

Myosin-IXA Regulates Collective Epithelial Cell Migration by Targeting RhoGAP Activity to Cell-Cell Junctions

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Summary

Background: Epithelial tissues undergo extensive collective movements during morphogenesis, repair, and renewal. Collective epithelial cell migration requires the intercellular coordination of cell-cell adhesions and the establishment of anterior-posterior polarity, while maintaining apical-basal polarity, but how this is achieved at the molecular level is not well understood.

Results: Using an RNA interference-based screen to identify Rho family GTPase regulators required for the collective migration of human bronchial epithelial cells, we identified myosin-IXA (gene name: *Myo9a*). Depletion of myosin-IXA, a RhoGAP and actin motor protein, in collectively migrating cells led to altered organization of the actin cytoskeleton and tension-dependent disruption of cell-cell adhesions, followed by an inability to form new adhesions resulting in cell scattering. Closer examination revealed that myosin-IXA is required during the formation of junction-associated actin bundles soon after cell-cell contact. Structure-function analysis of myosin-IXA revealed that the motor domain is necessary and sufficient for binding to actin filaments, whereas expression of the RhoGAP domain partially rescued the cell scattering phenotype induced by myosin-IXA depletion. Finally, a fluorescence resonance energy transfer biosensor revealed a significant increase in Rho activity at nascent cell-cell contacts in myosin-IXA depleted cells compared to controls.

Conclusion: We propose that myosin-IXA locally regulates Rho and the assembly of thin actin bundles associated with nascent cell-cell adhesions and that this is required to sustain the collective migration of epithelial cells.

Introduction

Collective cell migration is an important process in tissue morphogenesis, regeneration, and tumor dissemination [1]. The collective migration of epithelia is particularly interesting, because cells maintain cell-cell adhesion and apical-basal polarity, while establishing anterior-posterior polarity to promote directed migration. Collective epithelial migrations can be seen throughout development, for example, in the elongation of mammary ducts [2] or the formation of kidney nephrons [3]. During zebrafish gastrulation and in the migration of *Drosophila* border cells, cell-cell adhesion is essential for directional migration [4, 5]. A highly cooperative behavior has been seen during the migration of cancer cells in vitro, whereas in vivo imaging of primary breast tumors in mice has revealed collective migration of cancer cells toward and into lymph nodes [6–9].

The mechanisms that regulate cooperation between cells during collective migration are not well characterized. The assembly and disassembly of actin filaments is known to be the major driving force for cell migration, and actin-driven protrusive activity in the basal plane has been observed at the front of leading edge cells and follower cells during epithelial cell migration [10]. At the apical surface of epithelial cells, the actin cytoskeleton interacts intimately with cell-cell junction proteins, such as ZO-1 and vinculin. One suggestion is that communication between cells is transmitted by mechanical tension induced by leader cells acting through cell-cell contacts and affecting the actin cytoskeleton [11–13]. In combination with actin-based, myosin motor proteins, this could facilitate the transmission of tensile forces across adjacent cells [14–16]. Alternatively, biochemical signals could mediate coordinated changes in migrating cells, as during planar cell polarity (PCP), where molecular changes can be induced across a tissue, even in the absence of migration [17, 18].

Rho GTPases are important regulators of the actin cytoskeleton in eukaryotic cells [19]. They control many aspects of cell behavior but in particular are required for cell migration and for the formation of cell-cell junctions. Fluorescence resonance energy transfer (FRET) studies have revealed that Rac1 is recruited by E-cadherin during the initiation of cell-cell contact formation in epithelial cells and is required to promote actin polymerization [20–22]. RhoA and Cdc42 are activated at these early stages, as well as during cell-cell contact expansion [21–23]. Several guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) that regulate Rho proteins and several downstream effectors have been implicated in the assembly of cell-cell junctions [24–27]. Whether they are required to facilitate collective migration, however, is not known.

We have used a small interfering RNA (siRNA) screen to identify regulators of the Rho family that are required for junctional integrity in a human bronchial epithelial cell line (16HBE) undergoing collective cell migration, and we identified myosin-IXA, a RhoGAP and actin-binding motor protein [28]. In the absence of myosin-IXA, 16HBE cells have an altered actin cytoskeleton and are unable to maintain their adherens junctions during migration leading to cell scattering. Mutational studies of myosin-IXA revealed that the motor domain is required for its association with actin bundles, whereas the RhoGAP activity can partially rescue cell scattering induced by myosin-IXA depletion. Finally, a FRET biosensor revealed a significant increase in RhoA activity at nascent cell-cell contacts in myosin-IXA-depleted cells compared to control. We conclude that myosin-IXA is an important negative regulator of Rho at nascent cell-cell junctions and is required for collective migration of cells in the bronchial epithelium.

Results

Collective Cell Migration in Bronchial Epithelial Cells

Time-lapse analysis of cultured 16HBE cells revealed important features indicative of collective cell behavior: (1) complex cell flows within a monolayer (Figure 1A, arrows 2 and 3; see

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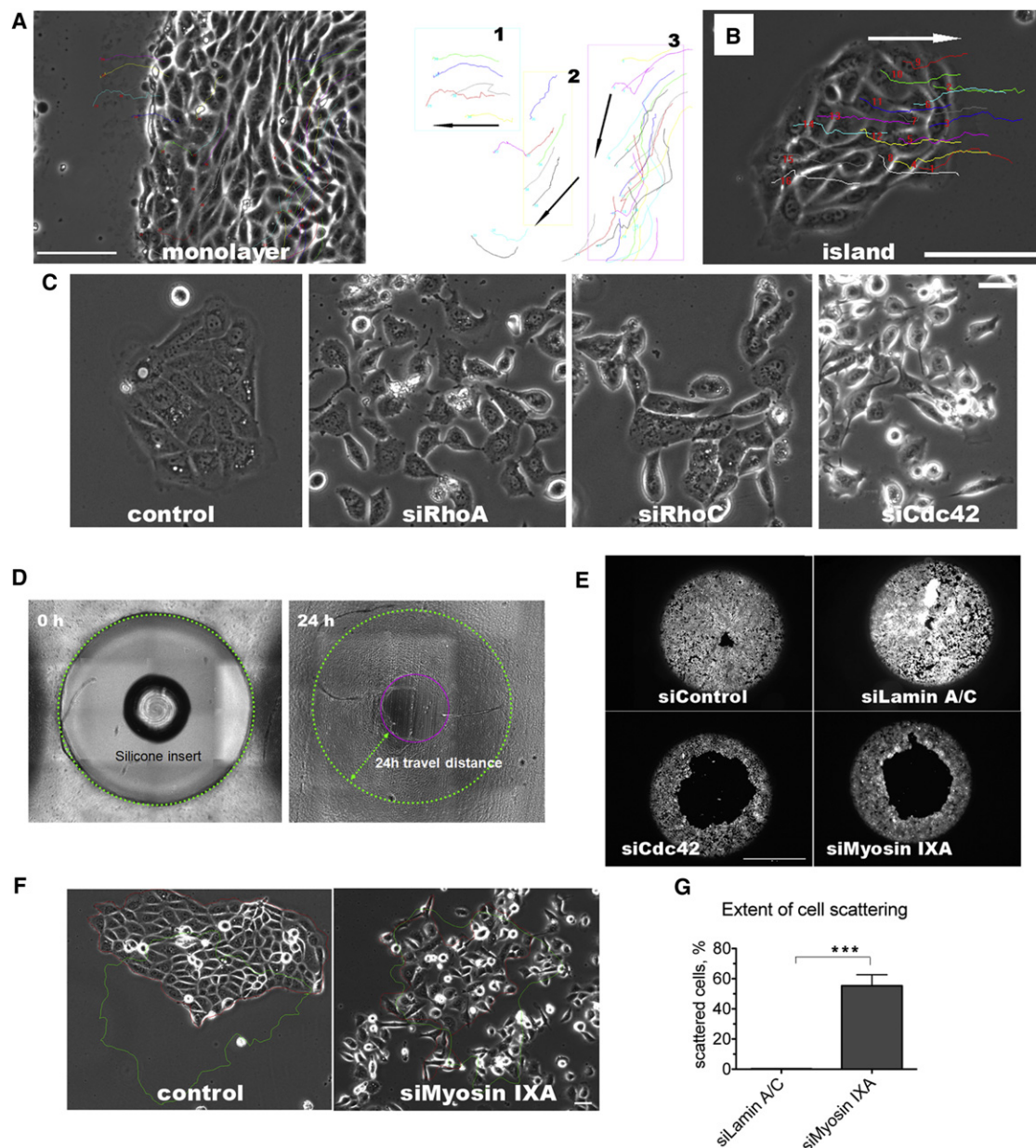


Figure 1. Identification of Myosin-IXA as a Regulator of Collective Epithelial Migration

(A) Migration of 16HBE cells in a monolayer is highly coordinated (140 min time course). Regions 1, 2, and 3 (black arrows) are streams distinguished by parallel tracks of cell groups (Movie S1). (B) Cell islands migrate in a highly coordinated manner (white arrow) with almost parallel cell tracks (140 min time course). Scale bar represents 100 μm . (C) Scattering of island cells after SMARTpool siRNA knockdown of RhoA, RhoC, or Cdc42. Scale bar represents 50 μm . (D) Schematic of 96-well migration assay used in siRNA screen (insert diameter = 1.5 mm). (E) Myosin-IXA depletion with siRNA inhibits forward migration. (F) Myosin-IXA depletion with siRNA causes cell scattering of migrating 16HBE islands (Movie S2). The initial (green) and last (red) position of the island is outlined. Scale bar represents 30 μm . (G) Quantification of cell scattering (error bars represent SEM for four independent experiments, *** $p = 0.0003$, unpaired t test).

also Movie S1 available online) and (2) coordinated movement of individual islands (Figure 1B). Within the monolayer, cell groups stream (Figure 1A, arrows) with similar directional trajectories, whereas in sparse cultures, cell islands move directionally as a single unit over period of a few hours. Migration rates of cells within the monolayer and in an island are similar (0.6–0.9 $\mu\text{m}/\text{min}$). These observations indicate a highly cooperative mode of collective migration in 16HBE cultures.

Identification of Myosin-IXA in a Screen for Collective Migration

To determine whether Rho GTPases are required for collective migration, we depleted RhoA, RhoC, and Cdc42 from 16HBE cells using siRNA and found disruption of cell-cell contacts leading to cell scattering (Figure 1C). This indicates that Rho and Cdc42 are not required for migration per se but are required to maintain collective migration. To identify regulators

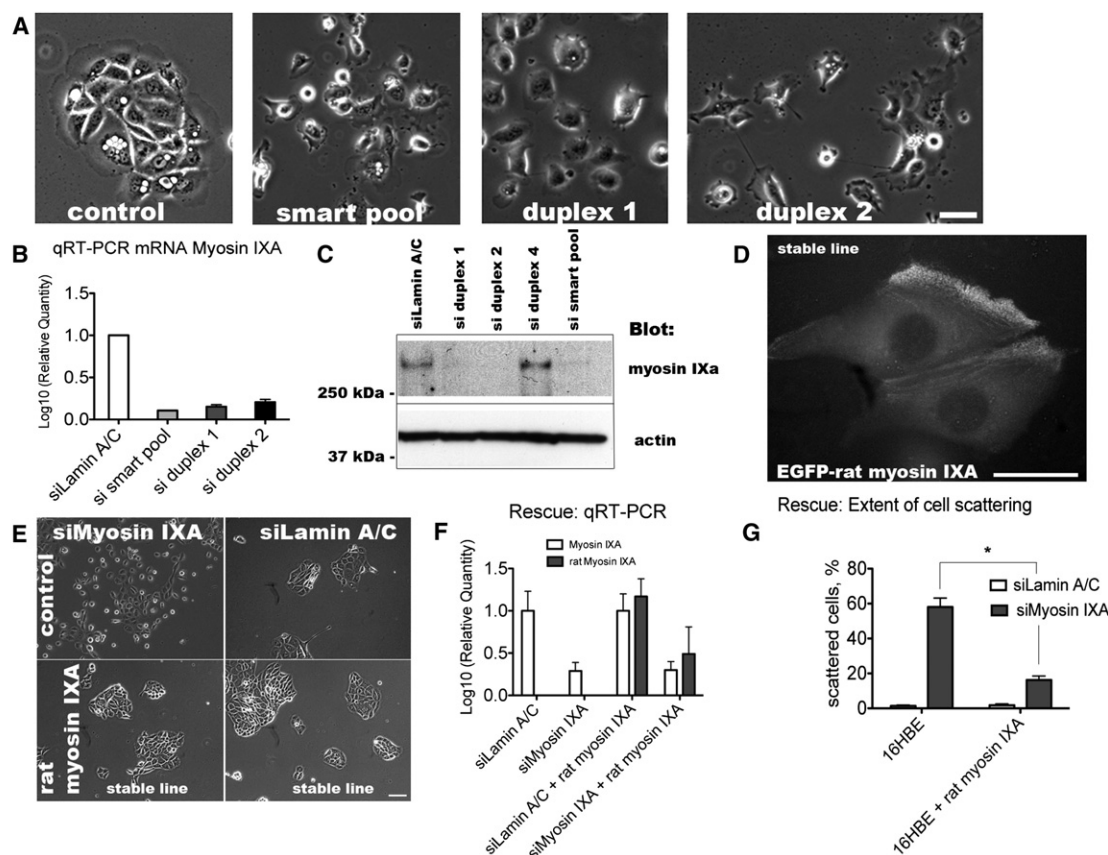


Figure 2. Validation of Myosin-IXA Phenotype

(A) Two siRNA duplexes and the SMARTpool cause cell scattering. Scale bar represents 50 μ m. (B) mRNA quantification by qRT-PCR after siRNA treatment (error bars represent SEM for three independent experiments). (C) Western blot analysis of myosin-IXA siRNA depletion. (D) 16HBE cells stably expressing EGFP-rat-myosin-IXA. Scale bar represents 30 μ m. (E) Migration of EGFP-rat-myosin-IXA-expressing cells before and after transfection with myosin-IXA siRNA. 16HBE stable pools expressing EYFP-actin were used as control. siRNA targeting lamin A/C (control) or myosin-IXA duplex-1 were used. Scale bar represents 100 μ m. (F) Quantification of endogenous human and exogenous rat myosin-IXA mRNA by qRT-PCR. Depletion of endogenous myosin-IXA by duplex-1 siRNA is seen in control cells and EGFP-rat-myosin-IXA-expressing cells (error bars represent SD for triplicate). (G) Quantification of cell scattering in control and rat myosin-IXA expressing cells, after depletion of endogenous myosin-IXA (error bars represent SEM for two independent experiments, *p = 0.0176, unpaired t test).

of Rho GTPases important for collective migration, we screened a library of siRNA SMARTpools (each pool = four distinct siRNAs) targeting the 67 known Rho family GAPs using a 96-well plate migration assay (Platypus Technologies) (Figure 1D). Myosin-IXA depletion caused a severe inhibition of forward cell migration (Figure 1E) (area of free space = $1.9 \text{ mm}^2 \pm 0.5$) similar to Cdc42 depletion ($2.2 \text{ mm}^2 \pm 0.2$), when compared to parental cells ($0.02 \text{ mm}^2 \pm 0.01$) or lamin A/C depleted cells ($0.05 \text{ mm}^2 \pm 0.03$) (three independent experiments).

Time-lapse microscopy revealed that myosin-IXA depletion induced cell scattering in migrating islands (Figures 1F and 1G, 4 hr time course; Movie S2) and in scratched monolayers (Figure S1A; Movie S3). Wound edge advancement was inhibited by 90% when compared to control cells (Figure S1B). Myosin-IXA depletion led to uncoordinated migration in monolayers and islands without significant changes in cell migration rates (Figures S1C and S1D).

Validation of Myosin-IXA Phenotype

The cell scattering phenotype was induced with siRNA duplex-1 and -2, whereas duplex-3 and -4 gave a partial effect

(Figure 2A; data not shown), in good agreement with the extent of messenger RNA (mRNA) depletion, as judged by qRT-PCR (Figure 2B), and protein levels, as judged by depletion of the expected band of 300 kDa on western blots (Figure 2C). To validate the phenotype, we performed rescue experiments. Stable pools of cells expressing EYFP-actin (control) or EGFP-rat-myosin-IXA were isolated. The level of EGFP-rat-myosin-IXA expression detected by live cell imaging (Figure 2D) was variable between individual cells, but nevertheless, cell scattering induced by siRNA duplex-1 was significantly inhibited (Figures 2E–2G). qRT-PCR analysis confirmed the efficiency of endogenous myosin-IXA depletion in this experiment (Figure 2F).

Myosin-IXA Accumulates at Nascent Cell-Cell Contacts

To explore the function of the protein at cell-cell contacts, we revealed via immunostaining the endogenous myosin-IXA and ectopic expressed EGFP-Myosin-IXA localize at cell-cell contacts in 16HBE monolayers (Figures S2A–S2C) [29]. Careful line scan analysis revealed that myosin-IXA colocalizes with ZO-1, but not with E-cadherin. After myosin-IXA

depletion, cells that still remained associated with neighbors showed a loss of ZO-1 staining, whereas E-cadherin adhesions became irregularly shaped (radial and punctate), in contrast to the tangential linear contacts observed in control cells (data not shown). Radial cell-cell contact geometry is characteristic of high tension between cells and time-lapse microscopy confirmed myosin-IXA-depleted cells pull away from adjacent cells (*Movie S2*). These results suggest that myosin-IXA is involved in stabilizing junctional complexes. In an analysis of cell-cell collisions, EGFP-myosin-IXA (*Figures S2D* and *S2F*), but not EGFP (*Figure S2E*), accumulates in regions of overlapping lamellipodia and filopodia, where nascent junctions form. The accumulation of myosin-IXA at nascent junctions can also be seen in calcium switch experiments (*Figure S2G*).

Myosin-IXA Is Required for the Assembly of Radial Actin Bundles at Cell-Cell Contacts

Cell scattering induced by myosin-IXA depletion could be a consequence of at least two events: (1) loss of mature cell-cell contacts and (2) inability to reform new adhesions as cells move relative to each other (*Movie S2*). To examine the role of myosin-IXA in contact formation, we isolated stable pools of cell expressing EYFP-actin, or GFP-E-cadherin and the assembly of cell junctions monitored after cell-cell collisions. In control cells, an overlapping zone between two lamellipodia formed within the first 3 min of contact (*Figure 3A*, arrows; *Movie S4*). During a period of 2–4 min postcollision, 16HBE cells established cell-cell contact expansion (*Figure 3B*, upper graph). In contrast, in colliding cells depleted of myosin-IXA, cell-cell contact expansion was inhibited, and cells were unable to form an overlapping zone (*Figure 3A*, arrowheads, *Figure 3B*; *Movie S4*). The average area of lamellipodial overlap in myosin-IXA-depleted cells is about four times less than in control (*Figure 3B*, lower graph). These data indicate that myosin-IXA is important during lamellipodial overlapping and early stabilization of cell-cell contacts.

Time-lapse sequences of EYFP-actin expressing cells revealed further details of cell-cell interactions in 16HBE cells upon collision. In control cells, junctional actin accumulated at contact sites within the first 2 min of collision (*Figure 3C*, upper panels, arrows), and over time, this transformed into radial actin bundles, or actin fingers, bridging the two colliding cells (*Figure 3C*, arrowheads, *Movie S5*). After myosin-IXA depletion, junctional actin initially accumulated similar to control (*Figure 3C*, lower panels, arrows), but although some minor bundles could be seen, these bundles did not bridge two colliding cells and cells retracted from each other (shown schematically in *Figure 3D*).

The formation of radial bundles or actin fingers can best be observed when imaging a collision between a fluorescent and a nonfluorescent cell (*Figure 3E*, arrows, blue line outlines nonfluorescent cell). In control cells (*Movie S6*), actin fingers emerged from an overlapping lamellipodium formed on top of its neighbor, supporting a recently proposed asymmetric push-pull mechanism of contact formation [12]. These filopodia-like structures contained GFP-E-cadherin signal (*Figure 3E*, arrowheads). In myosin-IXA-depleted cells (*Movie S7*), actin fingers either did not form, or if they did, they were very small (*Figure 3E*, arrow in EYFP-actin panel, *Figure 3F*). GFP-E-cadherin clustering did occur (*Figure 3E*), but clusters were unstable and disappeared as cells moved apart. These results suggest that myosin-IXA regulates the formation of cell-cell contacts via remodeling of the actin cytoskeleton.

Myosin-IXA Localizes with Actin Filaments at the Leading Edge

To examine further the relationship between myosin-IXA and actin, we examined individual 16HBE cells. EGFP-myosin-IXA localized in puncta associated with the prominent marginal bundle (MB) of actin filaments at the periphery (a pattern similar to nonmuscle myosin II but different from myosin IXB, which accumulated more diffusely) (*Figure S3A*) [30]. Myosin-IXA puncta in lamellipodia underwent retrograde flow (*Figure S3B*), whereas live imaging of cells coexpressing EGFP-myosin-IXA and mDsRed-actin, revealed colocalization of myosin-IXA puncta with actin bundles (*Figure S3C*, top panels), which were disrupted with latrunculin (*Figure S3C*, lower panels) or the Rho kinase inhibitor Y-27632 (data not shown).

To investigate the relationship between myosin-IXA and actin filament organization, we depleted myosin-IXA in EYFP-actin/16HBE cells. Whereas lamellipodia in control migrating cells have a smooth contour, in myosin-IXA-depleted cells, lamellipodia have irregular curvature and are rich in ruffles (*Figure S3D*). In addition, myosin-IXA depletion promoted reorganization of the marginal bundle into multiple actin bundles oriented perpendicular to the leading edge (*Figure S3E*, arrowheads). We conclude that myosin-IXA interacts with contractile actomyosin filaments and is involved in organizing actin filaments at the leading edge.

Myosin-IXA Is Recruited to Actin Bundles through Its Motor Domain

To characterize myosin-IXA function further, we generated mutants of EGFP-human-myosin-IXA (*Figure S4*) and expressed in 16HBE cells. The role of myosin-IXA motor function was evaluated using the ATP hydrolysis mutant, R297C, which is analogous to a myosin-IXB motor mutant, R295C [30]. Unlike wild-type (WT) EGFP-myosin-IXA, EGFP-myosin-IXA-R297C showed almost no localization to actin bundles, with most cells showing a cytoplasmic diffuse distribution (*Figure 4*, panels 1 and 2) suggesting that a functional motor domain is necessary to target myosin-IXA to actin. The punctate distribution of the WT, but not the motor mutant protein, is clearly seen in the line scan analysis (*Figure 4*, lower panels).

Class-IX myosins are single-headed plus-end-directed motors and have unique two-domain-motor separated by a large loop-2 insertion important for regulation of myosin-IX affinity to actin (*Figure S4A*) [30]. Deletion of this insertion (A701-H886 full) abolished binding to actin bundles (*Figure 4*, third panel). In contrast, expression of the N terminus of myosin-IXA (motor-neck) led to a cellular distribution similar to full-length myosin-IXA (*Figure 4*, fourth panel). This indicates that a functional motor domain is necessary and sufficient for localization of myosin-IXA to peripheral actin bundles.

Functional Analysis of RhoGAP Domain

Individual cells depleted of myosin-IXA showed blebbing and abnormal protrusive activity (*Figure 5A*, arrows), consistent with the loss of RhoGAP activity leading to Rho hyperactivation at the cell periphery. To confirm that increased Rho activity contributes to the myosin-IXA depletion phenotype, we used Y-27632 or blebbistatin to inhibit Rho-kinase, or myosin-II, respectively. Inhibition of actomyosin contractility in myosin-IXA-depleted cells led to cell flattening (*Figure 6A*, arrows) and disappearance of the blebs and protrusive swellings seen in the absence of the inhibitors (*Figure 6A*, arrowheads). Furthermore, upon collision and in the presence of

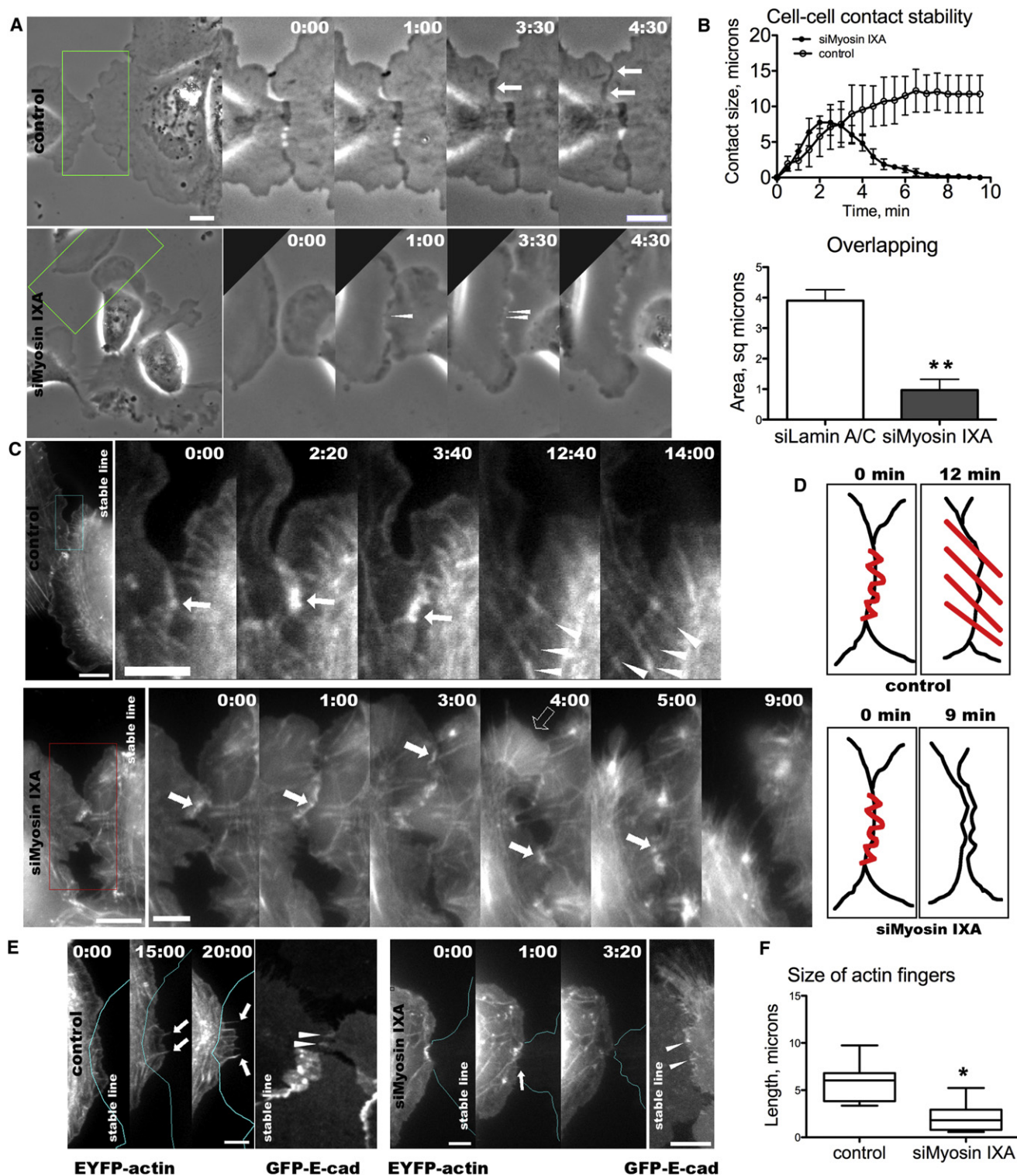


Figure 3. Myosin-IXA Is Required for Junctional Radial Actin Bundle Formation

(A) Time-lapse images of colliding 16HBE cells show lamellipodial overlaps (arrows) inhibited in myosin-IXA-depleted cells (arrowheads) (siMyosin-IXA) (Movie S4). Min:sec; scale bar represents 10 μ m.

(B) Cell-cell contact stability was quantified. Error bars represent SEM for three independent experiments, 13 collisions analyzed. The inability of siRNA SMARTpool myosin-IXA-depleted cells to establish stable contacts can also be seen in the area of lamellipodial overlap (error bars represent SEM for three independent experiments, **p = 0.004, unpaired t test).

(C) Epifluorescence microscopy of cells expressing EYFP-actin. Collision between control lamellipodia leads to actin enrichment (upper panel arrows) followed by the formation of radial actin bundles bridging two cells together (arrowheads). SMARTpool siRNA myosin-IXA depletion does not abolish actin enrichment (lower panel arrows) but inhibits radial actin bundle formation (Movie S5). Min:sec, scale bar represents 5 μ m.

(D) Schematic summary of time-lapse sequences in (C). Actin is shown in red.

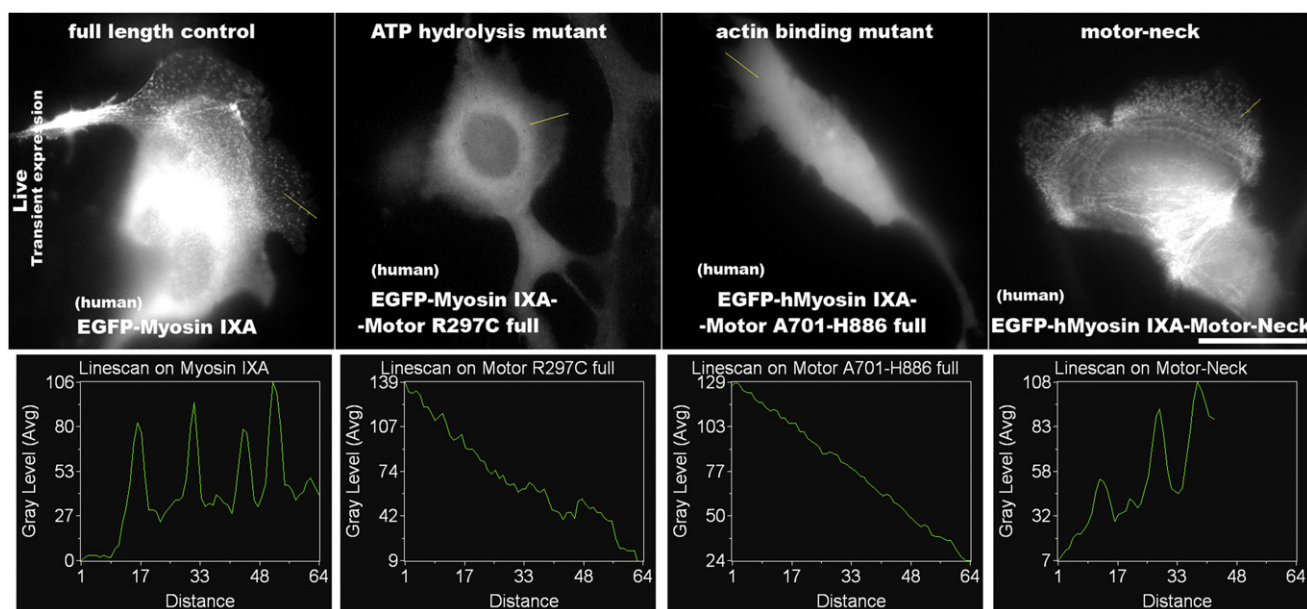


Figure 4. Motor Domain Is Necessary and Sufficient for Targeting Myosin-IXA to Actin Bundles

16HBE cells were microinjected with full-length EGFP-myosin-IXA, EGFP-myosin-IXA-motor R297C, EGFP-myosin-IXA-motor (deletion aa701–886) or EGFP-myosin-IXA-motor-neck plasmid expression constructs. Fluorescent cells were imaged live. Scale bar represents 30 μ m. Line scans through lamellipodia (yellow line, top panels) were used to analyze the presence of EGFP puncta shown as peaks on graphs in the lower panels. Note clear peaks found in full-length EGFP-myosin-IXA and EGFP-myosin-IXA-motor-neck expressing cells. Distance is in pixels.

inhibitor, cells maintained some contact and did not retract from each other (Figures 6A and 6B; Movie S8).

The overexpression of full-length myosin-IXA, on the other hand, resulted in the formation of long processes consistent with downregulation of Rho activity, whereas expression of myosin-IXA lacking RhoGAP domain (myosin-IXA- Δ GAP, Figure S4A) had little effect on cell morphology (Figure 5B) [31]. Expression of a construct lacking the motor domain (neck-tail, Figure S4A) induced a complete loss of actin bundles and dramatic morphological changes (Figure 5C, arrow), whereas a similar construct but with an inactivating point mutation in the GAP domain (neck-tail-R2098M, Figure S4A) had no effect (Figure 5C). We conclude that the N-terminal motor-neck region attenuates RhoGAP activity.

Finally, we attempted to rescue the myosin-IXA-depletion phenotype by stable expression of different myosin-IXA constructs in 16HBE cells followed by treatment with myosin-IXA siRNA. Low-level expression of the tail domain or the motor-neck domain did not disrupt cell-cell contacts and had only modest effects on cell morphology (Figure S5A). Treatment of these lines with myosin-IXA siRNA led to endogenous myosin-IXA depletion as expected (Figure S5B). EGFP-motor-neck did not significantly rescue junction formation during cell-cell collision, as observed by time-lapse microscopy (Figure 6C, top panels, Figure 6D; Movie S9) and did not prevent cell scattering (Figure 6E). Expression of EGFP-tail, however, partially rescued myosin-IXA-depleted cells from cell-cell retraction after collision, leading to increased

stability of contacts (Figure 6D). Time-lapse observations of cells (Figure 6C, lower panels) revealed extensive contact between adjacent cells, often lasting for up to 1 hr (Movie S9). The extent of cell scattering was significantly reduced in these cells (Figure 6E). Cell tracking of migrating islands revealed that random cell migrations induced by myosin-IXA depletion were partially rescued (Figure S5C). These results suggest that RhoGAP activity is required to prevent the cell scattering phenotype induced by myosin-IXA depletion in migrating cells.

Rho Activity at Nascent Cell-Cell Contacts

To investigate further the effect of myosin-IXA depletion on Rho activity, we analyzed phosphorylation levels of myosin-light-chain (MLC), a downstream target of Rho-dependent contractility signaling. Western blot analysis revealed a 10% increase in MLC phosphorylation in myosin-IXA-depleted cells compared to control cells (Figure 7A). We also detected a 25% increase in total cellular RhoGTP levels after myosin-IXA depletion, using G-linked immunosorbent assay (G-LISA), which likely reflects myosin-IXA regulating only a subset of endogenous Rho. As expected, expression of the GAP-containing EGFP-tail reduced Rho activity (by 37%) in comparison to control cells expressing EGFP-motor-neck (Figure 7B).

To explore the spatiotemporal activity of RhoA, specifically during cell-cell contact formation, we used a single-chain, FRET biosensor as a tool to detect RhoA activity in live cells [32]. In the activated GTP-bound form, the RhoA biosensor

(E) Epifluorescence microscopy of cells expressing EYFP-actin and GFP-E-cadherin. Development of actin radial fingers (arrows) during collision between a fluorescent and a nonfluorescent cell (outlined with blue line) in control (Movie S6) and myosin-IXA-depleted cells (Movie S7). In SMARTpool myosin-IXA-depleted cells, E-cadherin clustering (arrowheads) was observed between colliding cells but without significant lamellipodial overlap. Min:sec; scale bar represents 5 μ m.

(F) Quantification of actin fingers size (24 cell-cell contact zones analyzed in three independent experiments, * p = 0.036, unpaired t test). Top and bottom of a box indicates min and max values, line is the mean.

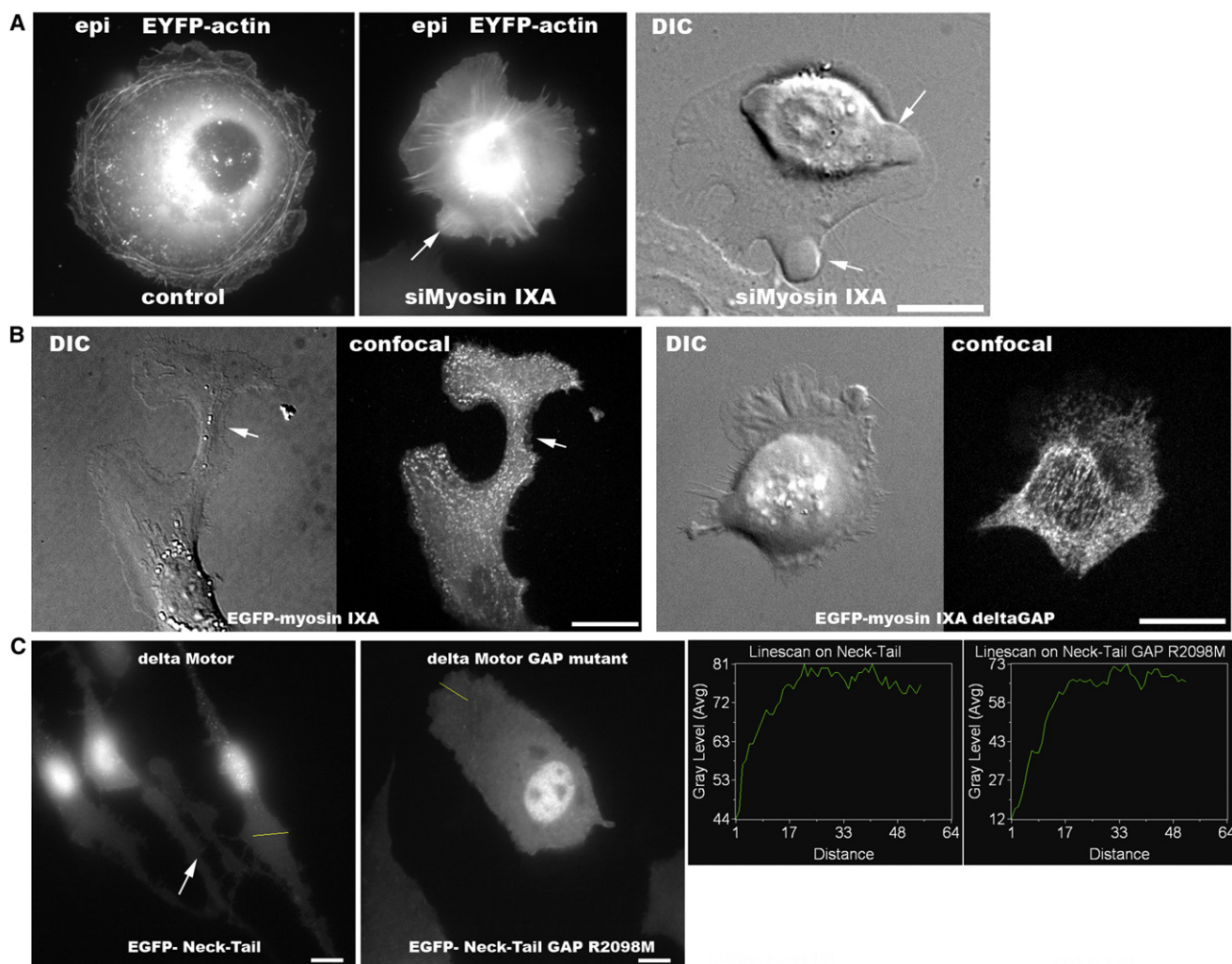


Figure 5. Functional Analysis of Myosin-IXA RhoGAP Domain

(A) Epifluorescence or differential interference contrast (DIC) microscopy of cells expressing EYFP-actin. Myosin-IXA depletion by siRNA causes blebbing and lamellipodia swellings (arrows).

(B) Confocal and DIC images of a cell expressing full-length, EGFP-rat myosin-IXA showing the formation of long protrusions (arrows). Expression of EGFP-myosin-IXA-deltaGAP has little effect on normal cell morphology. Scale bar represents 30 μ m.

(C) Diffuse cytoplasmic localization of EGFP-myosin-IXA-neck-tail leads to dramatic morphological changes, with long tails (arrow) characteristic of strong Rho inhibition. This phenotype is not induced by a GAP-defective mutant (R2098M) of EGFP-myosin-IXA-neck-tail. Live-cells were imaged by epifluorescence microscopy. Linescans (right panels) through lamellipodia were used to quantify presence of EGFP puncta shown as peaks on graphs. Note absence of clear peaks found in cells expressing either EGFP-myosin-IXA-neck-tail or its GAP-defective mutant (R2098M). Distance is in pixels.

shows increased FRET between cyan and yellow fluorescent proteins, and the FRET/CFP emission ratio reflects the level of RhoA activation [32]. Collisions between control or myosin-IXA-depleted cells were imaged over 5–30 min. After calculating FRET/CFP ratios, elevated signals were observed at nascent cell-cell contacts in myosin-IXA-depleted cells compared to control cells (Figure 7C, arrows). Normalized FRET efficiency at these cell-cell contact sites was increased about 2-fold after myosin-IXA depletion (Figure 7D). These results suggest that myosin-IXA negatively regulates RhoA activity specifically at nascent cell-cell junctions in 16HBE cells.

Discussion

To study the molecular mechanisms underlying collective epithelial migration, we used the human bronchial epithelial

cell line, 16HBE [33]. Scratching a 16HBE monolayer induces collective migration of cells into the space created. Cell streams, or flows, can be seen within the migrating epithelium, resulting in cells changing their position with respect to each other, a common characteristic of epithelial collective migration [34, 35].

Rho GTPases, particularly Rho, Rac, and Cdc42, regulate the actin cytoskeleton and cell polarity in epithelial tissues. Through effector proteins such as a Rho-kinase, Wiskott-Aldrich syndrome protein, and mDia, they promote actin polymerization and actin-myosin filament contraction, and through effectors such as Par6 and IQGAP, they mediate the establishment of apical-basal polarity and cell-cell junction formation [19]. They are, therefore, important regulators of epithelial morphogenesis and migration. A key feature of these signaling pathways lies in the ability of upstream activators (GEFs) and inactivators (GAPs) to regulate Rho GTPases in a spatially restricted way.

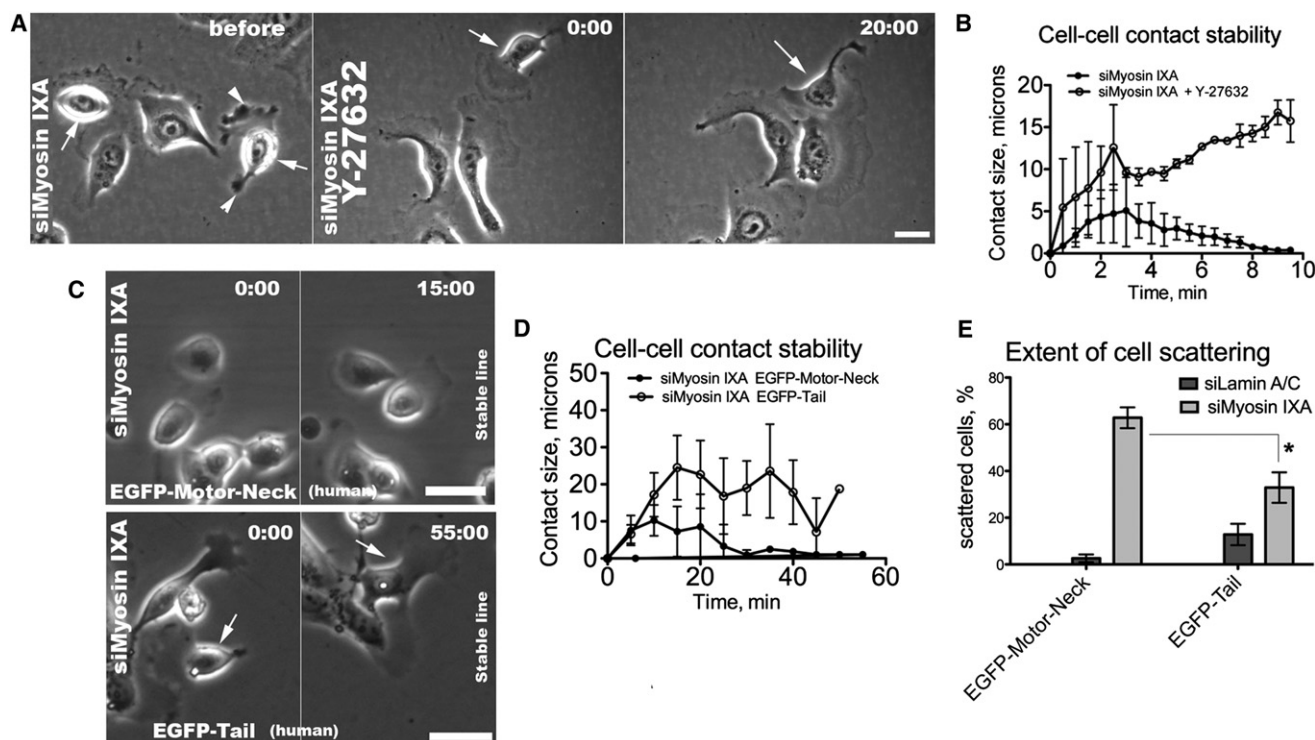


Figure 6. Rho-Dependent Activities of Myosin-IXA

(A) Partial rescue of cell-cell contact stability in myosin-IXA-depleted cells (SMARTpool siRNA) after inhibiting Rho-dependent actomyosin contractility, with Rho-kinase inhibitor Y-27632 (5 μ M). Cells with swollen lamellipodia (arrowheads) and rounded cell body in myosin-IXA-depleted cells become flattened (arrows) after treatment with Y-27632 (Movie S8). Time is in min:sec. Scale bar represents 30 μ m.

(B) Quantification of rescue of cell-cell contact stability by Y-27632 (error bars represent SEM for two independent experiments).

(C) Partial rescue of cell-cell contact stability by RhoGAP expression (EGFP-tail) (lower panels), but not by motor-neck expression (EGFP-motor-neck) (upper panels) in myosin-IXA-depleted cells. Depletion of endogenous myosin-IXA in EGFP-motor-neck expressing line by duplex-1 and in EGFP-tail expressing line by duplex-2 was performed. Upon cell-cell collision, cells remain attached for up to 1 hr (arrow) (Movie S9). Min:sec; scale bar represents 30 μ m.

(D) Quantification of rescue of cell-cell contact stability (error bars represent SEM for two independent experiments).

(E) Partial rescue of cell scattering phenotype in myosin-IXA-depleted cells after expression of EGFP-tail, but not EGFP-motor-neck, constructs (error bars represent SEM for two independent rescue experiments, * p = 0.03, unpaired t test).

To explore their contribution to collective cell migration, we screened an siRNA library targeting the 67 known Rho family GAPs, in 16HBE cells, and identified myosin-IXA, an actin-binding, RhoGAP-domain-containing protein, as an important player in collective migration.

Depletion of myosin-IXA by siRNA resulted in uncoordinated, random migration in scratched monolayers and in islands, through the inability of cells to maintain cell-cell contacts. A large number of proteins are recruited to adherens junctions and tight junctions at cell-cell contact sites to control their assembly, organization, maintenance and association with actin filaments, including numerous actin-based motors, such as myosin-II, -VII, -VI and -X. Myosins regulate cell-cell contacts by remodeling the actin cytoskeleton with their actin cross-linking activity, or by motor function creating tension between actin filaments, or by delivering cargo components. A myosin-IXA knockout mouse revealed a crucial role for this protein in the differentiation of ependymal cells, where it is highly expressed, with defects seen in morphology, gene expression, and junction formation, supporting our results on the importance of myosin-IXA function at cell-cell contacts [29].

Myosins are a large family of actin-binding motors, structurally diverse but sharing a conserved motor domain that binds to actin filaments and hydrolyzes ATP leading to translocation

along the filament [36]. Myosins bind selectively to actin filaments with different composition and spatial arrangement [37]. Endogenous myosin-IXA and EGFP-myosin-IXA localize in puncta at cell-cell contact sites, along the circumferential, contractile actin bundle [29]. The circumferential localization suggests that myosin-IXA harbors recognition motifs for contractile actin filaments, perhaps in the neck-tail region, similar to myosin-IIA and -IIB [38].

Rho signaling is important during cell-cell contact formation, and we recently described a role for the Rho target PRK2 in cell-cell junction maturation in 16HBE cells [39]. Localized activation of RhoA at the cell-cell contact zone, visualized by FRET, has a distinct spatiotemporal pattern upon cell-cell interactions suggesting tight regulation by both GEFs and GAPs [21, 22]. Here we present evidence that the RhoGAP activity of myosin-IXA facilitates the assembly of cell-cell contacts and that this is particularly important during collective cell migration. First, we found that the contractile phenotype induced by depletion of myosin-IXA can be partially reversed by inhibiting Rho-kinase. Second, the phenotype observed after overexpression of myosin-IXA is abolished by an inactivating mutation in the RhoGAP catalytic site. Third, low level expression of the constitutively active RhoGAP domain partially rescues the cell scattering phenotype induced by myosin-IXA depletion. Fourth, RhoA activity at

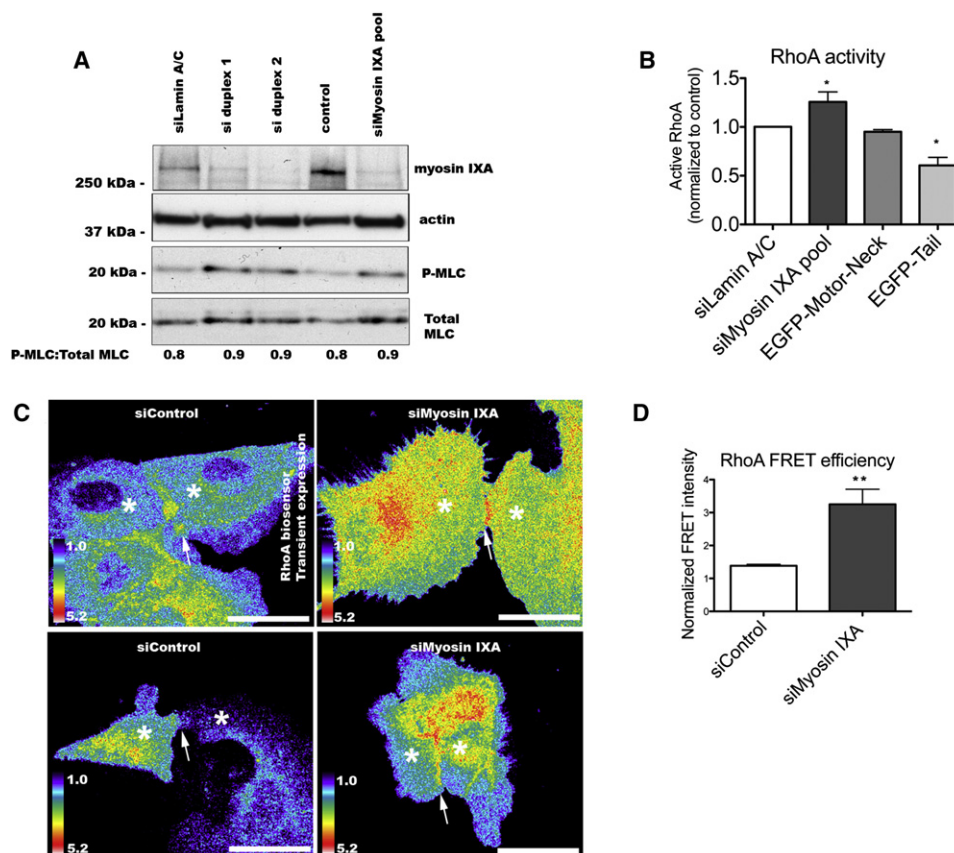


Figure 7. Quantification of Rho Activity after Myosin-IXA Depletion

(A) Western blot analysis of myosin-light-chain phosphorylation in myosin-IXA-depleted 16HBE cells. The ratio of phosphorylated over total levels of MLC is shown below.

(B) Levels of active RhoA were measured in control, SMARTpool myosin-IXA-depleted cells, and in cells expressing myosin-IXA mutants (error bars represent SD for two independent experiments, * $p < 0.004$, unpaired t test).

(C) Confocal time-lapse ratio imaging of RhoA FRET biosensor in control and SMARTpool myosin-IXA-depleted cells. Colliding cells are labeled with asterisks. Newly formed cell-cell contacts are indicated by arrows. Note the high FRET/CFP ratio signal at nascent cell-cell contacts after myosin-IXA depletion. Scale bar represents 10 μ m.

(D) Quantification of normalized FRET/CFP intensity ratio signal. Nascent cell-cell contacts were outlined using the MetaMorph Region tool (2–4 square microns) and similar-sized, contact-free edge regions were selected as controls. Normalized FRET was calculated as ratio between average pixel gray levels over cell-cell contact region relative to average pixel gray levels in control contact-free regions. A total of 14 cell-cell contacts were analyzed (error bars represent SEM for three independent experiments, ** $p = 0.008$, unpaired t test).

nascent cell-cell contacts, detected by FRET ratio imaging, is significantly increased in myosin-IXA-depleted cells. These data suggested that the GAP domain of myosin-IXA acts as a localized, negative regulator of Rho to control contractile forces during the early stages of cell-cell contact formation.

The interaction of lamellipodia from two colliding cells stimulates local clustering of E-cadherin and the recruitment of actin filaments, referred to as junctional actin. Junctional actin can be seen at very early stages of cell-cell interactions in both control and myosin-IXA-depleted colliding 16HBE cells, but in the absence of myosin-IXA, this did not reorganize into radial actin filaments. As a consequence, junctions were not stabilized and cells moved away from each other. These observations indicate that myosin-IXA is essential during the assembly of radial actin filaments (Figure 3D).

We conclude that myosin-IXA is required during the assembly of radial actin filaments, seen within a few minutes after cell-cell contact. In migrating 16HBE cells, these appear to be essential for subsequent stabilization of contact sites and depletion of myosin-IXA leads to cell scattering. Maturation

of cell-cell junctions is accompanied by Rho-dependent conversion of radial actin filaments into tangential actomyosin filaments. We speculate that myosin-IXA is required locally to downregulate Rho activity during early stages of junction formation, as well as during the dynamic reorganization of cell-cell contacts seen during collective migration.

Experimental Procedures

Cell Culture and Transfections

Human bronchial epithelial cells 16HBE14o- (16HBE) [33] were maintained as described [24]. We used 60–120 ng siRNA (Dharmacon) to transfect 0.5×10^5 16HBE cells. Duplex sequences were: duplex-1 GAAAGAAG CUUAGCCCUUAAU; duplex-2 GAUAAUACCGCAUAAUU; duplex-3 GAAC AUACAUUACGGAUAAUU; duplex-4 GAACAAAGGCUAAGAGAAUU. For transient expression, DNA (4 μ g) was nucleofected into 0.5×10^6 16HBE using kitT (Lonza), with program A-23, according to manufacturer's protocol. All stable lines were obtained by selection in G418.

RNAi-Based Screen

16HBE cells were plated at 1×10^4 cells/well in a 96-well plate each containing a silicon insert (Platypus Technologies). The next day, the medium

was replaced with OptiMEM reduced serum and antibiotic-free medium (Invitrogen) and cells were transfected using Oligofectamine with SMART-pool siRNAs (Dharmacon/ThermoFisher Scientific). Seventy-two hr post-treatment, silicon inserts were removed to allow migration for 24 hr. Cells were stained with Calcein AM (Invitrogen), fixed in 3.7% formaldehyde, and images were taken using Olympus dissection fluorescence microscope.

Antibodies, Immunofluorescence, Western Blot Analysis

Primary antibodies: anti-ZO-1 (1:100) (Zymed Laboratories, San Francisco, CA), anti-E-cadherin (1:100) (Zymed), anti-GFP (1:100) (Invitrogen, Eugene, OR), anti-myosin-IXA (1:1000-1:10,000) [31], rabbit polyclonal anti-MLC-2 (Cell Signaling) (1:500), anti-phospho-myosin-II-Ser19 (Cell Signaling) (1:1000), mouse monoclonal anti- β -actin IgG2a isotype (clone AC-74) (Sigma). Secondary antibodies: Alexa 488, Alexa 568 and Alexa 350 conjugated donkey, anti-rabbit, and goat anti-mouse (Molecular Probes/Invitrogen); horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG (1:5000) (Jackson ImmunoResearch Laboratories, West Grove, PA). For staining, cells were fixed in -20°C methanol or 3.7% formaldehyde and treated with blocking/permeabilization solution (0.1% saponin, 50 mM NH_4Cl in PBS) for 25 min prior to antibody application. For western blots, cells were lysed with sample buffer, sonicated, boiled, and separated by 3%–8% Tris-Acetate SDS-PAGE as described [24].

Image Analysis

Image analysis was performed using MetaMorph. Cell tracking analysis was performed by thresholding phase contrast images for dark regions (cytoplasm is dark, cell-cell contacts are bright), determining region centroids and automatic tracking. Alternatively, manual tracking was performed using cell centroids (track points function). Extent of cell scattering was calculated as the ratio of scattered cells over total number of cells. Scattered cells are defined as cells with no contacts. Cell-cell contact stability was calculated by plotting the length of the cell-cell contact (determined by the straight line, line region tool) over time. Actin fingers were defined in time-lapse sequences as actin-rich filopodia-like structures protruding after lamellipodia collisions.

FRET ratio analysis was performed using MetaMorph. FRET images were background corrected using the MetaMorph FRET application and the FRET/CFP ratio was calculated using an Arithmetic MetaMorph function. Normalized FRET efficiency was calculated from the FRET/CFP images: average pixel gray intensity of cell-cell contact region was divided by average gray intensity of protruding cell free edge region. The size of the region, the same for each image, was 2–4 square microns depending on the size of the cell-cell contact. Only images with cell-cell contacts formed within 5 min after collision were analyzed.

RhoA Activity Assay

16HBE cells were transfected with siRNAs or nucleofected with pEGFP-myosin-IXA mutants in six-well plates and at day 3 (myosin-IXA depletion), or day 2 (postnucleofection), cell lysates were collected and analyzed for levels of active RhoA using the G-linked immunosorbent assay (G-LISA) kits (Cytoskeleton). Equal amounts of protein were used for the different conditions.

Supplemental Information

Supplemental Information includes five figures, Supplemental Experimental Procedures, and nine movies and can be found with this article online at doi:10.1016/j.cub.2012.01.014.

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