SUPPLEMENTARY INFORMATION

Tumor-penetrating nanosystem strongly suppresses breast tumor growth

Shweta Sharma[†], Venkata Ramana Kotamraju[†,‡] , Tarmo Mölder[#], Allan Tobi[#], Tambet Teesalu[†,‡,#], Erkki Ruoslahti[†,‡*]

[†]Sanford-Burnham-Prebys Medical Discovery Institute, Cancer Research Center, La Jolla, CA, USA 92037

[‡]Center for Nanomedicine and the Department of Cell, Molecular and Developmental Biology, University of California, Santa Barbara, CA, USA 93106

[#]Laboratory of Cancer Biology, Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia 50411

*Correspondence to: Dr. Erkki Ruoslahti, Sanford-Burnham-Prebys Medical Discovery Institute, La Jolla, CA, 92037, USA; email: ruoslahti@sbpdiscovery.org

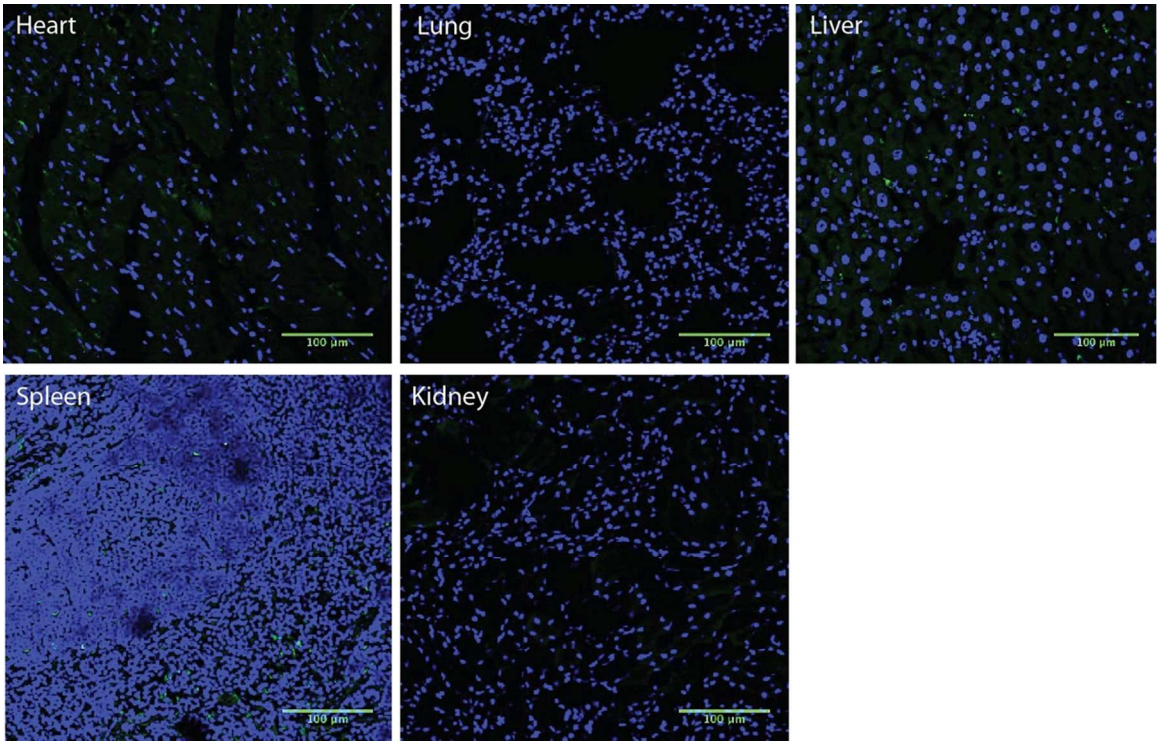
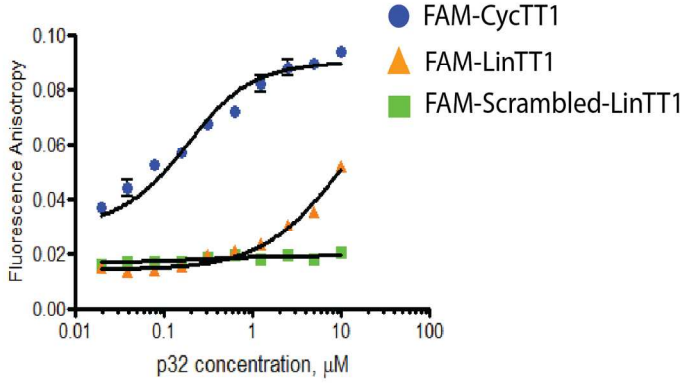


Figure S1. LinTT1-NWs do not accumulate to normal organs. Tumor bearing mice were i.v. injected with 7.5 mg/kg/wt of FAM-labeled TT1-NW. After 5 h the mice were perfused through the heart with PBS, and tissues were collected, sectioned, and analyzed for NW accumulation. Merged image: green, NWs; blue, nuclei. Scale bars, 100µm.

A



B

Peptide	kd (µM)
FAM-CycTT1	0.13 ± 0.034
FAM-LinTT1	8.7 ± 2.623
FAM-Scrambled-LinTT1	ND

Figure S2. TT1 binding affinity for p32. (A) Fluorescence anisotropy assay showing of binding affinity of FAM-cyclicTT1 and FAM-linearTT1 peptides for p32 protein. (B) Dissociation constants (K_D) based on binding curves fitted on Michaelis-Menten kinetics. Measurements were performed with PheraStar FS plate reader (BMG Labtech, Ortenberg, Germany) on 384-well plate.

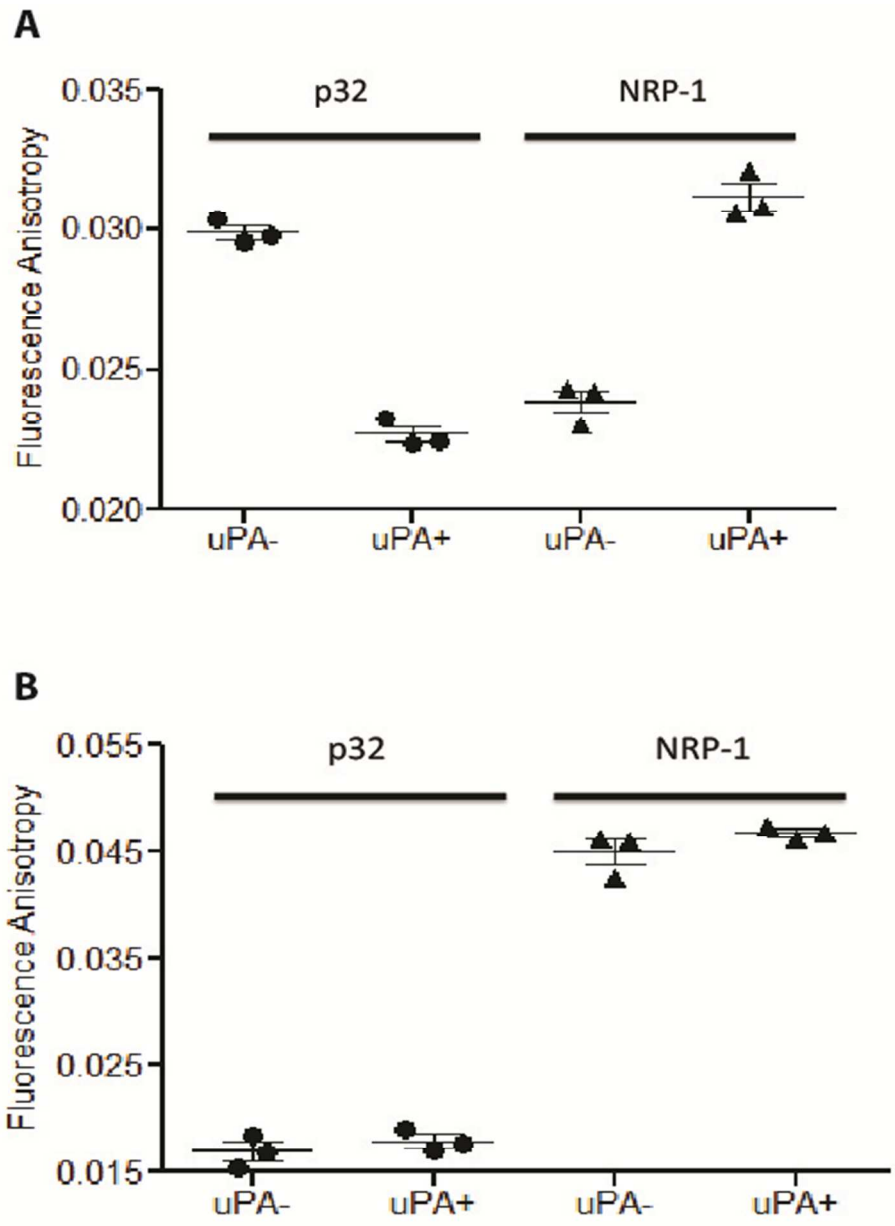


Figure S3. Binding of LinTT1 to p32 and NRP-1 before and after uPA treatment. Receptor switch caused by urokinase-type plasminogen activator cleavage. Fluorescence anisotropy assay showing binding of FAM-linearTT1 (A) or FAM-RPARPAR (B) peptide to p32 and neuropilin-1 b1b2 protein with or without urokinase-type plasminogen activator (uPA) cleavage. Measurements were performed with PheraStar FS plate reader (BMG Labtech, Ortenberg, Germany) on 384-well plate.

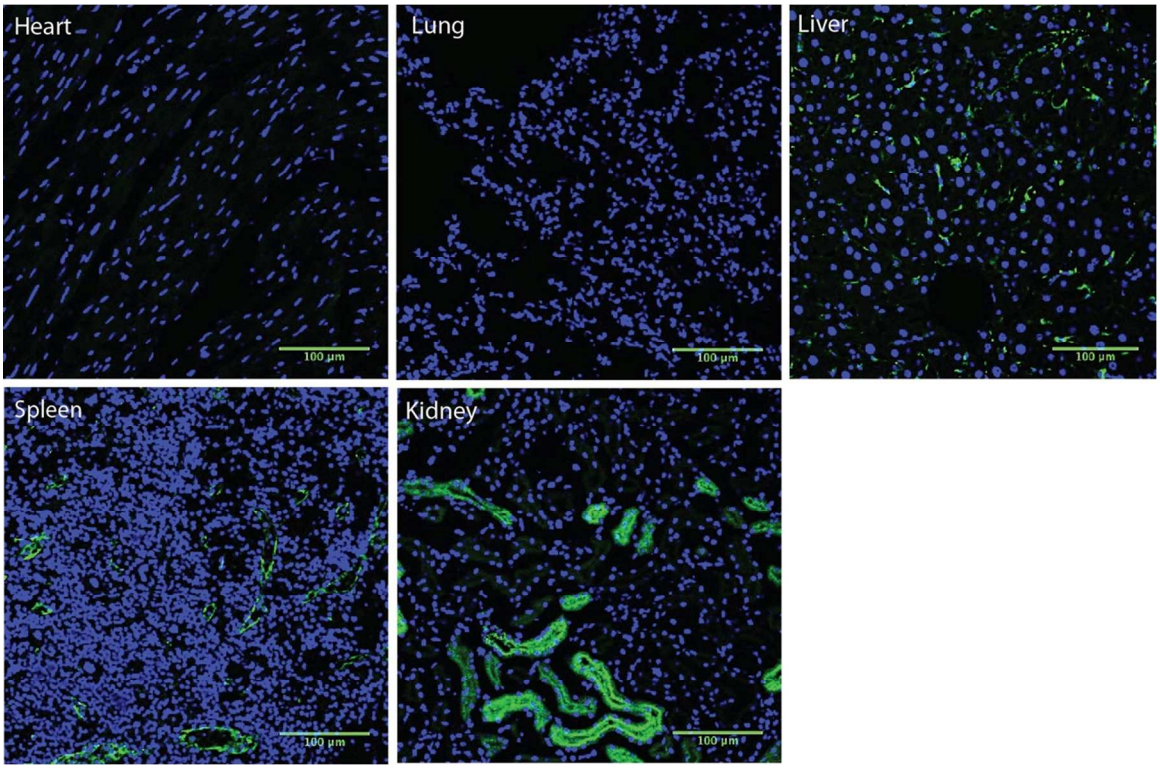


Figure S4. Distribution of TT1-$_D$(KLAKLAK)$_2$-NWs in normal organs. LinTT1-$_D$[KLAKLAK]$_2$-NW (green) were injected into tumor bearing mice, and tissues were collected and analyzed by confocal microscopy. Blue, nuclei stained with DAPI. (Scale bars, 100µm.)

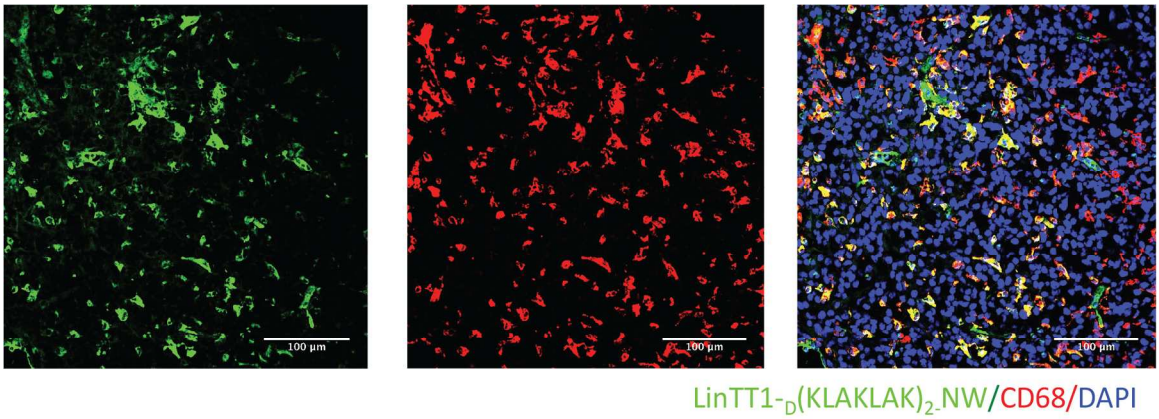
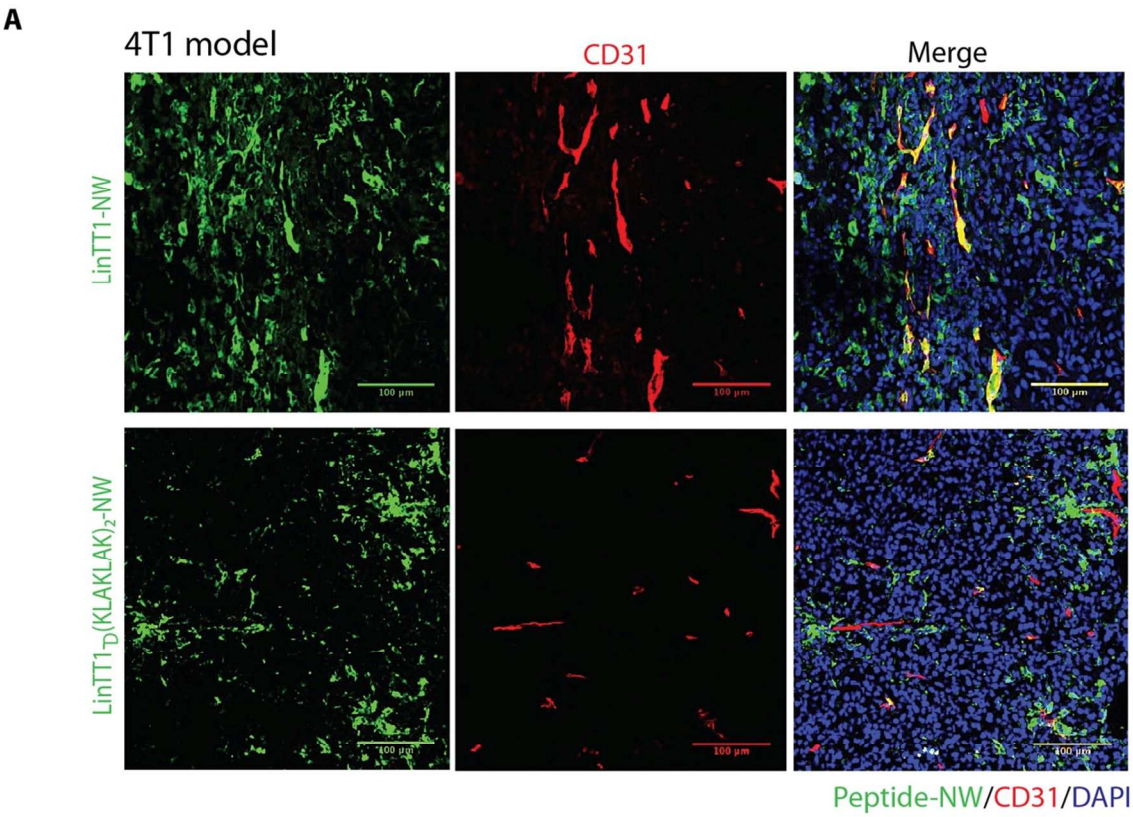


Figure S5. LinTT1-$_D$(KLAKLAK)$_2$-NWs target the tumor associated macrophages (TAM) /myeloid cells in breast cancer models. Mice bearing 4T1 tumors were injected with LinTT1-$_D$[KLAKLAK]$_2$-NW (green) (7.5mg iron/kg), and the NWs were allowed to circulate for 5 hours. The mice were perfused through the heart with PBS, and tumors and organs were collected and processed for fluorescence microscopy. Co-localization is visualized by yellow staining in the merged image. Green (peptide-NW) overlapping with red (CD68) is 47%, while red overlapping with green is 79%. Merged image: green, NWs; red, CD68; blue, nuclei. Scale bars, 100µm.


A
4T1 model
CD31
Merge
LinTT1-NW
LinTT1-D(KLAKLAK)2-NW
100 μm
Peptide-NW/CD31/DAPI

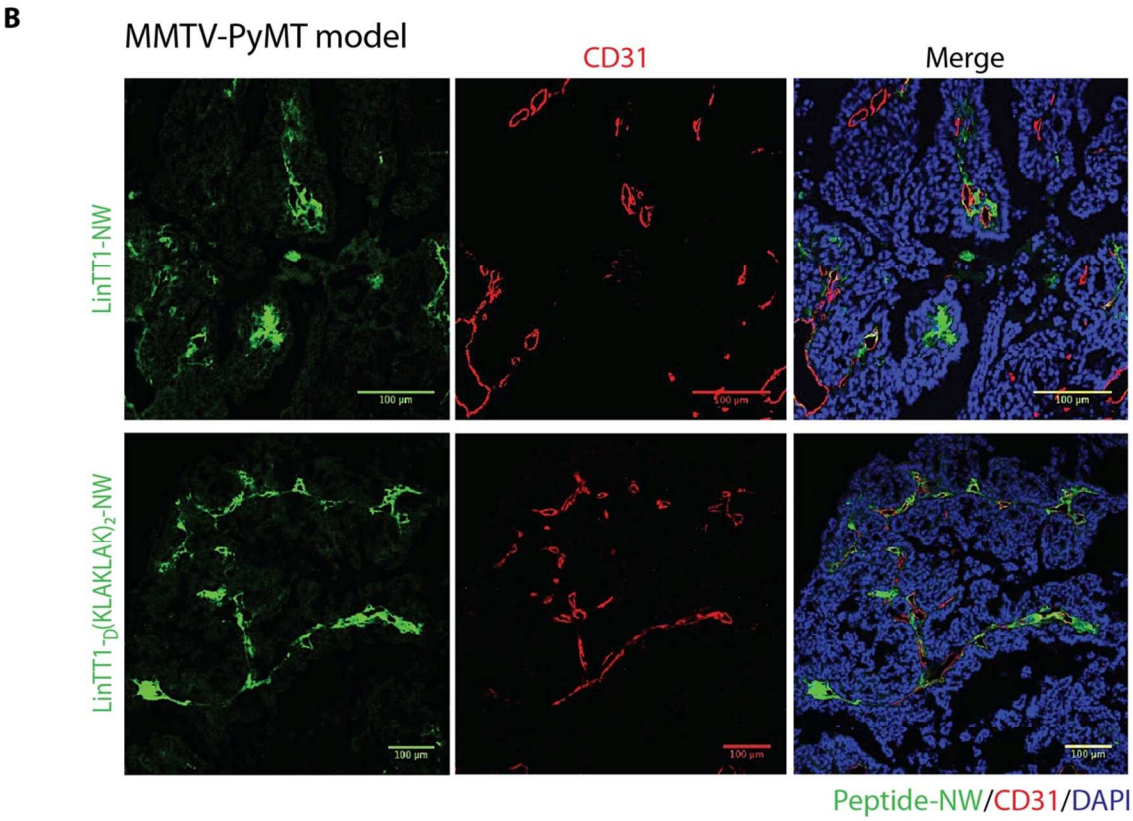

B
MMTV-PyMT model
CD31
Merge
LinTT1-NW
LinTT1-D(KLAKLAK)2-NW
100 μm
Peptide-NW/CD31/DAPI

Figure S6. Homing of LinTT1-NW and LinTT1-$_D$(KLAKLAK)$_2$-NWs to tumors in breast cancer models. Mice bearing (A) 4T1 and (B) MMTV-PyMT orthotopic tumors were injected with peptide coated-NWs (7.5mg iron/kg), and the NWs were allowed to circulate for 5 hours. The mice were perfused through the heart with PBS, and tumors and organs were collected and processed for fluorescence microscopy. Merged image: green, NWs; red, CD31; blue, nuclei. Scale bars, 100μm.

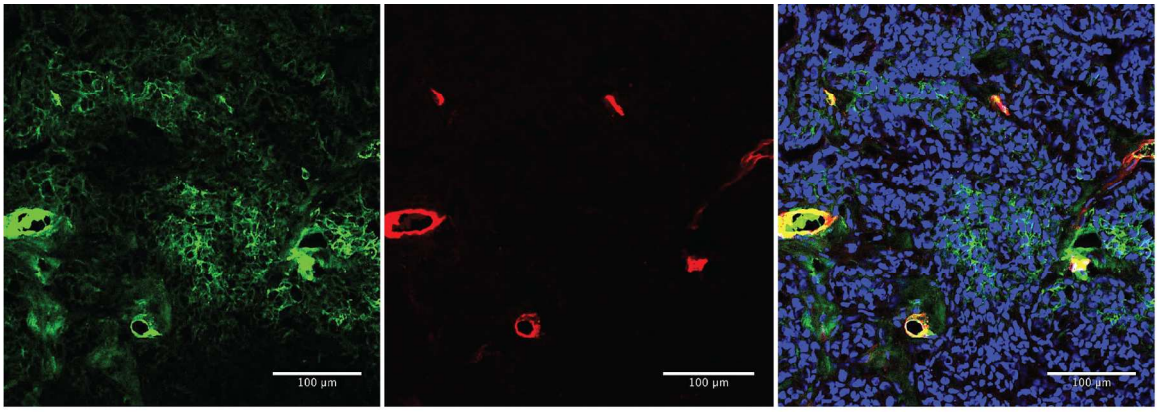


Figure S7. Homing of LinTT1-$_D$(KLAKLAK)$_2$-micelles to tumors in breast cancer models. Mice bearing MCF10CA1a orthotopic tumors were injected with peptide coated-micelles (200ug of micelles/mouse), and micelles were allowed to circulate for 3 hours. The mice were perfused through the heart with PBS, and tumors and organs were collected and processed for fluorescence microscopy. Merged image: green, micelles; red, CD31; blue, nuclei. Scale bars, 100μm.

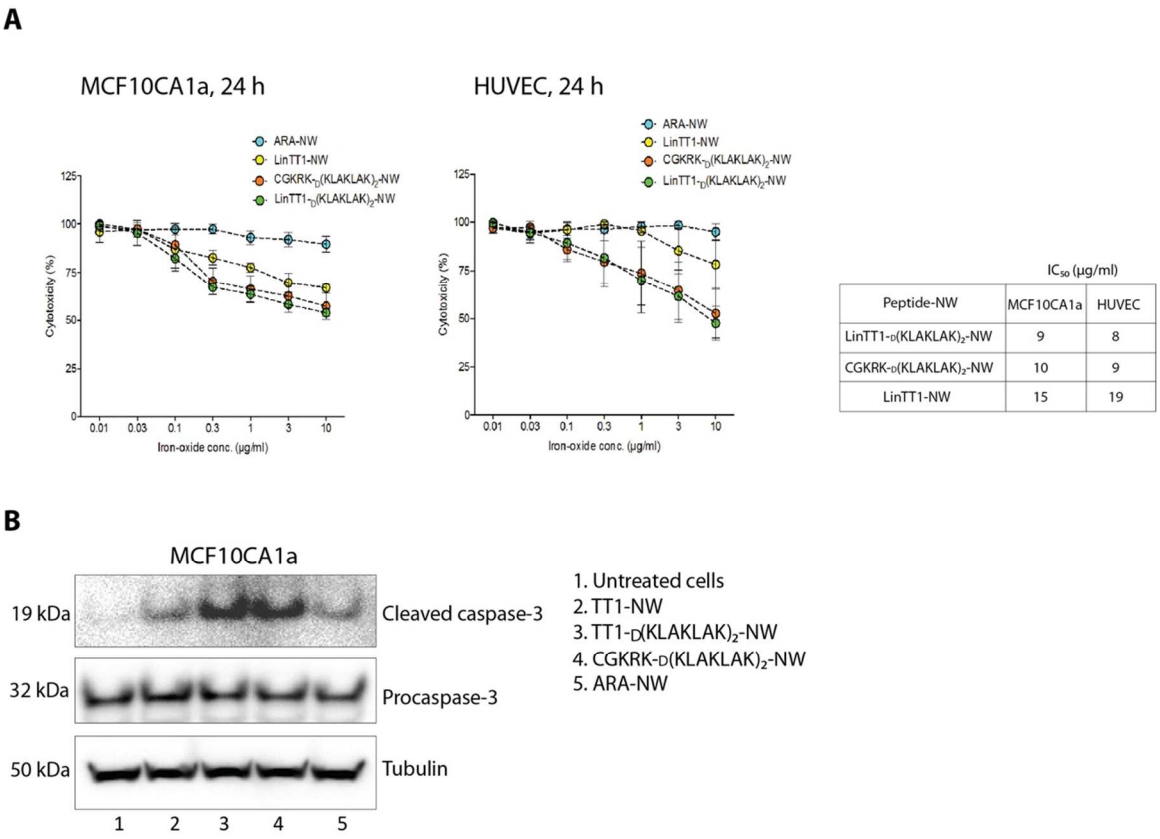


Figure S8. LinTT1$_D$(KLAKLAK)$_2$-NW induces apoptosis. (A and B) Cell viability of MCF10CA1a cells and actively growing HUVEC after 24 of incubation with peptide-NWs as measured by MTT assay. Both cell lines were highly sensitive to the targeted $_D$(KLAKLAK)$_2$-NWs. NWs coated with TT1 without $_D$(KLAKLAK)$_2$ showed slight activity, which is in agreement of the weak *in vivo* activity of these NWs. Non-targeted ARA-NWs were essentially inert. The inset shows the half-maximal cytotoxic concentrations (IC_{50}) of the various peptide-NWs after 24 h of incubation with cells.

(**C**) Immunoblot analysis of cleaved (active) and procaspase-3 in cells treated with peptide-NWs for 72 hrs. LinTT1$_D$(KLAKLAK)$_2$-NWs and CGKRK$_D$(KLAKLAK)$_2$-NWs effectively activated caspase-3, while TT1-NWs were less active paralleling the activity of these NWs in vivo and in the MTT assay.

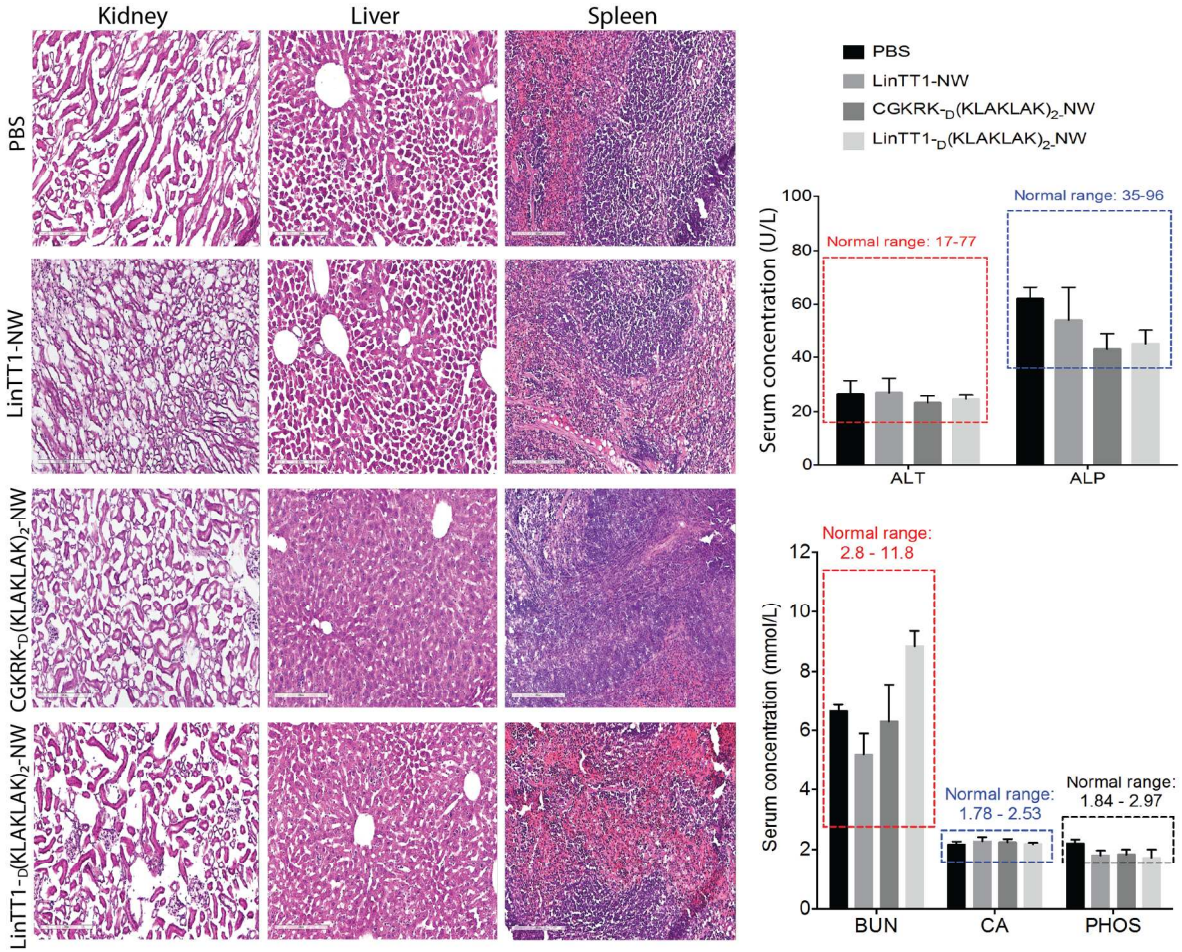


Figure S9. Lack of evidence for organ damage after NW treatment. Panels on the left show H&E staining of kidney, liver and spleen sections from mice injected with the indicated peptide-NWs every other day for 3 weeks (7.5 mg/ of iron/kg). Upper right panel, serum levels of the liver damage indicators alanine aminotransferase (ALT) and alkaline phosphatase (ALP); n=3 per group Lower right panel, kidney functional indicators serum blood urea nitrogen (BUN), calcium (CA), and phosphatase (PHOS); n=3 per group. The $LinTT1_D(KLAKLAK)_2$-NW BUN value appeared slightly elevated but the difference to the other NW preparations was not significant. The values for the remaining markers listed in the Supplemental Method section were all normal in

all samples. These results indicate that the NWs we tested are not overtly toxic upon systemic delivery at effective anti-tumor doses.

Methods

Peptide homing to tumors and tumor treatment

Mice bearing breast tumors were used in peptide-NW homing studies when they reached 0.5-1 cm in size. Peptide-conjugated NWs were injected into the tail vein (7.5 mg of iron/kg of body weight in 100µl of PBS). After 5-6 hours of NW circulation the mice were euthanized by cardiac perfusion with PBS under anesthesia, and tumors and organs were dissected and analyzed for NW homing.

Tumor treatment was started when the tumors were about 50-60 mm^3 in diameter. The tumor-bearing mice were intravenously injected with peptide-coated NWs at a dose of 7.5 mg /kg on alternate days for three weeks. A control group was similarly injected with 100µl of PBS. Tumor volume was calculated by using the following formula: volume = (d2 × *D)/2,* where *d* and *D* are the smallest and the largest tumor diameters, respectively.

Histological and immunostaining of tumors

Tissues from mice treated for three weeks with the various NW preparations were fixed in 4% paraformaldehyde overnight at 4°C, cryoprotected in 30% sucrose overnight, and frozen in optimum cutting temperature embedding medium. Tissue sections (5-7µm) were cut and stained with hematoxylin and eosin or processed for immunostaining. For immunostaining, tissue sections were first incubated for 1 hour at room temperature with 10% serum from the species in which the secondary antibody was generated, followed by incubation with the primary antibody overnight at 4°C. Rat monoclonal anti–mouse CD31 (10 µg/mL) was from BD Pharmingen. The primary antibody was detected with Alexa 594 goat anti–rat secondary antibody (1:1000; Molecular Probes). Each

staining experiment included sections stained only with secondary antibodies as negative controls. Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole; 5 µg/mL; Molecular Probes). The sections were mounted in gel/mount mounting medium (Biomeda) and viewed under a LSM 710 NLO Multiphoton Laser Point Scanning Confocal Microscope (Zeiss). Each staining experiment included sections stained with the secondary antibody only as a negative control. Immunoperoxidase staining with anti Ki67 was performed using DAB (MP Biomedicals) reaction and the sections were counterstained with hematoxylin (Sigma Aldrich).

Click-iT TUNEL 647 Alexa Fluor imaging assay (Invitrogen) was carried out according to the manufacturer's instructions. Nuclei were counterstained with DAPI. The sections were mounted in Gel/Mount mounting medium (Biomeda, Foster City, CA) and viewed under a Fluoview 500 confocal microscope (Olympus America, Center Valley, PA).

Assays for organ toxicity

Liver and kidney functions were evaluated by blood chemistry analyses after the three weeks of treatment. The analyses included alanine aminotransferase (ALT), amylase, alkaline phosphatase (ALP), albumin, total bilirubin, globulin, sodium, potassium, calcium, inorganic phosphorous, blood urea nitrogen, creatinine, glucose, and total protein. The tests were performed using VetScan VS2 machine at Sanford Burnham Prebys Medical Discovery Institute.

MTT assay

Cytotoxicity of peptide-NWs was estimated by using in vitro MTT assay (Molecular Probes). Cells were cultured in complete growth medium at a density of $5X10^3$ cells/well in 96-well plates. After overnight incubation at 37°C, culture medium was replaced with various concentrations of peptide-NWs and further incubated for 24 and 72h. MTT solution (10µL of 5 mg/mL) was added to each

well. After 3 h, the medium was removed, and the cells were mixed with 100μL of DMSO:MEOH (1:1 vol/vol). The absorbance was measured at 595 nm by a microplate reader (Thermo Scientific, USA).

Immunoblot analysis of caspase-3.

MCF10CA1a cells and HUVEC were seeded at a density of $2X10^5$ cells per well and cultured 24 h at 37°C. Cells were then treated with 100ug/mL peptide-NWs for 24 and 48 h and lysed with RIPA buffer (Pierce) according to the manufacturer's instructions. The lysates were separated by SDS/PAGE. After transfer of the proteins onto nitrocellulose membranes for 2 h at 200 mA, the membrane was treated for 1 h at room temperature with TBS-0.05% Tween containing 5% milk and incubated with 1 mg/mL anti-caspase 3 (Cell Signaling) and pro-caspase antibody (gift of Dr. Stan Krajewski).

Fluorescence anisotropy (polarization) assay

The FA assay was performed as described. [28]

Protease cleavage and fluorescence anisotropy assay

Protease cleavage and fluorescence anisotropy measurements were done in FA-buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 0.05% NP-40, 1 mM MgCl2, 1 mM CaCl2). 50 uL of 100 nM FAM-peptide (FAM-linearTT1 and FAM-RPARPAR) was mixed with 10 uL of uPA (10,000 U/mL, Calbiochem) and incubated for 1.5 hours at 37 ^oC. The proteins were used at 5 uM concentration. PheraStar FS plate reader (BMG Labtech, Ortenberg, Germany) with a 384-well plate was used for the fluorescence measurements.

Micelle synthesis

Micelles were synthesized and characterized as described[34,35.]