Endosomal Escape Vehicle (EEV) Platform to Enhance the Functional Delivery of Oligonucleotides

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TIDES EUROPE 2022
November 17th, 2022

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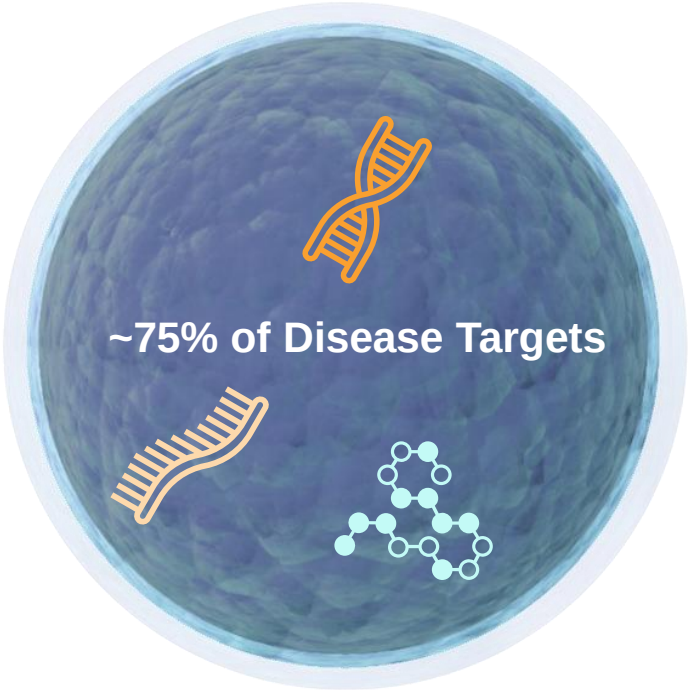
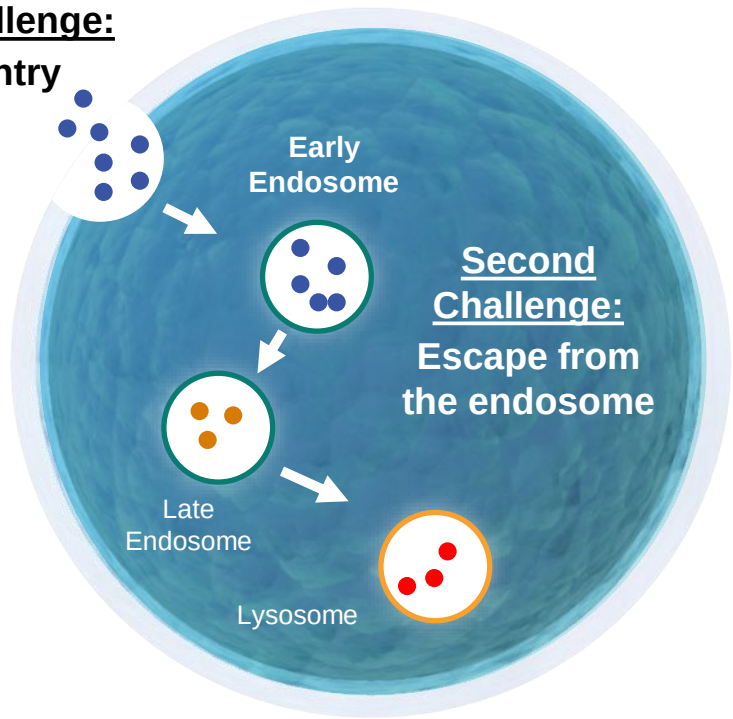
ENTRADA'S MISSION

Treating Devastating Diseases With Intracellular Therapeutics

THE NEED FOR INTRACELLULAR THERAPEUTICS

Approximately 75% of all disease-causing targets are intracellular and difficult to reach, representing a significant issue when developing effective therapies



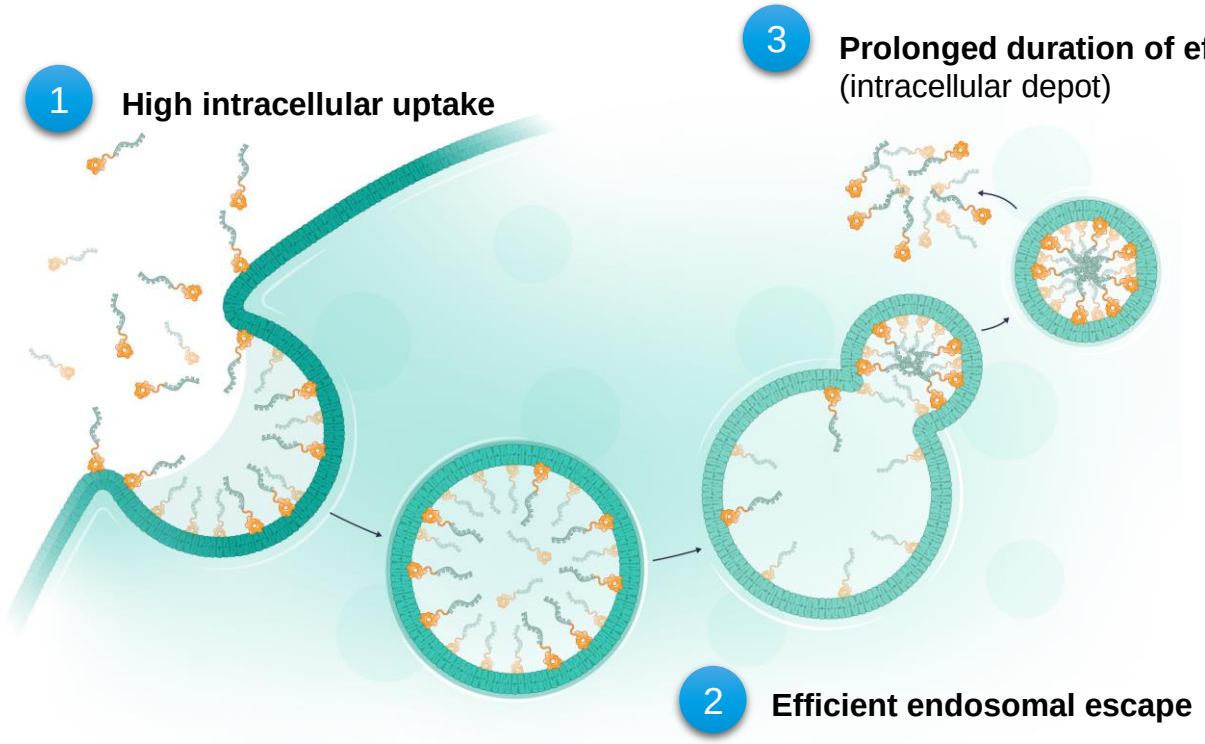


The Endosomal Escape Vehicle (EEV™) Platform aims to solve the fundamental problem: Lack of efficient cellular uptake and escape from the endosome

ENDOSOMAL ESCAPE VEHICLE (EEV™) PLATFORM

Entrada’s EEV platform consists of a library of proprietary cyclic peptides with unique chemistry that enable improved uptake and endosomal escape

- Unique chemistry results in **improved uptake and endosomal escape**
- Cyclic structure enhances **proteolytic stability**
- Small and cyclic structure may **reduce immunogenicity risk**
- Mechanism of internalization **conserved across species**

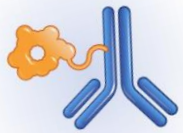


Qian, Z. et al. *ACS Chem. Biol.* 2013; Qian, Z. et al. *Biochemistry* 2014; Qian, Z. et al. *Biochemistry* 2016; Sahni, A. et al. *ACS Chem. Biol.* 2020; Pei, D. *Acc. Chem. Res.* 2022.

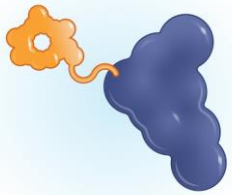
A BROADLY APPLICABLE PLATFORM

Entrada has demonstrated intracellular uptake of unique moieties ranging from 1 kDa to 600 kDa

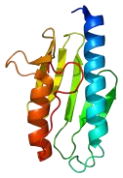
Antibodies



Enzymes

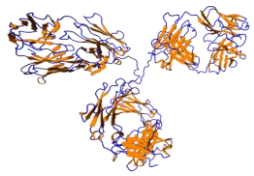


Oligonucleotides



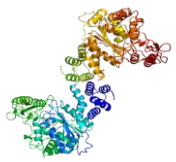
550-600 KDa

Hybrid frataxin



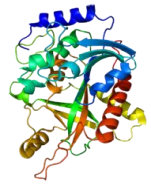
150 KDa

Antibody



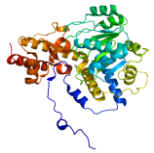
98 KDa

Thymidine phosphorylase



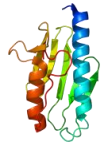
96 KDa

Purine nucleoside phosphorylase



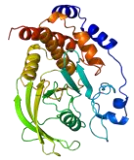
86 KDa

Alanine-glyoxylate aminotransferase



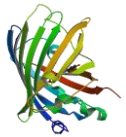
46 KDa

Human frataxin



37 KDa

PTP1B catalytic domain



32 KDa

EGFP

16 KDa

Nanobody

6 KDa

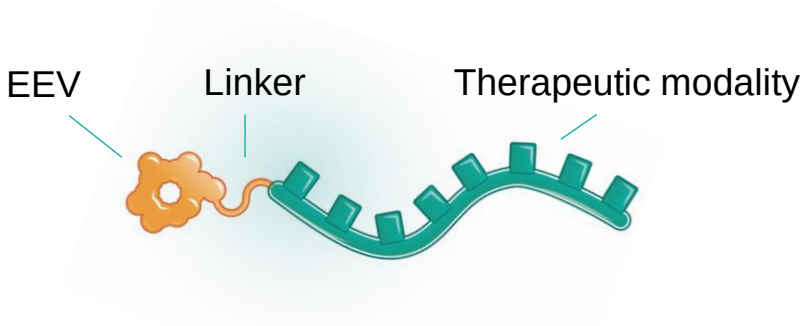
Oligonucleotide

1-3 KDa

Various peptide cargos

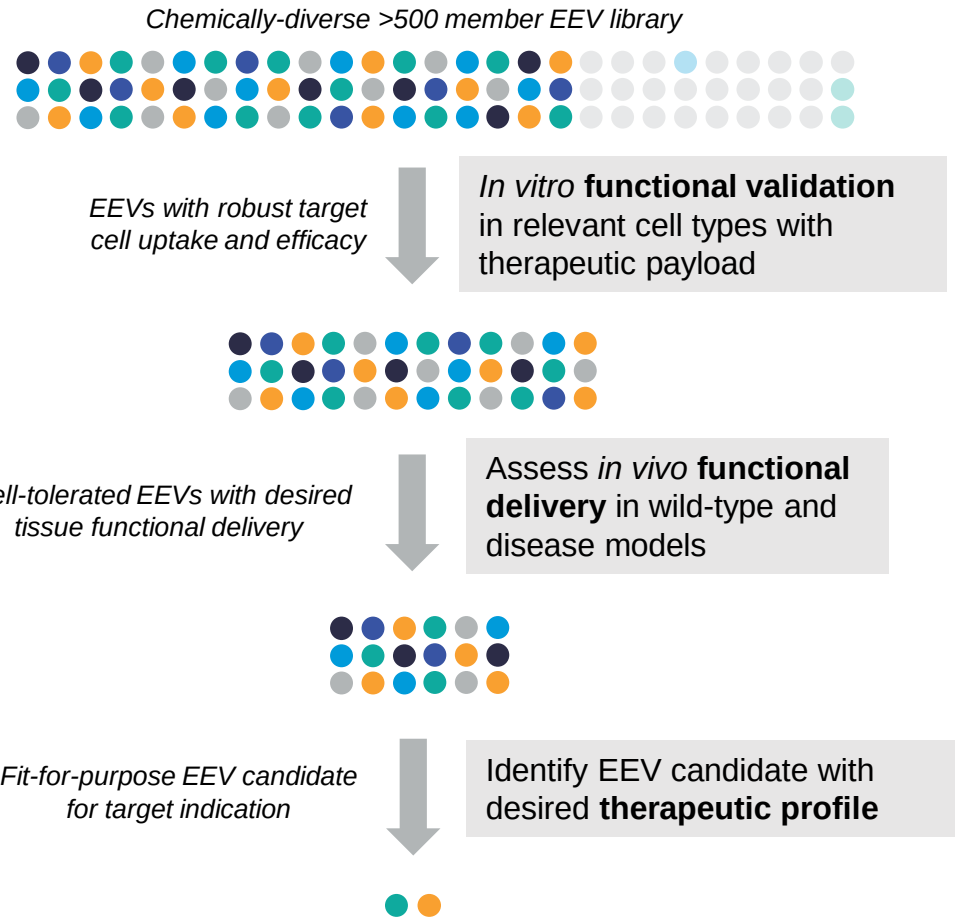
EEV LIBRARY: SCREENING AND OPTIMIZATION

Discovery Engine for Intracellular Therapeutics



- Cyclic peptide library design and combinatorial synthesis to generate **EEV library**
- Delivery and counter-screening assays enabled for *in vitro* **high throughput screening**
- Functional screening of lead EEVs *in vivo* to select for **pharmacodynamic activity** in target tissues
- Optimize **linker & conjugation chemistry** for desired therapeutic modality

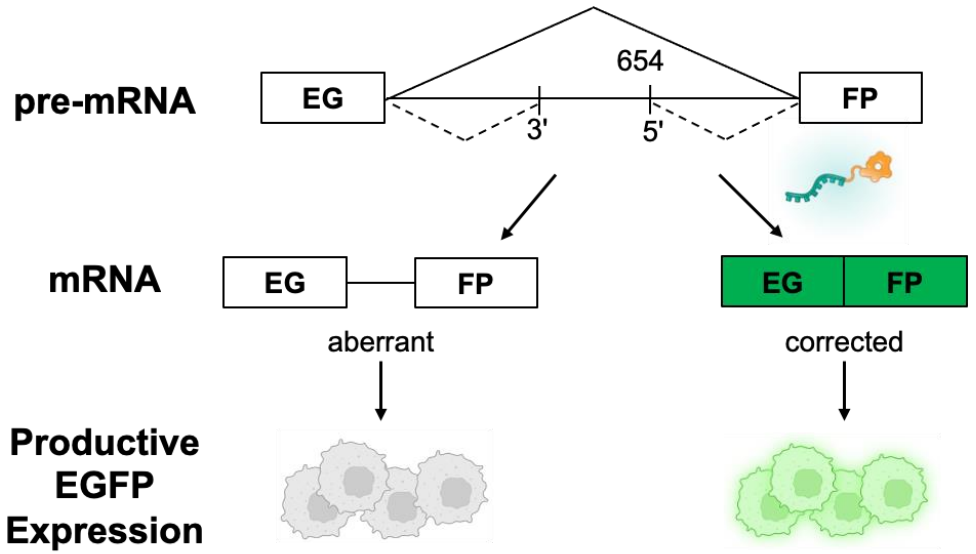
Screening Cascade for EEV Candidates



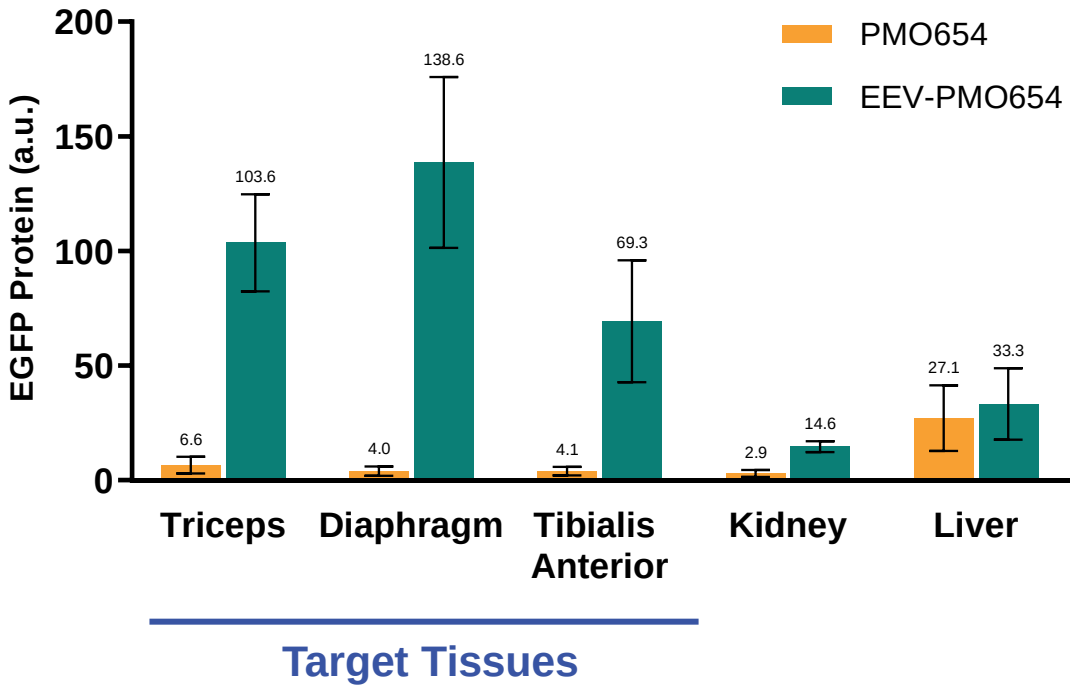
FUNCTIONAL DELIVERY FOR TARGET TISSUES

Entrada's EEV-therapeutics can be designed to enhance functional delivery to target tissues

EGFP-654 Transgenic Mice



Functional Delivery to Target Tissues



PMO, phosphorodiamidate morpholino oligomer (Summerton, J. et al. *Antisense Nucleic Acid Drug Dev.* 1997); **EGFP-654** transgenic mouse model contains an EGFP gene interrupted by human beta-globin intron 2 with mutated nt654 (Sazani, P. et al. *Nature Biotech.* 2002); **PMO654**, splicing switching PMO targeting nt654; shown as mean ± standard deviation.

TRANSLATION FROM UPTAKE TO OUTCOMES

EEV-therapeutics possess favorable pharmacological properties: significant uptake in target tissues, efficient intracellular delivery and potent pharmacodynamic outcomes

Tissue Uptake in Muscle

- ✓ Skeletal muscle
- ✓ Cardiac muscle

+

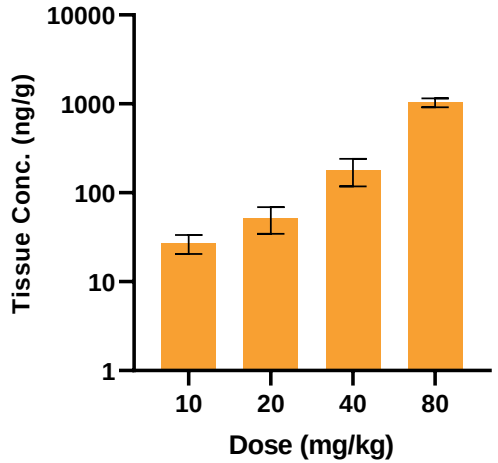
Intracellular Delivery

- ✓ Endosomal escape
- ✓ Nuclear localization

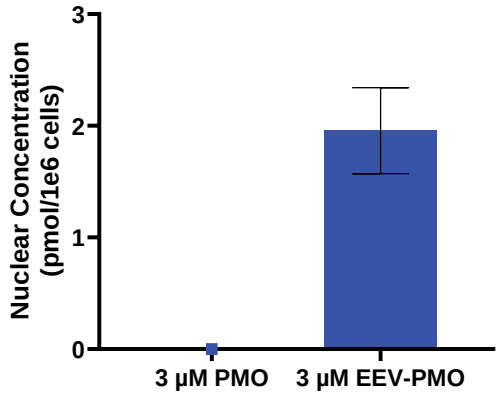
=

Pharmacodynamic Outcome

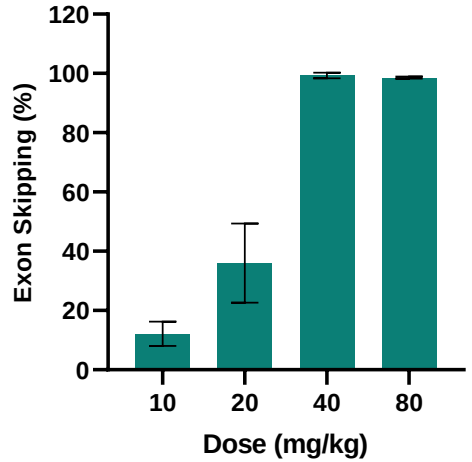
- ✓ Rapid, dose-dependent efficacy
- ✓ Duration of at least 12 weeks



IV, hDMD mice, 5-day p.i.



24-hour incubation



IV, hDMD mice, 5-day p.i.

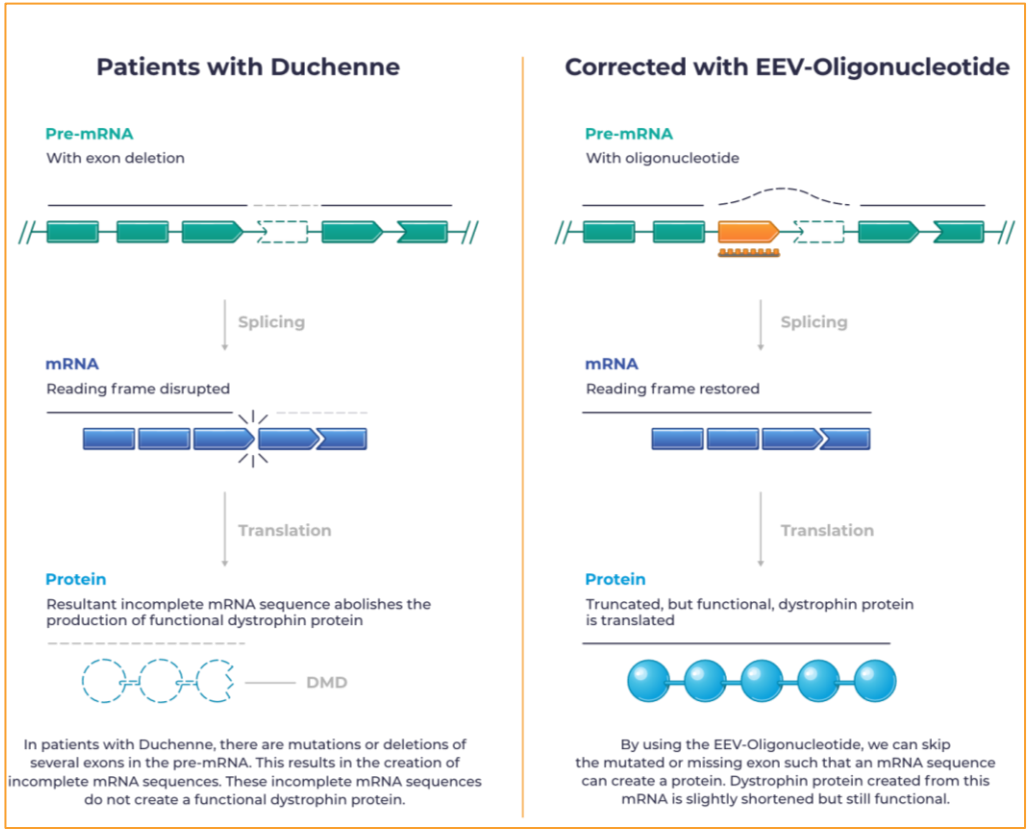
hDMD mice express full-length human dystrophin gene. **p.i.** post injection; shown as mean ± standard deviation.

DUCHENNE MUSCULAR DYSTROPHY (DMD)

DMD OVERVIEW

Duchenne muscular dystrophy (DMD) is a progressive, devastating muscle wasting disease with significant unmet need. Entrada's first DMD program is for exon 44 skipping

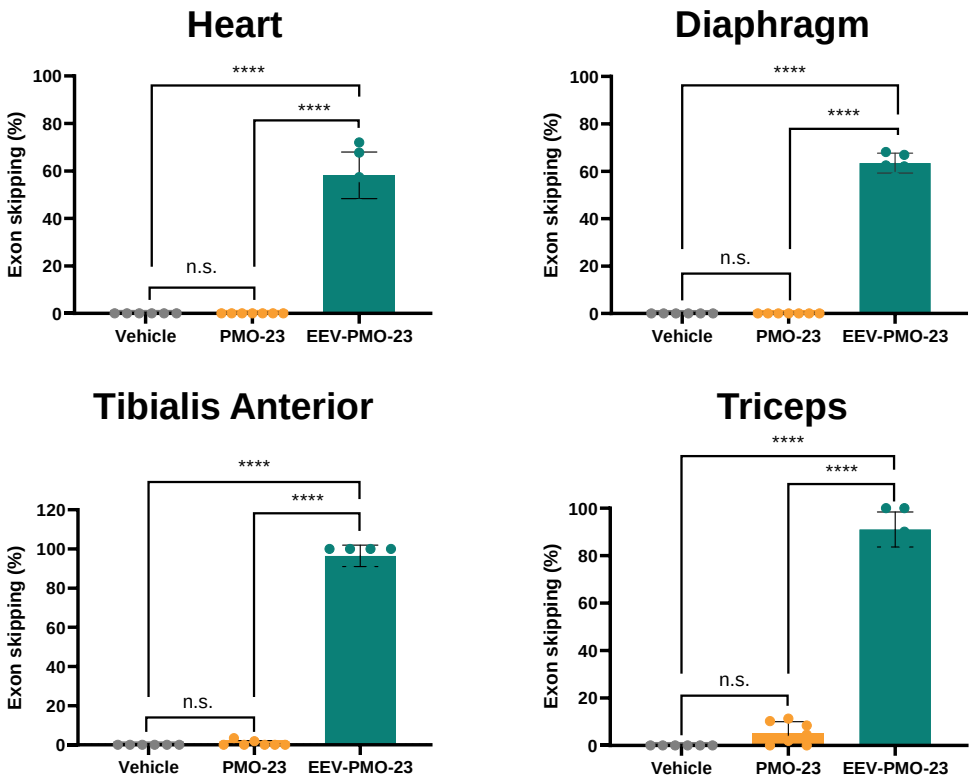
- DMD is caused by mutations in the *DMD* gene that encodes for dystrophin[1]
- Progressive muscle degeneration, wasting and paralysis generally lead to death via **respiratory and/or cardiac failure**[2]
- Exon skipping therapeutics (for exons 45, 51 and 53) using **PMO chemistry** were approved based on **a very modest improvement in dystrophin levels ranging from ~1 to 6%**
- 40% of patients with DMD have mutations amenable to exon skipping of exons 44, 45, 51 and 53.[3] **However, there is currently no approved therapy for patients with mutations amenable to exon 44 skipping**



[1]Hoffman, E.P. et al. *Cell* 1987; [2]Birnkrant, D.J. et al. *Lancet Neurol* 2018; [3]https://www.parentprojectmd.org/about-duchenne/what-is-duchenne/types-of-mutations/mutation-specific-therapies

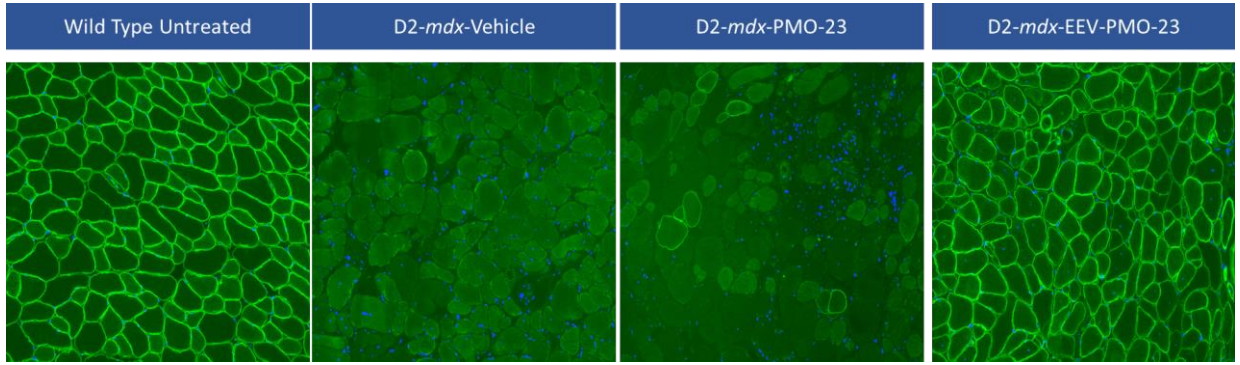
REPEAT EEV-PMO TREATMENT IN D2-*mdx* MICE

Robust exon 23 skipping after four monthly IV doses of EEV-PMO-23 in D2-*mdx* mice

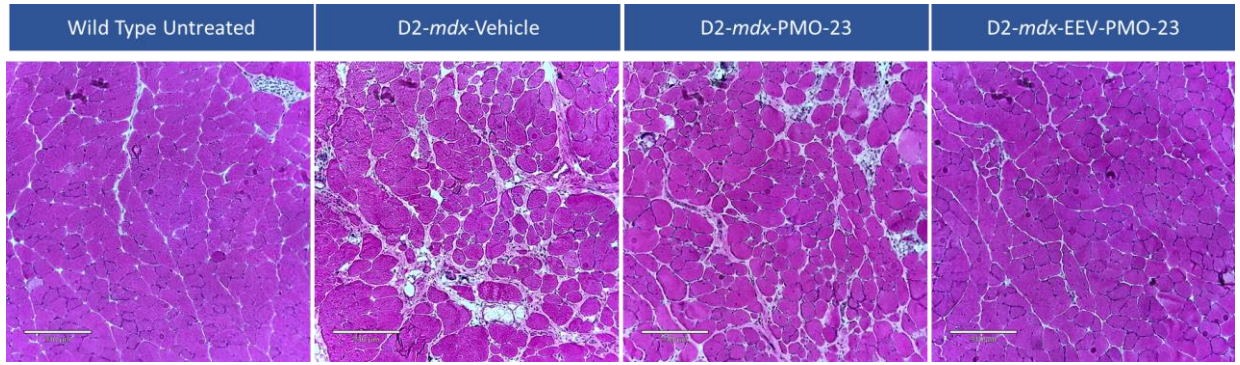


Broad dystrophin expression and restoration of muscle integrity after four monthly IV doses of EEV-PMO-23 in D2-*mdx* mice

Representative Immunofluorescence of Gastrocnemius Muscle (Dystrophin Staining in Green)



Representative Histopathology of Gastrocnemius Muscle (H&E Staining)



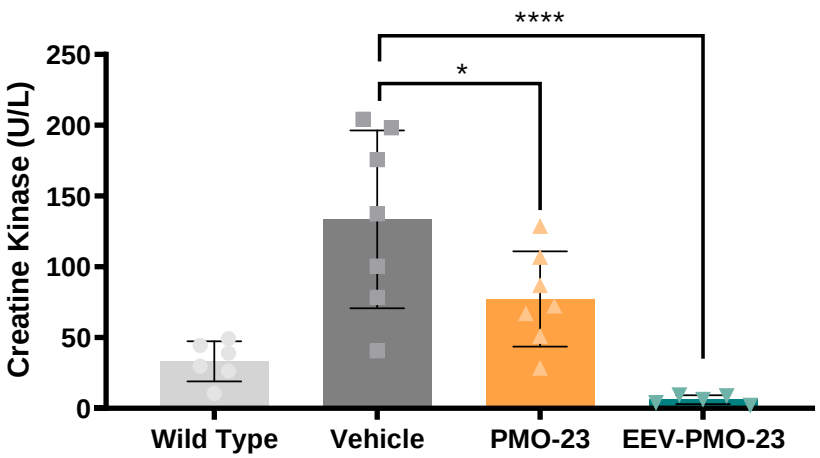
- D2-*mdx* mice (male, n=6-7) were treated with 4 monthly doses of either vehicle, 20 mg/kg PMO-23 or 20 mg/kg PMO-23 equivalent of EEV-PMO-23, and the data were collected ~4 weeks after the last dose

EEV, Endosomal Escape Vehicle; **PMO-23**, mouse *Dmd* exon 23 skipping phosphorodiamidate morpholino oligomer; **D2-*mdx*** is a DMD mouse model with a nonsense mutation in *Dmd* exon 23 (Coley et al. *Hum. Mol. Genet.* 2016); ****p<0.0001; **n.s.**, not significant; shown as mean ± standard deviation.

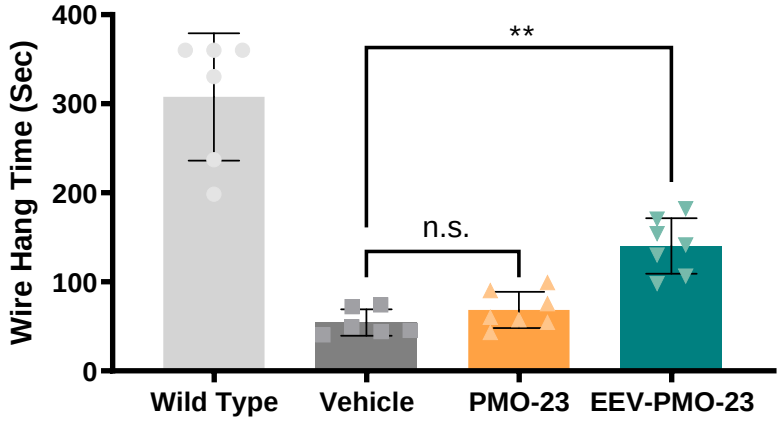
REPEAT EEV-PMO TREATMENT RESULTS IN CK CORRECTION AND FUNCTIONAL IMPROVEMENT

Repeat EEV-PMO-23 treatment normalized serum CK levels and showed significant improvements in muscle function when compared to PMO alone after four monthly IV doses in D2-*mdx* mice

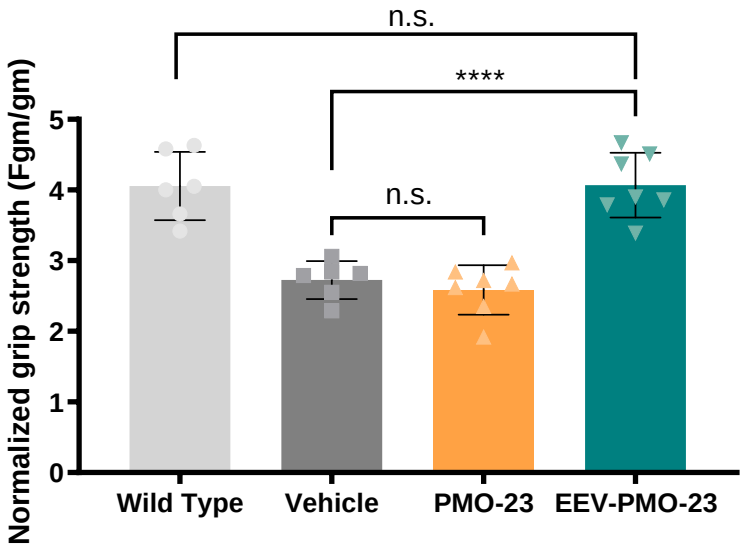
Serum CK Levels



Wire Hang Time



Grip Strength



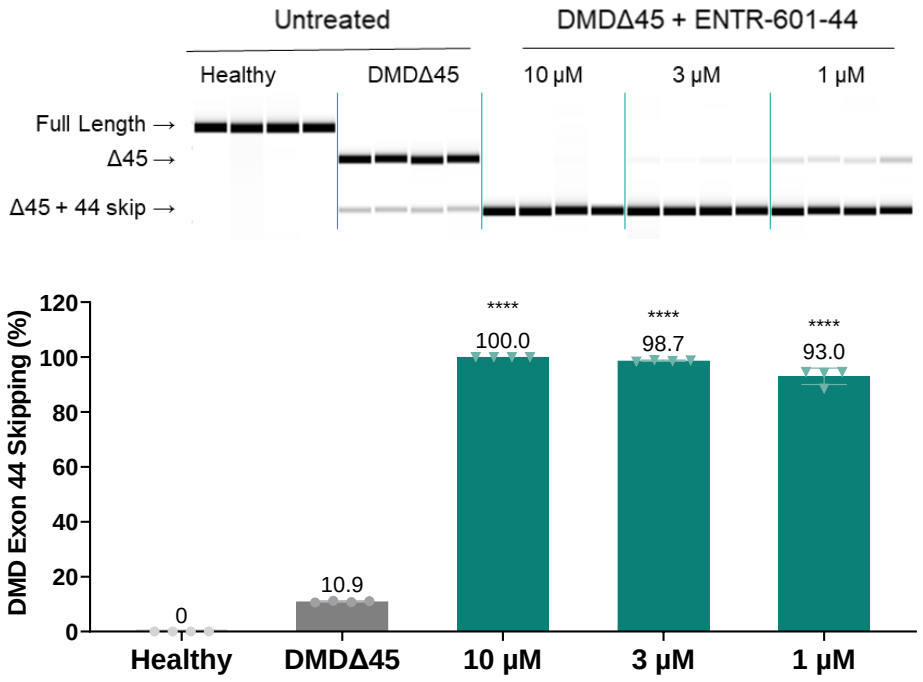
- **D2-*mdx*** is a DMD mouse model on DBA/2J background and better recapitulate disease pathology (Fukada et al. *Am. J. Path.* 2010)
- D2-*mdx* mice (male, n=6-7) were treated with 4 monthly doses of either vehicle, 20 mg/kg PMO-23 or 20 mg/kg PMO-23 equivalent of EEV-PMO-23, and the data were collected ~2 weeks after the last dose

CK, creatine kinase; *p<0.05, **p<0.01, ****p<0.0001; **n.s.**, not significant; shown as mean ± standard deviation.

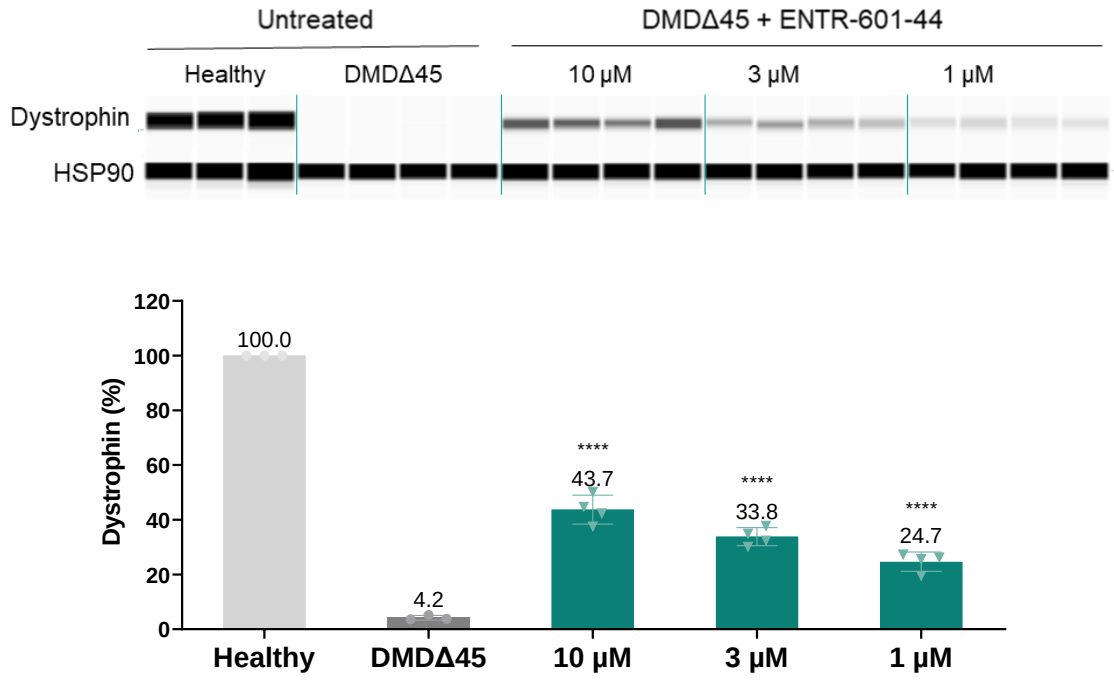
EXON SKIPPING AND DYSTROPHIN PRODUCTION IN PATIENT-DERIVED CELLS

Robust dose-dependent exon 44 skipping and dystrophin protein production were observed in DMD patient-derived muscle cells treated with clinical candidate, ENTR-601-44

Exon Skipping



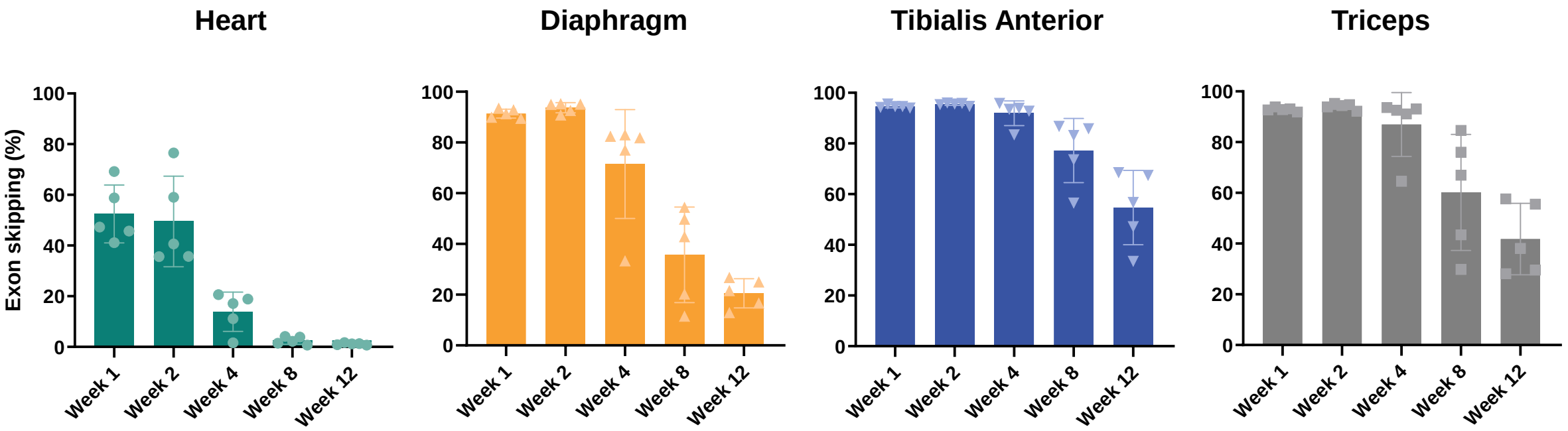
Dystrophin Protein Production



DMDΔ45, immortalized myoblasts from DMD patients harboring an out-of-frame exon 45 deletion and further differentiated into myotubes**; ENTR-601-44** is the clinical candidate selected for DMD exon 44 skipping EEV-oligonucleotide conjugate; ****p<0.0001; shown as mean ± standard deviation.

ENTR-601-44 IN HUMAN DMD MICE (hDMD)

A single IV dose of ENTR-601-44 resulted in high and durable levels of exon 44 skipping across major muscle groups in hDMD mice for at least 12 weeks



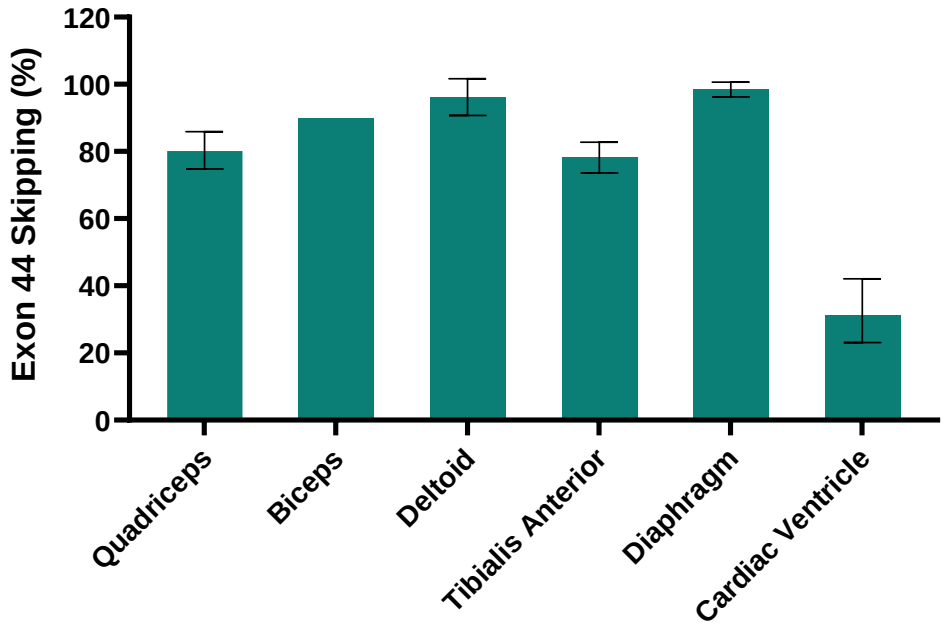
- Post single IV dosage of 60 mg/kg (PMO equivalent), robust exon skipping was observed in ENTR-601-44 treated hDMD mice

hDMD transgenic mice express full-length human dystrophin gene which allows for preclinical testing of human sequence-specific PMO for DMD transcript correction ('t Hoen et al. *J. Biol. Chem.* 2008); shown as mean ± standard deviation.

ENTR-601-44 IN NHP

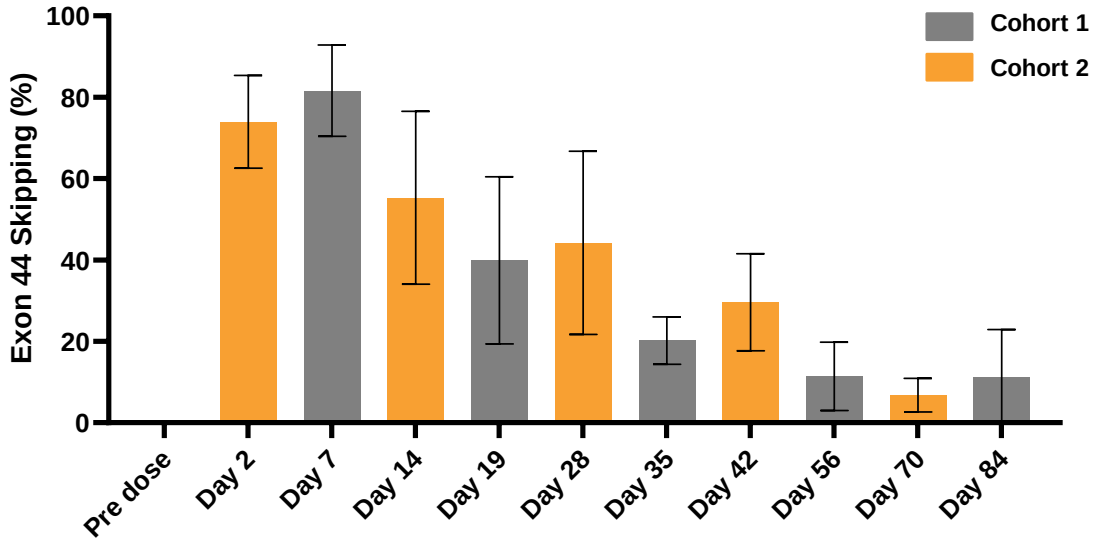
A single IV dose of ENTR-601-44 resulted in robust exon skipping in both skeletal and cardiac muscles in NHP, as well as prolonged duration of effect for at least 12 weeks

Exon Skipping in NHP Muscles at Day 7



- At 7 days post IV infusion of 30 mg/kg (PMO equivalent), robust exon 44 skipping across different muscle groups isolated from the ENTR-601-44 treated NHP

Duration of Effect in NHP Biceps for at Least 12 Weeks



- Post IV infusion of 35 mg/kg (PMO equivalent), robust exon 44 skipping observed in biceps in the ENTR-601-44 treated NHP (n=3 per cohort) for at least 12 weeks

NHP, non-human primates; shown as mean ± standard deviation.

ENTRADA DMD DATA SUMMARY

Entrada's murine and NHP data represent a robust set of translational data; IND application in Q4 2022

- High levels of exon skipping across *mdx*, D2-*mdx,* human dystrophin mouse and NHP studies
- Exon skipping translates to promising dystrophin production in heart and skeletal muscles
- Dystrophin production sufficient to result in functional improvement
- Extended circulating half-life and durable dystrophin production over 12+ weeks from a single injection of ENTR-601-44 was observed in the NHP
- ENTR-601-44 IND application planned in Q4 2022
- Exon 45 clinical candidate selection expected in Q4 2022

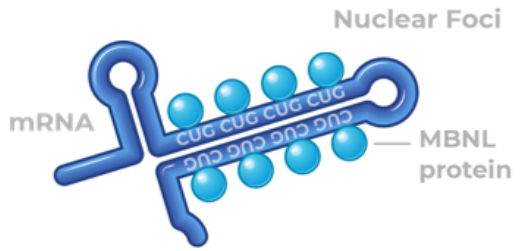
MYOTONIC DYSTROPHY TYPE 1 (DM1)

DM1 OVERVIEW

DM1 is a debilitating multi-systemic disease with no available treatments; CUG repeats in DMPK mRNA sequester MBNL proteins, resulting in nuclear foci, aberrant splicing, and disease

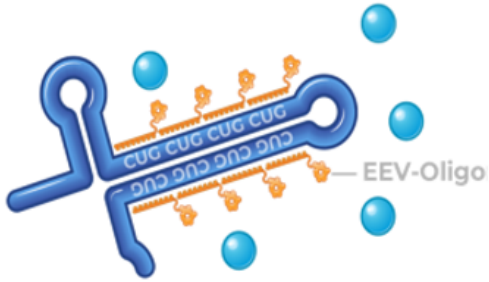
- **DM1 occurs in 1:8,000 people worldwide and affects ~40,000 patients in US and over 50,000 in Europe**
  - 75% of patients have adult-onset DM1
  - Multisystemic: including myotonia, muscle weakness and atrophy, cardiac conduction abnormalities, pulmonary complications, cataracts, and endocrine dysfunction[1]
  - **Currently there are no approved therapies**
- **DM1 is caused by CUG repeats in the mRNA that sequester MBNL proteins**[2]
  - Mutant *DMPK* mRNA and MBNL proteins form aggregates named nuclear foci[3]
- **MBNL activity is decreased as a result of sequestration leading to spliceopathy of downstream transcripts**[4,5]

Mutant *DMPK* mRNA



- Nuclear foci formation
- mRNA accumulation
- Reduced MBNL function
- Aberrant splicing

EEV-Oligonucleotide Approach



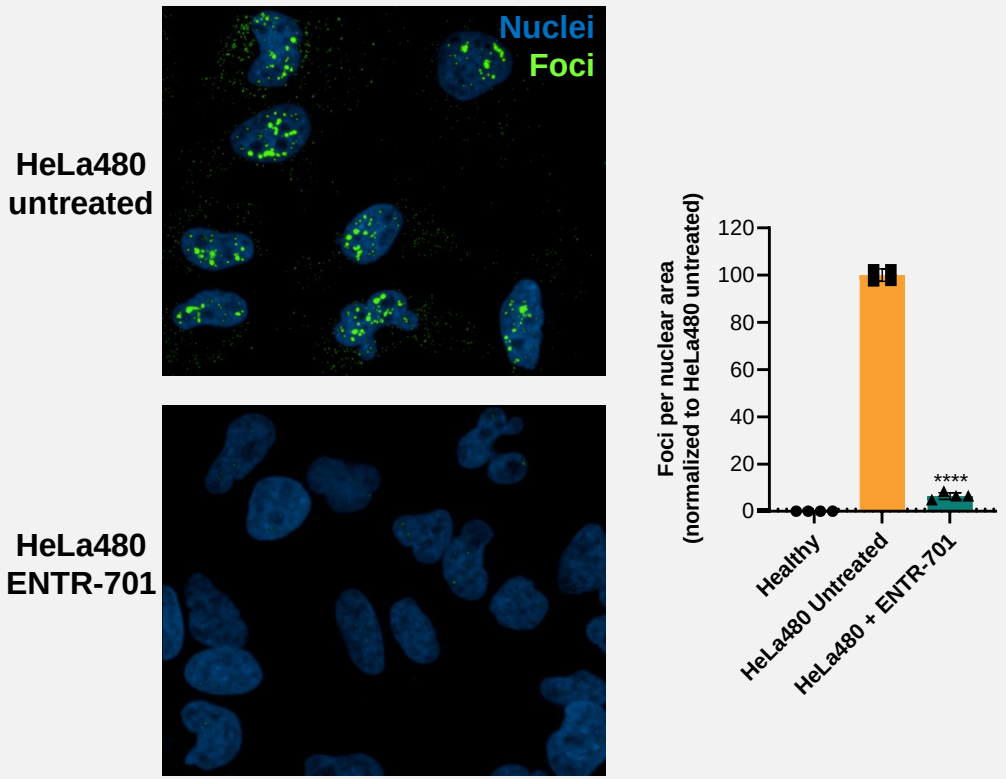
- Reduced nuclear foci
- Selective mRNA reduction
- Normal MBNL function
- Corrected spliceopathy

[1]Thornton C.A. *Neurol. Clin.* 2014, 705. [2]Philips, A.V. et al. *Science* 1998; [3]Pettersson, O.J. *NAR* 2015; [4]Wagner, S.D. et al. *PLoS Genet.* 2016; [5]Tanner, M. et al. *Nucleic Acids Res.* 2021.

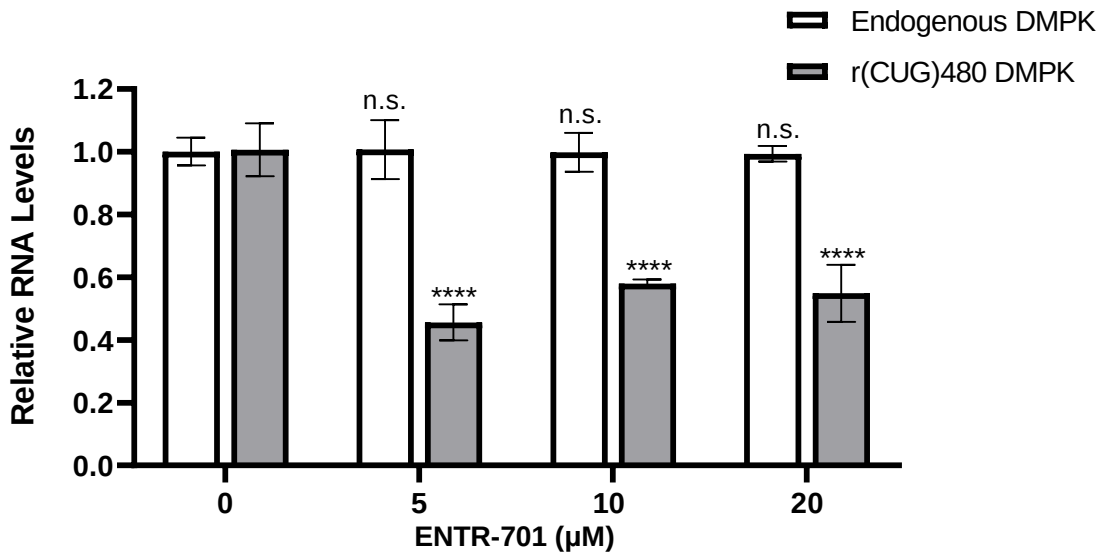
ENTR-701 IN DM1 CELL LINE WITH REPEAT EXPANSION

DM1 clinical candidate, ENTR-701, showed reduction of nuclear foci and selective reduction of repeat expansion-containing DMPK transcript in the HeLa480 cell line

Nuclear Foci Reduction



Selective Reduction of Mutant DMPK mRNA



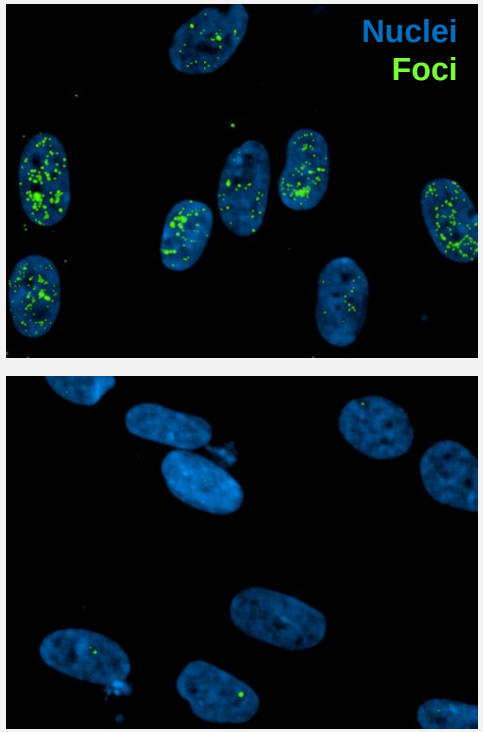
- Free uptake of ENTR-701 reduced nuclear foci and selectively reduced (CUG)480 containing DMPK mRNA

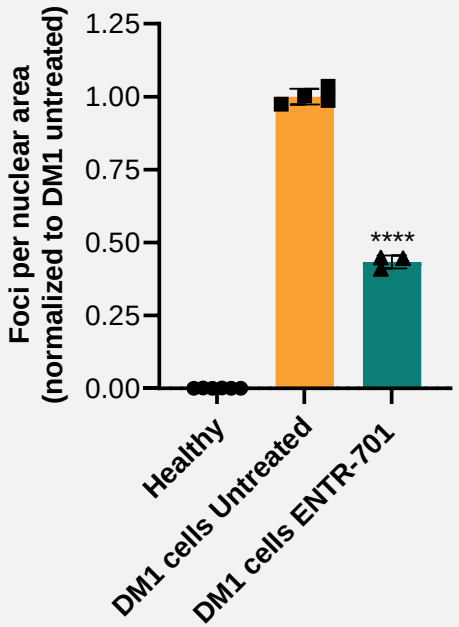
ENTR-701 is the clinical candidate selected for DM1, composed of CUG-repeat blocking PMO conjugated to EEV; **HeLa480** cell line was constructed by integrating (CTG)480 and (CTG)0 containing DMPK transgenes, which showed MBNL1-dependent aberrant splicing (Reddy, K. et al. *Proc. Natl. Acad. Sci.* 2019); ****p<0.0001, n.s., not significant vs. untreated cells (left) or endogenous DMPK (right); shown as mean ± standard deviation.

ENTR-701 IN DM1 PATIENT-DERIVED MYOTUBES

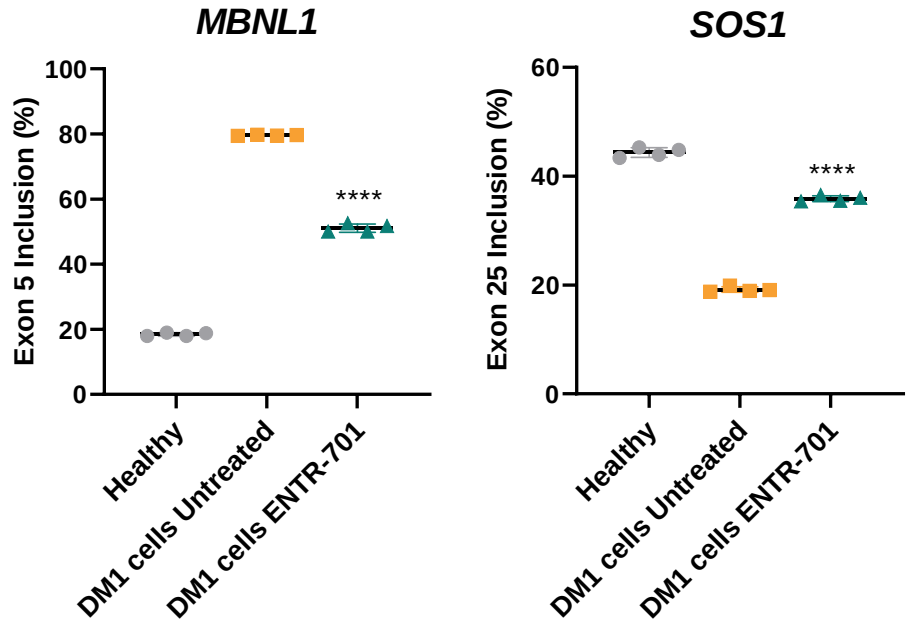
ENTR-701 treatment in DM1 patient-derived muscle cells resulted in significant nuclear foci reduction and correction of aberrant splicing

Nuclear Foci Reduction





Correction of Aberrant Splicing



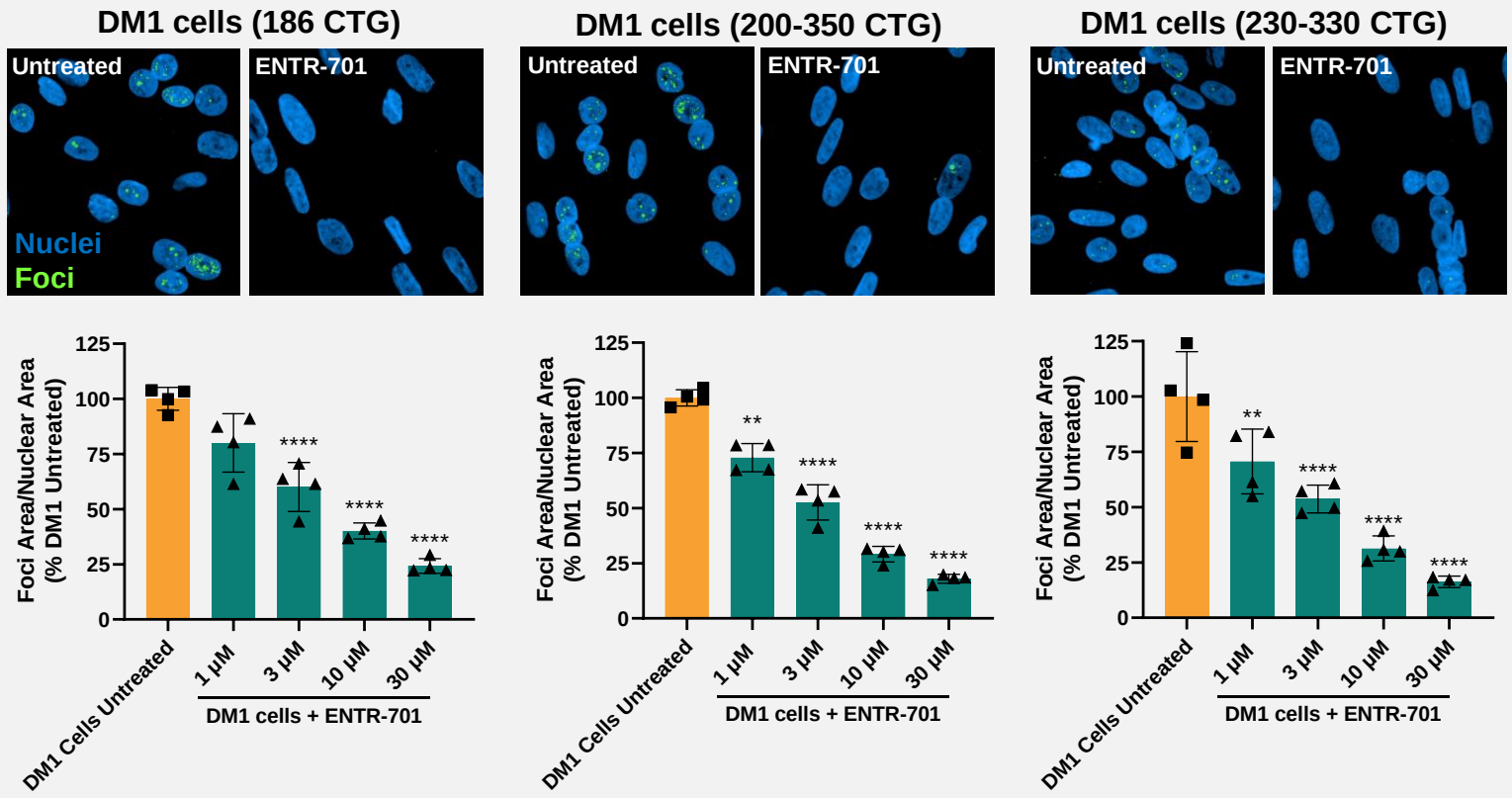
- Immortalized DM1 patient-derived (2,600 CUG repeats) muscle cells[1] were treated with ENTR-701 and analyzed for the reduction of nuclear foci and the correction of aberrant splicing

[1]Arandel, L. et al. *Dis. Model. Mech.* 2017. **ENTR-701** is the clinical candidate selected for DM1, composed of CUG-repeat blocking PMO conjugated to EEV; ****p<0.0001 for ENTR-701 compared to untreated; shown as mean ± standard deviation.

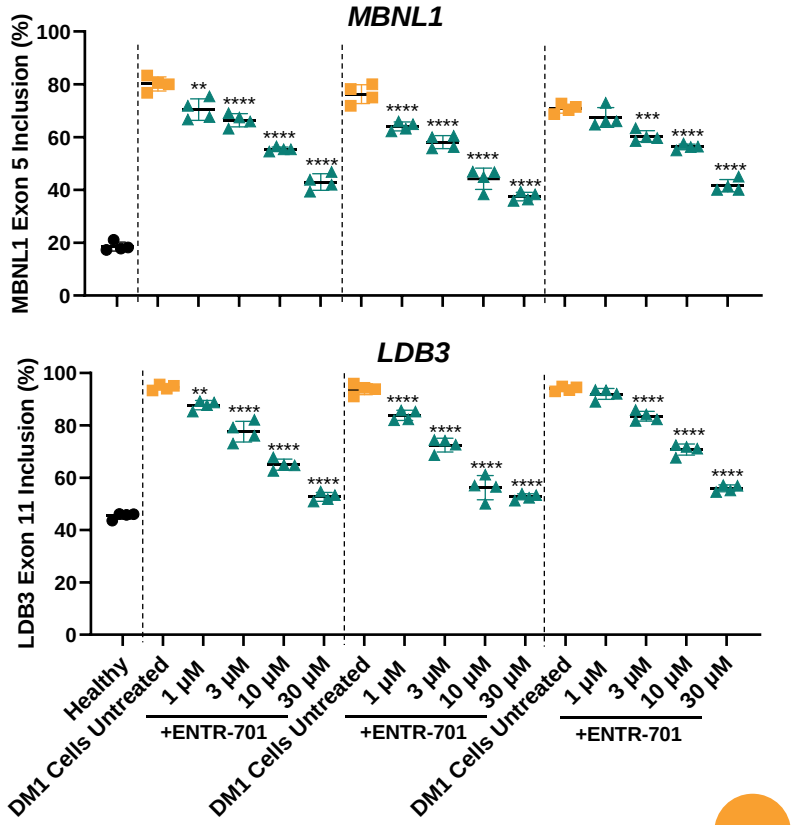
ENTR-701 EFFICACY IN DM1 PATIENT CELLS WITH DIFFERING CTG REPEAT NUMBER

Treatment of primary DM1 patient-derived cell lines with ENTR-701 confirmed nuclear foci reduction and correction of aberrant splicing regardless of CTG repeat number

Nuclear Foci Reduction



Correction of Aberrant Splicing

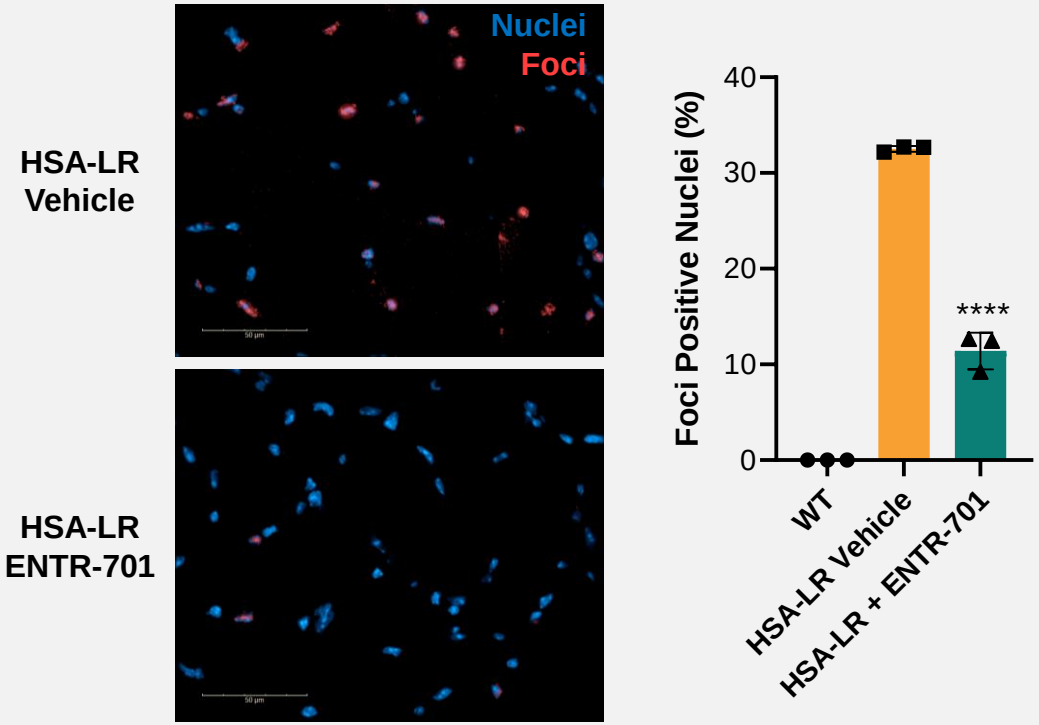


Primary DM1 patient-derived muscle cells were treated with an EEV-PMO conjugate and analyzed for the reduction of nuclear foci and the correction of aberrant splicing **ENTR-701** is the clinical candidate selected for DM1, composed of CUG-repeat blocking PMO conjugated to EEV; ****p<0.0001, ***p<0.001, **p<0.01 for ENTR-701 compared to untreated; shown as mean ± standard deviation.

EFFICACY OF ENTR-701 IN HSA-LR MICE

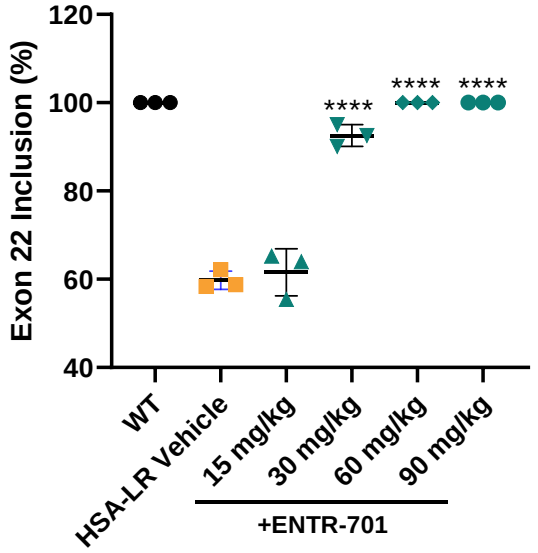
ENTR-701 treatment reduced nuclear foci and CUG-repeat expansion transcript level and corrected aberrant splicing in HSA-LR mice

Nuclear Foci Reduction

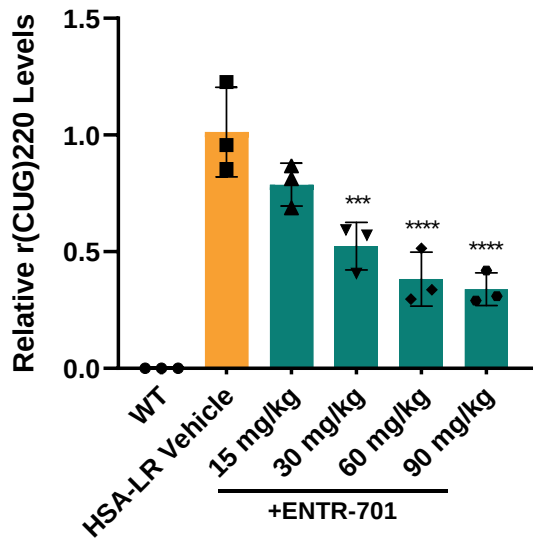


Tibialis anterior section

Atp2a1 Splicing Correction



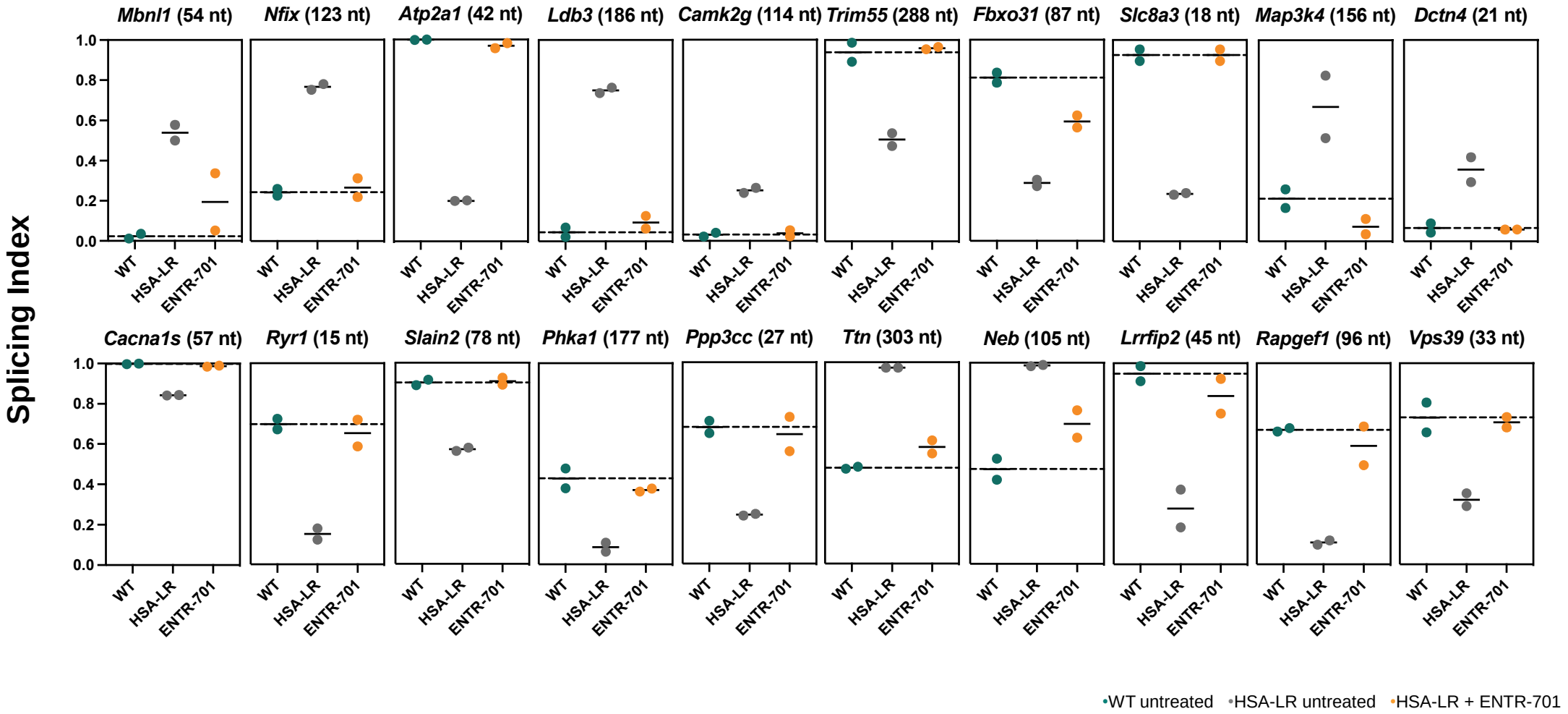
HSA-r(CUG)220 Reduction



- HSA-LR mice were dosed with vehicle or ENTR-701 and taken down 1-week post injection; tibialis anterior samples analyzed as a representative skeletal group

[1]**HSA-LR** mice carry a transgene resulting in CUG repeat expansion and recapitulates DM1 phenotype and molecular pathology (Mankodi, A. et al. *Science* 2000); **ENTR-701** is the clinical candidate selected for DM1, composed of CUG-repeat blocking PMO conjugated to EEV; ***p<0.001, ****p<0.0001, shown as mean ± standard deviation. **WT**, wild type.

ENTR-701 CORRECTED SPLICEOPATHY IN HSA-LR MICE

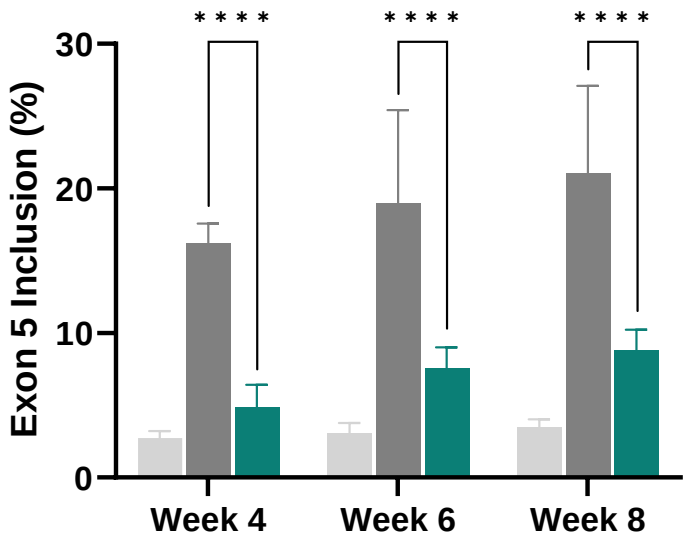


DM1-affected splicing events analyzed by RNA-seq; **ENTR-701** is the clinical candidate selected for DM1, composed of CUG-repeat blocking PMO conjugated to EEV

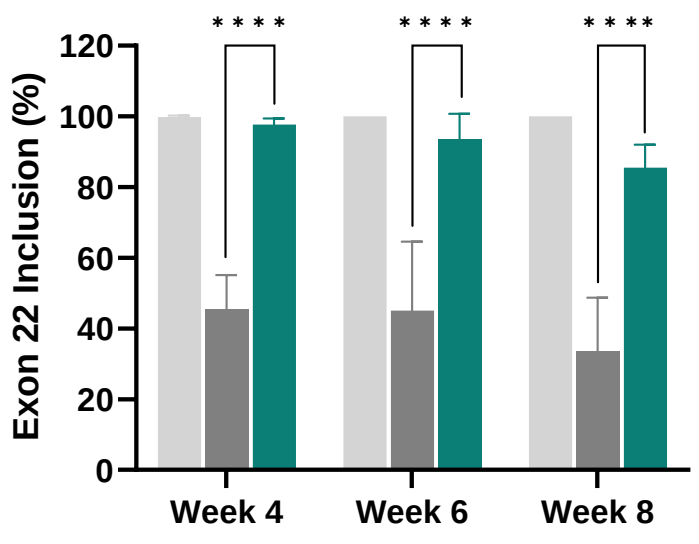
DURABILITY OF ENTR-701 IN HSA-LR MICE

A single dose of ENTR-701 resulted in splicing correction in HSA-LR mice for at least 8 weeks

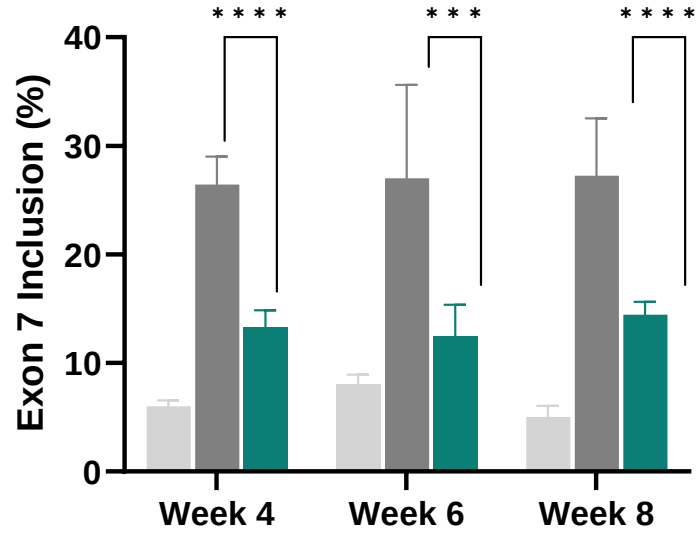
Mbnl1 Exon 5 Inclusion



Atp2a1 Exon 22 Inclusion



Nfix Exon 7 Inclusion



Wild type | HSA-LR + Vehicle | HSA-LR + ENTR-701

- Post single IV dosage of 60 mg/kg (PMO equivalent), robust splicing correction in the ENTR-701 treated HSA-LR mice 4, 6, or 8 weeks post injection

ENTR-701 is the clinical candidate selected for DM1, composed of CUG-repeat blocking PMO conjugated to EEV; ***Mbnl1***, muscleblind like splicing regulator 1; ***Atp2a1***, sarcoplasmic/endoplasmic reticulum calcium ATPase; ***Nfix***, nuclear factor I X; ***p<0.001, ****p<0.0001, shown as mean ± standard deviation.

MYOTONIA CORRECTION IN HSA-LR MICE

A single dose of ENTR-701 ameliorated pinch-induced myotonia for at least 8 weeks

HSA-LR Mouse: **Non-treated**

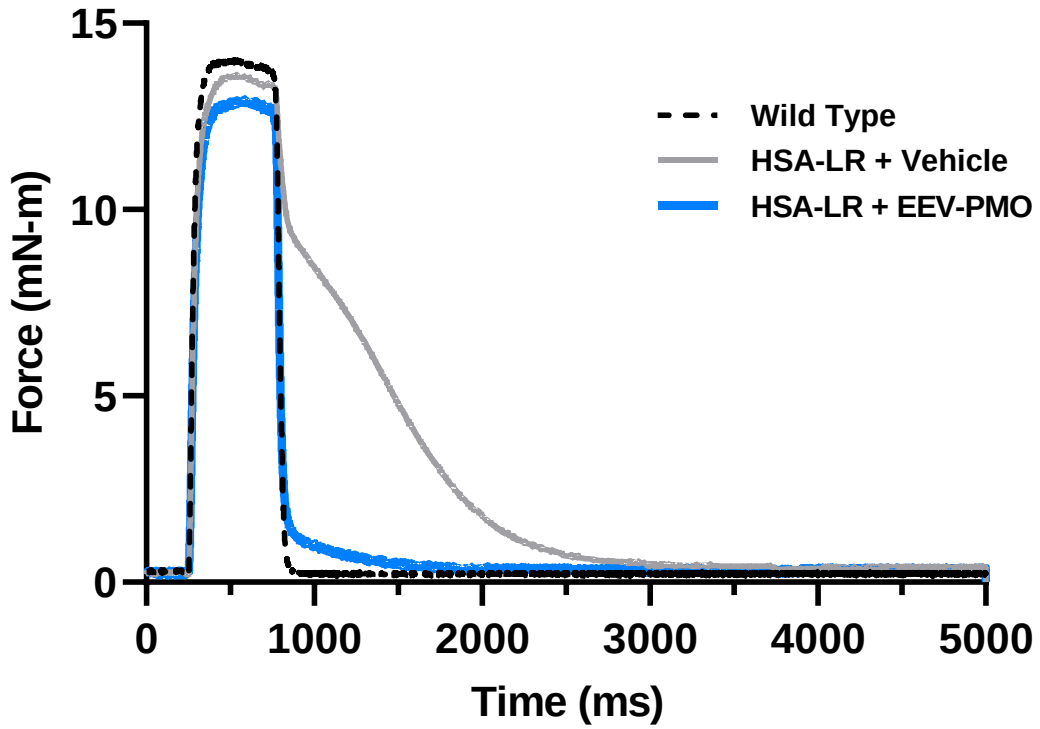
HSA-LR Mouse: **ENTR-701 Treated**

ENTR-701 is the clinical candidate selected for DM1, composed of CUG-repeat blocking PMO conjugated to EEV

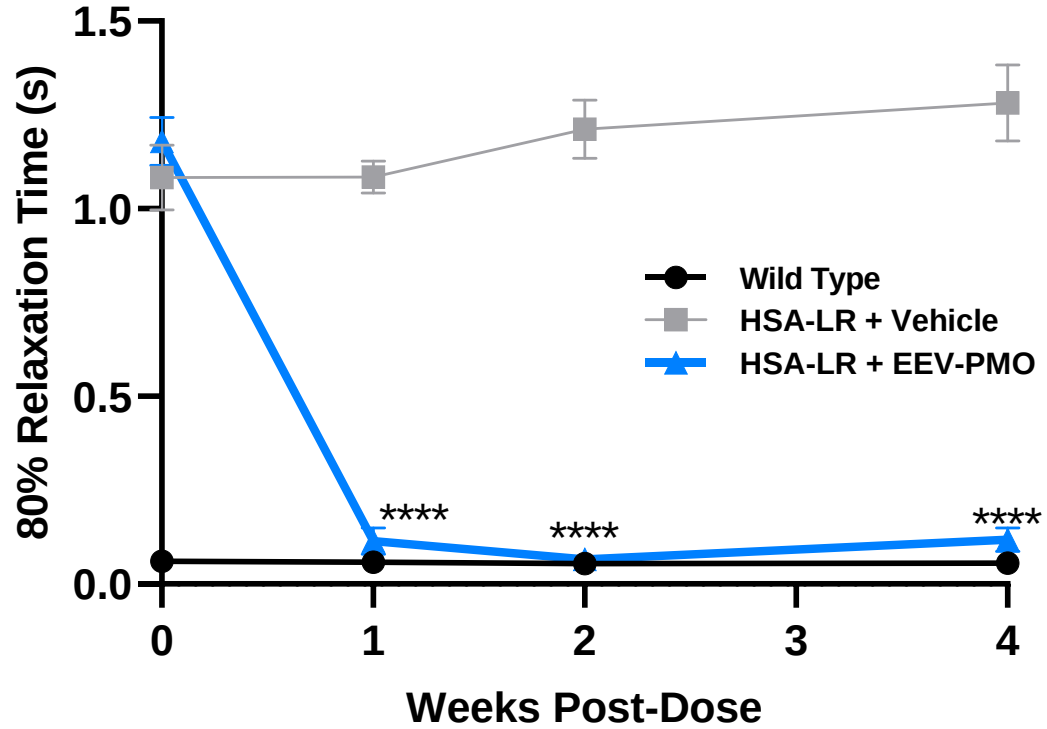
PROLONGED MUSCLE RELAXATION OBSERVED IN HSA-LR MICE

Muscle relaxation after tetanic contraction of plantar flexor muscle group was rescued in 7 days and sustained for 4 weeks following a single dose of a CUG-repeat blocking EEV-PMO

Muscle Relaxation



Durable Muscle Relaxation



EEV-PMO is composed of CUG-repeat blocking PMO conjugated our EEV platform; Muscle relaxation was assessed by stimulating sciatic nerve using 200 ms trains of supramaximal stimuli at 150 Hz. ****p<0.0001 for EEV-PMO compared to untreated; shown as mean ± SEM.

ENTRADA DM1 DATA SUMMARY

ENTR-701 demonstrated potential to treat DM1 via a CUG-repeat steric blocking approach both *in vitro* and *in vivo;* IND application planned in 2023

- Robust *in vitro* and *in vivo* data set demonstrating:
  - Highly specific reduction of pathogenic CUG-repeat containing mRNA
  - Reduction of nuclear foci
  - Correction of *Mbnl1* and downstream aberrant splicing
  - Correction of global transcriptome
- Single dose of ENTR-701 demonstrates durable splicing correction and amelioration of myotonia for at least 8 weeks post-dose in HSA-LR model
  - Importantly, HSA-LR model carries a transgene resulting in CUG repeat expansion and recapitulates DM1 phenotype and molecular pathology
- ENTR-701 IND submission planned in H2 2023

DISCOVERY PROGRAMS

ADDITIONAL PLATFORM OPPORTUNITIES

Entrada continues to invest in and build upon our EEV Platform to extend our efforts in developing novel EEV-therapeutic candidates

Target	Platform Approach	Goal
DNA	Gene editing	Deliver CRISPR enzyme and repair gene function with guide RNA
RNA	RNA editing	Deliver oligonucleotide therapeutics for RNA editing
	RNA splicing	Modify RNA via exon/intron splicing to activate protein expression
	RNA blocking	Block trinucleotide repeats in RNA to inhibit adverse binding
	RNA silencing	Silence or knockdown RNA to prevent protein expression
Protein	Protein replacement	Replace proteins and enzymes
	Protein inhibition	Inhibit protein signaling pathways
	Protein degradation	Degrade disease-causing proteins

ACKNOWLEDGEMENTS

Thank you!

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