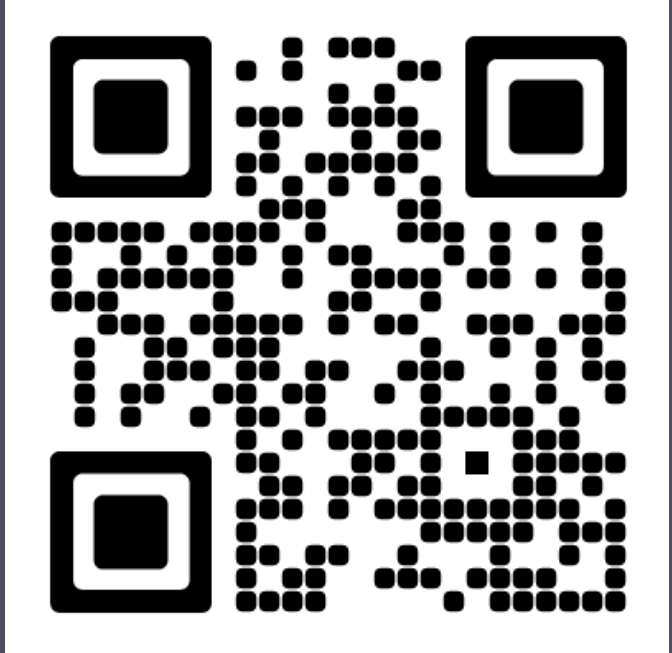




Development of the Endosomal Escape Vehicle (EEV™) Platform for Enhanced Delivery of Oligonucleotides to Skeletal and Cardiac Muscle

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INTRODUCTION

- Intracellular delivery of oligonucleotide therapeutics is challenging because of poor cell entry and limited escape from the endosome within the target cell.
- These limitations often necessitate high therapeutic doses and can be associated with less-than-optimal therapeutic activity.
- One strategy to overcome these limitations is conjugation of oligonucleotides to cell-penetrating peptides (CPPs).
- In comparison with linear CPPs, cyclic CPPs have been shown to efficiently penetrate the cell membrane, escape the endosome into the cytosol, and be more resistant to proteolytic degradation.^{1,2}
- To address these challenges, we designed a family of proprietary cyclic CPPs that form the basis of our EEV platform and are capable of intracellular delivery of covalently conjugated cargo.³
- In vivo efficacy of EEV-oligonucleotide conjugates was evaluated in *mdx*, EGFP-654, D2-*mdx*, del44hDMD.*mdx*, and del51hDMD.*mdx* mouse models.

OBJECTIVE

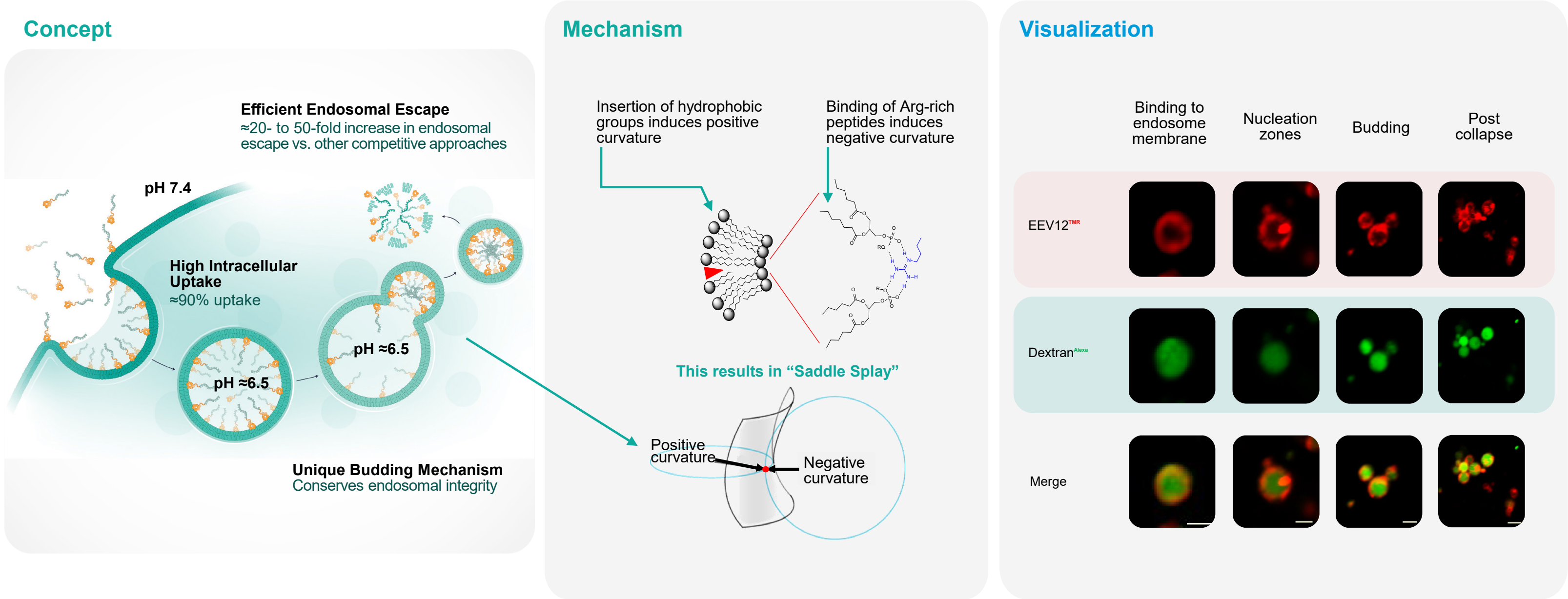
- To describe the design, optimization, and preclinical evaluation of the EEV platform for enhanced intracellular delivery of oligonucleotides to skeletal and cardiac muscle.

RESULTS

EEV Platform Mechanism of Action

- The overall mechanism of action of the EEV platform is shown in **Figure 1**.
- EEV peptides have a higher affinity to phospholipids in comparison with traditional linear CPPs; the interaction of EEV peptides with phospholipids is pH-dependent.
- The specific interaction of EEV peptides with the endosomal membrane facilitates the escape from early endosomes, as demonstrated by the correlation between endosomal escape efficiency and endosomal membrane-binding affinity.

Figure 1. pH-Dependent Binding Affinity Enhances EEV Endosomal Escape

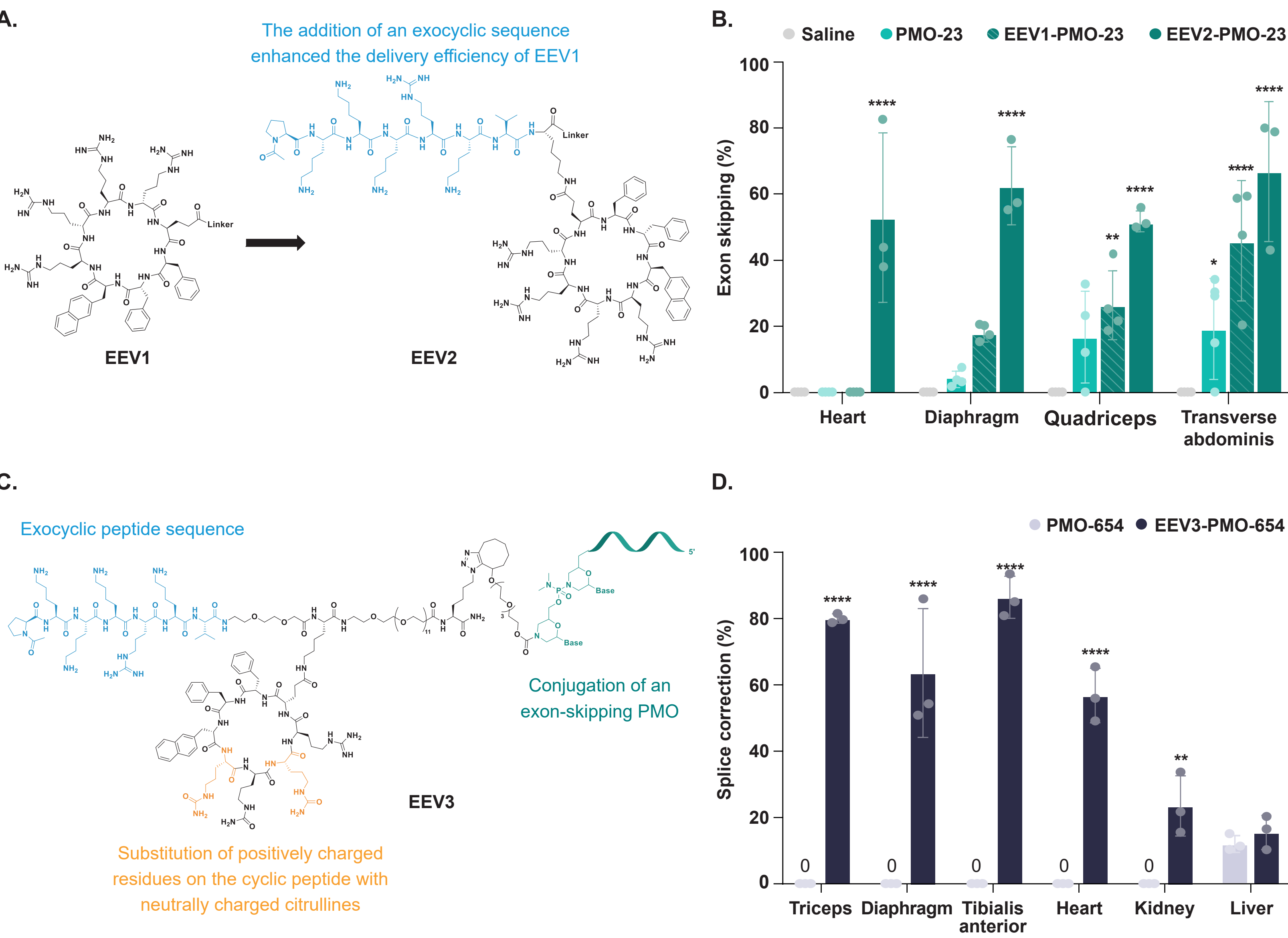


Midle panel: Used with permission of the American Chemical Society, from Dougherty PG, et al. Chem Rev. 2019;119(17):10241–10287; permission conveyed through Copyright Clearance Center, Inc. EEV, endosomal escape vehicle; TMR, tetramethylrhodamine.

EEV Peptides to Efficiently Deliver PMOs to Skeletal and Cardiac Muscle

- Optimization of the first-generation EEV cyclic CPPs identified specific chemical modifications that enhanced functional delivery of antisense oligonucleotides to skeletal and cardiac muscle³ (**Figure 2**).
- Addition of an exocyclic peptide sequence to a first-generation EEV construct (EEV1) (**A**) increased exon skipping in cardiac and skeletal muscle of *mdx* mice (**B**).
- The EEV platform was further optimized (EEV3) by replacing positively charged residues with neutrally charged residues (**C**). These modifications resulted in enhanced splice correction in both skeletal and cardiac muscle (**D**).
- The EEV3 construct was further optimized to generate the candidate EEV construct used in **Figure 3** and **Figure 4**.

Figure 2. EEV Peptides to Efficiently Deliver PMOs to Skeletal and Cardiac Muscle



(A) Development of a second-generation EEV (EEV2). (B) A single low dose of PMO-23 was administered by IV injection, and tissues were analyzed 7-days post-injection. EEV2 enhanced PMO-23 exon skipping in *mdx* mice ($p<0.0001$ vs. vehicle). (C) Development of a second-generation EEV (EEV3). (D) Three weekly low doses of PMO-654 were administered by IV injection, and tissues were analyzed 7 days after the last dose. EEV3 efficiently delivered PMO-654 to target tissues in EGFP-654 mice ($p<0.0001$ vs. PMO-654). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. EEV, endosomal escape vehicle; PMO, phosphorodiamidate morpholino oligomer.

ACKNOWLEDGMENTS

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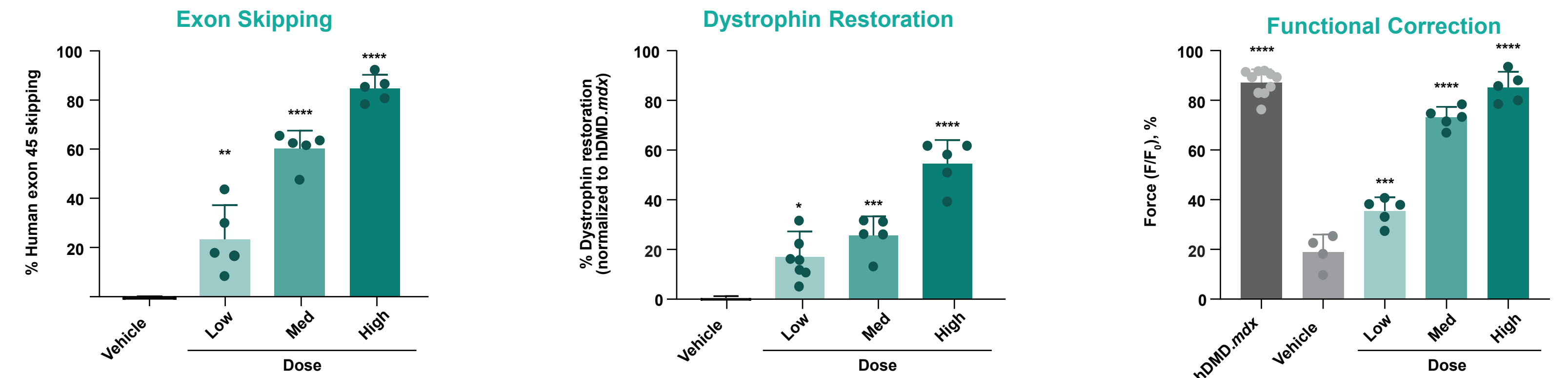
MATERIALS AND METHODS

- Exon skipping was quantified by reverse transcriptase-polymerase chain reaction in *mdx* and EGFP-654 mice and by digital droplet polymerase chain reaction in del44hDMD.*mdx* and del51hDMD.*mdx* mice.
- Immunohistochemistry was used to demonstrate muscle satellite cell uptake of EEV-oligonucleotide conjugates in D2-*mdx* mice.
- Dystrophin restoration was measured by automated capillary Western blot (Jess), and functional correction was measured by percentage force after 10 contractions in del44hDMD.*mdx* and del51hDMD.*mdx* mice.

Efficacy of ENTR-601-45 in Exon 45 Skipping Amenable Mice

- Administration of ENTR-601-45 led to a dose-dependent increase in exon skipping and dystrophin expression, which correlated to complete functional correction, in preclinical disease models of Duchenne muscular dystrophy (DMD) (**Figure 3**).

Figure 3. ENTR-601-45 Dose-Dependent Increase in Exon Skipping and Dystrophin Expression Correlated to Functional Correction

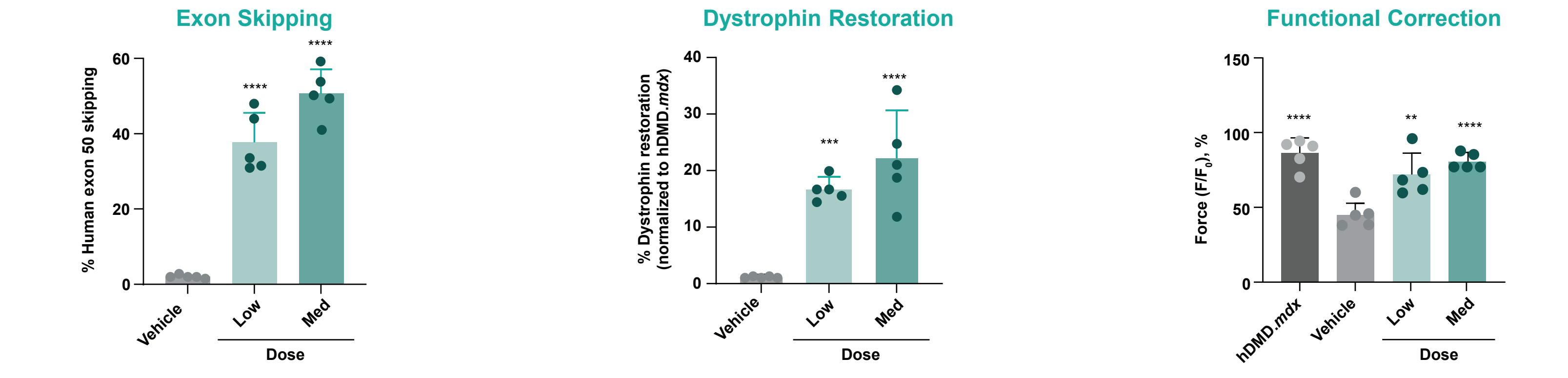


Active and vehicle del44hDMD.*mdx* mice, n=5 per cohort. ENTR-601-45 (Q6W × 3 doses). Control saline-treated hDMD.*mdx* mice, n=10 (Q6W × 3 doses). (Left) Skipping (ddPCR) and (Middle) dystrophin production (Jess) was significantly increased 6 weeks after the third dose of ENTR-601-45 (gastrocnemius muscle shown). ECC-induced muscle force loss generated by ECC contraction of the gastrocnemius muscle was assessed 5 weeks after the third dose. Data (mean ± standard deviation) is shown across 10 ECC contractions (right) normalized into a percentage of the initial force before any ECC contractions and as the percentage of force retained after the tenth contraction. One-way ANOVA was used for statistical comparison to vehicle-treated del44hDMD.*mdx* mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. ANOVA, analysis of variance; ddPCR, digital droplet polymerase chain reaction; ECC, eccentric force; EEV, Endosomal Escape Vehicle; hDMD, human dystrophin; Med, medium; PMO, phosphorodiamidate morpholino oligomer; Q6W, every 6 weeks.

Efficacy of ENTR-601-50 in Exon 50 Skipping Amenable Mice

- A single dose of ENTR-601-50 produced robust human DMD exon 50 skipping and dystrophin protein expression, which led to improvements in muscle function within 2 weeks post-dose (**Figure 4**).

Figure 4. ENTR-601-50 Dose-Dependent Increase in Exon Skipping and Dystrophin Expression Correlated to Functional Correction

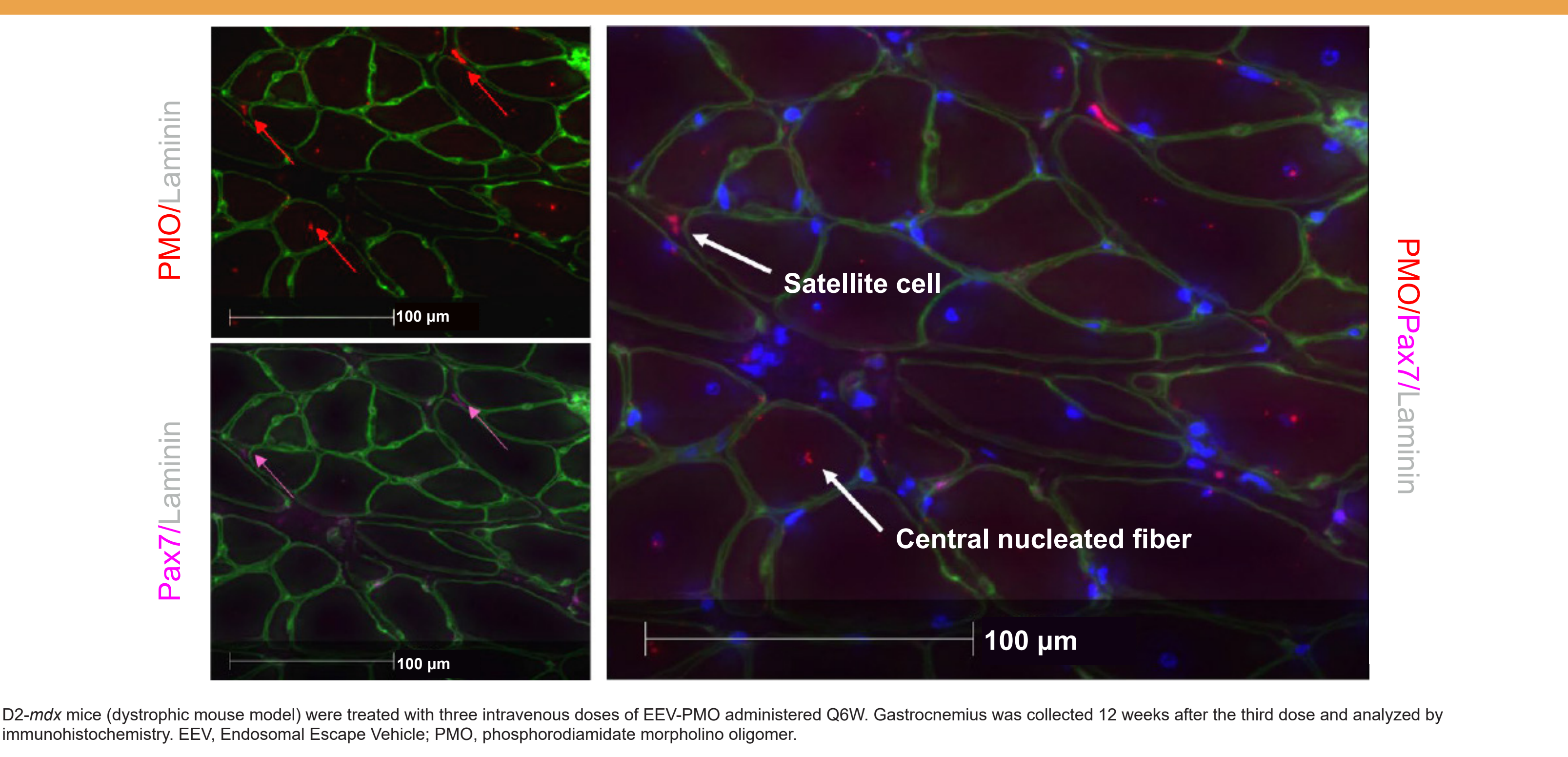


Active and vehicle del51hDMD.*mdx* mice, n=5 per cohort. ENTR-601-50 (single dose). Control saline-treated hDMD.*mdx* mice, n=5 (single dose). (Left) Skipping (ddPCR) and (middle) dystrophin production (Jess) were significantly increased 2-weeks post-dose of ENTR-601-50 (gastrocnemius muscle shown). ECC-induced muscle force loss generated by ECC contraction of the gastrocnemius muscle was assessed 2 weeks after dosing. Data (mean ± standard deviation) are shown across 10 ECC contractions (right) normalized into a percentage of the initial force before any ECC contractions and as the percentage of force retained after the tenth contraction. One-way ANOVA was used for statistical comparison to vehicle-treated del51hDMD.*mdx* mice. * $p<0.01$, ** $p<0.001$, *** $p<0.0001$ vs. Vehicle. ANOVA, analysis of variance; ECC, eccentric force; IV, intravenous; Med, medium.

PMO-23 Persists in Satellite Cells of D2-mdx Mice 12 Weeks After Final Dose

- Modified EEV constructs enabled muscle satellite cell uptake of phosphorodiamidate morpholino oligomers (PMOs) (**Figure 5**).
- PMO-23 colocalized with satellite cells and centrally nucleated fibers 12 weeks post washout after 3 doses every 6 weeks (Q6W).

Figure 5. PMO-23 Persists in Satellite Cells of D2-mdx Mice 12 Weeks After Final Dose



D2-*mdx* mice (dystrophic mouse model) were treated with three intravenous doses of EEV-PMO administered Q6W. Gastrocnemius was collected 12 weeks after the third dose and analyzed by immunohistochemistry. EEV, Endosomal Escape Vehicle; PMO, phosphorodiamidate morpholino oligomer.

CONCLUSIONS

- These studies suggest that the medicinal chemistry of EEV constructs may contribute to efficient intracellular delivery of therapeutic cargo, and highlight the potential of cyclic CPP-based approaches to address key barriers associated with oligonucleotide delivery.
- EEV-oligonucleotide conjugates demonstrated enhanced oligonucleotide delivery and functional activity across multiple preclinical models of DMD.
- Our lead EEV-conjugated oligonucleotides are currently in clinical development for DMD and myotonic dystrophy 1.
- Collectively, these findings support continued development of the EEV platform to enhance intracellular delivery of cell-impermeable therapeutics and address diseases with significant unmet medical needs.