Supplemental data to Aberg et al. “TF/FVIIa induces cell survival and gene transcription by transactivation of the IGF-1R” (Thromb Haemost 2014; 111.4)

Suppl. Figure 1: Activation of AKT in PAR2 siRNA silenced cells. A) To silence PAR2 expression, 90 000 MDA-MB-231 cells were seeded in to 12-well plates and Silencer® Select Validated individual siRNA duplexes toward PAR2 and scramble RNA (Ambion Inc.) were transfected into the cells at a concentration of 10 nM with the siPORT Amine transfection agent. mRNA (Assay on Demand, Applied Biosystems) was analyzed at 48 h with β2-microglobulin as house-keeping gene while the protein levels and functional experiments were assessed after 72 h by WB. n = 2. B) MDA-MB-231 cells were incubated with siRNA targeting PAR2. siRNA directed against a scrambled sequence was used as negative control. After 72 h, the cells were treated with 100 ng/ml TRAIL or TRAIL and 100 nM FVIIa as described in the figure. As compared to TRAIL-treatment alone (100%), FVIIa activated AKT in untransfected cells (175 ± 12%), in scramble transfected cells (234 ± 3%), as well as in PAR2 knocked down cells (269 ± 37%). Incubation with siPORT™ Amine Transfection Agent did not induce AKT phosphorylation by its own (105 ± 18%, n =2). n = 4. * = p≤0.05 ** = p≤0.01 *** = p≤0.01 vs TRAIL
Suppl. Figure 2: IGF-1R mRNA and protein levels after siRNA knockdown. In order to silence IGF-1R expression, 90 000 MDA-MB-231 cells were seeded in to 12-well plates and Silencer® Select Validated individual siRNA duplexes toward IGF-1R and scramble RNA (Ambion Inc.) were transfected into the cells at a concentration of 10 nM with the siPORT Amine transfection reagent. mRNA (Assay on Demand, Applied Biosystems) was analyzed at 48 h with β2-microglobulin as house-keeping gene while the protein levels and functional experiments were assessed after 72 h by WB. n = 2
Suppl. Figure 3: The TF/FVIIa-complex phosphorylates the IGF-1R in PAE cells independently of TF cytoplasmic domain. Porcine Aortic Endothelial (PAE) cells stably transfected with wild type TF or TF with the cytoplasmic domain deleted were maintained in DMEM/F12 supplemented with 10% FBS and 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humified chamber at 37°C, 5% CO₂. The cell lines express comparable amounts of TF or TF mutant, both with respect to functional TF and number of TF molecules per cell (Siegbahn et al, Thromb Haemost. 2005 Jan;93(1):27-34). The cells were verified for IGF-1R expression by using A) WB with an antibody toward the β-chain (rabbit, Cell Signaling Tech., USA) and B) PLA using an antibody pair targeting the α-(rabbit, Santa Cruz Biotech., USA) and β-chain (mouse, Merck Millipore, USA) as described in Methods. The images are magnifications from maximum intensity projections over the entire image volume, captured with a 20X objective on an AxioImager M2 fluorescence microscope. C) The cell lines were then treated with 100 nM FVIIa for 30 minutes and the proximity of antibodies targeting IGF-1Rβ (rabbit, Cell Signaling Tech., USA) and phosphotyrosine (mouse, Merck Millipore, USA) was assessed by PLA. D) The total PLA signals per cells were determined for the different treatments by analyzing the captured Z-stacks from C with Blob Finder software and normalized to untreated MDA-MB-231 cells. n (D) = 4. * = p≤0.05 vs. untreated cells.
Suppl. Figure 4: TF/FVIIa-dependent transactivation of the IGF-1R analyzed by WB.

MDA-MB-231 cells were left untreated or treated with FVIIa (50 nM) for the indicated times. The cells were then lysed in Triton X100 buffer and the IGF-1R was immunoprecipitated using Dynabeads® Protein A beads (Invitrogen). After IP, the proteins were detected by SDS-PAGE and immunoblotting using pY (Merck Millipore, USA) and IGF-1Rβ (Cell Signaling, Cat# 3194) antibodies. The membrane bound proteins were scanned with the Odyssey Infrared Imaging System (Licor) and quantified with the Odyssey V3.0 software. The image shows immunoreactivity from one representative blot. n = 3-6
Suppl. Figure 5: Thrombin is not involved in the TF/FVIIa-induced activation of the IGF-1R. MDA-MB-231 cells were pretreated with 5 U/ml of the thrombin inhibitor hirudin for 20 minutes prior 30 minutes of treatment with FVIIa (10 nM). The proximity of antibodies targeting IGF-1Rβ (rabbit, Cell Signaling Tech., USA) and phosphotyrosine (mouse, Merck Millipore, USA) was assessed by PLA. The total PLA signals per cell were determined for the different treatments by analyzing Z-stacks, captured with a 20X objective on an AxioImager M2 fluorescence microscope, with Blob Finder software and normalized to untreated MDA-MB-231 cells. n = 5. * = p≤0.05 vs. untreated cells.
Suppl. Figure 6A: FVIIa-treatment does not induce phosphorylation of tyrosine 612 of IRS1. MDA-MB-231 cells were left untreated or treated with either FVIIa (100nM), IGF-1 (100 ng/ml) or insulin (100 ng/ml). The cells were then washed and harvested for SDS-PAGE and immunoblotting using phospho-IRS1( tyr612) (Millipore, Cat.#09-432) and IRS1 (Cell Signaling, Cat# 3194) antibodies. The membrane bound proteins were scanned with the Odyssey Infrared Imaging System (Licor) and quantified with the Odyssey V3.0 software. Left panel shows immunoreactivity from one representative blot. The right panel shows results from densitometric analysis of band intensities from 5 independent experiments.

Suppl. Figure 6B: FVIIa treatment does not induce phosphorylation of tyrosine 612 of IRS1. MDA-MB-231 cells were left untreated or treated with either FVIIa (50 nM, 20 and 30 min.) or IGF-1 (100 ng/ml,10 min.). The cells were then lysed in Triton X100 buffer and IRS1 was immunoprecipitated using Dynabeads® Protein A beads (Invitrogen). After IP, the proteins were detected by SDS-PAGE and immunoblotting using pY ( Millpore, Cat# 05-1050) and IRS1 (Cell Signaling, Cat# 3194) antibodies and scanned as in fig S4A. The image shows immunoreactivity from one representative blot.
Suppl. Figure 7: Growth of MDA-MB-231 cells after treatment with FVIIa

MDA-MB-231 cells were seeded into a 6-well plate and 24 h later, the medium was changed and the cells were either left alone or treated with 100 nM FVIIa for 6 h. The cells were then detached and counted using Flow Count beads on a Cytomics FC 500 flow cytometer (Beckman Coulter Inc., Bromma Sweden). During the time course of the experiment, no increase in cell growth was detected in the FVIIa-treated group (756 800 ± 61 000) as compared to the control group treated with 10% FBS (776 800 ± 23 400). n = 2