Selected Oral Abstracts

1 Editing of Postmitotic Muscle and Satellite Cells for Continuous Correction of Dystrophin Expression in Duchenne Muscular Dystrophy

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Our longtime research focus has been centered on correcting dystrophin expression and pathophysiology in Duchenne muscular dystrophy (DMD) via gene therapy using adeno-associated virus (AAV) vectors. An integral part of this effort involves use of miniaturized muscle-specific gene regulatory cassettes (RCs) to express therapeutic proteins in striated muscle following adeno-associated-viral (AAV) vector-mediated delivery. RCs developed by our group have been used to effectively restore dystrophin expression and muscle function in cardiac and post-mitotic skeletal muscles via gene replacement, and via editing of the endogenous dystrophin gene. However, the lifelong workloads of skeletal muscles may cause therapeutic efficacy to decrease over time due to muscle remodeling in response to growth, maintenance, exercise, or trauma. Thus, therapeutic longevity could benefit greatly from effective and safe correction of DMD-causing mutations in satellite cells (SCs) to ensure a lifelong supply of skeletal muscle myonuclei that express functional dystrophins.

We have designed a panel of promising AAV-size-compatible regulatory cassettes with preferential activities in SCs (SC-RCs). These SC-RCs are comprised of highly conserved regulatory regions from the human *PAX7* and *MYOD1* genes and exhibit a range of activities in primary SC-derived myoblasts *in vitro* and in FACS-isolated SCs from wildtype versus dystrophic mice. *In vivo*, prime SC-RC candidates display varied myogenic transcriptional patterns, ranging from preferential activity in SCs to more widespread activity in both SCs as well as postmitotic cardiac- and skeletal muscle. Importantly, they are showing great promise for targeting SCs of dystrophic mice via systemic AAV-mediated base editing, so far achieving up to 50-fold enhanced correction compared to the low levels detected using our postmitotic muscle specific expression cassette CK8e. Ultimately, efficient gene correction in SCs constitutes an important element for addressing current limitations of skeletal muscle gene therapies aiming to treat DMD and potentially many other genetic muscle diseases.

2 CSF Delivery of INS1201 AAV9-Micro-Dystrophin as a Potential Therapy for DMD

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INS1201 is an AAV9-micro-dystrophin construct being evaluated for the potential treatment of Duchenne muscular dystrophy (DMD). INS1201 expresses a segment of the dystrophin protein that demonstrates efficacy in the *mdx* mouse and is delivered via a single intrathecal (IT) administration.

The nonclinical program for INS1201 comprises: proof of concept intrathecal route of administration and AAV9 biodistribution in mice and nonhuman primates (NHPs), histopathological and functional strength and electrophysiology efficacy in the *mdx* model, a GLP toxicology study in wild-type (WT) mice and a GLP toxicology study in NHPs.

Pharmacology: Nonclinical histopathological and functional efficacy studies with INS1201 demonstrated improvement in general muscle histology, increased fiber size, decreased inflammation and fibrosis, and quantitative improvement in muscle strength and physiology compared to control *mdx* animals. Dose ranging studies were conducted in *mdx* mice to determine a minimally efficacious dose as well as a dose that nearly corrected the diseased muscle phenotype relative to control *mdx* mice. These studies provide strong evidence to support further clinical studies of INS1201 to evaluate therapeutic benefit in DMD. Treatment of *mdx* mice, a commonly used mouse model of DMD, with INS1201 resulted in: 1) broadly transduced muscle tissues as analyzed via droplet digital polymerase chain reaction (ddPCR), as well as immunohisto-

chemistry and protein analysis of micro-dystrophin, 2) reduced DMD disease pathology as assessed by histopathological analyses, 3) improved forelimb and hindlimb strength and physiology indicating that INS1201 has biological activity and functional activity in a mouse model.

Pharmacokinetics: Biodistribution studies in both mice and NHPs demonstrated significant vector genome delivery to muscle groups throughout the body by delivery to the cerebrospinal fluid (CSF), as well as effective cardiac muscle targeting with limited distribution to the liver compared to systemic dosing.

Toxicology: A GLP toxicology study was undertaken in WT mice, testing INS1201 as compared to vehicle controls, for three different doses, i.e., minimally, medium, and most efficacious, in postnatal day (p) 28 male C57BL/6J mice.

The overall in-life health parameters, clinical pathology, and necropsy results indicate INS1201 was well tolerated at all doses administered and the apparent no observed adverse effect level (NOAEL) for INS1201 administered intracerebroventricularly in mice was the maximal dose administered in this study.

A 3-month GLP NHP toxicology study is nearing completion to evaluate safety in a larger animal model, which will potentially provide additional safety and tolerability.

3 Therapeutic Potential of ENTR-601-44, an Endosomal Escape Vehicle (EEV™) - Oligonucleotide Conjugate for the Treatment of Exon 44 skip Amenable DMD

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Currently approved antisense phosphorodiamidate morpholino oligomer (PMO)-mediated exon skipping therapies for Duchenne muscular dystrophy (DMD) produce only a very modest amount of dystrophin in skeletal muscle. To enhance PMO delivery to target tissues, we designed a family of proprietary cyclic cell-penetrating peptides that form the core of the Endosomal Escape Vehicle (EEV™) platform. Studies in D2-*mdx* mice demonstrated that EEV-PMO-23 (an EEV-exon 23 skipping PMO construct) dosed every four or six weeks produce dystrophin in skeletal and cardiac muscle.

Based on these findings, we examined the therapeutic potential of a DMD exon 44 skipping EEV-PMO construct (ENTR-601-44) in cell and animal models. ENTR-601-44 produced robust exon skipping and restoration of dystrophin protein expression in DMD patient-derived skeletal muscle cells harboring an exon 44 amenable mutation. In addition, single IV doses of ENTR-601-44 led to dose-dependent exon skipping in cardiac and skeletal muscle in human dystrophin-expressing (hDMD) mice and demonstrated robust exon skipping in cardiac and skeletal muscle of nonhuman primates.

Significantly, preliminary results from a clinical trial in healthy volunteers demonstrated that single doses of ENTR-601-44 showed dose-dependent exon 44 skipping, and there were no adverse events related to study drug. Together, these findings suggest therapeutic potential of ENTR-601-44 in preclinical models and support further study in patients with DMD amenable to exon 44 skipping.

4 Development of Gene Therapy for LGMD-R9

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Limb girdle muscular dystrophies (LGMD) refer to a heterogeneous group of rare genetic neuromuscular diseases, which is the fourth most frequent group of genetic muscle diseases (estimated prevalence of 1.63 per 100,000 people). LGMD subtypes are caused by genetic alterations of various genes playing a critical role in muscle function, maintenance, and repair. They shared the common clinical features of predominant impairment of the proximal skeletal muscles, leading to progressive weakness and atrophy and consequently progressive loss of motor function. Some of the subtypes can be associated with respiratory and cardiac impairment. There is no cure for any of the LGMDs.

We aim at developing a therapeutic product based on gene transfer for the most prevalent forms of LGMDs, including LGMD-R9.

This form of LGMD is due to mutations in the Fukutin Related Protein (*FKRP)* gene. FKRP is a rib-

itol-5-phosphate transferase, participating in the process of α-dystroglycan (αDG) glycosylation, which is important to ensure the cell/matrix anchor of muscle fibers. We demonstrated the efficiency of AAV-mediated gene transfer in two LGMD-R9 mouse models, showing restored biochemical defects, corrected histological abnormalities and improved resistance to eccentric stress. A non-GLP dose study was performed, showing efficacy at the dose of 5e12 vg/kg. Two toxicology studies were also perfomed: a GLP study in rat and a non-GLP study in non-human primates, demonstrating no adverse effects even at the dose 30 times higher than the efficient dose. CMC development and the transfer to a CDMO were completed. The dose escalation clinical trial was therefore initiated. Results for the first three patients will be presented.

This trial is sponsored by Atamyo Therapeutics.

6 GNT0004, Genethon's AAV8 Vector-delivered Microdystrophin Gene Therapy for Duchenne Muscular Dystrophy: First Data from Phase 1/2 Part of GNT-016-MDYF All-in-one Clinical Trial in Ambulant Boys

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Duchenne Muscular Dystrophy (DMD) is a rare, progressive, lethal, X-linked disease caused by mutations in the dystrophin gene, leading to progressive muscle degeneration and early death.

GNT0004 is a recombinant serotype 8 adeno-associated virus (AAV8) vector-based gene therapy containing a shortened functional version of dystrophin gene (hMD1). Driven by the Spc5.12 promoter, hMD1 transgene targets skeletal and cardiac muscles.

The pharmacodynamic (PD), safety/tolerability, and efficacy of a single IV administration of GNT0004 is evaluated in the clinical trial GNT-016-MDYF. This all-in-one phase 1/2/3 international trial combines: a first-in-human dose escalation phase (Part 1), a quadruple blind, placebo controlled, pivotal Phase 3, with a 1:1 randomisation (Part 2), at the end of Part 2, there is a cross-over administration of GNT0004 and a long-term follow-up (Part 3). The planned sample size of 64 patients will be reassessed at the interim analysis.

Ambulant DMD boys aged 6 to under 10 years, with a stable or early declining North Star Ambulatory Assessment (NSAA) score despite stable steroids treatment, and no detectable neutralising antibodies to AAV8 were included. Eligible participants were rolled over from the natural history / baseline study (GNT-014-MDYF) into GNT-016-MDYF. Participants received immune prophylaxis with sirolimus and add-on steroids. hMD1 expression was measured by immunohistochemistry and Simple Western at baseline and 8 weeks after GNT0004 administration (biceps brachii biopsies).

Here we report data from Part 1. Two and three participants received dose 1 ($1x10^{13}$vg/kg) and dose 2 ($3x10^{13}$vg/kg) respectively (follow-up per patient ranged from 26 weeks to 3 years). Post dosing, mean hMD1 positive fibres was 1.96% with dose 1, and 53% with Dose 2. Vector Copy Number per nuclei (VCN) was 0.4-2.5 (mean 1.2) with Dose 2. A decrease in serum creatine kinase (CK) of 50%-87% from baseline to week 16 (a timepoint after cessation of immune prophylaxis), with a sustained decrease observed with Dose 2. Administration of GNT0004 was safe and well tolerated in all subjects receiving sirolimus and steroid prophylaxis, which was initiated after dosing of patient 1 in cohort 1 (SUSAR case). Five Adverse Drug Reactions were reported in the two cohorts, including one serious adverse reaction (SUSAR) of immune-mediated myositis that occurred in the first patient in cohort 1 (dystrophin epitope-naive patient at risk of immunological complications, this subpopulation with mutations at risk epitopes was consequently excluded from the trial) and 4 mild adverse events (occurring in 3 patients).

GNT0004 at dose 2 provided significant transgene transduction and expression in skeletal muscle. hMD1 expression appeared to be correctly localised to the sarcolemma, which may contribute to stabilisation of the dystrophin-associated glycoprotein complex. The early and sustained decrease in CK suggests sarcolemma stabilisation with preliminary evidence of clinical benefit. GNT0004 administration was safe and well tolerated in the last 4 participants (including all 3 patients at dose 2). Therefore, dose 2 ($3x10^{13}$vg/kg) was selected to proceed to part 2 (pivotal phase 3).

58 RGX-202, an Investigational Gene Therapy for the Treatment of Duchenne Muscular Dystrophy: Interim Clinical Data

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Duchenne is a severe, progressive, degenerative muscle disease caused by mutations in the *DMD* gene which encodes for dystrophin, without which muscles degenerate and become weak, eventually leading to loss of movement and independence, required support for breathing, cardiomyopathy, and premature death.

RGX-202 is an investigational, one-time AAV gene therapy designed to deliver an optimized microdystrophin gene. RGX-202 is the only gene therapy that encodes for a novel microdystrophin protein that includes the C-Terminal (CT) domain found in naturally occurring dystrophin. In preclinical studies, the CT domain has been shown to protect muscles from contraction-induced stress and improves muscle repair.

The multicenter, open-label phase I/II AFFINITY DUCHENNE® trial is evaluating the safety, tolerability, and clinical efficacy of a one- time intravenous (IV) dose of RGX-202 at one of two dose levels ($1x10^{14}$ or $2x10^{14}$ genome copies (GC)/kg body weight) in boys aged 1-11 years old with Duchenne.

As of July 8, 2024, RGX-202 has been well tolerated with no serious adverse events in boys aged 4-11 years old. Patients across both dose levels demonstrated robust RGX-202 microdystrophin expression three months following RGX-202 administration. RGX-202 microdystrophin ranged from 11.1% to 83.4% at dose level 1 and from 20.9% to 77.2% at dose level 2 (n=4). In the subset of boys aged 8-11 years old, RGX-202 microdystrophin ranged from 20.9 to 75.5%.

Initiation of the pivotal trial is expected in the second half of 2024.

5 The Venture Philanthropy Model of Drug Development - Working with Non-Profit Foundations and Governments from Concept to Drug Approval

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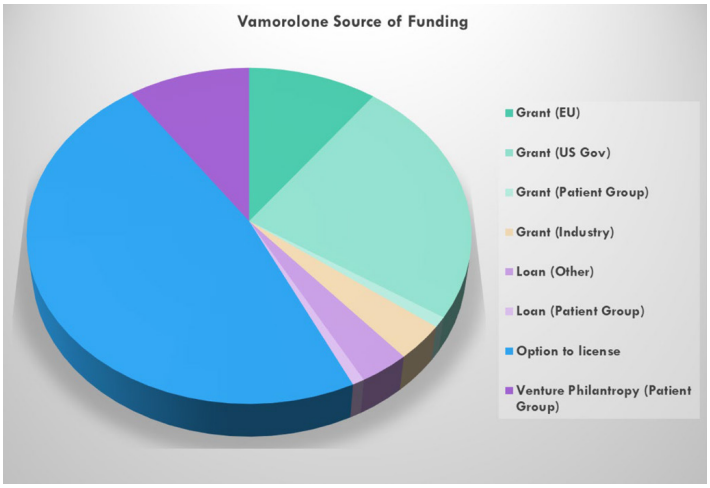
Regulatory authorities increasingly wish to hear and consider the voice of patient advocates and advocacy groups (foundations) in the development and marketing of new medicines. EMA encourages disease-specific Community Advisory Boards (CABs) to convene meetings with drug Sponsors during drug development EMA also includes trained patient advocates as members of EMA Scientific Advice Working Parties (SAWP) reviewing new medicines under development. Most efforts to date have focused on patient advocates engaged as advisors at arm's-length or external to the Sponsor (e.g.interactions with regulatory bodies that then interact with the Sponsor). In the vamorolone (Agamree) drug development program for Duchenne muscular dystrophy, patient advocates and advocacy groups (non-profit foundations) were directly involved in the design and day-to-day management of the drug development program from concept to drug approval. The multi-pronged approach to greater inclusion of the patient voice included foundation and government peer review and funding of many individual aspects of the vamorolone program. Much of this funding was under a 'venture philanthropy' model, where foundations receive 450% return on their investment based on later drug sales. Also, patient advocates advised on trial design, clinical trial site selection, and inspection readiness, again directly with the Sponsor. Indeed, the primary outcome successfully used in the vamorolone clinical trials, time to stand from the floor velocity, was chosen in large part due to patient advocates opinion that this was best related to their children's quality of life. While this motor outcome had not previously been used as a primary outcome in drug development programs previously, there was no pushback from competent authorities on clinical meaningfulness, in part due to the voice of the parent advocates. Critical government programs included European Commission Horizons 2020, NIH NINDS SBIR, and NIH NCATS TRND.



Poster Hall

7 U7snRNA as a Tool for Restoring Dystrophin Expression in Patients with Exon 17 Skip-Amenable Deletions

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Duchenne Muscular Dystrophy (**DMD**) is a severely debilitating disease defined by progressive skeletal muscle wasting and weakness that historically leads to loss of ambulation by age 12 and premature death due to cardiac and respiratory failure.

Despite the great progress made in the development of gene therapy to treat DMD, there is no cure that could show ability to halt or reverse the disease progression. Currently approved and experimental gene therapies have resulted in expression of shortened dystrophin isoforms, with overall limited efficacy. There are several alternative approaches to induce exon skipping and one of them is to incorporate antisense oligonucleotides into U7 small nuclear RNA (**U7snRNA**) and deliver them into cells using adeno-associated virus (**AAV**). This strategy has already shown significant potential for restoring either full-length dystrophin or its highly functional N-terminal truncated isoform in patients with exon 2 duplications (NCT04240314)—one of the most frequent single exon duplications identified.

Here we present the development and efficacy testing of rAAV.U7snRNA-mediated therapy, providing compelling evidence for correction of the disrupted open reading frame in DMD patients carrying skip-amenable mutations—duplications and deletions—that flank exon 17. Duplications of exon 17 (**Dup17**) are the second most common (~1.8%) of duplication mutations. The exclusion of a duplicated copy of exon 17 results in wild type DMD transcript and therefore expression of full-length dystrophin protein. In patients with variable deletions that flank exon 17 (~1.6%), the exclusion of this exon results in an In-frame (**InF**) DMD mRNA transcript that encodes an internally deleted, yet highly functional dystrophin protein. In the case of small deletions (one or several exons), we believe that the skipping only one exon could preserve most of the primary structure of the protein, retaining the biochemical function of dystrophin intact.

We designed several rAAV.snRNA constructs, each encoding a single copy of an antisense sequence targeting either splice acceptor, exon splicing enhancer, or splice donor sites. All vectors were tested *in vitro* and *in vivo*. For *in vitro* tests, two DMD patient-derived cell lines carrying exon 17 amenable deletions were used. These cells were treated with rAAV.U7snRNA vectors and then differentiated into myotubes to study exon skipping and dystrophin expression. The efficiency of AAV treatment was confirmed using RT-PCR, which showed robust dose-dependent skipping of exon 17 in both cell lines. Semi-quantitative analysis of agarose gel images revealed the presence of InF dystrophin transcripts in both cells.

To evaluate the efficiency of rAAV vectors to induce exon 17 exclusion *in vivo*, we have generated a novel DMD mouse model with a large deletion that includes exons 18 to 41. Del18-41 males were intramuscularly injected into the tibialis anterior (**TA**) and gastrocnemius (**Gast**) muscles and euthanized 4 weeks after rAAV administration. Exon 17 skipping and dystrophin restoration were assessed by RT-PCR and JESS capillary western blot (**WB**), revealing exon 17 exclusion for two of the three target sequences in both TA and Gast muscles, resulting in dystrophin protein levels up to 5.2% detected by JESS WB.

These results clearly show that rAAV.U7snRNA-mediated therapy is a powerful therapy that may benefit up to 3.4% of all DMD patients carrying amenable mutations that flank exon 17.

8 KT809 Dose-Dependently Improves Muscle Function in a Severe DMD Mouse Model and Results in High and Uniform Microdystrophin Expression in the Heart and Skeletal Muscles of Non-Human Primates

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Duchenne muscular dystrophy (DMD) is a fatal disease, and the most common muscular dystrophy, with 20,000 boys diagnosed worldwide each year. DMD is caused by loss of the dystrophin protein, an essential component of the dystrophin-glycoprotein complex that preserves myofiber membrane integrity during contraction. Adeno-associated virus (AAV) mediated microdystrophin (mDys) gene replacement therapy is a promising approach to treat DMD. Unfortunately, clinical stage AAV-mDys therapies produce low, nonuniform mDys expression in skeletal muscles, with no clinical data supporting mDys expression in the heart, leading to suboptimal clinical efficacy and limited potential to address cardiomyopathy, a major cause of mortality in DMD. To address these limitations, we used our capsid engineering and regulatory element technologies to develop KT809, a novel AAV-mDys therapy with the potential to be safer and more effective. KT809 expresses a codon-optimized mDys selectively in cardiac and skeletal muscles by using a skeletal muscle/heart-tropic and liver de-targeted capsid (MyoAAV-LD 6.1) and novel regulatory elements. A FLAG tag was added at the C-terminus of the mDys protein to facilitate immunofluorescent detection. To evaluate safety, efficacy, and mDys expression, 8-week-old male D2-mdx mice were injected intravenously with KT809 at 1E13, 2E13, and 4E13 vg/kg, and were evaluated 7 weeks later. KT809 produced a significant dose-dependent improvement in muscle pathology and fibrosis, grip strength, and mechanical properties of the extensor digitorum longus muscle ex vivo. Maximal efficacy occurred at 4E13 vg/kg, associated with mDys expression in nearly all myofibers at protein levels between 62 and 83% of normal human dystrophin levels. Further, KT809 was well tolerated and did not induce toxicity in any treated mice. To translate these findings to large animals and to guide human dose selection, we evaluated the biodistribution and safety of KT809 in juvenile male non-human primates (NHPs). Animals received a single intravenous dose of 4E13 vg/kg, and tissues were collected 28- and 90-days post-dose. KT809 was well tolerated, had no effects on chemistry, hematology, or histopathology up to 90 days, and produced robust, uniform mDys expression in almost all skeletal muscle fibers and cardiomyocytes. The average levels of mDys protein in heart and skeletal muscles relative to normal human dystrophin were 72% and 66%, respectively. Further, the expression of the mDys transgene was highly selective for skeletal muscle and heart, with no transgene expression detected in off-target tissues. In summary, our results illustrate the need for high mDys expression in the majority of myofibers to produce maximal efficacy, and for the first time demonstrate quantitative expression of mDys protein in NHP muscle at levels that are associated with maximal efficacy in a DMD mouse model. These results provide compelling preclinical data to support further development of KT809 to treat DMD.

9 Full-Length Dystrophin Gene Delivery Using Triple MyoAAV Vectors

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Adeno-associated virus (AAV)-mediated delivery of a shortened yet functional microdystrophin transgene that fits within the size constraints of AAV has recently been approved for Duchenne muscular dystrophy (DMD). However, current AAV.rh74-delivered microdystrophin does not provide full protection for striated muscles as it lacks many important functional domains of full-length (FL) dystrophin. Moreover, it requires high doses for systemic transduction, which could lead to serious adverse events. Here we develop a triple vector system to deliver FL-dystrophin into skeletal and cardiac muscles. We split FL-dystrophin into three fragments linked to two orthogonal pairs of split intein, allowing efficient assembly of FL-dystrophin via intein-mediated protein trans-splicing. The three fragments packaged in myotropic AAV (MyoAAV4A) restored FL-dystrophin expression in both skeletal and cardiac muscles in mdx4cv mice, which significantly improved muscle histopathology, contractility, and overall strength comparable to microdystrophin. We further optimized the triple vectors, allowing an over three-fold increase in the expression of FL-dystrophin compared to our recently published version. These optimization efforts also led to efficient assembly of mini-dystrophin (SR3-14 deletion) using dual vectors. Taken together, these data support the feasibility of a mutation-independent mini- and FL-dystrophin gene therapy for DMD, which could be implemented for many other diseases with large

gene payloads exceeding the packaging capacity of AAV to be delivered.

11 CRISPR/hfCas12Max DNA Editing Therapy Restores Dystrophin Protein and Muscle Functions in Duchenne Muscular Dystrophy

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Background: Duchenne muscular dystrophy (DMD) is a fatal, progressive, muscle-wasting neuromuscular disease caused by mutations in the dystrophin gene (*DMD*), with symptom onset before age 6 years in boys. These mutations abolish dystrophin production in the muscle, leading to dystrophin deficiency at the myofiber membrane, continued fiber degeneration, the need for assisted ventilation, and premature death. The approved antisense oligonucleotide medicines (Eteplirsen and Casimersen) treat DMD patients with specific exon 45–55 hotspot region mutations but only restore the dystrophin protein expression to <2% of the normal level after one year of weekly intravenous administration. Here, we developed CRISPR-based high-fidelity(hf)Cas12Max-mediated gene-editing therapy for DMD. **Methods:** We generated three humanized DMD mouse models. The first model replaced the mouse *Dmd* exon51 with the corresponding human *DMD* exon51 and deleted the mouse exon52 ($DMD^{\Delta mE51E52,hE51KI}$). The second model replaced the mouse exon45 with the human exon45 and removed the mouse exon46. The third model introduced a deletion of exon54 in the humanized transgenic DMD mice, disrupting the open reading frame necessary for producing dystrophin protein. We first investigated the genome-editing efficiency of hfCas12Max in human *DMD* exons 45, 51, and 53 in vitro. Then, a single adeno-associated virus (AAV) vector packaged with hfCas12Max (AAV-hfCas12Max) and a guide RNA (gRNA) targeting the splice donor (SD) site of human exon51 (AAV-hfCas12Max-sgRNA targeting exon51; HG302) was injected in DMD mice (Figure 1A) and wild-type non-human primates (NHPs) (Figure 1G). Then, we examined the dystrophin protein level in different muscles using immunohistochemistry, followed by muscle functional tests in DMD mice after a single injection of HG302. **Results:** All three humanized DMD mouse models exhibited phenotypes highly similar to humans with DMD, suggesting they are suitable disease models. Our hfCas12Max-mediated genome-editing targeting the SD sites of human *DMD* exons 45, 51, and 53 in vitro demonstrated an editing efficiency of over 60%. Additionally, systemic delivery of AAV-hfCas12Max effectively restored dystrophin protein expression in all three humanized DMD mouse models, a significant achievement that instills confidence in the potential of our approach. HG302 also rescued the pathological features of DMD, including histopathology, rotarod test, and grip strength (Figures 1C-F), to wild-type levels in DMD mice. Moreover, HG302 induced therapeutically acceptable editing efficiency at the exon51 SD site in healthy wild-type NHPs at a low dose with no observed hfCas12Max-related toxicity, suggesting its potential good safety profile (Figures 1H and I). **Conclusions:** Our innovative CRISPR/hfCas12Max-based approach opens new doors in the fight against this devastating disease. Our novel discovery of hfCas12Max nuclease supports the clinical development of an "all-in-one" single AAV with the CRISPR/hfCas12Max gene-editing therapy for DMD. Based on these preclinical data, we plan to conduct the first-in-human **MUSCLE** clinical study, enrolling 4-6 ambulant boys aged 4 years or older with confirmed deletion of exon 52, 52-61, or 52-63 and measurably impaired muscle function, and investigate the safety and efficacy of HG302. HG302 CRISPR-based DNA-editing therapy has been granted both orphan drug and rare pediatric disease designations for treating DMD by the U.S. FDA.

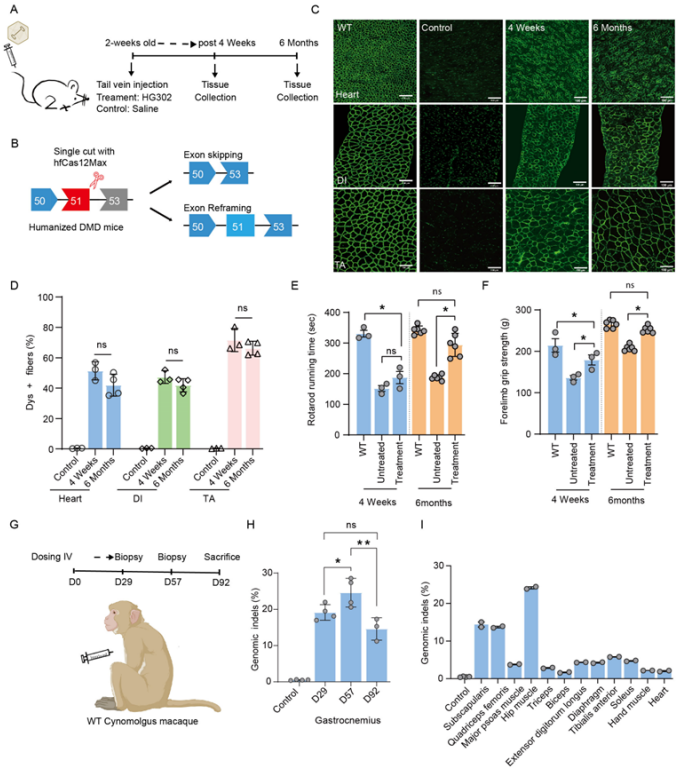

Figure 1. Intravenous delivery of the hfCas12Max system in humanized DMD mice and NHP. (A) Schematic of intraperitoneal injection of AAV particles (HG302). HG302 was injected intravenously into 2-week-old DMD$^{\Delta mE51E52,hE51KI}$ mice. (B) Schematic of the therapeutic single-cut strategy. (C) Immunohistochemistry for dystrophin in TA, DI, and heart of DMD$^{\Delta mE51E52,hE51KI}$ mice was performed at indicated time points. Dystrophin is shown in green. DI, diaphragm. TA, tibialis anterior. (D) Quantification of Dys+ in cross sections of TA, DI, and heart muscles (n=3-6). Rotarod rod (E) and forelimb grip strength (F) were measured in WT, DMD$^{\Delta mE51E52,hE51KI}$ mice, and DMD$^{\Delta mE51E52,hE51KI}$ mice treated with all-in-one AAV particles (n=3-6). (G) Systemic delivery of low-dose HG302 on 1-year-old wild-type cynomolgus macaque. Black nodes indicate time points for tissue collection after intravenous injection. (H) Gastrocnemius muscle was processed D29, D57, and D92 later by biopsy for editing efficiency analysis. D, Day. (I) Editing efficiency analysis (D92) in different tissues of wild-type cynomolgus macaque.

12 Precise Correction of Duchenne Muscular Dystrophy via Exon-Skipping Using Compact Base Editor

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Background: Duchenne muscular dystrophy (DMD) is a lethal, X-linked genetic muscle-wasting disorder. Antisense oligonucleotide (ASO)-based exon-skipping is a promising approach to restore dystrophin protein but requires weekly infusions. CRISPR-based editor-mediated exon-skipping is effective for many DMD cases. However, the base editor's coding sequence exceeds a single AAV capsid's packaging capacity. Using the dual-AAV vector to deliver the CRISPR/Cas9 system necessitates increased clinical dosages, posing significant AAV-related adverse events, including hepatoxicity, thrombotic microangiopathy, cardiac toxicity, and deaths. This dual-AAV approach also suffers low in-vivo recombination efficiency of the split CRISPR system, thus compromising editing efficiency and limiting its clinical application. As an evolutionary ancestor of the Cas9 nuclease, the IscB protein is a compact RNA-guided DNA endonuclease, positioning it as a potential curative treatment for DMD. **Methods:** We evaluated novel cytosine base-editor (aTdCBE) and adenine base-editor (ABE2) to separately modify the exon55 splice acceptor site (SAS) and exon50 splice donor site (SDS) of the *DMD* by intramuscularly injecting the dual-AAV vectors into two DMD models (Figures 1A,C). One replaced the mouse *Dmd* exon50 with the corresponding human *DMD* exon50 and deleted the mouse exon51 (DMD$^{\Delta mE5051,KI-hE50/Y}$); the other introduced a deletion of exon54 in the humanized transgenic DMD mice, disrupting the open reading frame for producing dystrophin protein. ABE2 was systemically delivered to DMD$^{\Delta mE5051,KI-hE50/Y}$ mice. The extent of exon skipping, dystrophin restoration, and motor function rescue was evaluated at 6 weeks and 10 months post-treatment. We then employed a computational pipeline to annotate IscB orthologs by data-mining assembled bacterial genomes. Finally, we investigated the potential of a compact cytosine base-editor (CBE) derived from the IscB variant to modify the SAS of the *DMD* exon 50. **Results:** Intramuscular administration of the dual-AAV system, encoding ABE and CBE components and utilizing a split-intein trans-splicing system, demonstrated over 90% targeted exon-skipping in DMD mRNA and restored up to 90% of dystrophin protein in the affected tissues (Figure 1B,D). Systemic delivery of ABE2 rescued motor function deficits and maintained motor function improvements for over 10 months (Figure 1E-G) in DMD$^{\Delta mE5051,KIhE50/Y}$ mice. Because using the dual-AAV vectors for these base editors, we engineered compact IscB-derived base editors. We identified 10 out of 19 uncharacterized IscB proteins from the uncultured microorganisms (Figure 1H) and developed IscB.m16*, an engineered IscB variant with editing efficiency of ~90% to induce DNA editing and a broad target-adjacent motif (TAM). We then created IscB-based compact CBEs and ABEs by fusing the deaminase domain with IscB.m16* nickase (Figure 1I,J). Subsequent single AAV vector delivery of the IscB-CBE to DMD$^{\Delta mE5051,KIhE50/Y}$ mice showed up to 30% level of exon-skipping by modifying the exon50 SAS, restoring the dystrophin protein expression (Figure 1K,L). **Conclusions:** Our results highlight that the compact base editor possesses sufficient editing efficiency, target specificity, and broad-TAM-scope DNA base editing capabilities to induce exon-skipping in mammalian cells and DMD mice. Combined with a single AAV delivery approach,

these base editors represent a significant step toward the clinical correctin of DMD.

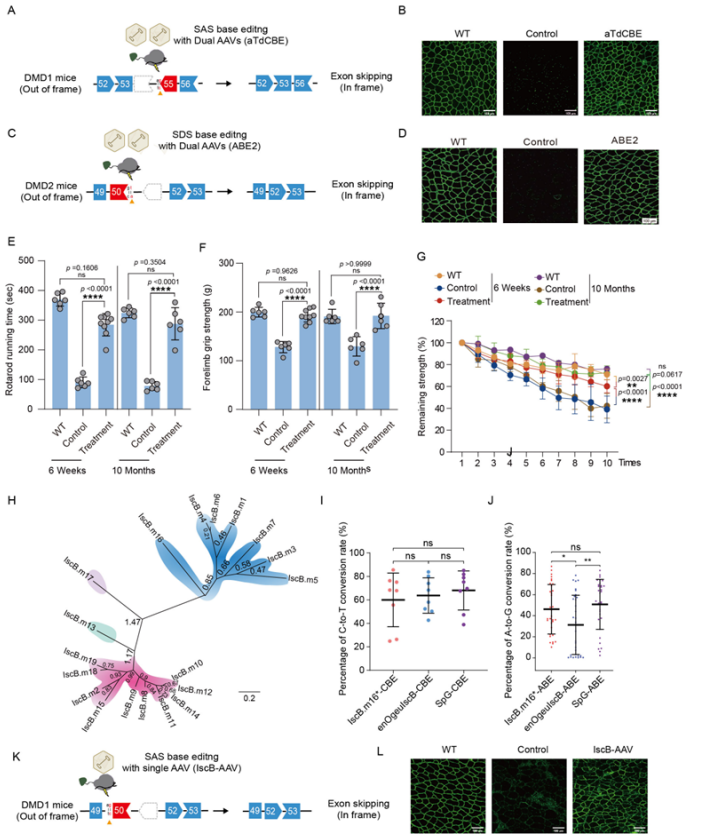

Figure 1. Base editing mediated robustly exon-skipping and rescued dystrophin expression in DMD mice.
(A, C, and K) Schematic illustrating exon-skipping by cytosine base editor (aTdCBE), adenine base editor (ABE2), and compact cytosine base editor (IscB-CBE) by modifying exon55 splice acceptor sites (SAS), exon50 splice donor site (SDS) and exon50 SAS. (B, D, and L) Base editing mediated exon-skipping efficiently rescues dystrophin expression. Rotarod rod running time (E), forelimb grip strength (F), and remaining strength (G) were measured for two days in WT and DMD mice treated or untreated with ABE2 particles. (H) Phylogenetic tree of 19 uncharacterized IscB orthologs. (I and J) Comparison of the C-to-T and A-to-G conversion efficiency of IscB termed CBE and SpG-CBE at multi-target sites.

13 A Comparative Analysis of Long-Term and Short-Term Outcomes of AAV-CRISPR Treatment in a Canine Model of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a lethal and devastating genetic disorder caused by a lack of dystrophin protein due to mutations in the *DMD* gene. DMD is mostly caused by out-of-frame mutations, leading to a premature stop codon. Genome editing holds the promise of treating DMD. Most studies, including ours, focus on removing specific parts of the DMD gene through exon-skipping or reframing, aiming to produce a truncated yet functional dystrophin protein. This approach is intended to shift the clinical prognosis toward its milder counterpart, Becker muscular dystrophy (BMD). Previous research has shown that genome editing can successfully restore dystrophin expression and mitigate the DMD phenotype in mouse models. In addition, single-cut genome editing was effective in restoring dystrophin expression in a canine model of DMD (deltaE50-MD). On the other hand, there have also been reports that Cas9-specific immune responses may compromise the efficacy of systemic AAV-mediated CRISPR therapy in dystrophic dogs. However, the interaction between treatment efficacy and immune responses, particularly in the short and long term, remains unclear and warrants further investigation.

In this study, we examined the effects of genome editing in Canine X-linked Muscular Dystrophy (CXMD) with a mutation in the N-terminal hotspot, with a focus on both efficacy and immune responses over short and long-term periods. We employed the CRISPR/Staphylococcus aureus Cas9 (SaCas9) system to excise exons 6-8 of the DMD gene, aiming to induce exon skipping and produce truncated dystrophin protein. This approach was validated in immortalized DMD canine muscle cells.

For the in vivo experiments, we delivered all-in-one recombinant AAV serotype 9 (rAAV9) vectors containing SaCas9 driven by the spc 5.12 promoter and guide RNAs into 4-12 week-old dystrophic dogs via limb perfusion or intravenous injection (2.0-2.7x10^{14} GC/kg). Short-term treatments (two and five weeks) demonstrated exon skipping at the RNA level, along with notable recovery of dystrophin protein expression in muscle tissues, as confirmed by RT-PCR, Western blotting, and immunohistochemistry.

However, in three dogs (4-5 weeks old) treated with an intravenous injection of 2.0x10^{14} GC/kg, little to no exon-skipping or dystrophin expression was observed one year post-injection. Despite this, isometric dorsiflexion torque showed some improvement, though it was not statistically significant. Notably, these long-term treated dogs exhibited a significant increase in CD4+ T cell infiltration compared to untreated controls, suggesting an elevated immune response. These findings suggest that the immune response could present a major challenge to the long-term efficacy of CRISPR/SaCas9-based genome editing therapies.

14 Rational Design and Rigorous Testing of Novel, Non-Immunogenic Dystrophin Mimetics Providing Superior Cardioprotection and Biomechanical Stability *in vivo*

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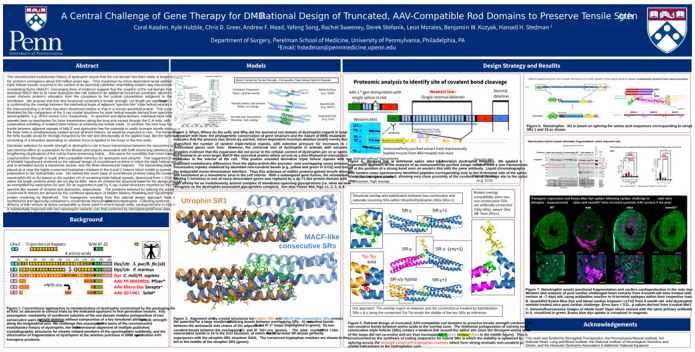
Introduction Duchenne and Becker muscular dystrophy (DMD & BMD) usually result from deletions in exons encoding the 427 kD isoforms of dystrophin. Despite groundbreaking progress, two enduring challenges have complicated the development of effective, durable, and safe gene therapy for DMD. 1) mRNAs encoding the native 427 kD isoforms of dystrophin are ~ 14 kb in length, vastly exceeding the < 5 kb cloning capacity of the AAV platform. Miniaturized variants of dystrophin encoded by internally deleted cDNAs have been advanced to the clinic by multiple groups, but data released to date suggest limited durability consistent with the pathobiology of BMD, including the instability of mutant dystrophin at the deletion junctions. 2) Reported findings in DMD patients receiving systemically administered adeno-associated virus (AAV)-dystrophin vectors include T cell mediated myositis/myocarditis and limited durability of therapeutic efficacy associated with progressive loss of transgene expression. Here we present the development and rigorous testing of a novel class of therapeutics providing compelling evidence for solutions to address these limitations.

Methods We addressed T cell immunity by substituting epitopes from the self-protein utrophin for those of the non-self protein dystrophin. We addressed the problem of protein functionality by avoiding a pitfall of previously used approaches to dystrophin "downsizing" for AAV: non-native protein folding geometry at the deletional splice junction(s) with the capacity to locally destabilize the rod domain as in BMD. Reconstruction of the evolutionary history of dystrophin enabled novel insight into structural and functional constraint on proteins in this superfamily, defining a source for the instability of the rod with load-bearing in the first-generation gene therapies. We harnessed structural biology tools to circumvent these limitations. We used novel, clinically relevant assays to investigate both physiological functionality and protein structural integrity under mechanical stress.

Results We constructed and tested in mdx mice a series of AAV vectors encoding dystrophin- and utrophin-based miniaturized proteins, including three nanotrophins in which the splice junctions in the rod domain share the unique capacity to preserve the three-dimensional folding geometry of adjacent triple helical repeats. To rigorously test our hypothesis, we performed randomized, blinded in vivo tests to provide quantitative indices of functionality conferred by each of the vectors. Following catecholamine-induced cardiac stress, multiple quantitative assays for acute myocardial injury revealed biologically and statistically significant differences between groups of mdx mice injected with microdystrophin or our best performing nanotrophin-5U (cardiac troponin I: 8.09 vs 1.07 ng/mL, p=0.0016 and Evans blue dye: 2.04 vs 0.25%, p=0.0100, respectively). Concordant with these functional results, western blot analysis showed that only the nanotrophins were highly resistant to load-dependent fragmentation in the rod domain.

Conclusions Rigorously designed and executed in vivo tests support the hypothesized correlation between load-dependent fragmentation and superior physiological performance of nanotrophin-5U, a novel class of candidate therapeutics for DMD based on the non-immunogenic utrophin sequence. These findings highlight a role for multidisciplinary approaches to pathobiology and investigational therapeutics.



15 Wearable Device Outcomes From EMBARK, a Pivotal Phase 3 Study in Patients With Duchenne Muscular Dystrophy Treated With Delandistrogene Moxeparvovec

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Delandistrogene moxeparvovec is an rAAVrh74 vector-based gene transfer therapy that delivers a transgene encoding an engineered, functional form of dystrophin shown to stabilize or slow disease progression in Duchenne muscular dystrophy (DMD); it is approved in the US and in other select countries. Stride velocity 95th centile (SV95C) is a wearable-derived digital endpoint representing the 5% fastest strides taken during everyday living; it is a European Medicines Agency-qualified primary endpoint in DMD trials of patients aged ≥4 years and is currently under U.S. Food and Drug Administration Clinical Outcome Assessment Qualification Program review. In the Phase 3 EMBARK trial (NCT05096221) of delandistrogene moxeparvovec versus placebo in patients aged ≥4 to <8 years, the primary endpoint (change from baseline to Week 52 in North Star Ambulatory Assessment) was not met, but multiple secondary functional endpoints (including SV95C, time to ascend 4 steps, Time to Rise, and 10-meter Walk/Run) showed stabilization of disease progression. We report SV95C and exploratory ambulation wearable endpoint data from EMBARK Part 1 (52 weeks).

Data were collected with Syde®, a valid and suitable device worn daily on the ankle for 3 weeks pre-infusion and for 3 weeks before Weeks 12, 24, 36, and 52. Outcomes are reported as between-group differences (95% confidence interval) in least squares mean change from baseline to Week 52 between delandistrogene moxeparvovec and placebo. Participant numbers were N=57 (delandistrogene moxeparvovec) and N=61 (placebo) for all parameters except stair-climbing velocity (N=36 and N=42, respectively).

At Week 52, SV95C (0.10 meters/second [0.00, 0.19]) and stair-climbing velocity (0.06 meters/second [0.02, 0.11]) showed a significant between-group difference (P<0.05, nominal). Stride length (0.02 meters [−0.01, 0.05]), stairs climbed (0.29 stairs/hour [−0.13, 0.71]), and distance walked (14.05 meters/hour [−7.11, 35.21]) numerically favored delandistrogene moxeparvovec. Patient compliance was excellent; a low level of missing data was observed across recording periods.

SV95C findings from EMBARK suggest improvement in mobility during normal activities of daily living following treatment, supporting stabilization or slowing of disease progression with delandistrogene moxeparvovec.

16 Turbo-Charging Gene Therapy for Genetic Muscle Disorders using Novel Transcriptional Cis-Regulatory Elements

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In vivo gene therapy based on adeno-associated viral vectors (AAV) is a promising modality to treat hereditary muscle disorders. Nevertheless, current AAV vectors are characterized by a relatively narrow therapeutic window and often result in low expression, toxicity and even mortality in clinical trials, especially when higher vector doses (10^{14} vg/kg or higher) are employed as in the case of gene therapy for Duchenne muscular dystrophy or myotubular myopathy. To overcome these limitations of muscle-directed AAV gene therapy, we aimed to maximize transcription of the therapeutic genes. To achieve this, we have identified and validation novel combinations of transcriptional *cis*-regulatory elements (CREs) that serve as "molecular turbo-chargers" to specifically boost expression of the therapeutic genes in the diseased target cells including skeletal muscle, heart and diaphragm. These CREs were identified by transcriptome-wide data-mining of genes expressed in human muscle, heart and/or diaphragm, providing a short-list of evolutionary conserved transcriptional elements. The potency and specificity of these CREs was validated based on in vivo AAV9 gene delivery in various murine disease models of severe genetic muscle disorders, including Duchenne muscular dystrophy (DMD), limb girdle muscular dystrophy (LGMD) and Pompe disease. Notably, incorporation of these CREs led to a significant increase in transcription of reporter genes (e.g. luciferase) or various therapeutically relevant genes (i.e. micro-dystrophin or myostatin in the case of DMD, beta-sarcoglycan for LGMD or alpha-glucosidase for Pompe). The extent of the increased transcription varied depending on the gene and/or target tissues attaining up to a 10 to 100-fold increase in transcriptional activity. The increased transcription correlated with a concomitant increase in protein expression levels of the respective therapeutic genes. Most importantly, systemic administration of the CRE-containing AAV9 vectors expressing the respective therapeutic genes resulted in sustained phenotypic correction in the DMD, LGMD or Pompe models based on different func-

tional assays. Finally, the elevation of gene expression by the CREs was not confined to these various murine models but was also confirmed when tested on transfected human primary cardiomyocytes. Taken together, these preclinical results underscores the potential broad therapeutic implications of using CRE-containing AAV vectors for the treatment of severe hereditary muscle disorders.

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18 Development of Pilot Potency Assessments for MyoDys$^{45-55}$, a Gene Editing Therapy for Duchenne Muscular Dystrophy

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Gene editing offers a solution for permanent *DMD* gene reframing as a potential treatment for Duchenne muscular dystrophy, a fatal muscle wasting disease. Duchenne is typically caused by out-of-frame mutations in the *DMD* gene resulting in a loss of the dystrophin protein whereas the milder Becker muscular dystrophy is typically caused by in-frame mutations. Thus, restoration of the reading frame by using gene editing to create an in-frame deletion offers a therapeutic approach to mimic a mild form of Becker. We have developed a gene editing therapy, MyoDys$^{45-55}$, that generates an in-frame deletion of *DMD* exons 45-55. Becker patients with a naturally occurring exon 45-55 deletion have one of the mildest phenotypes where some patients have been asymptomatic into their 60s. Importantly, deletion of this hotspot region would be applicable for 50% of Duchenne patients, significantly more than single exon targets, and generates a dystrophin protein 87% the size of normal. We are currently developing a dual vector adeno-associated virus (AAV) delivery approach for MyoDys$^{45-55}$ where one vector carries Cas9 and one carries the guide RNAs (gRNAs). The vectors will likely be manufactured and released separately, necessitating potency assays for each vector. Here we demonstrate initial potency feasibility using three assays on cardiomyocytes *in vitro*. One assay quantifies expression of Cas9, one quantifies levels of the gRNAs, and the third quantifies correct gene editing when both vectors are added. We have performed initial characterization of each assay which will be further developed in line with FDA recommendations as a potency outcome for MyoDys$^{45-55}$. This work is critical as we advance development of MyoDys$^{45-55}$ towards Duchenne patients since initial potency testing will need to be performed to release cGMP material.

19 A Transformative DMD Cytosine Base Editing Drug

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Duchenne muscular dystrophy (DMD) is the most severe form of muscular dystrophy affecting 1 in 3500 to 5000 boys at birth globally. Patients experience a progressive wasting of both skeletal and cardiac muscles attributable to the loss of dystrophin protein encoded by the DMD gene on chromosome Xp21. The milder symptoms in Becker muscular dystrophin (BMD) patients, who has an in-framed truncated but still functional dystrophin protein, provides a hereditary basis for exon skipping therapy against DMD. GEN6050 is a preclinical investigational drug which utilized Targeted AID-mediated Mutagenesis (TAM) cytosine base editor (CBE) to induce DMD exon 50 skipping via editing the 5'SS of DMD IVS50. GEN6050 is an intravenous dual AAV product containing two AAV9 drug products. One encodes muscle-specific promoter-driven TAM CBE protein while the other encodes 3 copies of hE50 sgRNA targeting the junction between DMD exon 50 and intron 50. Also, the sgRNA vector carries a human gamma-actin gene, which can bind de novo Dystrophin to rapidly facilitate costamere and Dystroglycan-associated complex remodeling and provide a synergistic therapeutic effect with TAM CBE. In vitro, GEN6050 exhibited high editing efficiency on target sites in different DMD iPSC-derived cardiomyocytes and myotubes. In vivo, the mouse surrogate demonstrated therapeutic effects of TAM CBE in a DMD E4* mice model with significant CK-MB reduction and the substantial pathway recovery in heart accompanied by the restoration of Dystrophin protein. The MOA of GEN6050 was confirmed in wild-type mice, which caused dose-dependent and time-accumulated editing efficiency and exon skipping. The proof-of-concept study using humanized GEN6050 is undergoing. The offtarget assessments in normal iPSC-derived cardiomyocytes or myotubes showed low to undetectable sgRNA-dependent or independent offtargets. No transcriptome offtargets were found, confirming that TAM CBE is an RNA edit-free DNA editor. We have successfully established the large-scale AAV9 process development using triple transfection, suspended HEK293 (vpc2.0) system. The IND will be filed at the end of 2024. Furthermore, an investigator- initiated trial will begin in China in June. In sum, GEN6050X has demonstrated therapeutic potential in vitro and in

vivo and may provide a cure to DMD exon 50 skipping patients.

20 Drug Metabolism and Pharmacokinetics in Mice Systemically Administrated with a Base Editing Drug for Duchenne Muscular Dystrophin (DMD)

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DMD is a progressive muscle-wasting disease caused by mutations in the gene encoding the dystrophin protein. Until now, there is no effective drug for DMD. GEN6050X is an intravenously cytosine base editing drug for DMD exon 50 amenable patients. GEN6050X contains two AAV9 vectors, ss.AAV9.oTAM and ss.AAV9.hE50-sgRNA. In DMD patients amenable to exon 50 skipping, GEN6050X can restore the dystrophin protein through inducing exon 50 skipping. GEN6050X also carries a weak therapeutic gene ACTG1, which can provide a synergetic effect with *de novo* synthesized Dystrophin protein. The understanding of GEN6050X Drug metabolism and pharmacokinetics (DMPK) will facilitate the interpretation of toxicity and prediction of in vivo efficacy of base editors.

In this study, we investigated the dynamics of vector DNA and transgene expression in wild-type B6 mice after a tail vein injection with 2E14 vg/kg GEN6050X for 3 months.

After administration, the two vectors level of GEN6050X in blood fell rapidly in mice. On Day 8, oTAM and sgRNA vector DNA decreased by 99.54% and 99.52% of the level of Day 1. The half-life of both components is 1.7 day.

The oTAM and hE50-sgRNA vectors were widely distributed in the tested tissues on Day 1. Since Day 8, the homeostatic tissue biodistribution of vector DNA was formed with predominant distribution in liver, adrenal gland, heart and injection site. The vector DNA exhibited different elimination rates in these four tissues. In liver, oTAM and hE50-sgRNA vectors DNA fell rapidly from Day 8 to Day 22 followed by slow elimination to Day 92. The half-life of oTAM and hE50-sgRNA in liver were 44.1 and 35.5 days. In heart and adrenal gland, vectors DNA maintained stable between Day 8 and Day 22, then slowly descend to Day 92. The oTAM and hE50-sgRNA half-life is 71.0days and 73.3 days in heart while 36.5 and 30.7 days in adrenal gland, respectively. From Day 8 to Day92, the vectors DNA in injection site and other low-distributed organs declined slowly with oTAM/ hE50-sgRNA half-life ranging from 26 to 73.3 days. No gender difference of elimination pattern in all tested tissues was observed. On Day 92 after administration, the vectors DNA is predominantly distributed in liver, followed by heart, adrenal gland, injection site and other tissues.

In mice dosed with 2E14 vg/kg GEN6050X, low-level of hE50-sgRNA transcription began to appear in the heart, liver and injection site of mice on day 8. Since day 22, hE50-sgRNA transcripts were predominantly distributed in the heart. Cardiac hE50-sgRNA transcript levels peaked on day 22 (127700 copies/ ng total RNA) and remained stable thereafter until day 92 (106650 copies/ ng total RNA). After the liver and injection site peaked on day 8, transcript levels continued to decline with half-life of 74.7 and 112.3 days in liver and injection site. The analysis of oTAM and ACTG1 protein in tested tissue is ongoing.

In conclusion, the up-to-date DMPK data of GEN6050X demonstrated the vector DNA will eliminate over time after administration and the transcription of sgRNA showed different dynamics with vector DNA.

21 Screening of Treatment-Emergent Anti-Dystrophin Antibodies: A Validated, Regulatory-Compliant LIPS Assay Covering 100% of Dystrophin

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Background: Therapeutic *de novo* dystrophin has the potential to elicit a humoral immune response (anti-dystrophin antibodies) as a neo-antigen, although any treatment-emergent anti-dystrophin antibodies may or may not be pathogenic. As a potential safety signal, all approved dystrophin replacement therapies show post-marketing commitments to screen treated patients for treatment-emergent anti-dystrophin antibodies. The method published by FDA, and typically preferred by regulators, is called Luciferase immunoprecipitation system (LIPS).[1] This method uses fusion proteins of the protein target to luciferase. When the luciferase-target fusion protein is mixed with patient sera, potential anti-target human antibodies bind, the antibody-fusion complexes are immunoprecipitated with Protein A/G resin, and luciferase activity assayed.

Objective: To develop a validated, regulatory-compliant LIPS assay for the viltolarsen/Viltepso program that is supportive of the post-marketing

program.

Methods: Eight luciferase-dystrophin fusion protein expression vectors were constructed and tested for suitability for expression in COS1 cells, and performance in the LIPS assay. We tested 4 dystrophin variants (full length dystrophin [427kDa] and 3 overlapping subfragments [exons 1-30, exons 30-52, exons 52-79]), each with nanoluciferase (Nluc) fused to both the amino- and carboxyl-termini. While all 8 dystrophin fusion constructs were shown to successfully express the expected fusion proteins after transfection in COS1 cells, the 4 carboxyl-terminal Nluc fusions expressed at higher levels and were carried forward. Three region-specific positive control anti-dystrophin antibodies were tested with each of the fusion constructs for performance in the LIPS assay.

Results: Full-length dystrophin was not efficiently pulled down by any antibody, whereas the 3 overlapping dystrophin subfragments were efficiently pulled down (immunoprecipitations). Each of the 3 overlapping subfragments and paired antibodies were validated in regulatory-compliant LIPS assays. A population-based cut-point was also established for each assay.

Conclusion: A regulatory-compliant LIPS assay was established for three overlapping regions of the complete dystrophin protein.

[1] Kumari S, et al. Clin Vaccine Immunol. 2014 Mar;21(3):383-90.

22 Development of a 248 Kilodalton Midlength-Dystrophin Gene Therapy for DMD using an AAV-Based RNA End-Joining Approach

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Several hereditary diseases including Duchenne muscular dystrophy (DMD) are caused by recessive loss of function mutations in a single gene. In such cases, gene replacement therapy (gene therapy) is a promising strategy. Adeno-associated virus (AAV) is a preferred vector for gene therapy, but this strategy for treatment of DMD has faced the challenge of incompatibility of the large size of the DMD gene with a limited packaging capacity of AAV. Strategies to overcome the packaging constraints of gene therapy are evolving and Insmed is evaluating a novel RNA end-joining (REJ) technology to test the delivery of multiple synthetic DNA molecules leading to the joining of RNA fragments within a single cell. This method could result in the expression of a larger protein that could not be expressed using a traditional single AAV strategy.

Insmed is developing an AAV9-midlength-dystrophin construct that creates a 248 kilodalton (kDA) dystrophin for the treatment of DMD versus the more common ~140 kDA micro-dystrophin by utilizing REJ technology to piece together two RNA fragments within a cell.

Early research studies have established that by delivering two separate AAV's carrying two separate synthetic DNA molecules, REJ technology is efficient in joining RNA molecules encoding fragments of the dystrophin protein in cells both in vitro and in vivo. Midlength-dystrophin expression in *mdx* animals is observed in a dose-dependent manner in cells transduced with a dual AAV9-mid-dystrophin construct. Robust mid-dystrophin expression is also observed in skeletal and cardiac muscle when administered to the *mdx* mouse model of DMD. Importantly, this mid-dystrophin expression confers biological activity, as *mdx* animals exhibit significant improvement in muscle strength at a range of doses, relative to *mdx* control mice, as measured by hindlimb muscle electrophysiology. Studies have demonstrated significant expression of mid-length dystrophin by immunohistochemistry as well as Western blotting, with no evidence of truncated or partial dystrophin fragment expression.

This work opens the possibility of developing potential improved dystrophin constructs that may have considerable therapeutic benefit in muscles affected by the dystrophic pathology experienced in patients with DMD.

23 Optidys, a New Gene Therapy Product for Duchenne Muscular Dystrophy, Shows Therapeutic Potential and Advantage over Current Treatments

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Duchenne Muscular Dystrophy (DMD) is a severe rare pediatric disease that affects the skeletal and cardiac muscles, leading to progressive muscle wasting and premature death. DMD is caused by X-linked mutations in the dystrophin gene that results in total lack of dystrophin, a crucial protein required for the biomechanical support of the muscle fibers and, as recently identified, for signaling function. Although DMD is a suitable disease for gene replacement therapy, the extent of Dystrophin transcript size exceeds by far the AAVs cargo capacity and only short versions of dystrophin can be accommodated. Several forms of μDys are currently being investigated in clinical trials. However, while the therapeutic effects are unequivocally met in the animal models, results from clinical trials highlighted an insufficient correction of the disease and have raised safety concerns due to the appearance of severe adverse events (AE) triggered by immunogenic reactions after vector delivery. This evidence supports the necessity to the identify strategies to face the challenges of safety and efficacy. It is still unclear to which extent microdystrophins can really replace the full-length dystrophin function in human patients. Our strategy is to generate a larger version of dystrophin (quasidystrophin-qDys EP2020/05837) including key additional structural and functional domains by a dual system based on homologous recombination of the overlapping region of two AAVs vectors. We evaluated efficiency and safety parameters of our system by employing an innovative ddPCR-based methodology by which we proved that our Dual AAV approach, systemically administered in DBA2mdx mice, led to high transgene reconstitution efficiency and negligible ITRs-dependent concatemerization, and consequently to remarkable protein restoration in muscles. Our data indicated that mice treated systemically with Dual AAV-qDys displayed global improvement of muscle pathology and function. In particular, DBA2 mdx mice treated with qDys have lower fibrosis and higher muscle force and grip strength as compared to mice expressing similar amount of microdystrophin protein. The histological and functional amelioration was also associated with increased stability of the dystrophin-associated complex. Of note, no adverse effects were observed in these experiments.

24 AUF1 Gene Therapy for DMD Increases Durable Endogenous Utrophin Expression, Muscle Regeneration, Muscle Function Performance and Synergizes With Microdystrophin Therapy

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Duchenne Muscular Dystrophy (DMD) is one of the most severe disorders of muscle degeneration, caused by mutations in the dystrophin gene. The absence of dystrophin leads to progressive muscle degeneration, chronic inflammation and fibrosis. While clinical trials indicate potential benefits of systemic AAV delivered microdystrophin (μDys) gene therapy, there remain major unmet needs. Remaining issues include the need to increase muscle stem (satellite) cells, muscle regenerative capacity, mitochondrial biogenesis and function, muscle innervation, prevent muscle atrophy and promote slow-type oxidative myofibers. Therefore, there remains an urgent need for approaches that address the full suite of pathophysiological features in DMD and can promote durable increased muscle strength and physical endurance.

AUF1 is an RNA binding protein that binds repeated AU-rich elements (AREs) located in the 3' untranslated region of approximately 3% of mRNAs, that in muscle encode the major regulators of myogenesis. We previously showed that AUF1 targets key myogenic inhibitory ARE-mRNAs, inflammatory cytokine ARE-mRNAs, and metalloproteinase ARE-mRNAs such as MMP9 that disrupt the satellite cell niche for rapid degradation, thereby stimulating myogenesis. We also showed that ARE-mRNAs that promote myogenesis are stabilized and increased in their translation by AUF1, thereby activating, orchestrating and coordinating the end-to-end process of myogenesis after satellite cell activation to terminal myofiber differentiation. Importantly, utrophin, the paralog of dystrophin, which can functionally substitute for dystrophin, is encoded by an ARE-mRNA that is strongly increased in both stability and translation by AUF1, providing a means to restore both myofiber stability and muscle function in DMD. Here we show that one-month-old *mdx* DMD mice administered systemic, skeletal muscle specific expression of AAV8.tMCK.AUF1 demonstrate strongly increased physical endurance and strength compared to *mdx* controls, which is durably retained at 6 months at study completion. AUF1 gene therapy strongly increased the satellite cell population, utrophin expression, dystrophin associated protein complexes, diaphragm and limb skeletal muscle integrity, mitochondrial biogenesis and function, neuromuscular innervation, type I slow twitch myofiber content, and almost eliminated, immune cell infiltration and necrosis at 3 months post-administration. There are no negative safety signals at 13 months post-administration.

When combined with systemic AAV8.μDys, AUF1 provided superior muscle function, strength and endurance compared to AUF1 or μDys therapy alone, and superior muscle morphology, protection of muscle from atrophy following muscle injury and durably increased exercise endurance. These results provide compelling evidence that AUF1 mono- or μDys combination gene therapy may offer an alternative single agent and improved combination therapy for the treatment of DMD.

25 AUF1 Gene Therapy Restores Muscle Morphology and Function in Limb Girdle Muscular Dystrophy Recessive 1 (LGMDR1, calpainopathy)

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The most common LGMD is LGMDR1, previously known as LGMD2A (calpainopathy), which represent up to 30% of all recessive LGMDs. LGMDR1 is a slowly progressive disorder where patients present proximal limb muscle wasting that does not involve cardiac or facial muscles, or sarcolemmal fragility. LGMDR1 is caused by mutations in the *CAPN3* gene, which encodes a skeletal muscle-specific, Ca2+ dependent, non-lysosomal cysteine protease, calpain 3 (CAPN3). Dysregulation of CAPN3 leads to the reduction of Ca2+ calmodulin kinase II beta (CaMKIIB) expression and activation that causes mitochondrial abnormalities, oxidative stress and impaired muscle regeneration. It has been shown that activation of CaMKIIβ phosphorylation in Capn3-deficient mice (C3KO mice) improves the oxidative program and muscle function. There are currently no FDA approved therapeutic interventions for LGMDR1. There is, therefore, a large unmet need to develop therapeutic approaches.

AU-rich mRNA binding factor 1 (AUF1) is an RNA binding protein that binds repeated AU-rich elements (AREs) located in the 3′ untranslated region of approximately 3% of mRNAs that in muscle are the major regulators of myogenesis. We previously showed that AUF1 targets myogenic inhibitory ARE-mRNAs for rapid degradation, and myogenic promoters for stabilization and increased translation, thereby orchestrating and coordinating the end-to-end process of myogenesis after satellite cell activation to terminal myofiber differentiation. Moreover, AUF1 supplementation promotes mitochondrial biogenesis and increased respiration activity, and slow myofiber transition, through the stabilization and increased translation of *Pgc1a* and other mitochondrial biogenesis ARE-mRNA in adult and sarcopenic mouse skeletal muscle.

Here, in preclinical mouse models, we show that skeletal muscle specific systemic supplementation of AUF1 by muscle specific AAV8-tMCK-AUF1, in 4 weeks old C3KO male and female mice strongly and durably increases muscle strength and endurance compared to untreated C3KO mice. Supplementation of AUF1 also shows an increase of CaMKIIβ levels and phosphorylation in skeletal muscles and restores muscle oxidative capacity. Transmission electron microscopy and Succinic dehydrogenase (SDH) immunohistochemistry staining also reveal the increase of mitochondria biogenesis and function along with a rescue of normal muscle anatomical and supramolecular structure upon AUF1 supplementation. Finally, safety and toxicity rodent studies indicate that AUF1 gene therapy is not associated with any negative safety signals after 6-12 months of expression.

In conclusion, AUF1 gene therapy is a potential clinical candidate for treatment of LGMDR1 calpainopathy.

26 An Engineered AAV Targeting Integrin Alpha V Beta 6 Presents Improved Myotropism Across Species

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Adeno-associated virus (AAV) has shown potential as a gene therapy vector due to its efficiency in delivering transgenes and low toxicity. However, its effectiveness can be limited by non-optimal tissue targeting and accumulation in the liver. High doses are often required to achieve therapeutic efficacy, particularly in the treatment of muscular diseases where the target tissue represents half the body mass. Such doses can lead to severe adverse effects, including hepatotoxicity as seen in recent clinical trials. With the primary aim to lower treatment doses, we present here a novel methodology for AAV computational rational design targeting skeletal muscle, resulting in a new myotropic cross-species AAV variant. First, integrin alpha V beta 6 (αVβ6) was selected from screening of potential receptors with high enrichment in skeletal muscle tissue compared to other organs. A liver-detargeting AAV capsid backbone, a hybrid between AAV-9 and -rh74 (AAV9rh74), was engineered to bind specifically to αVβ6. We modified the entire sequence and structure of variable region IV loop, in such a way that the designed capsids acquire

the RGDLxxL/I motif structure derived from TGFβ proteins with low estimated energy. All *in silico* designed AAVs show higher productivity and superior muscle transduction compared to their parent. The transduction improvement was shown to be dependent on binding to αVβ6 from multiple species. One notable variant, LICA1, greatly increases transgene expression at 16.6/25.0-fold greater in human myotubes, and murine skeletal muscle at 13.7/129.3-fold greater than AAV9 and AAV9rh74, respectively. The myotropic properties of LICA1 were further validated in non-human primate. We further examined its efficacy, in comparison with AAV9, in delivering therapeutic transgenes in mouse models of Duchenne muscular dystrophy and limb-girdle muscular dystrophy R3 at a low dose of 5E12 vg/kg. At this dose, AAV9 is suboptimal where it can only infect 22.1-58.1% of total muscle fibers. In contrast, LICA1 effectively delivered and expressed transgenes in 74.8% myofibers in severely affected diaphragm, or at almost 100% in other tested muscles. Consequently, LICA1 corrected pathohistology by lowering centro-nucleation index and fibrosis and restored global transcriptomic dysregulation. Furthermore, compared to other published state-of-the-art myotropic AAVs, LICA1 exhibits highest tropism towards the skeletal muscle. These results suggest the potential of our design method for AAV engineering and demonstrated the interest of the novel AAV variant for the gene therapy treatment of muscular diseases.

27 Overcoming Phenotypic Variability in Dystrophic Models: A Machine Learning Method for Refined Gene Therapy Evaluation

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Muscular Dystrophic diseases are debilitating pathologies. Among them, Limb-Girdle Muscular dystrophies (LGMD) affect specific skeletal muscles (scapular or pelvic muscle) and are caused by genetic mutations mostly leading to the absence of proteins at the membrane. One of the most prevalent forms is the LGMD-R1 (or LGMD2A) or calpainopathy which is an autosomal recessive muscular dystrophy due to mutations in the calpain 3 gene (CAPN3). Currently, there is no cure for this disease. Furthermore, the murine models of the disease exhibit a mild phenotype in opposite to human physiopathology. In this context, accurately evaluating therapeutic strategies, including gene therapy, becomes challenging. To tackle this issue, we initiated the development of a rat model using CRISPR technology to silence the CAPN3 gene. This new model presents dystrophic impairments, with the soleus and psoas being the most affected muscles. To evaluate the dystrophic state of muscles, we developed a histological analysis pipeline based on machine learning which demonstrated significant muscle damage from one month of age. At molecular level, in the soleus muscle, the deficiency of calpain 3 protein leads to dysregulated expression of genes involved in the mitochondrial respiratory, in fatty acid metabolism, neutrophil degranulation and cytoskeletal pathways. We then initiated a calpain 3 AAV mediated-gene transfer in deficient rats. Systemic injection of the vector led to the expression of calpain 3 in tested muscles except, as designed, in the heart. This expression is associated with an overall improvement in dystrophic signs. Taken together, the results present a new rat model for calpain 3 deficiency with a dystrophic phenotype reliable to evaluate therapeutic strategies, such as delivery of our AAV-calpain 3 transgene. The machine learning methodology used to assess the dystrophic state of muscles and to evaluate therapeutic strategies could be applied to any dystrophic model and to assay any therapeutic approach.

28 A Universal Approach to Potency Assay Development for AAVs Delivering Micro-RNAs

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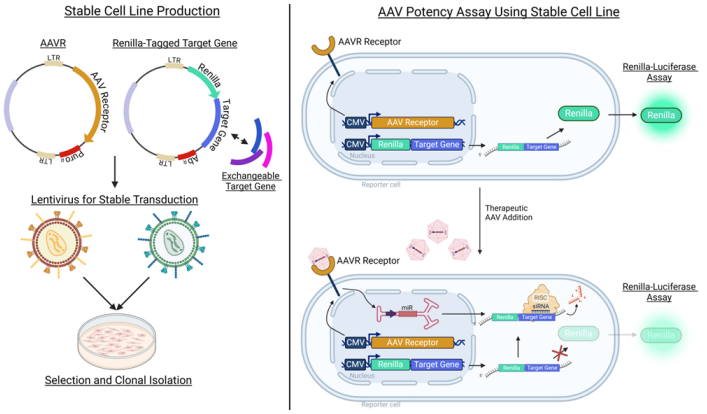
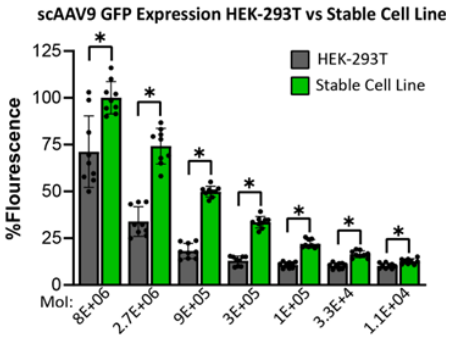
AAV is a cornerstone system for delivering gene therapies for several diseases, including two under development in our lab: Charcot-Marie-Tooth disease type 1A (CMT1A) and Facioscapulohumeral muscular dystrophy (FSHD). Despite the immense progress made in the field to develop AAV based gene therapies, many technological and manufacturing challenges remain that serve as bottlenecks to translation and contribute to the high price tag for a single dose of an AAV therapy. While most AAV therapies involve gene replacement for recessive disorders, CMT1A and FSHD are dominant diseases that would benefit from disease gene silencing. Specifically, we are using AAVs to deliver DNA cassettes expressing artificial miRNAs engineered to silence *PMP22* for CMT1A, and *DUX4* for FSHD. In addition to collecting extensive pre-clinical safety and efficacy data, we are also working to support

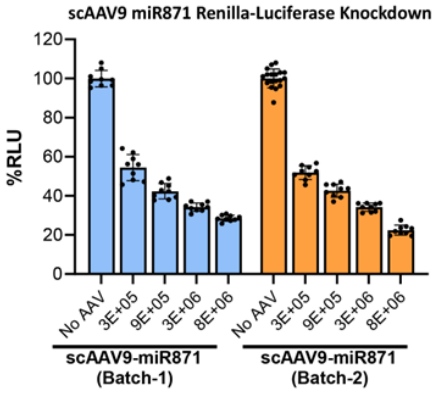
regulatory filings and future clinical product development. Here, we describe our approach for *in vitro* potency assays.

An effective potency assay can measure the strength and stability of a therapeutic product over time and under different storage conditions, making it a valuable tool during product release testing. Here, we describe a universal approach for developing a potency assay to assess AAVs delivering miRNAs. Two primary challenges needed to be addressed when developing our approach: (1) cell line permissibility to AAV transduction, and (2) a quantifiable, treatment-responsive readout.

An ideal cell line for an *in vitro* potency assay would be easily propagated in culture and permissive to AAV transduction. Unfortunately, such cell lines are scarce and highly dependent on the AAV serotype used. HEK293T cells grow well in culture but are not efficiently transduced by AAV vectors. We were concerned that low AAV permissibility in HEK293Ts could represent a hurdle to assay development and sought to improve *in vitro* transduction efficiency of HEK293Ts. To do this, we stably inserted an essential AAV receptor (AAVR) into HEK293T genomes using a lentiviral vector. The AAVR-transduced cells showed 2.7x increase in AAV transduction compared to untreated HEK293Ts. In the same cells, we used a second set of lentiviral vectors to insert reporter genes capable of producing a quantifiable, treatment-responsive readout of gene silencing. Specifically, these constructs contained a *Renilla luciferase* (Rluc) gene tagged to either *PMP22* or *DUX4* open reading frames. We generated a clonal, stable cell line expressing AAVR/Rluc-PMP22 for CMT1A, and are currently isolating clones expressing the AAVR and RLuc-DUX4 for FSHD. To measure treatment response, we delivered AAV vectors carrying therapeutic miRNAs targeting *PMP22* (miR871) and measured reduction in chemiluminescence by luciferase assay. Two different AAV9-miR871 batches were tested, and both produced robust, reproducible knockdown of the Rluc signal. We expect to report similar findings for a cell line containing a Rluc-DUX4 reporter at the November meeting, thereby demonstrating by simply swapping out the gene of interest tagged to Rluc, this approach can be applied to several AAV serotypes and gene therapies utilizing miRNAs or other gene silencing strategies. Overall, we believe this platform approach to potency assay development eases a significant hurdle for regulatory filing and will help streamline translation of new AAV gene therapies to the clinic.







29 Developing a Second Generation Proviral Plasmid to Reduce Aberrant Cross Packaging and ITR Promoter Activity in AAV Vector Preparations

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AAV preps largely contain correctly packaged genomes, with the intended ITR-flanked, therapeutic gene expression cassette inserted within the AAV capsid. However, a recent survey suggested AAV preps contain ~3.5% packaged DNA contaminants (N=30; <1 - 13%) derived from mispackaging of proviral plasmid backbone sequences instead of the intended payload. Thus, AAV contaminants, or

cross-packaged material, can include sequences required to propagate plasmids in bacteria, such as antibiotic resistance genes, bacterial sequences with immunostimulatory CpGs, and potential open reading frames. Risks of cross-packaged bacterial sequences in AAV vectors were highlighted in a recent study in non-human primates, where toxicity was linked to ITR-driven expression of transcripts arising from cross-packaged bacterial sequences in the plasmid backbone and not the intended payload (PMID: 34663988). These results suggested that improved production methods are needed to (1) increase correctly packaged AAV payloads and (2) decrease ITR-driven transcription of cross-packaged material.

To address these issues, we recently published (PMID: 39139628) a novel AAV proviral plasmid for vector manufacturing. Our first generation system (1) significantly decreased cross-packaged bacterial sequences by 70%; (2) increased correctly packaged AAV payloads; and (3) blunted ITR-driven transcription of cross-packaged material to avoid expressing potentially toxic bacterial sequences. The new plasmid had no impact on viral titers or expression from correctly packaged genomes. Although we believe this system may help improve the safety of AAV vector products, there is still room for improvement, and we are now optimizing the system. We expect to report results from several new, second-generation systems designed to further reduce cross-packaging, decrease the amount of packaged bacterial sequences in our AAV vector preps, and eliminate ITR-driven transcription of potentially harmful open reading frames. New modifications include: extending benign human intronic sequences to increase their size; testing potentially more powerful insulator sequences with and without CTCF binding sites outside the ITR regions; eliminating payload truncation hotspots; and testing strategies to decouple plasmid backbone sequences from the AAV proviral payloads. We hypothesize that these modifications will further alleviate toxicity in AAV preps generated with our manufacturing system, potentially having long-term effects on the safety of AAV therapies used for muscular dystrophies and other diseases.

30 CRISPR-Cas13d Gene Therapy for Facioscapulohumeral Muscular Dystrophy

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Facioscapulohumeral muscular dystrophy (FSHD) is a potentially devastating neuromuscular disease affecting ~1 in 8,000 individuals. It is the third most common type of muscular dystrophy, with no approved disease-modifying treatments currently existing. FSHD is caused by aberrant expression of the myotoxic double homeobox protein 4 (DUX4) gene in muscle. We propose that DUX4 gene silencing will offer a treatment for FSHD and are currently testing numerous strategies to accomplish this goal. In this study, we are utilizing a CRISPR-Cas13 system to knockdown DUX4 messenger RNA (mRNA) expression and develop a potential therapy for FSHD. Our attempts to accomplish this goal using Cas13b, one of the first Cas13 enzymes discovered, produced robust DUX4 silencing and improved myopathic phenotypes in FSHD mice at early timepoints in vivo. However, we found reduced efficacy over time that was associated with a CD8+ T cell response to the Cas13b protein. We are now developing several strategies to address Cas13 immunogenicity, including testing the universality of immune response to other Cas13 proteins from different bacterial species. In this study, we focused on the Cas13d enzyme isolated from gut bacteria. Cas13d is among the smallest Cas13 enzymes and as such, is capable of being co-packaged with guide RNA expression cassettes in the same AAV vectors. We designed 50 Cas13d guide RNAs (gRNAs) that target DUX4 and developed a luciferase screening assay to test efficacy in vitro. Specifically, we used a dual luciferase assay in which full length DUX4 was inserted as the 3' UTR of Renilla Luciferase, then we cotransfected this reporter plasmid with Cas13d and our various gRNAs. We identified 11 effective gRNAs and further narrowed down our leads to 3 candidates, based on efficacy in secondary outcomes measures that included ddPCR and western blot for DUX4 mRNA and protein, respectively. We are now testing both in vivo efficacy, safety, and immune responses to AAV-delivered Cas13b in our TIC-DUX4 mouse model of FSHD and wild-type mice. Our results support that Cas13 can form the basis of a robust gene silencing strategy for FSHD and other dominant neuromuscular disorders, but may require further optimization to improve long-term efficacy and reduce immune responses.

31 Development of KT323 as a Potent and Safe Gene Therapy Candidate for Facioscapulohumeral Muscular Dystrophy (FSHD) Through High Throughput AAV Capsid and Cargo Engineering

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Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal-dominant genetic disorder affecting approximately 1 in 8,000 individuals. FSHD patients typically exhibit progressive muscle wasting in the face, shoulders, upper arms, legs, and abdomen. Approximately 20% of patients will eventually require the use of a wheelchair. De-repression of the transcription factor DUX4 has been identified as the cause of muscle degeneration in FSHD. Insufficient epigenetic silencing of the *DUX4* gene in patient muscles results in aberrant expression of DUX4, which is toxic to mature myofibers. Therefore, knockdown of the *DUX4* transcript has been evaluated as a therapeutic strategy for FSHD and has shown efficacy in preclinical models.

Here, we demonstrate efficacy of a novel MyoAAV-LD-mediated RNAi therapy for FSHD, both in vitro in FSHD patient-derived myotubes and in vivo in the ACTA1-MCM;FLExDUX4 mouse model. Biodistribution and safety was evaluated in non-human primates (NHPs). First, we conducted a high throughput cell-based tiling screen to identify the most potent *DUX4*-targeting artificial miRNA sequences. Top hits from this screen were validated in FSHD patient-derived myotubes. These cells contain a disease-causing contraction in the D4Z4 region of chromosome 4q that is responsible for epigenetic silencing of *DUX4*, and upon differentiation, express DUX4 and its transcriptionally regulated target genes. Candidate miRNA sequences were ranked based on the magnitude of DUX4 and DUX4 target gene knockdown achieved in these cells, and the top miRNAs were evaluated for off-target effects in control human myotubes by RNAseq and small RNAseq. We selected our lead DUX4 targeting miRNA based on high potency in FSHD myotubes and lack of significant off-target effects in control human myotubes.

In separate experiments, we evolved a potent liver de-targeted, muscle-tropic MyoAAV-LD capsid variant (MyoAAV-LD 6.1) in non-human primates. We packaged the transgene encoding our lead miRNA into MyoAAV-LD 6.1 and tested the efficacy of this development candidate (KT323) in vitro and in vivo. We demonstrated dose-dependent knockdown of DUX4 and DUX4 target genes in FSHD patient-derived myotubes and minimal off-target effects in control human myotubes after transduction with KT323. To assess in vivo efficacy, we used ACTA1-MCM;FLExDUX4 mice, a bi-transgenic line with inducible expression of DUX4 in skeletal muscles, which reproduces disease-relevant biochemical, histological, and functional phenotypes of FSHD. We injected these mice with increasing doses of KT323 or vehicle, and induced DUX4 expression in the mice by injecting tamoxifen. Treatment with KT323 dose-dependently protected mice from severe impairment in treadmill time to exhaustion, protected their muscles from DUX4-mediated degeneration and regeneration, and blocked elevations in DUX4 and DUX4 target genes, all of which were observed in the vehicle-treated mice. Similar treatment also rescued biochemical and histological phenotypes in aged, non-induced ACTA1-MCM;FLExDUX4 mice. Finally, we assessed biodistribution and safety of KT323 in NHPs at 2E+13 vg/kg and 4E+13 vg/kg. We were able to achieve miRNA expression levels in NHP skeletal muscles comparable to those shown to be efficacious in the ACTA1-MCM;FLExDUX4 mice. KT323 was safe and well tolerated in the NHP study. Taken together, these data support further development of KT323 for FSHD.

32 Efficient Expression of Full-Length SMCHD1 in Muscles Using Split Inteins

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Facioscapulohumeral dystrophy (FSHD) is one of the most prevalent forms of muscular dystrophy, affecting approximately 870,000 people globally. FSHD is associated with inappropriate expression of *DUX4*, caused by contraction of the D4Z4 repeats on chromosome 4q or insufficient epigenetic silencing of the normal D4Z4 repeat arrays. Mutations in the chromatin modifier, *structural maintenance of chromosomes hinge domain containing 1* (*SMCHD1*) gene have been linked to hypomethylation of D4Z4 repeats and re-expression of *DUX4* in skeletal muscle. Thus, overexpression of SMCHD1 could be beneficial in promoting hypermethylation and repressing the *DUX4* locus in muscles. However, inserting the 6,018 base pair (bp) coding sequence of *SMCHD1* is limited by the AAV packaging capacity of 4,700 bps. In this study, we utilized a split intein-mediated protein trans-splicing approach to overexpress full-length SMCHD1 in muscles. We generated four pairs of AAV constructs using two different split inteins. For each vector pair, the first vector contains the coding sequence for the N-ter-

minal half of *SMCHD1* followed by the N-intein coding sequence, and the second vector contains the coding sequence of a C-intein in frame with the C-terminal half of *SMCHD1* and a terminal V5 epitope tag. The split-intein vector pairs that displayed efficient protein reconstitution *in vitro* were used for *in vivo* validation. Intramuscular injection of dual AAV split intein vectors in wild-type mice showed efficient reconstitution of the full-length SMCHD1 protein. We are currently performing *in vivo* efficacy studies in human FSHD2 myoblasts xenografted in mice, and the D4Z4-2.5 mouse model expressing human, FSHD-permissive D4Z4 repeats. Taken together, our data support the use of split inteins for large gene replacement therapies.

33 The Prevalence of Sleep Disorders among Individuals with Muscular Dystrophy: A Review of the Literature

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Problems in the quality, timing or amount of sleep are common comorbidities of progressive muscular dystrophy. Most studies focus on myotonic dystrophy (DM) or dystrophinopathies (Duchenne/Becker muscular dystrophy (DBMD)), but sleep disorders have also been reported in facioscapulohumeral dystrophy (FSHD), congenital muscular dystrophy (CMD) and limb-girdle muscular dystrophy (LGMD). Professional guidelines recommend periodic evaluation for sleep disorders and nocturnal hypoventilation for individuals with DBMD or DM but there are currently no recommendations regarding sleep evaluations for individuals with other muscular dystrophies. Our objective was to examine the prevalence of sleep disorders among individuals with DM, DBMD, FSHD, CMD, LGMD, or mixed or unspecified muscular dystrophy.

We systematically reviewed PubMed- and EBSCO-indexed literature on sleep disorders among individuals with muscular dystrophy. Two librarians conducted independent searches using the same strategies. Two investigators reviewed citations for eligibility using preset criteria 1) published in English at any time and 2) provided data on the prevalence of sleep evaluations, disorders, or treatments among individuals with an eligible muscular dystrophy. We calculated summary prevalence across all studies as the total affected/total sample size. All abstractions were reviewed by a senior investigator. We identified 514 unique citations, of which 95 were found to meet the inclusion criteria. The distribution of articles by muscular dystrophy was DM, 61; DBMD, 20; FSHD, 9; CMD, 1; LGMD, 1; and mixed or unspecified, 3. Hypersomnia, including excessive daytime sleepiness, and sleep apnea were the most studied and most consistently defined sleep disorders. Nine studies included healthy controls. As expected, the prevalence of hypersomnia was higher among individuals with myotonic dystrophy. Among 1,265 individuals with myotonic dystrophy (in 7 studies) who completed standard questionnaires on daytime sleepiness, 63% had abnormal daytime sleepiness. In comparison, only 9% of healthy individuals (199 individuals, 5 studies) and only 13% of individuals with FSHD (725 individuals, 4 studies) had abnormal levels of sleepiness. Sleep apnea was common in some but not all muscular dystrophies. The prevalence of sleep apnea of any severity was 65% among individuals with CMD (20 individuals, 1 studies), 47% among individuals with DBMD (448 individuals, 7 studies), 28% among individuals with FSHD (86 individuals, 3 studies) and 24% among individuals with myotonic dystrophy of unspecified type (259 individuals, 9 studies). Among 712 individuals with myotonic dystrophy type 1 (14 studies), 58% had at least mild sleep apnea. Among healthy individuals, the prevalence of sleep apnea was 11%. Our findings support current guidelines that recommend sleep studies for individuals with DBMD or DM. They suggest sleep studies may be useful for individuals with CMD and FSHD.

34 BIN1 Gene Replacement Reverts BIN1-Related Centronuclear Myopathy in a Preclinical Model

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Centronuclear myopathies (CNM) are severe genetic disorders characterized by generalized muscle weakness associated with organelle mispositioning in myofibers. Most CNM cases are caused by mutations in proteins involved in membrane remodeling, including amphiphysin 2 (BIN1). There is no treatment and the pathological mechanisms are not understood. Here, we aimed to cure the Bin1-CNM

mouse model (Bin1$^{mck-/-}$) via an adeno-associated virus (AAV)-based gene replacement strategy. Early systemic exogenous BIN1 expression efficiently prevented disease progression. BIN1 expression after disease onset reverted all disease signs four weeks after treatment, including motor defects, muscle weakness, muscle and myofibers hypotrophy, kyphosis, nuclei and mitochondria misposition, and altered T-tubules network. We then validated the most efficient construct combining a myotropic AAV serotype with the muscle BIN1 isoform. The rescue correlated with normalization of autophagy and excitation-contraction coupling markers. Cellular and in vivo investigations revealed that different BIN1 natural isoforms shared similar beneficial effects. Artificial constructs coding for separated protein domains rescued different CNM hallmarks. Only the muscle-specific BIN1 isoform combined the different cellular functions of BIN1 on membrane tubulation and dynamin (DNM2) regulation necessary for a full rescue. Overall, this study validates BIN1 gene replacement as a promising strategy to cure BIN1-related centronuclear myopathy.

35 Functional Benefit of CRISPR/Cas9-Induced Deletion for *RYR1* Dominant Mutation

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The ryanodine receptor 1 (RyR1) is a calcium channel that plays a pivotal role in the contraction of skeletal muscle cells. A large number of mutations in the gene encoding this channel have been identified as causative factors in a group of rare myopathies, collectively referred to as "RyR1-related myopathies." These conditions manifest as muscle weakness of varying degrees of severity in affected individuals. Despite ongoing research, there is currently no curative treatment available for these disorders. One of the primary challenges in developing gene therapy for these conditions is the extensive number of mutations found in the RYR1 gene, as well as their widespread distribution throughout the gene. In addition, gene replacement therapy is not a viable option for RYR1 due to its large size. In light of these challenges, we propose a versatile approach aimed at restoring RyR1 calcium release, irrespective of the specific patient mutation. Our methodology involves the targeted deletion of the pathogenic allele, enabled by the use of CRISPR- Cas9 technology. For the proof-of-principle, we concentrated our efforts on a dominant mutation identified in a family afflicted with Central Core Disease. Our approach entailed the design of a pipeline to select the most effective guide RNAs for targeting the pathogenic allele, using single nucleotide polymorphisms as the target. Subsequently, we transduced immortalized myoblasts derived from the patient using lentiviruses expressing both Cas9 and the selected guide RNA. Throughout the course of our study, we assessed the efficacy of the treatment at the DNA, RNA, and functional levels. Our findings represent the first successful application of genome editing techniques for the treatment of dominant RyR1-related myopathies.

36 Correcting Mutations in the DYSF Gene Using Prime Editing

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Dysferlinopathy is a limb-girdle muscular dystrophy that mainly affects the skeletal muscles of the legs and then the upper limbs. People with the disease use a cane to walk in their thirties and in a wheelchair in their forties or fifties. This inherited disease is caused by mutations in the dysferlin gene and I am fascinated by my research project on dysferlinopathy since it affects family members, including my mother and I am a carrier.

I study the effect of correcting these mutations by prime editing in cells derived from patients of different types (fibroblasts, myoblasts) and in mice.

I am working with the Jain Foundation to caracterize a mouse model with a patient point mutation added by Prime editing by the Jackson lab.

My results show that since this gene is not expressed in the skin, Prime editing is not effective in patient fibroblasts. The same trend is observed in our laboratory when we try to modify genes expressed mainly in muscles using fibroblast cell lines. The trend is also that editing works when using the same Prime editing construct in myoblasts. Indeed, a single treatment can correct 11% of the DNA and two treatments by electroporation in the cells make it possible to achieve 25% editing in the myoblasts. The yield is similar when a line is created by bringing a mutation of patients into healthy myoblasts with Prime editing.

In addition, a mouse model (DYSF R1925X) with

a patient mutation (DYSF R1905X) was developed using Prime editing to insert a mutation that replaces an arginine with a premature stop codon, as is the case in several patients with this disease. I confirmed by Sanger sequencing that the mice do indeed possess the desired homozygous mutation. Then, I verified that dysferlin is not expressed in these mice by observing the absence of the protein by immunohistochemistry and western blotting. I also observed some very interesting properties in these mice, which I will present in more detail. I will also have Prime editing correction results in the mice soon.

37 Multiplex Base Editing in Primary Human Muscle Stem Cells to Address Compound Heterozygous Muscular Dystrophy

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Autosomal recessive limb-girdle muscular dystrophies (LGMD) are severely debilitating and progressive muscle wasting disorders without any treatment. Autologous cell replacement therapies with gene repaired muscle stem cells (MuSC) are underway to the clinic. In case of compound heterozygous mutations, it is hypothetical whether repairing one mutation alone would suffice to provide adequate gene-dosage. Here, we demonstrate precise and highly efficient simultaneous multiplex gene editing in primary human MuSC using mRNA and ribonucleoprotein (RNP) mediated delivery of CRISPR tools to address compound heterozygous muscular dystrophy.

Loss-of-function mutations in the *SGCA* gene, encoding the muscle membrane protein α-sarcoglycan, cause LGMD2D/R3. We obtained pure MuSC populations from two patients carrying compound heterozygous *SGCA* mutations amenable to adenine base editing (ABE). *SGCA* c.739G>A is a frequent founder mutation common to both families. The second mutation differed between the families and was either *SGCA* c.229C>T, the most frequent LGMD2D-causing founder mutation, or *SGCA* c.101G>A. As a proof-of-concept, we examined the potential of delivering mRNA encoding the first (ABE7.10) and newer (ABE8e) generation ABE with a classical NGG PAM requirement, as well as ABE8e-SpRY, a PAM-relaxed variant, in combination with suitable single guide RNAs (sgRNAs) targeting the mutations to patient MuSCs. Single editing of each mutation individually resulted in up to >90% gene repair, with minimal bystander activity. Multiplex editing led to high nucleotide conversion rates (up to >90%) at each target site and resulted in efficient double repair of both mutations in MuSC from both patients. Moreover, multiplex ABE resulted in a robust rescue of α-sarcoglycan protein expression and localization in patient MuSC-derived myotubes.

In addition, we investigated delivery of ABE8e RNP to human MuSC using cell penetrating peptides (CPP). We used sgRNAs against two splice donor sites in exons 3 and 7 of the gene encoding the surface molecule *NCAM1*, which we previously established as a universal endogenous reporter locus to test gene editing approaches in human MuSC. Using simultaneous RNP:CPP-mediated delivery of ABE8e plus sgRNAs targeting both sites, we achieved up to approximately 90% base editing in *NCAM1* exon 7 and 70% in exon 3. Importantly, the myogenic and proliferative capacity of edited MuSC was preserved after both mRNA- and RNP:CPP-mediated base editing.

In summary, we report ABE-mediated multiplex precise gene repair in patient MuSC, which has immediate implications for autologous transplantation therapies in disorders that previously lacked any therapeutic perspective. In addition, we demonstrate highly efficient multiplex base editing by RNP:CPP-mediated delivery, introducing a novel platform for ex vivo delivery of CRISPR tools to human MuSC and paving the way for potential applications in in vivo genome editing.

38 Functional Efficacy of SRP-9004 in Young and Aged LGMD2D/R3 Mice

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Background: Limb-girdle muscular dystrophy subtype 2D/R3 (LGMD2D/R3) is an inherited neuromuscular disorder caused by mutations in the gene encoding for alpha-sarcoglycan (*SGCA*) that leads to loss of functional protein. In patients, LGMD2D/R3 leads to proximal muscle weakness and wasting, elevated creatine kinase (CK) levels, and eventual loss of ambulation.

Given the monogenic nature of LGMD2D/R3, gene therapy is a promising modality. SRP-9004 (patidistrogene bexoparvovec, rAAVrh74.tMCK.hSGCA), a rationally designed gene therapy, utilizes a self-complementary AAVrh74 vector (adeno-sso-ciated virus, rhesus subtype 74) driven by a muscle-specific triple-tandem muscle creatine kinase promoter (tMCK) to deliver a full-length human SGCA (hSGCA) transgene to reduce LGMD2D/R3 disease burden. Here, we explore the functional efficacy of SRP-9004 across the LGMD2D/R3 disease continuum using a mouse model for LGMD2D/R3.

Methods: C57BL/6J (wild-type) or B6.129S6-Sgca^{tm2Kcam}/J (SGCA -/-) mice of both sexes were intravenously dosed with either saline or SRP-9004 at age 4–6 weeks (young) or 20–22 weeks (aged) and observed for ~12 weeks. Functional improvements were assessed by treadmill testing, grip-strength evaluation, and muscle physiology assays. Serum was collected to evaluate CK and liver enzyme levels in response to SRP-9004 treatment. Vector biodistribution was assessed by droplet digital polymerase chain reaction (ddPCR). SGCA protein expression was determined by automated Simple Western (Jess). SGCA and dystrophin-associated protein complex (DAPC) protein localization were evaluated using immunofluorescent assays. Histopathology was performed on Masson's trichrome and hematoxylin and eosin-stained slides to assess fibrosis and other histopathologic changes associated with SRP-9004 administration.

Results: Biodistribution evaluation in SGCA -/- mice indicated robust skeletal and cardiac muscle transduction following systemic SRP-9004 administration. In young SGCA -/- mice, SRP-9004 led to restoration of SGCA protein expression. The functional activity of the SRP-9004 induced hSGCA transgene was further demonstrated through localization to the sarcolemma membrane and concomitant restoration of sarcoglycan components of the DAPC. Importantly, SRP-9004 treatment resulted in improved tibialis anterior and diaphragm function in SGCA -/- mice. Additionally, a reduction in the incidence and/or severity of SGCA -/- strain-related skeletal myofiber degeneration, myofiber necrosis, mononuclear cell infiltration, and fibrosis was evident in the skeletal muscles and diaphragm of SRP-9004 treated mice. No SRP-9004 related toxicity was apparent in the tissues examined microscopically. The lack of adverse effects in SRP-9004 treated SGCA -/- mice was supported by clinical pathology data which indicated normal liver enzyme and reduced CK levels. In aged SGCA -/- mice, SRP-9004 treatment led to improved functional outcomes on treadmill, grip strength, and tibialis anterior force assessments. Taken together, these data demonstrate the efficacy of SRP-9004 and highlight a mechanistic basis to reduce LGMD2D/R3 pathogenesis regardless of age or disease state.

Conclusion: Intravenous administration of SRP-9004 demonstrated functional efficacy in young and aged mouse models of LGMD2D/R3. These findings suggest that SRP-9004 may be an effective therapeutic option for patients with LGMD2D/R3 across different levels of disease severity or progression.

39 Patient-derived stem cells for *in vitro* modeling of LAMA2-related Congenital Muscular Dystrophy

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LAMA2-related congenital muscular dystrophy (LAMA2-CMD) is a progressive degenerative condition whereby skeletal muscle cells lose their structural integrity in the absence of a critical extracellular protein, laminin-211. Affected babies and children struggle to independently move, breathe, and eat, leading to early death from medical complications. There is no cure for this disease. Mouse models have provided significant preclinical advancements in developing potential therapeutics, however, the mouse phenotype differs from the human condition with more significant nerve involvement than is observed in children. We have developed patient-specific iPSC-derived muscle fibers in 2D- and 3D-cultures that can serve as a potential platform for studying disease mechanisms and testing novel therapeutics. LAMA2-CMD patient cells showed abnormal development of complex 3D networks (neural crest-like structures) during differentiation of skeletal muscle progenitor cells (SMPCs) from iPSCs. Despite this, normal-appearing SMPCs were generated to a similar degree as parent controls. When SMPCs were further generated into contractile myofibers in 2D- and 3D- cultures, LAMA2-CMD myofibers showed similar developmental timeline and muscle biomarker profile, but with a reduction in the ability to sustain contractility on multielectrode array analysis when compared to parent control samples. Here we further show 3D-muscle bundle formation using commercial and in-house

products, using fibroblasts for supporting cells, and LAMA2-CMD patient tissues showed reduced attachment and contractility compared to parent controls. This study demonstrates an alternative approach to further understanding human-specific disease mechanisms and test potential therapeutics on a patient-specific platform for children suffering from LAMA2-CMD.

40 AAV Delivery of ADAR Mediated RNA Editing for Treatment of Collagen VI Related Muscular Dystrophy

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Introduction: The collagen VI related muscular dystrophies (COL6-RD) are inherited disorders of muscle characterized by progressive weakness and a combination of distal joint laxity and proximal joint contractures. Missense mutations resulting in substitution of the glycine residue in the conserved Gly-x-y repeat of the triple helical (TH) domain are the most common mutation accounting for 30-40%. Mutations in the TH domain allow incorporation of abnormal chains into secreted tetramers resulting in a dominant negative effect. Allele-specific gene silencing has been proposed as a therapeutic mechanism for COL6-RD and several different approaches using antisense oligonucleotide (ASO) or small interference RNA (siRNA) are in early stages of development.

We report an alternative approach to therapy for COL6-RD using adenosine deaminases acting on RNA (ADAR) mediated RNA-editing. ADAR is a site-specific RNA editor converting adenosine (A) to inosine (I) in RNA transcripts. Since 'I' in the mRNA sequence is read by the translation machinery as guanine (G), A-to-I edits of G-to-A transitions result in a correction of the mutation at the transcript level. ADAR editing may allow targeting of multiple adjacent sites using the same guide-RNA. This is especially attractive for COL6-RD where dominant negative mutations are clustered in the N-terminal end of the TH domain.

Methods: We used cultured fibroblasts from skin biopsy samples from patients with COL6-RD due to the c.850G>A (p.G284A) missense mutation in the TH domain of the *COL6A1* gene. Candidate guide-RNA sequences were cloned into plasmids and transfected into cultured using lipofectamine. ADAR editing was measured using RNA-seq. In our first experiments, we first used linear synthetic RNA oligonucleotides or RNA transcribed from plasmid vectors specific to *COL6A1*, exon 9. Next, we designed two circularized guide-RNA's targeting exon 9 by flanking the sequences targeting either pre-mRNA or spliced mRNA with sequences from Twister ribozyme and stem-forming sequences from pre-tRNA. The Twister ribozyme sequences undergo rapid autocatalytic cleavage, leaving termini that are brought together by the stem-forming sequences and ligated by the ubiquitous endogenous RNA ligase RtcB resulting in a more stable guide-RNA with longer half-life. To increase efficiency of transfection we incorporated the circularized guide-RNA into an AAV delivery system.

Results: Using linear synthetic RNA oligonucleotides or RNA transcribed from plasmid vectors specific to *COL6A1*, exon 9, we were unable to demonstrate successful editing. Circular guide-RNAs showed improved stability and longer half-life than the linear counterparts with A-to-I editing efficiencies approaching 20% for both pre-mRNA and mRNA guide-RNAs without detectable bystander editing. Our best candidate guide-RNA to date shifted the A:G ratio from 0.88 in control samples to 0.56 in treated samples. The incorporation of the designed guide-RNA into AAV system greatly improved the delivery efficiency and improved the accuracy of editing efficiency evaluation. Based on these improvements, we expanded our target sites to neighboring dominant mutations on the TH domain and achieved encouraging editing results.

Conclusions: We demonstrate A-to-I editing in vitro using a circularized guide-RNA. These experiments support development of ADAR editing as a therapy for COL6-RD.

41 Etiology of a Mouse Model of CHKB mediated muscular dystrophy - A Platform for Gene Therapy for Disorders of Lipid Synthesis

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The *CHKB* gene encodes choline kinase β, the first enzyme in the biosynthesis of the major phospholipid phosphatidylcholine (PC) by the Kennedy pathway. Megaconial congenital muscular dystrophy (OMIM: 602,541) is caused by rare variants in the *CHKB* gene and is a rare autosomal recessive disorder with clinical presentations that manifest as newborn congenital muscular dystrophy and adolescent-onset limb-girdle muscular dystrophy. Dilated cardiomyopathy is also present in some *CHKB* patients. Animal models mimicking a human disease are essential to understand

the in depth etiology of the disease and are vital tools in preclinical drug development. We have studied *Chkb*$^{-/-}$ (*Chkb*$^{rmd/rmd}$) mice to investigate the pathomechanism of disease and to evaluate whether it represents a suitable model for developing treatment options. Using *Chkb*$^{-/-}$ mice, we demonstrated that Chkb deficiency does not directly affect the level of the end product of the Kennedy pathway, PC, and instead produces large fluctuations in tangential lipid metabolic pathways. We determined that there is an inability to use fatty acids for mitochondrial β-oxidation in affected muscles leading to an initial build up of fatty acyl-carnitine and subsequent shunting of fatty acids into triacyglyerol and their storage as lipid droplets. There is a decrease in peroxisome proliferator-activated receptors (Ppars) and Ppar target gene expression specific to *Chkb*$^{-/-}$ affected muscle. Ppar agonists enabled fatty acids to be used for β-oxidation and prevented triacyglyerol accumulation, while increasing expression of the compensatory choline kinase alpha (*Chka*) isoform, in *Chkb*$^{-/-}$ myoblasts in vitro. Loss of sarcolemma integrity is a hallmark of muscular dystrophies and we determined that in affected muscle from *Chkb*$^{-/-}$ mice the dystrophin-glycoprotein complex is intact whereas there is a failure of the vinculin-talin-integrin system due to α-actinin detaching from the plasma membrane and disruption of the integrin-mediated linkage between actin filaments and sarcolemma. Pharmaceutial inhibition of choline kinase activity in U2O2 cells resultsed in internalization of a fluorescent PI(4,5) P2 reporter from plasma membrane clusters to the cytosol with a corresponding decrease in plasma membrane vinculin localization at focal adhesions that was restored by overexpressing *Chkb*. Furthermore, similar to humans, there is cardiac hypertrophy in *Chkb*$^{-/-}$ mice along with a decrease in left ventricle size, internal diameter, and stroke volume compared with wildtype and *Chkb*$^{+/-}$ mice. Both homozygous and heterozygous *Chkb* mice have increased susceptibility to cardiac arrhythmias. Similar to affected muscle, the major change in lipid level in *Chkb*$^{+/-}$ and *Chkb*$^{-/-}$ hearts is an increase in the lipid acylcarnitine, which is known to cause arrhythmias. Our data from *Chkb* deficient mice are phenotypically similar to those observed in *CHKB* patients, and provide mechanistic and observational insights into how a defect in PC synthesis can result in muscular and cardiac defects. These findings suggest that the *Chkb*$^{-/-}$ mouse model is a promising platform for developing novel treatment options, including gene therapies, for *CHKB* mediated muscular dystrophy patients.

42 Treatment of a Severe DM1 Mouse Model with Verapamil, Amlodipine, and Ranolazine

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Myotonic dystrophy type 1 (DM1) is the most common form of muscular dystrophy in adults. In DM1, there are hundreds of aberrantly spliced transcripts making it challenging to connect splice variants to disease manifestations. The mechanism behind the leading cause of mortality, muscle weakness and atrophy, remains poorly understood. However, a correlation between altered splicing in transcripts central to excitation-contraction coupling and muscle weakness in DM1 patients has been made. With this in mind we recently demonstrated that the combination of aberrant splicing of the skeletal muscle voltage-gated calcium channel (Cav1.1) and chloride channel (ClC-1) contributes to severe muscle weakness, respiratory deficits and reduced survival in mice. As a therapeutic strategy, we wanted to determine if existing drugs that target the Cav1.1 and myotonia improve muscle function and survival in longitudinal studies. Further, we showed that the calcium-channel blocker verapamil significantly improved survival, reduced muscle strength, prolonged time of righting and myotonia, and severe transient weakness (Cisco et al., 2024). In this study we are interested in determining if calcium channel blockers verapamil and amlodipine, and anti-myotinc ranolazine treatment could improve survival and health in a severe DM1 mouse models that exhibit the full panoply of altered splicing and myopathic features. To this end, we chose the *HSA*^{LR}/*Mbnl1*$^{-/-}$ mouse model, which combines toxic gain-of-function CUG-repeat expansions with loss of MBNL1 protein. We measured muscle force generation, respiratory function, body weight, time of righting reflex, and survival. We found that verapamil, amlodipine, or ranolazine treatment improved survival, increased muscle strength, improved respiratory function, increased body weight, and reduced time or righting in the *HSA*^{LR}/*Mbnl1*$^{-/-}$ model. This work shows that targeting $Ca_V1.1$ conduction with verapamil or amlodipine has therapeutic benefit, even in a complex model for DM1. Further, long term treatment with ranolazine improved survival and health in the *HSA*^{LR}/*Mbnl1*$^{-/-}$ mice. The next step will be to test if there is a synergistic impact of combining a calcium channel blocker with an anti-myotonic. Since these drugs are FDA approved, the goal is rapid translation to the clinic.

43 Splicing is Improved Using a Novel AAV-microRNA Delivery Platform as a Treatment for Myotonic Dystrophy Type 1

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Myotonic dystrophy type 1 (DM1) is the most common form of adult muscular dystrophy. Characterized by life-threatening muscle weakness, compromised respiration, and often cardiac conduction abnormalities, DM1 has high unmet medical need. DM1 is caused by a CTG repeat expansion in the 3' untranslated region of the dystrophia myotonica protein kinase (DMPK) gene which causes aggregation of DMPK mRNA hairpin structures as insoluble ribonuclear foci and sequestration of several RNA-binding proteins. Sequestration of key splicing regulators such as muscleblind like splicing regulator 1 (MBNL-1) drives aberrant splicing of hundreds of downstream transcripts. These splicing defects are thought to be a key driver of DM1 disease pathogenesis. We have developed a novel myotropic delivery platform comprising a myotropic AAV capsid and a promoter with strong activity in all muscle cells. We tested the ability of this novel platform to deliver a DMPK-targeting miRNA (AAV-amiR^{DMPK}) and correct DM1-associated splicing defects. First, we treated immortalized human DM1 myotubes with AAV-amiR^{DMPK} and analyzed the splicing profile of 36 genes previously shown to be affected in DM1 by NanoString analysis. Here, we observed significant splicing improvement for a subset of the 36 genes (MBNL1, GOLGA4, SOS1, CLASP1, zTTN and PKM). Subsequently, we performed splicing analysis of cardiac and skeletal muscles of a mouse model of DM1 (DMSXL mice), treated with a single intravenous dose of AAV-amiR^{DMPK}, and we showed that treatment resulted in improved splicing in both tissues. Together, our data demonstrate that our novel myotropic delivery platform can improve a key marker of the disease in the cellular and animal models of DM1.

44 Enhancing Antisense Oligonucleotide Efficacy Using Small Molecule Oligonucleotide Activity Enhancers (OAEs)

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Antisense oligonucleotides (ASOs) are synthetic oligonucleotides engineered to bind RNA and modulate gene expression through various mechanisms, including RNA interference (RNAi), RNase H-mediated cleavage, splicing modulation, non-coding RNA inhibition, gene activation, and targeted gene editing. ASOs hold significant promise for treating a wide array of complex genetic and rare diseases. However, their clinical application is frequently constrained by challenges related to delivery and intracellular sequestration, particularly in tissues beyond the liver and kidneys, e.g., musculoskeletal and nervous tissues. Intracellular sequestration, including endosomal entrapment, further diminishes ASO efficacy by restricting their availability at the intended site of action.

We hypothesized that small molecule oligonucleotide activity enhancers (OAEs) can significantly amplify ASO efficacy and safety. We created a mini library of OAEs by repurposing molecules already approved by the FDA or extensively researched for other indications. These molecules are hypothesized to aid ASO escape from endosomal entrapment, and they might also influence ASO activity through additional, yet to be fully understood, mechanisms.

We observed significant improvements in ASO activity across three *in vitro* disease models. In dysferlin-deficient limb-girdle muscular dystrophy recessive type 2 (LGMDR2) patient-derived myoblasts, which harbour a splice site mutation in intron 26, causing the transcript to go out-of-frame, we achieved a marked (~3-fold) increase in exon-skipping efficiency using phosphorodiamidate morpholino oligomers (PMOs) targeting exon 27 of the *DYSF* gene. This intervention restored the out-of-frame transcript to in-frame, leading to the expression of functional dysferlin protein. Enhanced muscle differentiation and improved membrane resealing capacity confirmed this functional recovery. In facioscapulohumeral muscular dystrophy (FSHD) patient-derived myotubes, OAEs significantly increased the efficacy of *DUX4* transcript knockdown (71–97%) when combined with locked nucleic acid (LNA) and 2′-O-methoxyethyl ribose (MOE) gapmers. This resulted in a substantial reduction in the expression of *DUX4* downstream genes, including *ZSCAN4*, *TRIM43*, and *MBD3L2*. For spinal muscular atrophy (SMA), patient-derived fibroblasts were treated with ASOs aimed to modulate the inclusion of *SMN2* exon 7. Co-administration of OAEs with free PMOs, DG9-peptide-conjugated PMOs, or MOE-based ASOs (matching the sequence of nusinersen, an FDA-approved therapy) significantly increased the inclusion of *SMN2* exon 7 (30–63%). These treatments were well tolerated across all *in vitro* models. These treatments were generally well tolerated across all *in vitro* models, though we observed a slight increases in apoptosis with some OAE treatments, indicating that further optimization may be required to balance efficacy and safety.

Across all these *in vitro* disease models and ASO platforms, we demonstrate significant enhancements in ASO activity. Our findings underscore the potential of OAEs as a practical tool for enhancing the efficacy of diverse ASO chemistries, thereby ex-

panding the therapeutic applicability of ASO-based strategies. Current research is focused on assessing the *in vivo* efficacy and safety of these OAEs in relevant animal models, with the goal of translating these findings into clinically viable therapeutic strategies.

45 Development of a New DUX4-Responsive Reporter Mouse

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Autosomal dominant Facioscapulohumeral muscular dystrophy (FSHD) is among the most prevalent muscular dystrophies affecting approximately 870,000 people worldwide and this progressive muscle-wasting disease typically exhibits in early adulthood. There are currently no disease-modifying treatments for FSHD, however, recent years have seen a surge in drug discovery efforts and an expansion of clinical trial networks dedicated to FSHD. Although several FSHD animal models are now available, each has limitations. We propose that improved animal models could better facilitate the development of pre-FSHD therapies.

Our lab developed several FSHD models and extensively uses the TIC-DUX4 model for pre-clinical *in vivo* efficacy studies. Both sexes of animals develop molecular, histopathological, and functional myopathic phenotypes in a Tamoxifen- and DUX4-dose-dependent fashion. Due to low-level, stochastic leakiness in the Tamoxifen control system, older TIC-DUX4 mice also develop milder histopathology and molecular phenotypes lacking induction with Tamoxifen. We propose that the leaky DUX4 expression system more accurately models low-level DUX4 expression in humans, compared to Tamoxifen-induced mice that express higher levels of DUX4 in nearly all myonuclei. Unfortunately, at these low DUX4 levels, individual TIC-DUX4 mice show phenotypic variability, demanding relatively large experimental numbers in therapeutic DUX4-inhibiting studies.

To track DUX4 expression and phenotypes longitudinally *in vivo*, we generated a DUX4-responsive reporter mouse. This model contains a single copy dual-reporter transgene expressing *Renilla reniformis* Luciferase-P2A-mScarlet. As *Renilla* luciferase has not been extensively used as an *in vivo* reporter, we first optimized the *Renilla* luciferase substrate delivery methods, finding that retro-orbital injections produce the best luminescent signal. In preliminary assessments, we show the model produces (1) DUX4-responsive luciferase, which can be imaged in live mice over time without requiring euthanasia; and (2) mScarlet fluorescence, for tagging individual myonuclei upon sacrifice. We have characterized this mouse model to establish the peak luminescence of *Renilla* luciferase and are now assessing phenotypes in crosses of TIC-DUX4 and our reporter mouse, which we expect to present at the meeting. This new model may ultimately be useful for testing *DUX4*-silencing therapies over time in the same mice and contribute to streamlining overall therapy development in the field.

46 Spatial Transcriptomic Characterization of an AAV-DUX4 Mouse Model of FSHD Identifies Focal and Widespread Cell Type Expression Changes in Muscle

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Facioscapulohumeral dystrophy (FSHD) is a slow progressive adult muscle disease whose mechanism is difficult to understand due to the extent, variability, and timing of muscles affected by the aberrant expression of the *DUX4* gene in adult muscle. Expression of DUX4 is associated with cell death *in vitro* and *in vivo*. To gain a better understanding of the spatiotemporal changes affected by *DUX4* gene expression *in vivo* we explored changes across muscle following induced DUX4 expression. Spatial transcriptomics was performed using a mouse model of FSHD developed in the laboratory which is produced on demand by activation of the *DUX4* promoter and gene in wild type mouse muscle following local adeno-associated viral vector (AAV6-DUX4) administration. Expression libraries were made from 55 um spots across cryosections made from AAV-DUX4 injected tibialis anterior muscles at 2 weeks post injection with doses ranging from 0.2-1e10 vector genomes and from muscle injected with a non-expressing vector control. At sites of *DUX4* expression mapped onto the tissue sections we observe a significant suppression of genes involved in mitochondrial and muscle function and activation of protein translation and innate and humeral immune response. Single cell deconvolution of data collected as nearest neighbor sites from *DUX4* expression reveals an increase in B cell, dendritic cell, neutrophil, FAP and macrophage expression. Moving away from *DUX4* expression there are increases in endothelial cell, myoblast, neural, and NK cell expression. The changes in specific cell type expression away from

areas of DUX4 induced damage suggest a larger muscle regional response to DUX4 that alters the broader muscle environment. In a current study we are comparing the changes in DUX4 exposed muscle to single nuclei expression changes in *mdx* mice to investigate muscle disease-related responses. Through analysis of the spatially distant changes in DUX4-exposed muscle we aim to identify points of biochemical pathway intervention to limit the wider effects of repeated DUX4 expression to reduce FSHD muscle damage.

47 Development and Characterization of a Minipig Model of Facioscapulohumeral Muscular Dystrophy (FSHD)

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Facioscapulohumeral muscular dystrophy (FSHD) is a progressive skeletal muscle wasting disorder, affecting nearly one million people worldwide. Currently, there is no cure or treatment. FSHD is caused by the aberrant increased expression of the *DUX4* (double homeobox protein 4) gene in skeletal muscles, resulting in progressive muscle weakness and atrophy. Therapeutic strategies that target DUX4 expression, activity, or downstream pathways are currently being developed. Several mouse models of FSHD have been generated, which have been useful for understanding disease mechanisms and developing therapeutic proof-of-concepts. However, mice are increasingly being recognized as poor predictors of efficacy in patients. Thus, an improved preclinical model in a species with better predictive efficacy is needed. To address this, we have generated an inducible DUX4 transgenic model in Göttingen minipigs. The ROSA26$^{tm(DUX4)}$; H11$^{tm(ACTA1\text{-}mERcremER)}$ minipigs display tamoxifen-inducible, Cre-dependent induction of DUX4-fl mRNA and protein in skeletal muscles. Initial characterization demonstrates that DUX4 minipigs recapitulate DUX4-FL target gene expression and a myopathic phenotype, similar to that seen in patients, including fat infiltration, fibrosis, and immune infiltration. Current efforts to establish the DUX4-inducible minipig as a preclinical model, including the development of robust translational readouts (gait analysis and MRI), will be discussed.

48 Human Stem Cell-derived Engineered Skeletal Muscle Tissues to Test Epigenetic Modulators and Deimmunized Gene Transfer Strategies for Duchenne Muscular Dystrophy

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Introduction
Although Duchenne muscular dystrophy (DMD) is a progressive and degenerative muscle disease of young boys, pathological molecular signatures are already present during embryonic myogenesis. Transcriptomic and epigenetic changes occur immediately downstream of dystrophin deficiency which sets off a cascade of pathological and compensatory signaling pathways. Our goal is to identify the earliest molecular drivers of pathology downstream of dystrophin deficiency, thereby identifying novel therapeutic targets that complement second-generation gene transfer technologies. To that end, we have created human iPSC-derived 3D engineered muscle tissues (EMTs) carrying various dystrophin mutations to test novel epigenetic modulators and deimmunized micro-dystrophins predicted to evade T-cell mediated immune responses. Top candidates will be evaluated for efficacy and safety using the DMD^{mdx} rat model.

Methods
Human induced pluripotent stem cell (hiPSC) derived models recreate embryonic muscle development and allow contractile property characterization in engineered muscle tissues (EMTs). Dystrophin-deficient EMTs were utilized to track the initiation and progression of pathology and then screened for functional recovery following treatment with novel epigenetic compounds from our high throughput DMD zebrafish screen. Additionally, deimmunized micro dystrophin (or utrophin-based) vectors were designed to be non-immunogenic by incorporating *in silico* prediction tools with protein redesign methods aimed at preventing T cell recognition and maintaining function *in vivo*. EMTs were analyzed using the Magetometric Analyzer for eNgineered Tissue ARRAY (MANTARRAY) platform, enabling non-invasive longitudinal analysis of developing contraction kinetics with varied conditions.

Results
Dystrophin-null EMTs showed force deficits, contractile kinetics dysregulation, elevated resting calcium and blunted calcium transients. The histone deacetylase inhibitor Trichostatin A reversed the contractile deficit and corrected other aspects of the

pathology in a dose dependent manner, validating the model for screening our novel compound, EPI1. Preliminary data for EPI1 shows functional restoration in both EMT and zebrafish models, priming it for DMD rat studies. Novel vectors demonstrate efficient lentiviral transduction into hiPSC-derived myoblasts with consistent expression across a panel of regulatory cassettes. Incorporation of transduced cells into the 3D system enables functional comparisons and iterative design before costly *in vivo* studies.

Conclusion
We aim to develop safer, more effective therapeutics for DMD that can be used in a broader patient population, including those currently excluded from trials due to the risk of immune responses. By leveraging hiPSC-derived EMTs, high-throughput screening, and computational protein design, we're creating a pipeline for optimizing potential treatments. This approach allows iteration of small molecule and gene therapy candidates, potentially accelerating the path to clinical trials. Combining epigenetic modulation and deimmunized vectors offers a multi-pronged strategy for DMD's complex pathology. As we move forward with the most promising candidates in animal models, we anticipate that this integrated approach will yield therapies with improved efficacy and safety profiles, benefiting DMD patients across the disease spectrum.

49 Delandistrogene Moxeparvovec Promoter Activity in Human Cardiomyocytes and Cardiac Safety Assessment in Mice and Nonhuman Primates

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Background: MHCK7 is a cardiac and skeletal muscle-specific promoter, which was developed from the alphamyosin heavy chain (α-MHC) enhancer and muscle creatine kinase (CK) enhancer and promoter. MHCK7 has been incorporated within the recombinant adeno-associated virus rhesus isolate serotype 74 (rAAVrh74) vector for targeted delivery of a transgene encoding delandistrogene moxeparvovec micro-dystrophin, an engineered dystrophin that retains key functional domains for the treatment of Duchenne muscular dystrophy (DMD).

Cardiomyopathy is a significant contributor to disease burden in patients with DMD. Thus, the evaluation of MHCK7 activity in the heart is essential for understanding the cardiac efficacy of delandistrogene moxeparvovec. Previously, we demonstrated the effectiveness of MHCK7 in driving expression of micro-dystrophin in the hearts of dystrophin-deficient *DMD^{mdx}* mice, dystrophin-deficient *DMD^{mdx}* rats, and wild-type nonhuman primates. Here, we demonstrate the ability of the MHCK7 promoter to drive delandistrogene moxeparvovec micro-dystrophin expression in human cardiomyocytes. An additional histological assessment of delandistrogene moxeparvovec safety in cardiac tissue of mice and nonhuman primates is reported.

Methods: The delandistrogene moxeparvovec transgene was delivered via the rAAVrh74.MHCK7 vector and promoter to human induced pluripotent stem cell (hiPSC) cardiomyocytes. Protein expression in cells was determined via Western blot and electrochemiluminescence (Meso Scale Discovery). In addition, cardiac tissue histology of wild-type mice and nonhuman primates was evaluated to determine if treatment-related adverse effects were evident. The same rAAVrh74.MHCK7 vector and promoter was used to introduce a full-length, codon-optimized human gamma-sarcoglycan (*SGCG*) transgene to wild-type nonhuman primates for further evaluation of cardiac safety in a larger species.

Results: We detected MHCK7-driven expression of delandistrogene moxeparvovec micro-dystrophin in hiPSC cardiomyocytes. The highest level of micro-dystrophin expression was elicited from the highest multiplicity of infection. Histological data suggest that MHCK7 mediates safe cardiac expression levels of micro-dystrophin in wild-type mice and nonhuman primates at the clinical dose of 1.33×10^{14} vector genomes/kg and as high as 3-fold the clinical dose in murine toxicology studies. With another rAAVrh74.MHCK7 cassette, we detected MHCK7-driven SGCG protein expression in wild-type nonhuman primate cardiac tissue. No adverse cardiac histopathological effects were noted at the highest dose used in the study.

Conclusions: MHCK7 effectively drives delandistrogene moxeparvovec micro-dystrophin expression in hiPSC cardiomyocytes. Delandistrogene moxeparvovec-mediated micro-dystrophin expression in the cardiac tissue of wild-type mice and nonhuman primates was tolerated and did not lead to adverse cardiac remodeling. Expression of a separate MHCK7-driven human *SGCG* transgene was demonstrated in the cardiac tissue of nonhuman primates. These data illustrate the utility of MHCK7 as a promoter for adeno-associated, virus-based gene therapies targeting human cardiac tissue.

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50 Reduction of Dystrophin-Targeting microRNAs Increases Expression of Clinically Relevant Dystrophin Isoforms

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When dystrophic muscle expresses a truncated dystrophin isoform, either in exon skipping-treated DMD or in BMD, reduced/suboptimal levels of dystrophin are observed. Our laboratory has previously described microRNAs that are elevated in DMD and BMD muscle and found that some of these (miR-146a/miR-146b/miR-223/miR-31) bind to the dystrophin 3′ UTR and reduce dystrophin protein (termed dystrophin-targeting microRNAs or DTMs). These microRNAs are regulated by the pro-inflammatory transcription factor NF-kB. As inflammation is present in DMD and BMD and persists even after patients are treated with FDA-approved dystrophin restoration therapies, it is likely that these inflammation-regulated microRNAs oppose optimal dystrophin expression. Here, we performed proof-of-principal studies to determine if genetic deletion, reduction, or inhibition of DTMs could increase expression of truncated dystrophin protein levels in DMD or BMD. We first determined if genetic deletion of a single DTM could increase dystrophin restoration by generating mdx52 mice with body-wide deletion of miR-146a (146aX mice). We analyzed muscles of mdx52 and 146aX treated with an exon 51 skipping PMO via Western immunoassay or immunofluorescence and found higher levels of dystrophin in treated 146aX muscles. We next examined the effects of a more global DTM reduction using the FDA-approved anti-inflammatory drug vamorolone (Agamree). Becker Muscular Dystrophy model (bmx) mice were administered 20 mg/kg vamorolone (Agamree) daily from 6-16 weeks of age. We observed significant DTM reduction and increased dystrophin protein levels in bmx gastrocnemius and heart muscles treated with vamorolone. Supporting these data, we found that 1) intramuscular injection of a miR-146a inhibitor into bmx gastrocnemius muscles increased dystrophin, and 2) conversely the co-injection of miR-146a with an exon skipping PMO in mdx52 muscles decreased dystrophin protein. Additionally, miRNAscope demonstrated elevated miR-146a expression in both the myofibers and inflammatory cells of dystrophic muscles. Collectively, these data show DTMs such as miR-146a are expressed in dystrophic myofibers and are detrimental to truncated dystrophin expression. Thus, inhibition of miR-146a singly or with other DTMs could benefit dystrophin protein expression and warrants further investigation.

51 Template-Assisted Sequence Knock-In Rescues a Deletion Model of Duchenne Muscular Dystrophy

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Advancements in gene-editing techniques present promising therapeutic options for addressing the underlying cause of inherited disorders. In Duchenne muscular dystrophy (DMD), the complex role of dystrophin in maintaining muscle function and health presents significant challenges in therapy design, as the restoration of truncated proteins often fails to fully address the disease's pathophysiology. Most patients harbour multi-exonic deletions that can be corrected through targeted DNA insertions, allowing for the expression of full-length dystrophin. However, the low efficiency of previously tested strategies limits clinical translatability. Here, we present a robust alternative, termed the template-assisted sequence knock-in (TASK) strategy, which we employed to target and correct the *Dmd Δ52-54* mutation *in vivo* (Fig. 1A). By co-delivering a TASK donor template and the Cas9 nuclease using AAV9 vectors, the splice-competent coding sequence for *Dmd* exons 52–54 was efficiently integrated into the gene, resulting in the systemic restoration of dystrophin protein. TASK improves on previous knock-in systems tested *in vivo* by incorporating elements that recruit all available endogenous repair pathways, thereby boosting its efficiency.

Our TASK-based strategy successfully restored the expression of full-length dystrophin at therapeutically relevant levels, up to 14.5% ± 1.0% in the heart and between 2.3% ± 0.6% in skeletal muscles (Fig. 1B), leading to significant improvements in muscle function. Left ventricular hypertrophy (LVH) is a major phenotype of the *Dmd Δ52-54* model, and given the high efficiency of our system in correcting cardiomyocytes, we quantified improvements to heart function. The assessment of heart rate and ejection fraction in systemically treated animals 12 weeks after injection demonstrated significant improvements (Fig. 1D left).

When comparing the progression of LVH, we observed a significant reduction in the thickening of anterior and posterior ventricular walls as well as an increase in the ventricular lumen diameter of treated animals (Fig. 1C). These findings highlight that the restoration of full-length dystrophin not only reduces the progression of LVH at 12 weeks but also dramatically enhances cardiac function.

To evaluate improvements in skeletal muscle function, we conducted grip strength tests, which demonstrated a significant increase in the forelimb and hindlimb strength of the treated animals. We also evaluated their performance in an open-field test to determine whether the systemic restoration of dystrophin was adequate to improve overall functional mobility. Significant improvements were noted in several parameters, including longer travel distances (Fig. 1D right). Weight gain, often a consequence of pseudohypertrophy in both patients and mouse models of DMD, was also reduced in TASK-treated mice, suggesting reduced inflammation. The functional and behavioural test results highlight the systemic benefits of restoring full-length dystrophin through our TASK-based DNA knock-in strategy, showing quantifiable physiological improvements in the treated animals.

These findings showcase TASK's potential for adapting to other large deletion mutations, offering a transformative approach for DMD and similar disorders. By precisely correcting genetic defects, this strategy brings us closer to precision health, enabling tailored therapies that directly address the unique genetic needs of patients.

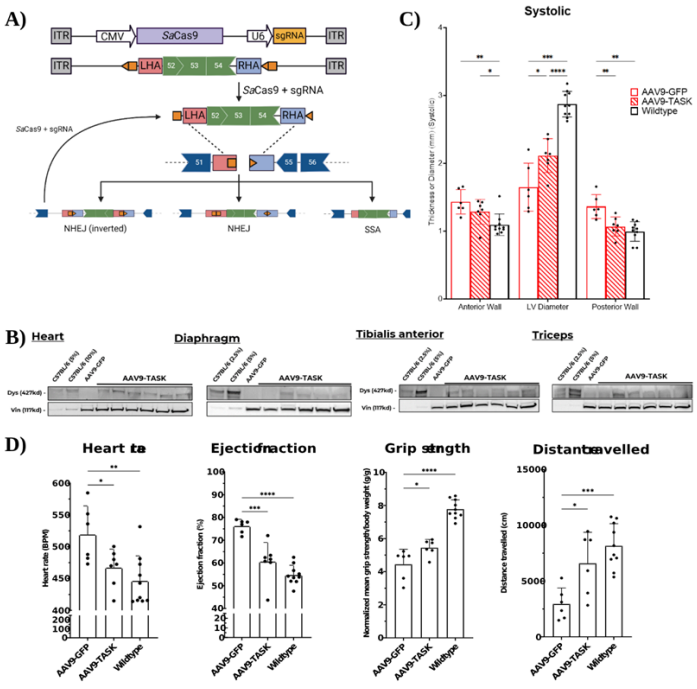

Figure 1. TASK-mediated insertion of *Dmd* exons 52-54 restores full-length dystrophin expression in a DMD deletion model, significantly improving skeletal and cardiac muscle function. A) Schematic of dual AAV vector approach for TASK-mediated exon 52-54 integration, orange pentagon represents the sgRNA target sequence, divided up into the spacer sequence (orange square) and the PAM (orange triangle). **B)** Western blot for dystrophin shows restoration of full-length protein expression in the heart as well as all skeletal muscles following systemic AAV9-TASK treatment. **C)** Echocardiogram analysis of the left ventricle shows reduced hypertrophy in the posterior and anterior walls as well as an increase in ventricular diameter of treated animals. Two-way ANOVA, followed by Tukey's post hoc multiple comparisons test (∗∗∗∗p < 0.0001, ∗∗∗p < 0.001, ∗∗p < 0.01 and ∗p < 0.05; mean ± SEM; n = 6-10 mice). **D)** AAV9-TASK treatment significantly improves cardiac and skeletal muscle function in *Dmd Δ52-54* mice. One-way ANOVA, followed by Dunnett's post hoc multiple comparisons test (∗∗∗∗p < 0.0001, ∗∗∗p < 0.001, ∗∗p < 0.01, ∗p < 0.05; mean ± SEM; n = 6-10 mice).

52 Correction of DMD Exon 45 Deletion Using Homology-Independent Targeted Integration

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Exon 45 deletion is the single most common mutation causative of Duchenne Muscular Dystrophy, accounting for 3-4% of patients. We developed a gene editing system based on CRISPR/Cas9 and homology-independent targeted integration to correct this mutation by inserting exon 45 into the native intron 44 in order to restore the full dystrophin coding sequence. The system is adaptable to other deletions beginning at exon 45. *In vitro* screening of guide RNAs (gRNAs) identified a lead candidate, and activity of the editing system was confirmed in patient-derived cells. A pair of AAV vectors were used for *in vivo* delivery. A single-stranded vector carried the SaCas9 enzyme, while a self-complementary vector carried 1-3 copies of the gRNA and a donor construct containing exon 45 along with short or long flanking intronic sequences. The donor construct was bounded by gRNA target sites in the reverse orientation relative to genomic DNA, thus resulting

in reconstitution of the target sites in the event of genomic integration in the incorrect orientation. Vectors containing multiple copies of the gRNA showed greater recombination during production, which was partially mitigated by using different promoters to drive each copy of the gRNA. Vectors were delivered by intramuscular injection in a humanized mouse model of exon 45 deletion. The donor construct containing long intronic sequences was least effective in restoring dystrophin expression due to unintended splicing events. The most efficient vector was used for systemic delivery in the same mouse model. Integration of the donor vector was detectable in all treated skeletal muscle, diaphragm and cardiac samples at 1 and 4 weeks. Editing efficiency was higher in the heart than in skeletal muscles and higher at 4 weeks than at one week, but the maximal efficiency remained low (0.15%). This was attributable to poor gRNA activity, as evidenced by a low rate of indel formation at the target site despite high Cas9 expression. Further studies will aim to optimize gRNA activity and donor vector design.

53 Ultrasound-Mediated Delivery of Non-Viral Gene Therapy Payloads to Skeletal and Cardiac Muscles in Mice and Non-Human Primates

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Introduction: Recent approval of viral gene therapy for Duchenne muscular dystrophy (DMD) has established a new standard of care paradigm for DMD patients. However, several significant limitations remain, including the inability to deliver full-size dystrophin gene, high product cost, pre-existing immunity to viral vectors, and lack of redosing capabilities. These challenges underscore the urgent need for developing alternative, non-viral gene therapy approaches for treating DMD patients. Transcutaneous ultrasound-mediated gene delivery (UMGD) represents a promising, noninvasive *in vivo* gene delivery method that could address the critical obstacles of viral-based genetic therapies. SonoThera is pioneering an innovative ultrasound-guided non-viral gene therapy platform, leveraging UMGD to enable selective targeting of specific organs and tissues within the body. This approach aims to offer a safe, redosable, durable, and titratable solution, potentially overcoming current state-of-the-art DMD treatment limitations.

Methods: To assess the potential of UMGD for safe and efficient transgene delivery to the heart and skeletal muscles, we developed an advanced technology platform that incorporates novel acoustic profiles, next-generation genetic payloads, and FDA-approved ultrasound components. This delivery process involves the intravenous co-administration of nucleic acid payloads and ultrasound contrast agents (microbubbles), combined with the targeted application of externally applied ultrasound energy to facilitate tissue-specific delivery via sonoporation. In vivo imaging, along with histological and ddPCR-based analyses, were employed to confirm transgene expression in both mouse and non-human primate (NHP) skeletal and cardiac muscles.

Results: Optimization of UMGD parameters resulted in the efficient delivery of non-viral genetic payloads to skeletal and cardiac muscles in NHPs. Parallel studies in mice demonstrated similarly effective genetic payload delivery, leading to sustained transgene expression. Notably, repeated treatments yielded a significant increase in gene expression compared to a single administration. Subsequent quantification of the payload copy numbers and histological analysis confirmed efficient and widespread biodistribution of transgene expression across muscle tissues.

Conclusion: The data support optimized UMGD as a promising method for delivering large transgenes to skeletal and cardiac muscles in both mice and NHPs. These findings suggest that sonoporation holds significant potential as a therapeutic approach for developing genetic medicines to treat muscular dystrophies.

54 Bioengineered Synthetic Chromosomes as Therapeutics for Muscular Dystrophies

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Introduction

Next-generation cell-based therapies for the treatment of muscular dystrophies will be buoyed by the development of portable, gene delivery systems that are capable of delivering large genetic inserts along with efficient safety switches. However, impediments remain as this approach is developed for precision medicine applications and broad utility in a clinical setting. These impediments include transgene genomic integration and increased mutagenesis, payload size limitations, and viral tro-

pism. Mammalian synthetic chromosomes (MACs) circumvent many of the limitations associated with plasmid and viral-based gene expression systems and provide an alternative means to introduce large segments of genomic DNA, sizeable cDNAs that exceed viral vector carrying capacity, developmentally regulated gene isoforms or splice variants, or multiple copies of two or more genes in fixed stoichiometry. We illustrate the utility of a portable synthetic chromosome to engineer cells for independently controlled delivery of multiple gene products.

Materials and Methods
We have developed a synthetic chromosome-based genetic platform, termed hSynC, engineered to carry the full-length cDNA corresponding to the human dystrophin gene (DMD). Placement of the DMD cDNA on the hSynC *via* a unidirectional, site-specific recombination event was catalyzed by a modified phage lambda integrase. Confirmation of the placement of the full-length DMD cDNA on the hSynC was demonstrated by PCR-based assay of recombination junctions as well as fluorescent *in situ* hybridization. In addition, expression of the DMD cDNA was confirmed by RT-PCR. As additional proof of concept of the utility of the hSynC bioengineering approach to DMD therapeutics, a splice variant of the DMD gene, Dp71ab, was isolated and engineered onto the hSynC platform. Finally, to address safety of the system in clinical applications a genetic safety switch was designed and included with the hSynC technology.

Results
Previously we have demonstrated the feasibility of engineering large and multiple genomic fragments onto a generated mouse synthetic chromosome as a curtain-raiser for human therapeutic applications (Greene *et al*., MTMCD (2019) 13:463-473). Our result in the present study builds upon previous work and demonstrates the ability of the human-derived hSynC platform for efficient and tractable engineering of the large human dystrophin cDNA as well as a transcript variant of the DMD gene, Dp71ab. As a further step in translating the hSynC platform technology for human therapeutic applications, we show that the hSynC system can incorporate a genetic safety switch capable of curbing potential side effects.

Discussion
The hSynC platform provides an adept bioengineering system enabling large genetic inputs onto a synthetic, chromosome-based vector without direct modification of the host genome. The hSynC proffers a novel cytoreagent system amenable to designing complex genetic circuits for multi-therapeutic biological delivery. Synthetic chromosomes, as engineerable modular platforms, interallied with advances in induced pluripotent stem cell production, could greatly streamline the process and broaden the utility of precision medicine for pharmaceutical manufacturing geared towards the treatment of muscular dystrophy.

55 Exon 45 Skipping and Dystrophin Production with ENTR-601-45 in Preclinical Models of Duchenne Muscular Dystrophy

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Intracellular delivery of oligonucleotide therapeutics for the treatment of Duchenne muscular dystrophy (DMD) is challenging because of poor cell entry and limited escape from the endosome in the target cell resulting in high therapeutic doses. To address this, we designed a family of cyclic cell-penetrating peptides that form the core of our Endosomal Escape Vehicle (EEV™) platform, which has been shown to efficiently deliver exon skipping phosphorodiamidate morpholino oligomers (PMOs) to skeletal and cardiac muscle. Studies in D2-*mdx* mice we showed robust exon skipping and dystrophin production in skeletal and cardiac muscle following administration of an EEV-exon 23 skipping PMO construct dosed every four or six weeks. These preclinical proof of concept studies confirmed the therapeutic potential of EEV-PMO conjugates for the treatment of DMD.

ENTR-601-45 is an EEV-exon 45 skipping PMO conjugate in development for the treatment of exon 45 skip amenable patients with DMD. We examined target engagement and efficacy of ENTR-601-45 in cell and animal models amenable to exon 45 skipping. Treatment of DMD patient-derived skeletal and cardiac muscle cells harboring an exon 45 amenable mutation deletion demonstrated robust, dose-dependent exon 45 skipping and dystrophin production.

To further examine the therapeutic potential of ENTR-601-45 in vivo, we utilized a newly generated del44-hDMDmdx mouse model containing a deletion mutation in the human *DMD* exon 44 transgene on an *mdx* mouse background (generated at Leiden University). These mice are amenable to human *DMD* exon 45 skipping and allow for quantification

of both exon 45 skipping, dystrophin production, and functional assessment *in vivo*. IV administration of ENTR-601-45 showed robust exon skipping translating to dystrophin production. Further, we demonstrate that the partially truncated dystrophin rescues muscle function comparable to the healthy control mice.

These findings demonstrate the robust preclinical efficacy of ENTR-601-45 in both cell and animal models and support further study in patients with DMD amenable to exon 45 skipping.

56 A Synergistic Oligonucleotide Therapy to Improve Muscle Health and Dystrophin Restoration in Duchenne Muscular Dystrophy

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Despite its proven safety, poor delivery of the dystrophin-targeting PMOs (phosphorodiamidate morpholino oligomers) limits the efficacy of dystrophin restoration by this Duchenne Muscular Dystrophy (DMD) gene therapy. Limited myogenesis and excessive fibrosis in DMD are features that contribute to the poor efficacy of PMO therapy. We show that the severe DMD mouse model (D2-*mdx*) replicates these features and poor PMO-mediated dystrophin restoration, as compared to the milder B10-*mdx* model of DMD. To address these features while benefiting from the safety of PMO-based therapy, we developed a TGFβ-inhibitory PMO and used it in combination with dystrophin exon skipping PMO for a synergistic therapy that simultaneously lowers macrophage TGFβ and enhances myofiber dystrophin level. Acute use of this therapy inhibited TGFβ signaling in injured areas and enhanced muscle regeneration and dystrophin restoration. Chronic use of this therapy reduced TGFβ signaling and ameliorated muscle loss. It also enhanced skeletal muscle functional capacity and improved dystrophin restoration in skeletal muscle and heart. These findings offer the first combination gene therapy for DMD that maintains the safety of PMOs and allows greater restoration of dystrophin while synergistically addressing fibrosis and poor myogenesis for greater improvement of muscle health in DMD.

57 A Novel IP-Mass Spectrometry Method to Characterize the Dystrophin Associated Protein Complex in Skeletal Muscle

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The dystrophin associated protein complex (DAPC) plays a pivotal role in the sarcolemma stabilization and cell signaling of a muscle fiber. The lack of dystrophin expression such as in Duchenne muscular dystrophy (DMD) results in severe and progressive muscle wasting which eventually leads to death due to cardiac and diaphragm deterioration. Promising therapies aimed at restoring the missing dystrophin in DMD have been developed in recent years and received accelerated or conditional approvals from the FDA. These include exon skipping therapies, micro-dystrophin gene therapy and stop codon read through therapies. However, the function and the turnover of these restored dystrophins, as well as their interaction with the DAPC components are not well characterized. Addressing these unmet gaps will provide a better understanding of the efficacy of these restored types of dystrophin and will help improve current and future dystrophin restoration therapies. We previously employed a targeted mass spectrometry approach to determine the turnover rate of exon-skipped dystrophin and DAPC components in mdx mice. Due to the inherent low abundance of dystrophin and DAPC proteins relative to total protein in muscle tissue homogenate, muscle lysates were fractionated by gel electrophoresis and bands containing target proteins were excised and subsequently subjected to proteolytic digestion and mass spectrometry analysis. This method, while robust, is highly labor intensive and thus unsuitable in studies involving the analysis of a large number of samples collected through pre-clinical and/or clinical studies. We have thus developed a sequential immunoprecipitation (IP) method in combination with targeted mass spectrometry to enrich and study the turnover of gene therapy restored micro-dystrophin and its effect on the turnover of the DAPC proteins using the mdx mouse model for DMD. Method standardization was performed on 4 different tissues (gastrocnemius, heart, diaphragm, and tibialis anterior) from untreated and treated mdx mice to determine the optimal lysis conditions using different SDS concentrations. A good reproducible extraction method that was compatible with IP was achieved using a modified RIPA buffer con-

taining 5% SDS. Muscle lysates were diluted with 1 mL of IP-MS Cell Lysis Buffer (Thermofisher Scientific), incubated overnight with 10 µL of anti-dystrophin antibody and antibody-protein conjugates bound by protein A/G magnetic beads. The remaining supernatant underwent a subsequent co-IP of three DAPC proteins (syntrophin, dystrobrevin, and dystroglycan). Tryptic peptides were generated for the two IP reactions and parallel reaction monitoring (PRM) was performed on six proteolytic peptides for dystrophin and 3, 2 and 1 respectively for syntrophin, dystrobrevin and dystroglycan. The method was found to be highly reproducible (CVs <20%) and shows parity with in-gel digestion analysis of the same samples. This method was found to be a highly specific, accurate and less time consuming way to quantify and study the turnover of low abundant proteins in muscle lysates. As proof of principle this method was applied to quantify DAPC components in muscle biopsies from DMD patients and healthy controls.

59 Cellular Mapping of DMD Transcript in Human Myogenic Cells and Skeletal Muscle by RNA in Situ Hybridization and Multiphoton Imaging

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Duchenne muscular dystrophy (DMD) is severe X-linked neuromuscular rare disease (1 in 5000 males) due to pathogenic variations in the dystrophin (DMD) gene, which lead to the reduction or absence of dystrophin protein (DYS). DMD is characterized by progressive weakness of skeletal, respiratory, and cardiac muscles with different phenotypes running from the severe Duchenne type to the Becker milder type and including other intermediate phenotypes. Deletions are the most common molecular defects (57%), followed by small mutations (32%), duplications (11%), and very rare, atypical rearrangements. Lack of dystrophin causes a cascade of pathological events impacting on cell division and differentiation and leading to failure of regenerate, depletion in muscle cell energy metabolism and signaling, cell death, and adipose and fibrous tissue replacement. All these steps cause disease progression and clinical outcome which is very severe and often fatal in Duchenne boys. Several gene corrective strategies are ongoing to restoring dystrophin protein via translation read-through therapy, exon skipping, or vector-mediated gene therapy, and some published results highlight success and failure. Despite these therapies target RNA and/or are related to the gene transcription, the precise mechanisms underlying DMD transcript dynamics, cellular compartmentalization, spatial gene expression, and nuclear export are unknown, though they are outstanding to design RNA-targeted therapies and gene therapies. We have done a fine characterization of DMD gene transcription dynamics and spatial localization by RNAscope, in both DMD myogenic cells and skeletal muscle and we showed that the global reduction of DMD mRNA is due to a dramatic decrease of the DMD mRNA in the cytoplasmic fraction, while a massive amount of DMD transcript is retained in the nucleus (up to 90%) with high accumulation in the nucleoli. This leads to severe unbalanced transcript spatial distribution with poor or absent mRNA availability in cytoplasm. Our data provide evidence that the DMD mature transcript export fails since of the incompleteness of the mutated mRNA maturation and since that both heterogenous RNA (hnRNA) and mRNA accumulate in the nucleus, possibly exerting a nuclear spatial hindrance impacting on hnRNA processing. Multiphoton imaging is an innovative label-free method recently used in DMD muscle to identify both muscle macro-morphology and new biomarkers, especially related to collagen displacement, abundance and fibers microarchitecture, avoiding the use of traditional staining. Indeed, collagen fibers, due to their non centro-symmetric morphology, can be imaged by acquiring the Second Harmonic Generation (SHG) signal, which is scattered by collagen at twice the frequency of the incident laser. We have innovatively combined multiphoton image to RNA scope (Figure 1) to further define the muscular spatial localization of both collagen and DMD transcript to better profile not only DMD RNA localization but its relationship with other muscle structures. Moreover, we preliminary demonstrated that using artificial intelligence (AI) we can correlate collagen-related morphological properties and dystrophin transcripts expression in DMD muscle biopsies. Further studies are in progress, and if successful, the combined RNA-scope-multiphoton image approach could be used as an ex-vivo or in vivo, very novel image-RNA biomarker tool.

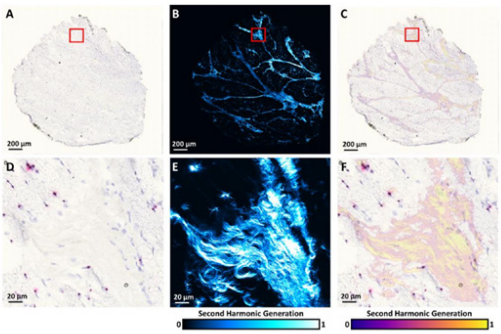

Figure 1
(A) shows a whole image of RNAscope ISH 37-42 probe on SKM tissue from a DMD patient (…). (B) reports the SHG signal scattered by collagen in the same biopsy, acquired with label-free multiphoton microscopy. (C) depicts the output of the elastic registration procedure involving (A) and (B). Red squares in (A), (B) and (C) highlight the same regions of interest, whose zoom is (D), (E) and (F), respectively.

60 Gene Transfer to Muscle Using HSV Vectors

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Duchenne muscular dystrophy (DMD) is an X-linked recessive, progressive muscle-wasting disease affecting approximately 1 in 5,000 male births. DMD is caused by frameshift or nonsense mutations in the dystrophin (*DMD)* gene that significantly reduce or eliminate expression of the dystrophin protein. As a monogenic disorder, DMD represents an ideal target for gene replacement therapy. However, the full-length dystrophin cDNA is >11 kb, limiting current gene therapy to micro-dystrophin proteins that fail to fully restore function. Replication defective (rd) herpes simplex virus (HSV) vectors are emerging as an ideal candidate for efficient transduction of myofibers *in vivo*. This non-toxic, high-capacity gene delivery platform accommodates large amounts of foreign DNA, overcoming the limitations of other viral vector-based platforms. We recently determined that transgene expression in rdHSV vectors requires strategic placement of insulators—small DNA elements that overcome the host's epigenetic silencing of foreign DNA—to maintain transgenes transcription. Moreover, the viral insulator flanking the latency-associated transcript (LAT) and the infected cell protein 4 (ICP4) loci showed to be responsible in maintaining transgene expression in a cell-specific manner. Hence, comparison of the expression profile of these two loci in muscle cells showed that the LAT locus expressed significantly higher levels of transgene compared to the ICP4 locus. Transgene expression in this location was protected in vivo up to 1 year upon direct injection in the mouse hind limb skeletal muscles. These data suggest that in rdHSV vectors the LAT locus is the most effective location for a gene therapy approach targeting muscle tissue. To further enhance transgene expression, we designed a novel insulator construct by combining cellular and viral insulators. The new design included mouse tRNA genes and scaffold/matrix attachment regions (S/MARs) known to function as chromatin barrier elements shielding the transgene from the spread of repressive heterochromatin. This novel combination of insulator elements restored transgene expression at the ICP4 locus and eliminated the difference in transgene activity observed between the two loci in muscle cells. We have previously shown that a rdHSV vector expressing the full-length dystrophin cDNA was able to restore dystrophin expression to differentiated muscle progenitor cells (MPCs) derived from dystrophin-deficient *mdx* mice. Here we expand this observation showing that a rdHSV vector can restore dystrophin expression in the D2.mdx dystrophin deficient mouse model. These findings encourage further studies to apply rdHSV vectors for treatment of mouse models of DMD.

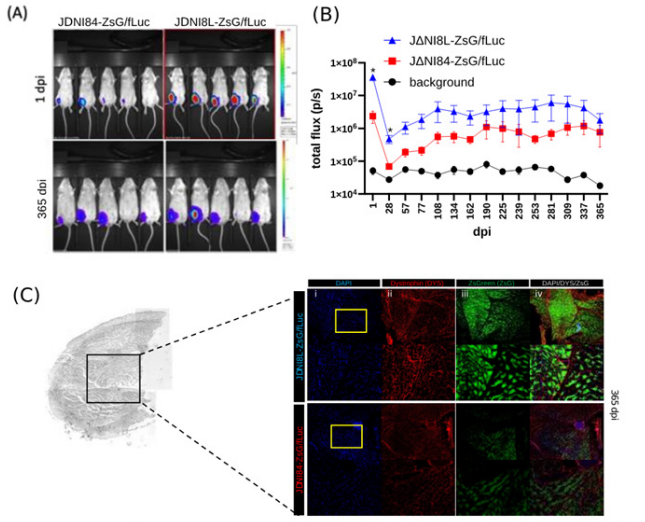

Figure 3. In vivo expression in the mouse hind limb muscles. Mice were injected with the indicated vector and assessed by BLI: **(A)** images at 1 dpi (upper) and 365 dpi (lower), **(B)** time course over 365 dpi as photons per seconds (p/s). (N=8 per group; p-value < 0.005 *). **(C)** ZsG distribution (365 dpi). Left: hematoxylin and eosin–stained full cross section of the mouse hind limb. Black box indicate the area where ZsG positive signal is localized. Right: upper panels show low magnification images (4X); lower panels show higher magnifications of the areas framed in yellow in the upper panels i (10X). (i) Nuclei, stained in blue with DAPI; (ii) dystrophin stained in red; (iii) ZsG positive fibers in green; (iv) DAPI, dystrophin and ZsG triple-fluorescence.

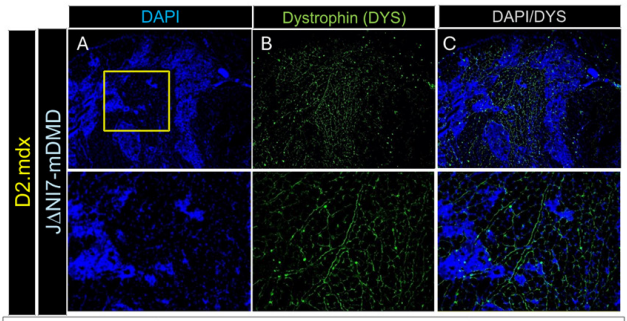

Figure 4. Dystrophin distribution in the hind limb muscles of D2.mdx mice upon DI of JΔNI7-mDMD. Representative cross section of hind limb muscles (8 sections/animals, 3 animals) injected with JΔNI7-mDMD. Upper panels show low magnification images (4X); lower panels show higher magnifications of the muscle area where abundant DYS transduced fiber are observed (framed in yellow in the upper panel A). (A) Nuclei, stained in blue with DAPI; (B) DYS positive fibers in green; (C) DAPI and dystrophin double-fluorescence.

61 Correction of Nonsense Variants in *DMD* Using AAV-Compatible Base and Prime Editing

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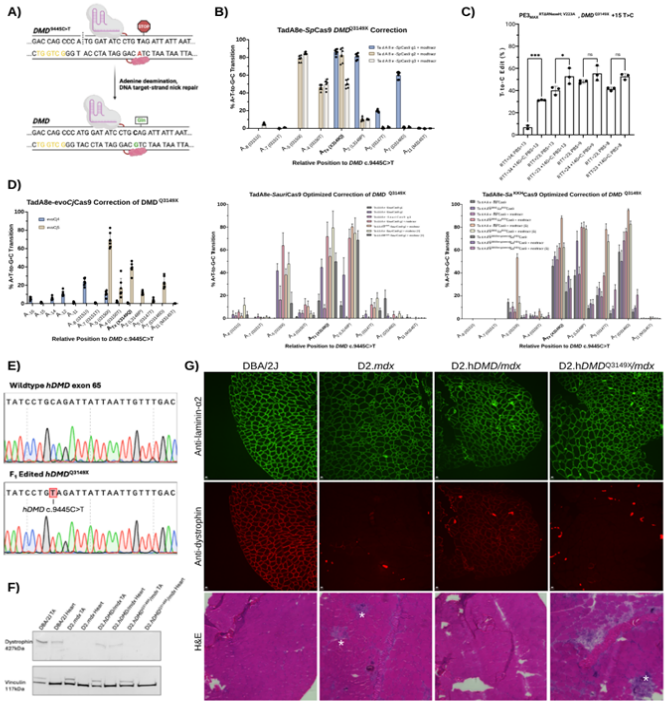
Duchenne muscular dystrophy (DMD) is an X-linked recessive neuromuscular disorder caused by perturbations of the *DMD* gene resulting in loss of functional dystrophin, a structural protein located at the sarcolemma. Dystrophinopathy compromises the mechanical integrity of skeletal/cardiac myofibers, culminating in progressive muscle wasting and cardiorespiratory dysfunction often fatal by the third decade of life. We performed extensive molecular characterization of a patient identified clinically harboring a truncating *de novo* nonsense variant *DMD* c.9445C>T (NM_004006.2) affecting all dystrophin isoforms. To characterize variant pathogenicity and potential treatment modalities, we established this nonsense variant in our novel fully-humanized D2.h*DMD*$^{9445C>T}$/*mdx* murine model which recapitulated key histopathological features of DMD including loss of dystrophin production and laminin-α2 co-localization, severe endomysial thickening with fibrotic infiltrates, and centralized nuclei throughout cardiac and skeletal muscles, respectively and concluded the variant was not amenable to canonical CRISPR/Cas9 nuclease-based exon-skipping or reframing approaches. Adenine base editors (ABEs) are a CRISPR-derived technology capable of performing programmable A•T-to-G•C transitions *in situ* via engineered TadA-mediated deoxyadenosine deamination, however their lack of target-adenine specificity (collateral DNA bystander and RNA editing) and large size has hindered translational applications due to off-target complications and packaging constraints of clinically validated myotropic viral vectors including AAV, respectively. Here, we provide the first demonstration of single AAV-compatible ABEs for precise correction of nonsense variants capable of restoring full-length dystrophin expression in DMD patient-derived myoblasts *ex vivo*. We evaluated ABE constructs via stable lentiviral transduction and differentiation of patient-derived skeletal myoblasts and established that simplifying the heterodimeric TadA8e deoxyadenosine deaminase to a monomeric configuration could efficiently correct *DMD*$^{9445C>T}$ up to 88.2±1.7% when coupled with diverse Cas9 nickase orthologues (*Sp*Cas9, *Sa*^{KKH}Cas9, *Sauri*Cas9, evo*Cj*Cas9). Notably, we demonstrate that rational engineering of the TadA8e deaminase, in combination with modified gRNA spacer and scaffold architectures, synergistically reduced bystander effects while eliminating off-target RNA editing with minimal compromise in therapeutic activity (49.9±7.4%). Furthermore, we benchmarked our patient-optimized ABE with prime editing (PE), an alternative search-and-replace DNA editing technology capable of directly reverse transcribing programmable edits into the genome. Engineering of the prime editor to reduce its size and improve transcriptase activity (PE3$_{MAX}^{RT\Delta RNaseH;V223A}$) and performing iterative optimizations of epegRNA and ngRNA design resulted in a dual-AAV compatible PE system with comparable levels of errorless *DMD*$^{9445C>T}$ correction (55.4±6.8%) and significant dystrophin rescue *ex vivo*. These results substantiate the utility of base and prime editors as efficacious tools for therapeutic genome editing and identify previously undescribed optimizations for enhancing precision and safety considerations required for clinical translation. As ~50% of all pathogenic variants are point mutations, the advancements described here expand the scope of variants amenable to therapeutic gene editing for conditions beyond DMD.



Adenine base editing and prime editing for correction of *DMD*$^{9445C>T}$ and characterization of the first fully-humanized mouse model of DMD. A) Schematic representation of the *DMD*$^{9445C>T}$ (p.Q3149X) nonsense variant caused by a pathogenic C>T transition (bold black base) of the glutamine codon (CAG>TAG). Deamination of the complementary A (red base) to G (green base) restores the wildtype glutamine codon and rescues full-length functional dystrophin expression. Selected example of TadA8e^{SpCas9} PAM (yellow bases) and nick position (vertical dotted line). **B)** Proof-of-concept ABE using TadA8e-*Sp*Cas9. **C)** Preliminary optimization of prime editing comparing efficiency of +14G>C MMR-evading mutation. One-way ANOVA with Tukey Post-Hoc (N=3). **D)** Efficiency of therapeutic ABE of three available *Sauri*Cas9 gRNA (*Sauri* g1-3) and one *Sa*^{KH}Cas9 gRNA. Distinct patterns of adenine (A) bystander edits occur at positions relative to the therapeutic (A $_{Tx}$) target (N=6-12). Modified gRNA scaffold (modtracr) and spacer (S) architecture. **E)** Electropherograms highlighting successful generation of the patient *DMD* ^{Q3149X} nonsense variant in the first fully-humanized whole-gene (~2.3Mbp) transgenic mouse model of DMD. **F)** Dystrophin immunoblot shows compensation by h*DMD* on a dystrophin-null (*mdx*) mouse line and loss of human dystrophin expression in the h*DMD* ^{Q3149X}/*mdx* model. **G)** Immunofluorescent characterization of dystrophin-laminin-α2 co-localization at the sarcolemma in skeletal muscle (tibialis anterior; similar patterning in myocardium not shown). Histological characterization of dystrophic phenotype in the h*DMD* ^{Q3149X} /*mdx* model recapitulated key hallmarks of disease including the presence of centralized nuclei, variable myofiber diameter, and fibrotic/immune infiltrates (asterisks) comparable to dystrophin-null (*mdx*) mice.

62 Extensor Digitorum Brevis Feasibility for Cell-Based Therapy for Duchenne Muscular Dystrophy

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Background: Duchenne muscular dystrophy (DMD) is an x-linked recessive disorder caused by a loss of function in dystrophin gene production1. To track muscle function less invasively for DMD patients, ultrasound (US) images of the extensor digitorum brevis (EDB) were captured to determine correlation with magnetic resonance (MR) EDB images2,3. US images of the EDB were patient-matched with the tibialis anterior (TA) to compare the EDB's feasibility for injection of induced pluripotent stem cells (iPSC).

Methods: Seven DMD patients, ages 10.5 to 17.0, had their EDB imaged by US and were quantified in terms of gray-scale using the ImageJ program4. Five individuals had EDB MR images captured and function levels graded by a radiologist5,6. Four individuals had both US and MR images captured. Six participants had both their TA and EDB imaged using US. Four participants with EDB images had their hand-grip strength compared, and all participants's ambulatory status was assessed.

Results: The coefficient of determination, R2, was 0.5746 between US and MR EDB images. The TA had a higher gray-scale value than the EDB in 100% of patients, ($t(5) = 3.14$, $p = 0.0257$). Ambulatory status was correlated with a lower mean EDB grayscale value but did not have a significant difference ($t(2.74) = 2.195$, $p = 0.077$). Functional hand-grip strength was correlated with lower mean EDB gray-scale values with an R2 value of 0.788.

Discussion: The study demonstrated a correlation between US imaging and standard research biomarkers of muscle function tracking for DMD patients. In DMD patients, the EDB muscle was significantly less atrophied than the TA muscle, promoting its feasibility as a target for cell-based therapies.

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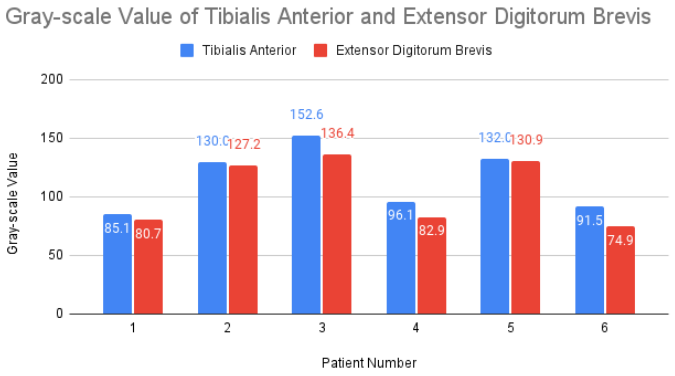

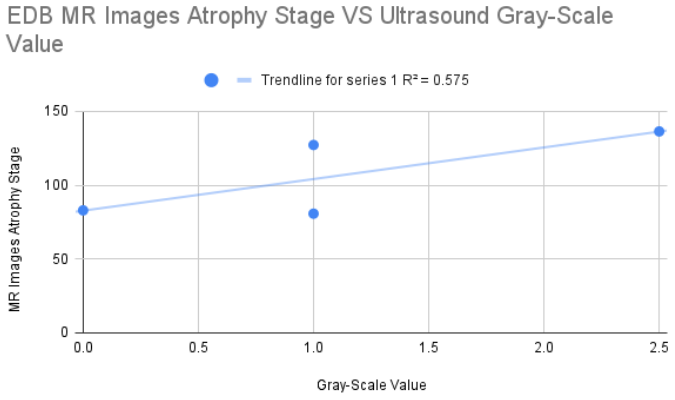

63 Transcriptomics Reveals DMD Driven Cell Dynamics and Mechanisms of Myofiber Death and Innate Immune Memory in Human Dystrophic Muscle; Potential Barriers to Microdystrophin Gene Therapy

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Single cell/nuclei technologies have revolutionized our understanding of the dramatic remodeling of complex multi-cellular tissue that accompanies injury, regeneration, and disease. Duchenne muscular dystrophy (DMD) is a fatal genetic disease of childhood characterized by progressive skeletal muscle weakness and resulting from mutation of *DMD* and loss of functional dystrophin protein. Here we discuss, at single nuclei/cell resolution, intramuscular cell and gene expression dynamics within a broad cohort of needle muscle biopsies obtained from DMD individuals with varying degrees of severity and includes a subset of DMD individuals with low levels of dystrophin. We report a strong correlation between expression levels of *DMD*/dystrophin and disease severity and substantial differences in cellularity and cell type-specific gene expression in DMD versus healthy muscle. Expression signatures indicate that DMD myofibers become immunologically alert, upregulating adaptive and innate immune sensors, including TLR4, TNF family receptors, and mediators of inflammation and pyroptosis. Niche cells, including fibroblast and myeloid cells, expand and diversify to encompass over 40% of the muscle nuclei. One immune responsive fibroblast subpopulation that induces complement C3 and TNC as well as three myeloid populations, are expanded in DMD muscle and express signatures of innate immune memory, epigenetically instilling tissue memory to perpetuate and amplify the hyper-inflammatory DMD state and contributing to myofiber death. We discuss longterm microdystrophin gene expression and cellular shifts from gene therapy in DMD. The compendium of single/cell nuclei DMD severity we report here serves as a valuable reference set and has immediate impact for biomarker discovery, clinical trial design, identification of barriers to dystrophin replacement therapies and novel druggable cell mechanisms operating in DMD.

64 Development of Functionalized Lipid Nanoparticles for Enhanced Micro-Dystrophin Delivery in Duchenne Muscular Dystrophy Therapy

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Duchenne Muscular Dystrophy (DMD) is a genetic disorder caused by mutations in the dystrophin gene, leading to muscle wasting and respiratory failure. Despite significant research efforts, developing effective treatments remains challenging due to the gene complexity and large size. A promising approach involves using micro-dystrophin variants, which are smaller segments of the dystrophin gene containing essential functional domains, optimized for gene delivery.

To meet the demand for efficient and protective nucleic acid delivery systems in gene therapy, we developed a library of ten DNA-loaded lipid nanoparticles (LNPs) using an advanced microfluidic platform. Initially, these LNPs were loaded with a luciferase reporter gene and characterized for their physicochemical properties. Biological efficacy tests in the C2C12 myoblast cell line identified LNP2 as the most promising candidate. We then enhanced LNP2 with a functional DNA coating, significantly boosting its performance compared to the unmodified version.

Next, we transfected primary muscle cells isolated from the dystrophic mdx^{4cv} mouse model. These cells present a challenge due to rapid differentiation, which hinders plasmid nuclear entry. To overcome this problem, we pre-condensed the DNA with Protamine Sulfate (P*), which naturally contains a Nuclear Localization Signal (NLS). After optimizing the P*-to-DNA weight ratio, we synthesized and characterized an LNP encapsulating pre-condensed DNA. Our subsequent screening revealed that combining pre-condensed DNA with DNA surface coating markedly enhanced the transfection efficiency of P*-LNP2. Confocal microscopy confirmed a significant increase in nuclear localization, a crucial result as efficient gene delivery to the nucleus is essential for effective gene therapy in non-dividing cells. Encouraged by these findings, we synthesized and characterized an LNP2 formulation encapsulating micro-dystrophin pre-condensed with P*. This complexation improved the physicochemical properties of the LNPs, which were then tested on primary dystrophic muscle cells. The system significantly increased dystrophin expression levels and reduced IL-6 expression levels, a critical player involved in the establishment of a chronic inflammatory response. As a last step, we validated the optimized formulation on a bioengineered three-dimensional vascularized skeletal muscle tissue, referred to as eX-vivo Muscle Engineered Tissue (X-MET). In conclusion, our innovative approach consisting in using P*-condensed micro-dystrophins within functionally coated LNPs has shown substantial potential in enhancing gene expression and reducing inflammation in mdx4cv-derived primary muscle cells. These findings support further exploration of this strategy in in vivo settings to fully realize its therapeutic potential for DMD.

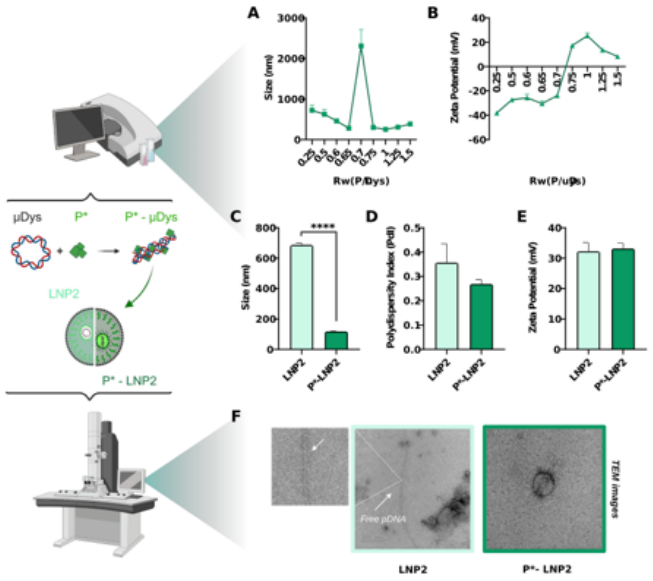

Figure 1. Size **(A)** andZeta Potential **(B)**of theProtamine (P*)– micro-dystrophin (μDys) complex atdifferent wight ratios (Rw). Forthe furthersteps theRw=0.65 was chosen.
Characterization ofthe size**(C)**, PdI**(D)** andzeta potential **(E)**through Dynamic Light Scattering (DLS). TEM images ofthe synthetized LNPswith andwithout μDys pre-condensation with Protamine.
Statistical analysis have beenperformed throughStudent's ttest.

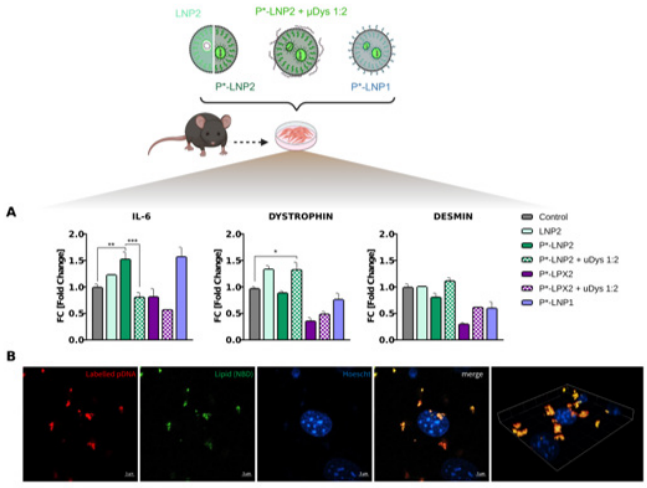

Figure 2. **(A)** The RealTime-PCR expression analysis ofIL-6, DYSTROPHIN andDESMIN onmdx ^{4cv} – derived primary muscle cells. Thesignificance was measured byOne Way Anova andTukey's multiple-comparison post test, *p<0.05. **(B)** Confocal microscopy images ofP*-LPX +pDNA 1:2 with three different staining: labelled pDNA (CY3), NBD(lipids) andHoescht (nuclei).

65 A Novel Engineered Membrane Repair Protein Therapeutic Increases Sarcolemma Repair by Producing Phosphatidylserine Binding in Models of Duchenne Muscular Dystrophy

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Introduction: Duchenne Muscular Dystrophy (DMD) is a fatal neuromuscular disease involving progressive cardiac and skeletal myocyte cell death and subsequent muscle degeneration. DMD and Becker muscular dystrophy (BMD) result from mutations in the dystrophin gene that compromise the structural integrity of the sarcolemma. While there has been important recent progress in the treatment of DMD, the currently available therapeutics have significant limitations. Thus, novel therapies that address the compromised sarcolemmal membrane integrity are an unmet medical need. Tripartite motif protein 72, or mitsugumin 53 (TRIM72/MG53), facilitates the sarcolemma repair response after disruption of the membrane. Loss of TRIM72/MG53 function in mice leads to a progressive myopathy. Our previous studies showed that exogenous delivery of recombinant human MG53 protein (rhMG53) can increase sarcolemmal membrane repair in many different cell types and ameliorate pathology in models of DMD and multiple limb girdle muscular dystrophies. Despite the promise of rhMG53 as a therapeutic invention the development of this protein to treat DMD has been complicated by findings showing that high levels of the protein correlate with metabolic dysfunction in animal models and some human patients. Deletion analysis of the major domains of the rhMG53 protein allowed us to design an improved version of rhMG53 called MyoTRIM.

Methods: Phosphatidylserine (PS) binding capacity was measured using a PS-coupled bead pull down assay followed by blotting for the recombinant protein using a specific antibody. Membrane repair was assessed in skeletal muscle in the mdx mouse using multiphoton microscopy. Briefly, the flexor digitorum brevis (FDB) muscle was isolated by dissection and attached to the bottom of a 35 mm glass bottom dish. Ca2+ Tyrode and 2.5 μM of FM4-64 dye (ThermoFisher) were transferred to the dish so that the FDB was completely submerged. Immediately after addition of MyoTRIM, individual muscle fibers were imaged on an Olympus Multiphoton FV1000 microscope, and an area of ~0.9 ×0.9 μm of the plasma membrane was vaporized by heat transferred by the infrared laser. The extent of FM4-64 dye entry was monitored by confocal microscopy and used as a proxy for membrane repair capacity.

Results: MyoTRIM contains amino acid replacements to eliminate metabolic effects and improve solubility while maintaining membrane repair effects. One set of modifications inactivate the E3 ligase activity of the protein, which revealed this function is dispensable for subcellular localization of the protein. TRIM72/MG53 is reported to mediate this effect by binding PS at membrane injury sites, so we demonstrate that MyoTRIM can bind PS-coated beads to illustrate that carboxy-terminal domains are required for efficient PS binding rather than the E3 ligase function. External application of MyoTRIM can increase membrane repair capacity in

a variety of cell-based assays using DMD and BMD patient myoblasts and myotubes. We also establish that MyoTRIM can increase membrane repair and prevent eccentric contraction induced injury in a dystrophin deficient mouse model.

Conclusion: Taken together, these results provide mechanistic insight into rhMG53 function and indicate that MyoTRIM can recapitulate the therapeutic effects on sarcolemmal membrane repair while eliminating E3 ligase activity and associated side effects.

66 Prime Editing Permits to Correct Point Mutations Responsible for Many Muscular Dystrophies

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Most muscular dystrophies are due to point mutations or to a frame shift deletion of one or several exons. Most of these mutations can be corrected by the Prime editing (PE) technology derived from the CRISPR/Cas9 technology (Anzalone et al. Nature 2019). PE uses a Streptococcus pyogene (SpCas9) nickase fused with an engineered reverse transcriptase (SpCas9n-RT) and a prime editing guide RNA (pegRNA) which is an extended single guide RNA (sgRNA). The pegRNA contains a spacer (i.e., a 20 nucleotides sequence which hybridizes with a protospacer sequence in the genome) which has to be followed by a Protospacer Adjacent Motif (PAM), which is NGG for the SpCas9n. The pegRNA also contains an extension of the constant sgRNA nucleotide sequence, which binds with the SpCas9n protein. This extension includes a Primer Binding Site (PBS) which hybridizes with the strand of DNA released by the SpCas9n and a Reverse Transcriptase Template (RTT). As indicated by its name the RTT see as a template for the RT to synthetize a new DNA strand. Thus, the RTT sequence permits the synthesis of a new DNA strand containing one or several nucleotide modifications. Any nucleotide may be replaced by any desired nucleotide. Moreover, the PE technique permits small deletions or small insertions. Initially PE was producing low percentage of precise gene editing but since its discovery in 2019 many improvements have been developed and it is possible to obtain high percentage of PE (up to 60%) in cells in culture

Our research group has used PE to correct point mutations responsible for Duchenne Muscular Dystrophy located in the *DMD* gene coding for dystrophin (Mbakam et al. MTNA 2022; Mbakam et al. Neurotherapeutics 2022; Mbakam et al. Int J Molecular Sciences 2022). Insertions or deletions of a few nucleotides also permitted to restore the normal reading frame of DMD gene deleted of one or several exons. We have also corrected mutations responsible for Congenital myopathy 1A or 1B in the Ryanodine Receptor 1 (*RYR1*) gene (Godbout et al. Cells 2023) and in the *DYSF* gene responsible for dysferlinopathies (Limb-Girdle Muscular Dystrophy LGMD2R and Miyoshi muscular dystrophy) (Bouchard et al. in preparation). PE may eventually be used to correct point mutations responsible for other hereditary muscle and non-muscle diseases.

For *in vivo* treatment the PE components may be eventually delivered using different vectors like a dual Adeno Associated Viruses (AAVs), Lipid Nanoparticles (LNPs), extra-cellular vesicles (EVs), and engineered Virus Like Particles (eVLPs).

The PE technology is promising. It indeed permits to specifically add, delete or replace selected nucleotide(s) in many mutated genes responsible for several muscular dystrophies. Modifications a low percentage of genes may eventually be enough to induce the presence of enough normal proteins to induce a phenotypic improvement.

67 Combinatorial in vitro and in vivo Phage Display Methodology to Identify Skeletal Myocyte Targeting Cell Penetrating Peptides

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Background: Cell penetrating peptides (CPPs) are 5-30 amino acid long peptides able to cross cell membrane barriers while carrying cargoes many times their size in an intact, functional form. Development of most of these CPPs has been hampered by ubiquitous uptake of these peptides by cells, as well as crossing of the blood brain barrier by some. Phage display is a methodology for identifying binding/uptake of peptides without a priori knowledge of the binding partner. Here we report utilizing a combinatorial in vitro and in vivo methodology to identify skeletal muscle cell targeting peptide(s).

Methods: A transformed human skeletal muscle cell line was obtained from ATCC (PCS-950-010) and cultured per protocol, 100k cells plated and incubated with 50uL ($5*10^{11}$) phage library (New England Biolabs; E8210) for 6hrs. Post incubation, media with non-specific phage was removed,

cells washed 6x with pre-warmed PBS, trypsinized, collected, and lysed. Recovered phage was titered, amplified and put through another cycle of in vitro phage display. Recovered phage from second cycle was amplified and injected into a wild-type adult mouse that had been pre-treated with Chloroquine (20mg/Kg IP) for 3 days. Injected phage was allowed to circulate for 24hrs, mouse euthanized, and skeletal muscle (along with multiple organs) dissected out. Skeletal muscle cells were digested, cells lysed, and recovered phage titered. At the end of 4 cycles, 10 plaques recovered from muscle tissue were sequenced. An emerging common sequence peptide was synthesized using solid state synthesis, fluorescently labeled at N-terminus with Cy5.5 and C-terminus amide capped (for enhancing peptide stability), injected into wild-type mice (5mg/Kg), allowed to circulate for 60 mins, after which mice were euthanized, organs dissected out and ex vivo IVIS imaging performed.

Results: With each cycle of phage display, the output to input ratio decreased steadily ($5.4*10^{-6}$, $3.2*10^{-8}$, $7.9*10^{-8}$, to $3.75*10^{-9}$), indicating loss of contaminating phage and recovery of more specific, targeting ones. After the fourth cycle, sequencing of 10 plaques revealed 4 out of 10 as being identical sequences. Intravenous injection of a wild-type mouse with fluorescently labeled peptide showed a high fluorescence signal from gluteal and femoral leg muscles (Fig. 1). Ex vivo imaging of the organs revealed a high fluorescence signal from skeletal muscle tissue at 60 mins (Fig. 2). Additional mouse injections as well as confocal imaging of multiple organs, in addition to the skeletal muscle, are ongoing.

Conclusions: Using a combinatorial in vitro and in vivo phage display methodology, we believe we have identified a skeletal muscle targeting peptide. Our previous work has shown the feasibility of conjugating oligonucleotides via a disulfide bond to the N-terminus of CPPs for targeted therapies. Use of skeletal muscle targeting CPP could represent a viable alternative to viral vectors.

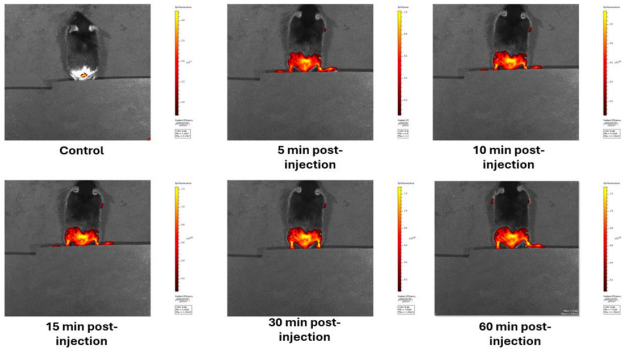

Fig 1. Wild-type B6, female mouse injected with PBS (Control) or Cy5.5 labeled peptide and imaged with
IVIS at the indicated time points.

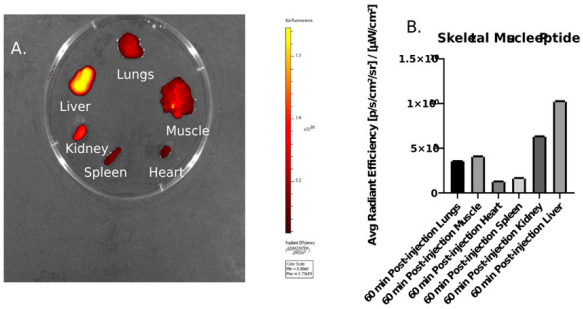

Fig 2. Ex vivo IVIS imaging of various organs after 60mins of circulation time with peptide (A) with measured average radiation efficiencies (B).