The PAR complex controls the spatiotemporal dynamics of F-actin and the MTOC in directionally migrating leukocytes

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RUNNING TITLE
PAR Complex in leukocyte motion

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SUMMARY
Inflammatory cells acquire a polarized phenotype to migrate toward sites of infection or injury.
A conserved polarity complex comprising PAR-3, PAR-6, and atypical protein kinase C (aPKC)
relays extracellular polarizing cues to control cytoskeletal and signaling networks affecting morphological and functional polarization. Yet, there is no evidence that myeloid cells use PAR signaling to migrate vectorially in 3D environments in vivo. Using genetically-encoded bioprobes and high-resolution live imaging we revealed the existence of F-actin oscillations in the trailing edge and constant MTOC repositioning to direct leukocyte migration in wounded medaka fish larvae. Genetic manipulation in live myeloid cells demonstrated that the catalytic activity of aPKC and the regulated interaction with PAR-3/PAR-6 are required for consistent F-actin oscillations, MTOC perinuclear mobility, aPKC repositioning and wound-directed migration upstream of Rho-kinase/ROCK/ROK activation. We propose that the PAR complex coordinately controls cytoskeletal changes affecting both traction force generation and directionality of leukocyte migration to sites of injury.

INTRODUCTION

Polarization allows cells to sense and to elicit the proper spatiotemporal responses to cues that arise from the surrounding microenvironment. Migrating cells are characterized by a sustained front-rear polarity. At the molecular level, polarized migration involves the establishment and maintenance of a spatial and functional asymmetry of molecular components between the anterior (leading) and posterior (trailing) edges of the migrating cell (Lauffenburger and Horwitz, 1996). How such coordinate partitioning of the cell migration and signaling machinery is controlled and maintained over time is largely unknown. Prevailing models evoke the creation of asymmetry in the distribution of key signaling molecules in the migrating cell, either through the production of rapidly diffusing inhibitory molecules by the front or through the sequestration of limiting polarity components to the front (Wang, 2009). Hydrostatic pressure-driven blebbing (Blaser et al., 2006) or local tension (Houk et al., 2012) generated in the leading edge membrane have been proposed as additional processes underlying polarization and directional migration in selected cell types. A further level of complexity in our understanding of the dynamics of cell polarization is due to the limitations of most in vitro models of cell migration, which fail to recreate the heterogeneous 3D environment in which cells polarize and migrate in vivo. Studies performed in 2D substrates can be highly detailed but often provide results that differ substantially from those obtained in vivo under 3D conditions (Lämmermann et al., 2008; Pouthas et al., 2008).
In various cell types and species, the PAR complex, consisting of a core of PAR-3, PAR-6, and atypical PKC (aPKC-λ/ι and aPKC-ζ), controls different aspects of cell polarity, ranging from cytoskeletal dynamics to sensing of positional cues. In a close interplay with Rho family members, the PAR complex presides over the architecture of the actin and microtubule cytoskeletons for polarized migration (Iden and Collard, 2008). The small GTPase Cdc42 activates aPKC, and the PAR complex, via the adaptor molecule PAR-6 (McCaffrey and Macara, 2009). PAR-3, in association with Cdc42-PAR-6-aPKC, binds directly to the Rac1-GEFs Tiam1/2, thus mediating Cdc42-induced Rac1 activation and lamellipodia formation (Nishimura et al., 2005).

Whether the PAR polarity complex is functionally required for chemokine-induced leukocyte polarization is unknown. Recent studies have shown that various polarity proteins are differentially localized throughout polarized T cells, suggesting that they may regulate T cell polarization (Gérard et al., 2007; Ludford-Menting et al., 2005). Further, PAR-3 knockdown impairs monocyte migration towards inflammatory signals in vitro (Tamehiro et al., 2009).

Using high resolution live imaging in genetically engineered medaka fish larvae (Oryzias latipes), we show that core components of the PAR complex regulate wound-induced directional migration of myeloid cells, through modulation of ROCK-dependent F-actin and MTOC dynamics. We also found that the catalytic activity of PKC-ζ is required for its own polarized distribution during migration, and that such relocation is a ROCK-independent phenomenon. Thus, we propose that the PAR complex, through the Rho pathway, coordinates the dynamics of both F-actin and MTOC/microtubules to support the efficient migratory profile of ameboid leukocytes in confined in vivo environments.

RESULTS

PAR proteins promote the directed migration of myeloid cells in vivo

To determine how PAR-3, PAR-6 and aPKC regulate directed migration of leukocytes in vivo in a 3D environment, we developed a model of wound-induced inflammatory cell migration in medaka fish, based on live imaging of tissue resident myeloid cells expressing membrane-tethered YFP (memYFP, using the transgenic (TG) line TG(FmpoP::memYFP) as, in medaka, myeloperoxidase (MPO) is expressed in mixed myeloid lineages that also contain sudanophilic material) (Fig. S1A) (Aghaallaei et al., 2010; Grabher et al., 2006). Since silencing
of the PAR components affects the morphogenesis of several embryonic tissues in zebrafish (Horne-Badovinac et al., 2001; Munson et al., 2008; Wei et al., 2004) and the use of morpholinos is not effective in juvenile medaka (~9-11 days post-fertilization) we adopted a meganuclease-driven, transient transgenesis approach based on injection of embryos at the one-cell stage (Rembold et al., 2006). We devised a strategy whereby a set of PAR complex-interfering mutants, expressed under the myeloid cell-specific Fmpo promoter (FmpoP), were co-injected with a nuclear-localized fluorescent marker (mCherry fused to histone H2A, H2AmCherry), to track the PAR mutant-expressing cells (Fig. 1A-C) (Souren et al., 2009). Using this established gene expression system, we first determined that two transgenes were co-expressed in >72% of cells (supplementary material Fig. S1B-D). As the transgenes were expressed in a mosaic fashion, we could directly compare the control subpopulation (H2AmCherry-) with the PAR mutant expressing subset (H2AmCherry⁺), to assess migration-associated parameters during the wound-response within the same animal (Fig. 1A).

To interfere with the PAR complex-associated catalytic activity, we ectopically expressed the kinase-inactive PKC-ζ-K281W (PKC-ζ-KW) mutant, or wild-type PKC-ζ (PKC-ζ-WT) as a control, specifically in myeloid cells. Ectopic expression of wild-type PKC-ζ in myeloid cells did not alter wound-directed migration, relative to cell straightness and directional speed (Fig. 1E-G). In contrast, PKC-ζ-KW expressing cells, even if fully motile, moved more randomly, in a "zig-zag" fashion, towards the wound, indicating that the catalytic activity of aPKC is dispensable for mobilisation of the cells, but promotes persistent migration in vivo (Fig. 1E-G; supplementary material Movies 1,2). These findings were confirmed using multicistronic viral 2A peptide-based vector, whereby PKC-ζ variants and the fluorescent reporter mCherry were stoichiometrically co-expressed in all cells as independent proteins (Fig. S1E-H).

Next, we evaluated whether aPKC was promoting leukocyte migration as part of its molecular and functional interaction with PAR-3 and PAR-6. To this aim, we ectopically expressed either the aPKC-binding region of medaka PAR-3 (PAR-3-aPKCBR) or the N-terminal (NT) domain of medaka PAR-6B specifically in myeloid cells, as overexpression of these deletion mutants has been shown to compete for the binding of endogenous aPKC with PAR-3 or PAR-6, respectively (Nakayama et al., 2008; Nishimura et al., 2005). Consistent with the PKC-ζ-KW data, both PAR-3-aPKCBR and PAR-6-NT-expressing cells still sensed the migration-inducing cues but moved less directionally to the wound-site, thus suggesting that
PAR-3 and PAR-6 contribute, together with aPKC, to the migrating response of leukocytes to the wound (Fig. 1E-G). Taken together, these findings establish that functional integrity of the PAR-6/aPKC/PAR-3 complex promotes the wound-directed migration of leukocytes *in vivo*.

**Defined spatial and temporal patterns of F-actin and MTOC dynamics occur during leukocyte directional migration *in vivo***

PAR proteins control cell polarity by establishing and maintaining asymmetry in the localization of both the actomyosin contractile system and microtubules (Iden and Collard, 2008). To perform simultaneous imaging and quantitation of both F-actin and microtubule dynamics in the living animal, we generated a double transgenic line TG(FmpoP::EB3-EGFP/FmpoP::RFP-Lifeact) in which medaka myeloid cells expressed simultaneously Ruby-tagged Lifeact (RFP-Lifeact) (Riedl et al., 2008), which detects polymerized actin, and the End-Binding Protein 3 (EB3) fused to EGFP, which binds to microtubule plus-end tips and also allows visualization of the MTOC. By applying the meganuclease system in this transgenic line, we expressed histone H2B fused to CFP (H2B-CFP), to allow visualization of nuclei in directionally migrating cells. RFP-Lifeact/EB3-EGFP/H2B-CFP triple positive cells migrated towards the wound as efficiently as memYFP positive cells (Fig. 2A,B) and displayed a highly variable and dynamic subcellular localization of the visualized cytoskeletal structures (supplementary material Movie 3).

To search for recurrent patterns in F-actin dynamics linked to migration we examined and quantitatively assessed the fluorescence intensity of RFP-Lifeact along the entire length of leukocytes during the migration process (Fig. 2D) (Solecki et al., 2009). Interestingly, F-actin displayed clear oscillatory patterns in migrating cells, with a temporally defined, alternate enrichment in the “front” and “back” edges of migrating cells (Fig. 2C,E; supplementary material Movie 4). Ratiometric analysis of RFP-Lifeact versus membrane-tethered YFP signals confirmed that these oscillations were not an artifact due to convolution of the plasma membrane, which can be particularly problematic in 3D environments (supplementary material Fig. S2). To better characterize F-actin oscillatory behavior and to circumvent the high variability in fluorescence levels between individual cells, we performed Fourier analysis (Geva-Zatorsky et al., 2010). Fourier analysis decomposes oscillatory signals in their basic frequencies and associated amplitudes. The outcome is the Fourier spectrum, which shows the
contribution, by means of amplitude, of each frequency to the oscillatory signal. Different oscillatory signals can be therefore compared based on their frequency components and associated relative contributions. Surprisingly, we found that both “front” and “back” F-actin waves displayed a main oscillation frequency common to all analyzed cells and that reflected their consistency. This oscillation frequency was centered between 0.005 and 0.0099 s\(^{-1}\) (oscillation period between 1.85 and 3.33 minutes) (Fig. 2F,G; supplementary material Movie 4). Notably, periodic accumulation of F-actin in the cell uropod paralleled boosts of speed in the migrating leukocyte. On the other hand, F-actin enrichment in the leading edge was linked to low-speed rates associated with lamellipodia extension and spatial exploration of the 3D context by the migrating cells (supplementary material Fig. S2). The robustness of the Fourier spectra in different cells suggests that periodic localization of F-actin in the cellular “front” and “back” during migration does not occur by chance, but is a conserved mechanism that can have a functional role in proper migration.

To assess the MTOC positioning during migration in our in vivo model, we determined the angular positioning of the MTOC around the nucleus during wound-induced directional migration. We assigned a “front” or “back” orientation when the MTOC was positioned within the 315°-45° or 135°-225° angular sections, respectively, as determined based on the vectorial axis of migration (Fig. 3B). The analysis of individual cells migrating towards the wound showed that the MTOC was highly dynamic and continuously shifted from the “front” to the “back” of the nucleus in directionally migrating cells (f events \(_{front} = 0.27\), f events \(_{back} = 0.34\)) (Fig. 3A,C,D; supplementary material Table S1, Movie 5). This mobility pattern may be caused by a marked anterior to posterior nuclear displacement combined with a strong rotational movement of both the MTOC and the nucleus (supplementary material Fig. S3A-D). Finally, and unlike F-actin waves, positioning of the MTOC either at the “front” or at the “back” of the nucleus did not correlate with overall speed rates of the migrating leukocytes (Fig. 3E).

**Regulation of anteroposterior polarity of F-actin by PAR proteins**

The observation that enrichment of F-actin in the uropod occurs when ameboid leukocytes increase speed rates raises the possibility that actin polymerization in the trailing edge functions as an oscillatory engine that cyclically propels the cell forward. In agreement with such a mechanism, we found a negative relationship between F-actin enrichment and...
nuclear positioning (supplementary material Fig. S3E,F). Hence, we tested if in our model expression of PAR complex dominant interfering mutants affects the oscillatory pattern of “back” F-actin in wound-directed migration of myeloid cells. We preliminarily determined that ectopic expression of wild-type PKC-ζ in myeloid cells did not alter the periodicity of F-actin waves in the uropod. Fourier spectra of F-actin signal in the cell rear displayed a dominant frequency of oscillation centered between 0.005 and 0.0099 s⁻¹, which was comparable to control cells. Conversely, Fourier profiles of PKC-ζ-KW, PAR-6-NT and PAR-3-aPKCBR cells did not show a clearly dominant frequency of oscillation, indicative of profound modifications in the nature of these oscillatory signals when the PAR complex is functionally perturbed (Fig. 4A-D; supplementary material Movie 6). Moreover, in contrast to the homogeneity of PKC-ζ-WT waves, we observed a huge variability in between single PAR-mutant cells, suggesting that the robustness of physiological oscillations was disrupted upon PAR complex interference. Altogether, our data suggests that the three core components of the PAR complex are coordinating the dynamics of actin polymerization in the rear of leukocytes migrating rapidly in vivo and that the catalytic function of PKC-ζ is required for such function.

As an indicator of morphological polarization, we analyzed cell shape changes (by means of cellular "roundness") in wound-migrating myeloid cells expressing the various PAR mutants. First, we observed that the shape profile of wild-type PKC-ζ leukocytes was typically migratory and was comparable to control cells (Roundnessₜᵢₓₜ₀₄₋₀₆ₙₜᵢₓ; CTR = 42% , PKC-ζ-WT = 48%; RoundCTR = 0.5351±0.0010; RoundPKC-ζ-WT = 0.5403 ± 0.0089). Next, we found that PKC-ζ-KW and PAR-6-NT cells adopted more rounded shapes, possibly due to multiple random protrusions (RoundPKC-ζ-KW = 0.5940 ± 0.0080; RoundPAR-6-NT = 0.6243 ± 0.0099). Surprisingly, cells expressing the aPKCBR of medaka PAR-3 showed minimal changes on mean roundness values (RoundPAR-3-aPKCBR = 0.5494 ± 0.0093) but, instead, revealed a wide range of shape changes, from extremely elongated to more rounded phenotypes (supplementary material Fig. S4, Table S2).

Regulation of MTOC perinuclear dynamics by PAR proteins

Prior reports have identified PAR components as key regulators of MTOC mobility and spatial localization in migrating cells (Etienne-Manneville and Hall, 2001; Etienne-Manneville and Hall, 2003; Gomes et al., 2005; Schmoranzer et al., 2009; Solecki et al., 2009; Solecki et al.,
Hence, we assessed whether perturbation of the PAR complex interferes with MTOC perinuclear dynamics in our in vivo model of wound-directed 3D migration. As with the analysis of F-actin dynamics, ectopic expression of wild-type PKC-ζ in myeloid cells did not alter MTOC mobility around the nucleus. Similar to control cells, the MTOC shifted from “front” to “back” with respect to the nucleus during the wound-response (PKC-ζ-WT: \( f_{\text{front}} = 0.29, f_{\text{back}} = 0.38 \)). Notably, in PKC-ζ-KW, PAR-6-NT and PAR-3-aPKCBR expressing cells the MTOC was markedly less mobile, and preferentially oriented towards the cell “front” (PKC-ζ-KW: \( f_{\text{front}} = 0.48, f_{\text{back}} = 0.16 \); PAR-6-NT: \( f_{\text{front}} = 0.53, f_{\text{back}} = 0.11 \); PAR-3-aPKCBR: \( f_{\text{front}} = 0.48, f_{\text{back}} = 0.17 \)) (Fig. 5A-C; supplementary material Table S1, Movie 7).

Rho-dependent actomyosin contractility is required for MTOC mobility around the nucleus in wound-induced myeloid cell migration

One obvious question that arises from the prior data is whether these F-actin and MTOC/microtubules act coordinately to promote cell migration in leukocytes, as it was previously reported in nocodazole-treated cell lines (Xu et al., 2005) and recently proposed for Drosophila hemocytes (Stramer et al., 2010). We did find a positive link between F-actin distribution and MTOC perinuclear orientation, meaning that when F-actin was enriched at the uropod the MTOC was behind the nucleus and oriented towards the “back” of the cell (supplementary material Fig. S3F). To further address how actomyosin contraction functionally contributes to MTOC perinuclear dynamics in migrating leukocytes, we pharmacologically inhibited Rho-kinase/ROCK/ROK, the RhoA effector controlling actin-myosin interaction in non-muscle cells, by exposing the larvae to Y-27632. Fourier analysis on F-actin fluctuations in the uropod of the cell confirmed that the periodicity of F-actin movement was profoundly modified upon treatment (Fig. 6A,B; supplementary material Movie 8). Interestingly, ROCK inhibition with Y-27632 strongly reduced the perinuclear mobility of the MTOC, which became markedly oriented towards the cellular “front” (Y-27632: \( f_{\text{front}} = 0.61, f_{\text{back}} = 0.11 \)) (Fig. 6A,C,D; supplementary material Table S1, Movie 9). In conclusion, primarily impairing Rho-dependent actomyosin contraction not only alters F-actin localization as expected, but also modifies MTOC mobility around the nucleus. This suggests a direct, causal link between these two cytoskeletal networks.
To unveil the functional relevance of the identified F-actin and MTOC dynamics to forward locomotion, we examined the relationships between directional speed and either the periodicity of F-actin oscillations in the cell “back” or the mobility of the MTOC in the perinuclear region. Interestingly, we found a statistically significant, positive correlation between the periodicity of F-actin oscillations (as determined by the existence of a dominant peak in Fourier transform) and the efficiency of directional migration to the wound (Fig. 6E). Likewise, the angular mobility of the MTOC around the nucleus positively correlated with the cells' directional speed, suggesting that cells need to constantly re-position the MTOC in the perinuclear region to be able to migrate more efficiently towards pro-migratory cues \textit{in vivo} (Fig. 6F).

The enzymatic activity of PKC-\(\zeta\) is required to establish and maintain a polarized distribution of the PAR complex

Neither the spatial and temporal dynamics of PAR complex distribution nor its functional role have been determined in leukocytes migrating \textit{in vivo}. To address this issue, we transiently expressed GFP tagged PKC-\(\zeta\) in a transgenic line expressing mCherry as cytoplasmic volume marker (Tg(FmpoP::mCherry)), using the myeloid cell-specific \textit{Fmpo} promoter. Ratiometric imaging of PKC-\(\zeta\)/mCherry revealed marked and transient local PKC-\(\zeta\) increases at the “front” of migrating cells (Fig. 7A; supplementary material Movie 10). Quantitative analysis on fluorescent protein distribution in ratiometric images (Melichar et al., 2011) confirmed that PKC-\(\zeta\) localized predominantly at the cell “front” (Fig. 7A,B,D,F).

Next, we investigated the molecular mechanisms underlying PKC-\(\zeta\) polarization in live leukocytes based on the hypothesis that the catalytic function of the PAR complex plays a role in such a polarized distribution. To this aim, we transiently co-expressed GFP-PKC-\(\zeta\) with the wild-type or kinase inactive PKC-\(\zeta\) in TG(FmpoP::mCherry) larvae. While ectopic expression of wild-type PKC-\(\zeta\) in myeloid cells did not perturb the GFP-PKC-\(\zeta\) distribution pattern, expression of PKC-\(\zeta\)-KW led to mislocalization of the fluorescent signal toward the uropod of migrating cells (Fig. 7C,E; supplementary material Movie 11). Thus, activated PKC-\(\zeta\) is required for its own polarized distribution in leukocytes migrating \textit{in vivo}. 
To investigate whether ROCK modulates PAR polarity in leukocytes migrating in vivo, we tested the effect of its specific inhibitor Y-27632 on GFP-PKC-ζ subcellular distribution. Notably, GFP-PKC-ζ localized predominantly at the cell “front” upon treatment with Y-27632, similar to control cells. Inhibition of ROCK actually increased the degree of front-rear asymmetry of the fluorescence signal (Fig. 7D,F; supplementary material Movie 10). These results indicate that PKC-ζ polarization at the “front” is independent of Rho-kinase activity when leukocytes migrate to wounds in vivo.

Regulation of RhoA activity by PKC-ζ catalytic function

Next, we explored whether the catalytic function of the PAR complex modulates RhoA/ROCK signaling in our in vivo model of leukocyte migration. To this aim, we co-expressed the cytosolic RhoA-FRET biosensor (Kardash et al., 2010) with the wild-type or the kinase inactive PKC-ζ variants in medaka leukocytes. FRET ratiometric imaging of the migration process revealed that RhoA activity is reduced in PKC-ζ-KW cells, when compared to cells expressing wild type PKC-ζ (Fig. 8A,B; supplementary material Movie 12), indicating that the catalytic activity associated with the PAR complex is upstream of RhoA activation in wound-directed myeloid cell migration.

DISCUSSION

This study was undertaken to address the in vivo relevance of the evolutionarily conserved PAR complex in inflammatory cell polarization and directional motility in response to tissue injury. Our results unveil the relevance of periodic rear edge enrichment of F-actin in directionally migrating tissue–resident myeloid cells, which is paralleled by a high degree of perinuclear mobility of the MTOC. Both of these features appear to be severely perturbed by the expression of dominant interfering PAR complex mutants, which results in impaired directionality and cell shape patterns associated with vectorial migration (Fig. 8C). An additional layer of complexity in this model is given by the adaptation of migrating cells to the constraints of the changing 3D environment they encountered on approaching the wound edge. The geometric complexity of the interstitium induces Cdc42-dependent pathways for leading-edge coordination in chemotactic leukocytes (Lämmermann et al., 2009). One of the most conserved functions of Cdc42 is the activation of aPKC and the PAR module through PAR-6
(McCaffrey and Macara, 2009), thus establishing a potential link between pro-migratory cue sensing and the activation/recruitment of the PAR complex. We found that PKC-ζ activity promotes persistent leukocyte movement as disruption of its catalytic function increased dispersive motion of cells. The same phenotype was observed in cells expressing mutants interfering with the binding of PAR-6 or PAR-3 to aPKC, although to a variable extent. Despite such difference, the expression of either PKC-ζ, PAR-6 and PAR-3 dominant-interfering mutants affected the same qualitative parameters of leukocyte migration, namely straightness and directional speed, consistent with the previously shown interdependence of the three core components of the complex in leukocytes migrating directionally to tissue injury in vivo.

Periodicity of trailing edge F-actin enrichment was a prominent feature of wound-directed migration of myeloid cells, as unveiled by Fourier transform analysis. Also of note, all PAR mutants not only impaired directional movement but also disrupted the periodicity of F-actin enrichment in the trailing edge of migrating leukocytes. Mathematical models predict that oscillatory behaviors arising from the onset of negative feedback mechanisms in cells responding to steady stimuli show a gradual dampening of the amplitude of the response, while a constant amplitude may reflect the existence of a process controlled by a fast oscillatory input, which could have evolved to generate biological responses only if the input has reached a certain threshold (Cheong and Levchenko, 2010). We speculate that trailing edge F-actin oscillations in directed migration are the outcome of signaling inputs reaching critical concentration thresholds in spatially-defined domains of the cells, namely the rear edge of polarized, directionally migrating leukocytes.

Another hallmark of polarity in most migrating cells is the orientation of the MTOC-nucleus axis relative to the front-rear cellular axis (Luxton and Gundersen, 2011). Most in vitro studies, generally performed in 2D systems, showed that leukocytes position the MTOC in the uropod and behind the nucleus (Krummel and Macara, 2006; Sánchez-Madrid and del Pozo, 1999). Surprisingly, we found that the MTOC is highly dynamic in leukocytes migrating to wounds, shifting continuously from “front” to “back” nuclear orientation. We also revealed that MTOC perinuclear mobility is PAR complex-regulated and correlates with directional movement. As the MTOC-nucleus orientation axis is linked to the dynamics of vesicular trafficking (Luxton and Gundersen, 2011), we reason that MTOC position around the nucleus might regulate directional migration by constantly adapting microtubule nucleation and vesicle
trafficking to the specific needs of 3D ameboid motility. We also found that ROCK-dependent actomyosin contraction controls MTOC perinuclear positioning, reminiscent of what happens during glial-guided neuronal migration (Solecki et al., 2009). One likely scenario would place ROCK as an upstream regulator of the PAR complex, as previously suggested for crawling cells (Nakayama et al., 2008). However, we do not favor this hypothesis since: a) inhibiting ROCK activity does not perturb the polarized distribution of GFP-PKC-ζ during migration and b) interfering with the catalytic function of the PAR complex reduces RhoA activity, as determined by FRET ratiometric imaging. This is consistent with studies in dendritic spines morphogenesis, which implicate the PAR complex in the control of regulators of Rho-GTP levels (Zhang and Macara, 2008).

What drives GFP-PKC-ζ polarization to the “front” of migrating leukocytes? Functional antagonism between PAR and Scribble complexes has been suggested in several settings to explain the spatial segregation of each of the polarity modules to opposite sides of the cells (Humbert et al., 2006). Such antagonism requires aPKC catalytic function. Importantly, in polarized T-cells in vitro, the PAR complex is distributed in the front of the cell while the Scribble complex is localized at the tail (Krummel and Macara, 2006; Ludford-Menting et al., 2005). Further studies addressing these issues will have to combine quantitative imaging in genetically tractable living organisms including the assessment of the stoichiometry and effector function of molecular complexes controlling cell polarization.

MATERIAL AND METHODS

Fish Stocks

Medaka (Oryzias latipes) and zebrafish (Danio rerio) stocks were maintained as previously described (Koster et al., 1997; Westerfield, 2000). Lines used in the study were medaka wild-type (wt) Cab, TG(FmpoP::memYFP) (Grabher et al., 2006) (both animals kindly provided by J. Wittbrodt), TG(FmpoP::EB3-EGFP/FmpoP::RFP-Lifeact), TG(FmpoP::mCherry) and zebrafish MPO::GFP (Renshaw et al., 2006). Experimental animals were kept and treated according to the German (Tierschutzgesatz) or Italian (decree 116/92) national guidelines and experimental procedures were approved by Institutional Animal Care and Use Committee.

Sudan Black staining and imaging
Sudan black staining was performed in larvae as reported (Le Guyader et al., 2007). Images were taken with an IX81 Olympus inverted microscope equipped with an Olympus LUCPlanFLN NA 0.6/40x dry objective, coupled to a Nikon DS-5Mc-U1 digital camera and driven by Nikon NIS Elements software.

**DNA Expression Vectors**

All the DNA expression vectors contain the fugu myeloperoxidase promoter fragment (FmpoP), I-SceI meganuclease recognition sites and an SV40 polyadenylation sequence. The construct FmpoP::memYFP is a kind gift from J. Wittbrodt (Grabher et al., 2006). Constructs which have each of the following sequences in the I-SceI-FmpoP backbone vector were engineered:

- nuclear-tagged H2AmCherry (a generous gift from J. Wittbrodt) (Brown et al., 2010); Lifeact-Ruby (RFP-Lifeact; a generous gift from M. Sixt) (Riedl et al., 2008); EB3-EGFP (a generous gift from V. Lecaudey) (Carvalho et al., 2009); nuclear-tagged H2B-CFP (a generous gift from R. Köster) (Distel et al., 2010); monomeric Cherry (mCherry; Clontech Laboratories, INC);
- cytosolic RhoA-FRET biosensor (generous gift from E. Raz) (Kardash et al., 2010); flag-tagged rat PKC-ζ-WT or PKC-ζ-KW (generous gifts from A. Toker) (Chou et al., 1998) and GFP-tagged PKC-ζ-WT (GFP-PKC-ζ); mCherry-P2A-memYFP; mCherry-P2A-PKC-ζ-WT; mCherry-P2A-PKC-ζ-KW; myc-tagged aPKC-binding region of medaka PAR-3 (PAR-3-aPKCBR) and myc-tagged N-terminal domain of medaka PAR-6 (PAR-6-NT).

**RT-PCR and PCR**

The aPKCBR of rat PAR-3 (NCBI Reference Sequence: NP_112514.1) and the N-terminal region of human PAR-6A (NCBI Accession: Q9NPB6) were previously described (Izumi et al., 1998; Nishimura et al., 2005). The myc-tagged corresponding regions of medaka PAR-3 (Ensemble: ENSORLP00000002648; amino acids: 653-879) or medaka PAR-6B (Ensemble: ENSORLP00000002315; amino acids: 1-124) were isolated by RT-PCR (Invitrogen) from wt cDNA. The myc-tagged cDNAs were amplified by double-rounded PCR, using the sequential set of primers:

PAR-3-aPKCBR - 1st forward, 5’-gagcgcagactccactccactc-3’; 1st reverse, 5’-tcacggcgtgactccacctcagtttgt-3’; 2nd forward, 5’-ttttgatcaggtagccatctccactcagtttgt-3’; 2nd reverse, 5’-aatctgccctcttttgactccactcagtttgt-3’;
PAR-6-NT: 1\textsuperscript{st} forward, 5'-ttttctcggagaatgaacaaaaaccacgcggc-3'; 1\textsuperscript{st} reverse, 5'-aaagaattctaggcgtccggcctcagaagaac-3'; 2\textsuperscript{nd} forward, 5'-ttttgaattcaccaccatggcatcaatgcagaagctgatctcagaggaggacctgggaaacaaaaaccaccgagtg-3'; 2\textsuperscript{nd} reverse, 5'-atagtttagcggccgcatcttatctagcgtccggcctcagaagaac-3'.

The GFP-PKC-ζ fusion was generated by cloning enhanced green fluorescent protein in frame at the N-terminal end of rat PKC-ζ cDNA.

P2A-linked multicistronic cassettes were designed and generated based on previous reports (Szymczak-Workman et al., 2012a; Szymczak-Workman et al., 2012b).

cDNAs were cloned into I-SceI-FmpoP backbone vector. Sequences were confirmed by DNA sequencing.

DNA injection

For injection of one or two constructs a solution containing 15-22.5 ng/μl of the single plasmid DNA or 7.5-11.25 ng/μl of each construct, I-SceI buffer (1x; New England Biolabs) and I-SceI meganuclease (0.25 units/μl; New England Biolabs) was injected into one-cell stage embryos (Rembold et al., 2006; Thermes et al., 2002). DNA plasmids were expressed transiently as referred in the results section.

TG(FmpoP::EB3-EGFP/FmpoP::RFP-Lifeact) and TG(FmpoP::mCherry) lines

Transgenic lines were created with I-SceI-FmpoP backbone vectors as reported (Rembold et al., 2006; Thermes et al., 2002). Double injection of EB3-EGFP and RFP-Lifeact or single injection of mCherry was performed into one-cell stage Cab embryos. Stable transgenic lines with double expression of EGFP and RFP or single expression of mCherry in myeloid cells were designated TG(FmpoP::EB3-EGFP/FmpoP::RFP-Lifeact) and TG(FmpoP::mCherry), respectively.

Tail fin wounding and live imaging

Imaging was performed on fish at 9-11 days post-fertilization wounded and mounted as reported (Grabher et al., 2006). Time-lapse fluorescence images were acquired with an UltraView VoX spinning disk confocal unit (Perkin Elmer) equipped with an inverted Nikon Eclipse Ti microscope, coupled to a C9100-50 emCCD camera (Hamamatsu) and a Yokogawa CSU-X1 scanning head and driven by Volocity software (Improvision, Perkin-Elmer). CFP/YFP or GFP/
RFP or Cherry imaging was performed using 405nm, 488nm and 568nm laser lines and a specific multiband pass emission filter. Image sequences were generated as following: YFP/Cherry or RFP every 20-25 seconds using a NA 0.75/20x objective and 1-3 μm step size; GFP/Cherry every 15 seconds using a NA 1.3/40x oil immersion objective lens and 1 μm step size; CFP/GFP/RFP every 15-18 or 7-8 seconds using, respectively, a NA 1.3/40x or NA 1.4/60x oil immersion objective lens and 1μm step size. DIC images were taken with low-level illumination with a halogen lamp. Where indicated, embryos were pretreated with 500 μM Y-27632 (Sigma) for 2 hr, and then images were taken with the drug in ERM/tricaine solution (Sigma; 0.16 mg/ml). Images were processed with ImageJ software (NIH). Upon background subtraction for each fluorescence channel, a gaussian blur filter was applied. Brightness and contrast were set and then multi-channel image sequences were overlaid. To make overlay images of DIC and fluorescence pictures, Z-stacks were overlaid onto a single DIC plane.

Co-expression analysis of two FmpoP-driven transgenes

The degree of coexpression of two transgenes was determined as the fraction of memYFP+/H2AmCherry+ cells in the fluorescent myeloid-cell population.

Cell dynamics analysis

Transgenic cells (H2AmCherry+) or internal control cells (H2AmCherry-) that were positioned at the same distance from the wound were tracked with the Manual Tracking plugin (ImageJ). Resulting two-dimensional coordinates were analyzed with a custom made algorithm based on MatLab Interface. Upon applying a rotation angle that centered control tracks on the y- or wound-directed axis, straightness, the y-directed (or wound-) speed (Vy) and the x-directed (or dispersive-) speed (Vx) were extracted for each track and finally averaged. Straightness (S) was defined as the ratio between the length of the segment connecting the first (t₁) and the last (tn) point of the path, and the entire displacement of the cell along the xy plane. It was calculated as:

\[ S = \frac{\sqrt{(y_n - y_1)^2 + (x_n - x_1)^2}}{\sum_{i=1}^{n-1} \sqrt{(y_{i+1} - y_i)^2 + (x_{i+1} - x_i)^2}} \]
The wound-speed \((V_y)\) and the dispersive-speed \((V_x)\) were defined, respectively, as the ratio between the displacement of the cell along the \(y\)-axis or its absolute displacement along the \(x\)-axis and the time spent by the cell during the whole migration. Values were calculated as:

\[
V_y = \frac{\sum_{i=1}^{n} (y_i - y_{i-1})}{t_n - t_0}
\]

\[
V_x = \frac{\sum_{i=1}^{n} (x_i - x_{i-1})}{t_n - t_0}
\]

The ratio between wound-speed and dispersive-speed \((V_y/V_x)\) was determined and designated directional speed ratio. Speed was calculated using the Chemotaxis Tool plugin (Ibidi, Germany). Straightness, directional speed ratio and speed were finally represented as fold increase over the internal control values.

Transgenic cells \(\text{mCherry-P2A}\text{^\textdagger}/\text{PKC-}\zeta\text{WT}\textsuperscript{\textdagger}; \text{mCherry-P2A}\text{^\textdagger}/\text{PKC-}\zeta\text{-KW}\textsuperscript{\textdagger}; \text{memYFP}\textsuperscript{\textdagger}; \text{H2B-CFP}\textsuperscript{\textdagger}/\text{EB3-EGFP}\textsuperscript{\textdagger}/\text{RFP-Lifeact}\textsuperscript{\textdagger}\) were tracked and resulting coordinates were analyzed as described above, upon applying a rotation angle that centered the talfin rays on the wound-directed axis.

Cytoskeletal and Shape analysis

Cell dynamics parameters were determined in an unbiased manner for all cells in the wound area. To assess shape and cytoskeletal dynamics in a more qualitative manner, control cells or a representative cohort of mutant-cells displaying uncoordinated movement to the wound-site was analyzed during migration. In the analyses, the direction of migration vector at \(t_i\) corresponded to the segment connecting the centroids of the cell at \(t_{i-1}\) and \(t_{i+1}\). Measurements were carried out using ImageJ software.

F-actin analysis

Cell shapes were subdivided in three equally long regions “front”, “center” and “back” based upon the direction of migration vector (Solecki et al., 2009). Mean RFP-Lifeact fluorescence intensity values were extracted and represented as percentage of total RFP-Lifeact fluorescence.

- Fourier analysis
Fourier analysis was applied to RFP-Lifeact fluorescence time profiles for individual cells using the Fast Fourier Transform (FFT) algorithm (MatLab Interface). The time interval analyzed was comprised between the first speed-up until the last speed-down processes. In rare cases, when the two limits were not obvious from the speed time profile, all the temporal series was used for the analysis. For each Fourier profile, the amplitude values from the five highest peaks were normalized to the highest amplitude value among the five. Normalized values were averaged in the correspondent ranges of frequencies to create a histogram showing the relative contribution of a certain range of frequencies to RFP-Lifeact oscillations. For each cell, the strength of F-actin main frequency in the “back” was defined as the amplitude value of the main Fourier peak normalized to the sum of the amplitude values from the five highest peaks.

- **F-actin polarity and total speed**

Percentage of total RFP-Lifeact fluorescence was obtained as described above and normalized to memYFP fluorescence levels. Values at $t_i$ were finally averaged in ranges of cellular speed ($v_i$). Cellular speed ($v_i$) at time $t_i$ was approximated by considering the positions at $t_{i-1}$ and $t_{i+1}$:

$$ v_i = \frac{\sqrt{(x_{i+1} - x_{i-1})^2 + (y_{i+1} - y_{i-1})^2}}{t_{i+1} - t_{i-1}} $$

- **MTOC and Nucleus analysis**

  - **Perinuclear MTOC dynamics**

A vector linking the nuclear centroid and the MTOC (MTOC-Nucleus vector) was determined. The angle $\alpha$ between the MTOC-Nucleus vector and the direction of migration vector was defined the MTOC/Nucleus angle. Absolute values for MTOC/Nucleus angles were then averaged in ranges of cellular speed ($v_i$). The MTOC perinuclear mobility per cell was represented by the standard deviation associated to the MTOC/Nucleus angles (Range: $-180^\circ$ counterclockwise to $180^\circ$ clockwise).

  - **MTOC and Nucleus position**

Analysis of MTOC and nucleus position was described previously (Gomes et al., 2005).

  - **MTOC and Nucleus orientation**
Vectors linking the centroid of the cell and the MTOC (MTOC vector) or the nuclear centroid (Nucleus vector) were determined. The angle between the MTOC vector or the nucleus vector and the direction of migration vector was defined the MTOC or the nucleus angle, respectively. The MTOC and the nucleus angular mobility per cell were calculated as the MTOC perinuclear mobility.

**Analysis on F-actin distribution and MTOC or Nucleus dynamics**

The distance between the two extremities of the cell that cross the direction of migration vector was calculated (cell length.) The orthogonal projection of the nuclear centroid onto the cell length was determined and its distance from the “front” extremity of the cell normalized to the cell length (nucleus A-P position). Nucleus A-P position and MTOC perinuclear orientation were then averaged in ranges of “back” F-actin fluorescence.

**Morphological analysis**

Roundness values during migration were obtained from cell shapes using the plugin Measurements/Shape descriptors of ImageJ.

**Ratiometric analysis of GFP-PKC-ζ**

2D ratiometric images were made after Z-series stacking (Yoo et al., 2010). To quantify the distribution of GFP-PKC-ζ signal, we based on a previously described methodology (Melichar et al., 2011). We used the mCherry mask to identify the centroid of the cell and the fluorescent-weighted center of GFP-PKC-ζ/mCherry ratiometric signal. The vector between the cell centroid and the fluorescent-weighted center was defined the asymmetry vector and its angle $\alpha$ with the direction of migration vector was defined the asymmetry–migration angle.

**Imaging and analysis of RhoA activity**

FRET ratiometric imaging was performed on a Leica TCS SP5 laser confocal scanner mounted on a Leica DMI 6000B inverted microscope equipped with HCX PL APO 40X/1.25-0.75NA oil immersion objective and driven by Leica LAS AF software. FRET sensor/mCherry were excited with Violet (405nm laser diode) and yellow (561nm laser diode) laser lines, respectively. Z-stacks were collected every 15 seconds with 1.3μm step size. ImageJ software was used to
generate YFP/CFP ratio images as previously described (Kardash et al., 2011). Mean emission ratio of YFP/CFP for the entire cell during migration was calculated to determine the average activation level of RhoA.

Statistical analyses
Statistical analyses were performed as described in the figure legends (Graphpad Prism, San Diego, CA). For circular statistics, inter-sample statistics and data visualization were performed using the Oriana software (Kovach Computing Services, Anglesey, Wales). When needed, Levene’s test of homogeneity of variances was used to test for equality of variances.

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AUTHOR CONTRIBUTIONS
C.L.C. designed and performed the experiments, generated and interpreted the data, prepared and wrote the manuscript. C.V. designed algorithms and simulations and contributed to data interpretation. P.J.K. designed the algorithm to analyze cellular dynamics. M.G. provided support on FRET ratiometric imaging and analysis. J.R.B. contributed to data interpretation and some of the experimental design. J.W. provided training and supervision on the model and key reagents, and contributed to data interpretation. R.P. designed the study, interpreted the data, wrote the manuscript and provided grant funding to support the study.

CONFLICT OF INTEREST DISCLOSURE
The authors declare no competing financial interests.

REFERENCES


**FIGURE LEGENDS**

**Fig. 1.** PAR complex promotes wound-directed migration of myeloid cells *in vivo.* (A) TG(FmpoP::memYFP) embryos were injected at the one-cell stage with a 1:1 mixture of DNA coding for H2AmCherry as a nuclear reporter and one of the PAR transgenes, driven by the myeloid-specific *Fmpo* promoter and flanked by I-SceI integration sites (Sc), in the presence of I-SceI meganuclease. Wounded larvae with mosaic expression of H2AmCherry in tailfin myeloid cells were imaged. The dashed line represents the wound. Inset shows a transgenic cell (Cherry+, PAR) and the built-in control (Cherry−, CTR). Scale bar = 50 μm. Inset: Scale bar = 10 μm. **(B)** Domains of interaction between members of the mammalian PAR complex. Connecting lines indicate regions of the proteins that interact with one another. PB1, Phagocyte
oxidase/Bem1 domain; Zn, Zinc finger motif; Kinase, catalytic domain; CRIB, Cdc42/Rac interactive binding motif; PDZ, PSD-95/Dlg/Zona occludens-1 domain; CR1, conserved region 1; aPKCBR, aPKC binding region; predicted coiled-coil region. (C) Schematics of the constructs used to perturb the function of the PAR complex in myeloid cells. Numbers refer to amino acid positions. *, K to W mutation at codon 281. NT – N-terminal domain. (D) 2D tracks of individual leukocytes migrating in the tailfin of unperturbed fish (left panel) or toward the tailfin wound (right panels) (No wound: n = 11; CTR/PKC-ζ-WT: n = 12; CTR/PKC-ζ-KW: n = 10; CTR/PAR-6-NT: n = 13; CTR/PAR-3-aPKCBR: n = 13). Tracks are from one representative experiment of at least three independent experiments. (E, F and G) Quantitation of 2D (E) speed, (F) straightness and (G) directional speed ratio of myeloid cells during the wound response. Data are expressed as means ± SEM of at least three separate experiments (PKC-ζ-WT: n = 27 cells in 3 larvae, PKC-ζ-KW: n = 27 cells in 3 larvae, PAR-6-NT: n = 45 cells in 4 larvae, PAR-3-aPKCBR: n = 64 cells in 5 larvae; * p < 0.05, ** p < 0.01, *** p < 0.001, two-tailed unpaired t-test). See also supplementary material Fig. S1 and Movies 1,2.

Fig. 2. F-Actin shows an oscillatory antero-posterior polarity during wound-directed migration of leukocytes in vivo. (A and B) Quantitation of 2D (A) speed and (B) straightness of memYFP⁺ or H2B-CFP⁺/EB3-EGFP⁺/RFP-Lifeact⁺ leukocytes migrating to wounds. Data are expressed as means ± SEM of all analyzed cells (memYFP⁺: n = 75 cells in 3 larvae, H2B-CFP⁺/EB3-EGFP⁺/RFP-Lifeact⁺: n = 64 cells in 3 larvae, two-tailed unpaired t-test). (C) Two consecutive cycles (C1 and C2) of “front” to “back” F-actin waves (lower panels) are visualized in transgenic larvae co-expressing RFP-Lifeact and EB3-EGFP in myeloid cells (TG(FmpoP:: EB3-EGFP/FmpoP::RFP-Lifeact)), following transient expression of H2B-CFP as a nuclear marker (upper panels). Shown are frames from representative movies of myeloid cells migrating in the wounded tailfin. The white arrows indicate direction of migration. Outlines of the cell are drawn with a grey line. Scale bar = 10 μm. (D) Schematics of the 2D geometric compartmentalization used to quantify F-actin subcellular distribution. Black dots indicate the cell centroid shown at three consecutive time points and the dashed red arrow indicates the direction of migration vector. (E) Time course of F-actin “front” and “back” fluorescence signal distribution in the cell depicted in (C). Cycle C1 and C2 are those visualized in (C). (E) Fourier spectra showing the time frequencies that compose the oscillatory signal of F-actin in the “back”
and “front” regions of the cell shown in (C). The peak with the highest amplitude value (main peak) corresponds to the predominant frequency of oscillation. Frequencies with lower amplitude values are also displayed and give a minor contribution to the oscillatory signal. (G) Histograms showing the relative contribution of ranges of frequencies to “back” and “front” F-actin oscillations. Note that a predominant range of oscillatory frequencies (main range) emerges from ranges of secondary frequencies. Data are expressed as means ± SEM of all analyzed cells (n = 8 cells in 4 larvae; statistical analysis was performed between the main range and each of the remaining ranges of frequencies, ** p < 0.01, *** p < 0.001, two-tailed paired t test). See also supplementary material Fig. S2 and Movie 4.

Fig. 3. The MTOC is highly mobile in the perinuclear compartment of leukocytes migrating to wounds in vivo. (A) The microtubules and the nucleus are visualized in the migrating leukocyte shown in Fig. 2. Top panel: fluorescence images; bottom panel: the green dots represent digitalized reconstruction of the MTOC position. The white arrows indicate direction of migration. Outlines of the cell are drawn with a grey line. Scale bar = 10 μm. (B) Schematics of 2D analysis on MTOC perinuclear positioning during migration. The dashed red arrow indicates the direction of migration defined as in Fig. 2. The yellow arrow originating from the nuclear centroid (white dot) to the MTOC is the MTOC-Nucleus vector. The angle α between the MTOC-Nucleus vector and the direction of migration vector is the MTOC/Nucleus angle (orange arc). (C) Rose diagram mapping the MTOC orientation and the respective spatial frequency of events in migrating myeloid cells (230 counts, 7 leukocytes in 5 larvae). Light or dark grey areas corresponds to 90º ranges for “front” or “back” orientations, respectively. (D) Quantification of MTOC perinuclear mobility in migrating myeloid cells. MTOC mobility for each cell is represented by the standard deviation associated to the angles MTOC/nucleus during wound-response. Red line shows the mean ± SEM for all cells analyzed (7 leukocytes in 5 larvae). (E) MTOC perinuclear orientation is plotted against cellular speed. For each range of speed, data represents means ± SEM (226 counts, 7 leukocytes in 5 larvae; R Spearman = 0.008, p (two-tailed) = 0.8932 (n.s.)). See also supplementary material Fig. S3A-D, Table S1 and Movie 5.
Fig. 4. PAR complex regulates anteroposterior polarity of F-actin during wound-directed migration of leukocytes in vivo. (A) F-actin distribution is visualized in transgenic larvae with RFP-Lifeact and EB3-EGFP in myeloid cells, following transient co-expression of nuclear H2B-CFP and each of the indicated PAR transgenes. Frames from representative movies of migrating leukocytes in the wounded tailfin are shown. The white arrows indicate direction of migration. Outlines of cells are drawn with a grey line. Scale bars = 10 μm. (B) Time course of F-actin “front” and “back” fluorescence intensity distribution in the cells shown in (A). (C) Fourier spectra of F-actin oscillations in the “back” region of the cells depicted in (A). (D) Comparison of histograms of F-actin oscillatory frequencies in the “back” region of the cell (Ranges (s⁻¹) are the same as shown in Fig. 2E). Data are expressed as means ± SEM of all analyzed cells (PKC-ζ-WT: 8 leukocytes in 5 larvae, PKC-ζ-KW: 8 leukocytes in 6 larvae, PAR-6-NT: 7 leukocytes in 4 larvae, PAR-3-aPKCBR: 6 leukocytes in 4 larvae; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, two-tailed paired t test). See also supplementary material Fig. S4 and Movie 6.

Fig. 5. PAR complex regulates the MTOC perinuclear positioning during wound-directed migration of leukocytes in vivo. (A) The microtubules and the nucleus are visualized in the migrating leukocytes shown in Fig. 4. The white arrows indicate direction of migration. Outlines of cells are drawn with a grey line. Scale bars = 10 μm. (B) Rose diagrams mapping the orientation of the MTOC and the respective spatial frequency in migrating myeloid cells (PKC-ζ-WT: 265 counts, 7 leukocytes in 6 larvae, PKC-ζ-KW: 347 counts, 7 leukocytes in 5 larvae, PAR-6-NT: 235 counts, 7 leukocytes in 5 larvae, PAR-3-aPKCBR: 307 counts, 7 leukocytes in 5 larvae). Grey areas indicate “front” or “back” orientation as in Fig. 3. (C) Comparison of MTOC perinuclear mobility in migrating myeloid cells as described in Fig. 3D (PKC-ζ-WT: 7 leukocytes in 6 larvae, PKC-ζ-KW: 7 leukocytes in 5 larvae, PAR-6-NT: 7 leukocytes in 5 larvae, PAR-3-aPKCBR: 7 leukocytes in 5 larvae; ** p < 0.01, *** p < 0.001, **** p < 0.0001 two-tailed unpaired t test). See also supplementary material Table S1 and Movie 7.

Fig. 6. Rho-kinase-dependent actomyosin contraction is required for MTOC dynamic positioning in leukocytes migrating to wounds in vivo. (A) F-actin (upper and middle panels), the microtubules and the nucleus (upper and lower panels) are visualized in control or Y-27632-
treated transgenic larvae generated as in Fig. 2. Frames from representative movies of migrating myeloid cells in wounded tailfins are shown. The white arrows indicate direction of migration. The arrowheads point to F-actin accumulation in the trailing edge of the cell. Outlines of cells are drawn with a grey line. Scale bar = 10 μm. (B) Comparison of histograms of F-actin oscillatory frequencies in the “back” region of the cell (Ranges (s⁻¹) as shown in Fig. 2E). Data are expressed as means ± SEM of all analyzed cells (CTR: 8 leukocytes in 4 larvae, Y-27632: 7 leukocytes in 3 larvae; * p < 0.05, ** p < 0.01, *** p < 0.001, two-tailed paired t test). (C) Comparison of MTOC mobility as described in Fig. 3D (CTR: 7 leukocytes in 5 larvae, Y-27632: 6 leukocytes in 4 larvae; ** p < 0.01 two-tailed unpaired t test). (D) Rose diagrams mapping the orientation of the MTOC and its spatial frequency (CTR: 230 counts, 7 leukocytes in 5 larvae, Y-27632: 524 counts, 6 leukocytes in 4 larvae). Grey areas indicate “front” or “back” orientation as in Fig. 3. (E and F) The strength of the main frequency of F-actin oscillations in the “back” region (E) is plotted against directional speed ratio. Shown is the normalized amplitude of the main frequency peak in the Fourier spectra (44 leukocytes, R Spearman = 0.5149). MTOC perinuclear mobility (F) is plotted against directional speed ratio (41 leukocytes, R Spearman = 0.5416). *** p (two-tailed) < 0.001. See also supplementary material Fig. S3E,F, Table S1 and Movies 8,9.

**Fig. 7. The catalytic activity of PKC-ζ is essential for its polarized localization in leukocytes migrating in vivo.** (A) Top panel, mCherry and GFP-PKC-ζ are visualized in TG(FmpoP::mCherry) larvae transiently expressing GFP-PKC-ζ in myeloid cells. Bottom panel, ratiometric GFP-PKC-ζ/mCherry images were generated using mCherry as a volumetric control. (B) Schematics of 2D analysis on protein asymmetry during cell migration. Black dots indicate the cell centroid shown at three consecutive time points and the dashed black arrow indicates the direction of migration. The asterisk represents the fluorescence center of ratiometric signal and the dashed red arrow from the cell centroid to the fluorescent center is the asymmetry vector. The angle α between the asymmetry vector and the direction of migration (blue arc) is defined the asymmetry–migration angle, which approaches 180° for a protein that is located at the “back” of the cell. (C) Top panel, mCherry and GFP-PKC-ζ are visualized in TG(FmpoP::mCherry) transgenic larvae transiently expressing GFP-PKC-ζ alone or together with PKC-ζ-WT or PKC-ζ-KW in myeloid cells. Bottom panel, ratiometric GFP-PKC-
ζ/mCherry images were created. (D) Top panel, mCherry and GFP-PKC-ζ are visualized in control or Y-27632-treated transgenic larvae established as in (A). Bottom panel, ratiometric images GFP-PKC-ζ/mCherry were generated. (E and F) Histograms show the polarized distribution of ratiometric GFP-PKC-ζ/mCherry images in migrating cells assessed using the asymmetry–migration angles. Data are expressed as means ± SEM of all analyzed cells (CTR: 15 leukocytes in 4 larvae, PKC-ζ-WT: 8 leukocytes in 4 larvae, PKC-ζ-KW: 10 leukocytes in 3 larvae, Y-27632: 11 leukocytes in 4 larvae; * p < 0.05 *** p < 0.001, **** p < 0.0001, two-tailed unpaired t test). Frames from representative movies of migrating myeloid cells in wounded tailfins are shown. The white arrows indicate direction of migration. Outlines of cells are drawn with a grey line. Scale bars = 10 μm. See also supplementary material Movies 10,11.

Fig. 8. PKC-ζ regulates RhoA activity in leukocytes migrating to wounds in vivo. (A) RhoA activity is visualized in TG(FmpoP::mCherry) larvae transiently expressing cytosolic RhoA-FRET biosensor together with PKC-ζ-WT or PKC-ζ-KW in myeloid cells. Ratio images of YFP/CFP emission for each cell are shown. (B) Average activation level of RhoA (mean emission ratio of YFP/CFP for the entire cell during migration) in wound-activated leukocytes. Data are expressed as means ± SEM of all analyzed cells (PKC-ζ-WT: 25 leukocytes in 15 larvae, PKC-ζ-KW: 15 leukocytes in 5 larvae; ** p < 0.01, two-tailed unpaired t test). Frames from representative movies of migrating myeloid cells in wounded tailfins are shown. The white arrows indicate direction of migration. Outlines of cells are drawn with a white line. Scale bars = 10 μm. See also supplementary material Movie 12. (C) A model illustrating the mechanism by which the PAR complex controls wound-directed leukocyte migration in vivo. PAR complex co-ordinately controls Rho-dependent F-actin dynamics and MTOC perinuclear mobility to support the persistent migration of leukocytes to wounds.
Fig. 1

A

[Image of Fig. 1A showing a diagram of molecular interaction and experimental setup]

B

[Diagram showing molecular domains and interactions]

C

[Diagram showing different protein constructs]

D

[Graphs showing directional speed and straightness ratios]

E

[Graph showing speed ratios]

F

[Graph showing straightness ratios]

G

[Graph showing directional speed ratios]
**Fig. 2**

**A**

![Graph showing speed (μm/min) for memYFP and H2B²/EB3²/Lifeact² with n.s. for both categories.](image)

**B**

![Graph showing straightness for memYFP and H2B²/EB3²/Lifeact² with n.s. for both categories.](image)

**C**

![Images of fluorescence over time showing CYCLE 1 and CYCLE 2 with RFP-LIFEACT, EB3-EGFP, and H2B-CFP.](image)

**D**

![Diagram showing motion at time = -1, time = 0, and time = +1 with arrows indicating BACK, CENTER, and FRONT.](image)

**E**

![Graph showing fluorescence percentage (% Fluorescence) over time (s) for CYCLE 1, CYCLE 2, and CYCLE 3 with BACK and FRONT lines.](image)

**F**

![Graphs showing Amplitude (a.u.) vs. f (s⁻¹) for BACK and FRONT with main peak and main range highlighted.](image)

**G**

![Graphs showing Relative Amplitude vs. f (s⁻¹) for BACK and FRONT with main range highlighted.](image)
Fig. 4

A

0s 30s 60s 90s 120s 150s 180s 210s 240s 270s 300s
PKC-ζ-WT
PKC-ζ-KW
PAR-6-NT
PAR-3-aPKCBR

B

% Fluorescence
PKC-ζ-WT
PKC-ζ-KW
PAR-6-NT
PAR-3-aPKCBR

C

PKC-ζ-WT
PKC-ζ-KW
PAR-6-NT
PAR-3-aPKCBR

D

Relative Amplitude (normalized to main peak)
**Fig. 6**

A

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B

**CTR**

**Y-27632**

C

D

E

F

Spearman correlation:

\[ p = 0.0003 \]
Fig. 7

A

mCherry 0s 15s 30s 45s 60s
PKC-ζ

PKC-ζ/mCherry

0.15 0.00

B
time = -1 time = 0 time = +1
Fluorescent weighted center
Asymmetry-migration angle (α)

C

PKC-ζ-WT PKC-ζ-KW

D

CTR Y-27632

E

% Events
Asymmetry-migration angle (degrees)
CTR PKC-ζ-WT PKC-ζ-KW
0-45 45-90 90-135 135-180

F

% Events
Asymmetry-migration angle (degrees)
CTR Y-27632
0-45 45-90 90-135 135-180
Fig. 8

A

RhoA-FRET - RhoA activation

Ratio: YFP/CFP

PKC-ζ-WT

PKC-ζ-KW

B

Mean Emission ratio: YFP/CFP

C

PAR complex
(PAR-6/catalytically active aPKC/PAR-3)

RhoA/RhoK

MTOC perinuclear mobility

F-actin front-back waves

In vivo leukocyte directional migration to wounds