Current Biology, Vol. 15, 732–736, April 26, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.cub.2005.03.042

Cortical Actin Turnover during Cytokinesis Requires Myosin II

Minakshi Guha, Mian Zhou, and Yu-li Wang*
Department of Physiology
University of Massachusetts Medical School
377 Plantation Street, Suite 327
Worcester, MA 01605

Summary

The involvement of myosin II in cytokinesis has been demonstrated with microinjection, genetic, and pharmacological approaches; however, the exact role of myosin II in cell division remains poorly understood. To address this question, we treated dividing normal rat kidney (NRK) cells with blebbistatin, a potent inhibitor of the nonmuscle myosin II ATPase. Blebbistatin caused a strong inhibition of cytokinesis but no detectable effect on the equatorial localization of actin or myosin. However, whereas these filaments dissociated from the equator in control cells during late cytokinesis, they persisted in blebbistatin-treated cells over an extended period of time. The accumulation of equatorial actin was caused by the inhibition of actin filament turnover, as suggested by a 2-fold increase in recovery half-time after fluorescence photobleaching. Local release of blebbistatin at the equator caused localized accumulation of equatorial actin and inhibition of cytokinesis, consistent with the function of myosin II along the furrow. However, treatment of the polar region also caused a high frequency of abnormal cytokinesis, suggesting that myosin II may play a second, global role. Our observations indicate that myosin II ATPase is not required for the assembly of equatorial cortex during cytokinesis but is essential for its subsequent turnover and remodeling.

Results and Discussion

Myosin II Is Partially Involved in Mitotic Cell Rounding

Blebbistatin is available as either the s-(–) active enantiomer or the s-(+) inactive enantiomer. As was reported previously, blebbistatin s-(–) at 100 μM caused complete inhibition of cytokinesis [1]. Furthermore, the s-(+) control isomer had no effect on cytokinesis or actin organization (data not shown). Under our experimental conditions, there was no detectable effect of blebbistatin on mitosis, and all observed mitotic cells entered anaphase onset without a noticeable delay. Immunofluorescence of microtubules also showed a normal organization (data not shown). However, furrow ingression failed within 3–4 min of exposure of anaphase cells to blebbistatin, indicating that the drug was quick to enter the cell and to inhibit the myosin II ATPase. In addition, recovery occurred within 15 min of the replacement of medium, indicating that the drug dissociated readily from the binding sites. The reversibility allowed us to apply the compound both globally and locally (described later) in order to probe the function of myosin II in various regions.

Morphological effects of globally applied blebbistatin on dividing cells were observed as early as prometaphase, when normal cells underwent striking retraction and rounding. Blebbistatin-treated cells showed persistent, disorganized retractions and ingressions, which generated multiple processes and a highly irregular cell shape (Figure 1A, Movie S1 in the Supplemental Data available with this article online). The irregular morphology was similar to what was found with the Rho inhibitor C3, which inhibits myosin II indirectly through deactivation of the Rho-dependent kinase [2]. However, cells treated with C3 failed to form a concentrated band of actin and myosin along the equator as discussed below.

Quantitative analysis of the spreading area indicated a decrease in the rate of mitotic retraction, the decrease lasting well beyond anaphase onset, whereas control cells reached maximal rounding by metaphase (Figure 1B). However, most blebbistatin-treated cells eventually reached a similar extent of rounding as did control cells (Figure 1B), suggesting that myosin-II-driven contractions facilitate mitotic rounding but do not exclusively account for the activity. Unlike cells treated with butadione monoxime [3], blebbistatin-treated cells showed no apparent inhibition of the postmitotic respreading process (Movie S1).

Myosin-II-ATPase Activity Is Not Required for Equatorial Actin and Myosin-II Assembly during Early Cytokinesis

We first stained cells treated with 100 μM blebbistatin for actin filaments and myosin II. Equatorial-actin assembly during early anaphase appeared unaffected by blebbistatin (Figure 2A), as indicated by the organization of actin filaments and the normal extent of actin localization along the equator of treated and control cells (Figures 2A–2C). Cells injected with a small amount of Alexa-546 phalloidin at prometaphase showed a similar concentration of fluorescence along the equator as soon as furrowing initiated (Figure S1). Assuming that phalloidin equilibrates very slowly once bound to actin filaments [4–6], the results suggest that myosin-II-ATPase activity is not required for the accumulation of preexisting actin filaments into the equator (see [4] for the rationale of imaging injected phalloidin). In the presence of blebbistatin, myosin II showed an equatorial concentration similar to that in control cells (Figures 2A and 2B).

The above observations suggest that myosin does not rely on its own motor activity to drive itself to the equatorial region, and this is consistent with the previous observation of normal equatorial organization of myosin with defective heads [7, 8]. They similarly argue

*Correspondence: yuli.wang@umassmed.edu

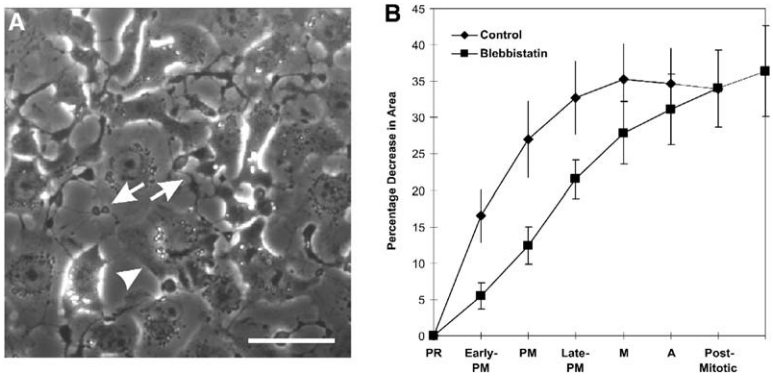

Figure 1. Impaired Mitotic Retraction of NRK Cells in the Presence of Blebbistatin

Blebbistatin was applied to a culture dish for 20 min, and a field containing mitotic cells was identified for time-lapse recording. A mitotic cell (A, arrowhead) shows a highly irregular shape and several strands of cytoplasm that failed to retract (A, arrows). However, chromosomal separation appears unaffected for all the mitotic cells in this field (see Movie S1). The bar represents 40 μm. Quantitative analysis, performed via the measurement of the percentage decrease in cell area relative to the corresponding cell area at prophase for five control cells and six blebbistatin-treated cells, indicates that blebbistatin induces a delay in rounding ([B]; the following abbreviations are used: PR, prophase; PM, prometaphase; M, metaphase; and A, anaphase). Error bars indicate the standard error of the mean (SEM). The retraction of treated cells continues well after anaphase onset and reaches a similar average extent as do control cells at a later time point.

against a myosin-II-driven equatorial contraction mechanism that causes the cortical actin network to collapse and concentrate into the equator [9]. Furthermore, the results suggest that the inhibition of cytokinesis by blebbistatin is caused not by a defect in equatorial cortical assembly but instead by defects in subsequent steps.

Myosin-II-ATPase Activity Is Required for Actin and Myosin Disassembly during Late Stages of Cell Division

We examined the dissociation of actin and myosin II from the equatorial region during and after late anaphase. In blebbistatin-treated cells, both actin and myosin II persisted along the equator long after (>30 min) the separation of chromosomes (Figures 2A and 2C). In contrast, control cells showed a striking decrease in the percentage of actin filaments along the equator after anaphase onset (Figures 2B and 2C). These results indicate that myosin-II-ATPase activity is required for the postmitotic dissociation of equatorial actin and myosin-II filaments. The accumulating filaments may inhibit not only cortical ingression but also retraction along the equator, causing the equatorial region to form a bulge as seen in some treated cells (Figure 2D).

To determine whether the inhibition of actin dissoci-

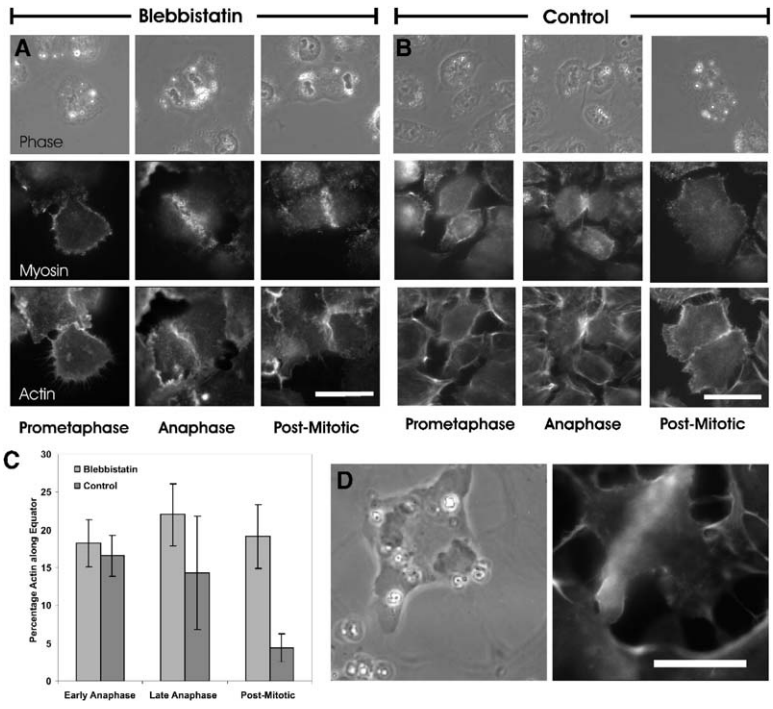

Figure 2. Effects of Blebbistatin on the Assembly and Disassembly of Equatorial Actin and Myosin

A dish was treated with blebbistatin for 20 min before fixation, and images of phalloidin or myosin-IIa immunostaining were collected from cells at various stages of division. Myosin-IIa and actin filaments show a similar increase in equatorial concentration during early anaphase in control (B) and blebbistatin-treated (A) cells. However, after the completion of mitosis, both actin and myosin II disappear from the cleavage site in control cells ([B], right column; the concentration of actin between the daughter cells is due to the formation of cell-cell junctions) but persist in the equatorial region of blebbistatin-treated cells ([A], right column). The bar represents 40 μm. Integration of the intensity of phalloidin staining in the equatorial region versus the whole cell indicates that equatorial-actin filaments persist in blebbistatin-treated cells ([C], left bars), whereas control cells show a striking decrease shortly after anaphase onset ([C], right bars). The graph was generated from the measurements of four to eight control or treated cells at each stage. Error bars indicate SEM. The persistent band of equatorial actin defines a region that resists anaphase retraction in blebbistatin-treated cells, causing some cells to form a bulge along the equator (D). The bar represents 40 μm.

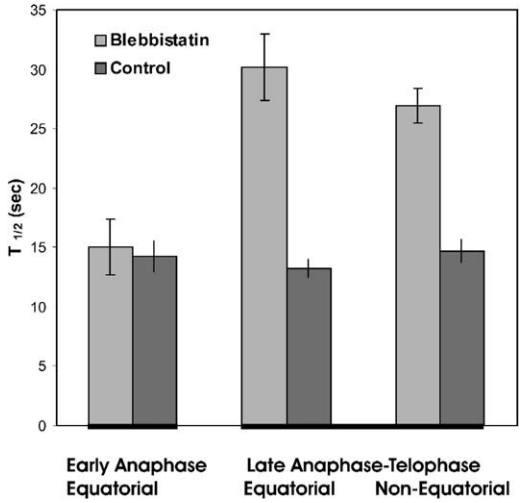

Figure 3. FRAP Analysis of Actin Dynamics in Control and Blebbistatin-Treated Cells

Cells were injected with rhodamine actin during prometaphase, treated with blebbistatin after 10–15 min of recovery, and bleached in the equatorial region within 1–2 min ("Early Anaphase") or after more than 5 min ("Late Anaphase-Telophase") of anaphase onset. The half-time of recovery shows no significant difference during early anaphase, but it does show a 2-fold increase in blebbistatin treated cells as compared to control cells during a later stage. Moreover, similar increases in half-time are observed both along and out of the equatorial region. Each half-time represents the average from four to six measurements. Errors bars indicate SEM.

ation was due to a decrease in the dynamics of the cortical-actin network, we performed fluorescence recovery after photobleaching (FRAP) analysis on cells microinjected with rhodamine-labeled actin. Fluorescence recovery for both experimental and control cells at early anaphase showed a half-time of about 12–15 s (Figure 3), indicating that equatorial cortex at this stage is highly dynamic and that the dynamics are independent of myosin motor activities. However, at later stages when the equatorial band became well organized, blebbistatin-treated cells showed a 2-fold increase in recovery half-time (to 25–30 s) whereas the half-time for control cells remained unchanged throughout cytokinesis (Figure 3). Similar differences in half-time between control and treated cells were observed outside the equator (Figure 3). Together, these observations suggest that mitotic exit has a global effect in stabilizing the actin cortex, and that myosin-II motor activities are required for maintaining the dynamics. It is worth noting that the amount of myosin in the furrow accounts for only about 10% of the total cellular myosin in *Dictyostelium* [10]. Although the concentration of myosin II is higher along the equator than in other cortical regions, the ratio of myosin II to actin filaments is likely to be similar throughout the cortex, which explains the similar rate of actin turnover in control cells.

The delayed recovery, together with the accumulation of equatorial-actin filaments, indicates that myosin-II motor activity is crucial for the turnover of actin filaments in the equatorial region. The process could involve either a "ripping" of actin filaments from the membrane by the contractile forces or an activation of an actin-severing or depolymerization mechanism. Such a cortical disassembly process has been implicated in an earlier ultrastructural analysis [11] and is likely responsible for removing the accumulating cortical materials in order for the ingression to continue.

Myosin II Functions Both along and outside the Equatorial Region during Cytokinesis

We found previously that local application of cytochalasin D at the equator not only failed to inhibit cytokinesis but also appeared to facilitate the furrowing process [12]. In contrast, application of cytochalasin D near the spindle poles inhibited cytokinesis. This raised the provocative possibility that crucial actin-myosin interactions for cytokinesis may occur outside the equatorial region. To address this question, we applied blebbistatin to a small (10–15 μm diameter) region at the equator by using a microneedle to release the drug close to the cell cortex while simultaneously removing the medium with a nearby suction needle (Figure 4A; [12]). The treatment caused the inhibition of ingression (~60% of cells), as did global applications (Figure 4B and Movie S2). Phalloidin staining of these cells showed a strong accumulation of actin filaments on the side facing the needle (Figure 4C), whereas local treatment with dimethyl sulfoxide (DMSO)-containing carrier solution had no effect on cytokinesis (data not shown; [12]). Although the inhibitory effect of blebbistatin is opposite that of cytochalasins, it may be explained if myosin II, like cytochalasins, promotes the disassembly of actin filaments during the force-generating interactions. The effect of blebbistatin is similar to that of jasplakinolide, which inhibits ingression by stabilizing the equatorial actin network [12].

To investigate whether myosin II also functions outside the equator, we applied blebbistatin near the polar region. The treatment caused only a small percentage of cells to fail cytokinesis (<10%). However, a large percentage of cells (~60%) showed various degrees of abnormality (Figures 4A and 4B, Movie S3), including asymmetric ingression, misplaced cleavage furrows (Movie S3), and top-to-bottom divisions with little lateral ingression; these effects are not observed with equatorial application even at suboptimal doses. These results suggest that cytokinesis involves not only contractions and cortical assembly and disassembly along the equator but also possibly a global balance among contractile forces, cortical rigidity, and structural integrity.

Conclusions

In summary, our results suggest that equatorial myosin II serves a dual role during cytokinesis: to generate the forces and to disassemble the cortex to allow ingression. However, motor activity of myosin II is not necessary for the assembly of the equatorial band of myosin and actin filaments. The possibility of coupled contraction and structural disintegration was proposed more than two decades ago [13], although myosin II was never suspected to play both roles. The action of myosin II may be compared to that of mitotic centro-

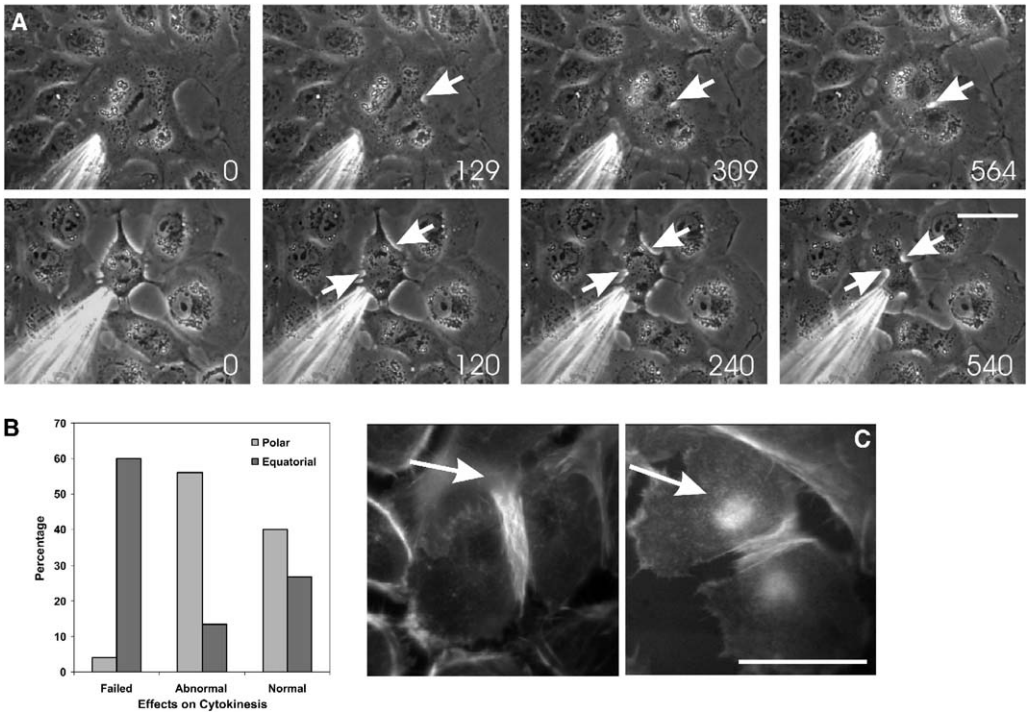

Figure 4. Effects of Localized Treatment with Blebbistatin

A cell treated at the equator shows inhibition of cytokinesis on the side facing the needle ([A], upper row). The other side shows normal cytokinesis as indicated by the arrows. A cell treated near the pole shows misplaced ingression ([A], lower row, upper arrows), located far away from the equator and near separated chromosomes. Numbers indicate time in seconds. Analysis of 15 cells treated along the furrow and of 25 cells near the pole shows a high percentage of cleavage failure with equatorial treatment and a high percentage of abnormal cleavage (top-down division and misplaced furrow) with polar treatment (B). A cell treated at equator ([C], left panel, arrow) shows strong accumulation of actin filaments along the side facing the needle. Cells treated near the pole ([C], right panel, arrow) show an equatorial-actin organization similar to that in control cells. The bar represents 40 μm.

mere-associated kinesin (MCAK) for microtubules [14]; MCAK is believed to induce microtubule disassembly while transporting the cargo in a "pacman"-like manner.

Experimental Procedures

Cell Culture, Drug Treatment, and Microscopy

Normal rat kidney (NRK) epithelial cells (NRK52E; American Type Culture Collection) were cultured as described previously [15]. They were subcultured onto cover-glass chamber dishes 12–24 hr before the experiment. Blebbistatin [s-(–) isomer; Toronto Research, Toronto, Canada] or the s-(+) control (EMD Biosciences, San Diego, California) was dissolved with 90% DMSO to make a stock solution of 100 mM, which was diluted 1:1000 with supplemented F-12K medium to obtain a working concentration of 100 μM. After replacement of the medium, the culture chamber was incubated for a minimum of 20 min prior to recording or fixation. Fixation and fluorescent staining were performed as described previously [2]. Antibodies against myosin IIa were obtained from Covance Research.

Because blebbistatin is known to be light sensitive [16], microscope lamp intensity was maintained at a minimum and red cellophane filters (Edmund Scientific, Barrington, New Jersey) were used during the observation of live cells. Images were acquired with a 40×, numerical aperture (NA) 0.75 Plan-Neofluar phase or a 100×, NA 1.30 Plan-Neofluar phase lens (Carl Zeiss, Thornwood, New York) and a cooled-CCD camera (NTE-CCD-EBFT; Roper Scientific, Trenton, New Jersey) and were processed with customized digital-imaging software for background subtraction. Integrated fluorescence intensity along the equator (defined as the region with a concave lateral border during anaphase and telophase) and within the whole cell was also obtained with custom software. Mitotic cell rounding was calculated by dividing the retracted area of the cell during mitosis, this area measured as the number of pixels within a manually drawn cell boundary, by the area of the same cell during prophase.

Micromanipulation and Local Drug Application

A stock solution of blebbistatin was mixed with 25 mg/ml red fluorescent dextran (Molecular Probes, Eugene, Oregon) in warm, supplemented F-12K medium to obtain a working concentration of 850 μM blebbistatin, 1 mg/ml dextran, and 1% DMSO. The solution was clarified at 25,000 RPM for 20 min in a Type 42.2 Ti rotor (Beckman Coulter, Fullerton, California). Localization of blebbistatin was achieved via a release needle paired with a suction needle [12]; the latter was prepared by breaking the tip of a microneedle and fire polishing with a microforge (Narishige USA, Sea Cliff, New York). The needles were mounted on a custom-made double micromanipulator, and the flow was regulated with a combination of regulated compressed air and vacuum. NRK cells were rinsed with filtered F-12K medium immediately before the experiment, and the culture was layered with mineral oil (embryo tested; Sigma, St. Louis, Missouri) after drug application to prevent evaporation. Alexa-546 phalloidin was prepared and microinjected as described previously [17]. Images of injected cells were acquired via spinning-disk confocal optics (Solemere Technology, Salt Lake City, Utah) with a diode-pumped solid-state laser at 532 nm.

FRAP Analysis

Rhodamine-labeled muscle actin (cytoskeleton) was microinjected at a concentration of 0.8 mg/ml after clarification in an ultracentrifuge. Fluorescence was bleached with the 514 nm line from an

Argon laser at a power of 300 mW and a duration of 50 ms. Images during fluorescence recovery were captured every 10 s with spinning-disk confocal optics. A 525 nm dichroic mirror (525DRLP; Omega Optical, Brattleboro, Vermont) was placed in the optical path to allow simultaneous bleaching with the 514 nm beam and imaging with the 532 nm laser. Fluorescence intensity was measured with custom software and analyzed with Excel (Microsoft). Photobleaching of control cells had no effect on division.

Supplemental Data

Three supplemental figures are available with this article online at http://www.current-biology.com/cgi/content/full/15/8/732/DC1/.

Acknowledgments

The research was supported by a grant GM-32476 from the National Institutes of Health to Y.L.W.

Received: January 10, 2005
Revised: March 7, 2005
Accepted: March 17, 2005
Published: April 26, 2005

References

1. Straight, A.F., Cheung, A., Limouze, J., Chen, I., Westwood, N.J., Sellers, J.R., and Mitchison, T.J. (2003). Dissecting temporal and spatial control of cytokinesis with a myosin II Inhibitor. Science *299*, 1743–1747.
2. O'Connell, C.B., Wheatley, S.P., Ahmed, S., and Wang, Y.L. (1999). The small GTP-binding protein rho regulates cortical activities in cultured cells during division. J. Cell Biol. *144*, 305–313.
3. Cramer, L.P., and Mitchison, T.J. (1995). Myosin is involved in postmitotic cell spreading. J. Cell Biol. *131*, 179–189.
4. Cao, L.G., and Wang, Y.L. (1990). Mechanism of the formation of contractile ring in dividing cultured animal cells. I. Recruitment of preexisting actin filaments into the cleavage furrow. J. Cell Biol. *110*, 1089–1095.
5. Wieland, T. (1977). Modification of actins by phallotoxins. Naturwissenschaften *64*, 303–309.
6. Wang, Y.L. (1987). Mobility of filamentous actin in living cytoplasm. J. Cell Biol. *105*, 2811–2816.
7. Yumura, S., and Uyeda, T.Q. (1997). Transport of myosin II to the equatorial region without its own motor activity in mitotic Dictyostelium cells. Mol. Biol. Cell *8*, 2089–2099.
8. Zang, J.H., and Spudich, J.A. (1998). Myosin II localization during cytokinesis occurs by a mechanism that does not require its motor domain. Proc. Natl. Acad. Sci. USA *95*, 13652–13657.
9. White, J.G., and Borisy, G.G. (1983). On the mechanisms of cytokinesis in animal cells. J. Theor. Biol. *101*, 289–316.
10. Robinson, D.N., Cavet, G., Warrick, H.M., and Spudich, J.A. (2002). Quantitation of the distribution and flux of myosin-II during cytokinesis. BMC Cell Biol. *3*, 289–316.
11. Schroeder, T.E. (1972). The contractile ring. II. Determining its brief existence, volumetric changes, and vital role in cleaving Arbacia eggs. J. Cell Biol. *53*, 419–434.
12. O'Connell, C.B., Warner, A.K., and Wang, Y. (2001). Distinct roles of the equatorial and polar cortices in the cleavage of adherent cells. Curr. Biol. *11*, 702–707.
13. Hellewell, S.B., and Taylor, D.L. (1979). The contractile basis of ameboid movement. VI. The solation-contraction coupling hypothesis. J. Cell Biol. *83*, 633–648.
14. Moore, A., and Wordeman, L. (2004). The mechanism, function and regulation of depolymerizing kinesins during mitosis. Trends Cell Biol. *14*, 537–546.
15. McKenna, N.M., and Wang, Y.L. (1989). Culturing cells on the microscope stage. Methods Cell Biol. *29*, 195–205.
16. Kolega, J. (2004). Phototoxicity and photoinactivation of blebbistatin in UV and visible light. Biochem. Biophys. Res. Commun. *320*, 1020–1025.
17. Wang, Y.-L. (1992). Fluorescence microscopic analysis of cytoskeletal organization and dynamics. In Cytoskeleton: A Practical Approach, K.L. Carraway and C.A.C. Carraway, eds. (Oxford: Oxford University Press), pp. 1–22.