Action at a distance during cytokinesis

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Introduction

The cytokinetic apparatus of animal cells consists of a transient, dynamic array of actin filaments and myosin-2—the contractile ring—and proteins that link it to the plasma membrane. Constriction of the contractile ring must draw the cell surface between the poles of the mitotic apparatus, separating centrosomes and sister chromosomes, to properly partition daughter cells. To ensure this, cytokinetic apparatus formation depends on spatial cues conferred upon the cortex by the mitotic apparatus.

Animal cells decide where to build the cytokinetic apparatus by sensing the position of the mitotic spindle. Reflecting a long-standing presumption that a furrow-inducing stimulus travels from spindle to cortex via microtubules, debate continues about which microtubules, and in what geometry, are essential for accurate cytokinesis. We used live imaging in urchin and frog embryos to evaluate the relationship between microtubule organization and cytokinetic furrow position. In normal cells, the cytokinetic apparatus forms in a region of lower cortical microtubule density. Remarkably, cells depleted of astral microtubules conduct accurate, complete cytokinesis. Conversely, in anucleate cells, asters alone can support furrow induction without a spindle, but only when sufficiently separated. Ablation of a single centrosome displaces furrows away from the remaining centrosome; ablation of both centrosomes causes broad, inefficient furrowing. We conclude that the asters confer accuracy and precision to a primary furrow-inducing signal that can reach the cell surface from the spindle without transport on microtubules.

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delivery (Mandato et al., 2000; Canman et al., 2003; Shannon et al., 2005; Strickland et al., 2005b; Foe and von Dassow, 2008; Odell and Foe, 2008; Vale et al., 2009). These cannot be dismissed as model system–dependent variation, as different studies in the same system have reached opposite conclusions (see Dechant and Glotzer, 2003 vs. Motegi et al., 2006 vs. Verbrugge and White, 2007).

Newer findings change the details but not the notion that microtubules deliver the cytokinetic signal. Specifically, recent work demonstrates at least two experimentally separable cytokinetic signals: one from the spindle midzone and one from the asters (Bringmann and Hyman, 2005; Werner et al., 2007; Baruni et al., 2008; von Dassow, 2009). Also, it is now clear that activation of the small GTPase Rho at the cell equator is a conserved feature of animal cytokinesis (Bement et al., 2005), and this event is attributed to the centralspindlin complex, which consists of the kinesin MKLP1 and MgcRacGAP, working with Ect2, a Rho GEF (Yüce et al., 2005; Zhao and Fang, 2005; Kamijo et al., 2006; Nishimura and Yonemura, 2006). A popular scenario posits that MKLP transports centralspindlin along microtubules to the equatorial cortex, where it meets and activates Ect2, locally activating Rho (Saint and Somers, 2003; Somers and Saint, 2003; D’Avino et al., 2006). Delivery of a positive signal to the equator may be complemented by provision of a negative signal by dynamic microtubules that contact the cortex elsewhere (Werner et al., 2007; Chen et al., 2008; Murthy and Wadsworth, 2008; Foe and von Dassow, 2008).

Surprisingly, however, no direct proof has ever been offered that microtubules must contact the cortex for transmission of the cytokinetic signal. This is partly because it is hard to disrupt microtubules before anaphase onset without inducing metaphase arrest. Also, in large cells where signal transport might be most important, the means to clearly image microtubules extending to the cell surface have been lacking.

Here, we demonstrate the means to image and manipulate microtubules in living embryos of echinoderms and amphibians. We find that a functional cytokinetic apparatus forms and closes in the proper position in the complete absence of contact or even approach of astral microtubules with the cortex in very large, round cells. We also find that pairs of asters without an intervening spindle can specify a functional cytokinetic apparatus only when they are separated sufficiently from each other. We show that the centrosomes or asters confine the signal or its effect. Based on these and other results, we propose that distinct subsets of the mitotic apparatus collaborate to make specification of the cytokinetic apparatus both accurate and precise.

Results

Spatial organization and behavior of live microtubules during embryonic cytokinesis

To image microtubules in living urchin or Xenopus laevis embryos, we used three tandem GFPs fused to the ensconsin microtubule-binding domain (EMTB-3G; Faire et al., 1999). This probe, unlike fluorophore-conjugated or GFP-tubulin, enabled high-contrast, high-resolution microtubule visualization throughout the cell cycle, from the eight-cell stage on in purple urchin or sand dollar embryos (Fig. 1 and Video 1) and X. laevis blastomeres (Fig. S1 D and Video 2), which revealed apparently single microtubules extending to the cortex, even in large cells. Rapid microtubule growth and shrinkage were clearly evident (Fig. 1 C’ and Fig. S1 E), with microtubule growth at 16.7 ± 11 µm/min (n = 50) and shrinkage at 9.9 ± 2.6 µm/min (n = 50) in X. laevis embryos, which is consistent with studies of cell free extracts (e.g., Belmont et al., 1990).

Immunostaining embryos expressing EMTB-3G showed that GFP overlapped nearly completely with tubulin (Fig. S2), the sole exception being the ends of spindle midzone microtubules and the center of the midbody, which were underlabeled by EMTB-3G. The only drawback to EMTB-3G was that rapid expression in the earliest embryos caused cell cycle arrest (Fig. S1 A) or defective mitosis. However, injection of 0.05–0.1 ng/ml EMTB-3G mRNA routinely produced embryos with robust microtubule labeling and normal cell cycle progression; such embryos cleaved with no sign of mitotic errors to late blastula, whereupon they began to swim, gastrulated normally, and became morphologically normal feeding pluteus larvae (Fig. S1, B and C).

We assessed microtubule dynamics in surface and axial views of sand dollar and purple urchin embryos (Fig. 1 and Fig. 2, A–C) and in surface views of X. laevis embryos (Fig. 2 D, Video 2, and Fig. S1 D). In all three species, regardless of imaging plane, three observations were remarkably consistent. First, microtubules arrived in every part of the cortex, including the equator, before furrowing (Fig. 1 A, B [ii–iv], and C; Figs. 2, A’–C; and Fig. S1 D’). Second, after anaphase onset, most astral microtubules at the cortex were stable, regardless of orientation (Fig. 2, A’–C; and Fig. S1 D’). Third, astral microtubules reached the cortex in greater density outside the furrow region (Figs. 1 C, 2, and S1 D). The last point was confirmed by imaging both microtubules (with 3C-EMTB) and active Rho, the earliest known marker for cytokinetic apparatus specification (with GFP–rhotein KTPase-binding domain [rGBD]; Benink and Bement, 2005) in X. laevis embryos (Fig. 2 D), which showed that microtubules arrived at the cortex before Rho activation even in very large cells, and that the Rho zone occupied a microtubule-poor region of the cortex.

Nocodazole-insensitive microtubules in living cells

Immunofluorescence analysis of fixed echinoderm zygotes (Foe and von Dassow, 2008) revealed a nocodazole-insensitive population of astral microtubules that arises at anaphase onset and that has a clear equatorial bias before furrowing. However, EMTB-3G detects apparently stable microtubules throughout the cortex in later embryos (eight-cell and beyond). We tested whether these are nocodazole insensitive by perfusing eight-cell and older EMTB-3G–expressing embryos with nocodazole. Consistent with previous results, 10–20-µM nocodazole treatment during anaphase prompted rapid loss of most astral microtubules but left a substantial fraction extending from each spindle pole (Fig. 3 and Video 3). However, in contrast to the results obtained with...
In the course of these experiments, we noticed that nocodazole treatment failed to prevent cytokinesis even when it prevented microtubules from approaching the cortex, either from the spindle midzone or the asters (Fig. 3 and Video 3). Every zygote, in these smaller cells, there was no obvious directional bias of nocodazole-insensitive microtubules toward the equator before furrowing (Fig. 3), which indicates that these stable microtubules cannot solely explain cytokinetic pattern formation.

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Figure 2. The furrow forms in a microtubule-poor region. (A) Single superficial confocal sections of a 16-cell sand dollar embryo, slightly compressed (EMTB-3G). (02:00) All three cells in metaphase; few microtubule ends are visible. (07:00) Left cell begins cleavage, right cell is still in metaphase, and the top cell has likely entered anaphase. (13:00) Right cell begins to cleave; fewer microtubules approach the cell surface (bright dots) in the incipient furrow than outside it. (A') “Kymocubes” made by 3D rendering sequence in A; white lines denote frames in A. The whole of each of three cells is shown.
cell that entered anaphase (as deduced from emergence of a dark gap between spindle halves), regardless of the extent of the surviving aster, established a well-placed furrow. This is epitomized by Fig. 3 B: cytokinesis occurred in one cell that entered anaphase just when nocodazole was added (and completely lacked long astral microtubules) but not in three metaphase-arrested cells.

Microtubule-independent delivery of the cytokinetic signal

The nocodazole results imply that cytokinetic signals can reach the cortex without the help of microtubules extending from the spindle to the cortex. But because nocodazole must be applied after anaphase onset to avoid metaphase arrest, it might be that signaling occurred in the narrow window before nocodazole took effect. Also, although nocodazole greatly shortened microtubules extending from the spindle to the cell surface, perhaps stable microtubules were still close enough to mediate equatorial delivery. To inhibit astral microtubule growth without cell cycle arrest, we therefore treated embryos with trichostatin A (TSA), an inhibitor of HDAC6 (a tubulin deacetylase) that selectively disrupts dynamic microtubules in mammalian cells (Matsuyama et al., 2002). Perfusion of urchin embryos with TSA in prophase or metaphase caused rapid collapse of the aster and usually a delay, but not an arrest, in metaphase (Fig. 4; Videos 4 and 5; and Figs. S3 and S4).

In metaphase, TSA caused the entire aster to collapse within minutes (Figs. 4, S3 B, and S4 A); as with nocodazole, anaphase cells retained a subset of stable astral microtubules (Fig. S3 B). The spindle itself often became much shorter in TSA-treated cells, and sometimes became deranged during a prolonged metaphase (e.g., Fig. S4 B). Even so, most TSA-treated cells underwent anaphase, as revealed by a widening gap in the midzone and emergence of nuclear vesicles near spindle poles (Fig. 4, A and C), or by coexpressing EMTB-3G and mCherry-histone H2B (mC-H2B; Figs. 4 B and S3 B). Although short microtubules often regrew from centrosomes in anaphase, and isolated microtubules formed in the cytoplasm (e.g., Fig. 4 C), extension of microtubules from either the spindle midzone or the poles to the cortex was not detected.

Astonishingly, the vast majority of TSA-treated cells completed cytokinesis, despite the near-total absence of astral microtubules (Fig. 4, Videos 4 and 5, and Figs. S3 B and S4). Moreover, cytokinesis occurred with unexpected fidelity; the furrow formed and closed equatorially between the modestly separated chromosomes even when the closest detectable approach by a spindle microtubule to the cell surface was >10 µm (Fig. 4 C and Fig. S4 B). Only failure to enter anaphase prevented furrowing. In a minority of cells, the ingressing furrow initially failed to separate the chromosome sets. However, in many of these, the cell corrected this error as the constricting furrow approached the spindle, either by the spindle being moved back and forth until it matched the furrow position, or by shifting the furrow toward the spindle midzone (not depicted).

These results, which were obtained by imaging a single medial optical plane, were confirmed by complete z series or surface views of live TSA-treated cells (not depicted). To complement live imaging, we treated embryos with TSA, then fixed and stained for tubulin and serine-19 phosphorylated myosin light chain (phospho-myosin; a marker for active myosin-2 and the cytokinetic apparatus). 3D reconstructions of these embryos showed clearly that a functional cytokinetic apparatus, replete with active myosin-2, forms at the equator of TSA-treated cells in the complete absence of approach of spindle microtubules to the cortex (Fig. 5), which eliminates the possibility that the apparent lack of astral microtubules seen by live cell imaging reflects either deficiencies in the EMTB-3G probe or the imaging regimen. Finally, the possibility that the cytokinetic pattern formed long before TSA took effect was ruled out by cases in which TSA was applied before metaphase; the cell depicted in Fig. S4 B, for example, never achieved long astral microtubules in this cell cycle. We find it impossible to escape the conclusion that abolishing contact between astral microtubules and the cell cortex simply does not prevent the cell from assembling a normally positioned, functional cytokinetic apparatus.

Astral microtubules focus the cytokinetic pattern

Although TSA-treated cells established accurate cytokinetic furrows, those furrows were abnormally broad, as was the distribution of active myosin-2 (e.g., Fig. S4 B and Fig. 5). We therefore assessed cytokinetic pattern formation using GFP-rGBD, alone or in combination with 3C-EMTB. The Rho zone was harder to detect in TSA-treated cells, but was definitely present (Fig. 6, C and D), which confirmed that cells can confine Rho activation to the equator without help from asters. TSA-treated cells, like normal cells, exhibited ubiquitous low-level cortical Rho activity that declined after anaphase onset (Fig. 6 D). However, the Rho zone was much wider than normal in TSA-treated cells (Fig. 6 A). Measurement in the kymocube. Bright dots (open arrowheads in A–C) indicate brief cortical visits by microtubule ends; these predominate in metaphase. Vertical streaks (closed arrowheads in A–C) indicate kinematically stable microtubule ends, which appear before furrowing, and are scarce in the equatorial zone. [B] Kymograph of 5-µm medial strip (indicated by box) ~1 µm beneath the surface of a slightly flattened eight-cell sand dollar embryo. The arrow indicates furrow initiation time; vertical streaks above this point indicate stable microtubules at the cortex before furrowing. These are notably fewer in the equator. (C) Surface rendering of single medial sections of uncompressed sand dollar blastomers, each covering anaphase through cytokinesis. Long streaks (stable microtubules at the cell surface) are evident well before furrowing. Broad smears appear during furrowing, notably in the area around the crotch; these reflect microtubules growing along the cortex (see also A and B). [D] Single superficial sections from a 6.5-h X. laevis embryo mosaically expressing GFP-rGBD (red) and 3C-EMTB (cyan). Surface microtubules disappear in metaphase, then reappear (16:52) ~90 s before active Rho appears in the equator (18:28). Microtubules are largely absent from a >20-µm-wide band inhabited by the Rho zone; arrows indicate equatorial microtubules that disappear during furrowing. Time is indicated in minutes:seconds.
of Rho zone width by curve fitting (see Materials and methods) showed that Rho activity in TSA-treated cells covered a latitude range along the cell surface on average twice as wide as in controls from the same batch of eggs (Fig. 6B; 0.28 ± 0.14 of the pole-to-pole cell diameter in TSA-treated cells [n = 23] vs. 0.14 ± 0.06 in controls [n = 29]; P < 0.0002). Notably, the intensity above background integrated across the entire Rho zone was not significantly less than normal in TSA-treated cells; thus, Rho activity is diluted, not diminished, by the absence of asters.

Figure 3. Nocodazole-insensitive microtubules exhibit no orientation bias in urchin blastomeres. (A–C) Single sections, all expressing EMTB-3G; (A’–C’) 2x enlarged views of cells indicated by the white marks in A–C. (A) Eight-cell purple urchin embryo; 20 µM nocodazole was added at time 00:00. Within 2 min, most astral microtubules disassembled; remaining microtubules are randomly oriented and most end well short of the cortex. Even so, furrowing is accurate and timely. Arrowheads indicate surviving nonequatorial astral microtubules. (B) 16-cell purple urchin embryo; 10 µM nocodazole was added at time 00:00. The top left cell entered anaphase around time 0; none of the other cells in view left metaphase nor furrowed. This one cell cleaved despite nearly complete absence of astral microtubules, and the furrow crossed the spindle midzone. (C) 16-cell sand dollar embryo; 10 µM nocodazole was added at time 00:00, at which time three of four macromeres have entered anaphase; the east cell enters anaphase shortly thereafter (03:00). In all cells, numerous astral microtubules, pointing in all directions, persist >5 min after nocodazole addition (arrowheads). Stable microtubules may approach the cortex in west and south cells (03:00), but none can be seen to do so in north and east cells; all commence furrowing at a normal time and complete cytokinesis. Note that in all cases, stable microtubules become brighter because EMTB-3G liberated by disassembly becomes available for binding. Video 3 includes A–C. Time is indicated in minutes:seconds.
Video 6). In all cases, regardless of cytokinetic behavior, astral microtubules penetrated the cortex everywhere (as in normal cells) during anaphase and telophase, and in no case did we detect anything like a spindle between two centrosomes in an anucleate cytoplast.

Asters alone can only induce a furrow if they are far enough apart.

The results of TSA treatment show that cells can accurately position the cytokinetic apparatus without the astral microtubule array. Yet numerous classical results identified the juxtaposition of two asters as the sufficient condition for furrow induction (e.g., Rappaport 1961). To resolve this contradiction, we created EMTB-3G–expressing cell fragments that lack nuclei (see Materials and methods). These anucleate cytoplasts conduct a normal centrosome cycle: duplication and separation, and cyclic alternation between a compact, dense, and dynamic aster (metaphase) and a loose aster of long, stable microtubules (interphase). Anucleate cytoplasts with two or more centrosomes exhibited a full spectrum of cytokinetic behaviors: some showed no sign of cleavage; some cleaved completely; and in some, furrows ingressed some distance before regressing (Fig. 7, A–C; and Video 6). In all cases, regardless of cytokinetic behavior, astral microtubules penetrated the cortex everywhere (as in normal cells) during anaphase and telophase, and in no case did we detect anything like a spindle between two centrosomes in an anucleate cytoplast.

The observed behavioral spectrum might suggest that asters alone can induce a furrow but that induction isn’t robust under these conditions. However, we noticed that anucleate cytoplasts that manifest deeply ingressing furrows have a greater separation between adjacent asters (Fig. 7, A vs. B). This is apparent even within a single case: in Fig. 7 C, deeply ingressing furrows develop between well-separated asters, and only shallow furrows develop between closer pairs. This correlation was quantified by comparing the distance between centrosomes versus the distance from the surface, versus the extent of furrowing (see Materials and methods). As shown in Fig. 7 E, if the distance between centrosomes exceeded...
Figure 5. Myosin occupies a wider-than-normal zone in asterless cells. Cyan, anti-tubulin; yellow, Hoechst; magenta, anti-phospho-myosin. (A and B) Untreated 16-cell sand dollar embryos. Projection of 24 0.5-µm sections, vegetal view (A); and 19 0.6-µm sections, side view (B). (C and D) 16-cell sand dollar embryos fixed 10 min after adding 25 µM TSA. (C) A projection of 18 0.5-µm sections through the middle portion of the macromeres. (D) A projection of 28 0.5-µm sections through the middle portion of the mesomeres. A–D are from the same batch fixed at the same time. Normally, phospho-myosin is virtually absent outside of ~10 µm furrow zone. In TSA-treated cells, phosphomyosin is not as thoroughly excluded from the poles but is still enriched equatorially, with definite zones around ingressing furrows (brackets in D), even though no spindle microtubules approach the cortex. A’–D’ are 2× enlarged views of the cells indicated by the white marks in A–D.

the distance to the cell surface, furrows ingressed deeply. If the two distances were roughly equal, only shallow furrows developed. If centrosomes were substantially closer than the distance to the surface, no furrow developed. These differences were highly significant (in both comparisons, P < 0.001).
Asters spatially confine the cytokinetic signal

The behavior of anucleate cytoplasts confirms that asters, or the centrosomes they grow from, somehow provide enough positional information to direct furrowing. Meanwhile, nocodazole and TSA treatment show that the astral microtubules need not reach the cortex for the cell to accurately identify the appropriate furrow site, although the cytokinetic apparatus is more broadly specified in the absence of asters. These results could be reconciled if the central spindle localizes a positive signal, and the astral microtubules or centrosomes suppress cortical contractility outside the equator, thereby sharpening this signal at the cortex. A second hypothesis is that a positive signal from the spindle midzone is spatially confined by the asters or centrosomes, perhaps by interacting with astral microtubules or because something associated with centrosomes depletes or inactivates the signal in the cell interior. If the first hypothesis were correct, one would predict destruction of a single aster to broaden and strengthen the signal; if the second were correct, relatively little change in signal level is expected. Both hypotheses predict that destroying a single aster should shift the signal away from the ablated aster. A third hypothesis is that asters or centrosomes generate positive signals that are summed at the equator, with or without synergistic signals from the midzone; in this case, destruction of a single aster should shift the signal away from the ablated aster, and the signal should be weakened.

We tested these predictions by destroying centrosomes in sand dollar blastomeres using a UV laser (see Materials and methods). Most singly ablated cells clearly underwent...
often partitioning both chromosome sets into the daughter cell with an intact centrosome. In most singly ablated cells, furrowing completed without significant attempts by the cell to correct furrow position; this contrasts with the results of physical spindle displacement (Bement et al., 2005) or TSA treatment (this paper).

We also conducted ablations in cells coexpressing GFP-rGBD with 3C-EMTB. Because of numerous experimental factors (see Materials and methods), double labeling was often faint. Even so, some crucial observations emerged: first, Rho zones appeared even in cells with extreme ablations; second, anaphase (e.g., Fig. 8 A), as confirmed by conducting ablations in cells coexpressing EMTB-3G and mC-H2B (Fig. S5 B and Video 8). Ablation of a single centrosome in metaphase consistently shifted furrows from the spindle midzone toward the ablated pole (Fig. 8, Video 7, and Fig. S5). If the aster regrew substantially, or if the ablation took place in anaphase, no displacement of the furrow resulted (see Fig. S5 C). Partial ablations, in which some microtubules regrew from the irradiated pole, led to modest displacement. Complete ablations caused cells to establish furrows that closed near (Fig. 8, A, B, and D) or beyond (Fig. S5, A and C) the ablated aster, often partitioning both chromosome sets into the daughter cell with an intact centrosome. In most singly ablated cells, furrowing completed without significant attempts by the cell to correct furrow position; this contrasts with the results of physical spindle displacement (Bement et al., 2005) or TSA treatment (this paper).

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We also ablated both centrosomes, which was difficult given the narrow time window and the likelihood of missing one or both poles. In several instances, we accomplished this operation with minimal aster regrowth and without preventing anaphase (Fig. 8, C and D; and Fig. S5 B). In some doubly ablated cells that executed anaphase, cleavage failed, and cells exhibited only incoherent surface ruffling. Other doubly ablated cells initiated broad, shallow furrows, approximately over the midzone (Fig. 8 C). Many of these completed cytokinesis, often (not always) partitioning daughter chromosomes to daughter cells (compare two doubly ablated cells in Fig. S5 B).

These zones were not notably different (broader, brighter, or dimmer) than zones in normal cells (Fig. 8 B and Video 9); and third, rather than appearing asymmetrically (as one would expect if asters only inhibited Rho activation), zones were centered on the eventual furrow site. Importantly, we did not find more Rho activity than normal on the polar cortex near ablated centrosomes, which implies that asters or centrosomes shape the distribution of the cytokinetic signal rather than only inhibiting it; if the latter were true, we would expect Rho activation to take place in a normal spatial relation to the intact centrosome instead of shifting distally, as we observed.

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This is consistent with results from TSA treatment, collectively implying that asters confer accuracy and precision to the cytokinetic signaling mechanism. To quantify this we scored all centrosome-ablated, EMTB-expressing cells that made credible attempts at cleavage into three categories: double ablations, partial single-pole ablations, and total single-pole ablations. In each, and in controls from the same films, we measured the concurrence of furrow plane and spindle midzone, normalized by cell diameter. The results, superimposed upon normal curves computed from mean and variance in each category (Fig. 8E), show that doubly ablated cells are less precise than controls but accurate in aggregate, whereas singly ablated cells are precisely inaccurate.

Discussion

Our most surprising finding is that astral microtubules are not needed to transmit the cytokinetic signal, even in large round cells where the nearest spindle microtubule is far from the cell surface. Although it could be argued that our experimental approaches account for this result, the EMTB probes reveal both dynamic and stable microtubules, and precisely colocalize with all astral and cortical microtubules revealed by anti-tubulin in fixed samples. The cortical distribution of microtubules seen in living cells with EMTB parallels that detected by careful EM analysis of urchin zygotes (Asnes and Schroeder, 1979). Thus, if there is some significant cortical microtubule population not detected by EMTB-based probes, it is also undetectable by any other means currently available. Moreover, the same results are obtained in fixed samples never microinjected with EMTB nor subjected to live imaging, and the TSA results are supported by those obtained with nocodazole. Collectively, these results compel the conclusion that neither astral microtubules extending from centrosomes to regions outside the equator, astral microtubules extending from centrosomes to the equator, nor microtubules extending from the midzone to the equator are necessary for delivery of the cytokinetic signal from the spindle to the cortex.

The second finding is that centrosomes, or the asters that grow from them, limit the spatial extent of the cytokinetic signal. This idea is not new, but the approach—centrosome ablation in urchin blastomeres—complements those used in other studies and affirms this principle in urchin embryos. The latter point is critical: it has been vigorously argued, based on micromanipulation studies of urchin zygotes, that asters provide a positive signal that is summed at the equator (for reviews see Rappaport, 1996; Burgess and Chang, 2005). If this were true, ablation of a single centrosome should shift cytokinetic apparatus assembly away from the ablated centrosome. In fact, exactly the opposite happens: the cytokinetic apparatus is displaced toward the ablated centrosome, often so far that all chromosomes end up in one daughter cell. An inhibitory signal from the asters is consistent with studies showing that myosin-powered cortical flow is directed away from microtubule-organizing centers (Hird and White, 1993; Benink et al., 2000; Chen et al., 2008), formation of furrows away from asters during cytokinesis in monopolar cells (Canman et al., 2003; Hu et al., 2008), and local symmetry breaking of a cortical myosin-2 network by displaced asters in Caenorhabditis elegans embryos (Werner et al., 2007). However, most of these studies, including ours, posit that this requires direct action of microtubules at the cortex, a presumption challenged by our results.

Asters could limit the cytokinetic cue either by inhibiting that signal locally—by providing an antagonist, by sequestration, or by degradation—or by spatially confining the signal to the equatorial region (e.g., through plus-end-directed transport of the signal or its generators away from the centrosomes). Simple inhibition would predict that the Rho zone would be broader and brighter after destruction of one or both asters, in contrast to what we observed. Instead, the Rho zone is diluted by reduction of the asters with TSA, and ablation of a single centrosome shifts the Rho zone away from the remaining aster without increasing the intensity or breadth. This argues that asters spatially confine the signal, thus enhancing it locally by preventing it from spreading away from the equator.

Our third finding is that although paired asters without a midzone can direct cytokinetic apparatus induction as previously described (Rappaport, 1961), they can do so only under limited geometrical conditions. The use of a live microtubule label reveals unambiguously and for the first time that microtubules from paired asters penetrate the cortex extensively, regardless of whether a furrow forms, and paired asters induce furrows only if there is a microtubule-poor corridor between them. This further argues that the centrosomes or asters limit, rather than create, the cytokinetic signal, as otherwise, closely separated asters would be more potent furrow inducers than distant ones. Anucleate cytoplasts lack chromosomes or midzone but likely retain the molecular species that normally generate the cytokinetic signal. Therefore, if the asters spatially confine the signal rather than simply inhibiting or promoting it, then perhaps juxtaposed asters, in anucleate cytoplasts or toroidal cells, elicit furrowing by concentrating enough of the participants between them.

If the cytokinetic signal can pattern the cortex without the aid of microtubules, how does it get there? Actin can support myosin-powered vectorial transport, but F-actin is dispensable for cytokinetic apparatus specification (Straight et al., 2003; Bement et al., 2005; Foe and von Dassow, 2008). Intermediate filaments and membrane systems permeate the cell but are inherently nonpolar, so it is hard to imagine how they could conduct signals from spindle to cortex. We are led to hypothesize that the primary signal for cytokinesis reaches the cell surface from the spindle by diffusion.

Diffusion seems a poor means to accurately deliver a signal from an interior structure to the cell periphery across large distances, but a diffusible cue arising from the cell interior could be sharpened by at least three nonexclusive mechanisms. First, if the signal is inactivated or sequestered at centrosomes or by astral microtubules (even if they do not reach the cortex), lateral spreading would be suppressed. Centrosomal inactivation could occur by microtubule-independent means as described previously for polarization in C. elegans embryos (Cowin and Hyman, 2004). Sequestration could result from microtubule binding by MKLP1 or other candidate signaling agents.
Second, the microtubule-poor corridor between asters could represent a path from the central spindle to the cell surface in which the putative diffusible signal is relatively mobile. Thus, an initial bias established by the spindle in the cell center would be maintained out to the cell surface. If the few microtubules penetrating this region tended to trap potential signal generators (e.g., chromosomal passenger proteins or centralspindlin), such an effect would be enhanced.

Third, an initially broad signal could be sharpened at or near the cortex. A microtubule-independent mechanism is indicated by the fact that Rho zones eventually focus even when microtubules don’t reach the cortex, whereas a microtubule-dependent mechanism is suggested because partial inhibition of astral microtubule outgrowth enhances myosin recruitment (Foe and von Dassow, 2008). Anillin, which binds F-actin, Rh, and MgcRacGAP, is a good candidate for the first type of amplifier (e.g., D’Avino et al., 2008); transport of centralspindlin or chromosomal passenger proteins along stable microtubules outside the equator (Canman et al., 2003; D’Avino et al., 2006; Odell and Foe, 2008), and inhibition of Rho activation by microtubules outside the equator (Werner et al., 2007; Odell and Foe, 2008; Murthy and Wadsworth, 2008), are good candidates for the second kind of amplifier.

The core proposition—that the primary cytokinetic signal is focused to the equator by the combined action of the spindle midzone and the centrosomes—requires that future attention be directed toward parameters that are not yet well established, such as realistic diffusion and transport rates for large macromolecular complexes (like centralspindlin) over long distances within the cytoplasm, and on distinctions that few studies of cytokinesis have made, i.e., to differentiate the role of the aster from the role of other centrosome-associated cellular constituents.

Materials and methods

Eggs and microinjections

Gametes of the purple urchin Strongylocentrotus purpuratus and the sand dollar Dendraster excentricus were obtained either by intracelomic injection of 0.56 M KCl or, for purple urchins, by alternately bouncing then shaking a gravid individual in the palm of the hand. Sperm were collected dry from the aboral surface and kept chilled until use. Purple urchin eggs were shed into a large volume of coarse-filtered seawater (FSW), rinsed twice in FSW, and stored settled at sea-table temperature (11–14°C) until use. Sand dollar eggs were shed into a large volume of FSW and left unrisen at sea-table temperature until use. All echinoderm embryo culture likewise took place at sea-table temperature.

Purple urchin eggs were prepared for injection by dejellying with two rapid washes in calcium/magnesium-free artificial seawater, then returned to FSW in dishes rendered nonsticky by washing with 1% BSA in FSW. Dejellied eggs were used within 2 h. For injections, coverslip-bottom dishes were coated by washing 30 s in 1% proteamine sulfate, rinsing 3x in distilled water, and air drying. Dejellied purple urchin eggs were arranged in a row in an injection dish filled with FSW + 1 mM 3-aminotriazole, which prevents hardening of the vitelline envelope, then fertilized by addition of several urchin sperm. For imaging, purple urchin eggs were dejellied with two washes in FSW, and stored settled at sea-table temperature until use. All echinoderm embryo culture likewise took place at sea-table temperature.

Purple urchin and sand dollar embryos were imaged with a laser scanning confocal microscope (Radiance 2000; Bio-Rad Laboratories) mounted on a compound microscope (E800; Nikon) using a 40x 1.3 NA Plan-Fluor oil lens. Room temperature was maintained at 14–17°C, which is tolerated well by both species. Some experiments were conducted on a spinning disc confocal microscope (CARV; BD) consisting of an inverted microscope (TE2000; Nikon) with a 60x 1.4 NA Plan-Fluor oil lens and a 60x 1.2 NA Plan-Apochromat water lens, the CARV spinning disc head, and a camera (ORCA-ER; Hamamatsu), controlled by MetaMorph software (MDS Analytical Technologies). X. laevis embryos were imaged on a laser scanning confocal microscope (1024; Bio-Rad Laboratories) mounted on an Axiovert microscope (Carl Zeiss, Inc.) using a 63x 1.4 NA Plan-Apochromat oil objective.

Fixation and antibody staining

Embryos were fixed by transferring 200–300 devitellinized sand dollar embryos in 100 μl of seawater into 900 μl of fixative at a final concentration of 2% formaldehyde (from 20% EM-grade stock), 0.2% glutaraldehyde, 0.2% Triton X-100, 100 mM Hepes, pH 7.0, 50 mM EGTA, 10 mM MgSO₄, and 400 mM dextrose. After 1 h in fixative, embryos were rinsed 3x in PB and once in PBS, treated for 2 h with 0.1% sodium borohydride in PBS to reduce autofluorescence, washed once in PBS, then blocked in...
Analysis of Rho zone width

As described previously (Bement et al., 2005), single frames from equivalent stages of furrow ingression were selected from sequences at medial planes within cells expressing GFP-GBD alone or with 3CEMTB. A freehand line selection was drawn along the cell edge and then straightened using ImageJ. Intensities were plotted along a 7-pixel-wide band covering the cell edge, yielding data points (position and intensity) spanning the Rho zone in the furrow. Intensity data were imported into Mathematica (Wolfram Research, Inc.) and fit with a Gaussian curve plus a quadratic equation with small coefficients. The Gaussian curve fits the Rho zone, whereas the quadratic fits the background cortical signal level. The width of the Rho zone was taken as twice the standard deviation of the fit Gaussian curve, which is equivalent to slightly less than the peak width at half maximum height. Total intensity of the Rho zone was taken as the integral under the Gaussian curve from the center plus or minus two standard deviations, minus the integral in the baseline fit over the same region. Zone widths plotted in Fig. 6 B were normalized by the pole-to-pole distance in each measured cell. Significance was judged using the mean difference test in Mathematica.

Creation and analysis of anucleate cytoplasts

Sand dollar eggs were bisected either freehand using a fine glass needle or using a needle mounted on a hanging-joystick oil hydraulic micromanipulator (Kaye Orthopaths Group). Bissection was conducted after pro-nuclear fusion for un injected eggs, or ~45 min after fertilization (and after injection) for injected eggs. Bissection yields half zygotes, which by random chance include some pairs in which one half contains the nucleus and a single centrosome and the other half contains only a centrosome. Such pairs were recognized by differential interference contrast microscopy or by staining live cells with Hoechst 33342. Anucleate, centrosome-containing cytoplasts conduct orderly centrosome duplication and separation on a normal schedule; the nucleated half skips first cleavage, then anucleate cytoplasts. Video 1 shows EMTB-3G in normal purple urchin and sand dollar embryos. Video 2 corresponds to Fig. 5 D: superficial view of EMTB-3G in normal X. laevis blastomere. Video 3 shows nocodazole-treated purple urchin and sand dollar embryos expressing EMTB-3G. Video 4 shows TSA-treated purple urchin and sand dollar embryos expressing EMTB-3G. Video 5 shows untreated and TSA-treated purple urchin embryos expressing EMTB-3G and mCh2B. Video 6 shows anucleate sand dollar cytoplasts expressing EMTB-3G. Video 7 shows sand dollar blastomeres expressing EMTB-3G, in which a single centrosome was ablated in metaphase. Video 8 shows sand dollar embryo expressing EMTB-3G and mCh2B, in which two cells suffered ablation of both centrosomes in metaphase, and two cells experienced ablation of a single pole. Video 9 shows centrosome-ablated sand dollar blastomeres expressing GFP-GBD and 3CEMTB. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200907090/DC1.

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