Supplementary File

Ultrasound molecular imaging as a non-invasive companion diagnostic for netrin-1 interference therapy in breast cancer

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Supplementary Material and Methods

Preparation of netrin-1-targeted MBs

Biotinylation of anti-netrin-1 antibody:

Monoclonal humanized anti-netrin-1 antibody (NET1-H-mAb, Netris Pharma) was biotinylated using EZ-Link hydrazide biotin (Thermo Fisher). Biotinylation degree was quantified with HABA/Avidin reagent (Sigma).

ELISA for functional testing:

To verify antibody functionality after biotinylation, the non-conjugated and biotinylated antibody were titrated by ELISA. A 96-well plate (Costar, Corning) was coated with 1 µg/ mL netrin-1 with Flag tag (Apotech) overnight at 4 °C. After three washing steps with 0.05% TWEEN20 in 1x PBS using the TECAN Strip Washer HydroFlex, the plate was blocked with 3% BSA in 1x PBS for 1 h at room temperature followed by series of three washing steps. Non-conjugated anti-netrin-1 antibody or biotinylated antibody was loaded at 1:3-serially diluted concentrations from 3 µg/mL to 0.0019 ng/mL for 1h at room temperature. After washing, anti-netrin-1 antibody was revealed using horseradish peroxidase-coupled goat anti-human IgG Fc antibody (A0170, Sigma) for 1 h at room temperature. After washing, Pierce ECL substrate (32106, Thermo Fisher) was added for 5-10 min in the dark at room temperature. Luminescence intensities were measured with the TECAN Infinite M1000 Pro microplate reader.

Functionalization of MBs:

MBs (Target-Ready MicroMarker, VisualSonics) were reconstituted and functionalized as described previously [1]. Briefly the lyophilized MB cake was re-suspended in 700 µL of 0.9% (w/v) NaCl in H_2O. To functionalize the avidin-coated MB shell with biotinylated anti-netrin-1 antibody, 1 - 100 µg of antibody was diluted in 300 µL of NaCl in H_2O and added to the MB suspension resulting in 1.6 x 10^9 functionalized MBs according to the manufacturer. The vial of MBs was incubated for 10 min at 25 rpm on the rotator (Stuart). To determine the optimal concentration for MB functionalization, i.e. the concentration of antibody that does neither require removal of excess antibody nor leave the MB shell

unsaturated, the functionalized MBs were centrifuged and at 300 xg for 5 min at 4 °C. The infranatant, i.e. the solution under the MB layer that had accumulated at the surface, was collected and quantified by ELISA including a calibration curve that was established with biotinylated anti-netrin-1 antibody (limit of detection at 0.01 µg/mL antibody). The number of antibody molecules/MB was determined via flow cytometry (FACS Calibur, BD; Quantum Simply Cellular, Bangs Laboratories).

Sizing and counting of MBs:

MB size distribution was determined by optical microscopy as described [1]. Briefly, MBs were diluted and loaded in a Malassez counting chamber (Marienfeld-Superior). Once, MBs had floated to the top, several images were acquired in an upright transmitted light microscope (LaborLux S with a 40x objective; Leitz) using an Axiocam Color 105 camera (Zeiss) above the large rectangles. MBs in microscopy images were automatically measured using MATLAB 2013 (The MathWorks) according to the following protocol: The optical images were first binarized, using a threshold estimated by Otsu's method ("graythresh" function of MATLAB). The diameter and eccentricity of each object on the binary image were computed using the "regionprops" function of MATLAB, and objects whose diameter was larger than 0.5 µm and whose eccentricity was lower than 0.5 were selected as MBs. This selection was found to exclude dust or noise on the image, as well as MB aggregates when present (Supp. Fig. S4). The number of MBs per surface area, and the diameter of each MB were then computed, to estimate the number of MBs/mm^2, MB concentration, and MB diameter distribution. MB sizes were indicated in micrometer with the standard error (STD).

The above MATLAB code was also used for counting of MBs in static binding assays to provide the number of MBs/mm^2. To determine the number of attached MBs in dynamic binding assays in parallel plate flow chambers, short image sequences (i.e. videos of 1 s length composed of 9-11 frames) were superposed to obtain an average image, in which only immobilized MBs are depicted. This averaged photomicrograph was then used to perform the above processing and calculate the number of MBs/mm^2 in dynamic binding conditions.

Zeta potential of MBs:

MB surface charge was measured as the Zeta potential on the Zetasizer Nano Series Nano-Zs (Malvern Instruments Ltd.). Water was used as dispersant. The Smoluchowski model was used for calculations. Surface charges were indicated in millivolt with the standard error (STD).

Netrin-1 expression analysis by Q-PCR

Cells were seeded at 1 x 10^6 cells/ 75 cm² flask. Cells were detached 3 days after seeding using 0.05% trypsin. Total RNA was extracted and 1 µg was reverse-transcribed using the iScript cDNA Synthesis kit (BioRad). Real-time quantitative RT-PCR was performed with a LightCycler 2.0 apparatus (Roche) using LightCycler® TaqMan® Master kit (Roche, Basel, Switzerland). Reaction conditions for all optimal amplifications, as well as primers selection were determined as already described [2]. The ubiquitously expressed human or murine GAPDH gene was used as an internal loading control. The sequences of the primers are available upon request.

Immunofluorescence staining of CD146

Endothelial cells were labeled with primary rabbit anti-CD146 antibody (ab75769, Abcam), followed by secondary Alexa Fluor 405-coupled goat anti-rabbit IgG (A31556, Invitrogen).

Receiver operating characteristic

To evaluate whether USMI allows differentiating netrin-1-positive and negative tumors, and to determine a cut-off threshold for netrin-1-targeted ultrasound imaging, a receiver operating characteristic (ROC) curve was plotted using the USMI signal in [%] derived from netrin-1-targeted MBs only. Using the pROC package for R [3], the sensitivity and specificity for potential imaging signal thresholds and corresponding 95% confidence intervals (CI) were computed based on $MB_{Netrin-1}$ signals obtained from MMTV-PyMT tumors and normal mammary glands. The optimal threshold to discriminate netrin-1-positive tumors from netrin-1-negative glands was estimated from the ROC curve by minimizing the distance of the curve to the top-left corner of the plot.

References

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Targeted Microbubbles in in Vitro Static Binding Assays. Ultrasound Med Biol. 2017; 43: 1506–19.

[2] Grandin M, Meier M, Delcros JG, Nikodemus D, Reuten R, Patel TR, et al. Structural Decoding of the Netrin-1/UNC5 Interaction and its Therapeutical Implications in Cancers. Cancer Cell. 2016; 29: 173–85.

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Supplementary Figure Legends

Supplementary Figure S1: Netrin-1 mRNA expression in human SKBR7 and MDA-MB-231 breast tumor tissue collected from nude mice. Netrin-1 mRNA was quantified by q-RT-PCR and presented as fold change relative to the HPRT housekeeping gene. N=4 for SKBR7 and MDA-MB-231; Error bars present SEM. Two-group comparison was performed with Student t-test.

Supplementary Figure S2: Western blot showed that HeLa-Ctrl cells were netrin-1-negative and HeLa-Net1 cells netrin-1-positive.

Supplementary Figure S3: Netrin-1 interaction on cell surfaces. (**A**) Netrin-1 presentation on the surface of model cells (HeLa-Ctrl: netrin-1-negative; HeLa-Net1: netrin-1 over-expression). (**B**) The effect of heparin on netrin-1 presentation on HeLa-Net1 cells. (**C**) The effect of enzymatic digestion of cell surface glycans on netrin-1 presentation on HeLa-Net1 cells. (**D**) The effect of netrin-1-containing supernatant on HeLa-Ctrl cells with or without prior enzymatic digestion of cell surface glycans. (**E**) Netrin-1 presentation on HUVEC endothelial cells in untreated condition or after incubation with either netrin-1-containing supernatant or a combination of netrin-1-containing supernatant and heparin. For enzymatic treatment, a cocktail containing Heparinase I, Heparinase III, Chondroitinase ABC and Glucorinidase was used. Netrin-1 supernatant was produced by HeLa-Net1 cells.

Supplementary Figure S4: Exemplarily representation of the size distribution, concentration, and binding analysis of MBs using MATLAB. The original microscopy image or an average microphotograph (composed of superposed video frames), in case of dynamic binding assays, was binarized using the "graythresh" function of MATLAB. Then, particles were filtered according to their diameter (>0.5 μm) and their eccentricity (<0.5) to exclude debris and MB aggregates from analysis. Scale bar indicates 10 μm.

Supplementary Figure S5: Preparation of netrin-1-targeted MBs. (**A**) Anti-netrin-1 antibody was biotinylated using hydrazide-biotin interacting with aldehyde groups of the antibody Fc. (**B**) Binding affinity of biotinylated anti-netrin-1 antibody was assessed. N=2; Error bars indicate SEM. (**C**) MBs (1.6 x 10^9 MBs/mL) were functionalized with increasing concentrations of antibody. Residual free antibody was quantified to determine the optimal concentration of antibody for functionalization. With a maximal concentration of 10 µg/mL of anti-netrin-1 antibody, no residual antibody was detected and 1.0 x 10^4 antibody molecules were coupled per MB. N=3. (**D**) Size profiles and surface charges of non-functionalized and functionalized MBs were compared. Size histograms (N=1) were plotted using 1814 non-functionalized MBs and 3062 functionalized MBs. Surface charge analysis N=3, and mean ± STD indicated.

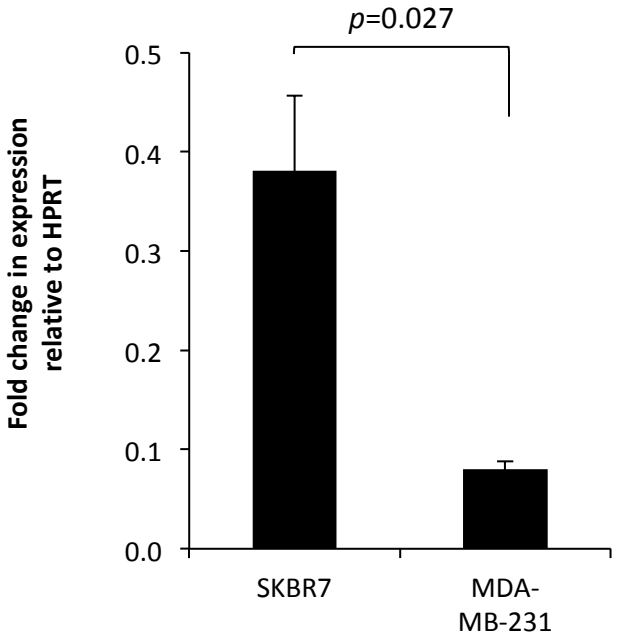
Supplementary Figure S6: Netrin-1 mRNA expression in human MDA-MB-231 and SKBR7 cell lines. Netrin-1 mRNA was quantified by q-RT-PCR and presented as fold change relative to the HPRT housekeeping gene. N=3. Two-group comparison was performed with Student t-test.

Supplementary Figure S7: USMI signal quantification of SKBR7, MDA-MB-231 and MMTV-PyMT tumors and normal mammary glands based on the calculation of the Targeted Enhancement ratio (T.E. (r)) in linear units (l.a.u.). The same data as presented in Figure 4 and 5 was analyzed using the VevoLab software. The T.E. (r) was calculated as: (pre-burst signal) / (post-burst signal). Thus, the minimum expected molecular imaging signal, in which case the pre-burst and post-burst signal intensities should be equal, has a value of 1.0 l.a.u. While this situation was nearly reached in normal mammary glands where the background signal was very low, the tumor models SKBR7, MDA-MB-231 and MMTV-PyMT showed T.E. (r) values that were higher than 1 l.a.u. in control conditions. Due to the presentation of the molecular imaging signals as T.E. (r) in l.a.u., differences between groups were smaller than what was

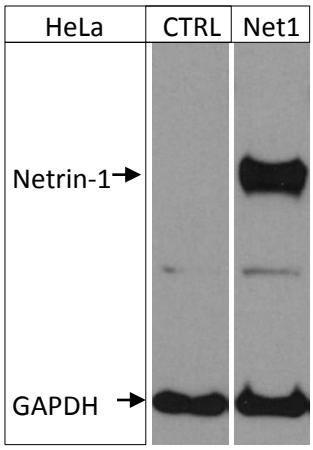
shown with the data depicted in % in Figure 4 and 5. In all figures, two-group comparisons were performed with Student t-test.

Supplementary Figure S8: IVIL analysis of CD146 and netrin-1 in (**A**) human SKBR7 and MDA-MB-231, and (**B**) transgenic MMTV-PyMT breast cancer and normal wildtype mammary glands. Scale bars indicate 20 µm.

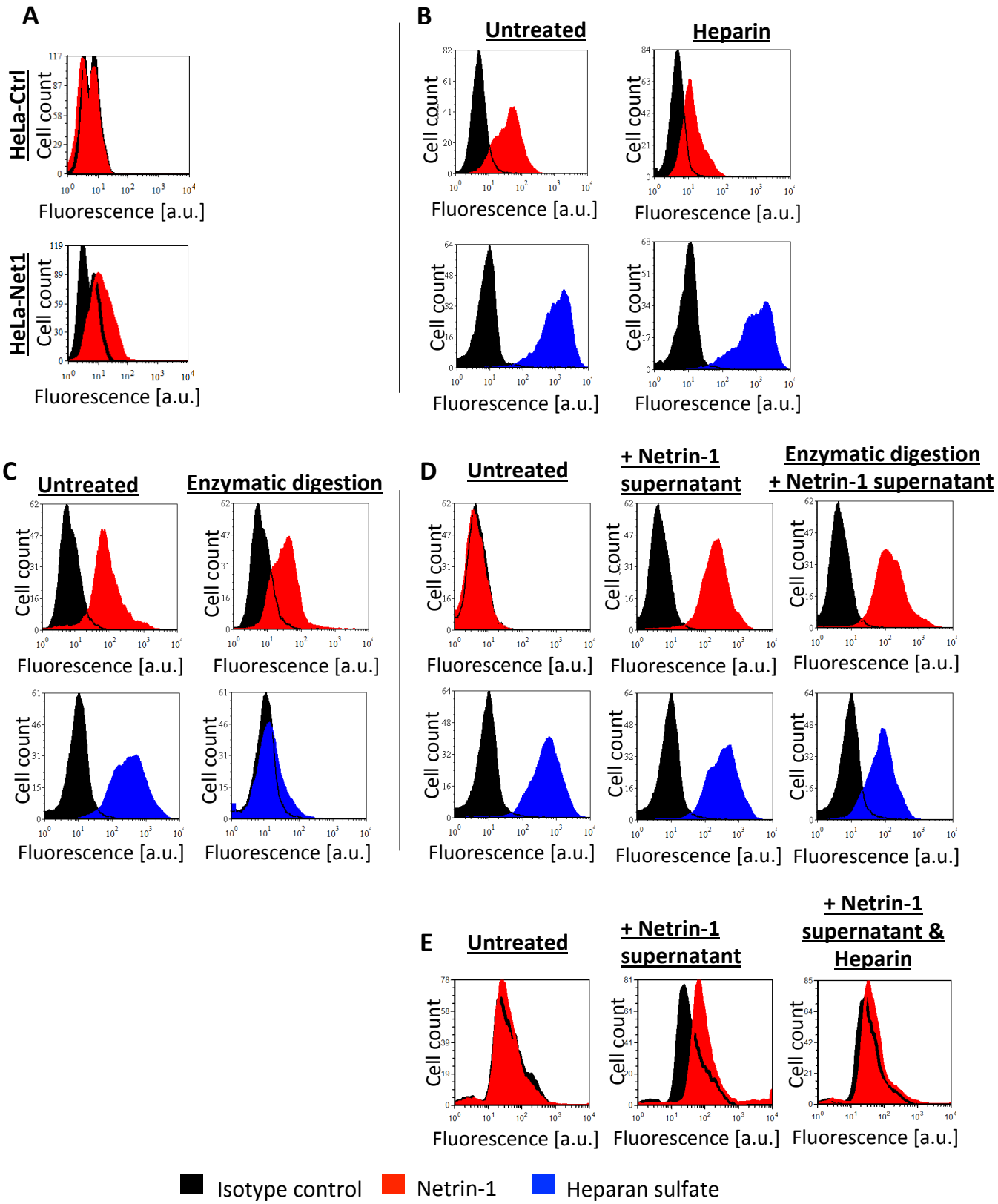
Supplementary Figure S9: Receiver operating characteristic (ROC) curve (black line) and its 95% confidence interval (blue area) based on USMI signal intensities [%] obtained with netrin-1-targeted MBs for MMTV breast tumor (N=30) vs. wildtype normal mammary glands (N=15). The area under curve (AUC), sensitivity and specificity and the respective 95% confidence interval (CI) are given. A red cross indicates the position of the optimal cut-off point discriminating netrin-1-positive tumors from negative mammary glands corresponding to a false positive rate (1-specificity) of 100% and a true positive rate (sensitivity) of 90%. The threshold value was at 33.3% of USMI signal, which was equivalent to a T.E. (r) of 1.47 l.a.u..



Supplementary Figure S1



Supplementary Figure S2



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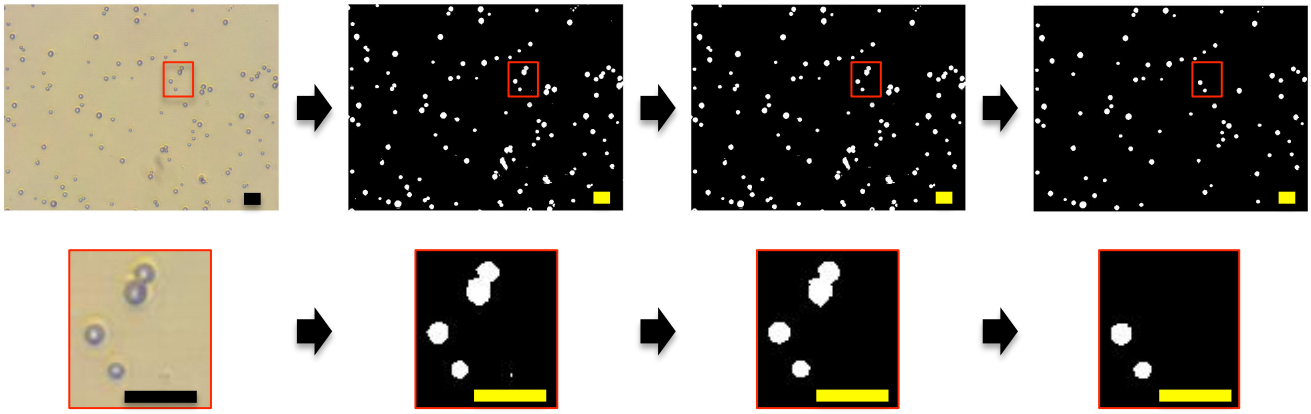
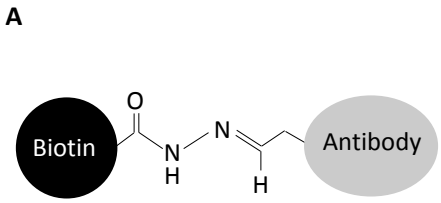
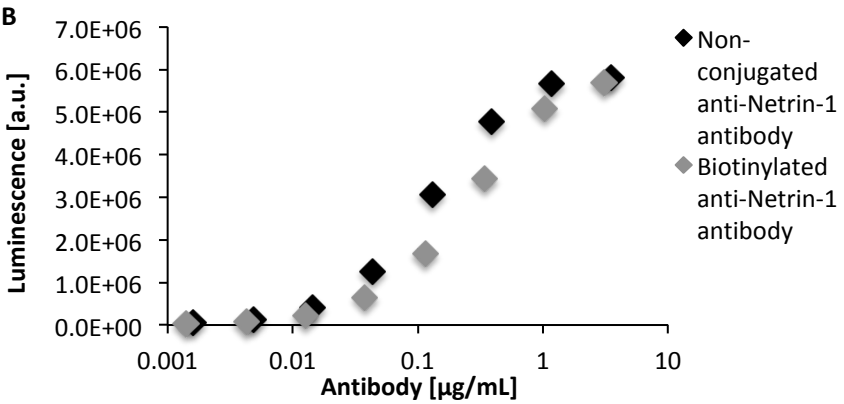
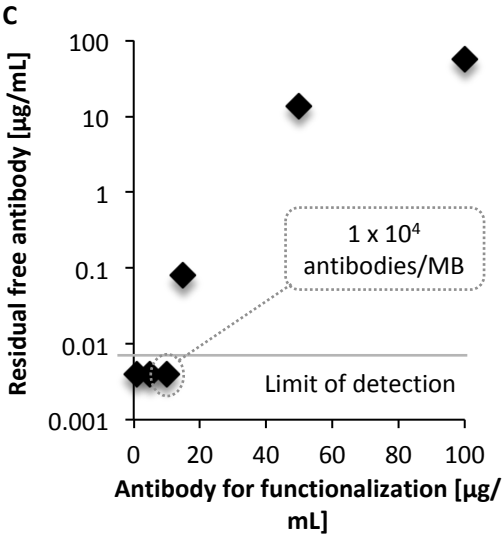


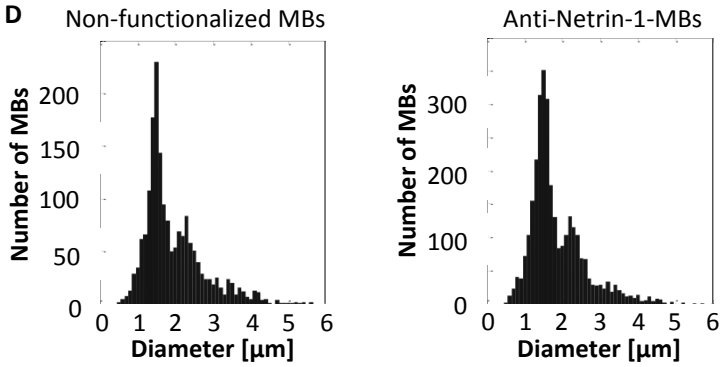
Supplementary Figure S4



Biotin linkage via covalent hydrazone bond:
4.7 biotins per antibody

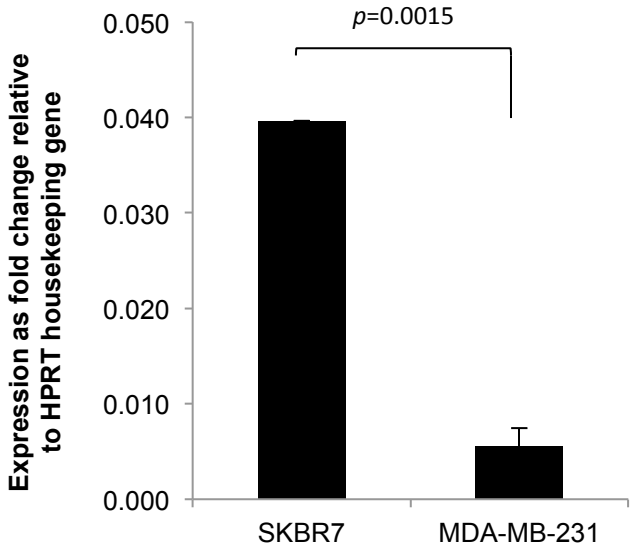




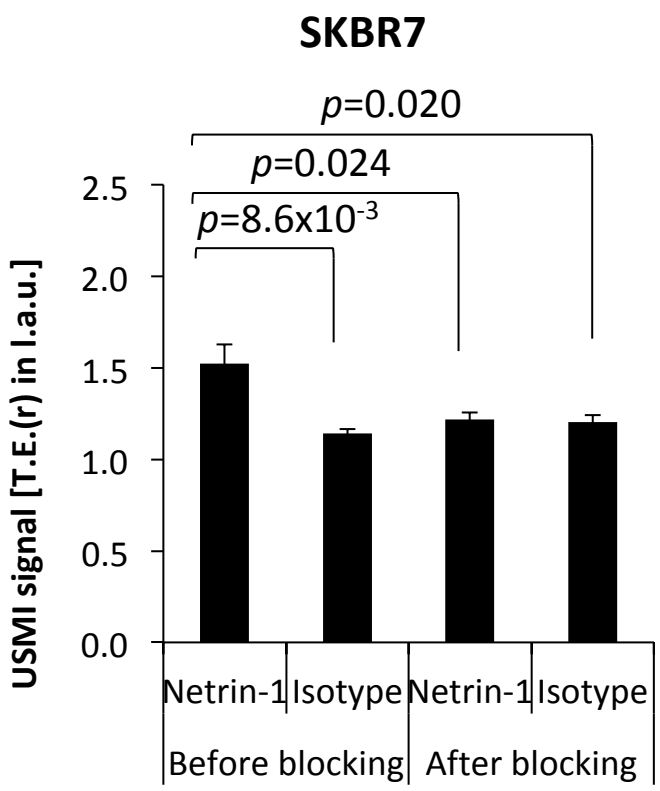


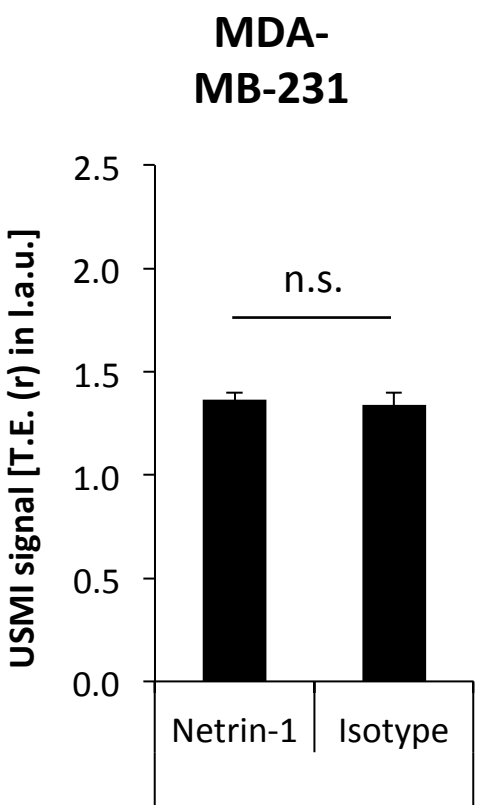
MB type	Non-functionalized	Netrin-1-targeted
Diameter [µm]	1.96 ± 0.82	1.85 ± 0.74
Surface charge [mV]	-14.8 ± 3.6	-18.8 ± 2.2

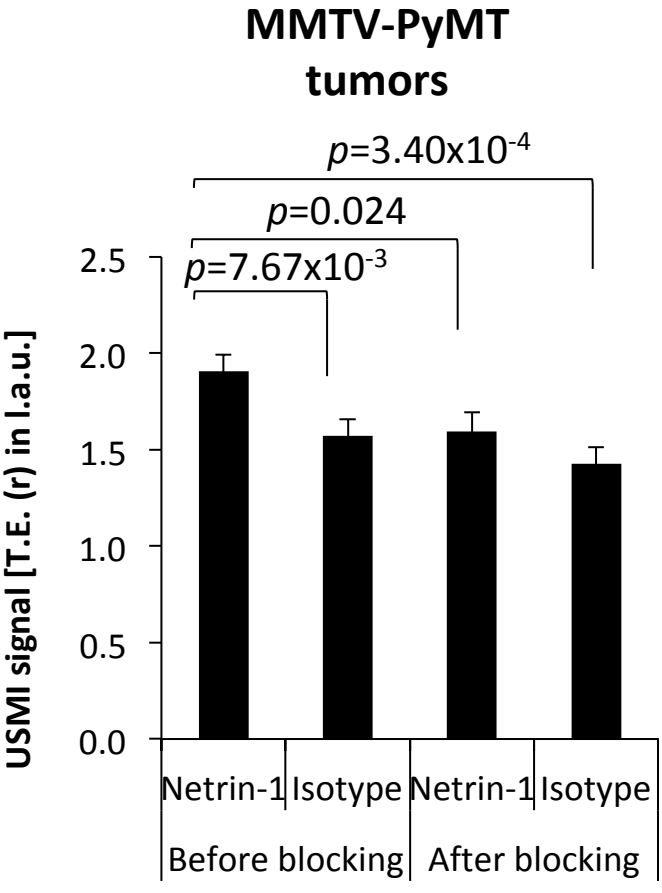
Supplementary Figure S4

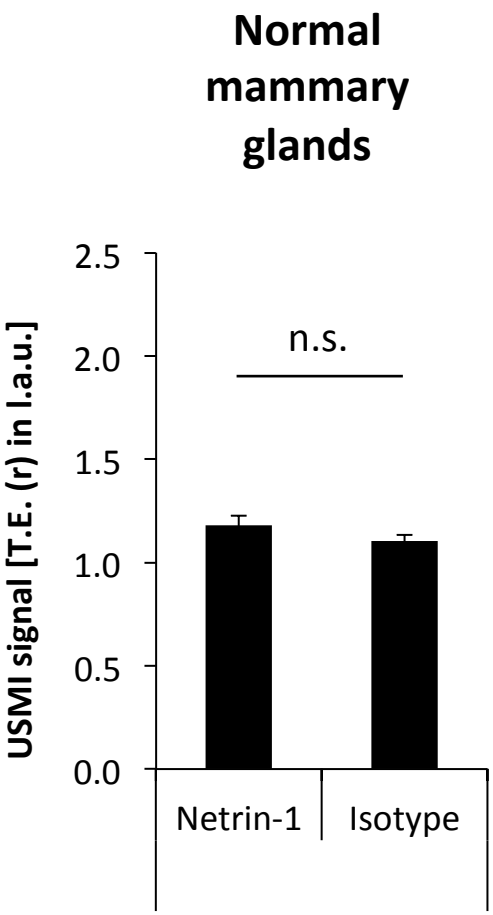


Supplementary Figure S6









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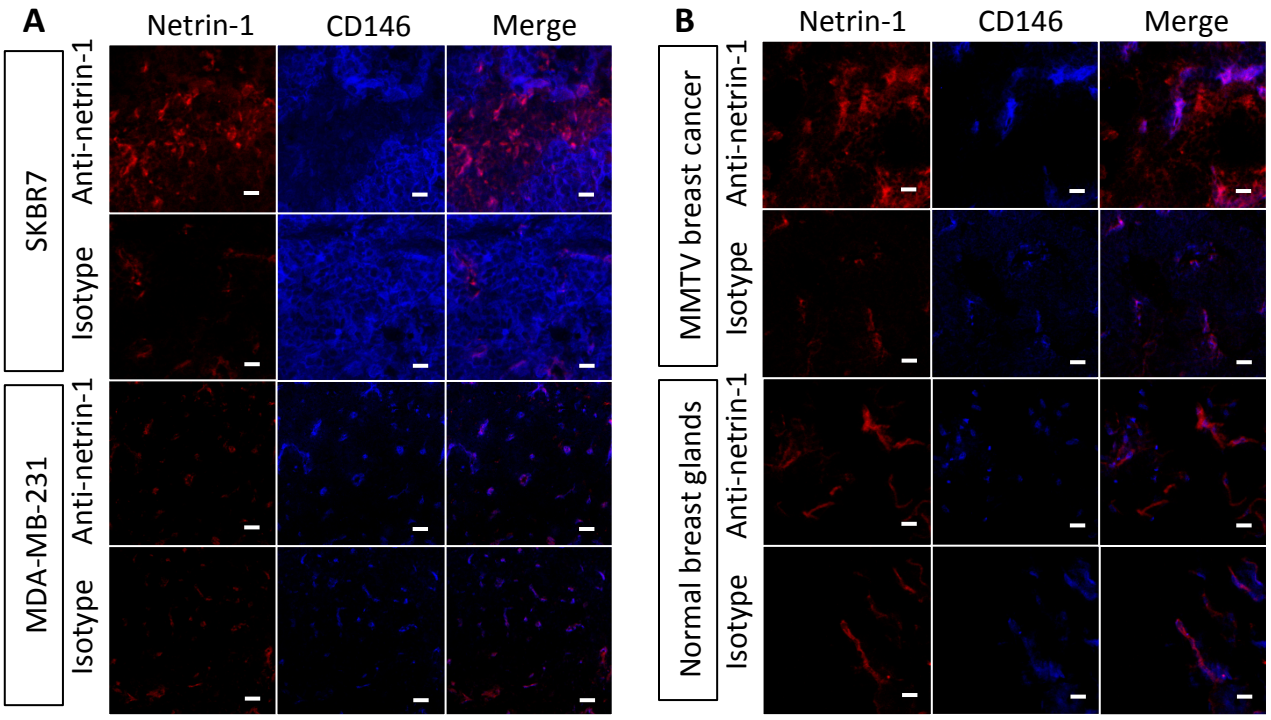
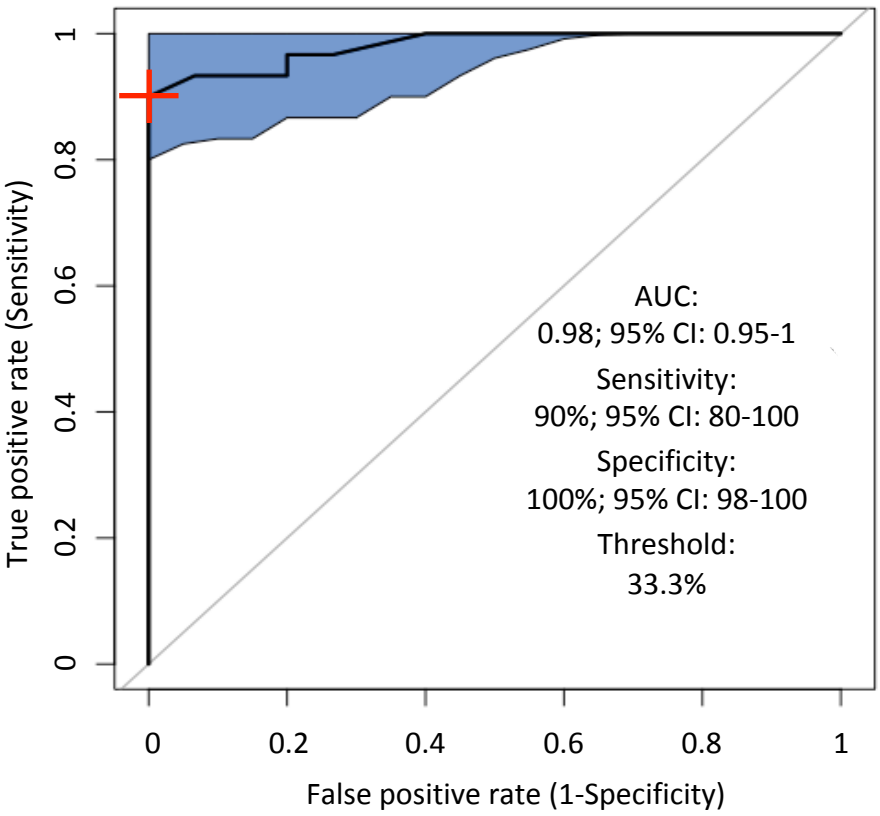


Supplementary Figure S8



Supplementary Figure S9