Sliding of kinetochore fibers along bridging fibers helps center the chromosomes on the spindle

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ABSTRACT

Chromosome alignment at the spindle equator during metaphase is the most remarkable feature of mitosis, which promotes proper chromosome segregation and depends on the forces exerted at the plus end of kinetochore microtubules and polar ejection forces. However, forces arising from lateral mechanical coupling of kinetochore fibers with non-kinetochore microtubules play a role in chromosome alignment, but the mechanism is unclear. Here we develop a speckle microscopy assay to measure the poleward flux of individual microtubules in spindles of human cells and show that bridging microtubules slide apart and undergo poleward flux at a higher rate than kinetochore microtubules. Depletion of the microtubule coupler NuMa increased the difference in the flux velocity of kinetochore and bridging microtubules, suggesting that sliding forces from the bridging fiber are transmitted largely through NuMa onto the associated kinetochore fibers. Depletions of Kif18A/kinesin-8, Kif4A/kinesin-4, as well as double depletions of Kif18A together with Kif4A or Kif18A together with the crosslinker of antiparallel microtubules PRC1 increased the flux velocity of kinetochore fibers up to the velocity of bridging fibers, due to the increased coupling resulting from the extended antiparallel overlap regions. We found severe kinetochore misalignment after double Kif18A and Kif4A as well as Kif18A and PRC1 depletions compared to a single Kif18A depletion, suggesting that forces within the bridging fiber have a centering effect on the kinetochores. We propose that lateral length-dependent sliding forces that the bridging fiber exerts onto kinetochore fibers drive the movement of kinetochores towards the spindle center, thereby promoting chromosome alignment.

INTRODUCTION

Chromosome alignment at the spindle equator in metaphase is a hallmark of mitosis and is important for proper completion of mitosis (Fonseca et al., 2019; Maiato et al., 2017). According to the prevailing view, kinetochore alignment is maintained by the forces exerted at the tips of kinetochore fibers (k-fibers) (Armond et al., 2015; Stumpff et al., 2008), with contribution of polar ejection forces acting on chromosome arms (Stumpff et al., 2012; Wandke et al., 2012). However, k-fibers are not isolated from other microtubules in the spindle, but mechanically coupled with non-kinetochore microtubules (McDonald et al., 1992; O'Toole et al., 2020) and other k-fibers (Vladimirou et al., 2013). In the central part of the spindle, a pair of sister k-fibers interacts with a bundle of antiparallel interpolar microtubules termed bridging fiber, which are crosslinked by PRC1 (Kajtez et al., 2016; Polak et al., 2017). Optogenetic experiments have shown that bridging fibers promote kinetochore alignment (Jagrić et al., 2020). Yet, the mechanism how these non-kinetochore microtubules regulate kinetochore positioning is still unclear.

K-fibers undergo poleward flux, a conveyor belt-like movement in which the whole k-fiber is being transported towards the pole while its minus ends depolymerize and plus ends polymerize (Forer, 1965; Hamaguchi et al., 1987; Hiramoto and Izutsu, 1977; Mitchison, 1989). Poleward flux is regulated by several motor proteins, including Kif2a/kinesin-13 that depolymerizes microtubule minus ends at the spindle poles (Ganem et al., 2005; Rogers et al., 2004). A recent comprehensive study showed that flux is driven by CENP-E/kinesin-7, Kif4A/kinesin-4, Eg5/kinesin-5, and Kif15/kinesin-12 (Steblyanko et al., 2020).

Poleward flux is typically studied by using photoactivation, a method that was used for the first demonstration of this process (Mitchison, 1989). In tubulin photoactivation experiments, all the microtubules within the illuminated region are photoactivated, thus the movements of kinetochore and non-kinetochore microtubules cannot be distinguished. Given that kinetochore microtubules provide the strongest tubulin signal in spindles of mammalian somatic cells (Mitchison, 1989), the photoactivated spots are interpreted as belonging to kinetochore fibers. However, it remains unclear whether other spindle microtubules experience flux, how the fluxes of different microtubule classes are related, and how they affect chromosome alignment.

In this study we developed a speckle microscopy assay to measure the movements of individual microtubules within the spindle of human cells, which allowed us to uncover that bridging microtubules undergo flux during metaphase at a rate higher that kinetochore microtubules. Sliding forces from the bridging fiber are transmitted to the k-fiber in a manner dependent on the coupling between bridging and k-fibers. We show that k-fibers flux slower when this coupling is weakened such as after depletion of NuMa, whereas the k-fiber flux speeds up when the coupling is stronger such as after depletion of Kif18A or Kif4A, which results in larger overlaps. Our results suggest a model in which lateral length-dependent sliding forces that the

bridging fiber exerts onto k-fibers promote the movement of kinetochores towards the spindle center.

RESULTS AND DISCUSSION

Assay to follow the movement of individual microtubules within the spindle

We developed a speckle microscopy assay to study the movements of individual microtubules within spindles of the human non-cancer immortalized epithelial cell line hTERT-RPE1 (from here on referred to as RPE1), by using a very low concentration (1 nM) of SiR-tubulin (Lukinavicius et al., 2014). This resulted in a speckled signal of SiR-tubulin in the spindle (**Fig. 1 A**), which results from a few dye molecules within a resolution-limited region (Waterman-Storer and Salmon, 1998).

To distinguish between different classes of microtubules within the spindle, we divided the speckles into groups: (1) the speckles that originate close to a kinetochore, at the pole-facing side, were defined as those on a kinetochore microtubule (**Fig. 1 B**), (2) the speckles that pass the region between sister kinetochores, moving close to the kinetochores, were defined as those on a bridging microtubule (**Fig. 1 C**), (3) all other speckles in the spindle region between the centrosomes, for which we cannot determine the type of microtubule they belong to, we refer to as "other" speckles (**Fig. 1 D**). This assay allowed us to study the movement of individual microtubules with respect to each other.

Bridging microtubules undergo poleward flux at a higher rate than kinetochore microtubules

We first analyzed speckles on k-fibers. The speckles appeared at various distances in the range 4 - 9 μm from the pole, moved poleward and could be followed for about 1 minute before disappearing, presumably due to photobleaching. We tracked each speckle and measured its linear velocity with respect to the centrosome over the first 30 seconds of the movement, and found that the speckles on kinetochore microtubules moved poleward at a velocity of 1.28 ± 0.13 μm/min (n = 32 speckles from 12 cells) (**Fig. 1 E-I; Table 1**). This velocity is similar to the rate of poleward flux previously measured by tubulin photoactivation on kinetochore fibers in RPE1 cells expressing photoactivatable-GFP-α-tubulin, 1.09 ± 0.11 μm/min (Dudka et al., 2018). This validates our identification of speckles on k-fibers.

A similar analysis revealed that speckles on bridging microtubules also undergo poleward flux. These speckles appeared in the range 5 - 10 μm from the pole and could be followed typically over 1 minute. Remarkably, the speckles on bridging microtubules moved poleward at a velocity of 1.96 ± 0.23 μm/min (n = 32 speckles from 15 cells) (**Fig. 1 E-I; Table 1**), which is significantly faster than the speckles on kinetochore microtubules ($p = 0.01$). Flux velocities were higher for speckles that started at a larger distance from the pole (**Fig. 1 J**).

Because this is the first measurement of poleward flux of bridging microtubules in human spindles, we decided to validate our method of identification of speckles in the bridging fiber. We used PRC1 siRNA, which is known to specifically reduce the number of bridging microtubule to ~50% of the original number (Jagrić et al., 2020; Polak et al., 2017). In cells treated with PRC1 siRNA, we observed 10 speckles on bridging microtubules in 8 cells, which is roughly a 2-fold reduction in comparison with untreated cells, 32 speckles in 15 cells, providing support for our method.

Given that the spindle length is constant during metaphase, the antiparallel bridging microtubules slide apart at twice the rate of their poleward flux, 3.9 ± 0.5 µm/min. This rate is comparable to the sliding rate of bridging microtubules in early anaphase measured by photoactivation, which is roughly 4.5 µm/min (Vukusic et al., 2019), suggesting that the bridging microtubule sliding is driven by a similar mechanism in metaphase and early anaphase. However, in contrast to early anaphase where kinetochore fibers and bridging fibers slide together at a similar rate (Vukusic et al., 2017), in metaphase kinetochore fibers move slower than bridging fibers. This difference is most likely due to the chromatin link between sister kinetochore fibers during metaphase. Thus, kinetochore fibers slip during their poleward movement with respect to bridging fibers in metaphase, but not in anaphase.

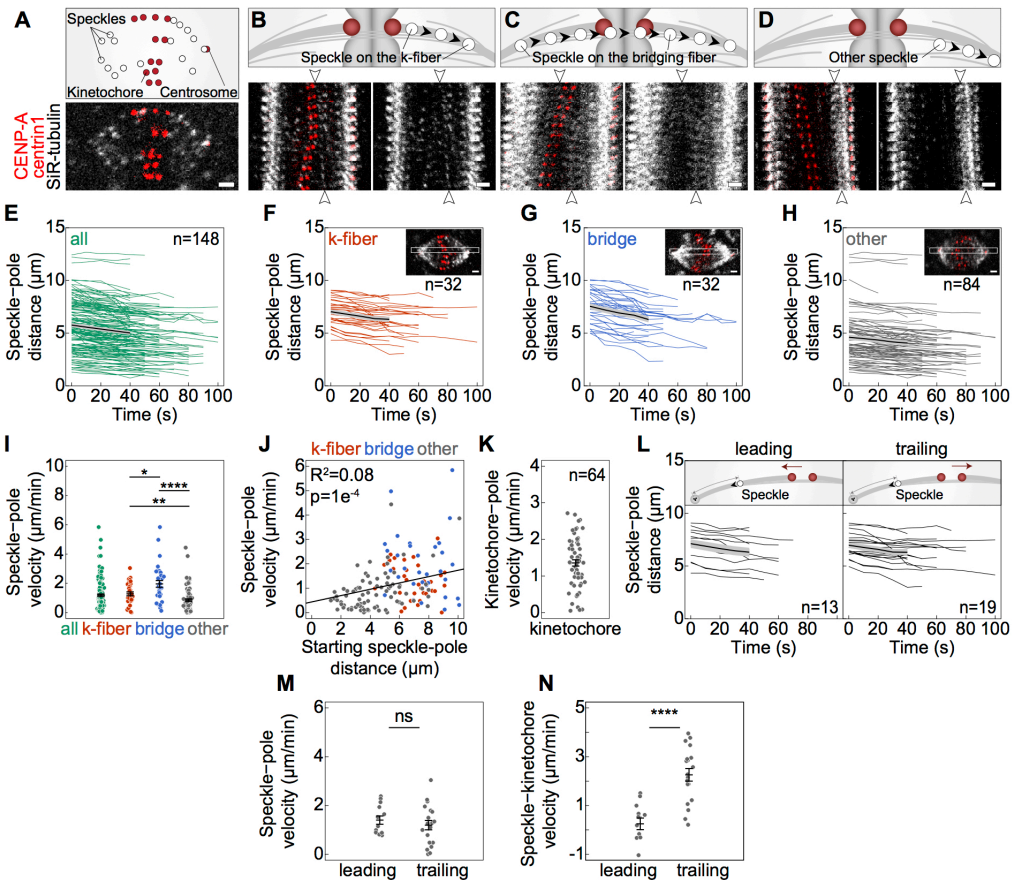

Figure 1. (**A**) Spindle in a RPE1 cell stably expressing CENP-A-GFP and centrin1-GFP (red) stained with 1 nM SiR-tubulin dye which appears as distinct speckles marking individual microtubules (gray). (**B**) Scheme of the speckle originating at kinetochore defined as the one marking the k-fiber microtubule (top). Montage over time demonstrating the movement of speckle belonging to the k-fiber microtubule (bottom). Merge (left); tubulin channel only (right). Arrowheads mark starting and ending positions of the tracked speckle. (**C**) Scheme of the speckle passing the region between sister kinetochores, moving close to the kinetochores, defined as the one marking microtubule within the bridging fiber (top). Montage over time demonstrating the movement of speckle belonging to the bridging fiber microtubule. Legend as in B. (**D**) Scheme of other speckles for which it could not be determined the type of microtubule they belong to (top). Montage over time demonstrating the movement of this group of speckles. Legend as in B. (**E-H**) Distance of all, k-fiber, bridging fiber and other speckles from the pole over time in untreated cells. Colored lines show individual speckles. Black line; mean. Grey area; s.e.m. Rectangles on the spindles inside graphs mark the regions for which montages in B-D are generated. (**I**) Poleward velocity of the speckles shown in E-H. Each dot corresponds to individual speckle. (**J**) Poleward velocity of k-fiber, bridging and other speckles versus the distance of their starting position from the pole. Black line shows linear regression. (**K**) Graph of kinetochore poleward velocity. Each dot corresponds to individual kinetochore pair. (**L**) Distance between speckles on the leading (left) and trailing (right) kinetochore microtubules from the pole. (**M**) Poleward velocity of the speckles shown in L. (**N**) Velocity of speckles on leading and trailing kinetochore microtubules moving away from the corresponding kinetochore. Error bars; s.e.m. n; number of measurements. Scale bars; 2 µm. Statistical analysis, t-test. R^2, coefficient of determination. p-value legend: < 0.0001 (****), 0.0001 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*), ≥ 0.05 (ns).

Leading kinetochore microtubules are largely in a pausing state, while trailing microtubules polymerize

Throughout metaphase, kinetochores oscillate around the equatorial plane of the spindle. During the periods of time when a pair of sister kinetochores moves at a roughly constant velocity towards one pole, this velocity was 1.4 ± 0.1 µm/min (n = 64 kinetochore pairs from 17 cells) (**Fig. 1 K**). The speckles on the leading kinetochore microtubules, which are linked to the pole towards which the kinetochores are traveling, moved poleward at a velocity of 1.4 ± 0.2 µm/min (n = 13 speckles from 11 cells) (**Fig. 1 L,M**). However, this value is likely overestimated because only those speckles that move poleward faster than the kinetochores and thus are not "chewed up" by the kinetochore can be tracked. These speckles on the leading k-fiber moved away from kinetochore at a velocity of 0.24 ± 0.23 µm/min (n =13 speckles from 11 cells), implying that kinetochore microtubules at the leading side are largely in a pausing state (**Fig. 1 N**). Even though we cannot exclude that in some cases of oscillations the leading k-fiber depolymerizes, this result indicates that kinetochores can move poleward while their leading k-fiber microtubules are in a pausing state or polymerize slowly.

Speckles on the trailing kinetochore microtubules moved poleward at velocity of 1.2 ± 0.2 µm/min (n =19 speckles from 10 cells), which was not different from the

leading side (p=0.4) (**Fig. 1 L,M**). These speckles moved away from the kinetochore at a velocity of 2.2 ± 0.3 µm/min (n =19 speckles from 10 cells), which corresponds to the polymerization rate of kinetochore microtubules at the trailing side (**Fig. 1 N**). Taken together, these result show that kinetochore microtubules are mainly in a polymerizing or a pausing state.

Sliding forces from the bridging fiber are transmitted onto the associated k-fiber

To explore how the sliding of the bridging microtubules and the poleward flux of k-fibers are related to each other and to kinetochore alignment, we performed a set of perturbations in which we depleted candidate microtubule-associated proteins by siRNA. First, we depleted non-motor proteins that are important for k-fiber and bridging fiber integrity and their crosslinking. Then we depleted motor proteins that localize to the bridging fiber and regulate microtubule dynamics and/or generate microtubule sliding.

We started with NuMa, a protein that is required for local load-bearing in the spindle (Elting et al., 2017) and for synchronous microtubule flux across the spindle (Steblyanko et al., 2020). Thus we hypothesized that NuMa may be one of the main crosslinkers of kinetochore and bridging microtubules extending from the same spindle half, i.e., aligned in a parallel orientation. To explore this hypothesis, we depleted NuMa by siRNA (**Fig. S1**) and found that the flux velocity of bridging fibers did not change, whereas the flux velocity of kinetochore microtubules decreased by ~34% (from 1.28 ± 0.13 in untreated cells to 0.84 ± 0.14 after NuMa depletion, **Fig. 2 A,B; Table 1**). These results support the model in which the sliding apart of bridging microtubules is transmitted through NuMa to kinetochore microtubules, which flux slower when this connection is weakened.

To perturb specifically bridging fibers, we depleted PRC1 (**Fig. S1**), which results in ~50% decrease of the number of microtubules in the bridging fiber without affecting the length of their antiparallel overlaps (Jagrić et al., 2020). We found that depletion of PRC1 did not change the flux velocity of bridging or kinetochore microtubules (**Fig. 2 C; Table 1**), in agreement with a previous result based on tubulin photoactivation (Steblyanko et al., 2020). This result suggests that the bridging microtubules that remain after PRC1 depletion are sufficient to generate sliding.

The augmin complex promotes nucleation of new microtubules at the wall of existing microtubules (David et al., 2019; Goshima et al., 2008; Kamasaki et al., 2013). Depletion of the Haus8 subunit of the augmin complex results in ~50% decrease of the number of microtubules in the bridging fiber and ~30% in the k-fiber, together with an elongation of the PRC1-labeled antiparallel overlaps of bridging microtubules (Manenica et al., 2020). When we depleted Haus8, we measured ~35% slower flux of the bridging microtubules, while the flux of kinetochore microtubules decreased ~15% (**Fig. 2 D; Table 1**). Given that after Haus8 depletion most of the

microtubules are nucleated at the spindle pole, we speculate that the mechanisms driving microtubules depolymerization at the pole limit their velocity of flux.

To explore how k-fibers affect the sliding of bridging microtubules, we depleted Ndc80, the main coupler of kinetochores to microtubule ends (Cheeseman et al., 2006; Cheeseman and Desai, 2008). Reduction of k-fibers was confirmed by the observation that speckles at the pole-facing side of the kinetochore were not found (n=8 cells). We found that the speckles on bridging microtubules fluxed at a similar velocity as in untreated cells (**Fig. 2 E; Table 1**), suggesting that k-fibers do not act as a brake for the sliding of bridging microtubules.

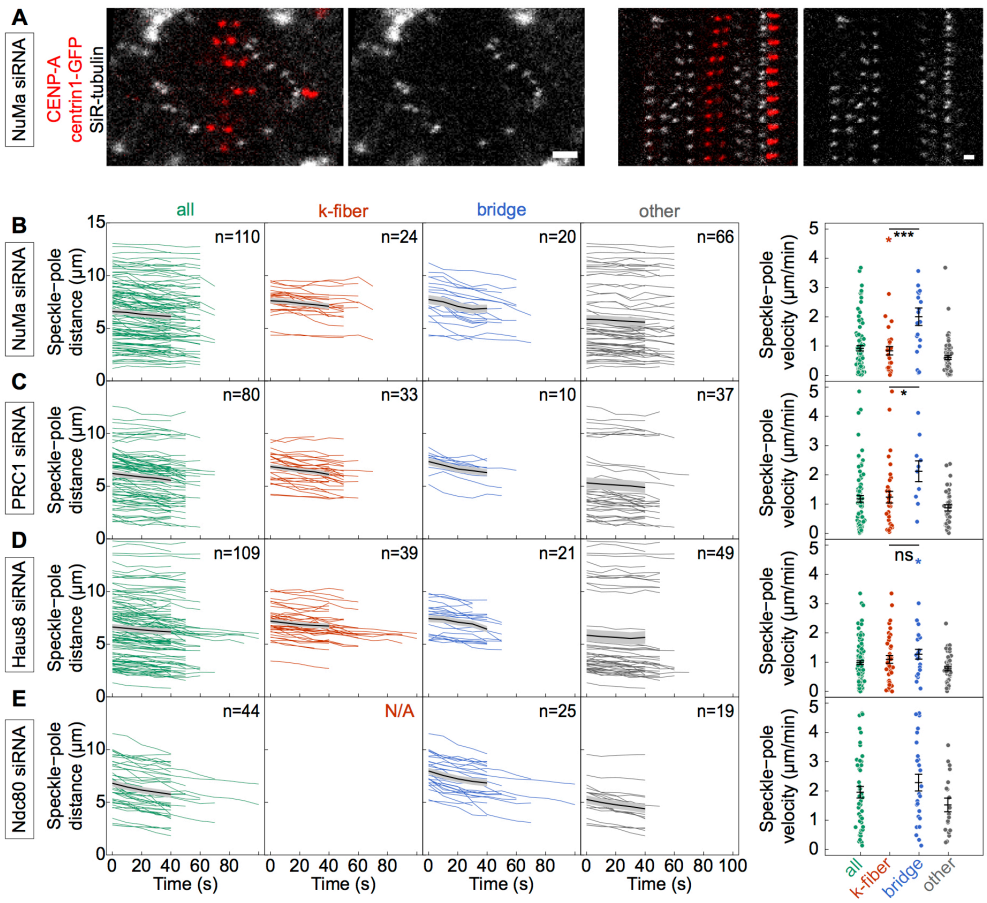

Figure 2. (**A**) Spindle in a RPE1 cell stably expressing CENP-A-GFP and centrin1-GFP (red) treated depleted for NuMa and stained with 1 nM SiR-tubulin dye (left). Montage over time for speckle movement in spindle on left (right). Merge (left); tubulin channel only (right). Speckles after depletion of NuMa (**B**), PRC1 (**C**), Haus8 (**D**) and Ndc80 (**E**). Graphs from left to right show: distance between speckles and the spindle pole over time for all speckles, speckles on kinetochore microtubules, speckles on bridging microtubules, other speckles. Colored lines show individual speckles. Black line; mean. Grey area; s.e.m. Poleward velocity of the speckles is shown at the right. Error bars; s.e.m. All treatments include at least three independent experiments. n; number of measurements. Scale bars; 2 µm. Statistical analysis, t-test. Coloured asterisks denote significance with respect to untreated cells. p-value legend as in Fig. 1.

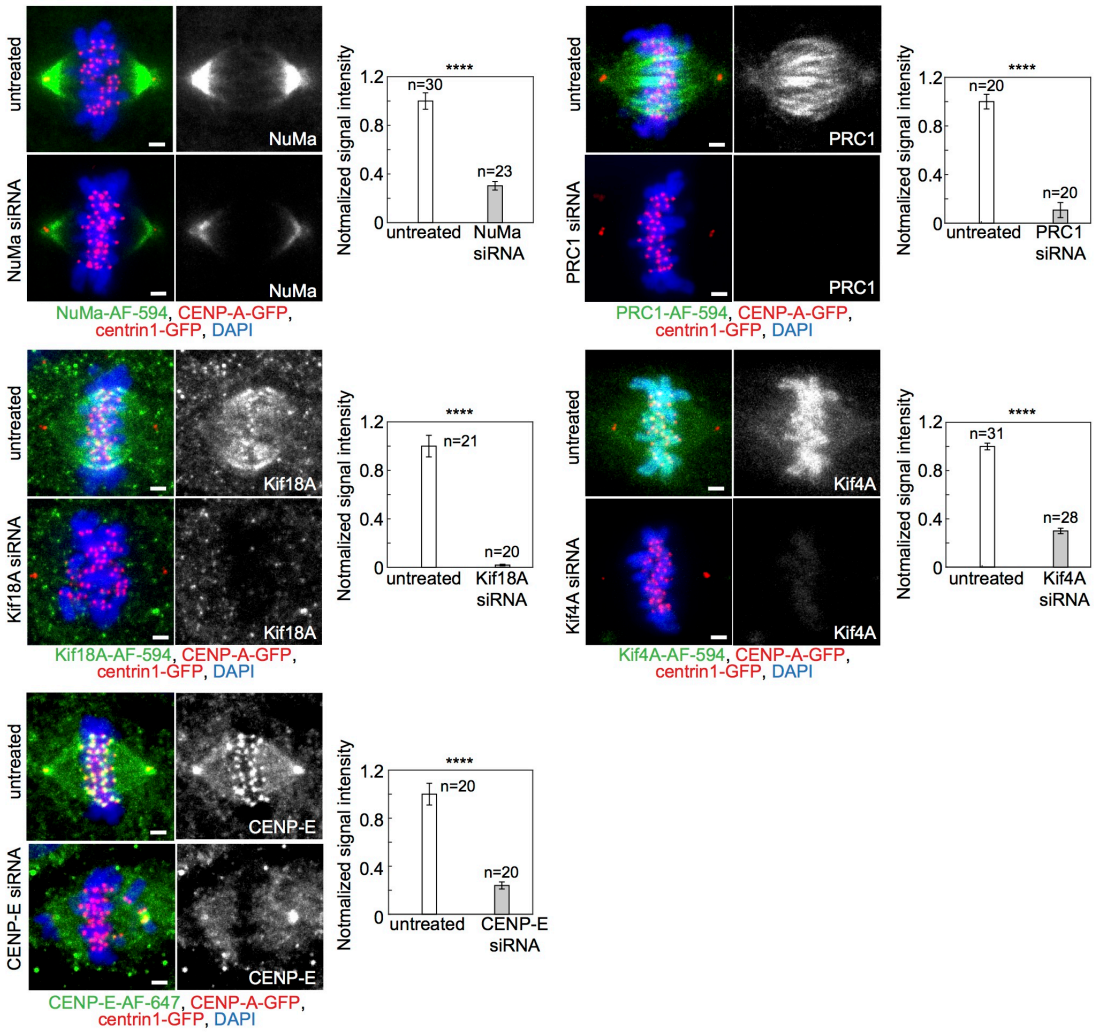

Figure S1. Fixed spindles in a RPE1 cell stably expressing CENP-A-GFP and centrin1-GFP (red) in cells immunostained for NuMa (AF-594, green) (upper left), PRC1 (AF-594, green) (upper right), Kif18A (AF-594, green) (middle left), Kif4A (AF-594, green) (middle right) and CENP-E (AF-647, green) (bottom left) in untreated (upper rows) and corresponding siRNA treated cells (bottom rows), with DNA stained with DAPI (blue). Left; merge; protein of interest; right (gray). Graphs showing intensities of indicated proteins in untreated (white bars) and siRNA treated (gray bars) cells are given on the right. All values are normalized on the mean intensity value of untreated cells for each protein. All treatments include at least two independent experiments. n; number of cells. Scale bars; 2 µm. All images are maximum intensity projections of five z-planes smoothed with 0.5-pixel-sigma Gaussian blur. Statistical analysis, t-test. p-value legend as in Fig. 1.

Motor proteins that localize within the bridging fiber modulate the flux velocity of bridging and kinetochore microtubules

Next, we depleted motor proteins that localize to the bridging fiber and regulate microtubule dynamics and/or generate microtubule sliding (**Fig. 3**). Kinesin-8

proteins are major regulators of chromosome alignment in various organisms from yeast to humans (Garcia et al., 2002; Stumpff et al., 2008; Wargacki et al., 2010). The human kinesin-8 Kif18A accumulates to the plus end of kinetochore microtubules, where it suppresses microtubules dynamics (Du et al., 2010; Stumpff et al., 2012). Kif18A also localizes within bridging fibers and regulates the length of their antiparallel overlap regions (Jagrić et al., 2020). When we depleted Kif18A (**Fig. S1**), we found that the flux velocity of bridging fibers did not change, whereas the flux velocity of kinetochore microtubules increased by ~54% (from 1.28 ± 0.13 in untreated cells to 1.97 ± 0.29 after Kif18A depletion, **Fig. 3 A; Table 1**).

Kinesin-4 proteins are chromokinesins that regulate microtubule dynamics (Bieling et al., 2010b; Bringmann et al., 2004; Stumpff et al., 2012; Wandke et al., 2012) and generate polar ejection forces (Bieling et al., 2010a; Brouhard and Hunt, 2005). Kif4A, a human kinesin-4, localizes during metaphase to bridging fibers in addition to its prominent localization on chromosome arms, and regulates the length of the antiparallel overlaps of bridging microtubules (Jagrić et al., 2020). After depletion of Kif4A (**Fig. S1**), we measured that the flux velocity of bridging microtubules did not change, whereas the flux velocity of kinetochore microtubules increased by ~52% (from 1.28 ± 0.13 in untreated cells to 1.94 ± 0.27 after Kif4A depletion, **Fig. 3 B; Table 1**), similar to Kif18A depletion. Double depletion of Kif18A and Kif4A and a double depletion of Kif18A and PRC1 also resulted in a similar phenotype, with no effect on the bridging fiber flux and an increase in the flux velocity of kinetochore microtubules (**Fig. 3 C-E; Table 1**).

Taken together, in Kif4A depletion and all depletions that include Kif18A kinetochore microtubules slide at a velocity that is indistinguishable from the sliding velocity of bridging microtubules, suggesting a stronger coupling between bridging and kinetochore microtubules. We hypothesized that this may be due to longer overlaps of bridging microtubules, which imply longer overlaps between a k-fiber and its bridging fiber. In agreement with previous results on HeLa cells (Jagrić et al., 2020), Kif4A depletion and Kif18A depletion in RPE1 cells resulted in an increased length of PRC1-labeled streaks marking antiparallel overlaps of bridging microtubules (**Fig. S2**; **Table 1**). Double depletion of Kif18A and Kif4A resulted in a similar increase, close to single Kif18A depletion (**Fig. S2**; **Table 1**). Thus, similar velocities of bridging and k-fiber flux are correlated with an increased overlap length, supporting the role of antiparallel overlaps in the coupling between bridging and k-fibers.

Both single Kif4A and Kif18A depletions led to an increased spindle length, similar to the results on HeLa cells (Jagrić et al., 2020), and the double depletion resulted in the largest increase in spindle length (**Table 1**). It is reasonable to assume that longer spindles have longer k-fibers and thus the overlaps between a k-fiber and the bridging microtubules extending from the same spindle half are also longer, which may further increase the mechanical coupling between bridging and k-fibers.

Finally, we explored the role of CENP-E in the flux of bridging and kinetochore microtubules. A role of this protein in flux has been demonstrated during prometaphase (Steblyanko et al., 2020). Depletion of CENP-E (**Fig. S1**) resulted in a

significant reduction of flux velocity both for bridging and kinetochore microtubules in metaphase (**Fig. 3 F; Table 1**). These two velocities were similar in CENP-E depleted cells. Given that CENP-E localizes to bridging fibers in metaphase (Steblyanko et al., 2020), we propose that CENP-E within the bridge contributes to the sliding of bridging microtubules during metaphase.

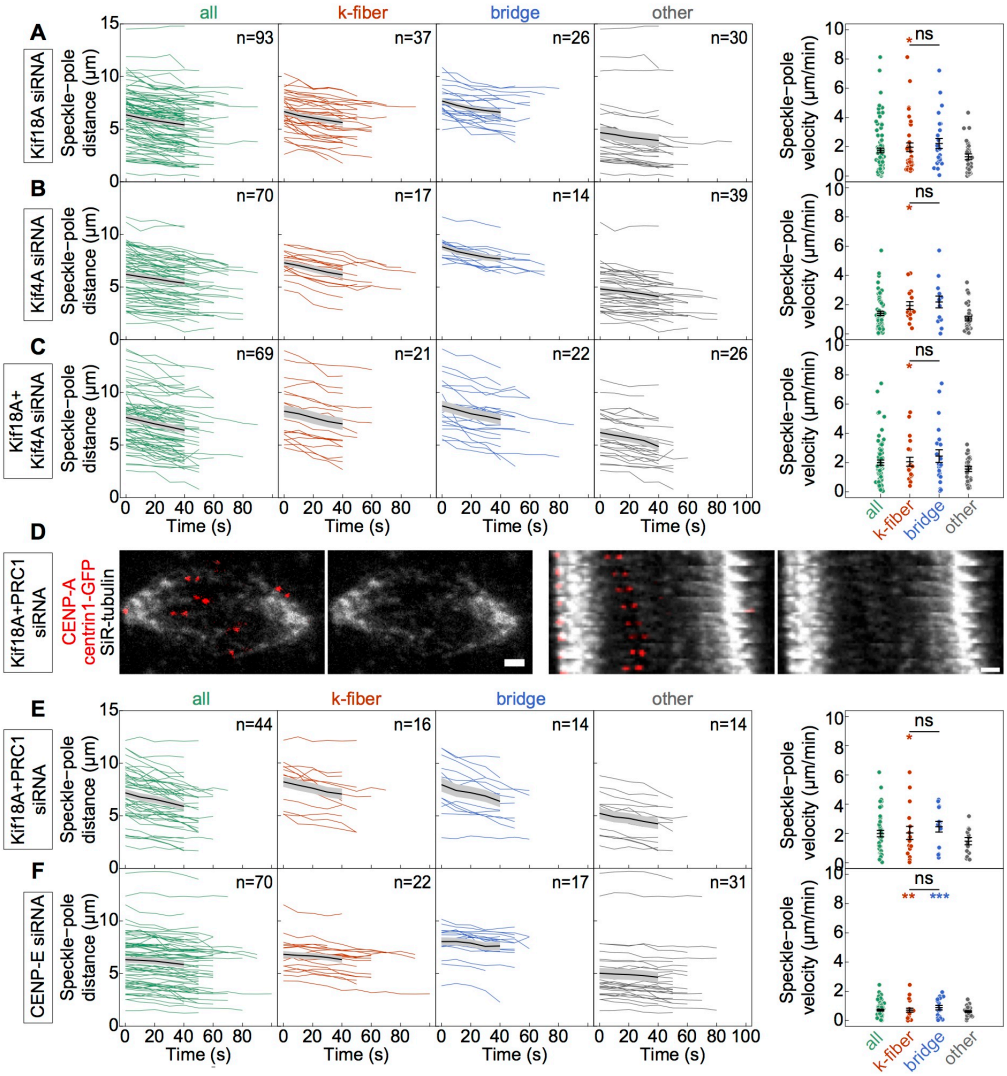

Figure 3. Speckles after depletion of Kif18A (**A**), Kif4A (**B**), Kif18A and Kif4A (**C**), Kif18A and PRC1 (**E**) and CENP-E (**F**). Graphs from left to right show: distance between speckles and the spindle pole over time for all speckles, speckles on kinetochore microtubules, speckles on bridging microtubules, other speckles. Colored lines show individual speckles. Black line; mean. Grey area; s.e.m. Poleward velocity of the speckles is shown at the right. Error bars; s.e.m. (**D**) Spindle in a RPE1 cell stably expressing CENP-A-GFP and centrin1-GFP (red) depleted for Kif18A and PRC1, stained with 1 nM SiR-tubulin dye (left). Montage over time for speckle movement in spindle on the left (right). Merge (left); tubulin channel only (right). All treatments include at least three independent experiments. n; number of measurements. Scale bars; 2 µm. Statistical analysis, t-test. Colored asterisks denote significance with respect to untreated cells. p-value legend as in Fig. 1.

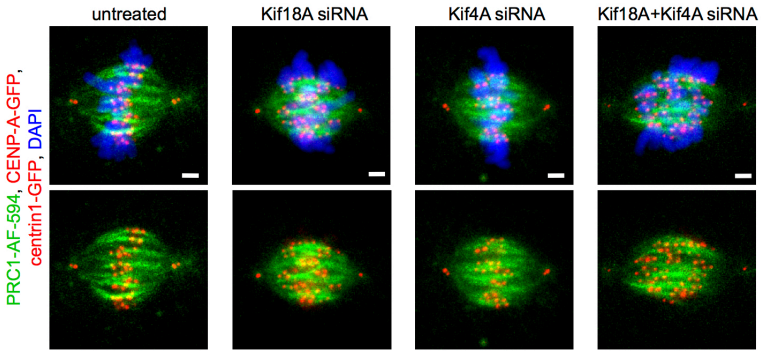

Figure S2. Fixed spindles in RPE1 cells stably expressing CENP-A-GFP and centrin1-GFP (red) in untreated (first column), Kif18A siRNA (second column), Kif4A siRNA (third column), and Kif18A and Kif4A siRNA treated cells (fourth column), immunostained for endogenous PRC1 (AF-594, green) and stained with DAPI (blue). Upper row; merge, bottom row; GFP and AF-594. Scale bars; 2 µm. All images are maximum intensity projections of five z-planes smoothed with 0.5-pixel-sigma Gaussian blur.

Forces within the bridging fiber have a centering effect on the kinetochores

To explore the role of the selected proteins in kinetochore alignment, we quantified the alignment efficiency by measuring the distances of sister kinetochore midpoints from the equatorial plane, d_{EQ} (**Fig. 4 A**). Among the single protein depletions, only Kif18A, as well as Ndc80 which does not have bioriented kinetochores, resulted in a significantly larger d_{EQ} implying kinetochore misalignment (**Fig 4 A; Table 1**). This is in agreement with the known major role of Kif18A in the positioning of bioriented kinetochore (Mayr et al., 2007; Stumpff et al., 2008).

To uncouple the role of forces generated within the bridging fiber from the function of Kif18A in kinetochore alignment, we used a double depletion of Kif18A and Kif4A. Strikingly, the double depleted cells showed remarkable kinetochore misalignment, reflected in a significantly increased d_{EQ} with respect to Kif18A depletion (2.86 ± 0.12 versus 1.50 ± 0.08, **Fig. 4 A,** see also **Fig. S2**). Thus, in the absence of Kif18A, Kif4A promotes kinetochore alignment, possibly through its activity on chromosome arms in generating polar ejection forces or within the bridging fiber in regulating the sliding forces.

To distinguish whether the effect of Kif4A depletion in the absence of Kif18A is due to its action on chromosome arms or within the bridging fiber, we depleted PRC1 together with Kif18A. PRC1 depletion results in significant removal of Kif4A from the bridging fiber, but not from the chromosome arms (Jagrić et al., 2020). Thus, if the effect of Kif4A is from its role on chromosome arms, kinetochore alignment after double Kif18A and PRC1 depletion is expected to be similar as after single Kif18A depletion. In contrast, if the role within the bridging fiber is crucial, double Kif18A and PRC1 depletion is expected to have stronger misalignment than single

Kif18A depletion. Our experiments showed significantly higher value of d_{EQ} after double Kif18A and PRC1 depletion than after single Kif18A depletion, supporting a role of Kif4A within the bridging fiber in the regulation of kinetochore alignment (**Fig. 4 A**; **Table 1**). We conclude that an important function of PRC1 in chromosome alignment is to recruit Kif4A to the bridging fiber.

The three depletion conditions with the strongest kinetochore misalignment, Kif18A, double depletion of Kif18A and Kif4A, and double depletion of Kif18A and PRC1, had larger spindle lengths than untreated cells, but not much larger than other conditions that did not induce misalignment (**Fig. 4 B,C**). Importantly, these three conditions had a significantly faster k-fiber flux than most other cases, whereas the bridging fiber flux was not exceptional (**Fig. 4 D,E**). Thus, we conclude that the relative sliding of the k-fibers with respect to the bridging fiber is important for kinetochore alignment.

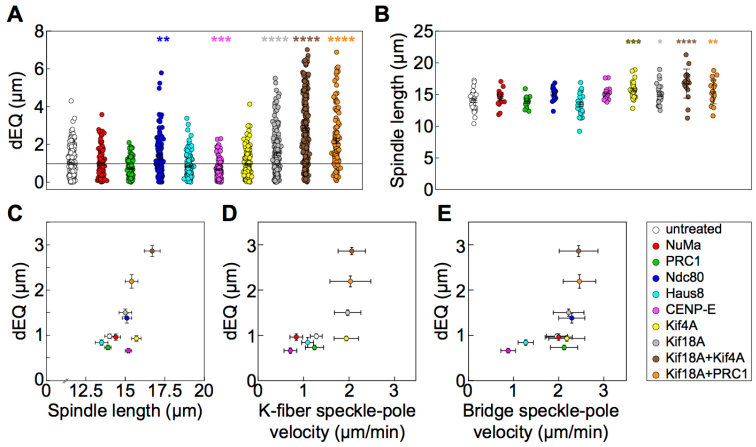

Figure 4. (**A**) Distance of sister kinetochore midpoints from the equatorial plate (d_{EQ}) for untreated and siRNA treated cells. Treatments are color-coded according to the legend (bottom right). Horizontal black line corresponds to the mean d_{EQ} value in untreated cells. (**B**) Spindle lengths for cells as in A. (**C**) d_{EQ} versus spindle length for cells as in A. (**D**) d_{EQ} versus k-fiber speckle poleward velocity for cells as in A. (**E**) d_{EQ} versus bridge speckle poleward velocity for cells as in A. Asterisks denote significance with respect to untreated cells. Statistical analysis; Mann-Whitney (A), t-test (B). p-value legend as in Fig. 1.

Table 1. Measurements of flux, spindle and kinetochore parameters.
Values are given as mean ± sem. The numbers in brackets denote the number of measurements (number of speckles for flux measurements or number of kinetochore pairs; for spindle length this number is not given because it is equal to the number of cells), number of cells, and p-value from a t-test for comparison with untreated cells. *n.a.*, not applicable; *n.d.*, not determined.

	Flux, all (µm/min)	Flux, k-fiber (µm/min)	Flux, bridge (µm/min)	Flux, other (µm/min)	Spindle length (µm)	Overlap length (µm)	Inter-kinetochore distance (µm)	Kinetochore distance to equatorial plane (µm)
Untreated	1.19 ± 0.08 (148, 23, n.a.)	1.28 ± 0.13 (32, 12, n.a.)	1.96 ± 0.23 (32, 15, n.a.)	0.86 ± 0.09 (84, 23, n.a.)	14.0 ± 0.3 (30, n.a.)	6.6 ± 0.2 (33, 11, n.a.)	0.94 ± 0.01 (258,44 , n.a.)	0.98 ± 0.05 (258, 44, n.a.)
NuMa	0.92 ± 0.09 (110, 13, 0.03)	0.84 ± 0.14 (24, 11, 0.02)	2.00 ± 0.30 (20, 10, 0.90)	0.62 ± 0.07 (66, 12, 0.03)	14.4 ± 0.3 (17, 0.40)	6.8 ± 0.1 (33, 10, 0.45)	0.95 ± 0.02 (130, 17, 0.7)	0.96 ± 0.07 (130, 17, 0.7)
PRC1	1.18 ± 0.11 (80, 11, 0.95)	1.24 ± 0.20 (33, 11, 0.86)	2.12 ± 0.35 (10, 8, 0.70)	0.87 ± 0.11 (37, 5, 0.9)	13.9 ± 0.2 (15, 0.71)	n.a.	0.87 ± 0.01 (93, 15, 3e-4)	0.73 ± 0.05 (93, 15, 0.01)
Haus8	0.98 ± 0.07 (109, 14, 0.05)	1.09 ± 0.13 (39, 13, 0.3)	1.27 ± 0.17 (21, 11, 0.01)	0.77 ± 0.07 (49, 11, 0.4)	13.5 ± 0.4 (23, 0.23)	n.d.	0.81 ± 0.01 (137, 23, 2.2e-16)	0.84 ± 0.06 (137, 23, 0.09)
Ndc80	1.96 ± 0.20 (44, 8, 6e-04)	n.a.	2.29 ± 0.29 (25, 8, 0.3)	1.52 ± 0.23 (19, 3, 0.01)	15.1 ± 0.3 (15, 0.03)	n.d.	0.81 ± 0.01 (105, 15, 7e-12)	1.38 ± 0.11 (105, 15, 0.003)
Kif18A	1.83 ± 0.17 (93, 21, 7e-04)	1.97 ± 0.29 (37, 18, 0.03)	2.22 ± 0.34 (26, 12, 0.5)	1.31 ± 0.20 (30, 8, 0 .04)	15.0 ± 0.4 (21, 0.04)	8.1 ± 0.3 (35, 14, 1e-04)	0.90 ± 0.01 (198, 28, 0.01)	1.50 ± 0.08 (198, 28, 9e-6)
Kif4A	1.48 ± 0.14 (70, 16, 0.07)	1.94 ± 0.27 (17, 8, 0.03)	2.18 ± 0.40 (14, 7, 0.6)	1.03 ± 0.14 (39, 8, 0.3)	15.7 ± 0.3 (25, 2e-04)	7.4 ± 0.2 (39, 10, 6e-04)	1.06 ± 0.02 (165, 25, 1e-7)	0.93 ± 0.06 (165, 25, 0.4)
Kif18A+ Kif4A	1.99 ± 0.18 (69, 11, 1e-04)	2.06 ± 0.30 (21, 10, 0.02)	2.44 ± 0.43 (22, 9, 0.3)	1.56 ± 0.17 (26, 6, 8e-04)	16.7 ± 0.5 (18, 2e-05)	8 ± 0.2 (35, 13, 1e-04)	0.83 ± 0.01 (235, 37, 9e-13)	2.86 ± 0.12 (235, 37, 2e-16)
Kif18A + PRC1	1.99 ± 0.22 (44, 4, 0.001)	2.03 ± 0.45 (16, 3, 0.04)	2.46 ± 0.36 (14, 4, 0.2)	1.47 ± 0.25 (14, 4, 0.03	15.4 ± 0.4 (18, 0.009)	n.d.	0.80 ± 0.01 (116, 18, 1e-14)	2.19 ± 0.15 (116, 18, 8e-15)
CENP-E	0.71 ± 0.06 (70, 9, 9e-06)	0.71 ± 0.14 (22, 8, 0.004)	0.88 ± 0.16 (17, 7, 3e-04)	0.63 ± 0.06 (31, 3, 0.02)	15.2 ± 0.2 (20, 0.007)	6.3 ± 0.2 (28, 11, 0.17)	0.94 ± 0.02 (122, 25, 0.9)	0.66 ± 0.05 (122, 25, 0.0001)

A model for kinetochore centering on the spindle by sliding forces from the bridging fiber

Based on our results from speckle microscopy that allowed us to measure the relative movements of kinetochore and bridging microtubules in normal and perturbed spindles, we propose that sliding forces from the bridging fiber facilitate kinetochore positioning at the spindle equator (**Fig. 5A**). We have shown that bridging microtubules slide apart, which is most likely driven by forces generated by motor proteins acting within the antiparallel overlap zones of bridging microtubules. One of these sliding motors is CENP-E based on our finding that its depletion leads to slower poleward flux of bridging microtubules.

The forces from the bridging fiber are transmitted to k-fibers through NuMa and similar proteins that crosslink kinetochore and bridging microtubules. When NuMa is depleted, this transmission is impaired and the k-fiber flux slows down, whereas the bridging fiber flux remains unchanged.

The length of the region of the antiparallel overlap of bridging microtubules is regulated by Kif18A and to a smaller extent Kif4A. This length is especially important because if overlaps between bridging microtubules are longer, then the antiparallel overlap zones between bridging and kinetochore microtubules are also longer, accumulate more motors that slide kinetochore microtubules with respect to the antiparallel bridging microtubules, thereby increasing the flux velocity of the k-fiber (**Fig. 5B**). This picture is based on our finding that when Kif18A or Kif4A or both are depleted, the overlap zones are longer and the k-fiber flux is equal to the bridging fiber flux.

How can sliding of bridging microtubules promote kinetochore centering? If a pair of kinetochores is misaligned, i.e., displaced towards one pole, then the k-fiber extending towards that pole is shorter than the opposite one and has a shorter overlap with the associated bridging microtubules, both those that extend from the same and from the opposite spindle half. This means that the transmission of the sliding forces from the bridging microtubules to the kinetochore microtubules is less efficient, causing this short k-fiber to slip along the bridging fiber and flux slower than the bridging fiber. In contrast, the k-fiber on the other side is longer and has a long overlap region with the bridging microtubules, including both those that originate from the same and from the opposite spindle half. Thus, the force transmission to the k-fiber is more efficient and this k-fiber fluxes at a velocity similar to the bridging fiber. Faster flux of the longer k-fiber results in the movement of the midpoint between the two k-fibers in the direction of the longer k-fiber, thereby promoting centering of the kinetochores (**Fig. 5A**).

We propose that this mechanism based on relative sliding of kinetochore and bridging microtubules works together with the known length-dependent regulation of microtubule dynamics by Kif18A to ensure alignment of kinetochores at the equatorial plane of the spindle in metaphase. This mechanism may also work during prometaphase to promote chromosome movement from polar regions of the spindle towards the equator and their congression at the metaphase plate.

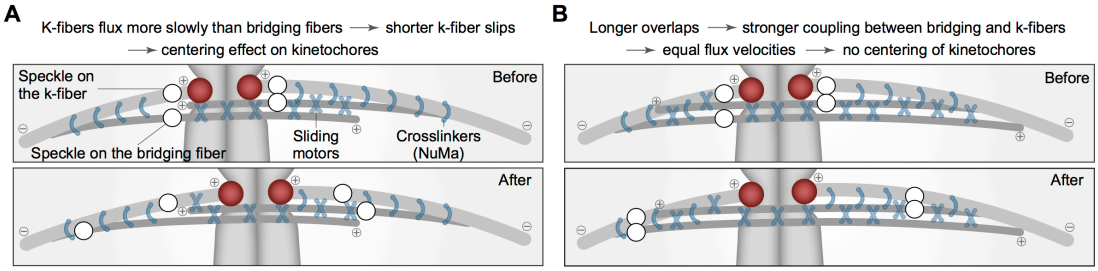

Figure 5. A model for how sliding forces exerted by the bridging fiber onto the sister kinetochore fibers help center the kinetochores on the spindle.
(**A**) The schemes depict how lateral forces acting along the k-fiber help kinetochore alignment at the spindle center. Top: A pair of kinetochores (red circles) is displaced towards the left pole. To visualize relative movements of the microtubules, four marks representing tubulin speckles are denoted, two on k-fibers and two on the antiparallel microtubules of the bridging fiber adjacent to the speckles on the k-fiber (white circles; plus and minus signs mark microtubule ends). Bottom: Over time, the speckles on the bridging fiber move poleward by a similar distance, due to sliding of bridging microtubules driven by motor proteins (blue X-shapes). These sliding forces are transmitted to the k-fibers through crosslinks between the fibers (blue C-shapes), but k-fibers flux more slowly than bridging fibers due to imperfect coupling. In particular, the longer k-fiber on the right side has a longer overlap with the bridging fiber and thus the coupling is stronger, leading to a higher flux velocity of this fiber in comparison with the shorter k-fiber. Thus, the midpoint between the marks on the sister k-fibers, and hence also the kinetochores, move towards the spindle center. We propose that this centering mechanism acts together with the mechanisms that regulate k-fiber plus-end dynamics (not depicted) to drive chromosome alignment.
(**B**) If the coupling between the k-fibers and the bridging fiber is too strong, such as in cases when the antiparallel overlap is excessively long, the k-fibers flux at the same velocity as bridging fibers. In this situation, the sliding forces do not promote kinetochore movement towards the spindle center, resulting in chromosome misalignment.

METHODS

Cell culture

hTERT-RPE-1 cell line with a stable expression of CENP-A-GFP and centrin1-GFP was a gift from Alexey Khodjakov (Wadsworth Center, New York State Department of Health, Albany, NY, USA) (developed in (Magidson et al., 2011). Cells were maintained in Dulbecco's Modified Eagle's Medium (containing 1 g/L d-glucose, l-glutamine, pyruvate; Lonza, Basel, Switzerland) supplemented with 10% Fetal Bovine Serum (Sigma-Aldrich, St. Louis, MO, USA), 100 IU/mL penicilin (Lonza) and 100 mg/mL streptomycin (Lonza). Cells were grown at 37 ºC in a Galaxy 170s humidified incubator (Eppendorf) with a 5% CO2 atmosphere.

RNA interference and transfection

One day before transfection, 120 000 cells were seeded on 35-mm glass coverslip dishes with 0.17-mm glass thickness (MatTek Corporation). siRNA constructs were diluted in Opti-MEM medium (Life Technologies) and transfection was performed with Lipofectamine RNAiMAX Reagent (Life Technologies) by following manufacturer's protocol. Constructs and their final concentrations used were: 100 nM NuMA siRNA (sc-43978; Santa Cruz Biotechnology), 300 nM PRC1 siRNA (L-019491-00-0010; Dharmacon), 100 nM Ndc80 siRNA (HA12977117-004; Merck), 20 nM Haus8 siRNA (L-031247-01-0005; Dharmacon), 100 nM CENP-E siRNA (L-003252-000010; Dharmacon), 100 nM Kif4A siRNA (sc-60889; Santa Cruz Biotechnology) and 100 nM Kif18A siRNA (4390825; Ambion). After 4 h of incubation with transfection mixure, medium was replaced with regular cell culture medium. All experiments on siRNA-treated cells were perfomed 24 h after transfection, except for Haus8 siRNA-depleted cells, where silencing was done for 48 h. All treatments include at least three independent experiments.

Speckle microscopy

Cells grown in glass coverslip dishes were stained with 1 nM SiR-tubulin dye (Spirochrome AG). After 15 min of staining, live imaging was performed on a spinning disk confocal microscope system (Dragonfly; Andor Technology) using 63x/1.47 HC PL APO glycerol objective (Leica) and Zyla 4.2P scientific complementary metal oxide semiconductor (sCMOS) camera (Andor Technology). During imaging, cells were maintained at 37 ºC and 5% CO2 within H301-T heating chamber (Okolab). Images were acquired using Fusion software (v 2.2.0.38).
For live imaging of RPE1 cells expressing CENP-A-GFP and centrin1-GFP, and stained with SiR-tubulin, 488-nm and 561-nm laser lines were used to excitate GFP, and SiR, respectively. In order to visualize SiR-tubulin speckles, images were acquired with 80% laser power and exposure of 1 s. Image acquisition was done on one focal plane every 10 s. Note that time-frame within which SiR-tubulin, at 1 nM concenration, can be visualized in patches on the mitotic spindle is between 15 and 75 min after SiR-tubulin staining.

Immunostaining

Cells were fixed in ice-cold methanol for 1 min and permeabilized for 15 min in 0.5% Triton X-100 in PBS. Following permeabilization, cells were blocked with 1% NGS in PBS for 1 h and incubated with primary antibodies at 4 ºC overnight. Primary antibodies were prepared in 1% NGS in PBS to 1:100 dilution. Following incubation with primary antibodies, cells were incubated with fluorescence-conjugated secondary antibodies at room temperature for 1 h. Secondary antibodies were prepared in 2% NGS in PBS to 1:250 dilution. To visualize DNA, cells were stained with DAPI for 10 min. After each step, cells were washed three times in PBS for 5 min. Primary antibodies used were: mouse anti-NuMa (sc-365532; Santa Cruz Biotechnology), mouse anti-PRC1 (sc-376983; Santa Cruz Biotechnology), rabbit anti-CENP-E

(C7488; Sigma-Aldrich), mouse anti-Kif4A (sc-365144; Santa Cruz Biotechnology), and rabbit anti-Kif18A (A301-080A; Bethyl Laboratories). Secondary antibodies used were: donkey anti-mouse IgG-Alexa 594 (Abcam), donkey anti-rabbit IgG-Alexa 594 (Abcam), and donkey anti-rabbit IgG-Alexa 647 (Abcam). Immunostained cells were imaged using Bruker Opterra Multipoint Scanning Confocal Microscope (Bruker Nano Surfaces) with a Nikon CFI Plan Apo VC 100x/1.4 numerical aperture oil objective (Nikon). 405/488/561/640-nm laser lights were used with following emission filters: BL HC 525/30, BL HC 600/37 and BL HC 673/11 (Semrock). Images were captured with an Evolve 512 Delta Electron Multiplying Charge Coupled Device (EMCCD) Camera (Photometrics) using a 200 ms exposure time.

Image analysis

Measurements were performed in Fiji/ImageJ (National Institutes of Health). Quantification and data analysis were performed in R (R Foundation for Statistical Computing) and MATLAB (MathWorks). Figures and schemes were assembled in Adobe Illustrator CC (Adobe Systems). Statistical analysis was performed using Student's t-test and Mann-Whitney test.

Upon inspection of tubulin speckle movement within the spindle, speckles which could be followed in at least four time frames were taken into account. For every tubulin speckle position, corresponding CENP-A and centrin positions, suggestive of the location of sister kinetochores and spindle poles, respectively, were also tracked. Tracking was done by using *Multi-point* tool. Speckles which started at proximal kinetochore were categorized as part of k-fiber, whilst speckles which started between sister kinetochores or proximal to the distal pole and passed through sister kinetochores were categorized as part of bridging fiber. Speckles which could not be categorized as part of k-fiber or bridging fiber are termed other. Speckle-pole velocity was calculated by fitting linear regression on distances between tubulin speckle and spindle pole towards which speckle is moving to during first 30 s of its trajectory. Similar was done to determine speckle-kinetochore velocity, however, distances between tubulin speckle and proximal sister kinetochore were used. Speckles were categorized as part of the leading k-fiber when moving in the same direction as sister kinetochores or trailing when moving in the opposite direction.

For kinetochore alignment and inter-kinetochore distance measurements, *Multipoint* tool was used to track positions of sister kinetochore pairs. The equatorial plane was defined with two points placed between outermost pairs of kinetochores on the opposite sides of the spindle. Kinetochore alignment was calculated as the distance between the midpoint of kinetochore pairs and equatorial plane, while inter-kinetochore distance as the distance between two sister kinetochores.

In cells immunostained for PRC1, 5-pixel-thick segmented line was used to track the pole-to-pole contour of PRC1-labeled overlap region. The pole-to-pole tracking was performed on single z-planes and the mean value of the cytoplasm was subtracted from the retrieved intensity profiles. The overlap length was determined as the width of the peak of the signal intensity in the central part of the contour in SciDavis (Free

Software Foundation Inc.). The width of the peak was measured at the base of the PRC1 intensity peak where the PRC1 signal is roughly equal to the mean value of the PRC1 signal along the contour on either side of the peak.
To determine percentage of protein depletion, we measured mean spindle intensity by encompassing the area of the spindle with *Polygon selection* tool. Mean background intensity in the cytoplasm, measured using 1x1 μm rectangle, was subtracted from mean spindle intensity.

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AUTHOR CONTRIBUTIONS

P.R. and M.J. performed all experiments and data analysis. I.M.T. conceived the project and supervised the experiments.

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