Supplementary Materials for

Targeting Oncofetal Fibronectin and Neuropilin-1 in solid tumors with PL2 peptide

Prakash Lingasamy[1,4]*, Allan Tobi[1], Kaarel Kurm[1], Olav Tammik[2], Tambet Teesalu[1,3]†

[1] Laboratory of Precision and Nanomedicine, Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia.

[2] General Surgery and Oncology Surgery Centre, North Estonia Medical Centre, Tallinn, Estonia

[3] Materials Research Laboratory, University of California, Santa Barbara, CA 93106, USA.

[4] Celvia CC AS, Tartu 50411, Estonia.

†Corresponding author:

Tambet Teesalu, PhD

Laboratory of Precision and Nanomedicine, Department of Biomedicine and Translational Medicine, University of Tartu, 50411 Tartu, Estonia. Phone: 372-5397-4441; Fax: 372-737-4268; E-mail: tambet.teesalu@ut.ee

The file includes:

Supplementary Materials and Methods

Cloning, expression, and purification of recombinant proteins

Vector Construction: The plasmids pASK75-Fn7B8 and pASK75-Fn789 were kindly provided by Prof. Dr. Arne Skerra. Gene fragments for Fn-EDB domains were amplified from pASK75-Fn7B8 plasmids using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific Inc # F-537L) with the primer pairs 5′-CTCCTCTCATATGGAGGTGCCCCAACTCA -3′ and 5′-CAGACACTCGAGTTATCACGTTTGTTGTGT -3′ for the domain EDB (the underline refers to restriction sites for NdeI and XhoI). The amplified fragments were then cloned into a pET28a+ plasmid containing a N-terminal His_6-tag for *E. coli* expression.

Expression and purification of Fn-EDB: The pET28a+ Fn-EDB plasmids were transformed into *E. coli* BL21 Rosetta 2 (DE3) pLysS strain (Novagen, #70956). Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma, # I6758) at a final concentration of 0.5 mM, and the bacteria were cultured at 18 °C for 16 hours. The bacterial cells were collected by centrifugation and lysed by sonication (Bandelin Sonopuls HD 2070, Germany). The cleared bacterial lysate was purified using HiTrap IMAC HP columns (GE Healthcare # 17-0920-05) on the ÄKTA purification system (GE Healthcare) according to the manufacturer's protocol. The eluates were analyzed by SDS-PAGE and dialyzed against PBS using 3.5 kDa cut-off 3 mL Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific #66330). The protein purity, size, and de novo sequence were analyzed by SDS-PAGE and mass spectrometry.

Single-chain antibodies: The EDB L19 Single-Chain Variable Fragment (ScFv) sequences were obtained according to US patent applications (US8455625 B2). The scFv gBlocks Gene

sequences were synthesized and cloned into a pET28a+ vector by GenScript USA Inc. (Piscataway, NJ USA) and termed ScFv-L19-EDB. The DNA sequence was confirmed by Sanger sequencing. The *Escherichia coli* strains BL21 Rosetta™ 2 (DE3) pLysS (Novagen, #70956) were transformed with the recombinant pET28a+ ScFv-L19-EDB plasmids. The soluble ScFV antibody fragments were expressed in *E. coli* and purified from soluble cytoplasmic fractions using rProtein A GraviTrap Sepharose (GE Healthcare # 28-9852-54). The purified proteins were analyzed by 12% SDS-PAGE, and a pull-down assay further verified ScFv interaction with the target protein.

Polyclonal antibody: Rabbits were immunized with injections of the recombinant Fn-EDB for rabbit polyclonal antibody (RPAbs) production and purified by protein G and affinity column (LabAs Ltd, Tartu, Estonia). The rabbit PAbs were validated with ELISA and Western blot, and stored at 4 °C in a storage buffer.

Supplementary Figures

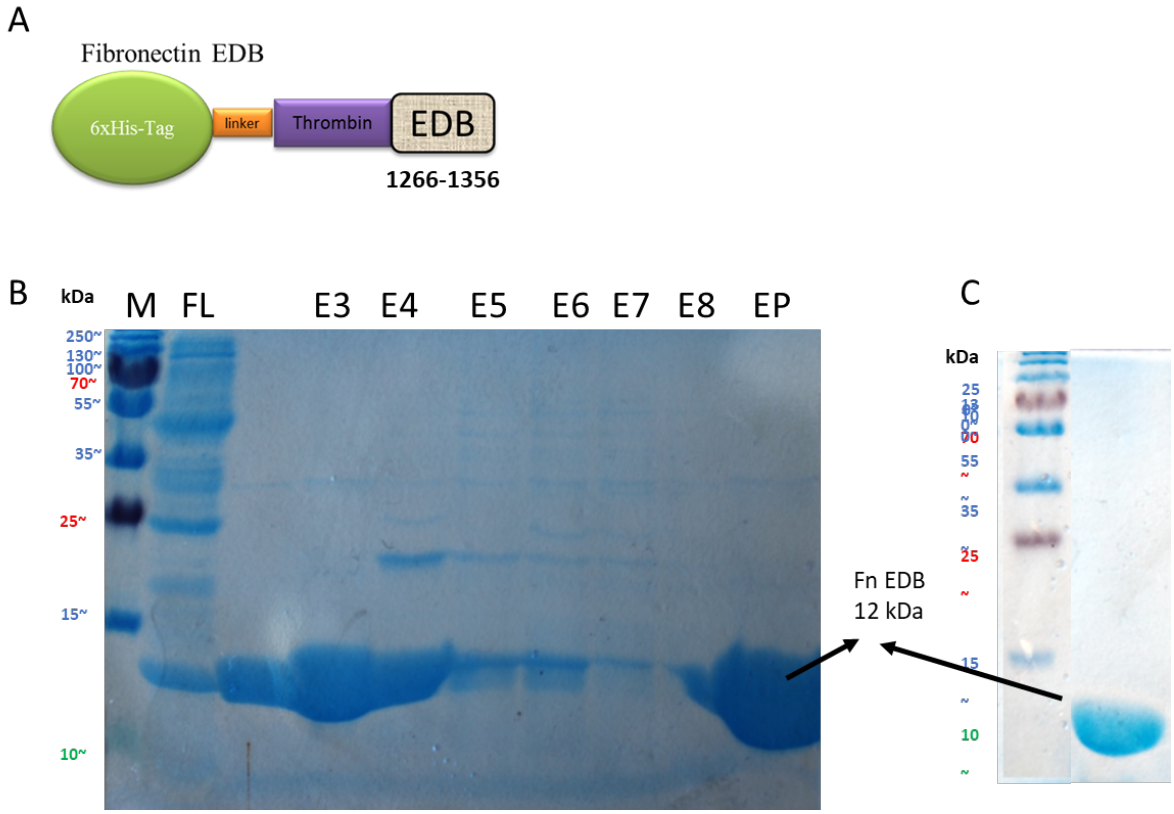

Figure S1. Expression and purification of Fn-EDB domain. (A) Schematic diagram of the Fn-EDB expression cassette cloned in the pET28a+ plasmid. (B) SDS-PAGE analysis of purified recombinant protein using HisTrap column on the ÄKTA system. Lane FL: Flow-through, lanes E2-E8: Elution fractions, and lane EP: Elution pool. (C) SDS-PAGE analysis of the final purified Fn-EDB protein after dialysis.

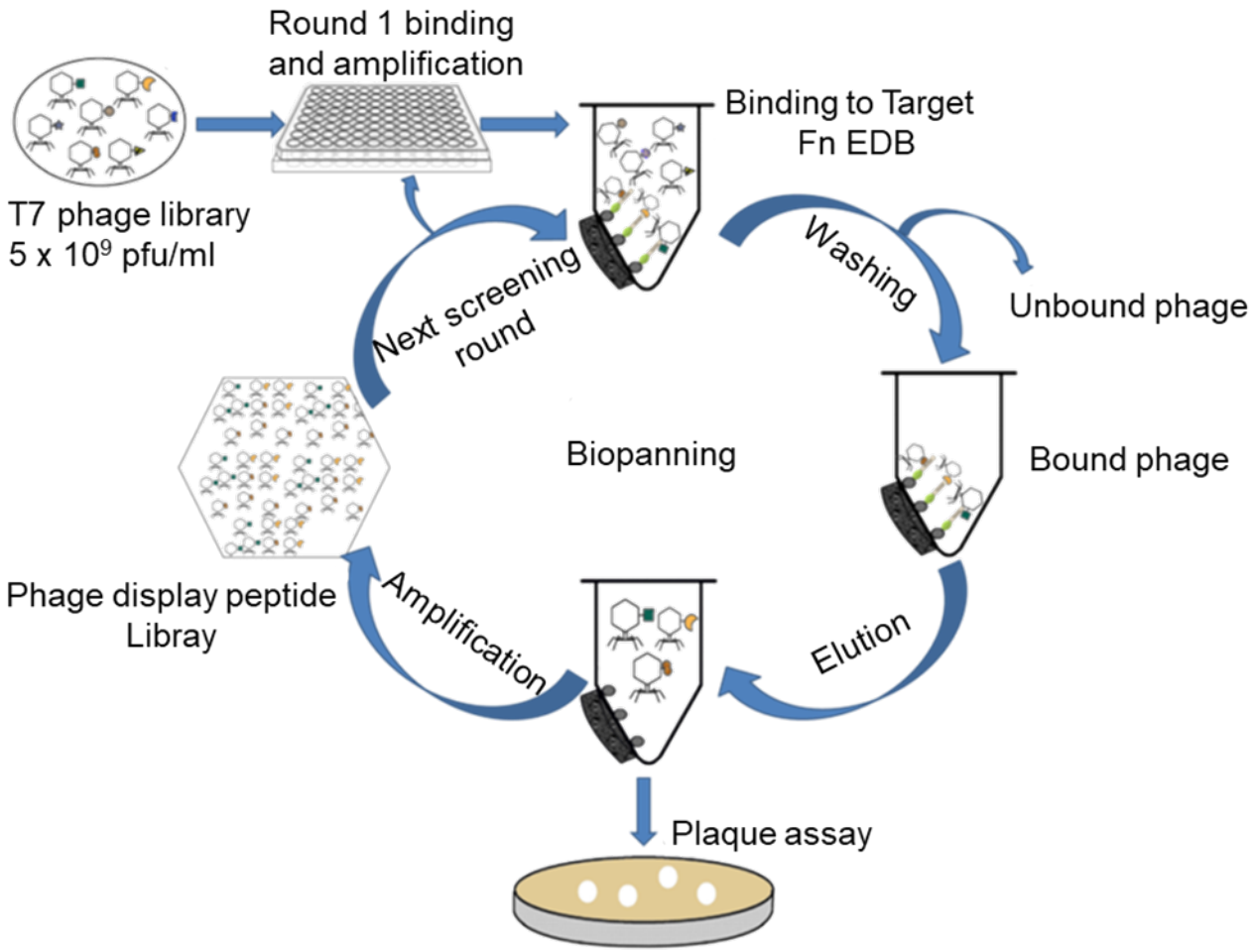

Figure S2. Fn-EDB Biopanning Workflow. This figure illustrates the biopanning process for selecting Fn EDB-specific phage clones using phage display technology. The first and fourth rounds of selection were screened on an ELISA plate, while subsequent rounds were screened on Ni-NTA agarose beads.

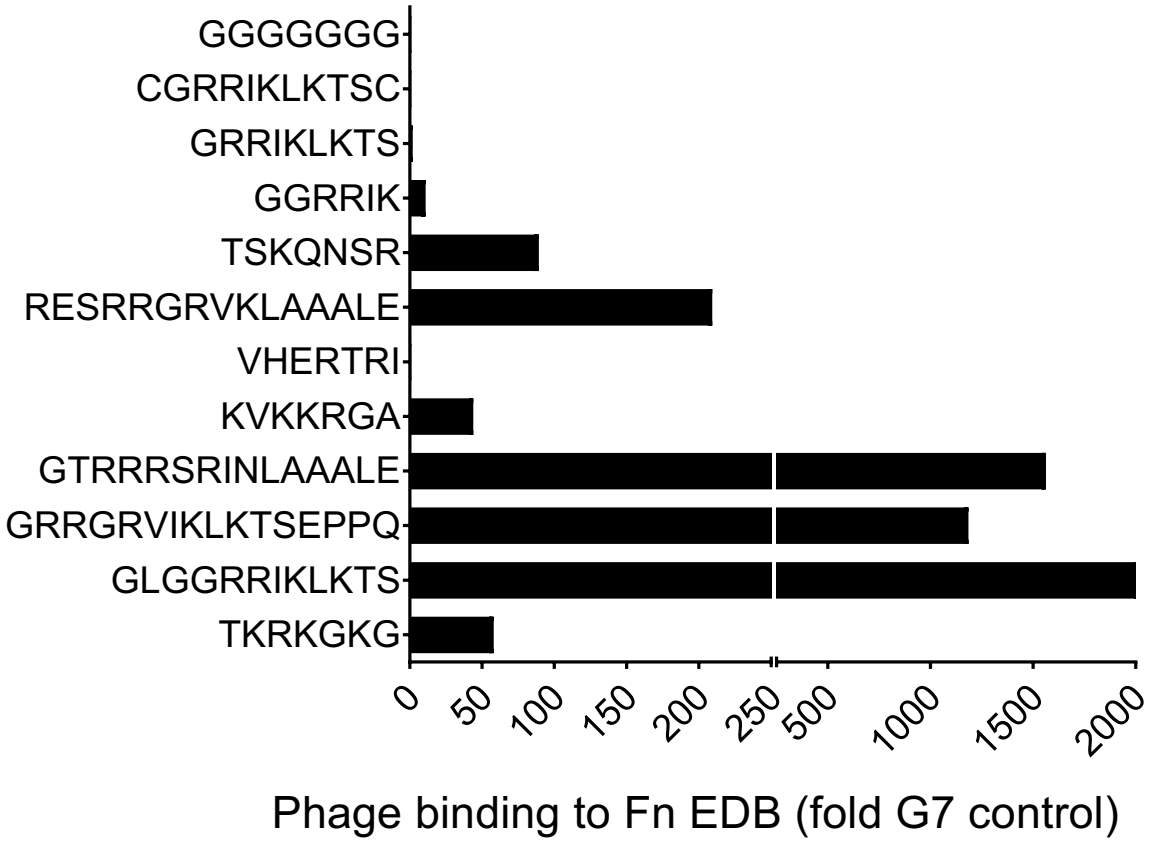

Figure S3. Identification of tumor specific Fn-EDB homing peptides. The bar charts depict a selection of candidate phage peptides obtained from the fifth round of Fn-EDB biopanning. The binding of peptide-phage is presented as fold over control phage G7.

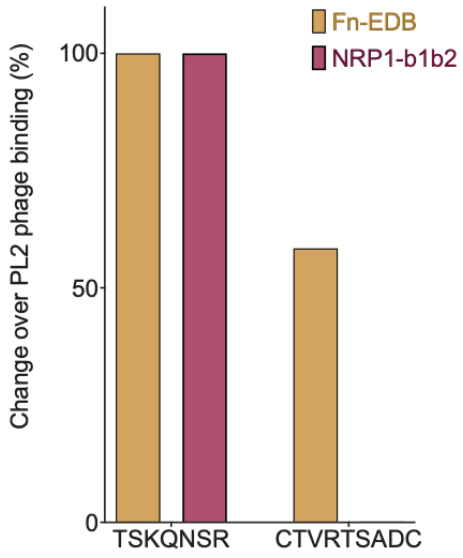

Figure S4. Comparison of Fn-EDB binding peptides PL2 and ZD2. The bar chart illustrates the binding of PL2 (TSKQNSR) and ZD2 (CTVRTSADC) peptide-phages to Fn-EDB and NRP-1. The Y-axis indicates the percentage change in binding relative to normalized PL2 peptide binding (set at 100%). The X-axis displays the binding of PL2 and ZD2 peptides to Fn-EDB and NRP-1.

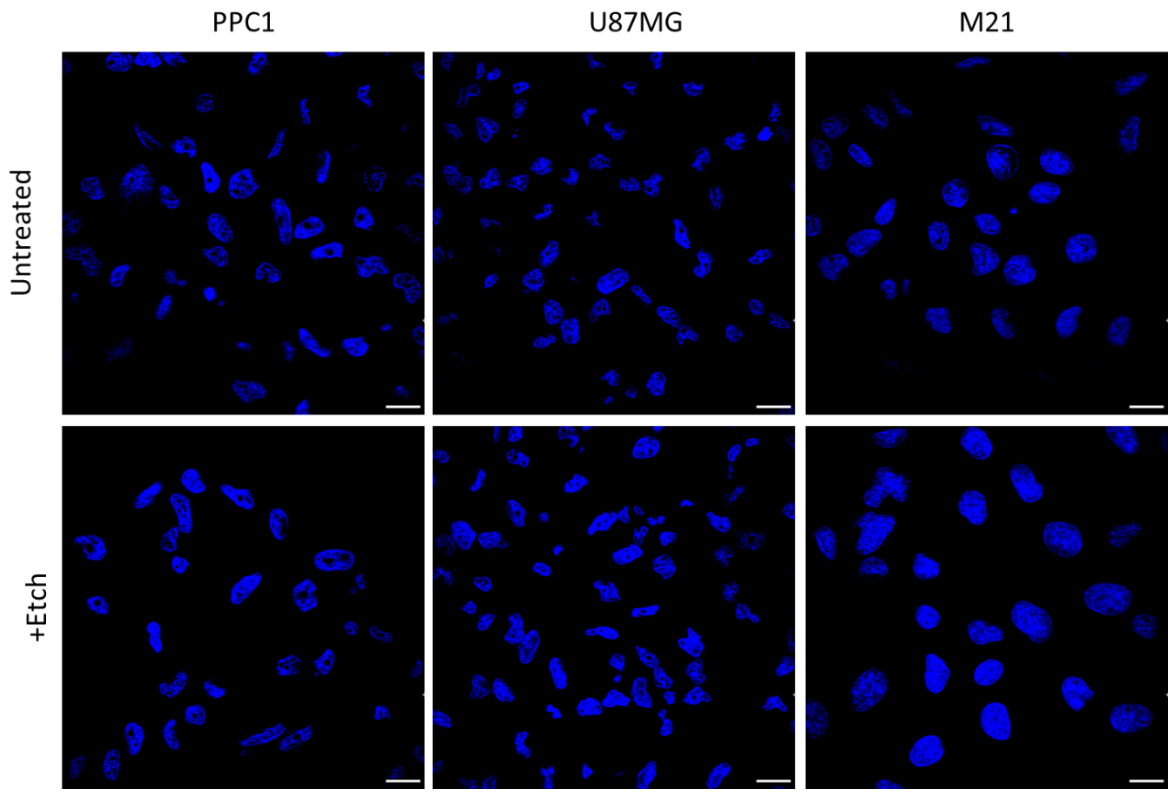

Figure S5. Negative control for PL2 AgNPs treatment. Confocal microscopy images of untreated and etched PPC1, U87-MG, and M21 cells show no detectable or internalized control biotin -AgNps (CF555 fluorophore dye) signal, confirming specificity of PL2 **AgNPs**. Scale bar: 20 µm.

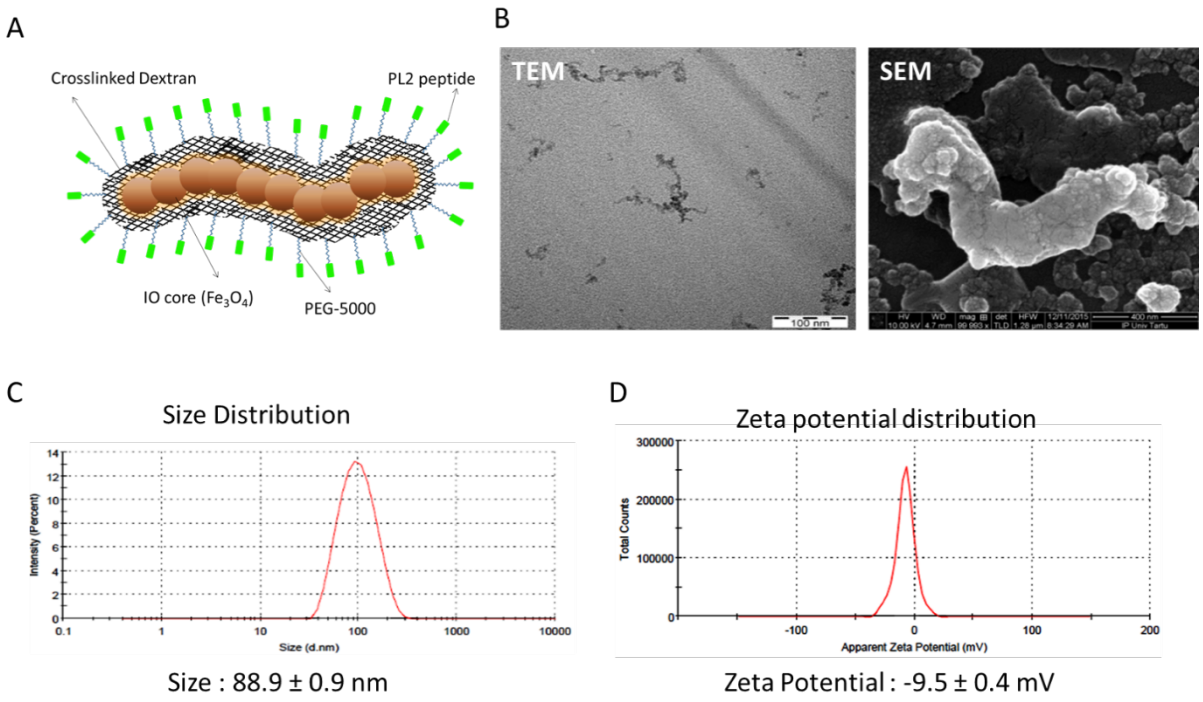

Figure S6. Characterization of iron oxide nanoworms (NWs). (A) Schematic representation of theranostic NWs, showing covalent coupling of FAM (carboxyfluorescein)-labeled PL2 peptide to NWs covered with PEG-5000 linker and cross-linked dextran. (B) Transmission Electron Microscopy (TEM) and Scanning Electron Microscope (SEM) images of non-functionalized NWs with increased in apparent size is attributed to the abundant contrast agent coating, scale bars of 100 nm and 400 nm, respectively. (C–D) Physicochemical properties of NWs, including zeta potential and size distribution, measured by DLS at 25 °C.

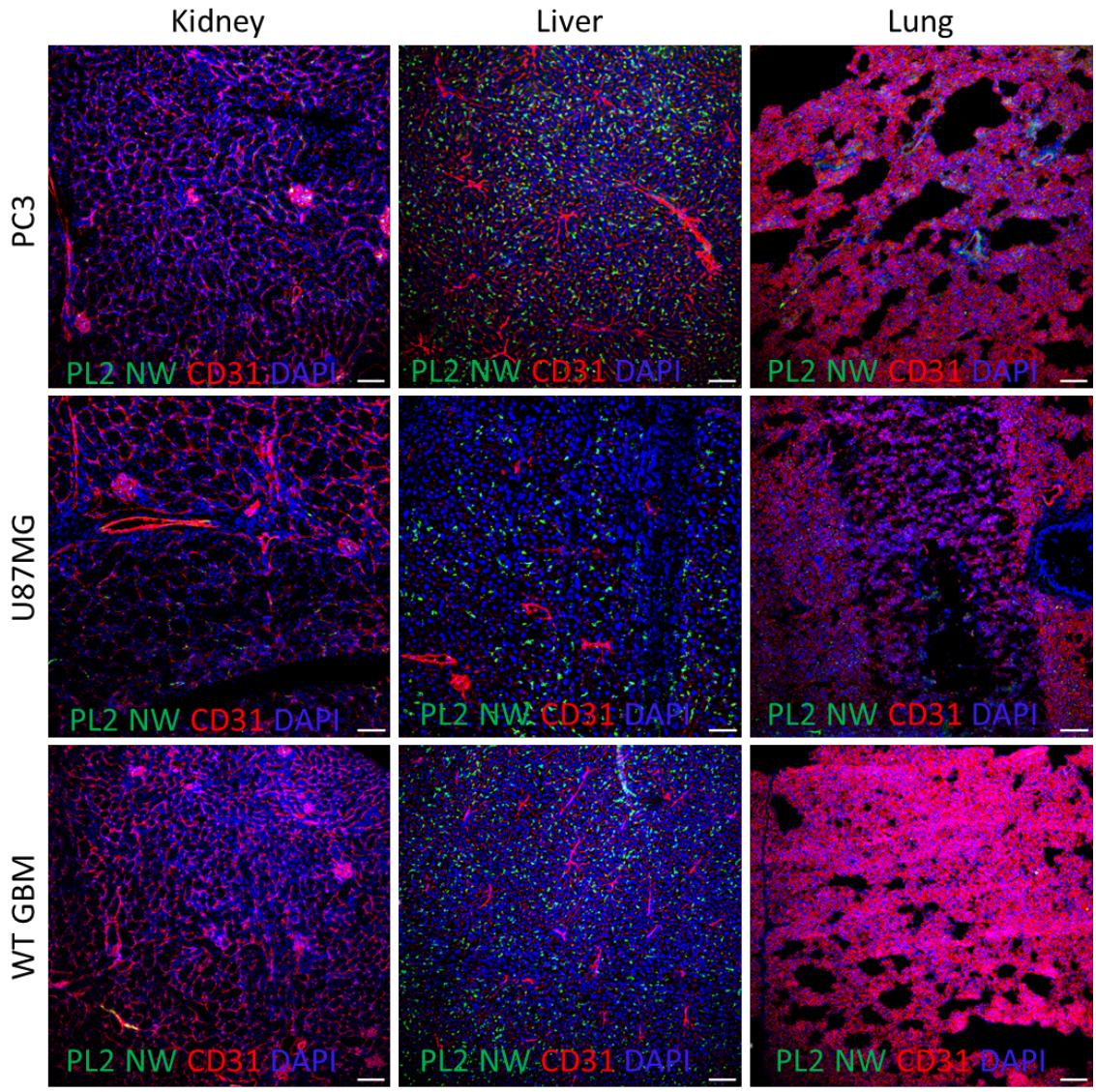

Figure S7. Biodistribution of PL2-NWs in healthy organs. FAM-labeled PL2 peptide-coated iron oxide NWs (or non-peptide NWs) were intravenously injected at 7.5 mg/kg into mice bearing PC3, U87-MG, and WT GBM tumors. Five hours after injection, the mice were perfused through the heart with PBS/DMEM. The collected tumors and control organs were immunostained with antibodies against FAM (green), CD31 (red), and DAPI (blue) was used to counterstain nuclei in control organ sections. N = 3; scale bars: represent 100 μm.

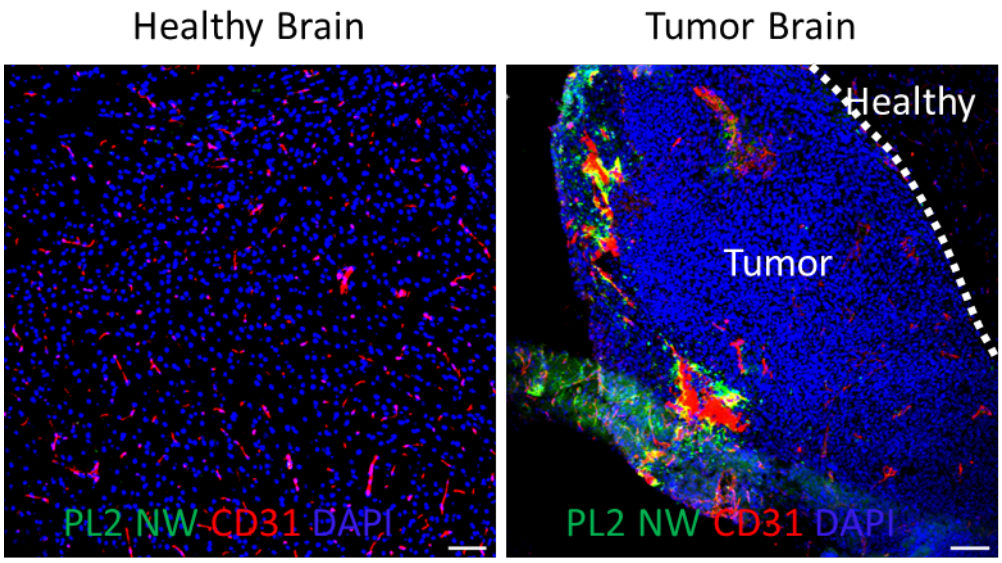

Figure S8. The PL2-NWs targetability in glioblastoma. FAM-labeled PL2-NWs were intravenously injected at a dose of 7.5 mg/kg into mice with WT GBM tumors and healthy mice. After 5 hours of circulation, the mice were perfused through the heart with PBS/DMEM, and the tumor and healthy parts of brain tissues were collected for immunostaining. The dashed line indicates the tumor region of the brain with high nuclei density, while the less dense nuclei area represents the healthy brain region. The anti-FAM antibody (green), anti-CD31 antibody (red), and DAPI-stained nuclei (blue) were used for staining. The study was conducted with n = 3 mice, and the scale bars represent 100 μm.

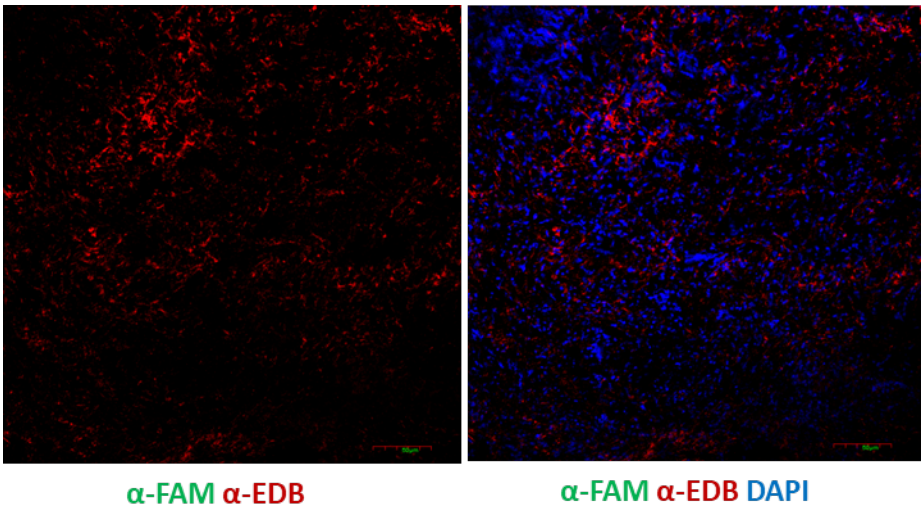

Figure S9. High expression of Fn-EDB receptor in human ovarian carcinoma cancer. Confocal fluorescence imaging of fresh surgical explants of human ovarian carcinoma cancer tissues stained with anti-Fn-EDB ScFv L19 (red) reveals strong expression of the Fn-EDB receptor. The negative control staining using anti-FAM antibody (green) shows no detectable signal in the cancer tissue. DAPI (blue) was used to counterstain nuclei. N = 3; scale bars: represent 50 μm.