E-Cadherin-Dependent Stimulation of Traction Force at Focal Adhesions via the Src and PI3K Signaling Pathways

Audrius Jasaitis,[†] Maruxa Estevez,[‡§] Julie Heysch,[†] Benoit Ladoux,[‡§] and Sylvie Dufour[†]

[†]Unité Mixte de Recherche 144, Centre National de la Recherche Scientifique, Institute Curie, Paris, France; [‡]Laboratoire Matière et Systèmes Complexes, Unité Mixte de Recherche 7057, Centre National de la Recherche Scientifique, Université Paris Diderot, Paris, France; and [§]Mechanobiology Institute, National University of Singapore, Singapore

SUPPORTING MATERIAL

MATERIALS AND METHODS

Cell lines, plamids and stable transfections. All cell lines were cultured at 37°C, under an atmosphere containing 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; lonza), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Confluent cultures were routinely replated, following treatment with 0.05% trypsin + 0.02% EDTA (Gibco, Invitrogen). Cells were plated overnight on µFSA arrays before experiments. Rho kinase inhibitor (Y27632, Calbiochem) and PI3K inhibitor (LY294002, Calbiochem) were used at a concentration of 15 µM and Src kinase inhibitor I (SKI-1; 4-(4-phenoxyanilino)-6,7-dimethoxyquinazoline, Sigma-Aldrich) was used at a concentration of 200 nM. All inhibitors were added to the sample 30 min before measurements were taken. Stock solutions of Y27632 and LY294002 were made in DMSO (20 mM). Cells were transiently transfected in the presence of Lipofectamine (Life Technologies), as previously described (23).

Antibodies and reagents. The mAbs recognizing LCAM and cadherin-7 were purchased from Hybridoma Bank. The mAb NCD2 directed against chicken N-cadherin was generously provided by M. Takeichi (Center for Developmental Biology, RIKEN, Kobe, Japan). The mAbs directed against β-catenin, paxillin, α5, αV and activated β1 integrin (clone 9EG7) were obtained from Becton Dickinson Biosciences. The mAb directed against α-catenin was purchased from AbCAM. The rabbit polyclonal antibody recognizing the phosphorylated form of FES was generously provided by M. Arpin (UMR144, Institut Curie, CNRS, France)(1). The mAb directed against Y397 FAK was obtained from Biosource. Fluorescent secondary antibodies and phalloidin were purchased from Molecular Probes. Src kinase inhibitor I, LY294002 and Rho kinase inhibitors were obtained from Calbiochem. Plasmids encoding GFP-tagged paxillin and GFP-UtrCH were generously provided by A. Bershadsky and W.M Bement (2), respectively.

Preparation of poly(dimethylsiloxane) (PDMS) micropillars for µFSA. A liquid silicone prepolymer, PDMS (Sylgard 184, Dow-Corning), was poured over the silicon template for pillar arrays, cured by heating at 65°C for 12 h (giving a Young's modulus of PDMS of 2±0.1 MPa), and was then peeled off the template in ethanol, to prevent the pillars from sticking. The ethanol was gradually replaced by PBS and the tops of the pillars were fluorescently labeled with a mixture (1:10 molar ratio) of bovine plasma FN (Sigma) and Alexa 594-FN (Molecular Probes) for 10 min. The excess liquid was then removed and the mold was dried with a stream of nitrogen and gently placed in contact with µFSA arrays for 10 min.

Traction force measurements. Cells were incubated in CO_2-independent medium (Gibco, Invitrogen) supplemented with 1% FBS and viewed with an Olympus BX51 upright microscope (Olympus, France) equipped with an on-stage chamber kept at 37°C. Image stacks were acquired with a frame delay of 30 s over a period of about one hour. Observations were made with an Olympus 60 x water immersion objective (NA 1.1). Images were acquired with a Photometrics Coolsnap ES CCD camera (Roper Scientific, France) and Metamorph software (Universal Imaging). Images were analyzed with ImageJ software. The stack of images was first aligned and the grayscale images were converted to binary (black and white) images with an appropriate threshold. The MTrack2 plugin for ImageJ was used to track the moving pillars. Further numerical analysis was performed with Excel (Microsoft Corp., USA). The displacement of all pillars in the field of view, both those below the cells and

those not covered by cells, was automatically analyzed by the ImageJ software. The spatial resolution for pillar displacement is 220 nm, which correspond to a traction force resolution of 5nN. From comparative analysis of pillar displacement on fields devoid of cells and fields containing Ecad, Cad7, Ncad and S180 cells, we plotted forces above 16 nN on graphs, to ensure clarity. The statistical significance of differences was assessed in two-tailed Mann-Whitney U tests.

Preparation of EcadFC-coated beads and cell stimulation. Polystyrene beads ($3x10^8$) were washed, suspended in 0.1 M borate buffer pH8 and then incubated with 100 µg of goat anti-FC fragment-specific antibody (Jackson ImmunoResearch Laboratories, Inc.) with gentle mixing for 18 h at room temperature. Beads were then washed three times with 2 ml PBS and incubated with PBS+5%BSA for 1 h at room temperature (control-beads). $6x10^7$ control-beads were then incubated with 30 µg of the Ecad-FC fragment (EcadFC-beads) for 2 h at room temperature washed 3 times in 2 ml PBS+BSA and suspended in PBS+BSA for storage (up to one week). Ecad cells were plated overnight on µFSA arrays at very low density in order to avoid cell-cell contacts on FN-pillars array. Then control-beads or EcadFC-coated beads (EcadFC-beads) were added to the cells (10/1 ratio) and let to settle onto cells. After 20-30 min sedimented beads were gently suspended in order to make more cell-bead interactions. The cycle was repeated twice and the non-adherent beads were removed prior to perform time-lapse imaging.

Immunofluorescence labeling viewing. Samples were viewed under a Leica DM6000 epifluorescence microscope or a confocal Nikon Eclipse Ti inverted microscope.

Polyacrylamide gels. Glass coverslips were activated by incubation with 0.5% glutaraldehyde in PBS before gel preparation. FN was bound to the gel (FN-PAG) by treatment with sulfo-SANPAH. After activation, we incubated the surface of the gel with 0.005% FN in PBS for 1 h at 37°C.

SUPPORTING FIGURES, THEIR LEGENDS AND MOVIE 1 LEGEND.

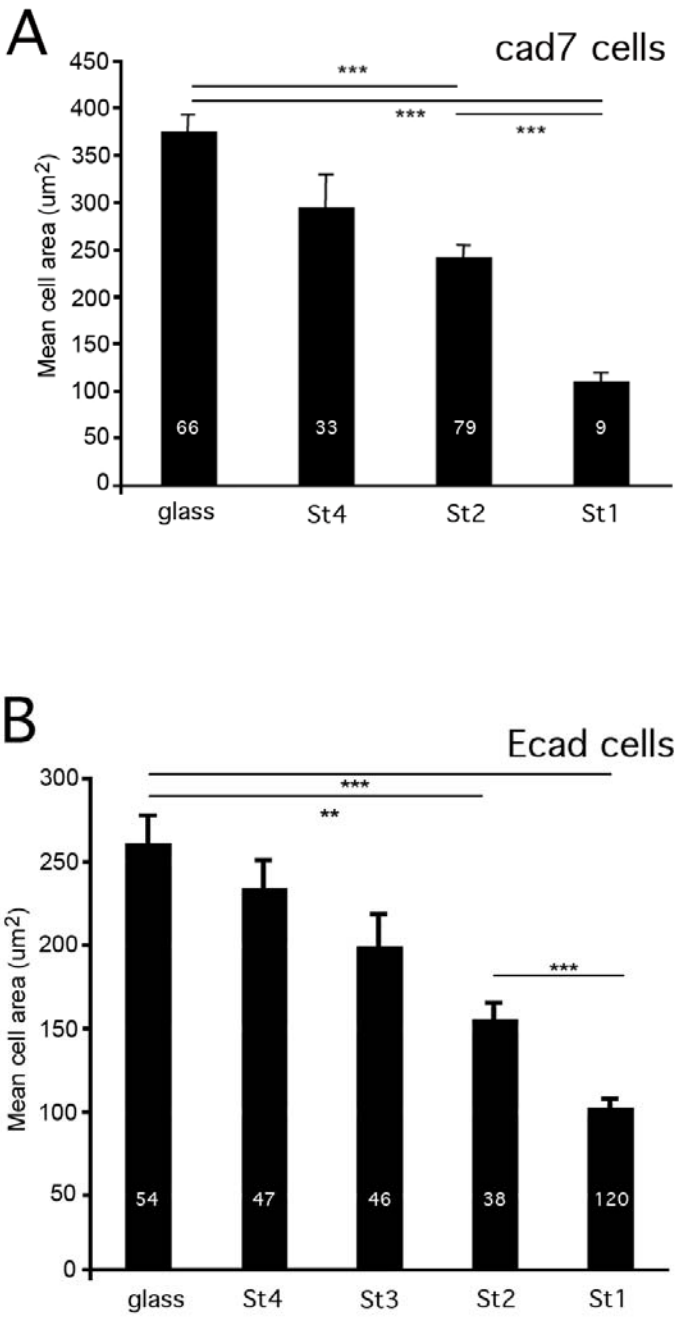

FIGURE S1. The adhesion behavior of cadherin-expressing cells depends on external rigidity. A, Histograms of the mean Cad 7 cell area in μm^2 on glass or FN-PAG of various stiffnesses (St1 to St4 represent stiffness 1, 2, 3 and 4 with a Young's modulus of 2.8, 7.5, 16.7 and 23.4 kPa, respectively). B. Histograms of the mean Ecad cell area in μm^2 on glass or FN-PAG of different stiffnesses. Error bars indicate the SEM. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ (two-tailed). The number indicated in the bars in A and B represents the number of cells analysed.

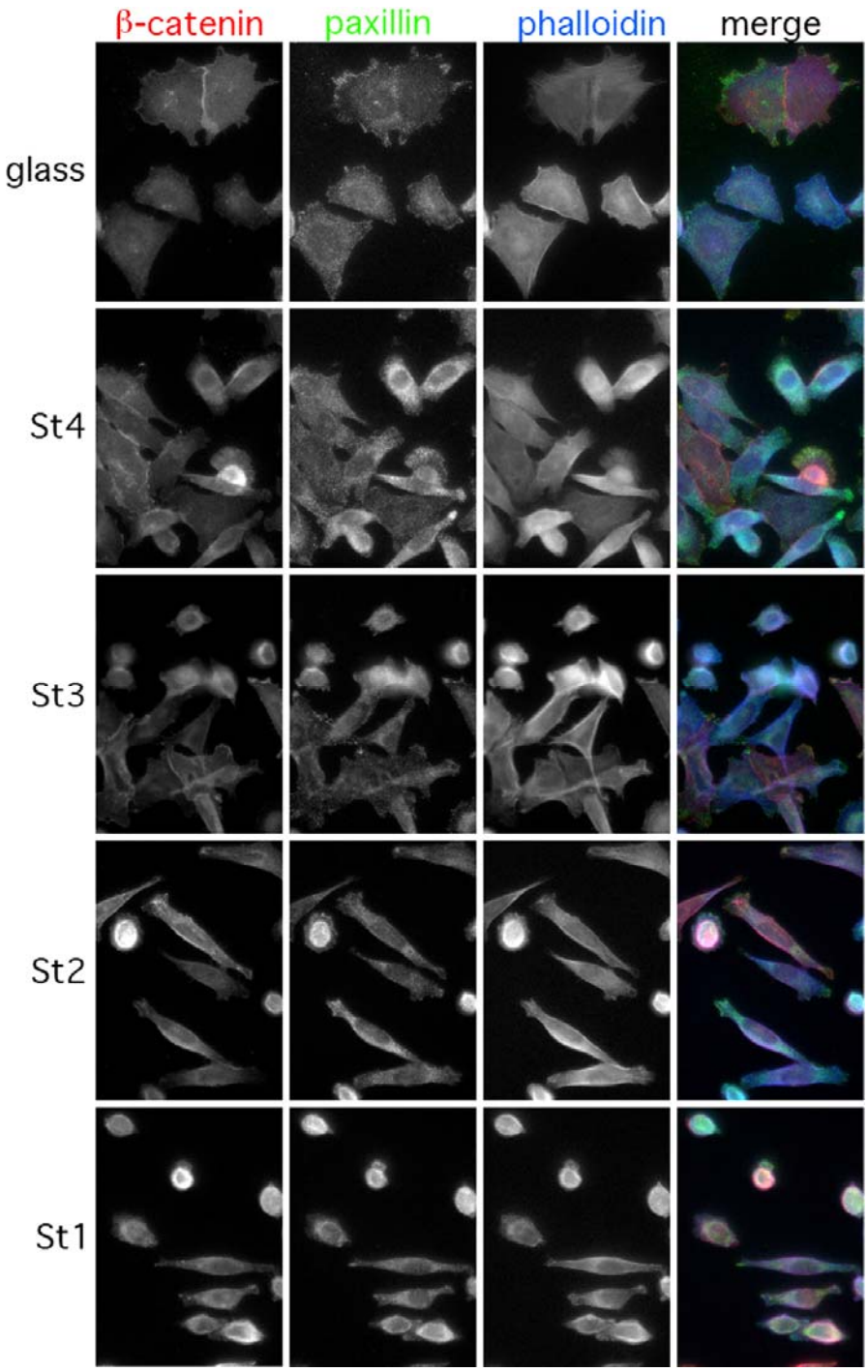

Figure S2. Localization of focal adhesion proteins on Ecad cells plated on FN-coated glass and on FN-PAG of different stiffnesses (st1, 2, 3 and 4 for stiffnesses 1, 2, 3 and 4, respectively). Black &White images show the immunofluorescence staining for β-catenin, paxillin, phalloidin and merge images (β-catenin (red), paxillin (green), phalloidin (blue)).

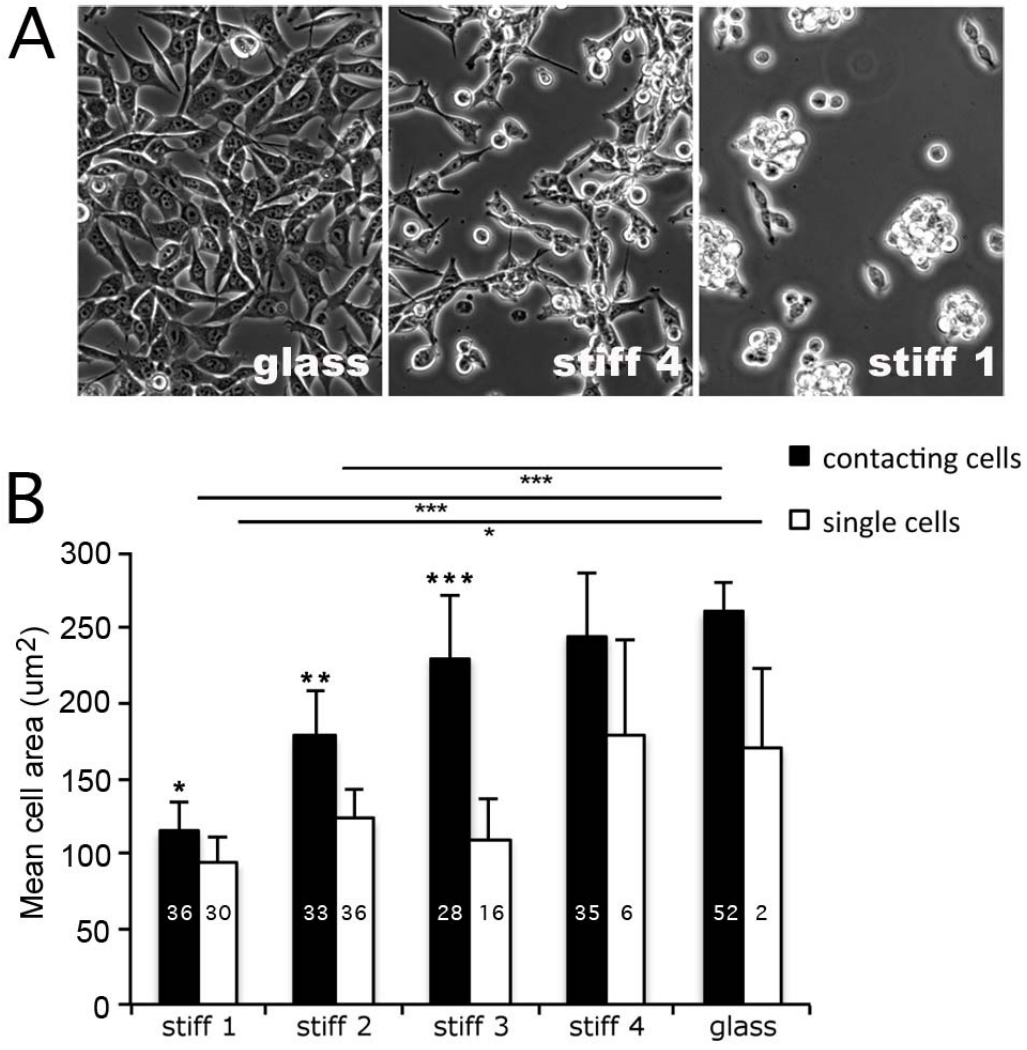

FIGURE S3. External rigidity modifies intercellular adhesive properties. A. Effect of substrate stiffness on Ecad cell agregation properties. B. Histograms of the mean spreading area in μm^2 of Ecad cells in contact with neighbors (black bars) and of single Ecad cells (white bars) on glass or FN-PAG of stiffness St1 to St4. Error bars indicate the SEM. ***, $p < 0.001$; *, $p < 0.05$ (two-tailed). The number indicated in the bars in B represents the number of cells analysed.

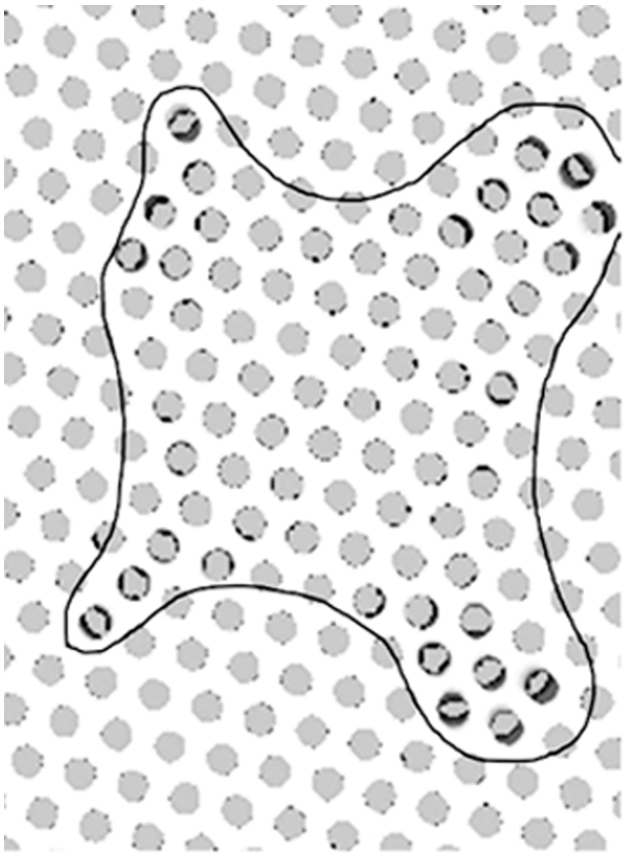

Figure S4. Schematic diagram of the moving pillars below an Ecad cell (the area of the cell is outlined in black) on FN-pillars (gray dots). The color code, extending from black to gray, indicates the extent to which the pillars (light gray dots) are displaced.

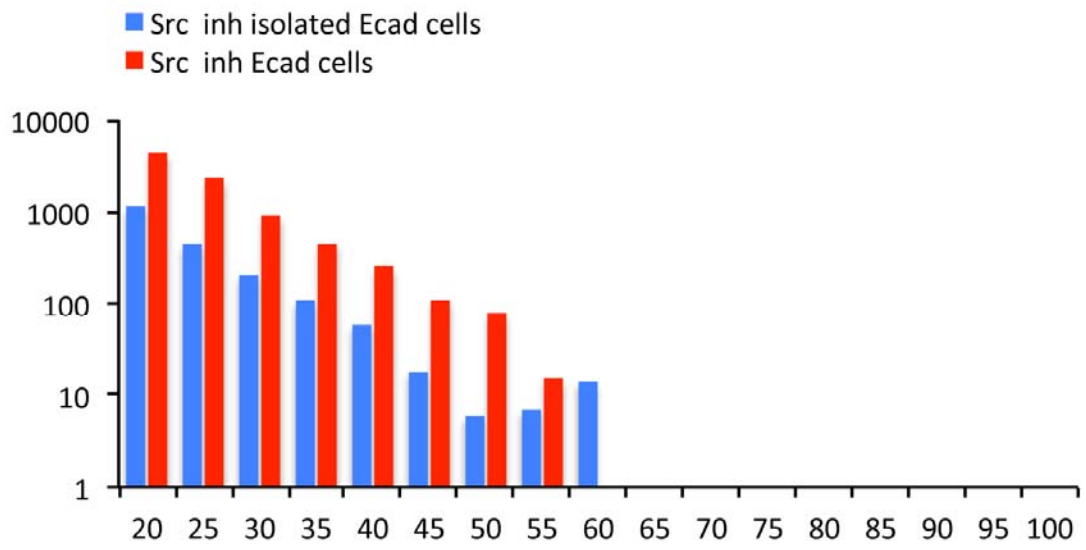

Figure S5. Effect of the Src inhibitor 1 on Ecad cells. Distribution of pulling forces for isolated Ecad cells (blue bars) and contacting Ecad cells (red bars) treated with the inhibitor.

Video S1: Actin dynamics (green) at the cell-FN pillar (red) adhesion sites, as revealed with the GFP-UtrCH probe (6). Images were taken using the Olympus 60 x water immersion objective (NA 1.1) every 15 seconds over a period of 21 minutes (time indicated in minutes on the upper left corner of the movie).

SUPPORTING REFERENCES

1. Naba, A., C. Reverdy, D. Louvard, and M. Arpin. 2008. Spatial recruitment and activation of the Fes kinase by ezrin promotes HGF-induced cell scattering. Embo J 27:38-50.
2. Burkel, B. M., G. von Dassow, and W. M. Bement. 2007. Versatile fluorescent probes for actin filaments based on the actin-binding domain of utrophin. Cell Motility and the Cytoskeleton 64:822-832.