Online supporting materials

Detailed Experimental Procedures

Cell culture. Spontaneously immortalized mouse embryo fibroblasts (MEFs) and MCF10A mammary epithelial cells were maintained as previously described [1]. MC3T3-E1 osteoblasts (clone 14; kind gift of Kurt Hankenson) were cultured in αMEM (lacking nucleosides) and 10% FBS. Primary mouse vascular smooth muscle cells (VSMCs) were isolated from 10-12 week old male C57BL/6 aortic explants, and used at passages 2–5. To synchronize cells in G0, MEFs and VSMCs were grown to ~90% confluence and serum-starved for 48-72 h in DMEM with 1 mg/ml heat inactivated, fatty-acid free BSA. MC3T3-E1 osteoblasts were synchronized by growing to ~90% confluence and starving for 48 h in αMEM with 1 mg/ml heat inactivated, fatty-acid free BSA. MCF10A cells were synchronized by growing to ~90% confluence and starving for 48 h in 1:1 DMEM:Ham's F12 nutrient medium with 1 mg/ml BSA. The quiescent cells were trypsinized and suspended in serum-free media for 30 min at 37°C prior to reseeding with mitogens. MEFs, VSMCs, and osteoblasts were stimulated with 10% FBS. MCF10A cells were stimulated with 10% FBS plus a growth factor cocktail containing 20 ng/ml epidermal growth factor (EGF; BD Biosciences), 10 μg/ml insulin (Sigma), 0.5 μg/ml hydrocortisone (Sigma), and 100 ng/ml cholera toxin (List Biologicals). The time points used for analysis were optimal for the particular readout and the cell type being studied.

In some experiments, trypsinized cells in DMEM, 1 mg/ml fatty acid-free BSA were pre-incubated in suspension (30 min at 37^{o}C) with 50 μM U0126 (a MEK inhibitor commonly used to inhibit ERK activity) or 10 μM PP1 (Src family kinase inhibitor) prior to reseeding. Adenoviruses were titered and used as described [1]. GFP-FRNK, CD2-FAK, cyclin D1, and

HPV-E7 adenoviruses were kind gifts from J. Thomas Parsons (University of Virginia), Craig Henke (University of Minnesota), Jeffrey Albrecht (Hennepin County Medical Center), and Meenhard Herlyn (Wistar Institute), respectively. FAK^{Y397F} and Rac^{V12} were kind gifts from Christopher Chen (University of Pennsylvania). Unless noted otherwise, adenoviruses were used at the following MOIs: FRNK (600), FAK^{Y397F} (1500), CD2-FAK (50), Rac^{V12} (900), Rac^{N17} (100), Rho^{V14} (30), cyclin D1 (100), and Cre (800). Control viruses (Ad-LacZ and Ad-GFP) were typically used at the highest MOI of the test virus.

Preparation of ECM-coated hydrogels. Polyacrylamide gels were covalently attached to 25-mm glass coverslips (Fisher) as described previously [1]. Unless noted otherwise, the acrylamide concentration remained constant at 7.5%, and the bis-acrylamide concentration was 0.03% for the low stiffness gels and 0.3% for the high stiffness gels. The elastic moduli under these conditions were 1800 and 24,000 Pa, respectively, as measured by rheology [2]. The coating efficiency of matrix protein is equivalent on hydrogels with differing stiffness [3]. Gels were placed in 6-well plates and coated overnight with either 2 ml collagen-I (6.12 μg/ml in PBS; for MCF10A cells) or fibronectin (3.05 μg/ml in PBS; for MEFs, VSMCs, and MC3T3-E1 osteoblasts). Control experiments showed that the inhibitory effects of low matrix stiffness on cell spreading (Suppl. Fig. 2B) as well as S phase entry and cyclin D1 mRNA induction (Suppl. Fig. 4A and B) were independent of the amount of matrix protein added to the activated hydrogels.

Measurement of mouse tissue stiffness by milliprobe indentation. Inguinal mammary glands were isolated from 3 month-old female C57BL/6 mice. We determined the elastic modulus of these tissues using a custom-built milliprobe indentation device. The device consists of a μN

resolution force probe and a 100-nm resolution micromanipulator (MLW-3, Narishige, Tokyo, Japan). The force probe is adapted from the surface tension measurement apparatus of a Langmuir trough (MicroTroughX, Kibron Inc., Helsinki, Finland) which consists of a tungsten wire (blunt-ended cylinder; radius = 275 μm) hung from a tensiometric cantilever. The probe acts as a Hookean cantilever whose deflection is directly proportional to its vertical displacement (calibrated with known weights and displacements). Briefly, the tissues were brought into contact with the probe by coarse adjustment of the stage followed by 7 successive 5-10 μm upward displacements of the sample towards the probe. These displacements lead to upward deflections of the cantilever as well as small indentations into the sample. The magnitude of indentation and force on the sample were calculated from cantilever calibrations and averaged to derive the average indentation and force on the sample. These were used to quantify sample stiffness by the method of Hayes et al. [4], which calculates stiffness corrected for sample thicknesses on the scale of the radius of the indenter.

$$E = \frac{P(1-\nu^2)}{2a\omega\kappa}$$

where E is the Young's modulus, P is the force on the cantilever, υ is the Poisson ratio, ω is the vertical displacement of the sample, and κ is the sample thickness correction factor. Measurements of polyacrylamide gel stiffness made using this method were consistent with previous bulk stiffness measurements done by rheology [2].

The thickness of the inguinal mammary gland (~1.5-2 mm) was measured with a caliper. A Poisson's ratio of 0.5 was assumed for the mammary glands. Multiple stiffness measurements were made on each tissue sample, and outliers were discarded according to Chauvenet's criterion. Although these tissues are viscoelastic rather than purely elastic, the viscous response

was small compared to the elastic response, and nearly constant values of deformation at a given stress were observed on a time scale of a few seconds. We report only the static elastic modulus E for comparisons of tissue and ECM compliance with the recognition that this comparison is valid for time scales on the order of seconds.

Atomic force microscopy (AFM) of control and injured arteries. Thoracic aortae were isolated from 4-6 month-old C57BL/6 mice or SMA-GFP mice on the C57BL/6 background (kind gift of Sanai Sato). Injured femoral arteries and uninjured contralateral control femoral arteries were isolated from 6-month, male SMA-GFP mice two weeks after fine-wire injury performed largely as described [5]. Each end of cleaned arteries was attached to a 35-mm culture dish using a very small amount of adhesive, the samples were submerged in 2 ml PBS and analyzed by AFM in force mode. The AFM was a DAFM-2X Bioscope (Veeco, Woodbury, NY) mounted on an Axiovert 100 microscope (Zeiss, Thornwood, NY). A silicon nitride cantilever (196 μm long, 23 μm wide, 0.6 μm thick) with a spherical tip (1 μm in diameter) was used for indentation. The spring constant of the cantilever was 0.06 N/m (Novascan, IA). To quantify stiffness (Young's modulus), the first 400-800 nm of tip deflection was fit with the Hertz model for a sphere.

$$f_{bead} = k * d_{cantilever} = \frac{4}{3}\frac{E}{1-\nu^2}\sqrt{R}\delta^{\frac{3}{2}}$$

where f_{bead} is the force on the bead, $d_{cantilever}$ is the deflection of the cantilever measured by the AFM, E is the Young's modulus, ν is the Poisson ratio, R is the radius of the bead and δ is the vertical indentation of the cantilever. To assess femoral artery stiffness at sites of vascular injury, the AFM was combined with fluorescence microscopy to distinguish GFP-positive and GFP-negative regions of injured femoral arteries.

Analytical methods. Total RNA was collected and analyzed by QPCR as described [1]. Taqman primer and probe sequences for human cyclin D1 mRNA, mouse cyclin D1 mRNA, and 18S rRNA (same primer/probe set for mouse and human samples) have been listed previously [1]. Fra-1, JunB, and INK4 mRNA levels were analyzed by SYBR Green QPCR using primers (900 nM) listed in Suppl. Table 3. Mouse KLF8 mRNA levels were analyzed using Assay-on-Demand Mm00620643_m1 (Applied Biosystems). RNA expression was quantified using a standard curve. QPCR results are shown as the mRNA levels normalized to 18S rRNA levels and plotted as the mean ± s.d. of duplicate PCR reactions. Samples for western blotting were collected as described [1] and probed using the following antibodies: cyclin D1 (sc-718, Santa Cruz), p27 (610241, BD Biosciences), p21 (sc-6246, Santa Cruz), phospho-ERK (9101S, Cell Signaling), ERK (610030, BD Biosciences), Rac (05-389, Upstate Biotechnology), phospho-FAK^{Y397} (44-624, Invitrogen), FAK (sc-558, Santa Cruz), Rb (28-0007, Zymed), cdk4 (sc-260, Santa Cruz), and GAPDH (sc-25778, Santa Cruz).

Rac and Rho GTPase assays. Serum-starved MEFs were reseeded on high and low stiffness hydrogels and stimulated with 10% FBS. VSMCs were seeded on hydrogels, serum-starved for 48 h, and stimulated with 10% FBS. Active Rac and Rho GTPase levels were measured using a G-LISA small G-protein activation assay kit (Cytoskeleton, Inc.) according to manufacturer's directions. Data were normalized to unstimulated GTPase activity and representative experiments are shown.

Immunofluorescence microscopy and colocalization analysis. S phase entry was determined by measuring BrdU- or Ki67-stained nuclei similarly to the procedure described [1].

Polymerized actin was visualized with FITC-conjugated phalloidin. BrdU and phalloidin staining were visualized by epifluorescence microscopy using a Nikon Eclipse 80i microscope, and images were digitally acquired (Hamamatsu camera C4742-95). For focal adhesion immunofluorescence, cells were fixed and permeabilized in 3.7% formaldehyde, 5% sucrose, and 0.5% Triton X-100 in PBS for 3 minutes followed by 3.7% formaldehyde and 5% sucrose in PBS for 15 minutes. All antibody incubations and washes were done in PBS with 1 mg/ml BSA and 0.1% Triton X-100. Coverslips were incubated with primary antibodies (FAK, BD Biosciences 610087; vinculin, Sigma V4505; paxillin, BD Transduction Labs 612405; and talin, a gift from Keith Burridge, University of North Carolina) for 1.5 h. After washing, coverslips were incubated with secondary antibodies (anti-mouse Alexa 488 and anti-rabbit Alexa 568, Invitrogen) for 1 h. Washed coverslips were mounted with anti-fade medium and images were acquired on a Leica TCS SP2 confocal microscope.

FAK and talin colocalization was determined by morphometric analysis of confocal images using ImagePro software. Briefly, intensities of FAK and talin signals were measured using the line intensity tool. Several lines were drawn across the edges of multiple cells, and the coincidence of signal peaks was determined. The pixel intensities for each color were normalized to the minimum intensity in order to superimpose peaks. Quantification of FAK/talin colocalization in wild-type cells was determined by the ratio of coincidental peaks to total talin peaks. A minimum of 24 lines per condition were analyzed and averaged. The level of coincidence due to noise was assessed by immunostaining FAK-knockout MEFs ([6]; obtained from ATCC) for FAK and talin using the same procedure. The average apparent colocalization in the knockout cells was defined as background and subtracted from the averaged colocalization in the wild-type cells.

Suppl. Table 1. Relative expression of transcription factors regulating cyclin D1 expression

Transcript	Expression level (relative to cyclin D1 mRNA)
JunB	25.7-46.0
Fra-1	4.3-15.4
KLF8	0.03-0.09

MEFs were starved, stimulated with 10% FBS, and reseeded on high stiffness FN-coated substrata for 1-3 hr. The mRNA expression of each gene was calculated using the comparative CT method [7] and is reported relative to the expression of cyclin D1 mRNA under conditions where cyclin D1 mRNA and protein are maximally expressed (9 h after seeding FBS-stimulated MEFs on stiff hydrogels). The ranges correspond to the relative expression seen in 3 independent experiments.

Suppl. Table 2. Measurement of tissue elastic moduli

Tissue	Methodology	Elastic modulus (Pa) ± SEM	n
Mammary gland	Milliprobe indentation	631 ± 48	3
Thoracic aorta	AFM	4311 ± 239	4
Femoral artery	AFM	3208 ± 332	4*

Freshly isolated tissue from C57BL/6 or SMA-GFP mice was analyzed by milliprobe indentation or AFM as described in Detailed Experimental Procedures. *Taken from control samples in Fig. 6C.

Suppl. Table 3. Primers for QPCR

mRNA	Forward Primer	Reverse Primer
Mouse Fra-1	ACCGAAGAAAGGAGCTGACA	CTGCTTCTGCAGCTCTTCAA
Mouse JunB	ATCCCTATCGGGGTCTCAAG	AGAGATGCGCCTGTGTCTG
Human Fra-1	CAGGCGGAGACTGACAAACT	CTTCCAGCACCAGCTCTAGG
Human JunB	TCTCTCAAGCTCGCCTCTTC	CGGGGGTAAAAGTACTGTCC
Mouse p15	CACCGAAGCTACTGGGTCTC	CTGTGGCAGAAATGGTCCTT
Mouse p16	CCCACTCCAAGAGAGGGTTT	ATGTTCACGAAAGCCAGAGC
Mouse p18	TCGACTTGGCCAGGTTCTAT	ACATTCACTGCAGGCTTGTG
Mouse p19	GCCACTGTCTCCAGCCTTAC	TCCCACTCCCTTCTTCAATG

Supplemental Figure Legends

Suppl. Figure 1. Near confluent MC3T3-E1 osteoblasts and MEFs were starved and reseeded on FN-coated glass coverslips or on low and high stiffness FN-coated hydrogels with 10% FBS and BrdU. Cells were fixed at 21 h and BrdU incorporation was measured by immunofluorescence.

Suppl. Figure 2. Cell spreading on high and low stiffness ECM. (**A**) Starved MEFs, MCF10A cells, and VSMCs were seeded on ECM-coated hydrogels with mitogens. The cells were fixed at 24 h, and f-actin was visualized with FITC-phalloidin. Scale bars = 50 μm. (**B**) Asynchronous MEFs were seeded on hydrogels coated with selected amounts of FN (1X=0.635 μg/cm^2) and incubated with 10% FBS. The cells were fixed at 9 h, and f-actin was visualized with FITC-phalloidin. Scale bar = 50 μm. The result shows that the impaired spreading at low ECM stiffness is not rescued by altering the amount of matrix protein used to prepare the hydrogel.

Suppl. Figure 3. ERK activity and ERK-dependent transcription is similar on high and low stiffness substrata. (A) Serum-starved MEFs were reseeded on low (L) and high (H) stiffness FN-coated hydrogels and stimulated with 10% FBS. Reseeded cells were collected at the indicated times and analyzed by western blotting. Band intensities from 3 independent experiments were measured using either LICOR Odyssey software or ImageJ: phospho-ERK signals were normalized to total ERK. Statistical significance was determined by t-test (# not significant, $p > 0.1$). (**B)** Serum-starved MEFs were trypsinized, pre-treated with DMSO (vehicle) or U0126 (U0) prior to reseeding on the high stiffness substratum with 10% FBS. Cells were collected at the indicated times and analyzed for Fra1 or JunB mRNA expression by QPCR.

Suppl. Figure 4. Similar mitogenic responses on rigid and high stiffness substrata

(A-B) Serum-starved MEFs were seeded on FN-coated glass coverslips or on low and high stiffness hydrogels coated with increasing amounts of FN (1X=0.635 μg/cm^2). The cells were stimulated with 10% FBS. **(A)** Cells were fixed after 24 h, and BrdU incorporation was measured by immunofluorescence microscopy. **(B)** Cells were collected after 9 h, and cyclin D1 mRNA expression was measured by QPCR. **(C-D)** Mouse VSMCs were serum-starved and reseeded with 10% FBS on FN-coated glass coverslips or on low (L) and high (H) stiffness FN-coated hydrogels for the times shown. Fra-1, JunB, cyclin D1, and cyclin A mRNA levels were determined by QPCR.

Suppl. Figure 5. Focal adhesions regulated by ECM stiffness. Asynchronous MEFs were seeded on glass coverslips or on low and high stiffness FN-coated hydrogels with 10% FBS. The cells were fixed 24 h after reseeding and individual coverslips were immunostained for vinculin, paxillin, FAK, and FAK^{pY397}. Regions of focal adhesions (some of which are indicated by arrowheads) were visualized by confocal microscopy. Scale bar = 30 μm. The FAK and FAK^{pY397} images were taken from independent experiments.

Suppl. Figure 6. Focal adhesions persist in cells expressing FRNK or FAK^{Y397F}. Asynchronous MEFs were infected with adenoviruses encoding GFP, FRNK, or FAK^{Y397F} and seeded on glass coverslips with 10% FBS for 24 h. Focal adhesions were identified by immunostaining for vinculin. GFP-tagged FRNK and IRES-driven GFP in the FAK^{Y397F} construct allowed for identification of infected cells (arrowheads) by epifluorescence microscopy. Scale bar = 50 μm.

Suppl. Figure 7. Effects of FAK deletion and FRNK. (A) Serum starved $FAK^{+/+}$ and $FAK^{-/-}$ MEFs (ATCC) were replated on FN-coated culture dishes and stimulated with a growth factor cocktail comprised of 10 ng/ml bFGF, 10 ng/ml PDGF, 1 μM insulin, and 2 nM EGF. Lysates were prepared at the indicated times and analyzed by western blotting for FAK, cyclin D1, and cdk4 (loading control). **(B)** Starved MEFs infected with adenovirus encoding GFP or GFP-FRNK were trypsinized, pre-treated with DMSO (vehicle) or PP1 (Src-family kinase inhibitor; 10 μM) in suspension (30 min, 37°C), reseeded on plastic culture dishes, and stimulated with 10% FBS. Collected cells were lysed and analyzed by western blotting at the indicated times. ERK activity is regulated by PP1 but not FRNK.

Suppl. Figure 8. CD2-FAK localizes to focal adhesions on high stiffness hydrogels. Floxed-FAK MEFs were infected with Cre adenovirus for 3 days to deplete endogenous FAK. The cells were then infected with adenoviruses encoding either lacZ or CD2-FAK, seeded on high or low stiffness hydrogels, fixed after 24 h, immunostained for FAK, and analyzed by confocal microscopy. CD2-FAK infected cells were identified using the IRES-GFP in the CD2-FAK adenovirus. Scale bar= 30 μm. Arrowheads indicate several areas with focal adhesion staining patterns.

Suppl. Figure 9. FAK localization to activated integrins is regulated by matrix stiffness. (**A**) Asynchronous MEFs were seeded on high and low stiffness hydrogels with 10% FBS, fixed at 24 h, and immunostained for FAK (red) and talin (green). The thin white line in the merged image shows a representative slice taken for FAK-talin colocalization analysis. The insets show 2X magnifications of the areas analyzed for colocalization. Scale bar = 15 μm. (**B**)

Representative line intensities of FAK (red) and talin (green) immunofluorescence signals are shown for wild-type and FAK-null MEFs on high and low stiffness FN-coated hydrogels. The tracings for the wild type cells correspond to the thin white lines shown in the merged images from panel A.

Suppl. Figure 10. Expression of constitutively active Rho does not rescue cyclin D1 expression on low stiffness hydrogels. (**A**) VSMCs were seeded on low and high stiffness FN-coated hydrogels and serum-starved for 48 h. The cells were stimulated with 10% FBS, and Rho activity was measured using the G-LISA assay. **(B-C)** MEFs were starved, infected with adenoviruses encoding LacZ, CD2-FAK, or Rho^{V14} and reseeded on FN-coated hydrogels with 10% FBS and BrdU. (**B**) Cells infected with Rho^{V14} were plated on the low stiffness substratum, fixed 24 h after reseeding, and f-actin was visualized with rhodamine-labeled phalloidin. The Rho^{V14} expression vector contained IRES-driven GFP allowing for identification of Rho^{V14} expressing cells by GFP fluorescence (~70% of cells were GFP-positive). Scale bar = 50 μm. (**C**) Total RNA was collected from cells incubated for 9 h on high (H) and low (L) stiffness hydrogels, and cyclin D1 mRNA levels were measured by QPCR.

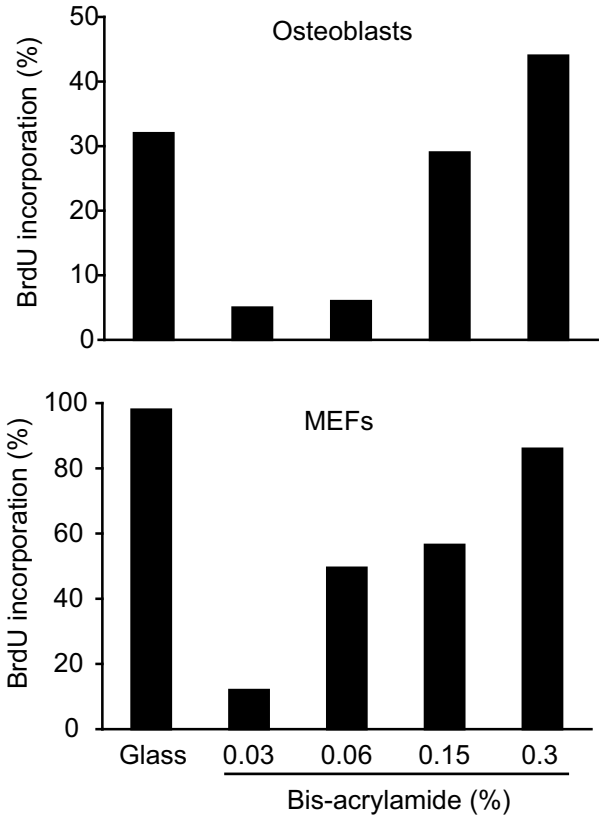
Suppl. Figure 11. Matrix stiffness regulates Rac-GTP loading in VSMCs. (**A**) VSMCs were seeded on high (H) and low (L) stiffness FN-coated hydrogels and serum-starved for 48 h. The cells were stimulated with 10% FBS, and Rac GTP-loading was measured. (**B**) The same conditions were used in an independent experiment to assess Rac protein levels by western blotting after 48 h on the hydrogels.

Suppl. Figure 12. Matrix stiffness regulates cyclin D1-dependent S phase entry in MCF10A cells. Starved MCF10A mammary epithelial cells infected with adenoviruses encoding GFP (100 MOI), cyclin D1 or HPV-E7 (30 or 100 MOI) were seeded on low (L) and high (H) stiffness collagen-coated hydrogels and stimulated with mitogens as described in Detailed Experimental Procedures. S phase entry was assessed by BrdU incorporation 24 h after mitogenic stimulation.

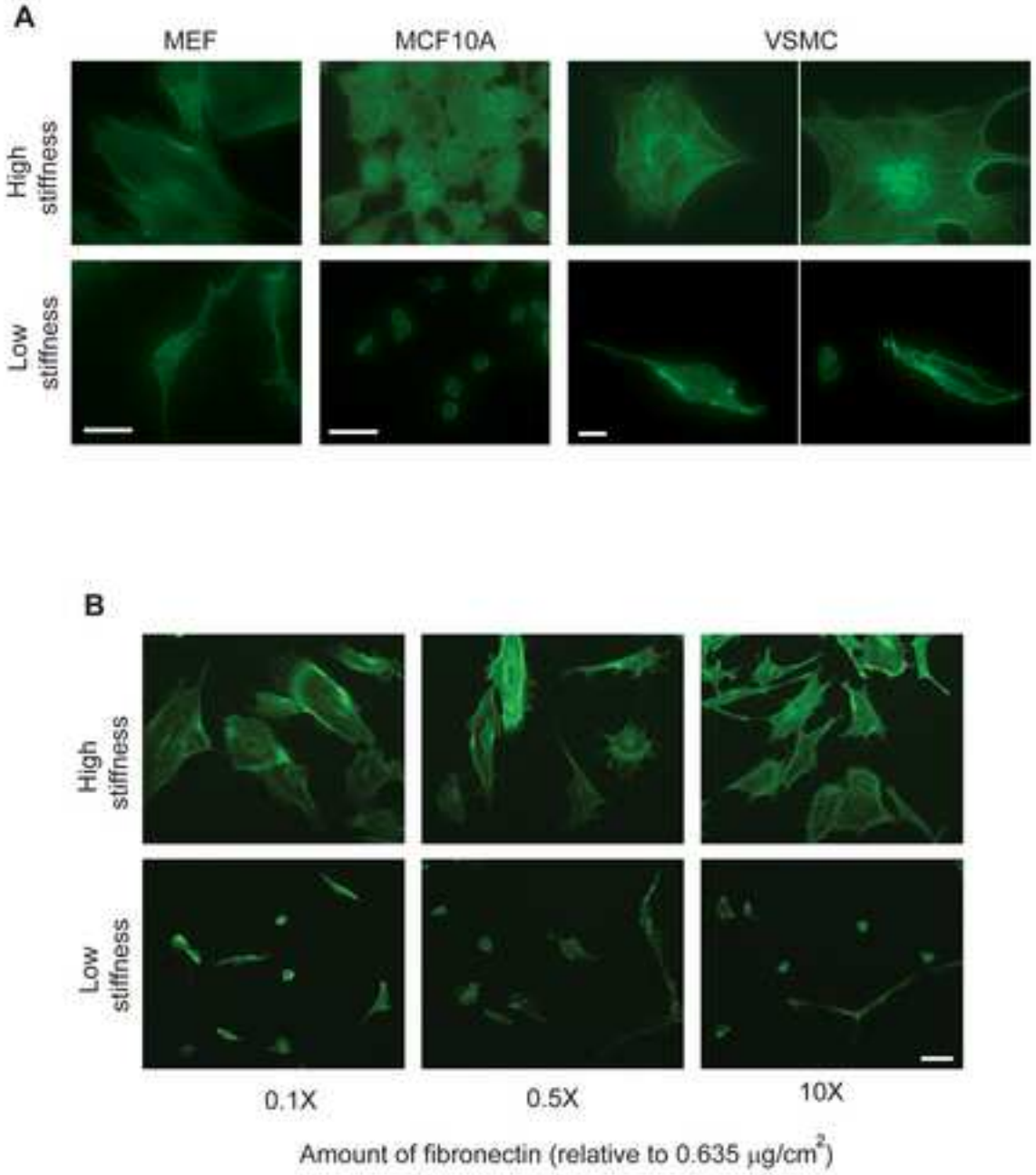
Supplemental References

1. Klein, E.A., Yung, Y., Castagnino, P., Kothapalli, D., and Assoian, R.K. (2007). Cell adhesion, cellular tension, and cell cycle control. Methods Enzymol *426*, 155-175.
2. Yeung, T., Georges, P.C., Flanagan, L.A., Marg, B., Ortiz, M., Funaki, M., Zahir, N., Ming, W., Weaver, V., and Janmey, P.A. (2005). Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. Cell Motil Cytoskeleton 60, 24-34.
3. Byfield, F.J., Wen, Q., Levental, I., Nordstrom, K., Arratia, P.E., Miller, R.T., and Janmey, P.A. (2009). Absence of filamin A prevents cells from responding to stiffness gradients on gels coated with collagen but not fibronectin. Biophys. J. 96:5095-102.
4. Hayes, W.C., Keer, L.M., Herrmann, G., and Mockros, L.F. (1972). A mathematical analysis for indentation tests of articular cartilage. J Biomech *5*, 541-551.
5. Kothapalli, D., Zhao, L., Hawthorne, E.A., Cheng, Y., Lee, E., Pure, E., and Assoian, R.K. (2007). Hyaluronan and CD44 antagonize mitogen-dependent cyclin D1 expression in mesenchymal cells. J Cell Biol *176*, 535-544.
6. Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature *377*, 539-544.
7. Bookout, A.L., and Mangelsdorf, D.J. (2003). Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. Nucl Recept Signal 1, e012.

Suppl. Figure 1

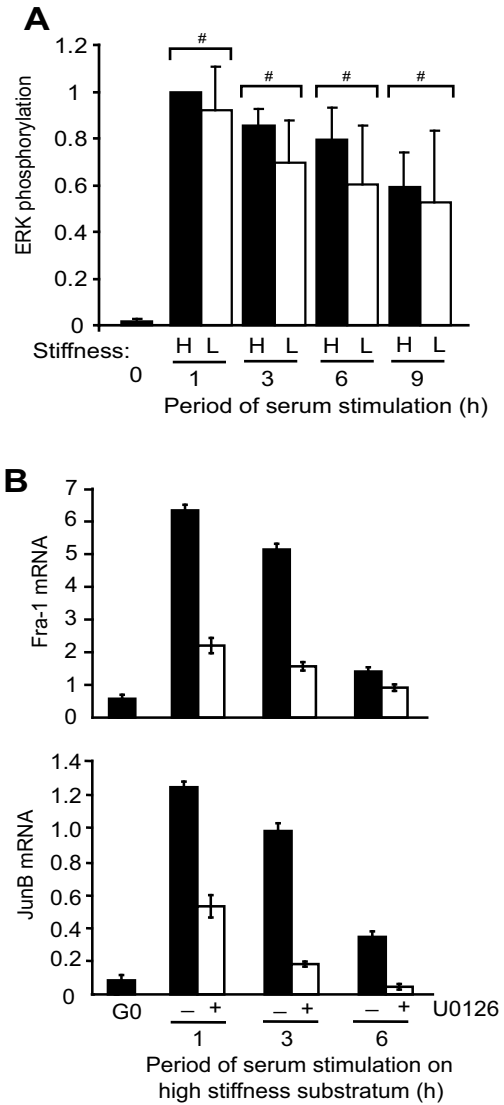


Suppl. Figure 2



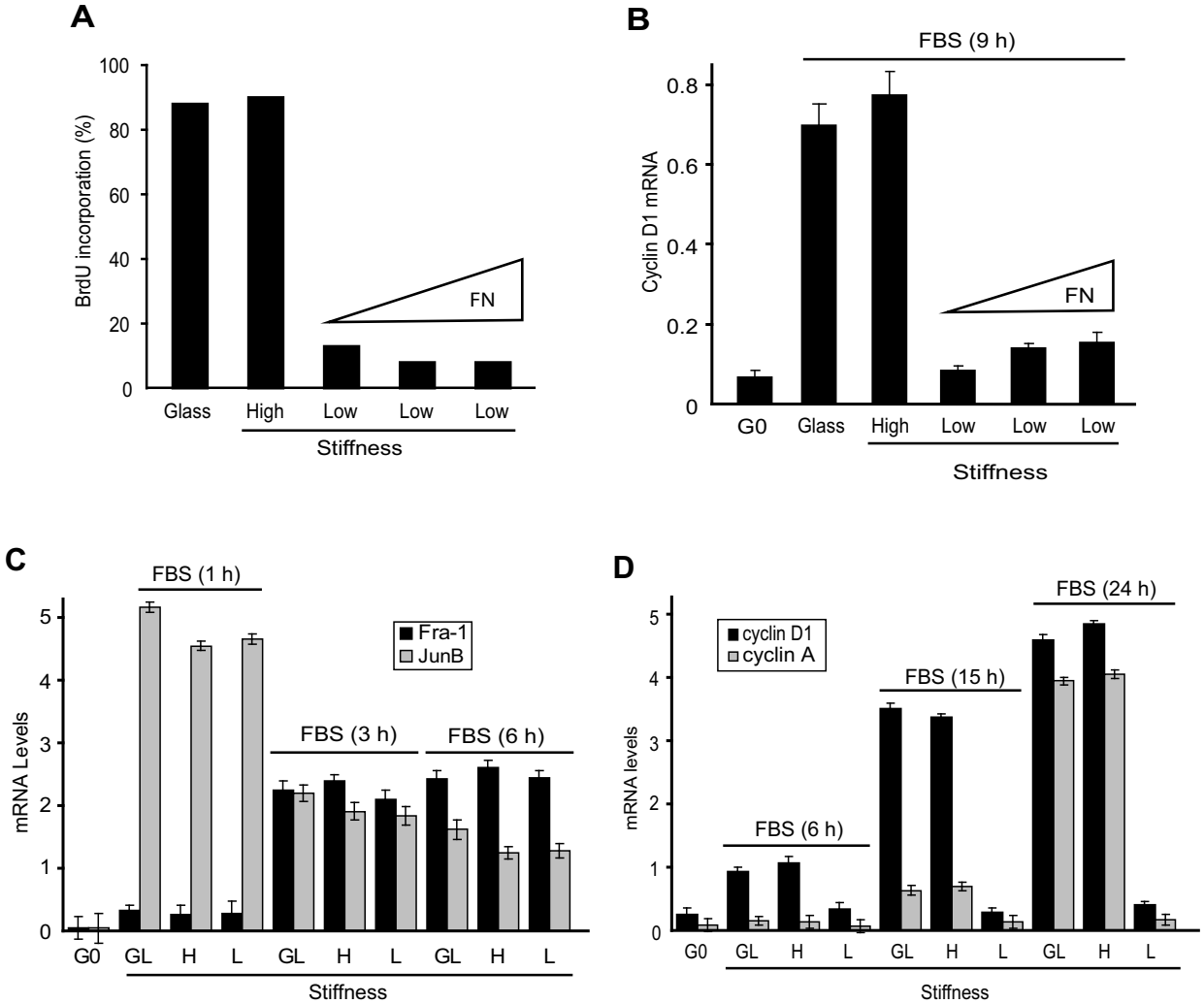
Supplemental Data

Suppl. Figure 3

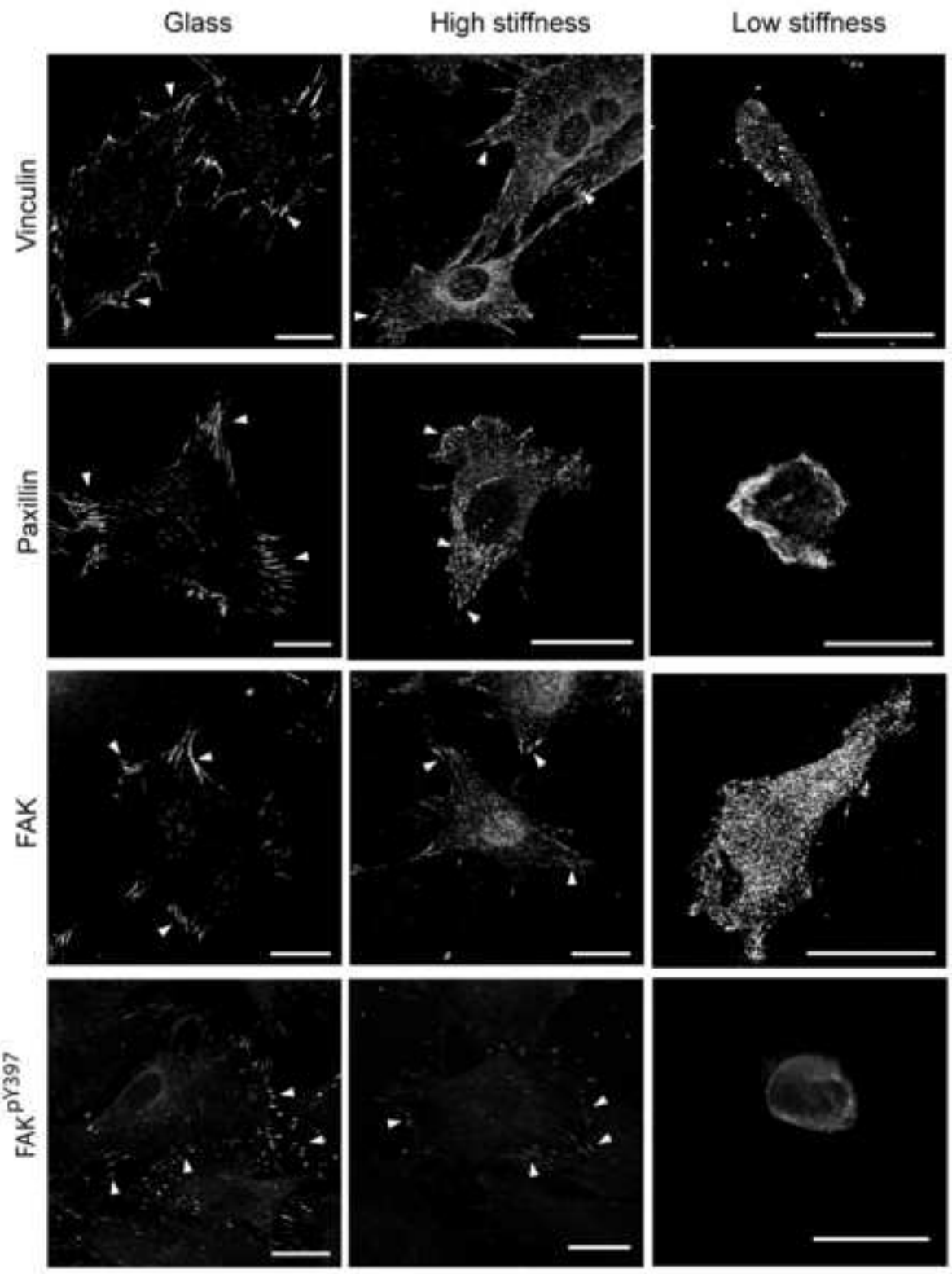


Supplemental Data

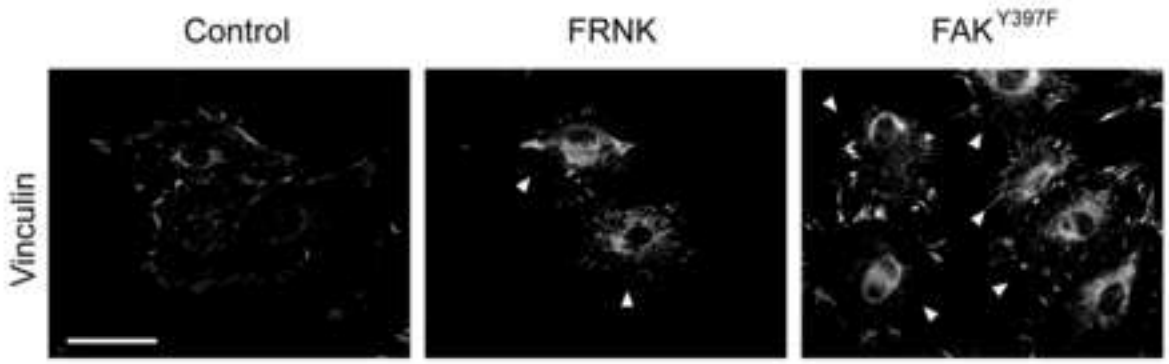
Suppl. Figure 4



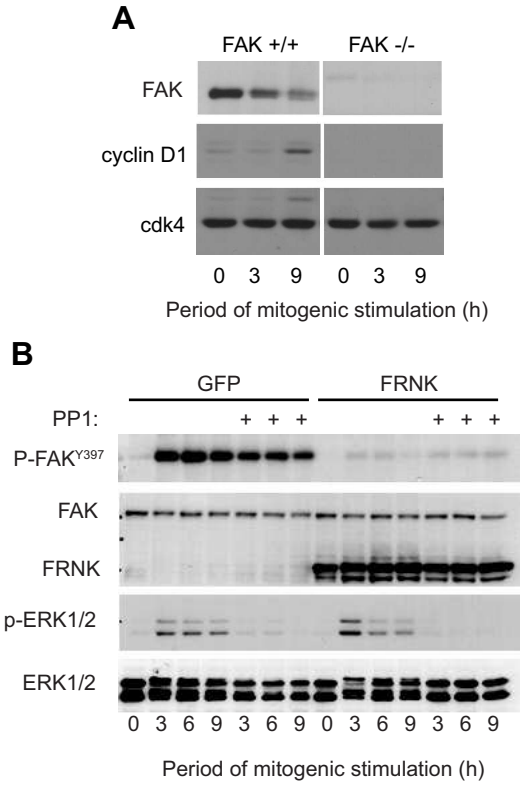
Suppl. Figure 5



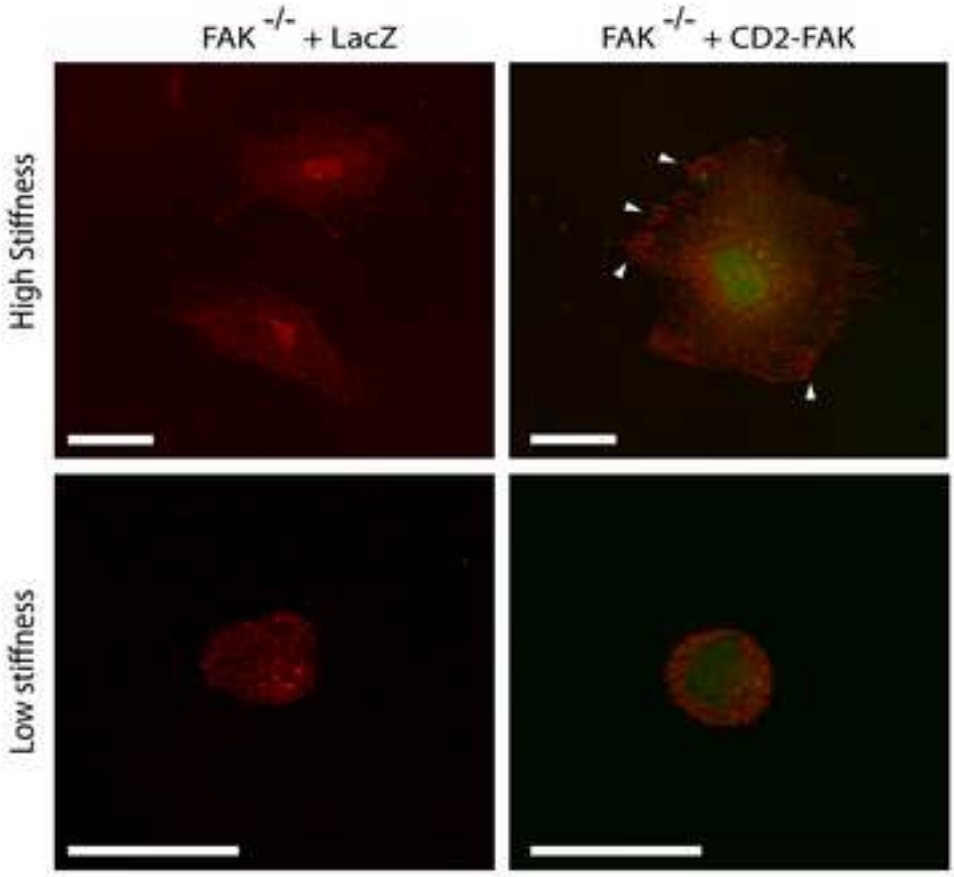
Suppl. Figure 6



Suppl. Figure 7

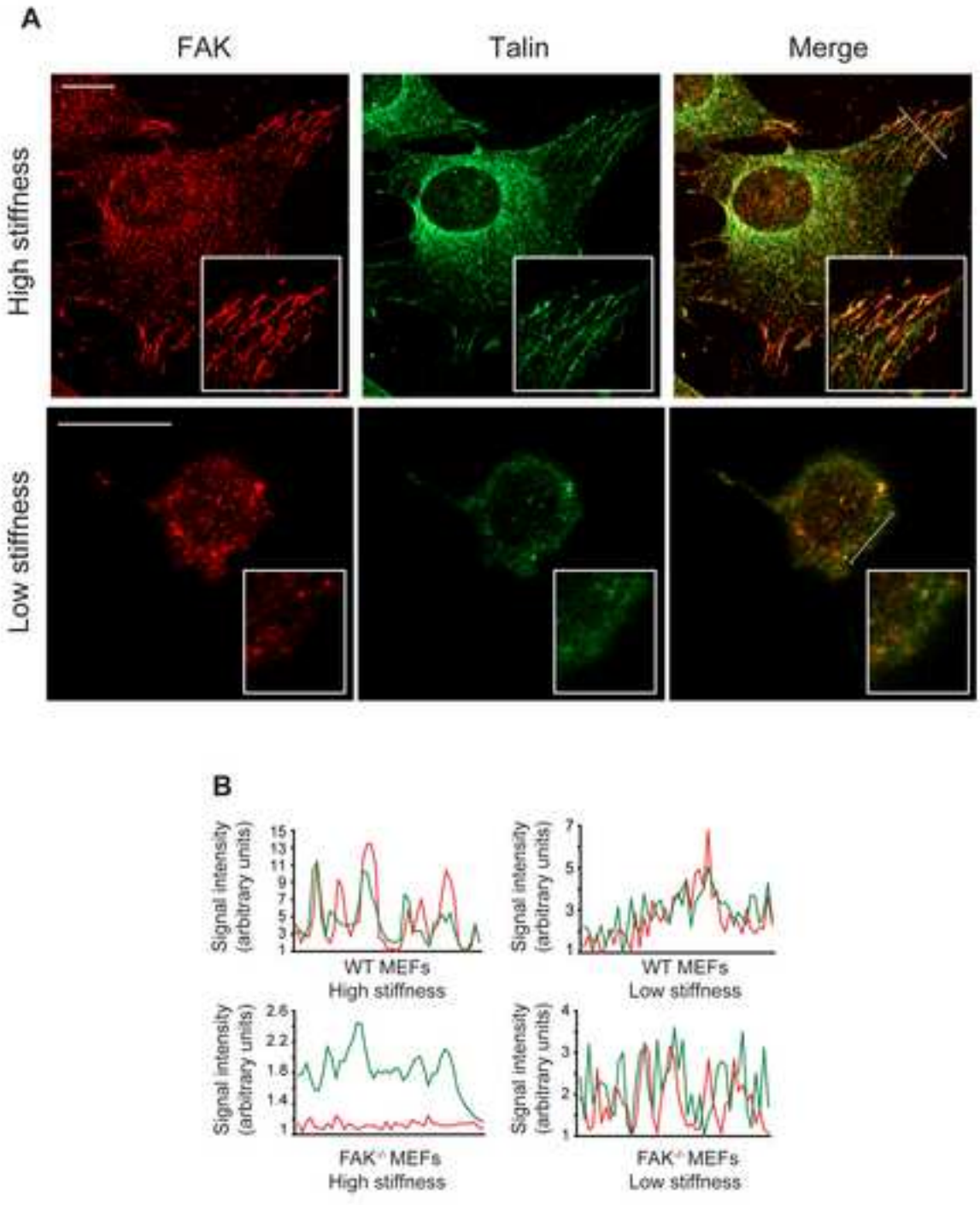


Suppl. Figure 8

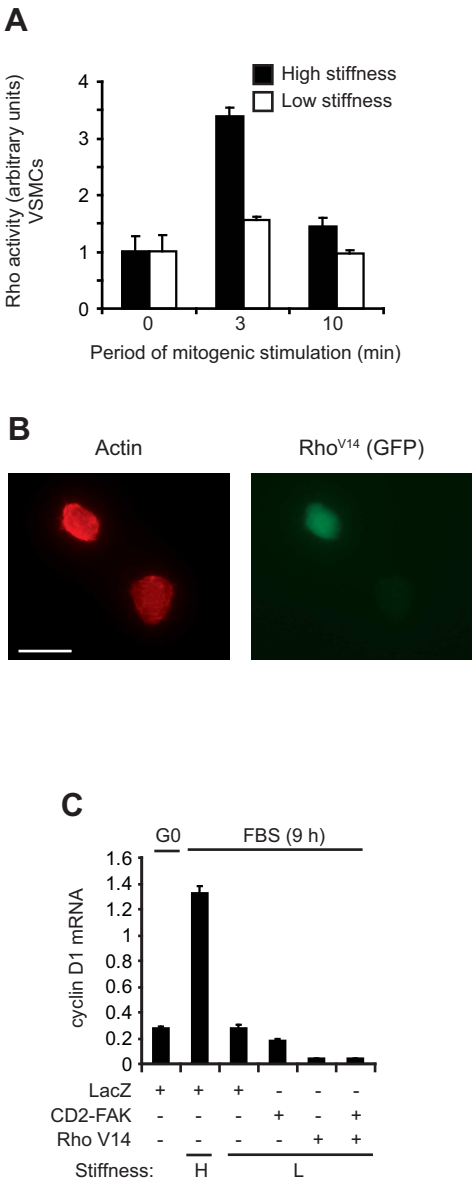


Supplemental Data

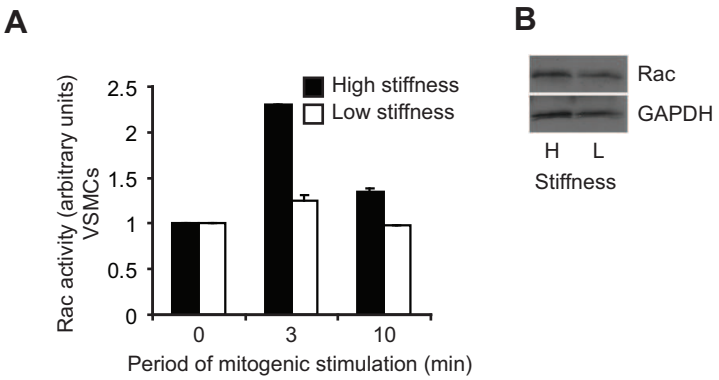
Suppl. Figure 9



Suppl. Figure 10



Suppl. Figure 11



Suppl. Figure 12