Compact DNA Demethylase-Activator combination Modulators for CRISPR-Mediated Epigenetic Gene Activation

X Yang [1] C Klappenbach [1] R Yeo [1] T Borman[1] MZaki Jawaid [1] D Hart [1]

1 Epicrispr Biotechnologies, South San Francisco, CA 94080, USA

Abstract

Epigenetic silencing of gene expression is a well-characterized mechanism that has been successfully harnessed for therapeutic applications, particularly in CRISPR-based approaches. In contrast, the development of epigenetic tools for robust and sustained gene activation has lagged, representing a significant barrier to advancing transformative therapies for human disease. While DNA demethylation, mediated by enzymes such as TET family members, has shown promise in de-repressing silenced genes, two major challenges remain: (1) the large size of current DNA demethylation editors, which precludes their delivery via adeno-associated viruses (AAVs), and (2) the variability in gene activation across distinct chromatin states and transcriptional contexts. To address these limitations, we developed a new class of compact, AAV-deliverable DNA demethylating enzymes, termed "miniTETs," which are 33% smaller than conventional catalytic domains. These miniTETs (epigenetic modulators, EMs) can be co-packaged with guide RNAs and tissue-specific promoters into a single AAV vector, facilitating efficient *in vivo* delivery. We demonstrate that dCas9-miniTET variants are capable of stable reactivation of silenced loci in vitro, with sustained target gene expression observed for up to 100 days in HEK293T cells following transient transfection. Additionally, we engineered an ultra-compact dCas variant, dCasONYX (<500 amino acids), and showed that a fusion to miniTET EM domain is highly active in human cells, and capable of epigenetically reactivating a silenced locus. Remarkably, dCas-miniTET fusions with transcriptional activators yielded synergistic reactivation of a silenced synthetic reporter gene, with stable expression maintained for 30 days post-transfection. These findings provide a foundation for the next generation of CRISPR-based tools for epigenetic gene activation, tailored for therapeutic applications. Our work establishes a robust platform for precise, durable, and tissue-specific control of gene expression in vivo.

Platform

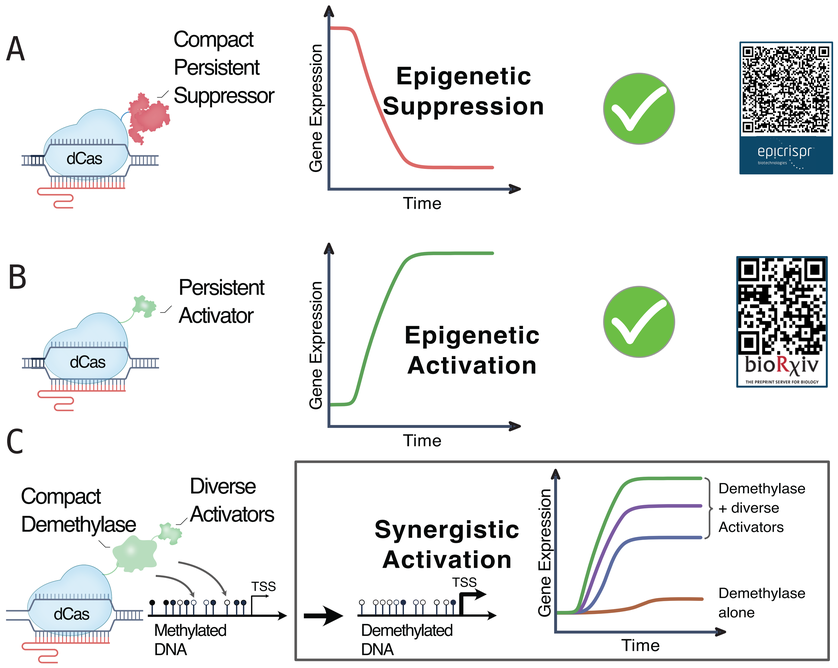

Figure 1. The ideal epigenetic editing platform is capable of inducing targeted, specific and durable changes in gene expression. **A** Epicrispr Bio has developed compact dCasONYX-based epigenetic suppressors for the targeted modulation of gene expression. EPI-321, a potential cure for FSHD, is an example of this. **B** We have previously reported on the epigenetic activation of numerous synthetic and endogenous human target genes using a compact Persistent Activator. **C** An idealized compact DNA Demethylase, or DNA Demethylase-Activator fusion is shown in the schematic. To the right is a representation of a demethylated locus (closed circles represent methylated CpG dinucleotides and open circles represent unmethylated CpG dinucleotides). Idealized results of DNA demethylation and synergistic gene activation are presented.

Compact, potent DNA Demethylase

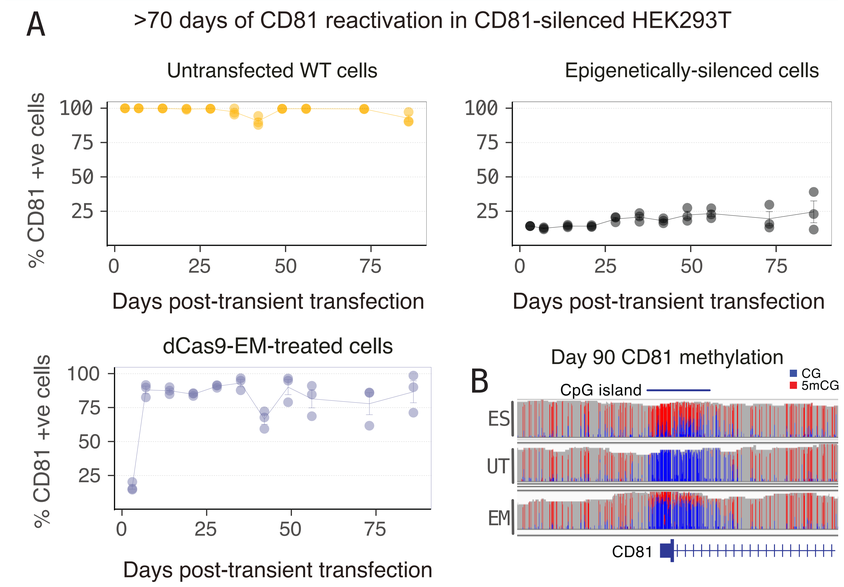

Figure 2. A. The ability to reactivate epigenetically silent loci is a critical to fulfilling the therapeutic potential of programmable epigenetic gene regulation. We have used rational protein engineering to generate a novel miniTET epigenetic modulator for gene reactivation (EM). We show that upon 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.** Long-read sequencing analysis of CpG methylation status upstream of CD81. Data represent a single time-point of analysis 90 days post-transient transfection of the indicated constructs. Epigenetically silenced cells (ES) show robust targeted CpG methylation that is reversed by the engineered compact epigenetic modulator EM. Untransfected cell were assessed for comparison (UT).

Synergistic Gene Activation Assay

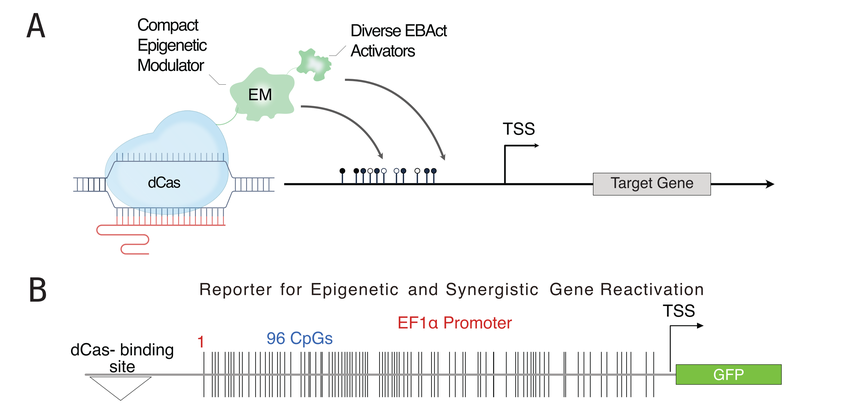

Figure 3. A. Schematic representation of the recruitment of a dCas fused to Epicrispr Bio's Epigenetic Modulators (dCas-EM) and one of various Epicrispr Bio Activators (EBAct, previously presented). EBActs are capable of activating endogenous human genes to different levels, and in different chromatin contexts. A subset of these activators can induce durable, mitotically stable target gene activation. **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 in total. 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.

Synergistic Gene Activation with diverse Demethylase-Activator fusions

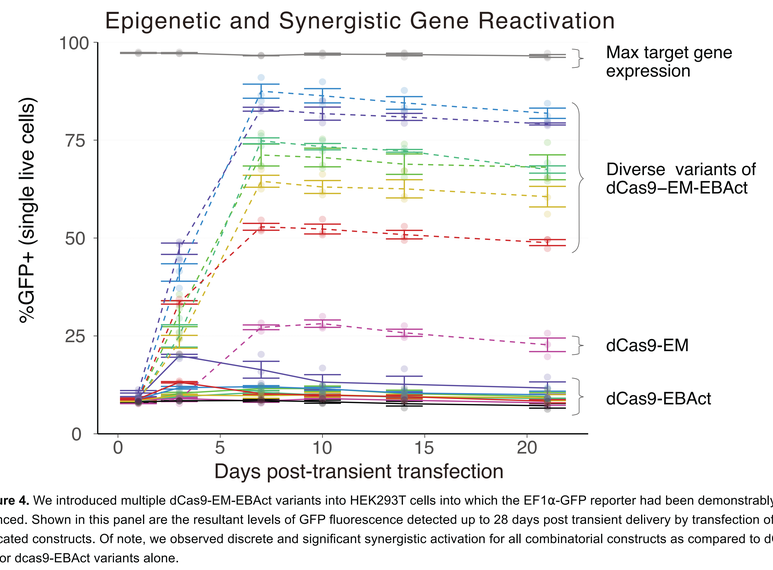

Figure 4. We introduced multiple dCas9-EM-EBAct variants into HEK293T cells into which the EF1α-GFP reporter had been demonstrably silenced. Shown in this panel are the resultant levels of GFP fluorescence detected up to 28 days post transient delivery by transfection of the indicated constructs. Of note, we observed discrete and significant synergistic activation for all combinatorial constructs as compared to dCas9-EM or dcas9-EBAct variants alone.

An AAV-deliverable Demethylase-Activator

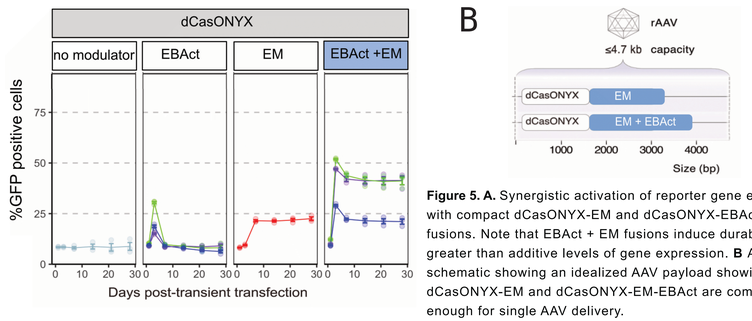

Figure 5. A. Synergistic activation of reporter gene expression with compact dCasONYX-EM and dCasONYX-EBAct + EM fusions. Note that EBAct + EM fusions induce durable and greater than additive levels of gene expression. **B** A schematic showing an idealized AAV payload showing that dCasONYX-EM and dCasONYX-EM-EBAct are compact enough for single AAV delivery.

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*.

Contact and References

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Poster contact: Dan Hart (dan.hart@epic-bio.com); **Business inquiry:** Benson Cheng (benson.cheng@epic-bio.com); **Media Contact:** Kimberly Ha (kimberly.ha@epic-bio.com)