

Microtubules are the only structural constituent of the spindle apparatus required for induction of cell cleavage

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Structural constituents of the spindle apparatus essential for cleavage induction remain undefined. Findings from various cell types using different approaches suggest the importance of all structural constituents, including asters, the central spindle, and chromosomes. In this study, we systematically dissected the role of each constituent in cleavage induction in grasshopper spermatocytes and narrowed the essential one down to bundled microtubules. Using micromanipulation, we produced “cells” containing only asters, a truncated central spindle lacking both asters and chromosomes, or microtubules alone. We show that furrow

induction occurs under all circumstances, so long as sufficient microtubules are present. Microtubules, as the only spindle structural constituent, undergo dramatic, stage-specific reorganizations, radiating toward cell cortex in “metaphase,” disassembling in “anaphase,” and bundling into arrays in “telophase.” Furrow induction usually occurs at multisites around microtubule bundles, but only those induced by sustained bundles ingress. We suggest that microtubules, regardless of source, are the only structural constituent of the spindle apparatus essential for cleavage furrow induction.

Introduction

Proper positioning of the cleavage furrow is essential for the equpartition of segregated chromosomes and other cellular constituents into daughter cells. It has long been recognized that the spindle apparatus dictates furrow position (Rappaport, 1996). It is unclear, however, which part of the spindle apparatus is the position determinant. Evidence from multiple cell types implies each of the structural constituents of the spindle apparatus, namely asters (including astral microtubules), the central spindle, and chromosomes, as the source of the furrow-positioning signal.

The evidence of asters in defining furrow position came largely from classical experiments in echinoderm embryos, cells with massive astral microtubule arrays as compared with their spindle (Larkin and Danilchik, 1999). When sand dollar eggs are experimentally distorted into a donut-like shape, subsequent cleavages occur not only at the spindle equators, but also between the asters of adjacent spindles (Rappaport, 1961). Furrow induction between the asters of two independent spindles has also been reported in fused somatic tissue culture cells (Eckley et al., 1997; Rieder et al.,

1997). Further, in sea urchin eggs, mechanical aspiration of the central spindle does not impede furrow induction (Hiramoto, 1971), whereas hydrostatic pressure-induced disassembly of astral microtubule arrays prevents the induction (Salmon and Wolniak, 1990).

In contrast, the central spindle appears more important for furrow positioning in insect or mammalian cells whose asters are relatively small. In cultured rat kidney epithelial cells (NRK), placing a perforation between the spindle midzone and cell cortex before anaphase results in the formation of a furrow at the site of the perforation, but not the cortex where astral microtubules are localized (Cao and Wang, 1996). Spermatocytes of the *Drosophila* mutant *asterless* fail to form normal asters, but are fully capable of positioning a cleavage furrow with respect to the midzone of a normal-appearing central spindle (Bonaccorsi et al., 1998). The central spindle is comprised of antiparallel, overlapping microtubules at the midzone or the “midbody,” which contains an amorphous deposit of electron-dense materials (McIntosh and Landis, 1971) that prevents binding of antitubulin antibodies. The midbody has been found to act as the site for accumulation of “chromosomal passenger proteins” (Earnshaw and Bernat, 1991) involved in furrow positioning (Martineau et al., 1995; Wheatley and Wang, 1996) and sustained cytokinesis (Wheatley and Wang, 1996; Savoian et al., 1999).

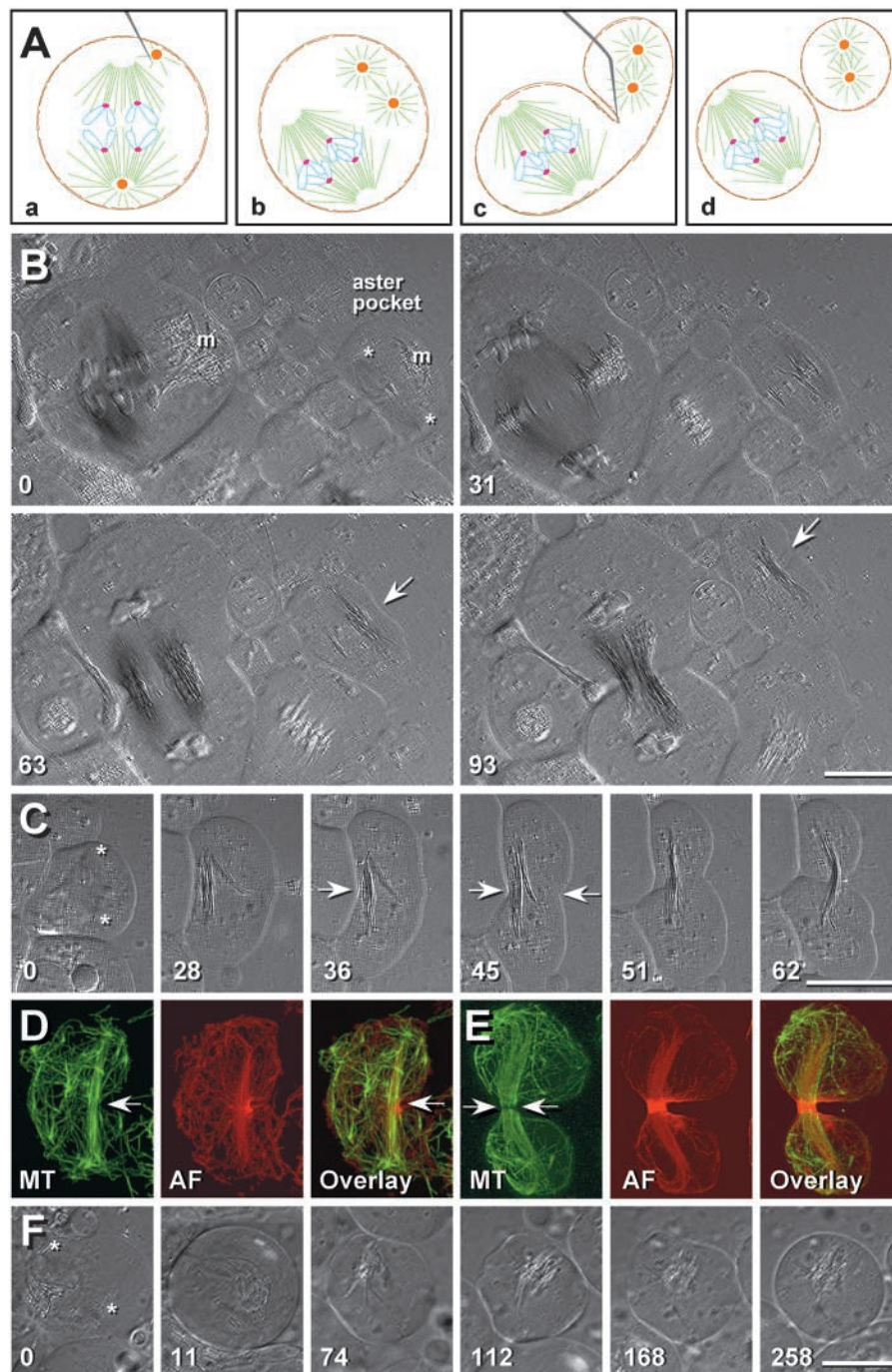
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Figure 1. Asters alone are sufficient for induction of cell cleavage.

(A) Asters are detached from spindle poles using a microneedle (a), placed at the cell periphery (b), and secluded into a membrane pocket cut off from the mother cell (c and d). (B) Polarization microscope images. Time is given in minutes. The membrane pocket contains two prominent asters (0 min, *) with mitochondria on one side (m). The mother cell (left) bears the spindle with truncated poles. Formation of microtubule-mitochondria bundles in the pocket (31), similar to the central spindle in its mother cell, corresponds with disappearance of asters. Furrow initiation of the pocket and mother cell occurs simultaneously (63). The pocket initiates a furrow where the bundled microtubule array is proximal to the cortex (63, arrow), ingressing asymmetrically on one side of the cell (93, arrow). (C) Symmetric furrow ingression can also occur in an aster-containing membrane pocket (0–62; see Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200301073/DC1>). (D) Microtubules (MT) at furrow initiation are less organized than observed in spindle-containing cells, but do exhibit a lightly stained midzone (MT, arrow) enriched with actin filaments (AF and Overlay, arrow). (E) Once ingressed, the bundled microtubule array establishes a clear midzone (MT, arrows) embraced with a tightly organized contractile ring (AF and Overlay). (F) Disassembly of astral microtubules (0, *) with nocodazole treatment (11 onward) prevents furrow induction in an aster-containing pocket (11–74) that bears only a disorganized mitochondrial mass. Although random contractions can still occur (74 onward), no furrow initiation is observed. Bars, 10 μm .



Chromosomal passenger proteins, such as INCENPs, TD-60, CHO-1, CENP-E, and CENP-F, are suggested to ride on chromosomes to the metaphase plate (Earnshaw and Mackay, 1994) where they redistribute at anaphase onset to the equatorial cortex, perhaps defining cleavage furrow position. However, removal of chromosomes from grasshopper spermatocytes during prometaphase does not affect cytokinesis (Zhang and Nicklas, 1996). Successful ectopic furrows in PtK1 cells appear to require the formation of a spindle midzone, which localizes INCENP and CHO-1, between disjointed asters that never possessed intervening chromosomes (Savoian et al., 1999). Such evidence suggests that spindle microtubules, but not chromosomes, are essential for

proper distribution of chromosomal passenger proteins. Surprisingly, *Caenorhabditis elegans* embryos do not appear to require the spindle midzone for furrow initiation, though the midzone is necessary for the completion of cytokinesis (Jantsch-Plunger et al., 2000).

Even as different spindle constituents appear sufficient to initiate a cleavage furrow in different cell types, it is unclear how microtubules that are always present in these cells contribute to initiation. Microtubules comprise spindle constituents such as asters or the central spindle and act as the scaffolding on which the midzone assembles and localizes factors critical for cytokinesis. It remains to be tested, however, whether microtubules are sufficient to position the furrow in

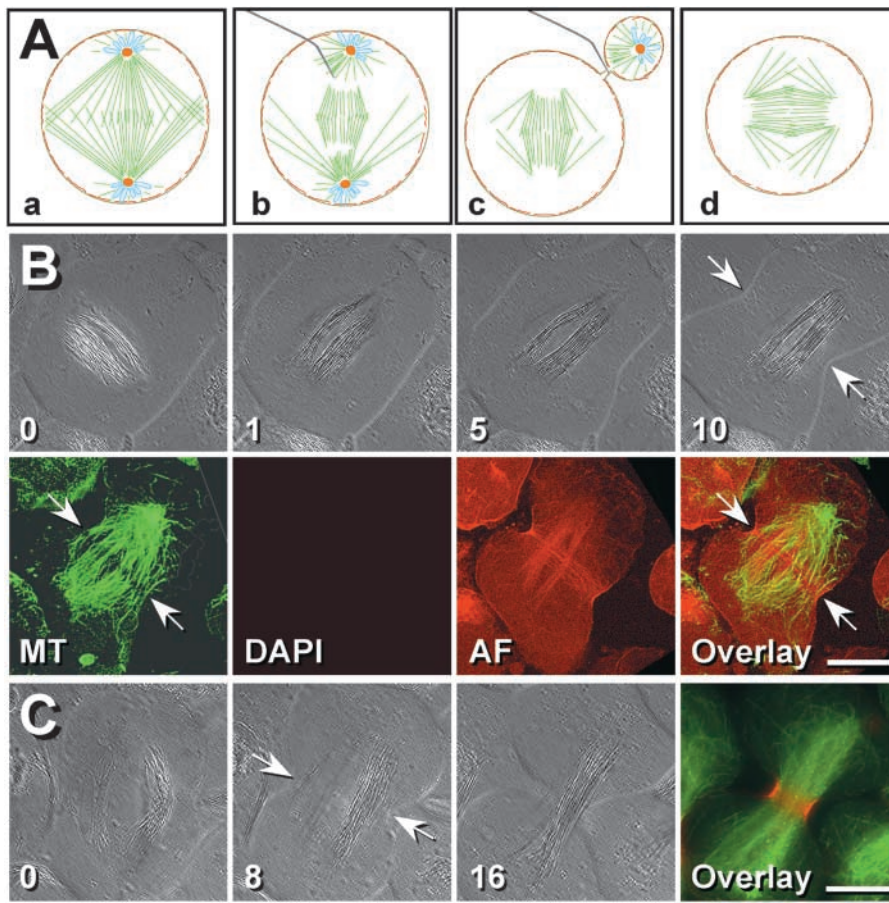


Figure 2. Truncated central spindles alone are sufficient for cell cleavage.

(A) Spindle poles, including asters and pronuclei, are removed from late anaphase or early telophase cells (a–c). The remaining central spindle fragment is then rotated $\sim 90^\circ$ from the original equator to avoid any predeposited furrow signals (d). (B) A truncated central spindle (0 min onward), lacking astral microtubules (MT) and chromosomes (DAPI), initiates a furrow (10 min, arrows) around the spindle midzone (MT, arrows) enriched with actin filaments (AF and Overlay, arrows). (C) Furrow ingression (0–16) is normal, bearing a distinct contractile ring at the midzone of the truncated spindle (Overlay). Bars, 10 μm .

the absence of other structural constituents of the spindle apparatus. In this study, we mechanically altered grasshopper spermatocytes to test one structural constituent of the spindle apparatus at a time, so as to systematically narrow down the minimal requirement for induction of cell cleavage.

Results and discussion

Cell cleavage can be induced independently by asters

To determine whether asters alone are sufficient for furrow induction in grasshopper spermatocytes, we produced membrane pockets that contain asters as the only spindle structural constituents and a small amount of mitochondria to provide energy. Using a microneedle, we detached asters from spindle poles in metaphase or early anaphase (Fig. 1 A, a and b) and secluded them into a membrane pocket (Fig. 1 A, c) cut off from the mother cell (Fig. 1 A, d). The pockets were monitored using a digital-enhanced polarization microscope. Several pockets were also fixed to observe the distribution of microtubules and actin filaments.

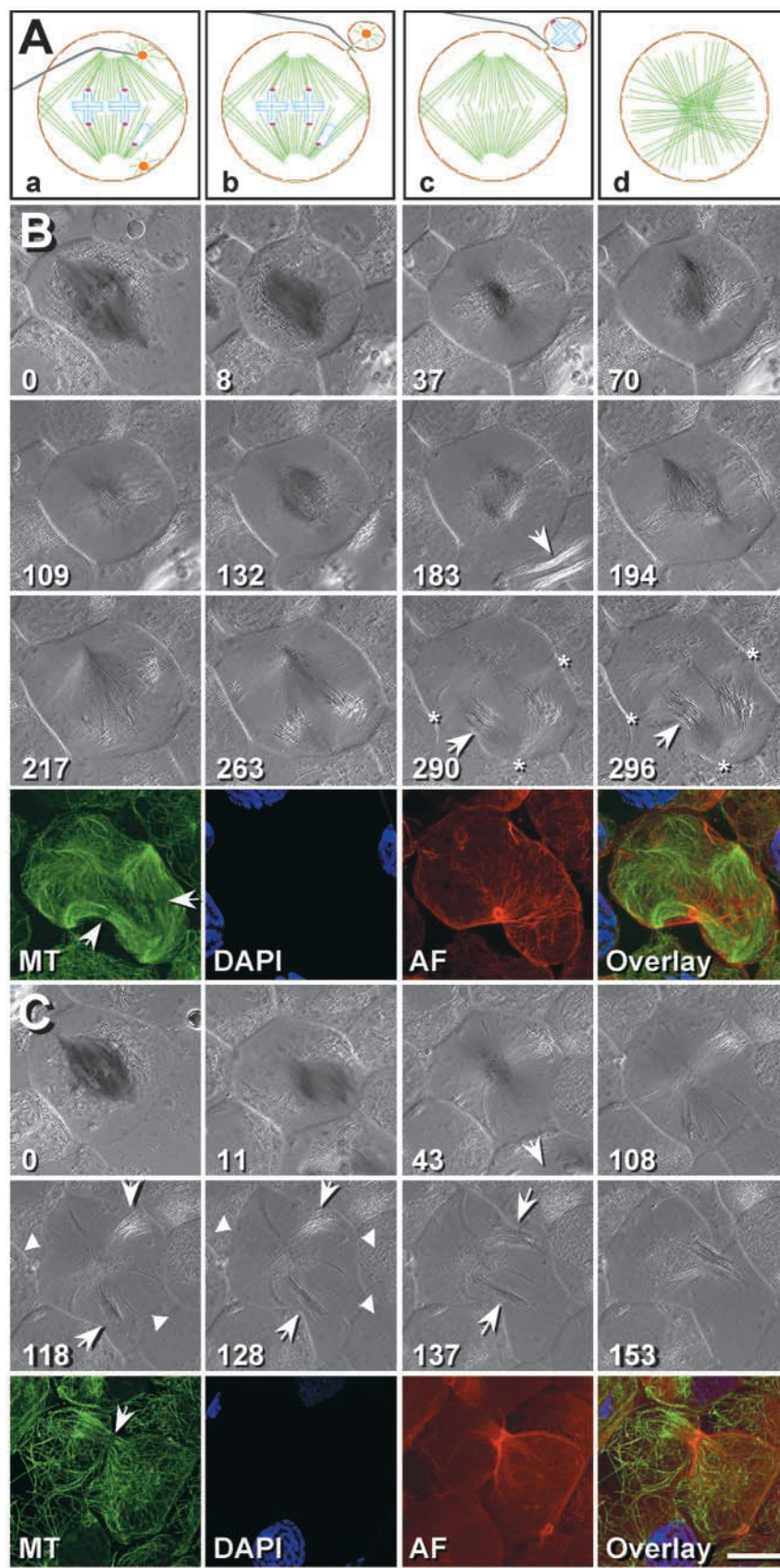
Detachment of asters using a microneedle is an all-or-none operation; successful manipulations (9 of 15 pockets had furrow initiation and ingression) seclude clearly visible asters in the pocket (Fig. 1 B, 0 min, *) and truncate the spindle in the mother cell (0, left). During late anaphase (Fig. 1 B, 31, left), asters in the pocket gradually disappear as astral microtubules bundle together with mitochondria (0, m) to form a central spindle-like structure similar to that in

the mother cell (31–63). Furrow initiation in the pocket occurs at about the same time as the mother cell and usually begins on the side proximal to the midzone of bundled microtubules (Fig. 1 B, 63, arrow; Fig. 1 C, 36, arrow, only the pocket is shown; see Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200301073/DC1>). Furrow ingression can occur either asymmetrically from the earlier initiation side (Fig. 1 B, 93, arrow), or rather symmetrically, even when bundled microtubules are acentric in the pocket (Fig. 1 C, 0–62; see Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200301073/DC1>). Bundled microtubules in an aster pocket, though disorganized, possess a visible midzone lightly stained by antitubulin antibody (Fig. 1 D, MT, arrow). The accumulation of actin filaments in the ingressing furrow corresponds precisely to the position of the midzone (Fig. 1 D, AF and Overlay, arrow). After ingression, bundled microtubules become more organized, exhibiting a distinct midzone (Fig. 1 E, MT, arrows) embraced with a tightly constricted contractile ring (Fig. 1 E, AF and Overlay). Disassembly of astral microtubules (Fig. 1 F, 0, *) with nocodazole (11 onward) in aster-containing pockets prevents the formation of bundled microtubules and furrow initiation (Fig. 1 F).

Cell cleavage can also be induced in the absence of asters

Despite the ability of asters to induce cell cleavage in grasshopper spermatocytes, we found that asters are not essential

Figure 3. Microtubules alone are sufficient for cell cleavage. (A) Asters and chromosomes are removed in metaphase (a–c), which induces disassembly of the spindle (d). (B) After manipulation, the spindle collapses (0–37 min; see Video 2, available at <http://www.jcb.org/cgi/content/full/jcb.200301073/DC1>), inducing transient and repeated formations of bipolar (70 and 194) or monopolar (217) pseudospindles, and eventually gives rise to relatively disorganized arrays of bundled microtubules bearing pseudopoles (290–296, *). Furrow initiation (290–296, arrows) and accumulation of actin filaments (AF and Overlay) correspond to the midzone (MT, arrows) of bundled microtubule arrays. Due to microtubule reorganizations, furrow initiation is delayed by nearly 2 h in comparison with an initially synchronized cell (183, arrow). (C) Often, newly assembled microtubule bundles radiate randomly (0–108) and induce furrow initiation at multiple sites (118–128, arrows and arrowheads; see Video 3, available at <http://www.jcb.org/cgi/content/full/jcb.200301073/DC1>). However, only furrows initiated by persistent bundles sustain (118–137, arrows), others eventually regress (arrowheads). Furrow ingression forces the bundled microtubule arrays together (128–153), bearing a single midzone (MT, arrow) with an ingressed contractile ring (AF and Overlay). Furrow initiation is delayed by ~ 1 h, judging by division in a neighboring cell (43, arrow). Bar, 10 μm .



for cytokinesis in these cells. We produced cells lacking asters by cutting and removing entire spindle poles from late anaphase or early telophase cells (Fig. 2 A, a–c). We then

rotated the remaining central spindle fragment $\sim 90^\circ$ from the equatorial cortex to avoid any predeposited furrow signals (Fig. 2 A, d). Despite being truncated (Fig. 2 B, 0 min

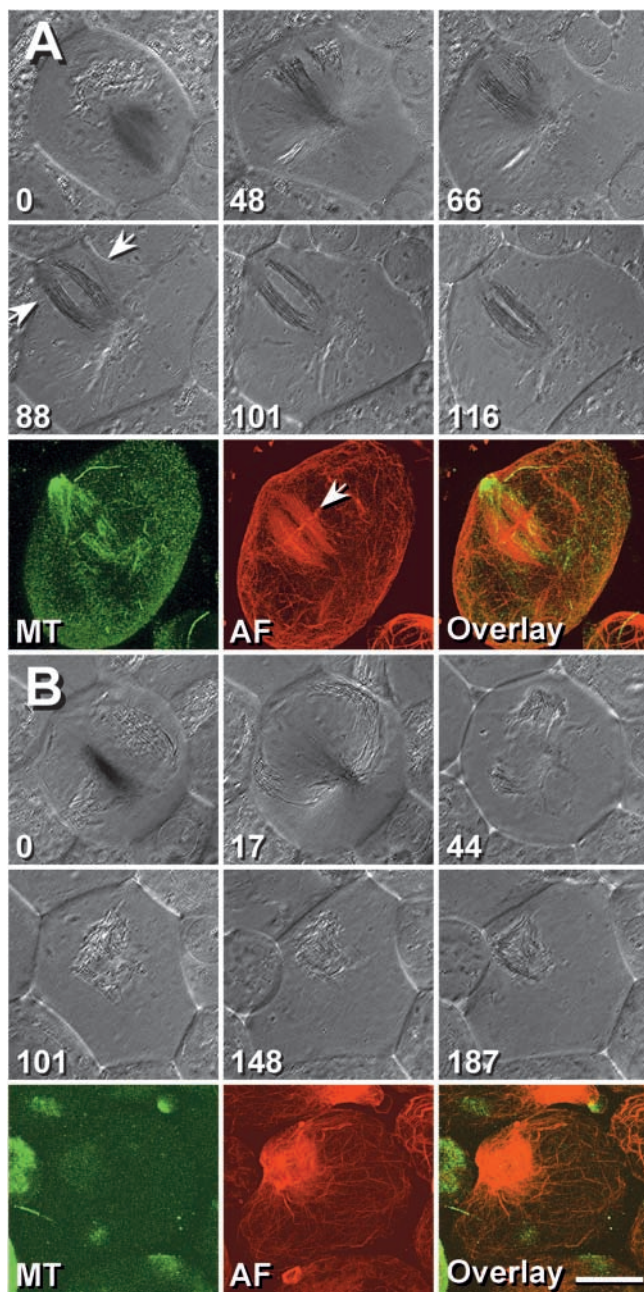


Figure 4. Bundled microtubule arrays are required for furrow induction. Cells were manipulated as in Fig. 3 to remove both asters and chromosomes, inducing assembly of bundled microtubules. (A) Partial disassembly of microtubules with nocodazole at furrow initiation (88 min) leads to regression of the furrow (101 onward). The fixed cell (116) retains remnants of bundled microtubule arrays (MT) and actin filaments from the disassembled contractile ring (AF and Overlay, arrow). (B) Complete disassembly of microtubules (MT) with nocodazole before furrow initiation (17) results in formation of a disorganized mitochondrial mass (44 onward). Although random contractions can still occur (101–187), no furrow initiation is observed. Actin filaments (AF and Overlay) are associated primarily with the mitochondrial mass. Bar, 10 μm .

onward; Fig. 2 C, 0 onward), lacking astral microtubules (MT and Overlay) and pronuclei (DAPI), the central spindle in all 21 cells produced remains well organized and initiates a cleavage furrow (Fig. 2 B, 10, arrows; Fig. 2 C, 8,

arrows). The cytoskeleton in cells fixed shortly after furrowing shows the expected stage-specific structures: bundled microtubule arrays with a distinct midzone (Fig. 2 B, MT, arrows) surrounded by actin filaments (AF and Overlay, arrows). Furrow ingression (Fig. 2 C, 16) in such manipulated cells is normal, showing a well-organized central spindle and a contractile ring enriched in actin filaments at the midzone (Overlay).

The ability of a truncated central spindle to induce cell cleavage (Fig. 2) may appear contradictory to findings that asters alone are sufficient for furrow induction in the same cell type (Fig. 1). Under both circumstances, however, microtubules are present and are able to organize into bundled arrays. It may be that the presence of microtubules, as opposed to other structural constituents of the spindle, is sufficient to induce cell cleavage.

Cell cleavage can be induced by microtubules as the only spindle structural constituent

To produce cells containing microtubules as the only spindle structural constituent, we removed both asters and all chromosomes (Fig. 3 A, a–c; Fig. 3 B, 0–8 min) from cells in metaphase (12 of 20 cells had furrow initiation and ingression). These manipulations not only remove other confounding spindle constituents, but also induce disassembly of the spindle and assembly of radiating microtubules bundled together with mitochondria (Fig. 3 A, d; Fig. 3 B, 37). Obviously, disassembly destroys bipolarity of the metaphase spindle and normal distribution of furrow signaling molecules, potentially from asters (Rappaport, 1996) or chromosomes (Earnshaw and Mackay, 1994). Radiating microtubules exhibit dynamics similar to that observed in metaphase (Cassimeris et al., 1988), repeatedly changing their length and distribution to transiently organize mono- or bipolar pseudospindles (Fig. 3 B, 70–217; see Video 2, available at <http://www.jcb.org/cgi/content/full/jcb.200301073/DC1>), and ultimately give rise to relatively disorganized arrays of bundled microtubules (Fig. 3 B, 290–296). Induction of cell cleavage occurs at midzones of bundled microtubule arrays (Fig. 3 B, 290–296, arrows) organized by randomly formed pseudopoles (290–296, \cdot). Cells fixed at initiation (296) show no chromosomes (DAPI) and exhibit central spindle-like bundled microtubule arrays with lightly stained midzones (Fig. 3 B, MT, arrows), where actin filaments are localized (AF and Overlay). Often, furrow induction occurs at multiple locations (Fig. 3 C, 118–128, arrows and arrowheads; see Video 3, available at <http://www.jcb.org/cgi/content/full/jcb.200301073/DC1>) due to random distribution of bundled microtubule arrays (43 onward). Many of these furrows, however, are transient and regress as microtubules reorganize (Fig. 3 C, 118–128, arrowheads). Successful furrows are usually induced independently at the midzones of persistent microtubule bundles (Fig. 3 C, 118–137, arrows), supporting earlier discoveries that furrow ingression does not require a complete contractile ring (Rieder et al., 1997). During ingression, these independent furrows can change directions of inward movement and force bundled microtubule arrays together (Fig. 3 C, 128–153, arrows). Cells fixed at completion of ingression

(153) exhibit one central microtubule bundle with a distinct midzone (Fig. 3 C, MT, arrow) colocalized precisely with a contractile ring (AF and Overlay).

Organization of actin filaments and furrow initiation in cells lacking both asters and chromosomes require the presence of bundled microtubule arrays; partial (Fig. 4 A, 101 onward; MT) or complete (Fig. 4 B, 44 onward; MT) disassembly of microtubules with nocodazole in such cells ($n = 5$) disrupts microtubule-associated distribution of actin filaments and prevents furrow initiation (Fig. 4, A and B, AF and Overlay). Random cell contractions, however, can still occur (Fig. 4 B, 101 onward), as is observed in mammalian cells that lack microtubules (Canman et al., 2000). We do not know how radiating microtubules organize midzones, as one would expect these microtubules to extend continuously from the cell center with their plus ends leading toward the periphery of the cell. A self-assembly process (Bonaccorsi et al., 1998; Bucciarelli et al., 2003) requiring midzone-associated motor proteins (Mishima et al., 2002) is probably involved in defining the midzone. Perhaps microtubules with opposite polarity are assembled at the distal ends of radiating microtubules. The elongation of newly assembled microtubules would shift antiparallel, overlapping microtubules along radiating microtubules to the midzone.

Cytokinesis in cells lacking both asters and chromosomes is variably delayed in comparison with naturally synchronized neighboring cells from the same cyst (Fig. 3 B, 183,

arrow). In some cells, the delay occurs in “metaphase” due to repeated assembly–disassembly of pseudospindles (Fig. 3 B, 37–290). In other cells, the delay happens in “anaphase” (Fig. 3 C, 43, arrow), judging by some anaphase-specific changes, such as fading of the collapsed spindle and outward movement of mitochondria (Zhang and Nicklas, 1996). In these cells, a lack of specific orientation of bundled microtubule arrays (Fig. 3 C, 108) perhaps delays cytokinesis, due to a random distribution of furrow signals and an increase of distance from the midzones of bundled microtubules to the cell cortex.

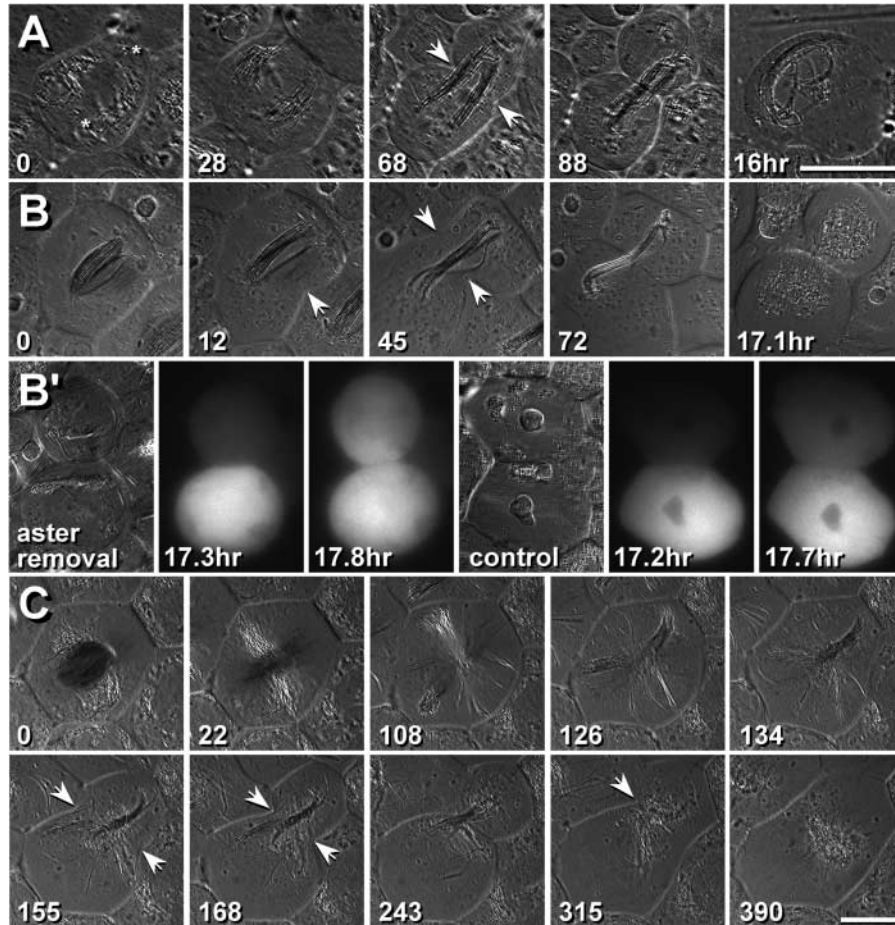
Cleavage furrow abscission in micromanipulated cells

Certain spindle constituents, such as centrosomes (Piel et al., 2001), may play a critical role during furrow abscission, resolution of the midbody and separation of daughter cells (Ou and Rattner, 2002), which can be tested in micromanipulated cells containing defined spindle constituents.

In cells containing asters (Fig. 5 A, 0, *) alone, furrow ingression (68, arrows) tightly constricts the bundled microtubule array (88). Surprisingly, despite the presence of centrosomes, such furrows ($n = 5$) eventually regress (Fig. 5 A, 16 h). In contrast, furrow ingression (Fig. 5 B, 12–72, arrows; see Video 4, available at <http://www.jcb.org/cgi/content/full/jcb.200301073/DC1>) in cells ($n = 11$) lacking asters and pronuclei sustains and leads to midbody abscission, yielding two daughter cells (17.1 h). Owing to observa-

Figure 5. Furrow abscission and regression in micromanipulated cells.

Unless indicated, time is given in minutes. (A) An aster-containing pocket, produced as in Fig. 1, undergoes successful furrow initiation (0–68 min, arrows) and ingression (88), but fails in abscission due to furrow regression (16 h). (B) A cell lacking both asters and pronuclei, manipulated as in Fig. 2, is fully capable of furrow induction (12–45, arrows), ingression (72), and normal abscission (17.1 h; see Video 4, available at <http://www.jcb.org/cgi/content/full/jcb.200301073/DC1>). (B') Rhodamine dextran microinjected into one daughter cell gradually flows into the other in both visually abscised experimental cells (aster removal, 17.3–17.8 h after cleavage initiation) and nonmanipulated controls (control, 17.2–17.7 h after cleavage initiation). (C) Despite normal furrow induction and ingression (155–243, arrows), cells containing only microtubules, manipulated as in Fig. 3, usually fail to separate (315–390, arrow; see Video 5, available at <http://www.jcb.org/cgi/content/full/jcb.200301073/DC1>). An exception is shown in Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200301073/DC1>. Bars, 10 μm .



tions that grasshopper spermatocytes remain connected after cytokinesis (Carlson and Handel, 1988) and cell cleavage regresses in *C. elegans* Zen-4 mutants even after daughter cells enter G1 phase (Severson et al., 2000), we compared visually abscised experimental cells ($n = 5$) with nonmanipulated controls ($n = 5$). In both cases, injection of rhodamine dextran (Bukauskas et al., 1992) into one daughter cell results in accumulation of fluorescence in the other (Fig. 5 B'), showing that micromanipulation does not alter the final stage of cell cleavage. When microtubules are the only remaining spindle constituent ($n = 6$; Fig. 5 C), furrow ingression (155–243, arrows) is usually followed by regression (Fig. 5 C, 315–390, arrow; see Video 5, available at <http://www.jcb.org/cgi/content/full/jcb.200301073/DC1>), except in one cell where successful abscission is observed (see Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200301073/DC1>; Video 5).

These experiments suggest that successful abscission in grasshopper spermatocytes does not require the presence of centrosomes. Failed abscission in cells containing only asters is probably due to a significant reduction in central spindle-associated proteins, such as *Cyk4* and *Zen4* (Jantsch-Plunger et al., 2000). Regression in cells lacking both asters and chromosomes is perhaps due to random distribution of bundled microtubule arrays that defuses midbody proteins required for furrow abscission. Perturbations to central spindle organization may also prevent abscission, as cells lacking asters but containing an unperturbed central spindle always abscise.

In conclusion, our results demonstrate that microtubules, whether from asters, the central spindle, or even a collapsed spindle lacking both asters and chromosomes, are sufficient to induce cell cleavage and maintain furrow ingression. We do not imply that microtubules act as an independent source of the furrowing signal, as a plethora of motor proteins and regulatory factors are critical for proper cytokinesis (for reviews see Earnshaw and Mackay, 1994; Laroche et al., 2000; Robinson and Spudich, 2000; Glotzer, 2001; Guertin et al., 2002). However, without asters and chromosomes, microtubules appear sufficient to mediate the distribution of associated cytokinetic factors, as judged by their ability to form midzone-bearing microtubule bundles and initiate cell cleavage. We therefore propose that microtubules, regardless of source, are the only structural constituent of the spindle apparatus required for induction of cell cleavage.

Materials and methods

Cell culture of grasshopper spermatocytes

Primary cultures of spermatocytes from the grasshopper *Melanoplus femurrubrum* were prepared by spreading a monolayer of cells on a glass coverslip under inert halocarbon oil (Zhang and Nicklas, 1999).

Micromanipulation with digital-enhanced polarization microscopy

Micromanipulations, such as chromosome/aster removal and cell cutting (Zhang and Nicklas, 1999), were performed using a fine glass needle with a tip diameter of $\sim 0.1 \mu\text{m}$, maneuvered using a Burleigh MIS-5000 series piezoelectric micromanipulator. Cells were observed with a high extinction/high resolution polarization microscope modified according to Inoué and Spring (1997), except from an Axiovert 100 microscope equipped with a 1.4 NA achromatic-aplanatic condenser, an infinity-corrected 1.4

NA/63x Plan-Apochromat objective lens (Carl Zeiss MicroImaging, Inc.), and a cooled CCD digital camera (ORCA-100; model C4742-95; Hamamatsu). Images were acquired and processed using Image Pro Plus software (Media Cybernetics).

Immunofluorescence microscopy

Cells were microfixed (Nicklas et al., 1979) at the moment of interest and stained for microtubules, actin filaments, and chromosomes. In brief, target cells on the coverslip were fixed by micropipetting microfixative (2% glutaraldehyde, 1% CHAPS, $0.33 \mu\text{M}$ rhodamine-phalloidin [Molecular Probes] in Pipes buffer) in cells' vicinity. The coverslip was then transferred into macrofixative (0.1% glutaraldehyde, 0.5% NP-40 in Pipes buffer). Microtubules were stained with anti- β tubulin (clone KMX-1; Chemicon) primary antibody and Alexa[®] Fluor 488-conjugated secondary antibody (Molecular Probes). Actin filaments were stained with $0.165 \mu\text{M}$ rhodamine-phalloidin (Molecular Probes). Coverslips were mounted in Vectashield (Vector Laboratories) containing DAPI to stain chromosomes. Image stacks were acquired using a confocal microscope (Leica TCS), processed in Adobe Photoshop 5.0[®], and reconstructed using Simple PCI software (C-imaging Systems).

Microinjection

We have succeeded in microinjection of grasshopper spermatocytes. In brief, micropipettes were pulled with a Flaming/Brown puller (model P-87; Sutter Instrument Co.), loaded with 2 mg/ml rhodamine-labeled dextran in a Pipes buffer, and maneuvered with a piezoelectric micromanipulator. A home-made high pressure (up to 60 psi) pneumatic system was used to simultaneously drive needle penetration and delivery of the fluorescent dye.

Nocodazole treatment

1 mg/ml nocodazole in Belars ringers was prepared from 10 mg/ml nocodazole in DMSO stock (Sigma-Aldrich) and micropipetted to the cell culture to a final concentration $\sim 20 \mu\text{g/ml}$.

Online supplemental material

The videos of polarization microscope sequences corresponding to Fig. 1 E (Video 1), Fig. 3 B (Video 2), Fig. 3 C (Video 3), Fig. 5 B (Video 4), and Fig. 5 C (Video 5) and Figs. S1 and S2 are available at <http://www.jcb.org/cgi/content/full/jcb.200301073/DC1>.

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