RNA-targeting Cas Systems Correct Molecular and Physiological Features of Myotonic Dystrophy Type I

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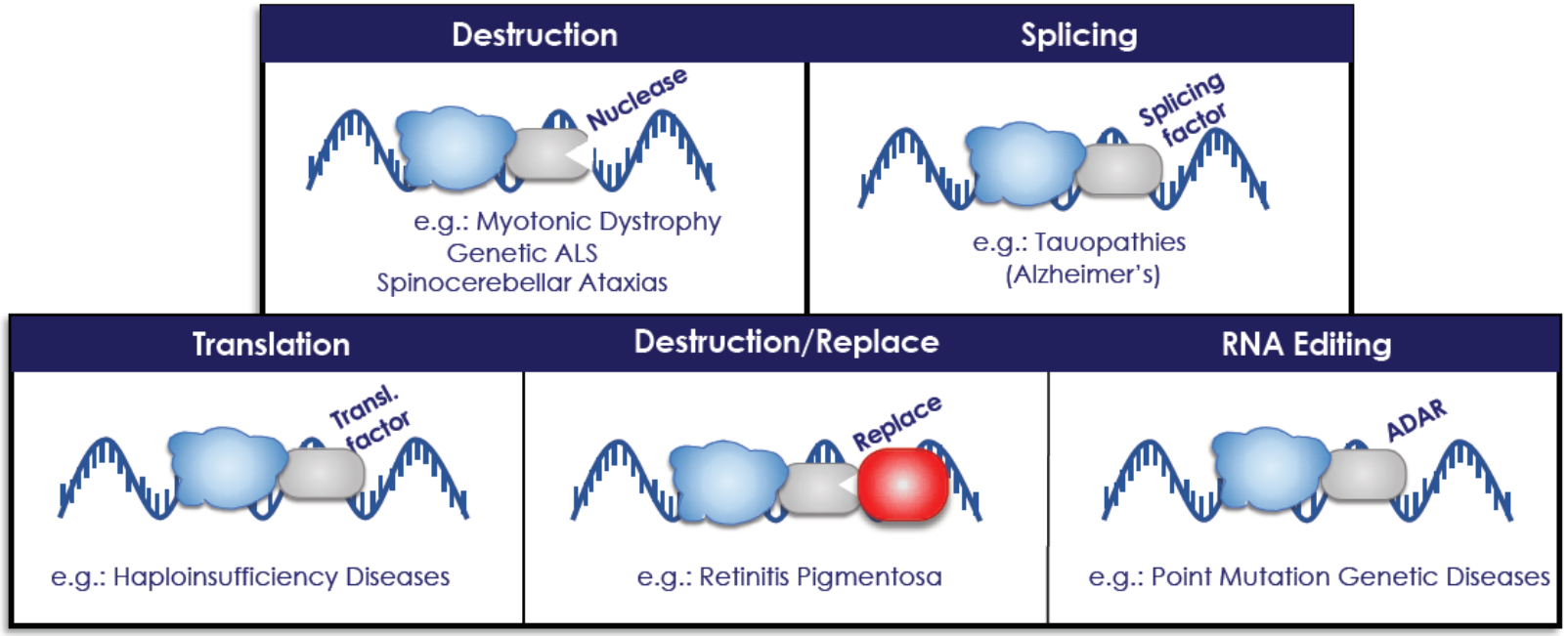
Introduction

Locana's RNA Targeting Platform

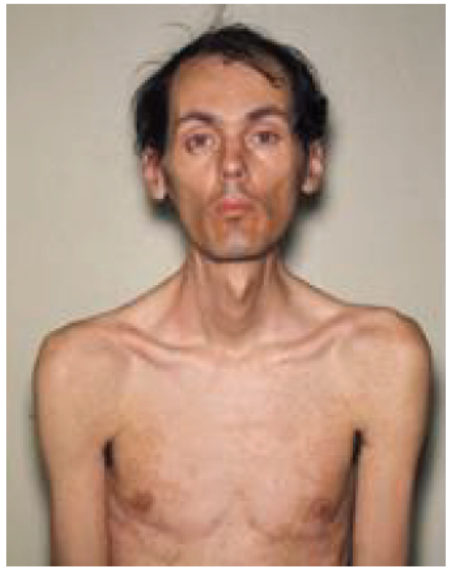


• Locana has families of RNA binding proteins and a menu of effector domains, which combined present an array of options to address human diseases.

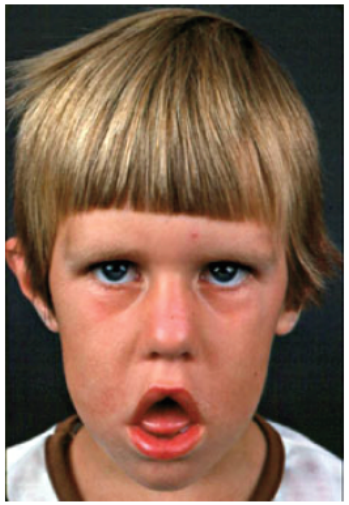
• Using different effector modules, the system can be built to knockdown toxic RNA, upregulate mRNA translation, alter splicing patterns, edit RNA, and knockdown mutant RNA while simultaneously replacing with a normal copy.

• In the current study, RNA Targeting Cas (RCas) systems were evaluated for their knockdown potential for disease related RNAs

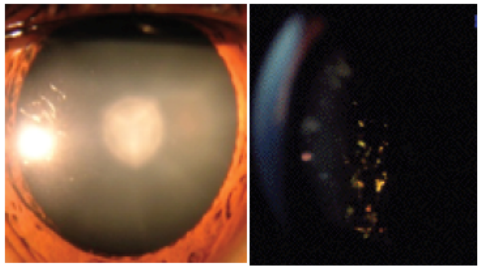
Myotonic Dystrophy is a Multisystemic Disease



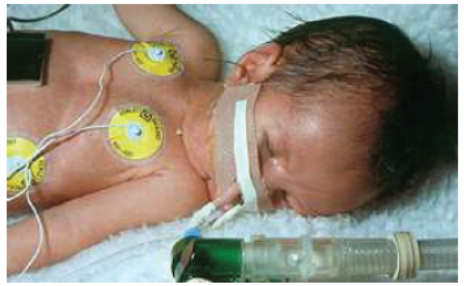
Myotonia
Muscular Atrophy



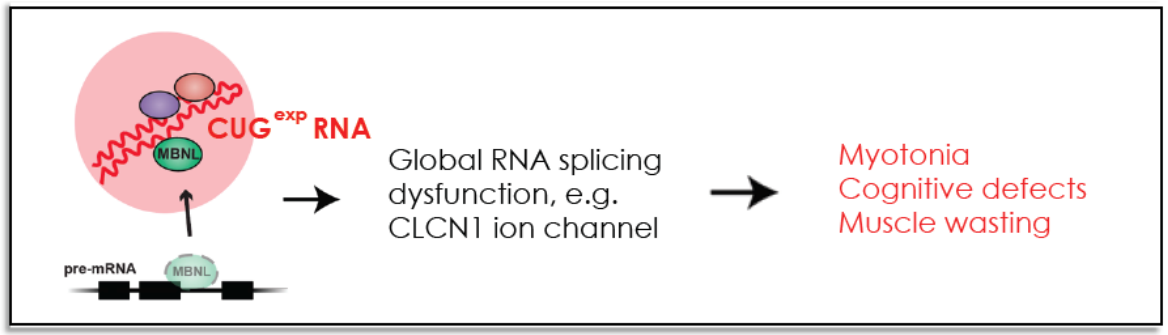
Facial muscle weakness
Developmental delays in brain function



Cataracts



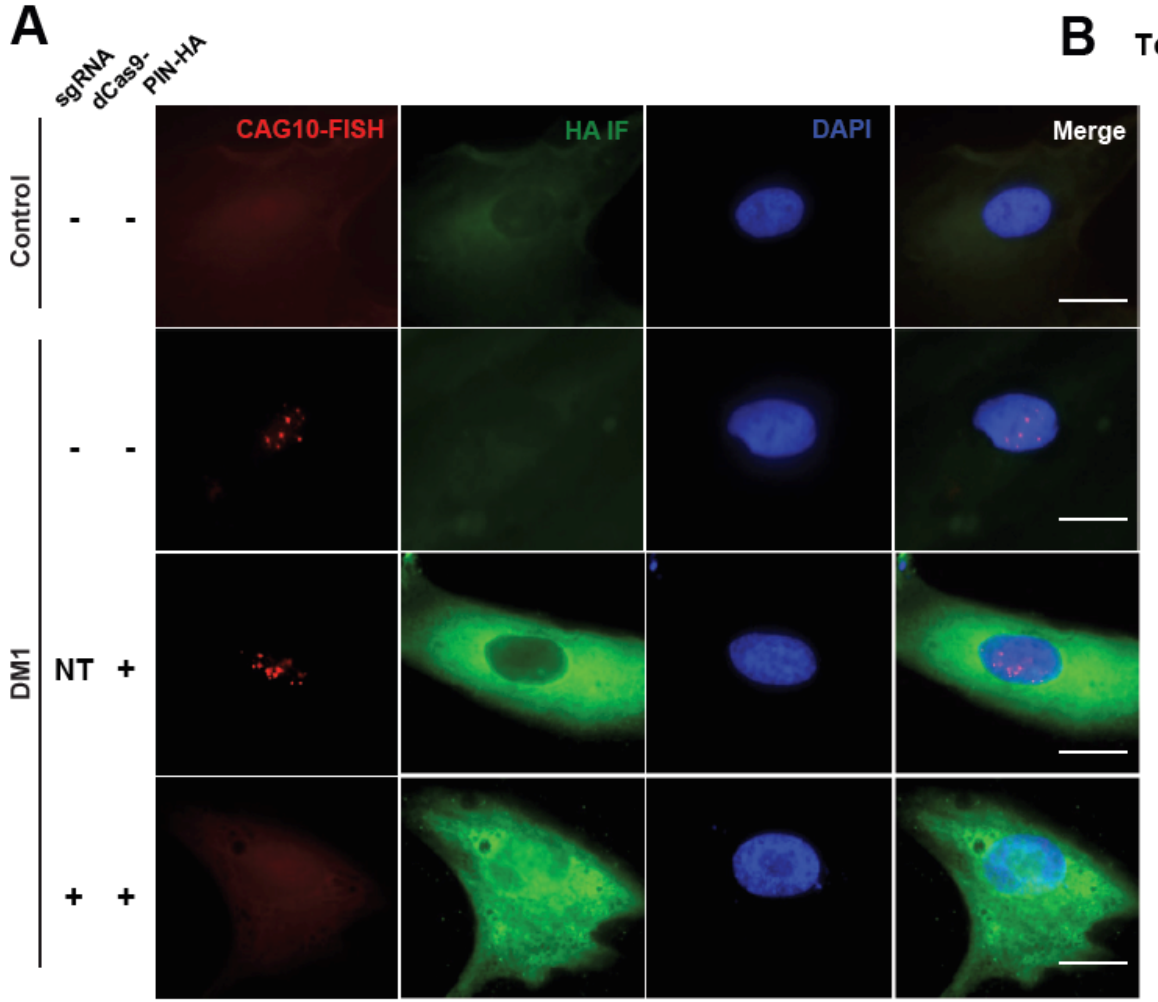
Congenital Myotonic Dystrophy

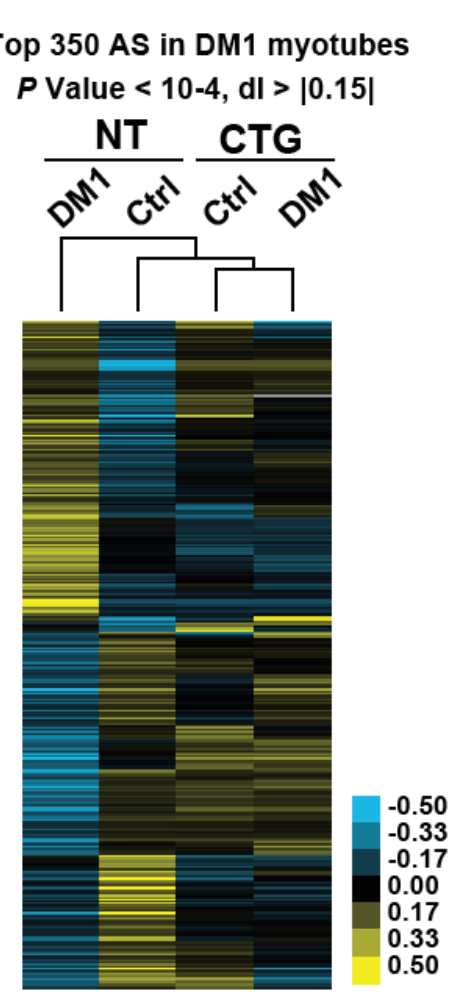


Myotonic Dystrophy Etiology

- Myotonic Dystrophy (DM1) is caused by a CTG repeat expansion in the 3' untranslated region of the DMPK gene.
- RNA transcripts containing the CUG repeat expansions sequester muscleblind-like (MBNL) proteins which are the regulators of the alternative splicing switch from fetal to adult isoforms.
- Loss of MBNL proteins in different tissues result in alternative splicing dysregulation which are the major cause of symptoms of DM1.
- Targeting and Eliminating expanded CUG repeats is a therapeutic strategy for DM1.

RCas9 Corrects DM1 Pathology in Patient Muscle Cells





Study Design and Results

- Primary myoblasts were isolated from bicep biopsies from DM1 patients and controls.
- RCas9 system targeting CUG repeats was introduced using Lentivirus.

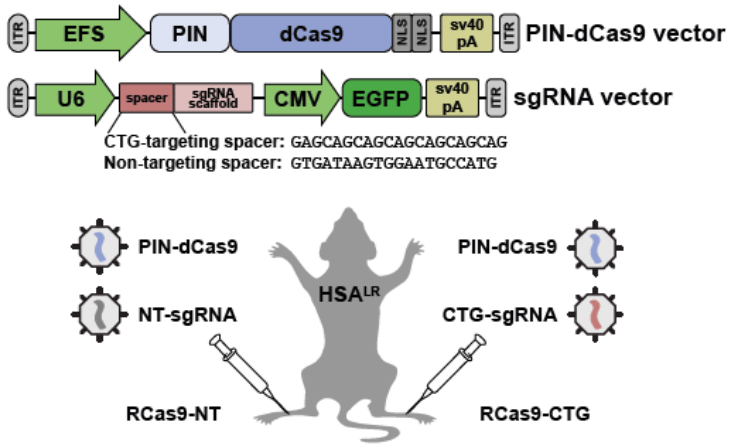
(A) RNA FISH was performed to image RNA-foci typical of DM1 and IF was used to visulaize Cas9 tagged with an HA tag. RCas9-CTG eliminated CUG repeats.

(B) Myoblasts differentiated into myotubes were subjected to RNA-seq and DM1-related alternative splicing (AS) changes were hierarchically clustered.

Intramuscular AAV9-RCas9 Eliminates CUG Repeat Foci and Reverses Hallmark Splicing Defects in Adult DM1 Skeletal Muscle

Study Design: HSA_{LR} mouse model of DM1 expresses ~250 CTG repeats under the control of human skeletal actin (ACTA1) promoter. Non-Targeting RCas9 (RCas9-NT) and CUG-Targeting RCas9 (RCas9-CTG) were injected in contralateral tibialis anterior (TA) muscles of HSA_{LR} mice. The RCas9 system was delivered with two AAV9 vectors ($2.5*10^{10}$ vg each; schematic A) containing Cas9 linked with the SMG6 RNA endonuclease PIN and CUG targeting single guide RNA (sgRNA) and GFP, respectively. Muscles were examined 4 weeks post injection.

A



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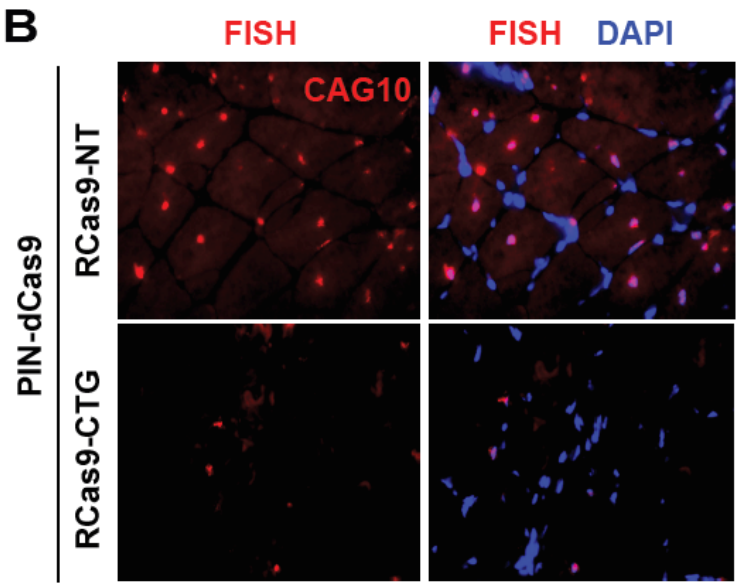

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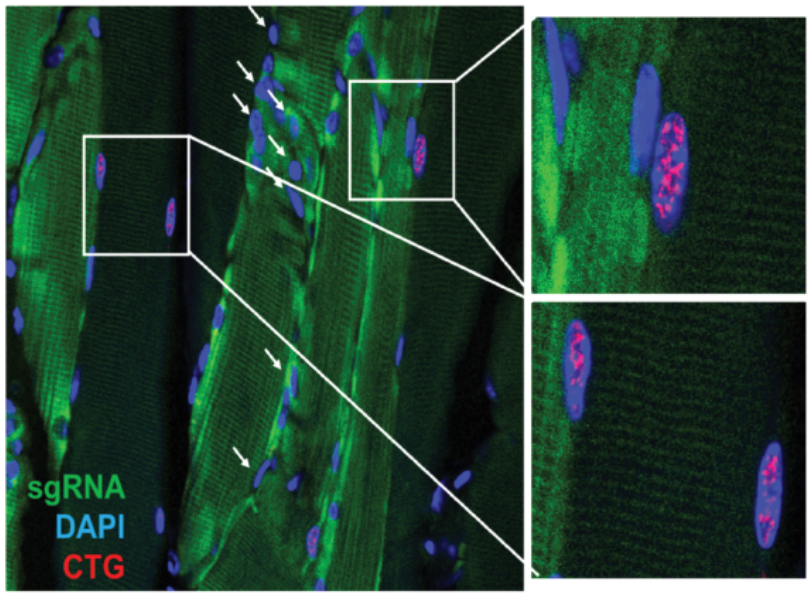

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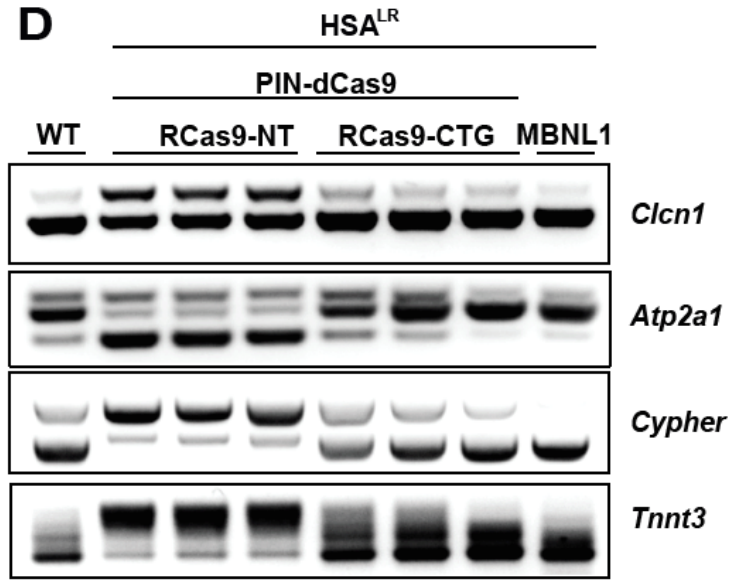
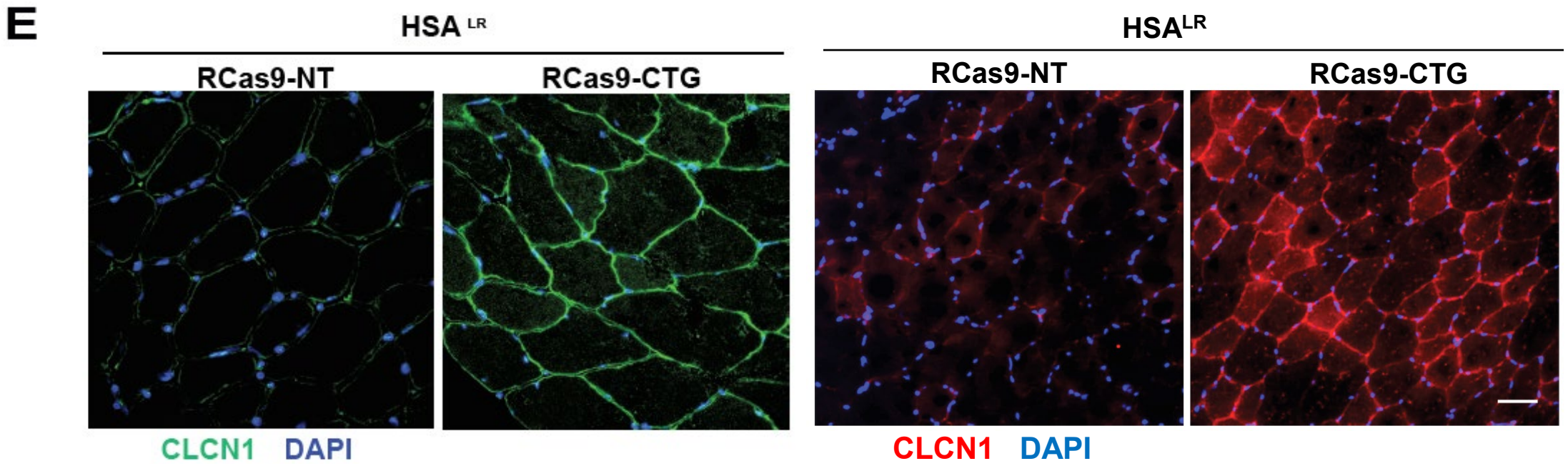
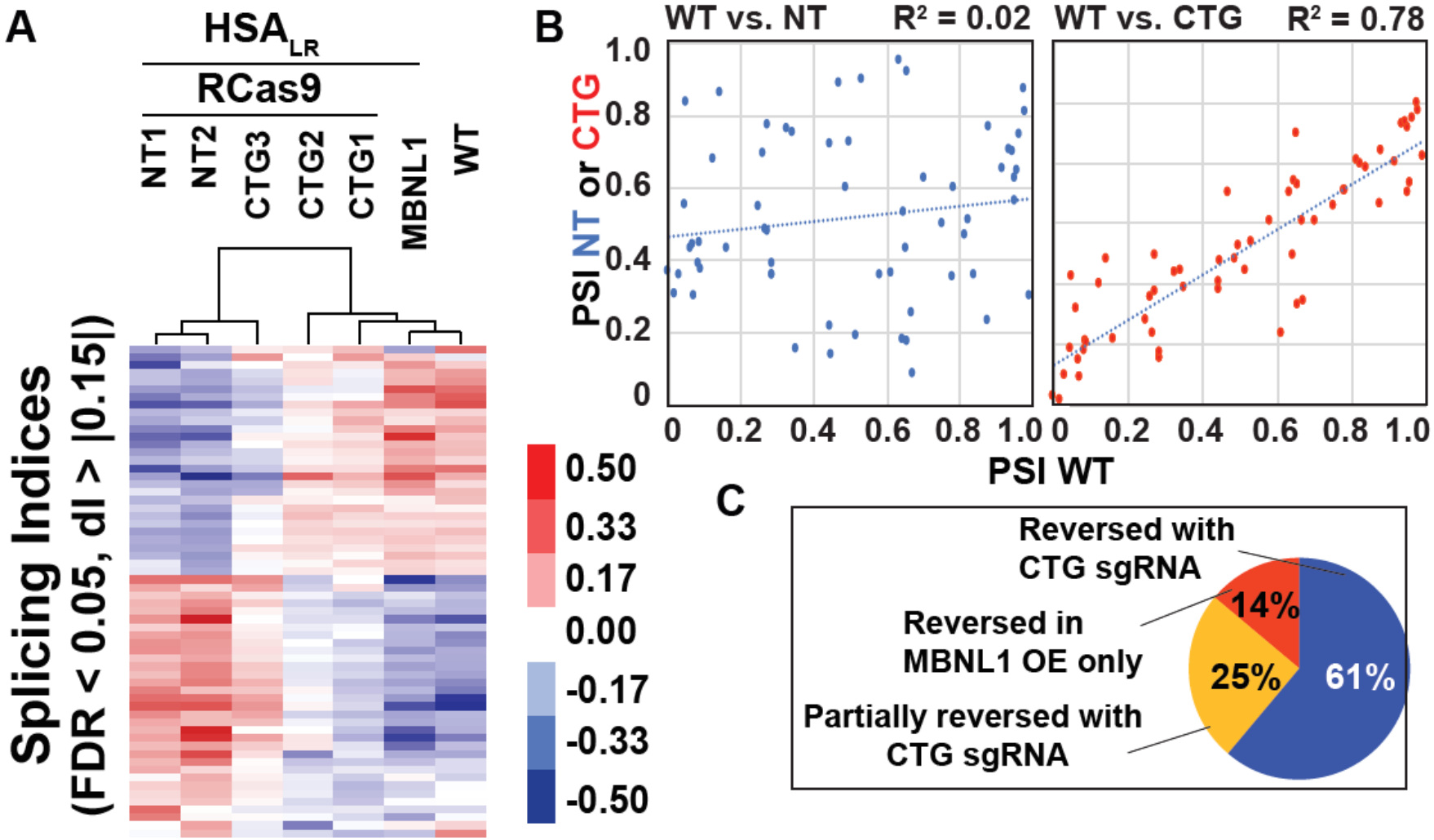


Intramuscular RCas9 Corrects CLCN1 Expression in DM1 Mice



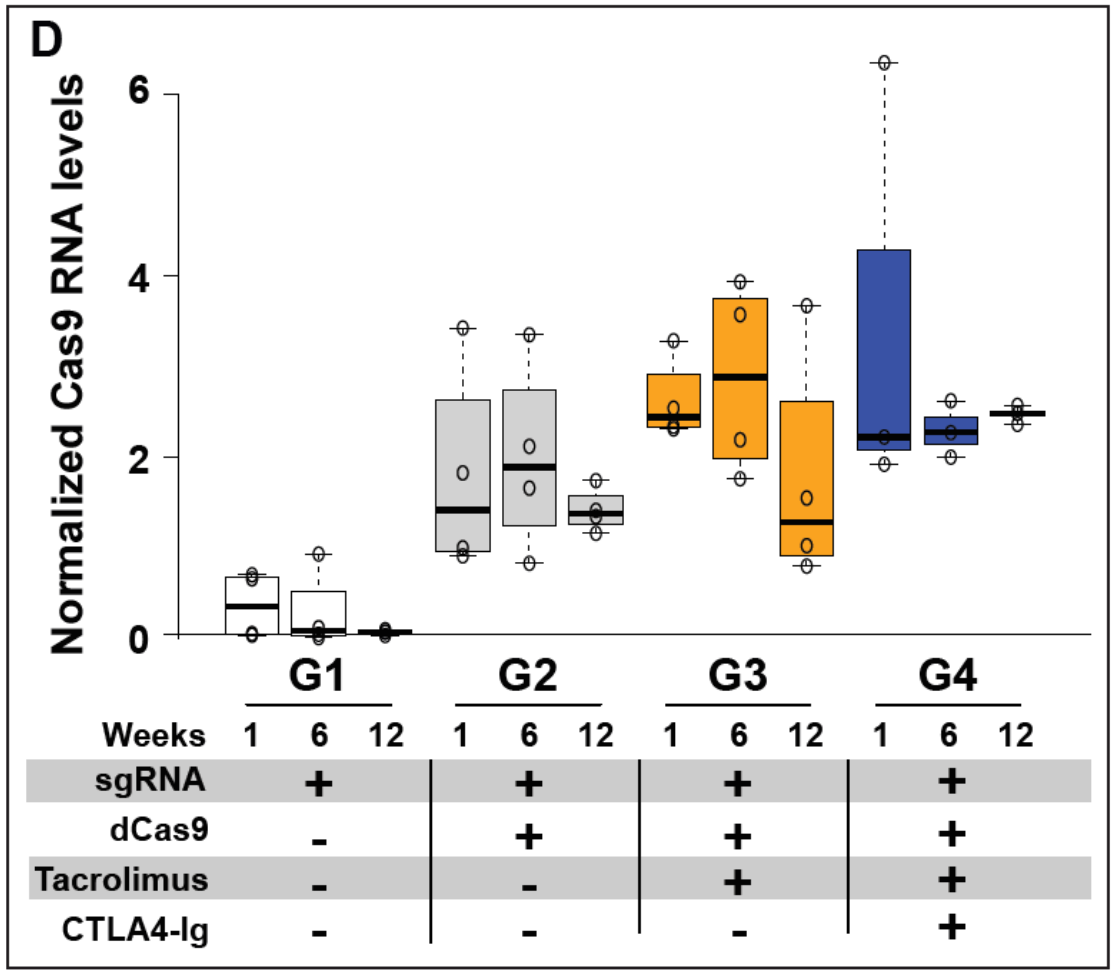
Results: (A) Schematic of Intramuscular Injection of RCas9-NT and RCas9-CTG into contralateral TA muscles. (B) RNA-FISH of contralateral TA muscles show elimination of toxic CUG RNA-foci with RCas9-CTG treatment. (C) Longitudnal section of TA shows that transduced fibers expressing RCas9-CTG (green) show clearance of CUG RNA foci whereas non-transduced fibers (dark) retain CUG RNA foci. (D) Reversal of DM1-related missplicing in RCas9-CTG treated muscle. Re-constituion of Chloride channel CLCN1 staining on RCas9-CTG treatment.

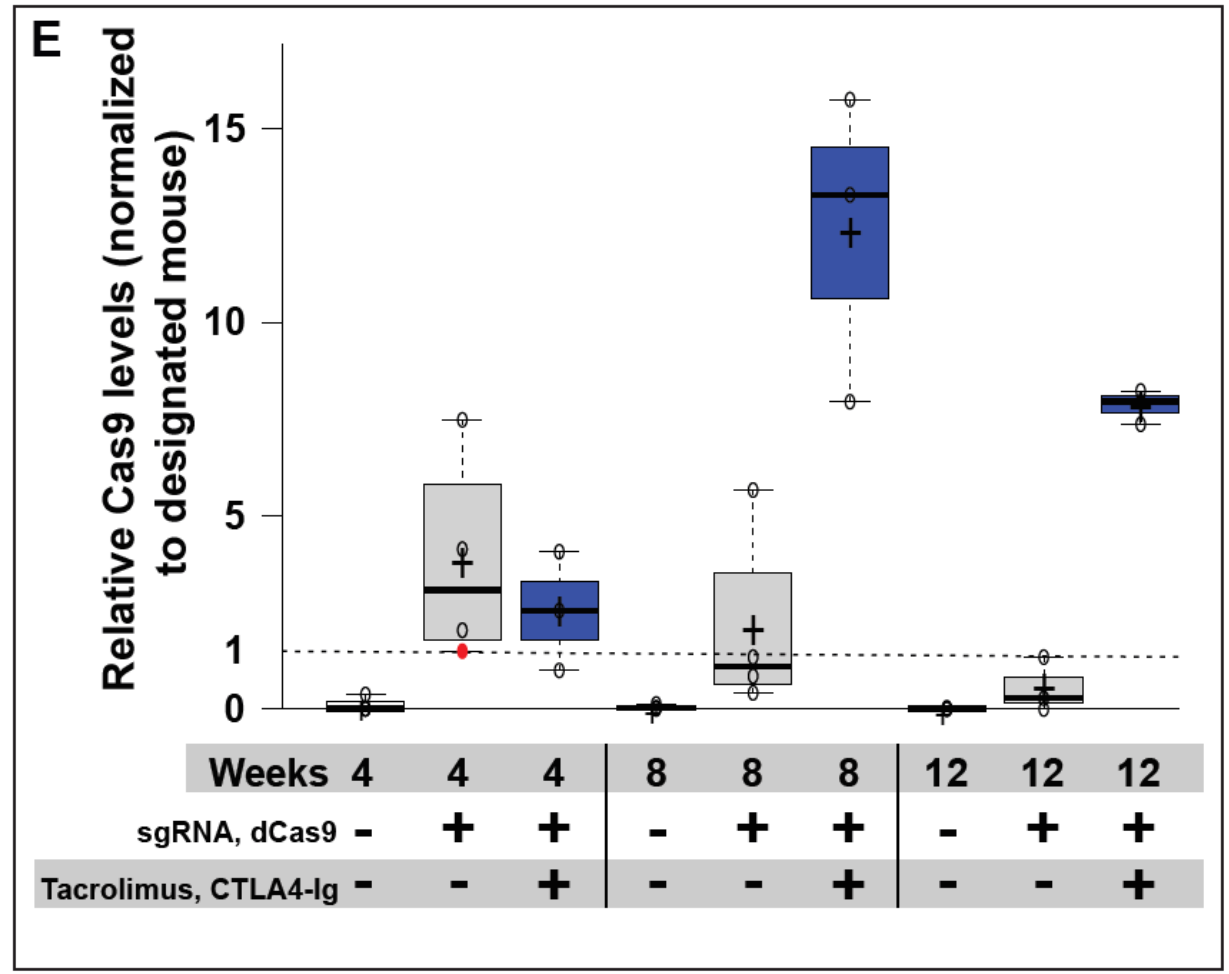
RCas9 Reverses Transcriptome-wide DM1-Associated Missplicing



Results: (A) Hierarchical Clustering of exon splicing indices (percent spliced in or PSI) of HSA_{LR} TA treated with either RCas9-NT (NT), RCas9-CTG (CTG) or MBNL1 (positive contrrol). RCas9-CTG treated muscle clusters with MBNL1 and WT groups. (B) Scatter plots of PSI values of wildtype (WT) vs. NT (HSA_{LR}) or WT vs. CTG (HSA_{LR}) shows marked reduction of splicing defects after RCas9-CTG (CTG) treatment. (C) ~86% of top 350 DM1-related AS changes show partial reversal (25%) or complete reversal (61%) of splicing defects.

Transient Immunosuppression Tolerizes RCas9-CTG Expression

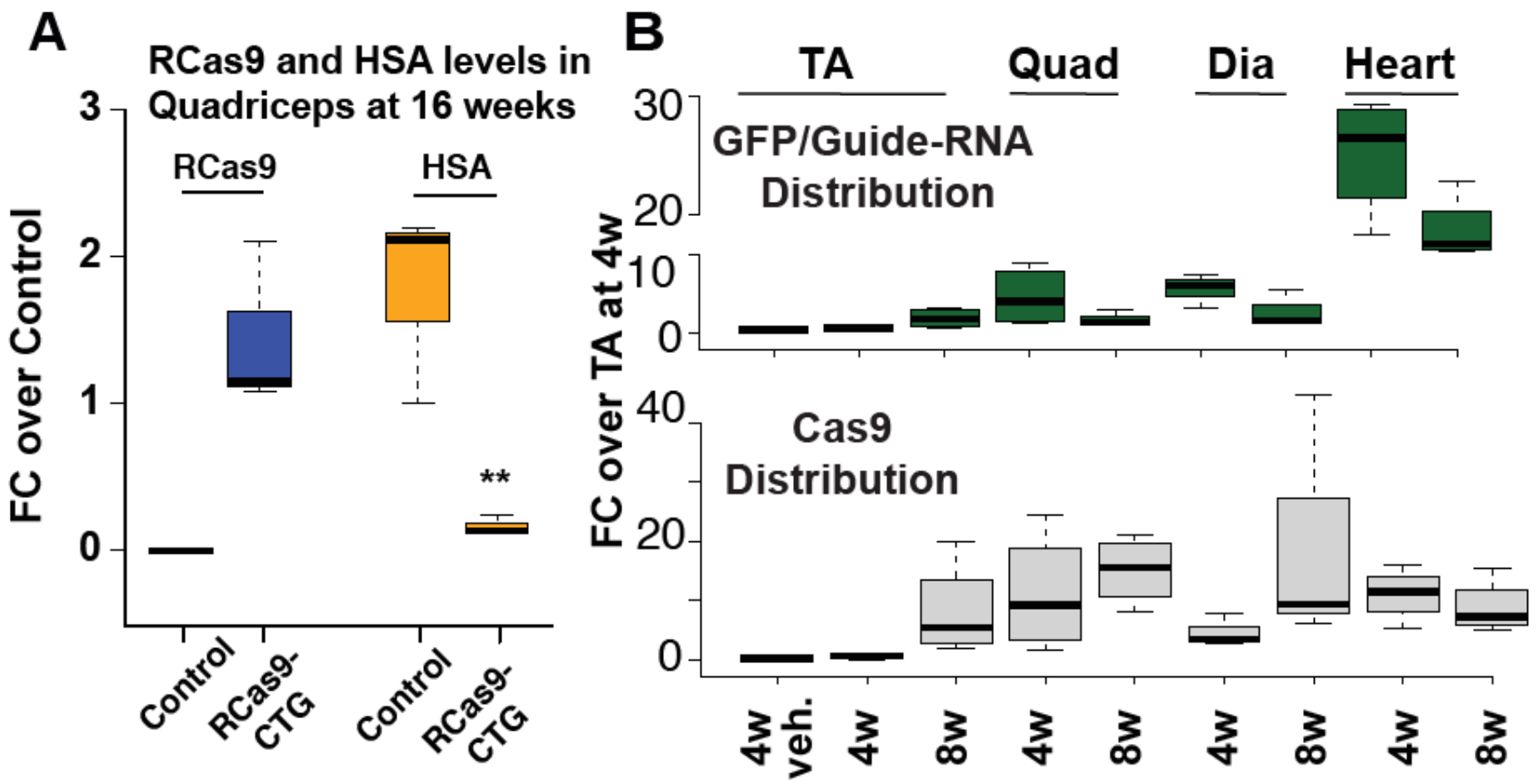




Results: (D) RCas9 levels were sustained in wildtype mice after TA injection for >12 weeks with or without immunosuppression with Tacrolimus and CTLA4-Ig (T-cell pathway). (E) RCas9 levels were sustained for >12 weeks post IM injection with immunosuppresion with Tacrolimus and CTLA4-Ig but not without immunosuppression.

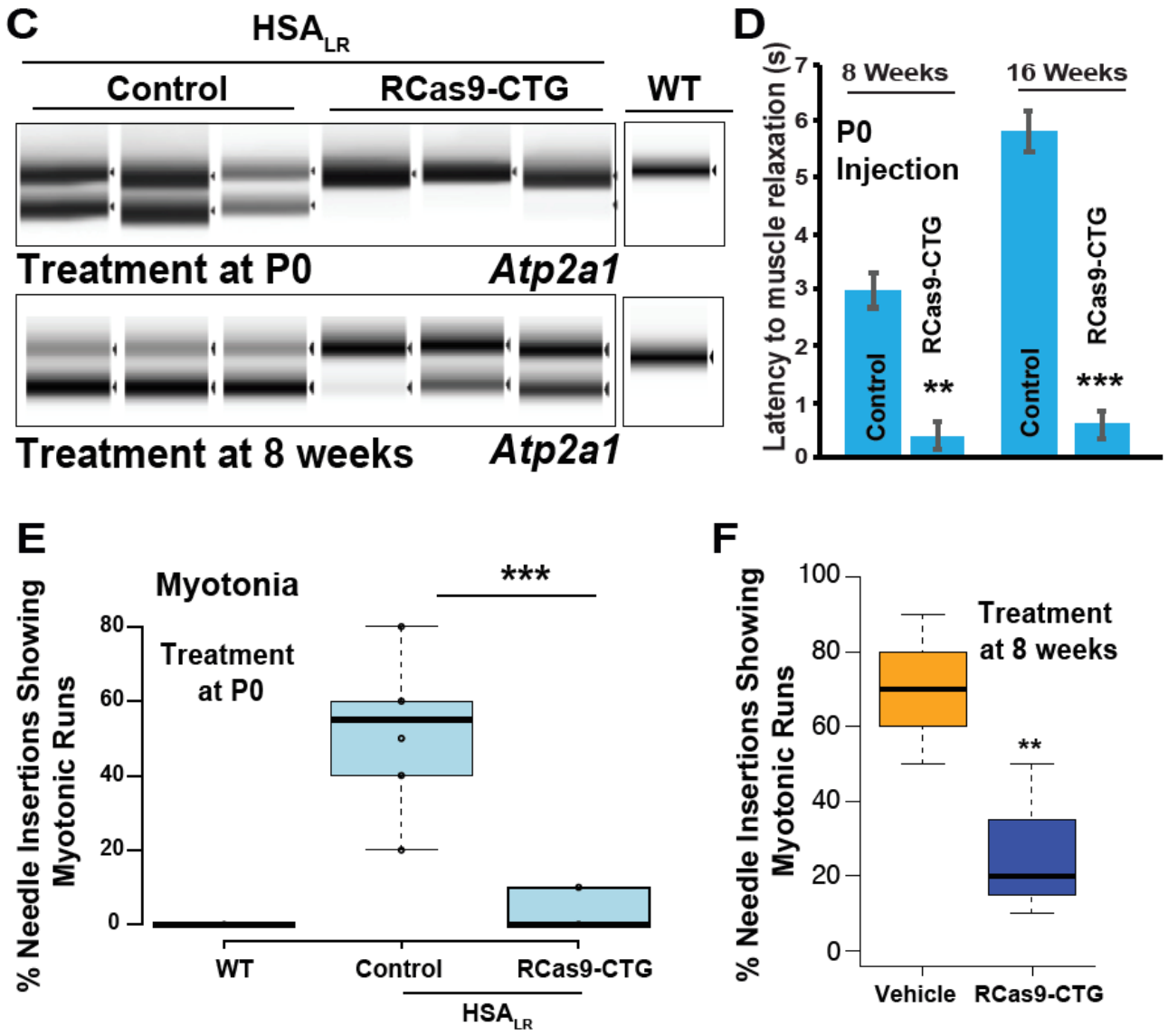
Systemic RCas9 Corrects DM1-related Abnormalities

Study Design: Either P0 neonatal or adult HSALR mice were injected with either vehilce (control) or RCas9-CTG systemically in the temporal vein (1011 vg) or lateral tail vein (5*1011 vg/kg) respectively. Mice were subjected to functional tests for mytonia and electromyography tests and tissues were processed at various time points (4-16 weeks) as indicated and analysis of RCas9-CTG expression and splicing biomarkers was carried out.



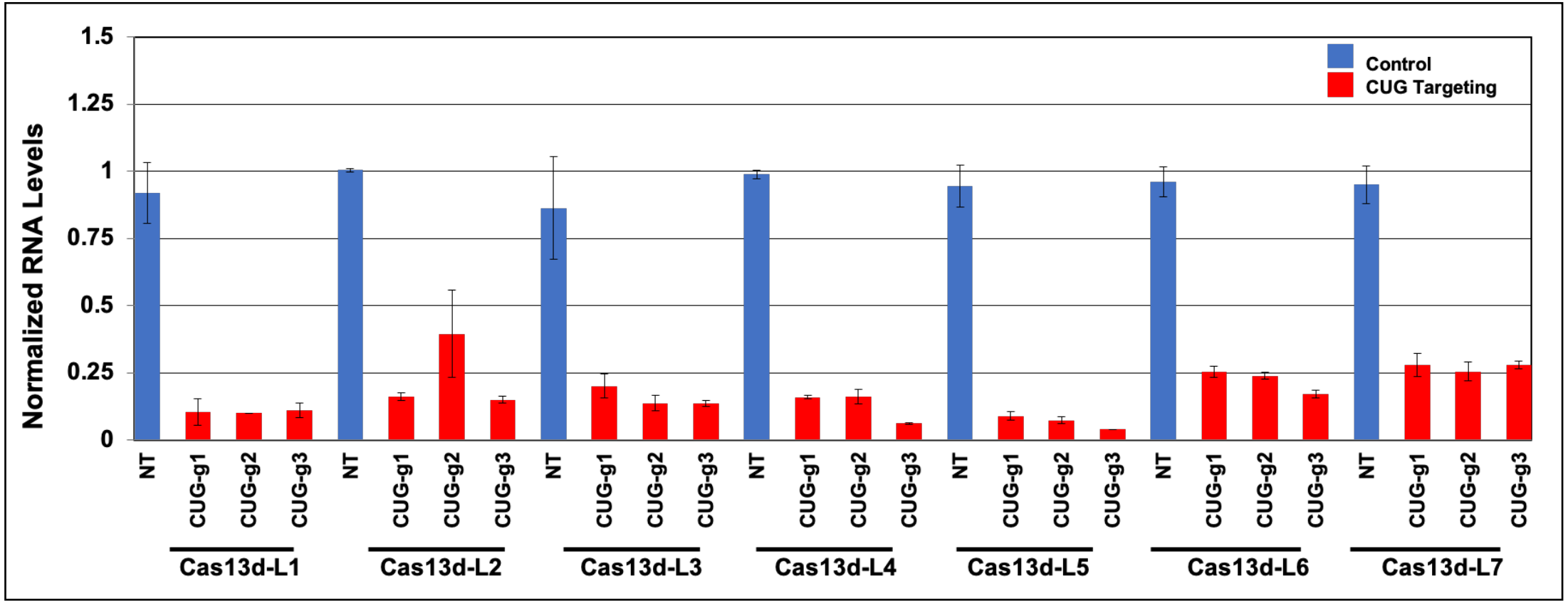
Results: (A) Boxplot shows mRNA levels of RCas9 and HSA (CUG repeat levels) in the quadriceps of treated HSALR mice 16 weeks after treatment of P0 HSALR mice. N=3; Student's T-test $p < 0.01$. (B) Box-plots show distribution mRNA levels of guide-RNA-GFP and Cas9 levels in various indicated tissues at 4 and 8 weeks after lateral tail vein injection (N=3).

Systemic RCas9 Corrects DM1-related Abnormalities



Results: (C) Splicing of alternative exon 22 in Atp2a1 assessed by RT-PCR in quadriceps. (D) Latency to hind-limb relaxation at 8 and 16w post-treatment of P0 HSALR mice. (E and F) Electromyography (EMG, Gastrocnemius) of wildtype, vehicle, and AAV9-RCas9-CTG treated P0 HSALR mice at 8 weeks post injection (E; N=5, Z-score = 2.8, $p < 0.01$) or vehicle and RCas9-CTG treated adult mice 4 weeks post injection (F; T-test p value < 0.05).

Locana's Proprietary RNA Targeting Cas Proteins are Effective in Eliminating Expanded CUG RNA

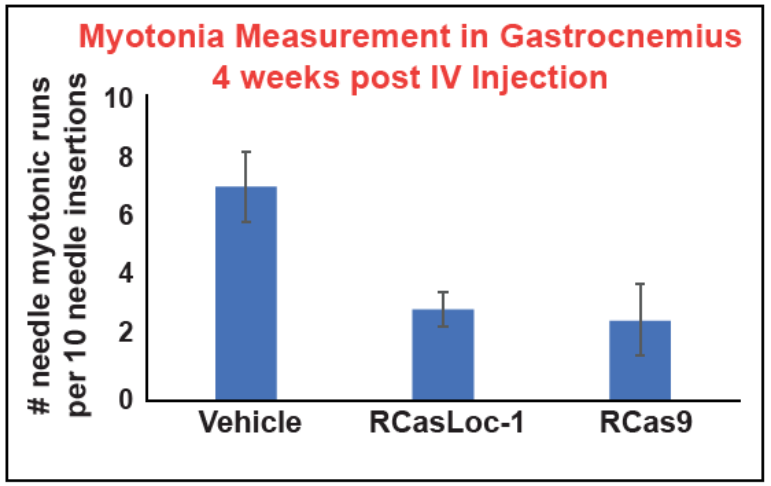


Study Design: Various RNA Targeting Cas proteins were tested with cognate guide RNAs for three different frames of CUG repeats in vitro (cells).

Results: (A) Various RCas systems were tested for their CUG-taregting activity in vitro with transient transfection and RT-qPCR.

Locana's Proprietary RNA Targeting Cas Proteins are Effective in Eliminating Expanded CUG RNA

B



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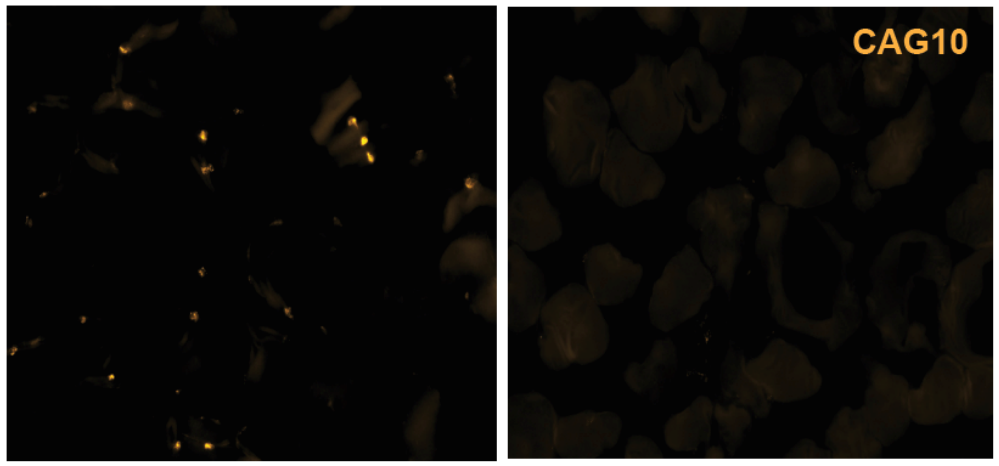

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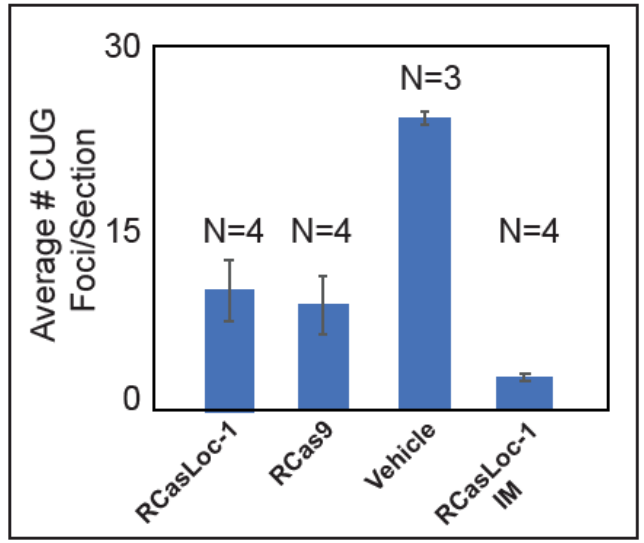

Study Design: Locana's proprietary unitary (packed in a single AAV) Cas13d-L2 was tested against the RCas9 system with intramuscular (tibialis anterior, 5*1010 vg) and systemic delivery (lateral tail vein, 1012 vg). Myotonia was measured with EMG and RNA foci were quantified 4 weeks post injection.

Results: (B) HSALR mice were injected in the lateral tail vein with AAV9 carrying RCas9 or RCasLoc-1. EMG was performed and number of myotonic runs were recorded for 10 needle insertions. Both RCas9 and RCasLoc-1 (Cas13dL2) decreased myotonia. (C) RNA-FISH was performed to stain for CUG RNA foci in HSALR TA and we observed a decrease in RNA foci with RCas9 and RCasLoc-1.

Conclusions

Conclusions

- RNA Targeting Cas9 targets and cleaves CUG RNA foci *in vitro* and *in vivo*.
- IM of RCas9 reverses DM1-related splicing abnormalities and restores CLCN1 staining.
- RCas9 levels were sustained for >12 weeks post IM injection in wildtype and HSA_{LR} mice when co-administered with Tacrolimus and CTLA4-Ig.
- Systemic injection of RCas9 leads to reduction in toxic CUG RNA foci, splicing defects, and myotonia.
- RNA Targeting Cas systems packaged in a **unitary AAV** vector show *in vitro* and *in vivo* CUG elimina-