Supplementary Information

RNAi suppressor P19 can be broadly exploited for enhanced adenovirus replication and oncolysis

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Supplementary Figures

Supplementary Figure S1. Design and characterization of the P19 expressing adenovirus Bwtp19ΔE3. (**a**) Construct used to generate the adenovirus Bwtp19ΔE3 containing a p19 expression cassette expressed under the control of the major late promoter. The spacer, the internal ribosomal entry site (IRES), P19 and a polyadenylation signal of the SV40 virus (polyA) containing fragment was inserted into a bacterial artificial chromosome (BAC) that harbors a E3-deleted adenovirus genome by homologous recombination. ITR: Inverted terminal repeats; Ψ: packaging signal. For a detailed description of the cloning procedure please refer to the materials and methods section. (**b**) PCR analysis of reconstituted virus. After viral plasmid transfection in HEK293 cells, viral lysates were used for subsequent infection step of HEK293 cells and generation of single plaques which were picked and amplified (plaques 1 to 13). In addition from a small part of the single cell plaque lysate genomic DNA was isolated and analyzed by PCR using primers “Fiber end forw” and “poly A rev” resulting in a virus specific band of 1710 bp. (**c**) P19 mRNA expression from the BAC-derived virus Bwtp19ΔE3 in comparison to stably p19 expressing B6 cells exemplified by virus clones 9 and 10. HEK293 cells were infected with the virus Bwtp19ΔE3 at an MOI of 1 and 24 hours post-infection RNA was isolated and reverse transcribed. The generated cDNA was then used as template for a PCR with p19 specific primers. As positive controls (+), reverse transcribed cDNA from B6 cells and genomic DNA of Bwtp19ΔE3 infected cells were used. (**d**) Quantification of p19 mRNA molecules during infection of HEK293 cells with Bwtp19ΔE3 and during infection of B6 cells with wild type adenovirus serotype 5 (wtAd5). HEK293 or B6 cells were infected with Bwtp19ΔE3 (black line) or wtAd5 (grey line) at an MOI of 3, respectively, and used to isolate RNA. Upon reverse transcription the cDNA was subjected to qRT-PCR reaction with p19 specific primers and normalized to 10 000 molecules of the internal control (human B2m). p.i.: post-infection.

Supplementary Figure S2. The control virus AdΔfiberIL and analysis of its replication profile. (**a**) DNA sequences contained in the viral vector AdΔfiberIL used as a control virus for replication analysis of Bwtp19ΔE3 and BwtΔE3 containing the matching deletion in the E3 gene and no 24 bp deletion in the E1 region. AdΔfiberIL expresses firefly luciferase (luc) under the major late promoter connected to the fiber via a spacer and an internal ribosomal entry side (IRES) and the SV40 polyadenylation signal (polyA). In addition, as an oncolytic virus, this virus has a 24 bp deletion in the E1a gene. ITR: inverted terminal repeat; Ψ: packaging signal. (**b**) To rule out that the 24 bp deletion in the E1 region of the control virus AdΔfiberIL, which is not contained in the P19 expressing virus Bwtp19ΔE3 (Supplementary Fig. 2a), negatively interferes with virus replication in HEK293 cells, we directly compared the replication profile of AdΔfiberIL with the E3-deleted adenovirus BwtΔE3. HEK293 cells were infected either with the E3-deleted virus BwtΔE3 or the oncolytic virus AdΔfiberIL at an MOI of 1 and viral genome copy numbers were quantified from whole genomic DNA using Hexon specific primers. Normalization was carried out using the human B2m gene. We found that the control virus AdfiberIL replicated even faster than BwtΔE3 demonstrating that the 24 bp deletion in the E1 region has no influence on virus genome replication in HEK293 cells and that this virus is suitable for a direct comparison with the virus Bwtp19ΔE3 not containing the 24 bp deletion. P-value:< 0.05.

Supplementary Figure S3. Binding of sva-RNAs to P19 and the fate of the svaRNAs during infection. Upper panel: Analysis of VAI-RNA (**a**) and VAII- RNA (**b**). B6 cells were infected with wtAd5 at an MOI of 10 and P19 protein was purified 24 hours post infection. Small RNA molecules were isolated from the protein fraction and inserted into a polyA polymerase reaction followed by cDNA synthesis (polyA-tailing Kit; Ambion). PCR was then performed (upper panel) with sva-RNA specific primers for the 3'´stem of VAI-RNA (3´Van-r) or the 5' stem of VAII-RNA (5´VAII) and a primer specific for the polydT adapter. M: Marker; 293: HEK293 cells; B6f/t: B6 cells were lysed by 4 consecutive freeze/thaw cycles; B6s: B6 cells were sonificated 5 times on ice; PK: Proteinkinase buffer was used to isolate miRNAs

from P19. Other miRNAs were isolated using Qiazol reagent and following the manufacturer instructions. Northern Blot analysis was performed (lower panel) to track the fate of sva-RNAs during adenovirus infection in HEK293 cells and B6 cells. HEK293 cells and the RNAi knockdown cell line B6 were infected at an MOI of 0.05 and small RNAs were isolated at time points as indicated in the figure. Equal RNA amounts were loaded on a Northern gel, blotted to a NylonBond+ membrane and detected with an α-P32 labeled probe specific for either the stem of the VAI-RNA (3´Van) (**a**) or the VAII-RNA (5´VA II) (**b**). Degradation products observed in the B6 cell line are highlighted in boxes. (**c**) Sequencing results obtained from the isolation of small RNAs from P19. The miRNA miR196a was used as a control and confirms the feasibility of the assay. Different svaRNA sequences either from VAI-RNA or VAII-RNA origin were obtained and exemplified sequencing results are listed. For details regarding the procedure please refer to **Supplementary Methods 5**.

Supplementary Figure S4. Schematic overview of high-capacity adenovirus amplification in 116 producer cell lines. (**a**) Basic strategy for generation of high-capacity adenovirus vectors (HCAs). Generation of HCA requires the HCA vector genome and a helper virus supplementing all necessary factors for particle production. Upon HCA transfection and helper virus co-transduction into 116 cells (a HEK293 based cell line stably expressing the Cre-recombinase) the helper virus genome expresses all necessary factors for genome replication and particle production whereas Cre-recombinase mediates excision of the packaging signal of the helper virus genome. Thus, only the HCA genome is packaged into viral capsids. (**b**) Large-scale amplification of HCAs using a 3 liter spinner flask system. Three serial passages of co-infection in 6 cm dishes (P0-P2) as well as in one step in 15 cm dish (P3) are required for large-scale amplification. After pre-amplification one 3 liter culture of 116 cells is infected with the lysate and co-infected with helper virus using an MOI of 2. Two days post-infection viral lysate is harvested and purified by CsCl centrifugation. For detailed information about large-scale amplification please refer to the publication from Jager and colleagues[1].

Supplementary Figure S5. Design and characterization of the p19 expressing helper virus BHVp19. (**a**) DNA sequences contained in the novel helper-virus BHVp19, an E3 deleted helper virus that contains the p19 expression cassette under the control of major late promoter connected via a spacer and an internal ribosome entry side (IRES) sequence to the open reading frame of the fiber. Additionally, the packaging signal is flanked by loxP sites. (**b**) PCR analysis of reconstituted virus BHVp19. After viral plasmid transfection in HEK293 cells using FuGeneHD (Roche), viral lysates were collected 10 days later and used for subsequent infection of HEK293 cells. Upon generation of single plaques by agarose overlay, single cell plaques were picked, amplified, genomic DNA isolated and analyzed by PCR using primers “Fiber end forw” and “poly A rev”. (**c**) P19 mRNA expression from the helper virus BHVp19. HEK293 cells were infected with BHVp19 single clone lysates, RNA was isolated and reverse transcribed. The generated cDNA was then amplified using p19 specific primers. M: Marker; g.DNA: genomic DNA from BHVp19 infection; 1, 2: two different single viral plaque lysates. (**d**) Upper panel: AdNG163R-2 standard helper virus deleted for the early viral genes E1 and E3 contains a loxP flanked packaging signal. Lower panel: HCA-luc, HCA, lacking all viral coding sequences, only containing the two inverted terminal repeats (ITR) and the adenovirus packaging signal (Ψ). This HCA harbors a luciferase expression cassette driven by the human α-1-antitrypsin promoter (hAAT) and the SV40 polyA signal. (**e**) Small-scale amplification of HCA-Luc using either helper virus BHVp19 or standard helper virus AdNG163R-2. 116 cells were co-infected with HCA-Luc (MOI 3) and either BHVp19 or AdNG163-2 (MOI 1). For small scale analysis the cellular lysate was harvested 24 hours post-infection and a 1:80 dilution was used to infect Huh7 cells. Luciferase assay to measure luciferase expression levels which directly correlated to vector production was performed 16 hours later (black horizontal stripes, left y-axis).

Supplementary Table S1. Oligonucleotides used in this study

a		
5´fiber forw	5´forward	TATGCCTAACCTATCAGCTTATCC
IRES p19 rev	3´reverse	GTCGTTTCCTTGTATAGCTCGTTCCATGGTATCATCGTGTTTTTCAA
IRES p19 forw	5´forward	´TTCCTTTGAAAAACACGATGATACCATGGAACGAGCTATACAAGGAA
Poly 3´fier rev	3´reverse	ACACAAACGATTACTCTACTAGTTACCACATTTGTAGAGGTTTTAC
P19 forw Xho	5´forward	ATTCTCGAGATGGAACGAGCT
P19 rev Xba	3´reverse	CGTCTAGATTACTCGCTTTCTTT
3´fiber polyA	5´forward	CAAGTAAAACCTCTACAAATGTGGTAACTAGTAGAGTAATCGTTTGTG
3´fiber rev	3´reverse	TGTCTGTTACCCATGATATGATC
Luc forw	5´forward	ATCCATCTTGCTCCAACACC
Luc rev	3´reverse	TTTTCCGTCATCGTCTTTCC
b		
IRES-p19_IC_5	5´forward	CATGGGACTGGTCTGGCCACAACTACATCAAGCTTGGTACCGAGCTCGG
IRES-p19_IC_3	3´reverse	GCTATGTGGTGGTGGGGCTATACTACTGAGGGCGAATTGGGCCCTCTAG
IRES-p19_IC_5_ne	5´forward	ACAACTCCAAGTGCATACTCTATGTCATTTTCATGGGACTGGTCTGGCC
IRES-p19_IC_3_ne	3´reverse	GAGTTTGATTAAGGTACGGTGATCTGTATAAGCTATGTGGTGGTGGGGC
Wt/Hv-BAC-Zeo	5´forward	ATGCAAGTGTGTCGCTGTCGAGTTTCGTGTCAGTCCTGCTCCTCGGCCAC
Wt/Hv-BAC-nested	5´forward	CGGCACGTTAACCGGGCTGCATCCGATGCAAGTGTGTCGCTGTCGAGTTT
Ng-Hv_Adv-pIX_3611	3´reverse	CTTCCATCAAACGAGTTGGTGCTC
c		
human B2m (hB2m)	5´forward	TGCTGTCTCCATGTTTGATGTATCT
	3´reverse	TCTCTGCTCCCCACCTCTAAGT
DBP	5´forward	ACTTGCCGGAAAACTGATTG
	3´reverse	GCACGTGATTGAAATGGATG
E1B55K	5´forward	TAGTGAAAAGCGTGGCTGTG
	3´reverse	GGAACAGCGGGTCAGTATGT
E4Orf6	5´forward	TTCAAAATCCCACAGTGCAA
	3´reverse	TACCGGGAGGTGGTGAATTA
Fiber	5´forward	CGTGCACGACTCCAAACTTA
	3´reverse	GGGCTCTTTCAAGTCAATGC
Hexon	5´forward	CTTACCCCCAACGAGTTTGA
	3´reverse	GGAGTACATGCGGTCCTTGT
d		
P19NcoI	5´forward	AGCTGCCATGGAACGAGCTATACAAGG
P19FseI	3´reverse	TCAAGTGGCCGGCCTTACTCGCTTTCTTTTTCGAAGG
phAAT-forw	5´forward	AGCTAGCATCGATACCGTCGAGGCCGC
polyA-rev	3´reverse	CTTTACCAACAGTACCGGAA
e		
polydT adaptor		GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTT
Reverse primer		GCGAGCACAGAATTAATACGAC
mTBP-f	5´forward	CCCCACAACTCTTCCATTCT
mTBP-r	3´reverse	GCAGGAGTGATAGGGGTCAT
f		

3´Van		AGGAGCGCTCCCCCGTTGT
3´Van-r		ACAACGGGGGAGCGCTCCT
5´VAI		ATCCACCAGACCACGGAAGA
3´VAII		CGCTCGTCCCTGTTTCCGGAG
3´VAII-r		CTCCGGAAACAGGGACGAGCG
5´VAII		AATAACCCTCCGGCTACAGGGAGCGAGCC
miR122		TGGAGTGTGACAATGGTGTTTGT

Supplementary Methods 1

Cloning of the replication-competent adenovirus Bwtp19ΔE3 and the helper virus BHVp19

The pCR2.1 BluntII-Topo based plasmid pFIPP contains the spacer, IRES, p19 and the poly A signal with the flanking homologous sequences to the adenovirus genome. The previously described plasmid pNEB.PK.fiberIL[2] was used as main template for several overlapping PCR steps to generate pFIPP. Primer sets were designed with 15-20 nt long overhangs if overlapping PCR products had to be generated and all primer sequences used during these steps are listed in the **Supplementary Table 1a**. To generate pFIPP, pNEB.PK.fiberIL was first inserted into a PCR reaction with the primer set "5´fiber forw" and "IRES p19 rev" using an annealing temperature of 56°C and an elongation time of 40 sec. To generate the fragment containing the *p19* expression cassette and the poly A signal a PCR reaction using the primer pair "IRES-p19 forw" and the "polyA-3´fiber rev" the pZacp19 plasmid was performed. To generate pZacP19 the *p19* expression cassette was amplified utilizing the primer pair "p19forw Xho" and "p19rev Xba" and introduced into the *Xho*I and *Xba*I sites of the pZac expression vector by direct cloning. The PCR for the p19-polyA fragment was performed using an annealing temperature of 57°C and an elongation time of 40 sec running for 35 cylces. Afterwards the 3´fiber fragment containing also homologous sequences to the adenovirus genome downstream of the *fiber* was generated with the primer "3´fiber-polyA forw" and "3´fiber rev" using the pNEB.PK.fiberIL as template and running 30 cycles with an annealing temperature of 56°C and 30 sec elongation time. The last two PCR products were purified by gel column purification (Promega) and subjected to a new PCR reaction with the outer primer "p19-IRES forw" and "3´fiber rev" using a 2-fold excess of the long p19-polyA fragment. Conditions for this PCR were as follows: the primer annealing temperature was set to 56°C for 10 sec and an elong ation time of 30 seconds starting with 16 cycles without adding primer. After these cycles, 1.5 µl of each primer was added to the reaction followed by another 35 cycles under the same conditions. The gel fragment containing the PCR product was purified and cloned into the pCR-BluntII-TOPO vector

following the manufacturer instructions resulting in the plasmid pTopop19big. Finally, the latter PCR product was inserted as a template into a PCR reaction together with a second PCR product containing the 5´fiber-spacer-IRES seuences. For this reaction 12.5 µl of each purified product, an annealing temperature of 56°C, an elongation time of 50 sec and the primers "5´fiber forw" and "3´polyA rev" were used. The whole fragment was cloned into the pCR-BluntII-Topo vector, resulting in pFIPP.

The Fiber-IRES-p19 construct within the pFIPP vector was incorporated into the genome of a first-generation adenovirus (ΔE1/E3) utilizing a bacterial artificial chromosome (BAC) cloning method as described elsewhere[3]. In brief, the galactokinase- kanamycin (galK-Kan) construct was amplified in two subsequent PCR steps using the primer pair IRES-p19_IC_5 and IRES-p19_IC_3 and the nested primers IRES-p19_IC_5_ne and IRES- p19_IC_3_ne. The purified PCR product from the second PCR step was then transformed into SW102 *E.coli* containing BAC pB-FG (kindly provided by Zsolt Ruzsics, Max von Pettenkofer-Institute, Munich, Germany). After induction of homologous recombination and selection for kanamycin the intermediate clone pB-FG-fib-GK was isolated containing the galK-Kan construct incorporated at the 3' end of the fiber encoding sequence. Subsequently, pFIPP was used as template to amplify the Fiber-p19-IRES fragment using primers IRES-p19_IC_5_ne and IRES-p19_IC_3_ne. After PCR amplification the generated product was purified and transformed into SW102 cells containing the intermediate BAC pB-FG-fib-GK. Final clone BFgp19 was isolated after recombination and selection against galK.

For generation of the replication-competent adenovirus Bwtp19ΔE3 containing a deletion in the early gene E3 region and the *p19* expression cassette, the 5´region of BAC clone BFgp19 was exchanged by the BAC cloning technology. In brief, a construct containing a zeocin resistance cassette and the 5' end of the wild type adenovirus was PCR-amplified in two steps using the plasmid T-PacI- 5'wtAd5-ΔKan (generated from DNA isolated from wtAd particles) as template for the first PCR and the primer pair Ng-Hv_Adv- pIX_3611 and wt/Hv-BAC-Zeo and the primers Ng-Hv_Adv-pIX_3611 wt/Hv-BAC-nested for the second PCR, respectively. The purified product was then transformed into SW102 bacteria containing the

BAC BFgp19. After induction of recombination and zeocin selection the BAC DNA Bwtp19ΔE3 was isolated and amplified.

Generation of the BHVp19 helper virus was also based on the BFgp19 construct and the cloning procedure was comparable to the strategy described above. In brief, a construct that consists of the zeocin resistance cassette and the 5´end of the helper virus genome of AdNG163R-2[4] was PCR amplified using the primers described above but T-PacI-5'Hv-ΔKan as template. After homologues recombination in SW102 bacteria containing BFgp19 and zeocin selection the BAC DNA BHVp19 was obtained. All PCRs were performed with 95℃ denaturation for 20 seconds, 60℃ annealing tempera ture for 20 sec and an elongation of for 25 sec/kb at 70℃ using 35 cycles. Primers used for generation of the BAC derived viruses are displayed in **Supplementary Table 1b**.

Production of the replication-competent adenoviruses Bwtp19ΔE3 and the helper virus BHVp19

For BHVp19 and Bwtp19ΔE3 virus reconstitution, 20 µg of the BAC vector was linearized by *Pme*I restriction enzyme digest and purified by phenol/ chlorophorm/ isoamylalcohol (25/24/1, Roth) extraction followed by ethanol precipitation. The complete digest was then transfected into HEK293 cells using FuGene6 (Roche) transfection. Medium was changed every 5 days, until cytopathic effect (CPE) appeared. Whole cellular lysates were then harvested and virus was released from the cells by four consecutive freeze/thaw cycles. Afterwards, a 95% confluent 6 cm dish with HEK293 cell was infected using 2/3 of the lysate and 2 hours later the medium was aspirated and cells were overlayed with a 1:1 mixture of 1% autoclaved agarose (diluted in H_2O; Peqlab) and 2x DMEM (Invitrogen) supplemented with 20% FBS and 2% P/S. The overlay was performed every 3 days (with 0.5 ml) until viral plaques were obtained. Single plaques were picked and stored in 500 µl of culture medium. After the release of the virus from the cells a 24-well dish of HEK293 cells was infected with 400 µl of the lysate. Approximately two days later CPE starts, the cells were harvested and one half was subjected to DNA isolation and PCR analysis to confirm the correct viral

construct. The other half was used to amplify the virus. Twenty 15 cm tissue culture dishes were sufficient for purification. Purification of all viruses used within this study was performed following the manuscript from Jager and colleagues[1].

Titration of viruses was performed by agarose overlay as shown in Curiel and Douglas[5]. In brief, 100% confluent HEK293 cells were infected with serial dilutions of virus stocks. Two hours post infection medium was aspirated and cells were supplemented with 2.5 ml of a 1:1 dilution of 1% autoclaved agarose (Peqlab) and 2x DMEM (see above). The overlay was then performed every 3 days with 0.5 ml of the mixture. After approximately 10 days plaques were counted and the titer was determined.

To determine the physical virus titer (OD-titer) 25 µl of purified virus was incubated 15 min in 475 µl lysis buffer[1] and the optical density was measured. An OD of 0.01 corresponds to $1*10^7$ transducing units per µl.

Supplementary Methods 2

Analysis of infection parameters on RNA, DNA and protein levels

Analysis of RNA expression levels

Adenoviral early and late RNA expression levels were analyzed upon infection of HEK293 cells with viruses Bwtp19ΔE3 and AdΔfiberIL at an MOI of 3. Cells of one 6 cm dish were resuspended in 500 µl TRIZOL (Invitrogen, Darmstadt, Germany) reagent, homogenized with a 21-gauge needle and RNA was isolated following the manufacturer instruction. RNA was resuspended in 40 µl of RNA-H_2O and 4 µl were used for cDNA synthesis. Reverse transcription was performed using the cDNA synthesis kit from New Engand Biolabs (Frankfurt, Germany) following the manufacturer instruction (ProtoScript®First strand cDNA synthesis kit). As shown in our previous study[6], to exclude cross-contamination from viral genomes we performed a respective control experiment without reverse transcriptase. For quantitative Real-Time PCR (qRT-PCR) 4 µl of the cDNA were used for a PCR reaction

using gene specific primers (**Supplementary Table 1c**). For RNA isolation from liver tissue, mice were sacrificed and 1/6 of the liver was removed, immediately kept on ice and treated with TRIZOL following the manufacturer instruction (for isolation of RNA from tissues). Further processing was accordingly to the procedure described above.

PCR analysis on DNA level

The cell pellet of one 6 cm dish or a 24-well was used to isolate whole genomic DNA. DNA amounts were quantified by measuring the optical density (OD) at 260 nm. Normal taq-polymerase (New Engand Biolabs, Frankfurt, Germany) based PCR was performed with 100 ng of genomic DNA following the manufacturer instructions.

Quantitative RT-PCR was performed using the Taq-man system (Applied Biosystems, Carlsbad, California) and FastStart Universal SYBR Green Master (Roche, Mannheim Germany) reagent following the manufacturer instruction. For qRT-PCR of genomic DNA, 50 ng of genomic DNA was used, for cDNA analysis 5 µl of a reverse transcription was subjected to the reaction. Oligonucleotides were either designed using a maximum template size of 150 bp or in case of human Beta-2 microglobulin (hB2m) a previous published primer pair was used[7]. **Supplementary Table 1c** shows all oligonucleotides used for qRT-PCR analyses. Each sample was normalized to either genomic B2m level (10 000 cells) or expression level of 10 000 RNA molecules of hB2m. Quantification was performed by calculating known hB2m levels from the plasmid pTopohB2m by the following calculation: 1 pg plasmid = [10^{-12} / plasmid size (bp) * 660 * 6.02 x 10^{23}] copies. Standard curves for all adenoviral proteins were performed using DNA purified from wtAd5 viral particles.

Western Blot analysis of adenoviral proteins

Protein lysates were generated from 3.2 x 10^6 cells using 300 µl of NP-40 lysis buffer (50mM Tris-HCL pH 8.0, 150mM NaCl, 1% NP-40, store at -20℃) supplemented with proteinase inhibitors without EDTA (Roche, Mannheim Germany) following a 30 min incubation step on ice. To determine protein amounts, the Bradford test was applied

following the manufacturer instructions (Carl Roth, Karlsruhe, Germany). Equal protein amounts were separated on a 10% SDS polyacrylamid gel. Afterwards proteins were blotted on a methanol treated PVDF membrane and incubated o/n in TBS buffer (10 mM Tris (pH 7.5), 150 mM NaCl) supplemented with 5% milk powder (Carl Roth, Karlsruhe, Germany) at 4℃. After three washing steps with TBST (TBS + 0.0 1 % Tween20 (Roth)) blots were incubated with either first anti-adenovirus antibodies against DBP (1:250, mouse derived), E1B55K (1:1000; mouse derived), both supplied by Matthew Weitzman (Salk Insitute, San Diego, USA), E4Orf6 (1:10 anti mouse) obtained from Thomas Dobner (Heinrich-Pette-Institut, Hamburg, Germany), Fiber (1:10 000 mouse derived; NeoMarkers), Hexon (1:1000 rabbit, Abcam) or GAPDH (1:1000, anti goat, R&D systems) as control for 1 hour at room temperature. This step was followed by another 3 washing steps with TBST and incubation with secondary peroxidase (POX) labeled antibodies specific to the first antibody. All secondary antibodies were obtained from Invitrogen. Finally, blots were washed 3 times with TBST and POX was activated by adding ECL reagent (GE Healthcare, München, Germany).

Supplementary Methods 3

Analysis of CAR expression on HEK293 cells and stably P19 expressing B6 cells

HEK293 and B6 cells were counted and $1*10^6$ cells were washed once with PBS supplemented with 1% BSA (PBS/BSA). After centrifugation of the cells at 500 g for 3 min they were resuspended in 100 µl PBS/BSA and 5 µl CAR antibody (Santa Cruz Biolaps) was added following an incubation step at 4℃ for 1 hou r. As controls each cell line was also incubated without supplementation of the primary antibody. Afterwards the cells were washed again with PBS/BSA, to remove unbound antibodies and resuspended in 100 µl PBS/BSA. To detect CAR expression using flow cytometry, 1 µl of an APC labeled anti-mouse secondary antibody (kindly provided by A. Baiker, Max von Pettenkofer-Institute, Munich, Germany) was supplied to the mixture and incubated for 1.5 hours at 4℃ with

continuous shaking. Cells were again washed with PBS/BSA and finally resuspended in 400 µl PBS for flow cytometry using FACS DIVA (Becton Dickinson, Heidelberg, Germany).

Supplementary Methods 4

Generation of high capacity adenoviral vectors HCA-luc and HCA-p19

For cloning of high-capacity HCA-luc two liver specific enhancer regions and the human α-1-antitrypsin promoter were PCR amplified using the plasmid pBS-ApoE-HCR-hAAT-hFIX[8] as template. The resulting 1.3 kb fragment was cloned into an upstream position of the luciferase gene of the plasmid pGL3 basic (Promega) by *Sac*I/*Nhe*I ligation resulting in the plasmid pGL3-ApoE-HCR-hAAT-luc. For HCA vector generation the shuttle vector pHM5 and the HCA vector pAdFTC were used[9]. The transgene was released from pGL3-ApoE-HCR-hAAT-luc by SacI/*Sal*I digest cloned into the same restriction enzyme recognition sites of the plasmid shuttle vector pHM5. By PI-*Sce*I and I-*Ceu*I restriction enzyme digest the liver-specific luciferase construct was cloned into the vector pAdFTC resulting into the vector pFTC-luc.

The high-capacity adenoviral vector HCA-p19 expresses p19 under the control of the human alpha-1 antitrypsin promoter (phAAT). For cloning of the p19 expression cassette, p19 was amplified from the plasmid pZacp19 using the primers p19FseI and p19NcoI (**Supplementary Table 1d**) and the obtained PCR fragment was digested with *Fse*I and *Nco*I. At the same time the firefly luciferase was excised from the plasmid pGL3-hAAT containing the luciferase cDNA expressed under the control under the hAAT promoter using the same restriction enzymes. Ligation of both fragments resulted into the pGL3-hAATp19 plasmid. The p19 expression cassette was then PCR amplified from the plasmid pGL3-hAATp19 using primers phAAT-forw and polyA-rev (**Supplementary Table 1d**) and cloned into the pGEM vector supplied by Promega (pGEM-p19). Since the p19 expression cassette alone is too small for proper virus production, a matrix attachment region (MAR) was inserted

as stuffer into the pHM5 shuttle vector. Therefore, the plasmid pBS-2-(B-1-CX)[10] and the pHM5 were both digested with *Sac*I and ligated, resulting in the plasmid pHM5sMAR. The p19 expression cassette was then excised from the pGEM-p19 utilizing *Nde*I and *Sph*I and the pHM5sMAR was digested with *Sal*I. Both plasmids were then supplied to a T4 DNA Polymerase reaction (NEB) to generate blunt ends. After purification blunt end fragments were ligated, resulted in the plasmid pHM5sMAR-p19. Finally, for HCA-p19 vector generation pHM5sMAR-p19 and the pAdFTC plasmids were digested with I-*Ceu* and PI-*Sce* and ligated resulting into the plasmid pFTC-p19[1]. Sequences contained in the final plasmid were confirmed by PCR and DNA sequencing.

For amplification and purification of high capacity adenoviral vectors HCA-luc and HCA-p19 we followed a detailed protocol which was published previously[1, 11]. The strategy of the production procedure is outlined in **Supplementary Figure 5**. In brief, the HCA genome was released by *Not*I restriction enzyme digest from plasmids pFTC-luc and pFTC-p19 and linear DNA was transfected into 116 cells. One day post-transfection cells were transduced with the standard helper virus AdNG163R-2[11] at an MOI of 5. After 3 pre-amplification steps in tissue culture dishes and simultaneous infection with the helper virus providing all adenoviral gene products in trans, a 3 liter suspension culture of 116 cells grown in spinner flasks was co-infected with the crude lysate of the last pre-amplification step and co-infected with the helper virus. Two days post-infection cells were harvested, resuspended in 28 ml of Dulbecco's modified eagle medium, and subjected to 3 freeze and thaw cycles. Released virus was purified using cesium chloride gradients and quantification of infectious and physical titers in the final vector preparation was performed by quantitative real-time PCR as described earlier[1].

Supplementary Methods 5

Isolation of small RNAs bound to P19 after His-tag purification

To investigate, if sva-RNAs from VAI-or VAII-RNA origin bind to P19, the His-tagged protein was purified from five 15 cm tissue culture dishes of B6 cells under native conditions after wtAd5 infection at an MOI of 3. Purification under native conditions was performed with magnetic beats (Qiagen, hildne Germany) following the manufacturer instruction. For the destruction of the cells, the lysate was either subjected to 3 consecutive freeze/thaw cycles or cells were sonificated at level 2 for 5 times on ice using a Branson 450 sonificator. For the isolation of siRNAs bound to P19 two methods were used. On the one hand Qiazol reagent (Qiagen, Hilden Germany) was supplied to the protein solution and standard RNA isolation was performed (see above). On the other hand protein lysates were incubated for 15 min with 2 x Proteinkinase K solution (200 mM Tris-Cl pH 7.4, 25 mM EDTA, 300 mM NaCl, 2% SDS, 2 mg/ml Proteinase K) and small RNAs were isolated by isopropanol precipitation over night at -20℃ . Small RNA preparations were resusp ended in 30 µl RNase free H_2O.

The purified small RNAs were polyadenylated using the polyAAA-tailing kit from Ambion. Reverse transcription was performed with polydT primer with an adaptor followed by a standard KOD (Novagen, Darmstadt, Deutschland) based PCR with the reverse primer (**Supplementary Table 1d**) 3´Van-r or the 5´VAII primer (**Supplementary Table 1e**). The PCR products were either purified by gel extraction (Promega) and send for sequencing or they were subcloned in the pCR-Blunt II-Topo (Invitrogen, Darmstadt, Germany) vector following the manufacturer instructions and send for sequencing (Eurofins, MWG, Ebersberg, Germany).

Isolation of small RNAs and Northern Blot analysis

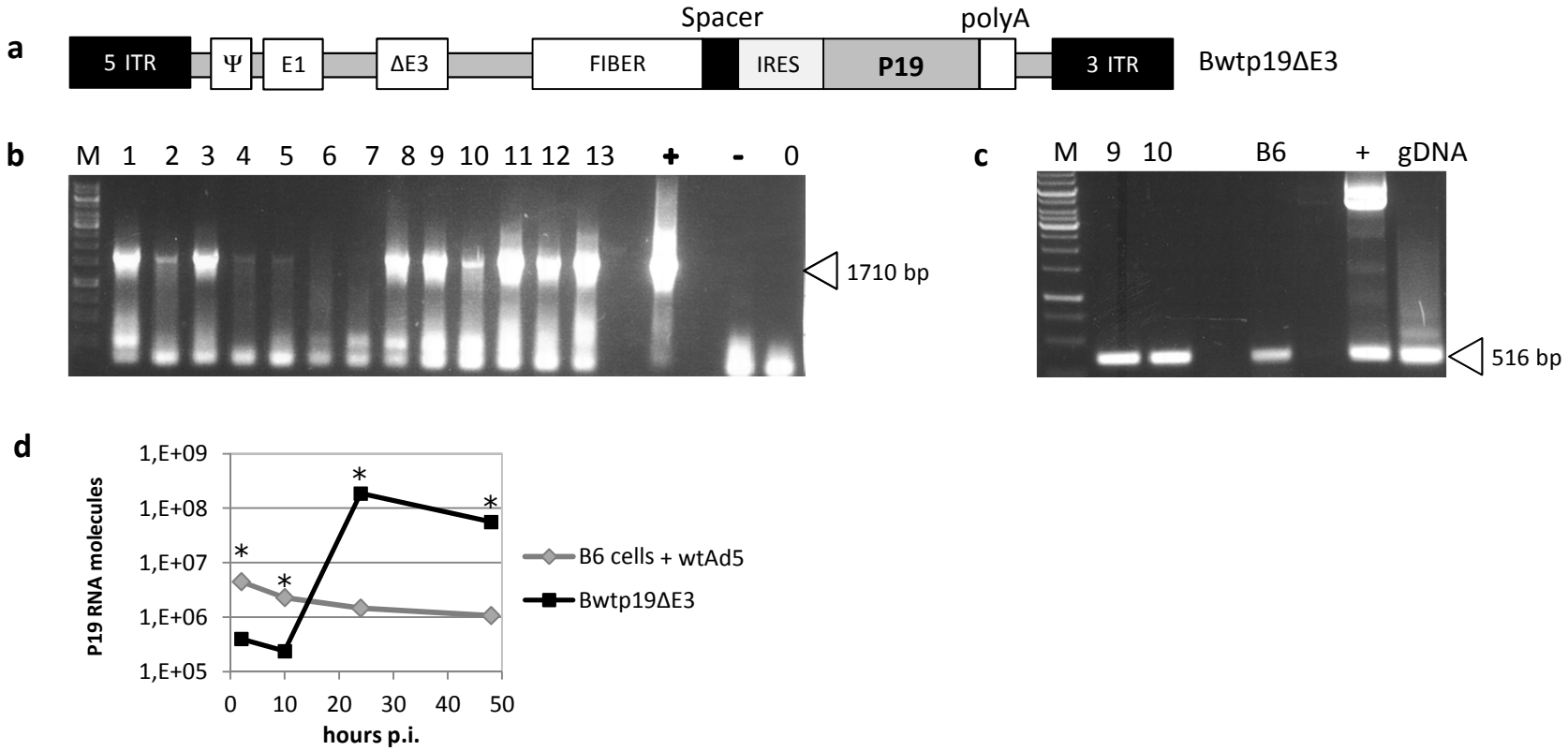
In order to monitor the VA-RNA profile under RNAi knockdown conditions, HEK293 or B6 cells were infected with wtAd5 at an MOI of 3 and small RNAs were isolated at different time points post infection following a protocol obtained from Rebecca Schwab (University of Tuebingen, Tuebingen, Germany). In brief, cells were treated with TRIZOL (Invitrogen, Darmstadt, Germany) reagent and cell lysates were homogenized using a 21-gauge needle. Afterwards, one third volume of chloroform was added and cells were centrifuged for 10 min

at 5000 g at 4 ℃. The upper phase was harvested and subjected to another phenol purification step using the same conditions as described before. Again the upper phase was collected and 1 volume of isopropanol was added to precipitate RNAs overnight at -20℃. The next day, RNA solution was precipitated by centrifugation for 30 min at 5000 g at 4℃ and the RNA pellet was washed with 70% EtOH. After centrifugation the cell pellet was air-dried and resuspended in 40 µl of RNase-free water. Isolated RNAs were then separated on a 17% vertical polyacrylamid gel containing 7 M urea in 0.05x tris-borate-ethylenediaminetetracetic (TBE) buffer. As a positive control for Northern Blot analysis also representing the length marker, we ran the reverse oligonucleotide of 3´Van (3´Van-r) or VAII-RNA (3´VanII and 3´VanII-r) oligonucleotide probe also published by Aparicio et al. (**Supplementary Table 1e**)[12]. We performed a semi-dry transfer in 0.5x TBE onto a Hybond-N+ membrane (GE Healthcare) and detected the VAI-RNA and VAII- RNA, or sva-RNAs by radioactive end labelling of the oligonucleotides with gamma-P32 (Quick spin columns, Roche) and incubation in Denhardt´s solution at 50°C for 1 hour. Blots were washed twice for 10 min with 5x SSC/0.1% SDS and once for 10 min with 1 x SSC/ 0.1% SDS at 50℃. Afterwards blots were exposed to phospho-imager plates for 10 min and analyzed in a phosphorimager.

Supplementary References

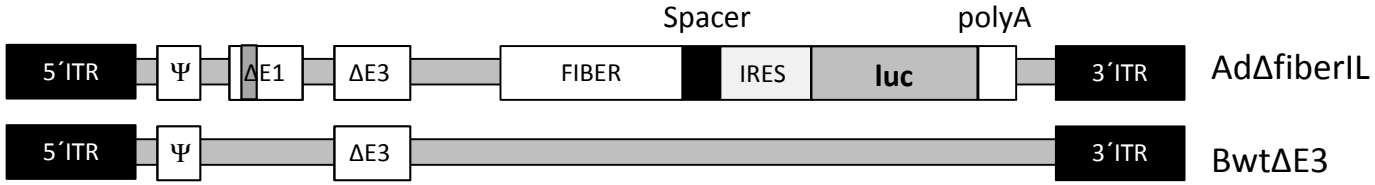
1. Jager, L. *et al.* A rapid protocol for construction and production of high-capacity adenoviral vectors. *Nature protocols* **4**, 547-564 (2009).
2. Rivera, A.A. *et al.* Mode of transgene expression after fusion to early or late viral genes of a conditionally replicating adenovirus via an optimized internal ribosome entry site in vitro and in vivo. *Virology* **320**, 121-134 (2004).
3. Warming, S., Costantino, N., Court, D.L., Jenkins, N.A. & Copeland, N.G. Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res* **33**, e36 (2005).
4. Ng, P., Parks, R.J. & Graham, F.L. Preparation of helper-dependent adenoviral vectors. *Methods Mol Med* **69**, 371-388 (2002).
5. Douglas, C.u. *Adenoviral Vectors for Gene Therapy*. (2002).
6. Rauschhuber, C. & Ehrhardt, A. RNA interference is responsible for reduction of transgene expression after Sleeping Beauty transposase mediated somatic integration. *PloS one* **7**, e35389 (2012).
7. Vandesompele, J. *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* **3**, RESEARCH0034 (2002).
8. Miao, C.H. *et al.* Inclusion of the hepatic locus control region, an intron, and untranslated region increases and stabilizes hepatic factor IX gene expression in vivo but not in vitro. *Molecular therapy : the journal of the American Society of Gene Therapy* **1**, 522-532 (2000).
9. Ehrhardt, A. *et al.* A gene-deleted adenoviral vector results in phenotypic correction of canine hemophilia B without liver toxicity or thrombocytopenia. *Blood* **102**, 2403-2411 (2003).
10. Phi-Van, L., von Kries, J.P., Ostertag, W. & Stratling, W.H. The chicken lysozyme 5' matrix attachment region increases transcription from a heterologous promoter in heterologous cells and dampens position effects on the expression of transfected genes. *Molecular and cellular biology* **10**, 2302-2307 (1990).
11. Palmer, D. & Ng, P. Improved system for helper-dependent adenoviral vector production. *Molecular therapy : the journal of the American Society of Gene Therapy* **8**, 846-852 (2003).
12. Aparicio, O., Razquin, N., Zaratiegui, M., Narvaiza, I. & Fortes, P. Adenovirus virus-associated RNA is processed to functional interfering RNAs involved in virus production. *Journal of virology* **80**, 1376-1384 (2006).

Supplementary Figure S1

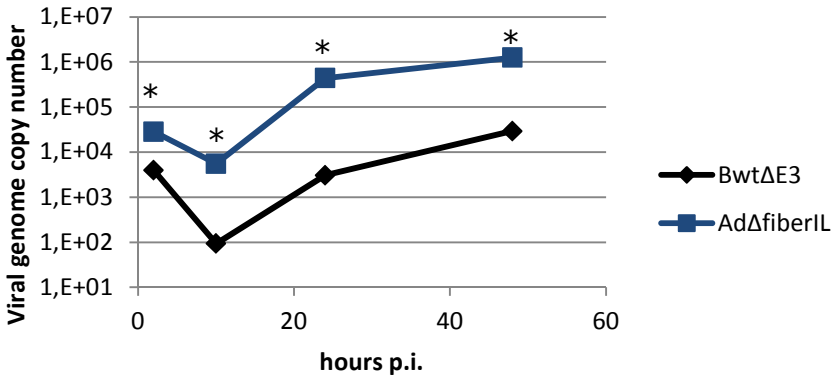


Supplementary Figure S2

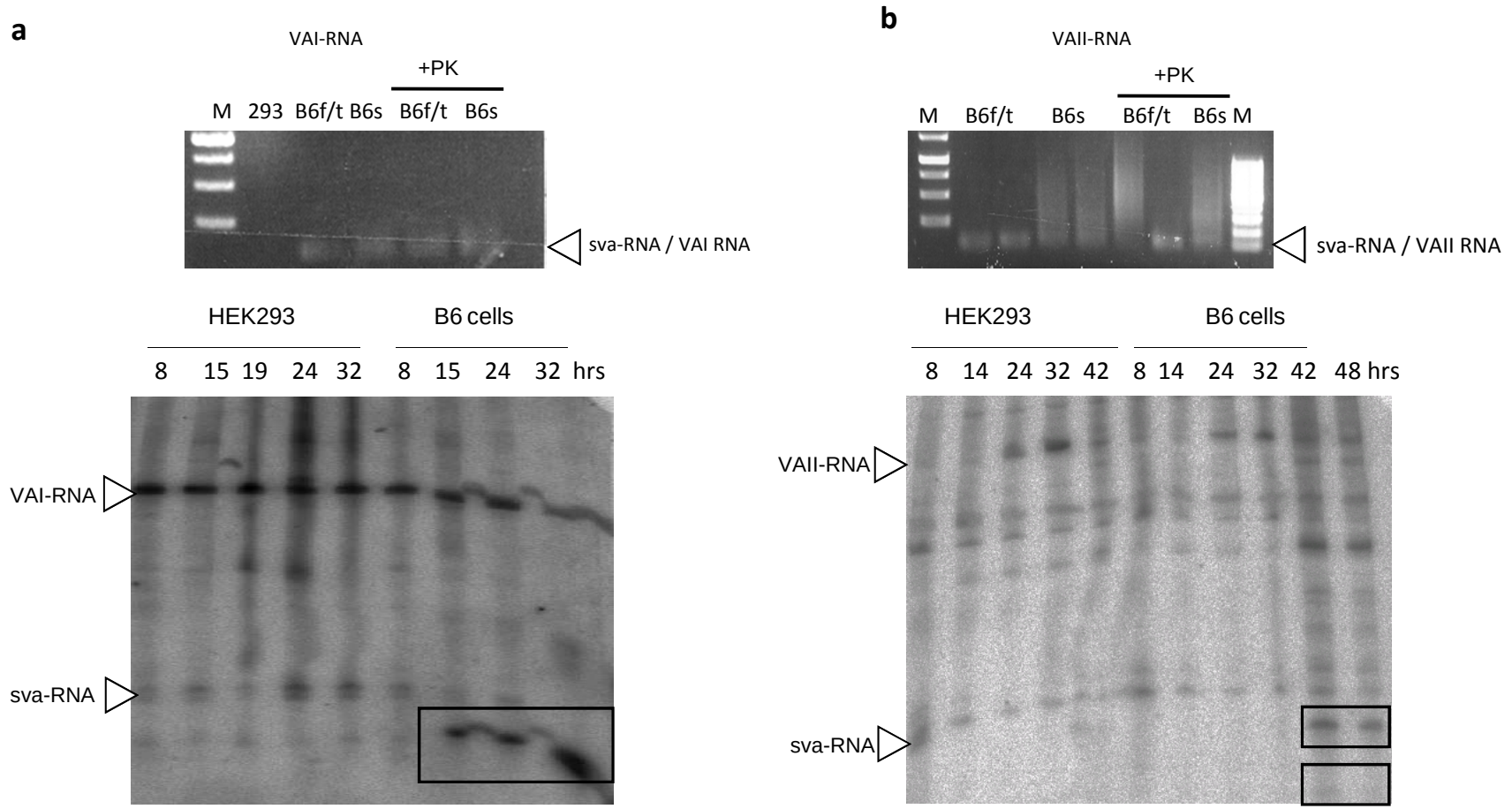
a



b



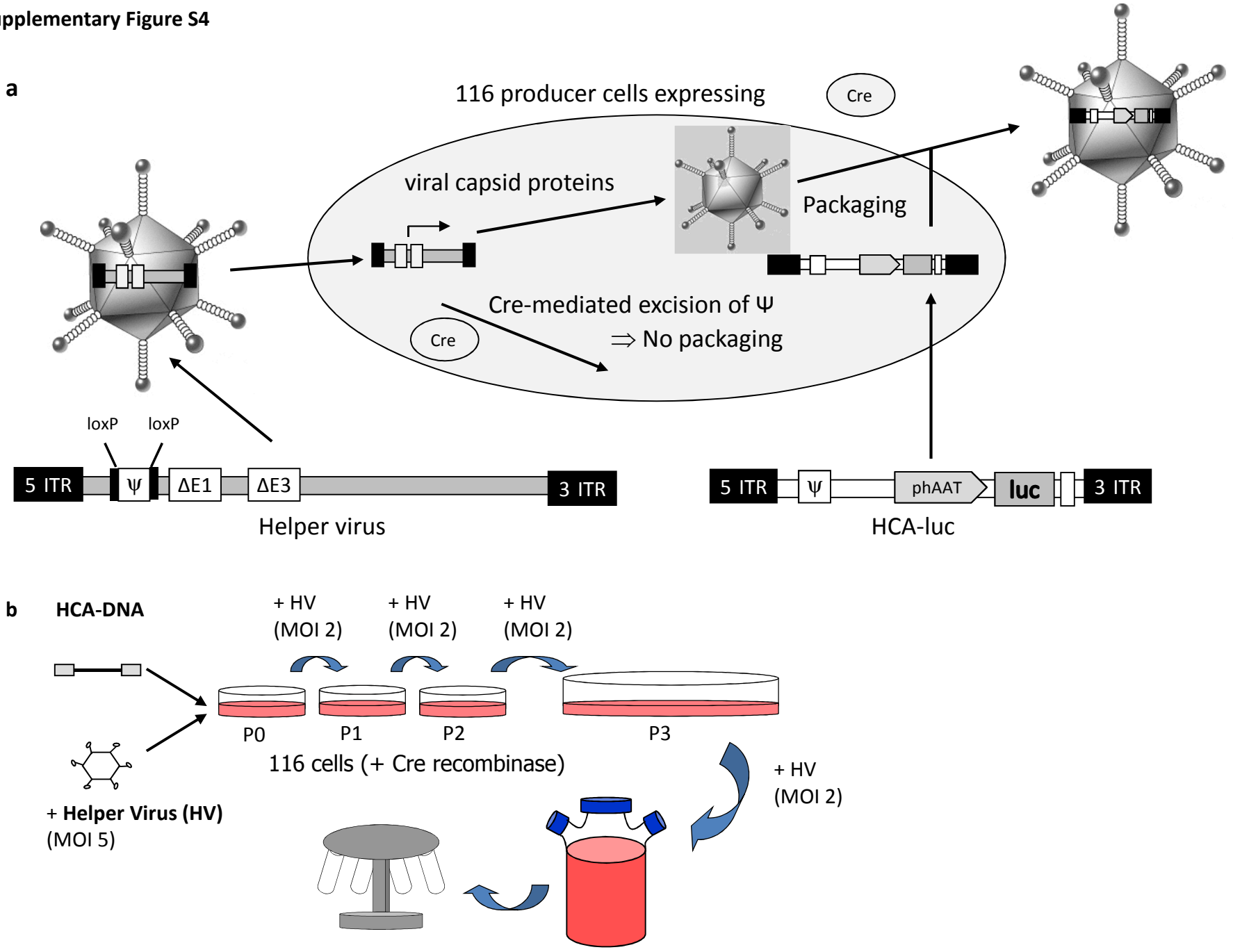
Supplementary Figure S3



c

	Sequencing results	location
VAI-RNA	GACGTCAGACAACGGGGGAGT	142-162 bp
	GACGTCAGACAACGGGGGAGTGCTCCTTTT	133-159 bp
VAII-RNA	CCCTTCGGCTACAGGGAGCGAGCC	2- 25 bp
	AATAACCCTTCGGCTACAGGGAGCGAGCC	2- 30 bp
	CCTCCGGAAACAGGGACGAGCCCCT	134- 160bp
miR196a	ACGTCAGACAACGGGGGAGCGCTCCA	

Supplementary Figure S4



Supplementary Figure S5