Supplementary Material: Breast Tumor Cell Invasion and Pro-Invasive Activity of Cancer-Associated Fibroblasts Co-Targeted by Novel Urokinase-Derived Decapeptides

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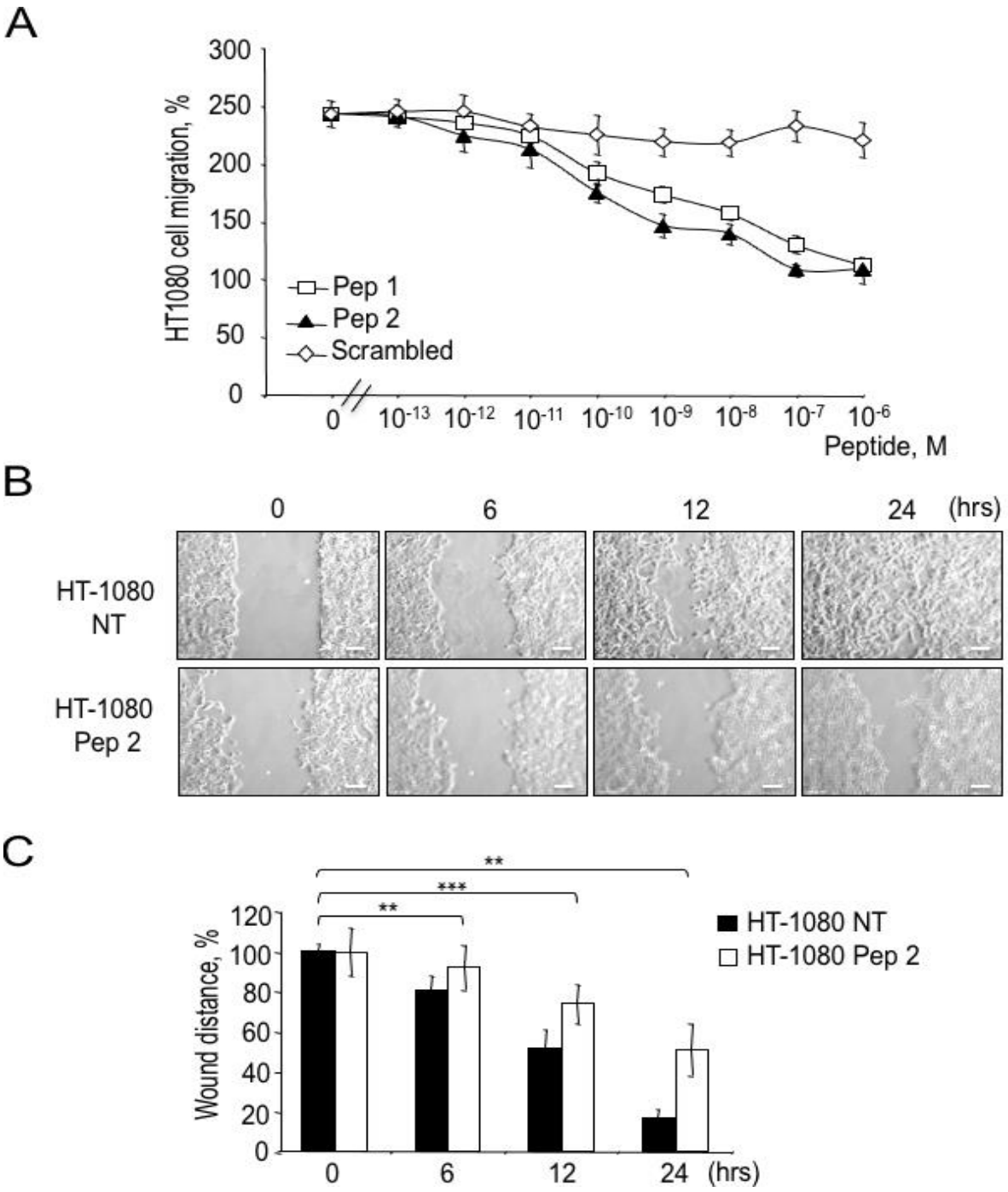


Figure S1. Inhibition of HT1080 wound healing closure by Pep 1 or Pep 2. (**A**) 2 × 10^4 HT1080 cells/sample were pre-incubated with or without Pep 1, Pep 2 or scrambled Pep 2 peptides at the

indicated concentrations for 1 h at 37 °C and then allowed to migrate toward DMEM-1% FBS in Boyden chambers, as described in the legend to Figure 3D. (**B**) HT1080 cells were grown to confluence in 12-well plates with inserts causing a defined 500 μm cell-free scratch (ibidi, GmbH, Martinsried, Germany), pre-incubated with 100 nM Pep 2 or diluents in serum-free medium for 1 h at 37 °C and then exposed to 1% FBS, in the presence of Pep 2. Images were taken every 6 h for 18 h and representative images are shown. 20× Magnification, Scale bar, 50 μm. (**C**) The wound width at time 0 is taken as 100% and the average wound width at each time point is calculated as relative to that. Bars represent the average of three separate experiments undertaken in triplicate. Differences between data sets were determined by the Student's *t*-test, (** $p < 0.005$; *** $p < 0.001$, Student's *t*-test).

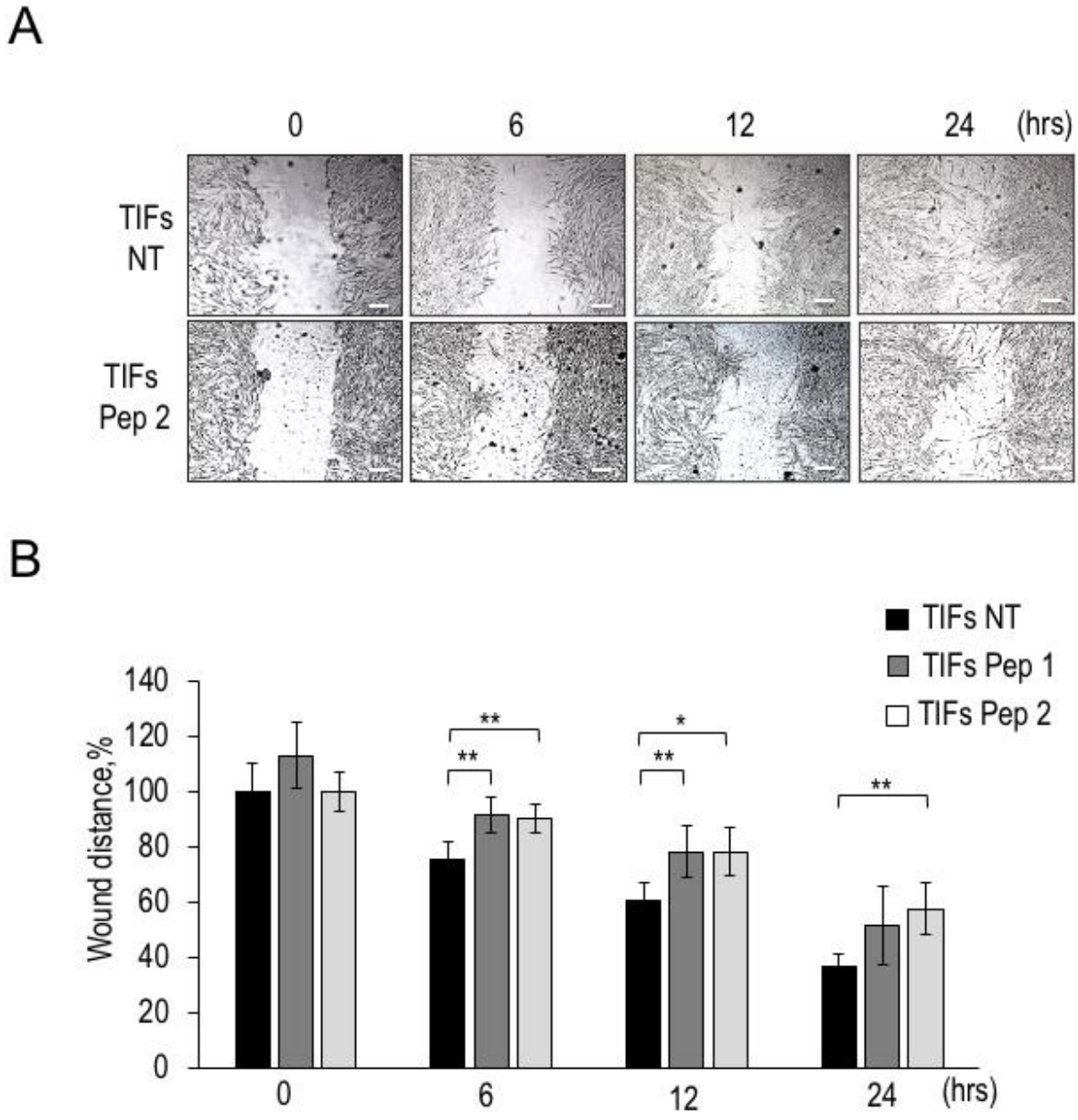


Figure S2. Inhibition of TIF wound healing assay closure by Pep 1 or Pep 2. (**A**) Wound healing assay of 1 × 10^5 TIF fibroblasts was conducted as described in the legend to figure S1B and monitored for the indicated time points. Representative images are shown, 20× Magnification, Scale bar, 50 μm. (**B**) The wound width was calculated as the average of three separate experiments undertaken in triplicate. (** $p < 0.005$; *** $p < 0.001$, Student's *t-test*).

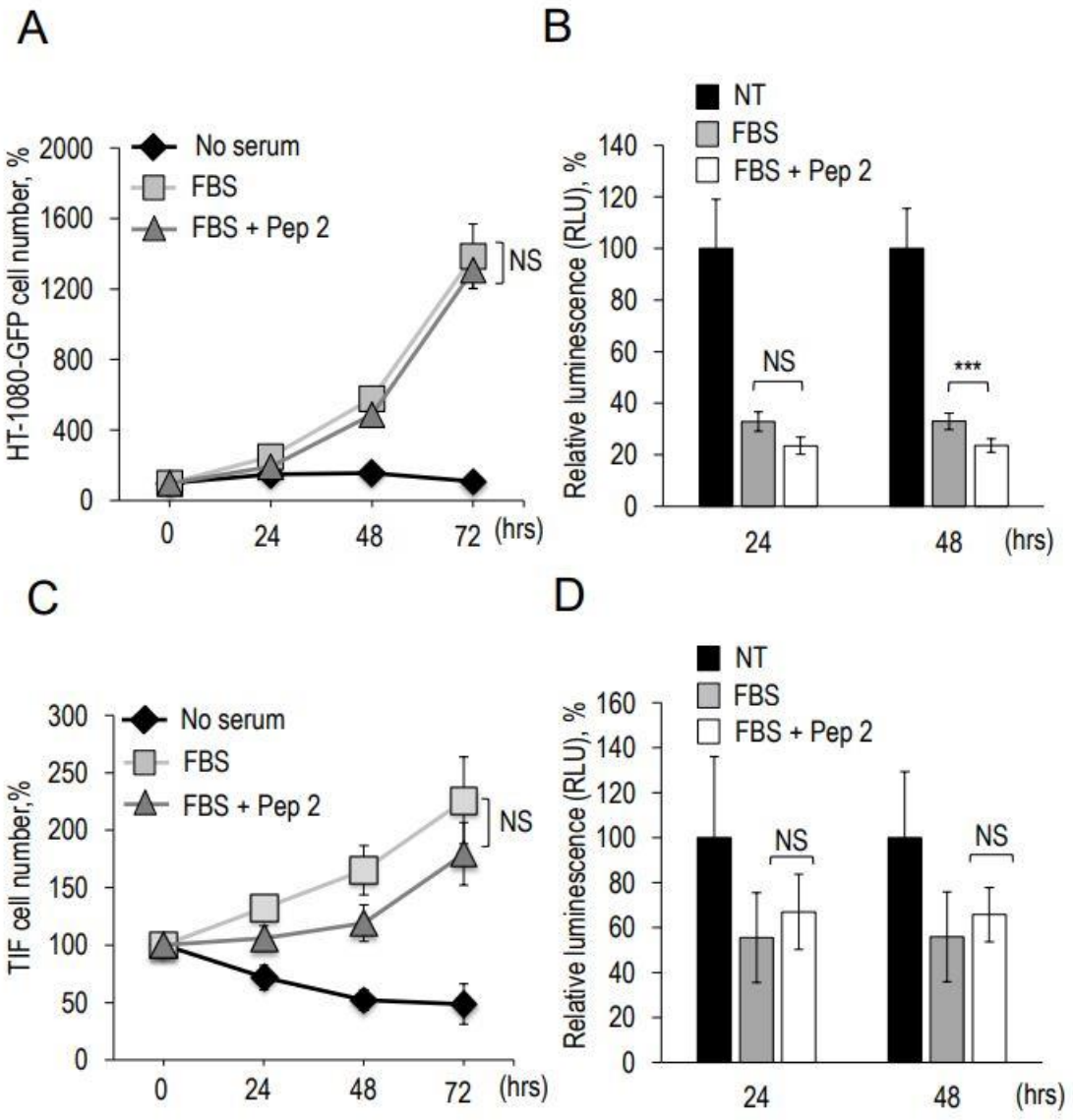


Figure S3. Unaffected proliferation and apoptosis of TIF fibroblasts and HT1080-GFP fibrosarcoma cells exposed to Pep 2. 2 × 10^4 HT1080-GFP (**A**) or TIFs (**C**) were seeded in 12 well plates for 24 h in DMEM-10% FBS and serum-starved for 6 hrs. Then, cells were counted (0 h = 100%) and grown in DMEM (No serum) or DMEM-10% FBS (FBS), in the presence of Pep 2 (FBS + Pep 2), for 72 h (NS, non-significant). For the caspase 3/7 luminometric apoptosis assay, 2 × 10^3 HT1080-GFP (**B**) or TIFs (**D**) were seeded in 96 well white plates for 24 h in DMEM-10% FBS, serum-starved for 6 h and grown in DMEM-10% FBS or DMEM, in the presence or in the absence of 100 nM Pep 2. Caspase 3/7 activity was measured by a luminometric assay after 24 and 48 h. Histograms represent the average of two separate experiments undertaken in triplicate (*** $p < 0.001$, Student's *t-test).*

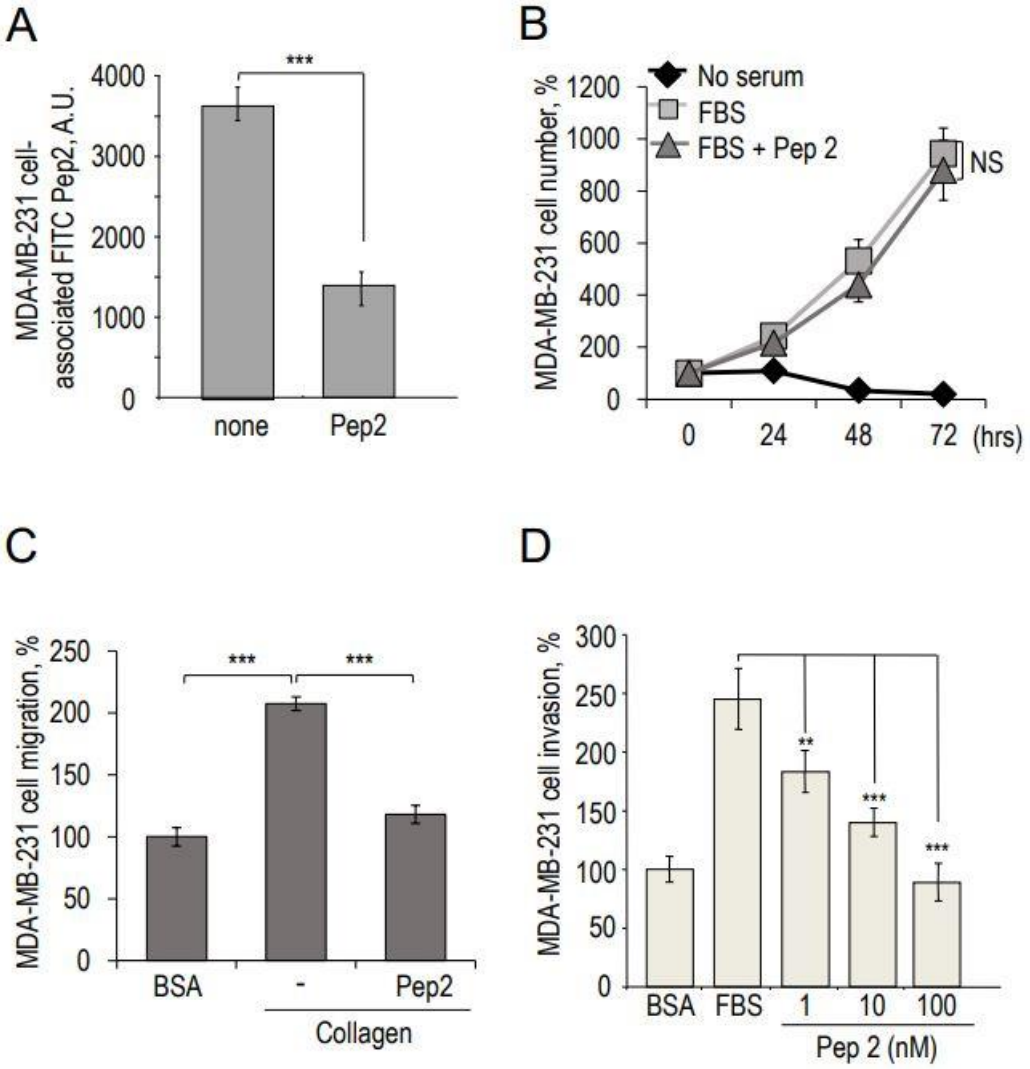
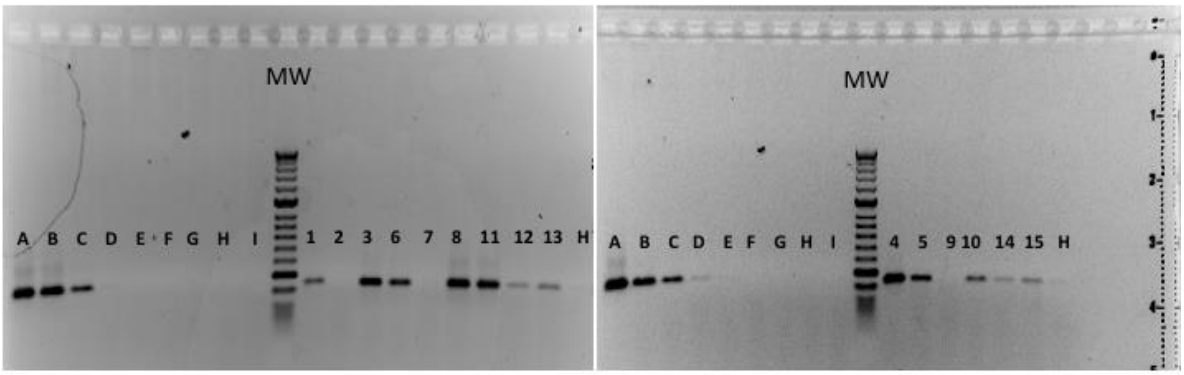


Figure S4. Interaction of Pep 2 with MDA-MB-231 breast adenocarcinoma cells. (**A**) 1.7 × 10^6 cells/sample were assayed for FITC-Pep 2 binding, as described in Figure 4C. Data represent a mean of three independent experiments performed in duplicate (*** $p < 0.001$, Student's *t*-test). (**B**) 2.5 x 10^4 MDA-MB-231 cells were seeded in 12 well plates for 24 h in RPMI-10% FBS and serum-starved for 24 h. Then, cells were counted (0 h = 100%) and incubated in RPMI (No serum) or RPMI-10% FBS (FBS) or in RPMI-10% FBS, in the presence of 100 nM Pep 2 (FBS + Pep 2), for 72 hrs. Cell counts represent the mean of three independent experiments performed in triplicate. (**C**) 2 × 10^4 MDA-MB-231 were pre-treated for 1 h at 37 °C with 100 nM Pep 2 peptide or diluents and allowed to migrate toward 20 µg/mL collagen type VI. Data represent a mean of two independent experiments performed in triplicate (*** $p < 0.001$, Student's *t*-test). (**D**) 2 × 10^4 MDA MB-231 cells/sample were pre-incubated with Pep 2 at the indicated concentrations and assayed for FBS-dependent invasion as described in Figure 4A (** $p < 0.005$; *** $p < 0.001$, Student *t*-test).



A= 10 000 000 HT1080
B= 1 000 000 HT1080 + 9 mln P19
C= 100 000 HT1080 + 9 900 000 P19
D= 10 000 HT1080 + 9 990 000 P19
E= 1 000 HT1080 + 9 999 000 P19
F= 100 HT1080 + 9 999 900 P19
G= 10 HT1080 + 9 999 990 P19
H= 1 HT1080 + 9 999 999 P19
I= 10 000 000 P19
H= Healthy lung

Figure S5. Uncropped image of the 1% agarose gel from Figure 2C.

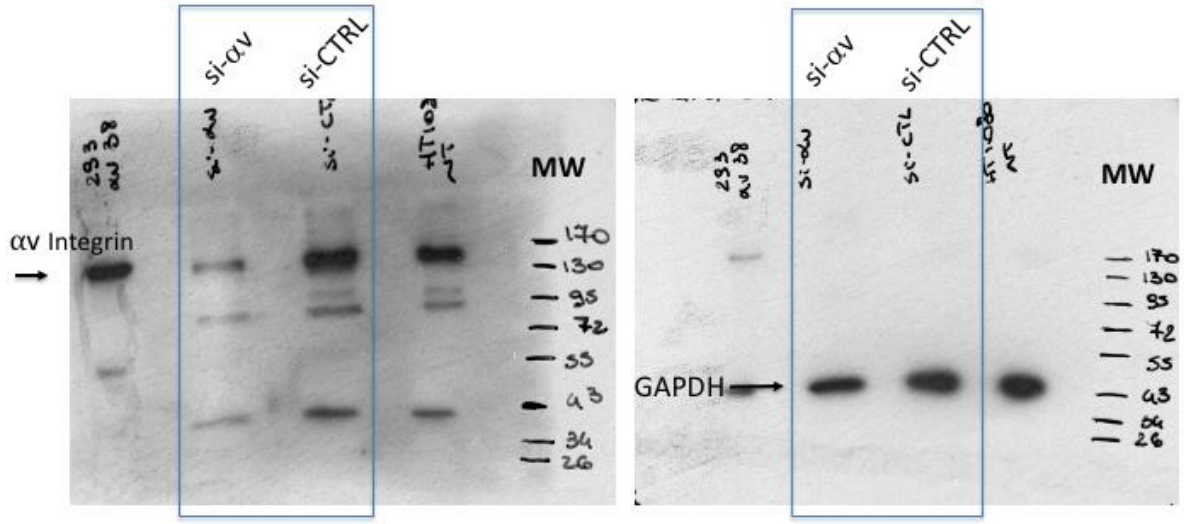


Figure S6. Whole blot image from Figure 4D.

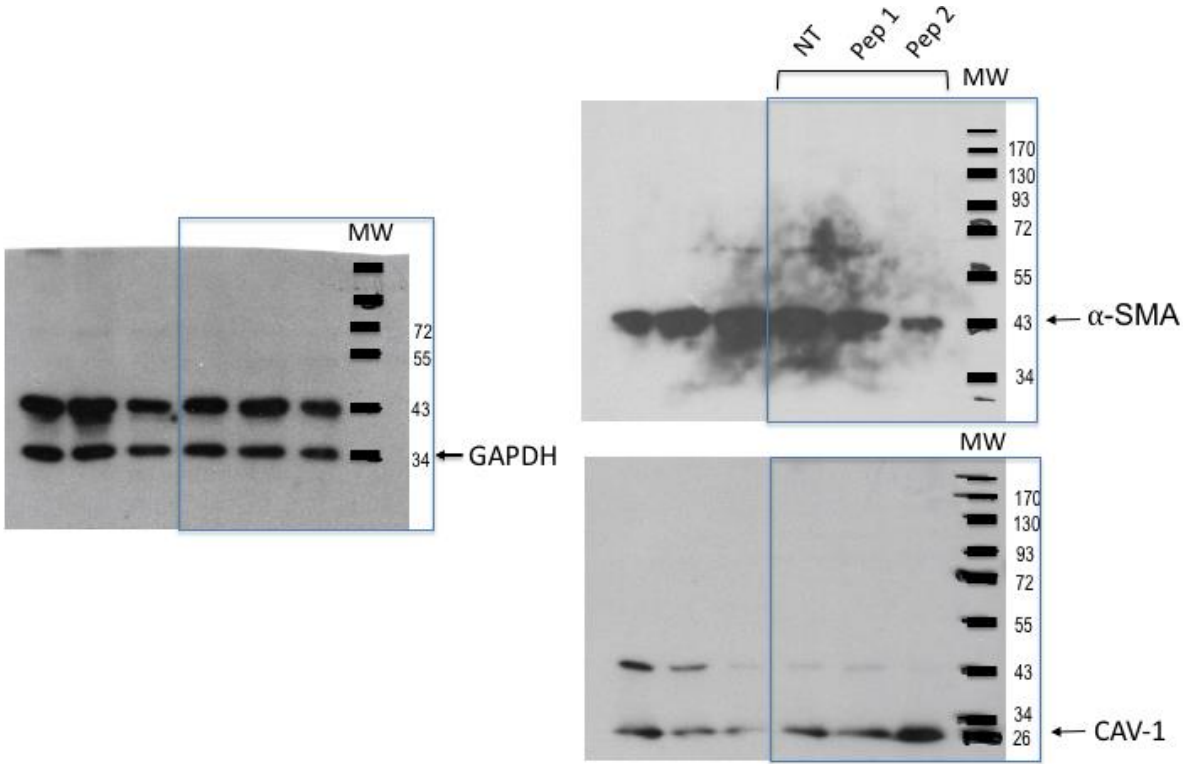


Figure S7. Whole blot image from Figure 5C.

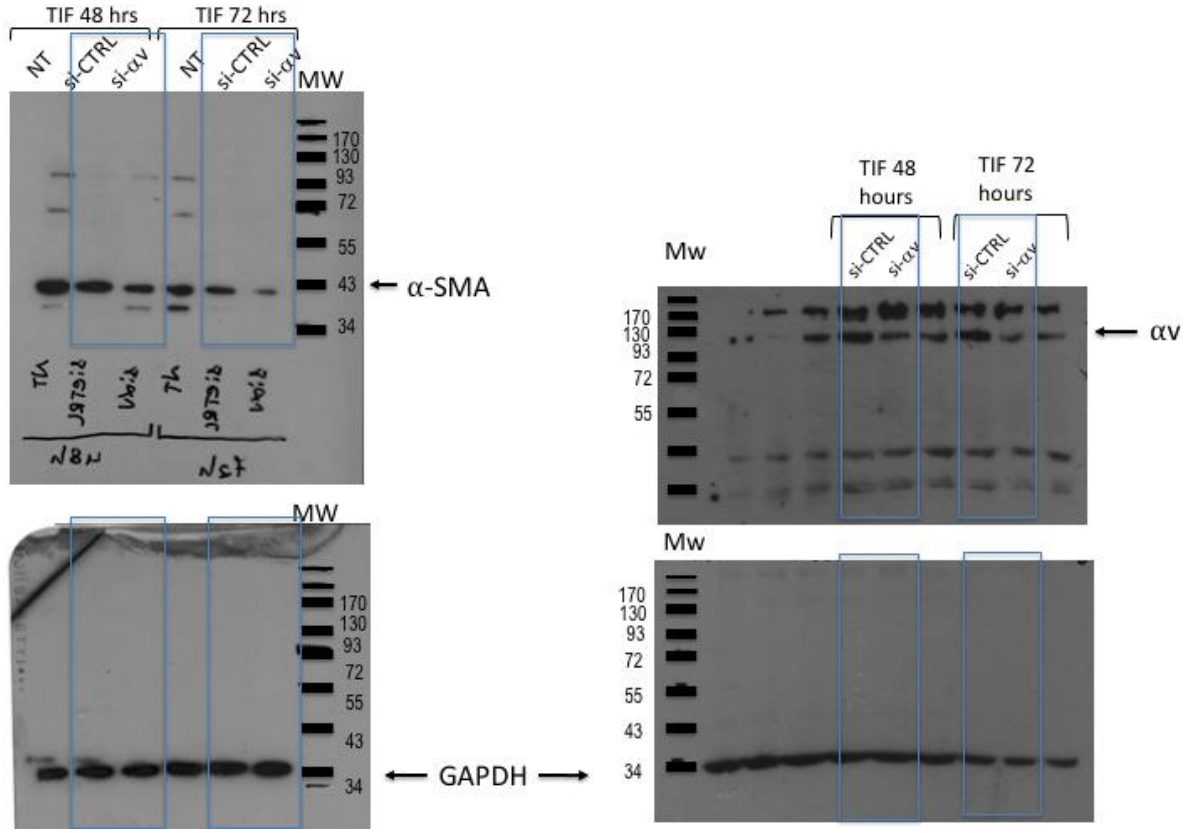


Figure S8. Whole blot image from Figure 6E.

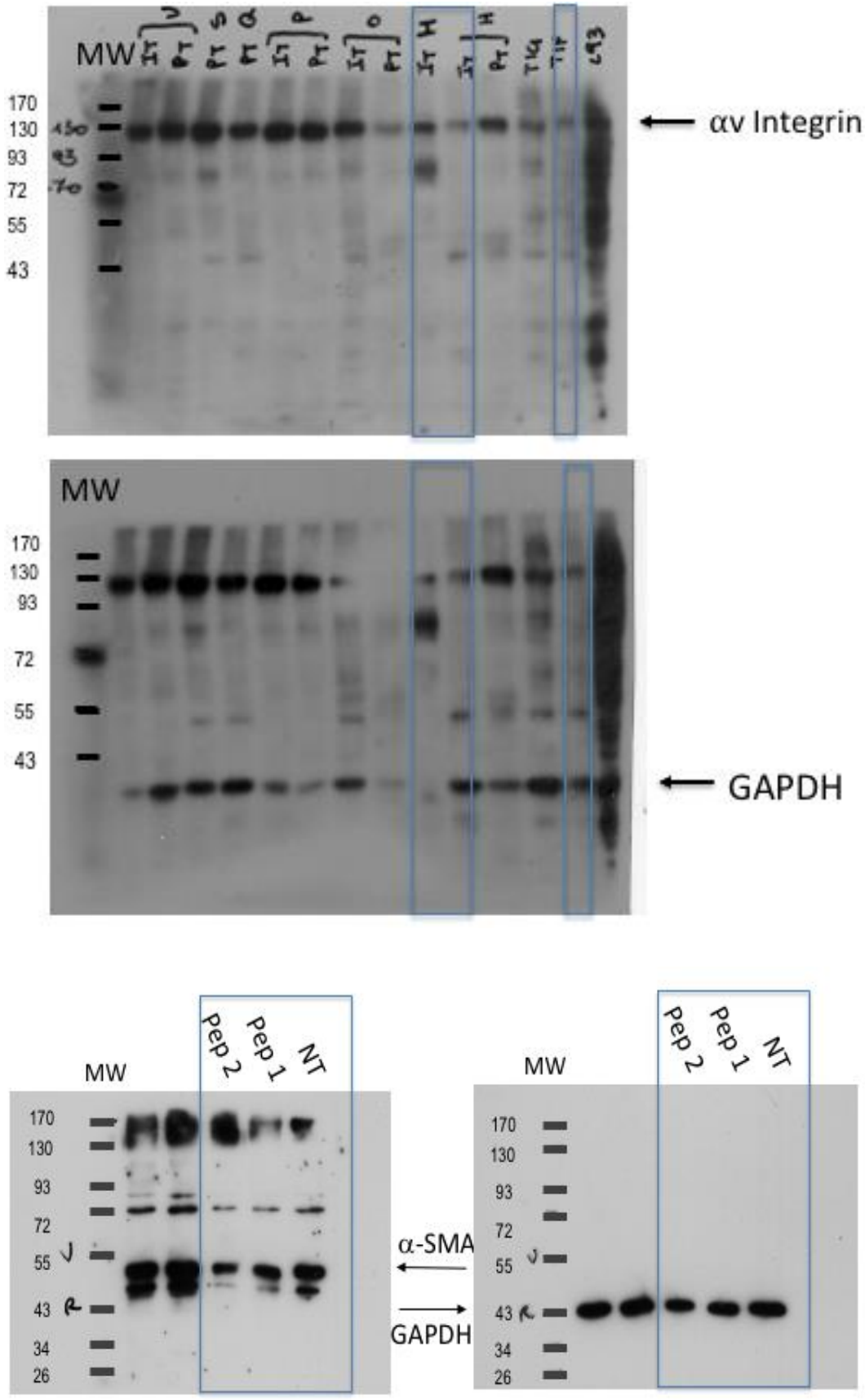


Figure S9. Whole blot image of Figure 7D,E.

Table S1. NMR resonance assignments [a] of Pep 1 in water solution at 10 °C.

Residue	NH ($^3J_{\alpha N}$, −Δδ/ΔT)[b]	$C^{\alpha}H$	$C^{\beta}H$	Others
Lys[136]	8.39 (6.6, 8.7)	4.58	1.72, 1.81	1.47(γ); 1.69(δ); 2.99(ε); 7.60 (ζ)
Pro[137]		4.44	2.32, 1.90	2.02(γ); 3.87, 3.65(δ)
Glu[138]	8.71 (6.3, 7.3)	4.29	2.04, 1.93	2.31(γ)
Ser[139]	8.52 (7.4, 9.5)	4.75	3.88, 3.75	
Pro[140]		4.73	2.38, 1.94	2.05(γ); 3.85, 3.68 (δ)
Pro[141]		4.40	2.34, 1.92	2.05(γ); 3.84, 3.67(δ)
Glu[142]	8.68 (5.9, 5.7)	4.21	2.02, 1.94	2.31(γ)
Glu[143]	8.55 (6.1, 6.2)	4.26	2.03, 1.93	2.30(γ)
Leu[144]	8.40 (7.2, 8.3)	4.34	1.66	1.60(γ); 0.94, 0.88 (δ)
Lys[145]	8.40 (7.2, 8.4)	4.29	1.79, 1.86	1.42, 1.47 (γ); 1.68 (δ); 2.99 (ε)

[a] Obtained at pH = 5.5, with TSP (δ 0.00 ppm) as reference shift. Chemical shifts are accurate to ± 0.02 ppm. [b] $^3J_{\alpha N}$ coupling constants in Hz−Δδ/ΔT = temperature coefficients (ppb/K) calculated in the range 10–25 °C. Further signals: CH_3CO, 2.01 ppm; $CONH_2$, 7.19, 7.61 ppm.

Table S2. NMR resonance assignments [a] of Pep 2 in water solution at 10 °C.

Residue	NH ($^3J_{\alpha N}$, −Δδ/ΔT)[b]	$C^{\alpha}H$	$C^{\beta}H$	Others
Lys[136]	8.40 (6.9, 9.7)	4.59	1.71, 1.81	1.49(γ); 1.71(δ); 2.99(ε); 7.60 (ζ)
Pro[137]		4.43	2.32, 1.88	2.03(γ); 3.88, 3.63(δ)
cGlu[138]	8.53 (7.3, 9.4)	4.29	2.07, 1.99	2.36, 2.44(γ)
Ser[139]	8.36 (8.9, 9.1)	4.52	3.60, 3.70	
Pro[140]		5.06	2.08, 2.54	1.78, 1.97(γ); 3.56 (δ)
Pro[141]		4.37	2.44, 1.87	2.13(γ); 3.91, 3.62(δ)
Glu[142]	8.90 (5.8, 4.8)	4.24	2.06, 1.98	2.29(γ)
Glu[143]	8.67 (5.8, 7.2)	4.18	2.05, 1.94	2.25, 2.33(γ)
Leu[144]	8.23 (7.1, 6.8)	4.34	1.64	1.59(γ); 0.96, 0.89 (δ)
cLys[145]	8.15 (7.4, 9.2)	4.21	1.78, 1.82	1.40,1.49 (γ); 1.55 (δ); 3.08,3.38 (ε); 8.16 (ζ)

[a] Obtained at pH = 5.5, with TSP (δ 0.00 ppm) as reference shift. Chemical shifts are accurate to ± 0.02 ppm. [b] $^3J_{\alpha N}$ coupling constants in Hz−Δδ/ΔT = temperature coefficients (ppb/K) calculated in the range 10−25 °C. Further signals: CH_3CO, 2.01 ppm; $CONH_2$, 7.14, 7.61 ppm.

Table S3. NOE derived upper limit constraints of Pep 1.

Residue number	Residue type	Atom type	Residue number	Residue type	Atom type	Upper Limit Distance
135	ACE	QH	136	LYS	HN	3.62
136	LYS	HN	136	LYS	QB	3.21
136	LYS	HN	136	LYS	QG	6.38
136	LYS	HN	137	PRO	HD2	5.50
136	LYS	HN	137	PRO	HD3	5.50
136	LYS	HA	137	PRO	HD2	3.61
136	LYS	HA	137	PRO	HD3	3.61
136	LYS	HA	137	PRO	QD	3.00
137	PRO	HA	138	GLU	HN	2.49
137	PRO	HA	139	SER	HN	4.97
137	PRO	HD2	138	GLU	HN	5.50
137	PRO	HD3	138	GLU	HN	5.50
138	GLU	HN	138	GLU	HA	2.86
138	GLU	HN	138	GLU	QB	3.21
138	GLU	HN	138	GLU	QG	5.82
138	GLU	HA	139	SER	HN	3.44
139	SER	HN	139	SER	HB2	3.39
139	SER	HN	139	SER	HB3	3.39
139	SER	HN	139	SER	QB	3.14
139	SER	HN	140	PRO	QD	5.34

Table S3. *Cont.*

Residue number	Residue type	Atom type	Residue number	Residue type	Atom type	Upper Limit Distance
139	SER	HA	140	PRO	HD2	3.11
139	SER	HA	140	PRO	HD3	3.11
139	SER	HA	140	PRO	QD	2.67
140	PRO	HA	141	PRO	HD3	2.99
140	PRO	HA	142	GLU	HN	5.13
141	PRO	HA	142	GLU	HN	3.36
141	PRO	HA	143	GLU	HN	3.00
141	PRO	HD2	142	GLU	HN	3.00
141	PRO	HD3	142	GLU	HN	5.50
142	GLU	HN	142	GLU	HA	2.83
142	GLU	HN	142	GLU	QB	3.50
142	GLU	HN	142	GLU	QG	5.42
142	GLU	HA	143	GLU	HN	3.25
143	GLU	HN	143	GLU	QB	3.12
143	GLU	HN	143	GLU	QG	6.25
143	GLU	HA	144	LEU	HN	2.52
143	GLU	HB2	144	LEU	HN	4.01
143	GLU	HB3	144	LEU	HN	4.01
143	GLU	QB	144	LEU	HN	3.49
143	GLU	QG	144	LEU	HN	6.38
144	LEU	HN	144	LEU	QB	4.18
144	LEU	HN	144	LEU	HG	4.19
144	LEU	HN	144	LEU	QD1	6.53
144	LEU	HN	144	LEU	QD2	6.53
144	LEU	HA	144	LEU	QD1	6.53
144	LEU	HA	144	LEU	QD2	6.53
145	LYS	HN	145	LYS	HB2	3.95
145	LYS	HN	145	LYS	HB3	3.95
145	LYS	HN	145	LYS	QB	3.18
145	LYS	HN	145	LYS	HG2	5.50
145	LYS	HN	145	LYS	HG3	5.50
145	LYS	HA	145	LYS	QD	6.38