EEV-mediated Delivery to Satellite Cells: Towards a Comprehensive Correction of Pathophysiology in a Preclinical Model of Duchenne Muscular Dystrophy

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entrada THERAPEUTICS

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DMD, Duchenne muscular dystrophy; **EEV**, endosomal escape vehicle.

OUR MISSION:

To Treat Devastating Diseases With Intracellular Therapeutics

We're proud to share the stories of JJ, Andrew, Max and Franklin – all living with Duchenne muscular dystrophy

EEV™ PLATFORM

ENDOSOMAL ESCAPE VEHICLE (EEV™) CONSTRUCT THERAPIES

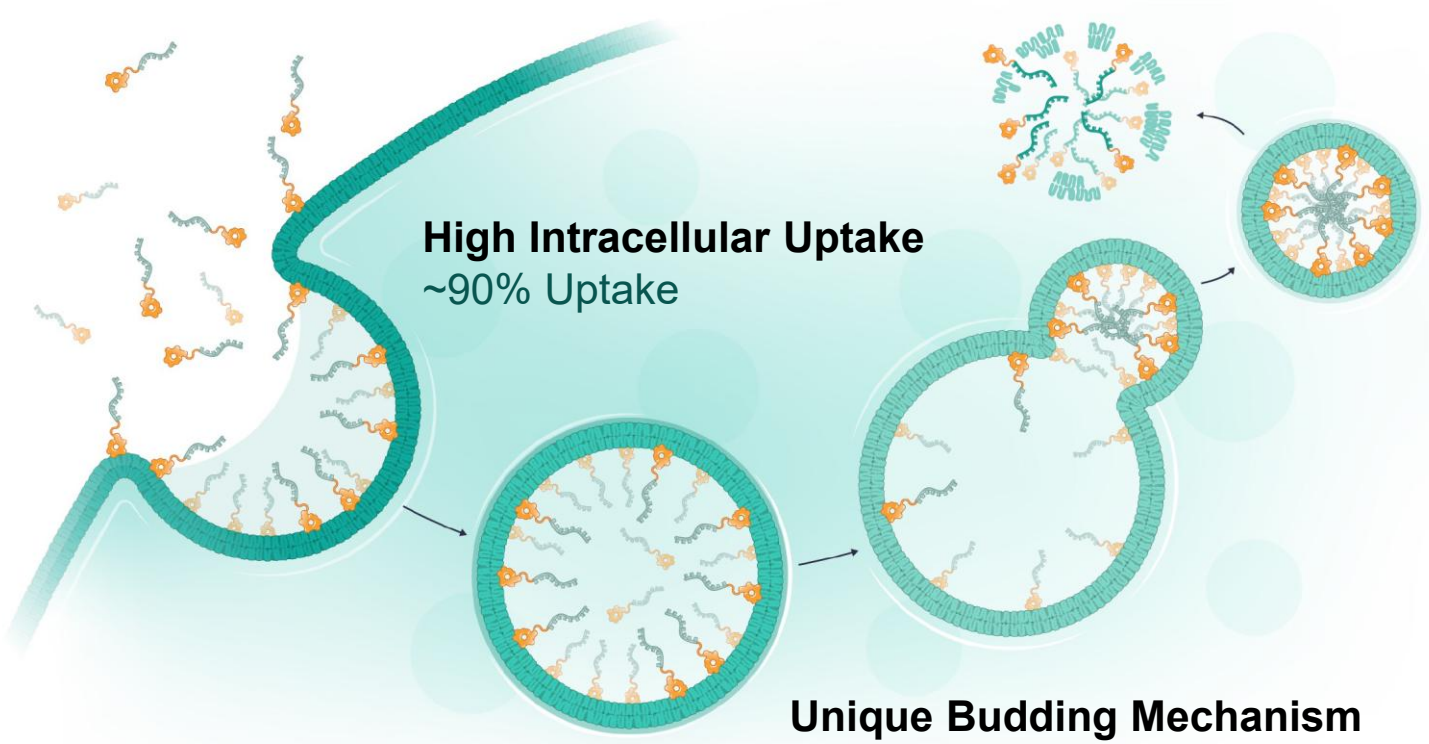
Unique chemistry
Improved uptake and endosomal escape

Cyclic structure
Extended half-life and increased stability

Phospholipid binding
Broad biodistribution to all cells

Consistent and predictable pharmacokinetics
Same EEV construct used across initial programs

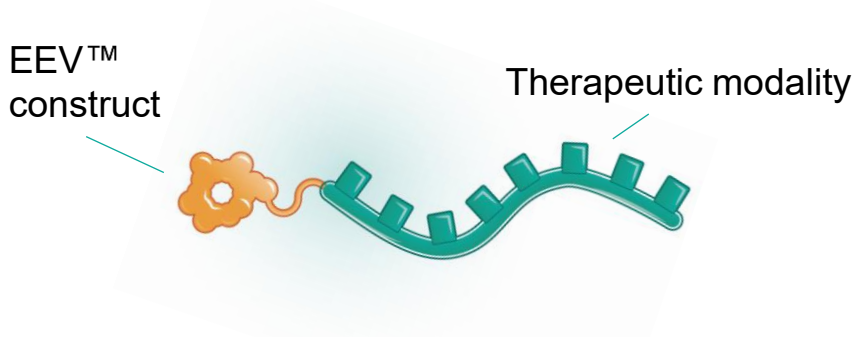
Efficient Endosomal Escape
~50% Escape vs. ~2% Standard



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.

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 EEV constructs in vivo to select for **pharmacodynamic activity** in target tissues
- Optimize **conjugation chemistry** for desired therapeutic modality

Screening Cascade for EEV Candidates

Chemically-diverse >500 member EEV library

EEV constructs with robust target cell uptake and efficacy

In vitro **functional validation** in relevant cell types with therapeutic payload

Well-tolerated EEV constructs with desired tissue functional delivery

Assess in vivo **functional delivery** in wild-type and disease models

Fit-for-purpose EEV candidate for target indication

Identify EEV candidate with desired **therapeutic profile**

EEV, endosomal escape vehicle.

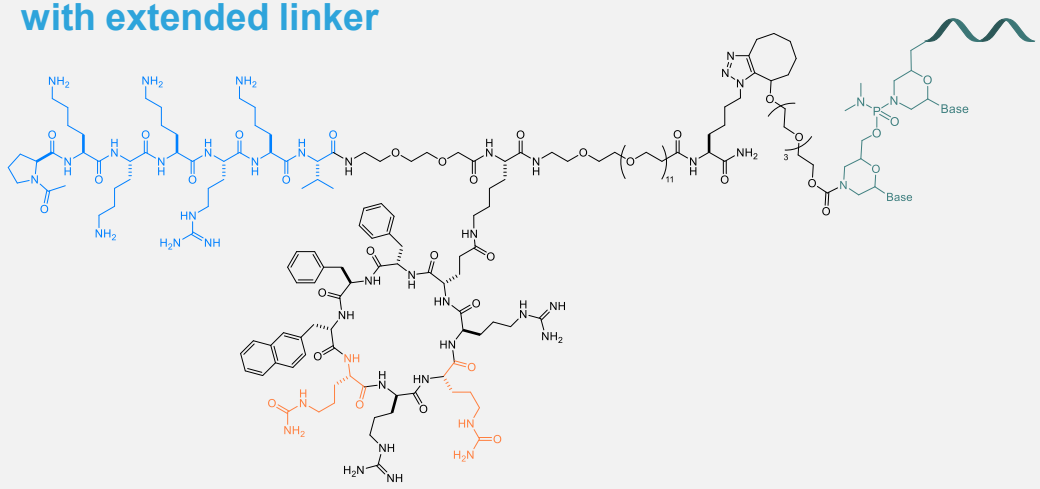
IMPROVED EEV-CONSTRUCTS FOR MUSCLE DELIVERY

Rational substitution of cationic residues with a surrogate results in robust functional delivery to skeletal and cardiac muscle

EEV3-PMO654 Structure and Medicinal Chemistry

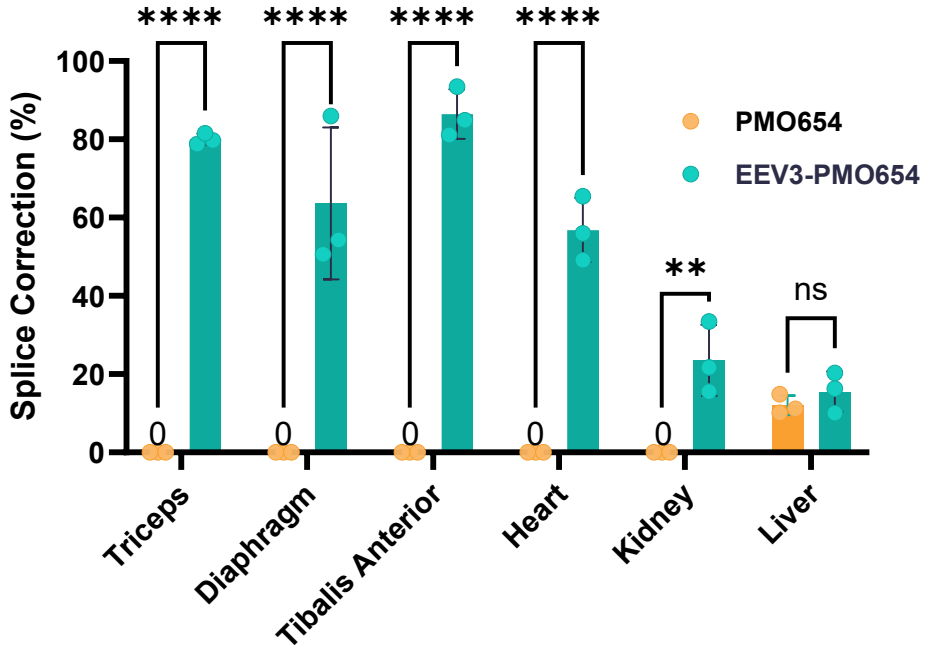
Exocyclic peptide sequence with extended linker

Conjugation with PMO



Substitution of positively charged arginine residues with neutral charged citrullines

Enhanced Functional Delivery to Muscle



- *EGFP654* mice were evaluated for splice correction 7 days following three weekly 10 mg/kg IV injections of PMO654 or EEV3-PMO654

p<0.01, ****p<0.0001; values are shown as mean ± standard deviation. **EEV, endosomal escape vehicle; **IV,** intravenous; **ns**, not significant; **PMO**, phosphorodiamidate morpholino oligomer.

DUCHENNE MUSCULAR DYSTROPHY

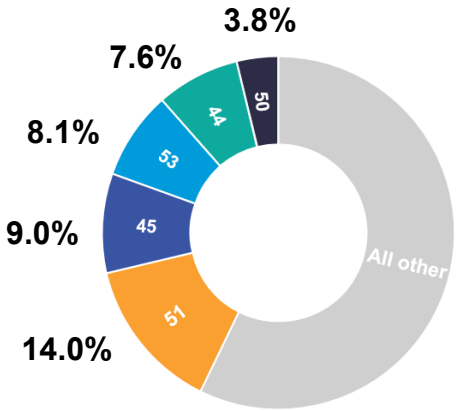
SIGNIFICANT THERAPEUTIC NEED EXISTS WITHIN A VALIDATED DUCHENNE MARKET

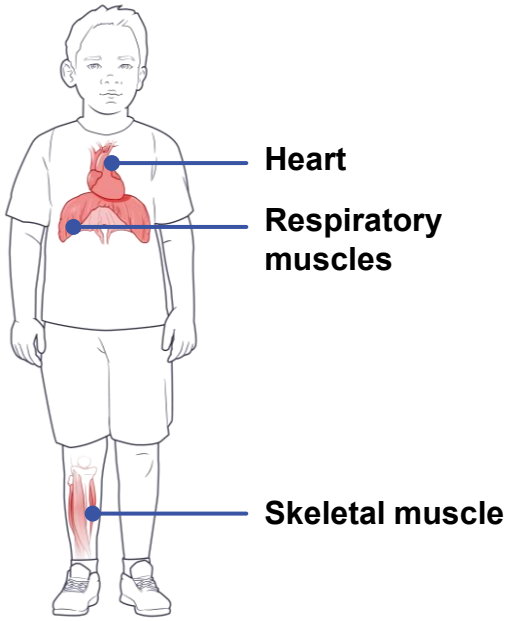
Duchenne is caused by **mutations in the *DMD* gene, which lead to a lack of functional dystrophin**, causing progressive loss of muscle function throughout the body

Exon skipping therapeutics have been approved based on **modest improvement in dystrophin levels ranging from ~1% to 6%**[4-7]

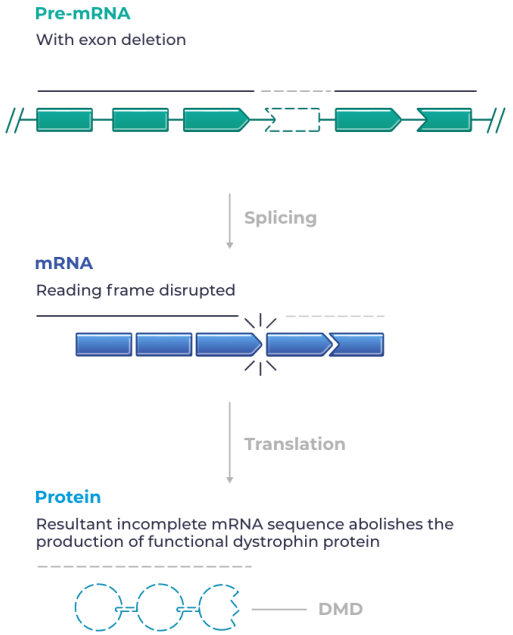
Approximately **41,000** people in the **U.S.**[1] **and Europe**[2] have Duchenne

>40% of patients with Duchenne[3] have mutations amenable to exon skipping of exons 44, 45, 50, 51 and 53

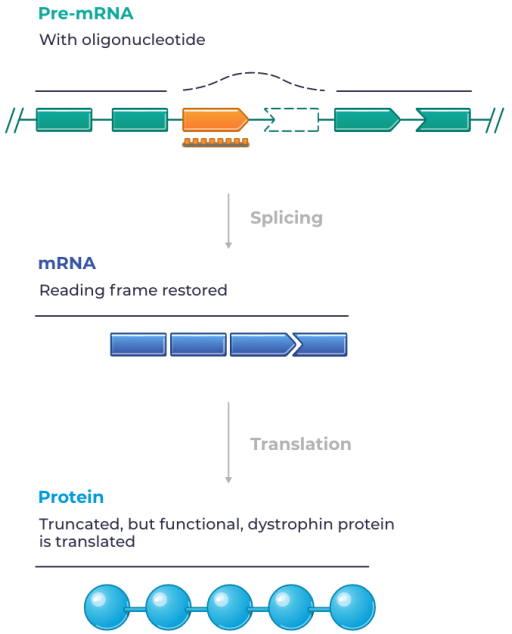




Patients with Duchenne



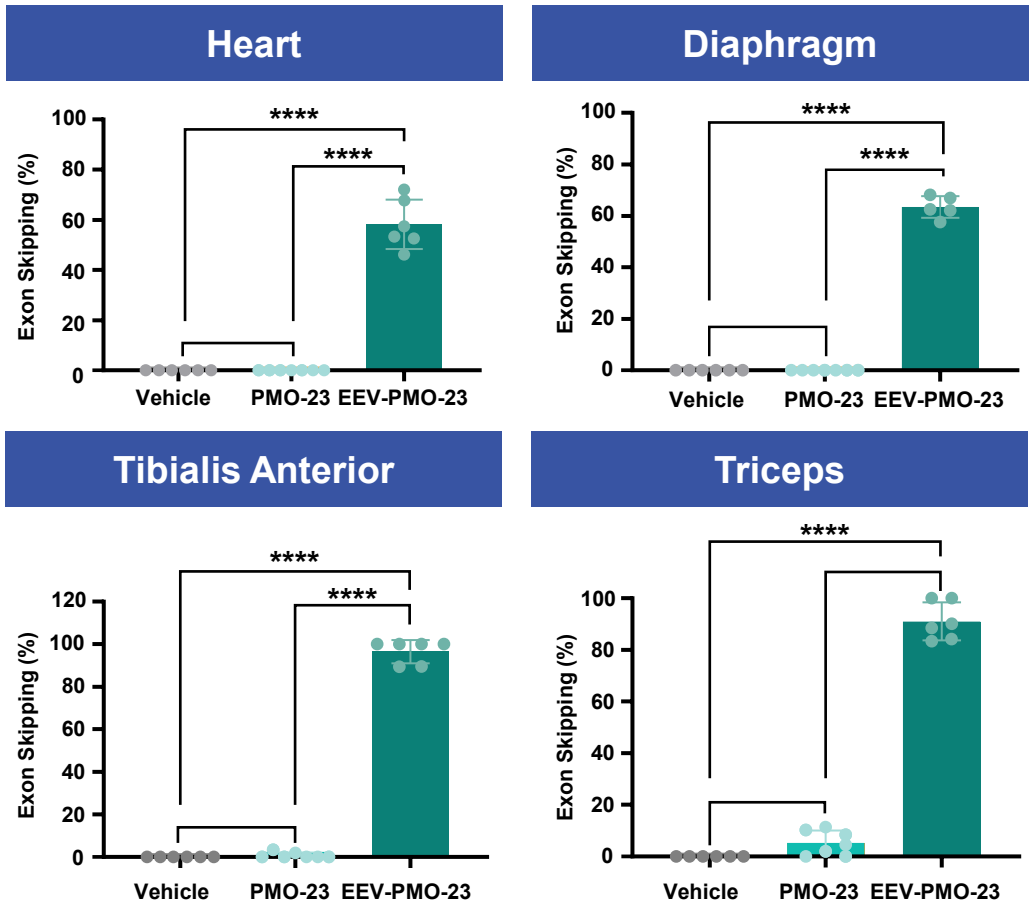
EEV-Oligonucleotide Approach



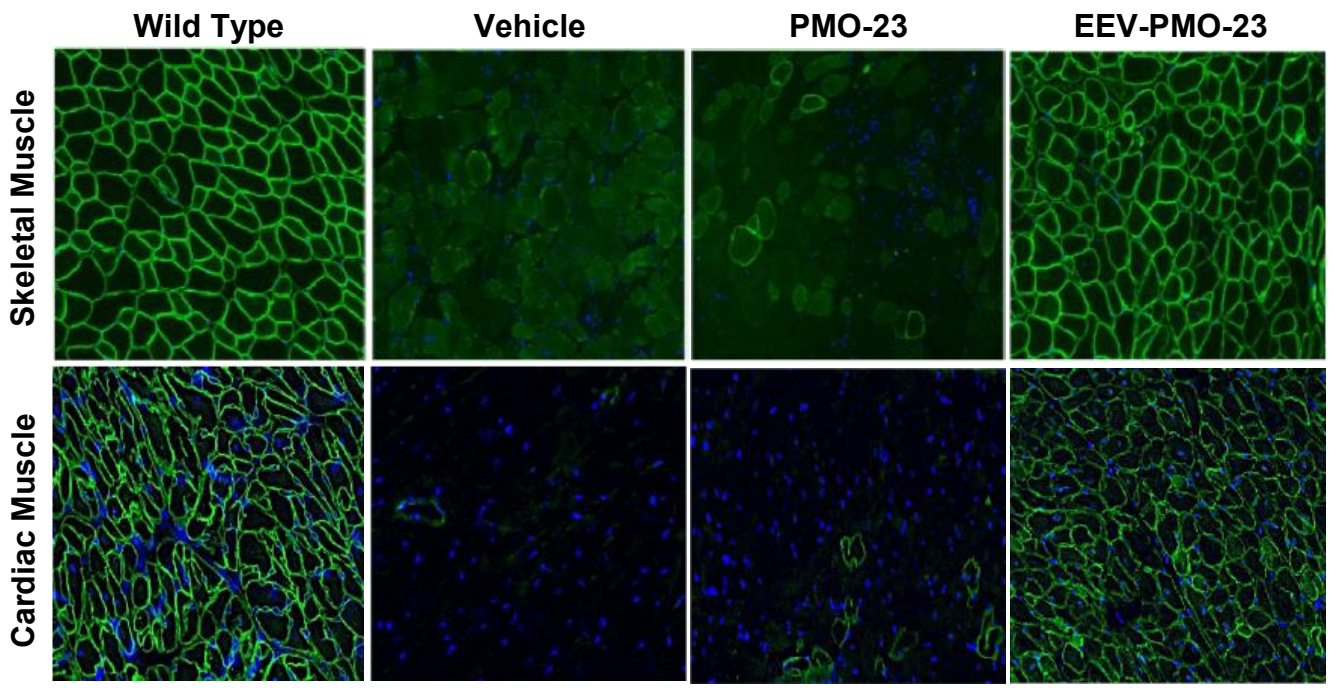
1. Parent Project Muscular Dystrophy. https://www.parentprojectmd.org/about-duchenne/. Accessed August 18, 2023. **2.** Europeans Medicines Agency. https://www.ema.europa.eu/en/medicines/human/orphan-designations/eu3202375. Accessed August 18, 2023. **3.** Bladen, C.L. et al. *Hum Mutat*. 2015. **4.** AMONDYS 45 PI. **5.** VILTEPSO PI. **6.** VYONDYS 53 PI. **7.** EXONDYS 51 PI. **DMD**, Duchenne muscular dystrophy; **EEV**, endosomal escape vehicle

REPEAT EEV-PMO-23 TREATMENT IN D2-*mdx* MICE

Robust exon 23 skipping after 4 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



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

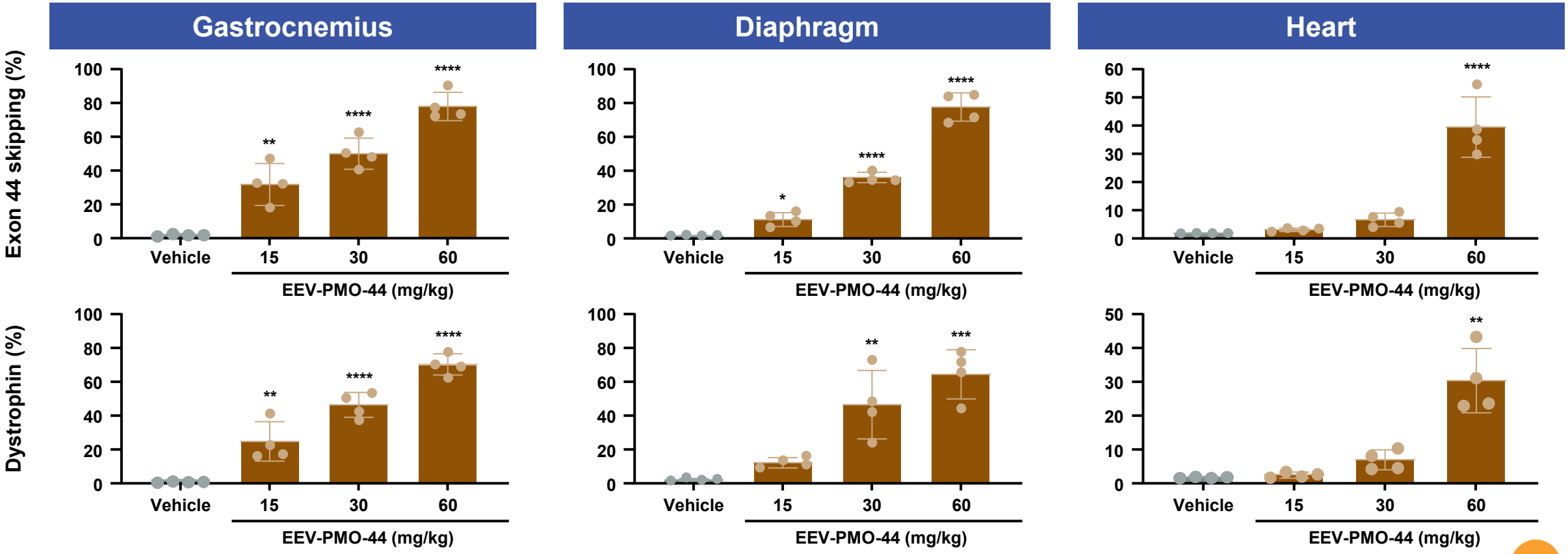
****$p<0.0001$. Values are shown as mean ± standard deviation. **D2-*mdx*** is a DMD mouse model with a nonsense mutation in *DMD* exon 23 (Coley, W.D. et al. *Hum. Mol. Genet.* 2016). Li, X. et al. *Mol. Ther. Nucleic Acids* 2023. **EEV**, endosomal escape vehicle; **DMD**, Duchenne muscular dystrophy; **IV,** intravenous; **ns**, not significant; **PMO**, phosphorodiamidate morpholino oligomer; **PMO-23**, mouse *DMD* exon 23 skipping phosphorodiamidate morpholino oligomer.

EEV-PMO PRECLINICAL STUDIES

EEV-PMO-44 EFFICACY IN del45hDMD.*mdx* MICE

A single dose of EEV-PMO-44 produced robust human *DMD* exon 44 skipping and dystrophin production 2 weeks post-dose in mice amenable to exon 44 skipping

*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ vs. Vehicle

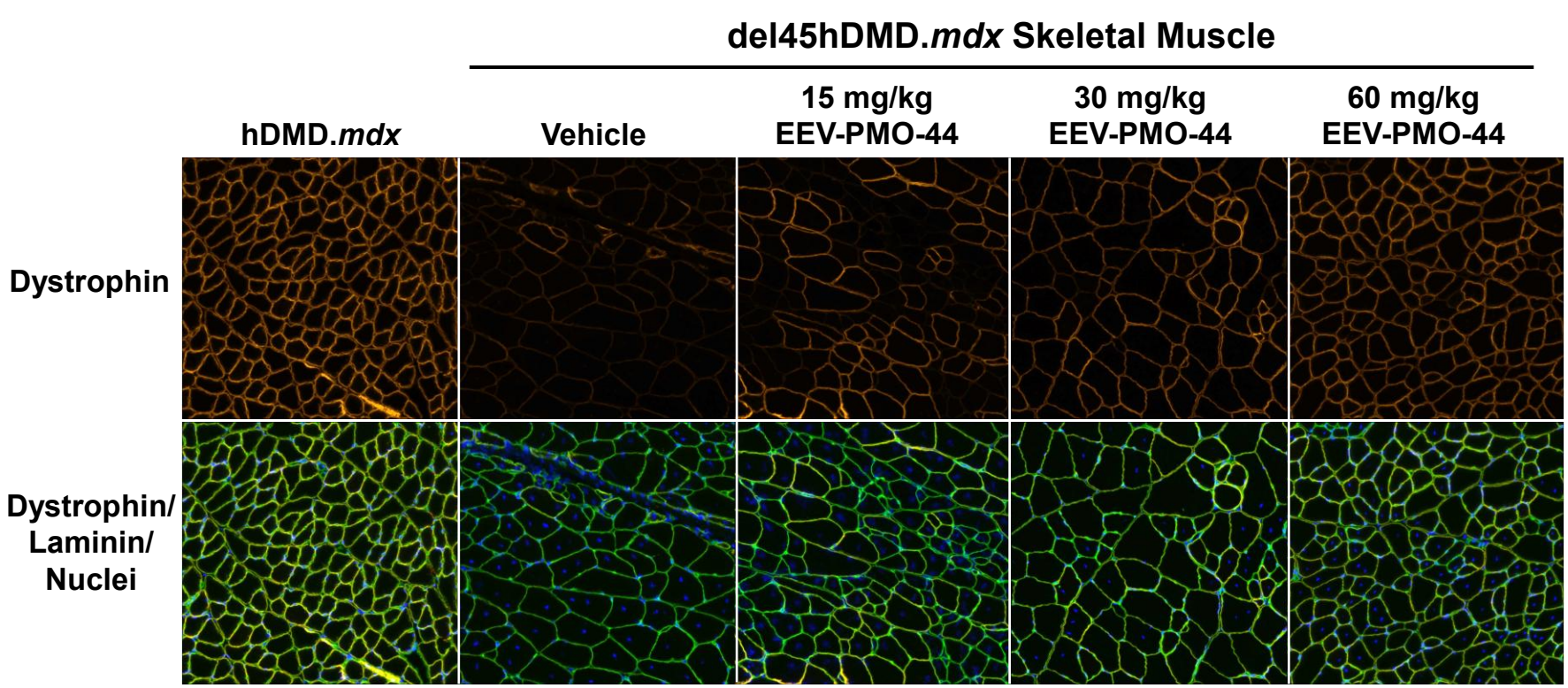


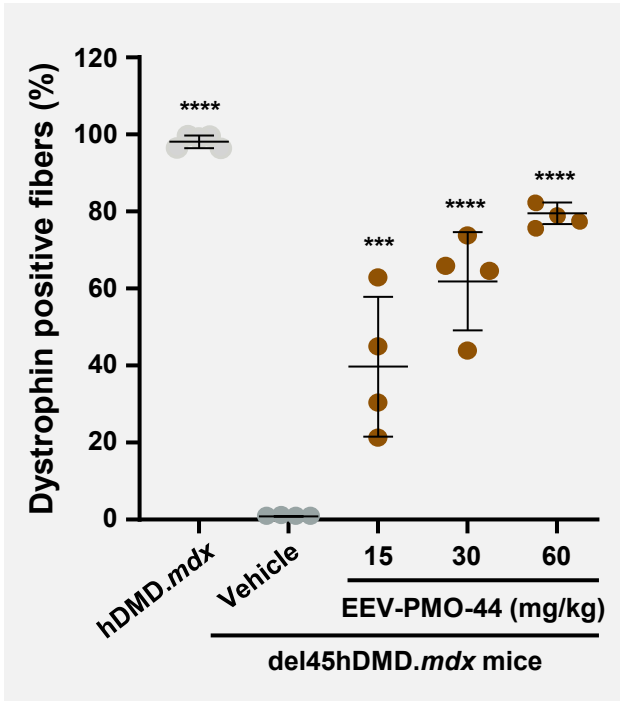
del45hDMD.*mdx* mice were treated with a single IV injection of EEV-PMO-44 (*DMD* exon 44 skipping EEV-oligonucleotide construct) or vehicle. Human *DMD* exon 44 skipping (top) and dystrophin protein expression (bottom) were analyzed in the gastrocnemius, diaphragm, and heart 2 weeks after the final dose. Percent dystrophin protein restoration is normalized to total protein and normalized to hDMD.*mdx* controls. Data shown as mean ± standard deviation. One-way ANOVA was used for statistical comparison; **ANOVA**, analysis of variance; **hDMD**, human dystrophin transgene; **IV**, intravenous.

DYSTROPHIN LOCALIZATION WITH EEV-PMO-44 IN del45hDMD.*mdx* Mice

EEV-PMO-44 produced dose-dependent increases in dystrophin-positive muscle fibers localized to the sarcolemma of del45hDMD.*mdx* mice 2 weeks post-dose

***$p \leq 0.001$, ****$p \leq 0.0001$ vs. Vehicle





- del45hDMD.*mdx* mice were treated with a single IV dose of EEV-PMO-44 or vehicle. Dystrophin protein distribution and cellular localization were analyzed by immunofluorescence in the gastrocnemius 2 weeks post-dose.

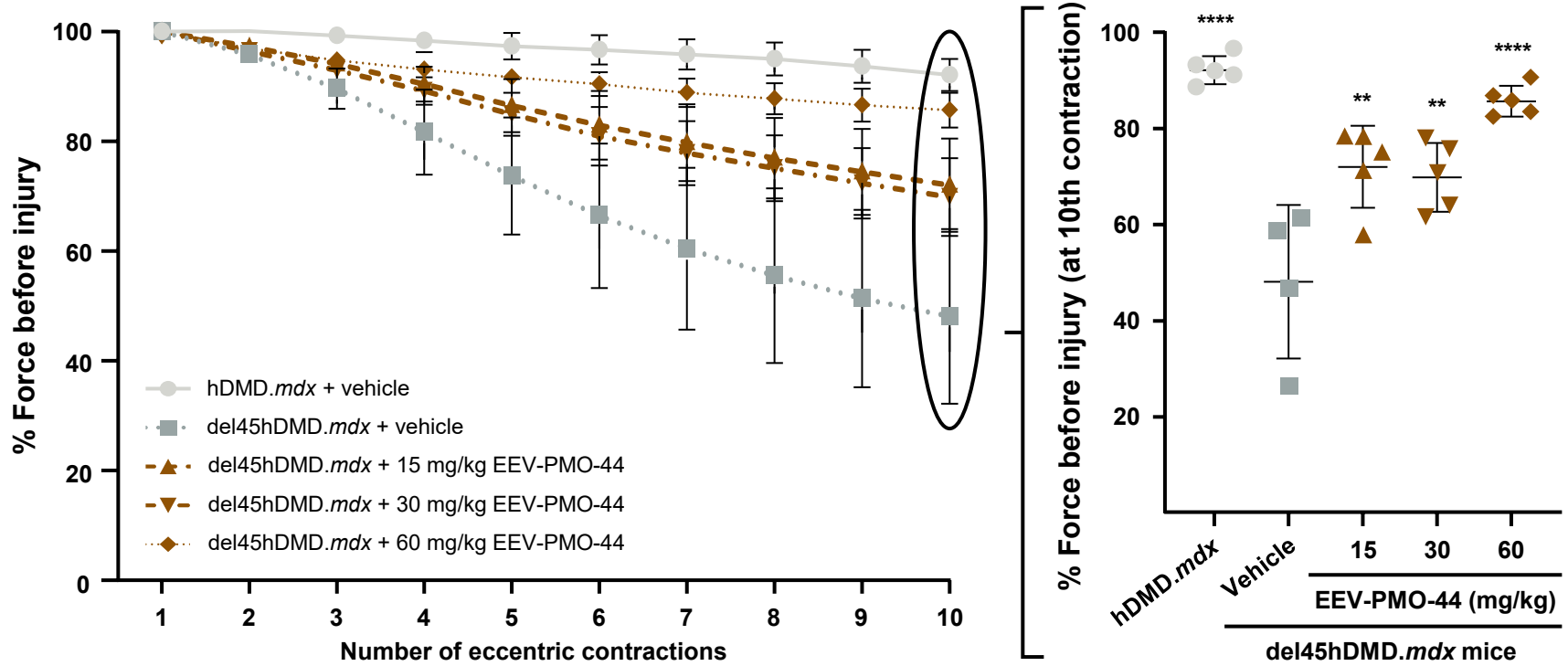
Quantification via Halo Image Analysis Software is shown as the percentage of dystrophin-positive muscle fibers relative to the total number of muscle fibers as determined by laminin staining (green) and dystrophin staining (red); co-localization to the sarcolemma appears yellow and nuclei appear blue. Data shown as mean ± standard deviation. One-way ANOVA was used for statistical comparison; **ANOVA**, analysis of variance; **hDMD**, human dystrophin transgene; **IV**, intravenous.

EEV-PMO-44 IMPROVES MUSCLE FUNCTION IN del45hDMD.*mdx* Mice

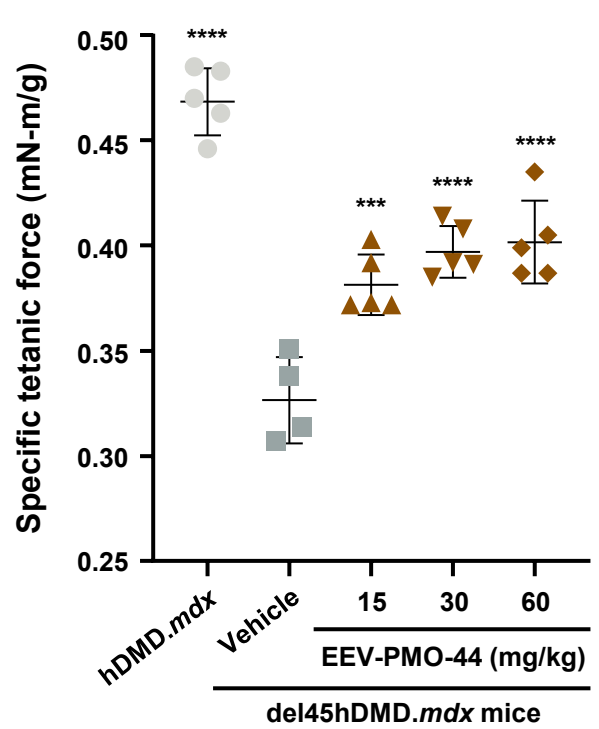
A dose-dependent increase in resistance to membrane damage was observed following the tenth contraction, as well as an increase in tetanic force 2 weeks post-dose of EEV-PMO-44

$^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ vs. Vehicle

Skeletal Muscle Membrane Stability



Specific Tetanic Force

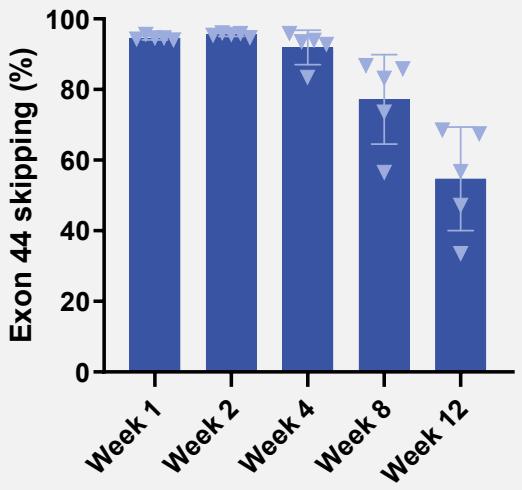


del45hDMD.*mdx* mice were treated with a single IV injection of EEV-PMO-44 or vehicle. ECC-induced muscle force loss generated by repeated ECC contraction and tetanic force of the gastrocnemius muscle was assessed 2 weeks post-dose. Data (mean ± standard deviation) shown across 10 ECC contractions normalized into a percentage of the initial force before any ECC contractions and as the percentage of force retained after the 10th contraction. Vehicle-treated hDMD.*mdx* mice were used as a control group for normal muscle function. One-way ANOVA was used for statistical comparison to vehicle-treated del44hDMD.*mdx* mice. **ECC**, eccentric force; **hDMD**, human dystrophin transgene; **IV**, intravenous.

CONSISTENT AND DURABLE EFFICACY OF EEV-PMO WAS DEMONSTRATED ACROSS SPECIES

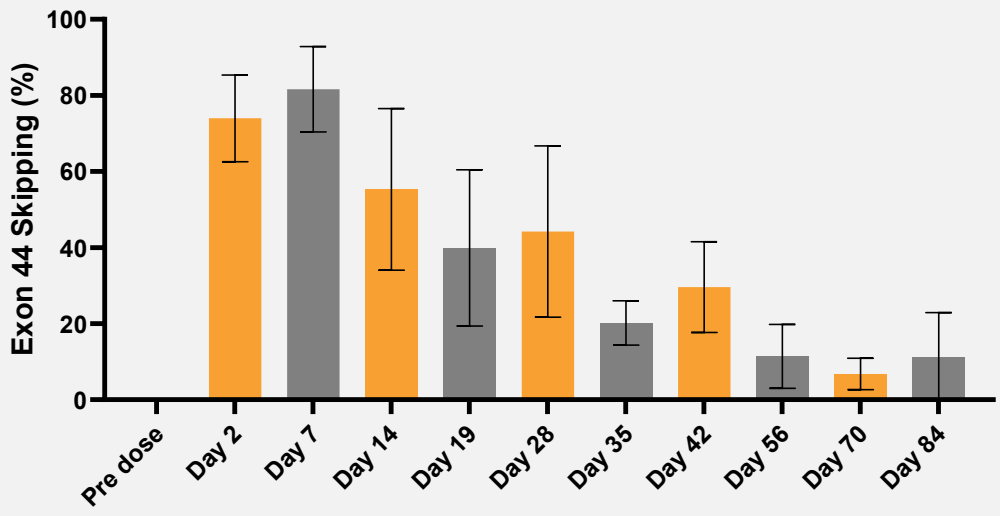
Significant patient benefit is implied by data in the mouse and the monkey at clinically relevant levels; *in vitro* data suggests much higher target engagement in patient cells

Exon 44 Skipping in hDMD Mouse



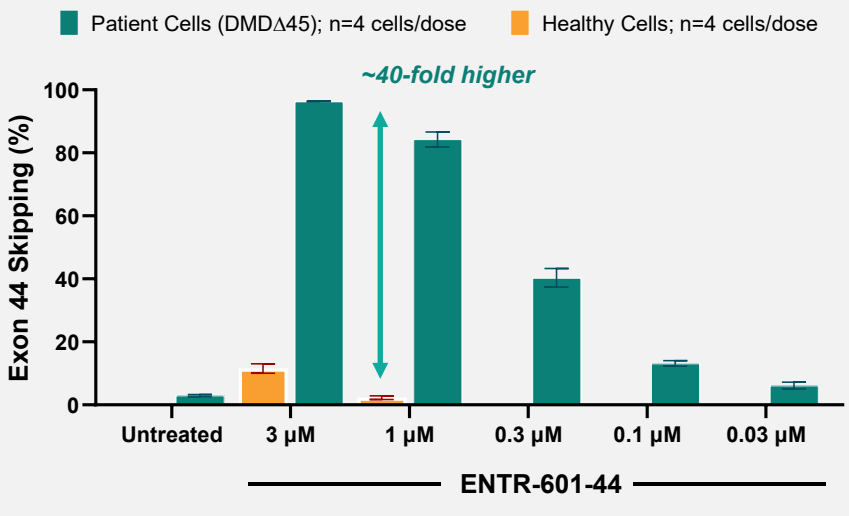
- Single IV 80 mg/kg dose of ENTR-601-44
- Tibialis Anterior

Exon 44 Skipping in Monkey



- Post IV infusion of single 45 mg/kg dose of ENTR-601-44, robust exon 44 skipping observed in biceps of treated monkeys (n=3 per cohort) for at least 12 weeks

Exon 44 Skipping in Healthy and Patient-Derived Muscle Cells



- Robust dose-dependent exon 44 skipping was observed in DMD patient-derived muscle cells harboring an exon 44 skip-amenable mutation

hDMD transgenic mice (left) express full-length human dystrophin gene ('t Hoen, A.C. et al. *J. Biol. Chem.* 2008). **DMDΔ45** (right) are immortalized myoblasts from DMD patients harboring an out-of-frame exon 45 deletion and further differentiated into myotubes. Values are shown as mean ± standard deviation. **ENTR-601-44** is a *DMD* exon 44 skipping EEV-oligonucleotide construct. **DMD**, Duchenne muscular dystrophy; **hDMD,** human Duchenne muscular dystrophy; **IV,** intravenous.

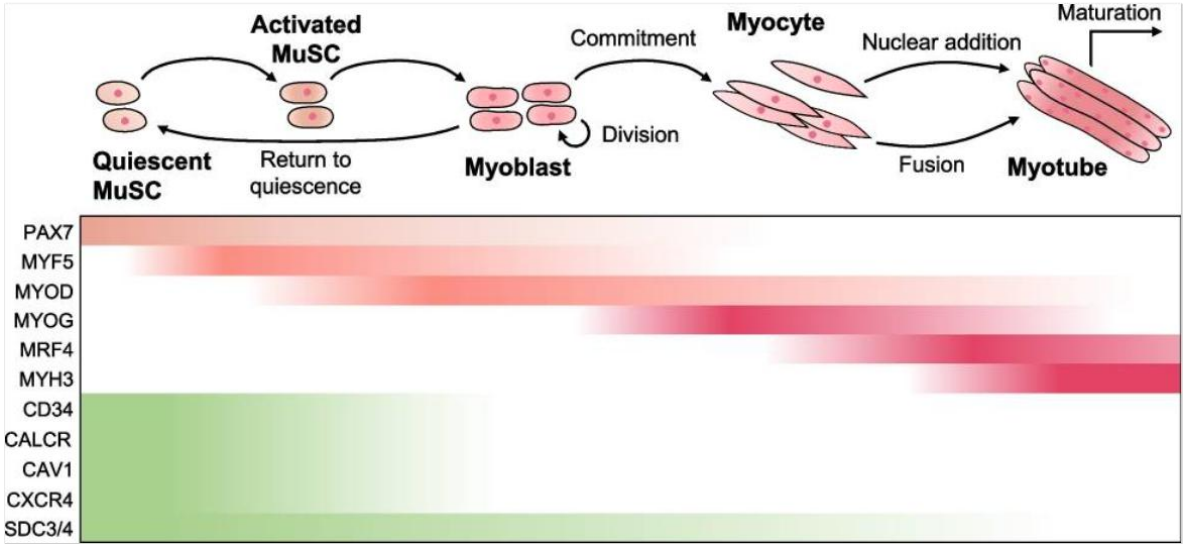
DELIVERY OF PMO TO SATELLITE CELLS

MUSCLE SATELLITE CELLS

Muscle satellite cells as a new therapeutic target with potential in several neuromuscular disorders

- **Quiescent satellite cells are historically challenging to access by therapeutic modalities**
  - Despite notable advancements in therapeutic strategies for DMD, current treatments primarily target dystrophic myofibers and show modest ability to generate dystrophin
  - The lack of dystrophin leads to changes in phenotype and function of satellite cells, exacerbating disease progression by impairing muscle regeneration[1]
- **Efficient delivery of EEV-PMO to quiescent satellite cells could enable early disease intervention**
  - EEV constructs have effectively facilitated the delivery of exon skipping PMOs to skeletal and cardiac muscle of mice
  - Targeted correction of satellite cell dysfunction in DMD has been shown to ameliorate pathophysiology in preclinical models[2]

Developmental Stages of Muscle Satellite Cells[3]



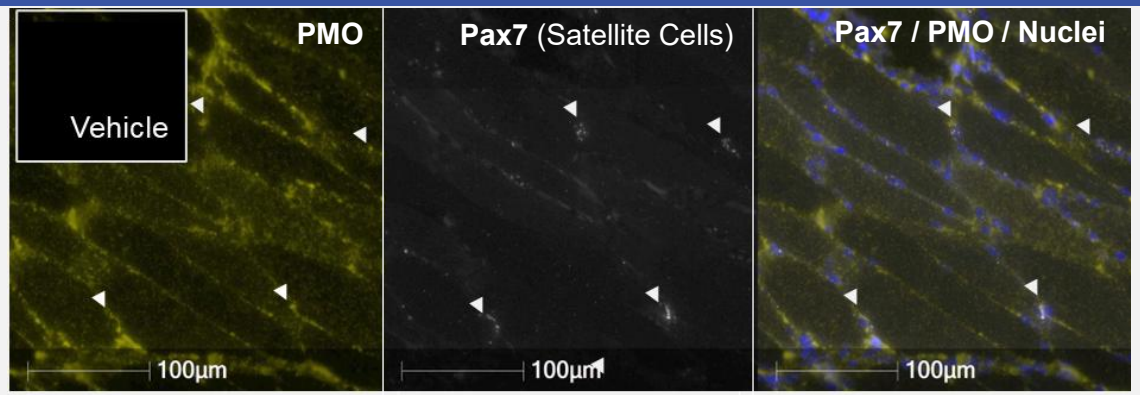
1. Kodippili & Rudnicki *Front Physiol*, 2023; 2. Kwon et al. *Mol Ther Meth Clin Dev*, 2020; 3. Byun et al., *Inflamm Regen*, 2024.

DISTRIBUTION OF EEV-PMO TO SATELLITE CELLS

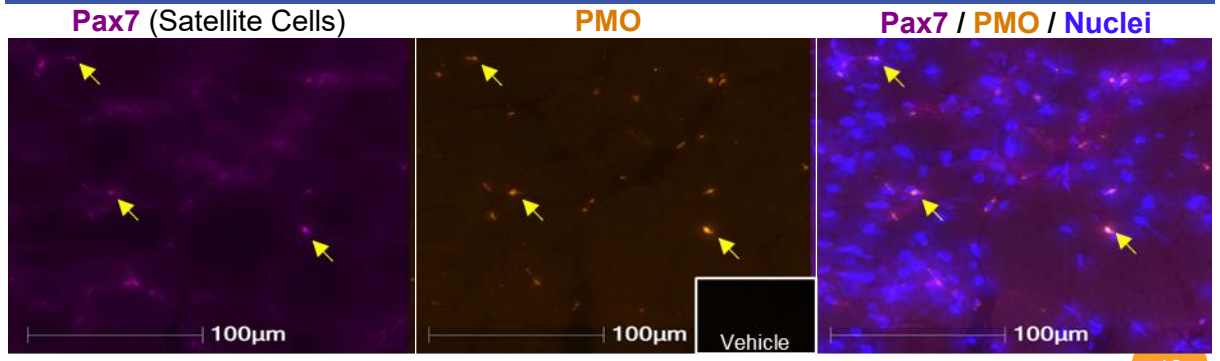
EEV-PMO efficiently co-localizes with quiescent satellite cells (Pax7 positive) at 48 hours; Qualitative data demonstrates that co-localization lasts at least 1-week post-dose

- **Two independent molecular techniques were utilized to determine EEV-PMO distribution within specific cell lineages across muscle tissue**
  - RNA-ISH: Highly selective and sensitive technique to assess specific cell lineages across muscle tissue (top panel)
  - Immunohistochemistry Assessment (bottom panel)
- **Analysis of RNA-ISH data confirms that EEV-PMO is co-localized in 100% of satellite cells at 48 hours**
  - Quantitative assessment confirms qualitative data (data not shown)
- **Qualitative assessment of IHC data demonstrates co-localization of satellite cells in hDMD mice with EEV-PMO at 7 days**

PMO Distribution (48 hours, RNA-ISH Quantitation)



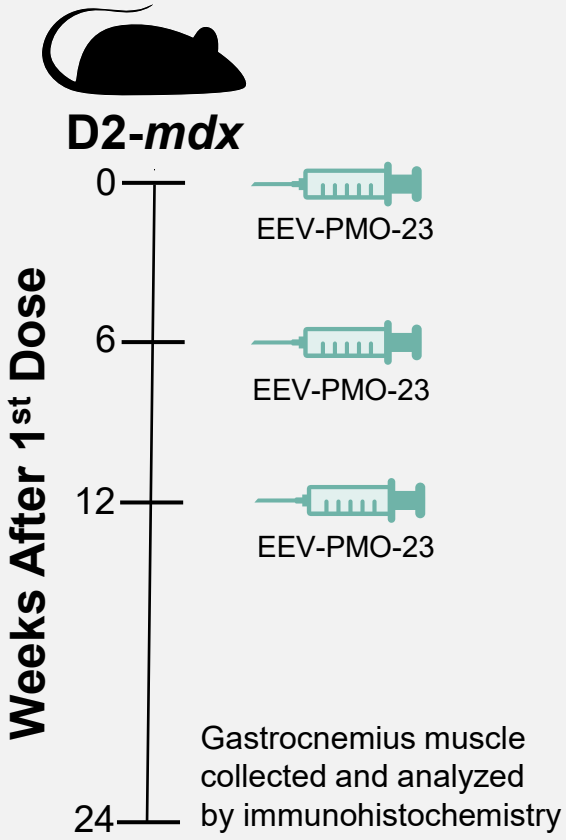
PMO Distribution in hDMD Mice (Day 7, IHC)

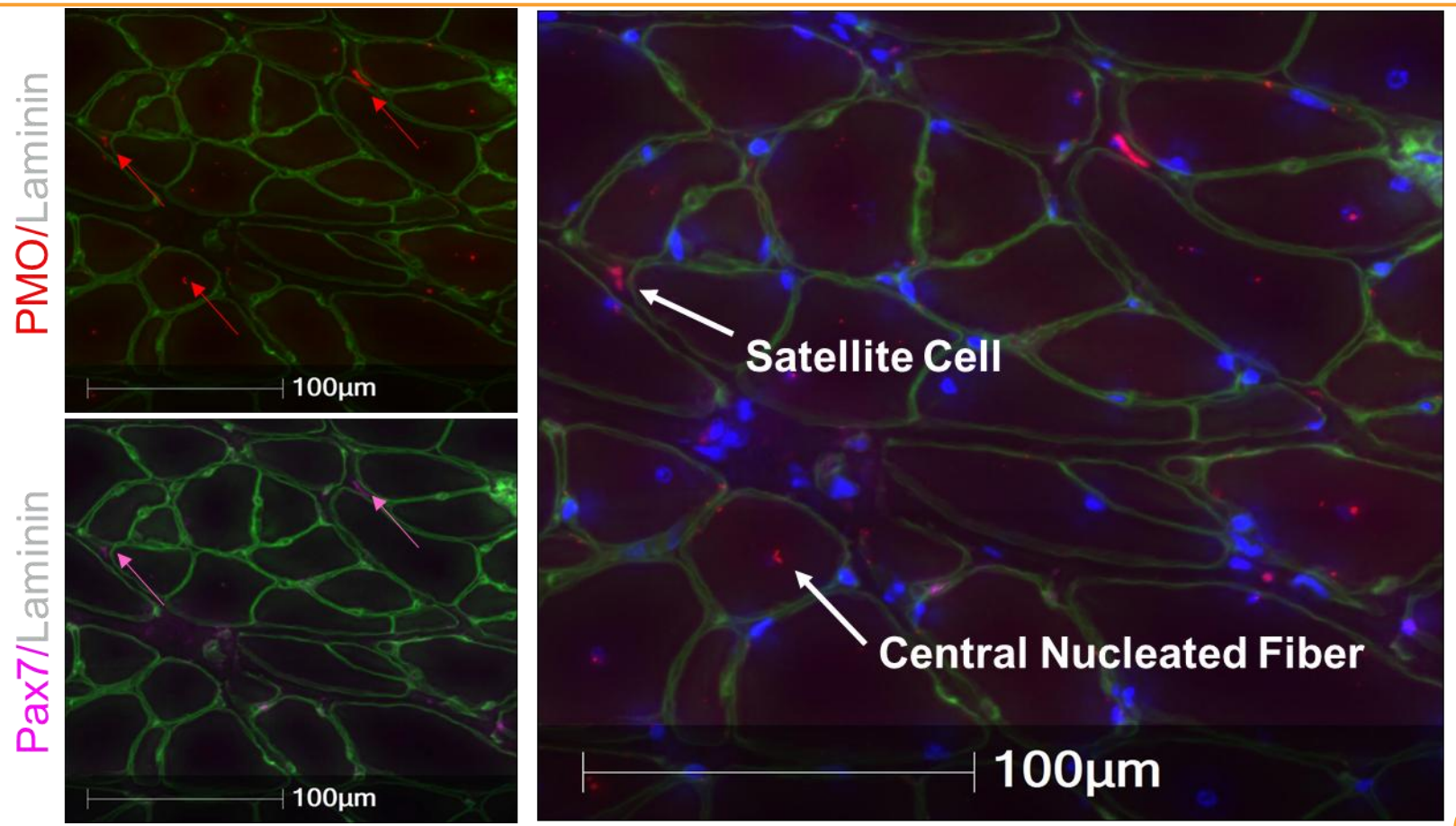


(Top right) D2-*mdx* mice were treated with a single IV dose of EEV-PMO; Gastrocnemius was collected at 48 hours and analyzed by RNA-ISH. (Bottom right) hDMD mice were treated with a single IV dose of EEV-PMO; Gastrocnemius was collected at 7 days and tissues analyzed by IHC. Pax7 is a satellite cell marker (Seale, P. et al *Cell* 2000). **hDMD,** human Duchenne muscular dystrophy; **IV,** intravenous; **RNA-ISH**: RNA *in situ* Hybridization image analysis; **IHC**: immunohistochemistry.

PMO-23 PERSISTS IN SATELLITE CELLS OF *D2-mdx* MICE 12 WEEKS AFTER FINAL DOSE

PMO-23 co-localizes with satellite cells and newly regenerated centrally nucleated fibers 12 weeks post washout after 3 Q6W doses

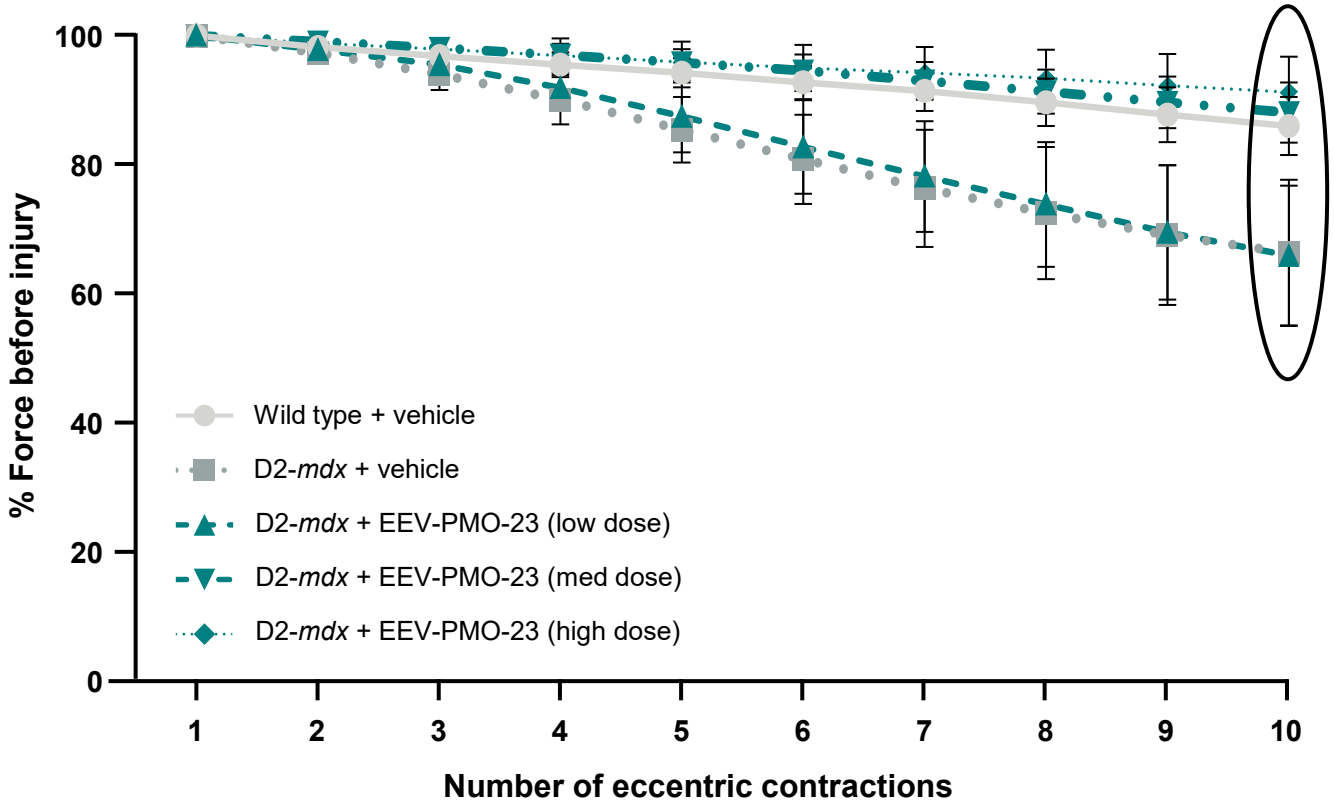


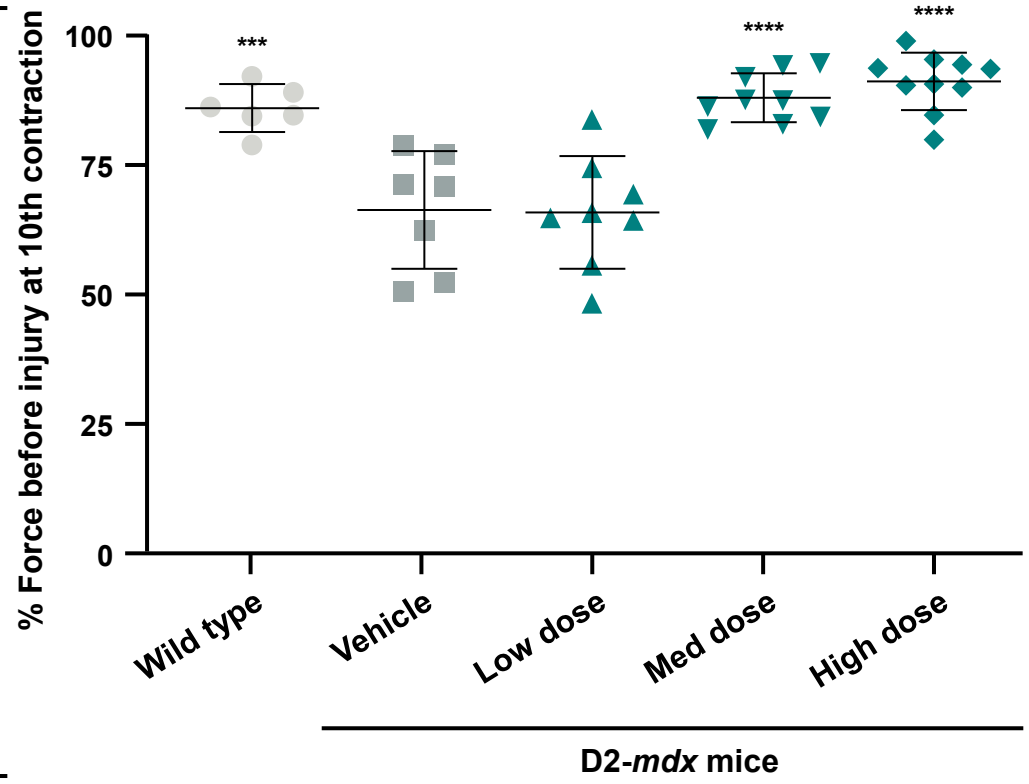


D2-*mdx* mice were treated with 3 IV doses of EEV-PMO-23 administered every six weeks; Gastrocnemius was collected 12 weeks after the 3rd dose and analyzed by immunohistochemistry. **Q6W**: every six weeks.

EEV-PMO-23 IMPROVES MUSCLE FUNCTION IN *D2-mdx* MICE

Three Q6W doses of EEV-PMO (medium and high dose) maintain a significantly improved tetanic force and restored the membrane stability to wild type mice

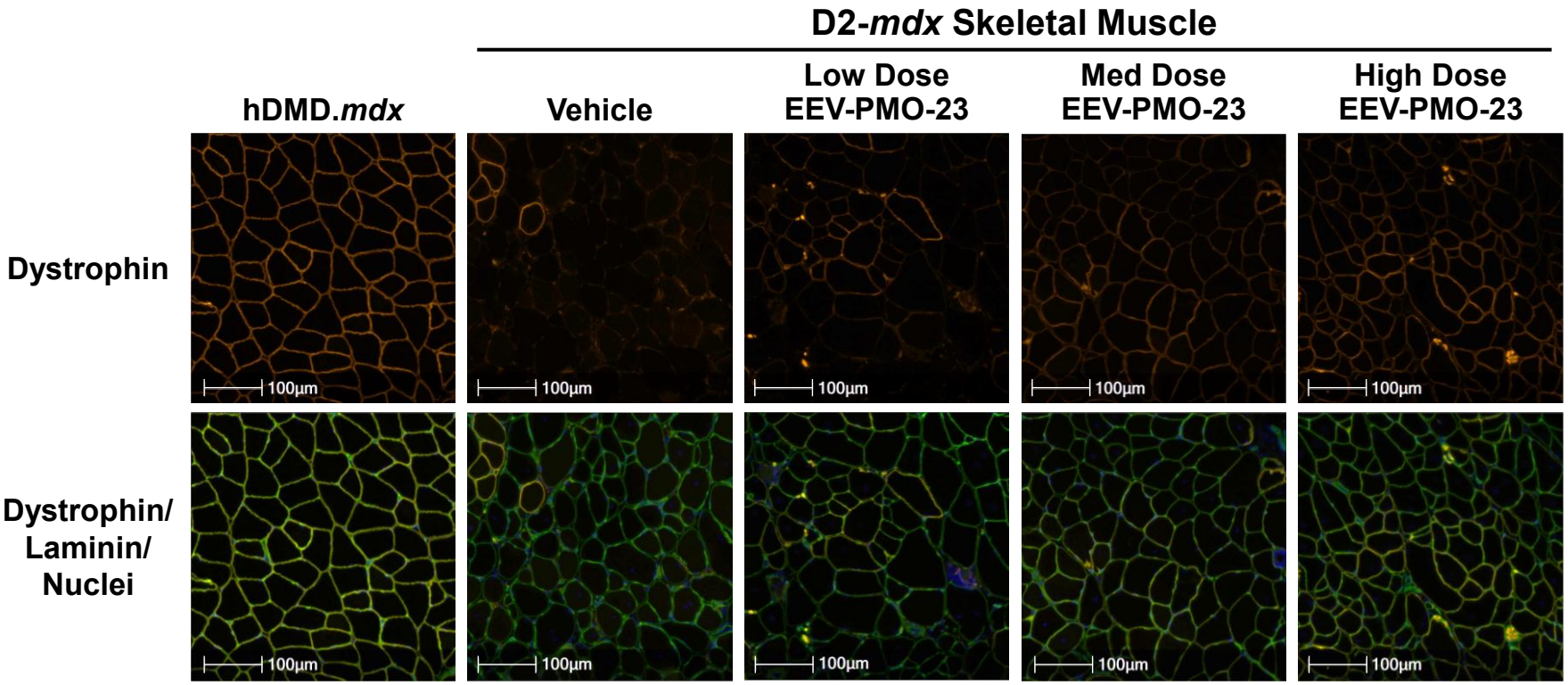


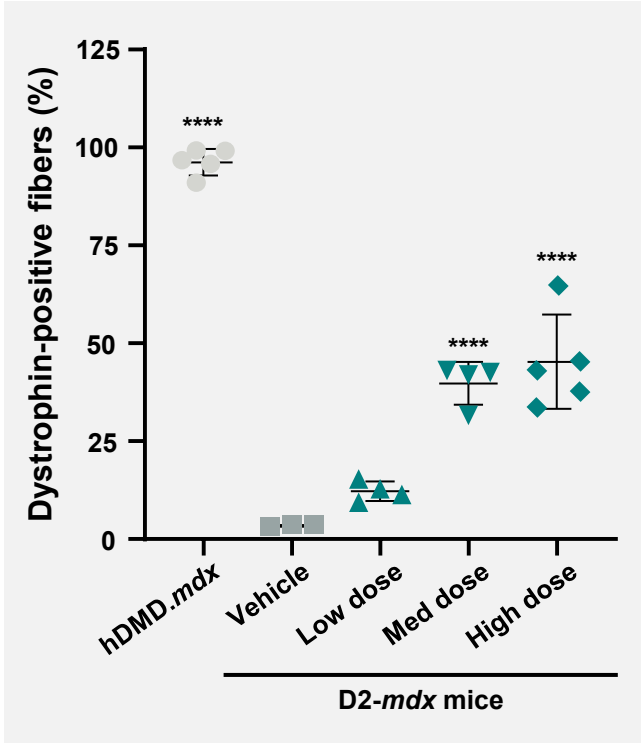


D2-*mdx* mice were treated with 3 Q6W IV injections of EEV-PMO-23 or vehicle. ECC-induced muscle force loss generated by repeated ECC contraction of the gastrocnemius muscle was assessed 12 weeks after the third dose. Data (mean ± standard deviation) shown across 10 ECC contractions normalized into a percentage of the initial force before any ECC contractions and as the percentage of force retained after the 10th contraction. One-way ANOVA was used for statistical comparison. ***$p<0.001$, ****$p<0.0001$. **ECC**, eccentric force; **IV**, intravenous; **med**, medium; **Q6W**, every six weeks.

DYSTROPHIN LOCALIZATION WITH EEV-PMO-23 IN D2-*mdx* MICE

Three Q6W doses of EEV-PMO-23 produced dose-dependent increases in dystrophin-positive muscle fibers localized to the sarcolemma of D2-*mdx* mice 12 weeks after the third dose





- D2-*mdx* mice were treated with a three Q6W IV doses of EEV-PMO-23 or vehicle. Dystrophin protein distribution and cellular localization were analyzed by immunofluorescence in the gastrocnemius 12 weeks post-dose.

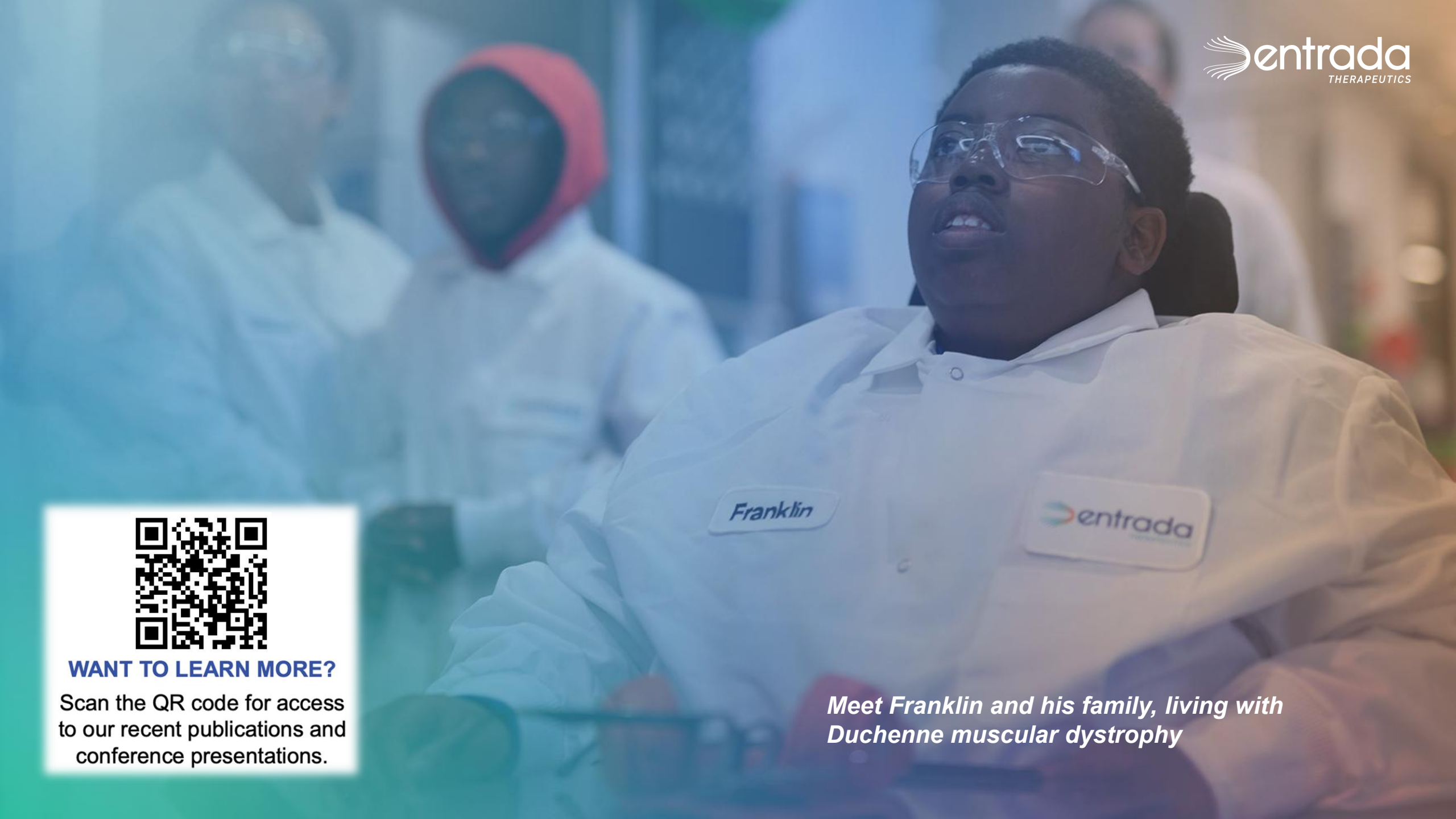
Quantification via Halo Image Analysis Software is shown as the percentage of dystrophin-positive muscle fibers relative to the total number of muscle fibers as determined by laminin staining (green) and dystrophin staining (red); co-localization to the sarcolemma appears yellow and nuclei appear blue. Data shown as mean ± standard deviation. One-way ANOVA was used for statistical comparison. ****$p \leq 0.0001$; **ANOVA**, analysis of variance; **hDMD**, human dystrophin transgene; **IV**, intravenous; **med,** medium.

SUMMARY

- **EEV-PMO constructs demonstrated significant exon skipping and dystrophin production across several models of DMD**
  - This suggests effective delivery of PMO therapeutics directly to muscle tissue by the EEV platform

- **PMO co-localizes with satellite cells and newly formed centrally nucleated fibers in skeletal muscle following EEV-PMO administration**
  - Improved dystrophin restoration and muscle function was observed following delivery of exon-skipping PMO therapeutics to the satellite cell compartment

- **The ability to deliver therapeutic PMOs to muscle fibers and to quiescent satellite cells holds potential for DMD and other neuromuscular disorders**



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