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14. ABSTRACT

The objective of this project is to achieve a single-vector CRISPR-dCas9 gene activation platform to upregulate near full-length muscle dystrophin isoform (Dp427m) as a therapeutic strategy to compensate for its reduction in candidate dystrophinopathy patients with in-frame deletions. Our lead construct design will be systemically delivered using AAV9 and tested for the optimal dose required to achieve therapeutic upregulation of the dystrophin gene from its endogenous locus in both skeletal and cardiac muscles. A transgenic humanized DMD/BMD mouse model harboring a deletion of exon 45-49 of muscle dystrophin will be generated to enable functional assessment of our construct and the therapeutic potential of upregulating truncated dystrophin.

15. SUBJECT TERMS
None listed.

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TABLE OF CONTENTS

1. INTRODUCTION

The objective of this project is to achieve a single-vector CRISPR-dCas9 gene activation platform to upregulate near full-length muscle dystrophin isoform (Dp427m) as a therapeutic strategy to compensate for its reduction in candidate dystrophinopathy patients with in-frame deletions. Our lead construct design will be systemically delivered using AAV9 and tested for the optimal dose required to achieve therapeutic upregulation of the dystrophin gene from its endogenous locus in both skeletal and cardiac muscles. A transgenic humanized DMD/BMD mouse model harboring a deletion of exon 45-49 of muscle dystrophin will be generated to enable functional assessment of our construct and the therapeutic potential of upregulating truncated dystrophin.

2. KEYWORDS

DMD, CRISPR activation.

3. ACCOMPLISHMENTS

What were the major goals of the project?

The major goals of the project for Year 1 taken from the approved statement of work are as follows:

SPECIFIC AIM 1
a. Identify optimal guide RNA targeting sequence for upregulation of Dp427m
b. Testing of rAAV-packaged construct in candidate in-frame DMD patient myogenic lines
c. Dose-escalation studies of Dp427m construct via AAV systemic delivery in hDMD/D2-mdx mice
d. Off-target assessment of Dp427m construct

SPECIFIC AIM 2
a. Generation of an exon 45-49 in-frame deletion of dystrophin in the hDMD/D2-mdx mouse

The major goals of the project for Year 2 requires the generation of the mouse model and are as follows.

SPECIFIC AIM 2
b. Characterization of muscle pathology in hDMD/D2-mdxdelEx45-49 mice
c. Assessment of Dp427m upregulation in hDMD/D2-mdxdelEx45-49 dosed with candidate construct.
d. Assessment of functional correction in hDMD/D2-mdxdelEx45-49 dosed with candidate construct.

What was accomplished under these goals?

SPECIFIC AIM 1

a. Identify optimal guide RNA targeting sequence for upregulation of Dp427m

The one year lag between proposing this project and starting is an extremely long time in CRISPR technology research. The novel CRISPR activation system based on CasMINI and a VPR transactivation domain (Figure 1A) was first compared to the original proposed SaCas9 using either single VP64 or dual VP64 (Figure 2A).

Potential guide targeting sites for CasMINI-VPR were predicted within 500bp upstream of the Transcription Start Site (TSS) of Dp427m or Dp427c, by CHOPCHOP (https://chopchop.cbu.uib.no/) and CRISPOR (http://crispor.tefor.net/). Guides containing the sequence TTTT, which cannot be transcribed with a U6 promoter were excluded; guides having any off-target transcripts outside of the target gene with 3 mismatches or less were also excluded (Table 1, Figure 1B).

The guides were screened in human cell line (HEK293) to select the best performing in terms of *DMD* expression (Figure 1C). The best performing Dp427c (cg3 and cg9) and Dp427m (mg5) targeting guides were compared to the best performing Dp427c targeting guide using either a single or dual VP64 activation domain fused to SaCas9 (Figure 2). The CasMINI-VPR Dp427c guides showed much better performance than SaCas9-VP64, while Dp427m expression was slightly increased. Due to the improved performance we switched to using **CasMINI-VPR activation constructs and proceeded to use cg3 and mg5 as the optimal guides RNAs** for subsequent aims.

Table 1. Target guides for Dp427m and Dp427c up-regulation

name	sequence
mg1	AATTCACAGAGCTTGCCATGCTG
mg2	GATATTAATCAGAACACAGTTGA
mg3	AAAGCAAGTACTATGTCCACTGT
mg4	TTAACAGAGCAGAAAATCCGGAT
mg5	AAATATCCGGGGGCCTCTACAGA
mg6	TCCTCTCAACAACACCAAAAATT
mg7	GTAGACAGTGGATACATAACAAA
mg8	AAGCCTTCTAAACACAAAAACTA
cg1	TTATGCAGCTGCTACAACGTCGG
cg2	CATTTAACGAGCATTCTTCCTGC
cg3	GGCAGGAAGAATGCTCGTTAAAT
cg4	ACGAGCATTCTTCCTGCCTAAAA
cg5	GCGAGGAACTCTACTATTGTTAC
cg6	AGGGTAAAAACTCTCTTCTGAAT
cg7	CAATAAGATGTTGTGTGCTCAGC
cg8	CAACGCAGAAATGTGGAGCTGAG
cg9	AATTCCAACAGCTCCCCTTTCGC
cg10	TTCGACTGACGTATCAGATAGTC
cg11	CATCTGTACAGAAGAGCGAGTAG
cg12	CCCTTAAAAGGCTGTGAAGATAA

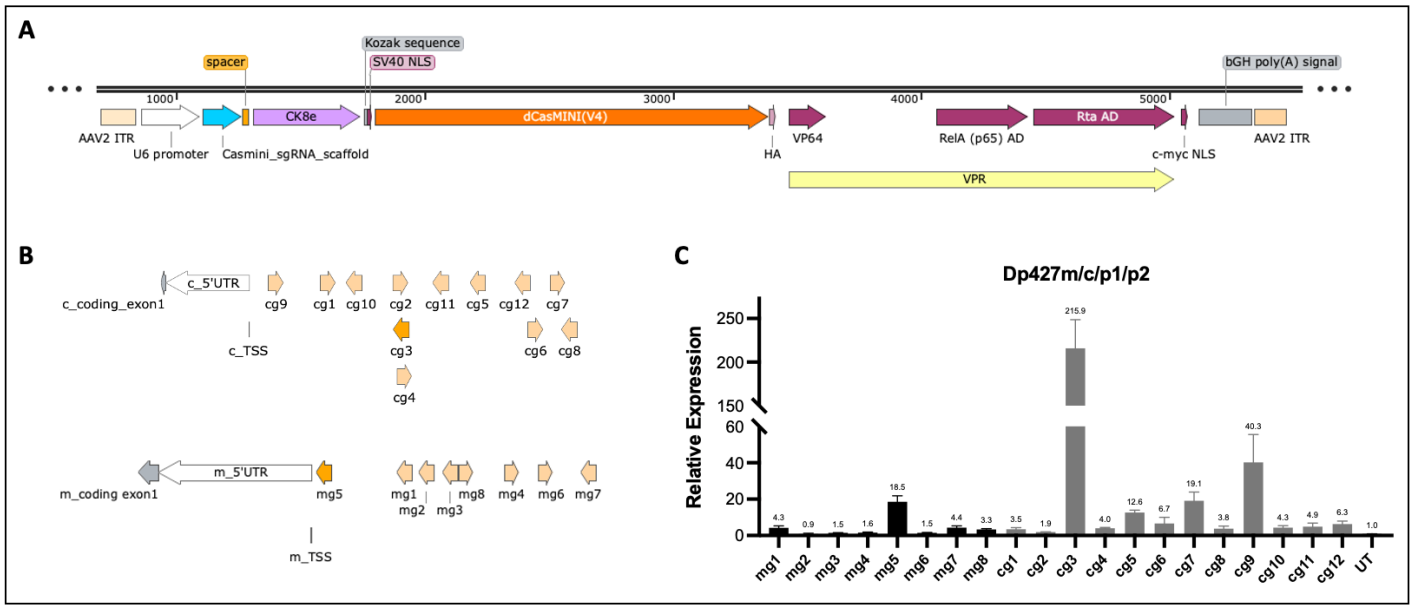

Figure 1. Guide RNA targeting sequence for dCasMINI-VPR to upregulate Dp427m, Dp427c.
A. The linear plasmid map of dCasMINI-VPR. B. Locations of the candidate dCasMINI guide RNA sites relative to the transcription Start Sites of Dp427c and Dp427m (within 500bps). Guides were predicted within 500bp upstream of the Transcription Start Site (TSS) of Dp427m or Dp427c, by CHOPCHOP (https://chopchop.cbu.uib.no/) and CRISPOR (http://crispor.tefor.net/), based on CasMINI PAM sequence TTTR. Guides containing the sequence TTTT, which cannot be transcribed with a U6 promoter, and guides having any off-target transcripts outside of the target gene with 3 or less than 3 mismatches were also excluded. C. Identifying optimal guide RNAs for dCasMINI-VPR to upregulate Dp427m, Dp427c. HEK293T cells were transfected with dCasMINI-VPR constructs with the candidate guide RNAs. Cells in each 12-well plate well were transfected with 2µg of plasmid in 2 biological replicates. For each biological replicate, 3 technical qPCR replicates were performed. The expression level of DMD hDp427m/c/p1/p2 isoforms was normalized to the expression of a housekeeping gene GAPDH. Values are presented as means ± SEM.

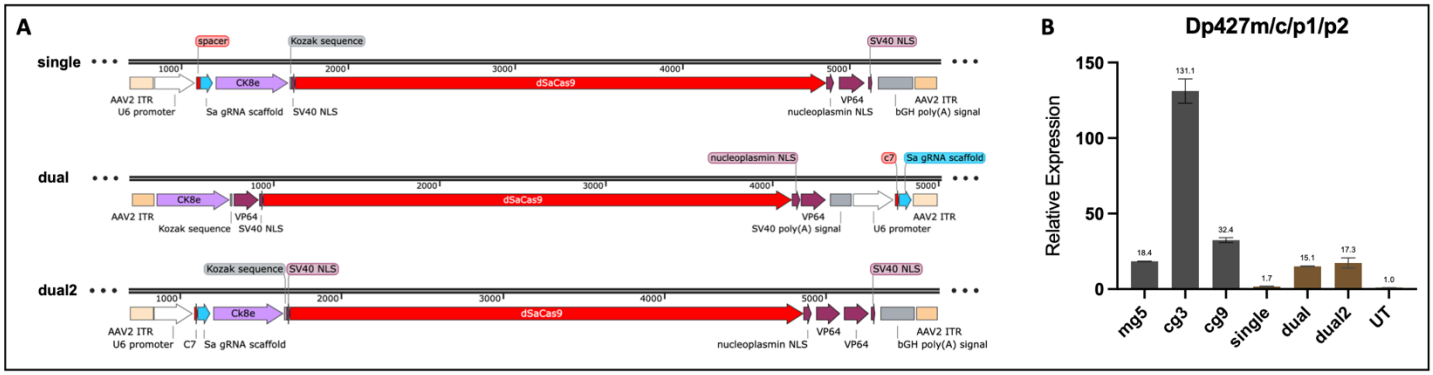

Figure 2. Testing the upregulation efficiency for dCasMINI-VPR, dSaCas9-single-VP64, and dSaCas9-dual-VP64 constructs.
A. Linear plasmid maps of dSaCas9-single-VP64 (single) and dSaCas9-dual-VP64 (dual, dual2) constructs with the same guide RNA c7. B. HEK293T cells were transfected with dCasMINI-VPR constructs, dSaCas9-single-VP64, and dSaCas9-dual-VP64 constructs with their optimized guide RNAs. mg5, cg3 and cg9 samples were transfected with dCasMINI-VPR constructs with guides targeting muscle isoform (mg5) and cortical isoform (cg3, cg9); single sample was transfected with dSaCas9-single-VP64 construct with the optimized guide targeting cortical isoform (c7); dual and dual2 samples were transfected with dSaCas9-dual-VP64 constructs with the optimized guide targeting cortical isoform (c7). Cells in each 12-well plate well were transfected with 2µg of plasmid in 2 biological replicates. For each biological replicate, 3 technical qPCR replicates were performed. The expression level of DMD hDp427m/c/p1/p2 isoforms was normalized to the expression of a housekeeping gene GAPDH. Values are presented as means ± SEM.

Materials and Methods
A human cell line was selected for testing (HEK293T) given that guide RNA targets are human specific. HEK293T cells were selected as they do not express endogenous levels of dystrophin and therefore any production is generated from the transfected constructs.

HEK293T cells (ATCC, CRL-3216) were cultured in DMEM with high glucose and sodium pyruvate (Gibco), additionally supplemented with 10% FBS (Atlanta Biologicals) and 1X Antibiotic-Antimycotic (Gibco). Cells were grown at 37°C and 5% CO2 and maintained at confluency below 80%.

HEK293T cells were plated in a 12-well plate with 25% confluency in 1ml culture medium per well one day before transfection. Transfections were performed the next day when cells reach a 70-90% confluency, with each well transfected with 2µg of plasmid in 2 biological replicates. Lipofectamine 3000 transfection reagent (Thermofisher Scientific) was used at a ratio of 4µl P3000 and 5µl Lipofectamine 3000 per 2µg of plasmid per 100µl volume of Opti-MEM reduced serum media (Gibco) in each 12-well plate well. Media were removed and fresh culture media were applied to the transfected cells 18-24hrs after transfection. The transfected cells were harvested 3 days post transfection.

RNA was extracted from harvested cell pellets using RNA purification kit (MACHEREY-NAGE). The RNA product was eZDNase enzyme (Thermofisher Scientific) treated to remove DNA, and reverse transcripted into cDNA

using PrimeScript RT Reagent Kit (Takara Bio). 150ng of cDNA was used to perform Quantative PCR reactions using primers to detect hDp427m/c/p1/p2 in 3 technical replicates for each biological sample.

The expression of hDp427m/c/p1/p2 was normalized to the expression of a housekeeping gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). A standard Ct-Expression curve was made for each pair of primers. For each pair of primers, 3 qPCR reactions were set for each biological replicate, and the mean of the Ct values were used to calculate the expression level. Normalized hDp427m/c/p1/p2 expression (i.e. hDp427m/c/p1/p2_expression : GAPDH_expression) was used for plotting with GraphPad Prism software. Values are presented as means ± SEM.

b. Testing of rAAV-packaged construct in candidate in-frame DMD patient myogenic lines

Two primary fibroblast lines harboring DMD in-frame deletions were purchased from Coriell biobank (GM04981: exons 45-53 del; GM05089: exons 3-5 del). Primary cells were expanded and subset were frozen for future usage as an early passage. Immortalization was done on both of the two fibroblast lines using lentivirus packaged Human telomerase reverse transcriptase (hTERT) (addgene plasmid #85140). MyoD conversion was sequentially performed on the immortalized fibroblast lines via VP64-human-MyoD lentivirus transduction (addgene plasmid #60629) to achieve myogenic cells.

The up-regulation of DMD full length transcripts (Dp427) by cg3 and mg5 is in progress. This will be completed once in vitro optimization experiments are complete.

c. Dose-escalation studies of Dp427m construct via AAV systemic delivery in hDMD/D2-mdx mice

Constructs containing the cg3 or mg5 guides were packaged into AAV9 for *in vivo* delivery. hDMD/mdxD2 mice were used in this study. These mice do not express dystrophin from the endogenous Dmd locus, but instead human isoforms are expressed from the transgene (i.e. hDMD) providing genetic compensation. The transgenic hDMD locus contains both muscle and cortical promoter sequence that can be targeted by cg9 and mg5 (Table 1).

Table 2. Study design at low and high dose AAV9 delivery.

Group	Dose (vg/kg)	Mice gender & number
cg3 high	3 E14	2 males, 1 female
cg3 low	1 E14	1 male, 2 females
mg5 high	3 E14	1 male, 2 females
mg5 low	1 E14	2 males, 2 females
UT	NA	3 males

hDMD/mdxD2 mice aged between 7-11 weeks were tail vein injected with AAV9 at a high dose of 3 E14 vg/kg and a low dose of 1 E14 vg/kg for both u6-scaffold-cg3-CK8e-dCasMINIV4-VPR and u6-scaffold-mg5-CK8e-dCasMINIV4-VPR constructs (Table 2). Mice health was monitored daily for one week after injection and then on a weekly basis. Mice were sacrificed and various tissues were harvested at 8-week time point.

The results show that the CasMINI-VPR construct packaged in AAV9 was successful in expressing CasMINI-VPR with heart having approximately 100-fold greater expression compared to liver. This demonstrates the muscle specificity of using the CK8e promoter (Figure 3A). The up-regulation of the hDp427c transcript isoform shows a dose-dependent response in heart and as expected very little up-regulation was achieved in liver representing a non-target tissue (Figure 3B). The full analysis of various skeletal muscles are in progress. The up-regulation achieved by mg5 was difficult to detect due to the strong expression of the hDp427m isoform. Due to the challenges of detecting hDp427m transcript and the absence of a reliable antibody to differentiate between hDp427m and hDp427c protein isoforms, we plan to use the cg3 guide for subsequent experiments within Specific Aim 2.

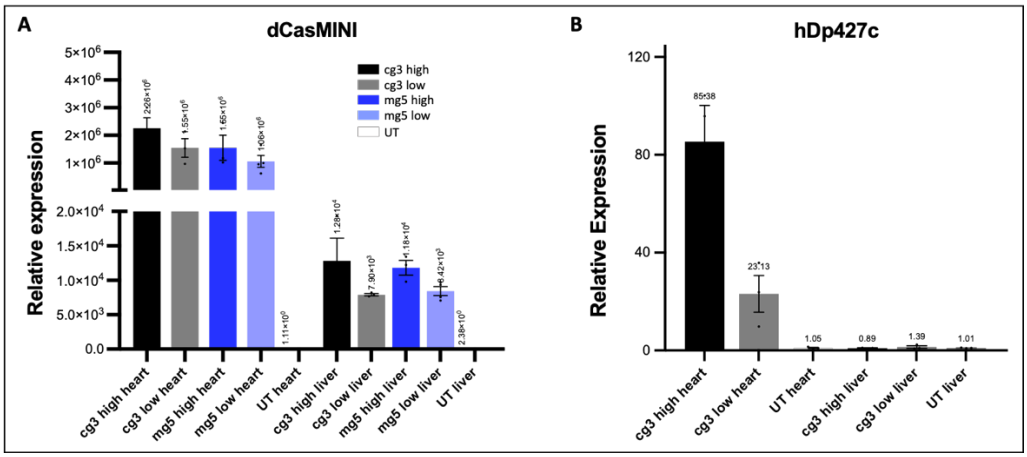

Figure 3. Dose-escalation studies of Dp427m and Dp427c constructs via AAV9 systemic delivery in hDMD/D2-mdx mice.

A. Expression of dCasMINI from heart and liver of AAX9 injected hDMD/D2-mdx mice at 8-week time point. B. Dp427c upregulation in cg3 injected and uninjected mice. Female or male hDMD/D2-mdx mice aged between 7-11 weeks were tail vein injected with AAV9 at a high dose of 3 E14 vg/kg and a low dose of 1 E14 vg/kg for both u6-scaffold-cg3-CK8e-dCasMINI-VPR and u6-scaffold-mg5-CK8e-dCasMINI-VPR constructs. 3 mice were used for each dose group and uninjected group (mg5 low dose group had 4 mice). Mice were sacrificed and tissues were harvested at 8-week time point. RNA was extracted from heart and liver, RT-qPCR was performed to determine expression of dCasMINI and hDp427c. For each biological replicate, 3 technical qPCR replicates were performed. The expression level of dCasMINI and DMD hDp427c was normalized to the expression of a housekeeping gene mHprt1. Values are presented as means ± SEM.

Materials and Methods

RNA was extracted using Trizol Reagent (Thermofisher Scientific) from heart and liver of the treated and untreated mice 8-week time point. RNA product was eZDNase enzyme (Thermofisher Scientific) treated to remove DNA. 150ng of RNA was used to perform RT-qPCR reactions using iTaq Universal SYBR Green One-Step Kit (BioRad) to determine expression of dCasMINI and hDp427m/c/p1/p2 for all mice, and expression of hDp427c for cg3 injected mice.

All qPCR reactions were performed in triplicates and Ct values were averaged. The house keeping gene, mHprt1 was used to normalize expression for dCasMINI, hDp427m/c/p1/p2 and hDp427c. A standard curve as a function of Ct values was created for dCasMINI, hDp427m/c/p1/p2, hDp427c and mHrpt1 to determine expression. Fold expression was determined relative to the mean of the untreated controls. Graphpad Prism was used to plot results from the data analysis. Values are presented as means ± SEM.

d. Off-target assessment of Dp427m construct

Three computation off-target prediction tools (CHOPCHOP, CRISPOR and Cass-offinder) were used to predict off-target sites for cg9 and mg3. CHOPCHOP and CRISPOR predicted no off target sites with 3 or less mismatches. Cas-offinder accounts for bulge formed by RNA-DNA-Cas complex, which allows for deletions in sequence matches along with base mismatches. Using a bulge size of 1, Cas-offinder detected 1 and 5 off-target sites for mg5 and cg3, respectively. Only two off-target sites from cg3 are within 10 kb of a gene and both lie within the introns (Table 3). The changes in expression levels of UBR4 and ARHGAP15 will be detected directly using qPCR.

Table 3. Off target sites predicted by Cas-offinder.

Guide	Chromosome	Position	Strand	Mismatches	Genomic Region
mg5	chr1	202099337	-	3	Intergenic
cg3	chr1	19101938	-	3	Intron UBR4
cg3	chr2	143408860	+	3	Intron ARHGAP15
cg3	chr6	167572749	-	3	Intergenic
cg3	chr10	57764166	-	3	Intergenic
cg3	chr3	112666177	-	3	Integenic

The unbiased detection of differentially expressed genes mediated by mg5 and cg3 guides are in progress and will be completed once in vitro optimization experiments are complete.

SPECIFIC AIM 2
a. Generation of an exon 45-49 in-frame deletion of dystrophin in the hDMD/D2-mdx mouse
Progress is delayed see 5. CHANGES/PROBLEMS section.

What opportunities for training and professional development has the project provided?
Nothing to report.

How were the results disseminated to communities of interest?
Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

SPECIFIC AIM 1
b. Testing of rAAV-packaged construct in candidate in-frame DMD patient myogenic lines
Cell lines have been established. We aim to demonstrate up-regulation of DMD transcripts and dystrophin protein expression. Progress was delayed in Year 1-2, see 5. CHANGES/PROBLEMS section.

c. Dose-escalation studies of Dp427m construct via AAV systemic delivery in hDMD/D2-mdx mice
Completion showing expression of the transgene (i.e. CasMINI-VPR) and downstream target, Dp427c transcript and protein expression on all tissues. Progress was delayed in Year 1-2, see 5. CHANGES/PROBLEMS section.

d. Off-target assessment of Dp427m construct
Direct qPCR on computationally predicted off target sites in genic regions as this has much higher sensitivity than RNA sequencing. RNA sequencing will be performed on cg3 mediated Dp427c isoform up-regulation, which will detect unbiased targets transcriptome-wide that may have large differential effect. Progress was delayed in Year 1-2, see 5. CHANGES/PROBLEMS section.

SPECIFIC AIM 2
a. Generation of an exon 45-49 in-frame deletion of dystrophin in the hDMD/D2-mdx mouse
The hDMD mouse embryonic stem cells have been successfully edited by Charles River Laboratory. They have planned injections in January/February 2024 to create chimeric mice. Progress was delayed in Year 1-2, see 5. CHANGES/PROBLEMS section.

The hDMD and hDMD/D2-mdx mouse models have been challenging to edit as it contains two copies of the human DMD (hDMD) gene in tandem. Directly editing embryos has not being successful as both copies needs to be edited and this is very low efficiency and that's why Charles River has editing the embryonic stem cells instead. Our collaborators in Sick Kids Hospital (Toronto, Canada) were able to remove one hDMD copy from this mouse making it higher efficiency and more viable to edit the embryos. We have designed guides and have used alternate source of funding to also generate the exon 45-49 in-frame deletion in parallel to the Charles River effort. These two approaches have given us two shots at goal to achieve this aim of generating a mouse model for this project.

Table 3. Design of candidate guides to create DMD exon 45-49 in-frame deletion.

Guide	Sequence	Predicted Editing Efficiency
BLg1	CGGTAGCCTGAAACACCTAG	78.35
BLg2*	ATTTCAACAAGACACCTCGG	73.66
BLg3*	CCATTTGACCTGCTATACCG	72.16
BLg4*	AGTAGGGCTAGACACGTCGT	65.91
BLg5	GAAACTTCTCCGGTATACAC	63.92
BRg1	CCTTGCGCTGCTACACACGG	73.79
BRg2*	GTGCATATCTAACTCCCGTG	73.54
BRg3	TCTCGGTACCAATAAAACGG	73.53
BRg4*	ACAGGAGACGTAATTGACGG	72.88
BRg5*	CAACGAGTATACAAAGATCG	69.81

Key: * denotes guides with minimal off target mismatch in the mouse genome. These six guides were selected for experimental validation of editing efficiency.

Table 4. Editing efficiency determined in vitro using HEK 293T cell line.

Guide	Replicates 1 - Indel %	Replicates 2 - Indel %
BLg2	39	40
BLg3	37	38
BLg4	36	35
BRg2	37	39
BRg4	20	31
BRg5	26	29

Based on highest editing efficiency and minimal off target, BLg2 and BRg2 were selected for the creation of the mouse model.

b. Characterization of muscle pathology in hDMD/D2-mdxdelEx45-49 mice
Will proceed as planned once mutant mouse is confirmed.

c. Assessment of Dp427m upregulation in hDMD/D2-mdxdelEx45-49 dosed with candidate construct.
Will proceed as planned once mutant mouse is confirmed.

d. Assessment of functional correction in hDMD/D2-mdxdelEx45-49 dosed with candidate construct.
Will proceed as planned once mutant mouse is confirmed.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?
Nothing to report.

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change
Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Aspects of Aim 1 were delayed due to the two personnel assigned to this grant leaving shortly after the project commenced (i.e. end of October 2021). This required me to reallocate personnel to this project as Yale University still had a hiring freeze due to the pandemic and so was not a timely option in Year 1. The assigned personnel, Dr Shushu Huang needed to leave country to look after sick family member in January 2023 and was stranded afterwards due to visa application issues until October 2023. This has had a dramatic impact on the progress of this project for 2023.

In Year 1, progress on Aim 2 have been delayed for two reasons. Firstly, Charles River Laboratory had a shortage of viable hDMD mouse embryonic stem cells and there was another project first in queue to receive their hDMD/mdx-D2 mutant mice. Second, Charles River Laboratory has increased the cost for mouse generation for this mouse model from $25,000 (budgeted in this grant) to approximately $50,000-$60,000 due to known challenges that was unanticipated earlier (Christopher Dowdy personal communication). This unanticipated two-fold increase in cost has drastically affected the plans for Aim 2. Proceeding with the original plan is not possible given the increase in budget is a large proportion of the total budget for this grant and also create a large delay in Year 2 aims.

We worked with Dr Lingling Kong (Scientific Officer) to discuss alternative approaches and she was able to get a budget increase approved along with a one year no cost extension due to the delays in the creation of the mouse model. Unfortunately, the official approval for budget increase was not received until months later and then further delay due to challenges in establishing the contract with Charles River Laboratory and Yale due to any IP that may be generated. This really delayed the start of the generation of the mouse model (i.e. Aim 2a).

Changes that had a significant impact on expenditures
Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

Significant changes in use or care of human subjects
Nothing to report.

Significant changes in use or care of vertebrate animals
Nothing to report.

Significant changes in use of biohazards and/or select agents
Nothing to report.

6. PRODUCTS

Publications, conference papers, and presentations
Nothing to report.

Website(s) or other Internet site(s)
Nothing to report.

Technologies or techniques
Nothing to report.

Inventions, patent applications, and/or licenses
Nothing to report.

Other Products
Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name	Project Role	Total Month effort	Contibution to project
Angela Lek	Senior Scientist	0.15	Initial design of CRISPR constructs. Left in Nov 2022
Keryn Woodman	Associate Research Scientist	1	Initial planning of in vivo experiments and obtained IACUC/ACURO approval. Left in Nov 2022.
Monkol Lek	Principal Investigator	3.6	Responsible for overall coordination and supervision of all aspects of the research goals proposed.
Shushu Huang	Associate Research Scientist	14	Design of CRISPR constructs. Responsible for completing Aims 1a-d. Designed and validated guides for mouse model (Aim 2a). Unavailable January - October 2023.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report.

What other organizations were involved as partners?
Not applicable.

8. SPECIAL REPORTING REQUIREMENTS
Not applicable.

9. APPENDICIES
Nothing to report.