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The DOCK180/Elmo Complex Couples ARNO-Mediated Arf6 Activation to the Downstream Activation of Rac1

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Summary

Cell motility requires extensions of the plasma membrane driven by reorganization of the actin cytoskeleton. Small GTPases, particularly the Rho family, are key regulators of this process [1–3]. A second class of GTPases, the ADP-ribosylation factors (ARFs), have also been implicated in the regulation of the actin cytoskeleton and motility [4]. ARF6 is intimately involved in the regulation of Rac activity [5–9]; however, the mechanisms by which ARF activation leads to activation of Rac remain poorly understood. We have previously shown that expression of the ARF-GEF ARNO in MDCK cells induces robust activation of Rac, the formation of large lamellipodia, and the onset of motility [9]. We report here that ARNO-dependent activation of Rac is mediated by a bipartite Rac GEF, the Dock180/Elmo complex. Both DOCK180 and Elmo colocalize extensively with ARNO in migrating MDCK cells. Importantly, both a catalytically inactive Dock180 mutant and an Elmo mutant that fails to couple to Dock180 block ARNO-induced Rac activation and motility. In contrast, a similar mutant of the Rac GEF β-PIX fails to inhibit ARNO-induced Rac activation or motility. Together, these data suggest that ARNO and ARF6 coordinate with the Dock180/Elmo complex to promote Rac activation at the leading edge of migrating cells.

Results and Discussion

We previously hypothesized [9] that active ARF6 could promote Rac activation by recruitment of a protein complex containing the Arf-GAPs GIT1/2 [10–12] and the Rac GEF β-PIX [13]. In this model, targeting of the GAP to sites of Arf activation would lead to local activation of Rac, which would promote membrane protrusion at the leading edge of the cell. A prediction of this hypothesis is that inhibition of β-PIX activity would reduce the robust activation of Rac observed in ARNO-expressing cells and would thereby inhibit protrusiveness and motility. To determine if this were the case, we made use of a catalytically inactive mutant of β-Pix (L238R, L239S, hereafter referred to as PIX-LL) that has previously been shown to function in a dominant-negative manner [14, 15]. As we have previously reported, expression of ARNO in MDCK cells leads to formation of large fan-shaped lamellipodia, separation of cells from the epithelium, and migration into open spaces created by wounding of epithelial monolayers (Figure 1A) [9]. Coexpression of wild-type β-Pix in these cells did not alter this morphology. Surprisingly, while PIX-LL colocalized extensively with ARNO, its expression did not inhibit the ARNO-induced extension of lamellipodia, suggesting that Rac activation was not impaired under these conditions (Figure 1A). Biochemical analysis of Rac-GTP levels revealed that this was indeed the case. As shown in Figure 1B, expression of ARNO led to a robust increase in Rac activation, and this was neither enhanced nor inhibited by coexpression of wild-type PIX. Additionally, we found that coexpression of PIX-LL did not significantly inhibit Rac activation in ARNO-expressing cells. Taken together, these data indicate that, while β-Pix may mediate Rac activation in other contexts, it does not facilitate the activation of Rac downstream of Arf6.

This finding led us to consider alternative pathways for crosstalk between the two GTPases. One such alternative is Dock180, a nonconventional Rac GEF that has been shown to function in numerous cell migration events, including dorsal closure in *Drosophila* [16, 17] and gonadal distal tip cell migration in *C. elegans* [18–20]. Dock180 lacks the typical *dbl*-homology domain (DH)/PH domain pair found in most Rac GEFs. Instead, catalytic activity resides in a conserved region called the DOCKER domain, and the PH domain (which is necessary for optimal catalytic activity) is provided in *trans* by a second protein, Elmo [21, 22]. Dock180 has long been known as a binding partner for the scaffolding protein Crk [23], and microinjection of CrkII into MDCK cells induces a morphology that is remarkably similar to that induced by ARNO expression [24]. Given the striking similarity in morphology between ARNO-expressing and CrkII-expressing MDCK cells, we reasoned that they might function in the same pathway.

To compare the subcellular localization of ARNO, Elmo, and Dock180, MDCK cells were transfected with myc-ARNO and GFP-Elmo (Figure 2A) or ARNO, Elmo, and flag-Dock180 (Figure 2B). Deconvolution microscopy demonstrates extensive colocalization of ARNO with both Elmo and Dock180 at the leading edge of migrating MDCK cells, as well as at other structures located more distally in the cell. Similar to cells expressing ARNO alone, cells coexpressing ARNO and wild-type Dock180 form large lamellipodia and migrate away from neighboring cells (Figure 3A). In contrast, cells coexpressing ARNO and Dock-ISP, a catalytically incactive mutant that acts in a dominant-negative fashion [22], fail to generate detectable lamellipodia, suggesting that inhibition of DOCK180 activity blocks the ability of ARNO to induce motility (Figure 3A). Similarly, Elmo T629, a truncation of Elmo lacking the Dock180 binding domain, also inhibits formation of lamellipodia when coexpressed with ARNO (Figure 3B). Together, these data support the hypothesis that Rac activation

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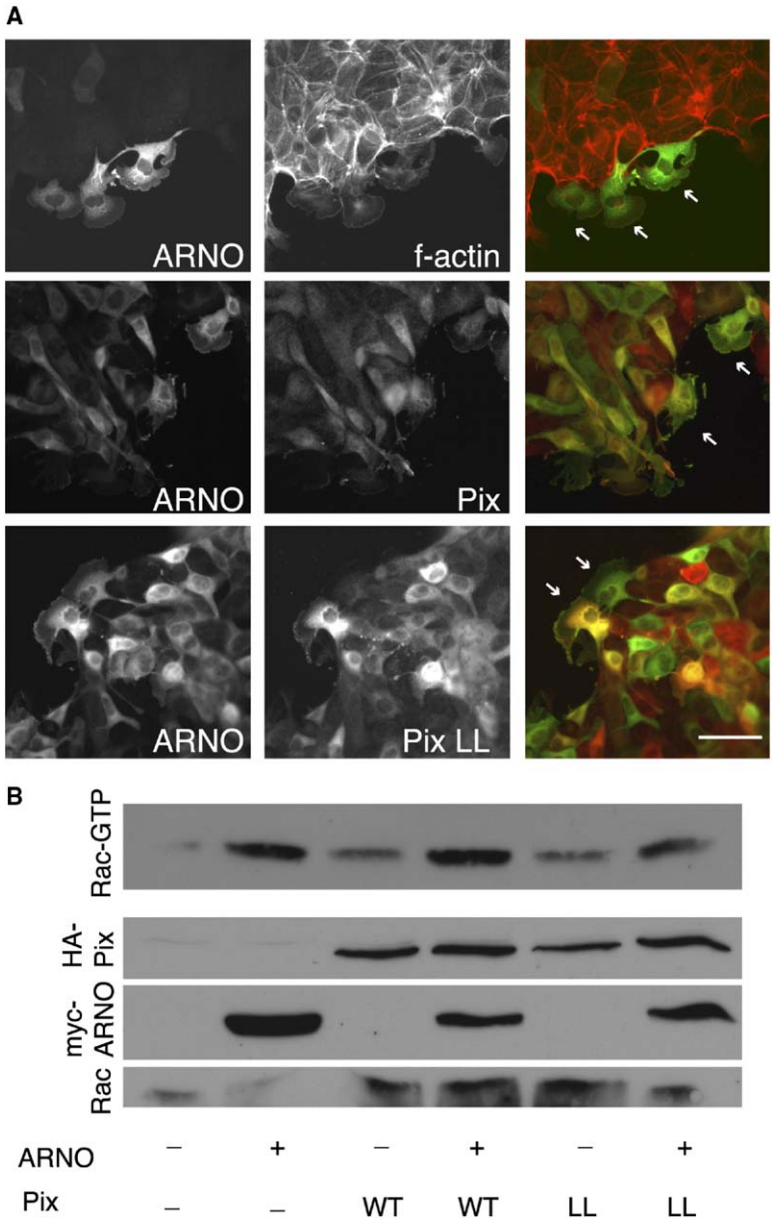

Figure 1. A Catalytically Inactive β-Pix Mutant Fails to Inhibit Arf6-Induced Formation of Lamellipodia or Rac Activation

(A) Monolayers of MDCK cells were infected with adenoviruses encoding myc-ARNO (row 1), ARNO and HA-Pix (row 2), or ARNO and HA-Pix (L238R, L239S) (row 3) under the control of a tetracycline-responsive promotor. After 15 hr in the presence of doxycycline, cells were washed and incubated for 3 hr in the absence of doxycycline to induce transgene expression. Wounds were introduced into the monolayers, which were incubated an additional 2 hr to allow migration into the wound. Cells were then fixed and stained with 9e10 anti-myc followed by cy2-conjugated anti-mouse and rhodamine-conjugated phalloidin (row 1) or with rabbit anti-HA and 9e10 anti-myc, followed by cy3-anti-rabbit and cy2-anti-mouse secondary antibodies (rows 2 and 3). Pix or f-actin is shown in red and ARNO in green in the merged images. Scale bar equals 50 μm.

(B) Subconfluent islands of MDCK cells were infected with adenoviruses encoding the indicated proteins for 3 hr. Cells were then lysed and GTP bound Rac was isolated by binding to GST-PBD as previously described [9]. Rac activation in ARNO-expressing cells ranged from 1.5- to 10-fold the levels seen in control cells, while Rac activation in cells coexpressing ARNO and Pix (L238R, L239S) ranged from 1.3- to 4-fold control levels. The difference in Rac activation between cells expressing ARNO and cells coexpressing ARNO and Pix (L238R, L239S) was not statistically significant (paired t test, $p = 0.22$, $n = 4$).

downstream of Arf6 is mediated by the Dock180/Elmo complex.

To directly test this hypothesis, we generated recombinant adenoviruses encoding wild-type Dock180 or Dock-ISP. The use of adenoviral vectors permits near 100% infection efficiency in epithelial cells, allowing us to quantitate endogenous Rac activation by using standard pull-down assays. As described above (Figure 1), expression of ARNO enhanced levels of active Rac in these cells, relative to controls. Coexpression of Dock-ISP but not wild-type Dock180 inhibited ARNO-induced Rac activation, and this inhibition was statistically significant (Figure 4A, 2.6- ± 1.4-fold enhanced Rac activation in ARNO-expressing cells versus 1.3- ± 0.6-fold ARNO-induced Rac activation in Dock-ISP expressing cells, $p = 0.006$, $n = 9$, paired t test).

We also tested the ability of the Dock-ISP mutant to block motility of MDCK cells in a transwell migration assay. Cells were infected with the appropriate adenoviruses for 4 hr and plated on fibronectin-coated transwell filters, and migration across the filters was assayed as previously described [9]. As shown in Figure 4B, expression of ARNO enhanced migration of MDCK cells more than 5-fold. Surprisingly, expression of DOCK180 alone did not lead to enhanced motility, despite its ability to activate Rac (Figure 3C). However, in agreement with its ability to block ARNO-induced Rac activation, Dock-ISP was able to significantly inhibit ARNO-induced motility in MDCK cells (Figure 4B). Additionally, cotransfection of MDCK cells with ARNO and GFP-Elmo T629 substantially inhibited migration in the transwell assay, relative to cells coexpressing ARNO

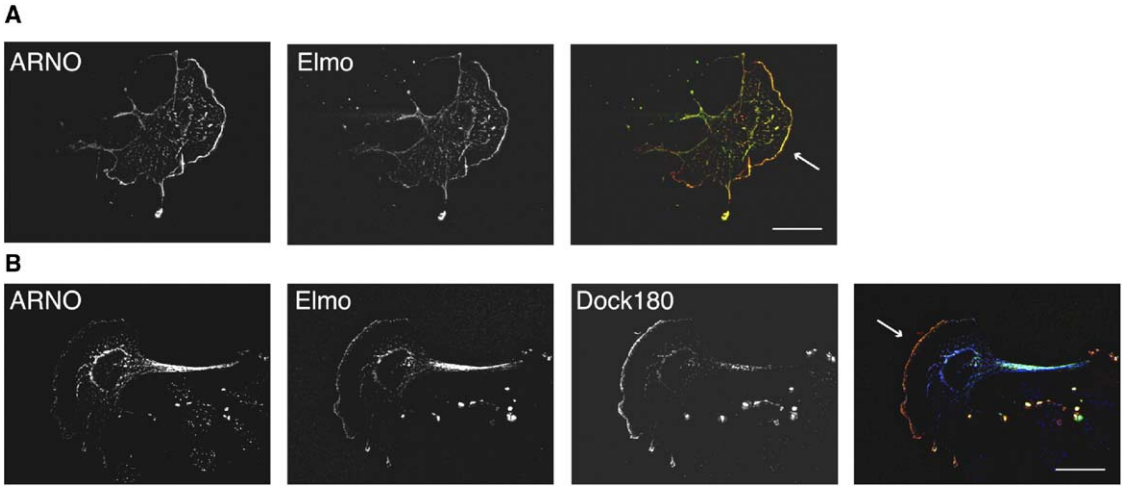

Figure 2. ARNO, Elmo, and Dock180 Colocalize in Migrating MDCK Cells

(A) MDCK cells, plated at subconfluent density, were transfected with myc-ARNO (in the tet-responsive plasmid pTRE) and GFP-Elmo by use of Lipofectamine2000 according to the manufacturer's directions. Cells were then incubated overnight in the presence of doxycycline to repress expression of ARNO. After a 3 hr incubation in the absence of doxycycline, cells were fixed and stained with 9e10 anti-myc followed by cy3-conjugated anti-mouse secondary. GFP-Elmo is shown in green and myc-ARNO shown in red in the merged image.

(B) MDCK cells at near confluence were transfected with flag-Dock180, GFP-Elmo, and myc-ARNO. After 15 hr expression, the monolayer was wounded to induce motility and incubated for 3 hr. Cells were then fixed and stained with goat anti-Dock180 (Santa Cruz Biotechnology, Santa Cruz, CA) and 9e10 anti-myc followed by Cy3 conjugated anti-goat and Cy5-conjugated anti-mouse secondary antibodies. In the merged image, Dock180 is shown in red, Elmo in green, and ARNO in blue. Optical sections were taken at 0.2 μm intervals throughout the depth of the cell, and deconvolution was used to remove out-of-focus information. The deconvolved slices were then combined into a single projection. Scale bar equals 20 μm.

and GFP. Most of the starting ARNO-expressing cells also expressed GFP or GFP-Elmo T629. However, after passing through the filter, only 34.5% ± 9.0% of ARNO-expressing cells coexpressed GFP-Elmo T629, while 77.8% ± 10% of the control cells coexpressed GFP with ARNO (see Figure S1 in the Supplemental Data available with this article online).

Taken together, these data suggest that the activation of Rac1 downstream of ARNO and Arf6 is mediated by the Dock180/Elmo complex. However, the exact mechanism by which this occurs remains to be elucidated. One possibility is that activation of Arf6 promotes delivery of endosomal Rac to the Dock180/Elmo complex at the plasma membrane [8, 25]. Arf6 has also been shown to directly activate phosphatidlyinositol-4P 5-kinase-α (PIP5Kα) [26], thereby enhancing local production of PIP_2. Locally high concentrations of PIP_2 could then recruit the DOCK180/Elmo complex by binding to the Elmo PH domain.

Another potential mechanism could involve a second Rho family GTPase, RhoG, which has been shown to bind Elmo in a nucleotide-dependent manner and promote recruitment of the Dock180/Elmo complex to the membrane [27, 28]. However, in our hands, ARNO-induced lamellipodia formation was not inhibited in the presence of either dominant-negative RhoG(T17N), RhoG(F37A) (an effector domain mutant that fails to bind Elmo), or RhoGIP122 (a RhoG binding domain that sequesters active RhoG) (Figure S2) [27–29]. These data suggest that RhoG most likely does not participate in signaling from ARNO to Dock180.

One of the early signals detected in migrating cells is the accumulation of $PI(3,4,5)P_3$ (PIP_3) at the leading edge. At least in some cells, this polarization is established by the overlapping actions of PI 3-kinases and the PTEN phosphoinositide phosphatase, whose complementary distribution leads to the production of PIP_3 at the leading edge and its hydrolysis more distally in the cell, thereby producing a steep gradient of PIP_3 [30, 31]. Both ARNO and the related protein GRP1 are recruited to membrane surfaces in a PI 3-kinase-dependent manner in response to growth factor receptor signaling [32–34]. Similarly, a gradient of PIP_3 would selectively recruit ARNO to the leading edge of migrating cells, thereby establishing a gradient of active ARF6 and, consequently, Rac activation.

The precise subcellular location of GTPase activation is an important component of directional motility. While expression of constitutively active ARF6 Q67L can induce membrane ruffling, it does not induce the migratory phenotype observed in ARNO-expressing cells [35]. Similarly, expression of an activated mutant of Rac1 also fails to promote migration in many contexts [36–39]. Additionally, a truncation mutant of Elmo lacking the N terminus failed to promote migration even though it efficiently enhanced levels of Rac-GTP [39]. One likely explanation for these observations is that mislocalized or unlocalized Rac activation is incompatible with efficient migration [36–39]. Although other Rac GEFs have been shown to respond to PIP_3-dependent signaling (e.g., VAV2, Tiam1), our data suggest that ARNO and the Dock180/Elmo complex can coordinate a localized activation of ARF6 and Rac at the leading edge of migrating MDCK cells.

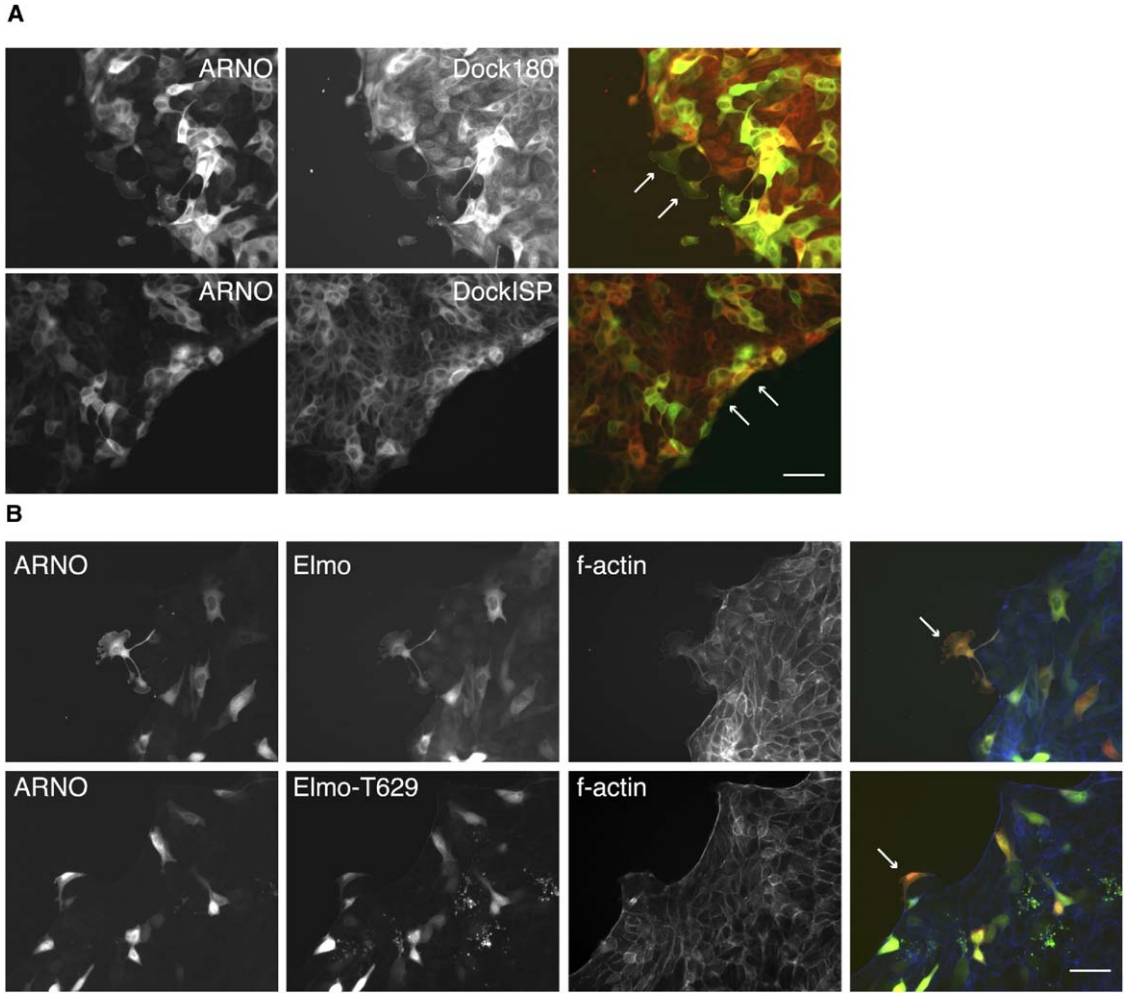

Figure 3. A Dock180 Mutant Lacking Rac GEF Activity Blocks the Induction of Lamellipodia by ARNO

(A) Monolayers of MDCK cells were infected with adenoviruses encoding myc-ARNO and either wild-type flag-Dock180 or flag-Dock-ISP. After overnight recovery in the presence of doxycycline, cells were washed out of doxycycline and incubated for 3 hr to induce transgene expression. Monolayers were then wounded and incubated 2 hr to allow cells to migrate into the wound area. The wounded monolayers were then fixed and stained with cy3-conjugated M2 anti-flag (Sigma, St. Louis, MO) and biotinylated 9e10 anti-myc primary antibodies followed by cy2-conjugated streptavidin. Dock180 is shown in red and ARNO in green in the merged image.

(B) MDCK cells were transfected with GFP-Elmo or GFP-Elmo T629 and myc-ARNO and wounded as described for Figure 2B. Cells were fixed and stained with 9e10 anti-myc followed by cy3 conjugated anti-mouse secondary antibody and Alexa647-conjugated phalloidin. In the merged image, ARNO is shown in red, Elmo in green, and phalloidin in blue. Scale bars equal 50 μm.

Although localized activation of specific GTPases at the leading edge limits protrusive events to this location, additional signals reinforce this localization by preventing protrusions along the sides and trailing edge of the cell. One such signal is the exclusive localization of phosphorylated α4-integrin to the leading edge of some cell types [40]. A recent study demonstrates that the scaffolding protein paxillin binds selectively to nonphosphorylated α4-integrins located at sites distal to the leading edge. In turn, paxillin binds the ARF-GAPs GIT1 and GIT2/Pkl whose presence leads to locally reduced levels of active ARF6, (and consequently Rac1) along the sides and trailing edge of these cells [41]. Although α4 integrins are not expressed in all cells, related mechanisms may function to concentrate the activities of both Arf6 and Rac1 at the leading edge.

Understanding these spatial and temporal aspects of GTPase regulation requires understanding the regulation of the GEFs responsible for GTPase activation during the onset of migration. We show here that ARNO, Elmo, and Dock180 work together to coordinate ARF6 and Rac activation at the leading edge of migrating epithelial cells, initiating some of the early events of motility.

Supplemental Data

Supplemental Data include two figures and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/19/1749/DC1/.

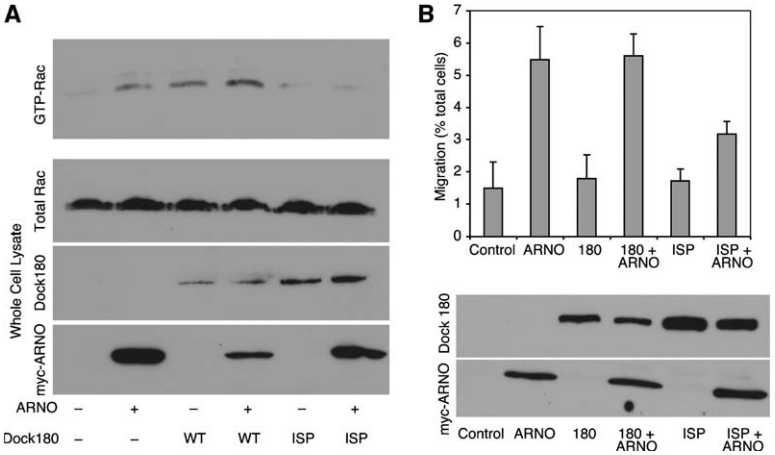

Figure 4. Mutant Dock180 Blocks ARNO-Induced Rac Activation and Motility

(A) Subconfluent islands of MDCK cells were infected with ARNO and Dock180 adenoviruses for 3 hr (180 = wild-type Dock180, ISP = DockISP). DockISP is a catalytically inactive mutant that acts in a dominant-negative fashion. Cells were then lysed and GTP bound Rac was isolated by binding to GST-PBD.

(B) MDCK cells were infected with ARNO and Dock180 adenoviruses for 4 hr. Transwell migration assays were performed as previously described [9]. In brief, cells were nonenzymatically lifted and replated onto transwell filters (6.5 mm, 8 μm pore, Costar) coated on the underside with fibronectin at a density of 2×10^5 cells/filter. Cells were allowed to migrate for 15 hr, then fixed and quantitated as described [9]. Data shown are mean ± standard deviation.

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