Supplemental material

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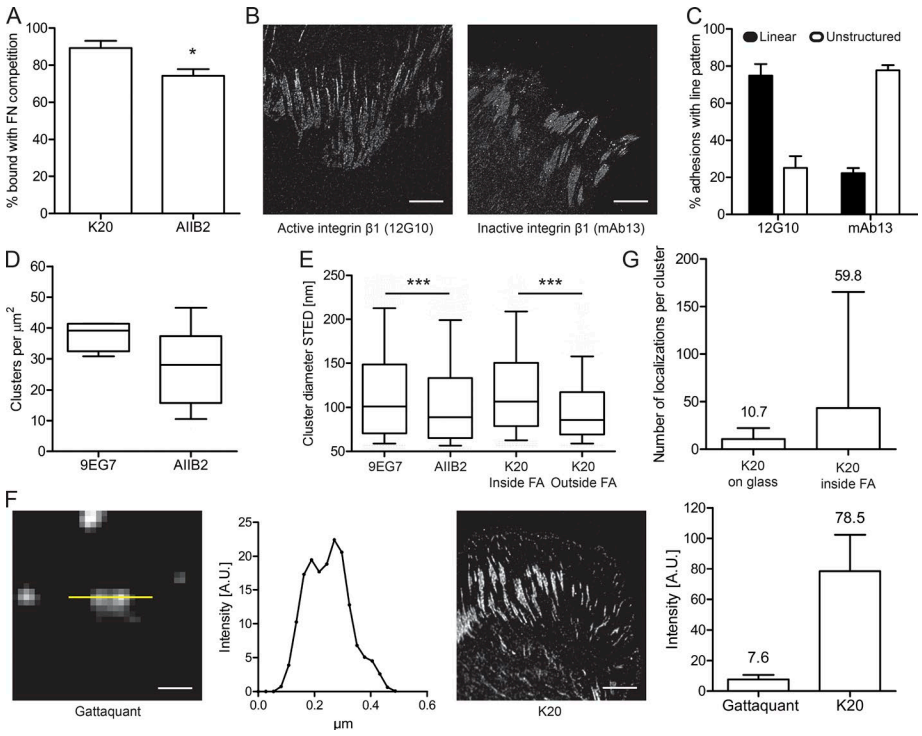

Figure S1. **Ab characterization and colocalization controls. (A)** AIIB2 recognizes a LABS. The percentage of AIIB2 bound to Hs578T cells preincubated with recombinant human fibronectin (FN) domain-10 compared with untreated cells was determined by FACS. The bars show medians ± SEM among four independent experiments. **(B)** Representative STED image of an Hs578T cell stained with anti–β1 (active β1) integrin mAb 12G10 (rhodamine) or anti–β1 (inactive β1) integrin mAb 13. **(C)** Manual scoring of individual adhesions for linear or unstructured patterns. The bars show the mean percentages (± SEM) of FAs displaying linear versus unstructured patterns for stainings using mAbs 12G10 (n = 10 images) and mAb 13 (n = 10 images). **(D)** Cluster density within FAs of STORM images labeled with mAb 9EG7 for active (n = 4 images) and mAb AIIB2 for inactive (n = 13 images) β1 integrins. The bars show the median cluster density with quartile distribution in boxes and whiskers for decile distribution. **(E)** Cluster size of STORM images of mAb 9EG7 (n = 14 images) and mAb AIIB2 (n = 14 images) labeling of active and inactive β1 integrins within FAs as well as mAb K20 labeling of total β1 integrins inside and outside FAs (n = 10 images). The bars show the median cluster size with quartile distribution in boxes and whiskers for decile distribution. t test: *, P ≤ 0.05; ***, P ≤ 0.001. **(F)** Far left: Representative STED image of the customized Gattaquant sample (n = 3 images). Center left: Intensity quantification along the yellow line in the Gattaquant sample. Center right: Representative STED image of an Hs578T cell stained with anti–β1 (total β1) integrin mAb K20 acquired with the same settings used for imaging the Gattaquant sample (OG488 label). Far right: Gattaquant-based STED-detected cluster intensity determination. Intensities of 50-nm-large regions of interest drawn on Gattaquant sample labeling locations of representative STED images were analyzed (n = 120 clusters) and compared with intensities of similarly large regions of interest drawn in representative STED images of K20 cell–staining nanoclusters (n = 120 clusters). Bars, 5 µm. **(G)** The number of localizations per cluster of STORM images of mAb K20 adsorbed on glass (n = 2 images) is smaller than the number of localizations per cluster of STORM images of mAb K20 (n = 6 images)–labeled total β1 integrins within FAs. **(F and G)** Bars show the mean numbers (± SD) with mean values above.

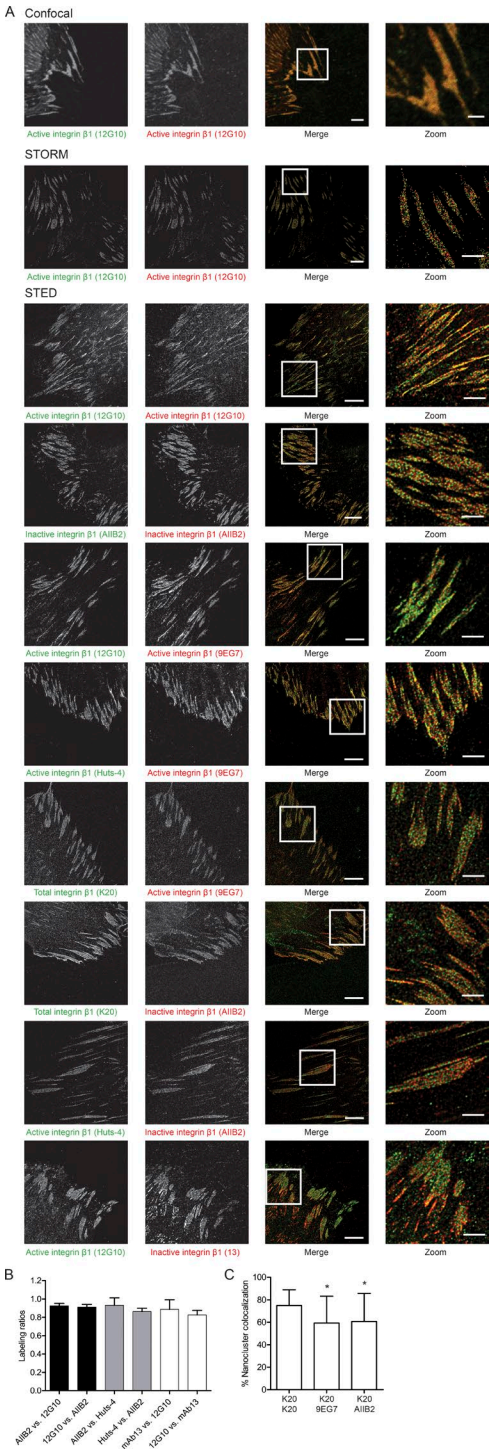

Figure S2. **Total, active, and inactive β1 integrin localization within FAs. (A)** Representative confocal, STED, and STORM images of Hs578T cells stained using mAb 12G10 and two differently colored secondary abs, both targeting mAb 12G10 (top three rows). Next rows: Representative STED images of Hs578T cells costained with anti–β1 (active β1) integrin mAb 12G10 (OG488) and anti–β1 (active β1) integrin mAb 9EG7 (rhodamine); anti–β1 (active β1) integrin mAb Huts-4 (OG488) and anti–β1 (active β1) integrin mAb 9EG7 (rhodamine); anti–β1 (total β1) integrin mAb K20 (OG488) and anti–β1 (active β1) integrin mAb 9EG7 (rhodamine); anti–β1 (total β1) integrin mAb 12G10 (OG488) and anti–β1 (inactive β1) integrin mAb AIIB2 (rhodamine); anti–β1 (active β1) integrin mAb Huts-4 (OG488) and anti–β1 (inactive β1) integrin mAb AIIB2 (rhodamine); and anti–β1 (active β1) integrin mAb 12G10 (OG488) and anti–β1 (inactive β1) integrin mAb 13 (rhodamine). The white boxes indicate the zoomed regions. Bars: (main images) 5 µm; (insets) 2 µm. **(B)** Anti–β1 integrin mAbs used in this study did not compete for ligand binding. The bars show the ratio of the background-corrected median intensities (± SEM; *n* = 3 experiments) of Hs578T cells labeled with the single mAb indicated first under each bar in the presence of the second mAb indicated, divided by the single labeling of the first mAb alone. **(C)** Percentage of overlapping nanoclusters defined by intensity peaks within adhesions in STED images of Hs578T cells costained with mAb K20 and mAb 9EG7 (*n* = 12 images) or mAb AIIB2 (*n* = 8 images). Hs578T cells stained using mAb K20, and two differently colored secondary abs targeting both mAb K20 were used as controls for maximal percentage of overlap (*n* = 9 images). The bars show the mean percentages (± SD). *t* test: *, P ≤ 0.05.

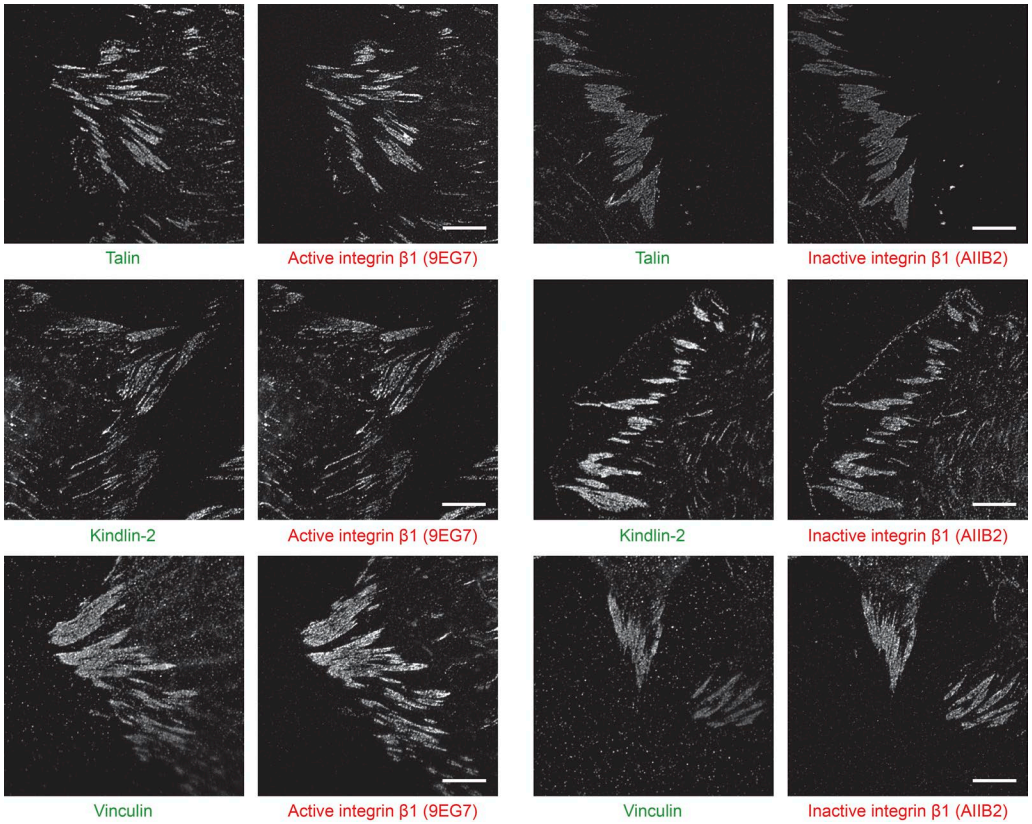

Figure S3. **Individual channels of STED colocalization images.** Left: Representative STED images of Hs578T cells costained with anti–β1 (active β1) integrin mAb 9EG7 (rhodamine) and antitalin mAb (OG488), anti–kindlin-2 polyclonal ab (OG488), and antivinculin polyclonal ab (OG488). Right: Representative STED images of Hs578T cells costained with anti–β1 (inactive β1) integrin mAb AIIB2 (rhodamine) and antitalin mAb (OG488), anti–kindlin-2 polyclonal ab (OG488), and antivinculin polyclonal ab (OG488). Bars, 5 µm.