Microtubules continuously dictate distribution of actin filaments and positioning of cell cleavage in grasshopper spermatocytes

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Summary

We systematically examined the impact of microtubules on distribution of actin filaments and positioning of cell cleavage using micromanipulation to progressively alter the symmetric distribution of spindle microtubules in grasshopper spermatocytes. The initial microtubule asymmetry was induced by placing a single chromosome at one spindle pole using a microneedle, which facilitates regional assembly of spindle microtubules. We augmented chromosome-induced microtubule asymmetry by further removing the aster from the achromosomal pole, producing unichromosome-bearing monopolar spindles. We created the highest spindle asymmetry by cutting early anaphase cells in two, each containing a full set of segregating chromosomes in a half-spindle. We demonstrate that the location of the spindle midzone, distribution of actin filaments, and position of cell cleavage depend on the amount of microtubule asymmetry generated, shifting up to 48.6±3.8% away from the spindle equator in cut cells. The positional shift is dynamic, changing incessantly as spindle microtubules reorganize during cytokinesis. These results suggest that microtubules continuously dictate the distribution of actin filaments and positioning of cell cleavage in grasshopper spermatocytes.

Movies available on-line

Key words: Cytokinesis, Microtubule, Actin filament, Cleavage furrow, Spindle midzone

Introduction

Cytokinesis in animal cells is brought about by the contractile ring, a belt of actin and myosin fibers attached to the cell cortex around the spindle equator (reviewed by Satterwhite and Pollard, 1992). The contractile ring assembles during late anaphase and constricts the cell by deforming the membrane around the spindle midzone [for an in-depth discussion see Rappaport (Rappaport, 1996)]. The molecular basis underlying the organization and contraction of the contractile ring is well documented (Mabuchi, 1986; Satterwhite and Pollard, 1992; Robinson and Spudich, 2000; Glotzer, 2001; Pelham and Chang, 2002; Silverman-Gavrila and Forer, 2003), yet how the ring is symmetrically positioned with respect to the spindle apparatus remains less well defined. Among different spindle constituents, microtubules, whether from asters or the central spindle, have been shown to be essential for cell cleavage (Tilney and Marsland, 1969; Hiramoto, 1971; Hamaguchi, 1975; Mullins and Snyder, 1981; Salmon and Wolniak, 1990; Larkin and Danilchik, 1999; Canman et al., 2000; Shuster and Burgess, 2002; Straight et al., 2003; Dechant and Glotzer, 2003). In grasshopper spermatocytes, microtubules can even be the only spindle structure necessary for cell cleavage to take place (Alsop and Zhang, 2003).

In cells that have relatively small asters compared with echinoderm oocytes, microtubules from the central spindle play a more important role than asters in initiation of contractile ring formation (for reviews, see Rappaport, 1996; Oegema and Mitchison, 1997; Field et al., 1999; Glotzer, 2001; Guertin et al., 2002; Scholey et al., 2003). The central spindle consists of both preexisting and newly assembled interzonal microtubules radiating from the region of reforming daughter nuclei (Julian et al., 1993; Shu et al., 1995). These microtubules extend toward the spindle equator where they overlap and interdigitate (Hepler and Jackson, 1968; McIntosh and Landis, 1971) at the antiparallel plus-ends (Euteneuer and McIntosh, 1980), forming a midzone that coincides with furrow position. In cultured rat kidney epithelial cells (NRK), a perforation placed between the spindle midzone and cell cortex prior to anaphase results in formation of a furrow at the site of perforation, but not the cortex where astral microtubules are localized (Cao and Wang, 1996). In addition, cortical ingression appears to correlate directly with the position of midzone microtubules, not astral arrays (Wheatley and Wang, 1996). Mutant *Drosophila* spermatocytes whose centrioles fail to form normal asters are fully capable of positioning a cleavage furrow at the midzone of a normal appearing central spindle (Bonaccorsi et al., 1998) and spindle midzone microtubules are continuously required for completion of cell cleavage (Wheatley and Wang, 1996; Powers et al., 1998; Raich et al., 1998; Jantsch-Plunger et al., 2000; Matuliene and Kuriyama, 2002).

One potential role of spindle microtubules is to dictate distribution of cytokinetic factors involved in furrow positioning by recruiting them to the midzone (Rappaport, 1996). Numerous proteins accumulate as an amorphous deposit of electron dense materials (McIntosh and Landis, 1971) at the

midzone or 'midbody' (Mullins and Biesele, 1973; Saxton and McIntosh, 1987; Rattner et al., 1992), which excludes anti-tubulin antibody from labeling the microtubules. A number of them, such as inner centromere proteins (INCENPs) and several kinesin-like motors, behave like 'chromosomal passengers' riding on congressing chromosomes to the spindle equator where they diffuse to the cell cortex prior to cytokinesis (Cooke et al., 1987; Andreassen et al., 1991; Yen et al., 1991; Williams et al., 1995; Adams et al., 1998; Martineau-Thuillier et al., 1998; Sellitto and Kuriyama, 1988; Schumacher et al., 1998; Skoufias et al., 2000). Several of these proteins have been shown essential for contractile ring assembly (Williams et al., 1995; Eckley et al., 1997; Adams et al., 1998; Giet and Glover, 2001) and cleavage furrow ingression (Savoian et al., 1999; Jantsch-Plunger et al., 2000; Severson et al., 2000; Matuliene and Kuriyama, 2002). For instance, TD-60, AIM-1 and INCENPs colocalize at the midzone and may play an important role in furrow positioning (Martineau-Thuillier et al., 1998). Disruption of midzone bound regulatory molecules, such as aurora B, Cyk-4, survivin and Cdc37, perturbs both midzone formation and cytokinesis (Jantsch-Plunger et al., 2000; Fraser et al., 1999; Giet and Glover, 2001; Lange et al., 2002). Chromosomes might facilitate protein accumulation at the midzone by mobilizing chromosomal passengers to the spindle equator. For instance, cytokinesis usually fails to occur in cells whose INCENP accumulation at the midzone and the equatorial cortex is prevented by expressing chimeric INCENP tethered to the centromere (Eckley et al., 1997), or through treatment with inhibitors that block microtubule assembly (Wheatley et al., 2001). Interestingly, in mammalian cells, a subpopulation of stable microtubules mediated by chromosomes in monopolar spindles is sufficient to distribute INCENP and dictate cell cleavage (Canman et al., 2003). The presence of chromosomes, however, is not essential for proper furrow positioning (Zhang and Nicklas, 1996; Rieder et al., 1997; Bucciarelli et al., 2003; Alsop and Zhang, 2003) or accumulation of chromosomal passengers at the spindle midzone (Wheatley and Wang, 1996; Eckley et al., 1997; Savoian et al., 1999).

Alternatively, spindle microtubules might mediate transport of contractile elements, such as actin filaments, toward microtubule plus-ends and thus accumulate at the overlapping zone. Observations in *Xenopus* egg extract have demonstrated that microtubules may interact with actin filaments (Sider et al., 1999). In the extract, actin filaments can be excluded toward plus-ends of astral microtubules, presumably driven by motor proteins or dynamic release of elongating microtubules (Waterman-Storer et al., 2000). During cellularization in the *Drosophila* syncytial blastoderm, actin filaments appear to be transported along microtubules toward their plus-ends (Foe et al., 2000). Recently, two kinesin-like motor proteins, CHO1 and KLP3A, localized at the spindle midzone, have been shown to have affinity for actin filaments (Sisson et al., 2000; Kuriyama et al., 2002). These findings suggest that certain motor proteins might link microtubules to contractile machinery (Gavin, 1997; Goode et al., 2000).

In this study, we systematically examine the impact of microtubule asymmetry on distribution of actin filaments and induction of cell cleavage by altering the bipolar symmetry of spindle microtubules in grasshopper spermatocytes. We produced cells containing various degrees of asymmetric distribution of spindle microtubules and analyzed the resulting effect on induction of cell cleavage. These alterations progressively shift the region of antiparallel, overlapping microtubules, which consequently affects the location of the spindle midzone, distribution of actin filaments, and ultimately position of the cleavage furrow in cells containing a bipolar spindle, monopolar spindle, or half-spindle. The dramatic repositioning of the midzone in cut cells containing a half-spindle permits direct observation of actin filament redistribution following reorganization of central spindle microtubules.

Materials and Methods

Micromanipulation of living grasshopper spermatocytes

Preparation and micromanipulation of spermatocytes of the grasshopper *Melanoplus femurrubrum* were performed as described previously (Zhang and Nicklas, 1999; Alsop and Zhang, 2003). In brief, primary spermatocyte cultures were prepared by spreading a monolayer of cells on a coverslip under halocarbon oil. Micromanipulations, such as chromosome/aster removal and cell cutting, were carried out using a glass needle (tip diameter ~0.1 μm) maneuvered with a Burleigh MIS-5000 series piezoelectric micromanipulator. Digitally enhanced polarization microscopy of cells was performed on an Axiovert 100 microscope (Carl Zeiss,) equipped with an Ellis optical fiber light scrambler (Technical Video, Woods Hole, MA) to provide uniform, high-intensity illumination, and a Glan-Thompson polarizer to increase transmission and extinction of polarized light (Inoue and Spring, 1997). A 1.4 NA achromatic-aplanatic condenser, infinity-corrected 1.4 NA/63× Plan-Apochromat objective lens (Carl Zeiss), and cooled-CCD digital camera (ORCA-100, Model C4742-95, Hamamatsu) were used in image acquisition with Image Pro Plus software (Media Cybernetics).

Immunofluorescence microscopy

Fixation and staining of cells were performed as described previously (Nicklas et al., 1979; Alsop and Zhang, 2003). In brief, a small amount of microfixative [2% glutaraldehyde, 1% Chaps, 0.33 μM Rhodamine labeled phalloidin (Molecular Probes) in potassium Pipes buffer] was micropipetted in the region of target cells. Following microfixation (5 minutes), the coverslip was transferred into macrofixative (0.1% glutaraldehyde, 0.5% NP-40 in potassium Pipes buffer) for 10 minutes. Microtubules were stained with a 1:500 dilution of anti-β tubulin (clone KMX-1, Chemicon) primary antibody, and a 1:100 dilution of Alexa-fluor 488-conjugated goat anti-mouse secondary antibody (Molecular Probes). Actin filaments were stained with 0.165 μM Rhodamine labeled phalloidin (Molecular Probes). Vectashield mounting medium (Vector Laboratories) containing DAPI was used to stain chromosomes and mount coverslips. A laser scanning confocal microscope (Leica TCS) was used to acquire image stacks, which were processed in Photoshop 5.0 (Adobe) and reconstructed in SimplePCI (C-imaging Systems).

Results

Cytokinesis in grasshopper spermatocytes

Cytokinesis in grasshopper spermatocytes is typical of cultured animal cells except for a unique distribution of mitochondria, which initially coincides with the equatorial accumulation of actin filaments during cleavage furrow formation. Mediated by redistribution of spindle microtubules (Fig. 1A, polarization microscope sequence; see also Movie 1, http://jcs.biologists.org/supplemental/), mitochondria that originally

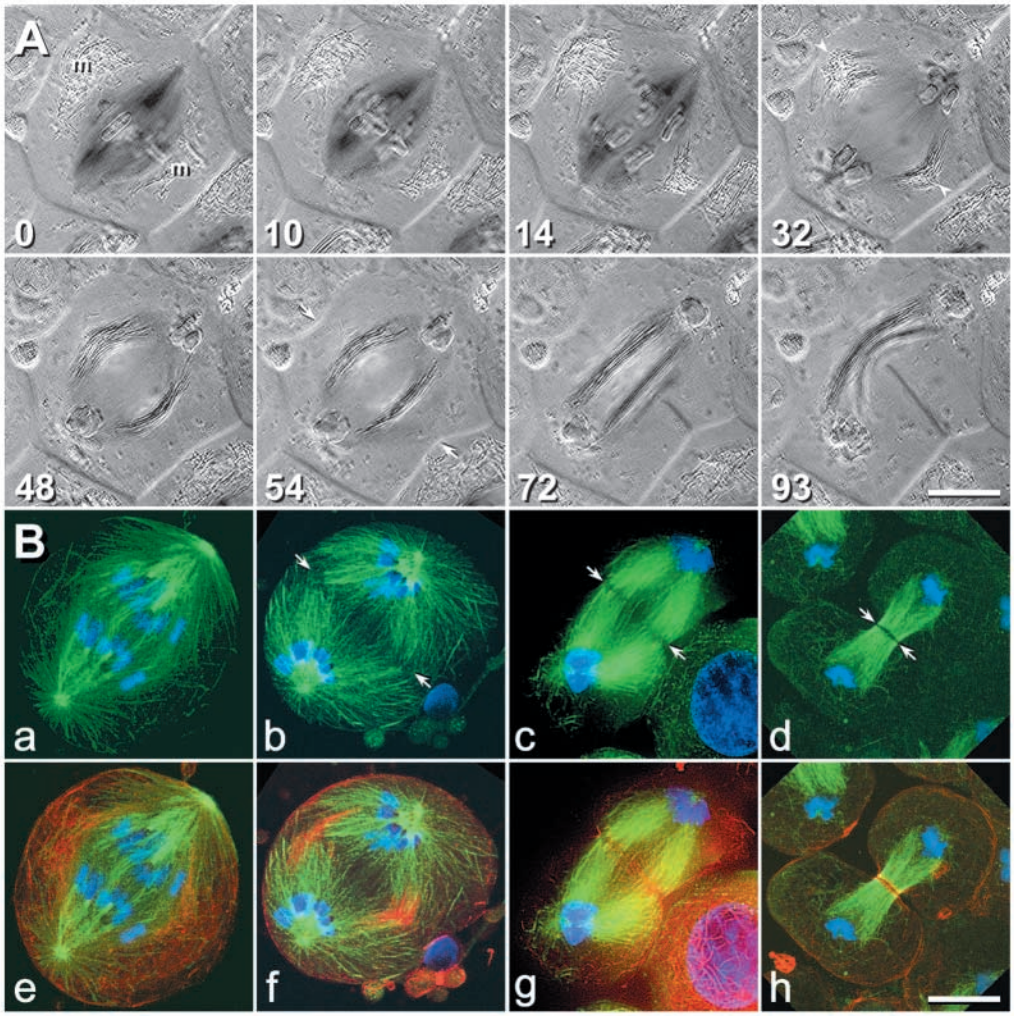

Fig. 1. Cytokinesis in grasshopper spermatocytes. (A) A polarization microscope sequence (Movie 1, http://jcs.biologists.org/supplemental/) showing behavior of mitochondria during cell cleavage in *Melanoplus femurrubrum* spermatocytes. Time is given in minutes. In metaphase (0 minutes), mitochondria (m) are scattered around the spindle apparatus (microtubules appear as black fibers). During early to mid anaphase (10-14 minutes), mitochondria accumulate around the spindle midzone (arrowheads, 32 minutes), and later bundle with and move poleward along peripheral microtubules of the central spindle (32-54 minutes). The cleavage furrow initiates (arrows, 54 minutes) and ingresses (72-93 minutes) at the midzone, which is symmetric to the bipolar spindle. (B) Localization of microtubules (green), actin filaments (red), and chromosomes (blue) in cells fixed at various stages during cell cleavage. Actin filaments, distributed randomly through the cell in early anaphase (e, red), relocate to the microtubule overlapping zone where mitochondria accumulate (b,f, arrows) in late anaphase, corresponding to the 32-minute stage shown in A. During furrow initiation, actin filaments (g, red) further accumulate at the light-stained midzone (c, arrows) of the newly assembled central spindle (c,g, green). During furrow ingression, actin filaments (h, red) become extremely condensed on both sides of the narrowed midzone (d, arrows). Scale bars: 10 μm.

flank the spindle in metaphase (0 minutes, m) aggregate around the spindle equator during early to mid anaphase (10-14 minutes). As chromosomes segregate to spindle poles, mitochondria bundle together with spindle microtubules that overlap at the interzone (32 minutes, arrowheads). Staining of cells fixed at the corresponding stages (14-32 minutes) shows that actin filaments (Fig. 1Be,f, red) accumulate at the same region as mitochondria prior to furrow initiation, around the equator of overlapping spindle microtubules (a and b, arrows). During central spindle formation (48-54 minutes), this correlated distribution departs: mitochondria extend poleward along peripheral microtubules of the central spindle (32-54 minutes), whereas actin filaments (Fig. 1Bg, red) bundle around the lightly stained spindle midzone (Fig. 1Bc, green; arrows depict anti-tubulin antibody exclusion gap) where the cleavage furrow initiates (54 minutes; Fig. 1Bc,g, arrows). During cleavage furrow ingression (54-93 minutes), bundled microtubules and mitochondria are pushed inward by the contractile ring at the furrow, symmetrically dividing the central spindle and cell (Fig. 1Bd,h).

Micromanipulation designs to produce asymmetric distributions of spindle microtubules

We generated a low level of microtubule asymmetry (Fig. 2A) by taking advantage of chromosomal impact on assembly of spindle microtubules (Zhang and Nicklas, 1995). By removing all 11 bivalents but the X chromosome (Fig. 2Aa-b) during

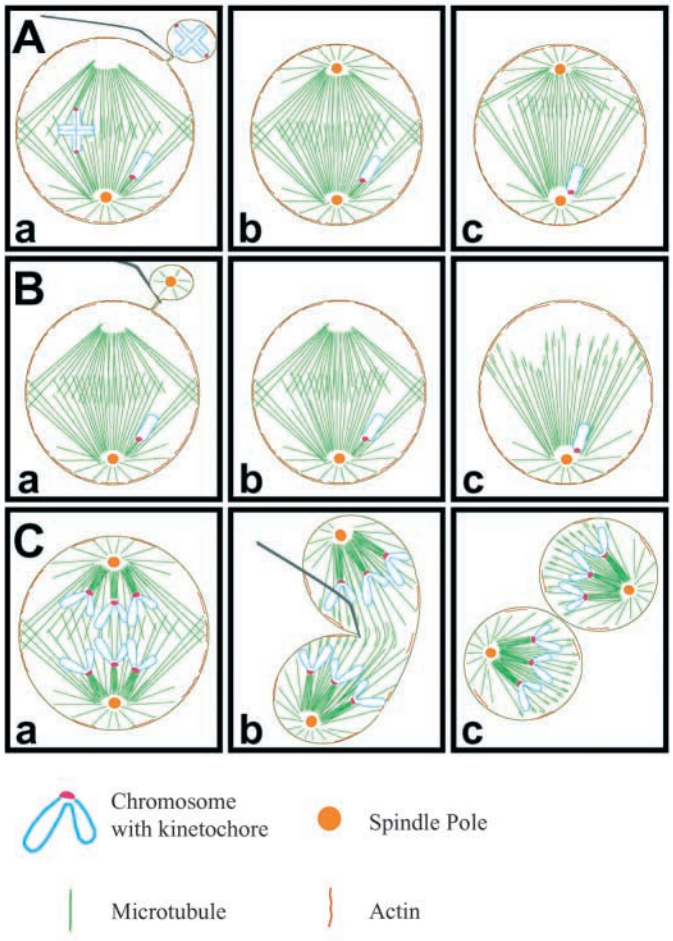

Fig. 2. Schematic illustration of the generation of microtubule asymmetry in grasshopper spermatocytes. (A) Bipolar spindles with an asymmetric microtubule distribution were produced by removing all chromosomes, except the X, in prometaphase of meiosis I cells (a,b). The X chromosome subsequently causes spindle asymmetry by promoting microtubule assembly at its proximal pole (c). (B) Greater microtubule asymmetry was produced by further removal of the aster from the achromosomal spindle pole (a,b) following extraction of all but the X chromosome, yielding a cell with a monopolar spindle bearing only the X chromosome (c). (C) The greatest spindle asymmetry was created by cutting cells between segregating chromosomes in anaphase (a,b), producing two cells, each containing a half-spindle with all segregated chromosomes (c).

meiosis I in *Melanoplus femurrubrum*, microtubule assembly was greatly enhanced at the spindle pole containing the X (Fig. 2Ac). A greater microtubule asymmetry was induced by further removal of the aster from the achromosomal spindle pole (Fig. 2Ba-b), producing a cell with a monopolar spindle bearing only the X (Fig. 2Bc). The highest microtubule asymmetry was created by surgically cutting a cell in two during early anaphase between segregating chromosomes (Fig. 2Ca-b). Since spindle microtubules from opposite poles overlap extensively during anaphase at the interzone (McIntosh et al., 1979; Inuoé, 1981; McIntosh, 1985) where they interact through kinesin in insect cells (Sharp et al., 1999), cutting through the equator using a fine microneedle inevitably produces two half-spindles with cut plus-ends overlapping with short severed microtubules from the opposite half-spindle (Fig. 2Cc). Cut cells provide a unique opportunity to test if and how reorganization of the spindle affects reformation of the midzone, distribution of actin filaments, and induction of cell cleavage.

Furrow positioning in cells with asymmetric microtubule distribution mediated by the X chromosome

A single chromosome, whether a bivalent or the X, positioned at one pole in a bipolar spindle can cause a fourfold spindle asymmetry in microtubule density in grasshopper spermatocytes (Zhang and Nicklas, 1995). We chose the X chromosome to induce microtubule asymmetry because it is a univalent and naturally attached only to one pole. Thus, removal of all bivalents but the X will not trigger spindle checkpoint control that halts cell division (Nicklas, 1997). Following micromanipulation (Fig. 2Aa-c), microtubule density in a polarization microscope sequence (Fig. 3A, 0-57 minutes; see also Movie 2, http://jcs.biologists.org/supplemental/; n=12) increases dramatically at the pole associated with the X chromosome. Immunostaining of cells fixed at corresponding stages (Fig. 3Ba,b, green) shows that the resulting asymmetric spindle assembles more and longer microtubules at the chromosome associated pole but fewer and shorter microtubules at the achromosomal pole. Elapse of ‘anaphase’ in these cells can be identified by split of X chromosome arms (82 minutes, circle; Fig. 3Ba,b, blue) and disassembly of spindle microtubules as a reduction in birefringence (82-104 minutes) and fluorescence (Fig. 3Ba,b, green) at the X chromosome-containing pole. The microtubule asymmetry has a profound effect on distribution of mitochondria (0-87 minutes, m and arrowheads) and actin filaments (Fig. 3Be,f, red) in anaphase, since they both accumulate at the shifted region of overlapping microtubules. As the central spindle forms (104 minutes; Fig. 3Bb,c, green), mitochondria bundle along microtubules and an off-equator ‘midzone’ (Fig. 3Bb,c, arrows) appears at the shifted region of overlapping microtubules. Cleavage furrow initiation is dislocated 16±2% from the genuine central position of the spindle (104 minutes, arrows; n=7) to the location of the shifted midzone (Fig. 3Bc, arrows) that is embraced with a well organized contractile ring (Fig. 3Bg, red; see also Movie 3, http://jcs.biologists.org/supplemental/). During ingression, the furrow (104 minutes onward) and contractile ring (Fig. 3Bg,h, red) reposition with the midzone back toward the chromosome-bearing pole (Fig. 3Bd, arrows) as spindle symmetry re-establishes following disassembly of X chromosome-associated microtubules (Fig. 3Bd,h, green). Owing to the initial asymmetric furrow initiation, however, the repositioned furrow does not divide the cell equally (152 minutes; Fig. 3Bd,h).

Furrow positioning in cells with a monopolar spindle associated with the X chromosome

To induce a greater amount of microtubule asymmetry, we further removed the aster from the achromosomal spindle pole (Fig. 2Ba-c) in metaphase cells containing only the X chromosome. This micromanipulation completely destroys spindle bipolarity and generates cells (n=5) containing a monopolar spindle with much enhanced microtubule density at the X chromosome-associated pole (Fig. 4A, 0 minutes; see also Movie 4, http://jcs.biologists.org/supplemental/). Initially, microtubules radiate exclusively from the remaining pole

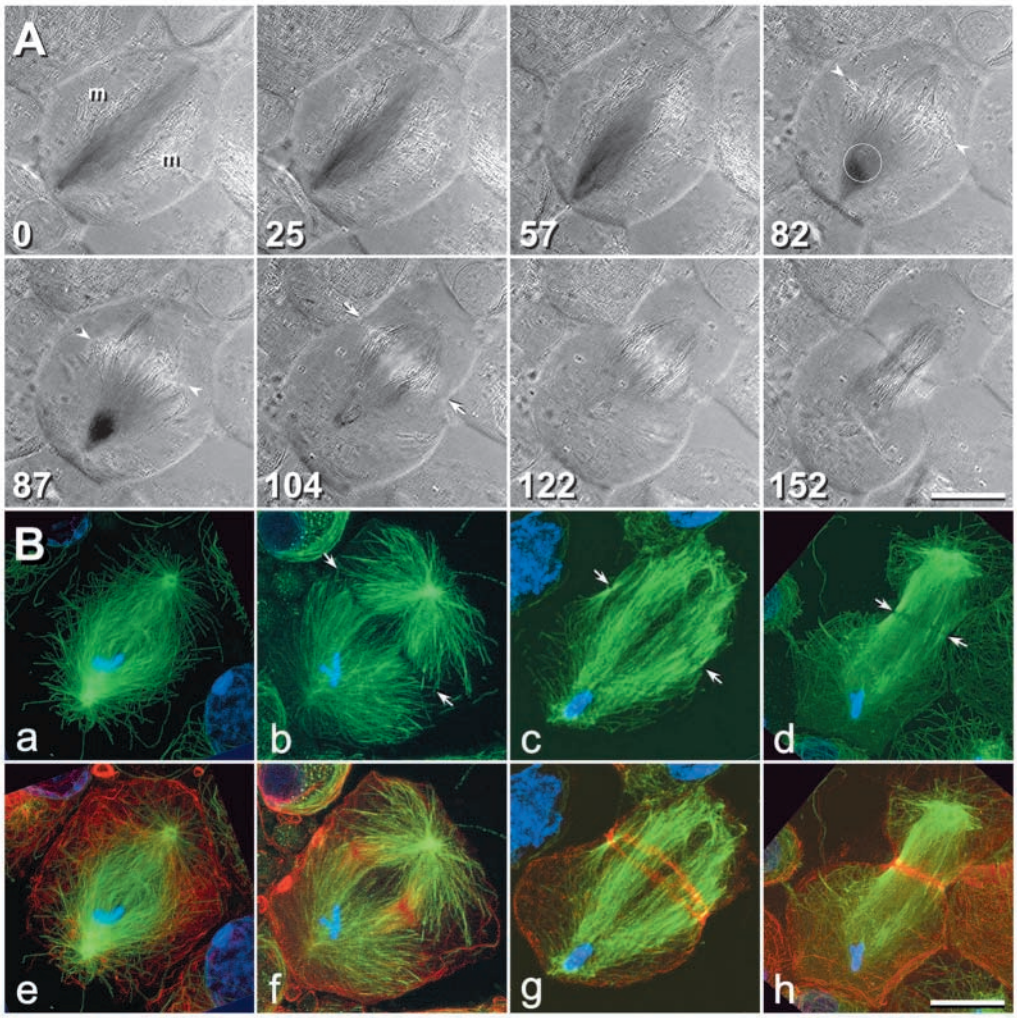

Fig. 3. Microtubule distribution and furrow induction in cells containing a bipolar spindle and the X chromosome. Following removal of all bivalents, microtubule density increases at the pole bearing the X chromosome, which is shown in both polarization microscope images (A, 0-57 minutes; see also Movie 2, http://jcs.biologists.org/supplemental/) and a cell fixed at the corresponding stage and stained (B) for microtubules (a, green), actin filaments (e, red), and the chromosome (a, blue). The asymmetric microtubule distribution shifts mitochondria (m) away from the chromosome bearing pole (0-57 minutes). Anaphase onset is recognized as a spilt of the X chromosome (82 minutes, circle in A) arms (b, blue) and disassembly of spindle microtubules, which appears as a reduction in spindle birefringence (82-104 minutes) and fluorescence (a,b, green). During anaphase (87 minutes) mitochondria (arrowheads) and actin filaments (f, red) accumulate at the midzone (82-87 minutes; b, arrows), which is shifted away from the pole with greater microtubule density. Following anaphase, mitochondria extend along microtubules (87-122 minutes) as actin filaments bundle into the contractile ring (f,g, red; see also Movie 3, http://jcs.biologists.org/supplemental/) around the shifted midzone (c, arrows), asymmetrically inducing cell cleavage 16±2% from the genuine central position of the spindle (104 minutes, arrows; *n*=7). Despite spindle reorganization (c,d, arrows) that symmetrically shifts the midzone and furrow (104 minutes onward; g,h, red) along the central spindle, the initial asymmetry to the cell remains (152 minutes; d,h). Scale bars: 10 μm.

toward the cell periphery (Fig. 4Ba) and mitochondria are scattered around the monopolar spindle (0 minutes). Shortly before anaphase, mitochondria become excluded to the frontier, or plus-ends, of radiating microtubule arrays (32 minutes, arrowheads). During anaphase, recognized by separation of the X chromosome (50 minutes, arrow) and reduction of spindle birefringence, microtubules gradually disassemble as the chromosome moves poleward (50-77 minutes). Not recognizable in polarization images but obvious in fixed cells is formation of a truncated new half-spindle (Fig. 4Bb, green) beyond the excluded mitochondria. Microtubules in the new half-spindle and existing monopolar spindle overlap and establish a significantly shifted ‘midzone’ (Fig. 4Bb, arrows). Actin filaments, distributed throughout the cell before anaphase (e, red), accumulate at the newly formed midzone (Fig. 4Bf, red) in accordance with distribution of mitochondria during anaphase (32-77 minutes). As a new bipolar central spindle forms, mitochondria gradually move back along microtubules toward the original pole (77-87 minutes). Cleavage furrow initiation (87 minutes, arrows) corresponds with the actin enriched midzone position (Fig. 4Bf, red), displaced 42±2.1% (*n*=5) from the equator of the central spindle. Meanwhile, microtubules at the new half-spindle elongate, shifting the midzone toward the spindle equator (c, arrows). Furrow ingression (87-142 minutes) follows the spindle midzone surrounded by the contractile ring (Fig.

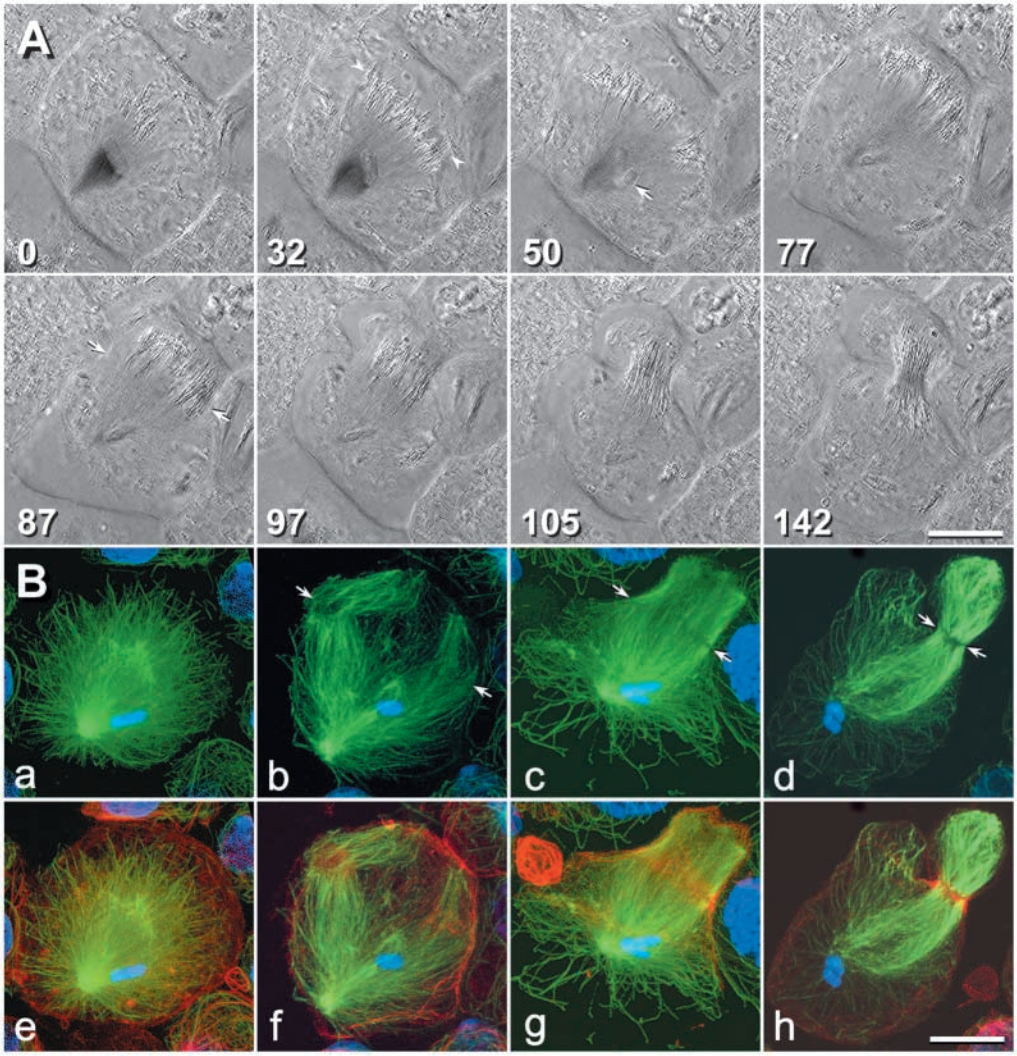

Fig. 4. Microtubule distribution and furrow induction in cells containing a monopolar spindle and the X chromosome. Following removal of all bivalents and one aster, the spindle becomes monopolar as microtubules are stabilized around the X chromosome and the remaining pole (A, 0 minutes; see also Movie 4, http://jcs.biologists.org/supplemental/; Ba, green). Mitochondria, scattered at the spindle periphery initially, accumulate at the plus-ends of radiating spindle microtubules (0-32 minutes, arrowheads; a, green). Following anaphase, as recognized by the separation of the X chromosome (50 minutes, arrow), mitochondria move back toward the original pole along central spindle microtubules (50-87 minutes). Accumulation of actin filaments (f, red) and furrow initiation are shifted 42±2.1% (87 minutes, arrows; *n*=5) from the central position to the 'midzone' (b, arrows), asymmetrically defined by microtubules from the original and newly established half-spindles. The furrow ingresses (87 minutes onward) as actin filaments accumulate and constrict (g,h, red) the midzone (c,d, arrows). Owing to microtubule elongation at the new half-spindle (c,d, green), the furrow eventually becomes more symmetric with respect to the central spindle (142 minutes) but retains its asymmetry with respect to the cell (97 minutes onwards). Scale bars: 10 μm.

4Bg,h, red), which becomes slightly more symmetric in the central spindle (142 minutes) because of microtubule elongation at the new half-spindle (Fig. 4Bb-d, arrows). However, the initial asymmetry to the cell remains during cleavage (142 minutes; Fig. 4Bd,h).

Furrow positioning in cut cells containing a half-spindle with all separating chromosomes

The highest microtubule asymmetry was generated by severing early anaphase cells (*n*=19) at the overlapping region of interzonal microtubules between segregating chromosomes (Fig. 2C). The microsurgery produces two small cells, each containing an extremely asymmetric half-spindle whose newly-exposed plus-ends of microtubules overlap with short severed microtubules from the opposite half-spindle (Fig. 2Cc). During microsurgery, chromosomes continue to move poleward at a relatively unperturbed rate (Fig. 5A, 0-40 minutes; see also Movie 5, http://jcs.biologists.org/supplemental/; 0.47±0.11 μm/minute; *n*=6) as compared to non-manipulated cells (0.58±0.16 μm/minute; *n*=19). Immediately following cutting, mitochondria (0 minutes, m) and actin filaments (Fig. 5Bf, red) are slightly disorganized by the needle to the region between the cut edge and chromosomes (8 minutes). As chromosomes move poleward, both mitochondria (15-25 minutes) and actin filaments (Fig. 5Bf,g,

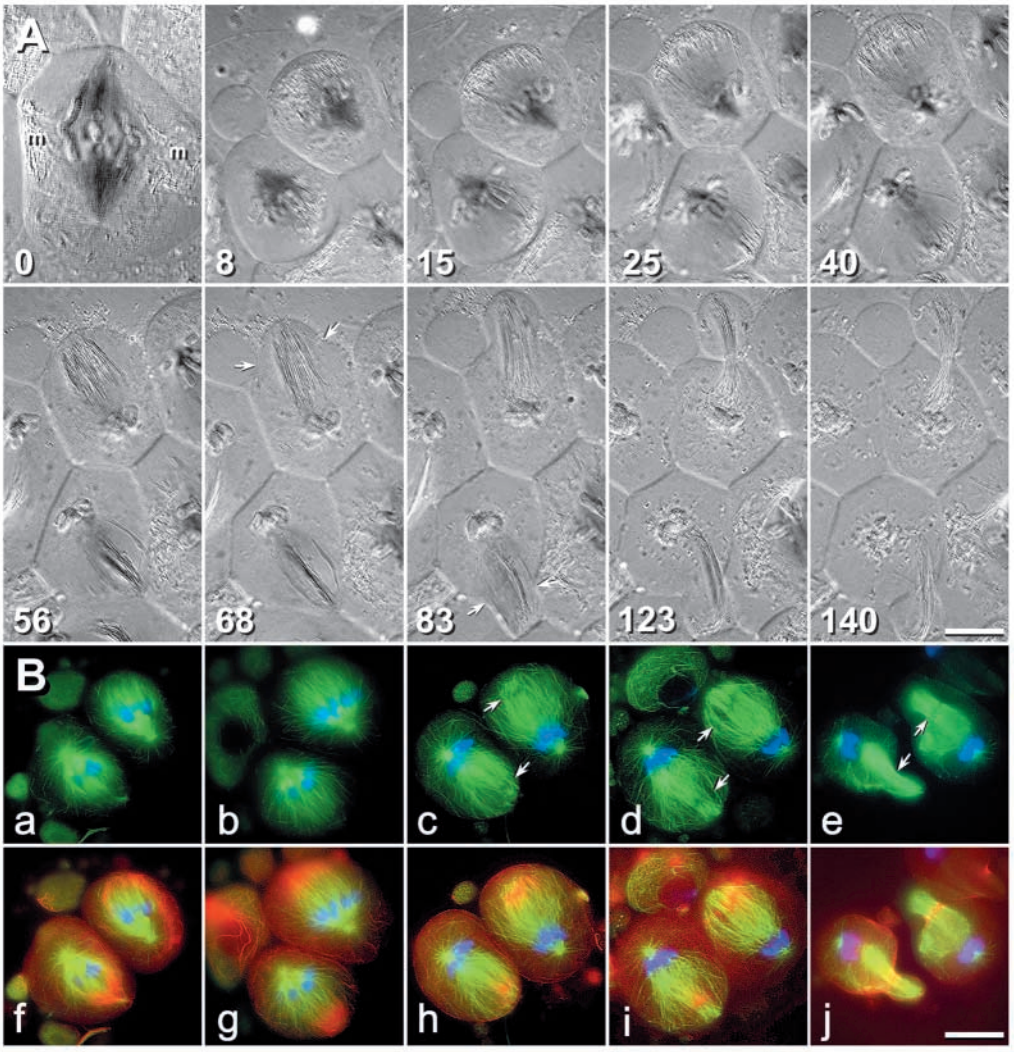

Fig. 5. Microtubule distribution and furrow induction in cut cells containing a half-spindle and segregated chromosomes. Spermatocytes (A, 0 minutes) were cut in two (8 minutes) between segregating chromosomes in early anaphase, exposing plus-ends of overlapping microtubules (Ba, green). As chromosomes continue to move poleward (8-40 minutes; see also Movie 5, http://jcs.biologists.org/supplemental/), both mitochondria (0-8 minutes, m) and actin filaments (f, red), slightly disorganized by cutting, are excluded toward microtubule plus-ends at the cell periphery (15-25 minutes; f,g, red). When chromosomes arrive at the pole (40 minutes), a new half-spindle emerges and overlaps with cut plus-ends of original half-spindle microtubules (c, green), forming an extremely asymmetric 'midzone' (c, arrows). Actin filaments (h, red) continue to follow microtubule plus-ends, now overlapping at the midzone, whereas mitochondria begin to extend along microtubules (40 minutes onwards). Furrow initiation (68-83 minutes, arrows) and contractile ring formation (i, red) occur at the asymmetric midzone, displaced 48.6±3.8% (n=8) away from the central position. As the furrow ingresses, the contractile ring (i,j, red) and furrow (83 minutes onward) follow the midzone position (c-e, arrows), becoming progressively more symmetric with respect to the central spindle because of elongation of the new half-spindle (c-e, green). But the furrow remains asymmetric to the cell because of the initial asymmetry at furrow initiation (140 minutes). Scale bars: 10 μm.

red) are excluded toward the cut plus-ends of half-spindle microtubules (Fig. 5Ba,b; f,g, green). Remarkably, a new half-spindle initiates as short, bundled microtubule arrays assemble in the region of microtubule overlap at the cut-end of the original half-spindle in both cut-cells (Fig. 5Bc,h, green; arrows). As spindle bipolarity reestablishes, actin filaments are restricted by microtubules from both poles to an off-equator 'midzone' (Fig. 5Bh, red). Mitochondria, originally excluded at the plus-ends of microtubules in the original half-spindle (15-25 minutes), gradually move back along spindle microtubules toward the original pole (25-68 minutes). The new half-spindle elongates while the original half slightly shortens, shifting the midzone back toward the equator of the newly-formed central spindle (Fig. 5Bc-e). Redistribution of actin filaments precisely follows relocation of the midzone, repositioning progressively toward the equator of the central spindle (Fig. 5Bf-h, red). Shortly after the new half-spindle appears, a cleavage furrow initiates around the newly established midzone (68 minutes; Fig. 5Bd, arrows), which is extremely asymmetric to the genuine equator of the spindle (48.6±3.8%; n=8). The furrow ingresses while the new half-spindle continues to elongate and reposition the midzone (Fig. 5Bc-e, arrows). Consequently, the contractile ring (Fig. 5Bi-j, red) and furrow (68-140 minutes) become progressively more

symmetric with respect to the central spindle, sufficient to correct the asymmetry caused by the presence of chromosomes and their potent impact on microtubule assembly in the original half-spindle.

An analysis of spindle elongation relative to cleavage furrow position in living cut cells (n=8) is shown in Fig. 6. We aligned furrow positions (Fig. 6A, line) at progressive time points (0-28 minutes) and plotted changes in length of the new (d_1) and the original (d_2) half-spindles in a bar graph (Fig. 6B). At furrow initiation, microtubule length at the new half-spindle (d_1) was 5.48±1.75 μm. Once ingressed, the length increases to 12.45±1.76 μm, matching that of the now slightly shorter original half-spindle (d_2). However, the initial furrow asymmetry with respect to the cell remains (Figs 5A, 140 minutes; B).

Discussion

Distribution of spindle microtubules defines the position of the spindle midzone

The spindle midzone acts as the site of accumulation of many cytokinetic factors and plays a critical role during cytokinesis (for reviews, see Glotzer, 2001; Adams et al., 2001; Guertin et al., 2002; Scholey et al., 2003). Our results demonstrate that midzone position is defined by distribution of spindle microtubules; it can form at any region in a spindle where microtubules overlap during late anaphase (Figs 3-5), even if the overlap is extremely asymmetric in the spindle. When compared at the time of furrow initiation, the initial midzone position correlates closely with the amount of microtubule asymmetry generated; deviating from the genuine spindle equator by 16±2% (n=7) in the X chromosome alone induced asymmetry (Fig. 3) of up to 48.6±3.8% (n=8) in cut cells (Fig. 5).

The dislocated midzone is dynamically defined by redistribution of microtubules during spindle reorganization and elongation, repositioning progressively back toward the genuine central position of the spindle (Figs 3-5). The most dramatic repositioning is observed in cut cells containing a half-spindle with a midzone initially formed at the remaining microtubule overlap where cutting was performed (Figs 5, 6). The assembly and elongation of a new half-spindle at the cut plus-ends of microtubules gradually shift the midzone back toward the true equator of the newly formed bipolar spindle during furrow ingression. The original half-spindle occasionally shortens (Fig. 6A), but statistically shows no significant change in length (6B). Notably, monastrol-induced monopolar spindles lack antiparallel microtubules and sustain monopolarity during cytokinesis (Canman et al., 2003). We think that cutting through the equator of an early anaphase spindle creates a microtubule overlap at the cut end of each half-spindle. The severed plus-ends of half-spindle microtubules overlap with cutting-exposed minus-ends of short severed microtubules left from the opposite half-spindle. It is not clear whether individual severed microtubules are statically associated with the tips of the half-spindle or undergo dynamic instability, disassembling and reassembling rapidly. In either case, however, as a population of original and/or newly assembled microtubules, they could be dynamically preserved at the overlap of the half-spindle. Presumably, these short severed microtubules can serve as nucleation seeds and assemble de novo into a new half-spindle. Because severed minus-ends of spindle microtubules are stable in grasshopper spermatocytes (Nicklas, 1989), we think that new half-spindle elongation occurs because of the growth of plus-ends of microtubules. Following its establishment, addition of tubulin at plus-ends along with motor-driven antagonistic sliding of microtubules (Masuda and Cande, 1987; Cande and Hogan, 1989; Nislow et al., 1992; Sawin and Endow, 1993; Sharp et al., 1999; Mishima et al., 2002; Dechant and Glotzer, 2003; Goshima and Vale, 2003) may extend the new half-spindle away from microtubule overlap at the midzone. This apparent microtubule extrusion process may occur much as described for formation of overlapping central spindle microtubules from chromatin beads in *Xenopus* egg extracts (Heald et al., 1996).

Assembly of a new half-spindle requires neither the centrosome nor chromosomes, since both are absent at the assembly site when the aster is removed from the

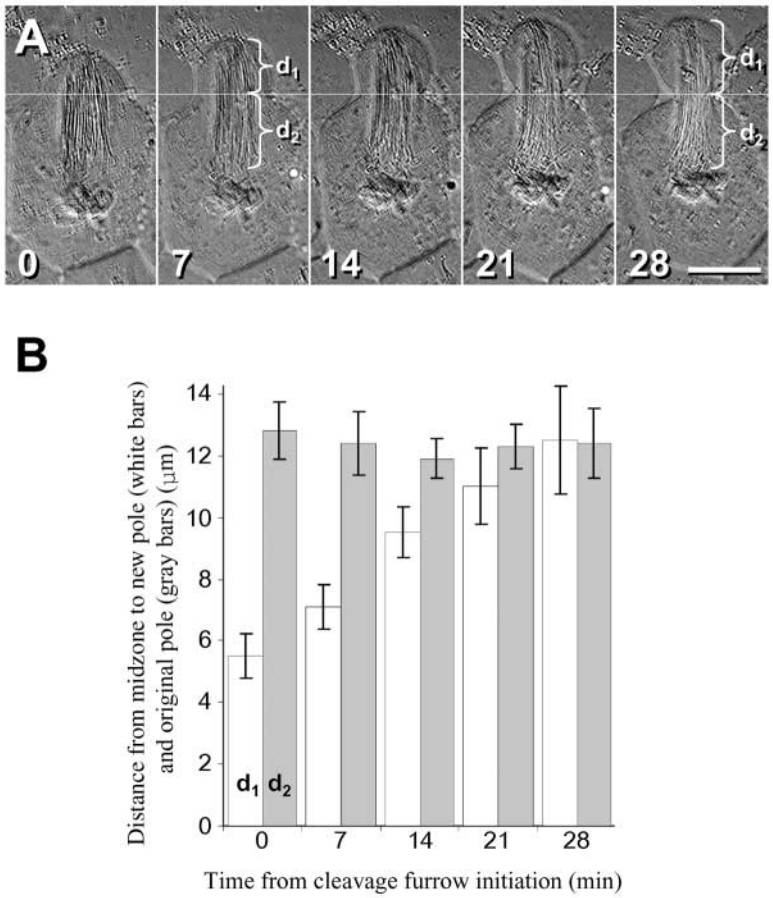

Fig. 6. Re-establishment of spindle bipolarity relative to changes in half-spindle length in cut cells. (A) Polarization microscope images illustrating length change measurements (n=8) of the new (d_1) and original (d_2) half-spindles with respect to the furrow position (line). Time is given in minutes. (B) Bar chart showing that during furrow ingression, the length of the new half-spindle (d_1) has more than doubled, from 5.48±1.75 to 12.45±1.76 μm, whereas that of the original half-spindle remains relatively unchanged (d_2). Scale bars: 10 μm.

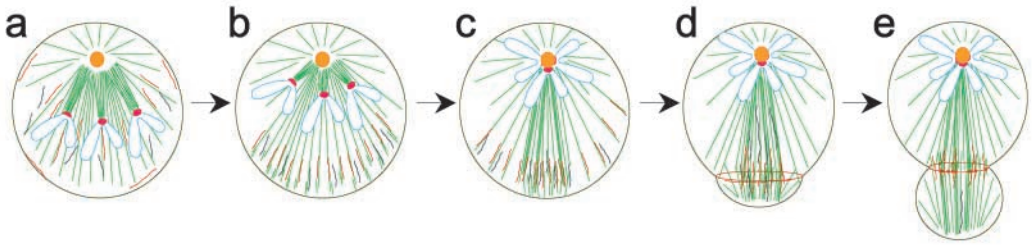

Fig. 7. Model for asymmetric furrow induction through microtubule-mediated distribution of actin filaments in cut cells. Following cutting in early anaphase (a), chromosomes (blue) continue to move poleward (b,c), whereas actin filaments (red) and mitochondria (gray) accumulate toward exposed plus-ends of spindle microtubules (green). Spindle bipolarity begins to re-establish when short antiparallel microtubules organize a new half-spindle and overlap with cut plus-ends of the original half-spindle to form a new 'midzone' (c). While mitochondria become aligned with spindle microtubules, actin filaments continue to follow microtubule plus-ends to the cell cortex where they bundle into a contractile ring and initiate an asymmetric furrow (d). The furrow ingresses as the new half-spindle elongates, shifting along with the midzone toward the middle of the central spindle (d,e).

achromosomal pole (Fig. 4) or when the spindle is cut in half into two separate cells (Fig. 5). Spindle self-assembly in living cells has been suggested from studies in *Drosophila* spermatocytes lacking asters (Bonaccorsi et al., 1998) or chromosomes (Bucciarelli et al., 2003); here we directly demonstrate that self-assembly can occur in the simultaneous absence of both spindle constituents. Microtubule arrays extruding from the midzone overlap may be bundled together at their minus-ends by spindle pole organizers into a truncated pole (Hatsumi and Endow, 1992; Vaisberg et al., 1993; Gaglio et al., 1996; Heald et al., 1996; Matthies et al., 1996; Merdes et al., 1996; Echeverri et al., 1996; Walczak et al., 1998; Goshima and Vale, 2003). Notably, however, chromosomes and centrosomes are only dispensable in grasshopper spermatocytes for post-anaphase spindle assembly, since they are required to form a metaphase spindle (Zhang and Nicklas, 1995). When chromosomes and centrosomes are removed in early metaphase, the spindle collapses and cannot reform until late anaphase (Alsop and Zhang, 2003). How might spindle assembly differ before and after anaphase? The answer may come from phase-specific distribution of midzone-associated proteins, such as CHO1 (Sellitto and Kuriyama, 1988), KLP3A (Williams et al., 1995) and Zen4/Cyk4 (Jantsch-Plunger et al., 2000; Mishima et al., 2002) that accumulate at the microtubule overlapping zone following anaphase. These proteins might crosslink antiparallel microtubules and establish bipolarity of the central spindle in the absence of asters and chromosomes. In fact, spindle bipolarity does not establish in monopolar spindles until cells enter anaphase (Fig. 4A, 50 minutes) when a dislocated microtubule overlapping zone appears (Fig. 4Bb), whereas a new half-spindle assembles shortly after micromanipulation in cells severed during anaphase (Fig. 5A, 25 minutes; B).

Since neither chromosomes nor centrosomes are required for post-anaphase spindle reassembly, neither is needed for formation and relocation of the midzone. Its position depends solely on distribution of spindle microtubules, which correlates closely with the amount of asymmetry generated (Figs 3-5). Chromosomes and centrosomes do, however, affect the initial dislocation of the midzone, but only through their impact on distribution of spindle microtubules. Once microtubules redistribute following establishment of a new half-spindle, the midzone can reposition toward the equator of the spindle, up against the presence of the entire complement of chromosomes (Figs 5, 6). Moreover, regardless of how disorganized a spindle is initially, owing to removal of both chromosomes and centrosomes, the midzone can always form symmetrically following organization of a central spindle-like structure (Alsop and Zhang, 2003). These findings do not rule out the possibility that asters (Rappaport, 1961; Rappaport, 1996; Hiramoto, 1971; Sanger et al., 1998) or chromosomes (Earnshaw and Bernat, 1991), when present, contribute to distribution of furrow signaling molecules to the midzone, but they do support the idea that neither of these spindle constituents is essential for distribution of furrow signals and induction of cell cleavage (Alsop and Zhang, 2003).

Central spindle organization persists through cytokinesis, continuously repositioning the midzone toward the equator of the central spindle while the cleavage furrow ingresses and constricts the midzone (Figs 3-5). This shows the tendency of the midzone to become symmetric in the central spindle. We are puzzled by how a central spindle can redefine its symmetry and properly position the midzone. Perhaps opposite half-spindles have a default length, determined by tubulin to polymer ratio and microtubule dynamic instability specific to the stage of the cell cycle (Mitchison and Kirschner, 1984).

Distribution of spindle microtubules defines position of actin filaments and cell cleavage

Superficially, our findings that cleavage furrow induction and ingression dynamically follow the location of the central spindle midzone (Figs 3-5) seem to agree well with the idea that the midzone organizes actin filaments into the contractile ring and induces cell cleavage (Cao and Wang, 1996; Gatti et al., 2000). This idea, however, cannot explain why the midzone may not be required for defining the cleavage plane. For example, cells mutant for Cyk4, Zen4 and CHO1 all fail to form a spindle midzone, yet they initiate a cleavage furrow at the central region of the spindle (Powers et al., 1998; Raich et al., 1998; Jantsch-Plunger et al., 2000; Matuliene and Kuriyama, 2002). Ectopic furrows that do not elaborate a spindle midzone can sometimes initiate a cleavage furrow (Savoian et al., 1999). Since the furrow eventually regresses in cells containing a disrupted midzone because of a lack of certain cytokinetic factors, theses findings suggest that the midzone plays a role in furrow ingression rather than its induction.

An important question remains as to how actin filaments accumulate at the central region of the spindle in the absence of the midzone. Our results suggest that spindle microtubules may exclude actin filaments to the equator before midzone

formation in the central spindle. The first indication comes from observations in non-manipulated control cells (Fig. 1). We find that actin filaments, as well as mitochondria, both align with radiating spindle microtubules at the equator as early as mid-anaphase, prior to the appearance of the midzone (Fig. 1A, 32 minutes; Be,f, red). Further evidence is obtained from analyzing distribution of actin filaments in cut cells (Fig. 5). As illustrated in a model (Fig. 7), cutting, performed in early anaphase before formation of the midzone, exposes a region of microtubule overlap at cut plus-ends in half-spindles (Fig. 7a, green). As chromosomes (blue) continue to move poleward, mitochondria (gray) and actin filaments (red) are excluded toward the plus-ends of half-spindle microtubules (Fig. 7a,b). This apparent movement of actin filaments and mitochondria occurs before the organization of a new half-spindle, thus in the absence of the central spindle midzone. Once a new half-spindle forms and overlaps with the original half (Fig. 7c), actin filaments are restricted at overlapping plus-ends of antiparallel microtubules where the midzone is defined by opposite half-spindles. As microtubules elongate at the new half-spindle (Fig. 7d-e), actin filaments shift with the midzone while bundling into the contractile ring (red), presumably by midzone factors (Larochelle et al., 2000; Robinson and Spudich, 2000; Glotzer, 2001; Guertin et al., 2002). This model entirely differs from the conclusion suggesting that furrow formation depends on a reduced microtubule density at the cell cortex in *Caenorhabditis elegans* (Dechant and Glotzer, 2003), but is consistent with the recent discovery that microtubules from monastrol-induced monopolar spindles are sufficient to mediate midzone factor distribution and induce a furrow (Canman et al., 2003). These investigators speculated that "cytokinesis in cells with bipolar spindles seems to occur as the sum of two asymmetric monopolar spindles". Here we provide a direct demonstration of their speculation by showing microtubule-dependent redistribution of actin filaments and repositioning of cell cleavage during mono to bipolar spindle transition (Fig. 5).

Microtubule-dependent distribution of actin filaments has been observed in a variety of cell types (reviewed in Gavin, 1997; Goode et al., 2000). Wound healing in *Xenopus* oocytes requires organization of microtubule arrays and formation of an actomyosin ring, a structure similar to the contractile ring in cytokinesis. Perturbations of microtubules prior to wounding inhibit actomyosin ring formation and wound closure (Bement et al., 1999). During cellularization of the *Drosophila* syncytial blastoderm, actin filaments appear to move along microtubules toward their plus-ends (Foe et al., 2000). Analysis of *Drosophila* embryonic extracts has revealed 21 proteins capable of interacting with both actin filaments and microtubules, seven of which have been previously implicated in cellularization and/or cytokinesis (Sisson et al., 2000). Two kinesin-like motor proteins, KLP3A and CHO1, have been shown to interact with actin filaments (Sisson et al., 2000; Kuriyama et al., 2002). In plant cytokinesis, while the preprophase band may predefine the division plane, distribution of actin filaments to the equatorial plane during phragmoplast formation coincides with dynamics of central spindle microtubules (Zhang et al., 1993). Analysis of the *Arabidopsis* genome has identified six kinesins that potentially interact with actin filaments (Reddy and Day, 2001). Studies in *Xenopus* egg extracts indicate that microtubules may move actin filaments by two distinct mechanisms. Astral microtubules released from asters (Keating et al., 1997) may bind and exclude actin filaments, or actin filaments may translocate along microtubules in the presence of oocyte microtubule-binding proteins (Sider et al., 1999; Waterman-Storer et al., 2000). Similar microtubule-dependent mechanisms might exist in grasshopper spermatocytes to exclude actin filaments toward the spindle midzone.

The unique colocalization of mitochondria with actin filaments following anaphase and with central spindle microtubules prior to cell cleavage makes one wonder whether the mitochondria in grasshopper spermatocytes, unlike tissue cells, play critical roles in promoting central spindle assembly and furrow initiation. A naturally occurring event during meiosis II, however, implies that mitochondria distribution is irrelevant to cell cleavage. Resolved from meiosis I, mitochondria are restricted only on one side of the meiotic II spindle, yet actin filament distribution is obviously not limited only to that side, suggesting that their colocalization is not required for distribution of actin filaments during cell cleavage in grasshopper spermatocytes. We think that the movement of mitochondria is coincidental to, but independent of, that of actin filaments, as mitochondria later bundle along microtubules, whereas actin filaments incorporate into the contractile ring around the midzone. Perhaps, mitochondrial movement is driven by a kinesin family motor protein similar to KLP67A that has been shown to be responsible for distribution of mitochondria along microtubules in *Drosophila melanogaster* (Pereira et al., 1997). Nonetheless, experiments are in progress to examine the specific role, if any, of mitochondria in cell cleavage in grasshopper spermatocytes.

In conclusion, our results suggest that microtubules, in addition to defining the spindle midzone position, may distribute actin filaments toward plus-ends of spindle microtubules at the midzone. These results not only support the notion that microtubules are the only structural constituent of the spindle apparatus required for induction of cell cleavage (Alsop and Zhang, 2003), but further suggest that microtubule distribution dictates the location of the midzone, accumulation of actin filaments, and ultimately position of cell cleavage. We do not know whether actin filaments move along microtubules (Sider et al., 1999; Bement et al., 1999; Waterman-Storer et al., 2000; Foe et al., 2000) or whether they are excluded by radiating microtubules to the overlapping zone by a force similar to polar ejection on chromosome arms (Rieder and Salmon, 1994). An attempt to test the mechanism of actin transport in living cells is now underway. Among many other remaining questions about the distribution of actin filaments, we are particularly puzzled by translocation of actin filaments from overlapping microtubules to the cell cortex. Especially obvious in cut cells, the majority of actin filaments, initially accumulated on central spindle microtubules, do not seem to reach the cortex (Fig. 5Bh, red), but later bundle into the contractile ring (i, red). Perhaps, bundled microtubule arrays that approach the cortex (i, green) laterally translocate actin filaments. Regardless of this, some midzone proteins must be critical for bundling of actin filaments into the contractile ring and proper cytokinesis (Larochelle et al., 2000; Robinson and Spudich, 2000; Glotzer, 2001; Guertin et al., 2002; Scholey et al., 2003).

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