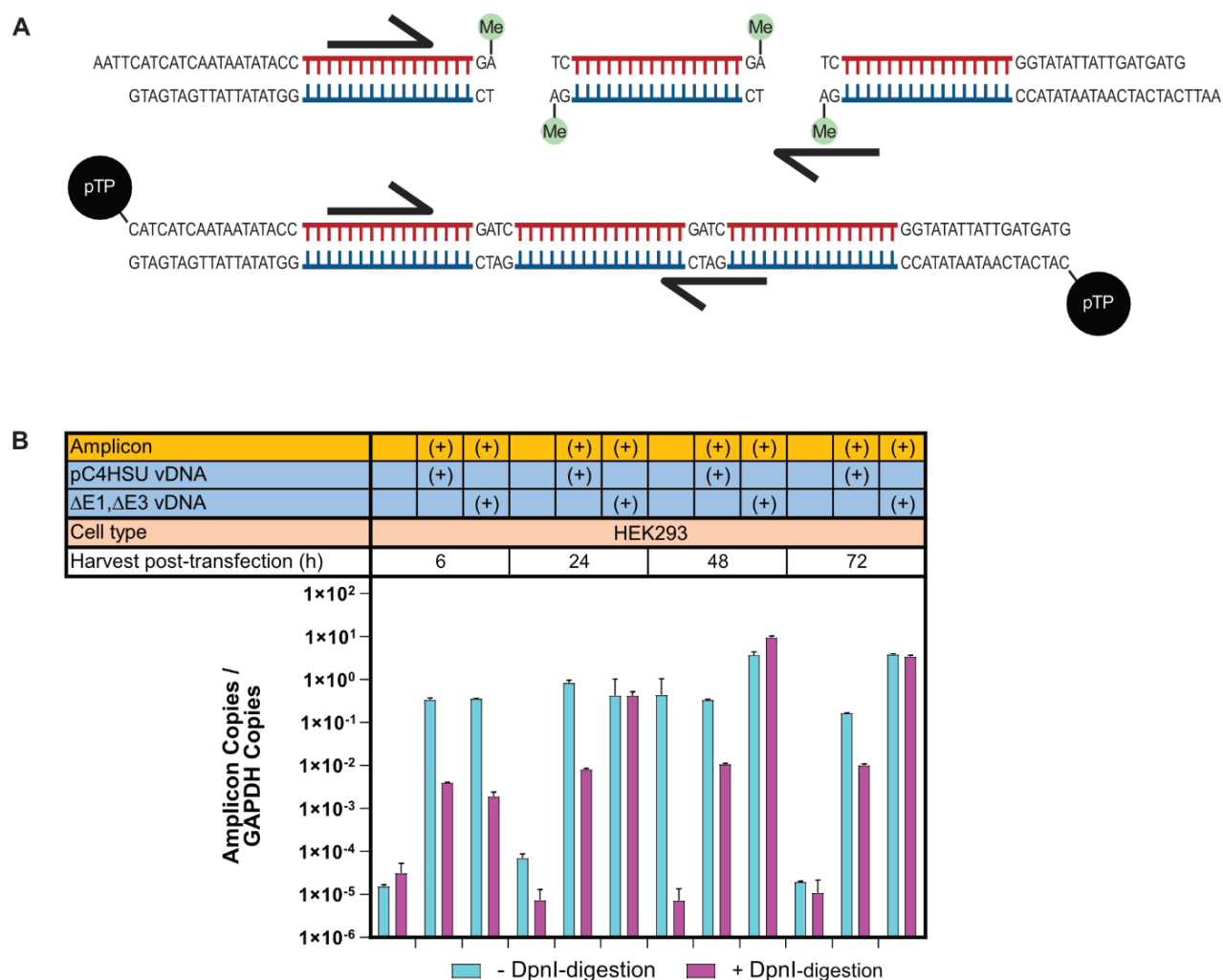
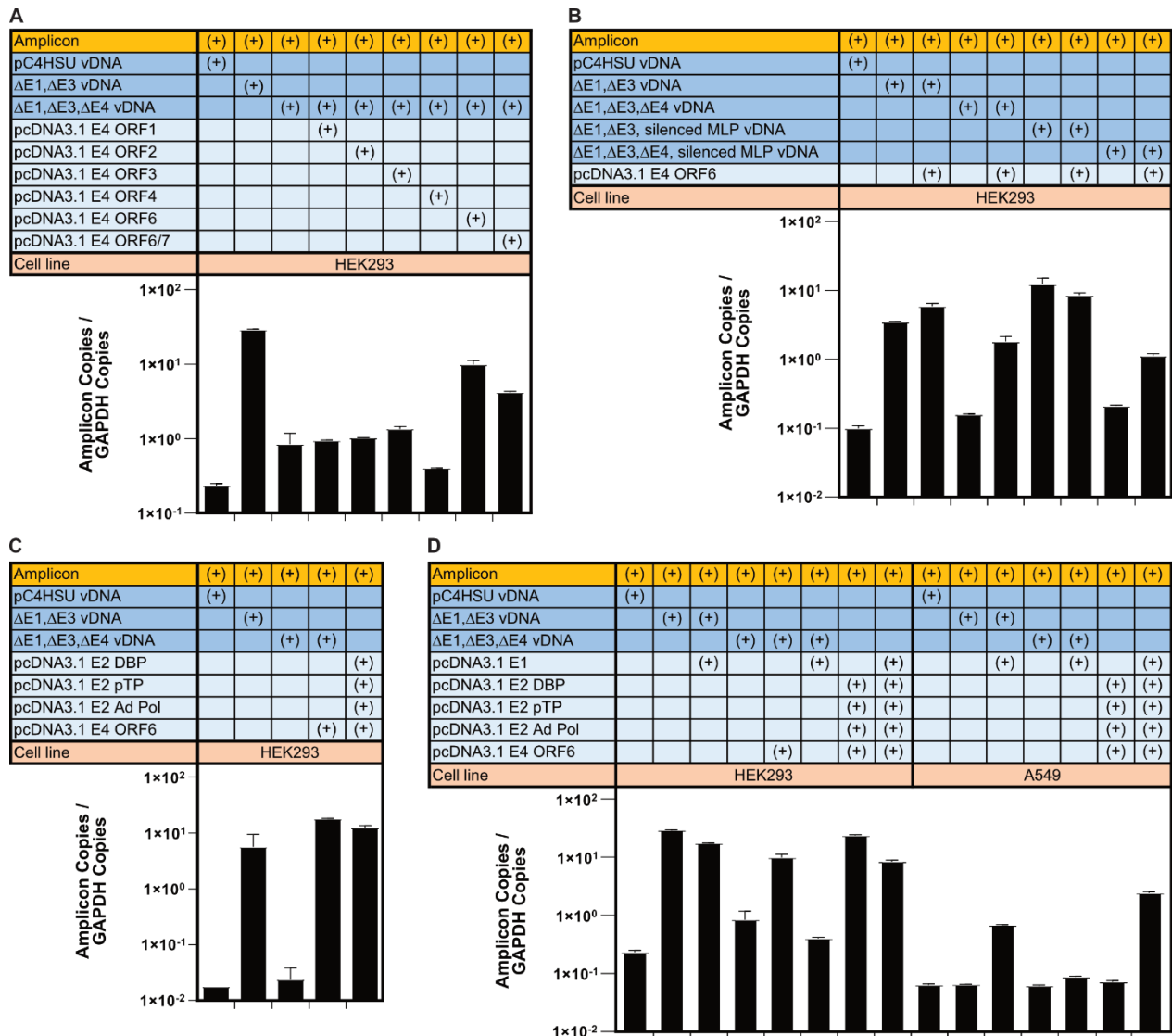


**Figure S1. Schematic illustration of the genetic architecture of the human wild-type AdV-C5 genome.** The double-stranded DNA genome is displayed as a pair of black lines, left and right inverted terminal repeats (ITRs) are shown in blue and the packaging signal  $\Psi$  in magenta. Transcriptional units (TUs) are depicted in gray (early TU 1-4 and intermediate TUs) and green (late TU 1-5) boxes with individual transcripts shown as black arrows with the thick bars denoting open reading frames (ORFs) and thin bars denoting introns and untranslated regions (UTRs). Intermediate transcripts are displayed in teal and adenoviral virus-associated RNA (VA RNA) I and II are illustrated in cyan. Transcription start sites (TSS) of TUs or individual genes are represented by red arrows.



**Figure S2. *DpnI*-digestion allows selective quantification of *de novo* synthesized amplicons via qPCR.** (A) Schematic depiction of transfected amplicon (upper panel) and *de novo* replicated amplicon (lower panel). Only the transfected amplicon is selectively depleted, as successful *DpnI*-digestion requires methylation of recognition site (green), mediated by the bacterial Dam methylase during production of DNA molecules in *E. coli*. The *de novo* synthesized amplicon is replicated in mammalian cells, not methylated and thus resistant to *DpnI* digestion and provides a valid template for qPCR analysis. qPCR primers (black) can bind to the correct DNA template for selective quantification of amplicon copy numbers. (B) *Trans*-replication leads to resistance of the amplicon towards *DpnI*-digestion, as determined by qPCR analysis. Amplicon copies per cellular GAPDH copies were determined after co-transfection with plasmids reconstituting a functional (e.g.,  $\Delta E1, \Delta E3$  vDNA) or non-functional replication machinery (pC4HSU vDNA). Quantification of amplicons was performed 6, 24, 48 and 72 h post-transfection via qPCR, after total DNA was left

untreated (cyan bars) or *DpnI*-digested (magenta bars). Bar graphs represent mean amplicon copy numbers per GAPDH copies  $\pm$  SD, n = 3 technical replicates.

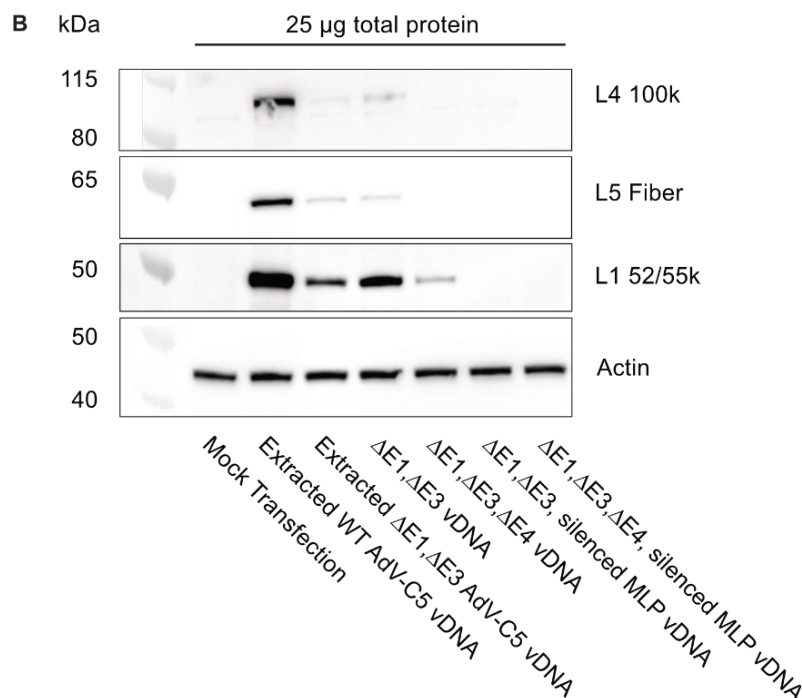
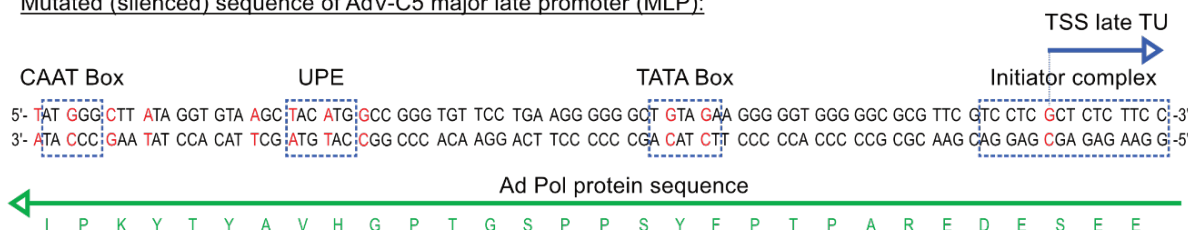


**Figure S3. qPCR quantification of amplicon copy numbers, only normalized to cellular GAPDH gene copies, without conversion to x-fold replication.** (A) Amplicon copy numbers per GAPDH copies, corresponding to fold-change data shown in Figure 1D. (B) Amplicon copy numbers per GAPDH copies, corresponding to fold-change data shown in Figure 1E. (C) Amplicon copy numbers per GAPDH copies, corresponding to fold-change data shown in Figure 2A. (D) Amplicon copy numbers per GAPDH copies, corresponding to fold-change data shown in Figure 2B. Bar graphs represent mean amplicon copy numbers per GAPDH copies  $\pm$  SD, n = 3 technical replicates.

**A** WT sequence of AdV-C5 major late promoter (MLP):

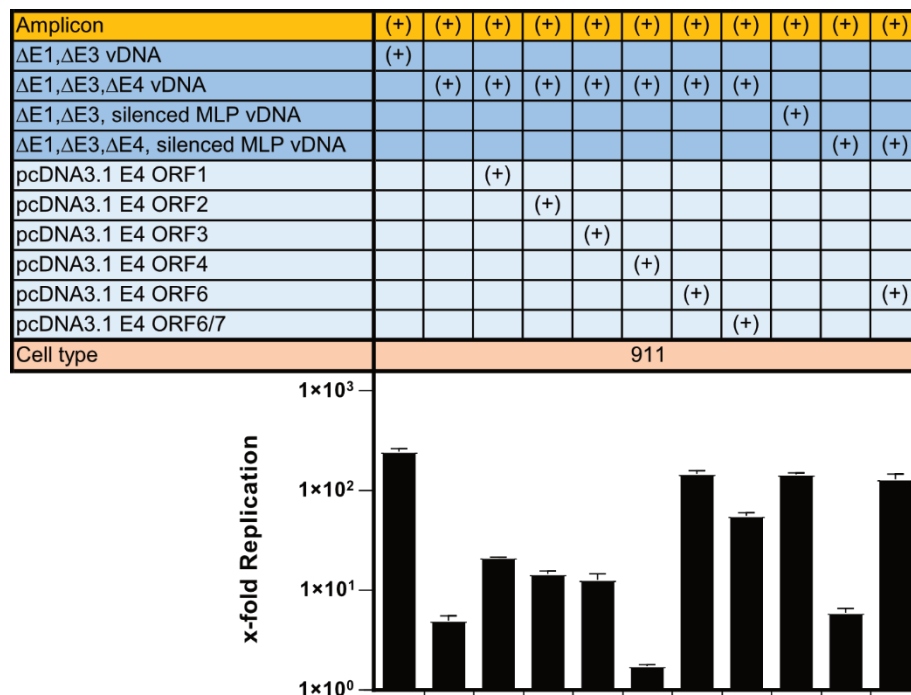


**Mutated (silenced) sequence of AdV-C5 major late promoter (MLP):**

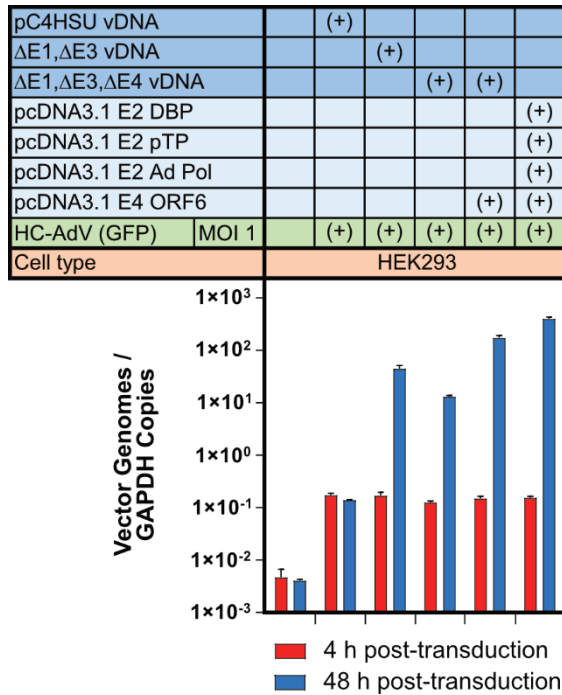


**Figure S4. Silencing of major late promoter (MLP).** (A) Sequence of the AdV-C5 MLP. The wild-type nucleotide sequences (upper panel) of transcription factor binding sites (CAAT Box, UPE, and TATA Box), binding sequence of the initiator complex, and transcription start site (TSS) of the major late transcript are denoted on the sense strand with a blue dashed box. The nucleotide sequence of the Ad Pol is encoded on the antisense strand, and the corresponding amino acid sequence is displayed below in green. The mutated nucleotide sequence of the silenced MLP is

illustrated in the lower panel. Multiple disrupting point mutations in transcription factor and initiator complex binding sites are highlighted in red. Point mutations preserved the protein-coding sequence of the Ad Pol, encoded on the antisense strand, as shown by the Ad Pol amino acid sequence (green). (B) Representative western blot analysis and immunostaining of three late gene products from the L1, L4, and L5 TU, 72 h post-transfection of HEK293 cells with different vDNAs. Cells were transfected with WT AdV-C5 vDNA and  $\Delta$ E1, $\Delta$ E3 AdV-C5 vDNA extracted from functional purified virions via proteinase K digestion and phenol/chloroform-extraction (lane 2 and 3). Additional transfections included  $\Delta$ E1, $\Delta$ E3 vDNA (lane 4),  $\Delta$ E1, $\Delta$ E3, $\Delta$ E4 vDNA (lane 5),  $\Delta$ E1, $\Delta$ E3, silenced MLP vDNA (lane 6), and  $\Delta$ E1, $\Delta$ E3, $\Delta$ E4, silenced MLP vDNA (lane 7), all produced as plasmids in *E. coli*.



**Figure S5. Characterization of E4 and late genes via *trans*-replication of amplicon in the cell line 911.** Quantification of x-fold *trans*-replication via qPCR, 48 h after co-transfection of 911 cells with amplicon (orange),  $\Delta E1, \Delta E3$  vDNA,  $\Delta E1, \Delta E3, \Delta E4$  vDNA,  $\Delta E1, \Delta E3$ , silenced MLP vDNA, and  $\Delta E1, \Delta E3, \Delta E4$ , silenced MLP vDNA (dark blue). Co-transfection of amplicon and  $\Delta E1, \Delta E3, \Delta E4$  vDNA was additionally *trans*-complemented with all different gene products from the E4 TU (light blue). Bar graphs represent x-fold amplification  $\pm$  SD, n = 3 technical replicates.

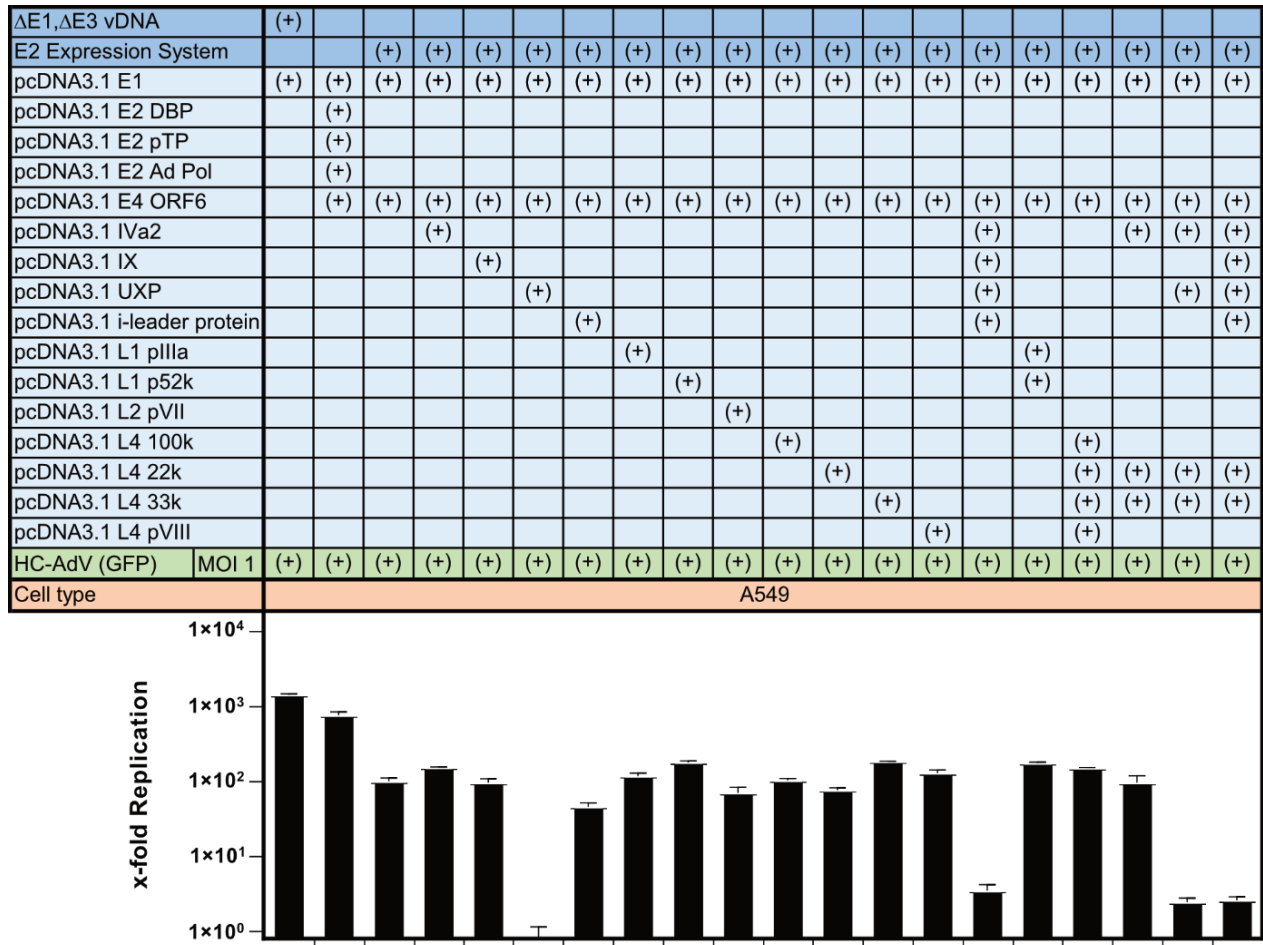


**Figure S6. *Trans*-replication of incoming, transduced HC-AdV genomes upon pre-transfection of HEK293 cells with replication systems.** Quantification of genome *trans*-replication of incoming, transduced HC-AdV genomes via qPCR upon pre-transfection of HEK293 cells with non-replicating pC4HSU vDNA, AdV replication machineries (dark blue), or individual AdV genes (light blue). HC-AdV copy numbers were quantified 4 h (red bars) and 48 h (blue bars) after transduction and normalized to cellular GAPDH copy numbers. Bar graphs represent mean HC-AdV copy numbers per GAPDH copies  $\pm$  SD, n = 3 technical replicates.

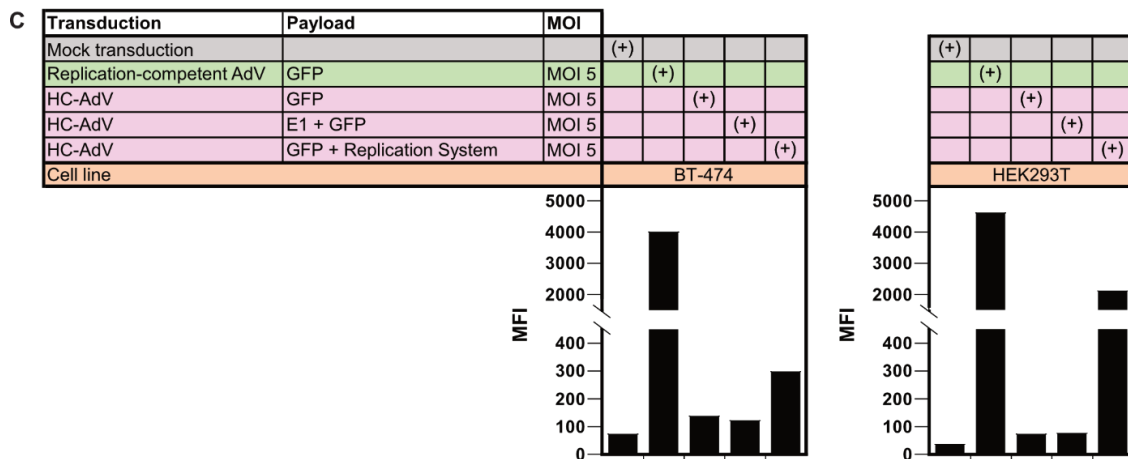
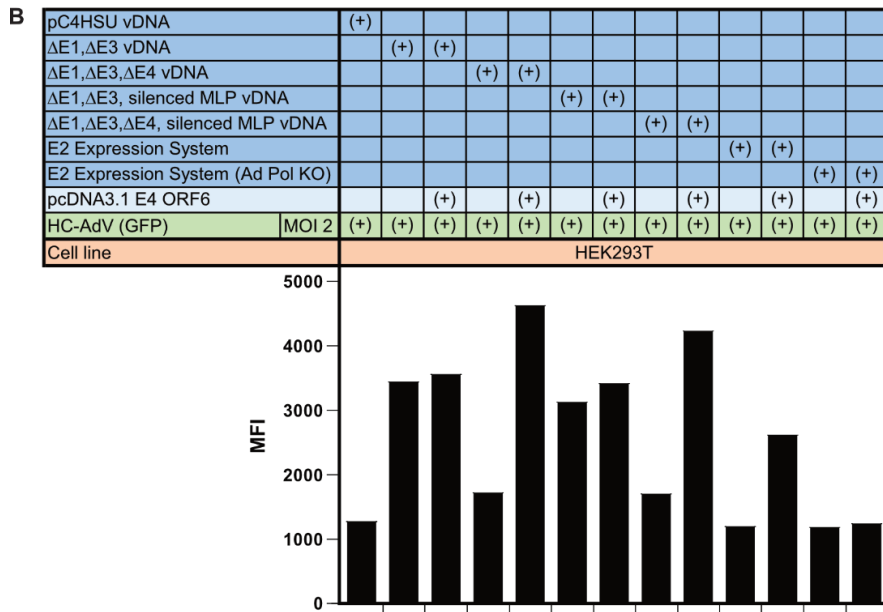
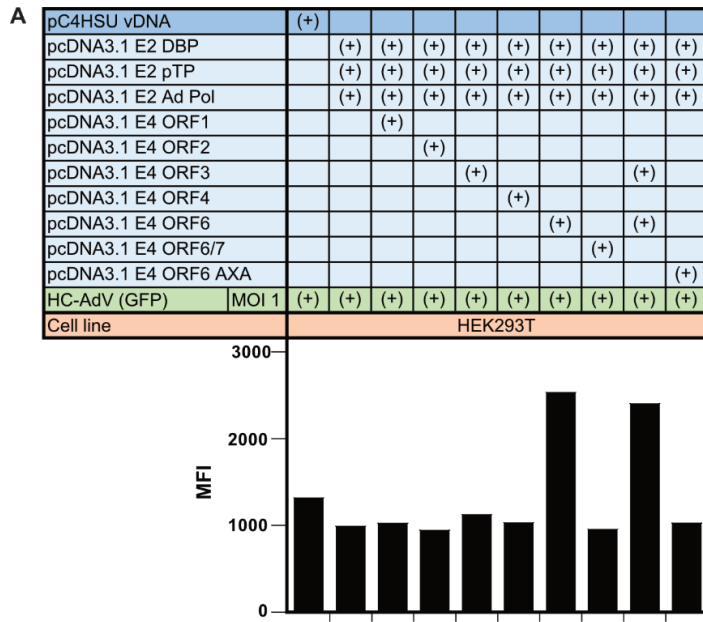




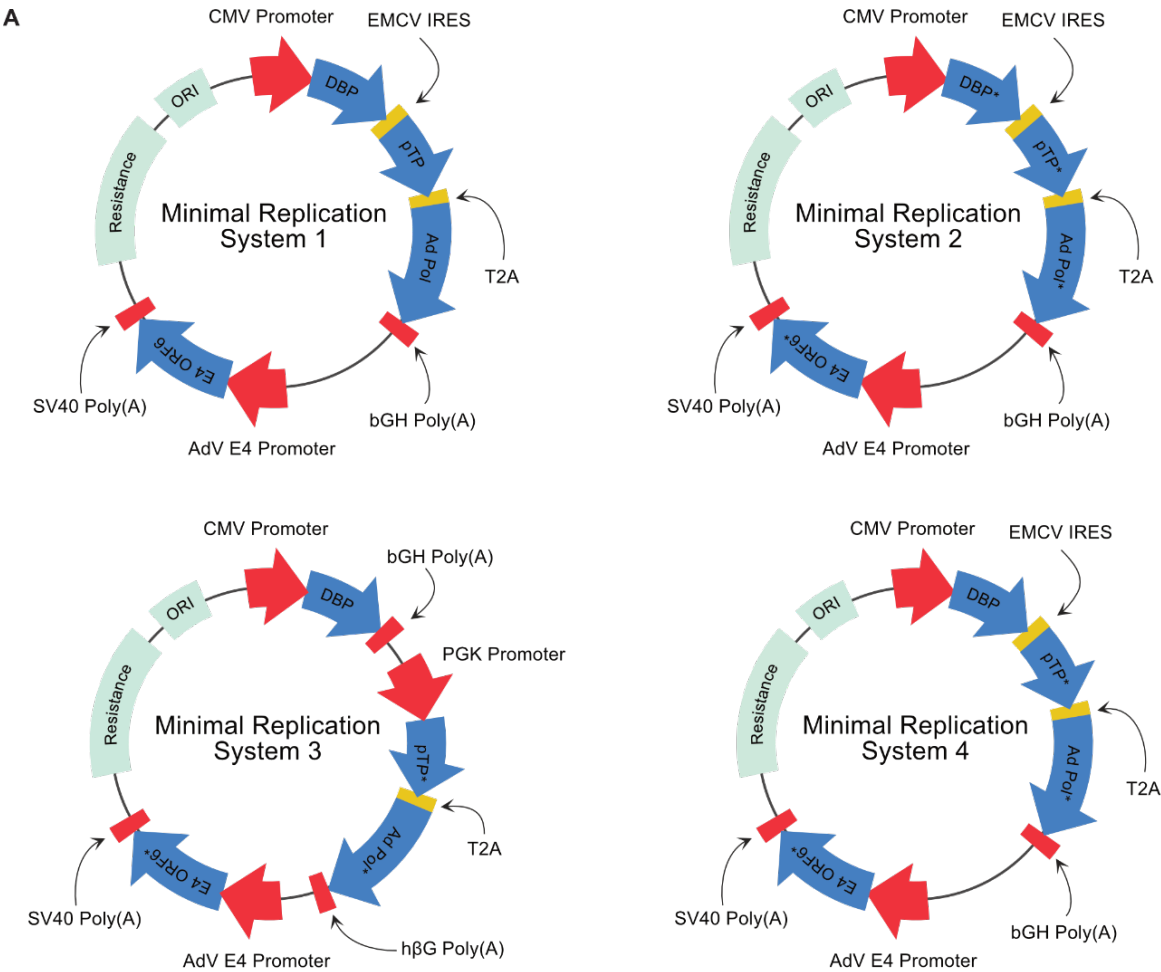
Figure 4C. Bar graphs represent mean HC-AdV copy numbers per GAPDH copies  $\pm$  SD, n = 3 technical replicates.



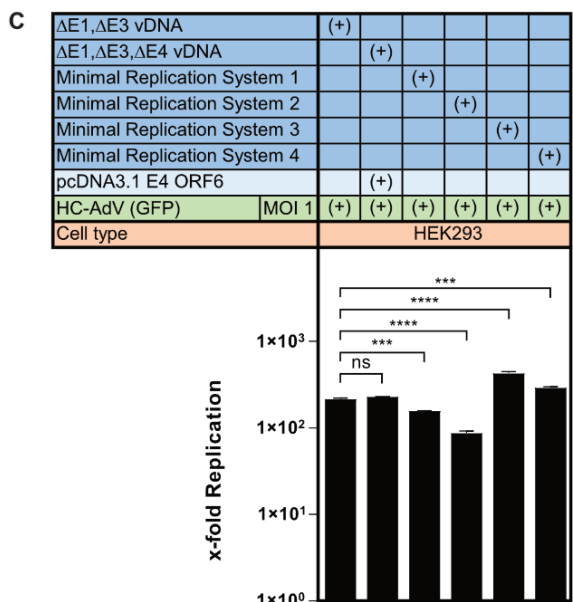
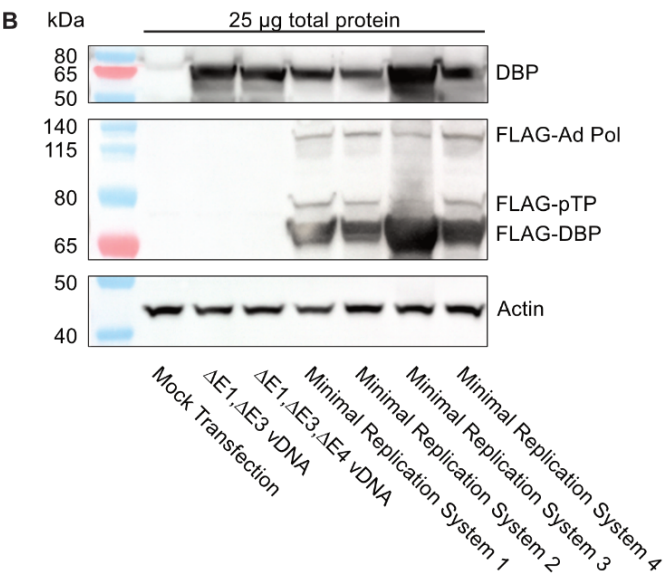
**Figure S8. *Trans*-replication of incoming, transduced HC-AdV genomes upon pre-transfection of A549 cells with replication systems and intermediate and/or late genes.** Quantification of x-fold amplification of incoming, transduced HC-AdV genomes via qPCR, as analyzed 48 h post-transduction of E1-non complementing A549 cells with different replication systems (dark blue). The E2 expression system was co-transfected with plasmids encoding E1, E4 ORF6, and individual or combined expression plasmids encoding relevant intermediate genes (IVa2, IX, UXP, and i-leader protein) or late genes (L1 pIIIa, L1 p52k, L2 pVII, L4 100k, L4 22k, L4 33k, and L4 pVIII) (light blue). Bar graphs represent x-fold replication  $\pm$  SD, n = 3 technical replicates.



**Figure S9. Independent repetition (biological replicates) of vector-encoded reporter quantification via flow cytometry following *trans*-replication of incoming, transduced HC-AdV virions.** (A) Flow cytometric quantification of GFP reporter expression, representing biological replicate of experiments shown in Figure 4B. (B) Flow cytometric quantification of GFP reporter expression, representing biological replicate of experiments shown in Figure 4C. (C) Flow cytometric quantification of GFP reporter expression, representing biological replicate of experiments shown in Figure 5D and 6A.

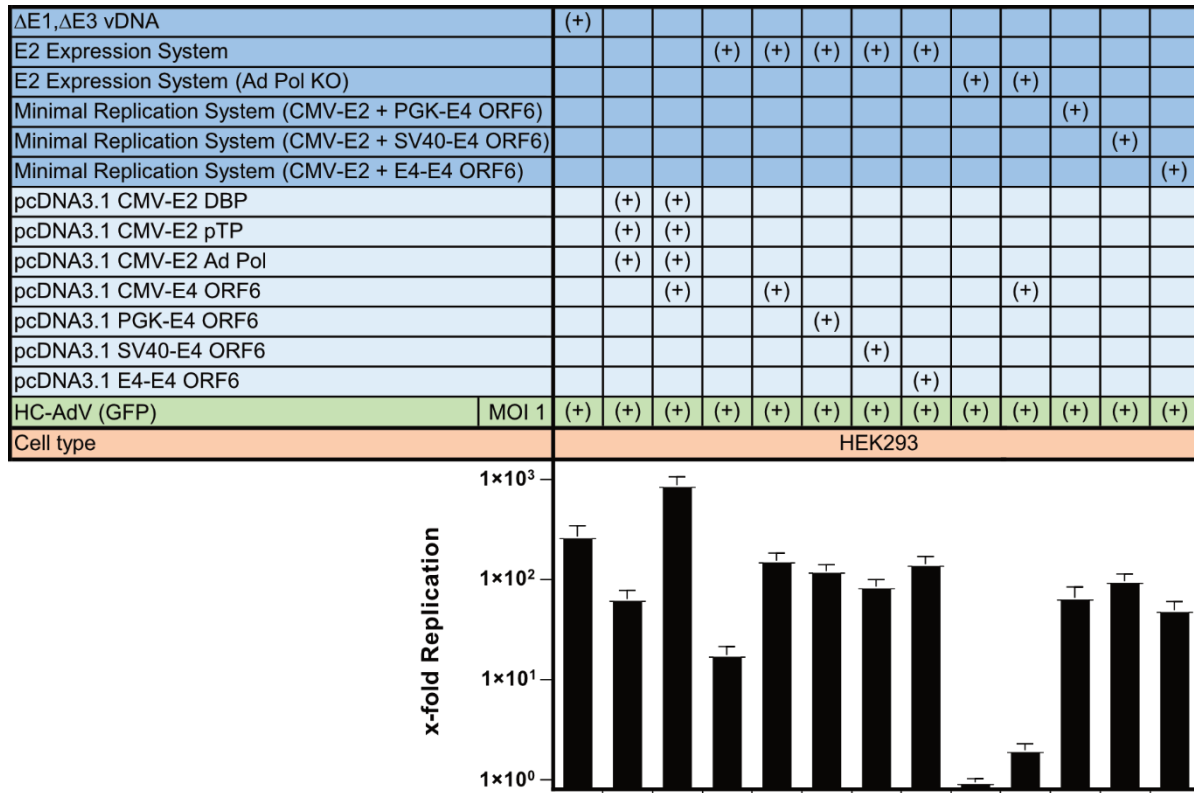


\*: Codon-optimized version of respective gene exhibiting reduced sequence homology to helper plasmid



**Figure S10. Development and characterization of different minimal replication systems. (A)**

Schematic depiction of engineered E2 expression systems additionally encoding E4 ORF6 in a separate expression cassette. In combination with E1 (supplied in *trans* or *cis*), these constructs reconstitute a minimal AdV replication system. Minimal Replication System 1 features a tricistronic cassette expressing all WT E2 proteins under a CMV promoter with a bovine growth hormone (bGH) transcription termination sequence, and a separate cassette expressing E4 ORF6 (WT) driven by the endogenous AdV E4 promoter and an SV40 polyadenylation signal. Minimal replication system 2 exhibits an identical configuration of expression cassettes as system 1, but with codon-optimized sequences for the three E2 genes and E4 ORF6 to reduce sequence homology with helper plasmids (denoted by asterisk). The modified nucleotide sequences only contain silent mutations and thus preserve the WT protein-coding sequences. Minimal Replication Systems 3 and 4 both encode WT DBP alongside codon-optimized pTP, Ad Pol, and E4 ORF6. In system 3, WT DBP is supplied from a separate expression cassette (using a CMV promoter and a bGH polyadenylation signal) while codon-optimized pTP and Ad Pol are expressed from a dicistronic cassette using a PGK promoter, a T2A self-cleavage sequence, and a human  $\beta$ -globin termination (h $\beta$ G) signal. Minimal replication system 4 is identical to system 2 except that it expresses WT DBP instead of the codon-optimized version. (B) Representative western blot analysis and immunostaining of DBP and FLAG-tagged E2 proteins, 36 h post-transfection of HEK293 cells with  $\Delta$ E1, $\Delta$ E3 vDNA,  $\Delta$ E1, $\Delta$ E3, $\Delta$ E4 vDNA, or minimal replication system 1-4. Detection of FLAG-tag allows evaluation of E2 protein expression across minimal replication systems, while DBP immunostaining enables direct comparison of DBP expression levels from replication systems to tag-less vDNAs.  $\beta$ -actin was immunostained as a loading control. (C) Quantification of incoming, transduced HC-AdV genome replication via qPCR upon pre-transfection of HEK293 cells with different vDNAs or minimal replication systems (dark blue). The  $\Delta$ E1, $\Delta$ E3, $\Delta$ E4 vDNA was co-transfected with plasmids encoding E4 ORF6 (light blue). Genome amplification was quantified 48 h post-transduction and is presented as x-fold replication. Bar graphs represent x-fold replication  $\pm$  SD, n = 3 technical replicates. Statistical significance was determined by one-way ANOVA with Dunnett's test for multiple comparisons to the control group of transfected  $\Delta$ E1, $\Delta$ E3 vDNA. Not significant (ns)  $P > 0.05$ ; \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; \*\*\*\* $P \leq 0.0001$ .



**Figure S11. Effect of different promoters driving E4 ORF6 expression in conjunction with CMV-driven E2 expression system on *trans*-replication of incoming, transduced HC-AdV genomes in HEK293 cells.** Quantification of x-fold HC-AdV genome replication via qPCR upon pre-transfection of HEK293 cells with AdV replication machineries (dark blue) or individual AdV genes (light blue). The CMV-driven E2 expression system was *trans*-complemented with individual plasmids expressing E4 ORF6 under the control of the CMV, PGK, SV40 or endogenous AdV E4 promoter. Alternatively, the plasmid encoding the CMV-driven E2 expression system was modified to additionally encode an E4 ORF6 expression cassette driven by either PGK, SV40, or AdV E4 promoter and pre-transfected for *trans*-replication of incoming HC-AdV genomes. Bar graphs represent x-fold replication  $\pm$  SD, n = 3 technical replicates.