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14. ABSTRACT

Background: Duchenne muscular dystrophy (DMD) is a severe recessive muscle wasting disease caused by mutations in the *dystrophin* gene. Aim: The two major goals of this grant are to (1) develop a fully AAV encoded split CRISPR prime editing approach to edit dystrophic mice, and (2) to generate a fully AAV encoded split dystrophin approach to deliver full-length dystrophin to dystrophic mice. Approach: Using an RNA-end joining technology we have developed, two- and three-way split protein coding RNAs can be assembled from individually AAV packaged expression cassettes. In this proposal, we use the RNA end joining technology to generate AAV encodable CRISPR genome editors to correct the mdx mouse mutation and to deliver a full-length Dystrophin replacement gene. Results: (1) AAV packable CRISPR prime editor constructs were generated and validated, but proved inefficient in correcting the disease causing premature stop codon in mdx mice. A latest generation AAV packable version of a CRISPR adenosine base editor was successfully developed to correct the mdx mutation. (2) AAV packable full-length dystrophin expression vectors were successfully generated. Conclusion: The goals for this funding period have been reached. In the next funding period we will proceed to the in vivo testing stage of the experiment.

15. SUBJECT TERMS

Gene therapy, Duchenne muscular dystrophy

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1. **INTRODUCTION:**

Duchenne muscular dystrophy (DMD) is a severe recessive muscle wasting disease caused by mutations in the *dystrophin* gene. Many approaches are being considered for treating DMD including: muscle-enhancing drugs, myoblast engraftment, gene exon skipping, gene repair using CRISPR/Cas9 variants, and gene replacement therapy. Proof-of-principle experiments in a variety of systems indicate that both gene repair and gene replacement therapy of *dystrophin* can prevent DMD, but a major limitation has been the lack of a suitable gene delivery system. Although adeno associated virus (AAV) has many features that make it a useful gene therapy vector leading to its approval by the FDA for the treatment of multiple diseases , AAV has a small cargo capacity of only ~5 kb (for review see Chamberlain et al., 2016, Patel, 2019). The limited cargo capacity of AAV represents a major hurdle for using this vector to treat DMD because a gene repair system such as the CRISPR prime editor is >6.3 kb, and a gene replacement that encodes full length dystrophin is >11.5 kb. Consequently, the goal of this grant is to use a novel RNA-end joining (REJ) system we recently identified to piece together fragmented genes to circumvent the size restriction of AAV. In preliminary in vivo experiments with reporter proteins we have demonstrated that our cargo-system is efficient, safe, and capable of targeting skeletal and cardiac muscle. In this grant we will test the efficacy and safety of our cargo-system to treat DMD in mice. Since both the CRISPR prime editor and full-length dystrophin are promising candidates for treating DMD, in Aim 1 we plan to express the CRISPR-repair system using our cargo-technology and in Aim 2 we will express full length dystrophin. The efficacy and safety of these two therapeutic approaches will be assayed in MDX mouse models of DMD in order to identify a lead candidate for clinical translation. In light of recent cautionary reports from an ongoing micro gene replacement trial (Solid Biosciences, NCT03368742), we will assess the safety of specific delivery routes and doses in non-human primates of the lead candidate so that the data from this grant will directly inform the feasibility of testing in humans.

2. **KEYWORDS:**

Gene therapy, Duchenne Muscular Dystrophy, Genome Editing, Adeno-associated virus

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

The two major goals of this grant are to (1) develop a fully AAV encoded split CRISPR prime editing approach to edit dystrophic mice, and (2) to generate a fully AAV encoded split dystrophin approach to deliver full-length dystrophin to dystrophic mice.

The following table is a list of our SOW, submitted with the application, that show our previously intended goals. In the column “Accomplished” we indicate what fraction of goals were accomplished. Milestone completion is shown. In parentheses we state if any given Milestone had to be modified (as will be discussed in a later section of the document).

(Aim 1) Develop a fully AAV encoded split CRISPR prime editing approach and demonstrate efficient correction of a premature stop codon in the dystrophic mdx mouse model.

Aim/ major goal, milestones	**Timeline (months** from approval)	**Accom-plished (%)**	**Adjusted timeline**
Specific Aim 1.1 – Establish split CRISPR prime editor AAVs by appending synthetic RNA dimerization and recombination domains (REJ-motifs) to two AAV packable fragments			
Milestone # 1 ACURO approval obtained	3	100%	
Milestone # 2 Selection of optimal split PE2 in vitro	4.5	100%	
Specific Aim 1.2 - Develop and establish an in vitro fluorescent protein reporter to assess editing efficiency for a mouse DMD (mdx mutant) mini gene.			
Milestone #3 Established gene editing screening constructs (mini-gene reporter)	2	100%	
Specific Aim 1.3 - Screen for prime editing guide RNA (pegRNA) configuration that allows for maximal mutation correction with minimal indel formation.			
Milestone #4 Establish all plasmid clones needed for pegRNA screening	4	100%	
Milestone #5 Identification of best pegRNA architecture.	6	100% (modified)	
Specific Aim 1.4 – Test optimized split prime editor constructs with optimized pegRNA in DMD mouse derived embryonic fibroblasts			
Milestone #6 Complete proof of principle in vitro in DMD fibroblasts after plasmid transfection.	8	100% (modified)	
Specific Aim 1.5 – Generate AAV constructs and produce high titer AAV preparations for in vivo testing.			
Milestone #7 Complete viral vector preparation and quality control	8	100%	

Specific Aim 1.6 – Systemically deliver the AAV mdx prime editor in the newborn DMD mouse model			
Specific Aim 1.7 – Assess treatment efficacy using dystrophin expression / localization			
Milestone #8 Successfully tested AAV delivered prime editing in dmd mouse model	13	ongoing	
Specific Aim 1.8 – Probe for possible unintended editing activity and adverse event			

Milestone #9 In depth safety profile of split AAV prime editor in mouse documented	17		
Specific Aim 1.9 – Generate an inducible muscle specific AAV configuration			
Milestone #10 Establish inducible muscle specific prime editing AAVs	10	0%	15m
Specific Aim 1.10 – Assess on target activity, efficacy, and toxicity, for the inducible prime editor configuration			
Milestone #11 Establishing of efficiency and efficacy profile of inducible system.			
Milestone #12 Establishing ON-OFF profile of inducible prime editor system	20		
Milestone #13 Compile overall data and prepare for publication	20-24		

(Aim 2) Develop a fully AAV encoded full-length dystrophin replacement gene, demonstrate efficacy in the mdx mouse model and assess efficacy of delivery in mouse and a small cohort of non-human primates**.**

Aim/ major goal, milestones	**Timeline** (months from approval award)	**Accom-plished** (%)	**Adjusted timeline**
Specific Aim 2.1 – Establish three-way split dystrophin AAVs by appending synthetic RNA dimerization and recombination domains (REJ-motifs) to three AAV packable fragments.			
Milestone # 14 Generate all dystrophin plasmid variants for initial screening	4	100%	
Milestone # 15 Selection of best triple AAV dystrophin delivery construct	8	100%	
Specific Aim 2.2 – Generate high titer AAVs and inject intramuscularly in the newborn DMD mouse model.			
Milestone # 16 Generate all dystrophin AAV preparations	9	50%	13m
Milestone # 17 Complete full-length AAV encoded intramuscular gene therapy	15		
Milestone #18 GO / NOGO for non-human primate experiment (contingent on favorable outcome of Milestone 17)	15		
Specific Aim 2.3 – Systemically deliver the AAV encoded dystrophin in the newborn DMD mouse model.			

Milestone # 19 Complete full-length AAV encoded intra venous gene therapy in mdx mice	20		
Subtask 3: Compilation of data for publication and preparation of manuscript	20-24		
Specific Aim 2.4 – Assess dystrophin expression in primate muscle after high density i.m. injection			
Milestone # 20 Complete assessment of full-length dystrophin expression in non-human primate	24		

What was accomplished under these goals?

Regarding Aim 1:

Milestone # 1 ACURO approval obtained
Major activities / specific objectives: Internal IACUC approval for all mouse experiments. ACURO approval for all mouse experiments.
Significant results / key outcomes: Approval was obtained.
Discussion: NA

Milestone # 2 Selection of optimal split PE2 in vitro
Major activities / specific objectives: Generate DNA clones to test reconstitution of split PRIME editor v2 (PE2). Generate full length control vectors. Identify efficient architecture/split point for reconstitution of PE2 while allowing for each fragment to be within 4.7-5kb packaging capacity of adeno associated virus.
Significant results / key outcomes:
Methodology: All proposed DNA clones were generated. The DNA clones were assembled using conventional cloning (restriction ligation, and homology based assembly) and gene synthesis. DNA clones were verified by Sanger sequencing and restriction digestion according to standard lab procedures.
Data / results: An architecture to split the PE2 into two AAV packable fragments was identified. In cell culture (HEK293T) the two-way split RNA end joining (REJ) constructs yielded ~60% reconstitution efficiency when compared to transfection-matched full-length control (see figure 1)

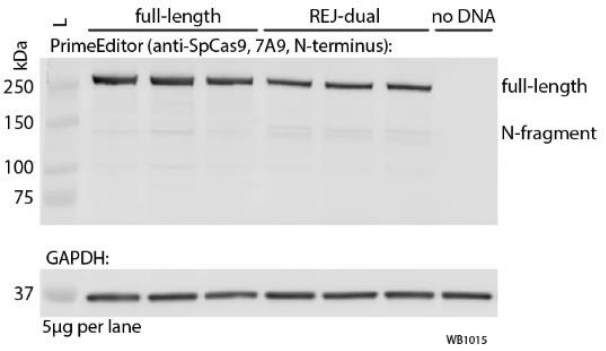

Figure 1: Western blot of three full length PE2 transfection control samples and three two-way split PE2 samples. Isolates were prepared from transfected HEK293T cells at 2 days post transfection. 5ug of total protein lysate were loaded per lane. A mouse monoclonal anti-SpCas9 antibody was used to probe for PE2. The antigen is located in the N-terminal fragment of the PE2 Cas9. Full length PE2 protein is predicted to run above 250kDa whereas unjoined N-terminal fragment is expected between 100 and 150kDA.

Conclusion / Discussion: An efficient PE2 split expression architecture was identified. Unjoined N-terminal fragment expression was minimal (fig 1). Due to the enzymatic nature of the PE2, a difference of 40% of expression levels is not expected to render the two way split version significantly less effective. The stated goal was met.

Milestone #3 Established gene editing screening constructs (mini-gene reporter):
Major activities / specific objectives: The main objective here was to identify a screening platform with which a stop codon correction in the dystrophin locus would get converted into a fluorescent readout.
Significant results / key outcomes:
Methodology: An enhanced yellow fluorescent protein was split in such a way as to produce two non fluorescent halves. The break point of the coding sequence was placed in an outer loop of the structure. In order to facilitate correct folding of the YFP independently of the "test locus" inserted in that outer loop, break point in the protein was equipped with short flexible linker sequences followed by an anti-parallel leucine zipper (to create distance between the split coding sequence and the "to be tested" sequence that contains the stop codon.

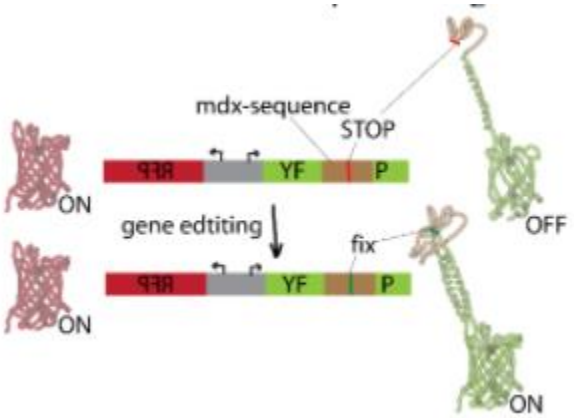

Figure 2: Schematic representation of the fluorescent reporter.

To test the functionality of the reporter, a plasmid was generated
which contained a corrected mdx locus (where the TAA stop codon was substituted to be a TAC).
Data / results: When transfected into HEK293T cells, the Stop codon containing construct yielded minimal to no YFP fluorescence (some very small amounts of YFP signal can be attributed for by a very low level of stop codon readthrough on a backdrop of very high transfection levels). If the TAA was substituted with TAC, an intense YFP signal could be detected in all transfected cells confirming that the YFP split zipper mdx reporter was functional in absence of the stop codon in the mdx segment.
Conclusion: The stop codon correction reporter works as predicted
Discussion: This reporter is used for the subsequent screening of peg-RNA.

Milestone #4 Establish all plasmid clones needed for pegRNA screening
Major activities / specific objectives: In addition to the CMV-PE2 expression plasmid (not the split version) and the mdx-editing reporter, the goal here was to generate a matrix of pegRNA expression plasmids (expressed from the U6 promoter) with different parameters.
Significant results / key outcomes:
Methodology: The two parameters that were varied were the template length and the primer length of the peg RNA. pegRNA designs were generated using the pegfinder software (http://pegfinder.sidichenlab.org/).
All plasmids were assembled using conventional golden gate assembly from synthesized oligonucleotides. The peg RNA designs were double checked independently by three scientists to make sure that the designs were in accordance with the original guidelines put forth in Anzalone et al. 2019.
Data / results: 5 sequence confirmed peg RNA expression plasmids were assembled for the mdx TAA to TAC.
Conclusion / Discussion: Plasmid libraries were generated.

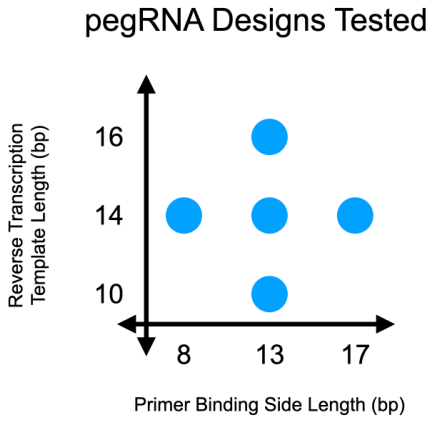

Figure 3: Schematic of the 5 constructs made for the pegRNA screen to edit mdx TAA to TAC

Milestone #5 Identification of best pegRNA architecture.
Major activities / specific objectives: The main goal here was to test which peg architecture would edit the mdx stop codon most effectively in HEK293T cells using the fluorescent gene editing reporter established in milestone #3.
Significant results / key outcomes:
Methodology: Triple plasmid transfection in HEK293T cells. The editing should result in YFP signal appearance which was to be quantified using flow cytometry and fluorescence microscopy.

Data / results: We failed to detect any YFP above background in any of the pegRNA variants we tested. We repeated the experiments several times to make sure the plasmid stocks used were good and to make sure that the failure to detect YFP was not based on a technical error. peg RNA designs were independently checked to make sure that no systematic errors have been committed. The conclusion of these efforts was that there was no significant editing activity when we used the PE2 architecture with the pegRNA designs we had come up with.
We troubleshot in the following way:

First we reasoned that the Prime editing reaction may require more time than e.g. enzymatic base editing since the reverse transcriptase reaction and the opposite strand repair may require a more complex cascade of events to unfold. Of note for this reasoning is that the original Anzalone et al. 2019 used a time point of 7 days post transfection for most of their experiments which in HEK cells is an untypically long time point. When we left the HEK cultures for up to seven days, we still failed to detect YFP fluorescence and concluded that no significant TAA to TAC editing had taken place.

Second we reasoned that a plasmid reporter may be reacting differently to a Prime editing event when compared to genomic DNA. To test this possibility we generated a lentivirus that contained the mdx stop codon reporter sequence and generated a stable cell line that expresses the YFP mdx reporter. We also generated a "sumulated" TAA to TAC cell line to confirm that fluorescence levels of YFP after stable integration are adequate for the flow cytometry and microscopy parameters which we used to assay prime editing induced YFP expression. After extensive testing of the pegRNAs in the reporter cells we concluded that we still did not observe any appreciable prime editing.

Next we reasoned that the mdx locus may not be particularly accessible and thus we tested prime editing in a different mdx mouse line which carries a premature stop codon in a different location in the dystrophin gene (the 4CV mdx mouse line). After generating YFP reporters and pegRNAs for the 4CV we concluded that again we did not observe any appreciable correction of the stop codon.

In order to confirm that the Prime editor constructs we were using were functional, we generated some of the published pegRNA constructs to edit the HEK cell genome (specifically the FANCF and the VEGFA3 loci). Testing the full length and the split constructs we concluded that indeed the desired edits could be induced.

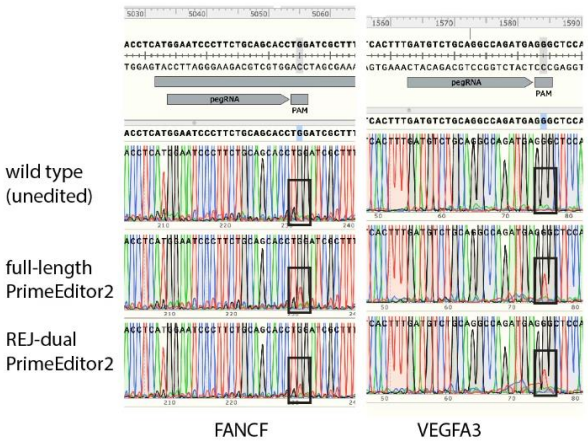

Figure 4: Reproducing some of the prime editing changes in the HEK genome that were reported in the original Anzalone et al 2019 publication. The bulk DNA sanger traces for the FANCF and VEGFA3 loci are shown in unedited and prime edited conditions.

Conclusions: We were able to confirm that the split base editor works at roughly the same efficiency as the full length constructs. These results were confirmed by analyzing amplicon sequencing results (data not shown). But in light of the negative results with the mdx locus editing, we had to conclude that the prime editor platform may perhaps be less universally applicable than we had hoped at the outset of the study.
Additional troubleshooting: Around the time of these experiments, significant advances have been made in the field of enzymatic base editing. Specifically an A to G base editor with remarkable efficiency (Abe8e) had been developed (https://www.nature.com/articles/s41587-020-0453-z?proof=t). In order to test the ability of these latest generation Adenosine base editors to correct the mdx stop codon mutation we generated a split version of the Abe8e and designed an mdx targeting sgRNA. We developed an efficient split Abe8e architecture that yielded on the order of 60-80% protein expression levels when compared to full length control in transiently transfected HEK293T cells.

Figure 5: A latest generation Abe8e base editor was split into two fragments to render it AAV packagable. The split version was reconstituted at remarkable efficiency of appx. 60-80% compared to full length control.

This Abe8e base editor was converted to an expanded PAM Cas9 variant (which is capable of targeting the TAA stop codon in the mdx locus and converting it to a CAA codon. Hence, this Abe8e variant could be used to achieve the goal initially intended for the CRISPR prime editor plattform.

To test this hypothesis we tested the ability of the split Abe8e base editor to result in YFP expression after triple transfection of the Abe8e plasmid, the mdx stop codon correction reporter, and the sgRNA expression plasmid.

In presence of the mdx locus targeting sgRNA strong YFP fluorescence was induced indicating efficient editing of the mdx stop codon to a non-stop CAA codon using the split Abe8e base editor platform.

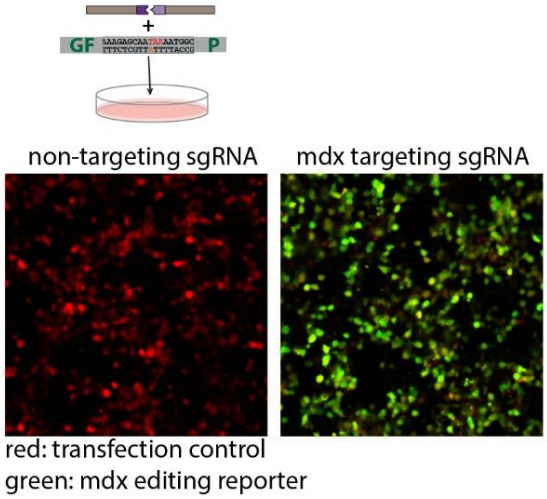

Figure 6: The Abe8e A to G base editor can efficiently convert a TAA stop codon to a CAA glutamine codon. In absence of the sgRNA, only the RFP reporter of the mdx stop editing reporter plasmid is observed. Upon introduction of the mdx targeting sgRNA most transfected cells express high levels of YFP indicating efficient editing of the stop codon.

Conclusion II / Discussion: In the course of the first year of funding on this grant, significant progress was made in the realm of enzymatic base editors. The targeting space was expanded by new PAM variants of Cas9 and in addition first enzymatic editors allowing for transversion mutations are being developed. Our efforts to get the prime editor plattform to edit the mdx locus did not yield a working configuration. The reasons for that may be several, but it appears that not all loci may be equally suited to be edited by the prime editor system. We were able to confirm the functioning of both the full-length Prime editor (and thus confirm the the published data) as well as to confirm that the two-way split prime editor we had generated was able to edit at comparable efficiency as the full length control. Hereby we confirmed that the split prime editor plattform we developed should result in efficient editing in scenarios where a functioning peg RNA were identified. To this end we are pursuing an in vivo validation in mice editing a locus for which peg RNA architectures were developed in the original publication.

In order to move forward with the mdx mutation correction, we have recently identified a well working Abe8e/sgRNA pairing to efficiently edit the TAA stop codon in the mdx mice. The nature of this construct is sufficiently similar to the originally proposed prime editor approach and is effective enough for us to move forward with the remainder of the proposed experiments using this Abe8e approach.

Milestone #6 Complete proof of principle in vitro in DMD fibroblasts after plasmid transfection.

Major activities / specific objectives: The main goal here is to test the genome editing approach in the mdx locus in a genomic context (as opposed to the transfected plasmid generated in Milestone 3).

Significant results / key outcomes:

Methodology: The transfected plasmid approach yielded good results using the Abe8e genome editing approach.

As outlined in the discussion of milestone 5, we have generated a HEK293T cell line which has a genomically integrated mdx reporter cassette (to test the prime editor approach in a genomic rather than a transfection context). Here we reasoned that the availability of a genomically integrated mdx stop reporter cell line would serve as an adequate stand in for a genomic editing in mdx derived embryonic fibroblasts and would give us sufficient confidence in the selected constructs to move forward with AAV vector production.
Data / results: As with the transfected reporter, transient transfection into the stable mdx stop codon reporter HEK293T cell line yielded efficient editing (and therefore YFP production) using the selected REJ dual AAV Abe8e and sgRNA variants.
Conclusion: These positive results warrant moving into the AAV production phase.
Discussion: NA

Milestone #7 Complete viral vector preparation and quality control.
Major activities / specific objectives: Viral vector production
Significant results / key outcomes:
Methodology: In the original proposal, packaging into AAV8 or AAV9 was considered. These are two serotypes which have been found to be efficient in infecting skeletal and cardiac muscle tissue. In the course of this study, additional AAV capsids with desirable properties have been published. One in particular, developed by Weinmann et al. 2020 - called AAV2/myo - is a derivative of AAV9 that has been selected for it's superior ability to infect muscle tissue while almost fully being excluded from the liver. Since our gene editing (and in Aim 2 gene replacement) approach is aimed at skeletal muscles, we tested the AAV2/myo capsid and selected it to package the split Abe8e base editors into.
Data / results: AAV2/myo was tested for its ability to infect skeletal and cardiac muscle

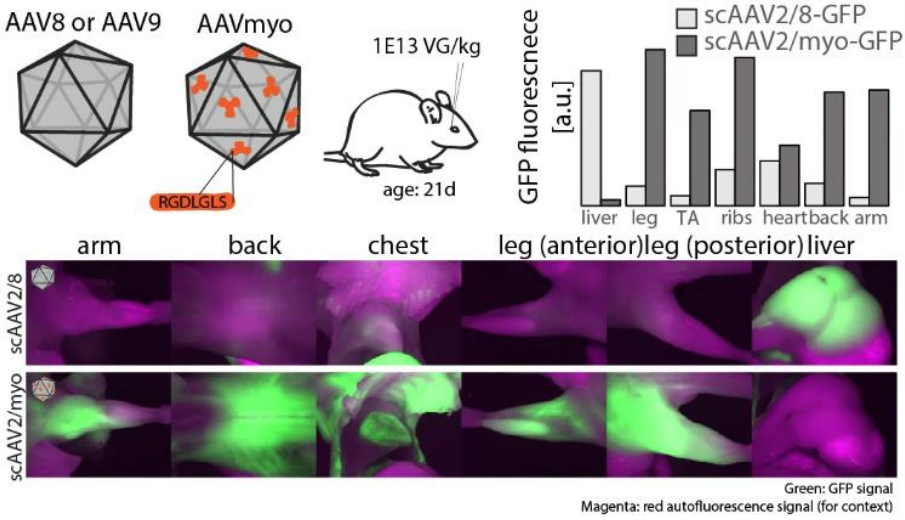

Figure 7: a GFP expressing scAAV2/myo was compared side by side with an scAAV2/8. AAV myo shows superior targeting to skeletal muscle compared to AAV2/8 while being nearly absent from the liver.

Viral vectors were produced at high titer:
AAV2/myo-N.Abe8e(2xsgRNA) at a titer of 4.43E+13 GC/mL
AAV2/myo-C.Abe8e(2xsgRNA) at a titer of 6.14E+13 GC/mL

Conclusion: Viral vector production was successful.
Discussion: NA

Milestone #10 Establish inducible muscle specific prime editing AAVs
Major activities / specific objectives: Generation of inducible AAV constructs
Significant results / key outcomes: NA
Methodology: Molecular cloning.
Conclusion: Inducible constructs have not yet been generated.
Discussion: Due to the delays that the prime editor screening caused, the inducible Abe8e genome editor plasmids have not yet been generated.

Regarding Aim 2:

Milestone # 14 Generate all dystrophin plasmid variants for initial screening
Major activities / specific objectives: The goal here was to generate full length control clones and three way split dystrophin clones to develop an efficient three way split dystrophin expression architecture that allows for AAV packaging.
Significant results / key outcomes:
Methodology: Standard molecular cloning and gene synthesis were used to generate DNA clones.
Data / results: The full-length dystrophin sequence was synthesized and codon optimized. Effective break sites were identified which allow for the full length sequence to be broken down into AAV packable fragments. These break points were successfully identified and RNA end joining domains were installed.
Conclusion: The generation of DNA clones for the screening of efficient three way split dystrophin architectures was successful.
Discussion: NA

Milestone # 15 Selection of best triple AAV dystrophin delivery construct
Major activities / specific objectives: Selection of optimal three way split constructs that allow for optimal reconstitution of full length dystrophin from three fragments in cell culture.
Significant results / key outcomes:
Methodology: DNA clones were generated using standard molecular biology techniques. Reconstitution efficiency was assessed by western blot after transient transfection in HEK293T cells.
Data / results: We tested a number of REJ domains for their ability to mediate efficiently on target joining while avoiding orthogonal joining between the first and second split points. An initial screen yielded relatively good orthogonality, but full length reconstitution was limited. In a second round, a more efficient architecture was identified. Although the orthogonal recombination of fragment 1 to fragment 3 was above background if the middle fragment was not provided, the off target joining appeared sufficiently low while the on target joining was sufficiently effective when all fragments were provided.
Conclusion: A construct architecture that yielded good full length dystrophin reconstitution was identified.
Discussion: Although the off target joining between fragment 1 and 3 is still higher than in a perfect scenario, the identified construct is considered sufficiently optimized to move forward with AAV production and subsequent in vivo testing.

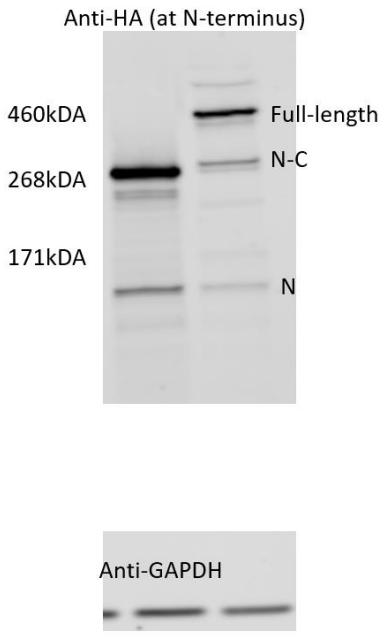

Figure 8: Western blot of only N and C-terminal fragment transfected (lane 1) and three fragment transfected (lane 2) HEK293T cells. The three fragment transfected condition shows the strongest band in the 460kDA range where full length dystrophin is predicted. The blot is probed for an N-terminal HA tag.

Milestone # 16 Generate all dystrophin AAV preparations
Major activities / specific objectives: The major objective here is to generate AAV preps for in vivo studies
Significant results / key outcomes:
Methodology: Standard plasmid preparation and standard AAV preparation by triple plasmid transfection.
Data / results: The DNA clones to package into AAV have been generated. The AAVs have not yet been packaged since we wanted to wait for a first pass with the AAV2/myo capsids used in Aim 1 (and other projects in the lab). If AAV myo proves to be effective for other applications, we will package the full length dystrophin constructs in AAV myo as well.
Conclusion: Waiting for confirmation of the AAV2/myo functional superiority before committing to producing the three fragment full length Dystrophin in AAV2/myo.

Discussion: Due to the superiority of AAV2/myo over conventional serotypes (AAV8 or 9) we wanted to confirm these assumptions before committing to a full scale production of the triple full-length Dystropin AAVs in this novel serotype. This should be concluded within a month of this report (i.e. month 14 of the project).

What opportunities for training and professional development has the project provided?

The funding of this project allowed Dr. Lukas Bachmann to critically advance his invention of the RNA end joining technology on several dimensions.
Furthermore it allowed for Dr. Lukas Bachmann to mentor/train Nicolas Criales, a student intern and Ryan Hsu, and MD-PhD student.

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?

Despite the COVID-19 pandemic, which caused delays in some of the day to day lab operations (e.g. distancing requirements, modified work schedules, and plasticware shortages) we have mostly stayed on course with this proposal. We will continue to work towards the goals as stated in the SOW and should be completing the work within the next reporting period.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

A key limitation for some oversized genes including dystrophin and CRISPR genome editors is their large size and therefore their inability to be packaged into adeno-associated vectors. The studies performed in this reporting period in this project contributed critically to reduce this approach to practice. The three way split full-length dystrophin is efficiently reconstituted in cell culture which represents a critical step towards oversized gene replacement therapy. If this approach proves to be effective, this might have implications for other diseases (specifically Leber congenital amaurosis 10 and Usher syndrome type 1D) which are caused by the loss of function of a large gene.

Furthermore, the broad applicability of CRISPR genome editor technology makes our experiments/results in Aim 1 highly relevant for currently ongoing translational efforts.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

The technology/approach developed in this study has a high potential to be valuable for technology transfer. If the gene therapies attempted in this proposal prove effective, it is conceivable that they would lend themselves to commercial/clinical development.

What was the impact on society beyond science and technology?

This project, if concluded successfully, could result in novel gene therapy approaches for the treatment of Duchenne muscular dystrophy.

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change

In the course of the first year of funding on this grant, significant progress was made in the realm of enzymatic base editors. The targeting space was expanded by new PAM variants of Cas9 and in addition first enzymatic editors allowing for transversion mutations are being developed. Our efforts to get the prime editor plattform to edit the mdx locus did not yield a working configuration. The reasons for that may be several, but it appears that not all loci may be equally suited to be edited by the prime editor system. We were able to confirm the functioning of both the full-length Prime editor (and thus confirm the the published data) as well as to confirm that the two-way split prime editor we had generated was able to edit at comparable efficiency as the full length control. Hereby we confirmed that the split prime editor plattform we developed should result in efficient editing in scenarios where a functioning peg RNA were identified. To this end we are pursuing an in vivo validation in mice editing a locus for which peg RNA architectures were developed in the original publication.

In order to move forward with the mdx mutation correction, we have identified a well working Abe8e/sgRNA pairing to efficiently edit the TAA stop codon in the mdx mice. The nature of this construct is sufficiently similar to the originally proposed prime editor approach and is effective enough for us to move forward with the remainder of the proposed experiments using this Abe8e genome editor approach.

Actual or anticipated problems or delays and actions or plans to resolve them

The COVID-19 pandemic resulted in changes in workplace occupancy which had a negative impact on overall productivity (workplace distancing measures) and has impacted the supply chain in a way that resulted in delays of gene synthesis products and has resulted in delivery delays in plasticware.

We have adjusted accordingly and were able to proceed largely according to plan.

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

No significant changes. All procedures on mice are currently approved as in our IACUC protocol 11-00020 and have ACURO approval.

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.

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6. **PRODUCTS:**

Publications, conference papers, and presentations

- **Journal publications.** Nothing to report.
- **Books or other non-periodical, one-time publications.** Nothing to report.
- **Other publications, conference papers, and presentations.** Nothing to report.

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

This project yielded technical confirmation and contributed to a reduction to practice for two applications of our previously established RNA end joining technology (REJ). The first technique relates to the two-way split

CRISPR genome editors (prime and base) and the second technique relates to the three-way splitting of full-length dystrophin.

Inventions, patent applications, and/or licenses

Some of the work funded by this grant resulted in data that is contained within:

- Provisional Patent Application No. 63/189,048 (filed on behalf of the Salk Institute for Biological Studies)
- And Patent Application WO2021096605 (filed on behalf of the Salk Institute for Biological Studies).

Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Prof. Samuel L. Pfaff, Ph.D.
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-2142-166X
Nearest person month worked:	2
Contribution to Project:	Plan and interpret experiments, ensure that regulatory and reporting requirements are met, prepare manuscripts and coordinate the sharing of reagents and communication of results.
Funding Support:	Institute funds from The Salk Institute for Biological Studies.

Name:	Lukas C. Bachmann, Ph.D.
Project Role:	SR RESEARCH ASSOCIATE
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	10
Contribution to Project:	Oversight of experiments and execution of in vitro and in vivo experiments.
Funding Support:	Institute funds from The Salk Institute for Biological Studies

Name:	Kip Hermann, Ph.D
Project Role:	RESEARCH SCIENTIST
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	8
Contribution to Project:	Cloning, virus production, in vitro cell culture experiments
Funding Support:	Institute funds from The Salk Institute for Biological Studies

Name:	Stella Kramer, Ph.D
Project Role:	RESEARCH SCIENTIST
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	2
Contribution to Project:	establishing a pipeline and conducting microscopy data analysis and western blotting
Funding Support:	Institute funds from The Salk Institute for Biological Studies

Name:	Ryan Hsu
Project Role:	GRADUATE STUDENT
Researcher Identifier (e.g. ORCID ID):	0000-0001-8221-224X
Nearest person month worked:	10
Contribution to Project:	Mr. Hsu does cloning and assists with in vivo experiments, focusing on building the prime editor REJ system and full-length dystrophin program
Funding Support:	In addition to DOD funding through this grand, Mr. Hsu was funded by Institute funds from The Salk Institute for Biological Studies

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Since this award was negotiated, the other DoD project ended ("MicroRNA Replacement Therapy for ALS Treatment") and three other grants were secured:

Tools for regulated expression control of miR-218; 5% effort; NIH/NINDS
Contact: Allison Bailey, 6001 Executive Boulevard, Suite 3290, MSC 9537, Rockville, MD 20852
05/15/2021-10/31/2022; annual direct
The major goal of this project is to generate and provide motor neuron-specific microRNA (miR-218) reagents to the ALS research community to accelerate the investigation of a promising new candidate for ALS-therapy.
Aim 1. Validation of mouse lines that conditionally express different levels of miR-218
Aim 2. Generation of mES lines that conditionally express miR-218
Overlap: None

MiR-218 regulatory networks in adult mice and its relationship to ALS; 8.33% effort; NIH/NINDS
Contact: Allison Bailey, 6001 Executive Boulevard, Suite 3290, MSC 9537, Rockville, MD 20852
05/15/2021-10/31/2022; annual direct
The major goal of this project is to identify the gene networks controlled by motor neuron-specific microRNA (miR-218) in adult motor neurons using genetics to decrease and elevate miR-218 with precise spatiotemporal control.
Aim 1. Identify the miR-218 gene network in adults and its relationship to ALS
Model miR-218 non-cell-autonomous gene network changes
Overlap: None

Characterization of spinal circuits underlying motor synergy function; 24% effort; NIH/NINDS
Contact: Allison Bailey, 6001 Executive Boulevard, Suite 3290, MSC 9537, Rockville, MD 20852
09/01/2021-08/31/2026; annual direct
The major goal of this project is to unravel the wiring and cellular constituents within motor synergy
circuits, and to examine how these circuits form during embryonic development and early postnatal life.
Aim 1: Create a spinal pre-motor interneuron atlas
Aim 2: Characterize the muscle specific proprioceptive inputs to MSE neurons
Aim 3: Dissect the intrinsic genetic programs that form MSE circuits
Aim 4: Examine the role of sensory feedback in building MSE circuits
Overlap: None

What other organizations were involved as partners?

Nothing to Report.

8. **SPECIAL REPORTING REQUIREMENTS**
   Not applicable
   - **COLLABORATIVE AWARDS:**Not applicable
   - **QUAD CHARTS:** Not applicable

9. **APPENDICES:**

   None