

GENE THERAPY

Use of split intein and myotropic AAV vectors enables effective preclinical gene therapy for dysferlinopathy

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Limb-girdle muscular dystrophy type R2 (LGMDR2) is a genetic disorder characterized by progressive skeletal muscle weakness and degeneration. It is caused by mutations in the *DYSF* gene, which prevent dysferlin expression. Dysferlin protein is essential for muscle cell membrane repair. Gene therapy using adeno-associated viral (AAV) vectors to restore full-length dysferlin expression is a promising therapeutic approach for LGMDR2. However, the large size of the dysferlin cDNA (6.2 kilobases) exceeds the ~4.7-kilobase packaging capacity of AAV vectors, posing a challenge to effective delivery of the full-length protein. Here, we describe the use of split inteins and myotropic AAV vectors for efficient systemic gene delivery and expression of full-length dysferlin in the muscles of an LGMDR2 mouse model. We generated and validated a potent split-intein version of dysferlin that enables reconstitution of scarless full-length dysferlin protein. In vitro assessment of these constructs demonstrated a high capacity of the reconstituted dysferlin to restore membrane repair defects in dysferlinopathic mouse myoblasts. Systemic administration of split-intein and dysferlin dual AAVMYO1 vectors improved muscle strength, increased contractile force, and reduced histopathological abnormalities in the LGMDR2 mouse model at both the presymptomatic stage and late stage of the disease. Compared with the homologous recombination dual-AAV vector strategy, the split-intein technology demonstrated superior efficiency in restoring the full-length dysferlin protein and resulted in greater functional improvements. These findings demonstrate the curative potential of this split intein-based gene therapy approach for LGMDR2.

INTRODUCTION

Dysferlinopathies, including limb-girdle muscular dystrophy R2 (LGMDR2; formerly LGMD2B) and Miyoshi myopathy, arise from defects in the sarcolemma repair process and are characterized by progressive muscle degeneration, inflammation, and weakness (1, 2). This is due to the inability of muscle fibers to efficiently repair and preserve myofiber integrity after mechanical activity-induced sarcolemmal damage (3). These autosomal recessive disorders are caused by mutations in the *DYSF* gene, which encodes dysferlin, a 237-kDa sarcolemmal membrane repair protein that contains seven calcium-binding C2 domains, two central ferlin domains, and a C-terminal transmembrane domain, all of which are required for proper sarcolemmal localization and function (4–6). Despite substantial advances in understanding dysferlin biology and disease pathogenesis, there are currently no curative therapies for dysferlinopathy.

Several pharmacological strategies have been explored to ameliorate downstream consequences of dysferlin deficiency, particularly impaired membrane repair and chronic muscle damage. These, including membrane-stabilizing molecules, proteins, and surrogate gene therapy, have demonstrated functional benefits in preclinical models (7–12). Additional small molecules that restore calcium and dysferlin trafficking and homeostasis have shown potential benefits (13–15). However, these therapies do not correct the underlying genetic defect, are expected to require chronic administration, and are unlikely to fully restore coordinated sarcolemmal repair or halt disease progression. As such, their therapeutic potential is inherently limited to symptomatic or adjunctive benefits.

Gene therapy offers a curative strategy for LGMDR2 by directly addressing the root cause of the disease, namely, the absence of functional dysferlin protein. Germline restoration of dysferlin can restore myofiber resistance to mechanical injury and prevent the cascade of secondary pathology that drives muscle degeneration (16). Adeno-associated viral (AAV) vector-mediated gene replacement has emerged as a leading approach for monogenic neuromuscular disorders given its favorable safety profile and long-term skeletal muscle expression. However, the ~4.7-kb packaging capacity of AAV vectors poses a challenge for dysferlinopathy, because *DYSF* is encoded by a 6.2-kb mRNA and, instead of a truncated or “micro-dysferlin” protein (17), the full-length protein is needed for therapeutic benefit.

To overcome these challenges, we previously used liver-targeted AAV gene therapy to address a major downstream consequence of dysferlin deficiency: reduced secretion of acid sphingomyelin (ASM) (12). However, this does not restore dysferlin expression, which we address here. To overcome the size limitation of AAV packaging capacity in restoring dysferlin expression, a dual-AAV vector strategy

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that relies on vector genome concatemerization via homologous recombination has been used to reconstitute full-length dysferlin (18–21). This dual-AAV vector strategy has been evaluated pre-clinically and used for intramuscular injection in human studies (clinicaltrials.gov/study/NCT02710500) (19, 20). Given the high viral vector dose, inefficient restoration, and potential formation of aberrant transcripts via unspecific conjugation and recombination of the viral genome, vector concatemerization-based gene therapy has not been approved for any disease, and no active clinical trials exist for dysferlinopathy. Thus, dysferlin-restoring gene therapy requires the development of new approaches to achieve full-length dysferlin expression.

Recently, protein trans-splicing (PTS) has emerged as an approach that, rather than relying on genome recombination or concatemerization, enables posttranslational reconstitution of two polypeptide halves using split inteins (22–26). This reduces reliance on rare DNA-level events and enables more predictable and precise protein reconstitution. Our use of split intein-based gene therapy demonstrated robust efficacy in models of Duchenne muscular dystrophy, achieving high-level expression of mid-size or full-length dystrophin using a dual- or triple-vector approach, with superior functional outcomes compared with miniaturized constructs at clinically relevant AAV doses (23, 24, 27).

Dysferlin is well suited for split intein-mediated reconstitution because of its modular domain architecture and cytoplasmic N terminus, which allow it to be divided into functional fragments without disrupting sarcolemmal targeting. Despite its therapeutic promise, split intein-mediated gene therapy has not previously been applied to dysferlinopathy, and its capacity to achieve high-level dysferlin expression, dose-dependent efficacy, and meaningful functional improvements in vivo has not been tested. Here, we address these needs, demonstrate preclinical use of split inteins for dysferlinopathy gene therapy, and compare this strategy with the vector concatemerization approach.

RESULTS

In vitro evaluation of split intein-based PTS to reconstitute full-length dysferlin

Dysferlin is a 237-kDa protein composed of seven C2 calcium-dependent phospholipid-binding domains, multiple FER and DYSF domains, and a C-terminal transmembrane domain (Fig. 1A). Given the limited packaging capacity of AAV vectors (~4.7 kb) and the large size of the dysferlin cDNA (6.2 kb), we attempted to reconstitute full-length dysferlin by using the conventional DNA-based homologous recombination strategy (Fig. 1B) and comparing it with split intein-mediated PTS (Fig. 1C).

On the basis of our prior characterization of a split-intein library (23), in silico analysis of the dysferlin protein sequence identified three potential split sites compatible with the insertion of either gp41.1 or inosine-5'-monophosphate dehydrogenase (IMPDH) split inteins (Fig. 1A). For gp41.1, partially matching split sites were identified in the linker region between the FerA and FerB domains (split site #1) and in the FerB domain itself (split site #2) (Fig. 1A). Because this intein catalyzes protein ligation using glycine-tyrosine residues at the N-terminal end and serine at the C-terminal half, insertion at sites #1 or #2 would result in substitution of histidine at position 762 or leucine at position 860, respectively, with tyrosine. In contrast, a perfect match for the catalytic residues required for split IMPDH was

identified in the C2D domain. At split site #3, glycine (position 1263) and the adjacent serine (position 1264) were selected as the split site for IMPDH insertion (Fig. 1A).

To evaluate the efficiency of these split inteins in reconstituting full-length dysferlin, human embryonic kidney (HEK) 293 cells were transfected with either a single plasmid expressing full-length dysferlin or equimolar dual plasmids encoding the split-intein and dysferlin (split-intein/dysferlin) constructs. Immunoblot analysis using antibodies targeting the N or C terminus of dysferlin demonstrated efficient reconstitution of full-length protein for all three split-intein combinations (Fig. 1D). The highest expression levels were observed with the scarless IMPDH intein inserted at split site #3 (Fig. 1D and table S1). In addition to full-length dysferlin, unspliced N- and C-terminal fragments were detected (Fig. 1D), indicating the stability of individual fragments and underscoring the importance of optimal vector stoichiometry for downstream applications.

In vitro assessment of PTS-mediated dysferlin expression and function to improve cell membrane repair

To assess whether the full-length dysferlin produced by the above split-IMPDH/dysferlin constructs (split site #3) is functional, we transfected dysferlin-deficient C2C12 mouse myoblasts with either a single plasmid expressing full-length dysferlin or the dual (N- and C-terminal at various ratios) split-IMPDH/dysferlin plasmids to encode full-length dysferlin protein. A robust expression of full-length dysferlin was detected with dual N- and C-terminal plasmid transfection at a ratio of 3:1 (Fig. 2A). Both cells transfected with either a single plasmid or split-intein plasmids showed Venus-tagged full-length dysferlin protein localized similarly, with a mix of cell membrane and vesicle localization (Fig. 2B).

We previously demonstrated that dysferlin-deficient C2C12 myoblasts exhibit poor cell membrane repair (10). Thus, to evaluate the functionality of the full-length dysferlin protein produced by the split-intein approach, we examined the repair ability of cells expressing the Venus-dysferlin protein and compared it with that of neighboring untransfected cells. This showed that cells expressing full-length dysferlin or both fragments of split intein and dysferlin successfully prevented dye entry after focal laser injury compared with untransfected cells (Fig. 2, C and D). The dye entry was significantly reduced in cells transfected with either full-length ($P = 0.0002$) or split intein/dysferlin ($P = 0.0075$), indicating improved membrane repair kinetics in dysferlin-deficient cells expressing these constructs (Fig. 2D). Next, we examined whether this improved membrane repair kinetics affected the ability of the injured cells to repair by quantifying the cells that took in excessive dye ($\Delta F/F > 5$, where F is the baseline intensity) or failed to prevent dye entry even in the final 30 s of repair. This identified a more-than-fourfold improvement in the repair ability of cells expressing the full-length and split-intein/dysferlin constructs (Fig. 2E). Together, these in vitro studies of cell membrane repair of dysferlinopathic C2C12 myoblasts demonstrate that the full-length protein produced via the split-intein approach is comparable to single vector-expressed full-length dysferlin in restoring cell membrane repair.

In vivo characterization of split-intein/dysferlin expression after local delivery of AAVMYO1

For in vivo assessment, the scarless split intein-IMPDH/dysferlin construct (referred to as SI-Dysf, hereon) and dysferlin fragments with overlapping sequence to allow homologous recombination (HR-Dysf)

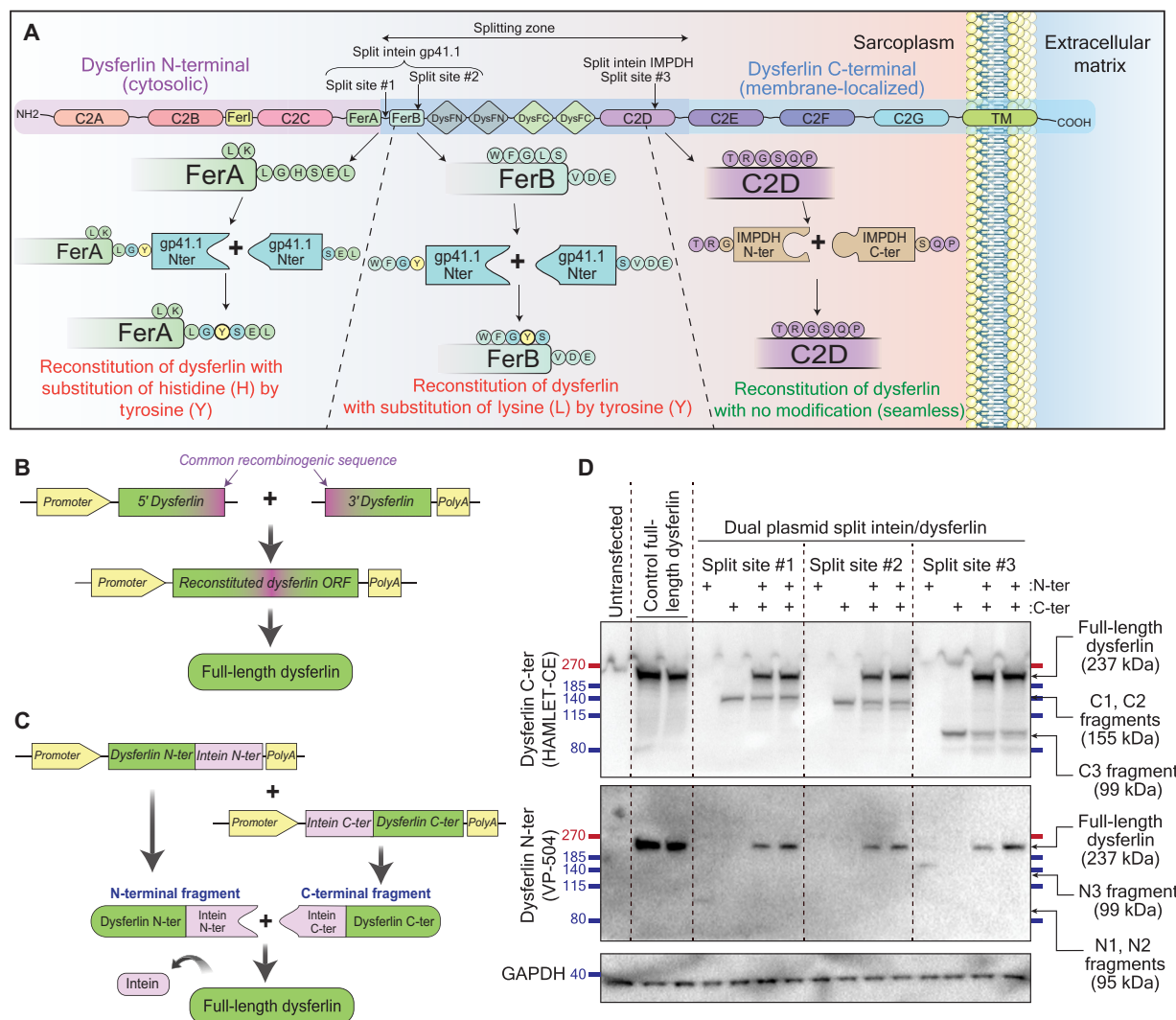
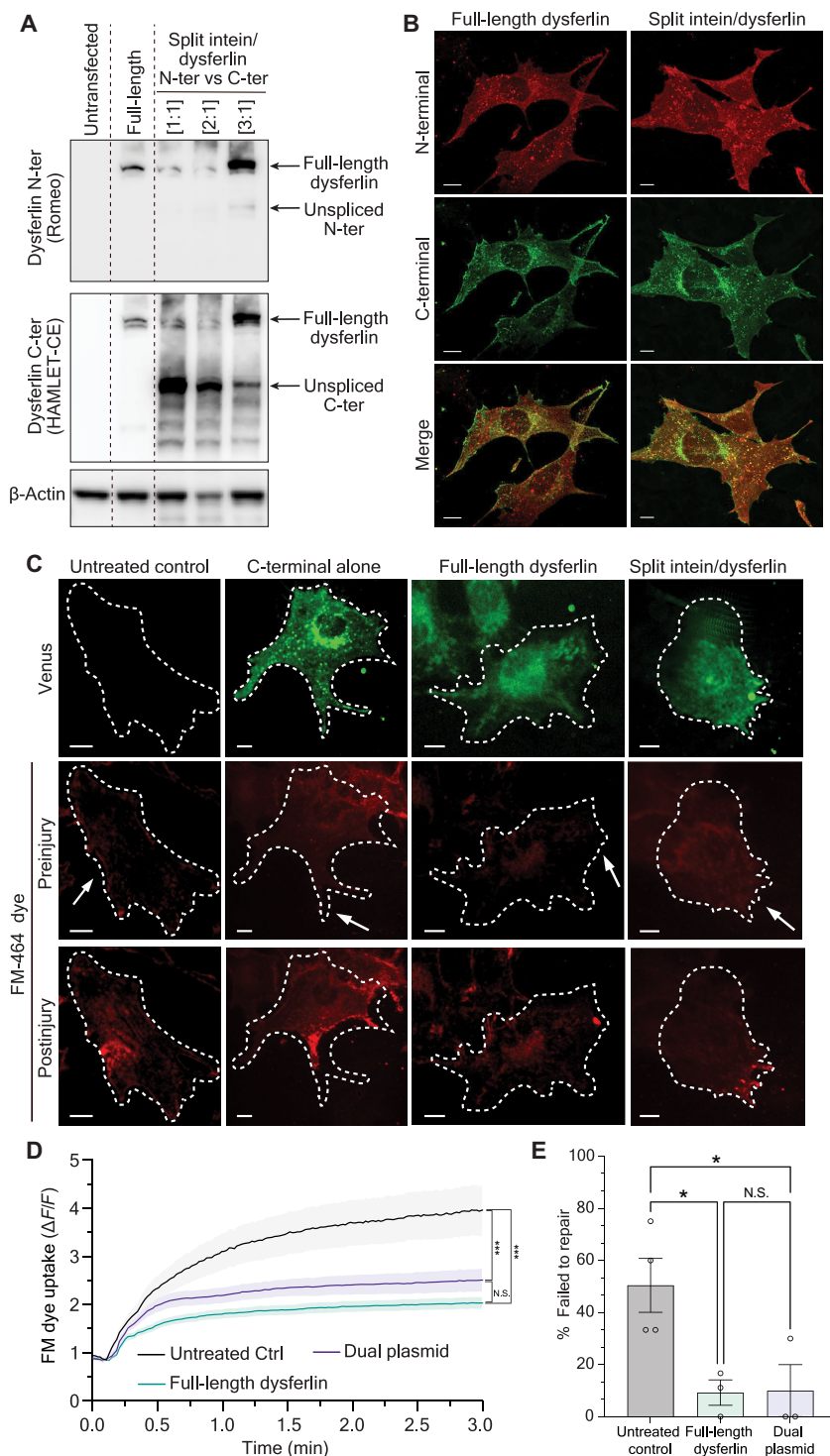


Fig. 1. In vitro assessment of PTS-mediated dysferlin expression using three splitting sites. (A) Dysferlin protein organization and location of the split sites where split intein gp41.1 or IMPDH was inserted. (B and C) Schematic representation of dual-vector strategy using DNA homologous recombination (B) or split intein-mediated PTS to express full-length dysferlin (C). (D) Representative immunoblots of protein lysates collected from HEK293 cells showing efficient expression of full-length dysferlin (237 kDa). In control full-length dysferlin, cells were transfected with a single plasmid containing the full-length human dysferlin sequence. In split intein/dysferlin, dual plasmids contained the split-intein/dysferlin sequences (N-terminal, C-terminal, or both). Densitometry quantification of full-length dysferlin bands is presented in table S1.

were packaged in the myotropic vector AAVMYO1 under the control of muscle-specific expression cassette M-creatine kinase 8e (CK8e) (Fig. 3, A and B). A total dose of 6×10^{10} viral genomes (vg) was administered into the tibialis anterior (TA) muscles of 8-week-old dysferlin-deficient BlaJ mice (Fig. 3C). For the HR-Dysf, this dose was equally divided between the 5' and 3' vectors, whereas five different N- to C-terminal (N:C) vector ratios were tested for the SI-Dysf approach to identify the optimal stoichiometry. After 4 months, muscle lysates were examined by immunoblotting using antibodies specific to N- or C-terminal dysferlin. This revealed robust reconstitution of full-length dysferlin with SI-Dysf dual AAV vectors. All SI-Dysf dual-AAV vector ratios resulted in higher expression of full-length dysferlin than that of HR-Dysf, with the 2:1 and 1:1 ratios yielding the highest full-length protein levels (Fig. 3, D and E). Nonetheless, HR-Dysf allowed nearly twofold higher levels of dysferlin

expression as compared with wild-type (WT) muscle. This in vivo expression in skeletal muscle resulted in nearly undetectable levels of unspliced N- or C-terminal fragments. The use of the N- and C-terminal dysferlin antibodies for immunostaining of muscle cross sections revealed mosaic expression of dysferlin with twofold higher numbers of dysferlin-positive myofibers and brighter signal in muscles with the SI-Dysf as compared with the HR-Dysf muscles (Fig. 3, F to H, and fig. S1). Both the SI-Dysf and HR-Dysf proteins showed dysferlin staining uniformly distributed across the myofiber sarcolemma indicated by the overlapping peaks of dysferlin and laminin/wheat germ agglutinin (WGA; marked by arrows in the line scan plots in Fig. 3H), which, in addition to the cytosolically localized dysferlin, recapitulated T-tubule localization known for dysferlin (Fig. 3H). These data demonstrate the native behavior of SI-Dysf and HR-Dysf proteins, with SI-Dysf allowing higher expression upon

Fig. 2. In vitro characterization of membrane repair of intein-mediated dysferlin. (A) Immunoblot analysis of protein lysates collected from dysferlin-deficient C2C12 myoblasts that were transfected with either a single full-length dysferlin or N- and C-terminal dysferlin plasmids. Immunoblots were probed with antibodies targeting either the N or C termini of dysferlin. Densitometry quantification of full-length dysferlin bands is presented in table S2. (B) C2C12 myoblasts were transfected with either Venus-tagged full-length dysferlin or N- and Venus-tagged C-terminal dysferlin plasmids. Cells were immunostained with an antibody targeting the N-terminal dysferlin. Confocal imaging of cells shows colocalization of N- and C-terminal dysferlin plasmids (scale bars, 10 μ m). (C) Transfected C2C12 myoblasts were subjected to laser injury in the presence of FM-464 dye and imaged for 3 min. The white arrows represent the site of injury. (D) Kinetics of FM-464 dye entry in myoblasts after laser injury. (E) Percentages of myoblasts that failed to repair after laser injury. $n = 3$ or 4 coverslips per group. Data represent the means $\pm$ SEM. N.S., not significant; * $P < 0.05$ and *** $P < 0.001$ using a one-way ANOVA followed by Tukey's multiple-comparisons test.



intramuscular injection of the dual AAV vectors into the BlaJ muscles.

In vivo characterization of AAVMYO1-mediated SI-Dysf expression after systemic delivery

Given that LGMDR2 affects multiple muscle groups, a critical consideration for gene replacement therapy is achieving broad and efficient transgene expression. We and others have previously demonstrated that systemic delivery of myotropic AAV vectors at low doses enables widespread transgene biodistribution using either single- or multi-AAV vector strategies (23–25, 28, 29). However, in addition to vector tropism, multiple factors can influence transgene expression and biodistribution, including the promoter activity, protein half-life, and stability.

To determine the optimal systemic dose required for efficient expression of dysferlin, three doses of dual SI-Dysf AAVMYO1, 1:1 ratio, were administered intravenously via the tail vein to 8-week-old BlaJ mice (Fig. 4A): 2×10^{12} vg/kg (low), 6×10^{12} vg/kg (medium), and 2×10^{13} vg/kg (high). It is noteworthy that the highest dose used in this dose-finding study was 10-fold lower than doses commonly administered in preclinical or clinical studies using conventional AAV serotypes for muscular diseases and is approximately fivefold lower than the dose used previously for HR-Dysf expression (20, 30).

Two months after injection, we harvested skeletal muscles for protein expression, including hindlimb muscles (gastrocnemius and quadriceps), pelvic girdle-associated muscles (psoas and gluteus), diaphragm, and heart. Although the low dose resulted in undetectable levels of full-length dysferlin, the medium dose produced a faint signal corresponding to ~10 to 40% of WT levels (Fig. 4, B and C). In contrast, robust expression of full-length dysferlin was observed in muscles from mice treated with the highest dose (2×10^{13} vg/kg), averaging ~70% of WT levels in quadriceps and psoas, with higher levels in the diaphragm and heart. Collectively, these results highlight the high efficiency of both the split-intein construct

and myotropic AAVMYO1 vector in delivering and expressing therapeutically relevant levels of full-length dysferlin across multiple muscles after systemic administration of relatively low AAV doses.

Assessment of SI-Dysf therapy in dysferlinopathic mice at disease onset

To evaluate disease progression and potential protection through expression of full-length dysferlin using either DNA homologous

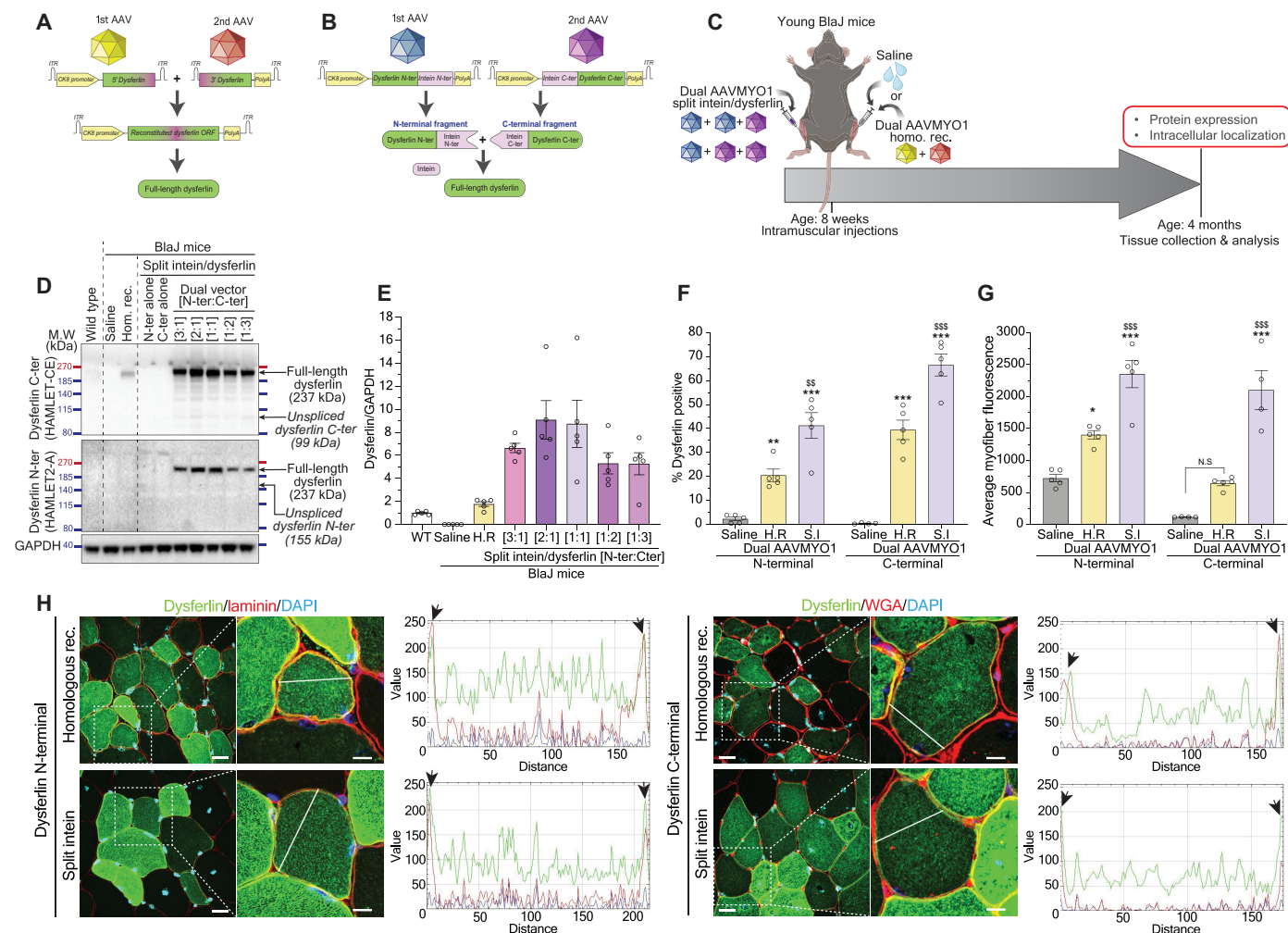


Fig. 3. In vivo comparison of the split-intein approach with DNA homologous recombination. (A and B) Schematic representation of the dual-AAVMYO1 vector strategy using DNA homologous recombination (A) or split intein-mediated PTS (B). (C) Experimental timeline used to compare the in vivo efficacy of the split-intein strategy (at various N:C vector ratios) with DNA-based homologous recombination after intramuscular AAVMYO1 administration. (D) Representative immunoblots showing reconstituted full-length dysferlin detected using two anti-dysferlin antibodies. (E) Quantification of dysferlin expression based on densitometric analysis of signals obtained with the C terminus-specific antibody. (F) Percentages of dysferlin-positive myofibers and (G) signal intensities of dysferlin expression after immunohistochemistry labeling of TA muscle cross sections with N- or C-terminal antibodies. $n = 5$ samples per condition. Data represent the means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus WT and $^{SS}P < 0.01$ and $^{SSS}P < 0.001$ versus BlaJ treated with saline using a one-way ANOVA followed by Tukey's post hoc test. (H) Representative TA muscle cross section labeled with specific antibodies against N- or C-terminal dysferlin, laminin (scale bars, 20 μ m), or WGA. To the right of the muscle section panels are signal scans across a single dysferlin myofiber (presented at the higher-magnification image; scale bars, 10 μ m). The signal shows homogeneous staining in the sarcoplasm, with the staining slightly enriched at the myofiber sarcoplasmic membrane, as indicated by the black arrows. H.R., homologous recombination; S.I., split intein; M.W., molecular weight.

recombination or split intein-mediated PTS, a cohort of 6-month-old BlaJ mice was treated with these dual AAVMYO1 vectors (Fig. 5A). For both strategies, a total dose of 2×10^{13} vg/kg was infused systemically via tail vein injection using equimolar ratios of 5' and 3' vectors for the HR-Dysf strategy or N- and C-terminal vectors for the SI-Dysf strategy.

Six months after treatment, gastrocnemius and quadriceps muscles were harvested to assess full-length dysferlin expression and bio-distribution. Vector genome quantification showed a similar presence of HR-Dysf (3' and 5') or SI-Dysf (N- and C-terminal) vectors (Fig. 5B). Again, SI-Dysf allowed the expression of a higher level of full-length dysferlin protein as compared with HR-Dysf and achieved 60 to 70% of

WT levels compared with 5 to 15% of WT dysferlin levels by HR-Dysf (Fig. 5, C and D). This trend was also reflected in the dysferlin immunostaining of the muscle cross sections (Fig. 5E and fig. S2). Analysis of gastrocnemius muscle cross sections showed improved muscle histology in mice treated with SI-Dysf (Fig. 5E and fig. S2). At this age, BlaJ gastrocnemius muscles treated with saline or HR-Dysf showed tissue with loss of myofibers, enrichment for adipogenic (perilipin) staining, and a high proportion of myofibers with central nuclei (Fig. 5, E and F). In contrast, muscles expressing SI-Dysf showed reduced histopathology, including fewer centrally nucleated myofibers and increased myofiber size (cross-sectional area) (Fig. 5G).

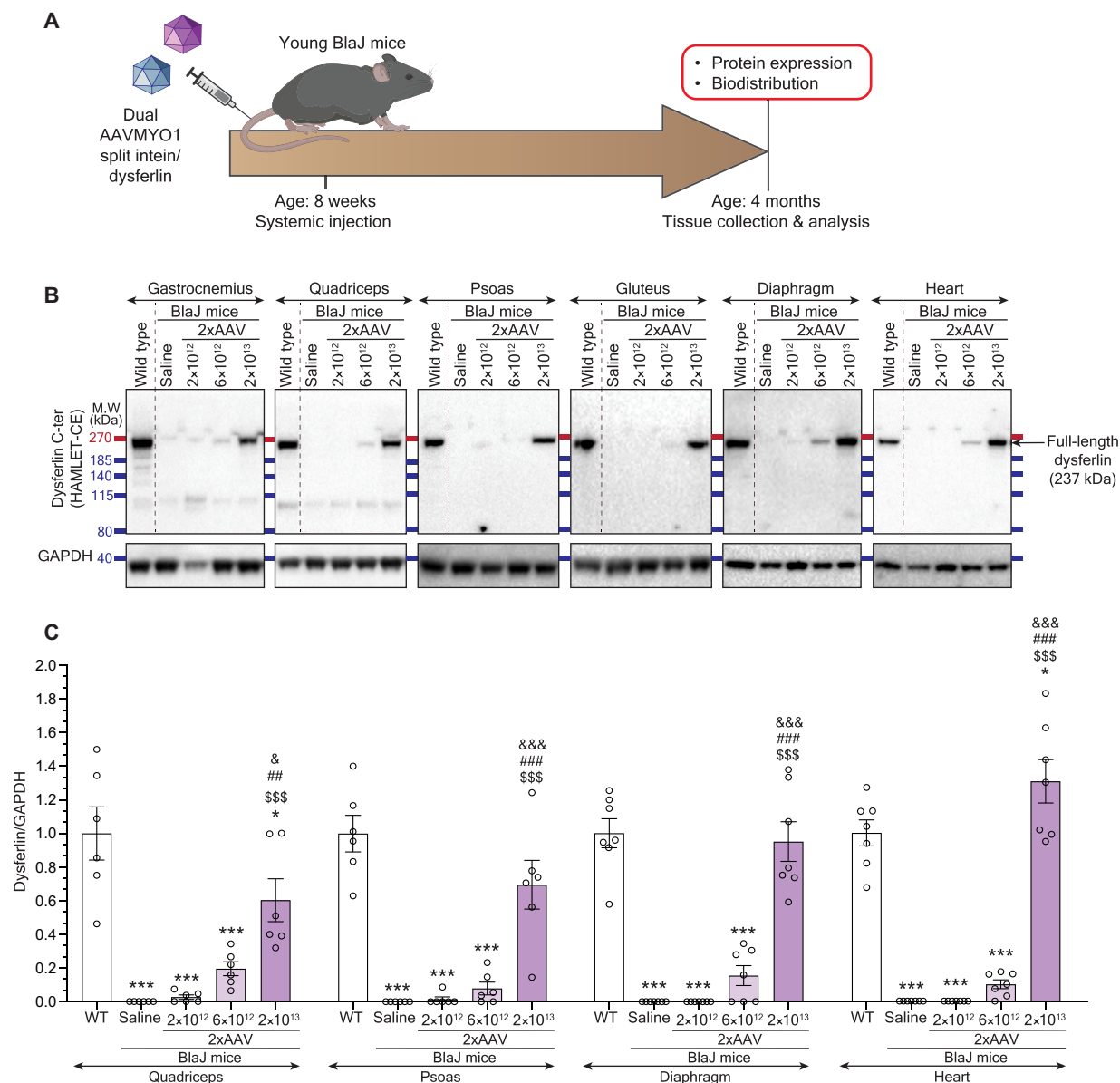


Fig. 4. Dose-finding study to determine the optimal systemic dose. (A) Experimental timeline used to evaluate three doses of dual AAVMYO1 SI-Dysf after systemic administration via tail vein injection of young BlaJ mice. (B) Representative immunoblots of protein lysates from hindlimb muscles (gastrocnemius and quadriceps), pelvic girdle-associated muscles (psoas and gluteus), and diaphragms and hearts collected from BlaJ mice treated with saline or different doses of dual AAVMYO1 split-intein/dysferlin vectors or age-matched WT mice. (C) Quantification of full-length dysferlin expression in the heart, diaphragm, quadriceps, and psoas muscles. $n = 7$ samples per condition. Data represent the means $\pm$ SEM. * $P < 0.05$ and *** $P < 0.001$ versus WT, \$\$\$ $P < 0.001$ versus BlaJ treated with saline, ## $P < 0.01$ and ### $P < 0.001$ versus BlaJ mice treated with 2×10^{12} vg/kg of dual AAVMYO1, and & $P < 0.05$ and &&& $P < 0.001$ versus BlaJ mice treated with 6×10^{12} vg/kg of dual AAVMYO1 using a one-way ANOVA followed by Tukey's post hoc test.

We next assessed whether restoration of dysferlin expression and muscle histology translated into improved muscle function. Dysferlin deficiency in BlaJ mice affects hindlimb and girdle muscles, resulting in reduced all-limb grip strength compared with age-matched WT mice. This deficit was prevented in mice treated with HR-Dysf and SI-Dysf (Fig. 6A). Furthermore, forelimb grip strength and hang time, which were not altered in BlaJ mice, remained unaffected in the HR-Dysf- and SI-Dysf-expressing mice (Fig. 6B and fig. S3A). To assess whether the improved grip strength is due to improved muscle contractile force,

we examined the twitch force in the hindlimb (gastrocnemius) muscle. This was significantly lowered in the saline-treated BlaJ mice as compared with the WT mice ($P = 0.0001$), but expression of full-length dysferlin with HR-Dysf or SI-Dysf prevented this deficit (Fig. 6C). We extended this analysis further and examined the force frequency curve and isometric force of the gastrocnemius before and 10 min after a bout of 20 repeated eccentric contractions (ECs) (Fig. 6, D and E, and fig. S3, B and C). Compared with WT controls, saline-injected BlaJ muscles exhibited reduced strength before any EC-induced injury. This deficit

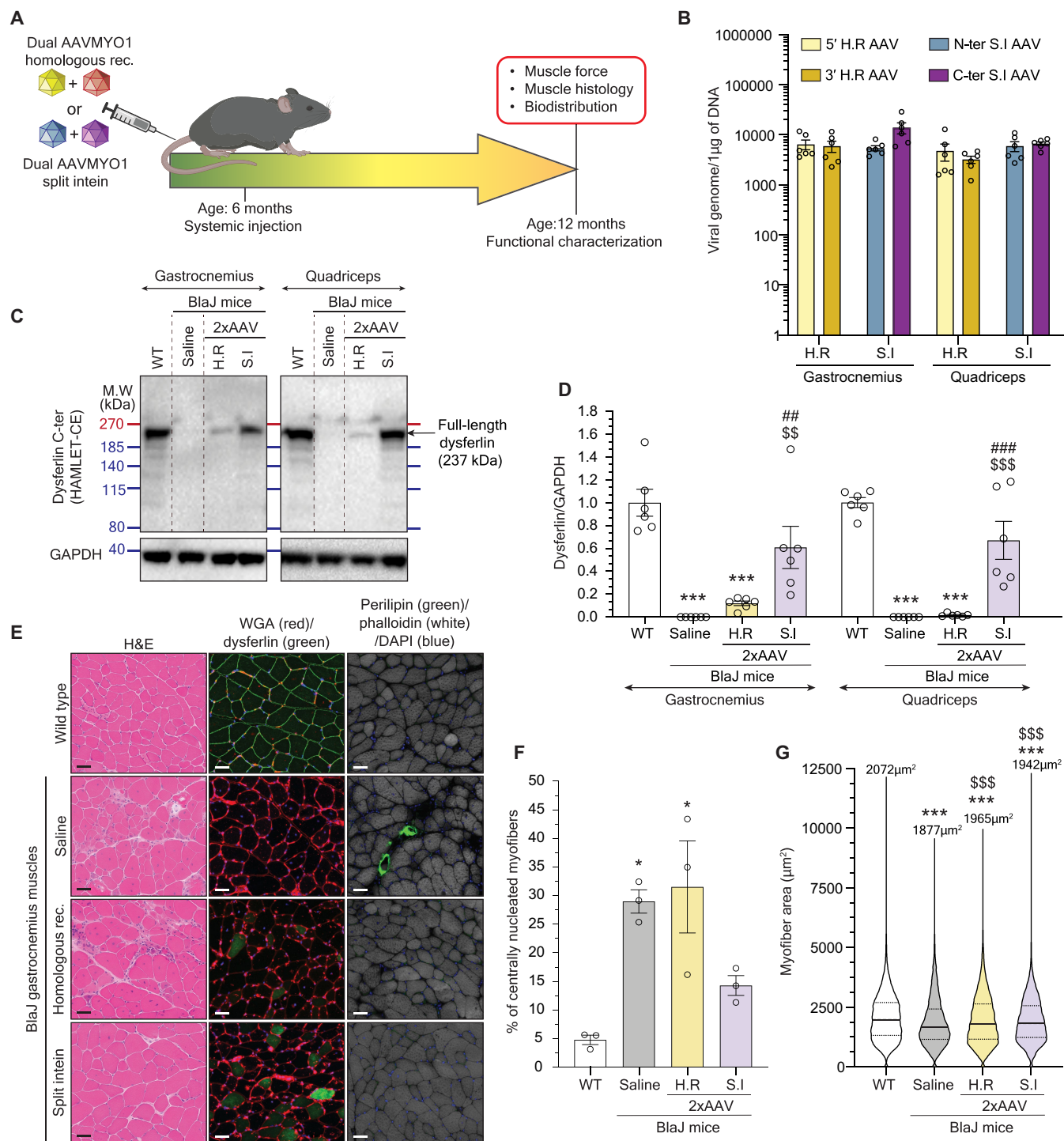
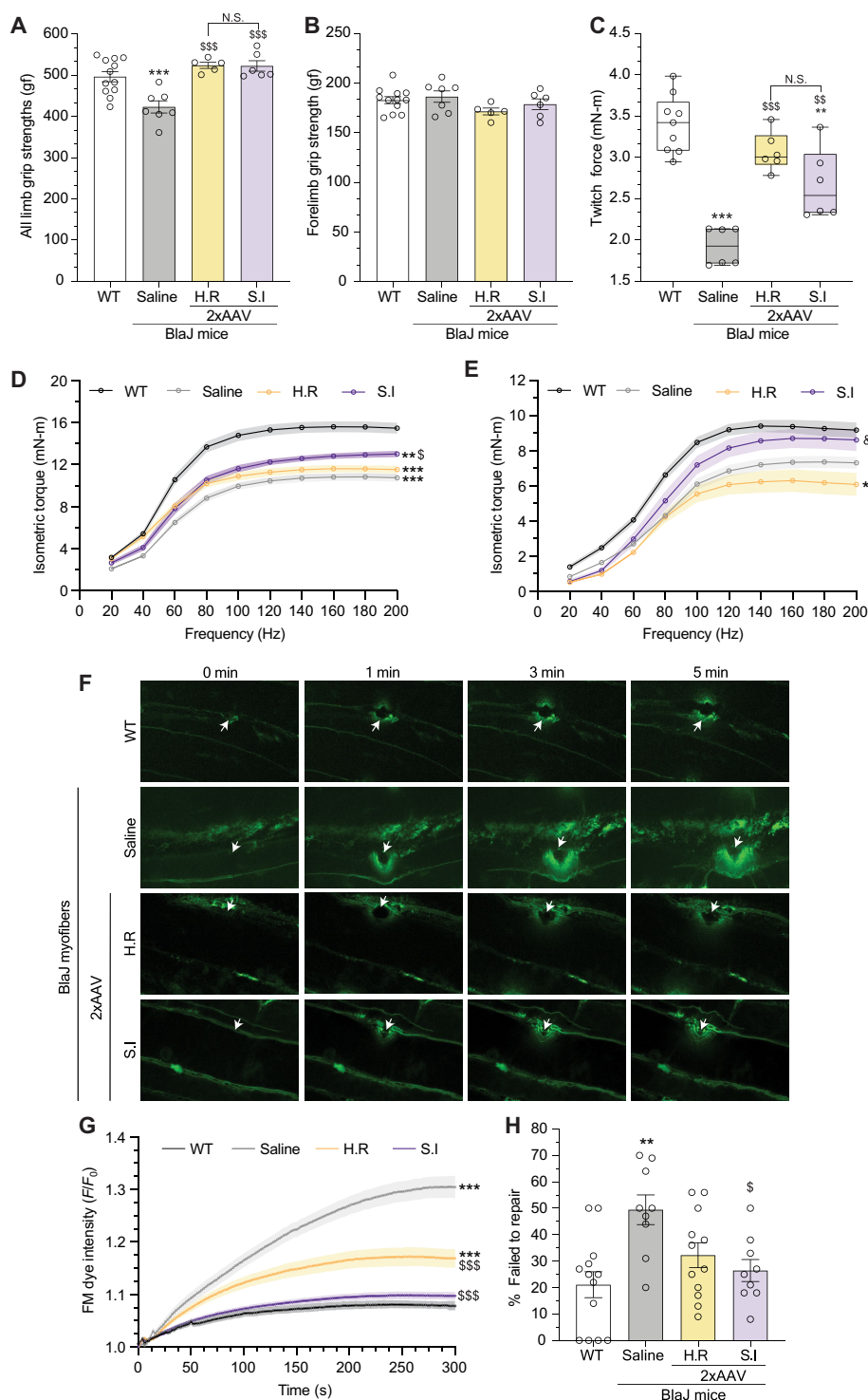


Fig. 5. Analysis of SI-Dysf therapy in early-stage dysferlinopathic mice. (A) Schematic representation of the workflow used to investigate the functional rescue after systemic administration of dual AAV vectors to express full-length dysferlin in young BlaJ mice. (B) AAV viral genome quantification using digital polymerase chain reaction. $n = 6$ samples. (C) Immunoblots showing successful expression of full-length dysferlin in gastrocnemius and quadriceps samples 6 months after AAV injections. (D) Quantification of full-length dysferlin expression was performed by densitometry and normalized to the housekeeping protein GAPDH. $n = 6$ per condition. (E) Gastrocnemius muscle cross sections stained with H&E (scale bars, 40 μ m), WGA and C-terminal dysferlin antibody (scale bar, 40 μ m), or perilipin and phalloidin (scale bars, 40 μ m). (F) Percentages of myofibers with central nuclei. $n = 3$ samples. (G) The myofiber area was measured in more than 700 myofibers per sample. The average values are shown above the violin bars. The solid line represents the median, whereas the dashed lines show the quartiles. $n = 5$ samples per group. For bar plots, the data represent the means $\pm$ SEM. * $P < 0.05$ and *** $P < 0.001$ versus WT, $^{SS}P < 0.01$ and $^{SSS}P < 0.001$ versus BlaJ treated with saline, and $^{##}P < 0.01$ and $^{###}P < 0.001$ versus BlaJ mice treated with dual AAVMYO1 HR-Dysf using one-way ANOVA followed by Tukey's post hoc test.

Fig. 6. Functional benefits of SI-Dysf therapy for early-stage dysferlinopathic mice.

(A and B) Quantification of all-limb (A) and forelimb (B) grip strength. $n = 5$ to 12 per group. **(C)** Twitch force produced by the gastrocnemius muscles. Data represent the means $\pm$ SEM. N.S., no significance; $**P < 0.01$ and $***P < 0.001$ versus WT and $^{SS}P < 0.01$ and $^{SSS}P < 0.001$ versus BlaJ treated with saline using a one-way ANOVA followed by Šidák's multiple-comparisons test. **(D and E)** Force-frequency curves before (D) and 10 min after 20 repeated ECs (E). $**P < 0.01$ and $***P < 0.001$ versus WT, $^SP < 0.05$ versus BlaJ treated with saline, and $^BP < 0.05$ versus BlaJ treated with HR-Dysf using a one-way ANOVA followed by Tukey's multiple-comparisons test. **(F)** Bicep myofibers were subjected to laser injury in the presence of FM-143 dye and imaged for 5 min. White arrows represent the site of injury. **(G)** Kinetics of FM-143 dye entry in myofibers after laser injury. Data represent the means $\pm$ SEM. $***P < 0.001$ versus WT and $^{SSS}P < 0.001$ versus BlaJ treated with saline using a one-way ANOVA followed by Tukey's multiple-comparisons test. **(H)** Percentages of myofibers that failed to repair after laser injury. Each data point is from one muscle. $n = 9$ to 13 muscles per condition. $**P < 0.01$ versus WT and $^SP < 0.05$ versus BlaJ treated with saline using a one-way ANOVA followed by Tukey's multiple-comparisons test.



was least pronounced in SI-Dysf muscles and, to a lesser extent, in HR-Dysf-expressing muscles (Fig. 6D and fig. S3B). The EC-injury bout reduced contractile force such that maximal force in SI-Dysf muscles was comparable to that of EC-injured WT muscle, whereas HR-Dysf muscles exhibited the lowest force, indicating the poorest recovery from EC injury (Fig. 6E and fig. S3C).

To directly assess the ability of myofibers to repair sarcolemmal injury, intact biceps muscles from control and treated mice were used to perform focal laser-induced injury on individual myofibers in the presence of the cell-impermeant FM1-43 dye (Fig. 6F). The kinetics of dye entry into the injured myofiber were monitored using live time-lapse imaging. This demonstrated the poor ability of the BlaJ myofibers to repair after injury, leading to greater dye uptake than in WT myofibers (Fig. 6G). Myofibers from mice treated with dual-vector HR-Dysf showed improved repair ability compared with control BlaJ mouse myofibers. The efficacy of sarcolemmal repair after focal laser injury was even greater for the myofibers from BlaJ mice expressing SI-Dysf and was comparable to the WT myofibers (Fig. 6G). This improved repair kinetics resulted in a nearly twofold decrease in the number of injured myofibers from BlaJ mice treated with SI-Dysf that failed to repair (Fig. 6H). These results collectively support the greater efficacy of the SI-Dysf approach in restoring full-length dysferlin expression and, consequently, greater improvement in BlaJ myofiber repair, muscle health, and function compared with HR-Dysf.

Assessment of SI-Dysf therapy in dysferlinopathic mice exhibiting advanced pathology

Given the superior expression of full-length dysferlin with the SI-Dysf strategy, we next sought to characterize its therapeutic potential in BlaJ mice at an advanced stage of the disease. For this, we intravenously administered a dose of 2×10^{13} vg/kg to 12-month-old BlaJ mice (Fig. 7A). Six months after treatment, we observed the equal presence of N- and C-terminal vectors in

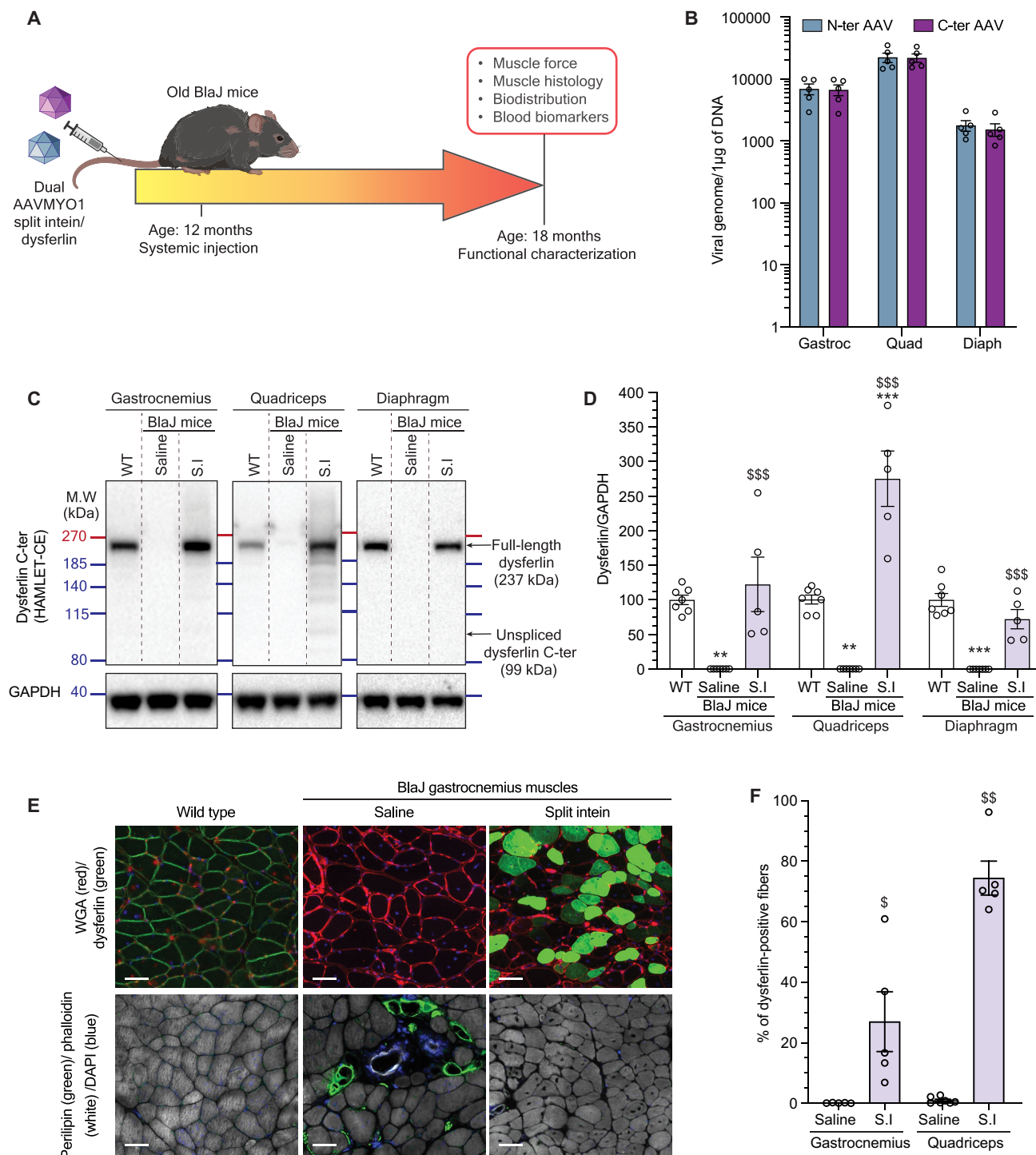


Fig. 7. Analysis of SI-Dysf therapy in late symptomatic stage dysferlinopathic mice. (A) Experimental timeline of systemic injections of dual AAVMYO1 SI-Dysf in old dysferlin-deficient BlaJ mice. (B) Viral genome quantification in gastrocnemius, quadriceps, and diaphragm muscles collected from BlaJ mice treated with dual AAV SI-Dysf. (C) Representative immunoblots using protein lysates from gastrocnemius, quadriceps, and diaphragm muscles of 18-month-old WT, saline-treated, or AAV-treated BlaJ mice. (D) Dysferlin protein levels measured by densitometry and normalized to GAPDH. $n = 7$ samples for WT and saline-treated BlaJ mice. $n = 5$ for AAV-treated BlaJ mice. (E) Gastrocnemius muscle cross sections stained with WGA and C-terminal dysferlin antibody (scale bars, 50 μ m) or perilipin and phalloidin (scale bars, 50 μ m). (F) Percentages of dysferlin-positive myofibers quantified on gastrocnemius or quadriceps muscle cross sections. Data represent the means $\pm$ SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus WT and $^sP < 0.05$, $^{ss}P < 0.01$, and $^{sss}P < 0.001$ versus BlaJ treated with saline using a one-way ANOVA followed by Tukey's post hoc test.

the different muscles collected from injected mice such that the overall vector levels were higher in quadriceps muscle, whereas the levels were lower in diaphragm muscles (Fig. 7B). This led to a robust expression of full-length dysferlin in these muscles, with the expression levels comparable to those of the WT mouse muscles in the gastrocnemius and diaphragm muscles and even higher (two to three times more) in the quadriceps (Fig. 7, C and D). Immunohistochemistry staining of gastrocnemius and quadriceps muscle cross sections revealed the widespread presence of dysferlin-positive myofibers (30 to 70%), which was higher in the quadriceps muscles compared with the gastrocnemius muscles (Fig. 7, E and F, and fig. S4). Furthermore, unlike the saline-treated muscle sections, which showed several perilipin-labeled areas, muscles treated with dual split-intein/dysferlin vectors showed no such staining (Fig. 7E).

Histological analysis of gastrocnemius and quadriceps muscle cross sections revealed morphological improvements in mice treated with SI-Dysf. Hematoxylin and eosin (H&E) staining showed that saline-treated muscles exhibited marked variability in fiber size, tissue remodeling, and inflammatory infiltration (Fig. 8A). Quantification of these features revealed reduced myofiber cross-sectional area and minimal Feret diameter in control BlaJ muscles (Fig. 8B and fig. S5A). In contrast, dual AAVMYO1 SI-Dysf treatment increased both parameters, but the values remained lower than those observed in WT muscles.

Quantification of fibrosis using trichrome staining revealed extensive fibrotic remodeling in saline-treated muscles, averaging ~18% of total muscle area in both gastrocnemius and quadriceps (Fig. 8C). SI-Dysf gene therapy significantly reduced these numbers to nearly 6 and 9% in gastrocnemius ($P = 0.0013$) and quadriceps muscles ($P = 0.0002$), respectively. In addition, assessment of adipogenic degeneration of these muscles by oil red O staining revealed widespread lipid-positive areas in saline-treated BlaJ gastrocnemius and quadriceps muscles (Fig. 8A), covering ~3 and ~7% of total muscle area, respectively (Fig. 8D). In contrast, muscles treated with SI-Dysf exhibited a significant reduction in both the number and area of lipid deposits ($P = 0.0004$ for gastrocnemius and $P = 0.0002$ for quadriceps), reaching levels comparable to those of the WT controls.

The histopathology observed in saline-treated BlaJ mice led to significantly reduced muscle-specific force in the gastrocnemius ($P = 0.0001$) compared with age-matched WT controls (Fig. 8E and fig. S5B). In contrast, treatment with SI-Dysf vectors improved both absolute muscle contractile force and specific muscle contractile force. Similarly, diaphragm muscles from saline-treated BlaJ mice exhibited weakness (Fig. 8F), which improved after SI-Dysf treatment, approaching WT levels. Last, analysis of circulating creatine kinase levels at the end point revealed a three- to fourfold increase in saline-treated BlaJ mice compared with WT controls (fig. S6A). These levels were lower in BlaJ mice treated with dual AAVMYO1 SI-Dysf but remained elevated relative to WT samples. Despite the disease severity and length of gene therapy treatment, we observed no elevation in liver injury biomarkers (fig. S6, B to D). Collectively, these data demonstrate substantial improvements in muscle function and histopathology after dual AAVMYO1 SI-Dysf treatment in BlaJ mice with advanced muscular dystrophy. These therapeutic benefits were achieved after a 6-month treatment period and were associated with robust, body-wide expression of full-length dysferlin.

DISCUSSION

Dysferlinopathies are monogenic loss-of-function disorders and represent compelling candidates for gene replacement therapy, where AAV-mediated dysferlin restoration in affected myofibers could ameliorate disease pathology. Advances in AAV technology, including the development of highly myotropic capsids with potent tissue-specific promoters and improved understanding of host immune responses, have helped advance the field of AAV-based gene therapy. Given the large size of many muscle structural proteins, a major recent challenge in the field has been the need to effectively produce such proteins that exceed the packaging capacity of a single AAV vector. A potential solution to this challenge is to make use of miniaturized proteins (17). However, such versions of dysferlin proteins suffer from limitations that range from poor functionality to poor localization (17, 31, 32). We and others have addressed this challenge by using approaches that take advantage of DNA heterodimerization, RNA trans-splicing, or ribozymes to produce large proteins such as dystrophin using dual AAV vectors (20, 23, 24, 33–36).

A dual-vector approach to generate full-length dysferlin in humans has used the homologous recombination approach (clinicaltrials.gov/study/NCT02710500) (19, 20), but the need for an efficient whole-body benefit from this approach through systemic delivery of the dual AAVs has limited its clinical potential. Our work shows that an alternative split intein-mediated PTS approach yields superior in vitro and in vivo production of full-length dysferlin and improved preclinical therapeutic outcomes. Despite using comparable levels of dual vectors for HR-Dysf and SI-Dysf therapy, the latter restored dysferlin expression to near WT levels, improving muscle histology, contractile force, and myofiber membrane repair after injury. These therapeutic benefits of the SI-Dysf approach were achieved at a five-fold lower vector dose than previously used for HR-Dysf (20). This likely reflects the combined contributions of the muscle-specific CK8 promoter, the high tropism of the AAVMYO1 capsid, the specificity of the trans-splicing mechanism, and the rapid splicing kinetics of the split IMPDH intein (37).

Although various split inteins could, in principle, be used to reconstitute full-length dysferlin, the selection of an optimal intein pair is critical to ensure seamless and efficient ligation of the N- and C-terminal fragments. Dysferlin appears particularly sensitive to residual sequence “scars” introduced during splicing, which may compromise protein stability or function. Among the candidates evaluated, the split IMPDH intein enabled precise ligation at native residues (Gly¹²⁶³ and Ser¹²⁶⁴), preserving the integrity of the protein. At this junction, coexpression of the N- and C-terminal dysferlin fragments resulted in stable split-intein/dysferlin fragments, leading to high levels of full-length protein reconstitution, as confirmed by immunoblot analyses in both plasmid-transfected nonmuscle and muscle cells. We observed similar efficacy when these fragments were delivered in vivo using AAV vectors, either by intramuscular injection or systemic intravenous delivery. Both delivery routes resulted in greater expression of the full-length dysferlin protein with the SI-Dysf strategy than with the HR-Dysf.

The stoichiometry of the N- and C-terminal AAV vectors delivered in vivo influenced expression levels, with an equimolar ratio yielding optimal levels of full-length protein and little to no unsplined N- or C-terminal fragments, as detected by immunoblotting using fragment-specific dysferlin antibodies. The lack of truncated dysferlin protein fragments with PTS offers an advantage of this approach. Similar advantages are also offered by the ribozyme-mediated full-length

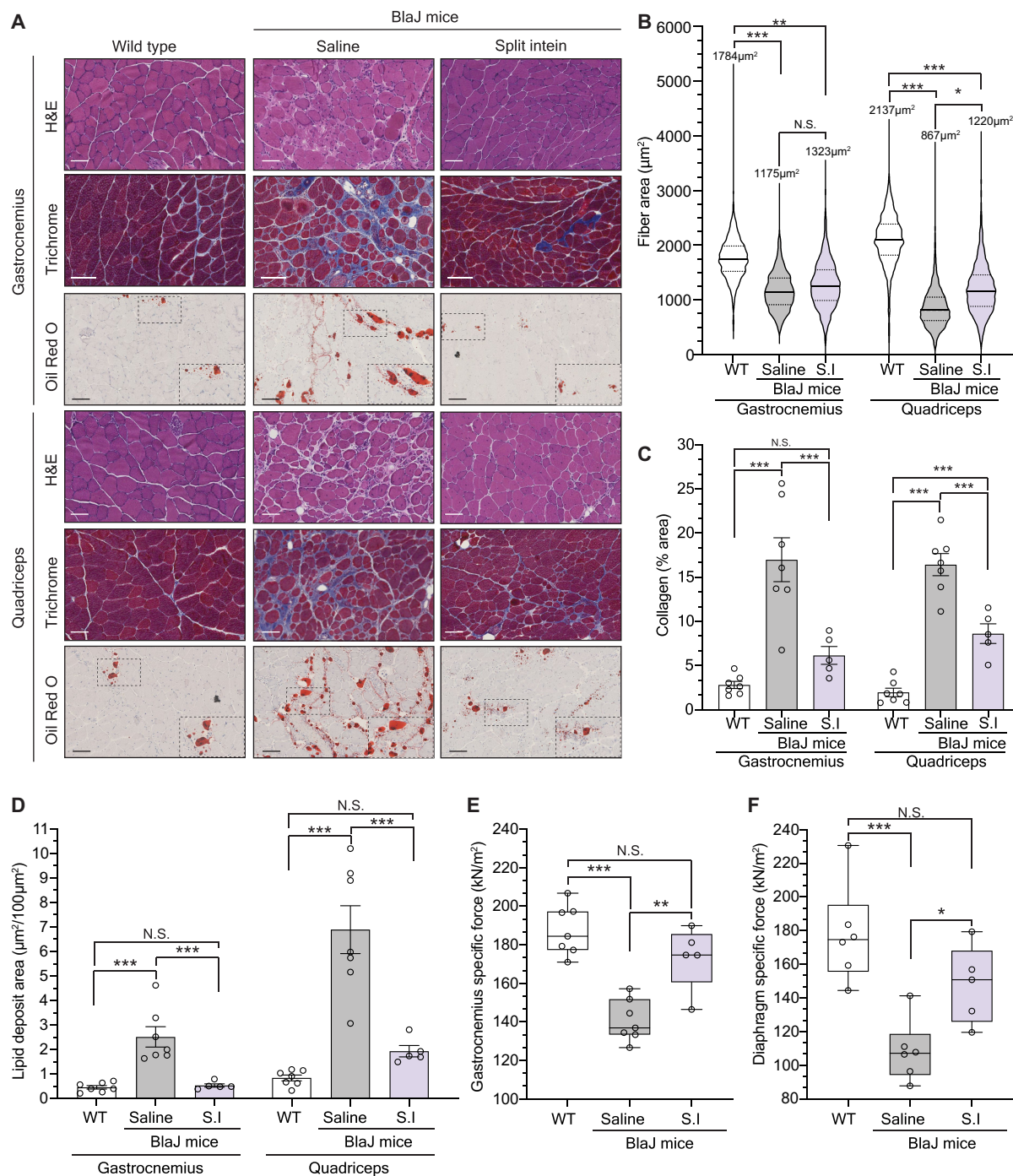


Fig. 8. Functional benefits of SI-Dysf therapy in late-stage and symptomatic dysferlinopathic mice. (A) Representative cross sections of gastrocnemius and quadriceps muscles stained with H&E, Masson's trichrome, or oil red O (scale bars, 100 μm). (B) Quantification of myofiber cross-sectional areas measured on transverse gastrocnemius and quadriceps sections. More than 800 myofibers per sample from $n = 7$ for WT and saline-treated BlaJ mice or $n = 5$ samples for AAV-treated BlaJ mice were analyzed. The average values are shown above the violin bars. (C) Fibrotic areas quantified from trichrome-stained gastrocnemius and quadriceps cross sections. (D) Lipid deposition areas quantified in gastrocnemius and quadriceps sections stained with oil red O. (E) Maximal developed specific forces of the gastrocnemius muscles were calculated by normalizing the absolute force to the muscle weight. (F) Specific forces of diaphragm muscle strips measured in vitro. For box plots, center horizontal lines represent medians, and box limits are the 25th and 75th percentiles. The whiskers extend to the minimum and maximum values. The solid line in the violin bars represents the median, whereas the dashed lines show the quartiles. $n = 7$ samples for WT and saline-treated BlaJ mice. $n = 5$ for AAV-treated BlaJ mice. Data represent the means $\pm$ SEM. N.S., no statistical significance. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ using a one-way ANOVA followed by Tukey's post hoc test.

dysferlin reconstitution (35). Further studies are needed to assess the long-term benefits of this advantage.

Using two independent dysferlin antibodies, immunohistochemical analysis of endogenous dysferlin confirmed its dual localization at both the sarcolemma and internal membranes. This distribution is consistent with a prior report showing dysferlin at the sarcolemma and in the T-tubule system (38). The relative abundance of dysferlin at these sites varied among myofibers and appeared to depend on overall protein expression, with higher-expressing fibers showing greater intracellular localization, as reported previously (19, 20, 35). The expressed dysferlin proteins not only recapitulated the localization pattern of the native protein but also demonstrated comparable functional activity. Muscles treated with the SI-Dysf approach exhibited faster sarcolemmal repair kinetics than those treated with HR-Dysf, with repair rates similar to those of WT myofibers, supporting improved recovery after focal injury. Consistent with enhanced repair, SI-Dysf muscles showed a reduced requirement for myofiber regeneration, likely reflecting decreased cumulative damage compared with untreated or HR-Dysf BlaJ muscles. Furthermore, the SI-Dysf approach improved contractile force generation during tetanic stimulation and conferred greater protection from, and recovery after, EC-induced injury. Improved myofiber repair and reduced need for myofiber regeneration mirror the benefits offered by liver-targeted AAV to produce secreted ASM enzyme, which is compromised by dysferlin deficiency (12). Future studies combining dysferlin restoration and ASM supplementation can determine whether this yields even greater improvement.

Dysferlinopathy is characterized by late onset and a substantial delay in diagnosis, with an average of ~6 years between symptom onset and clinical identification (9). This creates an important translational requirement for gene therapy approaches: They must be effective at both presymptomatic and more advanced stages. To address this, we evaluated the SI-Dysf gene therapy strategy in BlaJ mice at both disease onset (6 months of age) and a later advanced stage (12 months of age). SI-Dysf treatment enabled robust dysferlin expression in older BlaJ mice, which reduced fibroadipogenic muscle degeneration and improved contractile force production in both limb (gastrocnemius) and respiratory (diaphragm) muscles. These findings are translationally relevant, given that fibroadipogenic degeneration is the key driver of loss of ambulation in patients and greater disease severity correlates with reduced forced vital capacity (39). Overall, the efficacy of the SI-Dysf approach in mice at a later disease stage underscores its translational potential for treating patients with LGMDR2, including those presenting substantial disease progression.

Our study has several limitations. First, AAV-mediated delivery of transgenic dysferlin resulted in mosaic expression, with differences in expression levels among myofibers. This heterogeneity may reflect differences in the intracellular uptake of both vectors, variability in CK8e promoter activity across transduced myonuclei, and intrinsic differences in myofiber physiology and pathology. Second, dysferlin expression was assessed by immunohistochemistry on muscle cross sections, which does not fully capture the distribution of dysferlin along the entire length of individual myofibers. For optimal therapeutic benefit, full-length dysferlin should ideally be expressed and distributed uniformly throughout the muscle fiber. Further studies are therefore needed to determine whether more uniform expression can be achieved through the use of next-generation myotropic capsids, more potent muscle-specific expression cassettes, or modifications to the dysferlin protein sequence that enhance its distribution

in myofibers. Third, although the origin of the split IMPDH used in this study is not fully established, most split inteins are derived from microorganisms, raising the possibility that their expression or accumulation in human cells could elicit an immune response that may limit the safety and efficacy of this therapeutic approach. Moreover, the intracellular half-life, stability, and fate of split inteins in mammalian cells remain largely unknown. The development of specific antibodies and other sensitive methods to detect and characterize split-intein expression, localization, and persistence will therefore be important before clinical translation. Last, although our study provides evidence of therapeutic efficacy, we did not perform an in-depth characterization of the immune response to the AAV vectors, split-intein components, or reconstituted dysferlin. Comprehensive assessment of humoral and cellular immune responses, as well as long-term safety and durability of expression, will be important in future studies and during the development of this approach toward clinical application.

In conclusion, this study addresses a major limitation of AAV-mediated dysferlin gene replacement by overcoming vector packaging constraints and enabling efficient restoration of full-length protein expression. Our findings demonstrate that the combination of dual myotropic AAV vectors and split intein-mediated trans-splicing results in robust functional recovery and clinically relevant improvements in LGMDR2-associated pathology in a preclinical model, supporting the translational potential of this approach.

MATERIALS AND METHODS

Study design

Dysferlin deficiency causes muscle defects and degeneration that clinically manifest as LGMDR2 or Miyoshi myopathy. We hypothesized that efficient delivery and muscle-restricted expression of full-length dysferlin would restore muscle function and protect myofibers from contraction-induced mechanical damage. To test this hypothesis, we compared two strategies designed to reconstitute full-length dysferlin from dual AAV vectors. First, we evaluated the efficiency of split inteins to reassemble dysferlin *in vitro* using plasmid transfection. The split-intein/dysferlin construct that yielded the highest expression with scarless protein reconstitution was then selected and compared with a homologous recombination-based approach. Dual myotropic AAV vectors were produced, purified, and then injected locally into a dysferlin-deficient mouse model for *in vivo* validation and assessment of vector stoichiometry. Subsequently, systemic AAV administration was performed to assess therapeutic efficacy, biodistribution, and functional rescue after restoration of full-length dysferlin in young or old dysferlinopathic mice. Statistical analyses and sample sizes were determined on the basis of previous studies. Further details on sample size and replications (technical or biological) are provided in figure legends. All injected mice and obtained data were included in this study.

Animals

All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees of the University of Washington (protocol no. 3333-01) and Children's National Research Institute (protocol no. 30709). Sample sizes and experimental design were determined on the basis of previously published data from our laboratories. Homozygous BlaJ male and female mice (obtained from the Jain Foundation private colony at

the Jackson Laboratory) were used for breeding, whereas only males were used for injections and functional assessments. The BlaJ mouse carries a spontaneous insertion of the ETn retrotransposon in intron 4 of the *Dysf* gene. This disrupts normal mRNA splicing, resulting in a complete absence of dysferlin protein in skeletal muscle (40). Age-matched WT mice were purchased from the Jackson Laboratory and served as controls. The number of biological replicates, statistical methods, and *P* values are provided in the figure legends. Mice were randomized into experimental groups and assigned serial identification numbers on the day of injection to enable unbiased and blinded analyses. These identifiers were used throughout the study, and treatment assignments were revealed only after data collection was completed.

Cloning and AAV vector production

Codon-optimized split-intein pairs were selected from our previously characterized library (23). Split-intein sequences were cloned into N- or C-terminal dysferlin plasmids using the Golden Gate assembly method from a plasmid containing the human dysferlin cDNA (a gift from the Jain Foundation; Addgene plasmid no. 60216). For in vitro studies, plasmids expressing full-length dysferlin or split-intein/dysferlin constructs under the control of the cytomegalovirus promoter were used. For AAV production, the most efficient N- and C-terminal split-intein/dysferlin combination, as well as split-dysferlin constructs containing overlapping homologous DNA regions (21), were subcloned into a pAAV vector carrying the muscle-specific CK8e promoter and a synthetic polyadenylation signal, flanked by AAV2 inverted terminal repeats. Myotropic AAVMYO1 vectors were produced using the conventional triple-plasmid transfection method in HEK293 cells, as previously described (41). AAV vectors were purified by PEG-8000 (polyethylene glycol, molecular weight 8000) precipitation followed by cesium chloride (CsCl) and sucrose gradient ultracentrifugations. Vector quality, titer, and full/empty capsid ratios were assessed using Stunner analysis (Unchained Labs), DNA gel electrophoresis, and quantitative polymerase chain reaction before in vivo studies.

In vitro validation of split intein/dysferlin

HEK293 cells were seeded in 24-well plates and incubated at 37°C in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin-streptomycin (Gibco). At ~80% confluence, cells were transfected using Lipofectamine 3000 (Invitrogen) with either a control plasmid expressing full-length human dysferlin or N-terminal, C-terminal, or both split-intein/dysferlin plasmids. Forty-eight hours after transfection, cells were harvested for protein extraction and subsequent analysis.

WT and dysferlin-deficient C2C12 myoblasts (42) were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Millipore Sigma) and 1% penicillin-streptomycin (Gibco). Myoblasts were seeded in either 60-mm plates (for protein analysis) or coverslips (for staining) and transfected using Fugene (Promega) with a full-length dysferlin or N-terminal, C-terminal, or both split-intein/dysferlin plasmids. Seventy-two hours after transfection, cells were harvested for protein extraction, and coverslips were fixed with 4% paraformaldehyde (Electron Microscopy Sciences).

Laser injury

For myoblast laser injury experiments, C2C12 myoblasts were seeded on glass coverslips and transfected using Fugene with a full-length

dysferlin or N-terminal, C-terminal, or both split-intein/dysferlin plasmids. Seventy-two hours after transfection, coverslips were transferred to cell imaging media containing FM-464 dye (5 µg/ml; Thermo Fisher Scientific). A 1- to 5-µm² area was irradiated for 10 ms with a pulsed laser (Ablate!, 3i Intelligent Imaging Innovations Inc.) to create a cavitation bubble that caused focal mechanical injury as the bubble collapses. Cells were imaged at 2-s intervals using the Olympus IX81 microscope (Olympus America). Dye intensity ($\Delta F/F$) was quantified using SlideBook 2026 to measure the kinetics of cell membrane repair. For focally injuring myofibers in intact muscle, intact biceps muscles were mounted in prewarmed Tyrode's buffer [119 mM NaCl, 5 mM KCl, 25 mM Hepes, 2 mM CaCl₂, 2 mM MgCl₂, and glucose (6 g/liter), pH 7.4] with FM 1-43 dye (1 to 2 mg/ml) and imaged using the 40×/1.4-numerical aperture objective Olympus IX81 microscope similar to above. Myofiber sarcolemma was injured similarly to the above by the Ablate! laser, and myofiber repair kinetics, as well as successful repair, were determined as in the myoblast injury assays.

In vivo AAV administration

For intramuscular injections, 8-week-old BlaJ male mice were anesthetized with isoflurane (Piramal Critical Care). A total dose of 6×10^{10} vg of AAVMYO1 was injected into each TA muscle. Different ratios of N- and C-terminal vectors were tested to determine the optimal vector stoichiometry, and the contralateral leg received equimolar dual AAVMYO1 vectors carrying a split-dysferlin homologous recombination construct or sterile saline as a sham control. Two months after injection, mice were euthanized, and TA muscles were harvested for downstream analyses.

Three BlaJ cohorts were used for systemic delivery of dual AAVMYO1 vectors. A dose-finding study was performed in 8-week-old mice, whereas 6- and 12-month-old mice were used to evaluate therapeutic rescue of dysferlin expression after dual-AAV vector administration. Before injection, mice were anesthetized with isoflurane. AAV solutions were prepared individually by diluting the stock in sterile saline and administered intravenously via the lateral tail vein. After successful injection, mice were placed in a warmed recovery cage and monitored for 1 hour.

Grip strength

Forelimb and all-limb grip strength was assessed using a grip strength meter (Columbus Instruments). Approximately 1 week before testing, mice were acclimated to the grip strength meter for 5 days. On each day, the mice were subjected to five pulls on both forelimb and all-limb grip strength tests. For testing, mice were subjected to five pulls for each test daily for 3 consecutive days. The maximum force for each day was averaged and plotted for each mouse.

Wire hang

Wire hang test was performed in accordance with the Treat-NMD protocol (SOP DMD_M.2.1.004). A wire was suspended 35 cm above a cage containing bedding. Mice were placed onto the wire using only their forelimbs. Mice were allowed to swing and hang using all four limbs. Hang time was recorded with a maximum of 10 min used as a cutoff.

Gastrocnemius muscle force analysis

In vivo muscle force output measurements were made using the 1300A Whole Animal System (Aurora Scientific). Mice were anesthetized

using isoflurane, and the fur was removed from the lower hindlimb before the animal was placed in the apparatus on a heated pad and clamped at the knee. The animal's foot was fixed to the footplate, and electrodes were placed through the skin adjacent to the tibial branch of the sciatic nerve to induce plantarflexion. Optimal muscle length was then identified by adjusting until a maximal twitch force was produced. The force-frequency curve was then recorded using ascending stimulation frequencies from 20 to 200 Hz, followed by repeated ECs (stimulation: 140 Hz, 350 ms; stretch: 39° joint rotation, 200 ms). The data were plotted as the maximal tetanic force across 20 repeated eccentricities (30-s interval between repetitions). Last, the muscle was allowed to recover for 10 min before the post-eccentric force-frequency curve was measured.

In situ force generation of the gastrocnemius muscle was assessed after sciatic nerve stimulation (Aurora Scientific, model 701C). Briefly, mice were deeply anesthetized with isoflurane. After confirming a surgical plane of anesthesia, as indicated by the absence of response to tactile stimuli, the distal tendon of the gastrocnemius muscle was detached and secured to the lever arm of a force transducer (Aurora Scientific, model 305B-LR) using silk suture. The optimal muscle fiber length (L_0) that produced the maximal isometric twitch force was determined by incremental micromanipulation of muscle length. While maintained at L_0 , the maximal isometric tetanic force (P_0) was elicited by stimulating the muscle at 200 Hz. Specific muscle force was calculated by normalizing P_0 to the muscle cross-sectional area (43).

In vitro diaphragm muscle force analysis

Upon completion of gastrocnemius force measurements, anesthetized mice were euthanized, and the entire diaphragm with the surrounding rib cage was excised and immediately transferred to carbonated Tyrode's solution (43). Under a light microscope, diaphragm strips consisting of longitudinal, intact muscle fibers with attached portions of the central tendon and rib bones were carefully dissected. Both ends of each strip were securely tied with surgical silk, and the sutures were mounted in a temperature-controlled horizontal in vitro bath (Aurora Scientific, model 809A) filled with carbogen-bubbled Tyrode's solution. The L_0 that produced maximal isometric twitch force was determined by fine adjustments of muscle length (Aurora Scientific, model 300). Specific muscle force was calculated by normalizing the maximal isometric tetanic force (P_0) to the muscle cross-sectional area.

Muscle histology analysis

After euthanasia, muscles were rapidly dissected and weighed, whereas diaphragm samples (half of each diaphragm) were embedded in optimal cutting temperature compound. All tissues were snap-frozen in liquid nitrogen-cooled isopentane and stored at -80°C until further analysis.

To analyze general muscle morphology, transverse 10- μm cryosections of TA, gastrocnemius, and quadriceps muscles were stained with H&E, Masson's trichrome, or oil red O. Whole sections were imaged using a Hamamatsu NanoZoomer slide scanner. Collagen-positive and lipid deposition areas were quantified using Fiji image analysis software.

For dysferlin immunostaining, muscle cross sections were immunolabeled with antibodies against N-terminal dysferlin (Romeo, Millipore Sigma), laminin (Millipore Sigma), C-terminal dysferlin (Hamlet Leica), and WGA (Invitrogen). For C-terminal dysferlin

staining, muscle sections were subjected to antigen retrieval before blocking. Slides were placed in an antigen retrieval buffer (10 mM sodium citrate with 0.05% Tween, pH 6.0) and heated at 90°C for more than 10 min. Slides were then cooled in buffer for 1 hour to 30°C . Slides were mounted with Prolong Gold containing DAPI (4',6-diamidino-2-phenylindole) and imaged using a VS-200 (Olympus).

All images were analyzed in Qupath version 0.6.0. Myofibers were segmented using the Cellpose plug-in for Qupath using the Cyto3 model. Per-fiber measurements were exported from Qupath after Cellpose segmentation. After segmentation, a script to identify central nucleated fibers was run on the segmented fibers, which shrinks the fiber segmentation by 30% and identifies positive DAPI staining within the new region of interest. Positive dysferlin fibers were quantified by using the Object Classifier function in Qupath by training a model to discern between positive and negative segmented fibers.

Protein extraction and immunoblot

Total protein was extracted from HEK293 or C2C12 cell pellets or serial muscle cryosections using radioimmunoprecipitation assay buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and 5% protease inhibitor cocktail (P8340; Sigma-Aldrich). Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific). Samples were denatured at 100°C for 10 min, and 30 μg of total protein per sample was resolved on NuPAGE 4 to 12% bis-tris polyacrylamide gels (Invitrogen). Proteins were transferred to 0.45- μm polyvinylidene difluoride membranes (Amersham Hybond) at 120 V for 2 hours at 4°C . Membranes were blocked for 2 hours in tris-buffered saline containing 5% nonfat dry milk and 0.05% Tween 20, followed by overnight incubation at 4°C with primary antibodies against dysferlin N terminus (VP-D504, Vector labs), dysferlin C terminus (Hamlet Leica), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; rabbit; Sigma-Aldrich, G9545) as a loading control. Horseradish peroxidase-conjugated secondary antibodies included goat anti-rabbit IgG (Jackson ImmunoResearch, 111-035-144) and anti-mouse IgG1 (Jackson ImmunoResearch, 115-035-205). Membranes were incubated for 2 hours at room temperature and visualized using a ChemiDoc MP imaging system (Bio-Rad) with Clarity Western ECL substrate (Bio-Rad). Band densitometry was performed on unsaturated images using Fiji (ImageJ) software. Uncropped immunoblots are supplied in data file S1.

Blood biomarkers

Blood samples were collected by cardiac puncture from anesthetized mice. Clots were allowed to form while samples rested at room temperature for 15 min. Samples were then centrifuged at 3500g for 10 min at 4°C , and the serum was immediately collected and stored at -80°C for subsequent analysis by the HIC core at the University of Washington.

Statistical analysis

Individual-level data are presented in data file S2. Statistical analyses were performed using GraphPad Prism (version 10.2.0). Data are presented as the means $\pm$ SEM. Unless otherwise indicated, statistical significance was assessed using one-sided tests with a significance threshold of $\alpha = 0.05$. The statistical tests used, sample sizes (n), numbers of biological replicates, and independent experimental repeats are specified in the corresponding figure legends. Comparisons among more than two groups were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparisons

test. For longitudinal studies, data are presented as time-course curves and were analyzed using a one-way repeated-measures ANOVA, with multiple-comparisons correction as indicated. Each data point represents an independently processed biological sample. Parametric tests were applied when data met the assumptions of the selected analyses on the basis of assessment of data distribution, variance, and experimental design. No statistical methods were used to pre-determine sample size; instead, sample sizes were selected on the basis of prior experience with the experimental models and are consistent with accepted standards in the field.

Supplementary Materials

The PDF file includes:

Figs. S1 to S6

Tables S1 and S2

Other Supplementary Material for this manuscript includes the following:

Data files S1 and S2

MDAR Reproducibility Checklist

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Use of split intein and myotropic AAV vectors enables effective preclinical gene therapy for dysferlinopathy

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