SUPPLEMENTAL MATERIAL

Discoidin domain receptor 1 (DDR1)-RhoA Axis Senses Matrix Stiffness to Promote Vascular Calcification

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Supplemental Methods

Data, materials, and methods will be made available to others upon request for reproducibility purposes or replicating procedures. Requests can be made to the corresponding author.

Cell Culture

Primary *Ddr1*$^{+/+}$ (WT) VSMCs were isolated as previously described.[1] Cells were isolated from male mice because female mice have attenuated development of cardiovascular disease including vascular calcification, and we sought to study the condition of maximum calcification, VSMC transdifferentiation, and the pathogenesis of vascular calcification. Experiments were performed using VSMCs between passages 4 and 8. Cells were cultured in normal media (5.5mM glucose DMEM [11885084; Gibco], 10% fetal bovine serum (FBS) [12483020; ThermoFisher], and 1% penicillin-

streptomycin [15140122; ThermoFisher]) or calcifying media (25mM glucose DMEM [11995065; Gibco], 3% heat-inactivated FBS [12483020], 1% penicillin-streptomycin [15140122], and 2.4mM inorganic phosphate). High glucose and phosphate calcifying media was chosen because it produces minimal cell death, reliably elicits calcification in mouse VSMCs, and is consistent with previous studies from our laboratory.[2, 3] FBS was heat-inactivated at 56°C in a water bath for 60 minutes. Osteogenesis experiments with C3H10T1/2 cells were performed with osteogenic media (high glucose DMEM [11995065], 10% FBS [12483020], 1% penicillin-streptomycin [15140122], 10 μM Dexamethasone [D8893; Sigma-Aldrich], 70 μg/mL L-ascorbic acid [A4544; Sigma-Aldrich], 10 mM β-glycerophosphate [G9422; Sigma-Aldrich]).

Reagents and Treatments

Rho Activator II [CN03; Cytoskeleton] was added at a concentration of 0.25μg/mL for 3 hours and C3 exoenzyme [CT04; Cytoskeleton] at a concentration of 1μg/mL for 4 hours. DDR1-in-1 dihydrochloride [5077], (±)-Blebbistatin [1760], Jasplakinolide [2792], and Latrunculin A [3973] were bought from Tocris Bioscience. DDR1-in-1 is a selective DDR1 inhibitor that binds to the kinase domain and prevents ligand-induced autophosphorylation. DDR1-in-1 was used at a concentration of 1μM supplemented with each media change for osteogenesis experiments. Jasplakinolide was used at a concentration of 1μM for 3 hours and Latrunculin A at 1μM for 3 hours. Blebbistatin was used at a concentration of 25uM [1760].

Generating Silicone Substrates on Glass Slides

Glass slides were cleaned by sequentially washing with gentle shaking for 30 minutes with ddH_2O, 30 minutes with acetone, 30 minutes with methanol, 15 minutes in

ddH_2O, 1 hour with 0.05M NaOH, and finally 3x5 minutes with ddH_2O. Slides were dried at 70^oC for 1 hour before Sylgard 527 mixtures for the desired stiffness were spin-coated onto the glass. The slides were placed in the Spincoater Model P6700 [Specialty Coating Systems Inc.; Indianapolis, IN] and 1mL of Sylgard 527 mixture was slowly pipetted onto the centre of the slide. Spin-coating was performed at 350rpm for 70 seconds with a ramp time of 15 seconds. Once spin-coated, curing of the silicone substrate and collagen coating was performed as described.

In Vitro Osteogenesis

For osteogenesis of C3H10T1/2 cells, 30,000 cells were seeded on 5kPa or 100kPa collagen I-coated silicone substrates in 6-well plates and grown for 3 days before culturing in osteogenic media for 21 days with or without 1μM DDR1-in-1, changing media every 2-3 days.

Immunoblotting

Protein was collected with 1x cell lysis buffer [9803; Cell Signaling Technology] supplemented with 1μM phenylmethylsulfonyl fluoride (PMSF) from cells grown in 6-well plates. Protein concentration was measured by the DC protein assay kit [5000112; Bio-Rad]. Equal amounts of protein was added to 4x sample buffer (200mM Tris pH 6.8, 8% SDS, 40% glycerol, 4% β-mercaptoethanol, 0.08% bromophenol blue), boiled for 5 minutes, and separated by SDS-PAGE at 100V. The protein was transferred onto a polyvinylidene fluoride (PVDF) membrane [1620177; Bio-Rad] at room temperature for 90 minutes at 100V in transfer buffer (20% methanol, 25mM Tris, 192mM glycine, pH 8.3-8.5). Membranes were blocked for 1 hour with 5% skim milk in Tris-buffered saline with Tween-20 (TBST) or 5% BSA in TBST for phosphoproteins. Primary antibodies

were diluted 1:1000 in 1% BSA in TBST and incubated with the membranes at 4°C overnight. Horseradish peroxidase (HRP)-linked secondary antibodies were diluted at 1:5000 in 1% BSA in TBST and incubated with the membrane for 1 hour at room temperature. Antibodies are from Cell Signaling Technology: Rabbit DDR1 [5583], rabbit phospho-DDR1 (Y792) [11994], and HRP-linked anti-rabbit secondary [7074]. Western blots were imaged using the ChemiDoc Touch Imaging System [Bio-Rad] and quantified by Bio-Rad Image Lab software.

Subcellular Fractionation

500,000 WT and KO VSMCs were seeded on silicone substrates in 15cm dishes and grown to confluency in normal media before culturing in calcifying media for 2 days. Cell lysis was done with 1x cell lysis buffer [9803; Cell Signaling Technology] supplemented with 1μM PMSF and EDTA-free mini complete protease inhibitor cocktail [04693159001; Roche]. Cells were homogenized with a Dounce homogenizer and centrifuged at 228 x g for 5 minutes. Supernatants were collected as the cytoplasmic fraction. The pellet was re-suspended in 3mL of S1 solution (0.25mM sucrose, 10mM $MgCl_2$) supplemented with EDTA-free mini complete protease inhibitor cocktail tablet [04693159001; Roche]. 3mL of S3 solution (0.88mM sucrose, 0.5mM $MgCl_2$) was layered on top. Samples were centrifuged at 2,800 x g for 10 minutes at 4^oC to pellet the nuclear fraction. Nuclei were re-suspended in 1x RIPA buffer supplemented with EDTA-free mini complete protease inhibitor cocktail [04693159001; Roche].

Immunocytochemistry

To assess stiffness-dependent stress fibre formation, 100,000 VSMCs were seeded on spin-coated slides and allowed to attach for 24 hours before 48 hours of

culture in calcifying media. To examine collagen I-mediated stress fibre formation with Vav2 siRNA, VSMCs were seeded on uncoated or collagen I-coated coverslips and allowed to attach for 24 hours before control or Vav2 siRNA transfection for 24 hours. VSMCs were serum-starved overnight and serum-stimulated for 3 hours. For Runx2 immunostaining, WT VSMCs were seeded at 6,000 cells/well in 8-well chamber slides [0030742079; Eppendorf] and allowed to attach for 24 hours before 48 hours of culture in calcifying media supplemented with 0.25µg/mL ACT or 1µg/mL C3. VSMCs were fixed with 4% PFA for 10 minutes, washed 3x5 minutes with Ca^{2+} and Mg^{2+}-free PBS, then permeabilized with 0.25% Triton X-100 for 10 minutes. Blocking was done with 1% BSA in TBST for 1 hour at room temperature. VSMCs were immunolabelled for Runx2 (1:100) [12556; Cell Signaling Technology], 1x AlexaFluor488 Phalloidin [A12379; ThermoFisher], and AlexaFluor568 goat anti-rabbit secondary antibody (1:200) [A11011; ThermoFisher]. Nuclei were stained with Hoechst-33342. VSMCs on spin-coated slides or stained for Runx2 were imaged with the Zeiss AxioObserver.Z1 confocal microscope and VSMCs transfected with siRNAs were imaged with the Nikon Eclipse C*i* epifluorescence microscope. Zeiss Zen 3.0 blue was used to quantify total nuclear and cytoplasmic fluorescence intensity to calculate the nuclear to cytoplasmic ratio of Runx2. All samples were mounted with ProLong Gold Antifade mountant [P36930; ThermoFisher].

Plasmid Preparation

Plasmid was expanded by a standard protocol in MAX efficiency DH5-α competent cells [18258012; Invitrogen]. Briefly, 1-5ng of Ddr1b-YFP or Actin-mApple plasmid was transformed into 40µL *E.coli* by heat-shock at 42^{o}C for 45 seconds

followed by placing on ice for 2 minutes. 960µL of Super Optimal broth with Catabolite repression (SOC) media [15544034; Invitrogen] was added to transformed bacteria and put in a bacterial incubator at 37ºC rotating at 225rpm for 1 hour. Bacteria was streaked onto 50µg/mL Kanamycin [KAN201; BioShop] LB agar plates and incubated at 37ºC overnight. Single colonies were picked and used to inoculate 250mL Kanamycin LB agar broth before rotation at 225rpm at 37ºC overnight. Plasmid DNA was isolated with the GeneJET Plasmid Maxiprep Kit [K0492; ThermoFisher]. Plasmid DNA concentration was measured by NanoDrop 1000 [ThermoFisher].

Live Cell Imaging and Laser Cutting Stress Fibres

30,000 WT VSMCs were seeded on collagen I-coated 35mm^2 glass bottomed dishes and allowed to attach for 24 hours in normal media. Cells were transfected with 5µg of Ddr1b-YFP (gift from Dr. Christopher McCulloch and Dr. Nuno Coelho; University of Toronto) and Actin-mApple (gift from Dr. Sergey Plotnikov; University of Toronto) plasmid with Lipofectamine 3000 [L3000008; ThermoFisher] in Opti-MEM reduced serum medium [31985070; ThermoFisher] for 24 hours. Images were taken using a Revolution XD spinning disk confocal [Andor] with a 60x oil-immersion lens [NA 1.35; Olympus]. Wounds were created using a pulsed MicroPoint N_2 laser [Andor] tuned to 365nm. The laser produced 120µJ pulses with duration of 2–6ns. To sever stress fibres, ten pulses were delivered at a diffraction-limited spot on the fibre. Images were acquired immediately before and after cutting as well as every 10 seconds henceforth until 2 minutes post-cut. Images were captured with an iXon Ultra 897 camera [Andor] and Metamorph software [Molecular Devices] for image acquisition. 16-bit *Z*-stacks were acquired at 0.5µm steps (10-20 slices). For quantitative analysis of DDR1-YFP clusters

in response to laser cutting stress fibres, 2-3 z-stack slices were maximum-intensity projected for each time point. ROIs of 20 x 50 pixels (3.6 x 8.9 μm) were selected in three locations within each cell: at the cut site centered and aligned with the severed stress fiber (cut), at a random site opposite from the cut site containing DDR1 clusters (control), and in a region away from the cut site with no DDR1 clusters (background). Clusters located within cut and control ROIs were used for further analysis (5-6 per ROI). Fiducial markers were manually placed at the center of each cluster at each time point using the image analysis software SIESTA.[4] Using custom written code in MATLAB, fiducial markers were dilated to a circular ROI with a diameter of 5 pixels (0.9μm). The average intensity in each circular ROI was calculated and normalized to the mean intensity within the background ROI to correct for sample photobleaching for each time point. The resulting intensity curves were normalized to the initial (pre-cut) intensity to yield a relative intensity measurement over time.

Data and Statistical Analysis

Statistical analysis was performed with Graphpad Prism 5 software. The data have been analyzed for normality and equal variance. Statistical significance was assessed by Student's t-test, one-way ANOVA, or two-way ANOVA with Bonferroni post-hoc test. $p < 0.05$ was considered statistically significant and denoted by asterisks. A limitation of the study is the small sample sizes (N = 3-6) in many experiments.

References

1. Hou G, Vogel W and Bendeck MP. The discoidin domain receptor tyrosine kinase DDR1 in arterial wound repair. *J Clin Invest*. 2001;107:727-35.
2. Ahmad PJ, Trcka D, Xue S, Franco C, Speer MY, Giachelli CM and Bendeck MP. Discoidin domain receptor-1 deficiency attenuates atherosclerotic calcification and smooth muscle cell-mediated mineralization. *Am J Pathol*. 2009;175:2686-96.
3. Lino M, Wan MH, Rocca AS, Ngai D, Shobeiri N, Hou G, Ge C, Franceschi RT and Bendeck MP. Diabetic Vascular Calcification Mediated by the Collagen Receptor Discoidin Domain Receptor 1 via the Phosphoinositide 3-Kinase/Akt/Runt-Related Transcription Factor 2 Signaling Axis. *Arterioscler Thromb Vasc Biol*. 2018;38:1878-1889.
4. Fernandez-Gonzalez R and Zallen JA. Oscillatory behaviors and hierarchical assembly of contractile structures in intercalating cells. *Phys Biol*. 2011;8:045005.

Supplemental Figures and Figure Legends

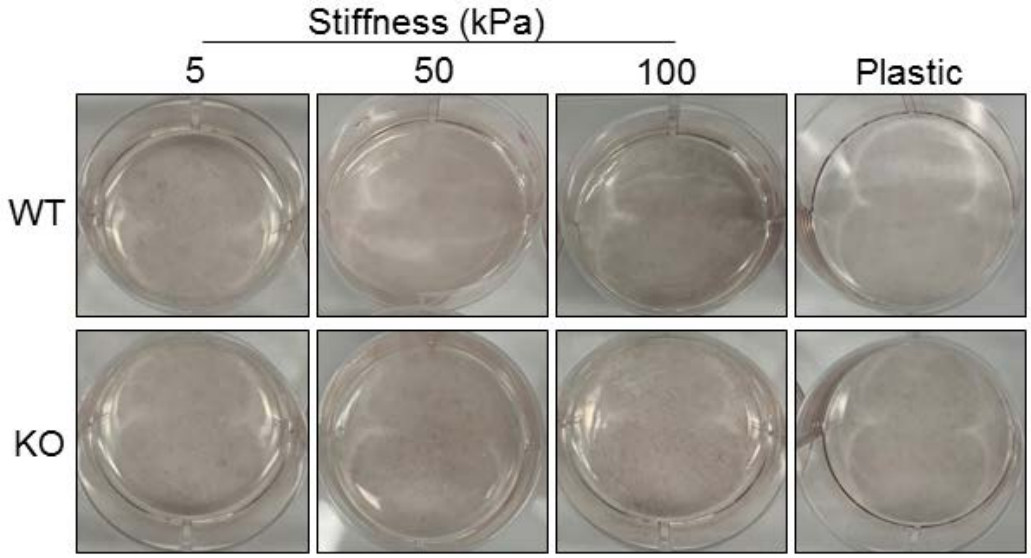

Figure SI: Increased matrix stiffness does not promote VSMC calcification in the absence of calcifying media. WT or KO VSMCs were cultured in normal media for 12 days before staining with Alizarin Red (n=3).

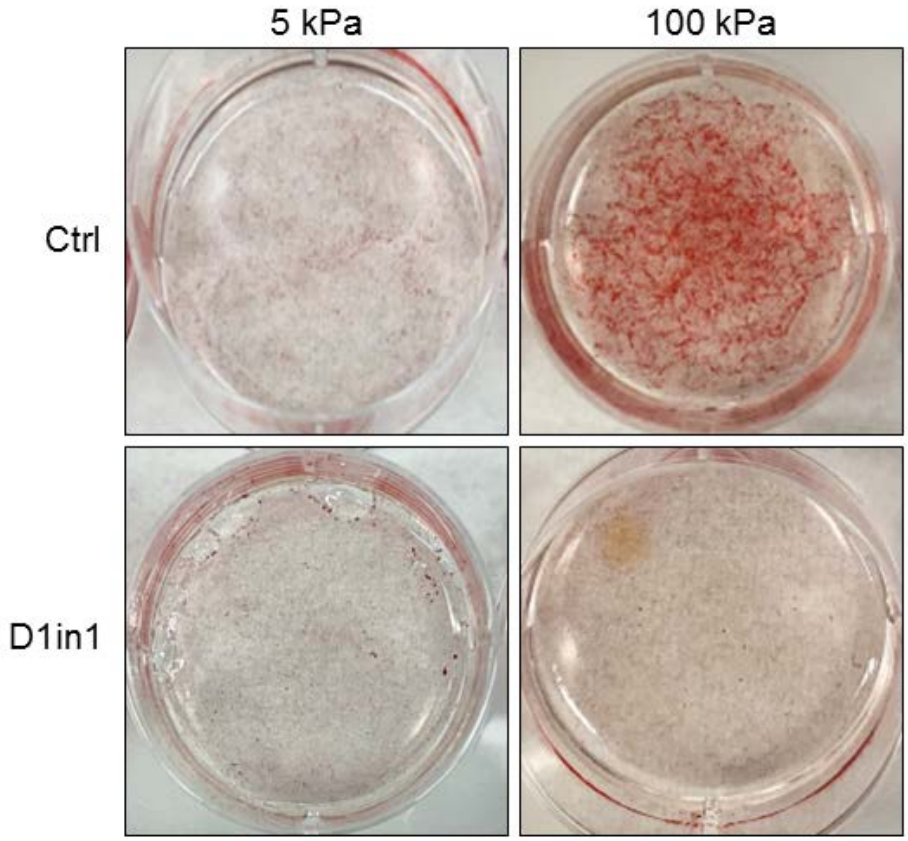

Figure SII: DDR1 inhibition prevented stiffness-mediated increase in osteogenesis of C3H10T1/2 murine mesenchymal cells. C3H10T1/2 mesenchymal stem cells were cultured in osteogenic media (β-glycerophosphate) for 21 days with or without 1μM DDR1-in-1 (D1in1) to inhibit DDR1, and stained with Alizarin Red (n=3).

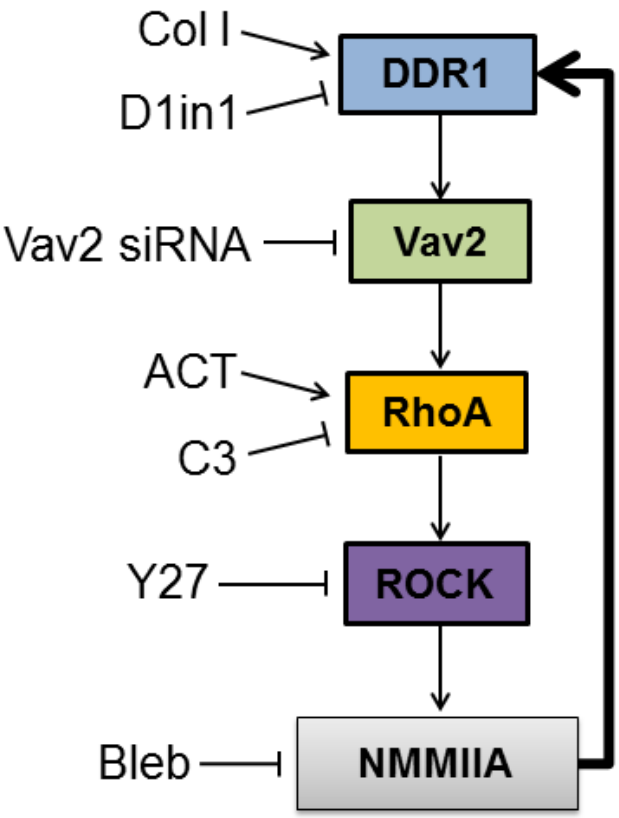

Figure SIII: Schematic representation of the proposed cyclical pathway in the regulation of RhoA and myosin contractility by DDR1 (expression and activation) and vice versa. The compounds used to activate and inhibit DDR1 (Col I, D1in1), RhoA (ACT, C3), ROCK (Y27), and myosin (Bleb) are depicted in the diagram.

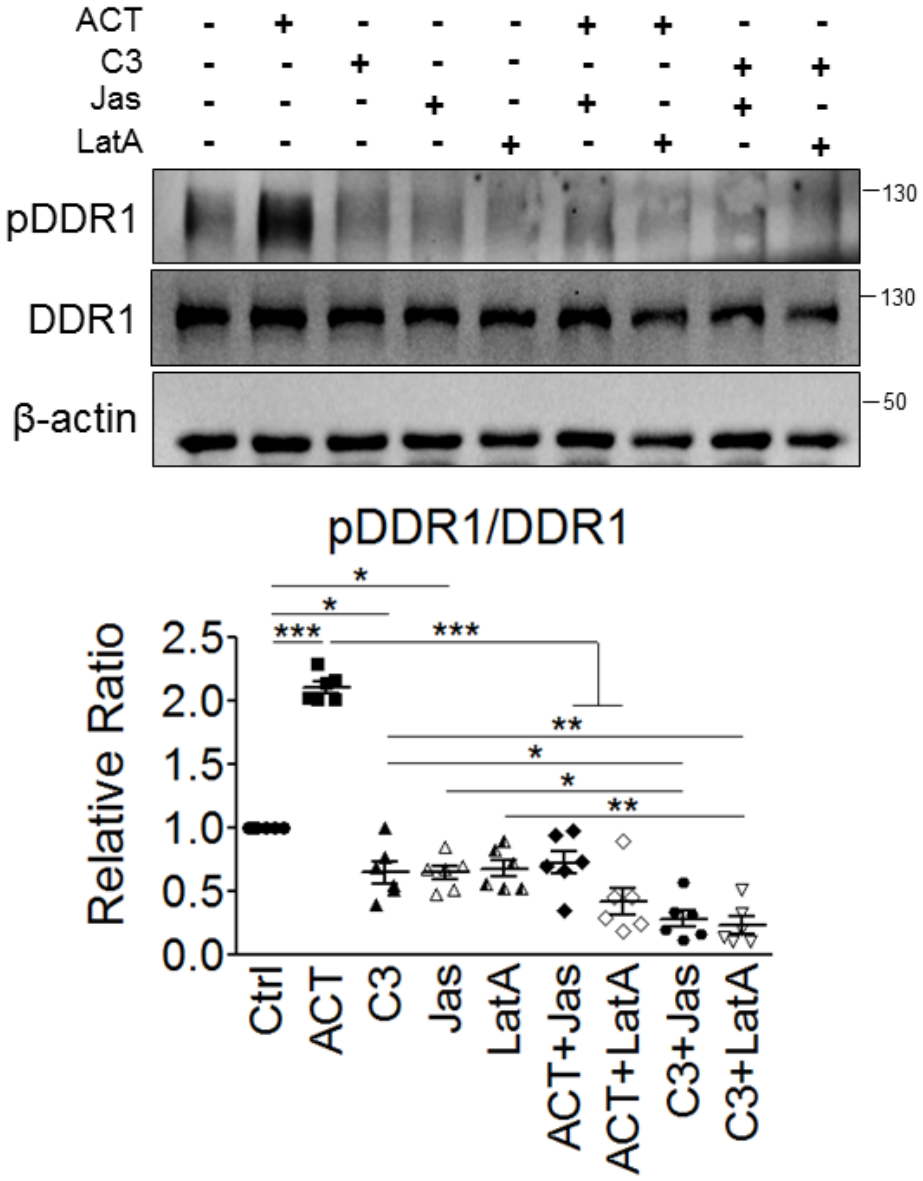

Figure SIV: Jasplakinolide and Latrunculin A inhibit RhoA-mediated activation of DDR1. WT VSMCs were treated with ACT, C3, Jasplakinolide (Jas), or Latrunculin A (LatA), or with varying combinations of these compounds (n=6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, bars represent means $\pm$ SEM. Statistics were done by one-way ANOVA with Bonferroni post-hoc test.

Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Genetically Modified Animals

Species	Vendor or Source	Background Strain	Other Information	Persistent ID / URL
Mouse	Michelle Bendeck	C57BL/6N	*Ddr1*$^{+/+}$, 6-8 weeks	
Mouse	Michelle Bendeck	C57BL/6N	*Ddr1*$^{-/-}$, 6-8 weeks	

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Persistent ID / URL
DDR1	CST	5583	1:1000 (WB) 1:100 (IP)	https://www.cellsignal.com/products/primary-antibodies/ddr1-d1g6-xp-rabbit-mab/5583
pDDR1 (Y792)	CST	11994	1:1000 (WB)	https://www.cellsignal.com/products/primary-antibodies/phospho-ddr1-tyr792-antibody/11994
Runx2	CST	12556	1:1000 (373ng/mL) (WB), 1:100 (3.73μg/mL) (ICC)	https://www.cellsignal.com/products/primary-antibodies/runx2-d1l7f-rabbit-mab/12556
Sox9	CST	82630	1:1000 (3.2ng/mL) (WB)	https://www.cellsignal.com/products/primary-antibodies/sox9-d8g8h-rabbit-mab/82630
Lamin A/C	CST	2032	1:1000 (7ng/mL) (WB)	https://www.cellsignal.com/products/primary-antibodies/lamin-a-c-antibody/2032
β-actin	CST	4967	1:1000 (9ng/mL) (WB)	https://www.cellsignal.com/products/primary-antibodies/b-actin-antibody/4967
Vav2	Abcam	ab52640	1:1000 (800ng/mL) (WB)	https://www.abcam.com/vav2-antibody-ep1067y-ab52640.html
pVav2 (Y172)	Abcam	ab86695	1:1000 (900ng/mL) (WB)	https://www.abcam.com/vav2-phospho-y172-antibody-ab86695.html
pY20	Abcam	ab10321	1:1000 (940ng/mL) (WB)	https://www.abcam.com/phosphotyrosine-antibody-py20-ab10321.html
BMP-2	Novus Biologicals	NBP1-19751	1:1000 (1.15μg/mL) (WB)	https://www.novusbio.com/products/bmp-2-antibody_nbp1-19751
Osteopontin	Invitrogen	PA5-34579	1:1000 (1μg/mL) (WB)	https://www.thermofisher.com/antibody/product/Osteopontin-Antibody-Polyclonal/PA5-34579
HRP-linked anti-rabbit	CST	7074	1:5000 (13.14ng/mL) (WB)	https://www.cellsignal.com/products/secondary-antibodies/anti-rabbit-igg-hrp-linked-antibody/7074
HRP-linked anti-mouse	CST	7076	1:5000 (30.6ng/mL) (WB)	https://www.cellsignal.com/products/secondary-antibodies/anti-mouse-igg-hrp-linked-antibody/7076
AF488 Goat anti-rabbit	Thermo Fisher	A11008	1:200 (10μg/mL) (ICC)	https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11008
AF568 Goat anti-rabbit	Thermo Fisher	A11011	1:200 (10μg/mL) (ICC)	https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11011

DNA/cDNA Clones

Clone Name	Sequence	Source / Repository	Persistent ID / URL
Ddr1b-YFP		Dr. Christopher McCulloch and Dr. Nuno Coelho (University of Toronto)	
Actin-mApple		Dr. Sergey Plotnikov (University of Toronto)	

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
Ddr1$^{+/+}$ Vascular Smooth Muscle Cell		M	
Ddr1$^{-/-}$ Vascular Smooth Muscle Cell		M	

Other

Description	Source / Repository	Persistent ID / URL
AF488 Phalloidin	ThermoFisher	https://www.thermofisher.com/order/catalog/product/A12379
Hoescht 33342	ThermoFisher	https://www.thermofisher.com/order/catalog/product/H3570#/H3570
Prolong Gold Antifade Mountant	ThermoFisher	https://www.thermofisher.com/order/catalog/product/P36930#/P36930
Lipofectamine 3000	ThermoFisher	https://www.thermofisher.com/order/catalog/product/L3000008#/L3000008
Lipofectamine RNAiMAX	ThermoFisher	https://www.thermofisher.com/order/catalog/product/13778150#/13778150
Kanamycin	BioShop	https://www.bioshopcanada.com/secure/detail.asp?offset=2340&Pin=KAN201
MAX Efficiency DH5-α cells	Invitrogen	https://www.thermofisher.com/order/catalog/product/18258012#/18258012
SOC Media	Invitrogen	https://www.thermofisher.com/order/catalog/product/15544034#/15544034
GeneJET Plasmid Maxiprep Kit	ThermoFisher	https://www.thermofisher.com/order/catalog/product/K0492#/K0492
Opti-MEM Reduced Serum Media	ThermoFisher	https://www.thermofisher.com/order/catalog/product/31985070#/31985070
Sylgard 527 A&B Silicone Dielectric Gel	Dow Corning	https://krayden.com/buy/dc-527-9kg-kit-2lb-dc1696742.html
PureCol bovine collagen I	Advanced BioMatrix	https://advancedbiomatrix.com/purecol/
Rat Tail Collagen I	Sigma-Aldrich	https://www.sigmaaldrich.com/catalog/product/sigma/c7661?lang=en&region=CA
Rho Activator II	Cytoskeleton	https://www.cytoskeleton.com/cn03
C3 Exoenzyme	Cytoskeleton	https://www.cytoskeleton.com/ct04
DDR1-in-1 dihydrochloride	Tocris Bioscience	https://www.tocris.com/products/ddr1-in-1-dihydrochloride_5077
Jasplakinolide	Tocris Bioscience	https://www.tocris.com/products/jasplakinolide_2792
Latrunculin A	Tocris Bioscience	https://www.tocris.com/products/latrunculin-a_3973
(±)-Blebbistatin	Tocris Bioscience	https://www.tocris.com/products/dl-blebbistatin_1760

Y-27632	CST	https://www.cellsignal.com/products/activators-inhibitors/y-27632/13624
Dexamethasone	Sigma-Aldrich	https://www.sigmaaldrich.com/catalog/product/sigma/d8893?lang=en&region=CA
L-Ascorbic Acid	Sigma-Aldrich	https://www.sigmaaldrich.com/catalog/product/sigma/a4544?lang=en&region=CA
β-glycerophosphate	Sigma-Aldrich	https://www.sigmaaldrich.com/catalog/product/sigma/g9422?lang=en&region=CA
2-amino-2-methyl-1-propanol	Sigma-Aldrich	https://www.sigmaaldrich.com/catalog/product/sial/a9199?lang=en&region=CA
o-Cresolphthalein Complexone	Sigma-Aldrich	https://www.sigmaaldrich.com/catalog/product/sial/p5631?lang=en&region=CA
8-hydroxyquinoline	Sigma-Aldrich	https://www.sigmaaldrich.com/catalog/product/sial/252565?lang=en&region=CA
Negative Control siRNA	ThermoFisher	https://www.thermofisher.com/order/catalog/product/AM4611#/AM4611
Vav2 siRNA	ThermoFisher	https://www.thermofisher.com/order/genome-database/details/sirna/186991?CID=&ICID=&subtype=