Supplementary Materials

Supplementary Table S1. Oligonucleotides used for optimized gRNA assembly and corresponding target sites.

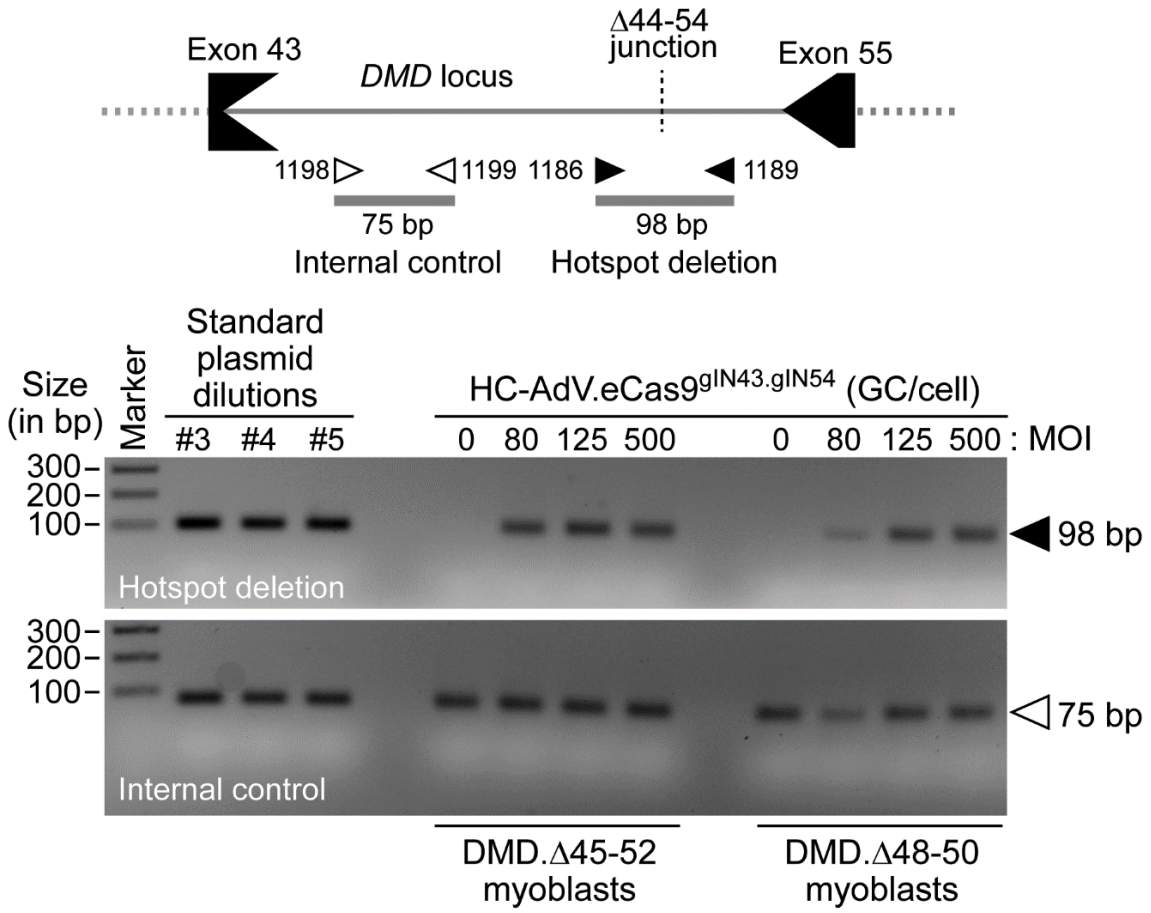
gRNA	Oligonucleotide Code	Oligonucleotide Sequence (5' → 3')	Target Site Sequence (5' → 3'), PAM Underlined	Target Site Region
gEX51	# 83	ACCGACCAGAGTAACAGTCTGAGT	ACCAGAGTAACAGTCTGAGTAGG	Exon 51
	# 84	AAACACTCAGACTGTTACTCTGGT		
gIN43	# 99	ACCGTTACATACAGGCTAGGGAG	GTTACATACAGGCTAGGGAGTGG	Intron 43
	# 100	AAACCTGCCTAGCCTGTATGTAA		
gIN54	# 105	ACCGGTACACAATAGGTACGGAA	GGTACACAATAGGTACGGAATGG	Intron 54
	# 106	AAACTTCCGTACCTATTGTGTAC		

Supplementary Table S2. Primer pairs and composition for qPCR mixtures used for the quantification of genomic deletions.

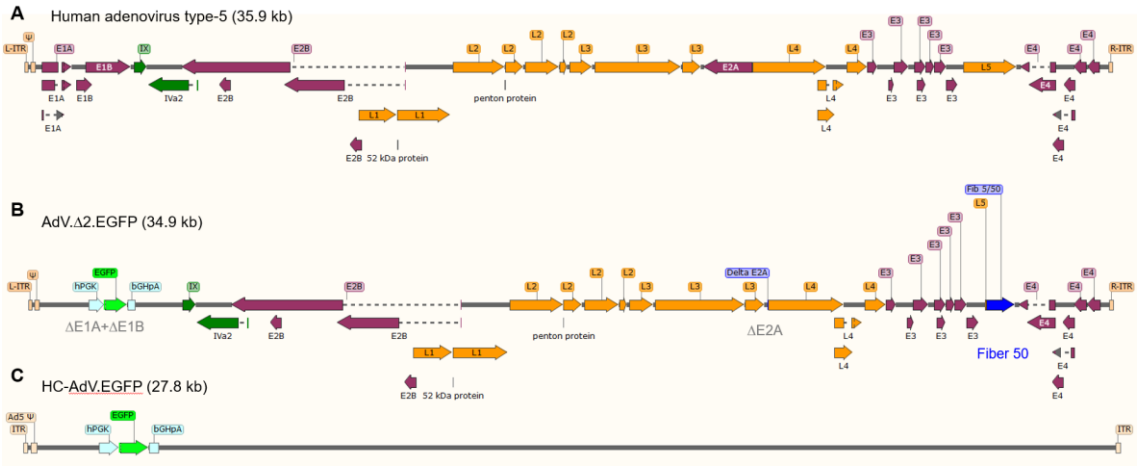
Target Site	Primer Code	Primers (5' → 3')	iQ SYBR Green Supermix	Amplicon Size
Intronic junction Δ44-54	# 1186	TGGTGTCATCCTGGAACTGC (150nM)	1×	99 bp
	# 1189	TCCACATGCCTACCAACATCT (150nM)		
Internal control *DMD* intron 43	# 1198	TCCCAGCACTTTGAGAGACC (150nM)	1×	75 bp
	# 1199	TCCATGTTGCTCAGTCTGGT (150nM)		

Supplementary Table S3. qPCR cycling parameters used for the quantification of genomic deletions.

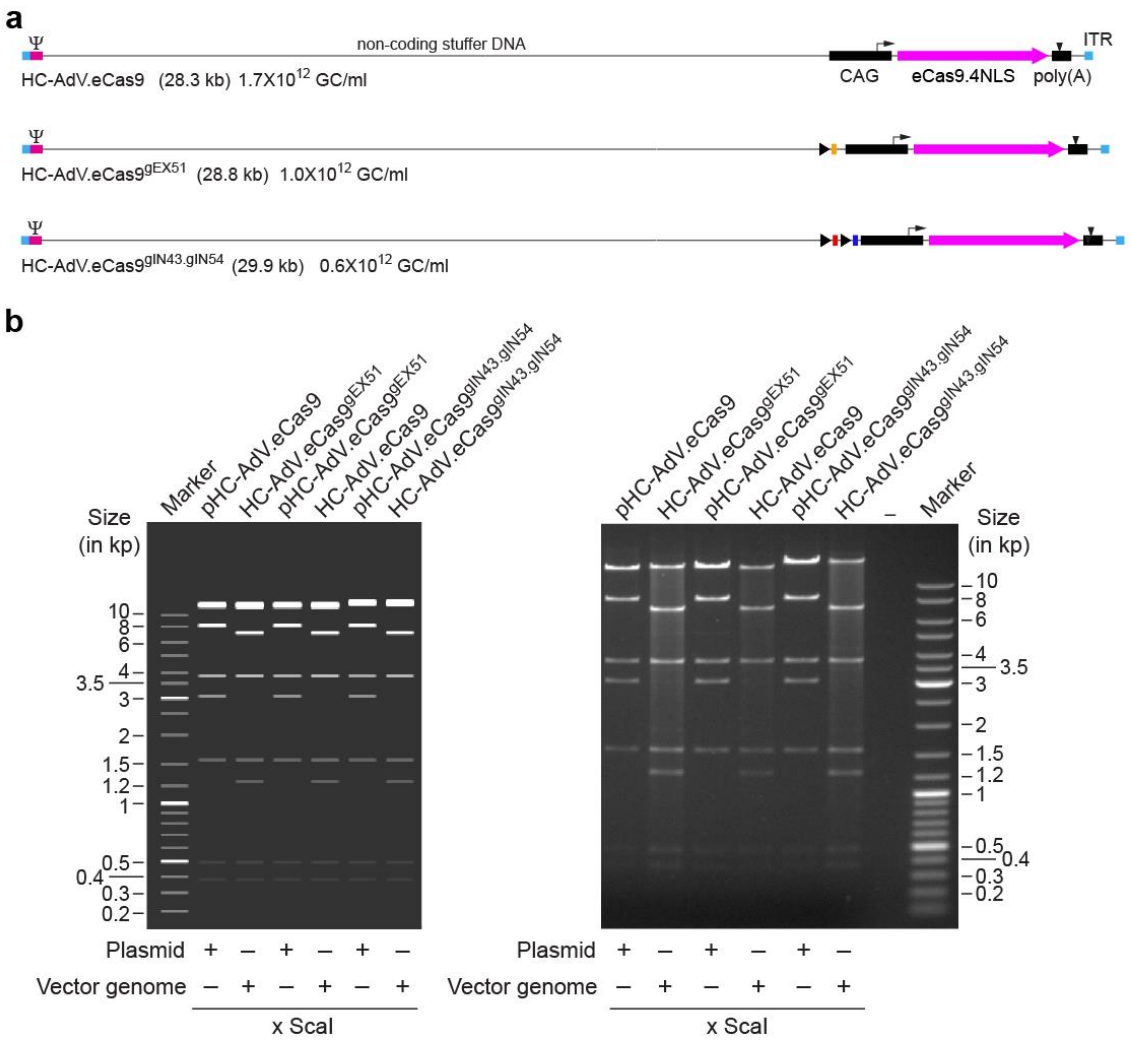
Target	Denaturation	Amplification			Melt Curve Analysis
		Denaturation	Annealing/Extension + Plate Read	# Cycles	
Intronic junction ⌾44–54	95 °C 5 min	95 °C 10 sec	65 °C 30 sec	40	55 to 95 °C (0.5 °C increment/step)
Internal control *DMD* intron 43	95 °C 5 min	95 °C 10 sec	65.5 °C 30 sec	35	55 to 95 °C (0.5 °C increment/step)



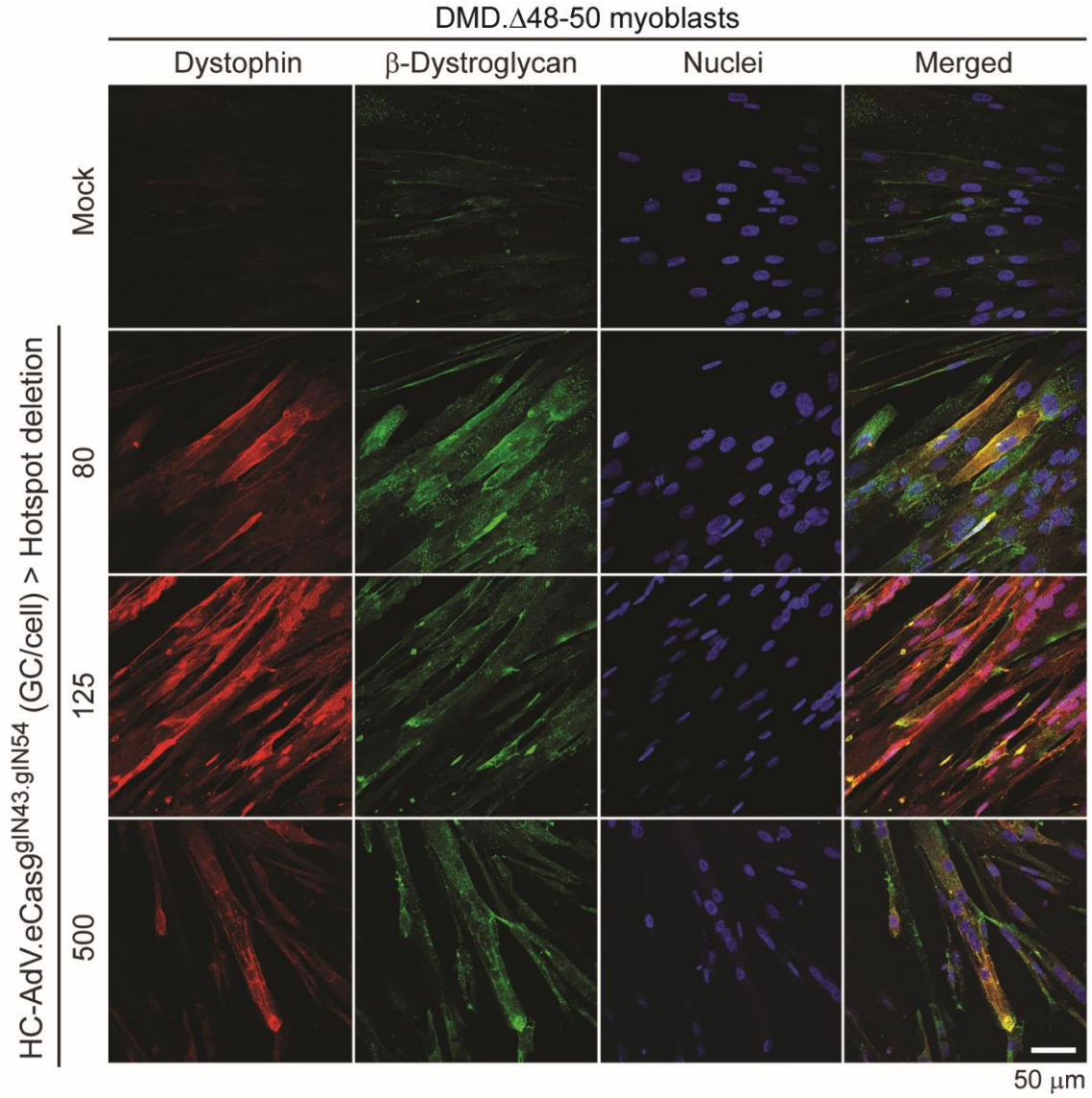
Supplementary Figure S1. Quality control of qPCR amplicons used for quantifying long-range genomic deletions induced by dual RGN-encoding HC-AdV particles. The qPCR assays were performed on genomic DNA from DMD.Δ45-52 and DMD.Δ48-50 myoblasts transduced with the "all-in-one" vector HC-AdV.eCas9$^{gIN43.gIN54}$ at the indicated multiplicities-of-infection (MOI). At 3 days post-transduction vector-transduced cells were sub-cultured and total cellular DNA was isolated at 7 days post-transduction for qPCR analyses. Mock-transduced cells provided for negative controls. qPCR products specific for the deletion encompassing *DMD* exons 44 through 54 (hotspot deletion) and intron 43 (internal control) are marked by open and solid arrowheads, respectively. Amplicons resulting from three serial dilutions of the standard plasmids AV24_jDEL.I43-I54 and AL05_pDMD, containing hotspot deletion and internal control target sequences, respectively, were taken along to confirm the size and specificity of the respective qPCR products generated *in cellula*. Serial dilutions #3, #4 and #5 of plasmid AV24_jDEL.I43-I54 correspond to 2.26×10^5, 2.26×10^4 and 2.26×10^3 DNA copies, respectively. Serial dilutions #3, #4 and #5 of plasmid AL05_pDMD correspond to 7.56×10^5, 7.56×10^4 and 7.56×10^3 DNA copies, respectively. Marker, GeneRuler DNA Ladder molecular weight mix (Fermentas).



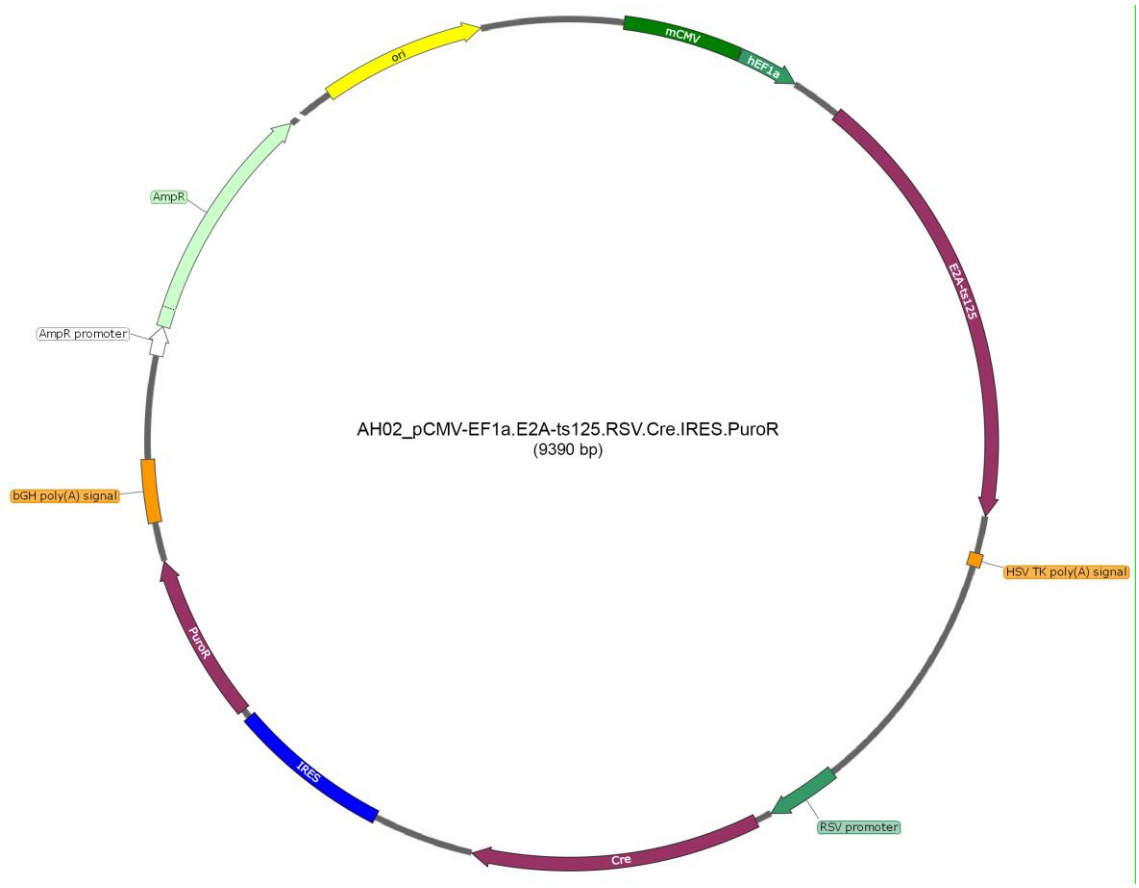
Supplementary Figure S2. Diagrammatic representation of wild-type and recombinant adenoviruses. Genome organization of the prototypic human adenovirus type-5 (**A**) drawn in relation to that of the second-generation adenoviral vector AdV.Δ2.EGFP (**B**) and to that of the high-capacity adenoviral vector HC-AdV.EGFP (**C**). The vectors encode the same expression unit consisting of the EGFP open reading frame under the transcriptional control of the human *PGK1* promoter (hPGK) and the bovine *GH1* polyadenylation signal (bGHpA). The vector particles are tropism modified as they display chimeric fibers consisting of basal shaft sequences from adenovirus type-5 fused to the apical shaft and knob domains from the CD46-interacting adenovirus type-50. The non-coding cis-acting elements: inverted terminal repeats (ITR) and packaging signal (Ψ), necessary for DNA replication and encapsidation, respectively, are indicated. Regulatory functions involved in the activation of the viral gene expression program (magenta arrows) are encoded by the early (E) regions *E1A*, *E1B*, *E2A*, *E3* and *E4*; Structural proteins (orange arrows) necessary for the assembly of mature virions are encoded by the late (L) regions *L1* through *L5*, with *L5* yielding the cell receptor-interacting fibers. Products from reading frames encoding the intermediate proteins IX and IVa2 are also depicted (green arrows). Second-generation AdVs are deleted in *E2A* and/or *E4* and, hence, are produced in the respective complementing packaging cell lines. HC-AdVs (a.k.a. "gutless", helper-dependent or third-generation AdVs) are devoid of all trans-acting coding sequences retain only the cis-acting inverted terminal repeats and packaging signal. As a result, HC-AdVs are generated in *E1*-complementing cell lines in the presence of a first-generation AdV. This so-called helper AdV provides in trans all the viral gene products required for assembling HC-AdV particles. The packaging signal of the helper is flanked by recognition sequences for a site-specific recombinase (e.g. Cre or FLP) that renders its genome unpackageable in recombinase-expressing and *E1*-complementing producer cells. Diagrams were assembled with the aid of SnapGene version 4.3.10. The human adenovirus type-5 source sequence was retrieved from GenBank accession number: AY601635.1.



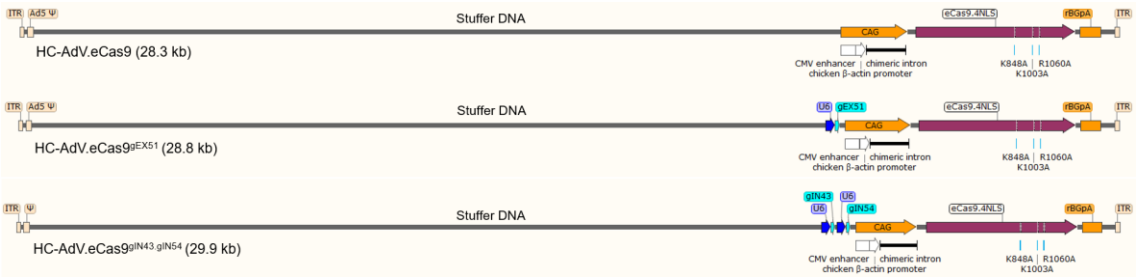
Supplementary Figure S3. Structural analysis of eCas9.4NLS-encoding HC-AdV genomes. (**A**) Schematics of the genome structure of control and *DMD*-targeting HC-AdVs. The high-specificity eCas9.4NLS nuclease is under the transcriptional control of the hybrid CAG promoter and the rabbit *β-globin* polyadenylation signal. Horizontal arrowheads, human *U6* promoters driving expression of optimized gRNAs opt-gRNA^{gEX51}, opt-gRNA^{IN43} and opt-gRNA^{IN54}, marked as orange, red and blue boxes, respectively. HC-AdV.eCas9^{EX51} was designed for inducing *DMD* repair via reading frame resetting or exon 51 skipping; HC-AdV.eCas9$^{IN43.IN54}$ was constructed for triggering *DMD* repair via removal of the major mutational hotspot region. All vector genomes were packaged in adenovirus type-5 capsids displaying CD46-interacting fibers motifs for efficient gene transfer into CAR-negative myogenic cells. ITR and Ψ, cis-acting adenovirus type-5 inverted terminal repeats and packaging signal. The vector genome lengths and titers in genome copies per ml are indicated. (**B**) Assessing the genetic integrity of control and *DMD* editing HC-AdVs. Restriction fragment length analyses was performed by agarose gel electrophoreses of ScaI-treated vector DNA isolated from purified vector particles. Marker, GeneRuler DNA Ladder molecular weight mix (Fermentas). Each of the parental plasmids were also digested with ScaI to serve as additional molecular weight references. *In silico* (SnapGene 4.3.10) and in-gel restriction fragment patterns (left and right panels, respectively).



Supplementary Figure S4. Assessing dystrophin rescue in DMD muscle cells after "all-in-one" HC-AdV transduction of dual RGNs. Immunofluorescence microscopy on muscle cells edited by HC-AdV particles encoding dual RGNs targeting *DMD* introns 43 and 54. Confocal microscopy for dystrophin and β-dystroglycan was performed on myotubes differentiated from DMD.Δ48-50 myoblasts transduced with HC-AdV.eCas9$^{gIN43.gIN54}$ at the indicated MOIs. GC/cell; genome copies per cell.



Expression plasmid co-expressing E2A-ts125, Cre and Puromycin used to generate PEC3.30 packaging cells. Regulatory sequences from the murine cytomegalovirus *immediate-early* and the human *EEF1A1* genes; E2A-ts125, adenovirus type-5 *E2A* open reading frame encoding a thermosensitive DNA-binding protein; RSV, Rous sarcoma virus enhancer/promoter; Cre, bacteriophage P1 *Cre* open reading frame encoding the site-specific Cre recombinase; IRES, internal ribosome entry site of the encephalomyocarditis virus (EMCV); PuroR, *puromycin N-acetyltransferase* selectable marker whose expression confers resistance to puromycin; bovine *GH1* polyadenylation signal; AmpR, *β-lactamase* selectable marker conferring resistance to ampicillin; ori, prokaryotic origin of replication.



Maps of HC-AdV genomes encoding eCas9.4NLS alone or together with optimized *DMD*-targeting gRNAs. The non-coding adenovirus type-5 *cis*-acting elements: inverted terminal repeats (ITR) and packaging signal (Ad5 Ψ), necessary for vector DNA replication and encapsidation, respectively, are indicated. CAG, CMV *immediate-early* enhancer/chicken *β-actin* promoter; eCas9.4NLS, optimized variant of eSpCas9(1.1) endonuclease derived from the type II CRISPR/Cas system from *Streptococcus pyogenes* with the point mutations conferring enhanced specificity annotated; rBGpA, rabbit *β-globin* polyadenylation signal; Stuffer DNA added to increase HC-AdV genomes to sizes over the minimum length required for efficient DNA packaging into AdV capsids (i.e. ~27 kb); U6, RNA polymerase III promoter from the human *U6* gene; gEX51, gIN43 and gIN54, guide RNAs with optimized scaffolds targeting exon 51, intron 43 and

intron 54 of *DMD*, respectively. The top to bottom HC-AdV genomes are cloned in plasmids AW71_pHC-AdV.eCas9, AW72_pHC-AdV.eCas9^{gEX51} and AW70_pHC-AdV.eCas9$^{gIN43.gIN54}$, respectively.

>AW71_pHC-AdV.eCas9 expression unit(6697 bp)

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>AW72_pHC-AdV.eCas9^{gEX51} expression units (7195 bp)

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>AW70_pHC-AdV.eCas9$^{gIN43.gIN54}$ expression units (7608 bp)

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