# Self-inactivating Cas9: a method for reducing exposure while maintaining efficacy in virally-delivered Cas9 applications

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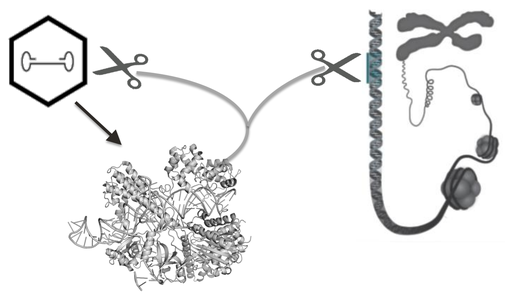
## Summary


- Some genetically defined diseases may be successfully treated by the in vivo delivery of Cas9 and gRNAs.
- AAV is a viable method of delivery to many tissues, and while it is considered to be generally non-integrating, it can persist for extended periods of time, providing sustained expression of its payload.
- It may be preferable to express Cas9 in the cells of interest only until the target locus has been modified at one or both alleles.
- Here we have engineered our AAV vector system such that it contains self-inactivating, universally applicable, tunable modules. These modules include the already-targeted endogenous sequence, obviating the need for any additional gRNAs.
- These modules can be tuned based on position within the viral genome, choice of gRNA, or PAM sequence.
- Our constructs provide for robust Cas9 expression and target locus modification while simultaneously self-targeting the vector encoding SaCas9.
- Both *in vitro* and tissue explant data show promise for this method and demonstrate the feasibility of attaining robust target modification with substantial reduction of exposure through time in a target tissue.


## Figure 1. Self-inactivating design embeds target sites in vector

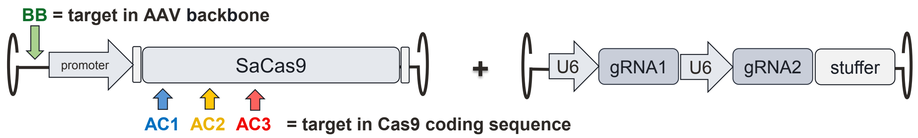


This self-inactivating design contains DNA sequences that are identical or nearly identical to that of the endogenous target locus.

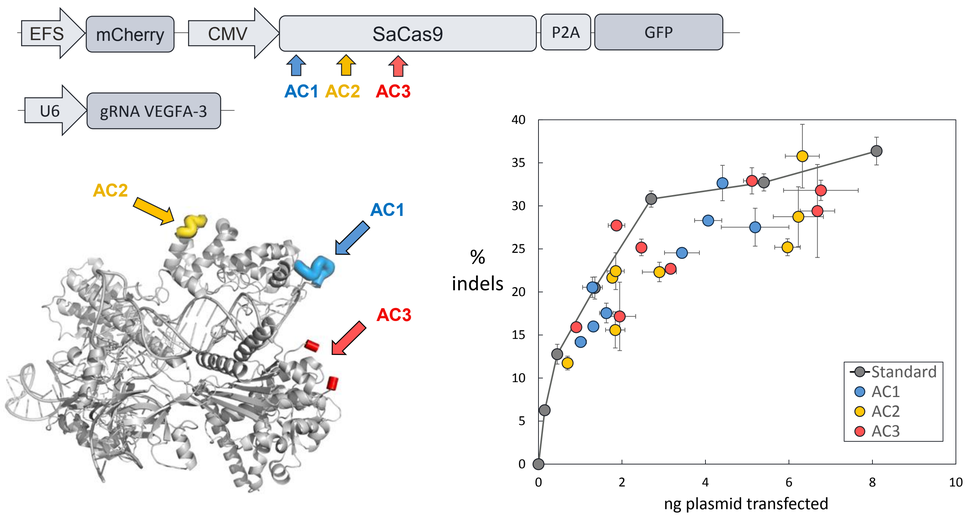
SaCas9-gRNA complexes that form will simultaneously target the endogenous locus and the AAVs that encode SaCas9.

Target sequences in the AAV are variably positioned, at either a site in the viral backbone or one of three regions in the SaCas9 coding sequence, and contain either canonical or suboptimal PAMs.

This self-inactivating design makes use of a 2-vector system in which SaCas9 and gRNAs are encoded on separate viral genomes; singe-vector systems are also in development.

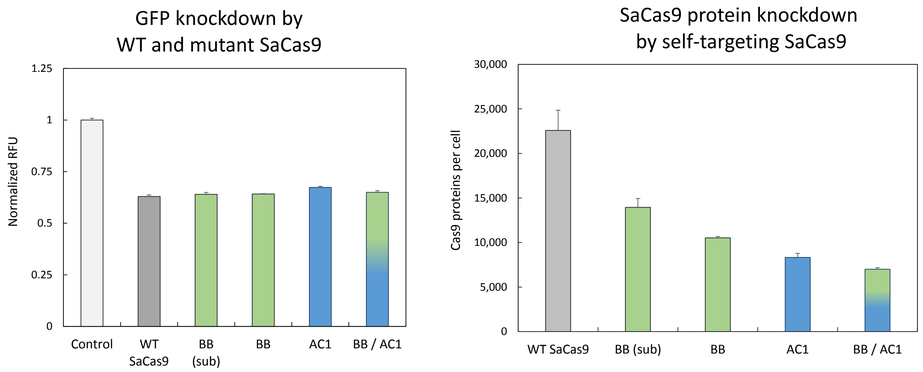



## Figure 2. Target sites in SaCas9 don't disrupt nuclease activity



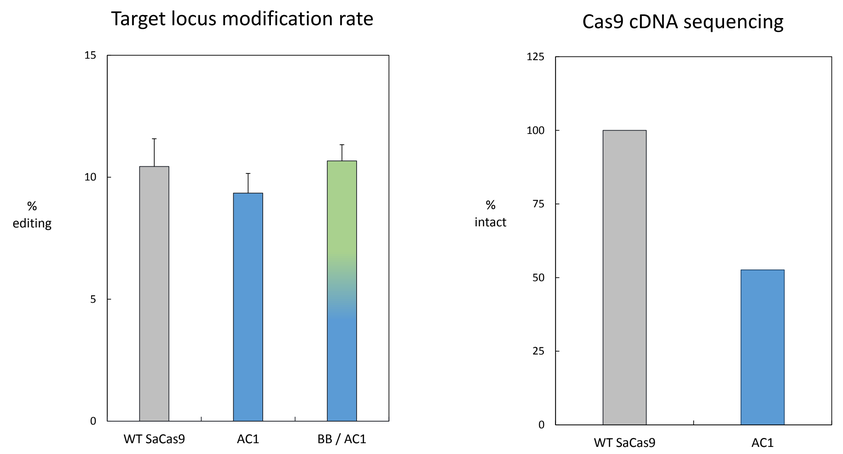

Twelve plasmids were constructed- with four different target sites at three different positions in the SaCas9 coding sequence. These plasmids were each transfected into HEK293 cells along with a gRNA expression plasmid targeting VEGFA site 3. mCherry, expressed off of a separate promoter, was used to normalize transfected amount of plasmid. GFP, expressed from the same transcript as SaCas9, was used to measure potential differences in transcription or translation rates. WT and mutant SaCas9 activity was measured by T7E1 assay.

## Figure 3. Self-inactivating AAVs maintain efficacy at target GFP plasmids while self-inactivating in HEK293s




HEK293 cells were seeded in 24-well plates and transfected with 500ng / well of GFP expression plasmids containing gRNA target sites embedded in the 5' end of the GFP coding sequence. They were transduced the next day with a mixture of gRNA AAV and either wild type or self-targeting SaCas9 AAV (as shown in Figure 1) at a total dose of 200,000 vg/cell. Two days later, cells were analyzed by FACS to determine knockdown of GFP expression (left) and protein was harvested for quantification of SaCas9 by alphaLISA (right).

## Figure 4. Self-inactivating AAVs maintain efficacy at target locus while self-inactivating in retinal explants




Retinal explants were extracted from BL6 mice and cultured in 24-well plates. They were transduced with a mixture of gRNA AAV and either wild type or self-targeting SaCas9 AAV (as shown in Figs. 1 and 3) at a total dose of 1E11 vg/retina. At Day 14 post extraction, DNA and RNA were harvested from explants. Endogenous target locus was amplified from extracted DNA by PCR and sequenced (left). cDNA was generated from extracted RNA, and SaCas9 sequence was amplified by PCR and sequenced.