Supporting Information

Bordeleau et al. 10.1073/pnas.1505421112

SI Materials and Methods

Reagents. Primary antibodies used were as follows: mouse monoclonal anti-fibronectin (A17, ab26245) and mouse monoclonal anti-human EDB-fibronectin (BC-1, ab154210; Abcam); primary goat polyclonal anti-VE-cadherin (C19, sc-6458), rabbit polyclonal anti CD31 (sc-1506), rabbit polyclonal anti-ROCK (H-85, sc-5560), rabbit polyclonal anti PKC βII (C-18, sc-210), rabbit polyclonal anti-fibronectin (H300, sc-9068), and goat polyclonal anti-fibronectin (N-20, sc-6953; Santa Cruz); mouse monoclonal anti–EDB-fibronectin from Richard O. Hynes (Massachusetts Institute of Technology, Cambridge, MA); mouse monoclonal anti-pan phosphoepithope SR proteins (1H4, MABE50), and primary mouse monoclonal anti-GAPDH (MAB374; Millipore); and primary rabbit polyclonal anti-S451 AKT (D9E, #4060; Cell Signaling) and primary rabbit polyclonal anti-phospho AKT substrate (RXXS*/T*) (#9614; Cell Signaling). Secondary antibodies used were as follows: Alexa 488-donkey anti-mouse IgG, Alexa 594-goat anti-mouse IgG, Alexa-594-goat anti-rabbit IgG (Invitrogen) and HRP-goat anti-rabbit IgG and anti-mouse IgG (Rockland). FBS, LSGS, and Fetal Clone III (FCIII) were purchased from Gibco (Life Technologies); PI3K inhibitor wortmannin, myosin inhibitor blebbistatin, myosin light chain kinase inhibitor ML-7, and ROCK inhibitor Y27632 were purchased from Calbiochem. RhoA inhibitor C3 transferase (CT04) was purchased from Cytoskeleton. siRNA against ROCK1 (sc-29473) and control siRNA (sc-37007) were purchased from Santa Cruz. All other chemicals were from Sigma-Aldrich.

Western Blotting. ECs were plated on 1- and 10-kPa PA gels and kept in culture for either 3 d or 24 h when inhibitors were used and rinsed with ice-cold PBS. Total proteins were extracted with preheated (90 °C) 2× sample buffer (52). For tumor samples, whole frozen tumors were ground using a liquid nitrogen-cooled mortar and pestle. The tumor was then collected with ice-cold RIPA buffer. The lysate was harvested, mixed, and spun at 14,000 × *g* at 4 °C, and the supernatant was removed for analysis. Laemmli buffer (1×) was added to the supernatant, and samples were heated for 5 min at 95 °C. Ten to 20 μg was subjected to SDS/PAGE with a Mini-PROTEAN Tetra System (BioRad) and electrotransferred onto a PVDF membrane. Membranes were blocked with 5% (wt/vol) milk (Nestlé) in Tris-buffered saline (TBS)-polyoxyethylene (20) sorbitan monolaurate (Tween; JT Baker) or 5% (wt/vol) BSA. Membranes were then incubated overnight 1:500 in TBS-Tween with an anti-fibronectin primary antibody [A17, H300, or BC1 for EDB-FN (Santa Cruz Biotechnology); or anti-EDB-FN (Hynes Laboratory) for mouse tumor samples] at 4 °C, and 1:2,000 in TBS-Tween with 0.1% milk with an HRP-conjugated secondary antibody for 1 h at room temperature. Samples were images with a Las-4000 imaging system (Fujifilm Life Science) after addition of Immobilon Chemiluminescent Substrate (Millipore). Samples were stripped with Restore Stripping Buffer (Thermo Scientific), reblocked with milk, incubated 1:1,000 in TBS-Tween with an anti-GAPDH primary antibody at room temperature for 1 h, incubated 1:2,000 in TBS-Tween with 0.1% milk with an HRP-conjugated secondary antibody for 45 min at room temperature, and reimaged. Densitometry was performed with ImageJ and expressed as the fold change of the ratio of the protein of interest to GAPDH or actin. Data were the result of three independent experiments.

Immunofluorescence of Fixed Samples. Endothelial cells on PA gels were processed for fluorescence imaging as described previously (63) with anti-FN primary antibodies (A17 or BC1 for EDB-FN only, Santa Cruz Biotechnology). Fluorescent images were acquired with a Zeiss Axio Observer Z1m or a Zeiss 710 confocal microscope, and z-stacks were reconstructed with Zen software (v. 2009; Carl Zeiss).

Immunohistochemistry and Immunofluorescence on Tissue Sections. Formalin-fixed, paraffin-embedded, invasive ductal carcinoma and patient-matched normal human breast tissue from New York Presbyterian Hospital-Weill Cornell Medical College was used for this study in accordance with protocols approved by the Institutional Review Board at Weill Cornell Medical College. For each tumor case, 4-μm-thick sections were deparaffinized in xylene and rehydrated in graded alcohols, and antigen retrieval was performed in a 10 mM citrate buffer (pH 6). Envision+System-HRP (DAB; Dako) was used for immunostaining according to the manufacturer's protocol. The sections were incubated overnight at 4 °C with mouse anti-human EDB-FN (BC1; Abcam) at a 1:200 dilution. Primary antibody was omitted for negative control. Slides were incubated with labeled polymer-HRP anti-mouse secondary antibodies and visualization was performed using a substrate-DAB+Chromogen solution. Slides were counterstained with Harris hematoxylin.

Snap frozen mouse tumor samples were embedded within OCT compound and processed in a cryostat to obtain either 8- or 30-μm-thick sections. For EDB-FN staining, the sections were air dried and fixed for 5 min in cold acetone. The sections were then washed two times in PBS for 10 min and air dried. Otherwise, the tumor samples were immediately fixed after sectioning in 4% (wt/vol) paraformaldehyde for 10 min at room temperature. The sections were then washed two times in PBS as above and kept in PBS. Before the staining procedure, air-dried sections were rehydrated for 5 min in PBS. All sections were blocked for 1 h using the Vectashield mouse-block (Vector Laboratories). The sections were washed once with PBS containing 2% (vol/vol) FBS and 0.01% Tween × 100 for 5 min. The sections were incubated overnight at 4 °C with mouse anti-EDB-FN (Hynes Laboratory), with goat anti-FN and rabbit anti-CD31 or with mouse anti-pSR proteins and rabbit anti-CD31. Primary antibody was omitted for the negative control. Slides were incubated for 1 h at room temperature with Alexa488-donkey anti-mouse, Alexa594-donkey anti-rabbit, Alexa488-donkey anti-rabbit, or Alexa594-donkey anti-goat as needed. Slides were mounted in DAPI containing Vectashield mounting media. Fluorescent images were acquired with a Zeiss 710 confocal microscope.

Quantitative Real-Time PCR. HUVECs were plated on 1- and 10-kPa PA gels, rinsed with PBS, and collected after the addition of 0.25% trypsin-EDTA (Gibco) for 10 min at 37 °C after 3 d. Cells were pelleted and rinsed with PBS, and total RNA was purified with an RNeasy Plus Mini Kit (Qiagen). To generate cDNA, 1 μg of total RNA per sample was mixed with 80 μM random primers (Invitrogen, Life Technologies), 10 mM deoxynucleotide solution mix (New England Biolabs), and nuclease-free water, and heated for 5 min at 75 °C. After the addition of 40 U/μL RNase inhibitor and 200 U/μL M-MuLV reverse transcriptase in M-MuLV reaction buffer (New England Biolabs), cDNA was synthesized in an iCycler thermal cycler (BioRad). cDNA was diluted 1:50 in deionized water, prepared for quantitative real-time PCR by the addition of 300 nM forward primer, 300 nM reverse primer (Integrated DNA Technologies), and 1× iQ SYBR Green Supermix (BioRad), and analyzed with a My iQ Real-Time PCR Detection System (BioRad). The primers used for EDB-FN were 5′-CCTGGAGTACAATGTCAGTG-3′ (forward) and

5′-GGTGGAGCCCAGGTGACA-3′ (reverse) (68). Primers for the reference gene GAPDH were 5′-CATGAGAAGTATGACAACAGCCT-3′ (forward) and 5′-AGTCCTTCCACGATACCAAAGT-3′ (reverse). The results were expressed as a fold change in mRNA expression of EDB-FN relative to GAPDH on 10-kPa gels compared with 1-kPa gels using the Livak method. Data were the results of three independent experiments.

ROCK Knockdown. ROCK-mediated cellular contractility was disrupted by plating BAECs in the presence of 10 μM Y27632 (65). Samples were fixed at 4–48 h after plating and stained to localize FN, actin, and nuclei as described above. ROCK was also disrupted with RNAi. BAECs were treated with an siRNA against bovine ROCK (Invitrogen) or a control siRNA (Santa Cruz Biotechnology). To knock down ROCK, BAECs in six-well plates were incubated with 20 pmol siRNA in 5 μL Lipofectamine 2000 in Optimem for 5 h. Transfected cells were plated on PA substrates 24 h after transfection, and samples were fixed at 24-h time points over the next 3 d and stained as described above. Separate experiments determined the expression of ROCK with Western blotting over the same 3-d period as described above but using an anti-ROCK primary antibody. Similarly, HUVECs were treated as described above for BAECs with siRNA against human ROCK or a control siRNA (Santa Cruz Biotechnology) and stained to localize EDB-FN on the third day after plating as described above. Western blotting was performed at this same time point as described above but using an anti-ROCK primary antibody (H-85; Santa Cruz Biotechnology).

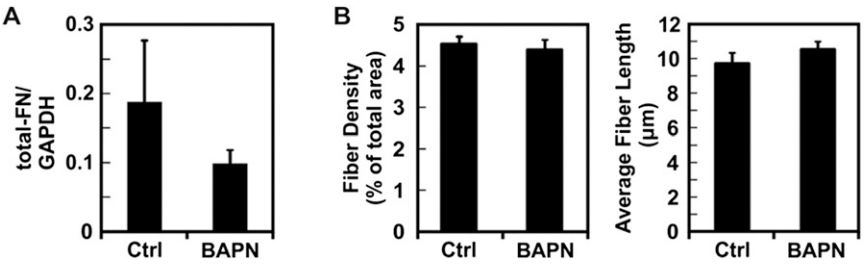

Fig. S1. Effects of BAPN treatment on total FN levels in vivo. (*A*) Quantification of total FN levels observed by Western blot of PyMT tumors from mice treated with BAPN or with the mock treatment (Ctrl). (*B*) Quantification of the average total FN fiber-like structure length and content from PyMT frozen tumor sections. Plots are mean ± SE.

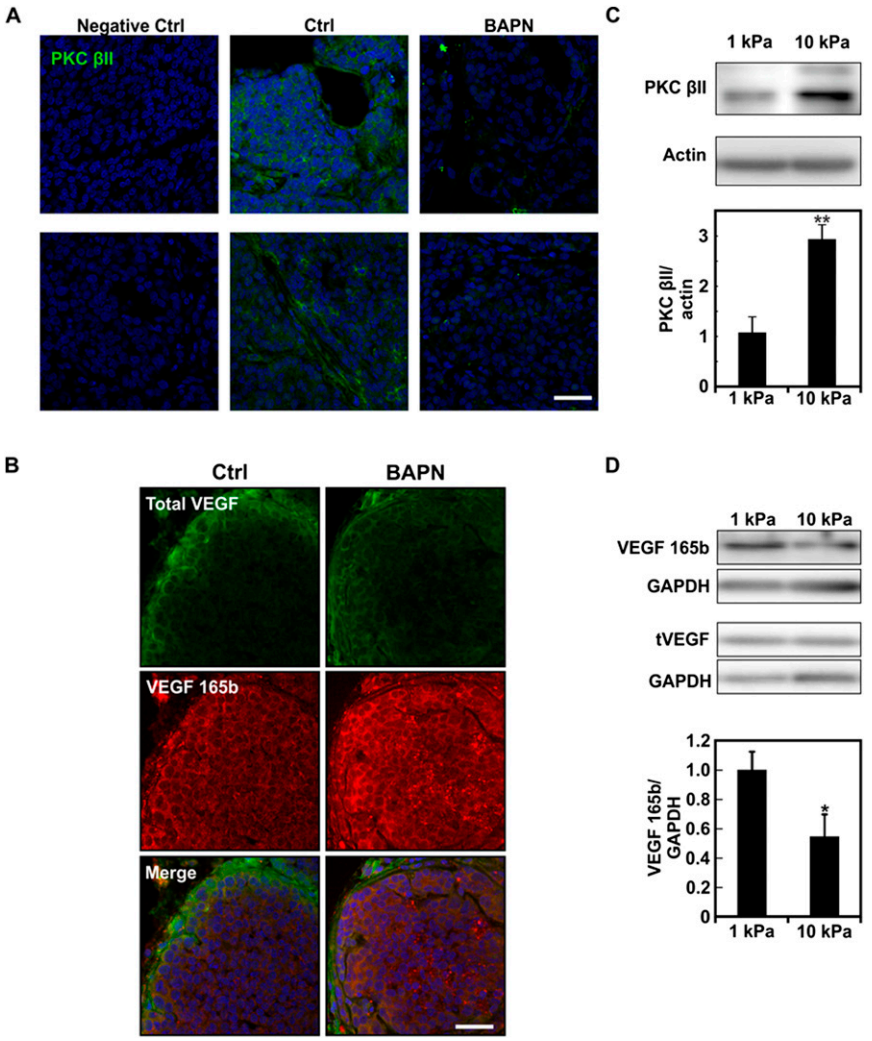

Fig. S2. ECM stiffness regulates the expression of the PKC βII and VEGF 165b isoforms. (*A*) Representative confocal images of frozen tissue sections from PyMT tumors with or without BAPN treatment and stained for PKC βII showing the overall increase of the PKC βII isoform with tumor tissue stiffness. (*B*) Representative confocal images of frozen tissue sections from PyMT tumors with or without BAPN treatment stained for VEGF 165b and total VEGF showing the overall decrease of the VEGF 165b isoform in stiffer tumor tissue. (*C*) Representative Western blot of total protein extract from ECs in culture on compliant and stiff substrates with the corresponding densitometric quantification showing increased PKC βII isoform expression. Actin was used as loading control. (*D*) Representative Western blot of total protein extract from ECs in culture on compliant and stiff substrates with the corresponding densitometric quantification showing increased VEGF 165b isoform expression. GAPDH was used as loading control. Plots are mean ± SE, Student *t* test: ***$P < 0.001$. (Scale bars, 30 μm.)

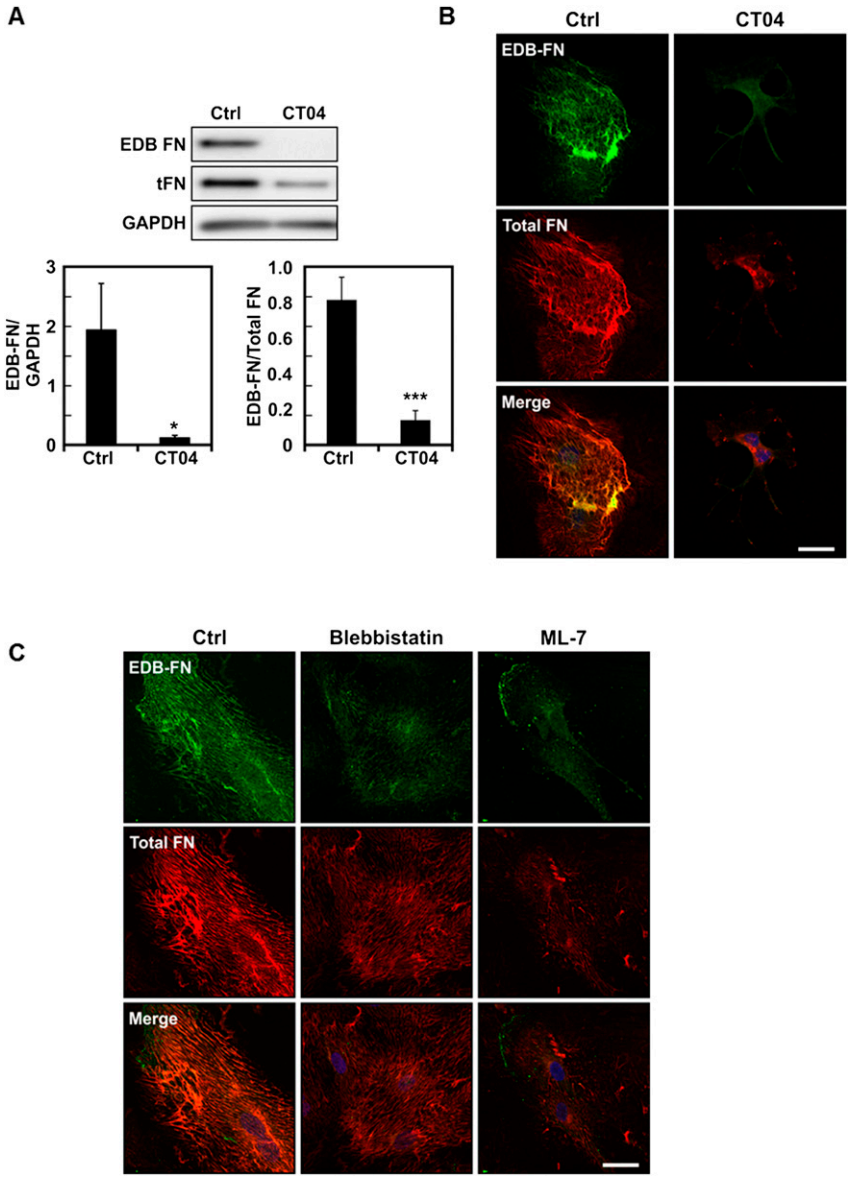

Fig. S3. EDB-FN splice variant expression is mediated by cell contractility. (*A*) Western blot of total protein extract from cells plated on collagen-coated substrates and treated with or without the RhoA inhibitor CT04 showing a decrease in EDB-FN compared with control. Plots are mean ± SE, Student *t* test: $*P < 0.05$, $***P < 0.001$. (*B*) Representative images of EDB-FN and total fibronectin of ECs cultured overnight on collagen-coated substrates and treated with or without CT04. Images were acquired using the same exposure settings. (*C*) Representative images of EDB-FN and total fibronectin of ECs cultured overnight on collagen-coated substrates and treated with blebbistatin or ML-7. Images were acquired using identical exposure settings. (Scale bars, 30 μm.)

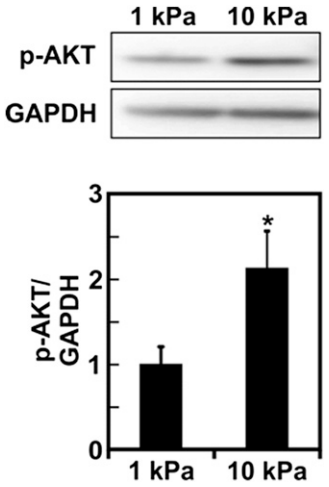

Fig. S4. Substrate stiffness regulates AKT activation. Western blot of whole protein extract from ECs plated on either compliant ($E = 1$ kPa) or stiff ($E = 10$ kPa) substrates showing an increase in AKT phosphorylation with increasing substrate stiffness with the corresponding densitometric quantification. GAPDH was used as the loading control. Plots are mean ± SE, Student t test: $^*P < 0.05$.

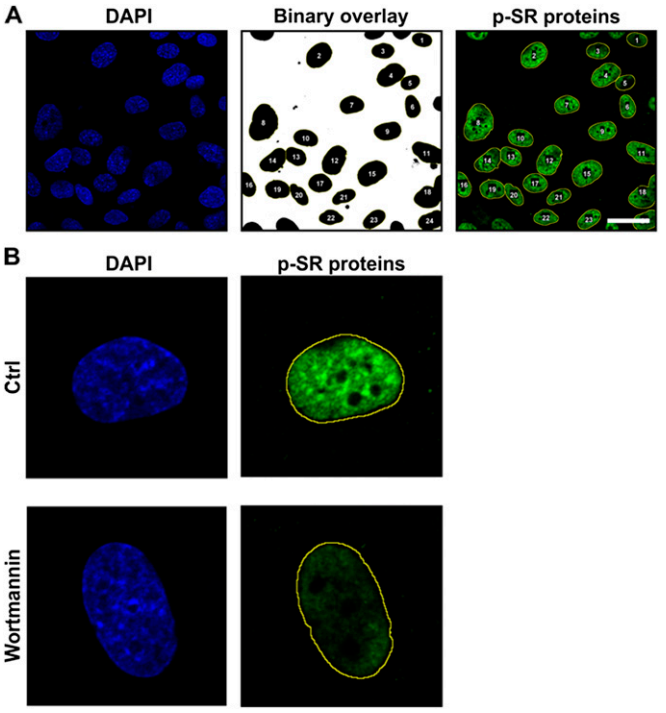

Fig. S5. Quantification of SR protein phosphorylation. (*A*) To perform single cell quantification of the SR phosphorylation signal, a threshold was applied on the DAPI-stained nucleus to obtain a binary image in ImageJ. The binary image was then used to select overlay regions that were then applied on the p-SR channel. The average signal intensity was measured for each region of interest. (Scale bar, 30 μm.) (*B*) Representative zoomed-in confocal images of cells seeded on 10-kPa substrates with or without a 1-h wortmannin treatment showing the overlay regions.