

# Translational Research and Therapeutic Perspectives in Dysferlinopathies

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Dysferlinopathies are autosomal recessive disorders caused by mutations in the dysferlin (*DYSF*) gene, encoding the dysferlin protein. *DYSF* mutations lead to a wide range of muscular phenotypes, with the most prominent being Miyoshi myopathy (MM) and limb girdle muscular dystrophy type 2B (LGMD2B) and the second most common being LGMD. Symptoms generally appear at the end of childhood and, although disease progression is typically slow, walking impairments eventually result. Dysferlin is a modular type II transmembrane protein for which numerous binding partners have been identified. Although dysferlin function is only partially elucidated, this large protein contains seven calcium sensor C2 domains, shown to play a key role in muscle membrane repair. On the basis of this major function, along with detailed clinical observations, it has been possible to design various therapeutic approaches for dysferlin-deficient patients. Among them, exon-skipping and minigene transfer strategies have been evaluated at the preclinical level and, to date, represent promising approaches for clinical trials. This review aims to summarize the pathophysiology of dysferlinopathies and to evaluate the therapeutic potential for treatments currently under development.

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## INTRODUCTION

Dysferlinopathies are autosomal recessive muscular dystrophies caused by mutations in the gene encoding dysferlin (*DYSF*; Online Mendelian Inheritance in Man [OMIM] gene number 603009, 2p13, GenBank NM\_003494.2) (1,2). Dysferlinopathies are rare muscular dystrophies, as the number of adult patients whose symptoms meet the full diagnostic criteria is estimated between 1/100,000 to 1/200,000 (3). Disease-causing mutations in *DYSF* mainly cause limb girdle muscular dystrophy type 2B (LGMD2B; OMIM number 253601) (1) and distal Miyoshi myopathy (MM; OMIM number 254130) (2). However,

myopathy (DACM) (alternatively called distal myopathy with anterior tibial onset [DMAT]; OMIM number 606768) and proximo-distal phenotypes (PD) (4,5). LGMD2B is characterized by proximal weakness, initially affecting the muscles of the pelvic and shoulder girdles, with age of onset in the late teens or early adulthood. For MM, however, the muscles initially affected are distal, in particular, the gastrocnemius. Age of onset is at the end of childhood (as with LGMD2B), and progression is typically slow. Proximodistal forms of dysferlinopathies are interesting as they display a range of symptoms, with a hypothesized influence of both genetic and

phenotype that combines both proximal and distal myopathies of the lower limbs. However, in every case, by late stages of disease progression, all limb-girdle muscles are affected.

The dysferlin gene is located on 2p13 and encodes several transcripts. The most common transcript is composed of 55 exons, and alternative splicing events (such as exon 1 for *DYSF\_v1* [GenBank DQ267935], exon 5a [GenBank DQ976379] and exon 40a [GenBank EF015906]) generate transcript diversity. Fourteen different splice isoforms, containing exon 5a, 17 or 40a, have been described for human *DYSF* (6). Seven of these isoforms are transcribed from the

variations were reported in the Leiden Muscular Dystrophy database (<http://www.dmd.nl>), including deleterious mutations, as well as nonpathogenic polymorphisms. Most of the reported disease-causing variants are point mutations and small insertions/deletions (7), but large exonic deletions and duplications have also been described (8). To date, no mutations have been identified in the alternatively spliced exon 1 of *DYSF\_v1*, 5a or 40 (9).

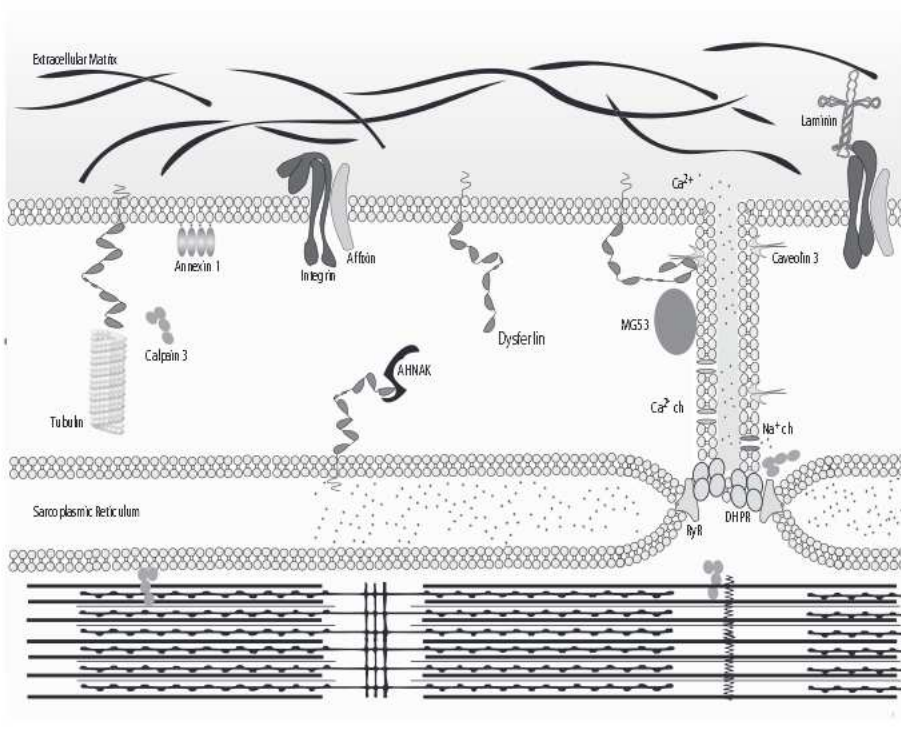
Translation of isoform 8 results in a 237-kDa transmembrane protein, named dysferlin. This protein belongs to the ferlin family, which includes otoferlin, myoferlin, fer-1-like 5 (FER1L5) and the originally identified *Caenorhabditis elegans* Fer-1. Fer-1 mediates calcium-dependent membrane fusion during spermatogenesis (10). Otoferlin is involved in vesicle exocytosis and is important for synaptic vesicle-plasma membrane fusion (mediated by  $Ca^{2+}$ ) at the inner hair-cell ribbon synapse (11). As such, mutations in otoferlin were shown to cause nonsyndromic deafness (deafness, autosomal recessive 9 [DFNB9; OMIM number 601071]) (12). Myoferlin is involved in both myoblast-myoblast fusion (13) and myoblast-myofiber fusion during muscle differentiation and maturation, respectively (14). Myoferlin has also been implicated in vesicle recycling during myoblast fusion because of its interaction with EH-containing domain 2 (EHD2) (15). Data published recently suggest that FER1L5 can interact, as previously described for myoferlin, with EHD1 and EHD2 to engage myoblast fusion (16). However, FER1L5 has not been associated with any human pathology to date.

cisely, it is important to determine ferlin C2 domain topology. Different three-dimensional shapes adopted by these protein domains allow ferlin members to interact with binding partners; therefore, C2 structural data would be very informative (18,19). Within the ferlin family, dysferlin is particularly well conserved among mammals (90% similarity between human and mouse [20,21]) and contains seven C2 domains. Multiple copies of C2 domains are frequently found in this particular protein family (22). Recently, their topology was described (C2A, C2B and C2E are type 1 C2 domains, while all the others are type 2), and it appears that C2 secondary structures are important for protein function (18,19). The C2 domains of dysferlin are similar to the well-characterized C2 domain of synaptotagmin VII, a protein predominantly involved in vesicle exocytosis in neurons (23,24). Besides C2 domains, two other motifs are found in this protein family: dysferlin sequences (3 of 6 human ferlin family members contain this sequence) and ferlin sequences (carried by all the members of the ferlin family). Both domains are of unknown function (20). Moreover, dysferlin sequences are conserved between orthologs. In addition, dysferlin also has several nuclear localization sequences (although there is no evidence for its nuclear localization). Finally, several proline, glutamic acid, serine and threonine (PEST) sequences, involved in protein-protein interaction, or in protein ubiquitinylation and degradation by the proteasome, have been described (25).

The *DYSF* gene, although expressed in a variety of tissues including brain,

complex (DGC), since in dysferlin null mice, no alteration of the DGC complex was evident (29,30). These data clearly indicate that mechanisms involved in dysferlinopathies must be different from the ones caused by DGC deficiencies. Interestingly, a role of dysferlin in the maintenance of muscle repair capabilities has been described. In dysferlin-deficient mice, muscle recovery after injuries is measurably slower than in wild-type mice, since recovery in the mutant tissue involves myogenesis rather than membrane repair (31). In fact, dysferlin was directly implicated in sarcolemmal resealing of muscle fiber damage resulting from mechanical stress, two-photon laser lesions or muscle elongation in humans and mice (29,32–35). The involvement of dysferlin in membrane repair is also supported by the numerous abnormalities in muscles of LGMD2B/MM patients, mainly subsarcolemmal vesicle accumulation (36,37). It was shown *in vitro* that *Dysf*<sup>-/-</sup> cultures have delayed expression of myogenin, resulting in defective muscle differentiation (38). Finally, two additional functions for dysferlin were described recently: (i) dysferlin is required for T-tubule homeostasis in humans (39) and (ii) dysferlin is associated with inflammation (40,41).

Because of its size and its numerous protein domains, dysferlin likely interacts with numerous partners, some of which have already been described. Identified interacting proteins are involved in diverse physiological processes including membrane maintenance (annexin 1 and 2, affixin and caveolin-3), cytoskeleton regulation (calpain-3, AHNAK [desmoyokin]) and membrane repair (mitsugumin 53



**Figure 1.** Binding partners of dysferlin. In normal myofibers, dysferlin is anchored by its C-terminal domain to the plasma membrane and membrane network to cytosolic vesicles. In these contexts, it associates with different partners according to its localization. Schematized here are the binding partners of dysferlin and their localization within the myofiber. ch, Channel.

and affixin (or  $\beta$ -parvin, a focal adhesion protein) have each been shown to interact with dysferlin in human skeletal muscles (48). Calpain-3 (mutated in LGMD2A) also interacts with dysferlin (49,50) (OMIM 253600). Calpain-3 is a calcium-dependent protease for which substrates are present in sarcolemme/sarcomeres and function as a regulator in muscle remodeling (51). Finally, AHNAK has been shown to interact with dysferlin. Interestingly, along with annexin I & II and affixin, AHNAK has not been directly im-

recent publication clearly demonstrated that AHNAK is also found in the costameric network and is not present in the T-tubule system, leading to the hypothesis that AHNAK is involved in fiber stiffness (59).

Finally, dysferlin has binding partners that play direct and indirect roles in membrane repair, such as MG53 (also called TRIM72). MG53 associates with phosphatidylserine in cell membranes and acts as a sensor for the entry of extracellular oxidative molecules. As a re-

same complex. Although dysferlin and MG53 (60) are the only proteins known to be involved in muscle repair, their precise function in this context remains largely unknown.

Dysferlin colocalizes with the dihydropyridine receptor in T-tubules, suggesting a role for dysferlin in T-tubulogenesis (39). The T-tubule system is an extensive internal membrane invagination that passes transversally from the sarcolemma across a myofibril of striated muscle. T-tubules are involved in sarcomere stimulation by triggering the action potential deep in the fiber, allowing the release of calcium ions from the sarcoplasmic reticulum (61). Because of binding partners such as the annexins and caveolin-3, dysferlin could play a role in “invagination/evagination” of the membrane. Interestingly, T-tubule biogenesis requires both fusion and “invagination/evagination” of the membrane (62) (see Figure 1). Recent work has shown that dysferlin’s plasma membrane localization is transitory; membrane anchorage never exceeds 3 hours, and the protein’s half-life is only 5 hours. The syntaxin-4-associated endocytosis pathway plays a critical role in regulating this process (63).

#### FROM SYMPTOMS TO DIAGNOSIS

Dysferlinopathies, for which onset generally occurs early in the second decade of life, are characterized by atrophy and weakness of the gastrocnemius muscle and/or the anterior tibial muscles. If a patient presents with these symptoms, the first step toward diagnosis is to measure serum creatine kinase (which is elevated in most cases of LGMD2B). In some cases, this test is supplemented

(LGMD2A). This step increases the complexity of dysferlinopathy diagnosis. Histology of affected muscles shows when extensive fiber degeneration-regeneration has taken place, revealed by centronuclear fibers of varying sizes and immune infiltration (predominantly CD8<sup>+</sup> cells and macrophages). No B cells are detected, probably because intracellular material is released after membrane lesion (64).

#### WHAT ARE THE MUTATIONS INVOLVED IN DYSFERLINOPATHIES?

Because the discovery that *DYSF* dysfunction underlies both LGMD2B (1) and MM (2), a wide spectrum of *DYSF* mutations has been identified, including missense, nonsense, frameshift deletions/insertions, splicing mutations and large deletions (1,2,7,8,46,65,66). Although no mutational hotspots were identified (UMD-DYSF database, Leiden muscular dystrophy pages: <http://www.dmd.nl>), five founder mutations were reported: c.2372C>G (p.Pro791Arg) for a native Canadian population, c.4872\_4876delinsCCCC (p.Glu1624AspfsX9) for a Libyan Jewish population, c.2875C>T (p.Arg959Trp) for an Italian population, c.5713C>T (p.Arg1905X) for a Spanish population (region of Sueca) and c.2779delG (p.Ala927LeufsX21) for a Caucasian Jewish population (67–71).

Loss of dysferlin is frequently associated with nonsense or frameshift mutations (7,66). However, except for rare cases (for example, exon 32 deletion causes mild dysferlinopathy [see below, 72]), no measurable phenotype to genotype correlation was established. In general, the dysferlin gene has a high level

of expression (ERAD) system and autophagy (74). ERAD is used to clear nonaggregated native or mutant dysferlin, whereas autophagy removes aggregated versions of the mutated protein. In situations where unfolded-dysferlin degradation is misregulated, the pathophysiology could be explained by the formation of amyloid-like aggregates (75). *In silico* analysis of dysferlin predicted a bi-partite nuclear localization sequence (NLS) near its N-terminus ([www.genscript.com/psort/psort2.html](http://www.genscript.com/psort/psort2.html)). Whereas endogenous dysferlin is typically not found in the nucleus, this motif could become relevant for truncated proteins that lack transmembrane domains. Indeed, truncated dysferlins may be directed to inappropriate cellular compartments, in particular the nucleus, because of the NLS sequence. Unfortunately, this result has not yet been observed because of the C-terminal specificity of the Hamlet antibody. Every *DYSF* mutation reported to date affects protein expression levels in skeletal muscle, except for one (TG573/574AT; p.Val67Asp), which affects dysferlin calcium-binding properties (76). Before effective therapeutic approaches can be developed, it is critical to fully characterize *DYSF* sequence variations that result in pathologies.

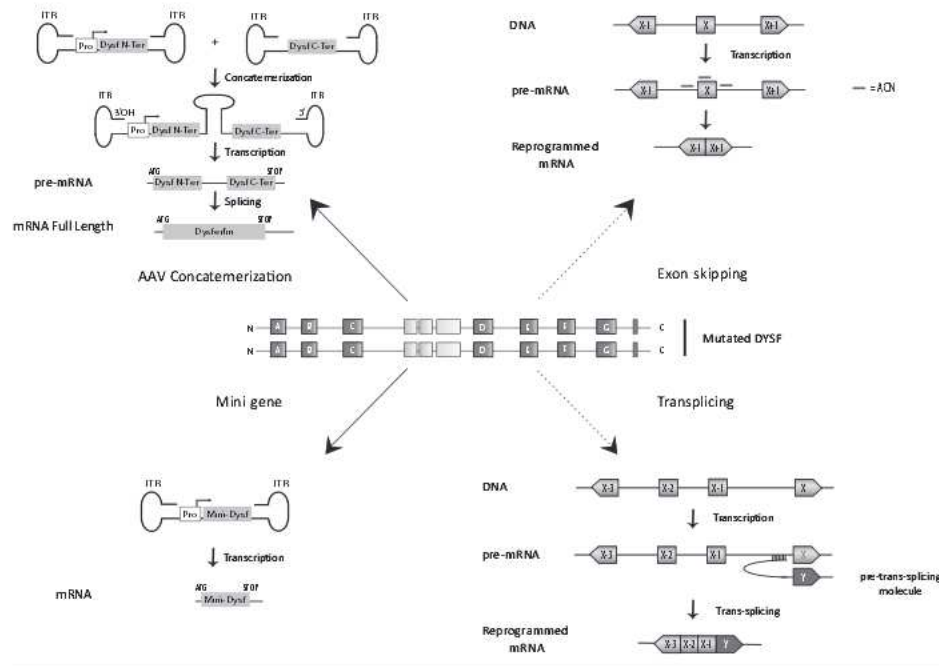
#### THERAPEUTIC APPROACHES FOR DYSFERLINOPATHIES

Today, no curative treatment for dysferlinopathies exists. In the following section, several validated approaches for dysferlinopathy therapy will be discussed, including synthesis of functional protein after adeno-associated virus (AAV) vector transfer, gene surgery

from A/J mice), which will be helpful when assessing novel therapeutic strategies. In addition to these cell culture systems, several animal model systems also exist. In particular, two natural mice strains (SJL/J and A/J) were identified that have dystrophic phenotypes. The SJL/J mouse strain has a splicing mutation in the dysferlin gene, making it a natural model for LGMD2B and MM (21,78). Genomic analysis identified the mutation as a 141-bp genomic deletion involving a tandem repeat and altering the 3' splicing site of exon 45, therefore leading to the splicing of this 171-bp long exon. Another strain of mice carrying a deficient dysferlin was fortuitously discovered in The Jackson Laboratory. The A/J strain presents mild progressive muscular dystrophy. Genetic analysis of this strain demonstrated that an early transposon (ETn) retrotransposon inserted itself in the intron 4 of the dysferlin gene. However, research involving these strains is not easy because of a mild phenotype and the absence of a matched control strain. Thus, genetically engineered and backcrossed mice (*dysf*<sup>-/-</sup>, C57BL/10.SJL-Dysf, B6.129-Dysf<sup>tm1Kcam/Mmmlh</sup>, B6.A/J-Dysf<sup>prmd</sup>) were created (29,78–80). Issues still remain with these dysferlinopathy models, since none truly recapitulate the human disease. Although not perfect, these models will serve as valuable tools to develop and test novel therapeutic approaches.

#### TRANSFER OF FULL-LENGTH DYSFERLIN

The ultimate goal is to alleviate the symptoms associated with dysferlin deficiency. Currently, gene transfer ap-



**Figure 2.** Therapeutic strategies currently being tested. Dysferlinopathies are an ensemble of pathologies, due to mutations in the *DYSF* gene. Wide spectrums of mutations are responsible for the pathologies, including missense, nonsense, frameshift, deletion, insertion and splicing mutations. Currently, two main types of strategies are envisaged: gene transfer (AAV concatemerization, or minigene) and mRNA surgery (exon-skipping and trans-splicing strategies). Black arrows indicate strategies available for all gene mutations, whereas dashed arrows indicate mutation-specific strategies (mutations in exon 32 for exon skipping, 3' mutations for trans-splicing). Inverted terminal repeats (ITRs) are the adeno-associated vector backbone.

encapsulation size is limited to approximately 4 kb, the equivalent of three C2 domains (83). To solve this problem, researchers used the concatemerization property of AAV (84,85). Two independent AAV vectors are used: one carrying 5' dysferlin cDNA fused with an intronic sequence that carries a donor splice site and the other containing the 3' cDNA-bearing intronic sequence and a splice acceptor site. This approach allows expression, after concatemerization and splicing, of a

and his colleagues have also tried to use one AAV to encode full-length dysferlin (using the AAV5 serotype) (86). They conclude that this strategy allows expression of dysferlin at detectable levels; however, dysferlin expression could be in part the result of a recombination event between different parts of the transgene carried by AAVs (87).

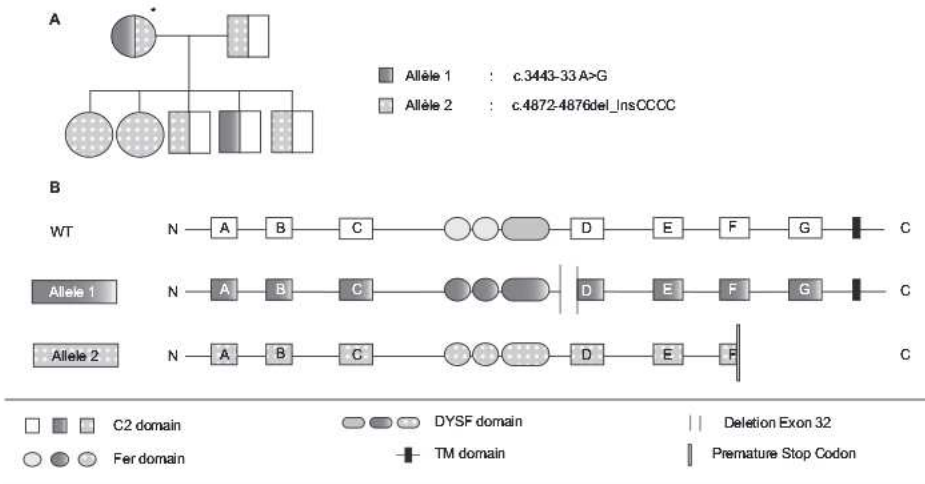
#### TRANSFER OF DYSFERLIN MINIGENES

Recently, a minidysferlin was discov-

ered and introduced into dysferlin-deficient mice and observed membrane insertion of minidysferlin, which restored membrane repair. Although this minidysferlin prevented the appearance of severe dysferlinopathies, it was not completely functional, since no histological improvements were seen in treated mice (88). Therefore, our experiments demonstrate that the minidysferlin retains membrane repair function, although functions associated with missing C2 domains are lacking. For example, the C2A domain interacts with phospholipids, AHNAK and  $\alpha$ -tubulin (58). C2A (as well as C2B, C2C, C2D and C2E) is absent from the natural minidysferlin expressed in this patient. This result certainly explains why restoring minidysferlin is not sufficient to rescue all aspects of the dysferlin phenotype. Nevertheless, these results indicate that both the AAV injection and the level of expression of a functional minidysferlin are not toxic. This is important information for future clinical developments, since we have shown that the overall function of dysferlin can be, at least partially, carried by a large deleted amino acid sequence protein. This result also pinpoints the necessity to develop genomic explorations in patients affected by muscular dystrophies to identify new natural truncated mutated proteins.

#### MESSENGER SURGERY BY EXON SKIPPING

In dysferlinopathies, correlations between genotype and phenotype are rare (as previously mentioned). However, the Sinnreich group identified a family in which two severely affected sisters were homozygous for a dysferlin null muta-



**Figure 3.** A natural proof of exon skipping. Depicted is a family studied by Sinnreich *et al.* (72), in which the index case (identified by an asterisk) carries two mutant alleles. The family tree (A) and the nature of the two mutant alleles (B) are shown.

exon-skipping therapeutic approach for dysferlinopathies. The use of an exon-skipping strategy was first applied in Duchenne muscular dystrophies (89), and clinical trials using antisense oligonucleotides (AONs) are underway (90). The results obtained in preclinical trials are promising and pave the way for similar approaches for dysferlinopathies (91). In particular, using an exon-skipping strategy to treat *DYSF* mutations lying in exon 32 is promising. According to the UMD database, approximately 4% of dysferlin patients have disease-causing mutations in this exon. AON directed against splicing regulatory elements of exon 32 were tested on myotubes transdifferentiated from patient fibroblasts previously infected by a lentivirus encoding MyoD. The efficiency of exon skipping induced by these AONs was validated by reverse transcription–polymerase chain

### PHARMACOLOGICAL AND IMMUNOLOGICAL APPROACHES

Pharmacological or immunological approaches have also been envisaged to treat dysferlinopathies. Recently, PTC124<sup>®</sup> (also known as Ataluren<sup>™</sup>) was shown to read through nonsense mutations. Wang *et al.* (94) showed that treating myotubes from a patient with a premature stop codon allowed the expression of a sufficient functional dysferlin to rescue myotube membrane blebbing. However, in dysferlinopathies, less than one-third of mutations are nonsense mutations.

In 2007, the use of Dantrolen<sup>™</sup> as a potential treatment for dysferlinopathies was described. CPK levels were reduced in response to Dantrolen (95); however, no amelioration of the muscle phenotype has been seen to date. In 2001, the presence of a complement deposit in non-necrotic muscle fibers deficient for dysfer-

lin for dysferlinopathy patients treated with Rituximab<sup>™</sup> (a human/murine chimeric monoclonal antibody directed against CD20-positive B cells) (98). The authors suggest a possible role for B cells in dysferlin pathophysiology. After the transcriptomics studies performed in EP Hoffman’s laboratory, demonstrating the upregulation of proteins involved in rho signaling, they started preclinical studies using an inhibitor of a rho kinase (Fasudil<sup>™</sup>) (99). This preclinical treatment demonstrated some benefits: notably, a lower level of inflammatory cells in the muscles and an increase in body weight, but no impact in another physiological process such as muscle fiber degeneration/regeneration or in muscle force (100). Nevertheless, this study is a starting point for drug-testing trials in dysferlin-deficient mice, by a standardized measurement of dysferlin functions that will be useful for high-throughput screening.

### CONCLUSION

It is essential to characterize dysferlin at the molecular level to functionally understand this sarcolemmal protein in both normal and pathological conditions. This knowledge is absolutely required if adapted therapeutic strategies are to be developed for dysferlinopathies (and possibly other forms of muscular dystrophies), where enhancement of sarcolemmal resealing could be of benefit. Researchers are still working to define functional roles for each C2 domain and to accurately describe dysferlin activity in a variety of physiological processes, such as T-tubule homeostasis and membrane repair. As molecular diagnostic technologies improve, a better under-

AFM and the Fondation pour la Recherche Médicale (FRM), respectively.

## DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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