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TITLE: Allele-Specific Protein-Based Therapeutics for Myotrophic Lateral Sclerosis Associated with C9ORF72 Repeat Expansions

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CONTRACTING ORGANIZATION: Enzerna Biosciences, LLC

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14. ABSTRACT:

Enzerna Biosciences is developing a novel treatment approach addressing the underlying disease mechanism of familial ALS. By modifying defective RNA molecules found in the neurons of ALS patients, Enzerna's technology will ameliorate the underlying cell pathologies that lead to neuronal death. As opposed to symptomatic treatment, Enzerna is treating the fundamental disease process to improve patient outcomes. Enzerna's therapeutic will help patients with an inherited form of the disease known as familial ALS. Mutation in the C9orf72 gene is the most common cause of familial ALS (~40% of cases) and is inherited dominantly, meaning only one copy of the gene is needed to cause disease. Patients with C9orf72 mutation also tend to have earlier disease onset and reduced survival. Currently, no disease-modifying treatments are available to these patients. Enzerna's treatment has the potential to change the disease trajectory for these patients by mitigating their underlying neurodegeneration, improving neuron survival and the patient's quality of life. Enzerna intends to develop this therapeutic as an FDA-regulated drug administered directly into the spinal cord. The benefit of our therapeutic is fundamental disease process modification to preserve patients' neurons, mitigating motor symptoms seen in patients. The primary contribution of this study is to develop a novel therapeutic mechanism to treat ALS. Enzerna's technology can provide patient-specific modification to mutant RNA molecules in patients' neurons. This targeted approach has never been attempted for ALS.

15. SUBJECT TERMS

NONE LISTED

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Although the association of expanded arrays of a GGGGCC (hereinafter referred to as G4C2) hexanucleotide sequence in intron 1 of the C9orf72 locus is observed in up to 40% of familial cases of ALS, the molecular pathways adversely impacted by the G4C2 expansion have not been clearly defined. Current hypotheses propose that three mechanisms (**Figure 1, Appendix**), perhaps in combination, lead to progressive neuronal loss: (1) gain of function due the production of truncated RNA transcripts that form nuclear foci that may trap factors needed for RNA metabolism (e.g., RNA binding proteins needed for splicing); (2) dipeptide production (DPR) from aberrant translation of G4C2 sequences in all three reading frames from RNAs transcribed in BOTH the sense and antisense directions (that form inclusion bodies in the cytoplasm that trap the transcription factor TDP-43 which inhibits TDP-43 function); and/or (3) haploinsufficiency due to epigenetic downregulation the major C9orf72 RNA species, V2. While therapies that target each specific mechanism individually show promise, current reviews argue that combinatorial therapies that at least two of the three mechanisms may yield more effective approaches for treating patients affected by ALS associated with G4C2 nucleotide expansions. Enzerna Biosciences is commercializing a novel approach to specifically bind any RNA molecule using **A**rtificial **S**ite-Specific **R**NA **E**ndonuclease**s** (**ASREs**) engineered with customized sequence specificity. ASREs are **human-based** chimeric proteins consisting of an RNA binding domain that specifically recognize different 8-nucleotide RNA sequences fused to an RNA endonuclease domain (**Figure 2, Appendix**). This unique binding mode makes PUF domain a programmable RNA-binding module that can bind to any sequence of choice. We proposed to leverage ASRE technology to create an innovative combinatorial therapy for ALS associated with G4C2 expansions. Specifically, we proposed to develop ASREs that can target two of the mechanisms that lead to the deleterious consequences of the expanded C9orf72 RNA with the goal of largely eliminating: (1) nuclear foci and (2) the truncated sense (G4C2) and antisense (C4G2) repeat containing transcripts that are translated into pathogenic dipeptides. Efficacy will be assessed assays can be a subset of the following: (a) decrease in RNAs that contain the expanded G4C2 sequences; (b) reduction in nuclear foci; (c) decrease in dipeptide levels both sense and antisense ASRE treatment); (d) release of TDP-43 from inclusion bodies (both sense and antisense ASRE treatment); and (e) motor neuron survival.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Nucleotide expansion, RNA editing, gene therapy

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Aim 1. Identify nuclear targeted ASREs that decrease dipeptide levels in ALS patient-derived cells

Aim 1a: Create sense and antisense ASREs targeted to expanded C9orf72 mRNA

- For the sense transcript, ASREs with a nuclear localization signal will be designed against the G4C2 repeat element. For the antisense transcript, gene ASREs with a will be designed against the G2C4 repeat element

Aim 1b: Use transposon-mediated transduction to create patient-derived iPSC or lymphoblast lines that express each candidate ASRE synthesized in Aim 1a

- Screen cultures for level of ASRE expression by Western blotting and confirm nuclear localization via IFC. Screen for targeted knockdown of expanded C9orf72 mRNA in ASRE-expressing cells

Aim 1c. Screen motor neurons derived from each engineered iPSC line for levels of dipeptides generated from sense and antisense RNAs

- Using standard protocols established in the Ichida lab, generate iPSC-derived motor neurons.
- After differentiation, assess levels of dipeptide proteins via ELISA.

Criteria for success: Identify at least one ASRE targeted against the sense and antisense RNAs that decrease dipeptide protein levels by >50%.

Aim 2. Create and assess efficacy of combinatorial treatment in patient derived iPSCs

- Patient-derived and non-affected iPSCs will be transduced with two ASREs (sense and antisense specific ASRE) to create stable ASRE-expressing lines
- iPSCs (transduced and non-transduced cells) will be differentiated into motor neurons for analysis: presence of C9orf72 nuclear foci via FISH, dipeptide levels via ELISA, release of TDP-43 from inclusion bodies, and restoration of "non-affected"-like expression profile in ASRE-transduced patient derived cells and analysis of ASRE-transduced non-affected cells for off-target effects on gene expression via RNAseq

Criteria for success: >10-fold reduction in nuclear foci in ASRE-treated patient-derived cells; changes in RNA expression in ASRE-treated cells that indicate a restoration to a "normal" RNA expression profile; <10% difference in RNA expression profiles in ASRE-treated non-affected cells.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Major Activities: We conducted the studies in Aims 1a and 6 outlined above. However, we observed that the ASRE candidates did not drive knockdown of the expanded RNAs. We therefore had to take a step back and determine why the ASREs were ineffective. Thus, we could not proceed to any of the subsequent proposed studies (Aim 1c and Aim 2) until we resolved the issue of ineffective ASRE activity.

Please keep the following two paragraphs . To assess binding efficiencies, we developed an RNA immunoprecipitation (RIP) assay – we discovered the RNA binding domain (PUF domain, see Figure 2) used in our studies could not immunoprecipitate the RNA target. Using a number of protein folding analysis programs, we observed that the "backbone" of the PUF (i.e., the left side of the PUF domain, see Figure 2) was predicted generate a domain with a low affinity to the RNA target. We then used Artificial Intelligence (AI) to create a backbone that would predict a higher affinity PUF binding domain. We choose to target the MS2 RNA target sequence because a natural protein MCP can bind to and be used to immunoprecipitated RNAs with the MS2 sequence – we sought to identify AI-generate PUF domains that can bind to and be used to immunoprecipitated RNAs with the MS2 sequence with better efficacy than the original PUF domain. The PUF code was modified to recognize the MS2 sequence.

As shown in Figure 3, Appendix, ~50% of input RNA (containing the MS2 target sequence) can be captured (immunoprecipitated with MCP); ~20% of input RNA can be captured by AI-generated PUF candidate 4t4. In contrast, the original PUF capture 0% of the target; another A)-candidate 4t3 also could not capture the target RNA. The ability of 4t4 to capture RNAs was reproducible and validates using an independent assay using a different RNA that contains the MS2 target sequence. These data have led to the creation of a new PUF backbone that has now been modified to recognize the G4C2 repeat so that we can proceed with Aim 1b, c and Aim 2 in the next report period.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased

knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We have now created RNA Binding Domains of the ASRE fusion protein that can be to be used to immunoprecipitate RNAs that contain the target sequences recognized by the RNA Binding Domain. Now that we have developed a new generation ASRE with higher affinity to the target RNA sequence, we have synthesized new generation ASREs against the G4C2 (sense) and C4G2 (anti-sense) repeats. We will also test the ability of the RNA binding domain alone (i.e., containing no endonuclease) to achieve the major goals outlined above. Given that the ASOs show promise as therapeutics for ALS associated C9orf72 G4C2 repeat expansions, we will test the hypothesis that binding of our new generation RNA binding domains represent a new therapeutic modality.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to report.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

No changes to objectives to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

We encountered one delay and one problem which were both addressed: Delay: Dr. Justin Ichida, academic subcontractor from USC, informed us in late 2022 that his lab would not have the bandwidth to participate in this project. Since all personnel at Enzerna (except Dr. Sazani) have experience in iPSC technology, Enzerna had the staff and expertise to conduct all the proposed aims in-house so we requested that the subaward tasks and budget be re-allocated to the Enzerna budget. While we did receive approval for our request, it was not granted until March 2023 and therefore we had a 4 month delay in the pursuing all of our proposed studies. However, we were granted a one-year no cost extension for this project. Problem:As outlined above, we observed in Aim 1 studies, that the ASRS candidates synthesized for this aim did not result in target knockdown (degradation) of the expanded C9of 72 RNA. We discovered that the binding affinity of the original RNA binding domain in our ASRE therapeutic to the was suboptimal which likely led to inability of ASREs to degrade the target expanded RNAs effectively. As outlined above, we have resolve this issue by using AI-generated RNA binding domains to dramatically improve the affinity of the RNA binding domain the target RNA sequence. We now are ready to proceed with the original studies outlined in the major goals section.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

Not applicable.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

  Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or Techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*

- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name:	*Joseph Ruiz*
Project Role:	*PI*
Nearest person month worked:	*4.8*
Contribution to Project:	*Dr. Ruiz provided general oversight to the project and conducted cell transduction studies and phenotypic analyses*

Name:	*Pete Sazani*
Project Role:	*co-investigator*
Nearest person month worked:	*2.4*
Contribution to Project:	*Dr. Sazani oversaw day-day research operations in the lab and analyzed data*

Name:	*Yuvabharath Kondaveeti*
Project Role:	*Senior Scientist*
Nearest person month worked:	*6*

Contribution to Project:	*Dr. Kondaveeti conducted all the iPSC studies and oversaw the technical research staff studies*
Name:	*Marcus Williams*
Project Role:	*Research Scientist*
Nearest person month worked:	*3*
Contribution to Project:	*Mr. Willams assisted with the iPSC studies and redesign of the RNA Binding domain of ASRE*
Name:	*Austin Marty*
Project Role:	*Research Scientisi*
Nearest person month worked:	*12*
Contribution to Project:	*Mr. Marty was the major researcher involved in the studies who generated the original ASREs, conducted cell transduction and phenotypic studies, and develop AI-based strategies to create a new generation of RNA binding domains to incorporate into ASREs*
Name:	*Julia Bay*
Project Role:	*Reseearch Scientist*
Nearest person month worked:	*3*
Contribution to Project:	*Ms. Bay assisted with redesign of the RNA Binding domain of ASRE*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
<u>Organization Name:</u>
<u>Location of Organization: (if foreign location list country)</u>
<u>Partner's contribution to the project</u> (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *N/A*

QUAD CHARTS: N/A

9. APPENDICES:

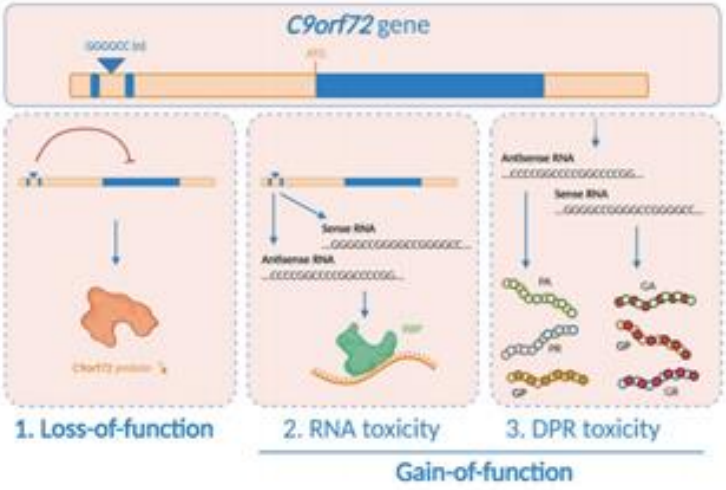

Figure 1. Proposed mechanisms for deleterious consequences of G4C2 expansions. Adapted from Braems et al. *C9orf72 loss-of-function: a trivial, stand-alone or additive mechanism in C9 ALS/FTD?* Acta Neuropathol, 2020. **140**(5): p. 625-643.

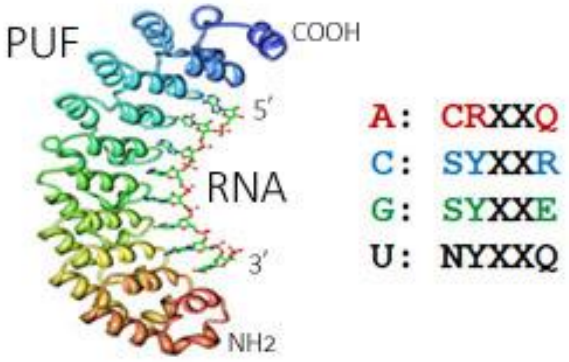

Figure 2. Overview of PUF domain binding code. PUFs bind the RNA target in a 3' to 5' direction relative to its amino terminus. Dong, S., et al., *Specific and modular binding code for cytosine recognition in Pumilio/FBF (PUF) RNA-binding domains.* J Biol Chem, 2011. **286**(30): p. 26732-42.

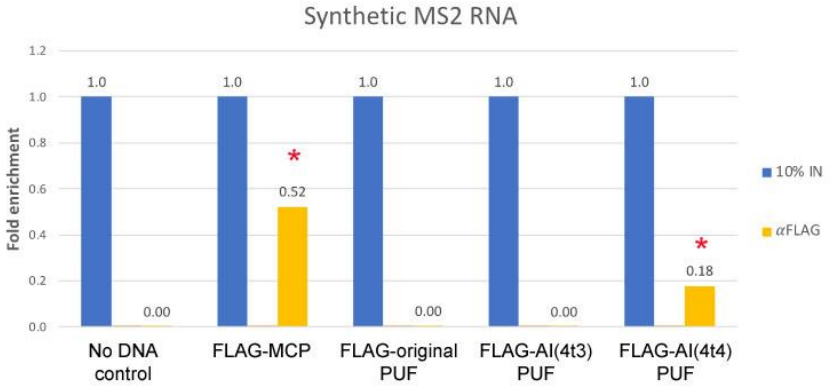

Figure 3. RIP using biotinylated MS2 RNA spiked into cell lysates. Blue Bar: input; Yellow Bar: RNA captured by Binding Protein after immunoprecipitation with anti-FLAG antibodies. MCP pulls down 52% of input RNA; AI-PUF (4t4) pulls down 18%; Original PUF pulled down 0% of input RNA; AI-PUF (4t3) pulls down 0%. AI-PUF(4t4) will be used as the foundation for all future PUFs (the amino acids that create the site specific RNA contacts shown Figure 2 have been incorporated into AI (4t4) to create the PUF domains outlined in Aim 1a. Please keep these data .