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Original Research Article

Optimized dual-AAV base editor delivery system with enhanced editing efficiency and virion production titer

Wenjia Yu [a,b,1], Yujie Wang [c,1], Siwei Li [b,1], Yingcai Dai [d,1], Yucheng Li [e], Xinyue Zhang [b], Bo Li [b], Siriguleng Qian [a,*], Xueli Zhang [b,**], Changhao Bi [b,***]

[a] School of Biological Engineering, Dalian Polytechnic University, Dalian, China
[b] Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin, 300000, China
[c] Binzhou Medical University, Shandong, China
[d] Shanghai Jiao Tong University, Shanghai, China
[e] Tianjin University of Science and Technology, Tianjin, China

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ABSTRACT

Base editors (BEs) are a promising tool for precise base conversion in human cells and animals, while the adeno-associated virus (AAV) is the major vector for human gene therapy. However, the size of the DNA cassette required for BE expression exceeds the 4.7 kb packing capacity of the AAV vector, making dual-AAV approaches based on *trans*-splicing intein necessary. Even with this approach, current split DNA cassettes are still larger than the AAV packing limit, posing a challenge for cellular production of AAV. Moreover, some split strategies yield variable editing results and target coverage. To address these limitations, 25 different split sets for BE4max and A3A-BE4max were tested at two target sites respectively, with splitting sites ranging from 493rd to 517th amino acids on the Cas9 peptide. Fortunately, the best Cas9 split site was identified between His511 and Ser512 and the arrangement of the AAV expression cassette was further manipulated to create evenly distributed CBE and ABE intein systems within 4.7 kb. These novel dual-AAV systems, designated 4.6AAV-CBE and 4.7AAV-ABE, were found to have base editing efficiencies similar to wild-type BEs, with a narrower editing window than the current 573 split system. Notably, 4.6AAV-CBE yield a higher AAV production titer, up to 2.1-fold in AAV-N and 1.5-fold in AAV-C, compared to the split-573BE system, likely due to the reduction of DNA cassette size within the AAV packaging capacity. Moreover, after packaging and infecting cells with AAV-N and AAV-C at the same volume and number of cells, the multiplicities of infection (MOI) and editing efficiency of 4.6 AAV-CBE were both higher than those of the split-573BE system. This study present advanced dual-AAV systems for ABE and CBE delivery, establishing a basis for safe and efficient BE therapies.

1. Introduction

The bacterial clustered regularly interspaced short palindromic repeats (CRISPR) associated system is making rapid advancements [1–6], with base editing being a promising method for efficiently changing single nucleoside without making double-stranded DNA breaks (DSBs) [7]. Cytosine base editors (CBEs) and adenine base editors (ABE) are molecular tools that can convert specific base pairs in DNA. Cytosine base editors (CBEs) convert C•G base pairs to T•A pairs, whereas adenine base editors (ABEs) convert A•T base pairs to G•C pairs [8–10]. Excluding the promoter and terminator, the main functional module of BE4 is approximately 5.5 kb, is composed of a cytidine deaminase (rat APOBEC1), a *Streptococcus pyogenes* Cas9 nickase (nCas9), and two copies of uracil glycosylase inhibitor (UGI). Similarly, ABEmax without promoter and terminator, ~4.8 kb in length, contains a single-chain heterodimer comprised of a wild-type noncatalytic TadA monomer and evolved ecTadA monomer (TadA-TadA*) fused with nCas9.

The successful therapeutic applications of base editors requires

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* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: srgl2000@163.com (S. Qian), zhang_xl@tib.cas.cn (X. Zhang), bi_ch@tib.cas.cn (C. Bi).
[1] These authors contributed equally.

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efficient and safe delivery to target cells or organs *in vivo*. Viral systems, such as adeno-associated virus (AAV), lentivirus, and adenovirus, have been used as delivery vectors for genetic therapies. While most *in vivo* gene editing applications have utilized AAVs, only a few preclinical studies have employed lentiviruses or adenoviruses [11]. Notably, ongoing clinical trials have used AAV vectors to deliver genes encoding many therapeutic proteins for the treatment of genetic diseases [12–14]. In comparison to non-viral delivery systems, AAV-based vectors provide significant advantages. AAV vectors are generally characterized by their ability to mediate stable and long-lasting gene expression, reducing the need for repeated administrations. Furthermore, AAV vectors possess a low immunogenic profile, which enhances their suitability for therapeutic applications in humans. This contrasts sharply with non-viral methods, which often suffer from low transfection efficiency and transient expression, necessitating multiple rounds of treatment [15].

However, the size of most base editors exceeds the AAV packing capacity of approximately 4.7 kb DNA cargo [16,17]. Despite the identification of compact Cas9 variants such as SaCas9 and CjCas9, which can be efficiently packaged within a single adeno-associated virus (AAV) vector, editing efficiency and stability persist as crucial determinants for the success of AAV-mediated gene therapy. While the huge size of SpCas9 (1368 amino acids) presents significant challenges for AAV delivery, it remains the most extensively studied and widely used Cas9 protein in gene therapy applications [18,19]. Therefore, investigating the optimal split sites of SpCas9 is critical for dual-AAV delivery strategy. To overcome this limitation, the BE expression cassette can be divided into two halves and packaged into two individual AAVs. In the same cell, the two AAVs are transfected simultaneously, and the full-length gene editing agent is reconstructed via RNA *trans*--splicing or intein-mediated protein *trans*-splicing mechanisms [20,21]. However, the editing efficiency of ABE delivered via RNA *trans*-splicing is low, as observed in a preclinical study using mice [22].

The intein-mediated protein *trans*-splicing approach has demonstrated high efficiency for *in vivo* genome editing. This technique involves the use of an autocatalytic internal protein domain, intein, which catalyzes self-splicing and ligation of two flanking peptides to generate a functional full-length protein at the posttranslational level. Previous studies have utilized split-Cas9 based on intein to achieve efficient genome editing, but the reported split sites of Cas9 are still limited and some base editors cassettes were not equally divided, leading to imbalanced N- and C-terminal parts [23,24]. Additionally, the split BE can affect reconstituted activity and structural stability, resulting in variable editing efficiencies and target scopes. Furthermore, current split DNA cassettes are still larger than 4.7 kb, especially the cellular factors fused BEs [25,26], which poses a challenge for cellular production of AAV.

Therefore, it is necessary to identify better split sites of Cas9 and redesign the system for dual AAV delivery of BEs. In this study, we screened 25 splitting sites and identified a novel split site that balances the CBE and ABE systems, resulting in even division of the dual AAVs and smaller cargo sizes. We developed novel dual-AAV systems of ABE and CBE that efficiently edited target sites and had higher AAV production titers and transduction efficiency.

2. Results

2.1. Design and screening of optimal split sites for Streptococcus pyogenes Cas9 (SpCas9)

To optimize the dual-AAV BE delivery systems, we constructed a group of split-BE candidates for genome editing based on the smaller CBE3.9max, which omitted the second UGI from BE4max without influencing the editing efficiency [27]. An editing intein$_N$ from *Nostoc punctiforme* (Npu) was fused to the C-terminal of the CBE3.9max N-terminal half, whereas the CBE3.9max C-terminal half contained an intein$_C$ at its N-terminal, resulting in a couple of fusion constructs, designated CBE-N and CBE-C. In the split-CBE system, the U6-gRNA expression cassette was inserted proximal to the 3′ ITR on the reverse strand of the Cas9-expressing strand. To accommodate the essential elements of the vector, including promoter, terminator poly(A), intein, and U6-gRNA within the loading capacity of AAVs (4.7 kb), we divided the CBE3.9max (5.3 kb) and a U6-gRNA expression cassette (0.35 kb) evenly into two parts by setting the split points in a small middle region (Fig. 1A). Twenty-five relatively even-split CBE3.9max sets were created with split sites ranging from 493 to 517 on the nCas9 peptide. The DNA cargo length of all the split AAV CBE systems was shorter than that of the previously reported 573-split system (Fig. 1A).

Previous studies have shown that Cas9 activity is highly dependent on the position of the intein split site [27,28]. To evaluate the activity of the 25 split-CBE sets, we measured their C-to-T conversion efficiency at the endogenous *ZNF410* target site9 in HEK293T cells. After 72 h of transfection with the 25 variants of the split-intein system (CBE-N and CBE-C) and a U6-expressed gRNA, the cells were analyzed by targeted high-throughput DNA sequencing. All the split nCas9 sets generated detectable C-to-T editing, although the efficiency varied widely, ranging from 1.6 % to 38 %, suggesting that the location of intein split sites greatly affected the activity of reconstituted BEs. Among the candidates, the split-498CBE and split-511CBE sets exhibited high editing efficiency, comparable to that of the wild-type full-length BE4max (Fig. 1B). To further validate the feasibility of this resolution, we conducted experiments on another target site, *ZNF410 site8*, utilizing 25 split-A3ABE4max base editors with the same split strategy. The results also demonstrated that the 511 splitting site exhibited the highest editing efficiency. In addition, it was reported that the N-terminal amino acid of Cas9C should be Cys, Ser or Thr to ensure high splicing efficiency, we selected split-511BE for further research [29].

2.2. Efficient gene editing by optimized split-CBEs at endogenous sites in mammalian cells

To analyze the performance of split-511CBE for general applications, we evaluated it at six endogenous genomic loci, including *RP11*, *PRNP*, *VIST*, *NONO5*, *NONO8* and *HERC5* in HEK293T cells. The split-CBE3.9max expressing plasmid and gRNA-expressing plasmid were transfected into HEK293T cells, and the editing efficiency was detected by next-generation sequencing (NGS). The C-to-T base editing efficiency is shown in Fig. 2A. The full length base editor and the split-573BE were tested as the positive control. Meanwhile, we also constructed the dual AAV split-A3ABE3.9max with the 511-split site. The editing efficiency from six target sites (*RP11*, *NONO site 3*, *ZNF410* site 9, *HDAC5*, *HEK1* and *HERC5*) were similar to that of the full-length base editors (Fig. 2B). These data indicated that split-511CBE could induce gene editing with high efficiency at endogenous genomic sites in human cells.

2.3. 46AAV-CBE has a higher virus production yield and delivery efficiency

With the split site between 511 and 512 on the spCas9 peptide, we divided the BE coding sequence evenly into two parts. Accompanied by rearrangement of the expression elements, two AAV production plasmids smaller than the previously reported split-573BE system were obtained (Fig. 1A). The two plasmids of split-511CBE were 4695 bp and 4688 bp, both within the 4.7 kb package capacity of AAV, designated 4.6AAV-CBE. The virus production titer of 4.6AAV-CBE was analyzed in comparison with the split-573CBE system, which was achieved via qPCR of episomal DNA isolated from producing cell lysate. The novel 4.6AAV-CBE enhanced AAV production by 2.1-fold for AAV-N, and 1.5-fold for AAV-C compared with the split-573CBE system (Fig. 3A and C). To further verify this conclusion, the titer of virus production with split A3ABE4max was tested and the novel 4.6AAV-A3ABE4max enhanced AAV production by 1.5-fold for AAV-N, and 2-fold for AAV-C compared with the split-573CBE system (Fig. 3B). Next, we performed a

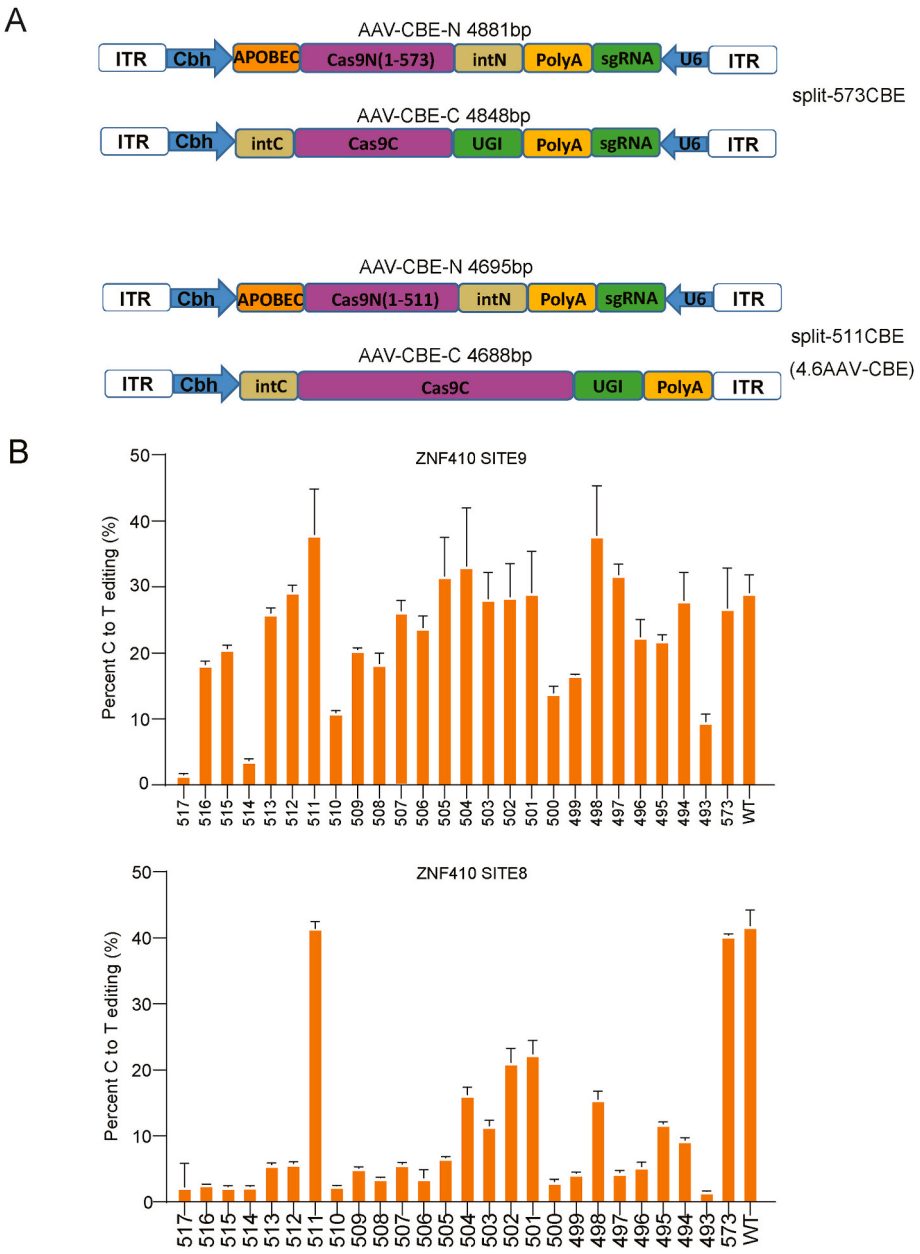

Fig. 1. Base editing efficiencies of the candidate split-CBE sets. (A) Schematic of intein-based split CBE constructs. To ensure high expression, a strong synthetic mammalian promoter (CBh, blue) and a bovine growth hormone (bGH, yellow) polyadenylation site are used. The inverted terminal repeats (ITRs) are shown in white; the intein is shown in earthy yellow; split Cas9 DNA is shown in red violet; deaminase is in orange, and UGI is in green. For gRNA expression (light green), the U6 promoter was chosen (blue). (B) The editing efficiencies of 25 split-CBE sets on genomic locus *ZNF410 site9*. Full-length wild-type base editor and split-573CBE are the controls. The error bars represent the standard deviation calculated from three experiments. (C) The editing efficiencies of 25 split-A3A-BE4max sets on genomic locus *ZNF410 site8*. Full-length wild-type base editor and split-573A3A-BE4max are the controls. The error bars represent the standard deviation calculated from three experiments.

comparative analysis of the split-573CBE system and the novel 4.6AAV-CBE system to evaluate their transduction efficiency and editing performance in HEK293T cells. The harvested AAV-Ns and AAV-Cs were combined at equivalent titer, cells were infected with viral compound at progressively increasing MOI to evaluate dose-dependent transduction efficiency. The best editing efficiency of split-511CBE system achieved 27.0 % ± 0.79 % at the ZNF410 SITE9 locus, demonstrating significant superiority ($p < 0.05$) compared to the split-573CBE system, which reached an average efficiency of 20.0 % ± 0.52 % (Fig. 3D). These results indicated that 4.6AAV-CBE could be produced with higher titers, transduction cell efficiency and editing efficiency relative previous split systems, probably due to the DNA cassette size was reduced within the AAV package capacity. The higher virus yield is highly applicable, which reduces the AAV production cost of genetic therapies.

2.4. *Efficient A-to-G editing by 4.7AAV-ABE in HEK293T cells*

Inspired by 4.6AAV-CBE, the 511-split site was used to construct the ABE dual-plasmid system. To ensure even loading length of AAV-N and AAV-C, we placed the U6 sgRNA expression cassette on the AAV-C vector proximal to the 3′ ITR on the reverse strand of the Cas9 coding sequence. With rearrangement of the expression elements, a dual-AAV system of the ABE was developed, with N- and C-terminal cargo sizes just above 4.7 kb, designated 4.7AAV-ABE (Fig. 4A). By contrast, the

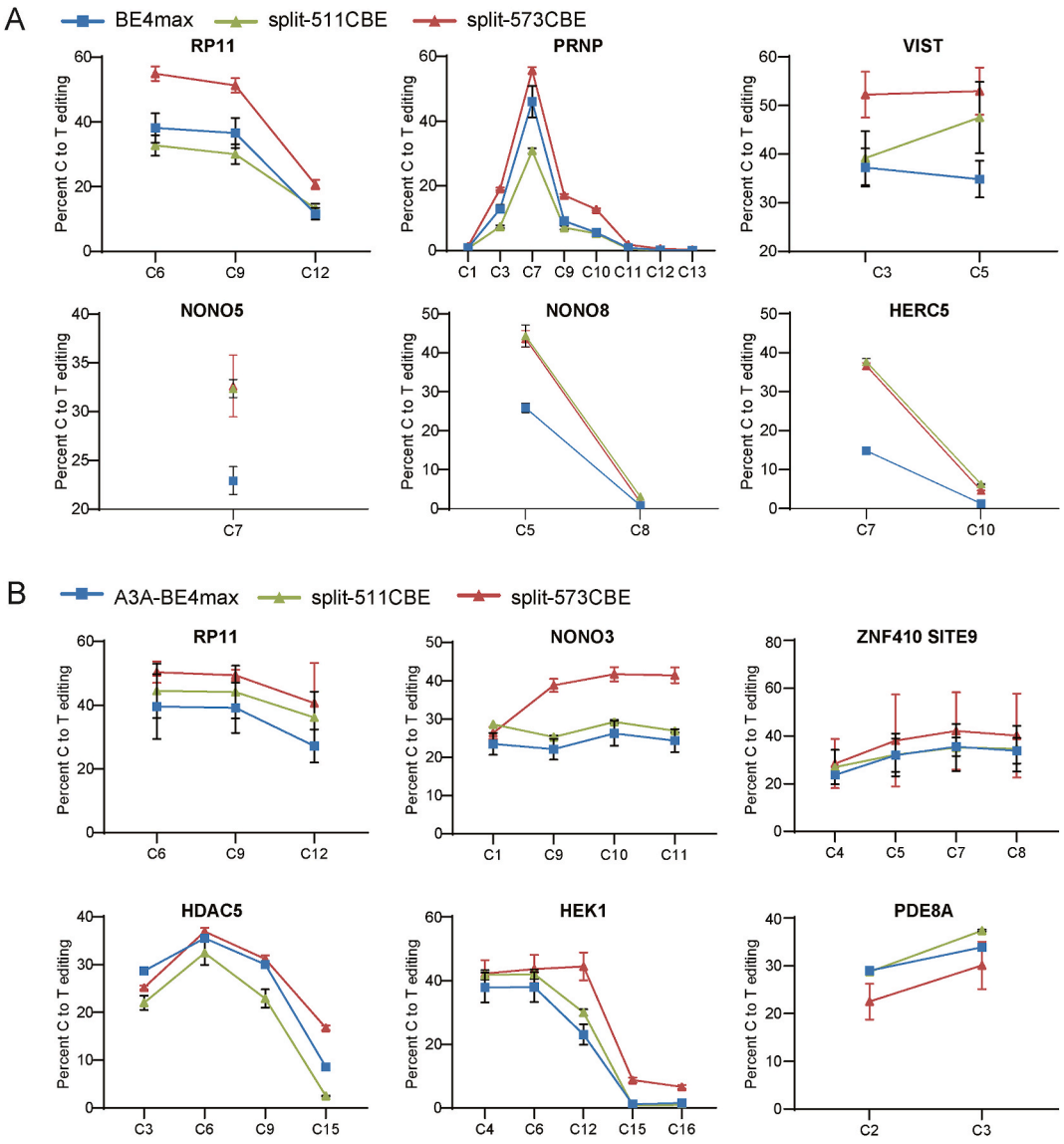

Fig. 2. Editing efficiency of split-511CBEs in HEK293T cells. (A) Comparison of the editing efficiency of wild-type BE4max, split-511BE3.9max and split-573BE3.9max at six genomic loci in HEK293T cells. (B) Comparison of the editing efficiency of wild-type A3A-BE4max, split-511A3ABE3.9max and split-573A3ABE3.9max at six genomic loci in HEK293T cells. The error bars represent the standard deviation calculated from three experiments.

AAV plasmid with the 573-split site was larger than 4.9 kb, which might greatly affect its virus production titer. We constructed both ABEmax and SpRY ABEmax based on 4.7AAV-ABE to test their editing efficiency at six genomic loci (*VISTA hs267 site1*, *ABCA3*, *PSMB2*, *EMX1*, *RP11* and *ANO5*) for ABEmax and six genomic loci (*RP1*, *HEK9*, *VISTA hs267 site2*, *PSMB2 site2*, *HEK2* and *VISTA hs267 site3*) for SpRY ABEmax in HEK293T cells. As expected, both the 4.7AAV-ABE systems showed similar gene editing efficiency as the full-length wild-type ABE (Fig. 4B and C). The results demonstrated that 4.7AAV-ABE systems efficiently edit human cells and can be applied to various applications, such as the genetic therapies.

2.5. Comparison of split-511BEs with split-573BEs

The split site 573 of Cas9 has been widely used in the delivery of base editors via AAV vectors, which was found to have a broadened editing window relative to the wild type in our previous studies. To compare the editing windows of WT, 4.6AAV-CBE and split 573CBE, A3ABE3.9max was split with the above strategies; the editing efficiencies and the target scopes of the three systems were tested at four endogenous sites. Split-573 based A3ABE3.9max revealed a wide, promiscuous editing window, with efficient editing (~33–45 %) occurring from C10 to C13. By contrast, the target scope of 4.6AAV-CBE based A3ABE3.9max was similar to that of the wild-type system at the four endogenous target sites (*ZNF410 site 8*, *PRNP*, *VIST* and *NONO site 8*) (Fig. 5A). These results demonstrated that the 4.6AAV-A3ABE3.9max system had a narrower editing window, similar to WT; and the increased specificity is an advantage for genetic therapies.

To determine the safety for better application of the splitting systems, we investigated the off-target editing potential and indel frequencies of AAV split systems with the wild-type base editors. To evaluate the Cas-dependent off-target editing frequencies of CBE, we amplified and detected the editing efficiencies of the potential off-target sites from two endogenous loci *ZNF410 site 9* and *VIST* with 4.6AAV-CBE, split-573CBE and BE4max in HEK293T cells. The potential off-target sites were generated using Cas-OFFinder. Similar off-target mutation rates were observed with the three tested systems (Fig. 5B). The same analysis was also performed for the NG. ABEmax systems, and similar off-target effect was observed at *VISTA hs267* and *ABCA3* loci (Fig. 5C). These results suggested that the 4.6AAV-CBE and 4.7AAV-ABE

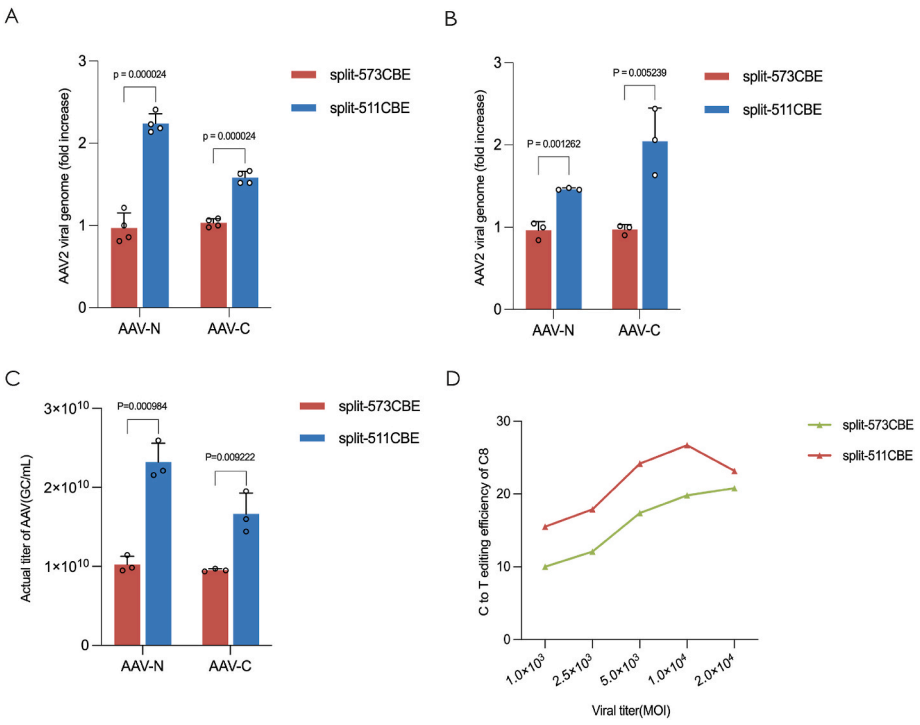

Fig. 3. Relative production titers and delivery efficiency of AAV vectors produced from 4.6AAV-CBE and split-573CBE. (A) The viruses were harvested 3 days after transfection, and the viral titer with split CBE was analyzed via real-time PCR. (B) The viruses were harvested 3 days after transfection, and the viral titer with split A3A-BE4max was analyzed via real-time PCR. (C) The actual viral titer. (D) The viruses were harvested 3 days after transfection, AAV-N and AAV-C were used to infect the cells at equal amounts and analyzed for editing efficiency through deep sequencing. The error bars represent the standard deviation calculated from three experiments. ***, $p < 0.001$.

strategies did not increase the off-target potential. Similarly, the difference of indel rates across the protospacer among the tested systems was not significant (Fig. 5D). The results demonstrated that the split-511BE systems did not induce higher indel frequencies than wild-type and split-573BE.

To test how the split-511BE performed in human cell lines other than HEK293T cells, we assayed the ability of split-511BE to edit six target loci in Hela cells. These targets could be edited by split-511BE system in Hela cells with the editing efficiency comparable to the wild type and split-573BE system (Fig. 6A and B).

3. Methods

3.1. Plasmid construction

AAV dual vectors used for HEK293T transfection were generated with the Gibson Assembly method. For rAAV experiments, the plasmids Cbh-V5 AAV-CBE-N-terminal (Addgene; #137175) and Cbh-V5 AAV-CBE-C-terminal (Addgene; #137176) were used to construct the different rAAV plasmids. A strong synthetic mammalian promoter CBh was used to drive the expression of the split CBE and ABE in the rAAV vectors. The AAV-N and AAV-C templates were subcloned from the BE4max, A3ABE4max, NG. ABEmax and SpRY ABEmax plasmids, and new split AAV sets were constructed by replacing the CBE sequence in the plasmids Cbh-V5 AAV-CBE-N-terminal and Cbh-V5 AAV-CBE-C-terminal plasmids. The gRNA expression plasmids were assembled using the Golden Gate method with the N20 sequence embedded in the primers. The expression of sgRNA was under the control of a U6 promoter. All PCR products were amplified with PrimeSTAR (TaKaRa). The AAV-RC and pHelper plasmids were purchased from Addgene, and all plasmid constructs were verified by sequencing.

3.2. Cell culture and transfection

HEK293T cells and Hela cells obtained from ATCC were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10 % (v/v) fetal bovine serum (FBS) at 37 °C with 5 % CO_2. Cell transfection was carried out by seeding cells in 24-well plates (Corning, USA) for approximately 24 h and transfecting at approximately 40 % confluence with 350 ng AAV-N plasmid, 350 ng AAV-C plasmid and 200 ng gRNA plasmid. Wild-type base editors (600 ng) and 300 ng gRNA were co-transfected as the control. Genomic DNA was extracted from the cells using QuickExtract DNA Extraction Solution (Epicentre, USA) 72 h after transfection.

3.3. rAAV production and transduction

For rAAV production, HEK293T cells were split 1:5 from 150-mm dishes for approximately 24 h on day 1. The following day, the cells were transfected using polyethylenimine (PEI, Polysciences) according to the manufacturer's protocol at approximately 70 % confluence. Transfection mix included 10 μg of ITR containing transfer plasmid, 10 μg AAV2 Rep/AAV2 Cap and 20 μg pHelper plasmid transfected into HEK293T cells. The medium was exchanged for fresh DMEM containing 10 % (v/v) FBS 6 h after transfection. Three days after transfection, the supernatant was harvested and processed with PEG8000 for virus precipitation, while the cells were harvested and lysed by repeated freezing and thawing. An iodixanol gradient was then prepared by ultracentrifugation for further purification of AAVs. Fractions containing AAVs were collected, pooled and dialyzed against 0.001 % Pluronic F68 in PBS. AAV titers were determined by quantitative PCR (qPCR) on vector genomes using the AAVpro® Titration Kit (TaKaRa). The purified virus was then aliquoted and stored at −80 °C.

For AAV-mediated transduction, HEK293T cells were cultivated under consistent conditions as previously described. Briefly, cells were

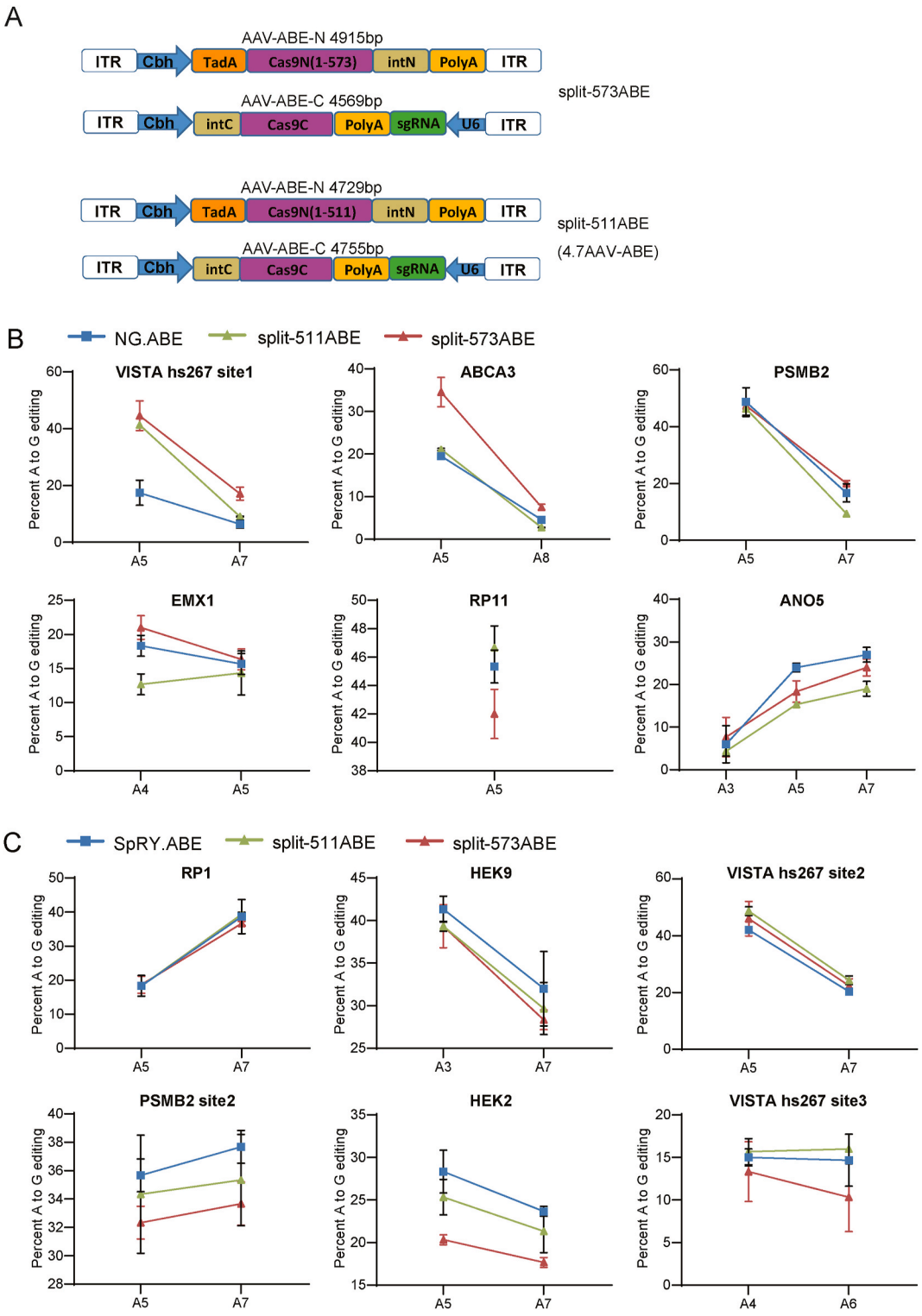

Fig. 4. A-to-G editing of endogenous sites in cultured cells by split-ABE pairs. (A) Schematic of intein-mediated ABE reconstitution through protein *trans*-splicing. To ensure high expression, a strong synthetic mammalian promoter (CBh, blue) and a bovine growth hormone (bGH, yellow) polyadenylation site were used. The inverted terminal repeats (ITRs) are shown in white. The intein is shown in earthy yellow. Split Cas9 cDNA is shown in red violet. Deaminase in orange. For gRNA expression (light green), the U6 promoter was chosen (blue). (B) Comparison of the base editing efficiency of NG. ABEmax, split-511NG.ABEmax and split-573NG.ABEmax at six target sites in HEK293T cells. (C) Comparison of the base editing efficiency of SpRY. ABEmax, split-511SpRY.ABEmax and split-573SpRY. ABEmax at six target sites in HEK293T cells.

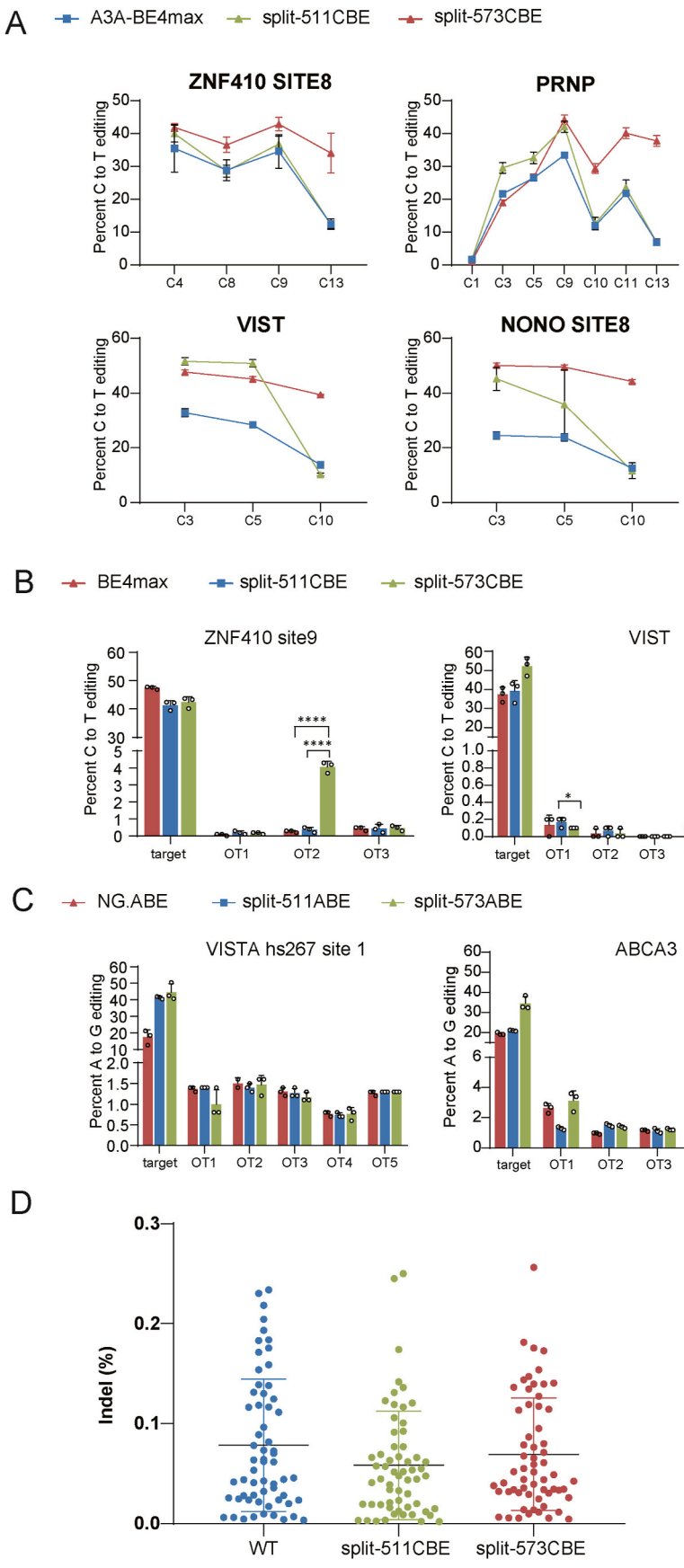

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Fig. 5. Comparison of editing characterization between intein-mediated split-BE and wild-type-BE. (A) Comparison of the efficiency and target scope of 4.6AAV-A3ABE3.9max, split-573BE and wild-type A3ABE4max at four target sites. (B) The off-target efficiency of 4.6AAV-CBE, split-573CBE and wild-type BE4max at *ZNF410 site9* and *VIST* loci in HEK293T cells. (C) The off-target efficiency of 4.7AAV-ABE, split-573ABE and wild-type ABEmax at the *ABCA3* and *VISTA hs267 site1* loci in HEK293T cells. (D) The indel efficiency of 4.6AAV-CBE, split-573CBE and wild-type BE across the protospacer at 12 loci in HEK293T cells. The error bars represent the standard deviation calculated from three experiments. ***, $p < 0.001$.

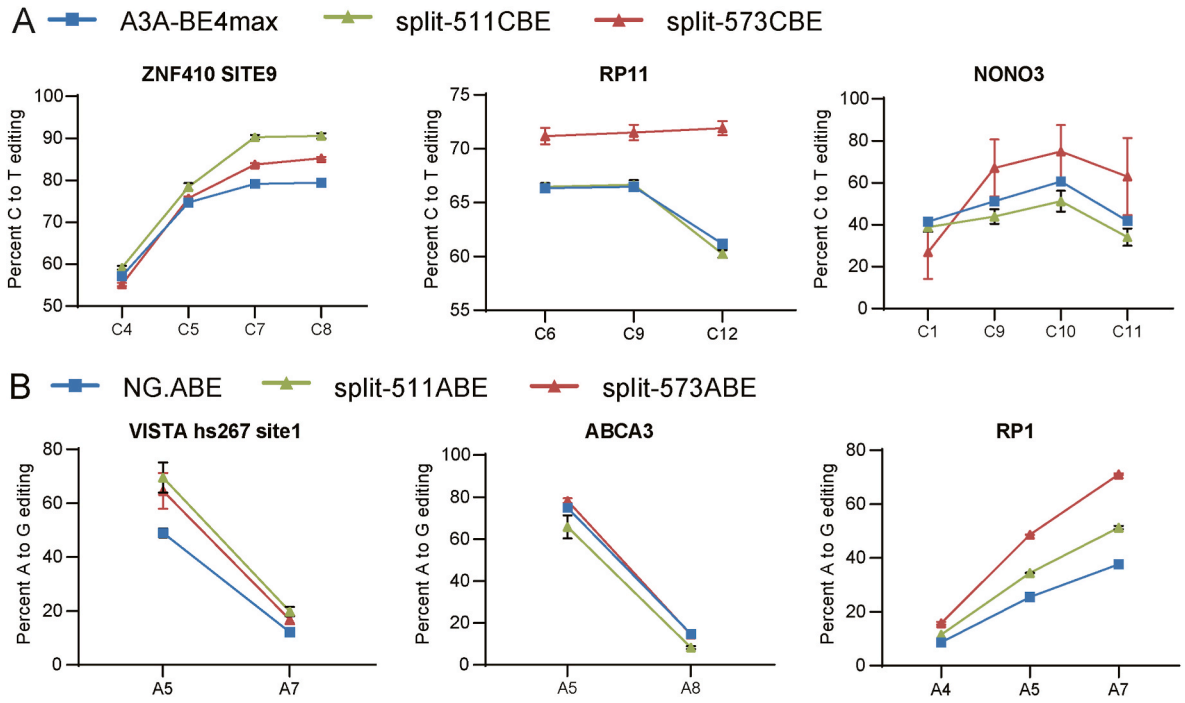

Fig. 6. Editing efficiency of split-511CBEs in Hela cells. (A) Comparison of the editing efficiency of wild-type A3A-BE4max, split-511A3ABE3.9max and split-573A3ABE3.9max at three genomic loci in Hela cells. (B) Comparison of the base editing efficiency of NG. ABEmax, split-511NG.ABEmax and split-573NG. ABEmax at three target sites in Hela cells. The error bars represent the standard deviation calculated from three experiments.

seeded in 48-well plates at a density of 5×10^4 cells per well. The harvested AAV-N and AAV-C viral stocks were combined at equivalent titers (1:1 ratio) and subsequently used to infect HEK293T cells, with MOI ranging from 1.0×10^3 to 2.0×10^4 viral genomes per cell. After 3 days of infection, cells were collected and genomic DNA was extracted using QuickExtract DNA Extract Solution (Epicentre, USA).

3.4. High-throughput sequencing and data analysis

Next-generation sequencing libraries were constructed and sequenced according to previous research [26,30]. For each sample, >50 ng of purified PCR fragments was used for the direct library. All the genomic regions of interest were amplified from 100 ng genomic DNA by PCR with the primers listed in Table S1. The qualified libraries were sequenced paired-end 150 bp on an Illumina Novaseq System. The PCR was performed at 95 °C for 3 min, 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 10 s, and a final extension at 72 °C for 5 min. Then, libraries with different indices were multiplexed and loaded on an Illumina HiSeq instrument according to the manufacturer's instructions. Base substitution frequencies were calculated according to the method used by the Bae group [31].

4. Discussion

The CRISPR/Cas9 systems has become a widely used tool for gene targeting in scientific research and gene therapy treatment due to its immense potential. However, the use of CRISPR/Cas9, especially base editors, for *in vivo* gene therapy is hindered by the challenged associated with efficient delivery. AAV has been successfully utilized to deliver spCas9 for genetic therapies in animal models [27,32]. The dual-AAV gene therapy method has emerged as a transformative strategy for delivering large therapeutic transgenes, achieving successful transduction across diverse tissues including eye, liver, and muscular systems in preclinical models. Notably, this approach has demonstrated therapeutic efficacy in addressing monogenic disorders such as retinitis pigmentosa (RP) [33], familial hypercholesterolemia [34], and amyotrophic lateral sclerosis (ALS) [35]. Based on these advances, dual-AAV systems are poised for expanded clinical application. However, comprehensive characterization of host immune responses remains critical. Immune responses and genome-wide integration analyses will be comprehensively evaluated in the future. In this study, we developed an optimized AAV delivery strategy via split intein mediated base editor reconstitution, which enabled high-efficiency genome editing. We first engineered and screened an efficient split site, 511–512, with high editing efficiency at the *ZNF410* target site9 and site8. We then demonstrated that 4.6AAV-CBE could mediate robust base mutation in mammalian cells through plasmid transfection. Furthermore, we confirmed that the target scope of 4.6AAV-A3ABE3.9max matched the wild-type full-length base editor perfectly, while split-573BE dose not. Importantly, the yield of 4.6AAV-CBE AAV production is higher than the previous split-573BE due to the slim loading. The split intein-mediated split-Cas9 *trans*-splicing system allows the coding sequence of Cas9 to be distributed on a dual-vector system and reconstituted post-translationally [24]. This system removes all exogenous sequences and regenerates a native peptide bond at the split site, resulting in a single reconstituted protein identical in sequence to the unmodified base editor. We tested 25 split sites for reconstitution of Cas9 and found the split position significantly affected the activity of reconstituted protein,

even theoretically the recombined protein shares the same peptide sequence. One explanation could be different reconstituting efficiency caused by the split site and local structure, which resulted different amount of full-length recombined protein. However, our results showed that the 573-split site influenced the target scope of A3ABE4max, which cannot be explained by the reconstitution efficiency. Here, we propose a novel hypothesis, that both the protein 3D structure formation and intein mediated reconstitution happen simultaneously at the post translational process; so that different split sites also result in different the protein maturation and form proteins in same peptide sequence but different, or slight different, structures, which in turn has different activities. However, this novel hypothesis for intein mechanism remains to be determined. Therefore, it is necessary to screen more rational split sites for using the intein with any proteins, including the genome editing proteins.

The dual AAV base editing strategy presents a promising approach for precise and effective genetic modifications, particularly in therapeutic contexts where high specificity and efficiency are paramount. By employing an optimized dual AAV system, we can effectively utilize two separate AAV vectors to deliver both the base editor and the necessary guide RNA to the target cells, potentially increasing the editing efficiency beyond what is achievable with single-vector systems. Optimization of the dual AAV base editing approach can be tailored to specific therapeutic environments by adjusting several factors, including the AAV serotype, the delivery route, and the ratio of the two AAV constructs.

In the development of our dual AAV base editing system, we focused on size-limited packaging strategies to keep AAV cargo within 4.7 kb, ensuring the structural integrity and catalytic activity of the base editor. Our optimization reduced unnecessary sequence elements and adopted a split-internal structure, resulting in an approximately 1.5-fold increase in viral titer (quantified by qPCR). Studies on the off-target effect show that the off-target character of base editor complexes. Theoretically, despite different Cas9 splitting strategies, the protein structure was barely affected after the recombining, so that the editor characterization, including off-target effect, was not affected. Off-target analysis based on Cas-OFFinder-predicted candidate sites has been widely adopted in base editing studies.

In this study, we have successfully demonstrated the feasibility of splitting the Cas9 protein at the 511 target site, and delivering it efficiently using a dual rAAV vector system. Our results indicated that the split-511BE can effectively simulate the wild-type base editors, with a higher AAV production yield. Furthermore, we have shown that the optimized split-511BE can function effectively in mammalian cells. Overall, our findings provide a novel split site of SpCas9 and offer more options for the delivery of CRISPR/Cas9 systems, such as base editors, prime editors and other emerging gene editing tools.

4.1. Statistical analyses

The experimental data are presented as the mean ± s.d. of n = 3 independent biological replicates, and the significant differences were conducted using two-tailed Student's t tests.

CRediT authorship contribution statement

Wenjia Yu: Writing – original draft, Visualization, Validation, Supervision, Formal analysis, Data curation. **Yujie Wang:** Writing – original draft, Visualization, Supervision, Formal analysis, Data curation. **Siwei Li:** Writing – original draft, Supervision, Resources, Formal analysis, Conceptualization. **Yingcai Dai:** Visualization, Validation, Supervision, Software, Formal analysis, Data curation. **Yucheng Li:** Visualization, Validation, Supervision, Formal analysis. **Xinyue Zhang:** Supervision, Formal analysis, Data curation. **Bo Li:** Funding acquisition, Project administration, Resources, Supervision. **Siriguleng Qian:** Project administration, Methodology, Investigation, Funding acquisition. **Xueli Zhang:** Methodology, Investigation, Funding acquisition, Conceptualization. **Changhao Bi:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

Data availability

There is no restriction on the data associated with this study. High-throughput sequencing data have been deposited in the NCBI database (accession code PRJNA904124).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2025.03.007.

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