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Report

Kif18A Uses a Microtubule Binding Site in the Tail for Plus-End Localization and Spindle Length Regulation

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Summary

The mitotic spindle is a macromolecular structure utilized to properly align and segregate sister chromatids to two daughter cells. During mitosis, the spindle maintains a constant length, even though the spindle microtubules (MTs) are constantly undergoing polymerization and depolymerization [1]. Members of the kinesin-8 family are important for the regulation of spindle length and for chromosome positioning [2–9]. Kinesin-8 proteins are length-specific, plus-end-directed motors that are proposed to be either MT depolymerases [3, 4, 8, 10, 11] or MT capping proteins [12]. How Kif18A uses its destabilization activity to control spindle morphology is not known. We found that Kif18A controls spindle length independently of its role in chromosome positioning. The ability of Kif18A to control spindle length is mediated by an ATP-independent MT binding site at the C-terminal end of the Kif18A tail that has a strong affinity for MTs in vitro and in cells. We used computational modeling to ask how modulating the motility or binding properties of Kif18A would affect its activity. Our modeling predicts that both fast motility and a low off rate from the MT end are important for Kif18A function. In addition, our studies provide new insight into how depolymerizing and capping enzymes can lead to MT destabilization.

Results and Discussion

Kif18A Perturbation Differentially Affects Spindle Length and Chromosome Congression

Spindle morphogenesis is important because defects in spindle organization often perturb the correct timing or the accuracy of chromosome segregation. One critical parameter of spindle morphogenesis is spindle length. For example, in *Xenopus* embryos, spindle lengths correlate with cell size in small cells; however, in larger cells, spindle length reaches an upper limit [13]. There are several models for how the spindle maintains a constant length, including regulation of microtubule (MT) dynamics, a balance of pushing and pulling forces, and a spatial gradient of diffusible morphogens [1].

It was previously shown that proteins that control spindle MT dynamics play a more critical role in spindle length determination than do proteins that mediate MT sliding [2]. Depletion of the human kinesin-8 Kif18A results in an increase in spindle length in addition to its more well-characterized role in mediating chromosome congression [4, 7, 9]. In contrast, depletion of the kinesin-14 HSET results in shorter spindles, which is mediated by HSET crosslinking and sliding of spindle MTs [14]. We therefore asked how Kif18A and HSET coordinately regulate the length of the spindle. Kif18A and HSET were depleted from HeLa cells individually and in combination, and the spindle lengths were measured (Figures 1A–1C). As previously described, depletion of Kif18A resulted in abnormally long bipolar spindles relative to control ($p < 0.001$) [4], whereas depletion of HSET caused shorter spindles ($p < 0.01$) [14]. When HSET was knocked down in combination with Kif18A, the long spindle length phenotype of Kif18A depletion was rescued to levels similar to control ($p = 0.06$). These results support the idea that MT dynamics and MT sliding are both important for spindle length regulation.

Previous studies suggest that the effect of Kif18A on chromosome oscillations is not a result of a change in spindle length but on threshold amounts of the protein [7]. Consistent with this idea, we found that although codepletion of Kif18A and HSET could restore spindle length, the chromosome misalignment phenotype caused by Kif18A knockdown alone could not be mitigated (Figure 1D). One possibility is that although the spindles in the double depletions are near control lengths, the MT organization in these spindles could be highly perturbed and indirectly affect chromosome congression. In addition, Kif18A acts upstream of astrin and Kif2B, which suggests that the effects of Kif18A on chromosome congression may be indirect by regulating astrin localization to kinetochores [15]. These results are consistent with the idea that Kif18A may use different mechanisms to control spindle length and congression at different times during mitosis and highlight the need for a better understanding of the role of Kif18A in spindle length regulation.

Full-Length Kif18A Is Needed for Microtubule Plus-End Targeting and Spindle Length Regulation

To ask how Kif18A controls spindle length, we generated a series of truncated versions of Kif18A to identify the protein domain requirements for proper spindle association. GFP-tagged Kif18A domain deletion constructs were expressed in HeLa cells and then analyzed for their ability to target to spindles and to regulate spindle length and chromosome alignment (Figure 2). Full-length Kif18A (G:Kif18A-FL) localized to the plus ends of MTs (Figure 2A) similar to endogenous Kif18A [4]. Mitotic cells with overexpressed Kif18A-FL had shorter spindles than control cells transfected with GFP-H2B (Figure 2B; $p < 0.05$), but overexpression did not affect the percentage of cells with aligned chromosomes (Figure 2C; $p = 0.48$). G:Kif18A-CD+Neck, which contains only the catalytic domain and the neck, was primarily localized in the cytoplasm of both interphase (data not shown) and mitotic cells with only a faint localization on spindle MTs, suggesting that domains within the stalk and tail are important in mediating proper localization of Kif18A to MT plus ends. Both the length of the spindle ($p = 0.37$) and the percentage of chromosome alignment ($p = 0.53$) in G:Kif18A-CD+Neck cells were similar to control cells.

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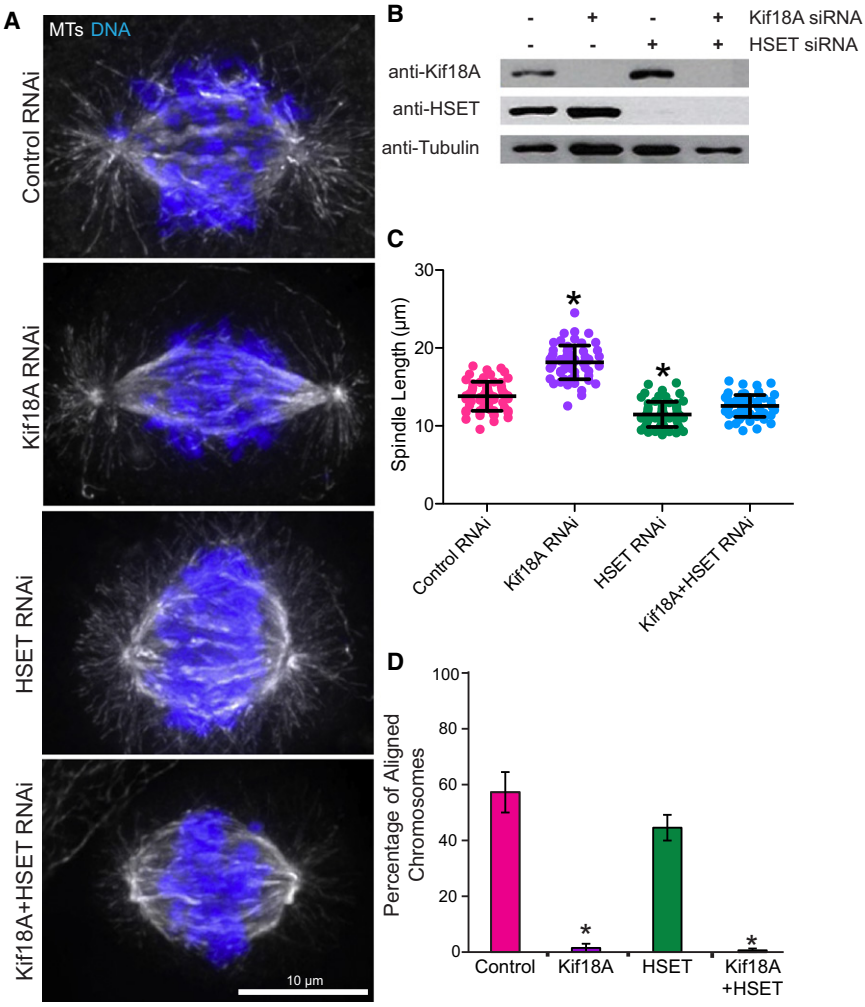

Figure 1. Kif18A and HSET Antagonistically Control Spindle Length

(A) HeLa cells transfected with luciferase (control) or the indicated siRNAs were stained for microtubules (MTs) (white) and DNA (blue). Scale bar represents 10 μm.

(B) Western blot of cells treated with luciferase, Kif18A, HSET, or Kif18A+HSET siRNAs and then probed with anti-Kif18A, anti-HSET, or anti-tubulin antibodies.

(C) Quantification of the spindle lengths from at least three independent experiments. For each knockdown condition, a total of at least 60 cells were scored for spindle length, and dot plots showing the lengths are graphed with the mean ± standard deviation (SD) indicated by the bar and whiskers.

(D) The percentages of cells containing bipolar spindles with aligned chromosomes for each condition were determined from >100 total cells in three independent experiments, and the mean ± standard error of the mean (SEM) is graphed. *p < 0.05 is relative to control.

G:Kif18A-CD, which contains only the catalytic domain, localized to MTs in interphase (data not shown). However, in contrast to G:Kif18A-CD+Neck, G:Kif18A-CD localized robustly along the length of the MTs in mitotic cells with no effect on spindle length ($p = 0.71$) or on chromosome alignment ($p = 0.95$). The construct containing only the stalk and tail domains (G:Kif18A-ST) was nuclear during interphase (data not shown) and surprisingly localized along MTs in mitotic cells. This result indicates the presence of an additional MT binding domain within Kif18A besides the catalytic domain. G:Kif18A-ST did not alter spindle length ($p = 0.26$) or chromosome alignment ($p = 0.11$) relative to control, suggesting that the stalk-tail domain may be necessary but not sufficient to target Kif18A to the plus ends of MTs. Together, these results show that proper targeting of Kif18A to the plus ends of MTs and proper spindle length control require both the catalytic domain and a second MT binding domain within the stalk-tail region. This requirement of proper localization for function was also observed for the *Drosophila* kinesin-8, Klp67A [6]. Although mediated by different mechanisms, proper localization of both Kif18A and Klp67A is required for regulation of spindle length.

The Additional Kif18A Tail MT Binding Domain Is Important for MT Plus-End Targeting and Spindle Length Regulation

The data above suggest that Kif18A contains an additional MT binding domain in its stalk or tail domain. Because other kinesins have second MT binding domains within the tail [16–18], we generated three tail deletion constructs of Kif18A and tested their ability to bind MTs in vitro (Figures 3A and 3B). All three of these proteins exhibited saturable binding to MTs in the absence of nucleotide with apparent K_d values that were indistinguishable (Figures 3C and 3D). These results suggest that amino acids 802–898 are sufficient for MT binding in vitro and constitute an ATP-independent MT binding site.

Based on the observation that G:Kif18A-CD+Neck was unable to localize to the plus ends of MTs, we hypothesized that the additional MT binding site in the tail domain was important for Kif18A localization and function. To test this idea, we compared the cellular localization and spindle lengths of HeLa cells transfected with the minimal MT binding domain alone, G:Kif18A(802–898), to cells transfected with Kif18A without the minimal MT binding domain, G:Kif18A(2–801) (Figures 3E and 3F). Consistent with our in vitro data, G:Kif18A(802–898) bound robustly to spindle MTs and did not alter spindle length relative to control G:H2B transfection ($p = 0.54$). Truncation of the additional MT binding domain of Kif18A in G:Kif18A(2–801) prevented MT localization and did not alter spindle length ($p = 0.17$). These results support the idea that the additional MT binding site in the tail domain is needed for proper localization of Kif18A at MT plus ends and suggest that proper localization is necessary for Kif18A regulation of spindle length.

Mathematical Modeling Predicts that High MT Plus-End Association Is Required for Kif18A Regulation of Spindle Length

It was shown previously that Kif18A, similar to the yeast ortholog Kip3p, pauses at the plus ends of MTs [11, 12], which may be important in the cooperative behavior of kinesin-8 proteins to induce MT destabilization [11]. We postulated that the tail domain of Kif18A would be important in facilitating Kif18A

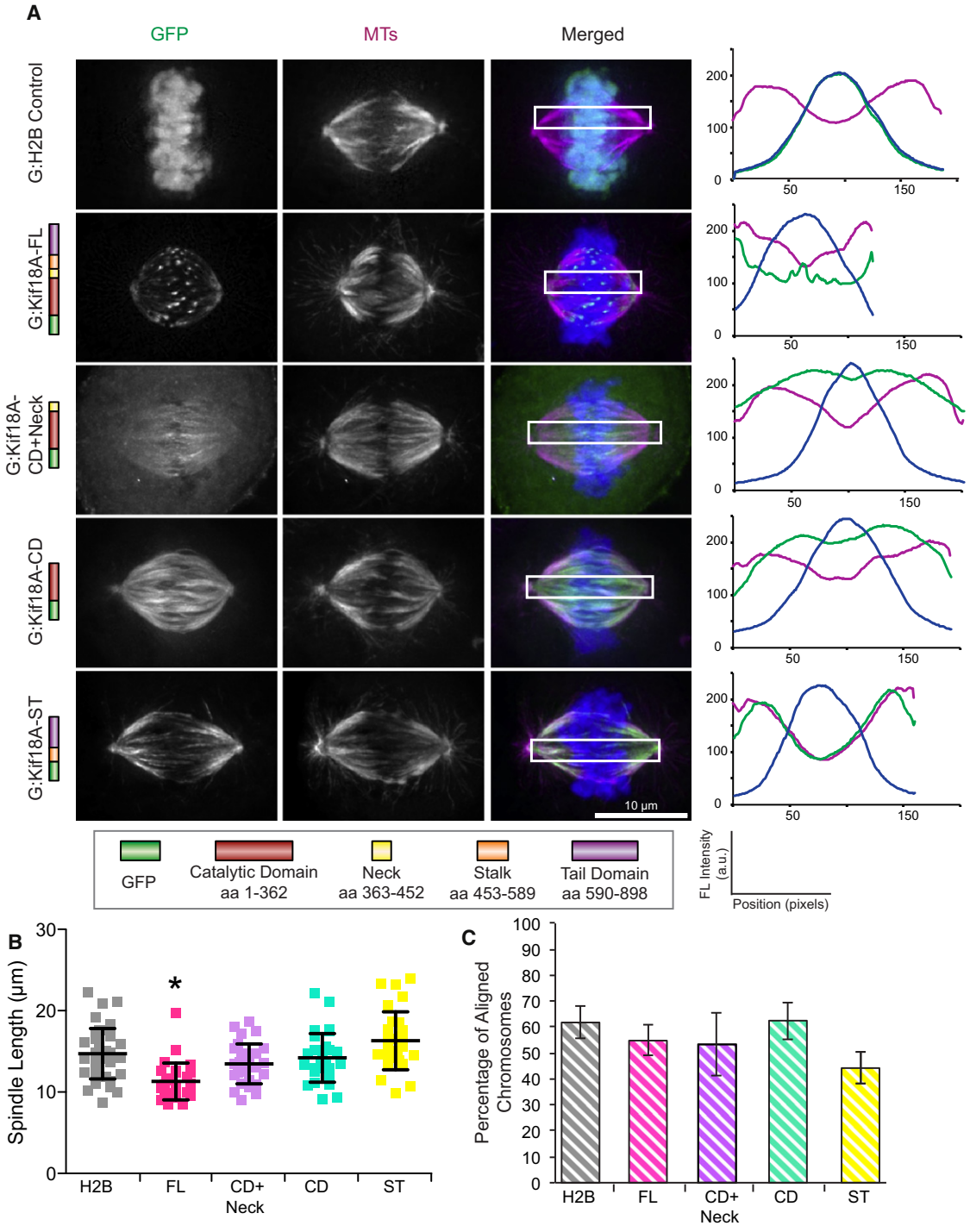

Figure 2. Overexpression of GFP-Kif18A Domain Deletion Proteins Does Not Alter Spindle Length or Chromosome Alignment

(A) HeLa cells were transfected with the indicated GFP-fusion construct (green) and stained for MTs (magenta) and DNA (blue). Scale bar represents 10 μm. The boxed region represents the region used to generate the line scans, which show the localization of Kif18A (green) relative to DNA (blue) and MTs (magenta).

(B) Spindle lengths from at least three independent experiments in which a total of 30 spindles for each Kif18A domain construct are graphed as dot plots with the mean ± SD indicated by the bar and whiskers. *$p < 0.05$ is relative to control.

(C) The average percentages of transfected cells with aligned chromosomes for each construct are indicated as the mean ± SEM from >100 total cells in three independent experiments.

accumulation at MT ends, either by increasing the efficacy of transport to the MT ends or by reducing dissociation from the MT end. To test these ideas, we developed a mathematical simulation to look at the effects of Kif18A on the steady-state length distribution of a dynamic MT population. Whereas other studies have modeled the behavior of kinesin-8s on stabilized

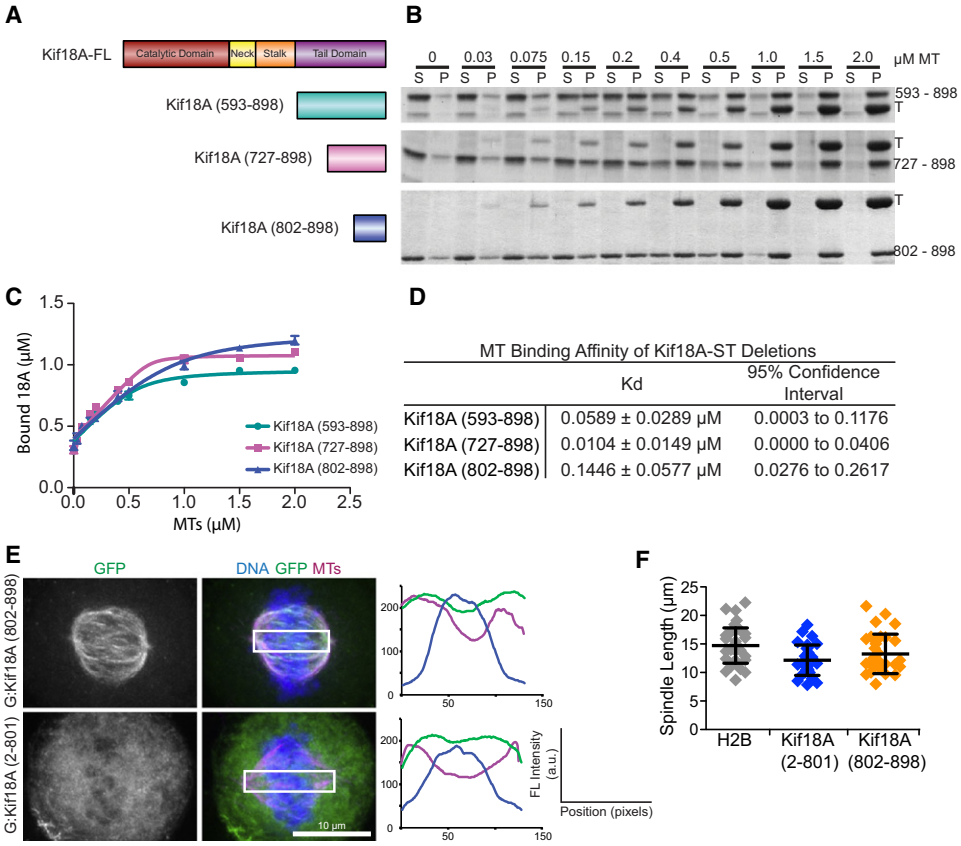

Figure 3. The Tail Domain of Kif18A Binds Microtubules In Vitro and In Vivo

(A) Schematic diagrams of Kif18A constructs.

(B) GST-Kif18A(593–898), GST-Kif18A(727–898), or GST-Kif18A(802–898) (1.3 μM) was incubated with increasing concentrations of preassembled MTs (0–2 μM) (T) for 15 min at room temperature in the absence of nucleotide. Soluble proteins were separated from MT-bound proteins by ultracentrifugation, and equivalent amounts of supernatant (S) and pellet (P) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining.

(C) The amount of protein in the S and P was quantified, and the binding curves were fit to the one-site quadratic MT binding equation. Each point on the curve represents the mean ± SEM from at least three individual experiments.

(D) Summary table of binding data in (C).

(E) HeLa cells were transfected with GFP fusions of Kif18A(802–898) or Kif18A(1–801) (green) and stained for DNA (blue) and MTs (magenta). The boxed region represents the region used to generate the line scans, which show the localization of Kif18A (green) relative to DNA (blue) and MTs (magenta). Scale bar represents 10 μm.

(F) The average spindle lengths for each construct relative to control are graphed as dot plots with the mean ± SD indicated by the bar and whiskers for >30 cells in at least three independent experiments.

MTs [10] or used automated image analysis to understand the behavior of kinesin-8s on interphase MTs [19], we were interested in testing how Kif18A would act on a population of dynamic mitotic MTs. In our simulation, 20 MTs were nucleated with a defined set of parameters governing dynamic instability that represented a mitotic state [20] (see Table S1 available online). The population of MTs reached a steady-state length of 10.7 ± 1.2 μm within 10 min (Figure S1). Fifty motors were included in the simulation with a plus-end-directed walking velocity (V_{motor}) that ranged from 50 nm s^{-1} to 400 nm s^{-1}, which covers the range of previously measured motor velocities of the kinesin-8 family [3, 8, 12]. The motor off rate from the MT lattice ($k_{off,lattice}$) was set at 0.013 s^{-1} for all simulations, as previously reported for Kif18A [12]. Because there are conflicting results in the literature as to whether Kif18A acts as a MT depolymerase or as a MT capping protein in vitro [4, 12], we ran simulations comparing these behaviors. We defined a MT depolymerase as a motor that increases the frequency of catastrophe by 4-fold, as described for the kinesin-13 protein MCAK [21, 22] (Figure 4A), whereas a capping protein was defined as a motor that stopped net growth at the MT end until the microtubule tip had a catastrophe event or until the motor stochastically dissociated from the MT end (Figure 4B).

With our simulation, we first tested how changes in the plus-end-directed velocity of the motor (V_{motor}) would alter the average length of the MTs. At lower velocities (50 to 175 nm s^{-1}), there was no effect on the average length of the MTs for either a depolymerase (Figure 4C) or a capping protein (Figure 4D). However, at V_{motor} = 200 nm s^{-1} or higher, both a depolymerase and a capping protein significantly decreased the average length of the MT population. Consistent with these predictions, using a fixed $k_{off,\ lattice}$ and increasing V_{motor} increases the processivity of the motor. This increased processivity allows the motor to reach the MT end and alter the MT length (Figure S2). It was previously shown that kinesin-8 motors need to walk along the MT at least as quickly as plus-end growth to be able to act on that MT end [3]. Thus,

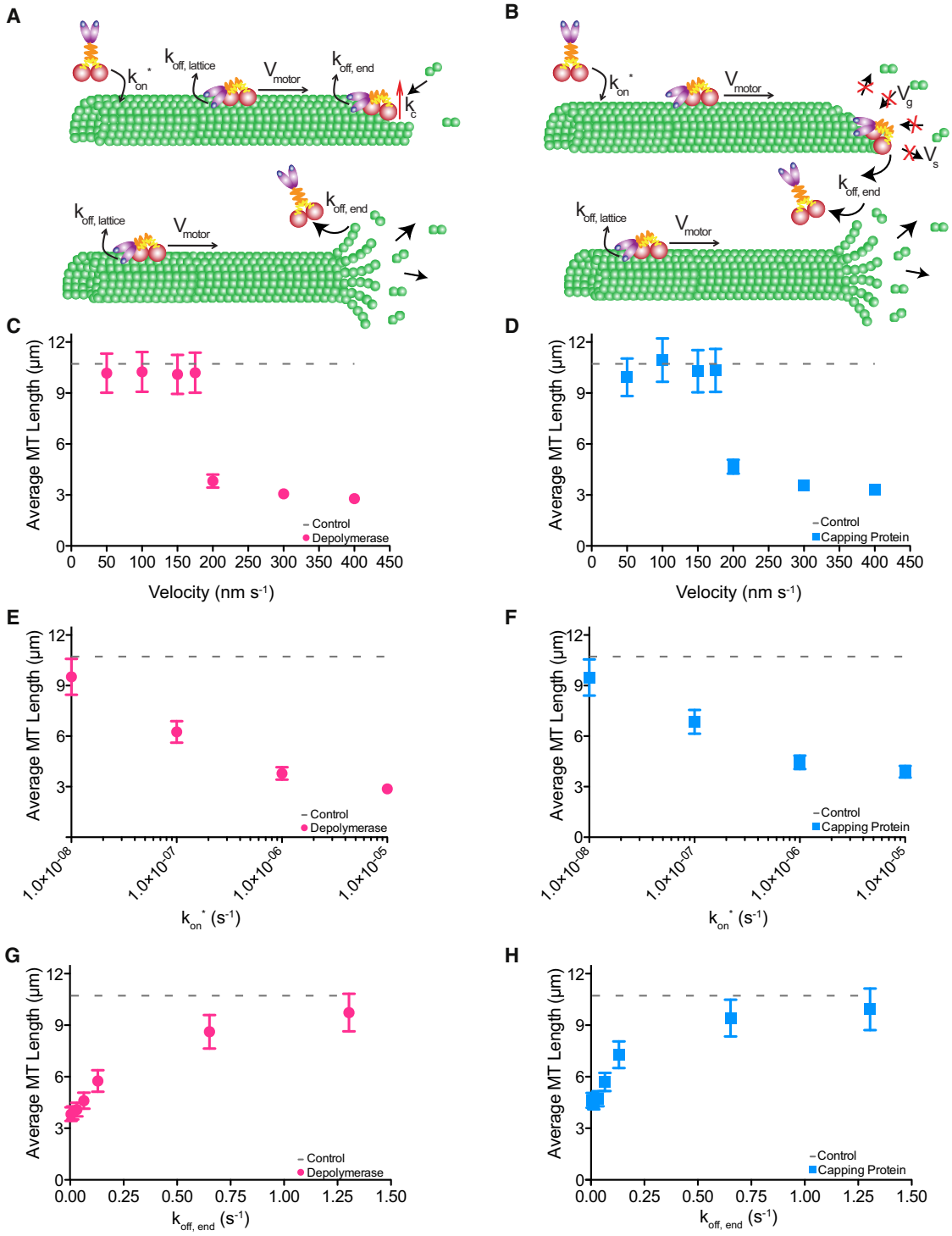

Figure 4. Mathematical Modeling Predicts that MT Plus-End Affinity Is Important for Regulation of MT Length

Simulations were performed in which 20 dynamic MTs were nucleated and 50 Kif18A motors were added with a variety of different dynamics parameters, including the k_{on}*, $k_{off,lattice}$, $k_{off,end}$, and motor velocity.

(A) A MT depolymerase was defined as a motor that increases the catastrophe frequency.

(B) A MT capping protein was defined as a protein that reduces MT growth velocity to 0 nm s^{-1}.

(C–H) Average MT length ± SEM of 75 trials recorded at the end of each 60 min simulation. The control value is indicated by the dashed line. The average MT length decreases with increasing V_{motor} for a MT depolymerase (C) or a capping protein (D). The average MT length decreases with increasing k_{on}* for a MT depolymerase (E) or a capping protein (F). Decreasing the $k_{off,end}$ from the end has only a modest effect on the activity of a MT depolymerase (G); however, as $k_{off,end}$ is increased, a MT capping protein has a decreased ability to destabilize MTs (H).

our simulation accurately depicts this aspect of kinesin-8 function.

Because it was shown that kinesin-8 proteins are cooperative [11], we next tested how varying the number of motors bound to the MT (on rate) would affect the length distribution of the MT population. To do this, we varied the motor on-rate constant (k_{on}*), to change the steady-state motor on rate (k_{on}) in the simulation (where $k_{on} = k_{on}$* [MT polymer]) [8, 23]. We used V_{motor} = 200 nm s^{-1} and the published k_{off} = 0.013 s^{-1}. When the k_{on}* was low, such that only a few motors bound to the MTs (Figures S3A and S3B), the average length of MTs in the presence of either a depolymerase or a capping protein was similar to the control with no added motor (Figures 4E and 4F). However, as we increased k_{on}* for the motor, there was a decrease in the average length of the MTs. Interestingly, the MT plus ends in the capping protein simulation accumulated higher motor numbers with increasing k_{on}* in comparison to the depolymerase simulation (Figures S3C and S3D). Although we did not simulate a cooperative motor effect, this result suggests that cooperative motors would work more efficiently for a capping protein that attenuates MT growth rate than for a depolymerase. Together, these simulation results predict that when more motors are bound to the MT, this increases the probability that more motors will reach the MT plus end and therefore alter the MT dynamics.

We were surprised to find that a depolymerase and a capping protein caused a similar decrease in the average MT length in the simulation. Although the overall effect of the two motors on the average MT population can result in similar phenotypes, the mechanism the motor utilizes to cause this phenotype is likely different. With a depolymerase, the motor would simply induce a catastrophe of a growing MT. However, when a simulated capping protein reaches the MT plus end, the dynamics of the MT are attenuated, halting MT growth. In the simulation, this attenuation of plus-end growth continues until the plus end has a catastrophe event, at which time the motor dissociates from the depolymerizing MT plus end. Therefore, the simulations demonstrate that a capping protein that halts net growth at the MT end may act to effectively limit the maximum length the MTs can achieve, similar to a MT depolymerase that acts to promote MT catastrophe events.

Our cellular results support the idea that the second MT binding site within the Kif18A tail facilitates MT plus-end localization that is necessary for Kif18A activity. Because Kif18A accumulates at MT plus ends, and because both Kip3p and Kif18A were shown to pause at the end of the MT [11, 12], one possibility is that the Kif18A tail could act by preventing dissociation of Kif18A from the MT end. To test this idea, we ran simulations with V_{motor} = 200 nm s^{-1} and k_{on}* = 1 × 10^{-6} s^{-1} μM^{-1}, but we varied the motor off rate at growing MT plus ends ($k_{off,end}$) from 2.5-fold lower to 500-fold higher than $k_{off,lattice}$ (Figures 4G and 4H; Table S1). For a depolymerase (Figure 4G), increasing $k_{off,end}$ from 0.013 s^{-1} to 6.5 s^{-1} resulted in a significant increase in the average MT length ($p < 0.001$). Similarly, increasing $k_{off,end}$ of a capping protein from 0.013 s^{-1} to 6.5 s^{-1} (Figure 4H) resulted in an increase in the average MT length ($p < 0.001$), suggesting that a motor that readily dissociates from the MT end would be ineffective as a capping enzyme. One interesting difference between a depolymerase and a capping protein was that increasing the $k_{off,end}$ for a capping protein had a more dramatic effect on the number of motors at the MT plus end (Figures S3F and S3G). These simulation results support the idea that the MT binding domain in the Kif18A tail helps Kif18A accumulate at growing MT plus ends to effectively modulate MT plus end dynamics. Therefore plus-end association could be critical for Kif18A to effectively control MT plus-end dynamics and for regulating spindle length in cells. We propose that the additional MT binding site in the tail domain acts as a tether at the MT plus end in order for multiple Kif18A motors to accumulate at MT plus ends, resulting in shorter MTs.

The existence of a second MT binding site in Kif18A was also recently identified by others [24, 25], who found that this MT binding site was necessary to confer effects of overexpressed Kif18A on chromosome oscillations. Consistent with these studies, a second MT binding domain in the tail of yeast Kip3p was identified, which is important for both its MT stabilizing and destabilizing effects in vivo [25]. Taken together, these data show that a secondary MT binding domain in the tail of kinesin-8 proteins is an important and conserved functional domain.

It is also important to understand the mechanisms by which Kif18A regulates MT plus-end dynamics and how the tail may affect its activity. Our simulations reveal that the on rate to the MT lattice increases the number of MTs that are bound by the motor. However, deletion of the Kip3 tail did not have a significant effect on the on rate of the motor to the MT [25], suggesting that this is not likely to be the major mechanism by which the tail regulates kinesin-8 activity. Another possibility is that the tail MT binding domain is important for kinesin-8 processivity, as was shown with single-molecule studies on Kif18A and Kip3 [24, 25]. Processivity reflects both the velocity of the motor and its relative off rate from the MT lattice (Figure S2). Interestingly, Stumpff and colleagues found that deletion of the tail actually speeds up motor velocity but reduces run length, consistent with the idea that the tail is needed to ensure that it reaches the MT end to act there [24].

Both our simulations and our cellular data highlight the important role of the tail in maintaining plus-end association of Kif18A. In cells, only Kif18A constructs with an intact tail could accumulate at MT plus ends and reduce spindle length, which is supported by our simulation outcomes, which predict that a decrease in the off rate of Kif18A from the MT end also reduced its ability to destabilize MTs. Consistent with this idea, a tail deletion of the yeast Kip3p decreases the plus-end pause time [25]. Given these results, it was surprising that Kif18A with or without the tail is effective in promoting MT destabilization activity in vitro [24]. One possibility is that Kif18A is a less robust MT destabilizing protein without the tail domain, which may register as a kinetic difference rather than the extent of MT destabilization seen.

Our findings provide new insight into how a capping protein and a depolymerase could each provide MT destabilizing activity, although they do not definitively resolve whether kinesin-8s are capping proteins or MT depolymerizing enzymes. One idea is that because the yeasts only have members of the kinesin-8 family but not the kinesin-13 family, yeast enzymes are in essence super motors that have to act in both capacities. This could be true for Kip3, which clearly depolymerizes MTs in vitro and regulates dynamics in vivo [3, 8], but there is no evidence that Klp5/6 depolymerizes MTs in vitro despite its ability to regulate dynamics in cells [26]. In contrast, in mammalian cells, which have much more complex cytoskeletal arrays, there exist both kinesin-8 and kinesin-13 families, which could readily modulate multiple aspects of MT dynamics. For example, previous studies showed that modulation of the catastrophe frequency is

a powerful way to make large changes in the structure of the MT array, such as those that occur upon the transition from interphase to mitosis [27]. In contrast, the role of Kif18A may be more important to fine-tune the lengths of MTs in response to normal mitotic progression, such as controlling chromosome oscillations [7, 9], and to provide modulation of spindle length during bipolar spindle assembly. These studies highlight the need for multiple dynamics regulators in cells to maintain the intricate control the MT cytoskeleton.

Supplemental Information

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.08.005.

Acknowledgments

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