Neutrophil microtubules suppress polarity and enhance directional migration

Jingsong Xu*, Fei Wang*[†], Alexandra Van Keymeulen*, Maike Rentel, and Henry R. Bourne*

Departments of Cellular and Molecular Pharmacology and Medicine and Cardiovascular Research Institute, University of California, San Francisco, CA 94143-0450

Contributed by Henry R. Bourne, March 18, 2005

How do microtubules, which maintain and direct polarity of many eukaryotic cells, regulate polarity of blood neutrophils? In sharp contrast to most cells, disrupting a neutrophil's microtubule network with nocodazole causes it to polarize and migrate [Niggli, V. (2003) *J. Cell Sci.* 116, 813–822]. Nocodazole induces the same responses in differentiated HL-60 cells, a model neutrophil cell line, and reduces their chemotactic prowess by causing them to pursue abnormally circuitous paths in migrating toward a stationary point source of an attractant, f-Met-Leu-Phe (fMLP). The chemotactic defect stems from dramatic nocodazole-induced imbalance between the divergent, opposed fMLP-induced “backness” and “frontness” signals responsible for neutrophil polarity. Nocodazole (*i*) stimulates backness by increasing Rho- and actomyosin-dependent contractility, as reported by Niggli, and also (*ii*) impairs fMLP-dependent frontness: pseudopods are flatter, contain less F-actin, and show decreased membrane translocation of PH-Akt-GFP, a fluorescent marker for 3'-phosphoinositide lipids. Inhibiting backness with a pharmacologic inhibitor of a Rho-dependent kinase substantially reverses nocodazole's effects on chemotaxis, straightness of migration paths, morphology, and PH-Akt-GFP translocation. Thus, microtubules normally balance backness vs. frontness signals, preventing backness from reducing the strength of pseudopods and from impairing directional migration.

chemotaxis | PIP3 | pseudopod | Rho GTPases

The critical roles of cell polarity and directional migration in host defense, wound healing, and development depend on complex interplay between the actin and microtubule cytoskeletons (1–7). Formation and breakdown of actin filaments create polarized asymmetry of the cell's front and back to generate forces required for movement and to regulate adhesion to extracellular matrix and other cells. Locations of the microtubule-organizing center (MTOC) and microtubules radiating from it are thought to determine or maintain orientation of cell polarity, in part by directing vesicular traffic and membrane localization of cytoskeletal regulators that control strength and spatial distribution of actin assemblies (4–6). In turn, actin-associated proteins at the cell cortex can stabilize or enhance depolymerization at the plus ends of microtubules, perhaps thereby designating destinations for delivery of regulatory molecules, such as guanine nucleotide exchange factors (GEFs) for Rho GTPases (1, 2).

Compared with most vertebrate cells, blood neutrophils polarize, move, and turn more rapidly over time scales of 30–90 sec rather than minutes or hours. As compared with the actin assemblies at the neutrophil's front and back that are critical for polarity, migration, and directionality (8–14), roles of the neutrophil's microtubule network in these processes are less well understood. Microtubules of a polarized neutrophil radiate toward its leading and trailing edges from an MTOC located just behind, just in front of, or between lobes of the nucleus (15–17). Microtubule-depolymerizing drugs like colchicine and nocodazole induce neutrophils to polarize and crawl in random directions, in the absence of chemoattractant (18, 19); in contrast, the drugs weaken or abolish polarity and migration induced by these

drugs in most vertebrate cells. The unusual response to microtubule disruption is accompanied by elevated activities of a small GTPase, Rho, and Rho-dependent phosphorylation of myosin light chains (18, 19), which probably account for the induction of polarity and migration, because both are substantially inhibited by exposure to Y-27632, a pharmacologic inhibitor of a Rho-dependent kinase, p160-ROCK (19). Finally, neutrophils whose microtubules were disrupted by colchicine oriented correctly in a shallow gradient of attractant (9) and reoriented correctly toward an attractant from a micropipette placed one cell length away from the cell (8); in a more stringent test of direction-finding ability, microtubule disruption caused neutrophils to migrate toward spores of *Candida albicans* in abnormally contorted migration paths with wide turns (20).

To explore the contributions of microtubules to orientation and directional migration, we have studied behavior of differentiated HL-60 (dHL-60) cells, a model neutrophil-like cell line, in gradients of f-Met-Leu-Phe (fMLP), a tripeptide attractant. We find that microtubules normally enhance accuracy and stability of orientation by balancing two spatially distinct and functionally opposed actin assemblies controlled by signaling pathways that diverge downstream of the fMLP receptor: “frontness,” or protrusive F-actin at the front of the cell, regulated by a trimeric G protein, Gi, the small GTPase Rac, and 3'-phosphoinositides such as PIP3 and protrusion of F-actin (11–13); “backness,” or contractile actin-myosin complexes at the back and sides, regulated by different trimeric G proteins, G12 and G13, Rho, p160-ROCK, phosphorylated myosin light chains, and myosin II (14). Effects of nocodazole on frontness and directionality are reversed by a p160-ROCK inhibitor, indicating that nocodazole-induced enhancement of backness impairs directionality by inhibiting frontness.

Materials and Methods

Most materials and methods used in this work have been described in detail in previous work from this laboratory (12, 14); these include culture and differentiation of HL-60 cells, expression of exogenous cDNAs by stable and transient transfection, micropipette assays, fixation and staining for actin, and the Rho-Rhotekin pull-down assay for Rho-GTP. Transwell chemotaxis was assessed as described in ref. 21. Cellular actin and particulate PH-Akt-GFP were measured as described by refs. 22 and 23, respectively. All methods are described in further detail in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

Freely available online through the PNAS open access option.

Abbreviations: dHL-60, differentiated HL-60; fMLP, f-Met-Leu-Phe; GEF, guanine nucleotide exchange factor; MTOC, microtubule-organizing center.

*J.X., F.W., and A.V.K. contributed equally to this work.

[†]Present address: Department of Stomatology, University of California, San Francisco, CA 94143-0512.

[‡]To whom correspondence should be addressed. E-mail: bourne@cmp.ucsf.edu.

© 2005 by The National Academy of Sciences of the USA

Fig. 2. Microtubules and the MTOC in migrating dHL60 cells. (A) Location and dynamic behavior of microtubules. *Left and Left Center* show a cell that was stimulated with a uniform concentration (100 nM) of fMLP for 3 min and then fixed and stained for tubulin (green) and F-actin (red) as described in *Supporting Materials and Methods*. Fluorescence and Nomarski images are shown. *Right Center and Right* show a cell that transiently expressed GFP-N-Clip170 2 min after challenge with a point source of fMLP (10 μ M). *, direction of the micropipette. GFP fluorescence and the corresponding Nomarski image are shown. GFP-N-Clip170 fluorescence in this migrating cell is shown in Movie 4. (B) Location of the MTOC in a polarizing, moving cell. A cell transiently expressing GFP-arrestin-3 was challenged with a micropipette containing 10 μ M fMLP, placed in the position shown at time = 0. GFP-arrestin-3 fluorescence and the corresponding Nomarski image are shown. The fMLP source was moved immediately after the 60-sec image to the position shown in *Left Center*; the pseudopod quickly followed the micropipette, whereas the MTOC remained stationary. (Scale bars: 10 μ m.)

inated at a common origin (presumably the MTOC) and moved at $\approx 3 \mu\text{m}/\text{min}$ toward the cell's periphery (Fig. 2*A Right*; Movies 3 and 4, which are published as supporting information on the PNAS web site), comets directed toward the back of the cell reached all of the way to the cell periphery and often traveled an additional distance ($\approx 1\text{--}3 \mu\text{m}$) in a direction parallel to the membrane, and comets directed toward the front of the cell stopped before entering the pseudopod (see Movie 4).

Although the MTOC plays a role in orienting polarity of many cells (4), such a steering role has not been stringently tested in neutrophils (previous studies located the neutrophil MTOC only in fixed cells; see refs. 15 and 16). We fortuitously discovered that a regulator of G protein-coupled receptors, GFP-tagged arrestin-3, serves as a useful MTOC marker in dHL-60 cells, where it colocalizes with the center of the microtubule array and with an MTOC protein (Fig. 5, which is published as supporting information on the PNAS web site).

Experiments with transiently expressed GFP-arrestin-3 (Fig. 2*B*; Fig. 6, which is published as supporting information on the PNAS web site) showed that the MTOC of dHL-60 cells follows the pseudopod and does not direct its orientation. In dHL-60 cells migrating toward a point source of fMLP, the MTOC marker was usually located behind the nucleus (10 of 12 cells; in two cells it overlapped the nucleus) and moved only after the pseudopod. The cell in Fig. 2*B* polarized and began to migrate toward the initial position of the micropipette, with its MTOC marker behind the nucleus; relocation of the micropipette at 60 sec induced a 90° change in orientation of the cell's pseudopod

within the ensuing 60 sec, whereas the marker remained in precisely its original position. Five other cells subjected to a moving fMLP source similarly showed reorientation of the pseudopod that preceded movement of the MTOC marker by at least 30 sec (one is shown in Fig. 6); *Dictyostelium discoideum* amoebae migrating toward a moving point source of attractant exhibited similar temporal lags in MTOC reorientation (26).

Microtubule Disruption Alters Morphology, F-Actin, and Behavior of dHL-60 Cells. Nocodazole substantially altered the distribution (Fig. 3*A*) and accumulation (Fig. 3*C*) of F-actin. In the absence of fMLP, nocodazole alone increased the total amount of cellular F-actin (Fig. 3*C*) and strikingly altered morphology: a prominent circumferential ring of F-actin was seen in almost all unpolarized nocodazole-treated cells but rarely in unpolarized controls (Fig. 3*A* and Table 1). These rings probably contain most of the increased accumulation of cellular F-actin (Fig. 3*C*), because pseudopods of most nocodazole-treated cells that polarized without fMLP appeared flat and thin, with little F-actin fluorescence. This phenotype contrasts with the prominent F-actin in the apparently thicker pseudopods of control cells that polarized in response to fMLP alone (arrows in Fig. 3*A* indicate the front edge of pseudopods). Pseudopods of nocodazole-treated cells exposed to fMLP showed either of two distinct morphologies (Fig. 3*A* and Table 1): (i) prominent, thick pseudopods with F-actin fluorescence like that of fMLP-treated control cells, or (ii) flat, thin pseudopods with little F-actin, similar to those of cells that polarized in response to nocodazole alone. In time-lapse movies, individual nocodazole-treated cells alternated between these two morphologies after polarizing in response to fMLP (data not shown).

The flat, actin-poor pseudopods of polarized nocodazole-treated cells suggested that microtubule disruption may weaken frontness signals. In accord with this idea, fluorescence of PH-Akt-GFP, a PIP3 probe, appeared weak or absent in pseudopods of cells that polarized in response to nocodazole alone, relative to control cells polarized in response to fMLP (Fig. 3*B*). In nocodazole-treated cells polarized in response to fMLP, PH-Akt-GFP accumulation was also weak in thin, flat pseudopods (Fig. 3*B*), but in thicker-appearing pseudopods (data not shown), accumulation was similar to that of fMLP-treated control cells.

Because fluorescence of PH-Akt-GFP in photomicrographs may not precisely reflect differences in PIP3 concentration at the plasma membrane, we assessed PIP3 accumulation more quantitatively: cells were treated with nocodazole and/or fMLP, followed by measurement of stably expressed PH-Akt-GFP in particulate extracts (Fig. 3*D*) or of PIP3-dependent phosphorylation of endogenous Akt (Figs. 3*E* and *F*). By both assays, nocodazole alone failed to activate the PIP3 pathway despite its ability to trigger morphologic polarity in a substantial proportion of cells; nocodazole treatment also reduced the PIP3 response to fMLP, in keeping with our inference from reduced intensities of F-actin or PH-Akt-GFP seen by microscopy.

Microtubule Disruption: Rho-Dependent Effects on Frontness Signals and Chemotaxis. Niggli (19) reported elevated accumulation of Rho and p160-ROCK in membrane extracts of cells treated with colchicine and that pharmacological inhibition of p160-ROCK prevented microtubule-disrupting agents from inducing neutrophil polarity and migration. Effects of microtubule disruption on key components of the backness response triggered by fMLP, in combination with the ability of backness signals to inhibit frontness in dHL-60 cells (14), suggested that nocodazole might impair frontness and chemotaxis of dHL-60 cells by enhancing Rho-dependent backness signals. In keeping with this idea, Rho activity in dHL-60 cells, like that of human neutrophils (19), increases in response to nocodazole (Fig. 3*G*): the drug alone

Fig. 3. Microtubule disruption alters backness and frontness signals. (A) Cellular distribution of F-actin. Cells preincubated with or without nocodazole (Noc) were exposed (where indicated) to 100 nM fMLP for 3 min (fMLP or fMLP + Noc) and then fixed and stained for F-actin (red). Fluorescence and the corresponding Nomarski images are shown. Arrows point to normal leading edges (irregular ruffles), and arrowheads point to flat, actin-poor lamellae seen at the front-most edges of nocodazole-treated cells. (Scale bar: 10 μ m.) Treatment protocols are described in *Supporting Materials and Methods*. (B) Cellular distribution of PH-Akt-GFP. Cells stably expressing PH-Akt-GFP were pretreated with or without nocodazole and exposed to fMLP as described in A. Arrows point to the leading edge. Fluorescence and Nomarski images are shown. (Scale bar: 10 μ m.) (C) Quantitation of F-actin. Cells pretreated with no drug, nocodazole, Y-27632, or nocodazole plus Y-27632 were exposed to 100 nM fMLP for 3 min, and F-actin in Triton X-100 insoluble fractions was quantitated as described in *Supporting Materials and Methods*. Results are normalized to the density of F-actin in unstimulated control cells (1.0). Bars show the mean $\pm$ 2 SEM of $n = 4$ experiments; **, statistical difference from control at $P < 0.01$. Coomassie-stained 42-kDa bands of actin from a representative experiment are also shown. (D) Association of PH-Akt-GFP with the particulate fraction. Suspensions of cells stably expressing PH-Akt-GFP were treated for 1 min with or without fMLP after exposure to inhibitors as described in C. PH-Akt-GFP in particulate fractions was assayed as described in the *Supporting Materials and Methods*. Results are normalized to the density of PH-Akt-GFP band in the unstimulated lane (1.0). The immunoblot shows the result of a representative experiment, and the bars show mean blot densities $\pm$ 2 SEM of $n = 4$ separate experiments. The double and single asterisks indicate pairs of conditions for which the respective results differ significantly at $P < 0.01$ or $P < 0.05$, respectively. (E) Time course of fMLP-stimulated Akt phosphorylation. Immunoblots show relative amounts of Akt phosphorylated at Thr-308. Upper and Lower show phospho-Akt densities in immunoblots of extracts from cells treated, respectively, without or with nocodazole and then stimulated with fMLP (100 nM) for the indicated times (seconds). Results are representative of two separate experiments. (F) Akt phosphorylation. Cell suspensions were pretreated, as indicated, without or with nocodazole, Y-27632, or nocodazole plus Y-27632 and then exposed to 100 nM fMLP for 1 min, where indicated. Results are normalized to the density of staining in the lane representing fMLP treatment of control cells (1.0). The immunoblot shows the result of a representative experiment; graphs show the mean blot densities $\pm$ 2 SEM of $n = 4$ separate experiments. *, pairs of conditions that differed significantly at $P < 0.05$. (G) Nocodazole increases Rho activity. Cell suspensions were treated with or without nocodazole and exposed to uniform fMLP for 1 min as indicated. Rho-GTP was determined by a GST-RBD pull-down assay as described in ref. 14. Results are normalized to the density of the Rho band in the unstimulated lane (1.0). The immunoblot shows the result of a representative experiment, and graphs show mean blot densities $\pm$ 2 SEM of $n = 4$ separate experiments. *, statistical differences from the control at $P < 0.05$.

increased cellular content of the GTP-bound active form of Rho 2.5-fold relative to control cells. Although fMLP alone elevated Rho-GTP in control dHL-60 cells as reported in ref. 14, the attractant slightly reduced the elevated Rho-GTP content of nocodazole-treated cells.

Effects of Y-27632, an inhibitor of p160-ROCK, indicated that nocodazole-induced elevation of Rho-dependent backness accounts for the decreased frontness responses to fMLP: Y-27632 prevented nocodazole from inhibiting fMLP-dependent PIP3 responses, including accumulation of PH-Akt-GFP in particulate fractions of fMLP-treated cells (Fig. 3D) and fMLP-stimulated phosphorylation of endogenous Akt (Fig. 3F).

Reversal of nocodazole-induced inhibition of frontness signals by Y27632 suggested that an excess of backness, relative to

frontness, could account for the chemotaxis defect of microtubule-disrupted cells. Indeed, in micropipette experiments Y-27632 strikingly improved the apparent straightness of migration trajectories of nocodazole-treated cells (Figs. 1B) and partially reversed nocodazole-induced reduction of the chemotactic index (Fig. 1C). The inhibitor also partially reversed the nocodazole-induced increase in migration speed (Fig. 1D).

Discussion

Contributions from both the actin and microtubule cytoskeletons are absolutely necessary for yeast cells, fibroblasts, neuroglia, and neurons to polarize and point in the right direction (1–6). In at least one case, microtubules are not only necessary, but can also suffice for a cell polarity and correct orientation:

cytochalasin D, an inhibitor of actin polymerization, fails to prevent cultured astrocytes from correctly orienting their MTOCs and microtubule-supported projections toward a wound in the cell monolayer (4). The neutrophil's microtubules clearly play a very different role, because microtubule disruption induces neutrophil polarity rather than preventing it, and impairs, but does not abolish, interpretation of chemoattractant gradients (Fig. 1; ref. 20). These findings raise three interesting questions, discussed below.

How Do Microtubules Suppress Neutrophil Polarity? Microtubules probably suppress neutrophil polarity in several ways, of which the most critical is inhibition of Rho activity: microtubule disruption causes Rho activation (Fig. 3G; ref. 19), inducing morphologic polarity and migration that are, in turn, prevented by inhibiting a Rho-dependent protein kinase (19). Microtubules may normally suppress Rho activity by sequestering (and thereby inactivating) Rho GEFs, several of which associate with intact microtubules in various cell types (27–30). Indirect evidence strongly supports the idea that microtubules do suppress activity of one such GEF, Rho GEF-H1 (31). Rho GEF-H1 mutants that cannot associate with microtubules activate cellular Rho in HeLa cells much more effectively than does the intact GEF, and a dominant-negative Rho GEF-H1 mutant prevents the Rho-dependent morphologic changes induced by microtubule disruption.

In contrast to sequestering and inhibiting Rho GEFs, however, intact microtubules are also thought to deliver such GEFs to appropriate membrane locations, thereby exerting positive regulation of Rho activity. A recent observation in *Drosophila* S2 cells led to a proposal that delivery of Rho GEF may be regulated by a trimeric G protein (7). In these cells DRhoGEF2 binds to EB1, a protein that specifically associates with the plus (growing) ends of microtubules; expressing a constitutively active mutant of *concertina*, the α subunit of a trimeric G protein in the fly, causes DRhoGEF2 to dissociate from microtubule plus ends and induces Rho-dependent morphologic changes, mediated by myosin II, identical to those induced by overexpressed exogenous DRhoGEF2. The proposal was not rigorously tested, partly because a ligand and receptor capable of activating endogenous *concertina* in these cells have not been identified. Moreover, although RNA interference-mediated knockdown of EB1 caused DRhoGEF2 to disappear from microtubule plus ends, the prediction that *concertina* cannot activate Rho-dependent morphology in the absence of EB1 was not tested (7).

This attractive possibility nonetheless merits further exploration in dHL60 cells, where fMLP triggers Rho-dependent backness through mammalian G12 α and G13 α (14), two homologs of *concertina*. To be explicit, we speculate that fMLP induces backness by activating G12 α and G13 α , which in turn induces dissociation and activation of Rho GEFs brought to the plasma membrane on growing plus ends of microtubules, where it is bound to EB1 (or an EB1-like protein). This idea would account for our observation (Fig. 2A and Movie 4) that most microtubule plus ends grow toward the plasma membrane at the back and sides of polarized dHL60 cells and for a report (19) that says abolishing microtubule dynamics with taxol reduces backness functions (membrane association of p160-ROCK and tail retraction) in human neutrophils.

Contrasting Roles of Microtubules in Neutrophils and Other Cells. In most cultured cells, Rho GEF isoforms bind to microtubules (27–30), and microtubule disruption increases Rho activity (32) and stimulates its downstream effects on the cytoskeleton (33). Why, then, does microtubule disruption impair polarity of most cells (34, 35) but induce it in neutrophils instead (18, 19)? The difference probably reflects the neutrophil's unusual capacity for rapid, self-organizing polarity, based on robust responses of

opposed actin assemblies, protrusive F-actin and contractile actin-myosin complexes, mediating frontness and backness, respectively (14), to spatially uniform stimuli. Such stimuli trigger distinct signals to activate frontness and backness, which segregate into spatially separate cell regions that oppose and balance one another in the new polarized steady state.

As noted above, nocodazole induces polarity primarily by activating backness (Rho- and myosin II-based contraction; refs. 18 and 19); frontness is detectable but weak, producing flat, thin pseudopods that contain less F-actin or PIP3 than those of normal cells polarized in response to fMLP (Fig. 3). Nocodazole may trigger polarity of a neutrophil just as transiently applied external pressure on one side induces polarity of a cytoplasm prepared from fish keratocytes (36). In the latter case, a radially symmetrical cell fragment rapidly polarizes by accumulating contractile actin-myosin complexes, parallel to the plasma membrane, on the side where the pressure is applied and protrusive F-actin, perpendicular to the membrane, on the other; the polarized cytoplasm then crawls away, its polarity sustained long after removal of the initiating stimulus. The different actin assemblies at the front and back of cytoplasm and, by extension, neutrophils, probably sustain polarity because they are mechanically incompatible locally but reciprocally reinforce one another at a distance.

In such a scenario, we propose that nocodazole-induced Rho activation in neutrophils, like localized external pressure on a cytoplasm (36), causes actin-myosin assemblies to array in parallel to the plasma membrane, thereby stimulating them to contract; initial slight, stochastic asymmetry in Rho-dependent contraction at one side of the cell, pushes the opposite side outwards, relieving membrane tension and enhancing formation of radially directed actin polymers in a protruding front (37). If protrusion at the front in turn enhances contraction at the back and *vice versa*, the cell maintains its polarity and migrates forward. Frontness of a cell polarized primarily by activating Rho would be weaker than that of a cell polarized in response to fMLP, which promotes not only Rho-dependent backness but in addition activates a PIP3- and actin-dependent positive feedback loop at the front.

The cytoskeletal machinery and cellular signaling pathways of professionally crawling cells like neutrophils and fish keratoplasts are poised to organize polarity in response to small perturbations. Neutrophils polarize in response not only to fMLP and microtubule disruption, but also to exposure to a uniform extracellular concentration of exogenous PIP3 (13, 38) or to a pharmacologic inhibitor of the μ isoform of calpain, a cytoplasmic protease (39). Mechanisms of these polarity responses are poorly understood but merit further study. We speculate that exogenous PIP3 may induce polarity in a fashion reciprocal to what we propose for nocodazole: that is, exogenous PIP3 stochastically stimulates protrusive frontness a bit more strongly on one side of the cell, and the mechanochemical coupling machinery responds by promoting contraction on the other side.

How Do Microtubules Enhance Directional Migration? The neutrophil's microtubule cytoskeleton plays ancillary roles not only in its ability to polarize in response to fMLP but also in its interpretation of an fMLP gradient. In a normal neutrophil with intact microtubules, the MTOC follows a turning pseudopod instead of pointing it in the correct direction (Fig. 2B). Moreover, nocodazole-treated neutrophils clearly sense the gradient of fMLP supplied by a stationary micropipette and can turn toward a moving fMLP source, although they follow meandering paths in doing so (Fig. 1B and C; ref. 20).

How, then, do the neutrophil's microtubules perform their ancillary, but not essential, role in direction finding? Although the mechanistic details remain poorly understood, our results strongly suggest that nocodazole impairs chemotactic efficiency

of dHL-60 cells by reducing the PIP3 and F-actin content of their pseudopods. We suspect that the weakening of pseudopods reflects their *local* inhibition by Rho-dependent backness, induced by microtubule disruption. In this scenario, by disrupting microtubules, nocodazole releases Rho GEFs indiscriminately throughout the cell, allowing them to promote backness everywhere, including the leading edge; in contrast plus ends of growing microtubules deliver Rho GEFs predominantly to the back and sides of normal polarized cells, where most of the plus ends are directed (Fig. 3). Accordingly, inhibiting nocodazole-induced backness with the p160-ROCK inhibitor increases PIP3 at the front of the cell (Fig. 3 *C* and *D*) and allows the cells crawl in straighter paths toward an fMLP source (Fig. 1 *B* and *C*). Because the backness-inhibiting agent does not completely restore directionality (Fig. 1*C*), it is possible that microtubule

disruption also diminishes microtubule-dependent transport to the pseudopod of vesicles carrying key frontness components, including phosphatidylinositol 3-kinases, GEFs for Rac and Cdc42, actin-polymerizing machinery, and membrane lipids.

We thank Ron Vale (University of California, San Francisco) for the GFP-N-Clip170 plasmid, Marc G. Caron (Duke University) for the GFP-arrestin-3 plasmid, and Wendell Lim and Dyrche Mullins for useful comments on the manuscript. This work was supported in part by National Institutes of Health Grant GM 27800 (to H.R.B.). A.V.K. was a postdoctoral researcher of the Fond National de la Recherche Scientifique of Belgium and is now a postdoctoral fellow of American Heart Association. F.W. was supported by National Institutes of Health Training Grant HL07713, and J.X. was supported by a fellowship from the Leukemia and Lymphoma Society.

- Rodriguez, O. C., Schaefer, A. W., Mandato, C. A., Forscher, P., Bement, W. M. & Waterman-Storer, C. M. (2003) *Nat. Cell Biol.* **5**, 599–609.
- Wittmann, T. & Waterman-Storer, C. M. (2001) *J. Cell Sci.* **114**, 3795–3803.
- Daub, H., Gevaert, K., Vandekerckhove, J., Sobel, A. & Hall, A. (2001) *J. Biol. Chem.* **276**, 1677–1680.
- Etienne-Manneville, S. & Hall, A. (2001) *Cell* **106**, 489–498.
- Etienne-Manneville, S. & Hall, A. (2003) *Nature* **421**, 753–756.
- Kodama, A., Lechler, T. & Fuchs, E. (2004) *J. Cell Biol.* **167**, 203–207.
- Rogers, S. L., Rogers, G. C., Sharp, D. J. & Vale, R. D. (2002) *J. Cell Biol.* **158**, 873–884.
- Zigmond, S. H., Levitsky, H. I. & Kreel, B. J. (1981) *J. Cell Biol.* **89**, 585–592.
- Zigmond, S. H. (1977) *J. Cell Biol.* **75**, 606–616.
- Weiner, O. D., Servant, G., Parent, C. A., Devreotes, P. N. & Bourne, H. R. (2000) in *Cell Polarity: Frontiers in Molecular Biology*, ed. Drubin, D. G. (Oxford Univ. Press, Oxford) pp. 201–239.
- Srinivasan, S., Wang, F., Glavas, S., Ott, A., Hofmann, F., Aktories, K., Kalman, D. & Bourne, H. R. (2003) *J. Cell Biol.* **160**, 375–385.
- Wang, F., Herzmark, P., Weiner, O. D., Srinivasan, S., Servant, G. & Bourne, H. R. (2002) *Nat. Cell Biol.* **4**, 513–518.
- Weiner, O. D., Neilsen, P. O., Prestwich, G. D., Kirschner, M. W., Cantley, L. C. & Bourne, H. R. (2002) *Nat. Cell Biol.* **4**, 509–513.
- Xu, J., Wang, F., Van Keymeulen, A., Herzmark, P., Straight, A., Kelly, K., Takuwa, Y., Sugimoto, N., Mitchison, T. & Bourne, H. R. (2003) *Cell* **114**, 201–214.
- Anderson, D. C., Wible, L. J., Hughes, B. J., Smith, C. W. & Brinkley, B. R. (1982) *Cell* **31**, 719–729.
- Schliwa, M., Pryzwansky, K. B. & Euteneuer, U. (1982) *Cell* **31**, 705–717.
- Eddy, R. J., Pierini, L. M. & Maxfield, F. R. (2002) *Mol. Biol. Cell* **13**, 4470–4483.
- Keller, H. U. & Niggli, V. (1993) *Cell Motil. Cytoskeleton* **25**, 10–18.
- Niggli, V. (2003) *J. Cell Sci.* **116**, 813–822.
- Allan, R. B. & Wilkinson, P. C. (1978) *Exp. Cell Res.* **111**, 191–203.
- Arai, H., Tsou, C.-L. & Charo, I. F. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 14495–14499.
- Lacalle, R. A., Gomez-Mouton, C., Barber, D. F., Jimenez-Baranda, S., Mira, E., Martinez, A. C., Carrera, A. C. & Manes, S. (2004) *J. Cell Sci.* **117**, 6207–6215.
- Steimle, P. A., Yumura, S., Cote, G. P., Medley, Q. G., Polyakov, M. V., Leppert, B. & Egelhoff, T. T. (2001) *Curr. Biol.* **11**, 708–713.
- Diamantopoulos, G. S., Perez, F., Goodson, H. V., Batelier, G., Melki, R., Kreis, T. E. & Rickard, J. E. (1999) *J. Cell Biol.* **144**, 99–112.
- Perez, F., Diamantopoulos, G. S., Stalder, R. & Kreis, T. E. (1999) *Cell* **96**, 517–527.
- Ueda, M., Graf, R., MacWilliams, H. K., Schliwa, M. & Euteneuer, U. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 9674–9678.
- van Horck, F. P., Ahmadian, M. R., Haessler, L. C., Moolenaar, W. H. & Kranenburg, O. (2001) *J. Biol. Chem.* **276**, 4948–4956.
- Ren, Y., Li, R., Zheng, Y. & Busch, H. (1998) *J. Biol. Chem.* **273**, 34954–34960.
- Tatsumoto, T., Xie, X., Blumenthal, R., Okamoto, I. & Miki, T. (1999) *J. Cell Biol.* **147**, 921–928.
- Glaven, J. A., Whitehead, I., Bagrodia, S., Kay, R. & Cerione, R. A. (1999) *J. Biol. Chem.* **274**, 2279–2285.
- Krendel, M., Zenke, F. T. & Bokoch, G. M. (2002) *Nat. Cell Biol.* **4**, 294–301.
- Ren, X. D., Kiosses, W. B. & Schwartz, M. A. (1999) *EMBO J.* **18**, 578–585.
- Enomoto, T. (1996) *Cell Struct. Funct.* **21**, 317–326.
- Glasgow, J. E. & Daniele, R. P. (1994) *Cell Motil. Cytoskeleton* **27**, 88–96.
- Goldman, R. D. (1971) *J. Cell Biol.* **51**, 752–762.
- Verkhovsky, A. B., Svitkina, T. M. & Borisy, G. G. (1999) *Curr. Biol.* **9**, 11–20.
- Kolega, J. (1986) *J. Cell Biol.* **102**, 1400–1411.
- Niggli, V. (2000) *FEBS Lett.* **473**, 217–221.
- Lokuta, M. A., Nuzzi, P. A. & Huttenlocher, A. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 4006–4011.