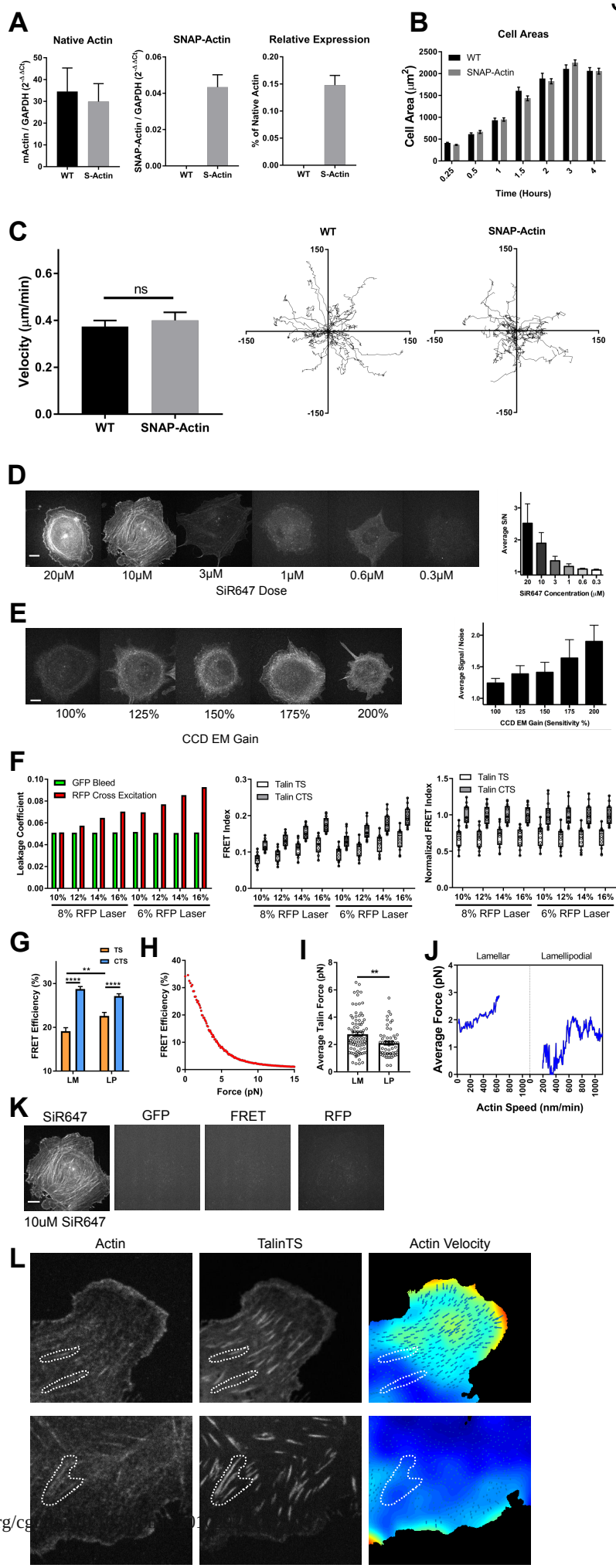
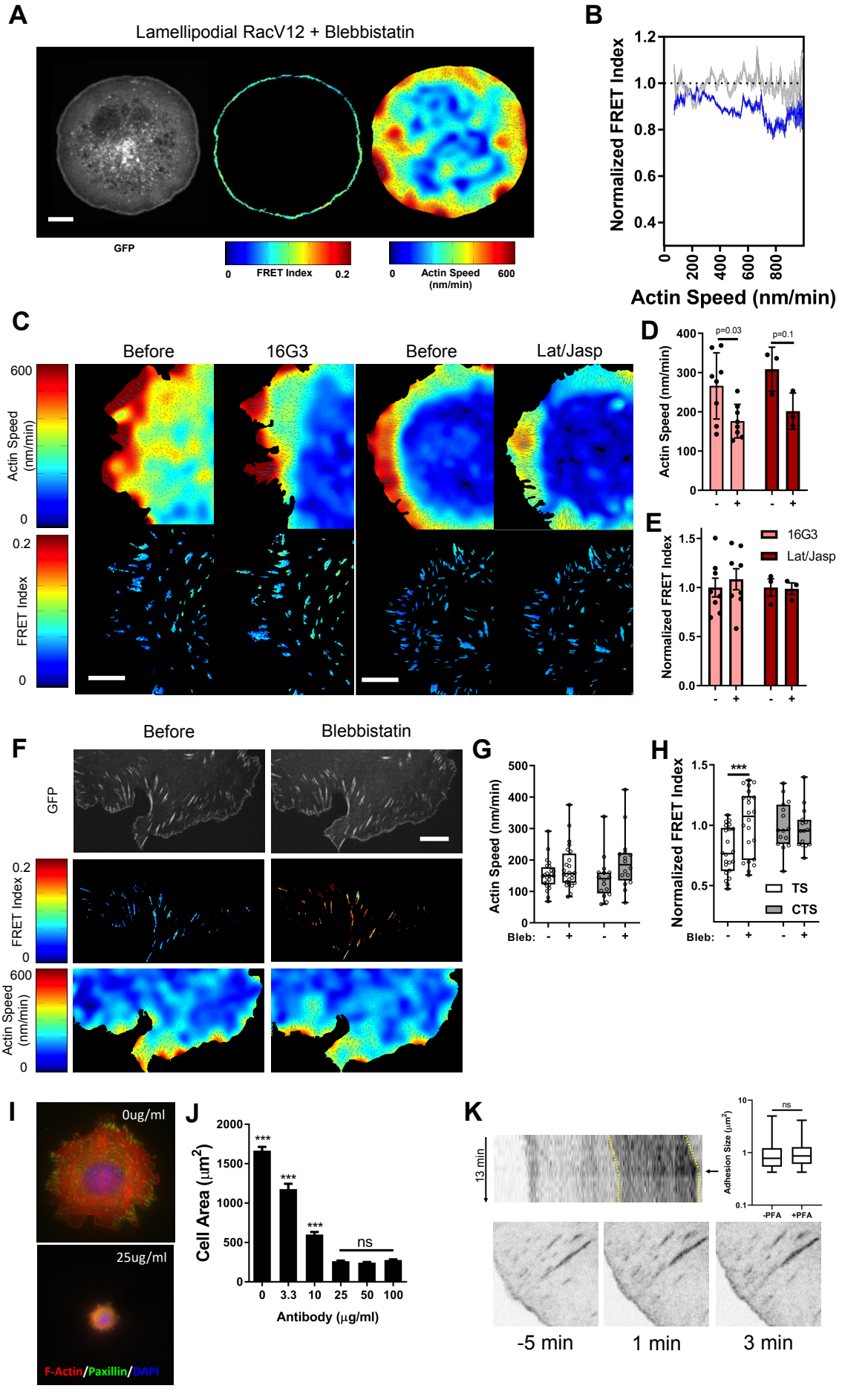
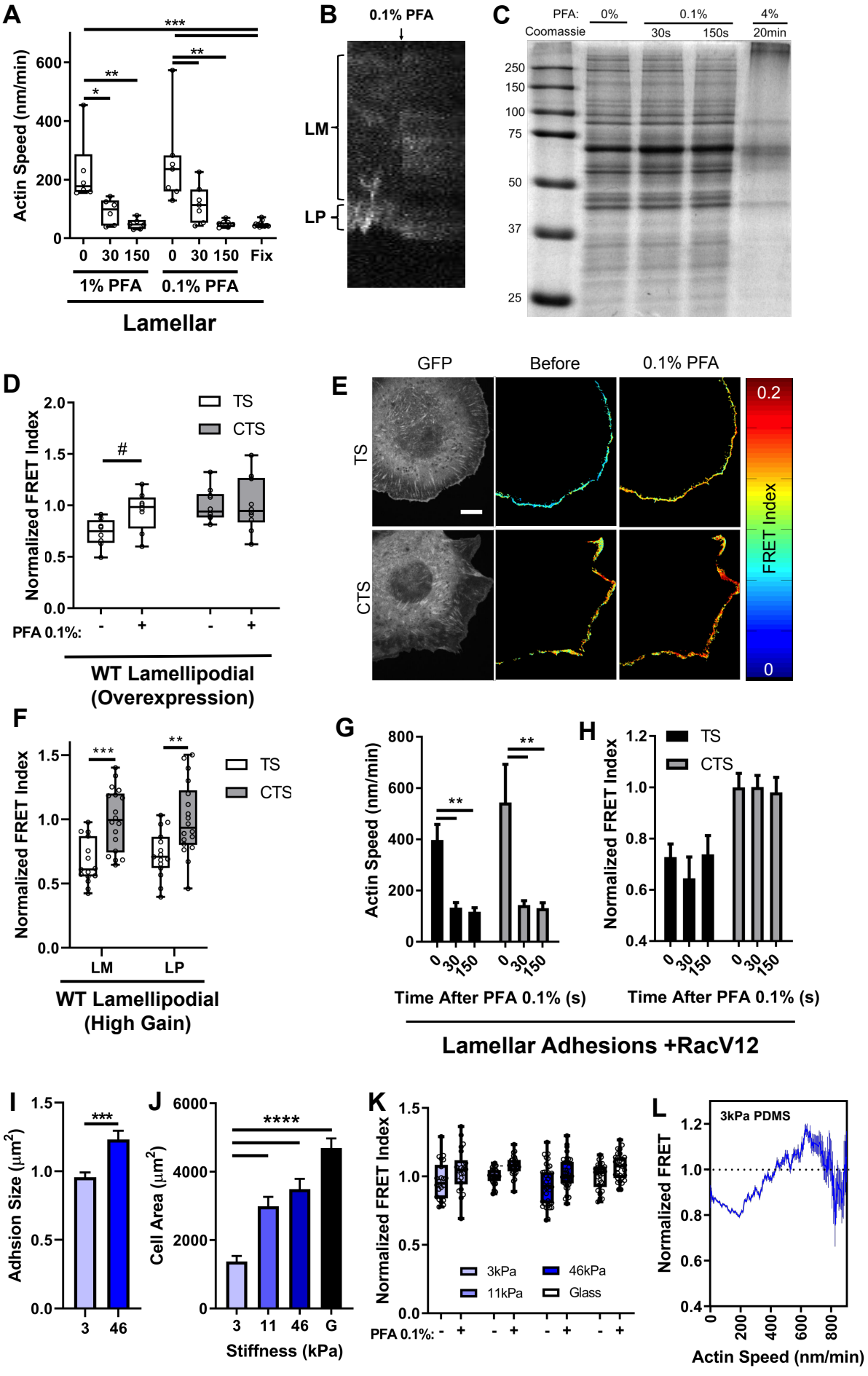

A
Native Actin
SNAP-Actin
Relative Expression
mActin / GAPDH ($2^{-\Delta\Delta Ct}$)
SNAP-Actin / GAPDH ($2^{-\Delta\Delta Ct}$)
% of Native Actin
WT
S-Actin
B
Cell Areas
WT
SNAP-Actin
Cell Area ($\mu m^2$)
Time (Hours)
C
Velocity (μm/min)
ns
WT
SNAP-Actin
D
20μM
10μM
3μM
1μM
0.6μM
0.3μM
SiR647 Dose
Average S/N
SiR647 Concentration (μM)
E
100%
125%
150%
175%
200%
CCD EM Gain
Average Signal / Noise
CCD EM Gain (Sensitivity %)
F
GFP Bleed
RFP Cross Excitation
Leakage Coefficient
Talin TS
Talin CTS
FRET Index
Normalized FRET Index
8% RFP Laser
6% RFP Laser
G
TS
CTS
FRET Efficiency (%)
LM
LP
H
FRET Efficiency (%)
Force (pN)
I
Average Talin Force (pN)
J
Lamellar
Lamellipodial
Average Force (pN)
Actin Speed (nm/min)
K
SiR647
GFP
FRET
RFP
10uM SiR647
L
Actin
TalinTS
Actin Velocity

**Supplemental Figure 1: (A)** rtPCR for native actin and SNAP-Actin in SNAP-actin expressing 3T3 cells. **(B)** Cell spreading on fibronectin-coated glass for WT and SNAP-actin expressing 3T3 cells (mean ± SEM, n=137-250 cells per group, two-way ANOVA with Sidak's multiple comparisons test indicated $p>0.25$ at all time-points). **(C)** Quantification of average migration velocity and traces of cell migration paths for WT and SNAP-actin expressing 3T3 cells for 24h on fibronectin coated glass (Mean ± SEM, n=30-32 cells per group tracked for 3 hours at 12 frames per hour). **(D)** Optimization of SNAP-Cell siR647 concentration. Cells in suspension were incubated with the indicated concentrations in phenol-free DMEM with 0.5% BSA, washed 3X and plated on fibronectin-coated glass for 45-90 min prior to live imaging. Signal-to-noise was quantified as average signal in the cell over background (scale bar = 10μm, mean ± SD, n=14 cells per group). **(E)** Optimizing CCD gain for low 640nm laser power on cells labeled with 3μM SNAP-Cell siR647 dye with quantification of signal to noise (scale bar = 10μm, mean ± SD, n=10 cells per group). **(F)** Bleed through and cross excitation coefficients for multiple laser powers calculated by fitting a line to pixel-wise data from n=12-15 cells per group. Raw FRET index for TS and CTS varies slightly depending on settings but the difference between TS and CTS is always ~40% so that normalized FRET Index is constant (box plots indicate median and quartiles, dots indicate single cell averages). **(G)** Calculation of average FRET efficiency per cell in lamellar (LM) and RacV12 lamellipodial (LP) adhesions for tension sensor (TS) and control sensor (CTS) (mean ± SEM, LM TS n=87, LM CTS n=52, LP TS n=57, LP CTS n=27, ** $p<0.01$, **** $p<0.0001$). **(H)** FRET efficiency vs. Force relationship based on a previously published worm-like chain model for tension sensor module force estimation (28). **(I)** Conversion of FRET efficiency to average estimated force on talin per cell. (mean ± SEM, dots indicate individual cells, LM n=87, LP n=57, ** $p<0.01$). **(J)** Correlation plots converted to force for tension sensor in Lamellar (n=49) and Lamellipodial (n=14) regions. **(K)** SiR647 labeled SNAP-Actin showed undetectable cross-excitation on FRET channels (GFP, FRET, RFP) that was at camera noise levels, allowing for simultaneous quantification of FRET with labeled actin imaging (scale bar = 10μm). **(L)** Some low flow regions showed stress fiber like structures (dotted outline), indicating that stress fibers may contribute to the slow moving actin population.


A
Lamellipodial RacV12 + Blebbistatin
GFP
0
FRET Index
0.2
0
Actin Speed (nm/min)
600
B
Normalized FRET Index
1.2
1.0
0.8
0.6
0.4
0
200
400
600
800
Actin Speed (nm/min)
C
Before
16G3
Before
Lat/Jasp
600
Actin Speed (nm/min)
0
0.2
FRET Index
0
D
p=0.03
p=0.1
Actin Speed (nm/min)
400
300
200
100
0
- + - +
E
16G3
Lat/Jasp
Normalized FRET Index
1.5
1.0
0.5
0.0
- + - +
F
Before
Blebbistatin
GFP
0.2
FRET Index
0
600
Actin Speed (nm/min)
0
G
Actin Speed (nm/min)
500
400
300
200
100
0
Bleb: - + - +
H
***
Normalized FRET Index
1.5
1.0
0.5
TS
CTS
Bleb: - + - +
I
0ug/ml
25ug/ml
F-Actin/Paxillin/DAPI
J
Cell Area (μm²)
2000
1500
1000
500
0
***
***
***
ns
0 3.3 10 25 50 100
Antibody (μg/ml)
K
13 min
Adhesion Size (μm²)
10
1
0.1
ns
-PFA +PFA
-5 min
1 min
3 min

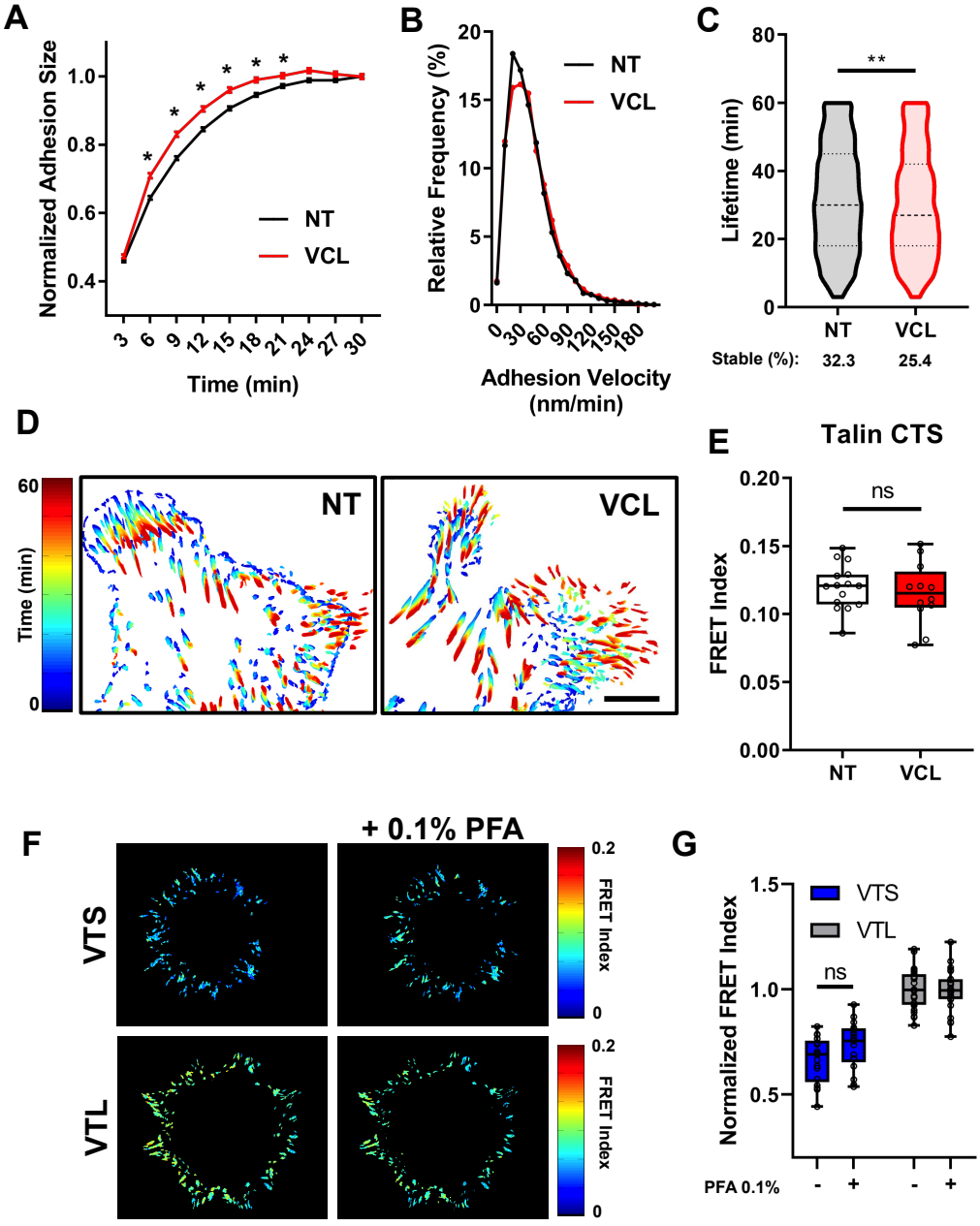
**Supplemental Figure 2:**

**(A)** Representative images for the talin tension sensor (GFP signal), Talin TS FRET index in lamellipodia, and a heat map of actin flow speed in cells expressing RacV12 and pre-treated with blebbistatin to inhibit myosin (10 μM, 1 hour). **(B)** Binned pixel-wise correlations of FRET vs. actin speed (mean ± SEM, TS n=26, CTS n=20). **(C)** Representative heat maps of actin flow speed and Talin TS FRET index for cells before and after treatment with fibronectin blocking antibody 16G3 to inhibit new adhesion formation, or latrunculin plus jasplikinolide (Lat/Jas) to stop polymerization but stabilize existing F-actin. **(D)** Average actin flow speed and **(E)** FRET index per cell. Values are means ± SEM, normalized to before addition, n=3-8 cells per group. Actin speed **(G)** and normalized FRET **(H)** before (-) and 10 minutes after (+) addition of 10μM Blebbistatin. **(F)** Representative images (box plots indicate median and quartiles, dots indicate single cell averages, n=15-23 cells per group from 2 independent experiments, *** $p<0.001$, two-way ANOVA with Tukey's post hoc). (All scale bars = 10μm). Fibronectin blocking with 16G3 at 25ug/ml is sufficient to completely prevent adhesion formation **(I)** and cell spreading **(J)** on fibronectin coated glass. (mean ± SEM, n=81-121 cells per group). **(K)** Kymograph of talin GFP before and after PFA treatment showing arrest of retrograde adhesion movement with quantification of adhesion size before and after treatment (bars indicate min, max and quartiles, n=844-921 adhesions).


A
Actin Speed (nm/min)
0 30 150 0 30 150 Fix
1% PFA
0.1% PFA
Lamellar
B
0.1% PFA
LM
LP
C
PFA: 0% 0.1% 4%
Coomassie
30s 150s 20min
250
150
100
75
50
37
25
D
Normalized FRET Index
TS
CTS
PFA 0.1%:
WT Lamellipodial
(Overexpression)
E
GFP
Before
0.1% PFA
TS
CTS
0.2
FRET Index
0
F
Normalized FRET Index
TS
CTS
LM
LP
WT Lamellipodial
(High Gain)
G
Actin Speed (nm/min)
Time After PFA 0.1% (s)
H
Normalized FRET Index
TS
CTS
Time After PFA 0.1% (s)
Lamellar Adhesions +RacV12
I
Adhsion Size (μm²)
3 46
J
Cell Area (μm²)
3 11 46 G
Stiffness (kPa)
K
Normalized FRET Index
3kPa
11kPa
46kPa
Glass
PFA 0.1%:
L
3kPa PDMS
Normalized FRET
Actin Speed (nm/min)

**Supplemental Figure 3:**

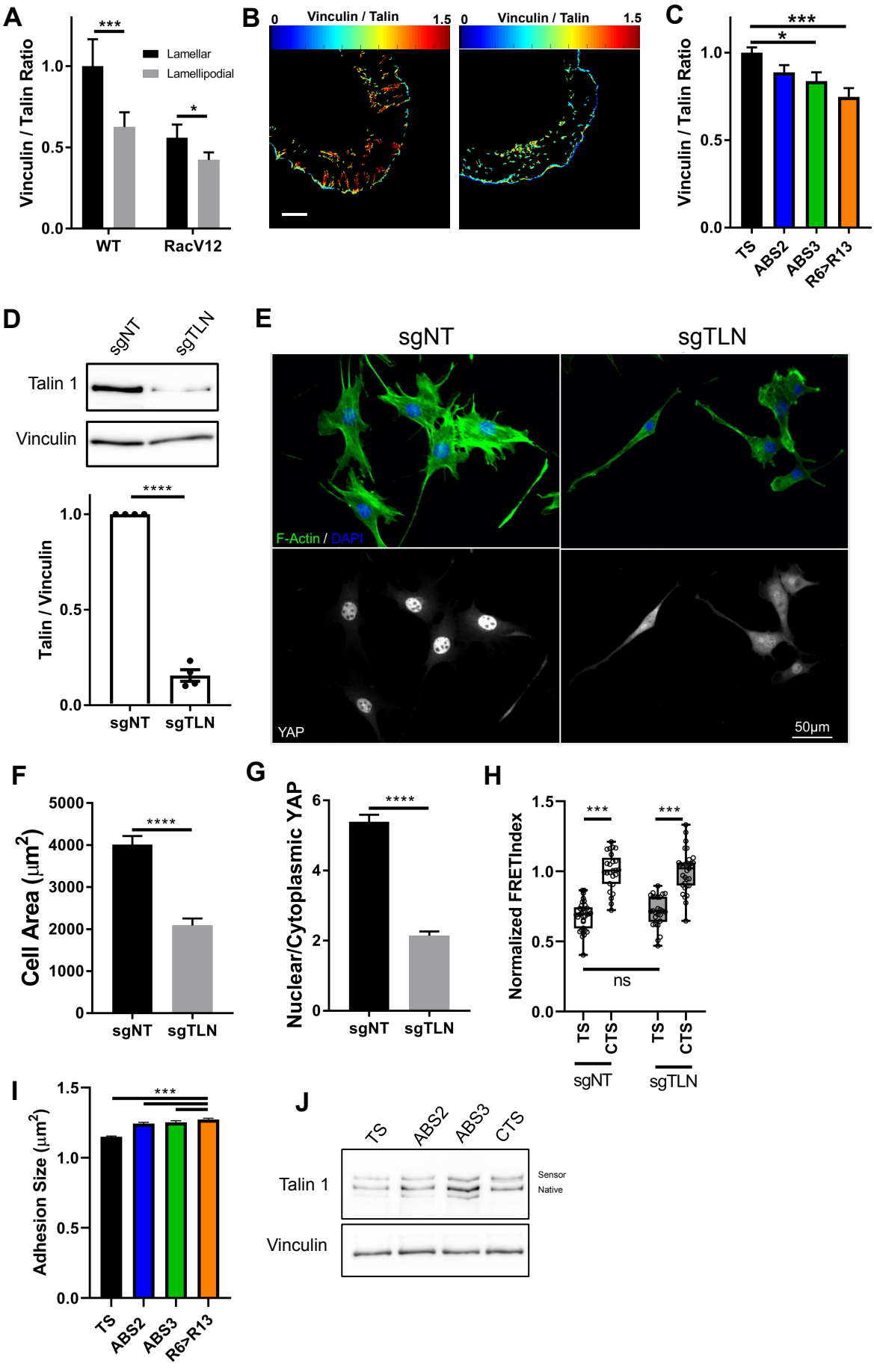
**(A)** Quantification of actin speed in cells before and after addition of 1% or 0.1% PFA. Cells fixed with 4% PFA for 15 minutes are included to assess analysis noise (box plots indicate median and quartiles, dots indicate single cell averages, n=6-15 cells per group, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). **(B)** Kymograph of actin flow before and after addition of 0.1% PFA with lamellipodial (LP) and lamellar (LM) regions indicated. **(C)** Imperial-stained SDS-PAGE for cell lysates. **(D)** FRET index (normalized to the control sensor) in lamellipodial adhesions in WT cells (high TS or CTS expression allowed analysis without RacV12) with representative images in **(E)**. (box plots indicate median and quartiles, dots indicate single cell averages n=8-10 cells per group before and after treatment with 0.1% PFA, # $p<0.05$, two-way ANOVA with Sidak's multiple comparisons test.) **(F)** Quantification of normalized FRET index for talin sensor (TS) and control sensor (CTS) wild type lamellipodial (LP) and lamellar (LM) adhesions was possible with high electron multiplying gain and higher (double) laser power (box plots indicate median and quartiles, n=15-18 cells, ** $p<0.01$, *** $p<0.0001$, two-way ANOVA with Sidak's multiple comparison). Quantification of actin speed **(G)** and normalized talin FRET index **(H)** after stoppage of actin flow with 0.1% PFA for lamellar adhesions in RacV12 expressing cells (mean ± SEM, n>9 cells per group, two-way ANOVA with Sidak's post hoc). **(I)** Quantification of focal adhesion size on soft (3kPa) and stiff (46kPa) gels (n=517-802 adhesions from 9-12 cells, *** $p<0.001$, two-sided t-test with non-parametric Mann Whitney test). **(J)** Quantification of cell spread area on 3, 11, 46 kPa fibronectin coated PA gels compared to fibronectin coated glass = G (mean ± SEM n=37-62 cells per group, **** $p<0.0001$ One-way ANOVA with Tukey's post hoc). **(K)** Control sensor data for 0.1% PFA stoppage experiments on surfaces of different stiffness (3kPa n=22, 11kPa n=28, 46kPa n=36, Glass n=30, matched cells per group from 5 independent experiments). **(L)** FRET versus flow correlation on 3kPa fibronectin coated PDMS gels binned pixel-wise correlations of FRET vs. actin speed (mean ± 95% confidence interval, TS n=19, cells from 2 independent experiments).


A
Normalized Adhesion Size
1.0
0.8
0.6
0.4
3 6 9 12 15 18 21 24 27 30
Time (min)
NT
VCL
B
Relative Frequency (%)
20
15
10
5
0
0 30 60 90 120 150 180
Adhesion Velocity (nm/min)
NT
VCL
C
Lifetime (min)
80
60
40
20
0
**
NT
VCL
Stable (%):
32.3
25.4
D
60
Time (min)
0
NT
VCL
E
Talin CTS
FRET Index
0.20
0.15
0.10
0.05
0.00
ns
NT
VCL
F
+ 0.1% PFA
VTS
VTL
0.2
FRET Index
0
0.2
FRET Index
0
G
Normalized FRET Index
1.5
1.0
0.5
VTS
VTL
ns
PFA 0.1%
-
+
-
+

**Supplemental Figure 4:**

**(A)** Adhesion growth in vinculin-depleted (VCL) and control (NT) cells. Adhesions that formed during the time course were tracked for 30 min (means ± SEM, normalized to their maximal size. NT n=3794 adhesions from 38 cells, VCL n=2034 adhesions from 33 cells, * $p<0.01$ vs. NT). **(B)** Adhesion velocity and **(C)** adhesion lifetime displayed as histograms for 1h time-course imaged at one frame per 3 minutes (violin plot with median and quartiles, NT n=38 cells, VCL n=33 cells). **(D)** Representative heat maps of adhesion dynamics over 1h (scale bar = 10µm). **(E)** Quantification of Talin TS FRET index for control sensor in non targeting (NT) and vinculin targeting (VCL) guide RNAs (box plots indicate median and quartiles, dots indicate individual cells, n=12 NT, n=15 VCL). **(F)** Representative heatmaps of vinculin tension sensor (VTS) and tail-less control sensor (VTL) before and after stoppage of actin flow with 0.1% PFA with quantification **(G)** of normalized FRET index (box plots indicate median and quartiles, dots indicate individual cells, n=17 VTS, n=24 VTL).

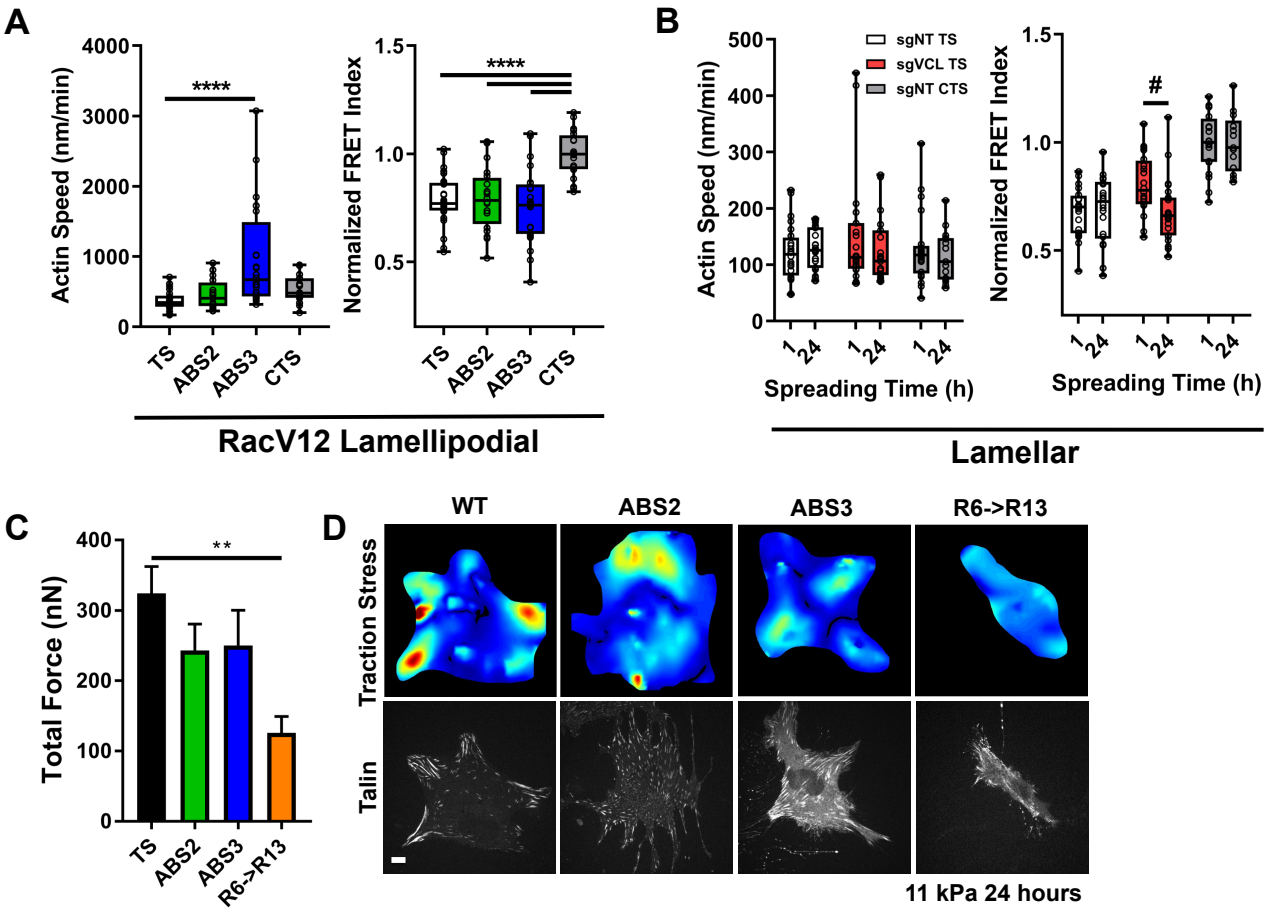
Supplemental Figure 5



**Supplemental Figure 5:**

**(A)** Vinculin-to-talin ratio in lamellar and lamellipodial adhesions of WT and RacV12 expressing cells (mean ± SEM, n=8-9 cells per group, * $p<0.05$, *** $p<0.001$) with **(B)** representative heatmaps. **(C)** Vinculin-to-talin ratio in lamellar adhesions of talin-depleted cells reconstituted with WT or mutated talin TS (mean ± SEM, n=46-49 cells per group, # $p<0.05$, **** $p<0.001$). **(D)** Western blot for talin1 after infection with Cas9 and guide RNAs directed at talin1; talin quantification normalized to vinculin (mean ± SEM, n=4 experiments, **** $p<0.0001$ two-sided t-test). **(E)** representative images of F-Actin (green), DAPI (Blue) and YAP/TAZ (white) for 3T3 cells treated with non-targeting (sgNT) or talin 1 targeting (sgTalin1A) small guide RNAs with quantification of cell area **(F)** and YAP nuclear/cytoplasmic ratio **(G)**. (mean ± SEM, n=54-58 cells per group, **** $p<0.0001$, two-sided t-test). **(H)** Quantification of normalized FRET index for talin TS and control cells treated with non-targeting (sgNT) or talin targeting (sgTLN) guide RNA (box plot indicates median and quartiles, n=25-37 cells per group). **(I)** Quantification of talin focal adhesion size for actin binding mutants (n>2504 adhesions per group, *** $p<0.001$, one-way ANOVA with Dunnett's multiple comparison). **(J)** Relative expression of tension sensor constructs compared to endogenous expression measured by western blot.

Supplemental Figure 6



**Supplemental Figure 6:**

**(A)** Quantification of actin speed and talin FRET index for lamellipodial adhesions in RacV12 cells depleted for talin and re-expressing WT (TS and CTS) or talin TS with actin binding site mutations (box plots indicate median and quartiles, dots indicate single cell averages, TS: n=26, ABS2: n=20, ABS3: n=24, CTS: n=20, cells/group from 3 independent experiments, **** $p<0.0001$, one-way ANOVA with Tukey's post hoc). **(B)** Actin speed and talin FRET for lamellar adhesions for cells 1h or 24h after plating. Cells were transfected with sgNT or sgVCL as indicated to deplete vinculin (mean ± SEM, n=13-24 cells per group, # $p<0.05$, two-way ANOVA with Sidak's post hoc). Quantification of total force per cell **(C)** and representative traction stress maps **(D)** for traction force microscopy performed on sgTLN cells expressing WT or mutant talin on 11kPa gels for 24 hours (mean ± SEM, n=13-23 cells per group, one-way ANOVA with Tukey's post hoc, scale bar = 10μm). **(E)** Focal adhesion images in talin mutant cells on fibronectin coated glass after 24 hours (scale bar = 10μm).

**Supplemental Videos**

**Supplemental Video 1:**

Video of 3T3 cells spreading on fibronectin-coated glass with SNAP-labeling of Actin with SiR-647 (green) and TalinTS (magenta). 20 min time-lapse video (played at 300x speed) with speckle images acquired at 6 frames per minute and talin images acquired at 1 frame per minute.

**Supplemental Video 2:**

Video of SNAP-labeled actin in a talin depleted 3T3 cell re-expressing WT talin. 2 min time-lapse video (played at 90x speed) with speckle images acquired at 10 frames per minute and talin images acquired at 1 frame per minute.

**Supplemental Video 3:**

Video of SNAP-labeled actin in a talin depleted 3T3 cell re-expressing the ABS3 talin. 2 min time-lapse video (played at 90x speed) with speckle images acquired at 10 frames per minute and talin images acquired at 1 frame per minute.

**Supplemental Video 4:**

Video of SNAP-labeled actin in a talin-depleted 3T3 cell re-expressing the R6-R13 swap talin. 2 min time-lapse video (played at 90x speed) with speckle images acquired at 10 frames per minute and talin images acquired at 1 frame per minute.

| PA Gels | | | | | | |
|---|---|---|---|---|---|---|
| Stiffness (kPa) | 40% aa (µl) | 2% bis-aa (µl) | Water (µl) | Beads (µl) | APS (µl) | TEMED (µl) |
| 3 | 125 | 50 | 803.5 | 10 | 10 | 1.5 |
| 11 | 187.5 | 50 | 741 | 10 | 10 | 1.5 |
| 46 | 300 | 75 | 603.5 | 10 | 10 | 1.5 |

**Supplemental Table 1:** Composition of polyacrylamide gels used for each stiffness.