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Rab11 regulates cell–cell communication during collective cell movements

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Abstract

Collective cell movements contribute to development and metastasis. The small GTPase Rac is a key regulator of actin dynamics and cell migration but the mechanisms that restrict Rac activation and localization in a group of collectively migrating cells are unknown. Here, we demonstrate that the small GTPases Rab5 and Rab11 regulate Rac activity and polarization during collective cell migration. We use photoactivatable forms of Rac to demonstrate that Rab11 acts on the entire group to ensure that Rac activity is properly restricted to the leading cell through regulation of cell–cell communication. In addition, we show that Rab11 binds to the actin cytoskeleton regulator Moesin and regulates its activation *in vivo* during migration. Accordingly, reducing the level of Moesin activity also affects cell–cell communication, whereas expressing active Moesin rescues loss of Rab11 function. Our model suggests that Rab11 controls the sensing of the relative levels of Rac activity in a group of cells, leading to the organization of individual cells in a coherent multicellular motile structure.

Collective cell migration emerges as a fundamental mode of migration that is widely used during the development of multicellular organisms¹ and in pathological conditions such as cancer^{2–5}. In *Drosophila*, border cells are a powerful model to study collective cell migration allowing the combination of cell biology and genetics *in vivo*^{6,7}. During *Drosophila* oogenesis, border cells perform a stereotypic migration between the germ cells towards the oocyte^{8–10} (Fig. 1a). Directional information is provided by ligands of two

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AUTHOR CONTRIBUTIONS

G.E. and D.R. conceived the project and G.E. directed it. D.R. designed and performed FRET experiments, staining of border cells and egg chambers, quantified migration, and developed the method for determination of the orientation of the protrusions. D.R. and X.W. performed photoactivation experiments. C.L. developed mass spectrometry analysis and immunoprecipitations. D.R., D.J.M. and G.E. wrote the manuscript.

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COMPETING FINANCIAL INTERESTS

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receptor tyrosine kinases (RTKs), the platelet-derived growth factor/vascular endothelial growth factor receptor (PVR; the *Drosophila* homologue of vertebrate PDGF and VEGF receptors) and the epidermal growth factor receptor^{9,11–14} (EGFR). A signalling cascade activated by both RTKs leads to the activation of Rac1, which is both necessary¹⁵ and sufficient to direct border cell migration when activated focally through the formation of protrusions¹⁶. Recently, we have shown that border cell migration requires the small GTPases Rab5 and Rab11 that regulate trafficking through the early and the recycling endosome, respectively¹⁷. Moreover, we identified a genetic interaction between Rac1 and Rab11 (ref. 17). Although these data highlight the importance of endocytosis for collective cell migration, the mechanistic links between Rac and endocytosis remain unknown.

Dominant negative forms of Rab5 and Rab11 (Rab5S43N and Rab11S25N, hereafter Rab5SN and Rab11SN) share the same phenotype in terms of active RTK localization¹⁷ (Supplementary Fig. S1a–c); however, Rab5SN expression induces a more severe phenotype than Rab11SN in terms of distance migrated in the egg chamber¹⁷. This effect was not due to a loss of general polarity as the staining of apical/basal polarity markers¹⁸ was unaffected by the expression of Rab11SN or Rab5SN (Supplementary Fig. S1d–i). Thus, Rab5 and Rab11 might regulate RTK polarization through two independent mechanisms impacting differently on cluster morphology. To test this hypothesis, we first performed a phenotypic analysis on clusters expressing Lifeact fused to GFP. At the onset of migration, control clusters exhibit one main protrusion towards the leading edge (Fig. 1b). In contrast, *Rab11SN* expression induces the formation of numerous ectopic protrusions (Fig. 1b,c). This pattern is conserved later during the migration process because *Rab11SN*-expressing clusters produce more protrusions after 25–50% of migration when compared with control clusters (Fig. 1c). After expression of *Rab5SN* we observed an absence of protrusion, a phenotype also observed when Rac activity is abolished by expression of dominant-negative Rac (RacN17, Fig. 1b,c). To quantify the spatial distribution of protrusions, we designed a radar map dividing the cluster into 8 sectors (Supplementary Fig. S2). At the onset of migration, control clusters exhibit a characteristic pattern of protrusions aligned towards the direction of migration, whereas *Rab11SN*-expressing clusters have lost this pattern and present a more spread orientation (Fig. 1d). After 25–50% of migration, protrusions have conserved a strong bias towards the direction of migration in control clusters, whereas they lost directionality in *Rab11SN*-expressing clusters (Fig. 1e). Together, these data highlight the different roles of Rab11 and Rab5 in regulation of protrusion dynamics.

As Rac is the main driving force for protrusion formation in border cells^{15,16}, and Rab5 endosomes are required for Rac activation in a single-cell migration model¹⁹, we next analysed Rac activity and localization in *Rab5SN*- and *Rab11SN*-expressing clusters. For this we took advantage of the Rac Förster resonance energy transfer (FRET) probe that has been adapted for *Drosophila*^{16,20}. We monitored Rac activation in time and space in living egg chambers at the onset of migration (Fig. 1f–t). In control conditions, the FRET signal exhibits a reproducible spatiotemporal pattern, with a higher signal in the leading cell (Fig. 1f–j). Expression of *Rab5SN* eliminated any detectable FRET signal, demonstrating that endocytosis is required for Rac activation in border cells (Fig. 1k–o,u). Expression of *Rab11SN* had no detectable effect on the overall level of activation of Rac (Fig. 1p–t,u).

However, the distribution of the FRET signal was abnormal. Indeed, high FRET signal alternated in different cells and did not stay at a fixed position or was present simultaneously in multiple cells of the cluster (Fig. 1p–t). To determine the persistence of Rac activity, we measured the FRET index specifically in the leading cell over time (as described in Supplementary Fig. S3). We found that Rac activity was fluctuating over time on expression of *Rab11SN*, as compared with the control (Fig. 1v,w). Still, we observed a strong Rac activity at the front of 20% of the clusters. Accordingly, some clusters expressing *Rab11SN* migrate at 75% to 100% of the migration distance¹⁷. Together, these data show that Rab11 is required for the polarization and the persistence of Rac activity at the leading cell of a collectively migrating cluster.

To further investigate the link between Rab5, Rab11 and Rac, we used the photoactivatable analogue of the active form of Rac (PA-RacQ61L), which is able to direct the migration of border cells (Supplementary Video S1 and Figs S2a–d) and was shown to inhibit the formation of protrusions in the rest of the cluster^{16,21}. We predicted that migration of *Rab11SN*-expressing clusters would not be rescued by local Rac activation because of competition from endogenous non-polarized Rac activity. In contrast, expression of *Rab5SN* should be rescued by PA-RacQ61L owing to the absence of competition from endogenous Rac. Consistent with the observation in fixed cells (Fig. 1), *Rab11SN*-expressing clusters exhibited protrusions in all directions before phototreatment (Supplementary Video S2). Photoactivation of Rac induced local protrusions but failed to rescue the migration of *Rab11SN*-expressing cells (Fig. 2e–h,u and Supplementary Video S2). Moreover, PA-RacQ61L did not inhibit the formation of protrusions outside the illuminated area, indicating that Rab11 is involved in the cell–cell communication process required for cells to sense the relative level of Rac activity in adjacent cells (Fig. 2e–h and Supplementary Video S2). In accordance with our model, photoactivation of Rac in a *Rab5SN*-expressing cluster was able to restore migration to the level of control clusters (Fig. 2m–p,u and Supplementary Video S3). These effects were indeed due to local Rac activation because a mutant form of PA-RacQ61L that is insensitive to light (C450M) had no effect (Fig. 2i–l,q–t and Supplementary Videos S4 and S5). Taken together, these data show that Rab5 and Rab11 act at two different levels for Rac regulation. Rab5 acts on the activation of Rac whereas Rab11 is implicated in the control of the spatiotemporal pattern of Rac activity in the entire cluster.

To assess the potential function of Rab11 in cell–cell communication, we considered the morphological consequences of local inactivation of Rac by light, using a photoactivatable dominant-negative form of Rac (PA-RacT17N). Illumination of the leading cells in a cluster expressing *PA-RacT17N* arrested migration¹⁶ and led to the formation of protrusions in the other cells of the cluster in all directions, but with a bias towards the leading edge (Fig. 3a–c,j,k and Supplementary Video S6). In contrast, the cluster shape is unchanged when PARacT17N is locally activated in a *Rab11SN* background. Indeed, before phototreatment *Rab11SN*-expressing clusters exhibit protrusions in all directions and this pattern is maintained after inhibition of Rac (Fig. 3d–f,j,k and Supplementary Video S7). These data show that there is a strong defect of communication between border cells in *Rab11SN*-expressing clusters. After *Rab5SN* expression, we observed that illumination of PA-RacT17N did not induce ectopic protrusions (Fig. 3g–i,k and Supplementary Video S8).

This effect reflects the absence of Rac activity in these clusters. However, we observed a contraction of the cluster (Fig. 3l), showing a global response to local inactivation of Rac, suggesting that communication is not affected. Together, these results demonstrate that Rab11 is a regulator of cell–cell communication during collective cell migration.

To investigate the molecular mechanism by which Rab11 regulates cell–cell communication, we searched for interactors of Rab11. We immobilized recombinant glutathione *S*-transferase (GST)–Rab11 on an affinity column. GST–Rab11 was then loaded with GDP or GTP γ S and cytosol of *Drosophila* embryos was applied. After extensive washes and elution with the converse nucleotide, the composition of the eluate was analysed by mass spectrometry (Supplementary Table S1). Our experimental conditions allowed us to identify nucleotide-specific interactors of Rab11. Accordingly, we found previously known interactors of Rab11 GTP such as Nuclear fallout^{22,23} (Nuf), and interactors of Rab11 GDP such as GDP-dissociation inhibitor²⁴ (GDI), validating our approach. Interestingly, we found that Moesin interacts specifically with the inactive form of Rab11. We confirmed this interaction in S2 cells, by pulldown of GST–Rab11WT, SN and QL and by western blotting with both exogenous GFP–Moesin and endogenous Moesin (Fig. 4a). Moesin belongs to the highly conserved ERM (Ezrin, Radixin, Moesin) protein family and is the sole ERM gene found in the genome of *Drosophila*. ERM proteins participate in the organization of the cell cortex by linking the plasma membrane with the actin cytoskeleton^{25,26}. We first assessed whether Moesin acts upstream or downstream of Rab11 in border cells. To test this, we downregulated *Moesin* by RNA-mediated interference (RNAi) and monitored the distribution and the volume of Sec15–GFP vesicles. Sec15 is a well-characterized Rab11 effector that is polarized through a Rab11-dependent mechanism¹⁷. Volume measurement of Sec15–GFP vesicles is a sensitive and specific readout of Rab11 activity²⁷. Downregulation of *Moesin* had no effect on the polarization and the volume of Sec15 vesicles (Supplementary Fig. S4a,b), demonstrating that Moesin does not act upstream of Rab11. We next examined whether Rab11 regulates Moesin activity. ERM proteins unfold and become activated on binding the phospholipid PtdIns(4,5)P₂ and phosphorylation of a conserved threonine residue within the carboxy-terminal domain (Thr 559; ref. 25). Thus, we determined whether Rab11 could regulate Moesin phosphorylation by examining the localization of Moesin and its level of activation using a specific phospho-Thr-559 antibody. In control conditions, activated Moesin is located at the periphery of the cluster (Fig. 4b). We found that expression of *Rab11SN* decreases the amount of activated Moesin, as measured by immunofluorescence microscopy of phospho-Moesin (Fig. 4b), suggesting that Rab11 controls the activation of Moesin. This decrease was not due to a reduction of the amount of Moesin as revealed by total Moesin staining and quantification at the nurse cell/ border cell interface (Fig. 4c,e). These effects were specific to Rab11SN because *Rab5SN* expression altered neither phosphorylation (Fig. 4b,d) nor localization (Fig. 4c,e,f) of Moesin. Moreover, we observed that *Rab11SN* expression induces the appearance of Moesin at the boundaries between border cells (Fig. 4c,f) correlating with redistribution of actin at these sites (Supplementary Fig. S4c,d). These data indicate that pMoesin is responsible for actin recruitment at the periphery of the cluster. To confirm this observation we decreased the amount of Moesin by RNAi and we also observed accumulation of actin at the boundaries between border cells (Supplementary Fig. S4c,d). We then investigated whether

a phosphomimetic mutant of Moesin (Moesin-TD) is able to restore the migration in a Rab11-deficient cluster by measuring migration and completion indices¹⁷. Expression of low levels of dominant-negative Rab11 (YFP-Rab11SN) leads to defects in migration (Fig. 4g and Supplementary Fig. S4e,h). Strikingly, although *Moesin-TD* expression affects migration on its own (Fig. 4g and Supplementary Fig. S4f), it was able to significantly rescue the migration defect induced by *YFP-Rab11SN* (Fig. 4g and Supplementary Fig. S4i). Consistently, expression of *Moesin-TD* in the *YFP-Rab11SN* background rescued the orientation of protrusions towards the direction of migration (Supplementary Fig. S5). Overexpression of *Moesin* WT alone had no effect (Fig. 4g and Supplementary Fig. S4g) and was not able to rescue *YFP-Rab11SN* (Fig. 4g and Supplementary Fig. S4j). Finally, Moesin-TD failed to rescue both RacN17 and Rab5SN (Fig. 4g) showing that Moesin is downstream of Rab11, but neither of Rac nor of Rab5. Together, these findings demonstrate that Rab11 regulates Moesin activity during collective cell migration.

To confirm that Moesin acts downstream of Rab11 to ensure correct communication between cells, we performed a phenotypic analysis of clusters expressing Lifeact fused to GFP in a *Moesin* loss-of-function mutant. Severe *Moesin* loss-of-function mutants are not viable²⁸; hence, we took advantage of the RNAi technology. We tested three different *Moesin* RNAi that induce partial depletion of *Moesin*. We measured by immunofluorescence analysis that we depleted 50% of the protein. The migration of clusters with a reduced Moesin level was significantly affected (Fig. 5a) showing that Moesin is important for border cell migration. At the onset of migration, control clusters exhibit one main protrusion towards the leading edge (Fig. 5b). In contrast, reduction of Moesin levels leads to the formation of protrusions in other directions (Fig. 5b). The spatial distribution of protrusions is also altered after 25–50% of migration by *Moesin* knockdown (Fig. 5b). Consistently, Rac activity, measured by FRET, was altered. We found that it was still polarized but present in different cells at the front of the cluster (Fig. 5c,d). Finally, analysing FRET signals in the leading cell revealed that Rac activity was inconsistent and fluctuated over time after *Moesin* depletion (Fig. 5e). Together, these data show that decreasing the level of *Moesin* phenocopies *Rab11* loss of function. Thus, we reason that *Moesin* is also implicated in the control of cell–cell communication. To investigate this, we analysed the effect of local inactivation of Rac after knockdown of *Moesin*, using PA-RacT17N. In control conditions, inactivation of Rac in the leading cell causes the formation of protrusions in other cells of the cluster (Fig. 5f–h,l and Supplementary Video S6). In contrast, *Moesin* RNAi impairs the ability of the non-illuminated cells to respond to phototreatment of the leading cell (Fig. 5i–k,l and Supplementary Video S9). These results confirm that *Moesin* is regulating cell–cell communication during collective cell migration. These observations provide evidence for a previously unknown mechanism coordinating Rac activity during collective cell migration. This mechanism involves Rab11, which regulates Moesin activity and the formation of a coherent actin–Moesin structure that surrounds the cluster. In turn, this ensures that Rac is spatially restricted to the leading cell.

Here, we demonstrated that Rab11 is a key regulator of cell–cell communication during collective movements, critical to achieve spatial restriction of Rac activation. Previous studies of border cell migration in *Drosophila* have indicated that endocytosis regulates

collective cell migration essentially through the regulation of guidance receptor localization. We have shown that Rab5 (this study), Rab11 and the exocyst complex control active RTK polarization at the leading edge¹⁷. From this, we initially reasoned that an endocytic cycle involving Rab5 and Rab11 is controlling collective cell migration. However, we demonstrated here that expression of *Rab5SN* and *Rab11SN* leads to different effects in terms of actin dynamics and Rac activity. The differential effects led us to propose that Rab5 and Rab11 target different mechanisms to regulate collective cell migration. Accordingly, we found that Rab5 controls Rac activity as previously shown in single-cell migration models¹⁹. In contrast, Rab11 acts on the whole cluster to spatially resolve Rac signalling, preventing it from becoming uniformly distributed and thus inefficient for migration.

The mechanism by which cells are able to sense the relative level of Rac activity in neighbouring cells remains unclear. Our study identifies both Rab11 and Moesin as major regulators of this process. We found that Moesin binds the inactive form of Rab11, suggesting that Rab11 activity is required to release Moesin from Rab11. Moreover, we observed that *Rab11SN* induces a decrease in the level of Moesin phosphorylation in border cells. How this is achieved is not known, but we can speculate that Rab11 is necessary to transport Moesin to domains where it can be phosphorylated by a kinase. Consistent with this idea, we observe that the distribution of total Moesin is different in control clusters when compared with *Rab11SN* clusters. Whereas in control clusters Moesin is almost exclusively localized at boundaries around the cluster (border cell-nurse cell), it is found at border cell–border cell boundaries in *Rab11SN* clusters. As a consequence, we observe that actin is also redistributed to internal boundaries. Moesin plays a critical role in organizing the epithelial architecture and in the regulation of cortical tension through regulation of the acto-myosin cytoskeleton²⁵. Hence, redistribution of Moesin and actin to internal boundaries might change the property of the cluster: in control clusters the acto-myosin cytoskeleton surrounds the entire cluster, which reacts as a group, whereas in *Rab11SN* clusters it surrounds every cell, which then seem to behave individually. Future work will be necessary to determine whether this is achieved through the regulation of cortical tension or through another mechanism.

METHODS

Fly genetics

slbo–Gal4 or *slbo*–Gal4, *UAS*–*CD8::GFP* drives UAS transgene expression in outer, migratory border cells, but not polar cells. Other stocks used were *UAS*–*Rab5SN*/CyO, *UAS*–*Rab11SN*/CyO, *UAS*–*RacN17* and *UAS*–*YFP*–*Rab11SN*, which have been described previously¹⁷. *UAS*–*PA*–*RacQ61L* and *UAS*–*PA*–*RacT17N* and the light-insensitive control *UAS*–*C450M*–*PA*–*RacQ61L* were described previously¹⁶. *UAS*–*Lifeact* *GFP*, *UAS*–*Moesin*–*TD*–*Myc* were from the Bloomington stock centre. *UAS*–RNAi lines against Moesin were from the Vienna *Drosophila* RNAi Center (RNAi #3, no. #37917, 5′-TGACCACAAT AAGACCACCC ACACAGCCGG CTTTCTGGCC AACGATCGCC TGCTGCCGCA GCGCGTCATC GACCAGCACA AGATGTCCAA GGACGAGTGG GAGCAGTCGA TTATGACCTG GTGGCAGGAG CATCGCAGCA TGCTGCGCGA GGATGCCATG ATGGAGTATC TGAAGATCGC CCAAGACCTG GAGATGTACG GCGTTAACTA

CTTTGAGATC CGCAACAAGA AGGGCACGGA TCTTTGGCTG GCGGTAGACG CACTGGGTCT GAACATTTAC GAGCAGGACG ATAGGTTGAC GCC-3') and Bloomington stock centre (RNAi #1, no 31135, TRIP, top oligonucleotide: 5'-AAGAATTCAAATGTTGCGAATCTCGGAC-3', bottom oligonucleotide: 5'-AGTCTAGATTCCGTGCCAAATTCTATCC-3' and #2, no 33936, TRIP, top oligonucleotide: 5'-CAGCAAGAGCAGATAATAATA-3', bottom oligonucleotide 5'-TATTATTATCTGCTCTTGCTG-3'). RNAi #3 was used for FRET and photoactivation experiments. All stocks were maintained at 25°.

Live-cell imaging

Living egg chambers were prepared for real-time imaging as described previously¹⁶. FRET images of live border cells were acquired with a Zeiss LSM510 inverted confocal microscope (Zeiss) equipped with a ×40/1.3 oil immersion objective as described previously¹⁶. CFP and YFP images were processed by ImageJ and Metamorph software. A Gaussian smooth filter was first applied to both channels with ImageJ. Then the YFP image was used to create a binary mask with the background set to zero. The final ratio image was generated with the ratio Image function of Metamorph using 8 ratios with 32 intensities²⁹. The FRET ratio was calculated in the entire border cell cluster or specifically at the leading edge by measuring the average intensity of FRET and CFP by the Region measurements function of Metamorph. Heat maps of FRET indices at the leading edge were generated with Excel. Rac photoactivation was performed as described previously. Briefly, the 458 nm laser was set at 10% power. After 30 s, border cells were imaged using a 568 nm laser. This series of steps was repeated for the duration of the time-lapse experiment.

Measurements of migration speed, protrusion number and distribution

Speed was determined as described previously¹⁶. For protrusion quantification, a circle was drawn around the cell body. Any actin extension more than 4 µm beyond this was defined as a protrusion. For distribution analysis, each protrusion defined as previously was aligned on a radar map divided into 8 different sectors of 45° each with the leading edge set at zero degrees and the trailing edge at 180°. The length and the width of the arrow represent the amount of protrusions in a given direction. Analysis and quantification were done with ImageJ and MATLAB and representations with Excel.

Quantitative analysis of RTK polarity

Images from fixed tissues were acquired with an LSM 510 META inverted microscope (Zeiss) using a ×63 objective. Images were acquired by sequential scans in multiple channels. Quantification of fluorescence intensities was performed as previously described^{17,27}.

Tissue staining and antibodies

Egg chambers were prepared and stained using standard techniques. All antibodies are previously described^{17,28} and were used at the indicated dilutions: mouse monoclonal anti-pTyr (1:10, 4G10), anti aPKC (1:50, Santa Cruz, #sc-216), rabbit anti phospho-Moesin (1:50) and total Moesin (1:100; kind gift from S. Carreno, IRIC, Université de Montréal,

Canada). Secondary antibodies were from Invitrogen and coupled to Alexa Fluor 555 (anti-mouse #A21422 and anti-rabbit #A21427) and used at 1:250 dilutions. Alexa Fluor 555-labelled phalloidin (Invitrogen, #A34055) was used at 1:250 dilutions to visualize F-actin. DAPI (Sigma-Aldrich) was used to stain nuclei. Egg chambers were mounted in Mowiol 4–88 (Sigma-Aldrich). Images from fixed tissues were acquired using an LSM510 (Zeiss), using either a $\times 40$ (full-size egg chambers) or $\times 63$ (border cells cluster alone) objective. Images were acquired by sequential scans in multiple channels. For figure assembly, images were processed with Photoshop (Adobe), using only the Gaussian blur and the level functions. For a better rendering of the blue channel, a post-treatment with the selective colour function was performed. Migration and completion indices were calculated as described previously^{17,27}.

Rab11 GST pulldown for mass spectrometry analysis

Cytosol extracts from *Drosophila* embryos were prepared in the following homogenization buffer (20 mM Tris-Cl at pH 8, 110 mM KCl, 5 mM MgCl₂ and 1 mM dithiothreitol). Recombinant GST–Rab11 (previously cloned in pgex-6P1) was purified from BL21 bacteria as described previously³⁰. An affinity column of GST–Rab11 loaded with GDP or with GTP S was prepared, loaded with cytosol extracts, washed and eluted with an excess of the converse nucleotide as in ref. 31. Total eluates were sent for analysis by mass spectrometry.

Liquid chromatography–mass spectrometry analysis

TCEP (tris(2-carboxyethyl)phosphine) was added to the protein samples to reach a concentration of 5 mM. Samples were incubated at 37 °C on a shaker rotating at 650 r.p.m. for 30 min. Trypsin (1 μ g) was added and the samples were digested overnight at 37 °C. The samples were dried down in a speed vac and resolubilized in 50 μ l of ACN 5%/formic acid (FA) 0.2%. A 20 μ l volume of each sample was injected onto a C18 precolumn (0.3 mm i.d. $\times$ 5 mm) and samples were separated on a C18 analytical column (150 μ m i.d. $\times$ 100 mm) using an Eksigent nanoLC-2D system. A 76-min gradient from (A/B) 10–60% (A: formic acid 0.2%, B: acetonitrile/0.2% formic acid) was used to elute peptides with a flow rate set at 600 nl min^{–1}.

The LC system was coupled to an LTQ-Orbitrap mass spectrometer (Thermo Fisher). Each full mass spectrometry (MS) spectrum was followed by three MS/MS spectra (four scan events), where the three most abundant multiply charged ions were selected for MS/MS sequencing. Tandem MS experiments were performed using collision-induced dissociation in the linear ion trap. The data were processed using the Mascot 2.2 (Matrix Science) search engine.

Rab11 GST pulldown for western blotting

Stable S2 cells expressing GST–Rab11 under the pMT promoter were cultured in Schneider medium supplemented with 10% FBS and transfected with Moesin–GFP using TransIT-LT1 (Mirus) on day 1. Protein expression was induced with 0.8 mM of CuSO₄ on day 2. On day 4, cells were lysed in Nonidet P-40 lysis buffer (20 mM Tris (pH 8.0), 137 mM NaCl, 1% Nonidet P40, 10% glycerol and 1 mM EDTA) with protease inhibitors. For GST pulldown assays, 50 μ l of 50% slurry of glutathione Sepharose beads equilibrated in lysis buffer was

added to protein lysates and rocked for 4 h at 4 °C. Beads were then washed three times with 1 ml of lysis buffer. Total protein lysates or eluted proteins were resolved on an 8–10% SDS–PAGE gel, transferred to nitrocellulose membranes and immune-detected using specific antibodies.

Vesicle analysis

Fluorescent images were analysed using semiautomated software custom written in MATLAB (MathWorks) as previously described²⁷. Briefly, images were first automatically thresholded using the GFP-Sec15 channel, and then the fluorescent signal was detected in three dimensions.

Statistical analysis

Statistical comparisons of means were made using the unpaired Student's two-tailed *t*-test for two data sets. For statistical tests, $P < 0.05$ was used as the criterion for statistical significance. Mean values are quoted $\pm$ s.e.m. in figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Rab proteins regulate actin dynamics and Rac activity and polarization. **(a)** Schematic representation of an egg chamber at stages 9 and 10. pTyr signalling and Rac activity are distinct events. **(b)** Border cell clusters expressing *UAS-Lifeact GFP* alone, with *UAS-Rab11SN* or *UAS-Rab5SN* at the onset of migration. The expression was driven by *slbo-Gal4* at 29 °C. Scale bars, 20 μ m. **(c)** Quantification of protrusion number in the same conditions at the onset and after 25–50% of migration of the indicated genotypes. $n = 30, 19, 10$ and 10 respectively for control, Rab11SN, Rab5SN and RacN17 at the onset of migration. $n = 15, 27, 10$ and 10 respectively for control, Rab11SN, Rab5SN and RacN17 at 25–50% of migration. $*P < 0.001$ (Student's *t*-test). **(d,e)** Analysis of protrusion distribution (see Supplementary Fig. S2 for details) in control and *Rab11SN*-expressing clusters at the onset **(d)** and after 25–50% **(e)** of migration. $n = 20$ clusters. **(f–t)** Processed FRET signal images of time-lapse series of border cells expressing *UAS-Rac Fret* alone or together with *UAS-Rab11SN* or *UAS-Rab5SN* at the onset of migration. The expression was driven by *slbo-Gal4* at 29 °C. Scale bars, 10 μ m. **(u)** Quantification of the total FRET index in the indicated genotypes. $n = 30$ for each condition. **(v)** Quantification of the ratio of the FRET index between the front and back of the cluster for the indicated conditions. $n = 30$ for each condition. $*P < 0.001$ (Student's *t*-test). **(w)** Heat maps representing the FRET index as a function of time at the leading edge of 10 WT and *Rab11SN* clusters. Error bars show s.e.m.

Figure 2.

Local activation of Rac does not rescue the Rab11 loss-of-function phenotype. (a–d) Selected still images from a time-lapse movie of photoactivation of a *UAS-PA-RacQ61L*-expressing cluster. $n = 8$. (e–h) Selected still images from time-lapse movies of photoactivation of *UAS-PA-RacQ61L* in a *UAS-Rab11SN* background. $n = 11$. (i–l) Control experiment with *UAS-PA-RacQ61L C450M* insensitive to light in a *UAS-Rab11SN* background. $n = 10$. (m–p) Selected still images from time-lapse movies of photoactivation of *UAS-PA-RacQ61L* in a *UAS-Rab5SN* background. $n = 8$. (q–t) Control experiment with *UAS-PA-RacQ61L C450M* insensitive to light in a *UAS-Rab5SN* background. $n = 9$. The expression was driven by *slbo*-Gal4 at 29 °C.

The blue circles indicate photoactivated regions. The white arrows indicate the direction of migration and the dashed blue arrows the expected direction after photoactivation. Scale bars, 20 μm . (u) Speed measurements for each genetic background presented in a–t. $n = 8, 8, 11, 10, 8, 9$ respectively for control PA-Rac C450M, PA-Rac control, PA-Rac C450M Rab11SN, PA-Rac Rab11SN, PA-Rac C450M Rab5SN and PA-Rac Rab5SN at the onset of migration. * $P < 0.01$; NS, not significant (Student's *t*-test). Error bars show s.e.m.

Figure 3.

Local inactivation of Rac reveals a role in cell–cell communication for Rab11. **(a–c)** Confocal images from an experiment of *UAS-PA-RacT17N* photoactivation in a control cluster. $n = 11$. **(d–f)** Confocal images of photoactivation experiments in a *UAS-Rab11SN* background. $n = 13$. **(g–i)** Confocal images of photoactivation experiments in a *UAS-Rab5SN* background. $n = 12$.

The red circles indicate photoactivated regions. The white arrows indicate the direction of migration. Scale bars, 20 μm. **(j)** Analysis of protrusion distribution in control and *UAS-Rab11SN*-expressing clusters. **(k)** Quantification of protrusions in *PA-RacT17N* experiments. $n = 11, 13$ and 12 respectively for control, *Rab5SN* and *Rab11SN* at the onset of migration. **(l)** Quantification of the area of clusters in *PA-RacT17N* experiments. $n = 11, 13$ and 12 respectively for control, *Rab5SN* and *Rab11SN* at the onset of migration. The expression was driven by *slbo*–Gal4 at 29 °C. * $P < 0.0001$ (Student's *t*-test). Error bars show s.e.m.

Figure 4.

Rab11 interacts with and controls Moesin activity. **(a)** Stably transfected S2 cells expressing GST, GST-Rab11SN, WT or QL and *GFP-Moesin* or not were lysed and submitted to GST pulldowns. Lysates and immunoprecipitates (IP) were analysed by western blotting using GFP, GST and Moesin antibodies (AB). Western blots are representative of three independent experiments. **(b)** Representative images showing the intensity and the distribution of pMoesin at the onset of migration in control (*CD8-GFP*; $n = 11$), and in *Rab11SN*- ($n = 16$) and *Rab5SN*- ($n = 10$) expressing clusters. Scale bars, 10 μ m. The expression was driven by the *slbo*-Gal4 promoter. **(c)** Representative images showing the intensity and the distribution of Moesin at the onset of migration in control (*CD8-GFP*; $n = 20$), and in *Rab11SN*- ($n = 20$) and *Rab5SN*- ($n = 10$) expressing clusters. Scale bars, 10 μ m. The expression was driven by the *slbo*-Gal4 promoter. **(d)** Quantification of the ratio of the mean fluorescence signal of pMoesin to the GFP mean intensity at the interface between border cells and nurse cells. $n = 20, 20$ and 10 respectively for control, *Rab11SN* and *Rab5SN*. **(e)** Quantification of the ratio of the mean fluorescence signal of Moesin at the interface between border cells (BC) and nurse cells (NC). $n = 20, 20$ and 10 respectively for control, *Rab11SN* and *Rab5SN*.

(f) Quantification of the ratio of the mean fluorescence signal of Moesin at the interface between border cells (BC) and nurse cells (NC) on the mean fluorescence signal of Moesin at BC–BC membranes. $n = 20, 20$ and 10 respectively for control, *Rab11SN* and *Rab5SN*. (g) Migration and completion indices after expression of the constructs indicated. n is indicated under each genotype. $*P < 0.0001$; NS, not significant (Student's t -test). Error bars show s.e.m. Uncropped images of blots are shown in Supplementary Fig. S6.

Figure 5.

Moesin regulates protrusion distribution, polarization of Rac activity and cell–cell communication. **(a)** Migration and completion indices after expression of mcherry RNAi (control) or three different RNAi lines against *Moesin*. The expression was driven by *c306*–Gal4 at 32 °C. n is indicated under each genotype. $*P < 0.0001$ (Student's *t*-test). **(b)** Analysis of protrusion distribution in RNAi-*Moesin*-expressing clusters at the onset and after 25–50% of migration. $n = 14$ clusters. **(c)** *UAS*–*Rac Fret* is expressed in border cells expressing RNAi *Moesin* and Rac activity is visualized by FRET in living egg chambers at the onset of migration. The expression was driven by *c306*–Gal4 at 32 °C. Scale bars, 10 μ m. **(d)** Quantification of the total FRET index in control and in RNAi-*Moesin*-expressing clusters. $n = 30$ in each condition. **(e)** Heat maps representing the FRET index as a function of time at the leading edge of 10 RNAi *Moesin* clusters. **(f–h)** Confocal images from an experiment of *UAS*–*PA-RacT17N* photoactivation in a control cluster. The expression was driven by *slbo*–Gal4 at 32 °C. **(i–k)** Confocal images of a photoactivation experiment in a *UAS*–RNAi *Moesin* background at 32 °C. The red circles indicate photoactivated regions. The white arrows indicate the direction of migration. Scale bars, 20 μ m. **(l)** Quantification of protrusions in *PA-RacT17N* experiments. $n = 11$ for each condition. $*P < 0.0001$ (Student's *t*-test). Error bars show s.e.m.