Directed Evolution and Characterization of Cas Effectors in Mammalian Cells for Expanded Epigenome Editing Space

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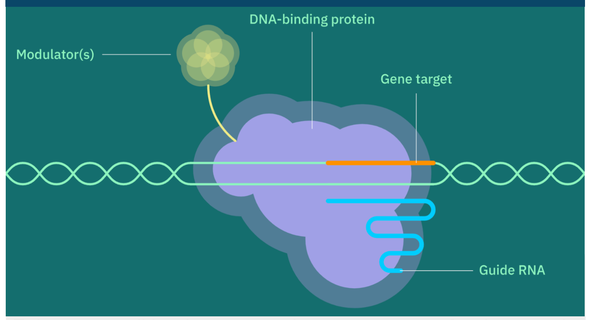
Gene Expression Modulation Systems (GEMs)



Epigenome editing is an emerging new strategy for therapeutic application by activating or silencing target gene expression. At Epicrispr Biotechnologies, we have developed a highly optimized CRISPR-based epigenome editing platform termed Gene Expression Modulation systems (GEMs). In this study, we describe a directed evolution platform in mammalian cells to alter and characterize the PAM recognition profile of our dCas effectors, which allows for wider epigenome targeting space.

gRNA and dCas Engineering for Improved GEMs Activity

We have engineered a panel of gRNA scaffold and dCas effectors for reduced size and improved CRISPRa activity.

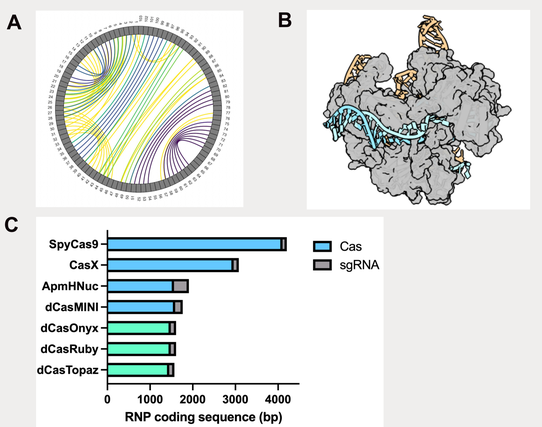


Figure 1 **A)** Selected gRNA scaffold variant (displayed as base pair probability map), **B)** Schematic representation of engineered gRNA-dCasONYX effector complex (ESMFold) and **C**) Summary of various gRNA-dCas effector complex size

Directed Evolution in Mammalian Cells to Alter dCas PAM Preference

We aim to alter the PAM preference of our dCas effectors to more G/C-rich for broader epigenome editing space. By tethering the activity of novel PAM recognition with the expression of a synthetic reporter, we were able to select and verify beneficial mutations from a pool of site-saturation mutagenesis library of over one thousand variants.

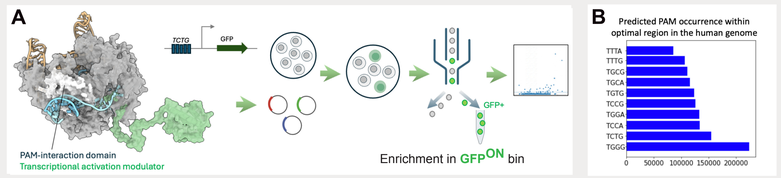


Figure 2 A) Cell-based reporter for directed evolution towards alternative PAM recognition (TCTG as example), **B)** Predicted PAM occurrence within -400 to +300 bp of TSS in the human genome

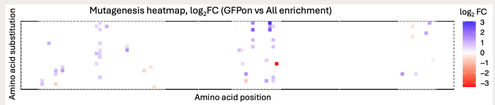


Figure 3 Mutagenesis heatmap showing 1st round hits with improved or decreased activity on GFPon population enrichment upon novel TCTG recognition (p_{adj} < 0.025)

Combinatorial engineering with both the strong and weak hits identified in the 1st round screen achieved synergistic enhancement on the novel PAM sequence recognition with over 500-fold improvement compared to the parent dCas effector.

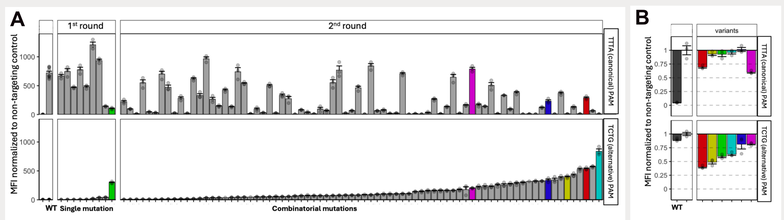


Figure 4 A) The 2nd round (combinatorial mutagenesis) engineering achieved significantly improved recognition towards alternative PAM in the context of CRISPRa when fused with a transcriptional activation modulator such as VPR, **B)** Alternative PAM recognition was also observed in the context of CRISPRi when fused with a compact epigenetic suppressor. (The TTTA and TCTG reporters are clonal cell lines engineered separately, thus cross-comparison of normalized MFI between the two cell lines is not recommended.)

Mammalian Cell-based Assay for PAM Characterization

We developed a mammalian cell-based pooled CRISPRa screen to query the PAM recognition profile of dCas variants against a diverse PAM library (4096 members)

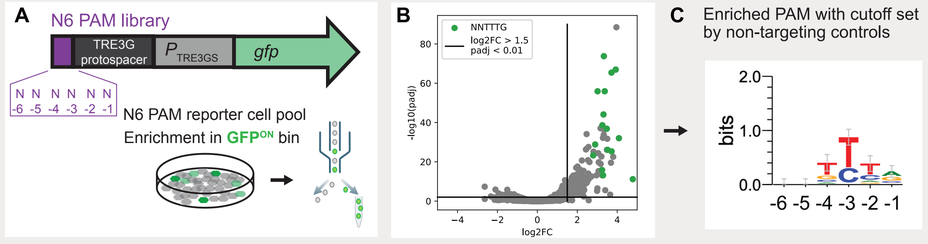


Figure 5 Cell-based PAM characterization screening indicates dCasONYX recognizes a T-rich 4nt PAM sequence with flexibility towards C at position -3 **A)** Schematic illustration of the N6 PAM screening workflow; **B)** Volcano plot showing enrichment (Log2FC) and p_{adj} for each 6nt PAM sequence. **C)** Sequence logo of all 6nt PAM hits

Epic's lead dCas proteins share an expanded PAM profile, enabling high coverage of genomic loci for effective targeting and therapeutic design. PAM characterization of our new dCas variants are ongoing.

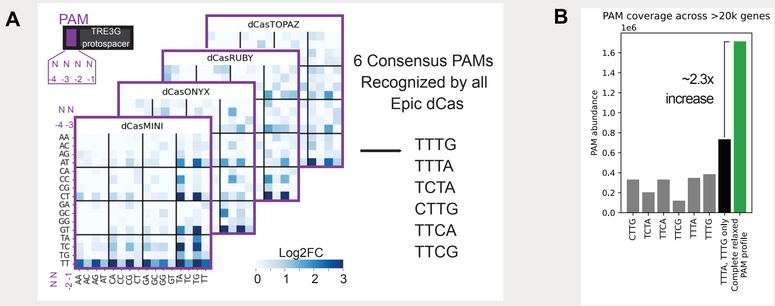


Figure 6 Full PAM characterization of dCasMINI, dCasONYX, dCasRUBY, and dCasTOPAZ reveals a shared pattern of relaxed PAM recognition, enabling easy design of therapeutics targeted to many disease causing genes. **A)** Heat-maps showing the strength of PAM recognition (Log2FC) measured in the cellular screen for each dCas across all possible N4 PAM sequences. The 6 consensus PAMs strongly recognized by all dCas are shown. **B)** PAM abundance within gRNA targeting window (-1kb to +1kb of TSS) for >20k genes in the human genome.

Summary and Future Direction

We are currently characterizing the full PAM recognition profile of the newly engineered dCas effectors with altered recognition towards TCTG PAM. Our preliminary results indicated broader or orthogonal epigenome targeting range when combining these dCas variants with the compact activation/suppression modulators we have developed in house.

Altogether, this work provides a novel mammalian cell-based platform for both directed evolution and characterization on the altered PAM recognition profile of dCas effectors, enabling a collection of novel proteins with expanded epigenome editing range with potential applications in both basic research and therapeutics.

References and Contact

Business inquiry: Benson Cheng (benson.cheng@epic-bio.com)
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2. Takeda, S. N. *et al,* Mol Cell, 2021, 81, 558.
3. Carroso, G. *et al*, bioRxiv, 2023.06.02.543492

Presentation: *Non-Human Primate (NHP) Safety Study of High-Dose EPI-321: A Novel AAV-Delivered Epigenetic Editing Gene Therapy for the Treatment of FSHD.* Wednesday 4:45 PM, NOLA Theater B

Poster #1110: *Compact DNA Demethylase-Activator combination Modulators for CRISPR-Mediated Epigenetic Gene Activation.* Wednesday, 5:30 - 7:00 PM

Poster #967: *Small Scale AAV Bioreactor Optimization Demonstrates Iterative Titer Gains of rAAVrh74 Serotype EPI-321, a CRISPR-mediated Epigenetic Therapy.* Tuesday, 6:00 - 7:30 PM

Poster #1939: *EPI-321 Development: Strategies to Establish a Scalable and Robust rAAVrh74 Upstream Manufacturing Process from 0.5 L to 1000 L Scale.* Thursday, 5:30 - 7:00 PM