Supplementary Materials

Targeted Nanofitin-drug conjugates achieve efficient tumor delivery and therapeutic effect in an EGFR^{pos} mouse xenograft model

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List of supplementary figures

D8-Cys

MVKVKFEVWGEEKEVDTSKITSVGRMGKHVSFYYDDNGKWGLGYVAEKDAPKELLDMLARAEREKC

B10-Cys

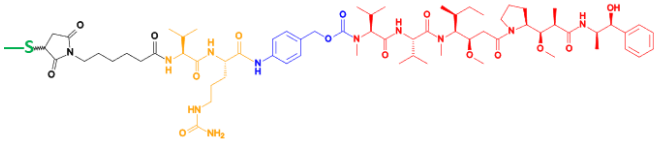
MVKVKFEVWGEEKEVDTSKIKEVFRLGKSVLFFYDDNGKTGVGFVSEKDAPKELLDMLARAEREKC

irrNF-Cys

MVKVKFFWNGEEKEVDTSKIVWVKRAGKSVLFIYDDNGKNGYGDVTEKDAPKELLDMLARAEREKC

D8-vc-MMAE

MVKVKFEVWGEEKEVDTSKITSVGRMGKHVSFYYDDNGKWGLGYVAEKDAPKELLDMLARAEREKC



B10-vc-MMAE

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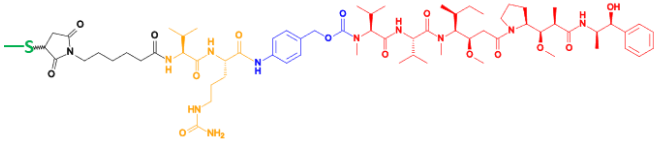

irrNF-vc-MMAE

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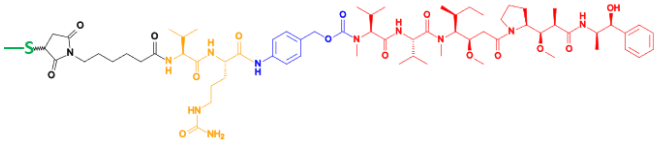

Fig. S1. Sequences of Nanofitins and Nanofitin-drug conjugates. Aminoacid sequences of D8, B10 and irrNF bearing a C-terminal cysteine are indicated in green. Attachment group, Val-Cit linker, spacer, and Monomethyl auristatin E moieties of the vc-MMAE payload are represented, respectively, as black, yellow, blue and red semi-developed structures.

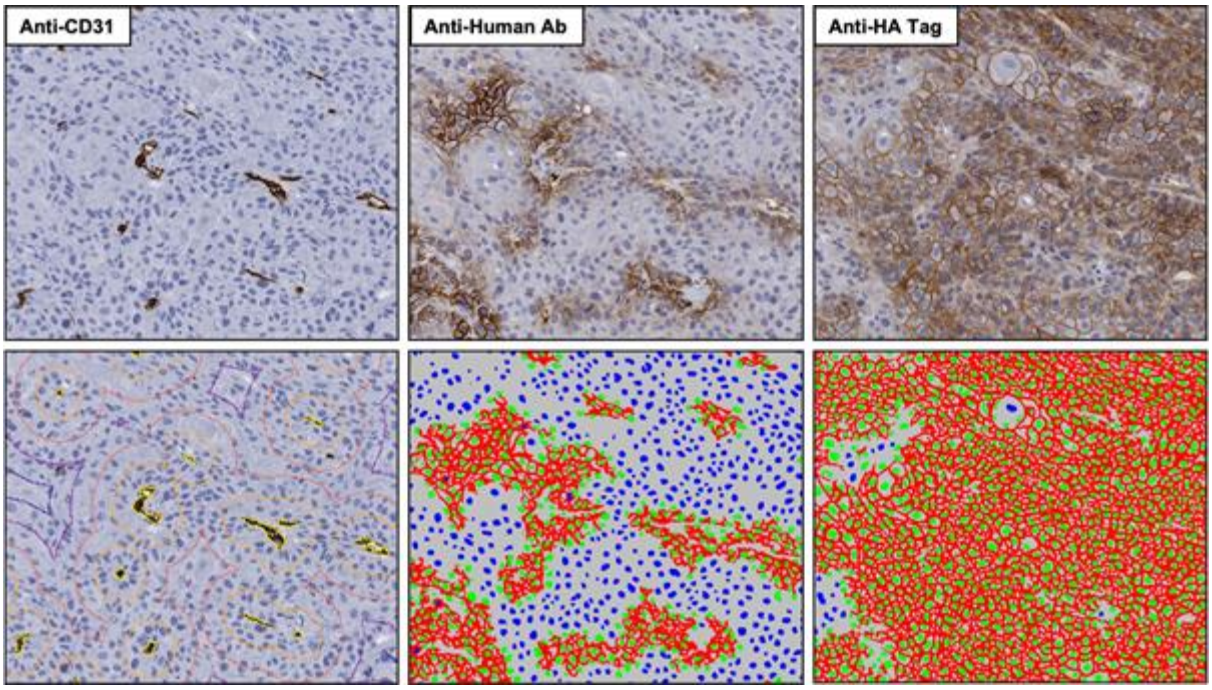

Fig. S2. Image processing to determine labeling indexes. Illustration of the image processing performed on the virtual slides from original images (top) to processed ones (bottom). Anti-CD31 IHC (left) was used to delineate blood vessels (in yellow) together with concentric tumor regions around them (in various colors). With regard to the compound (in red), which was evidenced by means of anti-human antibody IHC (targeting Cetuximab, center) or anti-HA tag IHC (targeting HA-tagged Nanofitins, right), DAB staining was used to identify the nuclei of positive cells (in green) as opposed to those of negative cells (in blue).

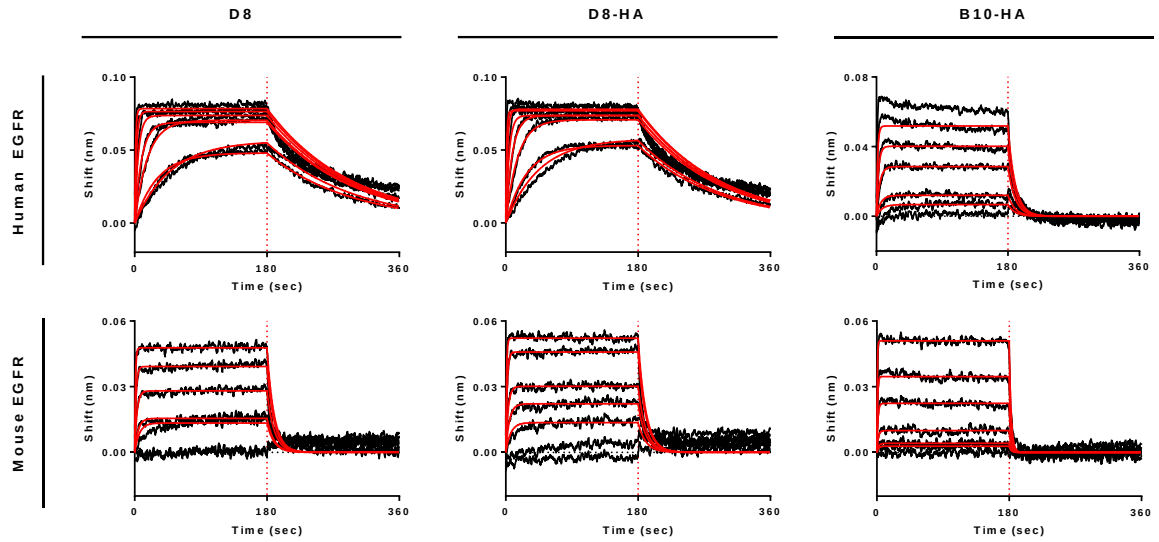

Fig. S3. Binding characteristics of the anti-EGFR Nanofitins on human and mouse EGFR. Determination of the binding characteristics of the anti-EGFR Nanofitins D8 (left), D8 with a C-terminal HA-tag (center), and B10 with a C-terminal HA-tag (right) by biolayer interferometry on human EGFR (top) or mouse EGFR (bottom). The experiment was run on Octet RED96 using the anti-EGFR Nanofitins at concentrations of 500, 250, 125, 62.5, 31.25, 15.625 and 7.81 nM. Fittings (1:1 model) are represented as solid red lines.

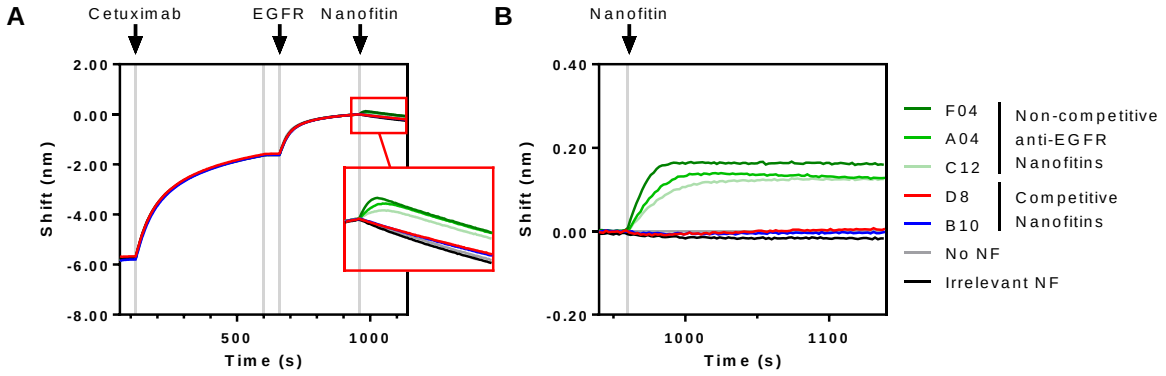

Fig. S4. Competition profiles with Cetuximab. Epitope competition with Cetuximab, determined in biolayer interferometry by loading Cetuximab (10 µg/mL) on protein A biosensors, followed by association with human EGFR extracellular domain (100 nM, 100001-H08H, Sino Biological) and by association with Nanofitins (125 nM). Each step, corresponding to baselines or addition of a molecule (indicated by arrows), is separated by a solid gray line. F04, A04 and C12 are non-competitive anti-EGFR Nanofitins, able to bind to EGFR captured by Cetuximab (dark to light green lines). D8 (red) and B10 (blue) are competitive anti-EGFR Nanofitins, unable to bind to EGFR captured by Cetuximab. Buffer alone (No NF, gray) and irrelevant Nanofitin (black) do not trigger binding to EGFR. (**A**) Complete sensorgrams aligned on the Nanofitin step, zoomed in the red rectangle. (**B**) Nanofitin association step, after subtraction of the reference signal (buffer alone).

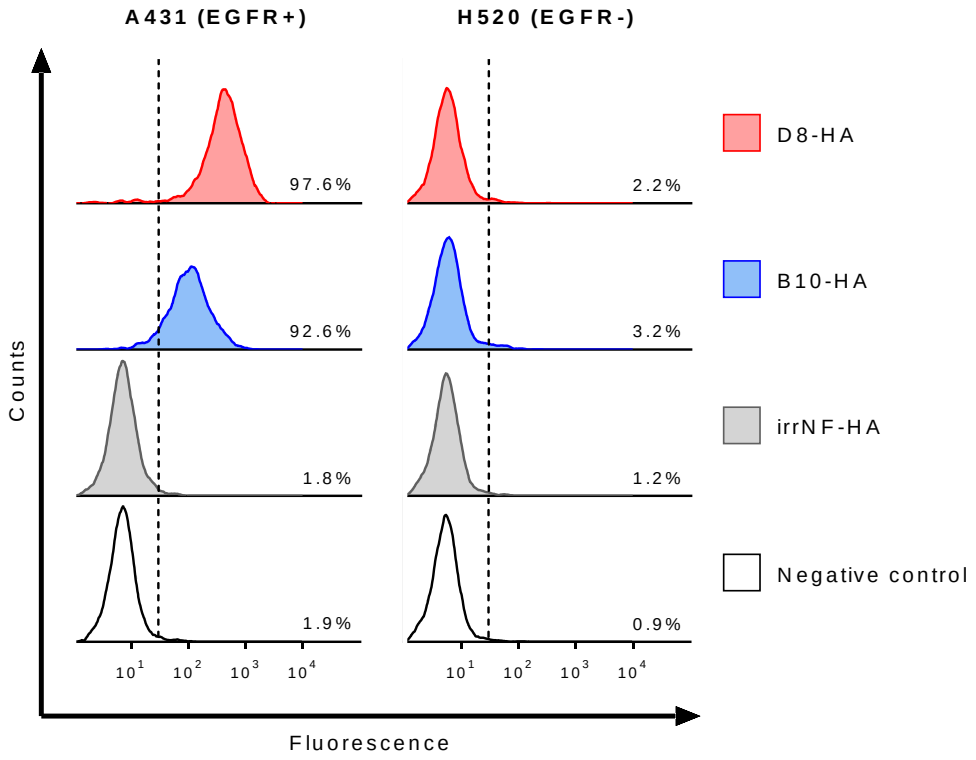

Fig. S5. Binding to cell surface EGFR. Cell staining of Nanofitins D8, B10 and irrNF was analyzed by flow cytometry using an antibody against C-terminus HA-tag on EGFR-positive (A431) or negative cells (H520). HA-tagged Nanofitins are named D8-HA, B10-HA and irrNF-HA for clarity. As a negative control, cells were stained with the detection anti-HA-tag antibody alone. Percentage (%) of positively stained cells above the fluorescence threshold (dashed lines) are indicated. Materials and methods: A431 (CRL-1555, ATCC) and H520 (HTB-182, ATCC) cells were resuspended in PBS containing 1% BSA and incubated on ice for ≥ 15 min in 96-well conical bottom microplates (2×10^5 cells/well). Supernatants were discarded after centrifugation at 4°C for 1 min at 750g. The next incubation steps were performed on ice in PBS containing 1% BSA, followed by 3 washing steps with ice-cold PBS discarded after centrifugation (4°C, 1 min, 750*g*). HA-tagged Nanofitins (100 nM, 100 µL) were allowed to bind on cells for 30 min. Then, primary HA-Tag Rabbit mAb (10 nM, 100 µL, #3724, Cell Signaling Technology) was incubated for 20 min to detect HA-tag. Secondary Alexa Fluor 488 AffiniPure Donkey Anti-Rabbit IgG (10 nM, 100 µL, 711-545-152, Jackson Immuno Research) was incubated for 20 min to allow fluorescent staining. Cells were acquired on the flow cytometer FC 500 (Beckman Coulter). Data analysis was carried out using Flowing Software 2.5.1 (Turku Bioscience Centre).