

# Rab27a-mediated protease release regulates neutrophil recruitment by allowing uropod detachment

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## Summary

Neutrophil migration is vital for immunity and precedes effector functions such as pathogen killing. Here, we report that this process is regulated by the Rab27a GTPase, a protein known to control granule exocytosis. Rab27a-deficient (Rab27a KO) neutrophils exhibit migration defects *in vitro* and *in vivo*, and live-cell microscopy suggests that delayed uropod detachment causes the migratory defect. Surface expression of CD11b, a key adhesion molecule, is increased in chemokine-stimulated Rab27a KO neutrophils compared with the control, suggesting a turnover delay caused by a defect in elastase secretion from azurophilic granules at the rear of bone marrow polymorphonuclear leukocytes (BM-PMNs). We suggest that Rab27a-dependent protease secretion regulates neutrophil migration through proteolysis-dependent de-adhesion of uropods, a mechanism that could be conserved in cell migration and invasion.

**Key words:** Rab27a, Chemotaxis, Cell migration, Neutrophil, Uropod

## Introduction

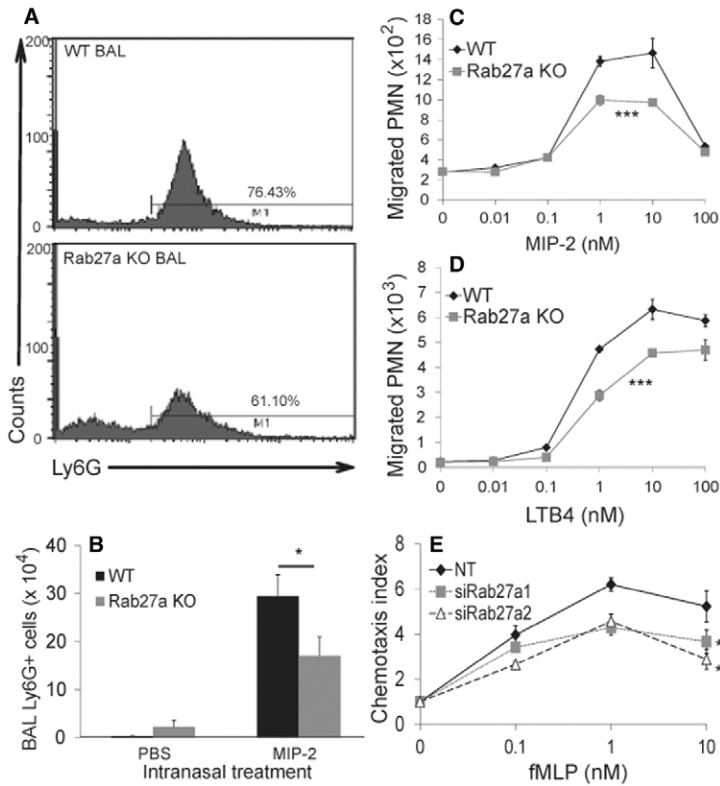
The small GTPase Rab27a regulates transport and exocytosis of secretory organelles in a variety of cell types e.g. melanosomes in melanocytes and lytic granules in cytotoxic T lymphocytes (CTLs) (Izumi, 2007; Tolmachova et al., 2004). Consistent with this, loss of Rab27a [in Griscelli syndrome type 2 (GS-2) patients and the ashen mutant mouse] results in partial albinism and immune dysfunction due to defects in pigment transport and lytic granule exocytosis in CTLs. Organism-wide expression studies highlight the abundance of Rab27a in cells of haematopoietic lineage e.g. mast cells, B cells, dendritic cells, eosinophils and neutrophils (Fukuda, 2008).

Neutrophils are a vital element of the innate immunity against bacterial infection (Nathan, 2006). Under homeostatic conditions neutrophils are abundant in the blood (35% of circulating leukocytes) but upon infection they rapidly extravasate and migrate through tissue to the infection site in response to a gradient of chemoattractants. Migrating neutrophils exhibit a polarised phenotype, and whereas integrin-mediated adhesion at the leading edge is crucial for migration, detachment through de-adhesion of the uropod is necessary for the cell to move forwards. The molecular mechanisms regulating this de-adhesion process are poorly understood, although there is evidence that it might involve ROCK (Alblas et al., 2001). Several studies indicate that Rab27a regulates the secretion of azurophilic granules at high chemoattractant concentrations; however, the

role of Rab27a-mediated azurophilic granule exocytosis in cell migration has not been investigated (Brzezinska et al., 2008; Munafó et al., 2007).

## Results and Discussion

To investigate the impact of Rab27a deficiency on neutrophil recruitment *in vivo*, the ELR-motif-containing CXC chemokine macrophage inflammatory protein (MIP)-2 was administered intranasally, and neutrophil recruitment into the bronchoalveolar fluid (BALF) was monitored in Rab27a-deficient ashen mice (Rab27a KO) and age and sex-matched C57BL/6 control (WT) mice. As expected, MIP-2 treatment led to a significantly greater number of Ly6G-positive neutrophils in the BALF as compared with vehicle (PBS)-treated mice of both genotypes (WT  $0.26 \pm 0.1$  versus  $29.43 \pm 4.5 \times 10^4$  cells, Rab27a KO  $2.26 \pm 1.36$  versus  $16.99 \pm 3.92 \times 10^4$  cells) (Fig. 1B). However, the effect of MIP-2 was significantly reduced in Rab27a KO compared with WT, both in terms of the number of BAL neutrophils (WT,  $29.43 \pm 4.5 \times 10^4$  cells versus Rab27a KO,  $16.99 \pm 3.92 \times 10^4$  cells) (Fig. 1B) and the proportion of BAL neutrophils (WT,  $76.43 \pm 4.09\%$  versus Rab27a KO,  $61.10 \pm 4.61\%$ ) (Fig. 1A). This suggests that Rab27a regulates chemokine-driven neutrophil migration. To test this directly we purified bone marrow polymorphonuclear leukocytes (henceforth BM-PMNs) of Rab27a KO and WT mice and tested their ability to migrate *in vitro*. Consistent with *in vivo* data (Fig. 1A,B) we observed a significant decrease in migrated Rab27a KO versus WT BM-PMNs in response to both the chemokine MIP-2 and the classical chemoattractant LTB<sub>4</sub>, over a range of concentrations (Fig. 1C,D). Similar results were observed in assays measuring chemoattractant-driven N-formyl-methionine-leucine-phenylalanine (fMLP) migration of Rab27a-knockdown



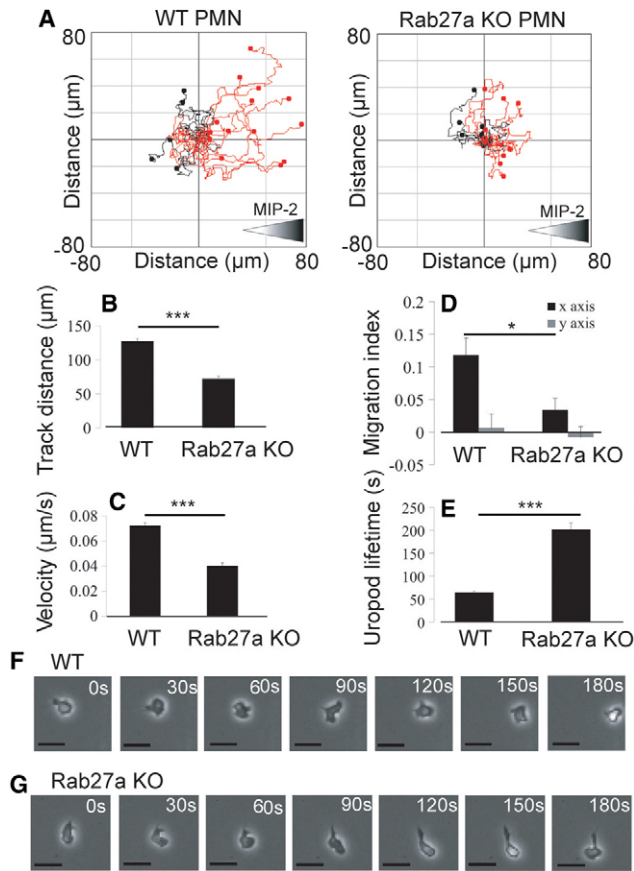
**Fig. 1. Rab27a promotes migration of neutrophils in vivo and in vitro.** (A) Leukocytes recruited to the bronchoalveolar space of WT and Rab27a KO mice treated intranasally with MIP-2; cells were stained with antibody against Ly6G and analysed by flow cytometry. Numbers indicate Ly6G-positive cells as a percentage of the total cell number. (B) Neutrophils recruited to the bronchoalveolar space of WT mice and Rab27a KO mice treated with MIP-2. Data represent the mean  $\pm$  s.e.m. ( $n=5-7$  for each treatment and genotype of mice). Transwell migration of WT versus Rab27a KO BM-PMN towards (C) MIP-2 or (D) LTB4 concentrations after 30 minutes, and (E) *Rab27a*-knockdown (siRab27) versus non-targeting (NT) control transfected differentiated HL-60 cells towards fMLP after 60 minutes. Data represent the mean from three independent experiments ( $n=3$  for each treatment and genotype of mice BM-PMN and knockdown HL-60 cells). \* $P \leq 0.05$ , \*\*\* $P \leq 0.001$  [Student's *t*-test (B) and two-way ANOVA (C-E)].

(achieved by using small interfering RNAs) versus control DMSO-differentiated HL-60 human neutrophils (Fig. 1E; supplementary material Fig. S1).

To understand the basis of the Rab27a KO migration defect we used video microscopy and cell tracking to record and compare the movement of WT and Rab27a KO BM-PMNs migrating towards a MIP-2 source in a Zigmond chamber (Fig. 2). We observed that both migration distance and velocity were significantly reduced in Rab27a KO compared with WT BM-PMNs (Fig. 2A–C). Analysis of the directionality of movement confirmed that migration both parallel to (shown on the *x*-axis, representing chemotaxis) and perpendicular to (shown on the *y*-axis, representing random migration and chemokinesis) the chemokine gradient was reduced in Rab27a KO versus WT BM-PMNs (Fig. 2D). However, the overall low level of random migration meant that it was only possible to confirm that chemotaxis towards chemokine was significantly impaired in the mutant compared with that in the control cells (Fig. 2D). Interestingly, a similar phenotype has been reported in a study that examined chemotaxis using neutrophils isolated from GS patients (Kurugöl et al., 2001). Close inspection of cell morphology during migration revealed that both WT and Rab27a KO BM-PMNs polarised successfully and made membrane protrusions at the front of the cell (Fig. 2F,G). However, we observed a striking delay in uropod release in migrating Rab27a KO compared with WT BM-PMNs (Fig. 2E–G; supplementary material Movies 1–4). In addition, inhibition of uropod release in Rab27a KO BM-PMNs was often accompanied by a loss of polarity and migration arrest (supplementary material Movies 3,4), which might explain the observed defects in directionality. These observations suggested that Rab27a KO BM-PMNs initially responded normally to chemoattractant by

polarising towards its source but that their subsequent movement was then retarded owing to delayed uropod release. Consistent with this, flow cytometry analysis revealed that surface levels of the MIP-2 specific receptor (CXCR2) and chemoattractant triggered F-actin polymerisation, a hallmark of the neutrophil response to chemoattractant, were similar in Rab27a KO and WT BM-PMNs (supplementary material Fig. S2a–d).

Serine proteases (elastase, proteinase 3 and cathepsin G) modulate PMN chemotaxis and inflammation (Kessenbrock et al., 2008; Lee and Downey, 2001). It is generally considered that they promote migration by cleaving the extracellular domain of adhesion molecules, thereby allowing dynamic cycles of PMN attachment and detachment in migrating cells (Pham, 2006). A recent study has shown that inhibition of serine-protease-dependent CD11b cleavage results in a uropod elongation and detachment phenotype resembling that of Rab27a KO BM-PMNs (Fig. 2E,F) (Zen et al., 2011). That study also reported that the elastase accumulated at the uropod of migrating PMNs, suggesting a model of protease release at the rear of the cell allowing localised cleavage of the extracellular domain of CD11b leading to uropod detachment and net forwards migration. We therefore hypothesised that uropod release and chemotaxis defects in Rab27a KO BM-PMNs results from an impairment of serine protease secretion and adhesion molecule cleavage at the rear of migrating PMNs. Consistent with this, by using fluorescence microscopy, we observed that EGFP–Rab27a-positive structures were enriched close to the rear of the cell in migrating BM-PMNs derived from EGFP–Rab27a-expressing transgenic mice (Fig. 3A–D). CD11b is transiently upregulated at the cell surface in response to chemokine stimulation (Zen et al., 2011). We observed that the chemokine-stimulated increase in CD11b expression is sustained on neutrophils in Rab27a KO



**Fig. 2. Rab27a regulates neutrophil migration by controlling uropod release.** (A) Tracks of WT and Rab27a KO BM-PMN migration measured from stacks of images taken every 30 seconds for 30 minutes in a Zigmond chamber with medium alone added to the left well and 10 nM MIP-2 added to the right well. Black and red tracks indicate cells with net leftwards or rightwards movement respectively. Migration distance (B), velocity (C) *x*- and *y*-migration index (D) and uropod lifetime (E) of WT and Rab27a KO BM-PMN. Data represent the mean  $\pm$  s.e.m. from 25 random cells per experiment from four independent experiments ( $n=4$  for each genotype of BM-PMNs). Still images of WT (F) and Rab27a KO (G) BM-PMN migration over a course of 180 seconds (from supplementary material Movies 2 and 4). Scale bars: 10  $\mu$ m. \* $P \leq 0.05$ , \*\*\* $P \leq 0.001$  (Student's *t*-test).

compared with WT BM-PMNs, suggesting that there is a defect in its downregulation (Fig. 3E). To test this hypothesis more directly we used a broad-spectrum inhibitor of serine proteases (AEBSF) to determine whether protease activity is required for neutrophil migration. At low doses (10  $\mu$ M) AEBSF reduced migration of Rab27a KO and WT BM-PMNs to a similar extent, indicating that serine protease activity facilitates migration in both cases (Fig. 3F). Strikingly at higher doses (50  $\mu$ M) the effect of AEBSF on migration was more pronounced on WT compared with Rab27a KO BM-PMNs. Indeed at this level, AEBSF reduced the number of migrating WT BM-PMNs to a level similar to that observed in Rab27a KO cells (Fig. 3F). These findings suggest that the Rab27a KO chemotaxis deficit results from defective release of a subset of serine proteases. Consistently, studies of disease models whose symptoms resemble Rab27a deficiency (GS-2 and ashen) such as the

beige Chediak–Higashi syndrome (CHS) model mouse and the ‘gray collie’ canine Hermansky–Pudlak syndrome type 2 (HPS-2) model, indicate that defects in elastase trafficking are responsible for PMN dysfunction in these animals (Benson et al., 2003; Zen et al., 2011). To test the hypothesis that reduced release of elastase underlies the defective migratory phenotype of the Rab27a KO BM-PMNs, we measured Rab27a KO and WT BM-PMN chemotaxis in the presence of the specific elastase inhibitor, elastatinal (Fig. 3G). Elastatinal caused a 30% inhibition of migration of WT BM-PMNs but no effect on the migration of Rab27a KO BM-PMNs, which is in contrast to the effect of AEBSF in the same cells (Fig. 3F,G). These observations support the hypothesis that migration defects in Rab27a KO BM-PMNs result from defects in elastase secretion.

In conclusion, we found that Rab27a regulates PMN chemotaxis *in vitro* and *in vivo* by regulating uropod detachment. We observed that Rab27a-positive membranes localised at the rear of migrating PMNs, suggesting that this protein has a role in cargo delivery to sites proximal to the uropod. Furthermore, we found that cell surface CD11b turnover was reduced in chemokine-stimulated Rab27a KO BM-PMNs and that Rab27a regulates the release of a subset of serine proteases. We propose that Rab27a regulates BM-PMN migration by stimulating elastase release from azurophilic granules at the rear of BM-PMNs, thereby allowing localised proteolysis of the extracellular domain of CD11b resulting in uropod detachment and net forwards movement of the cell. In view of recent data highlighting the role of Rab27a in T cell migration (Colvin et al., 2010) and Rab27b in breast cancer cell invasion (Hendrix et al., 2010), we suggest that Rab27 might play a general role in regulating cell migration by allowing localised detachment at the rear of migrating cells.

## Materials and Methods

### Mouse strains and purification of neutrophils

The ashen *Rab27a<sup>ash/ash</sup>* (Rab27a KO) and transgenic EGFP–Rab27a mice were as described previously (Barral et al., 2002; Tolmachova et al., 2004). Controls were age- and sex-matched C57BL/6 animals. All animals were treated humanely in accordance with the UK Home Office Regulations under PPL 70/7078. BM-PMNs were purified as described previously (Wengner et al., 2008). Typical preparations contained above 80% BM-PMNs.

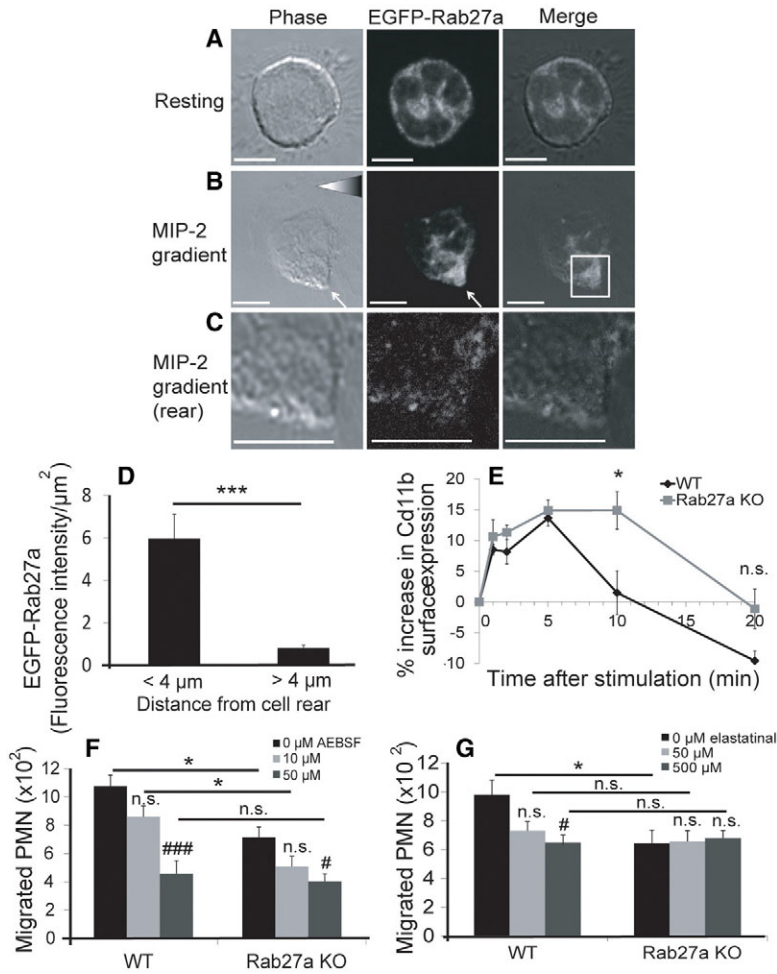
### Antibodies, immunoblot and flow cytometry

For immunoblot analysis, mouse monoclonal anti-Rab27a antibody (4B12) (Barral et al., 2002), anti-calnexin antibody (Stressgen) and HRP-conjugated secondary antibodies (DAKO) were used as described previously (Barral et al., 2002). Cells were lysed by resuspension in 50 mM Tris-HCl pH 7.4, 1  $\times$  protease inhibitors (Roche), 2% (w/v) SDS, and passed through a 26G needle 10 times on ice and processed for immunoblotting as previously described (Barral et al., 2002). For flow cytometry using a FACScalibur cytometer, phycoerythrin (PE)-conjugated anti-Ly6G (BD Biosciences), allophycocyanin (APC)-conjugated CXCR2 (R&D Systems), Alexa-Fluor-647-conjugated anti-Cd11b (BD Biosciences) antibodies were used at 1:100 dilution and Alexa-Fluor-647-conjugated phalloidin (Molecular Probes) used at 1:1000 dilution. For surface staining,  $2 \times 10^5$  BM-PMN cells were washed twice and resuspended in rat anti-Fc $\gamma$ R1I/RIII antibody (BD biosciences), then incubated with fluorophore-conjugated antibodies for 30 minutes on ice. For F-actin staining,  $3 \times 10^5$  BM-PMN cells were fixed for 30 minutes at room temperature in 4% paraformaldehyde (PFA) and permeabilised with 0.5% saponin for 15 minutes prior to Alexa-Fluor-647–phalloidin staining. For Cd11b measurements,  $2 \times 10^5$  BM-PMNs were resuspended in RPMI1640 plus 1% BSA, stimulated with 1 nM MIP-2 at 37°C for indicated times and placed on ice prior to Cd11b staining.

### Cell culture, transfection and differentiation

The human promyelocytic leukaemia cell line HL-60 (American Type Culture Collection) was cultured in DMEM, 20% (v/v) fetal bovine serum and 10 units/ml penicillin-streptomycin at 37°C under 5% CO<sub>2</sub>. HL-60 cells were nucleofected with non-targeting siRNA (Dharmacon) or two Rab27a-specific siRNA





**Fig. 3. Rab27a localises to the uropod in migrating neutrophils, and Rab27a-dependent secretion of serine proteases promotes neutrophil migration.** EGFP-Rab27a BM-PMNs from transgenic EGFP-Rab27a mice were plated onto coverslips for 20 minutes at 37°C, and fixed with 4% PFA (A) or placed into a MIP-2 chemokine gradient in a Zigmond chamber for 5 minutes and then fixed (B). (C) Detail of cell rear from B. Scale bars: 5  $\mu\text{m}$ . (D) Quantification of EGFP-Rab27a localisation close to (<4  $\mu\text{m}$ ) and far from (>4  $\mu\text{m}$ ) the cell rear in BM-PMNs migrating in a MIP-2 gradient. \*\*\* $P \leq 0.001$  (Student's *t*-test). Data are representative of three experiments ( $n=30$  cells). (E) Flow cytometry analysis of the increase in Cd11b surface expression in WT or Rab27a KO BM-PMN after stimulation with 1 nM MIP-2 for the indicated times. Transwell chemotaxis of WT and Rab27a KO BM-PMN towards 10 nM MIP-2 for 30 minutes in the presence of indicated concentrations of (F) AEBSF and (G) elastatinal. Data represent the mean  $\pm$  s.e.m. from four (D) or three (F,G) independent experiments ( $n=4$  or  $n=3$  for each genotype of mice BM-PMN) \* $P \leq 0.05$ , \*\* $P \leq 0.01$  (Student's *t*-test comparison of WT and Rab27a KO BM-PMN); # $P \leq 0.05$ , ### $P \leq 0.001$  (Student's *t*-test comparison of untreated and protease inhibitor treated BM-PMN). n.s., not statistically significant.

oligonucleotides using the Amaxa nucleoporator (Lonza) as described previously (Munafò et al., 2007). Nucleofected cells were then differentiated to mature neutrophils by suspension in medium containing 1.3% DMSO for 72 hours.

#### In vivo neutrophil recruitment assay

The recruitment of neutrophils to the bronchoalveolar space was performed as described previously (Fulton et al., 2002) with the following modifications. Mice were briefly anaesthetised with isoflurane and 0.8  $\mu\text{g}$  of recombinant murine MIP-2 (Peptrotech) or PBS was administered intranasally. 6 hours after challenge, mice were killed and the bronchoalveolar lavage collected. Neutrophils were identified as Ly6G<sup>high</sup> cells and by their characteristic high-side-scatter profile.

#### Transwell chemotaxis assay

Chemotaxis in vitro was measured as described previously (Chatterjee et al., 2005; Weller et al., 2005). BM-PMN or differentiated HL-60 cells ( $1 \times 10^5$ ) were placed on top wells of a chemotaxis plate with 3- $\mu\text{m}$  pores (Receptor Technologies, Adderbury, UK) and allowed to migrate towards recombinant murine MIP-2 (Peptrotech), LTB4 and fMLP (Sigma-Aldrich), at concentrations as indicated, for 30 minutes (or 1 hour for HL-60). Migrated cells were collected, resuspended in 200  $\mu\text{L}$  volume and counted for 30 seconds on the high setting using a FACScalibur system (BD Biosciences).

#### Zigmond chamber neutrophil chemotaxis assay

BM-PMNs ( $1 \times 10^5$ ) were resuspended in 50  $\mu\text{L}$  of RPMI plus 1% BSA and plated onto coverglass for 20 minutes at 37°C. Coverglass was washed and placed onto the inverted Zigmond chamber (Neuroprobe). The left well contained medium and the right contained 10 nM MIP-2. Brightfield images were taken every 30 seconds for 30 minutes using a 10 $\times$  objective on a widefield microscope (Zeiss Axiovert 200) with a heat-controlled stage (37°C). Stacks of images were analysed using ImageJ software (NIH) with the chemotaxis plug-in (Ibidi). Uropod lifetime measurements were derived by recording the elapsed time from uropod formation to release or disappearance. EGFP-Rab27a localisation was quantified using

ImageJ software by measurement of cell-associated fluorescence close to cell rear (<4  $\mu\text{m}$ ) and far from the cell rear (>4  $\mu\text{m}$ ). Fluorescence values were then normalised for the different surface areas by expressing the results as fluorescence units per unit of surface area ( $\mu\text{m}^2$ ).

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Supplementary material available online at

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