SUPPLEMENTARY FIGURES LEGENDS

Video1 (Figure 1c). Changes in cell morphology induced by engaging the uPAR/VN-interaction. 293 cells expressing $uPAR^{T54A}$ (293/$uPAR^{T54A}$) were treated with uPA (at time 2:30). Time-lapse phase contrast images were acquired for 12 minutes (4 frames per minute).

Video2 (Supplementary Figure 3b). α_5 integrin localisation during uPAR-mediated cell spreading. 293 cells expressing $uPAR^{T54A}$ were transiently transfected with GFP tagged α_5 integrin. Cells were treated with uPA (at time 0). TIR-FM time-lapse images were acquired for 20 minutes (3 frames per minute).

Supplementary Figure 1. (**a**) Real-time cell analysis experiment on 293 cells expressing different uPAR-variants seeded in a VN-coated E-Plate. Cells were plated on VN coated wells and treated at the indicated time point (vertical stripped line) with uPA. Cell adhesion was followed over time by recording the changes in electrical impedance. Curves represent the average cell index as a function of time from a representative experiment. (**b, c**) uPAR-induced phosphorylation of p130Cas and MAPK. Phosphorylated p130Cas **(b)** and ERK1/2 **(c)** in cells expressing uPAR variants stimulated with uPA for 30 min. Graphs show the quantified results from independent experiments merged by setting the phosphorylation ratio in uPA treated $uPAR^{WT}$ cells to 100% (means ± SEM, $n \geq 3$) . Representative blots are shown below the graphs. **(d)** Effect of inhibitory integrin antibodies on the adhesive properties of 293/$uPAR^{T54A}$ cells. Adhesion of 293/$uPAR^{T54A}$ cells treated with function blocking antibodies against $\alpha_v\beta_5$, $\alpha_5\beta_1$, or two different allosteric inhibitory antibodies against β_1 on VN (open columns), VN^{RAD} with uPA (grey columns) or FN (dotted columns)

for 30 minutes. The adhesion is presented in percentage of adhesion in the absence of antibody (means ± SEM, n = 3). **(e)** The uPAR/VN-interaction does not alter the surface expression of integrins. Cells expressing $uPAR^{T54A}$ were stimulated with uPA for 30 minutes and the surface expression of different integrins was analysed by flow cytometry. Representative histogram plots are shown. As negative control cells were incubated with secondary antibody only (Neg. Ctrl.).

Supplementary Figure 2. (**a, b**) The kringle domain of uPA is not required for the induction of p130Cas phosphorylation and cell spreading on VN^{RAD}. **(a)** $293/uPAR^{T54A}$ cells were seeded on VN^{RAD} in the absence or presence of 10 nM uPA or 10 nM growth factor-like domain (GFD). After a 30 minutes incubation cells were lysed and the levels of phosphorylated p130Cas, total p130Cas and vinculin determined by immunoblotting. The data represent technical triplicates (separate wells of cells) from a single experiment. Western blot data are represented as percentage of phosphorylated p130Cas SD of cells treated with uPA on VN (mean ± SEM). **(b)** For the analysis of cell spreading, $293/uPAR^{T54A}$ cells were seeded on VN^{RAD} in the presence of GFD, uPA or uPA plus Blebbistatin and cell matrix contact areas quantified. The indicated cell spreading represents data pooled from two independent experiments. The grey bars represent the range of cell area of not spread and fully spread $uPAR^{T54A}$ cells, respectively (see Fig. 2b.). Cell-matrix contact area measurements represent at least 50 cells with indicated geometrical means ± 95% CI, in two independent experiments. **(c)** uPAR-mediated VN adhesion increases the spreading of CHO cells. Where indicated, cells were stimulated for 30 minutes with uPA. Cell-matrix contact areas were assayed and quantified. The figure shows contact areas of individual cells with indicated geometric mean and 95% CI. Cells do not

adhere to VN^{RAD} in the absence of uPA and the quantification of cells spreading is therefore not applicable (n.a.). **(d, e)** Ligand-independent integrin signalling requires SRC kinase activity. p130Cas phosphorylation **(d)** and cell spreading **(e)** were assayed and quantified in 293 $uPAR^{T54A}$ cells pre-treated with Src inhibitors (PP1 and PP2) or MEK inhibitor (UO126) seeded on VN^{RAD} in presence of uPA for 30 minutes. Phosphorylation of p130Cas SD was quantified as described above with the level observed in cells seeded on VN^{RAD} with uPA in the absence of inhibitor set to 100% (n = 3, mean ± SEM). The cell area measurements represent at least 50 cells with indicated geometrical means ± 95% CI, in two independent experiments. The grey bars represent the range of cell area of not spread or fully spread $uPAR^{T54A}$ cells based on 95% confidence intervals on poly-D-lysine or on VN with uPA respectively (Fig. 2b).

Supplementary Figure 3. **(a)** MDA-MB-231 spreading on an anti-uPAR antibody is impaired by β_1 integrin inhibition. MDA-MB-231 cells were seeded on the indicated substrate in presence or absence of 4B4 for 30 minutes. Cell-matrix contact area was assayed and quantified. The figure shows the contact areas of individual cells and data are presented as geometric means with 95% CI. **(b)** Localisation of uPAR and β_1 integrin in MDA-MB-231 cells. MDA-MB-231 cells were plated on FN or VN and stained for uPAR (green) and β_1 integrin (red). Representative TIRF-FM images are shown. Scale bar: 10 mm (whole image), 5 mm (insert).

Supplementary Figure 4. **(a)** $\beta_1$integrin localisation during uPAR-mediated cell spreading on VN variants with intact or destroyed integrin binding site. 293 uPAR^{T54A} were seeded on the indicated substrate in presence of uPA for 30 minutes and stained with K20 antibody ($\beta_1$integrin: green) and actin (red). TIR-FM images are shown. Scale bar: 10 μm. **(b)** α_5 integrin localisation during uPAR-mediated cell spreading. Selected frames from a TIR-FM timelapse (Supplementary Movie 2) of 293 uPAR^{T54A} transiently transfected with GFP-tagged α_5 integrin and stimulated with uPA (time 0 min). Scale bar: 10 μm. **(c)** α_5 integrin is required for uPAR-induced cell spreading. 293 uPAR^{T54A} cells were transfected with the indicated siRNA and plated on VN^{RAD} in presence of uPA for 30 minutes or analysed for α_5 integrin expression by flow cytometry. Cell area measurement data represents at least 50 cells for each condition (two independent experiments, geometric means ± 95CI). A representative histogram plot for knock-down efficiency is shown.

Supplementary Figure 5. **(a)** The K218A substitution renders β_1 integrin insensitive to inhibition by 4B4. 293 cells transfected with empty vector (mock), β_1^{WT} (WT) and β_1^{K218A} (K218A) were allowed to adhere to FN for 30 min in the absence (no) or presence of the β_1 allosteric inhibitory antibodies 4B4 or mAb13. Specific cell adhesion is shown in % of adhesion to PDL (n = 3, mean ± SEM). (**b**) FACS analysis of β_1 expression in 239/uPAR^{T54A} cells engineered to express different β_1 integrin variants. Cells expressing the indicated integrin mutant were stained with 4B4 antibody and analysed by flow cytometry. Integrins carrying the K218A substitution are not recognised by mAb 4B4 and effective competition with endogenous β_1 therefore results in a reduction in surface staining with this antibody. Representative histogram plots are shown. As negative control cells were incubated with secondary

antibody only (Neg. Ctrl.). The reduction in 4B4 staining (mean fluorescence intensity ± standard deviation, n = 2) observed upon overexpression of the different integrin mutant are: β_1^{K218} (45 ± 5%), $\beta_1^{K218A/D130A}$ (50 ± 5%), $\beta_1^{K218/Y2A}$ (66 ± 1%) and $\beta_1^{K218A/Y2F}$ (49 ± 6%). **(c)** Real-time cell analysis experiment on 293 cells expressing different uPAR^{T54A} and selected β_1 variants seeded in VN^{RAD}, FN or poly-D-lysine coated wells. Cells were seeded on the indicated substrate and cell adhesion was followed over time by recording the changes in electrical impedance. After an initial adhesion phase of ~ 2 hours (vertical stripped line) the E-plate was removed form the instrument and cell seeded on VN^{RAD} were treated with uPA (solid line) or vehicle (stripped line). Wells coated with poly-D-lysine and FN were left untreated. The curves represent the average cell index and are from a representative experiment (each condition was analysed in triplicates). **(d)** Talin depletion impairs uPAR-mediated cell spreading. Cells were transfected with the indicated siRNA oligos, plated on VN^{RAD} for 30 minutes and cell spreading was determined. Cell area measurement data represents 50 cells for each condition (a representative experiment is shown, geometric means ± 95CI). The grey bars represent the range of cell area of not spread or fully spread uPAR^{T54A} cells based on 95% confidence intervals on poly-D-lysine or on VN with uPA respectively (Fig. 2b). Western blots for knock-down efficiency are shown.

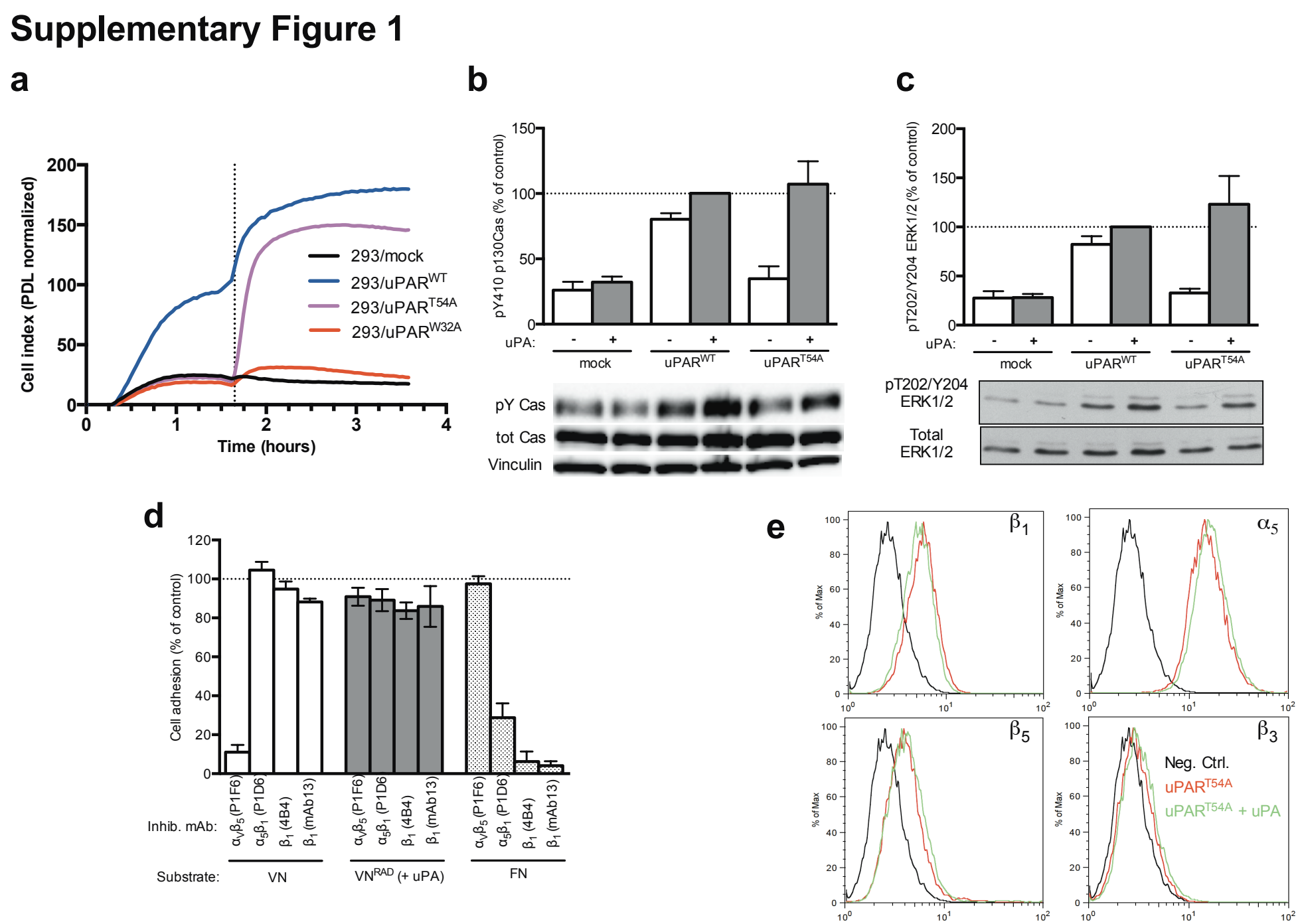
Supplementary Figure 6. **(a)** FACS analysis of β_3 integrin expression in 293/uPAR^{T54A} cells transfected with different β_3 integrin variants. Cells expressing the indicated β_3 integrin mutant were stained with LM609 antibody and analysed by flow cytometry. Representative histogram plots are shown. **(b)** Expression of a GPI-anchored PAI-1 (PAI-1/GPI) induces cell adhesion to VN. 293 cells expressing an engineered cell

surface VN receptor (PAI-1^{WT}/GPI), or a VN binding incompetent variant of the same receptor (PAI-1^{VN-}/GPI), were seeded on VN^{RAD} in the presence or absence of 4B4. Adhesion was quantified and is shown in percentage of adhesion to poly-D-lysine (n = 3, mean ± SEM). **(c)** The type of anchorage to the plasma membrane does not affect uPAR-induced VN adhesion. 293 cells expressing the ecto-domain of $uPAR^{T54A}$ fused to different GPI-anchoring signals (GPI-anchored) or transmembrane segments (TM-anchored) were assayed for cell adhesion on VN^{RAD} in the presence of uPA. The data is presented in percentage of the adhesion of cells expressing uPAR with its native GPI-anchoring signal (n = 3, mean ± SEM). **(d)** The type of membrane anchorage to the plasma membrane does not affect uPAR-induced signalling and spreading. Cell spreading (dots) and p130Cas SD phosphorylation (columns) of 293 cells expressing the indicated $uPAR^{T54A}$ chimeras were assayed and quantified. Cell spreading (50 cells, n = 2, geometric means, ± 95CI) and p130Cas phosphorylation expressed in percentage of the levels observed in $uPAR^{T54A}$ cells with WT GPI-anchor (n = 3, means ± SEM) are shown. The grey bars represent the range of cell area of not spread or fully spread $uPAR^{T54A}$ cells based on 95% confidence intervals on poly-D-lysine or on VN with uPA respectively (Fig. 2b). **(e)** Mechanical–induced β_3 integrin adhesion. 293 Cells expressing the indicated β_3 variants were plated on anti-$\alpha_v\beta_3$ (LM609) coated plates. Cell adhesion was expressed as percentage of poly-D-lysine. Data are means ± SEM, n=3.

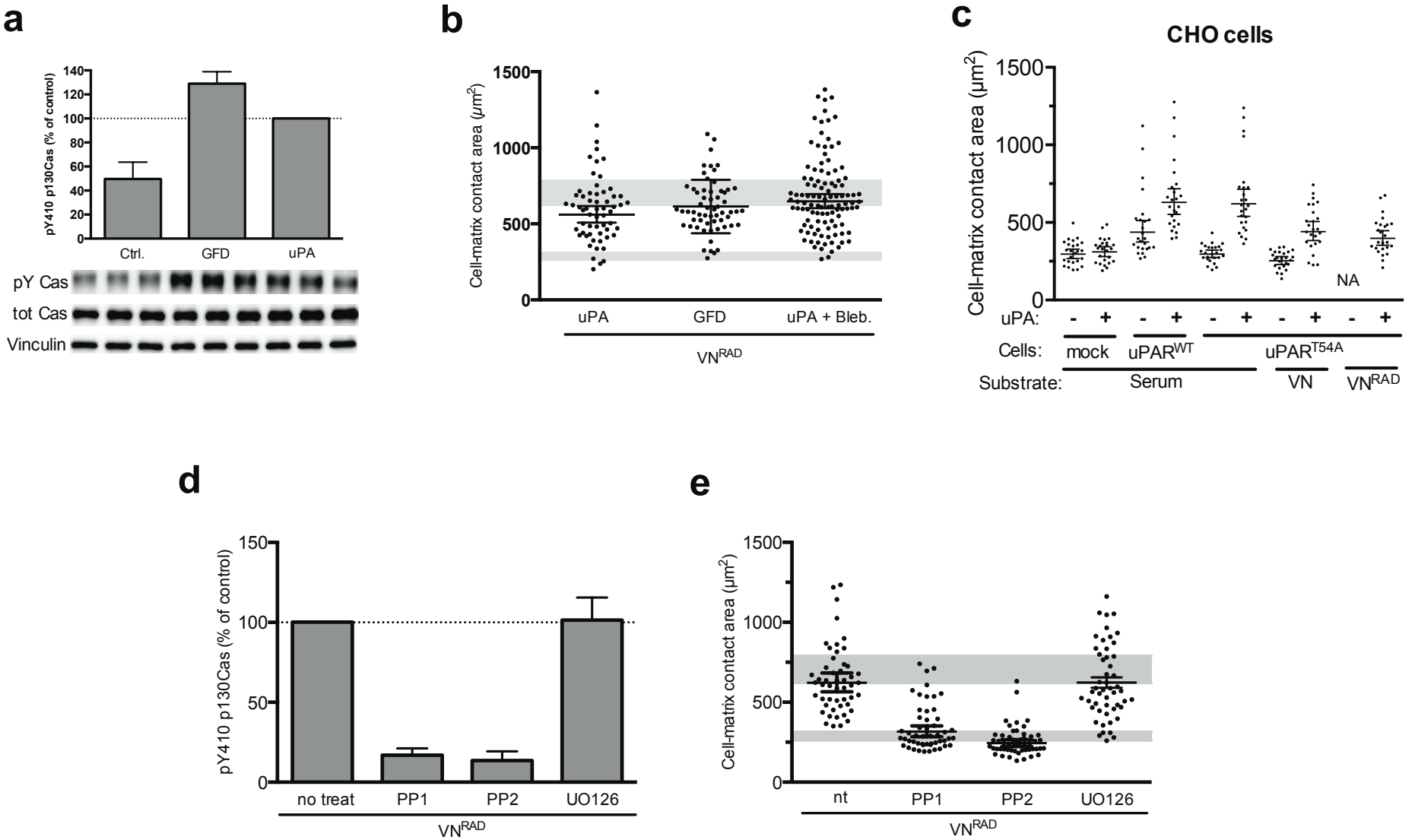
Supplementary Figure 7. Lamellipodial zones induced by uPAR-mediated VN adhesion are more rigid than the cellular body. (**a**) 293 cells expressing $uPAR^{T54A}$ were seeded on VN^{RGD} in the absence or presence of uPA. After adhesion the glass-bottomed plates were transferred to the atomic force microscope for the rigidity

measurement of the membrane/cytoskeletal layer. On the left side representative height images (in nm) of non uPA-stimulated (top) and uPA-stimulated cells (bottom) are shown and on the right side the corresponding elasticity/rigidity maps with the young's moduli of each force curve measurement represented in a color code. (**b**) The graph plots the distribution of the individual young's modulus values obtained by the force curve measurements of cells without (n = 5) or with uPA stimulation (n = 10). The white bars represent the values of the without uPA condition and the grey bars the cells with uPA stimulation. The black and the red lines show Gaussian distribution curves, the black ones for the values of the cell body and nucleus area (of both conditions, with or without uPA) and the red one for the values of the lamellipodial zones of the uPA-stimulated cells.

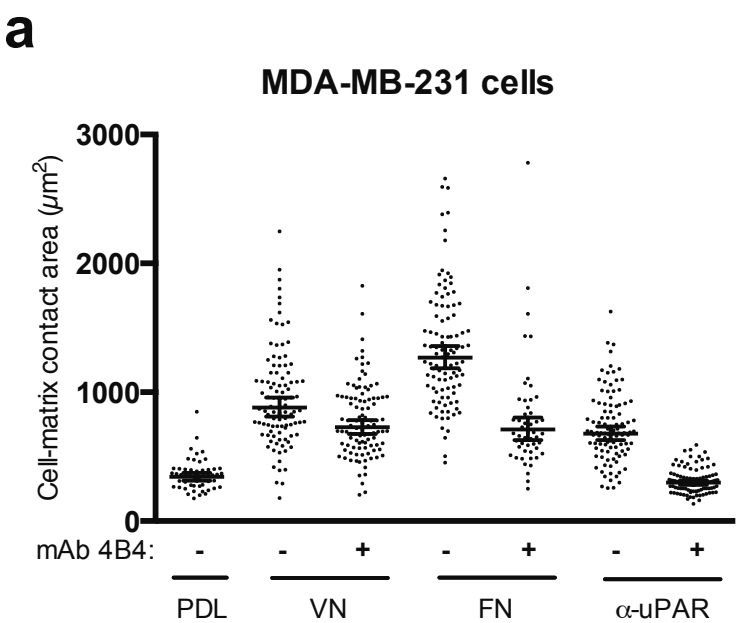
Supplementary Figure 1

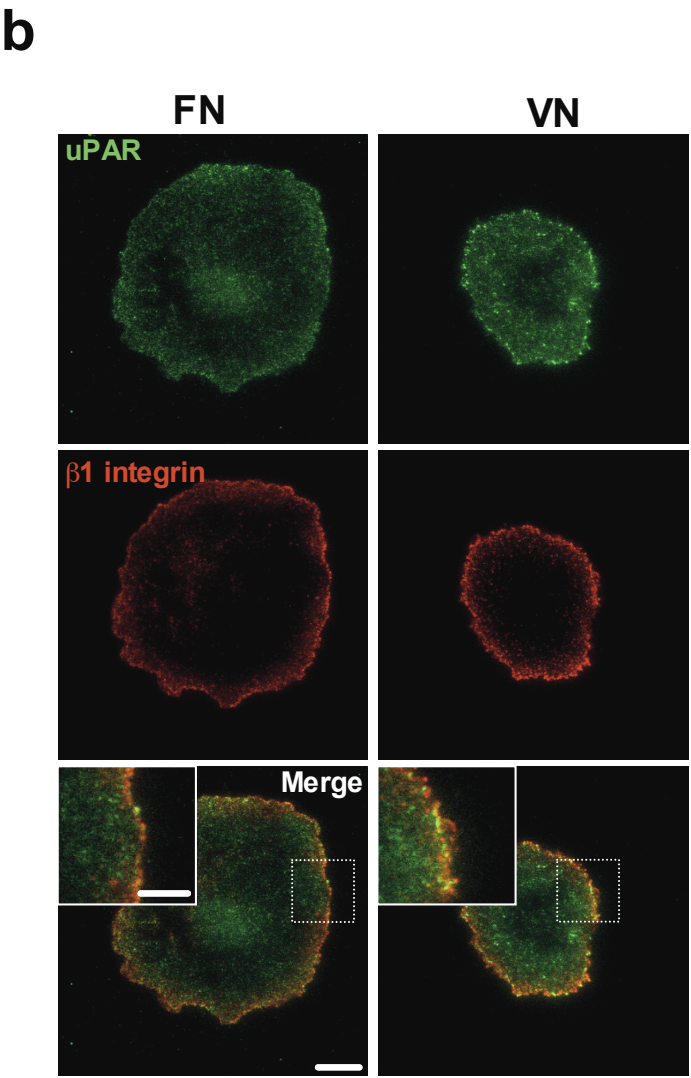


Supplementary Figure 2

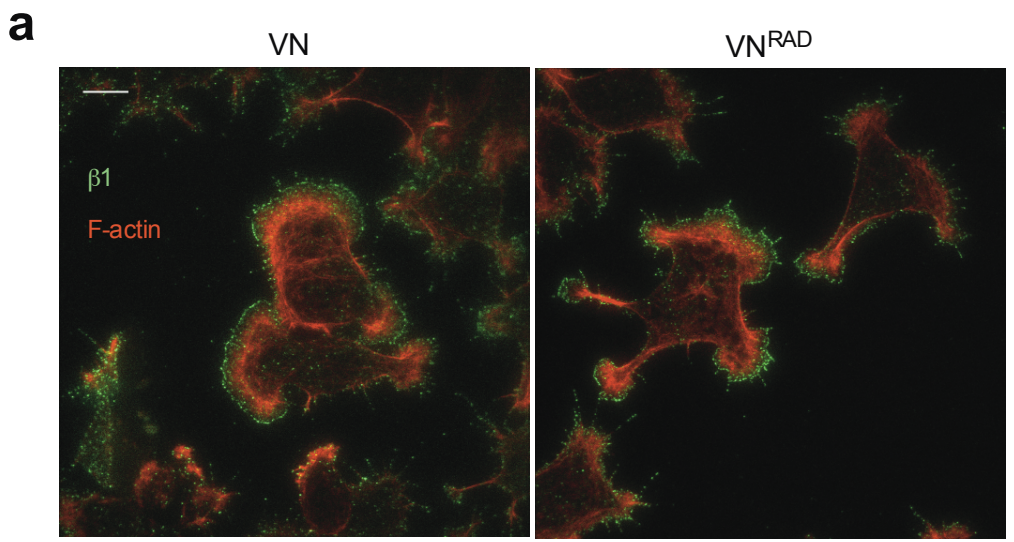


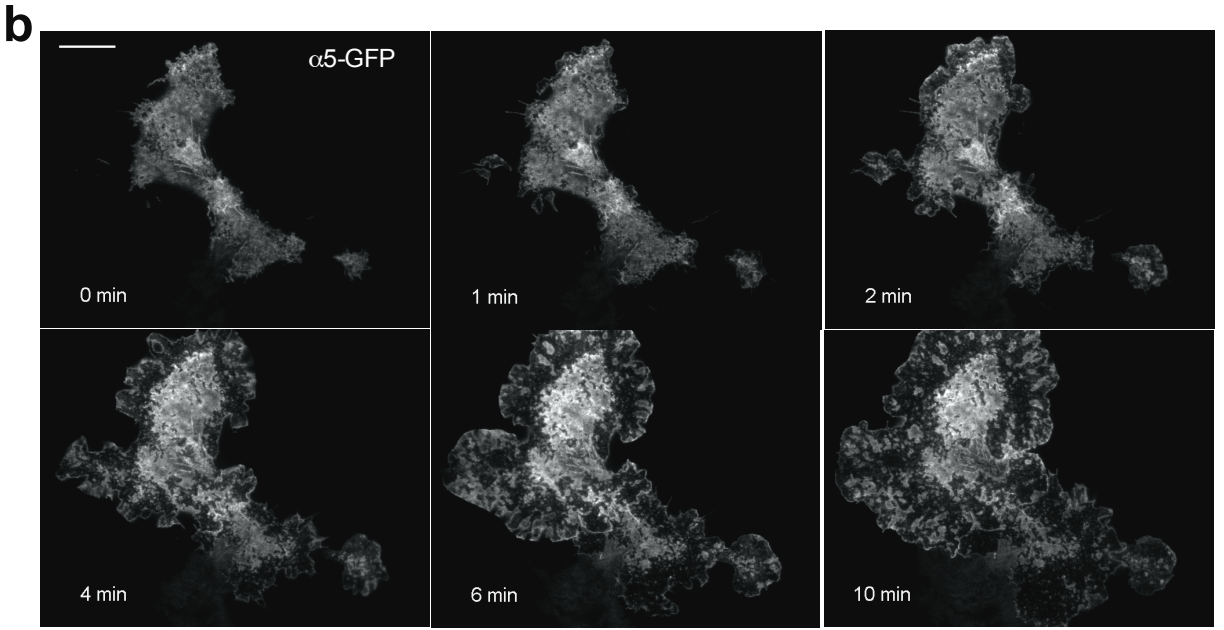
Supplementary Figure 3

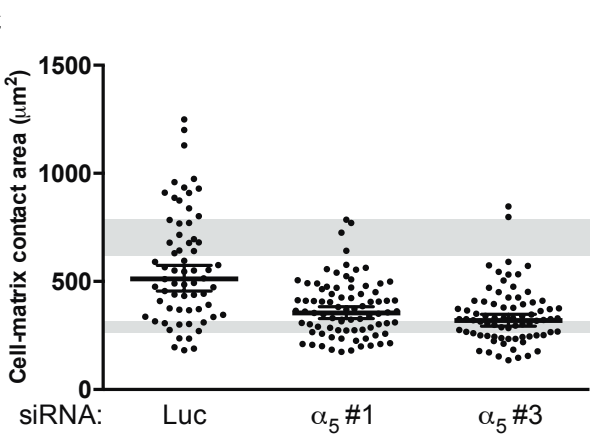


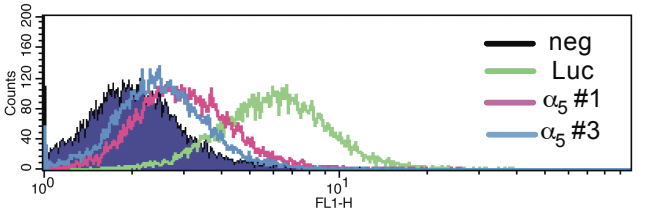


Supplementary Figure 4

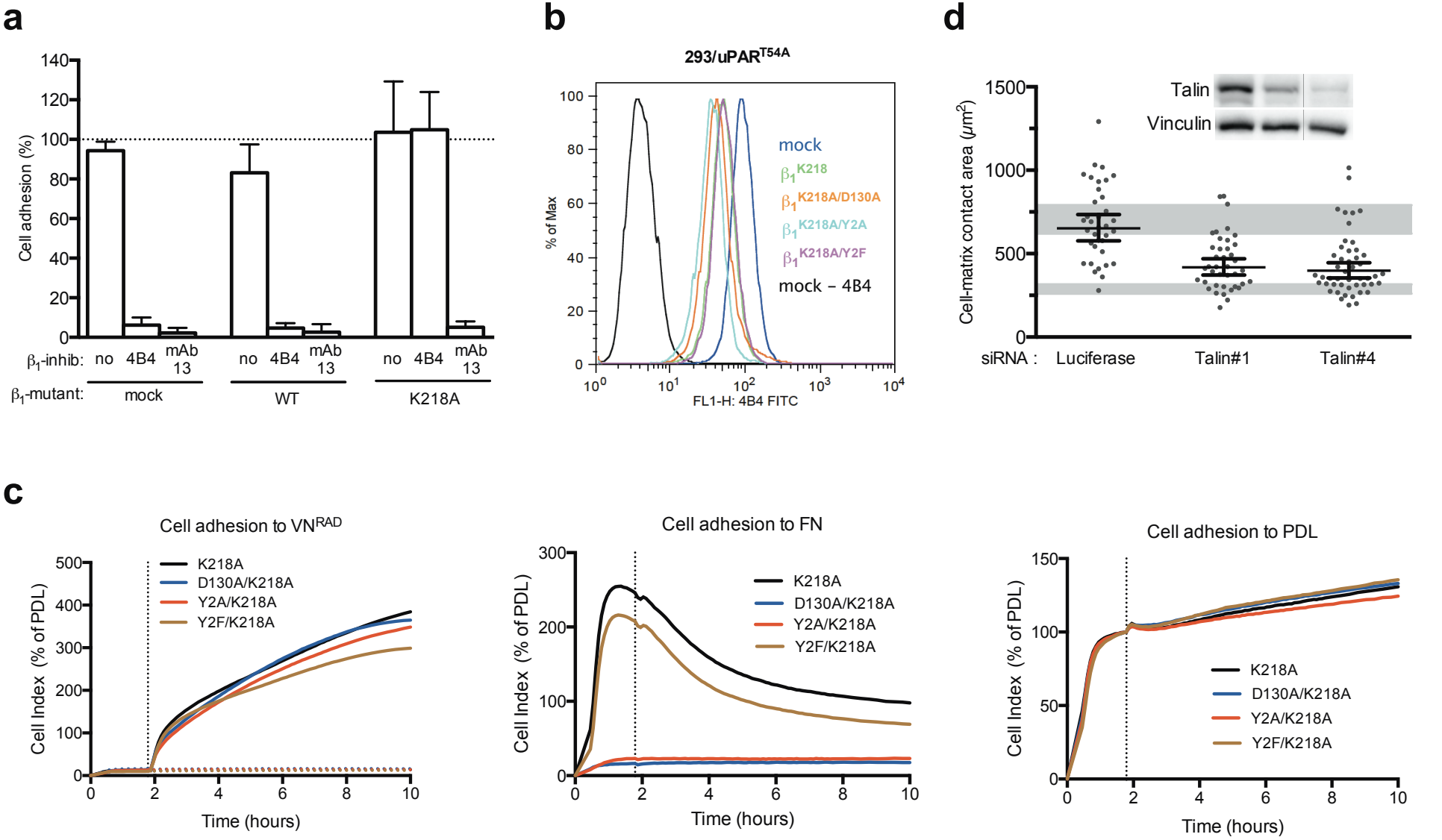




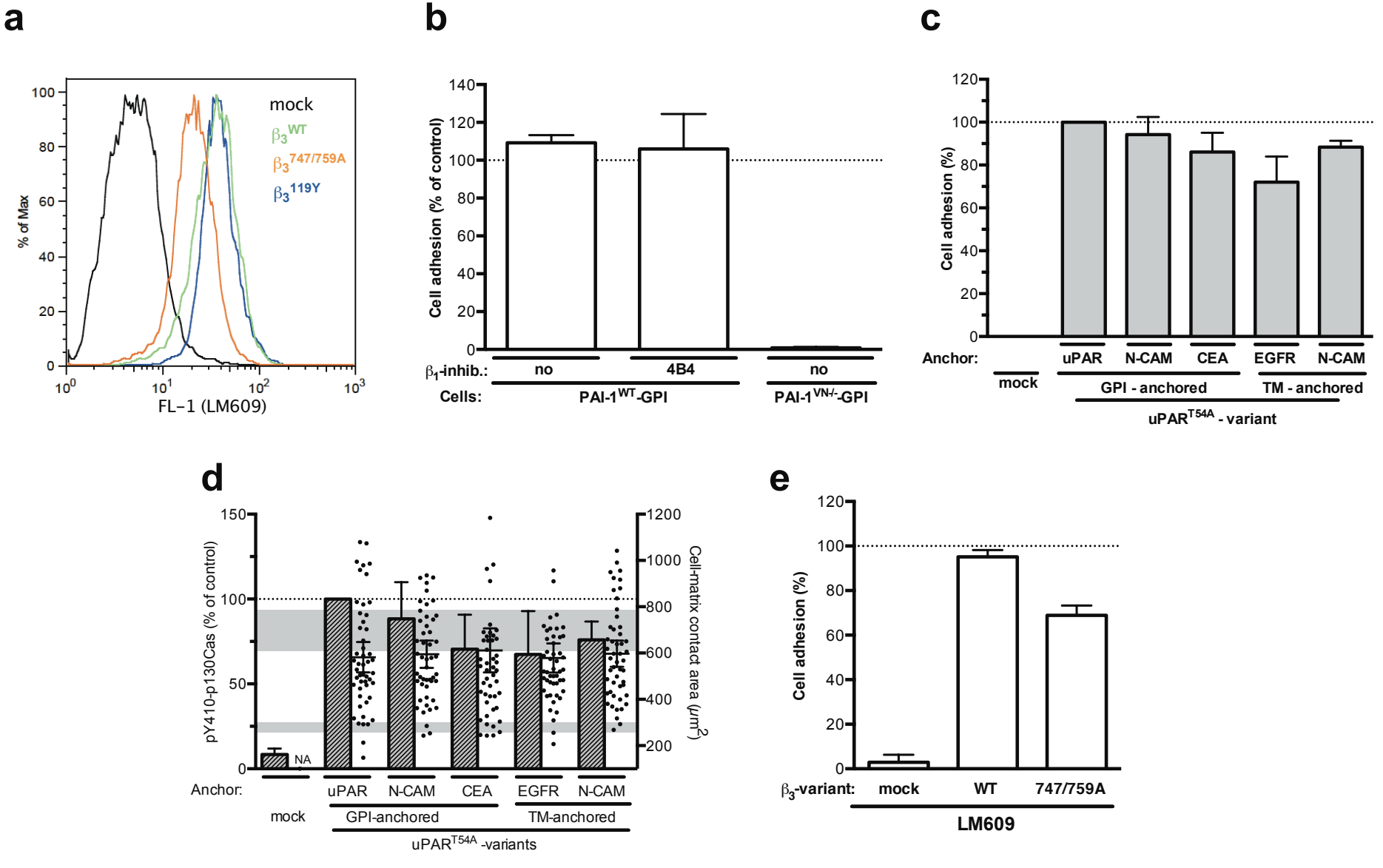




Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7