Molecular Therapy
Nucleic Acids
Brief report

Editing of homologous globin genes by nickase-deficient base editor mitigates large intergenic deletions in HSPCs

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Recent studies have shown that base editing, even with single-strand breaks, could result in large deletions of the interstitial regions while targeting homologous regions. Several therapeutically relevant genes such as *HBG*, *HBB*, *CCR5*, and *CD33* have homologous sites and are prone for large deletion with base editing. Although the deletion frequency and indels observed are lesser than what is obtained with Cas9, they could still diminish therapeutic efficacy. We sought to evaluate whether these deletions could be overcome while maintaining editing efficiency by using dCas9 fusion of ABE8e in the place of nickaseCas9. Using guide RNAs (gRNAs) targeting the γ-globin promoter and the β-globin exon, we evaluated the editing outcome and frequency of large deletion using nABE8e and dABE8e in human HSPCs. We show that dABE8e can edit efficiently while abolishing the formation of large interstitial deletions. Furthermore, this approach enabled efficient multiplexed base editing on complementary strands without generating insertions and deletions. Removal of nickase activity improves the precision of base editing, thus making it a safer approach for therapeutic genome editing.

INTRODUCTION

Base editing in the γ-globin promoter for the reactivation of fetal hemoglobin (HbF) or in the β-globin gene for direct correction of mutations is a promising approach for the treatment of β-hemoglobinopathies.[1–5] Unlike other loci, the globin locus is highly homologous with the probability of guide RNA (gRNA) binding simultaneously at two or more sites in the locus. Consequently, while using double-strand break (DSB)-mediated approaches, large deletions involving the intervening regions occur in addition to the intended edits.[6,7] Although base editing was expected not to cause large deletions due to the absence of DSBs, we and others have observed the occurrence of unintended large deletions in both gamma- and beta-globin genes, possibly because of simultaneous nicking at homologous sites.[6,8,9] Recent work also showed that even with a 15% large deletion in input cells, upon long-term engraftment, 50% of the mice harbored large deletions of one of the globin genes, which would mean less hemoglobin production per cell.[10] Additionally, the indirect consequences of large deletions such as chromothripsis, translocations, and p53 activation have not been investigated extensively.[11,12] Hence, it is important to develop genome editing strategies that generate minimal changes in the genome while achieving therapeutic benefits. Here, we sought to evaluate whether fusing dCas9 (dead Cas9) to ABE8e could overcome deletions generated because of DNA nicks. We show that it not only overcomes large deletions while editing homologous regions but also prevents the formation of insertions or deletions (indels) while base editing in complementary strands.

RESULTS

Frequency of 4.9-kb deletion in the γ-globin locus varies with gRNAs

Base editors were designed to introduce point mutations in the target region while avoiding the DSBs caused by Cas9 nucleases. The initial design of base editors using deaminase fused to a dCas9, however, was less efficient.[13,14] The use of D10A nCas9 (nickase Cas9) allowed the nicking of non-edited strands, thereby facilitating the effective installation of edits that resulted in significantly higher editing efficiency. Base editors were subsequently evolved to improve the activity, and the recently described hyperactive variant ABE8e was shown to edit, with conversion reaching ~100% in many target sites.[15] However, the use of nCas9 base editors in highly homologous regions resulted in the deletion of intervening regions.[8,9] We hypothesized that with its high processivity, ABE8e would be able to install mutations even when fused to catalytically dCas9 and thus can be used for base editing in homologous regions without risking deletion of the intervening region.

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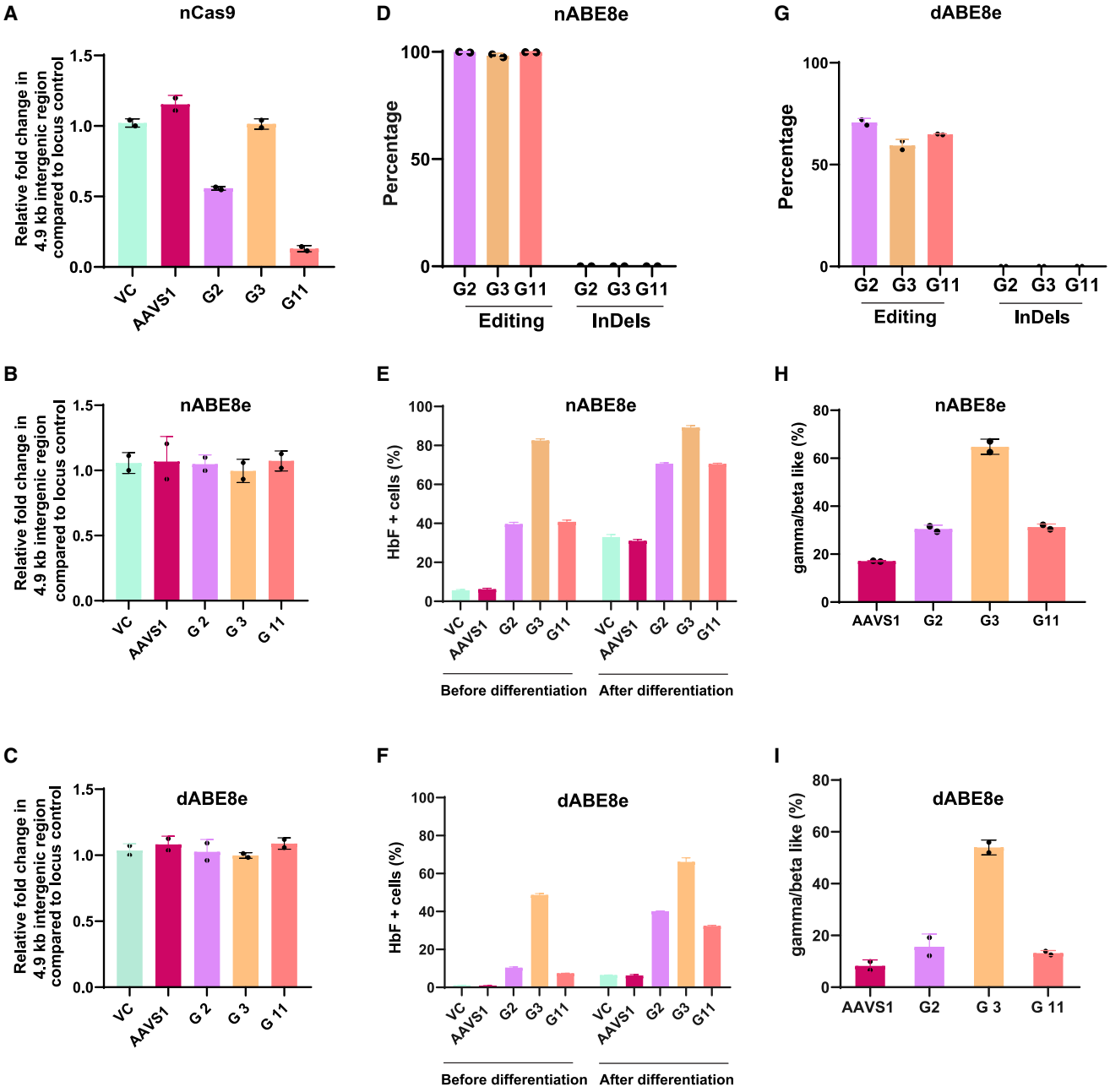
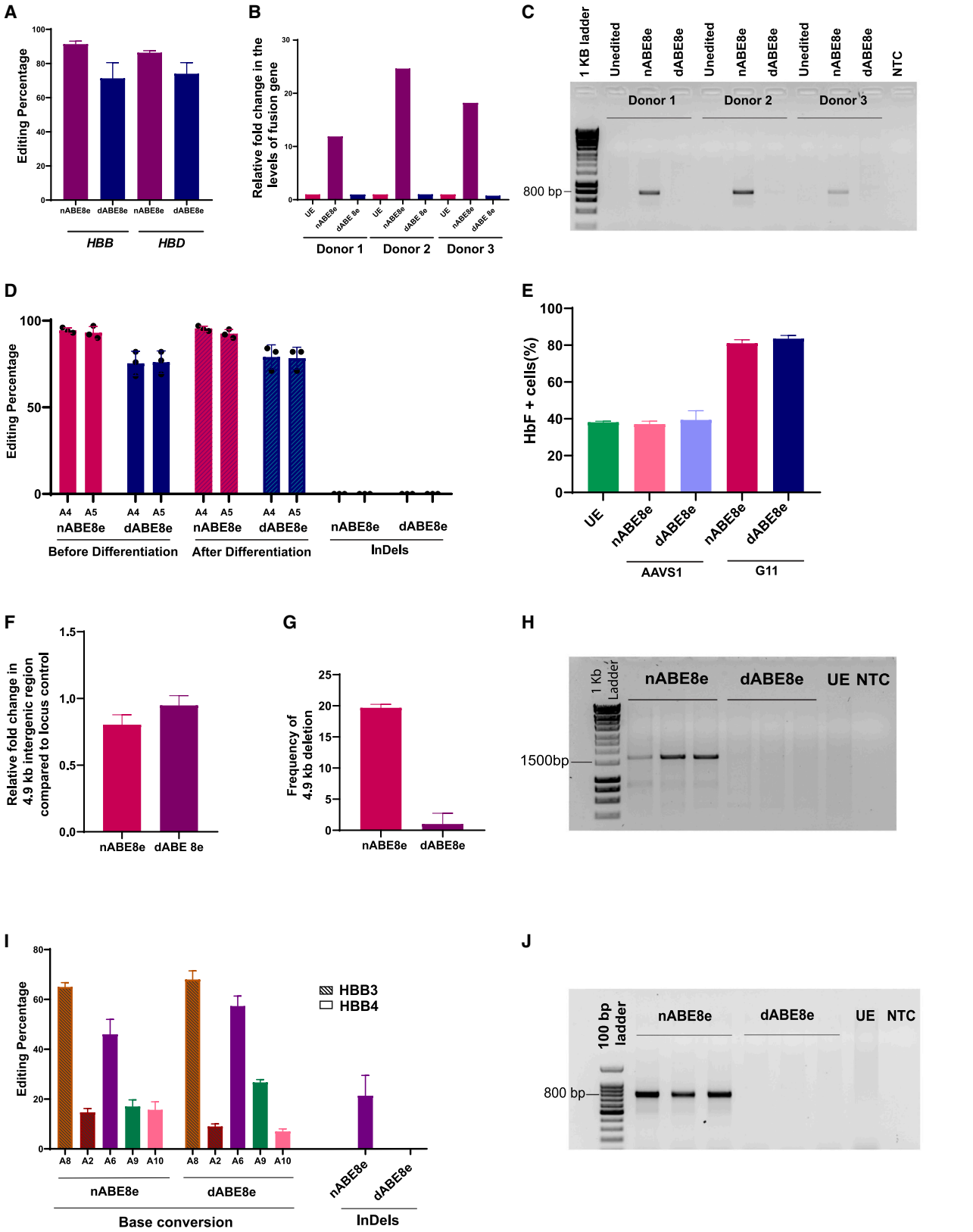


Figure 1. DNA nickases can introduce large interstitial deletions in homologous regions

(A–C) Quantification of changes in the 4.9-kb intergenic region upon targeting the γ-globin promoter by nCas9 (A), nABE8e (B), and dABE8e (C) in HUDEP 2 cells (by lentiviral delivery), measured as relative fold change compared to locus control by quantitative real-time PCR. (D and G) Editing efficiency and indels generated by nABE8e (D) and dABE8e (G) using gRNAs targeting γ-globin promoter evaluated by next-generation sequencing. (E and F) Percentage of HbF^+ cells evaluated by intracellular staining, followed by flow cytometry upon base editing with nABE8e (E) and dABE8e (F) measured before and after erythroid differentiation. (H and I) Measurement of globin chains after base editing in the γ-globin promoter using RP-HPLC in nABE8e (H) and dABE8e (I). All experiments were performed as biological duplicates. Data represented as mean ± SD. VC, vector control.

Using previously validated gRNAs[8] targeting the γ-globin locus (G2, G3, G11) with the potential for therapeutic applications, we sought to evaluate whether the frequency of 4.9-kb deletion varies between the gRNAs, irrespective of the base editor used (Figure S1). These gRNAs were delivered as lentivirus to D10A nickase HUDEP-2 stables, and the genomic alterations were evaluated. There was no appreciable



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base conversion or indels except in G11, which showed a very small percentage of indels (Figures S2A and S2D). However, by quantitative real-time PCR we detected large deletions occurring with the use of G2 and G11 but not with G3 (Figure 1A). We believe that gRNA efficiency might be a driving factor in determining the deletion frequency (Figures S2E and S2F; Note S1). While this level of deletion might not be reflected during base editing, it shows the potential for large deletion when the base editing components (gRNA and Base Editor mRNA) are available in excess as in therapeutic *ex vivo* editing of hematopoietic stem and progenitor cells (HSPCs).

Nickase-deficient ABE8e can edit efficiently in human erythroid cells

We introduced H840A mutation in nABE8e and tested whether it can edit efficiently to achieve therapeutic benefits. We tested this in HUDEP-2 cells stably expressing nABE8e or dABE8e using the same gRNAs. As expected, all three gRNAs resulted in >90% editing with nABE8e, while the efficiency was ∼60%–70% with dABE8e (Figures 1D, 1G, S2B, and S2C). No indels were detected with either construct, and there was no alteration in the editing window (Figure S2G). We did not observe any significant 4.9-kb deletion in any of the samples by quantitative real-time PCR (Figures 1B and 1C). This suggests that even with nickase activity in nABE8e, rapid kinetics of deaminase resulting in accelerated editing in HUDEP-2 cells likely reduced the deletion frequency, which was not picked up in the quantitative real-time PCR. Corresponding to the editing efficiency, we also observed a drop in HbF levels while editing with dABE8e, but the levels would be sufficient for therapeutic applications in β-hemoglobinopathies (Figures 1E, 1F, 1H, and 1I).

dABE8e can efficiently edit in human CD34$^+$ HSPCs and prevent the formation of interstitial deletions

As nicking by itself can generate large deletions in homologous regions, we evaluated whether the formation of large deletions during base editing in HSPCs can be overcome using dABE8e. We first tested base editing efficiency and the resulting large deletions in CD34$^+$ HSPCs in the therapeutically relevant *HBB* gene using gRNAs targeting exon-1 with (HBB1) and without (HBB2) homology to *HBD* gene (Figure S3A). Cells were nucleofected with nABE8e/dABE8e mRNA and the respective single-guide RNA, and as expected, the editing efficiency was slightly reduced while targeting with dABE8e (Figure 2A). Base conversion in *HBD* and resulting large deletion with nABE8e was observed only in HBB1 gRNA, which had binding sites at both genes and not with HBB2, which had binding sites only in *HBB* (Figure S3B). However, with dABE8e, large deletion was absent even when very high levels of editing were observed at both genes using HBB1 (Figure 2C). Quantitative real-time PCR with primers specific to large deletion between *HBD* and *HBB* showed that nABE8e had a 10-fold increase in amplicons with large deletion compared to unedited control and dABE8e edited samples (Figure 2B).

We further compared both of the editors in the *HBG* promoter. G11 was used along with AAVS1 targeting gRNA as the negative control. While the editing efficiency reached >90% in nABE8e, it was slightly lower in dABE8e (Figures 2D and S3C). As expected, we observed the 4.9-kb deletion with nABE8e but not in dABE8e (Figures 2F–2H). The edited cells upon differentiation showed similar elevation in F$^+$ cells reaching close to 80% in both nABE8e and dABE8e, suggesting that editing by dABE8e would be sufficient for therapeutically relevant HbF elevation (Figure 2E). We also tested two other gRNAs in the *HBG* promoter that were shown to elevate HbF to therapeutic levels and found that while editing efficiency with dABE8e was slightly lower than what was observed with nABE8e, it abolished the creation of the 4.9-kb deletion (Figures S3D–S3G).

Additionally, we tested the utility of dABE8e in preventing indel formation during multiplexed editing in complementary strands using two sgRNAs targeting the *HBB* gene (HBB3 and HBB4) (Figure S4A). While nABE8e resulted in indel formation due to nicking on opposite strands, dABE8e resulted in pure base conversion without any indels/large deletions (Figures 2I, 2J, and S4B). These data suggest that simultaneous nicking in the homologous site during base editing with nABE8e is responsible for large deletions and can be overcome using dABE8e.

Characterization of dABE8e activity in CD34$^+$ HSPCs

As dABE8e showed slightly reduced editing compared to nABE8e at all the sites, we evaluated whether this was due to the limited editing efficiency of dABE8e in the more primitive quiescent cells. The editing efficiency of nABE8e and dABE8e was compared in CD90$^+$ primitive HSPCs with that of bulk edited cells, and no significant difference was observed (Figures 3A and S5). We also tested whether increasing

Figure 2. dABE8e can edit efficiently in HSPCs without generating large deletions

(A) Base editing efficiency of HBB1 gRNA in *HBB* and *HBD* genes using ABE8e and dABE8e (by mRNA delivery) measured by Sanger sequencing (n = 3 individual donors). (B) Relative increase in HBD/HBB fusion gene generated by large deletions upon editing with nABE8e and dABE8e compared to unedited control measured by quantitative real-time PCR (performed in three individual donors). (C) Formation of fusion gene (HBD-HBB) due to large deletion upon base editing detected by agarose gel electrophoresis after PCR. A band at ∼800 bp indicates large deletion. (D) Editing efficiency of nABE8e and dABE8e using G11 in γ-globin promoter measured by Sanger sequencing before and after erythroid differentiation. (E) Percentage of HbF$^+$ cells evaluated by flow cytometry after intracellular staining of samples edited with G11 using nABE8e and dABE8e after erythroid differentiation. (F) Reduction in 4.9-kb intergenic region caused by interstitial deletions while targeting the γ-globin promoter using G11 measured as relative fold change compared to locus control by quantitative real-time PCR and normalized using unedited control. (G) Frequency of 4.9-kb deletion created by base editors while targeting the γ-globin promoter by G11 measured by probe-based droplet digital (ddPCR) and compared to unedited control. (H) Formation of fusion gene (HBG2-HBG1) due to large deletion on base editing detected by agarose gel electrophoresis following PCR. A band at ∼1,700 bp indicates large deletion. (I) Editing efficiency and indel formation upon multiplex editing with two sgRNAs targeting the complementary strands in β-globin exon 1(HBB3/HBB4) measured by Sanger sequencing. (J) Formation of fusion gene (HBD-HBB) due to large deletion after multiplexed base editing detected by agarose gel electrophoresis after PCR. A band at ∼800 bp indicates large deletion. (n= 3 for all experiments. Data represented as mean ± SD. NTC, non-templated control; UE, unedited control).

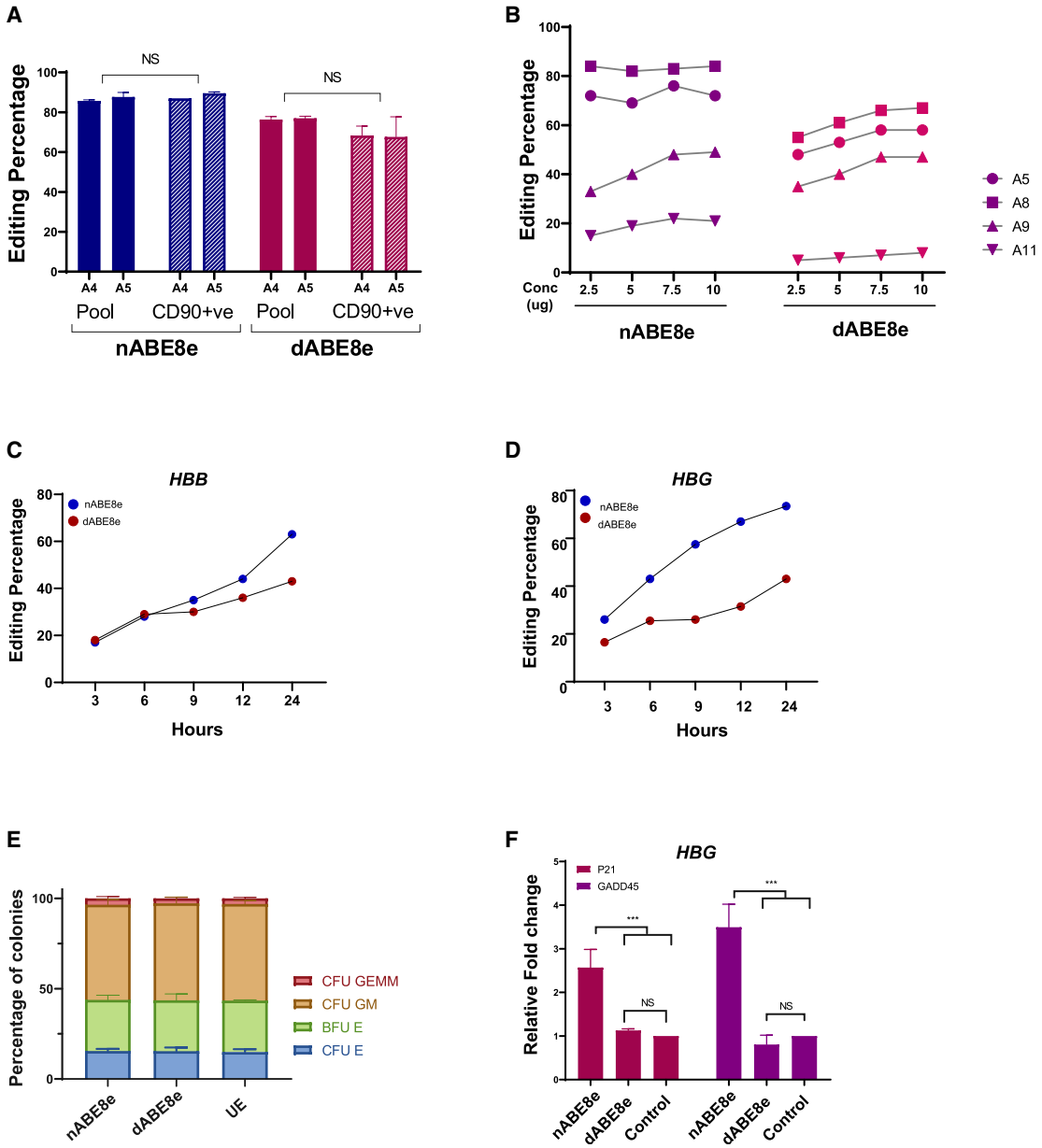

Figure 3. Characterization of nABE8e and dABE8e activity in HSPCs

(A) Base editing efficiency of nABE8e and dABE8e (by mRNA delivery) in a pool of HSPCs and in a primitive ($CD90^+$) population, with G11 targeting γ-globin promoter measured by Sanger sequencing ($n = 3$). Student t test was used for comparison between the groups. (B) Comparison of base editing efficiency with escalating doses of base editors in HSPCs using G2 targeting γ-globin promoter after 48 h of electroporation measured by Sanger sequencing ($n = 1$). (C and D) Evaluation of base editing efficiency over time after electroporation in HSPCs using HBB1 (C) and HBG G11 (D) gRNAs measured by Sanger sequencing ($n = 1$). (E) Percentage of colonies formed from base edited HSPCs compared to unedited (mock electroporated) control after 14 days of seeding in MethoCult medium ($n = 3$). (F) Relative fold change in mRNA expression of P21 and GADD45 genes after 48 h of electroporation (mRNA) by HBG G4 measured by quantitative real-time PCR. Data are normalized to mock electroporated control (G4 gRNA, $n = 3$). (ANOVA with multiple comparisons was used for evaluating statistical significance.) Data represented as mean ± SD. $^{***}p < 0.001$. NS, nonsignificant; UE, unedited control.

the base editor cargo would improve the editing efficiency in target sites that showed moderate editing efficiency, but we did not observe any appreciable improvement in editing, even with a 4-fold increase in the cargo, suggesting that reagent availability is not the limiting factor for editing at these sites (Figures 3B and S3H). It was also noted that both nABE8e and dABE8e started editing within 3 h of nucleofection, but the editing kinetics is much faster in nABE8e (Figures 3C and 3D). The edited cells were also subjected to clonogenic assay, and

we observed no difference in colony-forming potential between the samples (Figure 3E). Finally, we tested the levels of *P21* and *GADD45*, both markers of DNA damage response and observed that while nABE8e showed a slight elevation in RNA levels, dABE8e and the mock electroporated sample showed similar levels (Figure 3F). Thus, dABE8e would be a better approach, in terms of purity of outcomes, while editing regions that are highly homologous and during multiplexed editing for therapeutic applications.

DISCUSSION

As base editors are entering into clinical trials for the treatment of various disorders, there is an increasing interest in improving the precision of genome editing while minimizing undesired genomic alterations.[16,17] With precision genome engineering, there is often a very limited number of available gRNAs to generate the desired mutation, and hence the base editors must be precise, with minimal undesired outcomes.[3,18–20] It has been reported that base editors generate undesired genotoxic effects due to DNA nicking[11] and that single-strand breaks can have detrimental effects on cell fitness.[21] In this study, we show that large deletions are generated in homologous regions during base editing in a gRNA-dependent manner. These deletions can be overcome using nickase-deficient dABE8e that can edit efficiently even in $CD34^+$ HSPCs. The editing occurs even in $CD90^+$ HSPCs, and the edited cells show no lineage bias, suggesting the potential for long-term engraftment and repopulation. The editing efficiency by dABE8e is, however, lower than nABE8e by 20%–30% in all target sites tested. Hence, in non-homologous regions or sites that do not cause the formation of indels, nABE8e can be used because it would provide better editing efficiency. While here we demonstrate only the use of dABE8e, it is similarly possible to develop nickase-deficient cytosine base editors (CBEs) using hyperactive variants of CBEs.[22] A recent study has shown the proof of concept for overcoming indels using nickase-deficient CBEs.[23] In addition to targeting the homologous regions, dABE8e can be used for multiplexed editing in a single locus or simultaneous editing in complementary strands without risking the generation of large deletions or indels.

There are, however, certain limitations to our study. First, the approach was tested in two sites in the globin locus (*HBG* and *HBB* genes) to evaluate the abolishment of large deletions. There are other homologous loci such as *CCR5* and *CD33* that can be tested for the same. Additionally, cell-cycle dependence of dABE8e-mediated editing was not directly tested, and only an engraftment study would show the long-term repopulation potential of the cells edited with dABE8e. Considering the *in vitro* data, we believe that nABE8e is still a better option in non-homologous sites as the editing efficiency is evidently higher compared to dABE8e. However, dABE8e would be a suitable approach in terms of purity of outcomes while base editing in situations where two or more nicks are introduced in the same locus as in homologous regions or during multiplexed editing.

MATERIALS AND METHODS

Details of materials and methods used in the study can be found in the supplemental information.

DATA AND CODE AVAILABILITY

All data generated from this study have been made available in this paper.

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AUTHOR CONTRIBUTIONS

A.G.: conceptualization, methodology, investigation, formal analysis, visualization, data curation, and writing – original draft. P.S., N.S.R., and B.V.: methodology. S.M., S.T., and S.R.V.: resources. A.S.: resources and project administration. M.K.M.M.: conceptualization, supervision, formal analysis, funding acquisition, project administration, resources, and writing – review & editing. All authors read and approved the submitted version of the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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Supplemental information

Editing of homologous globin genes by nickase-deficient base editor mitigates large intergenic deletions in HSPCs

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Materials and Methods

Plasmid Constructs and gRNAs:

pLenti-ABE8e-puro vector was constructed as previously described[1] by inserting the base editor sequence from Addgene #138495 (Gift from David Liu) into lenti viral vector (Addgene #112675- Gift from Lucas Dow). pLenti-dCas9-ABE8e-puro vector was constructed by introducing H840A mutation in the Cas9 domain of pLenti-ABE8e-puro vector. dCas9 ABE8e was similarly constructed from Addgene #138495. lentiCas9n(D10A)-Blast (Addgene #63593- gift from Feng Zhang) was obtained from Addgene. All gRNAs were designed and cloned in pLKO5.sgRNA.EFS.GFP vector (Addgene #57822) as described previously[2]. Plasmids were isolated using NucleoBond Xtra Midi EF kit (Macherey-Nagel) according to the manufacturer's instruction. The synthetic gRNAs were purchased from Synthego. Details of gRNAs used in this study are listed in Supplemental Table 1

Cell Culture:

HEK 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% (v/v) FBS and 1× Pen-Strep. HUDEP 2 cells were cultured in Stemspan Media (SFEM II - STEMCELL Technologies) with 50ng/ml SCF (ImmunoTools), 1 µM dexamethasone (Alfa Aesar), 1 µg/ml doxycycline (Sigma-Aldrich), 1× L-glutamine 200 mM (Gibco), 3 U/ml EPO (Zyrop 4000 IU injection) and 1× Pen-Strep (Gibco) as per established protocols. After 8 days of editing, cells were set up for erythroid differentiation. Erythroid differentiation of HUDEP cells were carried out in a two phase protocol as described previously[3]. The peripheral blood mononuclear cells (PBMNCs) were obtained from a healthy donor according to the clinical protocols approved by the Intuitional Review Boards of Christian Medical College, Vellore. The PBMNCs were purified by density gradient centrifugation (Lymphoprep Density Gradient Medium|STEMCELL Technologies). CD34+

cells were isolated from the purified PBMNCs by EasySep Human CD34 positive selection kit II (STEMCELL Technologies) and expanded in HSC expansion media and further subjected to erythroid differentiation as per the 3 phase protocol described previously[4].

Lenti-virus production and transduction

Lenti-virus for stable cell lines and for gRNAs were prepared in HEK293T cells using Fugene HD as described previously[2]. The second generation packaging constructs pMD2.G and psPAX2 (Addgene #12259, 11260) were a gift from Didier Trono. The viral supernatant at 48hrs and 72hrs from 10 cm dish was collected, concentrated, and resuspend in 240ul of 1x PBS separately. From this 30ul was used to transduce 0.1 million HUDEP-2 cells taken in 24 well plate with 1% HEPES and 3µg polybrene (Sigma-Aldrich). Spinfection was carried out at 800G for 30 minutes at room temperature. Stable cell lines were prepared by maintaining the transduced cells in Puromycin or Blasticidine as appropriate. gRNA transduction efficiency was evaluated by measuring GFP expression using flow cytometry.

In Vitro transcription of base editor mRNA

nABE8e and dABE8e were synthesised using the plasmids described above using Takara IVTpro™ mRNA Synthesis System as per the manufacturer's protocol. Complete substitution of N-methyl pseudouridine (Trilink) was done and co-transcriptional capping was performed using ARCA (Jena Biosciences). mRNA was made into aliquots and stored in -80 until use.

Nucleofection of Base editing components

5ug of base editor mRNA along with 100pmol of gRNA was electroporated in 0.5 million cells using Maxcyte GtX electroporation system using the program HSC3 (HSPCs/HUDEP). The mRNA:gRNA:cells ratio was maintained while scaling up or down except for the dose dependency study where the cells number was maintained at 0.5 million and the cargo was

varied. Cells were allowed to recover at 37 degree Celsius for 15 minutes before transferring to prewarmed media.

Genomic DNA isolation

DNA was isolated from the cells on day 8 after transduction for lentiviral experiments or on day 5 after electroporation (unless specified) of HSPCs using QiaAMP DNA blood mini kit (Qiagen) as per the manufacturer's protocol and eluted in nuclease free water. For experiments requiring repeated sampling DNA was extracted using QuickExtract (LGC Biosearch Technologies).

Analysis of editing efficiency

The target region was amplified using primers listed in Supplemental Table 2. Sanger sequencing or next generation sequencing (Illumina Nova seq 6000) was performed as appropriate. Sanger sequencing data was analysed using EditR[5] or ICE(Synthego)[6] and NGS data was analysed using CRISPResso[7].

Evaluation of 4.9 kb deletion using qRT PCR

Primers were designed targeting deleted intervening region (on target) as well as the locus control (Supplemental table2). qRT PCR was performed as described previously using QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). SsoFast EvaGreen Supermix (Takara) was used along with 5 pmol each of forward and reverse primer and 50ng of DNA for the PCR.

Evaluation of 4.9kb deletion using ddPCR

BioRad ddPCR system was used for quantification of 4.9kb deletion using ddPCR supermix for probes. The primers and probes used are listed in supplemental table 2. The frequency of

deletion was calculated by normalizing the ratio of the deletion probe with locus control probe against the average ratio observed in unedited control samples

Evaluation of large deletion using PCR

Forward primer specific to HBG2/HBD and reverse primer specific to HBG1/HBB was designed to specifically amplify the fusion gene product generated after large deletion. 100 ng of DNA was amplified using the appropriate primer set using GoTaq Hotstart master mix (Promega) as per the manufacturer's protocol.

Intracellular HbF staining

0.5 million cells were processed and stained for intracellular HbF as described previously[8] using 2ul of antibody (Fetal Hemoglobin Monoclonal Antibody APC Invitrogen) per sample. The F positive cells were evaluated by Flow Cytometry (CytoFLEX LX Flow Cytometer – BC).

RP-HPLC

HUDEP2 cells were collected after erythroid differentiation, washed with 1x PBS, and resuspended in sterile water. The cells were lysed by sonication and the supernatant was used for reverse phase HPLC (Shimadzu Corporation-Phenomenex) to quantify globin chains as described earlier[3].

CD90 sorting

CD34+ve cells were stained using Antihuman CD90 antibody (BD)(5ul/10^6 cells), washed and taken for sorting using BD Aria III.

CFU assay

Colony forming assay was performed using Methocult Medium (Stem Cell technologies). 500 cells per sample were seeded in the medium as per the manufacturer's protocol. Colonies were enumerated after 14 days.

RNA isolation

RNA was isolated from the cultured cells using NucleoSpin RNA XS Kit (MN) as per the manufacturer's protocol. For Reverse transcription 1ug of RNA was used with iScript cDNA synthesis kit (BioRad).

qRT PCR

Relative gene expression (ΔΔCT) was measured using primers listed in supplemental table 2. GAPDH was used as internal control for normalising the gene expression. Commercially available primers targeting GAPDH and CDKN1a were purchased from Qiagen (QuantiTect primer assay) for the assay and used with TB green premix (Takara) for quantifying gene expression.

Statistical Analysis

All experiments are performed in triplicates unless specified otherwise. HUDEP2 lenti-virus experiments were performed as biological duplicates. Statistical analysis was performed using GraphPad Prism 8.1. The details of statistical methods are provided in the respective figure legends.

Supplemental Note 1

To test if the frequency of large deletion is in any way associated with the gRNA efficiency, we evaluated the efficiency score of each gRNA predicted by 2 different tools- E CRISP [12], a hypothesis driven tool and DeepSpCas9 [13], a machine learning based tool. Interestingly we

found that G3 had the lowest score in both tools while G11 had very high efficiency score and this observation is in line with the deletion frequency of each gRNA (Figure 1A). We also evaluated the microhomology score for the three gRNAs using CRISPR RGEN tool as MMEJ pathway was reported to reduce the NHEJ frequency while increasing the frequency of large deletion. Interestingly, G11 exhibited a very high MMEJ score compared to both G2 and G3 (Figure S1 E), suggesting that the presence of microhomology could be a contributing factor for increased deletion frequency. However, further evaluation with multiple gRNAs targeting different loci is required to reach any conclusion regarding the association of gRNA properties with frequency of deletion

Table S1: List of gRNAs used in the study

Sl No	Name	Sequence	Source
1	HBG G2	CTTGACCAATAGCCTTGACA	Ravi et al[1]
2	HBG G3	ATATTTGCATTGAGATAGTG	Ravi et al
3	HBG G4	GTGGGGAAGGGGCCCCCAAG	Ravi et al
4	HBG G11	GGCAAGGCTGGCCAACCCAT	Ravi et al
5	AAVS1	GGGGCCACTAGGGACAGGAT	Ravi et al
6	HBB1	CATGGTGCATCTGACTCCTG	This Study
7	HBB2	GTAACGGCAGACTTCTCCTC	This Study
8	HBB3	CCACGTTCACCTTGCCCCACA	Prasad et al[9]
9	HBB4	CGTGGATGAAGTTGGTGGTG	Venkatesan et al[10]

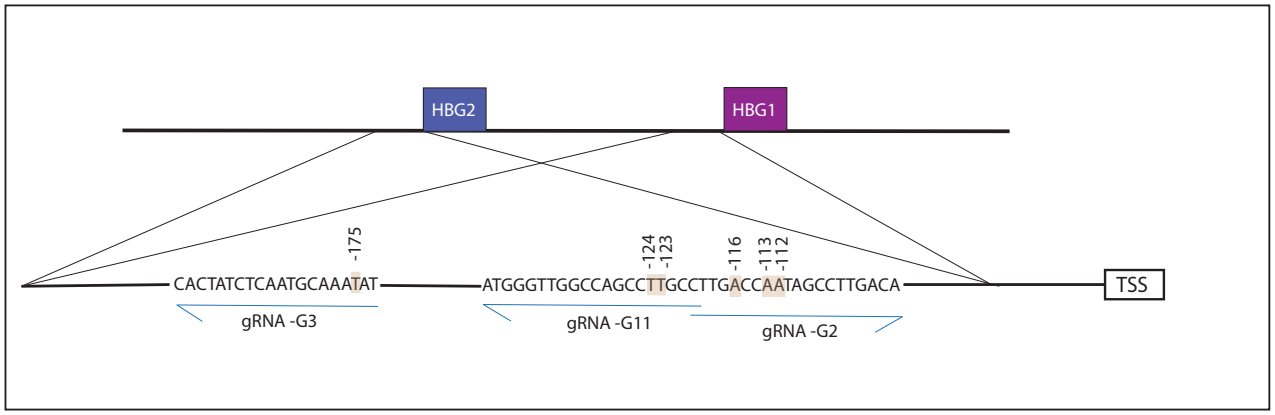
Table S2: List of primers used in the study

	Name	Sequence
1	HBG_NGS_F	TACACGACGCTCTTCCGATCTGGAATGACTGAATCG GAACAA
2	HBG_NGS_R	AGACGTGTGCTCTTCCGATCTGACGTTCCAGAAGCG AGTGT
3	HBG_SAN_F	ACAAAAGAAGTCCTGGTATC
4	HBG_SAN_R	CTTCCCAGGGTTTCTCCTCC
5	HBB_SAN_F	GTCATCACTTAGACCTCACC
6	HBB_SAN_R	CTGTACCCTGTTACTTATCC

7	HBD_SAN_F	ATGTAGAGGAGAACAGGGTT
8	HBD_SAN_R	CCATCACTAAAGGCACCTAG
9	AAVS1_SAN_F	GAGATGGCTCCAGGAAATGG
10	AAVS1_SAN_R	ACCTCTCACTCCTTTCATTTGG
11	HBG_RT_DEL_F	AGGGGCTCAACGAAGAAAAAGTGT
12	HBG_RT_DEL_R	CACTTCATTGTAGTTACCGTGGAAAGA
13	HBG_RT_LC_F	AAATGAATCAGCAGAGGCTCAC
14	HBG_RT_LC_R	ATGCACTAACATCCAACTATACAAAA
15	HBB_RT_DEL_F	CACTTTCTTCTGACATAACA
16	HBB_RT_DEL_R	CACAGGGCAGTAACGGCAGA
17	dd_HBG_DEL_F	ACGGATAAGTAGATATTGAGGTAAGC
18	dd_HBG_DEL_R	GTCTCTTTCAGTTAGCAGTGG
19	RT_GADD45_F[11]	GAGAGCAGAAGACCGAAAGGA
20	RT_GADD45_R	CAGTGATCGTGCGCTGACT

Figure S1

A)

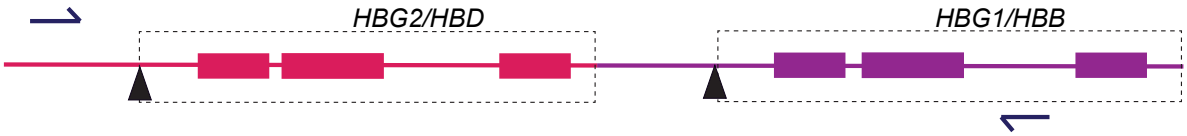


B) Strategy for detection of 4.9 kb deletion by qRT PCR

C) Strategy for detection of 4.9 kb deletion by probe based ddPCR

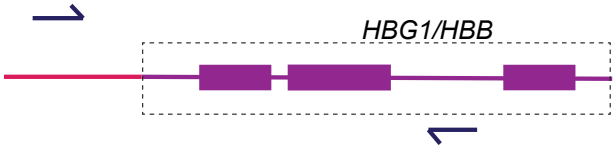
D) Strategy for deletion specific PCR

Intact Genes



No amplification

Fused Gene after interstitial deletion



Amplicon ~1690 bp (HBG2/HBG1)
~ 800 bp (HBD/HBB)
qRT PCR - ~96 bp

Figure S1: Strategies for detection of interstitial deletion

A) Schematic representation of gRNAs targeting the gamma globin promoter used in the study showing the position and target bases. **B)** Schematic representation of the method for detection of 4.9kb deletion in the gamma globin locus by qRT PCR. The black triangles represent the gRNA cut site. Green arrows represent the primers specific to the deleted region, the amplification of which will be reduced proportionate to deletion. Orange arrows represent the locus control primers. **C)** Schematic representation of the method for detection of 4.9kb deletion in the gamma globin locus by probe based ddPCR. The black triangles represent the gRNA cut site. Blue arrows represent the primers specific to the deleted region and green circle represents the probe (FAM) signal of which will be lost in DNA with 4.9kb deletion. Yellow arrows represent the locus control primers and red circle indicates the corresponding probe (HEX) **D)** Representation of the approach for PCR amplification of fusion gene formed by 4.9kb/7.4 kb deletion. If the gene is intact there will be no amplification with an extension time of 2 minutes while the presence of deletion will give a band at ~1690 bp in gamma globin locus and at ~800 bp in beta globin locus which can be detected by agarose gel electrophoresis.

Figure S2

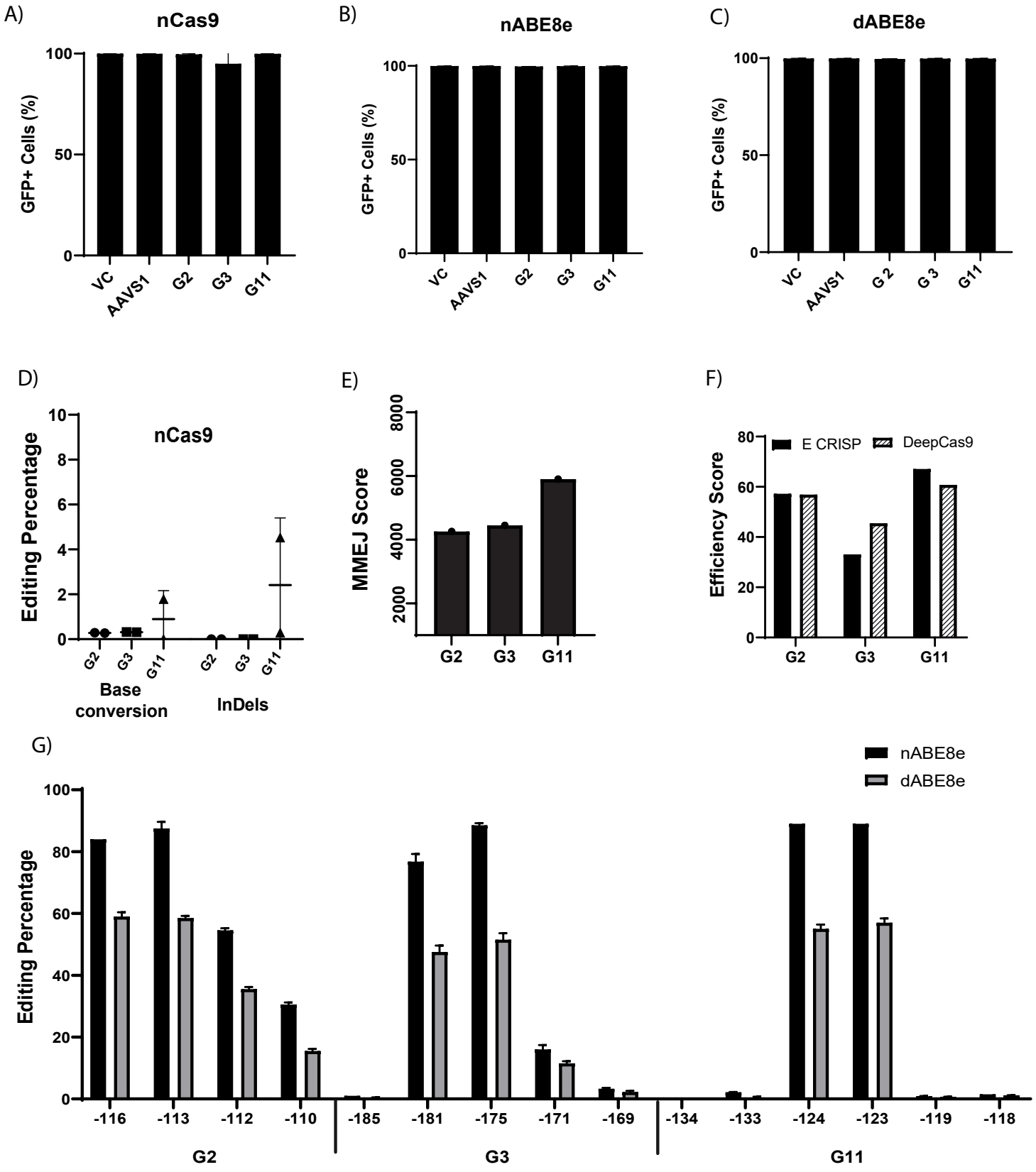


Figure S2: Evaluation of dABE8e in HUDEP2 cells

A-C) Percentage of GFP positive cells as a measure of transduction efficiency evaluated by flow cytometry in HUDEP nCas9 stables (A), nABE8e stables (B) and dABE8e stables (C). **D)** Base conversions and InDels obtained while editing in the nCas9 stables with respective gRNAs evaluated by NGS **E)** MMEJ score of the gRNAs predicted by CRISPR RGEN tool **F)** gRNA efficiency score predicted by ECRISP and deepSpCas9 algorithms **G)** Percentage of individual base conversions at the gamma globin promoter with each gRNA measured by NGS in nABE8e and dABE8e edited samples. (Data represented as mean± SD. n=2 for all samples)

Figure S3

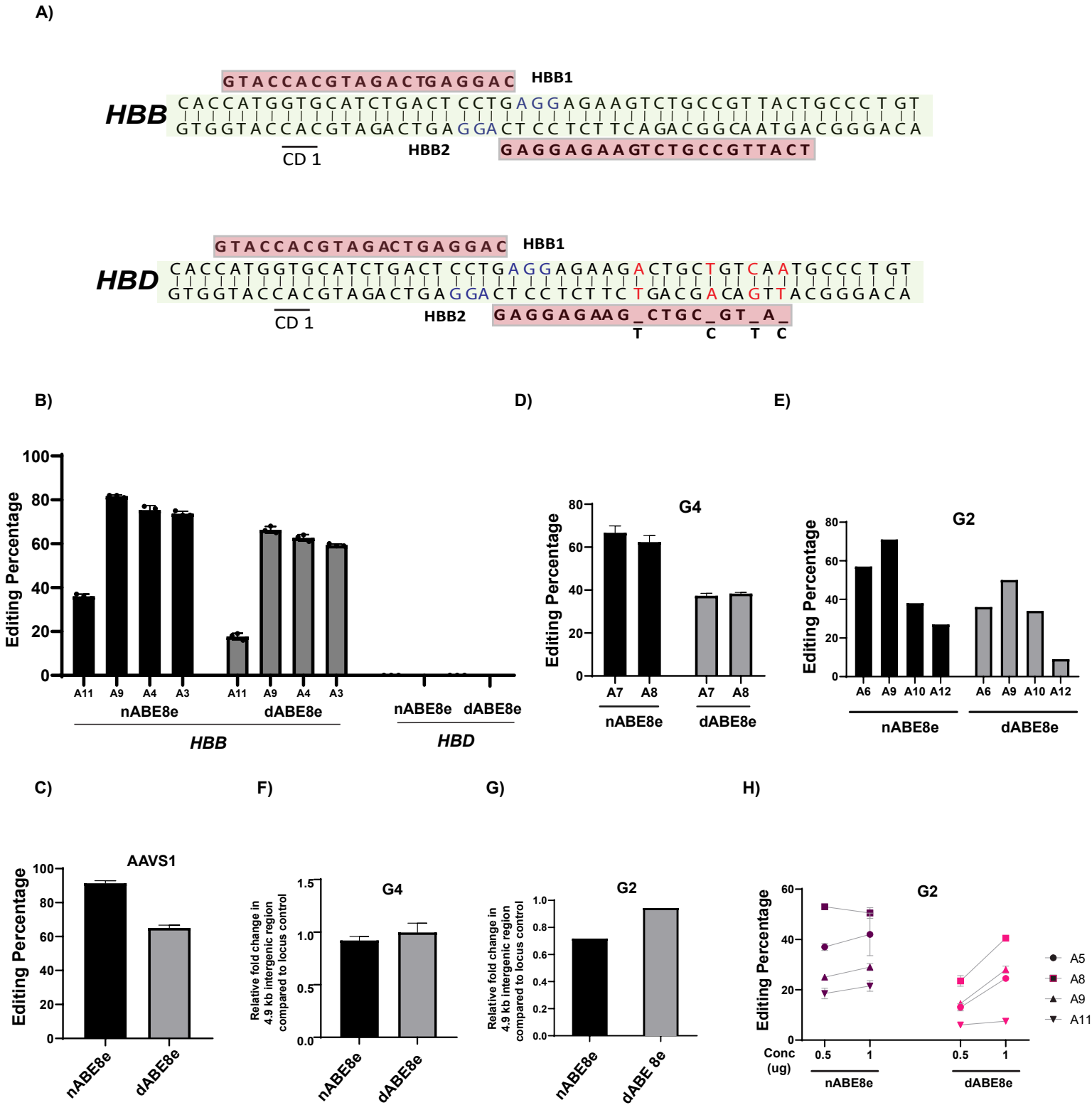


Figure S3: Evaluation of dABE8e in CD34+ve HSPCs

A) Schematic representation of the position of gRNAs in the beta globin and delta globin genes. Red colour indicating bases that differ in *HBD* gene and dashes representing the mismatches of HBB2 in *HBD*. **B-E)** Editing efficiency in HSPCs using HBB2 gRNA (B), AAVS1 gRNA (C), G4 (D) and G2 (E) measured by sanger sequencing. (n=3 for all data except E where n=1) **F-G)** Reduction in 4.9kb intergenic region caused by interstitial deletions while targeting the gamma globin promoter using G4 (F) and G2 (G) measured as relative fold change compared to locus control by qRT PCR and normalised using unedited control. **H**) Editing efficiency in HSPCs at 48 hrs after electroporation with varying concentrations of mRNA(nABE8e or dABE8e) evaluated using Sanger sequencing(n=2).

Figure S4

A)

B)

Figure S4: Multiplexed base editing in CD34+ve HSPCs

A) Schematic representation of the position of gRNAs in the beta globin gene used for multiplexed editing.

B) Representative InDel patterns generated after multiplexed base editing using nABE8e measured by sanger sequencing and analysed by Synthego ICE

Figure S5

A)

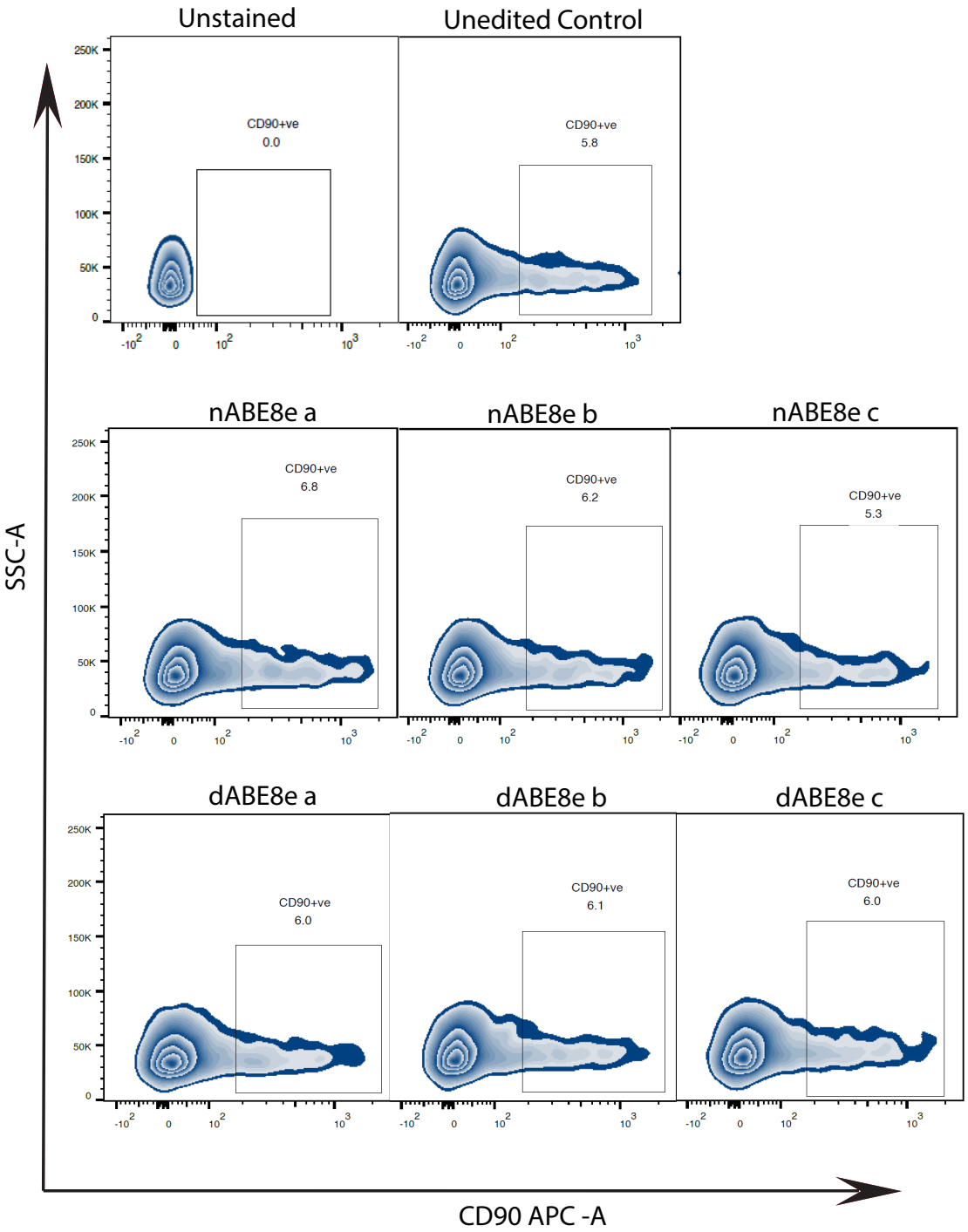


Figure S5: Representation of fluorescence activated cell sorting of HSPCs using CD90 APC conjugate

CD34 +ve HSPCs were edited with nCas9ABE8e /dCas9 ABE8e and editing was evaluated in bulk population and compared with CD90+ve HSPCs which were sorted from bulk population

Supplemental References

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