Prc1E and Kif4A control microtubule organization within and between large Xenopus egg asters

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ABSTRACT The cleavage furrow in Xenopus zygotes is positioned by two large microtubule asters that grow out from the poles of the first mitotic spindle. Where these asters meet at the midplane, they assemble a disk-shaped interaction zone consisting of anti-parallel microtubule bundles coated with chromosome passenger complex (CPC) and centralspindlin that instructs the cleavage furrow. Here we investigate the mechanism that keeps the two asters separate and forms a distinct boundary between them, focusing on the conserved cytokinesis midzone proteins Prc1 and Kif4A. Prc1E, the egg orthologue of Prc1, and Kif4A were recruited to anti-parallel bundles at interaction zones between asters in Xenopus egg extracts. Prc1E was required for Kif4A recruitment but not vice versa. Microtubule plus-end growth slowed and terminated preferentially within interaction zones, resulting in a block to interpenetration that depended on both Prc1E and Kif4A. Unexpectedly, Prc1E and Kif4A were also required for radial order of large asters growing in isolation, apparently to compensate for the direction-randomizing influence of nucleation away from centrosomes. We propose that Prc1E and Kif4, together with catastrophe factors, promote “anti-parallel pruning” that enforces radial organization within asters and generates boundaries to microtubule growth between asters.

INTRODUCTION

Symmetric cell division requires precise positioning of the cleavage furrow at the midplane of a cell. During anaphase, animal somatic cells assemble a bipolar microtubule array in the cell center called the cytokinesis midzone or central spindle (Glotzer, 2009). Midzone microtubules are oriented with plus ends toward the midplane and a small antiparallel overlap at the equator (McIntosh and Euteneuer, 1984; Mastronarde et al., 1993). The plus-end–directed kinesin-family motor Kif23 (a.k.a. MKLP1) transports the furrow-stimulating factor RacGap1 (together they constitute the centralspindlin complex) to the center of the midzone (Mishima et al., 2002), while the plus-end–directed motor Kif20A (a.k.a. MKLP2) transports the chromosome passenger complex (CPC) (Gruneberg et al., 2004). Centralspindlin and the CPC then signal locally to the equatorial cortex to initiate the furrow (Jantsch-Plunger et al., 2000; Yüce et al., 2005; Canman et al., 2008; Lewellyn et al., 2011; Argiros et al., 2012; Basant et al., 2015; Henson et al 2016). In addition, astral microtubules and/or kinetochores may relax the cortex and inhibit furrow formation at the poles in some systems (White and Borisy, 1983; Rodrigues et al., 2015). In cells forced to undergo monopolar cytokinesis, monopolar midzones assemble with microtubule plus ends oriented toward the side of the cell proximal to chromatin. Centralspindlin and CPC accumulate on that side, where they trigger highly asymmetric and incomplete cleavage (Canman et al., 2003; Hu et al., 2008; Shrestha et al., 2012).

An important question in cytokinesis mechanism is how midzone microtubules, or their equivalents in large embryo cells, are spatially organized. The conserved cytokinesis proteins Prc1 and Kif4A play important roles in promoting anti-parallel organization at the midplane and determining midzone microtubule length (Kurasawa et al., 2004; Hu et al., 2011; Subramanian et al., 2013). Their functions...
have been extensively studied in somatic cells, and using pure proteins, but not in very large egg cells. Prc1 (a member of the Ase1/ MAP65 family of microtubule cross-linkers) promotes anti-parallel bundling in the midzone (Jiang et al., 1998; Mollinari et al., 2002; Subramanian et al., 2010). Prc1 can also organize parallel microtubule bundles in dividing cells. In cells undergoing monopolar cytokinesis, Prc1 is required to polarize the midzone and trigger furrowing (Shrestha et al., 2012), and during normal division it transiently "tags" parallel bundles at plus ends (Subramanian et al. 2013). Kif4A (a member of the Kinesin-4 family of plus-end-directed motors with plus-end polymerization-inhibiting activity) antagonizes growth of midzone plus ends (Bringmann et al., 2004; Hu et al., 2011). Prc1 and Kif4A interact dynamically during cytokinesis but do not form a stable complex (Zhu and Jiang, 2005). Together, they can generate anti-parallel overlaps of defined length in a pure protein system (Bieling et al., 2010) and also "tag," and halt growth, of parallel bundles at plus ends (Subramanian et al. 2013).

The large size of frog eggs makes them an interesting system to study aster organization and cytokinesis signaling. The first mitotic spindle is small compared with the egg. At anaphase onset, large microtubule asters grow out from centrosomes at the spindle poles and expand toward the cortex, taking ∼15 min to reach it in frog zygotes (Mitchison et al., 2012). As the asters grow, the centrosomes and nuclei at their centers are pulled away from the midplane of the cell by dynein (Wühr et al., 2010). In a Xenopus zygote fixed between first anaphase and first cytokinesis, microtubules from the two asters overlap and interact, forming a disk of antiparallel bundles at the midplane. This disk, which we will call the aster interaction zone, recruits CPC and centralspindlin. It expands outward toward the cortex as the asters enlarge and initiates a furrow where, and when, it touches the cortex (Nguyen et al., 2014; Field et al., 2015). The interaction zone between asters is functionally equivalent to the midzone in a dividing somatic cell, but it extends out much further from the position previously occupied by the metaphase plate.

The interaction zone between asters in frog eggs appears to limit microtubule growth and prevent interpenetration of the two asters, as evidenced by lower microtubule density in the zone compared with nearby regions (Mitchison et al., 2012). Using EB1 tracking in an extract system, we directly observed that microtubules from one aster tend to stop at the interaction zone and do not cross between asters (Nguyen et al., 2014). This block to microtubule interpenetration between asters is presumably important for correct CPC and centralspindlin localization. It may also serve to generate microtubule length asymmetries that promote dynein-mediated movement of centrosomes away from the midplane (Wühr et al., 2010). Based on prior work in somatic cells and with pure proteins (cited above), Prc1 and Kif4A are candidates for mediating the block to microtubule growth between asters. Prc1 has been little characterized in Xenopus eggs, where it is present as the egg-specific homologue Prc1E (Nguyen et al. 2014). Xenopus Kif4A (previously called Xklp1) was shown to be expressed in eggs and essential for normal cleavage (Vernos et al., 1995). That paper focused on Kif4A localization to mitotic chromosomes and led to the concept of "chromokinesins."

Here we use the Xenopus egg extract system to probe the role of Prc1 and Kif4A in controlling microtubule dynamics within, and between, large interphase asters. In Nguyen et al. (2014) we reported that Prc1E and Kif4A are recruited to anti-parallel bundles between asters and that Kif4A is required for normal microtubule organization and CPC transport. We did not investigate the effect of Prc1E depletion on Kif4A localization or vice versa, and we did not investigate the effect of either depletion on EB1 comet dynamics. These experiments are reported below. In the course of this analysis, we made an unexpected finding: that Prc1E and Kif4A work together to enforce radial order in expanding single asters, presumably by recognition and pruning of accidental anti-parallel overlaps. Our findings extend understanding of the cell division biology of the conserved Prc1/Kif4A module and reveal interesting adaptions to promote microtubule organization and cleavage in very large egg cells.

RESULTS

**Prc1E and Kif4A expression in Xenopus zygotes**

We first identified the egg forms of Prc1 and Kif4A and generated antibodies to localize them in zygotes and deplete them in extracts. NCBI identifiers for each protein are listed in Supplemental Table S1. By proteomic analysis, Xenopus eggs express do not express detectable levels of somatic Prc1 (inferred from proteomics in Wühr et al. (2014). We confirmed this by Western blot with an anti-peptide antibody (not shown). Instead, eggs express a Prc1 isoform, called “Protein Regulator of Cytokinesis-Like (PRC1L)” in NCBI databases, that we will call Prc1E to reflect its egg specific expression. Prc1E is ∼45% identical to Prc1 and is present in eggs at ∼60 nM (inferred from proteomics in Wühr et al., 2014). Prc1E is gradually replaced by somatic Prc1 during development (inferred from mRNA and proteome analysis in Peshkin et al., 2015). Somatic Kif4A is present at ∼100 nM in eggs, and there is no evidence for egg-specific isoforms by proteomics (Wühr et al., 2014). We raised affinity-purified rabbit antibodies to a C-terminal peptide from Prc1E and to the entire nonmotor region of Kif4A. These antibodies were described and characterized previously using standard methods (Nguyen et al., 2014; Mitchison et al., 2013). For this study, we performed immunoprecipitation-mass spectrometry analysis, from which we concluded that our antibodies recognize native protein and are specific for their primary target over all cell division proteins and that Prc1E and Kif4A do not communoprecipitate, suggesting they do not form a stable complex in solution.

Having identified Prc1E and Kif4A as the form of this conserved cytokinesis module that is expressed in eggs, we probed their localization in fixed zygotes. We focused on the period between first mitosis and first cleavage, when a pair of asters grows out from the poles of the mitotic spindle to position the furrow. The boundary region between asters excluded our Prc1E antibody (not shown), even though this protein localizes to anti-parallel microtubule bundles in extract (see below). We suspect this negative result on localization in fixed zygotes was an artefact caused by lack of antibody penetration, which is known for somatic midzone staining (Saxton and McIntosh, 1987) or by failure of our antibody to recognize a phosphorylated form of Prc1E. Kif4A was enriched on anti-parallel bundles between asters, where it mostly colocalized with the AurkB subunit of the CPC (Figure 1). The CPC sometimes appeared to be more focused at the center of antiparallel bundles than Kif4A (Figure 1, A and C). Weak Kif4A staining was observed throughout asters, suggesting that Kif4A does not require anti-parallel bundles for recruitment to microtubules. Kif4A was also present on mitotic chromosomes shortly after anaphase onset, consistent with its function as a chromokinesin (Figure 1A).

**Prc1E and Kif4A localization in interphase egg extracts**

To more reliably localize Prc1E and Kif4A, and probe their dynamics and function, we turned to an actin-intact egg extract system that reconstitutes spatially organized cytokinesis signalling (Field et al., 2014). We used magnetic beads coated with anti-Aurora A kinase...
bright streaks of Prc1E and Kif4A at the free edges of growing asters (Figure 2a, zoom-ups within yellow square). These probably correspond to Prc1/Kif4A positive “plus end tags” observed in dividing somatic cells (Subramanian et al., 2013) that result from Kif4A transport to plus ends, followed by stabilization of plus ends by the two proteins. We also coimaged the CPC with Prc1E and tubulin, adding a GFP-tagged DasraA subunit of the CPC for visualization (Figure 2b and Supplemental Video 2). Unlike Prc1E and Kif4A, the CPC localized exclusively to a narrow region at the interaction zone and was not present in streaks elsewhere in asters. The tight localization at interaction zones was quantified using kymograph plots within the blue rectangle (Figure 2b′).

Plus-end dynamics at interaction zones between asters

To provide a quantitative assay for the block to microtubule growth between asters, we imaged growing plus ends between two asters with EB1-GFP and tracked them using PlusTipTracker software (Applegate et al., 2011). Figure 3a and Supplemental Video 3 show EB1 trajectories color-coded by the orientation of microtubule growth. Quantification of EB1 growth direction (Figure 3e) showed that more than 90% of the growing microtubules within each aster were oriented with their plus ends pointing radially away from the nucleating site, and orientation switched abruptly over ∼40 μm at the boundary between asters. Similar directionality data were reported previously using total internal reflection fluorescence (TIRF)
events, were uniformly distributed (Figure 3, c and h). Termination events, which correspond to catastrophes or pauses, were enriched at the center of the interaction zone (Figure 3, d and h). Comet density was lower near the aster centers but not specifically increased in the interaction zone (Figure 3g). From analysis of multiple representative zones, we observed no strong spatial regulation of either EB1 comet density (10 ± 39% reduction, mean ± SD from n = 5 interaction zones, p = 0.59 from one-sample t test) or of the spatial frequency of initiation events (15 ± 33% increase, p = 0.37). However, we saw a consistent increase in the frequency of termination events in or near the interaction zone midline (86 ± 33% increase, p = 0.004). Thus, plus ends tended to slow down and terminate more within interaction zones than elsewhere in asters, but the density of growing plus ends and the frequency of nucleation/rescue (which are hard to distinguish by EB1 tracking) are not spatially regulated. Spatially uniform nucleation of plus ends throughout large asters was quantified previously and shown to drive aster growth by mathematical modelling (Ishihara et al., 2014, 2016).

A limitation of EB1 tracking is that it does not report on catastrophes or depolymerization. To gain longer-time-scale information on dynamics at boundaries between asters, we imaged labeled tubulin for a prolonged time interval, using a lower magnification to minimize photobleaching and focal plane drift. We found that once asters grew into contact, the total microtubule density at boundaries remained approximately constant for 40 min (Figure 3i). We were not able to quantify microtubule density from tubulin images, but these long-time-scale data rule out a continuous buildup of anti-parallel bundles microtubules over time. This approximate steady state in total microtubule density implies that all plus-end polymerization reported by the EB1 probe must be balanced by an equal amount of depolymerization, most likely by catastrophe at plus ends. We previously estimated that individual microtubules inside interphase asters in the extract system undergo catastrophes at a rate of ∼3 min⁻¹ (Ishihara et al 2016).

Since the rates of EB1 comet initiation and termination are not very different in the boundary region between asters compared with the bulk aster (Figure 3h), and both regions are approximately at steady state in microtubule density (Figure 3i), we estimate that the catastrophe rate in anti-parallel bundles must be of similar or higher, that is, >1 min⁻¹. Thus, we infer that inhibition of plus-end growth in anti-parallel bundles must be followed by catastrophe. Alternatively, it is possible that anti-parallel bundles form early and then do not turn over or recruit new microtubules, but this seems unlikely given that EB1 comets appear to track into anti-parallel bundles continuously.

FIGURE 2: Localization of Prc1E and Kif4A in interphase egg extracts. (a) Spinning disk confocal time-lapse sequence of asters nucleated from AurkA beads in interphase extract. Probes: microtubules (Alexa 647-tubulin), mCherry-Prc1E, and Kif4A-GFP (Supplemental Video 1). (b) Widefield sequence using GFP-DasraA subunit to visualize the CPC (Supplemental Video 2). (a’, b’) Kymograph analysis along a 30-μm-wide line (cyan box in a and b). Note that the CPC is more focused in interaction zones than Prc1E or Kif4A.
Kif4A requires Prc1E for localization to anti-parallel bundles

To test the function of Prc1E and Kif4A in aster organization, we depleted each protein to less than 5% of its initial abundance. We depleted Kif4A previously in Nguyen et al. (2014) and noted strong disorganization of anti-parallel bundles between asters, and also defects in focusing the CPC at the center of the aster interaction zone. We did not deplete Prc1E or test the effect of Kif4A depletion on Prc1E localization or on EB1 comets trajectories. Figure 4 shows an overview of depletion effects and Figure 5 a detailed analysis of microtubule dynamics. Western blots quantification of depletions and add-backs are shown in Supplemental Figure 1, b–e, which is directly associated with Figure 5. Mass spectrometry analysis of these immunoprecipitates showed that Prc1E and Kif4A did not significantly coimmunoprecipitate (data not shown), so we could measure the effect of depletions independently.

Figure 4a shows an overview of the effects of depleting Prc1E or Kif4 or inhibiting the AurkB subunit of the CPC with the small molecule ZM447439. We observed no obvious effect on nucleation from AurKA beads or on microtubule polymerization rates (measured by EB1 tracking) for any of these perturbations. Formation of regions of low microtubule density between asters was inhibited by all three perturbations, suggesting that Prc1E, Kif4, and CPC activity are all required for formation of sharp boundaries between asters.

We next probed dependency relationships for localization to bundles using fluorescently labeled proteins. Kif4A no longer localized to bundles when Prc1E was depleted (Figure 4c). Prc1E still localized when Kif4A was depleted, though the bundles were longer and more disorganized (Figure 4d). Inhibition of AurkB caused profound randomization of bundle position relative to the midline between the asters, but Prc1E and Kif4A still localized to bundles and to some extent still co-localized (Figure 4e). These data suggest a model where Prc1E recognizes anti-parallel bundles and then recruits Kif4A, consistent with the model previously developed using pure proteins (Bieling et al., 2010). AurkB activity was not required for recruitment of either protein to bundles. However, AurkB activity was required for focusing Prc1E/Kif4A-positive bundles into a sharp line that bisects the distance between the asters.
Prc1E and Kif4A are required to block plus-end growth between asters

To quantify the effect of immunodepletion on microtubule growth between asters, we used EB1 comet tracking. The top panels in Figure 5a show tracking data and the bottom panels quantification of those data. The effects of depletions and add-backs are quantified as a bar graph in Figure 5g, where we report the distance over which EB1 directionality switches, using the arbitrary distance metric D60, the distance over which EB1 comet directionality switches from 60%: 40–40%:60%. For comparison, Figure 5g includes the effects of small molecule inhibition (ZM-447439) of the AurkB subunit of the CPC, which we previously showed increases D60 (Nguyen et al., 2014).

Depletion of either Prc1E or Kif4A caused microtubules from each aster to grow into the neighboring aster much more than depletion using control IgG or anti-Kif23 (the kinesin subunit of Centralspindlin) (Figure 5, a–d). There was still a switch in microtubule orientation between the asters when Prc1E or Kif4A was depleted, but the distance over which this occurred was much larger. Thus, both proteins are required for a tightly organized block to microtubule interpenetration at the boundary between asters. EB1 comet visualization was superior using TIRF imaging of plus ends tracking along the surface of partially passivated coverslips. Supplemental Videos 4–6 show EB1 and tubulin between asters in control, Prc1E-depleted and Kif4A-depleted extracts. The block to plus-end growth between asters in control extracts, and loss of this block after depleting either protein, are clearly visualized.

The Prc1E depletion phenotype was rescued by adding back the appropriate recombinant expressed proteins, either as a GFP fusion or unlabelled protein (Figure 5e). Kif4A add back also rescued (unpublished data). This confirms both the specificity of the immunodepletion and the functional attributes of the recombinant proteins. The effect of Prc1E depletion was also rescued by adding back the somatic isoform of Xenopus Prc1 protein (Figure 5f). In
Mock depleted asters maintained high radial order out to the largest radius measured with our camera (≈150–200 μm), with a slight decrease at large radii. Depletion of Kif4A or Prc1E strongly disrupted radial order. Limited order was maintained within ≈40 μm radius, presumably due to frequent renucleation from the AurkA bead. Radial order was progressively lost as radius increased, though growth direction never completely randomized. The effect of depleting both Prc1E and Kif4A on radial order was rescued by adding back appropriate recombinant proteins (Figure 6, d and e; data not shown for Kif4A add-back). Both the embryonic and somatic isoforms of Prc1 were able to rescue radial order. We conclude from these data that Kif4A and Prc1E are necessary to maintain radial order within large asters as they expand, presumably to counter directional randomizing effects of nucleation away from the centrosome. Loss of radial order probably caused the gross disorganization of microtubules between asters we observed in Nguyen et al. (2014) when Kif4A was depleted and could not account for in that paper.

Pruning of anti-parallel microtubule overlaps by Prc1E and Kif4A

We finally turned to single microtubule imaging to better understand the function of Prec1E and Kif4A in asters. We sought regions in two- or three-color confocal movies where the microtubule density was low enough to image individual microtubules and then analyzed them by visual inspection and kymographs. Finding regions that were interpretable at the single microtubule level was
difficult because microtubule density was high, and most microtubules occurred in bundles of more than two. Figure 7 shows two examples near the edge of growing asters where a microtubule growing in the wrong direction enters an anti-parallel overlap, and then the overlap recruits Kif4A. In Figure 7a (Supplemental Video 7) a single microtubule growing in the wrong direction is evident in the first frame. It then enters an anti-parallel bundle that first recruits Kif4A and then shrinks. In Figure 7b (Supplemental Video 8) the incorrect microtubule may nucleate within in a bundle, which we suspect is more common. The bundle recruits Prc1E and Kif4A, and then proceeds to shrink, with loss of microtubule density. These movies support an anti-parallel pruning model for Prc1E/Kif4A function in asters, though we were not able to find enough clear examples to make any statistically justified statements concerning single microtubule mechanism.

DISCUSSION
Our data confirm much of what is already known about Prc1 and Kif4A function from published work from our laboratory and many others, for example, the localization of both factors to anti-parallel bundles prior to cytokinesis (Figures 1 and 2), a Prc1E requirement for recruitment of Kif4A to anti-parallel bundles (Figure 4), a role for protein in both organizing anti-parallel bundles (Figures 4 and 5) and restricting plus-end growth in anti-parallel bundles (Figure 5). In some cases, these confirmatory findings come with greatly improved quantification compared with previous work or extend work from somatic cells to the Xenopus egg system with its huge spatial scale, for example, precise quantification of plus-end dynamics between asters (Figure 3) and showing that Prc1E and Kif4A keep asters distinct in frog eggs by inhibiting plus-end growth at shared boundaries (Figure 5). Data in this paper also reveal new mechanisms and allow us to build new hypothesis, for example, evidence for depolymerization as well as stabilization in anti-parallel bundles (Figure 3i) and an unexpected role of the two proteins in enforcing radial order in isolated asters (Figure 6).

In Figure 8 we propose an “anti-parallel pruning” model to account for the role of Prc1E and Kif4 in generating the boundary between asters and enforcing radial order within them. This model combines Bieling and Surrey’s molecular model from seminal biochemical reconstitution (Bieling et al., 2010) with new information from the egg extract system. We propose that after Prc1E and Kif4 recognize anti-parallel bundles and slow plus-end growth, the plus ends eventually undergo catastrophe, so at least one of the anti-parallel microtubules is removed. We were not able to image catastrophes and depolymerization directly, except in occasional movies (Figure 7). It must nevertheless occur on the majority of microtubules that join anti-parallel bundles in the interaction zone because new plus ends continually enter anti-parallel bundles, yet total microtubule density remains approximately constant for many minutes (Figure 3i and Supplemental Video 4). Catastrophes were...
Our model for the block to interpenetration between asters (Figure 8, red box) is essentially an extension of current ideas about somatic midzone organization applied to the much larger spatial scale of Xenopus zygotes, with the addition of a catastrophe step following recognition by Prc1E + Kif4A as discussed above. Our model for the role of Prc1E and Kif4A in enforcing radial order within asters (Figure 8, blue box) is a new idea and may be specific to very large egg cells. We propose radial order is enforced by the same anti-parallel pruning mechanism as the block to interpenetration between asters. Interphase asters must grow to radii of $>500 \, \mu$m to center the sperm pro-nucleus and orient the cleavage furrow in frog zygotes (Wühr et al., 2010; Mitchison et al., 2012). Radial order is presumably important to promote orderly aster growth and recruit cytokinesis signalling complexes selectively at the boundary between asters. Individual microtubules in the aster are short and unstable, with an estimated average length of $\sim16 \, \mu$m (Ishihara et al., 2016). We do not know the nucleation mechanism(s) of these microtubules.

Our model assumes that Prc1E and Kif4A transiently function together in anti-parallel overlaps, even though they do not strongly colocalize on average (Figure 2). Consistent with this assumption, we were able to observe examples of transient colocalization preceding anti-parallel pruning (Figure 7). Our model neglects other functions of both proteins. For example, Prc1E may contribute to the bundling of microtubules we observe throughout asters and may also help recruit CPC and centralspindlin to a subset of microtubules, as seen in somatic cells (Shrestha et al., 2012).

FIGURE 7: Examples of anti-parallel overlaps in growing asters being eliminated. Examples of anti-parallel microtubule overlap formation and elimination at the edge of growing asters imaged by spinning disk confocal microscopy (Supplemental Videos 7 and 8). Proteins visualized: (a) Alexa 647-tubulin and Kif4A-GFP, Alexa 647-tubulin; (b) Kif4A-GFP, and mCherry-Prc1E. Arrowheads indicate the following: likely plus ends (red), likely minus ends (cyan), microtubule growing out radially from aster (empty), and microtubule growing in opposite direction (full). (a’,b’) Kymographs of examples above along the radially growing microtubule. Events indicated: likely catastrophe (c), likely stabilization (st), and likely rescue (r).

FIGURE 8: Model for function of Prc1E and Kif4A within and between asters. Left box, general function of Prc1E and Kif4A in anti-parallel bundles. Middle box, block to interpenetration at the interaction zone between asters. Right box, enforcement of radial order within single asters by pruning of anti-parallel overlaps. Left box, general function of Prc1E and Kif4A in anti-parallel bundles. Middle box, Block to interpenetration at the interaction zone between asters. Right box, enforcement of radial order within single asters by pruning of anti-parallel overlaps.
From imaging EB1 track initiations, we suspect nucleation occurs mostly within bundles (Ishihara et al., 2014), and there is probably a preference for parallel nucleation, as observed in cytokstatic factor (CSF)-arrested egg extracts (Petry et al., 2013). However, the nucleation mechanisms likely differ, because TPX2 and Augmin, which play central roles in nucleating microtubules in meiosis-II spindles in CSF extract, did not seem to play a major role in interphase asters (Ishihara et al., 2014). We observed ~3–10% of plus ends growing with the incorrect orientation in control asters (Figure 3, a and e), so we suspect that nucleation away from centrosomes can occur with incorrect orientation, at least in extracts, and that the anti-parallel pruning function of Prc1E and Kif4A removes these incorrectly oriented microtubules.

Activity of the AurKB subunit of the CPC was required for assembly of spatially focused interaction zones between asters (Figures 4e and 5g). However, AurKB activity was not required for recruitment of Prc1E and Kif4A to microtubule bundles inside asters (Figure 4e) or for growth of well-organized asters with correctly oriented plus ends (Nguyen et al., 2014). We propose that the anti-parallel pruning function of Prc1E and Kif4A does not depend on AurKB activity, though the CPC helps focus it in CPC-positive interaction zones.

When asters from different mitotic spindles, which we term “nonsister” asters, meet in polyspermic Xenopus zygotes, they fail to recruit CPC and centralspinulin to their shared boundary, and they also fail to trigger cleavage furrows (Field et al., 2015). However, nonsister asters appeared not to cross into each other as they grew, and they formed distinct interaction zones at the shared boundary in microtubule images, albeit less focused than those between sister asters. We propose that Prc1E and Kif4A generate the boundary between nonsister asters in the same way they do between sisters, by anti-parallel pruning, though the boundary is less sharp in the absence of the spatial focusing activity of the CPC.

Many questions remain concerning the organization and dynamics of large interphase asters and the interaction zones that form between them. For example, we proposed that interaction zones constitute a bistable self-organizing system, which propagates outward with a memory of initial conditions, to explain how information on proximity to chromatin is propagated from the mitotic spindle to the cortex (Field et al., 2015). The egg extract system will be useful to further address spatial communication from spindle to cortex, which was first characterized in detail by Rappaport in echinoderm eggs (Rappaport, 1996) and lies at the heart of the spatial focusing activity of the CPC.

Passivation of glass coverslips

Fully passivated polyethylene glycol (PEG)-coated coverslips were prepared either by following previous relatively labor-intensive protocols for covalently coating coverslips with silane-PEG (Bieling et al., 2010) or by a much simpler coating with poly-[(−lysine)-PEG]-PLL (PLL-PEG). For the latter method, coverslips were stored in 95% ethanol. On the day of use, they were withdrawn from the ethanol with forceps, flame dried in a Bunsen burner for a few seconds, and then cooled to room temperature (RT). They were incubated on one side with 100 μg/ml PLL-PEG (PLL(20)-g[3.5]-PEG(2), SuSoS, Switzerland) dissolved in 10 mM HEPES, pH 7.7, for 10–30 min at room temperature. Coverslips were rinsed several times in deionized water by transferring to drops on parafilm and then blow dried with nitrogen gas and used within 24 h. For TIRF imaging where we needed microtubules to track along the glass we used either coated with kappa casein or partial PEG passivation according to a published protocol (Portran, 2014). For these experiments, we made squashed 22-mm² coverslip and a kappa casein coated, or partially passivated, 18-mm² coverslip (Portran, 2014) and imaged with less passivated coverslip.

Aster assembly assay

Metaphase-arrested Xenopus egg extracts with intact actin were prepared as described (Field et al., 2014). Interphase asters were assembled between PEG-passivated coverslips as previously described (Nguyen et al., 2014) with modifications in the coverslip passivation procedure described above. Briefly, M-phase extract was supplemented with fluorescent probes and treated with 0.4 mM CaCl₂ to mimic fertilization and induce metaphase-to-interphase transition. Activated extract was supplemented with Protein A Dynabeads (Life Technologies) coupled to Aurora kinase A antibody, which served as artificial centrosomes (Tsai and Zheng, 2005). Extract was then spread between two PEG-passivated glass coverslips. Aster assembly reactions were monitored with a widefield, spinning disk confocal, or a TIRF microscope at 20°C. For widefield and confocal imaging, asters were assembled between two fully passivated PEG-coated coverslips. For TIRF imaging, asters were assembled between a fully passivated PEG coverslip (top, 18 × 18 mm) and a partially passivated coverslip (bottom, 22 × 22 mm). Partial passivation allowed microtubules to track along the coated surface, within the TIRF illumination field. For visualization, fluorescent probes were
used at the following concentrations: 250 nM Alexa 488, 568, or 647-tubulin; 40 nM EB1-GFP or EB1-mCherry; 10 nM Kif4-GFP; 20 nM mCherry-Prc1E; and 20 nM GFP-DasraA.

**Immunodepletions, protein addbacks, and drug inhibition**

Prc1E, Kif4A, and Kif23 were depleted in two rounds using 2×20 μg of antibodies conjugated to 50 μl Protein A Dynabeads (Life Technologies) per 50 μl of extracts. Depletions using beads coated with random rabbit IgG served as controls. Depletions were confirmed by immunoblots. Kif4A depletion was rescued by adding back ~100 nM Kif4A-GFP, and Prc1E depletion was rescued by adding back ~65 nM purified Prc1E or Prc1. For inhibition of Aurora B kinase, 100 μM ZM447439 (Tocris Bioscience; 10 mM stock solution in 10 mM glycine, pH 7) was added to calculate activated extracts. Aster assembly assays were performed with treated extracts as described above.

All immunodepletion and drug inhibition experiments were performed with n ≥ 3 biological repeats, using extracts prepared from eggs produced by different female frogs. Ideally, all the treatments ought to be performed on the same extract to reduce extract-to-extract (and frog-to-frog) variability, and the samples from each treatment ought to be imaged at the same time to reduce the variability introduced by the “aging” of extracts while stored on ice. In practice, however, this is impossible due to limitations posed by the amount of extract produced from a single female frog and our ability to handle samples. Therefore, we routinely performed only three to four treatments (including control buffer addition or IgG depletion) per experiment. Although we aimed to acquire data for all treatments simultaneously, one to two samples of the three to four would often be destroyed via mishandling, and thus the data for those treatments had to be acquired in the next round of aster assembly reaction.

**Time-lapse fluorescence microscopy**

Widefield images were obtained using a 10× Plan Apo 0.45 NA objective lens (Nikon) on an upright Nikon Eclipse 90i microscope equipped with a Prior Lumen 200 metal arc lamp, a Prior ProScan III motorized XY stage, a Hamamatsu ORCA-ER-cooled charge-coupled device (CCD) camera, and driven by Metamorph image acquisition software (Molecular Devices). Spinning disk confocal images were obtained using a 40× oil Plan Apo 1.30 NA objective lens (Nikon) on an upright Nikon Eclipse E800 microscope equipped with a Merlecs Grift Krypton/Argon ion laser (488, 568, 647 nm), a Yokogawa CSU-10 spinning disk (Perkin Elmer), a Hamamatsu ORCA-ER-cooled CCD camera, and driven by Metamorph. TIRF images were obtained using a 60× Apo TIRF 1.49 NA objective lens (Nikon) on a Nikon Ti-E motorized inverted microscope equipped with a Nikon motorized TIRF illuminator, Perfect focus, a Prior ProScan II motorized stage, an Agilent MLC400B laser launch (488, 561, 647 nm), and an Andor DU-897 EM-CCD camera driven by NIS-Elements image acquisition software. Two separate TIRF set-ups were available at the Nikon Imaging Center at Harvard Medical School and the Marine Biological Laboratory.

**Fixed immunofluorescence of fixed Xenopus zygotes** (Figure 1)

Zygotes were fixed and stained ~100 min after fertilization as previously described (Field et al., 2015). Briefly, zygotes were fixed in a methanol/ethylene glycol-bis-[β-aminethanol ether]-N,N,N’,N’-tetraacetic acid (EGTA) solution for 24 h at room temperature, rehydrated in a series of methanol/Tris-buffered saline mixtures, hsiected, bleached, and then incubated with directly labeled antibodies for at least 24 h at 4°C. Antibodies were used at approximately the following concentrations: Alexa 488-anti-tubulin (1–2 μg/ml), Alexa 568-anti-Prc1E (1–2 μg/ml), and Alexa 647-anti-Kif4A (1–2 μg/ml). Fixed zygotes were mounted in a mixture of benzyl alcohol and benzyl benzoate (Aldrich) and imaged with a laser scanning confocal microscope at the Nikon Imaging Center at Harvard Medical School. Imaging used a Nikon Ti-E inverted microscope with a Nikon A1R point scanning confocal head, driven by NIS-Elements image acquisition software.

**Image analysis: microtubule plus-end dynamics from EB1 comet tracking** (Figures 3, 5, and 6)

Time-lapse image sequences of EB1-GFP were acquired, processed, and analyzed as described (Nguyen et al., 2014). Briefly, extract was supplemented with 40 nM EB1-GFP and 250 nM Alexa 647-tubulin. Aster images were assembled between fully passivated PEG coverslips as described above. Lower AurKA bead density was used to study isolated asters and higher bead density to study interaction zones. Multiple isolated asters or interaction zones were imaged between 20 and 50 min of the assembly reaction at 20°C, alternating between control and treatment conditions. A spinning confocal microscope with a 40× oil objective (NA = 1.30) was used to acquire images with 2×2 binning. Time-lapse image sequences were acquired of EB1-GFP with 1.5-s intervals for a total duration of 2 min (81 frames total). Tubulin images were acquired at the beginning and end of each sequence.

EB1 image sequences were registered using the StackReg ImageJ plugin with rigid body transformation (Thévenaz et al., 1998). The plusTipTracker MATLAB software (Applegate et al., 2011) was used to perform automated detection and frame-to-frame linking of EB1 comets. Tracks were filtered out and excluded from analysis based on the criteria and parameters listed in Nguyen et al. (2014). Remaining tracks were then plotted and colored according to their mean direction (Figure 3a) or instantaneous velocity averaged over three consecutive frames (Figure 3b).

The degree of interpenetration was quantified by dividing all EB1 comets detected in frames 2–81 into two groups based on the direction of their displacement relative to the previous frame (Figure 3a′, inset showing “blue” and “red” directions). Rectangular regions of interests (ROIs; 90 μm × 10 μm) parallel to the bead-bead axis and traversing the interaction zone were overlaid. Each ROI was divided into 9 cells (10 μm × 10 μm each), and the fraction of EB1 comets moving in the “blue” direction was determined and plotted as gray dots against the distance from the interaction zone midline (see Figure S3 in Nguyen et al. 2014). The procedure was repeated for multiple nonoverlapping, neighboring ROIs within an interaction zone, giving rise to all the gray dot data. The data points were fitted to a sigmoidal distribution (Figure 3a′, blue curve):

\[
 f(x) = Y_{\text{min}} + \frac{Y_{\text{max}} - Y_{\text{min}}}{1+\left(a + x\right)^b}
\]

where x is distance and \(f(x)\) is the fraction of EB1 comets moving in the “blue” direction. A complementary sigmoidal distribution described the fraction of EB1 comets moving in the other, “red,” direction (Figure 3a′, red curve). The exact position of the interaction zone midline was defined where the blue and red curves crossed 60%. This parameter served as a measure for the depth of interpenetration.

The instantaneous velocities associated with each EB1 comet detected in frames 2–81 were averaged within each square cell. The total number of EB1 comets was counted, and the comet density determined for each cell. The number of EB1 comets comprising...
the initiation or the termination of a growth track was counted and expressed as the fraction of total EB1 comets for each cell. EB1 comet speeds, densities, track terminations, and initiations were averaged for cells in neighboring ROIs at a given distance from the interaction zone midline. These mean values were plotted in Figure 3, b’ and c’, where the error bars indicate SEMs for EB1 speeds and SDs for densities, track terminations, and initiations.

To obtain the percentage difference in plus-end dynamics measurements inside an interaction zone compared with outside, the mean value of each measurement obtained at the exact interaction zone midline (0 μm) was divided by the mean of the mean values obtained at –40, –30, –20, 20, 30, and 40 μm from the interaction zone midline. The mean values obtained at –10 and 10 μm were considered transition values and thus were omitted from the calculations. The percentage difference measurements were averaged for multiple (n ≥ 3) interaction zones per condition; the mean values were plotted with standard deviations as error bars in Figure 4). All analyses were performed in MATLAB.

Image analysis: quantification of radial order within isolated asters

The first approach to quantifying radial order in asters involved tracking EB1 comets over 2 min (81 frames at 1.5-s intervals) and measuring their directions (angles of frame-to-frame displacement) within a quadrant of an isolated aster. Radial order heat maps were created by dividing the field of view into square cells measuring 5 × 5 μm² (Figure 6, a’–e’). A radial order parameter (R) was calculated from the distribution of angular deviations of EB1 comet directions from the radial direction within each square cell:

\[ R = \frac{1}{N} \sum_{i=1}^{N} \left( \cos(\theta_i) + i \sin(\theta_i) \right) \]

where \( \theta_i = \beta_i - \alpha_i \). The variable \( \alpha_i \) is the radial direction of each EB1 comet (i.e., angular coordinate within a polar coordinate system with the aster center as the pole), and \( \beta_i \) is the angle of displacement of each comet with respect to the previous frame. The variable \( \theta_i \) ranges from \(-\pi\) to \(\pi\) in radians. For the heat maps, all EB1 comets detected from frame 2–81 were considered within each square cell. In directional statistics, the circular variance is defined as (1 - R), which is a measure of the spread of a population of angles \( \theta_i \). R is the length of the mean resultant vector of the population of angles and ranges from 0 to 1. A MATLAB toolbox called “CircStat” was used to compute the resultant vector length (R) (Berens, 2009). To quantify the dependence of radial order on the distance from the aster center, the field of view was divided into concentric rings with a width of 5 μm, centering on the AurkA bead. Radial order parameters (R) were obtained for each ring area, considering EB1 comets detected within 10 consecutive frames (e.g., frames 2–11, 12–21, …, 72–81). Figure 6f shows the average values of the R parameter as a function of aster radius for different conditions. Each R value was averaged across all eight time ranges in the image sequence. The error bars are the standard deviation of these R values from the mean and represent the fluctuation of the R value over time, combined with measurement error.

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