Supporting Information

Differential Depth Sensing Reduces Cancer Cell Proliferation *via* Rho-Rac Regulated Invadopodia

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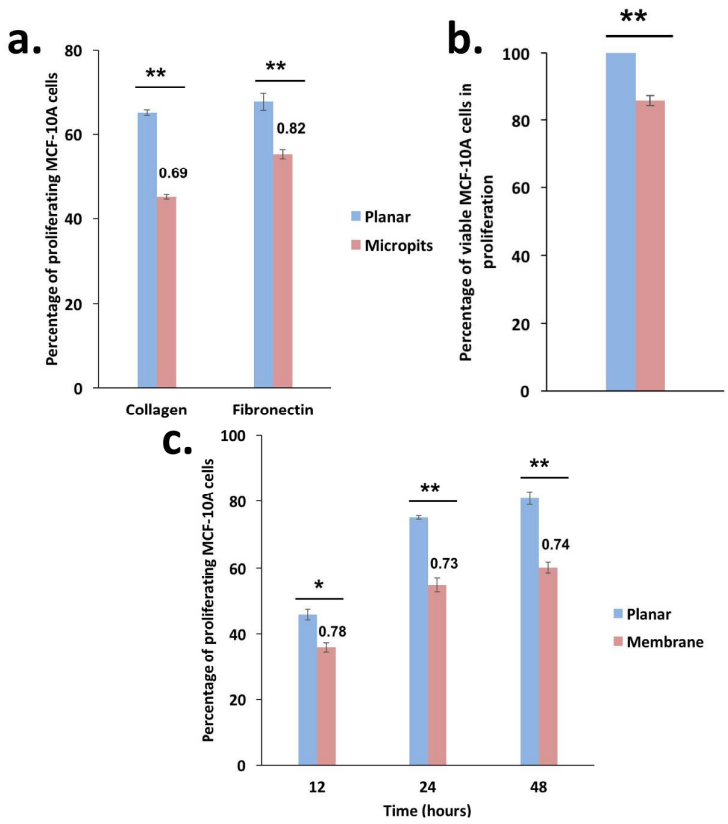

Figure S1: Micropits reduces MCF-10A proliferation across different ECM coatings and time points. (a) Micropits were coated with collagen and fibronectin to facilitate cell adhesion and spreading. Greatest reduction in MCF-10A proliferation occurred on collagen coated micropits. **(b)** Viable MCF-10A cells in proliferation reduces on 9 μm depth micropits, shown using MTS assay. **(c)** Membranes of 2 μm pore size induces sustained reduction of MCF-10A proliferation across different time points ranging from 12 to 48 hours. Data are means ± s.e.m. (n= 3). For each experiment, 300 cells were considered on an average. P values were obtained using student's unpaired t-test. **p< 0.01, *p< 0.05 with respect to planar. Blue bar indicates planar and red indicates micropits or membrane as indicated (W = 2 μm, D = 9 μm). The number on top of the respective bar indicates mean fold change with respect to planar.

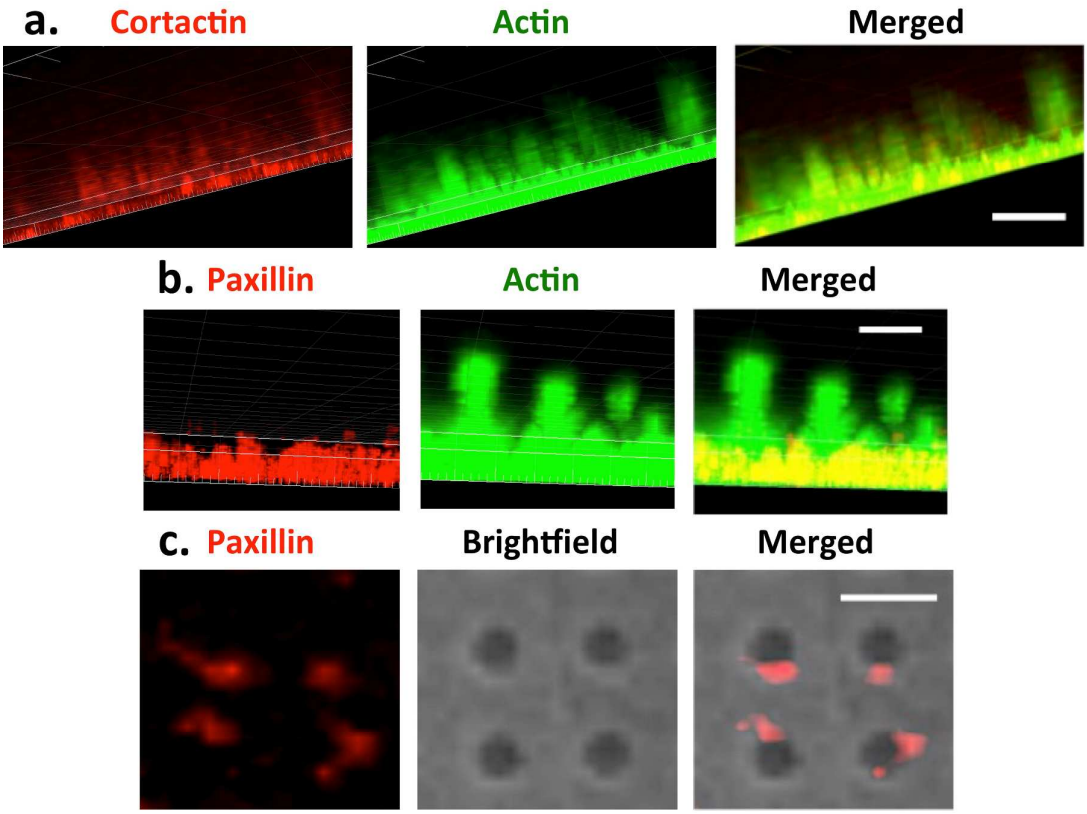

Figure S2: Cortactin co-localizes with actin along the protrusions and paxillin accumulates around the micropits. (a) MCF-10A protrusions on 1.5 μm depth micropits were immunostained for cortactin (red) and actin (phalloidin, green) (Scale 5 μm). **(b)** Cross-sectional view of paxillin (immunostained in red) accumulation near the base of the protrusions for micropits of 9 μm depth. F-actin is stained with phalloidin (green) (Scale 10 μm). **(c)** Paxillin (immunostained in red) accumulates near the base of the protrusions for micropits depth of 1.5 μm but do not form the adhesion ring (Scale 5 μm).

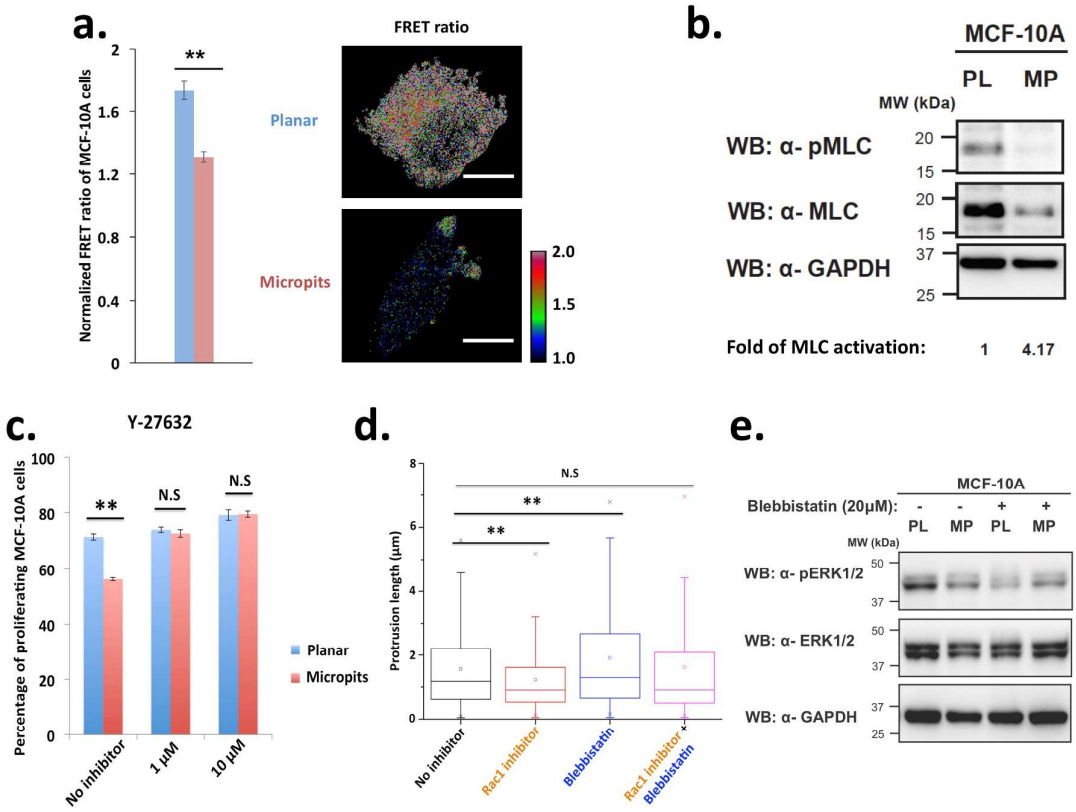

Figure S3: MCF-10A proliferation and invadopodia length is regulated by actomyosin contractility. (a) Micropits reduces Rac1 activity for MCF-10A cells. Cells were transfected with Rac1 biosensor (pTriEx4-Rac1-2G) and seeded on collagen coated substrates for 24 hours. FRET ratios were determined as described in Methods. (Scale 10 μm) **(b)** Lysates from MCF-10A cells grown on planar (PL) or micropits (MP) were separated by gradient gel and immunoblotted (IB) with antibodies for phospho-MLC (pMLC), total MLC and α-GAPDH as loading control. Under each condition, the level of Rho/ROCK activity is measured by the ratio of pMLC to total MLC. The fold of such activity in MP is deduced by conferring this ratio under MP to the ratio in PL. **(c)** 9 μm depth micropits decreases MCF-10A proliferation *via* activation of actomyosin contractility. Acto-Myosin contractility inhibitor treatment: Y-27632 (1, 10 μM). Data are means ± s.e.m. (n= 3). For each experiment, 300 cells were considered on an average. P values were obtained using student's

unpaired t-test. **p< 0.01 with respect to planar. Blue bar indicates planar and red indicates micropits (W = 2 µm, D = 9 µm). N.S. denotes non-significant difference compared to planar. **(d)** Actomyosin contractility decreases protrusion length. Acto-Myosin contractility modulating drug treatment: Rac1 inhibitor (NSC23766; 50 µM) and Blebbistatin (20 µM). **(e)** Lysates from MCF-10A cells grown on planar (PL) or micropits (MP) were separated by SDS-PAGE gel and immunoblotted (IB) with antibodies for phospho-ERK1/2 (pERK1/2), total ERK1/2 (pan ERK1/2) and α-GAPDH as loading control.

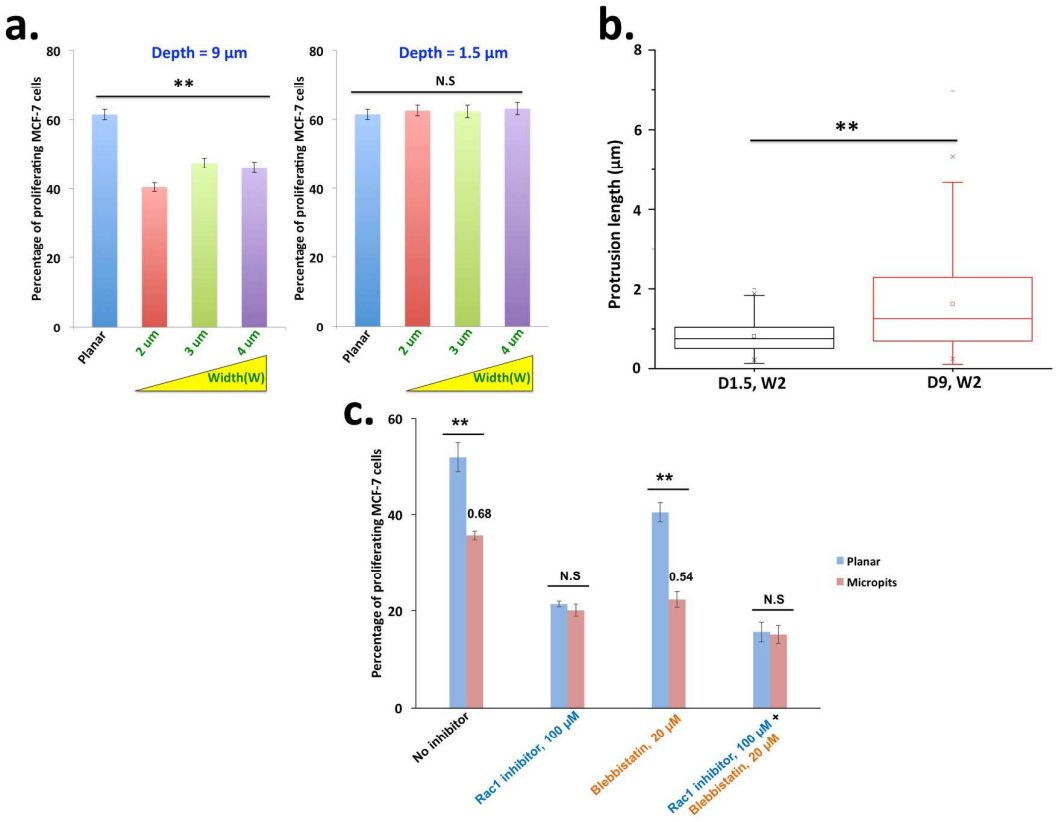

Figure S4: Non-metastatic breast cancer cell (MCF-7) proliferation decreases on 9 µm depth micropits but not on 1.5 µm depth. (a) Micropits depth of 9 µm (but not of 1.5 µm) reduces MCF-7 proliferation across different width (2, 3, 4 µm). **(b)** Protrusion length is greater on 9 µm depth micropits at a constant width of 2 µm. **(c)** Micropits decreases MCF-7 proliferation by inactivating actomyosin contractility. Acto-Myosin contractility modulating

drug treatment: Rac1 inhibitor (NSC23766; 100 μM) and Blebbistatin (20 μM). Data are means ± s.e.m. (n= 3). For each experiment, 300 cells were considered on an average. P values were obtained using student's unpaired t-test. **p< 0.01 with respect to planar. N.S. denotes non-significant difference compared to planar. The number on top of the respective bar indicates mean fold change with respect to planar.

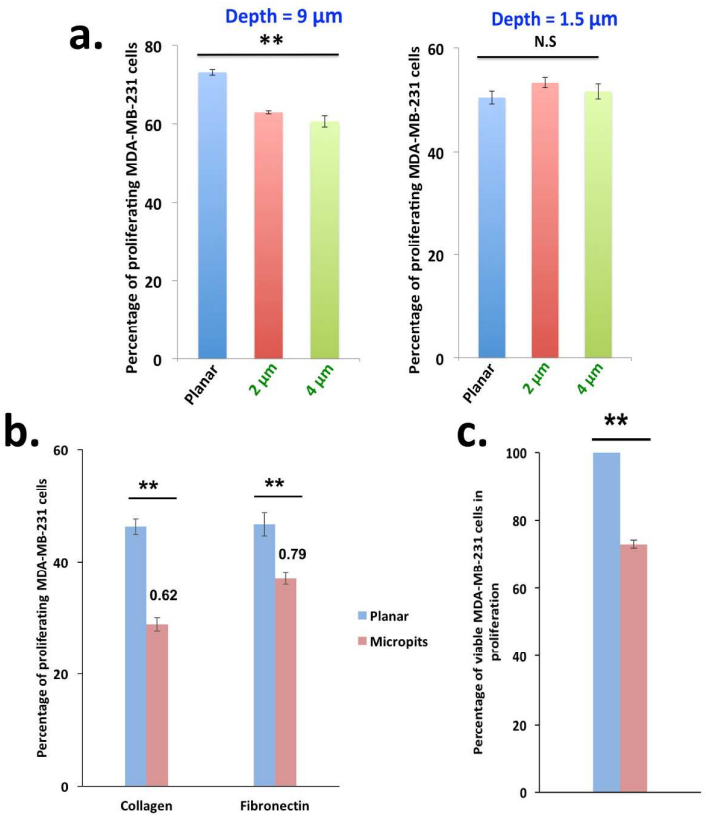

Figure S5: Metastatic breast cancer cell (MDA-MB-231) proliferation decreases on 9 μm depth micropits but not on 1.5 μm depth across different ECM coatings. (a) Micropits depth of 9 μm (but not of 1.5 μm) reduces MDA-MB-231 cell proliferation across different width (2, 4 μm). **(b)** Micropits were coated with collagen and fibronectin to facilitate cell attachment. Greatest reduction in MDA-MB-231 proliferation occurred on collagen coated micropits. **(c)** Viable MDA-MB-231 cells in proliferation reduces on 9 μm

depth micropits, shown using MTS assay. Data are means ± s.e.m. (n= 3). For each experiment, 300 cells were considered on an average. P values were obtained using student's unpaired t-test. **p< 0.01 with respect to planar. Blue bar indicates planar and red indicates micropits (W = 2 μm, D = 9 μm). The number on top of the respective bar indicates mean fold change with respect to planar.

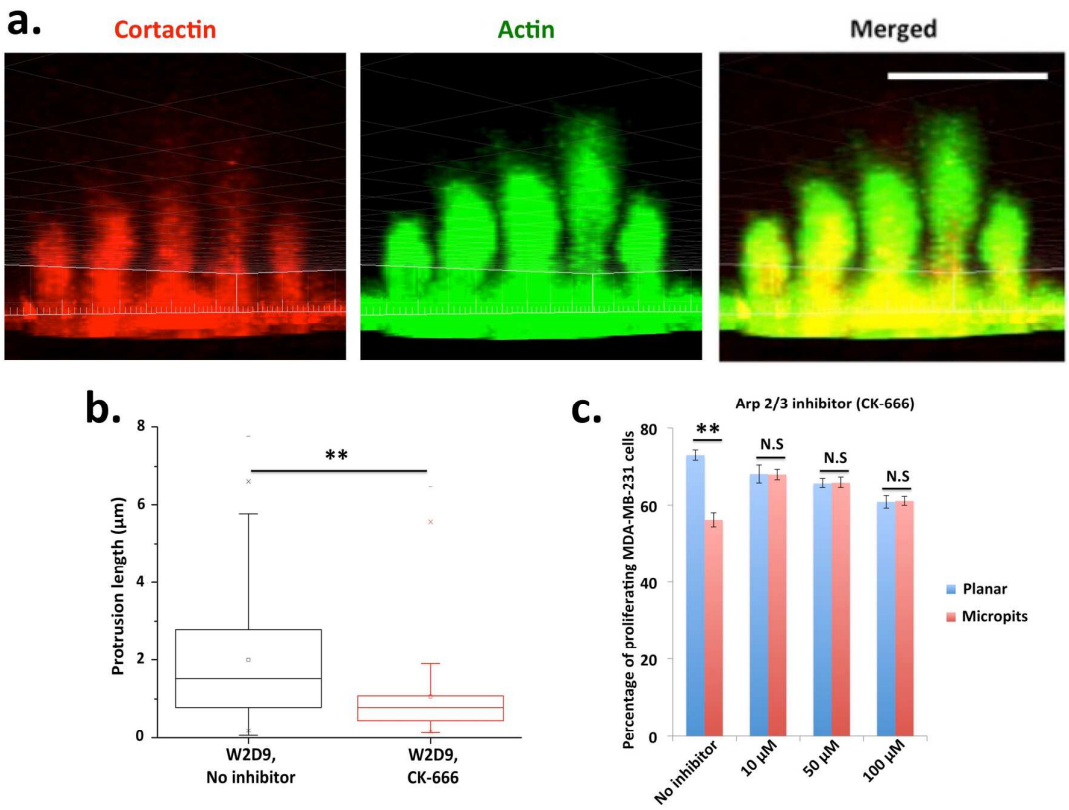

Figure S6: MDA-MB-231 proliferation decreases on micropits *via* invadopodia formation. (a) Cortactin (immunostained in red) co-localizes with actin (phalloidin in green) in the protrusions of 9 μm depth micropits (Scale 10 μm). **(b)** Arp 2/3 inhibitor (CK-666; 50 μM) partially inhibits the protrusion length. **(c)** Arp2/3 inhibitor (CK-666; 10, 50, 100 μM) prevents the depth induced proliferation reduction of MDA-MB-231 cells. Data are means ± s.e.m. (n= 3). For each experiment, 300 cells were considered on an average. P values were

obtained using student's unpaired t-test. **p< 0.01 with respect to planar. Blue bar indicates planar and red indicates micropits (W = 2 µm, D = 9 µm). N.S. denotes non-significant difference compared to planar.

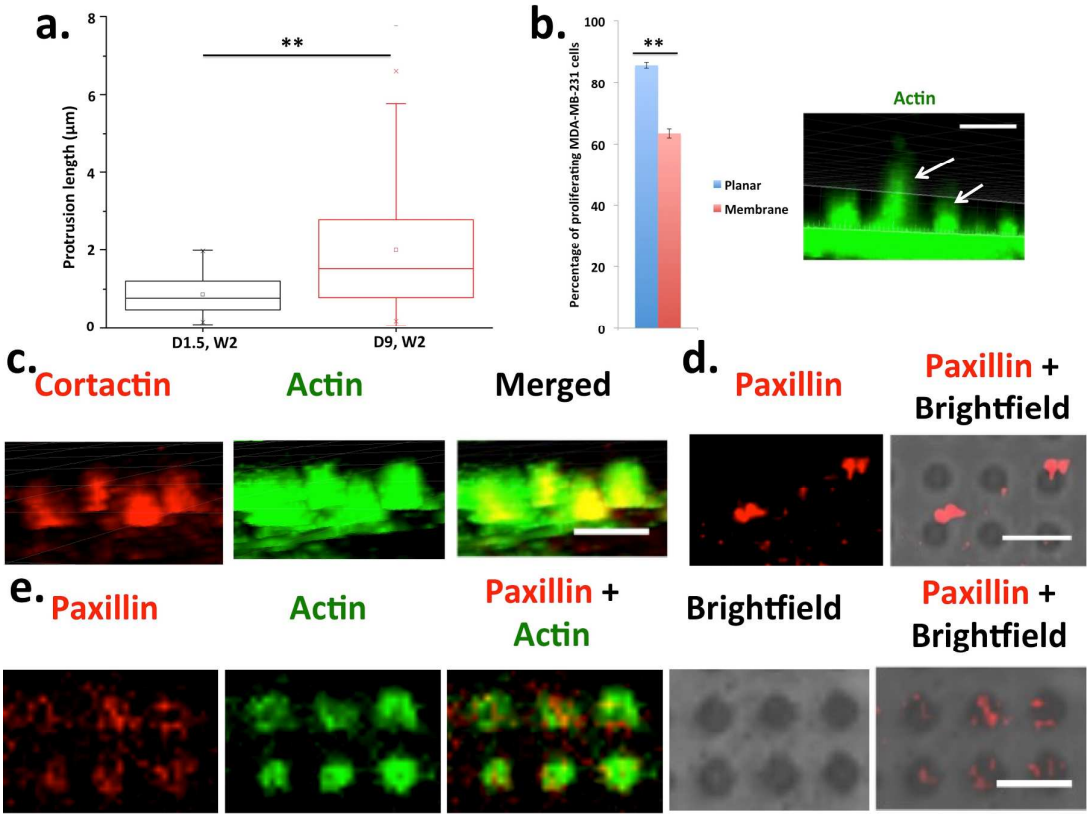

Figure S7: Invadopodia decreases MDA-MB-231 proliferation independent of biochemical cues. (a) Protrusion length is greater on 9 µm depth micropits at a constant width of 2 µm. **(b)** MDA-MB-231 proliferation is reduced by membranes of 2 µm pore size. Confocal microscopy images of the protrusion lengths (indicated with white arrows) stained with phalloidin (green) for actin filaments (Scale 10 µm). Data are means ± s.e.m. (n= 3). For each experiment, 200 cells were considered on an average. P values were obtained using student's unpaired t-test. **p< 0.01 with respect to planar. **(c)** Cortactin (immunostained in red) co-localizes with actin (phalloidin in green) in the protrusions of 1.5 µm depth micropits

(Scale 5 μm). **(d)** Paxillin (immunostained in red) accumulates near the base of the protrusions for micropits depth of 1.5 μm (Scale 5 μm). **(e)** Paxillin (immunostained in red) forms a ring at the base of the protrusions for micropits depth of 9 μm. Actin filaments are co-stained with phalloidin (green) (Scale 5 μm).

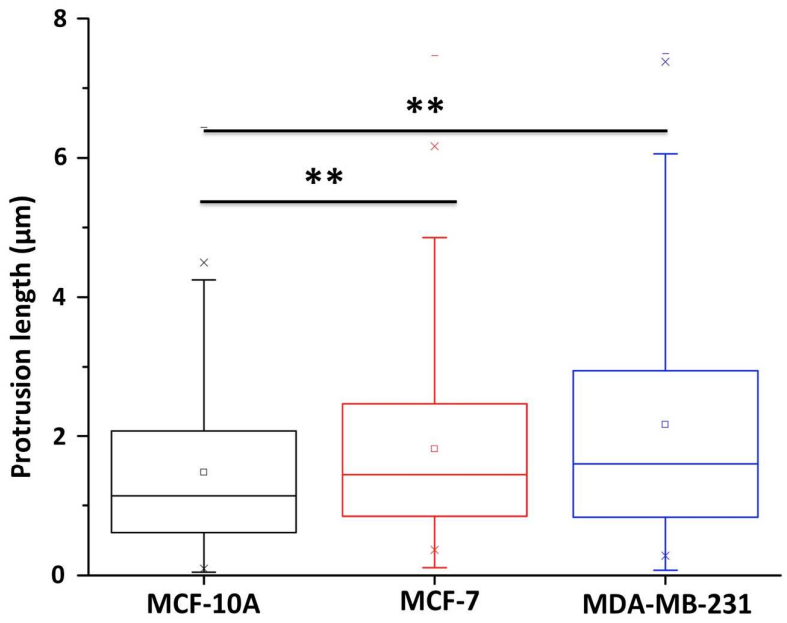

Figure S8: Protrusion length increases proportionally to metastatic potential. Cells were cultured on micropits of W = 2 μm, D = 9 μm. P values were obtained using student's unpaired t-test. **p< 0.01.

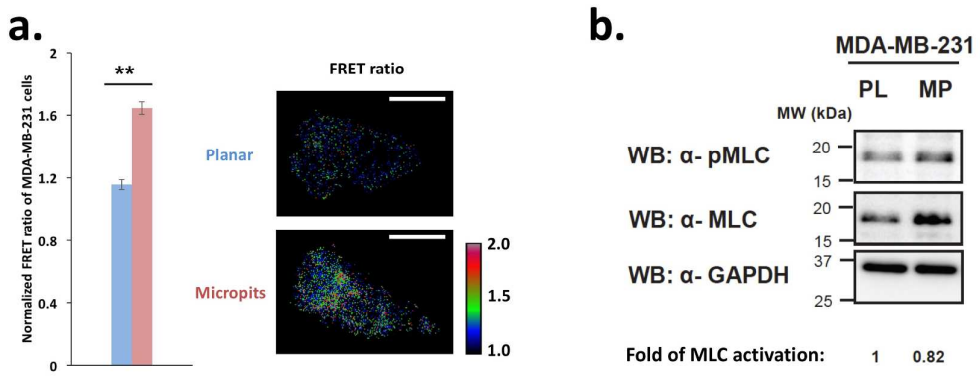

Figure S9: Micropits reduces MDA-MB-231 proliferation *via* differential regulation of Rac1 activity and actomyosin contractility. (a) Micropits induces Rac1 activity for MDA-MB-231 cells. Cells were transfected with Rac1 biosensor (pTriEx4-Rac1-2G) and seeded on collagen coated substrates for 24 hours. FRET ratios were determined as described in Methods. (Scale 10 μm) **(b)** Lysates from MDA-MB-231 cells grown on planar (PL) or micropits (MP) were separated by SDS-PAGE gel and immunoblotted (IB) with antibodies for phospho-MLC (pMLC), total MLC and α-GAPDH as loading control. Under each condition, the level of Rho/ROCK activity is measured by the ratio of pMLC to total MLC. The fold of such activity in MP is deduced by conferring this ratio under MP to the ratio in PL. P values were obtained using student's unpaired t-test. **$p < 0.01$ with respect to planar. Blue bar indicates planar and red indicates micropits (W = 2 μm, D = 9 μm).

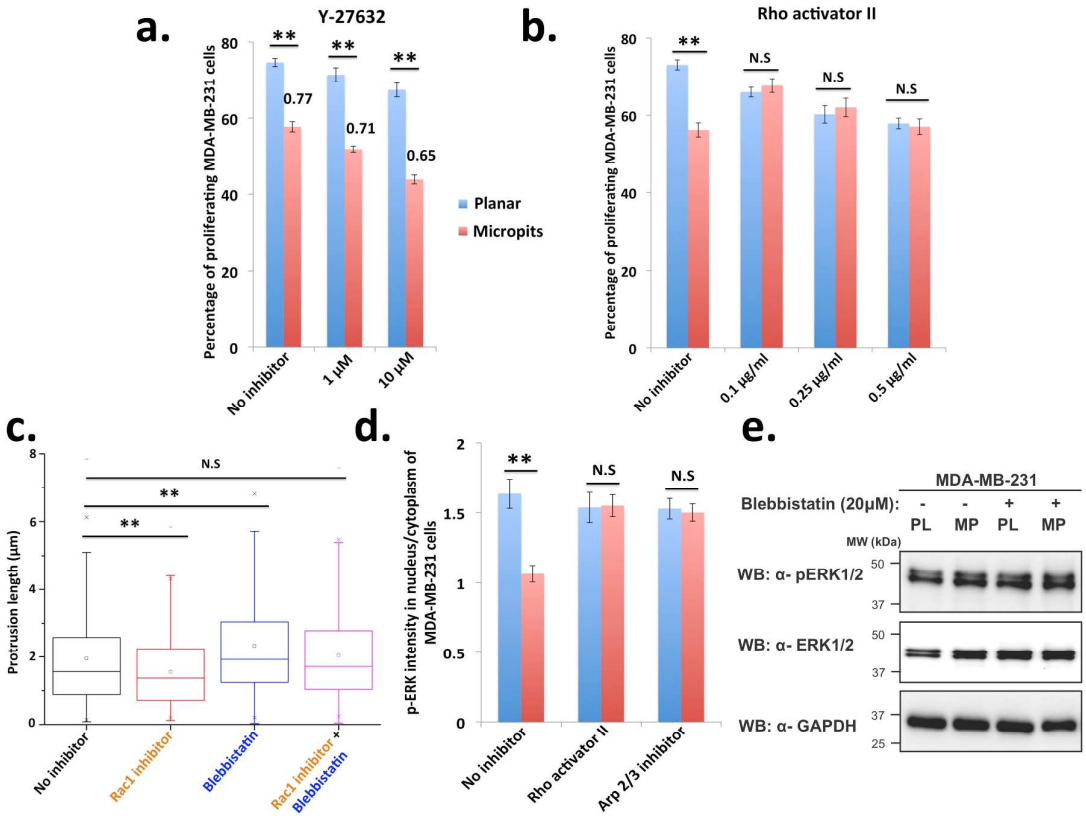

Figure S10: Actomyosin contractility and p-ERK signaling regulate MDA-MB-231 proliferation and invadopodia length. 9 μm depth micropits decreases MDA-MB-231 proliferation *via* inactivation of actomyosin contractility. Acto-Myosin contractility modulating drug treatment: **(a)** Y-27632 (1, 10 μM), **(b)** Rho activator II (0.1, 0.25, 0.5 μg/ml). Data are means ± s.e.m. (n= 3). For each experiment, 300 cells were considered on an average. The number on top of the respective bar indicates mean fold change with respect to planar. **(c)** Actomyosin contractility decreases protrusion length in MDA-MB-231 cells. Acto-Myosin contractility modulating drug treatment: Rac1 inhibitor (NSC23766; 50 μM) and Blebbistatin (20 μM). **(d)** p-ERK fluorescence intensity quantification in nucleus/cytoplasm of MDA-MB-231 cells in absence and presence of different drugs respectively: Rho activator II (0.25 μg/ml), Arp2/3 inhibitor (CK-666; 50 μM). Over 25 cells were analyzed for each condition. P values were obtained using student's unpaired t-test.

p< 0.01 with respect to planar. Blue bar indicates planar and red indicates micropits (W = 2 μm, D = 9 μm). N.S. denotes non-significant difference compared to planar. **(e) Lysates from MDA-MB-231 cells grown on planar (PL) or micropits (MP) were separated by SDS-PAGE gel and immunoblotted (IB) with antibodies for phospho-ERK1/2 (pERK1/2), total ERK1/2 (pan ERK1/2) and α-GAPDH as loading control.

Table I: Dimension of micropits patterns

No.	Width (μm)	Space (μm)	Depth (μm)
1	2	2	1.5
2	2	2	3.8
3	2	2	5.7
4	1.5	2.3	7.0
5	2	1.8	9.0