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Centrosome positioning in interphase cells

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The position of the centrosome is actively maintained at the cell center, but the mechanisms of the centering force remain largely unknown. It is known that centrosome positioning requires a radial array of cytoplasmic microtubules (MTs) that can exert pushing or pulling forces involving MT dynamics and the activity of cortical MT motors. It has also been suggested that actomyosin can play a direct or indirect role in this process. To examine the centering mechanisms, we introduced an imbalance of forces acting on the centrosome by local application of an inhibitor of MT assembly (nocodazole), and studied the resulting centrosome displacement. Using this approach in combination with microinjection of function-blocking probes, we found that a MT-dependent dynein pulling force plays a key role in the positioning of the centrosome at the cell center, and that other forces applied to the centrosomal MTs, including actomyosin contractility, can contribute to this process.

Introduction

The centrosome position is actively maintained at the cell center, but the nature of the forces that lead to this positioning have yet to be determined. Centrosome positioning requires a polarized radial array of cytoplasmic microtubules (MTs) (Euteneuer and Schliwa, 1992; Koonce et al., 1999). Several kinds of forces applied on the centrosome–MT complex may act separately or in combination with each other to maintain the position of the centrosome at the cell center. It has been suggested that the forces acting on cytoplasmic MTs may include pushing by MT growth at the plus ends (Inoue and Salmon, 1995), pulling by MT motors anchored at the cortex (Dujardin and Vallee, 2002; Gundersen, 2002) or in the cytoplasm (Hamaguchi and Hiramoto, 1986), and actin-dependent movement (Mikhailov and Gundersen, 1995; Waterman-Storer and Salmon, 1997; Yvon and Wadsworth, 2000; Salmon et al., 2002). One or more of these forces may be responsible for maintaining the position of the centrosome.

MT growth can produce significant mechanical force (Dogterom and Yurke, 1997). Free distal (plus) ends display dynamic instability and may push against the plasma membrane, creating the pushing force to displace the centrosome with attached MTs away from the cell edges. Since more MTs on average will reach the cortex closest to the centrosome, the resulting pushing forces will be stronger in the regions more proximal to the cell edges and will balance each other when the centrosome reaches a position in the cell center. Polymerization of MTs was proven to be sufficient for the centering of centrosome asters in vitro (Faivre-Moskalenko and Dogterom, 2002) or mitotic spindles in yeast cells (Tran et al., 2001).

MTs might also be pulled by minus-end MT motors such as cytoplasmic dynein, located at the cortex or anchored in the cytoplasm. Pulling on multiple MTs pointing in different directions may serve to keep the centrosome away from the cell margins. Dynein motors have been implied in the reorientation of the centrosome during cell polarization, in asymmetric positioning of mitotic spindles in *S. cerevisiae* and *C. elegans* (Eshel et al., 1993; Li et al., 1993; Skop and White, 1998; Gonczy et al., 1999), and in the positioning of astral MTs and mitotic spindles in *Dictyostelium* amoeba and mammalian cells (Koonce et al., 1999; O'Connell and Wang, 2000).

Centrosome positioning can also be maintained through the pushing on MTs by actomyosin-driven forces. MTs make physical contacts with the actin cytoskeleton and therefore experience force produced by actin centripetal flow. Constant growth of actin filaments at the cell margin, possibly coupled to the activity of a myosin motor, generates

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Abbreviation used in this paper: MT, microtubule.

a retrograde flow of actin filaments toward the cell center (Cramer, 1997; Wittmann and Waterman-Storer, 2001). Actin centripetal flow requires contractility of the actin network, which depends on myosin activity and is regulated by the small GTPase RhoA (Cramer, 1997; Wittmann and Waterman-Storer, 2001). Such flow can produce a significant mechanical force and has been shown to drive the centripetal movement of MTs anchored on the actin filaments (Salmon et al., 2002).

To examine the mechanism of centrosome positioning, we introduced an imbalance in the forces acting on the centrosome in nonmigrating mammalian cells by locally disrupting MTs through the local application of the MT-depolymerizing drug nocodazole. The results of our analysis of centrosome displacement in nocodazole-treated cells show that the MT-dependent forces involved in centrosome positioning are of a pulling rather than pushing nature. We have further demonstrated that the maintenance of the centrosome position requires the activity of a minus-end MT motor cytoplasmic dynein.

Results and discussion

Organization of the centrosome–MT complex in BS-C-1 cells was examined by injecting them with Cy-3 labeled tubulin and acquiring images of fluorescent MTs (Fig. 1, center). The position of the centrosome was easily traceable as the focal point of converging MTs. Immunostaining for ε-, γ-, and α-tubulins confirmed that such a focal point corresponded to the actual position of the centrosome and indicated that, similar to other cell types, MTs were attached to the less motile mother centriole (unpublished data), which we will refer to as the centrosome here.

The balancing of the centrosome position in the cell center is known to depend on the system of cytoplasmic MTs (Euteneuer and Schliwa, 1992). To introduce an imbalance in the centering forces, we locally disrupted MTs in cells by local application of an MT drug nocodazole (10 μg/ml). Time sequences of digital fluorescent images of MTs showed that within the first 10–15 min of the drug treatment MTs depolymerized, and the levels of soluble tubulin increased in the proximity of the micropipette (Fig. 1, left; Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200305082/DC1). Remarkably, MTs distal to the micropipette remained intact for at least 20 min of nocodazole treatment. Furthermore, the parameters of dynamic instability of the distal MTs were not affected in the drug-treated cells (Fig. 1, right; Video 2).

To confirm the local effect of nocodazole treatment, we developed a computational model for the disruption of MTs with nocodazole using Virtual Cell computational framework (see supplemental methods and Video 8). The model shows that the concentration of nocodazole on the side distal to the micropipette was ≤1 nM after 20 min of the local application of a concentrated nocodazole solution and is therefore below the minimum level that has been shown to affect MT dynamics (Vasquez et al., 1997).

Local application of nocodazole solution at the cell edge resulted in rapid (~0.3 μm/min) movement of the centrosome toward the micropipette tip in all examined cells ($n = 9$) (Fig.

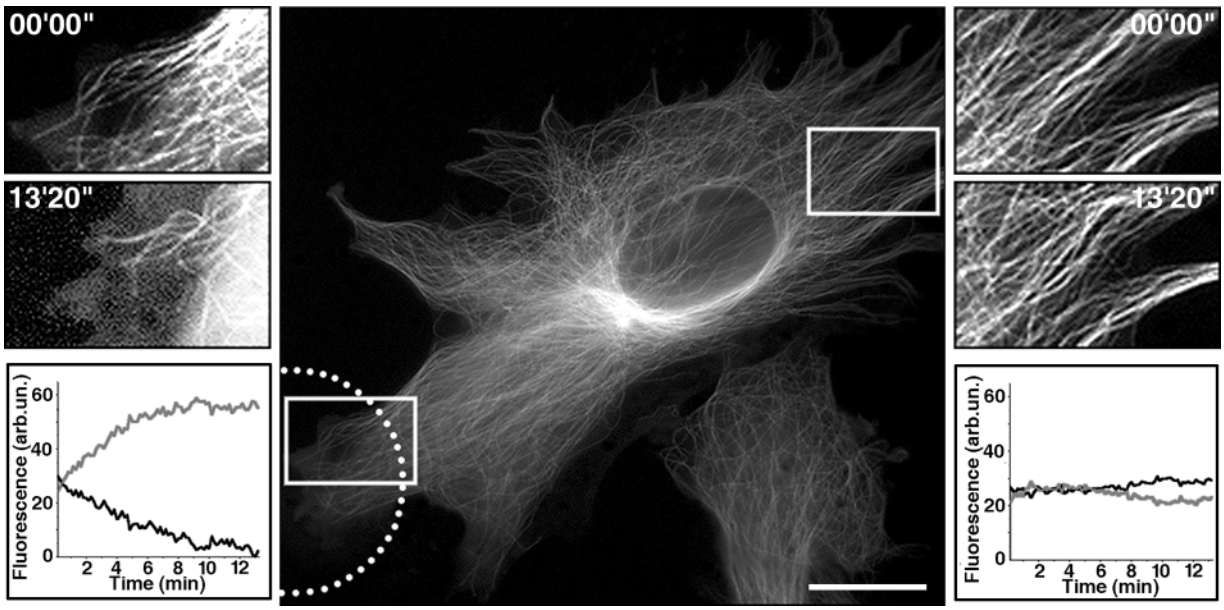


Figure 1. **Local disruption of MTs in a cell by the local application of nocodazole.** (Center) low magnification live fluorescence image of a cell with MTs labeled by injecting fluorescently tagged tubulin subunits. Image was obtained just before application through the micropipette of a nocodazole solution in the area depicted by the dashed line. Micrographs on the left and right are high magnification images of MTs in the boxed regions shown in the central panel acquired at the cell edge proximal to (left) or distal from (right) the micropipette tip, before (top) or after (bottom) the application of nocodazole. Time-lapsed series of MT dynamics corresponding to the micrographs on left and right are shown in Videos 1 and 2, available at http://www.jcb.org/cgi/content/full/jcb.200305082/DC1. The graphs show kinetics of changes in the levels of tubulin monomer (plots shown in gray) or polymer (plots shown in black) at the cell edges proximal (left) or distal (right) to the micropipette tip. Bar, 20 μm.

2, A and C; Video 3). In control experiments, local application of a drug-free medium did not induce significant changes in the centrosome position. The observed displacement was independent of the nucleus, since the same experiment in cell cytoplasts resulted in a similar centrosome behavior (Fig. 1 C). Thus, local disruption of MTs induces centrosome movement toward the site of nocodazole application.

The nocodazole-induced centrosome displacement may be caused by the pushing force produced by MT growth at the edge distal to the MT disruption site. However, MT depoly-

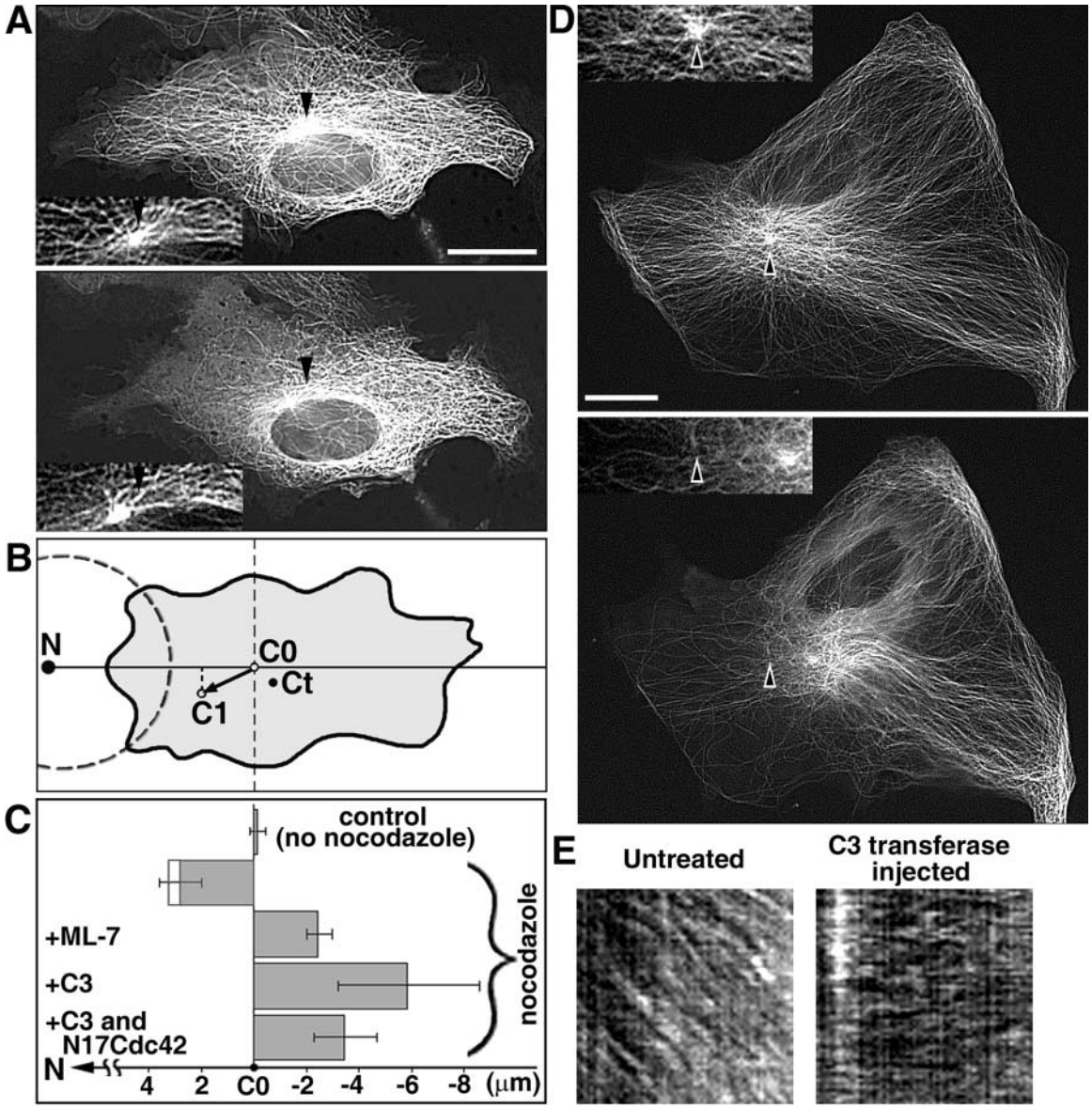


Figure 2. **Centrosome position is maintained through a MT mediated pulling force.** (A and D) Pairs of live fluorescence images of the centrosome acquired before (top image of each pair) and after (bottom image of each pair) the local application of nocodazole. Insets show the position of the centrosome in the same cells at higher magnification. The initial positions of the centrosome are indicated by black arrows. See also the corresponding movies (Videos 3 and 4) for full time-lapse series, available at http://www.jcb.org/cgi/content/full/jcb.200305082/DC1. (A) Noninjected cell. Local application of nocodazole induces centrosome movement toward the application site. (D) Cell injected with a Rho inhibitor C3 transferase (0.1 mg/ml). Inhibition of RhoA activity reverses the direction of the centrosome movement upon application of nocodazole. (B) Method for the quantification of the centrosome movement. The centrosome position (C) was determined as the focus of the MT fluorescence. Centroid position (Ct) was calculated by Metamorph software as the point equidistant from all the cell margins. Centrosome movement was calculated as the distance between the initial (C0) and final (C1) positions of the centrosome, projected onto a straight line connecting the nocodazole pipette tip (N) and the centrosome at time point zero. Observations were made in stationary cells, but cell shape changes during the time of the experiment (6–15 min) sometimes led to small changes in the calculated position of the centroid. In such cases, centroid displacement was projected onto the same straight line and the vector sum of the centroid, and centrosome displacement was calculated to obtain the final value of the centrosome movement. (C) Quantification of the centrosome movement. Positive value of movement was considered when the centrosome moved from the initial position (C0) toward the nocodazole pipette tip (N). Open bar shows centrosome displacement in cytoplasts. (E) Kymograph analysis of rhodamine F-actin speckles in control (left) and C3-transferase–injected (right) cell. The absence of the centripetal flow after the injection of C3-transferase is evident from the lack of directional movement of the fluorescent speckles (right), compared with the diagonal pattern of movement in the control cells (left). Bars, 20 μm.

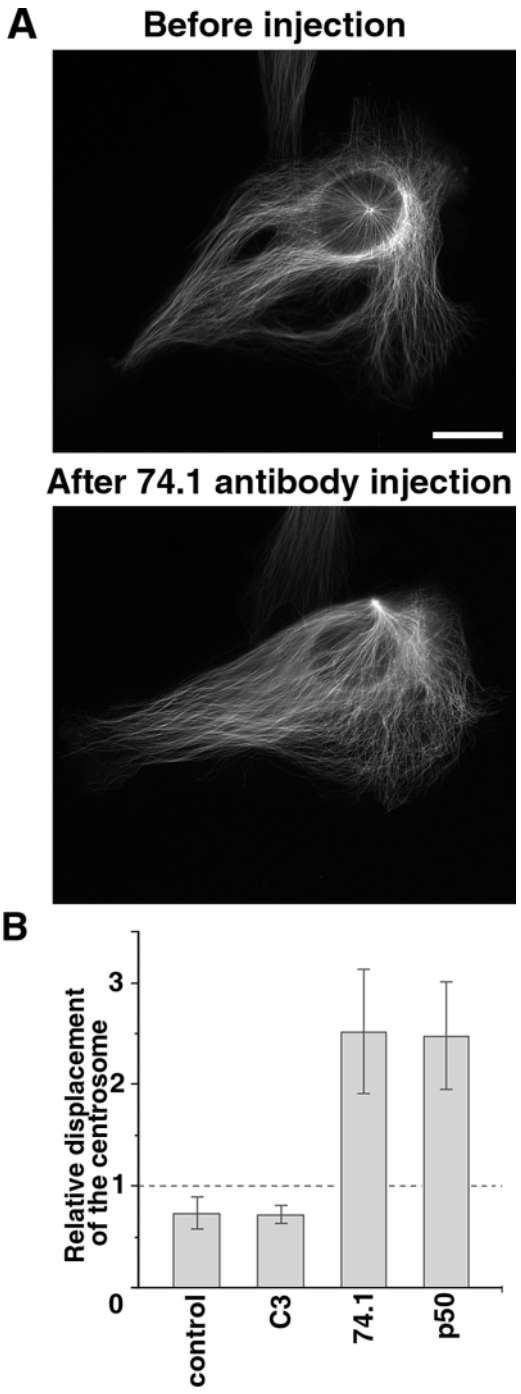
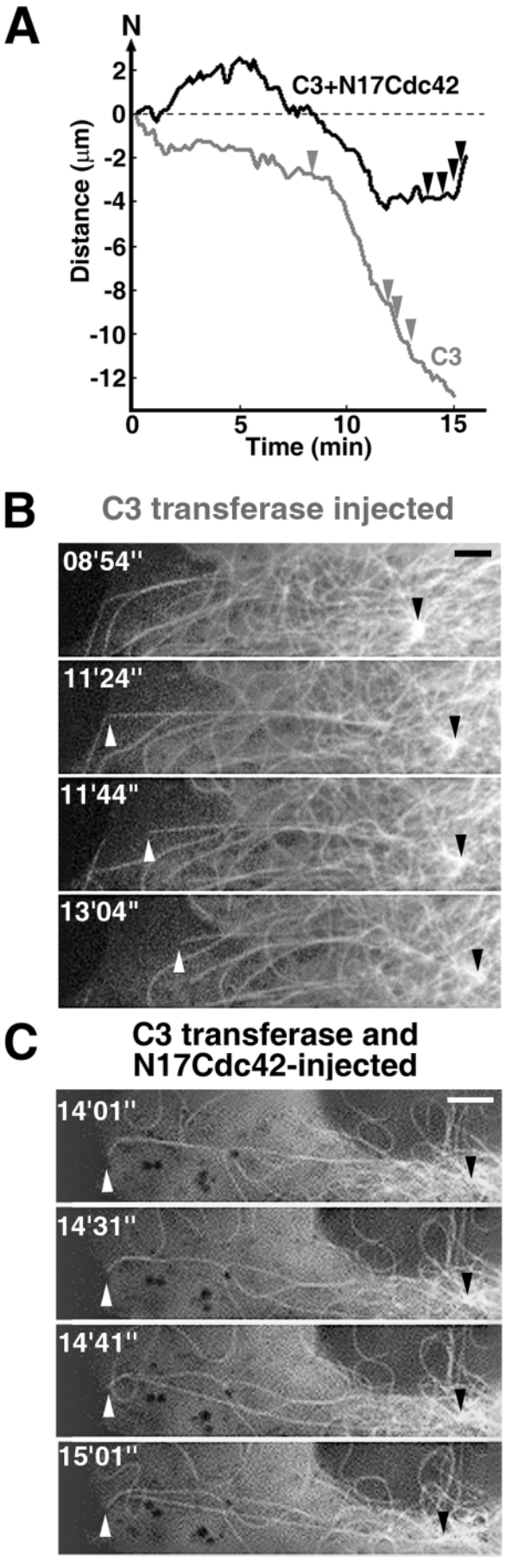


Figure 3. **Positioning of the centrosome in the cell center requires the activity of cytoplasmic dynein.** (A) Live fluorescence images of MTs in a cell before (top) or 45 min after (bottom) the injection of a dynein blocking antibody 74.1. After the injection of dynein blocking antibody, the centrosome moved to the cell margin. (B) Quantification of the displacement of the centrosome in cells injected with C3 transferase, antibody 74.1, or the p50 subunit of dynactin. Relative displacement of the centrosome was determined as the ratio between the initial (before injection) and the final (after injection) positions of the centrosome, calculated as the percentage of the cell radius drawn from the centroid through the centrosome. In control and C3-injected cells, where the centrosome remained in place, the ratios were close to 1. In cells injected with antibody 74.1 or the p50 subunit of dynactin, the centrosome traveled significantly toward the nearest cell margin, causing the greatly increased displacement values. Bar, 20 μm.

merization can also activate RhoA-dependent signaling pathway that is known to affect actomyosin contractility (for review see Wittmann and Wateman-Storer, 2001). Therefore, another explanation of the observed effect is that MT depolymerization by nocodazole mediates the induction of the local contraction of the actin cytoskeleton, and therefore facilitates the movement of the actin cytoskeleton with the attached MT aster toward the site of nocodazole application. To determine the role of actomyosin contractility in the movement of the centrosome, we either inhibited the activity of Rho A by injecting C3 transferase, or suppressed myosin activity by treating cells with myosin light chain kinase inhibitor ML7. Fluorescent speckle microscopy of actin in cells with intact MTs indicated that, as expected, C3 transferase (Fig. 2 E) or ML7 (unpublished data) completely suppressed actin centripetal flow. Inhibitors of actomyosin contractility did not significantly affect the position of the centrosome in intact cells, indicating that actomyosin contractility alone does not play a role in maintaining the centrosome position at the cell center (Fig. 3 B). However, local disruption of MTs in cells injected with C3 transferase or treated with ML7 induced profound displacement of the centrosome away from the micropipette tip (Fig. 2, C and D; Video 4). This result indicates that the displacement toward the pipette tip observed in the previous experiment was likely caused by the increase in actomyosin contractility, and that in its absence, when the forces applied to the centrosome are purely MT dependent, local depolymerization of MTs causes the pull on the centrosome from the distal end. We therefore conclude that the force exerted on the centrosome by MTs is of a pulling rather than a pushing nature.

A pulling force applied on MT arrays is generally produced by dynein motors bound to the cell cortex or anchored in the cytoplasm (Dujardin and Vallee, 2002; Gundersen, 2002). We therefore examined whether the centrosome positioning was dependent on cytoplasmic dynein by observing the centrosome position in cells injected with a dynein-blocking antibody 74.1 (Dillmann and Pfister, 1994). Inhibition of dynein activity resulted in a dramatic centrosome displacement from its normal position in the cell center to the nearest cell margin (Fig. 3, A and B). A similar effect was observed when cells were injected with recombinant dynamitin (p50) that has been shown to disrupt the dynactin complex and therefore perturb the normal localization of dynein (Echeverri et al., 1996; Burkhardt et al., 1997) (Fig. 3 B). Coinjection of C3 transferase and 74.1 antibody induced centrosome repositioning similar to the injection of dynein antibody alone (unpublished data). Inhibition of dynein activity did not affect the rate of centripetal movement of actin cytoskeleton (unpublished data), indicating that the dynein inhibition effect was independent of the actomyosin forces. These results indicate that generation of the centering force requires the activity of cytoplasmic dynein.

It has been shown that dynein-dependent centrosome repositioning during cell polarization requires the activity of Cdc42, a Rho family small GTPase (Etienne-Manneville and Hall, 2001; Palazzo et al., 2001). To test whether Cdc42 is involved in the MT pulling force applied to the centrosome, we have examined nocodazole-induced centrosome movement in cells coinjected with C3 transferase



and N17Cdc42 dominant–negative recombinant protein to inhibit both actomyosin contractility and the activity of Cdc42 (Fig. 4). In C3 transferase–injected cells, the centrosome moved continuously away from the pipette tip. Close observation showed that the pulling force applied to the centrosome from the distal side was strong enough to induce breakage of the proximal MTs anchored at the cortex, often resulting in an acceleration of the centrosome toward the distal end of the cell (Fig. 4, A and B; Video 5). In contrast, in cells coinjected with C3 transferase and Cdc42 inhibitor the centrosome wobbled around the central position, apparently held in place by MTs anchored at the cortex. Close observation revealed that the force of a few remaining MTs at the proximal end (often just one MT) was enough to hold the centrosome in place and/or reverse the direction of its movement (Fig. 4, A and C; Video 6), indicating the absence of a strong pull at the distal end. Thus, inhibition of the Cdc42 activity causes the reduction of the MT pulling force applied to the centrosome.

Since MT dynamics have been implied as a mechanism for generating a force that acts on the centrosomal MT array, we have checked the parameters of MT dynamics during the cell treatments used in this study and found that MTs continued to display normal dynamic behavior under these treatments (Table I). Therefore, it is possible that the centrosome displacement away from the cell center in the absence of dynein (Fig. 3) is driven by MT dynamics. To address this question, we looked at the centrosome displacement in cells where MT dynamics was suppressed by a combined treatment of low dozes of taxol and nocodazole (120 and 600 nM, respectively). This treatment dramatically inhibited MT growing and shortening as seen in live observations of MT dynamics (Video 7). Furthermore, it completely blocked the centrosome displacement induced by the injection of dynein-blocking antibody (unpublished data). Therefore, it is possible that the unbalanced force that acts

Figure 4. **Centrosome positioning is regulated by Cdc42-dependent signaling pathway.** (A) Kinetics of the centrosome movement in cells injected with Rho inhibitor C3 transferase (gray), or a combination of C3-transferase and Cdc42 dominant–negative recombinant protein N17Cdc42 (black). Arrowheads on the curves show the time points where images were taken in B and C. (B and C) Time series of the fluorescence images of MTs showing centrosome movement quantified in A, injected with C3 transferase (B) or double injected with C3 transferase and N17Cdc42 (C). See also the corresponding movies (Videos 5 and 6) for full time-lapse series, available at http://www.jcb.org/cgi/content/full/jcb.200305082/DC1. (B) In the absence of actin centripetal flow, the centrosome (black arrowhead) moves continuously away from the nocodazole pipette tip, driven by the pulling force on the MTs at the cortex. The pulling force on the end distal to the pipette tip is strong enough to induce breakage of the MTs at the proximal end (white arrowhead shows the position of the nascent end of the broken MT), accelerating the centrosome movement away from the pipette tip (as shown in A). (C) In the absence of both actin centripetal flow and the activity of Cdc42, centrosome moves back and forth around the central point. Pulling forces applied at the cortex are not enough to induce MT breakage. A single MT is enough to anchor the centrosome and pull it back toward the pipette tip, causing reversal of the direction of the movement (as shown in A). White arrowhead shows the position of the anchored MT end. Bars, 5 μm.

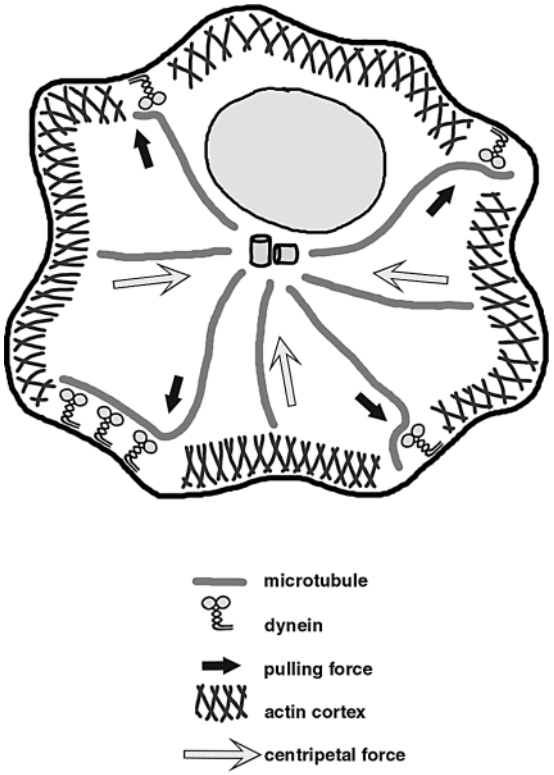


Figure 5. **Forces involved in the positioning of the centrosome.** Pulling forces applied to the MTs at the cell cortex by dynein act to position the centrosome at the cell center. Pushing forces, including actin centripetal flow and MT dynamics, are directed toward the cell center.

on the centrosome in the absence of dynein is exerted by MT dynamics.

The results of our study demonstrate that the centrosome in mammalian cells is positioned at the cell center predominantly by the MT pulling mechanisms. Furthermore, our results show that this pulling force in nonpolarized mammalian cells is exerted by cytoplasmic dynein (Fig. 5). Other forces that act on the centrosomal MTs can also contribute to the centrosome positioning. One such force is exerted by actomyosin contractility. It has been shown that actomyosin centripetal flow can move the anchored MTs toward the cell center with considerable force (Wittmann and Waterman-Storer, 2001) and can therefore contribute to the centering mechanisms. Our data that locally induced contractility can misplace the centrosome from its central position are consistent with these results. Another force that has been suggested to have a role in centrosome positioning is MT dynamics. However, as evident from experiments with dynein inhibition, actomyosin contractility and MT dynamics working separately or in combination with each other are not sufficient to position the centrosome at the cell center in the absence of dynein activity. Therefore, in stationary nonpolarized cells dynein pulling force appears to be a predominant centering mechanism.

During cell polarization, the forces acting on the centrosome are likely to be more complex. Dynein activity appears to predominate at the leading edge (Palazzo et al., 2001), balanced by the counteracting force at the rear of the cell, produced by actomyosin contractility. Therefore, in polarized cells centrosome could be positioned by the action of antagonistic forces exerted by dynein and myosin motors. It is possible that in our experiments local disruption of MTs with nocodazole in otherwise intact cells mimics the effect of cell polarization, causing the centrosome to experience dynein pull on one end and actomyosin pull induced by contractility on the other end.

Materials and methods

Cell culture

BS-C-1 cells were grown on a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F10 nutrient mixture supplemented with 10% fetal bovine serum, and antibiotics.

Microscopy and image analysis

For the fluorescence microscopy of MTs, cells were injected with bovine brain tubulin (6–7 mg/ml) conjugated with Cy3 as described previously (Rodionov et al., 2001). For the fluorescent speckle microscopy of actin cytoskeleton, rhodamine-labeled muscle actin (Cytoskeleton, Inc.) was injected at needle concentration 15 μM. Cells injected with tagged proteins were treated with Oxyrase (Oxyrase) and observed with a Nikon diaphot 300 inverted microscope. Images were collected with a slow-scan back-illuminated CCD camera (CH350, Roper Scientific) driven by Metamorph image acquisition and analysis software.

MT dynamics was measured by tracking individual MT ends and measuring the changes in the distance between a fixed point on the MT and its end in each frame. Periods of growth and shortening and frequencies

Table I. **Parameters of microtubule dynamics**

	Control untreated cells	Nocodazole-treated cells, distal edge	Cells injected with 74.1 antibody	Cells treated with ML-7
Rate of growth (μm/min)	7.25 ± 3.83	7.33 ± 3.97	7.38 ± 4.08	10.26 ± 6.51
Duration of growth (sec)	6.11 ± 4.53	6.48 ± 5.02	5.83 ± 4.54	8.83 ± 9.21
Rate of shortening (μm/min)	−15.78 ± 13.58	−14.08 ± 12.52	−15.33 ± 13.84	−17.35 ± 13.22
Duration of shortening (sec)	4.79 ± 3.17	4.66 ± 3.51	4.55 ± 2.77	5.56 ± 3.93
Rescue frequency (min^{-1})	3.85 ± 0.95	3.95 ± 0.93	3.90 ± 0.93	3.56 ± 1.46
Catastrophe frequency (min^{-1})	2.54 ± 1.23	2.26 ± 0.96	2.26 ± 1.02	2.15 ± 0.91

of rescues and catastrophes were averaged for all MTs included in the observation and used in calculating the parameters of MT dynamics shown in Table I.

The movement of the centrosome was calculated as the displacement along the straight line defined by the initial position of the centrosome and the position of the micropipette tip. Observations were made in nonmotile cells in a semiconfluent culture to exclude the influence of cell movement on the centrosome positioning. To account for the changes in cell shape, positions of the cell centroid before and after the drug treatment were calculated with Metamorph image analysis software, and the vector of the displacement of the cell centroid was subtracted from the vector of the centrosome displacement.

Local disruption of MTs

For the local disruption of MTs, cells were treated with nocodazole (10 μg/ml) applied through a micropipette placed at the cell margin at an angle to the substrate to produce a flow directed away from the cell. This arrangement avoided potential mechanical effect of nocodazole flow on MT organization in the lamella, reduced the cell retraction, and slowed down the diffusion of nocodazole toward the distal cell margin. To continuously monitor the flow of solution from the micropipette and to visualize the gradient of nocodazole concentration, the nocodazole solution was supplemented with fluorescein taken at a low (1 μg/ml) concentration.

Preparation of cytoplasts

Cytoplasts of BS-C-1 cells were prepared by centrifuging cells in the presence of the actin-depolymerizing drug Latrunculin B as described (Rodionov et al., 2001).

Inhibitor studies

To inhibit the activities of Rho family GTPases RhoA or Cdc42, cells were injected with bacterial toxin C3 transferase (0.1 mg/ml; Cytoskeleton Inc.) or a dominant–negative Cdc 42, N17Cdc42 (0.75 mg/ml; Cytoskeleton Inc.). The activity of myosin light chain kinase was inhibited by treating the cells with myosin light chain kinase inhibitor ML7 (50 μM; Sigma-Aldrich).

Online supplemental materials

Online supplemental materials include eight Quick-Time movies showing time-lapse series of the stills shown in Figs. 1–4 (Videos 1–7) and the computational simulation of local disruption of MTs with nocodazole (supplemental methods and Video 8). All supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200305082/DC1.

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