Pak3 inhibits local actin filament formation to regulate global cell polarity

Y. Asano,1 A. Jiménez-Dalmaroni,2 T. B. Liverpool,3 M. C. Marchetti,4 L. Giomi,4 A. Kiger,5 T. Duke,2 and B. Baum1

1MRC Laboratory for Molecular Cell Biology, University College London, Gower St., London, WC1E 6BT, United Kingdom
2London Centre for Nanotechnology, University College London, Gordon St., London, WC1H 0AH, United Kingdom
3Department of Mathematics, University of Bristol, Bristol, BS8 1TW, United Kingdom
4Department of Physics and Syracuse Biomaterials Institute, Syracuse University, Syracuse, New York 13244
5Department of Cell and Developmental Biology, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093

(Received 23 December 2008; accepted 27 February 2009; published online 10 April 2009)

Lamellipodia are broad actin-based structures that define the protruding edge of many motile animal cells. Here we identify a Drosophila homolog of the p21-activated kinases (Paks) as a novel inhibitor of Rac-mediated lamellipodial formation: Pak3 overexpression mimics a loss of Rac activity, while Pak3 RNAi-mediated silencing enhances lamellipodial dynamics. Strikingly, the depletion of Pak3 also polarizes the cellular distribution of actin filaments, is sufficient to induce nonmotile cells to migrate, and, in cells firmly attached to the substrate, gives rise to a wave of high actin filament density that encircles the cell periphery at a steady pace. To better understand these systems level phenomena, we developed a model of the cortical actin network as an active gel whose behavior is dominated by the rate of actin filament bundling and polymer synthesis. In the presence of filament treadmilling, this system generates a propagating density wave of actin filaments like that seen in Pak3 RNAi cells. This analysis reveals an intimate relationship between local regulation of actin filament dynamics and global cytoskeletal polarity, and suggests a role for negative regulators of lamellipodial formation, like Pak3, in the maintenance of a poised state, in which regulated directional cell movement can occur. [DOI: 10.2976/1.3100548]

CORRESPONDENCE
Buzz Baum: b.baum@ucl.ac.uk

Lamellipodia are broad, flat actin-based protrusions that constitute the extending leading edge of a wide range of motile animal cells. Their dynamic form is determined by the activity of a large set of actin binding proteins that modulate different steps in the cycle of actin filament formation and depolymerization; from filament nucleation, to elongation, cross-linking, and disassembly (Pollard and Borisy, 2003). Through their ability to modulate this diverse set of actin binding proteins in a spatially and temporally controlled manner, the Rho family GTPases Cdc42 and Rac are thought to act together with a set of downstream kinases, including the p21-activated kinases, to establish this dynamic lamellipodial architecture (Ridley, 2006). In order to induce cell migration, however, it is not enough to activate a pathway to induce the formation of actin-based protrusions. The actin cytoskeleton must be polarized if the forces generated by actin filament dynamics are to be translated into net forward cell movement, and the advancing protrusion must adhere to the substrate (Giannone et al., 2007), while adhesions at the cell rear are released (Cramer, 1999; Weiner et al., 2006). In a chemotactic cell migrating up a chemical concentration gradient, polarity is achieved by using receptor-mediated signaling to couple actin dynamics...
to differences in the local concentration of an extracellular cue (Devreotes and Janetopoulos, 2003). However, most motile cells spontaneously polarize even when maintained in a homogeneous environment (Xu et al., 2003; Devreotes and Janetopoulos, 2003). Moreover, small differences in local actin filament dynamics can be amplified and stabilized, so that an isotropic cell fragment will move persistently once pushed (Verkhovsky et al., 1999). This break in cytoskeletal symmetry and its maintenance are likely to result from interactions between local actin structures in different regions of the cell as they grow and compete for a globally limited pool of resources. Such systems level properties are hard to intuit, however. Because of this, several groups have constructed mathematical models to explore their behavior (Kozlov and Mogilner, 2007; Maree et al., 2006), to show, for example, how a given dynamic actin filament architecture could arise as a simple consequence of combining well-understood molecular processes when viewed in the context of an entire cell (Keren et al., 2008; Lacayo et al., 2007). In order to better understand actin dynamics and polarization of the actin cytoskeleton, we and others have employed RNAi screening as a genetic tool to identify novel regulators of both processes in nonmotile epithelial-derived Drosophila cells in culture (Kiger et al., 2003; Rogers et al., 2003). This approach identified Drosophila Pak3, one of the three Drosophila p21-activated kinases (Hofmann et al., 2004; Mentzel and Raabe, 2005), as a novel inhibitor of filament formation in a number of cell types (Liu et al., 2009). RNAi-mediated depletion of Pak3 resulted in an increase in lamellipodial width and, strikingly, was accompanied by a global change in the cellular distribution of actin filaments, which was sufficient to induce nonmotile cells to break symmetry and to migrate. Intriguingly, in cells forced to tightly adhere, this led to the appearance of a single lamellipodium that steadily encircled the cell periphery. In order to determine the macroscopic conditions under which the formation of these actin waves might occur, we developed a physical model in which the actin cytoskeleton is considered to be an active gel (Liverpool, 2006). Our theoretical description reveals how enhanced actin filament formation and actin bundling can drive a switch from isotropic to polar actin organization, as seen in Pak3 RNAi cells. This combined experimental and theoretical analysis shows that Paks are likely to regulate both actin filament formation and cell polarity by modifying local actin filament dynamics to regulate the switch between isotropic and polar states.

RESULTS
Pak3 knock down cells accumulate lamellipodial actin filament-based structures
We have carried out a series of RNAi screens to identify novel genes involved in actin filament organization and cell morphology in a variety of Drosophila cell lines (Liu et al., 2009, Kiger et al., 2003). These studies consistently identified Drosophila Pak3, one of the three Drosophila p21-activated kinases [Pak1, Pak2 (Mbt), and Pak3 (Mentzel and Raabe, 2005)], as a potent regulator of lamellipodial structure. RNAi-induced silencing of Pak3 led to the formation of polarized actin-filament-based protrusions in ML-DmD16-c1 cells (Fig. 1) and Kc cells (data not shown), and to the formation of unusually broad lamellipodia in S2R+ cells [Fig. 3(A)]. Although Pak1 and Pak2 RNAi were not identified as strong hits in these RNAi screens, and dsRNAs targeting these genes failed to generate a clear lamellipodial actin phenotype in ML-DmD16-c1 and S2R+ cells even when added in combination (data not shown), we note that siRNAs targeting mammalian Pak1 have also been reported to increase lamellipodial width (Smith et al., 2008) and to perturb lamellipodial dynamics (Machacek and Danuser, 2006).
In order to study the lamellipodial defects associated with Pak3 RNAi in more detail, we chose the adherent ML-DmD16-c1 wing-disk cell line as a model system (Baum and Cherbas, 2008). In the majority of symmetrical ML-DmD16-c1 cells, Pak3 depletion disturbed the uniform distribution of actin filaments, leading to the formation of a single actin-filament-based protrusion in cells on an uncoated glass substrate [Fig. 1(A)]. These polarized protrusions in Pak3 RNAi cells were dynamic [Figs. 1(B)–1(D)], and were sufficient to drive the forward movement of these nonmotile cells. This is visible in traces of ML-DmD16-c1 cell centroids over time [Fig. 1(C)]. Thus, while control cells remained in position over a period of 1 h, Pak3-RNAi cells migrated over significant distances with the lamellipodium extended in the direction of migration [Fig. 1(C)], changing their direction of movement approximately every 15 min [Fig. 1(D)]. It is clear from this analysis that Pak3 is not required for lamellipodial formation itself (Cau and Hall, 2005), but is required for normal lamellipodial architecture and to regulate the global distribution of actin filaments within the cell.

To facilitate a more detailed kinetic analysis of the actin-based protrusions in control and Pak3 RNAi cells it was important to uncouple the possible effects of Pak3 on actin filament organization from its effects on cell shape. To do this, we used Concanavalin A to adhere cells tightly to a coated glass substrate (Rogers et al., 2003). This was sufficient to control cell centroid cells depleted for Pak3 to spread and flatten. As before, control RNAi cells acquired an isotropic form under these conditions [Fig. 2(A)], whereas the majority of well-spread Pak3 RNAi cells (87%) retained a single, dominant actin-based lamellipodium [Figs. 2(A), 2(B), and 2(E)]. In the majority of these flat, polarized Pak3 RNAi cells imaged between 5 and 7 days after the addition of dsRNA, when RNAi is most potent (Baum and Cherbas, 2008), lamellipodia were seen encircling the cell periphery every 788±217 s at a relatively constant speed of 6.93±1.66 μm/min (n=9) [Figs. 2(B) and 2(C) Supplemental Movies 1 and 2]. The position of the nucleus remained relatively stationary throughout (data not shown). This propagating lamellipodium was most easily seen in kymographs representing evenly positioned points around the cell circumference [Fig. 2(C) and marked in Fig. 2(A)]. By tracking the progression of the front (F) and rear (R) margins of the lamellipodium over time [defined morphologically as shown in Fig. 2(A) right panel] and expressing its movement as an angular velocity [graph in Fig. 2(D)], it was clear that progress at the front margin was more erratic than the forward movement of the trailing margin. On average, however, the portion of the cell margin encompassed by the lamellipodium remained relatively constant over time [the distance between the traces in black and white in Fig. 2(D)]. In addition, this movement of the lamellipodium did not halt following an encounter with a break in the cell perimeter [break noted by arrow in Fig. 2(F)], corresponding to a region where the cell failed to adhere well to the substrate. Instead, the lamellipodial wave was observed pausing before jumping the gap [Fig. 2(F) and data not shown]. These data argue against the idea that a simple sliding mechanism underlies wave propagation, and suggest a role for forces within or at the back of the protrusion in the procession of the lamellipodia around the cell.

**Pak3 inhibits lamellipodial actin filament formation downstream of Cdc42 and upstream of Rac**

Pak3 physically interacts with Rac1 and Rac2 (Mentzel and Raabe, 2005), and p21-activated kinases (Paks) have long been known to act as effectors of Cdc42 and Rac [reviewed in Arias-Romero and Chernoff (2008)]. Because of this, in searching for a molecular function for Pak3, we carried out a series of epistasis experiments to test whether Pak3 contributes to Cdc42/Rac lamellipodial signaling in both S2R+ [Fig. 3(A)] and ML-DmD16-c1 cells (Fig. S1). First, we combined dsRNA targeting Pak3, Cdc42, and Rac1/2. As previously reported, both Cdc42 and Rac1/2 RNAi S2R+ cells lack the ability to generate lamellipodia, resulting in a characteristic spiky cell phenotype in both cases (upper panels of Fig. 3) (Kiger et al., 2003; Kunda et al., 2003; Rogers et al., 2003). When combined with Pak3 dsRNA, the Rac1/2 RNAi phenotype remained [lower panels of Fig. 3(A)]; that is Rac1/2 RNAi Pak3 RNAi cells remained spiky. By contrast, Pak3 RNAi rescued the loss of lamellipodia seen following Cdc42 RNAi [Fig. 3(A) middle of lower panels], so that Cdc42 RNAi Pak3 RNAi cells exhibited well-formed lamellipodia. Significantly, this effect of Pak3 silencing in Cdc42 RNAi cells mimics the effects of Rac activation (Kunda et al., 2003). Similar results were seen in ML-DmD16-c1 cells (Fig. S1). Taken together, these data suggest that Pak3 functions genetically downstream of Cdc42 and upstream of Rac1/2.

To extend this epistasis analysis, we next tested the effects of Pak3 overexpression. Significantly, Pak3-GFP expression (but not GFP expression) was found to be sufficient to limit lamellipodial formation in fly cells, causing cells to take on a spiky morphology like that seen in Rac RNAi cells [Figs. 3(B) and 3(C)]. If, as suggested by the epistasis analysis, Rac acts downstream of Pak3, it should be possible to rescue this Pak3 gain of function phenotype through Rac activation. Indeed, the coexpression of active Rac (Rac1-V12) restored lamellipodia to Pak3-GFP cells [Fig. 3(D), right hand panel, in 89% of cells, n=29]. These data show that Pak3 acts to inhibit Rac-dependent lamellipodial formation. In keeping with this conclusion, we observed a significant 1.6-fold increase in the speed of retrograde flow in Pak3 RNAi cells compared to the control [1.45±0.38 μm/min (n=10) in control RNAi cells and 2.3±0.47 μm/min (n=10) in Pak3 RNAi cells, p=0.00036, Fig. S2(a) and S2(b)]. This increase in flow within the lamellipodium was
Figure 2. Analysis of the lamellipodial wave generated in Pak3 RNAi cells. (A) Phase contrast images show control RNAi and Pak3 RNAi cells on a Concanavalin A-coated substrate. The lamellipodium of this Pak3 RNAi cell rotates anticlockwise. Black dots in Pak3 RNAi cell indicate the ruffling cell edge as the lamellipodium. Arrows with F and R indicate the front and rear margin of a rotating lamellipodium, respectively. (B) The proportion of cells that were polarized under these conditions was quantified. (C) Kymographs correspond to the movement of the margins of control of the RNAi and Pak3 RNAi cells at positions marked by numbers in (A) over a 20 min period. Arrows in the kymographs indicate the fully extended lamellipodium. (D) The positions of the front and rear margins of a rotating lamellipodium in a Pak3 RNAi cell were tracked over time and are expressed as an angle. In this example, the lamellipodium rotated through two revolutions in 20 min. (E) Control (DsRed RNAi) and Pak3 RNAi ML-DmD16-c1 cells were plated on Concanavalin A, before being fixed and stained for actin filaments using TRITC-Phalloidin. Asterisks in Pak3 RNAi cells indicate the area stained with Phalloidin. (F) Phase contrast images show the lamellipodial wave in a Pak3 RNAi cell with a gap in its periphery (arrow). Black dots indicate the ruffling cell edge as the lamellipodium rotates clockwise. Images show the cell at consecutive 90 s intervals. Scale bars=10 μm in (A) and (F), and 5 μm in (C). Cells in (E) were rescaled to enable comparisons of overall filament distribution.
Figure 3. Placing Pak3 in the Cdc42-Rac pathway. (A) Fluorescent images show dsRNA-treated S2R+ cells, fixed and stained for actin with TRITC-Phalloidin. Upper panels show cells incubated with lacZ (control), Cdc42, or Rac1/2 dsRNA. Lower panels show the effects of combining each with Pak3 dsRNA. (B) Confocal images of S2R+ cells expressing Pak3-GFP were used to quantify (C) the effects of Pak overexpression, which inhibited lamellipodial formation. (D) Constitutively active Rac1 (RacV12) was coexpressed together GFP (left) or Pak3-GFP (right) for one day prior to fixation. A fivefold excess of the RacV12 plasmid was used. RacV12 expression rescued lamellipodial formation in Pak3-GFP expressing S2R+ cells. (E) A lamellipodial signaling pathway based on the results of this epistasis analysis is shown. (F,G) Pak3 localizes to lamellipodia. Confocal microscopic images show TRITC-Phalloidin labelling in control S2R+ cells stained for Pak3 using the Pak3 antibody (see Fig. S3) and in cells expressing low levels of Pak3-GFP using a GFP antibody (G). Arrows in (F) and (G) indicate areas with high levels of Pak3. Stars indicate high levels of F-actin and low levels of Pak3. Scale bars=20 μm.
associated with a corresponding 1.4-fold increase in average lamellipodial width \(2.22 \pm 0.61 \mu m\) in control RNAi cells \((n=21)\) and \(3.11 \pm 0.84 \mu m\) in Pak3 RNAi cells \((n=38)\) \(p=0.0000086\), Figs. S2(c) and S2(d)]. This is not unexpected, since lamellipodial width is determined both by the rate of actin filament polymerization at the membrane and by the rate of filament disassembly at the rear of the lamellipodium. Thus, if the rate of actin filament disassembly remains unaltered, an increase in actin polymerization as measured by retrograde flow will result in a parallel increase in lamellipodial width, as observed.

Interestingly, although these data make a strong case for Pak3 playing an inhibitory role in the regulation of lamellipodial actin filament formation, both Pak3 [Fig. 3(F) and Fig. S3] and GFP-Pak3 [Fig. 3(G)] were found localized within a subset of lamellipodial extensions in fixed cells (cells expressing low levels of Pak3-GFP were imaged using an Alexa Fluor 488-conjugated anti-GFP antibody), as previously reported for GST-fused *Drosophila* Pak3 (Mentzel and Raabe, 2005). Moreover, in living cells in which membranes were visualized using red-fluorescent Cell Tracker (CMTPX), Pak3-GFP was seen accumulating in protrusions as they begin to retract [Fig. S3(c)]. Since Pak3 silencing leads to run-away lamellipodia, the recruitment of Pak3 to lamellipodia suggests a role for this inhibitor in the regulation of lamellipodial turnover.

### Physical description of actin wave propagation in the cell periphery

In order to better understand wave propagation, the dynamical equation for the local polarization similarly involves several contributions [Eq. (2) in the Supplemental Material]. First there is active convection of filament polarization along the direction of self-propulsion, which is along the direction of the polarization vector itself. Filament polarization dynamics are also affected by gradients in the gel. In general, polar liquid crystals, like the actin cytoskeleton, have additional contributions to their free energy from variations in the local polarization coupled to the Frank elasticity constant (De Gennes and Prost, 1993). Active processes, such as bundling or gel contraction, give contributions to the polarization dynamics similar to those arising from Frank elasticity, allowing one to define an “effective” local Frank elastic constant, which, unlike the equilibrium case, can be negative. A negative effective Frank elasticity leads to modulated phases with spatially varying polarization directions. Finally, there are terms that stabilize the modulated phases at high density, that is, slow down and stop the build-up of local variations in the gel polarization when the local density becomes high enough. The positive contributions to the effective Frank elasticity and the terms that stabilize the build-up of inhomogeneous states at high density result naturally from excluded volume interactions between the filaments. We emphasize that because the components of the coarse-grained dynamics described above can arise from a wide variety of different microscopic processes, microscopic mechanisms other than treadmilling and filament bundling could play a role.

In order to better understand wave propagation, the gel dynamics defined above were employed in a two-dimensional model of the cortical cytoskeleton in an ML-DmD16-c1 cell [Fig. 4(A)]. For simplicity, membrane fluctuations were not included in this analysis, even though they are observed [Figs. 2(C) and 4(B)] and could be usefully analyzed as part of a future model. Instead the cell boundary was modeled as a circle of radius \(R\) [Figs. 4(B) and 4(C)]. For the purposes of the model, it was also assumed that the actin cytoskeleton is concentrated in a thin peripheral ring around the cell circumference, such that the width \(d\) of the peripheral ring is much less than the cell radius \(d \ll R\) [Fig. 4(C)]. This
circular ring of actin is described in terms of 2d polar coordinates \((r, \phi)\), in which \(r\) is kept at a constant value, while \(\phi\) varies in the range \(0 \leq \phi \leq 2\pi\). Thus, the concentration and polarization of the actin gel can be written solely as a function of the angle \(\phi\) and time \(t\), \(c = c(\phi, t)\) and \(p = p(\phi, t)\). We also assume that the mean filament length \(\ell\) is constant, implying that on average depolymerization and polymerization are balanced.

The gel equations have a family of stable steady-state solutions when \(\beta = 0\), for which the concentration is spatially uniform and the filaments point at an arbitrary angle to the radial direction. When \(\beta \neq 0\), there is only one homogeneous state with filaments pointing tangentially to the cell perimeter. In the case of nonzero \(\beta\), a linear stability analysis shows that the homogeneous solution is unstable (Supplemental Material). We expanded the solution of the linearized equations in Fourier modes of varying wavelengths. The simplest inhomogeneous state is one in which a single localized region of high concentration occurs. This happens when the first Fourier mode of \(c\) becomes unstable, while polarization modes remain stable. We determined the set of parameter values under which this gel inhomogeneity occurs, and then investigated its temporal evolution. We considered the full nonlinear equation for the concentration and solved it to obtain the expression of the perturbation to the constant steady-state value of \(c\). A traveling wave solution of amplitude \(|b|\) was obtained, which depends on the bundling parameter \(\alpha\) [Eq. (25) in the Supplemental Material]. The full expression for the gel concentration is then

\[
c(\phi, t) \simeq \text{const} + |b|\cos(\phi + \omega t + \gamma),
\]

where the angular velocity \(\omega\) is \(\beta\) dependent [Eq. (25) in the Supplemental Material] and \(\gamma\) is an arbitrary constant. A wave solution obtained by solving numerically the nonlinear gel equations [Eqs. (6) and (7) in the Supplemental Material] is shown in Fig. 4(D).

In essence this model reveals that simple changes in actin filament dynamics and bundling can induce a change from a nonpolar to polar cytoskeleton. Therefore, it is not necessary to evoke the existence of a complex molecular mechanism underlying the observed switch in polarity following Pak3 silencing. Instead this type of behavior is a simple property of an active gel like the actin cytoskeleton, so that cells are necessarily prone to develop cytoskeletal inhomogeneities, leading to the formation of propagating waves of actin density, like those seen in Pak3 RNAi cells. When considered in this light, the model helps to explain the importance of negative regulators of actin filament dynamics in limiting local increases in actin filament density to maintain cells in a poised isotropic state. In this state, subtle changes in local Pak3 activity will be sufficient to alter the global distribution of actin filaments to give rise to random cell movement or, when coupled to an external cue, directed cell migration.

**DISCUSSION**

The findings described here, that (i) ectopic Pak3 expression inhibits lamellipodial formation; that (ii) the loss of Pak3 increases the rate of retrograde flow and lamellipodial width; and that (iii) Pak3 is recruited to protrusions as they turn over, suggest that Pak3 may act as a break to limit lamellipodial actin filament formation. Furthermore, because Pak3 RNAi mimics Rac1-V12 expression in rescuing the loss of Cdc42 (Kunda et al., 2003), and because the effects of Pak-GFP overexpression can be overcome by the expression of active Rac, Pak3 appears to inhibit Rac downstream of Cdc42 to negatively regulate Arp2/3-mediated protrusion formation. Although this goes against the view most commonly put forward in literature (Arias-Romero and

---

**Figure 4. Model of wave propagation in an actin gel in circular cells.** (A) Figure shows a rotating lamellipodium in a Pak3 RNAi treated ML-DmD16-c1 cell on Concanavalin A—arrow marks the region of maximum lamellipodial activity. (B) Phase contrast microscopy picture of a control cell fixed to a substrate with a typical circular shape. (C) In the theoretical model, the cell perimeter is represented by a circle of radius \(R\). (D) The concentration of active filaments as a function of the polar angle \(\phi\) and time. See theoretical supplement for model details.
in which Pak kinase family proteins function are assumed to be positive regulators of actin filament formation downstream of Rho family GTPases, our genetic data are supported by a number of recent studies in mammalian cells. Thus, an increase in lamellipodia width like that seen in Pak3 RNAi cells is seen in human cells treated with siRNAs targeting Pak1 (Smith et al., 2008). Moreover, a recent biochemical analysis demonstrated that Pak interferes with the ability of an exchange factor beta-PIX to activate Rac (ten Klooster et al., 2006), suggesting a function for Pak as an upstream inhibitor of Rac function. Our analysis also fits with the observation that Paks can act downstream of Cdc42 to modulate Rac activation, without their being absolutely required for lamellipodial formation (Cau and Hall, 2005), with observations suggesting that Cdc42 inhibits Pak activity (Weisz Hubsman et al., 2007), and by the presence of both Pak and Cdc42 in a single complex that regulates polarity in yeast (Kozubowski et al., 2008). The effects of Paks on changes in the direction of cell migration, e.g., during neural growth cone turning (Ang et al., 2008). The effects of Paks on changes in the direction of cell migration, e.g., during neural growth cone turning (Ang et al., 2008), suggest a function for Pak as an upstream inhibitor of Rac function. Our analysis also fits with the observation that Paks can act downstream of Cdc42 to modulate Rac activation, without their being absolutely required for lamellipodial formation (Cau and Hall, 2005), with observations suggesting that Cdc42 inhibits Pak activity (Weisz Hubsman et al., 2007), and by the presence of both Pak and Cdc42 in a single complex that regulates polarity in yeast (Kozubowski et al., 2008). The effects of Paks on changes in the direction of cell migration, e.g., during neural growth cone turning (Ang et al., 2003; Hing et al., 1999; Newsome et al., 2000), may reflect a similar function for Paks in damping local actin polymerization to facilitate the necessary redistribution of actin filaments.

Interestingly, the loss of this negative regulation in Pak3 RNAi cells does not lead to runaway actin polymerization [as seen in Cofilin RNAi cells (Jovceva et al., 2007)]. Instead, the relatively subtle change in lamellipodial actin dynamics in the absence of Pak3 are sufficient to induce a dramatic change in global actin organization, so that actin filament formation at a single site comes to dominate. While this might seem surprising, our theoretical analysis reveals how a minor increase in the rate of actin filament treadmilling can lead to the propagation of inhomogeneous cytoskeletal structures like those observed experimentally following the loss of Pak3. Moreover, these calculations show the difficulties of maintaining a simple active actin gel in an isotropic state, as is frequently seen in models of the actin cytoskeleton that include elements of positive feedback (Wedlich-Soldner et al., 2003). Once symmetry has been broken, both the model and our data [Fig. 2(F)] argue against the movement of the rotating lamellipodium being driven solely by spreading of the lamellipodial front, e.g., by the invasion of growing actin filaments into adjacent quiescent regions at the cell margin. Instead, they suggest that the net circular motion of the lamellipodium is determined by a combination of forces within the lamellipodium, described by α and β terms in the model, which enable the lamellipodium to traverse small blockades and large gaps. In future work, it will be important to experimentally probe the effects of quantitative changes in the rates of filament nucleation, extension, remodeling, and disassembly on the propagation of this wave, and to compare these data to the results of modeling.

Previous authors have reported the existence of actin waves in cells (Dobereiner et al., 2006) and have suggested a role for Pak in their suppression (Machacek and Danuser, 2006). Moreover, they have investigated the role of these waves in cell motility (Weiner et al., 2007) and have speculated about the need for either membrane-cytoskeleton interactions (Shlomovitz and Gov, 2007) or negative regulators (Weiner et al., 2007) to shape propagating lamellipodial actin waves in mammalian cells. In line with these previous studies, our analysis suggests that negative regulators of actin filament dynamics, like Pak3, are required to maintain the actin cytoskeleton in a poised isotropic state, since the higher the level of actin polymerization and filament bundling, the greater the extent of polarization. In metazoan cells, this may be achieved by Paks negatively regulating local Cdc42 and Rac-dependent lamellipodia formation to control the overall distribution of actin filaments within the cell. Our data also show how this type of systems property can induce a spontaneous break in symmetry, causing a cell to migrate even when placed in a homogeneous environment (Fig. 1). Interestingly, analogous studies in yeast have implicated Paks in the regulation of actin cytoskeletal polarity (Kozubowski et al., 2008). For example, germinating budding yeast cells lacking the Pak homolog Cla4 are unable to remodel their highly polarized actin cytoskeleton to undergo the normal switch from polar to isotropic growth (Kono et al., 2005), while Pak homolog Shk1/Orb6 in fission yeast cells is required for the switch from monopolar to bipolar growth (Sawin et al., 1999), and functions as part of a feedback loop in which polarized cytoskeletal organization helps to determine cell shape. Taken together, these data suggest a conserved role for Paks working together with Rho family GTPases in the control of local actin filament formation and global cell polarity.

EXPERIMENTAL PROCEDURES

Cell culture and RNAi

*Drosophila* cell lines were cultured in Shields and Sang M3 insect medium (Sigma) in 10% fetal bovine serum, supplemented with 1 µg/ml insulin for ML-DmD16-c1 cells. Cells were kept at 23 °C and all subsequent manipulations were carried out at room temperature. To prepare dsRNA to target Pak3, two sets of primers

\[
5'\text{-}CTATGCGAGCTCCCTTG-3' \\
5'\text{-}AAATCTGCCGAGCGTTAGAA-3' \\
5'\text{-}AACAAATACCGAACGCAAAC-3' \\
5'\text{-}CTGTGAGTTCATTAGGCGCA-3'
\]

with T7 sequence were used to amplify ~1000 and 300 bp DNA fragments. Then dsRNAs were synthesized from these PCR products using the MEGAscript T7 kit (Ambion). For...
RNAi, 0.3 ng dsRNA was added to 10 µl of 2 × 10^{14} cells in a 384-well plate in serum starved conditions for an hour, before supplementing with complete medium. Cells were processed for analysis 5–7 days later. DsRED or LacZ dsRNAs were used in control experiments. Western blotting was carried out as previously described (Kunda et al., 2003).

**Ectopic Pak-GFP expression analysis**

The pUAST-Pak3::EGFP fusion was made by PCR amplification from LD10376 Pak3 cDNA to lack the transcription stop and 3’UTR using primers GTACGAATTCCatatactaataactatataatctaagcgaaagtttcccacccttctggc, and then cloned into EcoRI and KpnI sites of pUAST, respectively. EGFP cDNA (Clontech) with stop sequences was subsequently added in-frame with Pak3 at downstream pUAST KpnI and NotI sites. Plasmid, UAS-Pak3-EGFP, UAS-GFP, UAS-Rac1V12 (a gift from Liqun Luo), were co-transfected with Actin5C-Gal4 plasmid. In each case, 0.2–1 µg plasmid was transfected into 6 × 10^5 cells in 0.3 ml serum free medium, in a mix containing 3 µl FuGENE HD transfection reagents (Roche). Cells were then reseeded with 0.2 ml complete medium 1 h later. Transfection was done 1 day before observation/fixation. For nonspecific fluorescence staining, Cell Tracker CMTPX (Molecular Probes) was added (2 µg/ml) to transfected cells in serum free medium for 30 min to mark the cell edge. The medium was then changed to complete medium to remove background signal.

**Cell fixation and fluorescent staining**

To prepare the Concanavalin A-coated substrate, coverslips were incubated with 50 µg/ml Concanavalin A solution for half an hour, before the remaining solution was removed and the glass dried completely. Other experiments were carried out on untreated glass or in plastic 384-well plates (Corning), before fixation cells were transfected in the relevant substrate and allowed to settle for a minimum of 30 min. Cells were then fixed with 4% formaldehyde in PBS for 20 min and washed with PBS. Before staining, cells were permeabilized with PBS including 0.1% TritonX-100 and 5% BSA for half an hour. To label F-actin and microtubules, we used TRITC-Phalloidin (Sigma) and a FITC-conjugated anti-α-tubulin antibody conjugate (Sigma). Alexa Fluor 488 conjugated rabbit polyclonal antibody against GFP (Molecular Probes) was used at 1:350 to enhance the signal in cells expressing low levels of Pak3-GFP. Pak3 rat polyclonal antibodies were raised against a peptide CVPNIKAKKVLRRNV (Eurogentec, Belgium) and used at 1:100. Alexa Fluor 647 goat anti-rat IgG (Molecular Probes) was used as the secondary antibody at 1:500. The specificity of Pak3 antibody was checked by staining Pak3-GFP expressing cells and by western blotting Pak3 RNAi cell extracts (Fig. S3).

**Microscopy**

For phase contrast live cell imaging, cells were transferred to glass bottom culture dishes (MatTek Corporation) one day before taking images. In the case of on the Concanavalin A-coated glass, cells were allowed to settle for 1 h before imaging. Phase contrast images were taken with a TE2000 microscope (Nikon) with a 100× or 40× objective lens or an Axiovert 135 TV (Zeiss) with a 100× or a 63× objective lens using a cooled CCD camera (Hamamatsu Orca ER). Intervals between time lapse images varied from 1.4 to 30 s depending on experiment. To measure the speed of retrograde flow, ten kymographs were derived from two control RNAi, and two Pak3 RNAi cells were used. Fluorescent microscopy was carried out using a TE2000-E Eclipse (Nikon) with a Plan Fluor 20 × /0.45 and 40 × /0.60 lenses and images were taken using a cooled CCD camera (Roper Scientific). Two systems, LSM510 (Zeiss) with a 63× objective lens and TCS SP5 (Leica) with a 40× objective lens, were used for confocal microscopic analyses. For confocal time lapse, images of live cells were taken every 5 s.

ImageJ software was used for quantitative analyses and for image processing.

Statistical significance was determined using the Student’s t-test.

**ACKNOWLEDGMENTS**

We thank A. Takesono, A. Ridley, and F. Jülicher for helpful discussions. YA received funding for this work from the Association for International Cancer Research, the Ludwig Institute for Cancer Research, and the MAIN EU FP6 network. AJD and TD were funded by Cancer Research UK. TBL acknowledges the support of the EPSRC under Grant No. EP/G026440/1. MCM and LG were supported by the U.S. National Science Foundation through Grant Nos. DMR-0705105 and DMR-0806511. BB thanks the Royal Society, the EMBO YIP program, the Ludwig Institute for Cancer Research, and UCL for support.

**REFERENCES**


See EPAPS Document No. E-HJFOA5-3-006904 for supplemental material. This document can be reached through a direct link in the online article’s HTML reference section or via the EPAPS homepage (http://www.aip.org/pubservs/epaps.html).
Liu, T, Sims, D, and Baum, B (2009). “Parallel RNAi screens across different cell lines identify generic and cell-type specific regulators of actin organisation and cell morphology.” Genome Biol. 10, R26.