Supplement Material

Materials and Methods:

Reagents: Antibodies used were as follows; goat anti-ROCK1, goat anti-ROCK2, rabbit anti-myosin light chain and anti-6xHis from Santa Cruz Biotechnology (Santa Cruz, CA), mouse anti-ROCK1, mouse anti-ROCK2, mouse anti-MYPT1 (MBS), anti-phosphothreonine 18, serine19 myosin light chain and anti-p116RIP from BD Biosciences (Franklin Lakes, NJ), MYPT1 anti-phosphothreonine 696 and 850 from Millipore (Billerica, MA), anti-GAPDH was from Sigma-Aldrich (St. Louis, MO). Mouse anti-vinculin was from Abcam (Cambridge, MA). 1-Oleoyl-*sn*-glycerol 3-phosphate (lysophosphatidic acid) was purchased from Sigma-Aldrich. Y27632 and blebbistatin were purchased from Calbiochem Biochemicals. 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI), Alexa Fluor 488 phalloidin, donkey anti-mouse Cy3 and Slowfade reagent were from Molecular Probes.

Co-immunoprecipitation Assays: A7r5 or primary rat aortic smooth muscle cells were maintained in DME with 10% serum, or were serum-deprived for 24 hours and treated with 10% serum or 1μM lysophosphatadic acid for 5 minutes. The cells were rinsed with cold PBS, then lysed in buffer A (50mM Tris pH 7.5, 150mM NaCl, 5% glycerol, 1% Triton X, 10mM $MgCl_2$, 1mM EGTA, 1mM dithiothreitol, 25mM NaF, 20mM β-glycerophosphate, 1mM Na_3VO_4, 0.01μg/ml each of aprotinin, leupeptin and pepstatin A, 2mM phenylmethylsulfonyl fluoride). Immunoprecipitations were performed as described[1]. The cell lysates were microcentrifuged at 4^{o}C for 5 minutes, then incubated with protein G beads for 15 minutes at 4^{o}C and centrifuged. The supernatants were then incubated with pre-coupled ROCK1 or ROCK2 antibodies bound to protein G

beads for 30 minutes at 4°C, rocking. The beads were centrifuged and washed three times in buffer A, then boiled in protein SDS sample buffer. Samples were resolved on 7.5% SDS PAGE gels and bound proteins determined by immunoblotting with the indicated antibodies.

GST-fusion protein Interaction Assays: The indicated sequences of human MBS (Full-length MYPT1, kind gift of Dr. Masumi Eto) were amplified by PCR and ligated into pGEX vectors for expression as GST-fusion proteins in bacteria. The PCR primers for each MBS domain are as follows (5' to 3'): 1-850-5' (amino acids 1-283): TAGGATCCAAGATGGCGGACGCGAAGCAGAAG, 1-850-3': GCGAATTCGCAACTCTTCTAAATATCCTAAAA, 800-1650-5' (amino acids 266-550): TAGGATCCCAAACAGCCTTTGATGTAGCAGAT, 800-1650-3': GCGAATTCATGATACGTTGATCCTTCATTAAC, 1620-2575-5' (amino acids 540-858): TAGGATCCAATAGCTCAGTTAATGAAGGATCA, 1620-2575-3': GCGAATTCTCCAAAATGAAACTCCTGTAGATC, 2050-2600-5' (amino acids 683-866): TAGGATCCAAAGCAAGATCTAGACAAGCAAGA, 2050-2600-3': GCGAATTCTGTTCATTTTCATCACTATCTTGT. MBS 2550-3090 (amino acids 850-1030) was from [2]. All PCR products were sequenced in both directions. GST-fusion proteins were expressed in bacteria and purified on glutathione-agarose beads as described [3]. Untransfected A7r5 cells and HEK293 cells transfected with mycROCK1 or mycROCK2 (plasmids kindly provided by Prof. Shuh Narumiya) were lysed in buffer A as above, microfuged and the supernatant incubated with GST-fusion proteins for 1 hour at 4°C, rocking. The beads were then washed three times with buffer A, then

boiled in SDS sample buffer. The bound proteins were resolved by 7.5% SDS-PAGE and immunoblotted with the indicated antibodies.

Direct Protein Domain Interaction Assays: The indicated sequences of human ROCK1 and 2 were amplified from the full length cDNA by PCR and ligated into pQE vectors for expression of 6xHis-tagged proteins in bacteria. The PCR primers for the ROCK domains are listed as follows (5' to 3'): ROCK1 1-1020 5' (amino acids 1-340): CGGATCCTCGACTGGGGACAGTTTTGAGACTCGA, ROCK1 1-1020-3': GCAAGCTTATTTTTGAAGAAGAGATGTCGTTTGAT, ROCK2 1-1080-5' (amino acids 1-360): CGGATCCAGCCGGCCCCCGCCGACGGGGAAAATG, ROCK2 1-1080-3': GCAAGCTTATTCCATTGATCATTCTTAAAGAAAGG, ROCK1 1014-2250-5' (amino acids 338-750): CGGATCCTTCAAAAATGACCAGTGGGCTTGGGAA, ROCK1 1014-2250-3': GCAAGCTTTTGCTTCAGATCAACGTCTAGCATGGA, ROCK2 1062-2325-5' (amino acids 354-775): CGGATCCAAGAATGATCAATGGAATTGGGATAAC, ROCK2 1062-2325-3': GCAAGCTTATTTAGCTTCTGCTGAGACTGTTTGAG, ROCK1 2100-3354-5' (amino acids 700-1118): CGGATCCAAACATCAATCTATTGAAGAGGCAAAG, ROCK1 2100-3354-3': GCAAGCTTCTCTGGGAGGTTACCATCAGTTTCATC, ROCK2 2175-3450-5' (amino acids 725-1150): CGGATCCGAAGAAGCTAAATCAGAAGCCATGAAA, ROCK2 2175-3450-3': GCAAGCTTTTCTGGAAATCCATCATCAGGCTCAGC, ROCK1 3354-4062-5' (amino acids 1118-1354): CGGATCCTCAAGAATTGAAGGTTGGCTTTCAGTA, ROCK1 3354-4062-3': GCAAGCTTACTAGTTTTTCCAGATGTATTTTTGAC, ROCK2 3450-4164-5' (amino acids 1150-1388): CGGATCCTCAAGATTAGAAGGATGGTTGTCATTG,

ROCK2 3450-4164-3': GCAAGCTTGCTTGGCTTGTTTGGAGCAAGCTGTCG. All ROCK constructs were fully sequenced in both directions. ROCK1 and 2 domains were expressed in bacteria, affinity purified and the 6xHis-fusion proteins eluted from the 6xHis beads with 250mM imidazole. The soluble 6xHis-fusion proteins were mixed with GST or GST-MBS683-866 for 1 hour at 4^{o}C in buffer B (50mM Tris pH 7.6, 7mM $MgCl_2$, 2mM EDTA, 2mg/ml *n*-dodecyl-B-maltoside, 0.4mg/ml cholesteryl hemisuccinate, 1% Triton X, 0.6M NaCl, 10mM Na molybdate, 0.01µg/ml each of aprotinin, leupeptin and pepstatin A, 2mM phenylmethylsulfonyl fluoride). The beads were washed three times in buffer B, and boiled in SDS sample buffer. Bound 6xHis-fusion proteins were detected by SDS-PAGE on 10% gels and immunoblotting with anti-6xHis antibodies.

Transfection: A7r5 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. HEK293 cells were transfected with Polyfect (Qiagen) according to the manufacturer's instructions.

MBS and MLC Phosphorylation Assays: A7r5 cells were maintained in DME with 10% serum following siRNA transfection. Some cells were serum-deprived for 24 hours before the assay, and others were serum-deprived then treated with 10% serum for the indicated times for the phosphorylation time course experiments. The cells were then rinsed once with cold PBS. To the cells was added 2mM EDTA, 10mM dithiothreitol and 10% trichloroacetic acid in DME. The cells were scraped on ice, microcentrifuged for 20 minutes at 4^{o}C, then washed three times in cold acetone with 2mM dithiothreitol. The

washed cell pellets were air dried, then solubilized by sonication in SDS sample buffer. The protein was resolved by SDS-PAGE followed by immunoblotting with phospho-specific antibodies against MBS Thr696 and Thr850 and MLC Thr18/Ser19.

Immunofluorescence microscopy: Cells grown on glass coverslips coated with fibronectin were rinsed in PBS, then fixed in 3.7% paraformaldehyde. The cells were then permeabilized with 0.3% Triton X in 10% donkey serum, and blocked an additional 1hr in 10% donkey serum. The cells were labeled with Alexa Fluor 488 phalloidin, anti-vinculin or anti-phospho-MLC for 1hr. Vinculin and phospho-MLC labeled cells were then rinsed in PBS and labeled with donkey anti-mouse Cy3 for 1hr, and all coverslips were incubated with DAPI stain for 5mins. The coverslips were mounted on slides with SlowFade reagent. Cells were imaged on a Nikon Optiphot-2 microscope with Nikon Plan 20/0.50 and Plan 40/0.70 objectives and coupled to a SPOT CCD camera. Cell area was measured using NIS Elements software. Stress fibers and focal adhesions were counted manually.

siRNA: A7r5 and primary rat aortic smooth muscle cells were transfected at 20% confluency using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Oligonucleotides for rat ROCK1 and ROCK2 were as described [1] and were synthesized by Dharmacon (Lafayette, CO) (ROCK1 UCG GCA GAG GUG CAU UUG G, ROCK2 CGU GGA, AAG, CCU, GCU, GGA, U). A second ROCK2 oligonucleotide (R2b), was purchased from Dharmacon HP GenomeWide siRNA (GUC UAU UAA UAC UCG UCU A).

Smooth muscle cell contractility assay: Measurement of contractility of single VSMCs was peformed by plating VSMCs on a polymer substrate consisting of microfabricated posts. The cells form adhesions on the tops of the flexible posts, and are thus free to contract, unhindered by their attachments, causing movement of the posts which can subsequently be measured allowing calculation of the mechanical forces exerted upon them. This method has been previously published for contractile force measurement in cardiomyocytes [4, 5] and traction force measurement [6-9]. The posts are made by replica molding from the polymer polydimethylsiloxane (PDMS), which is nontoxic and supports cell growth (see [4] for details). The tops of the posts were coated with fibronectin, to facilitate VSMC attachment. The diameter of the posts was 2-4μm and the height was 6-10μm. Primary rat aortic smooth muscle cells were seeded onto the microfabricated post structures in perfusion chambers in DME with 10% bovine growth serum overnight, then changed to serum-free media for 48 hrs. For imaging of contractions, the samples were moved to the microscope stage (Nikon TE2000-U microscope with Nikon S Fluor 20X/0.75 objective) and enclosed in a LiveCell environment chamber (Neue biosciences, Inc) where they were maintained at 37^{o}C and at 5% CO_2. After the addition of agonist, the cells were imaged every minute for 30 minutes using a Cool SNAP EZ CCD camera (Photometrics) with NIS Elements software. Cell length change following agonist stimulation was measured using Image Pro Plus 6.0 software.

Cellular Force Measurement: Background noise from captured images was filtered (MATLAB) and the displacement of microfabricated posts derived from comparison to a

reference array. A displacement map was generated and force calculated based on the relationship $F=kx$ where x is the lateral deformation and k is the spring constant of the post (determined from height, diameter and Young's Modulus of the cantilever)[4]. Force was measured in 20 cells from each silencing condition.

Statistics: One way ANOVA was used in Figures 2, 5, 6 and 7. A p value of < 0.05 was considered statistically significant.

Amino acid alignment was performed using the BLAST 2 sequences program from the National Center for Biotechnology Information (NCBI): http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi

Online Figure Legends:

Online Figure 1: GST and GST-fusion proteins encompassing the entire M-RIP molecule and the MBS domain that binds ROCK in Figure 3A were tested for ROCK2 binding from A7r5 cell lysates. Minimal ROCK binding to M-RIP was detected. ROCK2 bound MBS 683-866 as in Figure 3A.

Online Figure 2: Amino acid alignment of human ROCK1 338-750 with the ROCK2 MBS binding domain, 354-775. ROCK2 and ROCK1 sequences are labeled on the left. There is 58% homology between the two sequences. The alignment was performed using BLAST 2 sequences from the NCBI: http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi.

Online Figure 3: A7r5 cells transfected with GFP (top row) or Myc-ROCK-Rho-binding domain (Myc-ROCK-RBD, bottom row). The GFP transfected cells were immunostained with anti-phospho-MLC-Cy3 (top, left panel) and imaged for green fluorescence (top, right panel). The Myc-ROCK-RBD transfected cells were immunostained with both anti-phospho-MLC-Cy3 (bottom, left panel) and anti-Myc-FITC (bottom, right panel). The arrowheads indicated transfected cells. The scale bar is 30μm.

Online Figure 4: Phosphorylation of MBS at the known inhibitory site Thr696 in A7r5 cells, following the silencing conditions described in 5B (Top). Total MBS is shown as a loading control. Pooled data from 4 experiments of MBS phosphorylation at Thr696 following ROCK isoform silencing (Bottom). The values are represented as Thr696 phosphorylation normalized to MBS expression for each sample.

Online Figure 5: Immunofluorescence microscopy of representative primary rat aortic VSMCs following scrambled negative control (Scr, top row), ROCK1 (middle row) and ROCK2 (bottom row) silencing. The cells were plated on fibronectin coated coverslips, serum-deprived for 48 hours and treated with LPA for 30 minutes. Immunofluorescence labeling was performed with phalloidin (left column) to image actin fibers and vinculin (right column) to image focal adhesions. The scale bar is 30μm.

Online Figure 6: Primary rat aortic VSMCs after silencing of ROCK isoform expression as in Supplementary Figure 5 above, and treated with Y27632 for 30 minutes before fixation. The cells were labeled with phalloidin and vinculin as above. The scale bars are 30μm.

Online Figure 7: Inhibition of rat aortic smooth muscle cell contraction by Y27632. Untransfected cells were plated on micrfabricated posts, and stimulated with 1μM LPA with and without pretreatment with 10μM Y27632 for 30 minutes. The number of contracting cells per field were counted and averaged over three experiments. Control and Y27632-treated samples contained similar numbers of cells.

Online Figure 8: Inhibition of rat aortic smooth muscle cell contraction by blebbistatin. Untransfected cells were plated on micrfabricated posts, and stimulated with 1μM LPA with and without pretreatment with 20μM blebbistatin for 30 minutes. The number of contracting cells per field were counted and averaged over three experiments. Control and blebbistatin-treated samples contained similar numbers of cells.

Online Figure 9: Immunofluorescence microscopy of representative primary rat aortic VSMCs following scrambled negative control (Scr, top row), ROCK1 (middle row) and ROCK2 (bottom row) silencing. The cells were plated on fibronectin coated coverslips, serum-deprived for 48 hours and treated with LPA for 30 minutes. Immunofluorescence labeling was performed with phalloidin (left column) to image actin fibers and phospho-MLC (right column). The scale bar is 30μm.

Online Figure 10: Primary rat aortic VSMCs after silencing of ROCK isoform expression, and treatment with Y27632 for 30 minutes before fixation. The cells were labeled with phalloidin and phospho-MLC as above. The scale bar is 30μm.

Online video 1: Primary rat aortic smooth muscle cells, 48 hours after transfection with scrambled dsRNA were plated on microfabricated posts, serum deprived for 48 hours and stimulated with 1μM LPA. The image capturing begins when LPA is added, and continues to capture 1 frame per minute for 30 minutes.

Online video 2: Primary rat aortic smooth muscle cells, 48 hours after transfection with ROCK1 specific dsRNA were plated on microfabricated posts, serum deprived for 48 hours and stimulated with 1μM LPA. The image capturing begins when LPA is added and continues to capture 1 frame per minute for 30 minutes.

Online video 3: Primary rat aortic smooth muscle cells, 48 hours after transfection with ROCK2 specific dsRNA were plated on microfabricated posts, serum deprived for 48 hours and stimulated with 1μM LPA. The image capturing begins when LPA is added and continues to capture 1 frame per minute for 30 minutes.

Reference List

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(4) Zhao Y, Lim CC, Sawyer DB, Liao R, Zhang X. Cellular force measurements using single-spaced polymeric microstructures: isolating cells from base substrate. *J Micromech Microeng.* 2005;15:1649-56.

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(6) du Roure O, Saez A, Buguin A, Austin RH, Chavrier P, Siberzan P, Ladoux B. Force mapping in epithelial cell migration. *Proc Natl Acad Sci.* 2005;102:2390-5.

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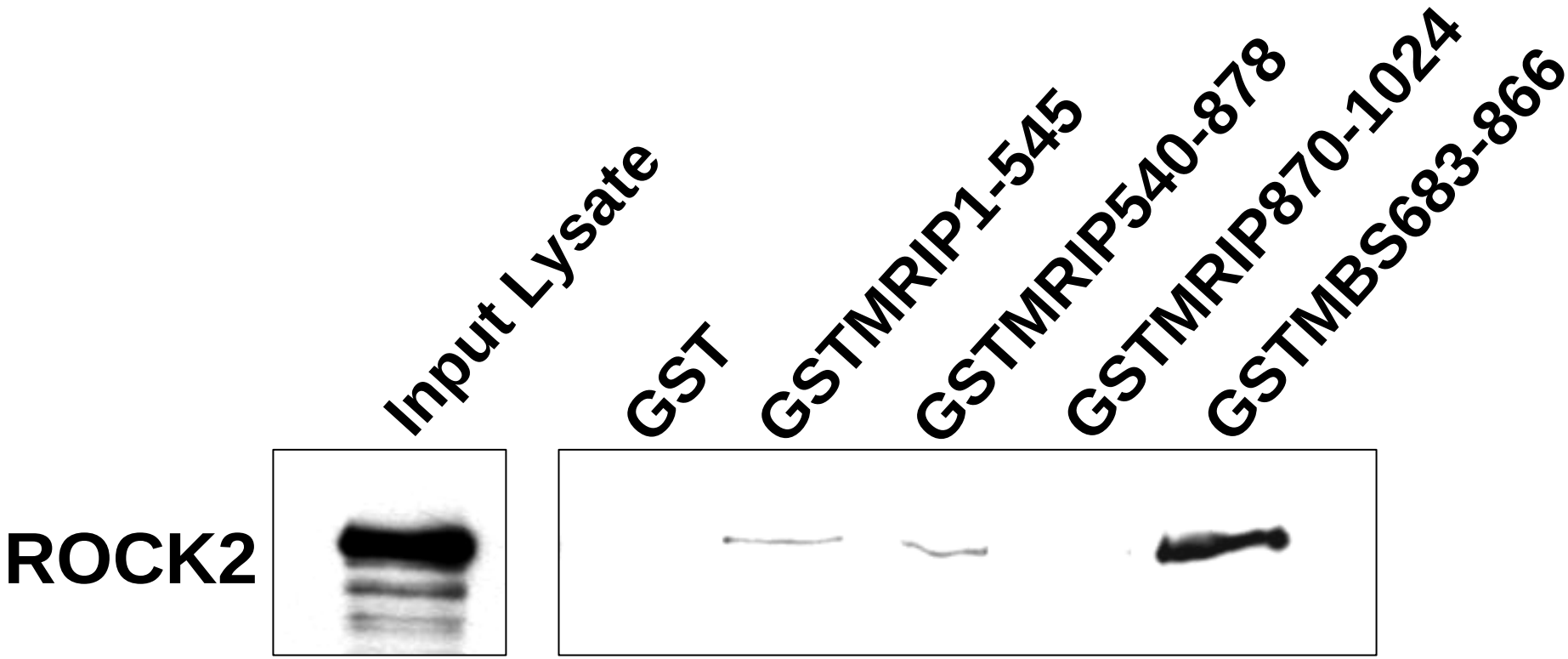
Online Figure 1



Online Figure 2

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ROCK1  2    KNDQWAWETLRDTVAPVVPDLSSDIDTSNFDDLEEDKGEEETFPIPKAFVGNQLPFVGFT  61
            KNDQW W+ +R+T APVVP+LSSDID+SNFDD+E+DKG+ ETFPIPKAFVGNQLPF+GFT
ROCK2  1    KNDQWNWDNIRETAAPVVPELSSDIDSSNFDDIEDDKGDVETFPIPKAFVGNQLPFIGFT  60

ROCK1  62   YYSNRRYLSSANPNDNRTSSNADKSLQ-ESLQKTIYKLEEQLHNEMQLKDEMEQKCRTSN  120
            Y+     LS + P     +    KS + + +QK +Y LEE L +E+Q K+E+EQKC++ N
ROCK2  61   YFRENLLLSDSPPCRENDAIQTRKSEESQEIQKKLYALEEHLSSEVQAKEELEQKCKSIN  120

ROCK1  121  IKLDKIMKELDEEGNQRRNLESTVSQIEKEKMLLQHRINEYQRKAEQENEKRRNVENEVS  180
             +L+K  KEL+EE   R+++EST+ Q+E+EK LLQH+  EYQRKA+ E +K+RN+EN+V+
ROCK2  121  TRLEKTAKELEEEITLRKSVESTLRQLEREKALLQHKNAEYQRKADHEADKKRNLENDVN  180

ROCK1  181  TLKDQLEDLKKVSQNSQLANEKLSQLQKQLEEANDLLRTESDTAVRLRKSHTEMSKSISQ  240
            +LKDQLEDLKK +Q+SQ++ EK++QLQKQL+EAN LLRTESDTA RLRK+  E SK I Q
ROCK2  181  SLKDQLEDLKKRNQSSQISTEKVNQLQKQLDEANALLRTESDTAARLRKTQAESSKQIQQ  240

ROCK1  241  LESLNRELQERNRILENSKSQTDKDYYQLQAILEAERRDRGHDSEMIGDLQARITSLQEE  300
            LES NR+LQ++N +LE +K + +K++  LQ+ LE+ERRDR H SE+I DLQ RI+ L+E+
ROCK2  241  LESNNRDLQDKNCLLETAKLKLEKEFINLQSALESERRDRTHGSEIINDLQGRISGLEED  300

ROCK1  301  VKHLKHNLEKVEGERKEAQDMLNHSEKEKNNLEIDLNYKLKSLQQRLEQEVNEHKVTKAR  360
            +K  K  L KVE E+++ Q+ L   EKEK+N+EID+ Y+LK +QQ LEQE  EHK TKAR
ROCK2  301  LKTGKALLAKVELEKRQLQEKLTDLEKEKSNMEIDMTYQLKVIQQSLEQEEAEHKTTKAR  360

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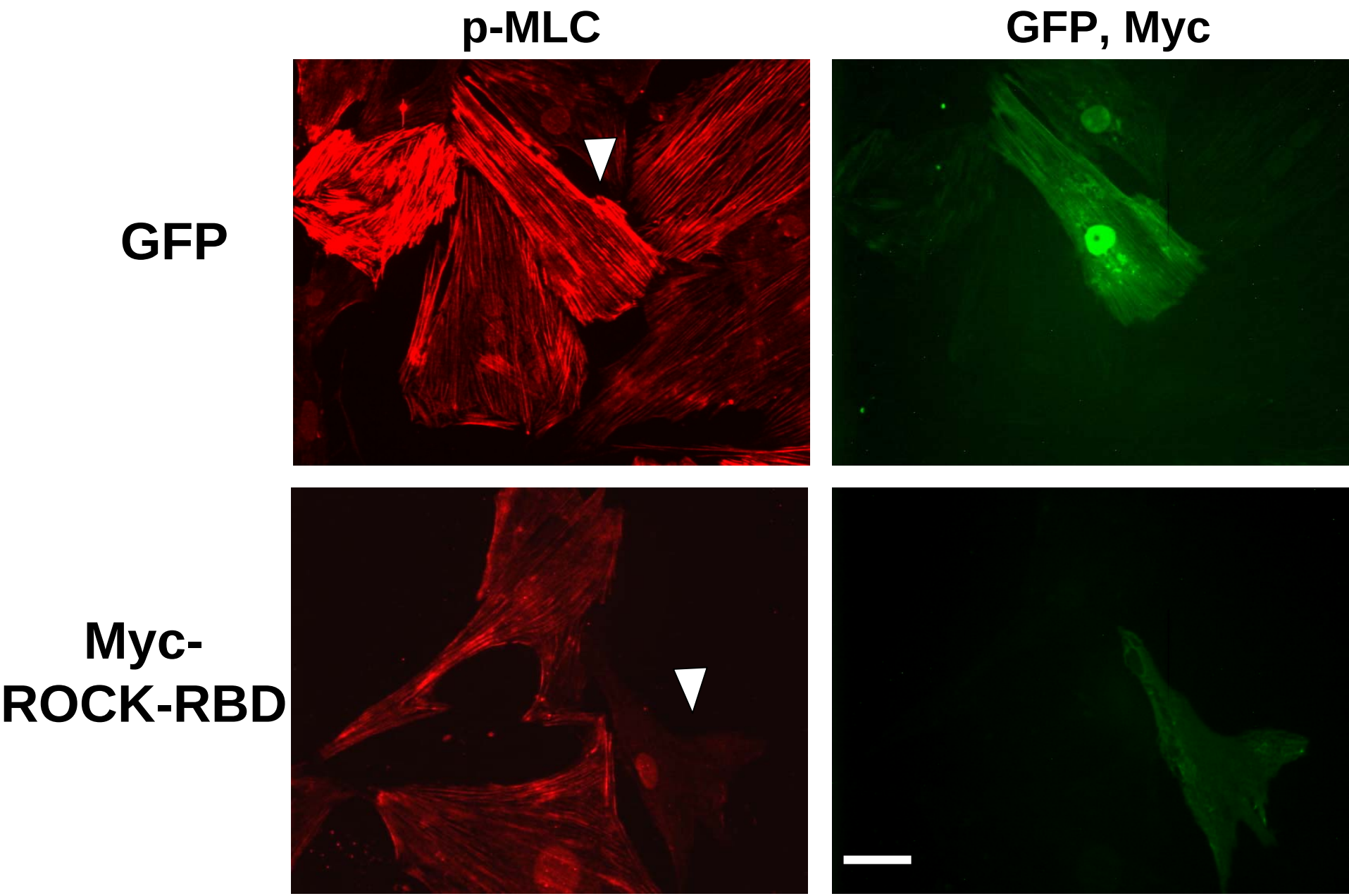
Online Figure 3



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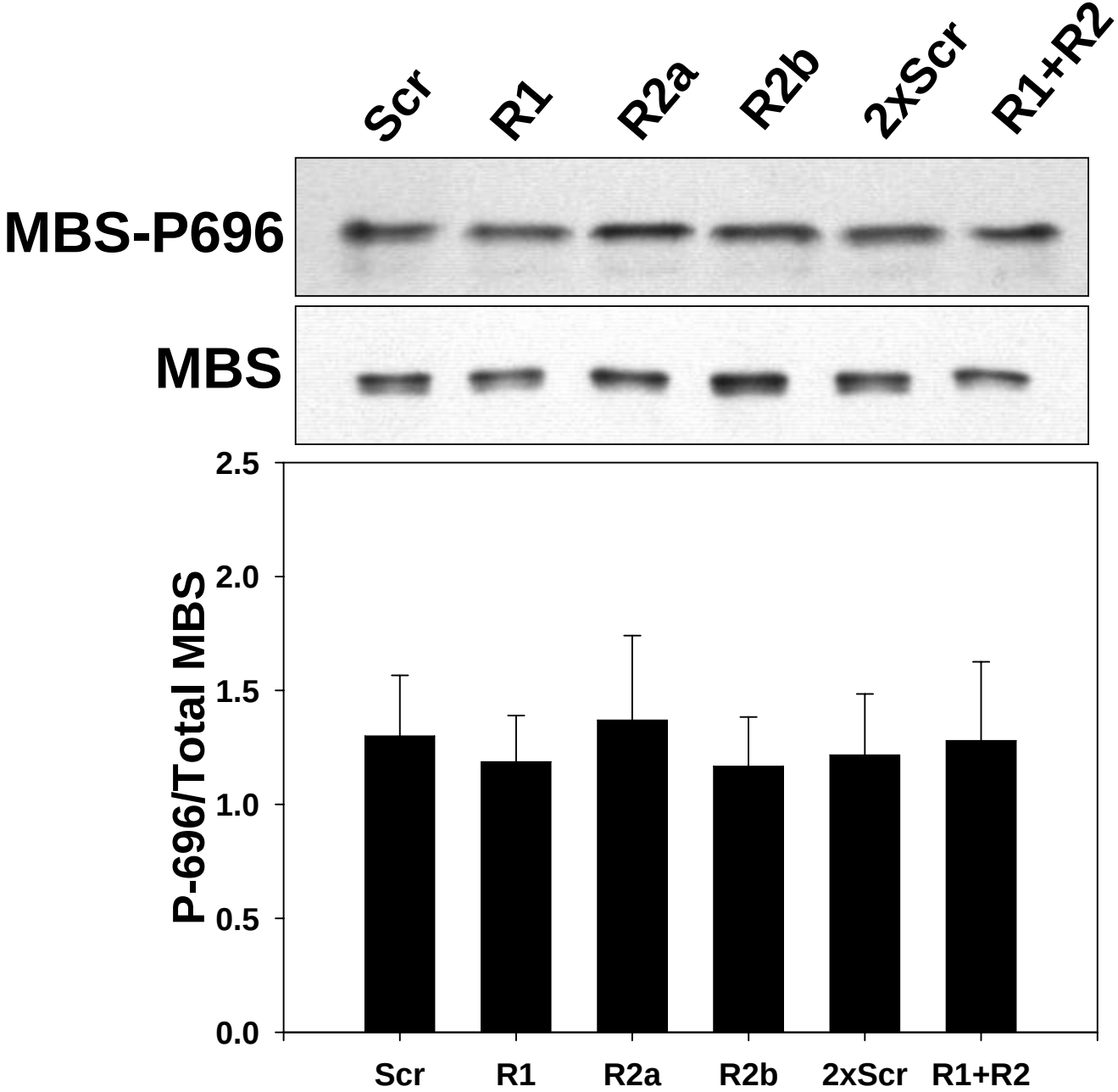

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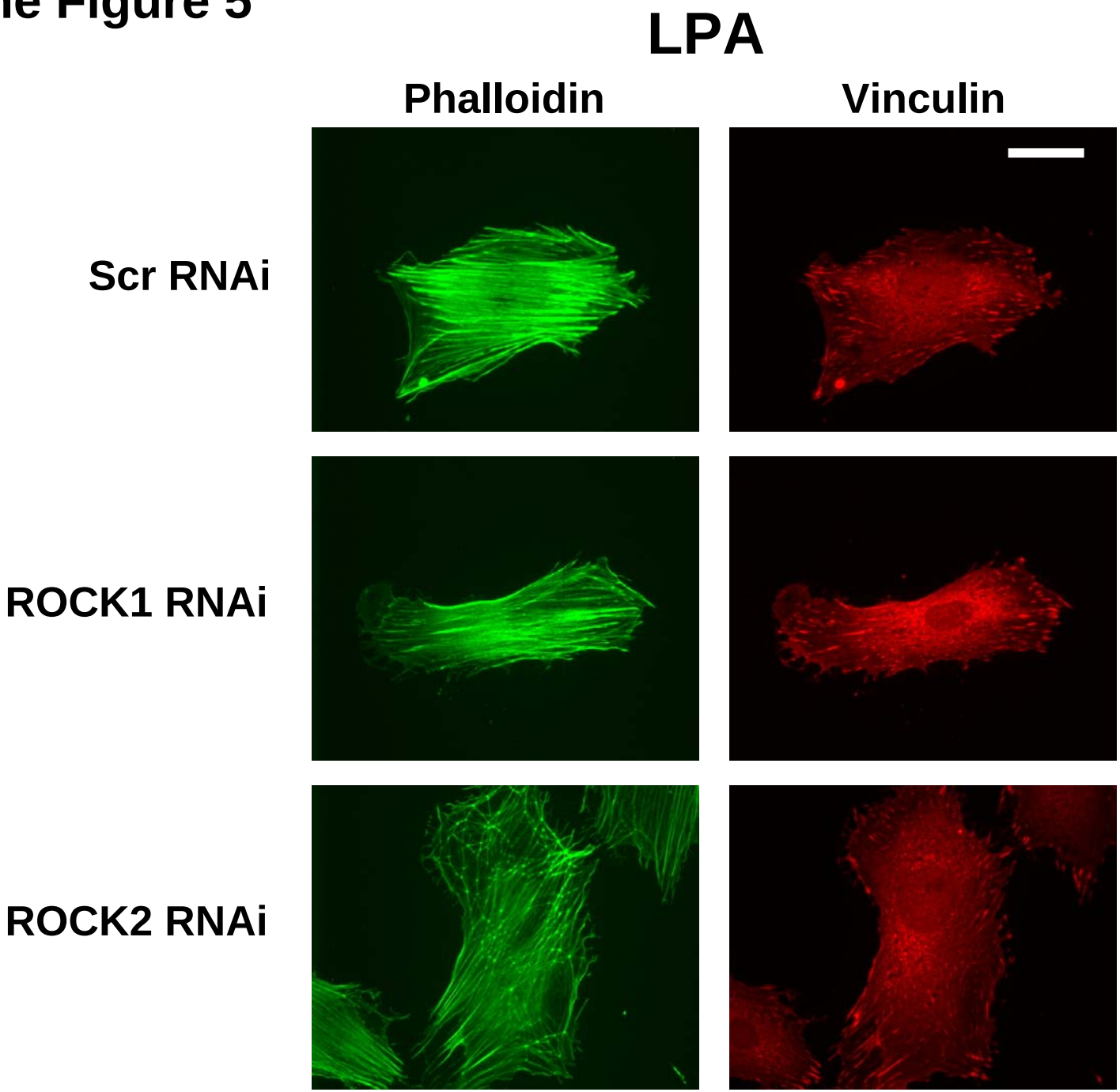

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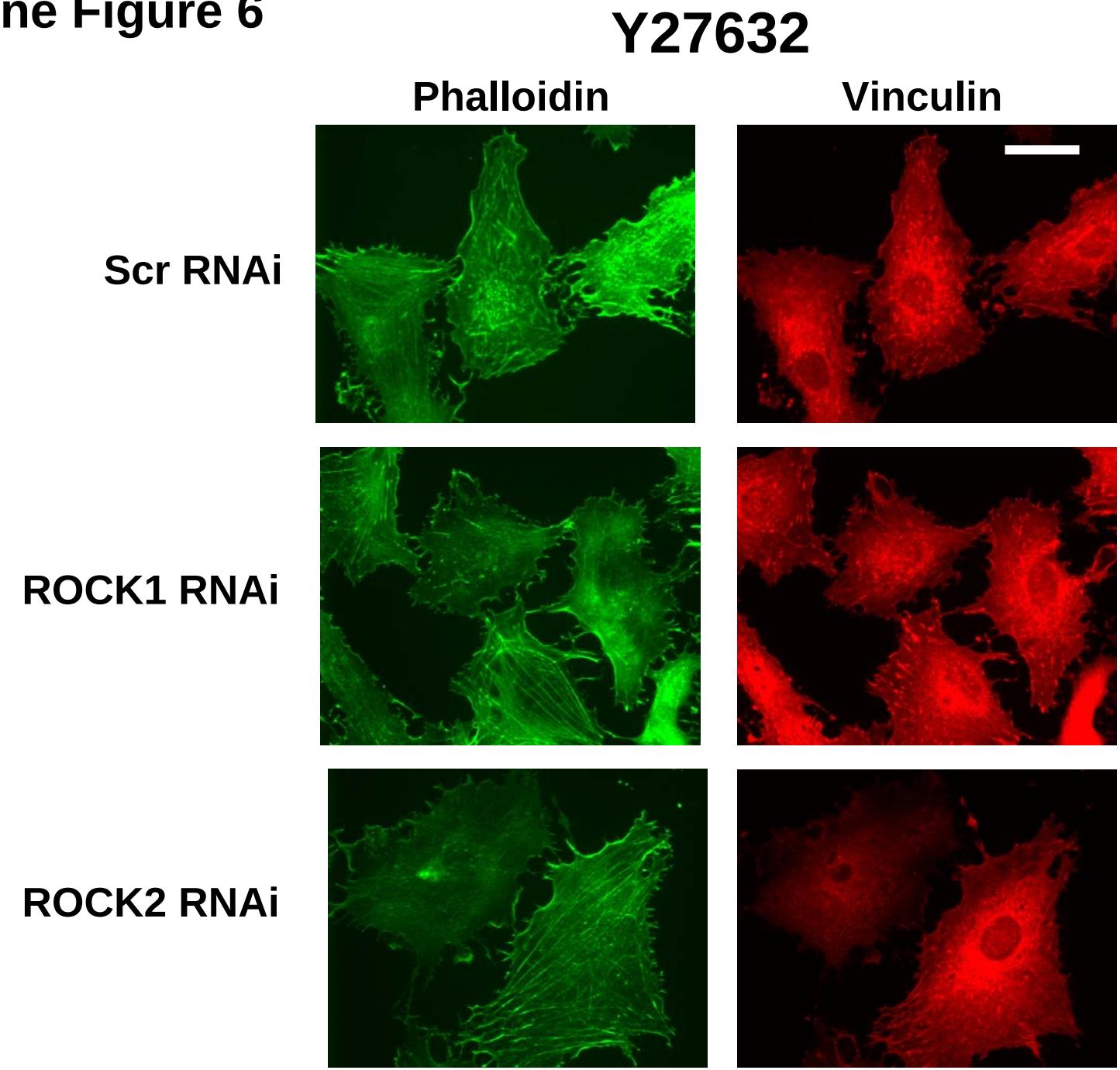

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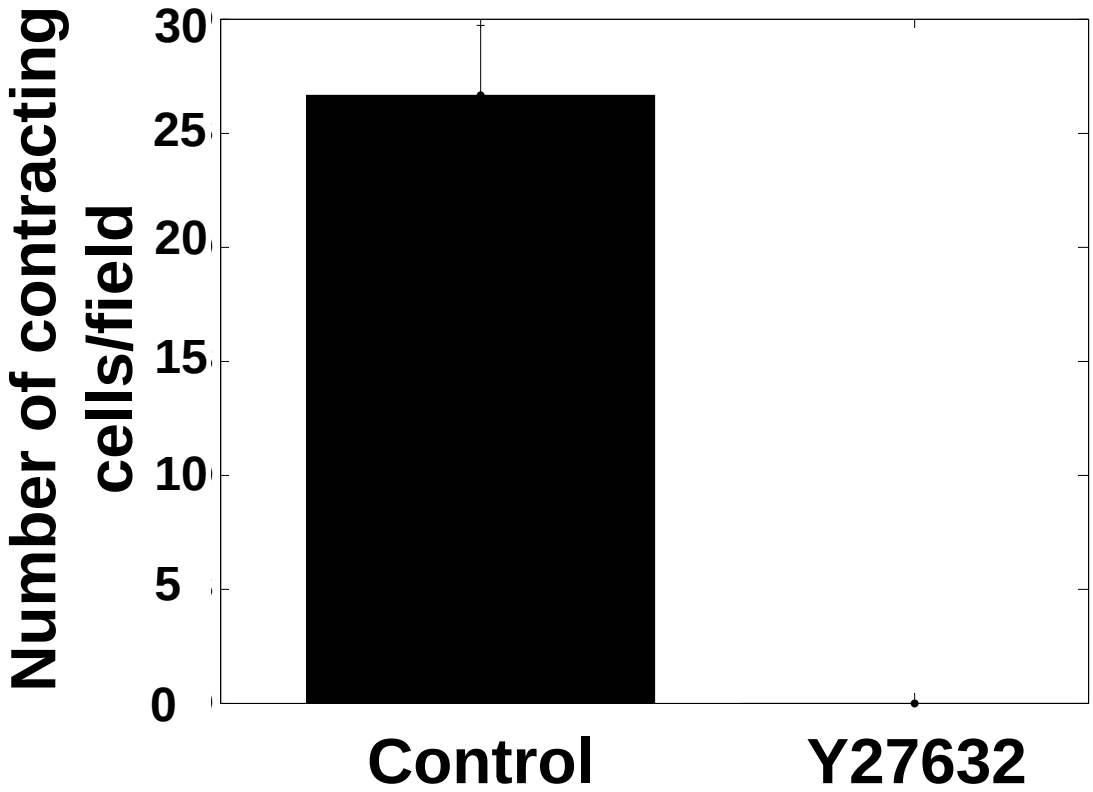

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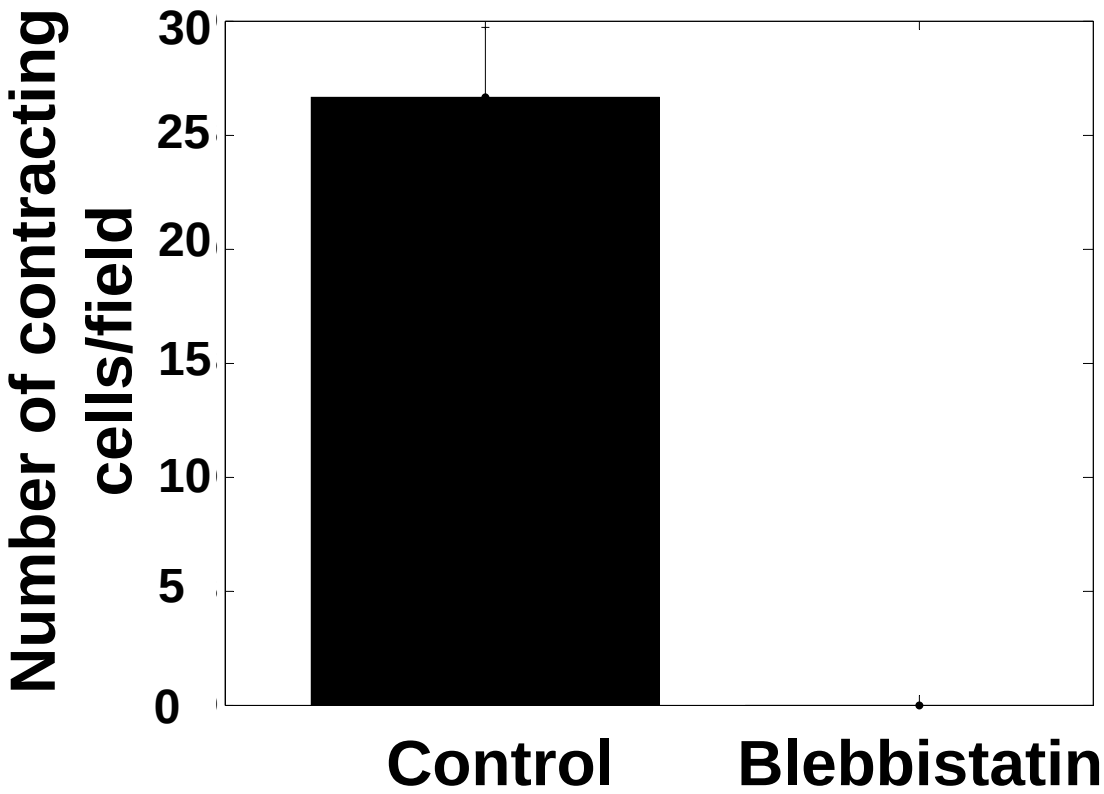

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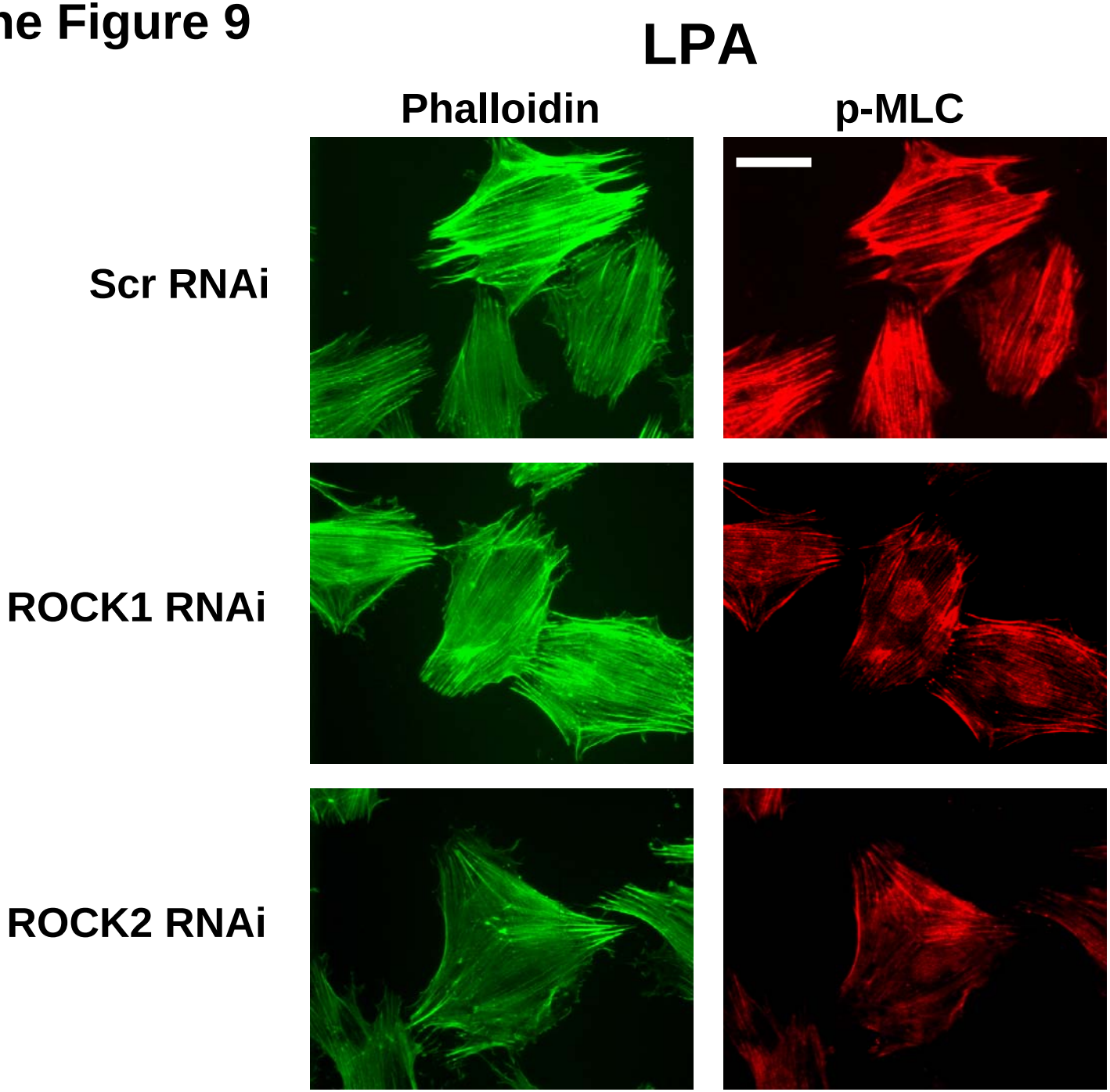

Online Figure 10