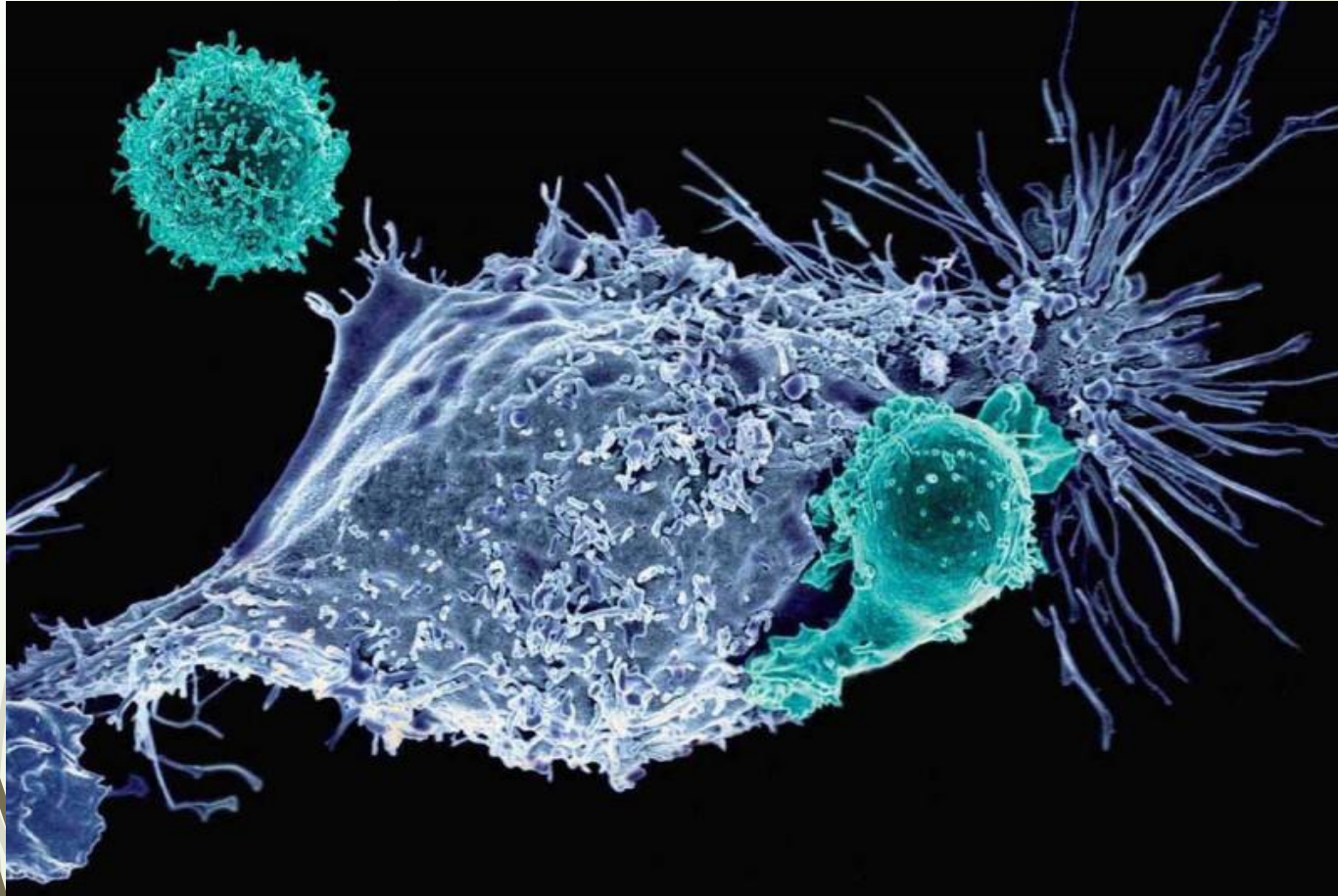


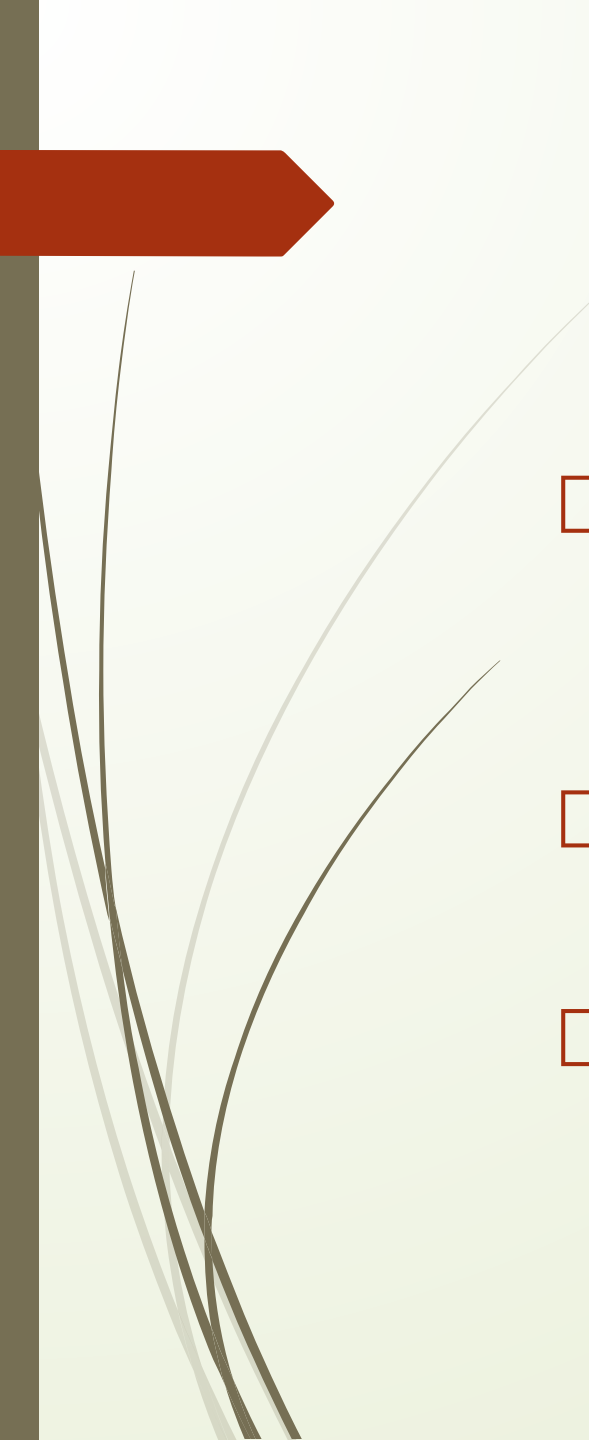


# Crispr Cas9 Genome Editing

# CRISPR Gene-Editing Tool against Tumors



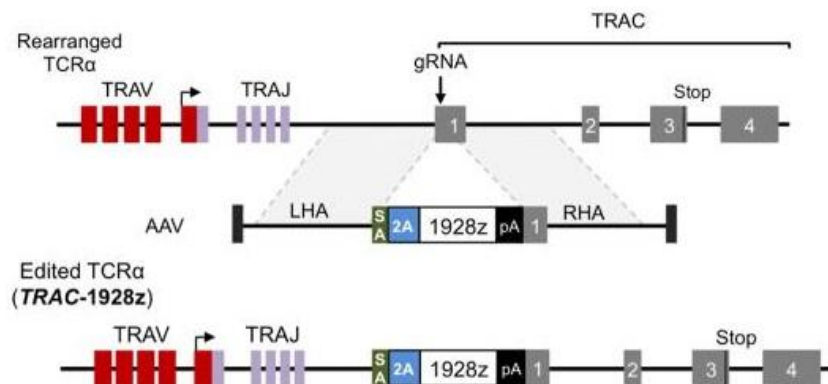
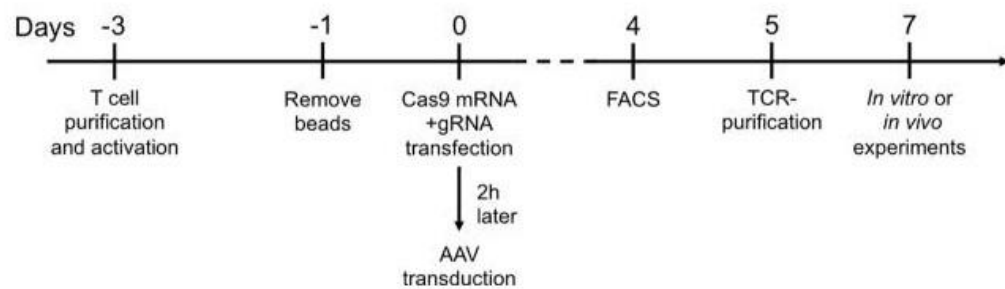
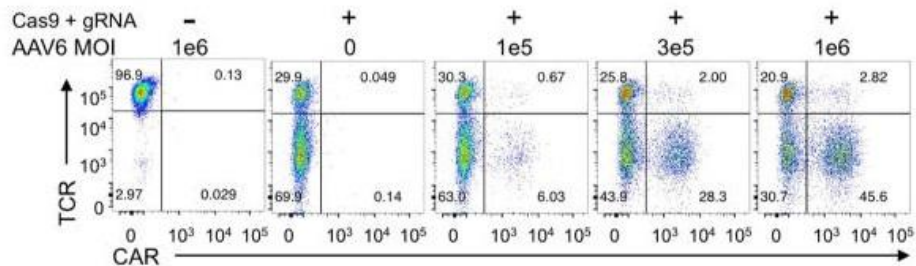
Mesothelin-specific  
CAR T cells  
attacking a  
cancer cell.  
Credit: Prasad  
Adusumilli /  
Memorial Sloan  
Kettering

- 
- The cells modified and express **Chimeric Antigen Receptors (CARs)** on the surface so that they can recognize and attack cancer cells.
  - CAR gene in the *T-cell receptor alpha chain (TRAC)* gene which includes the gene for the T-cell receptor
  - Effective at destroying tumor cells than those in which it was inserted randomly with a retrovirus

**a**

TCCTAACCTGATCCTCTTGTCCACAGATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAG...  
 AGGATTGGGACTAGGAGAACAGGGTGTCTATAGGCTCTGGGACTGGGACGGCACATGGTCGACTCTCTGAGATTTAGGTC...


TRAC exon 1

**b****c**

**a**, *TRAC* locus with the 5' end (grey) of the *TRAC* first exon, the *TRAC* gRNA (blue) and the corresponding PAM sequence (red). The two blue arrows indicate the predicted Cas9 double strand break. Bottom, CRISPR/Cas9-targeted integration into the *TRAC* locus. The targeting construct (AAV) contains a splice acceptor (SA), followed by a P2A coding sequence, the 1928z CAR gene and a polyA sequence, flanked by sequences homologous to the *TRAC* locus (LHA and RHA, left and right homology arm). Once integrated, the endogenous TCR $\alpha$  promoter drives CAR expression, while the *TRAC* locus is disrupted. TRAV, TCR $\alpha$  variable region; TRAJ, TCR $\alpha$  joining region; 2A, the self-cleaving *Porcine teschovirus* 2A sequence. pA: bovine growth hormone polyA sequence.

**b**, Timeline of the CAR targeting into primary T cells.

**c**, Representative TCR/CAR flow plots 4 days after transfection of T cells with Cas9 mRNA and *TRAC* gRNA and addition of AAV6 at the indicated multiplicity of infection.

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- CAR T cells created with CRISPR were less likely to stop recognizing and attacking tumor cells
  - prove safer than random integration
  - need not come from a patient's own T cells
  - easier and cheaper manufacture of CAR T cells.
  - implications for research on diseases other than cancer



## ARTICLE

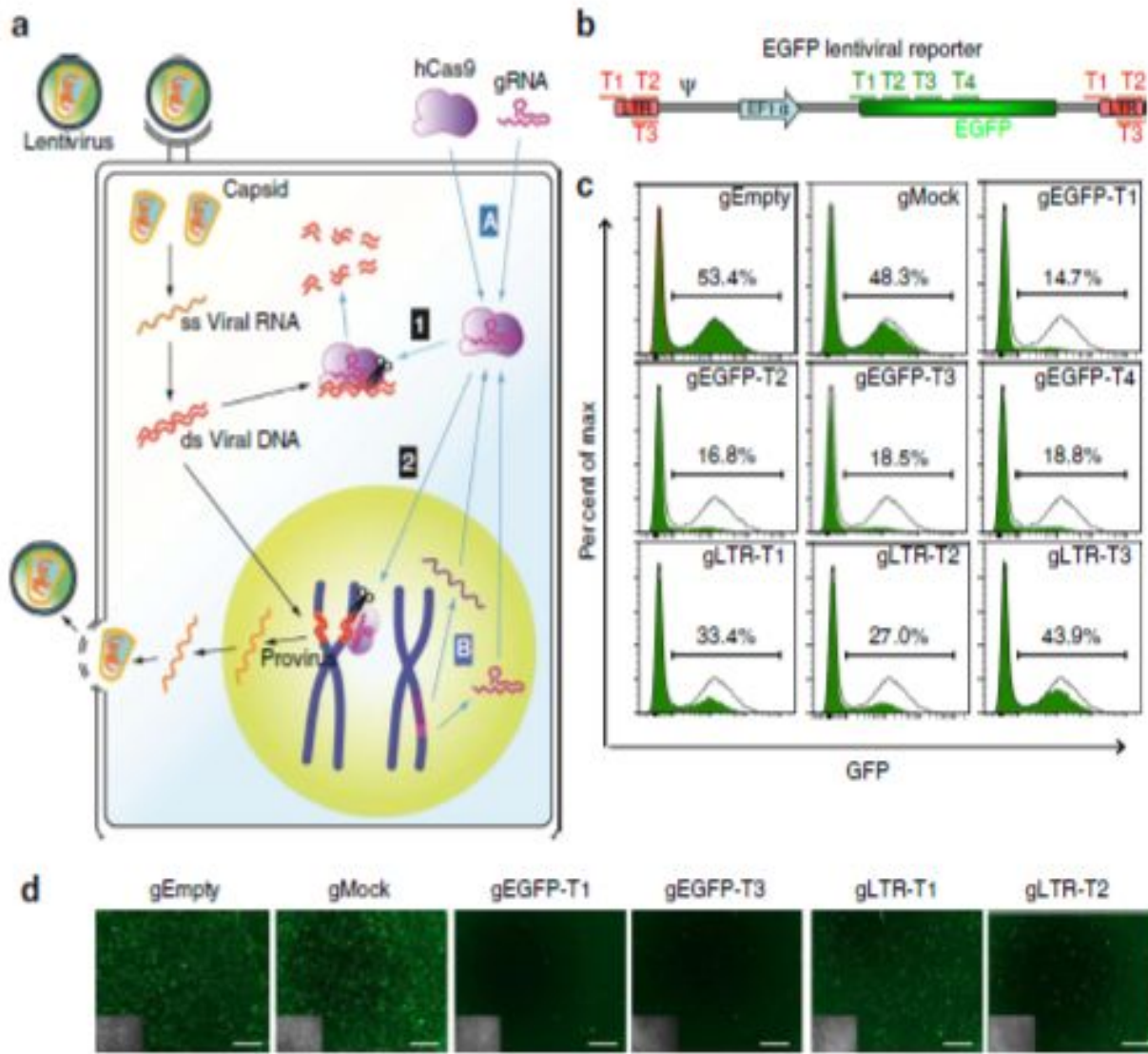
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# Use of the CRISPR/Cas9 system as an intracellular defense against HIV-1 infection in human cells

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& Juan Carlos Izpisua Belmonte<sup>1</sup>

To combat hostile viruses, bacteria and archaea have evolved a unique antiviral defense system composed of clustered regularly interspaced short palindromic repeats (CRISPRs), together with CRISPR-associated genes (Cas). The CRISPR/Cas9 system develops an adaptive immune resistance to foreign plasmids and viruses by creating site-specific DNA double-stranded breaks (DSBs). Here we adapt the CRISPR/Cas9 system to human cells for intracellular defense against foreign DNA and viruses. Using HIV-1 infection as a model, our results demonstrate that the CRISPR/Cas9 system disrupts latently integrated viral genome and provides long-term adaptive defense against new viral infection, expression and replication in human cells. We show that engineered human-induced pluripotent stem cells stably expressing HIV-targeted CRISPR/Cas9 can be efficiently differentiated into HIV reservoir cell types and maintain their resistance to HIV-1 challenge. These results unveil the potential of the CRISPR/Cas9 system as a new therapeutic strategy against viral infections.



**(a)** Model of CRISPR/Cas9 directed intracellular defense against lentiviral infection. hCas9 and its gRNA can be synthesized from either transfected plasmids (module A) or knockin expression cassettes (module B). After synthesis, hCas9 and gRNA assemble and work as an antiviral module in different steps. Once penetrating into the host cells, virally encoded reverse transcriptase uses the lentiviral RNA genome as a template for the synthesis of viral cDNA. Viral integrase binds and processes the LTR and assists in the insertion of the viral cDNA into the host genome. During these processes, the viral genome is unprotected by its envelope and capsid, rendering the viral cDNA vulnerable to cleavage by DNA endonucleases. In our study, the CRISPR/Cas9 system was adapted for human cells as an antiviral module, which bound and disrupted the viral genome of both pre-integration viral DNA (Step 1) and integrated provirus (Step 2).

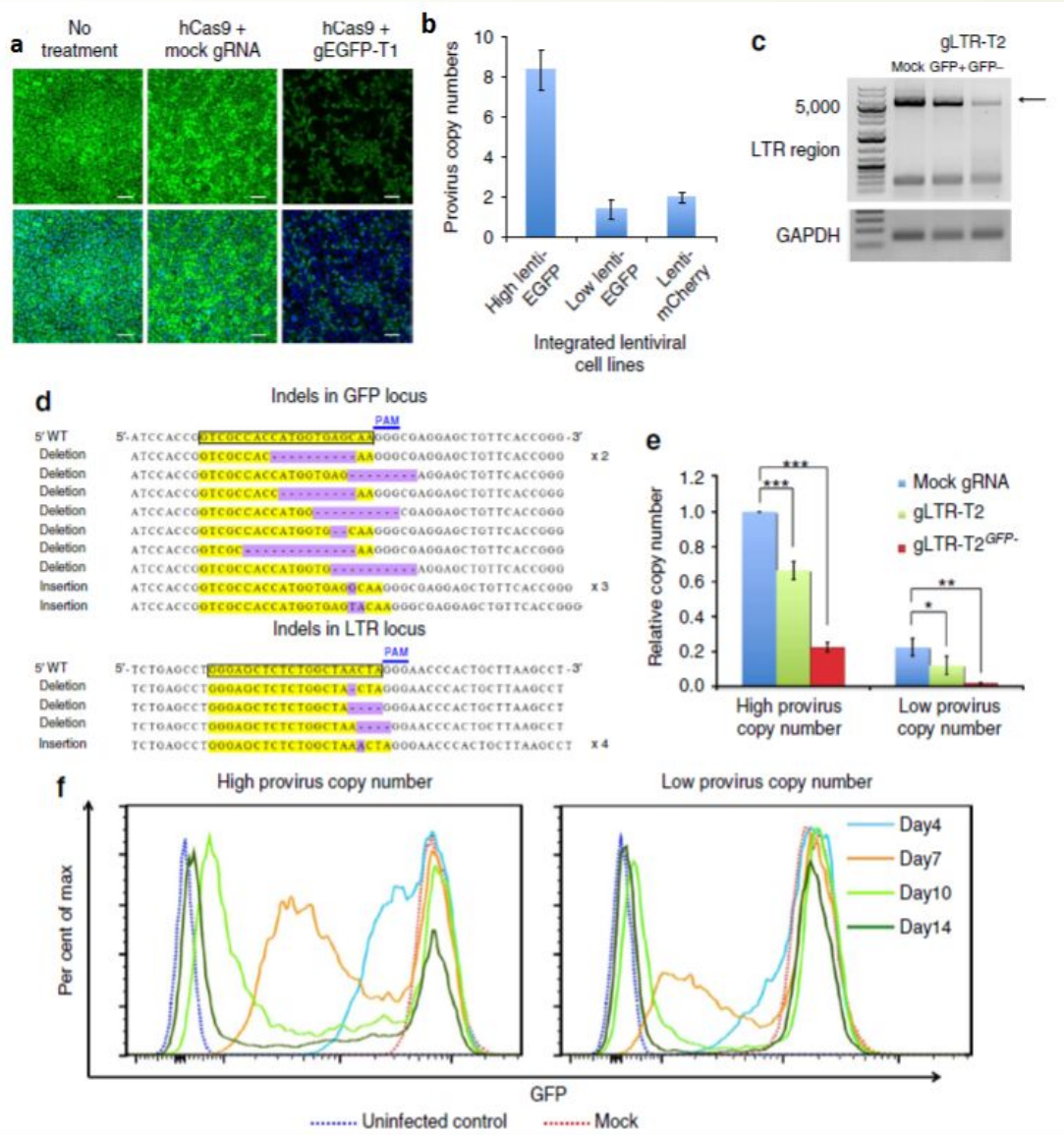
**(b)** Seven gRNAs were designed and used with hCas9 to target corresponding regions in the EGFP reporter lentivirus, including gEGFP-T1 to -T4 and gLTR-T1 to -T3.

**(c)** FACS analysis of HEK293 cells that were pretreated with hCas9-mCh and gRNAs for 20B24 h, and then challenged by EGFP reporter lentiviruses for 4 days. gEmpty is an empty gRNA vector and gMock (as the gS35 in Supplementary Fig. 1) is a non-targeting mock gRNA.

**(d–g)** The CRISPR/Cas9 system can direct targeted disruption of the nonintegrative lentivirus.



**(d)** Fluorescence microscopy images of HEK293 cells pretreated with CRISPR/Cas9 followed by non-integrative lentivirus infection for 3 days. Insets show bright field images. Scale bar, 500 nm.





**Figure 2 | CRISPR/Cas9 directed disruption of integrated lentivirus. CRISPR/Cas9-mediated disruption of integrated lenti-proviral DNA in infected HEK293 cells.** (a) Top: confocal images of cells with high copy numbers of EGFP proviral DNA that were co-transfected with hCas9 and either gEGFP-T1 (right panels) or mock gRNA (middle panels). Left panels show untreated cells. Images were taken at 14 days after transfection. Bottom: confocal images with DAPI staining (blue). Scale bars, 50  $\mu$ m. (b) Determination of provirus copy numbers in three different lentiviral integrated HEK293 cell lines. (c) PCR analysis of proviral DNA. The amount of full-length provirus (arrow) was reduced in the GFP-negative cells generated by CRISPR/Cas9-mediated lentiviral disruption. (d) DNA sequencing analysis of the CRISPR/Cas9 target sites in the EGFP proviruses. Two examples shown here were treated with hCas9 and either gEGFP-T1 (top) or gLTR-T2 (bottom). Sequences identified multiple times were marked accordingly on the right. (e) Proviral copy number quantitation by qPCR. Statistical analysis determined using unpaired t-test (\*\* $P < 0.01$ ; \* $P < 0.1$ ). (f) FACS analysis of proviral EGFP expression at different days after transfection of hCas9 and gRNAs into cells with either high or low provirus copy numbers as determined in b.

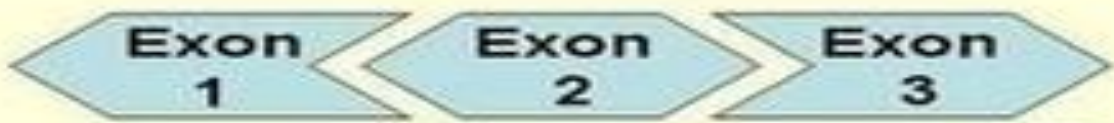


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- In summary, these results indicate that the CRISPR/Cas9 system can mediate targeted disruption of both pre-integration viruses and integrated proviruses with dsDNA in either linear or circular format.

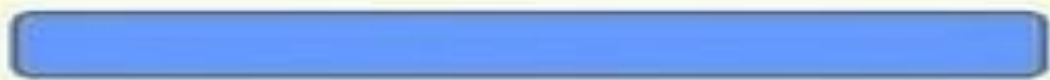
DNA



Antisense oligonucleotide



Myostatin gene



Myostatin protein



No protein produced

***Crispr/Cas9-Based Genome editing for correction of Dystrophin Mutations that cause Duchenne Muscular Dystrophy***

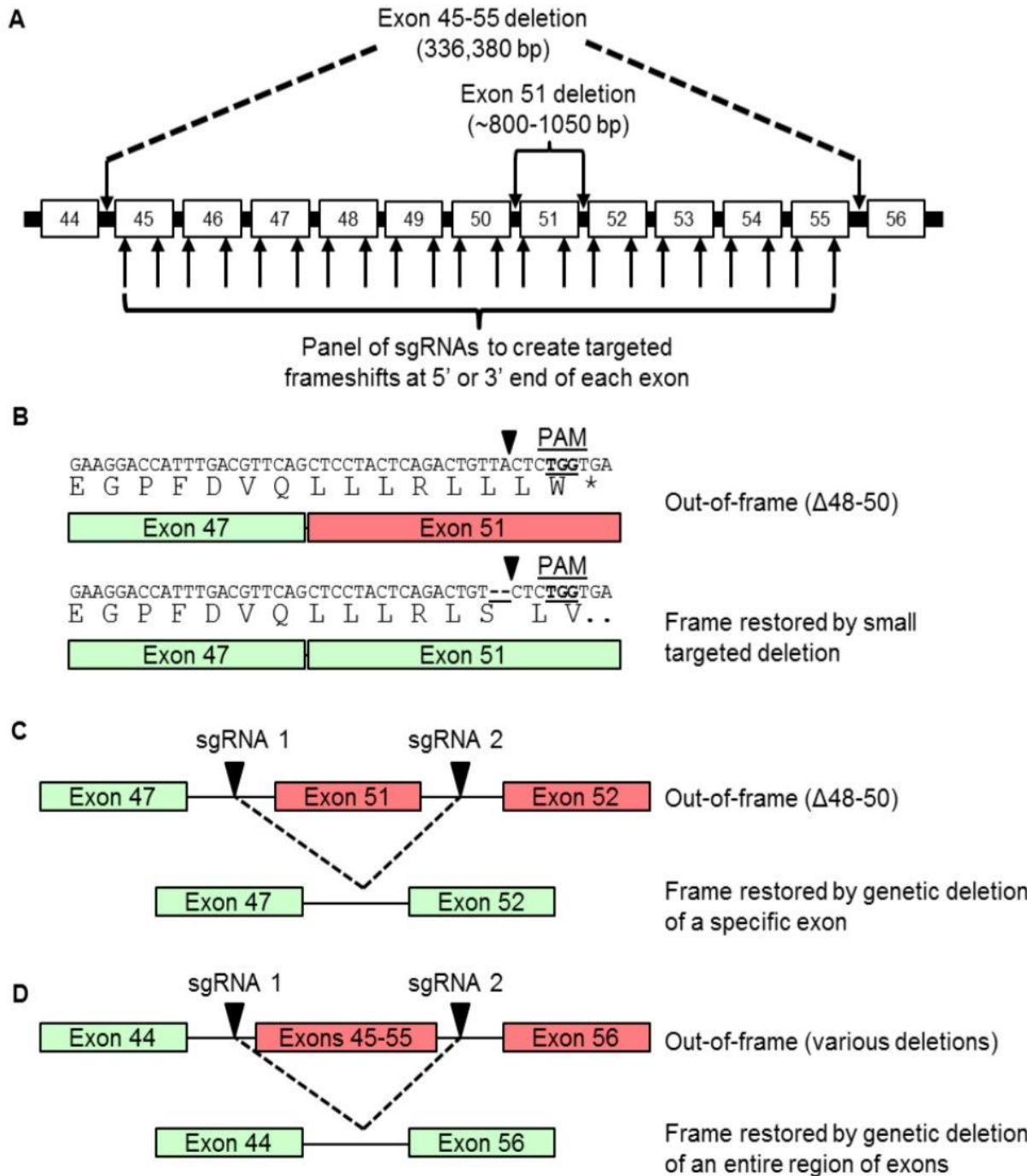
## CRISPR/Cas9 targeting of the dystrophin gene

(A) sgRNA sequences were designed to bind sequences in the exon 45–55 mutational hotspot region of the dystrophin gene, such that gene editing could restore dystrophin expression from a wide variety of patient-specific mutations. Arrows within introns indicate sgRNA targets designed to delete entire exons from the genome. Arrows within exons indicate sgRNA targets designed to create targeted frameshifts in the dystrophin gene.

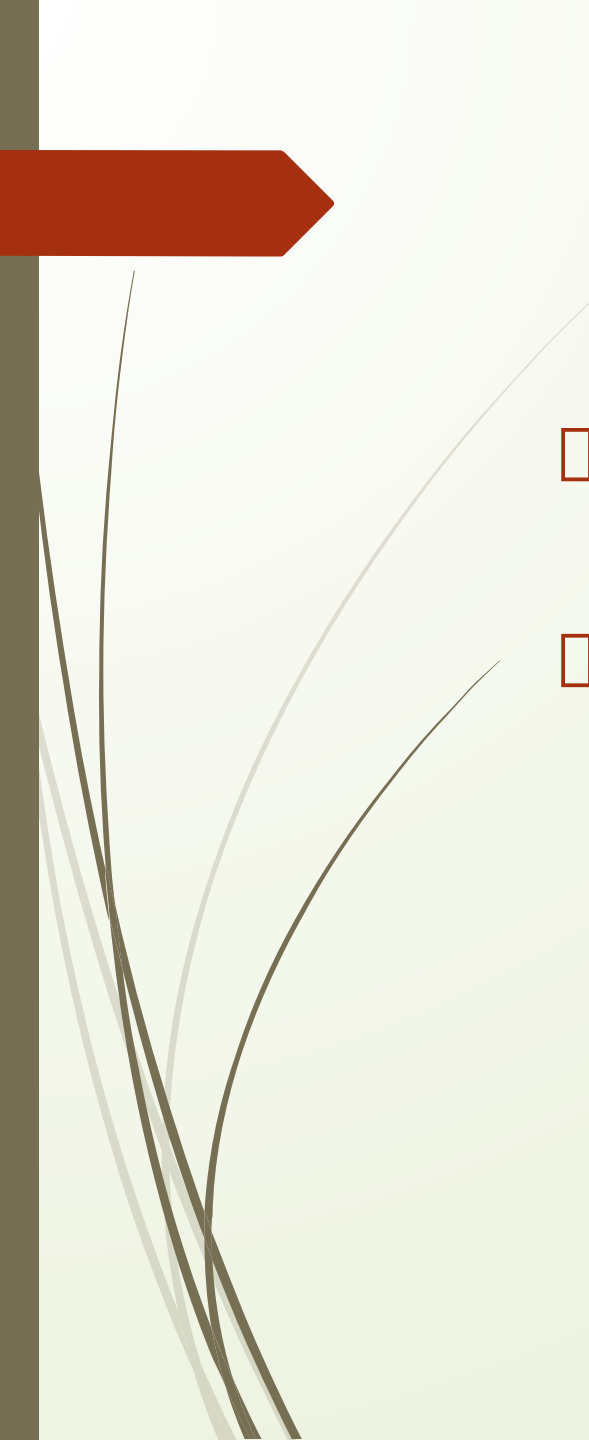
(B) Example of frame correction following introduction of small insertions or deletions by NHEJ DNA repair in exon 51 using the CR3 sgRNA.

(C) Schematic of multiplex sgRNA targets designed to delete exon 51 and restore the dystrophin reading frame in a patient mutation with the deletion of exons 48–50.

(D) Schematic of multiplex sgRNA targets designed to delete the entire exon 45–55 region to address a variety of DMD patient mutations.





- 
- Gene editing capabilities of CRISPR/Cas9 system can correct up to 62% of Duchenne Muscular Dystrophy
  - Collectively, this study provides proof-of-principle that the CRISPR/Cas9 technology is a versatile method for correcting a significant fraction of dystrophin mutations and with continued development may serve as a general platform for treating genetic disease.



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*Thank You* for your patient  
*listening*