Online Supplemental Material
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Supplemental methodological details

Transfection of HEK cells
For transfection with uPAR expression vectors, approx. 3 x 10^6 HEK cells, suspended in 250 µl of HEBS-buffer (20 mM HEPES, 0.7 mM Na_2HPO_4, 137 mM NaCl, 5 mM KCl, 6 mM D-Glucose, pH 7.5) including 15 µg of pRc/CMV-uPAR plasmid, were placed in a 0.2 cm Gene Pulse® electroporation cuvette (BioRad, Hercules, CA, USA) connected to a Gene Pulser apparatus equipped with a pulse controller and capacitance extender (BioRad). Electroporation was performed using unlimited resistance-mode and the settings 250 µF, 274 V, except for transfections with N17Rac plasmid (see below) where a setting of 500 µF, 274 V was used. For cloning of uPAR transfectants, cells were then resuspended by addition of 800 µl of serum-containing growth medium (MEM with penicillin/streptomycin, non-essential amino acids and 10 % FCS) and cultured over night before the addition of 800 µg/ml of geniticin. Cells were grown for two weeks after which single colonies were cloned by limiting dilution and further propagated before characterization by flow cytometry. Cells from the N17Rac transfection experiments were used directly without cloning. Evaluation of the transfection efficiency with these cells was done by standard methods using co-transfection with the GFP expressing plasmid, pEGFP-C1 (Invitrogen).

Flow cytometry
For flow cytometric analysis of uPAR expression of proliferating cells from the selected, transfected HEK clones (Mock, $uPAR_{wt}$, $uPAR_{W32A}$, $uPAR_{Y57A}$), the cells were transferred to new culture flasks one day before the assay. After detachment with trypsin/EDTA and washing in PBS with 0.5 % BSA, the cells were first incubated with either anti-uPAR antibody mAb R2 (Rønne E, Behrendt N, Ellis V, Ploug M, Danø K and Høyer-Hansen G. (1991) *FEBS Lett.* **288,** 233-236) or isotype-matched irrelevant monoclonal antibody (mouse IgG1 code X0931; Dako, Glostrup, Denmark), using 1 µg of antibody for 5 x 10^5 cells in 100 µl washing buffer, for 30 min at 4°C. After washing, all samples were stained by incubation with FITC-conjugated polyclonal rabbit-anti-mouse IgG $F(ab')_2$ antibody (a 50-fold dilution of Dako product F0313 in washing buffer), then washed and filtered through a 51 µm mesh nylon filter before analysis. Flow cytometry was performed using a FACSort flow cytometer (BD Biosciences), calibrated with standard fluorescent beads (Polyscience, Warrington, PA, USA). Histograms of FITC-fluorescence (FL1-H, 530/30 nm, log scale) were obtained for the cell cluster defined by simultaneously measured forward and orthogonal light scattering.

Adhesion assays
For vitronectin coating, the wells of a 96-well Falcon non-coated culture plate were incubated with 50 µl of a 5 µg/ml solution of human vitronectin in PBS at 37 °C. The wells were then blocked by addition of 50 µl of 2 % BSA in PBS and incubation for further 1 h at room temperature. After removal of the coating and blocking solution, the wells were washed twice with PBS. Before the assay, cells were trypsinized and incubated for 2 h at 37°C in pre-culture medium (MEM with 10 mM HEPES, 0.5 % BSA and penicillin/streptomycin). The cells were then washed and resuspended

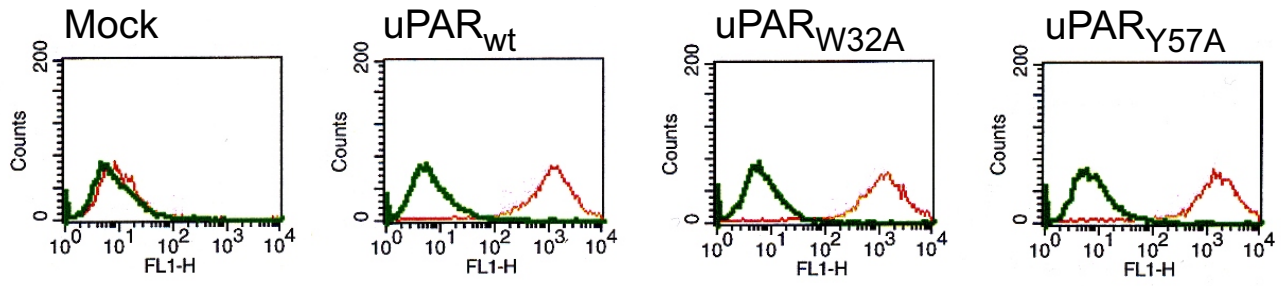
in adhesion assay buffer (PBS with 5 mM EDTA and 0.5 % BSA) at a cell density of 1 x 10^6/ml. In some experiments, pro-uPA or other reagents were included, as indicated. Cells were incubated in this solution for 15 min at 37°C after which aliquots of 100 µl of the cell suspension were placed in the vitronectin-coated wells. Each sample was measured in triplicate. Cells were then allowed to adhere for 1 h, after which non-adherent cells were removed by gently washing the wells with PBS, using an automated pipetting technique.
Adherent cells were quantified by MTT assay (31). For this purpose, a 0.5 mg/ml solution of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; "Thiazolyl Blue Tetrazolium bromide", Sigma, Saint Louis, MO, USA) was prepared in Dulbecco´s modified Eagle´s medium (without phenol red), supplemented with 10 % FCS and penicillin/streptomycin. 100 µl of this solution was added to each drained well with adherent cells. After incubation for 1 h at 37°C, the MTT solution was removed and the wells were washed twice, after which the cells were lysed by addition of 200 µl of lysis solution (0.04 M HCl, 0.5 % SDS in 91 % 2-propanol). The tray was gently shaken for 15 min at room temperature after which the absorbance was read at 570 nm. The readings were calibrated relative to wells with known cell counts that were not subjected to washing before the MTT assay.

Fixation and phalloidin-staining of permeabilized cells
For examination of lamellipodia and cytoskeletal rearrangements, cells were fixed and permeabilized followed by staining with FITC-labeled phalloidin. For this purpose, coverslips were washed gently in PBS/Ca/Mg at 4 °C, followed by fixation of the cells for 10 min at room temperature in 4 % para-formaldehyde in PBS/Ca/Mg. The wells with the coverslips were then washed twice in PBS. Subsequent incubations were performed by placing the coverslips upside-down in 50 µl droplets of the solutions to be used, placed on Parafilm sheets. Thorough washing in PBS was performed after each incubation step. Cells were permeabilized for 5 min at room temperature in detergent buffer (0.2 % Triton X-100 in PBS), followed by incubation in FITC-phalloidin (1 U/ml in PBS) for 30 min at room temperature. Finally, the coverslips were washed in H_2O and mounted in antifade reagent (ProLong® Gold, Invitrogen). The cells were then examined by fluorescence microscopy, using a Leica DM4000B fluorescence microscope with a Leica DFC-480 camera.

Quantification of cells with cytoskeletal rearrangements after dominant-negative Rac transfection
N17Rac-transfected and mock-transfected cell populations were cultured on vitronectin-coated coverslips in the presence or absence of pro-uPA and stained as described above. Since all microscopy fields would include a low number of untransfected cells, scoring for lamellipodia-positive cells was in these experiments done by randomly selecting microscopy fields representing at least 50 cells. These cells were scored individually for the presence of lamellipodia and the percentage of lamellipodia-positive cells was recorded.

Fig S1

Supplemental figures



Flow cytometric analysis of transfected cells

Mock-transfected cells or cells transfected with cDNAs encoding uPAR$_{wt}$ or the indicated uPAR mutant proteins were examined by flow cytometry using anti-uPAR mAb R2 as the primary antibody. Red line: anti-uPAR antibody. Green line: isotype-matched irrelevant mouse monoclonal antibody. The median intensities (arbitrary units) were 1.5 x 10^3 (uPAR$_{wt}$), 1.5 x 10^3 (uPAR$_{W32A}$), 1.5 x 10^3 (uPAR$_{Y57A}$) and 0.9 x 10^1 (Mock), respectively.

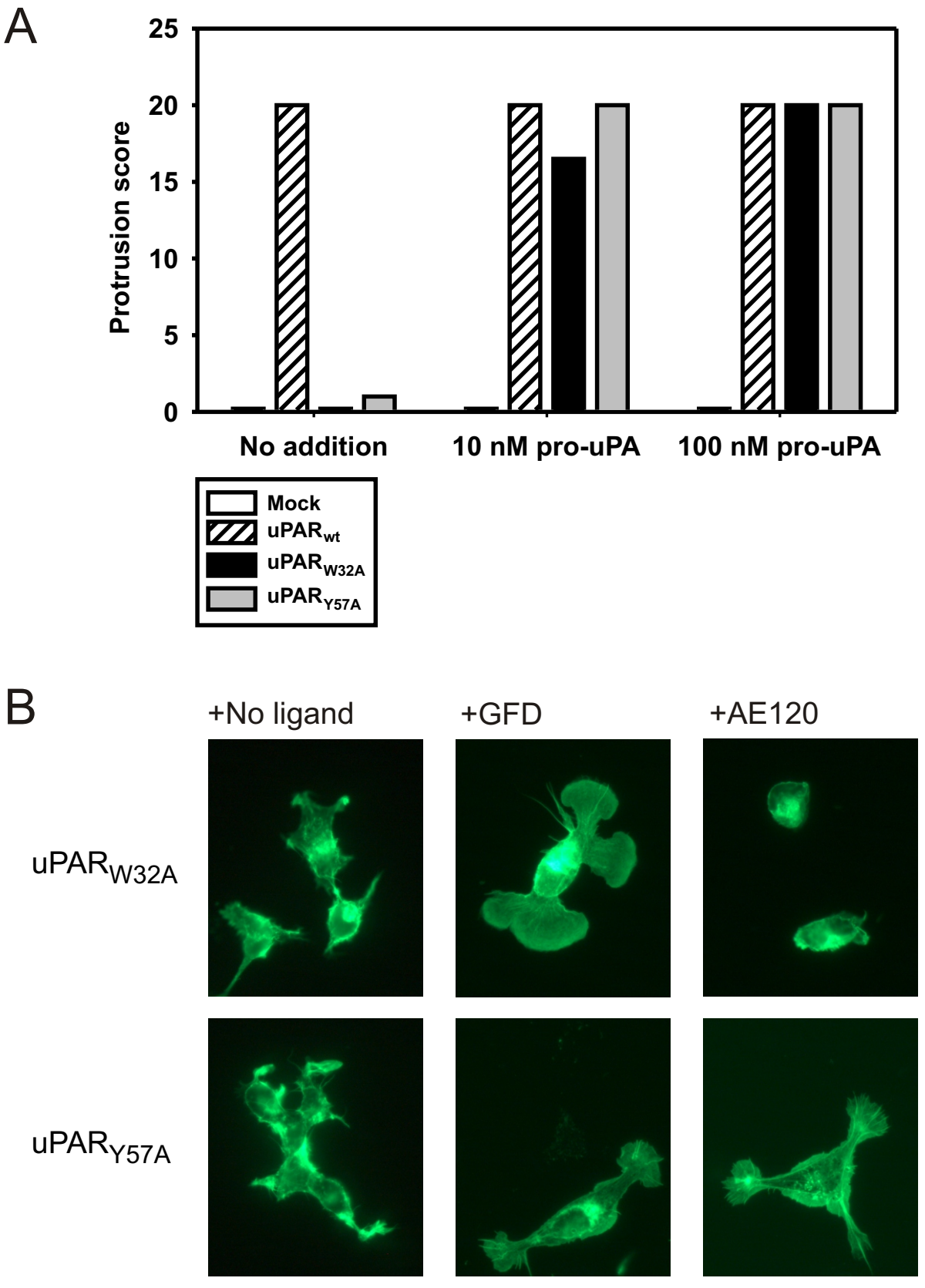


Fig. S2

Cell morphology of uPAR transfectants in the presence of various ligands

Transfected HEK cells were cultured in the presence of the indicated ligands and prepared for FITC-phalloidin staining followed by fluorescence microscopy as in Fig. 1B.

A Lamellipodia-positive cells after over-night culture in the presence of the indicated concentrations of pro-uPA. Blinded scoring of fields with lamellipodia-positive cells and representation of the cumulative score were done as in Fig. 1C.

B Morphology of $uPAR_{W32A}$ and $uPAR_{Y57A}$ cells cultured for five days without added ligand or in the presence of GFD (100 nM) or AE120 (1 μM).

Fig S3

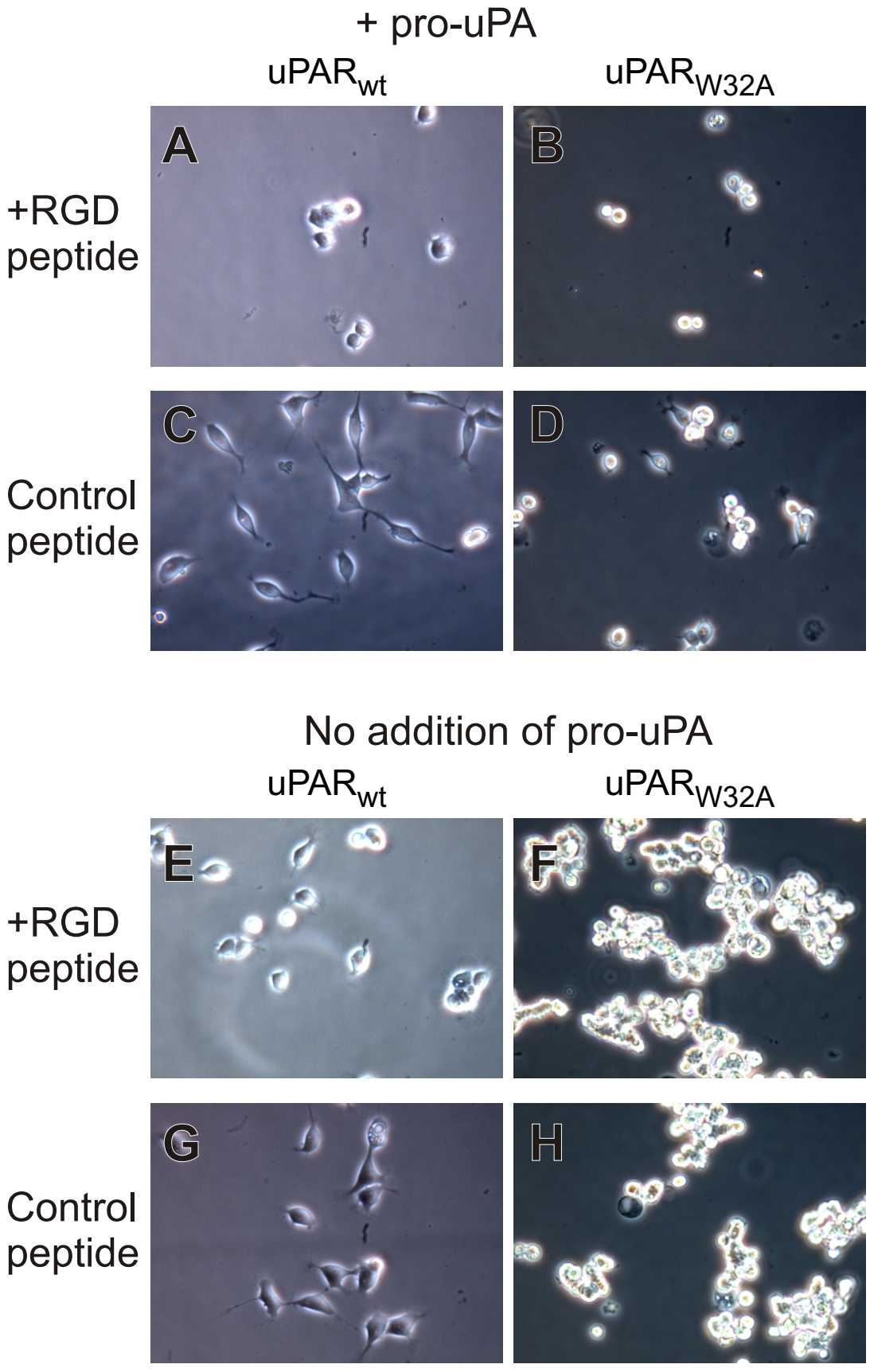
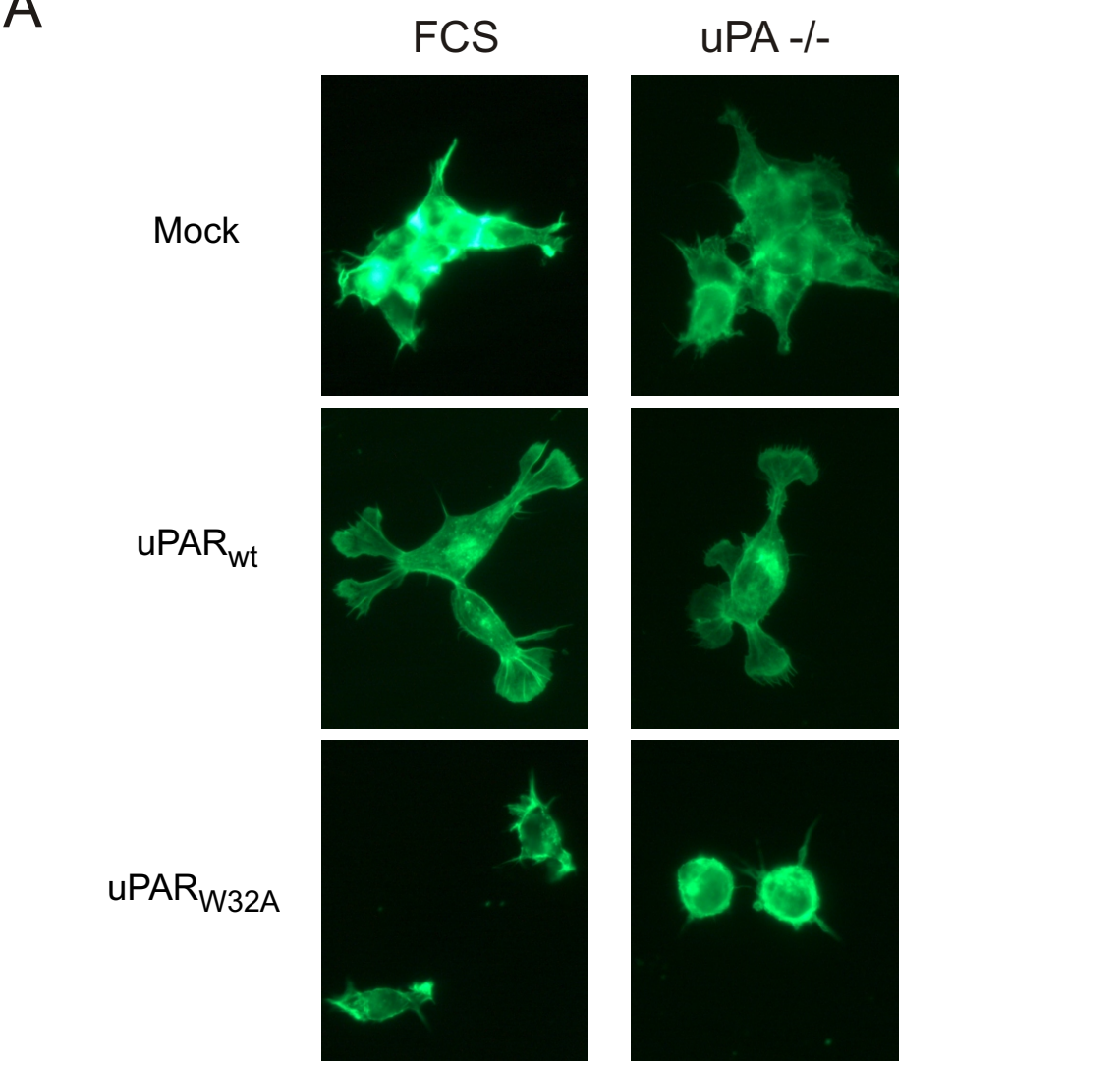


Fig. S3

Effect of an integrin-binding cyclic peptide

Transfected HEK cells (uPAR$_{wt}$ and uPAR$_{W32A}$) were cultured for five days in the presence of 200 μM of cyclo(Arg-Gly-Asp-**D**-Phe-Val) (RGD peptide) (A, B, E, F) or cyclo(Arg-Ala-Asp-**D**-Phe-Val) (Control peptide) (C, D, G, H). Pro-uPA (10 nM) was present during cell culture in A, B, C and D. Cells were examined directly by phase contrast microscopy. Mock-transfected cells (not shown) were lamellipodia-negative with rounded morphology with both peptides and both in the presence and absence of pro-uPA.

Fig S4



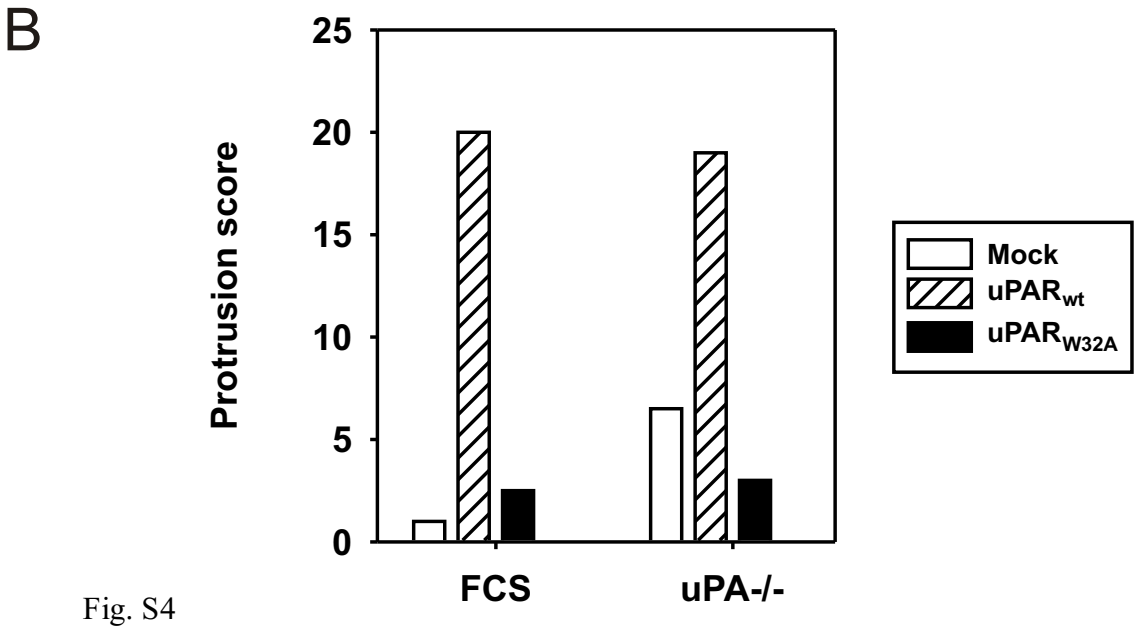


Fig. S4

Cytoskeletal rearrangements can be induced by a vitronectin-binding uPAR in the absence of uPA

Mock-transfected, $uPAR_{wt}$-transfected or $uPAR_{W32A}$-transfected cells were cultured on vitronectin-coated coverslips as in Fig. 1B (in medium including FCS), or in medium in which the FCS was substituted with uPA deficient mouse serum.

A Cell morphology after FITC-phalloidin staining. The transfectant cell types (Mock, $uPAR_{wt}$, $uPAR_{W32A}$) are indicated to the left. The growth conditions specified on top designate fetal calf serum (FCS) and uPA deficient mouse serum (uPA-/-), repectively.

B Quantification of fields with lamellipodia-positive cells. Blinded scoring and representation of the cumulative score were done as in Fig. 1C.