Supporting Information

High-Frequency Mechanostimulation of Cell Adhesion
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1. Preparation of push-pull azobenzene compounds

Organic starting materials were purchased from Aldrich, Grüssing, Riedel-de Haën, Alfa Aesar and Merck and used as delivered. Solvents were purified and dried by standard techniques. \(^1\)H-NMR and \(^{13}\)C-NMR spectra were recorded using a Bruker DRX 500. Mass spectra were recorded on a Finigan MAT 8200 (EI, 70 eV) / MAT 8230 (CI, Isobutan). UV-Vis spectra were recorded on a Perkin-Elmer Lambda 14. Elemental analysis was carried out on an Euro Vector EA 300 Series. The synthetic pathways to obtain the precursor and push-pull compounds 1, 2 and 3 (PP 1, PP 2, and PP 3) are schematically depicted in Figure S1.

First, \(N\)-ethyl-N-(2-(prop-2-yloxy)ethyl)aniline (PP 1) was prepared. Therefore 2.18 mg (90.8 mmol) sodium hydride (w/w 60 % in mineral oil) was provided under nitrogen atmosphere and cooled to 0 °C. 10.0 mg (60.5 mmol) 2-(ethyl(phenyl)amino)ethan-1-ol was diluted in 46.0 mL tetrahydrofuran, added to the sodium hydride and stirred for 30 min. After adding 9.80 mL (88.0 mmol) 3-Bromo-1-propyne (w/w 80 % in toluene) the reaction mixture was stirred for 2 h at 0 °C. After stirring for another 24 h at room temperature, 130 mL water were added and the mixture was extracted three times with 150 mL dichloromethane. The combined organic layer was dried over MgSO\(_4\), concentrated under reduced pressure and purified via column chromatography on silica gel (dichloromethane \(R_f = 0.77\)). The resulting product PP 1 was a clear liquid (9.28 g, 76 %). \(^1\)H-NMR (500 MHz, CDCl\(_3\)): \(\delta = 7.25 - 7.19 (m, 2H, H-3')\), 6.71 (d, \(\ ^3J_{2'-3'} = 8.1 \text{ Hz}, 2H, H-2'\)), 6.67 (t, \(\ ^3J_{3'-4'} = 7.2 \text{ Hz}, 1H, H-4'\)), 4.17 (d, \(\ ^4J_{3-5} = 2.4 \text{ Hz}, 2H, H-3\)), 3.70 (t, \(\ ^3J_{1-2} = 6.4 \text{ Hz}, 2H, H-2\)), 3.53 (t, \(\ ^3J_{1-2} = 6.4 \text{ Hz}, 2H, H-1\)), 3.43 (q, \(\ ^3J_{CH2-CH3} = 7.0 \text{ Hz}, 2H, CH_2\)), 2.43 (t, \(\ ^4J_{3-5} = 2.4 \text{ Hz}, 1H, H-5\)), 1.17 (t, \(\ ^3J_{CH2-CH3} = 7.1 \text{ Hz}, 3H, CH_3\)) ppm. \(^{13}\)C-NMR (126 MHz, CDCl\(_3\)): \(\delta = 147.7 (C_q, C-1'), 129.3 (C_t, C-3'), 115.8 (C_t, C-4'), 111.9 (C_t, C-2'), 79.7 (C_q, C-4), \)
74.5 (C, C-5), 67.7 (C, C-2), 58.5 (C, C-3), 49.9 (C, C-1), 45.4 (C, CH₂), 12.1 (C, CH₃) ppm. MS(EI): m/z (%) = 203 (11) [M]⁺. MS(Cl): m/z (%) = 204 (100) [M+H]⁺, 203 (20) [M]⁺.

Then 901 mg (4.95 mmol) 5-amino-2-nitrobenzoic acid in 9.00 mL hydrochloric acid-water (conc. HCl:H₂O, 1:2) was cooled to 0 °C. A solution of 376 mg (5.44 mmol) sodium nitrite in 1.50 mL water was added while the reaction temperature was kept below 5 °C. After addition the reaction mixture was stirred for 20 min at 0 -5 °C. Subsequently 1.01 mL (4.95 mmol) N-ethyl-N-(2-(prop-2-yn-1- yloxy)ethyl)aniline (PP 1) dissolved in 12.0 mL methanol was added while the reaction temperature was kept below 5 °C. After stirring for 2 h at 0 °C the reaction mixture was allowed to warm to room temperature and was stirred for another 24 h at room temperature before adding 2.00 g sodium acetate. The resulting precipitate was filtered off and washed with 300 mL water. The product PP 2 was a dark red solid and used without further purification (1.55 g, 80 %). ^1H-NMR (500 MHz, (CD₃)₂SO): δ = 8.14 (d, ^3J₃′,₄′ = 8.4 Hz, 1H, H-3′), 8.03 (d, ^4J₄′-₆′ = 2.2 Hz, 1H, H-6′), 8.02 (dd, ^3J₃′,₄′ = 8.4 Hz, ^4J₄′-₆′ = 2.2 Hz, 1H, H-4′), 7.84 (d, ^3J₂-₃ = 9.2 Hz, 2H, H-2), 6.89 (d, ^3J₂-₆ = 9.2 Hz, 2H, H-6), 4.18 (d, ^4J₈-₁₀ = 2.4 Hz, 2H, H-8), 3.67 (s, 4H, H-2), 3.54 (q, ^3J₉₂₃ = 6.9 Hz, 2H, H-2), 3.44 (t, ^4J₈₋₁₀ = 2.4 Hz, 1H, H-10), 1.16 (t, ^3J₉₂₃ = 6.9 Hz, 3H, CH₃) ppm. ^13C-NMR (126 MHz, (CD₃)₂SO): δ = 165.9 (Cq, C=O ), 154.5 (Cq, C-5′), 151.8 (Cq, C-4), 146.7 (Cq, C-2′), 142.5 (Cq, C-1), 129.5 (Cq, C-1′), 126.3 (Ct, C-2), 125.6 (Ct, C-3′), 124.9 (Ct, C-4′), 121.5 (Ct, C-6′), 111.7 (Ct, C-3), 80.2 (Cq, C-9), 77.3 (Ct, C-10), 67.1 (Cs, C-6), 57.8 (Cs, C-8), 49.4 (Cs, C-5), 45.3 (Cs, CH₂), 12.0 (Cp, CH₃) ppm. MS(EI): m/z (%) = 396 (18) [M]⁺, 327 (100) [M-CH₂CH₂CCH]⁺. MS(Cl): m/z (%) = 397 (67) [M+H]⁺, 396 (8) [M]⁺. UV-Vis (CH₃CN): ε(λ_max 481 nm) = 29639.6 L mol⁻¹cm⁻¹.
312 mg (1.51 mmol) \( N,N' \)-dicyclohexylcarbodiimide, 174 mg (1.51 mmol) \( N \)-hydroxysuccinimide and 15.4 mg (126 \( \mu \)mol) 4-dimethylaminopyridine were provided under nitrogen atmosphere and cooled to 0 °C. 500 mg (126 mmol) \( N \)-ethyl-\( N \)-(2-(propargyloxy)ethyl)-3-benzoic acid-push-pull-azobenzene (PP \( \text{2} \)) in 2.00 mL dry \( N \),\( N \)-dimethylformamide was added dropwise at 0 °C. After addition the reaction mixture was stirred 2 h at room temperature. The solvent was removed under reduced pressure and the raw product was purified via column chromatography on silica gel (1. dichloromethane \( R_f = 0.10 \), 2. ethyl acetate \( R_f = 0.63 \)). Further impurities of \( N \),\( N \)'-dicyclohexylcarbodiimide and 1,3-dicyclohexylurea were removed via recrystallization in ethyl acetate. The solvent was removed under reduced pressure and the product was obtained as highly viscous dark red oil (471 mg, 76 %).

\( ^1 \text{H-NMR} \) (500 MHz, (CD\(_3\))\(_2\)SO): \( \delta = 8.41 \) (d, \( ^3 J_{3'-4'} = 8.7 \text{ Hz}, 1 \text{H}, H-3' \)), 8.21 (dd, \( ^3 J_{3'-4'} = 8.7 \text{ Hz}, \) \( ^4 J_{4'-5'} = 2.2 \text{ Hz}, 1 \text{H}, H-4' \)), 8.10 (d, \( ^4 J_{4'-5'} = 2.2 \text{ Hz}, 1 \text{H}, H-6' \)), 7.89-7.85 (m, 2H, H-2), 6.94-6.89 (m, 2H, H-3), 4.19 (d, \( ^4 J_{8-10} = 2.4 \text{ Hz}, 2 \text{H}, H-8 \)), 3.68 (s, 4H, H-5, H-6), 3.56 (q, \( ^3 J_{CH_2-CH_3} = 7.0 \text{ Hz}, 2 \text{H}, CH_2 \)), 3.44 (t, \( ^4 J_{8-10} = 2.4 \text{ Hz}, 1 \text{H}, H-10 \)), 2.91 (s, 4H, H-8), 1.17 (t, \( ^3 J_{CH_2-CH_3} = 7.0 \text{ Hz}, 3 \text{H}, CH_3 \)) ppm. \( ^{13} \text{C-NMR} \) (126 MHz, (CD\(_3\))\(_2\)SO): \( \delta = 170.3 \) (C\(_q\), C-7'), 161.5 (C\(_q\), OC=O), 155.5 (C\(_q\), C-5'), 152.7 (C\(_q\), C-4), 146.0 (C\(_q\), C-2'), 143.2 (C\(_q\), C-1), 127.7 (C\(_t\), C-4'), 127.6 (C\(_t\), C-3'), 127.1 (C\(_t\), C-2), 124.0 (C\(_q\), C-1'), 121.9 (C\(_t\), C-6'), 112.3 (C\(_t\), C-3), 80.6 (C\(_q\), C-9), 77.8 (C\(_t\), C-10), 67.6 (C\(_s\), C-6), 58.3 (C\(_s\), C-8), 49.9 (C\(_s\), C-5), 45.8 (C\(_s\), CH\(_2\)), 26.1 (C\(_s\), C-8'), 12.5 (C\(_p\), CH\(_3\)) ppm. MS(EI): m/z (%) = 493 (15) [M]+. MS(CI): m/z (%) = 494 (2) [M+H]+. UV-Vis (CH\(_3\)CN): \( \epsilon(\lambda_{\text{max}} \text{500 nm}) = 27157.8 \text{ L mol}^{-1}\text{cm}^{-1} \).
Figure S1. Synthesis of PP 3. a) K$_2$CO$_3$, N$_2$, 80 °C, DMF; b) NaNO$_2$, 0-5 °C, HCl; c) DCC, DMAP, 0 °C -RT, DMF $[^{[S1]}]$. 

2. Preparation of self-assembled azobenzene monolayers

Reagents were purchased from ABCR, Alfa Aesar and Aldrich. CH$_3$-O-PEG-C≡CH (PEG-MW 2000 Dalton) was purchased from Rapp Polymere and c(RGDfK) peptide from Panatecs. All reagents were used as delivered. Self-assembled monolayers were prepared by chemisorptions of bromo-terminated alkyltrichlorosilane on silicon oxide surfaces, i.e. activated quartz or glass slides, which were subsequently functionalized with azobenzene and cyclic RGD peptide.

For surface cleaning and activation quartz and glass slides were immersed in a solution containing 98 % H$_2$SO$_4$ and 30 % H$_2$O$_2$ (volume ratio 7:3) for 1 h, rinsed thoroughly with bidest. water and dried under nitrogen gas. Then the slides were immersed in isopropanol and treated with ultrasound for 5 min. This procedure was
repeated once with isopropanol and twice with double destilled H$_2$O. Then the slides were dried at 130 °C in an oven for 45 min and finally treated with oxygen plasma (Expanded Plasma Cleaner PDC-002, Harrick Plasma) for another 45 min (29.6 W, 1-1.5x10$^{-1}$ mbar).

Surface coating with a coupling layer and the subsequent click reaction were carried out as reported in literature.$^{[S2]}$ In brief, cleaned and activated slides were immersed in a solution of 11-bromoundecyltrichlorosilane (750 µL) in anhydrous toluene (150 mL) for 30 min in a glove box under nitrogen atmosphere. Then the slides were rinsed once with anhydrous toluene under nitrogen atmosphere and were taken out of the glove box, and rinsed twice with toluene. Then the slides were immersed in toluene and treated twice with ultrasound for 5 min, followed by two rinsing steps with N,N-dimethylformamide. In order to obtain azide terminal groups on the monolayer, the slides with bromo terminal groups were immersed for 3 d in a saturated solution of sodium azide in anhydrous N,N-dimethylformamide at 70 °C. After 3 d the slides were immersed in fresh N,N-dimethylformamide, treated with ultrasound for 5 min and rinsed with N,N-dimethylformamide one more time. This procedure was repeated with double destilled H$_2$O and toluene. Afterwards the slides were dried under nitrogen gas.

For the click reaction (Figure S2 a), solutions of 1.10$^{-3}$ M PP 3 in ethanol and 1.10$^{-3}$ M CH$_3$-O-PEG-C≡CH (PEG-MW 2000 Dalton) in ethanol were prepared. 1.00 mg Copper(II) sulfate pentahydrate and 1.00 mg sodium ascorbate were suspended in double destilled H$_2$O (1 mL). 400 µL of the suspension were added to 11 mL of a mixture of the azobenzene solution and the polyethylene glycol CH$_3$-O-PEG-C≡CH solution (PP 3:PEG, 1:99). The slides were immersed into the reaction mixture and stirred under nitrogen atmosphere at room temperature for 3 d. After 3 d the slides
were immersed in ethanol, treated with ultrasound for 5 min and rinsed with ethanol. This procedure was repeated with acetone and isopropanol.

Figure S2. The NHS-activated acid reacts easily with the lysine amino function of the c(RGDfK) peptide.

3. Cyclic RGD peptide coupling

For coupling of cyclic RGD peptide (Figure S2 b),[S3] 2 mg of c(RGDfK) were dissolved in 1 mL phosphate buffered saline (PBS, pH = 7.2). The slides with activated ester azobenzene monolayer were incubated with 100 µL c(RGDfK) in PBS at room temperature. After an immersion time of 2 h, the slides were rinsed twice with double destilled H₂O, then immersed in double destilled H₂O and treated with ultrasound for 5 min. After a final rinsing step with acetone, the c(RGDfK) functionalized surfaces were dried under nitrogen gas and stored under nitrogen until further use.
4. Single-cell force spectroscopy (SCFS)

The detachment forces of cells were measured using an atomic force microscope (Nanowizard III, JPK Instruments) mounted onto an inverted light microscope (IX71, Olympus). All experiments were performed in HBSS buffer at room temperature. First, tipless Si₃N₄ cantilevers with Au reflective coating and nominal spring constant in the range of 0.06 to 0.35 N/m (NP-O10, Bruker) were calibrated using the thermal noise method implemented in the JPK AFM software. Then, the calibrated cantilevers were functionalized with concanavalin A (conA, Sigma-Aldrich, C2272) to immobilize single REF52 wt cells according to standard procedures. To capture a cell, a number of freshly trypsinised cells were injected into the sample chamber and the cantilever was immediately brought into contact with a cell with an applied force of a few nN for 8 s. Eventually, the cell-capturing cantilever was carefully lifted off the surface and held in place for a resting period of 12 min to guarantee stable attachment of the cell. Force-distance curves were recorded at an approach/retract speed of 30 µm/s. Experiments with 1 s and 3 s contact times between cell and surface were carried out at a constant force of 500 pN. The force curves were recorded on four different positions on the substrate as defined by the vertices of a 10 × 10 µm square. 20 and 10 force curves were taken on each of the four positions in the 1 s and 3 s contact time experiments, respectively. Force-distance curves were initially recorded in the dark, i.e. with the c(RGDfK)-azobenzene in the trans state, before turning on the green light (530 nm, 182 µW/cm², measured using a FieldMaxII Laser Power Meter, Coherent). Subsequently, the surface was set to the “active” state by switching on the 530 nm light, which mounts on an objective lens slot and illuminates from beneath. During exposure, force curves were taken at the same four positions of the sample. Each force-taking sequence (whether the light was switched on or off) was followed by a 3 min waiting time before commencing with new force measurements.
in order to let the cell recover from previously taken force curves. Each cell was used for two complete cycles of light OFF/light ON/light OFF sequences. Control experiments were conducted on glass substrates to show that cells are not influenced by irradiation. The analysis of force curves was conducted using JPK Instruments data processing software. Statistical differences were determined using Origin 9.1 (OriginLab).

5. Quantitative real-time PCR (qRT-PCR)

mRNA was extracted and cDNA was synthesized with SuperScriptTM III CellsDirect cDNA Synthesis System using oligo(dT)20 primer (Invitrogen). The qRT-PCR was performed using GoTaq® qPCR Master Mix (Promega) on Applied Biosystems 7300 Real Time PCR System (ABI) according to the manufacturer’s protocol. Template cDNA amounts were equilibrated by GAPDH. All primers were diluted to 10 pmol/µl. The concentrations were measured using a Nanodrop spectrophotometer (Thermo Scientific). Each analysis was repeated with at least two technical replicates. All samples were analyzed using GAPDH as the reference gene. Expression levels were calculated with the ∆∆Ct method.

6. Single-rupture analysis

In addition to the force analysis of the last rupture event, which was addressed in Figure 3, the position (d) (the distance along the x-axis at which rupture takes place) of this rupture event as well as the length of the tether (w) prior to rupture were analyzed (Figure S3). The results show a shift toward a higher rupture position and a longer tether length during RGD push-pull azobenzenes oscillation for 1 s and 3 s cell-surface contact time experiments. The shift in rupture position is significant at a level of p < 0.001 (Student’s t-test) for both cell-surface contact times (number of
force curves for 1 s contact time: 1318 (light OFF) and 868 (light ON); 3 s contact time experiments: 559 (light OFF) and 392 (light ON)). The shift in tether length is significant at a level of $p < 0.001$ for 1 s contact time experiments and at a level of $p < 0.01$ for 3 s contact time experiments for the same number of force curves shown above.

**Figure S3.** Relative frequencies of the position (d) and tether length (w) of the last rupture event in force-distance curves. a, representative force-distance curves taken during light is off (black) and light is on (green), including the definition of the position
(d) and the tether length (w) of the last rupture. b and c, the position of the last rupture event for 1 s and 3 s cell-surface experiments, respectively. d and e, the tether length of the last rupture event for 1 s and 3 s cell-surface experiments, respectively.

5. Primers used in the gene expression experiments

| Gene   | Primer | Sequence (5’ -> 3’)               |
|--------|--------|----------------------------------|
| Paxillin | Pxn-F  | AGGCTGTTTGTCCCTCTCAC             |
|         | Pxn-R  | GACGGACTTCTGGCTCTA               |
| Zyxin  | Zyx-F  | GCATGGCCTAGGACTGAG               |
|         | Zyx-R  | CAGCAGCACAAGGTGACCC              |
| Vincullin | Vcl-F  | GGACTAAGACCCCTGGATG              |
|         | Vcl-R  | AGGAAATAGGGAAGCCTGAG             |
| Talin  | Tln1-F | GAAGGAGAAATGGTTGGGGG             |
|         | Tln1-R | TCTCGAAGCTCTGAAGGCAAG            |

References for the Supporting Information

[S1] J. I. Gavrilyuk, U. Wuellner, S. Salahuddin, R. K. Goswami, S. C. Sinha, C. F. Barbas III, *Bioorganic & Medicinal Chemistry Letters* 2009, 19 (14), 3716–3720.

[S2] T. Lummerstorfer, H. Hoffmann, *J. Phys. Chem. B* 2004, 108, 3963.

[S3] S. Petersen, J. M. Alonso, A. Specht, P. Duodu, M. Goeldner, A. del Campo, *Angew Chem Int Edit* 2008, 47, 3192.