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Centralspindlin and Chromosomal Passenger Complex Behavior During Normal and Rappaport Furrow Specification in Echinoderm Embryos

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Abstract

The chromosomal passenger (CPC) and Centralspindlin complexes are essential for organizing the anaphase central spindle and providing cues that position the cytokinetic furrow between daughter nuclei. However, echinoderm zygotes are also capable of forming “Rappaport furrows” between asters positioned back-to-back without intervening chromosomes. To understand how these complexes contribute to normal and Rappaport furrow formation, we studied the localization patterns of Survivin and mitotic-kinesin-like-protein1 (MKLP1), members respectively of the CPC and the Centralspindlin complex, and the effect of CPC inhibition on cleavage in mono- and binucleate echinoderm zygotes. In zygotes, Survivin initially localized to metaphase chromosomes, upon anaphase onset relocalized to the central spindle and then, together with MKLP1 spread towards the equatorial cortex in an Aurora-dependent manner. Inhibition of Aurora kinase activity resulted in disruption of central spindle organization and furrow regression, although astral microtubule elongation and furrow initiation were normal. In binucleate cells containing two parallel spindles MKLP1 and Survivin localized to the plane of the former metaphase plate, but were not observed in the secondary cleavage plane formed between unrelated spindle poles, except when chromosomes were abnormally present there. However, the secondary furrow was sensitive to Aurora inhibition, indicating that Aurora kinase may still contribute to furrow ingression without chromosomes nearby. Our results provide insights that reconcile classic micromanipulation studies with current molecular understanding of furrow specification in animal cells.

Keywords

cytokinesis; mitotic spindle; sea urchin; microtubules; MKLP1; chromosomal passenger complex; centralspindlin; cleavage plane determination

Introduction

The classic micromanipulation experiments performed by Rappaport helped define the basic rules by which animal cells determine their future cleavage plane, and framed hypotheses regarding the basic cytokinetic mechanism still debated today [Rappaport, 1996; Pollard,

2004]. In the decades since Rappaport's seminal torus experiment [Rappaport, 1961], tremendous progress has been made in identifying the components of the contractile ring and the molecular players that specify the spatial and temporal activation of the small GTPase, RhoA, the master regulator of contractile ring assembly and furrow constriction [Glotzer, 2005; D'Avino and Glover, 2009]. Yet, despite these advances, there has not been a full reconciliation between current mechanistic models of cleavage plane specification and the classic experiments that originally captured the imagination of investigators in this field.

Following anaphase onset, the mitotic apparatus (MA) plays an essential role in positioning the cleavage plane and orchestrating the events of furrow initiation, ingression and abscission. Rappaport's elegant manipulations of the geometric relationship between the MA and the cell cortex provided experimental evidence for the existence of a positive cue, emanating from astral microtubules, that stimulates contraction [Rappaport, 1996]. On the other hand, manipulations carried out on much smaller cultured cells suggested that the chromosomes and microtubules of the anaphase central spindle were the source of the a stimulus responsible for determining the cleavage plane [Kawamura, 1977; Cao and Wang, 1996; Wheatley and Wang, 1996; Eckley et al., 1997], although studies using heterokaryons appeared to replicate the results of Rappaport's toroid cells [Rieder et al., 1997; Savoian et al., 1999; Oegema et al., 2000]. These studies left in dispute whether different populations of microtubules were deploying fundamentally different signals, or whether cell size dictated that different microtubule populations delivered the same stimulus [Wang, 2001].

Key to cytokinetic furrow formation is the localized activation of RhoA at the cortex, which in turn stimulates cortical contractility [for studies in echinoderms see Bement et al., 2005; Foe and von Dassow, 2008]. RhoA activation has been studied in detail, and a proposed model links chromosomes and the central spindle with equatorial activation of Rho [McCollum, 2004; Glotzer, 2005, 2009, 2010]. Central to this model is the recruitment of the Centralspindlin complex, consisting of mitotic- kinesin-like-protein1 (MKLP1) and MgcRacGap, to the central spindle. Centralspindlin not only organizes antiparallel microtubules [Mishima et al., 2002], but also recruits Ect2 protein [Yuce et al., 2005; Zhao and Fang, 2005; Nishimura and Yonemura, 2006]. Ect2 is thought to be the major RhoGEF mediating Rho activation during cytokinesis, thus suggesting a causal link between central spindle formation and the initiation of cortical contractility at the cell equator [Lehner, 1992; Tatsumoto et al., 1999; Kimura et al., 2000; O'Keefe et al., 2001; Somers and Saint, 2003; Yuce et al., 2005; Nishimura and Yonemura, 2006]. Another player key to the central spindle model for furrow specification is the chromosomal passenger complex (CPC; composed of Aurora B kinase, Inner Centromere Protein (INCENP), Survivin and Borealin), which is essential for both central spindle organization and cytokinesis [Ruchaud et al., 2007]. Although the CPC has additional targets, control of central spindle formation is thought to be mediated by the CPC-dependent phosphorylation of MKLP1 [Guse et al., 2005].

While the central spindle model suggests an appealing way for the cleavage plane to be positioned in the plane of the former metaphase plate in small diameter cells, where the cortex is immediately adjacent to the spindle midzone, it is unclear how this model would work in cells where chromosomes are either dispensable for cytokinesis, or are far from the cortex. Rappaport's experiments that concluded the furrow-stimulating cue emanates from opposed astral microtubule arrays were conducted in large echinoderm eggs where the distance between the cortex is tens of microns away from chromosomes and the central spindle [Rappaport, 1961, 1996]. More recent studies on grasshopper spermatocytes, echinoderm eggs and nematode embryos likewise show furrows forming far from chromosomes and the central spindle [Zhang and Nicklas, 1996; Shuster and Burgess, 2002b; Baruni et al., 2008; von Dassow et al., 2009]. Given that microtubules are essential

for the localized RhoA activation that drives furrowing [Bement et al., 2005], a motor-dependent mechanism for relaying the signal from the central spindle to the equatorial cortex is an attractive, though as yet unproven, candidate for Rappaport's elusive "cleavage stimulus."

To date there has been little analysis of the CPC and Centralspindlin in echinoderms. KRP110, the urchin homolog of MKLP1, has been identified and biochemically characterized [Chui et al., 2000], and microinjection of antibodies against CHO1 antigen disrupt cell division [Wright et al., 1993] in sea urchin embryos. In contrast to many other animal cells, Echinoderms have a single Aurora kinase, which localizes to the spindle poles as well as to metaphase centromeres and the anaphase central spindle, and therefore might assume functions that in other cell types are split between Aurora A and B [Abe et al., 2010]. While little is known about the behavior of CPC and Centralspindlin during normal cleavage in echinoderms, nothing at all is known about how these complexes behave under conditions analogous to those in Rappaport's toroid cells.

For over a century, the large size and optical clarity of the echinoderm egg has made it a popular experimental system for studying cytokinesis and in this report, we describe the localization dynamics of the CPC and Centralspindlin complexes in dividing echinoderm zygotes. We found that, as in other cell types, the CPC (visualized by Survivin localization) associated with chromosomes up until anaphase onset, at which time it relocated to the central spindle and then to equatorially aimed astral microtubules. During anaphase Centralspindlin (visualized by MKLP1 localization) was initially recruited to the central spindle and later to equatorially aimed astral microtubules, eventually concentrating on discreet anti-parallel microtubule bundles beneath the ingressing furrow. CPC signaling was required for equatorial microtubule organization, Centralspindlin localization and cytokinesis. Lastly, generation of binucleate eggs (which mimic the torus experiment by creating opposed astral arrays with and without chromosomes between them) revealed neither the CPC nor Centralspindlin complexes in the secondary (Rappaport) cleavage plane, yet secondary furrow ingression still required Aurora kinase activity. Our results provide new information on how, in large embryonic cells, chromosomal factors originating on the chromosomes and central spindle can act over long distances to induce localized cortical contractility and furrow ingression.

Results

Cytokinesis in Sea Urchin Zygotes Requires CPC Signaling

In contrast to other metazoans, echinoderms, ascidians and hemichordates possess only a single Aurora kinase, with homology to both Aurora A and B (Fig. 1A, Sup-F1 porting Information). Echinoderm Aurora binds INCENP and is capable of rescuing an Aurora B siRNA depletion in cultured cells [Abe et al., 2010]. In an effort to characterize the role of the CPC in cleavage plane establishment, we examined the sensitivity of sea urchin embryos to small molecule inhibitors of Aurora kinases. Recombinant *Lytechinus variegatus* Aurora phosphorylated histone H3 on Serine 10, and was sensitive to the Aurora inhibitor VX-680 (Fig. 1A), and treatment of eggs with VX-680 blocked serine 10 phosphorylation of histone H3 and cytokinesis (Figs. 1B–1D), with a half-maximal effective concentration EC₅₀ of 32 μ M. Timelapse imaging of eggs treated with 50 μ M VX-680 revealed that, in contrast to DMSO carrier controls, only 13% of cells successfully completed cytokinesis, with 44% undergoing furrow regression and 39% failing to initiate furrowing (Figs. 1C and 1D). Other Aurora drugs, such as Hesperadin and ZM 447439 also blocked cytokinesis with an EC₅₀ of 0.62 μ M, and 1.4 μ M respectively. However, VX-680 displayed the most consistent solubility and stability in seawater and therefore was used for all experiments described herein.

Histone H3 phosphorylation on serine 10 is an established marker for Aurora B kinase activity in a wide variety of organisms [Giet and Glover, 2001; Crosio et al., 2002; Goto et al., 2002; Ota et al., 2002; George et al., 2006], as is the organization of the central spindle through Aurora regulation of MKLP1 [Severson et al., 2000; Guse et al., 2005]. To verify that VX-680 treatment affected these processes in echinoderm zygotes, cells were treated with either DMSO carrier control or Aurora inhibitors and probed for chromatin and microtubule organization (Fig. 2). As detected by immunofluorescent F2 staining (Fig. 2, panels A–I) VX-680 treatment immediately after nuclear envelope breakdown blocked histone H3 phosphorylation without affecting mitotic entry or spindle assembly, although aberrant chromosome segregation was observed in some eggs (Fig. 2, panels G–I). However, postmetaphase microtubule organization was disrupted in Aurora-inhibited cells (Fig. 2, panels J–O): in VX-680-treated *Dendraster excentricus* zygotes, the normal anaphase swelling of the spindle poles did not occur (compare Fig. 2 panels J, L and N with panels K, M and O); the anaphase central spindle formed poorly if at all (Fig. 2 panels J vs. K); the dark zone (where epitope shielding prevents microtubule staining) that develops in the future cleavage plane did not appear or was greatly reduced (compare Fig. 2 panels L and N with panels M and O); in VX-680-treated cells astral microtubules extended rampantly across the cell midline, which does not occur in controls (compare Fig. 2 panels L and N with panels M and O). Finally, three-dimensional reconstructions of furrowing zygotes revealed that controls formed highly organized microtubule bundles beneath the ingressing furrow (data shown below), which Aurora-inhibited cells did not (data not shown). Thus, while cells were capable of initiating cleavage furrow formation in the presence of Aurora inhibitors, microtubule organization in the equatorial plane was clearly affected by compromised CPC activity.

CPC and Centralspindlin Organization in Dividing Sea Urchin Zygotes

To further examine how the CPC contributes to cleavage plane establishment in large embryonic cells, we developed antibody probes for Survivin and MKLP1, members respectively of the chromosomal passenger and Central-spindlin complexes. Immunolocalization with rabbit antibodies produced against full-length *S. purpuratus* Survivin revealed that Survivin localized to the chromosomes at metaphase (Fig. 3, panels A–C) and immediately upon F3 anaphase onset, migrated from the kinetochores to the central spindle (Fig. 3, panels D–I). Additionally, Survivin could be observed on astral microtubules adjacent to, but outside of the midzone microtubule bundle (Fig. 3, panels E and H). Finally, as cells completed cytokinesis, Survivin was associated with the forming midbody (Fig. 3, J–L). The components of the CPC are interdependent on each other for localization and function [Honda et al., 2003], and treatment of cells with VX-680 resulted in a large-scale loss of Survivin from anaphase microtubules (Fig. 2, panels G–O, Supporting Information). Survivin staining, which normally transfers from the metaphase chromosomes to the central spindle at anaphase, in VX-680 treated cells could still be detected early in anaphase as small puncta associated with chromosomes (Fig. 2, Panel H, Supporting Information).

Micromanipulation of echinoderm cell geometry implicated astral microtubules as the predominant influence on cleavage plane determination [Rappaport, 1996], but perforation studies by Rappaport suggested that the central spindle may also stimulate furrowing [Rappaport and Rappaport, 1974]. Our observations, as described above, show that the organization of central spindle microtubules, astral microtubules and furrow-associated microtubules bundles were all affected by CPC activity in echinoderm zygotes (Fig. 2 panels J–O, and Fig. 2, panels G–O, Supporting Information). In light of this, we sought to characterize one of the principle organizers of the central spindle, the Centralspindlin complex, comprising MKLP1 and MgcRacGAP [Glotzer, 2009; Douglas and Mishima,

2010; Hornick et al., 2010]. Immunolocalization of MKLP1 using a peptide antibody generated against a C-terminal epitope of *S. purpuratus* MKLP1 (also known as KRP110 in sea urchins) [Chui et al., 2000] revealed that MKLP1 localized not only to the central spindle but as anaphase progressed, it also concentrated along the lengths and then on the tips of astral microtubules aimed towards the equatorial cell cortex (Fig. 4, panels A–I). By the time the zygote had commenced furrowing, MKLP1 localization was largely restricted to two populations of microtubules: the spindle midzone and microtubule bundles associated with the ingressing furrow (Fig. 4, panels J–L and Fig. 5, panels F5 A–F). These bundles underlying the furrow were especially conspicuous in long-axis views of dividing cells, where each discrete microtubule bundle was heavily labeled with MKLP1 antibodies (Fig. 5, panels G–L). When furrowing was near completion, Survivin and MKLP1 distribution in echinoderm zygotes closely resembled midbody organization in cultured cells [Hu et al., 2012], with MKLP1 concentrated in the center of the midbody bundle while Survivin was detected throughout the central spindle remnant (Fig. 3, Supporting Information).

One of the principle mechanisms by which the CPC is thought to regulate central spindle assembly is through Aurora B phosphorylation of MKLP1 following anaphase onset [Severson et al., 2000; Guse et al., 2005; Douglas et al., 2010]. To determine whether sea urchin MKLP1 localization was dependent on Aurora kinase activity, we analyzed MKLP1 localization in embryos treated with the F6 Aurora kinase inhibitor, VX-680 (Fig. 6). Inhibition of Aurora signaling disrupted microtubule organization (as previously shown in Fig. 2 and Fig. 2, Supporting Information), and central spindle- and astral microtubule-associated MKLP1 observed in controls (Fig. 6 panels A–C) was completely lost in VX-680 treated cells (Fig. 6, panels E, H and K). In summary, chromosome passenger complex-based organization of the central spindle appeared to be the same in large echinoderm zygotes as in small cultured cells, with previously undescribed additional effects on the organization of these zygotes' large astral microtubule arrays.

Assessment of CPC-Centralspindlin in Midzone-Independent Furrowing

Inhibition of CPC signaling in sea urchin embryos resulted in disorganization of cleavage plane microtubules, loss of MKLP1 recruitment throughout the cleavage plane, and cleavage failure (Figs. 1–3, 6 and Supporting Information Fig. 2). Large echinoderm cells are capable of establishing a secondary cleavage plane between asters even when the cleavage furrow does not intersect a prior metaphase plate or a central spindle [Rappaport, 1961; Hiramoto, 1971; Salmon and Wolniak, 1990; Shuster and Burgess, 2002a], a finding in apparent conflict with a strict central spindle furrow specification model. To cast new light on this puzzling observation, we investigated CPC and Centralspindlin localization in cells where a cleavage furrow is induced between the two poles of independent spindles. To create a condition similar to the famous torus experiment, we took advantage of the temperature sensitivity of microtubules to create binucleate cells. Using a method similar to that described by Wilson [Wilson, 1925], eggs were transferred into ice-cold seawater at anaphase onset, which resulted in astral microtubule collapse and cleavage failure, although chromosome segregation proceeded normally producing binucleate cells. Once eggs were returned to normal culture temperatures, these binucleate cells resumed their second cell cycle and entered mitosis with two parallel spindles F7 (Fig. 7). During anaphase furrows formed not only in the plane of the former metaphase plates (the primary cleavage plane), but also in a secondary plane between the two parallel spindles (Fig. 7, panel A and Movie 2, Supporting Information). Timelapse analysis of furrow formation in binucleate cells revealed that, while 97% of primary furrows completed furrowing, 54% of secondary furrows ("Rappaport" furrows) ingressed completely and 44.7% regressed (Fig. 7B). Examination of the microtubule organization within these two cleavage planes revealed two

distinct populations of microtubules (Fig. 7C). A dense array of postanaphase microtubules bisected by a dark zone characteristic of central spindle arrays formed in the plane of the presumptive primary furrow (Fig. 7C, panels a and a'). In contrast, the overlapping antiparallel astral microtubules in the secondary cleavage plane lacked this characteristic central spindle organization (Fig. 7C, panels b and b'). Examination of Serine 19-phosphorylated myosin regulatory light chain (P-MLC) in these cells also revealed differences between the primary and secondary cleavage furrows (Fig. 7D). While activated myosin II could be detected in both cleavage furrows (Fig. 7D, panels b and e), P-MLC staining intensity was 4.6 ± 0.3 fold higher in the primary cleavage furrow than in the secondary furrow. Thus, while secondary furrows were capable of forming in the absence of a well-formed central spindle, there were marked differences in the cytoskeleton in the two cleavage planes, and in the ability of these furrows to ingress to completion.

To investigate CPC and Centralspindlin distribution during primary *versus* secondary furrow formation, binucleate embryos generated by cold-shock were cultured to mitosis of their second cell cycle, fixed and probed for Survivin and MKLP1 localization. Survivin localized to the spindle midzone in the primary cleavage plane F8 (Fig. 8, panels A–H) and could be detected on microtubule tips that extend outward from the central spindle toward the cortex and on microtubules extending between the two parallel central spindles (Fig. 8, panel G). As furrowing progressed, Survivin continued to be visible in the primary cleavage plane, on the microtubules that underlie the furrow and in the forming midbody (Fig. 8, panel K, and Fig. 4, panels G–I, Supporting Information). By contrast, in 66% of binucleate cells Survivin could not be detected in the secondary cleavage plane either prior to (Fig. 8, panels A–H) or following furrow initiation (Fig. 8, panels I–L, arrowhead; and Fig. 4, panels G–I, Supporting Information), and the MKLP1 localization pattern was similar (Fig. 4, panels A–F, Supporting Information). However, in 34% of binucleate cells, the spindles formed in sufficiently close proximity that chromosomes were trapped between the two parallel spindles at metaphase (Fig. 9, panels A–D). Under these conditions, segregating chromosomes were arranged in a distinctive “L” configuration and nuclei reformed in a manner (Fig. 9, panels E and I) that was easily distinguishable from cells where chromosomes were confined to their respective spindles (Fig. 8, panels A, E and I). Under these circumstances, Survivin could easily be observed in both planes of division (Fig. 9, panels C, G and K) indicating that the prior position of chromosomes strictly dictated where the CPC concentrates following mitotic exit.

The lack of visible CPC and Centralspindlin recruitment to the secondary furrow plane suggested that furrow initiation can occur in the secondary plane independent of positional information from the chromosomes. However, in the presence of VX-680, 92% ($n = 60$ cells scored over 10 experiments) of secondary furrows followed by timelapse microscopy regressed in comparison to controls, where only 44.7% of secondary furrows regressed (Movie 3, Supporting Information). Thus, while the CPC and Centralspindlin factors were not detectable in secondary furrows, CPC activity appeared to be required for successful furrowing.

Discussion

The classic experiments performed by Ray Rappaport on echinoderm cells are revered for providing the foundation for a mechanistic understanding of cleavage plane specification. His micromanipulation experiments gave rise to the proposal that antiparallel (astral) microtubule arrays impart stimulatory positional cues that induce the cortical cytoskeleton to form a contractile ring [Rappaport, 1961, 1985, 1996]. While the central spindle model for furrow specification can be thought of as a modification of Ray's hypothesis, the apparent requirement for chromosomal factors runs somewhat counter to Rappaport's original

proposition. Our own examination of Survivin localization and the sensitivity of furrow ingression to Aurora inhibition revealed that indeed, the CPC is required for cytokinesis in the echinoderm embryo, and our observation of MKLP1 accumulation on the tips of equatorially aimed astral microtubules, coincident with furrow initiation, leaves little doubt that Central-spindlin is a component of this signaling apparatus. Moreover, we observe that overlapping astral microtubules form bundles coated with MKLP1 all around the equatorial circumference in association with the ingressing furrow. We think these Centralspindlin-coated bundles function as miniature central spindles (Fig. 4, panels J–L and Fig. 5, panels G–L) and find that their formation depends on CPC activity. And while MKLP1 and Survivin could not be directly detected in secondary or “Rappaport” furrows, these furrows still required Aurora kinase activity, just as primary furrow formation does. These results provide for the first time a possible explanation of how Rappaport’s classical experiments may be reconciled with the central spindle furrow specification model and with our current molecular understanding of furrow induction.

Asters and Central Spindles in Large Embryonic Cells

Micromanipulation experiments on cultured cells identified the central spindle as being the population of microtubules most critical for furrow induction in small somatic cells [Cao and Wang, 1996; Wheatley and Wang, 1996; Eckley et al., 1997; Wheatley et al., 1998; Wang, 2001]. This contrasted with work by Rappaport, Hiramoto, Zhang and others, which implicated astral microtubules tips remote from chromosomes and the central spindle in furrow stimulation [Rappaport, 1961; Hiramoto, 1971; Zhang and Nicklas, 1996; Shuster and Burgess, 2002a; Baruni et al., 2008; von Dassow et al., 2009]. The stark differences in geometries between somatic cells and large echinoderm eggs could necessitate altogether different signaling mechanisms in cells of radically different size. Alternatively, astral microtubule-based signaling in large cells might simply involve the mobilization of the CPC and Centralspindlin complexes over long distances, and our data support this second proposition. In addition to localizing to the central spindle, MKLP1 could be clearly observed along the length and concentrating at the tips of equatorially aimed astral microtubules as these elongated towards the cell cortex (Fig. 4, panels B, E and H). Both MKLP1 and Aurora B localize along astral microtubules in cultured cells [Murata-Hori and Wang, 2002; Nishimura and Yonemura, 2006], and thus CPC/Centralspindlin signaling in echinoderm zygotes may be mechanistically identical to somatic cells, differing only by the scale of the distances across which they must convey spatial information. During furrow ingression, MKLP1 was incorporated into multiple furrow-associated bundles that were spatially distinct from the central spindle (Fig. 4, panels J–L and Fig. 5, panels G–L). The observation that formation of both the central spindle and the cortical microtubule bundles were dependent on CPC activity (Figs. 2 and 6), and that both structures greatly concentrate MKLP1 (Figs. 4 and 5) lends credence to the idea that, as regards furrow induction, the cortical bundles function like midzone bundles, overcoming the vast distances between the central spindle proper and the cell cortex in large cells.

Treating echinoderm zygotes with high concentrations of nocodazole has revealed a subpopulation of stable astral microtubules whose tips aim towards the future furrow [Odell and Foe, 2008] and agent-based computer modeling predicts a greater accumulation of MKLP1 on stable microtubules than on dynamic ones [Odell and Foe, 2008]. INCENP directly binds tubulin [Wheatley et al., 2001], and Survivin has also been implicated in regulating microtubule dynamics [Giardini et al., 2002; Rosa et al., 2006], raising the possibility the CPC may preferentially stabilize microtubules growing in the plane of the former metaphase plate. Thus, in addition to its direct role in regulating MKLP1 [Guse et al., 2005], the CPC may preferentially stabilize microtubules in the plane of the former

metaphase plate, and by this means also promote the accumulation of MKLP1 at the equator.

While CPC/Centralspindlin/Ect2 signaling may be the primary means of inducing furrow formation in sea urchin embryos, furrow initiation still occurred in the presence of Aurora inhibitors (Figs. 1, 2 and 6, Supporting Information Movies 1 and 3), and thus alternative, redundant, mechanisms may be involved in the early events of cytokinesis. It is quite possible that dynamic astral microtubules spatially restrict the zone of RhoA-dependent contractility through other as yet unidentified players [Lucero et al., 2006; Motegi et al., 2006; Werner et al., 2007; Chen et al., 2008; Murthy and Wadsworth, 2008]. Identifying these mechanisms will be essential for developing a comprehensive model for cleavage plane determination in animal cells.

Primary and Secondary Furrows in Binucleate Cells

To determine how CPC/Centralspindlin signaling may function in Rappaport furrows, we employed a simple technique dating back to the turn of the 20th century to generate eggs containing two parallel spindles [Wilson, 1925]. In a manner similar to Rappaport's toroid eggs, binucleate eggs demonstrated that cleavage furrows could form between asters whether or not central spindles and chromosomes occurred between these asters (Fig. 7 and Supporting Information Movie 2). However, to our surprise, neither marker for the CPC or Centralspindlin, so visible in the primary cleavage plane, was evident in the secondary cleavage plane (Fig. 8 and Fig. 4, Supporting Information). The exception to this observation was when chromosomes segregated improperly and were shared between closely apposed, parallel spindles (Fig. 9). Studies of heterokaryons in cultured mammalian cells have demonstrated that furrows may form between two poles of unrelated spindles [Rieder et al., 1997; Savoian et al., 1999; Oegema et al., 2000], but in contrast to our results, components of the CPC and Centralspindlin proteins localized to these secondary furrows [Savoian et al., 1999]. It is unclear if this implies significant cell type differences or, as we think more likely, occurs because the distances between neighboring spindles in these cultured cells were small enough to allow recruitment of these protein factors from the central spindle to the secondary furrow plane. The observation that Survivin could be detected in secondary furrows in echinoderm cells if there were (mis-segregated) chromosomes between the two spindles argues that chromosome proximity does strongly influence where the CPC concentrates following metaphase exit (Fig. 9). Our studies of binucleate cells cannot exclude the possibility that trace amounts of CPC or MKLP1 protein, undetectable by immunofluorescence, are being recruited to the secondary furrows. Indeed, the sensitivity of secondary furrows to Aurora inhibitors hints at this.

Echinoderms have only a single Aurora kinase, which localizes to the spindle poles, the metaphase centromeres and the anaphase central spindle [Abe et al., 2010], raising the possibility that pole-associated Aurora may participate in specifying secondary cleavage furrows. Indeed, enucleate blastomeres containing two asters were capable of initiating cleavage furrows [von Dassow et al., 2009], though it is not known whether these furrows were Aurora- sensitive. In vertebrate and insect cells, Aurora plays a role in centrosome maturation, mitotic entry, and spindle bipolarity [Glover et al., 1995; Giet and Prigent, 2000; Hannak et al., 2001; Giet et al., 2002]. While we did not examine the effects of Aurora inhibition prior to M phase onset, the characteristic expansion of the spindle poles during anaphase fails to occur in VX-680 treated cells (Fig. 2, panels K, M, and O), consistent with urchin Aurora playing a role in regulating centrosome organization during late mitotic events.

The higher rate of cleavage failure and weaker phosphomyosin staining observed in secondary furrows (Figs. 7B and 7D) implies that the secondary furrows are less robust than

those lying within the primary cleavage plane. The less robust nature of these secondary furrows however, does not imply a significantly lower probability of furrow initiation: 92.8% of the binucleate cells initiated secondary furrows (Fig. 7B). In the presence of Aurora kinase inhibitors a large percentage of normal (mononucleate) cells initiate furrows (but do not complete cleavage). This is consistent with a partially redundant CPC-independent mechanism for furrow initiation and we cannot rule out the possibility that furrow initiation in Rappaport furrows is attributable to a CPC-independent mechanism. However, the sensitivity of secondary furrow progression to Aurora inhibition, does suggest that CPC signaling is required for furrow persistence and completion, in Rappaport furrows just as in normal furrows.

If CPC localization is normally determined by the position of the chromosomes (Fig. 9), yet secondary furrows require CPC signaling (Fig. 1), how might Aurora influence secondary furrow formation? FRET reporters have detected a microtubule-dependent gradient of Aurora B radiating from the spindle midzone [Fuller et al., 2008; Tan and Kapoor, 2011]. Secondary furrows form where the antiparallel tips of astral microtubules overlap (Figs. 7 and 8). This region of overlap is less organized than the central spindle array (Fig. 7C), perhaps for lack of an abundant nearby supply of CPC (Fig. 8), and the furrows induced are conspicuously weaker (Fig. 7D). Our micrographs leave one with the clear impression that in normal furrow formation Centralspindlin reaches the cortex by moving down microtubules towards their plus ends (Fig. 4). Using plus end-directed transport, two opposed astral arrays, might be able to focus a small diffusing supply of CPC/ Centralspindlin/Ect2 not captured by the central spindle (with the CPC component perhaps coming in echinoderms from the spindle poles) and thus concentrate between them sufficient complex to induce a secondary furrow. This mechanism, with its lack of reliance on chromosomal position, would be similar to what Rappaport envisioned for the stimulation of all furrows.

Materials and Methods

Sea Urchin Embryo Culture

Strongylocentrotus purpuratus (purple urchin) and *Lytechinus pictus* (painted urchin) were obtained from Marinus Scientific (Garden Grove, CA) and maintained in artificial seawater (ASW) in a chilled saltwater aquarium at 9–12°C. At the Friday Harbor Laboratories *S. purpuratus* and the sand dollar *Dendraster excentricus* were collected at Clallam Bay, WA and Eastsound, WA, respectively, and kept in flowing seawater tanks. Eggs and embryos were cultured in coarse-filtered natural seawater at 9–13°C in a flowing seawater table. Adults were spawned by intracoelomic injection of 0.5M KCl and eggs were collected by inversion into a beaker of seawater while the sperm were collected dry. All gametes were used the same day of collection. Just prior to fertilization, sperm was diluted 1:1000 in ASW, and several drops of diluted sperm were added to a 10 ml suspension of eggs. Purple urchins eggs were fertilized in seawater containing 1mM 3-amino-triazole to prevent hardening of the fertilization membranes. Fertilization envelopes for all species were removed by pouring zygotes through Nitex mesh (73 µm mesh for *S. purpuratus*, 105 µm mesh for *L. pictus*; 130 µm mesh for *D. excentricus*). *D. excentricus* envelopes were stripped at 12 min postfertilization, prior to membrane hardening and *S. purpuratus* 45–60 min postfertilization after hyaline secretion has ceased.

Reagents, Drugs, and Antibodies

Unless specified otherwise, all reagents and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or ISC Bioexpress (Kaysville, UT). ZM447439 was obtained from Tocris Bioscience (Ellisville, MO); VX-680 from Selleck Chemicals LLC and Hesperadin

was a gift from Andrea Pawelak at Boehringer Ingelheim. Monoclonal anti- α tubulin (DM1A) was purchased from Sigma Co (St. Louis, MO), and anti-phosphohistone H3 (Ser10)- and anti-phospho Ser19 myosin regulatory light chain antibodies were purchased from Cell Signaling (Beverly, MA). Rabbit polyclonal anti-sea urchin MKLP1 antibodies were generated against an epitope mapping to residues 818–835 of sea urchin MKLP1 (GRSKRRVEDDPAEYSGEW), and antibodies were affinity purified using antigen conjugated to Aminolink Plus resin (Thermo Scientific, Rockford, IL). For generation of polyclonal anti-Survivin antibodies, the *S. purpuratus* Survivin cDNA was amplified from an EST (accession # CX689613, provided by James A. Coffman, Mount Desert Island Biological Laboratory) and subcloned into pExp-5 NT TOPO (Invitrogen). Protein expression in BL21DE3 STAR cells was induced with 1mM isopropyl β -D-1-thiogalactopyranoside (IPTG), after which cells were harvested and snap frozen in liquid nitrogen. Cells were thawed and resuspended on ice in lysis buffer (10 mM Tris pH 7.5, 500 mM NaCl, 1% Triton X-100) supplemented with 0.1mM phenyl-methylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail (Sigma). Clarified lysates were passed over nickel nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen). The column was washed with greater than 20 volumes of wash buffer (10 mM Tris pH 7.5, 500 mM NaCl) and recombinant protein was eluted with wash buffer containing 500mM Imidazole. Protein fractions were then dialyzed in a 1° phosphate-buffered saline (PBS)/10% glycerol and used as either antigen for antibody preparation or conjugated to Amiinolink columns for affinity purification.

Immunofluorescence

Indirect immunofluorescence was performed as previously described [Lucero et al., 2006; George et al., 2008]. Briefly, embryos were fixed for 30 minutes at room temperature in Millonig's solution composed of 3.7% formaldehyde, 200mM NaH₂PO₄, 136mM NaCl, pH 7.0. Cells were then washed three times with 1° PBS + 0.1% Triton X-100 (PBST) and blocked with 3% bovine serum albumin (BSA) diluted in 1X PBST for at least one hour. Cells were incubated with appropriate primary antibodies diluted in blocking buffer, and rocked overnight at 4°C. Cells were then washed three times with 1X PBS, probed with fluorescent secondary antibodies (Invitrogen), and rocked overnight at 4°C. Cells were again washed three times with 1° PBS and mounted onto slides in 90% glycerol. *Dendraster excentricus* zygotes treated in the absence or presence of VX-680 were fixed and processed for microtubule localization as previously described [Foe and von Dassow, 2008].

Generation of Binucleate Embryos

Binucleate embryos were generated by reversibly blocking the first embryonic cleavage using a cold shock treatment to block astral microtubule growth. Fertilized sea urchin eggs were stripped of their fertilization envelopes and cultured at 12–15°C until approximately 10% of embryos began to show furrow ingression. At this point, cells were transferred to an ice-cold seawater bath and incubated for an additional 20 minutes on ice. Cells were then transferred back to 12–15°C until the second round of cell division. Cells were then either imaged live or fixed and probed with appropriate antibodies at different cell division stages.

Microscopy, Image Acquisition, and Processing

Timelapse and fluorescence imaging was performed on either a Leitz Labovert microscope equipped with Hoffman modulation optics and a Diagnostic Instruments Insight camera or a Zeiss Axiovert 200M inverted microscope equipped for differential interference contrast (DIC), standard epifluorescence and Apotome structured illumination capabilities. Both microscopes were equipped with temperature-controlled heating and cooling stage inserts (Brook Industries, Lake Villa, IL). Fixed embryos were imaged using a 63X-Plan-

apochromat 1.4NA oil objective and images were collected with a 12-bit AxioCam MrM CCD camera driven by Axiovision 4.5 software (Zeiss, Thornwood, NY). Confocal imaging was performed on either a Radiance 2000 laser scanning confocal microscope (Bio-Rad Laboratories) mounted on a Nikon E800 microscope or an Olympus Fluoview1000 mounted on a Nikon 2000-U inverted microscope. Live cell imaging using polarization microscopy was performed on an Zeiss Axiovert200M equipped with a circular polarizer and 546 nm filter placed above the condenser and a liquid crystal universal compensator (LC-PolScope, Cambridge Research Instruments, Woborn MA) placed below the reflector turret. Images were acquired using a QImaging cooled CCD camera controlled by PSJ software (Marine Biological Laboratories). Figures were prepared from exported 8 bit images using Adobe Photoshop CS2 software, and time-lapse movies were prepared using ImageJ software (<http://rsbweb.nih.gov/ij/>). No nonlinear adjustments or convolution filters were applied.

Aurora cDNA Isolation and Recombinant Protein Purification

Lytechinus variegatus Aurora cDNA (Accession # AFN06392) was isolated by using starfish Aurora (*Patiria pectinifera*, Accession# AB530259) and *S. purpuratus* EST's (Accession # EC434552 and CX551041.1) to BLAST *L. variegatus* sequences available through the Baylor College of Medicine (<http://www.hgsc.bcm.tmc.edu/>) to design primers (Forward: ATGACTTCAGAATATGGCAAAGAGAATTCTTCTC; Reverse TCATGATTGTGATTCTGAGGAGGATGCTGAAGGCTTC). Full-length LvAurora cDNA was amplified from a plasmid library mass excised from a λ ZAP phage ovary cDNA library (a gift from Gary Wessel, Brown University), and subcloned into a pET101-TOPO vector (Invitrogen). Recombinant proteins were expressed in BL21DE3 STAR cells and purified over Ni-NTA as described above for Survivin. Additionally, The C-terminal domain of human INCENP (InBox, residues 791–918) was amplified from human reference mRNA (Clontech), subcloned into pEGFP-TOPO, and the resulting fusion protein was amplified by polymerase chain reaction (PCR) and subcloned into pET100-TOPO and purified as described above. To confirm the drug sensitivity of sea urchin Aurora, recombinant LvAurora was incubated with 1 μ g of recombinant Histone H3 in the presence of EGFP-InBox and 50 μ M ATP in a kinase buffer containing (5 mM 3-(N-morpholino)propane-sulfonic acid (MOPS), pH 7.2, 2.5 mM β -glycerophosphate, 1 mM ethylene glycol tetraacetic acid (EGTA), 5 mM MgCl₂, 0.5 mM dithiothreitol) and either DMSO or increasing concentrations of VX-680 for 30 minutes at 25°C. Reactions were stopped with hot sample buffer, and resolved by SDS-PAGE. Histone phosphorylation was detected by Western blotting with mouse anti-phospho (Ser10) Histone H3 (Cell Signaling), and a rabbit anti-Histone H3 was used to detect total histone levels per lane.

Mammalian Cell Culture

To compare CPC and Centralspindlin localization patterns between echinoderm embryos and cultured somatic cells, Human retinal pigmented epithelial 1 cells were transfected with pEGFP-Survivin (gift from Sally Wheatley, University of Nottingham), fixed and probed for MKLP1 localization (rabbit anti-MKLP1, Cytoskeleton, Inc) as previously described [Shrestha et al., 2012].

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Fig. 1. Cytokinesis in sea urchin embryos requires Aurora B kinase activity

A. Sensitivity of recombinant LvAurora kinase to the Aurora kinase inhibitor VX-680 was assayed using Histone H3 as a substrate, and phosphorylation was detected by Western blotting with mouse anti-phospho-(Ser10) histone H3. **B.** Fertilized *S. purpuratus* eggs were cultured up until nuclear envelope breakdown (NEB) and then transferred into either 0.1% dimethyl sulfoxide (DMSO) or 50 μ M VX-680. Extracts were then probed by Western blotting for phospho-(Ser10) histone H3. **C.** Quantification of furrowing phenotypes for *L. pictus* eggs cultured during mitosis in either 0.1% DMSO (control, $n = 143$) or VX-680 ($n = 343$). Bars denote standard error for 13 separate experiments. **D.** Timelapse imaging of *L. pictus* eggs treated beginning at NEB with either 0.1% DMSO (**a–e**) or 50 μ M VX-680 (**f–j**). Bar: 50 μ m.

Fig. 2. Aurora kinase inhibition blocks Histone H3 phosphorylation and alters anaphase microtubule organization in echinoderm zygotes

Panels A–I. *S. purpuratus* zygotes were treated with either 0.1% DMSO or 100 μ M VX-680 from NEB to anaphase onset, then fixed and probed for phospho-(Ser10) histone H3 and DNA, and imaged by confocal microscopy. **Panels J–O.** *D. excentricus* zygotes at NEB were transferred into either 0.1% DMSO (**panels J, L, N**) or 50 μ M VX-680 (**panels K, M, O**), cultured and fixed during anaphase (**panels J–M**) and telophase (**panels N,O**). Embryos were stained for tubulin and imaged by confocal microscopy. Bar: 20 μ m.

Fig. 3. Survivin localization during mitosis in sea urchin zygotes

S. purpuratus zygotes were fixed and probed for tubulin (green) and Survivin (red) at various stages of first mitosis, and imaged by confocal microscopy. **Panels A–C** show a metaphase cell: Survivin localized to chromosomes. **Panel D–I** depict embryos during early and late anaphase: Survivin localized to the central spindle midzone and weakly to equatorial astral microtubules. As cytokinesis progressed, Survivin concentrated at the ingressing furrow and midbody (**panels J–L**). Bar: 45 μ m.

Fig. 4. Mklp1 localization in *S. purpuratus* zygotes during furrow specification and ingression
 Zygotes were fixed at various stages of mitosis and processed for tubulin (green) and MKLP1 (red) localization, and imaged using confocal microscopy. During anaphase MKLP1 localized to the central spindle (**panels A–C**), but as anaphase progressed was increasingly found on the tips of astral microtubules in the equatorial cleavage plane (**panels D–I**). During furrow ingression, MKLP1 concentrated at the spindle midzone and on microtubule bundles associated with the ingressing furrow (**panels J–L**); see also Fig. 5. Bar: 45 μ m.

Fig. 5. MKLP1 concentrates on furrow-associated microtubule bundles

Dividing *S. purpuratus* zygotes fixed and probed for tubulin (green) and MKLP1 (red), and imaged by confocal microscopy. **Panels A–F** illustrate MKLP1 localization at microtubule tips during an early stage of furrowing. **Panels D–F** are high magnification views of **panels A–C**. **Panels G–L** illustrate a long axis view of MKLP1 localization in a dividing sea urchin zygote, where individual microtubule bundles are visibly associated with the ingressing furrow. **Panels J–L** are high magnification views of **panels G–I**. Bars: 20 μ m.

Fig. 6. Centralspindlin localization is dependent upon CPC function

Beginning at NEB *S. purpuratus* zygotes were cultured in either 0.1% DMSO or 50 μ M VX-680, then fixed and processed to reveal tubulin (green), MKLP1 (red) and DNA (blue) localization and imaged by confocal microscopy. **Panels D–F, G–I, and J, L** show progressive stages from anaphase through early telophase, when normal cells would be cleaving (compare with Fig. 4 panels G–L). Note not only the loss of normal MKLP1 localization, but also the altered microtubule organization (see also Fig. 2 panels J–O). Bar: 45 μ m.

Fig. 7. Cytokinesis in binucleate eggs

Binucleate *L. pictus* and *S. purpuratus* cells were generated by transferring late anaphase/early cleavage zygotes into ice cold seawater for twenty minutes to disrupt astral microtubule elongation and furrow specification. Eggs were then warmed to normal culture temperature whereupon they resumed development as binucleate cells. **Panel A.** Binucleate eggs undergoing second division imaged by polarization microscopy (also see Movie 2, Supporting Information). **Panel B.** Quantification of furrowing phenotypes of binucleate eggs where the primary furrow corresponds to cleavage in the plane of the former metaphase plate, and the secondary furrow is cleavage between the two parallel spindles. Furrowing was scored as either successfully completed (cleaved), furrows regressed, or no initiation; cold-shocked cells whose chromosomes failed to fully segregate to produce two discrete and separate nuclei were discarded. **Panel C.** Microtubule staining of cells containing two parallel spindles reveals the presence of a true central spindle in the primary cleavage plane. In contrast, microtubule tips in the secondary cleavage plane clearly overlap but, unless chromosomes are abnormally present there (see Fig. 9), they do not form a well defined midzone. Bars: 10 μ m. **Panel D.** En face views of microtubule and Ser19-phosphorylated myosin regulatory light chain (P-MLC) localization in cleaving binucleate cells. The primary cleavage furrow is clearly characterized by more robust P-MLC localization than the secondary cleavage furrow. Bar: 10 μ m.

Fig. 8. CPC preferentially localizes to the primary cleavage plane in binucleate cells
Survivin staining of binucleate cells at three successive stages of cleavage. Even when the primary furrow had ingressed to near completion and Survivin was at its most concentrated (**panels I–L**) Survivin was still not detectable in the ingressing secondary furrows (arrowhead, **panel J**). Color code as in Fig. 3. Bar: 25 μ m.

Fig. 9. Chromosome position determines CPC recruitment pattern

Survivin accumulation in both primary and secondary cleavage planes was seen in binucleate cells where the spindles were in sufficiently close proximity that chromosomes were captured by microtubules from both of the parallel spindles. **Panels A–D, E–H, and I–L** show successive stages. Staining and color code as in Fig. 3. Bar: 25 μm.