Online supplemental material

Calculation of the normalized paxillin in focal adhesions (FAs)

The derivation for the fluorescence of paxillin-EGFP in FAs as measured by TIRF follows the same line of reasoning as described elsewhere (Haugh and Schneider, 2004). Briefly, the fluorescence signal for paxillin-EGFP in FAs is the sum of two signals; the fluorescence from cytosolic paxillin-EGFP above FAs and the fluorescence from paxillin-EGFP within FAs. The background cytosolic fluorescence of paxillin-EGFP above FAs is given by

$$F_1 = \frac{aN_{pax,cyt}}{V_{cyt}} \int_{\delta}^{\infty} e^{-z/d_{cell}}\, dz = \frac{aN_{pax,cyt} d_{cell} e^{-\delta/d_{cell}}}{V_{cyt}}, \qquad \text{(A1)}$$

a is related to the power density of the incident beam, quantum efficiency of the camera, quantum yield of the fluorophore, extinction coefficient of the fluorophore and gap distance between the ventral membrane of cell and the glass surface. The gap distance is slightly different under FAs than it is under other regions of the ventral membrane of the cell (Truskey et al., 1992). However, because both cytosolic and FA fluorescences are averaged throughout the entire contact area of the cell and normalized to the prestimulus condition, we assume that the difference between the average gap distances before and after stimulation is small. Thus, *a* is approximated as a constant. $N_{pax,cyt}/V_{cyt}$ is the concentration of paxillin-EGFP inside the cell, z is the distance from the membrane, d_{cell} is the penetration depth of the evanescent wave (150nm) and δ is the average thickness of the FA plaques (15-65nm) (Franz and Muller, 2005). The fluorescence from paxillin-EGFP within FAs is given by

$$F_2 = \frac{aN_{pax,FA}}{\delta A_{FA}} \int_{0}^{\delta} e^{-z/d_{cell}}\, dz = \frac{aN_{pax,FA} d_{cell}}{\delta A_{FA}} \left[1 - e^{-\delta/d_{cell}}\right], \qquad \text{(A2)}$$

where $N_{pax,FA}$ is the total number of paxillin-EGFP molecules in all the FAs and A_{FA} is the total area of all the FAs. Summing these two fluorescence contributions gives the equation for the fluorescence of paxillin-EGFP in FAs as

$$F_{pax,FA} = F_1 + F_2 = a\left[\frac{N_{pax,cyt} d_{cell} e^{-\delta/d_{cell}}}{V_{cyt}} + \frac{N_{pax,FA} d_{cell}}{\delta A_{FA}}\left[1 - e^{-\delta/d_{cell}}\right]\right]. \quad \text{(A3)}$$

The cytosolic fluorescence of paxillin-EGFP in areas outside of FAs is given by

$$F_{pax,cyt} = \frac{aN_{pax,cyt}}{V_{cyt}} \int_0^\infty e^{-z/d_{cell}} dz = \frac{aN_{pax,cyt} d_{cell}}{V_{cyt}}. \quad \text{(A4)}$$

Using Eqns.A3 and A4 and solving for the number of paxillin-EGFP molecules in FAs results in the following expression:

$$N_{pax,FA} = \frac{\frac{F_{pax,FA}}{a} - \frac{F_{pax,cyt} e^{-\delta/d_{cell}}}{a}}{\frac{d_{cell}}{\delta A_{FA}}\left[1 - e^{-\delta/d_{cell}}\right]}. \quad \text{(A5)}$$

Using Eqns.A4 and A5, one can solve for the fraction of paxillin-EGFP in FAs, yielding

$$\hat{f}_{pax,FA} = \frac{N_{pax,FA}}{N_{pax,FA} + N_{pax,cyt}} = \frac{F_{FA}/F_{cyt} - e^{-\alpha}}{F_{FA}/F_{cyt} + \left[\beta\left(1 - e^{-\alpha}\right) - e^{-\alpha}\right]}, \quad \text{(A6)}$$

where $\alpha = \delta/d_{cell}$ and $\beta = V_{cyt}/(\delta A_{FA})$. Using the range of values for δ and the known value for d_{cell}, α is calculated to be 0.10-0.43, thus setting the range of $e^{-\alpha}$ to 0.65-0.90. Assuming a cellular volume of 1pL (1000μm^2) and an experimentally calculated range for A_{FA} as 10-100μm^2, one can estimate β as 130-6700 and $\beta(1\text{-}e^{-\alpha})$ as 13-2300. This value is most likely larger than either $e^{-\alpha}$ (0.65-0.90) or F_{FA}/F_{cyt} (1-4). Consequently, this equation can be reduced to

$$\hat{f}_{pax,FA} = \frac{F_{FA}/F_{cyt} - e^{-\alpha}}{\beta\left(1 - e^{-\alpha}\right)}. \quad \text{(A7)}$$

By assuming that V_{cyt} and δ do not change dramatically over the course of the experiment and by normalizing Eqn.A7 to the prestimulus value of the fraction of paxillin in FAs, one can derive the following expression used to calculate the normalized paxillin in FAs.

$$f_{pax,FA} = \left(\frac{F_{FA}/F_{cyt} - e^{-\alpha}}{F_{FA0}/F_{cyt0} - e^{-\alpha}} \right) \left(\frac{A_{FA}}{A_{FA0}} \right). \qquad \text{(A8)}$$

References

Franz, C.M., and Muller, D.J. (2005). Analyzing focal adhesion structure by atomic force microscopy. Journal of Cell Science *118*, 5315-5323.

Haugh, J.M., and Schneider, I.C. (2004). Spatial analysis of 3 ' phosphoinositide signaling in living fibroblasts: I. Uniform stimulation model and bounds on dimensionless groups. Biophysical Journal *86*, 589-598.

Truskey, G.A., Burmeister, J.S., Grapa, E., and Reichert, W.M. (1992). Total Internal-Reflection Fluorescence Microscopy (Tirfm) .2. Topographical Mapping of Relative Cell Substratum Separation Distances. Journal of Cell Science *103*, 491-499.

Video1: EGF-stimulated protrusion is temporally segregated from retraction: HaCat cells stimulated with 5nM EGF and imaged using time-lapse DIC microscopy. The images were background-subtracted and 5 x 5 median filtered. The video frame rate is 150x the experimental rate, which is labeled on the video in minutes and referenced to the time of EGF stimulation. The same cell is shown in Figure 1.

Video2: EGF stimulated increase in traction force generation: HaCat cells were plated on 3kPa polyacrylamide gels that were embedded with 0.2μm far red beads, stimulated with 5nM EGF and imaged using phase contrast microscopy. Bead displacements relative to the cell-free state were tracked and overlaid on the phase contrast images. The video frame rate is 150x the experimental rate, which is labeled on the video in minutes and referenced to the time of EGF stimulation. The same cell is shown in Figure 2.

Video3: EGF-stimulated retraction is blocked by blebbistatin and Y27632 but not ML-7: HaCat cells were pretreated with vehicle only (0.3% DMSO), 20μM ML-7, 30μM blebbistatin, or 10μM Y-27632, as indicated, and stimulated with 5nM EGF and imaged by time-lapse DIC microscopy. The images were background-subtracted and 5 x 5 median filtered. The video frame rate is 150x the experimental rate, which is labeled on the video in minutes and referenced to the time of EGF stimulation. The same cells are shown in Figure 4.

Video4: EGF stimulates FA assembly during protrusion followed by maturation of existingFAs and FA disassembly prior to retraction: HaCat cells were transfected with paxillin-EGFP, stimulated with 5nM EGF and imaged using time-lapse TIRF microscopy. The

images were scaled such that the cytosolic fluorescence remained approximately constant. The video frame rate is 150x the experimental rate, which is labeled on the video in minutes and referenced to the time of EGF stimulation. A zoomed region is shown on the right. The same cell is shown in Figure 6.

Video5: Blebbistatin and Y-27632 do not block EGF-mediated FA assembly, but inhibit EGF-mediated maturation of existing FAs: HaCat cells were transfected with paxillin-mCherry and pretreated with 30μM blebbistatin (left) or transfected with paxillin-EGFP and pretreated with 10μM Y-27632 (right). Both were then stimulated with 5nM EGF, and imaged by time-lapse TIRF microscopy. Bottom panels show zoomed views of regions of the top panels. All images were scaled such that the cytosolic fluorescence remained approximately constant. The video frame rate is 150x the experimental rate, which is labeled on the video in minutes and referenced to the time of EGF stimulation. The same cells are shown in figure 7 (blebbistatin) and supplemental Figure 2 (Y-27632).

Video6: Y-27632 tempers EGF-stimulated retraction even when administered simultaneously with EGF: HaCat cells were stimulated with either 5nM EGF alone (left) or 10μM Y-27632 and 5nM EGF (right) and filmed using time-lapse phase contrast microscopy. The video frame rate is 150x the experimental rate, which is labeled on the video in minutes and referenced to the time of EGF stimulation. The same group of cells is shown in Figure 7.

Video7: EGF-mediated retraction depends on paxillin dephosphorylation: HaCat cells treated with paxillin siRNA or paxillin siRNA and additionally expressing paxillin-EGFP,

Y31E/Y118E-paxillin-EGFP or Y31F/Y118F-paxillin-EGFP were stimulated with 5nM EGF and filmed using time-lapse phase contrast microscopy. The video frame rate is 150x the experimental rate, which is labeled on the video in minutes and referenced to the time of EGF stimulation. The same group of cells is shown in Figure 9.

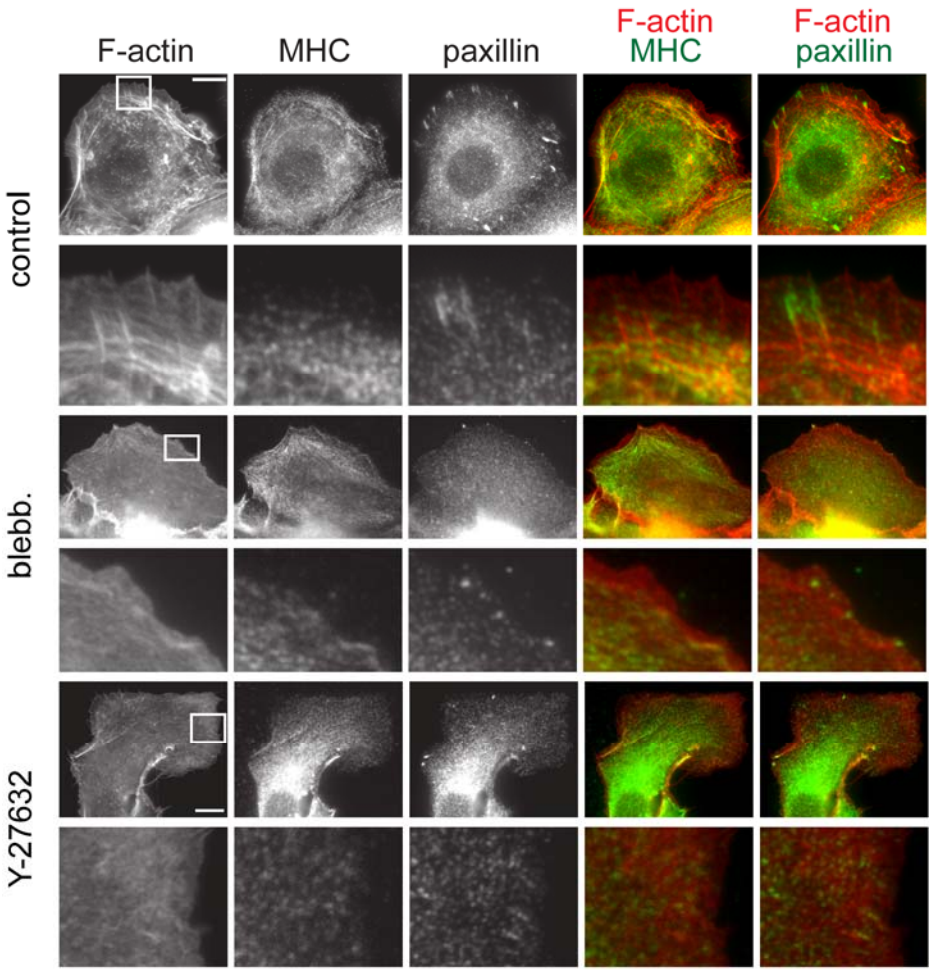
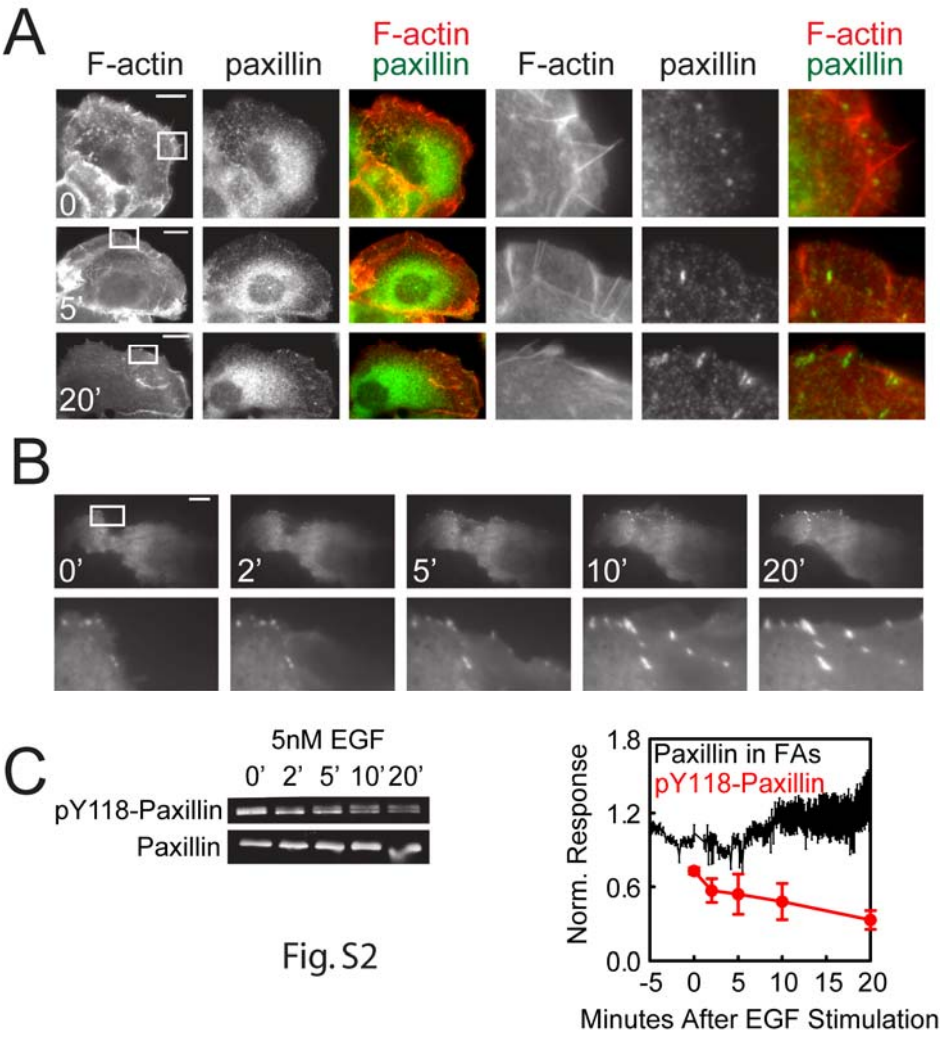


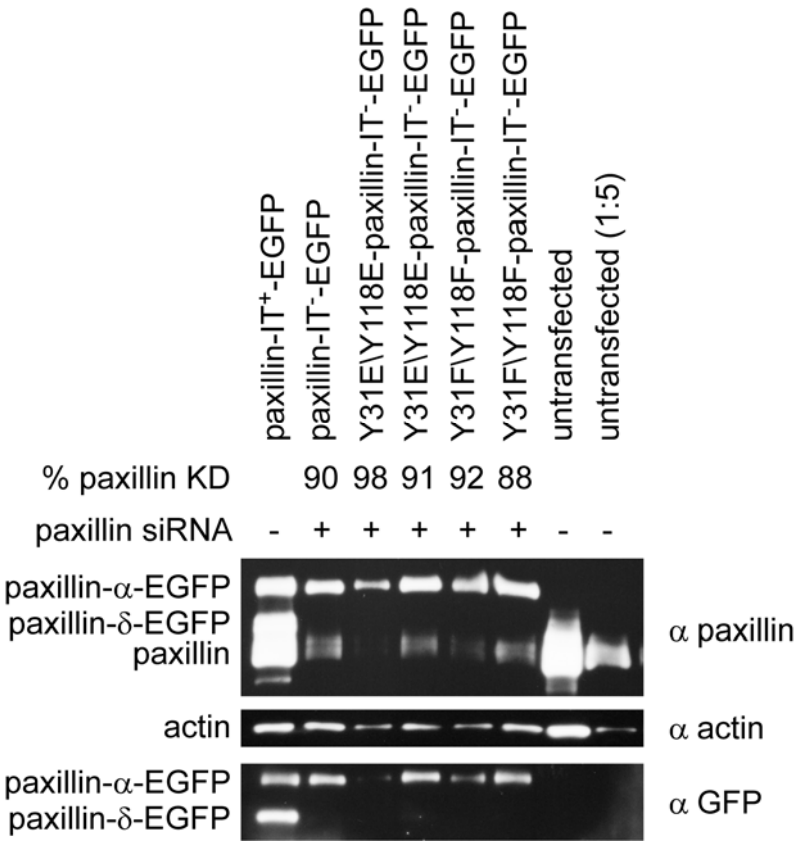
Fig. S1

Supplemental Figure S1. Myosin-II inhibition affects FA size and distribution. Epi-fluorescence images of HaCat cells that were pretreated with control media, 30μM blebbistatin or 10μM Y-27632 for 1 hour prior to fixation, and were processed for localization of F-actin, myosin IIA and paxillin. For each condition, a low magnification image is shown (top) along with enlarged views of areas outlined by white squares (bottom). Bars = 10μm.



Supplemental Figure S2. ROCK is required for EGF-mediated recruitment of paxillin to FAs. (A) Left: Epi-fluorescence images of HaCat cells that were pretreated with 10μM Y-27632, stimulated with 5nM EGF for the times (in mins) noted, fixed and processed for localization of F-actin and paxillin. Right: Enlarged views of the regions denoted by the white squares. (B) Top: TIRF images of paxillin-EGFP in cells pretreated with 10μM Y-27632, stimulated with 5nM EGF for the times (in mins) noted. Bottom: Enlarged views of the region highlighted by a white squares. (C) Left: Western blots of cell lysates probed for pY118-paxillin and total paxillin from cells pretreated with 10μM Y-27632 and lysed at the noted times after EGF

stimulation. Right: The average normalized paxillin-EGFP fluorescence in focal adhesions (n = 7 cells, black). The average of quantified western blots of pY118-paxillin level normalized to total paxillin level (n = 2, red) is shown on the same graph. Error bars mark ±S.E.M. Bars = 10μm.



Supplemental Figure S3. Paxillin siRNA substantially reduces endogenous paxillin level but not exogenously expressed paxillin-EGFP mutants. Cells were transfected with or without EGFP- tagged avian paxillin constructs or human paxillin siRNA oligos. Identical dishes were used for the imaging and the analysis of these cells are shown in Figure 9, A and B. Western blots were conducted probing lysates with anti-paxillin, -actin and –GFP antibodies. Mutation of the internal translation site of avian paxillin (denoted IT^-) eliminated the smaller paxillin-δ-EGFP band seen in the lane in which the expressed avian paxillin-EGFP construct did not bear this mutation (denoted IT^+). SiRNA treatment reduces endogenous paxillin by ~ 90% (after normalizing the paxillin level to actin level).

Supplemental Table S1. Parameters for a Phenomenological Model Fit to EGF-induced Contraction and Adhesion Processes

Process	A_{max}	k_1(s^{-1})	k_2(s^{-1})	t_{lag}(s)	k_{obs}(s^{-1})	t_{max}(s)
MRLC phosphorylation*	>5	>10	>0.8	0	>10	0.25-0.5
Paxillin phosphorylation Bl	0.5	1.3	0.042	1.1	1.4	3.0
Paxillin recruitment	0.2	0.86	0.0078	1.6	0.89	5.2
Traction force increase	1.1	0.37	0.015	0.34	0.38	7.4
Cell area increases	13	0.0063	0.054	1.9	0.010	7.8
Cell Retraction†	-	-	-	11.2	-	-

Process kinetics were fit to the phenomenological model equation

$f(t) = \left(1 + A_{\max}\left(1 - e^{-k_1(t-t_{lag})}\right)\right)e^{-k_2(t-t_{lag})}$, where $k_{obs} = (k_2 + k_1 A_{\max})/A_{\max}$ and

$t_{\max} = t_{lag} + \ln[A_{\max}(k_1 + k_2)/((1 + A_{\max})k_2)]/k_1$.

*estimated values due to poor fitting.

† lag times estimated from the average from control cells in Figure 9B.

Supplemental Table S2. Effects of Different Treatments on EGF-stimulated Cell Retraction

Treatments	Concentration	Target	Retraction
TS2/16	1-2ug/mL	β1 integrins	blocks slightly
P5D2	0.1-2ug/mL	β1 integrins	no affect
short Y-27632	10μM	ROCK	blocks
long Y-27632	10μM	ROCK	blocks
blebbistatin	30μM	MHC	blocks
ML-7	20μM	MLCK	no affect
C3-transferase	0.2ug/mL	rhoGTPases	no affect
calpain Inhibitor I	10μM	Calpain	no affect
calpeptin	10μM	Calpain	no affect
PD-098059	10μM	MEK	no affect
Fak Inhibitor	1μM	Fak	no affect
PP2	10μM	Src	no affect
DN-Shp2	-	Shp2	no affect
paxillin-EGFP	-	-	no affect
Y31E/118E-paxillin-EGFP	-	paxillin	blocks moderately
Y31F/118F-paxillin-EGFP	-	paxillin	no affect