# Supplementary Information

## Supplementary experimental procedures

*Vector 21tet(5) cloning*

The vector 21tet(5) is based on the phage vector fdg3p0ss21 and contains a 2.5 kb stuffer fragment instead of the gene region coding for D1 and D2 domains of phage p3. The vector was obtained by ligating two *Sfi*I-digested PCR products amplified with the primer pairs g3pNba/pelbsfifo and tetsfiba/tetsfifo from the vectors fdg3p0ss21 (around 7 kb) and fd-tet-DOG1[16] (around 2.5kb). The ligated DNA was transformed into TG1 cells.

*Production and biotinylation of human uPA*

The catalytic domain of human uPA (a N145Q mutant deficient in the glycosylation site) was expressed as pro-enzyme (pro-uPA) in mammalian cells and purified as previously described.[6] After the purification, pro-uPA showed an apparent molecular mass of about 32 kDa in SDS-PAGE. The protein was subsequently activated by plasmin cleavage: 12.3 mg pro-uPA were incubated with 49.4 µg plasmin (HPLM, from human plasma, 85 kDa, Molecular Innovations, Novi, MI, USA) in 50 mM HEPES, 150 mM NaCl, pH 8 for 6 hours at room-temperature. The activated protein (uPA) was purified by size exclusion chromatography using a HiLoad 16/60 Superdex 75 prep-grade column (GE Healthcare, Glattbrugg, Switzerland). The protein was eluted as a monomer giving a single band in SDS-PAGE, confirming the complete cleavage, with a molecular mass of about 28 kDa under reducing conditions (see Supplementary figure 1).

For biotinylation, uPA (10 µM) was incubated with EZ-link Sulfo-NHS-LC-biotin (200 µM; Pierce) in 50 µl PBS (pH 8) for 1 h at 25 °C. Excess of biotinylation reagent was removed by

gel filtration with a Sephacryl S100 column (GE Healthcare) using 50 mM NaAc buffer, 200 mM NaCl, pH 5.5. The ability of the biotinylated uPA to bind to either streptavidin or neutravidin was verified by incubating the protein with magnetic streptavidin and neutravidin beads respectively and analyzing the bound and unbound protein fraction by SDS-PAGE.

*Chemical synthesis of peptides*

Fmoc-protected amino acids and Fmoc-rink amide AM resin (0.26 mmol/g resin) were purchased from Iris Biotech GmbH (Marktredwitz, Germany). O-Benzotriazole-*N*,*N*,*N*',*N*'-tetramethyl-uronium-hexafluoro-phosphate (HBTU, ChemPep, Wellington, FL, USA), *N*,*N*-Diisopropylethylamine (DIPEA, Merck Schuchardt OHG, Hohenbrunn, Germany), trifluoroacetic acid (TFA, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 1,2-ethanedithiol (EDT, Fluka Chemie GmbH, Buchs, Switzerland), thioanisole (Fluka), piperidine (Fluka), phenol (Acros Organics, Geel, Belgium) and 1,3,5-tris(bromomethyl)benzene (TBMB) were used as received without further purification. Peptides were synthesized on an Advanced ChemTech 348Ω peptide synthesizer (Aapptec, Louisville, USA) by standard Fmoc (9-fluorenylmethyloxycarbonyl) solid-phase chemistry on a Rink Amide AM resin (0.03 mmol scale). Coupling steps were carried out twice, for each time amino acid (4 eq., 0.2 M solution in DMF), HBTU (4 eq, 0.45 M solution in DMF), OxymaPure (4 eq, 0.45 M solution in DMF) and DIPEA (6 eq, 0.5 M solution in DMF) were used. Fmoc groups were removed using a 20% (v/v) solution of piperidine in DMF (2.5 ml x 2). The final peptides were deprotected (side-chain protected groups) and cleaved from the resin using a TFA/thioanisole/$H_2O$/phenol/EDT mixture (90/2.5/2.5/2.5/2.5 v/v, 4 ml) for 3 hours at room temperature. The resin was removed by filtration under vacuum and the peptides were precipitated with cold diethyl ether (40 ml). The precipitated peptides were

resuspended and washed twice with diethyl ether (20 ml each time). Finally, the peptides were dissolved in $H_2O$: $CH_3CN$ (1:1) and lyophilized.

*Mass spectrometric analysis of synthetic peptides*

The molecular masses of synthetic peptides before and after chemical modification were determined with an Axima-CFR plus MALDI-TOF mass spectrometer (Axima-CFR plus, Kratos Shimadzu Biotech, Manchester, UK). HPLC-purified peptides (0.1-10 μM in 0.1% v/v TFA/10-30% v/v $CH_3CN$ in water) were mixed 1:1 with a saturated solution of matrixα-cyano-4-hydroxycinnamic acid (α-CHCA) in 50% v/v $CH_3CN$, 49.9% v/v $H_2O$, 0.1% v/v TFA and loaded onto a MALDI carrier plate for mass determination.

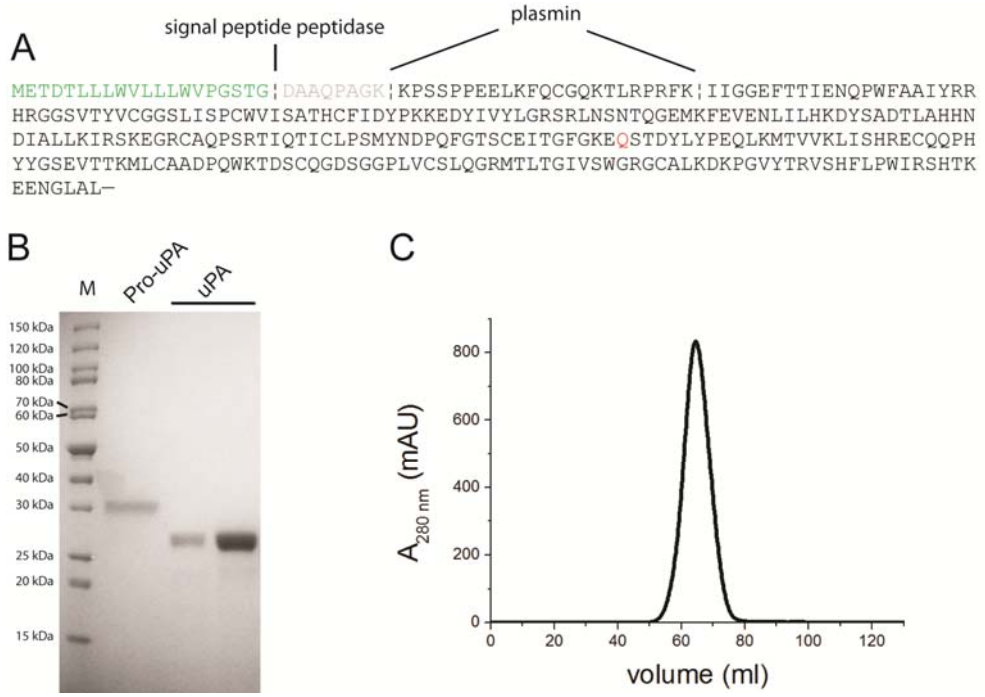
## Supplementary table

| Primer name | DNA sequence |
|---|---|
| g3pNba | 5'−CAGTCAGGCCTCGGGGGCCATGGCTTCTGGTACCCCGGTTAAC−3' |
| pelbsfifo | 5'−GACTGAGGCCGGCTGGGCCGCATAGAAAGGAACAACTAAAGGAAT−3' |
| tetsfiba | 5'−CAGTCAGGCCCAGCCGGCCGATCTCGGGAAAAGCGTTGGTCAC−3' |
| tetsfifo | 5'−GACTGAGGCCCCCGAGGCCTTCCCTTTGTCAACAGCAATGG−3' |
| prepcr | 5'−GGCGGTTCTGGCGCTGAAACTGTTGAAAGTAG−3' |
| sfi2notfo | 5'−CCATGGCCCCCGAGGCCGCGGCCGCATTGACAGG−3' |
| SfiIcx3cx4cba | 5'−TATGCGGCCCAGCCGGCCATGGCAGCTGCNNKNNKNNKTGCNNKNNKNNKNNKTGTGGCGGTTCTGGCGCTG−3' |
| SfiIcx4cx3cba | 5'−TATGCGGCCCAGCCGGCCATGGCAGCTGCNNKNNKNNKNNKTGCNNKNNKNNKTGTGGCGGTTCTGGCGCTG−3' |
| SfiIcx3cx5cba | 5'−TATGCGGCCCAGCCGGCCATGGCAGCTGCNNKNNKNNKTGCNNKNNKNNKNNKNNKTGTGGCGGTTCTGGCGCTG−3' |
| SfiIcx4cx4cba | 5'−TATGCGGCCCAGCCGGCCATGGCAGCTGCNNKNNKNNKNNKTGCNNKNNKNNKNNKTGTGGCGGTTCTGGCGCTG−3' |
| SfiIcx5cx3cba | 5'−TATGCGGCCCAGCCGGCCATGGCAGCTGCNNKNNKNNKNNKNNKTGCNNKNNKNNKTGTGGCGGTTCTGGCGCTG−3' |
| SfiIcx3cx6cba | 5'−TATGCGGCCCAGCCGGCCATGGCAGCTGCNNKNNKNNKTGCNNKNNKNNKNNKNNKNNKTGTGGCGGTTCTGGCGCTG−3' |
| SfiIcx4cx5cba | 5'−TATGCGGCCCAGCCGGCCATGGCAGCTGCNNKNNKNNKNNKTGCNNKNNKNNKNNKNNKTGTGGCGGTTCTGGCGCTG−3' |
| SfiIcx5cx4cba | 5'−TATGCGGCCCAGCCGGCCATGGCAGCTGCNNKNNKNNKNNKNNKTGCNNKNNKNNKNNKTGTGGCGGTTCTGGCGCTG−3' |
| SfiIcx6cx3cba | 5'−TATGCGGCCCAGCCGGCCATGGCAGCTGCNNKNNKNNKNNKNNKNNKTGCNNKNNKNNKTGTGGCGGTTCTGGCGCTG−3' |
| SfiIcx4cx6cba | 5'−TATGCGGCCCAGCCGGCCATGGCAGCTGCNNKNNKNNKNNKTGCNNKNNKNNKNNKNNKNNKTGTGGCGGTTCTGGCGCTG−3' |
| SfiIcx5cx5cba | 5'−TATGCGGCCCAGCCGGCCATGGCAGCTGCNNKNNKNNKNNKNNKTGCNNKNNKNNKNNKNNKTGTGGCGGTTCTGGCGCTG−3' |
| SfiIcx6cx4cba | 5'−TATGCGGCCCAGCCGGCCATGGCAGCTGCNNKNNKNNKNNKNNKNNKTGCNNKNNKNNKNNKTGTGGCGGTTCTGGCGCTG−3' |
| SfiIcx5cx6cba | 5'−TATGCGGCCCAGCCGGCCATGGCAGCTGCNNKNNKNNKNNKNNKTGCNNKNNKNNKNNKNNKNNKTGTGGCGGTTCTGGCGCTG−3' |
| SfiIcx6cx5cba | 5'−TATGCGGCCCAGCCGGCCATGGCAGCTGCNNKNNKNNKNNKNNKNNKTGCNNKNNKNNKNNKNNKTGTGGCGGTTCTGGCGCTG−3' |

**Supplementary table 1.** DNA sequences of the primers (5' to 3') used for 21tet(5) vector generation and library cloning. *Sfi*I restriction sites are underlined. Diversity was introduced

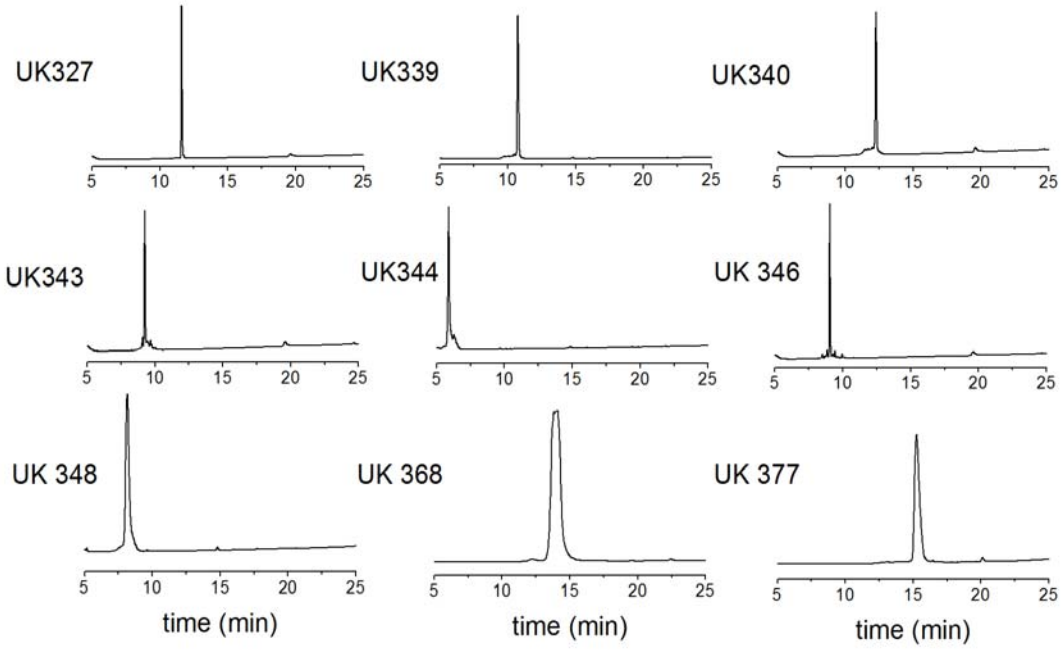
by using the degenerate codon NNK in the synthetic primers. N represents any of the 4 nucleotides and K thymidine (T) and guanosine (G).

## Supplementary figures

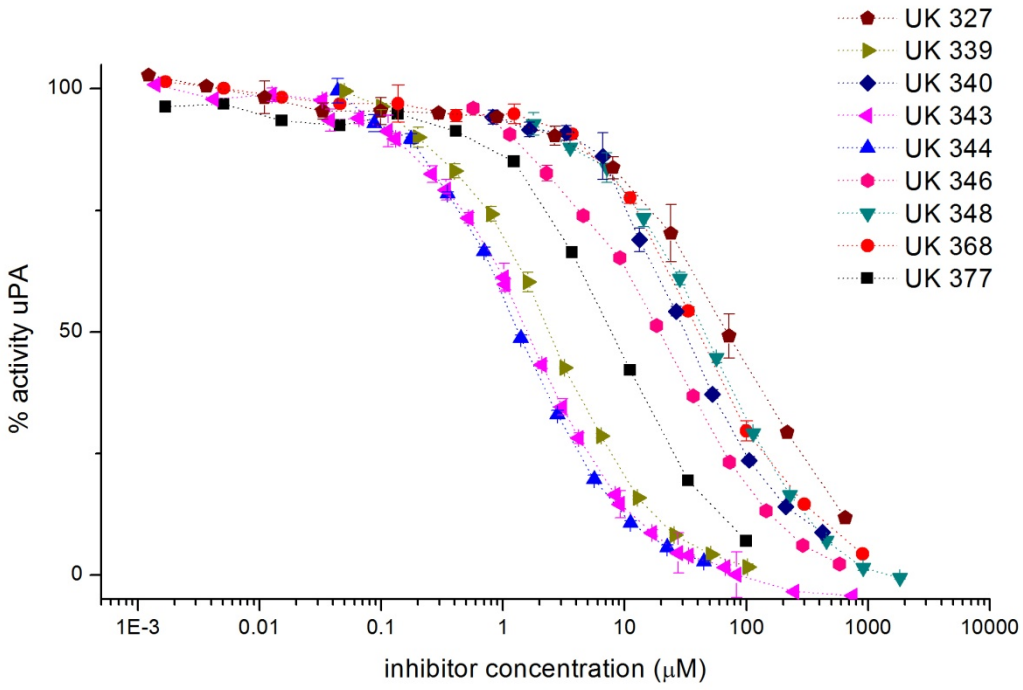



**Supplementary figure 1.** Expression and purification of the catalytic domain of human uPA. (A) Amino acid sequence of the protein expressed in mammalian cells. The Ig κ-chain leader sequence is highlighted in green, 8 random amino acids inserted due to the cloning strategy are shown in grey and the C-terminal fragment of chain A as well as the catalytic domain of human uPA (chain B) are shown in black wherein a mutated residue (N145Q) is highlighted in red. Signal peptide peptidase and plasmin proteolysis cleavage sites are indicated by black broken lines. (B) SDS-PAGE analysis of the protein secreted by mammalian cells before (Pro-uPA, approximately 31 kDa) and after activation with human plasmin (uPA, approximately 28 kDa) under reducing conditions. The protein was stained with Coomassie blue dye. M: molecular weight marker. (C) Active uPA-N145Q (uPA), analyzed by size exclusion chromatography, showed a high degree of purity.

| **Name** | **Peptide sequence** | ***m/z* expected (TBMB modified)** | ***m/z* found (TBMB modified)** |
|---|---|---|---|
| UK327 | ACTARTCPATQVLCG | 1607.7 | 1608.0 |
| UK339 | ACNWKFSLCETQRNQCG | 2100.9 | 2101.6 |
| UK340 | ACNSRFALCSPSSQMCG | 1874.8 | 1875.3 |
| UK343 | ACTEFQTDCRGRSSICG | 1946.8 | 1948.3 |
| UK344 | ACNHAATDCRGRGGPCG | 1758.7 | 1759.0 |
| UK346 | ACKQSVCTARTLCG | 1553.7 | 1554.5 |
| UK348 | ACKHSDCTARFPCG | 1608.7 | 1608.9 |
| UK368 | ACRGGCKFTMCG | 1346.6 | 1346.8 |
| UK377 | ACLQGERGCENRRPSCG | 1948.9 | 1948.9 |




**Supplementary figure 2.** Sequence, mass values predicted and experimentally determined by MALDI-TOF (top) and analytic HPLC chromatograms (bottom) for selected peptides after TBMB modification.




**Supplementary figure 3.** Inhibition of human uPA by the nine TBMB modified peptides tested. The residual activity of the protease was measured with a fluorogenic substrate and plotted against the concentration of bicyclic peptide (logarithmic scale).