APPENDIX

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APPENDIX SUPPLEMENTARY FIGURES AND FIGURE LEGENDS
Appendix Figure S1.

- A Analysis of the effect of plasminogen activation on cell adhesion to VN measured by plate-and-wash assay. 293/uPAR cells were plated on VN and treated with subsequent additions of 10 nM sc-uPA and 30 nM Plg. 2 h after Plg treatment, cell adhesion was quantified as described in the Appendix Supplementary Methods. Results are presented as % of no treated cells.

- B sc-uPA and Plg do not affect the cell adhesion to VN of mock-transfected HEK293 cells. RTCA experiments were performed on 293/uPAR cells and mock-transfected cells seeded on VN and treated with 10 nM sc-uPA, 30 nM Plg and 100 nM α2AP at the indicated time-points. A representative experiment is shown. Cell indexes values were normalized on the average cell index of 293/uPAR cells prior to the first treatment.

- C The direct contribution of tc-uPA to the negative feedback requires uPAR binding. RTCA experiments were performed on 293/uPAR cells seeded on VN and treated with the different uPA variants at a final concentration of 10 nM and 300 nM aprotinin. A representative experiment is shown.

- D The different uPAR variants are expressed at comparable levels on the cell surface. The expression of uPAR was evaluated by FACS. The negative control (neg cntrl), meaning no primary antibody, is indicated.

- E Cells expressing the different uPAR variants display largely comparable cell adhesion to VN. RTCA experiments were conducted on HEK293 cells expressing the different uPAR variants seeded on VN and treated with 10 nM sc-uPA or vehicle. The cell index 2 h after treatment of a representative experiment is reported.

- F The cleavage site(s) responsible for the negative feedback are located in the N-terminal region of VN. RTCA experiments were performed on 293/uPAR^{R83/89A} cells seeded on
VN(1-64)-Fc and treated with 10 nM sc-uPA and tc-uPA, 30 nM Plg and 100 nM α2AP. 
A representative experiment is shown.
Appendix Figure S2.

- **A-B** VN<sup>G46A</sup> is more prone to uPA-mediated proteolytic cleavage compared to VN<sup>WT</sup>. (A) The different VN variants were immobilized in 96-well plates and incubated with 3 nM tc-uPA and 10 nM soluble Fc-tagged uPAR. The supernatants were recovered at different time-points and analysed for the presence of N-terminal VN-fragments by immunoassay. Data points represent mean ± SD of a representative experiment. (B) A concentration curve of the VN variants was immobilized in 96-well plates and detected with an anti-VN polyclonal antibody (Molecular Innovations). The specific signal was calculated subtracting the background signal coming from control wells coated with buffer only. The
data show that the different VN preparations contain a virtually identical amount of VN antigen. The results shown in panel A are therefore not an artefact caused by imprecise estimation of the VN concentration.

- **C-D** The R45A substitution impairs the negative feedback mediated by plasmin. RTCA experiments were conducted on 293/uPAR<sup>R33/89</sup> cells seeded on VN(1-64)-Fc WT or R45A. Cells were treated with 10 nM GFD and 30 nM Pli at the indicated time-points. A representative experiment is shown.
Appendix Figure S3.

- A tc-uPA mediated release is not due to trace amount of plasmin in the preparation. 293/uPAR cells were plated on VN and treated with 10 nM tc-uPA or with a combination of 10 nM sc-uPA and 30 nM Plg in the presence or absence of plasmin inhibitors (100 nM α2AP and 300 nM aprotinin). Supernatants were analysed as described in the legend to Fig. 4A. Values represent the mean ± SD of 3 independent experiments. The statistical significance was probed using Student’s t-test (two-tailed).
• **B** The R45A substitution impairs the SMB release mediated by tc-uPA and Pli. 293/uPAR cells were plated on the indicated substrates and treated with 10 nM sc-uPA, 10 nM tc-uPA, 30 nM Pli or with a combination of 10 nM sc-uPA, 30 nM Plg and 100 nM α2AP. Supernatants were analysed as described in the legend to Fig. 4A. Means ± SD of a representative experiment are shown.
Appendix Figure S4.

Plasmin is more efficient than tc-uPA in cleaving VN.

VN immobilized in 96-well plates was incubated with 10 nM Pli or tc-uPA in the presence or absence of 10 nM soluble Fc-tagged uPAR. After 1 h, the supernatants were recovered and analysed for the presence of N-terminal VN-fragments by immunoassay. Means ± SD of a representative experiment are shown.
Figure S5

A

![Graph showing normalized cell index over time](image)

B

![Scatter plot showing cell matrix area](image)

C

![Western blots for R2 and R3](image)
Appendix Figure S5.

• **A** Effect of PAI-1 on cell adhesion to VN. RTCA experiments were conducted on 293/uPAR cells seeded on VN. Cells were treated with wild-type or VN-binding deficient (VN-) PAI-1 at the indicated time-point. A representative experiment is shown.

• **B** Effect of agonists and antagonists of the uPAR-VN interaction on cell spreading. Cell-matrix contact area was quantified in the DIC images collected in the experiment described in the legend to Fig 5B, using ImageJ. Twenty-five cells were quantified for each condition. Means ± SD of a representative experiment are shown. Statistical significance was probed using Student’s t-test (two-tailed).

• **C** PAI-1 inhibits the cleavage of uPAR mediated by tc-uPA. 293/cells were seeded on FN and allowed to adhere before treatment with 10 nM PAI-1 and tc-uPA. After 1 h, cells were lysed, resolved by SDS-PAGE and analysed by immunoblotting.
Figure S6

HOP92

A

B

OVCAR5

C

no cells
Appendix Figure S6.

Identification of the VN(1-45) fragment in cell supernatants of cancer cells treated with Plg and α2AP. HOP92 (A) and OVCAR5 (B) cells (or no cells (C) as control) were plated on VN and treated with 30 nM Plg and 100 nM α2AP. Cell supernatants were collected after 16 h and immunoprecipitated with HU3-conjugated beads. Eluted material was analyzed by MALDI-TOF mass spectrometry. Representative spectra are shown.
Appendix Figure S7.
Plasminogen activation induces SMB release and cell adhesion inhibition in a wide variety of cancer cell lines.

• **A-B** The epithelial cancer cell lines belonging to the NCI-60 panel were seeded on VN in the absence (A) or presence (B) of 30 nM Plg and 100 nM α2AP. The supernatants were harvested after 16 h and the level of SMB-containing VN-fragments was measured by immunoassay. Values represent the mean ± SD. The vertical dashed lines indicate the background (0.27 nM) and the background + 2SD (0.35 nM). The cancer types are shown using different colours.

• **C** The cancer cell lines were seeded on VN and treated with 30 nM Plg and 100 nM α2AP after 4 h of adhesion. The graph shows the reduction of cell adhesion for the individual cell lines after 16 h of treatment, reported in percentage of the normalized cell index measured in control wells treated with the vehicle.
Appendix Figure S8.

Treatment with exogenous tc-uPA does not induce a reduction in cell adhesion. The indicated cell lines were plated on VN, allowed to adhere for about 4 h and treated with 10 nM tc-uPA (A). The data represented in panel B are from the experiment described in the legend to Fig 6C. The cell index measured after 16 h of treatment is shown in % of the one measured for cells seeded on the same substrate, but treated with vehicle.
APPENDIX SUPPLEMENTARY METHODS

Expression vector construction. pcDNA5/FRT/TO-uPARR83/89A was made by site-directed mutagenesis as previously described [1]. For the generation of pcDNA5/FRT/TO-VN-6xHis, a human VN cDNA (RZPD Clone ID: IRAUp969G1135D6) was amplified with oligos hVNu/hVNd and the PCR product cloned BamHI/XbaI in pBluescript. A 6xHis tag was introduced at the C-terminal by digestion with XbaI/NotI and insertion of a linker made by annealing oligos XbNhisf and XbNhisR. The His-tagged VN coding region was transferred BamHI/NotI to pcDNA5/FRT-TO generating the expression vector pcDNA5/FRT/TO-VN-His. pcDNA5/FRT/TO-VN*R45A*-His and pcDNA5/FRT/TO-VN*G46A*-His were generated in a two-step PCR overlap amplification procedure. Firstly, pcDNA5/FRT/TO-VN-His was amplified with oligos hVnR45AR or G46AR and with oligos R45AF or G46AF/HISNOTR. Secondly, the two PCR products were mixed, co-amplified using oligos hVnu/HISNOTR and cloned BamHI/NotI in pcDNA5/FRT-TO. The expression vectors for recombinant proteins tagged with a human IgG constant region (Fc) are based on the pcDNA5/FRT/TO-Fc plasmid [1], however, a number of modifications was introduced to facilitate the shuffling of different coding regions as well as to improve protein yields. Firstly, an XhoI restriction site located in the vector sequence downstream of the Fc coding region was destroyed by site-directed mutagenesis using oligos dXu/dXd. Secondly, a linker encoding a cleavage sequence for the PreScission protease, made by annealing oligos PreF/PreR, was inserted in the XhoI site located at the signal peptide/Fc junction. Since the removal of the intron present in the Fc region of the construct was found to increase the yield of recombinant protein, the vector was transfected into CHO cells, RNA extracted and reverse transcribed. The cDNA was then amplified with oligos hVNukpn/FcNr and cloned KpnI/NotI into pcDNA5/FRT-TO (Invitrogen corp.) to generate pcDNA5/FRT/TO-Fc.
pcDNA5/FRT/TO-VN(1-64)-Fc, pcDNA5/FRT/TO-VN(1-64)^R45A-Fc, pcDNA5/FRT/TO-VN(1-64)^G46A-Fc were generated amplifying pcDNA5/FRT/TO-VN-6XHis, pcDNA5/FRT/TO-VN^R45A-6XHis and pcDNA5/FRT/TO-VN^G46A-6XHis, respectively, with oligos hVNukpn/VN64dx and cloning the product KpnI/XhoI in pcDNA5/FRT/TO-Fc. To introduce the K78Q mutation in the tag, pcDNA5/FRT/TO-VN(1-64)-Fc was amplified using oligos FcSuKQ/FcNrr and the PCR product was digested XhoI/NotI and cloned in pcDNA5/FRT/TO-VN(1-64)-Fc, pcDNA5/FRT/TO-VN(1-64)^R45A-Fc to generate pcDNA5/FRT/TO-VN(1-64)-Fc^K78Q and pcDNA5/FRT/TO-VN(1-64)^R45A-Fc^K78Q, respectively. Construct encoding human soluble uPAR tagged with a Fc, pcDNA5/FRT/TO-uPAR-Fc, was made by amplification of a full-length uPAR cDNA with oligos urskf/upre2d and cloned KpnI/XhoI. To obtain mutant uPAR-Fc constructs, pcDNA5/FRT/TO-uPAR^R83/89A and pcDNA5/FRT/TO-uPAR^W32A/R91A were amplified with oligos urskf/upre2d and cloned KpnI/XhoI in pcDNA5/FRT/TO-Fc to generate pcDNA5/FRT/TO-uPAR^R83/89A-Fc and pcDNA5/FRT/TO-uPAR^W32A/R91A-Fc, respectively. To generate pcDNA5/FRT/TO-VN(1-66), pcDNA5/FRT/TO-VN(1-66)-Fc was amplified with oligos hVNuKpn/VN66rn, digested KpnI/NotI and cloned in pcDNA5/FRT/TO-VN(1-64)-Fc.

Oligonucleotide sequences.

hVnu 5’-GCGGATCCAGCCCTGCCATGGCACCCTGAG-3’
hVNd: 5’-CGGGGTACCATGGCACCCTGAG-3’
XbnhisF 5’-CTAGAGGCGCATCATCCACCACCATTGAGC-3’
XbnhisR 5’-GGCCGCTCAATGGTGATGGTGATGATGCCCT-3’
G46AF 5’-CCCCAAGTGACTCGCGGATGTGTTCACTATG-3’
G46AR 5’-CATAGTGACACATCCGCGGAGTCACTTGGG-3’
Adhesion assays. Clear 96-well plates were coated with 5 μg/mL recombinant VN and blocked with 5% BSA in PBS for 1 h at 37°C. uPAR cells (15000 cells/well) were plated and treated with subsequent additions of 10 nM sc-uPA and 30 nM Plg. After 2 h, cells were extensively washed. Adherent cells were fixed, stained with crystal violet and quantified by measuring absorbance at 540 nM. The specific signal was calculating by subtraction of the non-specific signal measured in BSA-coated wells.
FACS analysis. Cell surface expression of the different uPAR variants was analysed by flow cytometry. Cells were harvested and stained using the anti-uPAR monoclonal antibody R2 (2 µg/mL) and anti-mouse FITC secondary antibody. Stained cells were acquired on a FACSCalibur (BD Pharmigen, USA). Data were analysed using the FlowJo software (Tree Star, USA).

REFERENCES