DETAILED MATERIALS AND METHODS

Animal Studies

The DNmDia cDNA variant was cloned into a previously described pBSCX1-LEL plasmid vector(1, 2) (see Figure 2a), and transgenic mice were generated by the University of North Carolina Animal Models Core. All animals were housed in a university animal care facility accredited by the American Association for Accreditation of Laboratory Animal Care, and all procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee. DNmDia mice were bred with either SM22α-Cre mice (Jax) (3) or SM MHC-CreER^{T2} mice(4) to express DNmDia in a SMC-dependent manner. Some DNmDia mice were crossed to the R26R Cre-recombinase reporter mouse line to visualize where Cre is expressed. To see *in vivo* LacZ expression, whole tissues were fixed in 4% paraformaldehyde for 15 min, incubated in X-gal staining solution for 16 hr, fixed for another 24 hr, dehydrated in increasing concentrations of methanol, and visualized by X-gal clearing solution. For injury studies, carotid artery ligation was performed as previously described(5). Briefly, a suture was tied around the left common carotid artery just below the bifurcation and the right carotid artery served as a sham control. Animals were sacrificed at various times following injury, and tissues were embedded in either paraffin or OCT. To determine neointimal area, we used ImageJ (NIH) to measure circumference of the lumen, internal elastic lamina (IEL), and external elastic lamina (EEL). Intimal area = IEL are minus lumen area. Medial area = EEL area minus IEL area.

Immunofluorescence/Immunohistochemistry

For IHC in either transfected or knockdown cells, cells were fixed in 4% paraformaldehyde for 20 minutes, permeabilized in 0.5% Triton X-100 for 3 min, blocked in 10% goat serum/3% BSA in PBS for 1 hr, then exposed to antibody in blocking solution for 1 hr. Alexa Fluor secondary antibodies were used for visualization at 1:1000 and DAPI (200 nM) was used to detect nuclei. TUNEL staining was performed with the In Situ Cell Death Detection Kit, TMR red (Roche) as per the manufacturer's instructions. For DAB staining, HRP-conjugated secondary antibodies (Sigma) were used and signals were visualized by development using ImmPACT DAB Peroxidase Substrate (SK-4105, Vector labs). For MRTFA-DAPI colocalization, a confocal microscope was used to visualize 8 μm sections of ligated and uninjuted carotid arteries. The ImageJ Plug-In "Colocalization Threshold" was used to quantify percent pixilation overlap of DAPI and endogenous MRTF-A.

Western Blotting and Antibodies

To examine protein levels, lysates from cells or tissues were prepared by lysing in RIPA buffer with protease and phosphatase inhibitors as previously described(5). The following primary antibodies were used: Flag M2 (F1804, Sigma), SM MHC (ab53219, Abcam), SM α-actin (A5228, Sigma), Calponin/CNN1 (LS-B7497, LifeSpan Bio), SM22/Transgelin (sc-271719, Santa Cruz), α-Tubulin (T6074, Sigma), phospho-histone H3 (6-570, Millipore), and MRTF-A (sc-32909, Santa Cruz). Blots were next incubated with horseradish peroxidase secondary antibodies (Sigma). Blots were visualized after incubation with Luminol Enhancer Solution (Thermo Scientific).

Plasmids, Cell Culture, and Transient Transfections
DNmDia cDNA (amino acids 567–1182, minus the 20AA from 750–770) was subcloned into flag pcDNA3.1 and/or pmCherry-C3 (Clontech). LifeAct (MGVADLIKKFESISKEE) was cloned into the N-terminus of pEGFP-C1 (Clontech). 10T1/2 cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS) and 0.5% penicillin-streptomycin. SMCs from rat or mouse thoracic aorta were isolated and cultured as previously described(6) and maintained in DMEM:F12, also with 10% FBS and 0.5% penicillin-streptomycin. For transfections, cells were maintained in 10% FBS media, and transfected 24 hr after plating at 70-80% confluency, using the transfection reagent, TransIT-LT1 (Mirus), as per protocol. For sphingosine-1-phosphate (S1P) treatment, 24 hr post-transfection cells were serum-starved in DMEM with 0.5% FBS for 16 hr and then treated with S1P (Mateya) at 10 μM for 24 hr (Immunoblot) or 4 min (MRTFA localization).

Small Interfering RNA (siRNA) Mediated Knockdowns and in vitro assays
The following short interfering (si)RNAs were obtained from Invitrogen: control (GFP) 5'-GGUGCGCUCCUGGACGUAGCC-3' , mDia1 5'-GGACCUCUAUUGCCCUCAATT- 3', and mDia2 5'- GCAUGACAAGUUUGUGAUATT-3'. 10T1/2 or SMCs were split and replated at 48 hr post knockdown for transwell assays, scratch wound assays, cytotoxicity assays, and tetrazolium MTT assays.

- **Cytotoxicity.** Cells were split and replated at 20K cells/well into 48-well cell culture plates for cytotoxicity assays. Assays were carried out as per protocol of Cytotoxicity Detection Kit: LDH (Roche). Cells were maintained in 1% serum. Negative control = untransfected cells. Positive control = NTC-siRNA cells in media with 1% Triton-X-100. Absorbance at 490 nm was measured in three separate experiments.
- **Proliferation.** Cells were split and replated at 5K/well into 96-well cell culture plates for tetrazolium MTT assays. Cells were serum-starved for 16 hr and treated with either 10% serum, 1 ng/ml TGF-β, or 20 ng/ml PDGF-BB for 24 hr. MTT assays were carried out as per protocol of Cell Proliferation Kit I: MTT (Roche). Absorbance at 600 nm was normalized to serum-starved NTC-siRNA for each cell type for three separate experiments.
- **Flow Cytometry.** 10T1/2 or SMCs were split and resuspended at a concentration of ~1x1^{06} cells/ml in 0.1% Triton X-100 in PBS. DAPI was added to cell suspension at 1 ug/ml, and cells were incubated on ice for 30 min prior to cell cycle analysis on a Becton Dickinson LSRII. At least 50,000 cells were scored per siRNA condition, per cell type, in three separate experiments**.**

Migration Assays
For transwell assays, SMCs and 10T1/2 cells were trypsinized and resuspended in serum-free media or 0.5% media, respectively. Approximately 20K cells were plated on transwell filters (8 μm pore size) precoated with fibronection, using the same media with 20 ng/ml PDGF-BB as a chemoattractant. After 7 hours, the cells were fixed in 4% paraformaldyhyde and the remaining cells in the upper chamber were removed with a cotton swab. Migrated cells were stained with 1% Crystal Violet and visualized by

microscopy. For scratch wound assays, 10T1/2 cells were split and replated post-knockdown at high confluence, scratched with a P1000 pipette tip, and visualized every 3 hr until wound closure. For live cell imaging, 10T1/2 cells were transfected with both LifeAct-GFP and mCherry or mCherry-DNmDia and imaged every 5 min for both unstimulated (random) migration (~8 hr) and scratch wound-stimulated (directional) migration (~24 hr). Cell movement was quantified using ImageJ.

Statistical Analyses

All values are presented as means with standard deviation. Comparisons were performed by using unpaired Student t test or X^2 analysis, as appropriate. All tests were 2-tailed, and significance was accepted at $P < 0.05$.

SUPPLEMENTAL REFERENCES

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