Online methods

Statistics. Statistical significance was determined by Student's t-test, one-way or two-way ANOVAs, as appropriate. Post-hoc testing was done with Dunnet's or Tukey-b test as indicated. Calculations were performed with SPSS (SPSS Inc.) or Excel (Microsoft).

Antibodies. Antibodies used in this study include: mAb paxillin (BD Transduction), AlexaFluor 647 goat-anti-mouse, mAb tubulin E7 (Developmental Studies Hybridoma Bank), mAb vinculin (Sigma), mAb GFP (Santa Cruz), non-muscle myosin heavy chain IIa (Covance), anti-mouse-HRP and anti-rabbit-HRP (Jackson ImmunoResearch Lab.). AlexaFluor 568-phalloidin was from Molecular Probes.

Cell culture conditions and live cell microscopy. Vinculin$^{-/-}$ cells, BAECs, MEFs and HEK293 cells were cultured in DMEM containing non-essential amino acids, penicillin/streptomycin (Gibco) and 10 % calf serum (Atlanta Biologicals). Live cell experiments used phenol-red free DMEM (Gibco). Cells were seeded on No.1.5 microscope cover glasses (Corning) coated with 2.5 μg/ml FN (for BAECs), 10 ug/ml FN or 20 μg/ml poly-L-Lysine (for vinculin$^{-/-}$ cells). To inhibit contractility, cells were treated with 10 μM Y-27632 (Sigma) for 2 h. Images for FRET, FRAP, and FA stability analysis were obtained using a Zeiss LSM510 confocal microscope with a Plan-Neofluor 40x NA=1.3 DIC objective or a Plan-Apochromat 100x NA=1.4 objective and an argon laser featuring 458 nm and 514 nm laser lines. For FRET experiments a HFT 458/514 beam splitter and the following filters were used: mTFP1, BP 470-500, venus/FRET, BP 530-600.

Traction force imaging and analysis. Polydimethylsiloxane (PDMS)-based micropost arrays (mPADs) were used to report cellular traction forces as previously described[16]. To measure traction forces, fluorescent images of the microposts were acquired at focal planes passing through the tip and base of the microposts. Images were taken with a Nikon TE 300 equipped with a violet-corrected Plan Apo 60x NA=1.4 DIC objective and

655 long pass filter set. A custom-written program (Matlab) was used to extract the centroids of the microposts at the tip and base, representing the deflected and undeflected positions. Micropost deflections were converted into forces by multiplying with a spring constant of 7.22 nN/μm.

FLIM experiments and analysis. Time-domain FLIM was performed with a multi-photon microscope system as described previously[23]. The system is based on a modified Bio-Rad MRC 1024MP workstation, comprising a solid-state-pumped femto-second Ti:Sapphire laser system (Tsunami, Spectra-Physics), a focal scan-head and an inverted microscope (Nikon TE2000), and a 40x Nikon CFI Plan Fluor NA=1.3 objective. Enhanced detection of the scattered component of the emitted photons was afforded by the use of fast response (Hamamatsu R7401-P) non-descanned detectors, developed in-house, situated in the re-imaged objective pupil plane. Fluorescence lifetime imaging capability was provided by time-correlated single photon counting (TCSPC) electronics (Becker & Hickl, SPC 700). Data were collected at 500±20 nm through a bandpass filter (Coherent Inc. 35-5040). Laser power was adjusted to give average photon counting rates of the order 10^4–10^5 photons s^{-1} (0.0001 to 0.001 photon counts per excitation event) to avoid pulse pile up. Acquisition times up to 150 s achieved sufficient photon statistics for fitting. Excitation was at 890 nm. FRET efficiency (E) is related to the molecular separation of donor and acceptor and the fluorescence lifetime of the interacting fraction by:

$$E = R_0^6 / (R_0^6 + R^6) = 1 - (\tau_{DA} / \tau_D)$$

where R_0 is the Förster radius, R the molecular separation, τ_{DA} is the lifetime of the interacting fraction and τ_D the lifetime of the donor in the absence of acceptor. mTFP1 demonstrated an average fluorescence lifetime in the un-complexed state of 2.98 ns.

Expression and purification of F40 for force calibration. F40 flanked by a sequence containing a single cysteine and a thrombin cleavage site was inserted into pGEX-4T3. BL21(DE3) pLysS competent cells were transformed and expression was induced with 0.5 mM IPTG. The cell pellet was lysed using 2 mg/mL lysozyme and affinity purification of GST-F40 was performed using GSTrap 4B prepacked columns (GE Healthcare). GST-F40 was eluted in 10 mM glutathione and thrombin was added to a final concentration of 10 mg/mL for 6 h at 4˚C. The thrombin/GST-F40 mixture was then separated on a Superdex75 column and purified with reverse phase chromatography. The F40 elution peak was analyzed and confirmed by mass spectrometry and speed vacuumed to dryness.

Preparation of the TSModCy for fluorescence-force spectroscopy. Two handle DNAs with 5'-amine modifications (5'-CCCACGCGCGACTACCCAGC -3' and 5'-GCCTCG CTGCCGTCGCCA-3') were reacted with 200x molar excess of succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (SMCC). Un-reacted SMCC was removed by fast protein liquid chromatography (FPLC) purification and modified DNA handles were incubated with dried F40 peptide (1:1) in 50 mM Tris buffer (pH 7.5) overnight at 4˚C. Annealing with Cy3- and Cy5-labelled strands was performed by incubation of 250 pmol of the DNA modified F40 peptide, 250 pmol of biotinylated strand (5'-/biotin/-TGGCGACGGCAGCGAGGC -/ Cy5/ 3') and 250 pmol of single stranded (ss) DNA containing a λ-DNA cos site (5'-GGGCGGCGACCTGCTGGGTAGTCGCGCG TGGG/Cy3/-3') in 10 mM Tris-HCl (pH 8.0) and 50 mM NaCl overnight at room temperature. For fluorescence-force experiments, λ-DNA (Promega) was attached to the pre-annealed product as described previously[2]. Subsequently, the digoxigenin-labelled oligonucleotide complementary to the cohesive end-site of λ-DNA was added (5'-AGGTCGCCGCCCTTT /digoxigenin/-3'). Thus, the complete construct for the force

sensor calibration contained a single digoxigenin-tag on λ-DNA and a biotin-tag at the DNA-F40 construct.