Table of Contents

Supplementary Figures .......................................................................................................................... 2
- Figure S1. In vivo imaging of luminescence mediated by recombinant AAV2 vectors. .......................................................... 2
- Figure S2. Longterm transgene expression mediated by rAAV2-ESGHGYF. ........................................... 3
- Figure S3. In vivo imaging of luminescence mediated by rAAV2-ESGHGYF after intraperitoneal injection ........................................................................................................ 4
- Figure S4. Functional consequences of alanine scan or sequence scrambling of the peptide ESGHGYF .................................................................................................................. 5
- Figure S5. Competition assay with soluble ESGHGYF peptide .......................................................... 6
- Figure S6. Biodistribution of AAV2-ESGHGYF, four hours after administration ...... 7
- Figure S7. Prevalence of neutralizing antibodies against AAV2-WT and -ESGHGYF ............................................................................................................................ 8
- Figure S8. Neutralizing antibody response after injection of mice with AAV2-WT and -ESGHGYF .................................................................................................................................. 9

Supplementary Notes ............................................................................................................................ 10
- 1: Bioinformatical data processing .................................................................................. 10

Supplementary Materials and Methods ............................................................................................ 11
- Peptide competition assay ......................................................................................... 11
- Neutralizing antibody assay ..................................................................................... 11

Supplementary References .................................................................................................................. 12
Supplementary Figures

Figure S1. In vivo imaging of luminescence mediated by recombinant AAV2 vectors.

Images were taken 14 days after i.v. administration of rAAV2-CMV-Luciferase vectors at a dose of 5x10^{10} genomic particles/FVB/N mouse. Recombinant AAV2 vectors displaying the lung enriched peptide ESGHGYF mediate strong (≥10^{5} p/sec/cm^2/r) luciferase expression, specifically in the lung, with almost no differences between the tested mice. Recombinant AAV vectors displaying the random control peptide CVGSPCG mediate weak (≥10^4 p/sec/cm^2/r) expression in heart and some spots in the abdomen. Recombinant wild-type AAV2 vectors mediate moderate (≥10^4 p/sec/cm^2/r) expression mainly in the liver.
Figure S2. Longterm transgene expression mediated by rAAV2-ESGHGYF

Transgene expression was measured at 14 different time points after i.v. administration of recombinant AAV2 vector displaying the lung enriched peptide ESGHGYF (5x10^{10} gp/mouse; n = 2 FVB/N mice/group). Luminescence in the region of interest (ROI) was quantified when the signal reached the highest intensity.
Figure S3. In vivo imaging of luminescence mediated by rAAV2-ESGHGYF after intraperitoneal injection

Images were taken 5 days after i.p. administration of rAAV2-ESGHGYF-CMV-LUC at a dose of $5 \times 10^{10}$ genomic particles/mouse ($n = 3$ FVB/N mice). Recombinant AAV2 vectors displaying the lung enriched peptide ESGHGYF mediate strong ($\geq 10^5$ p/sec/cm$^2$/r) luciferase expression, specifically in the lung.
Figure S4. Functional consequences of alanine scan or sequence scrambling of the peptide ESGHGYF

Images were taken 14 days after i.v. administration of rAAV2-CMV-luciferase vectors displaying modified versions of the peptide ESGHGYF (5x10^{10} gp/mouse; n = 3 FVB/N mice/group). Conversion of either one of the first two amino acids (E,S) to A does not abrogate the lung tropism. Conversion of amino acids 3 or 4 (G, H) to A leads to almost complete transduction deficiency. The conversion of amino acids 5; 6 and 7 (G, Y, F) results in de-targeting to heart and skeletal muscles. A scrambled version of the peptide ESGHGYF (FHEYGSG) also mediates strong transduction of heart and skeletal muscles, in addition to the liver.
Figure S5. Competition assay with soluble ESGHGYF peptide

Images were taken 17 days after i.v. administration of rAAV2-ESGHGYF-CMV-LUC at a dose of 2x10^{10} genomic particles/mouse (n = 3 SCID mice/group). Animals received either vector alone (upper left panel) or in combination with 200 µg (= 1.5x10^{17} molecules) soluble peptide NH₂-ESGHGYF-COOH (lower left panel). No significant blocking of gene transfer was observed in terms of luminescence quantified in the lungs as ROI = region of interest (left and right panels).
Figure S6. Biodistribution of AAV2-ESGHYGF, four hours after administration

The amount of vector in seven analyzed organs was determined by quantitative real time PCR of vector genomes, 4 hours after i.v. administration of $5 \times 10^{10}$ gp/mouse. Data are shown as bars (mean) with plotted individual data points ($n = 3$ FVB/N mice/group).
Figure S7. Prevalence of neutralizing antibodies against AAV2-WT and -ESGHGYF

(a) Screening of human sera for neutralizing antibodies against AAV2. Four out of nine donors (#1-4) were seronegative for wild-type AAV2 (AAV2-WT) and the capsid modified variant AAV2-ESGHGYF. Five out of nine donors were seropositive, with no significant (#5-9) or little (#7) differences between AAV2-WT and AAV2-ESGHGYF. (b) Pooled human intravenous immunoglobulin (IVIG) is highly reactive against AAV2 with no significant differences between AAV2-WT and AAV2-ESGHGYF. All data show mean values, +SD (technical triplicates).
Figure S8. Neutralizing antibody response after injection of mice with AAV2-WT and –ESGHGYF

(a) No neutralizing antibodies against AAV2-WT or AAV2-ESGHGYF were detected in the serum of naïve, untreated mice. (b) Four days after injection of AAV2-ESGHGYF-CMV-LUC vector (2x10^{10} vg/mouse), neutralizing antibodies against AAV2-ESGHGYF were detected (>1/128), which showed cross-reactivity for AAV2-WT, although at a lower titers (<1/128). (c) Four days after injection of AAV2-WT-CMV-LUC vector (5x10^{10} vg/mouse), neutralizing antibodies against AAV2-WT were detected (<1/512), which showed cross-reactivity for AAV2-ESGHGYF, although at lower titers (<1/64). (c) High titers (>1/1024) of cross-reactive neutralizing antibodies against AAV2-ESGHGYF and AAV2-WT were detected in the serum of mice that were pre-immunized with AAV2-ESGHGYF virus like particles (1x10^{11} VLP/mouse), 23 days before treatment with AAV2-ESGHGYF-CMV-LUC vector (5x10^{10} vg/mouse). (d) High titers (>1/528) of cross-reactive neutralizing antibodies against AAV2-WT and AAV2-ESGHGYF were detected in the serum of mice that were pre-immunized with AAV2-WT virus like particles (1x10^{11} VLP/mouse), 23 days before treatment with AAV2-WT-CMV-LUC vector (5x10^{10} vg/mouse). (f) Area Under the Curve (AUC) analysis was used to quantify the aforementioned observations. The data were grouped based on the seropositivity for each vector. Groups were compared to naïve, non-treated by two-way ANOVA. All data show mean values, +SD (n = 3 animals per Group).
Supplementary Notes

1: Bioinformatical data processing.

Demultiplexing of short-reads into individual samples was conducted by sorting reads according to their prefix, keeping only those whose prefix was identical to one of the custom index sequences incorporated during library preparation. The random oligonucleotide sequences were then identified by searching the demultiplexed reads for exact matches of the two flanking sequences GAGAGCCAGAGAGGC and GCCCAGGCAGCCACCG. The obtained sequences were sorted into clusters of identical sequences and subsequently different filters were applied to remove possible artifacts. Given that the observed mean Phred quality score of the samples was above 32, it can be expected that approximately 1% of all random 21mers contain sequencing errors. To identify these erroneous sequences it was checked for every sequence if it was at least 100 times less abundant than any other sequence. If the Levenshtein distance between two such sequences was 1, we excluded the less abundant sequence from further analysis. Additionally, we removed sequences if they were shorter than 12 bp or if their length was not a multiple of three. The remaining sequences were translated into peptides. During this process sequences containing ambiguous bases and stop codons were filtered out. Owing to the method applied for randomization, the last base of a codon should either be guanine or thymine. Sequences containing codons whose last base was either an adenine or cytosine were therefore also removed before we ultimately obtained a set of high-quality oligopeptide sequences.

The described functionality was implemented in the programming language Ruby. The developed software provides detailed statistics for each sample including comprehensive information on filtering results, codon usage, and position-based amino acid usage.
Supplementary Materials and Methods

Peptide competition assay

Animals received either vector alone at a dose of $2 \times 10^{10}$ vector genomes (vg)/mouse or in combination with 200 µg soluble ESGHGYF peptide (1 mg/ml in 200 µl 0.9 % NaCl; 1.26 mM peptide solution). Luciferase reporter gene expression was assessed 17 days after vector administration as described in “Material & Methods” of the main manuscript (n=3 animals per group).

Neutralizing antibody assay

To test for neutralizing antibody response against viral capsids, mice were intravenously injected with $1 \times 10^{11}$ virus like particles (VLPs) of wild-type AAV2 or AAV2-ESGHGYF. Twenty-three days later, the pretreated animals and untreated control mice intravenously received $5 \times 10^{10}$ vg of wild-type AAV2 or AAV2-ESGHGYF luciferase vectors. Four days after vector administration, neutralizing antibody titers were determined in (n=3 animals per group). The titers of neutralizing antibodies in murine sera and the prevalence of neutralizing antibodies in pooled human intravenous immunoglobulins (IVIG) and patient sera were determined as follows.

Determination of neutralizing antibodies: The assay was performed as previously described with minor modifications. 293T cells were plated at a density of $1 \times 10^5$ cells per well in a 96 well plate using only DMEM and Penicillin/Streptomycin (1%) as medium. Four to five hours later, the assay was performed. An equal volume of $8 \times 10^8$ vg (viral genomes) in DMEM was mixed with seven serial dilutions of serum from individual donors giving informed consent (Biobank, Heidelberg University Hospital) in human immunoglobulin depleted serum (HIGDS) (SF505-2, BBI Solutions, Cardiff, UK). The starting dilution was 1/8 (25% test serum, 25% HIGDS and 50% viral solution). For the mouse sera due to limitations with the starting volume a starting dilution of 1/16 was chosen. For the experiments with IntraVenous ImmunoGlobulin (IVIG, Kiobig, Baxter, Illinois, USA), IVIG was serially diluted in PBS and mixed with an equal volume of AAV vectors in HIGDS. All samples were incubated at 30°C for 30 minutes and the
volume containing $2 \times 10^8$ vg was added in triplicate onto cells for a final $2 \times 10^3$ vg/cell infection. Cell were incubated at $37^\circ C$, 5% CO$_2$ in an incubator for 2 days. Cells were observed to attest viability prior to the luciferase assay.

Luciferase assay: The assay was performed as previously described$^1$. Briefly, cells were lysed in-well using a 2x Lysis buffer (10mM Tris•HCl pH 8.00, 150mM NaCl, 1% NP40, 10mM DTT). An equal volume of lysis buffer was added to the cells and the cells were incubated for 10 minutes at room temperature. 30ul of cell lysate was transferred to luminometer plates (Z3291, Promega, Wisconsin, USA) and 100ul of luciferin containing reaction mixture (25 mM Tricine•HCl pH 7.80, 5 mM MgSO$_4$, 0.5 mM EDTA (pH 8.0), 3.3 mM DTT, 0.5 mM ATP (A26209, Sigma, Munich, Germany), 1 mg/ml BSA, 0.05 mg/ml D-luciferin

(StayBriteTM D-Luciferin, Sodium Salt, 7902-1G, Biovision, Milpitas, CA, USA), 0.05 mM CoEnzyme A (13787, Affymetrix, Buckinghamshire, England) was added. The plate was read using a Luminoskan™ Ascent Microplate Luminometer (Ascent Software Version 2.6). The statistical analysis was performed in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) using “grouped” table format using the "ROC curve" for the IVIG and the patient sera experiments and samples were compared to the AAV2-ESGHGYF. The mouse sera experiments were analyzed using Area Under the Curve (AUC) and two-way ANOVA to compare the different groups (n = 3 animals/group).

Supplementary References