Engineered ultracompact epigenetic editors for DNA and histone modifications enable durable epigenetic gene activation and suppression

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ABSTRACT

The rapid advancement in the discovery and characterization of epigenetic editors is paving the way for innovative therapeutic approaches to human genetic disorders. These developments are highlighted by the significant and durable modulation of multiple therapeutically relevant gene expression, both *in vitro* and *in vivo*, underscoring the potential of epigenetic editing. Despite these advances, two major challenges limit the full realization of this potential: (1) the large size of current epigenetic editors for targeted DNA methylation and demethylation, which hampers effective tissue-specific delivery *in vivo*, and (2) the inconsistent modulation of gene expression by DNA methyltransferases and demethylases across different chromatin states and gene expression levels.

To address these challenges, we have engineered ultracompact yet highly effective DNA epigenetic modulator domains, along with compact and efficient and compact epigenetic gene activators. When combined with compact dCas proteins (<500aa), this new toolkit provides a versatile and potent platform for gene expression modulation. These novel efficacious engineered epigenetic suppressor tools are nearly 50% smaller than CRISPRoff, and 33% smaller than the conventional functional domains used for DNA demethylation. This results in a set of tools that can be packaged together with guide RNAs and tissue-specific promoters for delivery via AAVs for in vivo epigenome editing.

We demonstrate that these compact domains are functional in human cells and at an endogenous human gene, thereby showing substantial potential for use in future epigenetic editing therapeutic payloads.

GEMS

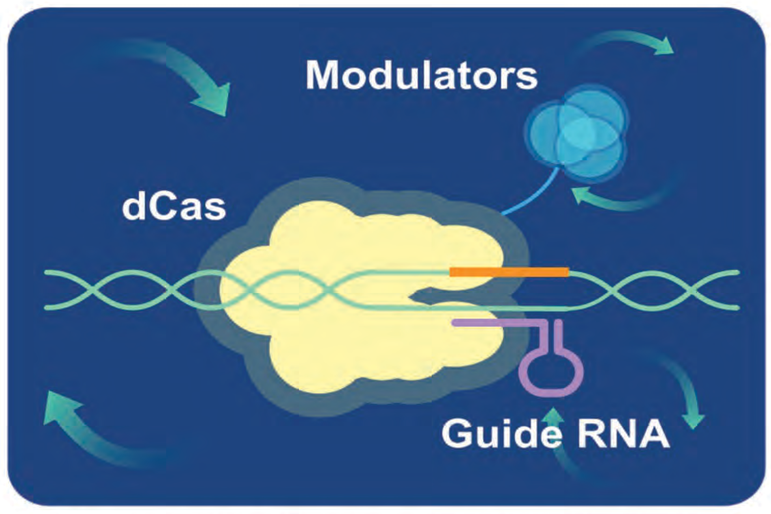


Figure 1. Epicrispr Bio's Gene Expression Modulation System (GEMS) A modular system comprising highly compact "nuclease dead" **Cas proteins**, transcriptional and epigenetic **Modulators,** and engineered compact guide RNAs. GEMS fits within the packaging limits of rAAVs (~4.7kb) leaving extra cargo space. GEMS can therefore be delivered to target organs *in vivo* for therapeutic applications.

Compact Cas Proteins

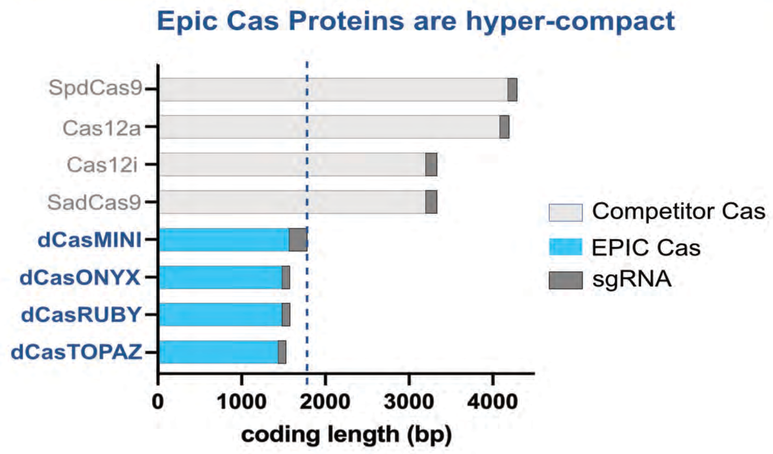


Figure 2. Size comparison of Epicrispr Biotechnologies' Cas proteins to benchmark Cas molecules. CasONYX, CasRUBY, and CasTOPAZ all fall under 1500 nt in length (less than 500 amino acids). Together with CasMINI, they constitute a suite of compact and active proteins suitable for *in vivo* gene therapeutic applications which can be delivered by rAAVs and other delivery modalities.

Compact Epigenetic Suppressors

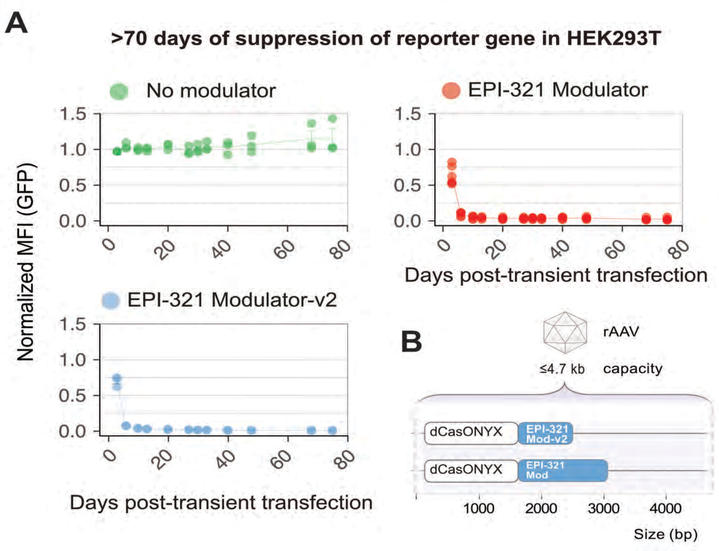


Figure 3. A. We have previously reported the development of an AAV-deliverable GEMS product, called EPI-321, for the epigenetic suppression of the DUX4 gene in the proposed treatment of Facioscapulohumeral muscular dystrophy (FSHD). Here we present data showing the successful development of a more compact variant of the suppressor used in EPI-321. When transiently delivered to HEK293T cells EPI-321 Modulator V2 is capable of epigenetically suppressing reporter GFP expression for greater than 70 days post-transient delivery (~140 cell divisions). **B.** Schematic showing the compactness of EPI-321 Modulator V2, representing a near 50% reduction in size. This compactness is achieved through rational protein engineering, without loss of gene suppressive activity.

Epigenetic Modulator for gene reactivation

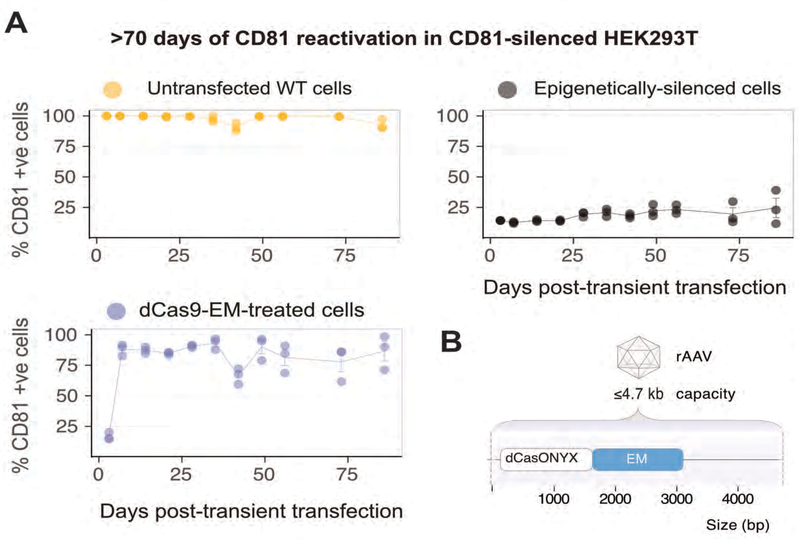
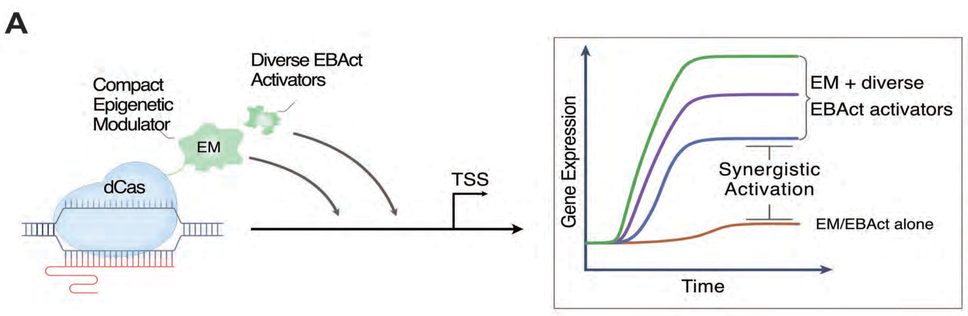


Figure 4. A. The ability to reactivate epigenetically silent loci is a critical to fulfilling the therapeutic potential of programmable epigenetic gene regulation. We have used rational protein engineering to generate a novel epigenetic modulator for gene reactivation (EM). We show that upon transient delivery to HEK293T cells in which the CD81 gene has been previously silenced, a dCas9-EM construct is able to reactivate CD81 to near 100%. This activation is sustained through >75 days (~150 cell divisions). **B.** Schematic showing the compactness of EM in a conceptualized fusion to dCasONYX. This highlights the potential deliverability of this epigenetic modulator for gene activation via AAV, and for therapeutic reactivation of human genes.

Synergistic Epigenetic Gene Activation



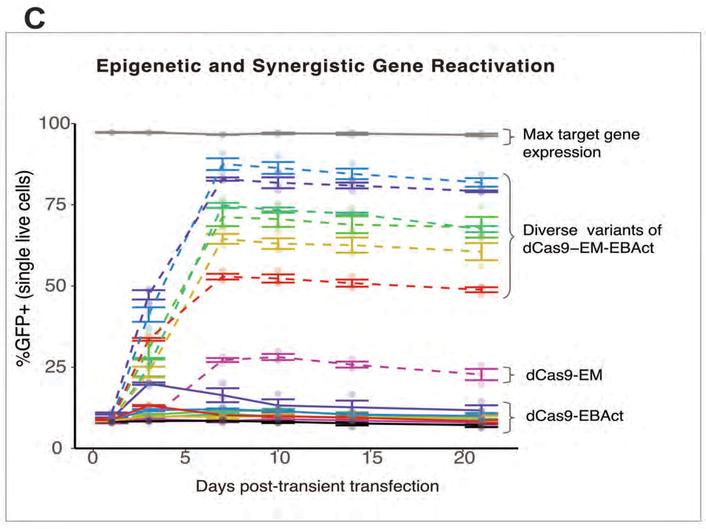


Figure 5. A. Schematic representation of the recruitment of a dCas fused to Epicrispr Bio's Epigenetic Modulators (dCas-EM) and one of various Epicrispr Bio Activators (EBAct, previously presented). EBActs are capable of activating endogenous human genes to different levels, and in different chromatin contexts. A subset of these activators can induce durable, mitotically stable target gene activation. To the right is an idealized experimental outcome of successful synergistic interaction between dCas-EM and EBAct in epigenetically reactivating a silenced target gene. **B.** A synthetic reporter was designed and genetically encoded within HEK293T cells to allow the study of gene reactivation potential of the Synergistic Epigenetic Gene Activation system. A dCas binding sequence was cloned upstream of the EF1α promoter. Vertical lines indicate individual CpG residues contained within the EF1α promoter fragment, numbering 96. This dCas binding site and promoter arrangement were in turn cloned upstream of the GFP fluorescent reporter gene and stably integrated into HEK293T cells. This reporter was first silenced epigenetically via CpG methylation in the reporter cell line. **C.** We introduced multiple dCas9-EM-EBAct variants into HEK293T cells into which the EF1α-GFP reporter had been demonstrably silenced. Shown in this panel are the resultant levels of GFP fluorescence detected up to 28 days post transient delivery by transfection of the indicated constructs. Of note, we observed discrete and significant synergistic activation for all combinatorial constructs as compared to dCas9-EM or dcas9-EBAct variants alone.

Conclusions

At Epicrispr Bio we have focused on the generation of reagents and tools to enable the precise and programmable regulation of target genes. Previously, we have been able to demonstrate therapeutically-relevant target gene suppression (e.g by targeting DUX4 in FSHD). Here, we have focussed on enhancing the tools we have previously discovered, in part by making them more compact and deliverable, but also by rationally combining different activities to elicit synergistic activities. In so doing we have refined our epigenetic editing tools to increase their practical use in genetic medicine. By more completely leveraging epigenetic editing we can now durably suppress, activate and reactivate human genes. This opens up the possibilities for therapeutic applications *ex vivo* and *in vivo*.

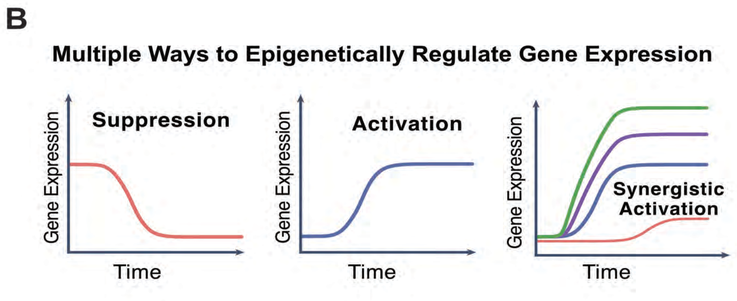


Figure 6. A. Epicrispr Bio GEMS are designed to be both efficacious and compact enough for therapeutic delivery by any single vector for *in vitro* and *in vivo* applications, schematically shown here are AAV, lentivirus and LNP. **B.** Modulator discovery and rational protein engineering approaches have allowed us to build a comprehensive set of epigenetic editing tools capable of target gene suppression, activation and synergistic gene activation.

References

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