Current Biology 21, 1356–1365, August 23, 2011 ©2011 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2011.07.017

Article

A Complex of Kif18b and MCAK Promotes Microtubule Depolymerization and Is Negatively Regulated by Aurora Kinases

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Summary

Introduction: Spindle assembly requires tight control of microtubule (MT) dynamics. This is dependent on a variety of MT binding proteins and their upstream regulators. The Aurora kinases have several well-described functions during cell division, but it remains unclear whether they control global spindle microtubule dynamics.

Results: Here, we find that simultaneous inhibition of Aurora A and B results in a dramatic decrease in spindle MT stability, and we identify the uncharacterized kinesin-8 Kif18b as a mediator of this effect. In interphase, Kif18b is nuclear, but upon nuclear envelope breakdown, Kif18b binds to astral MT plus ends through an interaction with EB1. Surprisingly, Kif18b also binds to the kinesin-13 motor MCAK, and this interaction is required for robust MT depolymerization. Furthermore, the Kif18b-MCAK interaction is negatively regulated by Aurora kinases through phosphorylation of MCAK, indicating that Aurora kinases regulate MT plus-end stability in mitosis through control of Kif18b-MCAK complex formation.

Conclusion: Together, these results uncover a novel role for Aurora kinases in regulating spindle MT dynamics through Kif18b-MCAK and suggest that the Kif18b-MCAK complex constitutes the major MT plus-end depolymerizing activity in mitotic cells.

Introduction

Microtubules (MTs) are dynamic filaments, which are tightly controlled by MT binding proteins (both stabilizing and destabilizing proteins) and their upstream regulators [1]. Whereas many MT stabilizing proteins have been identified, the number of MT destabilizers identified to date is limited. In mammalian cells, MT motors of the kinesin-8 and kinesin-13 families have been implicated in MT destabilization. Human kinesin-13 motors—Kif2a, Kif2b, and MCAK—have an internally positioned motor domain, which cannot walk along MTs but rather uses ATP hydrolysis for MT depolymerization [2–4]. These motors have the ability to depolymerize MTs from both ends [2, 3] and can diffuse along the MT lattice to reach the ends [5]. In addition, MCAK can target MT plus ends directly through an interaction with EB1 [6–8], which enhances its overall MT destabilizing activity [9]. In contrast, kinesin-8 motors have an N-terminal motor domain with plus-end-directed motility [10–12], similar to conventional kinesin. However, in addition to their motile activity, both the budding yeast kinesin-8 motor Kip3 [10, 12, 13] and the human kinesin-8 motor Kif18a [11] have intrinsic MT plus-end depolymerizing activity. Indeed, loss of kinesin-8 motors results in increased MT length in various organisms [14–16], suggesting that they generally act to destabilize MTs. However, whether all kinesin-8 motors possess intrinsic MT depolymerizing activity remains to be established.

Human somatic cells contain two Aurora kinases, Aurora A and Aurora B, which are known to control spindle assembly during cell division. Aurora A localizes to spindle poles and has been implicated in spindle pole formation [17]. Aurora B, on the other hand, localizes to centromeres and chromosome arms (although a weak localization to the spindle has been observed [18]) and is involved in spindle checkpoint signaling, correction of erroneous kinetochore-MT attachments and cytokinesis [19–21]. Among the known substrates of Aurora A and B, many are MT binding proteins, of which the MT binding properties are often modulated by Aurora phosphorylation [17, 20]. Although many substrates of Aurora A and B are unique to one of the two isoforms, several common substrates have been identified, suggesting that they might be partially redundant (for example, [22–25]).

Results

Kif18b and MCAK Act Downstream of Aurora Kinases to Control Microtubule Stability

Although loss-of-function phenotypes of Aurora A and B have been intensively studied, much less is known about the phenotype of simultaneous inhibition of both kinases. To address this, we treated U2OS cells with the Aurora B-specific inhibitor AZD1152, with Aurora A small interfering RNA (siRNA), or with a combination of both. Consistent with known roles for Aurora A and B, inhibition of Aurora B resulted in chromosome alignment defects, whereas loss of Aurora A resulted in defects in spindle morphology (Figure 1A). Surprisingly, combined inhibition of Aurora A and B resulted in a dramatic loss of spindle MTs, with only a few very short MTs remaining (Figure 1A; see also Figure S1A available online). Similar effects were observed after treatment with 1 μM VX-680 or 1.2 μM hesperadin (Figures S1B and S1C), concentrations that inhibit both Aurora A and B, but not with 2 μM ZM447439 or with 0.8 μM MLN8054, which specifically inhibit Aurora B or A, respectively (data not shown). Thus, simultaneous inhibition of Aurora A and B results in a dramatic reduction in MT stability in mitosis.

Because kinesin-8 and kinesin-13 motors have been implicated in MT depolymerization, we asked whether they were involved in the reduced MT stability induced by Aurora inhibition. Strikingly, depletion of either the kinesin-13 member MCAK or the novel kinesin-8 member Kif18b, but not the other members of the kinesin-8 and kinesin-13 families (siRNAs were

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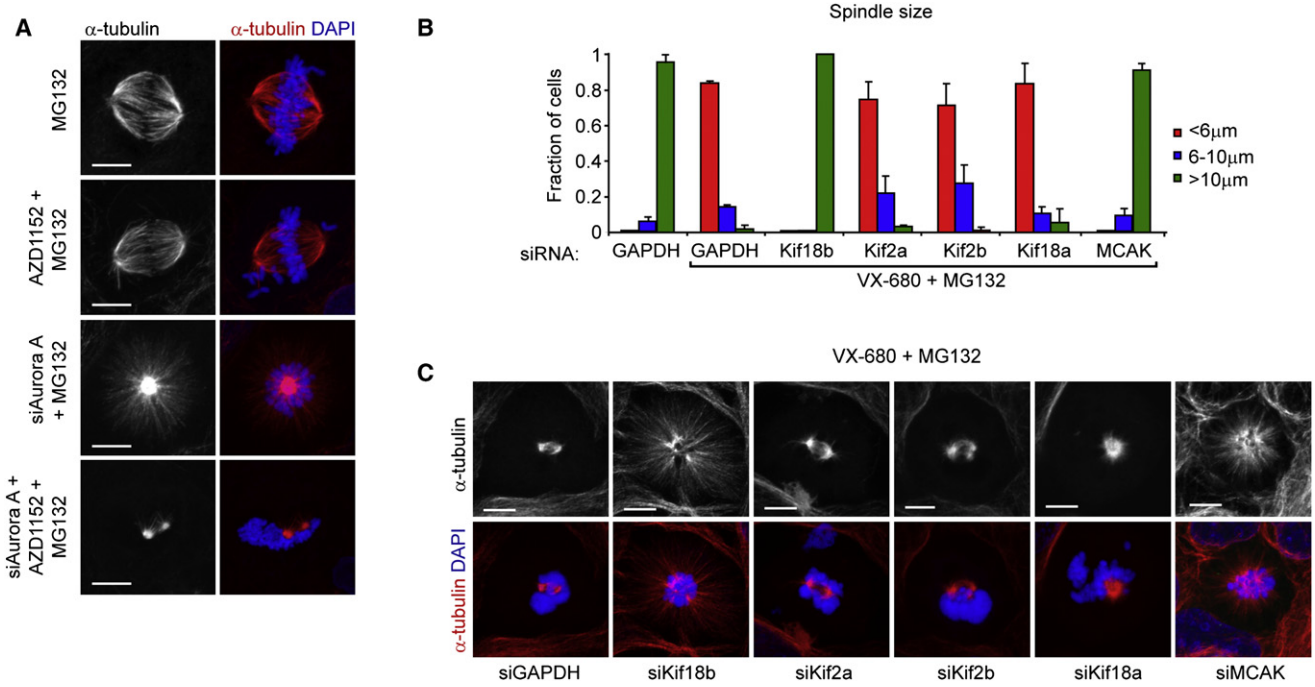

Figure 1. Aurora Kinases Prevent Excessive Microtubule Depolymerization by Kif18b and MCAK

(A–C) U2OS cells were transfected with indicated small interfering RNAs (siRNAs) and were treated with MG132 alone or in combination with indicated Aurora kinase inhibitors for 45 min. Cells were fixed and stained with indicated antibodies.

(B) Spindle size was scored in >25 cells per experiment. Graph shows average of three independent experiments. Error bars represent standard deviation. Scale bars represent 5 μm.

validated previously [26]), completely restored MT length in Aurora-inhibited cells (Figures 1B and 1C; Figures S1B–S1D). Codepletion of Kif18b and MCAK did not result in a further increase in MT length (Figures S1E and S1F), indicating that combined activity of Kif18b and MCAK promotes excessive MT depolymerization in mitosis when Aurora kinases are inhibited. Depletion of Kif18b or MCAK did not restore spindle bipolarity or chromosome alignment (Figure 1C), consistent with the fact that Aurora kinases have additional essential functions during cell division.

Kif18b Localizes to MT Plus Ends in Mitosis

Like other kinesin-8 members, Kif18b has an N-terminal motor domain, a short coiled-coil segment, and a large C-terminal tail (Figure 2A). Staining with an antibody raised against the C terminus of Kif18b produced a pattern in mitotic cells reminiscent of plus-end binding proteins (Figure 2B), which was specific for Kif18b (Figure 2C; Figure S2A). Indeed, in mitosis, Kif18b staining overlapped with EB1 staining (Figure 2B), and time-lapse analysis of GFP-Kif18b-expressing U2OS cells revealed comet-like structures moving through mitotic cells at 10–15 μm per minute (Figure S2B; Movie S1), consistent with MT growth speeds in these cells [27]. Interestingly, whereas all Kif18b comets overlapped with EB1 comets, a substantial amount of EB1 comets showed weak or no Kif18b staining, indicating that Kif18b preferentially localizes to a subset of MT plus ends (Figure 2B; Figure S2C). Specifically, astral MT plus ends showed high Kif18b staining, especially during early prometaphase (Figure 2B; Figures S2C and S2D). Similar results were obtained with a GFP-tagged version of Kif18b (Figure S2E), confirming that this was not due to masking of the epitope recognized by the Kif18b antibody. In interphase, Kif18b did not localize to MT plus ends: in G1, Kif18b was not expressed, and in G2, it was sequestered in the nucleus (Figures S2D and S2F). Time-lapse analysis of GFP-Kif18b showed that Kif18b was completely degraded at the metaphase-anaphase transition (Figure 2D; Figure S2F), which was confirmed by staining of endogenous Kif18b (data not shown) and consistent with a recent report [28]. Together, these results show that Kif18b is a mitosis-specific plus-end tracking protein that preferentially localizes to astral MT plus ends.

Kif18b Uses Its Motor Activity and Binding to EB1 to Target to MT Plus Ends

Kinesin-8 motors were shown to accumulate at MT plus ends through their plus-end-directed motility [10–12, 29], whereas conventional plus-end tracking proteins target directly to MT plus ends through binding to EB1 [30]. Disrupting the motor activity of Kif18b strongly reduced plus-end accumulation (Figure 2E). In addition, depletion of EB1 almost completely eliminated Kif18b staining on MT plus ends (Figure 2F), suggesting that Kif18b not only requires its motor activity, but also requires EB1 binding for robust plus-end accumulation. Indeed, recombinant GST-EB1 interacted directly with the tail of Kif18b (Figure 2G; Figure S2G). We found three sites in Kif18b that were similar to the SxIP consensus sequence recently shown to bind directly to EB1 [31] (Figure 2A). Mutation of site 1 together with the deletion of a small region encompassing sites 2 and 3 (hereafter called EB1-binding domain, EBBD) abolished EB1 binding and plus-end accumulation of Kif18b (Figures 2G–2I), whereas mutation of individual sites did not (data not shown). Thus, Kif18b accumulation at plus ends requires its motor activity and a direct interaction with EB1 through multiple sites in the tail of Kif18b.

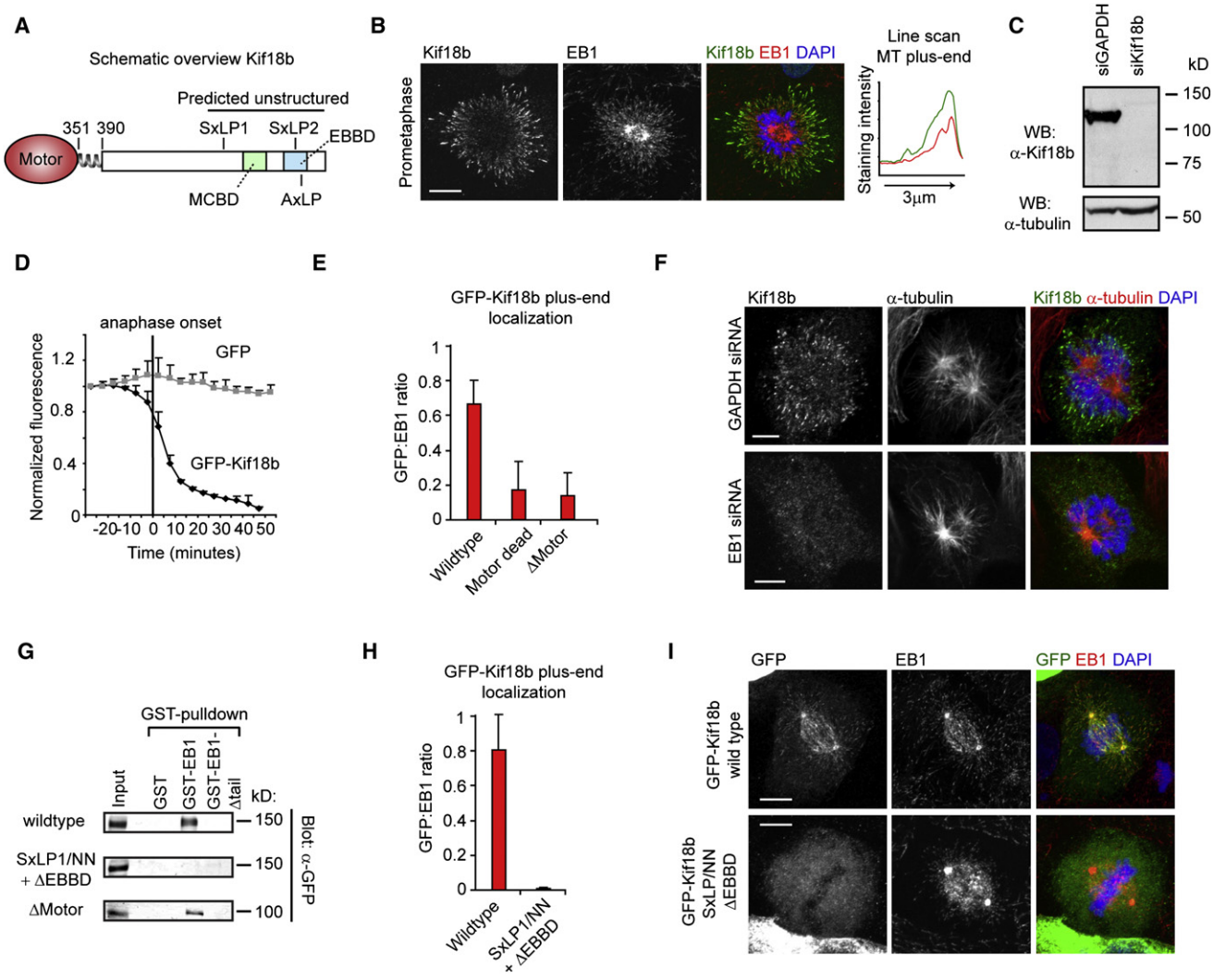

Figure 2. Kif18b Is a Mitosis-Specific Plus-End Tracking Protein

(A) Schematic overview of Kif18b domains. Motor domain, coiled-coil region, SxLP1 (aa 656–659), MCAK binding domain (MCBD), and EB1 binding domain (EBBD) containing SxLP2 (aa 777–780) and AxLP (aa 803–806) are shown.

(B) U2OS cells were fixed and stained with indicated antibodies. Line scans were made along the distal 3 μm of a microtubule (MT) plus end. Graph shows average of eight line scans.

(C) Cells were transfected with indicated siRNAs and were lysed 48 hr after transfection. Whole-cell lysates (WCLs) were probed for Kif18b or α-tubulin.

(D) Cells were transfected with either GFP or GFP-Kif18b and filmed 48 hr after transfection. Fluorescence intensity was measured over time, and the intensity at t = 0 was set to 1. Graph represents average of ten cells.

(E, H, and I) Cells were transfected with Kif18b siRNA and after 24 hr with indicated RNAi-insensitive GFP-Kif18b constructs. Forty-eight hours later, cells were fixed and stained for GFP and EB1 to determine plus-end staining intensity of the GFP-tagged constructs. (I) shows representative images. One hundred and fifty plus ends were quantified in ten cells (E and H).

(F) U2OS cells were transfected with indicated siRNAs, and 48 hr after transfection, cells were fixed and stained with indicated antibodies.

(G) HEK293 cells were transfected with GFP-Kif18b or with indicated GFP-Kif18b mutants. After 24 hr, lysates were prepared, and GST pull-downs were performed with GST alone, GST-EB1, or GST-EB1Δtail. All error bars present standard deviations. Scale bars represent 5 μm.

Kif18b Promotes MT Depolymerization during Mitosis

We next tested whether Kif18b might also affect MT stability, similar to what was shown for the kinesin-8 motors kip3 and Kif18a [10–12, 32]. Indeed, high-level overexpression of Flag-Kif18b resulted in a dramatic decrease in MT length and spindle size (Figures 3A and 3B). Time-lapse analysis of GFP-Kif18b-overexpressing cells revealed that MTs were formed normally before nuclear envelope breakdown (NEB) but rapidly depolymerized as soon as Kif18b was released from the nucleus (Figure S3A).

To determine the contribution of endogenous Kif18b to MT depolymerization in mitosis, we analyzed MT length in control and Kif18b-depleted cells. In control cells, MTs are relatively long in interphase, frequently reaching all the way to the cell cortex, but as soon as cells enter mitosis, these long MTs are lost (Figures 3C and 3D). In contrast, in early prometaphase cells depleted of Kif18b (see Figure 2C for knockdown), many MTs were still observed that reached the cortex (Figures 3C and 3D), indicating that Kif18b is required for rapid disassembly of the interphase MT network as cells enter mitosis.

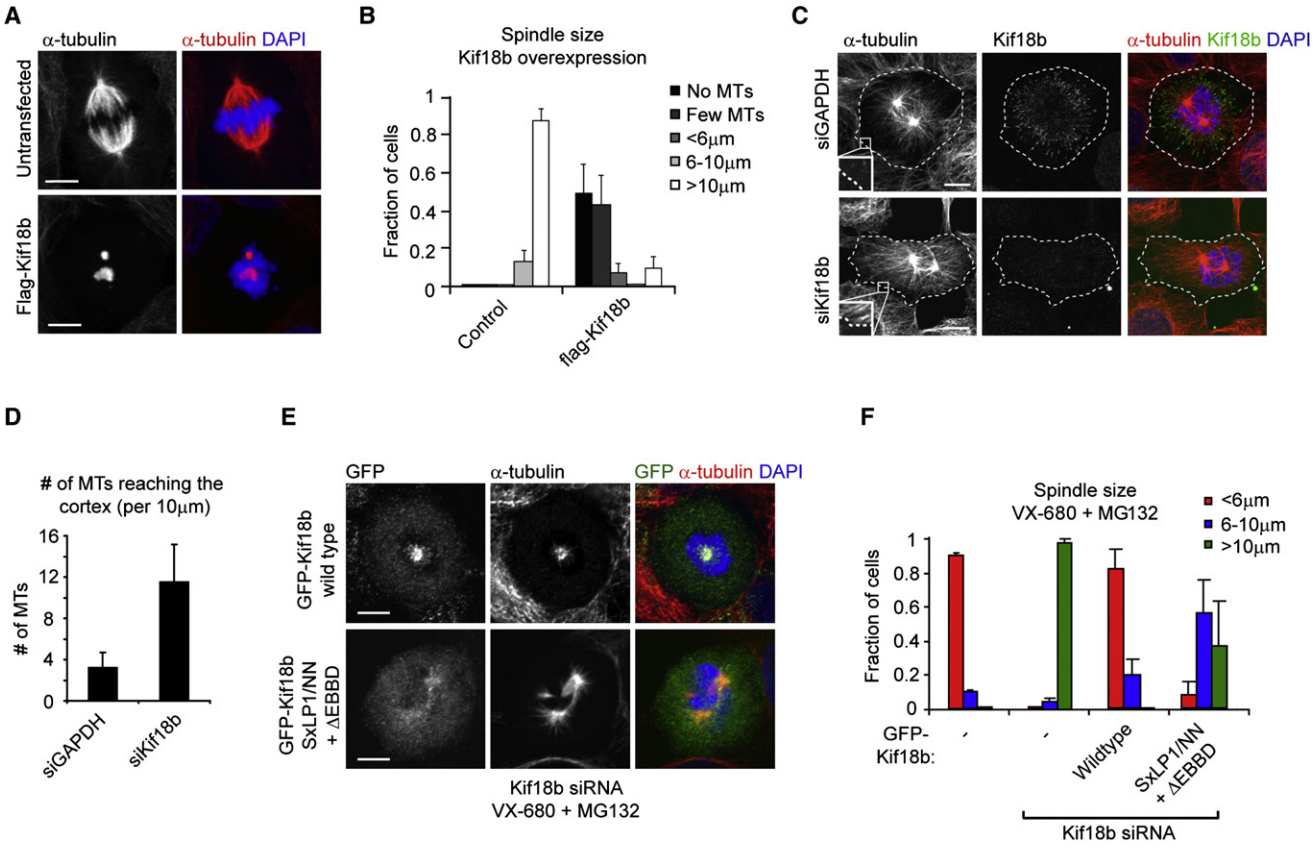

Figure 3. Kif18b Promotes Mitosis-Specific MT Depolymerization

(A and B) U2OS cells were transfected with Flag-Kif18b, fixed 48 hr after transfection, and stained for Flag and α-tubulin.

(B) Spindle size was quantified for >10 cells per experiment.

(C) U2OS cells transfected with indicated siRNAs. Forty-eight hours after the first transfection, cells were fixed and stained with indicated antibodies. Early prometaphase cells were selected based on DAPI staining. Dotted line indicates cell perimeter. Boxed area is enlarged in the inlay.

(D) Quantification of (C). A 10 × 1 μm area adjacent to the cortex furthest from the centrosomes was selected, and the number of MTs in this area was scored. Five cells were scored per experiment.

(E and F) Cells were transfected with Kif18b siRNA where indicated and subsequently with indicated plasmids. Forty-eight hours after plasmid transfection, cells were treated with 1 μM VX-680 + MG132 for 45 min and fixed and stained for GFP and α-tubulin. Spindle size was determined in 15 cells per condition. Graphs show averages of five (B) or three (E and F) independent experiments. All error bars represent standard deviations; scale bars represent 5 μm.

Interestingly, expression of RNAi-insensitive wild-type GFP-Kif18b was able to restore MT depolymerization in Kif18b-depleted cells, but a Kif18b mutant lacking all EB1 binding sites was significantly less active (Figures 3E and 3F; Figure S3B). This demonstrates that direct recruitment to MT plus ends by EB1 is required for efficient MT depolymerization by Kif18b, similar to MCAK [9]. Taken together, these results show that release of Kif18b into the cytoplasm upon NEB allows Kif18b to rapidly target long astral MT plus ends through binding to EB1 and induce their depolymerization. In addition, Kif18b's MT depolymerization activity is continuously counteracted by Aurora kinases to prevent excessive MT depolymerization of all spindle MTs.

Kif18b and MCAK Interact and Promote Each Other's MT Plus-End Accumulation

We next investigated whether Kif18b and MCAK act independently or together to control MT stability. Depletion of MCAK decreased Kif18b recruitment to MT plus ends (Figure 4A; Figure S4A), and knockdown of Kif18b decreased MCAK levels at MT plus ends (Figure 4B; Figure S4B). Thus, although both Kif18b and MCAK can target to MT plus ends independently of each other, they increase the affinity of each other for MT plus ends. In line with this, MCAK and Kif18b coimmunoprecipitated (Figure 4C). This interaction is not mediated via EB1, because an MCAK mutant that cannot bind EB1 [33] binds Kif18b as well as wild-type MCAK (Figure S4C). Furthermore, recombinant full-length MCAK could bind purified Kif18b in vitro, suggesting that Kif18b and MCAK interact directly (Figure 4D).

To determine how MCAK promotes the accumulation of Kif18b to MT plus ends, we depleted MCAK by siRNA and expressed RNAi-insensitive wild-type or mutant GFP-MCAK from a tetracycline-inducible promoter. Interestingly, both wild-type and motor-dead MCAK promoted Kif18b accumulation at plus ends (Figures S4D and S4E). However, a MCAK mutant unable to bind EB1 (SxIP/NN) that did not accumulate at MT plus ends itself ([31]; data not shown) did not promote accumulation of Kif18b at plus ends (Figures S4D and S4E), despite normal binding to Kif18b (Figure S4C). Thus, MCAK needs to be at MT plus ends to promote the accumulation of Kif18b, indicating that MCAK and Kif18b interact at MT tips. Taken together, Kif18b and MCAK share a common function, physically interact, and enhance each other's affinity for MT

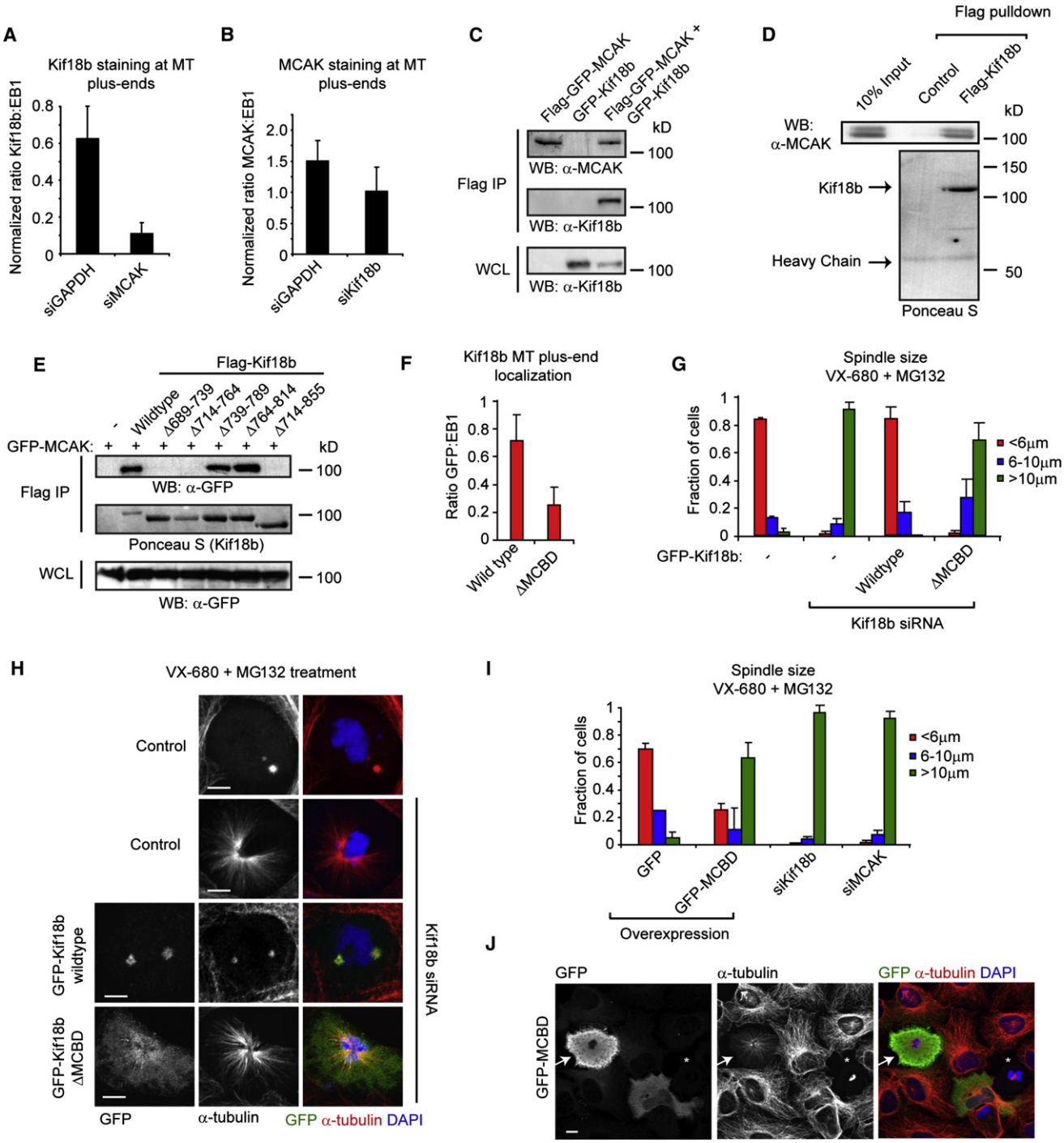

Figure 4. Kif18b and MCAK Interact at the Microtubule Plus End

(A and B) U2OS cells were transfected with indicated siRNAs and were fixed and stained for EB1 and Kif18b (A) or MCAK (B), and plus-end staining was quantified. Graph shows average of 150 plus ends in 10 cells (A) or 300 plus ends in 20 cells (B).

(C and E) HEK293 cells were transfected with indicated constructs and were treated with STLC for 18 hr to synchronize cells in mitosis. Cells were lysed, and α-Flag immunoprecipitations (IPs) were performed. IPs and WCLs were probed with indicated antibodies.

(D) HEK293 cells were transfected with Flag-Kif18b where indicated and treated with STLC as in (C). Cells were lysed and Flag immunoprecipitations were performed in high salt (0.5 M NaCl) to prevent binding of interacting proteins. IPs were washed and incubated with recombinant mCherry-MCAK-His (30 min/200 mM NaCl).

(F) U2OS cells were transfected with Kif18b siRNA and 24 hr later with GFP-Kif18b wild-type or mutant and were fixed and stained for GFP and EB1. Plus-end staining of 150 plus ends in ten cells was quantified.

(G–J) U2OS cells were transfected with Kif18b or MCAK siRNA where indicated. After 24 hr, cells were transfected with indicated expression vectors, and 48 hr after the DNA transfection, cells were treated with 1 μM VX-680 + MG132 for 45 min before fixation. Cells were then stained for GFP and α-tubulin, and spindle size was scored. Arrow in (J) indicates a transfected cell; asterisk indicates an untransfected cell. Graphs in (G) and (I) are averages of four independent experiments with 25 cells scored per experiment. All error bars represent standard deviations; scale bars represent 5 μm.

plus ends, strongly suggesting that they act together to promote MT plus-end depolymerization.

The Interaction between Kif18b and MCAK Is Required for MT Depolymerization

We could map the MCAK interaction site in Kif18b to a small region in the predicted unstructured tail of Kif18b (Figure 4E; Figure S4F) (amino acids 714–739, hereafter called the MCAK binding domain [MCBD]). Deletion of the MCBD reduced the accumulation of Kif18b at MT plus ends (Figure 4F), similar to what we observed after depletion of MCAK. Importantly, expression of Kif18b-ΔMCBD at the level of wild-type Kif18b (Figure S3B) failed to promote robust MT depolymerization (Figures 4G and 4H). This reduction in activity of Kif18b-ΔMCBD could not simply be explained by its reduced MT plus-end accumulation, because Kif18b-ΔMCBD accumulated at MT plus ends more efficiently than the EB1-binding mutant of Kif18b but promoted MT depolymerization less efficiently at similar expression levels (compare Figures 4F and 4G to Figure 2H and Figure 3F). To further confirm the importance of the Kif18b-MCAK interaction, we overexpressed a small fragment of Kif18b encompassing the MCBD to disrupt the interaction between endogenous Kif18b and MCAK. Overexpression of the MCBD blocked MT depolymerization almost as potently as depletion of either MCAK or Kif18b (Figures 4I and 4J), confirming the importance of the Kif18b-MCAK interaction for MT depolymerization.

Kif18b Requires Motor Activity and MCAK Binding for Depolymerization

To test whether the motor domain of Kif18b is directly responsible for MT depolymerization or whether it is mainly needed for movement along MTs, we engineered a Kif5b/18b chimeric motor protein. This chimera contains the motor domain, neck, and first coiled coil of the kinesin-1 Kif5b (lacking MT depolymerization activity), fused to the tail of Kif18b (Figure 5A). Strikingly, this Kif5b/18b chimera was as active in MT depolymerization as wild-type Kif18b in cells treated with Aurora kinase inhibitors (Figures 5B and 5C), suggesting that the major role of Kif18b's motor domain is to provide plus-end-directed movement along MTs. Importantly, deletion of the MCDB domain in the tail of the Kif5b/18b chimera completely blocked its ability to depolymerize MTs, even when highly expressed (Figures 5B and 5C). Similarly, although overexpression of a Kif18b mutant lacking the motor domain did not promote MT depolymerization in untreated cells, overexpression of the Kif5b/18b chimera severely reduced MT length, although the effect was slightly less prominent compared to wild-type Kif18b (Figure 5D). Deletion of the MCBD in wild-type Kif18b also strongly reduced the ability to promote MT depolymerization when overexpressed, and deletion of the MCBD in the Kif5b/18b chimera completely abolished the activity (Figure 5D). Taken together, these results show that the major function of the motor domain of Kif18b is to walk along MTs while binding to MCAK, suggesting that Kif18b acts to transport MCAK along MTs. It should be noted though that the motor domain of Kif18b may also have a minor, direct role in MT depolymerization, because Kif18b-ΔMCBD still shows residual activity in MT depolymerization, which is not seen with the Kif5b/18b-ΔMCBD chimera.

All this suggests that Kif18b contains three activities that are required for MT depolymerization: motor activity, EB1 binding, and MCAK binding. To determine whether these activities are sufficient for MT depolymerization, we generated minimal domain constructs (Figure S5A). Neither the Kif5b motor domain nor a fragment encompassing the EB1 and MCAK binding domains (EBBD-MCBD) was able to promote MT depolymerization, but a fusion of Kif5b and EBBD-MCBD robustly promoted MT depolymerization (Figure 5E). Thus, no other domains in Kif18b are needed for its MT depolymerization activity, and motile activity needs to be physically coupled to the EB1 and MCAK binding domains to promote MT depolymerization, again suggesting that Kif18b acts as a transporter of MCAK and/or EB1.

Aurora Kinases Negatively Regulate the Interaction between Kif18b and MCAK

Because the interaction between Kif18b and MCAK is required for efficient MT depolymerization upon inhibition of Aurora kinases, we wondered whether Aurora kinases directly inhibit the interaction between Kif18b and MCAK. Strikingly, endogenous coimmunoprecipitations (coIPs) revealed that the interaction between Kif18b and MCAK was much stronger in the absence of Aurora kinase activity (Figure 5F), demonstrating that Aurora kinases indeed negatively regulate the interaction between Kif18b and MCAK. Consistent with this, Kif18b and MCAK robustly localized to all MT plus ends in Aurora-inhibited cells (Figure S5B and S5C). We therefore examined phosphorylation of both Kif18b and MCAK by Aurora kinases. Both Aurora A and B phosphorylate Kif18b's tail in vitro; however, mutation of the six phosphorylation sites identified by mass spectrometry to either nonphosphorylatable or phosphomimicking residues did not affect the ability of Kif18b to bind to MCAK or to promote MT depolymerization (unpublished data). In contrast, mutation of five Aurora kinase phosphosites on MCAK [22] to phosphomimicking residues strongly reduced the ability of MCAK to bind to Kif18b (Figure 5G). Together, these results indicate that Aurora kinases control MT dynamics through negative regulation of Kif18b-MCAK complex formation via phosphorylation of MCAK.

Discussion

Here, we identify a novel kinesin-8 motor, Kif18b, which acts together with MCAK to promote MT depolymerization in mitosis. Our results show that the function of Kif18b-MCAK is inhibited by Aurora kinases, at least in part through disruption of the Kif18b-MCAK complex. Based on our results, we propose that a complex of Kif18b and MCAK constitutes the major MT plus-end depolymerase activity in mitotic cells and that the activity of this complex is tightly regulated in space and time.

EB1 binding is essential for plus-end accumulation of Kif18b, but it is not sufficient, because Kif18b also requires its motor activity for maximal accumulation at MT plus ends. This suggests that Kif18b might initially be recruited to plus ends by EB1 and subsequently use its plus-end-directed motility to walk along with the growing MT plus end. Alternatively, Kif18b molecules might be initially recruited to the MT lattice and walk to the plus end where they are maintained by EB1 as the MT grows. In both cases, the longer a MT spends in a growing state, the more Kif18b can accumulate at the plus end. This would allow for selective depolymerization of long MTs, keeping overall MT length in mitosis limited. Both MCAK and Kif18b need each other for robust accumulation at MT plus ends, suggesting that, after initial EB1-dependent loading at plus ends, Kif18b and MCAK can bind each other to increase each other's affinity for the MT plus end.

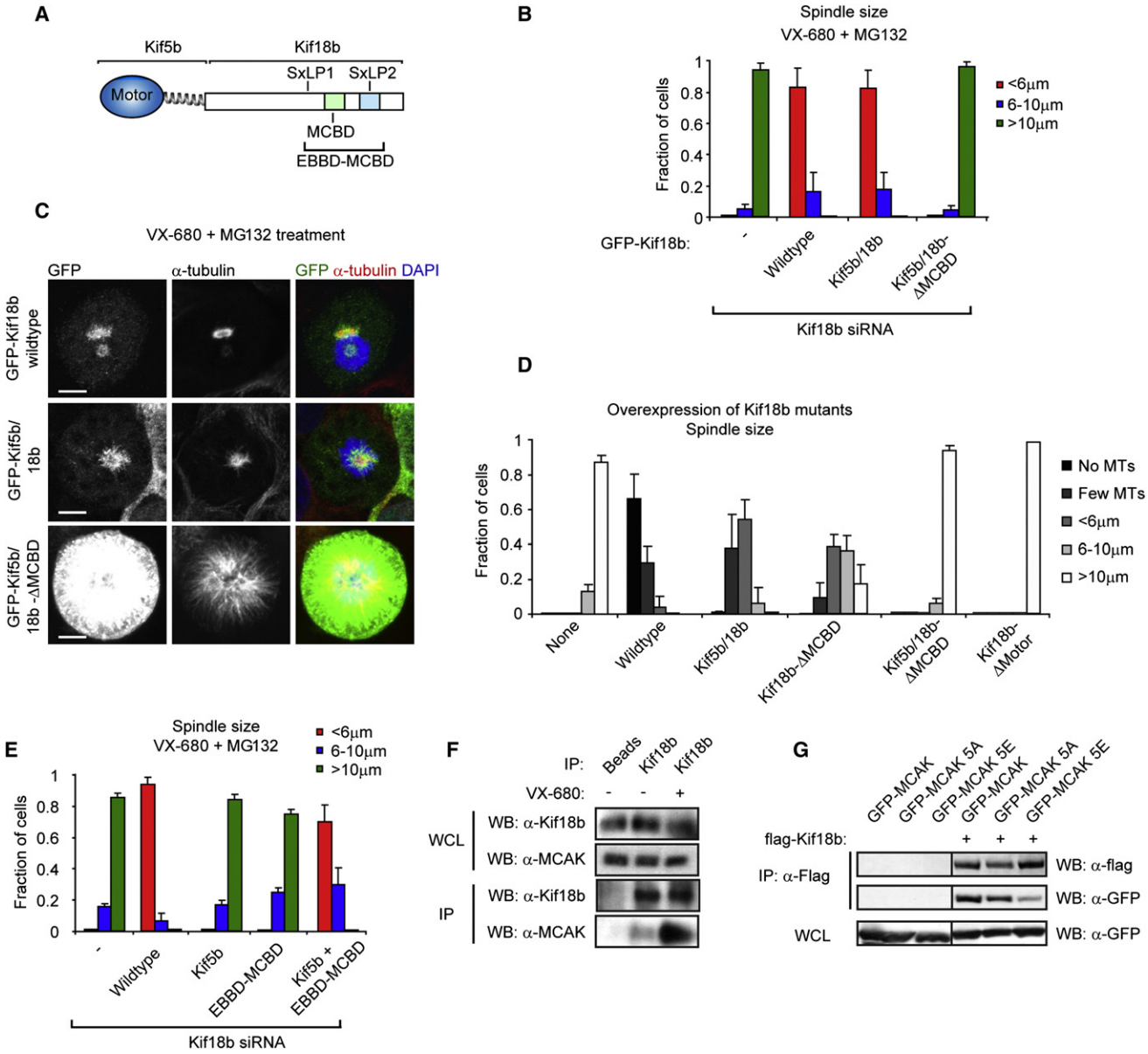

Figure 5. Aurora Kinases Promote Microtubule Stabilization through Negative Regulation of Kif18b-MCAK Binding

(A) Schematic representation of Kif5/18b fusion.

(B, C, and E) U2OS cells were transfected with Kif18b siRNA and after 24 hr were transfected with indicated GFP-Kif18b constructs. Forty-eight hours after DNA transfection, cells were treated with 1 μM VX-680 + MG132 for 45 min and were fixed and stained for GFP and α-tubulin. MT length was scored in cells expressing low levels of the GFP-tagged constructs. Twenty-five cells were quantified per experiment.

(D) Cells were transfected with Flag-Kif18b or mutants thereof. Forty-eight hours after transfection, cells were fixed and stained for Flag and α-tubulin, and spindle size was determined. Between 10 and 25 cells were scored per experiment. Graphs represent the averages of four (B) or three (D and E) independent experiments. Error bars represent standard deviations; scale bars represent 5 μm.

(F) U2OS cells were synchronized in mitosis by 18 hr STLC treatment, and mitotic cells were collected by shake-off. Mitotic cells were then treated with either MG132 or VX-680 + MG132 for 45 min. Cells were lysed, and Kif18b was immunoprecipitated. WCL and IPs were probed for MCAK and Kif18b.

(G) HEK293 cells were transfected with Flag-Kif18b and either wild-type GFP-MCAK or GFP-MCAK-5A or 5E. Flag IPs and WCLs were probed for Flag or GFP.

The similar phenotype observed after depletion of Kif18b or MCAK indicates that both have an equally important and nonredundant contribution to MT depolymerization in mitosis. Moreover, Kif18b needs to bind to MCAK and EB1 and be able to walk along MTs to promote robust MT depolymerization. Therefore, we propose that Kif18b is initially recruited to MT plus ends (note that the "plus end" is defined here as the distal 1–2 μm of the MT to which EB1 binds, not the terminal tubulin dimer) through EB1 and subsequently binds to MCAK, which is also directly recruited to the MT plus end by EB1. Complex formation increases the affinity of both proteins for the plus end and can result in transport of MCAK to the very tip of the MT, where MCAK needs to be to promote MT depolymerization (Figure 6). In addition, Kif18b may also make a minor contribution to MT plus-end depolymerization directly through its motor domain.

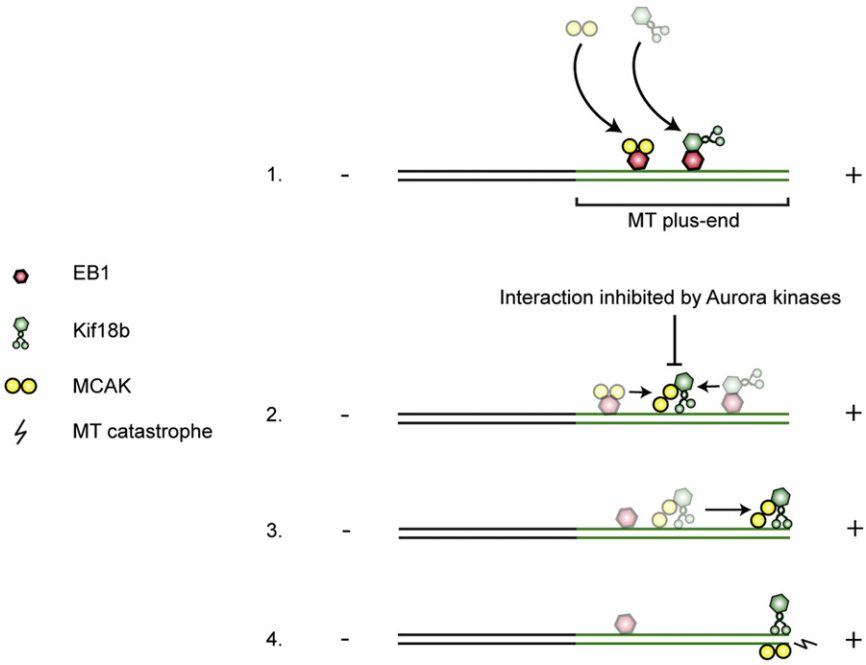

Figure 6. Model of EB1, Kif18b, and MCAK Function at Microtubule Plus Ends

(1) Kif18b and MCAK are recruited to the distal 1–2 μm of the MT by EB1.

(2) Kif18b and MCAK then bind each other on the MT, increasing each other's affinity for the MT. This interaction is inhibited by Aurora kinase-dependent phosphorylation of MCAK.

(3) If the complex persists, Kif18b can transport MCAK to the very distal tip of the MT, so the extent of this transport depends on the level of MCAK phosphorylation by Aurora kinases.

(4) Once Kif18b and MCAK arrive at the very tip, MCAK can promote MT depolymerization. Although the major function of Kif18b is to transport MCAK, Kif18b may also make a minor contribution to MT depolymerization through its own motor domain.

We find that in the absence of Aurora kinase signaling, MT destabilizing activities become dominant, and depleting either MCAK or Kif18b reverts this. Indeed, Aurora-dependent phosphorylation is known to inhibit MCAK's MT depolymerization activity [22, 23, 25, 34] and to inhibit binding of MCAK to EB1 [7, 31]. Here, we show that the interaction of MCAK and Kif18b is also inhibited by Aurora kinases and that this is largely due to phosphorylation of MCAK. Thus, Aurora kinases control MCAK's intrinsic activity, its plus-end recruitment by EB1, and its binding to Kif18b, to firmly inhibit its MT depolymerase activity. Our data show that binding of MCAK to Kif18b remains essential for efficient depolymerization, even when all other negative effects of Aurora activity on MCAK are alleviated, underlining the importance of Kif18b as a modulator of MCAK activity in mitosis. Aurora kinases can also promote the activity of MT stabilizing proteins through phosphorylation (for example, [35]). Thus, Aurora kinases probably act at multiple levels to counteract the potent MT destabilizing activity of Kif18b-MCAK to promote proper spindle assembly.

In interphase, Kif18b is sequestered in the nucleus, and robust MT depolymerization by Kif18b-MCAK cannot occur until after NEB, when MTs need to become highly dynamic (Figure S6). Other mechanisms, including CDK1-dependent phosphorylation of MT binding proteins, will likely also be important to change the dynamics of MTs in mitosis, but our results show that rapid disassembly of the interphase MT network requires nuclear release of Kif18b at NEB. Furthermore, Kif18b is rapidly degraded in anaphase at the time when the MT network is stabilized. Thus, Kif18b nuclear release and subsequent degradation could define two molecular switches that couple MT dynamics to cell-cycle progression. Taken together, our results uncover a novel molecular complex consisting of Kif18b and MCAK that robustly promotes MT depolymerization in mitosis and show that its activity is fine tuned by Aurora kinases to allow for proper spindle assembly and cell division.

Experimental Procedures

Cell Culture, Transfection, and Drug Treatments

U2OS, HeLa, RPE, and HEK293 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. All siRNA information is summarized in Table S1. DNA transfections were performed with FuGENE 6 (Roche) according to the manufacturer's guidelines. STLC (Sigma), VX-680, AZD-1152, and hesperadin (Selleck) were used at the indicated concentrations. MG132 (Sigma) was used at 5 μM.

Quantification of Immunofluorescence

Plus-end staining was quantified by line scans through the distal 2 μm of the MT, and the ratio of MCAK:EB1 staining was determined after background subtraction. For experiments in which cells were treated with Aurora kinase inhibitors, combined with different RNAis and expression constructs, the diameter of the spindle was measured. If two distinct asters were observed, the sum of the two diameters was used.

Immunofluorescence

Cells were grown on 10 mm glass coverslips and fixed with cold methanol for 5 min. α-tubulin antibody (Sigma) was used at 1:7500, α-GFP (custom-made) was used at 1:500, α-EB1 (BD) was used at 1:400, α-MCAK was used 1:1000 [36], and α-Flag (Cell Signaling) was used at 1:500. The rabbit polyclonal Kif18b antibody was raised against a GST-tagged protein encompassing amino acids 432–818 of human Kif18b 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 of images were adjusted with Adobe Photoshop 6.0. Images are maximum-intensity projections of all Z planes.

Time-Lapse Microscopy

U2OS cells stably expressing mCherry-α-tubulin [37, 38] 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 3–5 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 GFP-Kif18b fluorescence intensities were quantified using MetaMorph software (Molecular Devices). For live-cell imaging of GFP-Kif18b plus-end tracking, cells were imaged on a Zeiss LSM510 confocal microscope as described above, with 2.4 s intervals and a single Z plane.

Immunoprecipitation, GST Pull-Downs, and Western Blotting

For coimmunoprecipitations, HEK293 cells were transfected with indicated constructs using PEI transfection reagent, and STLC was added 24 hr after transfection to synchronize cells in mitosis. Cells were then lysed in buffer containing 50 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM EDTA, 200 mM NaCl, 5 mM NaF, 10% glycerol, 1% NP-40, and protease inhibitors. Proteins were precipitated by incubation with Flag-agarose beads (Sigma) for 30 min. Proteins were detected on western blots by incubation of indicated primary antibodies overnight and secondary horseradish peroxidase (HRP)-conjugated antibodies for 1 hr. For analysis of Kif18b knockdown, whole-cell lysates were prepared 48 hr after siRNA transfection, and Kif18b was detected with an affinity-purified Kif18b antibody (1:200 dilution). GST-EB1 pull-downs were performed as described previously [39]. For endogenous coimmunoprecipitation, U2OS cells were treated for 24 hr with STLC to synchronize cells in mitosis. Mitotic shake-offs were then performed, and mitotic cell populations were treated with either MG132 or MG132 + VX-680 for 45 min. Cells were then lysed as above, and Kif18b was immunoprecipitated using α-Kif18b antibody for 30 min.

Plasmid Construction and Protein Production

See Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes six figures, one table, Supplemental Experimental Procedures, and one movie and can be found with this article online at doi:10.1016/j.cub.2011.07.017.

Acknowledgments

We thank C. Walczak, L. Wordeman, and J. Swedlow for reagents and E. Geers and R. van Rijssel for technical assistance. We thank D. Pellman and members of the Medema, Kops, and Lens laboratories for helpful discussions and G. Kops, S. Lens, M. Alvarez, A. Janssen, and A. Saurin for critical reading of the manuscript. This work was supported by the Netherlands Organization for Scientific Research (NWO-VICI, ZonMw 918.46.616 and NWO-ALW, 81802003). L.M. was supported by grants from the Dutch Cancer Society (UU2006-3579) and the Grant Agency of the Czech Republic (P301/10/1525 and P305/10/P420). R.H.M. was funded by the Netherlands Genomics Initiative of the Netherlands Organization for Scientific Research.

Received: May 26, 2011
Revised: June 20, 2011
Accepted: July 14, 2011
Published online: August 4, 2011

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