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Combinatorial testing of viral vector and CRISPR systems for precision genome editing

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Chapter 3

AAV-vectored base editor *trans*-splicing delivers dystrophin repair

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Duchenne muscular dystrophy (DMD) (MIM: 310200) is a severe and frequent neuromuscular disorder (incidence of ~1 in 5,500 boys) caused by mutations in the vast X-linked *DMD* gene (~2.2 Mb), whose largest product, the long rod-shaped 427-kDa dystrophin isoform, is a key structural component of the striated musculature [1]. Contributing to the urgency in the development of currently inexistent DMD therapies is the observation that ~1/3 of cases arise *de novo* through germline mutations, often intragenic deletions that disrupt the mRNA reading frame. Critically, naturally occurring *DMD* gene deletions resulting in in-frame transcripts coding for internally truncated, yet partially functional, dystrophins cause the milder Becker muscular dystrophy (MIM: 300376). Hence, *DMD* gene manipulations yielding Becker-like dystrophins via direct coding sequence reframing or exon skipping have the potential of offering long-lasting therapeutic effects [1]. Toward this end, among other technologies, CRISPR-Cas9 nucleases and adeno-associated viral (AAV) vectors are being investigated for rescuing dystrophin expression upon double-strand DNA break (DSB) formation and ensuing chromosomal end-joining [1]. These experiments demonstrate that AAV/CRISPR-Cas9-based dystrophin restoration can improve striated muscle function in mice; however, a potentially insidious outcome is the identification of prevalent capture of Cas9-encoding AAV at nuclease target sites, including at *Dmd* exons 51 and 53 [2,3]. Moreover, programmable nucleases can trigger other untoward effects, *e.g.*, locus- or chromosome-wide rearrangements [4]. There is, therefore, a pressing need to expand candidate DMD genetic therapies to those based on DSB-independent genome editing systems. In a timely study published in *Molecular Therapy – Nucleic Acids*, Chai and coworkers [5] identify adenine base editors (ABEs) and guide RNAs (gRNAs) (**Figure 1**) that, after implementing single base-pair substitutions (*i.e.*, A·T-to-G·C transitions) at splicing motifs, a process that they name "single-swap" editing, lead to genotype-specific *DMD* repair through exon skipping. Next, the authors assemble a dual AAV ABE *trans*-splicing system to demonstrate in dystrophin-defective mice the amelioration

of dystrophic traits at the cellular and organismal levels upon intramuscular or systemic administrations. This study identifies ABE:gRNA complexes compatible with ~30% of DMD-causing genotypes and, notwithstanding its inherent complexity, establishes dual AAV ABE *trans*-splicing as a DSB-free *DMD* gene correction option.

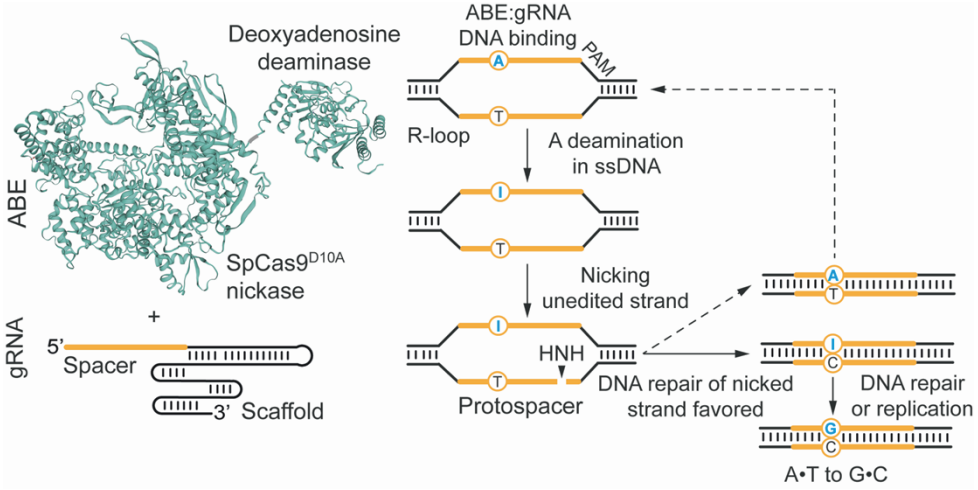

Figure 1. Adenine base editing. Adenine base editors (ABEs) catalyze A·T-to-G·C substitutions and consist of a fusion product between a disabled or nicking Cas9, or ortholog protein, and an evolved deoxyadenosine deaminase, *e.g.*, *Escherichia coli* tRNA adenosine deaminase (TadA) derivatives. Upon PAM binding, ABE:gRNA complexes form an R-loop at a gRNA-defined target sequence exposing a region of single-stranded DNA. A nucleotides in this single-stranded protospacer "bubble" become targets for the ABE effector domain that converts A nucleotides to inosine (I) intermediates preferentially within an "activity window." Subsequently, nicking of the unedited strand induces DNA repair that installs C nucleotides opposite I intermediates with additional DNA repair events (or replication) establishing the final A·T-to-G·C transitions.

Chai and colleagues start by testing in HEK293T cells and DMD iPSCs, ABE:gRNA complexes that, depending on their ABE component, *i.e.*, ABE8e [6] or ABEe-NG, recognize, respectively, canonical NGG or NG PAMs. DNA sequencing assays in DMD iPSCs identified ABE:gRNA complexes yielding high-frequency target-base editing at *DMD* exons 51 and 45 splice acceptor (SA) motifs (71.6% and 79.3%–83.3%, respectively). As a result,

robust expression of Becker-like dystrophins was detected in cardiomyocytes differentiated from base-edited DMD iPSCs. Interestingly, it was found that *DMD* exon 51 targeting serendipitously established an in-frame 11-nucleotide deletion instead of the intended exon skipping, presumably due to internal cryptic splice site usage. This finding *per se* stresses the importance of carefully assessing gene-edited products even when using subtle DSB-independent systems. Moreover, various amounts of bystander A·T-to-G·C transitions were also detected (range: 3.3%–91%). These bystander changes might have limited consequences as they map to either spliced-out intron or, if accompanied with the intended SA edits, to skipped exon sequences. Further investigations will be, nonetheless, necessary to probe for slight (or otherwise) splicing alterations in different cell types or contexts. Regardless, base editors with narrower "editing windows" should facilitate more favorable target-to-bystander ratios.

AAV vectors are attractive *in vivo* gene-editing tool delivery vehicles owing to their lack of viral genes and serotype diversity of their parental viruses. Indeed, packaging vector genomes in AAV serotype capsids with a strong tropism for certain cell types (pseudotyping) facilitates tissue-directed transductions. However, the limited AAV packaging capacity (<4.7 kb) permits delivering neither base editing nor other large constructs. To obviate this limitation, researchers are developing base editors with compact architectures [7], testing alternative delivery systems or applying dual AAV strategies in which split constructs linked to N- and C-terminal intein domains are packaged in different AAV vectors (**Figure 2**). Upon target cell co-transductions, intein-mediated protein *trans*-splicing results in the *in situ* assembly of full-length proteins. Indeed, dual AAV-vectored base editor *trans*-splicing is currently undergoing intense investigation for tackling various disease-causing mutations, including *DMD* mutations [8,9]. In Chai *et al*. [5], a dual AAV ABE *trans*-splicing system is assembled to address frequent *DMD* deletions through exon skipping modulation. By exploiting high human-murine conservation over intron 44 to exon 45 junctions and

guided by their earlier *in vitro* experiments, the authors apply dual AAVs to deliver a split version of an ABE8e variant (*i.e.*, ABE.TadA-8e^{V106W}), selected for its reduced off-target nucleic acid deaminase activities [6], together with a gRNA targeting the exon 45 SA region (**Figure 2**).

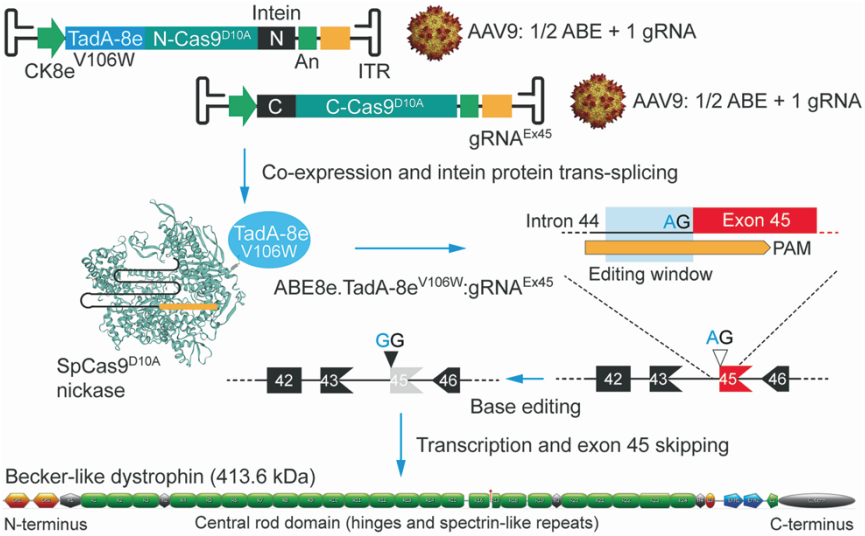

Figure 2. Dual AAV ABE *trans*-splicing system for dystrophin repair. Two AAV serotype-9 vectors expressing separated portions of an adenine base editor (ABE.TadA-8e^{V106W}) and a gRNA (gRNA^{Ex45}) lead to intein-mediated assembly of complete ABE:gRNA complexes. Base editing involving A·T-to-G·C transitions at the splice acceptor site of exon 45 establishes permanent exon 45 skipping in striated muscle cells. In dystrophin-defective muscle cells lacking exon 44, exon 45 skipping restores the reading of mature transcripts that code for a truncated Becker-like dystrophin with therapeutic potential for DMD patients. ITR, T-shaped hairpin-structured AAV serotype-2 inverted terminal repeats (*cis*-acting elements needed for vector DNA replication and packaging in producer cells). CK8e and An, synthetic striated muscle-specific promoter and polyadenylation signal, respectively. The dystrophin diagram was generated via: http://edystrophin.genouest.org/index.php?page=home.

To favor base editing in striated muscles over non-target organs of *Dmd* exon 44-deleted mice, the vector constructs were packaged in AAV serotype-9 capsids with the split ABE.TadA-8e^{V106W} moieties being expressed through the tissue-specific CK8e promoter. Intramuscular dual AAV co-administrations of 1 x 10^{11} total vector genomes (VGs) per tibialis anterior

(TA) led to 29.5% ± 2.7% A·T-to-G·C edits with minimal indel formation (*i.e.*, 0.2% ± 0.1%), as determined by deep sequencing at 3 weeks post injection. Systemic dual AAV co-administrations of 1.5 x 10^{14} VG kg^{-1} and 3 x 10^{14} VG kg^{-1} via the temporal facial veins of postnatal day 2 (P2) mice yielded, in TA muscles, 5.5% ± 1.2% and 8.1% ± 3.0% edits and, in hearts, 22.0% ± 2.2% and 26.2% ± 4.4% edits, respectively, with <0.1% indels detected at 8 weeks post injection. Critically, a general dose-dependent improvement of disease-associated molecular, cellular, and functional endpoints is reported. In this regard, up to 31% and 60% of wild-type dystrophin levels estimated in treated TA and heart muscles corresponded to over 76% and 95% of dystrophin-positive myofibers and cardiomyocytes, respectively. Partial dystrophin rescue translated, in turn, in noticeable reduction of myofiber central nucleation, diameter distribution, and fibrosis, all hallmarks of muscle degeneration. Finally, *Dmd* exon 44-deleted mice systemically treated at P2 with low and high doses of dual AAV ABE *trans*-splicing particles registered 31% and 41% grip strength augmentation, respectively, when compared with their untreated counterparts. Follow-up studies will be instructive to determine the long-term effects of the local and systemic gene-editing procedures in the treated animals. Of notice, despite the aforementioned transductional and transcriptional targeting measures, significant base editing was detected in the liver (*i.e.*, 11.1% ± 5.9%), which correlated with high VG copy numbers present specifically in this organ. These data confirm the importance of developing liver de-targeting protocols and strictly myotropic AAV capsids [10]. Indeed, as dose-dependent toxicity and immunological constrains have emerged during AAV clinical applications, optimization of tissue tropism and expression will be particularly important for dual AAV *trans*-splicing approaches due to the necessarily higher particle amounts required for maximizing co-expression and full-length protein assembly. Moreover, besides seeking the elimination of the observed editing at two of five top-ranked *in silico*-predicted candidate off-target sites [5], unbiased genome- and transcriptome-wide assessments of

off-target effects, including gRNA-independent deamination, will complement the safety profile of *DMD*-targeting ABE:gRNA complexes. Concluding, Chai and colleagues demonstrate that AAV-vectored ABE *trans*-splicing can induce robust synthesis of Becker-like dystrophins in striated muscles of dystrophic mice upon DSB-free splice site knockout and exon skipping [5], The resulting improvement in pathological traits measured at the molecular, tissue, and functional levels validates the use of this platform for the efficacious testing and optimization of the growing number of ABE reagents *in vivo* and expands the range of potential treatment modalities for DMD patients.

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Declaration of interest

The authors declare no competing interests.

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