Engineered Compact Epigenetic Editors Enable Synergistic and Durable Gene Activation and Suppression

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ABSTRACT

CRISPR-mediated epigenetic editing is a promising new technology with the potential to greatly enhance the growing arsenal of gene therapy modalities. However, a number of challenges remain to be overcome: (1) epigenetic editors are typically large, creating hurdles for efficient delivery for targeted epigenome engineering in vivo, (2) the epigenome targeting range is limited by the PAM recognition specificity of the CRIPSR mechanism, and (3) the risk of inconsistent modulation of gene expression across different chromatin states by a single editing mechanism such as DNA or histone modification alone.

To address Challenges (1) and (2), we have engineered compact yet highly effective DNA methylation modulators, DNA demethylases, ultracompact transcriptional modulators as well as compact deactivated Cas effectors with altered PAM recognition. We demonstrate that these compact protein domains are functional in human cells and at endogenous human genes. In addition, when combining these domains as CRISPR-mediated epigenetic editors, the size of these compact epigenetic activators or suppressors enables single AAV packaging for in vivo epigenome engineering.

To address Challenge (3), we took advantage of the synergistic relationship of transcriptional regulation in eukaryotes, where the transcriptional output driven by two or more mechanisms is often greater than the sum of the output driven by each mechanism individually. For example, when combining the engineered DNA demethylase with the ultracompact transcriptional activators, upon transient delivery in human cells, we were able to achieve both synergistic and durable re-activation of an epigenetically silenced loci, where the fold activation from such combination was greater than the predicted additive effect by a factor of two or more.

Our engineered compact epigenetic editors provide a versatile and potent platform for gene expression modulation, thereby showing substantial potential for use in future epigenetic editing therapeutic payloads.

GEMS

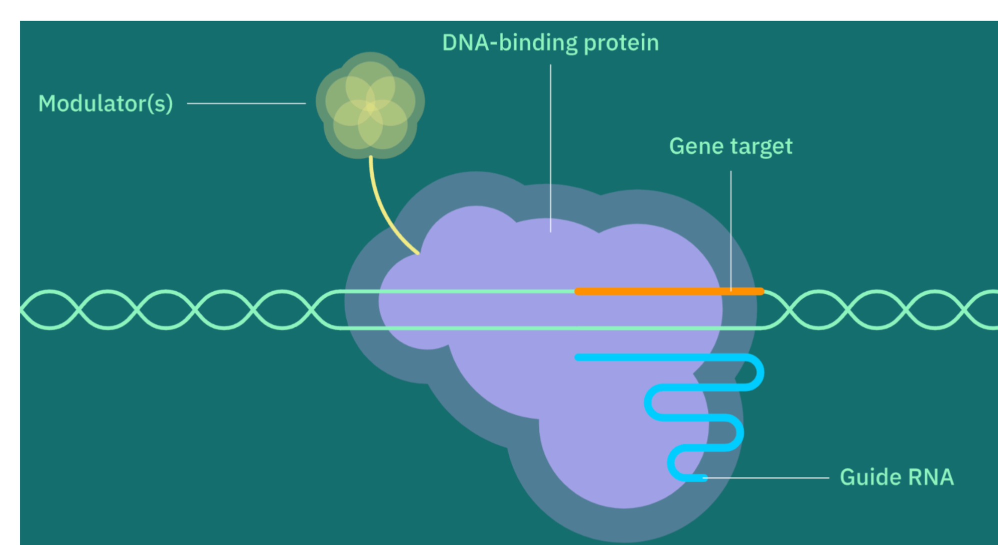


Figure 1 Epicrispr Bio's Gene Expression Modulation System (GEMS) is a modular system comprising highly compact "nuclease dead" Cas effector, transcriptional and epigenetic modulators, and engineered compact guide RNAs.

COMPACT CAS EFFECTORS WITH ENGINEERED PAM RECOGNITION

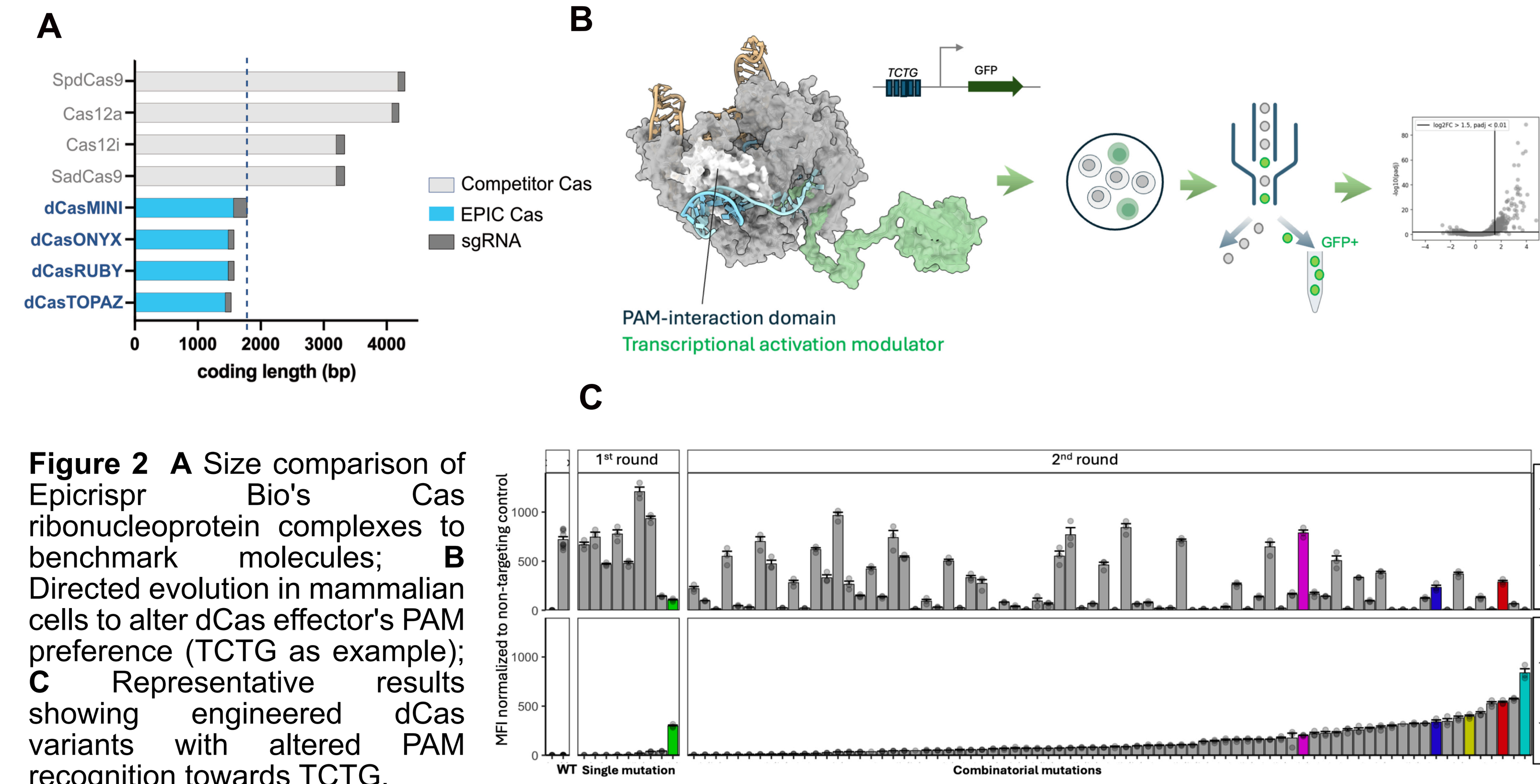
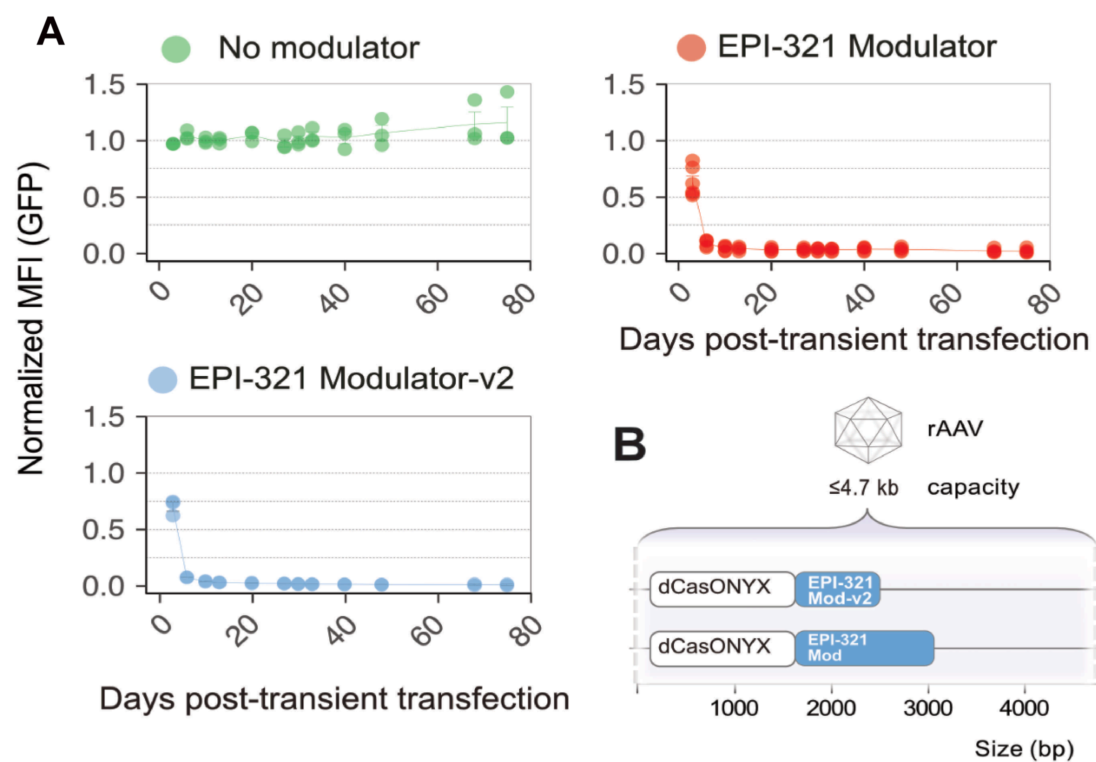


Figure 2 A Size comparison of Epicrispr Bio's Cas ribonucleoprotein complexes to benchmark molecules; **B** Directed evolution in mammalian cells to alter dCas effector's PAM preference (TCTG as example); **C** Representative results showing engineered dCas variants with altered PAM recognition towards TCTG.

COMPACT EPIGENETIC SUPPRESSORS

> 70 days of suppression of GFP reporter in HEK293T post transient delivery

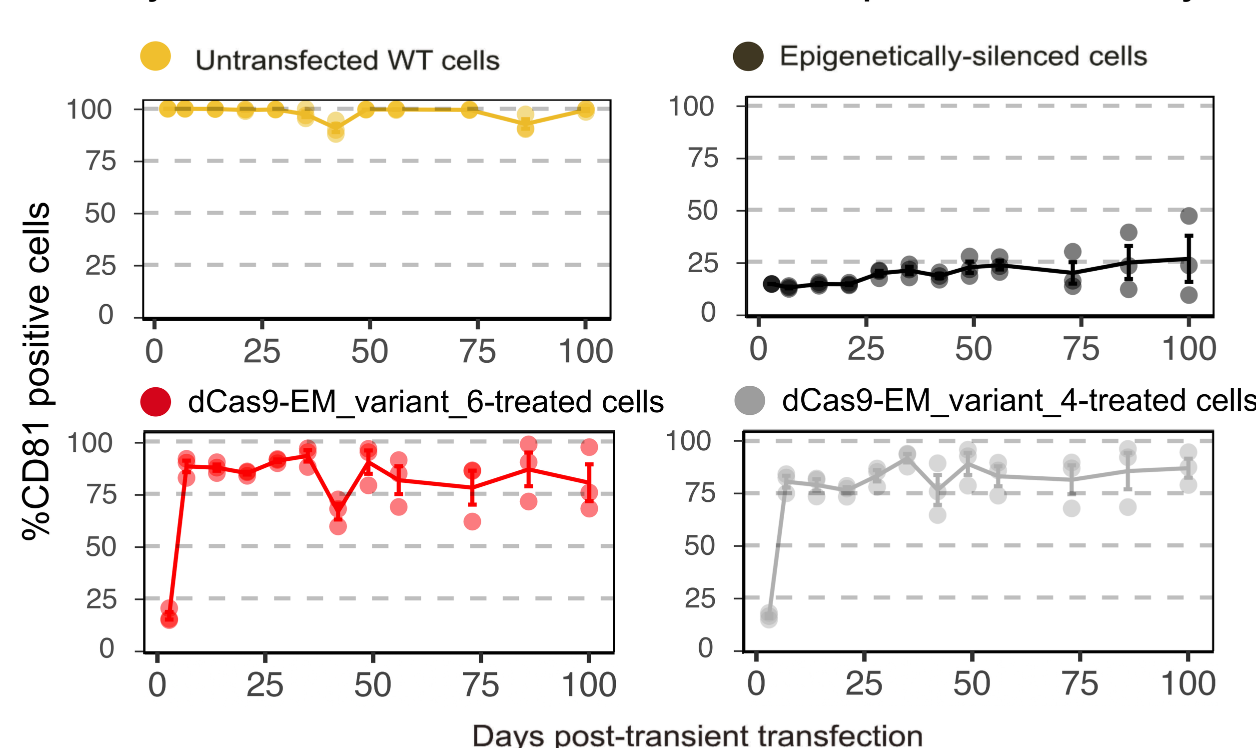


We have previously reported the development of an AAV-deliverable GEMs product, called EPI-321, for the epigenetic suppression of the DUX4 gene in the proposed treatment of Facioscapulohumeral muscular dystrophy (FSHD). Here we represent data showing the successful development of a more compact variant of the suppressor used in EPI-321.

Figure 3 A When transiently delivered to HEK293T cells, EPI-321 modulator-v2 is capable of suppressing reporter GFP expression for more than 70 days (~70 cell divisions). **B** Schematic showing the compactness of EPI-321 modulator-v2, representing a ~40% reduction in size. This compactness was achieved through rational protein engineering, without loss of gene suppression activity

COMPACT EPIGENETIC MODULATORS FOR GENE REACTIVATION

> 100 days of reactivation of silenced CD81 in HEK293T post transient delivery



The ability to reactivate epigenetically silenced gene is an important proof of concept for programmable epigenetic gene regulation. Via rational protein engineering, we've generated several DNA demethylase variants (EM) with an average protein size under 500 amino acid.

Figure 4 Upon transient delivery to HEK293T cells in which the CD81 gene expression has been exogenously silenced via methylation, selected compact dCas9-EM variants were able to reactivate CD81 to near 100%. This activation is sustained through >100 days (~100 cell divisions).

DURABLE AND SYNERGISTIC GENE REACTIVATION

> 28 days of synergistic activation of silenced GFP reporter in HEK293T post transient delivery

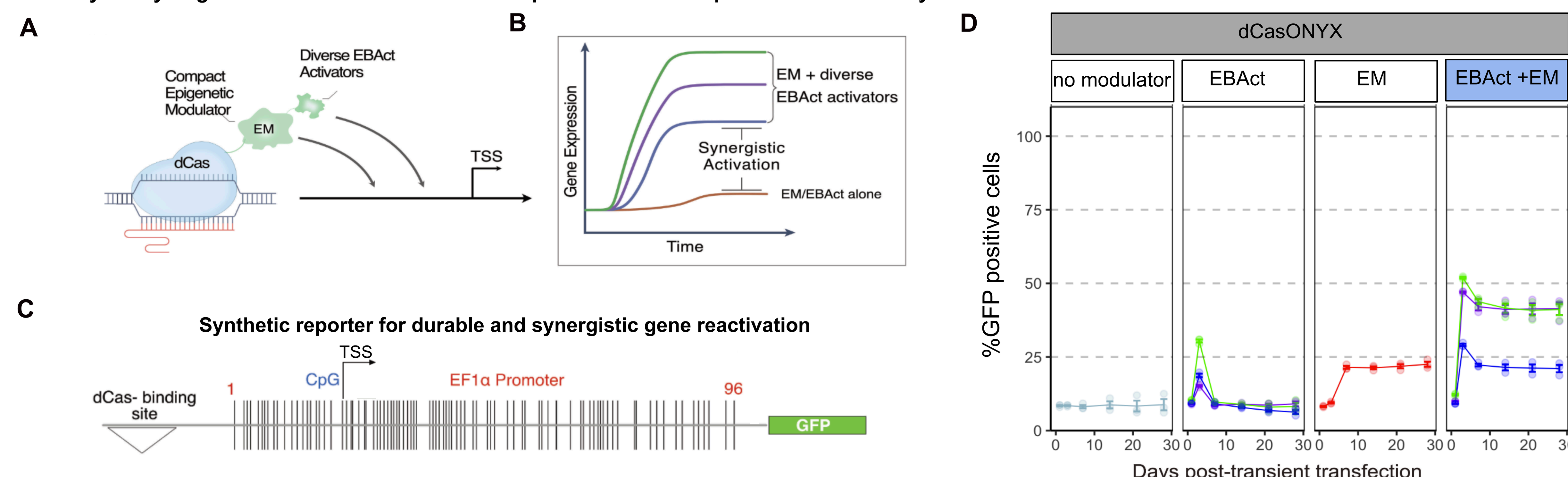


Figure 5 Durable and synergistic gene reactivation via compact GEMS comprising of dCasONYX, DNA demethylase and transcriptional activator. A Schematic representation of the recruitment of dCasONYX fused with one of Epicrispr Bio's Epigenetic Modulators (EM, in this case DNA demethylase) and Epicrispr Bio Activators (EBAct). EBActs are capable of transiently activating endogenous genes to different levels depending on chromatin context as previously presented, while EM (DNA demethylase) can induce durable and mitotically stable epigenetic modification. **B** To overcome the risk of inconsistent gene expression modulation across different chromatin states, we took advantage of the synergistic relationship of transcriptional regulation in eukaryotes, where the transcriptional output driven by two or more mechanisms is often greater than the sum of the output driven by each mechanism individually. By tethering EM (DNA demethylase) and EBAct to dCasONYX as fusion protein, we hypothesize a idealized experimental outcome with synergistic and durable activation of an epigenetically silenced target gene. **C** A synthetic reporter was designed and genetically integrated into HEK293T cell to allow the study of durable and synergistic gene reactivation potential, where dCas gRNA binding site was introduced upstream of EF1α promoter driving GFP reporter expression. Vertical lines indicate individual CpG residue within the promoter fragment. This reporter was first epigenetically silenced via CpG methylation by DNMT. **D** We introduced multiple dCasONYX-EM-EBAct variants into this silenced reporter cell line via transient transfection, and observed various level of GFP expression detected up to 28 days. Of note, we observed durable and synergistic reactivation at various levels, as compared to dCasONYX-EM and dCasONYX-EBAct alone.

rAAV ≤4.7 kb capacity

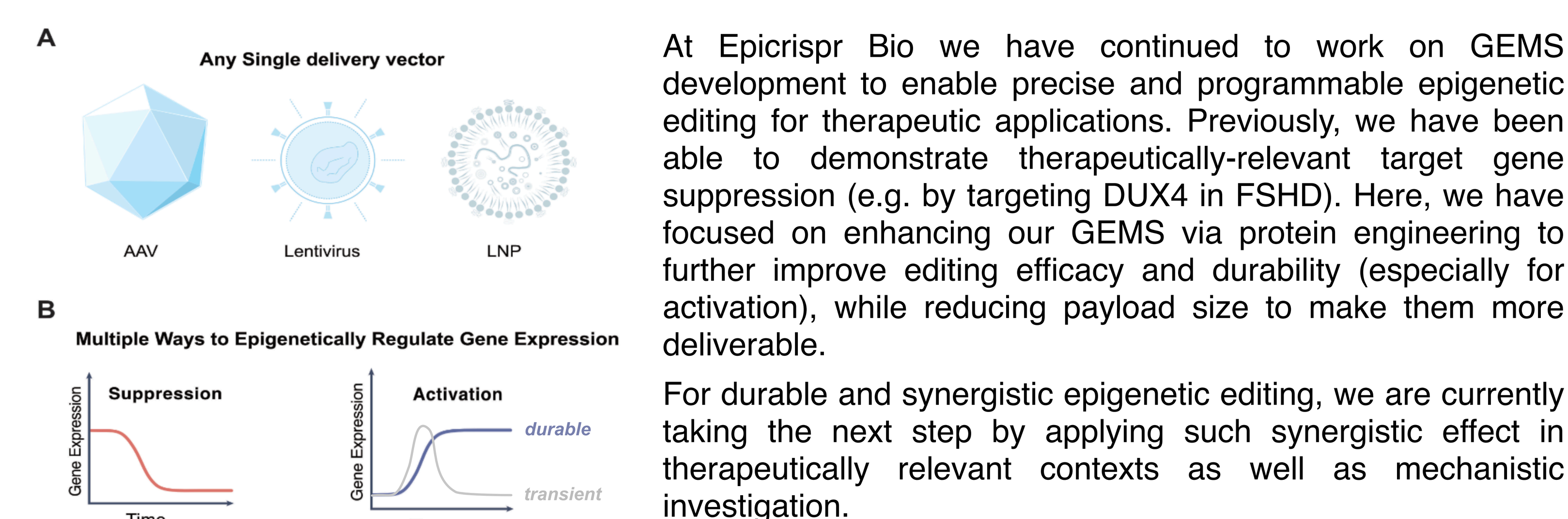
dCasONYX — EM

dCasONYX — EM + EBAct

1000 2000 3000 4000 Size (bp)

Figure 6 Schematic showing the compactness of dCasONYX-EM, and dCasONYX-EM-EBAct constructs. This highlights the potential deliverability of this GEMS for durable and synergistic epigenetic editing via single AAV, and for therapeutic reactivation of human genes.

CONCLUSION AND NEXT STEPS



At Epicrispr Bio we have continued to work on GEMS development to enable precise and programmable epigenetic editing for therapeutic applications. Previously, we have been able to demonstrate therapeutically-relevant target gene suppression (e.g. by targeting DUX4 in FSHD). Here, we have focused on enhancing our GEMS via protein engineering to further improve editing efficacy and durability (especially for activation), while reducing payload size to make them more deliverable.

For durable and synergistic epigenetic editing, we are currently taking the next step by applying such synergistic effect in therapeutically relevant contexts as well as mechanistic investigation.

Figure 7 A Epicrispr Bio GEMS are designed to be both efficacious and compact enough for therapeutic delivery by any single vector for both *in vitro* and *in vivo* applications. **B** Modulator discovery and protein engineering approaches have allowed us to build a comprehensive set of epigenetic editing tools capable of transient or durable gene suppression and activation.

CONTACT AND REFERENCES

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