Electronic Supplementary Information

Yellow fluorescent protein-based label-free tension sensors for monitoring integrin tension

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Materials and Methods

Protein engineering

The genes encoding $(GB1)_4$-YFP-$(GB1)_4$, RGD-YFP-Cys and YFP-RGD-Cys were constructed following a well-established iterative strategy, which is based on the identical sticky ends generated from BamHI and BglII restriction sites,[1, 2] and cloned into the pQE80L vector (for $(GB1)_4$-YFP-$(GB1)_4$) or a modified pQE80L vector (for RGD-YFP-Cys, YFP-RGD-Cys and YFP-Cys) linearized with restriction enzymes BamHI (Cat. No.: R3136S, NEB) and KpnI (Cat. No.: R3142S, NEB). The modified pQE80L vector carries a Cys codon and a stop codon located 3' to the KpnI site and in-frame with the protein to be expressed. Protein expression was carried out in the E. coli strain BL21 following standard protocols. Briefly, 20 mL starter culture was grown in 800 mL 2.5% lysogeny broth (LB) with 0.1 g/L ampicillin at 37 °C until OD600 reached 0.8-1.0. Protein overexpression was induced by 1 mM isopropyl-1-β-D-thiogalactoside (IPTG, Thermo Fisher) and then continued for 4 h at 37 °C. The bacteria cells were harvested by centrifugation at 3000 *g* for 10 min at 4 ºC. The pellet was stored at –80 ºC overnight. Then the cells were lysed using 1mg/mL lysozyme. DNAs and RNAs were digested by using 5 μg/mL DNase I and 5 μg/mL RNase A. Soluble proteins were purified using Co^{2+} affinity chromatography and eluted in phosphate buffer saline (PBS) containing 250 mM imidazole. The purity of the purified proteins was examined via SDS-PAGE analysis (Fig. S3).

Single-molecule AFM experiments

Single-molecule AFM experiments were carried out on a custom-made AFM, which was constructed as previously described[3]. Each cantilever (MLCT, Si_3N_4, Bruker, Santa Barbara, CA) was calibrated in buffer (PBS, 10 mM, pH 7.4) by using the equipartition theorem. The nominal spring constant is 40 pN/nm. In a typical experiment, ~1 μL of protein sample at the concentration of ~1 mg/mL was deposited onto a clean glass coverslip covered with ~100 μL of PBS buffer to allow the protein to adsorb onto the glass cover slip nonspecifically. To stretch the polyprotein, the AFM tip was brought into contact with the glass surface at a contact force of ~1 nN and then withdrawn from the surface at a constant velocity. Due to non-specific interactions, a polyprotein molecule could be picked up and stretched by the AFM tip in about 2% of the force-extension curves. Pulling experiments were performed at pulling speeds of 100, 400, 1000 and 4000 nm/s. Data analysis was carried out using a custom written code in Igor Pro (6.7).

Tension sensor (TS) immobilization on glass surface

Glass surfaces were activated with chromic acid overnight and washed with ultrapure water. The activated glass was immersed in 1.5% (v/v) (3-aminopropyl)-triethoxysilane (APTES, Cat. NO.: 440140-100ML, Sigma) in toluene for 2 h. The aminated glass was heated at 80 ºC for 40 min. 50 mg per ml maleimide-PEG-succinimidyl carboxymethyl ester (Cat.NO.: 22102, Thermo Fisher) dissolved in toluene was deposited onto the glass surface and incubated for 3 h, and then washed with toluene, ethanol, and dried with nitrogen gas. The RGD-YFP-Cys solution (~1 mg /mL) (or YFP-RGD-Cys in the negative control experiment) was then drop cast on the maleimide-glass and incubated at 4 ºC overnight for conjugation via the thiol-maleimide coupling chemistry. The functionalized glass surface functionalized with tension sensors were then washed with fresh PBS prior to use in cell plating experiments.

Cell culture and plating

Swiss 3T3 cells and human lung fibroblast (HLF) cells were cultured with DMEM medium (Cat. No.: 11995065, Thermo Fisher) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Chinese Hamster Ovary (CHO) cells were cultured with MEMα medium with 10% FBS. PC-3 cells were cultured with Ham's F-12K medium (Cat. No.: 211272022, Thermo Fisher) with 10% FBS. The cells reaching *ca.* 60–80 % confluence were used in the plating experiment. Briefly, the cells were detached from the culture dishes with EDTA solution and spun down at 300 g for 3 min, and then resuspended with fresh medium. The cells were re-plated on the tension sensor-conjugated glass surface and cultured for 2 h prior to the imaging experiments.

Inhibition test for actomyosin

To test whether the YFP tension sensor can sense the integrin tension change, the nonmuscle myosin II inhibitor blebbistatin (50μM) (Cat. No.: 13013, Cayman Chemical), and MLCK inhibitor (Y27632, 100 μM) (Cat. No.: 10005583, Cayman Chemical) was applied upon the re-plating experiment, and the fluorescence loss was recorded at 2 h with an IX83 inverted microscope (Olympus, Richmond Hill, ON, Canada), which is equipped with an X-Cite 120XL metal-halide light source (Excelitas Technologies, Mississauga, ON, Canada), a white-LED transmitted light source, an Orca-Flash 4.0 V2 sCMOS camera (C11440; Hamamatsu Photonics, Hamamatsu, SZK, Japan), motorized filter wheels (Sutter Instruments, Novato, CA, USA), and MetaMorph/MetaFluor software (Molecular Devices, Sunnyvale, CA). Filters and dichroic mirrors were from Chroma (Bellows Falls, VT). The cells were imaged using a 60x objective lens under differential-interference-contrast and fluorescence mode. The filters used for fluorescence imaging were: Exc. Filter 490/20, Em. Filter 540/50, Dichroic Mirror T510.

Fluorescence loss analysis

The relative average fluorescence loss was carried out via the cross-sectional analysis of the fluorescence intensity near focal adhesion regions or the cell edges using a

custom written Matlab code. The relative average fluorescence loss is defined as $(I_{Background} - I_{loss})/I_{Background}$, where $I_{background}$ is the greyscale fluorescence intensity outside the focal adhesion region, and I_{loss} is the greyscale fluorescence intensity of the focal adhesion region that shows fluorescence quenching.

Immunostaining

The cells were fixed with 3.7% (w/v) formaldehyde for 20 min, and permeabilized with 0.05% Triton X-100. Bovine serum albumin (2% w/v) was used to block non-specific binding. The anti-vinculin (FAK100, Millipore) was added with 1:400 dilution and incubated for 1 h at room temperature, which was followed with secondary antibody (goat anti-mouse IgG & IgM, AP130C, Millipore) binding for 1 h at room temperature. Washing with PBS was applied to between steps. The prepared samples were then imaged using an IX83 inverted microscope. The optical filters used for immunostained vinculin fluorescence imaging were a 550/15 (center wavelength/bandwidth) excitation filter, a dichroic mirror with a 565 nm cutoff, and a 570 nm-longpass filter for emission (Chroma).

Statistical analysis

Student t-test was used for the comparison between two groups of experimental data. A p-value less than 0.05 is deemed statistically significant.

Full length of the engineered proteins (YFP sequence is colored in blue, RGD-containing sequence is underlined)**:**

RGD-YFP-Cys:
MRGSHHHHHHGSTVYAVTGRGDSPASSRS*SAEELFTGVVPILVELDGDVNGHK*
FSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTFGYGLMCFARYPDHMKQH
DFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNIL
GHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGP
*VLLPDNHYLSYQSKLSKDPNEKRDHMVLLEFVTATGITLGMDELYK*RSGLNDIFEA
QKIEWHERSGGTKC

YFP-RGD-Cys:
MRGSHHHHHHGS*SAEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTL*
KFICTTGKLPVPWPTLVTTFGYGLMCFARYPDHMKQHDFFKSAMPEGYVQERTIF
FKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMA
DKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSYQSKLSK
*DPNEKRDHMVLLEFVTATGITLGMDELYK*RSTVYAVTGRGDSPASSRSGLNDIFE
AQKIEWHERSGGTKC

$(GB1)_4$-YFP-$(GB1)_4$:
MRGSHHHHHHGS(MDTYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDN
GVDGEWTYDDATKTFTVTERS)$_4$*SAEELFTGVVPILVELDGDVNGHKFSVSGEGE*

GDATYGKLTLKFICTTGKLPVPWPTLVTTFGYGLMCFARYPDHMKQHDFFKSAM
PEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYN
YNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNH
YLSYQSKLSKDPNEKRDHMVLLEFVTATGITLGMDELYK(RS(MDTYKLILNGKTL
KGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDDATKTFTVTE)$_4$RSGGTP
GRPAAKLN

YFP-Cys:
MRGSHHHHHHGS*SAEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTL*
KFICTTGKLPVPWPTLVTTFGYGLMCFARYPDHMKQHDFFKSAMPEGYVQERTI
FFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIM
ADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSYQSKLS
*KDPNEKRDHMVLLEFVTATGITLGMDELYK*RSGLNDIFEAQKIEWHERSGGTKC

The refolding of YFP does not affect the performance of the YFP tension sensor

In our AFM experiments, we observed that only a small fraction (~30%) of unfolded YFP refolded when the unfolded polypeptide was relaxed to zero force. Residual stretching force can effectively inhibit the refolding of YFP. In the cell experiments, the YFP tension sensor is under tension generated by the integrin, as evident from the co-localization of the YFP fluorescence loss and vinculin. Thus, most of unfolded YFP was not able to refold in the focal adhesion and the YFP remained quenched. Moreover, it appeared that the loss of YFP fluorescence persisted for an extended period of time. Even if the cells were migrated, fluorescence loss remained, as fluorescence loss and vinculin were not completely co-localized in some images (Fig. S5, ESI), suggesting that these YFP remained unfolded after the tension was removed.

References

1. Y. Cao and H. Li, *Nat Mater*, 2007, **6**, 109.
2. M. Carrion-Vazquez, A. F. Oberhauser, T. E. Fisher, P. E. Marszalek, H. Li and J. M. Fernandez, *Prog Biophys Mol Biol*, 2000, **74**, 63.
3. H. Li, H. C. Wang, Y. Cao, D. Sharma and M. Wang, *J Mol Biol*, 2008, **379**, 871.

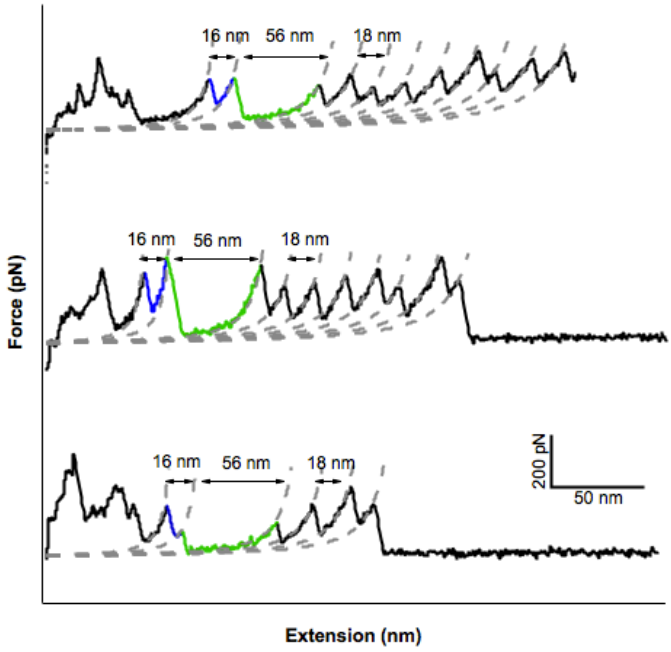

Figure S1. Representative force-extension curves of the mechanical unfolding of YFP via a three-state unfolding pathway. In the three-state unfolding pathway, the unfolding of YFP shows a ΔLc1 of ~16 nm (colored in blue) and ΔLc2 of ~56 nm (colored in green). Dashed lines are worm-like chain (WLC) fits to the force-extension curves.

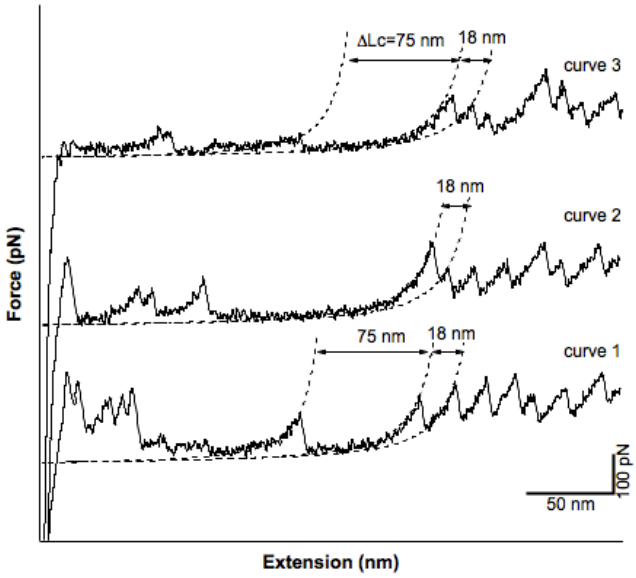

Figure S2. A small portion of YFP domains can refold at zero force. Three consecutive force-extension curves from stretching a single $(GB1)_4$-YFP-$(GB1)_4$ polyprotein are shown. After the unfolding of the polyprotein shown in curve 1), the molecule was relaxed to zero force. In subsequent stretching traces, most of the curves (such as curves 2) did not show the unfolding signature of YFP, suggesting that YFP did not refold at zero force. A small portion (~30%) of the unfolded YFP can refold, resulting the observation of the unfolding event of YFP again (such as curve 3). And residual stretching force can effectively inhibit the refolding of YFP.

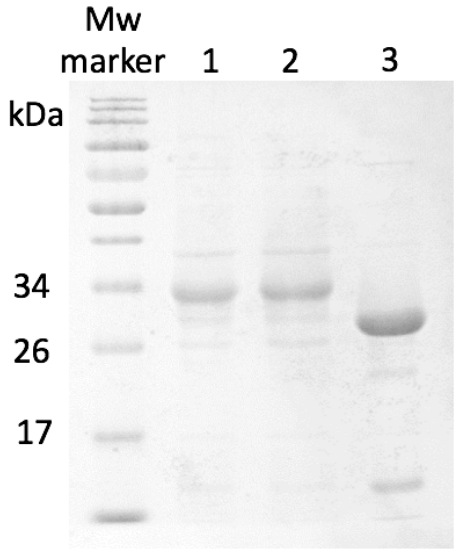

Figure S3. Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis (PAGE) analysis of RGD-YFP-Cys (lane 1), YFP-RGD-Cys (lane 2) and YFP (lane 3). The theoretical molecular weight is 32.6 kDa for RGD-YFP-Cys and YFP-RGD-Cys, and 30.6 kDa for YFP, respectively. It is evident that both RGD-YFP-Cys and YFP-RGD-Cys are full length proteins, and there is no C-terminal proteolytic cleavage.

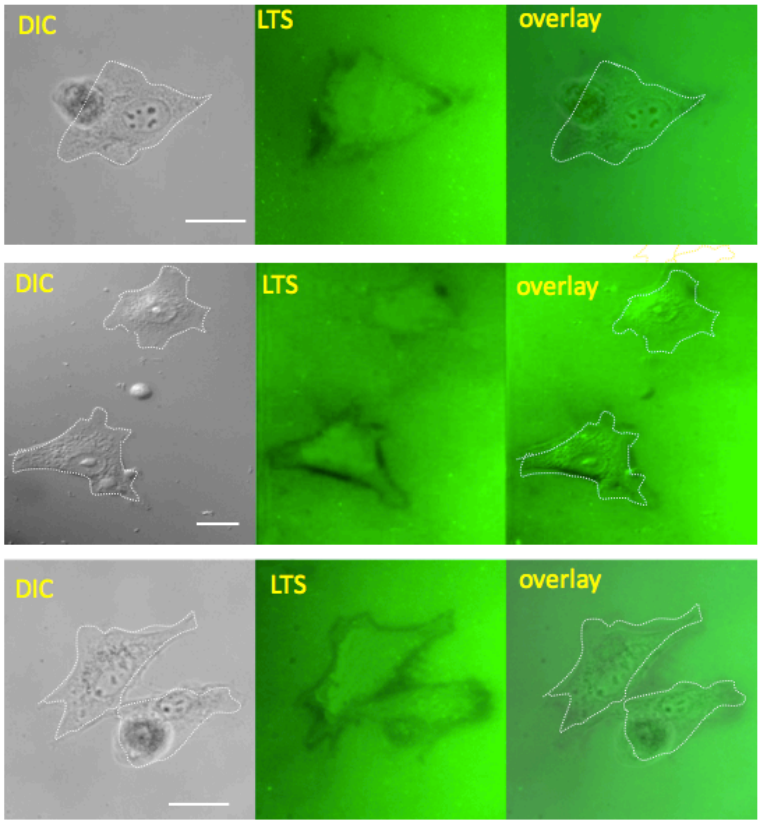

Figure S4. Additional images showing the YFP fluorescence loss when Swiss 3T3 cells are cultured on RGD-YFP coated surfaces. Scale bar: 20 μm.

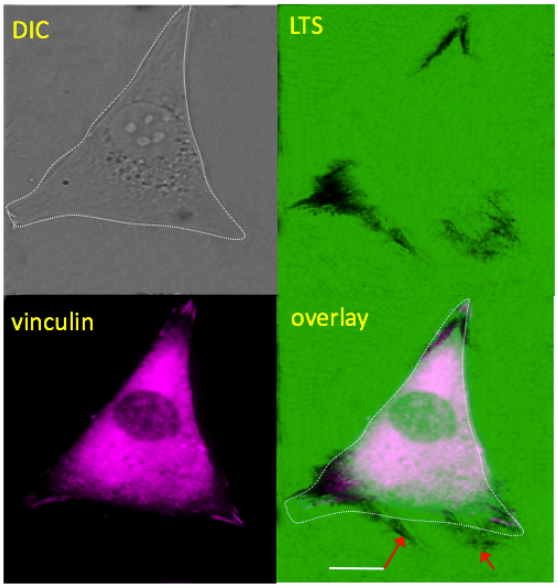

Figure S5. The YFP fluorescence loss can appear in regions outside of the 3T3 cells. These areas are indicated by arrows. Scale bar: 10 μm.

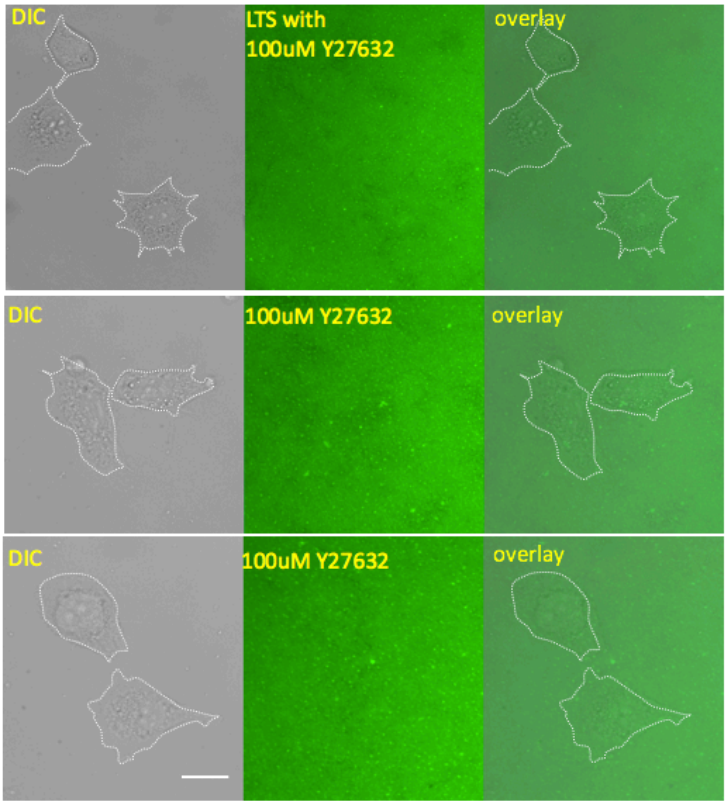

Figure S6. Additional images showing the decreased fluorescence loss after inhibition with 100 μM Y27632 after 2 h cell plating. Scale bar: 20 μm.

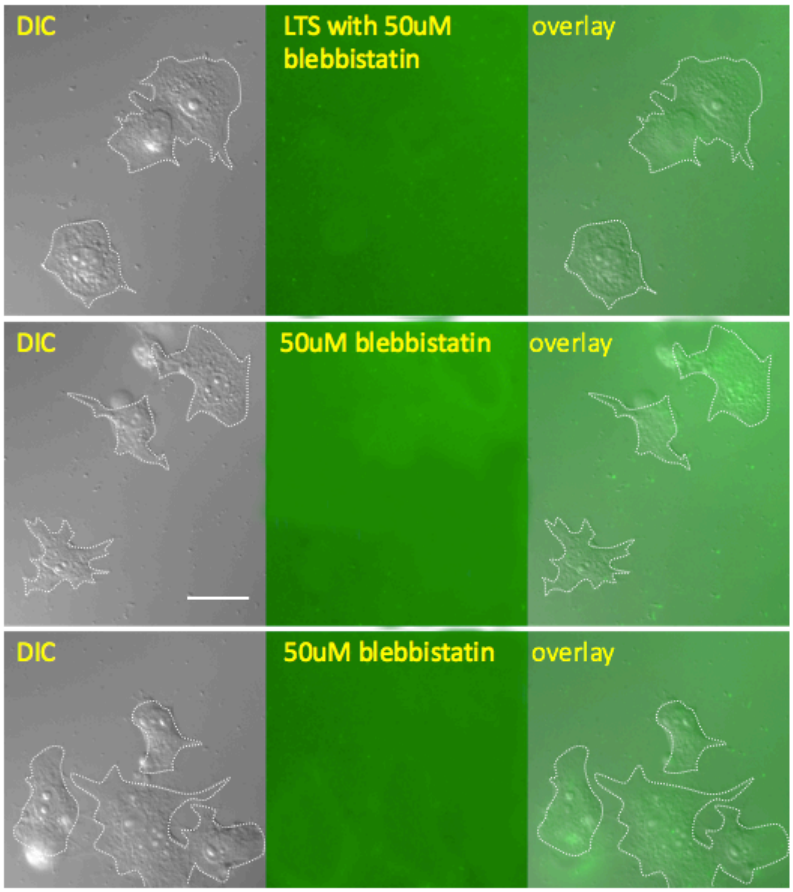

Figure S7. Additional images showing the decreased fluorescence loss after inhibition with 50 μM blebbistatin after 2 h cell plating. Scale bar: 20 μm.

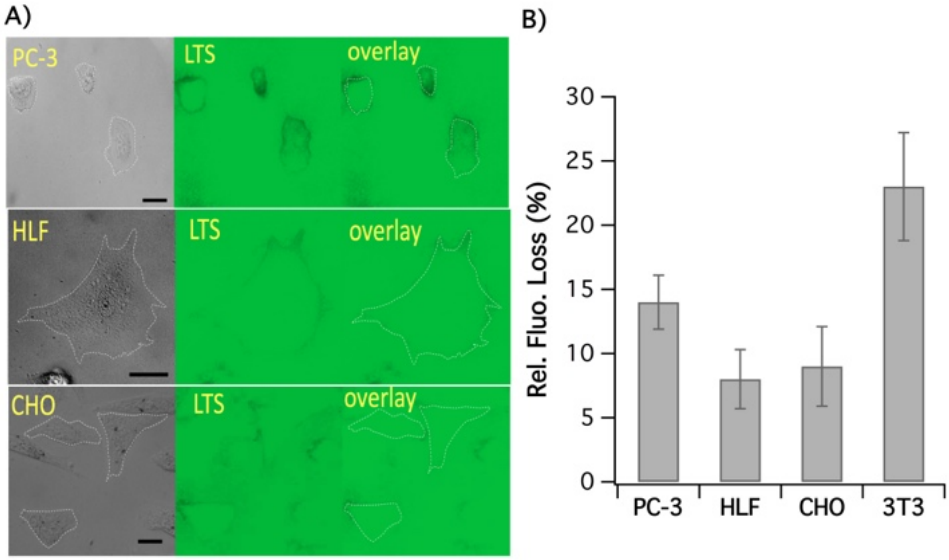

Figure S8. YFP tension sensor maps integrin tension in different cell lines. A). The integrin tensions were monitored with LTS in different cells. The cells were imaged 2 h after cell re-plating. The highest relative fluorescence loss was compared in (B). n=15, Scale bar: 10 μm.

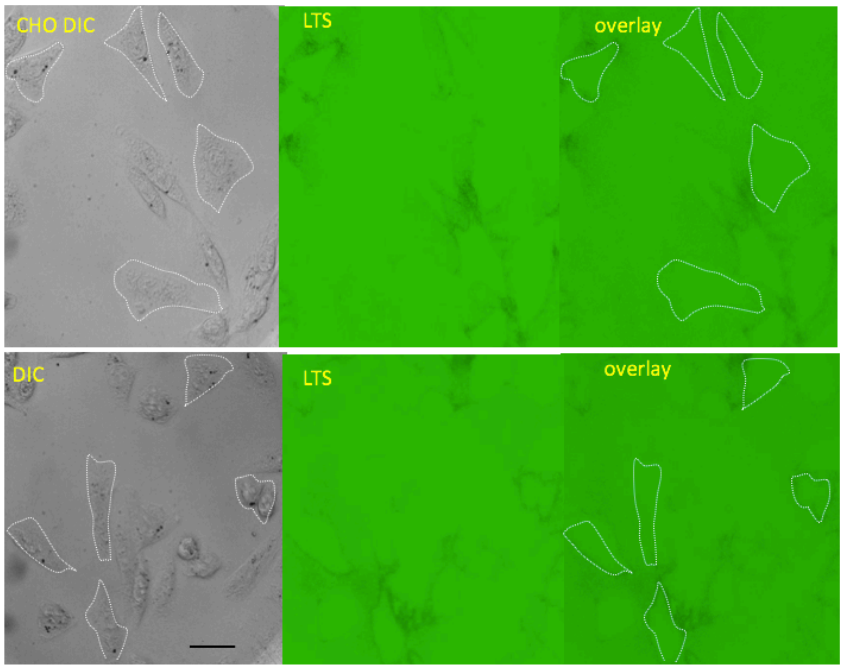

Figure S9. Additional images showing the reduced YFP fluorescence when CHO cells are cultured on RGD-YFP coated surface. Scale bar: 10 μm.

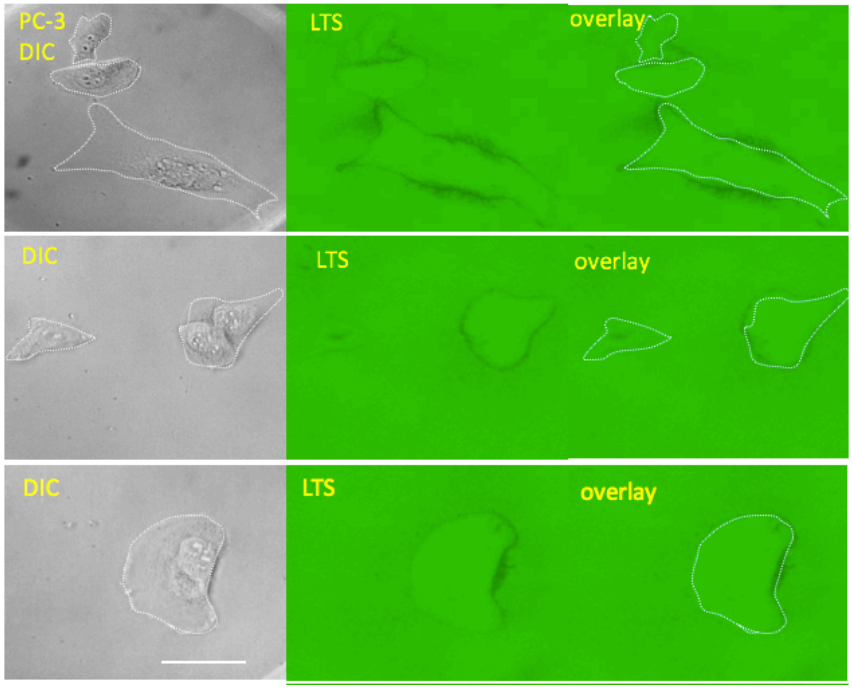

Figure S10. Additional images showing the reduced YFP fluorescence when PC-3 cells were cultured on RGD-YFP coated surface. Scale bar: 20 μm.

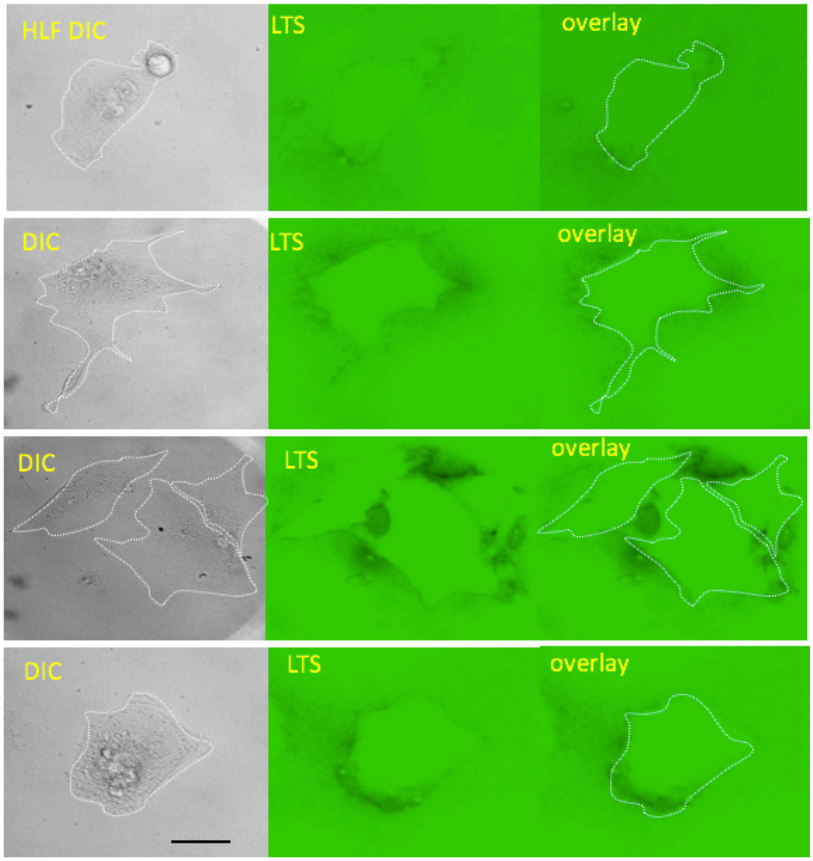

Figure S11. Additional images showing the reduced YFP fluorescence when HLF cells were cultured on RGD-YFP coated surface. Scale bar: 5 μm.