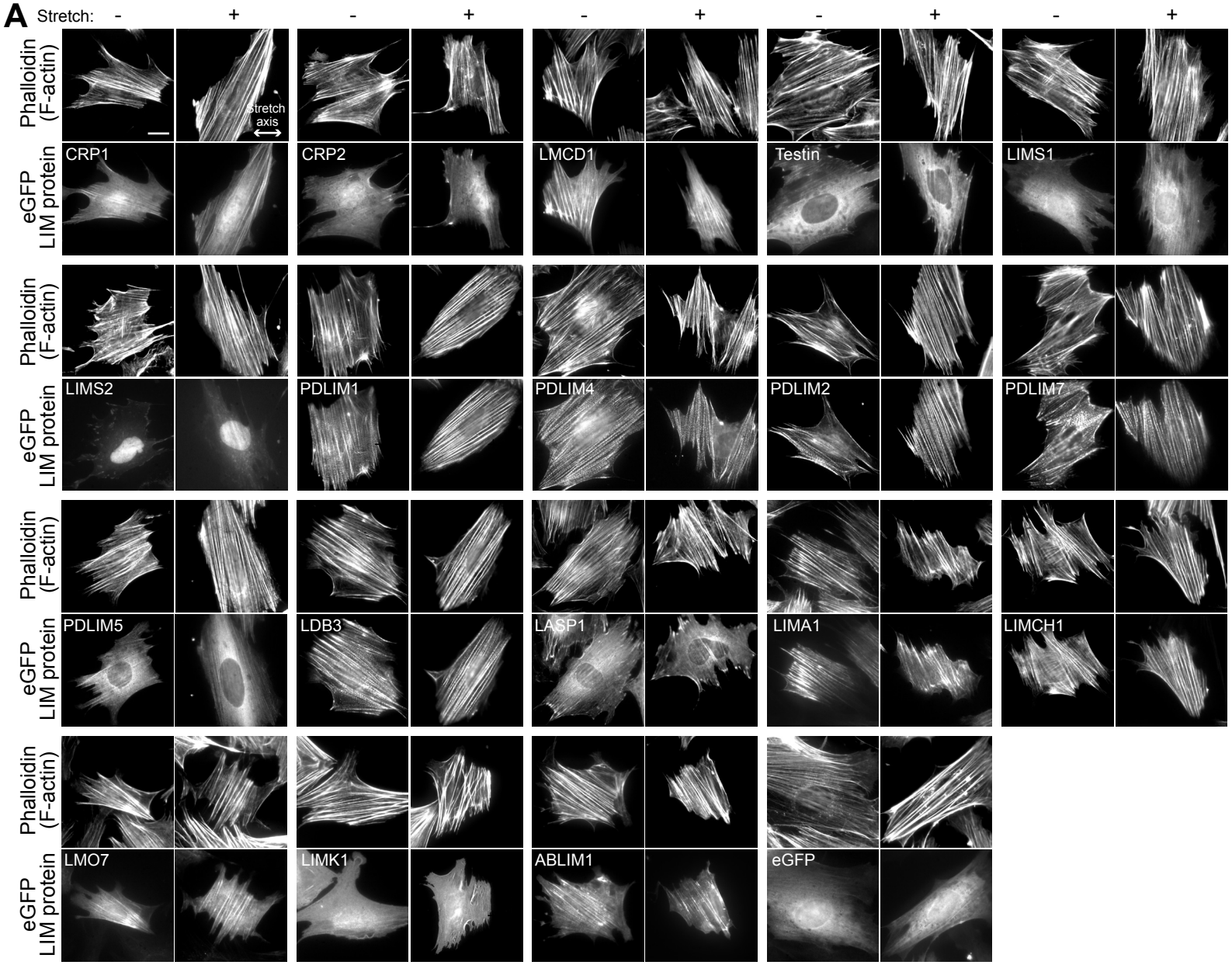
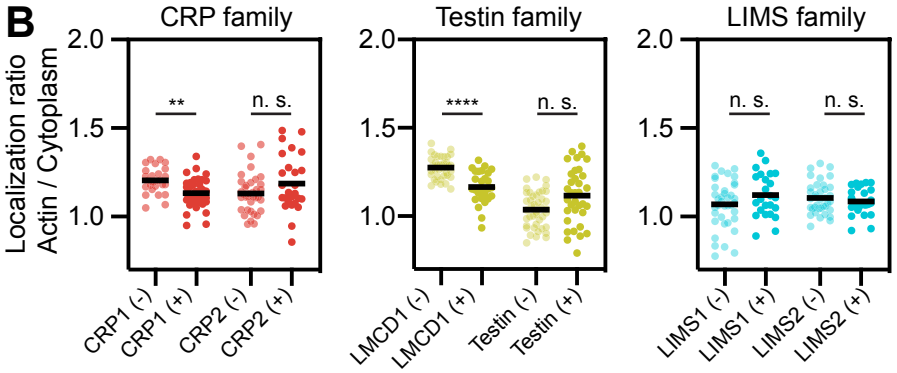
	Number of data points	Number of outliers
FHL1 (-)	28	0
FHL1 (+)	80	1
FHL2 (-)	69	5
FHL2 (+)	142	4
FHL3 (-)	166	9
FHL3 (+)	226	2
Paxillin (-)	72	2
Paxillin (+)	134	2
HIC5 (-)	102	1
HIC5 (+)	161	1
Zyxin (-)	74	5
Zyxin (+)	140	3
FBLIM1 (-)	91	2
FBLIM1 (+)	132	1
TRIP6 (-)	50	2
TRIP6 (+)	89	4
LPP (-)	39	2
LPP (+)	101	3
LIMD1 (-)	28	1
LIMD1 (+)	37	2
Paxillin LDO (-)	198	14
Paxillin LDO (+)	244	1
HIC5 LDO (-)	111	2
HIC5 LDO (+)	170	3
Zyxin LDO (-)	71	3
Zyxin LDO (+)	115	2
FBLIM1 LDO (-)	100	2
FBLIM1 LDO (+)	146	0
TRIP6 LDO (-)	45	1
TRIP6 LDO (+)	69	1
LPP LDO (-)	129	3
LPP LDO (+)	222	2
LIMD1 LDO (-)	35	2
LIMD1 LDO (+)	93	2

Table S1. Number of data points and outliers identified by ROUT method in GraphPad Prism for SF enrichment quantification in unstretched (-) and stretched (+) MEFs. Related to Figure 2.

Table S2. Additional oligonucleotides and recombinant DNAs used in this paper. Related to the Key Resources Table.


A
Stretch: - + - + - + - + - +
Phalloidin (F-actin)
eGFP LIM protein
stretch axis
CRP1
CRP2
LMCD1
Testin
LIMS1
LIMS2
PDLIM1
PDLIM4
PDLIM2
PDLIM7
PDLIM5
LDB3
LASP1
LIMA1
LIMCH1
LMO7
LIMK1
ABLIM1
eGFP


B
CRP family
Testin family
LIMS family
Localization ratio Actin / Cytoplasm
2.0
1.5
1.0
**
n. s.
****
CRP1 (-)
CRP1 (+)
CRP2 (-)
CRP2 (+)
LMCD1 (-)
LMCD1 (+)
Testin (-)
Testin (+)
LIMS1 (-)
LIMS1 (+)
LIMS2 (-)
LIMS2 (+)

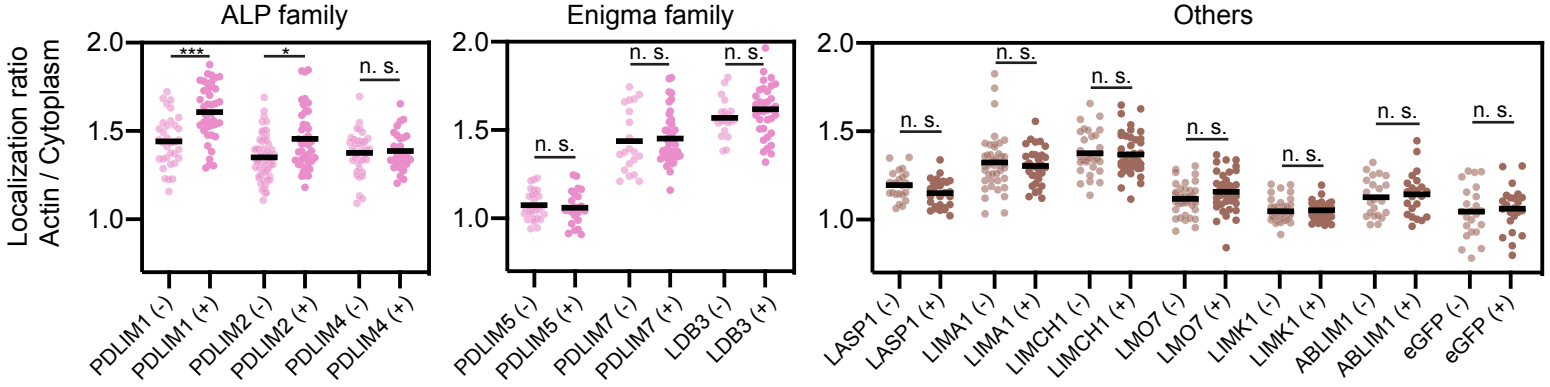

ALP family
Enigma family
Others
Localization ratio Actin / Cytoplasm
2.0
1.5
1.0
***
*
n. s.
PDLIM1 (-)
PDLIM1 (+)
PDLIM2 (-)
PDLIM2 (+)
PDLIM4 (-)
PDLIM4 (+)
PDLIM5 (-)
PDLIM5 (+)
PDLIM7 (-)
PDLIM7 (+)
LDB3 (-)
LDB3 (+)
LASP1 (-)
LASP1 (+)
LIMA1 (-)
LIMA1 (+)
LIMCH1 (-)
LIMCH1 (+)
LMO7 (-)
LMO7 (+)
LIMK1 (-)
LIMK1 (+)
ABLIM1 (-)
ABLIM1 (+)
eGFP (-)
eGFP (+)

Figure S1. Non-mechanoaccumulating LIM proteins identified in the cell-stretching screen. Related to Figure 1. (A) Epifluorescence micrographs of eGFP-labeled LIM proteins in unstretched (-) and stretched (+) MEFs stained with phalloidin to label F-actin. Double-headed arrow indicates the uniaxial stretch direction. Scale bar, 20 μm. **(B)** Whole-cell actin enrichment of LIM proteins in unstretched (-) and stretched (+) MEFs ($20 \leq n \leq 60$). Bars represent means. Dunnett's T3 multiple comparisons test after Welch's ANOVA is performed by comparing the actin enrichments of each LIM protein in unstretched cells with those in stretched ones simultaneously for all the LIM proteins included in the screen. NS, $p > 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

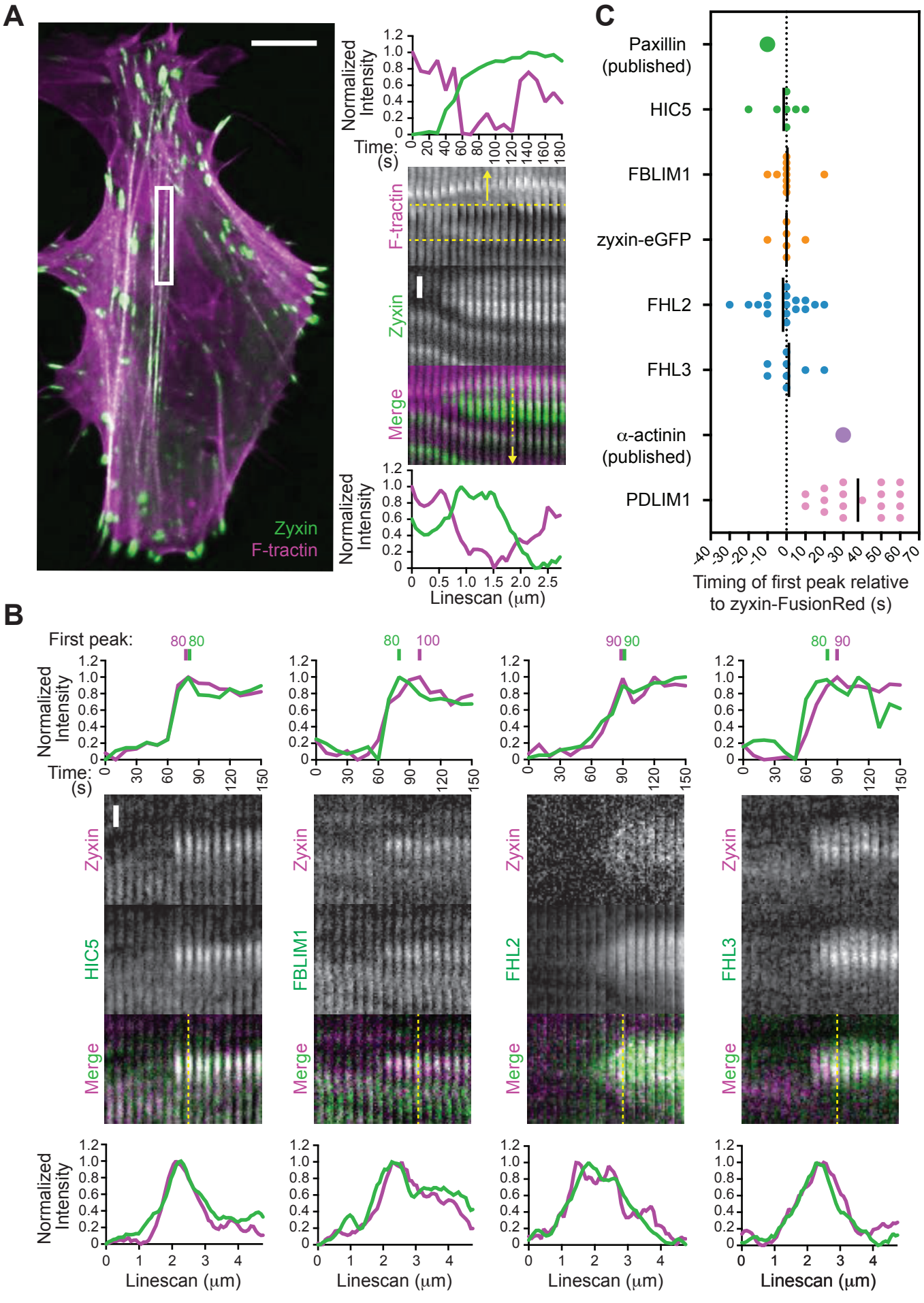

A
Zyxin
F-tractin
Normalized Intensity
Time: (s)
F-tractin
Zyxin
Merge
Linescan (μm)
C
Paxillin (published)
HIC5
FBLIM1
zyxin-eGFP
FHL2
FHL3
α-actinin (published)
PDLIM1
Timing of first peak relative to zyxin-FusionRed (s)
B
First peak:
80 80
80 100
90 90
80 90
Zyxin
HIC5
FBLIM1
FHL2
FHL3
Merge

Figure S2. FHL-, paxillin-, and zyxin-family proteins localize to SF strain sites with similar kinetics, supporting recognition of a common molecular mark. Related to Figure 1. (A) Left, spinning disk confocal snapshot of a U2OS cell co-expressing F-tractin-mApple (magenta) and zyxin-eGFP (green). Scale bar, 5 μm. Box highlights an SF strain site. Right, intensity versus time plot (top), time-lapse montages (middle), and intensity line scan (bottom) of F-tractin and zyxin at an SF strain site. Scale bar, 1 μm. **(B)** Intensity vs. time plots (top), time-lapse montages (middle), and intensity line scans (bottom) of representative SF strain sites marked by zyxin-FusionRed and the indicated eGFP-labeled LIM protein. Magenta, zyxin-FusionRed. Green, eGFP-labeled LIM protein. Scale bar, 1 μm. The time of first intensity peak is indicated for each protein. **(C)** Timing of first intensity peak of indicated LIM proteins at $6 \leq n \leq 17$ SF strain sites relative to zyxin-FusionRed. Bars represent means. Dashed line indicates simultaneous recruitment of zyxin-FusionRed and the eGFP-labeled LIM protein.

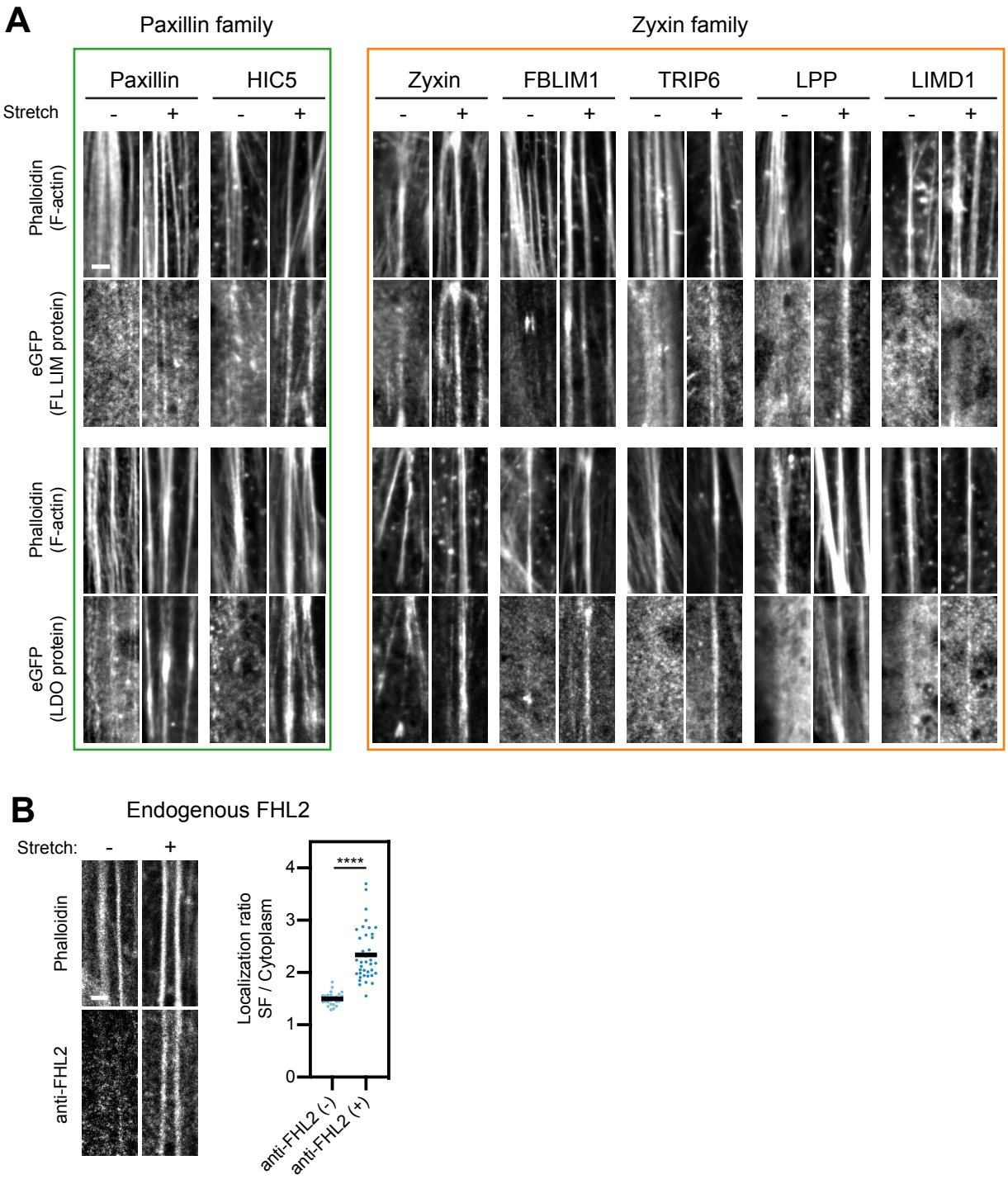

A
Paxillin family
Zyxin family
Paxillin
HIC5
Zyxin
FBLIM1
TRIP6
LPP
LIMD1
Stretch
-
+
Phalloidin (F-actin)
eGFP (FL LIM protein)
Phalloidin (F-actin)
eGFP (LDO protein)
B
Endogenous FHL2
Stretch: - +
Phalloidin
anti-FHL2
Localization ratio SF / Cytoplasm
****
0
1
2
3
4
anti-FHL2 (-)
anti-FHL2 (+)

Figure S3. Mechanoresponsive LIM proteins accumulate on SFs in stretched MEFs. Related to Figure 2. (A) Confocal slices of phalloidin-stained SFs and eGFP-labeled paxillin- and zyxin-family proteins in unstretched (-) and stretched (+) MEFs. Top panel, full-length (FL). Bottom panel, LIM domain only (LDO). Scale bar, 2 μm. **(B)** Left panel, confocal slices of SFs stained by phalloidin (top) and endogenous FHL2 (bottom) visualized by immunofluorescence. Scale bar, 2 μm. Right panel, SF enrichment of endogenous FHL2 in unstretched (-) and stretched (+) MEFs. Each data point is obtained from a single SF ($25 \leq n \leq 38$, $N = 2$ biological replicates). Bars represent means. Welch's unpaired t-test: **** $p < 0.0001$.

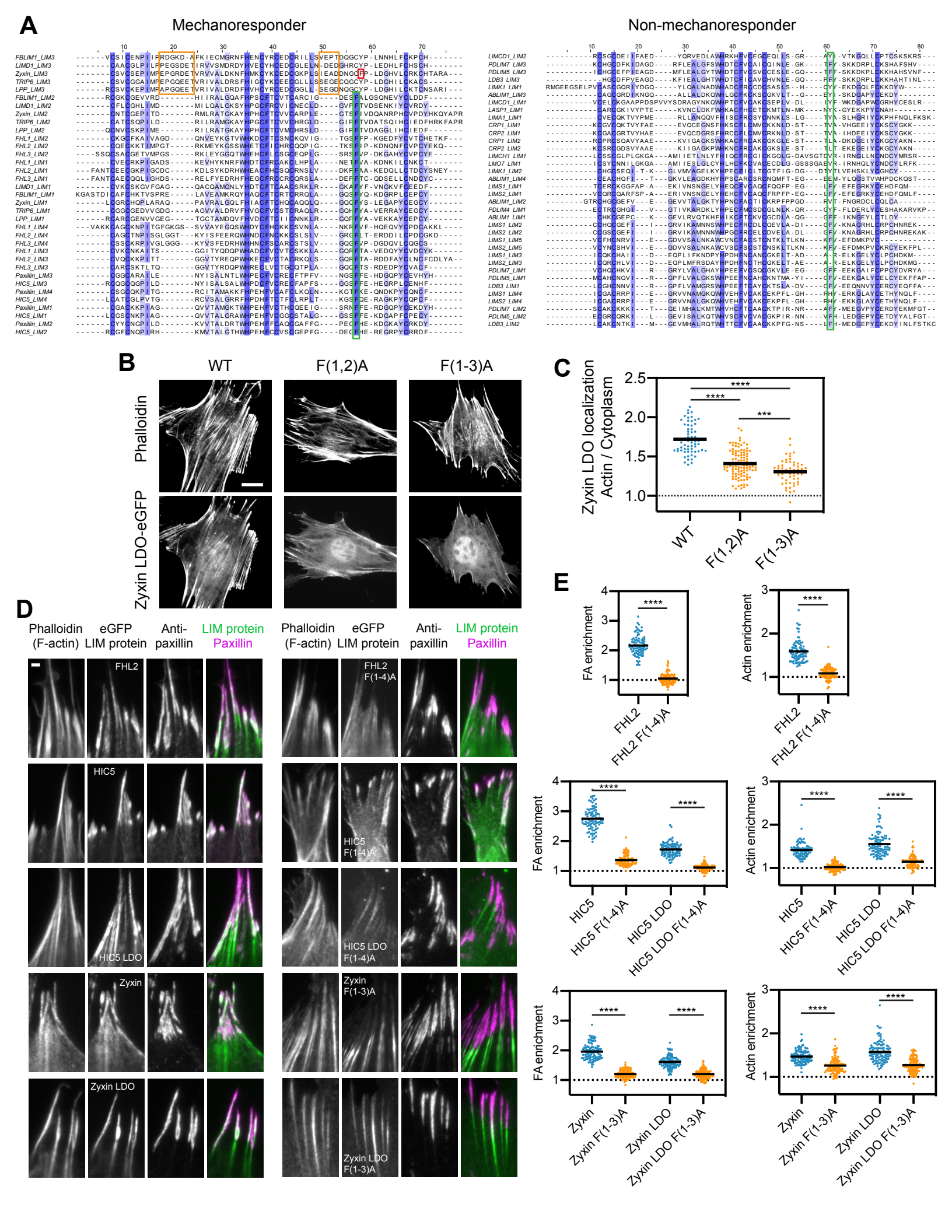
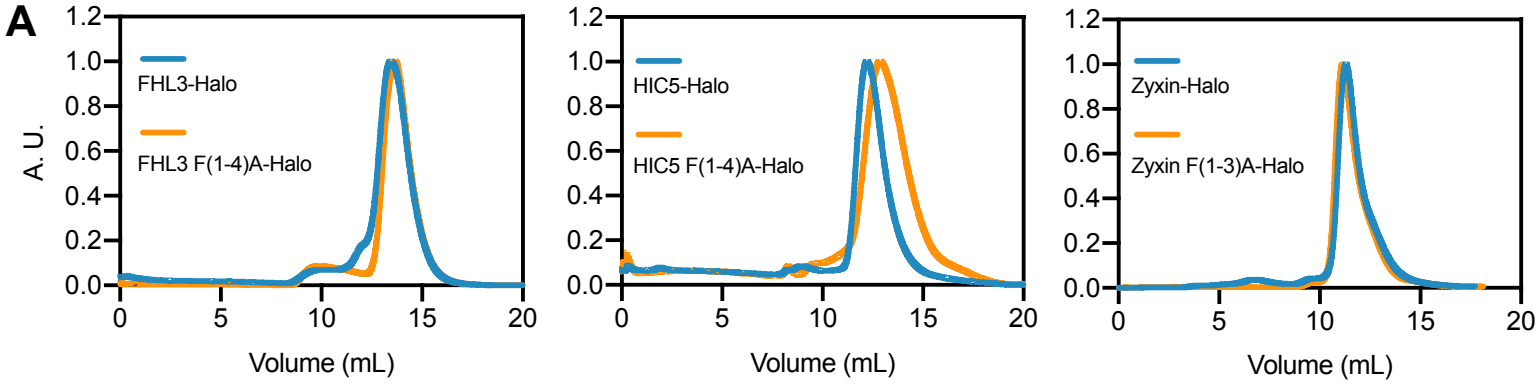
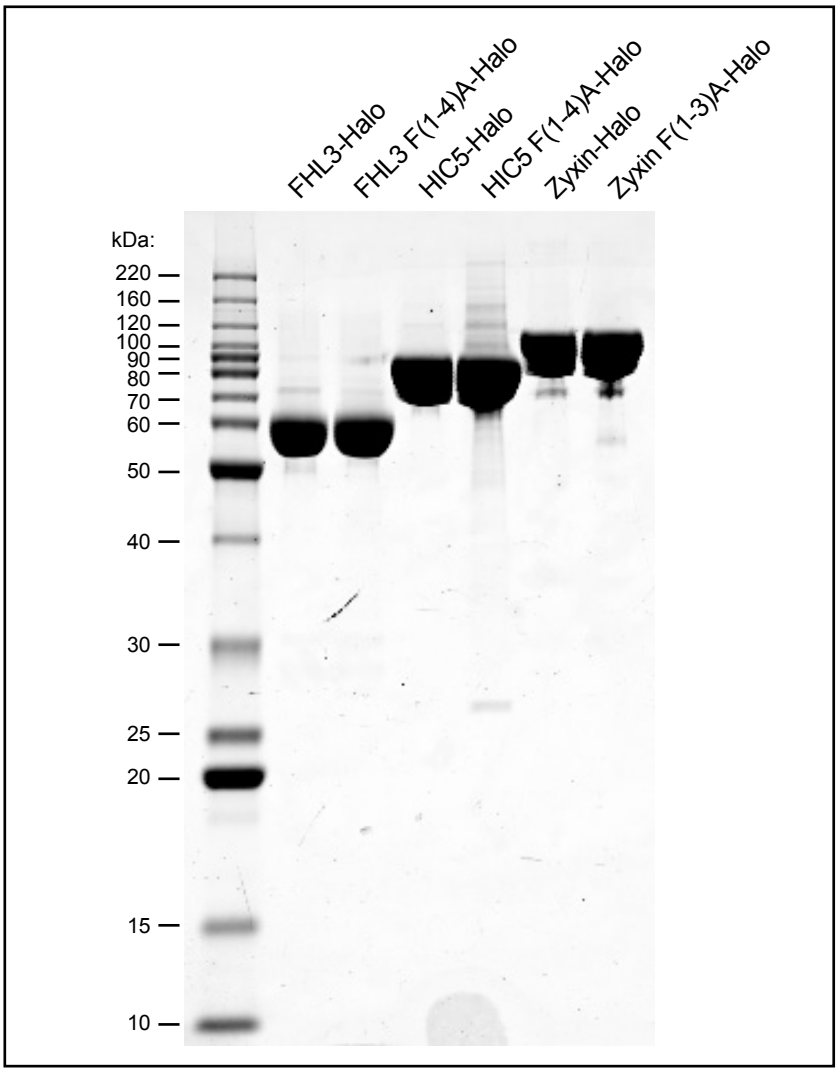

A
Mechanoresponder
Non-mechanoresponder
B
WT
F(1,2)A
F(1-3)A
Phalloidin
Zyxin LDO-eGFP
C
Zyxin LDO localization
Actin / Cytoplasm
WT
F(1,2)A
F(1-3)A
D
Phalloidin (F-actin)
eGFP LIM protein
Anti-paxillin
LIM protein Paxillin
FHL2
FHL2 F(1-4)A
HIC5
HIC5 F(1-4)A
HIC5 LDO
HIC5 LDO F(1-4)A
Zyxin
Zyxin F(1-3)A
Zyxin LDO
Zyxin LDO F(1-3)A
E
FA enrichment
Actin enrichment

Figure S4. Role of the divergent LIM3 of zyxin in SF mechanoaccumulation and role of actin mechanoaccumulation in LIM protein recruitment to FAs. Related to Figure 4. (A) Multiple sequence alignments of mechanoresponder (left) and non-mechanoresponder (right) LIM domains, colored by conservation (shades of purple). Green boxes highlight the conserved phenylalanine in mechanoresponder LIM domains (left) and residues at the same position in non-mechanoresponder LIM domains (right). Red box highlights the phenylalanine next to the conserved position in zyxin LIM3. Orange boxes highlight extended insertions in LIM3 of zyxin-family members. **(B)** Epifluorescence micrographs of MEFs expressing eGFP-labeled WT zyxin LDO (left), zyxin LDO F(1,2)A (middle), and zyxin LDO F(1-3)A (right), stained with phalloidin to label F-actin. Scale bar, 20 μm. **(C)** Whole-cell actin enrichments of wild-type (n = 62), F(1,2)A (n = 102), and F(1-3)A (n = 56) zyxin LDO. N = 2 biological replicates. Games-Howell's multiple comparison test after Welch's ANOVA: *** $p < 0.001$; **** $p < 0.0001$. **(D)** Epifluorescence micrographs of FAs in MEFs expressing eGFP-labeled LIM proteins, stained with phalloidin and anti-paxillin antibody. Scale bar, 2 μm. **(E)** Whole-cell focal adhesion and actin enrichments of wild-type and F(1-4)/(1-3)A full-length and LDO constructs. n = 81-115. N = 2 biological replicates. Welch's unpaired t-test: **** $p < 0.0001$.


A
A. U.
FHL3-Halo
FHL3 F(1-4)A-Halo
HIC5-Halo
HIC5 F(1-4)A-Halo
Zyxin-Halo
Zyxin F(1-3)A-Halo
Volume (mL)


B
FHL3-Halo
FHL3 F(1-4)A-Halo
HIC5-Halo
HIC5 F(1-4)A-Halo
Zyxin-Halo
Zyxin F(1-3)A-Halo
kDa:
220
160
120
100
90
80
70
60
50
40
30
25
20
15
10

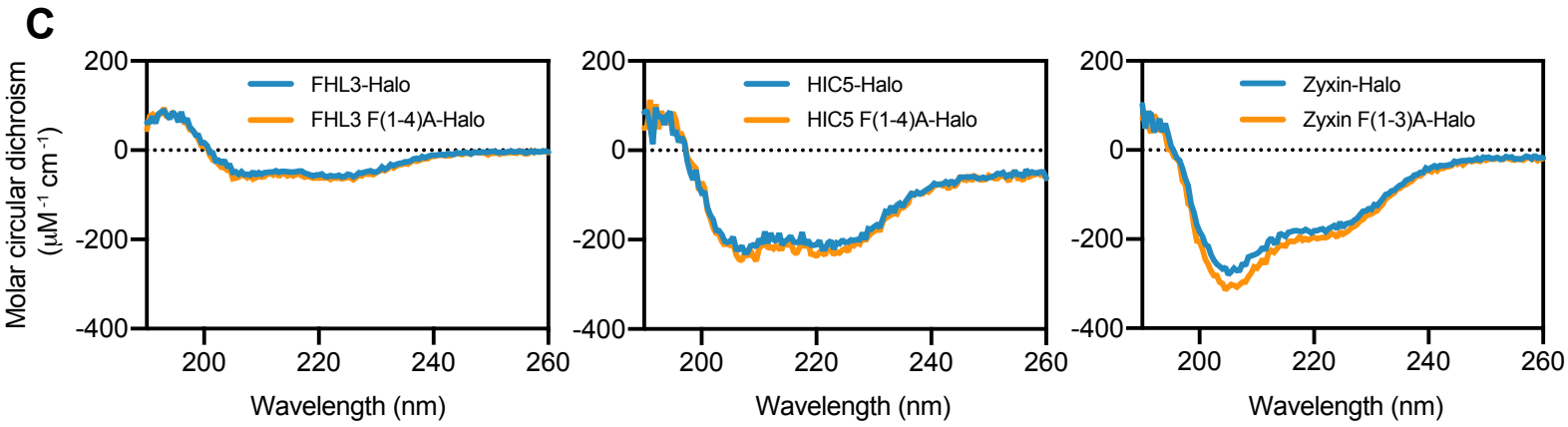

C
Molar circular dichroism
(μM⁻¹ cm⁻¹)
FHL3-Halo
FHL3 F(1-4)A-Halo
HIC5-Halo
HIC5 F(1-4)A-Halo
Zyxin-Halo
Zyxin F(1-3)A-Halo
Wavelength (nm)

Figure S5. Purified LIM proteins featuring phenylalanine mutations preserve their folded structures. Related to Figures 5, 6. (A) Size-exclusion chromatograms of Halo-tagged wild-type and mutant FHL3 (left), HIC5 (middle), and zyxin (right). **(B)** Coomassie-stained SDS-PAGE (3 μg) and **(C)** circular dichroism spectra of the indicated purified proteins.

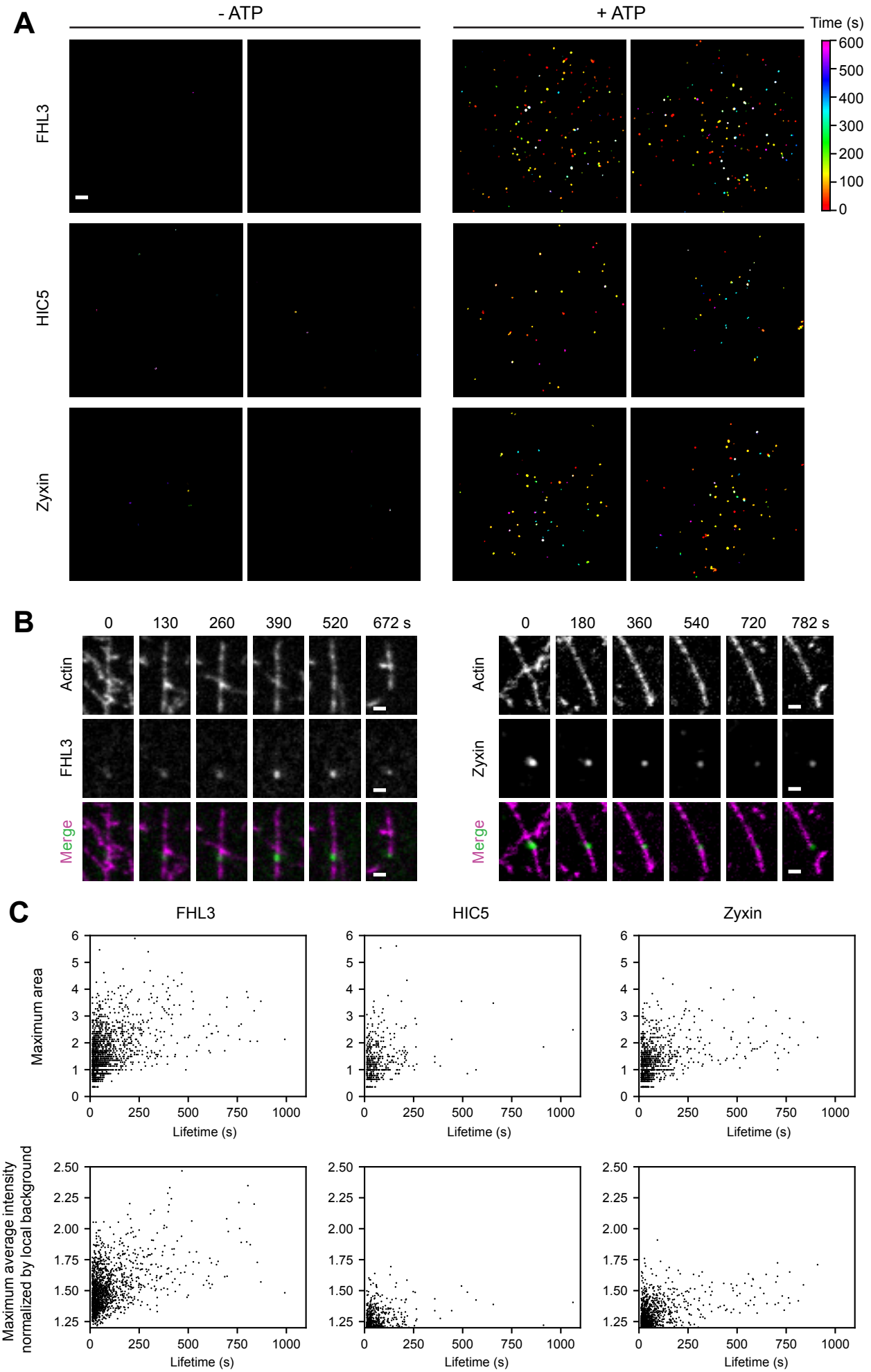

A
- ATP
+ ATP
Time (s)
600
500
400
300
200
100
0
FHL3
HIC5
Zyxin
B
0
130
260
390
520
672 s
0
180
360
540
720
782 s
Actin
FHL3
Merge
Actin
Zyxin
Merge
C
FHL3
HIC5
Zyxin
Maximum area
Maximum average intensity normalized by local background
Lifetime (s)

Figure S6. Additional analyses of LIM protein patches. Related to Figures 5, 6. (A) Two additional examples of the cumulative projections of wild-type FHL3 (top), HIC5 (middle), and zyxin (bottom) in the absence and presence of ATP. **(B)** Montages showing the rebinding of FHL3 (left) and zyxin (right) after filament breakage. Scale bars, 2 μm. **(C)** Scatter plots of maximum area (top) and maximum normalized average fluorescence intensity (bottom) of patches versus their lifetime for the indicated proteins.

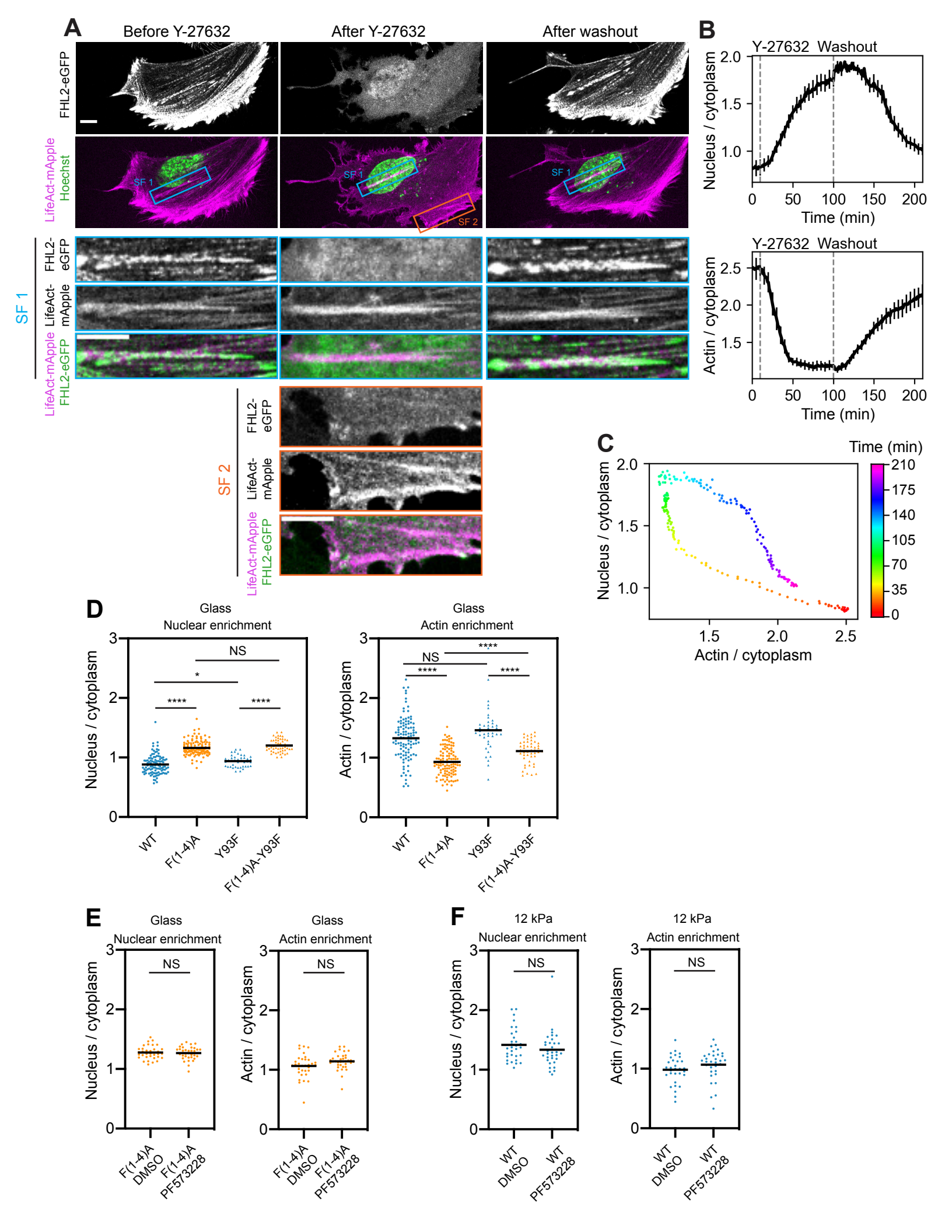

A
Before Y-27632
After Y-27632
After washout
FHL2-eGFP
LifeAct-mApple
Hoechst
SF 1
SF 2
SF 1
FHL2-eGFP
LifeAct-mApple
LifeAct-mApple
FHL2-eGFP
SF 2
FHL2-eGFP
LifeAct-mApple
LifeAct-mApple
FHL2-eGFP
B
Y-27632 Washout
Nucleus / cytoplasm
Time (min)
Y-27632 Washout
Actin / cytoplasm
Time (min)
C
Time (min)
Nucleus / cytoplasm
Actin / cytoplasm
D
Glass
Nuclear enrichment
Nucleus / cytoplasm
NS
*
****
****
WT
F(1-4)A
Y93F
F(1-4)A-Y93F
Glass
Actin enrichment
Actin / cytoplasm
NS
****
****
****
WT
F(1-4)A
Y93F
F(1-4)A-Y93F
E
Glass
Nuclear enrichment
Nucleus / cytoplasm
NS
F(1-4)A DMSO
F(1-4)A PF573228
Glass
Actin enrichment
Actin / cytoplasm
NS
F(1-4)A DMSO
F(1-4)A PF573228
F
12 kPa
Nuclear enrichment
Nucleus / cytoplasm
NS
WT DMSO
WT PF573228
12 kPa
Actin enrichment
Actin / cytoplasm
NS
WT DMSO
WT PF573228

Figure S7. Strained actin binding is the dominant upstream process that mediates FHL2 mechanosensation in MEFs, which does not require the phosphorylation of FHL2 by FAK. Related to Figure 7. (A) Top panel, maximum intensity projections (MIPs) of expressed FHL2-eGFP localization in a MEF before (left) and after (middle) Y-27632 treatment, and after washout (right). MIPs were constructed from three confocal z-slices. Bottom panel, zoomed-in views of the boxed regions. Scale bars, 10 μm. **(B)** Average nuclear (top) and actin (bottom) enrichments obtained from n = 16 cells from N = 3 biological replicates as a function of time, aligned by the timepoint of Y-27632 addition. Error bar represents SEM. Dash lines indicate the time points of Y-27632 addition (10 minutes) and washout (100 minutes). **(C)** Replotting of average nuclear enrichment versus average actin enrichment, displayed as a scatterplot where points are color-coded by time. **(D)** Nuclear (left) and actin (right) enrichment of eGFP-tagged wild-type (n = 106), F(1-4)A (n = 110), Y93F (n = 41), and F(1-4)A-Y93F FHL2 (n = 50) expressed in MEFs plated on glass. N = 2 biological replicates. Games-Howell's multiple comparison test after Welch's ANOVA: NS, $p > 0.05$; * $p < 0.05$; **** $p < 0.0001$. **(E)** Nuclear (left) and actin (right) enrichment of eGFP-tagged FHL2 F(1-4)A expressed in MEFs plated on glass with (n = 34) and without (n = 36) FAK inhibition. N = 2 biological replicates. Welch's unpaired t-test: NS, $p > 0.05$. **(F)** Nuclear (left) and actin (right) enrichment of eGFP-tagged wild-type FHL2 expressed in MEFs plated on 12-kPa hydrogels with (n = 31) and without (n = 32) FAK inhibition. N = 2 biological replicates. Welch's unpaired t-test: NS, $p > 0.05$.