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Characterization of mid-spindle microtubules during furrow positioning in early cleavage period zebrafish embryos

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Summary

We report evidence to suggest that during the first few meroblastic cell divisions in zebrafish embryos a dynamic population of central-spindle microtubules serve a crucial function in positioning the cleavage furrow at the surface of the blastoderm. Originating from the mid-zone of the mitotic spindle they develop into what we term a mid-spindle 'pre-furrowing microtubule array' that expands upward and outward from the spindle mid-zone towards the blastodisc surface. We suggest that this structure transmits positional information to the blastodisc cortex that results in the correctly positioned assembly of the cytokinetic contractile apparatus. We also propose that the pre-furrowing microtubule array then develops into a furrow-ingression microtubule array that helps direct and assemble the deepening furrow as it cuts its way through the blastodisc. Due to the location of its origin, the pre-furrowing microtubule array serves to successfully separate the daughter nuclei and thus equally divide the blastoderm. Furthermore, co-localization with elements of the cortical endoplasmic reticulum and their inositol 1,4,5-trisphosphate receptors suggests that the pre-furrowing microtubule array may also play a role in organizing localized Ca^{2+} transients that have been shown to be essential to the furrow positioning, propagation and deepening process during cytokinesis in zebrafish embryos.

Keywords: Calcium, Central spindle, Cytokinesis, Pre-furrowing microtubule array, Zebrafish

Introduction

Cytokinesis, the final stage in cell division, is a tightly regulated and complex process (for reviews see Mabuchi, 1986; Salmon, 1989; Satterwhite & Pollard, 1992; Rappaport, 1996; Robinson & Spudich, 2000; Glotzer, 2003). It is generally accepted that microtubules (MTs) determine the position of the cleavage plane midway between the poles of the mitotic spindle (Straight & Field, 2000; Alsop & Zhang, 2003). However, the precise mechanism(s) by which they transmit this spatial information to the correct location at the cell surface is still a matter of intense investigation. How they do so may vary from system to system in response to cell geometry and volume, as well as in relation to the location of the mitotic spindle within the dividing cell. In addition to positioning the cleavage furrow at the cell surface, it has also been shown that MTs are important, and perhaps essential, for furrow ingression and the ultimate separation of the daughter cells (Wheatley & Wang, 1996; Danilchik *et al.*, 1998; Jesuthasan, 1998; Larkin & Danilchik, 1999; Takayama *et al.*, 2002). Thus, as far as the process of cytokinesis is concerned, MTs are involved in dictating the cleavage plane and transmitting this information to the cell surface, as well as participating in the furrowing process itself. It has been suggested that two separate populations of MTs, those of the mitotic asters and the central spindle, may contribute to these processes and once again the extent of their individual participation may depend on the particular cytokinetic system under consideration (Straight & Field, 2000). Thus, one of these populations may be essential for cytokinesis in one system and partially or even fully dispensable in another (Glotzer, 1997). Over the years, good evidence has been collected from different systems to show that either of these MT populations is able to specify the site of cell division (for reviews, see Rappaport, 1996; Robinson & Spudich, 2000; Straight & Field, 2000).

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The first few meroblastic cell divisions in the optically clear zebrafish embryo provide a good model system to explore the role played by these MT populations in positioning cleavage furrows at the cell surface in large zygotic cells. In this report, we provide evidence to suggest that a population of central-spindle MTs serves both positional and directional functions. Initially they develop into what we have called a 'pre-furrowing microtubule array' (pf-MTA) that extends upward and outward from the spindle midzone towards the blastodisc surface. We suggest that this structure initially transmits positional information from the spindle to the cortex, which results in the correct positioning of the cleavage furrow. We also propose that the pf-MTA then develops into the recently described furrow-ingression MT array (fi-MTA; Jesuthasan, 1998) that helps direct and assemble the ingressing furrow as it cuts through the blastodisc to successfully separate the daughter nuclei and meroblastically divide the cell. Finally, we show that a portion of the corticular endoplasmic reticulum (ER) (with its accompanying inositol 1,4,5-trisphosphate receptors, IP_3Rs) co-localizes with the pf-MTA in the blastoderm cortex prior to the appearance of the cleavage furrow. We suggest, therefore, that the pf-MTA may play a role in organizing some of the elements required to generate localized Ca^{2+} transients that have been shown to be essential to the furrow positioning, propagation and deepening process in zebrafish embryos (Chang & Meng, 1995; Webb *et al*., 1997; Créton *et al*., 1998; Lee *et al*., 2003).

Materials and methods

Egg collection

Zebrafish (*Danio rerio)* were maintained on a 14 h light/10 h dark cycle to stimulate spawning (Westerfield, 1994). Fertilized eggs were collected by placing a breeding trap (consisting of a plastic box with a steel mesh insert) into a fish tank at the start of the light period. Fish normally lay eggs in the trap within 10 min of the lights coming on. Eggs were retrieved by emptying traps through a fine-mesh net, after which they were immediately transferred to a 90 mm Petri dish containing 30% Danieau's solution (19.3 mM NaCl; 0.23 mM KCl; 0.13 mM $MgSO_4 \cdot 7H_2O$; 0.2 mM $Ca(NO_3)_2$; 1.67 mM Hepes, pH 7.2). To enhance optical clarity, embryos were dechorionated by treatment with pronase (Sigma; at 1 mg/ml in 30% Danieau's solution) for several minutes at room temperature and then washed several times in 30% Danieau's solution.

Immunocytochemistry

Embryos were dechorionated as described above and fixed at different times during the first cell division cycle with 4% paraformaldehyde in phosphate-buffered saline (PBS; Westerfield, 1994) overnight at 4 °C (McMenamin *et al*., 2003). Fixed embryos were washed initially with PBS (>5 washes) to remove excess fixative, then with PBS containing 0.1% Tween-20 (PBT) for four 5 min washes, and finally with PBT containing 1% dimethylsulfoxide (PBTD) for 5 min. Embryos were then incubated in blocking buffer (PBTD containing 10% bovine serum albumin, BSA) for 2 h.

Labelling microtubules and nucleic acids

In some experiments the microtubules alone were labelled via direct immunocytochemistry, by incubating embryos with fluorescein isothiocyanate (FITC)-conjugated anti-β-tubulin (Sigma; diluted 1:100 in blocking buffer) for 1 h followed by washing for 3 × 1 min then 3 × 20 min with PBTD containing 1% BSA (PBTD/BSA). In another set of experiments, both microtubules and nucleic acids were labelled. For these experiments, embryos were incubated for 1 h with the mouse monoclonal anti-β-tubulin (Sigma; diluted 1:100 in blocking buffer) after which they were washed with PBTD/BSA using the wash protocol described above. Embryos were then incubated for 1 h with a mixture of rhodamine (TRITC)-conjugated AffiniPure $F(ab')_2$ fragment goat anti-mouse IgG (H + L; Jackson ImmunoResearch Laboratories; diluted 1:100 in blocking buffer) to label the microtubules, and SYTOX Green (Molecular Probes; diluted 1:200 000) to label the nucleic acids, followed by the PBTD/BSA wash protocol described earlier.

Labelling the ER and IP_3R

Embryos were incubated for 1 h with either anti-calnexin (Stressgen Biotechnologies) diluted 1:100 in blocking buffer to label the ER, or else anti-IP_3Rs (Type 1; Sigma), diluted 1:200 in blocking buffer. They were then washed for 3 × 1 min then 3 × 20 min with PBTD containing 1% BSA (PBTD/BSA) and incubated for 1 h with FITC-conjugated goat anti-rabbit IgG (H + L; Zymed Laboratories) diluted 1:100 (for the ER) or 1:500 (for the IP_3Rs) in blocking buffer, after which they were washed using the wash protocol described above, again with PBTD/BSA.

Fluorescence was visualized using a Bio-Rad MRC-600 laser scanning confocal microscope equipped with a krypton/argon laser, mounted on a Zeiss Axioskop upright microscope. FITC and SYTOX Green fluorescence was visualized using 488 nm excitation/520 nm emission, whereas rhodamine fluorescence was visualized using 568 nm excitation/585 nm emission. Images were collected using Zeiss water immersion Achroplan 20×/0.5 NA or 40×/0.75 NA objectives. Embryos were observed from facial and animal pole views (as described in Webb *et al*., 1997) and z-series of confocal sections were scanned through the blastodisc.

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Aequorin imaging

An attempt was made to visualize the initiation of localized changes in the level of intracellular calcium prior to the appearance of the cleavage furrow at the blastoderm surface (i.e. the furrow-positioning calcium signal). To do this, we utilized *hcp*-aequorin, a semi-synthetic aequorin with an improved sensitivity to calcium ions and a relative intensity around 190 times that of natural aequorin (Shimomura *et al*., 1989). Throughout experimental protocols, therefore, extra care was taken to ensure that the *hcp*-aequorin was not accidentally discharged due to contact with calcium ions.

Thus, embryos were dechorionated in calcium-free 30% Danieau's solution and were then placed, again in calcium-free solution, in custom-made holding/viewing chambers for *hcp*-aequorin microinjection and subsequent imaging. Calcium-free solution was prepared by omitting $Ca(NO_3)_2$ and using ultrapure deionized water to dissolve the remaining Danieau's reagents. In addition, EGTA (5 mM) was added to chelate any trace levels of contaminating calcium. These calcium-free conditions were employed to minimize the risk of discharging the *hcp*-aequorin during the microinjection process.

The microinjection pipettes, the pressure injection system and the other protocols used for injecting embryos with aequorin are described in detail elsewhere (Webb *et al*., 1997). Approximately 1.5 nl of recombinant *hcp*-aequorin (1.0% in 100 mM KCl, 5 mM MOPS and 50 μM EDTA) was injected into the centre of the embryo's yolk cell. Following microinjection, the calcium-free Danieau's solution was replaced with normal 30% Danieau's solution and the embryos were orientated to provide either an animal pole or facial view. They were then transferred to our custom-designed photon imaging microscope (PIM; Science Wares, East Falmouth, MA, USA; for details see Webb *et al*., 1997; Lee *et al*., 1999) for data acquisition. Aequorin-generated and bright-field images were collected using a Zeiss Fluar 20 × /0.75 NA objective. At the end of data acquisition, files were downloaded into Corel DRAW 11 and PHOTO PAINT 11 (Corel) for figure preparation and presentation.

Results

Microtubule array precedes the appearance of the cleavage furrow on the blastodisc surface

Embryos were fixed at different stages of the first cell division cycle and then the microtubules were labelled by direct immunocytochemistry. Fig. 1 is a representative embryo ($n = 19$) that was fixed at approximately 40 min post-fertilization (mpf). It is clear from the transmission image of the facial view of the embryo that there is no obvious furrow visible (see Fig. 1A); however, a projection of confocal sections taken through the top of the same embryo shows an array of microtubules in the cortex of the blastodisc (see Fig. 1B, C). As this structure is apparent before the first morphological sign of the cleavage furrow, we have called it the 'pre-furrowing microtubule array' or pf-MTA.

The representative embryos shown in Fig. 2 confirm a previous report (Jesuthasan, 1998) that an array of microtubules is also present during furrow ingression ($n = 19$; Fig. 2A) and zipping ($n = 9$; Fig. 2B). Fig. 2B shows the facial view of a representative embryo, which is at the end of cytokinesis of the first cell division cycle. Here, two pf-MTAs that are associated with the second cell division cycle, are already visible during furrow zipping of the first cell division cycle, i.e. these pf-MTAs again appear to precede the appearance of the second cleavage furrows on the blastodisc surface.

Origin and formation of the pre-furrowing microtubule array

Fig. 3 shows a series of confocal sections taken from the animal pole and then every 10 μm through the blastodisc of a representative embryo that was fixed during the metaphase/anaphase transition of the first cell division cycle and labelled by direct immunocytochemistry. At this early stage the mitotic spindle is located some 50 μm from the top of the blastodisc. Subsequently, embryos were fixed shortly after the metaphase/anaphase transition and both the microtubules and DNA were labelled. In the single confocal section shown in Fig. 4, the pf-MTA is clearly located between the chromosomes, with the spindles (attached to the chromosomes) and the microtubule organization centres (MTOCs; at the poles of the spindle) also being visible in this optical plane.

The development of the pf-MTA during the first cell division cycle is illustrated in Figs. 5 and 6. Fig. 5 shows a series of embryos that were fixed at metaphase and at different times following metaphase of the first cell division cycle and then both the microtubules and DNA labelled. This figure demonstrates that when the chromosomes separate as mitosis progresses through anaphase and telophase, so the pf-MTA grows outward towards the edge of the blastodisc. The pf-MTA also grows upward towards the animal pole and this is illustrated in the montage of images in Fig. 6. Fig. 6A illustrates a montage of images of the embryo shown in Figs. 4 and 5C, and demonstrates that at this stage of the cell division cycle the pf-MTA has only developed approximately 4 μm above the plane of

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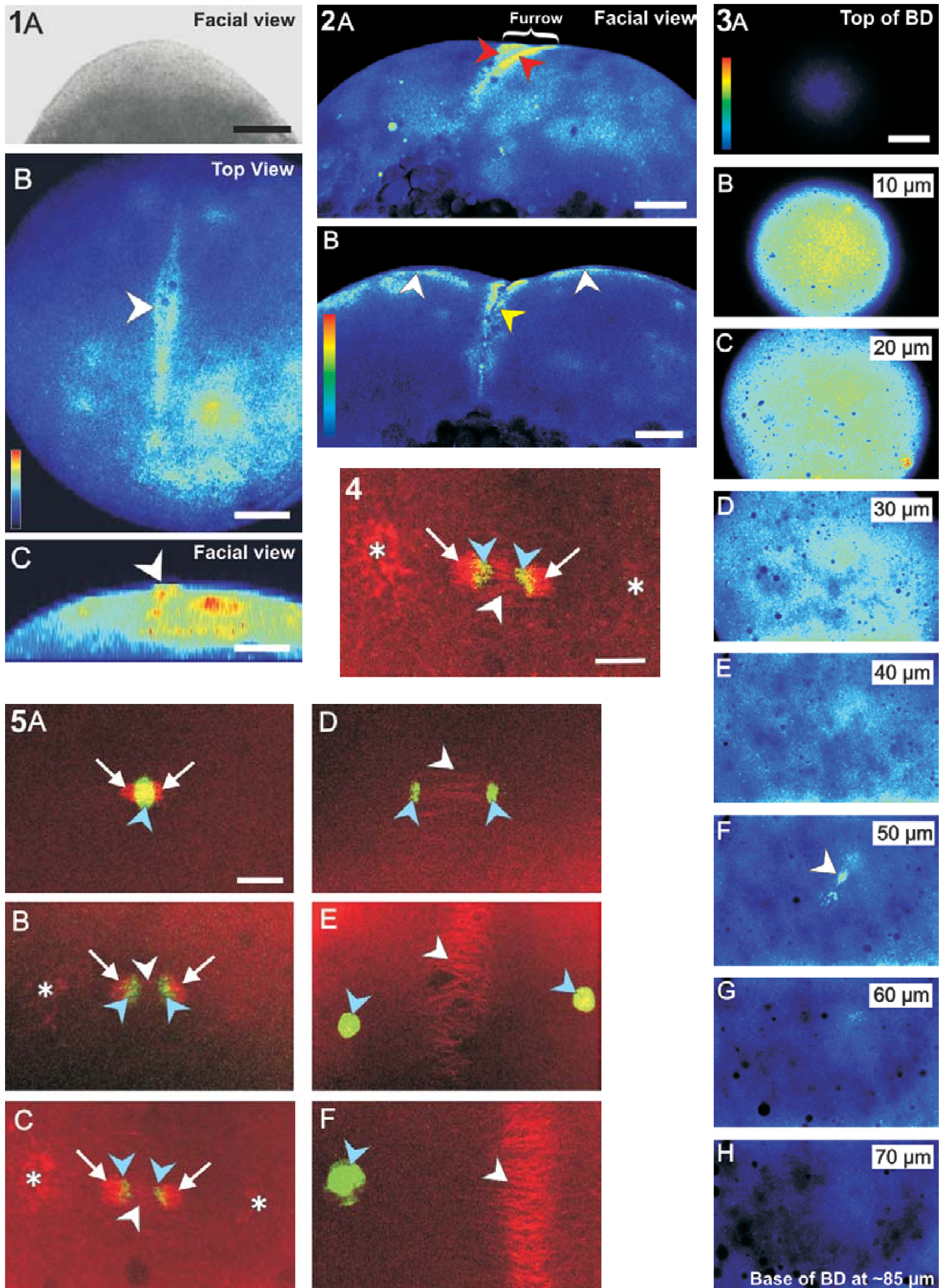

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the chromosomes towards the animal pole. Fig. 6B illustrates a montage of images of the embryo shown in Fig. 5F and demonstrates that at this stage of the cell division cycle the pf-MTA has grown some 20 μm above the plane of the chromosomes towards the animal pole.

Visualizing the distribution of the ER, IP_3 receptors and Ca^{2+} during furrow positioning

Embryos were either fixed during furrow zipping of the first cell division cycle and the ER and IP_3 receptors labelled by indirect immunocytochemistry, or were injected with *hcp*-aequorin and the Ca^{2+} signals imaged during the first two cell division cycles. Fig. 7 shows the top views of three representative embryos and illustrates the co-localization of the ER (see Fig. 7A), IP_3Rs (see Fig. 7B) and Ca^{2+} (see Fig. 7C) just prior to the morphological appearance of the cleavage furrows of the second cell division cycle.

Time correlation between the positioning calcium transient and the appearance of the furrow

Embryos injected with *hcp*-aequorin were continuously imaged with the photon imaging microscope (PIM) in photon collection mode using a high NA 20× objective until the first signs of the localized rise in Ca^{2+} that makes up the furrow-positioning transient were observed. The PIM was then rapidly switched to acquire a bright-field image, after which it was set up to collect bright-field images every 30 s during the subsequent photon collection. Fig. 8 shows a series of photon and bright-field images collected just after the first appearance of the furrow-positioning transient and then 2 min after its appearance in a representative embryo ($n = 5$). It is clear that when a calcium rise is first observed, there is no obvious indentation at the surface of the blastodisc. Indeed, the first sign of an indentation at the animal pole of the blastodisc is only observed approximately 30 s after the initiation of the furrow-positioning transient.

Figure 1 A microtubule array precedes the appearance of the furrow on the blastodisc (BD) surface. (*A*) A transmission image of a representative embryo fixed at approximately 40 mpf. In this facial view of the embryo (i.e. the best orientation to observe the first appearance of the cleavage furrow on the surface of the blastodisc), a furrow is not yet visible at the animal pole. (*B*), (*C*) However, when this embryo was fixed and labelled by immunocytochemistry, a pre-furrowing microtubule array (pf-MTA; white arrowhead) is clearly apparent. These panels show a stack of confocal images (5 μm apart) taken through the blastodisc from the animal pole, and shown as single projected images (*B*) viewed in the same orientation as the sections were acquired (i.e. from a top view) and (*C*) viewed when the projected section is rotated 90°. The colour scale indicates the relative level of fluorescence where blue is the lowest and red the highest intensity of fluorescence observed. Scale bars represent 100 μm (*A*, *B*) both horizontally and vertically and (*C*) horizontally alone.

Figure 2 An MTA is also associated with furrow ingression and zipping. Two representative embryos that were fixed: (*A*) as the furrow was starting to deepen during cytokinesis of the first cell division cycle and (*B*) during the zipping up of the first cleavage furrow. The microtubules were labelled by direct immunocytochemistry. Each image is a stack of confocal sections (z-step = 5 μm) that have been projected as a single image. (*A*) The cleaving furrow is clearly visible (as an indentation in the blastodisc surface) as is the furrow-ingression MTA, which extends laterally on either side of the furrow (red arrowheads). (*B*) An MTA associated with the zipping of the first cleavage furrow (yellow arrowhead) and the two pre-furrowing MTAs (white arrowheads) of the second cell division cycle. The colour scale indicates relative level of fluorescence where blue is the lowest and red the highest intensity of fluorescence observed. Scale bars represent 100 μm.

Figure 3 The pf-MTA forms from the spindle mid-zone deep within the blastodisc. This is a representative embryo that was fixed during the metaphase/anaphase transition and the microtubules labelled by direct immunocytochemistry. In this series of eight optical sections (10 μm apart) taken through the blastodisc from the animal pole, the mitotic spindle (white arrowhead) is first observed some 50 μm from the top of the blastodisc. Scale bar represents 100 μm.

Figure 4 The pf-MTA appears to arise from the mid-zone spindle. This is a representative embryo that was fixed during the metaphase/anaphase transition of the first cell division cycle. The microtubules (red) were labelled by indirect immunocytochemistry and the DNA (green) was labelled with SYTOX Green. In this single optical section through the blastodisc, the pf-MTA (white arrowhead) is located between the separating chromosomes (blue arrowheads) and originates from remnants of the mitotic spindle (white arrows). The microtubule organizing centres (white asterisks) at the spindle poles are also shown. Scale bar represents 20 μm.

Figure 5 Lateral development of the pf-MTA. Six representative embryos that were fixed at various times between metaphase to telophase of the first cell division cycle show the lateral development of the pf-MTA. The microtubules (red) were labelled by indirect immunocytochemistry and the DNA (green) was labelled with SYTOX Green. The pf-MTA, chromosomes, mitotic spindle and microtubule organizing centres are shown by white arrowheads, blue arrowheads, white arrows and white asterisks, respectively. Scale bar represents 20 μm.

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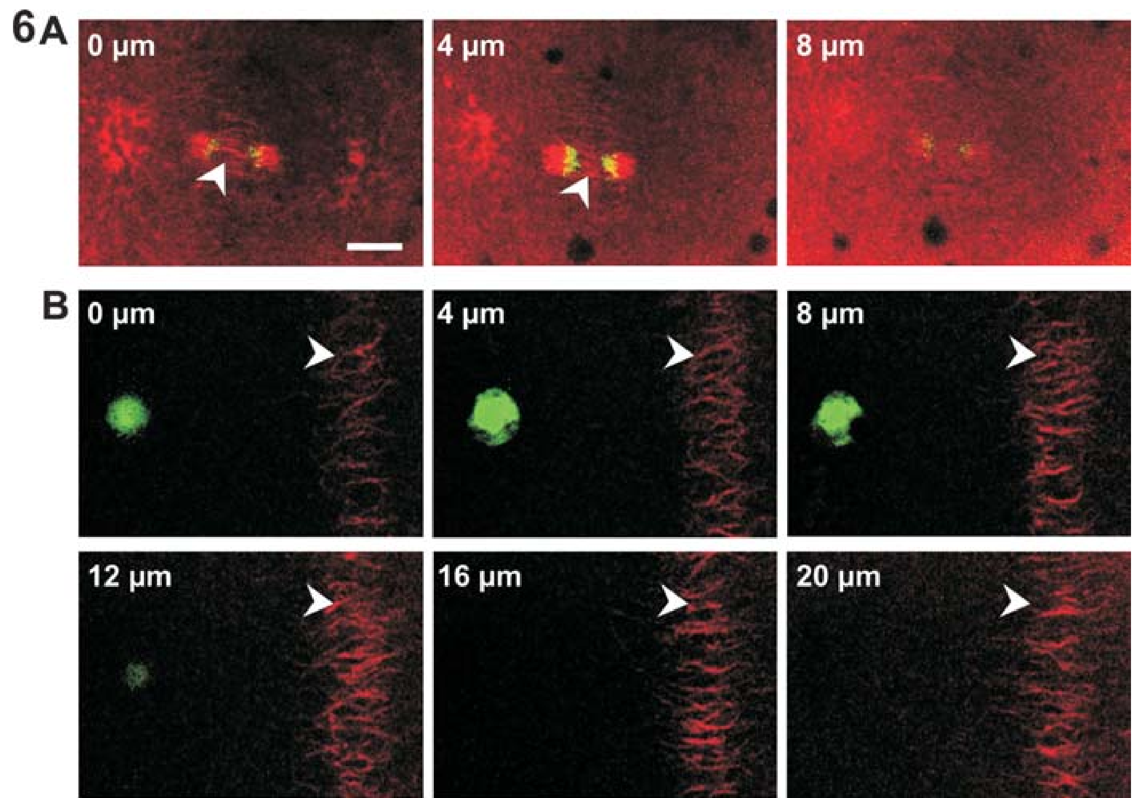

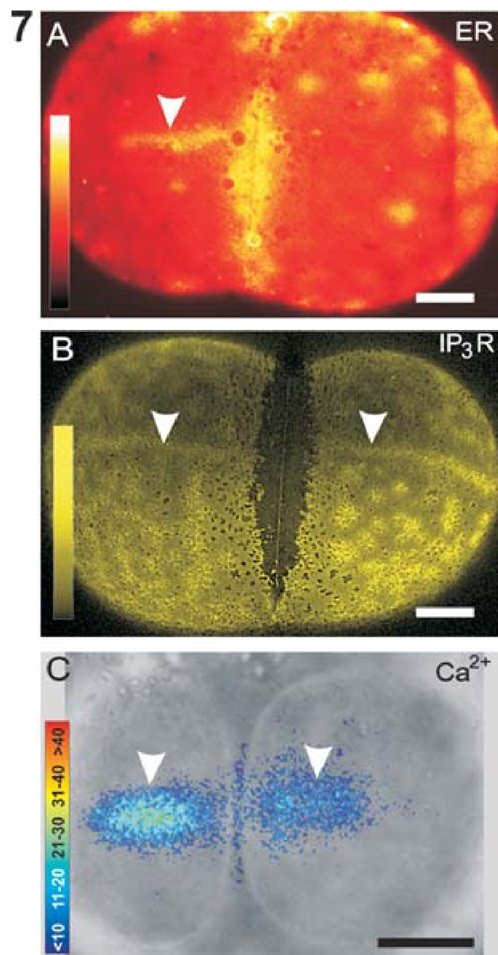

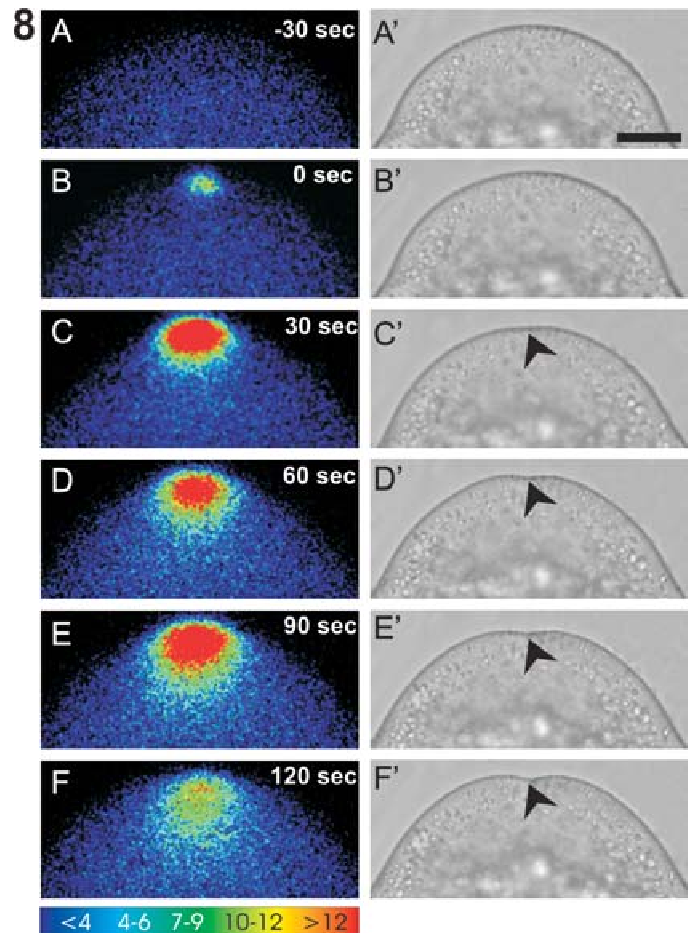

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Discussion

The challenge in cleavage furrow positioning during cytokinesis in large zygotic cells is to generate a mechanistic connection that conveys a positioning signal from the mitotic spindle to the cell surface where the cleavage furrow forms. In the embryonic cells of certain fish and amphibian species, the distances involved may be quite considerable and in the case of the first few cell divisions of the zebrafish blastodisc, the mitotic apparatus lies about 50 μm beneath the surface (see Fig. 3). It has been shown that in the large embryonic cells of some species such as *Xenopus*, astral microtubules play an important role in determining the plane of cell division (Takayama *et al*., 2002). These findings support earlier work from smaller embryonic cells, such as those of echinoderms, where manipulation of mitotic spindles provided insight into the role of the astral microtubules (MTs) in specifying the position of cleavage furrows (Rappaport, 1961, 1996; Rappaport & Rappaport, 1974). However, there is increasing evidence from cultured mammalian and insect cells that MT bundles in the spindle mid-zone, the so-called central spindle that arises after chromosome separation, may also play an important role in positioning the cleavage furrow (Wheatley & Wang, 1996; Adams *et al*., 2000). Here, we demonstrate that the furrow-positioning function of central-spindle MTs is not restricted to small, flat cultured cells, but that they also play a crucial role in correctly positioning the cleavage furrow during the early meroblastic divisions of the relatively yolk-free large blastoderm of zebrafish embryos. However, the reliance on either astral or mid-zone MTs conveying the furrow-positioning signal to the cell surface need not be mutually exclusive, as it has been shown that in some cells it is a combination of the two populations of MTs that delivers the cytokinetic signal (Murata-Hori & Wang, 2002).

We report that after chromosome separation during the first cell division in zebrafish embryos, a parallel array of short MTs remains at the spindle mid-zone (Figs. 4–6). When single 10 μm optical z-sections through the blastodisc are examined, it is clear that the array does not initially extend either upward towards the blastodisc surface or downward towards the yolk cell. Neither does it extend outward towards the sides of the blastodisc. It forms some 50 μm below the blastodisc surface in approximately the same focal plane as the mitotic MTOCs (i.e. the centrioles) at the spindle poles (Fig. 3F). We suggest that initially this structure is analogous to the so-called central spindle or mid-zone MT bundle described in tissue culture cells (Wheatley & Wang, 1996; Straight & Field, 2000). In the time period between the separation of the chromosomes and the appearance of the cleavage furrow at the surface of the blastodisc, the parallel array of short central-spindle MTs extends upward and outward towards the blastodisc cortex (Figs. 5, 6). It thus develops into a structure analogous to what has been previously described as a telophase disc (Andreassen *et al*., 1991; Margolis & Andreassen, 1993; Wheatley & Wang, 1996). Such a MT-based structure might also be a component of what was described in the early cytokinesis literature as a 'diastema' (reviewed by Rappaport, 1996).

We have called this structure a 'pre-furrowing microtubule array' (pf-MTA) as it arrives in the blastodisc cortex before there is any visible furrow-induced physical deformation of the cell surface. We propose that the central spindle forms a scaffold and that the pf-MTA develops from it, perhaps via a combination of polymerization and stabilization

Figure 6 Vertical development of the pf-MTA. Two representative embryos that were fixed (*A*) during anaphase and (*B*) during telophase of the first cell division cycle. The microtubules (red) were labelled by indirect immunocytochemistry and the DNA (green) was labelled with SYTOX Green. Here are two series of optical sections (4 μm apart) taken through the blastodisc to show the vertical extension of the pf-MTA starting at the plane of the chromosomes. The pf-MTA is shown by a white arrowhead. Scale bar represents 20 μm.

Figure 7 Localization of the ER, IP_3Rs and Ca^{2+} before the first morphological appearance of the cleavage furrow at the start of the second cell division cycle. Three representative examples of embryos viewed from the top show the co-localization of (*A*) the ER, (*B*) IP_3Rs and (*C*) Ca^{2+} at the site of the future furrows of the second cell division cycle. (*C*) The aequorin-generated image (in pseudocolour) is superimposed on the appropriate bright-field image of the embryo (acquired just prior to the aequorin image) to show the position of the Ca^{2+} signal more clearly. This panel represents 60 s of accumulated luminescence. The colour scale indicates luminescent flux in photons/pixel. Scale bar represents 10 μm.

Figure 8 Representative facial view of an embryo loaded with *hcp*-aequorin demonstrating the changes in intracellular free calcium that occur during furrow positioning of the first cell division cycle. The photon images (coloured panels *A* to *F*) represent 10 s of accumulated luminescence and were acquired (*A*) 30 s prior to the appearance of the furrow-positioning transient, (*B*) in the first 10 s of the furrow-positioning transient and then (*C*) 30 s, (*D*) 60 s, (*E*) 90 s and (*F*) 120 s after the initiation of the furrow-positioning transient. Corresponding bright-field images (*A'*–*F'*) were grabbed immediately after the photon images. The physical appearance of the cleavage furrow is shown by black arrowheads. The colour scale indicates luminescent flux in photons per pixel. Scale bar represents 100 μm.

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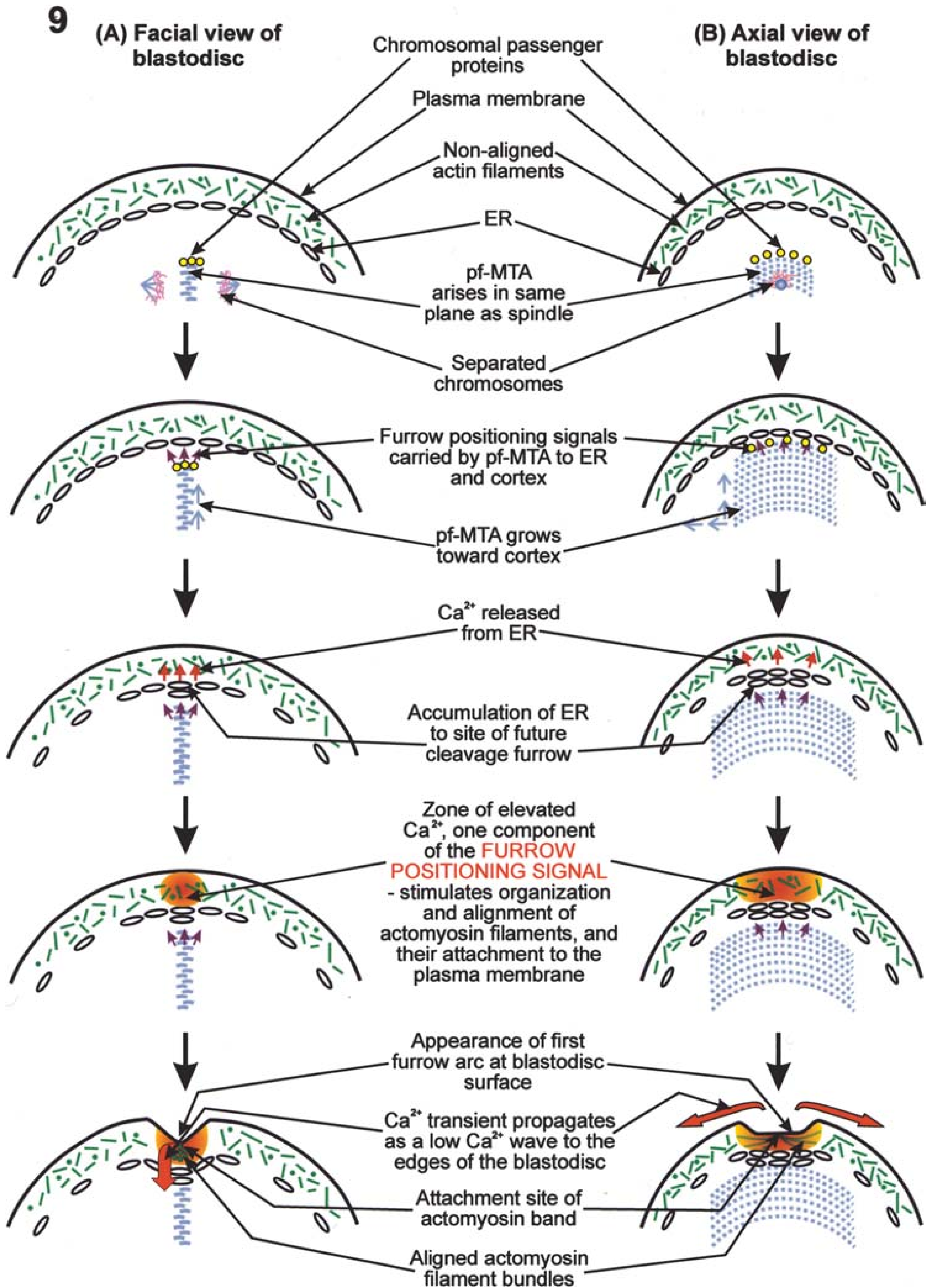

Figure 9 Hypothetical model to explain the dynamic nature of the pf-MTA during the first cell division cycle, and propose how it might play a role in positioning the cleavage furrow. (*A*) Facial and (*B*) axial views of the blastodisc are shown; however, in (*B*) the centrioles and astral array have been removed for clarity.

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between the positive ends of the spindle MTs that failed to make contact with chromosomal centromeres (Gatti *et al*., 2000). Through this expansion process, the pf-MTA then extends upward and outward towards the top and sides of the blastodisc, respectively.

From studies using tissue culture cells, it has been extensively reported that chromosome passenger proteins play a key role in positioning the cleavage furrow (Eckley *et al*., 1997; Terada *et al*., 1998; Skoufias *et al*., 2000; Wheatley *et al*., 2001). Early in mitosis, it is proposed that these proteins are located on the chromosomes. However, prior to and during anaphase they are transferred to the central spindle, where they play a crucial role in positioning the cleavage furrow. For example, the chromosome passenger protein, TD-60, found in the telophase disc, is known to play an important role in positioning the cleavage furrow in cultured epithelial cells (Wheatley & Wang, 1996). We suggest, therefore, that the pf-MTA we have described in early zebrafish embryos might also prove to function in a similar manner in order to transfer chromosome passenger proteins from the chromosomes first to the central spindle, then to the cortex via the pf-MTA.

Furthermore, our data suggest that the pf-MTA might also function in organizing the corticular endoplasmic reticulum (ER) network to predominantly localize to the position where the future cleavage furrow forms. It has been previously reported that MTs and the ER are highly interdependent structures (Terasaki *et al*., 1986). In addition, MTs have also been implicated in the process of release from the ER (Tasaka *et al*., 1991). Thus, this accumulation of the ER along the presumptive cleavage plane would subsequently result in the positioning of an intracellular Ca^{2+} store in the right place and at the right time for the release of Ca^{2+} ions (via inositol 1,4,5-trisphosphate receptors; see Fig. 7B and C), thus providing the means for locally raising intracellular Ca^{2+}. This is a process shown to be essential for cell division in zebrafish (Chang & Meng, 1995; Webb *et al*., 1997; Créton *et al*., 1998). Such a structural arrangement of the Ca^{2+} store would also help to explain the linear nature of the slow Ca^{2+} waves associated with furrowing in zebrafish embryos (Webb *et al*., 1997). In support of the link between the release of Ca^{2+} and furrow induction, it has been reported that the microinjection of Ca^{2+}-store-enriched microsomal fractions into dividing amphibian zygotes can induce extra cleavage furrows at the site of injection via IP_3-mediated Ca^{2+} release (Mitsuyama *et al*., 1999; Mitsuyama & Sawai, 2001). Our data also indicate that like the pf-MTA and the positioning of the ER, a localized elevation of intracellular free Ca^{2+} precedes the physical appearance of the furrow at the blastodisc surface (see Fig. 8). We propose that this Ca^{2+} elevation is also a key component of the furrow-positioning signal. We have previously suggested that the Ca^{2+} transient may act to initiate and organize the assembly of the actomyosin contractile band via Ca^{2+}-sensitive elements such as myosin light chain kinase (Webb *et al*., 1997).

An extensive MT array has also been reported to be associated with the ingression and apposition of daughter cells during cell division in zebrafish (Jesuthasan, 1998) and *Xenopus* (Danilchik *et al*., 1998, 2002). It was suggested that the array serves as a transport mechanism bringing new membrane and apposition components (e.g. cadherins and catenins) that support furrow ingression and daughter cell adhesion. Indeed, our data suggest that the pf-MTA we report in zebrafish might go on to develop into a furrow-ingression MTA (fi-MTA) and then a furrow-apposition MTA (fa-MTA; see Fig. 2B). As the cleavage furrow ingresses into the pf-MTA, this results in components of the pf-MTA being located on either side of the deepening furrow, i.e. in the same location as the ingression and apposition MT array described by Jesuthasan (1998). It has been reported that bundled MTs are an essential component required to sustain furrow ingression (Alsop & Zhang, 2003).

We have summarized our current data into a hypothetical furrow-positioning model illustrated in Fig. 9. In this model, we propose that the pf-MTA develops from the central spindle and acts to correctly position the cleavage furrow by acting as a transport mechanism to convey key chromosome carrier proteins to the blastodisc cortex as well as acting to organize the ER calcium store. The latter provides a means of generating a localized, linear cytoplasmic Ca^{2+} transient. It then develops into the fi-MTA when the leading edge of the ingressing furrow makes contact with it. The fi-MT array then forms the fa-MTA when furrow ingression is complete. Once apposition is complete, the array disassembles. In summary, we propose that during the early cell division cycles in zebrafish embryos, MTs of the central spindle play a key role in correctly positioning the cleavage furrow at the blastodisc surface.

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