Cas13d Multi-Targeting Efficiently Targets Sense and Antisense HRE Containing Toxic RNAs and Poly-GP DPR in C9ALS Patient Cells and in C9-BAC500 Mouse Model

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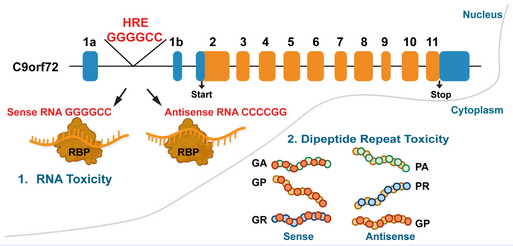
Introduction

- Amyotrophic lateral sclerosis (ALS) is a progressive, fatal, neurodegenerative disorder characterized by loss of upper and lower motor neurons, leading to muscle weakness, atrophy and eventual respiratory failure within 2-5 years from diagnosis.
- The C9ORF72 mutation is the most common genetic cause of ALS (C9ALS), and it is caused by a G4C2 hexanucleotide repeat expansion (HRE) in the first intron of *C9ORF72* gene (most patients will carry several 100s to 1000s repeats)[1].
- Bidirectional transcription produces both sense (G4C2) and antisense (C4G2) HRE-containing RNAs, which form nuclear RNA foci and sequester RNA binding proteins, disrupting RNA homeostasis[2,3].
- These HRE RNAs are also translated by repeat associated non-ATG (RAN) translation to dipeptide repeat proteins (DPR) that tend to aggregate leading to toxicity specially in motor neurons[4].
- To date there is no cure or effective treatments that can slow down disease progression for C9ALS patients.

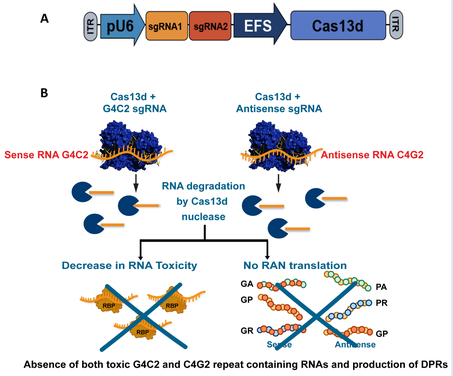


1. RNA Toxicity
Secondary RNA structures sequester RNA binding proteins (RBPs) affecting RNA homeostasis

2. Dipeptide Repeat Toxicity
Repeat-associated non-ATG (RAN) translation forms toxic dipeptide repeat proteins from sense and antisense toxic RNAs

Locanabio's Cas13d-Multi-Targeting Strategy

- CRISPR/Cas13d is a type VI programmable nuclease with the ability to process guide-RNA arrays and target multiple RNAs for degradation.
- We developed a multi-targeting (MT) Cas13d construct with 2 sgRNAs to target sense (G4C2) and antisense (C4G2) C9ORF72 transcripts and packaged it in a single AAV9 vector (**A**).
- The Cas13d-MT construct degrades both C9ORF72 sense and antisense pathological RNA transcripts decreasing RNA toxicity as well as reducing production of toxic DPRs (**B**).

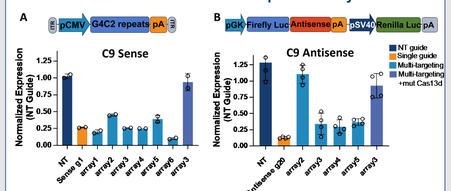


Absence of both toxic G4C2 and C4G2 repeat containing RNAs and production of DPRs

Results

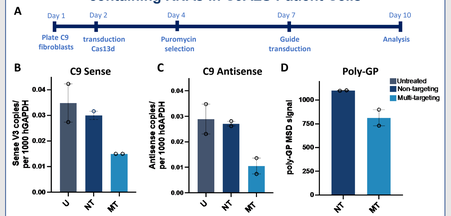
in vitro

Multiple Cas13d-MT Arrays Efficiently Knockdown C9 Sense and Antisense RNAs in Reporter Assays



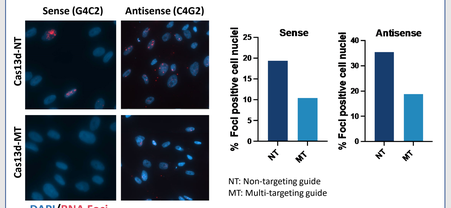
(**A**) Sense RNA expression determined by qRT-PCR (reporter expresses 80 G4C2 repeats). (**B**) Antisense expression determined by luminescence (reporter contains 800 nucleotides of the antisense strand). Non-targeting (NT) guide (dark blue), single-targeting guide (orange) or multi-targeting (MT, light blue). Purple bar refers to catalytic dead mutant Cas13d.

Cas13d-MT Efficiently Targets Sense and Antisense HRE containing RNAs in C9ALS Patient Cells



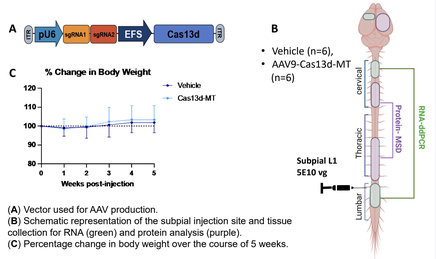
(**A**) Transduction timeline. ddPCR RNA quantification for C9 sense (**B**) or antisense (**C**) pathological transcripts. (**D**) Poly-GP DPR protein levels determined by MSD (MesoScale Discovery) assay.

Cas13d-MT Decreases Sense and Antisense RNA Foci in C9ALS Patient Cells



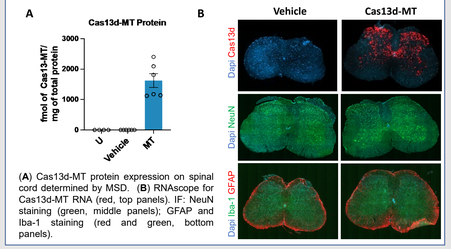
in vivo - Spinal Cord

Subpial Injection of AAV9-Cas13d-MT in C9-500 Mouse Model



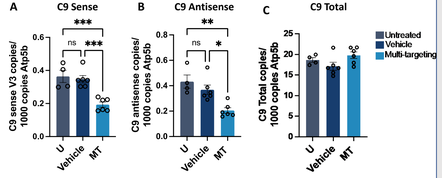
(**A**) Vector used for AAV production.
(**B**) Schematic representation of the subpial injection site and tissue collection for RNA (green) and protein analysis (purple).
(**C**) Percentage change in body weight over the course of 5 weeks.

Robust Cas13d-MT Transgene Expression with no Toxicity in Spinal Cord after 5 Weeks Post-Injection



(**A**) Cas13d-MT protein expression on spinal cord determined by MSD. (**B**) RNAscope for Cas13d-MT RNA (red, top panels). IF: NeuN staining (green, middle panels); GFAP and Iba-1 staining (red and green, bottom panels).

Cas13d-MT Significantly Decreases Toxic Sense and Antisense HRE containing RNAs Post-Subpial Delivery

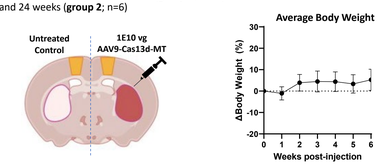


Expression of pathological transcripts, sense (**A**) and antisense (**B**) and normal isoform (C9 total, **C**) in lumbar spinal cord of C9-500 mice determined by ddPCR. Significant differences ($p \leq 0.05$) determined by one-way ANOVA, Tukey.

in vivo - Brain (intrastriatal)

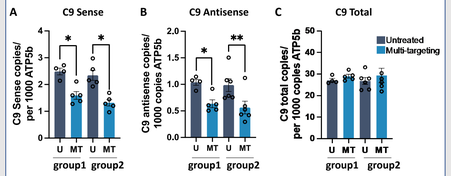
AAV9-Cas13d-MT Delivered Intrastriatal is Safe and Well Tolerated in C9-500 Mouse Model

- Treated 2 age groups: at 17 weeks (**group 1**; n=10) and 24 weeks (**group 2**; n=6)



- No decline in body weight observed over 6 weeks of treatment.
- Robust transgene expression and no signs of neuronal loss and inflammation observed.

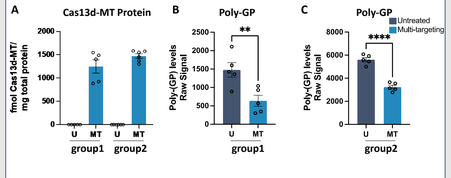
Cas13d-MT Significantly Decreases Toxic Sense and Antisense HRE containing RNAs Post Intrastriatal Delivery



group1: treated at 17 weeks
group2: treated at 24 weeks

Expression of pathological transcripts, C9 sense (**A**) and antisense (**B**) as well as non-pathological isoform (C9 total, **C**) determined by ddPCR. Significant differences ($p \leq 0.05$) determined by t-test.

Cas13d-MT Significantly Decreases Toxic Poly-GP DPR 6 Weeks Post Intrastriatal Delivery



Cas13d-MT protein expression (**A**) and Poly-GP DPR levels (**B/C**) determined by MSD. Significant differences ($p \leq 0.05$) determined by t-test.

Conclusions

- We developed a Cas13d-MT strategy to target both sense and antisense C9ORF72 toxic transcripts.
- Cas13d-MT is compact and was efficiently packaged into a single AAV vector.
- Reporter assays enable selection of best guide array combinations to target both toxic transcripts.
- Our Cas13d-MT efficiently knocks down both sense and antisense HRE-containing toxic RNAs, and decreases RNA foci in C9ALS patient fibroblasts.
- Subpial and intrastriatal delivery of AAV9-Cas13d-MT in C9-500 mouse model show no signs of toxicity and exhibit robust transgene expression.
- Significant decrease of sense and antisense HRE RNAs was observed *in vivo* in both the spinal cord and the brain, with maintenance of total non-pathological C9 RNA levels, crucial to avoid exacerbation of haploinsufficiency. This highlights the specificity of our Cas13d-MT for the pathological C9ORF72 toxic transcripts.
- Cas13d-MT significantly decreased poly-GP DPR peptides in mouse brain over 6 weeks of treatment.
- Targeting toxic sense and antisense HRE sequences with a single Cas13d AAV vector could be an effective therapeutic strategy for C9ALS as well as C9ORF72 associated FTD.

Acknowledgements

This study is supported by Locanabio, Inc. We thank the investors and colleagues that provided support for this study.

References

1. DeJesus-Hernandez M, Mackenzie IR, Boeve BF, Boxer AL, Baker M, Rutherford NJ, et al. Expanded GGGGCC hexanucleotide repeat in noncoding region of *C9ORF72* causes chromosome 9p-linked FTD and ALS. *Neuron* 2011; 72, 245–256.
2. Xu Z, Poidevin M, Li X, Li Y, Shu L, Nelson DL, et al. Expanded GGGGCC repeat RNA associated with amyotrophic lateral sclerosis and frontotemporal dementia causes neurodegeneration. *Proc. Natl. Acad. Sci. U.S.A.* 2013; 110, 7778–7783.
3. Chew J, Gendron TF, Prudencio M, Sasaguri H, Zhang YJ, Castanedes-Casey M, et al. C9ORF72 repeat expansions in mice cause TDP-43 pathology, neuronal loss, and behavioral deficits. *Science* 2015; 348, 1151–1154.
4. Cook CN, Wu Y, Odeh HM, Gendron TF, Jansen-West K, Del Rosso G, et al. C9orf72 poly (GR) aggregation induces TDP-43 proteinopathy. *Sci. Transl. Med.* 2020; 12 (559).

Presented at: **ASGCT 26th Annual Meeting | May 16-20, 2023**