Rac regulates PtdInsP₃ signaling and the chemotactic compass through a redox-mediated feedback loop

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Introduction

Neutrophils are important mediators of the innate immune system and are rapidly recruited from the circulation to sites of infection to eliminate pathogenic threats. Key to this recruitment is the release of chemoattractants by infected host tissue or pathogens, which subsequently form a chemical gradient that attracts neutrophils to the appropriate sites. In response to a chemoattractant gradient, neutrophils polarize and form a leading edge pointing toward the chemoattractant source and a posterior structure called the uropod. Actin polymerization within the leading edge drives up-gradient protrusion, whereas myosin activity in the uropod detaches the rear of the cell. The proper coordination of these polarized events is essential for directional migration and depends on the asymmetrical distribution of specific molecular determinants. A hallmark of neutrophil polarization is the asymmetrical accumulation of phosphatidylinositol(3,4,5)-trisphosphate [PtdIns(3,4,5)P₃] in the leading edge plasma membrane. How shallow gradients of chemoattractants trigger and maintain a much steeper intracellular gradient of PtdIns(3,4,5)P₃ is a critical question in the study of leukocyte chemotaxis. Our data demonstrate that the migration of neutrophils toward the chemoattractant N-formyl–L-methionyl–L-leucyl–L-phenylalanine depends on the generation of reactive oxygen species by the phagocytic NADPH oxidase (NOX2) and subsequent oxidation and inhibition of phosphatase and tensin homolog.

Methods

Abs and reagents

Rabbit polyclonal Ab against phosphorylated AKT (Thr308; #2965), total AKT (#9272), and PTEN (#9559) were purchased from Cell Signaling Technology. A mouse mAb against Rac was purchased from Upstate Cell Signaling Solutions. An mAb against CapZ (SB12.3) was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Percoll, (+)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), N-acetyl-L-cysteine (NAC), DTT, VO-OHpic, and fMLP were purchased from ThermoFisher Scientific. 

The online version of this article contains a data supplement.
Sigma-Aldrich. EZ-Link Iodoacetyl-PEG2-Biotin was purchased from Pierce (Thermo Fisher Scientific).

**Transgenic mice and cell preparation**

All procedures were carried out in accordance with the Guide for the Humane Use and Care of Laboratory Animals and were approved by the University of Toronto Animal Care Committee. Rac1-null, Rac2-null, Rac1/2-null, and wild-type primary neutrophils were isolated from BM as described previously.14,15 In short, mice were killed by CO2 inhalation and femurs and tibias were removed. BM cells were layered onto discontinuous Percoll gradients of 80%/65%/55%. Mature neutrophils were recovered at the 80%/65%/55% interface.

**Zigmond chamber analysis**

Analysis of chemotaxis was performed as described previously.16 Where indicated, cells were pretreated with inhibitors for 15 minutes at 37°C before adhering to BSA-coated coverslips. fMLP (1μM) was used as a chemoattractant and time-lapse video microscopy was used to image chemotaxing cells for 15 minutes (3 frames/min). Captured images were analyzed using cell-tracking software (Retrac Version 2.1.01 freeware).

**Live imaging**

Neutrophils were transfected with a plasmid encoding green fluorescent protein (GFP)—tagged pleckstrin homology (PH) domain of AKT (kind gift from Dr Sergio Grinstein, Hospital for Sick Children, Toronto, ON) using the Amaxa Nucleoporator as described previously.16 Imaging of transfected neutrophils and data analysis were performed as described previously.16,17 Briefly, a single region of interest (see Figure 4 in Magalhaes and Glogauer18) was masked (leading-edge front, cytosol or rear back) and the mean fluorescence intensity within that region was determined using the Velocity 4.2 platform. Ratios in which each cell were determined with the denominator for both ratios being the same cytosol mean fluorescence intensity.

**PTEN-activity assay**

Primary neutrophils were partially permeabilized with 0.2% octyl glucoside for 15 seconds at room temperature using the procedure from Glogauer et al.19 resuspended in HBSS, and treated with 100μM Trolox (or mock-treated) for 15 minutes at 37°C. Cells were then stimulated with 1μM fMLP for 1 minute and incubated with 1μM of the PTEN substrate 3-methylenephosphonate, diC8 (Echelon Biosciences). Control reactions were set up using identical treatments omitting substrate. Reactions were incubated for 30 minutes at room temperature before measuring the released phosphate using the Malachite Green phosphate detection assay according to the manufacturer’s protocol (Echelon Biosciences). A sodium phosphate standard curve was used to calculate the release of phosphate. Relative PTEN activity was calculated by subtracting the values of the samples with substrate minus background the background release (no substrate).

**TAT-protein transduction**

Murine wild-type Rac1 and Rac2 open reading frames were cloned into pTAT-HA containing a HIS tag (a kind gift from Dr G. Bokoch) using standard molecular techniques. Rac1-A27K (a kind gift from Dr E. Pick) was subcloned in pTAT-HA. Point mutations were introduced in the wild-type constructs using site-directed mutagenesis to obtain Rac1-T17N and Rac2-A27K. The resulting constructs were transformed into BL21(DE3)-pLysS (Stratagene). Protein expression and purification was performed as described previously.19 In short, 500-mL cultures were inoculated 1:100 and grown for 3–4 hours at 37°C. Protein expression was induced overnight at 28°C after induction with isopropyl β-D-1-thiogalactopyranoside (1mM). Cell pellets were resuspended in lysis buffer (100mM NaH2PO4, and 10mM Tris, and 8M urea) and sonicated briefly. Cell debris was removed by centrifugation and HIS-tagged proteins were isolated from cleared lysates using Ni-NTA Superflow columns (QIAGEN) according to the manufacturer’s protocol, and subsequently dialyzed for 24 hours 2× at 4°C against PBS using 15-mL Slide-a-Lyzer cassettes (Pierce Protein Research Prod-

ucts; ThermoFisher). Finally, proteins were concentrated in Pierce protein concentrators, and protein concentration and purity were assessed by SDS-PAGE followed by Coomassie staining. Control experiments and data from previous studies20,21 have verified that addition of TAT-Rac-WT rescues ROS generation and chemotaxis in leukocytes (data not shown).

**SDS-PAGE and immunoblotting**

Lysates were prepared in 4× Laemmli loading buffer, boiled for 10 minutes at 99°C, subjected to reducing SDS-PAGE, and transferred to nitrocellulose membranes. Each membrane was blocked and incubated with the indicated primary Ab according to the manufacturer’s specifications (1:2000 for Rac1 1:1000 for p-AKT and AKT, and 1:1000 for PTEN) at 4°C overnight. Membranes were washed 3 times for 10 minutes with TBS-T, followed by incubation with donkey anti-rabbit or sheep anti-mouse IgG peroxidase conjugates (Amersham Pharmacia Biotech). Ag-Ab complexes were visualized in X-ray film by enhanced chemiluminescence (ECL Plus; Amersham, GE Healthcare).

**Oxidative modification PTEN**

Oxidized and reduced forms of PTEN were detected by a modified method adapted from Silva et al.22 Cell pellets were resuspended in deoxygenated lysis buffer containing SDS (2%), Tris (50mM, pH 7.5), EDTA (0.5mM), iodoacetamide (50mM), and catalase (5000 u/mL), and incubated for 10 minutes in the dark. The insoluble fraction was sedimented by ultracentrifugation for 20 minutes at 175 000g at 8°C, and the soluble fraction was transferred to a new tube, mixed with nonreducing loading buffer, and separated by nonreducing SDS-PAGE. PTEN was detected by immunoblotting.

**Rac activation assay**

Active Rac1 was quantified using the PAK-binding domain pull-down assay as described previously.14

**CapZ quantification**

CapZ were measured as described in Sun et al.15 In short, 1 million murine neutrophils were permeabilized with 0.2% OG buffer (PHM buffer containing 10μM phallacidin, 42mM leupeptin, 10mM benzamidine, and 0.125mM aprotinin) and then stimulated with 1μM fMLP for 60 seconds. The supernatants and lysates of the remaining cells were analyzed by SDS-PAGE and subsequent immunoblotting for CapZ. The release of capping proteins was evaluated by the calculating the ratio of CapZ in the supernatant divided by CapZ in the total lysate, as determined by densitometry using ImageJ 1.32j software in 3 independent experiments.

**Results**

Neutrophils migrating toward a chemoattractant source display polarized ROS production at the front of the cell10 (supplemental Figure 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). Because PTEN is a known target of oxidation,12,13 we analyzed the redox state of PTEN by nonreducing SDS-PAGE. Stimulation of neutrophils with fMLP increased the fraction of oxidized PTEN by 32% ± 12%, and this effect could be blocked by pretreating cells with Trolox, a potent ROS scavenger23 (Figure 1A). Cells treated with H2O2 or lysates incubated with DTT were used as positive and negative controls for oxidative modification of PTEN, respectively. Interestingly, cells that lack the catalytic subunit (gp91) of the NADPH oxidase NOX224 also displayed reduced PTEN oxidation in response to fMLP (supplemental Figure 2). It has been reported that oxidation of PTEN inhibits enzymatic activity.12,13,22 To assess the impact of fMLP-induced oxidation on enzymatic activity, we measured dephosphorylation of the PTEN substrate 3-methylenephosphonate (diC8) in partially permeabilized neutrophils.19 diC8...
A mMLP - + + - - 
Trolox - - + + - 
H2O2 - + + - + 
DTT - + + + + 
Red Ox - + + + + 

B Control 100 50 0 P=0.006 
Control 125 100 75 50 25 0 P=0.002 
mlMLP 100 75 50 25 0 
mMLP + Trolox 125 100 75 50 25 0 

Figure 1. PTEN is oxidized and inhibited in response to mMLP. (A) Mouse neutrophils were preincubated with Trolox (100μM) or mock-treated for 10 minutes before exposure to 1μM mMLP or 1mM H2O2 for 1 minute. Lysates were analyzed by SDS-PAGE under nonreducing conditions and immunoblotted for PTEN to detect oxidized and reduced forms of PTEN. mMLP-induced oxidation of PTEN was prevented by Trolox. Treatment of lysate with DTT was used as a positive control for the reduced form. Densitometry was used to calculate the ratio between oxidized and reduced PTEN (n = 3) and the averages ± SD are shown. (B) Partially permeabilized neutrophils were preincubated with Trolox (100μM) or mock-treated for 10 minutes before stimulation with 1μM mMLP. A PTEN-enzymatic activity assay was performed with the soluble PTEN substrate diC8 for 30 minutes. Pi-release was quantified using a Malachite Green assay, and the means ± SD of 3 independent experiments are shown.

was dephosphorylated at a significant lower rate in cells treated with mMLP (53% ± 12%) compared with control untreated cells (Figure 1B). This decrease in activity was prevented by pretreating cells with Trolox, suggesting that the effect was due to oxidation-dependent events. We noted an inverse correlation between the fraction of oxidized PTEN and the dephosphorylation of diC8 (Figure 1A-B). Treatment with the selective PTEN inhibitor VO-OHpic25 demonstrated the specificity of the assay for PTEN activity (supplemental Figure 3).

Oxidative modification of PTEN and subsequent decreased activity could result in increased PtdIns(3,4,5)P3 levels. We quantified phosphorylation of AKT, a downstream target of PtdIns(3,4,5)P3 that is widely used as an indicator of PdIns(3,4,5)P3 generation.26 Stimulation of neutrophils with mMLP resulted in a robust increase in AKT phosphorylation (Figure 2A-B). Because a decrease in AKT activity by oxidation would enhance PtdIns(3,4,5)P3 accumulation, preventing oxidation by ROS scavengers would have the opposite effect: decreasing phosphorylation of AKT. Indeed, pretreatment of cells with the ROS scavengers NAC and Trolox decreased phosphorylation of AKT significantly (Figure 2A-B). To confirm that decreased AKT phosphorylation in response to ROS scavengers was because of decreased PtdIns(3,4,5)P3, we applied live-cell imaging using the PH domain of AKT fused to GFP (PH-AKT-GFP) as a reporter for subcellular PtdIns(3,4,5)P3 accumulation and, to a lesser extent, PtdIns(3,4)P2.27,28 In response to mMLP, PH-AKT-GFP was recruited to the leading edge in neutrophils (Figure 2C). However, the distinct asymmetrical accumulation rapidly dissipated upon treatment with NAC, suggesting that ROS are required for robust PtdIns(3,4,5)P3 levels at the front of the cell. mMLP stimulates ROS production by activating NOX2 in polymorphonuclear cells, and loss-of-function mutations in NOX2 lead to defective respiratory burst in neutrophils and macrophages, causing chronic granulomatous disease in humans.29 To determine whether phosphorylation of AKT depends on mMLP-mediated activation of NOX2, we used mice that are deficient in gp91, the catalytic subunit of NOX2.30 In neutrophils derived from C57/BL6 control mice, phosphorylation of AKT was strongly induced by mMLP (Figure 2D and supplemental Figure 4), whereas in Gp91−/− cells, phosphorylation remained at basal levels (Figure 2E and supplemental Figure 4). Intriguingly, activation of AKT in gp91−/− neutrophils could be (partially) rescued by the addition of exogenous H2O2, whereas in the presence of ROS scavengers (NAC and Trolox) phosphorylation of AKT was suppressed (Figure 2E). We conclude from these experiments that ROS produced by NOX2 play an important role in modulating PtdIns(3,4,5)P3 and its downstream target, AKT, upon mMLP stimulation.

Accumulation of PtdIns(3,4,5)P3 at the leading edge of migrating neutrophils has been implicated in regulating directional cell migration by mediating the polarized recruitment of factors required for local actin polymerization and cell advancement up the chemotactic gradient.3 Therefore, decreased PtdIns(3,4,5)P3 levels induced by ROS scavengers could affect directionality. To test this, neutrophil chemotaxis toward mMLP was analyzed using Zigmond chambers in the presence of the ROS scavenger NAC (Figure 3A,B,D). The majority of untreated control cells (74% ± 6%) migrated up the mMLP gradient. However, pretreatment of cells with NAC inhibited directional sensing significantly, with only 56% ± 4% of the cells migrating toward mMLP. We then tested directionality in neutrophils derived from gp91-deficient mice (Figure 3C-D). Only 49% ± 6% of the gp91−/− cells migrated toward mMLP, whereas 75% ± 6% of their wild-type counterparts (C57/BL6) displayed directional migration toward mMLP. Migration velocity was not affected in these experiments (data not shown). These data demonstrate that directional sensing in neutrophils requires ROS generated by NOX2. To demonstrate that ROS are important in directionality by inhibiting PTEN, we treated neutrophils with the ROS scavenger Trolox and the PTEN inhibitor VO-OHpic. Trolox inhibited directional migration of neutrophils to a similar extent as NAC (Figure 3B,E), whereas the PTEN inhibitor VO-OHpic did not alter directionality compared with untreated cells (Figure 3F). Cells treated with both Trolox and VO-OHpic did migrate normally toward mMLP. This demonstrates that inhibition of directionality by scavenging ROS can be overruled by inhibition of PTEN activity, supporting a role for redox regulation of PTEN in directional migration.

Because Rac is a principal regulator of NOX2 in neutrophils,30 it is possible that Rac regulates PtdIns(3,4,5)P3 levels through redox-mediated inhibition of PTEN. In earlier studies, we found that phospho-AKT levels were decreased in Rac1-null and, to a lesser extent, in Rac2-null neutrophils.14 Moreover, directional migration was completely lost in Rac1-null cells. This led us to
investigate the possibility that Rac1 is specifically required for activation of NOX2 during ROS-dependent directional migration. By making use of the Rac1 mutant (A27K), which is defective in p67phox binding,31,32 we were able to selectively block Rac1-mediated NOX2 activation while maintaining other Rac-effector functions. Rac1-null neutrophils were transduced with 200nM recombinant TAT-tagged wild-type Rac1, inactive Rac1-T17N, or Rac1-A27K before chemotaxis was analyzed using a Zigmond chamber (Figure 4 and supplemental Figure 5). Transduction of wild-type Rac1 completely rescued directionality toward fMLP in Rac1-null cells, whereas inactive Rac1-T17N did not. However, Rac1-A27K also rescued directional migration, suggesting that ROS generation required for directionality does not depend on Rac1 and that Rac1 has alternative essential function(s) in directionality that are not related to ROS signaling.15,33 To determine whether there is redundancy between Rac1 and Rac2 in ROS generation, we transduced neutrophils that are deficient for both Rac1 and Rac2 (double knockout) with combinations of Rac1-WT, Rac1-A27K, Rac2-WT, and Rac2-A27K (Figure 4B,D). Transduction efficiencies were verified (supplemental Figure 6). Transduction of the wild-type versions of both Rac isoforms or a single wild-type isoform in combination with the A27K version of the other isoform was able to rescue directionality in Rac1/2-null cells.

However, when both isoforms contained the A27K mutation, cells displayed random migration, confirming that Rac-mediated generation of ROS through at least one Rac isoform is required for directional sensing during chemotaxis. Because H$_2$O$_2$ has been reported to act as a chemoattractant for neutrophils in zebrafish34 and murine neutrophils,35 we investigated whether H$_2$O$_2$ could serve as an exogenous source of ROS to rescue directionality in Rac1/2-null cells. Indeed, Rac1/2-null cells transduced with Rac1-A27K and Rac2-A27K regained some ability to migrate toward a combined gradient of fMLP and H$_2$O$_2$, whereas fMLP or H$_2$O$_2$ alone was not sufficient for directional migration (Figure 4C-D).

To determine whether Rac-mediated ROS generation is important for inhibiting PTEN activity, we performed enzymatic activity assays using neutrophils from Rac1-null and Rac2-null mice. In agreement with the chemotaxis results, PTEN activity was increased in both Rac1-null and Rac2-null cells after fMLP stimulation, whereas activity was decreased in wild-type cells (supplemental Figure 7). This is in accordance with earlier data showing that phospho-AKT was decreased in Rac1-null and Rac2-null cells,14 which can be explained by increased PTEN activity. Indeed, cells deficient for Rac1/2 or gp91 display decreased oxidation of PTEN upon fMLP stimulation (supplemental Figure 2). We conclude from
Figure 3. ROS are required for direction migration. Neutrophil directional migration toward fMLP was determined using a Zigmond chamber. XY-plots represent the end points of migrating neutrophils in respect to the origin. (A) The majority of wild-type neutrophils migrate toward fMLP. (B) Neutrophils incubated with NAC (10mM) migrated in a random fashion. (C) Neutrophils lacking the NOX2 subunit Gp91 (defect in ROS production) also did not show directionality toward fMLP. (D) The average percentage of neutrophils migrating toward fMLP was calculated from 3 independent experiments ± SD. Neutrophils derived from C57/BL6 were used as control for gp91null cells. (E-H) Directionality of WT neutrophils toward fMLP was determined in the presence of (E) Trolox (100μM), (F) VO-OHpic (75nM) or (G) Trolox + VO-OHpic. VO-OHpic could rescue Trolox-mediated inhibition of directionality. The XY-plots represent the end points of migrating neutrophils in respect to the origin. (H) The average percentage of neutrophils migrating toward fMLP was calculated from 3 independent experiments ± SD.

Figure 4. Rac1 and Rac2 regulate ROS formation required for directional migration. Neutrophil directional migration toward fMLP was determined using a Zigmond chamber. XY-plots represent the end points of migrating neutrophils with respect to the origin. (A) Rac1-null control neutrophils exhibit random migration. TAT-mediated transduction (200nM) of Rac1-null cells with wild-type Rac1 was able to rescue directionality. Transduction with TAT-Rac1-A27K also rescued directionality toward fMLP. Rac1-T17N–transduced cells behaved similarly to Rac1-null control cells. (B) Rac1/2-null double-knockout cells were transduced with equal amounts (200nM each) of TAT-tagged Rac1 wild-type or A27K and Rac2 wild-type or A27K. All combinations except Rac1-A27K/Rac2-A27K were able to rescue the directional migration defect. (C) Rac1/2 double-knockout cells were transduced with TAT-Rac1-A27K and TAT-Rac2-A27K. Subsequently, the cells were exposed to an fMLP gradient or to a combined fMLP/H2O2 gradient. (D) The combined results of at least 3 experiments ± SD are presented in the bar diagram. Asterisks represent significance (P < .05). XY plots depict representative results of 2-3 independent experiments.
rulates actin filament–uncapping activity. Uncapping of actin filaments was assessed in neutrophils pretreated with Trolox or mock treated and stimulated. Upon stimulation with fMLP, cells were partially permeabilized and the capping protein CapZ was quantified in both supernatant and cell pellet. Upon stimulation with fMLP, CapZ was released from actin filaments (Figure 6A-B). However, when cells were treated with Trolox, fMLP-induced uncapping decreased significantly, demonstrating a requirement for ROS in uncapping. To confirm this, we quantified uncapping activity in gp91<sup>−/−</sup> cells and wild-type control cells (C57/BL6). fMLP induced uncapping of filaments in polymorphonuclear cells derived from C57/BL6, which could be inhibited by the ROS scavenger Trolox (Figure 6C). Moreover, exogenous addition of H<sub>2</sub>O<sub>2</sub> increased uncapping activity. In gp91<sup>−/−</sup> cells, the release of CapZ in response to fMLP was decreased compared with C57/BL6 wild-type cells (Figure 6C). Treatment with H<sub>2</sub>O<sub>2</sub> was able to partially restore uncapping activity. These experiments demonstrate that ROS generation and uncapping activity are functionally linked in fMLP-stimulated neutrophils. We therefore postulate a redox-regulated feedback mechanism in which PTEN activity is inhibited by ROS, which promotes the accumulation of PtdIns(3,4,5)P<sub>3</sub> and subsequent Rac activation and uncapping (Figure 6C).

**Discussion**

Our data support a model in which Rac-mediated NOX2 activation is required to inhibit PTEN, resulting in the accumulation of PtdIns(3,4,5)P<sub>3</sub>, and this positive feedback loop stimulates Rac activation itself and promotes directional migration in neutrophils (Figure 6C). The role of PTEN in chemotaxis has been somewhat controversial, with some studies suggesting a role for PTEN in directionality or migration and others finding PTEN to be dispensable for chemotaxis. These discrepancies likely reflect differences in experimental setup, such as in vitro versus in vivo studies, differences in chemoattractant gradients, and the activation state of cells. In addition, the time frame of observation is important. We analyzed directionality during the first 15 minutes after fMLP exposure. Indeed, PI3K activity was shown to be specifically important during the initial polarization of neutrophils, but dispensable during later phases of migration. Furthermore, parallel PtdIns(3,4,5)P<sub>3</sub>-independent mechanisms, such as the p38 MAPK pathway, add additional complexity to the signaling events regulating directional migration in neutrophils. We propose a model in which active PTEN would normally negatively contribute to regulation of directional sensing by blocking the development/maintenance of the PtdIns(3,4,5)P<sub>3</sub> gradient at the leading edge. PTEN therefore acts as the "brake" to PI3K’s "accelerator" role. Our data support the conclusion that ROS are required at the earliest phase of chemotactic compass activation to deactivate PTEN, which is initially distributed throughout the cytoplasm before cell polarization. We propose that the initial activation of the chemotactic compass—the PtdIns(3,4,5)P<sub>3</sub> gradient—requires ROS-mediated inhibition of PTEN activity to allow for the initial early development of the PtdIns(3,4,5)P<sub>3</sub> gradient and the subsequent directional sensing. Our data using the PTEN inhibitor to rescue activation of the chemotactic compass when ROS are sequestered or their generation is blocked support our conclusion that ROS-mediated deactivation of PTEN is required for initial chemotaxis directional sensing.

Feedback loops are very common in biologic systems and can amplify internal or external signals. It has been proposed that...
To elucidate the role of Rac GTPases in ROS-mediated endogenously produced ROS and trigger additional signaling, actual subcellular concentrations and localization might differ from the NOX2 phenotype. However, it is important to note that although we used relatively low concentrations of exogenous H₂O₂, actual subcellular concentrations and localization might differ from endogenously produced ROS and trigger additional signaling pathways. To elucidate the role of Rac GTPases in ROS-mediated PtdIns(3,4,5)P₃ accumulation, we used Rac-effector mutants that are unable to activate NOX2. Although Rac1 is required for the chemotactic compass in neutrophils and Rac2 is mainly responsible for actin polymerization and ROS generation, we did not identify a unique role for Rac1 in activating NOX2 in directional migration (Figure 4). Instead, Rac1 and Rac2 were both able to activate the ROS generation required for directional sensing and are therefore upstream regulators of PtdIns(3,4,5)P₃. This might not be entirely surprising, because it has been shown that both Rac1 and Rac2 can activate NOX2, and that in Rac2-null neutrophils, Rac1 is still able to generate ROS albeit at lower levels. The fact that Rac1-null cells are defective in directional migration is therefore due to isoform-specific functions that are independent of the ability of Rac1 to activate NOX2. Because most guanine nucleotide exchange factors for Rac require PtdIns(3,4,5)P₃, their activation, Rac GTPases are also downstream effectors of PtdIns(3,4,5)P₃. The implication that downstream Rac activation consequently depends on ROS was indeed supported by our data (Figure 5). Moreover, fMLP-induced uncapping of actin filaments is a downstream event of Rac1 and is also inhibited by scavenging of ROS. These observations support a dual role of Rac in chemotaxis, both upstream (activation of NOX2) and downstream of ROS. These observations support a dual role of Rac in chemotaxis, both upstream (activation of NOX2) and downstream of ROS. These observations support a dual role of Rac in chemotaxis, both upstream (activation of NOX2) and downstream of ROS.

Figure 6. ROS signaling is required for uncapping activity. (A) Uncapping of actin filaments was measured by the fMLP-induced release of CapZ from partially permeabilized neutrophils. Cells were pretreated with Trolox or mock-treated for 15 minutes before stimulation with 1μM fMLP for 1 minute. Cells and supernatant were separated by centrifugation and analyzed by SDS-PAGE and immunoblotting for CapZ. (B) CapZ in pellets and supernatant were quantified by immunoblotting and subsequent densitometric analysis. Uncapping activity was quantified in 3 independent experiments by determining the ratio between CapZ in supernatant and pellet. The bar diagram depicts the averages ± SEM (n = 3). (C) CapZ-uncapping experiments were performed as described above using neutrophils from Gp91⁻/⁻ and C57/BL6 control mice. Cells were stimulated with fMLP (1μM) and H₂O₂ (50μM) and pretreated with Trolox (100μM) where indicated. Bar diagrams depict averages ± SD (n = 3). Asterisks indicate significance, P < .05. (D) Model describing the redox-mediated amplification loop. Activation of Rac induces ROS formation by NOX2, which oxidizes PTEN. The decrease in PTEN activity results in increased PtdIns(3,4,5)P₃ levels, facilitating guanine nucleotide exchange factor activity toward Rac. Subsequent Rac activity induces uncapping of actin filaments and local actin polymerization required for directional migration.

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Authorship
Contribution: J.W.P.K. conceived of the project and prepared the manuscript and figures; C.S. and J.W.P.K. performed all experiments except the live-imaging experiments; M.A.O.M. performed the live-imaging experiments; and M.G. conceived of and supervised the project and revised the manuscript.

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Rac regulates PtdInsP₃ signaling and the chemotactic compass through a redox-mediated feedback loop

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