Direct interaction between centralspindlin and PRC1 reinforces mechanical resilience of the central spindle

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During animal cell division, the central spindle, an anti-parallel microtubule bundle structure formed between segregating chromosomes during anaphase, cooperates with astral microtubules to position the cleavage furrow. Because the central spindle is the only structure linking the two halves of the mitotic spindle, it is under mechanical tension from dynein-generated cortical pulling forces, which determine spindle positioning and drive chromosome segregation through spindle elongation. The central spindle should be flexible enough for efficient chromosome segregation while maintaining its structural integrity for reliable cytokinesis. How the cell balances these potentially conflicting requirements is poorly understood. Here, we demonstrate that the central spindle in C. elegans embryos has a resilient mechanism for recovery from perturbations by excess tension derived from cortical pulling forces. This mechanism involves the direct interaction of two different types of conserved microtubule bundlers that are crucial for central spindle formation, PRC1 and centralspindlin.
Microtubule bundle structures play crucial roles in animal cell cytokinesis. During anaphase, the central spindle is formed between segregating chromosomes, and, in cooperation with astral microtubules, it determines the position of the cleavage furrow by forming a zone of active Rho GTPase, a master regulator of contractile ring formation. It also serves as a precursor to the midbody, which recruits factors crucial for the final separation of two daughter cells by membrane abscission. Dynein at the cell cortex generates ‘cortical pulling force’, a mechanical force that pulls astral microtubules towards the cell cortex. As a consequence, the spindle poles are pulled outwards. This force determines spindle positioning and drives chromosome segregation through elongation of the pole-to-pole distance (anaphase B).

After anaphase onset, when sister chromatid cohesion has dissolved, the central spindle becomes the only structure that links the two halves of the mitotic spindle. Thus, it is under mechanical tension from the cortical pulling force, the only structure that links the two halves of the mitotic spindle. This mechanical tension is important for the formation and function of the central spindle. The central spindle forms an equatorial zone of active Rho, which is a major activator of Rho during cytokinesis, accumulates at the cell cortex, and determines the position of the spindle poles. As a consequence, the spindle becomes more dynamic, and the plus ends of the two sets of interpolar microtubules from the two pole-tips are pulled outwards. This force determines spindle positioning and drives chromosome segregation through elongation of the pole-to-pole distance (anaphase B).

To examine the response of the central spindle to increased pulling forces (Fig. 1a), we generated transgenic C. elegans strains expressing mCherry-tagged tubulin and green fluorescent protein (GFP)-tagged CYK-4. During the first mitotic division of control embryos, the bipolar spindle formed promptly following nuclear envelope breakdown (NEBD, time = 0). NEBD was monitored by the loss of exclusion of the tubulin signal from pronuclei (Fig. 1b and Supplementary Fig. 1) for grayscale images covering the whole embryos. Approximately 120–150 s following NEBD, the distance between the two spindle poles (Fig. 1c) started to increase, indicating the onset of anaphase, as previously reported (Fig. 1d).

At the same time, CYK-4 began to accumulate at the spindle midzone (Fig. 1b, control), where the plus ends of the two sets of interpolar microtubules from the two spindle poles form an interdigitating overlap, as confirmed by an accelerated increase in the peak intensity (Fig. 1d, control and Supplementary Fig. 2a for examples of the line profiling of CYK-4:GFP intensity). Within the next 30–60 s, the central spindle was established, with CYK-4 enriching rapidly in the central overlap zone and remaining there for ~300 s until the cleavage furrow fully ingressed and the central spindle was compacted to form the midbody.

EFA-6 is a negative regulator of the dynein-based cortical pulling force. In embryos depleted of EFA-6, pole-to-pole elongation was accelerated (from 80 nm s⁻¹ in control embryos to 120 nm s⁻¹; Fig. 1d, efa-6(RNAi)), as expected. In these embryos, CYK-4 accumulated normally until 180 s after NEBD (Fig. 1b,d, efa-6(RNAi)), when the peak corresponding to its accumulation suddenly broadened (Fig. 1b,d, arrowheads). Thereafter, the total amount of CYK-4 detected between the two poles continued to increase, and the tight localization of CYK-4 fluorescence at the midzone was gradually restored (Fig. 1d, black arrows in the ‘CYK-4 peak width’ panels). A similar pattern of CYK-4 accumulation was observed in the embryos depleted of the kinesin-5 BMK-1, which has been reported to function as a brake during spindle elongation in C. elegans embryos and other cell types. Reciprocally, the dependency of SPD-1 midzone localization on centralspindlin has also been reported, implying that the cooperative interaction between the two different types of microtubule bundling proteins might be important for the mechanical resilience of the central spindle.

Direct interaction between PRC1 and centralspindlin. A direct interaction between human orthologues of SPD-1 and CYK-4 has been reported, and a physical interaction between Asel, a yeast PRC1 homologue, and Klp9, a kinesin that is distantly related to ZEN-4/MKLP1 (although more closely to MKLP2), has also been shown. We therefore examined whether SPD-1 and CYK-4 physically interact in vitro using pull-down and yeast two-hybrid assays. In vitro translated full-length CYK-4 was pulled down by beads coated with bacterially expressed full-length SPD-1 (Fig. 2a and Supplementary Fig. 3a). Reciprocally, SPD-1 was bound to beads coated with CYK-4 as well as the CYK-4/ZEN-4 centralspindlin holocomplex (Fig. 2b and Supplementary Fig. 3b). This interaction was also observed in yeast two-hybrid assays between CYK-4 and the N-terminal fragment of SPD-1 and was drastically weakened by deletion of the C-terminal tail of CYK-4 (Fig. 2c,d CYK-4 ΔTail), which is dispensable for both centralspindlin complex assembly and microtubule bundling. In contrast, the CYK-4 C-terminal tail fragment alone showed an interaction with SPD-1 that was enhanced by dimerization by adding back the coiled coil (Fig. 2c and d, CYK-4 Tail and CC + Tail). A similar requirement of the C-terminal tail of CYK-4 was also observed in pull-down assays (Fig. 2e and Supplementary Fig. 4). SPD-1 1–228 was

Results Resilience of central spindle under mechanical perturbation. To examine the response of the central spindle to increased pulling forces (Fig. 1a), we generated transgenic C. elegans strains expressing mCherry-tagged tubulin and green fluorescent protein (GFP)-tagged CYK-4. During the first mitotic division of control embryos, the bipolar spindle formed promptly following nuclear envelope breakdown (NEBD, time = 0). NEBD was monitored by the loss of exclusion of the tubulin signal from pronuclei (Fig. 1b and Supplementary Fig. 1) for grayscale images covering the whole embryos. Approximately 120–150 s following NEBD, the distance between the two spindle poles (Fig. 1c) started to increase, indicating the onset of anaphase, as previously reported (Fig. 1d). At the same time, CYK-4 began to accumulate at the spindle midzone (Fig. 1b, control), where the plus ends of the two sets of interpolar microtubules from the two spindle poles form an interdigitating overlap, as confirmed by an accelerated increase in the peak intensity (Fig. 1d, control and Supplementary Fig. 2a for examples of the line profiling of CYK-4:GFP intensity). Within the next 30–60 s, the central spindle was established, with CYK-4 enriching rapidly in the central overlap zone and remaining there for ~300 s until the cleavage furrow fully ingressed and the central spindle was compacted to form the midbody.

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In contrast, in embryos depleted of SPD-1, the C. elegans orthologue of PRC1, the spindle was broken into two halves, and the accumulation of CYK-4 at the midzone dropped drastically (Fig. 1b,d, arrow in spd-1(RNAi)) and Supplementary Fig. 2b and c). Once it was lost from the spindle midzone, CYK-4 could not re-accumulate there, although much later, it localized to the midbody via the cortical/astral route independent of the central spindle (Fig. 1b,d, double-headed arrows in spd-1(RNAi) panels). Reciprocally, the dependency of SPD-1 midzone localization on centralspindlin has also been reported, implying that the cooperative interaction between the two different types of microtubule bundling proteins might be important for the mechanical resilience of the central spindle.

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robustly pulled down by CYK-4 fragments containing the C-terminal tail region (FL, 34–681, 121–681 and CC+Tail) but much more weakly by fragments lacking the C-terminal tail (34–618 and CC). These data indicate that the SPD-1–CYK-4 interaction is mediated mainly by the N-terminal region of SPD-1 and the C-terminal tail of CYK-4.

The spd-1 gene was originally isolated in a forward genetic screen for temperature-sensitive cell division defects. Interestingly, the oj5 allele isolated in this screen as the cause of central spindle defects has been shown to be an R83W mutation, which lies within the CYK-4-binding region of SPD-1 defined above. We tested whether this mutation affected the SPD-1–CYK-4 interaction and found that it was drastically weakened in both yeast two-hybrid (Fig. 2d) and in vitro pull-down assays (Fig. 2e and Supplementary Fig. 4). Importantly, however, this mutation did not affect other known properties of PRC1, such as dimerization/oligomerization and microtubule bundling. In yeast two-hybrid assays, the interaction between SPD-1 and SPD-1 as prey was not affected by the R83W mutation (Fig. 2d, bottom row). Consistently, this mutation did not affect the mobility of full-length recombinant SPD-1 in size exclusion chromatography (Fig. 2f). In addition, SPD-1 with the R83W mutation could bind microtubules (Fig. 2g) and bundle microtubules (Fig. 2h) in solution as efficiently as its wild-type counterpart. These data indicate that the R83W mutation specifically affects the interaction between SPD-1 and CYK-4, although we cannot formally exclude possible influences on as-yet-unknown functions of SPD-1/PRC1. These findings strongly suggest that the central spindle defects observed in spd-1(oj5) mutants are caused by the defective binding between PRC1 and centralspindlin, the two major microtubule crosslinking/bundling proteins of the central spindle.

Figure 1 | Resilience of central spindle against excess tension. The central spindle in C. elegans can recover from near disruption during the first embryonic division. (a) Schematic of an animal cell in anaphase. The central spindle is under mechanical tension from cortical pulling forces. (b) Spinning disk confocal time-lapse images of embryos expressing mCherry::tubulin (magenta) and a major organizer of the central spindle, CYK-4::GFP (green), after the depletion of EFA-6, BMK-1 and SPD-1 by RNAi. The arrowheads indicate the sudden broadening of CYK-4 midzone accumulation associated with the mid-anaphase acceleration of pole-to- pole elongation caused by the depletion of these molecules. The central spindle was not completely broken in efa-6(RNAi) (enhanced outward pulling force) or in bmk-1(RNAi) (less drag against the pulling force) embryos, and CYK-4 accumulation was later restored. This recovery did not occur in embryos depleted of SPD-1/PRC1, another major central spindle organizer. The midzone accumulation of CYK-4 was largely lost (arrow), although weak accumulation via the furrow-dependent pathway was observed later (double-headed arrow). Scale bar, 10 μm. (c, d) Quantitative analysis of morphological changes in the mitotic spindle. The positions of the spindle poles were determined as the peak mCherry::tubulin signals, and the distance between them (pole-to-pole distance) is plotted in magenta. The intensity of CYK-4::GFP in individual embryos was standardized using a uniform cytoplasmic signal during the pre-mitotic stage. Line profiles of 4.4 μm in width between the two poles were measured for each time point, and the width at half-maximum (CYK-4 peak width), height (CYK-4 peak intensity) and area (total CYK-4 on spindle) of the peak were measured and are plotted in green. The dotted lines on the graphs of CYK-4 peak width indicate the pole-to-pole distance during metaphase. In the efa-6(RNAi), bmk-1(RNAi) and spd-1(RNAi) panels, the values for the control are also plotted in grey. Recovery after near disruption in efa-6(RNAi) and bmk-1(RNAi) embryos was detected as gradual tightening of the CYK-4 peak width (arrows) after sudden widening (arrowheads). The grey arrows indicate the loss of CYK-4 accumulation in spd-1(RNAi) embryos and the central spindle-independent late recruitment. The mean and standard error were determined from 33, 7, 6 and 6 embryos for the control, efa-6(RNAi), bmk-1(RNAi) and spd-1(RNAi), respectively.
Rupture of central spindle caused by CYK-4 tail mutations. To further assess the physiological importance of the interaction between SPD-1 and CYK-4, we introduced point mutations in CYK-4 that affected its interaction with SPD-1. Sequence comparison of the C-terminal tails of CYK-4 orthologues revealed the presence of two motifs, SILGPVTT and K/R-X-K/R, which have been widely conserved throughout metazoan evolution (Fig. 3a and Supplementary Fig. 5). Mutations in these motifs weakened the SPD-1–CYK-4 interaction, either moderately (IL to NN, IL to AA and SIL to AAA) or more severely (RAR to EAE; Fig. 3b). Similar effects were also observed in an in vitro pull-down assay (Fig. 3c). We generated transgenic animals carrying these mutations (AA and EAE) as well as a deletion of the tail domain (ΔTail) in a cyk-4::gfp transgene and expressed the transgenes in a strain in which endogenous cyk-4 had been deleted. Although the wild-type transgene efficiently suppressed the embryonic lethality...
Figure 3 | Mutations in the C-terminal tail of CYK-4 affect the SPD-1–CYK-4 interaction and cause mid-anaphase spindle rupture. (a) An alignment of the CYK-4 C-terminal tail region, which corresponds to amino acids 644–675 of the C. elegans protein. The conserved motifs with the mutations assessed are highlighted. Ce, C. elegans; Ch, C. briggsae (nematode); Nv, N. vectensis (sea anemone); Hv, H. vulgaris (hydra); Ct, C. teleta (annelid); Cg, C. gigas (oyster); Sp, S. purpuratus (sea urchin); Dr, D. rerio (zebrafish); Xl, X. laevis (frog); Cl, C. livia (bird) and Hs, H. sapiens. (b) Yeast two-hybrid assay assessing the effects of mutations within the above conserved motifs. (c) An SPD-1 1-228 fragment was evaluated for in vitro binding with wild-type or mutant (AA and EAE) CYK-4 constructs immobilized on beads via a maltose-binding protein (MBP) tag (bottom panel, Coomassie staining) and detected with an anti-SPD-1 antibody (top panel). (d) Rescue of the embryonic lethality of cyk-4 null embryos by the indicated cyk-4::gfp transgenes. For each strain (two independent strains each for wild-type, AA, EAE and ΔTail), at least 690 embryos produced by seven hermaphrodites homozygous for the cyk-4 null allele and the cyk-4::gfp transgene were scored. (e,f) Spinning disk confocal time-lapse imaging of embryos expressing mCherry::tubulin and the indicated cyk-4::gfp transgene in the null background for the cyk-4 endogenous locus (n = 25 and 23 for EAE and ΔTail, respectively). Mid-anaphase spindle rupture was observed in the mutants defective for the SPD-1–CYK-4 interaction (t = 210 s). No recovery of the midzone accumulation of CYK-4 mutants was observed, although in some embryos, the transient association of mutant CYK-4 near the microtubule plus ends was detected (arrows). Scale bar, 10 μm. In f, the data for the mutants are displayed in colour, whereas those for the wild-type control (same as in Fig. 1; n = 33) are plotted in grey.
caused by the deletion of endogenous cyk-4, embryos expressing the mutant transgenes showed different levels of lethality, according to the severity of the SPD-1-binding defect (Fig. 3d). Although the mild AA mutation caused mild lethality, the more severe EAE mutation caused much higher lethality. The ΔTail mutation resulted in the most severe phenotype (>90% lethality). This close correlation between the in vitro and in vivo phenotypes clearly shows that the SPD-1–CYK-4 interaction plays a crucial role in early C. elegans development.

Next, we examined the influences of the defective SPD-1–CYK-4 interaction on cell division, especially on the robustness of the central spindle. The fluorescence intensities of uniform cytoplasmic CYK-4::GFP during the pre-mitotic stage confirmed that the protein expression level was not affected by the EAE and ΔTail mutations (Supplementary Fig. 6). Consistent with the normal behaviour of CYK-4 in spd-1(RNAi) embryos up to mid-anaphase (NEBD ~180 s), in the EAE and ΔTail mutant embryos, the bipolar spindle formed normally, and mutant CYK-4 began to accumulate at the spindle midzone at the same time as in wild-type embryos (Supplementary Fig. 7). However, once it formed, the midzone microtubule bundle was disrupted within 30–60 s. As a consequence, the anaphase spindle was split into two half-spindles, and the poles moved rapidly apart. At the same time, accumulated mutant CYK-4 was lost, detected as a rapid broadening of the peak width and a gradual decrease in the peak intensity (Fig. 3f) of CYK-4::GFP fluorescence, indicating that in some embryos, mutant CYK-4 was retained near the tips of the broken half-spindles for a while (Fig. 3e, arrows). These observations clearly indicate that the SPD-1–CYK-4 interaction is necessary for the robust maintenance of the central spindle after its initial formation.

Suppression of spindle rupture by reduction of tension. Because enhancement of the cortical pulling force by efa-6(RNAi) caused pole separation to accelerate (Fig. 1), we hypothesized that spindle breakage in the absence of the SPD-1–CYK-4 interaction might be suppressed if cortical pulling forces were reduced by the depletion of cortical activators of dynein\(^5^\). Under these conditions, an apparently normal central spindle was formed in cyk-4 ΔTail mutant embryos and was maintained thereafter much more stably than in control embryos (Fig. 4a). The peak intensity of CYK-4 ΔTail at late anaphase (240 s after NEBD) was restored to almost 100% of the level of wild-type embryos without RNA interference

Figure 4 | Mid-anaphase spindle rupture in the cyk-4 mutants defective for the SPD-1–CYK-4 interaction is suppressed by reduction of the cortical pulling force. (a,b) Time-lapse imaging of the cyk-4 ΔTail mutant embryos depleted of an activator of the cortical pulling force, GPR-1/2 or LIN-5 (n = 6 and 11, respectively). Although the central spindle was broken in control embryos (same as in Fig. 3, n = 23), in gpr-1/2(RNAi) or lin-5(RNAi) embryos, the central spindle and the midzone accumulation of the CYK-4 mutant (arrowheads) were stably maintained. Scale bar, 10 μm. In b, the data for the reduced pulling force (gpr-1/2(RNAi) or lin-5(RNAi)) are plotted in bold colour, whereas those for the ΔTail mutant and wild-type embryos with unperturbed cortical pulling forces (control) are depicted in pale colour and grey, respectively. Similar effects of the reduction of the cortical pulling force were observed in EAE mutant embryos (Supplementary Fig. 7).
(RNAi; Fig. 4b). The width of midzone accumulation also became comparable to that of the control, although later, it was slightly broadened (Fig. 4a,b). However, consistent with the role of the G-protein pathway in the regulation of the cortical pulling force in the astere-dependent pathway of cleavage furrow ingestion\cite{1,2}, cytokinesis failure in cyk-4 ΔTail mutant embryos (13/23) was not suppressed but was rather enhanced by the depletion of GPR-1/2 or LIN-5 (6/6 and 11/11, respectively). Similar restoration of the stability of the central spindle following a reduction in cortical pulling forces was also observed in CYK-4 EAE mutant embryos (Supplementary Fig. 7).

To further examine whether the mechanical fragility of the central spindle in the cyk-4 tail mutants was caused by the lack of SPD-1–CYK-4 interaction or by possible defects in an as-yet-unknown function of the CYK-4 tail, we tested the effect of the SPD-1–CYK-4 interaction or by possible defects in an as-yet-unknown function of the CYK-4 tail, we tested the effect of the reduction of the cortical pulling force in spd-1(oj5) embryos (Fig. 5a,b). As previously reported\cite{3}, the central spindle was disrupted in spd-1(oj5) embryos, accompanied by the dispersion of transiently accumulated wild-type CYK-4::GFP. In contrast, LIN-5 depletion suppressed the sudden rupture of the central spindle and prolonged the accumulation of CYK-4. These data further indicate that the SPD-1–CYK-4 interaction through the CYK-4 C-terminal tail plays an important role in maintaining the mechanical integrity of the central spindle under tension. This interaction is dispensable for lateral microtubule bundling per se and for the tight localization of CYK-4 to the spindle midzone but becomes crucial when the whole spindle is under mechanical tension equivalent to that experienced during the normal asymmetric first cell division.

### Discussion

Here, we have revealed a molecular mechanism for the mechanical resilience of the central spindle, which is key to reconciling the conflicting requirements for efficient cytokinesis versus spindle positioning and chromosome segregation. Interestingly, this mechanism has been implemented into the core machinery of central spindle formation via the direct interaction of two conserved major microtubule bundlers, PRC1 and centralspindlin. The motifs critical for SPD-1 binding in the C-terminal tail of CYK-4 seem to have been widely conserved throughout metazoan evolution\cite{4}, with the exception of arthropods and platyhelminthes (Supplementary Fig. 5). Interestingly, arthropods carry out embryonic cleavage using a unique strategy, and the contribution of the cortical pulling force on anaphase B is limited in Drosophila embryos\cite{5}. Because a direct interaction between centralspindlin and PRC1 has been demonstrated in humans and C. elegans, a pair of distantly related species, it is reasonable to speculate that the binding between them might also be widely conserved. The interaction between centralspindlin and PRC1 in metazoans might be an evolutionary adaptation to the coupled sliding and polymerization mechanism\cite{6,7}, which originally occurred to drive spindle elongation in eukaryotic ancestors, functioning as an ‘engine brake’ to counteract excess external forces.

External pulling force applied to the central spindle accelerates the anti-parallel sliding of microtubule bundles, which would cause constant ‘leakage’ of microtubule-bundling proteins from the zone of anti-parallel microtubule overlap into the non-bundled part of the microtubules, where binding of the bundling proteins to them would be less stable (Fig. 6a). We speculate that the interaction between different types of microtubule bundlers might compensate for their limitations, such as the inability of PRC1 to carry out active unidirectional transport\cite{8,9} and the low processivity of centralspindlin as a motor, especially when it is not clustered\cite{10}, potentially helping them return to the anti-
parallel microtubule overlap (Fig. 6b). In the absence of the PRC1-centralspindlin interaction, slowing anti-parallel sliding would reduce the leakage of bundling proteins from the overlap zone but not completely prevent it. This notion is consistent with the gradual loss of CYK-4 signals observed at the later time points (Figs 4 and 5, ~300 s after NEBD). This model also predicts that even when the interaction between PRC1 and centralspindlin is intact, if the cortical pulling force is too strong, the central spindle will be broken. This situation might have occurred in the case of the midzone rupture reported following KLP-7/CeMCAK depletion.4,46 Direct testing of this model would require precise measurement of the distribution of microtubule plus ends and the resilience of the central spindle in cooperation with the PRC1–centralspindlin interaction investigated here.

Embryonic lethality assay. To score the rescue efficiency of cyk-4::gfp transgenes, the cyk-4::gfp MosSCI strains were crossed into the cyk-4(ok1034) deletion strain (VC859) to obtain worms homozygous for both cyk-4(ok1034) and cyk-4::gfp, which were verified by PCR using a pair of primers, 5’-TTATGGACGCTGTGTGTTGAGGC-3’ and 5’-GAGACTGTCACCAGGTTG-3’, and a combination of three primers, 5’-GGCCGCCCATCCTCCTTCTGATG-3’, 5’-GACGTAACAGATATAAATGAGTGATG-3’ and 5’-GCGAATTTGCTGACAGAAT-3’. In the absence of a cyk-4 transgene, homozygous cyk-4 deletion (ok1034) causes 100% embryonic lethality. The hatching rates of the embryos laid during the first 36 h from late L4 stage by the cyk-4(ok1034); cyk-4::gfp null embryonic cyk-4 deletion (ok1034) were kindly given by J. Ahringer (Gurdon Institute, UK). The engineered transgenes were confirmed by sequencing and injected into the EG0803 strain for insertion into chromosome IV. Transgene integration was verified by sequencing, mCherry-tubulin strain (JA1559) was a gift from J. Ahringer (Gurdon Institute, UK).

RNAi. RNAi depletion was performed by feeding L4 hermaphrodites with Escherichia coli clones expressing double-stranded RNA from L4440 plasmid containing a DNA fragment specific for the target gene. All RNAi clones were used in this study (except for that for spd-1, which was made by a primer pair, 5’-GGCCGCCCATCCTCCTTCTGATG-3’ and 5’-GACGTAACAGATATAAATGAGTGATG-3’). To score the rescue efficiency of rescue transgenes, the cyk-4::gfp transgenes were amplified with a pair of primers, 5’-GGCCGCCCATCCTCCTTCTGATG-3’ and 5’-GACGTAACAGATATAAATGAGTGATG-3’;

Figure 6 | A model for the mechanical robustness of the central spindle, as achieved by interaction between two different types of microtubule-bundling proteins. (a) A coupled sliding and polymerization model and the fate of a microtubule bundler at the edge of the sliding overlap zone. Relative sliding between an anti-parallel pair of microtubules by external pulling forces causes stress on a bundler and destabilizes its interaction with microtubules. This stress can be released by the detachment of either one of the microtubule-binding sites from the bundler. When the detachment occurs on the microtubule-binding site that is at the plus end of the microtubule, the bundler cannot return to the overlap zone easily because the site on which it was originally positioned (yellow) moves away due to sliding. (b) A non-motor diffusing bundler can remain on a non-bundled microtubule without detachment. However, because the distance that it can move by diffusion is limited, the chance that this molecule can return to the overlap zone is low when rapid sliding is occurring. Even a motor that can move along a microtubule faster than the rate of sliding might fail to return to the overlap zone by dropping off the microtubule. If the two types of microtubule-bundling proteins are associated, they can compensate for each other’s limitations and have a greater chance of returning to the overlap zone without detachment and re-establishing crosslinkage.

Methods

C. elegans strains and culture. The C. elegans strains used in this study are listed in Supplementary Table 1 and were maintained at 20 °C, except for strains containing temperature-sensitive spd-1(aj5) allele, which were maintained at 15 °C. To generate the cyk-4::gfp strains by Mos1-mediated single-copy insertion (MosSCI),45 a 4.5-kb cyk-4 genomic fragment was amplified with a primer pair, 5’-AATCCG GCGGCTACACAAACACACACATCGCT-3’ and 5’-ACTATCCCAGCGGTTACT CACCGGAAAACAGAGTC-3’ and cloned into the PspOME/Xmal site of the pBlueScript vector. Following insertion of the gfp sequence between the coding region and the stop codon, the BoW1/Xmal fragment (from 990 bp upstream of the start codon to 1,072 bp downstream of the stop codon) was subcloned into a MosSCI targeting vector, pCFJ178. The point mutations and deletion were introduced with primer pairs, 5’-GCGGCTACACAAACACACACATCGCT-3’ and 5’-GGATGATGTTGTAATGCTTACCGCA GCGCTAGATACAGAGACG-3’ (‘AA’), 5’-GGCCGCCAAGCGGACTGAA GCAGAAGGTGCTCATCGC-3’ and 5’-GCGATGAGCACTTCTCGCTCA GTGGGTTGGCCCGAC-3’ (‘EAE’), and 5’-TGCAAGTTGCCCATCTTCTGATG-3’. The engineered transgenes were confirmed by sequencing and injected into the EG0803 strain for insertion into chromosome IV. Transgene integration was verified by sequencing, mCherry-tubulin strain (JA1559) was a gift from J. Ahringer (Gurdon Institute, UK).
with an iXon EMCCD DV885KCS-VP camera (Andor) at 100 ms exposure with no binning, controlled using Metamorph software (Molecular Devices). For the experiments shown in Fig. 2A, embryos were isolated on an aluminum stage with the FC52 cooling device (Biotechps) connected to a Nano-Therm System (Biotechps) set at 18 °C and filmed with a Revolution XD system (Andor) equipped with an iXon Ultra 897 EMCCD camera (Andor).

Time-lapse images were analysed using ImageJ (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/; 1997–2011) and R (http://www.r-project.org) software. Equal expression of the wild-type and mutant cyk-4::gfp transgenes was confirmed by measuring the intensities of the GFP signals (Supplementary Fig. 6). For the mCherry::tubulin channel, the cy2 slice with the best focus of the two spindle poles was identified for each time frame, and using this slice and the three slices above and below it, an average z-projection was produced. For the CYK-4::GFP channel, the z-slice with the best focus of the two spindle poles was identified for each time frame, and using this slice and the three slices above and below it, an average z-projection was produced by applying the same colour look-up tables to the standardized images. To quantify the accumulation of CYK-4 at the spindle midzone, a line profile along a spline curve that passed these three points (4.4-μm width, 6-μm z-depth) was obtained and the peak height, the peak width at half maximum and the peak area of the line profile were measured.

Protein–protein interaction and other biochemical assays. SPD-1 N-terminally tagged with a chitin-binding domain (CBD) was expressed from pCB-TEV vector in bacteria and purified by immobilized chitin beads (New England Biolabs). CYK-4 N-terminally tagged with glutathione S-transferase was expressed from pGEX-TEV vector in bacteria and purified with glutathione Sepharose 4B (GE Healthcare). The complex of glutathione S-transferase-CYK-4 and CBD-ZEN-4 was expressed from a bicistronic pGEX-6b vector in bacteria and purified by sequential binding to chitin column and Human immunodeficiency virus (HIV) 1B subunit cleavage tag. For the CBD tag, and according to glutathione Sepharose 4B. For experiments with radioactive probes, 35S-labelled proteins were produced by TNT T7 Cooled Reticulocyte Lysate System (Promega). A volume of 10 μl of the lysates were mixed with 5 μl of beads with immobilized bacterially expressed proteins in 170 μl SD150 buffer (20 mM Tris-HCl [pH 8.0], 20% [v/v] glycerol, 5 mM MgCl2, 150 mM NaCl, 0.1% [v/v] NP-40, 1 mM dithiothreitol, 10 μg ml–1 leupeptin, 10 μg ml–1 pepstatin and 0.2 μM phenylmethylsulfonylfluoride). For experiments without radioactive probe, purified SPD-1 1–228 fragments were incubated with azoylase beads immobilized with fusion proteins of maltose-binding protein and CYK-4 frag-

References
1. Green, R. A., Paluch, E. & Oegema, K. Cytokinesis in animal cells. Annu. Rev. Cell Dev. Biol. 28, 29–58 (2012).
2. Agromayor, M. & Martin-Serrano, J. Knowing when to cut and run: mechanisms that control cytokinetic abscission. Trends Cell Biol. 23, 433–441 (2013).
3. Green, R. A. et al. The midbody ring scaffolds the abscission machinery in the absence of midbody microtubules. J. Cell Biol. 203, 505–520 (2013).
4. Gull, M. K., Goetz, P., Pelletier, H. & Hyman, A. A. Polarity controls forces governing asymmetric spindle positioning in the Caenorhabditis elegans embryo. Nature 409, 630–633 (2001).
5. Gonczy, P. Mechanisms of asymmetric cell division: flies and worms pave the way. Nat. Rev. Mol. Cell Biol. 9, 355–366 (2008).
6. Grill, S. W., Howard, J., Schaffer, E., Stelzer, E. H. & Hyman, A. A. The distribution of active force generators controls mitotic spindle positioning. Science 318, 518–521 (2007).
7. Kozlowski, C., Srayko, M. & Nedelec, F. Cortical microtubule contacts position the spindle in C. elegans embryos. Cell 129, 499–510 (2007).
8. Collins, E. S., Balchand, S. K., Faraci, J. L., Wadsworth, P. & Lee, W. L. Cell cycle-regulated cortical dynein/dynactin promotes asymmetric cell division by differential pole motion in anaphase. Mol. Biol. Cell 23, 3380–3390 (2012).
9. Kajita, S., Bussé, C. & Goetz, P. Cortical dynein is critical for proper spindle positioning in human cells. J. Cell Biol. 199, 97–110 (2012).
10. Kiyonitisu, T. & Cheeseman, I. M. Cortical dynein and asymmetric membrane elongation coordinately position the spindle in anaphase. Cell 154, 391–402 (2013).
11. Douglas, M. E. & Mishima, M. Still entangled: assembly of the central spindle by multiple microtubule modulators. Semin. Cell Dev. Biol. 21, 899–908 (2010).
12. Lee, K. Y., Davies, T. & Mishima, M. Cytokinesis microtubule organizers at a glance. J. Cell Sci. 125, 3495–3500 (2012).
13. Mishima, M., Kaitna, S. & Glotzer, M. Central spindle assembly and cytokinesis (2005).
14. White, E. A. & Glotzer, M. Centralspindlin: at the heart of cytokinesis. Cytochemistry 69, 882–892 (1992).
15. Jiang, W. et al. PRC1: a human mitotic spindle-associated CDK substrate protein required for cytokinesis. Mol. Cell 2, 877–885 (1998).
16. Mollinari, C. et al. PRC1 is a microtubule binding and bundling protein essential to maintain the mitotic spindle midzone. J. Cell Biol. 157, 1175–1186 (2002).
17. Mastronarde, D. N., McDonald, K. L., Ding, R. & McIntosh, J. R. Interpolar microtubule tubules in PTK cells. J. Cell Biol. 123, 1475–1489 (1993).
18. Kurasawa, Y., Earnshaw, W. C., Mochizuki, Y., Dohme, N. & Todokoro, K. Essential roles of Kif4 and its binding partner PRC1 in organized central spindle midzone formation. EMBO J. 23, 3327–3348 (2004).
19. Zhu, C. & Jiang, W. Cell cycle-dependent translocation of PRC1 on the spindle by Kif4 is essential for midzone formation and cytokinesis. Proc. Natl Acad. Sci. USA 102, 343–348 (2005).
20. Gruneberg, U. et al. KIF4A and citron kinase act together to promote efficient cytokinesis. J. Cell Sci. 172, 363–372 (2009).
21. D’Avino, P. P. et al. Recruitment of Polo kinase to the spindle midzone during cytokinesis requires the Feo/Klpa3A complex. PLoS ONE 2, e572 (2007).
22. Somers, W. G. & Saint, R. A RhoGEF and Rho family GTPase-activating protein complexes link the contractile ring to cortical microtubules at the onset of cytokinesis. Dev. Cell 4, 29–39 (2003).
23. Royce, O., Piekay, A. & Glotzer, M. An ECT2-centralspindlin complex regulates the localization and function of RhoA. J. Cell Biol. 170, 571–582 (2005).
24. Tomatsu, T., Xie, X., Blumenthal, R., Okamoto, I. & Miki, T. Human ECT2 is an exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in cytokinesis. J. Cell Biol. 147, 921–928 (1999).
25. Zhao, W. M. & Fang, G. MgcRacGAP controls the assembly of the contractile ring and the initiation of cytokinesis. Proc. Natl Acad. Sci. USA 102, 13158–13163 (2005).
26. Kiem, J. et al. Dissecting the role of Rho-mediated contractile ring formation. Mol. Biol. Cell 17, 43–55 (2006).
27. Ishiyama, Y. & Yonemura, S. Centralspindlin regulates ECT2 and RhoA accumulation at the equatorial cortex during cytokinesis. J. Cell Sci. 119, 101–114 (2006).
28. Bement, W. M., Benink, H. A. & von Dassow, G. A microtubule-dependent zone of active RhoA during cleavage plane specification. J. Cell Biol. 170, 91–101 (2005).
29. Ban, R., Irino, Y., Fukami, K. & Tanaka, H. Human mitotic spindle-associated protein PRC1 inhibits MgcRacGAP activity toward Cdc42 during the metaphase. J. Biol. Chem. 283, 16394–16402 (2004).
30. Oegema, K., Desai, A., Rybina, S., Kirkham, M. & Hyman, A. A. Functional analysis of kinetochore checkpoint in Caenorhabditis elegans. J. Cell Biol. 153, 1209–1226 (2001).
31. O’Rourke, S. M., Christensen, S. N. & Bowerman, B. EFA-6 limits microtubule growth at the cell cortex. J. Cell Biol. 189, 1235–1241 (2010).
32. Saunders, A. M., Powers, J., Strome, S. & Saxton, W. M. Kinesin-5 acts as a brake in anaphase spindle elongation. Curr. Biol. 17, R453–R454 (2007).
33. Tikhonenko, I., Nag, D. K., Martin, N. & Koonce, M. P. Kinesin-3 is not essential for mitotic spindle elongation in D. c. Cell Mot. Cytoskeleton 65, 853–862 (2008).

34. Collins, E., Mann, R. J. & Wadsworth, P. Eg5 restricts anaphase B spindle elongation in mammalian cells. Cytoskeleton 71, 136–144 (2014).

35. Verbrugge, K. J. & White, J. G. SP-1/PRC1 reinforces mechanical resilience of the central spindle. Curr. Biol. 179, 1575–1580 (2014).

36. Bringmann, H. & Hyman, A. A. A cytokinesis furrow is positioned by two consecutive signals. Nature 430, 731–734 (2004).

37. Fu, C. et al. Phospho-regulated interaction between kinesin-6 Klp9p and microtubule bundler Ase1p promotes spindle elongation. Dev. Cell 17, 257–267 (2009).

38. Pavicic-Kaltenbrunner, V., Mishima, M. & Glotzer, M. Cooperative assembly of CYK-4/MgcRacGAP and ZEN-4/MKLP1 to form the centralspindlin complex. Mol. Biol. Cell 18, 4992–5003 (2007).

39. O’Connell, K. F., Leys, C. M. & White, J. G. A genetic screen for temperature-sensitive cell division mutants of C. elegans. Genetics 149, 1303–1321 (1998).

40. Zhu, C., Lau, E., Schwarzenbacher, R., Bossy-Wetzel, E. & Jiang, W. Spatiotemporal control of spindle midzone formation by PRC1 in human cells. Proc. Natl. Acad. Sci. USA 103, 6194–6201 (2006).

41. Subramanian, R., Si, C. Y., Tan, L., Darst, S. A. & Kapoor, T. M. Marking and measuring single microtubules by PRC1 and kinesin-4. Cell 154, 377–390 (2013).

42. Knoblich, J. A. Asymmetric cell division: recent developments and their implications for tumour biology. Nat. Rev. Mol. Cell Biol. 11, 849–860 (2010).

43. Pecreaux, J. et al. Spindle oscillations during asymmetric cell division require a threshold number of active cortical force generators. Curr. Biol. 16, 2111–2122 (2006).

44. Srinivasan, D. G., Fisk, R. M., Xu, H. & van de Heuvel, S. A complex of LIN-5 and GPR proteins regulates G protein signaling and spindle function in C. elegans. Genes Dev. 17, 1225–1239 (2003).

45. Nguyen-Ngoc, T., Afshar, K. & Gönczy, P. Coupling of cortical dynein and G alpha proteins mediates spindle positioning in C. elegans. Genetics 149, 1303–1321 (1998).

46. Gotta, M., Dong, Y., Peterson, Y. K., Lanier, S. M. & Ahringer, J. Asymmetrically distributed C. elegans homologs of AGS3/PINS control spindle position in the early embryo. Curr. Biol. 13, 1029–1037 (2003).

47. Bringmann, H., Cowan, C. R., Kong, J. & Hyman, A. A. LET-99, GOA-1/GPA-16, and GPR-1/2 are required for aster-positioned cytokinesis. Curr. Biol. 17, 185–191 (2007).

48. Hedges, S. B. The origin and evolution of model organisms. Nat. Rev. Genet. 3, 838–849 (2002).

49. Brust-Mascher, I., Civelekoglu-Scholey, G., Kwon, M., Mogilner, A. & Scholey, J. M. Model for anaphase B: role of three mitotic motors in a switch from poleward flux to spindle elongation. Proc. Natl. Acad. Sci. USA 101, 15938–15943 (2004).

50. Goshima, G. & Scholey, J. M. Control of mitotic spindle length. Annu. Rev. Cell Dev. Biol. 26, 21–57 (2010).

51. Wang, H., Brust-Mascher, I., Civelekoglu-Scholey, G. & Scholey, J. M. Patronin mediates a switch from kinesin-13-dependent poleward flux to anaphase B spindle elongation. J. Cell Biol. 203, 35–46 (2013).

52. Kapitein, L. C. et al. Microtubule-driven multimerization recruits ase1p onto overlapping microtubules. Curr. Biol. 18, 1713–1717 (2008).

53. Bieling, P., Telley, I. A. & Surrey, T. A minimal midzone protein module controls formation and length of antiparallel microtubule overlaps. Cell 142, 420–432 (2010).

54. Subramanian, R. et al. Insights into antiparallel microtubule crosslinking by PRC1, a conserved nonmotor microtubule binding protein. Cell 142, 433–443 (2010).

55. Braun, M. et al. Adaptive braking by Ase1 prevents overlapping microtubules from sliding completely apart. Nat. Cell Biol. 13, 1259–1264 (2011).

56. Hutterer, A., Glotzer, M. & Mishima, M. Clustering of centralspindlin is essential for its accumulation to the central spindle and the midbody. Curr. Biol. 20, 2043–2049 (2009).

57. Hu, C. K., Coughlin, M., Field, C. M. & Mitchison, T. J. KIF4 regulates midzone length during cytokinesis. Curr. Biol. 21, 815–824 (2011).

58. Nunes Bastos, R. et al. Aurora B suppresses microtubule dynamics and limits central spindle size by locally activating KIF4A. J. Cell Biol. 202, 605–621 (2013).

59. Froyen-Jensen, C. et al. Single-copy insertion of transgenes in Caenorhabditis elegans. Nat. Genet. 40, 1375–1383 (2008).

60. Kamath, R. S. & Ahringer, J. Genome-wide RNAi screening in Caenorhabditis elegans. Methods 30, 313–321 (2003).

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Author contributions

B.Z. and M.M. initiated the research. K.-Y.L. and M.M. designed the experiments. K.-Y.L. and M.M. performed the experiments. K.-Y.L. and M.M. analysed the data. K.-Y.L. and M.M. wrote the manuscript.

Additional information

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