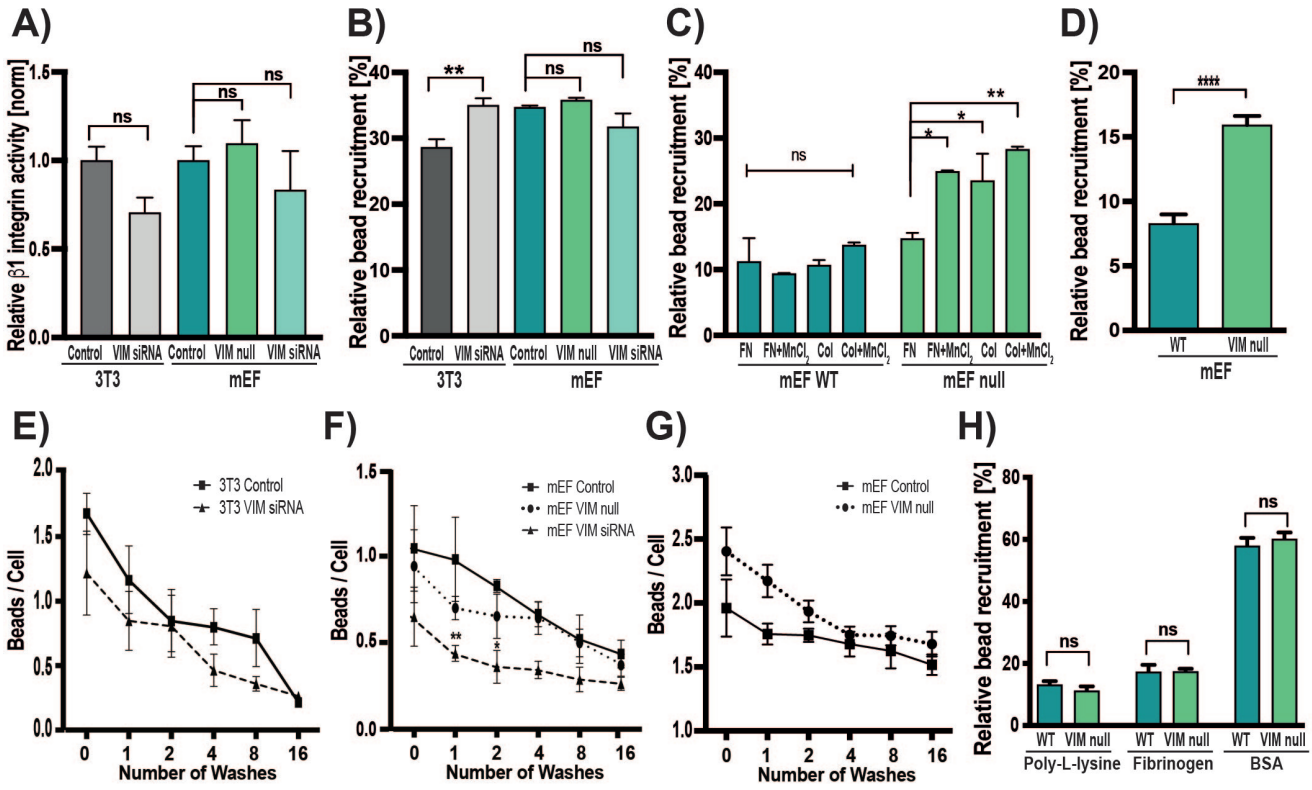

Fig. S1. (A) Estimation of relative β1 integrin activation levels after 3h incubation on fibronectin-coated tissue culture dishes. Cells were immunostained for β1 integrin with 9EG7 clone antibody. (B) Histogram shows relative recruitment of fibronectin-coated 2 μm fluorescent beads for all indicated cell types from flow cytometry analyses. 3T3 control cells (dark gray) and Vim siRNA (light gray), mEF control (teal), mEF Vim null (green) and mEF Vim siRNA (turquoise). (C) Recruitment of fibronectin or collagen-coated beads for mEF WT and Vim null cells after β1 integrin activation by 0.2 mM $MnCl_2$. (D) Quantification of fibronectin-coated beads recruitment by mEF WT and null plated on 0.01 mg/ml fibronectin-coated dishes. Quantification of the relative binding strength of control cells (square, solid line) and vimentin-depletion (Vim siRNA - triangle, dashed line; Vim null- circle, dotted line) 3T3 (E) and mEF cells (F). Fluorescent beads (2 μm) were coated with 1 mg/ml fibronectin and incubated with cells (8 beads/cell) for 2h. (G) Fibronectin-coated beads binding strength for mEF WT (square, solid line) and null (circle, dotted line) cells plated on 0.01 mg/ml fibronectin-coated dishes. Cells with bounded beads were subjected to 0, 1, 2, 4, 8, and 16 washes. Plots represent the number of bounded beads per cell after each wash. Quantification was done by ImageJ software. All data are reported as a mean ± S.E.M. Assessment of fluorescent beads recruitment by mEF WT and mEF null cells were done by flow cytometry. (H) Yellow-green (2 μm) beads were coated with poly-L-lysine, fibrinogen, or BSA, respectively. Quantification was done by FCS Express 6 software. All experiments were repeated four times, and data are reported as a mean ± S.E.M.

Fig. S2

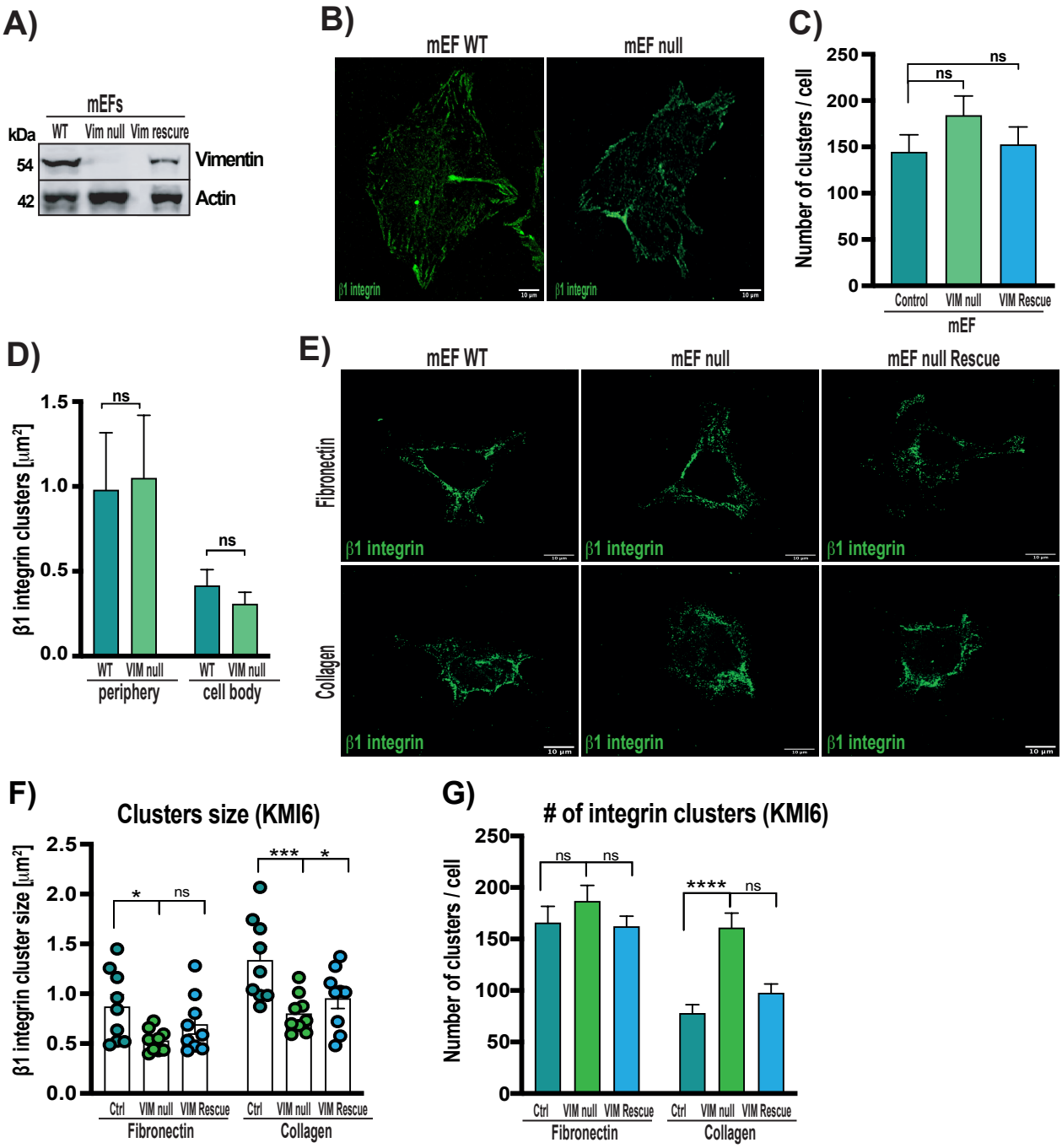

Fig. S2. (A) Western blot represents vimentin expression in mEF WT, mEF VIM rescue and mEF null cells. β-actin was used as a loading indicator. (B) Confocal images of β1 integrin in mEF WT and null cells plated on fibronectin-coated surfaces. Scale bar 10 μm. Quantification of β1 integrin cluster numbers (C) and cluster size (D) for mEF control (teal), mEF null (green), Vim rescue (blue). (E) Representative confocal images for mEF WT, Vim null and Vim rescue cells plated on fibronectin or collagen-coated surfaces staining for the total β1 integrin (KMI6 clone antibody). MEF control (teal), mEF Vim null (green) and mEF Vim siRNA (turquoise) and mEF Vim rescue cells (blue bars) were analyzed for: (F) β1 integrin cluster mean size (μm^2), and (G) β1 integrin cluster number per cell. All quantifications are from three experiments. *$p<0.05$, ****$p<0.0001$. All data are reported as a mean ± S.E.M, (n=3, at least 30 cells per group).

A)

2020 Scaffold Results from Orbitrap LC-MS					Abundance
Description	Accession	Number of Peptides	MW in kDa	Gene Symbol	Ratio mEF Null vs WT
Vimentin OS=Mus musculus (Mouse) OX=10090 GN=Vim PE=1 SV=3	**P20152**	**73**	**53.7**	**Vim**	**0.03**
Clathrin heavy chain 1 OS=Mus musculus (Mouse) OX=10090 GN=Cltc PE=1 SV=3	Q68FD5	52	191.4	Cltc	1.141
Alpha-actinin-1 OS=Mus musculus (Mouse) OX=10090 GN=Actn1 PE=1 SV=1	Q7TPR4	42	103	Actn1	1.405
Actin, cytoplasmic 1 OS=Mus musculus (Mouse) OX=10090 GN=Actb PE=1 SV=1	P60710	32	41.7	Actb	1.886
Collagen alpha-1(III) chain OS=Mus musculus (Mouse) OX=10090 GN=Col3a1 PE=1 SV=4	P08121	24	138.9	Col3a1	1.969
Talin-1 OS=Mus musculus (Mouse) OX=10090 GN=Tln1 PE=1 SV=2	**P26039**	**133**	**269.7**	**Tln1**	**2.491**
Integrin-linked protein kinase OS=Mus musculus (Mouse) OX=10090 GN=Ilk PE=1 SV=2	O55222	20	51.3	Ilk	2.636
Myosin regulatory light chain 12B OS=Mus musculus (Mouse) OX=10090 GN=Myl12b PE=1 SV=2	Q3THE2	12	19.8	Myl12b	2.97
Spectrin beta chain, non-erythrocytic 1 OS=Mus musculus (Mouse) OX=10090 GN=Sptbn1 PE=1 SV=2	Q62261	133	274.1	Sptbn1	3.722
Myosin-10 OS=Mus musculus (Mouse) OX=10090 GN=Myh10 PE=1 SV=2	Q61879	160	228.9	Myh10	4.103
Spectrin alpha chain, non-erythrocytic 1 OS=Mus musculus (Mouse) OX=10090 GN=Sptan1 PE=1 SV=4	P16546	166	284.4	Sptan1	4.525

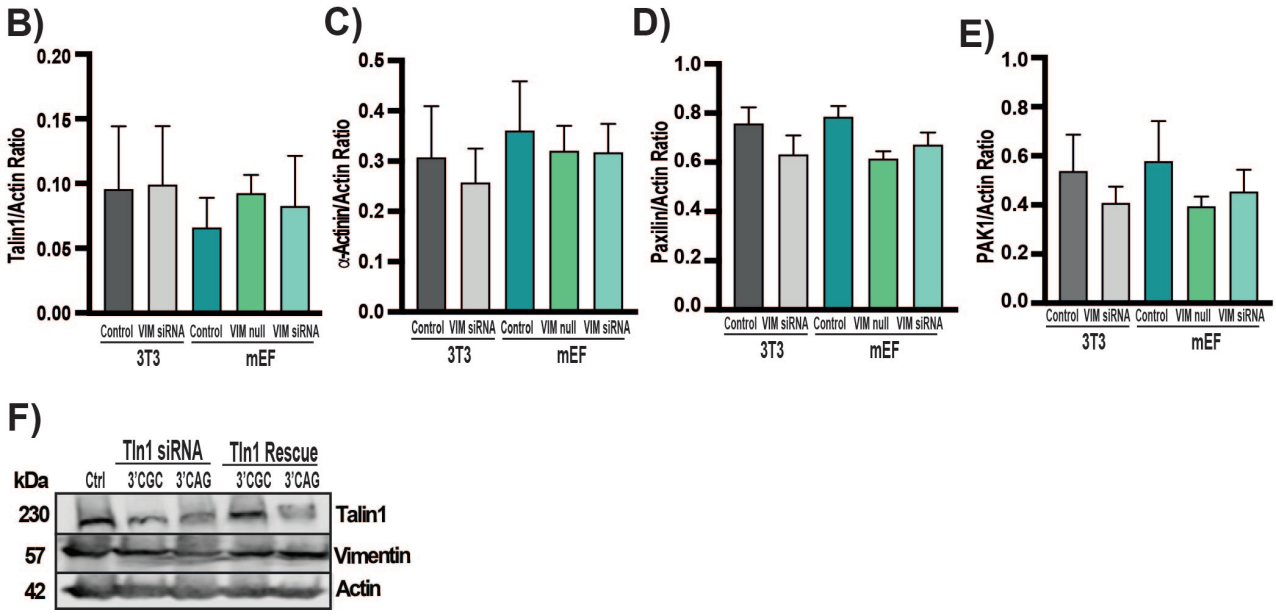

Fig. S3. (A) Mass spectrometry data of collagen bead-associated proteins analyzed presented as an abundance ratio for mEF WT and mEF null cells. Scaffold 4.10 (Proteome Software, Portland, OR, USA) was used for analyzing search results, calculating P values for each peptide match and matching peptide spectra. (B-E) Bar graphs represent talin:β-actin ratio (B); α-actinin:β-actin ratio (C); Paxillin:β-actin ratio (D) and PAK1:β-actin ratio (E) in the analyzed cell lines that were plated on fibronectin (black bars) or collagen (green bars) matrices. Quantifications were done based on western blot analysis (Fig. 4B) immunostained for talin, α-actinin, paxillin, PAK1 and β-actin (loading control) with appropriate antibodies. All data are reported as a mean ± S.E.M. (F) Immunoblotting represents Talin expression after 3'UTR CGC and CAG Tln siRNA and Tln re-expression (Tln-GFP plasmid), compared with control samples treated with scramble siRNA. Vimentin and β-actin were used as a loading control. All data are reported as a mean ± S.E.M, n=3.

Fig. S4

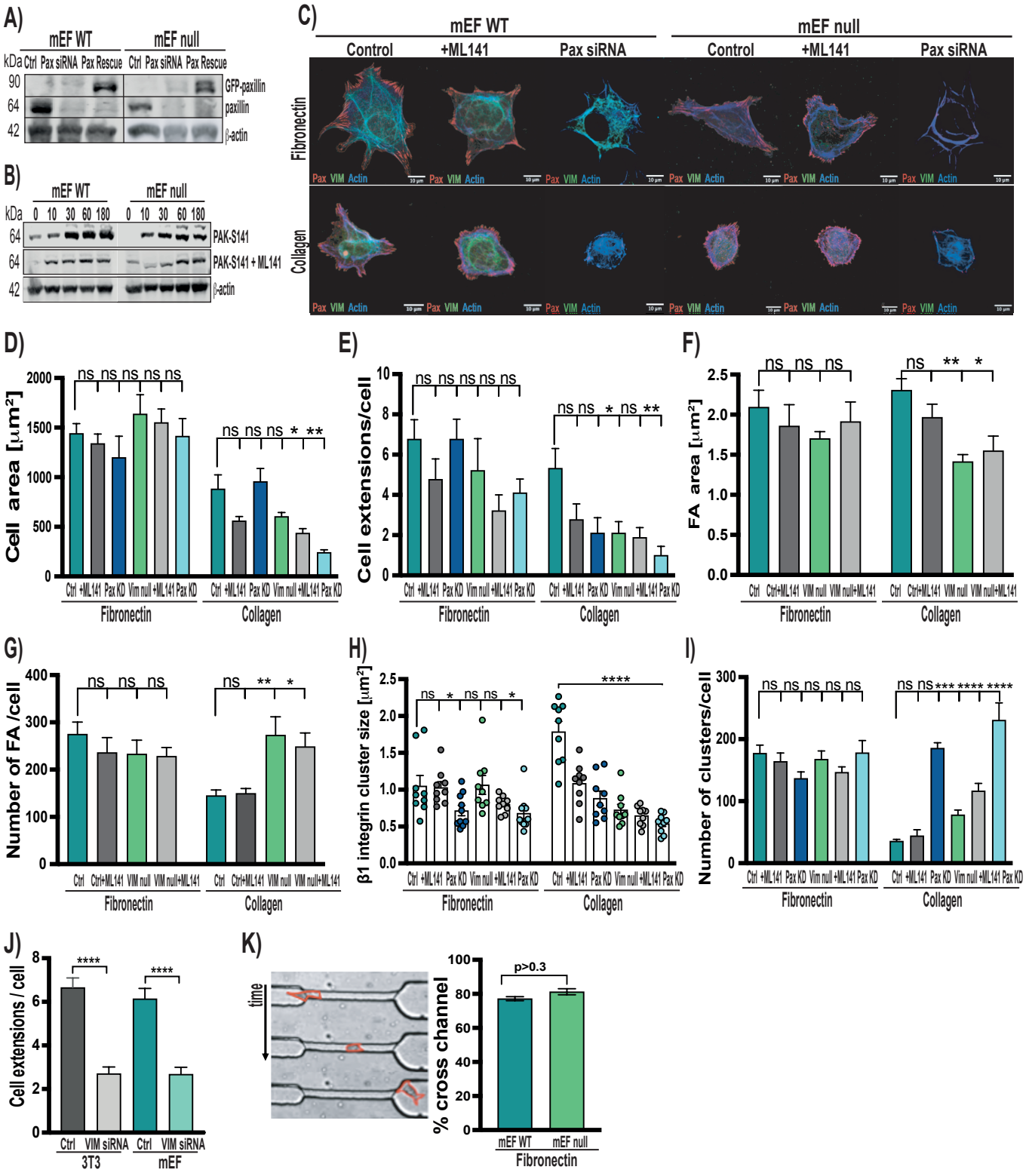

Fig. S4. Immunoblotting shows (A) paxillin (Pax) expression efficiency in vimentin WT and null mEFs. Cells were treated with scrambled siRNA (Ctrl), ON-TARGET paxillin siRNA (Pax siRNA) and paxillin-GFP plasmid. β-actin was used as a loading control. (B) mEFs were plated for the indicated times (0,10, 30, 60, and 180 min), lysed and immunoblotted for the indicated proteins, which include actin and PAK phospho-serine 141. The upper panel shows vehicle- treated samples (control). The middle panel shows cells treated with ML141, a specific Cdc42/Rac1 inhibitor (10 μM). Actin was used as a loading control. Experiments were performed in triplicates. All data are reported as a mean ± S.E.M, n=3. (C) Representative confocal images of mEFs treated with ML141 or paxillin siRNA cultured on fibronectin or collagen-coated surfaces and staining for paxillin (red), vimentin (green) and actin (blue). Scale bar 10 μm. Assessment cell morphology defined by (D) the mean area of cell and (E) the number of cells extensions per cell for mEF WT (control-teal), mEF WT+ML141 (dark gray bar), mEF Pax KD (dark blue), mEF null (green), mEF null+ML141 (light gray), mEF null Pax KD (light blue) plated on fibronectin or collagen-coated surfaces. Quantification of the focal adhesion size (F) and number (F) form mEF WT and null cells treated with 10 ⌈M ML141. Estimation of the β1 integrin cluster size (H) and average number (I) for mEF WT (control-teal), mEF WT+ML141 (dark gray bar), mEF Pax KD (dark blue), mEF null (green), mEF null +ML141 (light gray), mEF null Pax KD (light blue) plated on fibronectin or collagen-coated surfaces. (J) Number of cell protrusions per cell for 3T3 cells and mEFs after vimentin deletion and then cultured on fibronectin. All data are reported as a mean ± S.E.M, n=3. (K) Vimentin WT and null mEF migration through fibronectin-coated microchannel (at least 45 cells were counted; n=3). Experiment was conducted as described in (Patteson et al., 2019).

Fig. S5

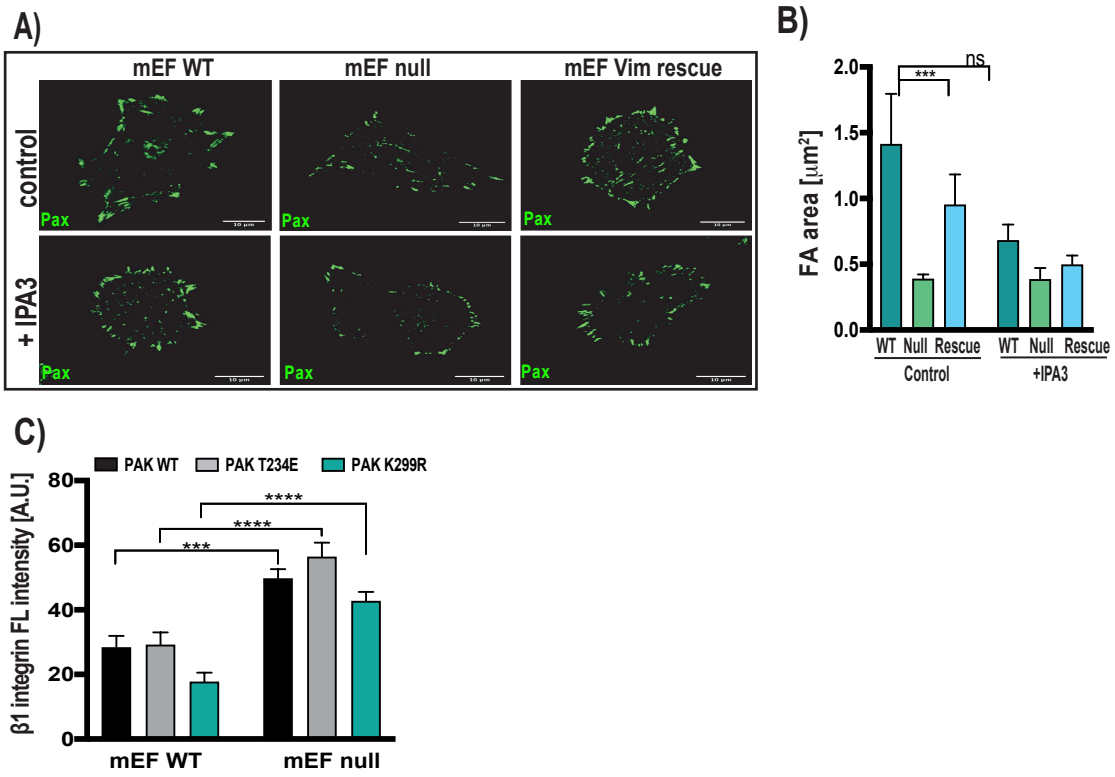

Fig. S5. Inhibition of PAK1 activity and vimentin expression alters focal adhesion size. (A) Representative fluorescence confocal images of cells treated with vehicle (control) or IPA3 (5 mM) and plated on collagen for 3h. Cells were fixed and immunostained with paxillin antibody (green). Scale bar, 10 μm. (B) Bar graphs represent size (μm^2) of focal adhesion in mEFs cells treatment with vehicle (control) and IPA3 (5 μM). MEF WT (tile bars), mEF null (green bars), and mEF VIM rescue (blue bars). Quantifications were done based on confocal images (Fig. 5A). (C) Impact of PAK1 kinase mutants on the β1 integrin activity for mEF WT and null cells. PAK1 WT (black), PAK1 constitutively active T423E (gray), PAK1 dominant negative mutant K299R (teal). Data are obtained from three independent experiments and reported as a mean ± S.E.M, *** $p<0.001$, **** $p<0.0001$.