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Interplay of microtubule dynamics and sliding during bipolar spindle formation in mammalian cells

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Summary

Accurate chromosome segregation during mitosis relies on the organization of microtubules into a bipolar spindle. Kinesin-5 proteins play an evolutionarily conserved role in establishing spindle bipolarity [1,2] and clinical trials are currently evaluating inhibitors of human kinesin-5 (i.e. Eg5) for chemotherapeutic potential. However, in mammalian somatic cells Eg5 activity is dispensable for maintenance of bipolar spindles once they are formed [3,4], suggesting distinct requirements for establishment versus maintenance of spindle bipolarity. By combining Eg5 inhibition with RNA interference of other spindle proteins, we show that mitotic cells deficient in MCAK fail to maintain spindle bipolarity in the absence of Eg5 activity. Collapse of bipolar spindles in MCAK-deficient cells is driven by pole focusing activities and is independent of MCAK function at centromeres, implicating hyperstabilized non-kinetochore microtubules in spindle collapse. Conversely, destabilizing non-kinetochore microtubules in early mitosis reduces the reliance on Eg5 for establishment of spindle bipolarity and renders cells partially resistant to Eg5 inhibitors. Thus, the temporal requirement for microtubule sliding generated by Eg5 activity during bipolar spindle assembly in mammalian cells is regulated by changes in the dynamic behavior of microtubules during mitosis.

Results and Discussion

To examine mechanisms contributing to spindle bipolarity in human cultured cells we utilized monastrol to inhibit Eg5 activity [5] at different stages of mitosis. Addition of monastrol to human U2OS cells before nuclear envelope breakdown induces centrosomes to collapse resulting in a monopolar spindle, whereas monastrol addition after nuclear envelope breakdown has no deleterious effect on spindle bipolarity or mitotic progression (Fig. 1A, B;Suppl. Movies 1,2). Likewise, monastrol induces monopolar spindles in 80% of mitotic cells in populations of unsynchronized U2OS cells but only ~20% of mitotic cells if cells are synchronized in metaphase (accumulated by treatment with MG-132; Fig. 1C). These percentages are consistent with previous values generated by inhibition of Eg5 function by antibody injection [3]. These data confirm that Eg5 activity is required for establishment but not maintenance of bipolar spindles in human somatic cells [3,4].

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Supplemental Data
Supplemental data include Experimental Procedures, two figures, and three movies and can be found with this article.

Since a mechanism for maintaining spindle bipolarity has not been described in somatic cells, we reasoned that a mechanism may be revealed under "sensitized" conditions where Eg5 is inhibited with monastrol. We considered various forces that could contribute to maintaining bipolar spindles in the absence of Eg5 activity including force generated by kinetochores, chromokinesins, anti-parallel microtubule crosslinkers, and microtubule-associated proteins. To identify which of these mechanisms is responsible for maintenance of spindle bipolarity in the absence of Eg5 activity, we designed an assay that scores only bipolar spindle maintenance and not establishment. Candidate proteins were depleted in U2OS cells using RNA interference followed by MG-132 treatment to accumulate bipolar spindles, which were then subjected to monastrol treatment (Fig. 1D). Immunoblots show the efficiency of depletion of each candidate protein by RNA interference (Fig. 1E). Populations of untransfected control cells displayed 80% bipolar spindles and 20% monopolar spindles under these conditions (Fig. 1F). For ease of comparison, we converted the population percentages into a "monopolarity index" which is the percentage of monopolar spindles in the RNAi treated samples divided by the percentage of monopolar spindles in the control sample (Fig. 1G; raw population data is presented in Suppl. Fig. 1).

Kinetochores have been proposed to increase the rate of bipolar spindle formation [6] suggesting a possible role in bipolar spindle maintenance. To examine this, we applied our assay to the protein Nuf2, a component of the outer kinetochore Ndc80 complex essential for stable kinetochore-microtubule interactions [7,8]. Populations of cells depleted of Nuf2 displayed 73% bipolar and 24% monopolar spindles under our assay conditions (Fig. 1F), resulting in a monopolarity index not significantly different from control cells (Fig. 1G; $p=0.056$, Fisher's exact test). Therefore, inhibition of stable kinetochore-microtubule interactions does not affect bipolarity maintenance in our assay. Similarly, when we tested the role of anti-parallel microtubule crosslinking in bipolarity maintenance by depleting PRC1 [9], the percentage of mitotic cells with monopolar spindles did not increase significantly; indicating that anti-parallel microtubule crosslinks provided by PRC1 do not account for the stability of bipolar spindles in the absence of Eg5 function (Fig. 1G). Likewise, depletions of either a chromokinesin (Kif4) [10], a pole focusing motor (HSET) [11], or microtubule-associated proteins TOG [12] and EB1 [13] did not significantly change the monopolarity index, ruling out those proteins in maintaining spindle bipolarity in the absence of Eg5 activity as well (Fig. 1G).

In stark contrast, cells with even modest reductions of the microtubule depolymerizing enzyme MCAK (Fig. 2B) displayed ~60% mitotic cells with monopolar spindles under our assay conditions, yielding a monopolarity index that is significantly elevated relative to control cells (Fig. 2A; raw population data is presented in Suppl. Fig. 2; $p<0.0001$, Fisher's exact test). Moreover, unlike control prometaphase cells that are impervious to Eg5-inhibition after bipolar spindle formation (Fig. 1B), time lapse imaging of MCAK-deficient prometaphase cells shows nascent bipolar spindles treated with monastrol to collapse rapidly (Fig. 2C, Suppl. Movie 3). These data demonstrate that altering microtubule dynamics by depletion of MCAK renders human somatic cells reliant on Eg5 activity to maintain spindle bipolarity.

MCAK regulates the dynamics of both kinetochore and non-kinetochore spindle microtubules [14–18], and therefore, suppressing dynamics of either population may be responsible for the failure to maintain spindle bipolarity in MCAK-deficient cells lacking Eg5 activity. To determine if MCAK's activity on kinetochore microtubule dynamics contributes to its role in maintaining spindle bipolarity, we subjected cells simultaneously depleted of Nuf2 and MCAK to our bipolarity maintenance assay (Fig. 2A and B). It has been shown that Nuf2-deficient cells lack stable kinetochore microtubule attachments [7]. Similar to cells lacking MCAK under our bipolarity maintenance assay conditions, mitotic cells simultaneously deficient in Nuf2 and MCAK fail to maintain spindle bipolarity and display a high monopolarity index (Fig. 2A,

raw population data is presented in Suppl. Fig. 2). Thus, MCAK activity is essential to maintain spindle bipolarity in the absence of Eg5 activity, but these data suggest that it fulfills this role independent of stable kinetochore microtubules.

Next, we examined the role of MCAK in maintaining spindle bipolarity through regulation of non-kinetochore spindle microtubules. Most non-kinetochore spindle microtubules are depolymerized by calcium treatment prior to fixation, leaving only kinetochore microtubules (Fig. 3A) consistent with previous electron microscopy studies in PtK cells showing very few overlapping interpolar microtubules during metaphase [19]. Strikingly, MCAK-depleted metaphase spindles possess numerous calcium-stable microtubules that are not associated with kinetochores (Fig. 3B). These calcium-stable microtubules form bundles that frequently appear to extend from one half of the spindle into the other, exaggerating the population of overlapping spindle microtubules. These long overlapping microtubules may be subject to inappropriate pole focusing activities that would impair maintenance of spindle bipolarity in our assay. Pole focusing activities acting on these microtubule bundles could pull centrosomes together and collapse a bipolar spindle. To test this idea, we depleted the minus end-directed motor protein HSET which plays an established role in pole focusing [11]. Cytoplasmic dynein provides the dominant pole focusing activity, but depletion causes spindle poles to splay [11,20], and so we were forced to specifically target HSET in these experiments. Depletion of HSET alone does not induce bipolar spindle collapse when Eg5 activity is inhibited under our assay conditions (Fig. 1E and G,Suppl Fig. 1D). Simultaneous depletion of MCAK and HSET shows a significant decrease in monopolarity index compared to cells depleted of MCAK alone (Fig. 2A and B, $p<0.0001$, compared to MCAK alone, Fisher's exact test). Thus, whereas most mitotic cells lacking MCAK alone fail to maintain spindle bipolarity under our assay conditions, bipolarity maintenance is partially rescued if pole focusing is simultaneously inhibited by HSET depletion. The limited size of this effect most likely reflects the minor contribution that HSET makes to pole focusing in somatic cells [11,20].

The data presented thus far indicate high microtubule dynamics ensured by MCAK renders the microtubule sliding activity of Eg5 unnecessary for maintenance of spindle bipolarity in human cells. This suggests the existence of a functional relationship between microtubule dynamics and Eg5-generated microtubule sliding. These results predict that decreasing microtubule stability early in mitosis would reduce the requirement for Eg5 activity during bipolar spindle establishment at the time of nuclear envelope breakdown. To test this prediction, we reduced non-kinetochore microtubule stability and then determined the percentage of mitotic cells with monopolar spindles formed upon monastrol treatment followed by MG-132. Due to the duration of this experiment we could not use nocodazole to destabilize microtubules, because, even at low doses (33nM –132nM), it caused spindle disorganization. Instead, we depleted cells of the spindle-associated protein astrin, a mitosis-specific microtubule-associated protein [21]. Immunoblots show the efficiency of astrin depletion by RNA interference (Fig. 4A). The half-life of non-kinetochore microtubules in astrin-deficient cells in prometaphase is 7.40 ± 2.60 seconds (n=9 cells, $R^2 > 0.99$), a significant reduction from control cells where the half-life of non-kinetochore microtubules in prometaphase is 16.04 ± 1.14 seconds (Fig. 4B; n=9 cells, $R^2 > 0.98$, $p < 0.0005$, Student t-test), indicating that astrin depletion is an efficient way to decrease microtubule stability. When astrin is depleted, fewer mitotic cells display monopolar spindles when treated with a range of monastrol concentrations (60, 80, or 100 μM) compared to control cells. For example, 85.4% of control mitotic cells form monopolar spindles in the presence of 80 μM monastrol, but only 50.7% of astrin-deficient mitotic U20S cells have monopolar spindles at this concentration of monastrol (Fig 4C). Several roles have been proposed for astrin in mitosis [22,23]. To verify that this change in monastrol sensitivity is caused by the role of astrin in microtubule dynamics, we stabilized microtubules in astrin-deficient cells by addition of 5 nM taxol [24]. A majority of astrin-deficient mitotic cells treated with taxol and monastrol show monopolar spindles. At the

concentrations used here, the established effect of taxol is to stabilize microtubules indicating that the primary effect of astrin depletion was to destabilize spindle microtubules (Fig. 4C). These data demonstrate that the role of Eg5 in establishing spindle bipolarity in U2OS cells is influenced by the dynamic state of microtubules, and that sensitivity to Eg5 inhibition with monastrol can be reduced by destabilizing microtubules in early mitosis.

The microtubule sliding activity of Eg5 is only required to establish bipolar spindles in mammalian somatic cells [3,4]. Here we show that Eg5 activity becomes essential to maintain spindle bipolarity following disruption of microtubule dynamics through loss of MCAK. Multiple mechanisms influence centrosome separation and spindle bipolarity in mammalian cells (Fig. 4D) and many of those could be influenced by changes in microtubule dynamics. For example, kinetochores have been proposed to expedite centrosome separation, and stabilizing microtubule dynamics may augment that activity. However, the perseverance of spindle bipolarity observed upon disruption of stable kinetochore microtubule interactions through depletion of Nuf2 argues against kinetochore activity being the primary site of MCAK function for spindle bipolarity. Thus, it is more likely that the exaggerated non-kinetochore microtubules resulting from MCAK depletion renders Eg5 essential to maintain spindle bipolarity. Exaggerating the length and density of non-kinetochore microtubules could influence spindle bipolarity either by increasing cortical microtubule interactions or increasing the extent of microtubule overlap between the two halves of the spindle. We disfavor the former possibility because increasing microtubule contacts with the cell cortex would enhance outward forces pulling centrosomes apart rather than collapsing them, which is contrary to our observations. Instead, we favor the latter possibility because we observe calcium-stable microtubule bundles extending between the two spindle halves in MCAK-deficient cells and spindle bipolarity in MCAK-deficient cells can be preserved by simultaneously depleting the pole focusing motor HSET. Based on these observations, we propose a model for how microtubule dynamics influences the temporal requirement for the sliding activity of Eg5 (Fig. 4D). Microtubules have been shown to be relatively long and stable at the time of nuclear envelope breakdown[25], whereupon pole focusing factors (HSET, NuMA) are released from the nuclear compartment. The model suggests that Eg5 is essential at this early stage of mitosis to establish spindle bipolarity by sliding antiparallel microtubules to generate an outward force to prevent pole focusing activities from drawing spindle poles together into monopolar spindles. Microtubules then become shorter and less stable as cells transition into prometaphase after nuclear envelope breakdown[25], reducing the extent of overlapping microtubules. This renders Eg5 activity irrelevant for the maintenance of spindle bipolarity in somatic mammalian cells and other, as yet unknown, factors take over responsibility for maintaining spindle bipolarity. Thus, temporal changes in microtubule dynamics determine the temporal requirement for Eg5 activity to establish spindle bipolarity early in mitosis. This model provides a straight forward explanation for the disparities reported in the requirements of kinesin-5 motor activity in different experimental systems. For example, Eg5 activity is required for both the establishment and maintenance of spindle bipolarity in frog egg extracts [4]. Spindles in those extracts contain a network of short, crosslinked microtubules surrounded by a barrel array of astral microtubules that form antiparallel overlap [26]. Recent data shows that reducing the overlapping barrel array of microtubules in that system eliminates the requirement for Eg5 activity to maintain spindle bipolarity [27]. Moreover, spindles in *C. elegans* embryos do not require kinesin-5 activity for bipolarity [28], and it may be that those spindles have few overlapping non-kinetochore microtubules as judged by electron microscopy [29]. Thus, the model suggests that the difference in requirement for Eg5 activity to maintain spindle bipolarity between different model systems arises through disparities in the degree of spindle microtubule overlap governed by dynamics and not through inherently unique mechanisms. Finally, our data reveal that increasing microtubule dynamics in early mitosis reduces the sensitivity of cells to the inhibition of Eg5 activity and unveils pathways that are

independent of mutation of the Eg5 gene through which tumor cells may be (or become) recalcitrant to Eg5 inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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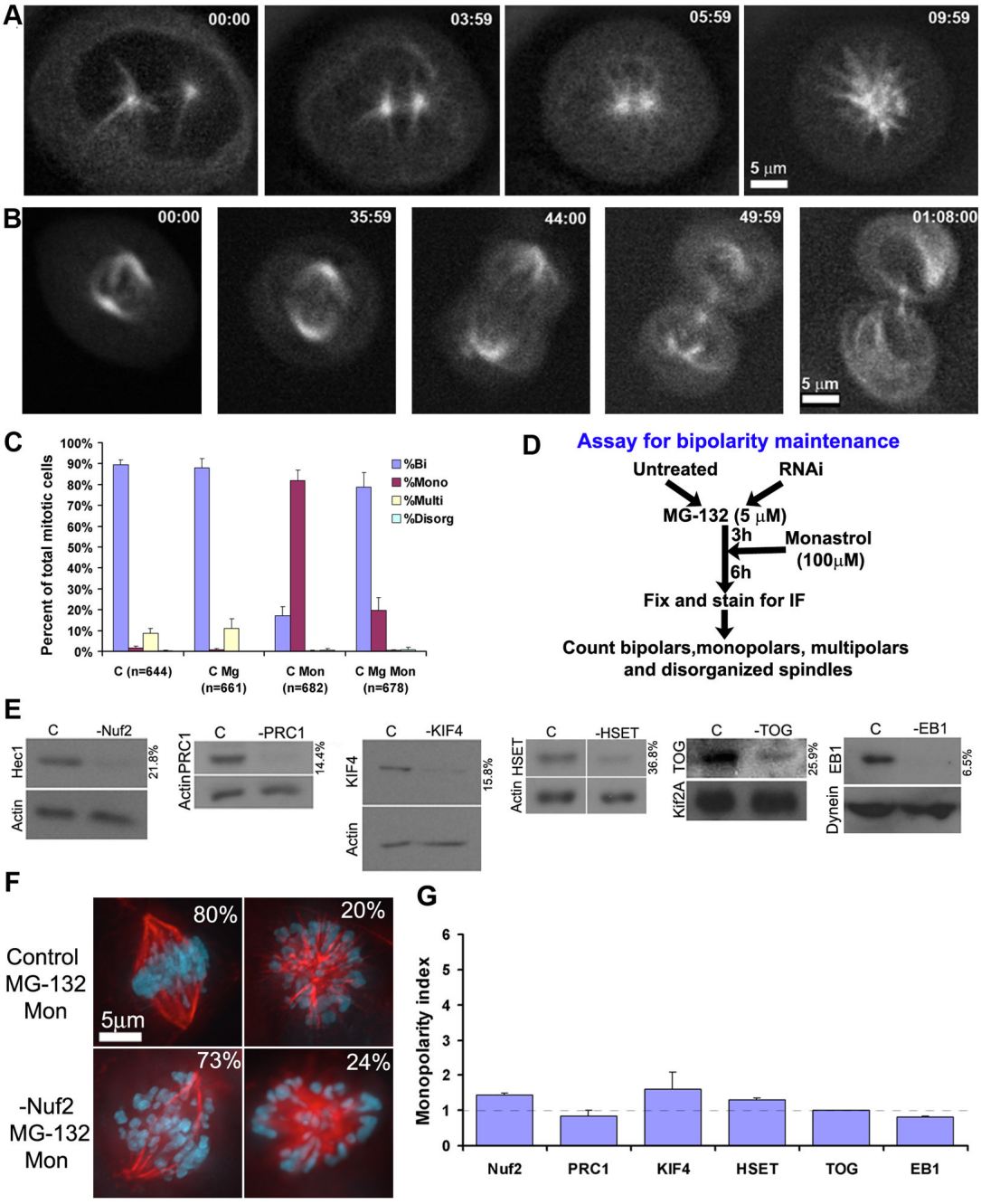

Figure 1.

Eg5 is dispensible for maintenance of spindle bipolarity in human U2OS cells. Time lapse imaging of monastrol-treated U2OS cells expressing GFP-tubulin (A) prior to or (B) after nuclear envelope breakdown. Time is presented in minutes:seconds. Scale bar as indicated. (C) Percentages of mitotic U2OS cells with bipolar, monopolar, multipolar or disorganized spindles in populations that were either untreated (C), treated for nine hours with 5 μM MG-132 alone (C MG), nine hours of 100 μM monastrol alone (C Mon), or with MG-132 for three hours followed by monastrol for six hours (C Mg Mon). N is total numbers of mitotic cells counted for each condition. Error bars represent standard deviations. (D) Design of the assay used for the analysis of maintenance of spindle bipolarity. (E) Immunoblots demonstrating the

efficiency of protein depletion using RNA interference for control cells (C) or cells depleted of PRC1, TOG, Kif4, Nuf2, HSET, or EB1 as indicated. Loading controls are identified as either actin, Kif2a, or dynein, and the numbers to the right of each blot indicate quantity of each protein remaining after RNAi compared to control. (F) Immunofluorescent images of fixed U2OS cells that were either untreated (Control MG-132 Mon) or depleted of Nuf2 (-Nuf2 MG-132 Mon) under our assay conditions. Percentages indicate the fraction of cells with bipolar or monopolar spindles in each population. (G) The monopolarity index refers to the percent of monopolar cells in the RNAi- treated population divided by the percent of monopolar cells in the control sample. Error bars represent standard errors.

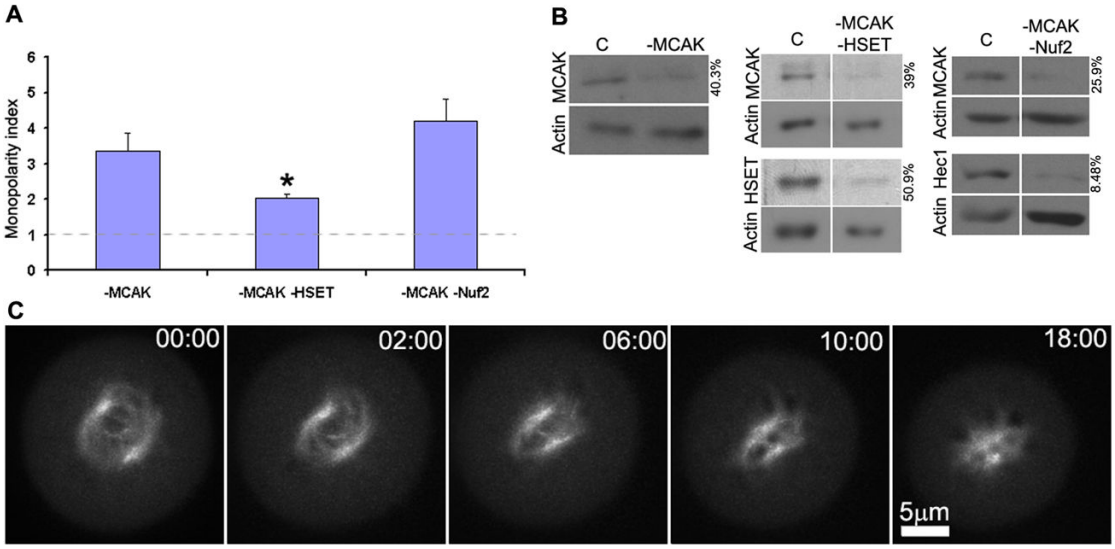

Figure 2.

MCAK contributes to maintaining spindle bipolarity in human U2OS cells. (A) Monopolarity indices of mitotic cells depleted of MCAK alone, MCAK and HSET, and MCAK and Nuf2 as indicated. Asterisk denotes significant reduction in monopolarity index compared to cells deficient in MCAK alone ($p<0.0001$, two tailed Fisher's exact test). Error bars show standard errors. (B) Immunoblots demonstrating the efficiency of protein depletion using RNA interference for control cells (C) or cells depleted of MCAK, MCAK and HSET, and MCAK and Nuf2 as indicated. Actin serves as a loading control and the numbers to the right of each blot indicate quantity of each protein remaining after RNAi compared to control. (C) Time lapse imaging of monastrol-treated, MCAK-deficient U2OS cells expressing GFP-tubulin after nuclear envelope breakdown. Monastrol was added after nuclear envelope breakdown. Time is presented in minutes:seconds. Scale bar as indicated.

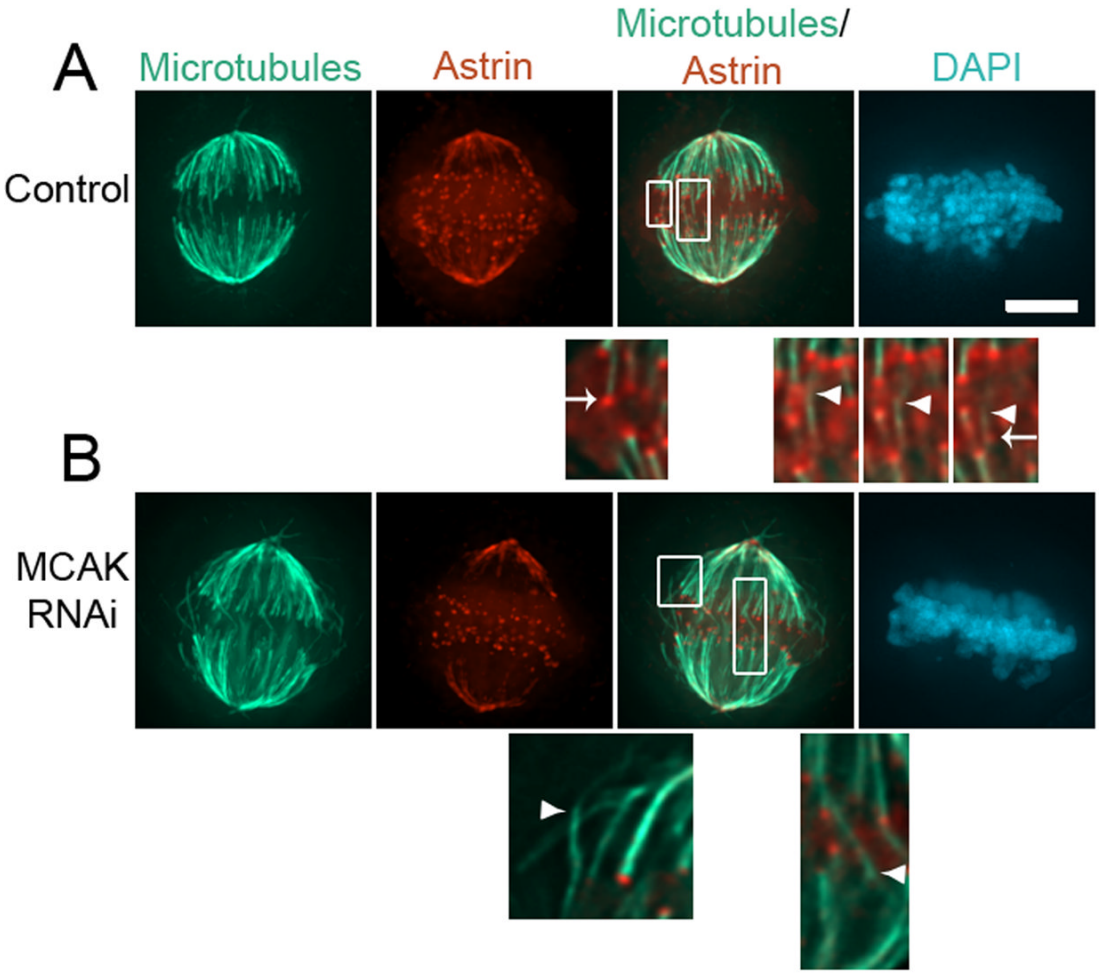

Figure 3.

Calcium-stable spindle microtubules. (A) Full volume projection of an untreated U2OS cell in metaphase following calcium treatment prior to fixation stained for microtubules, astrin, and DNA (DAPI) as indicated. Boxed regions are 4X magnifications and are shown as insets from single focal planes in the z-axis. Arrowheads indicate microtubule bundles and arrows indicate kinetochores. The 3 insets on the right represent sequential focal planes in the z-axis to highlight a single microtubule bundle traversing the midzone and ending at a kinetochore. (B) Full volume projection of an MCAK-deficient U2OS cell in metaphase following calcium treatment prior to fixation stained for microtubules, astrin, and DNA (DAPI) as indicated. Boxed regions are 4X magnifications and are shown as insets from single focal planes in the z-axis. Arrows indicate kinetochores. Scale bar, 5 μm.

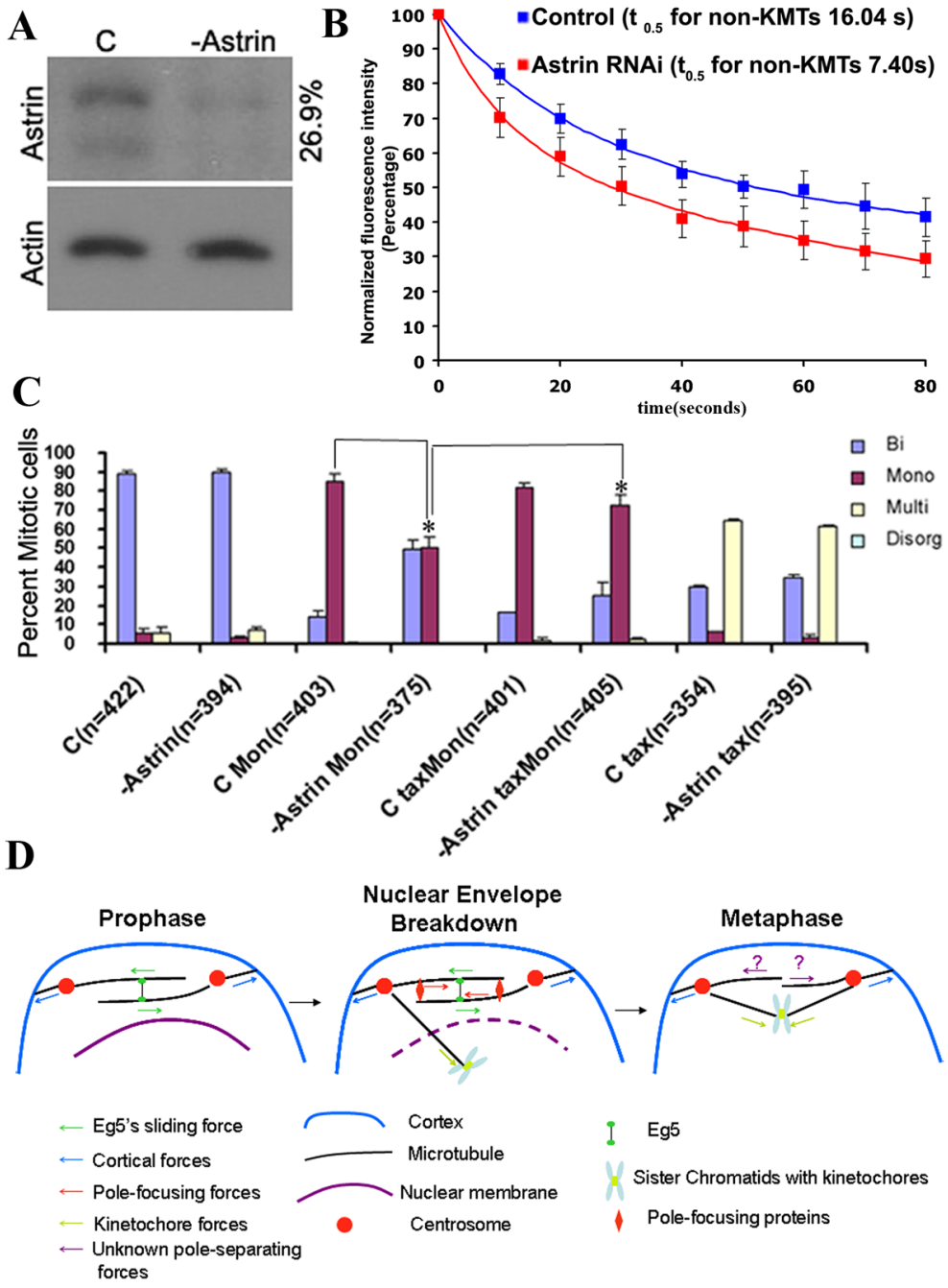

Figure 4.

Decreasing MT stability alters U2OS cell sensitivity to monastrol. (A) Immunoblots demonstrating the efficiency of protein depletion using RNA interference for control cells (C) or cells depleted of astrin as indicated. Loading controls are identified as actin and the numbers to the right of the blot indicate quantity of each protein remaining after RNAi compared to control. (B) Normalized fluorescence intensity of non-KMTs over time (seconds) after photoactivation of spindles in untreated (blue squares) and astrin-depleted (red squares) prometaphase cells. Data represent mean ± s.e.m, *n* = 9 cells for both conditions. (C) Percent of mitotic cells with bipolar, monopolar, multipolar, or disorganized spindles in untreated cells (C) and cells depleted of astrin (-astrin) with or without treatment with 80 μM monastrol (Mon)

or 5 nM taxol (tax). Asterisk (*) indicates two-tailed p-value <0.0001 using Fisher's exact test. Error bars represent standard deviations. (D) Model for forces acting during spindle bipolarity during different stages of mitosis in mammalian somatic cells. Arrows denote the direction of microtubule movement in response to applied force. During prophase, Eg5 activity slides apart antiparallel microtubules to separate centrosomes. Centrosome separation is assisted at this time by pulling forces generated at the cell cortex. Pole focusing activities (HSET, NuMA) are released into the cytosol at nuclear envelope breakdown, and these generate inward force on overlapping microtubules between the two centrosomes which is opposed by the outward force generated by Eg5. In metaphase, microtubules are relatively dynamic and lack extensive overlap. At this time, Eg5 is not necessary for spindle bipolarity because overlapping microtubules are not present for pole focusing activities and other, as yet unknown, proteins take responsibility for maintaining spindle bipolarity (question mark).