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Supporting Material

Decoding Cytoskeleton-Anchored and Non-Anchored Receptors from Single-Cell Adhesion Force Data

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SUPPLEMENTARY INFORMATION

Materials and methods

Cell culture

PC3 were obtained from ATCC (Wesel, Germany). PC3 cells were maintained in RPMI-1640 cell culture media (PAA, Cölbe, Germany) and 10% FBS (Sigma-Aldrich, Munich, Germany). The immortalized human MSC line SCP1, which is fully described in Böcker et al (1), was cultured in MEM GlutaMAX culture media (Life Technologies, Karlsruhe, Germany) supplemented with 10% FBS. During routine cell culture, the two cell types were grown up to 80% confluency in a humidified incubator. Culture medium was changed three times per week and for cell passaging, cells were detached with 1x trypsin/EDTA solution (PAA).

Quantitative reverse transcriptase (RT)-PCR

Quantitative RT-PCR was performed as described in Popov et al (2). Briefly, total RNA was extracted from PC3 cells with RNeasy Mini Kit (Qiagen, Hilden, Germany). For cDNA synthesis, 1 µg total RNA and AMV First-Strand cDNA Synthesis Kit (Life technologies) were used. LightCycler Fast Start DNA Master SYBR Green kit (Roche, Munich, Germany) and primer kits for α1, α2, α11, β1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (all Search-LC, Heidelberg, Germany) were applied. The PCR was performed in a LightCycler 1.5 instrument (Roche) equipped with LightCycler 3.5.3 software. Crossing points for each sample were determined by the second derivative maximum method and relative quantification was performed using the comparative ΔΔCt method according to the manufacturer's protocol. The relative gene expression was calculated as a ratio to GAPDH.

Substrate preparations

We used Col-I and bovine serum albumin (BSA)-coated glass cover slips and SCP1 monolayers as substrates for AFM force spectroscopy experiments. Sterile glass cover slips were coated with Col-I (100 µg/ml) or BSA (100 µg/ml) at 4 °C overnight. The full protocols used for the preparation of Col-I coated cover slips and SCP1 monolayers were described in Sariisik et al (3). Prior to use, the substrates were washed with and covered by 1.5 ml fresh serum-free MEM-Alpha medium supplemented with 15 mM Hepes. The Col-I and BSA coated cover slips were placed on top of the SCP1 monolayer in the culture dish lids. BSA coated glass cover slips were also used for cell capture.

AFM setup and force spectroscopy

Fresh serum-free Alpha-MEM/Hepes medium was used as measurement media throughout all force spectroscopy experiments. The culture dish lid, containing BSA and Col-I coated substrate as well as the SCP-1 monolayer, was mounted on a temperature-controlled stage in the AFM and was left to equilibrate for 10 min at 37 °C. Force Spectroscopy experiments were conducted using a NanoWizard II with CellHesion module (JPK Instruments, Berlin, Germany), mounted on a Zeiss Axiovert-200-M (Carl Zeiss, Göttingen, Germany) with a custom made temperature unit. The force sensors used were tip-less silicon nitride cantilevers with a nominal spring constant of 0.01 N/m (Tipless, MLCT-O10, Veeco, USA). Prior to cell adhesion experiments, the force sensors were coated overnight with 100 mg/ml Poly D-Lysine (PDL, Millipore, USA). The spring constants of the force sensors were determined individually by the thermal noise method.

A single PC3 cell resting on the BSA coated cover slide was gently contacted for a few seconds by the PDL-coated tip-less force sensor and after lifting the attached cell it was allowed to firmly adhere to the force sensor for about 3 minutes. Subsequently, each substrate (BSA, Col-I and SCP1 in varied order) was contacted by this cell between 100 and 200 times for as short as possible (0.3 ms) at 100 pN. By scanning a list of 0.5 µm spaced points of preselected grids sampling repeatedly the same spot was avoided. Partially identical experiments with identical results (within the statistical error) were conducted in Sariisik et al (3). Therefore more detailed protocols and schematics of the experimental setup are given there.

Force-distance curves were recorded while the piezo traveled in a closed loop up to 20 µm at an approach velocity of 7 µm/s, until a trigger force of 100 pN was reached. Subsequently, the adhesion force signature was recorded at a retraction velocity of 3 µm/s.

Application of integrin blocking antibody

After detachment of PC3 cells, the released cells were collected and washed with PBS (lacking calcium and magnesium). Prior to force spectroscopy and cell adhesion measurements, PC3 cells were suspended with fresh serum-free culture medium supplemented with 15 mM Hepes (Sigma-Aldrich). A monoclonal antibody against CD29/Integrin β1 (Acris Antibodies, Inc. San Diego, CA USA, BM2540) in a concentration of 4.8 µg/ml was added into 0.5 ml cell suspension containing $2x10^5$ cells and incubated for 30 minutes at 37 °C in a humidified incubator.

Latrunculin-A treatment of a cell on the cantilever (control experiment).

PC3 cells were prepared as described above for force spectroscopy experiments. One of the cells was captured from the BSA surface and attached to the PDL coated cantilever. Initially 60 force curves were collected on Col-I substrate with this cell, to check for normal adhesion properties (data not shown). (Subsequently Latrunculin-A (Lat-A, Sigma-Aldrich, USA) was added into the measurement medium until a final concentration of 0.2 µM was reached. After a period of 15 minutes for allowing Lat-A to disrupt the actin cytoskeleton, 60 additional curves were recorded with the same cell. In total 4 different cells were treated and measured in this way.

Force curve analysis

For data analysis, only the retraction part of the approach-retract cycles of the cell bearing cantilevers was evaluated (blue force traces in Fig. 1). In order to obtain characteristic quantitative information from the force-distance curves, a custom-designed data analysis and step detection software (4) was used to smooth the signal (black line on top of the blue line in Fig. 1), find the baseline (dashed lines in Fig. 1), correct for hydrodynamic drag and possible drift, and extract the following parameters (see also Sariisik et al (3)):

step height [pN] describing the difference in force measured before and after an individual detachment event, visible as a force step. The algorithm identifies such a step by maxima in the derivative of the smoothed signals, which surmount a certain threshold and marks it with a red cross (cf. also Fig. 1). Force steps smaller than 8 pN are not counted as steps.

adhesion rate [%] describing the fraction of curves with at least one detected force step.

number of steps describing the average number of steps detected per curve (only counting curves with at least one detected force step).

step position [µm] describing the distance between the contact point (black circle at the intersection of baseline and force curve in Fig.1) and a force step.

dissipated work [aJ] describing the energy dissipated during that force experiment by integrating the area between baseline (zero force) and force curve. (Note, this has no trivial relation to the adhesion energy as, velocity dependent viscous and plastic deformation of the cell and the cell membrane strongly contribute to the work of detachment)

detachment force [pN] describing the highest measured adhesion (global maximum) per curve.

We also analyzed force-loading rates (slope of the force trace) prior to each step by a line-fit to the force data points within the last 500 nm prior to the step. Due to the constant velocity of 3 µm/s a loading rate [pN/s] could be directly derived from the force-distance trace.

In this study, we classified steep steps as **jump steps** at slopes below -10 pN/µm (~loading rates of -30 pN/s and steeper; Fig. 1A) and plateau steps as **tether steps** between slopes of ±10 pN/µm (~loading rates between ±30 pN/s) see Fig. 1B and Fig. 2C&D).

For illustrating the advantage of the 2D-plots, the fraction of steps originated from tethers -as selected by eye in the classic manner- were marked in grey in the histogram of Fig 2C. According to this histogram, there is an area between -8 and -14 pN/µm where jumps and tethers are not clearly discernible by their slope.

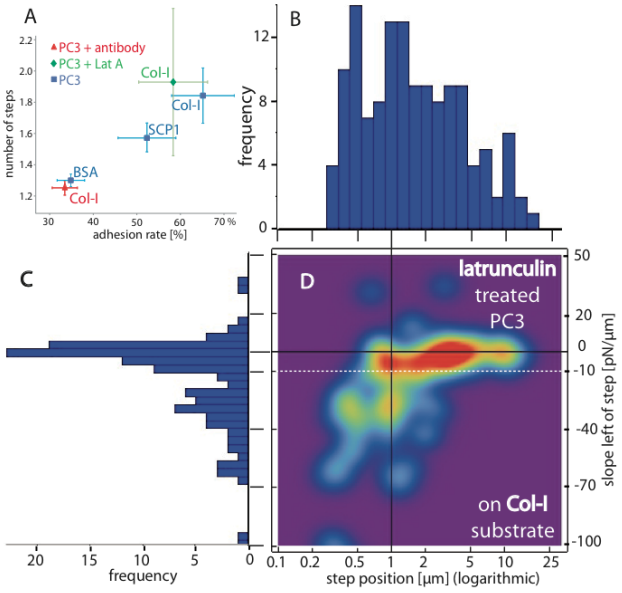
At the slope of -10 pN/µm we chose to set a guiding line for separating between tethers and jumps in case for the PC3 cell line studied here. Note that the values of the slopes may vary between different cell types and that cells are visco-elastic objects. Therefore, the slopes of jumps will depend on the cantilever velocity (in this study the velocity was 3µm/s).

Note, that the fit range of 500 nm for analyzing the slopes is a compromise between a reliable fit range to cope with the intrinsic noise of the force curves and the distance between individual steps. In case of steps closer than 500 nm in distance he algorithm detecting the slopes prior to each step generates positive slopes even larger than the noise level of about -10 pN/µm by fitting through more than one step. Such positive slopes larger than +10 pN/µm are therefore neglected when interpreting the density plots.

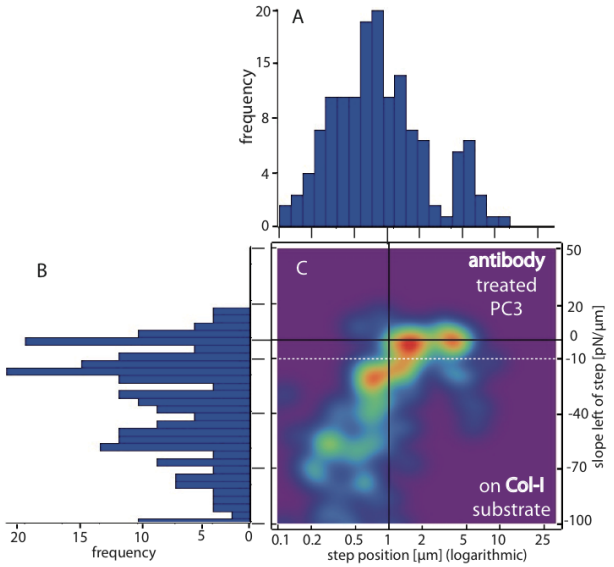
Preparation of 2D density plots

The slope versus peak position 2D density plots were smoothed using a Gaussian kernel density estimation (σ_x = log (x)*0,1 µm σ_y = 5p N/µm) for better visualization of the otherwise checkerboard patterned 2D-histograms. The density plots were normalized in order to compare tethers and jumps in the different experiments, i.e. on the different substrates.

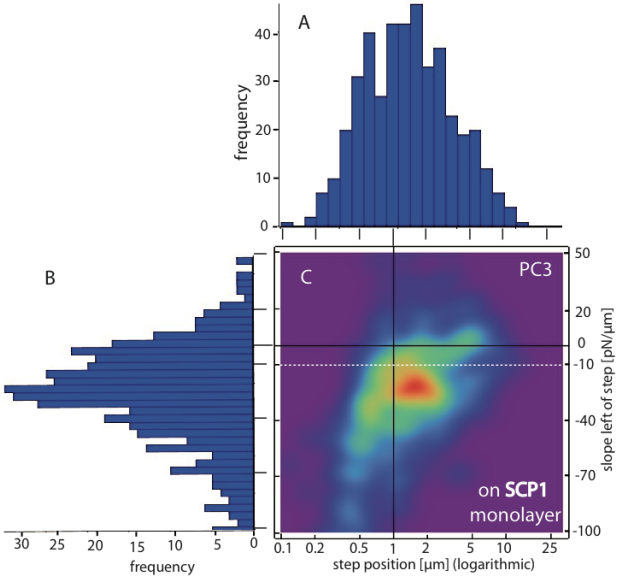
Supplementary Data



Supplementary figure 1: Adhesion rate vs. number of steps (A), histograms (B, C), and density plot (D) of latrunculin-A treated cells. In the adhesion rate vs. number of steps plot (A), the Lat-A treated cells appear in a similar range as the untreated cells, as the type of interaction is not changed but only the anchoring to the cytoskeleton is affected. Due to the smaller number of experiments (4 cells were measured instead of 10), the error bars are larger. The histogram of the position (B) shows a broad distribution with a maximum above 1 µm. The histogram of the slopes (C) shows a prominent peak close to 0 pN/µm indicating that the receptors are anchored predominately via membrane tethers, as expected for a disrupted cytoskeleton when only the cell membrane can anchor the interacting cell surface receptors. The density plot (D) clearly visualizes this broad distribution of mainly tethers (Note that the color code is identical to Fig 2 D; black lines mark "1 µm" and "0 pN/µm" slope, whereas the white dotted line marks "-10 pN/µm" as guide for the eye to separate tethers from jumps).

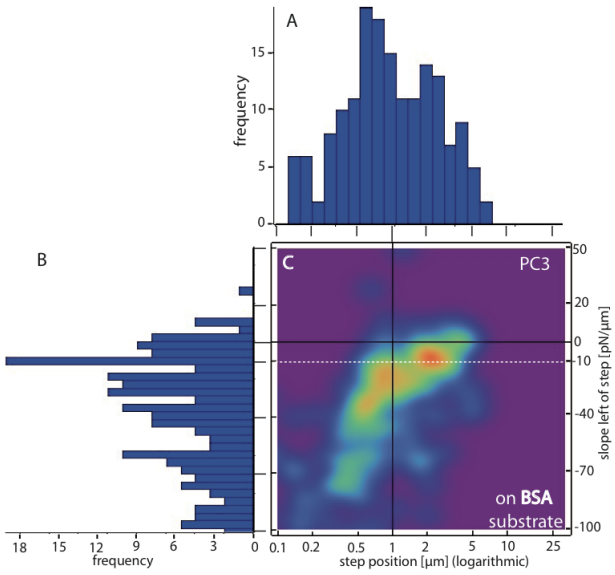


Supplementary figure 2: histograms behind the density plot of Figure 3B. The histogram of the position (A) shows one peak below 1 µm and a smaller peak above 3 µm. The histogram of the slopes (B) shows a two peaks close to 0 pN/µm and -20 pN/µm respectively. Due to the low adhesion rate the absolute number of points is small. The density plot (C) reveals a concentration of interactions to the tether region, but not as completely as Lat A. (The color code is identical to Fig 2 D; black lines mark "1 µm" and "0p N/µm" slope, the white dotted line marks "-10 pN/µm" as guide for the eye to separate tethers from jumps)



Supplementary figure 3: histograms behind the density plot of Figure 3C. The histogram of the position (A) shows a broad distribution (similar to Lat-A treated

cells in Figure S1) with a maximum above 1 µm. The histogram of the slopes (B) shows a broad maximum close to -30 pN/µm. The density plot (C) shows a concentration of all interactions in the region marked with the white dotted circle, which is shifted from below 1 µm in the case of collagen substrate (figure 2C) to values above 1 µm here. (The color code is identical to Fig 2 D; black lines mark "1 µm" and "0 pN/µm" slope, the white dotted line marks "-10 pN/µm" as guide for the eye to separate tethers from jumps)



Supplementary figure 4: histograms behind the density plot of Figure 3D. The histogram of the position (A) shows a broad distribution with one maximum below 1 µm and a weaker one above 2 µm (not as separated as in Fig S2). The histogram of the slopes (B) shows a broad distribution with a peak at -10 pN/m. Due to the low adhesion rate, the absolute number of points is small. The density plot (C) shows a concentration of most interactions in the tether region with some short ranged interactions. (The color code is identical to Fig 2D; black lines mark "1 µm" and "0 pN/µm" slope, the white dotted line marks "-10 pN/µm" as guide for the eye to separate tethers from jumps)

Step position and bond lifetime

At constant pulling velocity, the length of membrane tethers directly correlates to the lifetime of the bond(s) that led to the pulling of tethers. Unfortunately, in many cases, only the step position can be unambiguously extracted from the force curves; in the case of multiple tethers pulled or tethers originating from a preexisting filopodium, the tether-length does not necessarily coincide with the step position. To avoid over- or misinterpretations, we recommend thorough analysis of the datasets for those steps, where the step positions coincides with the true tether-length, whenever the bond lifetimes under force are in the focus of the investigation.

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