ROCK1 but not ROCK2 contributes to RhoA signaling and NMIIA-mediated contractility at the epithelial zonula adherens

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ABSTRACT Rho kinases (ROCK1 and ROCK2) function downstream of the small GTPase RhoA to drive actomyosin cytoskeletal remodeling. It has often been believed that ROCK1 and ROCK2 may be functionally redundant, as they share a highly conserved kinase domain. However, in this study, we report differential functional effects for these ROCKs at the epithelial zonula adherens (ZA). Using specific siRNA, we found that ROCK1 depletion disrupted cadherin organization at the ZA, accompanied by loss of F-actin and NMIIA, whereas ROCK2 knockdown had no significant effect. Further, ROCK1, but not ROCK2, was necessary to stabilize GTP-RhoA at the ZA, thereby sustaining junctional tension and inhibiting intraepithelial cell movement. We also found that nonmuscle myosin IIa is a major determinant of ROCK1 cortical stability. Thus, despite sharing the catalytic domain with ROCK2, ROCK1 appears to be the dominant kinase essential for junctional integrity and contractile tension at epithelial ZA.

INTRODUCTION Rho-dependent kinases (ROCKs) are major regulators of the actomyosin cytoskeleton and thus govern a variety of cellular processes, including cell division, migration, polarity, and epithelial homeostasis (Bishop and Hall, 2000; Riento and Ridley, 2003). They belong to the family of Ser/Thr kinases, and two proteins have been reported in mammalian cells: ROCK1 and ROCK2 (Nakagawa et al., 1996). Broadly, these proteins are constitutive dimers, each subunit containing an N-terminal kinase domain followed by a coiled-coil domain and a C-terminus that contains a canonical Rho-binding domain (RBD) and a pleckstrin homology (PH) domain (Jacobs et al., 2006; Garg et al., 2008; Truebestein et al., 2015). Overall they share 62% identity, with the kinase domain being highly conserved (92% identity) and the coiled-coil domain being the most divergent (55% identity; Amano et al., 2010; Julian and Olson, 2014). Two different modes for ROCK activation have been described. One widely accepted model suggests that the C-terminus of ROCK binds to the N-terminus, leading to inhibition of ROCK activity. Association of GTP-Rho to the RBD releases this autoinhibitory conformation to activate the kinase (Amano et al., 1996; Matsui et al., 1996). In the second mode of activation, particularly relevant for ROCK2, Rho signaling is not necessary for ROCK2 activation, and instead the length of its coiled-coil domain is a key determinant of its kinase activity (Truebestein et al., 2015).

Because the two ROCK proteins share a highly conserved kinase domain, it has been postulated that they may perform similar biological functions by phosphorylating common substrates. Indeed, using conditional knockout mice, it has been reported that ROCK1 and ROCK2 act redundantly to modulate MYPT phosphorylation and actomyosin contractility (Kumper et al., 2016). However, there are various reports suggesting that ROCK proteins exhibit distinct functions and regulation. In the context of cell–matrix adhesion, ROCK1 was required for the maturation of focal adhesion complexes and stress fiber formation, whereas ROCK2 knockdown enhanced the formation of stress fibers and focal adhesions (Yoneda et al., 2005; Lock et al., 2012). In fibroblasts, depletion of ROCK2 led to a clearly defined front and rear polarity, whereas ROCK1 knockdown resulted in a complete loss of polarity.
interacts with ROCK-2 but not ROCK1 (Wang et al., 2009).

ROCK is an important regulator of cadherin organization and function in cell–cell adhesion. ROCK inhibition perturbed apical F-actin and E-cadherin organization and disrupted epithelial adherens junction assembly in a calcium-switch assay (Anderson et al., 2002; Smith et al., 2012; Andreeva et al., 2014). One of the principal downstream effectors of ROCK at adherens junctions is nonmuscle myosin II (NMII; Shewan et al., 2005). ROCK activates NMII by phosphorylating the Ser-19/Thr-18 residues of the NMII regulatory light chain (MRLC) and also by inhibiting the MRLC phosphatase MYPT-1 (Amano et al., 1996; Kimura et al., 1996). This stimulates the ATPase activity of NMII, leading to generation of contractile forces and stability of cadherin junctions (Ratheesh et al., 2012; Priya and Gomez, 2013; Priya et al., 2015; Wu et al., 2014). Further, ROCK was recently found to participate in a feedback network that supported RhoA signaling at cadherin junctions (Priya et al., 2015, 2016). However, distinct contributions of ROCK1 and ROCK2 to junctional biology have yet to be investigated in detail. Accordingly, in the present study, we sought to dissect the selective functions of ROCKs at epithelial cadherin junctions. Our data demonstrate that ROCK1, but not ROCK2, is required for zonula adherens (ZA) organization, RhoA signaling, and cortical tension, which in turn restricts epithelial motility. Of interest, ROCK1 selectively supports nonmuscle myosin IIA (NMIIA) localization at the ZA, which in turn acts as a principal regulator of ROCK1 stability.

FIGURE 1: ROCK1 depletion affects NMIIA localization and stability. (a) GFP-ROCK1/ROCK2 localization in MCF-7 cells. (b, c) Endogenous ROCK1/ROCK2 localization in MCF-7 cells; yz images indicate the apical concentration of ROCKs with NMIIA and E-cadherin. (d) ROCK1, ROCK2, and β-tubulin immunoblots from the Ctrl, ROCK1 KD, or ROCK2 KD MCF-7 cell lysates. (e, f) NMIIA localization in Ctrl, ROCK1 KD, and ROCK1 KD + GFP ROCK1 cells and the corresponding line-scan analysis (n = 3). (g) NMIIA, NMIIIB, and β-tubulin immunoblots from the Ctrl, ROCK1 KD, or ROCK2 KD MCF-7 cell lysates. (h, i) NMIIA localization in Ctrl and ROCK2 KD cells and the corresponding line-scan analysis (n = 3). (j, k) FRAP of apical GFP-NMIIA in Ctrl, ROCK1 KD, and ROCK2 KD MCF-7 cells; mobile fraction values were calculated in GraphPad Prism (n = 3). (l, m) NMIIIB localization in Ctrl, ROCK1 KD, and ROCK2 KD cells and the corresponding line-scan analysis (n = 3).

To gain insight into the potential effect of individual ROCKs on the ZA, we examined how ROCK depletion affected the cortical pool of phosphorylated myosin regulatory light chain (pMRLC) and NMII, as these are canonical effectors of the RhoA-ROCK pathway that support junctional contractility and integrity.

RESULTS AND DISCUSSION
ROCK1, but not ROCK2, determines cortical myosin II localization and stability

We began by characterizing the localization of ROCK proteins at the ZA in MCF-7 and Caco-2 epithelial cells. Green fluorescent protein (GFP)–tagged transgenes of ROCK1 and ROCK2 localization in MCF-7 cells. (b, c) Endogenous ROCK1/ROCK2 localization in MCF-7 cells; yz images indicate the apical concentration of ROCKs with NMIIA and E-cadherin. (d) ROCK1, ROCK2, and β-tubulin immunoblots from the Ctrl, ROCK1 KD, or ROCK2 KD MCF-7 cell lysates. (e, f) NMIIA localization in Ctrl, ROCK1 KD, and ROCK1 KD + GFP ROCK1 cells and the corresponding line-scan analysis (n = 3). (g) NMIIA, NMIIIB, and β-tubulin immunoblots from the Ctrl, ROCK1 KD, or ROCK2 KD MCF-7 cell lysates. (h, i) NMIIA localization in Ctrl and ROCK2 KD cells and the corresponding line-scan analysis (n = 3). (j, k) FRAP of apical GFP-NMIIA in Ctrl, ROCK1 KD, and ROCK2 KD MCF-7 cells; mobile fraction values were calculated in GraphPad Prism (n = 3). (l, m) NMIIIB localization in Ctrl, ROCK1 KD, and ROCK2 KD cells and the corresponding line-scan analysis (n = 3).
either cell line (Figure 1, h and i, and Supplemental Figure S1, g and h). To further pursue this, we analyzed the effect of ROCK knockdown on the junctional dynamics of NMIIA using fluorescence recovery after photobleaching (FRAP). ROCK1, but not ROCK2, depletion led to a significant increase in the mobile fraction of the apical pool of NMIIA, confirming that ROCK1 was selectively required to stabilize NMIIA at ZA (Figure 1, j and k).

Of note, junctional NMIIb content was unaffected by depletion of either ROCK1 or ROCK2, both in MCF-7 (Figure 1, i and m) and Caco-2 cells (Supplemental Figure S1, j and k). Because both myosin paralogues have a similar RLC, this raises the question of how ROCK1 can selectively support the junctional localization of NMIIA. One possibility is that this reflects the higher expression of NMIIA than NMIIb in MCF-7 cells (Smutny et al., 2010). Further, our recent finding that ROCK1 can selectively immunoprecipitate NMIIA (Priya et al., 2015) raises the possibility that ROCK1 might interact more strongly with NMIIA than with NMIIb. By whatever mechanism(s), the apparently dominant effect of ROCK1 on NMIIA localization supports earlier evidence that the junctional localizations of NMIIA and NMIIb are subject to different upstream signals, with the RhoA-ROCK pathway being the key determinant of NMIIA localization (Sandquist et al., 2006; Smutny et al., 2010; Gomez et al., 2015).

ROCK1 regulates ZA organization to support junctional tension and restricts epithelial motility

Because NMIIA is a key determinant of ZA integrity and contractility (Ratheesh et al., 2012; Priya et al., 2013; Wu et al., 2014), we then asked how ROCK1 might affect these properties of the junction. Compared with control cells, E-cadherin and F-actin failed to concentrate into tight, ring-like structures at the apical junctions in ROCK1 KD cells, and this could be restored by RNAi-resistant full-length ROCK1 (Figure 2, a–d, and Supplemental Figure S2, a–d). Quantification revealed a significant decrease in the fluorescence intensity of E-cadherin and F-actin, although protein expression and cell density were unchanged (Figure 2, a–d, and Supplemental Figure S2, a–g). Further, tight junction integrity, as reflected byZO-1 staining, was not overtly compromised, suggesting a selective effect on ZA (Supplemental Figure S2h). ROCK2 depletion did not affect either E-cadherin or F-actin, consistent with the dominant effect of ROCK1 that we observed for NMIIA (Figure 2, e–h, and Supplemental Figure S2, a–d).

The reduction of E-cadherin, F-actin, and NMIIA at the ZA led us to predict that ROCK1 depletion would also affect junctional contractility. To test this, we cut the apical junctions (marked by expression of E-cadherin–GFP) using a femtosecond pulsed

![Image](image-url)
Finally, we asked what functional effect might arise from the ability of ROCK1, but not ROCK2, to regulate junctional tension. For this, we focused on epithelial motility and assessed the role of ROCK proteins on the movement of MCF-7 cells grown as confluent monolayers. Using particle-tracking software to quantitate the movement of nuclei (Jaqaman et al., 2008; Ng et al., 2012), we found that control cells showed low levels of locomotility (measured as mean squared displacement; Figure 2, l and m, and Supplemental Movie S1), and this was substantially increased in ROCK1 but not in ROCK2 KD cells. This result correlates well with the role of ROCK1 but not ROCK2 in promoting junctional tension, which retards intraepithelial cell movements.

Overall these data identify ROCK1 as a dominant architect of cadherin/actomyosin organization that establishes junctional tension, thus inhibiting epithelial motility.

**ROCK1, but not ROCK2, supports RhoA signaling at the ZA**

In addition to its role as an effector, we recently reported that ROCK can support junctional RhoA signaling itself. This involved a signaling feedback network in which ROCK phosphorylated Rnd3 to ultimately abrogate the junctional localization of the RhoA antagonist p190B RhoGAP (Priya et al., 2015). Consistent with this, we found that inhibiting ROCK with Y-27632 destabilized the junctional pool of GFP-RhoA, reflected by an increase in GFP-RhoA mobile fraction measured by FRAP, and this was prevented by p190B KD (Figure 3, a–c). This provided further evidence that ROCK supports junctional Rho signaling by antagonizing p190B GAP. What effect the different ROCK proteins might have on this signaling network, however, was unknown.

To explore this question, we used endogenous Rho localization as a useful proxy to assess the integrity of the RhoA zone at the ZA (Ratheesh et al., 2012). In control MCF-7 cells, RhoA exhibited prominent staining at the ZA, and this was substantially perturbed by ROCK1 siRNA without affecting total RhoA levels (Figure 3, d and e, and Supplemental Figure S3i). In contrast, depletion of ROCK2 had no effect on RhoA localization (Figure 3, d and e). To substantiate this notion, we then used a location biosensor for GTP-RhoA, GFP-AHPH (Piekny and Glotzer, 2008; Priya et al., 2015; Yu et al., 2016). This biosensor has been validated to faithfully recapitulate the dynamics of endogenous GTP-Rho (Priya et al., 2015), and depletion of RhoA by small interfering RNA (siRNA) abolishes its junctional localization (Supplemental Figure S3, a–c). In control cells,
GFP-AHPH showed a prominent junctional localization, and this was significantly reduced in ROCK1 siRNA cells (Figure 3, f and g). However, consistent with what we observed with endogenous RhoA, ROCK2 did not affect the AHPH signal at the ZA (Figure 3, f and g), implying that ROCK1 is predominately responsible for supporting RhoA signaling at the ZA.

Of note, ROCK1 inhibition can stimulate Rac1 activity via Tiam1 and thereby potentially down-regulate RhoA by cross-talk (Tang et al., 2012). However, we did not see any difference in Rac1 localization or activity when Rho kinase was inhibited or depleted (Figure 3, h and i, and Supplemental Figure S3, d–f). Alternatively, RhoA at epithelial cadherin junctions can be inactivated by either loss of the GEF ECT2 or gain of the GAP, p190B (Ratheesh et al., 2012). ROCK inhibition with Y27632 or its depletion by siRNA prompted us to further discriminate between these possibilities. One potential analytical difficulty, however, is that disruption of NMII also compromises its scaffolding properties, thus attenuating its cortical association (Truebestein et al., 2015).

This mutational analysis also indicates a previously unidentified role for the N-terminus in localizing ROCK1 at junctions, as the mutant Δ7, which lacks the first 76 amino acids, showed only weak cortical enrichment (Figure 4, a and b). The N-terminus of ROCK1 is very distinct from that of ROCK2 and is required for its dimerization, kinase activity, and Rnd3 regulation (Garg et al., 2008). Thus it is possible that this region regulates ROCK1 binding to effectors required for its cortical localization. Overall these results suggest that multiple domains, including the N-terminus, RBD, SBD, and PH domains, cooperate to confer junctional localization upon ROCK1.

A potential explanation for the foregoing findings is that multiple mechanisms cooperate to promote junctional recruitment of ROCK1. These include GTP-RhoA, which binds directly to the RBD of ROCK1 and is required to relieve its autoinhibition (Amano et al., 1996; Matsui et al., 1996), as well as NMIIA, which we recently found to interact with ROCK (Priya et al., 2015). Accordingly, we sought to further discriminate between these possibilities. One potential analytical difficulty, however, is that disruption of NMII also down-regulates RhoA signaling by inhibiting the junctional recruitment of p190B (Priya et al., 2015). To overcome this limitation, we delocalized NMIIA from junctions with blebbistatin (Smuty et al., 2010) and also prevented the concomitant down-regulation of junctional RhoA with p190B GAP siRNA, a maneuver that sustains junctional GTP-RhoA (Priya et al., 2015). Blebbistatin abrogated cadherin junctions and further suggest that it exerts this effect by antagonizing p190B recruitment (Priya et al., 2015).

**Mechanisms of ROCK1 localization at the ZA**

Because ROCK1 appeared to be the major regulator of RhoA signaling and epithelial contractility at the ZA, we then sought to better understand how ROCK1 is localized at the ZA. We created GFP-tagged deletion mutants of ROCK1 and expressed them in MCF-7 cells. ROCK1 is structurally composed of a distinct N-terminal domain, a highly conserved serine-threonine kinase domain, a coiled-coil domain (CC), a RBD, a Shroom-binding domain (SBD), and a PH domain (Figure 4a). Wild-type GFP-ROCK1 showed a predominant localization at the apical junctions of cells (Figure 4, a and b). In contrast, the mutants Δ1, Δ2, and Δ4, which lack the RBD, SBD, and PH domains, were completely cytoplasmic (Figure 4, a and b), indicating that these three domains contribute to junctional localization of ROCK1. However, the mutant Δ5, comprising the RBD, SBD, and PH domains, was still poorly localized at the ZA (Figure 4, a and b), indicating that these previously identified domains were essential but not sufficient to confer junctional localization to ROCK1 (Simões Sde et al., 2014), and possibly, as for ROCK2, deletion of the CC domain in this mutant compromises its scaffolding properties, thus attenuating its cortical association (Truebestein et al., 2015).

FIGURE 4: ROCK1 deletion-mutant analysis. (a) Domains of ROCK1 and the deletion constructs. (b) Representative confocal images of MCF-7 cells expressing various GFP-ROCK1 deletion constructs and immunostained for GFP and ZO-1.
In conclusion, our study identifies ROCK1 as the dominant ROCK that supports the ZA as a contractile junction in MCF-7 and Caco2 cells. NMIIA ensures the cortical stability of ROCK1, which in turn maintains the GTP-Rho zone, actomyosin organization, and junctional tension. Although ROCK-2 has been predicted to influence actin organization via cofilin (Shi et al., 2013) or coronin1B (Rana and Worthylake, 2012) and tight junction assembly via cingulin (Terry et al., 2011), we could not see any significant effect on actomyosin filament organization and tight junctions in cells depleted of ROCK2. Thus our findings contribute to the growing awareness that, despite having highly conserved enzymatic activities, ROCK1 and ROCK2 perform nonredundant functions. Future functional studies of Rho kinases should account for these biological differences and dissect them by incorporating selective inhibitors/reagents.

MATERIALS AND METHODS

Cell culture and transfections

MCF-7 cells were obtained from the American Type Culture Collection and cultured in DMEM (11995-073; Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were maintained with low-dose Plasmocin (Invitrogen, Grand Island, NY) and routinely examined for the presence of mycoplasma. For siRNA transfections, cells were cultured to 40–50% confluence and then transfected using RNAiMAX (Invitrogen) according to the manufacturer’s recommendations. At 48–72 h posttransfection, cells were harvested for immunofluorescence assay or protein-expression analysis.

For transfection of plasmids, cells were cultured to 80–90% confluent, transfected using Lipofectamine 3000, and then fixed after 24 h for immunofluorescence assay. For live-cell experiments, cells were grown on 29-mm glass-bottom dishes (Shengyou Biotechnology) and imaged in clear Hanks’ balanced salt solution (HBSS) supplemented with 5% FBS, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), and 5 mM CaCl₂.

siRNA and short hairpin RNA

ROCK1 cortical localization at the ZA, and this was not rescued by p190B siRNA (Figure 5, a and b), suggesting that RhoA signaling was not sufficient to stably localize ROCK1 at the ZA in the absence of an adequate pool of NMII. These findings reinforce the idea that NMII is one of the major determinants for ROCK1 junctional localization under these circumstances.

Therefore, to better evaluate the role of NMIIA and NMIIB in stabilizing ROCK1, we performed FRAP assays to test their effect on the junctional stability of ROCK1. Fluorescence recovery profiles indicate a significant increase in the mobile fractions of GFP-ROCK1 when NMIIA is depleted, whereas depletion of NMIIB has no effect (Figure 5, c, d, f, and g). This suggests that NMIIA is necessary to maintain a stable pool of ROCK1 at apical junctions. Consistent with this, we found that NMIIA depletion abolished the junctional localization of ROCK1, a phenotype that could be rescued by overexpressing NMIIA but not NMIIB in NMIIA KD cells (Figure 5, e, h, and i), reinforcing the selective contribution of NMIIA on ROCK1 localization.
**CACGCCGGAGGAUUGGUUGAAAU**; and **NM_005406.2_stealth_904 sense, UGGUCGGUAGAGGCAUUGUCA**, and **NMIIα (S′-UAUAGCCAGGACCUGAACCUGAUC-3′ and S′-UUUA-GAAUCAAGGAGGAGCAGCGG-3′).**

ROCK2 and RhoA were depleted using commercially available SMARTpool siRNA (Dharmacon, Lafayette, CO), which is a pre-mixed pool of four separate siRNAs. The sequences for ROCK2 siRNA are 5′-GCACGCAUGUAAGCGGUA-3′, 5′-CACAUGGCUCGGUCAUU-3′, 5′-GUAGAACUCCUCCCUAUC-3′, and 5′-GCAAGAGUUAAUCUGG-3′, and those for RhoA siRNA are 5′-GCACAGCCGAGUAGUUAU-3′, 5′-GACAAGAUGGAGUGAGA-3′, 5′-GCAGAGAAUGGCAACAG-3′, and 5′-GGAAUAUGAGCGACAACAG-3′.

NMIIα and NMIIβ were depleted using lentiviral shRNA, as described previously (Smutny et al., 2010).

**Antibodies and inhibitors**

Primary antibodies used in this study were mouse monoclonal antibody (mAb) HECD-1 against the ectodomain of E-cadherin (1:50; a gift from P. Wheelock, University of Nebraska, Omaha, NE; with the permission of M. Takeichi); rabbit polyclonal antibody (pAb) for nonmuscle myosin IIα heavy chain (1:1000; PRB-440P; Covance, Dedham, MA); rabbit pAb for nonmuscle myosin IIb heavy chain (1:1000; PRB-445P; Covance); mouse mAb (1:200; A-11120, clone 3E6; Molecular Probes/Invitrogen) against GFP; rabbit pAb (1:100; 61-7300; Invitrogen) and mouse mAb against human ZO-1 (1:300, 133-9100, clone ZO1-1A12; Invitrogen); mouse mAbs against RhoA (1:100; clone 26C4, sc418; Santa Cruz Biotechnology, Dallas, TX); rabbit pAb against Ect2 (1:50; 07-1364; Millipore, Billerica, MA); mouse mAbs against p190B (1:50, 611612, clone 54/P190-B; BD Biosciences, San Jose, CA); mouse mAb against β-tubulin (1:500, T4026, clone TUB 2.1; Sigma-Aldrich, St. Louis, MO); rabbit pAb against ROCK1 (1:300, AB134181; Abcam, Cambridge, MA); rabbit pAb to ROCK2 (1:100, AB71598; Abcam); mouse mAb against actin (1:100; MAB1501, clone number C4; Millipore); rat mAb E-cadherin (1:500, 13-1900, clone ECD-2; Invitrogen); mouse mAb NMIIα (1:500, ab55456; Abcam); mouse mAb Rac1 (1:300, 05-389; Merck, Kenilworth, NJ); and mouse mAb pMRLC (1:100, 3675; Cell Signaling, Beverly, MA).

Secondary antibodies were species-specific antibodies conjugated with Alexa Fluor 488, 594, or 647 (1:500; Invitrogen) for immunofluorescence or horseradish peroxidase (1:5000; Bio-Rad Laboratories, Hercules, CA) for immunoblotting.

Cells were treated with blebbistatin to inhibit myosin (100 μM, 2 h; US1203390-5MG; Merck) or Y-27632 (30 μM, 1 h; Y0503; Sigma-Aldrich) or US1203390 US1203390-5MG; Merck) or Y-27632 (30 μM, 1 h; Y0503; Sigma-Aldrich) to inhibit ROCK.

**Plasmids**

GFP-ROCK2 construct was generated by PCR-amplifying mouse ROCK2 using FLAG-tag mouse ROCK2 (a kind gift from M. Samuel, Centre for Cancer Biology, University of South Australia, Adelaide, Australia) as a template and cloned into mEGFP-C1 (Clontech) using EcoR1 and BamH1 restriction sites. GFP-RhoA (12965), GFP-NMIIα (11347), and GFP-NMIIβ (11348) were obtained from Addgene.

GFP-ROCK1, E-cad–EGFP, and GFP-AHPH (used as a reporter for GTP-RhoA) have been described previously (Smutny et al., 2011; Wu et al., 2014; Priya et al., 2015). To generate GFP-ROCK1 deletion constructs, the coding region for ROCK1 (1–375 Δ1; 1–727 Δ2, 1–1080 Δ3, 375–727 Δ4, 727–1354 Δ5, 375–1354 Δ6, and 76–1354 Δ7) were amplified from ROCK1 and cloned into pEGFP-C1 using Sac1 and Kpn1 restriction sites.

**Immunofluorescence microscopy**

MCF-7 cells were fixed with ice-cold methanol for 5 min at −20°C or with freshly made 10% trichloroacetic acid (TCA; T0699-100ML; Sigma-Aldrich) on ice for 15 min or with 4% paraformaldehyde (PFA) in cytoskeleton stabilization buffer (10 mM 1,4-piperazinediethanesulfonic acid at pH 6.8, 100 mM KCl, 300 mM sucrose, 2 mM ethylene glycol tetraacetic acid, and 2 mM MgCl2) on ice for 20 min. The cells fixed with TCA were subsequently washed three times with 30 mM glycine. To permeabilize TCA- and PFA-fixed cells, cells were incubated with 0.25% Triton X-100 for 5 min at room temperature and washed three times with phosphate-buffered saline (PBS). Confocal images were acquired using a Zeiss LSM-710 microscope or Zeiss LSM-S10 META inverted microscope. For representation purposes, both control and test images were processed identically using ImageJ by applying a median filter of 0.5-1-pixel radius or by using the rolling-ball background subtraction function of ImageJ.

**Quantification of fluorescence intensity at junctions**

Fluorescence intensity at junctions was quantified using the plot profile function of ImageJ. Briefly, a line 10–20 μm in length was drawn orthogonal to the junctions at its center with the junctions. The plot profile function of ImageJ was then used to obtain the numerical values for the fluorescence intensity. Background correction was performed by subtracting a constant value from each of the intensity profiles and then fitted to Gaussian function using GraphPad Prism software. A nonlinear regression function was used to determine the peak values. For each experiment, a minimum of 40 contacts were analyzed per condition.

For the Rho biosensor experiment, the mean-gray values of GFP-AHPH junctional and cytoplasmic intensity were obtained using ImageJ, and their ratio was used for statistical analysis. This normalized ratio is referred to as junctional GTP-RhoA.

Although the GFP-AHPH biosensor does not distinguish between the RhoA/RhoB/RhoC proteins, crystal structure data and biochemical results show that the AH domain of anillin directly binds to GTP-RhoA (Piekkny and Glotzer, 2008; Sun et al., 2015). Further, using a series of mutants and pharmacological/genetic ablation studies, we validated that the junctional enrichment of AHPH strictly depends on its interaction with GTP-RhoA and thus recapitulates the endogenous localization of GTP-RhoA (Priya et al., 2015; Supplemental Figure S3, b and c).

**Laser ablation**

To measure junctional tension in the steady state, MCF-7 cells were transduced with lentivirus encoding E-cadherin–EGFP (Wu et al., 2014) to identify apical junctions. Then individual ROCK proteins were depleted using siRNA for 48–72 h. An LSM 510 Meta Zeiss confocal microscope equipped with a 37°C heating stage was used for the experiments, and images were acquired and analyzed as described previously (Wu et al., 2014; Priya et al., 2015).

Differences in recoil are useful indicators of contractile tension, as long as the viscous drag remains unchanged or is a minor contributor to the recoil (Sugimura et al., 2016). We assessed this, we modeled the mechanics of junctions as Kelvin–Voigt fibers and extracted the rate constants (k values), which reflect the ratio of elasticity to viscosity, from the recoil measurements (Michael et al., 2016). We found no significant changes in k values in ROCK1 and ROCK2 KD cells compared with control cells (Supplemental Table S1), suggesting that the differences in the initial recoil velocities were principally due to changes in contractile tension.
FRAP
MCF-7 cells were transfected with GFP-NMIIA, GFP-RhoA, or GFP-ROCK1 and imaged in clear HBSS. Image acquisition and analysis was described previously (Priya and Gomez, 2013; Priya et al., 2015).

Fluorescence resonance energy transfer
MCF-7 cells were transfected with a Raichu-Rac fluorescence resonance energy transfer (FRET) biosensor (Itoh et al., 2002) and 24 h posttransfection were treated with either PBS or Y-27632 (30 μM, 1 h; Y0503; Sigma-Aldrich). For live-cell imaging, cells were incubated at 37°C in an LSM710 Zeiss confocal microscope equipped with a 63× oil immersion objective (Plan Apochromat 63×/1.4 numerical aperture [NA]; Zeiss). Images were acquired by sequential line acquisition. For the acceptor (A) channel, a 514-nm laser line was used for excitation, and emission was collected in the acceptor emission range (bandpass [BP] 530–590 nm). Donor and FRET channels were acquired using a 458-nm laser line, and emission was collected in the donor emission region (BP 470–490 nm) and the acceptor emission region (BP 530–590 nm, FRET). FRET measurements have been described previously (Rathesh et al., 2012).

Time-lapse imaging and nuclei tracking
Time-lapse live-cell imaging of MCF-7 cells stably expressing both Histone2b-GFP and a plasma membrane–targeted mCherry by fusing it to the C-terminal 14 amino acids of human K-Ras4B (mCherry-Mt) was performed on a Nikon Ti-E deconvolution microscope (40×/0.95 NA Plan Apo objectives) driven by NIS-Elements AR software (version 4.3; Nikon) equipped with a 37°C, 5% CO2 chamber. Images were acquired every 3 min for 5 h on cells cultured in DMEM in the presence of 10% FBS. Movies of nuclei in movies were then tracked using the u-track software developed by the Danuser lab (lccb.hms.harvard.edu/software.html; Jaqaman et al., 2008; Ng et al., 2012), and mean square displacements for different time intervals were calculated using a custom-made MATLAB script, which is available upon request.

Statistics
Unless otherwise stated, data represent mean ± SEM, and n is the number of independent experiments. Precisely, n states the number of times each individual experiment was repeated at different times, thus accounting for the variability of the biological process.

For line-scan analysis, at least 40 contacts were analyzed per experiment, and for laser ablation and FRAP, a minimum of 20 contacts were analyzed per experiment, except for Rho-FRAP, for which 10–15 contacts were analyzed per experiment. Student’s t test was used to compare two groups, and one-way analysis of variance (ANOVA) with corrections for multiple comparisons applied was used to compare more than two groups. GraphPad Prism 6 was used to determine the p values and perform all statistical analyses. Student’s t test was used for Figures 1i and 2, f and h, and Supplementary Figures S2g and S3e, f, and h. One-way ANOVA was used for Figures 1, f, k, and m, 2, k and m, 3, b, e, g, i, k, and m, and 5, g and i, and Supplementary Figures S1, e, h, and k, and S2, b and c.

NS, not significant. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

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