

Spatiotemporal Constraints on the Force-Dependent Growth of Focal Adhesions

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ABSTRACT Focal adhesions (FAs) are the predominant mechanism by which cells mechanically couple to and exert traction forces on their extracellular matrix (ECM). It is widely presumed that FA size is modulated by force to mediate changes in adhesion strength at different levels of cellular tension. However, previous studies seeking correlations between force and FA morphology have yielded variable and often conflicting results. Here we show that a strong correlation between adhesion size and traction force exists only during the initial stages of myosin-mediated adhesion maturation and growth. For mature adhesions, no correlation between traction stress and size is observed. Rather, the tension that is sustained at mature adhesions is more strongly influenced by proximity to the cell edge, with peripheral adhesions transmitting higher tension than adhesions near the cell center. Finally, we show that mature adhesions can withstand sixfold increases in tension without changes in size. Thus, although a strong correlation between adhesion size and mechanical tension is observed during the initial stages of myosin-mediated adhesion maturation, no correlation is observed in mature, elongated adhesions. This work places spatiotemporal constraints on the force-dependent growth of adhesions and provides insight into the mechanical regulation of cell-ECM adhesion.

INTRODUCTION

Adherent cells exert traction stresses on the surrounding extracellular matrix (ECM) at focal adhesions (FAs), hierarchical protein assemblies that facilitate mechanical interactions between the actin cytoskeleton and the ECM (1–4). Spatiotemporal regulation of tension sustained at FAs is essential for directed cell migration and ECM remodeling (4,5). In turn, force-mediated FA signaling regulates numerous cellular processes, including cell proliferation and differentiation (6–9). Thus, understanding the interdependencies between FA assembly and cell-ECM force transmission is essential for many aspects of cell physiology.

FAs assemble near the cell periphery within the lamellipodium in an actin polymerization-dependent process (3,5,10). After the assembly of myosin-independent adhesions, myosin-mediated tension within the lamellar actin cytoskeleton drives changes in FA composition and morphology in a process termed maturation (4,5,11). During maturation, FA morphology changes from a circular, submicron dot to an elongated plaque with a 0.5–1 μm width and lengths up to several micrometers (3,5). Concomitantly, posttranslational modifications of regulatory proteins (e.g., paxillin and FAK) occur (12,13) and structural proteins are recruited (e.g., vinculin and zyxin) (14,15). Changes in FA protein composition and posttranslational modification are known to be important for the regulation of FA dynamics (13,16), force transmission (14,17–19), and adhesion strength (20,21). However, the role of morphological changes in modulating cell-ECM force transmission during FA maturation is less certain.

Changes in FA size may occur to modulate changes in the adhesive strength at different levels of tension, such that FA size will be small at low tension and large under high tension. Indeed, in the presence of the activated formin mDia1, FA elongation can occur as a result of myosin-mediated internal force or the application of external force (22). Consistent with this idea, several studies have found a direct correlation between FA size and local traction force, indicating that the force per unit area, or stress, remains constant during FA maturation (23–25). However, other measurements have illustrated a more complex relationship. One study reported an inverse relationship between size and traction stress in the front of migrating cells, but found no such relationship in the cell rear (26). Moreover, it has been shown that small FAs ($\sim 1 \mu\text{m}$) exhibit a widely variable stress (25), and extremely large supermature FAs exert a high stress (24). The published data therefore provide an unclear picture of the relationship between traction force and FA morphology. Such knowledge is critical for our understanding of force transmission at FAs and cellular mechanosensing.

Here we show that these inconsistencies can be resolved if the FA assembly history is considered. We first show that, independently of traction force methodology, no robust correlation exists between FA size and traction force across an entire cell. By utilizing microcontact printing, we find that even similarly sized FAs do not exert a constant stress. Instead, we show that a strong positive correlation between FA size and traction stress persists only during the initial stages of myosin-mediated FA maturation. After this period, the FA size remains constant, whereas the local traction stress can either increase or decrease depending on the proximity of the FA to the cell edge. Finally, by both modulating

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cellular contractility and applying external forces, we show that mature FAs can withstand as much as sixfold increases in their endogenous tension without subsequent changes in size. Together, our data show that the strong correlations between FA size and traction stress occur only during the initial stages of myosin-mediated maturation. These results resolve many of the differences among previous reports and reveal important time- and space-sensitive characteristics of the force-dependent growth of FAs.

MATERIALS AND METHODS

Cell culture

U2OS human osteosarcoma cells (American Type Culture Collection, Manassas, VA) were cultured in McCoy's 5A media (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (HyClone, Waltham, MA), 2 mM L-glutamine (Gibco, Carlsbad, CA), and penicillin-streptomycin (Gibco). National Institutes of Health (NIH) 3T3 fibroblasts (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin. Cells were transfected with plasmid DNA constructs encoding for GFP-actin (a gift of the Gary Borisy laboratory, Northwestern University), mApple-paxillin (a gift of the Mike Davidson laboratory, Florida State University), GFP-talin (a gift of Clare Waterman, NIH), and EGFP-paxillin (27) using the transfection reagents FuGENE 6 or FuGENE HD (Roche, Madison, WI) for U2OS or NIH 3T3 fibroblasts, respectively. After 24 h, the cells were replated onto polyacrylamide (PAA) gels coupled to glass coverslips for 16–20 h. Coverslips were then mounted in a Warner perfusion chamber (Warner Instruments, Hamden, CT) and imaged in culture media supplemented with 10 mM HEPES and 30 μ l/ml Oxyrase (Oxyrase, Mansfield, OH).

PAA substrates for traction force microscopy

PAA substrates containing far-red 40 nm fluorescent microbeads (Invitrogen, Carlsbad, CA) serving as fiducial markers for substrate deformation were prepared on glass coverslips according to previously published methods (28,29). To accommodate differences in the cellular contractility, PAA substrates with shear elastic moduli of 2.8 kPa and 16 kPa were used for traction force microscopy of U2OS and fibroblast cells, respectively. Previous work indicated that these substrates permit the formation of stress fibers and elongated FAs in these cell types (30–33). To create PAA gels with shear elastic moduli of 2.8 kPa and 16 kPa, the weight percentages of the acrylamide/bis-acrylamide used to form the PAA gels were 7.5%/0.1% and 12%/0.15%, respectively (28,31). For the data shown in Figs. 1, *e–h*, and 3–5, fibronectin was coupled to the PAA gels uniformly with the use of sulfo-SANPAH (28,29).

Microcontact printing of PAA gels was performed as described previously (34,35). Briefly, the PAA gel surface was activated by incubation in pure hydrazine hydrate (Sigma) overnight for 16 h, followed by a 1-h incubation in 5% acetic acid (Fisher Scientific, Waltham, MA) and a 1-h wash in double-distilled water. A 10 μ g/mL fibronectin (Sigma) solution was prepared in sodium acetate buffer (pH 4.5), and oxidation of carbohydryl groups on the fibronectin was achieved by addition of 40 μ g/ml sodium meta-periodate (Thermo Scientific, Waltham, MA) and incubation at room temperature for 30 min. For the data shown in Fig. 1, *a–d*, and Fig. 6, *e–g*, oxidized fibronectin solution was pipetted directly onto the gel surface and incubated for 1 h. For microcontact printing, the top surface of a polydimethylsiloxane stamp containing 1- μ m-diameter circular features separated by 3 μ m center to center (a gift of Kweku Addae-Mensah, Columbia University) was immersed in the fibronectin solution for 1 h, after which the stamp was dried with a stream of lab air. Excess water was

removed from the PAA gel by an in-house-built coverslip spinner, and the polydimethylsiloxane stamp was pressed gently onto the gel for 90 s. After fibronectin coupling was achieved, the PAA gels were washed thoroughly in phosphate-buffered saline (PBS) and sterilized for 1 h under the UV-lamp of a tissue culture hood.

Live cell imaging

Cells adhered to PAA substrates were imaged in a perfusion chamber at 37°C on a multispectral spinning disk confocal microscope consisting of a Ti-E microscope body (Nikon, Tokyo, Japan), 60 $\times$ 1.2 NA Plan Apo WI objective (Nikon), CSU-X confocal scan head (Yokogawa, Tokyo, Japan), and an HQ2 cooled CCD camera (Roper Scientific, Sarasota, FL) controlled with Metamorph acquisition software (MDS Analytical Technologies, Sunnyvale, CA).

For blebbistatin (Sigma) washout experiments, cells were mounted as above and 50 μ M of blebbistatin-containing media was perfused into the chamber and incubated for 30 min. Fresh media was then perfused into the chamber, and imaging of beads and fluorescent proteins commenced. For calyculin (Sigma) experiments, cells were mounted as above and 5 nM calyculin A-containing media was perfused into the chamber after 10 min of imaging in normal media. To obtain an image of the cell-free bead positions for traction force analysis, 0.05% trypsin (Gibco) was perfused into the cell chamber to detach cells from the PAA substrate after completion of time-lapse imaging. The experimental methodology we used is described elsewhere (28). Figs. 1 and 2 reflect data obtained from a single time point in a time-lapse sequence. All other data reflect data obtained over the time series indicated.

Displacement analysis and force reconstruction

The methods we used for traction force microscopy have been previously described (29,32,35). Briefly, we aligned images of fluorescent beads embedded in the PAA gel to compensate for experimental drift, and calculated the bead displacement field between pairs of images by comparing the unstrained bead images obtained after the cell had been removed with images obtained with an attached cell. We calculated the displacement fields with the use of particle imaging velocimetry (PIV) software in MATLAB (The MathWorks, Natick, MA; available at <http://www.oceanwave.jp/software/mpiv/>), using the minimum quadratic differences algorithm, which calculates the shift necessary to produce the minimum cross-correlation coefficient between a small region of the experiment image and the reference image. We found higher-resolution displacements iteratively by computing the displacement in a smaller grid spacing using information from the previous computations to filter noisy and spurious displacement vectors. We then filtered and interpolated the displacement vectors using the kriging interpolation method, with a displacement grid size of 0.86 μ m.

After obtaining the displacement data, we used Fourier transform traction cytometry (FTTC) (36) and traction reconstruction with point forces (TRPF) to estimate the traction stress and force at FAs (29). The FTTC method uses a discrete computational mesh to compute the stress as a function of position on the image. This method solves the problem of reconstructing the stress from given displacement data by assuming that the substrate is an infinite elastic half-space and converting the problem into Fourier space, where it is more easily solved. The solution is transformed back to real space, and zeroth-order regularization is then used. This has been shown to yield traction force measurements consistent with the boundary element method (29). The regularization parameters remained constant for all data sets directly compared.

To experimentally estimate the error in traction stress measurement, we imaged cells at 1-s intervals and computed the FTTC traction stress field. We then measured the standard deviation in the traction stress magnitude at individual grid points over 20 s, a timescale in which forces are not expected to vary significantly. The average standard deviation was found to be 20 Pa for cells plated on 2.8 kPa gels, and was independent of the magnitude of the traction stress.

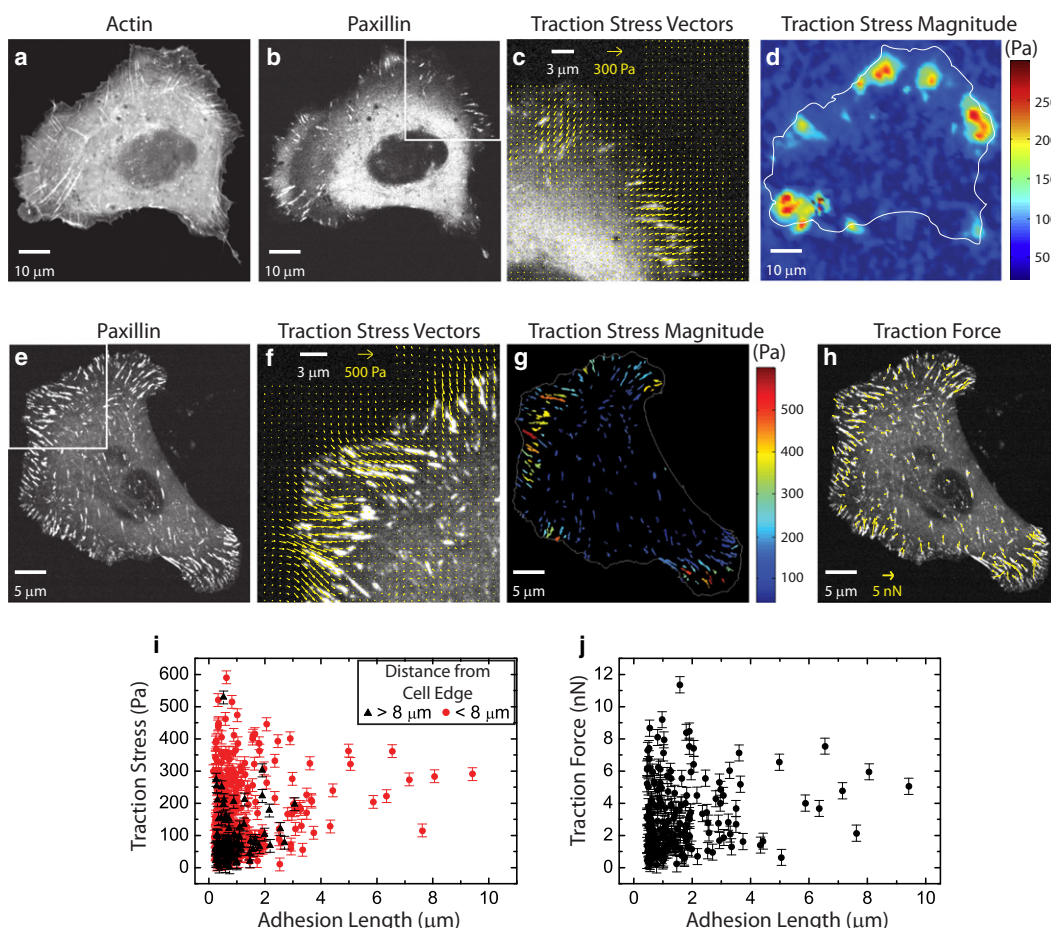


FIGURE 1 (a–d) Images of (a) GFP-actin, (b) mApple-paxillin, (c) traction stress vectors of the white boxed region from b, and (d) heat map of traction stress magnitude of U2OS cells plated on uniformly coated PAA gel. (e–h) Images of (e) mApple-paxillin in U2OS cells, (f) traction stress vectors of the white boxed region from panel e, (g) average stress in segmented FA, and (h) traction forces at FA. (i) Plot of FA length versus traction stress, calculated with FTTC, with color sorting for $<8 \mu\text{m}$ and $>8 \mu\text{m}$ away from the cell edge. (j) Plot of FA length versus traction force, calculated with TRPF. The data set in panels i and j contains 256 FAs from three cells. All traction force data in Fig. 1 were obtained on 2.8 kPa PAA gels.

The TRPF method relies on user-supplied points where high traction force is expected, as identified by a fluorescently tagged FA protein (37). The traction force at each point is computed assuming that the displacement field arises only from forces at the given points. The same regularization scheme as in FTTC is also used here to suppress noise-dominated, high-frequency contributions to the result.

Image analysis

For the static cells, we converted FA images into a binary mask by intensity thresholding of GFP- or mApple-paxillin images, and analyzed them using MATLAB (The MathWorks, Natick, MA) to extract the FA lengths and centroids. We then determined the stress at the FA centroids by interpolating traction stress vectors using a Gaussian weight function. Correlation coefficients cited in the text are the Pearson sample correlation coefficients.

Micropipette manipulation

For the micropipette manipulation experiments, cells were transfected and plated as described above. The coverslips were then mounted in an open-air imaging chamber and placed on the stage of a multispectral wide-field epifluorescence microscope consisting of a Nikon Ti-E microscope body,

60 $\times$ 1.2 NA Plan Apo WI objective and an HQ2 cooled CCD camera controlled with Metamorph acquisition software.

Micropipette needles were loaded into the manipulation arm of a motorized micropipette manipulator (MP-285; Sutter Instruments, Novato, CA). The tip of the needle was located and placed near the surface of the substrate, inserted $\sim 5 \mu\text{m}$ deep into the gel in the vicinity of an FA, and displaced in 5 μm steps up to a maximum of 20 μm . At each step, an image of the paxillin and beads was obtained. The total time for the pull was ~ 10 s. After imaging, 0.05% trypsin was added to the cell chamber to detach cells from the PAA substrate, and an image of the cell-free bead positions was obtained for analysis.

RESULTS

FAs exert varied levels of stress on the ECM

Previous studies have reported disparate relationships between FA size and local traction force, with cell migratory state, cell type, and traction force methodology providing potential variables to account for these discrepancies (23,25,26). To identify inconsistencies that might arise from using different traction force methodologies, we used

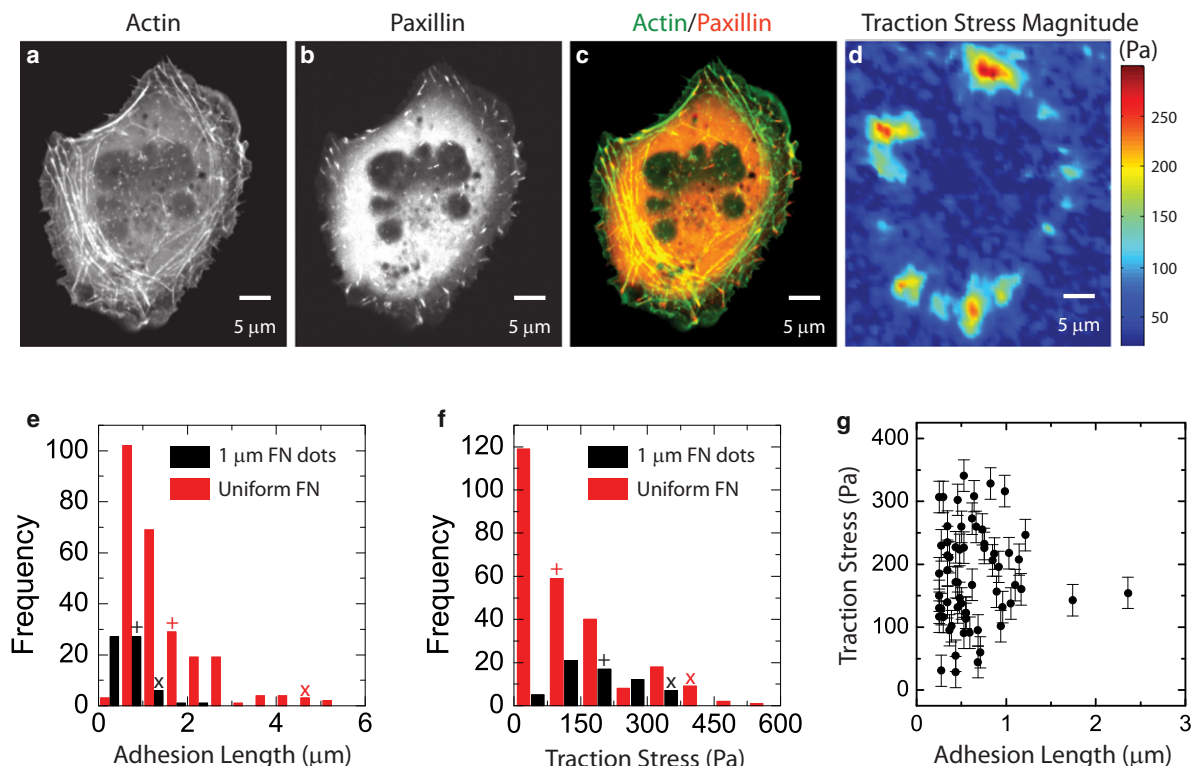


FIGURE 2 (a–d) Images of (a) GFP-actin, (b) mApple-paxillin, (c) color combined actin and paxillin, and (d) heat map of traction stress magnitude of U2OS cell on a micropatterned substrate. (e) Histogram of FA length on uniformly coated FN gels and gels micropatterned with 1 μm dots of FN. The bin size for both data sets is 0.25 μm . The mean and 98% quantile are indicated by the plus sign and cross, respectively. On patterned FN, the mean and 98% quantile of FA length are 0.63 μm and 1.17 μm , respectively. On uniform FN, the mean and 98% quantile of FA length are 1.74 μm and 4.52 μm , respectively. (f) Histogram of stresses at FAs on control and patterned substrates. The bin size for both data sets is 75 Pa. The mean and 98% quantile are indicated by + and $\times$, respectively. On patterned FN, the mean and 98% quantile of traction stress are 179 Pa and 328 Pa, respectively. On uniform FN, the mean and 98% quantile are 125 Pa and 420 Pa, respectively. (g) Plot of FA length versus traction stress for cells on FN dots. The data set contains 62 FA from four cells. All traction force data in Fig. 2 were obtained on 2.8 kPa PAA gels.

two algorithms to reconstruct cell traction: 1), FTTC with zeroth-order regularization; and 2), TRPF. The FTTC method was chosen for its computational efficiency and because it yields results consistent with the boundary element method (29), which was used previously (26). TRPF has the advantage of not being affected by undersampling effects (35), whereas with FTTC, undersampling effects can result in an underestimation of forces exerted at small FAs (29).

U2OS human osteosarcoma cells were transfected with GFP-actin and mApple-paxillin to visualize the actin cytoskeleton and FAs, respectively (Fig. 1, a, b, and e). FAs were identified by creating a binary mask from mApple-paxillin images. Each FA was approximated as an ellipse with a short and long axis measuring its width and length, respectively. The widths of FAs were similar across the cell ($\sim 0.5 \mu\text{m}$). By contrast, FA length varied significantly, ranging from $<0.5 \mu\text{m}$ to nearly 10 μm in U2OS cells.

Using FTTC, we calculated the traction stress vector field (Fig. 1, c, d, and f) and determined the traction stress at each FA centroid. The binary mask image with each FA color-mapped to the traction stress determined at the FA centroid

is shown in Fig. 1 g. The traction stress exerted at FAs with lengths $< 4 \mu\text{m}$ varied considerably, from 0 Pa to 600 Pa (Fig. 1 i). These data are consistent with a previous study that reported poor correlations between focal size and traction force for small FAs (25). The largest FAs (4–10 μm) were less variable, ranging from 200 to 350 Pa (Fig. 1 i).

We calculated the force exerted at each FA centroid using TRPF (23) (Fig. 1 h). Consistent with the data obtained with FTTC, no strong correlations between traction force and FA size were observed (Fig. 1 j). Thus, the force reconstruction algorithm did not qualitatively change the distribution of forces generated at FAs.

Previous data indicated that a strong correlation between traction stress and FA size may exist only near the cell periphery (38). To isolate peripheral FAs, we calculated the distance of the FA centroid from the cell edge. Consistent with these results, we found that central FAs located more than 8 μm from the cell periphery were typically smaller ($< 2 \mu\text{m}$ long) and exerted low traction stress (< 200 Pa; Fig. 1 i, black triangles, and Fig. S1 in the Supporting Material). By contrast, we found that the traction stress exerted by FAs within 8 μm of the cell edge varied

widely, ranging from 50 Pa to 600 Pa, and showed no strong correlation between length and stress (Fig. 1 *i*, red circles, and Fig. S1). Thus, although our data recapitulate previous results regarding the variability of forces exerted at small FAs (25) and weak forces exerted by central FAs (38), we observed no strong correlation between FA size and local traction force across the cell.

Well-separated FAs of similar size exert varied levels of stress

One difficulty in assigning traction stresses to individual FAs on uniform substrates is that they can occur within close proximity to each other ($\sim 0.5 \mu\text{m}$), below the spatial resolution of the traction stress reconstruction algorithms (29,35). To create a population of well-separated FAs with similar sizes, we used a micropatterned traction force substrate containing an array of $1 \mu\text{m}$ dots of fibronectin with a center-to-center spacing of $3 \mu\text{m}$. On these substrates, the FAs were constrained to $<1 \mu\text{m}$ in length and were found only near the cell periphery where actin bundles terminated (Fig. 2, *a–c*, and *e*) (35). The mean traction stress exerted at constrained adhesions was similar to those of control cells, ~ 150 Pa (Fig. 2, *d, f*, and *g*). By contrast, the 98% quantile of traction stresses exerted on the patterned substrate was significantly lower, ~ 330 Pa, as compared with ~ 420 Pa for control cells (Fig. 2, *e* and *f*).

For cells plated on patterned substrates, we found that the traction stresses exerted by FAs varied widely, from 20 to 300 Pa, which is significantly larger than our measurement error of 20 Pa. Similar to results obtained with unconstrained FAs, no correlation between FA length and traction stress was observed (Fig. 2 *g*). Thus, even in cells whose FAs were well separated to enable clear resolution of traction stress at individual adhesions near the cell periphery, the stress exerted at FAs of similar size was highly variable.

FA length is proportional to traction stress during initial stages of maturation

Our measurements yielded no robust correlations between traction force and size in peripheral FAs. To explore whether such correlations depended on the age or maturation state of the FA, we used time-lapse imaging to observe changes in traction stress during the assembly and maturation of newly formed adhesions near the leading cell edge. For these experiments, we used NIH 3T3 fibroblasts because they exhibit highly protrusive behavior and thus create many new adhesions.

Near the cell periphery, small paxillin-rich puncta appeared and elongated over a period of 5 min (Fig. 3 *a*, red ellipse), a timescale consistent with previous measurements of FA assembly (5,27). Subsequently, the length of FAs remained stable for at least an additional 5 min (Fig. 3 *b*). As the FA elongated from 0.6 to $1.7 \mu\text{m}$, the local traction

stress exerted increased from 200 Pa to ~ 600 Pa (Fig. 3, *a* and *b*). Once the FA length stabilized, the traction stress continued to increase to 900 Pa over the next ~ 10 min.

During FA assembly, a strong correlation between adhesion length and traction stress was observed (Fig. 3 *c*, top; $r = 0.73$). By contrast, a poor correlation was observed in stable FAs, as the traction stress typically continued to increase without further changes in FA size (Fig. 3 *c*, bottom; $r = 0.30$). Thus, the length of FAs is directly proportional to the traction stress they exert during FA assembly; however, the correlation diminishes thereafter.

A strong correlation between FA size and traction stress is observed during the initial stages of myosin-II mediated maturation

To study numerous newly forming FAs simultaneously, and to elucidate the role of myosin-mediated contractility in these two stages of force buildup at FAs, we used the pharmacological inhibitor of myosin ATPase activity, blebbistatin (39). U2OS cells expressing an FA marker, GFP-talin, were plated and spread on traction force substrates. After a 30-min incubation in $50 \mu\text{M}$ blebbistatin, FAs appeared as small puncta near the cell periphery, and traction stresses decreased to ~ 60 Pa above background levels (Fig. 4 *a*). Myosin activity was restored by washing out blebbistatin-containing media and rapidly inactivating the remaining blebbistatin by blue-light imaging (32,40). Myosin-mediated FA elongation occurred over the first 4 min of blebbistatin removal and corresponded to an increase in traction stress exerted from 60 Pa to 250 Pa (Fig. 4, *a* and *b*). After this time, the FA length remained constant, whereas the traction stress exerted at the FA continued to increase to a maximum of ~ 450 Pa. Therefore, the dynamics of FA growth and traction stress buildup after blebbistatin washout were similar to those observed in newly assembling FAs near the cell periphery.

We observed a strong correlation between FA growth and traction stress during FA growth (Fig. 4 *c*, top; $r = 0.87$). By contrast, the peak stresses exerted by FAs at later times varied widely and showed no strong correlation (Fig. 4 *c*, bottom; $r = 0.29$). Thus, the strong correlation observed between increased traction stress and FA elongation occurred in the initial stages of myosin-dependent growth and was independent of other mechanisms occurring during cell protrusion.

In rapidly protruding cells, traction stress decreases in large adhesions

Our previous measurements identified a strong correlation between FA size and traction stress growth during assembly of peripheral FAs, but indicated that once the FA length stabilized, the traction stress could continue to increase by as much as 100%. Furthermore, our results also showed

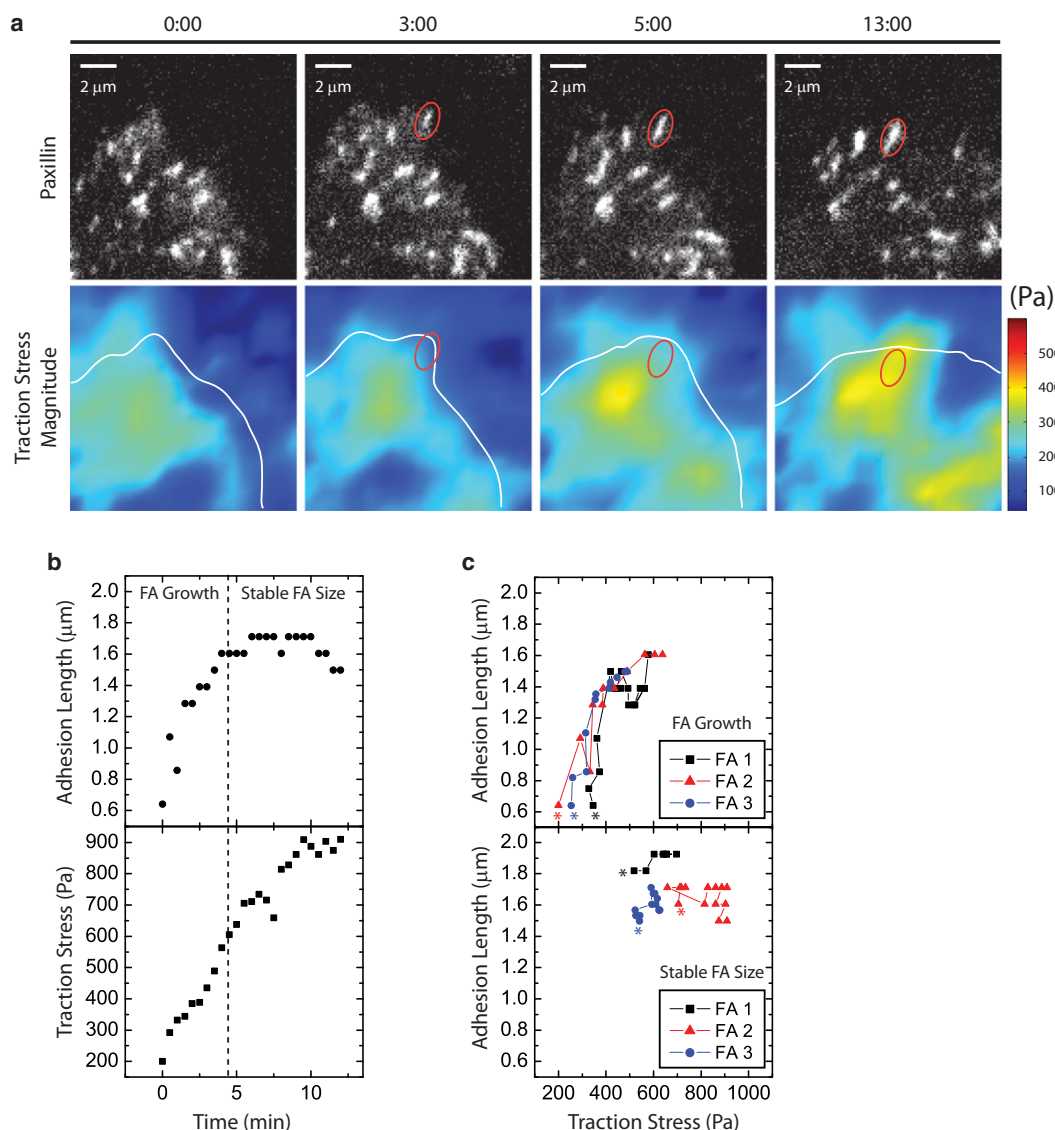


FIGURE 3 (a) Top row: Time course of FA assembly and maturation in an NIH 3T3 fibroblast cell expressing GFP-paxillin. The red outline indicates the formation and elongation of a new FA. Bottom row: Heat map of traction stress magnitude for the same cell, with the red outline indicating the location of the FA in the top row. Time indicated in min:s. (b) Plot of FA length and traction stress as a function of time for a characteristic FA. The dashed line indicates the transition between FA growth and stabilization of FA length. (c) Top: Plot of FA length versus traction stress during FA growth. The line connecting the data points links data points over time, with the asterisk indicating the earliest time. The Pearson correlation coefficient is 0.73 for this data set. Bottom: Plot of FA length versus traction stress while the FA size remains stable. The line connecting the data points indicates the flow of time, with the asterisk showing the earliest time. The Pearson correlation coefficient averaged for each FA is 0.30 for this data set. All traction force data in Fig. 3 were obtained on 16 kPa PAA gels.

that central FAs generated significantly less traction stress (Fig. 1, *g* and *i*, and Fig. S1). Therefore, we examined how the stress exerted at individual FAs changed as the distance between a newly formed FA and the cell edge varied, as is the case in rapidly protruding cells.

We identified rapidly protruding NIH 3T3 fibroblasts where the cell edge advanced at a rate of $\sim 1 \mu\text{m}/\text{min}$. Thus, newly forming FAs that appeared at the cell periphery were localized $10 \mu\text{m}$ from the cell edge 10 min after their assembly (Fig. 5 *a*). In these cells, the timescale of FA elongation was similar to that observed for cells with stationary

cell edges (Figs. 3 *b* and 4 *b*), ~ 5 min. After the initial period of cell protrusion, FA size remained stable for at least 10 min (Fig. 5 *b*).

As in our other experiments, the traction stress exerted by FAs increased during FA elongation (Fig. 5, *a* and *b*). During this time, a strong correlation was observed between FA length and traction stress (Fig. 5 *c*, top; $r = 0.76$). By contrast, after the FA length stabilized, the traction stress at the FA either remained steady or decreased slowly as the cell edge advanced (Fig. 5, *a* and *b*). Thus, a similar lack of correlation between traction stress and changes in

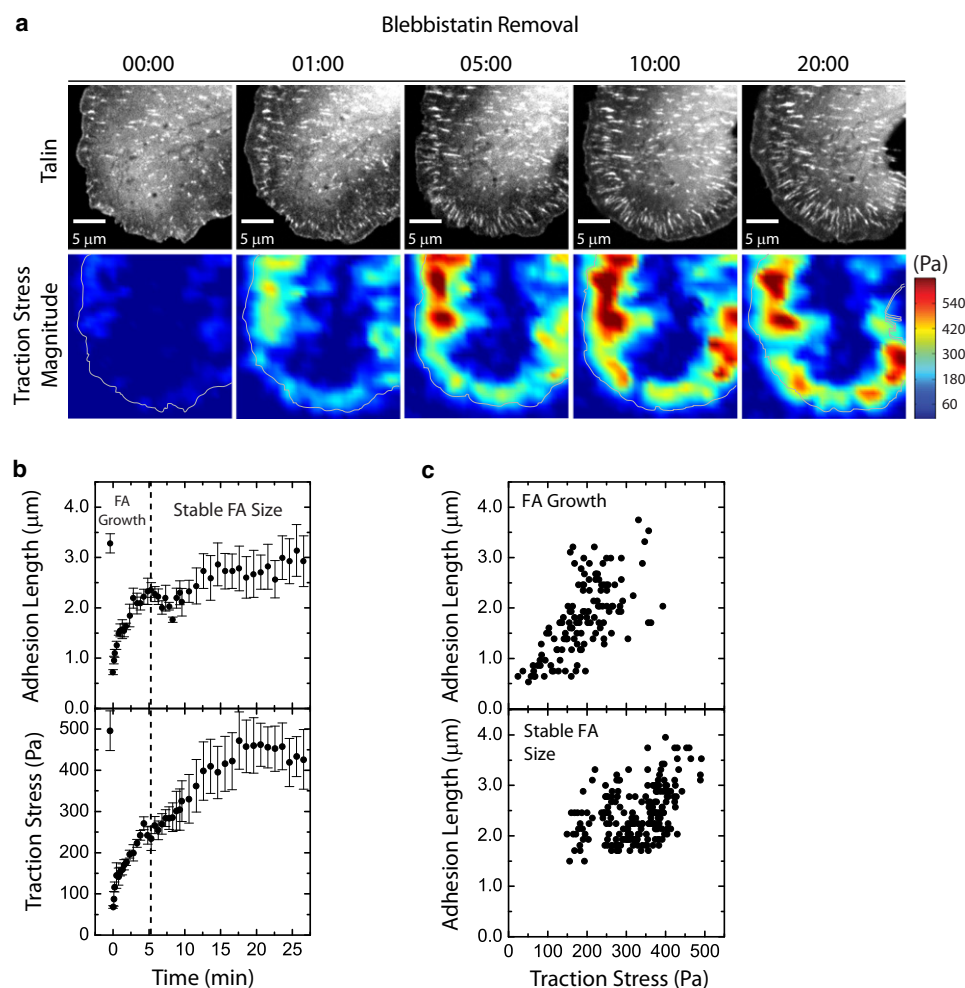


FIGURE 4 (a) Time-lapse images of a U2OS cell expressing GFP-talin (top) and the corresponding traction stress magnitude (bottom) at times after blebbistatin removal and inactivation. Time is indicated in min:s. (b) Plot of FA length and traction stress as a function of time. Data are given as the mean and standard error. The dashed line indicates the transition between FA elongation and stabilization of FA length. The data set contains four FAs from the cell shown in panel *a*. (c) Top: Plot of FA length versus traction stress during FA growth. The Pearson correlation coefficient is 0.87 for this data set. Bottom: Plot of FA length versus traction stress while the FA size remains stable. The Pearson correlation coefficient is 0.29 for this data set. The data set for panel *c* contains nine FAs from four cells. All traction force data in Fig. 4 were obtained on 2.8 kPa PAA gels.

adhesion size was observed in assembled FAs (Fig. 5 *c*, bottom; $r = 0.08$). However, in contrast to FAs in stationary cells, the stress exerted at mature FAs in rapidly protruding cells diminished without a reduction in FA size (Fig. 5 *b*).

These data speak to the dependence on location of the traction force exerted by FAs in different regions of the cell, which can be observed by plotting all the traction force data during FA elongation from Figs. 3 *c* and 5 *c* as a function of distance away from the cell edge (Fig. 5 *d*). As long as the FA remains near the cell edge, the traction stress builds rapidly and remains very high (Fig. 5 *d*). By contrast, the traction stresses increase weakly as the distance between the FAs and the protruding cell edge increases up to 8 μm. When FAs are >8 μm away from the cell edge, the traction stress they exert decreases. We also observed this phenomenon in data at single time points, as FAs farther than 8 μm from the cell edge bear less stress than adhesions closer than 8 μm to the cell edge (Fig. S1). These results suggest that the rate of cell protrusion and location of the FA can markedly affect the force transmitted at FA in a fashion that is independent of the FA size.

FAs can sustain large external loads without further elongation

In models of FA mechanosensitivity, FAs operate near their yield (or breaking) stress such that small changes in applied force will result in the stretching of bonds and promote further accumulation of proteins (7,41). To determine whether mature FAs are maintained near their critical rupture stress, we used a micropipette to apply stresses to FAs of U2OS cells plated on uniform and micropatterned PAA. We then used the micropipette needle to displace the PAA gel and subsequently released it over a period of ~10 s while imaging GFP-paxillin and the beads embedded in the substrate (Fig. 6 *a*). The time traces of two representative mature, elongated FAs show the initial endogenous FA stress and the stress sustained at the FA after each incremental micropipette pull (Fig. 6, *b* and *c*). The FAs did not show any significant immediate change in size on this short timescale. Despite having up to 1200 Pa of external stress and up to 8 times their endogenous stress applied to them (Fig. 6 *d*), the FAs withstood these manipulations without

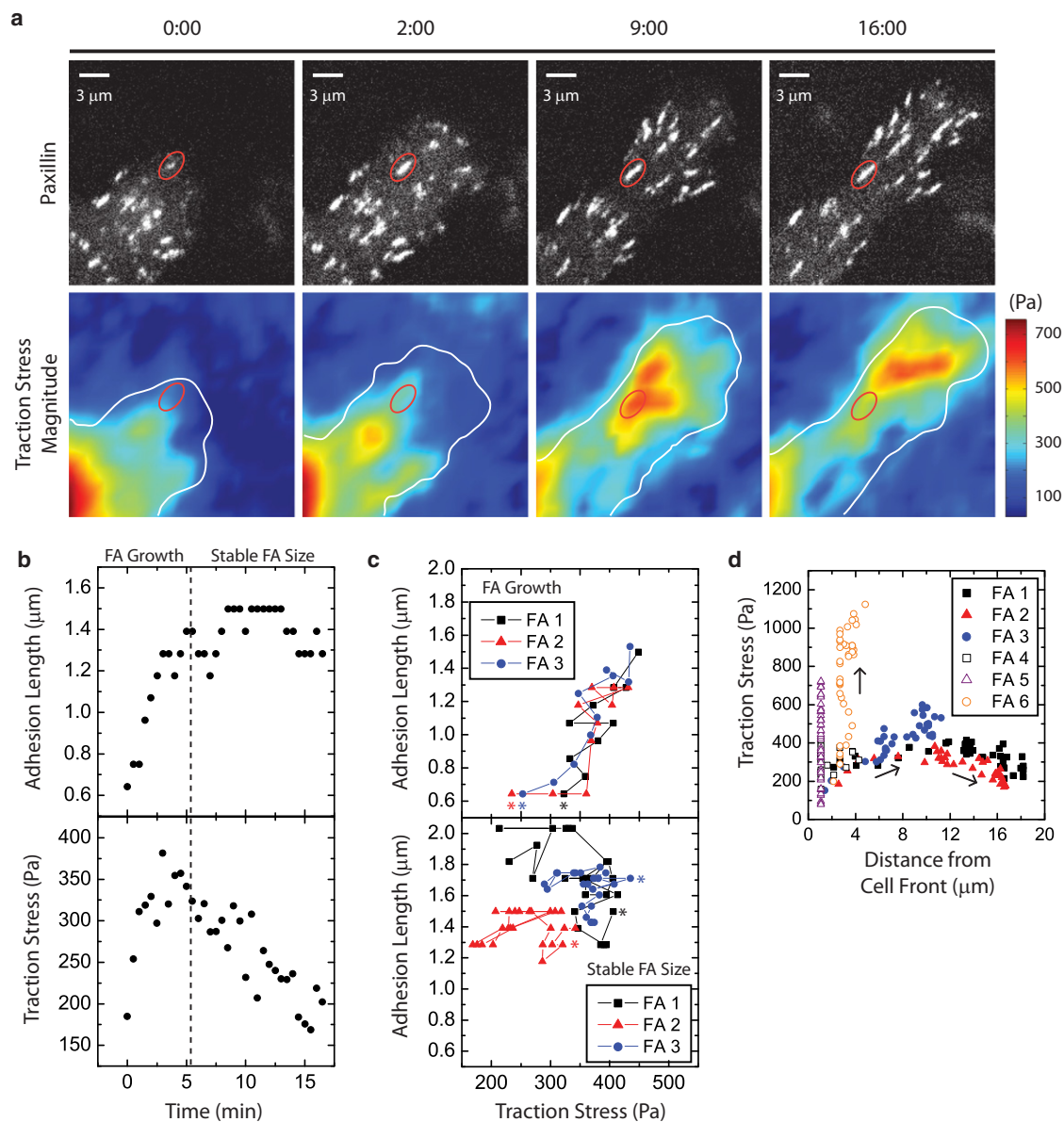


FIGURE 5 (a) Top: Images of rapidly protruding NIH 3T3 cells expressing GFP-paxillin. Bottom: Corresponding heat maps of traction stress magnitude. The red outline indicates the location of an elongating FA. (b) Plot of FA length and traction stress as a function of time for a characteristic FA. The dashed line indicates the transition between FA elongation and stabilization of FA length. (c) Top: Plot of FA length versus traction stress during FA growth. The lines connecting the data points indicate the progression of time, with the asterisks showing the earliest times. The Pearson correlation coefficient is 0.76 for this data set. Bottom: Plot of FA length versus traction stress while the FA size remains stable. The line connecting the data points indicates subsequent time points, with the asterisk showing the earliest time. The Pearson correlation coefficient averaged for each FA is 0.08 for this data set. (d) Data for the FA shown in Figs. 3 c and 5 c, now plotted as a function of distance from the leading cell edge. The arrows indicate the approximate flow of time. All traction force data in Fig. 5 were obtained on 16 kPa PAA gels.

disassembling or retracting from the point of adhesion. Even when the FAs were constrained on micropatterns, the FAs remained stable, indicating that adhesions as small as 1 μm are able to bear considerable stress without further elongation.

To test for changes in internal stress, we stimulated cellular contractility by perfusion of 5 nM of calyculin A to inhibit myosin light chain phosphatase and thus enhance

myosin phosphorylation (42). The addition of calyculin A stimulated an increase in traction stress at large FAs (Fig. 6, e–g), consistent with previous results (43). However, increased stress could occur in the absence of further growth of mature, elongated FAs (Fig. 6, f and g). Together, these results indicate that mature FAs are not near their critical rupture stress, because they are able to withstand very high stresses without changes to their morphology.

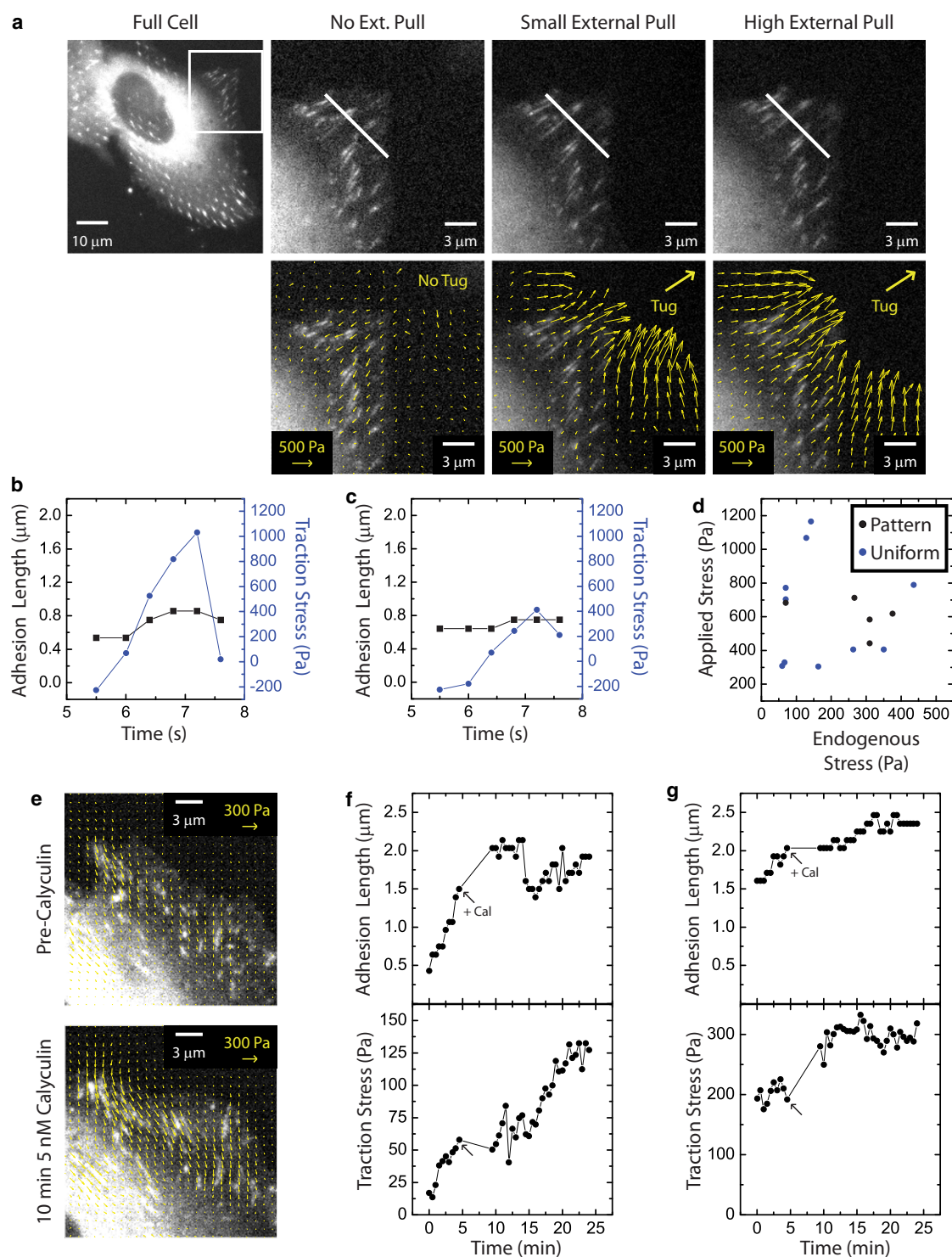


FIGURE 6 (a) Top left: Images of a U2OS cell expressing GFP-paxillin on patterned FN, with the region of analysis indicated by a white square. Top right: Magnified region of the cell during the course of the micropipette pulling experiment, showing a change in the position of the FAs and in the traction stress vectors as the external pull increases. (b and c) Plot of the FA length and traction stress for two characteristic FAs over the course of the micropipette manipulation. The negative stress indicates the cell's endogenous stress directed toward the cell center, and positive values indicate the displacement due to the external pulling directed away from the cell center. (d) Plot of initial endogenous stress versus externally applied stress, showing that FAs can sustain significant stress without failure. (e) Images of a U2OS cell expressing GFP paxillin showing the FAs and traction stress vectors before and 10 min after the addition of 5 nM calyculin. (f and g) Plot of FA length versus traction stress for two characteristic FAs during the course of 5 nM calyculin treatment. The arrow indicates the time at which calyculin was added. All traction force data in Fig. 6 were obtained on 2.8 kPa PAA gels.

DISCUSSION

Our data identify a variable relationship between FA size and traction force during FA assembly in both quiescent and rapidly protruding cells. This data provides a framework for resolving discrepancies found in the current literature, and places spatiotemporal constraints on the mechanosensory nature of FA growth.

At the initial stages of myosin-mediated FA maturation, a strongly positive correlation exists between FA size and traction stress exerted on the ECM (Figs. 3 c and 4 c). Accounting for differences in the absolute magnitude of forces calculated from FTTC and TRPF (35), and taking into account the FA width ($0.5\ \mu\text{m}$), we obtain a slope of $2\text{ nN}/\mu\text{m}^2$ and $4\text{ nN}/\mu\text{m}^2$ for U2OS and NIH3T3 fibroblasts, respectively, which is consistent with previous reports (23,25). This increase in cell-ECM force transmission may result from either myosin-induced changes to the FA composition, which could facilitate more efficient force transmission from the actin cytoskeleton to the ECM, or local remodeling of the F-actin network into a compact bundle, which could alter the spatial distribution of actomyosin forces within the cell. Although FA growth is intimately tied to myosin-mediated FA maturation on a uniform ECM, it is not clear to what extent FA growth is necessary to sustain large tensions at FAs. Our data show that FAs across the cell exert highly variable stress, or force per unit area. This variability is undiminished on micropatterned substrates, where the FAs are well separated and constrained to small sizes. The large variation of forces transmitted at small adhesions that we and others (25,38) have observed suggest that small and large FAs can sustain similar levels of tension. Thus, the steps during FA assembly and maturation by which force transmission to the ECM is modulated can occur in the absence of FA morphological changes. These results indicate that FA size alone is not a predictor of the local tension exerted at the adhesion.

Approximately 5 min after the FAs make their initial appearance, their growth stops and they maintain a near-constant length. When elongated FAs remain within $8\ \mu\text{m}$ of the cell periphery, the force exerted at the FA continues to increase with no further modifications to FA size. By contrast, the force sustained at FAs decreases as they become close to the cell center, consistent with previous data (26,44). Thus, as FAs in rapidly protruding cells become further back in the lamella, the local tension that is sustained decreases. This spatial dependence of FA tension may be a simple mechanical effect of the actomyosin cortex (45,46) or it may be indicative of local regulation of actomyosin contractility (4). Understanding the correlations between tension and FA size during FA disassembly will require further work. However, previous work has shown a strong correlation between diminished traction force and FA size upon treatment with the myosin inhibitor 2,3-butanedione monoxime (23).

Finally, our data indicate that elongated, mature FAs can withstand substantially higher stresses than the endogenous tension applied by actomyosin contractility. This is consistent with adhesion strength measurements indicating that the strength of cell-substrate adhesions is on the order of $\sim 200\text{ nN}$ (21). Thus, after the initial stages of maturation, the FA strength vastly exceeds the level of actomyosin tension exerted locally ($1\text{--}5\text{ nN}$). This calls into question the extent to which the adhesion compliance dominates the force transmission at mature FAs. Current models of force-dependent FA growth propose a compliant FA structure that is sensitive to variable forces (41,48,49). Although these models accurately capture the force-dependent growth of newly assembled FAs, future models should also consider the saturation of FA size and the stability of mature, elongated FA over the large range of tensions.

Altogether, our results demonstrate strict spatial and temporal constraints to the force-dependent growth of FAs. Without knowledge of FA assembly history or location within the cell, FA size is a poor predictor of the degree of tension exerted on the ECM. Moreover, our data indicate that FA growth is not necessary to transmit large traction stresses to the ECM. Because of the interdependent nature of FA elongation, actin bundle formation, and traction force buildup, dissecting out the structural and mechanical aspects underlying FA elongation will require further work. However, such information is necessary to obtain a quantitative understanding of how mechanical signals regulate the morphology and maturation of FAs.

SUPPORTING MATERIAL

A figure is available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(11\)00593-5](http://www.biophysj.org/biophysj/supplemental/S0006-3495(11)00593-5).

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