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REVIEW

Targeting IRES-dependent translation as a novel approach for treating Duchenne muscular dystrophy

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ABSTRACT

Internal-ribosomal entry sites (IRES) are translational elements that allow the initiation machinery to start protein synthesis via internal initiation. IRESs promote tissue-specific translation in stress conditions when conventional cap-dependent translation is inhibited. Since many IRES-containing mRNAs are relevant to diseases, this cellular mechanism is emerging as an attractive therapeutic target for pharmacological and genetic modulations. Indeed, there has been growing interest over the past years in determining the therapeutic potential of IRESs for several disease conditions such as cancer, neurodegeneration and neuromuscular diseases including Duchenne muscular dystrophy (DMD). IRESs relevant for DMD have been identified in several transcripts whose protein product results in functional improvements in dystrophic muscles. Together, these converging lines of evidence indicate that activation of IRES-mediated translation of relevant transcripts in DMD muscle represents a novel and appropriate therapeutic strategy for DMD that warrants further investigation, particularly to identify agents that can modulate their activity.

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1. Introduction

The regulation of protein translation is the basis for maintenance of homoeostasis for many cellular mechanisms. In some disease states and under stress conditions, classical cap-dependent protein synthesis can be halted or down-regulated. Thus, alternative translational mechanisms become activated to promote protein production. One such mechanism is the internal ribosome entry site (IRES)-driven initiation. Regulation at the initiation translation step permits a rapid response to changes in cellular physiological conditions. Internal ribosome entry mechanisms were first discovered in encephalomyocarditis virus (EMCV) and Poliovirus [1,2]. Following this discovery, many more viral IRES-harbouring mRNAs were identified along with several cellular mRNAs [3–6]. Previous studies have shown that ~10% of the total mRNA pool within given cells are predicted to contain IRESs. However, many of these remain to be identified [7].

IRES-containing cellular mRNAs are found to be preferentially translated under various conditions that cause inhibition of cap-dependent initiation including for example, cellular stress, episodes of hypoxia, nutrient limitation, pharmaceutical stimulation, cell cycle or differentiation [4,8]. Of particular interest is the fact that IRESs can also be activated under disease conditions thereby revealing their therapeutic potential. Indeed, the development of new methods to target IRES-mediated translation of disease-relevant transcripts via pharmacological interventions, small molecules or genetic manipulation, is gaining attention in order to create novel therapies for a variety of diseases [9–13]. Up until now, the most commonly targeted disorders through IRES regulation are: i) infectious diseases which use small inhibitors to reduce viral activity [12,13]; and ii) tumorigenesis via targeting cellular IRESs within oncogenes, growth factors and programmed cell death regulators [10,11,14]. As the discovery of more cellular IRESs emerged in recent years, IRES-harbouring mRNAs involved in other disorders have also been investigated for their therapeutic potential [15]. Among these, targeting IRES-mediated translation in Alzheimer's disease [16,17], Parkinson's disease [18–20], and neuromuscular diseases [21–25] has arose as a novel line of work for therapeutic intervention.

In the context of neuromuscular diseases, many laboratories including our own have been interested in identifying therapeutic strategies for Duchenne muscular dystrophy (DMD) [21–23,26–41]. Recently, a novel IRES was identified in utrophin A mRNAs and considerable efforts were focused in characterizing mechanisms of activation [21–23,27]. Utrophin A is highly relevant for DMD given that it can serve as a surrogate for the missing dystrophin protein in DMD muscles [42,43 and see details below]. Moreover, scanning of the available literature reveals that several other DMD-relevant transcripts also contain IRESs which make them similarly important for developing appropriate DMD therapies based on IRES-mediated translation [24,44–47]. Here, we review our current knowledge of DMD-relevant transcripts containing IRESs and discuss their therapeutic potential for improving sarcolemmal integrity, muscle regeneration and repair as well as angiogenesis in dystrophic muscle. Collectively, this body of work leads us to propose IRES-

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mediated translation as a key cellular mechanism amenable to appropriate therapeutic interventions for treating DMD.

2. Mechanisms for initiating translation

There are two main mechanisms known to promote translation of mRNAs, namely, cap-dependent and cap-independent translation. Both share similarities in that they each involve four distinct stages: initiation, elongation, termination and ribosomal recycling. Many excellent reviews describe in detail the fundamental regulatory checkpoints of translation (see, for example, Shatsky et al. [48] Yang et al. [49], Shirokikh and Preiss [50]). Therefore, only a summary is provided below.

Cap-dependent translation is the most conventional mechanism to translate mRNAs into functional protein products. The indispensable step for cap-dependent translation initiation of all eukaryotic mRNAs, is the association of the eukaryotic initiation factor (eIF) 4 F protein complex to the 7-methylguanylate cap (m^7G), also known as the 5′cap. Before this binding, a series of eIFs form a pre-initiation complex. eIF4E is bound by the scaffolding protein eIF4G and the RNA helicase eIF4A. This complex forms eIF4F which is important for 40S recruitment to the 5′end of the target mRNA. Additional initiation factors, including eIF3, which recruit the small ribosomal submit to the 5′ end of the mRNA as well as eIF2-GTP that delivers the first transfer RNA (tRNA methionine) to the 40S ribosome, are involved in translational initiation. The poly(A) binding protein (PABP) associates with eIF4F and binds to the poly(A) tail of the mRNA to promote its circularization to facilitate translation [51–53]. Cap-dependent translation can be repressed in a variety of ways including for example: i) through the cleavage of eIF4G by viruses and during apoptosis, thereby preventing binding of eIF4E to the m^7G cap; ii) phosphorylation of eIF2 that circumscribes recruitment of tRNAs to the ribosome; and iii) increasing the eIF4E-binding protein (4E-BP) which inhibits eIF4E's ability to bind eIF4G [54–57]. In fact, many physiological, pathological and pharmacological conditions such as during the cell cycle, hypoxia, apoptosis, drug treatments as well as in different disease states, may lead to inhibition of cap-dependent translation and usage of an alternate translation mechanism, i.e., IRES-mediated translation [4,24,27,48,49,58].

A rising number of mRNAs are believed to possess IRESs which permits translation of a transcript independently of its 5′ end cap. Conventional scanning from the 5′ end is likely not efficient for most IRES-containing cellular mRNAs because their 5′UTRs are typically long, GC-rich and highly structured [48,49,59]. In cap-independent translation, the ribosome interacts with the IRES with the help of some but not all canonical initiation factors, and allows for continued and/or enhanced expression of proteins when cap-dependent translation is suppressed [4,48,59]. In fact, IRES elements are believed to participate in various interactions with components of the translational machinery including the canonical initiation factors, IRES trans-acting factors (ITAFs) and the 40S ribosomal subunit [4]. ITAFs are suggested to increase the binding affinity between IRESs and canonical initiation factors as well as with the ribosome. ITAFs have been hypothesized to act as RNA chaperones to help the IRES primary sequence attain the appropriate conformational state to promote ribosome binding [60]. Interestingly, it has been reported that some IRESs require a combination of two or three ITAFs to reach efficient translational activity [61–64]. Examples of some of the most studied ITAFs include the human La autoantigen (LA), upstream of N-ras (Unr), poly-(rC) binding protein-2 (PCBP2) and polypyrimidine tract-binding protein (PTB) [62–73]. However, many more ITAFs have so far been identified with one or multiple targets. IRES activity is regulated positively or negatively by ITAFs and their relative cellular abundance, as well as, according to tissue specificity and exogenous stimuli [74–76]. Previous reports also demonstrate that the proteins controlling IRES-dependent translation initiation are modulated by their subcellular localization [76]. However, the precise mechanisms regulating protein translation by IRES are still largely unknown and requires additional studies.

3. Therapeutic potential of IRES-mediated translation in Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is caused by mutations in the gene that encodes dystrophin, a key protein to maintain the structural integrity of muscle fibres. DMD is one of the most common inherited paediatric neuromuscular diseases and accounts for over 80% of cases of muscular dystrophies around the world [77]. DMD patients suffer from severe muscle degeneration which results in muscle weakness, respiratory impairment, and cardiomyopathy [78–80]. Skeletal muscle fibres of these patients undergo cycles of degeneration and regeneration provoking a devastating domino effect of secondary symptoms such as surges in calcium uptake, critical inflammation and functional ischaemia [78,81,82]. Eventually, the ability of muscle fibres to regenerate fibres runs out leading to the replacement of muscle by adipose and connective tissues and, consequently, loss of muscle strength and function. As the disease progresses, DMD patients suffer from ambulation impairments at approximately 12 years of age and, typically, they will completely lose ambulation in their late teens [77,83]. Assisted ventilation becomes necessary usually around the age of 20 [80,83,84] and death occurs due to respiratory and/or cardiac complications when patients approach their thirties [78,80,85].

The dystrophin gene located on chromosome Xp21.1 contains 79 exons and encodes the 427-kDa dystrophin protein. The DMD gene produces different transcripts encoding various dystrophin isoforms (Fig. 1) that are generated by tissue-specific promoters (brain, muscle and Purkinje cells), alternative splicing and polyA-additional sites [86–92]. Mutations in the *DMD* gene include deletions (65%-70%) around the 2 hot areas near the N-terminus (exons 3–7) and within exon 45–55 [93–95], duplications (6–10%), small mutations (10%), or point mutations (nonsense or missense) [96–98]. These mutations disrupt the translational reading frame of dystrophin, which may lead to severe Duchenne muscular dystrophy (DMD) with an unfunctional protein or result in a short

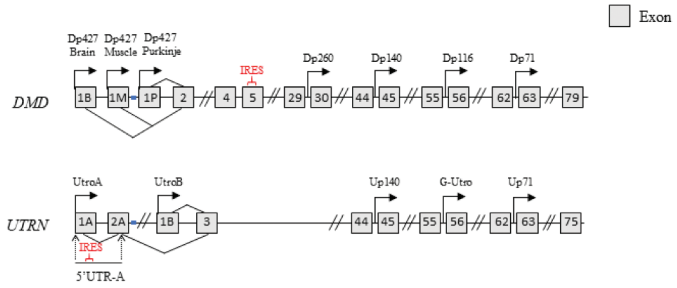

Figure 1. Utrophin and dystrophin genes. Dystrophin (*DMD*) and utrophin (*UTRN*) genes with associated promoters and exons. Full-length dystrophin transcription is controlled by 3 promoters (brain, muscle, Purkinje) [91,127,128]. Four alternate promoters result in short isoforms dp260, dp140, dp116, and dp71 [86,129,130]. An IRES is found in exon 5. Dp427 is the main variant produced in muscle [127]. The two isoforms of utrophin (A and B) have distinct promoters and 5′UTR regions [131,132]. Utrophin A is expressed primarily at the myotendinous and neuromuscular junction in mature skeletal muscle, in neurons, astrocytes as well as in the brain (choroid plexus and pia mater), whereas utrophin B is primarily localized in vasculature [131–135] The utrophin A transcript contains an IRES between nucleotides 71 and 152 in its 5′UTR [22]. The utrophin B promoter is located within the second intron of the utrophin gene resulting in a unique 5′ end [131,132]. Three short utrophin isoforms Up140, G-Utrophin and Up71 were reported similar to the dystrophin gene [129,130,136].

functional form of the dystrophin protein resulting in the mild disorder Becker muscular dystrophy (BMD) [77].

The dystrophin protein plays a critical role in supporting muscle fibre integrity by linking the intracellular actin network to the extracellular matrix [99,100] via binding through the dystrophin-associated protein complex (DAPC) at the muscle fibre membrane (also known as the sarcolemma) [101,102]. Such structural organization provides stability to the sarcolemma during repeated cycles of muscle contraction. In the absence of dystrophin, this physical link between the intra- and extracellular compartments of muscle fibres is lost, making the fibres highly susceptible to damage and hence, inducing degeneration. Many potential therapies for DMD have been investigated over the last years including, for example, cell therapy [103–107], gene editing [108–115], gene therapy [116–118], exon skipping [119–123] and suppression of premature stop codons [124–126]. An alternative strategy consists in utilizing a protein highly homologous to dystrophin and to induce its upregulation pharmacologically to compensate for the absence of dystrophin in muscle fibres. An ideal candidate for this role is utrophin A.

3.1. Targeting the utrophin A IRES for treating DMD

Utrophin A is an attractive candidate for treating DMD since it can functionally compensate for the absence of the dystrophin protein in dystrophic muscle. In normal mature skeletal muscle, utrophin A expression is restricted to the neuromuscular and myotendinous junctions. The upregulation of the utrophin protein from these sites to the entire muscle fibre membrane has been shown, to have beneficial effects on the performance, integrity and morphology of dystrophic muscle fibres. Thus, many laboratories have focused their efforts in increasing utrophin A expression through genetic or pharmacological stimulations as a therapeutic approach for the treatment of DMD [26,30–32,137–141].

3.1.1. Utrophin A as a surrogate for dystrophin

Similarities between utrophin A and dystrophin clearly highlight the potential of utrophin A to functionally compensate for dystrophin [142,143]. Utrophin A is expressed primarily at the myotendinous and neuromuscular junctions (NMJ), in neurons, astrocytes as well as in the brain (choroid plexus and pia mater) [131–135]. Utrophin A displays varying localization patterns in skeletal muscle through embryonic to adult development. In fact, utrophin A is expressed throughout the sarcolemma during embryogenesis, reaching maximal expression levels at approximately 17 weeks' gestation, and gradually decreasing to negligible levels at week 26, which coincides with increasing levels of dystrophin [144,145]. In adult tissues, utrophin A is mostly restricted to the NMJ but in regenerating muscles, utrophin A is expressed along the entire sarcolemma as a part of the repair process [146–148]. More interestingly, utrophin A is up-regulated in dystrophin-deficient muscle fibres and, in part, localizes to the sarcolemma [132]. Thus, these studies suggest that utrophin A plays a role similar to that of dystrophin during certain developmental stages of muscle fibres and could compensate for the loss of dystrophin in DMD.

Utrophin A has similar protein binding functions to dystrophin and has approximately 80% sequence homology in the actin-binding domain [43,142,149–152] and the carboxy terminus, including the cysteine-rich domain [142,149–151] (Fig. 2). In addition, utrophin A has been shown to associate to F-actin filaments with its N-terminal actin-binding domain and interacts with members of DAPC, notably α-syntrophin, β-dystroglycan and α-dystrobrevin-1 at its C-terminal area [153–157]. The major structural difference between dystrophin and utrophin A is in the rod domain at an identity less than 30%, where utrophin is missing repeats 15 and 19 as well as two hinge regions [142,157]. These differences could explain why utrophin is unable to perform all of dystrophin's

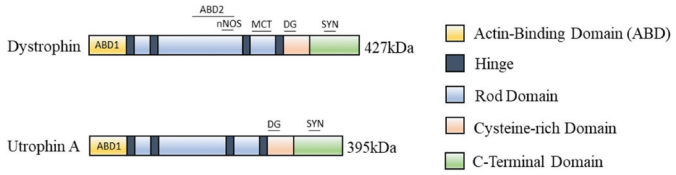

Figure 2. Utrophin A and dystrophin protein domains. Similar to full-length dystrophin, utrophin A can bind DAPC members at the sarcolemma of muscle fibres to provide a link between the ECM and the intracellular cytoskeleton. Utrophin A has a rod domain and four hinge regions (H1, H2, H3, and H4) and a cysteine-rich region (CR) that binds β-dystroglycan. Only the N-terminal domain (N) of utrophin A can interact with F-actin filaments [171]. The main difference between utrophin and dystrophin is the lack of the spectrin-like repeats 15 and 19, and 2 hinge regions [142,157]. Utrophin A and dystrophin associate with α-dystrobrevin, syntrophins α and β at their C-terminal domains [153–157]. Via their PDZ domain, the syntrophins recruit nNOS at the muscle fibre membrane to mediate blood flow to the muscle and this is also supported by dystrophin which binds to nNOS via its spectrin repeats 16 and 17 for proper localization to the muscle membrane [172]. However, utrophin A cannot recruit nNOS at the sarcolemma via direct binding like to dystrophin [161,162]. Additionally, utrophin A is unable to bind microtubules (MCT) to form an organized lattice at the sarcolemma [163–165].

functions. For instance, transgenic utrophin overexpression in mdx mouse models (an x-linked muscular dystrophy mouse mutant model widely used in DMD research) does not restore the localization of neural nitric oxide synthase (nNOS) to the sarcolemma of muscle fibres, which is the main source of nitric oxide in skeletal muscles and is indispensable for skeletal muscle integrity as well as its contractile performance [158–160]. Whereas dystrophin can bind nNOS to promote angiogenesis [161,162]. Additionally, unlike dystrophin, utrophin A is unable to bind microtubules to form an organized lattice at the sarcolemma which also protects against contraction-induced injury [163–165].

Despite these differences, increasing utrophin A expression throughout the sarcolemma of dystrophic muscle fibres has proven to be an efficient approach to compensate for the lack of dystrophin [139]. In dystrophic muscles, there is a slight increase in utrophin A at the sarcolemma, that occurs naturally as part of the repair process [146,166,167]. Transgenic overexpression of a truncated utrophin using the human skeletal α-actin (HSA) promoter improved the dystrophic muscle phenotype of mdx mice significantly. This includes a decrease in the serum levels of the enzyme creatine kinase (CK) that leaks out of damaged muscle, a reduction of centrally nucleated myofibers (indicative of fewer regenerating fibres), as well as a rescue of utrophin A and DAPC members at the sarcolemma [140]. Transgenic mouse models expressing full-length utrophin A elicited full recovery of mechanical functions of the diaphragm with only a 2-Fold induction of utrophin A as compared to the mdx control and with no adverse effects on non-muscle cells [139,168]. As little as a 1.5-fold increase of utrophin A can be beneficial as long as the localization of utrophin A is at the level of the sarcolemma in dystrophic muscle fibres [139]. Furthermore, a recent study showed that AAV9-mediated micro-utrophin expression can also strongly alleviate the cardiac defects of a more severe mdx mouse model [169]. Similar beneficial effects were also observed in the golden retriever muscular dystrophy (GRMD) dog model of DMD with AAV-mediated mini-utrophin gene transfer [170]. Thus, it appears that utrophin A is a suitable replacement for dystrophin and, accordingly, it represents an attractive therapeutic target for DMD.

3.1.2. Utrophin A 5′UTR contains an IRES

To date, transcriptional regulation of utrophin A has been the focus of most studies interested in upregulating its expression levels in dystrophic muscle [36,38,39,43,134,173–178]. However, over the past 15 years, a growing number of reports have highlighted the fact that regulation of utrophin A expression is also under the tight control of post-transcriptional events [33,34,179–182]. In this context, the notion of potential alternative translational regulation of utrophin A arose several years ago from studies conducted in our lab and that of others, showing elevated utrophin A protein levels in muscle, with little to no increase in mRNA expression [36,37,132,183,184]. The utrophin A protein of 395 kDa is encoded by a 13kb transcript which consists of 74 exons and is located on chromosome 6q24 [143,150]. Moreover, utrophin A has a long 5′UTR consisting of 560 nucleotides for humans (508 for murine), that expands from the transcriptional start site through exons 1A and 2A to the translation initiation site for utrophin A [131,134]. Similar to other IRES-containing mRNAs, the long utrophin A 5′UTR is predicted to have a high degree of secondary structure and is expected to be translationally repressed through both its 5′ and 3′UTR's [21,181,185]. The repression via the 3′UTR has been attributed to miRNAs and ARE-mediated degradation, but the exact inhibitory mechanisms that operate through its 5′-UTR remain nebulous [181].

Studies have shown that utrophin A can be translated through both cap-dependent and IRES-dependent translation [21–23,186]. With the use of a bicistronic reporter construct containing the utrophin A 5′UTR, Miura *et al.* demonstrated that in intact muscles, little utrophin A IRES activity is detected. However, a ~ 9-fold increase in reporter activity was observed in muscles subjected to degeneration and regeneration cycles by cardiotoxin injections, thus first revealing the possible cap-independent translational regulation of utrophin A in regenerating muscles [21]. Control experiments were performed in parallel to ensure that the bicistronic mRNA did not undergo aberrant splicing, and that the utrophin A 5′UTR did not contain cryptic promoter activity [21]. In addition, a study shows that glucocorticoids, the main intervention used for treating DMD patients [77,187,188], can drive IRES-mediated translation of utrophin A [22]. In fact, a 2–3 day treatment of C2C12 myotubes, an immortalized skeletal muscle cell line, with 6α-methylprednisolone-21 sodium succinate, promotes utrophin A IRES activation as detected using a bicistronic reporter assay [22]. Additionally, a polysome profiling assay demonstrates that the treatment with 6α-methylprednisolone-21 sodium succinate reduced global protein synthesis, while increasing the polysome association of the reporter mRNA harbouring the utrophin A 5′UTR IRES [22]. This indicates that the activity of this IRES can indeed be modulated pharmacologically. This work also suggests that the glucocorticoid corticosteroid, prednisolone, known for its anti-inflammatory actions [189,190], may promote combinatorial benefits in dystrophic muscle by reducing inflammation an increasing utrophin A expression in parallel.

To determine whether the utrophin A IRES displayed tissue-specific activity *in vivo*, another study aimed to generate transgenic mice harbouring control (CMV/betaGAL/CAT) or utrophin A 5′UTR (CMV/betaGAL/UtrA/CAT) bicistronic reporter transgenes. Examination of multiple tissues from two CMV/betaGAL/UtrA/CAT lines revealed that the utrophin A 5′UTR preferentially drives cap-independent translation of the reporter gene in skeletal muscles [23].

Since DNA-based bicistronic constructs used for IRES identification can sometimes give false positives, a study from Ghosh *et al.* took a different approach to confirm the presence of an IRES in the mouse utrophin-A 5′UTR with an alternate strategy [186]. In this study, the relative contribution of cap-independent and cap-dependent translation with the mouse utrophin A 5′UTR was compared through an m7G-capped and A-capped mRNA transfection-based reporter assay. As A-capped mRNA is poorly translated through cap-dependent events, its expression provides reliable estimation of cap-independent translation. The results confirm that

indeed utrophin A 5′UTR can be translated through a cap-independent manner but also demonstrate that the 5′UTR plays a key role in the inhibition of cap-dependent translation of utrophin A [186].

3.1.3. eEF1A2 regulates utrophin A-IRES activity

Recent work using RNA-affinity chromatography, identified the elongation factor 1A2 (eEF1A2) as a putative ITAF. The initial results showed that eEF1A2 associates to the 5′UTR of utrophin A and mediates utrophin A's IRES-dependent translation in skeletal muscles [23]. In addition, ectopic expression of eEF1A2 in tibialis anterior muscle of mdx and wild-type mice results in upregulation of utrophin A protein levels and utrophin A IRES-activation in CMV/betaGAL/UtrA/CAT bicistronic reporter harbouring transgenic mice [27]. These data are in agreement with a recent study reporting that another elongation factor, eukaryotic elongation factor 2 (eEF2), acts as an ITAF to regulate IRES-mediated translation of XIAP and FGF-2 mRNAs [191].

There are two eEF1A isoforms: eEFlAl and eEFlA2 which share ~92% sequence identity [192]. Both eEF1A isoforms function in regulating transport of aminoacyl-tRNA throughout translation elongation, however, eEF1A2 also plays a role in skeletal muscle, where it prevents apoptosis [193]. Several other functions have also been attributed to eEF1A including interaction with the cytoskeleton, binding and bundling of actin filaments, as well as inducing a severing effect on microtubules [194,195]. In contrast to eEF1A1 that is generally expressed ubiquitously, eEF1A2 is preferentially expressed in skeletal muscle, heart and brain [196,197], thereby suggesting that eEF1A2 could indeed play a distinct role in skeletal muscle. Given these observations, it seemed important to identify pharmacological compounds that target eEF1A2 in order to increase endogenous levels of utrophin A expression, via IRES-mediated translation. Such an approach could serve as a drug-based therapy to treat DMD, which offers several advantages over other methods including, ease of administration, positive impact on all skeletal muscles, and applicability to all DMD patients regardless of their dystrophin mutation.

With this in mind, a recent study set-out to identify and characterize FDA-approved drugs that target eEF1A2 and cause upregulation of utrophin A in skeletal muscle. Using FDA-approved drugs has the distinct benefit of potentially greatly accelerating translation of the findings into the clinical setting. Using an in-cell ELISA-based high-throughput screening approach, 11 FDA-approved drugs were identified that significantly and concomitantly raised protein expression levels of eEF1A2 and utrophin A in skeletal muscle cells. Among these drugs, many had common therapeutic roles including anti-diabetic, anti-peptic ulcer, cholesterol-lowering and beta-adrenergic blocking agents [27]. Five lead drugs (Acarbose, Betaxolol, Labetalol, Pravastatin, and Telbivudine) were able to stimulate reporter activation from a bicistronic reporter construct harbouring the utrophin A 5′UTR IRES, showing that, indeed, these two drugs act via IRES-mediated regulation of utrophin A translation.

This work focused on the two most promising candidates, the selective beta-1 adrenergic blocker Betaxolol, and the cholesterol-lowering agent Pravastatin. A 7-day treatment of transgenic mice containing the bicistronic utrophin A 5′UTR IRES reporter construct with Betaxolol or Pravastatin, demonstrated the potential of both drugs to activate utrophin A IRES-dependent translation *in vivo*. Chronic treatment of mdx mice with either of these two drugs resulted in increased expression of utrophin A via IRES activation and, localization of utrophin A at the sarcolemma of muscle fibres, while causing significant beneficial effects on the dystrophic phenotype. In fact, a grip strength assessment and *ex-vivo* force drop analysis demonstrated that the 4-week treatment with Betaxolol and Pravastatin significantly improved muscle strength. In addition, histological and immunofluorescence experiments showed a decrease in central nucleation, a marker of muscle fibre regeneration [198], and an improvement of muscle fibre integrity [27]. Finally, Pravastatin treatment of 'wasted' mice, which are genetically deficient in eEF1A2, failed to induce utrophin A expression in skeletal muscle, thus clearly demonstrating the key role of this ITAF in IRES-mediated translation of utrophin A. Altogether, these proof-of-principle studies illustrate the feasibility of specifically targeting the utrophin A IRES with pharmacological agents, as a therapeutically viable approach for treating DMD.

3.2. IRES-mediated translation of dystrophin

Recent studies revealed that a possible approach to recover partial dystrophin expression in DMD muscles is to target an IRES at the 5′end of the dystrophin transcript [24]. In fact, this finding aligns well with the original observation on the presence of an IRES in the utrophin A 5′UTR since the utrophin and dystrophin genes have common ancestral origins [142,143]. Such findings further suggest that the DMD disease state can suppress cap-dependent translation and that IRES-mediated translation has evolutionarily been conserved for both gene products.

The severity of the symptoms of muscular dystrophy patients is known to be dependent on the nature of the dystrophin gene mutation. More specifically, DMD, the most severe form, occurs when the mutation provokes a complete absence of the dystrophin protein whereas occasionally, the mutation still allows for production of a truncated, yet functional protein. For example, there is evidence of patients affected by a nonsense mutation in exon 1 of the dystrophin gene, who show mild symptoms and are known to be afflicted with the milder form of DMD, BMD. In some cases, ambulation of Becker patients over the age of 60 years has been reported. Although this nonsense mutation at exon 1 is predicted to result in no protein translation, muscle biopsies indicated that these patients produced a short but functional N-terminally truncated isoform of dystrophin, thus suggesting alternate translation initiation [199]. A similar outcome was recently reported in a patient affected by a nonsense mutation in exon 2 of the DMD gene, and who demonstrated mild BMD symptoms [200]. In 2014, Wein *et al.* reported that the production of a short dystrophin isoform protein in a situation where the mutation is present in the second exon of the DMD gene, is due to translation resulting from an IRES within exon 5 of the DMD gene [24]. Results from this study further demonstrated

that IRES-mediated translation of the short dystrophin isoform is achieved *in vitro* by expressing N-terminally truncated dystrophin reporter constructs or by promoting exon skipping of the upstream reading frame. In addition, exon skipping of exon 2 in a mouse model affected by a duplicated exon 2 mutation in the DMD gene, resulted in sarcolemmal recovery of the dystrophin complex, morphological improvements of the skeletal muscle fibres and amelioration of muscle strength [24].

Similar to utrophin A, IRES activation of dystrophin is stimulated by glucocorticoid treatment [24]. However, IRESs are not defined by a consensus sequence or distinct RNA structure [201] and, consequently, despite the many similarities between utrophin A and dystrophin, the regulation of the dystrophin IRES may not be comparable to utrophin A's. More specifically, an ITAF that regulate the dystrophin IRES has yet to be identified, but it would seem unlikely, given the current knowledge of ITAFs and their degree of specificity, that eEF1A2 could both regulate the utrophin A and dystrophin IRESs. Nonetheless, targeting IRES-mediated dystrophin protein translation, represents an attractive target for patients containing mutations at the 5′end of the DMD gene which consists of 6% of DMD patients [93].

3.3. IRES-mediated translation of growth factors

In disease state such as muscular dystrophies where cap-dependant translation may be compromised, targeting IRES activity of key growth factors may be another attractive strategy to improve the dystrophic muscle phenotype. Over the years, many laboratories worldwide have examined the potential therapeutic impact of various growth factors for dystrophic muscles [202–206]. Interestingly, several of these highly DMD-relevant growth factors, including VEGF, FGF-1, FGF-2, IGF-2 and IGF-1 R have been previously shown to contain IRESs.

3.3.1. Targeting IRES-mediated translation of VEGF

It has been established that there are beneficial effects of rising tissue perfusion by increasing the density of the vasculature in dystrophic muscles as a therapy for DMD. DMD patients are known to also suffer from abnormal blood flow after muscle contraction, thus, reducing the effects of functional ischaemia might decrease muscle damage [207–209] and improve dystrophic muscle function. One potential target in this case is vascular endothelial growth factor (VEGF), a known angiogenesis regulator. Interestingly, VEGF contains a long and structured 5′UTR containing two independent IRESs involved in its translation [210–213]. VEGF-A ligand can associate with the VEGF receptors to promote vascular permeability while also inducing proliferation and survival of newly formed endothelial cells, providing the basic structure of novel vasculatures [214]. Four-week post-intramuscular injection of a rAAV-VEGF vector in the bicep and tibialis anterior (TA) muscles of mdx mice, resulted in increased capillary density, reduced necrotic fibres, and significant improvements in forelimb strength, compared to AAV-LacZ-injected control mdx mouse muscle [215]. Thus, VEGF is an interesting and potentially important therapeutic target for DMD. VEGF-A expression has been shown to be regulated by IRES-mediated translation during ischaemic and hypoxic stress [213,216] via the ITAF heterogeneous nuclear ribonucleoprotein (hnRNP)L [216]. Although the implication of hnRNP L in DMD has yet to be examined, one can certainly envisage nonetheless, designing pharmacological interventions aimed at boosting VEGF expression via this translational mechanism.

3.3.2. IRES activation of the fibroblast growth factors: FGF-1 and FGF-2

Several other growth factors regulated by IRES translation are members of the fibroblast growth factor (FGF) family including FGF-1 and FGF-2. FGFs have been closely investigated over the years in muscular disorders due to their ability to promote muscle fibre regeneration and tissue damage repair [202,203,217,218]. More specifically, FGF-1 has been shown to be involved in muscle development and regeneration of muscle fibres. The FGF-1 gene is comprised of four tissue-specific promoters able to produce four transcripts that vary at their 5′ end. One such transcript, FGF-1A, contains an IRES that is activated during myoblast development and muscle regeneration, and is further stimulated by a cis-acting element in its promoter. This reveals a combined mechanistic process of mRNA transcription and translation coupling, in regenerating muscle fibres [204]. FGF-1A's IRES is activated in hypoxic cells where expression of its known ITAF, vasohibin1, increases in the nucleus, is translocated to the cytoplasm (may be translocated in part to stress granules) and binds the IRES of FGF-1A mRNA to recruit the transcript to the ribosome [219]. In agreement with these findings, FGF-1 is upregulated in regenerating muscle cells of mdx mice and in skeletal muscles of Facioscapulohumoral muscular dystrophy patients [205,220]. Considering that DMD patients suffer from muscle degeneration, this suggests that targeting the FGF-1 IRES may be of therapeutic benefit to stimulate regeneration of dystrophic muscle fibres. Currently, it is unknown whether vasohibin1 plays a role in DMD muscle but its regulation of FGF-1 suggests that it may be a good target to increase FGF-1 expression levels in dystrophic muscles via cap-independent translation.

Another key FGF that contains an IRES is FGF-2. Translation of FGF-2 results in four isoforms differentially localized in cells, with all isoforms being potentially synthesized through IRES-mediated translation [221,222]. FGF-2 is embedded within the basal lamina and extracellular matrix of uninjured skeletal muscle tissue where it plays a role in skeletal muscle regeneration by maintaining the pool of muscle precursor cells [217,223]. By repressing myogenesis, FGF-2 permits satellite cell self-renewal and is required for maintenance and repair of skeletal muscle [217]. FGF-2 is available to satellite cells following its production by myofibers, fibroblasts and other satellite cells [202,224–227].

Considering the key role of FGF-2 in muscle fibre repair [202,203,217], it may thus represent another attractive target for DMD therapy. In fact, like FGF-1, expression of FGF-2 is increased in regenerating muscle fibres including dystrophic muscles [225,228]. IRES-mediated translation of FGF-2 has been shown to be activated *in vivo* during limb ischaemia and

in hypoxic conditions [229]. FGF-2 IRES activity is also sensitive to oxidative stress. These conditions promote the nuclear-cytoplasmic translocation of eukaryotic eEF2 where it then assumes the role of an ITAF to drive FGF-2 IRES-mediated translation [191]. This suggests that this cellular IRES may be a good target for FGF-2 protein expression in different pathological conditions such as muscle atrophy, ischaemia and apoptosis, conditions often seen in muscular disorders where cap-dependent translation may be repressed. Along those lines, genetic ablation of FGF-2 in mdx mice promotes a more severe dystrophic phenotype than that seen in mdx mice, demonstrating its importance for proper muscle maintenance [206]. In addition, FGF-2 expression has also been shown to have protective effects against glucocorticoid-induced deterioration of bone through inhibition of the Wnt antagonist sclerostin [230]. Keeping in mind that glucocorticoid-treated DMD patients frequently develop osteoporosis, resulting in reduction of bone strength and long-bone fractures, IRES-mediated expression of FGF-2 in these patients might offer therapeutic benefits for improving muscle repair and bone reinforcement [78].

3.3.3. Targeting IRES-mediated Translation of IGF-2

Insulin-like growth factors (IGF) are amongst the growth factors that are the most studied in the DMD context [44–47]. One such IGF, IGF-2 has been studied in muscular dystrophies for its ability to modulate regulatory networks involved in programmed cell death [47]. IGF-2 is a potent growth factor with anti-apoptotic capacity in a variety of cell types [231–233]. IGF-2 can interact with the IGF-1 receptor (IGF-1 R) to activate phosphatidylinositol 3-kinase/Akt signalling, which in turn blocks proteolytic processing of key caspases and prevents initiation of the apoptotic cascade [231,234]. During foetal development, the IGF-2 gene produces three mRNAs containing identical coding regions but varying only at their 5′UTR. One isoform, IGF-2 leader 2, has a long 5′UTR containing an IRES which is activated in dividing cells [235]. The IGF-2 mRNA-binding protein 2 (IMP2) has been identified as an ITAF, phosphorylated by mTOR to modulate IGF-2 IRES activity in a mouse embryogenesis model [236].

The beneficial impact of IGF-2 has previously been investigated in the mdx mouse. For example, IGF-2 overexpression in mdx mice inhibits the normally seen elevated levels of programmed cell death in DMD muscle stem cells. This ectopic expression of IGF-2 results also in significant improvements in the initial histopathological changes observed in dystrophic skeletal muscles [47]. Thus, this provides evidence that that IGF-2 is a strong candidate for therapeutic intervention in DMD. In addition, its receptor IGF-1 R harbours an IRES at the 5′UTR [237] that is activated by thiazolidinediones antidiabetic compounds (rosiglitazone and pioglitazone) in smooth muscle cells and promotes beneficial effects on cell survival potentially via the ITAF HuR [238,239]. As the effects of IGF-2 can be mediated through IGF-1 R, pharmacologically promoting translation of IGF-1 R, through IRES activation in addition to targeting IRES-mediated translation of IGF-2, might represent another therapeutic approach for DMD. This seems particularly attractive given the known roles of HuR in skeletal muscle development and plasticity [240–242].

4. Conclusion and perspectives

Directing IRES-mediated translation of key transcripts involved in various diseases is gaining greater interest as an alternative therapeutic approach. This alternative translation avenue permits a rapid protein response to changes in physiological and pathological cellular conditions, bypassing the steps of transcription and post-transcription. In addition, this targeting approach achieves stimulation of protein expression or in turn, attenuates expression levels of deregulated or damaging signalling molecules in a stress and disease state, when cap-dependent translation might be compromised. In fact, laboratories around the world have been identifying and developing new drugs or small molecules to regulate IRES-mediated translation of target mRNAs in order to create new therapies for an ever-growing array of diseases [9–13,22,24,27]. Many of these molecules target and inhibit stress-induced, IRES-mediated translation such as antisense oligonucleotides, peptide nucleic acids, ribozymes, short hairpin RNAs and small interfering RNAs [243].

An important aspect to consider with targeting IRES-translation for therapy, is the potential for off-target effects. ITAFs likely have multiple mRNA targets and altering their normal expression patterns in certain tissues might result in detrimental secondary effects. For instance, VEGF has been shown to be involved in tumour survival through inducing tumour angiogenesis [244,245] whereas eEF1A2 has been shown to be upregulated in many types of cancers including breast, ovarian, pancreatic, prostate and lung cancers [246–252]. Similarly, growth factors such as FGF-2 which is particularly widespread, are known to be elevated in many tumours including melanomas as well as in breast, pancreas, bladder, head and neck, prostate, and hepatocellular tumours [253,254,255]. Thus, this type of therapy will need proper safety assessments and may ideally be designed to target-specific tissue(s).

In the context of neuromuscular disorders, a variety of DMD-relevant transcripts have been shown to contain IRESs as described above. Moreover, for some of these mRNAs, ITAFs have also been identified and characterized. All of these thus represent potential targets for pharmacological interventions aimed at modulating their activity leading to protein production (Fig. 3). This therapeutic concept seems well advanced for the utrophin A IRES and its ITAF eEF1A2, with several FDA-approved drugs recently identified and now known to be able to activate utrophin A expression. One could even envisage that, therapeutically, a rationally designed pharmacological intervention could potentially target several DMD-relevant IRES-containing transcripts thereby leading to multiple complementary benefits in dystrophic muscle. The fact that up to 10% of cellular mRNAs are predicted to contain an IRES [254], suggests that the search for additional DMD-relevant, IRES-containing transcripts, and their regulating

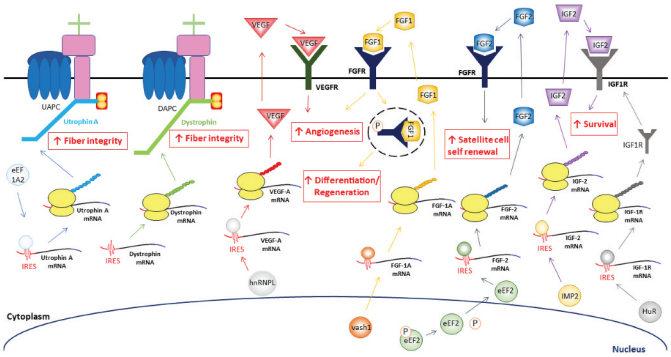

Figure 3. Potential DMD-relevant mRNAs that contain IRESs for the treatment of DMD. eEF1A2 acts as an ITAF, driving utrophin A IRES-mediated translation. This results in increased levels of utrophin A, rescue of the utrophin-glycoprotein complex (UAPC) at the sarcolemma of muscle fibres and improvement of fibre integrity. Dystrophin's IRES can drive its translation in dystrophic muscles and also provides stability at the sarcolemma with the DAPC. VEGF-A expression has been shown to be regulated by IRES-mediated translation via the ITAF hnRNP L [216]. VEGF-A interacts with the VEGF receptor (VEGFR) and induces angiogenesis, cell proliferation and survival [214]. FGF-1A's IRES is activated under stress conditions where vasohibin1 (VASH1) expression increase in the nucleus, translocate to the cytoplasm and bind the IRES of FGF-1A mRNA to recruit the transcript to the ribosome [219]. FGF-1A protein can either bind an external FGF receptor (FGFR) to promote angiogenesis or an intracellular FGFR to induce cell differentiation and regeneration [225,228]. FGF-2 IRES activity is sensitive to oxidative stress. This promotes translocation of nuclear eEF2 to the cytoplasm where it acts as an ITAF and drives FGF-2 IRES-mediated translation. FGF-2 protein binds FGFR, induces satellite cell self renewal by maintaining the pool of muscle precursor cells and inhibits myogenesis [191]. The IRES of IGF-2 has been shown to be activated via ITAF IMP2 [236]. The IGF-2 protein weakly binds the IGF-1 receptor (IGF-1 R) and promotes cell survival by modulating the regulatory networks involved in programmed cell death [47]. This is further stimulated by increasing the number of available IGF-1 R which in part is also regulated through IRES-mediated translation via HuR.

ITAFs, should continue, perhaps even accelerate given the promising findings obtained to date. In fact, this may well be important and extended to other neuromuscular conditions including for example amyotrophic lateral sclerosis, in which IRES-mediated translation of VEGF may play a modifying role [255,256]. Overall, our knowledge of cellular IRESs, their structure, mechanisms of regulation, and underlying signalling pathways is still in its infancy. Accordingly, increased efforts in this area appear warranted particularly if the goal is to capitalize and exploit the full therapeutic potential of IRESs and ITAFs for multiple diseases including neuromuscular conditions such as DMD which, despite the discovery of the dystrophin gene as causative of the disease more than 30 years ago, remains today without a cure or effective treatment.

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Disclosure statement

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