SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Cell culture and transfection

CHO-K1 cells, which do not express endogenous alpha V or beta 3 integrins (ATCC) and CHO-B2 cells, which do not express endogenous alpha 5 were grown in DMEM-F12 supplemented with 10% FBS. Cells were transfected with either human alpha V and beta 3 (CHO-K1) or alpha 5 and beta 1 (CHO-B2) using a Nucleofector II (Lonza) and Ingenio (Mirus) transfection reagents following manufacturer's protocols. Untagged integrins were in either pcDNA3.1 vectors (alpha v, beta 3, and alpha 5) or pRK5 (beta 1), and tagged vectors (mEos2 or Emerald) were constructed as previously described[1]. The tagged vectors are available through Addgene as mEos2-Alpha-V-integrin-N-25, mEmerald-Alpha-V-integrin-N-25, mEos2-Integrin-Beta3-N-18, mEmerald-Beta3-N-18, mEmerald-Alpha5-Integrin-12, and mEmerald-Beta1-N-18. The untagged alpha v and beta 3 subunits were gifts from Mark Ginsberg (UCSD). Cells were plated on plasma-etched cover glass that had been silanized and coated overnight with either 5μg/ml human plasma fibronectin (CHO-K1) or 10μg/ml human plasma fibronectin (CHO-B2).

Cell Spreading and Integrin Activation Assays

Approximately 24 hrs after transfection, cells were trypsinized and plated for 30, 60, or 90 min for spreading assays or 4 hrs for spreading assays. Cells were then fixed with 2% paraformaldehyde in PHEM[2]. Cells that were transfected with untagged integrins were co-transfected with an empty Emerald vector for visualization of the cell perimeter. For activation, treatment with 0.05mM Mn^{2+} initiated 5 min prior to plating and was maintained throughout the spreading assay[3]. To detect adhesions in cells transfected with untagged integrins, cells were labeled with LM609 (Millipore) prior to secondary labeling with Alexa 488.

To quantify the amount of integrin activation transfected cells were plated overnight prior to fixation and labeling with 9EG7 (BD Pharmigen) prior to secondary labeling with Alexa 647. 9EG7 interacts with high affinity mouse and human beta 1 integrin (Galbraith), binding the ligand-induced binding epitope exposed by treatment with Mn2+ (Bazzoni), 9EG7 does not interact with endogenous hamster integrin. Activation with 0.05mM Mn^{2+} was initiated 1 hr prior to fixation.

Microscopy

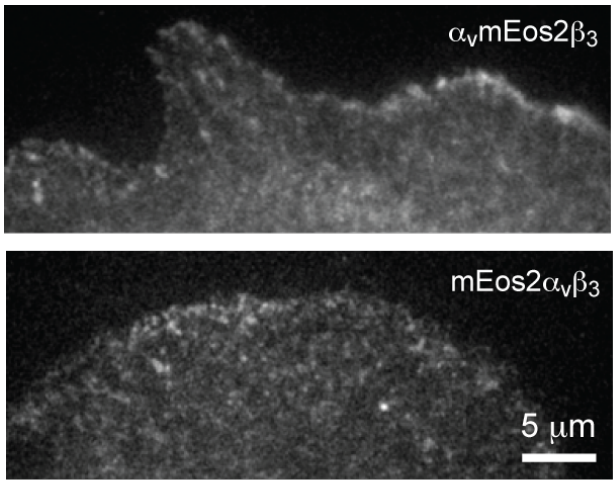
All imaging experiments were performed on an Olympus IX71 with a 60X 1.49 NA objective using TIRF illumination. To create the TIRF beam four laser lines (405, 488, 561, 633 nm) (Coherent) were merged and introduced through free space into the TIRF illumination port of the microscope. Position of the beam in the back aperture of the objective was motorized to ensure repeatability of the penetration depth of the evanescent TIRF wave. For the single molecule experiments a subpopulation of the mEos2 labeled molecules was stochastically excited with a low level of 405 nm activation and 561 nm excitation light (5 μW and 2.5 mW at the back aperture respectively). In these experiments cell edges were identified by collection of an image of unconjugated EGFP. In the case of live cell single-molecule experiments, every 10 sec (400 frames), the excitation light was switched to 488 nm (100 μW at the back aperture) using an acousto-optic tunable filter (AOTF, AA Opto-Electronic). Live cell experiments were imaged at 37° C for a minimum of 5 min and cells did not display any abnormal morphology or decreased motility at the end of this interval. Images were acquired at a final magnification of 111 nm/pixel with an Andor 897 EMCCD camera using an exposure time of 25 ms. For the activation quantification experiments, the laser power was maintained constant for all images.

Image processing, single molecule analysis, and statistics

The cell edge and adhesions were detected by thresholding images in Fiji[4] after smoothing with a 1 pixel Gaussian kernel sigma to reduce noise. Canny edge detection was used on the whole cell images after thresholding to obtain cell contours.

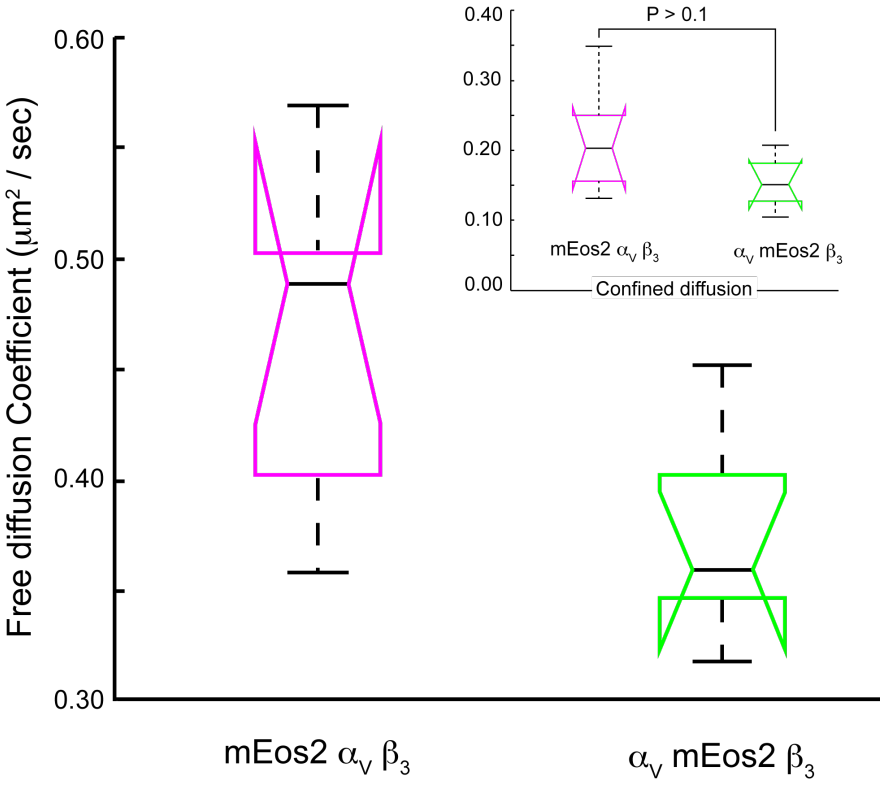
Single molecule analysis was performed using uTrack software[5] to localize and track individual mEos2 integrin molecules. Only molecules localized to better than 25nm precision were used for mobility analysis. Diffusion coefficients for tracks greater than 20 frames were analyzed as previously described and classified as either confined, freely diffusing or undergoing directed movement (i.e. drift)[1,6]. An average of between 6000 and 7000 molecules per cell was analyzed with a minimum of 6 cells per experimental group.

One-way ANOVA analysis was performed on all experimental groups. Sheffer post-hoc multiple comparison test was used to identify which treatments significantly differ from each other.



Supplementary Figure 1:

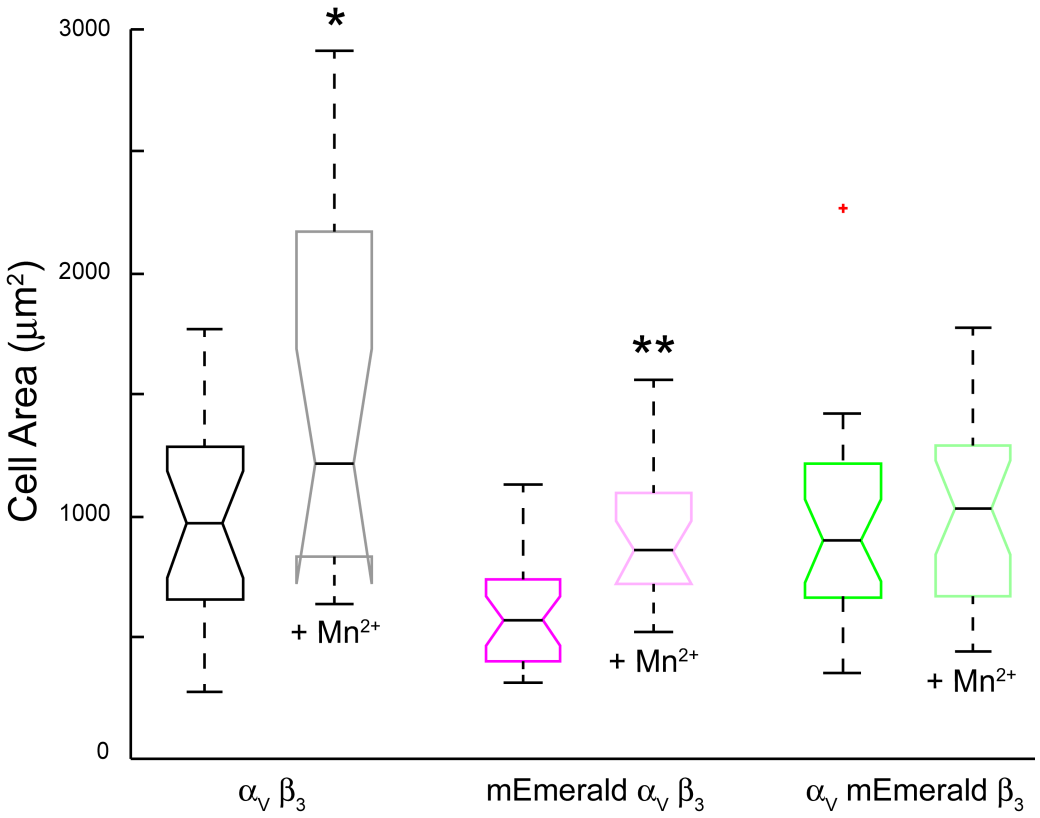
Tagging the Beta Subunit Increases Adhesion Size and Organization. CHO-K1 cells expressing $\alpha_v\beta_3$ integrin with either the beta (top) or the alpha (bottom) subunit tagged. Adhesions are larger and more organized at the leading edge in cells expressing tagged beta subunits.



Supplementary Figure 2:

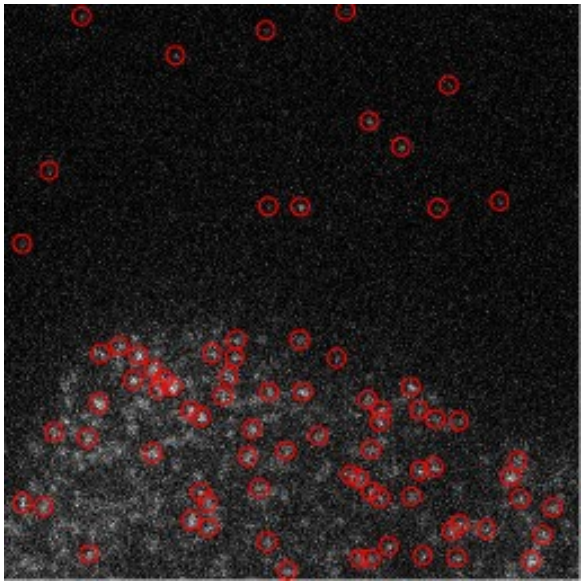
Tagging the Beta Subunit Does Not Change the Mobility of Confined Integrins.

Diffusion analysis derived from single molecule tracking of integrin mobility reveals that diffusion coefficients for unconfined movement are significantly lower for integrins with beta tagged subunits. $P < 0.025$, n=5048, 4164 molecules from N = 6 cells, alpha tagged and beta tagged integrins, respectively. Inset: Diffusion coefficients for integrins showing confined movement (integrins within adhesions) are not statistically different, $p > 0.1$, n=1844, 1323 molecules from N = 6 cells for alpha tagged and beta tagged subunits, respectively.



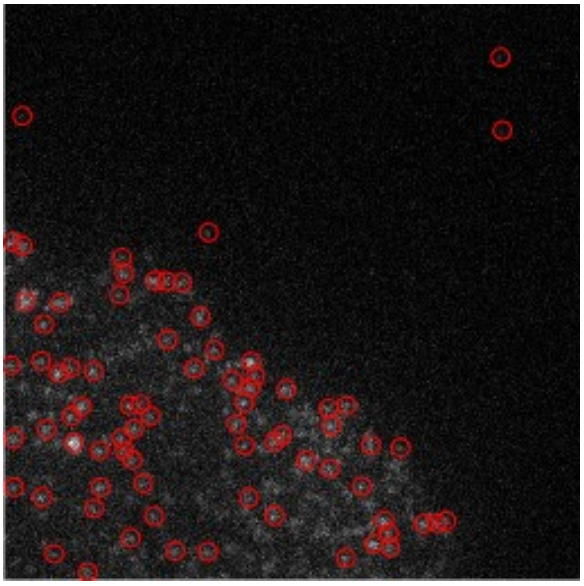
Supplemental Figure 3: Cell Response to Ligand Increases when Beta Subunit is Tagged. Cell spreading increases with integrin activation and expression of integrins with tagged beta subunits. In cells expressing integrins with various combinations of tagged and untagged integrins that were allowed to spread for 90 min, Mn^{2+} treatment yielded the expected increase in cell spreading (untagged: $p<0.03$*, tagged-alpha: $p<0.0005$**), except in cells that were expressing integrins with tagged beta subunits. Cells expressing tagged beta subunits did not increase their area in response to Mn^{2+} treatment ($p>0.5$). N=20, 18, 27, 21, 24, 24 cells for untagged, untagged Mn^{2+}, tagged-alpha, tagged-alpha Mn^{2+}, tagged beta, and tagged-beta Mn^{2+}, respectively.

Supplemental Movie S1



Movie S1. Live cell single molecule video of CHO-K1 cell transfected with mEos2 $\alpha_v\beta_3$ imaged at 25 ms per frame for 2 min (4800 frames). Red circles indicate detected molecules. Interspersed every 10s (400 frames) is a GFP fill which allows determination of the cell outline. Field size – 28.4μm x 28.4μm.

Supplemental Movie S2



Movie S2. Live cell single molecule video of CHO-K1 cell transfected with α_v mEos2 β_3 imaged at 25 ms per frame for 2 min (4800 frames). Red circles indicate detected molecules. Interspersed every 10s (400 frames) is a GFP fill which allows determination of the cell outline. Field size – 28.4μm x 28.4μm

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