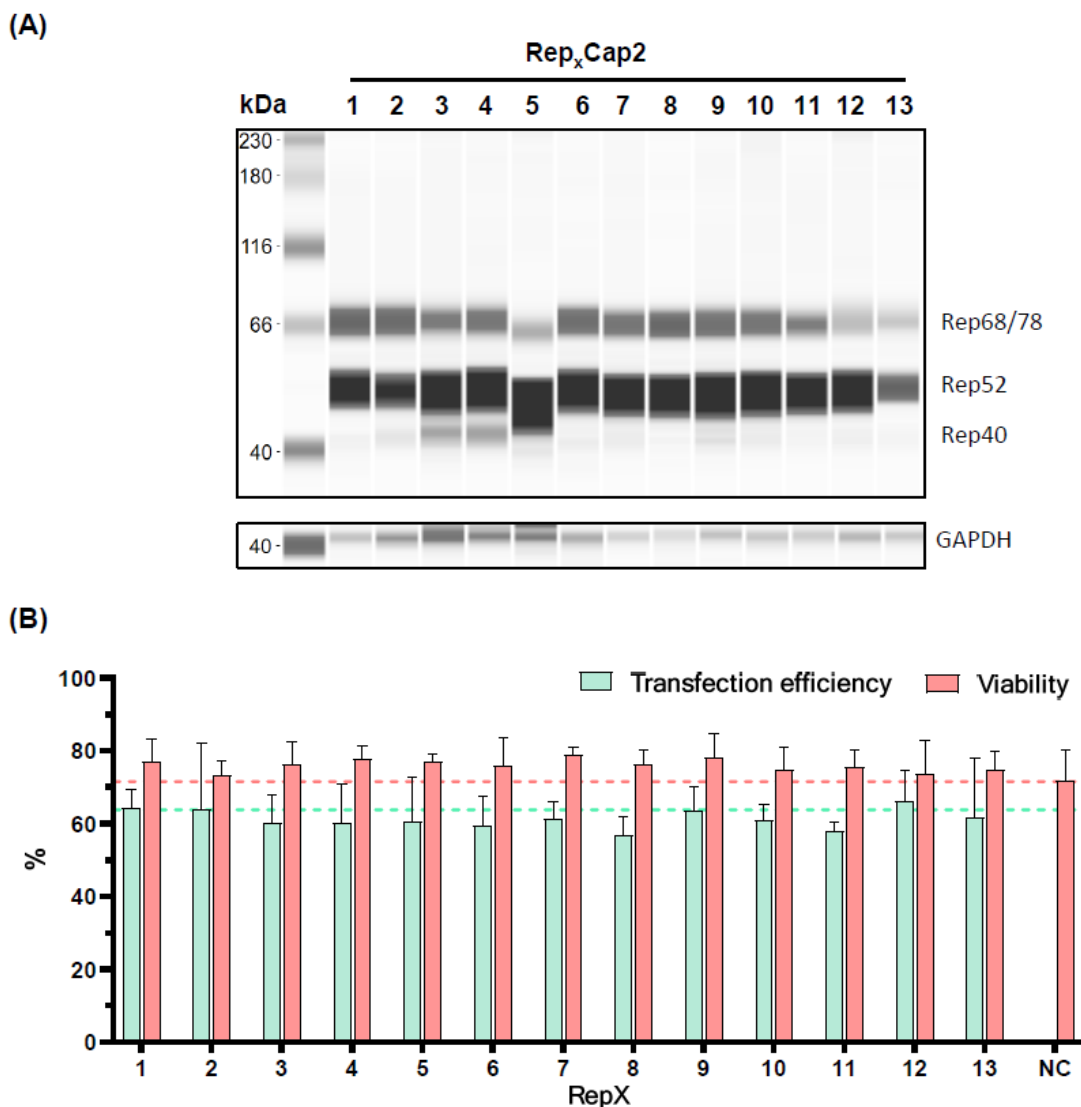
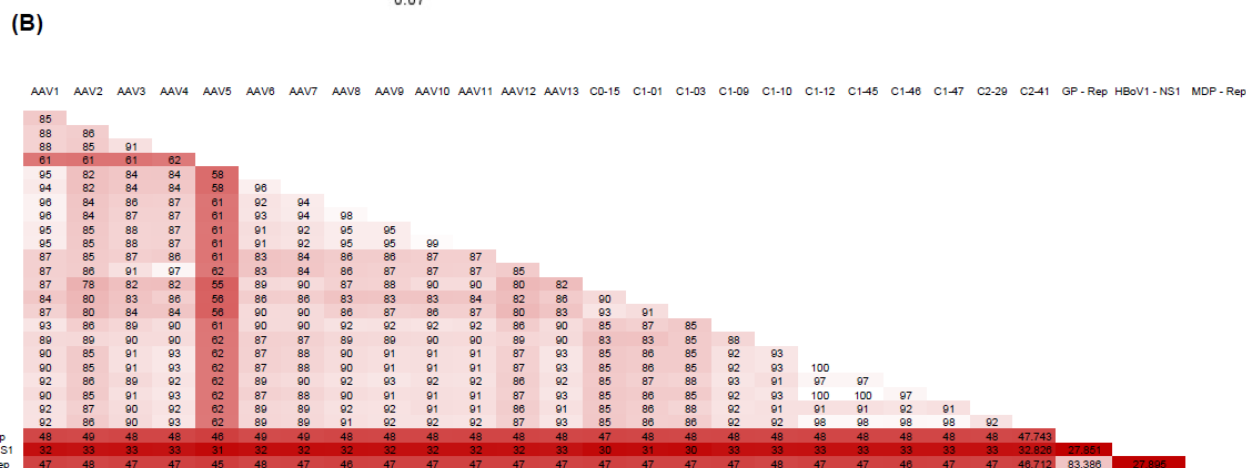
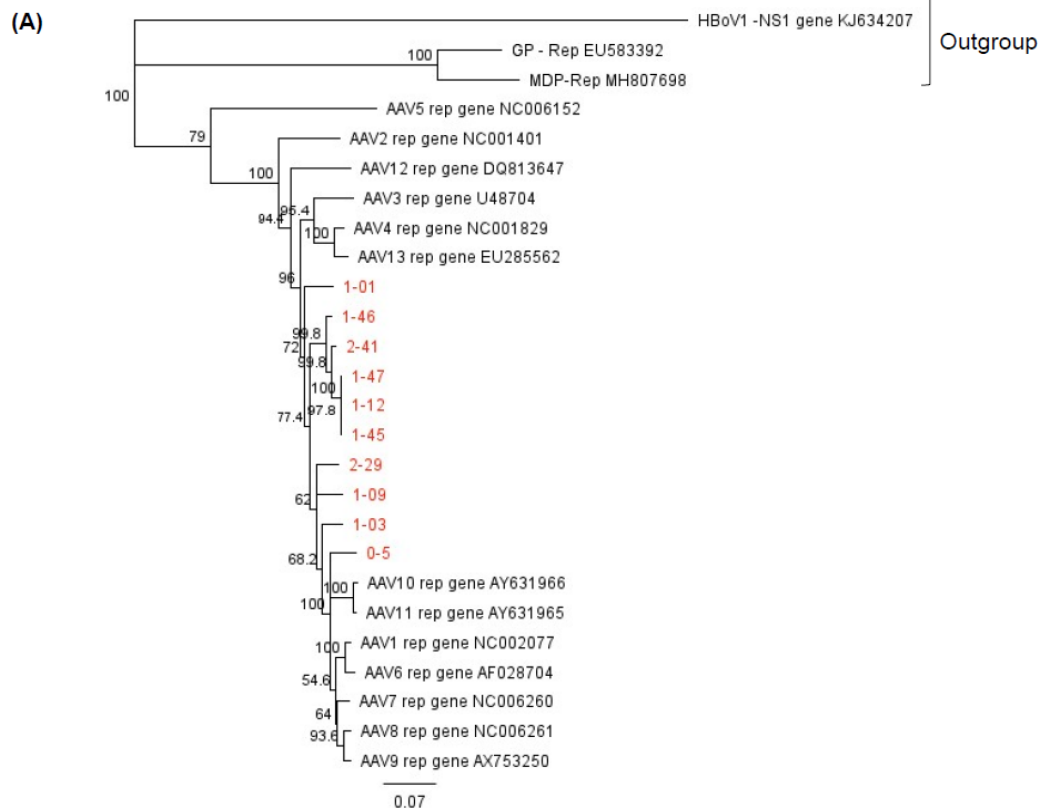




Assessment of AAV Rep variants in HEK293 cells. A) Western Blot analysis using the JESS Simple Western™ system. Expi293F™ suspension cells were transfected with the indicated Rep_xCap2 plasmids ($x = 1-13$), alongside the adenohelper and an AAV-GFP reporter plasmid. Cells were harvested three days post-transfection, and lysates were analyzed for Rep protein expression (top panel) and GAPDH expression (bottom panel) as a loading control. The expected Rep protein variants are indicated. **B)** Cell viability and transfection efficiency were assessed for the samples used in the analyses shown in Figure 1B–D. HEK293T cells were harvested in PBS three days post-transfection, followed by viability and flow cytometry analyses. NC = non-transfection control.

Figure S1



Phylogenetic and distance analysis of Rep hybrid clones. A) Phylogenetic analysis of the indicated *rep* variants (colored red). The evolutionary history was inferred in Geneious Prime by using the Neighbor-Joining algorithm based on the Tamura- Nei model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The scale bar represents the average number of nucleotide substitutions per site.

B) Nucleotide identity matrix. The percentage of nucleotide identity among the indicated nucleotide sequences.

Figure S2

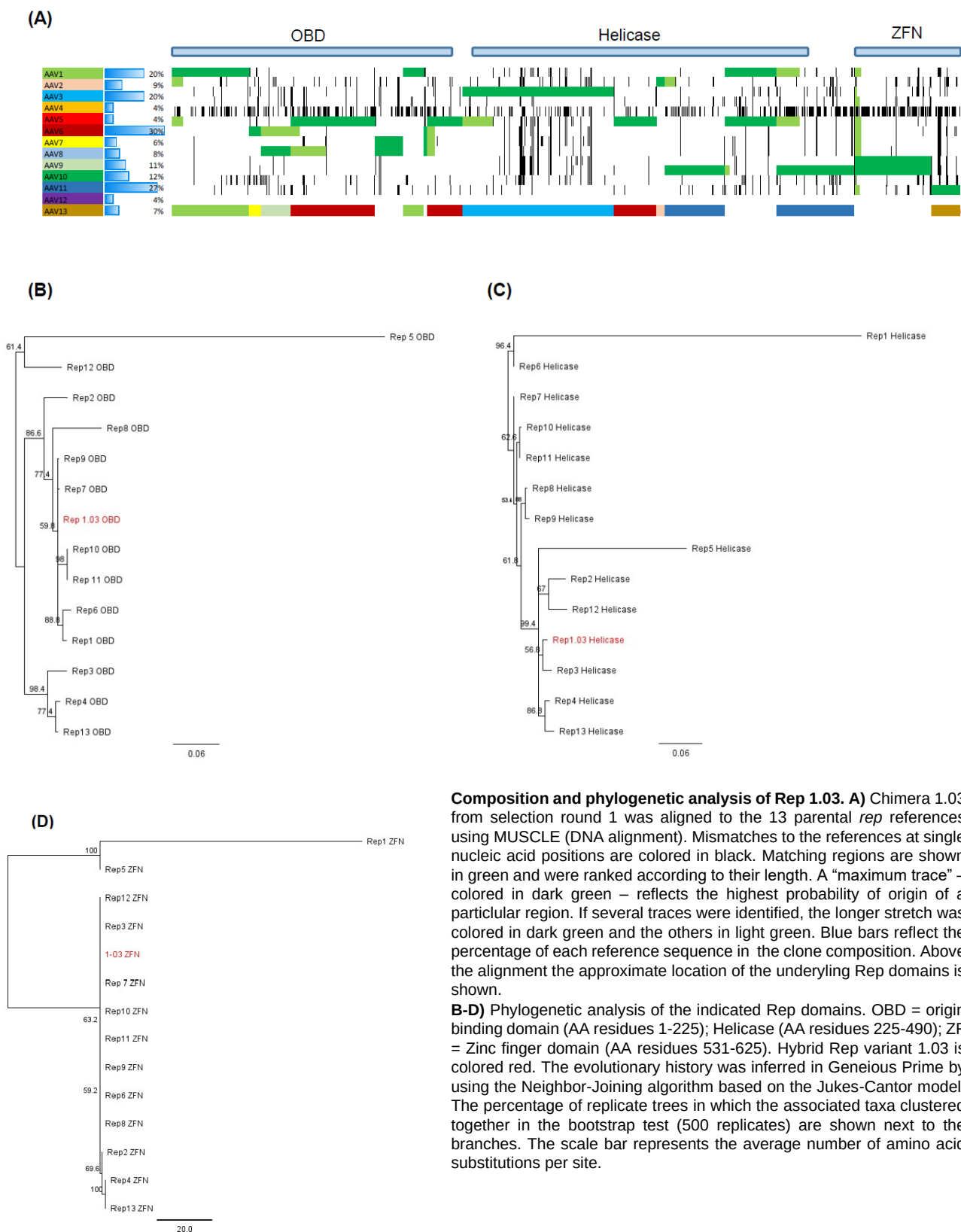
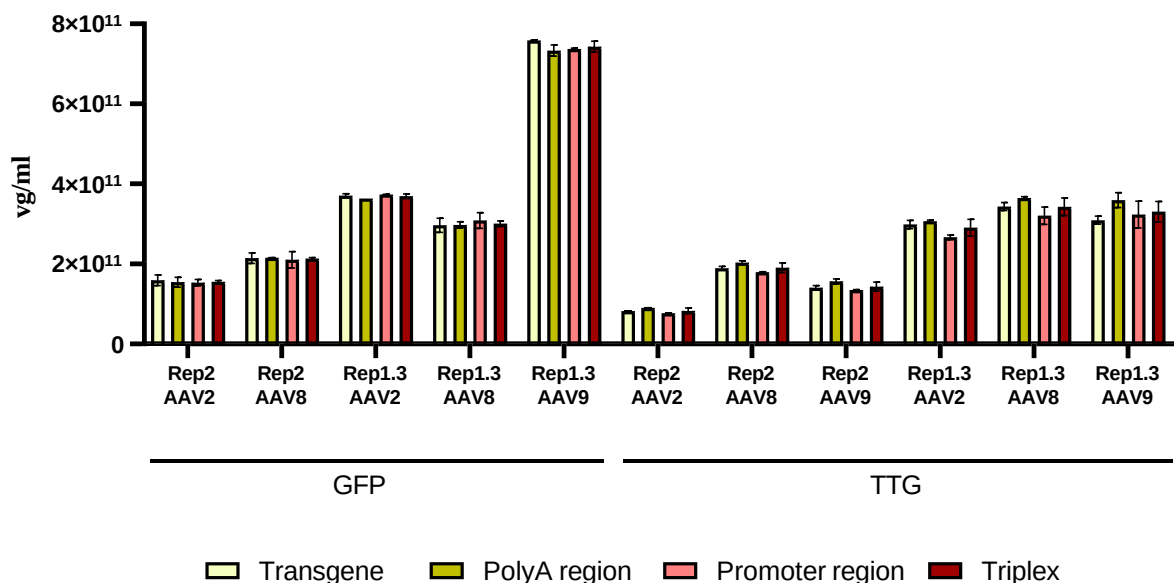


Figure S3



(B)

Transgene	Rep Hybrid	Capsid	Intact genomes (Ratio)
GFP	Rep2	AAV2	1
GFP	Rep1.3	AAV2	1.004
GFP	Rep2	AAV8	1
GFP	Rep1.3	AAV8	1.005
TTG	Rep2	AAV2	1
TTG	Rep1.3	AAV2	0.82
TTG	Rep2	AAV8	1
TTG	Rep1.3	AAV8	0.91
TTG	Rep2	AAV9	1
TTG	Rep1.3	AAV9	0.80

Genomic titers and genome intactness using Rep2 and Rep1.3. A) Absolute quantification of rAAV titers using dPCR. A total of 15 mL of the indicated rAAVs, produced in the AMBR 250 system, were purified using AAVX affinity resin. Singleplex and multiplex titer analyses were conducted using primer/probe sets targeting different regions of the recombinant genome: the 3' end, 5' end, and the middle. **B)** Estimation of viral genome intactness. The genome intactness of the rAAV preparations shown in panel A was assessed using the multiple-occupancy analysis feature of the QIAcuity Software Suite (version 2.5.0.1), as described in the Materials and Methods section. Percentages derived using Rep2 were averaged and normalized to a reference value of 1.

Figure S4

Table S1. Production of rAAV encoding for GFP or a therapeutic transgene (TTG) in an AMBR250 system

Parameter	vg/ml		vp/ml		%full	
Transgene	GFP	TTG	GFP	TTG	GFP	TTG
Rep1.03_Cap2	0.7	0.5	0.53	0.2	2.0	3.5
Rep1.03_Cap8	1.1	1.25	0.61	0.5	1.67	2.3
Rep1.03_Cap9	ND	0.65	ND	0.4	ND	1.3

^aGenomic (vg/ml) and capsid (vp/ml) titers were assessed using ddPCR and ECLIA, respectively. The calculated ratio of both parameters represents the percentage of full capsids. Data were normalized to the Rep2-control (set to 1).

Table S2. Semi-quantitative Western blot analysis of Cap protein expression (shown in Figure 5)

Protein	VP1		VP2		VP3	
Transgene	GFP	TTG	GFP	TTG	GFP	TTG
Rep1.03-AAV2	0.99	0.86	0.89	0.67	1.07	0.99
Rep1.03-AAV8	0.77	0.67	0.75	0.65	0.81	0.65
Rep1.03-AAV9	0.80	0.57	0.70	0.50	0.77	0.56

^bThe area under the peak was calculated using the Compass for SW Version 6.3.0 and corrected to the total protein content. Data of each condition were normalized to the respective Rep2 control construct.

Table S3. Semi-quantitative Western blot analysis of Rep protein expression (shown in Figure 5)

Condition	Rep 1.03-AAV2		Rep 1.03-AAV8		Rep 1.03-AAV9	
Transgene	GFP	TTG	GFP	TTG	GFP	TTG
Rep40	2.9	0.78	1.5	0.48	0.9	0.74
Rep52	4.8	1.25	2.03	0.6	1.5	1.3
Rep68/78	1.58	0.35	0.32	0.12	0.44	0.39

^cThe area under the peak was calculated using the Compass for SW Version 6.3.0 and corrected to the total protein content. Data of each condition were normalized to the respective Rep2 control construct.

Table S4. Overview of the % full of the rAAV harvest as depending on Rep, Cap and transgene combination

Rep	Cap	Transgene	Nr. of analyzed AAVs	% Full
1.3	2	GFP	637	25
2	2	GFP	2376	21
1.3	2	Therapeutic	413	37
2	2	Therapeutic	672	25
1.3	8	GFP	1411	18
2	8	GFP	1220	15
1.3	8	Therapeutic	1972	36
2	8	Therapeutic	1437	26
1.3	9	GFP	ND	ND
2	9	GFP	2724	22
1.3	9	Therapeutic	1171	21
2	9	Therapeutic	917	27

Table S5. Primer/probe sets used for singleplex and multiplex dPCR analysis of rAAV titer

Target	Primer/Probe name	Sequence (5'→3')
5' end	5'GFP_CMVenh5_F	TTGACGTCAATGGGTGGAGT
	5'GFP_CMVenh5_R	CGGGCCATTTACCGTCATTG
	5'GFP_CMV3nh5_P	ACATCAAGTGTATCATATGCCAAGTACGC
3' end	3'GFP_BGHpolA_F	[CY5]CATTGTCTGAGTAGGTGTCATT[C[BHQ2]
	3'GFP_BGHpolA_R	TGCCTGCTATTGTCTTCCCA
	3'GFP_BGHpolA_P	[HEX]CCTCCCCCTTGCTGTCCTGC[BHQ1]
Middle	eGFP_mid_F	TAGCCGCTACCCTGATCATA
	eGFP_mid_R	TAAAGAAGATGGTCCGCTCC
	eGFP_mid_P	[FAM]TCTTTAAGTCCGCTATGCCAGAAGG[BHQ1]