

CRISPR GENOME EDITING FOR *IN UTERO* TREATMENT OF MONOGENIC
DISORDERS

Eric Lee

A THESIS

in

Chemistry

Presented to the Faculties of the University of Pennsylvania in Partial
Fulfillment of the Requirements for the Degree of Master of Science

2026



WILLIAM PERANTEAU, MD
Supervisor of Research



JEFFERY SAVEN, PhD
Supervisor of Thesis



ZAHRA FAKHRAAI, PhD
Graduate Group Chairperson



ELIZABETH RHOADES, PhD
Reader

Dedication

To Shuangping Shi and Libin Lee, my greatest advocates and first-ever mentors in science and in life.

Acknowledgements

The scientific work embodied in this thesis would not be possible without the laboratory resources and institutional support of the Children's Hospital of Philadelphia (CHOP) or mentorship and collaboration of current and former members of the William Peranteau Laboratory in CHOP, including Grace Chau; Marco Carpenter, PhD; John Sincavage, MD; Jackson Bauer; Samantha Wang, Rosa Choi; Philip Zoltick, MD; Kara Kennedy, MD; Andrew Mudreac, MD; Natalie Berger, MD; Hanbing Cao; Aarsha Shah; Sriya Teerdhala; and William Peranteau, MD, as well as Mohamed Gabriel Alameh, PhD from the University of Pennsylvania's Engineered mRNA and Targeted Nanomedicine core. Additionally, this work was supported by institutional funds from the University of Pennsylvania's Ernest M. Brown, Jr., M.D. College Alumni Society Undergraduate Research Grant, the Roy and Diana Vagelos Science Challenge Award, and the Roy and Diana Vagelos Molecular Life Sciences.

This thesis is also supported by faculty advisors William Peranteau, MD; Jeffery Saven, PhD; and Elizabeth Rhoades, PhD, as well as undergraduate administrative coordinators Zoe Nguyen and Candace Adams.

I offer my deepest thanks to all the mentors, readers, collaborators, and organizers who made this thesis possible.

Abstract

CRISPR-Cas9-based tools offer opportunities to correct aberrant genes implicated in monogenic diseases with unprecedented efficiency and precision. In this thesis, I describe the optimization and evaluation of these tools as gene therapy candidates across four projects: CRISPR-Cas9 editing in the central nervous system, CRISPR-based epigenetic and genetic knockdown of a pathology-associated gene in skeletal and cardiac muscle tissue, CRISPR base editing in a muscle-specific disease gene, and CRISPR prime editing in hematopoietic stem cells. Each of these cases expands upon technologies among currently available gene editing tools while summarizing the design and challenges of experiments in generating genetic disease models, correcting pathological mutations, and assessing functional readouts of gene editing strategies.

Table of Contents

<i>Dedication.....</i>	<i>ii</i>
<i>Acknowledgements.....</i>	<i>iii</i>
<i>Abstract.....</i>	<i>iv</i>
<i>Tables.....</i>	<i>vi</i>
<i>Figures.....</i>	<i>x</i>
<i>Introduction.....</i>	<i>1</i>
<i>Methods.....</i>	<i>4</i>
<i>I. CRISPR-Cas9 Excision of FXN GAA Repeats in Friedreich’s Ataxia Models....</i>	<i>19</i>
Results.....	20
Discussion.....	23
<i>II. dCas9-based Methods for PIK3C2B Knockdown for Treatment of X-linked Myotubular Myopathy.....</i>	<i>25</i>
Results.....	26
Discussion.....	31
<i>III. Base Editing of DMD for Treatment of Duchenne Muscular Dystrophy.....</i>	<i>32</i>
Results.....	35
Discussion.....	41
<i>IV. Prime Editing of HBA2 for Treatment of Alpha Thalassemia.....</i>	<i>44</i>
Results.....	44
Discussion.....	46
<i>Conclusion.....</i>	<i>46</i>
<i>Appendix.....</i>	<i>47</i>
<i>References.....</i>	<i>61</i>

Tables

Table 1: <i>FXN</i> -targeting sgRNA guide sequences used by Rocca <i>et al.</i>	4
Table 2: Top 10 CRISPRoff sgRNA candidates tiling the 1-kb region containing the <i>PIK3C2B</i> promoter sequence, as determined by the Eukaryotic Promoter Database. sgRNA candidate spacer sequences and PAMs obtained with CRISPOR; MIT Specificity Score denotes uniqueness of sgRNA to target sequence and inversely correlated to off-target Cas9 binding.	5
Table 3: Top 10 ABE8.8 sgRNA candidates targeting splice donor or acceptor sites across 34 exon-intron junctions in <i>PIK3C2B</i> . sgRNA candidate spacer sequences, PAMs, and corresponding adenine base editor (ABE) scores obtained with SpliceR; ABE scores denote predicted editing. MIT Specificity Scores cross-referenced with CRISPOR.	6
Table 4: Mouse analogs of human <i>PIK3C2B</i> sgRNAs in Table 3.	7
Table 5: sgRNA candidates targeting the <i>DMD</i> exon 45 splice acceptor site (SAS) in mouse, as validated by Chai <i>et al.</i> ²² Corresponding sheep, nonhuman primate, and human sgRNA. Desired adenine edit underlined.	7
Table 6: Top 6 pegRNA 19-nt RTT sequences carrying silent mutations based on Shao <i>et al.</i> All pegRNAs used the same spacer and PBS sequences.	8
Table 7: Chemically modified sgRNAs targeting <i>DMD</i> exon 45 splice acceptor site. No prefix = DNA base; r_ = RNA base; m_* = Phosphorothioated 2'-O-methyl RNA base (3' and 5' end modifications); m_ = 2' O-methyl RNA bases (scaffold base pairing; terminal Uridine modification).....	12
Table 8: Illumina Miseq NGS adapters ligated to primer sequences for each project.	12
Table 9: <i>FXN</i> primers for long-range PCR, as per Rocca <i>et al.</i>	13

Table 10: <i>PIK3C2B</i> primers for RT-qPCR, as per Sabha <i>et al.</i> , for quantification of <i>PIK3C2B</i> following treatment with CRISPRoff.....	13
Table 11: Primers flanking human <i>PIK3C2B</i> exon splice donor and acceptor sites, designed using NCBI's Primer-BLAST.....	14
Table 12: Primers flanking mouse <i>PIK3C2B</i> exon 8, designed using NCBI's Primer-BLAST.....	15
Table 13: Primers flanking the <i>DMD</i> exon 45 splice acceptor site in mouse, sheep, nonhuman primate, and human, designed using NCBI's Primer-BLAST.....	15
Table 14: Exon skipping efficiency = (amount of skipped transcript)/(amount of skipped transcript + amount of unskipped transcript). qPCR primers were designed using NIH Primer Blast. The forward primer spans the exon-exon junction while the reverse primer lies at the 3' end of the second exon. Primers are designed with maximum specificity, melting temperatures between 55-70 degrees Celsius, and GC content between 40-60%. Due to a lack of pyrimidine bases at the exon 44-exon 46 junction in the skipped transcript, a GC content of 33% was tolerated (melting temperature = 54.61 degrees C).	16
Table 15: Forward and reverse primers for sequencing <i>HBA2</i> in NGS, as designed with Primer-BLAST	16
Table 16: <i>PIK3C2B</i> expression table for top 10 predicted sgRNAs in Huh7 CRISPRoff sgRNA screen.....	48
Table 17: Mouse analogs to top 3 human <i>PIK3C2B</i> -targeting CRISPRoff sgRNAs.....	48
Table 18: <i>PIK3C2B</i> expression table for murine analogs of top 3 human sgRNAs screened in Huh7 cells. Murine CRISPRoff sgRNA screen conducted in C2C12 cells...	48

Table 19: AàG editing efficiencies of ABE sgRNA candidates targeting <i>PIK3C2B</i> exon splice sites in Huh7 cells.....	48
Table 20: AàG editing efficiencies of the mouse analog of the top ABE sgRNA candidate targeting the <i>PIK3C2B</i> exon 8 splice donor site in fetal (E14) and adult (6 weeks) mice at 1 or 2 mg/kg IV LNP-delivered ABE mRNA.....	49
Table 21: Adenine base editing at <i>DMD</i> exon 45 splice acceptor site in bulk mouse liver tissue. Livers from first eight cohorts displayed (including control) obtained 1 month after IV injection of LNPs containing ABE8.20 or ABE8e mRNA and sgRNA adapted from Chai <i>et al.</i> ²² at time points (E14, E16, Adulthood [6 weeks]) and dosages (2 mg/kg, 3 mg/kg) specified. Livers of last cohort collected at 12 months to assess long-term proliferation of edited cells. HI MOD indicates “highly chemically modified” sgRNA for enhancement of CRISPR-Cas9 editing, as discussed in Table 7, Methods.....	50
Table 22: Adenine base editing at <i>DMD</i> exon 45 splice acceptor site in bulk mouse heart tissue. Reference Table 21, Appendix for remarks on notation.	50
Table 23: Adenine base editing at <i>DMD</i> exon 45 splice acceptor site in bulk mouse diaphragm tissue. Reference Table 21, Appendix for remarks on notation.	51
Table 24: Adenine base editing at <i>DMD</i> exon 45 splice acceptor site in bulk mouse quadriceps tissue. Reference Table 21, Appendix for remarks on notation.....	51
Table 25: Adenine base editing at <i>DMD</i> exon 45 splice acceptor site in bulk mouse tibialis anterior tissue. Reference Table 21, Appendix for remarks on notation.....	52
Table 26: ABE sgRNAs for targeting exon splice sites in sheep <i>DMD</i> . The entries for which “% Alignment with Human Guides” are filled as “NA” have no corresponding available PAMs in human <i>DMD</i>	55

Table 27: ABE sgRNAs for targeting exon splice sites in monkey <i>DMD</i>	56
Table 28: ABE sgRNAs for targeting exon splice sites in human <i>DMD</i>	58
Table 29: ABE sgRNAs for targeting exon splice sites in mouse <i>DMD</i> , including Chai <i>et al.</i> 's ²² sgRNA and an sgRNA targeting the exon 8 SDS in the <i>DMD</i> mouse model <i>Mdx-4cv</i> (mutation rescuable with exon skipping strategy).	58
Table 30: Editing rates assessed from NGS among control and experimental tissue and cell samples.....	60
Table 31: Editing efficiencies of PE6c and various pegRNAs carrying silent mutations, including Shao <i>et al.</i> 's original pegRNA, targeting <i>HBA2</i> in HbCS K562 cells.....	60

Figures

Figure 1: Timeline of murine development with all relevant injection time points. Mouse organs harvested 1 month after injection.....	10
Figure 2: DNA harvested from HEK293T cells treated with Cas9 mRNA and UP4 and DN4 sgRNAs, run on an agarose gel. 2-kb excised <i>FXN</i> fragment circled in red. (A) mRNA nucleofection with UP4 only; DN4 only; or 0 (control replicate), 1, 3, or 5 μ L mRNA. (B) LNP transfection with 0 (control replicate), 1 (replicate), 5, or 10 μ L mRNA. Figure courtesy of M. Carpenter.....	21
Figure 3: Wide-field fluorescence microscopy imaging of brain tissue sections in Sun1-GFP injected with LNPs containing Cre-recombinase mRNA at E14 (A) ICV or (B) IV. Figure courtesy of M. Carpenter.....	22
Figure 4: DNA harvested from YG8sR mouse organs following ICV or IV injection of LNPs containing Cas9 mRNA and UP4 and DN4 sgRNAs at E14, E15, or P0. <i>FXN</i> cleavage measured in (A) mouse pup livers (L), cerebella (Ce), and cortices (co) after E14 ICV injection; (B) cortices after P0 or E14 ICV injection; (C) livers after E14 ICV, E14 IV, or P0 IV injections; and (D) cerebella after E15 ICV or E14 IV injections. Mice brains harvested as described in Methods. Figure courtesy of M. Carpenter.....	23
Figure 5: (A) Top 10 predicted most efficient CRISPRoff sgRNAs tiling the human <i>PIK3C2B</i> promoter. (B) Relative <i>PIK3C2B</i> mRNA expression following CRISPRoff treatment in Huh7 cells. Figure courtesy of J. Sincavage.....	27
Figure 6: (A) Top 3 human CRISPRoff sgRNA candidates superimposed with murine analogs. Human sgRNA2, sgRNA5, and sgRNA10 correspond with mouse sgRNA1, sgRNA2, and sgRNA3, respectively. (B) <i>PIK3C2B</i> mRNA expression profiles 3, 14, and	

30 days after nucleofection of C2C12 cells with CRISPRoff mRNA and either mouse sgRNA1, sgRNA2, or sgRNA3. Figure courtesy of J. Sincavage.....	28
Figure 7: Editing efficiencies of human <i>PIK3C2B</i> splice site-targeting ABE sgRNAs screened in Huh7 cells.....	29
Figure 8: Measurement of PI3K-C2 β levels in Huh7 cells 30 days after nucleofection of ABE sgRNAs targeting human <i>PIK3C2B</i> splice sites. (A) Western blot, PI3K-C2 β bands boxed in red with GAPDH designated as housekeeping protein. g8 (1) refers to the first replicate of sgRNA targeting the splice donor site of <i>PIK3C2B</i> exon 8 (as in Table 3). Precision Plus Protein Dual Color Standards on far left measure protein size. (B) Quantified PI3K-C2 β expression.....	30
Figure 9: IV injections of LNPs containing ABE mRNA and the mouse analog of the sgRNA targeting the <i>PIK3C2B</i> exon 8 splice donor site. Editing results are shown in Table 20, Appendix. Figure courtesy of J. Sincavage.....	31
Figure 10: Schematic of the DMD exon 45 skipping strategy via adenine base editing at the splice acceptor site to restore an open reading frame, resulting in a truncated but functional dystrophin product. (A) In a disease model, a mutation at exon 44 in DMD may cause splicing errors that exclude exon 44 from the mature transcript. Splicing of exons 43 and 45 fails to produce a functional open reading frame. Skipping exon 45 by mutating its splice acceptor site causes exon 43 to splice with exon 46, which does create a functional open reading frame. (B) Functional tests for exon skipping may be conducted in wildtype models.....	34
Figure 11: <i>In vitro</i> experiments to screen optimal editor for DMD studies. (A) Selected sgRNA at the <i>DMD</i> locus with the targeted adenine in the exon 45 SAS highlighted at	

position 0. Editing evaluated for (B) human, (C) sheep, and (D) nonhuman primate cells.	
Species-appropriate primers in Table 7 (Methods) used.....	36
Figure 12: Whole-tissue-level editing for various tissues, mRNA compositions, LNP-mRNA dosages, and time points of injection. It should be noted that one mouse cohort injected with ABE8.20 mRNA was also included. Key: Control = Control, 1 = 2 mg/kg ABE 8.20 at E14; 2 = 2 mg/kg ABE 8e at E14; 3 = 2 mg/kg ABE 8e at E14 + chemically modified sgRNA; 4 = 2 mg/kg ABE 8e at E16; 5 = 2 mg/kg ABE 8e at E16 + chemically modified sgRNA; 6 = 3 mg/kg ABE 8e at 6 weeks + chemically modified sgRNA; 7 = 3 mg/kg ABE 8e at E14 + chemically modified sgRNA; 8 = 2 mg/kg ABE 8e at E14 + chemically modified sgRNA harvested at 12 months. Adenine base editing rates in Tables 21-25, Appendix. Individual tissue plots in Figure 18, Appendix.....	37
Figure 13: Gating of nuclei (DAPI+) for various tissues: heart (A), quadricep (B), diaphragm (C) following fluorescence-activated nuclear sorting (FANS). Sample preparation described in Methods. Nuclei are filtered from all events to distinguish from debris, and singlets are filtered from nuclei to distinguish individual nuclei from doublets. DAPI is used as a confirmatory nuclear marker.....	39
Figure 14: Gating of nuclear markers among nuclei (DAPI+). (A) Nuclei from quadricep (left) and diaphragm (right) tissues, where upper left quadrant contains PAX7+ satellite cells and lower right quadrant contains PCM1+ myocytes. (B) PCM1+ myocyte nuclei in heart (left), quadricep (middle), and diaphragm (right) tissues. (C) PAX7+ satellite cell nuclei from quadricep (left) and diaphragm (right) tissues.....	40
Figure 15: Editing efficiencies of PE6c and various pegRNAs carrying silent mutations, including Shao <i>et al.</i> 's original pegRNA, targeting <i>HBA2</i> in HbCS K562 cells.....	45

Figure 16: Measurement of PI3K-C2 β levels in C2C12 30 days after nucleofection of mouse analogs of top 3 human CRISPRoff sgRNAs. (A) Western blot, PI3K-C2 β bands boxed in red with GAPDH designated as housekeeping protein. Duplicates of controls and conditions with 3 <i>PIK3C2B</i> -targeting sgRNAs, from left to right with Precision Plus Protein Dual Color Standards on either end to measure size. (B) Quantified PI3K-C2 β expression.....	49
Figure 17: (A) Western blot of PI3K-C2 β levels in heart (control + 3 replicates, from left to right) and quadricep (control + 3 replicates, from left to right) tissues of control mice or mice injected IV with LNPs containing ABE mRNA and an sgRNA targeting the <i>PIK3C2B</i> exon 8 splice donor site at E14. Precision Plus Protein Dual Color Standards used to measure protein size. (B) Quantification of protein.....	49
Figure 18: Adenine base editing efficiency at <i>DMD</i> exon 45 SAS separated by organ: (A) livers, (B) hearts (C) quadriceps, (D) tibialis anterior, and (E) diaphragms. For key, reference Figure 11, Main Text.....	52

Introduction

Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR-Cas9) are a powerful genome engineering tool that can be customized for different gene editing applications. With a single-guide RNA (sgRNA) containing a 20-nucleotide (nt) spacer complementary to a target genetic sequence directly upstream of a 3-nt protospacer adjacent motif (PAM) and a trans-activating CRISPR RNA acting as a scaffold for Cas9, Cas9 is directed to the target sequence, where it induces a double-stranded DNA break with its RuvC and HNH nuclease domains 3 nt upstream of the PAM (NGG nucleotide sequence for the commonly used Cas9 variant from *Streptococcus pyogenes*, SpCas9)¹. The double-stranded break is subsequently repaired by either endogenous non-homologous end-joining (NHEJ), which results in a high rate of spontaneous nucleotide insertions and deletions (indels), or, if an exogenous repair template to be integrated into the host DNA is provided, homology-directed repair². With this function, CRISPR-Cas9 exhibits useful applications in knocking down, excising, inserting, or correcting genes implicated in disease: indeed, therapeutic CRISPR-Cas9-based candidates, such as a construct targeting the *BCL11A* enhancer to reverse γ -globin suppression in β -thalassemia and sickle cell disease patients, have already advanced to human use³.

However, CRISPR-Cas9 is not without risks. Unintended indels can perversely affect gene expression across the genome, wherever Cas9 happens to inadvertently make contact with the host DNA⁴. Novel iterations of CRISPR-Cas9-based tools enable edits in the genome without double-stranded breaks with the use of catalytically “dead” Cas9 (dCas9), which attaches to the target sequence without cleaving the DNA, or Cas9

nickase (Cas9n), which induces a single rather than a double-stranded break at the target sequence, both by way of mutating the Cas9 RuvC and HNH nuclease domains^{5,6}. As opposed to an indel or cleavage event, Cas9n enables precise single-base edits through attached effector domains, as in adenine base editors (ABEs) that use the adenosine deaminase TadA to convert an adenine base to an inosine that is recognized by DNA polymerase as guanine. The nickase action on the unedited strand forces the cell's mismatch repair machinery to prioritize the edited strand as the template, thereby converting the initial A:T base pair to G:C⁷. These tools are useful candidates for treating genetic diseases caused by pathological single nucleotide polymorphisms (SNPs), as has already been witnessed in the clinical administration of an adenine base editor for the treatment of liver CPS1 deficiency⁸. Furthermore, Cas9n enables any of the 12 nucleotide base changes to be executed in the prime editor, whose effector protein is a reverse transcriptase that synthesizes any letter or sequence encoded by a reverse transcriptase template (RTT) in the prime editing guide RNA (pegRNA). In addition to the RTT and sgRNA components, the pegRNA also includes a primer binding site (PBS) directly upstream of the desired site to be edited, which serves as the primer for the attached reverse transcriptase. The reverse-transcriptase-synthesized product then displaces the single-strand flap created by Cas9n to create the desired DNA edit⁹. Prime editors have already been deployed in clinical contexts for diseases caused by genetic mutations beyond SNPs, such as a 2-nt deletion in exon 2 in the *NCF1* locus in chronic granulomatous disease patients¹⁰.

Base and prime editors, though more precise than CRISPR-Cas9 nucleases, may still induce off-target or bystander editing at sites beyond the target sequence or at non-

target bases within the target sequence¹¹. However, changes in gene expression may still be modulated without having base changes induced by CRISPR effectors. CRISPRoff, which contains a dCas9 fused to a repressive Krüppel-associated box (KRAB) and the DNA methyltransferases Dnmt3A and Dnmt3L, suppresses gene expression by methylating CpG sites within gene promoters without modifying any bases¹², thereby avoiding both indels and off-target base editing. Though not yet advanced to clinical use cases, CRISPRoff demonstrates how CRISPR-Cas9 technology can be tailored for diverse gene modifications and enhanced safety profiles.

There are several gene therapy delivery vectors currently in development, among them adeno-associated viruses (AAVs) and lipid nanoparticles (LNPs). Of these vectors, LNPs are particularly favorable due to their ability to accommodate repeat dosing of large CRISPR-Cas9 constructs¹³, low immunogenicity, and customizability for tropism to specific organs. Furthermore, whereas AAVs mediate integration of their genetic contents into the host genome, resulting in potentially undesired longitudinal expression of AAV constructs, LNPs enable the transient expression of delivered mRNA in the host to achieve therapeutic effects with fewer long-term risks¹⁴.

In this thesis, I present the application of various CRISPR-Cas9-based tools as candidates for treating the following monogenic diseases: Friedreich's ataxia, X-linked myotubular myopathy, Duchenne muscular dystrophy, and alpha-thalassemia. The Peranteau Lab's work demonstrate how these tools are selected and deployed in versatile disease contexts, specifically through the perspective of how unique loci and physiological factors shape gene editing strategies. With further clinical maturation, the

toolbox of gene editing tools and strategies presented in this thesis aims to enable the development of precision therapies for patients with rare monogenic diseases.

Methods

For all tables below, sequences are displayed in 5' to 3' order. Primer sequences are displayed without their ligated Nextera adapter sequences.

Selection of sgRNAs/pegRNAs. sgRNA/pegRNA spacers upstream of PAMs that were favorably located near the desired site to be edited, and which exhibited the highest predicted CRISPR-Cas9 editing efficiency and lowest predicted likelihood of editing similar sites with few base mismatches, were used for each project.

In the Friedreich's ataxia (FRDA) project, Rocca *et al.*'s UP4 and DN4 sgRNAs fit these criteria well and were consequently used. UP4 and DN4 respectively target regions on opposite strands that are directly upstream (5') and downstream (3') of the GAA repeats located in intron 1 of the FXN locus implicated in FRDA (Table 1), as optimized by Doench *et al.*'s Rule Set 2 and *in vitro* experiments with hematopoietic stem cells^{15,16}.

sgRNA Name	Target	Spacer Sequence/PAM
UP4	<i>FXN</i> forward strand	TTACGCCACGGCTTGAAAGG/AGG
DN4	<i>FXN</i> reverse strand	ACCGGGCGTCATATGGTAAG/CGG

Table 1: *FXN*-targeting sgRNA guide sequences used by Rocca *et al.*

The X-linked myotubular myopathy (XLMTM) project used epigenetic and base editing approaches targeting *PIK3C2B*. In the epigenetic approach, the human *PIK3C2B* promoter sequence was first found on the Eukaryotic Promoter Database¹⁷, and the promoter region was defined as the 1-kilobase (kb) region surrounding the promoter. The sequence for the human *PIK3C2B* promoter region, Chromosome 1: 204423028 -

204424028, consisting of a CpG island and exon 1, was input into the sgRNA design webtool CRISPOR¹⁸. A list of ten candidate CRISPRoff sgRNAs with the highest MIT specificity scores and optimal coverage of the promoter was subsequently obtained (Table 2).

Human sgRNA ID	Spacer Sequence/PAM	MIT Specificity Score
sgRNA_1_CRISPRoff	ATTACGGTAGCGATGTTCGG/GGG	99
sgRNA_2_CRISPRoff	CGAATCCGCCGCGAGCCGGA/GGG	97
sgRNA_3_CRISPRoff	CCTCCGGCTCGCGGCGGATT/CGG	97
sgRNA_4_CRISPRoff	GAACATCGCTACCGTAATTA/TGG	96
sgRNA_5_CRISPRoff	CTGCACGGCGACGCGACGCT/CGG	96
sgRNA_6_CRISPRoff	GTGCGCGAGCTCGCAGAGTC/AGG	95
sgRNA_7_CRISPRoff	GCCTCTTGCACCAGCGCGAG/AGG	95
sgRNA_8_CRISPRoff	ACGCATCCTGCGGGTTGCAT/CGG	94
sgRNA_9_CRISPRoff	CGCCCCACTTGCCATTGCGT/GGG	94
sgRNA_10_CRISPRoff	GAGCTCGCGCACGCTCTCGG/CGG	94

Table 2: Top 10 CRISPRoff sgRNA candidates tiling the 1-kb region containing the *PIK3C2B* promoter sequence, as determined by the Eukaryotic Promoter Database. sgRNA candidate spacer sequences and PAMs obtained with CRISPOR; MIT Specificity Score denotes uniqueness of sgRNA to target sequence and inversely correlated to off-target Cas9 binding.

In the base editing approach, sgRNAs were selected for the adenine base editor ABE8.8, which exhibits deaminase activity in base pair positions 3 to 9 in the target DNA sequence complementary to the spacer¹⁹. The human *PIK3C2B* transcript, which consists of 33 exons, was located on the Ensembl genome database²⁰, and its identification number was input into SpliceR, a web tool developed by the Moriarity Lab at the University of Minnesota Medical School²¹. sgRNAs editing splice donor and acceptor sites at *PIK3C2B* intron-exon junctions were generated, and potential off-target effects of the sgRNAs were again cross-checked with MIT specificity scores from CRISPOR (Table 3).

Targeted Exon	Splice Site	Human sgRNA Spacer Sequence/PAM	ABE Position	ABE Score	MIT Specificity
---------------	-------------	---------------------------------	--------------	-----------	-----------------

					Score
27 (1)	donor	CACTTACATAGCCTTTGTTG /GGG	6	0.828	63
29 (1)	donor	CACCCACCTCGGCCACCTCA /GGG	6	0.755	0
32	acceptor	TTGTAGCAACTGCTCCAGGA /TGG	5	0.607	61
14	acceptor	CTTCTTAGGGTCCTGACCTG /TGG	7	0.619	64
27 (2)	donor	ACACTTACATAGCCTTTGTT /GGG	7	0.529	78
8	donor	ACCCACCTCGGCCACCTCAG /GGG	5	0.447	50
29 (2)	donor	ACCCACCTCGGCCACCTCAG /GGG	5	0.472	46
17	acceptor	TTGCAGCTTCCCGGACCAGG /AGG	5	0.43	78
10	acceptor	CCAACAGGATCCCAGTGTCT /TGG	6	0.737	63
19	donor	CACTAACCTGCCTTGCAGAT /GGG	6	0.556	71

Table 3: Top 10 ABE8.8 sgRNA candidates targeting splice donor or acceptor sites across 34 exon-intron junctions in *PIK3C2B*. sgRNA candidate spacer sequences, PAMs, and corresponding adenine base editor (ABE) scores obtained with SpliceR; ABE scores denote predicted editing. MIT Specificity Scores cross-referenced with CRISPOR.

Corresponding mouse *PIK3C2B* sgRNAs were found at corresponding exons (Table 4).

Targeted Exon	Splice Site	Mouse sgRNA Spacer Sequence/PAM	Similarity to Human sgRNA Spacer
27 (1)	donor	CACTTACATAGCCCTTGCTG /GGG	90%
29 (1)	donor	CACTCACCTCAGCCACCTCA /GGG	90%
32	acceptor	TTGTAGCAACCGCTCCAGGA /TGG	95%
14	acceptor	TGCCTTAGAGTCTTGACCTG /TGG	75%
27 (2)	donor	ACACTTACATAGCCCTTGCT	90%

		/GGG	
8	donor	ACTCACATCAGCCAGCAGGA /GGG	100%
29 (2)	donor	ACTCACCTCAGCCACCTCAG /GGG	90%
17	acceptor	TTGCAGCTTCCCGGACCAGG /AGG	100%
10	acceptor	CCAACAGGATCCCAGCGTGT /TGA	Not applicable—non-NGG PAM in mouse
19	donor	CACTCACCTGGCGGGCGGAT/ GGG	75%

Table 4: Mouse analogs of human *PIK3C2B* sgRNAs in Table 3.

The Duchenne muscular dystrophy (DMD) project modified Chai *et al.*'s²² highest-performing 18-nt sgRNA spacer targeting the *DMD* exon 45 splice acceptor site in the authors' *DMD* mouse model by adding two nucleotides to the 5' end that maintain mouse DNA sequence homology. For future sheep, nonhuman primate, and human studies, we modified the sequence to target analogous sites in each respective genome. In each species, selected sgRNAs target the adenine at position 8 in the spacer (Table 5).

Species	Site	Spacer Sequence/PAM
Mouse (Chai <i>et al.</i> ²²)	Exon 45 SAS	GGCTTAC <u>A</u> GGA ^A CTCCAGGA/TGG
Sheep	Exon 43 SAS	ATCTCAC <u>A</u> GGA ^A CTCCAGGA/TGG
Nonhuman Primate	Exon 45 SAS	ATCTTAC <u>A</u> GGA ^A CTCCAGGA/TGG
Human	Exon 45 SAS	ATCTTAC <u>A</u> GGA ^A CTCCAGGA/TGG

Table 5: sgRNA candidates targeting the *DMD* exon 45 splice acceptor site (SAS) in mouse, as validated by Chai *et al.*²² Corresponding sheep, nonhuman primate, and human sgRNA. Desired adenine edit underlined.

In the alpha-thalassemia project, the pegRNA spacer, PBS, and RTT used by Shao *et al.*²³ to correct the human Hemoglobin Constant Spring (HbCS) mutation served as a starting scaffold for subsequent optimization. RTTs carrying silent mutations

encoding the restored termination codon and preceding wildtype amino acids were designed to optimize prime editing efficiency (Table 6).

pegRNA Spacer Sequence/PAM	CTACCGAGGCTCCAGCTTGA/CGG
pegRNA PBS	AGCTGGAGCCTCG
pegRNA RTT	RTT Sequence
Shao <i>et al.</i> RTT	TGACCTCCAAATACCGTTA
RTT Mutant 1	TGACCTCCAAATATCGTTA
RTT Mutant 2	TGACCTCCAAATACAGATA
RTT Mutant 3	TGACCTCCAAATATAGGTA
RTT Mutant 4	TGACCTCCAAGTATCGTTG
RTT Mutant 5	TGACCTCCAAGTATAGGTG
RTT Mutant 6	TGACCTCCAAGTATAGGTA

Table 6: Top 6 pegRNA 19-nt RTT sequences carrying silent mutations based on Shao *et al.* All pegRNAs used the same spacer and PBS sequences.

sgRNAs and pegRNAs were obtained from Integrated DNA Technologies' (IDT) Alt-R platform.

Cells. Species-appropriate cell lines were grown in culture for each project and nucleofected with project-specific editors and sgRNAs/pegRNAs using Lonza nucleofection cassettes. Cells were harvested upon becoming confluent and lysed to obtain DNA or mRNA for assessment of editing efficiency using the appropriate primers. Protein products were quantified using western blot whenever applicable.

For the FRDA project, cells from the immortalized human embryonic kidney cell line HEK293T were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine. HEK293T cells were either nucleofected with 1, 3, or 5 ng/μL of Cas9 mRNA and the UP4 and DN4 sgRNAs or transfected with 1, 5, or 10 ng/μL of ALC-315 LNPs containing Cas9 mRNA from IDT and the UP4 and DN4 sgRNAs prior to DNA harvest.

For the XLMTM project, cells from the immortalized human hepatocellular carcinoma cell line Huh7 were grown in DMEM supplemented with 10% FBS and 1%

penicillin-streptomycin, then used to evaluate CRISPRoff sgRNA candidates. Huh7 cells were nucleofected with sgRNAs obtained from IDT and CRISPRoff mRNA synthesized by the University of Pennsylvania's Institute for RNA Innovation. Successful CRISPRoff sgRNA candidates were validated using cells from the mouse skeletal muscle myoblast cell line C2C12, grown in high-glucose DMEM supplemented with 10% FBS and 1% L-glutamine. ABE8.8 sgRNAs were meanwhile evaluated in Huh7 cells to determine which *PIK3C2B* exon splice donor or acceptor sites are most favorable to target for effective knockdown via DNA sequencing with *PIK3C2B* splice site-specific primers.

To determine the optimal adenine base editor (ABE8.20¹⁹, ABE8.8, or ABE8e⁷) for the top candidate sgRNA in the DMD project, different editors synthesized by the University of Pennsylvania's Institute for RNA Innovation were transfected with the candidate sgRNA used for this study into primary sheep fibroblasts, primary nonhuman primate mesenchymal stem cells, and HEK293T cells, adjusting the sgRNA sequence appropriately for each species.

For the alpha-thalassemia project, cells from the human immortalized erythroleukemia cell line K562 edited by ABE8e to contain the HbCS mutation in both *HBA2* alleles were held in stock in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS and 1% penicillin-streptomycin. The HbSC K562 cells were nucleofected with mRNA for the next-generation prime editor PE6c⁹ and various pegRNAs to determine the most efficient corrective prime editing strategy.

Animal models. ALC-315 LNPs were used for all animal injections.

The Sun1-GFP reporter mouse model was used to evaluate the biodistribution of injected LNPs at prenatal and postnatal time points. When injected with mRNA for Cre-

recombinase, an enzyme that excises gene segments flanked by loxP sites, a loxP-flanked termination codon is excised in Sun1-GFP mice, enabling the expression of the downstream gene encoding a Sun1 fusion protein fused to GFP²⁴. For embryonic or adult biodistribution studies, Sun1-GFP mice were injected at embryonic day 14 (E14) or at 6 weeks, respectively. In projects without a disease model, BALB/c mice were used to assess gene editing efficiency following injection of the appropriate editors and sgRNAs/pegRNAs. Suffolk sheep and rhesus monkeys (nonhuman primates) will be used for future large animal studies.

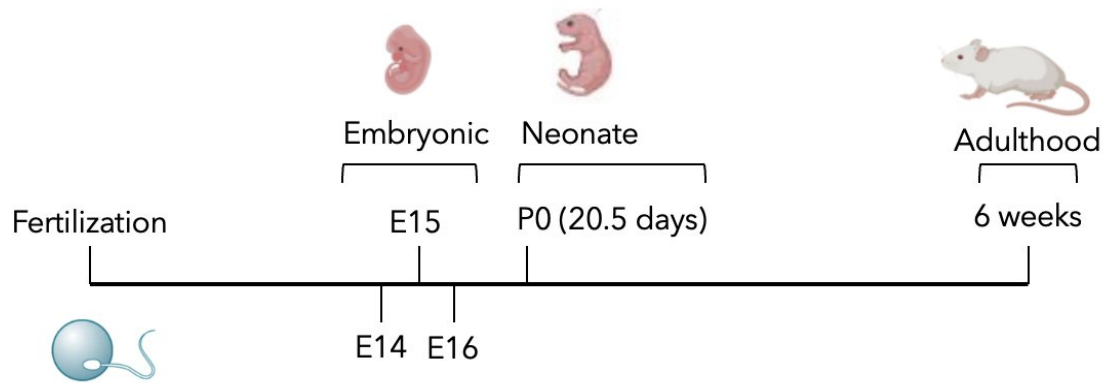


Figure 1: Timeline of murine development with all relevant injection time points. Mouse organs harvested 1 month after injection.

YG8sR mice were used as the disease model for the FRDA project. Mice were injected with LNPs containing Cas9 mRNA and the UP4 and DN4 sgRNAs (courtesy of University of Pennsylvania Institute of RNA Innovation) at either E15, E14 or postnatal day 0 (P0) intravenously (IV) or intracerebroventricularly (ICV). Specifically, E15 ICV, E14 IV, P0 ICV, and P0 IV injections were carried out.

In both the XLMTM and DMD projects, ABE8.8 mRNA synthesized by the University of Pennsylvania's Institute for RNA Innovation was packaged in LNPs together with unmodified or chemically modified²⁵ sgRNA targeting the exon splice sites

of interest, using PBS as a control. LNPs were injected intravenously at 2.0 mg/kg through the vitelline vein of E14 and E16 mice or the retroorbital sinus of 6-week-old mice. Diaphragm, heart, liver, quadricep, and tibialis anterior tissues were collected.

For the DMD project, mice were sacrificed at one- or twelve-months post-injection to investigate short-term and long-term retention of edited myonuclei. In sheep, intravenous injections of ABE8e mRNA and chemically modified sgRNA encapsulated in LNPs at 2.0 mg/kg via the vitelline vein will be carried out at 60-, 90-, and 150-days gestational age in accordance with our previous protocols, while nonhuman primate fetuses will similarly receive injections at 80 days gestational age. Sheep and nonhuman primate fetuses will be sacrificed one week after injection. Chemically modified sgRNA sequences for the DMD project are displayed in Table 7:

Species	Site	mRNA Sequence (PAM Excluded)
Mouse (Chai <i>et al.</i> ²²)	Exon 45 SAS	mG*mG*mC*rUrUrArCrArGrGrArArCrUrCrCrArGrGrArGr rUrUrUrUrArGrAmGmCmUmAmGmAmAmAmUmAmGm CrArArGrUrUrArArArArUrArArGrGrCrUrArGrUrCrCrGrU rUrArUrCrAmAmCmUmUmGmAmAmAmAmAmGmUmG mGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*m U*mU*U
Sheep	Exon 43 SAS	mA*mU*mC*rUrCrArCrArGrGrArArCrUrCrCrArGrGrArGr UrUrUrUrArGrAmGmCmUmAmGmAmAmAmUmAmGmC rArArGrUrUrArArArArUrArArGrGrCrUrArGrUrCrCrGrUr UrArUrCrAmAmCmUmUmGmAmAmAmAmAmGmUmG mGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*m U*mU*mU
Nonhuman Primate	Exon 45 SAS	mA*mU*mC*rUrUrArCrArGrGrArArCrUrCrCrArGrGrArG rUrUrUrUrArGrAmGmCmUmAmGmAmAmAmUmAmGm CrArArGrUrUrArArArArUrArArGrGrCrUrArGrUrCrCrGrU rUrArUrCrAmAmCmUmUmGmAmAmAmAmAmGmUmG mGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*m U*mU*mU

Human	Exon 45 SAS	mA*mU*mC*rUrUrArCrArGrGrArArCrUrCrCrArGrGrArGrUrUrUrUrArGrAmGmCmUmAmGmAmAmAmUmAmGmCrArArGrUrUrArArArUrArArGrGrCrUrArGrUrCrCrGrUrUrArUrCrAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
-------	-------------	--

Table 7: Chemically modified sgRNAs targeting *DMD* exon 45 splice acceptor site. No prefix = DNA base; r_ = RNA base; m_ = Phosphorothioated 2'-O-methyl RNA base (3' and 5' end modifications); m_ = 2' O-methyl RNA bases (scaffold base pairing; terminal Uridine modification)

For the alpha-thalassemia project, a HbCS mouse model is currently in development but will not be elaborated further in this thesis.

Quantification of editing efficiency. For each project, DNA was isolated from cells or tissues using the QIAGEN DNEasy Blood and Tissue DNA Isolation Kit, then amplified with an initial round of PCR using Platinum Superfi II DNA Polymerase and appropriate primers modified with adapter overhangs (Table 8). The product was then subjected to an additional round of PCR to ligate Nextera sequencing indices for Illumina Next Generation Sequencing (NGS). DNA was then subjected to NGS by the Illumina Miseq system, and sequences were output as FASTA files. The FASTA files for each sample, sgRNA sequence, and amplicon sequence were input into the webtool CRISPResso²⁶ to assess the efficiency of the applied editor at the appropriate locus.

Oligonucleotide Name	Sequence
Nextera Forward Adapter	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
Nextera Reverse Adapter	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

Table 8: Illumina Miseq NGS adapters ligated to primer sequences for each project.

For the FRDA project, long-range PCR was performed to amplify DNA from control HEK293T cells or cells treated with Cas9 and the UP4 and DN4 sgRNAs using the following primers at the *FXN* locus in Table 9:

Primer	Sequence
<i>FXN</i> Forward	CGTGGCTTTGTTTTCTGTAGG
<i>FXN</i> Reverse	CCTGCTCATGGGATGCATT

Table 9: *FXN* primers for long-range PCR, as per Rocca *et al.*

CRISPR-Cas9 cleavage efficiency was analyzed qualitatively on a DNA agarose gel. PCR samples of interest were loaded into wells, and DNA size was assessed by the GeneRuler 1 kb Plus DNA Ladder following gel electrophoresis.

Specifically for the evaluation of CRISPRoff sgRNAs in the XLMTM project, total mRNA rather than DNA was used. *PIK3C2B* knockdown efficiency was therefore assessed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In Huh7 cells nucleofected with human CRISPRoff sgRNAs, total mRNA was harvested using the RNEasy Mini RNA Extraction Kit 3, 14, or 30 days after the initial nucleofection, then reverse transcribed into cDNA using the iScript cDNA Synthesis Kit. RT-qPCR was then conducted with *PIK3C2B* primers used by Sabha *et al.*²⁷ to quantify *PIK3C2B* transcripts produced at each time point after CRISPRoff nucleofection (Table 10). In C2C12 cell validation using mouse CRISPRoff sgRNAs, total RNA was harvested, reverse transcribed, and subjected to RT-qPCR with appropriate primers 3, 14, 20, or 24 days after the initial nucleofection.

Primer	Sequence
<i>PIK3C2B</i> Forward	ACCTGCAACTGTTCTCCAC
<i>PIK3C2B</i> Reverse	ACAGGGCTTCAGCACAAAGT

Table 10: *PIK3C2B* primers for RT-qPCR, as per Sabha *et al.*, for quantification of *PIK3C2B* following treatment with CRISPRoff.

For the evaluation of ABE8.8 in the XLMTM project, primers flanking each of the investigated splice donor or acceptor sites were used to assess A $\rightarrow$ G editing efficiency in DNA harvested from Huh7 cells following ABE8.8 nucleofection (Table 11).

Targeted Exon	Splice Site	Primer	Sequence
27 (1)	donor	Forward	AAGTCAGGATCCCAGCAAGC
		Reverse	GCAACATCCGACGTGTCTTG
29 (1)	donor	Forward	TCCTGACTCCTAGTGCCTCC
		Reverse	CCAGCTTCCCTAGTCGCTTC
32	acceptor	Forward	CCTACCCCACCATTTTCCCC
		Reverse	GCATTGCAAGATGCCAGATGG
14	acceptor	Forward	ATTAGGTGCACTCCAACGGG
		Reverse	ACGGTGAGAATGAGAGGGGA
27 (2)	donor	Forward	AAGTCAGGATCCCAGCAAGC
		Reverse	GCAACATCCGACGTGTCTTG
8	donor	Forward	TCCACCCAGACCCCTCATAG
		Reverse	GGGCACTGGGTCTTTCATGA
29 (2)	donor	Forward	TCCTGACTCCTAGTGCCTCC
		Reverse	CCAGCTTCCCTAGTCGCTTC
17	acceptor	Forward	CCAATCCACCCACCTGTACC
		Reverse	CTGAAGCCCAGGTGTGAG
10	acceptor	Forward	ATAAATCAAGGGGCCAGGC
		Reverse	GCAGCTGTGAAAAGCCCTTC
19	donor	Forward	GCCCTGGATGAGAAATGGGT
		Reverse	ACCCTTGCCTTTGATCCCTG

Table 11: Primers flanking human *PIK3C2B* exon splice donor and acceptor sites, designed using NCBI's Primer-BLAST.

The sgRNA that resulted in the greatest editing and knockdown of *PIK3C2B*, as evaluated through NGS of *PIK3C2B* and western blot quantification of PI3K-C2 β , was subsequently used for mouse studies, where the primers in Table 12 were applied:

Primer	Sequence
<i>PIK3C2B</i> Exon 8 Donor Forward	TCTGATCCTCCAACAGAGAG
<i>PIK3C2B</i> Exon 8 Donor Reverse	CAAGGAAGGAACTTCCAATAGT

Table 12: Primers flanking mouse *PIK3C2B* exon 8, designed using NCBI's Primer-BLAST.

In the DMD project, DNA was harvested from sheep, nonhuman primate, and human cells after nucleofection of either ABE8.20, ABE8.8, or ABE8e together with Chai *et al.*'s²² sgRNA targeting the *DMD* exon 45 splice acceptor site and sequenced using species-appropriate primers (Table 13) to assess A→G editing efficiency. The best-performing editor was injected with Chai *et al.*'s²² sgRNA in mice, and DNA was subsequently harvested from tissues and evaluated for editing efficiency.

Primer	Sequence
Mouse <i>DMD</i> Forward	ACATGTCAGGTTCATTGAGCAAAT
Mouse <i>DMD</i> Reverse	TTCCCCAGTTGCATTCAGTGT
Sheep <i>DMD</i> Forward	AGAACTGCATGGGGTAGCAC
Sheep <i>DMD</i> Reverse	CTCTGACAACAGTTTGCCGC
Nonhuman Primate <i>DMD</i> Forward	GACACGGGGCTTCATTTTTGT
Nonhuman Primate <i>DMD</i> Reverse	AGCTGTTTGCAGACCTCCTG
Human <i>DMD</i> Forward	GTCTTTCTGTCTTGTATCCTTTGG
Human <i>DMD</i> Reverse	TCTGACAACAGTTTGCCGCT

Table 13: Primers flanking the *DMD* exon 45 splice acceptor site in mouse, sheep, nonhuman primate, and human, designed using NCBI's Primer-BLAST.

In addition, for assessment of exon skipping efficiency, total RNA will be isolated from harvested tissues and reverse transcribed into cDNA. Forward primers targeting the 5'

end of exon 44 or the exon 44-exon 46 junction in the *DMD* transcript will be used to amplify cDNA, and efficiency will be measured by dividing the number of exon-skipped transcript reads by the total number of transcript reads, as measured relative to transcription of the housekeeping gene GAPDH in RT-qPCR (Table 14).

Exon-exon junction	Forward Primer Sequence	Reverse Primer Sequence
44-46	GGTATCTTAAGGATTGAAGAACAA	TGCTGCT CATCTCC AAGTG
Transcript Sequence	GCGATTTCGACAGATCAGTTGAAAAATGGCGACACTTTCATTAT GATATGAAGGTATTTAATCAATGGCTGAATGAAGTTGAACAGT TTTTCAAAAAGACACAAAATCCTGAAAACATGGGAACATGCTAA ATACAAATGGTATCTTAAGGATTGAAGAACAAAAGAATGTCTT GTCAGAATTTCAAAGAGATTTAAATGAATTTGTTTTGTGGCTGG AAGAAGCAGATAACATTGCTATTACTCCACTTGGAGATGAGCA GCAGCTAAAAGAACAACCTTGAACAAGTCAAG	
Primer in 5' end of Exon 44	TCAGTTGAAAAATGGCGACAC	

Table 14: Exon skipping efficiency = (amount of skipped transcript)/(amount of skipped transcript + amount of unskipped transcript). qPCR primers were designed using NIH Primer Blast. The forward primer spans the exon-exon junction while the reverse primer lies at the 3' end of the second exon. Primers are designed with maximum specificity, melting temperatures between 55-70 degrees Celsius, and GC content between 40-60%. Due to a lack of pyrimidine bases at the exon 44-exon 46 junction in the skipped transcript, a GC content of 33% was tolerated (melting temperature = 54.61 degrees C).

For the alpha-thalassemia project, DNA was harvested from HbCS K562 cells following nucleofection with PE6c and various pegRNAs using QuickExtract, then PCR amplified and purified for assessment of editing with Sanger sequencing and NGS (Table 15).

Primer	Sequence
<i>HBA2</i> Forward	CGCCGAGTTCACCCCTG
<i>HBA2</i> Reverse	GCCGGTGCAAGGAGGG

Table 15: Forward and reverse primers for sequencing *HBA2* in NGS, as designed with Primer-BLAST

NGS forward primers are mixed with PCR-amplified and purified QuickExtract DNA samples for Sanger sequencing. Editing is analyzed either by the Sanger Sequencing Core at Genewiz (for Sanger sequencing) or on CRISPResso (for NGS).

Quantification of protein. Protein was quantified in the XLMTM and alpha-thalassemia projects to respectively characterize loss of a protein implicated in disease and restoration of an essential protein mutated in disease.

For both CRISPRoff and ABE8.8 studies in the XLMTM project, the protein product of *PIK3C2B* (PI3K-C2 β) was quantified via western blot to assess knockdown efficiency at the protein level. Protein lysates were isolated from Huh7 cells with RIPA buffer, denatured with reducing buffer and heat, and run in SDS-PAGE using 3-8% Tris-Acetate gels. Protein was transferred to western blot membranes for ten minutes, and blots were blocked with 5% milk in 1x Tris-buffered saline with tween (TBST) for two hours. Blots were incubated overnight with anti-human PI3K-C2 β primary antibodies raised in mice diluted at 1:1000 in 2% milk in TBST. After washing with TBST, blots were incubated in anti-mouse horseradish peroxidase-conjugated secondary antibodies at room temperature for an hour, diluted 1:2000 in 2% milk in TBST, washed once again with TBST, and then soaked in SuperSignal West Femto substrate in preparation for chemiluminescence imaging. PI3K-C2 β signals at 178 kD normalized against signals of the housekeeping protein anti-human GAPDH raised in mouse (diluted at 1:100,000 in 2% milk-TBST) at 37 kD were confirmed using the Precision Plus Protein Dual Color Standards and quantified with ImageJ software after verifying that protein concentrations fell within a loading sample linear range.

For the quantification of restored wildtype alpha-globin in the alpha-thalassemia project, following prime editor nucleofection, protein lysates were obtained from the HbCS K562 cells using RIPA buffer and protease inhibitors. 1.5 µg protein samples were run through a Native Bis-Tris gel at 150V and transferred onto a nitrocellulose membrane using a low molecular weight program on an iBlot machine. Total Protein No Stain Reagent was first applied to visualize total protein before blocking in 2% BSA for two hours. After a subsequent overnight incubation in 1:2000 primary anti-alpha globin antibody raised in mice, the blot was washed with TBST and incubated in 1:4000 anti-mouse-IgG HRP-linked secondary antibody for one hour. West Pico chemiluminescent substrate was used to visualize alpha globin on an iBright machine. Alpha globin signal intensities were normalized against those of the total protein stain. Quantification was conducted with ImageJ.

Fluorescence-activated nuclear sorting (FANS) of skeletal muscle myocytes and progenitor satellite cells. In the DMD project, nuclei from skeletal muscle myocytes, cardiac myocytes, and skeletal muscle satellite cells in skeletal and cardiac muscle tissues harvested from mice injected with LNPs containing ABE8e mRNA and an sgRNA targeting the *DMD* exon 45 splice acceptor site were isolated using mechanical dissociation by gentleMACS, washing with NF-1 buffer containing detergent and sucrose, washing with FANS buffer containing bovine serum albumin, fixation in paraformaldehyde, quenching with glycine and filtration through a 70 µm strainer. Aside from fluorescence-minus-one, single-stain, and unstained controls, all nuclei were stained with DAPI and counted using the blue light emission channel with flow cytometry. Nuclei isolated from skeletal and cardiac muscle tissues were stained with a primary

antibody against PCM1, a transcription factor found in myocytes. Nuclei isolated from skeletal muscle were also stained with a primary antibody against PAX7, a transcription factor specific to satellite cells and absent from cardiac muscle tissue. PCM1-stained nuclei were incubated with a secondary Alexa Fluor 647 stain, while the PAX7-stained nuclei were incubated with a secondary Alexa Fluor 555 stain. GFP fluorescence was assessed in the nuclear populations positive for DAPI and the listed transcription factors to assess the transfection of skeletal muscle myocytes, cardiomyocytes, or skeletal muscle satellite cells. DNA from these cells will be further isolated and sequenced with NGS to evaluate cell-type-specific editing.

Imaging. Microscopy and visualization of DNA agarose gels and western blots were the primary methods of imaging used.

In Sun1-GFP studies, immunohistochemistry samples of brain tissue were stained with DAPI, visible through the blue channel in fluorescence microscopy, to visualize nuclei. GFP is visible through the green channel in fluorescence microscopy. In all microscopy experiments, a wide-field fluorescent microscope was used.

DNA agarose gels and western blots were visualized with iBright.

I. CRISPR-Cas9 Excision of FXN GAA Repeats in Friedreich's Ataxia Models

Friedreich's ataxia (FRDA) is an inherited genetic disorder that affects one in 50,000 children. It is caused by a homozygous trinucleotide expansion of over thirty GAA repeats in intron 1 of the *FXN* gene, which results in epigenetic silencing of the *FXN* promoter and deficiency of the frataxin protein in mitochondrial iron metabolism. Subsequent oxidative stress induced by iron accumulation causes defects in skeletal

muscle, cardiomyocytes, and neurons¹⁵. Though treatments are palliative and there exists no one-time cure, one therapeutic strategy proposes using CRISPR-Cas9 to cleave upstream and downstream of the GAA repeats, thus excising the trinucleotide repeats from *FXN* and allowing repair of the gene through NHEJ. Rocca *et al.* have demonstrated successful gene editing in human FRDA lymphoblastic cell lines as a model for hematopoietic stem cells, which differentiate into macrophages that transfer frataxin to frataxin-deficient cells¹⁵. Furthermore, one study established the efficacy of *in vivo* mRNA delivery through IV injection of mice with cargo-carrying lipid nanoparticles (LNPs)¹⁴. As our lab proposes that the fetus is the ideal target for gene editing due to low immunogenicity and rapid cell proliferation²⁸, we aim to demonstrate that *in utero* treatment of FRDA patients could effectively excise GAA repeats from *FXN* through IV or ICV injection of LNPs containing *FXN*-targeting sgRNAs and Cas9 mRNA, even in the notoriously inaccessible and immunoprivileged central nervous system²⁹.

Results

To validate the ability of CRISPR-Cas9 to cleave *FXN*, we transfected HEK293T cells with Cas9 mRNA and a pair of sgRNAs created by Rocca *et al.*¹⁵, which flank a 3-kb segment of *FXN* that contains trinucleotide repeats in the diseased gene, either via nucleofection (Figure 2A) or treatment with LNPs (Figure 2B). *FXN* cleavage is indeed demonstrated in cells *in vitro*, as evidenced by the appearance of a 2-kb band in an electrophoresed DNA agarose gel.

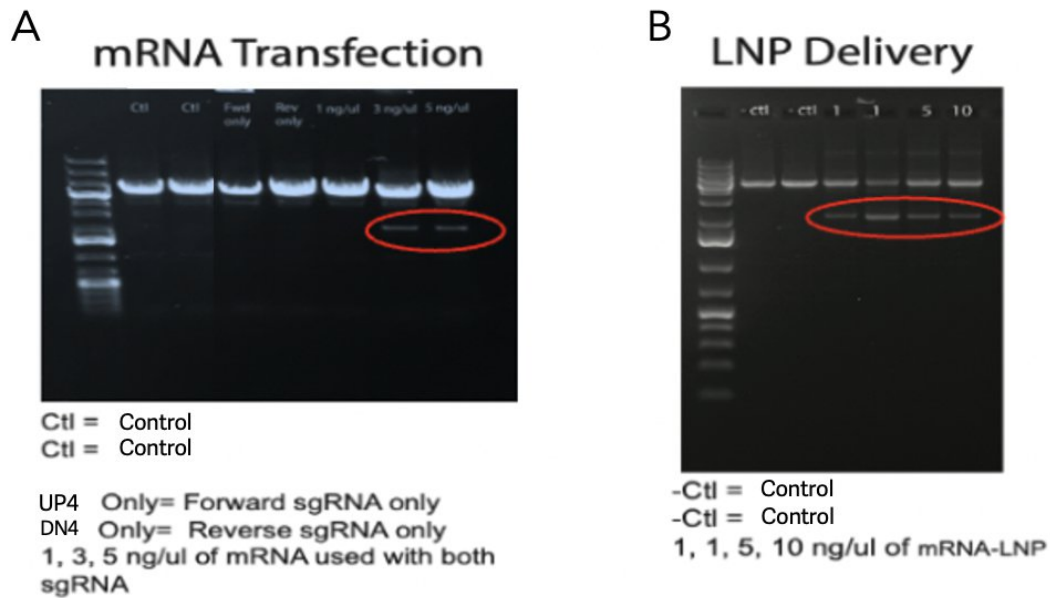


Figure 2: DNA harvested from HEK293T cells treated with Cas9 mRNA and UP4 and DN4 sgRNAs, run on an agarose gel. 2-kb excised *FXN* fragment circled in red. (A) mRNA nucleofection with UP4 only; DN4 only; or 0 (control replicate), 1, 3, or 5 μ L mRNA. (B) LNP transfection with 0 (control replicate), 1 (replicate), 5, or 10 μ L mRNA. Figure courtesy of M. Carpenter.

Having validated the ability of CRISPR-Cas9 to cleave *FXN* *in vitro*, we next sought to validate LNPs as an effective gene therapy vector. Sun1-GFP reporter mice were injected with LNPs containing Cre-recombinase mRNA. In addition to IV injections, among the safest and most efficient drug delivery strategies, ICV injections were conducted to ensure LNP access to the central nervous system (CNS), where FRDA-impacted neurons are located (Figure 3). Moreover, the incidence of gene editing in an environment as difficult to access as the CNS, owing to the protective blood-brain barrier, was also of principal interest. ICV and IV injections both demonstrated greater transfection of LNPs in fetal mice than adult mice, indicated by the overlap of green GFP and blue DAPI fluorescent signals, although IV injection resulted in lower rates of transfection overall as compared to ICV injection.

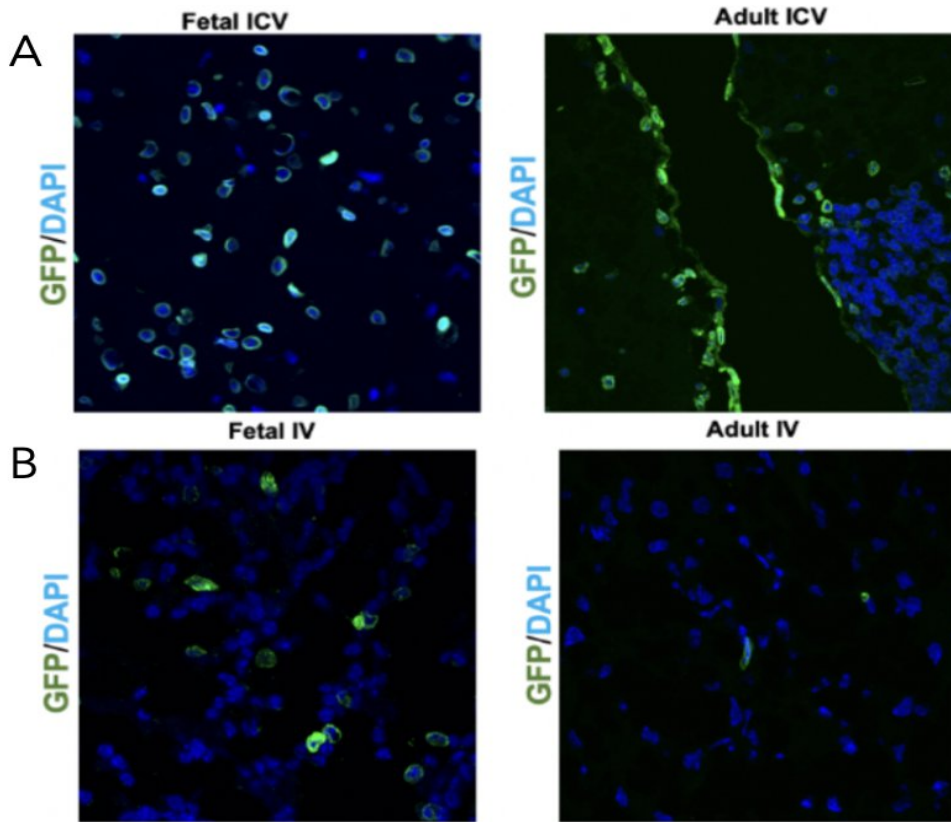
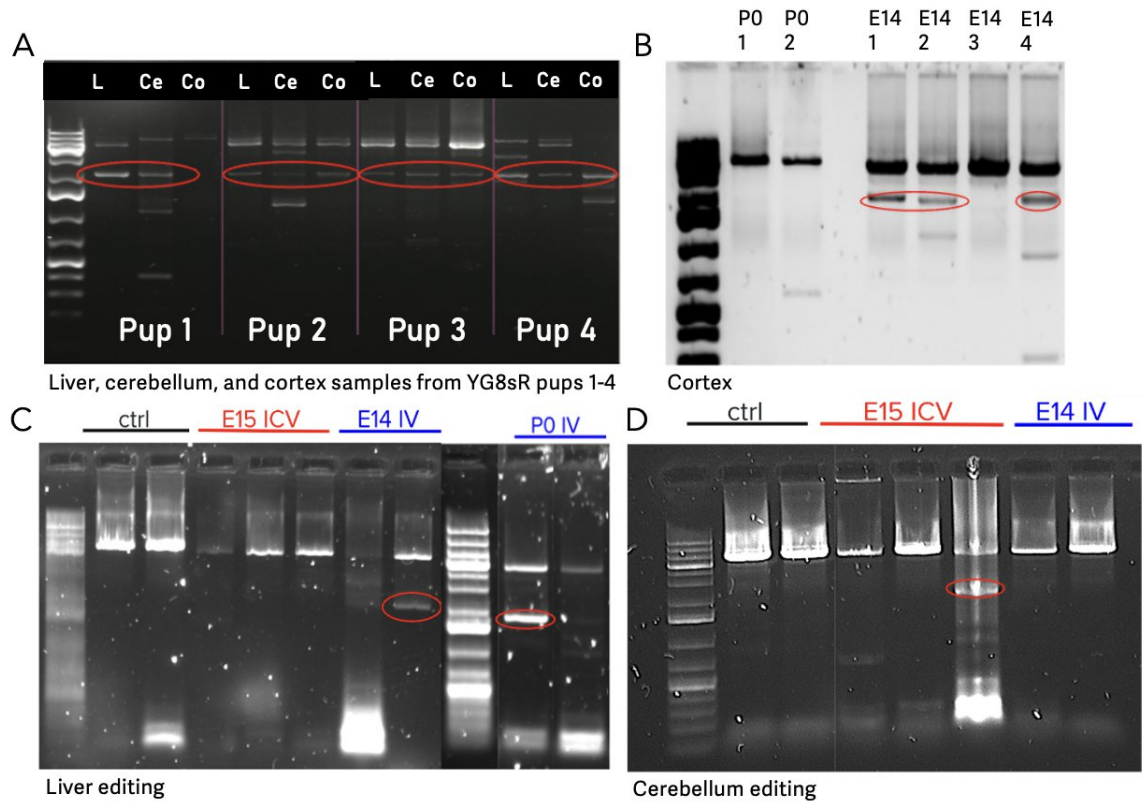


Figure 3: Wide-field fluorescence microscopy imaging of brain tissue sections in Sun1-GFP injected with LNPs containing Cre-recombinase mRNA at E14 (A) ICV or (B) IV. Figure courtesy of M. Carpenter.

Bridging preliminary *in vitro* and *in vivo* studies, we next injected YG8sR fetal or neonatal FRDA mouse models ICV or IV with LNPs containing CRISPR-Cas9 together with UP4 and DN4. Collection of brain and liver tissue revealed that ICV injections resulted in editing in the livers, cerebella, and cortices of fetal mice, as evidenced by the presence of 2-kb bands in an electrophoresed DNA agarose gel (Figure 4A). *FXN* cleavage was further demonstrated to be unique to mice given E14 injections, as mice injected at P0 did not exhibit *FXN* cleavage in the cortex (Figure 4B). In contrast, IV injections only resulted in editing in fetal and neonatal livers (Figures 4C and 4D),

suggesting that ICV injection is a superior method for *in utero* CNS editing.



Discussion

We demonstrate that *in utero* injection of LNPs containing Cas9 mRNA and sgRNAs targeting *FXN* removes repressive GAA repeats in various organs. While IV injection is most effective at removing GAA repeats in the liver, ICV injection is superior at editing the CNS, where disease-relevant cells reside. However, biodistribution studies with Sun1-GFP demonstrated that LNPs injected IV can reach CNS cells, suggesting that vector delivery is not the primary bottleneck to efficient gene editing. Therefore, the observation that IV injections do not exhibit noticeable *FXN* cleavage in the CNS

indicates inherent limitations of delivering two separate sgRNAs to direct Cas9 nucleases to cleave DNA.

In future work, cortices of fetal and neonatal mice given IV injections and cerebella of neonatal mice given ICV injections remain to be analyzed—it is possible that LNPs and their gene editing cargo reach some CNS regions more efficiently than others. Moreover, delivery of LNPs to the CNS could be further enhanced by decorating LNPs with antibodies against markers specific to CNS cells or cells implicated in CNS-specific pathways, as has been demonstrated previously in hematopoietic stem cells¹⁴. This possibility offers a redeeming hypothetical for IV CRISPR-Cas9 delivery. Extrapolating from previous findings that hematopoietic stem cells differentiate into microglia that transfer frataxin to frataxin-deficient neurons¹⁵, we further propose that *in utero* IV injection of LNPs containing CRISPR-Cas9 targeted towards HSCs—for example, through LNP conjugation with anti-CD117 antibodies—would activate *frataxin* to functional levels. Nevertheless, more precise CRISPR-based tools may enable safer and more efficient therapeutic gene editing modalities, as shall be discussed in the next chapter.

The FRDA project was my first formal introduction to gene editing. Though the HEK293T cell culture experiments and Sun1-GFP and YG8sR injections had already been conducted by my mentor, Dr. Marco Carpenter, I assisted with immunohistochemistry experiments for imaging of Sun1-GFP tissues, conducted polymerase chain reactions (PCR) for *FXN* in harvested YG8sR samples, and ran amplified DNA through agarose gels via electrophoresis for analysis of *FXN* cleavage.

II. dCas9-based Methods for PIK3C2B Knockdown for Treatment of X-linked Myotubular Myopathy

X-linked myotubular myopathy (XLMTM) is a rare genetic disease of the skeletal muscle that manifests in 1 in 50,000 males. It is characterized by abnormal centrally located nuclei in skeletal myocytes, and symptoms include muscle weakness as well as dysfunction of the respiratory system and liver. Some infants die very early in life, and patients who live often rely on palliative treatment, such as ventilator, feeding, and wheelchair assistance³⁰.

XLMTM is caused by mutations to the *MTM1* gene, which encodes for the phosphoinositide (PIP) phosphatase myotubularin. In XLMTM patients, who are myotubularin-deficient, the accumulation of phosphatidylinositol-3-phosphate (PI3P) due to a loss of phosphatase activity alters cell signaling and normal metabolism in skeletal myocytes. The resulting phenotype presents abnormal muscle triad structure and impaired excitation-contraction coupling, which relays neuronal action potentials to myocytes for proper contraction in healthy individuals²⁷. Loss of *MTM1* also results in a cholestatic liver phenotype by disrupting endosomal trafficking of bile transport proteins³¹.

Beyond palliative treatment, a current experimental gene therapy aims to alleviate XLMTM symptoms with gene replacement. From 2017 to 2021, the ASPIRO clinical trial sought to treat children with XLMTM with an exogenous adeno-associated virus (AAV)-delivered *MTM1* gene. Though intravenous infusions of the AAV-bound gene reduced ventilator dependence and increased motor function, the four participants who ultimately succumbed presented with a severe cholestatic liver phenotype³⁰.

Alternative gene therapy strategies for treating XLMTM seek to edit genes associated with pathways myotubularin participates in rather than gene replacement or editing *MTM1* itself. One such gene is *PIK3C2B*, which encodes for PI3K-C2 β , a phosphatidylinositol (PI) kinase that catalyzes PI3P formation and is not required for normal skeletal muscle function. Previous studies have demonstrated that inhibiting PI3K-C2 β or knocking out *PIK3C2B* results in rescue of the XLMTM phenotype in mouse models²⁷.

Advancements in CRISPR-Cas9 technology present an arsenal of gene editing tools that present alternatives to total gene knockout for effectively targeting genes such as *PIK3C2B*. Unlike other genes discussed in this thesis, *PIK3C2B* is a target for knockdown in the treatment of XLMTM; a unique use case thus arises for CRISPRoff¹², which enables the suppression of the genes it targets. Another tool in the CRISPR-Cas9 arsenal is the adenine base editor (ABE), which can specifically and efficiently target exon splice sites to disrupt the functional full-length protein¹⁹. Furthermore, delivery of these gene editing tools via LNPs may enable the circumvention of the ASPIRO trials' adverse events, thanks to LNPs' favorable immunogenicity properties.

In utero gene therapy presents a promising opportunity for delivering gene therapy to treat XLMTM before disease symptoms present, effectively serving as a prevention strategy with a lasting therapeutic effect. We therefore aim to present LNP-delivered ABEs targeting *PIK3C2B* exon splice sites or CRISPRoff targeting the *PIK3C2B* promoter as novel gene therapy strategies for treating XLMTM.

Results

To examine epigenetic silencing of *PIK3C2B* with CRISPRoff nucleofection, we first generated a list of CRISPRoff sgRNA candidates targeting the *PIK3C2B* promoter region (Table 2, Methods), which contains a CpG island rife with opportunities for methylation, and screened the predicted top 10 most efficient sgRNAs (Figure 5).

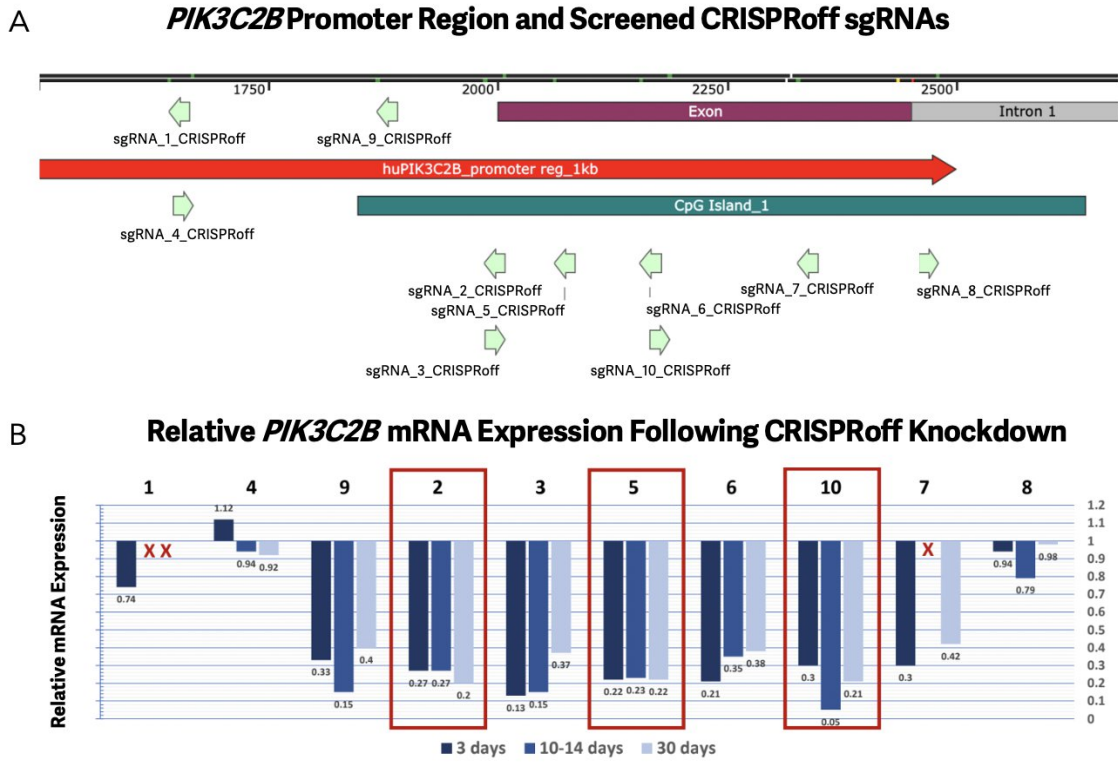


Figure 5: (A) Top 10 predicted most efficient CRISPRoff sgRNAs tiling the human *PIK3C2B* promoter. (B) Relative *PIK3C2B* mRNA expression following CRISPRoff treatment in Huh7 cells. Figure courtesy of J. Sincavage.

Upon finding the top 3 most efficacious CRISPRoff sgRNAs (Table 16, Appendix), we sought analogous mouse sgRNAs to prepare for preliminary *in vivo* studies (Table 17, Appendix). Mouse-specific CRISPRoff sgRNAs were assessed for longitudinal *PIK3C2B* knockdown efficiency in C2C12 mouse myoblasts (Figure 6). Notably, *PIK3C2B* knockdown persisted one month after nucleofection, implying that the CRISPRoff targeting strategy is not threatened by a gradual reversion of *PIK3C2B*

expression (Table 18, Appendix). This result was also corroborated by PI3K-C2 β knockdown in protein expression profiles (Figure 16, Appendix).

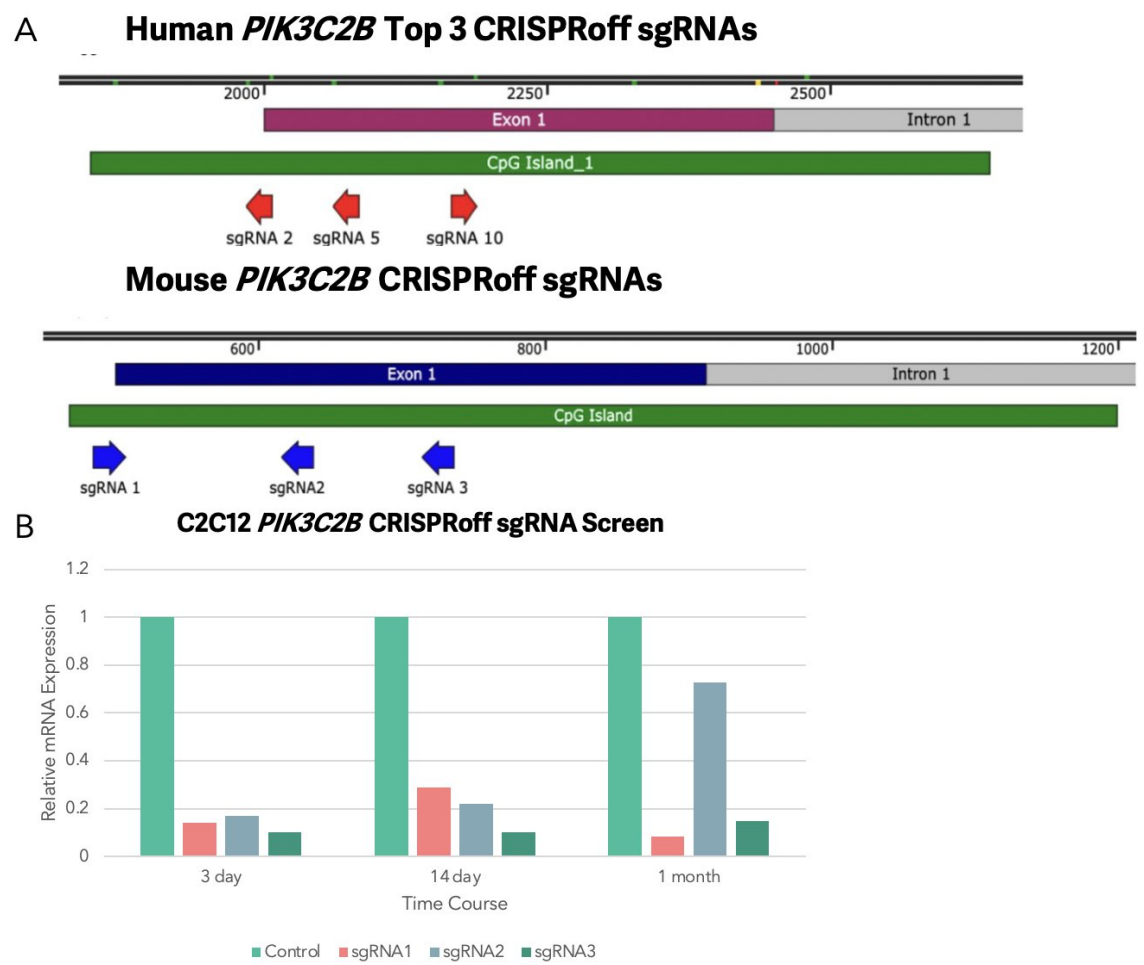


Figure 6: (A) Top 3 human CRISPRoff sgRNA candidates superimposed with murine analogs. Human sgRNA2, sgRNA5, and sgRNA10 correspond with mouse sgRNA1, sgRNA2, and sgRNA3, respectively. (B) *PIK3C2B* mRNA expression profiles 3, 14, and 30 days after nucleofection of C2C12 cells with CRISPRoff mRNA and either mouse sgRNA1, sgRNA2, or sgRNA3. Figure courtesy of J. Sincavage.

Encouraged by this longitudinal suppression of *PIK3C2B* by CRISPRoff in mouse cells, we next examined *in vivo* transcriptional suppression following IV injection of CRISPRoff at E14. Unfortunately, no positive results were produced, possibly because of the complexity of fetal epigenetic regulation and the difficulty of artificial epigenetic modulation *in vivo*. Many experiments could have further investigated the causes of failure at this point. However, it was collectively decided that time would best be spent

focusing on the second *PIK3C2B*-targeting strategy: knockdown via splice site disruption using ABEs²¹.

Testing the top 10 predicted most efficient ABE sgRNAs targeting ten splice donor and acceptor sites in human *PIK3C2B* (Table 3), we found that sgRNAs targeting splice donor sites of exons 8, 27, and 29, as well as the splice acceptor site of exon 32, exhibited the highest editing efficiencies and MIT specificity scores (Figure 7). These sgRNAs were therefore selected for evaluation of PI3K-C2 β knockdown.

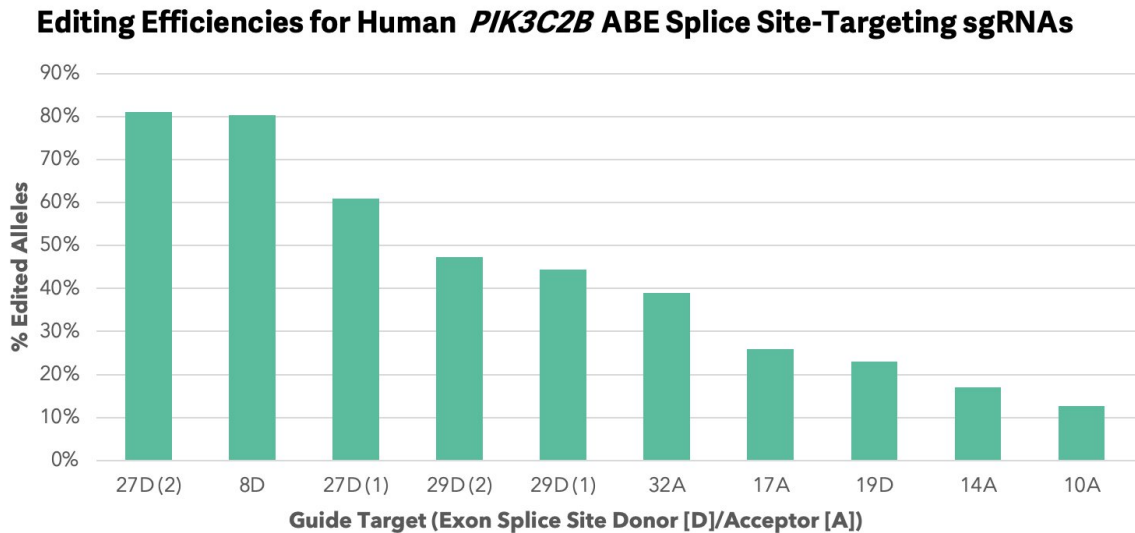


Figure 7: Editing efficiencies of human *PIK3C2B* splice site-targeting ABE sgRNAs screened in Huh7 cells.

Western blot protein analysis demonstrated that sgRNAs targeting the splice donor sites of exons 8, 27, and 29 effectively knocked down PI3K-C2 β in Huh7 cells, with the disruption of the exon 8 splice donor site being the most effective (Figure 8). Indeed, when exon 8 is skipped, a premature stop codon is generated, possibly leading to incomplete translation or nonsense-mediated decay of PI3K-C2 β .

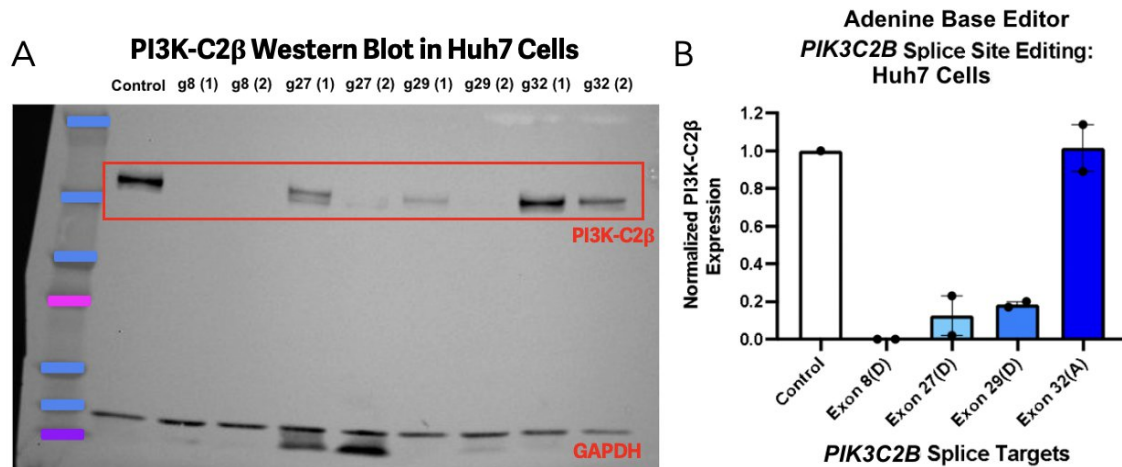


Figure 8: Measurement of PI3K-C2β levels in Huh7 cells 30 days after nucleofection of ABE sgRNAs targeting human *PIK3C2B* splice sites. (A) Western blot, PI3K-C2β bands boxed in red with GAPDH designated as housekeeping protein. g8 (1) refers to the first replicate of sgRNA targeting the splice donor site of *PIK3C2B* exon 8 (as in Table 3). Precision Plus Protein Dual Color Standards on far left measure protein size. (B) Quantified PI3K-C2β expression.

Our western blot results led us to nominate the ABE sgRNA disrupting the *PIK3C2B* exon 8 splice donor site for *in vivo* studies. IV injection of LNPs containing ABE mRNA and the mouse analog of the sgRNA targeting the *PIK3C2B* exon 8 splice donor site in fetal and adult mice indicates that editing is most efficient in the liver but remains appreciable at higher doses in fetuses (Figure 9). This result is corroborated by western blot results of total protein harvested from heart and quadricep tissues after injection of *PIK3C2B*-targeting sgRNAs (Figure 17, Appendix), but further optimization of the western blot remains to be done. It should be further noted that the mice injected in this study were not disease models, but rather ordinary healthy mice that were used as a proof of concept for editing the *PIK3C2B* locus *in vivo*.

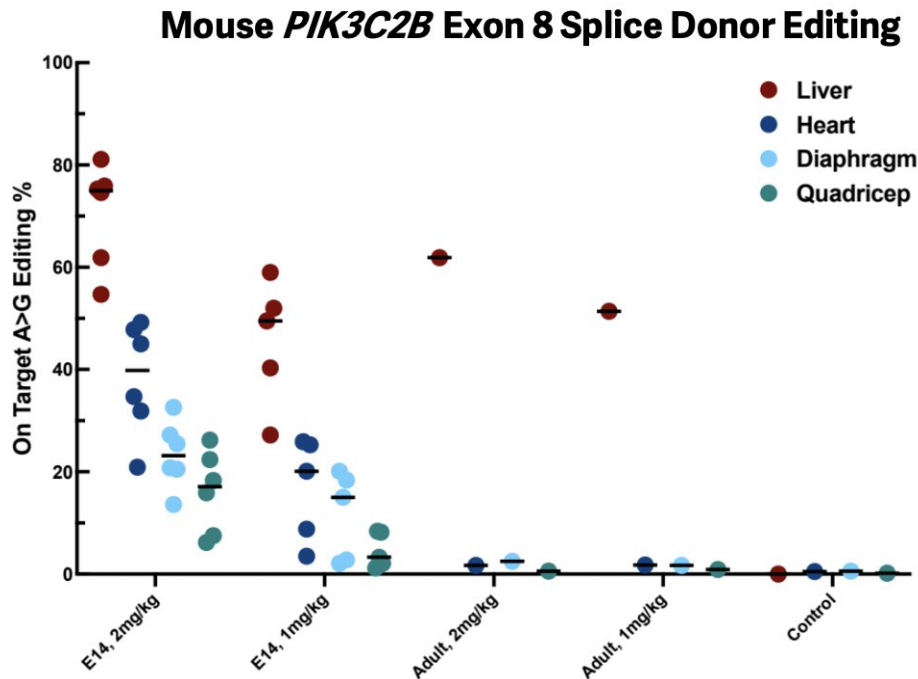


Figure 9: IV injections of LNPs containing ABE mRNA and the mouse analog of the sgRNA targeting the *PIK3C2B* exon 8 splice donor site. Editing results are shown in Table 20, Appendix. Figure courtesy of J. Sincavage.

Discussion

CRISPRoff and adenine base editing offer two gene editing methods for targeting *PIK3C2B* beyond CRISPR-Cas9 cleavage for the treatment of XLMTM: epigenetic suppression of expression and exon splice site disruption. In the latter approach, ABE8.8 mRNA and an sgRNA targeting the *PIK3C2B* exon 8 splice donor site demonstrated knockdown of PI3K-C2 β , which was most effective in IV injections in mouse embryos. ABEs thus exhibit potential as efficient and precise *in utero* gene therapies. In future studies, we hope to further refine PI3K-C2 β western blot assays to yield more interpretable results and conduct our *PIK3C2B* adenine base editing studies in *MTM1*-knockout mice from the Dowling Lab²⁷, which has recently moved to CHOP.

While epigenetic suppression of *PIK3C2B* by CRISPRoff was successful in cells, IV injection of CRISPRoff mRNA and an sgRNA targeting the *PIK3C2B* promoter failed

to elicit transcriptional suppression *in vivo*, likely due to endogenous epigenetic compensation or dynamic fetal epigenetic regulation that cannot be accounted for in cell culture alone³². Several epigenetic profiling methods that probe various layers of gene regulation may be used to elucidate the nature of *PIK3C2B* promoter methylation *in vivo*, shedding light on possibilities for improving methylation and knockdown efficiency. Furthermore, epigenetic suppression is locus-dependent³³ and has been demonstrated to be successful *in vivo* for the targeting of the hypercholesterolemia-associated gene *PCSK9*³⁴. Further studies on *in utero* CRISPRoff at disease-associated loci are therefore warranted.

Beyond *PIK3C2B*, there is evidence that inhibition of *DNM2* by even 50%—in contrast to the 100% *PIK3C2B* knockout mice studied by Sabha *et al.*²⁷— is sufficient to prevent the onset of XLMTM symptoms³¹. *DNM2* may therefore be an additional attractive knockdown target for therapeutic editing.

Before my introduction to the project, my mentor, Dr. John Sincavage, conducted all of the sgRNA candidate screens. I independently validated his choice of sgRNAs, passaged C2C12 cells, optimized the Western blot and protein quantification protocol, designed primers for prospective bisulfite sequencing experiments and NGS, conducted RT-qPCR, and optimized lab assays by synthesizing protocols I found in the literature.

III. Base Editing of DMD for Treatment of Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD), resulting from deleterious mutations of the dystrophin protein encoded by the *DMD* locus, is the most common inherited form of muscular dystrophy and a significant burden to patients. Total costs associated with DMD treatment and care, including those incurred outside of the clinic, range from

\$80,000 to \$100,000 per patient annually across developed Western countries³⁵. Medical management for DMD include expensive strategies such as palliative care, which addresses the patient's comfort rather than root cause of disease, and antisense oligonucleotide treatment, which targets mechanisms of DMD disease progression but must be administered repeatedly. There is an evident need for a “one-and-done” DMD treatment that targets the genetic basis of disease, which CRISPR-Cas9 technology offers to meet.

Previous work has detailed the use of CRISPR to induce insertions and deletions (indels) at splice donor sites (SDS) and splice acceptor sites (SAS) in the *DMD* locus, mimicking the action of antisense oligonucleotides that restore an open reading frame through exon skipping³⁶. However, CRISPR creates indels via double-stranded breaks, which are error-prone and may damage functional genes. As an improvement upon this strategy, ABEs allow precise targeting of *DMD* without double-stranded breaks, specifically through disrupting exon splice sites, which contain an adenine that is eligible for editing. As opposed to the adenine base editing strategy in the XLMTM project, wherein exon splice sites were disrupted to knock down expression of the targeted gene, the DMD project strategy aims to mediate exon skipping, which reframes the *DMD* transcript to produce a shorter yet still functional dystrophin protein when transcribed (Figure 10). Furthermore, ABE bystander edits are not deleterious in exon skipping strategies, as they are usually located in introns that are spliced out of the mature *DMD* transcript²².

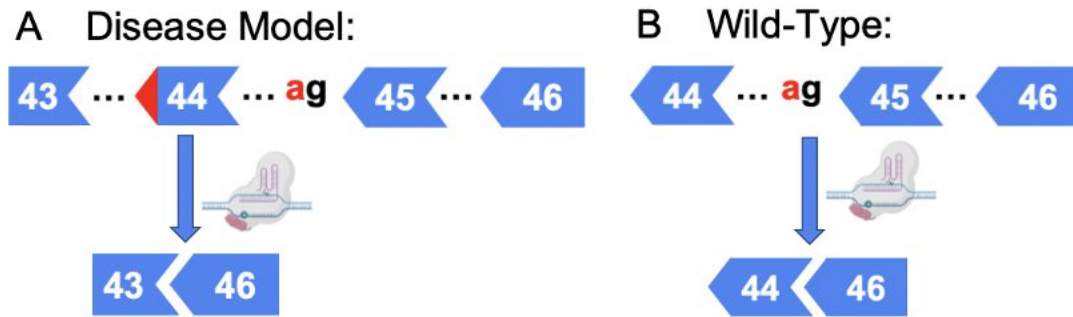


Figure 10: Schematic of the DMD exon 45 skipping strategy via adenine base editing at the splice acceptor site to restore an open reading frame, resulting in a truncated but functional dystrophin product. **(A)** In a disease model, a mutation at exon 44 in DMD may cause splicing errors that exclude exon 44 from the mature transcript. Splicing of exons 43 and 45 fails to produce a functional open reading frame. Skipping exon 45 by mutating its splice acceptor site causes exon 43 to splice with exon 46, which does create a functional open reading frame. **(B)** Functional tests for exon skipping may be conducted in wildtype models.

In DMD, mutations that are curable with skipping of exon 45, which account for 8% of all cases³⁷, may be addressed with modification of the adenine in the canonical AG sequence in the exon splice acceptor site (SAS) using an ABE. The *DMD* exon 45 SAS is a particularly favorable DNA sequence to target because it lies upstream of an NGG protospacer adjacent motif, which the canonical SpCas9 nickase in ABE8e recognizes with the greatest efficiency. Our study on DMD thus aims to target the exon 45 SAS of *DMD*.

Recent work has examined the delivery of ABEs packaged in adeno-associated viruses (AAVs) together with cardiac- and skeletal muscle-specific promoters via local intramuscular or systemic intravenous injections in mouse models of DMD^{13,22,38}. These AAVs have demonstrated near-complete rescue of dystrophin in cardiac tissue. However, AAVs are limited by their small plasmid packing size and tendency to elicit neutralization antibodies, which restricts their efficacy upon repeated dosages. Lipid nanoparticles (LNPs) exhibit larger packing capacity and deliver mRNA, which may be preferable due to its safety and transient expression compared to DNA plasmids. One

study further found that LNPs can be repeatedly dosed via intramuscular injection to mediate long-term editing, highlighting the potential of LNP delivery as a therapeutic platform for musculoskeletal diseases³⁹.

Both AAVs and LNPs exhibit predominant tropism to the liver, making targeting specific tissues such as cardiac and skeletal muscle a formidable challenge. *In utero* gene therapy may present an effective alternative method for extrahepatic delivery of gene editing tools, as has been evidenced in the CNS in the FRDA project. However, to date, the literature has yet to address systemic delivery of LNPs and *in utero* gene editing for the treatment of cardiac and skeletal muscle. While one grant from California has funded an unpublished 2024 study investigating *in utero* systemic delivery of LNPs for the treatment of DMD, it is not clear what the loaded editor design is, and the proposed study is limited to investigating only DMD mouse models and human patient cells⁴⁰. Beyond addressing *DMD* exon-45-skippable cases, the goal of our study on DMD is to fill this knowledge gap, thus providing an additional possible modality for safe and effective DMD treatment.

Results

We first aimed to select an optimal ABE for our studies. Using an adapted version of the sgRNA used by Chai *et al.*²², we assessed base editing in sheep, nonhuman primate, and human cells (Figure 11). ABE8e appears to be the most efficient editor in HEK293T cells and sheep fibroblasts, while ABE 8.20 appears to dominate in nonhuman primate mesenchymal stem cells.



Figure 11: *In vitro* experiments to screen optimal editor for DMD studies. (A) Selected sgRNA at the *DMD* locus with the targeted adenine in the exon 45 SAS highlighted at position 0. Editing evaluated for (B) human, (C) sheep, and (D) nonhuman primate cells. Species-appropriate primers in Table 7 (Methods) used.

We chose to proceed with ABE8e because it exhibited the highest editing efficiency at the adenine of interest in human cells, which are the most clinically relevant. In subsequent *in vivo* studies, we evaluated whole-tissue editing of mice injected with LNPs containing ABE8e mRNA and the sgRNA adapted from Chai *et al.*²² DNA was isolated from the liver, a good control for LNP delivery due to the inherent affinity of LNPs for the liver, as well as heart, diaphragm, quadriceps, and tibialis anterior tissues, then assessed for adenine base editing at the *DMD* exon 45 SAS (Figure 12).

Adenine Editing Efficiency at Mouse *DMD* Exon 45 Splice Acceptor Site

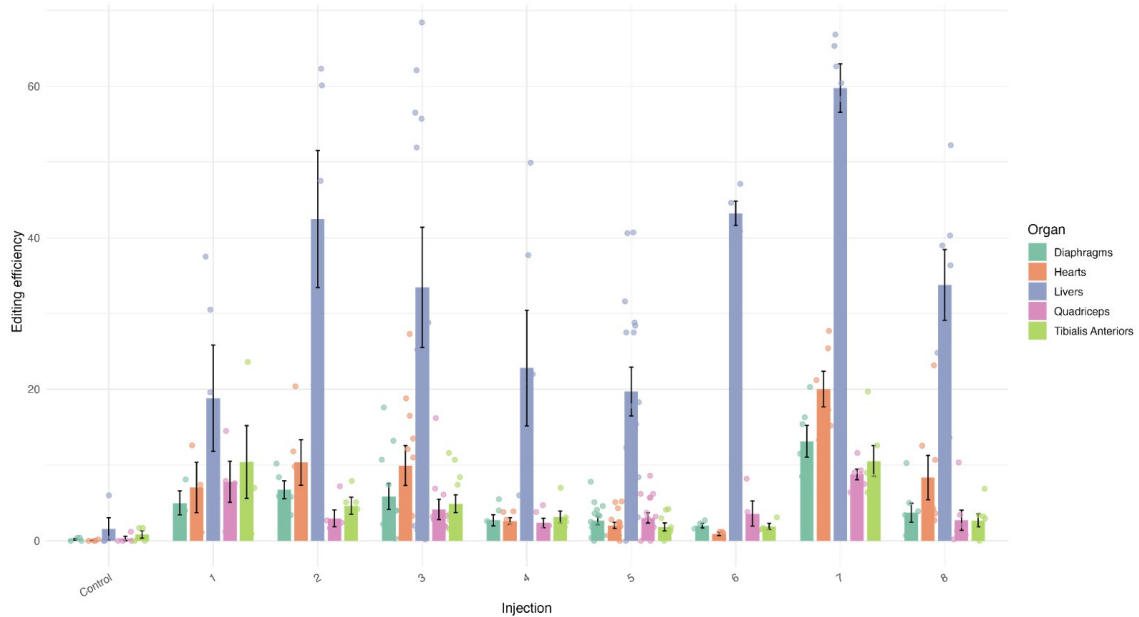


Figure 12: Whole-tissue-level editing for various tissues, mRNA compositions, LNP-mRNA dosages, and time points of injection. It should be noted that one mouse cohort injected with ABE8.20 mRNA was also included. Key: Control = Control, 1 = 2 mg/kg ABE 8.20 at E14; 2 = 2 mg/kg ABE 8e at E14; 3 = 2 mg/kg ABE 8e at E14 + chemically modified sgRNA; 4 = 2 mg/kg ABE 8e at E16; 5 = 2 mg/kg ABE 8e at E16 + chemically modified sgRNA; 6 = 3 mg/kg ABE 8e at 6 weeks + chemically modified sgRNA; 7 = 3 mg/kg ABE 8e at E14 + chemically modified sgRNA; 8 = 2 mg/kg ABE 8e at E14 + chemically modified sgRNA harvested at 12 months. Adenine base editing rates in Tables 21-25, Appendix. Individual tissue plots in Figure 18, Appendix.

Across all injections, ABE8e exhibited the greatest editing efficiency in the liver.

Of note, editing in organs other than the liver is significantly greater for mice injected at E14 and E16 compared to mice injected as adults (six weeks), even if the livers of mice injected as adults exhibit greater editing. Cardiac muscle tissue is edited at a greater frequency than skeletal muscle tissue. The injection that resulted in the greatest editing frequencies across all harvested organs was 3 mg/kg ABE8e mRNA and a chemically modified sgRNA at E14, indicating that a higher dosage, chemical modification of the sgRNA, and injection at E14 all contribute to higher adenine base editing efficiency in cardiac and skeletal muscle tissue.

However, the above sequencing reads were obtained from bulk tissue, which limits insights into editing of the dystrophin locus in specific cell types. Of note, satellite cells, which serve as stem cells in skeletal muscle tissue that proliferate and donate their nuclei to myocytes upon myocyte injury, are of particular interest, as editing satellite cells may be key to replacing dystrophic skeletal muscle fibers with healthy myocytes⁴¹. Sequencing a small number of satellite cells is difficult, since it may be subject to background artifacts that indicate spurious editing events, as demonstrated in sequencing readouts of control satellite cell DNA that exhibited supposed editing at the locus of interest (Table 30, Appendix). Therefore, we aimed to pool satellite cells from several animals and increase input DNA while sequencing to reduce background editing reads.

To obtain enough satellite cells for representative sequencing results, optimal isolation of satellite cells, and therefore proper gating of their markers, is necessary. All fluorescence-activated nuclear sorting (FANS) was handled by Cytex's Aurora CS System.

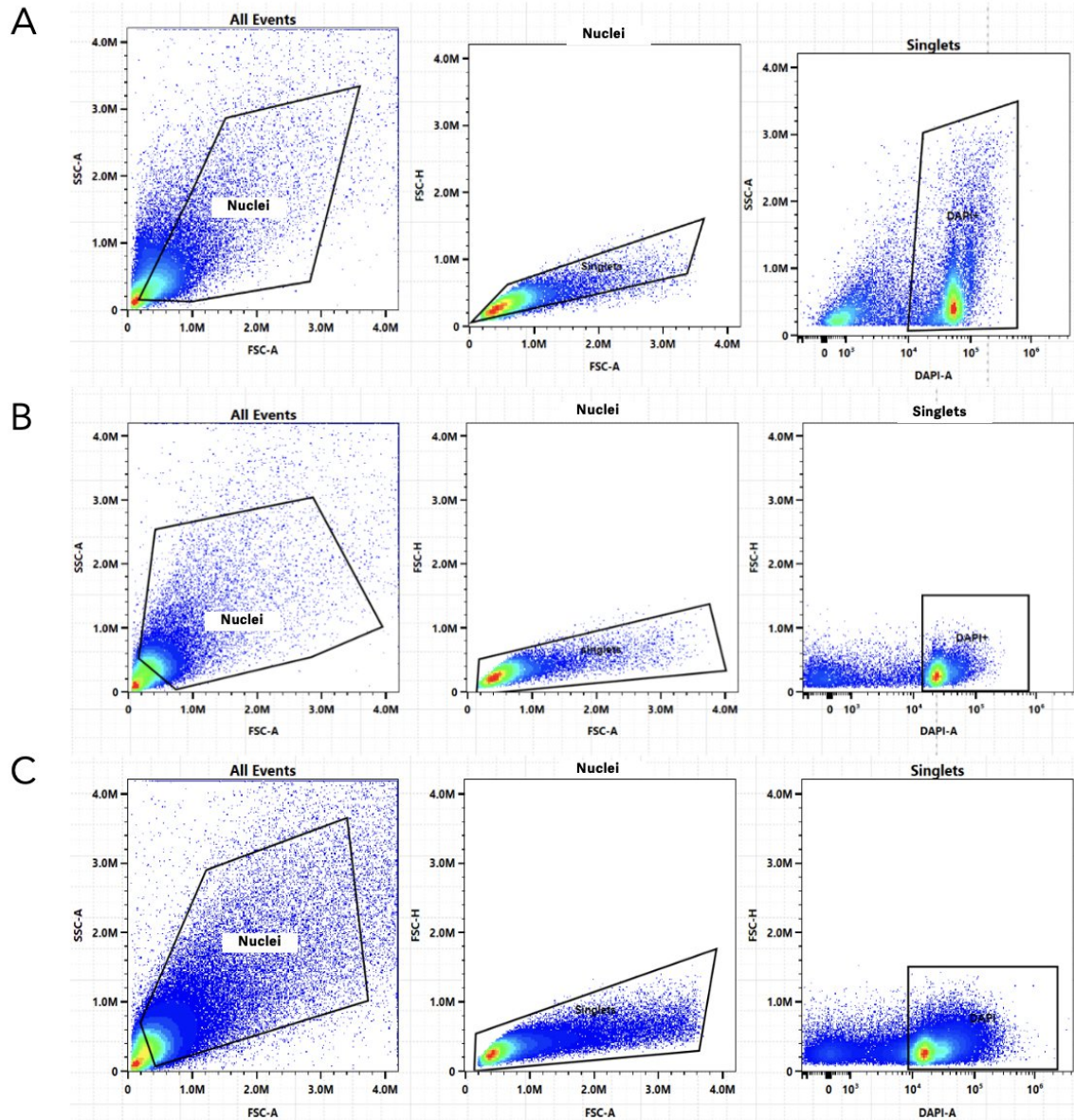


Figure 13: Gating of nuclei (DAPI+) for various tissues: heart (A), quadricep (B), diaphragm (C) following fluorescence-activated nuclear sorting (FANS). Sample preparation described in Methods. Nuclei are filtered from all events to distinguish from debris, and singlets are filtered from nuclei to distinguish individual nuclei from doublets. DAPI is used as a confirmatory nuclear marker.

Using nuclear (DAPI), myocyte (PCM1), and satellite cell (PAX7) markers (Methods), gates were created, and distinct populations of nuclear markers were separated (Figures 13 and 14).

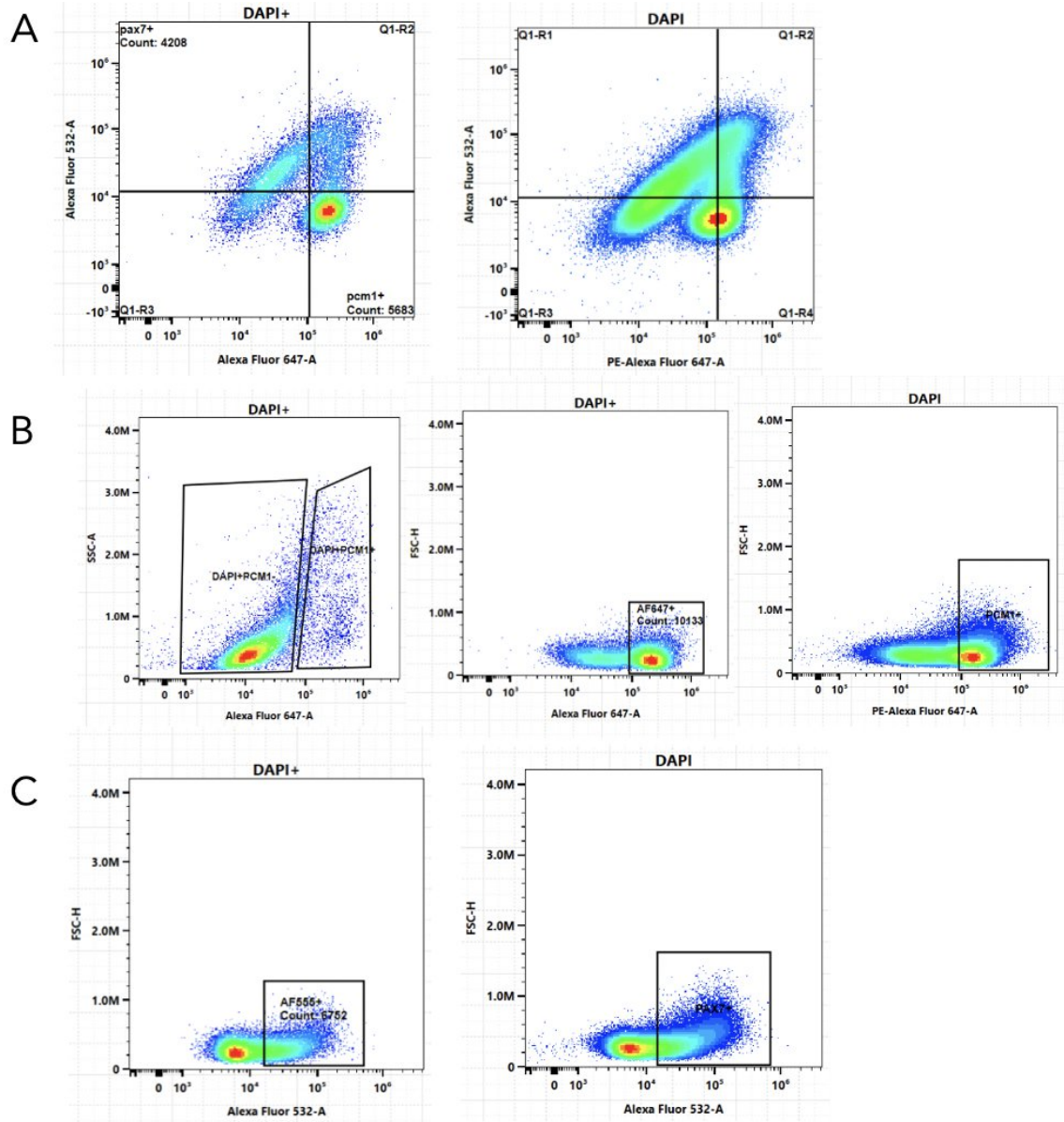


Figure 14: Gating of nuclear markers among nuclei (DAPI+). (A) Nuclei from quadriceps (left) and diaphragm (right) tissues, where upper left quadrant contains PAX7+ satellite cells and lower right quadrant contains PCM1+ myocytes. (B) PCM1+ myocyte nuclei in heart (left), quadriceps (middle), and diaphragm (right) tissues. (C) PAX7+ satellite cell nuclei from quadriceps (left) and diaphragm (right) tissues.

Because formalin fixation is required in the nucleus isolation step, de-crosslinking of DNA with nuclear proteins is necessary. Furthermore, formalin can shear DNA and cause unwanted base modifications, which may explain the unexpected edited reads. It was thus necessary to isolate DNA from nuclei shortly after sorting and remove formalin

through prolonged heating and incubation with proteinase K⁴². NGS with single satellite cell nuclei remains to be optimized further due to constraints with booking cell sorter machines and low DNA reading quality.

Discussion

In another demonstration of the utility of ABEs in gene editing applications, we show that A-to-G editing at the *DMD* locus for splice site disruption is more significant in the fetus than the adult, which indicates that *in utero* delivery may be a key consideration for the development of DMD gene therapy. Optimization of the gene editor, sgRNA, and delivery method is necessary for improving gene therapy outcomes. From both *in vitro* and *in vivo* studies, we identify optimal sgRNA composition, ABEs, drug dosages, and gene therapy delivery time points. Our *in vivo* studies are limited to the mouse, but results from *in vitro* studies using sheep, monkey, and human cells indicate that results may be translatable to larger animal models.

Administration of gene therapy in the fetus may be a strategy for overcoming bottlenecks in conventional gene therapy methods. Our experiments show that editing in fetal tissues beyond the liver is universally greater than editing in adult tissues, despite adult liver editing exhibiting the second greatest mean editing efficiency out of all injections. This result suggests that even if injections in adult mice are successful, most gene editing occurs in the liver; in contrast, all organs of injected fetuses are more amenable to gene editing, causing relatively high editing efficiency in organs beyond the liver. The fetus is therefore the ideal recipient for gene therapy targeting diseases that affect organs other than the liver, such as DMD.

Our study reports results on the systemic delivery of LNPs *in utero* to mediate base editing, which complements existing work on other gene therapy delivery modalities present in the literature. One study that examined repeated local administration of LNPs in the limb to treat DMD found that injection of 1 mg/kg LNPs containing CRISPR (LNP-CRISPR) via the tibialis anterior resulted in 15% exon 45 skipping efficiency, as measured by the percentage of transcripts lacking exon 45 produced. In the same study, injection of 10 mg/kg LNP-CRISPR in the dorsal saphenous vein with a tourniquet placed at the quadriceps resulted in total limb perfusion and editing ranging from 5-20% in muscle groups throughout the limb, including 20% editing in the tibialis anterior¹³. In Chai *et al.*'s²² study, on which we based our sgRNA design, a dual split intein-mediated protein trans-splicing AAV containing an ABE targeting the DMD exon 45 SAS was delivered systemically via injection in the temporal facial vein in a mouse model with a deletion of exon 44 to mediate exon skipping and restoration of an open reading frame. The highest dosage was 3×10^{14} vector genomes per kilogram bodyweight (vg/kg), which corresponds roughly to 1.5 mg/kg. The study found 8% A-to-G editing in the tibialis anterior and 25% editing in the heart, corresponding with a 75% and 95% restoration of dystrophin in tibialis anterior skeletal muscle fibers and cardiac muscle fibers, respectively²². The authors' results indicate that even relatively low-level editing mediates broad dystrophin restoration in tissues affected by DMD, a promising prospect for gene therapy strategies that are unable to edit muscle tissues with very high efficiency.

To our knowledge, *in utero* gene therapy for DMD is not reported extensively in the literature. In our study, the most potent experimental gene therapy dose was 3 mg/kg

dose of LNPs containing AB 8e and chemically modified sgRNA targeting the DMD exon 45 SAS, which were delivered systemically to fetal mice via the vitelline vein. This delivery strategy resulted in 20% editing in the heart, 13% editing in the diaphragm, 9% editing in the quadriceps, and 10% editing in the tibialis anterior, roughly matching editing efficiencies reported in the literature. Furthermore, systemic delivery of LNPs may be a particularly favorable gene therapy delivery strategy due to broad tissue tropism, safety, and dosage repeatability of LNPs.

In future studies, we seek to evaluate base editing via systemically delivered LNPs in sheep and monkeys, as findings in large animal models are more clinically translatable to human therapeutic applications. We expect similar to higher editing efficiency in injected sheep and monkeys, as we plan to harvest sheep and nonhuman primate tissues while still in gestation due to these animals' long gestational periods. Specifically, sheep and monkey fetuses will be sacrificed one week after injection. We will additionally test our anti-PAX7 and anti-PCM1 antibodies on sheep and monkey tissues to conduct FANS and evaluate cell-type-specific editing. We further aim to use our delivery and editing strategy in a DMD disease model, the *Mdx-4cv* mouse (see mouse sgRNA targeting DMD exon 53 SDS in Table 29, Appendix). We will assess longitudinal editing, production of transcripts that exhibit exon skipping (see Appendix D for qPCR primers), dystrophin restoration, and muscle function to progress closer towards a gene therapy for DMD.

My mentor, Dr. John Sincavage, conducted all injections in mice and sheep. I independently harvested tissues from mice, conducted NGS to evaluate base editing, created graphs of our data, and designed sgRNAs for future large animal or disease

model studies. I also conducted tissue preparation for FANS, operated the cell sorter, and pursued optimization of our new single-satellite-cell DNA sequencing protocol independently.

IV. Prime Editing of HBA2 for Treatment of Alpha Thalassemia

The Hemoglobin Constant Spring (HbCS) mutation in the blood disorder alpha-thalassemia is a condition in which a T>C mutation at the termination codon in *HBA2* locus produces an abnormally long and non-functional protein product. *HBA2*, together with *HBA1*, is expressed (four alleles total) to produce the alpha globin (HbA) subunit of hemoglobin. The more alleles are mutated, the more fatally alpha-thalassemia manifests²³.

In HbCS, the pathogenic mutation of the HbA termination codon is amenable to editing by cytosine-to-guanine conversion. One study has reported the use of prime editors to edit the HbCS mutation in hematopoietic stem cells *in vitro*²³, but further optimization could be conducted to enhance the efficiencies of pegRNAs, for example through incorporation of silent edits in the pegRNA reverse transcriptase template to bias cellular machinery away from mismatch repair⁴³.

HbCS thus serves as a valuable case study for using an additional tool beyond the base editor—the prime editor—to install DNA edits ever more precisely and open new opportunities for editing efficiency optimization.

Results

HBA2-targeting prime editors were evaluated using a previously generated K562 cell line harboring the HbCS mutation in *HBA2*. We designed pegRNAs with a spacer

sequence (CTACCGAGGCTCCAGCTTGA) targeting the HBA2 locus (also used by Shao *et al.*), a 13-nucleotide primer binding site (AGCTGGAGCCTCG) three base pairs upstream of the PAM on the strand complementary to the spacer-bound strand, and variable 19-nucleotide reverse transcriptase templates (RTTs) directly downstream of the primer binding sites encoding the therapeutic C>T mutation to restore the termination codon. The variations in these RTTs consist of silent mutations—one or more single nucleotide polymorphisms that encode the same amino acids as the wildtype sequence. Over 200 RTTs with silent mutations spanning the termination codon and its downstream sequences were designed. When nucleofected into our previous HbCS K562 cell line together with the prime editor PE6c⁹, pegRNAs containing these RTTs mediate markedly more efficient editing compared to pegRNAs with non-modified RTTs (Figure 15).

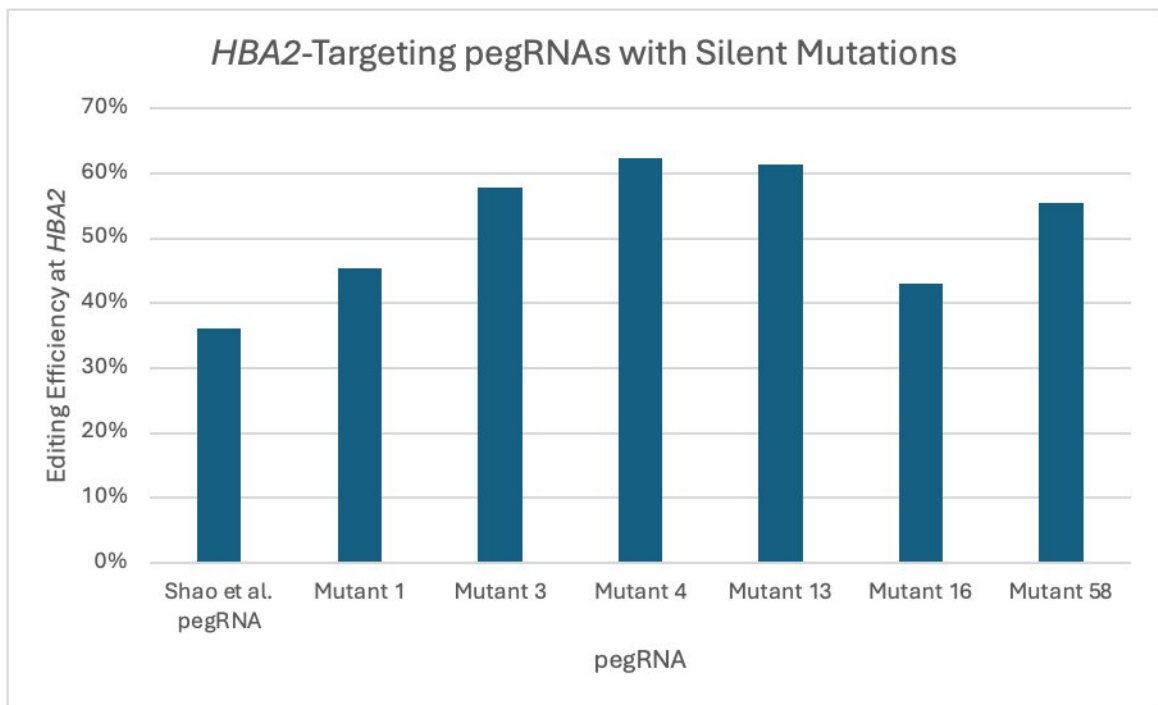


Figure 15: Editing efficiencies of PE6c and various pegRNAs carrying silent mutations, including Shao *et al.*'s original pegRNA, targeting *HBA2* in HbCS K562 cells.

Discussion

Though my contributions to this project were few, the mutant pegRNA library designed above nevertheless presents a new type of editor that completes this thesis's discussion of a therapeutic gene editing toolbox. From the above experiment, it appears that the Mutant 4 pegRNA enables the most efficient editing of the HbCS mutation. However, this selection may as well be arbitrary—though machine learning techniques may predict which prime editor components may be most important for maximizing gene editing efficiency⁴⁴, pegRNAs carrying silent mutations must be tested on a case-by-case basis. This tendency echoes several patterns from other projects discussed in this thesis: different injection modes and target organs in the FRDA project, different loci amenable to CRISPRoff methylation as well as different splice site disruptions yielding *PIK3C2B* knockdown in the XLMTM project, and different ABEs tested on cells for the DMD project all required case work without the benefit of certain prediction. Thus, in clinical applications, gene therapies must be personalized specifically to each disease and physiological context and will require iterative testing for the identification of an optimal therapy.

My mentor, Jackson Bauer, conducted most of the preliminary work for the alpha-thalassemia project. I designed the pegRNAs carrying silent mutations, nucleofected them in the previously generated HbCS K562 cells, and assessed editing efficiency for each of the pegRNAs.

Conclusion

In this thesis, I present projects on four monogenic diseases—Friedreich's ataxia, X-linked myotubular myopathy, Duchenne muscular dystrophy, and alpha-thalassemia.

Each project features a key CRISPR-Cas9-based genome editor, highlighting the breadth of gene therapy strategies, each with their own strengths and limitations. For the FRDA project, CRISPR-Cas9 nucleases are the most appropriate tool for excising pathogenic GAA repeats, yet inherent bottlenecks in DNA cleavage due to IV delivery of CRISPR-Cas9 persist. Alternatively, one could investigate epigenetic mechanisms of the gene of interest, which tools like CRISPRoff (and its TET-based twin, CRISPRon¹²) can leverage for therapeutic purposes. Beyond CRISPR-Cas9 nucleases and CRISPRoff, which are not always successful at editing their target loci, adenine base editors and prime editors offer methods for precise and efficient base modifications that can be used in applications such as splice site disruption or exon skipping.

Through all these examples, the Peranteau Lab demonstrates that *in utero* gene editing is a viable treatment modality that enables gene therapies to treat patients with higher efficiency at organs that are difficult to target with typical methods. Though some of the discussed projects do not use disease models or investigate the relief of disease-specific symptoms, this body of work may serve as a proof of concept for *in utero* therapeutic gene editing in a range of organ systems, across a multitude of editing strategies, for the early and personalized treatment of monogenic diseases.

Appendix

sgRNA Name	Relative <i>PIK3C2B</i> mRNA Expression		
	Day 3	Day 10-14	Day 30
sgRNA 1 CRISPRoff	74%	NA	NA
sgRNA 2 CRISPRoff	27%	27%	20%
sgRNA 3 CRISPRoff	13%	15%	37%
sgRNA 4 CRISPRoff	112%	94%	92%
sgRNA 5 CRISPRoff	22%	33%	22%
sgRNA 6 CRISPRoff	21%	35%	38%
sgRNA 7 CRISPRoff	30%	NA	42%

sgRNA 8 CRISPRoff	94%	79%	98%
sgRNA 9 CRISPRoff	33%	15%	40%
sgRNA 10 CRISPRoff	30%	5%	21%

Table 16: *PIK3C2B* expression table for top 10 predicted sgRNAs in Huh7 CRISPRoff sgRNA screen.

Mouse CRISPRoff sgRNA ID	Spacer Sequence/PAM
sgRNA1	GCCCTCTGGCTCCCGCGACT/AGG
sgRNA2	TAGCGCGAACGGCGAGCGAG/CGG
sgRNA3	CACGGGCAAGCGACGGGCGT/GGG

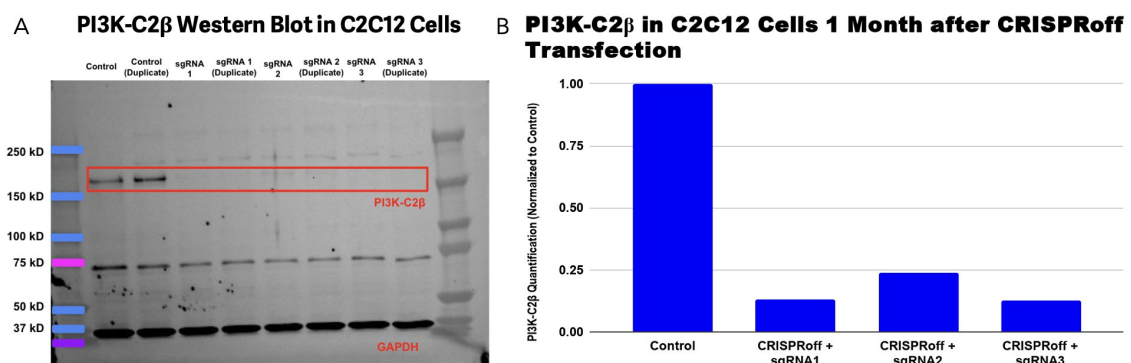
Table 17: Mouse analogs to top 3 human *PIK3C2B*-targeting CRISPRoff sgRNAs.

sgRNA	Relative mRNA expression at 3 days	Relative mRNA expression at 14 days	Relative mRNA expression at 1 month
Control	100%	100%	100%
sgRNA1	14%	29%	8.4%
sgRNA2	17%	22%	72.9%
sgRNA3	10%	10%	15%

Table 18: *PIK3C2B* expression table for murine analogs of top 3 human sgRNAs screened in Huh7 cells. Murine CRISPRoff sgRNA screen conducted in C2C12 cells.

<i>PIK3C2B</i> Exon Splice Site (Donor/Acceptor) Targeted	Editing Efficiency at Target Adenine in Splice Site
27D (2)	81%
8D	80%
27D (1)	61%
29D (2)	47%
29D (1)	44%
32A	39%
17A	26%
19D	23%
14A	17%
10A	13%

Table 19: A Δ G editing efficiencies of ABE sgRNA candidates targeting *PIK3C2B* exon splice sites in Huh7 cells.



Replicate		2mg/kg E14	2mg/kg E14	2mg/kg E14 HI MOD	2mg/kg E16	2mg/kg E16 HI MOD	3mg/kg Adult HI MOD	3mg/kg E14 HI MOD	2mg/kg E14 HI MOD
1	0%	37.5%	47.5%	25.2%	49.9%	27.5%	40.9%	65.3%	36.36%
2	6%	30.5%	20.6%	56.5%	20.8%	3.1%	40.3%	60.4%	38.97%
3	0.3%	5.6%	60.1%	28.8%	37.7%	31.6%	44.6%	58.3%	13.63%
4	0%	19.6%	62.3%	1.4%	0.4%	40.6%	47.1%	66.8%	40.29%
5		0.9%	21.8%	2%	22%	15.4%		45.1%	24.81%
6				51.9%	6%	27.5%		62.6%	30.09%
7				68.4%		17.8%			52.21%
8				62.1%		28.8%			
9				0.2%		40.7%			
10				15.7%		18.3%			
11				55.7%		3.8%			
12						28.4%			
13						12.3%			
14						0%			
15						8.4%			
16						11%			

Table 21: Adenine base editing at *DMD* exon 45 splice acceptor site in bulk mouse liver tissue. Livers from first eight cohorts displayed (including control) obtained 1 month after IV injection of LNPs containing ABE8.20 or ABE8e mRNA and sgRNA adapted from Chai *et al.*²² at time points (E14, E16, Adulthood [6 weeks]) and dosages (2 mg/kg, 3 mg/kg) specified. Livers of last cohort collected at 12 months to assess long-term proliferation of edited cells. HI MOD indicates “highly chemically modified” sgRNA for enhancement of CRISPR-Cas9 editing, as discussed in Table 7, Methods.

Replicate	Control	1 mo ABE8.20 2mg/kg E14	1 mo ABE8e 2mg/kg E14	1 mo ABE8e 2mg/kg E14 HI MOD	1 mo ABE8e 2mg/kg E16	1 mo ABE8e 2mg/kg E16 HI MOD	1 mo ABE8e 3mg/kg Adult HI MOD	1 mo ABE8e 3mg/kg E14 HI MOD	12 mo ABE8e 2mg/kg E14 HI MOD
1	0%	7.4%	7.8%	11%	3.9%	2.1%	1.2%	17.3%	12.55%
2	0.2%	12.6%	9.8%	12.1%	2.2%	1.1%	0.3%	15.2%	4.24%
3	0%	1.1%	11.8%	3.2%	3.8%	5.1%	1%	25.4%	1.42%
4	0%		20.4%	0.4%	1.1%	4.3%	1.2%	27.7%	10.68%
5			1.9%	1.7%	2.8%	2.4%		21.2%	2.71%
6				16.5%	1.8%	5.2%		13.3%	3.63%
7				27.3%		2.5%			23.16%
8				18.8%		1.9%			
9				1.5%		1.4%			
10				3.3%		1.2%			
11				13.5%		0.6%			
12						2.8%			
13						0.7%			
14						0%			
15						0.7%			
16						0.7%			

Table 22: Adenine base editing at *DMD* exon 45 splice acceptor site in bulk mouse heart tissue. Reference Table 21, Appendix for remarks on notation.

	Control	1 mo ABE8.20	1 mo ABE8e	1 mo ABE8e	1 mo ABE8e	1 mo ABE8e	1 mo ABE8e	1 mo ABE8e	12 mo ABE8e
--	---------	-----------------	---------------	---------------	---------------	---------------	---------------	---------------	----------------

Replicate		2mg/kg E14	2mg/kg E14	2mg/kg E14 HI MOD	2mg/kg E16	2mg/kg E16 HI MOD	3mg/kg Adult HI MOD	3mg/kg E14 HI MOD	2mg/kg E14 HI MOD
1	0%	8.1%	5.9%	2.9%	2.6%	1.8%	2.3%	15.4%	10.26%
2	0.4%	2.9%	8.4%	4%	5.5%	2.5%	1.6%	16.3%	3.4%
3	0%	4%	5.8%	2.2%	1.2%	5.1%	1.4%	11.5%	0.69%
4	0.4%		10.2%	0.3%	4%	3.6%	2.7%	20.3%	1.46%
5			3.4%	4%	2.4%	1.1%		6.8%	1.21%
6				7.4%	0.6%	2.2%		8.5%	3.91%
7				17.6%		3.4%			5%
8				10.7%		7.8%			
9				0.6%		3.3%			
10				1.6%		4.6%			
11				13.2%		0.4%			
12						4.1%			
13						0.7%			
14						0%			
15						0.4%			
16						1.5%			

Table 23: Adenine base editing at *DMD* exon 45 splice acceptor site in bulk mouse diaphragm tissue.
Reference Table 21, Appendix for remarks on notation.

Replicate	Control	1 mo ABE8.20 2mg/kg E14	1 mo ABE8e 2mg/kg E14	1 mo ABE8e 2mg/kg E14 HI MOD	1 mo ABE8e 2mg/kg E16	1 mo ABE8e 2mg/kg E16 HI MOD	1 mo ABE8e 3mg/kg Adult HI MOD	1 mo ABE8e 3mg/kg E14 HI MOD	12 mo ABE8e 2mg/kg E14 HI MOD
1	0%	7.6%	2.7%	3.2%	0.9%	1.7%	8.2%	9%	10.34%
2	0%	14.5%	1.7%	2.3%	3.8%	6.2%	3.8%	7.5%	0.87%
3	1.2%	7.9%	2.4%	1.8%	1.5%	6.2%	1%	9.3%	1.35%
4	0%	1.2%	7.2%	0.5%	4.7%	5.7%	1.4%	11.6%	2.04%
5			0.8%	2.8%	1.2%	5.7%		8.8%	0.2%
6				3.3%	2%	3.7%		6.4%	0.8%
7				16.2%		8.6%		3.43%	
8				6.9%		3%			
9				0.5%		3.2%			
10				2%		1.8%			
11				6.1%		0%			
12						1%			
13						0.7%			
14						0%			
15						0.4%			
16						0.5%			

Table 24: Adenine base editing at *DMD* exon 45 splice acceptor site in bulk mouse quadriceps tissue.
Reference Table 21, Appendix for remarks on notation.

Replicate	Control	1 mo ABE8.20 2mg/kg E14	1 mo ABE8e 2mg/kg E14	1 mo ABE8e 2mg/kg E14 HI MOD	1 mo ABE8e 2mg/kg E16	1 mo ABE8e 2mg/kg E16 HI MOD	1 mo ABE8e 3mg/kg Adult HI MOD	1 mo ABE8e 3mg/kg E14 HI MOD	12 mo ABE8e 2mg/kg E14 HI MOD
-----------	---------	----------------------------------	--------------------------------	--	--------------------------------	--	---	--	---

1	0%	10.1%	4.2%	2.7%	2.5%	4.1%	1.3%	12.6%	6.88%
2	0%	7%	5.1%	2%	1.8%	1.8%	3.1%	6.4%	2.94%
3	1.7%	0.9%	5.1%	2.9%	2.4%	4.2%	1.7%	9%	0.71%
4	1.7%	23.6%	7.9%	1.6%	7%	0.5%	1.5%	19.7%	1.72%
5			0.9%	1.6%	2.1%	3%		7.1%	0%
6				7.4%	3.1%	0.5%		8.5%	3.4%
7				11.6%		0%			3.23%
8				10.7%		1.1%			
9				1.5%		1.3%			
10				3.5%					
11				8.4%					

Table 25: Adenine base editing at *DMD* exon 45 splice acceptor site in bulk mouse tibialis anterior tissue. Reference Table 21, Appendix for remarks on notation.

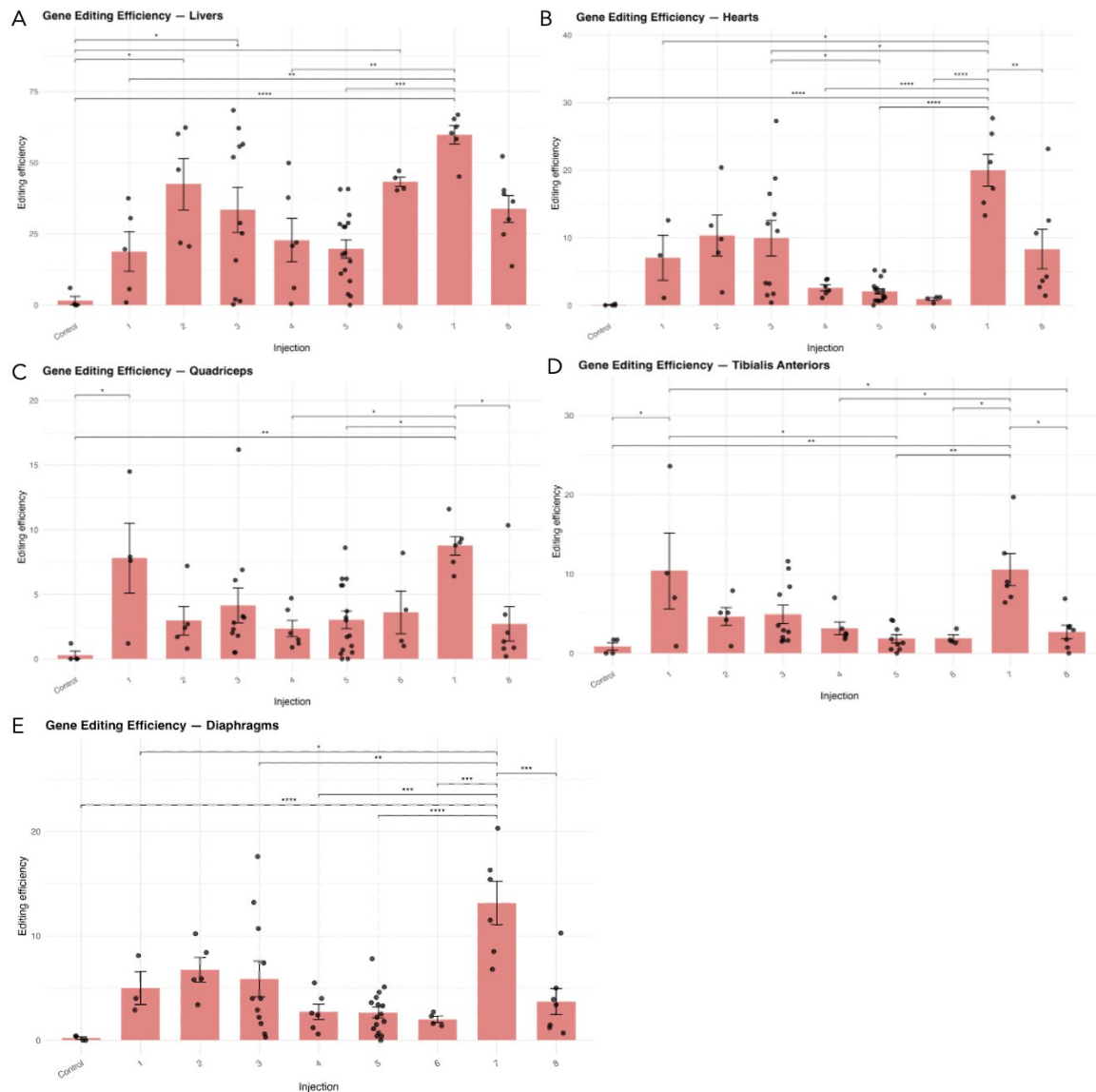


Figure 18: Adenine base editing efficiency at *DMD* exon 45 SAS separated by organ: (A) livers, (B) hearts (C) quadriceps, (D) tibialis anterior, and (E) diaphragms. For key, reference Figure 11, Main Text.

Skipped Exon	Splice Site	PAM	Guide Sequence	Position of Edit (A→G)	Potential Bystander Edit Positions	% Alignment with Human Guides	% of patients affected	MIT Off Target Score (Ideally > 50)
8	SDS	NGN	CTTTTA CCTGTT GAGAGT AA	6	NA	77.27%	2%	27
41	SDS	NGN	TACCTA CCCTTG TCGGTC CT	6	2	100%	4%	82
42	SAS	NGN	TTATTT CTAGGC AATTTG AC	9	3	76.19%	6%	37
42	SAS	NGN	TCTAGG CAATTT GACAGA TC	4	8, 9	85.71%	6%	52
43	SAS	NGG	TGGTAT CTCACA GGAACT CC	12	5, 10	NA	8%	76
43	SAS	NGG	ATCTCA CAGGAA CTCCAG GA	8	1, 6, 11, 12	95%	8%	56
43	SDS	NGG	ACCCTA CCTCTT TTTTCT CT	6	1	NA	8%	54
48	SAS	NGN	CTATTA AAGAGG AAGTTA GA	8	3, 6, 7	NA	4%	30
48	SDS	NGN	GCATAC TTACCT GCTCCA	9	3, 5	80%	4%	31

			GT					
48	SDS	NGN	TACTTA CCTGCT CCAGTT GC	6	2	71.43%	4%	42
49	SAS	NGN	AAAAAA TTTAGC TCCTAG TC	10	1, 2, 3, 4, 5, 6	77.27%	13%	50
49	SAS	NGN	AATTTA GCTCCT AGTCAG AC	6	1, 2	85.71%	13%	62
49	SDS	NGN	CCCCCA TACCTT CTGCTT GA	8	6	NA	13%	45
50	SAS	NGG	GTTCCT TCAGGC AACACT GC	9	•	85.71%	4%	38
50	SDS	NGN	AGACTT ACTTCG ATCAGT AA	7	1, 3	85.71%	4%	64
50	SDS	NGG	CTTACT TCGATC AGTAAT GA	4	10	NA	4%	81
51	SAS	NGG	TCTTCC AGTTGA AAGAAT TC	7	12	NA	8%	57
51	SAS	NGG	CTTCCA GTTGAA AGAATT CA	6	11, 12	NA	8%	57
51	SDS	NGN	GATACT AACCTT GGTTTC TG	8	2, 4, 7	100%	8%	40

53	SAS	NGN	ATTTGC AGAGTG AGTGAG CG	7	1, 9	85.71%	2%	33
----	-----	-----	----------------------------------	---	------	--------	----	----

Table 26: ABE sgRNAs for targeting exon splice sites in sheep *DMD*. The entries for which “% Alignment with Human Guides” are filled as “NA” have no corresponding available PAMs in human *DMD*.

Skipped Exon	Splice Site	PAM	Guide Sequence	Position of Edit (A→G)	Potential Bystander Edit Positions	% Alignment with Human Guides	% of patients affected	MIT Off Target Score (Ideally > 50)
8	SDS	NGN	ACTTTA CCTGTT GAGAAT AG	6	1	100%	2%	36
8	SDS	NGN	ACACTT TACCTG TTGAGA AT	8	1, 3	100%	2%	36
43	SDS	NGN	TACCTA CCCTTG TCGGTC CT	6	1	100%	4%	44
44	SAS	NGN	TTACCT GCAGGC GATTTG AC	9	3	100%	6%	52
44	SAS	NGN	TGCAGG CGATTT GACAGA TC	4	9	100%	6%	62
44	SDS	NGN	ACTTAC CTTAAG ATACCA TT	5	1, 10	100%	6%	39
45	SAS	NGG	ATCTTA CAGGAA CTCCAG GA	8	1, 6	100%	8%	31
50	SDS	NGN	GTATAC	9	3, 5	95%	4%	70

			TTACAG GCTCCA AC					
50	SDS	NGG	ATACTT ACAGGC TCCAAC AG	7	1, 3	95%	4%	82
51	SAS	NGN	AAATAT TTTAGC TCCTAG TC	10	1, 2, 3, 5	95%	13%	40
51	SAS	NGN	ATTTTA GCTCCT AGTCAG AC	6	1	95%	13%	42
52	SAS	NGG	GTTCTT ACAGGC AACAGT GC	9	7	95%	4%	77
52	SDS	NGN	AAACTT ACTTCG ATCCGT AA	7	1, 2, 3	100%	4%	84
53	SDS	NGN	GATACT AACCTT GGTTTC TG	8	• 2, 4, 7	100%	8%%	38
55	SAS	NGN	CTTCAC AGGGTG AGTGAG CA	7	5	85%	2%	29
55	SAS	NGG	TCACAG GGTGAG TGAGCA AG	5	3	80.95%	2%	52

Table 27: ABE sgRNAs for targeting exon splice sites in monkey *DMD*.

Skipped Exon	Splice Site	PAM	Guide Sequence	Position of Edit (A→G)	Potential Bystander Edit Positions	% Alignment with Human Guides	% of patients affected	MIT Off Target Score (Ideally
--------------	-------------	-----	----------------	------------------------	------------------------------------	-------------------------------	------------------------	-------------------------------

								> 50)
8	SDS	NGN	ACTTTA CCTGTT GAGAAT AG	6	1	2%	34	8
8	SDS	NGN	ACACTT TACCTG TTGAGA AT	8	1, 3	2%	36	8
43	SDS	NGN	TACCTA CCCTTG TCGGTC CT	6	1	4%	86	43
44	SAS	NGN	TTACCT GCAGGC GATTTG AC	9	3	6%	76	44
44	SAS	NGN	TGCAGG CGATTT GACAGA TC	4	9	6%	65	44
44	SDS	NGN	ACTTAC CTTAAG ATACCA TT	5	1, 10	6%	46	44
45	SAS	NGG	ATCTTA CAGGAA CTCCAG GA	8	1, 6, 11, 12	8%	67	45
50	SDS	NGN	GTATAC TTACAG GCTCCA AT	9	3, 5	4%	66	50
50	SDS	NGG	ATACTT ACAGGC TCCAAT AG	7	1, 3	4%	86	50
51	SAS	NGN	AAATAT TTTAGC TCCTAC TC	10	1, 2, 3, 5	13%	29	51

51	SAS	NGN	ATTTTA GCTCCT ACTCAG AC	6	1	13%	32	51
52	SAS	NGG	GTTCTT ACAGGC AACAAAT GC	9	7	4%	78	52
52	SDS	NGN	AAACTT ACTTCG ATCCGT AA	7	1, 2, 3	4%	84	52
53	SDS	NGN	GATACT AACCTT GGTTTC TG	8	• 2, 4, 7	8%	35	53
55	SAS	NGN	CTTTGC AGGGTG AGTGAG CG	7	5	2%	42	55
55	SAS	NGG	TTGCAG GGTGAG TGAGCG AG	5	3	2%	26	55

Table 28: ABE sgRNAs for targeting exon splice sites in human *DMD*.

Skipped Exon	Splice Site	PAM	Guide Sequence	Position of Edit (A→G)	Potential Bystander Edit Positions	% Alignment with Human Guides	% of patients affected	MIT Off Target Score (Ideally > 50)
45 (Chai <i>et al.</i> ²²)	SAS	NGN	GGCTTACA GGAAGTC CAGGA	8	6, 11, 12	90%	8%	69
53 (<i>Mdx-4cv</i>)	SDS	NGN	GACACTA ACCTTGGT TTCTG	8	1, 3	95%	8%	31

Table 29: ABE sgRNAs for targeting exon splice sites in mouse *DMD*, including Chai *et al.*'s²² sgRNA and an sgRNA targeting the exon 8 SDS in the *DMD* mouse model *Mdx-4cv* (mutation rescuable with exon skipping strategy).

Sample	Percentage of NGS Reads Edited	One-sided P-value: Editing in cells - Editing in corresponding tissues	One-sided P-value: Editing in edited samples - Editing in corresponding control samples
Control Heart Tissue Rep. 1	2.3%	0.0287 *	-
Control Heart Tissue Rep. 2	5.0%		
Control Cardiomyocytes Rep. 1	5.0%		
Control Cardiomyocytes Rep. 2	4.0%		
Control Cardiomyocytes Rep. 3	5.0%		
Control Quadricep Tissue Rep. 1	4.0%		
Control Quadricep Tissue Rep. 2	3.0%		
Control Quadricep Myocytes Rep. 1	5.5%		
Control Quadricep Myocytes Rep. 2	5.0%		
Control Quadricep Satellite Cells Rep. 1	7.0%		
Control Quadricep Satellite Cells Rep. 2	5.0%		
Edited Heart Tissue Rep. 1	14.0%	-	0.0001 ***
Edited Heart Tissue Rep. 2	10.7%		

Edited Quadricep Tissue Rep. 1	6.0%		
Edited Quadricep Tissue Rep. 2	14.0%		

Table 30: Editing rates assessed from NGS among control and experimental tissue and cell samples.

pegRNA Name	pegRNA RTT Sequence	Editing Efficiency in HbCS K562 Cells
Shao <i>et al.</i> pegRNA	TGACCTCCAAATACCGTTA	36%
Mutant 1	TGACCTCCAAATATCGTTA	45.40%
Mutant 3	TGACCTCCAAATACAGATA	57.77%
Mutant 4	TGACCTCCAAATATAGGTA	62.34%
Mutant 13	TGACCTCCAAGTATCGTTG	61.38%
Mutant 16	TGACCTCCAAGTATAGGTG	42.97%
Mutant 58	TGACCTCCAAGTATAGGTA	55.42%

Table 31: Editing efficiencies of PE6c and various pegRNAs carrying silent mutations, including Shao *et al.*'s original pegRNA, targeting *HBA2* in HbCS K562 cells.

References

- (1) Tremblay, F.; Xiong, Q.; Shah, S. S.; Ko, C.-W.; Kelly, K.; Morrison, M. S.; Giancarlo, C.; Ramirez, R. N.; Hildebrand, E. M.; Voytek, S. B.; El Sebae, G. K.; Wright, S. H.; Lofgren, L.; Clarkson, S.; Waters, C.; Linder, S. J.; Liu, S.; Eom, T.; Parikh, S.; Weber, Y.; Martinez, S.; Malyala, P.; Abubucker, S.; Friedland, A. E.; Maeder, M. L.; Lombardo, A.; Myer, V. E.; Jaffe, A. B. A Potent Epigenetic Editor Targeting Human PCSK9 for Durable Reduction of Low-Density Lipoprotein Cholesterol Levels. *Nat Med* **2025**, *31* (4), 1329–1338. <https://doi.org/10.1038/s41591-025-03508-x>.
- (2) Jinek, M.; Chylinski, K.; Fonfara, I.; Hauer, M.; Doudna, J. A.; Charpentier, E. A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science* **2012**, *337* (6096), 816–821. <https://doi.org/10.1126/science.1225829>.
- (3) Lin, J.; Jin, M.; Yang, D.; Li, Z.; Zhang, Y.; Xiao, Q.; Wang, Y.; Yu, Y.; Zhang, X.; Shao, Z.; Shi, L.; Zhang, S.; Chen, W.; Wang, N.; Wu, S.; Yang, H.; Xu, C.; Li, G. Adenine Base Editing-Mediated Exon Skipping Restores Dystrophin in Humanized Duchenne Mouse Model. *Nat Commun* **2024**, *15* (1), 5927. <https://doi.org/10.1038/s41467-024-50340-x>.
- (4) Leckie, J.; Zia, A.; Yokota, T. An Updated Analysis of Exon-Skipping Applicability for Duchenne Muscular Dystrophy Using the UMD-DMD Database. *Genes* **2024**, *15* (11), 1489. <https://doi.org/10.3390/genes15111489>.
- (5) Pinello, L.; Canver, M. C.; Hoban, M. D.; Orkin, S. H.; Kohn, D. B.; Bauer, D. E.; Yuan, G.-C. Analyzing CRISPR Genome-Editing Experiments with CRISPResso. *Nat Biotechnol* **2016**, *34* (7), 695–697. <https://doi.org/10.1038/nbt.3583>.

- (6) Tasfaout, H.; Buono, S.; Guo, S.; Kretz, C.; Messaddeq, N.; Booten, S.; Greenlee, S.; Monia, B. P.; Cowling, B. S.; Laporte, J. Antisense Oligonucleotide-Mediated Dnm2 Knockdown Prevents and Reverts Myotubular Myopathy in Mice. *Nat Commun* **2017**, *8* (1), 15661. <https://doi.org/10.1038/ncomms15661>.
- (7) Moussa, H. F.; Bsteh, D.; Yelagandula, R.; Pribitzer, C.; Stecher, K.; Bartalska, K.; Michetti, L.; Wang, J.; Zepeda-Martinez, J. A.; Elling, U.; Stuckey, J. I.; James, L. I.; Frye, S. V.; Bell, O. Canonical PRC1 Controls Sequence-Independent Propagation of Polycomb-Mediated Gene Silencing. *Nat Commun* **2019**, *10* (1), 1931. <https://doi.org/10.1038/s41467-019-09628-6>.
- (8) Lazzarotto, C. R.; Malinin, N. L.; Li, Y.; Zhang, R.; Yang, Y.; Lee, G.; Cowley, E.; He, Y.; Lan, X.; Jividen, K.; Katta, V.; Kolmakova, N. G.; Petersen, C. T.; Qi, Q.; Strelcov, E.; Maragh, S.; Krenciute, G.; Ma, J.; Cheng, Y.; Tsai, S. Q. CHANGE-Seq Reveals Genetic and Epigenetic Effects on CRISPR–Cas9 Genome-Wide Activity. *Nat Biotechnol* **2020**, *38* (11), 1317–1327. <https://doi.org/10.1038/s41587-020-0555-7>.
- (9) Long, C.; Li, H.; Tiburcy, M.; Rodriguez-Caycedo, C.; Kyrychenko, V.; Zhou, H.; Zhang, Y.; Min, Y.-L.; Shelton, J. M.; Mammen, P. P. A.; Liaw, N. Y.; Zimmermann, W.-H.; Bassel-Duby, R.; Schneider, J. W.; Olson, E. N. Correction of Diverse Muscular Dystrophy Mutations in Human Engineered Heart Muscle by Single-Site Genome Editing. *Sci. Adv.* **2018**, *4* (1), eaap9004. <https://doi.org/10.1126/sciadv.aap9004>.
- (10) Concordet, J.-P.; Haeussler, M. CRISPOR: Intuitive Guide Selection for CRISPR/Cas9 Genome Editing Experiments and Screens. *Nucleic Acids Research* **2018**, *46* (W1), W242–W245. <https://doi.org/10.1093/nar/gky354>.

- (11) Kluesner, M. G.; Lahr, W. S.; Lonetree, C.; Smeester, B. A.; Qiu, X.; Slipek, N. J.; Claudio Vázquez, P. N.; Pitzen, S. P.; Pomeroy, E. J.; Vignes, M. J.; Lee, S. C.; Bingea, S. P.; Andrew, A. A.; Webber, B. R.; Moriarity, B. S. CRISPR-Cas9 Cytidine and Adenosine Base Editing of Splice-Sites Mediates Highly-Efficient Disruption of Proteins in Primary and Immortalized Cells. *Nat Commun* **2021**, *12* (1), 2437. <https://doi.org/10.1038/s41467-021-22009-2>.
- (12) Frangoul, H.; Altshuler, D.; Cappellini, M. D.; Chen, Y.-S.; Domm, J.; Eustace, B. K.; Foell, J.; De La Fuente, J.; Grupp, S.; Handgretinger, R.; Ho, T. W.; Kattamis, A.; Kernysky, A.; Lekstrom-Himes, J.; Li, A. M.; Locatelli, F.; Mapara, M. Y.; De Montalembert, M.; Rondelli, D.; Sharma, A.; Sheth, S.; Soni, S.; Steinberg, M. H.; Wall, D.; Yen, A.; Corbacioglu, S. CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β -Thalassemia. *N Engl J Med* **2021**, *384* (3), 252–260. <https://doi.org/10.1056/NEJMoa2031054>.
- (13) Rocca, C. J.; Rainaldi, J. N.; Sharma, J.; Shi, Y.; Haquang, J. H.; Luebeck, J.; Mali, P.; Cherqui, S. CRISPR-Cas9 Gene Editing of Hematopoietic Stem Cells from Patients with Friedreich's Ataxia. *Molecular Therapy - Methods & Clinical Development* **2020**, *17*, 1026–1036. <https://doi.org/10.1016/j.omtm.2020.04.018>.
- (14) Gaudelli, N. M.; Lam, D. K.; Rees, H. A.; Solá-Esteves, N. M.; Barrera, L. A.; Born, D. A.; Edwards, A.; Gehrke, J. M.; Lee, S.-J.; Liquori, A. J.; Murray, R.; Packer, M. S.; Rinaldi, C.; Slaymaker, I. M.; Yen, J.; Young, L. E.; Ciaramella, G. Directed Evolution of Adenine Base Editors with Increased Activity and Therapeutic Application. *Nat Biotechnol* **2020**, *38* (7), 892–900. <https://doi.org/10.1038/s41587-020-0491-6>.

- (15) Ran, F. A.; Hsu, P. D.; Lin, C.-Y.; Gootenberg, J. S.; Konermann, S.; Trevino, A. E.; Scott, D. A.; Inoue, A.; Matoba, S.; Zhang, Y.; Zhang, F. Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity. *Cell* **2013**, *154* (6), 1380–1389. <https://doi.org/10.1016/j.cell.2013.08.021>.
- (16) Fletcher, S.; Adams, A. M.; Johnsen, R. D.; Greer, K.; Moulton, H. M.; Wilton, S. D. Dystrophin Isoform Induction In Vivo by Antisense-Mediated Alternative Splicing. *Molecular Therapy* **2010**, *18* (6), 1218–1223. <https://doi.org/10.1038/mt.2010.45>.
- (17) Shao, C.; Liu, Q.; Xu, J.; Zhang, J.; Zhang, C.; Xin, Y.; Ye, Y.; Lin, B.; Zhang, X.; Cheng, L.; Xu, X.; Xu, P. Efficient and in Situ Correction of Hemoglobin Constant Spring Mutation by Prime Editing in Human Hematopoietic Cells. *Molecular Therapy Nucleic Acids* **2024**, *35* (4), 102371. <https://doi.org/10.1016/j.omtn.2024.102371>.
- (18) Xu, L.; Zhang, C.; Li, H.; Wang, P.; Gao, Y.; Mokadam, N. A.; Ma, J.; Arnold, W. D.; Han, R. Efficient Precise in Vivo Base Editing in Adult Dystrophic Mice. *Nat Commun* **2021**, *12* (1), 3719. <https://doi.org/10.1038/s41467-021-23996-y>.
- (19) Martin, F. J.; Amode, M. R.; Aneja, A.; Austine-Orimoloye, O.; Azov, A. G.; Barnes, I.; Becker, A.; Bennett, R.; Berry, A.; Bhai, J.; Bhurji, S. K.; Bignell, A.; Boddu, S.; Branco Lins, P. R.; Brooks, L.; Ramaraju, S. B.; Charkhchi, M.; Cockburn, A.; Da Rin Fiorretto, L.; Davidson, C.; Dodiya, K.; Donaldson, S.; El Houdaigui, B.; El Naboulsi, T.; Fatima, R.; Giron, C. G.; Genez, T.; Ghattaoraya, G. S.; Martinez, J. G.; Guijarro, C.; Hardy, M.; Hollis, Z.; Hourlier, T.; Hunt, T.; Kay, M.; Kaykala, V.; Le, T.; Lemos, D.; Marques-Coelho, D.; Marugán, J. C.; Merino, G. A.; Mirabueno, L. P.; Mushtaq, A.; Hossain, S. N.; Ogeh, D. N.; Sakthivel, M. P.; Parker, A.; Perry, M.; Piližota, I.; Prosovetskaia, I.; Pérez-Silva, J. G.; Salam, A. I. A.; Saraiva-Agostinho, N.;

Schuilenburg, H.; Sheppard, D.; Sinha, S.; Sipos, B.; Stark, W.; Steed, E.; Sukumaran, R.; Sumathipala, D.; Suner, M.-M.; Surapaneni, L.; Sutinen, K.; Szpak, M.; Tricomi, F. F.; Urbina-Gómez, D.; Veidenberg, A.; Walsh, T. A.; Walts, B.; Wass, E.; Willhoft, N.; Allen, J.; Alvarez-Jarreta, J.; Chakiachvili, M.; Flint, B.; Giorgetti, S.; Haggerty, L.; Ilsley, G. R.; Loveland, J. E.; Moore, B.; Mudge, J. M.; Tate, J.; Thybert, D.; Trevanion, S. J.; Winterbottom, A.; Frankish, A.; Hunt, S. E.; Ruffier, M.; Cunningham, F.; Dyer, S.; Finn, R. D.; Howe, K. L.; Harrison, P. W.; Yates, A. D.; Flicek, P. Ensembl 2023. *Nucleic Acids Research* **2023**, *51* (D1), D933–D941. <https://doi.org/10.1093/nar/gkac958>.

(20) Whittaker, M. N.; Testa, L. C.; Quigley, A.; Jindal, I.; Cortez-Alvarado, S. V.; Qu, P.; Yang, Y.; Alameh, M.-G.; Musunuru, K.; Wang, X. Epigenome Editing Durability Varies Widely Across Cardiovascular Disease Target Genes. *ATVB* **2023**, *43* (10), 2075–2077. <https://doi.org/10.1161/ATVBAHA.123.319748>.

(21) Mo, A.; Mukamel, E. A.; Davis, F. P.; Luo, C.; Henry, G. L.; Picard, S.; Urich, M. A.; Nery, J. R.; Sejnowski, T. J.; Lister, R.; Eddy, S. R.; Ecker, J. R.; Nathans, J. Epigenomic Signatures of Neuronal Diversity in the Mammalian Brain. *Neuron* **2015**, *86* (6), 1369–1384. <https://doi.org/10.1016/j.neuron.2015.05.018>.

(22) Nuñez, J. K.; Chen, J.; Pommier, G. C.; Cogan, J. Z.; Replogle, J. M.; Adriaens, C.; Ramadoss, G. N.; Shi, Q.; Hung, K. L.; Samelson, A. J.; Pogson, A. N.; Kim, J. Y. S.; Chung, A.; Leonetti, M. D.; Chang, H. Y.; Kampmann, M.; Bernstein, B. E.; Hovestadt, V.; Gilbert, L. A.; Weissman, J. S. Genome-Wide Programmable Transcriptional Memory by CRISPR-Based Epigenome Editing. *Cell* **2021**, *184* (9), 2503–2519.e17. <https://doi.org/10.1016/j.cell.2021.03.025>.

- (23) Riesenberg, S.; Helmbrecht, N.; Kanis, P.; Maricic, T.; Pääbo, S. Improved gRNA Secondary Structures Allow Editing of Target Sites Resistant to CRISPR-Cas9 Cleavage. *Nat Commun* **2022**, *13* (1), 489. <https://doi.org/10.1038/s41467-022-28137-7>.
- (24) Rossidis, A. C.; Stratigis, J. D.; Chadwick, A. C.; Hartman, H. A.; Ahn, N. J.; Li, H.; Singh, K.; Coons, B. E.; Li, L.; Lv, W.; Zoltick, P. W.; Alapati, D.; Zacharias, W.; Jain, R.; Morrissey, E. E.; Musunuru, K.; Peranteau, W. H. In Utero CRISPR-Mediated Therapeutic Editing of Metabolic Genes. *Nat Med* **2018**, *24* (10), 1513–1518. <https://doi.org/10.1038/s41591-018-0184-6>.
- (25) Musunuru, K.; Chadwick, A. C.; Mizoguchi, T.; Garcia, S. P.; DeNizio, J. E.; Reiss, C. W.; Wang, K.; Iyer, S.; Dutta, C.; Clendaniel, V.; Amaonye, M.; Beach, A.; Berth, K.; Biswas, S.; Braun, M. C.; Chen, H.-M.; Colace, T. V.; Ganey, J. D.; Gangopadhyay, S. A.; Garrity, R.; Kasiewicz, L. N.; Lavoie, J.; Madsen, J. A.; Matsumoto, Y.; Mazzola, A. M.; Nasrullah, Y. S.; Nneji, J.; Ren, H.; Sanjeev, A.; Shay, M.; Stahley, M. R.; Fan, S. H. Y.; Tam, Y. K.; Gaudelli, N. M.; Ciaramella, G.; Stolz, L. E.; Malyala, P.; Cheng, C. J.; Rajeev, K. G.; Rohde, E.; Bellinger, A. M.; Kathiresan, S. In Vivo CRISPR Base Editing of PCSK9 Durably Lowers Cholesterol in Primates. *Nature* **2021**, *593* (7859), 429–434. <https://doi.org/10.1038/s41586-021-03534-y>.
- (26) Musunuru, K.; Chadwick, A. C.; Mizoguchi, T.; Garcia, S. P.; DeNizio, J. E.; Reiss, C. W.; Wang, K.; Iyer, S.; Dutta, C.; Clendaniel, V.; Amaonye, M.; Beach, A.; Berth, K.; Biswas, S.; Braun, M. C.; Chen, H.-M.; Colace, T. V.; Ganey, J. D.; Gangopadhyay, S. A.; Garrity, R.; Kasiewicz, L. N.; Lavoie, J.; Madsen, J. A.; Matsumoto, Y.; Mazzola, A. M.; Nasrullah, Y. S.; Nneji, J.; Ren, H.; Sanjeev, A.; Shay, M.; Stahley, M. R.; Fan, S. H. Y.; Tam, Y. K.; Gaudelli, N. M.; Ciaramella, G.; Stolz, L. E.; Malyala, P.; Cheng, C. J.;

Rajeev, K. G.; Rohde, E.; Bellinger, A. M.; Kathiresan, S. In Vivo CRISPR Base Editing of PCSK9 Durably Lowers Cholesterol in Primates. *Nature* **2021**, *593* (7859), 429–434.

<https://doi.org/10.1038/s41586-021-03534-y>.

(27) Breda, L.; Papp, T. E.; Triebwasser, M. P.; Yadegari, A.; Fedorky, M. T.; Tanaka, N.; Abdulmalik, O.; Pavani, G.; Wang, Y.; Grupp, S. A.; Chou, S. T.; Ni, H.; Mui, B. L.; Tam, Y. K.; Weissman, D.; Rivella, S.; Parhiz, H. In Vivo Hematopoietic Stem Cell Modification by mRNA Delivery. *Science* **2023**, *381* (6656), 436–443.

<https://doi.org/10.1126/science.ade6967>.

(28) Sousa, A. A.; Terrey, M.; Sakai, H. A.; Simmons, C. Q.; Arystarkhova, E.; Morsci, N. S.; Anderson, L. C.; Xie, J.; Suri-Payer, F.; Laux, L. C.; Roze, E.; Forlani, S.; Gao, G.; Frost, S.; Frost, N.; Sweadner, K. J.; George, A. L.; Lutz, C. M.; Liu, D. R. In Vivo Prime Editing Rescues Alternating Hemiplegia of Childhood in Mice. *Cell* **2025**, *188* (16), 4275–4294.e23. <https://doi.org/10.1016/j.cell.2025.06.038>.

(29) Palanki, R.; Bose, S. K.; Dave, A.; White, B. M.; Berkowitz, C.; Luks, V.; Yaqoob, F.; Han, E.; Swingle, K. L.; Menon, P.; Hodgson, E.; Biswas, A.; Billingsley, M. M.; Li, L.; Yiping, F.; Carpenter, M.; Trokhan, A.; Yeo, J.; Johana, N.; Wan, T. Y.; Alameh, M.-G.; Bennett, F. C.; Storm, P. B.; Jain, R.; Chan, J.; Weissman, D.; Mitchell, M. J.; Peranteau, W. H. Ionizable Lipid Nanoparticles for Therapeutic Base Editing of Congenital Brain Disease. *ACS Nano* **2023**, *17* (14), 13594–13610.

<https://doi.org/10.1021/acsnano.3c02268>.

(30) Karolczak, S.; Deshwar, A. R.; Aristegui, E.; Kamath, B. M.; Lawlor, M. W.; Andreoletti, G.; Volpatti, J.; Ellis, J. L.; Yin, C.; Dowling, J. J. Loss of Mtm1 Causes

Cholestatic Liver Disease in a Model of X-Linked Myotubular Myopathy. *Journal of Clinical Investigation* **2023**, *133* (18), e166275. <https://doi.org/10.1172/JCI166275>.

(31) Kenjo, E.; Hozumi, H.; Makita, Y.; Iwabuchi, K. A.; Fujimoto, N.; Matsumoto, S.; Kimura, M.; Amano, Y.; Ifuku, M.; Naoe, Y.; Inukai, N.; Hotta, A. Low Immunogenicity of LNP Allows Repeated Administrations of CRISPR-Cas9 mRNA into Skeletal Muscle in Mice. *Nat Commun* **2021**, *12* (1), 7101. <https://doi.org/10.1038/s41467-021-26714-w>.

(32) Cong, L.; Ran, F. A.; Cox, D.; Lin, S.; Barretto, R.; Habib, N.; Hsu, P. D.; Wu, X.; Jiang, W.; Marraffini, L. A.; Zhang, F. Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* **2013**, *339* (6121), 819–823. <https://doi.org/10.1126/science.1231143>.

(33) Mochida, T.; Fujimoto, N.; Asahina, M.; Asano, S.; Araki, S.; Inukai, N.; Hotta, A. Muscle Satellite Cell Editing by LNP-CRISPR-Cas9 to Resist Muscle Injury. *Cell Reports* **2026**, *45* (1), 116695. <https://doi.org/10.1016/j.celrep.2025.116695>.

(34) Doench, J. G.; Fusi, N.; Sullender, M.; Hegde, M.; Vaimberg, E. W.; Donovan, K. F.; Smith, I.; Tothova, Z.; Wilen, C.; Orchard, R.; Virgin, H. W.; Listgarten, J.; Root, D. E. Optimized sgRNA Design to Maximize Activity and Minimize Off-Target Effects of CRISPR-Cas9. *Nat Biotechnol* **2016**, *34* (2), 184–191. <https://doi.org/10.1038/nbt.3437>.

(35) Musunuru, K.; Grandinette, S. A.; Wang, X.; Hudson, T. R.; Briseno, K.; Berry, A. M.; Hacker, J. L.; Hsu, A.; Silverstein, R. A.; Hille, L. T.; Ogul, A. N.; Robinson-Garvin, N. A.; Small, J. C.; McCague, S.; Burke, S. M.; Wright, C. M.; Bick, S.; Indurthi, V.; Sharma, S.; Jepperson, M.; Vakulskas, C. A.; Collingwood, M.; Keogh, K.; Jacobi, A.; Sturgeon, M.; Brommel, C.; Schmaljohn, E.; Kurgan, G.; Osborne, T.; Zhang, H.; Kinney, K.; Rettig, G.; Barbosa, C. J.; Semple, S. C.; Tam, Y. K.; Lutz, C.; George, L.

A.; Kleinstiver, B. P.; Liu, D. R.; Ng, K.; Kassim, S. H.; Giannikopoulos, P.; Alameh, M.-G.; Urnov, F. D.; Ahrens-Nicklas, R. C. Patient-Specific In Vivo Gene Editing to Treat a Rare Genetic Disease. *N Engl J Med* **2025**, *392* (22), 2235–2243.

<https://doi.org/10.1056/NEJMoa2504747>.

(36) Doman, J. L.; Pandey, S.; Neugebauer, M. E.; An, M.; Davis, J. R.; Randolph, P. B.; McElroy, A.; Gao, X. D.; Raguram, A.; Richter, M. F.; Everette, K. A.; Banskota, S.; Tian, K.; Tao, Y. A.; Tolar, J.; Osborn, M. J.; Liu, D. R. Phage-Assisted Evolution and Protein Engineering Yield Compact, Efficient Prime Editors. *Cell* **2023**, *186* (18), 3983–4002.e26. <https://doi.org/10.1016/j.cell.2023.07.039>.

(37) Richter, M. F.; Zhao, K. T.; Eton, E.; Lapinaite, A.; Newby, G. A.; Thuronyi, B. W.; Wilson, C.; Koblan, L. W.; Zeng, J.; Bauer, D. E.; Doudna, J. A.; Liu, D. R. Phage-Assisted Evolution of an Adenine Base Editor with Improved Cas Domain Compatibility and Activity. *Nat Biotechnol* **2020**, *38* (7), 883–891. <https://doi.org/10.1038/s41587-020-0453-z>.

(38) Sabha, N.; Volpatti, J. R.; Gonorazky, H.; Reifler, A.; Davidson, A. E.; Li, X.; Eltayeb, N. M.; Dall’Armi, C.; Di Paolo, G.; Brooks, S. V.; Buj-Bello, A.; Feldman, E. L.; Dowling, J. J. PIK3C2B Inhibition Improves Function and Prolongs Survival in Myotubular Myopathy Animal Models. *Journal of Clinical Investigation* **2016**, *126* (9), 3613–3625. <https://doi.org/10.1172/JCI86841>.

(39) Mathis, N.; Allam, A.; Kissling, L.; Marquart, K. F.; Schmidheini, L.; Solari, C.; Balázs, Z.; Krauthammer, M.; Schwank, G. Predicting Prime Editing Efficiency and Product Purity by Deep Learning. *Nat Biotechnol* **2023**, *41* (8), 1151–1159. <https://doi.org/10.1038/s41587-022-01613-7>.

- (40) Gori, J. L.; Haddad, E.; Frangoul, H.; Kohn, D. B.; Morris, E. C.; Martin, B. N.; Deary, B. A.; Nickerson, M.; Scholz, R. L.; Fernandez, I.; Leveille, K.; De Ravin, S. S.; Kang, E. M.; Pierzynski, M.; Estwick, T.; Littel, P.; Kuhns, D. B.; Long Priel, D. A.; Teira, P.; Turvey, S.; Arnold, J.; Evans, M. D.; McManus, M.; Carpenter, B.; Waterman, D. P.; Anzalone, A. V.; Petrusich, A.; Osuna, C. E.; O'Malley, T. T.; Stewart-Ornstein, J.; Heath, J. M.; Nehilla, B. J.; Asmal, M.; Malech, H. L. Prime Editing for P47^{phox} - Deficient Chronic Granulomatous Disease. *N Engl J Med* **2026**, *394* (12), 1195–1203. <https://doi.org/10.1056/NEJMoa2509807>.
- (41) Qi, L. S.; Larson, M. H.; Gilbert, L. A.; Doudna, J. A.; Weissman, J. S.; Arkin, A. P.; Lim, W. A. Repurposing CRISPR as an RNA-Guided Platform for Sequence-Specific Control of Gene Expression. *Cell* **2013**, *152* (5), 1173–1183. <https://doi.org/10.1016/j.cell.2013.02.022>.
- (42) Shieh, P. B.; Kuntz, N. L.; Dowling, J. J.; Müller-Felber, W.; Bönnemann, C. G.; Seferian, A. M.; Servais, L.; Smith, B. K.; Muntoni, F.; Blaschek, A.; Foley, A. R.; Saade, D. N.; Neuhaus, S.; Alfano, L. N.; Beggs, A. H.; Buj-Bello, A.; Childers, M. K.; Duong, T.; Graham, R. J.; Jain, M.; Coats, J.; MacBean, V.; James, E. S.; Lee, J.; Mavilio, F.; Miller, W.; Varfaj, F.; Murtagh, M.; Han, C.; Noursalehi, M.; Lawlor, M. W.; Prasad, S.; Rico, S. Safety and Efficacy of Gene Replacement Therapy for X-Linked Myotubular Myopathy (ASPIRO): A Multinational, Open-Label, Dose-Escalation Trial. *The Lancet Neurology* **2023**, *22* (12), 1125–1139. [https://doi.org/10.1016/S1474-4422\(23\)00313-7](https://doi.org/10.1016/S1474-4422(23)00313-7).
- (43) Anzalone, A. V.; Randolph, P. B.; Davis, J. R.; Sousa, A. A.; Koblan, L. W.; Levy, J. M.; Chen, P. J.; Wilson, C.; Newby, G. A.; Raguram, A.; Liu, D. R. Search-and-

- Replace Genome Editing without Double-Strand Breaks or Donor DNA. *Nature* **2019**, 576 (7785), 149–157. <https://doi.org/10.1038/s41586-019-1711-4>.
- (44) Lazzarotto, C. R.; Katta, V.; Li, Y.; Manquen, G.; Wood, R. K.; Chyr, J.; Urbina, E.; Matsubara, A.; Lee, G.; Wu, X.; De Ravin, S. S.; Tsai, S. Q. Sensitive and Unbiased Genome-Wide Profiling of Base-Editor-Induced off-Target Activity Using CHANGE-Seq-BE. *Nat Biotechnol* **2026**. <https://doi.org/10.1038/s41587-025-02948-7>.
- (45) Chai, A. C.; Chemello, F.; Li, H.; Nishiyama, T.; Chen, K.; Zhang, Y.; Sánchez-Ortiz, E.; Alomar, A.; Xu, L.; Liu, N.; Bassel-Duby, R.; Olson, E. N. Single-Swap Editing for the Correction of Common Duchenne Muscular Dystrophy Mutations. *Molecular Therapy - Nucleic Acids* **2023**, 32, 522–535. <https://doi.org/10.1016/j.omtn.2023.04.009>.
- (46) Landfeldt, E.; Lindgren, P.; Bell, C. F.; Schmitt, C.; Guglieri, M.; Straub, V.; Lochmüller, H.; Bushby, K. The Burden of Duchenne Muscular Dystrophy: An International, Cross-Sectional Study. *Neurology* **2014**, 83 (6), 529–536. <https://doi.org/10.1212/WNL.0000000000000669>.
- (47) Koshiba, M.; Ogawa, K.; Hamazaki, S.; Sugiyama, T.; Ogawa, O.; Kitajima, T. The Effect of Formalin Fixation on DNA and the Extraction of High-Molecular-Weight DNA from Fixed and Embedded Tissues. *Pathology - Research and Practice* **1993**, 189 (1), 66–72. [https://doi.org/10.1016/S0344-0338\(11\)80118-4](https://doi.org/10.1016/S0344-0338(11)80118-4).
- (48) Périer, R. C.; Praz, V.; Junier, T.; Bonnard, C.; Bucher, P. The Eukaryotic Promoter Database (EPD).
- (49) Yeyha, N. A. UC Davis Health to Develop In-Utero Therapy for Duchenne Muscular Dystrophy. UC Davis Health March 24, 2023.