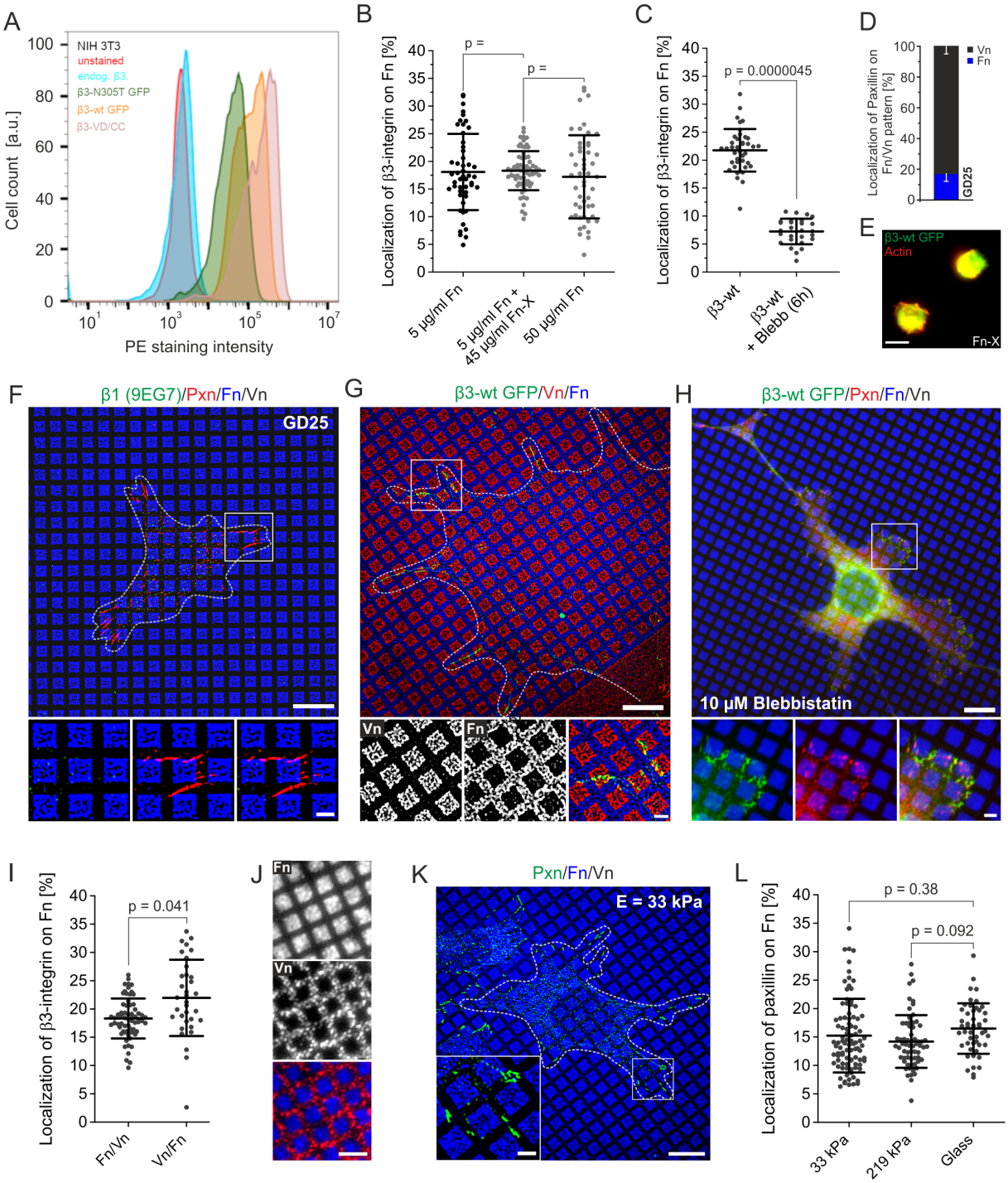


Figure S1: (**A**) Profile of anti-β3 staining measured with flow cytometry of a subclone of the NIH3T3 cell line that was selected for low β3 expression (Pinon et al., 2014). Cells were either mock transfected ('unstained' and 'endogenous β3') or transiently transfected with mouse β3 GFP (wt, N305T or V80C/D241C = VD/CC) followed by a hamster anti-mouse β3 (antibody clone HMβ3-1, staining total β3) and anti-hamster phycoerythrin (PE) staining. The number of all cells (mock transfection) or cells gated for β3 GFP expression are plotted against their PE signal. Category 'unstained' refers to mock transfected cells without any antibody staining. (**B**) The Fn content in the solution for producing Fn/Vn substrates was varied to analyze the effect of Fn concentration and activity on αVβ3 – Fn binding. The stamped solution contained as indicated either 5 µg/ml plasma Fn, 5 µg/ml plasma Fn + 45 µg/ml heat-inactivated plasma Fn (Fn-X), or 50 µg/ml plasma Fn. After stamping, substrates were backfilled with Vn to fill the free areas between Fn squares. NIH3T3 cells expressing β3-wt GFP were cultured on the respective substrates for 2 hrs, fixed, and the colocalization of β3-wt GFP with Fn was quantified (5 µg/ml Fn + 45 µg/ml Fn is a replot from Fig. 1d, 5 µg/ml Fn: n = 50, N = 3; 50 µg/ml Fn: n = 46, N = 3). (**C, H**) NIH 3T3 cells transfected with β3-wt

GFP integrin were incubated on Fn/Vn substrates for 6 hrs in presence of 10 μM blebbistatin. Colocalization of β3-wt GFP with Fn was quantified and compared to untreated control condition cultured for 2 hrs (β3-wt is a replot of Fig. 3g; β3-wt + Blebb (6h): n = 27, N = 3). (**D**) Quantification of paxillin colocalization with Fn in GD25 cells treated as described in **F** (n = 36, N = 3). (**E**) NIH 3T3 cells transfected with β3-wt GFP integrin cultured on heat-inactivated Fn (Fn-X) that was used together with native Fn during stamping (see Material & Methods and Supplementary Fig. 1b). Cells are unable to spread and to cluster integrins on this substrate. (**F**) GD25 cells cultured on Fn/Vn substrates (Fn in blue) and immunostained for β1 integrin (green) and paxillin (red). GD25 cells express no β1 integrin (shown by the immunostaining for β1 integrin; left zoom in and merge with paxillin on the right). (**G**) Vn labeled with Alexa Fluor 568 (red) was stamped and then overlaid with Alexa Fluor 647 labeled Fn (blue). NIH 3T3 cells were transfected with β3-wt integrin (green) and cultured on these Vn/Fn substrates. (**I**) Quantification of β3 integrin colocalization with Fn in NIH3T3 cells treated as described in **G**. Category "Fn/Vn" is a replot of Fig. 1D (Vn/Fn: n = 38, N = 3). (**J**) Protein separation of Fn/Vn patterns tested after transfer of the pattern to polyacrylamide gels by immunostaining for Fn (blue) and Vn (red). (**K**) Immunostained GD25 cell (paxillin: green; Fn: blue) on a gel with a Young's modulus of E = 33 kPa functionalized with Fn/Vn patterns. The zoom-in highlights the preferred localization of adhesions on Vn. (**L**) Quantification of the colocalization of paxillin with Fn for cells grown on Fn/Vn pattern printed on glass (n = 36, N = 3), gels with E = 33 kPa (n = 93, N = 3), or gels with E = 219 kPa (n = 64, N = 3). (**F**,**G**,**K**) White dashed lines in overview images indicate cell outlines. Scale bar: 10 μm in the overview (**E-H**,**K**) and 2 μm for the zoom-in and in (**J**). All fluorescent images except **E, H, J** were acquired with SR-SIM.

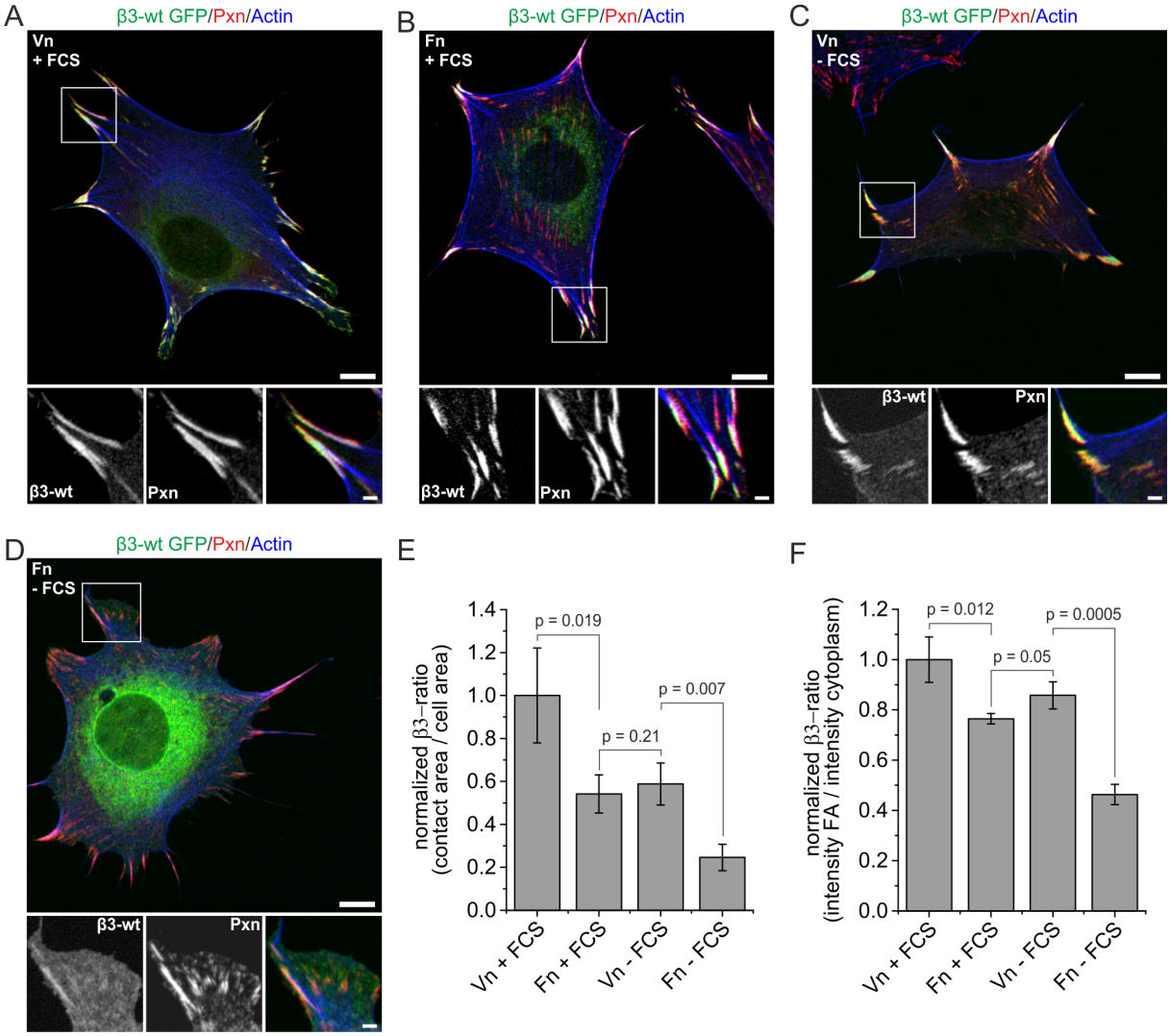


Figure S2: αVβ3 integrin favors Vn compared to Fn on homogenous substrates. (**A-D**) NIH 3T3 cells transfected with β3-wt GFP (green) cultured on homogenously coated substrates (Vn or Fn as indicated) and stained for anti-paxillin (Pxn; red) and actin (blue). Throughout the experiment, fetal calf serum (FCS) was present in the medium at 10% v/v (+FCS) or not (-FCS). (**E**) Quantification of the size ratio of β3-wt GFP mediated cell-matrix adhesions compared to the cell area for the conditions described in **A-D**. Ratios are normalized to the ratio for Vn + FCS (Vn+FCS: n = 67, Fn+FCS: n = 69, Vn-FCS: n = 70, Fn-FCS: n = 67; N = 3 in all cases). (**F**) Quantification of the intensity ratio of β3-wt GFP mediated cell-matrix adhesions compared to the plasma membrane around the adhesions for the conditions described in **A-D**. Ratios are normalized to the ratio for Vn + FCS (same cells as in **E** were used for quantification; N = 3 in all cases). All images were acquired with diffraction limited microscopy. Scale bars: 10 µm in the overview, 2 µm in the zoom-ins.

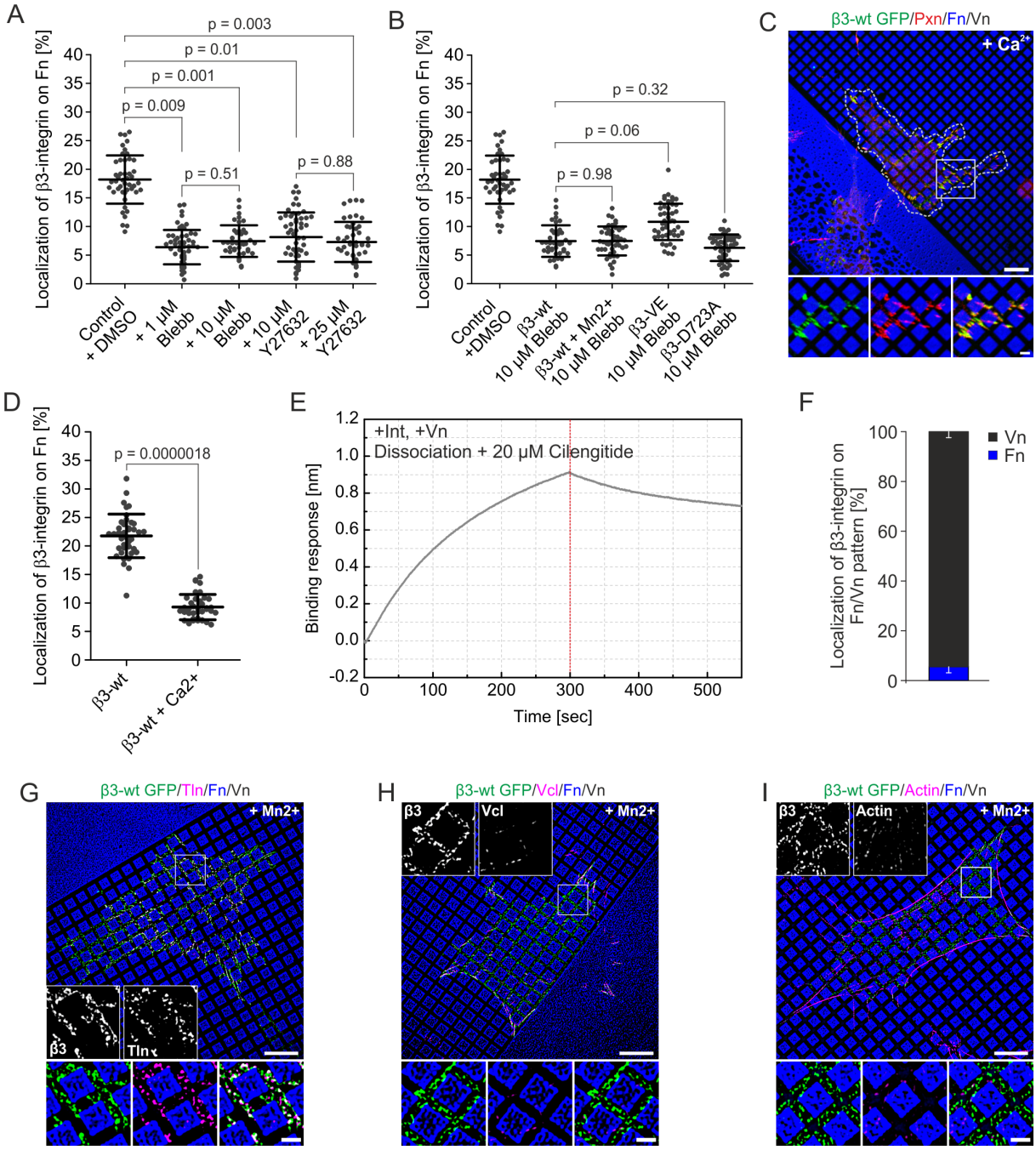


Figure S3: (**A**) Quantification of β3 integrin colocalization with Fn in NIH3T3 cells transfected with β3-wt integrin and cultured on Fn/Vn substrates. Cells cultured in presence of blebbistatin or Y27632 at the indicated concentrations. Values of 10 µM inhibitor concentration and DMSO control are replotted from Fig. 2G (+ 1 µM Blebb: n = 51, N = 3; + 25 µM Y27632: n = 56, N = 3). (**B**) Quantification of β3 integrin colocalization for the indicated mutations with Fn in NIH3T3 cells transfected with β3-wt integrin, cultured on Fn/Vn substrates, and in presence of blebbistatin where indicated. 30 min before fixation Mn^{2+} was added where indicated. Values of DMSO control and "β3-wt +10 µM Blebb" are a replot of Fig. 2G (β3-VE+10 µM Blebb: n = 55, N = 3; β3-wt+Mn^{2+} +10 µM Blebb: n = 45, N = 3; β3-D723A+10 µM Blebb: n = 49, N = 3). (**C**) NIH 3T3 cells transfected with β3-wt GFP (green) cultured on Fn/Vn substrates for 2 hrs. 1 mM Ca^{2+} was added for the last 30 min. Cells were stained for paxillin (red) after fixation. (**D**) Quantification of β3 integrin colocalization with Fn for cells treated as described in Fig. 1C (β3-wt) or in Supplementary Fig. 3C (β3-wt +Ca2+, n = 38, N = 3). (**E**) Association and dissociation curve for Vn interaction with purified αVβ3 integrin. Cilengitide was present in the dissociation buffer at 20 µM concentration (representative curve from n = 9, N = 3 measurements). (**F**) Quantification of β3-wt GFP in central areas of Mn^{2+} treated cells cultured as described in **G-I** but immunostained for paxillin. Only β3-wt GFP not colocalizing with paxillin was used for analysis to ensure exclusive measurement of nascent integrin clusters (n = 42; N = 3). (**G-I**) NIH

3T3 cells transfected with β3-wt GFP (green), cultured on Fn/Vn substrates and treated with 1mM Mn^{2+} 30 min before fixation. Additionally, cells were immunostained for (**G**) talin, (**H**) vinculin, or (**I**) for actin (magenta). (**C, G-I**) Scale bars: 10 µm in the overview, 2 µm in the zoom-ins. Images for (**C**, **D**) were acquired with diffraction-limited microscopy, (**F-I**) with SR-SIM.

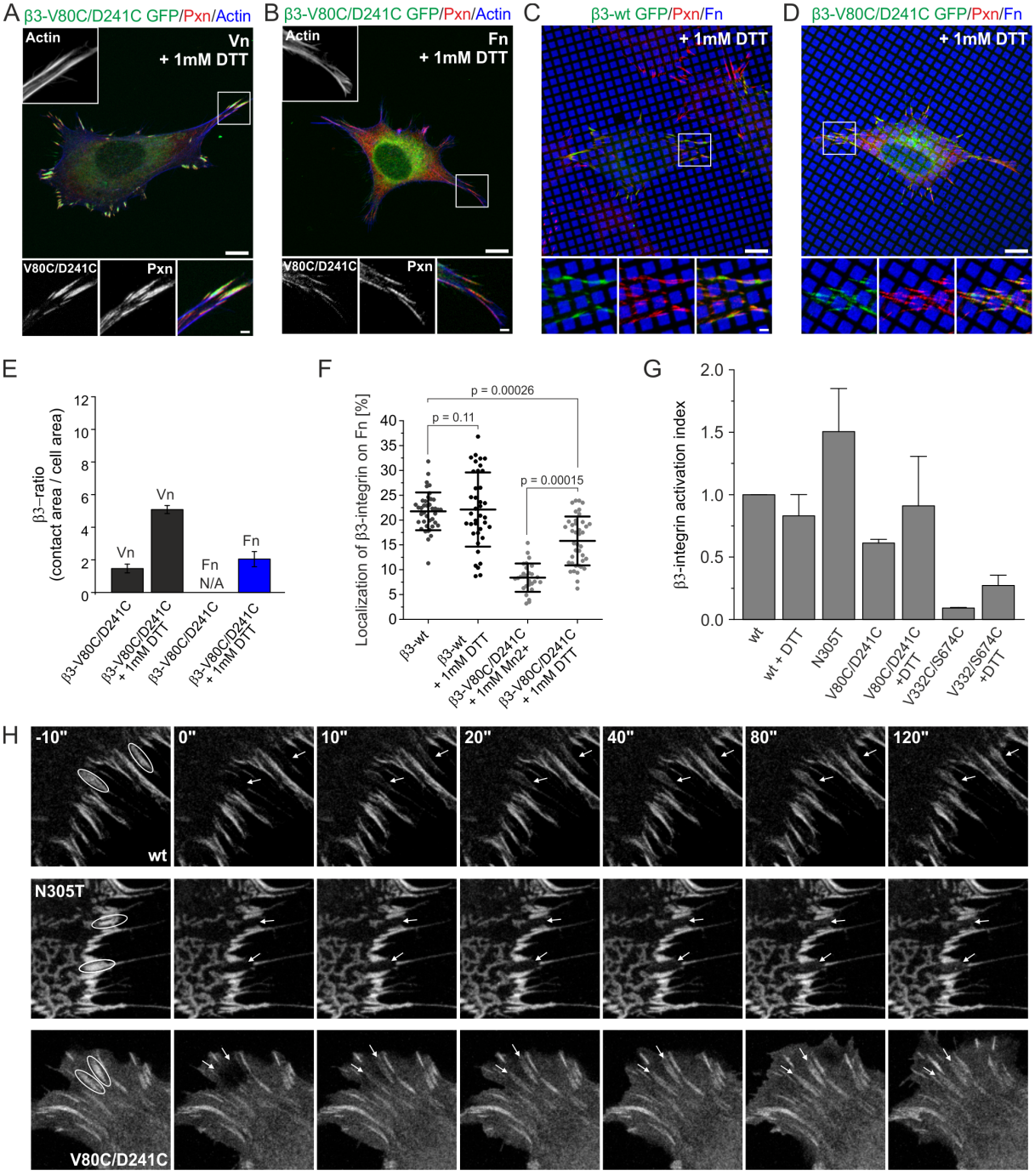


Figure S4: (**A, B**) NIH 3T3 cells transfected with β3-V80C/D241C GFP were cultured on Fn or Vn coated coverslips for 2 hrs and stained for paxillin (red). 1 mM dithiothreitol (DTT) was added 10 min before fixation to open disulfide bridges. (**C**, **D**) NIH 3T3 cells transfected with (**C**) β3-wt GFP or (**D**) β3-V80C/D241C GFP were cultured on Fn/Vn substrates and 1 mM DTT was added 10 min before fixation. (**E**) Quantification of β3-wt GFP recruitment into adhesions (β3-wt GFP adhesion area divided by cell area) for cells treated as described in Fig. S4 A, B and Fig. 4 B, C (β3-V80C/D241C on Vn: n = 28, N = 3; β3-V80C/D241C + 1 mM DTT on Vn: n = 32, N = 3; β3-V80C/D241C on Fn: n = 30, N = 3; β3-V80C/D241C + 1 mM DTT on Fn: n = 37, N = 3.). (**F**) Quantification of cells treated as described in Fig. S4 C, D (β3-wt + 1 mM DTT: n = 38, N = 3; β3-V80C/D241C + 1 mM DTT: n = 40, N = 3) and in Fig. 4G (β3-wt and β3-V80C/D241C + 1 mM Mn2+). (**G**) NIH3T3 cells expressing the indicated β3 constructs were stained for total β3 integrin (hamster anti-β3; clone HMβ3-1) or for active β3 with a high-affinity RGD-ligand (Ski7; (Pinon et al., 2014)). The respective stainings were analyzed with flow cytometry and the ratio of active/total β3 integrin was calculated and normalized to β3-wt GFP. This ratio represents the β3-activation index shown in the graph. Where indicated 1 mM DTT was added 10 min before cell detachment for subsequent staining. All conditions were analyzed in at least three independent experiments and typically more than 15 000 cells were analyzed per

experiment. (**H**) Representative time series of FRAP experiments as described in Fig. 4I. White ellipsoids indicate regions that were bleached at time point 0. These regions are indicated with white arrows for later timepoints. Scale bar: 10 µm in the overview and 2 µm for the zoom-in. All fluorescent images were acquired with diffraction limited microscopy.

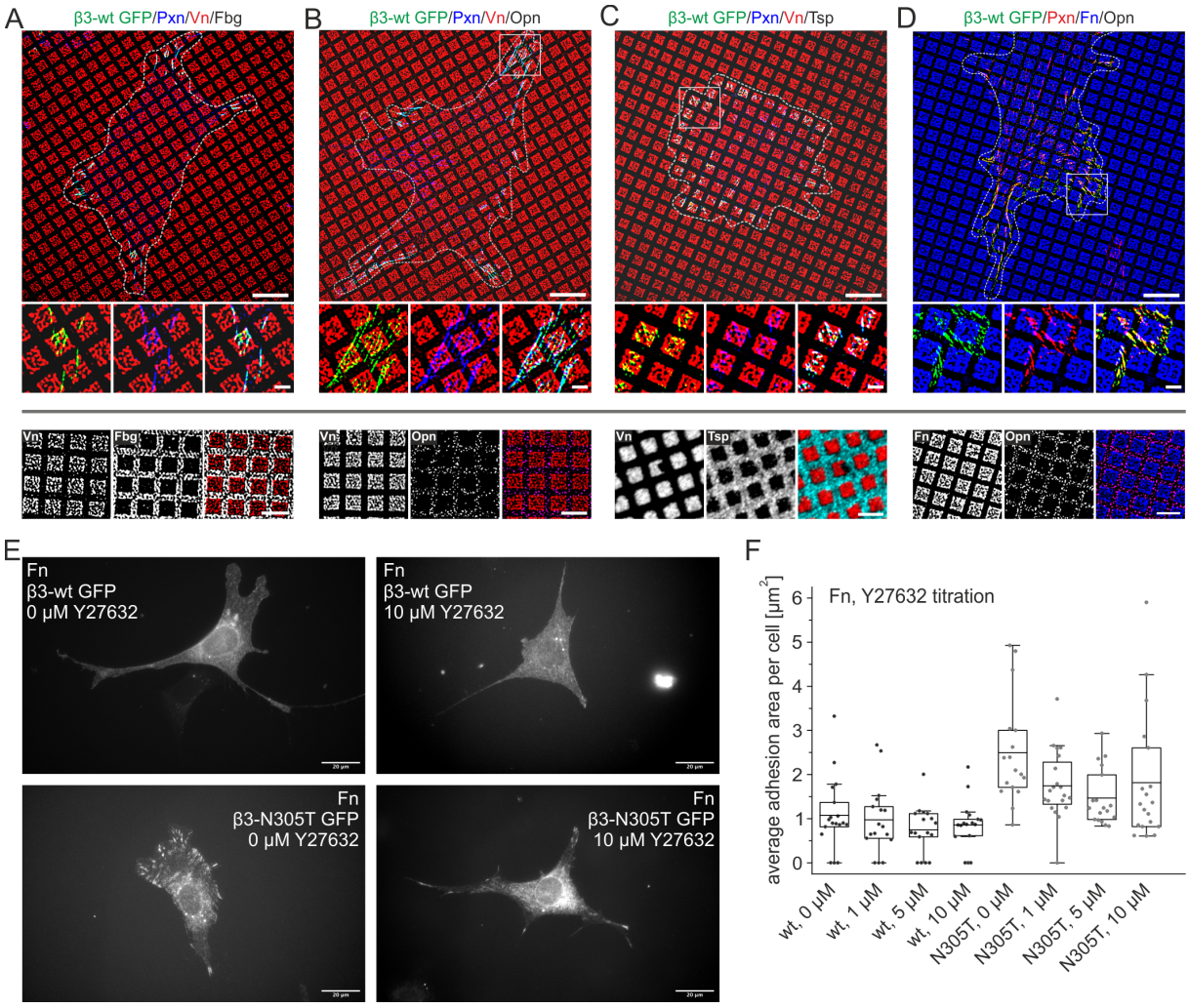


Figure S5: (**A**) Vn squares (red) were stamped onto cover slips and the remaining area was backfilled with Fbg (black). NIH 3T3 cells transfected with β3-wt GFP integrin (green) cultured on Vn/Fbg substrates and immunostained for paxillin (blue). (**B**) Cell treated as in (**A**) but on a Vn/Opn or (**C**) a Vn/thrombospondin (Tsp) or (**D**) a Fn/Opn pattern. The first protein mentioned was the one that was stamped, the second one was backfilled. Successful protein separation is shown by staining the respective proteins as indicated below **A-D**. (**E, F**) NIH3T3 cells transfected with β3-wt GFP (upper row) or β3-N305T GFP (lower row) were cultured on Fn coated cover slips for 4 hrs and in presence of Y27632 with concentrations as indicated. Average focal adhesion size (focal adhesion defined as adhesion area >= 0.5 μm^2) per cell was calculated as a measure for adhesion maturation and plotted for all conditions (n >= 17; N = 3). Cell regions with high background fluorescence were excluded from analysis; conditions were blinded during analysis. Scale bars: (**A-D**) 10 µm in the overview, 2 µm in the zoom in. (**E**) 20 µm. White dashed lines indicate cell outline. All images acquired with SR-SIM except Vn/Tsp pattern below c.

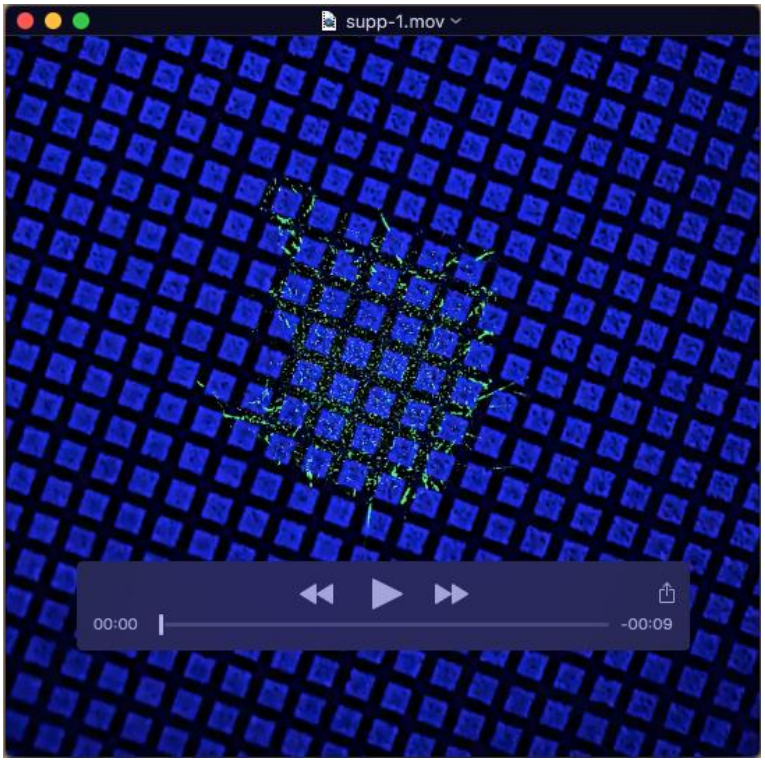
Table S1: Polyacrylamide gel rigidities measured with AFM. Number of analyzed cells refers to Fig. 5D, E.

Young's module [Pa] mean ± standard deviation	**acrylamide, final concentration [%]**	**bisacrylamide, final concentration [%]**	**analyzed cells on Fn**	**analyzed cells on Vn**
0.4 ± 0.08	4	0.03	44	37
3.0 ± 0.98	5.5	0.04	38	52
6.7 ± 1.95	7.46	0.04	60	73
20.1 ± 4.87	7.49	0.10	54	64
32.6 ± 9.38	7.52	0.16	55	50
61.7 ± 27.03	12	0.15	58	61

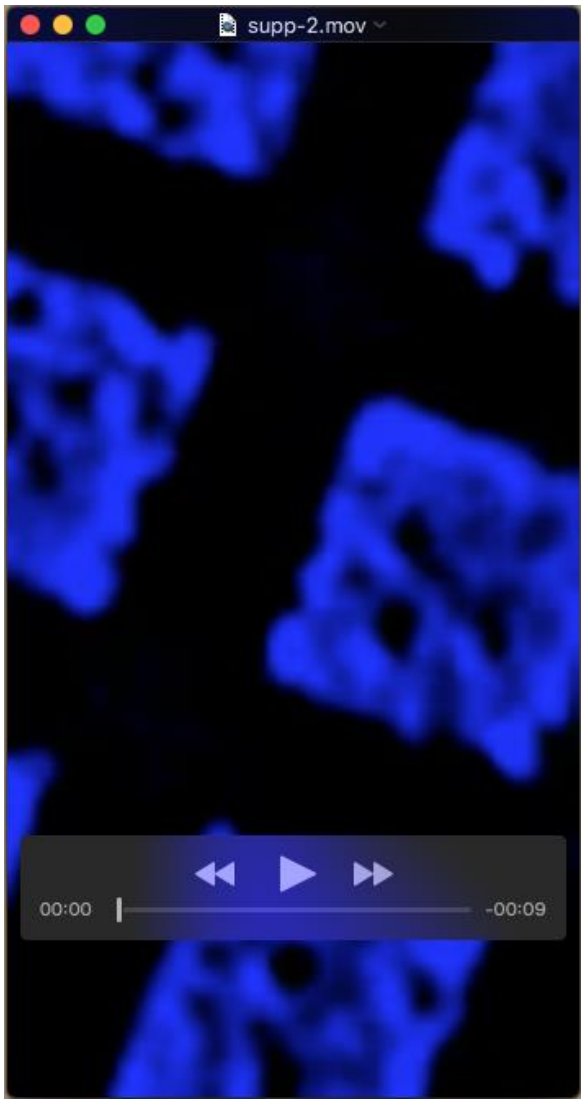
Table S2: p-values of student t-test (two tailed) for the indicated conditions referring to Fig. 5D, E.

Young's module [Pa] mean ± standard deviation	**Fn vs. Vn, cell area**	**Fn vs. Vn, adhesion length**
0.4 ± 0.08	0.59	0.53
3.0 ± 0.98	0.57	0.95
6.7 ± 1.95	0.09	0.02
20.1 ± 4.87	0.69	0.39
32.6 ± 9.38	0.51	0.92
61.7 ± 27.03	0.57	0.76

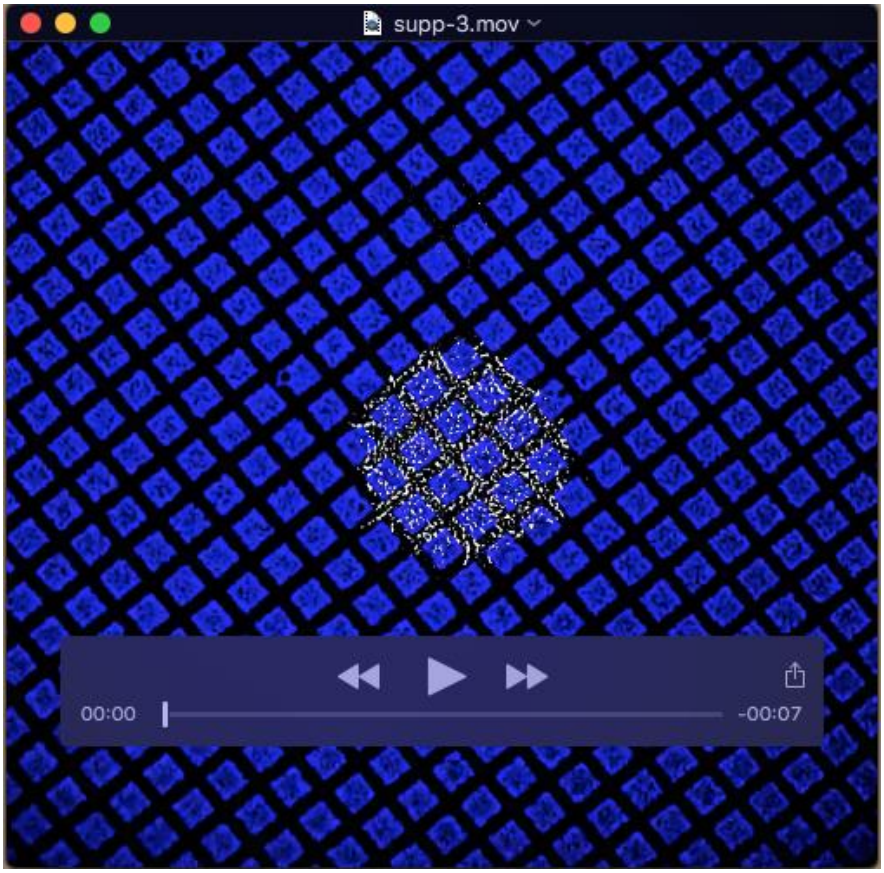
Supplementary Movies



Movie 1: NIH 3T3 cell expressing β3-wt GFP (green) was monitored during spreading on Fn/Vn substrate (Fn in blue) with live-cell SR-SIM with 1 frame per minute. The imaging medium contained 10% FCS. See also Fig. 1G.

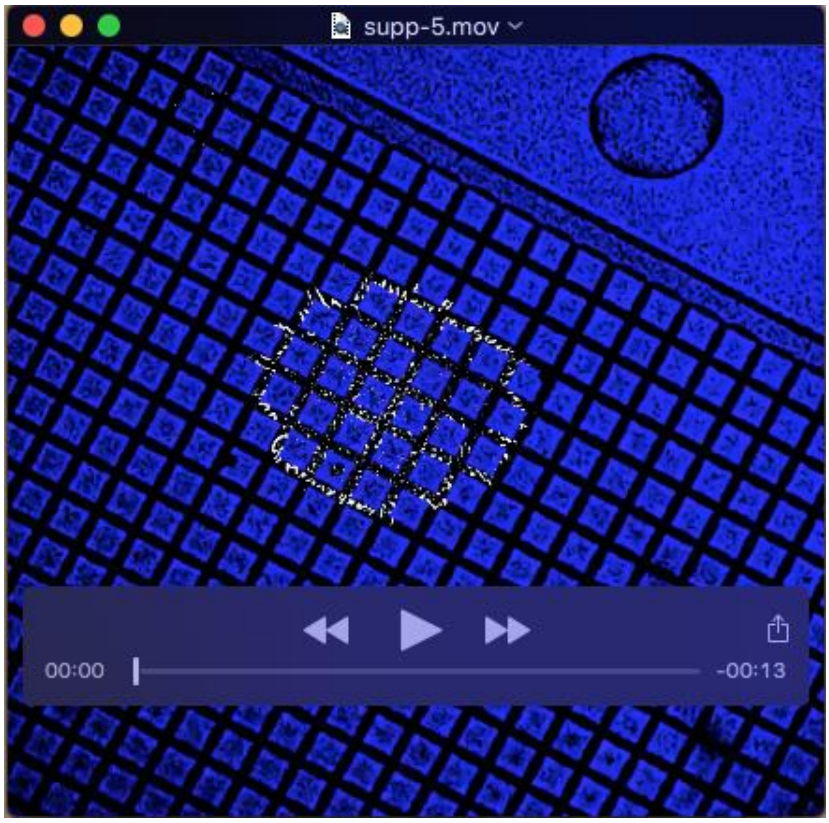


Movie 2: Zoom-in of Supplementary Movie 1.

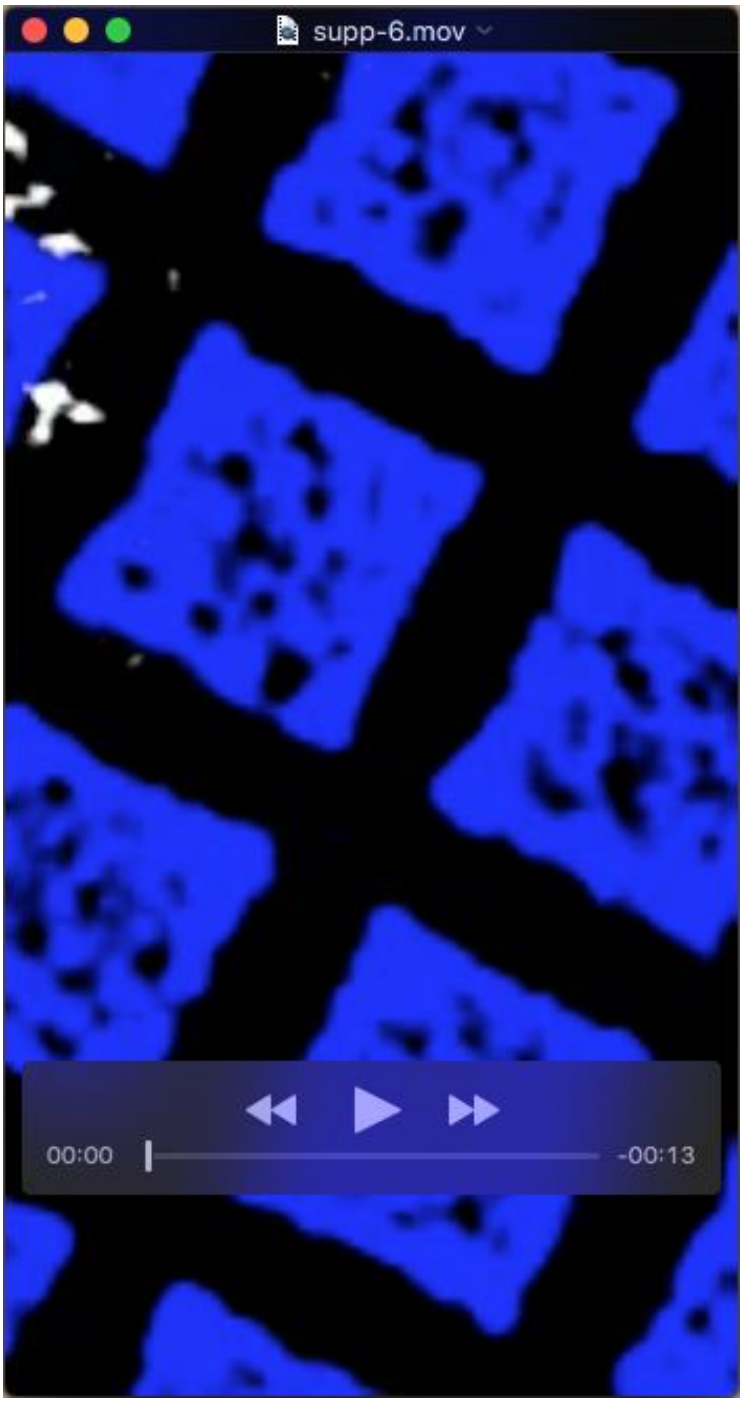


Movie 3: NIH 3T3 cell expressing β3-N305T GFP (white) was monitored during spreading on Fn/Vn substrate (Fn in blue) with live-cell SR-SIM with 1 frame per minute. The imaging medium contained 10% FCS. See also Fig. 3J.

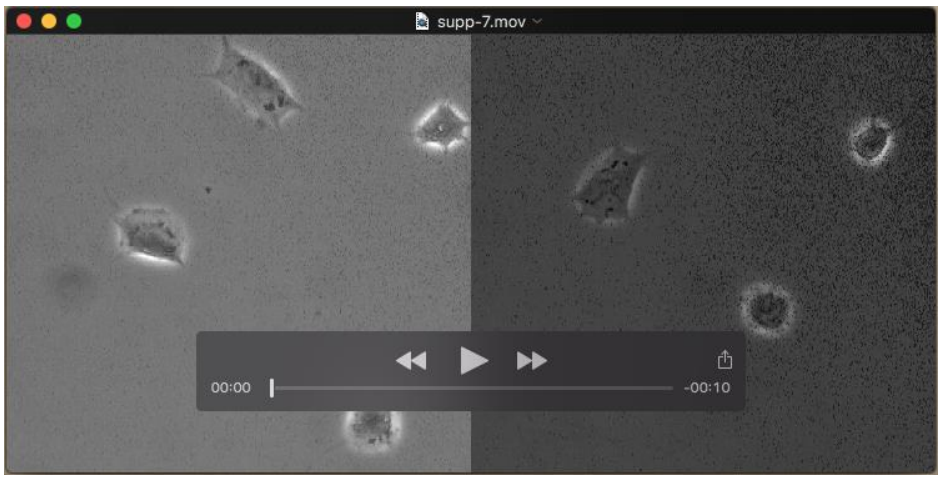
Movie 4: Zoom-in of Supplementary Movie 3.



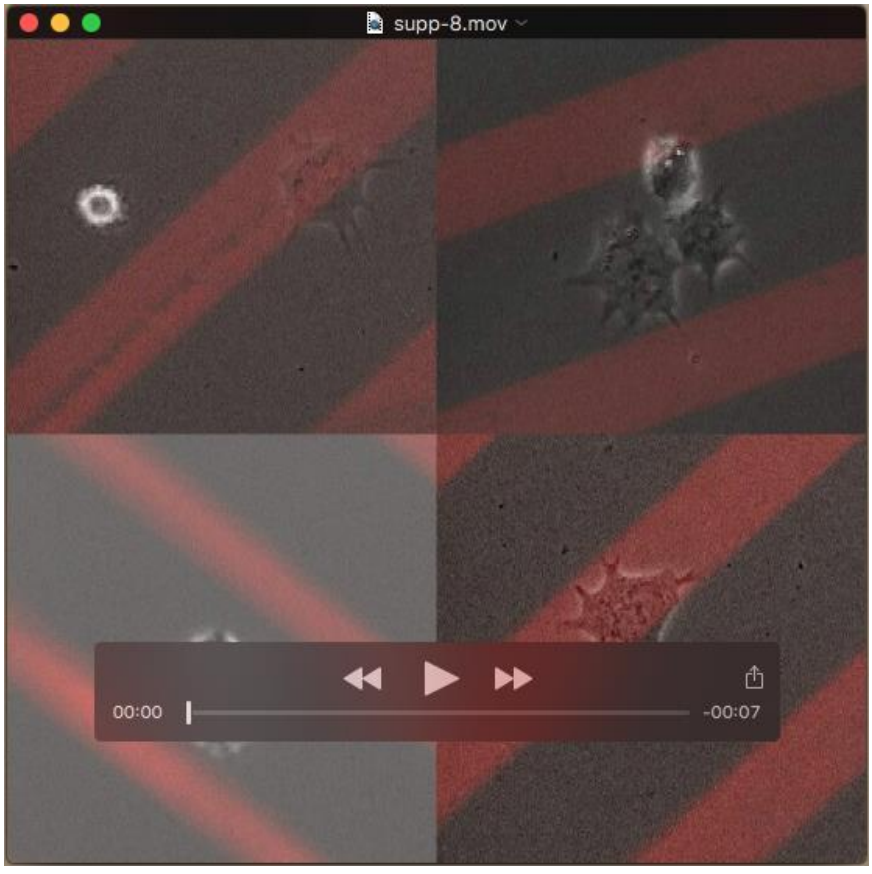
Movie 5: NIH 3T3 cell expressing β3-VE GFP (white) was monitored during spreading on Fn/Vn substrate (Fn in blue) with live-cell SR-SIM with 1 frame per minute. The imaging medium contained 10% FCS.



Movie 6: Zoom-in of Supplementary Movie 5.



Movie 7: GD25 cells cultured on homogenous Fn (left) or Vn (right) (10 µg/ml) and imaged with phase contrast microscopy with 1 frame per 20 minutes. 34 cells from three independent experiments were analyzed in both cases. The imaging medium contained 1% FCS.



Movie 8: GD25 cells cultured on Vn/Fn stripes and imaged with phase contrast microscopy with 1 frame per 20 minutes. Stripes of Vn (red) have a width of 20 µm. The imaging medium contained no FCS.