Novel Mechanism for Negatively Regulating Rho-Kinase (ROCK) Signaling through Coronin1B Protein in Neuregulin 1 (NRG-1)-induced Tumor Cell Motility*§

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Manish K. Rana and Rebecca A. Worthylake*§1

From the Departments of Pharmacology and Oral and Craniofacial Biology, Louisiana State University Health Sciences Center, New Orleans, Louisiana 70119

Background: Negative regulatory mechanisms of ROCK signaling are poorly understood. We have discovered a novel mechanism to attenuate ROCK signaling.

Results: Coronin1B is a novel binding partner of ROCK2 that inhibits ROCK signaling to myosin.

Conclusion: Coronin1B regulates ROCK by a novel signal attenuation mechanism.

Significance: Coronin1B attenuation of ROCK signaling is associated with NRG-1-induced tumor cell motility.

Although many mechanisms that activate ROCK are known, corresponding negative regulatory mechanisms required for cytoskeletal plasticity are poorly understood. We have discovered that Coronin1B is a novel attenuator of ROCK signaling. We initially identified Coronin1A in a proteomics screen for ROCK2-binding proteins, and here we demonstrate that Coronin1A/B bind directly to ROCK2 through its PH (Pleckstrin Homology) domain. The consequence of the ROCK2-Coronin1B interaction was tested and revealed that increased expression of Coronin1B inhibited, whereas knockdown of Coronin1B stimulated, phosphorylation of the ROCK substrate myosin light chain phosphatase and subsequently, myosin light chain. Thus, Coronin1B is a previously unrecognized inhibitor of ROCK signaling to myosin. Furthermore, we found that the phosphatase Slingshot 1L (SSH1L) was required for Coronin1B to inhibit ROCK signaling. To test the significance of this novel mechanism in tumor cell motility, we investigated its role in neuregulin 1 (NRG-1)-induced cell scattering. Importantly, we found that attenuation of the ROCK signaling by Coronin1B was required for NRG-1 stimulated scattering. Our data support a model in which Coronin1B fine-tunes ROCK signaling to modulate myosin activity, which is important for tumor cell motility.

Aberrant signal transduction has been linked to various pathophysiological processes including cancer (1–5). Post-translational modification such as phosphorylation of key signaling proteins influences the downstream signaling outcomes. Rho-kinases (ROCKs)2 are Ser/Thr kinases that regulate cell adhesion, migration, invasion, cytokinesis, apoptosis, and oncogenic transformation (6). ROCKs induce these effects by phosphorylation of various substrates, which ultimately determine the particular biological outcome (6–8). The integration of multiple upstream signaling inputs present in a particular cellular environment could determine the balance between phosphorylation and dephosphorylation of ROCK substrates, thereby controlling ROCK signaling outcomes.

The majority of studies on regulation of ROCK signaling have concentrated on the role of its kinase activity by either inhibiting its activity with a pharmacological inhibitor or forcing its activity through overexpression of the isolated kinase domain (6, 7, 9–20). Whereas the upstream signals that promote ROCK kinase activity are well described, mechanisms that modulate the levels of substrate phosphorylation to control downstream signaling appropriately are poorly understood (6). This is important during cell motility because it provides a potential mechanism for signaling plasticity to allow dynamic response of the cytoskeleton in response to upstream signals. We reasoned that ROCK may form complexes with other proteins to regulate its downstream signaling. We have previously used the ROCK2 PH domain as bait to identify novel binding partners that may serve as negative regulators of ROCK2 signaling (21). One of the proteins, Coronin1A, promotes membrane protrusions, whereas ROCK2 is known to limit membrane protrusions (22, 23); thus, we investigated Coronin1A and its more ubiquitous relative Coronin1B as potential negative regulators of ROCK2 signaling.

Coronins are actin-binding proteins, known to regulate cytoskeletal dynamics during cell migration (24–26). Coronin1A/B is known to regulate the recycling of actin at the leading edge, through inhibiting the Arp2/3 complex and by activating cofilin (23, 27, 28). Activity of cofilin is controlled by its phosphorylation status (29, 30). Kinases, such as LIM-kinase (LIM domain kinase), inactivate cofilin, whereas Coronin1B reactivates cofilin by promoting its dephosphorylation (23, 31, 32). Although Coronin1B does not contain any intrinsic enzyme activity, it has been shown to promote cofilin dephosphorylation through the recruitment of the phosphatase Slingshot 1L (SSH1L) (23).
Thus, one way that Coronin1B regulates cytoskeletal dynamics is by influencing the balance between kinase and phosphatase activities.

Here, we have discovered a novel signaling interaction between two previously unlinked cytoskeletal regulators: ROCK2 and Coronin1B. Our data show that Coronin1B attenuates ROCK signaling by reducing the phosphorylation levels of MYPT-1 and myosin light chain (MLC). We further determined that the mechanism by which Coronin1B decreases ROCK signaling is through the phosphatase SSH1L. Importantly, this Coronin1B attenuation of ROCK signaling is associated with neuregulin-1 (NRG-1)-induced scattering of tumor cells.

EXPERIMENTAL PROCEDURES

Cells and Transfection—MCF7-CXCR4 cells were cultured and transfected as described (33). Phoenix ecotropic retrovirus packaging cell lines (Garry Nolan laboratory via National Gene Vector Biorepository) were used for retroviral production and selection of stable pools of cells as described (34).

Antibodies and Growth Factors—Anti-phospho-MYPT-1 (Thr-696 or Thr-853), anti-phospho-cofilin (Ser-3), phospho-MLC (Ser-18/Ser-19), cofilin, myc, actin, and MYPT-1 were purchased from Bethyl Laboratories (Montgomery, TX), anti-MYC, and anti-phospho-cofilin (Ser-3), phospho-MLC (Ser-18/Ser-19), cofilin, myc, actin, and MYPT-1 were obtained from Cell Signaling. Anti-V5 antibody was purchased from Invitrogen. Anti-Coronin1B and anti-ROCK2 antibodies were purchased from Santa Cruz Biotechnology and Proteintech, respectively.

Plasmids and siRNA—The nGFP-DEST vector (33) was used to subclone ROCK2 constructs, generated using the Gateway system (Invitrogen) by PCR amplification of full-length ROCK2, ROCK2ΔPH (amino acids 1–1149), the PH domain (amino acids 1150–1388), and PHΔC1 (amino acids 1150–1350) as GFP, myc, or GST fusion proteins. Myc-SSH1L plasmids were kindly provided Dr. Bamburg (Colorado State University). Coronin1A (NM 007074) or Coronin1B (BC006449) was subcloned into the C-terminal V5 destination vector (Invitrogen) and mCherry DEST vector.

ROCK2 PH domain mutant, PH(A), (mutations: K1171A, K1337A, K1338A, R1334A) (rat) was obtained from Wen et al. (39) and subcloned as GST-PH(A), GFP-PH(A), and myc-PH(A). Based on the structure of the ROCK2 PH domain (A), we mutagenized a cluster of three positively charged amino acids in the full-length ROCK2 to generate GFP-ROCK2(A), (R1343A, and K1346A/K1347A) using the QuickChange™ kit (Stratagene), according to the manufacturer’s instructions. Nontargeting, control siRNA, siRNA against Coronin1B and SSH1L (40) were purchased from Dharmacon (Lafayette, CO).

Immunoblotting and Immunoprecipitation—Immunoblotting and immunoprecipitation were performed as reported previously (20, 32).

Recombinant His-Coronin1B and GST-ROCK2-PH—His-Coronin1B and GST fusion proteins were produced in Rosetta (DE3) pLysS expression bacteria (EMD Biosciences), lysed in 300 mM NaCl, 20 mM Tris-HCl, pH 7.8, 5% glycerol, 5 mM MgCl₂, 20 mM imidazole, and purified by nickel-Sepharose 4B or glutathione-Sepharose beads (GE Healthcare).

In Vitro Protein Binding Assay—Equal amounts of GST and GST-ROCK2 PH domain immobilized on glutathione-Sepharose beads were incubated with the purified His-Coronin1B recombinant protein for 2 h at 4 °C. The precipitates were resolved by SDS-PAGE, and binding was assessed by Coronin1B immunoblot analysis.

Microscopy—For immunofluorescence microscopy, MCF7-CXCR4 cells were transfected with Coronin1B siRNA for 72 h. Cells were then stained with rabbit anti-p-MLC (1:200; Cell Signaling), anti-MYC (1:200; Sigma) subsequently processed as described in Refs. 20, 32.

RESULTS

Coronin1B: Novel Binding Partner of ROCK2

ROCK2 and Coronin1A Form Complex in Cells—Our previous ROCK2 PH domain proteomics screen had identified Coronin1A as a potential negative regulator of ROCK2 (21). To test whether ROCK2 and Coronin1A form a complex in cells, we performed co-immunoprecipitation studies. Expression vectors encoding myc-ROCK2 and V5-Coronin1A were co-transfected into COS-7 cells, and V5-Coronin1A was immunoprecipitated using a V5 antibody. The presence of myc-ROCK2 was specifically detected in the V5-Coronin1A immune complex (Fig. 1A, top). Thus, the data show that ROCK2 formed a complex with Coronin1A within cells, demonstrating that Coronin1A is a novel binding partner for ROCK2.

ROCK2 also Forms Complex with Coronin1B—We then tested whether the more ubiquitous Coronin1B interacted with ROCK2. Fig. 1B shows that myc-ROCK2 (top) specifically co-

FIGURE 1. ROCK2 and Coronin1A/B form a complex in cells. A, COS-7 cells expressing myc-ROCK2 and V5-Coronin1A were immunoprecipitated (IP) with anti-V5 antibody or control IgG. Co-immunoprecipitation of myc-ROCK2 was determined by immunoblotting (IB). B, COS-7 cells expressing myc-ROCK2 and V5-Coronin1B were analyzed as above. C, MCF7-CXCR4 cells were immunoprecipitated with anti-ROCK2 antibody or control IgG. Co-immunoprecipitation of endogenous Coronin1B was determined by immunoblotting. Blots shown are representative of at least three independent experiments and show that both Coronin1A/B form a complex with ROCK2 in cells.
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immunoprecipitated with V5-Coronin1B (Fig. 1B, bottom). Our laboratory has studied the migration of MCF7 cells overexpressing the CXCR4 receptor, so we also tested the interaction of ROCK2 and Coronin1B in that system (33). To determine whether endogenous ROCK2 and Coronin1B form a complex, lysates of MCF7-CXCR4 cells were subjected to co-immunoprecipitation studies, and we found that Coronin1B specifically precipitated with the ROCK2 immune complex (Fig. 1C, bottom). These data show that both Coronin1A and 1B interact with ROCK2, verifying our proteomics screen data, thereby identifying Coronin1A/B as a novel ROCK2-binding partner.

Coronin1B Binds Directly to ROCK2 PH Domain in Vitro—Because the proteomics screen that identified Coronin1 used the ROCK2 PH domain and several studies have shown direct binding between PH domains and the WD40 domains that form the core structure of Coronin1 proteins, we tested whether the binding between ROCK2 and Coronin1B was direct (24, 25, 27, 41–44). We tested this by using recombinant His-Coronin1B and GST-PH(WT) in an in vitro binding assay. Fig. 2A shows that Coronin1B specifically precipitated (Fig. 2A, top) with the GST-PH(WT) (Fig. 2A, bottom) but not with GST alone. This result shows that Coronin1B interacts directly with the ROCK2 PH domain, as predicted by our proteomics study. To more specifically define the region of the ROCK2 PH domain that interacts with Coronin1B, we used the known structure of the GRK2-Gβ complex and the structure of the individual ROCK2 PH and Coronin WD40 proteins to predict a model of the ROCK2 PH-Coronin1B complex (39, 41, 45). Our analysis suggested that the negatively charged patch on Coronin1B will be available to interact with the positively charged region proline-rich C1 domain in the ROCK2 PH domain (39, 45). Based on this we created two PH domain mutants: one with positive residues mutated to alanine, PH(A), the second lacking the proline-rich C1 domain, PH(ΔC1). These mutants were used to test whether either the positive patch or the C1 domain is important for binding to Coronin1B. Fig. 2A shows that there was a marked decrease in the precipitation of Coronin1B (Fig. 2A, top) with the GST-PH(A) or GST-PH(ΔC1), compared with GST-PH(WT). These data demonstrate that Coronin1B binds directly to the PH domain of ROCK2 and more specifically, that both the positive patch and the C1 domain in the ROCK2 PH domain are important for the ROCK2 PH-Coronin1B interaction. Because the PH(A) mutant is more effective in disrupting the binding and only has a few point mutations instead of a deletion, we chose to perform the functional studies with the PH(A) mutant.

ROCK2 PH Domain Is Required for ROCK2-Coronin1B Interaction in Cells—We next investigated whether the ROCK2 PH domain was required for ROCK2-Coronin1B interaction in cells. Myc-ROCK2 and a mutant lacking the PH domain, myc-ROCK2(ΔPH), were transfected into MCF7-CXCR4 cells, and the presence of endogenous Coronin1B in the myc immunoprecipitate was determined by Western blotting. We found that Coronin1B specifically co-immunoprecipitated with full-length myc-ROCK2, whereas myc-ROCK2ΔPH failed to co-immunoprecipitate Coronin1B (Fig. 2B, bottom). This shows that the PH domain of ROCK2 is required for the interaction of ROCK2 and Coronin1B in cells. Based on our in vitro binding data where ROCK2 PH(A) showed notably less binding to Coronin1B, we tested whether the positive patch at the ROCK2 C terminus was required for the ROCK2-Coronin1B interaction in cells. To test this we mutated the corresponding positively charged amino acids in full-length ROCK2. Either GFP-ROCK2(WT) or GFP-ROCK2(A) was transfected into MCF7-CXCR4 cells, and Coronin binding was tested by co-immunoprecipitation, as above. The plots shown are representative of three independent experiments and show that ROCK2 PH domain is required for ROCK2-Coronin1B interaction in cells. The Coronin1B model data where ROCK2 PH(A) showed notably less binding to Coronin1B determined by co-immunoprecipitation, as above, are shown. The plots are representative of three independent experiments and show that ROCK2 harboring a mutation in its PH (Pleckstrin Homology) domain fails to interact with Coronin1B.
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Knockdown of Coronin1B stimulates MYPT-1 and MLC phosphorylation. A, cells were treated with control or the Coronin1B siRNA and then blotted (IB) for p-MYPT-1 (Thr-696 antibody). Effectiveness of the Coronin1B knockdown is shown by Coronin1B immunoblotting, and equal loading is demonstrated by total MYPT-1 and actin immunoblots. B, to assess whether the increase in MYPT-1 (Thr-696 or Thr-853) and MLC phosphorylation were dependent on ROCK activity, the Coronin1B knockdown cells were treated with ROCK pharmacological inhibitor H1152 30 min before lysis. The effectiveness of the Coronin1B knockdown is shown by Coronin1B immunoblotting, and equal loading is demonstrated by total MYPT-1 and actin immunoblotting. C, blots from B are quantitated. Bars represent the average ± S.E. (error bars) from three independent experiments. D, cells treated with control or Coronin1B siRNA were analyzed by immunofluorescence microscopy for phosphorylated MLC (red), MLC (green), and actin (gray). Cells were treated with ROCK pharmacological inhibitor H1152 30 min before fixation to test the dependence on ROCK activity. Scale bar, 10 μm. E, blots from D are quantitated. Bars represent the average ± S.E. (error bars) from three independent experiments and show that knockdown of Coronin1B increases phosphorylation of MYPT-1 and MLC and organized actin in a ROCK-dependent manner.

Knockdown of Coronin1B Increases MYPT-1 and MLC Phosphorylation—We next asked whether Coronin1B negatively regulates ROCK signaling. Activation of ROCK signaling is known to phosphorylate and inactivate the regulatory subunit of MYPT-1, resulting in increased phosphorylation and activation of MLC (6, 11, 19, 46–49). MCF7-CXCR4 cells were transfected with Coronin1B siRNA or nontargeting siRNA, and phosphorylated MYPT-1 and MLC were measured by immunoblotting and immunofluorescence, respectively. Two of the siRNA duplexes (#1 and #2) comprising the Coronin1B smartpool were efficient in down-regulating Coronin1B expression similar to the Coronin1B smartpool siRNA duplexes, and both resulted in increase in phosphorylated MYPT-1 levels, suggesting that the effect is specifically due to targeting Coronin1B (Fig. 3A). Smartpool targeting Coronin1B was used for further studies.

Knockdown of Coronin1B increased phosphorylated MYPT-1 (1.7-fold) (Fig. 3, B and C) and MLC (1.6-fold) (Fig. 3, D and E) and organized actin compared with control siRNA-transfected cells. To test directly whether the increase in phosphorylation of MYPT-1 (Thr-696 or Thr-853), MLC, and organized actin was dependent upon ROCK signaling, we used the ROCK pharmacological inhibitor H1152. Coronin1B knockdown cells were treated with H1152 (0.5 μM) for 30 min before lysis or fixation. The increase in p-MYPT-1, MLC levels, and organized actin in response to Coronin1B knockdown was inhibited by H1152 (Fig. 3, B and D), demonstrating that the increase in MYPT-1 and MLC phosphorylation and organized actin is dependent upon ROCK signaling. These results show that knockdown of Coronin1B increases phosphorylation of MYPT-1 and MLC, suggesting that Coronin1B is a negative regulator of ROCK signaling.

Increased Expression of Coronin1B Inhibits MYPT-1 Phosphorylation—We next used the complementary approach to the knockdown studies and asked whether increasing the levels of Coronin1B influences ROCK signaling. Stable expression of GFP-Coronin1B in MCF7-CXCR4 cells resulted in a 50% decrease of phosphorylated MYPT-1 (Fig. 4A) compared with GFP controls. GFP control cells treated with H1152 demonstrated that the phosphorylated MYPT-1 is dependent upon ROCK activity. These results show that increased expression of Coronin1B reduces phosphorylation of MYPT-1 and MLC. Together, our data show that increased expression of Coronin1B inhibits (Fig. 4) whereas knockdown of Coronin1B stimulates (Fig. 3) MYPT-1 and MLC phosphorylation, which is dependent upon ROCK. These results demonstrate a novel functional relationship between ROCK2 and Coronin1B, in which ROCK signaling is attenuated by Coronin1B.
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ROCK2 PH domain acts as dominant negative for ROCK2-Coronin1B interaction

Based on our direct interaction between Coronin1B and ROCK2 PH domain in vitro, we investigated whether overexpression of the ROCK2 PH domain could act as a dominant negative for the ROCK2-Coronin1B interaction in cells. Myc-ROCK2 was co-transfected with either GFP-ROCK2-PH(WT), GFP-ROCK2-PH(A), or GFP alone into MCF7-CXCR4 cells, and the presence of endogenous Coronin1B in the myc immune complex was determined by Western blotting. We found a marked decrease in the amount of Coronin1B that co-immunoprecipitated with myc-ROCK2, when the ROCK2-PH(WT) was co-expressed (Fig. 5A, top). This dominant negative effect was specific to expression of the WT PH domain because neither GFP nor GFP-PH(A) expression inhibited the co-immunoprecipitation of Coronin1B with myc-ROCK2 (Fig. 5A, top). Consistent with our previous results, ROCK2-PH(A) failed to block the ROCK2-Coronin1B interaction in cells whereas the WT ROCK2 PH domain blocked this interaction in cells. This shows that the overexpression of the PH domain of ROCK2 blocks the interaction of ROCK2 and Coronin1B in cells and can be used to test whether the ability of Coronin1B to attenuate ROCK signaling is dependent upon the interaction between Coronin1B and ROCK2.

Mechanism of Coronin1B Attenuation of ROCK Signaling

Negative Regulation of ROCK Signaling by Coronin1B Requires Binding to ROCK2—We then tested whether overexpression of the ROCK2 PH domain would act as a dominant negative to block inhibition of ROCK signaling by Coronin1B. Fig. 5 shows that the reduction in myosin phosphorylation in GFP-Coronin1B cells was reversed by transfection of GFP-PH by both blotting (Fig. 5, B and C) and immunostaining (supplemental Fig. 1). This supports a model in which Coronin1B attenuates ROCK signaling to myosin through a direct interaction between the two proteins. To strengthen this conclusion further, we overexpressed the PH domain mutant that we found did not interfere with ROCK2 binding to Coronin1B. Transfection of GFP-PH(A) did not block the inhibition of p-MLC by Coronin1B (Fig. 5, B and C, and supplemental Fig. 1), providing further evidence supporting a mechanism in which the direct interaction between ROCK2 and Coronin1B is required for Coronin1B to attenuate ROCK signaling through the myosin pathway.

SSH1L Dephosphorylates ROCK Substrate MYPT-1—Coronin1B is known to target the phosphatase SSH1L to regulate the levels of cofilin phosphorylation at the lamellipodium (23). Because we had also identified SlingShot as a potential ROCK2-associated protein in our initial proteomics screen (21), we reasoned that SSH1L might also reverse the phosphorylation of ROCK targets, such as MYPT-1.

We first asked whether SSH1L regulated p-MYPT-1 levels and found that overexpression of SSH1L reduced the phosphorylation of MYPT-1 by 50% (Fig. 6, A and B) compared with control cells. To test the effect of SSH1L on ROCK signaling further, we used siRNA to knockdown SSH1L. Knockdown of SSH1L was checked by quantitative PCR (Fig. 6E), and as an internal control, the effect of SSH1L knockdown on phosphorylation of a known SSH1L substrate, cofilin, was measured (40, 50, 51). Cells were transfected with SSH1L or control siRNA for 72 h, and levels of cofilin and MYPT-1 phosphorylation were determined by Western blotting. Fig. 6C shows that SSH1L siRNA increased cofilin phosphorylation, consistent with previous findings. We then measured the effect on MYPT-1 and found that knockdown of SSH1L also increased MYPT-1 phosphorylation compared with control (Fig. 6C, lane 2, top, and D), identifying a previously unrecognized role for SSH1L in regulating signaling through the myosin pathway.

Mechanism: Coronin1B Requires Phosphatase SSH1L to Inhibit ROCK Signaling—We then asked whether attenuation of ROCK signaling by Coronin1B requires SSH1L. To test this possibility, Coronin1B was overexpressed in cells treated with either control or SSH1L siRNA. The decrease in p-MYPT-1 and...
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p-cofilin induced by Coronin1B expression (Fig. 6C, compare lanes 1 and 3) was blocked by knockdown of SSH1L (Fig. 6C, compare lanes 3 and 4); quantitation of relative p-MYPT-1 levels is shown in Fig. 6D. These data demonstrate that Coronin1B attenuation of ROCK signaling requires the phosphatase SSH1L.

We then asked whether Coronin1B requires the phosphatase SSH1L to inhibit ROCK-dependent organization of p-MLC by immunofluorescence. GFP control or GFP-Coronin1B stable cells were transfected with SSH1L siRNA or nontargeting siRNA, and phosphorylated MLC and actin were measured by microscopy. Consistent with our previous results, we observed a decrease in both p-MLC and organized actin in GFP-Coronin1B cells compared with GFP control cells (Fig. 6F). Also, the morphology of GFP-Coronin1B cells was similar to that typically observed with the ROCK inhibitor. Interestingly, knockdown of SSH1L in GFP-Coronin1B cells restored their normal morphology, which was accompanied by an increase in p-MLC and organized actin (Fig. 6F). Collectively, our data sup-

FIGURE 5. ROCK2 PH domain acts as a dominant negative for Coronin1B attenuation of ROCK signaling. A, MCF7-CXCR4 cells expressing GFP-ROCK2 PH(WT), GFP ROCK2 PH(A), or GFP alone with myc-ROCK2, and the presence of endogenous Coronin1B in the myc immune complex were determined by Western blotting (IB). B, MCF7-CXCR4 GFP or GFP-Coronin1B cells were transfected with GFP-PH(WT), GFP-PH(A), or vector control and p-MLC determined by immunoblotting. Actin and total MLC are loading controls, and the expression of GFP-PH(WT) or GFP-ROCK2 PH domain(A) is shown with GFP blotting. C, quantitation of the band intensities of p-MLC levels are shown in the graph. Bars represent the average ± S.E. (error bars) from three independent experiments and show that ROCK2 PH domain acts as a dominant negative for Coronin1B-mediated inhibition of ROCK signaling to myosin.

FIGURE 6. Overexpression of SSH1L reduces MYPT-1 phosphorylation, and Coronin1B requires SSH1L to inhibit MYPT-1 and MLC phosphorylation. A, cells expressing myc-GFP or myc-SSH1L or V5-Coronin1B were immunoblotted (IB) for p-MYPT-1. For comparison, a sample with Coronin1B overexpression was included. Total MYPT-1 and actin immunoblots are shown as loading controls; the expression of myc-SSH1L or V5-Coronin1B is indicated by immunoblotting for the respective epitope tags. B, blots from A are quantified. Bars represent the average ± S.E. (error bars) from three independent experiments. C, cells were treated with control and SSH1L siRNA and then transfected with myc-GFP or V5-Coronin1B. Levels of phosphorylated MYPT-1 and cofilin were assessed by Western blotting. Lane 2 shows that knockdown of SSH1L increases p-MYPT-1 compared with control siRNA-treated cells (lane 1). Lane 4 shows that knockdown of SSH1L prevents inhibition of MYPT-1 phosphorylation when V5-Coronin1B is overexpressed (compared with lane 3). D, blots from C were quantified. Bars represent the average ± S.E. from three independent experiments. E, quantitation of relative expression of SSH1L detected by quantitative PCR is shown. F, MCF7-CXCR4 GFP or GFP-Coronin1B cells treated with control or SSH1L siRNA were analyzed by immunofluorescence microscopy for p-MLC (red) and actin (gray), and GFP fluorescence is shown. Collectively, these data demonstrate that SSH1L is required for Coronin1B to attenuate ROCK signaling to myosin. Scale bar, 10 μm.
port a mechanism by which Coronin1B attenuates ROCK signaling through the phosphatase SSH1L.

**Coronin1B Attenuation of ROCK Signaling Is Required for NRG-1-induced Cell Scattering**

The breast tumor-promoting factor NRG-1 triggers lamellipodia formation and scattering of MCF-7 cells (52, 53). Interestingly, NRG-1 stimulates lamellipodia formation and cell scattering by modulating the phosphorylation status of the cytoskeletal regulator, cofillin, via SSH1L (53, 54). Because our data show that Coronin1B uses SSH1L to reduce the phosphorylation of both cofillin and MYPT-1, we asked whether NRG-1 also reduced the levels of p-MYPT and p-MLC. We found that treating cells with NRG-1 induced the dephosphorylation of both MYPT-1 and MLC (Fig. 7, A and B) in a time-dependent manner. This prompted us to investigate whether NRG-1 negatively regulates ROCK signaling through Coronin1B.

To determine whether NRG-1 regulated the interaction between ROCK2 and Coronin1B, we performed co-immunoprecipitation in cells transfected with GFP-ROCK2(WT) or GFP-ROCK2(A)-transfected cells were treated with or without NRG-1 for 120 min, and time lapse phase contrast pictures were taken every 5 min. To test whether increased ROCK signaling prevents cell scattering by NRG-1 in Coronin1B knockdown cells, the cells were treated with H1152. "Quantitation of the percent increase in the area of the cell cluster is shown in the graph. The bars represent the average ± S.E. from three independent experiments."

**FIGURE 7.** Coronin1B is required for NRG-1-mediated inhibition of ROCK signaling to myosin for cell scattering. A, MCF7-CXCR4 cells treated with NRG-1 and levels of p-MYPT-1 and p-MLC were assessed by immunoblotting (IB). B, blots from A were quantified. Bars represent the average ± S.E. (error bars) from three independent experiments. C, MCF7-CXCR4 cells expressing GFP-ROCK2(WT) or GFP-ROCK2(A) were treated with NRG-1 for 40 min and immunoprecipitated (IP) with anti-GFP antibody or control IgG, and co-immunoprecipitation of Coronin1B was measured by immunoblotting. D, MCF7-CXCR4 cells expressing GFP-PH(WT) or GFP-PH(A) or myc-GFP control were treated with NRG-1 for 60 min, and p-MYPT-1 levels were determined by immunoblotting. E, MCF7-CXCR4 cells treated with control or Coronin1B siRNA were treated with NRG-1 for 120 min, and time lapse phase contrast pictures were taken every 5 min. To test whether increased ROCK signaling prevents cell scattering by NRG-1 in Coronin1B knockdown cells, the cells were treated with H1152. F, quantitation of the percent increase in the area of the cell cluster is shown in the graph. The bars represent the average ± S.E. from three independent experiments. G, MCF7-CXCR4 cells were treated with control or Coronin1B siRNA and then stimulated with NRG-1 and analyzed by immunofluorescence microscopy, as in Fig. 3. To determine the dependence on ROCK activity, cells were pretreated with H1152. H, micrographs from G were quantified. Bars represent the average ± S.E. from three independent experiments. Scale bar, 10 μm.

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NRG-1 increases the interaction of ROCK2 and Coronin1B in cells, which is correlated with a reduction in p-MYPT and p-MLC.

To determine whether the ROCK2-Coronin1B binding was required for the down-regulation of p-MYPT levels, we overexpressed the ROCK PH domain to block the interaction between ROCK2 and Coronin1B. Overexpression of ROCK-PH(WT) prevented NRG-1 from down-regulating p-MYPT levels (Fig. 7D). As a control, overexpression of ROCK-PH(A) that does not inhibit ROCK2-Coronin1B binding was unable to prevent NRG-1 from reducing p-MYPT. Taken together, these data show that NRG-1 induces the association between ROCK2 and Coronin1B to attenuate signaling to myosin.

We further reasoned that NRG-1-induced cell scattering might involve attenuation of ROCK signaling through the myosin pathway by Coronin1B. First, we tested whether Coronin1B was required for NRG-1 to induce cell scattering. We found that knockdown of Coronin1B blocked NRG-1-induced scattering, as measured by the increase in area of isolated cell colonies (Fig. 7, E and F). Because regulation of the myosin pathway is essential for cell motility, we performed immunostaining for p-MLC in cell clusters undergoing NRG-1 scattering. NRG-1 decreased p-MLC in cell clusters (Fig. 7, G and H, left). However, knockdown of Coronin1B blocked the reduction of p-MLC levels in the cell clusters, and no scattering was observed (Fig. 7, G and H, middle). Thus, in the absence of Coronin1B, NRG-1 cannot down-regulate p-MLC or induce scattering, suggesting that negative regulation of ROCK signaling by Coronin1B is required for cell scattering. To test this, we used the ROCK inhibitor H1152. Indeed, H1152 restored NRG-1-induced scattering activity to Coronin1B knockdown cells, with a concomitant reduction in p-MLC levels (Fig. 7G, right). Collectively, our data support a model in which NRG-1 scattering requires Coronin1B attenuation of ROCK signaling, demonstrating the significance of this novel mechanism in cell motility.

DISCUSSION

ROCK is implicated in multiple cellular processes, but how the appropriate signaling pathways are activated in response to a particular cellular context is not known (6, 7). In particular, little is known about how ROCK signaling is negatively regulated, or which phosphatases act on ROCK substrates to ensure dynamic regulation of the cytoskeleton. We selected Coronin1A/B as a potential negative regulator of ROCK signaling from a proteomics screen for novel ROCK2-binding partners (21). Here, we identify and establish Coronin1B as a previously unrecognized attenuator of ROCK2 signaling. Furthermore, we find that Coronin1B inhibits ROCK signaling through the phosphatase SSH1L. Importantly, we find that the Coronin1B attenuation mechanism is required for NRG-1-induced cell scattering, demonstrating the importance of this mechanism in tumor cell motility.

Coronin1B and ROCK2 are both well established regulators of cytoskeleton (6, 24, 25), but their interaction as signaling partners is novel. Endogenous Coronin1B and ROCK2 form a complex (Fig. 1C), and our experiments with the recombinant proteins demonstrate that this is a direct interaction between Coronin1B and the ROCK2 PH domain (Fig. 2A). Further, our co-immunoprecipitation results show that the ROCK2 PH domain is required for the ROCK2-Coronin1B interaction in cells (Fig. 2B). Coronins are WD40 repeat proteins, and there are several other examples of WD40 domains interacting directly with PH domains (41–43). Thus, interactions between PH domains with WD40 repeat proteins may be a conserved protein-protein interaction interface (44). Interestingly, our data show that the positive charged residues in the C terminus of ROCK2 PH domain are important for the interaction of ROCK and WD40 repeat protein: Coronin1B (Fig. 2, A and C).

Activation of ROCK via RhoA binding is well established, and although many signaling pathways that stimulate ROCK signaling have been described, only a few mechanisms that down-regulate its signaling are known (6, 7, 55, 56). One of the known mechanisms by which ROCK signaling is inactivated is through RhoE (56, 57). Thus, the ratio between active RhoA and RhoE may balance the degree of ROCK substrate phosphorylation to fine-tune its signaling in cells (3). Here, we have discovered a new mechanism to down-regulate ROCK signaling through the novel signaling partner Coronin1B (Figs. 3–5). This is important because it provides a potential mechanism for signaling plasticity to allow dynamic response of the cytoskeleton in response to upstream signals.

It is known that Coronin1B regulates the balance between phosphorylation and dephosphorylation of the cytoskeletal regulator cofilin by recruiting the phosphatase SSH1L (23). Our studies demonstrated that SSH1L is able to reduce phosphorylation of MYPT-1 (Fig. 6), identifying SSH1L as a previously undescribed negative regulator of ROCK signaling. Phosphorylation inactivates MYPT-1, leading to increased phosphorylation of MLC (11, 14, 46–49, 58) which promotes myosin contractility (6). Similarly, phosphorylation inactivates cofilin, which directly regulates actin dynamics. These examples illustrate that the balance between kinases and phosphatases is a pivotal regulatory point for controlling the activity of key cytoskeletal elements.

Upstream signals could trigger the active interplay between kinases and phosphatases to remodel the cytoskeleton during cell motility. Physiologically, NRG-1 is known to promote motility and invasiveness of breast cancer cells (52) and that NRG-1 triggers cofilin dephosphorylation by activating SSH1L to cause cell motility events such as cell scattering (53, 54). Interestingly, our data show that NRG-1 stimulates dephosphorylation of p-MYPT-1 and p-MLC, which is accompanied by enhanced co-immunoprecipitation between ROCK2 and Coronin1B (Fig. 7, A–C). The binding between ROCK2 and Coronin1B is then required for NRG-1 to reduce p-MYPT and p-MLC (Fig. 7D), suggesting that NRG-1-induced motility involves attenuation of ROCK2 signaling through Coronin1B.

Supporting that model, we have discovered that NRG-1-induced cell scattering requires inhibition of ROCK signaling by Coronin1B (Fig. 7, E and F). Our results show that the increase in ROCK signaling through the myosin pathway (Fig. 7, G and H) by Coronin1B knockdown prevents NRG-1-induced cell scattering, suggesting that the balance between the phosphorylated and dephosphorylated MLC is critical for NRG-1 function in motility. In summary, the data presented in this paper sug-
gest that Coronin1B counterbalances ROCK signaling to modulate myosin activity and control cytoskeletal organization. We propose a model in which Coronin1B coordinately regulates cofilin activity and ROCK signaling through SSH1L to fine-tune the behavior of the cytoskeleton during NRG-1-induced cell motility.

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