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Clinical potential of microdystrophin as a surrogate endpoint

Jessica F Boehler[a], Kristy J Brown[a], Margaret Beatka[b], J Patrick Gonzalez[a], Roxana Donisa Dreghici[a], Meghan Soustek-Kramer[a], Sharon McGonigle[a], Annie Ganot[a], Timothy Palmer[a], Caitlin Lowie[a], Jeffrey S. Chamberlain[c], Michael W Lawlor[b], Carl A Morris[a,*]

[a] Solid Biosciences, 500 Rutherford Avenue 3rd Floor, Boston, MA 02129, United States
[b] Diverge TSL, 247 Freshwater Way Suite 610, Milwaukee, WI 53204, United States
[c] Department of Neurology, University of Washington School of Medicine, Seattle, United States

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ABSTRACT

Accelerated approval based on a likely surrogate endpoint can be life-changing for patients suffering from a rare progressive disease with unmet medical need, as it substantially hastens access to potentially lifesaving therapies. In one such example, antisense morpholinos were approved to treat Duchenne muscular dystrophy (DMD) based on measurement of shortened dystrophin in skeletal muscle biopsies as a surrogate biomarker. New, promising therapeutics for DMD include AAV gene therapy to restore another form of dystrophin termed mini- or microdystrophin. AAV-microdystrophins are currently in clinical trials but have yet to be accepted by regulatory agencies as reasonably likely surrogate endpoints. To evaluate microdystrophin expression as a reasonably likely surrogate endpoint for DMD, this review highlights dystrophin biology in the context of functional and clinical benefit to support the argument that microdystrophin proteins have a high probability of providing clinical benefit based on their rational design. Unlike exon-skipping based strategies, the approach of rational design allows for functional capabilities (i.e. quality) of the protein to be maximized with every patient receiving the same optimized microdystrophin. Therefore, the presence of rationally designed microdystrophin in a muscle biopsy is likely to predict clinical benefit and is consequently a strong candidate for a surrogate endpoint analysis to support accelerated approval.

1. Introduction

In the United States, the Food and Drug Administration (FDA) requires randomized controlled clinical trials to demonstrate both safety and clinical benefit for approval of a drug product. The timeline from drug development to approval can take more than a decade [1]. In addition, large sample sizes are often needed to demonstrate safety, while clinical endpoints must be carefully selected that capture the drug's mechanism of action, have disease relevance and are meaningful to the patient. The generation of clinical functional endpoints, which are often considered the "gold standard" for efficacy, may present challenges to drug development and approval. Some clinical outcome measures can take years to complete. Others, particularly when the assessments are somewhat subjective, may be difficult to measure or are hard to interpret. To gain additional confidence in clinical endpoints, a surrogate endpoint can be highly informative and beneficial in clinical trial designs. Surrogate endpoints are objective, non-biased measures that can act as a substitute for clinical outcomes when they correlate with or predict future clinical benefit [2]. As such, surrogate endpoints have the potential to hasten efficacy readouts as they usually demonstrate change more quickly than the primary functional endpoint. Ideally, all surrogate endpoints are to be validated to confirm correlation to clinical outcome; but most accepted surrogate endpoints are not validated due to ethical or time considerations. Yet, to date, the FDA has accepted dozens of surrogate endpoints across a wide spectrum of different diseases [3].

The use of surrogate endpoints can be extremely powerful for rare diseases, which generally have limited to no treatment options. In these disorders, drug approvals are desperately needed, but can be the most difficult to achieve using traditional trial designs that rely on large, randomized patient populations. Further, many rare diseases are progressive and involve a worsening of symptoms over time. This not only leads to the need for faster

* Corresponding author.
E-mail address: carl@solidbio.com (C.A. Morris).

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approval for effective therapies, but also hinders measuring drug efficacy due to mixed populations of patients in different stages of disease progression. Trying to control for disease progression can be equally as difficult as patients with a rare disease often have variable natural history so predictions for degeneration of function are not always reliable.

Since rare diseases do not fit the typical drug approval paradigm, the FDA started an Accelerated Approval Program, which greatly aids approval for drugs aimed at treating serious conditions with unmet medical need through use of a surrogate endpoint [4]. Given that the underlying disease mechanism is known in most rare diseases, leveraging a surrogate endpoint for accelerated approval can enable more rapid and robust therapeutic development while also speeding growth of next generation treatments [5].

To date, there have been several accepted surrogate endpoints used for rare disease drug approval under the Accelerated Approval Program pathway [3]. An example of the power of surrogate endpoints for rare diseases was demonstrated in patients with Fabry Disease, which is a genetic lysosomal storage disorder caused by mutations in the galactosidase alpha (*GLA*) gene. When mutated, *GLA* cannot properly encode α-galactosidase A, which is an important enzyme that is needed for the breakdown of globotriaosylceramide (GL-3). Without enzyme function, GL-3 builds up in cells and causes organ dysfunction that manifests as pain and progresses to cardiac and renal involvement, leading to death [6]. One promising treatment for Fabry disease is Fabrazyme, an enzyme replacement gene therapy that delivers *GLA* to restore loss of function and reduce GL-3 levels. In 2003, the FDA granted accelerated approval to Fabrazyme based on GL-3 as a surrogate endpoint [3]. Since the approval was based on GL-3 levels, ongoing studies continued to assess long-term benefit of functional changes as GL-3 has been difficult to correlate to disease severity across all patients [7]. It took almost 20 years for Fabrazyme to show functional changes needed for full approval in patients two and older [8]. In the meantime, the approval based on the surrogate endpoint allowed patients faster access to a potential life-altering therapy that not only extended lifespan significantly, but also improved their quality of life [9].

Another well-known accepted surrogate endpoint for drug approval in rare disease is dystrophin, a protein absent in patients with Duchenne muscular dystrophy (DMD). The accelerated approval based on dystrophin was accepted more than a decade later than the surrogate endpoint for Fabry Disease. As a result, data are still being gathered to firmly establish the link between function and dystrophin. Another complicating factor is that the surrogate is not the full-length dystrophin protein. Multiple approaches have evolved to restore a shortened, but functional version at both the preclinical and clinical levels [10]. Two approaches that have been highly characterized in DMD patients include exon-skipping and AAV-gene therapy. Exon-skipping aims at correcting the dystrophin gene (*DMD*) mutation through administration of oligonucleotides to impact dystrophin pre-mRNA splicing [11], while AAV-gene therapy delivers a shortened form of the *DMD* gene, termed mini- or microdystrophin, through systemic administration of packaged adeno-associated virus [12]. The functional differences between exon-skipped dystrophins (there are numerous possible versions) and microdystrophin have not been fully characterized, but both aim to treat the dystrophin deficiency that causes DMD. To date, only exon-skipped dystrophins have been used as a surrogate endpoint for drug approval, but ongoing trials are measuring microdystrophin in anticipation for use as a surrogate endpoint. However, it has not been approved based on these results yet. To delve into this rationale, the aim of this review will outline the foundations for microdystrophin as a reasonably likely surrogate endpoint by highlighting the factors that lead to functional dystrophin. Overall, microdystrophins are designed to be a highly functional, shortened form of dystrophin and therefore are likely to predict clinical benefit if restored to muscle (Table 1).

1.1. Shortened dystrophin as therapeutic approach to mitigate disease severity in DMD

The *DMD* gene is one of the largest in the human genome; it spans over 2 megabases on the X chromosome [13]. It encodes an important functional muscle protein called dystrophin, which links the actin cytoskeleton to the muscle fiber membrane [14]. This link helps to facilitate proper muscle contractions and to dissipate contractile force into the extracellular matrix, while its absence leads to myofiber injury, subsequent degeneration, and overall muscle loss [15]. When the dystrophin protein is missing, progressive skeletal muscle damage results in loss of ambulation, followed by reduced pulmonary and myocardial function that can greatly shorten the lifespan of patients with a *DMD* loss of function mutation.

In general, patient phenotypes can be stratified according to the amount and/or functionality (i.e. quantity/quality) of dystrophin protein produced. For example, Duchenne muscular dystrophy (DMD) is generally associated with more severe phenotypes because dystrophin is absent (<5%), whereas Becker muscular dystrophy (BMD) patients tend to present with milder phenotypes due to the presence of some dystrophin [16–18]. However, this is a large generalization as BMD patients can vary dramatically in disease presentation [18–21]. Whether functional dystrophin protein is produced depends on whether the mutation preserves the translational reading frame for dystrophin; although there is evidence that a set of in-frame mutations impacting specific domains may also reduce or eliminate protein function [22,23]. Therefore, dystrophin deficiency can be described as a clinical spectrum from severe to no disease that is inclusive of no dystrophin (DMD), some functional dystrophin (BMD), or enough dystrophin (healthy muscle).

This clinical spectrum paved the path for restoration of dystrophin as a therapeutic to alleviate disease phenotypes. Multiple therapeutic approaches have evolved with the aim to restore a functional dystrophin protein and thus lessen disease severity [24]. Restoration of the dystrophin protein is accomplished at the gene level, which is complicated due to the *DMD* gene's large size, 2.4 million bps[13]. The most promising models that have emerged as drug development targets aim to shift the phenotype by converting low to absent dystrophin protein to an expressed smaller dystrophin [25]. The potential therapeutic usefulness of this approach is evident by looking at genotype-phenotype correlations of human dystrophinopathy patients, as different mutations in the dystrophin gene can lead to a broad range of phenotypic severity.

However, it also raises many questions regarding the quantity and necessary components of a shortened dystrophin needed to produce functional benefits. To answer these questions, preclinical experiments using transgenic *mdx* mice overexpressing mini- or microdystrophins became a useful tool to study the functional effects of rationally designed shortened dystrophins [26–37]. One of the benefits of using transgenic models is that the protein expression is not reliant on gene delivery method, so expression is more uniform across all skeletal and cardiac muscle tissues. Additionally, transgenic mice express the protein from birth so they are a better model for studying phenotypes as the presence of muscle damage will be a direct consequence of the shortened dystrophin. In general, these studies demonstrated that restoration of a variety of shortened dystrophins could improve functional readouts, but interestingly, the specific components that make up

Table 1
Dystrophin as a Reasonably Likely Surrogate Endpoint.

	Exon-Skipped Truncated Dystrophin	Rationally Designed Functional Microdystrophin
Qualifying Criteria for Accelerated Approval		
Whether a Condition is Serious (Duchenne muscular dystrophy)	Yes	Yes
Meaningful Advantage over Available Therapy	Mutation Specific	Not mutation specific
Demonstrates an Effect on an Endpoint That Is Reasonably Likely to Predict Clinical Benefit	Yes	Yes
Evidentiary Criteria for Accelerated Approval		
Understanding of the Disease Process	Yes	Yes
Understanding of the Relationship Between the Drug's Effect and the Disease Process	Yes	Yes
Additional Comparitors		
Established Safety Profile	Robust	Under investigation
Dosing Regime	Weekly infusion	Single infusion
Unmet Medical Need	Yes	Yes
Clinical evidence of naturally occuring (BMD) dystrophin	Yes	No

U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Biologics Evaluation and Research (CBER) [100].

the miniaturized dystrophin – referred to as the quality of the protein – were important for driving functional benefits as some smaller proteins had no change or worsened disease severity in *mdx* mice [28,29,38,39].

The translation of these findings to current clinical therapeutic approaches of exon skipping and AAV-gene therapy has been challenging. First, the impact of gene delivery method on expression can obscure data interpretation, as exon-skipped dystrophins and AAV-microdystrophins differ in both quantity and type of shortened dystrophin that can be produced. Second, both gene delivery methods have different biodistributions, with AAV having enhanced cardiac expression when compared to morpholinos [12,40,41]. Further, the direct comparison of microdystrophin expression to exon skipped shortened dystrophin is not straightforward due to differences in quantitative assays and the domain organization within the dystrophins generated by each method. The quantity of dystrophin has been reported as a percentage of healthy control skeletal muscle tissue using Western blot or mass spectrometry methods as readouts [42,43]. However, there is no universal standard that exists so cross comparison between assays is difficult. To date, comparisons have been made with traditionally qualitative assays, such as immunofluorescence, which can also be problematic due to differences in antibody selection and exposure settings. Although both quantitative and qualitative methods report information about dystrophin expression, they provide complimentary pieces of data with immunofluorescence reporting the total number of fibers expressing dystrophin, while Western blot or mass spectrometry reveal how much of the protein is present in the muscle. This distinction highlights that immunofluorescence displays important information regarding the uniformity of expression with better functional outcomes associated with a more even distribution of expression across all muscle fibers [33,44]. The quantity needed to produce an even fiber distribution – even with very low expression – is currently unclear. Both exon-skipped and microdystrophins have been shown to produce non-homogenous expression across all muscle fibers, which makes evaluation of truncated dystrophins difficult as expression may vary depending on location in the biopsy, as well as muscle type. To address this, quantitative measures like Western blot and mass spectrometry become useful because a larger portion of the muscle can be sampled. Historically, the presence – not the distribution or the amount– of exon-skipped dystrophins was considered enough to provide surrogacy; however, more clinical data regarding fiber distribution and protein quantity is needed to see if the preclinical findings are replicated in a clinical setting. Despite these caveats, clinical data from these two therapeutic approaches suggests that both microdystrophins and exon skipping dystrophins have the potential to produce clinically meaningful changes, but it raises the question of how to define microdystrophin as a surrogate endpoint.

Given the large number of ways that smaller various truncations of dystrophins can be generated, there are many different types of dystrophin proteins that can be formed. For example, the smaller dystrophins that are formed from exon-skipping are based on a patient's deletion mutation breakpoint such that several patients receiving the same drug can produce structurally different proteins as they may not all carry the same exon-encoded protein subdomains. Additionally, microdystrophins can be engineered multiple ways with variations of different dystrophin domains. Each of these dystrophins may not be functionally equivalent and could be one of the multiple contributing factors that leads to lack of correlation between BMD phenotype and shortened dystrophin quantity [45]. Furthermore, microdystrophins are synthetically generated via molecular cloning and are composed of carefully designed structures that do not depend on the breakpoints of patient-specific deletion mutations. Therefore, defining the regions that are necessary for dystrophin function is important when considering whether a non full-length dystrophin will provide reasonably likely clinical benefit.

1.2. Identification and characterization of regions essential for function: refining the 'spectrum' hypothesis

After the identification of the dystrophin protein, the primary structure was quickly determined and it was revealed that dystrophin is part of larger protein complex often referred to as the dystrophin associated protein complex (DAPC) [46]. The DAPC includes dystroglycan, sacroglycans, syntrophins and dystrobrevin, which can only localize properly when dystrophin is at the membrane. The importance of the DAPC and its relationship to dystrophin is highly studied and has been reviewed extensively [47,48]. For proper DAPC formation, the dystrophin protein needed four domains: the N-terminal domain, the spectrin-like repeat or rod domain, the cysteine-rich domain, and the C-terminal domain [47]. These domains were correlated with clinical characteristics in BMD patient muscle to define their functional capabilities with a strong emphasis on dystrophin quantity [18,20,49]. From these studies, general trends emerged; however, there was always high phenotypic variability as patients did not always exhibit clear genotype-phenotype correlations. Potential reasons for this include differences in protein quantity, cryptic mRNA splicing, an internal ribosome entry site (IRES) in exon 5, localization profile, or other factors independent of dystrophin that have been shown to modify the disease [50,51]. Despite this nuance, there were clear

teachings that did emerge from these clinical correlations involved three domains [17]. Mutations in the N-terminal actin binding domain were typically associated with more severe phenotypes while mutations in the cysteine-rich domain (which binds to dystroglycan) generally fully inactivates the protein [18,23]. Given that these domains are the key to dystrophin protein primary function, as they are required for linkage between the contractile apparatus and the muscle sarcolemmal membrane, they are essential for proper dystrophin function. To demonstrate whether these domains alone were sufficient to drive muscle function, in vivo testing with dystrophins that solely contained these linkage domains resulted in mice that exhibited severe pathology, indicating that these regions by themselves were not enough to drive functional changes and that other portions of the protein are required [32]. In contrast, disruption to the C-terminal domain has generally been associated with both mild and nonprogressive BMD symptoms [18,52]. However, mutations exclusive to this region are quite rare [23,53]. The association with milder phenotypes was strengthened when multiple in vivo studies using both transgenic mice models and AAV-delivered microdystrophins showed that full functional abilities were retained in the absence of the C-terminal domain [29,34,54–58]. However, the C-terminal domain is known to bind both syntrophin and dystrobrevin, while recent preclinical evidence has suggested that it could potentially be important for preventing cardiomyopathy [59–61]. More data regarding the functionality of this region would be helpful to guide further optimized microdystrophin proteins.

Although the characterization of both the essential N-terminal and cysteine-rich domains was relatively straightforward, defining the rod domain's function was more challenging. Becker patients with rod domain mutations were generally associated with milder symptoms, but they exhibited the most phenotypic variability [18–21,49]. This is not surprising given that the rod region is encoded by 50 exons and contributes to a large portion of the dystrophin protein [14,62]. In addition to the domain's considerable size, portions throughout the rod domain did not seem to be functionally equivalent as disruption by either a deletion or duplication resulted in similar symptomology [18]. Many of these deletions and duplications disrupted the normal dystrophin open reading frame resulting in non-functional proteins [17]. Furthermore, some small deletions led to worse phenotypes than larger deletions, suggesting differential effects on protein stability and/or function [18,63]. This was established when further characterization of the rod region showed that it contained 24 spectrin-like repeats with a low level of similarly between each repeat (10–25%) [62], suggesting that the rod domain was more than just a spacer region and could induce additional functional properties of dystrophin. The rod domain's role as a simple spacer was also brought into doubt when it was shown that as few as four of the dystrophin spectrin-like repeats (SRs) were needed to maintain dystrophin function, but that function was lost when the dystrophin SRs were replaced with four similar SRs from alpha-actinin [31]. Another perplexing factor associated with the rod region was the lack of correlation between dystrophin quantity and phenotype, further pointing to other components besides the protein amount that are important for function [18].

As previously mentioned, the dystrophin structure is typically defined as four domains, but it can be redefined from a quality perspective by grouping portions according to binding domains, spectrin-like repeat number and phasing, and hinge domains (Fig. 1). By viewing dystrophin restoration from this quality perspective, the predicted function of a non full-length dystrophin is strongly driven by these quality attributes. Further, it raises additional questions around dystrophin quantity in the context of quality, such as whether only small amounts of dystrophin may be needed to improve function if the quality of the shortened dystrophin is high.

1.3. Maximizing function through rational design: using human data to guide pragmatic design of highly functional microdystrophins

Based on BMD and transgenic mdx data, rational design of a highly functional microdystrophin requires incorporation of the essential N-terminal and cysteine-rich domains along with a partial rod domain. An important distinction that should be kept in mind when drawing conclusions from BMD patient and transgenic mice data is that truncated dystrophin has been expressed from birth, which is very different than restoring a truncated or shortened dystrophin protein using exon-skipping or AAV approaches. As such, restoring truncated dystrophins to heavily damaged muscle may have less of an impact on phenotype compared to patient data. Nevertheless, patient data can still be an informative approach as it can identify portions of the protein that are associated with clinical benefit for dystrophin restoration.

In BMD, mild phenotypes can exist with major modifications to the spectrin-like repeats (SRs) of the rod domain. SRs are domains composed of three α-helices that have both structural and signaling functions [64]. In general, SR size or length of the rod region has been viewed as an important trait for maintaining protein function under the simple spacer hypothesis. Under this hypothesis, many exon-skipped proteins should have greater function when compared to microdystrophin proteins as they often have a longer rod region that more closely mimics the full-length protein. However, more literature in recent years has suggested that SRs regulate function by maintaining protein stability through SR phasing and by preserving cell signaling through binding domains. The discovery of SR phasing and binding domains challenges the simple spacer hypothesis, as well as raises questions around the degree of contribution of each component – SR length, phasing or binding domains – on protein function. To further investigate this, BMD patient data can be informative, but many semi-functional dystrophins that are expressed in BMD muscle are predicted to have better function than any restorative dystrophin therapy as it is have been expressed from birth, assuming preservation of critical subdomains. Additionally, the correlation between BMD protein abundance and clinical course is not well developed with some studies suggesting that quantity is associated with better function [18,65], while others claim that there is a threshold effect [21].

The contribution of SR length was first characterized by discovery of very large genomic deletion (Δexon17–48) that led to an extremely mild BMD phenotype in a family segregating with this mutation [66]. This mutation illustrated that dystrophins can be smaller and still maintain functional capabilities, paving the way for exon-skipping and AAV-microdystrophin therapies. However, upon further study in transgenic mice, it was discovered that the Δexon17–48 shortened dystrophin required greater quantities of protein expression to reach similar functional capabilities as full-length dystrophin [33,67]. This difference was attributed to stability as the Δexon17–48 protein localized correctly in muscle according to immunofluorescence, but was enriched in the cytosolic fraction, while full-length protein was enriched in the microsomal or membrane fraction [37]. As the exon-intron structure of the dystrophin gene became delineated and was compared with the coding regions for individual SRs, it became clear that some deletions, such as Δexon17–48, generated proteins with partial SRs in their sequence, which affected folding and stability (i.e. SR phasing was disrupted) (Fig. 2A) [32].

SR phasing is dictated by the spectrin-like repeats that make up the rod domain [62,68]. Although each repeat has low sequence similarity, they all contain highly conserved hydrophobic

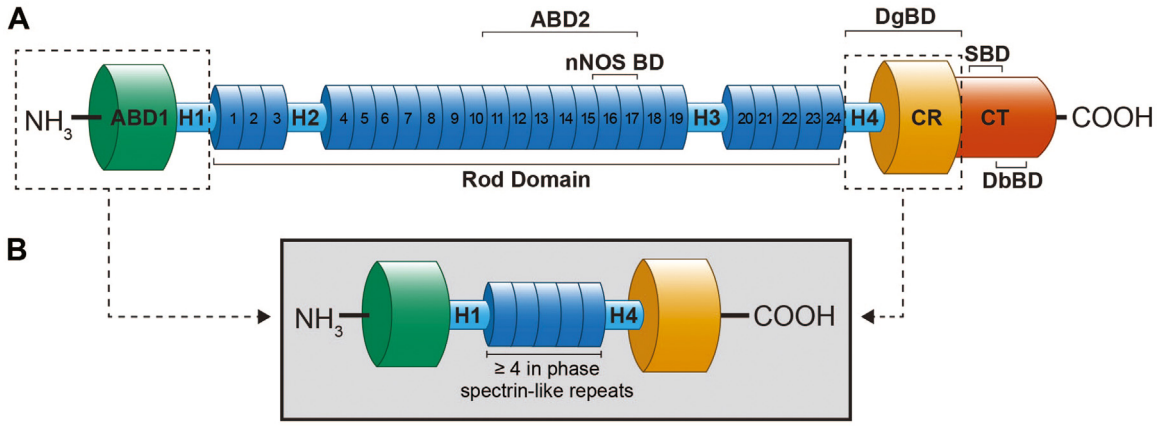


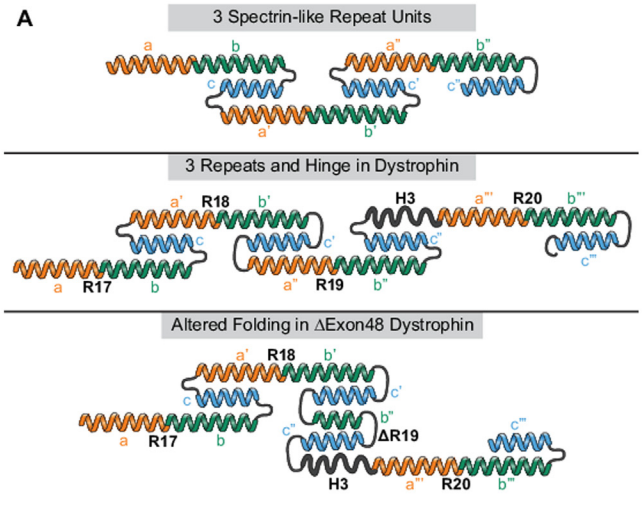
Fig 1. (A) Full-length dystrophin protein includes the N-terminal actin binding domain, 4 hinges, 24 in phase spectrin-like repeats, cysteine rich dystroglycan binding domain, and the C-terminal domain. (B) Essential elements for dystrophin functionality are shown including the N-terminal actin binding domain, hinges 1 and 2, at least 4 in phase spectrin repeats and the cysteine rich dystroglycan binding domain. The rational design of microdystrophin proteins allows for retention of portions that ensure functional properties. ABD1= Actin binding domain 1, ADB2 = Actin binding domain 2, nNOS BD= neuronal nitric oxide synthase binding domain, DgBD = dystroglycan binding domain, SBD = syntrophin binding domain, DbBD = dystrobrevin binding domain.

heptad repeat motifs characterized as an alpha-helical coiled coil conformation [62,68–71]. This conformation is thought to be important for protein folding into triple helical structures as the heptad motif localizes hydrophobic residues appropriately and allows for dynamic properties of wild type dystrophin to be retained [45]. Importantly, each spectrin-like repeat is typically encoded on 2 exons, and individual SRs interdigitate with adjacent SRs [62,71,72]. When odd (i.e. 1, 3, 5) numbers of exons are removed by deletions in the dystrophin gene, the various heptad repeats are not able to align and fold into a normal triple helical structure, resulting in protein structural abnormalities that affect stability and function [32,45,71,73,74].

The importance of this phasing was first demonstrated preclinically when the Δexon17–48 protein, which models an out-of-phase repeat, was tested head-to-head against proteins that maintained SR phasing (Fig. 2) [32]. These findings indicated that SR phasing was a variable that affected function and prompted scientists to improve the design of the Δexon17–48 protein. This resulted in multiple rod domain microdystrophin iterations with some having no function, illustrating that not all SR combinations function equally [32,75]. A key finding from these preclinical studies was that a minimally sized rod domain was required for function [32]. This replicated clinical findings where large in-frame deletions of the rod domain (>36 exons) have been associated with DMD phenotypes [23,76]. On the other hand, there have been extremely mild BMD patients described with deletions of up to 35 exons in this region [23,66]. This rod length relationship to phenotype required additional investigation as it raised the question of why severity greatly increases once a deletion threshold is reached. Additionally, duplications, which made the rod domain longer, can cause disease, pointing to other factors besides the overall rod domain length [16,18]. It is difficult in the clinical setting to rigorously interrogate these questions due to the small number of patients that fit these criteria. The limited data available suggest that important quality attributes of dystrophin are less likely to be retained if the deletion exceeds a certain size. However, phenotype is also likely affected by whether deletions or duplication remove or add entire SRs or partial SRs as each SR is encoded on more than one exon. Preclinical work has found that four to five spectrin-like repeats are sufficient to mitigate the dystrophin pathology and improve function [32,54], but an advantage of these studies allows for control over these quality attributes that may not be retained in BMD and DMD patients, such as by including full SRs rather than partial SRs (i.e. maintaining phasing) (Fig. 2). The rod length in relation to phenotype points to a potential symmetry argument that SR phasing can affect function as patients with sizeable deletions (>30 exons) in the rod domain can present with either severe or mild phenotypes [23]. Clinically, when BMD patients are stratified based on the conformation of repeat arrays, in-phase repeats were associated with a slower disease progression. For example, the age of onset for dilated cardiomyopathy was found to be delayed by 10 years (p = 0.003) if spectrin-like repeats were kept in-phase [73]. This work was validated in a different cohort to show similar trends with delay in cardiomyopathy, as well as a delay in loss of ambulation, and clearly links the functional quality of the shortened dystrophin to improvements in patient quality of life [45]. However, this study points out that other factors in combination with repeat phasing are important drivers of protein quality and function [45]. Interestingly, while exon skipping therapies do not always maintain proper phasing, synthetic dystrophins such as microdystrophins are designed specifically to maintain normal phasing (Fig. 2B, C) [32,57]. Therefore, while dystrophin produced through exon-skipping produces larger dystrophin protein than microdystrophin, the quality and functionality of that dystrophin is not consistent or guaranteed.

Additional dystrophin domains that need to be considered for understanding dystrophin quality are the four major hinges (Fig. 3). Hinges can also affect dystrophin quality through assistance in mechanical properties, such as flexibility or stability during contraction or relaxation of muscle fibers [62,77]. Hinges 1 and 4, which flank the rod domain, are thought to be essential as they provide sufficient physical flexibility of the protein and link the N-terminal and the cysteine-rich domains to the rod domain. Consequently, they are included in all microdystrophin construct designs currently being tested in clinical trials [54]. Within the rod domain are the hinge 2 and 3 regions. These two hinge regions have been shown to be strong drivers of function in preclinical models [32,57,77], while the presence of hinge 3 has been associated with milder phenotypes in BMD patients [78]. Further, *in silico* protein hydrophobicity profiles identified the hinge 3 as an important region within full length dystrophin protein that was associated with more BMD-like than DMD-like phenotypes when disrupted [79]; although, the majority of in-frame deletions in DMD tend to incorporate larger deletions of the gene which can confound the results [23,76] At least two smaller hinges have also been identified by alignment of the 24 SR domains [71]. This enabled incorporation of one of these small hinges in the μDys5 protein, which lacks hinges 2 & 3 [57]. A

A



B

Exon #	Mutation	SR Phasing Maintained?	Patients Reported in Database (n)	Percentage
44	del 43-44	Yes	1	0.16
	del 38-44	No	1	0.16
	del 42-44	No	1	0.16
45	del 45-48	Yes	173	27.46
	del 45-51	Yes	11	1.75
	del 45-53	Yes	42	6.67
	del 45-47	No	251	39.84
	del 45-49	No	55	8.73
50	del 50-51	No	3	0.48
	del 50-53	No	3	0.48
51	del 45-51	Yes	11	1.75
	del 49-51	Yes	5	0.79
	del 48-51	No	15	2.38
	del 50-51	No	3	0.48
	del 51-52	No	2	0.32
52	del 51-52	No	2	0.32
53	del 45-53	Yes	42	6.67
	del 48-53	No	5	0.79
	del 50-53	No	3	0.48
55	del 48-55	No	1	0.16
		Totals	630	100

*Exon-skipped proteins for del 18-45, 40-44, 44-45, 44-54, 47-51, 47-53, 47-55, 49-53, 49-55, 50-55, 52-53, 52-55, 54-55, 55-56, 55-62 had 0 patients reported in database

C

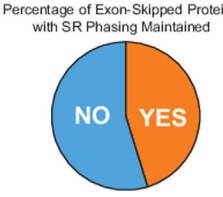


Fig 2. (A) Individual SRs can couple with adjacent SRs to form a repeat (depicted as portion a, b, c) with an alpha-helical coiled coil conformation. For some in-frame deletions, SR phasing may not be maintained. For example, the Δexon48 deletion results in a partial SR that disrupts correct protein folding and impacts the stability of the protein. (B) Analysis of SR phasing in exon-skipped proteins from The eDystrophin database (edystrophin.genouest.org). (C) Pie chart from Fig 2B denotes that 45% of exon-skipped proteins are predicted to conserve SR phasing. Not all exon-skipped proteins will result in proteins that have SR phasing maintained, which will impact the stability and functionality of the protein.

comparison of μDys proteins carrying the nNOS domain plus or minus hinge 3 revealed subsets of functional advantages with each construct [57,80]. However, adding both the NOS domain and hinge 3 creates size issues that impact the ability to produce high titer vectors. Consequently, it was concluded that the mini-hinge upstream of SR16 effectively substitutes for hinge 3 [57].

In addition to hinge regions, another important factor found to drive dystrophin quality is binding domains. Binding domains are located throughout the dystrophin protein [81] and include the neuronal nitric oxide synthase (nNOS) domain, a second actin binding domain, and domains for binding membrane lipids, microtubules, and synemin [26,80,82–87]. Overall, these domains have been discovered within a laboratory setting and have been studied experimentally, with little patient data describing their necessity for function, apart from the nNOS domain [88]. The nNOS domain has been shown to have potential impacts on patient function [89] as it is needed for localization of nNOS to dystrophin and is thought to prevent functional ischemia through enhancing blood flow to the muscle during activity [80,90–92]. When differentiating in-frame deletions from DMD and BMD phenotypes, more patients with deletions in the nNOS domain had a DMD phenotype, suggesting that lack or disruption of this domain may have a greater impact on disease severity. However, as mentioned above, these data are difficult to interpret as larger deletions tend to be associated with DMD phenotypes [23,76,79]. To further investigate the impact of the nNOS domain, comparisons in BMD phenotypes would be more informative and have been previously described elsewhere [89]. Interestingly, BMD patients with mutations in exon 44–45, which encodes for the nNOS domain, were documented to have the most variable phenotypes [18]. Given that differences in mutation breakpoints within this region can result in the disruption of the binding domain in different ways, and that such deletions often lead to unpredictable pre-mRNA splicing, multiple iterations of the binding domain can be formed and may help to explain the wide phenotypic variability [82,93].. These patient data highlight that some dystrophin domains enhance overall function but are not absolutely necessary for basic protection from necrosis, while illustrating that retention of binding domains within dystrophin may be an important driver for function.

1.4. Selection and characterization of the microdystrophins in clinical trials: using rational design to maximize function and predict clinical benefit

The characterization of the dystrophin protein structure and functional domains was the foundation for the optimization of microdystrophin's functional capabilities, illustrating that it is essential to retain certain quality attributes to produce a functional dystrophin [75]. Taken together, these studies showed that indispensable portions for function include the N-terminal actin binding domain, the dystroglycan binding cysteine-rich domain, hinges 1 and 4, and a rod domain with at least 4 spectrin-like repeats that are in-phase or model a hybrid repeat [18,23,32,45,54,73,74,79]. Function can be further maximized with addition of an internal hinge and the nNOS domain (Fig. 3) [57,62,77–80,89–92]. One major difference between exon-skipped and AAV truncated dystrophins is that skipped products will carry a full C-terminus, which will allow for slightly different ratios of syntrophin and dystrobrevin to associate with the DAPC. However, a functional consequence of altered ratios of syntrophin and dystrobrevin isoforms within the DAPC has not been observed [29,32,58] As microdystrophins have been shown to also improve cardiac function, more data are needed to draw conclusions on these differences between skipped semi-functional and microdystrophin.

Microdystrophins delivered by AAV have optimized quality through rational design. This approach allows for retention of portions that improve function, while limiting size through removal of nonessential regions. Further, it maximizes function because it relies on both preclinical and patient data that have demonstrated clinical benefit. In contrast, exon-skipped proteins will depend on the patient mutation, such that phasing of spectrin-like repeats and inclusion/exclusion of hinge regions and the nNOS domain may not be maintained. As such, internally deleted proteins formed by exon-skipping are not guaranteed to contain

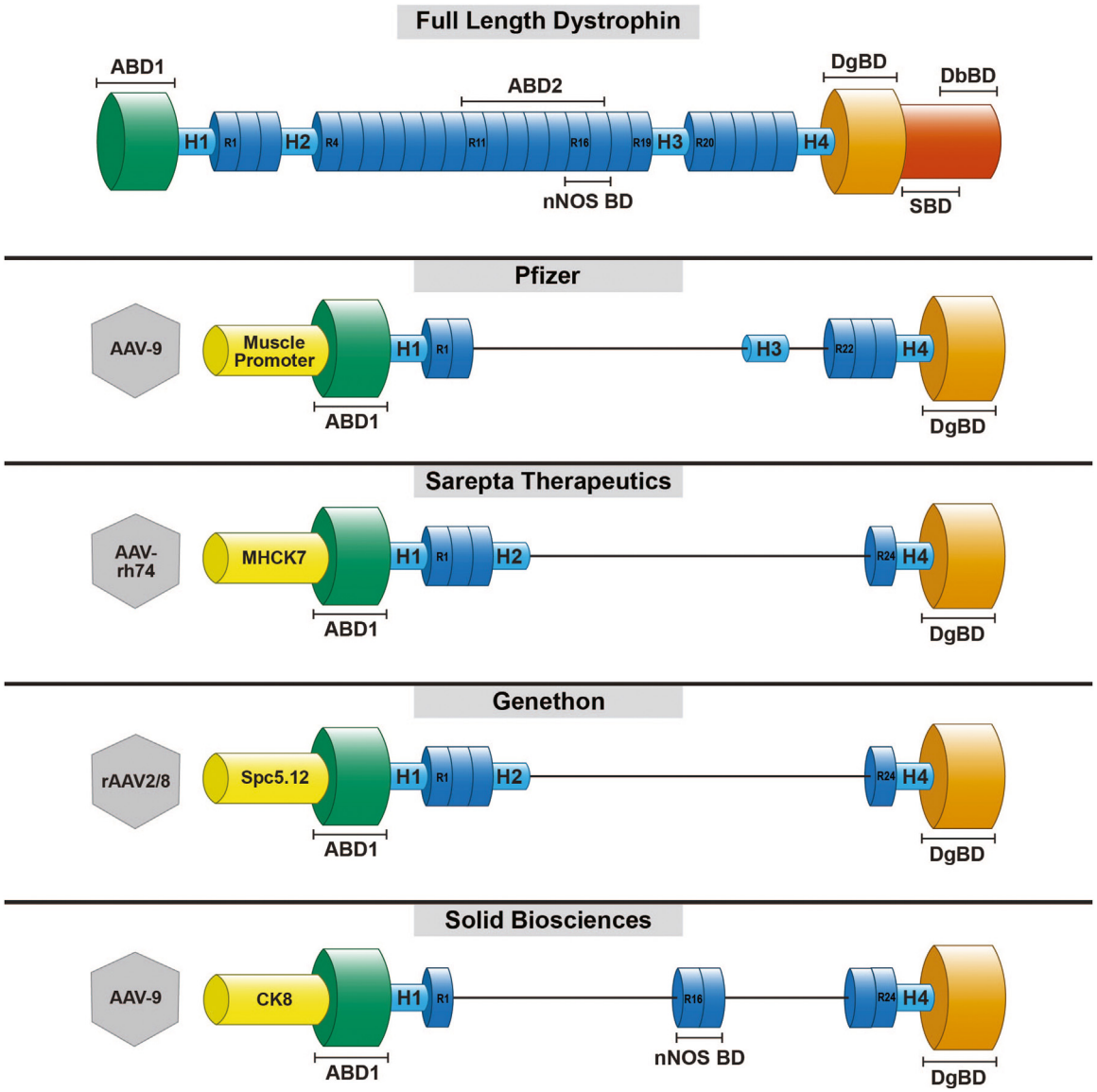


Fig 3. Comparison of current microdystrophin constructs in clinical trials show that each protein contains key quality attributes that have been demonstrated to provide functional qualities in both preclinical models and patient mutation data.
Table 2 [95,98,99,101–105].

the domains needed to maximize function. From this perspective, microdystrophins have the potential to be a reasonably likely surrogate markers as the dystrophin protein structure has been optimized from human data and confirmed through in vivo models to ensure that function is not only retained but maximized (Table 1). Furthermore, these synthetic microdystrophin genes have a precise structure that is not affected by mutations in individual patients.

Currently there are four different microdystrophins that have been advanced to clinical testing (Table 2). All of these microdystrophins contain the key elements for functionality (Fig. 1). One of these clinical programs developed by Bamboo, which was later acquired by Pfizer, advanced a microdystrophin termed Δ3990 or "Hinge 3″. This construct contains 5 spectrin-like repeats that maintained correct phasing in addition to the hinge 3 domain [54]. Functional improvements were confirmed in *mdx* mice and preliminary clinical data show functional improvements in DMD patients [54,94]. Two additional programs led by Sarepta and Genethon have a microdystrophin, termed ΔR4–23/ΔC or "Hinge 2″, which shows robust changes in preclinical models as well as suggestive preliminary data that it also improves function in patients [95–97]. These functional changes can be attributed to 4 in-phase spectrin-like repeats in combination with hinge 2. However, during development and testing of this construct, it was noted that it seemed to be associated with lower muscle mass in *mdx* mice and further investigations at the molecular level revealed that it caused the formation of ringed fibers around the sarcolemma that were associated with myotendious junction tears and NMJ fragmentation [32,77]. To date, there has been no clinical evidence of this in DMD patients, although these ultrastructural abnormalities are difficult to detect, and clinical evaluation of both constructs is ongoing in Phase 3 trials.

To further improve functionality, second generation constructs were made that not only considered the number and phasing of spectrin-like repeats, but also incorporated the nNOS domain to counteract muscle ischemia [57,80]. Incorporation of the nNOS domain involved the removal of both hinge 2 and 3 regions to

Table 2
Summary of Clinical Findings from DMD patients treated with rAAV-microdystrophin.

Sponsor	Drug	Key Efficacy Results Disclosed	Anti-Transgene Related Serious Adverse Events	Citation
Pfizer	PF-06,939,926 (fordadistrogene movaparvovec)	Mini-dystrophin expression and membrane localization	Muscle weakness	[98,99,101]
		NSAA improvement	Myocarditis	
Sarepta Therapeutics	SRP-9001 (delandistrogene moxeparvovec)	Microdystrophin expression and membrane localization	Immune myositis	[95,99,102,103]
		Restoration of β-sarcoglycan	Myocarditis	
		NSAA improvement		
Genethon	GNT 0004	N/A	Muscle weakness	[99,104]
Solid Biosciences	SGT-001 (zildistrogene varoparvovec)	Microdystrophin expression and membrane localization	N/A	[105]
		Restoration of β-sarcoglycan		
		Restoration of nNOS		
		NSAA improvement		

accommodate size for packaging into an AAV capsid, but with inclusion of a smaller mini-hinge upstream of SR 23. In preclinical models, second generation constructs were highly associated with the microsomal fraction, which shows major improvements over the initial testing of the Δexon17–48 protein [80]. Further, second generation constructs also increased function in preclinical models through improved force development and sarcolemmal localization of nNOS with clinical investigation as SGT-001 by Solid Biosciences currently in progress [57]. Importantly, all three microdystrophins currently in clinical trials maintain the quality attributes that have been defined to maximize function. As such, patients that receive a microdystrophin will be receiving a protein with high quality that has been optimized to ensure that function is maximized and likely to predict clinical benefit. However, one important consideration that needs to be further explored for all dystrophin restoration trials is the impact of anti-transgene related safety events seen in a subset of patients with certain mutations and therapeutic ways to mitigate those responses [98,99].

2. Conclusions

Both exon-skipping and gene therapy approaches aim to restore missing dystrophin protein, the primary genetic defect of DMD, and therefore using restored mini-dystrophins as a reasonably likely surrogate endpoint within the accelerated approval program would be advantageous. To date, exon skipped dystrophins have been accepted by the FDA to likely predict clinical benefit as several accelerated approvals have been based on exon-skipped dystrophins. Based on the data presented in this review, the rational design of dystrophin quality is a key driver of overall protein function. However, the design and quality of the dystrophin protein is currently not part of the endpoint biomarker evaluation. Microdystrophins are predicted to be highly functional as they have the required domains characterized from human data and are confirmed through in vivo animal model studies. Therefore, the rational design of microdystrophins positions them as a strong surrogate candidate for accelerated approval.

To further explore the functional benefit of microdystrophins, two clinical programs lead by Pfizer and Sarepta have initiated Phase 3 clinical trials that will test the functional efficacy of microdystrophin in randomized placebo-controlled studies. These traditional designs, while time-consuming, will acquire important data that could formally validate the functional correlation to microdystrophin protein expression, thereby enabling the future use of microdystrophin as a surrogate endpoint for accelerated approval and ultimately giving hope to patients and families with limited treatment options.

Declaration of Competing Interest

Jessica F Boehler, Kristy J Brown, J Patrick Gonzalez, Roxana Donisa Dreghici, Meghan Soustek-Kramer, Sharon McGonigle, Annie Ganot, Timothy Palmer, Caitlin Lowie, and Carl A Morris are employees and hold equity from Solid Biosciences, Inc.

Michael W. Lawlor is the founder, CEO, and owner of Diverge Translational Science Laboratory. MWL is or has recently been a member of advisory boards for Solid Biosciences, Taysha Gene Therapies, Astellas Gene Therapies (formerly Audentes Therapeutics), and Ichorion Therapeutics. MWL is also a consultant for Astellas Gene Therapies (formerly Audentes Therapeutics), Encoded Therapeutics, Modis Therapeutics, Lacerta Therapeutics, Dynacure, AGADA Biosciences, Affinia Therapeutics, Biomarin, Locanabio, and Vertex Pharmaceuticals. MWL receives research support from Astellas Gene Therapies, Solid Biosciences, Kate Therapeutics, Prothelia, Cure Rare Disease, Rocket Pharma, Taysha Gene Therapies, and Ultragenyx.

Margaret Beatka is an employee of Diverge Translational Science Laboratory.

Jeffrey S. Chamberlain is a member of the scientific advisory board for Solid Biosciences and an equity holder of Solid Biosciences. JSC is an inventor on several patents related to microdystrophin design.

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