Replacing the SpCas9 HNH domain by deaminases generates compact base editors with an alternative targeting scope

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Abstract: Base editors are RNA-guided deaminases that enable site-specific nucleotide transitions. The targeting scope of these Cas-deaminase fusion proteins critically depends on the availability of a protospacer adjacent motif (PAM) at the target locus and is limited to a window within the CRISPR-Cas R-loop, where single-stranded DNA (ssDNA) is accessible to the deaminase. Here, we reason that the Cas9-HNH nuclease domain sterically constrains ssDNA accessibility and demonstrate that omission of this domain expands the editing window. By exchanging the HNH nuclease domain with a monomeric or heterodimeric adenosine deaminase, we furthermore engineer adenine base editor variants (HNHx-ABEs) with PAM-proximally shifted editing windows. This work expands the targeting scope of base editors and provides base editor variants that are substantially smaller. It moreover informs of potential future directions in Cas9 protein engineering, where the HNH domain could be replaced by other enzymes that act on ssDNA.

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Base editors are RNA-guided deaminases that enable site-specific nucleotide transitions. The targeting scope of these Cas-deaminase fusion proteins critically depends on the availability of a protospacer adjacent motif (PAM) at the target locus and is limited to a window within the CRISPR-Cas R-loop, where single-stranded DNA (ssDNA) is accessible to the deaminase. Here, we reason that the Cas9-HNH nuclease domain sterically constrains ssDNA accessibility and demonstrate that omission of this domain expands the editing window. By exchanging the HNH nuclease domain with a monomeric or heterodimeric adenosine deaminase, we furthermore engineer adenine base editor variants (HNHx-ABEs) with PAM-proximally shifted editing windows. This work expands the targeting scope of base editors and provides base editor variants that are substantially smaller. It moreover informs of potential future directions in Cas9 protein engineering, where the HNH domain could be replaced by other enzymes that act on ssDNA.

INTRODUCTION

CRISPR-Cas systems provide adaptive immunity to bacteria and archaea to defend them against foreign genetic elements. A small number of these systems have successfully been adopted for genome editing in mammalian cells, transforming biomedical research and therapeutics. A paradigmatic example is the Streptococcus pyogenes Cas9 (SpCas9) nuclease. Recruitment of SpCas9 to a desired genomic locus via a single guide RNA (sgRNA) allows facile and efficient genome editing by generating site-specific double-stranded DNA (dsDNA) breaks. Precise genome editing via such dsDNA breaks, however, relies on template DNA for homology-directed repair (HDR) that is inefficient in most mammalian cell types and leads to repair via alternative, error-prone end-joining pathways that generate frameshift mutations and gene knockouts.

Recent modifications to the highly modular SpCas9 protein resulted in sophisticated genome-editing technologies, such as base editors (BEs), which enable genome editing without dsDNA break formation. BEs allow precise conversion of A-to-G and C-to-T nucleobases or vice versa via nucleotide deamination independent of dsDNA breaks and HDR and thus have great potential for research and therapeutic applications. They are monomers or heterodimers of ssDNA-specific deaminases fused N-terminally to catalytically impaired CRISPR class II effectors, such as Cas9 or Cas12. Adenine BEs (ABEs) are based on laboratory-evolved E. coli tRNA adenine deaminase (TadA), and cytidine BEs (CBEs) have been generated from various deaminases, including APOBEC1, APOBEC3, AID, and CDA. Upon binding to a protospacer element, Cas9 complexed with the sgRNA forms an R-loop, making the non-target DNA strand (the target DNA strand is bound to the sgRNA) accessible to ssDNA-specific deaminases.

Because Cas9 and Cas12 proteins require protospacer adjacent motif (PAM) sites for DNA binding, the targeting scope of BEs is restricted to nucleobases that lie within a defined proximity to these motifs. To address this limitation, deaminases have been fused to Cas variants with less restrictive PAM requirements. However, because no PAMless Cas9 and Cas12 variants are yet available, several pathogenic T>C and G>A single-nucleotide polymorphisms (SNPs) remain untargetable. Pursuing a different approach, recent work led to the expansion of the BE targeting scope by generating variants with slightly extended editing windows: Huang et al. fused deaminases to circularly permuted Cas9 proteins, and Wang et al. incorporated the cytidine deaminase rAPOBEC1 into the PAM-interacting (PI) domain of SpCas9. Broadening the editing window, however, can induce additional bystander mutations, and this approach is therefore applicable only at certain loci.

Here, we report novel ABE variants, where the HNH nuclease domain of SpCas9 is replaced by monomeric or heterodimeric (HD) TadA. These variants are significantly reduced in size and have a PAM-proximally shifted editing window, enabling correction

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Figure 1. Approaches to increase substrate accessibility for base editing at PAM-proximal bases

(A) Schematic domain organization of ABEmax7.10 and ABEmax7.10 PI1-3. ABEmax7.10 PI1-3 comprise an engineered SpCas9 (D10A) construct, where the TadA deaminase is integrated within the PI domain. ABEmax7.10 PI1, PI2, and PI3 use different linker lengths flanking the TadA deaminase.

(B) Editing efficiencies of ABEmax7.10 PI constructs after transfection into HEK293T cells are quantified by high-throughput sequencing at three different endogenous loci. Values represent mean of three independent biological replicates performed on separate days ± SD.

(C) Structural data (PDB: 6VPC) of adenine base editors with (left) and without (right) an HNH domain (indicated in red). TadA deaminases are indicated in orange and blue and target the ssDNA substrate (turquoise).

(D) Schematic domain organization of ABEmax7.10 ΔHNH, where the HNH domain is replaced by a glycine-serine linker (Gly-Gly-Ser-Gly-Gly-Ser).

(E) High-throughput sequencing data compare editing efficiencies of ABEmax7.10, dABEmax7.10, and ABEmax7.10 ΔHNH, where the HNH domain is replaced by a glycine-serine linker (Gly-Gly-Ser-Gly-Gly-Ser).

(legend continued on next page)
of several pathogenic SNPs that were not well targetable by classical ABEs.

RESULTS

The HNH domain of SpCas9 restrains ssDNA accessibility at PAM-proximal positions

In our first attempt to shift the editing window of BEs, we applied the strategy of Wang et al. to ABEs and incorporated the codon-optimized TadA7.10 deaminase into the PI domain of SpCas9. Similar to CBEs, also for ABEs this approach did not lead to a shift but rather to a PAM-proximal extension of the editing window (ABEmax7.10 PI; Figures 1A and 1B). However, no editing was observed at nucleotides beyond position 12 (counting from PAM-distal end of the protospacer), suggesting that ssDNA accessibility in this region is blocked. Structural data suggest that the HNH nuclease domain (amino acids 775–908) in SpCas9 is likely responsible for preventing the deaminase from accessing the ssDNA substrate at positions 13–15 (Figure 1C). Importantly, the HNH domain is not required for Cas9 to bind its target DNA and form an R-loop, and nickase activity is not essential for base editing (Figure 1E). We therefore reasoned that omission of the HNH domain might improve accessibility and editing at PAM-proximal positions. To test this hypothesis, we engineered ABEmax7.10 ΔHNH, where a heterodimer of wild-type (WT) and evolved TadA7.10 is linked to the N terminus of an SpCas9 variant that lacks the HNH domain (Figure 1D). Although the highest editing rates remained at positions that are also efficiently targeted by ABEmax7.10, ABEmax7.10 ΔHNH allowed editing at positions 12 and 14 (Figure 1E). Our data therefore suggest that the HNH domain of SpCas9 indeed constrains access of TadA to PAM-proximal ssDNA nucleobases.

Replacing the HNH domain with TadA7.10 shifts the ABE editing window PAM proximally

Simply expanding the editing window of BEs has the disadvantage that it can generate additional undesired non-target nucleotide (bystander) edits within the ssDNA of the R-loop. This led us to investigate whether directly replacing the HNH domain with the adenine deaminase (HNHx-ABE) leads to a shift rather than a broadening of the editing window (Figures 2A and 2B). Before incorporating a deaminase domain in place of the HNH domain in SpCas9, we first exchanged the HNH domain for a superfolder GFP (sfGFP). Transfection of these constructs into HEK293T cells demonstrated localization of GFP to the nucleus (Figure 2C), indicating that the HNH domain can be exchanged with another protein domain without substantial misfolding and degradation of Cas9. In a next step, we engineered HNHx-ABEmax7.10 by incorporating the deaminase domain from ABEmax7.10 with 20 different peptider linker combinations into SpCas9 lacking the HNH domain. Using high-throughput sequencing (HTS), editing efficiencies of constructs with different linker combinations were compared 2 days after transfection into HEK293T cells (Figure 2D). In the most promising construct, a GGS-linker was used to join SpCas9 S793 to the TadA7.10 N terminus, and a SGG-linker was used to join the TadA7.10 C terminus to SpCas9 R919. This construct (HNHx-ABEmax7.10) exhibited a distinct shift in the editing window PAM-proximally, with up to 13.7% editing at positions >12 at 5 days after transfection (Figure S1A) and 32.5% editing at 7 days after transfection (Figure 3A). A similar shift in the editing window was obtained when HNHx-ABEmax7.10 was transfected into the K562 human erythroleukemic cell line (Figure 3A). Notably, when we exchanged the HNH domain with the cytosine deaminases FERNY, a laboratory-evolved APOBEC variant,21 we observed a similar shift in the editing window, albeit with very low editing rates (Figure S2A). Although these could be increased by exchanging FERNY with the cytidine deaminase PmCDA1, HNHx-PmCDA1 constructs induced broad deamination across the entire protospacer region, leading to significant bystander edits (Figure S2B). Likewise, when we adapted the HNHx-ABE architecture to the smaller Cas9 ortholog from Staphylococcus aureus (SaCas9-KKH22) using various linker combinations, we also did not obtain substantial deamination in the protospacer (Figure S3).

Replacing HNH with TadA8e increases the efficiency and broadens the editing window of HNHx-ABE

Phage-assisted non-continuous and continuous evolution has enabled the development of TadA variants with increased deaminase activity.23 To assess whether the use of these variants would improve editing rates of HNHx-ABEs, we next exchanged the TadA7.10 deaminase domain with hyperactive TadA8e, resulting in HNHx-ABE8e. As expected, we observed higher editing rates with HNHx-ABE8e compared with HNHx-ABEmax7.10, up to 19.8% at 5 days after transfection (Figure S1B) and up to 40.4% at 7 days after transfection (Figure 3B). Higher editing efficiencies, however, came at the cost of lower specificity, because the editing window was significantly broadened (Figures 3B and S1B). Although this is a potential limitation due to the increase in bystander editing, targeting the pathogenic c.3188G>A mutation in FANCA provides an example for a disease-causing locus where HNHx-ABE8e has a benefit over classical ABEs with N-terminally fused TadA. Here, the only available canonical NGG PAM site positions the disease-causing mutation outside of the editing window of classical ABEs, and HNHx-ABE8e led to considerably higher on-target editing compared with ABEmax7.10 (Figure 3C). Notably, similar on-target editing rates were obtained when using ABEmax7.10ΔHNH within reach of classical ABEs (Figure 3C), but compared with HNHx-ABE8e this variant resulted in more non-synonymous bystander mutations.

\[ \text{dABEmax7.10, and ABEmax7.10 ΔHNH in endogenous loci in HEK293T cells. ABEmax7.10 retains nickase activity, nicking the target strand. Elimination of the nickase activity in ABEmax7.10 by introducing a H840A mutation in the HNH domain leads to dABEmax7.10. Complete removal of the HNH domain (that also abolishes nickase activity) in ABEmax7.10 results in ABEmax7.10 ΔHNH. Values represent mean of three independent biological replicates performed on separate days ± SD.} \]
High levels of ABE expression can lead to substantial sgRNA-independent off-target deamination on the transcriptome. Considering that in the HNHx-ABE architecture TadA is not terminally fused but instead integrated into Cas9, and thus potentially constrained by the HNHx-ABE architecture, we reasoned that off-target editing on the transcriptome might be reduced. However, RNA sequencing (RNA-seq) did not show significant differences in adenine deamination between HNHx-ABE8e- and ABE8e-treated cells and between HNHx-ABEmax7.10- and ABEmax7.10-treated cells (Figure S4). Thus, also with HNHx-ABE variants, excessive overexpression should be avoided in applications where off-target deamination is of concern.
Replacing the HNH domain with HD TadA increases HNHx-ABE efficiency

Recent studies demonstrate that in ABE variants with monomeric TadA, dimerization occurs with a second TadA domain contributed in trans from another ABE molecule, suggesting that TadA dimerization is essential for ABE activity. Because in trans TadA dimerization might be impeded by steric hinderance in HNHx-ABE constructs (Figure S5), we hypothesized that replacement of the HNH domain by HD TadA could improve editing rates. We therefore incorporated WT TadA linked to TadA7.10 or TadA8e into Cas9 lacking the HNH domain, resulting in HNHx-ABEmax7.10HD and HNHx-ABE8eHD (Figure 4A). Importantly, at the three analyzed loci, both variants still led to a PAM-proximal shift in adenine deamination (Figures 4B and 4C), but with an elevation in editing rates compared with monomeric TadA HNHx-ABE variants (Figures 3A, 3B, 4B, and 4C). To further refine editing characteristics of the different HNHx-ABE constructs generated in our study, we next transfected a pool of HEK293T cells carrying a stably integrated self-targeting library, where each cell contains one of the 100 target loci and also expresses the corresponding sgRNA (Figure S6). HTS of the target loci 10 days post-transfection confirmed the PAM-proximal shift of the editing window in all HNHx-ABE constructs, with substantially higher editing when the HNH domain was replaced with TadA heterodimers compared with monomers (mean editing rates with HNHx-ABE8eHD and HNHx-ABEmax7.10HD were 33.1% and 20.5% compared with 14.8% and 4.9% with HNHx-ABE8e and HNHx-ABEmax7.10; Figures 4D; Figures S7 and S8). Taken together and exemplified at two different pathogenic loci (c.3612G>A in CFTR and c.14911C>T USH2A) where disease-causing mutations were repaired with higher efficiency and precision (Figure 4E), the here-generated HNHx-ABE variants are a valuable extension to the ABE toolkit.
DISCUSSION
In this work, we demonstrate that replacing the HNH domain of SpCas9 with a deaminase domain shifts the editing window of BEs from positions 4–8 to 7–14 for TadA7.10. This expands the targeting scope of currently available BEs and enables targeting of additional disease-causing mutations. Replacement of the HNH domain with TadA furthermore reduces the size of BEs from 5.4 kb to below 4.4 kb, allowing the construction of single adeno-associated virus (AAV) vectors with minimal promoters for in vivo delivery. Although SaCas9 fused to a TadA monomer, in principle, also fits on a single AAV, applicability of such variants is limited by their broader editing window and the requirement of NNGRT PAM sites. Interestingly, editing rates of HNHx-ABEs were lower compared with classical ABEmax7.10 lacking the HNH domain (ABEmax7.10 ΔHNH) also exhibited a reduction in editing rates as compared with classical ABEmax7.10. However, because replacing the HNH domain with HD TadA increased editing rates to levels comparable with N-terminally fused TadA ABEs, and because TadA dimerization is likely essential for enzymatic activity, we speculate that in trans TadA dimerization from another ABE molecule was inefficient in monomeric HNHx-ABE variants, potentially due to inaccessibility of the dimerization interface.

In the future, directed protein evolution or rational protein engineering strategies may refine HNHx-ABE constructs and further increase editing specificity or efficiencies. In addition, our approach of replacing the HNH domain with proteins up to 370 amino acids could be extended to other effector enzymes that act on ssDNA, thereby further expanding the Cas9-based genome editing toolbox.

MATERIALS AND METHODS
General methods and cloning
PCR was performed using Q5 High-Fidelity DNA Polymerase (New England Biolabs). All BE constructs were assembled using NEBuilder HiFi DNA Assembly (New England Biolabs). pCMV_ABEmax, ABEmax, pB7280, and SaKKH-ABEmax were a gift from David Liu (Addgene plasmid no. 112095, 138489, 122610, and 119815). pcDNA3.1_pCMV-nCas-PmCDA1-ugi pH1-gRNA(HPRT) was a gift from Akihiko Kondo (Addgene plasmid no. 79620). Plasmids expressing sgRNAs were cloned using T4 DNA Ligase (New England Biolabs). Lent guiding Puro was a gift from Hyongbum Kim (Addgene plasmid no. 84752). LentiGuide-Puro was a gift from Feng Zhang (Addgene plasmid no. 52963). sgRNA sequences used are listed in Table S1.

Cell culture and HTS
All cell lines were cultured at 37°C and 5% CO2 in tissue culture incubators. HEK293T cells (ATCC CRL-3216) were cultured in Dulbecco’s modified Eagle’s medium GlutaMAX and K562 cells in RPMI-1640 medium (Thermo Fisher Scientific), both supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin (Thermo Fisher Scientific). Cells were maintained at confluence below 90% and seeded on 96-well cell culture plates (Greiner). At 12–16 h after seeding, at approximately 70% confluency, cells were transfected using 0.5 µL Lipofectamine 2000 (Thermo Fisher Scientific) and 400 ng BE plasmid DNA and 100 ng sgRNA plasmids. Cells were incubated for 5 days, unless specified otherwise. Genomic DNA was isolated by adding 10 µL lysis buffer (10 mM Tris–HCl at pH 8.0, 2% Triton X and 1 mM EDTA, and 25 µg/mL Proteinase K) to 30 µL cell suspension. The lysate was incubated at 60°C for 60 min, followed by a 95°C incubation for 10 min. The lysate was diluted with double-distilled H2O (ddH2O) to a final volume of 100 µL. 2 µL of the diluted lysate was used for subsequent PCRs of 10 µL using NEBNext High-Fidelity 2× PCR Master Mix. The PCR product was purified using Agencourt AMPure XP beads (Beckman Coulter) and amplified with primers containing sequencing adapters. The products were gel purified and quantified using the Qubit 3.0 fluorometer with the dsDNA HS assay kit (Thermo Fisher Scientific). Samples were sequenced on an Illumina MiSeq.

HTS data analysis
Sequencing reads were demultiplexed using MiSeq Reporter (Illumina) and analyzed using a MATLAB script as previously described.

Values are shown as n = 3 independent biological replicates over different days with mean ± SD.

Target library screen
One hundred self-targeting constructs were designed and ordered as gene blocks via TWIST Bioscience (Table S2). Subsequently, the gene blocks were pooled together in equimolar ratio, and PCR amplification was performed. Amplified pool was Gibson assembled into a BsmBI-digested Lenti_gRNA-Puro backbone. Assembly was electroporated with a BioRad GenePulser II Electroporation System into ElectroMAX SbiL4 (Thermo Fisher Scientific). Growth colonies (>100,000 colonies for 1,000× coverage) were harvested by scraping colonies from LB agar plates and performing Mini Prep (GeneJET)
for plasmid extraction. Lentivirus was produced in HEK293T by lipofection of the plasmid library together with pMD2.G and psPAX2 in a 1:3:8.8 (weight) ratio. pMD2.G and psPAX2 were a gift from Didier Trono (Addgene plasmid no. 12259 and 12260). After 3 days, supernatant-containing lentivirus library was harvested and filtered (0.45 µm) and used for subsequent transduction of HEK293T at low MOI (<0.3). HEK293T cells containing library sequences were transduced (n = 2 independent biological replicates on separate days) with ABE max7.10, ABE8e, HNHx-ABEmax7.10, HNHx-ABEmax7.10HD, or HNHx-ABE8eHD on a Tol2-backbone together with pCMV-Tol2. p2T-CMV-ABEmax-BlastR was a gift from David Liu (Addgene plasmid no. 152989), and pCMV-Tol2 was a gift from Stephen Ekker (Addgene plasmid no. 31823). After 1 day, medium was changed to selection medium containing Blasticidin S (7.5 µg/mL; InvivoGen), and selection was continued for 9 days. During the whole experiment, live cell number was always kept above 100,000 to maintain a 1,000× coverage. Genomic DNA was extracted as described above and continued with PCR and NGS on a MiSeq (Illumina) system. Sequencing data were analyzed by using an in-house Python script available on GitHub (https://github.com/mathinic/HNHx_ABE_Library). All sequences with less than 50 reads per editor construct were omitted.

Stable integration of FANCA (c.3188G>A) locus into HEK293T

The sequence of the FANCA (c.3188G>A; chr16:89,749,762–89,749,797) mutation was cloned between LTR sequences of a lentiviral plasmid (containing Hygromycin B resistance gene). Lentivirus was produced as stated above, and the filtrate was used to transduce HEK293T with low MOI (<0.3), which was subsequently selected with Hygromycin B.

Microscopy

HEK293T cells were transfected with 50 ng GFP-expressing plasmids in a 96-well plate, counterstained with Hoechst 33342, and imaged using a Zeiss Apotome. Imaging conditions and intensity scales were matched across replicates to control for differences in focal plane. Lentivirus was produced in HEK293T with a low MOI (<0.3), which was subsequently selected by Hoechst staining. Linker determination and testing

Zeiss Apotome. Imaging conditions and intensity scales were matched across replicates to control for differences in focal plane. Lentivirus was produced in HEK293T with a low MOI (<0.3), which was subsequently selected by Hoechst staining.

RNA-seq experiments and data analysis

RNA library preparation was performed using the TruSeq Stranded Total RNA kit (Illumina) with a ribosomal RNA (rRNA) deletion step. RNA-seq libraries were sequenced on an Illumina Novaseq machine with a sequencing depth of 140–200 Mio reads per sample.

Quality control, pre-processing, and alignment of RNA-seq reads

Quality control of Illumina PE RNA-seq reads was evaluated by FastQC v0.11.7 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Possible contaminations (genomic DNA, rRNA, Mycoplasma, etc.) were screened for using FastxScreen v0.11.1 (https://www.bioinformatics.babraham.ac.uk/projects/fastx_screen/) against a customized database that consists of SILVA rRNA (https://www.arb-silva.de/), UniVec (https://www.ncbi.nlm.nih.gov/tools/vecscreen/univec/), refseq mRNA sequences, and selected genome sequences (human, mouse, Arabidopsis, bacteria, virus, phix, lambda, and mycoplasma) (https://www.ncbi.nlm.nih.gov/refseq/). Illumina PE reads were pre-processed using Trimmomatic version 0.36 to trim off sequencing adaptors and low-quality ends (average quality lower than 20 within a 4-nt window). Flexible version 3.0.3 was used to remove the first 6 bases of each read, which showed priming bias introduced during library preparation. PE RNA-seq reads were generated with different read length (2X51 and 2X151). After adapter and quality trimming, if the read length was longer than 50 nt, only the first 50 nt were kept for downstream STAR mapping and variant calling. Quality-controlled reads (average quality 20 and above, read length 20 and above) were aligned to the reference genome (human reference genome: GRCh38.p10, Ensembl release 91) using STAR version 2.7.0e with 2-passes mode. PCR duplicates were marked using Picard version 2.9.0. Read alignments were comprehensively evaluated in terms of different aspects of RNA-seq experiments, such as sequence quality, gDNA and rRNA contamination, GC/PCR/sequence bias, sequencing depth, strand specificity, coverage uniformity, and read distribution over the genome annotation, using R scripts in ezRun (https://github.com/uzh/ezRun/) developed at the Functional Genomics Center Zurich.

RNA-seq sequence variant calling and filtering

Variant calling from RNA-seq reads was performed according to GATK Best Practices https://gatkforums.broadinstitute.org/gatk/discussion/3891/calling-variants-in-rnaseq. In detail, GATK (version 4.1.2.0) tool SplitNCigarReads was applied to post-processed read alignments. Afterward, variants were called using HaplotypeCaller (GATK version 4.1.2.0) on PCR-deduplicated, post-processed aligned reads. Variant loci in BE overexpression experiments were filtered to exclude sites without high-confidence reference genotype calls in the control experiment. For a given SNV, the read coverage in the control experiment should be >90 percentile of the read coverage across all SNVs in the corresponding overexpression experiment. Only loci having at least 99% of reads containing the reference allele in the control experiment were kept. Only sites with more than 10 reads mapping in the overexpression experiment were kept. The cut-off for RNA A-to-I conversion frequency was set to 0.1.

Data availability

All data will be made available upon reasonable request. Amino acid sequences of BE constructs are shown in Data S1. Plasmids encoding
HNHx-ABEmax7.10HD and HNHx-ABE8eHD are deposited on Addgene: 176477 and 176478, respectively.

Accession numbers
HTS data from all experiments are deposited online under accession number GEO: GSE161293.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.08.025.

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AUTHOR CONTRIBUTIONS
L.V. and L.S. designed the study, performed experiments, analyzed data, and wrote the manuscript. N.M., T.R., and K.M. performed experiments and analyzed data. G.S. designed and supervised the research and wrote the manuscript. All authors approved the final version.

DECLARATION OF INTERESTS
L.V. and G.S. have filed a patent application based on these constructs.

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20. L.V. and L.S. designed the study, performed experiments, analyzed data, and wrote the manuscript. N.M., T.R., and K.M. performed experiments and analyzed data. G.S. designed and supervised the research and wrote the manuscript. All authors approved the final version.

DECLARATION OF INTERESTS
L.V. and G.S. have filed a patent application based on these constructs.

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