Interchanging Cas enzymes, deaminases, and aptamer-guide RNA combinations to achieve optimal editing with the modular Pin-point™ base editing platform

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1 Abstract

Base editing was first described in 2016 as a powerful tool to introduce precise genomic changes by avoiding DNA double-strand breaks and it has rapidly progressed to the clinic. However, its original Cas9 configuration is not able to address all genetic changes due to PAM limitations and the available deaminases. The uniquely modular Pin-point base editing platform is a three-component system consisting of either a nuclease-deficient or nickase Cas enzyme, plus an extended guide RNA with an aptameric scaffold, and an aptamer binding protein fused to a deaminase. These three components can be efficiently delivered as mRNA and synthetic sgRNA in primary T cells, iPSCs, and HSPCs to efficiently edit DNA targets of interest. Using the basic configuration of the system with a nickase S. pyogenes Cas9 (nSpCas9) and rat-APOBEC deaminase, the targeting capacity for base editing is limited due to the NGG PAM requirement of the nSpCas9 enzyme along with an editing window primarily targeting positions 4-8 within the protospacer for the rat-APOBEC deaminase. To target additional genomic locations, we have leveraged the modular nature of the Pin-point platform for an easily adapted “plug-and-play” approach in which we introduce different combinations of nucleases, deaminases, and sgRNAs containing varying aptamer positions within the gRNA scaffold. We have developed an arrayed screening platform for high-throughput characterization of these additional configurations as a way to demonstrate the unique editing characteristics of these novel systems. Using alternative Cas enzymes such as members of the Cas12 family and its T-rich PAM requirements enables editing at target sites that are not readily addressable using Cas9. Additionally, use of different species of deaminases such as the lizard derived Anolis-APOBEC enables editing with a wider editing window. When used in combination with different aptamer binding proteins and sgRNA scaffolds, we show that regions of the genome not previously attainable with previous configurations can now be efficiently edited. We also show that when using a dual aptameric approach, simultaneous editing with multiple deaminases can be performed in a single transfection as a way to edit two or more target sites and without the need for introduction of orthogonal Cas enzymes to deliver the different deaminases. This modular approach to base editing enables highly specific and efficient editing that can be custom tailored to fit specific cell and gene therapy programs and their unique editing requirements.

2 The Pin-point base editing platform

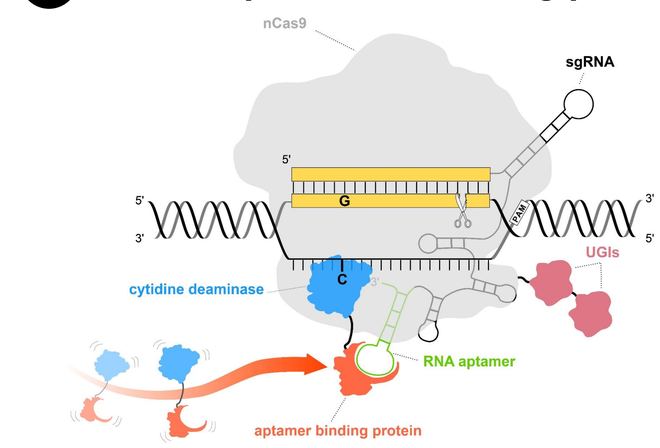


Figure 1: In one possible configuration of the Pin-point platform, a Cas9 nickase (nCas9) is guided to DNA target site via a guide RNA with an additional aptameric region engineered into the scaffold. The aptamer recruits a deaminase via fusion with an aptamer binding protein. The combination of nCas9, an aptamer binding protein fused to a deaminase, and an aptameric guide RNA efficiently base edit a DNA target of interest.

3 Efficient multiplex base editing and enhanced safety in T cells and iPSCs

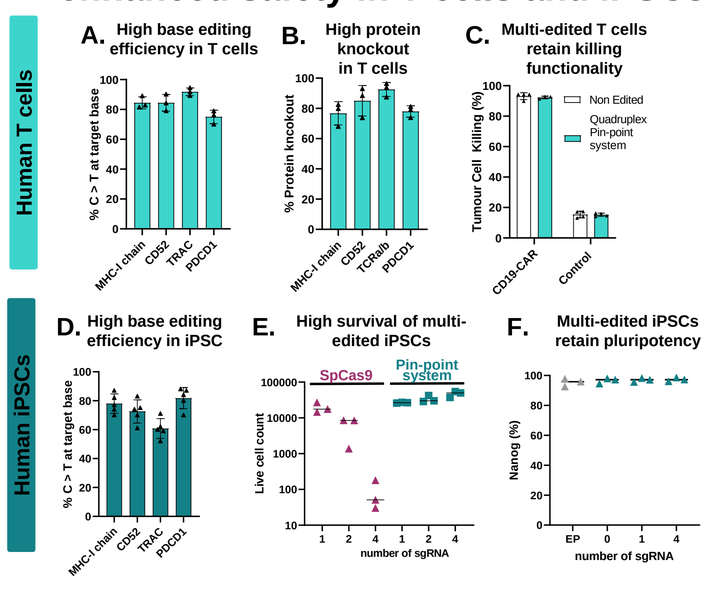


Figure 2: A) Four genes are edited simultaneously in T cells after delivery of Pin-point mRNAs (nCas9 and rAPOBEC1) and synthetic aptameric sgRNAs. Percentages of C to T conversion are reported. **B**) Protein knockout of 4 targets analysed by flow cytometry. **C**) In vitro tumour cell killing assay performed with either empty (control) or CD19-CAR expressing lentiviral vector transduced cells and mock electroporated (Non Edited) or T cells edited with a Pin-point system (Quadruplex Pin-point System). n = 3 T cell donors for A-B, n = 2 T cell donors for C. **D**) Four genes are edited simultaneously in iPSCs after delivery of Pin-point mRNAs (nCas9 and rAPOBEC1) and synthetic aptameric sgRNAs. Percentages of C to T conversion are reported. **E**) Viability of iPSCs edited with single or multiple sgRNAs using the Pin-point system or Cas9 analysed by flow cytometry 48hrs post electroporation. Viability was normalized to a no sgRNA control. **F**) Expression of the pluripotency marker Nanog in cells edited with the Pin-point reagents analysed by flow cytometry 4 days post electroporation. Data shown in D, E, F are from two independent experiments with 2 iPSC lines.

4 Versatile screening platforms developed to assess base editing for multiple deaminases

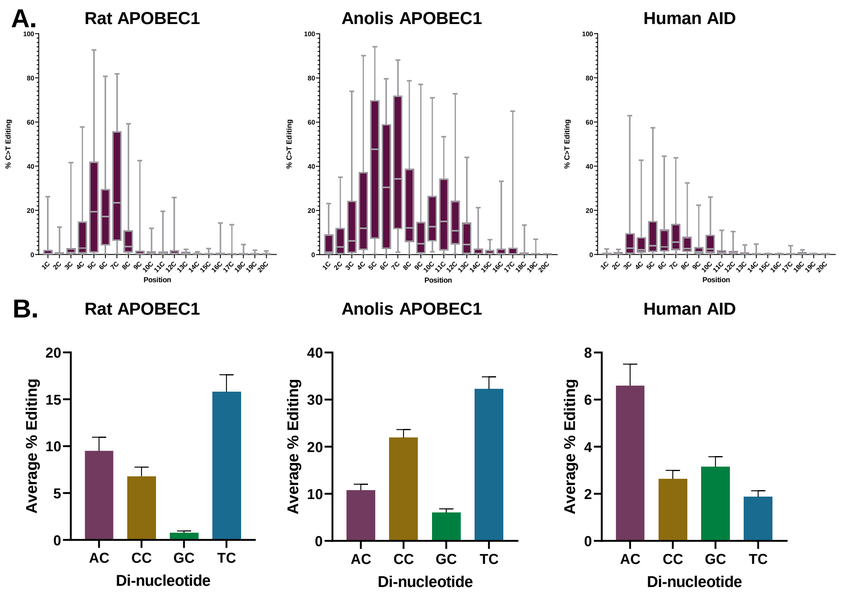


Figure 3: A base editing arrayed screening platform was designed to assess editing across each position of the protospacer for a gRNA library with control targets that have been well established in the literature or comprehensively validated internally. gRNAs were also designed to tile a protein coding region of STAG2 and required at least one C to be present within positions 1 to 18 of the 20 bp protospacer. Following transduction with nCas9, HEK293T cells were reverse transfected in 384-well format with gRNA & MCP-deaminase mRNA. Following NGS analysis of base editing, **A**) the percentage C to T conversion at each position across the protospacer is plotted as a box plot with the min/max of the distribution. The data show that a wide range of editing can be achieved at each position, and is both guide and aptamer dependent, with key differences in optimal editing window for the different deaminases screened. **B**) The data also reveal an impact of the preceding base on editing efficiency, with unique signatures amongst the different deaminases screened.

5 Robust editing using a Type V version of the Pin-point platform

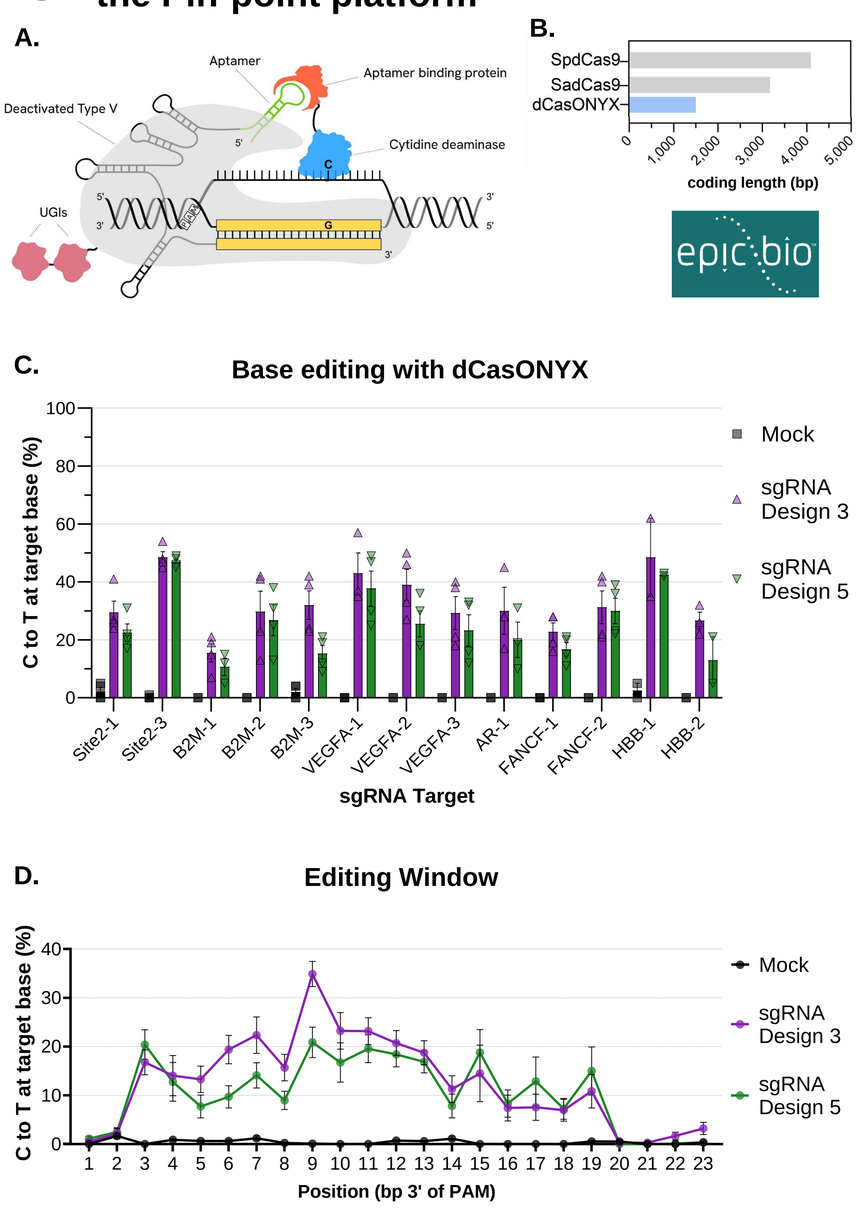
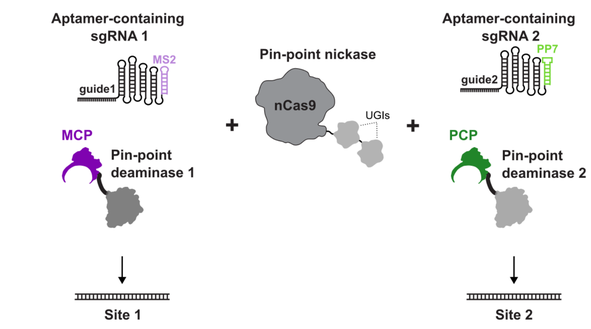


Figure 4: A) Graphical representation of Pin-point base editing system with Type V nuclease. **B**) dCasONYX is a compact Type V protein less than half the size of SpCas9. Figures and data courtesy of Epicrispr Biotechnologies (Epic Bio).
Two aptamer-sgRNA designs with placement of the aptamer at differing positions within the gRNA scaffold (design 3 and 5) were tested across a variety of genomic targets in HEK293T cells. **C**) Percentages of target C to T conversion in samples analysed by Sanger sequencing are reported across target sites. n≥3; error bars ±SEM. **D**) The C to T editing efficiency at each position of the 23bp target was averaged over each replicate of each sgRNA tested. n≥7 for each position; error bars ±SEM.

6 Dual aptameric base editing with the Pin-point platform

A. Dual aptameric base editing overview



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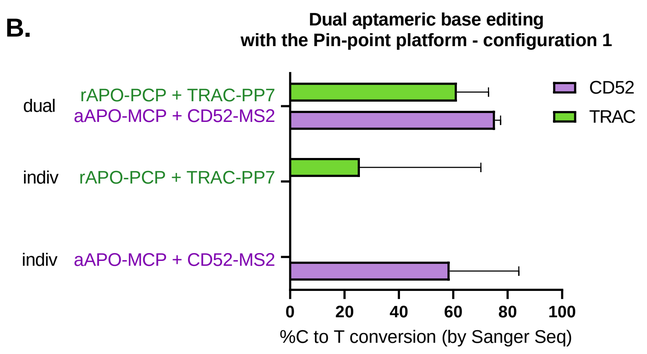
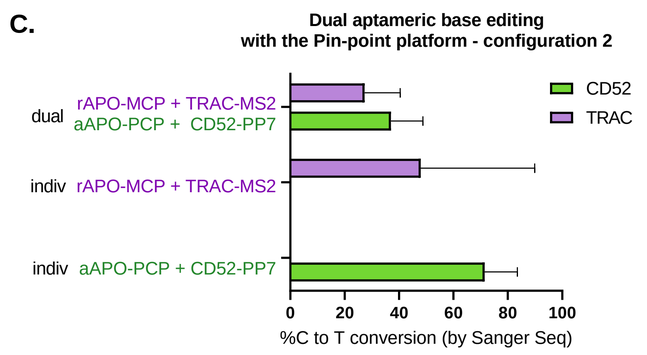


C.



D.

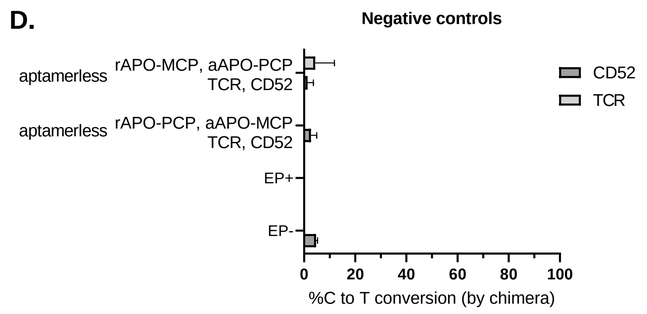


Figure 5: A) Diagram of dual aptameric base editing with the Pin-point system. Utilizing two different aptamers allows to simultaneously target different sites with different deaminases without the need for orthogonal nucleases. **B-C**) Dual aptameric editing efficiencies in primary human T cells with either Rat APOBEC1 (rAPO) or Anolis Apobec (aAPO) at CD52 and TRAC targets. Dual editing with two different configurations (rAPO-PCP + aAPO-MCP, and rAPO-MCP + aAPO-PCP) resulted in similar editing to individual (indiv) controls. **D**) No editing observed in aptamerless negative controls, as expected. Bars are mean ± SD. N=3 technical replicates.

8 Summary

We applied multiplex base editing using the Pin-point system to the development of engineered CAR-T cells and hypoimmunogenic iPSCs.

The Pin-point base editing system achieved greater than 70% knockout efficiency without any selection or enrichment and high purity at therapeutically relevant target sites in T cells and iPSCs.

Using arrayed screening platform developed for high-throughput study of gRNA function and design, we are able to further characterize novel Pin-point system configurations containing additional deaminase and Cas components.

dCasONYX is a novel evolved Type V nuclease generated by Epic Bio, with unique characteristics when used in the Pin-point system.

We have engineered two aptameric sgRNA molecules compatible with a dCasONYX-based Pin-point platform that allow efficient base editing at multiple target sites across the human genome.

Utilizing two aptamers with the Pin-point base editing system allows for simultaneous editing at two sites in the genome without the loss of efficiency.