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# Regulation of cofilin phosphorylation and asymmetry in collective cell migration during morphogenesis

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## SUMMARY

During *Drosophila* oogenesis, two actin dynamics regulators, cofilin and Rac, are required for the collective migration of a coherent cluster of cells called border cells. Cell culture data have shown that Rac and cofilin are both essential for lamellipodium formation, but Rac signaling results in phosphorylation and hence inactivation of cofilin. So it remains unclear whether cofilin phosphorylation plays a promoting or inhibitory role during cell migration. We show here that cofilin is required for F-actin turnover and lamellipodial protrusion in the border cells. Interestingly, reducing the dosage of cofilin by half or expressing a phospho-mimetic mutant form, S3E, partially rescues the migration and protrusion defects of Rac-deficient border cells. Moreover, cofilin exhibits moderate accumulation in border cells at the migratory front of the cluster, whereas phospho-cofilin has a robust and uniform distribution pattern in all the outer border cells. Blocking or overactivating Rac signaling in border cells greatly reduces or increases cofilin phosphorylation, respectively, and each abolishes cell migration. Furthermore, Rac may signal through Pak and LIMK to result in uniform phosphorylation of cofilin in all the outer border cells, whereas the guidance receptor Pvr (PDGF/VEGF receptor) mediates the asymmetric localization of cofilin in the cluster but does not affect its phosphorylation. Our study provides one of the first models of how cofilin functions and is regulated in the collective migration of a group of cells in vivo.

**KEY WORDS: Cofilin (Twinstar), Rac, Pvr, Cell migration, Border cells, Chemotaxis, *Drosophila***

## INTRODUCTION

Chemotaxis, or guided cell migration, is vital for a spectrum of physiological and pathological events, including embryogenesis, immune response, wound healing and tumor metastasis. One of the first steps in chemotaxis is directional sensing in which cells sense an extracellular gradient and, in response, send out a lamellipodial protrusion in a directional fashion (Devreotes and Janetopoulos, 2003; Sidani et al., 2007). Cofilin is one of the key actin dynamics-promoting factors that have been shown to control the protrusion process (DesMarais et al., 2004; Kiuchi et al., 2007). Recent biochemical and cell culture studies indicate that the dual F-actin severing and depolymerizing activities of cofilin not only generate free barbed ends that provide a burst of actin polymerization essential for directed lamellipodium formation in chemotaxis, but can also quickly replenish the dwindling monomeric actin pool, which ensures continuous actin filament extension and thus lamellipodial protrusion (Carlier et al., 1997; Wang et al., 2007). Furthermore, it has been shown that localized active cofilin may determine the direction of migrating tumor cells in vitro (Ghosh et al., 2004; Mouneimne et al., 2006). The small GTPase Rac is another key actin dynamics regulator that is known to promote lamellipodial extension during chemotaxis (Burridge and Wennerberg, 2004; Heasman and Ridley, 2008). Rac can signal downstream to Pak, which in turn phosphorylates and activates LIM kinases (LIMKs). Members of the LIMK family (which include LIMK1/2, TESK1/2) can inactivate the F-actin binding and depolymerizing activities of cofilin by phosphorylating its serine 3 residue, which is the major mode of regulation of cofilin activity (Bamburg, 1999). Members of the Slingshot family (SSH) and Chronophin (CIN) are two types of phosphatases that have been shown to dephosphorylate and thus reactivate cofilin in various cellular contexts (Gohla et al., 2005; Huang et al., 2008; Kligys et al., 2007; Niwa et al., 2002).

This evidence suggests that Rac might act through cofilin to determine the migration direction and promote lamellipodial protrusion during chemotaxis. However, one puzzling fact is that the end result of Rac signaling would be phosphorylation of cofilin at serine 3, which inhibits cofilin activity. Moreover, recent studies have reported that cofilin phosphorylation is increased in migrating tumor cells that are stimulated by chemokines or growth factors (Mouneimne et al., 2004; Nishita et al., 2005), whereas other studies have shown that stimulation with growth factors or chemotactic agents induces dephosphorylation of cofilin in various cell lines (Meberg et al., 1998; Suzuki et al., 1995). Therefore, it is still unclear what role cofilin phosphorylation plays in cell migration and chemotaxis.

The majority of cofilin functional studies in cell migration and chemotaxis have focused on various tumor or non-tumor cell lines, but conclusions from these studies need to be corroborated with in vivo data from model organisms. Border cell (BC) migration in the *Drosophila* ovary is a genetically tractable system with several unique features that are conducive to studies of chemotaxis (Fig. 1A) (Montell, 2003). First, epithelia-derived BCs exhibit a tumor-like invasive migration through germline-derived nurse cells. Second, BCs migrate in a highly directional fashion as a coherent cluster: beginning from the anterior end of an early stage-9 egg chamber, they migrate ~150 μm posteriorly and stop at the border between the nurse cells and oocyte by early stage 10 (Fig. 1A,D).

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Therefore, BCs can serve as a good model for the collective migration of a group of cells. Third, BCs chemotax through tissues using oocyte-secreted growth factors such as Pvf (PDGF/VEGF homolog) and Egfr ligands as attractive guidance signals (Duchek et al., 2001; McDonald et al., 2006).

We have previously shown that *twinstar* (*tsr*), the gene encoding the only cofilin homolog known in *Drosophila*, is required for BC migration (Chen et al., 2001). Here, we demonstrate that cofilin is required for actin turnover and lamellipodial protrusion of BCs. One of the functions of Rac signaling, which is mediated by Pak and LIMK, is to ensure a uniform cofilin phosphorylation pattern in BCs, whereas the guidance receptor Pvr induces an increase in cofilin protein levels at the front of the cluster.

## MATERIALS AND METHODS

### *Drosophila* genetics

All fly stocks were obtained from the Bloomington *Drosophila* Stock Center, except for the following: *slbo-Gal4,UAS-DNPVR/Cyo* [gift of Denise Montell (Prasad and Montell, 2007)], *UAS-DNDER/Cyo* [gift of Pernile Rorth (Duchek et al., 2001)], $tsr^{ntf}$/*Cyo*, $tsr^{1}$/*TSTL* and $tsr^{null}$/*TSTL* ($tsr^{\Delta 96}$/*TSTL*) (Chen et al., 2001). To perform flip-out experiments, *UAS-tsrWT (UAS-tsrS3A* or *UAS-tsrS3E);AyGAL4 UAS-GFP* was crossed to *hs-Flp*. Newly eclosed progeny were heat shocked at 37°C for 5 minutes, transferred to fresh food with yeast, and dissected after 1-2 days. BC clusters with 1, 2 or 3 cells per clone were counted for each genotype and their clone size ratios found to be very similar: for the GFP control, 53:31:16%; for tsrWT, tsrS3A and tsrS3E, the ratios were 51:33:15%, 53:28:19% and 52:35:13%, respectively.

### Immunostaining and microscopy

Ovary dissection was carried out in PBS and ovaries then fixed in a 1:6 devitellinizing buffer (7% formaldehyde):heptane (Sigma) mix for 10 minutes. After washes in PBS, ovaries were incubated in blocking solution [PBT (PBS with 0.3% Triton X-100) containing 10% goat serum] for 30 minutes and then stained overnight at 4°C. Primary antibodies were as follows: rabbit anti-cofilin, rabbit anti-phospho-cofilin (1:100, Signalway Antibody) and mouse anti-Armadillo (1:100, Developmental Studies Hybridoma Bank). The total cofilin and phospho-cofilin antibodies were raised against the non-phosphorylated and phosphorylated versions of the highly conserved N-terminal epitope MASGVAVSD and MAS$^{(p)}$GVAVSD. After extensive washes, Cy5 goat anti-rabbit and Cy3 goat anti-mouse (Jackson ImmunoResearch) secondary antibodies (1:100) were incubated with ovaries for 2 hours at room temperature. TRITC-conjugated phalloidin was used to visualize the actin cytoskeleton (1:100, Sigma). Confocal images were obtained with a Leica TSL SL confocal microscope and fluorescent images taken with an Olympus BX51 microscope. Fluorescence intensity was quantitated using Image J (NIH); details are given in the legends of Figs 3 and 5.

### Latrunculin A treatment

Ovaries were dissected and incubated in Schneider's medium cocktail, prepared as described previously (Prasad et al., 2007). After dissection, egg chambers were incubated in Latrunculin A (2 μM, dissolved in Schneider's medium cocktail) at room temperature for 30 minutes before fixation. Controls were treated in parallel with an equal volume of DMSO instead of Latrunculin A.

## RESULTS

### Cofilin is required for actin turnover and lamellipodial protrusion of BCs

Both our previous work and this study show that partial loss-of-function *tsr* mutants display a severe block in BC migration, with some failing to migrate at all, whereas others prematurely stop in the middle of the migration route (Fig. 1E,F) (Chen et al., 2001). In rare cases, mutant BCs may take a different path and travel a short distance along the surface of the egg chamber, suggesting a defect in directional sensing (Fig. 1G). In addition, mutant BC clusters that are able to initiate migration but experience a migration delay or arrest display a much less polarized and more rounded morphology than wild-type clusters, and the leading edge has no distinct shape and is not tapered (Fig. 1L-N′).

F-actin levels were substantially increased in the mutant BCs, but not in a uniform manner, with very strong F-actin staining largely localized near the outer cortical region of migratory BCs and especially at those regions that abut the nurse cells and could potentially form protrusions (Fig. 1L,M,N). By contrast, the increase in F-actin was very mild in the two central polar cells within the cluster that do not migrate but are pulled along by the

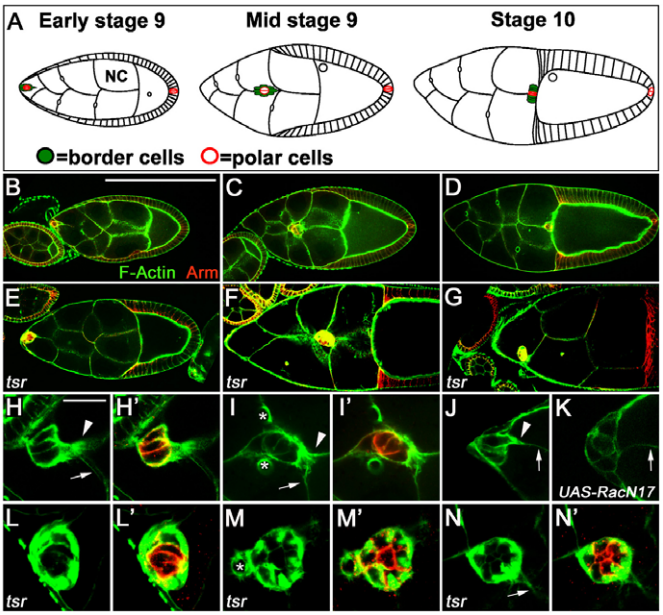


**Fig. 1. *Drosophila* cofilin is required for actin turnover, lamellipodial protrusion and polarization of BCs during migration.** (**A**) Diagram of border cell (BC) migration. NC, nurse cells. (**B-G**) Low-magnification views of BC clusters stained with TRITC-phalloidin (green), which detects F-actin, and with anti-Arm antibody (red), which detects β-catenin. (B-D) Wild-type BC clusters in early stage-9 (B), mid stage-9 (C) and stage-10 (D) egg chambers. (E-G) *twinstar* (*tsr*) mutant ($tsr^{ntf}$/$tsr^{1}$) BCs at late stage 9 (E) and stage 10 (F,G). (**H-N′**) High-magnification images of wild-type (H-J) and *tsr* mutant (L-N′) BC clusters at early (H,H′,L,L′) or mid (I,I′,M-N′) stage 9. Lamellipodial protrusions at the leading edge (arrowheads) and ring canals (asterisks) are indicated, and membrane actin staining between nurse cells (arrows) is indicated to distinguish them from lamellipodia. (K) RacN17 BCs (late stage 9) lack F-actin asymmetry and fail to invade and extend the leading lamellipodial protrusion between nurse cells, which is seen in wild-type BCs (J) at the anterior tip at early stage 9. (L-N′) *tsr* mutant BCs display substantially increased F-actin levels compared with wild-type BCs (H-J). The images in L-N′ were taken at a lower exposure than those in H-J to avoid overexposure of phalloidin staining and to reveal the subcellular details of F-actin accumulation, which is most dramatic in the outer BCs. (L-N′) The two polar cells located in the center of the BC cluster display the least increase in F-actin levels. In outer BCs, F-actin accumulation is mainly cortical and is more pronounced near the cortical regions abutting the nurse cells than at the cell-cell (both outer BC-outer BC and outer BC-polar cell) contact regions. For this and subsequent figures, anterior is to the left and posterior to the right, and the direction of migration is to the right. Scale bars: 200 μm in B; 20 μm in H.

DEVELOPMENT

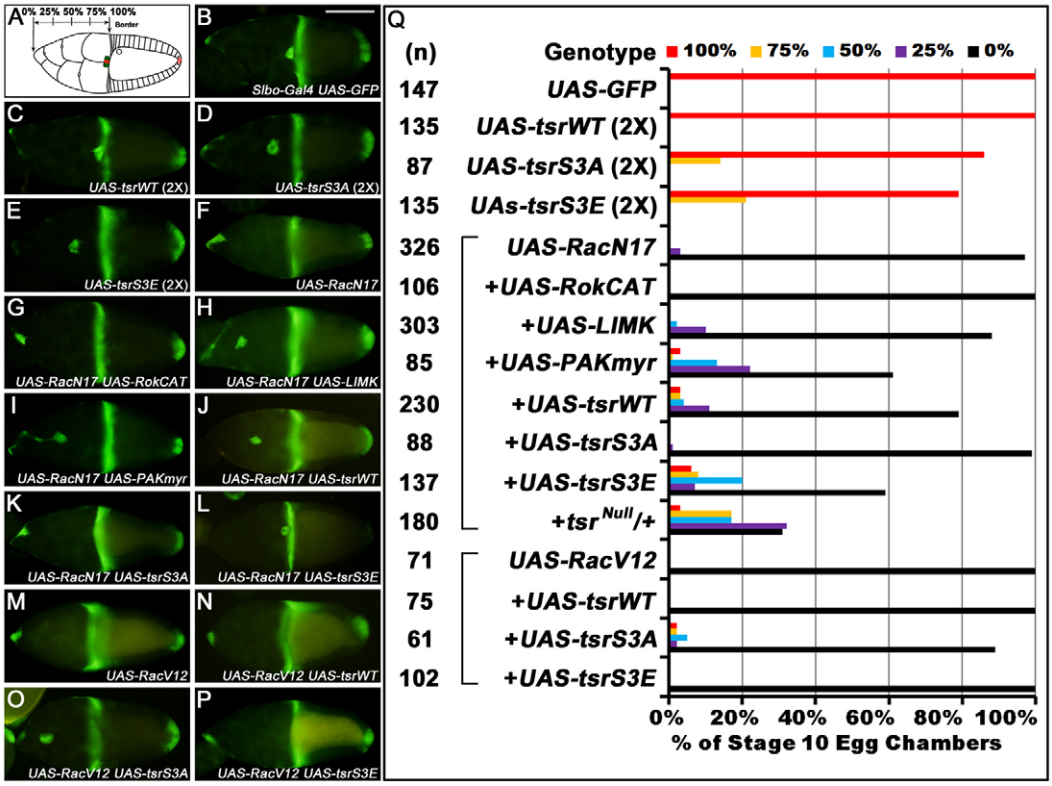


**Fig. 2. Genetic rescue of migration defects of RacN17 and RacV12 BCs by various *tsr* transgenes.** (**A**) The extent of migration for all stage-10 egg chambers examined was categorized as 0% (no migration), 25%, 50%, 75% or 100% (reaching the border) for all subsequent quantitative analyses of BC migration. (**B-P**) Low-magnification images of BC clusters in stage-10 egg chambers labeled by GFP; the border is also marked by GFP. Representative images show BCs expressing the various transgenes indicated. Scale bar: 100 μm. (**Q**) Quantitation of BC migration. The *x*-axis denotes the percentage of stage-10 egg chambers examined for each genotype that exhibited each degree of migration, as represented by the five color-coded bars for each genotype. For example, 96% of RacN17 BCs exhibit 0% migration (black bar) and the other 4% of BC clusters show 25% migration (purple bar). The number of egg chambers examined for each genotype is given (n).

outer BCs (Fig. 1A,L,M,N). This result suggests that cofilin not only functions to generally promote F-actin turnover and thus provide a certain level of actin dynamics throughout BCs, but also acts specifically at the outer cortical region to provide an environment of high actin dynamics that is conducive to lamellipodial protrusion. The highly excessive F-actin accumulation in cortical regions reveals a particularly high local rate of actin polymerization, given that the counteracting effect of actin depolymerization is largely removed due to the *tsr* mutation. Furthermore, the normal polarized distribution pattern of F-actin [higher at the front (leading edge) than at the back; see Fig. 1H-J] was mostly missing and the lamellipodial protrusion at the leading edge was very short and sometimes absent altogether (Fig. 1L-N′).

## Cofilin may function downstream of Rac during BC migration

Rac has also been shown to be required for BC migration. Previous work by others, as well as our study here, has shown that expressing a dominant-negative form of Rac, RacN17, from a BC-specific *slbo-Gal4* driver almost completely inhibits migration, with ~96% of BC clusters failing to initiate migration, whereas the other 4% exhibit a migration delay or arrest phenotype (Fig. 1K, Fig. 2F,Q) (Geisbrecht and Montell, 2004). These migration defects have been confirmed using Rac loss-of-function alleles (Geisbrecht and Montell, 2004).

To test whether cofilin acts downstream of Rac signaling to promote BC migration, we performed a series of overexpression experiments to determine which form of cofilin can rescue the RacN17 defects. The results indicated that the phospho-mimetic and dominant-negative mutant form of cofilin (tsrS3E), with the serine at position 3 changed to glutamate, display better rescuing ability than wild-type cofilin (tsrWT) or the constitutively active form (tsrS3A) with serine 3 changed to alanine. BCs expressing both tsrS3E and RacN17 initiated migration in 41% of stage-10 egg chambers, compared with 4%, 21% or 1% in those that expressed RacN17 alone, RacN17 and tsrWT, or RacN17 and tsrS3A, respectively (Fig. 2L,F,J,K,Q). Furthermore, reducing the endogenous dosage of cofilin by heterozygosity of $tsr^{null}$ ($tsr^{null}$/+) resulted in even stronger rescue of RacN17 migration defects (Fig. 2Q), consistent with the effect of the dominant-negative tsrS3E. $tsr^{null}$/+ BCs expressing RacN17 initiated migration in 69% of stage-10 egg chambers, whereas $tsr^{null}$/+ BCs themselves displayed no migration defects (data not shown).

Expressing a constitutively active form of Rac (RacV12) resulted in a more severe defect than RacN17, with migration completely blocked in all stage-10 egg chambers examined (Fig. 2M,Q) (Duchek et al., 2001). RacV12 rescue experiments using the above set of *tsr* constructs showed that the *tsrS3A* transgene had a moderate rescuing effect, whereas the *tsrWT* and *tsrS3E* transgenes had no detectable rescuing activity (Fig. 2M-Q). The emerging trend from these RacV12 rescue results (*tsrS3A*>*tsrWT*,*tsrSE*) is

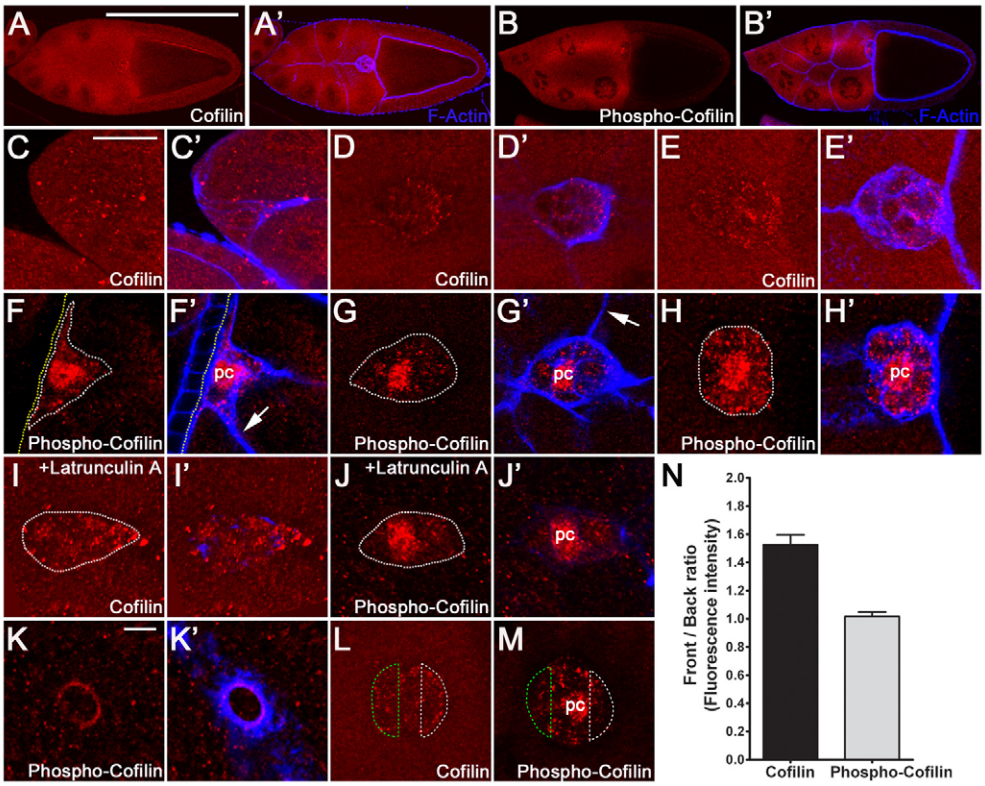


**Fig. 3. The distribution patterns of p-cofilin and total cofilin in wild-type BC clusters.** (**A-B′**) Low-magnification views of stage-10 egg chambers stained for total (A,A′) or phosphorylated (p-) cofilin (B,B′). (**C-H′**) High-magnification views of total cofilin (C-E′) and p-cofilin (F-H′) distribution within individual BC clusters that are initiating migration at early stage 9 (C,C′,F,F′), undergoing migration at the 50% position at mid stage 9 (D,D′,G,G′), or just reaching the border at late stage 9 (E,E′,H,H′). The outline of clusters (white dotted line), anterior boundary of egg chamber (yellow dotted line), nurse cell actin (arrow) and polar cells (pc) are indicated. (**I-J′**) Patterns and levels of total cofilin and p-cofilin are unaffected by treatment with the F-actin-destabilizing drug Latrunculin A. (**K**,**K′**) p-cofilin (red) localizes to the inner region of a ring canal. (**L-N**) Quantitation of fluorescence intensity for total cofilin (L) or p-cofilin (M) staining in the front and back regions of BC clusters. An area around the leading edge of the cluster, but excluding polar cells, was chosen as the front region (white dotted line), and an area including the lagging end and excluding polar cells was chosen as the back region (green dotted line). The original image was first processed in Photoshop (Adobe) to subtract the background region of interest and then thresholded and converted to a binary image in Image J. Fluorescence intensity (FI; the integrated density in each image) and area were measured in Image J for each region; the front/back ratios were calculated as [front FI/front area] divided by [back FI/back area] and the values plotted (N). Error bars indicate s.e.m. of 20 BC clusters. Scale bars: 200 μm in A; 20 μm in C; 5 μm in K.

consistent with there being an opposite trend from the RacN17 results (*tsrS3E>tsrWT>tsrS3A*). In addition, we quantified the leading edge protrusion of each BC cluster extending between two nurse cells for all the transgene combinations above. The results indicated that the ability of each transgene to rescue RacN17 or RacV12 leading protrusions corresponded to its ability to rescue RacN17 or RacV12 migration defects (see Fig. 5D).

Taken together, the above data suggest that *Drosophila* cofilin functions downstream of Rac and that one of the roles of Rac signaling during BC migration might be to ensure a proper level of phosphorylation and thus inhibition of cofilin activity.

## Rac is required for uniform phosphorylation but not asymmetric accumulation of cofilin in the outer BCs during migration

To better understand the role of cofilin phosphorylation in BC migration, wild-type migrating BCs were stained with two antibodies that detect phospho-cofilin (p-cofilin) or total cofilin (i.e. both cofilin and p-cofilin) (see Figs S1 and S2 in the supplementary material). In stage 9-10 egg chambers, total cofilin staining was strong in nurse cells and follicle epithelium, whereas p-cofilin stained strongly in the nurse cell cytoplasm and ring canals but weakly in follicle epithelium (Fig. 3A-B′,K,K′). Staining of migrating BCs showed that both total and p-cofilin were present at high levels but in distinct patterns. During the course of posterior migration, total cofilin exhibited a moderately asymmetric staining pattern, such that the leading edge of the BC cluster had more staining than the lagging end and the staining sometimes localized near the proximal (towards the cell body) region of the leading edge (Fig. 3C-E′). Measurements of signal intensity of front and back regions within individual BC clusters indicated a front/back ratio of 1.52±0.07 (*n*=20) for total cofilin (Fig. 3L,N). By contrast, p-cofilin staining was uniform throughout the cytoplasm of BCs and they lacked the asymmetric pattern exhibited in total cofilin stainings (Fig. 3F-H′); the front/back ratio was 1.01±0.02 (*n*=20; Fig. 3M,N). In addition, p-cofilin staining was especially high in the two non-migratory polar cells within the cluster (Fig. 3F-H′).

We then examined whether blocking Rac signaling by expressing RacN17 would reduce the phosphorylation level of cofilin in BCs. Indeed, expressing RacN17 in BCs from *slbo-Gal4* dramatically reduced the p-cofilin staining, as compared with that of wild-type BCs (Fig. 4B,D,H). Furthermore, because *slbo-Gal4*

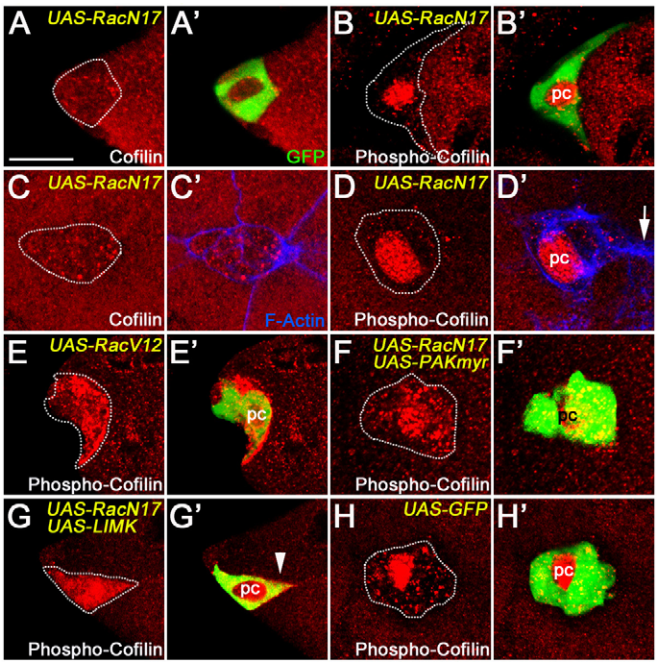


**Fig. 4. Rac signaling promotes global cofilin phosphorylation but not asymmetry of distribution.** (**A-D′**) RacN17-expressing BCs display an unchanged asymmetric staining pattern of total cofilin (A,C) but a greatly reduced p-cofilin level (B,D). The arrow indicates nurse cell actin. (**E**,**E′**) Large increase in p-cofilin staining in RacV12 BCs. (**F**,**F′**) The *Pak-myr* transgene restores p-cofilin to wild-type levels in RacN17 BCs. (**G**,**G′**) *LIMK* substantially increases p-cofilin levels and partially rescues the RacN17 lamellipodial protrusion (arrowhead) defects at 0% migration. (**H**,**H′**) Wild-type control expressing GFP, which labels the outer BCs but not the two central polar cells (pc). Scale bar: 20 μm.

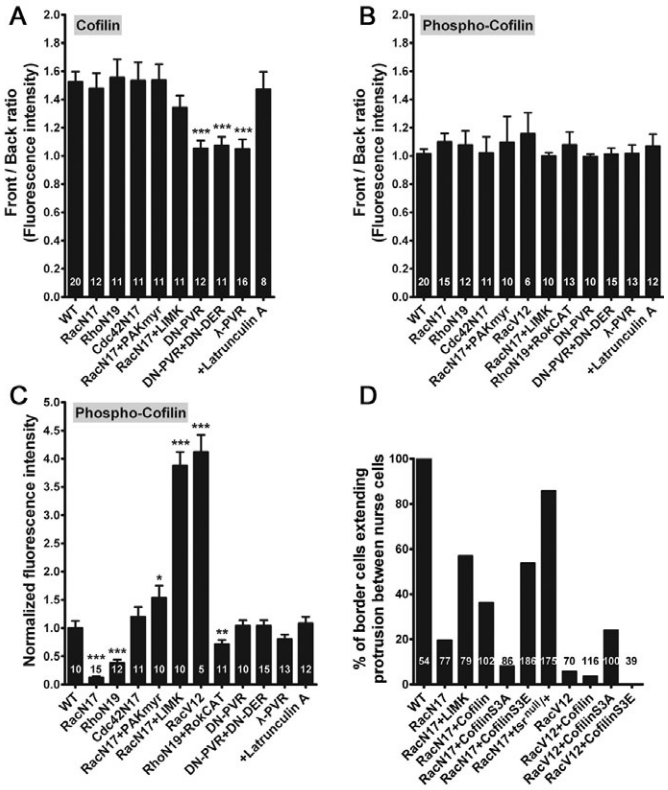


**Fig. 5. Quantitation of p-cofilin and total cofilin immunofluorescence intensity and BC lamellipodial protrusion.** (**A**,**B**) Fluorescence intensity (FI) was measured for cofilin (A) or p-cofilin (B) in both front and back regions of BC clusters (genotypes and treatments shown on the *x*-axis), and the values were used to calculate the front/back ratios, as in Fig. 3N. The number of BC clusters measured is indicated within each bar; error bars indicate s.e.m. (**C**) FI of p-cofilin measured for each BC cluster, except for the polar cells; the FI of adjacent nurse cells (chosen for their uniform levels) was also measured and used for normalization of the FI for each BC cluster. (**D**) Leading edge protrusion quantitated for BC clusters of various genotypes. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

only drives expression in the outer BCs and not in the central polar cells, the polar cells still retained very high levels of staining, in contrast to the much reduced levels of p-cofilin staining in the surrounding outer BCs (Fig. 4B,B′,D,D′). Interestingly, the cofilin accumulation at the front was unchanged in migration-blocked or delayed RacN17 BCs (Fig. 4A,C). If Rac signaling results in cofilin phosphorylation, overactivating Rac should increase the p-cofilin level in BCs. Indeed, we observed a large increase in p-cofilin staining in BCs expressing RacV12, such that p-cofilin staining in outer BCs was comparable to that in polar cells (Fig. 4E).

Finally, to rule out the possibility that phosphorylation of cofilin is a secondary consequence of changes in the actin cytoskeleton resulting from Rac deficiency, we tested the effect of the F-actin-destabilizing drug Latrunculin A (Lat-A) on the levels and localization of cofilin and p-cofilin. Lat-A treatment induced a drastic reduction of overall F-actin levels in BCs, whereas p-cofilin levels and cofilin or p-cofilin distribution patterns were virtually unaffected (Fig. 3I-J′, Fig. 5A-C), indicating that changes in p-cofilin levels and cofilin asymmetry are not secondary consequences of changes in the actin cytoskeleton. Taken together, the above results show that Rac signaling promotes robust and uniform cofilin phosphorylation throughout the outer BCs.

## Pvr activity is required for cofilin accumulation but not for its phosphorylation

We next explored which signaling might be responsible for the increase in cofilin protein levels at the front of the cluster. Pvr and Egfr are two receptor tyrosine kinases (RTKs) that have been reported to act redundantly as guidance receptors for BCs: their ligands were shown to be secreted from the oocyte and guide BCs to migrate posteriorly towards the border (between oocyte and nurse cells) along their concentration gradients (Montell, 2003). BCs expressing dominant-negative forms of Pvr (DN-PVR) and Egfr (DN-DER) exhibit severe defects in chemotactic migration (Duchek et al., 2001) (Fig. 6D-D″). Interestingly, BCs expressing both DN-PVR and DN-DER completely lose the asymmetric accumulation of total cofilin (its front/back ratio was 1.07±0.06; $n$=11; $P<0.001$), as compared with wild type (1.52±0.07; $n$=20) (Fig. 5A, Fig. 6C-D″). In addition, we found that Pvr alone is sufficient to mediate this asymmetry, as expressing DN-PVR alone in BCs also resulted in complete loss of cofilin asymmetry (1.05±0.05; $n$=12; $P<0.001$) (Fig. 5A, Fig. 6B). Moreover, p-cofilin levels and distribution in BCs expressing DN-PVR or DN-PVR and DN-DER were unaffected (Fig. 5B,C, Fig. 6F-G″), suggesting that Pvr and Egfr do not regulate cofilin phosphorylation. Levels of total cofilin in BCs expressing DN-PVR or DN-PVR and DN-EGFR were similar to those of the wild type (see Fig. S3 in the supplementary material), suggesting that the guidance receptors regulate the localization, rather than the total level, of cofilin. This was confirmed by the complete loss of cofilin

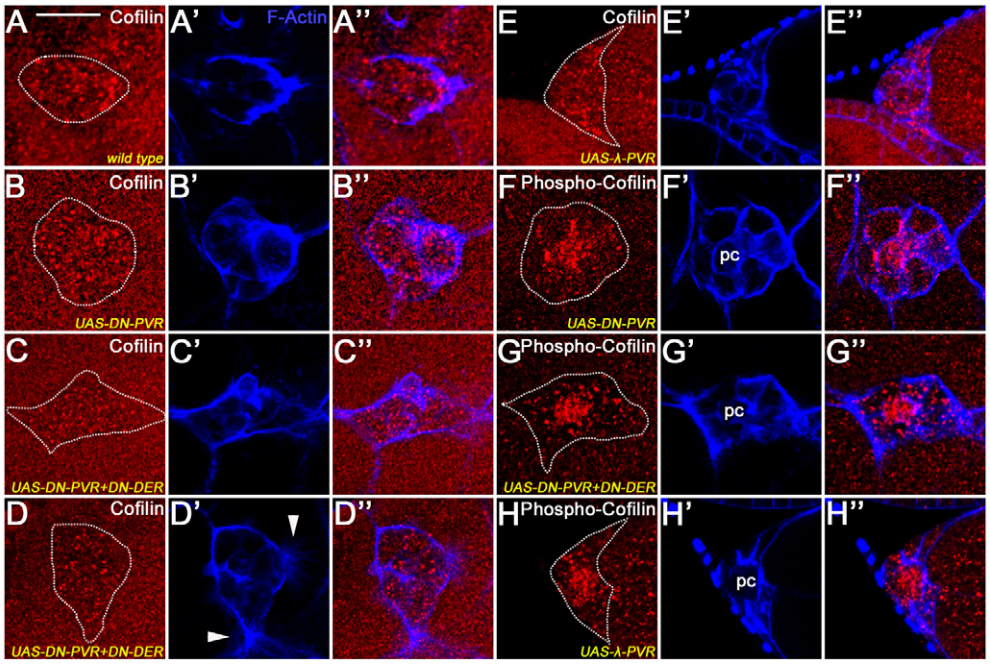


**Fig. 6. Pvr activity is required for cofilin asymmetry but not phosphorylation.** (**A-E″**) The asymmetric localization of total cofilin in the leading edge, as observed in the wild type (A-A″), is abolished in BCs expressing DN-PVR and DN-DER (C-D″), DN-PVR (B-B″) or λ-PVR (E-E″). Two predominant protrusions are marked in D′ (arrowheads) to indicate loss of direction. (**F-H″**) p-cofilin levels remain unchanged in outer BCs expressing DN-PVR and DN-DER (G-G″), DN-PVR (F-F″) or λ-PVR (H-H″). Scale bar: 20 μm.

asymmetry (Fig. 5A, Fig. 6E-E″) but unaltered levels of p-cofilin (Fig. 5C, Fig. 6H-H″) and total cofilin (see Fig. S3 in the supplementary material) observed in BCs expressing λ-PVR, a constitutively active form of Pvr, supporting the notion that only localized Pvr activity can result in an asymmetric cofilin distribution. Finally, the evidence that overactivation of Pvr results in no increase in total cofilin levels implies that Pvr signaling regulates cofilin not at the level of transcription/translation, but at the post-translational level.

## Effects of altering cofilin levels in all outer BCs

Our observations of uniform cofilin phosphorylation and asymmetric accumulation patterns suggest that the activity and amount of cofilin need to be closely regulated in BCs during chemotactic migration of the whole cluster. Indeed, we showed above that the substantial reduction of cofilin levels in *tsr* mutants results in severe migration defects that are associated with very short, or almost no, protrusions at the leading edge. We examined whether the alteration in cofilin levels or activity throughout the migratory outer BCs would have any effects on their migration or lamellipodial protrusions. Detailed analyses of *z*-series of confocal sections and their projected images for individual BC clusters revealed that a typical wild-type BC cluster extended, on average, 4.33±0.67 ($n$=6) protrusions of significant size, including a predominant protrusion at the leading edge with a length of 16.12±2.98 μm ($n$=6; Fig. 7J) and three shorter protrusions with an average length of 6.11±0.76 μm ($n$=20; Fig. 7K). Upregulation of the levels of wild-type cofilin in all outer BCs, using *slbo-Gal4* to drive expression of *tsrWT*, resulted in no obvious migration delay (Fig. 2Q), but caused each cluster to send significantly more non-leading protrusions than in the wild type. The cofilin-overexpressing cluster on average extended 7.50±0.50 protrusions ($n$=8; Fig. 7I), including one or two predominant protrusions at the leading edge of 8.37±0.78 μm ($n$=14; Fig. 7J) and about six other non-leading protrusions with an average length of 4.83±0.40 μm ($n$=50; Fig. 7K).

Similar effects were observed in S3A-overexpressing clusters (Fig. 7I-K; see Fig. S4 in the supplementary material), but unlike cofilin overexpression, a mild migration delay was apparent (Fig. 2Q), suggesting that phosphorylation of serine 3 is important for cofilin function. S3E overexpression also resulted in mild migration defects, with each cluster extending a similar number of protrusions, on average, as the wild type (4.87±0.85, $n$=8; Fig. 7I), but the predominant leading protrusion was substantially reduced in size (6.24±1.36 μm; $n$=12; $P$<0.01; Fig. 7J) and the non-leading protrusions were significantly shorter than those of the wild type (4.10±0.43 μm, $n$=40; $P$<0.05).

## Local alteration of cofilin levels in a small clone of BCs

We have shown that more cofilin is present in the 2-3 cells that occupy the leading position than in cells in other positions within a BC cluster, and this asymmetry requires the activity of the guidance receptor Pvr. To test whether local accumulation of cofilin plays an active role in determining which BCs within the cluster extend the predominant protrusion and thus set the direction for migration, we altered the amount or activity of cofilin in a subset of BCs within an otherwise wild-type cluster, using the genetic flip-out technique to drive expression of *tsrWT*, *tsrS3A* or *tsrS3E* in a small clone of 1-3 cells. Because wild-type BC clusters undergo active rotation during migration and, as a result, each BC can take its turn to lead the entire cluster (Bianco et al., 2007; Prasad and Montell, 2007), this local up- or downregulation of cofilin activity could bias whether the clone of 1-3 BCs becomes the leading cells. The wild-type control showed that cells in clones expressing only the GFP marker became leading cells in 40% of mosaic clusters ($n$=45), whereas clones overexpressing wild-type cofilin, the S3A form and the S3E form lead in 51% ($n$=39), 41% ($n$=32) and 26% ($n$=23) of mosaic clusters, respectively (Fig. 8). These results are consistent with the predictions for wild-type cofilin and S3E, but not for S3A, which did not show a significant increase in leading over controls, suggesting that cofilin needs to go through

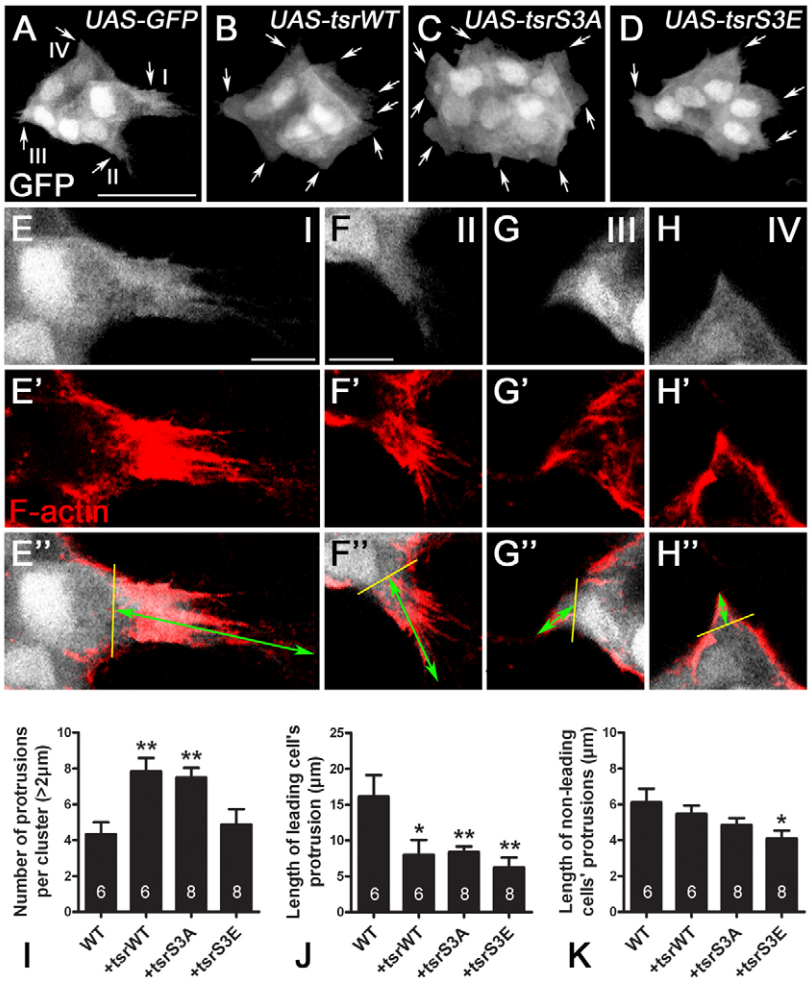


**Fig. 7. Global up- or downregulation of cofilin activity affects the number and length of lamellipodial protrusions.** (**A-D**) A *z*-series of ~25 confocal sections was taken for each BC cluster and an average projection performed for each *z*-series. The projected images show protrusions (arrows) in control BCs (A) and those expressing *tsrWT* (B), *tsrS3A* (C) and *tsrS3E* (D). (**E-H″**) Single confocal sections showing enlarged images of the actin-rich and GFP-marked leading edge protrusion (E-E″) and three non-leading protrusions (F-H″) labeled in A. The yellow line marks the boundary between protrusion and cell body; the green arrowhead indicates the length of each protrusion. (**I-K**) The number and length of protrusions (longer than 2 μm) were measured and quantitated for 6-8 clusters of each genotype. Error bars indicate s.e.m. *, $P<0.05$; **, $P<0.01$.

dynamic cycles of phosphorylation and dephosphorylation on serine 3 to be most effective, as previously proposed (Chen et al., 2000).

## Rac may act through Pak and LIMK to regulate BC migration

It is known that Rac can signal through Pak and then LIMK to remodel the actin cytoskeleton during cell migration (Burridge and Wennerberg, 2004). To test whether this signaling pathway is conserved in BC migration, the RacN17 rescue experiment was repeated by overexpressing *Drosophila* Pak and LIMK. The *Pak-myr* transgene (which expresses a membrane-tethered form of Pak) displayed a strong rescuing activity on the RacN17 BC migration phenotype, whereas *LIMK* showed a moderate rescuing effect (Fig. 2H,I,Q). Significantly, an active form of Rok, a likely downstream signal transducer of Rho signaling, failed to rescue RacN17 migration defects but did rescue RhoN19 migration defects (Fig. 2G,Q and see Fig. S5F in the supplementary material), indicating specific genetic interactions between Rac and Pak. The percentage of BCs that initiate migration increased from 4% in RacN17 alone to 39% in RacN17 plus Pak and 12% in RacN17 plus LIMK (Fig. 2Q).

Consistently, cofilin phosphorylation was restored to wild-type levels in BCs expressing RacN17 and Pak (Fig. 4F,F′, Fig. 5C), but cells expressing RacN17 and LIMK displayed much increased levels of p-cofilin (3.9-fold, relative to wild-type levels; Fig. 4G,G′, Fig. 5C), which were comparable to the high levels induced by RacV12 (4.1-fold; Fig. 4E,E′, Fig. 5C). This result suggests that LIMK is less effective in rescue than Pak owing to overphosphorylation of cofilin by LIMK, in turn implicating that only a proper and regulated level of phosphorylation is beneficial to BC migration. Furthermore, a majority of the BC clusters expressing RacN17 and LIMK, including those that failed to completely delaminate from the anterior end and thus did not show migration rescue (Fig. 2Q, Fig. 4G′), displayed lamellipodial protrusion or invasion between two adjacent nurse cells (Fig. 4G′, Fig. 5D). By contrast, most RacN17-expressing BC clusters did not extend their lamellipodial protrusions between the nurse cells (Fig. 1K, Fig. 4B′, Fig. 5D). Thus, LIMK overexpression strongly rescues the lamellipodial extension phenotype of RacN17 during the initial phase of cell migration. Taken together, these results suggest that like mammalian migratory cells, *Drosophila* BCs employ a conserved Rac-Pak-LIMK-cofilin signaling pathway to promote directional cell migration.

## Rho but not Cdc42 signaling moderately promotes cofilin phosphorylation in BCs

Mammalian cell culture data also implicate cofilin as a likely target for Cdc42 or Rho signaling (Bamburg, 1999). Consistent with previous reports (Bastock and Strutt, 2007; Llense and Martin-Blanco, 2008), overexpressing dominant-negative forms of either

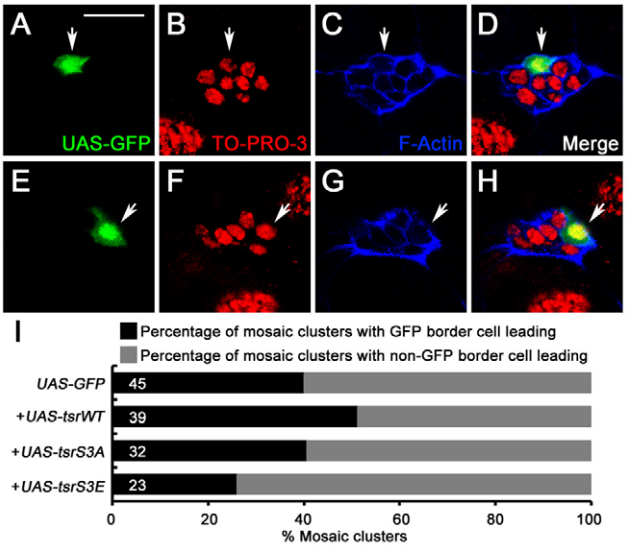


**Fig. 8. Local increases or decreases in cofilin levels in small clones bias them to become leading or non-leading cells, respectively, for BC clusters.** (**A-H**) Two examples of mosaic clusters containing a GFP-marked 1-cell clone at the leading (E-H) or non-leading (A-D) position. *Ay-Gal4/UAS-GFP,UAS* transgene generates a flip-out clone co-expressing GFP and the desired transgene within a wild-type cluster. (**I**) Mosaic clusters with clones expressing each transgene were scored for the percentage of flip-out clones occupying the leading position.

Cdc42 or Rho in BCs resulted in a migration defect that was much less severe than that of RacN17 (Fig. 2F,Q and see Fig. S5A,B,F in the supplementary material), with Cdc42N17 presenting the mildest phenotype of the three.

The three *tsr* constructs mentioned above were used to test whether cofilin could genetically rescue the dominant-negative Cdc42N17 or RhoN19 mutant phenotypes. We did not observe significant rescue by these *tsr* transgenes, with the exception that *tsrS3E* had a moderate rescuing effect on RhoN19 (see Fig. S5F in the supplementary material). Moreover, the p-cofilin level was not reduced in BCs expressing Cdc42N17 (see Fig. S5I-J′ in the supplementary material; quantitated in Fig. 5C), suggesting that Cdc42 signaling is not involved in setting up the uniform cofilin phosphorylation pattern. Interestingly, in those RhoN19 BCs that exhibited migration delay, a moderate reduction of p-cofilin (as compared with the dramatic reduction in RacN17 cells) was often observed (see Fig. S5L,L′ in the supplementary material; quantitated in Fig. 5C), and this reduction was partially rescued by an active form of Rok (RokCAT). However, compared with the degree of phosphorylation rescue achieved by LIMK and Pak-myr in RacN17-expressing BCs, the cofilin phosphorylation rescuing ability of RokCAT was only moderate (Fig. 5C). Together with the modest rescuing ability of *tsrS3E* on RhoN19, these results suggest that Rho signaling might partly contribute to uniform cofilin phosphorylation, albeit to a much lesser extent than Rac signaling.

## DISCUSSION

We have shown that cofilin is required for the lamellipodial protrusion and migration of BCs. The F-actin accumulation phenotype in the *tsr* mutant BCs suggests that cofilin promotes actin turnover both throughout BCs and specifically at the outer cortical region of the migratory outer BCs. When below wild-type levels, the amount of cofilin seems to positively correlate with the length of lamellipodial protrusions, as *tsr* mutations result in very short or no protrusions as well as severe migration defects, and expression of the weakly dominant-negative S3E form of cofilin leads to a significant reduction in the length of both leading and non-leading protrusions and in mild migration delays. Overexpression of cofilin or its active S3A form also results in significantly shorter protrusions of the leading edge, but no difference in non-leading protrusions, suggesting that above a certain threshold (i.e. wild-type) amount, too much cofilin activity could cause excessive actin turnover, possibly resulting in shorter actin filaments and thus shorter protrusions. These results suggest that cofilin activity has to be properly regulated, and that there is a need to tune the rate of actin depolymerization (turnover), as mediated by cofilin, to the endogenous rates of actin polymerization and nucleation to form protrusions of optimal length. Furthermore, we found that the amount of cofilin also positively correlates with the number of protrusions. We observed that wild-type BCs usually display one predominant protrusion at the front and about three minor protrusions at the lateral or back position of the cluster, whereas *tsr* mutations result in few or no protrusions. By contrast, overexpression of the wild-type or S3A form of cofilin throughout outer BCs causes increased numbers of minor protrusions, with only the S3A-expressing BCs presenting a mild migration defect (Fig. 2Q). We did not observe a reduction in the total number of protrusions in S3E-expressing BCs, probably owing to the moderate effect of S3E on knocking down cofilin activity, which only leads to a mild migration delay. These results suggest that ectopic cofilin activity could cause ectopic protrusions, explaining the need to limit cofilin activity throughout the BC cluster.

Indeed, our study shows that the migratory outer BCs exhibit a uniform cofilin phosphorylation pattern during the course of migration, and Rac is the major signaling pathway responsible for the phosphorylation. Partial rescue of the strong cofilin phosphorylation, migration and protrusion defects of RacN17 by S3E, Pak or LIMK further indicates that Rac partly promotes BC migration by signaling through Pak and LIMK to effect uniform cofilin phosphorylation. Interestingly, recent data suggest that there is a basal level of Rac activity throughout the BC cluster that is not induced by guidance receptor signaling from Pvr and Egfr (Wang et al., 2010), which is consistent with our data that the uniform phosphorylation of cofilin is not mediated by Pvr and Egfr. Furthermore, they found that there is a high level of Rac signaling at the leading edge of the BC cluster that is activated by Pvr and Egfr (Wang et al., 2010). In light of their data, the uniform cofilin phosphorylation we observed could be due to the uniform basal Rac signaling, which might be activated by some non-directional signal(s) as proposed in their study. Moreover, there needs to be some factor(s) at the leading edge to remove the excessive cofilin phosphorylation that could presumably result from high Rac activation at the front. Indeed, the cofilin phosphatases SSH and CIN, which are conserved across species, have been reported to be specifically localized at the leading edge of cultured mammalian cells (Gohla et al., 2005; Nagata-Ohashi et al., 2004). It remains to be determined whether SSH or CIN is localized at the leading edge of BC clusters to play such a role and whether its localization towards the front is effected by the guidance receptors PVR and EGFR.

However, the question remains as to why cofilin needs to be phosphorylated and thus inhibited in order to promote migration given that previous work, as well as this study, show that cofilin depolymerization activities are essential for the initiation and

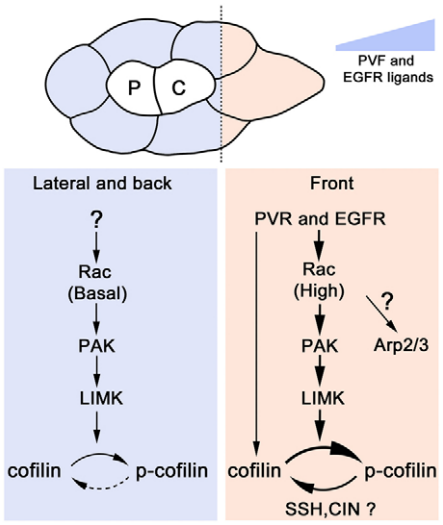


**Fig. 9. A model showing how cofilin functions and is regulated in the chemotactic migration of a BC cluster.** The pink region of the BC cluster shows the outer BCs at the front (leading edge) position, whereas light blue marks the lateral and back regions. The two central non-migratory cells are the polar cells (PC). In this model, non-directional signal(s) elicit a basal level of Rac signaling in the outer BCs that effects a uniform cofilin phosphorylation (through the mediators Pak and LIMK), which serves to inhibit and limit some of cofilin's activity in the bulk of the cluster. The extracellular gradient of guidance molecules activates PVR signaling, which in turn induces an increase in cofilin levels in the outer BCs at the leading edge. (See Discussion for more mechanistic details.)

continuous extension of lamellipodial protrusions. It was previously suggested that this paradox can be resolved by spatially separating active and inactive cofilin in a chemotactic cell or, in our study, in a cluster of cells, with active cofilin localized towards the cortical area and leading edge and inactive cofilin localized more towards the non-migratory region (Burridge and Wennerberg, 2004; Wang et al., 2007). Indeed, our results suggest that although cofilin is essential for lamellipodial protrusion and migration, its activity has to be limited to produce the optimal number and length of protrusions, and uniform phosphorylation of cofilin might serve to limit some of its activity throughout BCs. Our data showing that highly excessive F-actin tends to localize in mutant BCs at the outer cortical region abutting nurse cells suggest that cofilin activity might be much more essential in this potential protrusion-forming region than in the non-migratory regions (including the cell body of outer BCs and polar cells) to counteract, or keep pace with, the fast local actin polymerization rates. Furthermore, the finding that the guidance receptor Pvr mediates a moderate accumulation of cofilin in 2-3 cells around the leading edge is significant because it further supports the view that cofilin activity needs to be spatially separated within a single cluster of cells, with more cofilin present in cells at the migratory front.

Alternatively, a basal level of phosphorylation mediated by Rac signaling could be beneficial to actin dynamics near the cortical region by working together with phosphatases such as SSH and CIN to drive phosphocycling of cofilin, as previously proposed (Chen et al., 2000). In such a process, transient phosphorylation of cofilin within the cofilin-G-actin-ADP complex (resulting from depolymerization from the pointed ends of actin filaments) would dissociate cofilin from the complex and prevent it from binding to the complex again, allowing the monomeric G-actin-ADP to exchange its nucleotide to become G-actin-ATP, a form that can be readily polymerized onto actin filament barbed ends. Subsequent dephosphorylation of cofilin by phosphatase would then reactivate it to bind and depolymerize actin filaments, thus completing its phosphocyling and driving efficient actin dynamics. Indeed, we found that overexpression of cofilin in a small clone (1-3 cells) within a BC cluster significantly biases the cells within the clone to be the leading cells, whereas this effect is lost when the active S3A form of cofilin is overexpressed in the clone, suggesting that dynamic cycles of phosphorylation and dephosphorylation on serine 3 are required for cofilin to be most effective in promoting actin dynamics and sustaining lamellipodial protrusion at the leading edge.

Finally, our work provides one of the first working models of how cofilin functions and is regulated in the collective migration of a cluster of cells in vivo (Fig. 9). First, cofilin functions within individual BCs to provide a basal level of actin dynamics to limit excessive F-actin accumulation throughout the cell body. Second, cofilin acts at or near the outer cortical region (the potential protrusion-forming region) of outer BCs to specifically promote fast actin turnover and thus a high level of actin dynamics, possibly with the help of phosphatases including SSH and CIN. Third, non-directional signal(s) elicit a basal level of Rac signaling in the outer BCs that effects uniform cofilin phosphorylation (through the mediators Pak and LIMK), which serves to inhibit and limit some cofilin activity in the bulk of the cluster or to promote its phosphocycling and thus high actin dynamics at the cortical region with the help of SSH and CIN, or perhaps to perform both functions. It is noteworthy that Rac not only acts at the leading edge but also in cells at the lateral and back regions of the cluster to induce cofilin phosphorylation. Fourth, an extracellular gradient of Pvf activates Pvr signaling in the BCs near the leading edge, which in turn induces a moderate accumulation of cofilin in these cells, providing an asymmetry of cofilin localization in the context of a cluster. In addition, a previously reported high level of Rac activity that is responsive to strong guidance receptor signaling at the leading edge might cause substantial phosphorylation, which could be removed by high levels of SSH or CIN enriched at the leading edge to keep the overall p-cofilin levels in the leading cells similar to those at the lateral and back regions (Fig. 9). This could also result in increased phosphocycling of cofilin, which when coupled with the increased amount of cofilin and presumably increased actin polymerization (owing to elevated Rac signaling through Arp2/3) in the leading cells, could promote the extension of the prominent, long protrusion towards the direction of guidance signals.

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**Competing interests statement**
The authors declare no competing financial interests.

**Supplementary material**
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**References**
**Bamburg, J. R.** (1999). Proteins of the ADF/cofilin family: essential regulators of actin dynamics. *Annu. Rev. Cell Dev. Biol.* **15**, 185-230.
**Bastock, R. and Strutt, D.** (2007). The planar polarity pathway promotes coordinated cell migration during Drosophila oogenesis. *Development* **134**, 3055-3064.

**Bianco, A., Poukkula, M., Cliffe, A., Mathieu, J., Luque, C. M., Fulga, T. A. and Rorth, P.** (2007). Two distinct modes of guidance signalling during collective migration of border cells. *Nature* **448**, 362-365.
**Burridge, K. and Wennerberg, K.** (2004). Rho and Rac take center stage. *Cell* **116**, 167-179.
**Carlier, M. F., Laurent, V., Santolini, J., Melki, R., Didry, D., Xia, G. X., Hong, Y., Chua, N. H. and Pantaloni, D.** (1997). Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility. *J. Cell Biol.* **136**, 1307-1322.
**Chen, H., Bernstein, B. W. and Bamburg, J. R.** (2000). Regulating actin-filament dynamics in vivo. *Trends Biochem. Sci.* **25**, 19-23.
**Chen, J., Godt, D., Gunsalus, K., Kiss, I., Goldberg, M. and Laski, F. A.** (2001). Cofilin/ADF is required for cell motility during Drosophila ovary development and oogenesis. *Nat. Cell Biol.* **3**, 204-209.
**DesMarais, V., Macaluso, F., Condeelis, J. and Bailly, M.** (2004). Synergistic interaction between the Arp2/3 complex and cofilin drives stimulated lamellipod extension. *J. Cell Sci.* **117**, 3499-3510.
**Devreotes, P. and Janetopoulos, C.** (2003). Eukaryotic chemotaxis: distinctions between directional sensing and polarization. *J. Biol. Chem.* **278**, 20445-20448.
**Duchek, P., Somogyi, K., Jekely, G., Beccari, S. and Rorth, P.** (2001). Guidance of cell migration by the Drosophila PDGF/VEGF receptor. *Cell* **107**, 17-26.
**Geisbrecht, E. R. and Montell, D. J.** (2004). A role for Drosophila IAP1-mediated caspase inhibition in Rac-dependent cell migration. *Cell* **118**, 111-125.
**Ghosh, M., Song, X., Mouneimne, G., Sidani, M., Lawrence, D. S. and Condeelis, J. S.** (2004). Cofilin promotes actin polymerization and defines the direction of cell motility. *Science* **304**, 743-746.
**Gohla, A., Birkenfeld, J. and Bokoch, G. M.** (2005). Chronophin, a novel HAD-type serine protein phosphatase, regulates cofilin-dependent actin dynamics. *Nat. Cell Biol.* **7**, 21-29.
**Heasman, S. J. and Ridley, A. J.** (2008). Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nat. Rev. Mol. Cell Biol.* **9**, 690-701.
**Huang, T. Y., Minamide, L. S., Bamburg, J. R. and Bokoch, G. M.** (2008). Chronophin mediates an ATP-sensing mechanism for cofilin dephosphorylation and neuronal cofilin-actin rod formation. *Dev. Cell* **15**, 691-703.
**Kiuchi, T., Ohashi, K., Kurita, S. and Mizuno, K.** (2007). Cofilin promotes stimulus-induced lamellipodium formation by generating an abundant supply of actin monomers. *J. Cell Biol.* **177**, 465-476.
**Kligys, K., Claiborne, J. N., DeBiase, P. J., Hopkinson, S. B., Wu, Y., Mizuno, K. and Jones, J. C.** (2007). The slingshot family of phosphatases mediates Rac1 regulation of cofilin phosphorylation, laminin-332 organization, and motility behavior of keratinocytes. *J. Biol. Chem.* **282**, 32520-32528.
**Llense, F. and Martin-Blanco, E.** (2008). JNK signaling controls border cell cluster integrity and collective cell migration. *Curr. Biol.* **18**, 538-544.
**McDonald, J. A., Pinheiro, E. M., Kadlec, L., Schupbach, T. and Montell, D. J.** (2006). Multiple EGFR ligands participate in guiding migrating border cells. *Dev. Biol.* **296**, 94-103.
**Meberg, P. J., Ono, S., Minamide, L. S., Takahashi, M. and Bamburg, J. R.** (1998). Actin depolymerizing factor and cofilin phosphorylation dynamics: response to signals that regulate neurite extension. *Cell Motil. Cytoskeleton* **39**, 172-190.
**Montell, D. J.** (2003). Border-cell migration: the race is on. *Nat. Rev. Mol. Cell Biol.* **4**, 13-24.
**Mouneimne, G., Soon, L., DesMarais, V., Sidani, M., Song, X., Yip, S. C., Ghosh, M., Eddy, R., Backer, J. M. and Condeelis, J.** (2004). Phospholipase C and cofilin are required for carcinoma cell directionality in response to EGF stimulation. *J. Cell Biol.* **166**, 697-708.
**Mouneimne, G., DesMarais, V., Sidani, M., Scemes, E., Wang, W., Song, X., Eddy, R. and Condeelis, J.** (2006). Spatial and temporal control of cofilin activity is required for directional sensing during chemotaxis. *Curr. Biol.* **16**, 2193-2205.
**Nagata-Ohashi, K., Ohta, Y., Goto, K., Chiba, S., Mori, R., Nishita, M., Ohashi, K., Kousaka, K., Iwamatsu, A., Niwa, R. et al.** (2004). A pathway of neuregulin-induced activation of cofilin-phosphatase Slingshot and cofilin in lamellipodia. *J. Cell Biol.* **165**, 465-471.
**Nishita, M., Tomizawa, C., Yamamoto, M., Horita, Y., Ohashi, K. and Mizuno, K.** (2005). Spatial and temporal regulation of cofilin activity by LIM kinase and Slingshot is critical for directional cell migration. *J. Cell Biol.* **171**, 349-359.
**Niwa, R., Nagata-Ohashi, K., Takeichi, M., Mizuno, K. and Uemura, T.** (2002). Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. *Cell* **108**, 233-246.
**Prasad, M. and Montell, D. J.** (2007). Cellular and molecular mechanisms of border cell migration analyzed using time-lapse live-cell imaging. *Dev. Cell* **12**, 997-1005.
**Prasad, M., Jang, A. C., Starz-Gaiano, M., Melani, M. and Montell, D. J.** (2007). A protocol for culturing Drosophila melanogaster stage 9 egg chambers for live imaging. *Nat. Protoc.* **2**, 2467-2473.
**Sidani, M., Wessels, D., Mouneimne, G., Ghosh, M., Goswami, S., Sarmiento, C., Wang, W., Kuhl, S., El-Sibai, M., Backer, J. M. et al.** (2007). Cofilin determines the migration behavior and turning frequency of metastatic cancer cells. *J. Cell Biol.* **179**, 777-791.
**Suzuki, K., Yamaguchi, T., Tanaka, T., Kawanishi, T., Nishimaki-Mogami, T., Yamamoto, K., Tsuji, T., Irimura, T., Hayakawa, T. and Takahashi, A.** (1995). Activation induces dephosphorylation of cofilin and its translocation to plasma membranes in neutrophil-like differentiated HL-60 cells. *J. Biol. Chem.* **270**, 19551-19556.
**Wang, W., Eddy, R. and Condeelis, J.** (2007). The cofilin pathway in breast cancer invasion and metastasis. *Nat. Rev. Cancer* **7**, 429-440.
**Wang, X., He, L., Wu, Y. I., Hahn, K. M. and Montell, D. J.** (2010). Light-mediated activation reveals a key role for Rac in collective guidance of cell movement in vivo. *Nat. Cell Biol.* **12**, 591-597.