

## Supporting Information

# Modular adapters utilizing binders of different molecular types expand cell-targeting options for adenovirus gene delivery

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## Material and Methods

### Virus and cell lines

The replication-deficient HAdV-C5 first-generation vector used contains an E1/E3 deletion and 4 mutations in the HVR7 (I421G, T423N, E424S and L426Y) and was generated as previously described.<sup>1</sup> The vector encodes the infrared fluorescent protein (iRFP670) or the firefly luciferase (*luc*) gene in the E1 region under the control of the cytomegalovirus major immediate early promoter (CMV). CHO cells, stably expressing human NTR1, were generated using the CHO Flp-In cell system (Thermo Fisher Scientific product number). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with tetracycline-free fetal calf serum (FCS product number) with a final concentration of 10%. NTR1 expression was induced by addition of 1 µg/mL tetracycline to the medium. KB and SKBR3 cells were obtained from the American Type Culture Collection (ATCC; [www.atcc.org](http://www.atcc.org)) and cultures were grown according to ATCC recommendations. 24 h before transduction, KB cells were starved using folate-free DMEM and 1% FCS. All cell lines have been verified to be free of mycoplasma contaminations.

### Plasmid construction and expression of bacterial produced adapters

For introducing a single N-terminal cysteine for thiol-maleimide conjugation, the plasmids pQIq, encoding the trimeric adapters with DARPins G3 or E2\_5, were modified at their 5'-end by introducing a gene fragment encoding a TEV protease-cleavable His<sub>6</sub>-tag followed by a GCG spacer for site-specific conjugation (5'-MRGSHHHHHENLYFQGCG-3'), introduced via EcoRI and BamHI restriction sites. All single-cysteine-containing and non-cysteine containing trimeric adapters were expressed in *E. coli* XL1-Blue as previously described.<sup>2</sup> After expression, the cultures were centrifuged (4000 x g, 10 min, 4°C) and washed by resuspension of the cell pellets in ice-cold PBS, pH 7.4. Cell pellets were then resuspended in 50 mM Tris-HCl pH 8.0, 400 mM NaCl (TBS400), supplemented with 3 mg/ml lysozyme, 100 µg/mL DNase I and lysed by sonication and French Press. The obtained lysates were centrifuged (21,000 x g, 30 min, 4°C) and the supernatants applied to Ni-NTA Superflow (Qiagen) metal ion affinity columns (4 mL). All columns were washed with each 15 column volumes (CV) of 50 mM Tris-HCl pH 8.0, 20 mM imidazole supplemented with 400 mM NaCl, 1 M NaCl or 20 mM NaCl, respectively. Then the adapters were eluted in 5 CV PBS pH 7.4, 500 mM imidazole. The eluted proteins were then transferred into dialysis tubes with a MW cutoff of 6000-8000 Da, supplemented with 100 µg/mL TEV protease produced in-house and dialyzed overnight at 4°C in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT. The next morning, the TEV protease-cleaved His-tags and non-cleaved adapters were removed by running the content of the dialysis tubes over Ni-NTA Superflow metal ion affinity columns (1 mL). The flow-through was collected and concentrated by ultrafiltration (Amicon Centrifugal Filter Units, Millipore). Protein concentrations were determined by UV-Vis spectroscopy and purity was confirmed by SDS-PAGE analysis.

### Expression and purification of adapters expressed in mammalian cell

The trimeric adapters were cloned into pcDNA3.1, using the scFv 4D5 sequence previously reported<sup>3</sup>. The scFv adapter was encoded with an HSA leader peptide, an N-terminal 3C-cleavable His<sub>6</sub>- and FLAG-tag. The retargeting domain is flanked by a BamHI and a HindIII site for ready exchange of the domain. The scFv-containing adapters were expressed in CHO-S cells as described by Hacker et al.<sup>4</sup> Following expression for seven days, adapters were purified from the filtered supernatant using a Protein-L affinity chromatography (GE Healthcare) with subsequent 3C cleavage of the tags during dialysis

against 20 mM Hepes at pH 7.4. As an additional purification step, an anion exchange chromatography using a MonoQ column (GE Healthcare) was performed. The purified protein was shock-frozen in liquid nitrogen and stored at -80°C until usage.

Maleimide coupling of a small molecule or peptide to the cysteine-containing retargeting adapters

For site-specific conjugation to the N-terminal cysteines of purified adapters, folic acid was coupled to form an amide between its glutamyl carboxylate and the N-terminus of a spacer peptide (sequence H<sub>2</sub>N-ASPASPASPASPASPASP-COOH / MW = 2164.96 g/mol). The C-terminus forms an amide with N-(2-aminoethyl)maleimide, all custom synthesized by Wuxi Apptech. Similarly, neurotensin (custom synthesis by Bachem AG, peptide-NT<sub>8-13</sub> = H<sub>2</sub>N—SGSGGSGSGGSGGG-RRPYIL-COOH / MW = 2004.98 g/mol) was derivatized at the N-terminus to form an amide with N-maleoyl-beta-alanine. Both compounds were dissolved in anhydrous DMSO to a concentration of 15 mM for subsequent coupling.

Protein samples were spiked with freshly dissolved DTT to a final concentration of 5 mM and reduced while shaking at 25°C for 30 min. To remove DTT from the reduced samples, the buffer was exchanged to rigorously degassed PBS pH 7.4, 1 mM EDTA on a HiPrep™ 26/10 desalting column (GE Healthcare) connected to an Äkta Explorer (GE Healthcare) FPLC system. Desalted Protein samples (6-21 μM) were mixed with a 5-fold molar excess of maleimide-activated folate, or maleimide-activated neurotensin over reduced cysteine. For conjugation, the reaction was incubated for 3 h at 25°C with shaking. The conjugation mixtures were then quenched by the addition of a 5-fold molar excess of DTT over maleimide and incubated for 15 min at 25°C with shaking. All steps were carried out under a nitrogen atmosphere. For purification of the conjugates from residual DTT, EDTA and quenched maleimide-activated FA and NT, the conjugation reactions were dialyzed in PBS pH 7.4 using dialysis tubes with a MW cutoff of 7-14 kDa. During 24 h, the buffer was exchanged four times. The dialyzed conjugates were then concentrated to 15-45 μM. The purity of conjugates was monitored by SDS-PAGE and the identity confirmed by ESI-MS.

ESI-MS analysis

Protein masses were determined by time-of-flight (TOF) ESI-MS at the Functional Genomics Center Zurich (FGCZ). Prior to ESI-MS analysis, samples were desalted by C4 ZipTip (Millipore, USA) reversed phase chromatography and eluted in MeOH:2-propanol:0.2% formic acid (30:20:50). The eluates were infused through a fused silica capillary (inner diameter 75 μm) at a flow rate of 1 μL/min and sprayed through a PicoTip with an inner diameter of 30 μm (New Objective, USA). Nano ESI-MS analysis of the samples was performed on a Synapt G2\_Si mass spectrometer (Waters, UK) and the data were recorded with MassLynx 4.2. Software (Waters, UK). Mass spectra were acquired in positive-ion mode by scanning an *m/z* range from 100 to 5000 Da with a scan duration of 1 s and an interscan delay of 0.1 s. The spray voltage was set to 3 kV, the cone voltage to 40 V, and the source temperature to 80 °C. The recorded *m/z* data were then deconvoluted into mass spectra by applying the maximum entropy algorithm MaxEnt1 (MaxLynx) with a resolution of the output mass of 0.5 Da/channel and Uniform Gaussian Damage Model at the half height of 0.7 Da.

### Flow cytometry analysis of expression

Cells were trypsinized for 20 min at 37°C and washed with DMEM, centrifuged at 300 *g* for 3 min and resuspended in flow cytometry buffer (FC buffer) (PBS + 1% BSA + 0.05% azide) or FC buffer including receptor-staining reagent. Cells were incubated for 30 min at 4°C in the dark, and then washed 3x with FC buffer before analyzed with the flow cytometer. HER2 was detected using a 1:200 dilution of FAB1129G (R&D systems), folate receptors with 1:50 dilution of FolateRSense™ 680 (Perkin Elmer) and NTR1 was detected using fluorescently labeled neurotensin peptide HL488-NTS8–13<sup>5</sup> in a 1:2 dilution.

### Analysis of viral gene delivery

Cells were seeded with  $1.5 \times 10^4$  cells per well of a 96-well plate, 24 h prior to infection. Adenoviral vectors encoding firefly luciferase or iRFP670 under the control of a CMV promoter were incubated with a retargeting adapter (retargeted), a non-binding adapter that only contained the knob-binding DARPin E2\_5 (non-binding), resulting in blocking of the CAR mediated uptake, or without any retargeting adapter (untargeted) for one hour at 4°C. The ratio of viral knob to adapters was 1:20 with a MOI of 2.5 plaque-forming units (PFU/cell). Viral particle-containing supernatants were removed 16 h post addition to the 96-well plate and replaced by fresh culture medium. Transgene expression was determined 72 h post transduction. Luciferase activity was determined by a luciferase assay (Promega) according to the manufacturer's instructions. iRFP fluorescence was measured by flow cytometry. Cells were washed with PBS and then detached using trypsin. Cells were then washed twice using PBS and then fixed using 2.5% PFA in PBS. After 20 min incubation at room temperature, cells were again washed with PBS containing 1% BSA and analyzed using a FACSCanto II 2L (BD).

## Supporting Figures

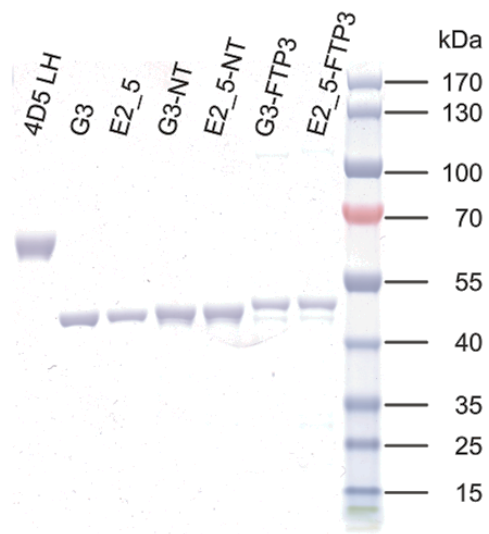


Figure S1: Purity analysis with SDS-PAGE of 2  $\mu$ g purified adapter protein, heated to 92°C with 0.1 M DTT. The label indicates the retargeting domain incorporated into the trivalent adapter, 4D5 LH (scFv), G3 and E2\_5 (DARPin), G3-NT and E2\_5-NT (DARPin coupled to neurotensin), G3-FTP3 and E2\_5-FTP3 (DARPin coupled to folate).

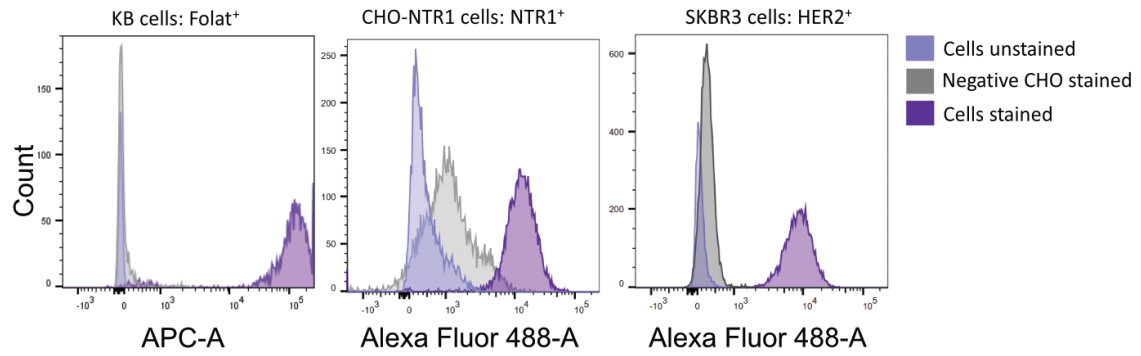


Figure S2 Expression of folate receptor on KB cells was confirmed using FolateSense<sup>TM</sup> 680. NTR1 expression in the CHO Flp-In NTR<sup>+</sup> cells was confirmed, after tetracycline induction, with fluorescently labeled peptide HL488-NTS8-13.<sup>5</sup> The expression of HER2 on SKBR3 cells was detected using the Alexa-488 labeled FAB1129G antibody. All staining agents were tested on non-expressing CHO parental cells (grey) and compared with the autofluorescence of the cells (light blue) and the stained cells (violet).

## Supporting Table

**Table S1: Overview of the theoretical mass and electrospray ionization mass spectrometry measured mass of monomeric adapters after bioconjugation**

Construct:	Theoretical MW (Da)	Measured MW (Da)	Difference MW (Da)
G3-1D3nc-SHP1	30872		
Folate-G3-1D3nc-SHP1	44113	44113	0
NT-GS-G3-1D3nc-SHP1	43953	43953	0
E2_5-1D3nc-SHP1	42103		
Folate-E2_5-1D3nc-SHP1	44268	44269	+1
NT-GS-E2_5-1D3nc-SHP1	44107	44107	0

## References

1. Schmid, M.; Ernst, P.; Honegger, A.; Suomalainen, M.; Zimmermann, M.; Braun, L.; Stauffer, S.; Thom, C.; Dreier, B.; Eibauer, M.; et al. Adenoviral vector with shield and adapter increases tumor specificity and escapes liver and immune control. *Nat. Commun.* **2018**, *9* (450), 1-16.
2. Dreier, B.; Honegger, A.; Hess, C.; Nagy-Davidescu, G.; Mittl, P.R.; Grütter, M.G.; Belousova, N.; Mikheeva, G.; Krasnykh, V.; Plückthun, A. Development of a generic adenovirus delivery system based on structure-guided design of bispecific trimeric DARPin adapters. *Proc. Natl. Acad. Sci.* **2013**, *110* (10), E869-E877.
3. Ramm, K.; Gehrig, P.; Plückthun, A. Removal of the conserved disulfide bridges from the scFv fragment of an antibody: effects on folding kinetics and aggregation. *J. Mol. Biol.* **1999**, *290* (2), 535-546.
4. Hacker, D. L.; Kiseljak, D.; Rajendra, Y.; Thurnheer, S.; Baldi, L.; Wurm, F. M. Polyethyleneimine-based transient gene expression processes for suspension-adapted HEK-293E and CHO-DG44 cells. *Protein Expr. Purif.* **2013**, *92* (1), 67-76.
5. Deluigi, M.; Klipp, A.; Klenk, C.; Merklinger, L.; Eberle, S. A.; Morstein, L.; Heine, P.; Mittl, P. R. E.; Ernst, P.; He, T. M. K. Y.; et al. Complexes of the neurotensin receptor 1 with small-molecule ligands reveal structural determinants of full, partial, and inverse agonism. *Sci. Adv.* **2021**, *7* (5), 5504.