15, the indicated concentrations of paclitaxel and STLC were added to the culture medium. Cells were then grown for 5 days, and fresh medium including drugs was added every 2 days. Cells were then fixed with methanol and stained with crystal violet.

Supplemental Data

Supplemental Data include eight figures and two tables and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01601-7.

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