Central Spindle Self-Organization and Cytokinesis in Artificially Activated Sea Urchin Eggs

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Abstract. The ability of microtubules of the mitotic apparatus to control the positioning and initiation of the cleavage furrow during cytokinesis was first established from studies on early echinoderm embryos. However, the identity of the microtubule population that imparts cytokinetic signaling is unclear. The two main—and not necessarily mutually exclusive—candidates are the central spindle and the astral rays. In the present study, we examined cytokinesis in ammonia-activated sea urchin eggs, which lack paternally derived centrosomes and undergo mitosis mediated by unusual anastral, bipolar mini-spindles. Live cell imaging and immunolabeling for microtubules and the centralspindlin constituent and kinesin-related protein, MKLP1, demonstrated that furrowing in ammonia-activated eggs was associated with aligned arrays of centralspindlin-linked, opposed bundles of antiparallel microtubules. These autonomous, zipper-like arrays were not associated with a mitotic apparatus, but did possess characteristics similar to the central spindle region of control, fertilized embryos. Our results highlight the self-organizing nature of the central spindle region and its ability to induce cytokinesis-like furrowing, even in the absence of a complete mitotic apparatus.

Introduction

The rapidly dividing early embryos of echinoderms have long served as a model system for studying the mechanism and regulation of cytokinesis. A number of landmark studies involving either physical or pharmacological manipulation of echinoderm eggs established the ability of the microtubules of the mitotic apparatus to specify the cleavage plane (reviewed in Rappaport, 1996; Burgess and Chang, 2005). One key discovery was that astral microtubules from two separate mitotic apparatuses of a torus-shaped, binucleate sand dollar embryo were able to signal the formation of a secondary cleavage furrow between them (Rappaport, 1961). Since that time, the formation of these so-called "Rappaport furrows" have also been reported in binucleate cultured cells (Rieder *et al.*, 1997; Savoian *et al.*, 1999; Oegema *et al.*, 2000) and in micromanipulated embryos of *Caenorhabditis elegans* (Baruni *et al.*, 2008). These findings suggest that it was not a phenomenon restricted to echinoderm embryos. Furthermore, they propose that the chromosomes and the non-astral, midzone component of the mitotic apparatus—often referred to as the central spindle—may be unnecessary, and that astral microtubules alone can direct the activation of Rho GTPase (Bement *et al.*, 2005), the master regulator of actomyosin contractile ring assembly (reviewed in Green *et al.*, 2012; Jordan and Canman, 2012).

Although the astral microtubule signaling hypothesis for furrow specification is well supported by a number of studies using echinoderm embryos, it has been interpreted as running counter to the large body of evidence in other

Abbreviations: CPC, chromosomal passenger complex; CYK-4, cytokinesis gene 4; DAPI, 4′,6-diamidino-2-phenylindole; DTT, dithiothreitol; GAP, GTPase-activating protein; INCENP, inner centromere protein; MgcRacGAP, male germ cell Rac GTPase-activating protein; MKLP1, mitotic kinesin-like protein 1; Rho, Ras homolog family member; RhoA, Ras homolog family member A; RhoGEF Ect2, Rho guanine nucleotide exchange factor epithelial cell transforming sequence 2.

experimental systems showing that signals derived from the chromosomes and the central spindle are responsible for Rho activation and subsequent cytokinesis (reviewed in Glotzer, 2009; Green *et al.*, 2012; White and Glotzer, 2012). This central spindle signal model is based on the activity of two key regulatory complexes required for central spindle assembly, Rho regulation, abscission, and, ultimately, successful cytokinesis. These complexes are 1) centralspindlin, consisting of the mitotic kinesin MKLP1 (also known as kinesin 6) and the Rho family GAP CYK-4 (also known as MgcRacGAP); and 2) the chromosomal passenger complex (CPC), consisting of the Aurora B kinase and three additional proteins involved in its localization and activation: INCENP, Survivin, and Borealin. These two complexes interact; CPC-mediated phosphorylation of MKLP1 allows for the accumulation of centralspindlin in the midzone. This concentration of centralspindlin, in turn, recruits the Rho GEF Ect2, which mediates the activation of Rho required for the functioning of the contractile ring that drives furrowing during cytokinesis (Yüce *et al.,* 2005; Green *et al.*, 2012). Despite the preponderance of evidence supporting the central spindle signal model in other systems, the lack of a requirement for central spindle regions and chromosomes for cytokinesis in echinoderm—and *C. elegans* (Baruni *et al.*, 2008)—embryos has led to speculation that central spindle signaling may not be relevant to these large-volume, spherical cells. However, more recent work has indicated that astral microtubules are not required for echinoderm cytokinesis, suggesting that central spindle signaling is taking place, perhaps in a microtubule-independent manner (von Dassow *et al.*, 2009).

In a recent work (Argiros *et al.*, 2012), we attempted to reconcile the astral microtubule and central spindle models in echinoderm embryos by examining the requirement for, and distribution of, the centralspindlin (tracked *via* anti-MKLP1 localization) and CPC (tracked *via* anti-Survivin localization) complexes during early division cycles. We demonstrated that CPC activity (in the form of Aurora kinase function) is required for cytokinesis, and provided the first evidence that CPC and centralspindlin components both accumulate on equatorially aimed and overlapping astral microtubules. We also showed that secondary Rappaport furrows required CPC activity. Among our observations, particularly striking was the accumulation of centralspindlin on antiparallel bundles of overlapping astral microtubules that accompany the ingressing cleavage furrow. This finding raised the possibility that these bundled astral microtubules serve as miniature central spindles, thus overcoming the great distances between the cell surface and the central spindle during the early phases of furrow ingression.

In the present study, we sought to extend our earlier work by examining cytokinesis in sea urchin eggs that were artificially activated *via* cytoplasmic alkalinization induced by ammonia treatment (Epel *et al.*, 1974; Mazia, 1974;

Mazia and Ruby, 1974). Earlier, we showed that these eggs form anastral, bipolar "mini-spindles" that can segregate chromosomes, along with non-spindle-associated microtubule arrays (Henson *et al.*, 2008; see also Paweletz and Mazia, 1979; Bestor and Schatten, 1982; Harris and Clason, 1992). These ammonia-activated eggs lack a paternally derived centrosome (Paweletz and Mazia, 1979; Schatten *et al.*, 1986). While these eggs do not form the classic aster-dominated, bipolar spindles present in fertilized embryos (Henson *et al.*, 2008), we have observed—and others have reported (Brandriff *et al.*, 1975; Harris and Clason, 1992)—cytokinesis-like activity in these cells.

Using live cell and immunofluorescence microscopy, we tested the hypothesis that a central spindle-like organization of microtubules mediated cytokinesis in these ammonia-activated eggs, even in the absence of a canonical, centriole-organized, aster-dominated mitotic apparatus. We demonstrate that furrowing in these activated eggs is associated with highly ordered, aligned arrays of centralspindlin-linked, opposed bundles of antiparallel microtubules that are not associated with a mitotic apparatus, but which do display characteristics similar to the central spindle region of control, fertilized embryos. Our correlative results lend support to the generally held hypothesis in the field (White and Glotzer, 2012) that the centralspindlin-linked tips of bundles of overlapping microtubules can function as central spindles and stimulate cell division. These results also underscore the self-organizing nature of the central-spindle and associated cytokinesis machinery (Bonaccorsi *et al.*, 1998; Mitchison *et al.*, 2013; Nguyen *et al.*, 2014), and suggest that activated eggs can undergo chytokinesis-like furrowing in the apparent absence of astral microtubules.

Materials and Methods

Animals, gametes, and reagents

Sea urchins of the species *Lytechinus pictus* (Verrill, 1867) were purchased from Marinus Biological Supply Company (Long Beach, CA), and kept in either running seawater or closed artificial seawater systems at 10–15 °C. Gametes were collected *via* intracoelomic injection with 0.5 mol l^{-1} KCl, with sperm collected dry and eggs collected in MBL artificial seawater (ASW; 423 mmol l^{-1} NaCl, 9 mmol l^{-1} KCl, 9.27 mmol l^{-1} CaCl$_2$, 22.94 mmol l^{-1} MgCl$_2$, 25.5 mmol l^{-1} MgSO$_4$, 2.14 mmol l^{-1} NaHCO$_3$, pH 8.0), and then dejellied by multiple washing with ASW.

Antibodies used included a mouse monoclonal and a rabbit polyclonal anti-α tubulin (Sigma-Aldrich Chemical Company, St. Louis, MO); MKLP1/KRP-110, a rabbit polyclonal anti-sea urchin kinesin (gift of Dr. Jonathan Scholey, University of California, Davis); a mouse monoclonal antibody against the Ser19 phosphorylated form of myosin II regulatory light chain (Cell Signaling Technology, Danvers, MA); and a rabbit polyclonal anti-actin (Sigma-Aldrich).

Secondary antibodies conjugated to Alexa Fluor 488 and 568 were obtained from Thermo Scientific, Waltham, MA. The majority of the other chemicals and/or reagents used in this study were purchased from Sigma-Aldrich.

Fertilization, ammonia activation, and live cell imaging

Two methods were used to remove the fertilization envelopes of the eggs. In one method, eggs, before fertilization, were exposed to 10–20 mmol l^{-1} dithiothreitol (DTT), then washed in ASW prior to the addition of sperm. In the other method, the fertilization envelopes were removed post-fertilization by passing the zygotes through a Nitex filter (Sefar, Inc., Buffalo, NY). Ammonia activation was performed by adding NH$_4$Cl at concentrations of 5–15 mmol l^{-1} to ASW and raising the pH to 8.5–9.0, using NaOH. Unfertilized eggs were exposed to the ammonia-containing solutions for 15–30 minutes, then washed in either ASW or calcium-free seawater (CFSW; 423 mmol l^{-1} NaCl, 9 mmol l^{-1} KCl, 22.94 mmol l^{-1} MgCl$_2$, 25.5 mmol l^{-1} MgSO$_4$, 2.14 mmol l^{-1} NaHCO$_3$, 1 mmol l^{-1} EGTA, pH 8.0, using NaOH). In some experiments, unfertilized eggs had been previously exposed to DTT prior to treatment in ammonia. Fertilized and activated eggs were allowed to develop at a range of temperatures between 15 and 22 °C, and progress through the cell cycle was monitored using a Nikon (Tokyo, Japan) TS100 Inverted Phase Contrast Microscope or an Olympus (Tokyo, Japan) IX70 inverted relief contrast microscope. Cells undergoing cytokinesis-like furrowing were quantified for comparison between fertilized and activated conditions.

Immunofluorescent localization and imaging

Control embryos and ammonia-activated eggs were allowed to adhere to poly-L-lysine-coated coverslips, fixed in either –20 °C methanol for 5 min (after Harris and Clason, 1992), or 3% formaldehyde (using the method of Wong *et al.,* 1997) for 30 min. Next, they were hydrated in phosphate-buffered saline (PBS), and blocked >2 h in PBS containing 2% goat serum and 1% bovine serum albumin. Blocked eggs were then incubated >2 h to overnight in primary antibodies, followed by a 2-h incubation period in the appropriate fluorophore-conjugated, secondary antibodies, then mounted in ProLong antifade reagent containing 4′,6-diamidino-2-phenylindole (DAPI; Thermo Scientific) in order to stain chromosomes. In some experiments, chromosomes were labeled with TO-PRO-633 (Thermo Scientific). Slides were then viewed using a 60×, 1.4 NA Plan Apo objective lens on either a Nikon E600 conventional epifluorescence microscope or an Olympus FluoView 500 laser scanning confocal microscope. For conventional wide-field microscopy, images were captured using a Photometrics CoolSNAP cf CCD Camera (Photometrics, Tucson, AZ) and processed using ImageJ/Fiji (Schindelin *et al.,*

2012) and Adobe Photoshop (San Jose, CA). To quantify the percentage of activated cells that exhibited furrowing activity and aligned bundles of microtubules, 100 total furrowing eggs were counted for each of 3 separate experiments. From these, the percentage of eggs containing the microtubule array was determined.

Results

Cytokinesis in activated eggs is associated with ordered arrays of aligned microtubule bundles

We previously reported that ammonia-activated eggs undergo a form of mitosis, in which the female pronucleus chromosomes duplicate and condense, the nuclear envelope breaks down, and the chromosomes align and then segregate *via* unusual, anastral "mini-spindles" (Henson *et al.*, 2008). The time to nuclear envelope breakdown in these eggs is typically 30%–50% longer than the time to first division in control, fertilized embryos. In the present study, we focused on observing these activated eggs past the time of mini-spindle formation, and discovered that a subset attempted cytokinesis (Fig. 1). This included a wide spectrum of furrowing responses, such as the formation of unidirectional (Fig. 1A, B) and bidirectional cleavage furrows (Fig. 1A, C, D, F, G); some activated cleaving eggs (Fig. 1D, F) appeared very similar to the fertilized controls. Furrows in the activated eggs often regressed; however, some cells completed either symmetric or asymmetric cleavages. The percentage of cells undergoing some type of furrowing averaged 20% in the ammonia-activated eggs *versus* 96% in the fertilized embryos over the course of 5 separate experiments (Fig. 1E). The percentage of activated cells exhibiting furrowing tended to increase with elevated incubation temperatures (as was reported by Harris and Clason, 1992). The temperature range of 15–21 °C that we used was adopted from Harris and Clason (1992), who established that it represents the normal physiological range for eggs of *Lytechinus pictus*, and avoids the aberrant tubulin polymerization induced by higher temperatures.

Microtubules in control, fertilized embryos undergoing cytokinesis were organized into the expected central spindle plus large asters (Fig. 2A, B). In contrast, cleaving, ammonia-activated eggs displayed significantly altered microtubule morphology, characterized by extensive parallel bundles of opposed, non-astral microtubules (Figs. 2, 3). Both wide-field (Fig. 2C–E) and confocal (Fig. 2F–H) fluorescence microscopy demonstrated that activated eggs undergoing furrowing contained arrays of aligned bundles of microtubules organized in a zipper-like pattern, with the presumed plus ends of the microtubules adjacent to a cleared zone in the middle. This unstained zone was also present in control embryos (Fig. 2A, B), and has been inferred to result from anti-tubulin antibody epitope shielding due to the dense packing of overlapping microtubules
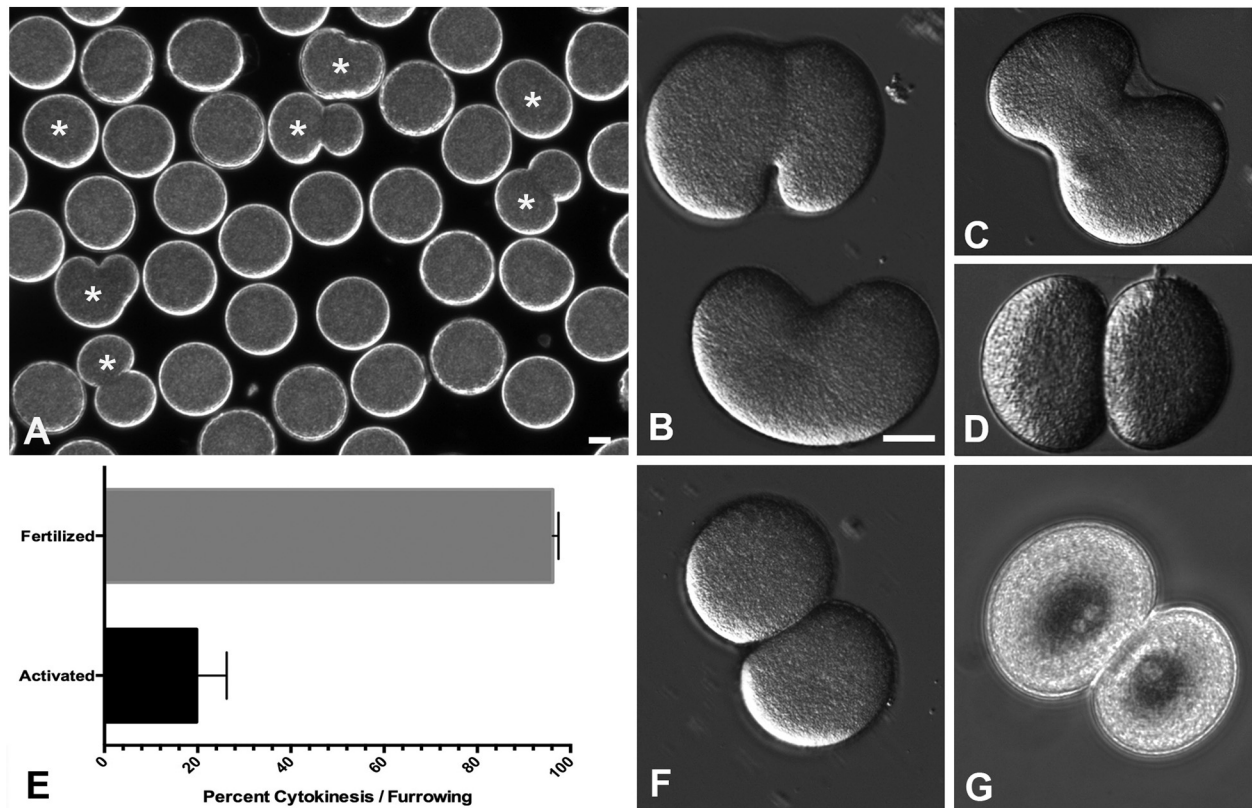
Figure 1. A subset of ammonia-activated eggs undergo a range of cytokinesis-like furrowing. Low-magnification, dark-field image of ammonia-activated eggs showing (A) a subset of cells undergoing some level of constriction/furrowing (eggs marked with *). Activated eggs can display unidirectional (B) and bidirectional furrowing (B)(C) activity. Activated eggs can also display symmetric (D)(F) and asymmetric (B)(C)(G) cleavages, as shown by relief-contrast (B–D)(F) and phase-contrast (G) microscopy. (E) The percentage of activated eggs exhibiting furrowing is in the range of 15%–25%, much lower than the close to 100% cytokinesis seen in control, fertilized embryos. (Note: graph is based on the results of five separate experiments; error bars represent standard deviations.) Scale bar = 20 μm.

complexed with central spindle components (Foe and von Dassow, 2008; Glotzer, 2009).

The pattern of microtubule bundle distribution was particularly striking in maximum-intensity, through-focus projections of confocal Z stacks (stacks of images collected *via* optical sectioning of the specimen in the z-axis) (Fig. 2G). These microtubule arrays in activated eggs were reminiscent of the aligned microtubule bundles present in the central spindle or spindle midzone of control, telophase-stage embryos (Fig. 2A, B), although they were generally more exaggerated and extensive. The presumed minus ends of the microtubule bundles in activated eggs were unfocused and splayed, a characteristic feature of central spindle poles (Glotzer, 2009). These unfocused, central spindle microtubule bundle ends were often some distance away from the reformed nuclei of the "daughter" cells (Fig. 2C–H; Fig. 3C, D)—unlike the close proximity of nuclei and central spindle microtubule minus ends present in control embryos (Fig. 2A).

Our previous work on mitotic spindles in ammonia-activated eggs demonstrated a wide spectrum of microtubule organizations (Henson *et al.*, 2008). In the present study, a similarly broad range of microtubule organizations was observed in activated eggs undergoing cytokinesis-like furrowing (Fig. 3). In some furrowing activated eggs, the centrally aligned microtubule bundles were accompanied by dense, cortical microtubule arrays (Fig. 3A, D), while other eggs had cytasters instead of bundles (Fig. 3F). Quantification of furrowing cells that displayed zipper-like microtubule bundles in 3 separate experiments (graph in Fig. 3B) revealed that a significant majority of constricting cells (mean = 72%) contained this type of microtubule organization. This finding suggests that aligned microtubule bundles are the structure most closely correlated with furrowing activity. Note that this microtubule organization was present in eggs displaying both bidirectional (Fig. 3A, C, D) and unidirectional (Fig. 3E) furrowing. In all of these experiments, nuclear staining indicated that the cells undergoing furrowing typically contained two or more nuclei, showing that some form of chromosome segregation had occurred (Figs. 2, 3).
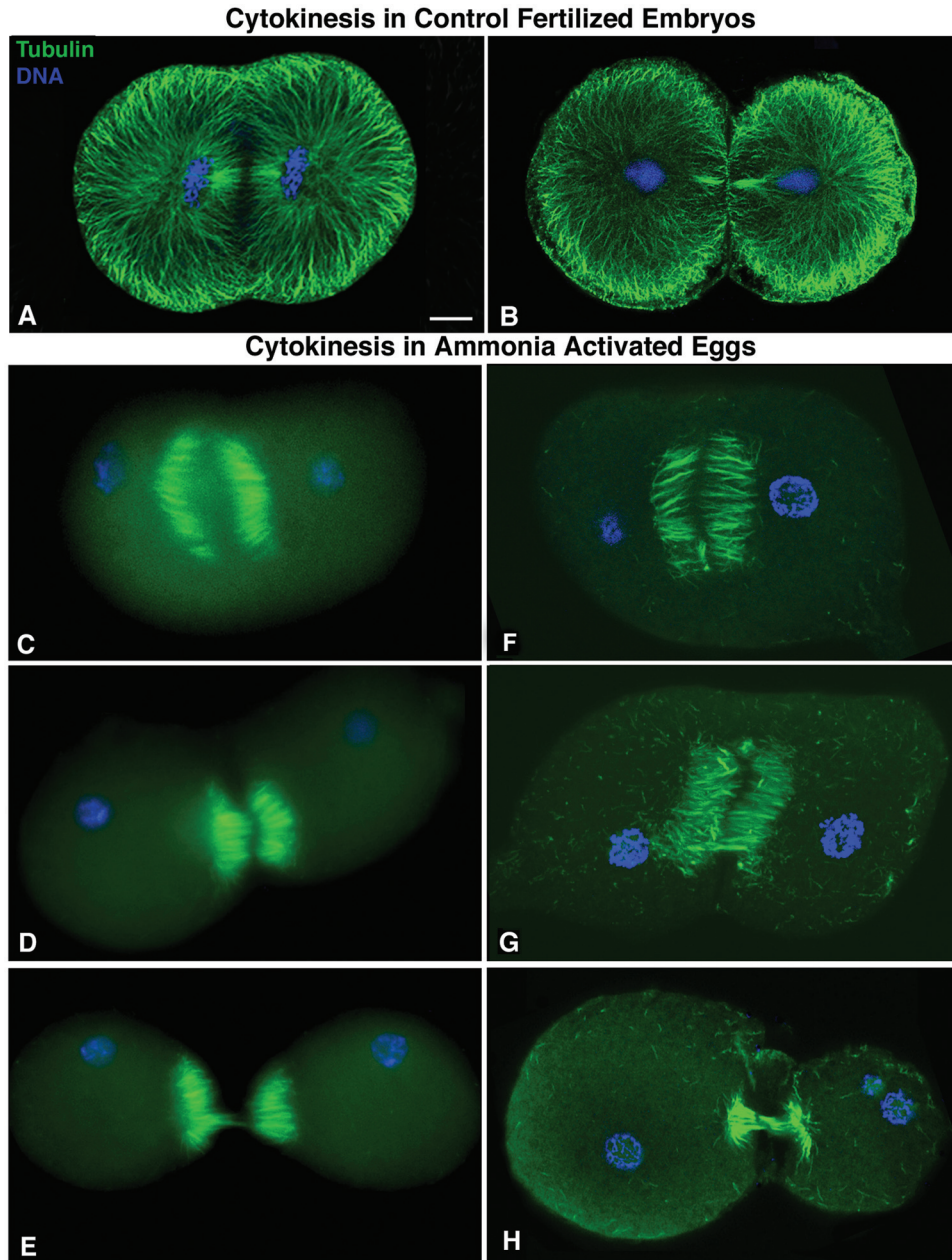
Cytokinesis in Control Fertilized Embryos

Cytokinesis in Ammonia Activated Eggs

Figure 2. Cytokinesis in ammonia-activated eggs is associated with aligned bundles of microtubules. In control, fertilized embryos undergoing cytokinesis (A)(B), confocal imaging shows that the microtubules (green; DNA, blue) of the central spindle are arranged in aligned bundles, and the microtubules of the asters fill the entire cytoplasm. In dividing, activated eggs, both conventional (C–E) and confocal (F–H) imaging show that furrowing is associated with ordered arrays of aligned bundles of microtubules. Note the presence of a cleared zone between the bundles (C)(D)(F)(G) and the complete absence of astral microtubules. (G) is a through-focus projection of a confocal Z stacks, emphasizing the extensive and highly organized nature of these zipper-like microtubule bundles. Note that the aligned bundles present in activated eggs are often far removed from the reformed nuclei. Scale bar = 20 μm.

MKLP1/centralspindlin cross-links aligned microtubule bundles in activated eggs

Centralspindlin, a complex of MKLP1 and CYK-4, is thought to help organize post-anaphase microtubules into the central spindle, and also to provide the scaffold for recruit-ment of the RhoGEF Ect2, which mediates the equatorial activation of RhoA and assembly of the contractile ring (Jantsch-Plunger *et al.*, 2000; Yüce *et al.,* 2005; Zhao and Fang, 2005; Nishimura and Yonemura, 2006; White and Glotzer, 2012). A double-headed Kinesin-6 motor protein,
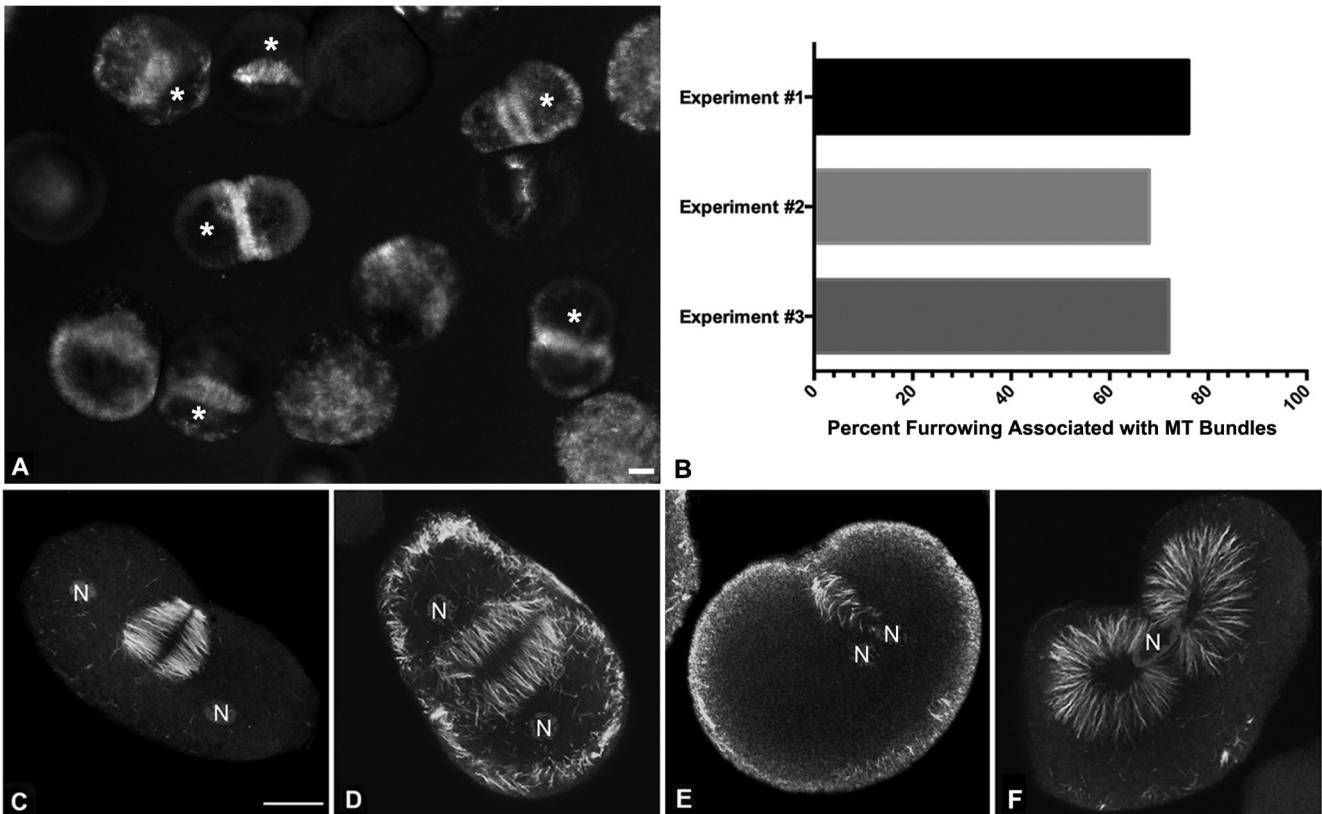
Figure 3. Furrowing ammonia-activated eggs often display aligned bundles of microtubules, although there is a diversity of microtubule patterns. A low-magnification fluorescence micrograph (A) shows that aligned microtubule bundles are associated with a majority of constricting, activated eggs (*). Quantification of the results of 3 separate experiments indicated that this association is present in some 72% of constricting, activated eggs (B). Note that the microtubule bundles present in isolation in some activated cells undergoing cytokinesis-like constriction (C) can be accompanied by a dense array of cortical microtubules (D). In addition, microtubule bundles are often associated with eggs displaying unilateral constrictions (E). Finally, furrow-like constrictions can be found between two opposed cytasters that do not include a central spindle-like region of aligned microtubule bundles (F). The letter N is used to designate the TO-PRO-based labeling of nuclei (C–F). Scale bar = 20 μm.

MKLP1 (also known as KRP110 in sea urchins) associates with antiparallel microtubules and accumulates not only at the spindle midzone, but also along astral microtubule tips at the cell equator (Chui *et al.*, 2000; Henson *et al.*, 2008; Argiros *et al.*, 2012). MKLP1 in fertilized control embryos of *Lytechinus pictus* was associated with the midzone/central spindle starting in anaphase (Fig. 4A). By telophase (Fig. 4B), this central spindle localization was more prominent, and MKLP1 was also present on overlapping astral rays of the mitotic apparatus in the equatorial cortex (Fig. 4B, arrows). As cytokinesis progressed, MKLP1 was found linking the microtubule bundles associated with the ingressing cleavage furrow (Fig. 4C), eventually forming a ring around the midbody (Fig. 4D). We recently reported similar patterns of MKLP1 distribution in the fertilized, first-division embryos of another sea urchin species (*Strongylocentrotus purpuratus*), using a different MKLP1 antibody (Argiros *et al.*, 2012).

Confocal microscopy of sea urchin MKLP1 staining in ammonia-activated eggs indicated that this kinesin-related protein started to accumulate in the midzone region of the bipolar, anastral mini-spindles at metaphase (Fig. 5A). By anaphase, MKLP1 had become localized to the tips of the overlapping bundles of microtubules within the central spindle region equivalent (Fig. 5B). Following the reformation of daughter nuclei, MKLP1 was found to be associated with the central overlap region of zipper-like patterns of aligned microtubule bundles in activated eggs (Fig. 5C). In furrowing eggs with associated microtubule bundles, MKLP1 was localized in the central region of overlap between these bundles (Fig. 5D, E). Localization of sea urchin MKLP1 on the tips of interacting regions of these bundles provided evidence that these zipper-like arrays of aligned bundles consist of anti-parallel microtubules. In addition, it suggested that these bundled arrays were
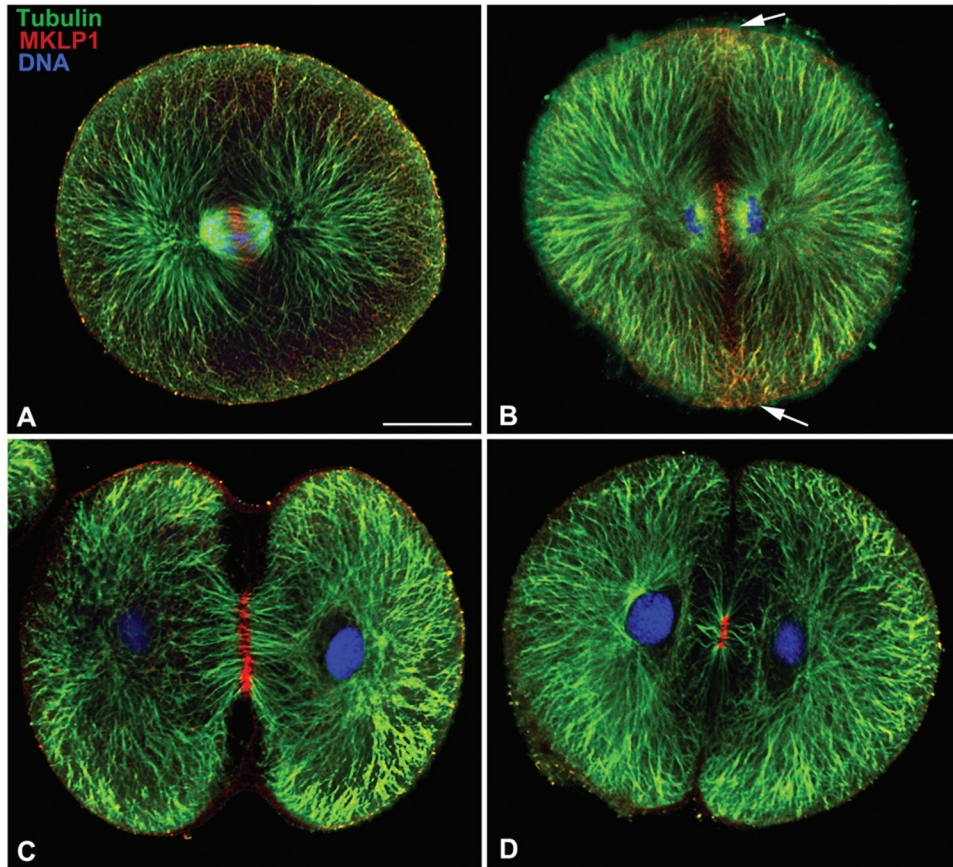
Figure 4. In dividing, fertilized embryos, MKLP1 is associated with regions of overlap between anti-parallel microtubule bundles in the central spindle and the equatorial cortex. In fertilized embryos, MKLP1 (red) is associated with the regions of overlapping microtubule (green; DNA, blue) bundles present in the central spindle starting in anaphase (A). At telophase (B), MKLP1 localizes to the central spindle midzone and to the overlapping bundles of astral microtubules present at the equatorial cortex (arrows). As the furrow ingresses, MKLP1 labeling intensifies in the region of overlapping central spindle microtubules (C), eventually forming a ring surrounding the midbody (D). Scale bar = 20 μm.

organized in a manner analogous to the central spindle observed in control embryos (Fig. 4B, C). Furthermore, using phospho-myosin II staining as a marker for contractile-competent myosin II in the contractile ring, activated eggs demonstrated a ring structure that was associated with the microtubule bundles (Fig. 5F). Activated myosin II in the contractile ring serves as an indicator of active RhoA (Jordan and Canman, 2012), suggesting that the RhoA-based furrowing induction system was being stimulated in activated eggs. In other immunofluorescent staining experiments, we have also demonstrated that actin filaments accumulate in the furrow regions of activated eggs, as expected (data not shown).

Discussion

It has been known for over a century that parthenogenetically activated eggs of marine invertebrates are capable of varying degrees of cleavage and developmental progression (Loeb, 1899; Wilson, 1925; Brandriff *et al.*, 1975). But despite the recent advances in our knowledge of the molecular details of furrow specification, there have been few investigations into cytokinesis in parthenogenetically activated embryos that use aster-based spindle assembly and cleavage plane determination. In the present study, we combined and extended our previous work on the role of centralspindlin in sea urchin embryo cytokinesis (Argiros *et al.*, 2012), and the ability of ammonia-activated sea urchin eggs to undergo assembly of a mitotic spindle (Henson *et al.*, 2008), by examining the process of cytokinesis in activated eggs. We demonstrated that aligned bundles of anti-parallel microtubules are associated with furrow formation in these activated eggs (Figs. 2, 3, 5). The notion that these bundles are functionally and structurally similar to the central spindle was supported by the localization of centralspindlin (Fig. 5). Centralspindlin was present in the dark midzone region between the aligned microtubule bundles (Figs. 2, 3,
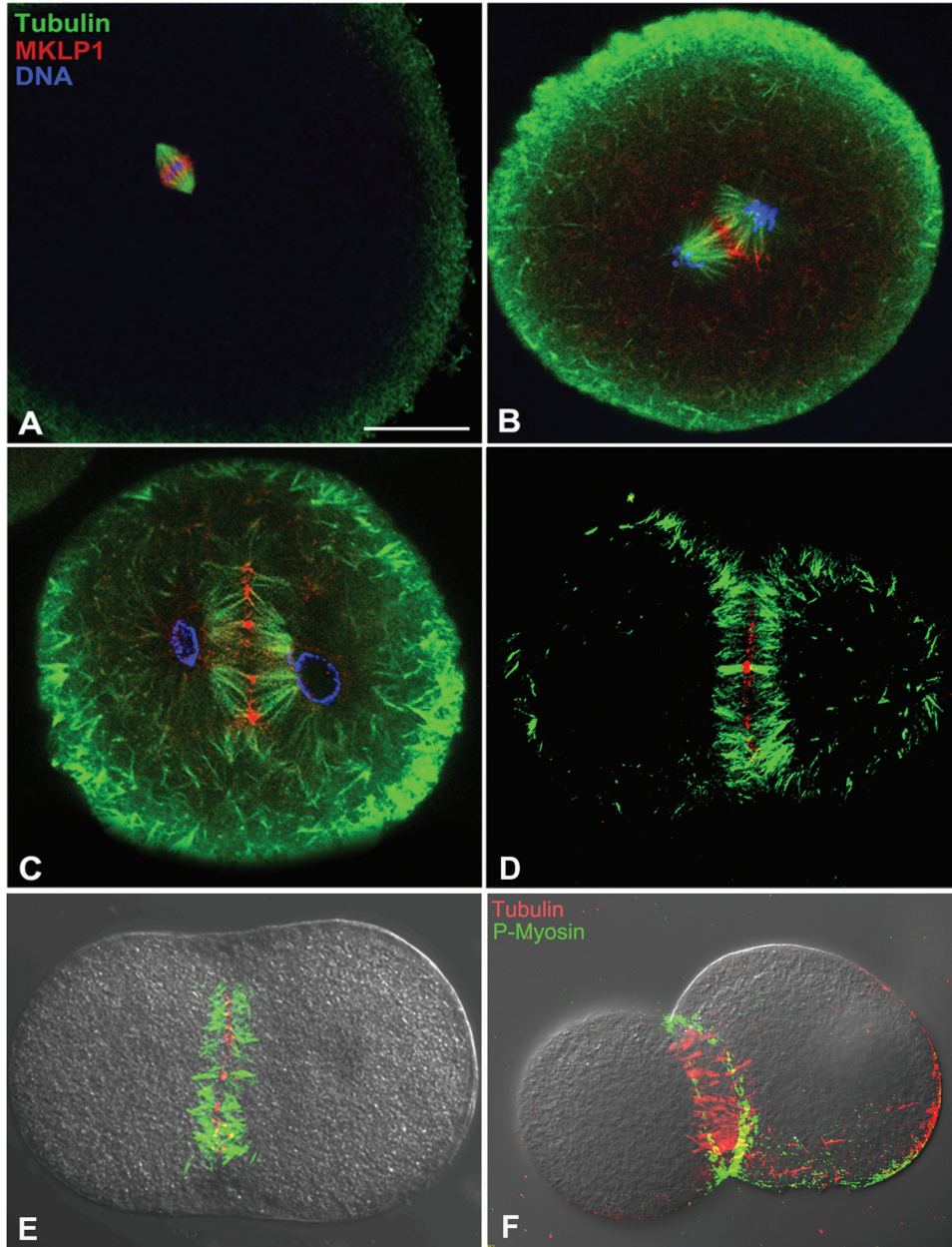
Figure 5. MKLP1 in ammonia-activated eggs is associated with regions of overlap between aligned, anti-parallel microtubule bundles. Confocal imaging shows that in ammonia-activated eggs, (A) MKLP1 (red; microtubules, green; DNA, blue) is localized in the midzone of the anastral mini-spindles at metaphase. (B) By anaphase, MKLP1 is affiliated with the overlapping regions of microtubule bundles in the central spindle region. (C) Following reformation of the nuclei during telophase, MKLP1 remains associated with the microtubule bundle overlap region as the microtubule minus ends become defocused. (D) and (E) In activated eggs exhibiting furrowing activity and zipper-like arrays of aligned microtubule bundles, MKLP1 localizes to the region of bundle overlap. In (E) and (F), a transmitted light differential interference contrast (DIC) image is superimposed on the through-focus series confocal fluorescence image. (F) The actomyosin contractile ring, as labeled *via* activated phosphorylated myosin II (green) staining, is also found in the furrows affiliated with microtubule red zippers in activated eggs. In (F), a transmitted light DIC image is superimposed on the through-focus series confocal fluorescence image. Scale bar = 20 μm.

5), which, in the control central spindles (Figs. 2, 4), resulted from centralspindlin-based epitope shielding in cells stained with anti-tubulin antibodies (Foe and von Dassow, 2008; Glotzer, 2009). These results suggest that the extensive bipolar astral arrays of microtubules that are so prominent in the mitotic apparatus of control, fertilized embryos

(Figs. 2, 4), are not essential in generating furrowing-like activity in ammonia-activated eggs.

The results of our present study support the results of von Dassow *et al.* (2009), who demonstrated that cytokinesis still occurred in control, fertilized sea urchin embryos, in which astral microtubules were reduced by treatment with either the general microtubule depolymerization agent, nocodazole, or a tubulin deacetylase inhibitor that destabilizes dynamic microtubules. However, our results differ from the von Dassow *et al.* (2009) study in that their pharmacological manipulation experiments used blastomeres from 16-cell-stage embryos that still contained traditional centrosome/centriole-organized mitotic spindles. The aligned microtubule bundles in our study were not part of a canonical mitotic apparatus, and the distances from the microtubule arrays to the cortex in our first-division activated eggs were larger than those present in 16-cell-stage blastomeres. Nevertheless, both studies suggest that astral microtubules may not be required for cytokinesis, although the processes in operation are complex; and it is difficult to absolutely rule out the importance of astral microtubules in control embryos, based on the available evidence.

In our earlier study on furrow ingression in control and Rappaport furrows (Argiros *et al.*, 2012), we demonstrated that once furrowing commenced, centralspindlin-bundled overlapping microtubules from opposite asters in the equatorial cortex appeared to function as "miniature central spindles" in cytokinetic signaling, and accompanied the furrow as it ingressed to completion. Other investigators have identified microtubule bundles in the cleavage furrow of sea urchin embryos (Larkin and Danilchik, 2001), and reported that microtubules were essential for cytokinesis completion (Larkin and Danilchik, 1999).

In our present study, we support and extend the miniature central spindle hypothesis by demonstrating that aligned arrays of centralspindlin-bundled microtubules not associated with a normal mitotic apparatus, were capable of performing cytokinetic signaling. In the activated eggs, it is possible that the central spindle-like arrays of microtubules arise *de novo* in a subset of cells; previous studies have suggested that the central spindle is a self-organizing structure. For example, microtubule bundles that resemble central spindles elicit cytokinesis in cells lacking spindle poles/centrosomes and/or chromosomes (Zhang and Nicklas, 1996; Canman *et al.*, 2000; Alsop and Zhang, 2003; Bucciarelli *et al.*, 2003), and also in the region of overlap between centrosomes from separate spindles within a single cell (Savoian *et al.*, 1999; Baruni *et al.,* 2008).

In addition, earlier work on *asterless* mutant *Drosophila* spermatocytes (Bonaccorsi *et al.*, 1998), and recent studies in *Xenopus* cell-free extracts (Mitchison *et al.*, 2013; Nguyen *et al.*, 2014) indicated that functional central spindles can self-organize and mediate cytokinetic signaling. It is likely that a similar phenomenon is occurring in activated sea urchin eggs. Finally, another recent study showed that a pool of chromosomal passenger complex (CPC)-regulated and membrane-bound centralspindlin can activate RhoA at the ingression site *via* oligomerization (Basant *et al.*, 2015). This mechanism may help to explain how furrowing can be initiated in activated eggs that lack astral microtubules.

The relatively low percentage of activated eggs observed undergoing cytokinesis in our study was probably a function of the wide spectrum of microtubule arrays present in the cells (Fig. 3). The ability to induce (Alsop and Zhang, 2003) and complete (Larkin and Danilchik, 1999) cytokinesis is dependent on the extent and appropriate organization of the microtubules present. We earlier showed that only a low number of eggs actually complete mitosis-like chromosome segregation following ammonia activation (Henson *et al.*, 2008), which may explain why successful division was rare in the activated eggs. Finally, furrow initiation appears to be more robust than ingression and abscission, as only a small subset of the activated eggs that showed furrow activity tended to complete cytokinesis. Our previous work has shown that furrow initiation is resistant to the application of CPC inhibitors, implying that alternative redundant mechanisms must exist for the early events of furrow establishment (Argiros *et al.*, 2012).

In summary, our results indicate that aligned arrays of microtubule bundles, cross-linked by centralspindlin and not associated with a normal mitotic apparatus, can mediate a cytokinesis-like process within ammonia-activated sea urchin eggs, providing a potential mechanism by which these parthenogenetically activated eggs may proceed through divisions. These results also suggest that, while the asters of the mitotic apparatus can help shape the location and extent of the cleavage furrow (Foe and von Dassow, 2008; von Dassow *et al.*, 2009), and augment cytokinesis signaling in control fertilized embryos by acting as cortical, central spindle-like structures (Argiros *et al.*, 2012), they may not be required for carrying the cytokinetic signal to the cortex in ammonia-activated eggs.

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