Centrosome-independent mitotic spindle formation in vertebrates

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Background: In cells lacking centrosomes, the microtubule-organizing activity of the centrosome is substituted for by the combined action of chromatin and molecular motors. The question of whether a centrosome-independent pathway for spindle formation exists in vertebrate somatic cells, which always contain centrosomes, remains unanswered, however. By a combination of labeling with green fluorescent protein (GFP) and laser microsurgery we have been able to selectively destroy centrosomes in living mammalian cells as they enter mitosis.

Results: We have established a mammalian cell line in which the boundaries of the centrosome are defined by the constitutive expression of γ-tubulin–GFP. This feature allows us to use laser microsurgery to selectively destroy the centrosomes in living cells. Here we show that this method can be used to reproducibly ablate the centrosome as a functional entity, and that after destruction the microtubules associated with the ablated centrosome disassemble. Depolymerization–repolymerization experiments reveal that microtubules form in acentrosomal cells randomly within the cytoplasm. When both centrosomes are destroyed during prophase these cells form a functional bipolar spindle. Surprisingly, when just one centrosome is destroyed, bipolar spindles are also formed that contain one centrosomal and one acentrosomal pole. Both the polar regions in these spindles are well focused and contain the nuclear structural protein NuMA. The acentrosomal pole lacks pericentrin, γ-tubulin, and centrioles, however.

Conclusions: These results reveal, for the first time, that somatic cells can use strong enough to drive bipolar spindle assembly even in the presence of a single functional centrosome.

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Background

According to E.B. Wilson [1], the centrosome was first seen by Flemming in 1875 but named by Bovery in 1888. At that time it was described as an intensely stained particle located preferentially near the geometrical center of the interphase cell and at the spindle poles during mitosis. Over the past 125 years, the centrosome has been implicated in a variety of cellular processes including locomotion, intracellular transport, development and division. All of these functions are derived from its role as the cell's primary, or in some cases only, microtubule-organizing center (MTOC) [2].

It is now evident that some functions traditionally ascribed to the centrosome can be achieved in the absence of this organelle. A good example is the formation of 'acentrosomal' meiotic spindles in many oocytes (for example of mice [3], *Xenopus* [4] and *Drosophila* [5]) and in some spermatocytes [6]. In such systems, microtubule polymerization and/or stabilization is promoted by chromatin [7–9], perhaps via the RCC1/Ran pathway [10]. These microtubules are then organized into a functional spindle by molecular motors [11,12] including Xklp1 [13], Nod [14], Eg5 [15], cytoplasmic dynein [4], and Ncd [5]. Thus, in systems where the centrosome is not present, its MTOC activity is replaced during spindle assembly by the combined action of chromatin (to stabilize microtubules and/or promote their assembly) and motor proteins (to sort and organize microtubules into a fusiform array).

An important question is whether these mechanisms, which have been elucidated primarily in acentrosomal meiotic systems, are also present during mitosis but are masked by the presence of the centrosome [8,9,16,17]. The resolution of this question has, in the past, been hampered by the paucity of methods for selectively and reproducibly removing centrosomes from cells as they are entering mitosis. Here we describe the effect on spindle formation of destroying one or both centrosomes by laser microsurgery in CVG-2 cells (green monkey fibroblasts, derived from CV-1 cells). These cells constitutively express a γ-tubulin–green-fluorescent-protein fusion (γTGFP), which becomes concentrated in and delineates the boundaries of the centrosome throughout the cell cycle [18].

As shown previously, this feature, when combined with laser microsurgery, allows one to completely and selectively destroy the centrosome without damaging surrounding structures [19]. Here we report that when one or both centrosomes are destroyed during prophase in CVG-2 cells, a functional bipolar spindle is formed. These new data reveal that spindle formation can occur by a centrosome-independent pathway, even in cells that normally contain centrosomes.

Results

Laser irradiation destroys the centrosome

We reported previously that a 3–4 second series of laser pulses (10 pulses/sec; 7 nsec pulse duration, 500 nJ at the specimen) is sufficient to convert the centrosome into an electron-opaque mass of denatured protein. As this ablative photo-decomposition process also abolishes the GFP fluorescence, the disappearance of the γTGFP signal can be used to assay for complete centrosome destruction [19].

To prove that this operation destroys not just the γTGFP, but also other centrosomal components, we irradiated one of two separated centrosomes in CVG-2 cells, and then stained these cells for either γ-tubulin or pericentrin. Like γ-tubulin, pericentrin is a *bona fide* centrosomal component that colocalizes with γ-tubulin [20,21]. Without exception, after the operation neither pericentrin (Figure 1f) nor γ-tubulin (Figure 2f) were detected in the irradiated centrosome, whereas the non-irradiated (control) centrosome was always brightly stained. In interphase CVG-2 cells a focal array of microtubules is associated with every centrosome, as is typical for animal cells. Once a centrosome was ablated by the laser, however, its associated microtubule array disappeared in less than 15 minutes, while normal numbers of microtubules remained associated with the control centrosome in the same cell (Figure 1).

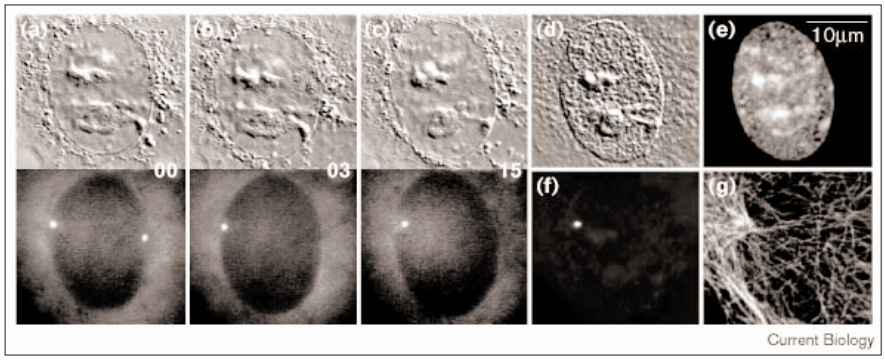
To determine whether irradiated centrosomes recover over time, we used the laser to ablate one or both centrosomes and then, at various times (2–120 minutes) after irradiation, chilled the cells to 5°C. Under this condition, all the microtubules in CVG-2 cells are completely depolymerized in approximately 15–20 minutes (data not shown). After a 30 minute incubation at 5°C, the cells were re-warmed to 37°C to induce microtubule reassembly. In cells in which only one centrosome was irradiated a typical focal array of microtubules was always seen to be associated with the remaining control centrosome around 2 minutes after re-warming (Figure 2). Along with the centrosomal array, individual microtubules were also present within the cytoplasm of these cells (Figure 2g). In those cells in which both centrosomes were destroyed, numerous individual microtubules were found randomly distributed throughout the cytoplasm around 2 minutes after re-warming (Figure 3). By contrast, the great majority of microtubules in neighboring non-irradiated cells were focused onto the centrosome(s) (see Supplementary material).

From these experiments we conclude that our laser microsurgery approach completely destroys the structural and functional properties of the centrosome. As non-labeled centrosomes respond similarly to laser microsurgery (data not shown), the ablative photo-decomposition does not require this organelle to be labeled with a fluorophore. Rather, we use the GFP label simply to define the boundaries of these organelles clearly, for complete and reproducible destruction.

Destroying one or both centrosomes in prophase CVG-2 cells does not prevent bipolar spindle formation

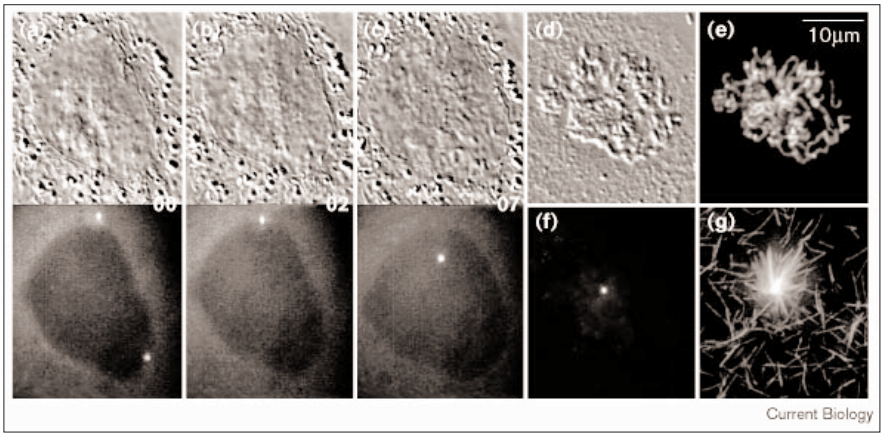
An important issue is whether vertebrate somatic cells can form a spindle in the absence of centrosomes, as occurs during meiosis in some organisms. To address this question, we conducted a series of experiments in which we selectively destroyed one or both centrosomes prior

Figure 1



Laser irradiation destroys centrosomal proteins and induces the associated microtubule array to disassembly. **(a–c)** Selected (top) differential interference contrast and (bottom) GFP-fluorescence frames of a CVG-2 cell containing two separated centrosomes. The right-hand centrosome was destroyed between (a) and (b), and the cell was fixed 15 min later, just after (c). **(d)** The same cell viewed by DIC after fixation. **(e–g)** The same cell shown by epifluorescence after staining for (e) DNA, (f) pericentrin and (g) α-tubulin. As revealed by (f) a lack of anti-pericentrin staining, laser irradiation of the centrosome ablates not only the γ-tubulin but also other centrosomal proteins. (g) Note also that ablating the centrosome induced its radial array of microtubules to disappear within 15 min after the operation. The numbers on the panels refer to time in minutes after the beginning of the experiment.

Figure 2



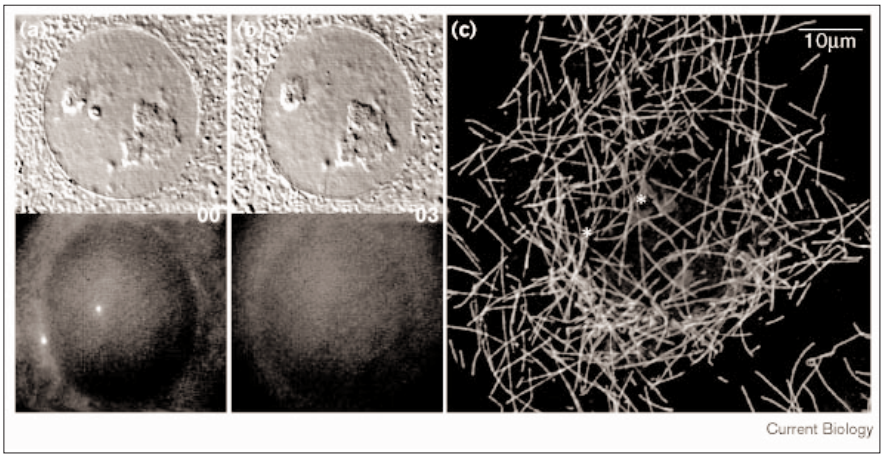
Ablated centrosomes lose their ability to nucleate microtubules. **(a–c)** Selected (top) DIC, and (bottom) GFP-fluorescence frames depicting the laser ablation (between (a) and (b)) of one (the bottom) centrosome during prophase. Immediately after (c) the cell was chilled to 5°C for 30 min to depolymerize microtubules. The cell was then warmed to 37°C for 2 min prior to fixation. **(d)** The same cell pictured by DIC after fixation. **(e–g)** The same cell shown by epifluorescence after staining for (e) DNA, (f) γ-tubulin and (g) α-tubulin. In this cell the nuclear envelope broke down during the cooling and/or warming steps. (g) Note that a radial array of microtubules has formed in association with the non-irradiated centrosome, and that a number of randomly oriented microtubules are also found in the cytoplasm. The numbers on the panels refer to time in minutes after the beginning of the experiment.

to spindle formation in CVG-2 cells and then followed the cells as they progressed into and through mitosis.

In the first set of studies we ablated just one of two centrosomes during prophase. Our prediction was that these cells would then form monopolar spindles in which the chromosomes would become attached to and grouped around the single remaining aster. Surprisingly, however, all of these cells ($n > 10$) formed a normal bipolar spindle (Figures 4,5). In most cells, monopolar spindles were initially formed in association with the single remaining centrosome, but over time asymmetric bipolar spindles began to appear and became progressively more symmetrical (Figures 4f–j,6e–h). The duration of prometaphase in these cells (around 1 hour) was similar to that of non-irradiated controls, and both anaphase chromatid separation and cytokinesis occurred normally.

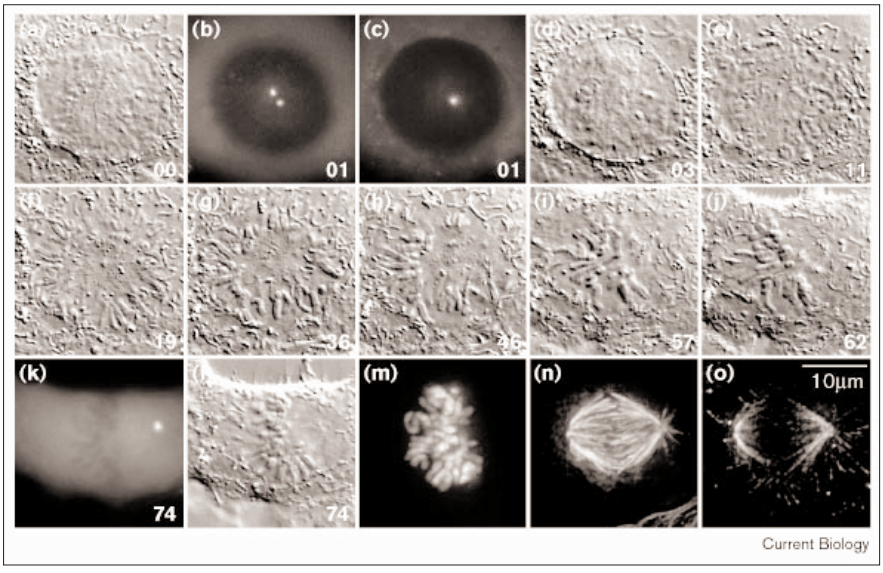
A structural analysis of these cells ($n > 10$), fixed at or near metaphase, revealed that the acentrosomal pole was well focused and contained an abundance of NuMA. This is a structural protein derived from the nucleus that has been implicated in focusing microtubule arrays during cell division [22]. We fixed four cells during anaphase for a three-dimensional electron micrographic analysis. In all cases, one of the half-spindles focused onto a pole that contained a pair of centrioles (that is, a diplosome; Figure 5o–q). By contrast, although the microtubules in the opposing half-spindle similarly converged to form a well defined and tapered pole, this region always lacked centrioles (Figure 5l–n). Although these acentrosomal spindle poles contained some electron-opaque deposits, this material was not γ-tubulin (Figure 5i) or pericentrin (data not shown;

Figure 3



Destroying both centrosomes does not prevent random microtubule polymerization in the cytoplasm. **(a,b)** DIC (top) and GFP-fluorescence (bottom) frames (a) before and (b) after ablation of both centrosomes. The procedure was similar to that in Figure 2. After chilling to 5°C for 30 min the cell was fixed for 2 min upon re-warming. **(c)** The same cell stained for α-tubulin. Note that numerous microtubules have formed throughout the cytoplasm in the absence of functional centrosomes. Asterisks mark the approximate positions of irradiated centrosomes. The numbers on the panels refer to time in minutes after the beginning of the experiment.

Figure 4

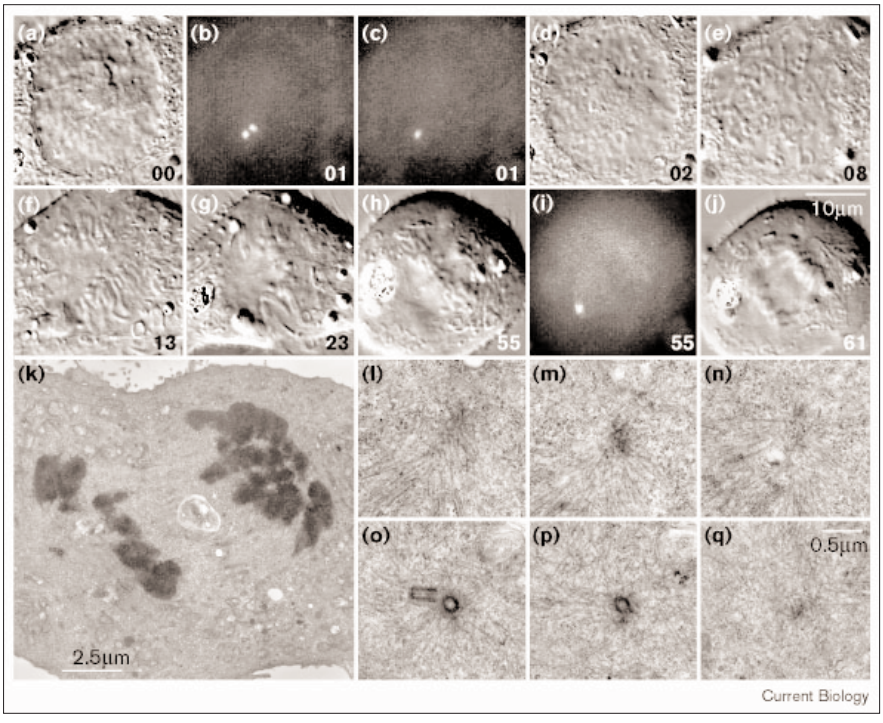


Destroying one of two prophase centrosomes does not prevent bipolar spindle formation. **(a–l)** Selected (a,d–j,l) DIC and (b,c,k) epifluorescence frames from a recording showing a prophase cell in which one of the two centrosomes was destroyed (between (b) and (c)). (e) The nuclear envelope broke down ~11 min later and (f) the spindle began to form in the presence of only one centrosome. (f,g) Initially, the forming spindle appeared monopolar, but (h–l) over time it adopted a typical bipolar organization. **(m–o)** In late prometaphase (shortly after (k,l)) this cell was processed for the localization of (m) DNA, (n) α-tubulin and (o) NuMA. Despite the presence of a single centrosome the spindle was bipolar, both poles were similarly focused, and each contained NuMA. The numbers on the panels refer to time in minutes after the beginning of the experiment.

see Figure 1), which are invariably absent from these poles. In this regard it should be noted that the presence of a non-centrosomal electron-opaque material containing, for example, NuMA protein, is a characteristic feature of acentrosomal spindle poles formed in cell-free extracts [23].

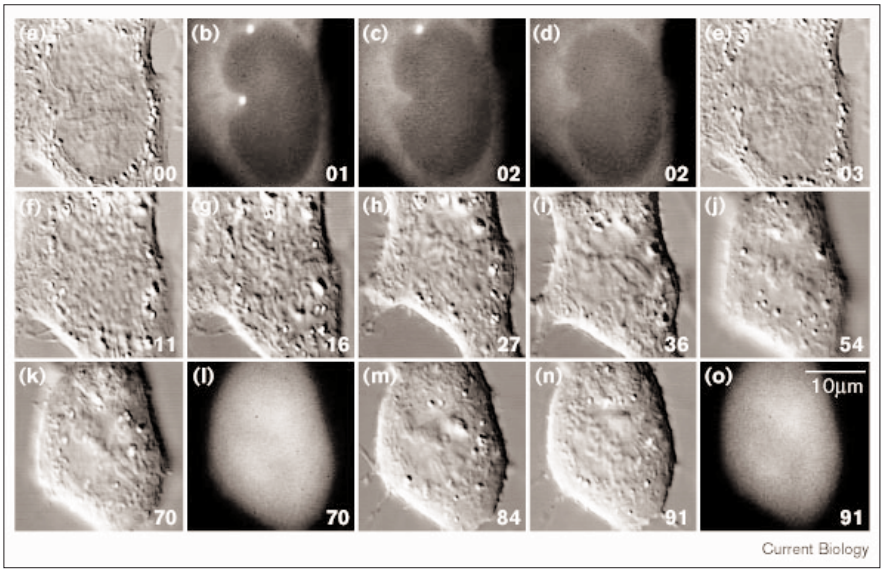
We next sought to determine whether CVG-2 cells would form a bipolar spindle when both centrosomes were destroyed before nuclear envelope breakdown. Despite the absence of centrosomes, these cells subsequently entered prometaphase and formed, over a period of 45–80 minutes, a normal bipolar spindle (Figure 6).

Figure 5



Acentrosomal spindle poles lack centrioles. The procedure was similar to that described in Figure 4 except that this cell was fixed ~7 min after the onset of anaphase for serial-section electron microscopy. **(a–h)** In this cell one centrosome was destroyed (between (b) and (c)) and (d–h) the cell ultimately formed a bipolar metaphase spindle containing a centrosomal and (i) an acentrosomal spindle pole which then (h) entered anaphase. **(k)** One of the sections cut through both poles. **(l–q)** A complete serial series through each spindle pole: (l–n) and (o–q) respectively. The control pole (o–q) contains a typical diplosome (a pair of centrioles), whereas the acentrosomal pole lacks any identifiable centrosomal components. The numbers on the panels refer to time in minutes after the beginning of the experiment.

Figure 6

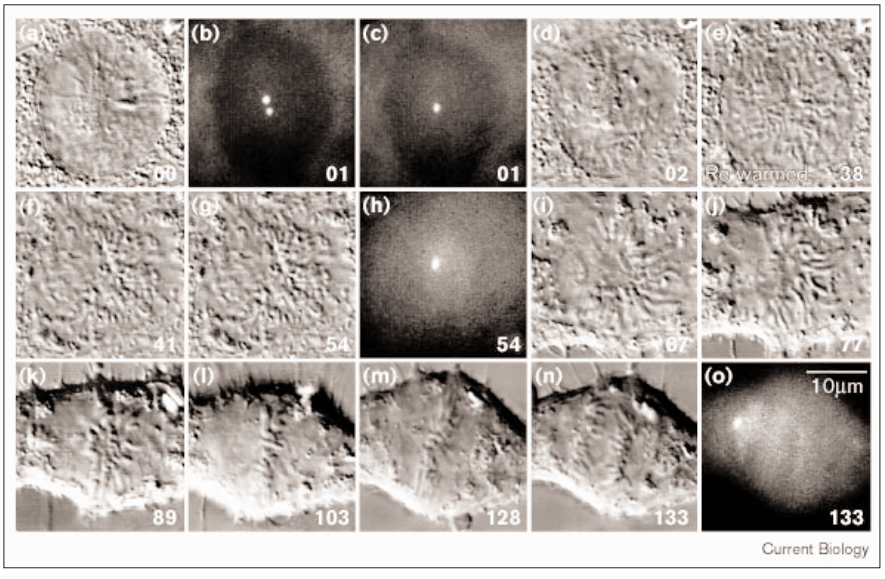


Destroying both centrosomes in prophase CVG-2 cells does not inhibit the subsequent formation of a functional bipolar spindle. The procedure was the same as in Figure 4 except that both centrosomes were destroyed. **(a–d)** The cell (a,b) before and (c,d) after the operation. **(e–m)** The cell subsequently formed a functional bipolar spindle and **(n,o)** then entered anaphase. It was fixed shortly after anaphase onset (after (n)) for serial-section electron microscopy. This analysis revealed that the structure and organization of both poles was similar to that of the acentrosomal pole in cells in which only one centrosome was destroyed (Figure 4l–n). The numbers on the figures refer to time in minutes after the beginning of the experiment. (a,e,f–k,m,n) DIC images; (b–d,l,o) GFP fluorescence.

A serial-section electron micrographic analysis revealed that both poles were similar in structure to those acentrosomal poles described above (data not shown).

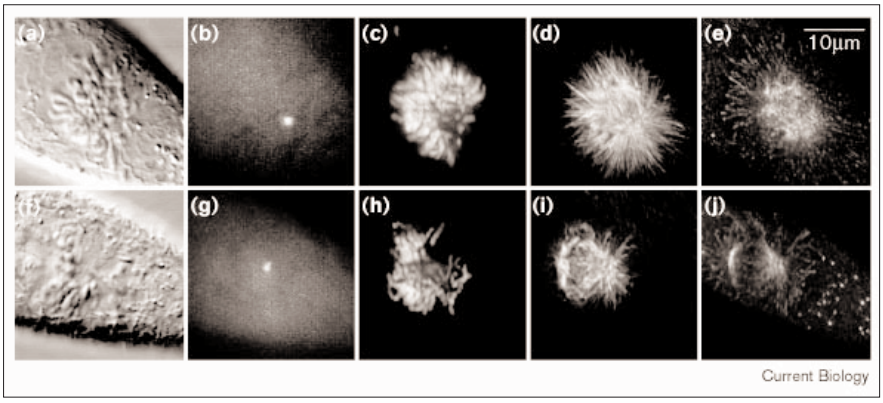
Our experiments on the redistribution of microtubules after centrosome ablation during interphase revealed that the aster associated with the irradiated centrosome ultimately disappeared within 15 minutes. As we often destroyed centrosomes during late prophase, it was possible, however, that by the time of nuclear envelope breakdown a remnant astral microtubule array(s) was still present to seed the formation of a spindle pole. To address this possibility, we chilled prophase cells to 5°C immediately after destroying one centrosome, and then re-warmed them 30 minutes later. Under this condition all microtubules were disassembled and, upon re-warming, spindle formation was initiated in association with only one astral microtubule array (see Figure 2). Nevertheless, all of these cells ($n > 10$) ultimately formed a normal bipolar spindle and entered anaphase (Figure 7). Cells containing only one centrosome also exhibited normal cytokinesis.

Figure 7



Upon re-warming, a normal bipolar spindle forms in CVG-2 even when centrosome ablation is followed by depolymerization of the residual microtubules with cold. Selected **(a,d–g,i–n)** DIC and **(b,c,h,o)** epifluorescence frames from a time-lapse sequence depicting an operation similar to that shown in Figure 4. Shortly after destroying one of the centrosomes (between (b) and (c)), however, this cell was chilled to 4°C for 30 min. (e) The nuclear envelope broke down shortly after re-warming. As in Figure 4, (f–i) the cell formed an asymmetric spindle which then (k–m) became more symmetrical over time. (n,o) Approximately 90 min after nuclear envelope breakdown the cell entered anaphase after which time it was fixed for electron microscopy. A serial-section analysis revealed that the spindle poles were organized as shown in Figure 4l–o. The numbers on the figures refer to time in minutes after the beginning of the experiment.

Figure 8



Intermediate stages of spindle assembly in CVG-2 cells lacking one centrosome. Two examples (**(a–e)** and **(f–j)**) of cells in which one centrosome was destroyed during prophase and the microtubules depolymerized by chilling. These cells were then fixed for an immunofluorescence analysis 15 min after re-warming. They were then stained for (c,h) DNA, (d,l) α-tubulin, and (e,j) NuMA. The cell shown in (a–e) contains numerous microtubules that are loosely focused on a robust sphere of NuMA protein which is not centered over the remaining centrosome. In the cell shown in (f–j), the spindle is highly asymmetric but clearly bipolar and the centrosome is associated with the more robust pole (on the right). Note that NuMA is associated with both centrosomal and acentrosomal poles.

In these cells, the forming spindle appeared to be asymmetric, as if proceeding through a transient monopolar stage (for example Figure 7i,j; see also above). This impression was validated by subsequent studies in which cells containing a single centrosome were fixed as they were forming spindles around 15 minutes after re-warming. Immunofluorescence analysis revealed two predominant phenotypes. In some cells, the microtubules radiated from a single large diffuse area. In these cells the single remaining centrosome was located within, but not necessarily centered in, the microtubule array (Figure 8b,d) and this region contained NuMA (Figure 8a–e). Other cells contained a bipolar array of microtubules that was often asymmetric (Figure 8f–j). The centrosome in these cells was associated with the more robust half-spindle (Figure 8g,i), and both poles contained NuMA (Figure 8j).

Discussion

Several methods have been used to disrupt or remove the centrosome from vertebrate somatic cells, including microinjection of antibody [24] and micromanipulation [25]. When antibodies to polyglutamylated tubulin are loaded into interphase HeLa cells the centrioles disappear over a 12 hour period, after which the pericentriolar material becomes scattered within the cytoplasm. In these experiments, some cells appeared to form a bipolar spindle and proceeded through mitosis with no identifiable centrosomes at the poles [24]. However, as the centrosome is only transiently disrupted by this approach, and recovers gradually over time, it is impossible to conclude with certainty that the spindle formed via an acentrosomal pathway. In fact, the spindle poles in these cells often contained widely different amounts of known spindle-pole components, including γ-tubulin and the pericentriolar antigen (stained by serum 013 [24]). Corresponding serial-section electron microscopy studies of mitotic cells also showed different degrees of centriole recovery.

Vertebrate somatic cells lacking centrosomes can also be produced by removing the centrosome by micromanipulation. This operation can, however, only be conducted during interphase and the acentrosomal cells produced never proceed into mitosis [25]. The only method currently available for destroying centrosomes during mitosis *in vivo* is laser microsurgery. Initial attempts at using this method, which were reported in the late 1970s before the development of anti-centrosome antibodies and *in vivo* labeling techniques, produced conflicting results [26,27]. As the boundaries of the centrosome could not be clearly defined, the success or failure of the operation could not be assayed until after the cells were fixed and analyzed by electron microscopy. Quite often these structural studies revealed that the centrosome was not completely destroyed but just damaged [26,27].

The centrosome is clearly visible *in vivo* in cells expressing γTGFP [18]. The progressive disappearance of the γTGFP signal observed during laser microsurgery directly corresponds with the progressive destruction of the irradiated centrosome. As a result, one can ablate part or all of the centrosome by targeting some or all of the centrosome-associated γTGFP signal [19]. It is noteworthy that photoablation destroys the centrosome as an organelle but not those centrosomal proteins distributed elsewhere in the cytoplasm. This feature distinguishes the laser-microsurgery approach from gene knock-outs in which a specific component is eliminated from the entire cell.

In our studies, the astral array of microtubules associated with a CVG-2 centrosome disappears < 15 minutes after ablating the centrosome. Microtubule repolymerization experiments reveal that the laser-irradiated centrosome does not recover during mitosis. Thus, when centrosomes are destroyed during prophase, the spindle must be assembled in the absence of any contributing microtubule

foci. We addressed the possibility that some remnants of the microtubule asters are still present, and subsequently act as foci after the operation, by depolymerizing any residual microtubules using cold. Nevertheless, when re-warmed, these cells always formed functional bipolar spindles and proceeded through a normal mitosis. Thus our experiments prove that at least some vertebrate somatic cells possess the capacity to form a functional bipolar spindle in the absence of centrosomes and preformed focal microtubule arrays.

A kinetic analysis of spindle formation with and without centrosomes in *Xenopus* egg extracts demonstrates that centrosomes dominate in spindle formation, and outcompete the centrosome-independent pathway [28]. Indeed, when replicated centrosomes fail to separate in vertebrate cells they function as a single unit, and form a persistent monopolar spindle [29]. Monopolar spindles are also formed when sea urchin blastomeres enter mitosis with only a single centrosome [30]. Thus, we were surprised to find that bipolar spindles form in CVG-2 cells when only one centrosome is destroyed. To our knowledge there are only two other reports suggesting that bipolar spindles can form in the presence of a single functional centrosome. Larvae of *Drosophila* KLP61F mutants contain a high incidence of 'monastral bipolar spindles' in their somatic cells [31]. As these cells appear to enter mitosis with two separated centrosomes, however, the origin of these spindles is ambiguous without *in vivo* data. Functional bipolar spindles also form in crane-fly spermatocytes when centrosome separation is mechanically inhibited [32]. In this classic meiotic system, an acentrosomal route for half-spindle formation clearly competes with the centrosomal route [33,34].

In order for a centrosome-independent pathway of spindle formation to work in the presence of centrosomes, free microtubules must form in the cell at the same time and in similar numbers to centrosomal microtubules [9]. In this regard, when we chill and re-warm CVG-2 cells in which one or both centrosomes have been destroyed, numerous free microtubules appear randomly within the cytoplasm (Figures 2,3). This is in sharp contrast to neighboring non-irradiated cells in which the great majority of microtubules reform in association with the centrosomes (see Supplementary material). Thus, after destruction of one centrosome, the number of free microtubules formed in the cytoplasm relative to those associated with the remaining centrosome increases dramatically. In turn, this is likely to provide sufficient numbers of free microtubules to allow a bipolar spindle to form despite the presence of the remaining centrosome. The reason why so many free microtubules form in the cytoplasm of CVG-2 cells in the presence of a remaining functional centrosome is unclear. It is possible that the numbers of microtubules nucleated by the surviving centrosome are not sufficient to drop the tubulin concentration below that required for spontaneous microtubule polymerization. Alternatively, destruction of one centrosome may trigger a mechanism that decreases the 'critical concentration' at which random microtubule assembly becomes possible. In this context it has been shown that the level of microtubule polymerization in centrosome-free cytoplasts differs depending upon the physiological conditions of the cell [35].

We assume that the pathway by which the spindle assembles in acentrosomal CVG-2 cells is similar to that described for meiotic systems, and that it requires the same motors and structural components [8]. In this regard, some of the components used to form acentrosomal spindles during meiosis have been implicated in focusing microtubules into a spindle pole during mitosis [22]. Microinjecting antibodies against cytoplasmic dynein, a microtubule minus-end motor, disrupts the focused organization of spindle pole microtubule minus ends in CV-1 cells [36]. As a result the centrosomes separate from the ends of the spindle, which then loses its typical fusiform shape. Abnormal spindles, with deformed polar regions, are also formed when p50 (a dynactin-complex component) is overexpressed in COS-7 cells [37].

The nuclear protein NuMA also has an important structural role in maintaining the fusiform arrays of half-spindle microtubules in both acentrosomal [38] and centrosomal [39] spindles. We note that NuMA protein is also associated with the acentrosomal polar regions in CVG-2 cells from the earliest detectable stage of its formation (Figure 7). Thus, it is highly likely that after destruction of one or both centrosomes, the formation of bipolar spindles in CVG-2 cells requires the activities of cytoplasmic dynein and NuMA. As bipolar spindles are formed even after destroying just one centrosome, this acentrosomal pathway is clearly sufficiently robust to compete favorably with the remaining centrosome.

Conclusions

Our results reveal that a centrosome-independent pathway for spindle assembly does exist in vertebrate somatic cells. We can now state that the mechanisms shown to drive spindle assembly in meiotic systems are applicable to mitosis in vertebrates. Moreover, at least in some cells (CVG-2), these mechanisms are strong enough to compete with the centrosomal pathway and drive bipolar spindle assembly in the presence of a single centrosome.

Materials and methods

Cell culture

The CVG-2 clone was isolated from the parental CV-1 (green monkey kidney, fibroblastic) cell line (American Type Culture Collection) after transforming with a γTGFP plasmid [18]. The cells in this clone constitutively express γ-tubulin–GFP fusion protein (γTGFP). Cells were maintained in a DMEM/F-12 (1:1) media mixture supplemented with

10% FCS in a humidified 37°C 5% CO_2 atmosphere. For laser microsurgery experiments cells were seeded on 24 × 24 mm number 1½ coverslips. After 24 h these coverslip cultures were mounted in Rose chambers in Phenol Red-free L-15 media (Gibco BRL) supplemented with 10% FCS. During experiments cells were maintained on the microscope stage at 37°C using a custom-built heater [40].

Laser microsurgery

The details of our laser microsurgery system, as well as the principles of using a GFP signal to target organelles for destruction with the laser, have been described previously [19,41]. Briefly, our system is based on an inverted light microscope (Diaphot 200, Nikon) equipped with De Senarmont-compensation DIC. The 1064 nm output of the Nd–YAG pulsed laser is frequency doubled to 532 nm, filtered, attenuated, and routed into the epi-port of the microscope. When focused by a 60× 1.4 NA lens the beam has an effective waist of 0.4–0.5 μm in the specimen plane [41]. To completely destroy a centrosome we expose it to the laser pulses while simultaneously focusing up and down and translating the sample in the *xy* plane. We do this until the γTGFP fluorescence is completely abolished. In CVG-2 cells this typically takes ~10 sec and requires two to three series of 20–30 laser pulses.

For DIC imaging cells were illuminated with light from a 100 W tungsten filament and time-lapse images were captured every 10–30 sec using a video-rate CCD camera (Model 100, Paultek Imaging). For GFP fluorescence, cells were illuminated with light from a 100 W mercury burner and images captured using a SIT camera (Model 68, Dage-MTI). Both light sources were filtered with GG400 (to eliminate ultraviolet) and KG5 (to eliminate infrared) filters. Transmitted light was additionally filtered by GIF546 (green) filter during DIC imaging. For the GFP mode a XF77 filter cube was used (Omega Optical). Both the fluorescence and DIC light sources were shuttered by UniBlitz shutters (UniBlitz Electronics), so that cells were illuminated only during image acquisition. The entire imaging system was driven by Image I software (Universal Imaging) running on a PC.

Immunofluorescence microscopy

Immunofluorescence staining and imaging of stained preparations were performed as previously described [18]. The following primary antibodies were used: polyclonal anti-pericentrin (serum M, a kind gift of Stephen Doxsey, University of Massachusetts) at 1:200; polyclonal anti-NuMA (kind gift of Duane Compton, Dartmouth College); anti-α-tubulin monoclonal antibody (T5168, Sigma) at 1:300; and anti-γ-tubulin antibody (T6557, Sigma). As the two centrosomes within the same cell were often located in different focal planes, all images were collected as Z-series (200 nm steps), and then deconvoluted using Delta Vision 2.1 deconvolution software (Applied Precision). All fluorescence images presented in this paper are maximal intensity projections computed from these three-dimensional datasets.

Electron microscopy

Cells were fixed and prepared for electron microscopy according to standard protocols [42]. After flat-embedding [43] they were relocated using by phase-contrast light microscopy and serially thick-sectioned (0.25 μm). The sections were imaged and photographed in a Zeiss 910 microscope operated at 100 kV.

Supplementary material

Supplementary material relating to Figure 3 is available at http://current-biology.com/supmat/supmatin.htm.

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