

Supplemental Materials and Methods

TGF β bioassay. To quantify the levels of active and total TGF β , we used mink lung epithelial cells (MLEC) that produce luciferase under the control of the PAI-1 promoter in response to TGF β (gift from Daniel Rifkin, New York University) (Abe *et al.*, 1994). MLEC were plated at 7.8×10^4 cells/cm², allowed to attach for 4 hours, and treated with culture-derived medium. Levels of total TGF β were assessed by heat-activating culture-derived medium for 10 min at 80°C. After exposure, the MLEC were incubated for 18 hours at 37°C. MLEC were then lysed and luciferase activity was measured with a luciferase assay system kit (Promega) using a luminometer (Promega).

Subcellular protein fractionation. The cell membrane, cytoplasmic and nuclear components of whole cell lysates were isolated using a Qiagen Qproteome Cell Compartment Kit (Qiagen) according to the manufacturer's instructions. Lysates were resolved by SDS-PAGE and electroblotted onto nitrocellulose membranes. Immunoblotting analyses were performed using antibodies against Rac1b (Radisky *et al.*, 2005), Rac1 (Cytoskeleton), caveolin (Cell Signaling), GAPDH (Cell Signaling), and lamin A/C (Cell Signaling). The signals were visualized with the ECM Plus Western Blotting Detection System (GE Healthcare).

Knockdown of integrin-linked kinase (ILK). SCp2 cells were transduced with lentiviral particles specific for shILK (Santa Cruz) or scrambled shRNA (Santa Cruz) and stable clones were selected using puromycin (1 μ g/ml; Invitrogen). Knockdown was confirmed using immunoblotting to detect ILK (**Supplemental Figure S7F**).

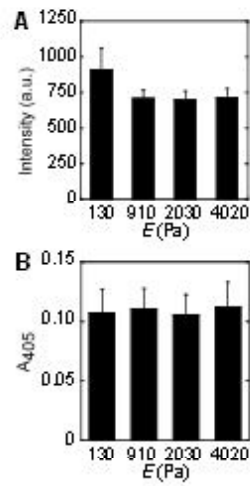
Supplemental References

Abe, M., Harpel, J.G., Metz, C.N., Nunes, I., Loskutoff, D.J., and Rifkin, D.B. (1994). An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Anal. Biochem.* *216*, 276-284.

Radisky, D.C., Levy, D.D., Littlepage, L.E., Liu, H., Nelson, C.M., Fata, J.E., Leake, D., Godden, E.L., Albertson, D.G., Nieto, M.A., Werb, Z., and Bissell, M.J. (2005). Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature* *436*, 123-127.

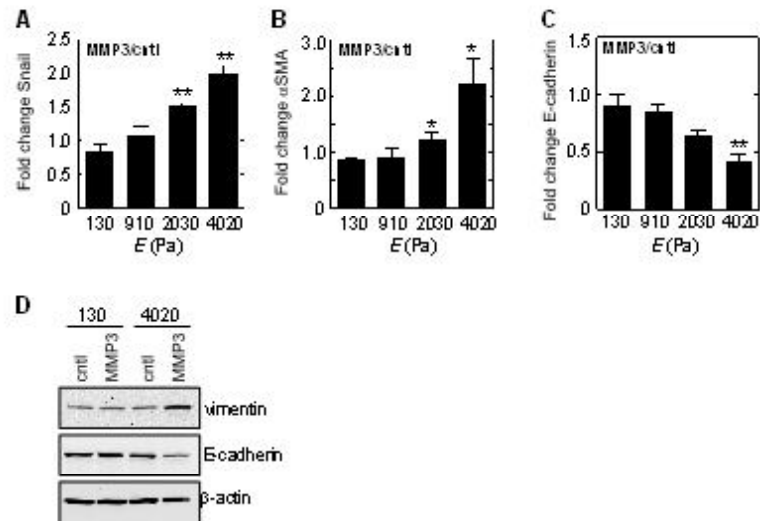
Supplemental Figure Legends

Supplemental Figure S1



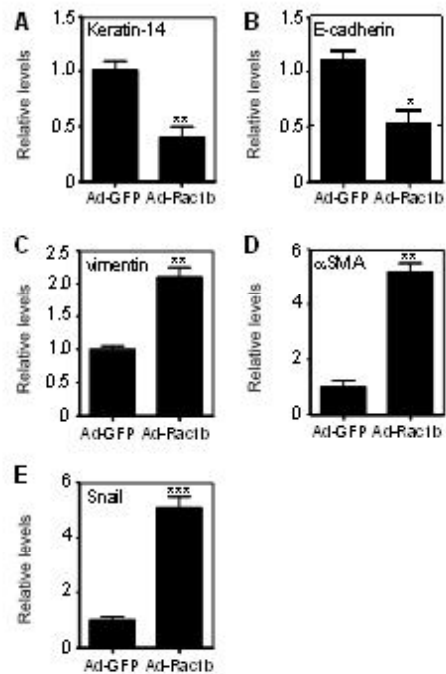
Supplemental Figure S1. Characterization of polyacrylamide substrata. (A) Fluorescence intensity from fibronectin-coated gels showing equivalent coating with fibronectin on different gels. (B) ELISA for fibronectin showing equivalent amounts conjugated to gel surfaces. (n = 4) Error bars represent SEM.

Supplemental Figure S2



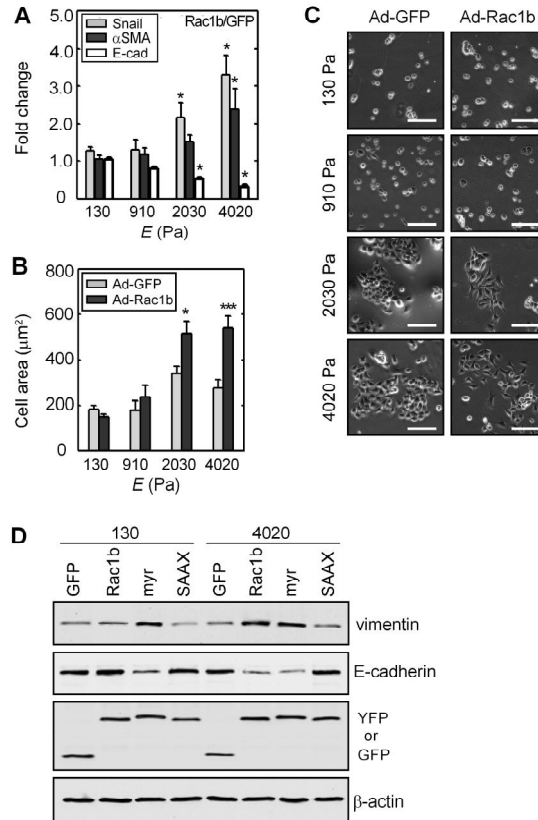
Supplemental Figure S2. Characterization of MMP3-induced EMT on PA gels. Shown are changes in the transcript levels of (A) Snail, (B) α SMA, and (C) E-cadherin in MMP3-treated mammary epithelial cells on substrata of various compliances. (n = 4) Error bars represent SEM (* p<0.05, **p<0.01). (D) Immunoblot analysis of vimentin, E-cadherin, and β -actin for MMP3-treated mammary epithelial cells cultured on soft (130 Pa) or stiff (4020 Pa) substrata.

Supplemental Figure S3



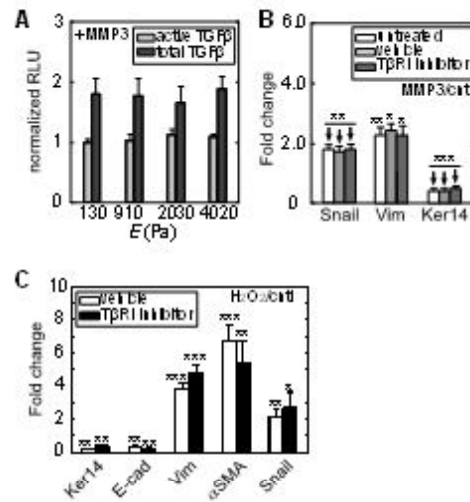
Supplemental Figure S3. Characterization of changes in the expression of EMT markers as a function of ectopic expression of Rac1b (Ad-Rac1b), as compared to control (Ad-GFP) in cells cultured on polystyrene. Shown are relative levels of (A) keratin-14, (B) E-cadherin, (C) vimentin, (D) α SMA, and (E) Snail. (n = 4) Error bars represent SEM (*, p<0.05; **, p<0.01; ***, p<0.001).

Supplemental Figure S4



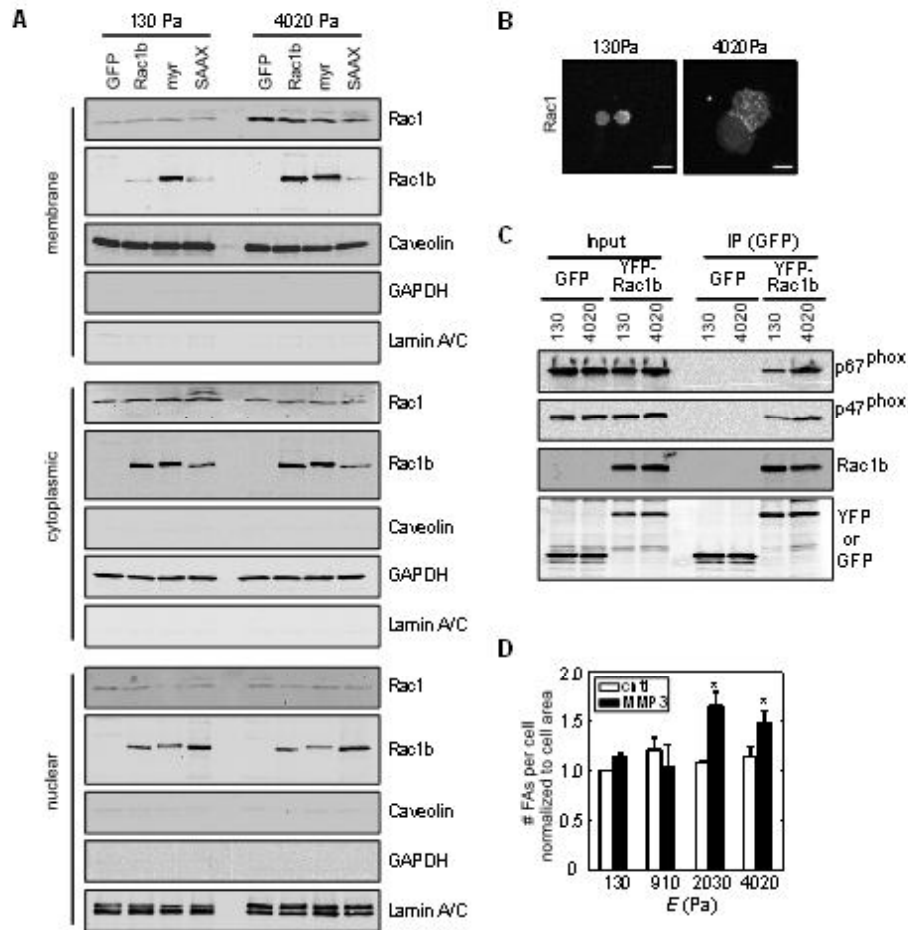
Supplemental Figure S4. Ectopic expression of Rac1b in cells on polyacrylamide gels. (A) Fold change in the transcript levels of Snail, α SMA, and E-cadherin in cells cultured on substrata of various compliances. (n = 4) (B) Changes in projected cell area on substrata of various compliances. (n = 3, 50 cells/n) (C) Phase contrast images of mammary epithelial cells cultured on substrata of various compliances. Scale bars, 100 μm . Error bars represent SEM (*, $p < 0.05$; **, $p < 0.01$). (D) Immunoblot analysis of vimentin, E-cadherin, GFP, and β -actin in SCp2 cells transduced with GFP, YFP-Rac1b, YFP-Rac1b-SAAX, or YFP-Rac1b-myr and cultured on soft (130 Pa) or Stiff (4020 Pa) substrata.

Supplemental Figure S5



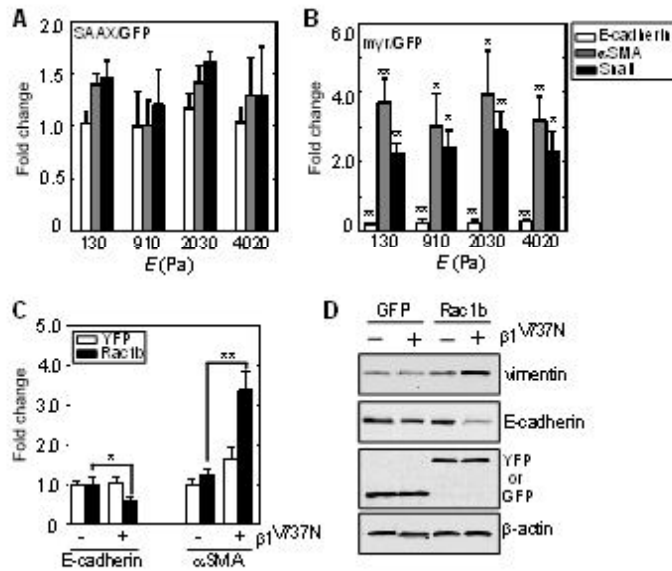
Supplemental Figure S5. TGFβ does not mediate MMP3-induced EMT. (A) Normalized levels of active and total TGFβ in the medium of MMP3-treated mammary epithelial cells cultured on substrata of various compliances. (B) Fold change in transcript levels of Snail, vimentin, and keratin-14 in SCp2 cells treated with or without MMP3 and vehicle or TβRI inhibitor. (C) Fold change in transcript levels of keratin-14, E-cadherin, vimentin, αSMA, and Snail in SCp2 cells treated with or without H₂O₂ in the presence of vehicle or TβRI inhibitor.

Supplemental Figure S6



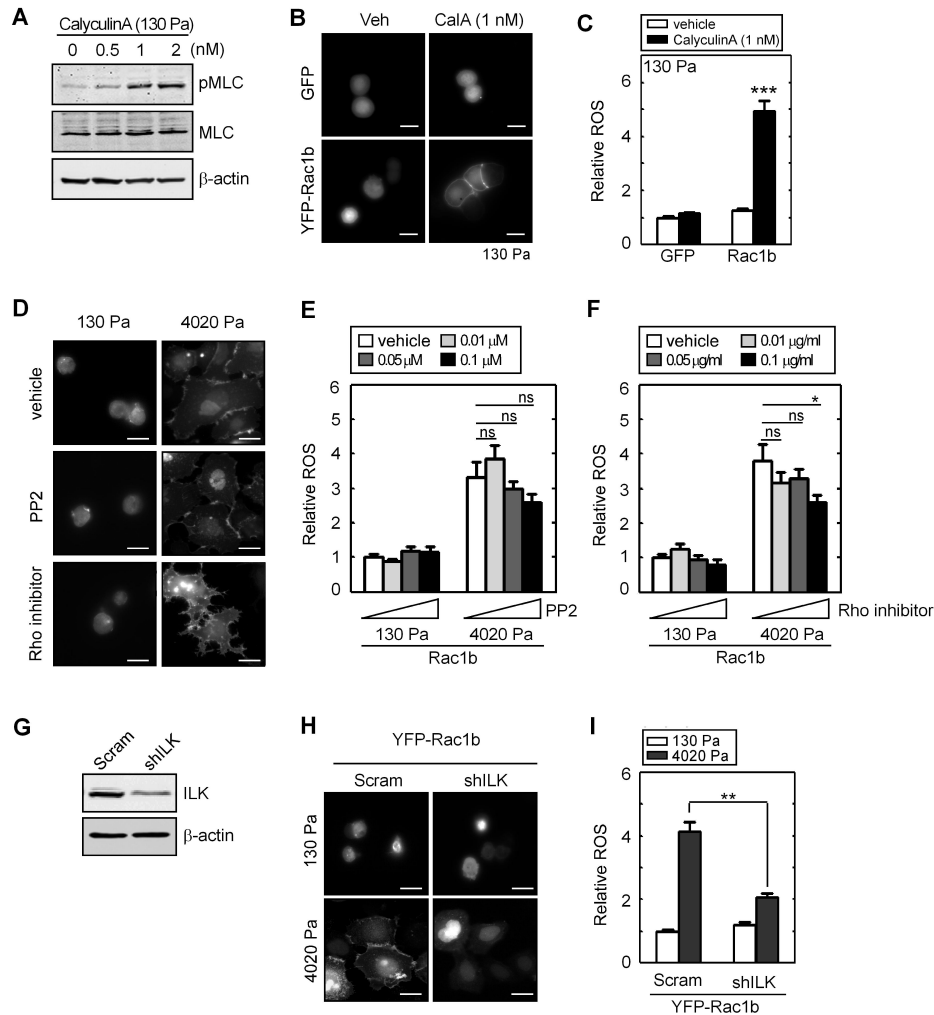
Supplemental Figure S6. Subcellular localization of Rac1 and Rac1b. (A) SCp2 cells were transduced with YFP, YFP-Rac1b, YFP-Rac1b-SAAX, or YFP-Rac1b-myr and grown on soft (130 Pa) or stiff (4020 Pa) substrata. Cells were fractionated and subcellular protein extracts were analyzed by immunoblotting to detect Rac1, Rac1b, caveolin, GAPDH, and lamin A/C. (B) Fluorescence images of GFP-Rac1 in SCp2 cells on soft (130 Pa) or stiff (4020 Pa) substrata. (C) MCF10A cells were transduced with YFP or YFP-Rac1b and grown on soft (130 Pa) or stiff (4020 Pa) substrata. Immunoprecipitation was performed using anti-GFP antibody. Whole cell lysates and immunoprecipitation products were assayed by immunoblotting analysis to detect p67^{phox}, p47^{phox}, Rac1b, and GFP. (D) Quantification of the average number of focal adhesions (FA) per cell area in cells treated with or without MMP3 on substrata of various compliances.

Supplemental Figure S7



Supplemental Figure S7. Membrane localization of Rac1b and clustering of β 1-integrin are required for EMT. SCp2 cells were transduced with GFP, YFP-Rac1b-SAAX, or YFP-Rac1b-myr and cultured on substrata of various compliances. Fold change in the transcript levels of E-cadherin and α SMA for cells ectopically expressing YFP-Rac1b-SAAX (A) or YFP-Rac1b-myr (B). (n = 4) (C) Fold change in the transcript levels of E-cadherin and α SMA for cells simultaneously expressing Rac1b and β 1^{V737N} and cultured on soft substrata. (n = 4). Error bars represent SEM. * p<0.05; ** p<0.01 (D) Immunoblot analysis of vimentin, E-cadherin, GFP, and β -actin in SCp2 cells simultaneously expressing Rac1b and β 1^{V737N} and cultured on soft substrata.

Supplemental Figure S8



Supplemental Figure S8. The role of cell contractility in stiffness regulation of Rac1b

localization and ROS production. (A) Immunoblot analysis of pMLC, MLC, and β-actin in SCp2 cells treated with vehicle or CalyculinA. (B) Fluorescence images of GFP and YFP-Rac1b in SCp2 cells cultured on soft substrata and treated with vehicle or CalyculinA (1 nM). Scale bars, 10 μm. (C) Fold change in cellular ROS levels in SCp2 cells cultured on soft substrata and treated with vehicle or CalyculinA. (n=3, 40 cells/n) (D) Fluorescence images of YFP-Rac1b in SCp2 cells cultured on soft (130 Pa) or stiff (4020 Pa) substrata and treated with vehicle, PP2

(0.1 μ M), or Rho inhibitor (0.1 μ g/ml). Scale bars, 10 μ m. (E-F) Fold change in cellular ROS levels in SCp2 cells cultured on soft (130 Pa) or stiff (4020 Pa) substrata and treated with vehicle, PP2 (E) or Rho inhibitor (F). (n=3, 40 cells/n) (F) Immunoblot analysis of ILK and β -actin in Scp2 cells transduced with shILK or scrambled shRNA. (G) Fluorescence images of YFP-Rac1b in SCp2 cells transduced with shILK or scrambled shRNA and cultured on soft (130 Pa) or stiff (4020 Pa) substrata. Scale bars, 10 μ m. (H) Fold change in cellular ROS levels in SCp2 cells transduced with shILK or scrambled shRNA and cultured on soft (130 Pa) or stiff (4020 Pa) substrata (n=3, 40 cells/n) Error bars represent SEM. * p<0.05; ** p<0.01; *** p<0.001.

Supplemental Table S1. Primers used for quantitative real-time PCR

Gene	Sequences
18S rRNA	Fwd: TCAGATACCGTCGTAGTTC Rev: CCTTTAAGTTTCAGCTTTGC
Keratin 14	Fwd: TTACCTCCTCTCGCTTCTC Rev: AAGACCACCACCAAGACC
E-cadherin	Fwd: CAACGAATCCCTCAAAGACC Rev: GATAGAGAAGCCATTGAAAA
Snail	Fwd: CCTGGTTCCTGCTTGGCTCTC Rev: GGCTCTGGGCGGGTACAA AG
vimentin	Fwd: CCCTGAACCTGAGAGAACTA AC Rev: GGTCATCGTGATGCTGAGAAG
αSMA	Fwd: GGCACCACTGAACCCTAAG Rev: CCAGAGTCCAGCACAATACC
Rac1b	Fwd: GGCACCACTGAACCCTAAG Rev: CCAGAGTCCAGCACAATACC