Positive feedback between Cdc42 activity and H^+ efflux by the Na-H exchanger NHE1 for polarity of migrating cells

Christian Frantz, Anastasios Karydis, Perihan Nalbant, Klaus M. Hahn, and Diane L. Barber

Department of Cell and Tissue Biology, University of California, San Francisco, San Francisco, CA 94143
Department of Immunology, The Scripps Research Institute, La Jolla, CA 92037
Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC 27599

A fundamental feature of cell polarity in response to spatial cues is asymmetric amplification of molecules generated by positive feedback signaling. We report a positive feedback loop between the guanosine triphosphatase Cdc42, a central determinant in eukaryotic cell polarity, and H^+ efflux by Na-H^+ exchanger 1 (NHE1), which is necessary at the front of migrating cells for polarity and directional motility. In response to migratory cues, Cdc42 is not activated in fibroblasts expressing a mutant NHE1 that lacks H^+ efflux, and wild-type NHE1 is not activated in fibroblasts expressing mutationally inactive Cdc42-N17. H^+ efflux by NHE1 is not necessary for release of Cdc42–guanosine diphosphate (GDP) from Rho GDP dissociation inhibitor or for the membrane recruitment of Cdc42 but is required for GTP binding by Cdc42 catalyzed by a guanine nucleotide exchange factor (GEF). Data indicate that GEF binding to phosphatidylinositol 4,5-bisphosphate is pH dependent, suggesting a mechanism for how H^+ efflux by NHE1 promotes Cdc42 activity to generate a positive feedback signal necessary for polarity in migrating cells.

Introduction

Positive feedback loops function as signaling devices to sustain or amplify biological processes. For eukaryotic cell polarity, positive feedback loops involving low molecular weight GTPases of the Rho family generate asymmetric amplification of signaling molecules. In chemotaxing neutrophils, a positive feedback loop between phosphatidylinositol-3-OH kinase, the Rho family GTPase Rac, and F-actin sustains amplification of phosphatidylinositol 3,4,5–trisphosphate at the cell front, which is necessary for polarity, and the loop maintains actin assembly at the leading edge for driving membrane protrusion (Wang et al., 2002; Weiner et al., 2002; Srinivasan et al., 2003). In budding yeast, cell polarity during bud formation requires a positive feedback loop between the Rho family GTPase Cdc42, the adaptor protein Bem 1, and actin assembly (Wedlich-Soldner et al., 2004). Although Cdc42 plays an evolutionarily conserved role in establishing cell polarity including specification of anterior–posterior axis patterning in Caenorhabditis elegans, asymmetric zygotye division and epithelial polarity in Drosophila melanogaster, directed migration in mammalian cells, and epithelial and neuronal polarity in many species (Etienne-Manneville, 2004; Macara, 2004), whether or not it functions in positive feedback loops in these cell processes is unclear. Moreover, although positive feedback loops are important in spatial sensing during amoeboid chemotaxis (Charest and Firtel, 2006), their significance in spatial sensing for haptokinetic migration of mesenchymal cells has been questioned (Schneider and Haugh, 2005). We report a positive feedback loop between Cdc42 and H^+ efflux by Na-H^+ exchanger 1 (NHE1) for maintaining polarity in migrating fibroblasts. In migrating fibroblasts (Denker and Barber, 2002) and epithelial cells (Stock and Schwab, 2006), and in chemotaxing Dictyostelium discoideum cells (Patel and Barber, 2005), a leading-edge H^+ efflux by NHE1 is necessary for polarity and directional movement. These processes are impaired by distinct mutations in NHE1 that abolish H^+ efflux or mislocalize NHE1 away from the leading edge. We previously reported that Cdc42 acts upstream of NHE1 to stimulate H^+ efflux (Hooley et al., 1996), and we now find that Cdc42 activation and localization of Cdc42-GTP at the leading edge of migrating cells...
cells is downstream of H\(^+\) efflux, which suggests a bistable positive feedback regulation between Cdc42 and NHE1 activities in cell polarity.

**Results and discussion**

To determine whether H\(^+\) efflux by NHE1 is necessary for activation of Cdc42, we used NHE1-deficient fibroblasts expressing wild-type (WT) NHE1 or a mutant NHE1 that contains an E266I substitution and lacks H\(^+\) efflux (Denker et al., 2000). To biochemically determine Cdc42 activity in migrating cells, multiple wounds were created in a confluent monolayer with a multichannel pipette. The abundance of active Cdc42-GTP at the indicated times before and after wounding was determined by precipitation with GST–p21-activated kinase (PAK)–Cdc42/Rac-interactive binding domain (CRIB) and immunoblotting for Cdc42 (Fig. 1 A). After wounding, the abundance of Cdc42-GTP in WT cells increased twofold at 5 min and remained elevated at 5 h. In E266I cells, the abundance of Cdc42-GTP before wounding was 60% of that in WT cells, and after wounding there was no increase in Cdc42-GTP. The abundance of total Cdc42 was similar in WT and E266I cells before and after wounding.

Monolayer wounding triggers multiple stimuli, including activation of integrins and release of growth factors. Integrin engagement with extracellular matrix proteins activates Cdc42 (Price et al., 1998) and stimulates NHE1 activity (Schwartz et al., 1991; Tominaga and Barber, 1998). We found that H\(^+\) efflux by NHE1 is necessary for haptokinetic migration toward fibronectin (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200704169/DC1) and for integrin-induced activation of Cdc42 (Fig. 1 B). Plating on fibronectin for 1 h increased Cdc42-GTP fourfold in WT cells compared with cells in suspension.

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**Figure 1.** H\(^+\) efflux by NHE1 is necessary for activation of Cdc42 by different extracellular cues. (A) Time course of Cdc42 activity in WT and E266I cells after multiple wounding of a confluent monolayer. At the indicated times, Cdc42-GTP was determined by affinity precipitation with GST-PAK-CRIB and immunoblotting for Cdc42. Total Cdc42 was determined in cell lysates. Representative immunoblots are shown, and data, expressed relative to the abundance of Cdc42-GTP in WT monolayers \(t = 0\), represent means ± SEM of six cell preparations. (B) Total Cdc42 and Cdc42-GTP in cells in suspension (susp) or at the indicated times after plating on 10 \(\mu\)g/ml fibronectin were determined as in A. Data are expressed as means ± SEM of three separate cell preparations. (C and D) Abundance of Cdc42-GTP and total Cdc42 in subconfluent quiescent cells \(t = 0\) and at the indicated times after treating with PDGF were determined as described in A for WT and E266I cells (C) and for CCL39 and PS120 cells (D). Data for Cdc42-GTP are expressed relative to the abundance of precipitated Cdc42 in quiescent WT cells \(t = 0\) and represent means ± SEM of four separate cell preparations. White lines indicate that intervening lanes have been spliced out.
In E266I cells, the abundance of Cdc42-GTP in cell suspension was less than in WT cells and there was no increase after plating on fibronectin. Integrin affinity for fibronectin, determined by binding FITC-labeled fibronectin, and expression of β1 integrin, determined by immunoprecipitating lysates of biotinylated cells with β1 antibodies, were similar in WT and E266I cells (Fig. S1).

PDGF also increases Cdc42-GTP (Jimenez et al., 2000) and NHE1 activity (Yan et al., 2001), and we found that H⁺ efflux by NHE1 is necessary for activation of Cdc42 by PDGF (Fig. 1 C). 50 ng/ml PDGF increased the abundance of Cdc42-GTP in WT cells, with a maximum of 2.2-fold increase at 2 min. In contrast, PDGF did not activate Cdc42 in E266I cells, although the abundance of total Cdc42 was similar in subconfluent WT and E266I cells. PDGF-induced activation of Cdc42 also was inhibited in NHE1-deficient PS120 cells, which were used to make WT and E266I cells, but not in parental CCL39 cells that express NHE1 (Fig. 1 D). Hence, H⁺ efflux by NHE1 is necessary for maintaining Cdc42 activity in quiescent cells and for increased Cdc42 activity with monolayer wounding, integrin engagement, and PDGF.

In migrating cells, Cdc42-GTP is predominantly localized at cell protrusions (Nalbant et al., 2004). NHE1 is localized at the leading-edge membrane of migrating cells (Denker and Barber, 2002; Patel and Barber, 2005), which suggests that H⁺ efflux might be necessary for Cdc42 activity at cell protrusions. This was confirmed by using a merocyanine–Cdc42-binding domain (MeroCBD) biosensor for Cdc42-GTP (Fig. 2 A; Nalbant et al., 2004). MeroCBD, an environmentally sensitive fluorescent dye covalently coupled to the Cdc42/Rac binding domain of the neural Wiskott-Aldrich syndrome protein, increases fluorescence intensity upon binding activated Cdc42, which enables detection of spatially localized endogenous Cdc42-GTP in living cells. EGFP attached to the Cdc42/Rac binding domain allows ratiometric image analysis, thereby normalizing for cell thickness and concentration artifacts. The MeroCBD probe, which is insensitive to pH 4.5–8.0, was injected into cells at the edge of a wounded monolayer 15 h after wounding, and images were acquired after 30 min. In WT cells, Cdc42-GTP was elevated in cell protrusions (n = 22 cells), however, in E266I cells, Cdc42-GTP was more uniform and notably reduced even where cells protruded (n = 52 cells; Fig. 2, B and C). Acquired images were also used to quantify active Cdc42 in microinjected cells, and, like biochemical assays with GST-PAK-CRIB, they indicated attenuated Cdc42-GTP in E266I cells (Fig. 2 D). The ratio intensity was 344.4 ± 28.1 U in WT cells and 170.9 ± 7.0 U in E266I cells. The Mero/EGFP fluorescence ratio of cells injected with an insensitive control probe (MeroCBD mutated to greatly reduce Cdc42 binding; Nalbant et al., 2004) was 196.9 ± 24.8 U (n = 5 cells; unpublished data), indicating that activation in E266I cells was near the minimum level detectable by the biosensor. Hence, biochemical and imaging analyses indicate that H⁺ efflux by NHE1 is necessary to maintain the abundance of Cdc42-GTP in quiescent and stimulated cells and to maintain the localization of Cdc42-GTP in migrating cells.

We previously reported that H⁺ efflux by NHE1 increases in fibroblasts expressing an active GTPase-deficient Cdc42-V12 and that H⁺ efflux by NHE1 stimulated by a constitutively active Ga13-QL is suppressed by coexpression of mutationally inactive Cdc42-N17 (Hooley et al., 1996). In wound-edge WT cells, expression of Cdc42-N17 inhibited H⁺ efflux by NHE1, resulting

![Image](https://via.placeholder.com/150)

Figure 2. H⁺ efflux by NHE1 is necessary for active Cdc42-GTP at the front of migrating cells. (A) Probe used as a biosensor for endogenous Cdc42-GTP. MeroCBD is composed of the CRIB domain of neural Wiskott-Aldrich syndrome protein (blue), covalently labeled with the merocyanine dye Mero (red), and fused to EGFP (green). (B) Representative ratio images of EGFP fluorescence and MeroCBD probe fluorescence in cells at the edge of a wounded monolayer as determined by microinjecting the MeroCBD probe 15 h after wounding. Arrows indicate direction of migration. (C) Percentage of WT (n = 22) and E266I (n = 52) cells with active Cdc42-GTP in wound-edge protrusions. (D) Relative abundance of Cdc42-GTP in wound-edge cells determined by the Mero/EGFP fluorescence ratio. Data are expressed as means ± SEM of fluorescence ratios in WT (n = 12) and E266I (n = 18) cells. Bar, 15 μm.
in decreased intracellular pH (pH$_i$) of 7.10 ± 0.09 (n = 43 cells), compared with pH$_i$ of 7.30 ± 0.10 (n = 30 cells) in cells not expressing Cdc42-N17. In subconfluent WT cells, PDGF stimulated NHE1 activity and increased pH$_i$ from 7.15 ± 0.03 to 7.47 ± 0.05, which was attenuated in cells expressing Cdc42-N17 to 6.99 ± 0.02 and 7.10 ± 0.03 (n = 3 cell preparations).

Activation of Cdc42 requires release of inactive Cdc42-GDP from Rho GDP dissociation inhibitor (RhoGDI) in the cytosol, recruitment to the plasma membrane, and activation at the plasma membrane by a guanine nucleotide exchange factor (GEF) that catalyzes the exchange of GDP for GTP. Immunoprecipitating RhoGDI and immunoblotting for total Cdc42 indicated that PDGF induced a decrease in the abundance of coprecipitating Cdc42 that was similar in WT and E266I cells (Fig. 3 A), which suggests that NHE1 activity is not necessary for the regulated dissociation of Cdc42 from RhoGDI. Immunoblotting particulate fractions (P100) for Cdc42 showed that abundance in E266I cells was comparable to that in WT cells after PDGF stimulation (Fig. 3 B), which indicates that H$^+$ efflux by NHE1 is not necessary for membrane recruitment of Cdc42.

These findings suggest that H$^+$ efflux by NHE1 might be necessary for GEF-induced guanine nucleotide exchange, which we confirmed by determining GEF activity in cell lysates (Fukuda et al., 2002). Exchange of [$^{32}$P]GTP by Cdc42 for cold GTP was used as an index of GEF activity. The amount of Cdc42-[$^{32}$P]GTP decreased to 49.2% in lysates from WT cells treated with PDGF (Fig. 3 C). Lysates from PDGF-stimulated E266I cells did not show a marked exchange of [$^{32}$P]GTP for cold GTP compared with unstimulated cells (Fig. 3 C). These data suggest that GEF activity is impaired in E266I cells.

We asked whether GEF activity might be pH sensitive because in WT cells the quiescent pH$_i$ of 7.15 increases to 7.45 with growth factors, but in E226I cells, the quiescent pH$_i$ of 7.00 does not change (Denker et al., 2000; Yan et al., 2001). At least 20 members of the Dbl family of GEFs stimulate guanine nucleotide exchange by Cdc42, and we tested whether the activity of two GEFs for Cdc42, Dbs (Dbl’s big sister) and intersectin, is pH dependent. Because Dbs contains a His residue (H814) in the α6 helix critical for interacting with switch two of Ccd42 and because it contacts a His residue in Cdc42 (H103 in the α3b region; Rossman et al., 2002), we reasoned that pH-dependent titration of these histidines might regulate GEF activity or contact with Cdc42. Using the minimal Dbl homology (DH)–pleckstrin homology (PH) segment of Dbs necessary for activity and a GST-fusion of Cdc42 loaded with the fluorescent analogue methylanthraniloyl (mant)-GDP, which has reduced fluorescence when not bound to Cdc42 (Nomanbhoy and Cerione, 1996), we found no change in activity from pH 6.5 to 8.0 for Dbs (Fig. 4 A) or intersectin (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200704169/DC1). Additionally, in the absence of Dbs, the release of mant-GDP from Cdc42 was similar from pH 6.5 to 8.0 (Fig. 4 A), indicating that guanine nucleotide exchange by Cdc42 was pH insensitive.

Although in most Rho family GEFs the DH domain is sufficient to catalyze nucleotide exchange, a tandem PH domain that binds phosphoinositides is invariant. In cells, phosphoinositide binding by the PH domain can regulate activity of the DH domain of some GEFs (Rossman et al., 2005). We asked whether phosphoinositide binding to Dbs or intersectin might be pH sensitive because phosphoinositides bind to positively charged residues in PH domains, which might titrate with changes in pH,
and phosphates on phosphoinositides have \( pK_a \) near neutral (van Paridon et al., 1986). Additionally, FYVE domains, which share structural similarity with PH domains for binding phosphoinositides at loops between \( \beta \) strands, have pH-dependent affinity for phosphoinositides (Kutateladze, 2006). By using liposome sedimentation, we found that the DH-PH domain of Dbs bound phosphotidylinositol 4,5–bisphosphate (PI(4,5)P2), as previously reported (Russo et al., 2001; Snyder et al., 2001), and that binding was pH dependent (Fig. 4 B). Maximal specific binding seen at pH 6.5 (52 ± 13%) was significantly reduced at pH 7.5 and 8.0 (\( P < 0.05; n = 4 \)), suggesting a lower affinity at higher pH. Although PI(4,5)P2 binding by the DH-PH domain of intersectin was maximal at pH 6.5 (42 ± 8%), binding was relatively insensitive to pH (Fig. 4 C). We speculate that pH-sensitive binding of PI(4,5)P2 to Dbs is caused by the presence of a His (H843) in the same position as H355 in the Arf GEF Grp1 that is critical for binding phosphoinositides (Lietzke et al., 2000; Barriero, G., and M. Jacobson, personal communication). Computational modeling (unpublished data) indicates that a spatially conserved His in close proximity to predicted PI(4,5)P2–binding sites is present in other GEFs activating Cdc42, including Fgd1 (H985), αPix (H38), ASEF (H513; H505), and Dbl (H701; H756), but is absent in intersectin, Fgd3, Tiam1, and PDZ-RhoGEF (unpublished data; Barriero, G., and M. Jacobson, personal communication). Hence, whether Dbs or another predicted pH-sensitive GEF mediates NHE1-dependent activation of Cdc42 remains to be determined.

It also remains to be determined whether pH-dependent PI(4,5)P2 binding by GEFs contributes to NHE1-dependent activation of Cdc42. PI(4,5)P2 binding to Sos2, a Ras GEF, inhibits nucleotide exchange activity, possibly by retaining a cis-inhibition of the DH domain by the adjacent PH domain (Jefferson et al., 1998; Das et al., 2000). The functional significance of PI(4,5)P2 binding to Rho family GEFs, however, is less clear. PI(4,5)P2 binding to recombinant DH-PH domains in vitro is reported to stimulate (Crompton et al., 2000), inhibit (Han et al., 1998; Russo et al., 2001), or not affect (Fleming et al., 2000; Snyder et al., 2001) activity. Additionally, we cannot rule out other pH-dependent mechanisms, such as scaffolding or conformation changes independent of phosphoinositide binding, because attenuated GEF activity is retained in lysates of E266I cells (Fig. 4 C) and for some proteins conformational changes, ligand-binding affinities, and macromolecular assemblies are sensitive to small changes in physiological pH (Srivastava et al., 2007). A suggested pH-dependent scaffolding by NHE1 (Baumgartner et al., 2004) is also a putative mechanism because NHE1 binds PI(4,5)P2 (Aharonovitz et al., 2000) and the ezrin-radixin-moesin protein ezrin (Denker et al., 2000), and ezrin is suggested to sequester Dbl to plasma membrane microdomains (Prag et al., 2007) and to regulate Dbl activation of Cdc42 (Batchelor et al., 2007). Moreover, H\(^+\) efflux by NHE1 could regulate an upstream activator of Cdc42 (Schwamborn and Puschel, 2004), was not impaired in E266I cells compared with WT cells (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200704169/DC1).

Our data indicate positive feedback signaling between Cdc42 and NHE1 activity that is likely critical for polarity in migrating cells by asymmetrically amplifying both signals at the leading edge. Our findings also suggest that RhoGDI dissociation and membrane recruitment of Cdc42 are distinct...
signaling events that can be regulated independently of guanine nucleotide exchange. Beyond our current focus on regulated Cdc42 activity our data raise the possibility that activity of other GTPases and GEFs, and the affinities of protein modules for binding phosphoinositides, might be pH sensitive and regulated by NHE1 activity.

Materials and methods

Pulldown assay for activated Cdc42
Activated Cdc42-GTP was determined by precipitation with a GST fusion of the PBD domain of PAK (GST-PAK-CRIB) as previously described (Benard et al., 1999). CCL39, PS120, WT, and E266I fibroblasts were maintained as previously described (Denker et al., 2000). For monolayer wounding, confluent cells were wounded with a multichannel pipette. Detached cells were removed by medium exchange, and lysates were prepared from adherent cells. For experiments with integrin activation, quiescent cells maintained in DME containing 0.2% FBS for 18–24 h were trypsinized, incubated for 10 min with 0.5 mg/ml of soybean trypsin inhibitor (Sigma-Aldrich), collected by centrifugation, and resuspended in serum-free DME. Resuspended cells were plated on dishes coated with 10 μg/ml of bovine plasma fibronectin (Sigma-Aldrich). Cells treated with 50 ng/ml PDGF-BB (Roche Diagnostics) were grown to 70% confluence and maintained for 18–24 h in DME containing 0.2% FBS (Yan et al., 2001). At the indicated times, cells were lyzed in 500 μl of lysis buffer (Benard et al., 1999) and lysates were clarified by centrifugation. A 20 μl aliquot of the supernatant was saved for determining total Cdc42, and the remaining lysate was incubated with 20 μg of GST-PAK-CRIB bound to Sepharose beads. Precipitated proteins were separated by 12% SDS-PAGE and immunoblotted with anti-Cdc42 antibodies (1:200; BD Biosciences). Immunoabsorbs were analyzed by densitometry using Image (National Institutes of Health). Because of variations in cell density between preparations and GST-PAK-CRIB, which was freshly generated for each experiment, the absolute values of Cdc42-GTP and total Cdc42 for each cell preparation, determined from the immunoblotting signal by Image, were expressed as a ratio of Cdc42-GTP/total Cdc42. The ratios from each cell preparation were expressed relative to the ratio of control WT cells. Hence, although there was variability in Cdc42-GTP/total Cdc42 ratios between cell preparations, the cell-type and condition-specific relative changes in ratios were consistent.

Cell migration
Cell migration was determined on nucleopore filters (8-μm pore; Costar; Corning Inc.) coated on the lower side of the membrane with 10 μg/ml fibronectin or BSA, and chambers were filled with growth medium. Cells treated with 50 ng/ml of bovine plasma fibronectin (Sigma-Aldrich) or, for experiments with integrin activation, quiescent cells were cocultured with 0.5 mg/ml of soybean trypsin inhibitor (Sigma-Aldrich) to prevent cell loss and were then fixed in 4% paraformaldehyde, washed with PBS, and stained for 5 min in the absence of PI(4,5)P2 was minimal and pH independent. To correct for baseline fluorescence, the fluorescent pH-sensitive dye BCECF (Invitrogen) was introduced into each cell suspension and was allowed to distribute. This allowed the fluorescence to be corrected for background. The amount of peptide bound to vesicles was determined by measuring the decrease in fluorescence (excitation, 360 nm; emission, 440 nm) with release of mant-GDP from Cdc42 using a spectrofluorometer (SpectraMax M5; Invitrogen).

Phospholipid binding
Lipid micelles were prepared as previously described (Ikeboshi et al., 2006) using a MiniExtruder (Avanti Polar Lipids) and contained phosphatidyl choline/Pl(4,5)P2 [86:10:4 molar ratio; Avanti Polar Lipids]. Vesicle suspensions adjusted to the indicated pH with KOH or HCl were incubated with 10 μg (3 μM) of recombinant DH/PH protein for 15 min at room temperature and then collected by centrifugation at 100,000 g for 60 min. Supernatants and pellets were analyzed by SDS-PAGE and Coomassie staining. The amount of protein on the gel was determined by densitometry analysis using Image. Specific binding was calculated as the abundance of peptide bound to vesicles containing Pl(4,5)P2 minus binding to vesicles in the absence of Pl(4,5)P2. The abundance of peptide bound to vesicles in the absence of Pl(4,5)P2 was minimal and pH independent. To correct for variations in lipid vesicle preparations, data were expressed relative to binding at pH 6.5 for each determination.

RhoB activation
Activated RhoB-GTP was determined in subconfluent cells by precipitation with a GST fusion of the Rho binding domain of RalGDS (GST-RalGDS-RBD) as previously described (Hochbaum et al., 2003). At the indicated times after treating with 50 ng/ml PDGF (Roche Diagnostics), cells were washed in ice-cold PBS and lysed in 500 μl of lysis buffer (Hochbaum et al., 2003), and then 20 μl of lysate was saved for determining total RhoB activity. The remaining
lyase was incubated for 1 h at 4°C with 10 μg of GST-RalGDS-RBD bound to Sepharose beads. Precipitated proteins were separated by 12% SDS-PAGE and immunoblotted with anti-Rap1B antibodies [1:200; Santa Cruz Biotechnology, Inc.]. Immunoblots were analyzed by densitometry using ImageJ.

Online supplemental material
Fig. S1 shows that H+ efflux by NHE1 is necessary for hepatokinin migration toward fibroblast but not for integrin affinity or expression. Fig. S2 shows that activity of the DH-PH domain of intersectin is pH insensitive. Fig. S3 shows that H+ efflux by NHE1 does not regulate activity of Rap1B. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200704169/DC1.

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Cruz Biotechnology, Inc.). Immunoblots were analyzed by densitometry using ImageJ. Cytosolic proteins were separated by 12% SDS-PAGE and immunoblotted with anti-Rap1B antibodies (1:200; Santa Cruz Biotechnology, Inc.). Immunoblots were analyzed by densitometry using ImageJ.

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