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## **N-terminus-mediated dimerization of ROCK-I is required for RhoE binding and actin reorganization**

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Short title: ROCK-I dimerization is required for RhoE binding and actin reorganization

## Synopsis

ROCK-I is a serine/threonine kinase that can be activated by RhoA and inhibited by RhoE. ROCK-I has an N-terminal kinase domain, central coiled-coil region, and a RhoA-binding domain near the C-terminus. We have previously shown that RhoE binds to the N-terminal 420 amino acids of ROCK-I, which includes the kinase domain as well as N-terminal and C-terminal extensions. Here, we show that N-terminus-mediated dimerization of ROCK-I is required for RhoE binding. The central coiled-coil domain can also dimerize ROCK-I in cells, but this is insufficient in the absence of the N-terminus to allow RhoE binding. The kinase activity of ROCK-I 1-420 is required for dimerization and RhoE binding, however, inclusion of part of the coiled-coil domain compensates for lack of kinase activity, allowing RhoE to bind. N-terminus-mediated dimerization is also required for ROCK-I to induce the formation of stellate actin stress fibres in cells. These results indicate that dimerization via the N-terminus is critical for ROCK-I function in cells and for its regulation by RhoE.

Keywords: Rho GTPases, Rho-kinases, GTP-binding proteins, Rnd3, actin cytoskeleton

## Introduction

Rho family proteins regulate cytoskeletal organization and thus play important roles in a number of processes including cell migration, phagocytosis and vesicle trafficking [1]. RhoA stimulates actomyosin contractility and the assembly of actin stress fibres in a variety of cell types by binding to and activating ROCKs (also known as Rho-kinases) [2]. Mammals have two ROCK isoforms, ROCK-I and ROCK-II, which are serine/threonine kinases and have multiple domains. ROCKs are approximately 160 kDa in size, and both have a highly homologous kinase domain near the N-terminus, an extensive coiled-coil region containing a Rho-binding domain (RBD), and a C-terminal section containing a pleckstrin homology (PH) domain and cysteine-rich region [2]. The RBD binds as a parallel coiled-coil dimer to two RhoA molecules [3], and two additional segments of the coiled-coil region of ROCKs are proposed to contribute to RhoA binding [4]. ROCKs are members of the AGC family of protein kinases [5], and most closely related to the myotonic dystrophy kinase DMPK and the myotonic dystrophy kinase-related Cdc42-binding kinases MRCK $\alpha$  and MRCK $\beta$ , which share a similar domain organization [2]. ROCKs are regulated by the auto-inhibitory C-terminal region containing the RBD and PH domains, which binds to the N-terminal kinase domain and inhibits kinase activity [6]. Interaction of RhoA with the RBD of ROCK-I can disrupt the auto-inhibitory constraints between the kinase domain and the C-terminal region. Alternatively, ROCK-I can be activated by caspase 3 cleavage during apoptosis, which removes the C-terminus [7, 8].

Most Rho proteins function as molecular switches, cycling between an active GTP-bound and an inactive GDP-bound form. They are activated by guanine nucleotide exchange factors (GEFs) and inactivated by GTPase activating proteins (GAPs) [1]. RhoE (also known as Rnd3) was originally identified as a binding partner for p190RhoGAP in a yeast two-hybrid screen [9]. RhoE/Rnd3 is most closely related to Rnd1 and Rnd2 [10]. In contrast to other Rho family members, Rnd proteins lack intrinsic GTPase activity and are constitutively bound to GTP in cells [9]. RhoE acts antagonistically to RhoA, inducing disassembly of stress fibres and associated focal adhesions, causing cell rounding and stimulating cell migration [11]. RhoE opposes RhoA at least in part by binding to and inhibiting ROCK-I [2]. It also binds to p190RhoGAP and increases its GAP activity towards RhoA, thereby reducing the level of active RhoA in cells [12]. RhoE can itself be phosphorylated by ROCK-I, increasing RhoE stability and activity [13].

Although ROCK-I and ROCK-II are highly homologous in their kinase domains, their N-terminal sequences are very different. ROCK-I but not ROCK-II interacts with RhoE [13]. The minimum RhoE-binding domain of ROCK-I identified to date encompasses amino acids 1 to 420, which includes the unique N-terminal region, the kinase domain and amino acids C-terminal to the kinase domain, but does not contain the three RhoA-interacting domains in the coiled-coil region [4, 14]. Interestingly, ROCK-I and ROCK-II may have different functions in cells: in fibroblasts, ROCK-I but not ROCK-II is required for stress fibre assembly [15].

Homodimerization or oligomerization regulates the activity of many kinases. For example, native MRCK $\alpha$  and DMPK are multimeric proteins and this is critical for their transautophosphorylation and activation [16-18]. Both MRCKs and ROCKs can exist in an inactive state where the kinase domain interacts with a negative auto-regulatory domain located near the C-terminus [6, 19]. Recently, the crystal structures of the kinase domains of ROCK-I and ROCK-II were solved, and in both cases these regions crystallize as dimers [20, 21]. For ROCK-I, amino acids 6-415 dimerize primarily through the helical region N-terminal to the kinase domain, although parts of the kinase domain also contribute to dimer formation [20]. The ROCK-II structure includes additional amino acids C-terminal to the kinase domain, which contribute to dimerization by interacting with the N-terminal region [21].

Here we show that endogenous ROCK-I exists as a dimer in cells, and that two distinct regions of ROCK-I can mediate its dimerization, the N-terminal region and the kinase-proximal part of the central coiled-coil region. We demonstrate that dimerization via the N-terminus of ROCK-I is critical both for RhoE binding and for ROCK-I to induce actin reorganization in cells.

## Experimental

### Antibodies

Mouse monoclonal anti-myc (9E10) and rabbit polyclonal anti-myc antibodies, rabbit polyclonal anti-GST antibody and mouse anti-ROCK-I (G6) antibody were purchased from Santa Cruz Biotechnology.

### Expression vectors

Expression vectors encoding myc epitope-tagged full-length ROCK-I (pCAG-myc-p160wt), full-length kinase-dead ROCK-I with a mutation in the RhoA-binding domain (pCAG-myc-KDIA) and three C-terminally truncated mutants (pCAG-myc 1-1080 ( $\Delta 1$ ), 1-727 ( $\Delta 3$ ), and 1-375 ( $\Delta 5$ )) were kindly provided by Shuh Narumiya (Kyoto University, Kyoto, Japan). The coding residues for ROCK-I 375-727, 1-76, 1-420, 14-420, 35-420, 76-420, 35-727, 76-727, 1-420KD and 1-727KD were amplified from pCAG-myc-160wt or pCAG-myc-KDIA by PCR and cloned with an N-terminal myc epitope into the KpnI and XhoI sites of pCAG. The mammalian expression vector pEF-Bos-GST was kindly provided by Dr Giles Cory (Ludwig Institute for Cancer Research, London, UK). ROCK-I fragments were amplified from pCAG-myc-ROCK-I vectors by PCR and ligated into the SmaI sites of pEF-Bos-GST. All constructs were checked by DNA sequencing (MWG Biotech). pCMV5-FLAG-V14RhoA was described previously [14].

### Cell culture, transfection and microinjection

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and penicillin-streptomycin (Invitrogen). For transfection, cells were washed with 5 ml of cold electroporation buffer (120 mM KCl, 10 mM  $K_2PO_4$ . $KH_2PO_4$  [pH 7.6], 25 mM HEPES [pH 7.6], 2 mM  $MgCl_2$ , 0.5% Ficoll). The buffer was removed and cells were resuspended in 250  $\mu$ l of cold electroporation buffer and electroporated at 250 V and 960  $\mu$ F with 5  $\mu$ g of DNA. The cells were then plated on 10 cm diameter dishes and incubated for 24 h prior to lysis. Swiss 3T3 cells were grown in DMEM containing 10% FCS. For microinjection, cells were seeded on coverslips at  $3 \times 10^4$  cells/ml. Cells were microinjected with 100 ng/ $\mu$ l of DNA. After microinjection, cells were incubated for 4 h to allow expression of the protein.

### Native gel electrophoresis

Precast 3-8% Tris-acetate gels (Invitrogen) or NativePAGE 3-12% Bis-Tris gels (Blue Native gels; Invitrogen) were used to detect ROCK-I dimers in cell extracts [22-24]. Cell lysates were loaded onto Tris-acetate or Blue Native gels and electrophoresis was performed according to the manufacturers' instructions. Protein samples for Tris-acetate gels were loaded in Novex native Tris-glycine sample buffer (Invitrogen), and for Blue Native gels were treated with NativePAGE 5% G-250 sample additive (Invitrogen) and NativePAGE sample buffer and loaded prior to filling the upper chamber with Dark Blue Cathode Buffer for ease of visualisation. Electrophoresis was performed at 4°C initially at 150 V, and increased to 250 V after 60 min. After electrophoresis the gel was detached from its plastic cassette and inverted

into a staining chamber containing Colloidal Blue (Invitrogen) or equilibrated in transfer buffer containing 0.05% SDS for 30 min prior to transferring to PVDF membrane (Millipore).

### DSS cross-linking

COS-7 cells with or without transfection of various ROCK-I constructs were extracted in conjugation buffer (20 mM HEPES [pH 7.2], 1% Triton X-100). Extracts were clarified at 14,000 g and protein concentrations of the soluble fraction were adjusted to 1 to 3 mg/ml. Cross-linking reactions were initiated by the addition of 0.05 to 0.5 mM disuccinimidyl suberate (DSS) (Pierce) at room temperature for 30 min. Reactions were stopped by adding 1 M Tris-HCl [pH 7.5] and incubation for 15 min at room temperature. An equal volume of Laemmli sample buffer was added to each of the cross-linked samples. Cross-linked products were detected by immunoblotting with either anti-ROCK-I antibody or anti-myc antibody for the detection of endogenous ROCK-I and exogenously expressed myc-tagged ROCK-I proteins respectively.

### GST pull-downs with recombinant Rho proteins

Recombinant V14RhoA and RhoE were expressed as glutathione-S-transferase (GST) fusion proteins in *E. coli* and purified as previously described [14]. Recombinant GST was used as a control. Transfected COS-7 cells were washed and lysed in lysis buffer (1% Triton-X100, 20 mM Tris-HCl [pH 8], 130 mM NaCl, 10 mM NaF, 1% aprotinin, 10 µg/ml leupeptin, 1 mM dithiothreitol, 0.1 mM Na<sub>3</sub>VO<sub>4</sub> and 1 mM phenylmethylsulfonylfluoride). Insoluble material was removed by centrifugation and the cell lysates were incubated for 2 h at 4°C with the recombinant GST-fusion proteins on glutathione-sepharose beads. Beads were washed extensively with lysis buffer before the proteins were eluted in Laemmli sample buffer.

### GST pull-downs with GST-ROCK-I expressed in COS-7 cells

COS-7 cells were co-transfected with GST-myc-ROCK-I and myc-ROCK-I constructs as indicated in figure legends. Clarified cell lysates were incubated with 20 to 50 µl of glutathione-sepharose beads to pull down the GST-myc-ROCK-I protein and incubated with rotation for 2 h at 4°C. The beads were washed extensively, proteins eluted in Laemmli sample buffer, resolved by SDS-PAGE and immunoblotted with anti-myc antibody to determine if GST-myc-ROCK-I associated with myc-ROCK-I.

### *In vitro* kinase assays

COS-7 cells transfected with myc-ROCK-I constructs were lysed, and myc-tagged ROCK-I proteins immunoprecipitated with anti-myc antibody (9E10) covalently linked to agarose (Santa Cruz) for 2 h at 4°C. The beads were washed extensively and then subjected to a high salt wash to remove non-specifically bound proteins. Kinase assays were carried out in kinase buffer (50 mM Tris-HCl [pH 7.5], 10 mM MgCl<sub>2</sub>, 1mM DTT, 30 µM ATP, 0.1 uCi/µl γ[<sup>32</sup>P]-ATP) at 30°C for 30 min. For transphosphorylation studies, two different ROCK-I proteins bound to myc antibody-agarose beads were combined and washed extensively before *in vitro* kinase assays were performed. Proteins were eluted from beads in Laemmli sample buffer and resolved by SDS-PAGE. Gels were fixed in 10% acetic acid for 30 min, then dried and autoradiographed.

### Immunoblotting

For immunoblotting, proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher and Schuell). For immunoblotting of Blue Native gels, the Dark Blue Cathode Buffer was replaced with Light Blue Cathode Buffer during electrophoresis. For Tris-acetate and Blue Native gels, the resolved proteins were then transferred to PVDF (Millipore)

after equilibration in transfer buffer containing 0.05% SDS. Membranes were blocked with TBS (20 mM Tris-HCl [pH 7.6], 137 mM NaCl) containing 5% non-fat dried milk and 0.05% Tween 20. Mouse anti-myc antibody was used for detection of myc-tagged proteins, mouse anti-ROCK-I (G6) antibody for full-length ROCK-I and mouse anti-GST antibody for GST-fusion proteins. The bound antibodies were visualised with horseradish peroxidase-conjugated goat anti-immunoglobulin G antibodies and enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

### Immunofluorescence microscopy

Swiss 3T3 cells on coverslips were fixed with 3.7% formaldehyde solution in PBS for 15 min, permeabilised with 0.2% Triton X-100 in PBS for 5 min and incubated for 1 h with anti-myc antibody to detect myc-tagged ROCK-I proteins followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch). Actin filaments were localised by incubating cells with 0.1 µg/ml of tetramethyl rhodamine isothiocyanate (TRITC)-labelled phalloidin (Sigma-Aldrich). Coverslips were mounted in Mowiol (Calbiochem) containing the anti-quench *p*-phenylenediamine (Sigma-Aldrich) and images were generated with a Zeiss LSM 510 confocal microscope using a x40/1.3 NA objective.

## Results

### ROCK-I exists as a homodimer in cells

*In vitro*, various fragments of ROCK-I can homodimerize [20, 25], but whether ROCK-I exists as a dimer in cells has not been tested. To investigate this, COS-7 cell lysates were treated with the lysine-specific chemical cross-linker disuccinimidyl suberate (DSS). Immunoblotting with anti-ROCK-I antibody detected endogenous monomeric ROCK-I, which migrates with an apparent molecular weight of 160 kDa, as well as a slower migrating form at approximately 320 kDa, consistent with the presence of a ROCK-I dimer (Figure 1A). Interestingly, the antibody to ROCK-I also detected an additional protein at 130 kDa, which is likely to be C-terminally truncated or cleaved ROCK-I as reported by others [7, 8]. This cleaved form of ROCK-I could also form dimers, as an additional band was detected by the anti-ROCK-I antibody at approximately 260 kDa in lysates treated with DSS (Figure 1A, arrows). Analysis on native 3–8% Tris-acetate gels (Figure 1B) or 3–12% Blue Native gels (data not shown) indicated that ROCK-I exists primarily as a dimer in COS-7 cells, migrating between 242 and 480 kDa (Figure 1B). No tetramer was detected, and as expected DSS treatment did not affect the size of the dimer. Using DSS cross-linking, exogenously expressed full-length ROCK-I and constitutively active C-terminally truncated ROCK-I 1-1080 (ROCK-I Δ1) [26] were also detected as dimers in COS-7 cells (Figure 1C).

### Two distinct regions of ROCK-I can mediate dimerization

ROCK-I is a multi-domain protein with a number of regions that could contribute to its ability to form dimers (Figure 2A). In the crystal structure of ROCK-I 6-415, the N-terminal residues 6-72 form a dimerization domain [20]. The central residues 423–1096 are predicted to dimerize as a coiled coil [27], and the RBD domain crystallizes as a parallel coiled-coil dimer [3, 28]. In COS-7 cells, ROCK-I 1-727 (ROCK-I Δ3 [26]) and ROCK-I 1-420 formed dimers, while a region including part of the coiled-coil domain, ROCK-I 375-727, formed dimers and higher molecular-weight oligomers (Figure 2B), similar to the coiled-coil region of MRCKα [16]. In contrast, ROCK-I 1-375 was unable to form dimers (Figure 2B), indicating that amino acids

375-420, C-terminal to the kinase domain, are necessary for dimerization in cells. This is consistent with the observation that ROCK-I 6-379 is primarily monomeric *in vitro* [25]. Interestingly, two bands were observed at the size of the dimer of ROCK-I 375-727. The upper band is probably due to cross-linking of a small protein or protein fragment to the dimer. So far, no proteins that bind to this region of ROCK-I have been identified, and this approach may therefore allow the identification of potential novel interacting partners.

Further evidence that ROCK-I dimerizes in cells was provided by co-expressing GST-myc-ROCK-I 1-420 with myc-ROCK-I 1-420. Association of the two proteins was detected by pulling down GST-myc-ROCK-I with glutathione-sepharose beads, followed by western blotting (Figure 2C).

To determine the contribution of the N-terminal 76 amino acids to ROCK-I dimerization, deletion mutants were constructed that progressively removed amino acids from the N-terminus (Figure 2A). Deletion of only 14 amino acids from the N-terminus of ROCK-I 1-420 prevented dimerization (Figure 2D), whereas dimerization and oligomerization of ROCK-I 1-727 was not affected by removal of the N-terminal 76 amino acids (Figure 2E). This indicates that dimerization of the central coiled-coil region is sufficiently strong to overcome the need for the N-terminal dimerization domain.

### **N-terminus-mediated dimerization of ROCK-I is required for RhoE binding**

We have previously shown that ROCK-I 1-420 is sufficient to bind RhoE, but that deletion of residues 1-76 abrogates RhoE binding [14]. To determine whether dimerization is required for RhoE binding, cell lysates from COS-7 cells transfected with various different ROCK-I constructs were incubated with GST-RhoE immobilised on beads (or GST as a control). All of the ROCK-I deletion mutants containing an intact N-terminus bound to RhoE, except for ROCK-I 1-375, which was unable to form dimers (Table 1, Figure 2B). ROCK-I 14-420, which does not dimerize (Figure 2D), was unable to bind to RhoE. Dimerization mediated by the coiled-coil region was insufficient to allow RhoE binding in the absence of the N-terminus, since ROCK-I 35-727 and ROCK-I 76-727 were unable to bind to RhoE (Table 1). These results show that the N-terminal 14 amino acids are required for ROCK-I to bind RhoE, indicating that dimerization of ROCK-I via its N-terminus is required for RhoE binding.

### **Kinase-dead ROCK-I 1-420 cannot form dimers or bind to RhoE**

We have shown previously that the kinase activity of ROCK-I 1-727 is not necessary for RhoE binding, since kinase-dead ROCK-I 1-727 (ROCK-I 1-727KD) binds to RhoE [14]. Interestingly, although ROCK-I 1-727KD could form dimers, ROCK-I 1-420KD did not dimerize detectably under identical conditions (Figure 3A, B). ROCK-I 1-420KD could, however, dimerize weakly with kinase-active ROCK-I 1-420, since GST-myc-ROCK-I 1-420 was able to associate with ROCK-I 1-420KD (Figure 3B, left panel), and GST-myc-ROCK-I 1-420KD bound to ROCK 1-420 but not ROCK 1-420KD (Figure 3B, right panel).

ROCK-I 1-420KD was unable to bind to GST-RhoE in pull-down assays (Figure 3C), consistent with the requirement for N-terminus-mediated ROCK-I dimerization in order to bind RhoE. These results suggest that, in the absence of the central coiled-coil region, auto- and/or trans-phosphorylation is required for N-terminus-mediated dimerization of ROCK-I 1-420, whereas the coiled-coil region mediates dimerization independently of phosphorylation, and this then allows the N-terminal domain to dimerize and interact with RhoE.

### **ROCK-I autophosphorylation and RhoE phosphorylation require ROCK-I dimerization**

The ROCK-I-related protein MRCK $\alpha$  can undergo dimerization via trans-phosphorylation [16]. To assess if kinase activity of ROCK-I is linked to dimerization, the ability of various ROCK-I deletion mutants to autophosphorylate or phosphorylate RhoE was tested. Myc-tagged ROCK-I



proteins were immunoprecipitated with anti-myc antibodies and subjected to *in vitro* kinase assays. ROCK-I 1-420 could autophosphorylate but only very low levels of ROCK-I 1-375 phosphorylation were observed (Figure 4A). Deletion of the N-terminal dimerization domain reduced ROCK-I phosphorylation and phosphorylation of RhoE to background levels (Figure 4B). Deletion of the N-terminal 14 amino acids was sufficient to reduce phosphorylation significantly, coinciding with loss of N-terminal dimerization and RhoE binding.

To determine whether the two molecules in ROCK-I dimers were able to transphosphorylate, we co-expressed ROCK-I 1-420 with ROCK-I 1-727 (Figure 4C). Kinase-dead versions of each protein did not become phosphorylated in *in vitro* kinase assays when expressed alone in COS-7 cells. However, ROCK-I 1-727 could phosphorylate ROCK-I 1-420KD, and ROCK-I 1-420 phosphorylated ROCK-I 1-727KD (Figure 4C). Together with the results in Figure 3, these results indicate that transphosphorylation occurs between ROCK-I dimers, and that transphosphorylation is required for dimerization of ROCK-I 1-420 but not ROCK-I 1-727.

### ROCK-I dimerization is required for its effects on the actin cytoskeleton

Previous studies have shown that deletion of the C-terminus of ROCK-I results in constitutive activation, and that this active mutant, ROCK-I  $\Delta$ 1, induces stress fibre assembly in fibroblasts [26]. ROCK-I  $\Delta$ 1 induces formation of stellate stress fibres, whereas ROCK-I acts together with a second RhoA target, mDia, to induce parallel stress fibres similar to the response to constitutively active RhoA [29]. In order to explore which regions of ROCK-I are required for actin reorganization, Swiss 3T3 cells were microinjected with different C-terminally or N-terminally deleted ROCK-I constructs (Figure 5, Table 1). Expression of full-length ROCK-I induced only a small increase in stress fibres, consistent with it being less catalytically active than proteins that lack an intact C-terminus (Figure 5). ROCK-I C-terminal deletions each induced stellate stress fibres, except for ROCK-I 1-375 (ROCK-I  $\Delta$ 5), which had no effect on the actin cytoskeleton, as previously reported [26]. The ROCK-I-induced stress fibres were clearly organized differently from the largely parallel stress fibres induced by activated V14RhoA. Catalytic activity of ROCK-I was required for stress fibre assembly, as described previously [26], and ROCK-I 375-727 did not alter the actin cytoskeleton, so did not act as a dominant-negative inhibitor of ROCK-I. Deletion of the N-terminus of ROCK-I 1-420 or ROCK-I 1-727 prevented stellate stress fibre formation (Figure 5, Table 1), although ROCK-I 14-420 retained partial activity, consistent with its low residual kinase activity *in vitro* (Figure 4). Since N-terminal deletions of ROCK-I 1-727 can still dimerize via the coiled coil domain (Figure 2C), these results indicate that N-terminal dimerization of ROCK-I is specifically required for its ability to induce stellate stress fibres. Interestingly, expression of N-terminally deleted ROCK-I 35-420 and 76-420 induced lamellipodia and in some cases reduced stress fibre levels (Figure 5, arrows indicate lamellipodia). These proteins could therefore act in a dominant negative manner, inhibiting the activity of endogenous ROCK-I.

### Discussion

The ROCK-I N-terminal region is known to dimerize via its N-terminal kinase extension *in vitro*, but the ability of ROCK-I to dimerize in cells and the functional consequences of this dimerization have not been addressed. Here we report that full-length ROCK-I exists as dimers in cells and that dimerization mediated by the N-terminal extension of ROCK-I is required both for RhoE binding and for its ability to induce actin reorganization in cells.

ROCK-I is a multi-domain protein similar to the related serine/threonine kinases ROCK-II, MRCK $\alpha$  and  $\beta$ , DMPK and citron-kinase. These proteins can all form dimers and/or multimers and possess N-terminal kinase domains and central coiled-coil regions [6]. Our results indicate that two separate regions of ROCK-I are independently able to mediate

dimerization: the N-terminal kinase extension (1-76), and the C-terminal kinase extension together with part of the central coiled-coil domain (375-727). Dimerization of the N-terminal extension is shown in the crystal structures of ROCK-I and ROCK-II [20, 21]. The central coiled-coil region of ROCK-I is predicted to be approximately 600 amino acids (423–1096). Based on the structure of the N-terminal domain and the parallel coiled coil structure of the RhoA-binding domain, it is presumed that the coiled-coil region of ROCKs will form a parallel dimer [21, 28]. Interestingly, ROCK-I 1-420 can dimerize with ROCK-I 375-727, indicating that amino acids 375-420, C-terminal to the kinase domain, are sufficient for dimerization. In the ROCK-I crystal structure, the C-terminal kinase extensions (357-405) interact with the N-terminal dimerization domain, but there is no evidence for dimeric interactions between the two C-terminal extensions [20]. However, residues 405-420 are not present in the structure and could therefore be critical for C-terminal dimerization. This region of ROCK-I is not very homologous to ROCK-II [2, 30], and thus it will be interesting to know whether the equivalent region in ROCK-II can dimerize.

The role of the coiled-coil domain in dimerization appears to vary between different family members [6]. For example, the coiled-coil domain of MRCK $\alpha$  mediates multimerization of the full-length protein in cells [16], whereas we did not detect multimers of full-length ROCK-I. Elimination of the coiled-coil region of full-length DMPK was reported to prevent dimerization [17], whereas ROCK-I and ROCK-II are able to dimerize via their N- and C-terminal kinase extensions in the absence of the coiled-coil region. However, recently the N-terminal region of DMPK was also found to dimerize without the coiled-coil region, whereas the coiled-coil domain by itself forms a trimer [18]. Whether trimerization is physiologically relevant is not known, but since the coiled-coil region of ROCK-I forms oligomers, it would be interesting to determine whether it also forms trimers.

The catalytic activity and dimerization of ROCK-I 1-420 are closely linked: catalytic activity is required for it to form dimers and presumably involves trans-autophosphorylation, since kinase-active ROCK-I 1-420 and kinase-dead ROCK-I 1-420 can dimerize. Conversely, progressive shortening of the N-terminal and C-terminal kinase extensions reduces catalytic activity and dimerization (our data; and [25]). Similarly, the N-terminus of MRCK $\alpha$  mediates kinase domain dimerization and trans-autophosphorylation, and hence its activation [16], and trans-autophosphorylation of the N-terminal kinase domain of ROCK-II appears to be essential for its catalytic activity [19]. In contrast, kinase-dead ROCK-I 1-727 can dimerize, and thus trans-autophosphorylation is probably not essential for dimerization of full-length ROCK-I, although it could modulate the efficiency of dimer formation. At present the phosphorylation sites involved in ROCK-I 1-420 dimerization are not known: no standard active site residues are phosphorylated in the crystal structure of ROCK-I [20].

We have previously shown that RhoE binds to ROCK-I 1-420, and that N-terminal deletions inhibit binding [14]. Here we demonstrate that this correlates with N-terminus-mediated dimerization. RhoE binding to ROCK-I does not appear to require ROCK-I catalytic activity since ROCK-I 1-727KD binds to RhoE, however, the shorter ROCK-I 1-420KD is unable to dimerize or interact with RhoE, suggesting that auto/transphosphorylation is required for its dimerization and hence RhoE binding, yet addition of the coiled-coil domain strengthens dimerization sufficiently to bypass the need for phosphorylation. The binding site for RhoE could include residues from two N-terminal regions of ROCK-I, and thus two ROCK-I molecules would be required to bind one RhoE molecule. Alternatively, dimerization could stabilize the conformation of the ROCK-I binding site for RhoE, and in this case each ROCK-I molecule could bind to a RhoE molecule.

Our results show that N-terminus-mediated dimerization of ROCK-I 1-420 is required for its catalytic activity and hence its ability to induce stress fibre reorganization in cells. Previous studies have shown that C-terminally truncated ROCK-I is more active than full-

length ROCK-I in promoting the formation of thick stellate stress fibres, correlating with its catalytic activity [26], whereas we find that ROCK-I lacking only a few N-terminal amino acids is completely inactive. In contrast, ROCK-I dimerized only via the C-terminal kinase extension and coiled-coil region did not induce stress fibres. It is possible that N-terminus-mediated dimerization of ROCK-I regulates binding not only to RhoE but also to its downstream targets involved in stress fibre formation, for example myosin light chain phosphatase [2]. Interestingly, N-terminally deleted ROCK-I proteins induced lamellipodia at the cell edge, similar to the effect of small-molecule ROCK inhibitors such as Y-27632 [31], and consistent with a model where Rho/ROCK signalling inhibits Rac and hence lamellipodium extension [32]. This suggests that ROCK-I constructs lacking the N-terminus act as dominant inhibitors, possibly by dimerizing with endogenous ROCK-I via the coiled-coil domain and thereby preventing N-terminal dimerization and activity.

In conclusion, ROCK-I N-terminus-mediated dimerization but not coiled-coil-mediated dimerization is required for RhoE binding, as well as for ROCK-I catalytic activity and stellate stress fibre formation in cells. The relative levels of RhoE versus other targets binding to ROCK-I will determine whether ROCK-I induces actin stress fibres in cells.

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ROCK fragments	RhoE binding	stellate stress fibres
1-1364 (full-length)	+	+/-
1-1080 ( $\Delta 1$ )	+	+
1-727 ( $\Delta 3$ )	+	+
1-727 KD	+	-
1-578	+	+
1-540	+	+
1-455	+	+
1-420	+	+
1-420 KD	-	-
1-375 ( $\Delta 5$ )	-	-
1-76	-	-
14-420	-	+/-
35-420	-	-
76-420	-	-
375-727	-	-
35-727	-	-
76-727	-	-

**Table 1. N-terminal dimerization of ROCK-I is required for RhoE binding and stellate stress fibre formation**

To measure RhoE binding, COS-7 cells were transfected with ROCK-I constructs. Cells were lysed after 24 h and lysates were incubated with GST or GST-RhoE beads and immunoblotted with anti-myc antibody to detect myc-tagged ROCK-I proteins. To assess stress fibre formation, Swiss 3T3 fibroblasts were microinjected with ROCK-I constructs. After 4 h, cells were fixed and stained for actin filaments and myc-tagged ROCK-I proteins.

## Figure legends

### Figure 1. ROCK-I forms dimers in cells

COS-7 cell lysates from untransfected cells (A,B) or cells expressing full-length myc-ROCK-I or constitutively active myc-ROCK-I ( $\Delta 1$ ) (C) were exposed to the indicated concentrations of the cross-linker DSS. Lysates were resolved by SDS-PAGE (A,C) or on a 3-8% Tris-acetate native gel (B). Endogenous ROCK-I and exogenously expressed myc-ROCK-I were identified by western blotting with anti-ROCK I antibody (A,B) or anti-myc (9E10) antibody (C). Fl, full-length.

### Figure 2. Two regions of ROCK-I mediate dimerization

A. Schematic diagram of ROCK-I constructs used in this study. ROCK-I contains a unique N-terminal domain, a serine-threonine kinase domain, an extensive coiled-coil region, which includes the RhoA-binding domain, and a C-terminal pleckstrin homology domain and cysteine-rich motif. All constructs have a myc tag at the N-terminus. B-E. COS-7 cells were transfected with constructs encoding the indicated regions of ROCK-I. Cell lysates were treated for 30 min with DSS at the indicated concentrations, and myc-tagged ROCK-I proteins were detected by immunoblotting with anti-myc antibody (B,D,F). Alternatively, lysates were incubated with glutathione-sepharose beads to pull down GST-myc-ROCK-I 1-420 and immunoblotted with anti-myc antibody to detect GST-myc-ROCK-I 1-420 and myc-ROCK-I (1-420) (C). Doublets (D) are most likely due to proteolysis of ROCK-I fragments in the protease-free conjugation buffer used for DSS cross-linking (see Experimental).

### Figure 3. Role of ROCK-I kinase activity in RhoE binding and dimerization

COS-7 cells were transfected with the indicated ROCK-I constructs. A. Lysates were treated with the indicated concentrations of DSS, and proteins were detected with anti-myc antibody. B. Lysates were incubated with glutathione-sepharose beads to pull down GST-myc-ROCK-I (1-420) and immunoblotted with anti-myc antibody to detect GST-myc-ROCK-I 1-420 and myc-ROCK-I 1-420 proteins. C. Lysates were incubated with GST or GST-RhoE on glutathione beads and immunoblotted with anti-myc antibody to detect myc-tagged ROCK-I proteins. KD, kinase-dead.

### Figure 4. ROCK-I auto-phosphorylation and RhoE phosphorylation requires dimerization

COS-7 cells were transfected with the indicated ROCK-I constructs. A. Cell lysates were immunoprecipitated with anti-myc antibodies coupled to agarose beads and subjected to an *in vitro* kinase assay. B. Lysates were immunoprecipitated using anti-myc antibody, and these ROCK-I immunoprecipitates were combined with lysates from COS-7 cells transfected with FLAG-RhoE and immunoprecipitated on anti-FLAG agarose beads. An *in vitro* kinase assay was performed. C. Transphosphorylation of kinase-dead ROCK-I by kinase-active ROCK-I. COS-7 cells were transfected with the indicated ROCK-I constructs. Cell lysates were immunoprecipitated using anti-myc antibody, then the immunoprecipitates were subjected to an *in vitro* kinase assay. KD, kinase-dead.

### Figure 5. Effects of N and C-terminally deleted ROCK-I proteins on the actin cytoskeleton

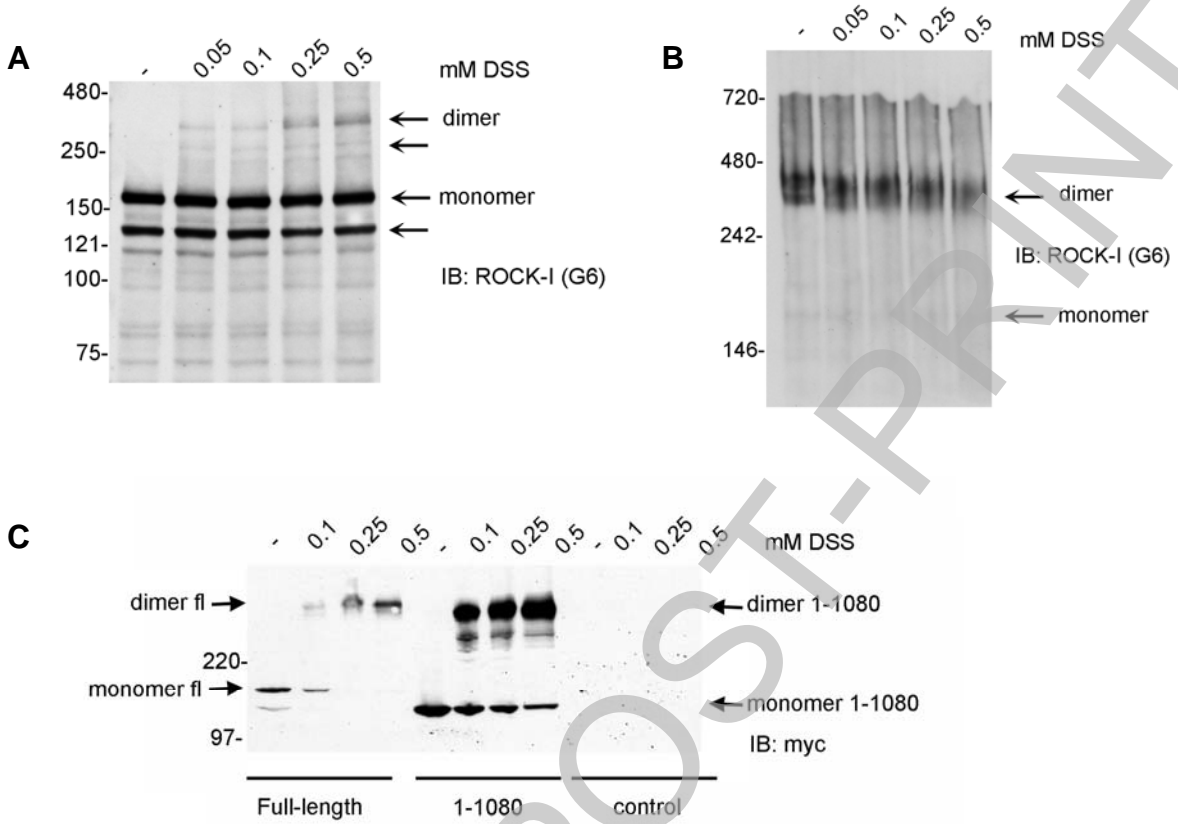
Swiss 3T3 fibroblasts were microinjected with the indicated myc-tagged ROCK-I constructs or with constitutively active V14RhoA. After 4 h, cells were fixed and stained with anti-myc antibody to identify cells expressing ROCK-I proteins and TRITC-labelled phalloidin to show actin filaments. Arrowheads indicate examples of ROCK-I-expressing cells. Arrows (F-actin

panels) indicate lamellipodia in cells expressing ROCK-I 35-420 and ROCK-I 76-420. Bar = 30  $\mu$ m.

Stage 2(a) POST-PRINT

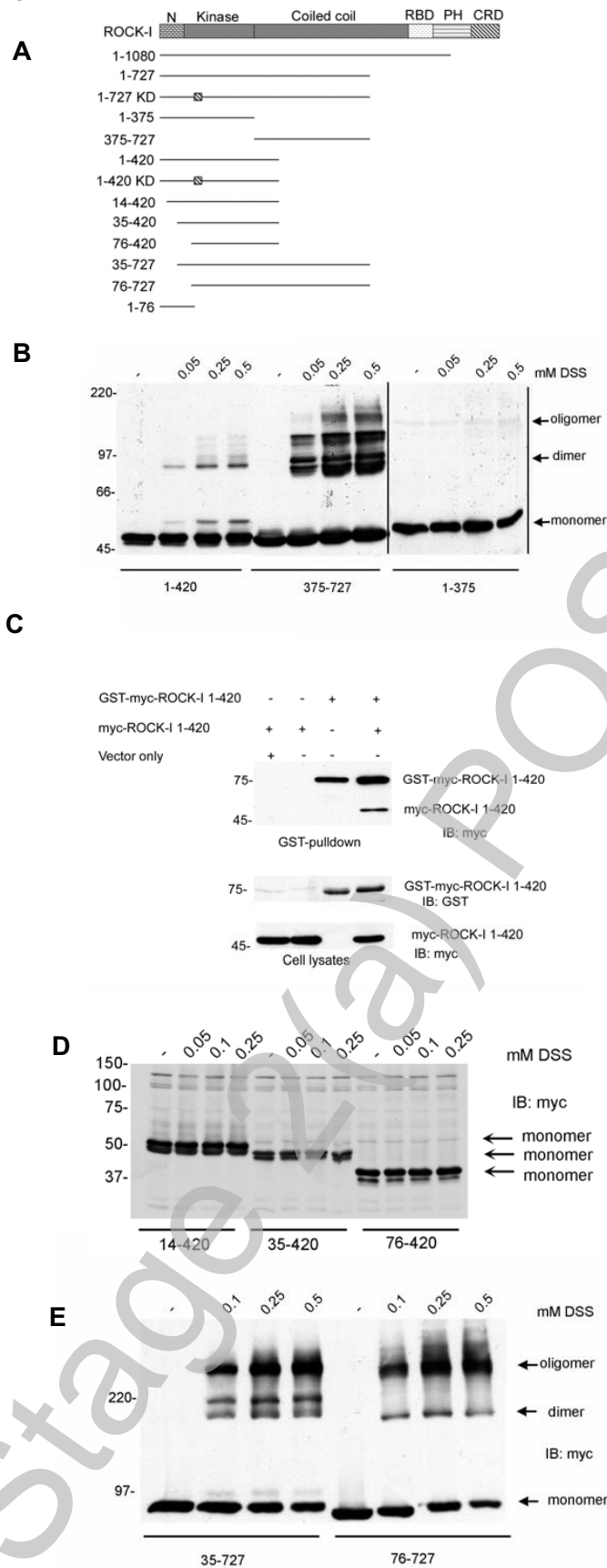


**Figure 1**



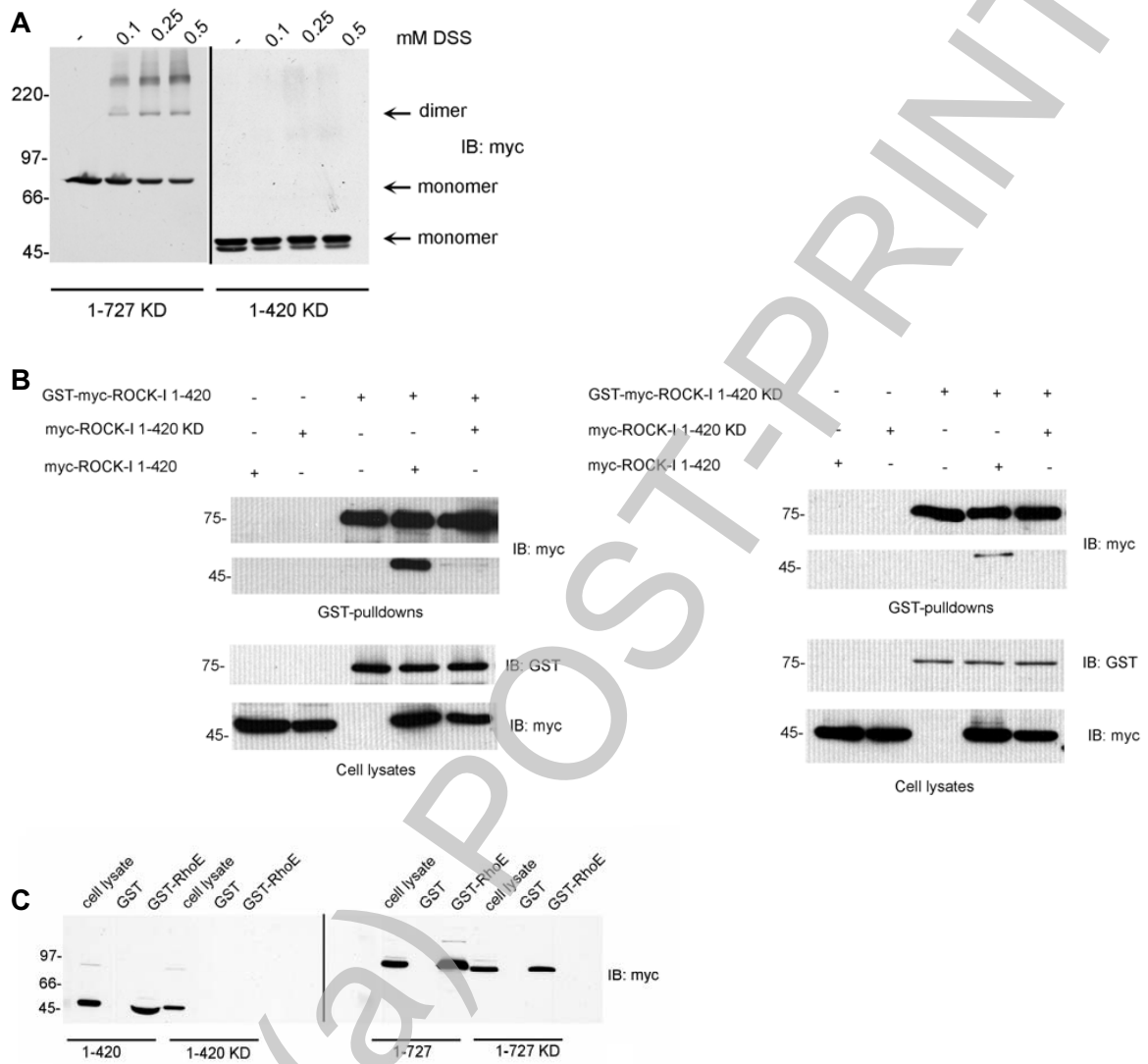
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Figure 2



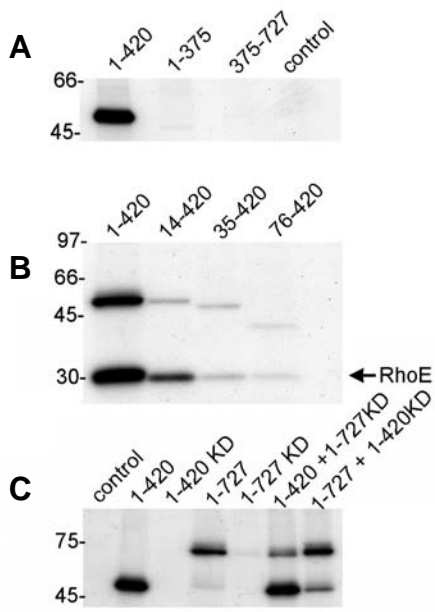
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Figure 3

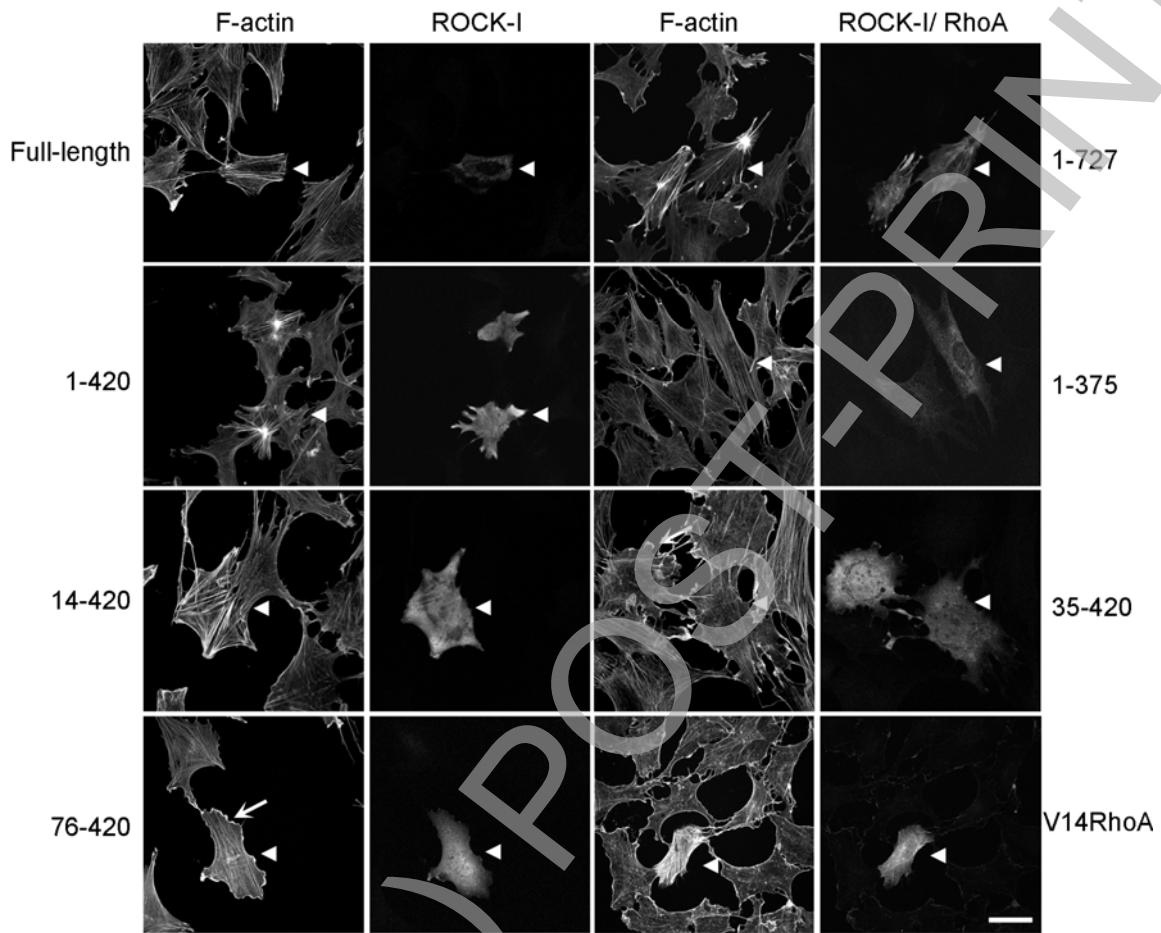


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**Figure 4**



**Figure 5**



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