Electronic Supplementary Material (ESI) for Nanoscale.

Supplemental Information

Viral nanoparticles decorated with novel EGFL7 ligands enable intravital imaging of tumor neoangiogenesis

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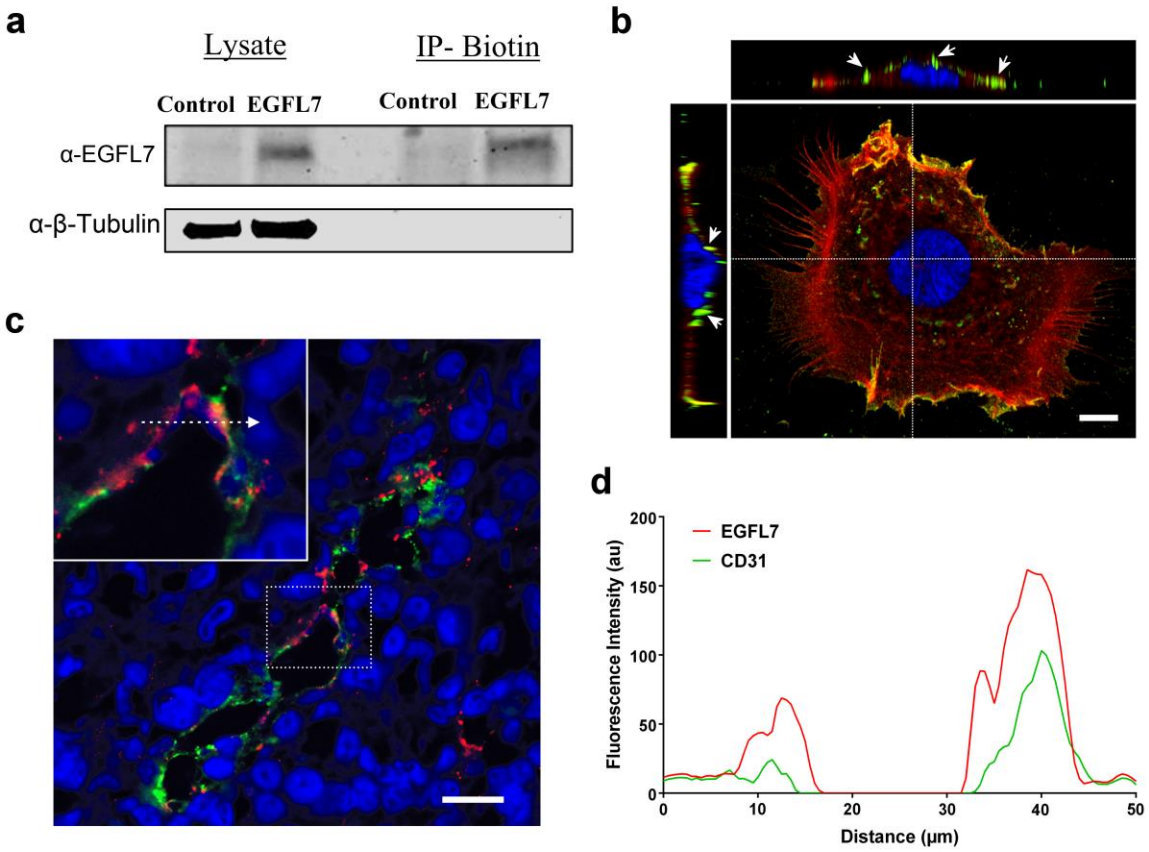
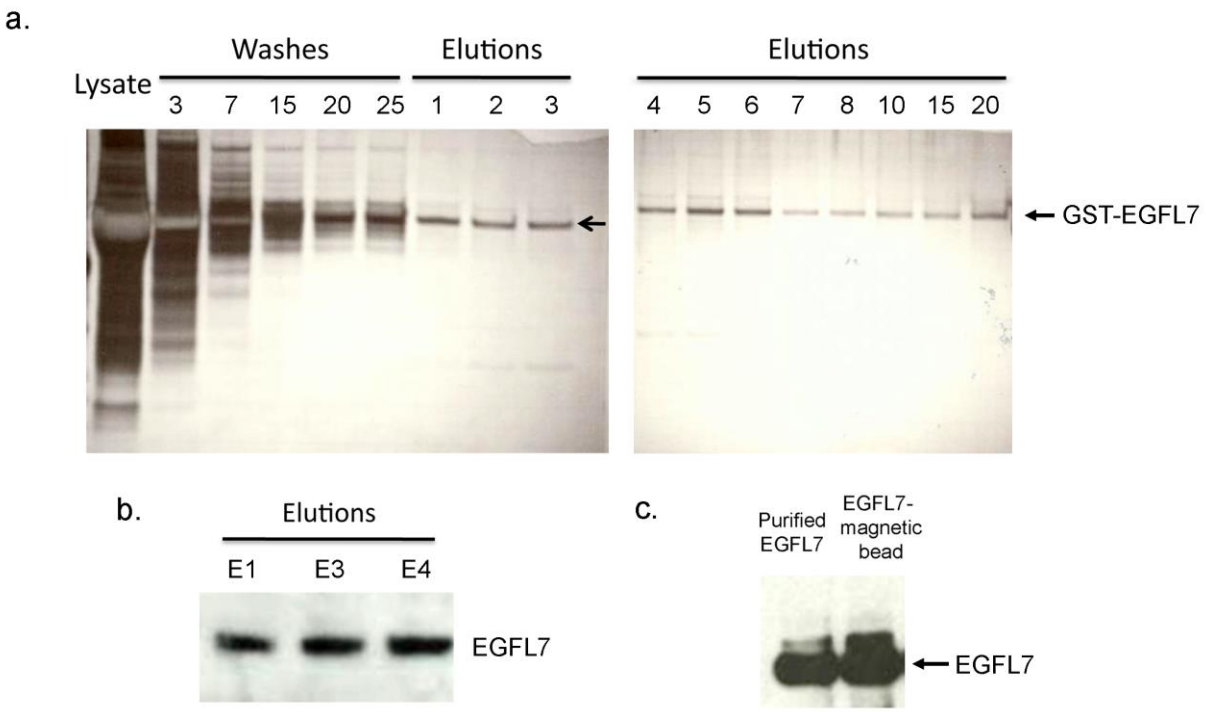
Supplementary Movie 1. Real-time intravital confocal microscopy showing the accumulation of CPMV-PEG-E7p72 nanoparticle in HT1080 neovasculature *in vivo* in an avian embryo model. CPMV (white), endothelium (red), and HT1080 fibrosarcoma cells (green).

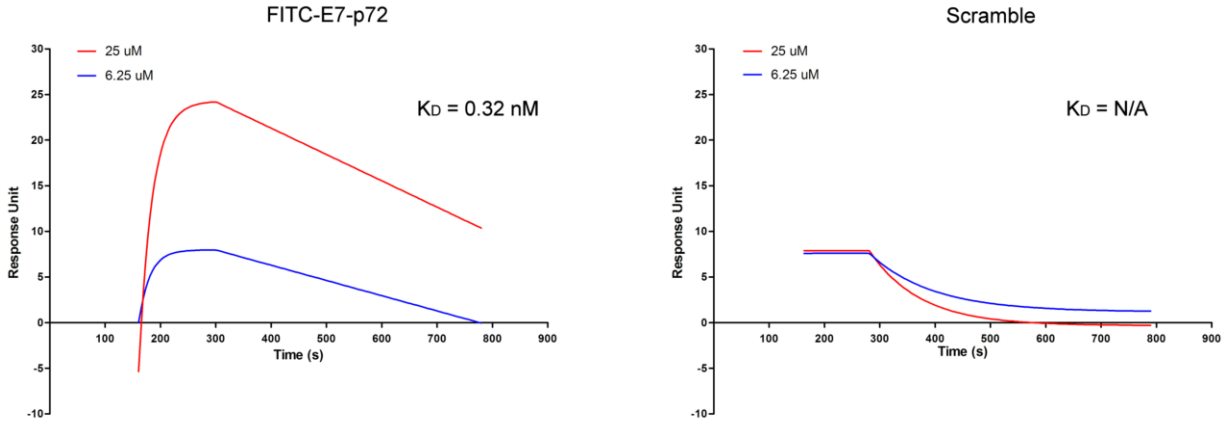
Supplementary Movie 2. Real-time intravital confocal microscopy of HT1080 neovasculature *in vivo* after the administration of control CPMV-PEG nanoparticle in an avian embryo model. CPMV (white), endothelium (red), and HT1080 fibrosarcoma cells (green).



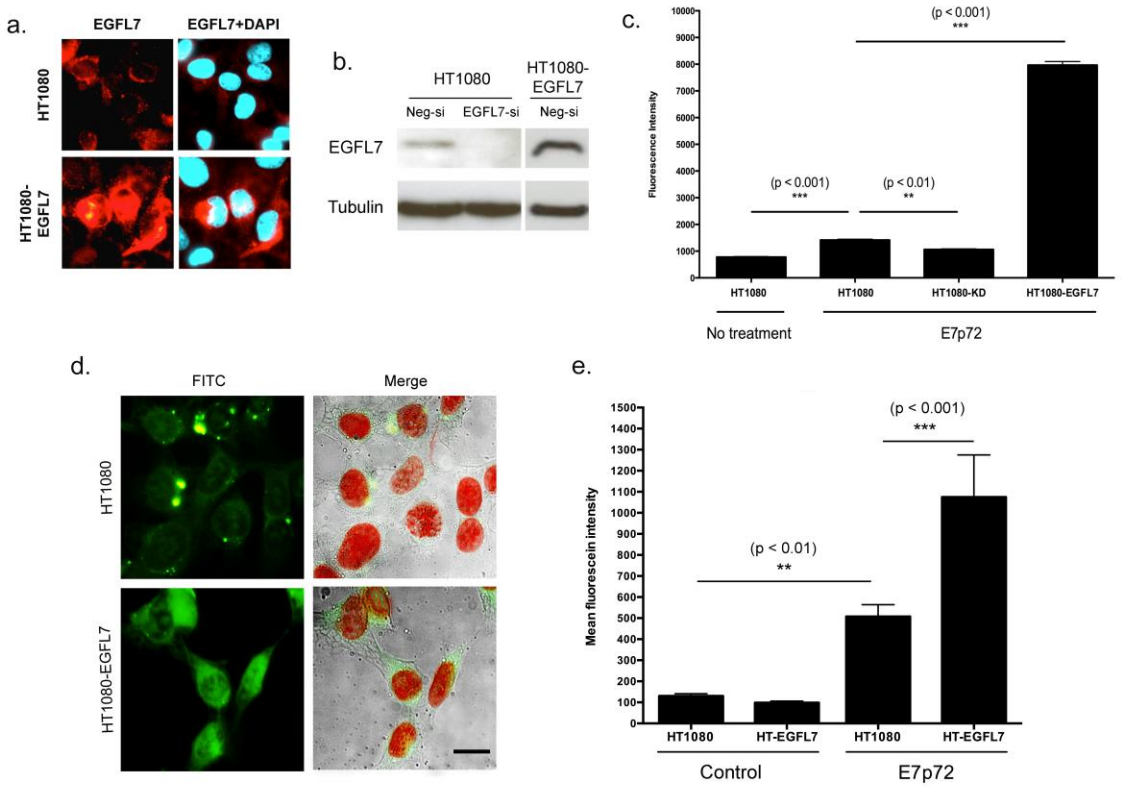
Supplementary Figure 1. EGFL7 is expressed on the tumor cell surface and the endothelial cell lumen. (a) Western blot analysis of immuno-precipitation of biotinylated EGFL7 using anti-Biotin antibody on HT1080 cells. HT1080 (control) or HT1080-EGFL7 cells were biotinylated using the cell-impermeable Sulfo-NHS-Biotin so that only proteins being expressed at the cell surface can be selectively precipitated. (b) Confocal images of non-permeabilized Control-HT1080 and EGFL7-expressing HT1080 fibrosarcoma cells captured at 63x magnification. Phalloidin-AF 647 (red), white arrows indicating cell surface EGFL7 (green) and nuclei staining (blue). Scale bar, 5 microns. (c) Fluorescence images of MDA-MD-468LN tumor tissues sections showing the expression of EGFL7 in the endothelial cell lumen. Immunofluorescence staining was performed to detect mouse EGFL7 (red), CD31 vascular marker (green), and nuclei (blue). Scale bar, 20 microns. (d) Line scan of a selected plane (dashed arrow, panel c) representing the expression of EGFL7 in the endothelial cell lumen alongside the vascular marker, CD31.



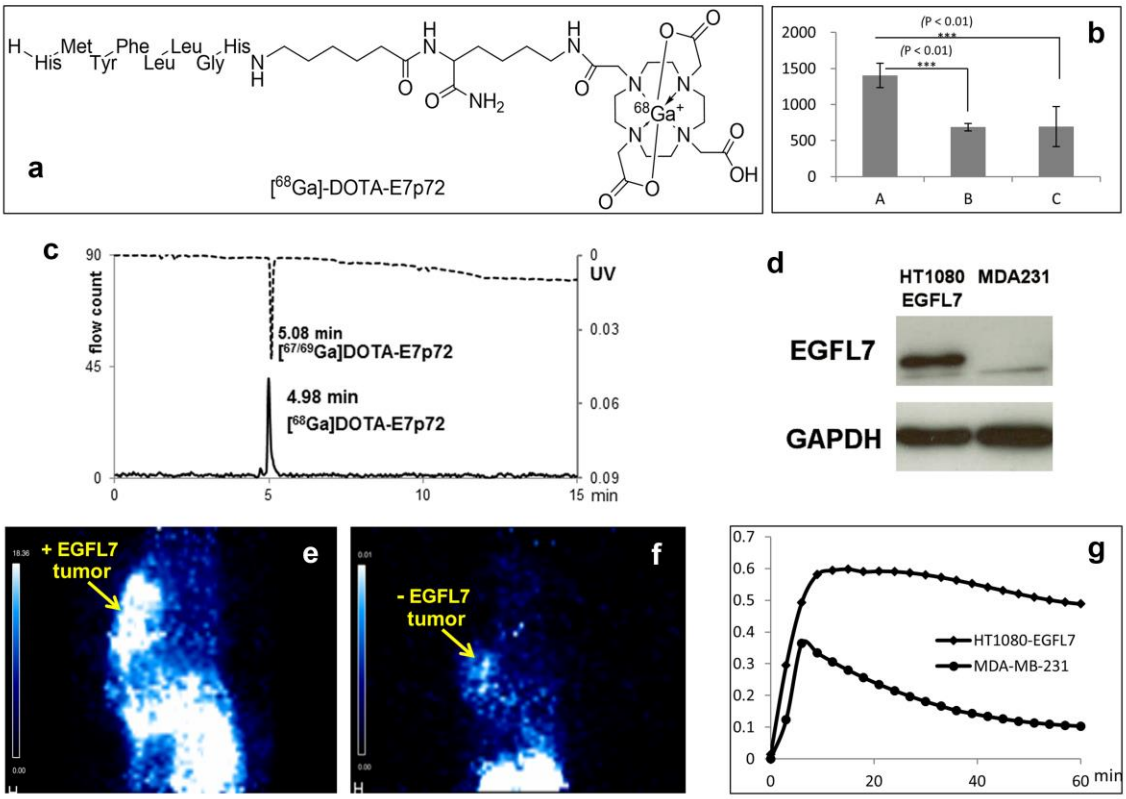
Supplementary Figure 2. Purification of EGFL7 fusion protein (a) Silver-stained gel showing the purification of recombinant GST-EGFL7 protein. (b) The eluted fractions from (a) were subjected to western blot analysis using a polyclonal anti-EGFL7 antibody. (c) Western blot analysis confirming the conjugation of purified EGFL7 protein onto the magnetic beads.



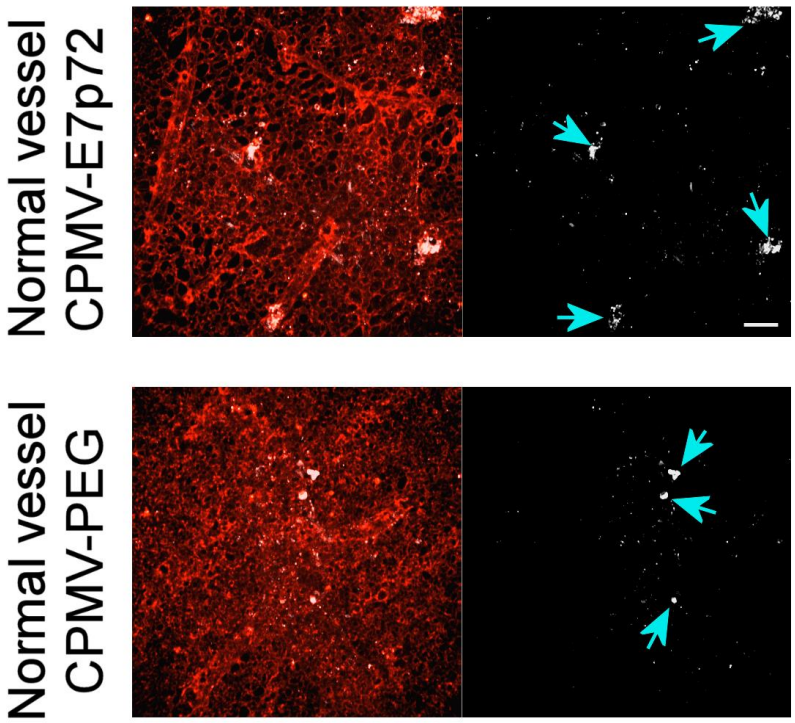
Supplementary Figure 3. Affinity of FITC-labeled E7p72 for EGFL7. Association and dissociation curves generated using the Biacore 3000 instrument to evaluate the affinity of FITC-E7p72 for immobilized EGFL7. Association curves were obtained by exposing EGFL7 immobilized onto the SPR chip with FITC-E7p72 or negative (control) peptide. At t = 280 s, the dissociation curves were generated when the injection of peptide was stopped, and switched to plain buffer. The fitted curves were generated using the 'association-then-dissociation' equation in GraphPad Prism. The K_D value is indicated in this plot.



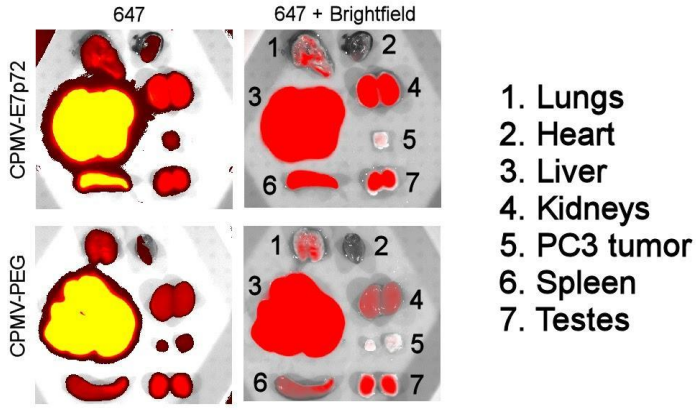
Supplementary Figure 4. Fluorescently-labeled E7p72 peptide is taken up by HT1080 fibrosarcoma cells in an EGFL7-dependent manner. (a) Fluorescence images showing the expression of EGFL7 in regular HT1080 and HT1080-EGFL7 overexpressing cells. (b) Western blot analysis showing the knockdown of EGFL7 by siRNA and overexpression of EGFL7 in HT1080 cells. (c) Bar graph indicating the level of FITC-E7p72 labeling in regular HT1080, HT1080-KD (EGFL7 knockdown) or HT1080-EGFL7 (overexpressing) cells by flow cytometry (n = 10,000) using the COPAS flow cytometer. All statistics were performed using a one-way ANOVA and Tukey post hoc test. (d) Fluorescence images showing the uptake of FITC-E7p72 (final concentration of 3.3 microM) in HT1080 cells and HT1080-EGFL7-overexpressing cells. Left panels: FITC (green). Right panels: merged images of brightfield image, nuclei staining (red) and FITC-conjugated peptide (green). Scale bar, 10 microns. (e) Bar graph showing the mean fluorescence intensity of cells from each group. Peptide uptake was quantified as described above. The binding of FITC-E7p72 to EGFL7-overexpressing HT1080 cells was significantly higher compared to regular HT1080 cells (n = 30, $p < 0.001$).



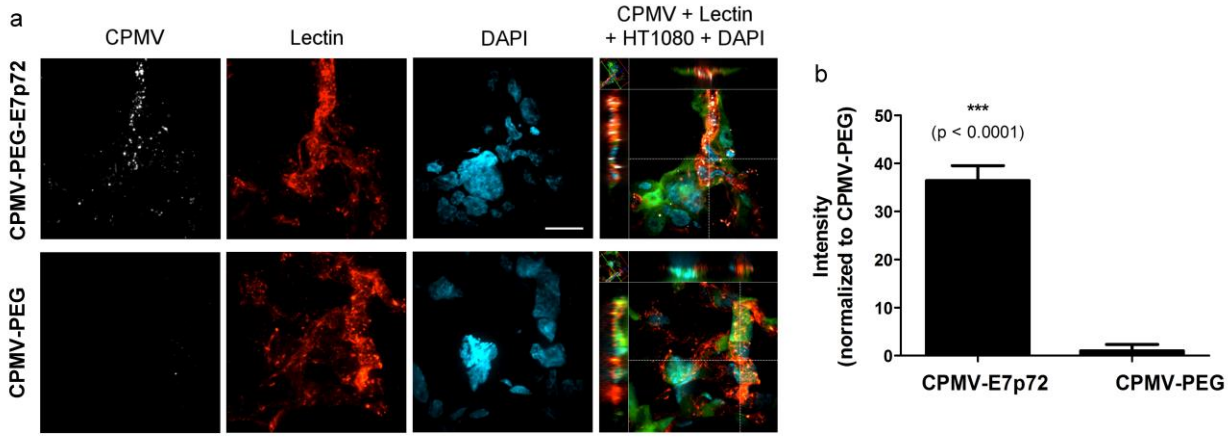
Supplementary Figure 5. PET imaging of EGFL7-expressing tumor using [^{68}Ga]-DOTA-E7p72 in mouse xenograft model. (a) Chemical structure of [^{68}Ga]-DOTA-E7p72; (b) Cell uptake of [^{68}Ga]-DOTA-E7p72 in (A) HT1080-EGFL7 cells, (B) HT1080-control cells and (C) HT1080-EGFL7 cells with E7p72 blocking. (c) HPLC chromatogram for [^{68}Ga]-DOTA-E7p72 (radiotracer, solid) and [$^{69/71}$Ga]-DOTA-E7p72 (UV at 254 nm, dashed). Conditions: mobile phase acetonitrile/H2O, gradient 10/90 to 90/10 (v/v) with TFA 0.1%, 10 min; flow rate 1.5 mL/min, Sunfire RP-C18 analytical column (Waters). (d) Western blot showing EGFL7 expression in HT1080-EGFL7 overexpression cells, but not in MDA-MB-231 breast cancer cells. (e) PET image of xenograft HT1080-EGFL7 tumor (EGFL7 positive) in NOD/scid mouse; (f) PET image of xenograft MDA-MB-231 tumor (EGFL7 negative) in NOD/scid mouse. (g) SUV of [^{68}Ga]-DOTA-E7p72 in both xenograft tumor models over time.



Supplementary Figure 6. CPMV-PEG-E7p72 and CPMV-PEG nanoparticles have similar distribution in quiescent vasculature. Blood vessels were labeled with lectin rhodamine and visualized using intravital imaging. Minimal uptake is observed in the endothelium of mature vessels at 90 minutes, although significant uptake by macrophages is observed (arrows). CPMV (white), lectin (red). Scale bar, 40 microns.



Supplementary Figure 7: The distribution of CPMV-PEG-E7p72 in normal and PC3 tumor tissues. Mice established with flank PC3 tumors were injected with CPMV-PEG-E7p72 or control CPMV-PEG nanoparticles. After 2 hrs, the mice were sacrificed, and their organs harvested and imaged using the IVIS instrument.



Supplementary Figure 8. CPMV-PEG-E7p72 nanoparticle labels HT1080 tumor neovasculature in avian embryo. (a) Z-stacks confocal images of HT1080 tumor tissue section showing that CPMV-PEG-E7p72 are internalized by endothelial cells. CPMV (white), endothelium (red), HT1080 (green) and nuclei (light blue). Scale bar, 10 microns. (b) Bar graph displaying the mean fluorescence intensity of tumor endothelium in chicken embryos injected with CPMV-PEG-E7p72 or CPMV-PEG (control).

Supplemental Experimental Procedures

Cloning recombinant EGFL7

EGFL7 cDNA was amplified by PCR (sense primer: (5'CACCATGAGGGGCTCTCAGGAGGTG3' and anti-sense primer: 5'CTACGAGTCTTTCTTGCAGGAGCAG3') and cloned into the pENTR/TEV/D-TOPO vector (Invitrogen). The TEV-EGFL7 region was cloned into pDEST20 plasmid (Invitrogen) by recombination and the recombinant bacmid DNA was isolated. All cloning procedures were carried out according to the Invitrogen manual's instruction.

Expression and purification of recombinant GST-EGFL7

Recombinant baculovirus was generated from *Spodoptera frugiperda*, Sf21 cells that were transfected with 1μg of bacmid DNA using CellFECTIN transfection reagent (Invitrogen). Plaque titration of the virus was performed according to the standard protocol described in the Invitrogen manual. Sf21 cells were seeded onto a T175 cell culture flask with approximately 80% confluency. Upon cell attachment, the medium was removed and the cells were infected with the recombinant baculovirus at a multiplicity of infection (MOI) of 20 for 72 h at 27°C. The cell lysate was harvested in 0.1% NP40, and passed through 1mL of glutathione-agarose resin. The column was then washed with 25 mL of wash buffer (150 mM, NaCl, 20 Tris–HCl, pH 7.9), and eluted with elution buffer supplemented with 20mM reduced glutathione. Elutions were collected in 1mL fractions and stored at 4^{o}C. All eluted fractions were analyzed using SDS-PAGE and visualized by silver staining.

Generation of EGFL7-coated magnetic screening beads and

Thirty microgram of anti-GST antibody (Thermo Scientific) was mixed into the purified fractions of EGFL7 recombinant protein, and incubated with 370 mg of MagnaBind Protein A/G magnetic beads (Thermo Scientific) for 15 minutes at 4^{o}C. After several washes with wash buffer, the conjugation of protein onto magnetic beads was analyzed by treatment of the

screening beads with SDS loading buffer and heat, followed by western blot analysis using anti-EGFL7 polyclonal antibody (R&D Systems).

Generation of OBOC peptide library

An octapeptide library was synthesized on ANP-TentaGel resin adopting a "split and mix" strategy on an automated synthesizer, APEX396 (AAPPTEC), using standard Fmoc chemistry in DMF [28]. Fmoc amino protecting groups were removed by 20% piperidine in DMF (two cycles) prior to coupling cycles. Each coupling cycle protocols implemented HBTU/DIPEA chemistry. Between deprotection and coupling steps, the resin was thoroughly washed sequentially with DMF, DCM and DMF (five cycles). During all procedures, mixing was accomplished using a synthesizer shaker set to 500 rpm. Removal of all side-chain protecting groups from the synthesized peptides was accomplished by treatment with cleavage cocktail consisting of TFA : Phenol: H2O: triisopropylsilane (87.5 : 5.0 : 5.0 : 2.5) for 2 h at room temperature, in the dark under constant shaking (800–1000 rpm, IKA-Vibramax-VXR).

siRNA knockdown of EGFL7

EGFL7 siRNA (sense: 5'- UGAAGGAAGAAGUGCAGAGUU -3', antisense: 3`-UUACUUCCUUCUUCACGUCUC -5`) (Sigma, Canada) or scrambled siRNA (negative control) (sense: 5` GAAGTAACACCCGCACCTAUU 3`, antisense: 3`-UUCUUCAUUGUGGGCGUGGAU -5`) (Sigma, Canada) were used to transfect HT1080 cells. Cells were seeded onto a 6-well plate and transfected with either EGFL7 siRNA at a final concentration of 40 nM using INTEFERin transfection reagent (Polyplus transfection). After 48 hrs, Western blot analysis of transfected cells was performed to confirm knockdown using the anti-EGFL7 polyclonal antibody (R&D systems).

Deconvolution of hit peptides

A bead containing the peptide was suspended in 50 µl of MilliQ H_2O in a 96-well polypropylene plate. Irradiation was accomplished under a UV lamp (λ: 365 ± 10 nm; UV Products, Upland, CA, model EL25, 8 $mWcm^{-2}$) for 2 hours. The supernatant was collected and subjected to mass

analysis. For MALDI-TOF/TOF deconvolution experiment, 10 µl of supernatant were mixed with 10 µl of CHCA matrix (5mg/ml; 50% acetonitrile, 6 mM ammonium phosphate monobasic, 0.1% TFA). Then, 0.75 µl of this mixture was deposited onto the MALDI target and air-dried. A 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) with TOF/TOF optics was used for all MALDI-MS and MS/MS applications using acquisition conditions that was previously described [31].

Synthesis of fluorescein-labeled peptides

A fluorescent group carrier was introduced to the peptides through a Lys residue. The acid labile protective methytrityl (Mtt) group of the Lys can be removed selectively in presence of standard protecting groups. The Rink amide resin was loaded with Fmoc-Lys(Mtt)-OH, followed sequentially by the coupling of the linker Fmoc-Ahx-OH and the amino acids corresponding to either E7-p72 or E7-p74. The peptide synthesis was carried out using Fmoc chemistry with HBTU /DIPEA for each coupling step and 20% piperidine in DMF solution for Fmoc deprotection step on an automated synthesizer (Biotage Syro Wave™). Upon completion, the peptidyl-resin was treated with 1% TFA, 4% TIS, 95% DCM at room temperature for 15 minutes (3 times) for the selectivey removal of the Mtt group. The peptides were then fluorescently labeled by mixing the resin with 6 equiv of fluorescein-5-isothiocyanate (FITC) and DIPEA (14 equiv) in minimal DMF for 12 hours in the dark. Fluorescein-peptides were cleaved using a cocktail comprised of TFA:EDT:TIS (95:2.5:2.5) and precipitated with ice-cold t-butyl methyl ether. The crude peptides were isolated by centrifugation, washed with cold ether, solubilized in minimum amount of water and freeze-dried. Purification was accomplished by HPLC (C_{18} column, 220-nm monitoring, gradient: CH_3CN + 0.1% TFA 30 to 60% over 20 min, H_2O + 0.1% TFA). Chemical identities of the peptides labeled with fluorescein were confirmed by ESI mass spectrometry. A negative peptide (NHGVVAPF) was labeled and purified using the same method. Fluororescein-E7-p72-NH_2: m/z 823.3 (Calcd. 823.9), HPLC purity (93%); and Fluorescein-E7-p74-NH_2: m/z 852.1 (Calcd. 852.8), HPLC purity (96%).

Synthesis of DOTA and $^{69/71}$Ga-Coordinated Peptide

The peptide synthesis followed the procedure outline for the fluorescein-labeled peptides, with the exception of the FITC conjugation step. Instead, DOTA(^{t}Bu)$_3$ was coupled to the side chain of the lysine using standard HBTU/DIPEA coupling conditions. The crude DOTA-E7p72 was recovered as previously described, purified by preparative HPLC-MS and lyophilize to a white powder (32 mg, 10% yield). MS (ESI+): *m/z* calcd 822.4, found 822.6 $[M+2H]^{2+}$.

DOTA-E7p72 (10 mg) was dissolved in NaOAc/HOAc (pH=5, 0.1 M), $GaCl_3$ (5 mg) was added and heated for 70 ˚C for 30 min. the reaction mixture was purified by HPLC and lyophilized to provide [$^{69/71}$Ga]-DOTA-E7p72 (white solid, 2 mg, 19% yield). MS (ESI+): *m/z* calcd 855.4, 856.4, found 855.5, 856.6 $[M+2H]^{2+}$.

Radio-synthesis of [^{68}Ga]-DOTA-E7p72

[^{68}Ga]-DOTA-E7p72 was prepared using an automated radiosynthesis unit (Modular Lab, Eckert & Ziegler), with $^{68}Ga^{3+}$ being eluted from a ^{68}Ge/^{68}Ga generator, passed through a cation exchange column (Strata TM-X-C 33 μm polymeric strong cation 30 mg/1mL cartridge, Phenomenex) and eluted with 0.02 M HCl in 98% acetone. The precursor DOTA-E7p72 (10 μg) in 100 μL of HEPES buffer (1M, pH = 3.5) was mixed with the eluted $^{68}Ga^{3+}$ (800 μL, 126 MBq) and was heated at 90 ˚C for 10 min., then cooled to RT and the contents were trapped on a Sep-Pak C-18 cartridge (Waters Inc.) Ethanol was used to elute the product, providing 55 MBq of [^{68}Ga]-DOTA-E7p72 in a 53% radiochemical yield (d.c.) The ethanol was removed under vacuum with a V-10 speed-vap (Biotage Inc.) The dry [^{68}Ga]-DOTA-E7p72 was reconstituted in saline for biological evaluation. Analysis was performed by HPLC (Waters Inc.), which was equipped with two detectors (UV, λ_{max} =220 nm and radioactivity) using a Sunfire RP-18 analytical column (4.6 × 250 mm, 5 μm, Waters). The identity of the ^{68}Ga-labelled radiotracer was confirmed by coinjection with the reference [$^{69/71}$Ga]-DOTA-E7p72 compound. The radiochemical purity was > 95% (**Figure S7**) and the specific activity was determined to be 9.4 GBq/μmol.

Gel electrophoresis of CPMV nanoparticles

Denaturing gel electrophoresis was used to analyze protein subunits. Specifically, 10 μg of proteins were analyzed on denaturing 4-12% NuPAGE gels (Invitrogen) in 1x MOPS running buffer (Invitrogen). After separation, the gel was stained with Coomassie Blue and imaged using the AlphaImager (Biosciences) imaging system. Intact CPMV nanoparticles were analyzed on native gel electrophoresis using 1.2 % (w/v) agarose gel in 1x TBE buffer. The gel was stained with ethidium bromide, and imaged under UV light using the AlphaImager (Biosciences) imaging system.

Evaluation of CPMV uptake using flow cytometry

CPMV-PEG (control) and CPMV-PEG-E7p72 were stored in PBS at 4 ^{o}C before further use. EGFL7 knockdown in EA.hy926 endothelial cells and HT1080 fibrosarocma cells were achieved using siRNA using jetPRIME transfection reagent (Polyplus Transfection). After 48 hrs of tranfection with either control or EGFL7 siRNA, cells were seeded onto a 6-well tissue culture plate to approximately 80% confluency. Cells were incubated with CPMV-PEG (control) or CPMV-PEG-E7p72 (1 μg/mL) at 37^{o}C for 3 hrs. Cells were washed three times with PBS, and then detached using 2.5 mM EDTA. Cells were washed once, fixed with 4% paraformaldehyde, resuspended in flow buffer (PBS with 2mM EDTA, 2% FBS and 0.05% sodium azide) and evaluated using the BD FACS calibur flow cytometer. Data analysis was performed using FCS express (version 3).

Biotinylated immunoprecipitation of EGFL7

EGFL7-HT1080 and control HT1080 cells were incubated with membrane impermeable Sulfo-NHS-Biotin for 1 hour at 37°C in 10 cm plates. Cells were washed three times with 1x PBS and lysed with RIPA buffer with 1% protease inhibitor. Anti-biotin antibody. (10μg/ml) was added to each remaining cell lysate and incubated for 1 hour at room temperature. Protein-A magnetic beads were added to the lysate for protein immunoprecipitation. Samples resolved by electrophoresis in SDS on 10% polyacrylamide gels. Western blots were stained with anti-EGFL7 and anti-β-Tubulin antibodies and analysed using the Odyssey LI-COR imaging system

Immunofluorescence imaging of EGFL7 in fibrosarcoma cells

EGFL7- HT1080 and control HT1080 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) in 10% FBS onto the 18 mm glass coverslips at approximately 80% confluency. Cells were fixed with 4% paraformaldehyde and stained with antibodies against EGFL7 for 1 hour at room temperature followed by secondary antibody. Cell nuclei and actin were stained with Hoechst 33342 and Phalloidin-Alexa 647 respectively for 10 minutes prior to mounting with ProLong Gold (Invitrogen). The Nikon A1+ confocal microscope was used to visualize the slides. 3D Z-stacked images were captured and images were generated using the NIS Elements AR software

EGFL7 expression in the endothelial lumen of mouse tumors

All studies were performed according to protocols approved by the University of Alberta Animal Care and Use Committee. 8-weeks old mice were established with MDA-MB-468LN breast adenocarcinoma tumors. MDA-MB-468LN cells (1 x 10^6 cells) were injected subcutaneously into the flank. After two weeks (or when the tumor size reached 1000 mm^3), animals were euthanized and tumors were excised, fixed in 4% formaldehyde and 10% sucrose overnight at 4 ^oC, and embedded in OCT medium. Frozen sections (10 μm) were prepared on a Leica cryomicrotome, and samples were stored at -80 ^oC for further analysis. For immunofluorescence staining, tumor sections were fixed in 4% formaldehyde at 25 ^oC. Samples were blocked using 10% goat serum and 1% BSA (in PBS + 0.02% Tween-20) for one hr at 25 ^oC. Tissues were incubated with a rat anti-CD31 antibody and a goat anti-EGFL7 antibody in blocking buffer (1 hr at 25 °C) and washed four times (PBS + 0.02% Tween-20). Tissues were then incubated with the anti-rat AlexaFluor 488 antibody and anti-goat AlexaFluor 594 antibody (Invitrogen) in blocking buffer (1 hr at 25°C in dark). Tissues were washed four times (PBS + 0.02% Tween-20), and mounted in ProLong Gold mounting media containing DAPI (Life Technologies). Tissues were imaged using the Zeiss Nikon A1+