Breaking Size and Silencing Barriers: A Compact CRISPR Epigenetic Activator for Durable AAV-Compatible Gene Activation

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ABSTRACT

Introduction: While traditional CRISPR-Cas systems continue to show significant promise in addressing multiple disease modalities, especially those amenable to permanent sequence correction, they face important limitations in treating diseases rooted in epigenetic dysregulation. Current approaches in DNA demethylation, such as those that utilize TET-based family members, are too large to fit into AAV delivery vehicles. Furthermore, activation efficacy is highly dependent on local chromatin accessibility and DNA methylation status, making consistent activation across diverse genomic contexts difficult with existing epigenetic editors.

Methods: Building on our prior work, we have now engineered a next-generation class of epigenetic modulators (EMs) that are substantially smaller than previously reported miniTETs, now achieving up to ~40% reduction in size relative to canonical TET catalytic domains while improving demethylase activity. These compact EMs are compatible with single-vector AAV packaging alongside guide RNAs, tissue-specific promoters, and optional transcriptional activation domains, enabling efficient in vivo delivery and supporting sustained epigenetic remodeling across variable chromatin contexts.

Results: When EMs are paired with our clinically-derisked and ultra-compact dCas variant (dCasONYX, <500 aa), we demonstrate sustained and robust endogenous gene activation for several genes of therapeutic and research interest. These data demonstrate successful activation across a panel of 11 human target genes, each with significant baseline methylation (>50%) within promoter-proximal regions. Additionally, when EMs are paired with transcriptional activators, these fusions yielded durable synergistic activation, with stable expression maintained significantly post transient transfection.

Conclusion: Together, these findings lay the groundwork for next-generation CRISPR-based epigenetic gene activation technologies by demonstrating broad efficacy at therapeutically relevant genes in their native chromatin context. Thereby, furthering the versatility of our GEMS platform (Gene Expression Modulation System) for precise, durable, and tissue-specific regulation of gene expression in-vivo.

GEMS: Gene Expression Modulation System

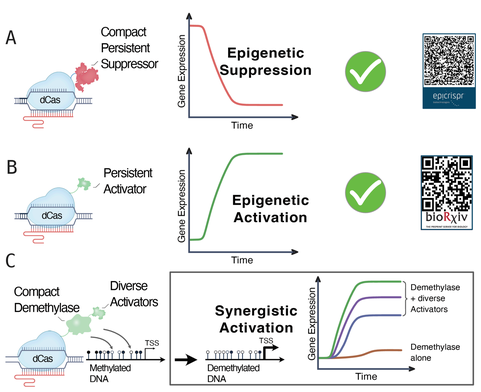

Figure 1: The ideal epigenetic editing platform is capable of inducing targeted, specific and durable changes in gene expression.

A, Epicrispr Bio has developed compact dCasONYX-based epigenetic suppressors for the targeted modulation of gene expression. **EPI-321 [2]**, a potential cure for FSHD currently being evaluated in a Phase 1/2 trial, 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. QR Code redirects to bioRxiv preprint [3].

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.

Robust and Durable Endogenous Gene Activation

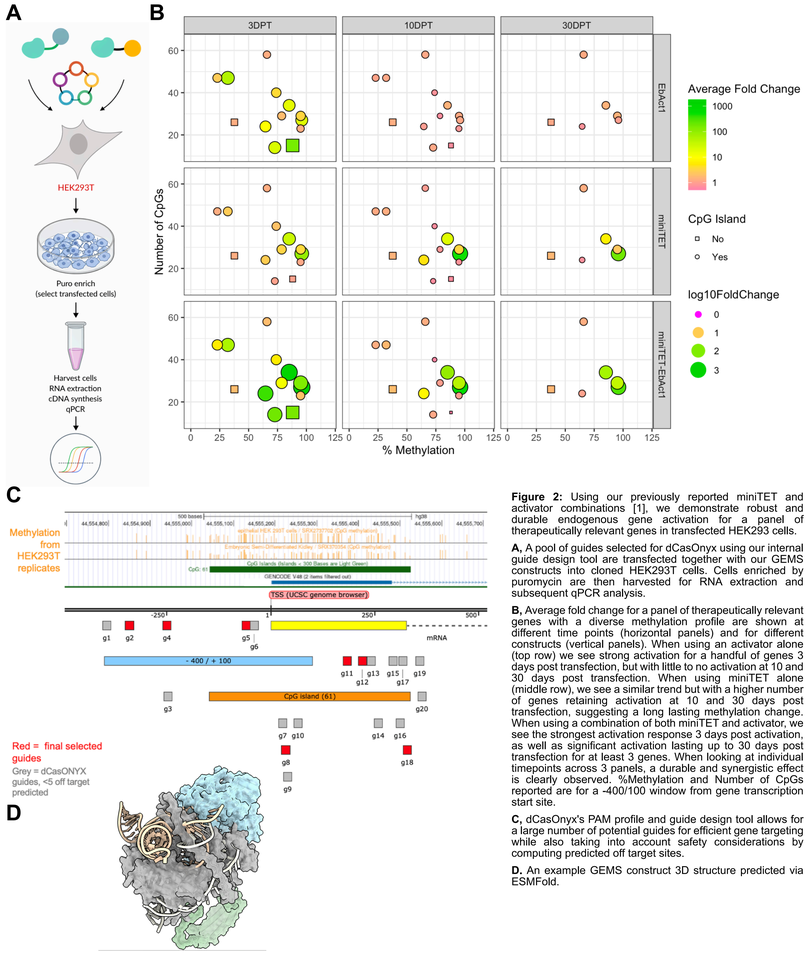

Figure 2: Using our previously reported miniTET and activator combinations [1], we demonstrate robust and durable endogenous gene activation for a panel of therapeutically relevant genes in transfected HEK293 cells.

A, A pool of guides selected for dCasOnyx using our internal guide design tool are transfected together with our GEMS constructs into cloned HEK293T cells. Cells enriched by puromycin are then harvested for RNA extraction and subsequent qPCR analysis.

B, Average fold change for a panel of therapeutically relevant genes with a diverse methylation profile are shown at different time points (horizontal panels) and for different constructs (vertical panels). When using an activator alone (top row) we see strong activation for a handful of genes 3 days post transfection, but with little to no activation at 10 and 30 days post transfection. When using miniTET alone (middle row), we see a similar trend but with a higher number of genes retaining activation at 10 and 30 days post transfection, suggesting a long lasting methylation change. When using a combination of both miniTET and activator, we see the strongest activation response 3 days post activation, as well as significant activation lasting up to 30 days post transfection for at least 3 genes. When looking at individual timepoints across 3 panels, a durable and synergistic effect is clearly observed. %Methylation and Number of CpGs reported are for a -400/100 window from gene transcription start site.

C, dCasOnyx's PAM profile and guide design tool allows for a large number of potential guides for efficient gene targeting while also taking into account safety considerations by computing predicted off target sites.

D. An example GEMS construct 3D structure predicted via ESMFold.

Engineering the Next Generation Demethylase Activator Combinations

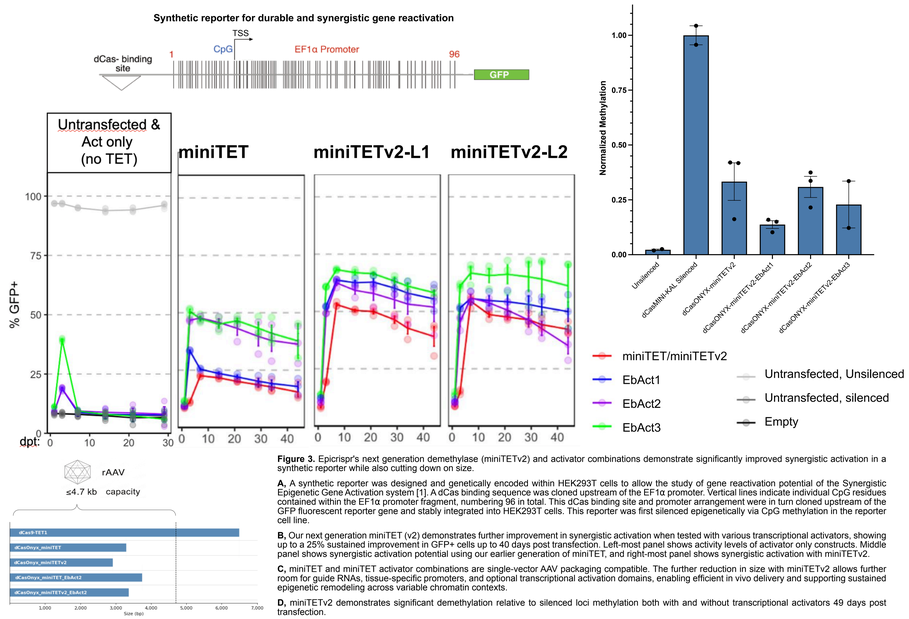

Figure 3. Epicrispr's next generation demethylase (miniTETv2) and activator combinations demonstrate significantly improved synergistic activation in a synthetic reporter while also cutting down on size.

A, 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 [1]. 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.

B, Our next generation miniTET (v2) demonstrates further improvement in synergistic activation when tested with various transcriptional activators, showing up to a 25% sustained improvement in GFP+ cells up to 40 days post transfection. Left-most panel shows activity levels of activator only constructs. Middle panel shows synergistic activation potential using our earlier generation of miniTET, and right-most panel shows synergistic activation with miniTETv2.

C, miniTET and miniTET activator combinations are single-vector AAV packaging compatible. The further reduction in size with miniTETv2 allows further room for guide RNAs, tissue-specific promoters, and optional transcriptional activation domains, enabling efficient in vivo delivery and supporting sustained epigenetic remodeling across variable chromatin contexts.

D, miniTETv2 demonstrates significant demethylation relative to silenced loci methylation both with and without transcriptional activators 49 days post transfection.

Presentations of Interest, References, and Contact

Please visit our oral presentation: **Epigenetic Editing Restores Frataxin Expression to Physiological Levels in Friedreich's Ataxia Cells** | Kaitlin Pensabene & Jackson Powell | Date/Time: May 14 | 4:45 PM

As well as our other poster presentations:

1. **APPRAISR: A Model for Predicting Guide RNA Efficacy for a Hypercompact CRISPR-Cas Epigenetic Editor Using Machine Learning**| Presenter: Tyler Borrman | Abstract #: 2360 | Date/Time: May 12 | 5:00–6:30 PM
2. **Rescuing Lost Titer: Identifying and Eliminating Transfection-Driven Failure Modes at Scale** | Presenter: Surabhi Godbole | Abstract #: 2149 | Date/Time: May 13 | 5:00–6:30 PM
3. **Epigenetic Editing Activates SORLA (Sortilin-Related Receptor with A-Type Repeats) to Reduce Soluble Beta Amyloid (Aβ) and Tau in Sporadic Alzheimer's Disease Patient Neurons |** Presenter: Melanie Silvis | Abstract #: 3318 | Date/Time: May 14 | 5:00–6:30 PM
4. **EPI-331: A CRISPR-Based Epigenome Editing as a Mutation-Agnostic Treatment of Duchenne Muscular Dystrophy (DMD)** | Presenter: Abhinav Adhikari, Ph.D. | Abstract #: 3313 | Date/Time: May 14 | 5:00–6:30 PM

Investor Contact: Benson Cheng (benson.cheng@epic-bio.com)
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1. Daniel Hart *et al*, Compact DNA Demethylase-Activator combination Modulators for CRISPR-Mediated Epigenetic Gene Activation. ASGCT 2025.
2. A Adhikari *et al*, EPI-321: a potential cure for Facioscapulohumeral Muscular Dystrophy (FSHD) targeting D4Z4 epigenome. ESGCT 2023.
3. G Carrosso *et al*, Discovery of hypercompact epigenetic modulators for persistent crispr mediated gene activation. BioRxiv 2023.
4. S R McCutcheon *et al*, Epigenome editing technologies for discovery and medicine. Nature Biotechnology 2024.
5. JK Nuñez *et al*, Genome-wide programmable transcriptional memory by CRISPR-based epigenome editing. Cell 2021.