Published in final edited form as:

Phys Biol. ; 14(2): 026005. doi:10.1088/1478-3975/aa59a2.

Analysis of microtubule growth dynamics arising from altered actin network structure and contractility in breast tumor cells

Eleanor C Ory¹, Lekhana Bhandary^{2,3}, Amanda E Boggs^{2,3}, Kristi R Chakrabarti^{2,3}, Joshua Parker¹, Wolfgang Losert^{1,2,5}, and Stuart S Martin^{2,4,5}

¹Department of Physics, IPST, and IREAP, University of Maryland, College Park, MD, United States of America

²Marlene and Stewart Greenebaum NCI Comprehensive Cancer Center, University of Maryland School of Medicine, 22 S. Greene Street, Baltimore, MD 21201, United States of America

³Program in Molecular Medicine, University of Maryland School of Medicine, 655 W. Baltimore Street, Baltimore, MD 21201, United States of America

⁴Department of Physiology, University of Maryland School of Medicine, 655 W. Baltimore Street, Bressler Bldg. Rm 10-29, Baltimore, MD 21201, United States of America

Abstract

The periphery of epithelial cells is shaped by opposing cytoskeletal physical forces generated predominately by two dynamic force generating systems—growing microtubule ends push against the boundary from the cell center, and the actin cortex contracts the attached plasma membrane. Here we investigate how changes to the structure and dynamics of the actin cortex alter the dynamics of microtubules. Current drugs target actin polymerization and contraction to reduce cell division and invasiveness; however, the impacts on microtubule dynamics remain incompletely understood. Using human MCF-7 breast tumor cells expressing GFP-tagged microtubule end-binding-protein-1 (EB1) and coexpression of cytoplasmic fluorescent protein mCherry, we map the trajectories of growing microtubule ends and cytoplasmic boundary respectively. Based on EB1 tracks and cytoplasmic boundary outlines, we calculate the speed, distance from cytoplasmic boundary, and straightness of microtubule growth. Actin depolymerization with Latrunculin-A reduces EB1 growth speed as well as allows the trajectories to extend beyond the cytoplasmic boundary. Blebbistatin, a direct myosin-II inhibitor, reduced EB1 speed and yielded less straight EB1 trajectories. Inhibiting signaling upstream of myosin-II contractility via the Rho-kinase inhibitor, Y-27632, altered EB1 dynamics differently from Blebbistatin. These results indicate that reduced actin cortex integrity can induce distinct alterations in microtubule dynamics. Given recent findings that tumor stem cell characteristics are increased by drugs which reduce actin contractility or stabilize microtubules, it remains important to clearly define how cytoskeletal drugs alter the interactions between these two filament systems in tumor cells.

⁵Author to whom any correspondence should be addressed. smartin@som.umaryland.edu and wlosert@umd.edu.

Supplementary material for this article is available online

Keywords

cytoskeleton; cancer; dynamic; microtubule; actin; EB1

Introduction

A delicate interaction between the physical forces of microtubule expansion and actin contraction is often disrupted as epithelial cells transform into carcinomas [1, 2]. More than 90% of human solid tumors arise as carcinomas from epithelial cells [3], emphasizing the importance of defining how the loss of epithelial cytoskeletal architecture could impact the understanding and treatment of cancer. Microtubule polymerization initiates from the centrosome, also known as the microtubule-organizing center (MTOC), which is found in the center of eukaryotic cells (figure 1). Extension of microtubules toward the cell surface is counteracted in several ways by the cortex of actin filaments that lies beneath the plasma membrane [4, 5].

First, the mesh size of the actin cortex is estimated to range from 20 to 250 nm [6], potentially providing a significant physical barrier for microtubule bundles, since individual microtubules are 25 nm in diameter. Second, continuous contraction of the actin cortex through the action of the motor protein, non-muscle myosin-II (NMII) provides an inward contractile force that opposes microtubule expansion from the cell center [4]. Third, the end-binding protein-1 (EB1), which is attached to the growing ends of microtubules, is captured at the actin cortex through association with the adenomatous polyposis protein (APC) [5, 7]. This combination of cortical network mesh [6], actomyosin contractility [4] and microtubule capture [5] ensure that the physical forces of microtubule extension and actin cortical contraction are interacting against each other [1]. In addition, the mechanical coupling of microtubules to the actin cytoskeleton allows micro-tubules to bear much larger compressive forces within cells than is possible with purified microtubules *in vitro* [8]. Mechanotransduction in epithelial cells is known to be regulated by this interaction of opposing forces between microtubules and actin filaments; toxins that alter either microtubules or actin can disrupt the ability of epithelial cells to respond to mechanical stimuli [9]. Alterations of cytoskeletal structure and regulation in epithelial tumor cells disrupt the counteracting stability between microtubules and actin cortex [10, 11].

It is clear that interactions between microtubules and actin exist, however complete understanding of the mechanisms and full implications are an active question of research. Previous studies in the literature show direct binding interactions between F-actin, actin associated proteins, microtubules, and microtubule associated proteins [12–16]. Additionally, research shows that microtubules attach to F-actin via formins and increase actin polymerization [12, 15]. Interactions between microtubules and actin have been documented in a variety of different cells including mouse fibroblasts, neurons, *Dictyostelium discoideum*, *Giardia intestinalis*, Sertoli cells, HeLA cells [12–18]. The work outlined in this paper looks at the impact of changes on actin filaments on microtubule growth dynamics. Several studies show that microtubules are load bearing and resist compression [19–21]. However, much of the literature demonstrates microtubules in tension,

including neuronal cells [17, 22, 23] or cells undergoing cell division [24, 25]. Additionally, microtubules of cells in Interphase can also undergo tension [26–32]. Microtubules can exert tensional forces on the nucleus [26, 28, 30, 31]. Severing microtubules can increase the curvature in microtubules [28, 32]. Many studies propose that tips are still in compression near the membrane and actomyosin cortex [28, 32].

Given the role of actin filaments in cell division and motility, actin has also become a target for the development of cancer therapies [33, 34]. Inhibition of actin assembly and contractility are effective at reducing the growth and invasion of tumor cells [35–37]. However, growing evidence also emphasizes the need to further understand the consequences of targeting the actin cytoskeleton on other aspects of cancer progression. Microtentacles (McTNs) on the surface of circulating tumor cells are supported by microtubules and counteracted by actin contractility [1, 38, 39]. Compounds or genetic alterations that stabilize microtubules [38, 40] or reduce actin integrity [38, 41] promote microtentacle formation and increase the retention of circulating tumor cells in the lung capillaries of living mice [40, 41]. Recently, it has also become clear that reducing actin contractility can actually increase stem cell characteristics in epithelial tumor cells [42–44], resulting in greater tumor-initiating capability.

The ability of patient-derived tumor cells to grow *in vitro* has historically been very limited, with months often required for patient tumor cells to proliferate in culture, raising concerns that the difficult adaptation to culture is imposing strong selective pressures that will alter behavior of tumor cells relative to the original patient. In contrast, cells with increased stem cell characteristics are able to grow much more efficiently *in vitro* or after transplantation in mice, with as few as 20 cancer stem cells being required to regenerate a tumor compared to more than a million non-stem cells. Very recent studies now show that even relatively short-term treatment with compounds which reduce actin contractility by targeting ROCK (Y-27632) or myosin-II (Blebbistatin) [45] can directly induce stem cell characteristics in epithelial tumor cells [42] that promote long-term growth of patient-isolated cancer cells [43, 44] and tumor formation in mice [42]. These results suggest that altering the mechanical tension of epithelial tumor cells can regulate their ability to proliferate *in vitro* and *in vivo*.

To improve the understanding of how altering actin integrity and contractility affects the cytoskeletal interactions with microtubules, we used time-lapse confocal microscopy to track the movement of EB1 in the epithelial MCF-7 breast cancer cell line. Since EB1 is so tightly coupled to the growing microtubule plus end [5, 46, 47], we could infer microtubule growth from the presence and motion of EB1. The schematic shown in figure 1 shows how manipulations of the actin cytoskeleton and actin cortex would be predicted to lead to different dynamics in EB1 trajectories. Dynamic actin filaments in the cytosol may provide obstacles for growing microtubules. Normally, the actin cortex enhances membrane tension [4] and resists EB1-capped, growing microtubules from extending beyond the cell membrane or continuing on a straight line [5]. Complete disruption of actin polymerization with Latrunculin-A should make it easier for microtubules to grow beyond the cell boundary. Targeting the Rho-kinase (ROCK) with the small molecule, Y-27632, inhibits activation of myosin-II and thereby reduces contractility of actin filaments within the cortex [48]. Likewise, Blebbistatin binds directly to myosin-II forcing an actin-detached state that

disrupts actomyosin contractility [4, 45]. Both Y-27632 and Blebbistatin should promote greater extension of microtubules and EB1 by reducing the counteracting force of the actin cortex [1, 4, 45], but without fully disrupting actin. Since Y-27632 and Blebbistatin were also both shown to increase tumor stem cell characteristics [42–44], it will be interesting to compare their effects on microtubule dynamics.

Materials and methods

Breast cancer cells and culture conditions

Human MCF-7 breast cancer cells, which retain many epithelial characteristics [49, 50], were used to model the effects of actin disruption on cytoskeletal interactions in epithelial carcinoma cells and obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). MCF-7 cells were cultured in DMEM (Life Technologies) containing 10% FBS (Life Technologies) and 1% Pen/Strep (Gemini bioproducts) [40]. At 80% confluency, cells were detached by trypsinization (Mediatech) and replated onto dishes with glass coverslip bottoms to enable imaging with confocal microscopy [40].

Indirect Immunofluorescence

MCF-7 cells grown on glass coverslips were treated with vehicle (DMSO 0.1%) or the indicated drug for 30 min, 25 μ M Blebbistatin (Sigma), 5 μ M Latrunculin-A (Sigma) or 10 μ M Y-27632 (Sigma). Cells were then fixed (3.7% formaldehyde/PBS, 15 min), permeabilized (0.25% Triton X-100/PBS, 10 min), and blocked for 1 h (PBS/5% bovine serum albumin (BSA)/0.5% NP40). Immunostaining was performed for 1 h at 4 °C with monoclonal α -tubulin DM1A (1:1000, Sigma), along with Hoescht 33342 (1:5000, Sigma) to label DNA and Alexa594-Phalloidin (1:200, Molecular Probes) to visualize polymerized actin. Images were acquired on an Olympus FV1000 laser scanning confocal microscope (Olympus, Center Valley, PA).

Transfection and confocal microscopy

To observe the growth of microtubules, cells were transfected using Exgen-500 (Fermentas) with full-length APC-associated end-binding protein-1 (EB1) that was fused at its C-terminus to green fluorescent protein (EB1-GFP) [47]. To compare cells with relatively similar levels of EB1 expression, cells were imaged at a short time point (16 h post-transfection) and cells with comparably low levels of particulate EB1-GFP fluorescence were used for analysis. Cells with bright and diffuse cytoplasmic EB1-GFP were excluded from the analysis, since this level of expression presumably saturates the microtubule binding sites for EB1. Cells were imaged on an Olympus FV-1000 point scanning confocal microscope, and live images were taken every 2 s in 50 sets of 256×256 image frames, using a 60 $\times$ oil immersion objective. GFP fluorescent signal was collected using excitation from a 488 nm laser line (10% power) and FITC emission filter set (Olympus, 41001). For boundary analysis datasets only, cells were co-transfected with mCherry to serve as a non-targeted cytoplasmic marker. Time-lapse videos were collected from at least 10 cells for each of the 4 different treatment conditions: Vehicle control (0.1% DMSO), Blebbistatin (25 μ M), Latrunculin-A (5 μ M) and Y-27632 (10 μ M).

Image analysis, particle tracking and computation

All image analysis, tracking, and further computation was conducted and written in MATLAB. EB1 localizes into clusters bound to the tips of growing microtubules. Trajectories of the EB1 clusters were computed using established particle tracking methods [51]. To remove noise, a bandpass filter was applied to the images with a lower threshold at 1 pixel and higher bandpass threshold at 4 pixels. The position of tips was determined with subpixel accuracy via peak fitting as the center of bright peaks. Finally, the locations on successive images are linked based on minimizing the total displacement between all particles. To reduce noise from centroid computation, a moving average of 3 was applied to the trajectory of each EB1 tip, truncating the first and last location of each tip. Furthermore, only particles that moved at a minimum displacement of 1 pixel were included in velocity vector computation. The average speed was taken by calculating the mean speed across individual EB1 tip speeds; each individual EB1 tip's speeds was calculated by dividing the cumulative distance traveled by the total time. The average velocity organized by regions was calculated by taking the mean of all instantaneous velocities between two frames.

In order to find the outline of the cytoplasmic boundary, we modified methods developed previously [52]. The outline was taken from the mean image of the mCherry stack of 50 frames, which resulted in an objective measure of cytoplasmic edge and defined a boundary for which cellular extensions (microtentacles, filopodia, etc) could be considered 'outside' the main cell shape for further analysis. As a threshold for cells to be included for further analysis, more than 85% of identified particles had to be successfully tracked; also 85% of the trajectories tracked through subsequent frames had to move at the minimum displacement of 1 pixel. Stacks of images that did not meet the above criteria were considered too noisy for inclusion.

For further analysis, the distance from the boundary was considered positive, if the tip was located inside the cytoplasmic boundary, but negative if outside the cytoplasmic boundary. Particle tip locations were then divided into 3 different locations: cell bulk, boundary, or outside the cell. Cell cytoplasmic boundary was defined as 10% of the cell area closest to the boundary from the inside, the area further inside is defined as bulk; and all protrusions beyond the boundary were considered outside.

Using image stacks without mcherry, autocorrelation functions of the orientation were computed based on the dot product of normalized velocity vectors with time lag τ ; normalized dot products were then binned temporally. In order to look at the straightness of EB1 tip trajectories spatially, the distribution of the same temporal correlation values were rebinned spatially. Standard error across cell populations is shown for each set of conditions. Using a least squares approximation, an exponential fit was computed for each condition's autocorrelation, as well as each individual cell's autocorrelation; error is the standard error for exponential decay across individual cells.

Statistics

For all statistical analysis, code was written in MATLAB. In order to determine whether the data had a normal distributions, measurements of skew and kurtosis were conducted. Data

was accepted as normal if the skew had a value within the range ± 2 and the kurtosis value was between 0 and 6. For comparing multiple conditions, anova tests measured for p -values less than 0.05. In the event that the data did not meet the criteria for a normal distribution, an additional Kolmogorov–Smirnov (ks -test) was conducted in parallel and both the anova and Komongorov–Smirnov p -values had to be less than 0.05 to be accepted as significant.

Results

Actin cytoskeleton integrity affects the structure of the microtubule network

Qualitative changes in the appearance of the microtubule network were investigated in conjunction with perturbations of the actin network using three different drugs: Latrunculin-A [38], Blebbistatin [45, 53], and Y-27632 [54]. Network morphology and colocalization were observed in fixed cells stained for DNA with Hoescht 33342, F-actin with Alexa594-phalloidin, and α -tubulin monoclonal antibody (DM1A). When the actin cytoskeleton was disrupted via the three drugs, no significant effect on nuclear structure and integrity was found, but blinded observations of at least 25 cells per condition revealed qualitative changes in the microtubule network. Figure 2 shows representative cells for control and the three treatments.

The morphology of the MCF-7 cells under control conditions was consistent with the relatively flat shapes associated with cultured epithelial breast tumor cells [38]. The membrane edges appeared tightly anchored to the surface and the cytoskeletal fibers were distributed throughout the cytoplasm. Polymerized actin was enhanced in diffuse spots within the cytoplasm and near the nucleus, but was most concentrated along the cell periphery where the denser actin cortex is localized. Thin α -tubulin fibers were visible as a network throughout the cytoplasm with some characteristic accumulation of α -tubulin filaments near the nucleus.

In cells treated with 5 μM of the actin depolymerizing compound, Latrunculin-A [17], only minimal residual polymerized F-actin was observed with phalloidin staining, as expected. Filaments of α -tubulin were thicker and more highly concentrated throughout the cell. Most interestingly, the stain for tubulin showed straight protrusions beyond the bulk of the cytoplasm; these protrusions did not contain F-actin due to the depolymerizing effect of Latrunculin-A. The results indicated that actin depolymerization can promote microtubule extension, likely by removing the barrier of the actin cortex. For cells treated with 25 μM Blebbistatin [53], F-actin clumped both within the cytoplasm and near the cell body boundary. In Blebbistatin treated cells, The α -tubulin in these cells appeared denser and to accumulate near the nucleus. Small, straight local protrusions containing both actin and α -tubulin were also visible outside the cell's cytoplasmic boundary. Finally, in cells treated with 10 μM of Y-27632 [54] the overall morphology of the cells had fewer localized protrusions, but also had several regions with actin membrane 'ruffles' along the cell periphery, consistent with reports that Y-27632 can result in Rac activation which promotes lamellipodial membrane ruffles [33]. Overall, fixed slides confirmed successful changes in cytoskeletal morphology for the 3 different drug treatments.

Live cell imaging of microtubule growth dynamics

In order to track moving EB1 tips on microtubule ends, images of GFP-labeled EB1 proteins were collected via fluorescence time-lapse microscopy [46, 47]. Taking one image every 2 s allowed following of the growing trajectories of the labeled microtubule plus ends. In control movies, EB1 tips appeared uniformly distributed across the inside of the cell (supplemental figure 1 top (stacks.iop.org/PhysBio/14/026005/mmedia)). A maximum intensity time projection written in MATLAB of images across all 50 time points illustrated the microtubule tip trajectories throughout the cell (supplemental figure 1-bottom).

Particle tracking microtubule end dynamics

The tip finding algorithm successfully picked out EB1 tips from images (figure 3(A)). We analyzed image sequences if the tip tracking algorithm tracked at least 85% of visible tips for a minimum of 4 frames (figure 3(B)), and at least 85% of tips moved more than 1 pixel. For control and drug treated cells, typical trajectories are shown both in the cell bulk and near the cell body boundary (figure 3(C)). For all 4 conditions, an overlay of all trajectories are shown in figures 3(D)–(G). As expected, trajectories are generally in concordance with time-projected images shown in supplemental figure 1 (bottom panels), indicating that the microtubule tip finding and tracking algorithm output was reasonable. The trajectories are color-coded to reveal the overall outward motion of microtubules—blue indicated the starting-point of each trajectory and red the endpoint (figures 3(D)–(G)). It is worth noting that Blebbistatin treated trajectories appeared shorter and less directed compared to control condition (figure 3(F)). Previous tip tracking methods use algorithms that give preference to pairing tracks with a low bending angle [55]; whereas, the tracking algorithms utilized in this study are better able to track brownian motion [51]. Having an algorithm that is not biased towards low bending angle allowed us to better compare directedness of motion for treatments like blebbistatin.

Vehicle-treated cells display new microtubule growth throughout the cytoplasm, with very few EB1 trajectories extending beyond the cell cytoplasmic boundary (figure 3(D)). Likewise, Blebbistatin-treated cells appeared to have the vast majority of EB1 trajectories in short segments throughout the cytoplasm, consistent with the time-projected images (supplemental figure 1-bottom). For cells treated with Blebbistatin, the tracking algorithm finds few EB1 trajectories beyond the cytoplasmic boundary unlike the fixed images. The lack of dynamic EB1 comets in these Blebbistatin-induced extensions suggested that the microtubules extending the cytoplasmic edge under these conditions (figure 2) were more stable.

In Latrunculin-treated cells, the microtubule longevity seemed homogenous near the nucleus, and long microtubules extended beyond the cytoplasmic edge, both in fixed cells (figure 2) and in live cell imaging (figure 3(E)), indicating that localized tubulin containing protrusions in this case did not contain significantly more stable microtubules than other regions. Similar to Blebbistatin treated cells, Y-27632 treated cells had short-lived tubulin growth near the edge of the cell body boundary (figure 3(G)). Beyond the cell body boundary, Y-27632 treated cells had less pronounced α -tubulin in fixed imaging than after Latrunculin-A treatment (figure 2), but clearly α -tubulin followed EB1 trajectories to extend

the cytoplasm in these cells (figure 3(G)). This suggests that in Blebbistatin treated cells, stable microtubules may have been preferred beyond the boundary, while Y-27632 and Latrunculin-A allowed continued, active microtubule growth beyond the cortical boundary.

Analysis of microtubule end boundary distance

To objectively define the outer boundary of the cytoplasm, we cotransfected MCF-7 cells with a non-targeted mCherry fluorescent protein. Figure 4(A) shows a representative cell, outlined by a tracing algorithm modified from methods developed previously [52]. The outline was measured based on the mean image from a 50 frame stack of mCherry stained cells; this approach allowed an objective measure of the cytoplasmic edge and defined the boundary for which cellular extensions (microtentacle, filopodia, etc) could be considered 'outside' the cytoplasmic cell boundary. Tips found outside the cytoplasmic cell boundary were assigned a negative value for distance. Analysis of 950 image frames from 19 independent cells (50 frames each) and 48 497 independent tip distances treated with drug vehicle (0.1% DMSO) showed EB1-GFP had an average distance of $1.70 \pm 0.16 \mu\text{m}$ (figure 4(B)).

Similar analysis of Latrunculin-A treated cells (18 cells, 900 frames, 45 148 tips) showed that the average distance of microtubule tips from the cell body boundary decreased significantly to only $0.16 \pm 0.23 \mu\text{m}$ compared to control (anova $p < 0.0001$ and $ks\text{-test } p = 1.0178 \times 10^{-6}$). Blebbistatin treated cells (16 cells, 800 frames, 41 161 tips) had microtubule tip locations at an average distance comparable to controls at $1.52 \pm 0.24 \mu\text{m}$ with no significant difference (anova $p = 0.9397$ and $ks\text{-test } p = 0.0528$). Cells treated with Y-27632 (19 cells, 950 frames, 44 821 tips) also exhibited a significantly smaller microtubule tip distance from the boundary at $0.83 \pm 0.26 \mu\text{m}$ $0.8 \pm 0.3 \mu\text{m}$ compared to control (anova $p = 0.0263$ and $ks\text{-test } p = 0.0181$). Additionally, microtubule tip distance from the boundary for Latrunculin-A treated cells was significantly less than Blebbistatin treated cells (anova $p = 0.0004$ and $ks\text{-test } p = 6.4616 \times 10^{-6}$).

Subcellular distribution of EB1 tips

To better understand the location EB1 tips in relationship to the cytoplasmic boundary, the percentages of EB1 tips in three key subcellular localizations were measured (figure 4(C)): (1) Bulk—positive distance from the boundary comprised 90% of the cell's cytoplasmic area, but not touching the boundary (2) Boundary—the 10% of the cell's cytoplasmic area closest to the boundary and (3) Outside—a negative value of pixels from the cytoplasmic boundary.

For control treated cells the percentages of tips in the regions outside the cell body boundary, near the cell body boundary, and in the cytoplasmic bulk were $3 \pm 2\%$, $19 \pm 2\%$, and $78 \pm 2\%$ where percentages for all 3 regions were significantly different (anova $p < 0.0001$). For Latrunculin-A treated cells the percentages of tips in the regions outside the cell body boundary, near the cell body boundary, and in the cytoplasmic bulk were $28 \pm 2\%$, $20 \pm 2\%$, and $52 \pm 2\%$. For cells treated with Latrunculin-A outside the cell body boundary, the distribution had a skew of 12, indicating a non-normal distribution; thus, an additional $ks\text{-test}$ was conducted on all comparisons including the population of Latrunculin-A treated

EB1 tips outside the cell body boundary to check for significance. For Latrunculin-A treated cells outside the cell body boundary, there was no significant difference when compared to EB1 tips near the cell body boundary (anova $p = 0.2663$ and ks -test $p = 0.4255$). For Latrunculin-A treated cells, there were significantly fewer EB1 tips outside the cell body boundary and near the boundary compared to inside the cell body bulk with anova computed p -values less than 0.0001 for both (ks -test $p = 2.9003 \times 10^{-7}$ and $p = 3.8098 \times 10^{-8}$ respectively). For Blebbistatin treated cells the percentages of tips in the regions outside the cell body boundary, near the cell body boundary, and in the cytoplasmic bulk were $4 \pm 2\%$, $23 \pm 2\%$, and $73 \pm 2\%$ where percentages for all 3 regions were significantly different (anova $p < 0.0001$). For Y-27632 treated cells the percentages of tips in the regions outside the cell body boundary, near the cell body boundary, and in the cytoplasmic bulk were $12 \pm 2\%$, $33 \pm 2\%$, and $55 \pm 2\%$ where percentages for all 3 regions were significantly different (anova $p < 0.0001$). For the region outside the cell body boundary, Latrunculin-A had a significantly higher percentage of EB1 tips compared to all other treatments, Blebbistatin, and Y-27632 (anova $p < 0.0001$ for all 3 and ks -test $p = 2.6693 \times 10^{-9}$, 1.3309×10^{-8} , and 1.6487×10^{-4} respectively). Additionally, while Y-27632 treated cells had significantly less EB1 tips outside the cell body boundary compared to Latrunculin-A, there were significantly more EB1 tips outside the cell body boundary compared to control treated cells (anova $p = 0.0334$ ks -test $p = 4.6625 \times 10^{-4}$). In the region closest to the boundary, only Y27632 had significantly more EB1 tip localization compared to control, Latrunculin-A, and Blebbistatin (anova $p = 0.0011$, 0.0031 , 0.0391 respectively). Both the control and Blebbistatin had over 70% of the EB1 tips in the cytoplasmic bulk and significantly more EB1 tips in the bulk region compared to Latrunculin-A (anova $p < 0.0001$ for both) and Y27632 (anova $p < 0.0001$ and 0.0008 respectively).

Measuring microtubule tip speed

In addition to quantifying EB1 tip localization, speeds of EB1 tips were measured to see whether actomyosin cortex integrity changed the speed of the EB1 tips for different drug treatments. For speed and dynamics experiments, videos without mcherry staining were used. Analysis for control was comprised of 24 independent cells and a total of 4979 individual EB1 tip trajectories measured over time. The average speed of microtubule tips in untreated cells was $0.075 \pm 0.002 \mu\text{m s}^{-1}$ (figure 5(A)). When broken down by regions outside, boundary, and bulk, the average speeds were $0.057 \pm 0.003 \mu\text{m s}^{-1}$, $0.065 \pm 0.003 \mu\text{m s}^{-1}$, and $0.084 \pm 0.003 \mu\text{m s}^{-1}$ respectively. For cells treated with $5 \mu\text{M}$ Latrunculin-A there was a total of 22 cells and 3703 trajectories where the average speed was $0.065 \pm 0.004 \mu\text{m s}^{-1}$; when broken down by regions outside, boundary and bulk: $0.048 \pm 0.003 \mu\text{m s}^{-1}$, $0.058 \pm 0.004 \mu\text{m s}^{-1}$, and $0.071 \pm 0.005 \mu\text{m s}^{-1}$ respectively. For cells treated with $25 \mu\text{M}$ Blebbistatin there was a total of 18 cells, and 4240 trajectories moving an average speed of $0.053 \pm 0.002 \mu\text{m s}^{-1}$; when the trajectories were broken down into their regions of outside, boundary and bulk, the speeds were $0.037 \pm 0.003 \mu\text{m s}^{-1}$, $0.046 \pm 0.002 \mu\text{m s}^{-1}$, and $0.056 \pm 0.003 \mu\text{m s}^{-1}$ respectively. For cells treated with $10 \mu\text{M}$ Y-27632, there was a total of 25 cells, and 5117 trajectories moving an average speed of $0.077 \pm 0.003 \mu\text{m s}^{-1}$; when the trajectories were broken down into their regions of outside, boundary and bulk, the speeds were $0.059 \pm 0.004 \mu\text{m s}^{-1}$, $0.062 \pm 0.002 \mu\text{m s}^{-1}$, and $0.088 \pm 0.003 \mu\text{m s}^{-1}$ respectively. For cells treated with $25 \mu\text{M}$ Blebbistatin, the average speed of EB1 tips moved significantly

slower than Control, 5 μ M Latrunculin-A, and 10 μ M Y-27632 ($p < 0.0001$, $p = 0.0228$, and $p < 0.0001$ respectively). Additionally, cells treated with Latrunculin-A had an average EB1 tip speed moving significantly slower than trajectories in cells treated with Y-27632 ($p = 0.0130$).

Within each cell treatment, the speed of trajectories was compared between all 3 regions: outside the cell body boundary, near the cell body boundary, and inside the cytoplasmic bulk. For the control and all drug treatments: Latrunculin, Blebbistatin, and Y-27632 EB1 trajectories outside the cell body boundary moved significantly slower than trajectories inside the cytoplasmic bulk ($p < 0.0001$, $p = 0.0002$, $p = 0.0001$, and $p < 0.0001$ respectively). It is evident from this change in speed that the membrane can exert a force that counteracts microtubule growth, even without intact actin or actomyosin contraction. For the control and all drug treatments: Latrunculin, Blebbistatin, and Y-27632 boundary region trajectories moved significantly slower than bulk region trajectories ($p < 0.0001$, $p = 0.0475$, $p = 0.0394$, and $p < 0.0001$ respectively), but no significant difference in speed between trajectories near the boundary and those outside the cell body boundary. However the differences in speed between the EB1 trajectories in the bulk region of the cell versus boundary region of the cell were more pronounced in control conditions and Y-27632 compared to Latrunculin-A treated cells and Blebbistatin treated cells suggesting that when contractility of the actomyosin cortex is compromised, there is less barrier to slow down the speed of microtubule growth.

Autocorrelation of EB1 particle trajectories

To determine the straightness of trajectories of growing microtubules, the orientation autocorrelation function was measured separately for each microtubule tip trajectory. We measured both the change in direction of motion over time, which yielded a characteristic turning time for growing tips, and the distribution of the temporal orientation autocorrelation as a function of position, which yielded a characteristic length-scale over which trajectories bent. We analyzed all trajectories in 24 control cells, 22 cells treated with Latrunculin-A, 18 cells treated with Blebbistatin, and 25 cells treated with Y-27632. The orientation correlations, averaged over all trajectories for each condition are shown as a function of time delay (figure 6) and as a function of cumulative distance (figure 7). We found that microtubules in cells treated with Y-27632 maintained their direction similar to control cells or no significant difference ($p = 0.9619$). In contrast, microtubules in Latrunculin-A and Blebbistatin treated cells changed direction more quickly, and over shorter distances than control cells (anova $p = 0.0383$ and $p < 0.0001$ respectively). Note that Blebbistatin and Latrunculin-A treated cells also had microtubule tips that move notably slower (figure 5). An exponential fit of the time delay autocorrelation function yielded a characteristic time and distance over which microtubule tips change direction, as shown in table 1. The higher τ and lower $1/\kappa$ decay constants in Control and Y-27632 indicate less change in direction and curving over time and space. Orientation autocorrelations by time and distance parameters are shown in table 1.

Discussion

Previous research suggests that microtentacles form in suspended cells when the actomyosin cortex is compromised supporting a model where microtentacle formation is the result of counteracting forces particularly between the actomyosin cortex and microtubules [1, 56–58]. Here we present results that show, changes in microtubule growth for attached cells as the result of changes in the actomyosin cortex. We focused on the drugs listed above that target the actin cytoskeleton yet induce changes in cell behavior that are strongly microtubule dependent (e.g. proliferation). The drugs are small molecule inhibitors of the actin cytoskeleton, both perturbing its integrity (Latrunculin-A) and contractility (Blebbistatin, Y-27632) [24]. Latrunculin-A binds directly to β -actin monomers, sequestering them and preventing their assembly into F-actin filaments [34, 35]. Blebbistatin and Y-27632 do not alter actin polymerization, but reduce actomyosin contractility through independent mechanisms. Blebbistatin binds to non-muscle myosin-II (NMII) and slows down the release of phosphate that primes myosin-II for reattachment to actin filaments [24]. As a result, NMII is blocked in an intermediate state with low actin affinity, effectively releasing the actin-myosin cross-bridges that promote contractility [24]. The small molecule drug Y-27632 suppresses Rho-associated kinase (ROCK) which in turn stimulates actomyosin contractility. Y-27632 competes with ATP in the ROCK active site, reducing the kinase activity of ROCK and therefore reducing NMII-induced actomyosin contractility [4]. Additionally, Y-27632's inhibition of ROCK results in the activation of cofilin an actin severing protein [16]. Therefore, while each of the three compounds target actin function, Latrunculin-A broadly disrupts actin polymerization, Blebbistatin targets actomyosin contractility, and Y-27632 targets both the actin filaments and actomyosin contractility (figure 1).

To observe the differences in microtubule dynamics that were induced by these actin-targeting compounds, we used live-cell confocal microscopy of MCF-7 breast tumor cells expressing the microtubule end-binding-protein-1 (EB1) fused to green fluorescent protein [46, 47]. EB1 binds preferentially to the GTP-tubulin cap found at microtubule plus-ends, forming comets within cells that track growing microtubule ends. Additionally, EB1 binding to the APC protein found within the actin cortex has been shown to play a role in microtubule capture at the cortex [5, 7, 14]. Differences between results observed via immunofluorescence in fixed cells (figure 2) and the dynamic measurements with EB1 provide an indication that microtubule stability can vary based on location within the cell. While there was a high concentration of microtubules surrounding the nucleus observed with fixed cell immunofluorescence, comparatively little dynamic EB1 was found in this area of the cell. Time-lapse confocal microscopy (supplemental figure 1), coupled with particle detection and tracking (figure 3) showed that control cells had the most EB1 tips only a few microns from the cell body boundary (figure 4(B)) and that the average distance of EB1 from the boundary was approximately 2 microns (figure 4(B)). Blebbistatin treatment did not significantly alter the average distance of EB1 from the cell boundary, but Y-27632 did decrease the average distance to the boundary slightly, but measurably. Dismantling the actin cortex through depolymerization of actin filaments with Latrunculin-A dramatically decreased the average distance to the boundary (figure 4(B)), but analysis of EB1 tips in

different regions (figure 4(C)) clarified that LA-treated cells did not have more tips closer to the boundary, but rather a significant increase in microtubule extension beyond the cell boundary and a large fraction of EB1 tips outside the boundary ($28 \pm 2\%$). In comparison, control cells showed less than $3 \pm 2\%$ of EB1 tips beyond the cell boundary (figure 4(C)), confirming the role of the cortex as a barrier to microtubule extension, either as a direct mechanical barrier or due to its effect on cell membrane tension. Reducing actomyosin contractility with Blebbistatin did not yield significant microtubule extension beyond the boundary. These compounds would leave actin filaments intact, but the reduction in actomyosin contractility could weaken the barrier provided by the actin cortex. Since microtubules will buckle and break when encountering a barrier [59], the relative softening of the actin cortex by Blebbistatin and Y-27632 could produce a more flexible actin cortex that improves cortical capture of growing microtubules, rather than breakage. For Y-27632 which severs the actin filaments in addition to softening it, there is a pronounced increase in the percentage of EB1 tips near the cell body boundary supporting the possibility of cortical capture. As would be expected, both Y-27632 and Latrunculin-A increased microtubule extension beyond the cell body boundary, and removing actin filaments completely via Latrunculin-A increased microtubule extension far more than Y-27632 (supplemental figure 1).

EB1 trajectories in cells treated with Latrunculin-A move significantly slower than control cells or cells treated with Y-27632. This suggests that the membrane tension beyond the cell body boundary is the largest inhibitor of speed and is further supported by the fact that EB1 trajectory speeds are slower outside the cell body boundary across all conditions where Latrunculin-A has significantly more EB1 tips beyond the cell body boundary than all other conditions. Interestingly, Blebbistatin moves significantly more slowly than any other conditions despite not having more EB1 tips beyond the cell body boundary. It is worth considering that while other results provide evidence supporting the notion of growing microtubules contained by a contractile actomyosin cortex, all the above experiments were conducted on attached cells. One of the key, widely accepted models for attached cells posits that actin based protrusions form via actin polymerization pushing the plasma membrane outward [60, 61]. Also, studies show that Blebbistatin weakens the protrusive force of lamellipodia [62]. Furthermore, the speed of actin in the lamellipodia is around speed 25 nm s^{-1} and 2 nm s^{-1} for retrograde flow [63]. The difference in the speed for EB1 tips between control ($75 \pm 2 \text{ nm s}^{-1}$) conditions and blebbistatin ($53 \pm 2 \text{ nm s}^{-1}$) is about 22 nm s^{-1} and comparable to the speed of F-actin on the cell edge.

In terms of the directionality changes in microtubule growth, the EB1 autocorrelation analysis (figures 6 and 7) demonstrated that actin depolymerization and inhibition of actomyosin contractility can influence directionality of microtubule growth. Broad actin depolymerization (Latrunculin-A) or the inhibition of actomyosin contractility with Blebbistatin caused trajectories that were less straight (figures 6 and 7). The less straight trajectories for blebbistatin treated cells suggested that the cortical barrier, though loosened, remained effective at changing microtubule direction. Since actin filaments would remain intact but with reduced contractile force, it is possible that this yields a flexible barrier within the cytoplasm that provides less guidance to growing microtubules. In support of this model, previous studies have shown that the cytoplasm provides a barrier to microtubule

persistence length, compared to *in vitro* microtubule growth [64]. Since microtubules follow the path of least resistance [54], the reduction in cytoplasmic actin filament resistance could then yield less differential between barrier and open space that would serve to confuse the choice of the less resistant path. Microtubule motor proteins (dynein, kinesin) can also cause deviation of microtubule trajectories, indicating coupling between microtubules and other components of the cytoplasmic cytoskeleton [65]. Interestingly, depolymerization of actin with Latrunculin-A also decreased the straightness of the EB1 trajectories overall, but trajectories were still deflected outside the boundary. This supports a model where even for cells with a largely depolymerized cortex, the plasma membrane may still be able to cause deflection of microtubule trajectories, possibly through residual forces of membrane surface tension.

Cells sense their mechanical microenvironment and the presence of wounds through both actin contraction and microtubule extension [1, 66, 67]. Persistent pulling of actomyosin contraction on both cell–matrix and cell–cell adhesion points serve as signals for appropriate cell attachment [68]. Imbalances between actin cortical contraction and microtubule extension from the cell center can also influence metastatic potential through the formation of microtentacles on the surface of free-floating tumor cells [1]. Reducing actin contractility with Blebbistatin enhances microtentacles that promote tumor cell reattachment and aggregation [53]. Further supporting a role for opposing cytoskeletal interactions, microtentacles are induced by either inhibition of actin polymerization with Latrunculin-A or hyperstabilization of microtubules with Paclitaxel [38, 40]. The importance of these opposing microtubule-actin forces is also demonstrated by several studies that have shown that compounds which reduce actomyosin contractility, including Blebbistatin and Y-27632, are capable of allowing long-term *in vitro* culture growth of patient-derived tumor cells from many cancers [43, 44]. Historically, very few patient-derived cells have been capable of growing in culture. The new method is termed ‘conditional reprogramming’, because cells treated with cytoskeletal inhibitors divert from their differentiation program and become capable of growing efficiently and for many generations [43, 44]. These results suggest that some stem cell characteristics may be regulated by mechanical changes in the cytoskeleton. In support of this model, both Blebbistatin and Y-27632 induce stem cell characteristics in primary colon cancer cells, including spheroid formation, CD44v expression and increased tumorigenicity in mice [42]. If the opposing cytoskeletal interactions of microtubule expansion against actin contraction is partly responsible for these effects, it would be predicted that microtubule stabilization would also be influenced by cell detachment and produce increased stem cell characteristics. In fact, both increased microtubule stabilization upon cell detachment [69] and the induction of stem cell characteristics by the common microtubule stabilizing compound, Paclitaxel, have already been observed [70]. It will be important to more clearly understand how proposed cancer drugs alter the opposing cytoskeletal interactions between microtubule extension and actin cortical contraction, since cytoskeletal compounds which promote actin disruption or microtubule stabilization could inadvertently induce surviving cancer cells to elevate microtentacles and increase stem cell characteristics.

Currently, many clinical chemotherapeutic drugs target the cytoskeleton such as Vinblastine, Paclitaxel, and Demecolcine [58, 70]. Cytoskeletal compounds which promote actin

disruption or microtubule stabilization could inadvertently induce surviving cancer cells to elevate microtentacles in circulating tumor cells, or localized protrusions in attached cells, and increase stem cell characteristics. Overall, our results show that it will be important to more clearly understand how proposed cancer drugs alter the opposing cytoskeletal forces between microtubule extension and actin cortical contraction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research supported by the Kahlert Foundation, the National Cancer Institute (R01-CA154624, R01-CA124704) and an Era of Hope Scholar award from the Department of Defense (BC100675). KRC is supported by F30-CA196075 from NCI. WL was supported by NIH grant R01GM085574. We thank Lynne Cassimeris and Michelle Piehl for generously providing the EB1-GFP expression vector.

Abbreviations

EB1	End-binding-protein-1
GFP	Green fluorescent protein
MTOC	Microtubule-organizing center
APC	Adenomatous polyposis protein
NMII	Non-muscle myosin-II
ROCK	Rho-kinase
DMEM	Dulbecco's modified eagle medium
EMT	Epithelial-to-mesenchymal transition
McTN	Microtentacle

References

1. Matrone MA, et al. Microtentacles tip the balance of cytoskeletal forces in circulating tumor cells. *Cancer Res.* 2010; 70:7737–41. [PubMed: 20924109]
2. Marshall TW, et al. The tumor suppressor adenomatous polyposis coli controls the direction in which a cell extrudes from an epithelium. *Mol Biol Cell.* 2011; 22:3962–70. [PubMed: 21900494]
3. Birchmeier C, Birchmeier W, Brand-Saberi B. Epithelial-mesenchymal transitions in cancer progression. *Acta Anat.* 1996; 156:217–26. [PubMed: 9124038]
4. Even-Ram S, et al. Myosin IIA regulates cell motility and actomyosin-microtubule crosstalk. *Nat Cell Biol.* 2007; 9:299–309. [PubMed: 17310241]
5. Wen Y, et al. EB1 and APC bind to mDia to stabilize microtubules downstream of Rho and promote cell migration. *Nat Cell Biol.* 2004; 6:820–30. [PubMed: 15311282]
6. Salbreux G, Charras G, Paluch E. Actin cortex mechanics and cellular morphogenesis. *Trends Cell Biol.* 2012; 22:536–45. [PubMed: 22871642]
7. Moseley JB, et al. Regulated binding of adenomatous polyposis coli protein to actin. *J Biol Chem.* 2007; 282:12661–8. [PubMed: 17293347]

8. Brangwynne CP, et al. Microtubules can bear enhanced compressive loads in living cells because of lateral reinforcement. *J Cell Biol.* 2006; 173:733–41. [PubMed: 16754957]
9. Alenghat FJ, et al. Global cytoskeletal control of mechanotransduction in kidney epithelial cells. *Exp Cell Res.* 2004; 301:23–30. [PubMed: 15501441]
10. Ingber DE. Cancer as a disease of epithelial-mesenchymal interactions and extracellular matrix regulation. *Differentiation.* 2002; 70:547–60. [PubMed: 12492496]
11. Yamaguchi H, Condeelis J. Regulation of the actin cytoskeleton in cancer cell migration and invasion. *Biochim Biophys Acta.* 2007; 1773:642–52. [PubMed: 16926057]
12. Henty-Ridilla JL, et al. Accelerated actin filament polymerization from microtubule plus ends. *Science.* 2016; 352:1004–9. [PubMed: 27199431]
13. Alberico EO, et al. Interactions between the microtubule binding protein EB1 and F-Actin. *J Mol Biol.* 2016; 428:1304–14. [PubMed: 26854759]
14. Bartolini F, et al. The formin mDia2 stabilizes microtubules independently of its actin nucleation activity. *J Cell Biol.* 2008; 181:523–36. [PubMed: 18458159]
15. Bartolini F, Ramalingam N, Gundersen GG. Actin-capping protein promotes microtubule stability by antagonizing the actin activity of mDia1. *Mol Biol Cell.* 2012; 23:4032–40. [PubMed: 22918941]
16. Robert A, et al. Microtubule-dependent transport of vimentin filament precursors is regulated by actin and by the concerted action of Rho- and p21-activated kinases. *FASEB J.* 2014; 28:2879–90. [PubMed: 24652946]
17. Hutchins BI, Klenke U, Wray S. Calcium release-dependent actin flow in the leading process mediates axophilic migration. *J Neurosci.* 2013; 33:11361–71. [PubMed: 23843509]
18. Tang EI, et al. EB1 regulates tubulin and actin cytoskeletal networks at the sertoli cell blood-testis barrier in male rats: an *in vitro* study. *Endocrinology.* 2015; 156:680–93. [PubMed: 25456071]
19. Robison P, et al. Detyrosinated microtubules buckle and bear load in contracting cardiomyocytes. *Science.* 2016; 352:aaf0659. [PubMed: 27102488]
20. Ingber DE, Wang N, Stamenovic D. Tensegrity, cellular biophysics, and the mechanics of living systems. *Rep Prog Phys.* 2014; 77:046603. [PubMed: 24695087]
21. Soheilypour M, et al. Buckling behavior of individual and bundled microtubules. *Biophys J.* 2015; 108:1718–26. [PubMed: 25863063]
22. Anava S, et al. The regulative role of neurite mechanical tension in network development. *Biophys J.* 2009; 96:1661–70. [PubMed: 19217881]
23. Heidemann SR, Lamoureux P, Buxbaum RE. Cytomechanics of axonal development. *Cell Biochem Biophys.* 1995; 27:135–55. [PubMed: 9279454]
24. Pinsky BA, Biggins S. The spindle checkpoint: tension versus attachment. *Trends Cell Biol.* 2005; 15:486–93. [PubMed: 16084093]
25. Hawkins T, et al. Mechanics of microtubules. *J Biomech.* 2010; 43:23–30. [PubMed: 19815217]
26. Bicek AD, et al. Anterograde microtubule transport drives microtubule bending in LLC-PK1 epithelial cells. *Mol Biol Cell.* 2009; 20:2943–53. [PubMed: 19403700]
27. Kent, IA., Lele, TP. *Microtubule-Based Force Generation.* New York: Wiley; 2016.
28. Kent IA, et al. Transient pinning and pulling: a mechanism for bending microtubules. *PLoS One.* 2016; 11:e0151322. [PubMed: 26974838]
29. Shekhar N, et al. Cytoplasmic dynein: tension generation on microtubules and the nucleus. *Cell Mol Bioeng.* 2013; 6:74–81. [PubMed: 23646068]
30. Wu J, Dickinson RB, Lele TP. Investigation of *in vivo* microtubule and stress fiber mechanics with laser ablation. *Integr Biol.* 2012; 4:471–9.
31. Wu J, et al. How dynein and microtubules rotate the nucleus. *J Cell Physiol.* 2011; 226:2666–74. [PubMed: 21792925]
32. Wu J, et al. Effects of dynein on microtubule mechanics and centrosome positioning. *Mol Biol Cell.* 2011; 22:4834–41. [PubMed: 22013075]
33. Rao J, Li N. Microfilament actin remodeling as a potential target for cancer drug development. *Curr Cancer Drug Targets.* 2004; 4:345–54. [PubMed: 15180500]

34. Fritz G, Kaina B. Rho GTPases: promising cellular targets for novel anticancer drugs. *Curr Cancer Drug Targets*. 2006; 6:1–14. [PubMed: 16475973]
35. Itoh K, et al. An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. *Nat Med*. 1999; 5:221–5. [PubMed: 9930872]
36. Vigil D, et al. ROCK1 and ROCK2 are required for non-small cell lung cancer anchorage-independent growth and invasion. *Cancer Res*. 2012; 72:5338–47. [PubMed: 22942252]
37. Wang L, et al. Effects of ROCK inhibitor, Y-27632, on adhesion and mobility in esophageal squamous cell cancer cells. *Mol Biol Rep*. 2010; 37:1971–7. [PubMed: 19649725]
38. Balzer EM, et al. Antimitotic chemotherapeutics promote adhesive responses in detached and circulating tumor cells. *Br Cancer Res Treat*. 2010; 121:65–78.
39. Whipple RA, et al. Vimentin filaments support extension of tubulin-based microtentacles in detached breast tumor cells. *Cancer Res*. 2008; 68:5678–88. [PubMed: 18632620]
40. Matrone MA, et al. Metastatic breast tumors express increased tau, which promotes microtentacle formation and the reattachment of detached breast tumor cells. *Oncogene*. 2010; 29:3217–27. [PubMed: 20228842]
41. Balzer EM, et al. c-Src differentially regulates the functions of microtentacles and invadopodia. *Oncogene*. 2010; 29:6402–8. [PubMed: 20956943]
42. Ohata H, et al. Induction of the stem-like cell regulator CD44 by Rho kinase inhibition contributes to the maintenance of colon cancer-initiating cells. *Cancer Res*. 2012; 72:5101–10. [PubMed: 23002207]
43. Liu X, et al. ROCK inhibitor and feeder cells induce the conditional reprogramming of epithelial cells. *Am J Pathol*. 2012; 180:599–607. [PubMed: 22189618]
44. Supryniewicz FA, et al. Conditionally reprogrammed cells represent a stem-like state of adult epithelial cells. *Proc Natl Acad Sci USA*. 2012; 109:20035–40. [PubMed: 23169653]
45. Kovacs M, et al. Mechanism of blebbistatin inhibition of myosin II. *J Biol Chem*. 2004; 279:35557–63. [PubMed: 15205456]
46. Piehl M, Cassimeris L. Organization and dynamics of growing microtubule plus ends during early mitosis. *Mol Biol Cell*. 2003; 14:916–25. [PubMed: 12631713]
47. Piehl M, et al. Centrosome maturation: measurement of microtubule nucleation throughout the cell cycle by using GFP-tagged EB1. *Proc Natl Acad Sci USA*. 2004; 101:1584–8. [PubMed: 14747658]
48. Velasco G, et al. Phosphorylation of the regulatory subunit of smooth muscle protein phosphatase 1M at Thr850 induces its dissociation from myosin. *FEBS Lett*. 2002; 527:101–4. [PubMed: 12220642]
49. Willipinski-Stapelfeldt B, et al. Changes in cytoskeletal protein composition indicative of an epithelial-mesenchymal transition in human micrometastatic and primary breast carcinoma cells. *Clin Cancer Res*. 2005; 11:8006–14. [PubMed: 16299229]
50. Gordon LA, et al. Breast cell invasive potential relates to the myoepithelial phenotype. *Int J Cancer*. 2003; 106:8–16. [PubMed: 12794751]
51. Crocker JC, Grier DG. Methods of digital video microscopy for colloidal studies. *J Colloid Interface Sci*. 1996; 179:298–310.
52. Driscoll MK, et al. Cellular contact guidance through dynamic sensing of nanotopography. *ACS Nano*. 2014; 8:3546–55. [PubMed: 24649900]
53. Vitolo MI, et al. Loss of PTEN induces microtentacles through PI3K-independent activation of cofilin. *Oncogene*. 2012; 32:2200–10. [PubMed: 22689060]
54. Balzer EM, et al. Physical confinement alters tumor cell adhesion and migration phenotypes. *FASEB J*. 2012; 26:4045–56. [PubMed: 22707566]
55. Matov A, et al. Analysis of microtubule dynamic instability using a plus-end growth marker. *Nat Methods*. 2010; 7:761–8. [PubMed: 20729842]
56. Bhandary L, et al. ROCK inhibition promotes microtentacles that enhance reattachment of breast cancer cells. *Oncotarget*. 2015; 6:6251–66. [PubMed: 25749040]

57. Chakrabarti KR, et al. Pharmacologic regulation of AMPK in breast cancer affects cytoskeletal properties involved with microtentacle formation and re-attachment. *Oncotarget*. 2015; 6:36292–307. [PubMed: 26431377]
58. Chakrabarti KR, et al. Molecular pathways: new signaling considerations when targeting cytoskeletal balance to reduce tumor growth. *Clin Cancer Res*. 2015; 21:5209–14. [PubMed: 26463706]
59. Hendricks AG, et al. Dynein tethers and stabilizes dynamic microtubule plus ends. *Curr Biol*. 2012; 22:632–7. [PubMed: 22445300]
60. Ponti A, et al. Two distinct actin networks drive the protrusion of migrating cells. *Science*. 2004; 305:1782–6. [PubMed: 15375270]
61. Mattila PK, Lappalainen P. Filopodia: molecular architecture and cellular functions. *Nat Rev Mol Cell Biol*. 2008; 9:446–54. [PubMed: 18464790]
62. Sayyad WA, et al. The role of myosin-II in force generation of DRG filopodia and lamellipodia. *Sci Rep*. 2015; 5:7842. [PubMed: 25598228]
63. Gardel ML, et al. Traction stress in focal adhesions correlates biphasically with actin retrograde flow speed. *J Cell Biol*. 2008; 183:999–1005. [PubMed: 19075110]
64. Brangwynne CP, MacKintosh FC, Weitz DA. Force fluctuations and polymerization dynamics of intracellular microtubules. *Proc Natl Acad Sci USA*. 2007; 104:16128–33. [PubMed: 17911265]
65. Shekhar N, et al. Fluctuating motor forces bend growing microtubules. *Cell Mol Bioeng*. 2013; 6:120–129. [PubMed: 24039637]
66. Kumar S, Weaver VM. Mechanics, malignancy, and metastasis: the force journey of a tumor cell. *Cancer Metastasis Rev*. 2009; 28:113–27. [PubMed: 19153673]
67. Seetapun D, Odde DJ. Cell-length-dependent microtubule accumulation during polarization. *Curr Biol*. 2010; 20:979–88. [PubMed: 20493705]
68. De Santis G, et al. How can cells sense the elasticity of a substrate? An analysis using a cell tensegrity model. *Eur Cells Mater*. 2011; 22:202–13.
69. Whipple RA, Cheung AM, Martin SS. Detyrosinated microtubule protrusions in suspended mammary epithelial cells promote reattachment. *Exp Cell Res*. 2007; 313:1326–36. [PubMed: 17359970]
70. Gupta PB, et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell*. 2009; 138:645–59. [PubMed: 19682730]

Figure 1.

Opposing forces of microtubule expansion and actin cortex contraction. In a normal, adherent epithelial cell, there is a cortex of cross-linked actin filaments beneath the plasma membrane that is under contraction mediated by the non-muscle myosin-II motor protein (red arrows). Microtubules are nucleated at the microtubule-organizing center (MTOC) and grow outward from the cell center toward the plasma membrane (green arrows). Microtubule end-binding protein-1 (EB1) binds preferentially to the growing GTP-capped microtubule plus end and forms comets projecting from the cell center. Microtubules grow outward to reach the network of cortical actin filaments, which have an estimated mesh size of 100 nm. Interaction of EB1 with cortical proteins, such as APC, also mediate capture of microtubules. Latrunculin-A depolymerizes actin, reducing the barrier to microtubule expansion. Phosphorylation of myosin-II by the Rho-kinase (ROCK) increases contractility, but can be inhibited by the small molecule ROCK inhibitor, Y-27632. Similarly, Blebbistatin binds directly to myosin-II and reduces contractility.

Figure 2.

Actin disruption promotes microtubule extension beyond the cytoplasmic boundary. Human MCF-7 breast cancer cells were treated with vehicle control (0.5% DMSO), 5 μ M Latrunculin-A, 25 μ M Blebbistatin or 10 μ M of Y-27632 for 30 min, fixed with formaldehyde and fluorescently stained for actin localization (red), microtubules (green) or DNA (blue). Confocal microscopy images are shown for each channel and condition, along with an overlay of all channels (right column; scalebar = 10 μ m).

Figure 3.

Tracking algorithm identifies dynamic EB1-GFP tips and trajectories. (A) Example of EB1 tips found. (B) Example of tips tracked. (C) Individual trajectories: (i) normal trajectory in cell bulk. (ii) Trajectory in cell bulk treated with 25 μM blebbistatin. (iii) Trajectory in cell bulk treated with 5 μM latrunculin. (iv) Trajectory in cell bulk treated with Y27632. (v) Normal trajectory near cell edge. (vi) Trajectory in near cell edge treated with 25 μM blebbistatin. (vii) Trajectory near cell edge treated with 5 μM latrunculin. (viii) Trajectory near cell edge treated with Y27632. (D) Trajectory overlays for normal cell where blue represents earlier time frames and red represents later time frames (scalebar = 10 μm). (E) Trajectory overlays for cell treated with 25 μM blebbistatin. (F) Trajectory overlays for cell treated with 5 μM Latrunculin-A. (G) Trajectory overlays for cell treated with Y-27632 (10 μM).

Figure 4.

Analysis of localization and distribution of dynamic microtubule ends. (A) Cell body boundary from mean mCherry image taken across 50 frames (left). (B) Mean distance from the boundary for all 4 conditions (bottom left). Control ($n = 19$) treated cells (0.1% DMSO) show an average distance from cell body boundary of measured $1.7 \pm 0.2 \mu\text{m}$. Cells treated with $5 \mu\text{M}$ Latrunculin-A ($n = 18$) show an average distance of $0.16 \pm 0.2 \mu\text{m}$ which is a significant decrease when compared to control (anova $p < 0.0001$ and $ks\text{-test } p = 1.0178 \times 10^{-6}$). Cells treated with $25 \mu\text{M}$ Blebbistatin ($n = 16$) have an average distance of $1.5 \pm 0.2 \mu\text{m}$ comparable to controls with no significant difference (anova $p = 0.9397$ and $ks\text{-test } p = 0.0528$). Cells treated with $10 \mu\text{M}$ Y-27632 ($n = 19$) have an average tip distance of $0.8 \pm 0.3 \mu\text{m}$ which is significantly smaller than control (anova $p = 0.0263$ and $ks\text{-test } p = 0.0181$). Additionally, microtubule tip distance from the boundary for latrunculin treated cells was significantly less than blebbistatin treated cells (anova $p = 0.0004$ and $ks\text{-test } p = 6.4616 \times 10^{-6}$). (C) Percentage of particles outside the boundary (left), near the cell body boundary (within 10% of mCherry-defined cell body boundary) and in the cell bulk (right).

Figure 5.

Average speed ($\mu\text{m s}^{-1}$) for all 4 conditions. (A) Average speed across all regions: control (black, $n = 24$) at $0.075 \pm 0.002 \mu\text{m s}^{-1}$, $5 \mu\text{M}$ Latrunculin-A (purple, $n = 18$), at $0.065 \pm 0.004 \mu\text{m s}^{-1}$, $25 \mu\text{M}$ Blebbistatin (red, $n = 22$) at $0.053 \pm 0.002 \mu\text{m s}^{-1}$, and $10 \mu\text{M}$ Y-27632 (yellow, $n = 25$) at $0.077 \pm 0.003 \mu\text{m s}^{-1}$. (B) Break down of speed for all 4 conditions broken into 3 regions from left to right: outside the cell body boundary, near the cell body boundary (the 10% of points inside the cell body closest to the boundary) and within the cell bulk (remaining area inside the cell body): speeds for control (black) were $0.057 \pm 0.003 \mu\text{m s}^{-1}$, $0.065 \pm 0.003 \mu\text{m s}^{-1}$, and $0.084 \pm 0.003 \mu\text{m s}^{-1}$ respectively; speeds for $5 \mu\text{M}$ Latrunculin-A (purple), were $0.048 \pm 0.003 \mu\text{m s}^{-1}$, $0.058 \pm 0.004 \mu\text{m s}^{-1}$, and $0.071 \pm 0.005 \mu\text{m s}^{-1}$ respectively; speeds for $25 \mu\text{M}$ Blebbistatin (red) were $0.037 \pm 0.003 \mu\text{m s}^{-1}$, $0.046 \pm 0.002 \mu\text{m s}^{-1}$, and $0.056 \pm 0.003 \mu\text{m s}^{-1}$ respectively; and speeds for $10 \mu\text{M}$ Y-27632 (yellow) were $0.059 \pm 0.004 \mu\text{m s}^{-1}$, $0.062 \pm 0.002 \mu\text{m s}^{-1}$, and $0.088 \pm 0.003 \mu\text{m s}^{-1}$ respectively. Error bars represent SEM.

Figure 6. Orientation autocorrelation as a function of time for control (black squares), Blebbistatin (red squares), Latrunculin-A (purple squares), and Y-27632 (yellow squares).

Figure 7. Orientation autocorrelation as a function of space for control (black squares), Blebbistatin (red squares), Latrunculin-A (purple squares), and Y-27632 (yellow squares).

Table 1

Orientation autocorrelation coefficients as a function of time (top) and spatial distribution of temporal orientation autocorrelation (bottom) for control, Latrunculin-A, Blebbistatin, and Y-27632.

Orientation autocorrelation as a function of time			
$Ae^{-t/\tau} + B$			
	A	B	τ (s)
Vehicle	0.79	0.22	3.05
Latrunculin	0.77	0.24	2.14
Blebbistatin	0.89	0.11	1.71
Y-27632	0.74	0.28	2.92
Spatial distribution of temporal orientation autocorrelation			
$Ae^{-\kappa x} + B$			
	A	B	$1/\kappa$ (μm)
Vehicle	0.62	0.37	0.25
Latrunculin	0.67	0.31	0.32
Blebbistatin	0.87	0.12	0.34
Y-27632	0.55	0.44	0.29