Skeletal muscle specific IRES activity of Utrophin A is enhanced by eEF1A2

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Abstract

Duchenne Muscular Dystrophy (DMD) is a severe degenerative muscle disease caused by the lack of dystrophin in muscle tissues of young boys. A potential therapeutic strategy for DMD is to increase endogenous levels of utrophin, an autosomal homologue of dystrophin. One of the molecular mechanisms in which utrophin A expression was shown to be regulated is via the internal ribosome entry site (IRES) located within the 5'untranslated region (UTR), where it was shown to drive cap-independent translation of utrophin A under several stress conditions (Miura et al., 2005;Miura et al., 2008). Transgenic mice harbouring either control or the utrophin A 5'UTR bicistronic reporter transgene were generated. Utrophin A IRES activity was found exclusively in skeletal muscles and not in other tissues examined. In addition, the eukaryotic elongation factor 1A2 (eEF1A2) was identified as a muscle specific trans-factor that associates with the 5'UTR of utrophin A. Regions of the utrophin A 5'UTR that bound eEF1A2 also mediated cap-independent translation of utrophin A in rat fibroblast cells that overexpressed eEF1A2. The direct interaction of eEF1A2 to endogenous utrophin A mRNA was demonstrated in differentiated myotubes by RNA-Immunoprecipitation. Moreover, the physiological role of eEF1A2 on utrophin A expression was further assessed in wasted mice (mouse model that does not express eEF1A2). The expression analysis of utrophin A in skeletal muscle and brain of wasted mice revealed that while no difference was evident in utrophin A mRNA levels, utrophin A protein levels were decreased. Together, these findings demonstrate the important role of eEF1A2 in regulating the cap-independent translation of utrophin A. In addition, eEF1A2 was found to direct the localization of utrophin A to extra-synaptic regions of skeletal muscle. Thus,

targeting eEF1A2 might be a potential therapeutic strategy to treat DMD, with the aim of upregulating utrophin A expression in dystrophic tissues.

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List of Abbreviations

3'UTR- 3'Untranslated Region

5'UTR- 5'Untranslated Region

AChE- acetylcholinesterase

AChR- acetylcholine receptor

AON- antisense oligonucleotides

APF-1- apoptotic protease-activating factor protein 1

ARE- AU-rich element

βGAL- β-Glactosidase

BiP- human immunoglobulin heavy chain binding protein

BMD- Becker muscular dystrophy

BTX- α-bungarotoxin

CaM- calmodulin

CaMBP- CaM-binding peptide

CAT- Chloramphenicol acetyltransferase

CT GalNAcT- Cytotoxic T cells β1-4-*N*-acetylgalactosamine transferase

CTX- cardiotoxin

DGC- dystrophy-glycoprotein complex

DMD- Duchenne muscular dystrophy

DUE- downstream utrophin enhancer

ECM- extracellular matrix

EDL- extensor digitorum longus

eEF1A/1A1/1A2- eukaryotic elongation factor 1A/1A1/1A2

eIF- eukaryotic initiation factor

ERF- ets-2 repressor factor

FGF-II- fibroblast growth factor 2

GAPB- GA binding protein

Grb2- growth factor receptor-bound protein 2

GRMD- Golden retriever muscular dystrophy

hnRNP A1- heterogeneous nuclear ribonucleoprotein A1

IGF-I- insulin growth factor

IgG- immunoglobulin G

IP- immunoprecipitation

IRES- internal ribosome entry site

ITAF- IRES trans-acting factor

Jazz- artificial zinc-finger based transcription factor

La- lupus autoantigen

mdx- X-linked muscular dystrophy

miR- micro RNA

MRF- myogenic regulatory transcription factor

MyHC- myosin heavy chain

NFAT- nuclear factor of activated T-cell

NF-kB- nuclear factor kappa B

NMJ- neuromuscular junction

nNOS- neuronal nitric oxide synthase

ORF- open reading frame

PABP- poly-A binding protein

PDN- 6a-methylprednisolone-21 sodium succinate

PGC-1α- peroxisome proliferator-activated receptor γ coactivator 1α

PPAR- peroxisome proliferator-activated receptor

PPRE- PPAR- response element

PTB- polypyrimidine-tract binding protein

rAAV- recombinant adeno-associated virus

RIP- RNA-IP

RT-PCR- reverse transcription polymerase reaction

SP- side population

TA- tibialis anterior

TNF-α- tumor necrosis factor a

unr- upstream of N-ras

Utr- utrophin

ZPR1- zinc finger-containing transcription factor

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Chapter 1

1. General Introduction

1.1. Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is a devastating hereditary muscle disorder that affects 1 in 3500 male births (Emery, 1991;Moser, 1984). The onset of clinical symptoms usually occurs before the age of 5, where these patients are often unable to run, climb stairs or even rise up from the floor. These symptoms are usually a result of weak skeletal muscle development, generally those in their calves and pelvis (Anderson and Kunkel, 1992;Hoffman et al., 1987). As time progresses, their symptoms become more severe. Between the ages of 7 and 12, patients lose the ability to walk and become wheelchair bound until their second or third decade of life when death typically ensues from cardiac or respiratory failure (Jennekens et al., 1991). Approximately 75% of dystrophic patients die from respiratory dysfunction indirectly caused by the progressive degeneration of the diaphragm, making them prone to infectious diseases such as pneumonia (Finsterer and Stollberger, 2003).

There is still no effective treatment for this incapacitating neuromuscular disease. Currently, the most common drug treatment for DMD is glucocorticoids. These drugs are associated with many side effects such as significant weight gain, thinning of the bone and suppression of the immune system (Biggar et al., 2004;Fenichel et al., 1991b;Manzur et al., 2008). In addition, these compounds are only effective for a short period of time. Despite their inevitable side effects, patients treated with glucocorticoids show improved muscle strength and in some cases, pulmonary function (Balaban et al., 2005;Manzur et al., 2008;Monaco et al., 1988).

1.1.1. Genetic defects and pathophysiology of DMD

DMD is an X-linked recessive disease caused by mutations in the dystrophin gene, resulting in the lack of the cytoskeleton protein dystrophin (Hoffman et al., 1987;Monaco et al., 1988;Worton and Thompson, 1988). Dystrophin is one of the largest genes known, composing about 0.1% of the human genome (Blake et al., 2002). Due to its remarkable size, the dystrophin gene is likely to be subjected to mutations (Coffey et al., 1992). These mutations can be grouped into 3 classes, deletion (60%), point mutation (32%) and duplication (8%), all of which cause disruption of the reading frame and thus lead to DMD (Koenig et al., 1987;Muntoni et al., 2003).The most common indications of the DMD pathology are elevated serum creatine kinase, abnormal fiber diameter variation, centrally nucleated myofibers and an accumulation of necrotic muscle fibers (Emery, 1977;Emery and Holloway, 1977).

In some instances, mutational events occur that do not disrupt the reading frame of dystrophin. These patients exhibit a milder form of muscular dystrophy called Becker muscular dystrophy (BMD) (Monaco et al., 1988). BMD patients express a truncated, but functional, form of dystrophin. Despite the fact that they exhibit some forms of muscle weaknesses, they live a normal lifespan (Hoffman et al., 1988).

1.1.2. Dystrophin and the dystrophin-glycoprotein complex

Located on chromosome Xp21, the dystrophin gene is comprised of approximately 2.5 Mb (Blake et al., 2002). Distributed and assembled into 79 exons, the dystrophin gene is regulated by 7 independent promoters (Muntoni et al., 2003). As a result of three independent promoters, the dystrophin gene can be transcribed into 3 full-

length isoforms, which differ only by their amino-terminal sequences (Ahn and Kunkel, 1993). The full-length dystrophin transcript measures approximately 14 kb and is found in the brain, skeletal and cardiac muscle (Sadoulet-Puccio and Kunkel, 1996). The presence of internal promoters within the dystrophin gene gives rise to four additional isoforms, which differ in their N-terminals, known as Dp260, Dp140, Dp116 and Dp71, where the numbers reflect the molecular weights (**Figure 1A**) (Byers et al., 1993;D'Souza et al., 1995;Feener et al., 1989;Lidov et al., 1995). These isoforms are expressed in non-muscle tissues such as the central and peripheral nervous system, kidney and lung (Schofield et al., 1994).

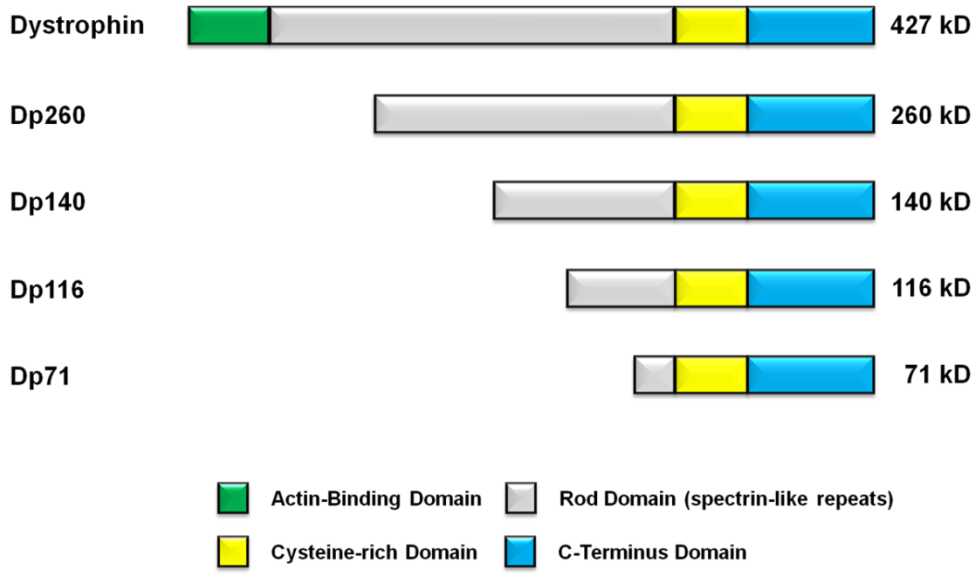
With a molecular weight of 427 kD and a total of 3685 amino acids, dystrophin is a member of the β-spectrin/α-actinin family of proteins (Ahn and Kunkel, 1993;Kapsa et al., 2003;Koenig et al., 1988). Dystrophin protein is composed of four distinct structural domains: N-terminal domain, central rod domain, cysteine-rich domain and the C-terminal domain (**Figure 1B**) (Koenig et al., 1988). The N-terminal region is a crucial domain composed of two calponin homology modules (sequences of approximately 100 residues each) that are required for binding to cytosolic actin filaments (Korenbaum and Rivero, 2002). The central rod domain is composed of 24 spectrin-like repeats and 4 hinge regions which are important to ensure proper flexibility and elasticity of the membrane, and thus preventing muscle cells from contraction-induced damage (Grum et al., 1999). Dystrophin also has a cysteine-rich domain (composed of two EF hand-like domains, a WW domain and a ZZ domain) located upstream of the carboxyl-domain that is critically important for linking dystrophin to the extracellular matrix by interacting with the intracellular tail of β-dystroglycan, thus preserving the entire dystrophin-

glycoprotein complex (DGC), a group of membrane proteins (**Figure 2**) (Ervasti and Campbell, 1991;Ponting et al., 1996;Roberts, 2001).

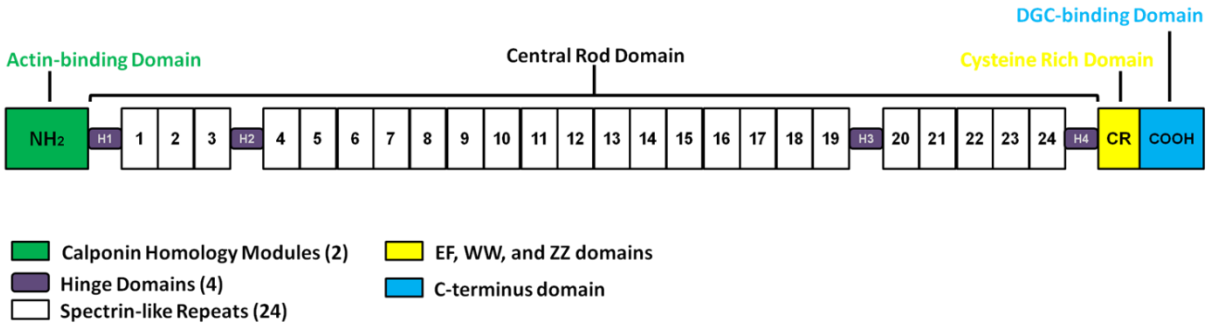
Figure 1. Schematic presentation of dystrophin isoforms.

A) Different isoforms of dystrophin. Shown are the actin-binding domains in green, spectrin-like repeats in gray, cysteine-rich domains in yellow, carboxy-terminal domains in blue, isoform names on the left and molecular weights on the right. B) Structural characterization of the full-length dystrophin protein. Dp, dystrophin protein; kD, kiloDalton

A)


Dystrophin
427 kD
Dp260
260 kD
Dp140
140 kD
Dp116
116 kD
Dp71
71 kD
Actin-Binding Domain
Rod Domain (spectrin-like repeats)
Cysteine-rich Domain
C-Terminus Domain

B)


DGC-binding Domain
Actin-binding Domain
Central Rod Domain
Cysteine Rich Domain
NH2
H1
1
2
3
H2
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
H3
20
21
22
23
24
H4
CR
COOH
Calponin Homology Modules (2)
Hinge Domains (4)
Spectrin-like Repeats (24)
EF, WW, and ZZ domains
C-terminus domain

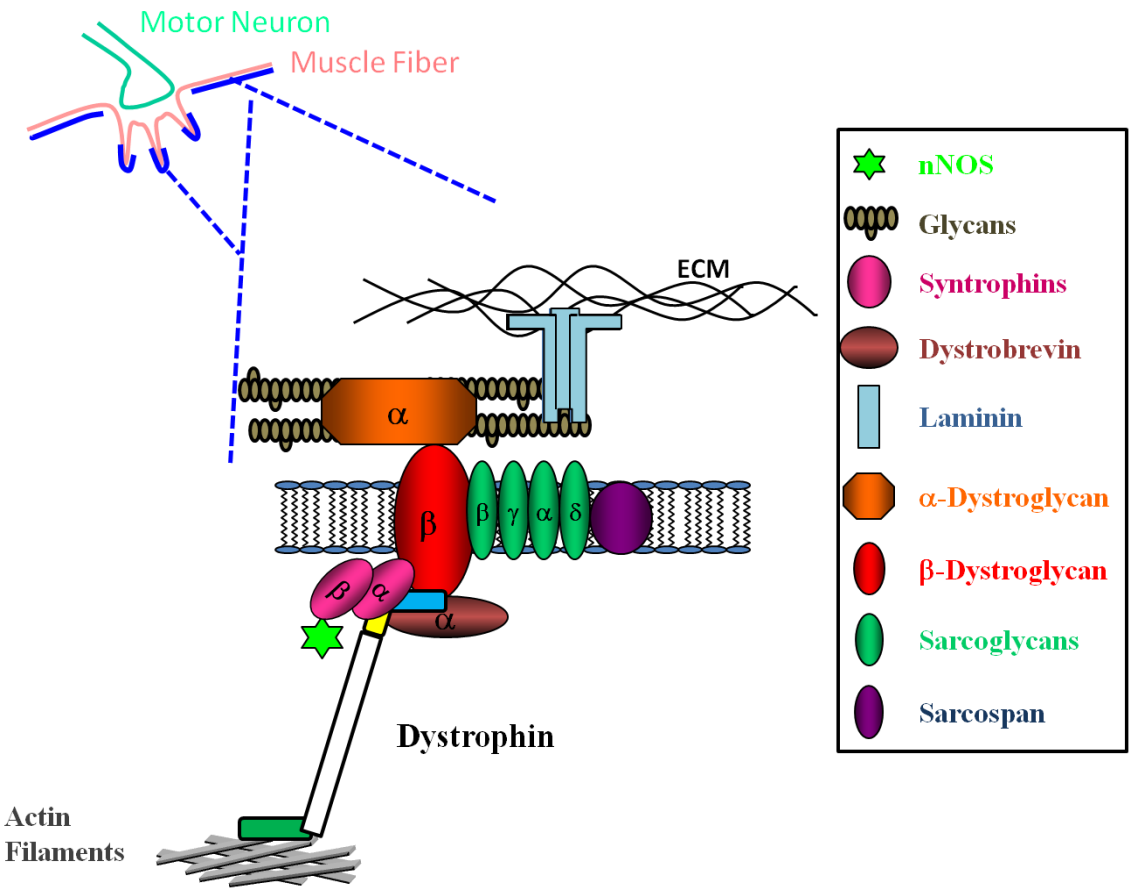
Dystrophin embodies approximately two percent of all the sarcolemmal proteins (Ohlendieck et al., 1991). In healthy adult muscle fibers, dystrophin is expressed along the entire length of the sarcolemma and at the troughs of the neuromuscular junctions (NMJs) (O'Brien and Kunkel, 2001;Watkins et al., 1988)(**Figure 2**). In addition, full-length dystrophin is also found in the myotendinous junctions where it plays a role in facilitating the transmission of forces generated in muscle fibers to nearby tendons (Tidball and Law, 1991). The main functional role of dystrophin is to provide structural integrity by interacting with the DGC. (**Figure 2**)(Moens et al., 1993;Petrof et al., 1993).

The main components of the DGC are dystroglycans (α and β), syntrophins (α1, β1 and β2), dystrobrevins, sarcospan and sarcoglycans (α, β, γ and δ), all of which have distinctive roles (Ervasti and Campbell, 1991). β-dystroglycan interacts with dystrophin (via its C-terminal intracellular domain) and with its counterpart α-dystroglycan (via its N-terminal extracellular domain), enabling interaction with the basal lamina protein laminin-2 (Ervasti and Campbell, 1991;Ervasti and Campbell, 1993). Neuronal nitric oxide synthase (nNOS), in addition to dystrophin, is also a binding partner for the syntrophins (Adams et al., 2001;Stamler and Meissner, 2001). Stabilization of the DGC to the sarcolemma is partly attributed to the sarcoglycan/sarcospan complex (Crosbie et al., 1999) (**Figure 2 and 3B**). Dystrophin and proteins of the DGC are not only involved in stabilizing the cell membrane, but are also indirect regulators of signalling events. Signalling molecules that bind dystrophin and members of the DGC include integrins (Cote et al., 2002), nNOS (Brenman et al., 1995) and growth factor receptor-bound protein 2 (Grb2) (Oak et al., 2001).

The absence of dystrophin has been shown to affect the formation of the DGC, causing damage to the cell membrane which leads to significant leakiness of calcium ions. The resulting intracellular accumulation of calcium can alter the activity of several signalling pathways involved in the regulation of proteolysis (Alderton and Steinhardt, 2000;Fong et al., 1990;Turner et al., 1988;Turner et al., 1991). Thus, in the absence of dystrophin, the cell membrane becomes more fragile and mechanically damaged during muscle contraction (Cohn and Campbell, 2000;Rando, 2001). Overtime, severe necrosis usually occurs due to the diminished regenerative capacity of muscle fibers (Allikian and McNally, 2007;Menke and Jockusch, 1991;Weller et al., 1990).

Figure 2. Association of dystrophin with the dystrophin-glycoprotein complex in healthy muscle fibers.

Interaction of dystrophin with the dystrophin-glycoprotein complex (DGC) and how it links the extracellular matrix (ECM) via its C-Terminal to the intracellular actin filament through its N-terminal. Dystrophin expression in healthy adult skeletal muscle along the sarcolemma and at the troughs of the neuromuscular junctions (NMJ) is shown in blue. Dystrophin associates with members of the DGC (not all shown) to provide structural stability. nNOS, neuronal nitric oxide synthase.


Motor Neuron
Muscle Fiber
ECM
α
β
β
γ
α
δ
β
α
α
Dystrophin
Actin
Filaments
nNOS
Glycans
Syntrophins
Dystrobrevin
Laminin
α-Dystroglycan
β-Dystroglycan
Sarcoglycans
Sarcospan

1.1.3. Other forms of muscular dystrophies

Although DMD and BMD are the most common genetic neuromuscular disorders, mutations in members of the DGC can also lead to other forms of muscular dystrophies. Since the initial characterization of dystrophin as the target gene for DMD and BMD, over 30 genes have been identified and associated to other types of muscular dystrophies (Costanza and Moggio, 2010). Other forms of muscular dystrophies include limb-girdle, facioscapulohumeral, myotonic, oculopharyngeal, distal and Emery-Dreifuss (Ozawa et al., 1998). Also, patients deficient in either signalling molecules, integrin or laminin, are diagnosed with congenital muscular dystrophy (Campbell, 1995). No curable therapies have been established for any of these muscular dystrophies.

1.1.4. Animal models of DMD

In addition to humans, dystrophin homologues have been identified in a number of animals such as dogs (Zeiss et al., 1998), mice (Geng et al., 1991), fish (Chambers et al., 2001) and invertebrates (Roberts and Bobrow, 1998). The most extensively studied animal model of DMD is the X-linked muscular dystrophy (*mdx*) mouse model. The dystrophin-deficient *mdx* mouse has a naturally occurring point mutation in exon 23 of the Dmd gene. This mutation results in the introduction of a premature transcriptional stop codon, such as the ones observed in one third of DMD patients (Bulfield et al., 1984;Sicinski et al., 1989). Despite signs of muscular dystrophies during the first six weeks of life, when the diaphragm undergoes several cycles of degeneration followed by regeneration, these mice live a relatively healthy life (Muntoni et al., 1993;Stedman et al., 1991). To assess the important physiological role of dystrophin in the *mdx* mouse model,

transgenic *mdx* mice expressing the full-length dystrophin gene were generated and further showed partial elimination of dystrophic symptoms (Cox et al., 1993).

Another animal model is the Golden Retriever Muscular Dystrophy (GRMD) dog which is physiologically more representative of the human DMD pathology. These dogs are symptomatic early in life. Their muscle tissues undergoe repeated rounds of degeneration followed by regeneration, which eventually leads to irreversible muscle wasting. The GRMD dogs suffer a premature death resulting from cardiac or respiratory failure (Cozzi et al., 2001;Willmann et al., 2009). Only a small amount of work has been done using the dog model due in part to financial reasons.

1.2. Potential therapeutic strategies to treat DMD

Several laboratories around the world have been researching different strategies for potential DMD treatment. It was shown that levels of dystrophin as low as 30% in dystrophic tissues is enough to alleviate the pathology (Neri et al., 2007). Therapy-based research for DMD can be categorized as gene-based, cell-based, or drug-based.

1.2.1. Gene-based therapy

Gene-based therapies use a variety of compatible viral vectors to either insert a functional dystrophin gene into dystrophic muscle cells or to repair the mutation in the endogenous dystrophin gene (Gregorevic and Chamberlain, 2003). When attempting to deliver a large gene such as dystrophin using viral vectors, several factors must be considered such as the maximum carrying capacity, duration of gene expression, specificity of cell population targeted and most importantly the immune response upon its introduction. To eliminate issues related to the overall size of the dystrophin gene and

carrying capacity of viral-type vehicles, highly functional ‘mini’ and ‘micro’ versions of dystrophin have been created. In fact, delivery of both truncated versions of dystrophin (mini and micro) into *mdx* mice significantly ameliorated several aspects of the dystrophic pathology (Phelps et al., 1995;Sakamoto et al., 2002;Wang et al., 2000). The most prominent virus-based gene therapy to this day involves a recombinant adeno-associated viruses (rAAV) carrying a functional truncated form of dystrophin (Odom et al., 2008). Despite the fact that it could be efficiently expressed in skeletal and cardiac muscles of *mdx* mice, continuous expression in proliferating cells was not successful.

Ideally, a promising therapy is one where delivery of oversized genes, potential immune response and specific tissue targeting are not problematic. A widely used strategy to encompass these limitations is exon-skipping. Exon-skipping is considered when mutations resulting from splice-site or deletions/duplication alter the reading frame and introduce a stop codon, therefore producing dysfunctional proteins (Wilton and Fletcher, 2008). To correct for these mutations, specific antisense oligonucleotides (AONs) are created to avoid internal splicing and enable the production of partially functional dystrophin, such as the ones found in BMD patients. AONs are single stranded DNA or RNA oligonucleotides that specifically target a complementary sequence of nucleotides. In fact, clinical studies in DMD patients normally carrying an exon 50 deletion showed restored expression of truncated dystrophin by therapeutically skipping the exon 51 with specific AONs, and thus restoring the open reading frame (van Deutekom et al., 2007). To this day, the exon-skipping strategy is presumably one of the most promising gene-based therapies considering the fact that it could be applicable to 83% of DMD patients, in addition to other genetic diseases (Aartsma-Rus et al.,

2009;Aartsma-Rus and van Ommen, 2009;van Deutekom et al., 2001;van Deutekom et al., 2007).

1.2.2. Cell-based therapy

Another convenient and promising therapy is to directly transplant healthy skeletal muscle precursor (stem or progenitor) cells into dystrophic muscle tissues so they can fuse with existing myofibers and form new ones that express dystrophin in order to improve muscle strength. Studies on *mdx* mice have shown that injecting precursor myoblast cells into regenerating muscle resulted in the production of newly formed cells that expressed dystrophin (Partridge et al., 1989). There have been reports of several negative consequences using this therapy which include cell death of injected myoblasts, severe immune response and more importantly the inability to be systemically delivered into the circulation (Irintchev et al., 1995;Labrecque et al., 1992;Mouly et al., 2005;Skuk et al., 2002).

In contrast to myogenic precursors, stem cells are capable of self renewing themselves indefinitely, differentiate into a vast range of specialized cells and easily disperse throughout the systemic circulation (Gussoni et al., 1999;Meregalli et al., 2010). In fact, stem cells originating from bone marrow had the potential to successfully populate cardiac and skeletal muscle of *mdx* mice and further regenerate not only cardiac cells, but skeletal muscle cells, as demonstrated by the upregulation of myogenic factors myogenin and myf-5 (Bittner et al., 1999). To this day, stem cell therapy is one of the principle therapies under investigation for DMD patients. There are several research laboratories that aim to combine gene-based therapy, such as AONs with stem cells, in hopes of eliminating the major secondary effects and ultimately reverse the dystrophic

phenotype (Benchaouir et al., 2007). Also, side population (SP) cells, a subpopulation of muscle-derived cells with stem cell-like characteristics (Asakura et al., 2002), derived from *mdx* mice that have underwent correctional genetic modifications (expressing micro-dystrophin) were shown to be successful in repairing damaged muscles (Bachrach et al., 2004).

1.2.3. Drug-based therapy

1.2.3.1. Pharmacological Compounds

Some drug-based therapies are aimed at introducing pharmacological agents or molecules that have been shown to correct the dystrophic pathology and improve quality of life of DMD patients. Preferential therapy outcomes would be to maintain calcium homeostasis, improve the regenerative capacity of dystrophic muscles, decrease inflammation and increase muscle strength. Treatment of DMD patients with Oxandrolone and Prednisone showed improvement of skeletal muscle function and strength for a relatively long period of time (Balagopal et al., 2006;Fenichel et al., 1991b;Fenichel et al., 1991a;Manzur et al., 2008;St Pierre et al., 2004). These specific glucocorticoids are generally used to conserve muscle mass and prevent muscle wasting by increasing overall protein synthesis, even thought their primary use is to treat chronic inflammatory diseases (Wagner et al., 2007). Although glucocorticoid-treatment shows significant alleviation of the dystrophic phenotype, short-term (behavioural abnormalities) and long-term (early inset of osteoporosis) side effects cannot be ignored (Manzur et al., 2008;Straathof et al., 2009).

To directly reduce inflammatory responses associated with the dystrophic pathology, immunosuppressants such as the TNF-α antibodies (Infliximab) and osteopontin were shown to be very efficient at increasing muscle strength and reducing necrosis in *mdx* mice (Grounds and Torrisi, 2004;Pierno et al., 2007;Vetrone et al., 2009;Wagner et al., 2007;Wehling-Henricks et al., 2004). Administration of L-arginine in tissues of *mdx* mice resulted in an ameliorated dystrophic pathology (Chaubourt et al., 1999). More specifically, L-arginine administration led 1) to the rearrangement of both utrophin and nNOS to the sarcolemma, 2) reduced accumulation of NF-κB and metalloproteinase, 3) inhibited β-dystroglycan cleavage, which ultimately caused the reassembly of the DGC, and 4) decreased immune response by preventing macrophage infiltration (Archer et al., 2006;Hnia et al., 2008). Studies have also shown that intraperitoneal injection of *mdx* mice with a small peptide encoding a specific region of heregulin, improved the mechanical properties of dystrophic muscles and reduced the severity of muscle pathology (Krag et al., 2004).

1.2.3.2.Booster Genes

Apart from trying to alleviate the dystrophic pathology by directly correcting the absence of dystrophin, manipulating specific genes involved in skeletal muscle growth and maintenance have also been shown to improve the pathology of dystrophic muscles. Booster genes include nNOS, α7β1-integrin, myostatin and utrophin (Barton et al., 2005;Burkin et al., 2001;Tinsley et al., 1996;Wehling et al., 2001). In dystrophic muscle, nNOS was found to be decreased and dislocated from the DGC (Chang et al., 1996). Overexpressing nNOS in *mdx* mice decreased the dystrophic pathology, including

substantial decreases in membrane damage and muscle inflammation (Wehling et al., 2001). Transgenically overexpressing α7β1-integrin, the predominant form of integrin in skeletal muscles and one of the principal binding proteins for laminin (beside α-dystroglycan), in the *mdx*/utrn$^{-/-}$ mice (animal model deficient in dystrophin and utrophin) can ameliorate the dystrophic pathology and increase lifespan of these mice (Burkin et al., 2001;Burkin et al., 2005). A well-characterized growth factor, produced primarily in skeletal muscles and shown to inhibit muscle differentiation and growth, is myostatin (Joulia-Ekaza and Cabello, 2006). The improved regenerative capacity, as well as increased strength and growth of dystrophic muscles in *mdx* mice, could be achieved by blocking endogenous myostatin expression using anti-myostatin antibodies (Bogdanovich et al., 2002). Perhaps the most promising and studied target gene shown to improve the dystrophic pathology seen in DMD patients is utrophin. Because of its high sequence similarity to dystrophin and its similar association with members of the DGC, utrophin can potentially serve as an alternative for dystrophin.

1.3. Utrophin, the autosomal homologue of dystrophin

1.3.1. Molecular biology of utrophin

Initially named the dystrophin-related protein, utrophin is the autosomal homologue of dystrophin (Love et al., 1989). The utrophin gene was first discovered as a coding DNA in fetal muscle where it displayed high homology with the dystrophin gene (Love et al., 1989). Located on chromosome 6q24, the utrophin gene is approximately 1 Mb in length and contains 74 exons (Pearce et al., 1993). Similar to dystrophin, the ~13 kb utrophin transcript also encodes a massive cytoskeletal protein with a molecular mass of approximately 395 kD (**Figure 3A**) (Love et al., 1989;Tinsley et al., 1992). Utrophin is

ubiquitously expressed. In fact, high levels of utrophin have been described in most tissues, including the heart and at the crest of the NMJs in skeletal muscle (**Figure 3B**), with noticeably higher expression in kidney, lung, nervous system, and vascular endothelial and smooth muscle cells (Helliwell et al., 1992;Khurana et al., 1990).

Utrophin was thought to have a similar functional role as dystrophin since it displays 73% amino acid sequence identity with dystrophin. Bioinformatical analysis of coding sequences revealed that the N-terminal domain and C-terminal domain (which include the cysteine-rich region) between utrophin and dystrophin are 85% and 83% similar, respectively (Love et al., 1989;Pearce et al., 1993;Tinsley et al., 1992). Not surprisingly, the C-terminal domain of utrophin interacts in a similar fashion as dystrophin with the proteins of the DGC (Winder et al., 1995). In addition, both utrophin and dystrophin have similar binding affinities for F-actin via their N-terminal domain (Matsumura et al., 1992;Morris et al., 1999;Winder et al., 1995). The central rod domain is composed of 22 spectrin-like repeats and four hinge regions and plays the same functional role as dystrophin, despite the fact that it's the least conserved region, with 35% similarity (Amann et al., 1999;Pearce et al., 1993;Rybakova et al., 2006) **(Figure 3A-B)**.

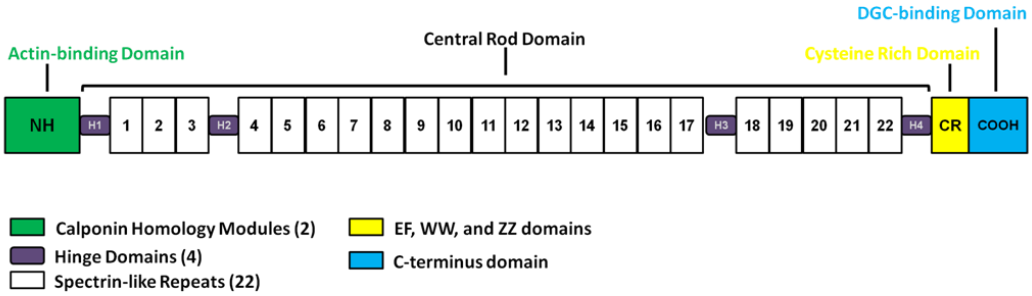
The utrophin gene contains two predominant promoters, the second of which is located within the second intron, that have been shown to translate into two full-length isoform: utrophin A and utrophin B. The utrophin A 5' untranslated region (UTR) is significantly longer (508 nucleotides) then the utrophin B 5'UTR (74 nucleotides). In addition to having different N-termini, utrophin A and B arise from different initial exons and are quantitatively different (Burton et al., 1999) (**Figure 4A-B**). Utrophin A is mostly

expressed in peripheral nerves, in vascular smooth muscle and at the crest of NMJs in skeletal muscle fibers, whereas utrophin B expression is limited to vascular endothelial cells (Weir et al., 2002). In dystrophic muscles, utrophin A is the major isoform that is upregulated (Weir et al., 2002). Due to the presence of an internal promoter within the utrophin locus, G-utrophin, a shorter isoform of utrophin that weighs 113kD and lacks the majority of the central rod domain, can be found in distinct regions of the adult brain, such as the amygdale (**Figure 4A**). It was also shown to be regulated during embryonic development, particularly in the sensory dorsal root and cranial nerve ganglia (Blake et al., 1995;Davies et al., 1995;Jimenez-Mallebrera et al., 2003).

Figure 3. Utrophin protein and its association with the dystrophin-glycoprotein complex in healthy muscle fibers.

A) Structural characterization of the utrophin protein. B) Physical interaction of utrophin with the dystrophin glycoprotein-complex (DGC) and how it links to the extracellular matrix (ECM), by binding to β-dystroglycan through its C-Terminal, and to the intracellular actin filament through its N-terminal. The expression profile of utrophin at the crests of the neuromuscular junctions (NMJ) in healthy adult skeletal muscle is shown in blue. Utrophin associates with members of the DGC (not all shown) to provide structural stability. nNOS, neuronal nitric oxide synthase; AChR, acetylcholine receptor

A)


Actin-binding Domain
Central Rod Domain
DGC-binding Domain
Cysteine Rich Domain
NH
H1
1
2
3
H2
4
5
6
7
8
9
10
11
12
13
14
15
16
17
H3
18
19
20
21
22
H4
CR
COOH
Calponin Homology Modules (2)
Hinge Domains (4)
Spectrin-like Repeats (22)
EF, WW, and ZZ domains
C-terminus domain

B)

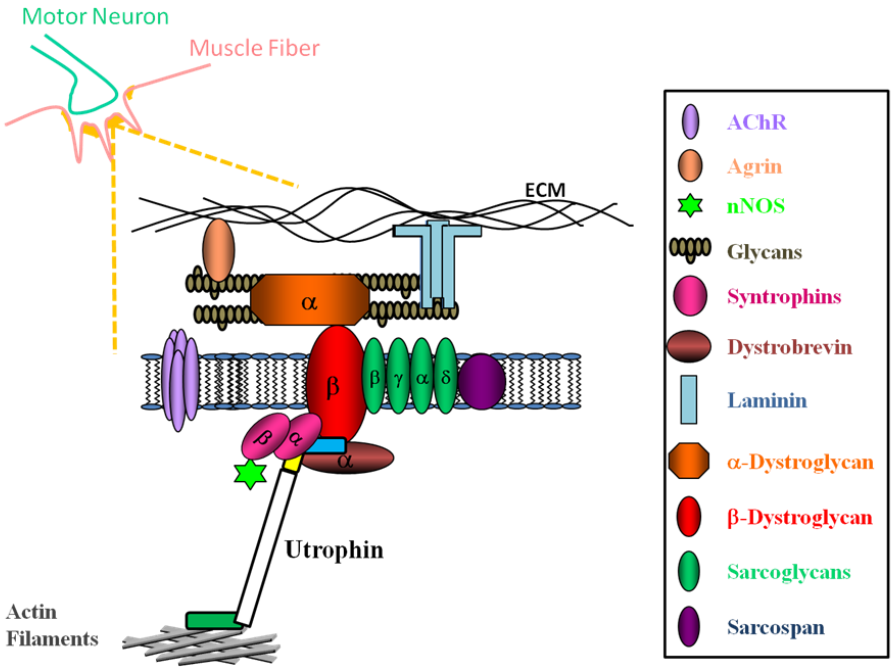
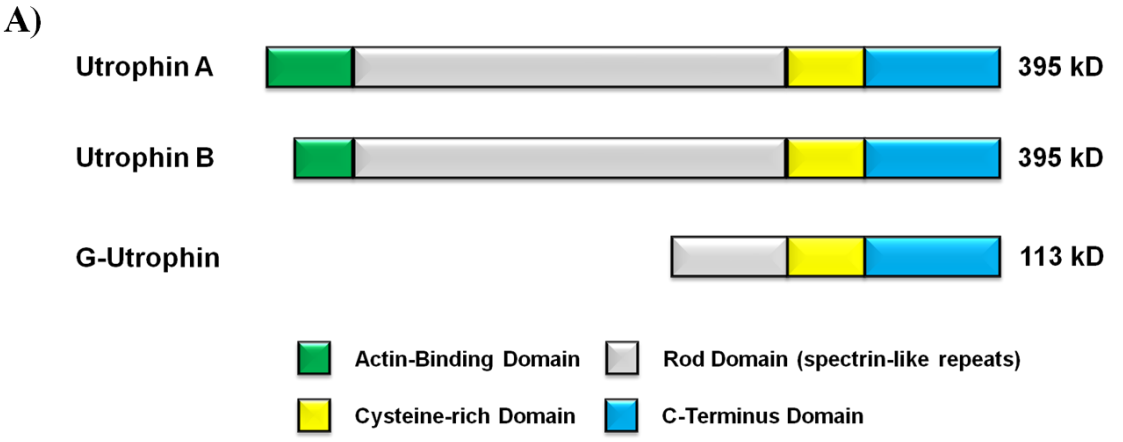
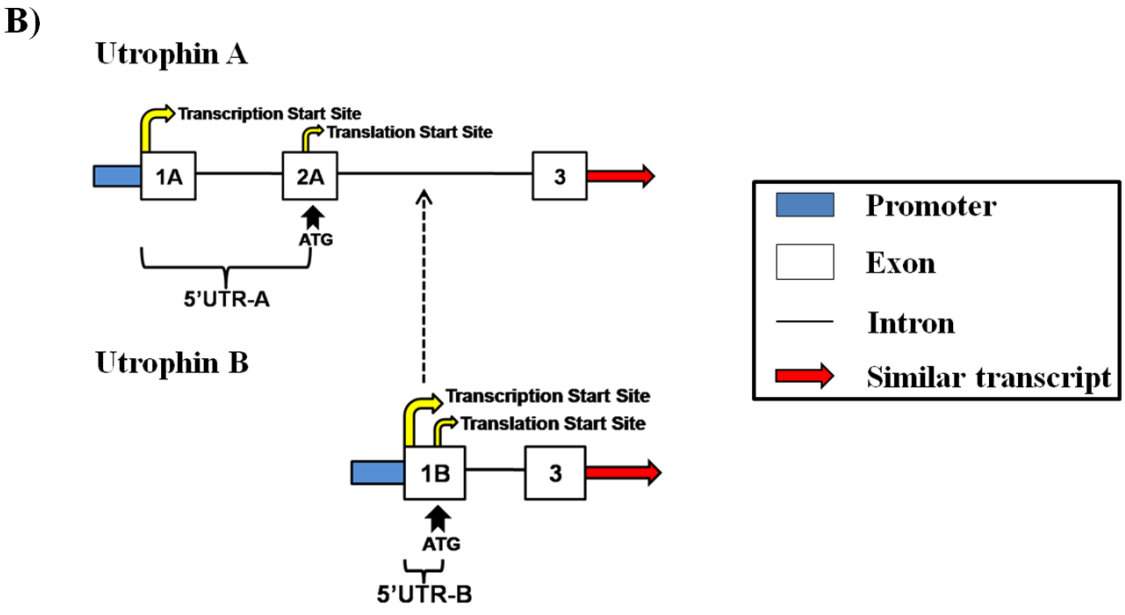

Motor Neuron
Muscle Fiber
ECM
α
β
β
γ
α
δ
β
α
α
Utrophin
Actin
Filaments
AChR
Agrin
nNOS
Glycans
Syntrophins
Dystrobrevin
Laminin
α-Dystroglycan
β-Dystroglycan
Sarcoglycans
Sarcospan

Figure 4. Schematic presentation of utrophin isoforms.

A) Different isoforms of utrophin. Shown are the actin-binding domains in green, spectrin-like repeats in gray, cysteine-rich domains in yellow, carboxy-terminal domains in blue, isoform names on the left and molecular weights on the right. B) Illustration of utrophin A and B promoters with the distinct 5'untranslated regions (UTR) found in the utrophin A and B transcripts, respectively. Note that the promoter B is located within the second intron of the utrophin gene, thus resulting in a unique 5' end and a full length mRNA expressing the third exon. Also, the utrophin A 5'UTR is significantly longer (508 nucleotides) then the utrophin B 5'UTR (74 nucleotides).


A)
Utrophin A
395 kD
Utrophin B
395 kD
G-Utrophin
113 kD
Actin-Binding Domain
Rod Domain (spectrin-like repeats)
Cysteine-rich Domain
C-Terminus Domain


B)
Utrophin A
Transcription Start Site
Translation Start Site
1A
2A
3
ATG
5'UTR-A
Utrophin B
Transcription Start Site
Translation Start Site
1B
3
ATG
5'UTR-B
Promoter
Exon
Intron
Similar transcript

1.3.2. Utrophin, a qualified replacement for dystrophin

Studies have demonstrated using transgenically modified *mdx* mice expressing the full-length or truncated form of utrophin, that introducing utrophin into dystrophic tissues led to the restoration of the DGC and significant functional improvement (Tinsley et al., 1996). In fact, transgenically-modified *mdx* mice overexpressing the full-length utrophin in the sarcolemma alleviated the dystrophic pathology by preventing dystrophy to occur in muscle tissues (Tinsley et al., 1998). Also, adenoviral-dependent delivery of full-length utrophin into dystrophic limb muscles of *mdx* mice greatly ameliorated the dystrophic phenotype (Deol et al., 2007). To further understand the physiological role of utrophin in dystrophic muscles, a mouse model that lacks both dystrophin and utrophin was generated by breeding the utrophin deficient mouse with the *mdx* mouse (*mdx*/utrn$^{-/-}$). Not surprisingly, the *mdx*/utrn$^{-/-}$ mice reproduced nearly all symptoms of DMD, such as muscle weakness, reduced overall skeletal muscle growth, heart muscle disease, myofibrosis, and most importantly premature death, compared to healthy mice (Deconinck et al., 1997;Deconinck et al., 1998;Grady et al., 1997). Also, delivery of truncated forms of utrophin into dystrophic tissues of *mdx* mice, *mdx*/utrn$^{-/-}$ mice and GRMD dogs, significantly ameliorated the dystrophic pathophysiology (Cerletti et al., 2003;Deconinck et al., 1997;Tinsley et al., 1996;Wakefield et al., 2000).

More recently, studies have shown that *mdx* mice injected with utrophin or micro-utrophin recombinant proteins conjugated to the HIV cell-penetrating TAT domain displayed restoration of membrane integrity and function by lowering creatine kinase serum levels, decreasing centrally nucleated fibers and improving contractile properties of dystrophic muscles (Sonnemann et al., 2009). It becomes very clear that the principal

advantages of using utrophin as a surrogate for dystrophin is the possibility of alleviating the dystrophic phenotype by restoring the DGC without activating the immune response. Thus, it's important to identify the mechanisms that regulate expression and localization of endogenous utrophin to potentially compensate for the absence of dystrophin in dystrophic muscles.

1.4. Regulation of the utrophin A promoter

Studies investigating the regulation of utrophin A at the NMJ have put a major focus on transcriptional regulatory mechanisms (All regulatory elements are summarized in **Figure 5**). Previously, the *mdx*/utrn$^{-/-}$ mice showed that both structural proteins, dystrophin and utrophin, were essential for full differentiation and maintenance of postsynaptic membranes (Deconinck et al., 1997). Also, only a 2 fold increase of full-length utrophin was sufficient to correct for the dystrophic pathology in skeletal muscle (Tinsley et al., 1998).

1.4.1. Cis-regulatory elements in the promoter region of utrophin A

Similar to the promoter region of the acetylcholinesterase (AChE), sequence analysis of the utrophin A promoter region revealed the absence of TATA and CAAT motifs generally present in eukaryotic genes (Dennis et al., 1996;Li et al., 1993). A number of genes lacking these motifs in their promoter regions encode for highly regulated proteins (Li et al., 1993). Instead, the basal promoter of utrophin contains a CpG-rich region that seems to drive transcription of utrophin in almost all cell types, such as human HeLa cells, human rhabdomyosarcoma IN157 cells, mouse C_2C_{12} myoblasts and monkey kidney COS-7 cells (Dennis et al., 1996).

Sequence analysis of the corresponding promoter region revealed the presence of an N-Box motif, which has a core TTCCGG sequence and is known to direct transcription of post-synaptic specific genes (Koike et al., 1995). To further demonstrate the important role of the N-box in mediating post-synaptic specific expression of utrophin, mutational analysis of the motif, along with agrin treatment, diminished the activity in muscle cells (Gramolini et al., 1997;Gramolini et al., 1998). Interestingly, mouse myotubes treated with heregulin, another nerve-derived trophic factor, resulted in an increase in utrophin expression by upregulating phosphorylation of the Ets-related transcription factor growth associated binding protein (GABP) α/β (Gramolini et al., 1999a;Khurana et al., 1999). In this context, phosphorylated GABPα/β would bind to the core Ets sequence contained within the N-Box region and further induce synapse-specific transcription of utrophin A. In fact, Ets-2 repressor factor (ERF) was shown to associate with the utrophin A promoter, via the N-Box motif, and downregulate its expression in the extrasynaptic regions of skeletal muscle fibers (Perkins et al., 2007). Additional studies have demonstrated that Sp1 and Sp3 transcription factors target the C/G region contained within the promoter and enhance transcriptional activation of utrophin by associating with GABP (Galvagni et al., 2001;Gyrd-Hansen et al., 2002).

Mutational analysis of the 1.3 kb promoter region led to the identification of a conserved muscle specific DNA consensus element, CANNTG, termed the E-Box (Gramolini et al., 1997;Gramolini and Jasmin, 1999;Perkins et al., 2001). Generally, myogenic regulatory transcription factors (MRFs), such as MyoD and myogenin, containing basic-helix-loop-helix motifs, bind to E-boxes and enhance transcription of muscle specific genes. Binding of MRFs to the E-Box sequence was shown to directly

regulate synaptic utrophin A expression during myogenesis (Perkins et al., 2001). In fact, utrophin mRNA levels were increased two fold in differentiating muscle cells (Gramolini et al., 1999b;Gramolini and Jasmin, 1999;Nudel et al., 1988). Moreover, the downstream utrophin enhancer (DUE) located within the second intron, 9kb downstream of the transcription start site, was shown to regulate transcriptional activity of utrophin in cardiac and skeletal muscles of transgenic mice (Tanihata et al., 2008).

Further examination of the utrophin A promoter region revealed that the muscle fiber specific abundance of utrophin A in slow muscle can be in part explained by the activation of calcineurin (Chakkalakal et al., 2003;Gramolini et al., 2001b). Calcineurin is a protein phosphatase that primarily activates the T cells of the immune system. Within the murine utrophin A promoter region is the nuclear factor of activated T-cell (NFAT)-binding site, which was shown to be conserved in the human utrophin A promoter (Chakkalakal et al., 2003). Both calcineurin and NFAT expression were shown to be highly expressed at the NMJ (Angus et al., 2005). Calcineurin-dependent dephosphorylation of NFAT ultimately causes the translocation of NFAT from the cytoplasm to the nucleus to bind specific genes, such as utrophin A, and enhance transcriptional activation in post-synaptic regions. Also, mutation of the NFAT binding site decreased transcriptional activity of utrophin A at the synapse (Angus et al., 2005).

1.4.2. Transcriptional activators of the utrophin A promoter

A special feature of slow oxidative fibers is the high level of intracellular calcium, which consequently activates calcineurin via calmodulin to promote the slower more oxidative phenotype (Meinhardt, 2000;Olson and Williams, 2000). Transgenic upregulation of calcineurin activity in *mdx* mice consequently increased the sarcolemmal

expression of utrophin A, displayed a restoration of the DGC at the sarcolemma, alleviated the inflammatory response and reduced contraction-mediated injury in dystrophic muscles (Chakkalakal et al., 2004;Stupka et al., 2006). To further understand the importance of calcineurin in mediating utrophin A expression, inhibition of calcineurin in healthy mice with either cyclosporin A or FK506 pharmacological compounds resulted in a reduction in utrophin A expression at synaptic regions (Angus et al., 2005).

Extensive analysis of the GABP/N-box pathway led to the identification of a transcriptional coactivator, peroxisome proliferator γ co-activator 1α (PGC-1α), which drives the expression of GABP specifically in slow muscle fibers, ultimately enhancing utrophin A transcription (Angus et al., 2005). In fact, *mdx* mice transgenically expressing PGC-1α exhibited improved dystrophic pathology such as decreased creatine kinase level and minimized muscle damage (Handschin et al., 2007). Interestingly, alteration of PGC-1α expression causes a shift in fiber type from slow to fast (Handschin et al., 2007;Lin et al., 2002). CaM-binding peptides (CaMBP) major role is to inhibit downstream signalling events of calcineurin. Inhibiting calmodulin in slow muscle of *mdx* mice by crossing with transgenic mice overexpressing CaMBP led to the downregulation of PGC-1α, GABP, MyHCI as well as utrophin A and resulted in a more severe dystrophic pathology with a shift in fiber type toward the faster phenotype (Chakkalakal et al., 2006). Furthermore, heregulin was shown to induce the phosphorylation of PGC-1α, which in turn activates the utrophin A promoter indirectly by stimulating GABP activity (Angus et al., 2005;Gramolini et al., 1999a). In fact, *mdx* mice treated with a small peptide comprising the epidermal growth factor-like region of heregulin ectodomain displayed upregulated

levels of utrophin at the sarcolemma and amelioration of the dystrophic pathology, making it a good candidate for drug-based therapy (Krag et al., 2004).

The expression profile of utrophin A in different fibre types led to the identification of an additional regulatory sequence in the promoter region of utrophin A known as the peroxisome proliferator-activated receptor (PPAR) response element (PPRE) (Grimaldi, 2003;Luquet et al., 2003). Nuclear receptor PPARβ/δ, the predominant form in skeletal muscles, was shown to induce the conversion of fibers to slow type and to upregulate transcriptional activity of utrophin A in muscle tissues (Miura et al., 2009;Muoio et al., 2002). Furthermore, when *mdx* mice were treated with a PPARβ/δ agonist (GW50156), muscle analysis revealed a restoration of the DGC, upregulation of utrophin A protein, an increase in the number of oxidative fibers and most importantly, improved muscle function (Miura et al., 2009).

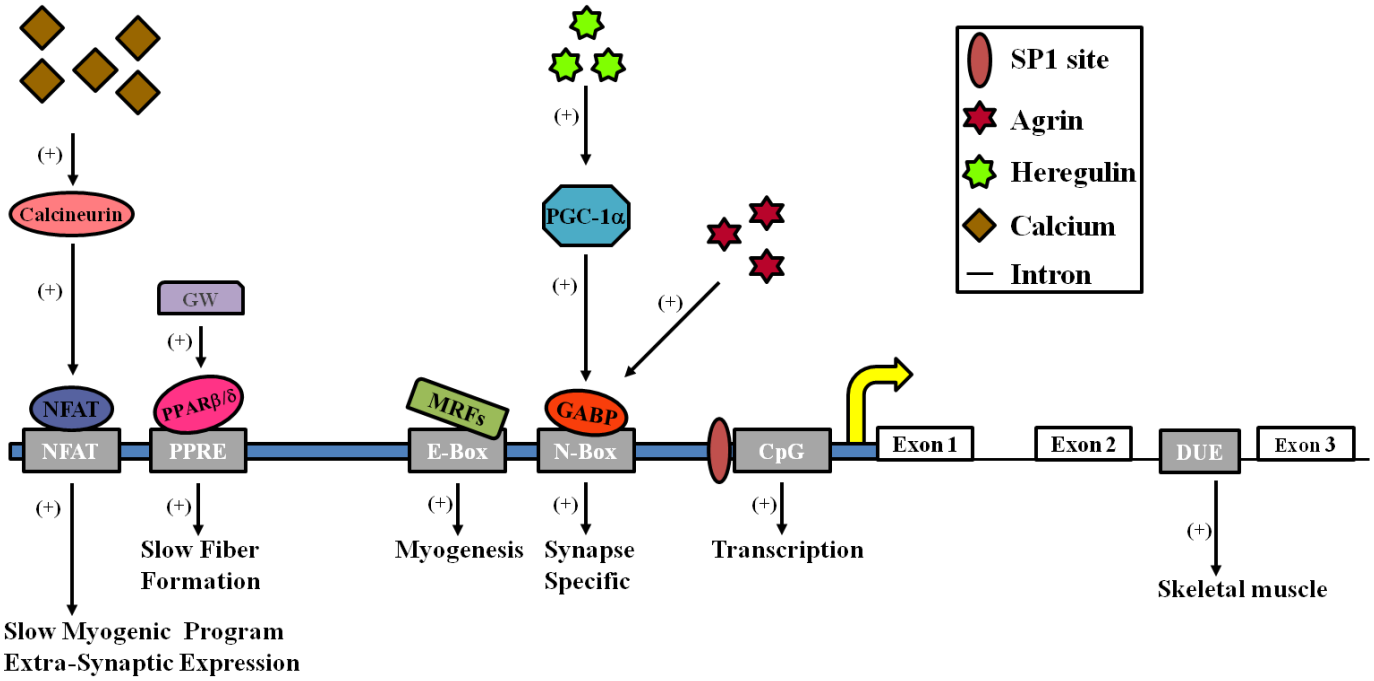
In this context, utrophin expression at the sarcolemma has been previously shown to be increased in muscle of *mdx* mice treated with L-arginine, a substrate for the signalling DGC-associated protein nNOS, the enzyme that catalyzes the generation of NO and citrulline (Chaubourt et al., 1999). In fact, L-arginine administration resulted in significant improvement of the contractile properties in *mdx* mice (Barton et al., 2005). In addition, drug-treatment of *mdx* mice with molsidomine, a therapeutic drug that increases NO production, caused an increase in utrophin levels, a decrease in serum creatine kinase levels and amelioration in muscle morphology (Voisin et al., 2005). Thus, stimulating expression of nNOS constitutes a potential therapy for DMD since it upregulates utrophin expression and ameliorates the dystrophic phenotype in *mdx* mice.

Based on the findings that upregulating utrophin A in dystrophic muscle can alleviate the disease pathology, it became important to develop different strategies to regulate its expression. A potential therapeutic strategy to upregulate utrophin A expression in dystrophic tissues is the use of engineered artificial zinc-finger based transcription factors (ZF ATFs). One potential ZF ATF named Jazz was generated to specifically targets a nine-base pair DNA sequence contained within the promoter of both the human and the mouse utrophin A genes (Corbi et al., 2000). Transgenic mice overexpressing the muscle specific artificial Jazz gene showed successful upregulation of utrophin A expression specifically in muscle tissues, in addition to the relocalization of utrophin A to extrasynaptic regions (Passananti et al., 2010). Crossing of the Jazz transgenic mice with the *mdx* mice further increased utrophin A levels by 1.8 fold in dystrophic tissues (Di Certo et al., 2010). In order for Jazz-based therapy to be applicable, the factor has to be combined with gene-based methods to ensure successful delivery into dystrophic muscle.

Another alternative mechanism shown to upregulate utrophin A expression in extrasynaptic regions of healthy and dystrophic muscles is by overexpressing a glycosylating enzyme called Cytotoxic T cells β1-4-*N*-acetylgalactosamine transferase (CT GalNAcT). When CT GalNAcT is transgenically overexpressed in *mdx* mice, it was shown to increase endogenous levels of utrophin A in extrasynaptic regions and consequently improve the dystrophic pathology, making it a potential targeting candidate for DMD therapy (Durko et al., 2010;Nguyen et al., 2002).

Figure 5. C*is*-regulatory promoter elements that control expression of utrophin A.

Cis-Regulatory elements located in the utrophin A promoter that regulate its transcriptional activity in slow muscle fibers, in synaptic and extra-synaptic localization, and in myogenesis. Arrows accompanied with (+) means that the element positively regulates the targeted molecule. NFAT, nuclear factor of activated T cells; PPRE, PPAR response element; PPARβ/δ, peroxisome proliferator-activated receptor β/δ; MRF, myogenic regulatory factors; GABP, Ets-related transcription factor growth associated binding protein; PGC-1α, peroxisome proliferator γ co-activator 1α; DUE, downstream utrophin enhancer; GW, PPARβ/δ agonist.


SP1 site
Agrin
Heregulin
Calcium
Intron
(+)
Calcineurin
GW
PGC-1α
MRFs
GABP
NFAT
PPARβ/δ
NFAT
PPRE
E-Box
N-Box
CpG
Exon 1
Exon 2
DUE
Exon 3
Slow Fiber Formation
Myogenesis
Synapse Specific
Transcription
Skeletal muscle
Slow Myogenic Program
Extra-Synaptic Expression

1.5. Post-transcriptional regulation of utrophin A

In order for a gene to become a functional protein, it has to undergo a series of steps. First, the gene has to be transcribed into a pre-messenger RNA in the nucleus. Then, in order for the pre-mRNA to become a mature RNA, it undergoes splicing of introns, capping of the 5'end with a methylated guanosine, and polyadenylation of the 3'end. These additions prevent the mRNA from being degraded in the nucleus while enabling transport out of the nucleus. When the mature mRNA reaches the cytoplasm, several regulatory elements within the mRNA control their stability and localization therein. Finally, when the mRNA reaches its destination, translational machinery enable the production of its prospective protein. Regulation of any of these steps can have an effect on gene expression (Day and Tuite, 1998;Saini et al., 1990). Over the past decade, post-transcriptional regulation has been one of the major topics in molecular biology. Post-transcriptional regulation describes the interaction of trans factors with cis-elements in either the 3'UTR or the 5'UTR of transcripts that could in turn control their localization, stability or even the efficiency of translation (Keene, 2010;Saini et al., 1990).

In addition to transcriptional regulation, utrophin A can also be regulated at the post-transcriptional level via its 3'UTR (Chakkalakal and Jasmin, 2003). Comparison of the full-length utrophin 3'UTR among different species revealed that the global sequence identity of the utrophin 3'UTR between human and mouse is around 80%, where specific regions in the 3'UTR show higher homology. More specifically, nucleotides within regions 352 and 583 share 87% homology (Chakkalakal et al., 2008;Gramolini et al., 2001a)(**Figure 6**).

1.5.1. Localization

It is now well established that utrophin A protein and mRNA levels are higher in slow soleus as compared to fast extensor digitorum longus (EDL) muscles. Immunofluorescence and in situ hybridization experiments revealed that this heightened utrophin expression occurred specifically in extrasynaptic regions of slow muscle fibers (Gramolini et al., 2001b). Subcellular fractionation experiments demonstrated that the ~2 kb murine utrophin A 3'UTR contains regulatory elements, more specifically within nucleotides 332 and 969 (**Figure 6**), responsible for appropriate localization of the utrophin transcript to the cytoskeleton in differentiated myotubes (Gramolini et al., 2001a).

1.5.2. Stability

In addition to localization, deletion analysis revealed that a destabilizing element was contained within nucleotides from positions 332 to 596 of the utrophin A 3'UTR that was responsible for preferentially suppressing reporter mRNA levels in fast muscles (**Figure 6**) (Chakkalakal et al., 2008). Furthermore, sequence analysis of that specific region revealed that 72% of the nucleotides contained within that locus are either adenosine or uridine (Chakkalakal et al., 2008). Moreover, there are three potential AU-rich elements (ARE), efficient determinants of RNA stability, within these 265 nucleotides, one of which is conserved between mouse and human transcripts (Barreau et al., 2005;Bevilacqua et al., 2003). In addition to enhancing transcription of utrophin in extrasynaptic regions of slow muscle fibers, calcineurin was also capable of regulating

the stability of utrophin A mRNA through the conserved ARE contained within the 3'UTR (Chakkalakal et al., 2008).

Another known post-transcriptional regulatory mechanism controlling utrophin expression involves micro-RNA 206 (miR-206) and the myogenic factor MyoD (Rosenberg et al., 2006). Characterized as post-transcriptional regulators of gene expression, miRNAs are specific complementary sequences that usually target the 3'UTR and induce silencing of the gene (Williams et al., 2009). It was shown that MyoD activation led to the induction of miR-206, which further resulted in the downregulation of utrophin expression (Rosenberg et al., 2006).

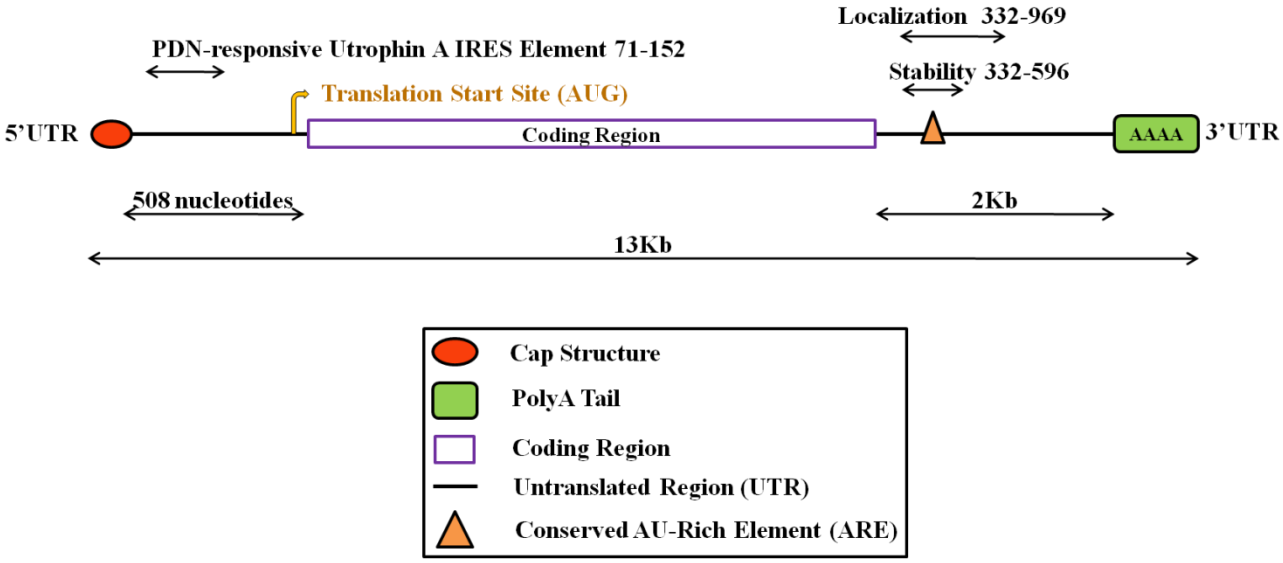
Figure 6. Schematic representation of the utrophin A transcript and its *cis*-regulatory elements.

The region within nucleotides 71 and 152 in the 5'untranslated region (UTR) was shown to enhance cap-independent translation initiation upon glucocorticoid treatment (PDN, α-methylprednisolone-21 sodium succinate). The region within nucleotides 332 and 969 in the 3'UTR was shown to regulate utrophin A localization to the cytoskeleton in differentiated myotubes. Regions within nucleotides 332 and 596 in the 3'UTR is responsible for controlling stability of utrophin A in slow muscle fibers, specifically through conserved AU-rich element (ARE). Both the 5'UTR and 3'UTR of human and mouse share high sequence identity. IRES, internal ribosome entry site; Kb, Kilo bases

Sequence Identity 5'UTR
Human and Mouse 63%

Sequence Identity 3'UTR
Human and Mouse 80%

GC nucleotides in 5'UTR of mouse: 58%



1.6. Translational regulation of the utrophin A 5'UTR

1.6.1. The utrophin A 5'UTR

The human utrophin A 5'UTR, a possible target for translational control mechanisms, is comprised of 560 nucleotides whereas the mouse homologue has 508 nucleotides, where 58% of the nucleotides are GC. Overall, the full-length human and mouse 5'UTR are 63% identical, despite the 77.5% identity that they share in the first 298 nucleotides (**Figure 6**) (Burton et al., 1999;Dennis et al., 1996). Converging lines of evidence have suggested that transcriptional mechanisms cannot explain for the discordant levels of utrophin A protein and mRNA observed under different physiological conditions. A contrast between protein and mRNA levels of utrophin was first identified when comparing muscle biopsies from DMD patients. While utrophin protein was higher in skeletal muscles from DMD patients, utrophin mRNA levels were similar to healthy muscles (Gramolini et al., 1999b). Similar reports indicating transcription-independent regulation of utrophin A was identified in the *mdx* mouse model. Skeletal muscles excised from *mdx* mice showed an upregulation of utrophin A protein when compared to wildtype mice. The small increase in utrophin A mRNA was not consistent with the massive increase of utrophin A protein (Weir et al., 2002).

Additional evidence of transcription-independent regulation of utrophin A was identified in glucocorticoid-treated muscle cells. Glucocorticoid treatment was shown to improve the pathology of dystrophic muscles in DMD patients (Bonifati et al., 2000;Fenichel et al., 1991b) and in *mdx* mice (Keeling et al., 2007;Passaquin et al., 1993). Glucocorticoid-treated muscle cells showed an increase in utrophin A protein without a corresponding increase in transcript level when compared to untreated muscles

(Courdier-Fruh et al., 2002;Pasquini et al., 1995). Overall, the aforementioned studies showing variability in utrophin A expression (protein versus mRNA) in various conditions suggest the involvement of regulatory events targeting either translational mechanisms or post-translational mechanisms, such as protein stability.

1.6.2. Cap-Dependent translation in Eukaryotic Cells

The major ribosome-scanning mechanism of translation initiation in eukaryotes is dependent on a 7-methyl guanosine cap structure situated on the 5'UTR. In normal circumstances, translation is initiated when the eukaryotic initiation factor (eIF) 4F cap-binding complex recognizes and binds the cap structure. The eIF4F complex is composed of a scaffolding protein, eIF4G, which bind eIF4E, the cap-binding component, and eIF4A to the mRNA. Necessary to unwind the RNA secondary and tertiary structures, eIF4A is an ATP-dependent helicase activated upon association with eIF4B. Meanwhile, the 43S pre-initiation complex is formed by binding of the 40S ribosomal subunit to a protein complex composed of eIF1, eIF2-GTP-Met-tRNA (also known as the ternary complex), eIF3 and eIF5. When formation is complete, the 43S complex recognizes and binds to the central domain of eIF4G via eIF3 to form the 48S ribosomal complex and further initiate scanning of the mRNA (**Figure 7A**). Recognition of the first AUG start codon enables the hydrolysis of GTP to GDP by eIF5, followed by dissociation of initiation factors. Protein synthesis is initiated when the 80S ribosome is formed by binding of the large ribosomal subunit to the small ribosomal subunit. Following binding of the poly-A binding protein (PABP) to the 3' poly-A region, it interacts with eIF4G to promote proper folding of the mRNA and further allow several rounds of translation initiation (Holcik et al., 2000;Van Der et al., 2009).

A number of additional mechanisms regarding eukaryotic cap-dependent translation were shown to target the 5'UTR and synthesize specific proteins. When the ribosome bypasses the first AUG codon because it is localized in a poor nucleotide-containing region, it is forced to continue scanning and to initiate translation at a further downstream AUG codon. This particular method of scanning is called 'leaky scanning'. Another well studied alternative to translation initiation is ribosomal shunting, where the ribosome encounters a large secondary structure and ultimately bypasses this region to scan for a downstream AUG codon. Also, a number of mRNAs possess a second open reading frame (ORF) and generally, a fraction of ribosomes continue scanning even after passing the first stop codon and reinitiate at the second AUG codon. This process is called the 'reinitiation mechanism' (Kozak, 1999).

1.6.3. Internal Ribosome Entry Site

One of the most studied cap-independent translation initiation mechanisms present both in prokaryotic and eukaryotic cells is through internal ribosome entry sites (IRES) located in the 5'UTR of specific mRNAs. The first IRESs were originally discovered in picornavirus mRNAs where they initiate translation of uncapped viral mRNAs (Jang et al., 1988;Pelletier and Sonenberg, 1988). Three years after this discovery, the first cellular IRES was found in the transcript encoding immunoglobulin heavy-chain binding protein (BiP) which was shown to be translated upon poliovirus infection, where the canonical method of translation initiation is down-regulated (Macejak and Sarnow, 1991). To date, more than 85 cellular IRES have been identified and the list is constantly growing (Baird et al., 2006).

IRESs are untranslated regions of mRNAs that initiate protein synthesis during cellular stresses (Merrick, 2004). They are considered as alternatives to cap-dependent translation (Hellen and Sarnow, 2001). Interestingly, stress-induced silencing of cap-dependent translation enhances the recruitment of ribosomal sub-units, independently of the cap structure, to 5'UTRs containing IRES to initiate protein synthesis of necessary survival genes (**Figure 7B**) (Holcik and Sonenberg, 2005). The main conditions where IRESs have shown to function are in cellular perturbations such as mitosis, viral infections, apoptosis and hypoxia (Hellen and Sarnow, 2001;Holcik and Sonenberg, 2005;Johannes et al., 1999;King et al., 2010). There is no specific core sequence or secondary/tertiary structure that would allow the identification of potential cellular IRESs, although this does not seem to be the case for viral IRESs (Baird et al., 2006).

Various stress-dependent molecular modifications of translation initiation factors have been shown to silence cap-dependent translation and ultimately induce IRES-dependent translation initiation. Biochemical modifications induced by environmental stresses include cleavage of eIF4G or PABP, phosphorylation of eIF2α and dephosphorylation of eIF4E-binding protein 1 (4E-BP1) (Blaszczyk et al., 2007;Fitzgerald and Semler, 2009). Depending on the targeted IRES, there has been characterization of several IRES trans-acting factors (ITAFs) that were shown to either enhance or silence IRES-dependent translation by acting as chaperone or stabilize/remodel the mRNA (Fitzgerald and Semler, 2009;Lewis et al., 2007;Lewis and Holcik, 2008;Spriggs et al., 2005). The polypyrimidine tract-binding protein (PTB) seems to target a wide range of IRESs (Bushell et al., 2006;Mitchell et al., 2005). Other ITAFs

known to regulate IRESs include DAP5 and Lupus autoantigen (La) (**Figure 7B**) (Spriggs et al., 2005).

The most widely used method to assess IRES activity is by generating bicistronic vectors where the IRES of interest is inserted between two reporter genes. The second cistron located downstream of the IRES of interest would be expressed only if cap-independent translation is enhanced under cellular stress. There are several advantages in using IRESs to study gene expression such as the coexpression of two different genes in the same cell population at a fixed expression ratio. Moreover, in addition to studying expression of RNAs lacking a 5'cap structure, IRESs can mediate protein synthesis in stress-induced cells (Filbin and Kieft, 2009). It is very important to provide proper controls when engineering IRES containing bicistronic vectors in order to eliminate controversies on cryptic promoter activity and possible internal splicing events.

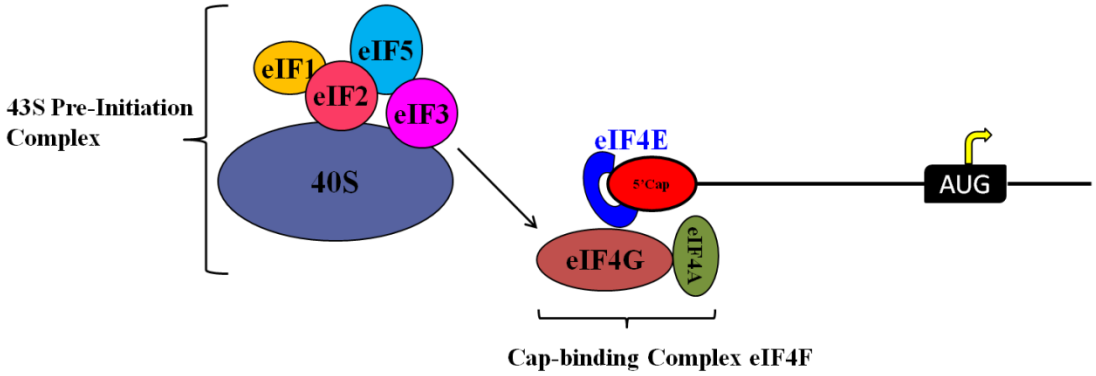
Several studies have shown that the 5'UTR of utrophin A contains an IRES which is activated under different physiological stress conditions (Miura et al., 2005;Miura et al., 2008). Cardiotoxin-treatment of skeletal muscles showed an increase in utrophin A protein levels when compared to untreated muscles. This massive increase of utrophin A protein levels was in stark contrast with the small increase in utrophin A transcript levels. This same study showed that, in healthy skeletal muscles, the 5'UTR of utrophin A caused the translational suppression of a reporter transcript. Conversely, additional experiments showed that the 5'UTR of utrophin A could mediate expression of a reporter protein in regenerating muscles and that this translational effect involves an IRES element located within the 5'UTR of utrophin A (Miura et al., 2005). A later study demonstrated the activation of the utrophin A IRES in glucocorticoid-treated muscle

cells. Moreover, C_2C_{12} muscle cells transfected with several truncated variants of the utrophin A 5'UTR bicistronic vector and then treated with 6α-methylprednisolone-21 sodium succinate (PDN) led to the identification of the PDN-responsive IRES element located within nucleotides 71-152 of the utrophin A 5'UTR (**Figure 6**)(Miura et al., 2008). Taken together, understanding the mechanisms regulating utrophin A expression at the transcriptional and post-transcriptional levels will allow development of therapies that would ameliorate the dystrophic pathology in DMD patients.

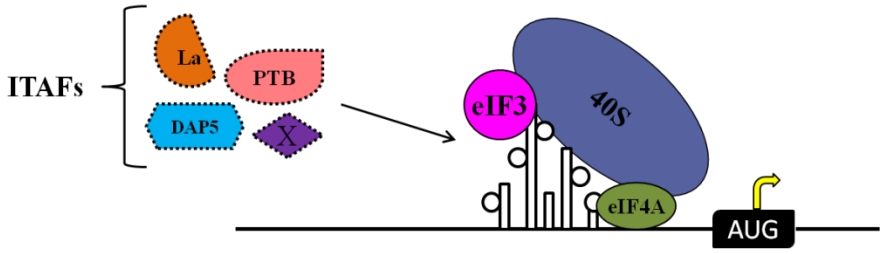
Figure 7. Cap-dependent versus IRES-dependent translation initiation in eukaryotic cells.

A) Translation is initiated when eIF4F complex recognizes and binds to the cap structure. The 43S pre-initiation complex recognizes and binds to the cap-binding complex, ultimately forming the 48S complex. The 48S complex then scans along the mRNA until it finds the first AUG. Binding of the 60S ribosomal subunits to 48S allows for translation elongation to take over. B) The 5'untranslated regions (UTR) of IRES containing mRNAs are usually long non-specific sequences with high secondary/tertiary structures. For IRES-dependent translation to occur, it does not require a cap structure at the 5'UTR. IRES structures are commonly recognized by the 40S subunit and some of the canonical translation initiation factors. In addition, various ITAFs are thought to bind IRES structures and enhance/inhibit translation initiation. eIF, eukaryotic initiation factor; 40S-43S, 40-43 kDa subunit; IRES, internal ribosome entry site; ITAF, IRES trans-acting factor; PTB, polypyrimidine tract-binding protein; La, Lupus autoantigen.

A) Cap-dependent translation Initiation



B) IRES-dependent translation Initiation



2. Statement of Problem, Hypothesis, Objectives

DMD is a lethal neuromuscular disorder caused by the lack of functional dystrophin. To date, there is no curative therapy but accumulating studies have shown that utrophin could compensate for the lack of dystrophin in dystrophic muscle fibers, thus making it a good therapeutic target (Blake et al., 2002). To this end, it becomes important to understand the mechanisms regulating utrophin A levels, in order to enhance its expression at the sarcolemma of dystrophic muscles and potentially alleviate the dystrophic pathology.

Based on previous findings that the utrophin A 5'UTR contains an IRES that is highly active under various stress conditions, and that cellular IRESs can be differentially regulated in different tissues (Creancier et al., 2000;Miura et al., 2005;Stoneley et al., 2000), we hypothesize that utrophin A IRES activity is specifically regulated in different tissues of transgenic mice expressing the utrophin A 5'UTR bicistronic reporter. Furthermore, as mentioned in the introduction, various cellular and viral IRESs were shown to be regulated by ITAFs. Thus, we further hypothesize that specific ITAFs bind to the 5'UTR of utrophin A and further regulate IRES-dependent translational activity.

To this end, our main objectives are to:

1) Characterize utrophin A IRES activity in different tissues of transgenic mice expressing the utrophin A 5'UTR bicistronic reporter.
2) Identify ITAFs that can regulate utrophin A IRES activity.
3) Study the physiological role of the identified ITAFs on utrophin A IRES activity and utrophin A expression.

Chapter 2

The utrophin A 5'UTR drives cap-independent translation exclusively in skeletal muscles of transgenic mice and interacts with eEF1A2

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Contribution from authors

Adèle Coriati, Pedro Miura and Guy Bélanger performed the experiments. More specifically, Adèle Coriati conducted all the transgenic mice experiments as well as the qRT-PCR analysis of eEF1A1 and eEF1A2 in all tissues, whereas Pedro Miura and Guy Bélanger performed the remaining *in vitro* experiments (Northwestern, RNA-Chromatography, Western, UV Cross-Linking and cell culture). Adèle Coriati, Pedro Miura, Martin Holcik and Bernard J. Jasmin analyzed the data. Pedro Miura and Bernard J. Jasmin wrote the manuscript. Edited by Adèle Coriati, Guy Bélanger, Rashmi Kothary, Martin Holcik, and Jonathan Lee. Yves de Repentigny performed the microinjections. Reagents and expertise provided by Rashmi Kothary, Jonathan Lee and Martin Holcik.

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Abstract

The molecular mechanisms regulating expression of utrophin A are of therapeutic interest since upregulating its expression at the sarcolemma can compensate for the lack of dystrophin in animal models of Duchenne Muscular Dystrophy (DMD). The 5'UTR of utrophin A has been previously shown to drive cap-independent internal ribosome entry site (IRES)-mediated translation in response to muscle regeneration and glucocorticoid treatment. To determine whether the utrophin A IRES displays tissue specific activity, we generated transgenic mice harboring control (CMV/βGAL/CAT) or utrophin A 5'UTR (CMV/βGAL/UtrA/CAT) bicistronic reporter transgenes. Examination of multiple tissues from two CMV/βGAL/UtrA/CAT lines revealed that the utrophin A 5'UTR drives cap-independent translation of the reporter gene exclusively in skeletal muscles and no other examined tissues. This expression pattern suggested that skeletal muscle-specific factors are involved in IRES-mediated translation of utrophin A. We performed RNA-affinity chromatography experiments combined with mass spectrometry to identify *trans*-factors that bind the utrophin A 5'UTR and identified eukaryotic elongation factor 1A2 (eEF1A2). UV-crosslinking experiments confirmed the specificity of this interaction. Regions of the utrophin A 5'UTR that bound eEF1A2 also mediated cap-independent translation in C2C12 muscle cells. Cultured cells lacking eEF1A2 had reduced IRES activity compared to cells overexpressing eEF1A2. Together, these results suggest an important role for eEF1A2 in driving cap-independent translation of utrophin A in skeletal muscle. The *trans*-factors and signaling pathways driving skeletal-muscle specific IRES-mediated translation of utrophin A could provide unique targets for developing pharmacological-based DMD therapies.

Introduction

The fatal neuromuscular disease Duchenne Muscular Dystrophy (DMD) is caused by loss of dystrophin expression in the muscle fibers of affected patients (Blake et al., 2002). Utrophin is the autosomal homologue of dystrophin, and through multiple approaches it has been demonstrated that enhancing its expression can compensate for the lack of dystrophin in animal models of DMD and alleviate the muscle pathology (Cerletti et al., 2003;Chakkalakal et al., 2004;Sonnemann et al., 2009;Tinsley et al., 1996). A major difference between dystrophin and utrophin A (the utrophin isoform expressed in skeletal muscle) is that while dystrophin is expressed along the entire sarcolemma, utrophin A expression in mature fibers is primarily restricted to post-synaptic regions of the sarcolemma (Baby et al., 2010;Weir et al., 2002). It is thus of considerable therapeutic interest to identify mechanisms by which utrophin A expression can be enhanced along the entire sarcolemma of DMD patient muscle fibers.

We have found that post-transcriptional mechanisms play an important role in enhancing expression of utrophin A at the sarcolemma. For instance, in addition to transcriptional mechanisms involving calcineurin/NFAT signaling, post-transcriptional events targeting the utrophin A 3'UTR can modulate the stability of utrophin A transcripts, thus contributing to the enhanced expression of utrophin A in extrasynaptic regions of slow-twitch, oxidative fibers as compared to fast-twitch, glycolytic fibers (Chakkalakal et al., 2003;Chakkalakal et al., 2008). Increased sarcolemmal expression of utrophin A also occurs in skeletal muscles undergoing regeneration. Interestingly, this enhancement of utrophin protein expression is not accompanied by concomitant increases in utrophin A mRNA levels, as observed both in regenerating versus control mouse

skeletal muscles and in muscle biopsies of DMD patients compared to healthy subjects (Gramolini et al., 1999b). Enhancement of utrophin A protein expression in response to muscle regeneration can be at least partially explained by increased translation initiation mediated by an internal ribosome entry site (IRES) located in the utrophin A 5'UTR (Miura et al., 2005).

IRES elements are thought to recruit the ribosome, IRES *trans*-acting factors (ITAFs), and other components of the translational machinery to the 5'UTRs of some cellular mRNAs (Baird et al., 2006). IRES-mediated translation initiation occurs independently of the 7-methyl guanosine cap at the 5' end of the mRNA, thus providing an alternative to the canonical, cap-dependent mechanism of translation initiation. This alternative mechanism allows for the cell to express a subset of proteins under stress conditions where global cap-dependent translation is suppressed, such as during viral infection, hypoxia, and apoptosis (Spriggs et al., 2008). In the case of the utrophin A IRES, its activity is enhanced during muscle regeneration and glucocorticoid treatment when global cap-dependent translation may be suppressed (Miura et al., 2005;Miura et al., 2008).

In this study, we set out to determine the tissue distribution of utrophin A IRES activity by generating and characterizing utrophin A 5'UTR reporter transgenic mice. We also initiated experiments to identify ITAFs that interact with the utrophin A 5'UTR and regulate its IRES activity.

Results

Generation of transgenic mice harboring CMV/βGAL/CAT and CMV/βGAL/UtrA/CAT reporters

Previous work on the FGF-2 IRES has shown that tissue specificity of IRES activity *in vivo* is not necessarily correlated with activity in cell lines (Creancier et al., 2000). We thus generated transgenic mice harboring a bicistronic utrophin A 5'UTR reporter. For the creation of utrophin A 5'UTR reporter transgenic mice, we used the CMV/βGAL/CAT bicistronic vector (**Figure 8A**). This vector was chosen because it does not exhibit splicing or internal promoter activity under a variety of conditions when harboring the utrophin A 5'UTR (Miura et al., 2005;Miura and Jasmin, 2006), and because previous studies have employed bicistronic reporters driven by the CMV promoter to evaluate IRES activity in transgenic mice (Creancier et al., 2000). From 3 founder mice identified by genotyping, full analysis was performed on two utrophin A 5'UTR reporter transgenic lines (CMV/βGAL/UtrA/CAT), namely lines 863 and 876. To serve as a control, we generated a transgenic line harboring a control bicistronic reporter (CMV/βGAL/CAT).

The utrophin A 5'UTR drives muscle-specific translation in transgenic mice

We examined multiple tissues of the transgenic mouse lines for IRES activity. The reporter transgene provides a read-out of cap-dependent translation as β-galactosidase activity (βGAL), while cap-independent, IRES-mediated translation, is reported as chloramphenicol acetyltransferase activity (CAT). In three transgenic lines harboring the utrophin A 5'UTR reporter, we detected appreciable levels of IRES activity (reported as a ratio of CAT to βGAL) in hindlimb skeletal muscles. Interestingly, we did

not detect IRES activity in any other organs examined, including the kidney, heart, lung, liver and brain (**Figure 8B and C**). In line 863, IRES activity in the tibialis anterior (TA) and gastrocnemius (gastroc) skeletal muscles was 14.5-fold and 10.7-fold above background brain levels (**Figure 8B**). In line 876, a similar trend was observed, with IRES activity 7.3-fold and 6-fold greater than background brain levels in the TA and gastroc, respectively (**Figure 8C**). A third line harboring the CMV/βGAL/UtrA/CAT (line 881) also displayed IRES activity exclusively in skeletal muscles (data not shown).

Examination of the above data expressed as individual βGAL (**Figure 9A-C**) and CAT (**Figure 9D-F**) values revealed that the increase in CAT to βGAL ratio in TA and gastroc muscles of the 863 and 876 lines is due to enhanced CAT expression, and not merely a result of low βGAL levels stemming from low CMV driven transcription. These findings indicate that, *in vivo*, cap-independent translation driven by the utrophin A IRES occurs exclusively in skeletal muscles and no other tissues.

We performed several control experiments to ensure that the skeletal muscle-specific expression of the CAT reporter could be attributed to genuine utrophin A IRES activity. Examination of the control transgenic line (CMV/βGAL/CAT) revealed that while βGAL activity was detectable in all tissues (**Figure 9C**), appreciable levels of CAT activity could not be detected (**Figure 9F**). This result demonstrates that the CAT activity detected in skeletal muscles of transgenic lines 863 and 876 can be attributed solely to the utrophin A 5'UTR.

Figure 8. Utrophin A 5'UTR bicistronic reporter transgenic mice display IRES activity exclusively in skeletal muscles.

A) Schematic representation of utrophin A 5'UTR (CMV/βGAL/UtrA/CAT) reporter transgene used for generation of transgenic mice. B) Relative IRES activity in various tissues of CMV/βGAL/UtrA/CAT reporter transgenic line 863. Values are standardized to the brain IRES activity ratio. *$P < 0.05$, relative to brain, n=6. C) Relative IRES activity in various tissues of CMV/βGAL/UtrA/CAT reporter transgenic line 876. Values are standardized to the brain IRES activity ratio. #$P < 0.05$, relative to heart, n=5. See fig. 9 for individual CAT and βGAL reporter levels D) qPCR analysis using βGAL and CAT primers was performed on reverse transcriptase-amplified RNA from various tissues of lines 876 and 863. The ratio of amplified βGAL and CAT cDNAs is not significantly different among the different tissues in both lines, providing evidence for the exclusive presence of an intact bicistronic transcript ($P > 0.05$, relative to brain, n=3). TA-tibialis anterior, Gastroc-gastrocnemius.

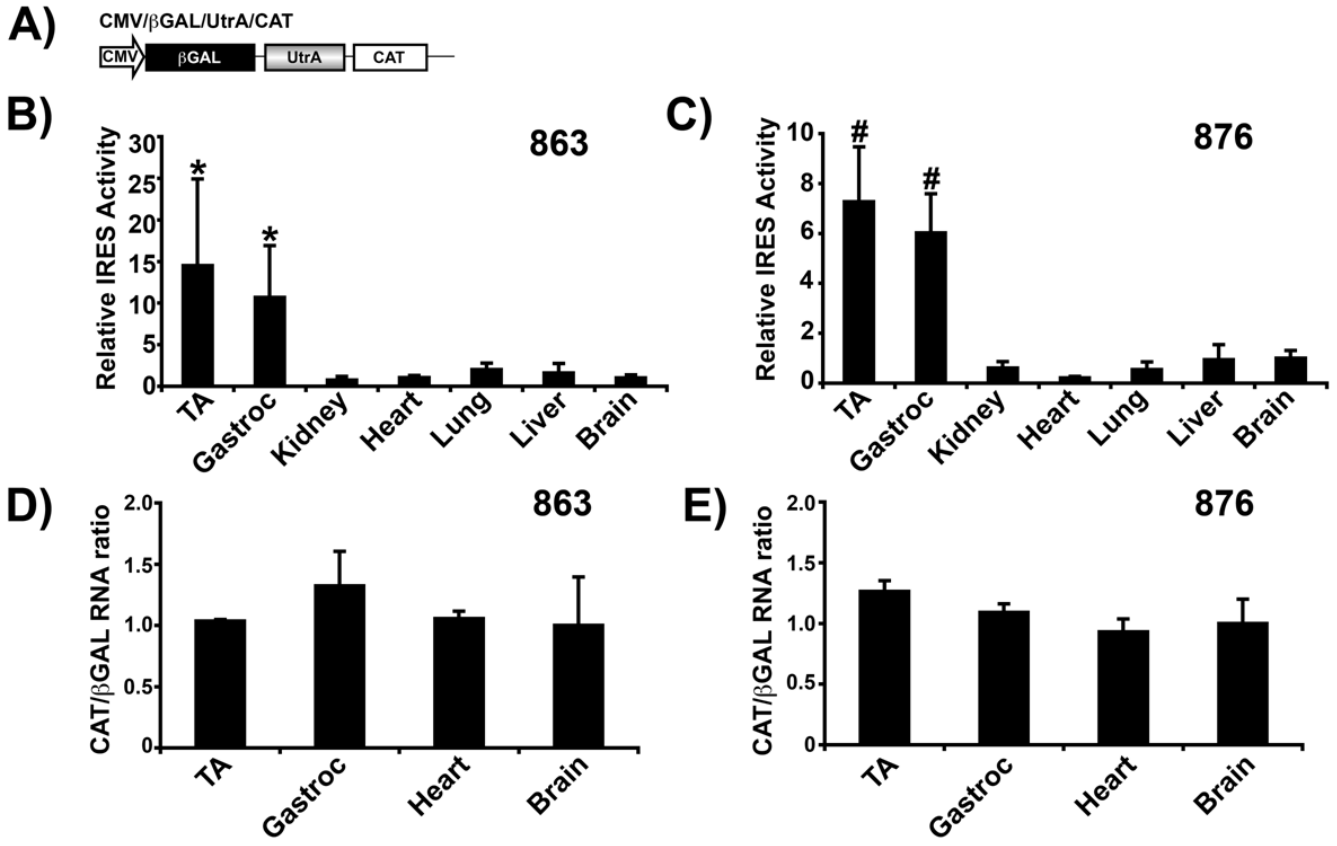
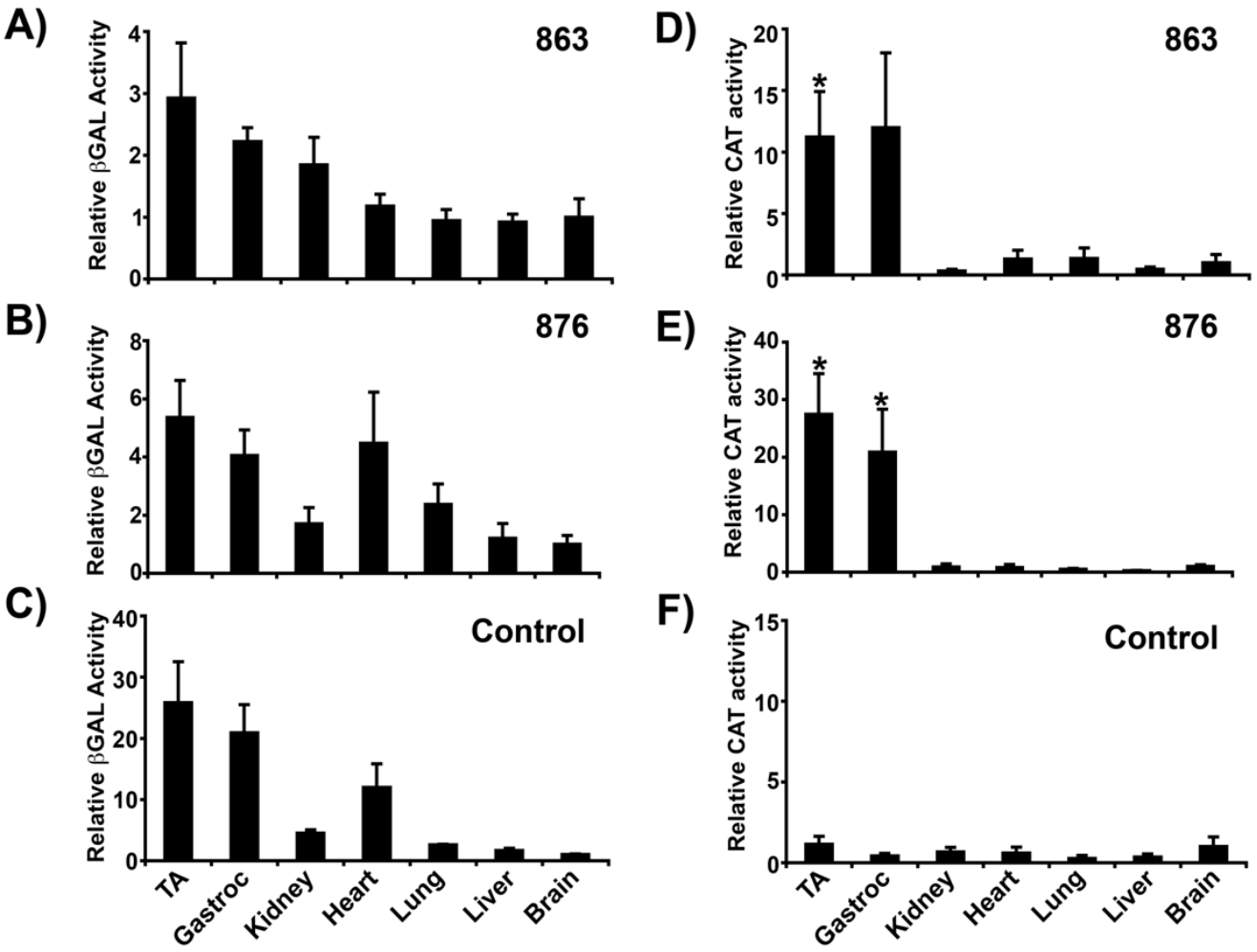

A)
CMV/βGAL/UtrA/CAT
CMV
βGAL
UtrA
CAT
B)
863
Relative IRES Activity
30
25
20
15
10
5
0
*
*
TA
Gastroc
Kidney
Heart
Lung
Liver
Brain
C)
876
Relative IRES Activity
10
8
6
4
2
0
#
#
TA
Gastroc
Kidney
Heart
Lung
Liver
Brain
D)
863
CAT/βGAL RNA ratio
2.0
1.5
1.0
0.5
0
TA
Gastroc
Heart
Brain
E)
876
CAT/βGAL RNA ratio
2.0
1.5
1.0
0.5
0
TA
Gastroc
Heart
Brain

Figure 9. Individual levels of βGAL and CAT reporter activity in utrophin A 5'UTR and control bicistronic reporter transgenic mice.

A,B,C) Relative βGAL activity in transgenic mice from transgenic lines 863, 876 and control normalized to reporter levels in brain. D,E,F) Relative CAT activity in transgenic mice from transgenic lines 863, 876 and control normalized to reporter levels in brain. Note that CAT activity is found at high levels only in skeletal muscles (TA and Gastroc) in both 863 and 876 lines and that no tissue of the control line displays CAT activity, although βGAL activity can be detected. $*P < 0.05$, relative to brain. Control line, n=4; 876 line, n=5; 863 line, n=6.


A)
863
Relative βGAL Activity
0
1
2
3
4
B)
876
Relative βGAL Activity
0
2
4
6
8
C)
Control
Relative βGAL Activity
0
10
20
30
40
TA
Gastroc
Kidney
Heart
Lung
Liver
Brain
D)
863
Relative CAT activity
0
5
10
15
20
*
E)
876
Relative CAT activity
0
10
20
30
40
*
*
F)
Control
Relative CAT activity
0
5
10
15
TA
Gastroc
Kidney
Heart
Lung
Liver
Brain

Additional control experiments were performed to ensure that IRES activity observed in skeletal muscle was not due to an aberrant splicing event or cryptic promoter activity. The presence of internal promoter activity or aberrant splicing could result in the expression of transcripts other than the intact, full-length bicistronic mRNA, potentially leading to a false-positive indication of IRES activity. We performed qRT-PCR analysis on RNA extracted from the 876 and 863 lines (CMV/βGAL/UtrA/CAT) to ensure that βGAL and CAT cDNA could be amplified in an equal ratio in both tissues that displayed IRES activity (gastroc and TA) and tissues that had no IRES activity (heart and brain). Indeed, we found that the ratio of amplified CAT to βGAL cDNA in all tissues examined was not statistically different ($P > 0.05$) (**Figure 8D and E**). The enhanced CAT activity in skeletal muscles of the CMV/βGAL/UtrA/CAT reporter mice can thus be attributed to increased cap-independent translation driven by events that target the utrophin A 5'UTR.

Identification of RNA-binding proteins that interact with the utrophin A 5'UTR

Since IRES-mediated translation is regulated by the binding of *trans*-factors, we set out to identify RNA-binding proteins that interact with the utrophin A 5'UTR. We first examined whether proteins isolated from regenerating muscle versus control muscle could bind selectively to the utrophin A 5'UTR since utrophin A IRES activity is enhanced in regenerating muscle (Miura et al., 2005). To induce skeletal muscle degeneration and regeneration, we injected TA muscles of mice with the snake venom cardiotoxin and isolated the muscles after 7 days. At this time-point, utrophin A IRES activity is induced and its protein levels are ~14-fold above control levels (Miura et al., 2005). Using northwestern analysis, we found that a RNA probe corresponding to a portion of the utrophin A 5'UTR (nucleotides 147-363) associated with several proteins

preferentially in regenerating muscle extracts (CTX) (**Figure 10A**). Thus, conditions under which the utrophin A IRES is highly activated are associated with the binding of multiple proteins to the utrophin A 5'UTR.

eEF1A2 interacts with the utrophin A 5'UTR

Various RNA-binding proteins are involved in cellular IRES-mediated translation; however, each particular IRES appears to be regulated by a distinct set of *trans*-factors, and no single universal factor has been shown to be necessary for cap-independent translation of all IRES. An RNA affinity chromatography approach (Kim et al., 2004;Lewis et al., 2007) was employed to isolate and identify proteins that interact with the utrophin A 5'UTR. For these experiments, we used a biotinylated RNA probe containing the same region of the utrophin A 5'UTR found to associate with several proteins in regenerating skeletal muscles (nucleotides 147-363, **Figure 10A**). The probe was linked to avidin-agarose beads and incubated with regenerating skeletal muscle protein lysates. After extensive washing, SDS PAGE was performed. Analysis of the SYPRO ruby-stained gel revealed the presence of a band, migrating at ~50 kDa, that was only present in the sample containing the 5'UTR probe and not in the sample lacking the biotinylated RNA (**Figure 10B**). The band was excised and analyzed by MALDI-TOF, which identified the protein to be eukaryotic elongation factor 1A2 (eEF1A2).

To confirm that the 50 kDA protein was indeed eEF1A2, we performed additional RNA affinity chromatography experiments, transferred the denatured proteins to a PVDF membrane and performed western analysis using an anti-eEF1A antibody that recognizes both eEF1A1 and eEF1A2. Western analysis revealed the presence of a band at the expected molecular mass in samples that were incubated with the utrophin A 5'UTR

probe (**Figure 10C**). We verified that this interaction between the utrophin A 5'UTR and eEF1A protein(s) was not caused by non-specific binding to RNA by performing additional experiments in which a non-related RNA probe was used (**Figure 10C**). We also performed the chromatography experiment using lysates from HEK293T cells. Association of the probe to eEF1A was not detected using these lysates (**Figure 10D**). Since HEK293T cells express eEF1A1 and not, or very limited amounts of, eEF1A2 (~12-fold difference as determined by qRT-PCR), these experiments provide evidence that eEF1A2 is a factor that interacts with the utrophin A 5'UTR.

Regions of the utrophin A 5'UTR that contain IRES activity also bind eEF1A2

In order to delineate the regions of the utrophin A 5'UTR that interact with eEF1A2, purified eEF1A2 was incubated with radiolabelled RNA probes containing various truncations of the utrophin A 5'UTR and then cross-linked by UV radiation. A specific interaction was identified for probes 1-228 and 147-363, but not 1-70, as indicated by the presence of a shifted complex (**Figure 11**). This interaction was specific since the shifted complexes detected using the 1-228 and 147-363 probes could be competed away by the addition of cold probe.

Figure 10. eEF1A-2 interacts with the utrophin A 5'UTR.

A) Northwestern analysis of control and cardiotoxin (CTX)- injected tibialis anterior (TA) muscles using a P^{32} UTP-labeled RNA probe corresponding to nucleotides 147-363 of the utrophin A 5'UTR. Note the presence of several bands in regenerating muscles. Blot is representative of experiments performed with muscles of three mice. B) RNA-affinity chromatography isolation of utrophin A 5'UTR-binding proteins. Precleared extracts from cardiotoxin-treated TA muscles were incubated with agarose beads coated with biotinylated utrophin A 5'UTR RNA (147-363) or agarose beads alone. Beads were washed extensively, eluted by boiling and resolved by SDS-PAGE. Sypro Ruby stained gel shows a 50 kDA protein species that was identified as eukaryotic elongation factor 1A2 (eEF1A2) by mass spectrometry analysis. C) Samples prepared as in B) were separated by SDS-PAGE, transferred to PVDF membrane, and western blot was performed using an anti-EF1A antibody. This antibody detects both eEF1A1 and eEF1A2 isoforms. eEF1A was detected in CTX muscle lysate incubated with the utrophin A 5'UTR biotinylated probe, but not to a no RNA control, or an unrelated biotinylated RNA probe (corresponding to the utrophin A 3'UTR). D) Biotinylated 5'UTR probe (147-363) does not bind to eEF1A from HEK293T protein lysate (HEK).

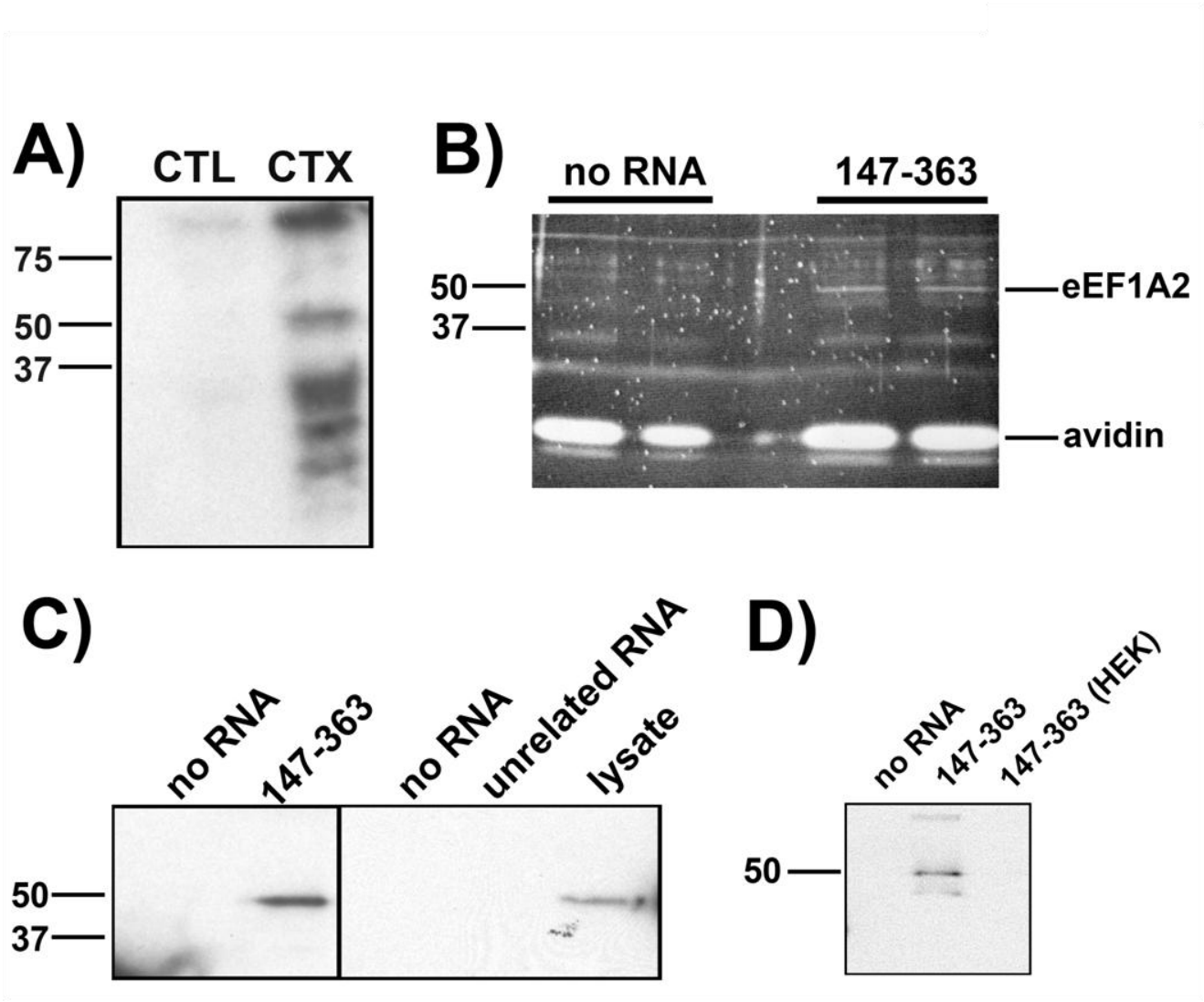
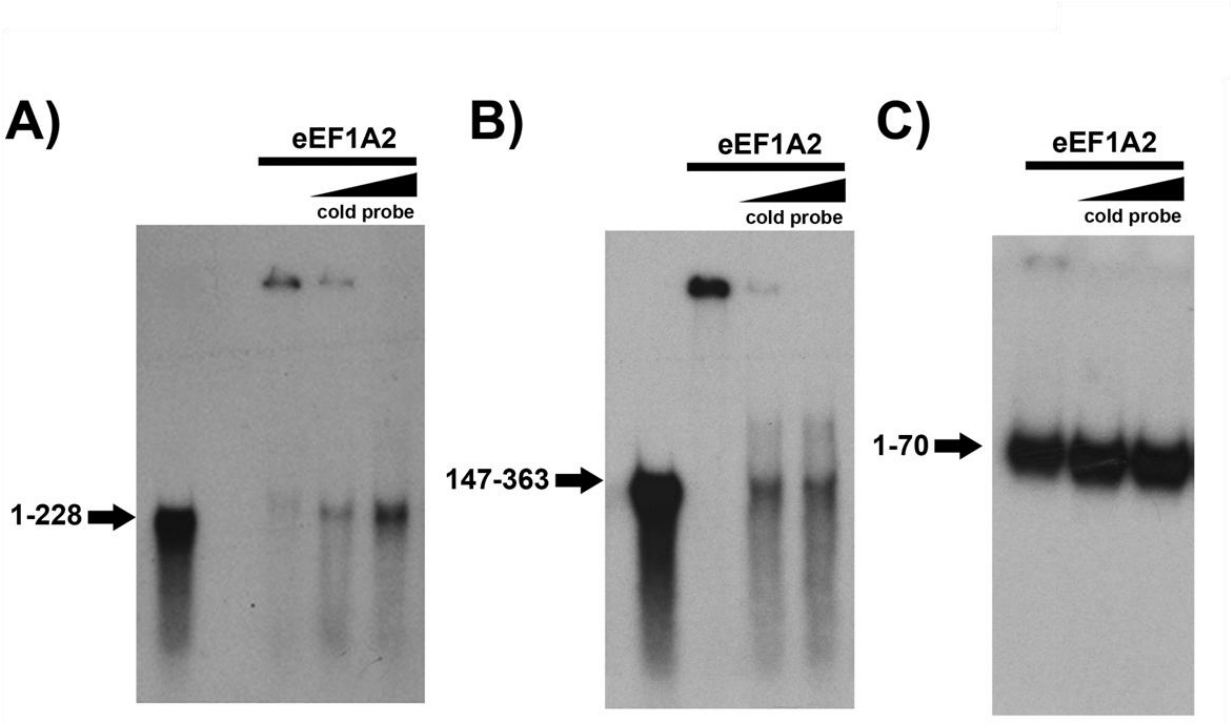

A)
CTL
CTX
75
50
37
B)
no RNA
147-363
50
37
eEF1A2
avidin
C)
no RNA
147-363
no RNA
unrelated RNA
lysate
50
37
D)
no RNA
147-363
147-363 (HEK)
50

Figure 11. eEF1A-2 directly interacts with particular regions of the utrophin A 5'UTR.

Recombinant eEF1A2 was incubated with P^{32} UTP probes corresponding to various regions of the utrophin A 5'UTR, including A) 1-228, B) 147-363 and C) 1-70. Samples were UV cross-linked and separated on Tris-glycine-polyacrylamide gels. The presence of a shifted complex is indicative of a specific interaction between the probe and the purified eEF1A2. Note that the addition of an increasing concentration of cold probe (150 ng to 600 ng) successfully competed away shifted bands (A, B).


A)
eEF1A2
cold probe
1-228
B)
eEF1A2
cold probe
147-363
C)
eEF1A2
cold probe
1-70

To determine whether the regions of the utrophin A 5'UTR that bind to eEF1A2 also harbor IRES activity, we performed transient transfections of utrophin A 5'UTR bicistronic reporters or an empty vector control in C2C12 myoblasts. The full-length utrophin A 5'UTR displayed ~14-fold IRES activity (normalized to empty vector control) (**Figure 12A**). The 1-228 region showed 59% of the activity of the full-length utrophin A 5'UTR and the 147-363 region showed 25% of full-length activity (8.3-fold for 1-228, 3.4-fold for 147-363 above empty vector control levels) ($P < 0.05$). In contrast, the 1-70 region showed no significant IRES activity over control levels ($P > 0.05$). These experiments demonstrate that regions of the utrophin A 5'UTR that bind to eEF1A2 are the same regions able to drive cap-independent translation in C2C12 myoblasts.

eEF1A2 enhances utrophin A IRES activity and its expression pattern correlates with IRES activity in transgenic mice

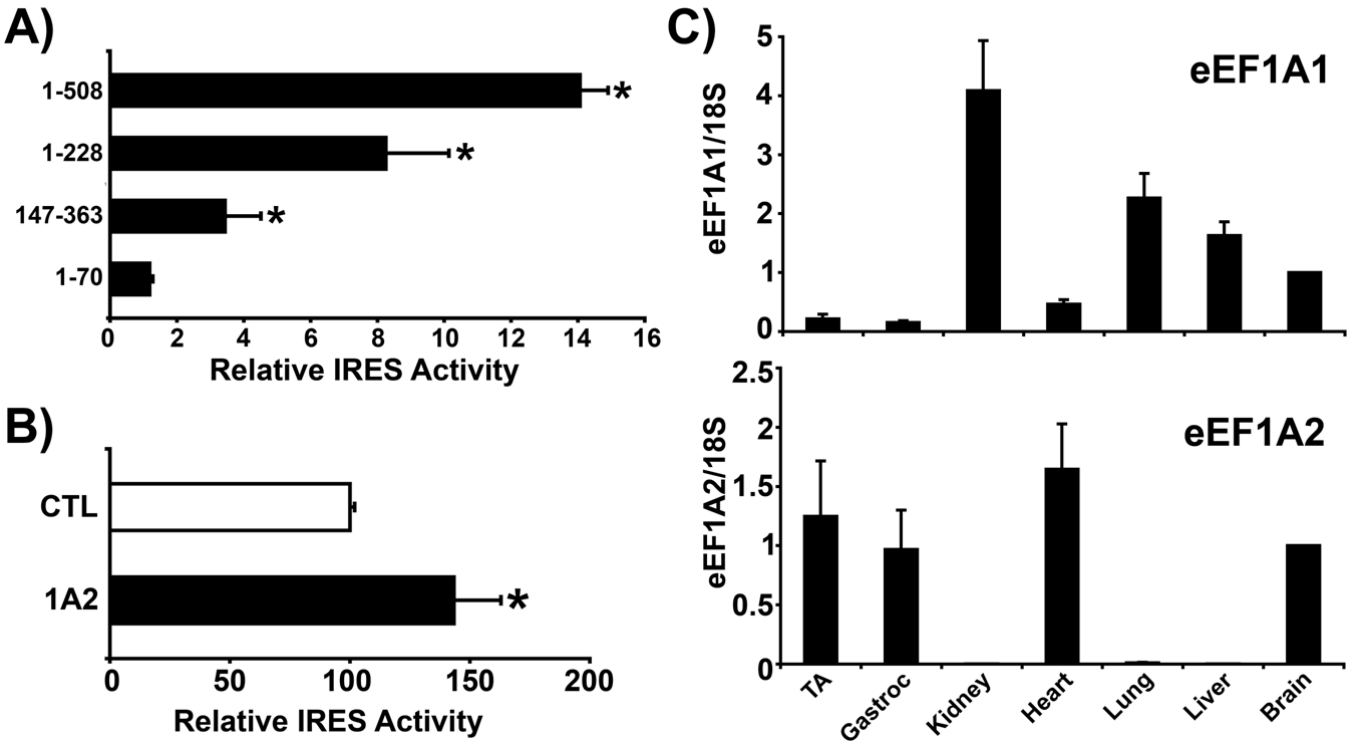
To ascertain whether eEF1A2 can directly regulate utrophin A IRES activity, we attempted to knockdown eEF1A2 in C2C12 cells by using siRNA and shRNA based protocols. Due to the abundant expression of eEF1A2 in C2C12 cells, we were unable to achieve significant knockdown of this protein (data not shown). As an alternative approach, we obtained a rat fibroblast cell line that lacks eEF1A2, and one that is stably transfected to overexpress eEF1A2 (Amiri et al., 2007). Transient transfection experiments revealed that the utrophin A IRES had ~1.4 fold greater activity in the cells expressing eEF1A2 (1A2) compared to controls lacking eEF1A2 (CTL) ($P < 0.05$) (**Figure 12B**).

To assess whether eEF1A2 expression correlates with utrophin A IRES activity in tissues of the utrophin A 5'UTR reporter transgenic mice, we examined mRNA levels of

eEF1A2 and eEF1A1 in various tissues by real-time quantitative RT-PCR. This technique was used because antibodies that distinguish between the two isoforms are not available. In accordance with previous studies (Chambers et al., 1998;Lee et al., 1992), we found that eEF1A2 was expressed in skeletal muscle, heart and brain, but not kidney, lung and liver (**Figure 12C**). Examination of the relative abundance of eEF1A2 to eEF1A1 revealed that although eEF1A2 is expressed in heart and brain (tissues that do not contain utrophin A IRES activity), there are higher levels of eEF1A1 in these organs compared to skeletal muscle, which contains nearly undetectable levels of eEF1A1 (**Figure 12C**). The relative abundance of the two eEF1A isoforms shown as a ratio in **Table 1** reveals that skeletal muscles contain ~ 2- and 6- fold the ratio of eEF1A2 to eEF1A1 as compared to heart and brain tissues, respectively.

Figure 12. Role of eEF1A-2 in regulating utrophin A IRES-mediated translation.

A) Transient transfection of bicistronic vectors harboring various truncations of the utrophin A 5'UTR into C2C12 myoblasts reveals that regions of the utrophin A 5'UTR that bind to eEF1A2 (see Fig. 11) also display significant levels of IRES activity. IRES activity is reported as a ratio of CAT to βGAL, values are standardized to control reporter levels. *, $P < 0.05$. B) Rat2 fibroblast cells (CTL) or Rat2 fibroblast cells stably transfected with eEF1A2 (1A2) were transfected with pβGAL/UtrA/CAT and relative IRES activity was measured. *$P < 0.05$. For A and B, values were determined from two or more independent transfections. C) qRT-PCR was performed on various tissues of mice from the 863 line to determine expression of eEF1A-1 or eEF1A-2 relative to 18S ribosomal RNA. Note the lowest levels of eEF1A1 are found in the skeletal muscle samples (n=3). Ratios of eEF1A2 to eEF1A1 mRNA are shown in Table 1.


A)
1-508
1-228
147-363
1-70
0
2
4
6
8
10
12
14
16
Relative IRES Activity
B)
CTL
1A2
0
50
100
150
200
Relative IRES Activity
C)
eEF1A1
eEF1A1/18S
0
1
2
3
4
5
eEF1A2
eEF1A2/18S
0
0.5
1
1.5
2
2.5
TA
Gastroc
Kidney
Heart
Lung
Liver
Brain

Tissue	eEF1A2/eEF1A1 mRNA ratio ± sem
TA	6.6 ± 0.5
Gastroc	7.8 ± 3.9
Kidney	0
Heart	3.4 ± 1.1
Lung	0
Liver	0
Brain	1

Table 1: Ratio of eEF1A2 to eEF1A1 mRNA levels in various tissues of transgenic mice.

Data was obtained by qRT-PCR. Ratio of eEF1A2 to eEF1A1 was calculated for each individual mouse prior to calculating the mean (n=3). For raw data of eEF1A1 and eEF1A2 standardized to 18S ribosomal RNA, see Figure 12C.

Discussion

In recent work, we have shown that the utrophin A 5'UTR contains an IRES that is activated in response to a variety of conditions (Miura et al., 2005;Miura et al., 2008). Here, we analyzed transgenic mice harboring utrophin A 5'UTR reporter constructs to determine whether the activity of the utrophin A IRES is tissue specific. Interestingly, we found that the utrophin A 5'UTR can direct cap-independent translation exclusively in skeletal muscles, and no other tissues. This expression pattern suggested that factors specifically expressed or activated in skeletal muscle could be important in mediating utrophin A IRES activity. In accordance with this prediction, RNA-affinity chromatography experiments identified eEF1A2, the isoform of eEF1A expressed in mature skeletal muscle, as a factor that interacts with the utrophin A 5'UTR. Additional binding experiments and reporter activity assays demonstrated the importance of this interaction in mediating utrophin A IRES activity.

Utrophin A is expressed in a variety of different organs and tissues, including lungs, kidney, brain, heart and skeletal muscle (Baby et al., 2010;Matsumura et al., 1992;Weir et al., 2002). At the transcriptional level, *cis*-elements in the promoter region direct expression of utrophin A to multiple tissues, and an intronic enhancer appears to contribute to utrophin A mRNA expression in heart and skeletal muscle (Stocksley et al., 2005;Takahashi et al., 2005;Tanihata et al., 2008). In contrast to the wide expression pattern of utrophin A mRNA and protein, here we found utrophin A IRES activity only in skeletal muscles. While this observation does not rule out the involvement of translational control mechanisms in regulating utrophin A in other tissues, it does strongly suggest that translational regulation of utrophin A by its 5'UTR is particularly important in skeletal

muscle. Indeed, we have already uncovered that, like several other IRES regulated transcripts, utrophin A cap-independent translation can be driven under conditions of stress where the overall levels of cap-dependent translation are reduced (Miura et al., 2005;Miura et al., 2008;Spriggs et al., 2008). Given these results, we predict that in skeletal muscle, additional physiologically important "stress" stimuli might target the utrophin A 5'UTR to allow for the rapid synthesis of protein from a pre-existing pool of utrophin A transcripts.

It is well established that utrophin A is more highly expressed in slow-oxidative muscles, such as the soleus, compared to fast-glycolytic muscles, such as the extensor digitorum longus (EDL) (Chakkalakal et al., 2003;Chakkalakal et al., 2008). Interestingly, we observed that IRES-activity in line 876 was 6.8-fold greater in the more fast-glycolytic EDL muscles as compared to soleus muscles ($P < 0.05$). A similar trend was observed in line 863, however the difference was not statistically significant. These observations suggest that utrophin A IRES activity is greater in muscle fibers expressing fast, type II myosin heavy chain isoforms. Future experiments using bicistronic vectors harboring fluorescent protein reporters will be necessary to confirm whether there are clear fiber type differences in utrophin A IRES activity. This question could not be addressed using our transgenic mice since antibodies do not reliably detect the CAT reporter by indirect immunofluorescence. Fluorescent bicistronic reporters will also be useful to investigate the subcellular localization of IRES activity within skeletal muscles; in particular to determine whether IRES-activity is enhanced at the neuromuscular junction.

A limited number of studies have examined the relevance of cellular IRES elements *in vivo*. Studies performed on *Drosophila* harboring bicistronic reporters for ultrabithorax and antennapedia 5'UTRs display spatial and temporal regulation of IRES activity (Ye et al., 1997). Mice harboring a c-myc IRES reporter display high activity in embryonic tissues, but low or undetectable activity in adult tissues (Creancier et al., 2001). Several studies have examined activity of the FGF-2 IRES in transgenic mice. IRES-activity in these mice is regulated in response to hyperglycemia and aging (Gonzalez-Herrera et al., 2006a;Teshima-Kondo et al., 2004), and activity is particularly high in the brain, with certain structures displaying more activity than others (Audigier et al., 2008;Creancier et al., 2000). Interestingly, a factor highly expressed in brain, hnRNP A1, is important for FGF-2 IRES activity (Bonnal et al., 2005). From these limited number of studies, it appears that cellular IRES elements are highly regulated in a tissue-specific manner, suggesting an important role for *trans*-factors that have tightly controlled expression patterns.

Since the utrophin A 5'UTR drives IRES-mediated translation only in skeletal muscles, it is likely that the complex of proteins that permits this type of translation is present or active exclusively in skeletal muscles. We provide evidence suggesting that one of the factors is eEF1A2. There are two eEF1A isoforms- eEF1A1, and eEF1A2. These share 92% sequence identity and appear to be functionally equivalent with regard to their effects on protein synthesis activity *in vitro* (Kahns et al., 1998). While the canonical role for eEF1A isoforms is in shuttling aminoacyl-tRNA during translation elongation, distinct "moonlighting" roles for these proteins have also been identified (Ejiri, 2002). eEF1A1 is important in regulating cytoskeletal organization (Gross and

Kinzy, 2005), protein nuclear export (Khacho et al., 2008) and mRNA stability (Yan et al., 2008;Zhang et al., 2009). In contrast, eEF1A2 protects against apoptosis (Chang and Wang, 2007;Ruest et al., 2002), and plays a role in actin remodeling and oncogenesis (Amiri et al., 2007;Jeganathan et al., 2008). Distinct roles for these two proteins are also suggested by their tissue specific expression pattern. While eEF1A1 is expressed almost ubiquitously, eEF1A2 is expressed in skeletal muscle, heart and brain (Knudsen et al., 1993;Lee et al., 1992). Interestingly, mice lacking functional eEF1A2 (wasted mice, *wst/wst*) exhibit muscle wasting and motor neuron degeneration resulting in premature death (Chambers et al., 1998;Newbery et al., 2007).

It could be expected that since eEF1A2 is expressed in skeletal muscle, heart and brain, utrophin A 5'UTR reporter transgenic mice would show IRES activity in all these tissues. One explanation for the lack of IRES activity in brain and heart tissue can be inferred from the greater ratio of eEF1A2 to eEF1A1 found in skeletal muscle compared to heart and brain (see **Figure 12C** and **Table 1**). In tissues that express both isoforms, competition for binding to the utrophin A 5'UTR may occur, with eEF1A1 opposing the positive effect of eEF1A2 on IRES-mediated translation. On the other hand, skeletal muscle-specific accessory factors that bind eEF1A2 (for example, see (Mansilla et al., 2008)), might be needed for IRES-meditated translation to occur. Alternatively, eEF1A2 might be only one of several proteins needed for skeletal muscle-specific utrophin A IRES activity. Based on expression pattern, it seems that eEF1A2 is not responsible for the enhancement of utrophin A IRES activity during muscle regeneration, since it is not upregulated after myotoxin injection (Miura, Coriati & Jasmin, unpublished observations;

(Khalyfa et al., 1999)). Thus, additional factors or protein modifications are likely required for enhancement of utrophin A IRES activity under various stress conditions.

It is unknown how eEF1A2 is involved in the mechanism of utrophin A IRES-mediated translation and what additional factors are required. Given the ability of eEF1A2 to bind aminoacyl-tRNA, and the presence of tRNA like structures in several viral IRES elements (Lyons and Robertson, 2003;Wilson et al., 2000), we hypothesized that the protein might interact with structural elements in the utrophin A 5'UTR that resemble tRNA; however, using the bioinformatic analysis of Baird et al. (Baird et al., 2006), we could detect no such structural elements. Cricket paralysis virus (CrPV) and hepatitis C virus (HCV) both contain tRNA-like structures and can interestingly be translated via their IRES elements in an *in vitro* system that contains ribosomes, tRNA and elongation factors, but lacks translation initiation factors (Lancaster et al., 2006;Pestova and Hellen, 2003). In light of our findings presented here, one could speculate that eEF1A2 plays a role in translation initiation for these viruses. Further investigation into regulation of IRES-mediated translation by eEF1A2 may provide insights into the mechanistic commonalities of translation initiation of viral and cellular IRESes.

Several groups, including our own, have been performing pre-clinical screening of small molecules for their ability to enhance activity of the utrophin A promoter in hopes of finding an effective drug treatment for DMD. Our results presented here, combined with previous work demonstrating that glucocorticoid treatment can stimulate utrophin A translation via its 5'UTR (Miura et al., 2008), provide a rationale to screen for compounds that can activate expression of utrophin A at the translational level. In this

regard, utrophin A 5'UTR reporter transgenic mice could serve as a useful tool to validate promising compounds *in vivo*. Given the skeletal-muscle specific pattern of utrophin A IRES activity, therapeutics that target the utrophin A 5'UTR would have the advantage of stimulating protein expression specifically in skeletal muscles and no other tissues.

Materials and Methods

Cell culture and Transfections

C2C12 cells were cultured under standard conditions (Miura et al., 2005). Rat2 cells and Rat2 cells overexpressing eEF1A2 were cultured as previously described (Amiri et al., 2007). The bicistronic reporter constructs containing various truncations of the utrophin A 5'UTR were transiently transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA) as previously described (Miura et al., 2008).

Bicistronic Transgenic mice

Six to eight week old C6B3F1 mice were purchased from Charles River Laboratories (Boston, MA). They were cared for in approval with the University of Ottawa Animal Care and Use Committee who is compliant with the Guidelines of the Canadian Council on Animal Care and the Animals for Research Act. The bicistronic vectors, pβGAL/UtrA/CAT and pβGAL/CAT were digested with SalI and ApaI restriction enzymes to generate 6528 and 6020 nucleotide products respectively. These fragments included the CMV promoter and both reporter genes. Products were electrophoresed on 1% agarose gels containing ethidium bromide. Bands were excised and purified using Qiagen gel extraction kit (Qiagen, Chatsworth, CA). To generate transgenic mice, hybrid C6B3F1 mice were used as donors for fertilized one-cell embryos. CMV/βGAL/UtrA/CAT and CMV/βGAL/CAT DNA fragments were separately microinjected into the pronucleus of donor embryos. Pseudopregnant females were used as recipients for the modified zygotes. Potential founders were weaned at 3 weeks after birth. Tail biopsies were collected, minced and incubated overnight in proteinase K (20 mg/ml) at 55°C, genomic DNA was extracted by a standard

phenol/chloroform/isoamyl alcohol extraction and subjected to PCR genotyping. PCR was performed using a 5' primer spanning 142 nucleotides of the LacZ gene and a 3' primer spanning 459 nucleotides of the CAT gene (5'-TTTTTCCCGATTTGGCTACA-3'; 5'-TGAAACTCACCCAGGGATTG-3'). PCR products were visualized on 2% agarose gel containing ethidium bromide. Founders were bred with C6B3F1 wild-type mice and their progeny were examined for transgene expression using β-galactosidase assay. The three CMV/βGAL/UtrA/CAT (876, 881, and 863) and one CMV/βGAL/CAT transgenic lines were backcrossed with C6B3F1 wild-type mice for several generations.

Reporter Activity Assays

Various tissues were excised from transgenic mice and immediately frozen in liquid nitrogen after euthanizing the mice with CO_2. For reporter assays, protein from tissues was extracted with reporter lysis buffer (Promega) as previously described (Miura et al., 2005) and protein concentration was quantified by Bradford Standard Assay. Protein samples were diluted to a final concentration of 4 μg/μl prior to reporter assays. β-galactosidase (βGAL) enzymatic assays were performed using the β-galactosidase enzyme assay system as recommended by the manufacturer (Promega). To measure chloramphenicol acetyltransferase (CAT) activity, we analyzed the conversion of chloramphenicol to butyryl chloramphenicol by incorporation of [^{14}C] butyryl coenzyme A (Miura et al., 2005). Background levels for both reporter assays were determined by analyzing reporter activity in tissues from mice not harbouring a transgene.

RNA Extraction and qRT-PCR Analysis

Total RNA was isolated from tissues of CMV/βGAL/UtrA/CAT and CMV/βGAL/CAT transgenic mice using TRIzol reagent (Invitrogen) as recommended by

the manufacturer. TRIzol extraction was followed by a one hour DNAse I (Invitrogen) treatment to eliminate possible plasmid and genomic DNA contamination. To control for the presence of an intact bicistronic reporter transcript in CMV/βGAL/UtrA/CAT transgenic mice, qRT-PCR was performed with previously described βGAL and CAT primers (Miura et al., 2005). Negative controls consisted of an RT mixture that had the reverse transcriptase replaced by sterile water. Quantitative real-time RT-PCR was performed on reverse transcribed RNA using QuantiTect SYBR green PCR kit (Qiagen) on a Stratagene MX3005p, and the delta delta Ct method was used to quantify expression of endogenous mRNAs relative to 18S ribosomal RNA. The following primer sets were used to detect eEF1A2- 5'-CCAGGCCGGAGGATACCG-3'; 5'-GAAATGCAAGCGGCGTGT-3'; and eEF1A1- 5'-GGCAGTGCCAGTGGCACCA-3'; 5'-CCAGGCTTGAGAACACCAGT-3'. 18S ribosomal RNA was detected using previously described primers (Ma et al., 2007). For detection of human eEF1A1 and eEF1A2 in HEK293T cells, previously published primer sequences were used (Kim et al., 2009).

RNA binding experiments

Cardiotoxin-treatment of C57BL/10 mice was performed as previously described (Miura et al., 2005) and tibialis anterior (TA) muscles were excised 7 days after toxin injection. Isolation of proteins binding the utrophin A 5'UTR was performed using an RNA-affinity chromatography protocol (Lewis et al., 2007). For these experiments, 60 μg of biotinylated RNA was conjugated to avidin-agarose beads and incubated with 4 mg of protein extract from regenerating TA muscle extracts. In-gel trypsin digestion and mass peptide fingerprinting was performed at the Protein Function Discovery Centre (Queen's

University, Kingston, ON, Canada). To confirm eEF1A2 as an interacting protein, we performed RNA-affinity chromatography using 15 μg of biotinylated RNA and 1.6 mg of TA muscle or HEK293T cell extracts, transferred proteins from the gel to a PVDF membrane, and performed western blot using an eEF1A antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

RNA probes were transcribed from PCR templates containing T7 RNA polymerase binding site using the MAXIScript in vitro transcription kit (Ambion, Austin, TX, USA), and α-P^{32}-UTP (800 Ci/mmol; Perkin Elmer, Boston, MA, USA). For northwestern analysis, 60 μg of cardiotoxin-treated TA muscle extract was run on a 10% SDS PAGE gel and transferred to PVDF membrane. Equal loading was confirmed by staining with Ponceau S. Membranes were presoaked, incubated with the $1x10^6$ cpm P^{32} labeled utrophin A 5'UTR RNA probes and washed as previously described (Sela-Brown et al., 2000). Binding of the RNA probe to proteins was visualized by autoradiography. For UV-crosslinking experiments, recombinant eEF1A2 was produced as previously described (Kulkarni et al., 2007), desalted with a Sepharose G25 column (Sigma, USA) and quantified by BCA kit (Pierce, Rockford, IL, USA). MEGAShort Script kit (Ambion, Austin, TX, USA) was used to produce non-radiolabelled RNA probes. Unincorporated nucleotides were removed by Sepharose G25 column and purified by electrophoresis on 6% Tris-glycine-polyacrylamide gels. Desalted eEF1A2 protein (100 ng) and 50000 cpm of RNA probe were pre-incubated for 30 minutes at room temperature, in UV-binding buffer (10 mM Tris-HCl pH 7.5, 50 nM KCl, 10% glycerol, 5 mM DTT and 10 ng/ul of yeast tRNA). For competition experiments, non-radiolabelled RNA was added 15 min prior to the end of the pre-incubation step. Samples were then exposed to 254 nm UV

light for 30 minutes at 4°C and resuspended in RNA loading buffer. Samples were separated on Tris-glycine-polyacrylamide gels using 4% w/v acrylamide/bis-acrylamide (19:1). Following electrophoresis, the gels were dried and subjected to autoradiography.

Statistical Analysis

For transgenic mice reporter activity analysis, one-tailed *t*-tests were used to determine statistical significance. For all other experiments, two tailed *t*-tests were used. Level of significance was set at $P < 0.05$.

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Conflict of Interest: None declared.

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Chapter 3

eEF1A2 regulates expression of utrophin A in skeletal muscle: preliminary study

A similar manuscript is to be completed and submitted for publication:

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Contribution from authors

Adèle Coriati conducted all the experiments shown. Adèle Coriati wrote the manuscript. Edited by Steven Hillock and Bernard J. Jasmin. Tissues of wasted mice, as well as the eEF1A1 and eEF1A2 specific antibodies were provided by Helen J. Newbery and Catherine M. Abbott. Steven Hillock helped with the RNA-immunoprecipitation experiments presented in figure 13.

Abstract

Understanding the regulatory mechanisms controlling utrophin A expression at the sarcolemma of dystrophic muscles will facilitate the development of therapeutic strategies to ameliorate the pathophysiological features of Duchenne Muscular Dystrophy (DMD). It is well established that the 5'untranslated region (UTR) of utrophin A contains an internal ribosome entry site (IRES) that is expressed preferentially in skeletal muscle of the utrophin A 5'UTR transgenic mice. In addition, the eukaryotic elongation factor 1A2 (eEF1A2) was previously identified as a muscle-specific factor shown to associate with the 5'UTR of utrophin A *in vitro* and mediate cap-independent translation of utrophin A (Miura et al., 2010).

In the present study, we show a direct interaction of eEF1A2 to endogenous utrophin A transcripts. To investigate the physiological role of eEF1A2 in regulating utrophin A expression in muscles, we characterized utrophin A protein and mRNA levels in wasted mice (mice lacking eEF1A2). We show that eEF1A2 mediates the expression of utrophin A *in vivo*. Moreover, eEF1A2 was found to direct the localization of utrophin A to extra-synaptic regions of skeletal muscle. Together, these findings demonstrate the physiological importance of eEF1A2 in enhancing utrophin A expression and localization in skeletal muscle. Identifying pharmacological compounds that would specifically target eEF1A2 and increase endogenous levels of utrophin A expression could serve as a drug-based therapy to treat DMD.

Introduction

Duchenne muscular dystrophy (DMD) is a severe hereditary muscle disease that is caused by the absence of full-length dystrophin protein in skeletal muscles (Blake et al., 2002). The major functional role of the cytoskeletal dystrophin protein is to provide structural integrity to the membrane by linking cytoskeletal actin to the extracellular matrix (Petrof et al., 1993). Skeletal muscles lacking dystrophin, as seen in DMD patients, display a higher susceptibility to stress-induced sarcolemmal injury and fibrosis. DMD patients eventually die by early adulthood because of cardiac and pulmonary failure (Allikian and McNally, 2007;Weller et al., 1990). To this day, there is no effective treatment for DMD, but one possible therapy would be to increase sarcolemmal levels of utrophin, the autosomal homologue of dystrophin (Blake et al., 1996). Investigations have demonstrated using different strategies that enhancing the expression of utrophin can compensate for the absence of dystrophin and alleviate pathophysiological dystrophic features in animal models of DMD (Cerletti et al., 2003;Chakkalakal et al., 2004;Tinsley et al., 1996).

Previous studies have demonstrated that both transcriptional and post-transcriptional mechanisms play important roles in modulating the expression of utrophin A, an isoform of utrophin primarily expressed in skeletal muscle fibers, both in synaptic regions, via the utrophin A promoter, and extra-synaptic regions, via the 3'untranslated region (UTR) (Miura and Jasmin, 2006). Interestingly, studies have also shown that in regenerating muscle fibers, such as in muscle biopsies of DMD patients and in skeletal muscles of *mdx* mice, utrophin A protein expression was enhanced, without concomitant increases in its mRNA (Gramolini et al., 1999b;Weir et al., 2002). One explanation for

the heightened protein expression of utrophin A in regenerating muscles is an increase in translation initiation conferred by an internal ribosome entry site (IRES) located within the 5'UTR of utrophin A (Miura et al., 2005).

IRESs are present in untranslated regions of the mRNA and are thought to control initiation of protein synthesis independently of the canonical 7-methyl guanosine cap-mediated translation initiation (Van Der et al., 2009). Certain 'stressors', such as cell cycle, amino acid starvation, hypoxia, and apoptosis, can cause the activation of IRES-dependent translation of specific mRNAs while simultaneously silencing the canonical method of translation initiation (King et al., 2010). In addition to ribosomes and a subset of initiation factors, IRES-trans acting factors (ITAFs) have been shown to be recruited to the IRES region of several cellular and viral mRNAs to regulate their activity (Spriggs et al., 2008). Recently, we characterized a transgenic mice expressing a utrophin A 5'UTR bicistronic reporter and showed that the utrophin A IRES is specifically activated in skeletal muscle (Miura et al., 2010). In addition, RNA-binding experiments and reporter activity assays suggested that the eukaryotic elongation factor 1A2 (eEF1A2) was a possible ITAF responsible for the muscle specific activation of the utrophin A IRES (Miura et al., 2010).

In this study, we examined the physiological role of eEF1A2 in regulating the endogenous expression of utrophin A in skeletal muscle. We first determined if eEF1A2 directly interacts with endogenous utrophin A transcripts, using eEF1A2 specific antibodies (Newbery et al., 2007) to precipitate associated mRNP complexes. We then examined utrophin A expression and localization in skeletal muscle of wasted mice (a mouse model naturally deficient in eEF1A2). We show that eEF1A2 directly interacts

with endogenous utrophin A mRNA. Also, our preliminary results suggest that eEF1A2 plays a role in controlling the endogenous expression of utrophin A.

Results

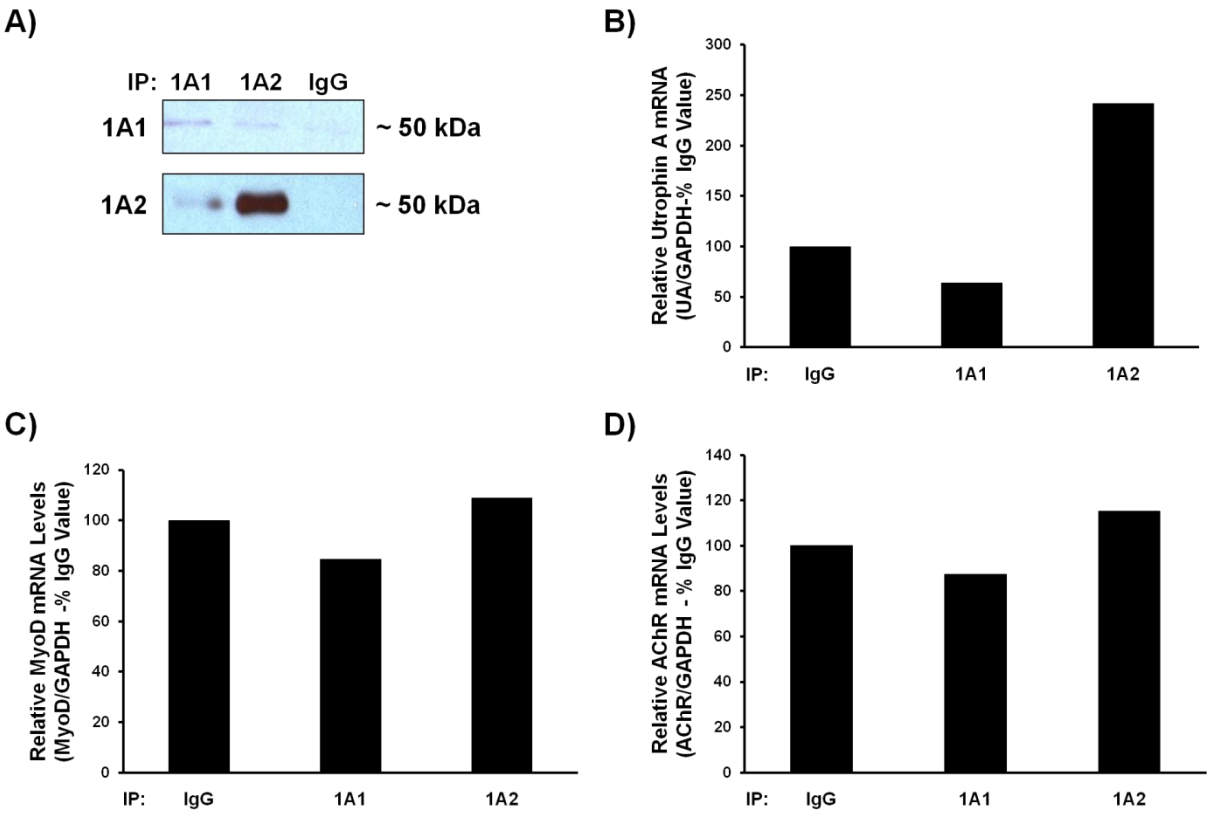
eEF1A2 interacts with utrophin A mRNA in differentiated mouse muscle cells

To examine the direct interaction of eEF1A2 with endogenous utrophin A mRNA, an RNA-Immunoprecipitation (IP) was performed. Cell lysates from differentiated C_2C_{12} myotubes were cross-linked with 1% formaldehyde and were used for IP with specific eEF1A2 antibodies (Newbery et al., 2007). Nonspecific binding of utrophin A mRNA with IP reagents was controlled for by additional IP reactions using specific eEF1A1 (Newbery et al., 2007) and mouse IgG antibodies. Western blot analysis was conducted to verify the IP of eEF1A1 and eEF1A2 protein (**Figure 13A**).

To determine the relative binding of eEF1A2 to endogenous utrophin A mRNA, qRT-PCR was conducted. Utrophin A transcripts were significantly more enriched in the eEF1A2 IP fraction than in the eEF1A1 and IgG fractions (**Figure 13B**). Transcript levels of MyoD, a muscle specific gene and acetylcholine receptor (AchR), a synapse specific gene, were assayed to control for nonspecific binding of mRNAs to eEF1A2 or the IP reagents. MyoD and AchR mRNA levels were not significantly different when compared to all IP fractions, thus confirming the specific and direct interaction of eEF1A2 with utrophin A transcripts. (**Figure 13C,D**).

Figure 13. eEF1A2 directly interacts with endogenous utrophin A mRNA in C_2C_{12} myotubes

C_2C_{12} myotubes were cross-linked with 1% formaldehyde prior to immunoprecipitation (IP) with eEF1A1 (1A1), eEF1A2 (1A2), or mouse IgG specific antibodies. A) Western blot analysis revealed the IP of 1A1 and 1A2. Neither 1A1 nor 1A2 was precipitated by the IgG antibodies. Total RNA was extracted from IP lysates and relative enrichment of B) utrophin A (UA), C) MyoD, and D) acetylcholine receptor (AChR) was examined by qRT-PCR. UA mRNA was significantly enriched in the 1A2 IP, when compared to 1A1 and IgG IPs, while MyoD and AChR mRNAs were used as negative controls. Transcript levels were standardized to the housekeeping gene GAPDH.


A)
IP: 1A1 1A2 IgG
1A1
~ 50 kDa
1A2
~ 50 kDa
B)
Relative Utrophin A mRNA (UA/GAPDH-% IgG Value)
300
250
200
150
100
50
0
IP: IgG 1A1 1A2
C)
Relative MyoD mRNA Levels (MyoD/GAPDH -% IgG Value)
120
100
80
60
40
20
0
IP: IgG 1A1 1A2
D)
Relative AChR mRNA Levels (AChR/GAPDH - % IgG Value)
140
120
100
80
60
40
20
0
IP: IgG 1A1 1A2

Utrophin A protein expression is decreased in skeletal muscle of the wasted mouse

To begin investigating the physiological role of eEF1A2 in regulating utrophin A expression, mice that do not naturally express functional eEF1A2, called wasted mice, were employed. The wasted mouse possesses a naturally occurring 15.8kb deletion that eliminates the first non coding exon and every regulatory promoter elements of the gene encoding eEF1A2 (Chambers et al., 1998;Shultz et al., 1982). These mice appear phenotypically normal at birth, but by day 21 of post natal development they manifest severe gait abnormalities, weight loss, muscle wasting, tremors and motor neuron degeneration which is usually fatal by day 28 (Abbott et al., 1994;Shultz et al., 1982). Interestingly, in neuronal, cardiac and skeletal tissues of normal mice, eEF1A1 is highly expressed until birth and gradually declines until it is undetectable by day 21. Conversely, eEF1A2 expression in neuronal, cardiac and skeletal tissues begins shortly before birth and steadily increases until peaking around day 21, and remains relatively constant throughout adulthood (Chambers et al., 1998;Khalyfa et al., 2001). Thus, it is thought that the absence of either eEF1A isoforms by day 21 in skeletal muscle of the wasted mice can in part explain the observed pathology (Chambers et al., 1998;Khalyfa et al., 2001).

Expression of eEF1A2 in skeletal muscle, brain, heart and kidney of wasted mice was analyzed by western blot. As expected, eEF1A2 was undetectable in all tissues of the wasted mice (**Figure 14A,B**), while eEF1A1 expression was relatively low when compared to the respective control (data not shown). qRT-PCR analysis further confirmed the absence of eEF1A2 in all tissues of the wasted mice (**Figure 14C**). Also, qRT-PCR analysis revealed that compared to the brain and the kidney, eEF1A1 transcript levels were relatively low in skeletal muscle of both control and wasted mice (data not

shown). The kidney was used as a negative control since it does not normally express eEF1A2.

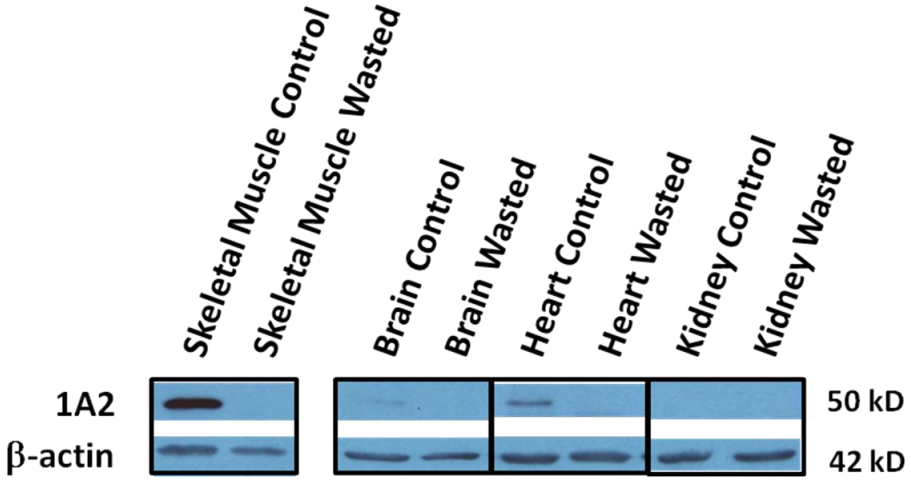
To determine whether the lack of functional eEF1A2 has an effect on utrophin A expression, we examined utrophin A protein levels in skeletal muscle of wasted mice. Western blot analysis revealed a decrease in utrophin A protein expression in skeletal muscle and in the brain of the wasted mice, when compared to control mice (**Figure 15A,B**). However, utrophin A protein levels in the heart (an organ that normally expresses eEF1A2) was not significantly different from the control (**Figure 15A,B**).

We further investigated these results by performing qRT-PCR experiments to determine if the decrease in utrophin A protein expression observed in skeletal muscle and the brain of the wasted mice could be explained, at least in part, by a result of decreased translational activity. In comparison to control mice, there was no significant difference in utrophin A transcript levels in the skeletal muscle or the brain of the wasted mice (**Figure 15C**). Similarly, utrophin A transcript levels were also unchanged in the heart and kidney of the wasted mice (**Figure 15C**). These findings suggest that the absence of functional eEF1A2 in skeletal muscle and brain of the wasted mouse, leads to decreased utrophin A protein levels without affecting utrophin A transcript levels, further reinforcing the suggested role of eEF1A2 in regulating cap-independent translational activity of utrophin A.

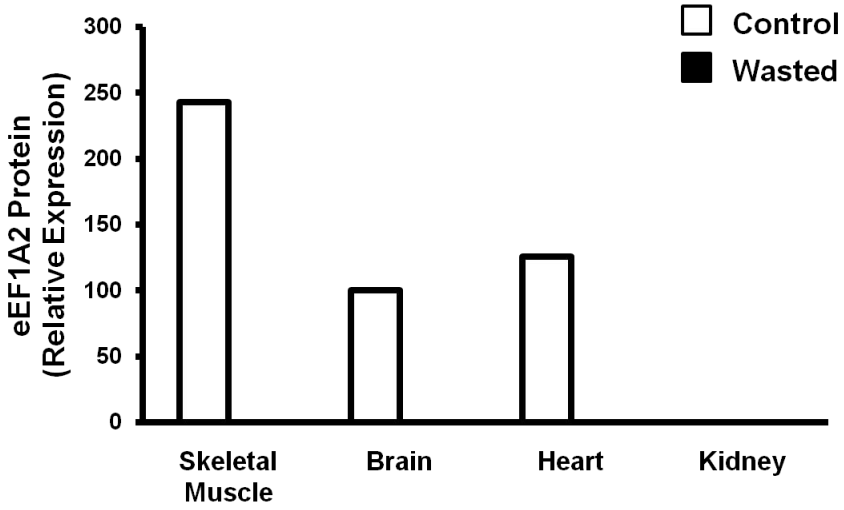
Figure 14. eEF1A2 expression in tissues of 21 day old wasted mice.

Skeletal muscle, brain, heart and kidney were collected from 21 day old wasted and control mice. Total cell lysates were isolated and analyzed by western blot experiments. A) Representative western blot analysis of eEF1A2 (1A2) expression in tissues of control and wasted mice. β-actin was used as a loading control. Analysis of 1A2 expression confirmed the absence of 1A2 protein in all tissues of the wasted mice. B) Graphical representation of 1A2 expression in wasted and control mice. Densitometry values were normalized to β-actin, relative to brain control value (n=1). C) Total RNA was subsequently extracted and analyzed by qRT-PCR. Analysis of eEF1A2 mRNA levels confirmed the absence of eEF1A2 transcript in all tissues of the wasted mice. qRT-PCR values are normalized to 18S ribosomal RNA. eEF1A2 mRNA levels in wasted mice are relative to the brain control. Transcript data represent one of two independent experiments, performed in duplicate. kD (kilo Dalton)

A)


Skeletal Muscle Control
Skeletal Muscle Wasted
Brain Control
Brain Wasted
Heart Control
Heart Wasted
Kidney Control
Kidney Wasted
1A2
β-actin
50 kD
42 kD

B)


Control
Wasted
eEF1A2 Protein
(Relative Expression)
300
250
200
150
100
50
0
Skeletal Muscle
Brain
Heart
Kidney

C)

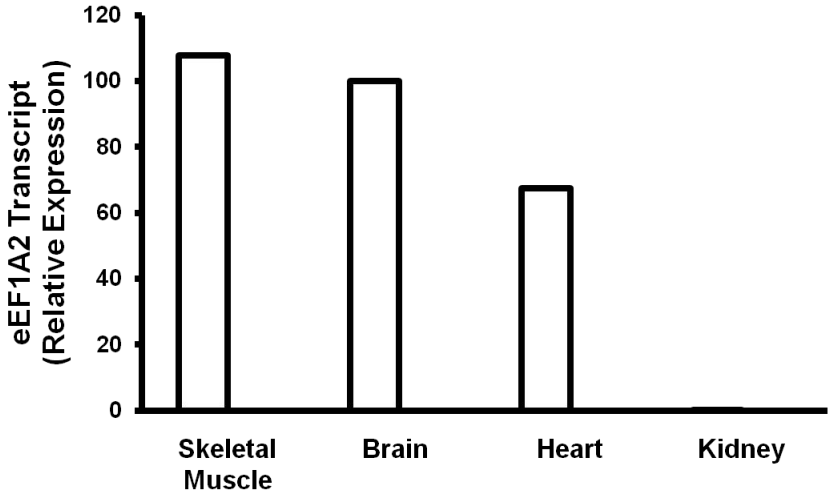
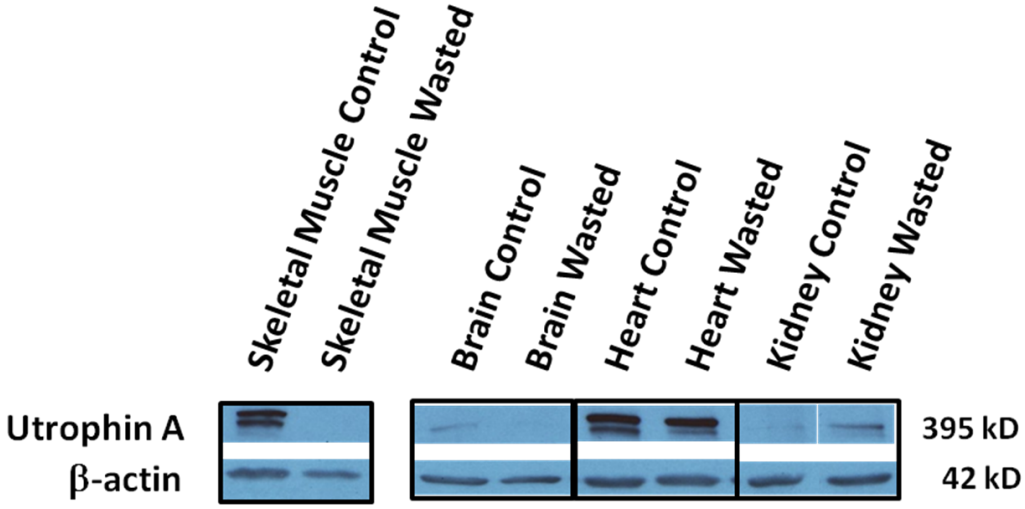

eEF1A2 Transcript
(Relative Expression)
120
100
80
60
40
20
0
Skeletal Muscle
Brain
Heart
Kidney

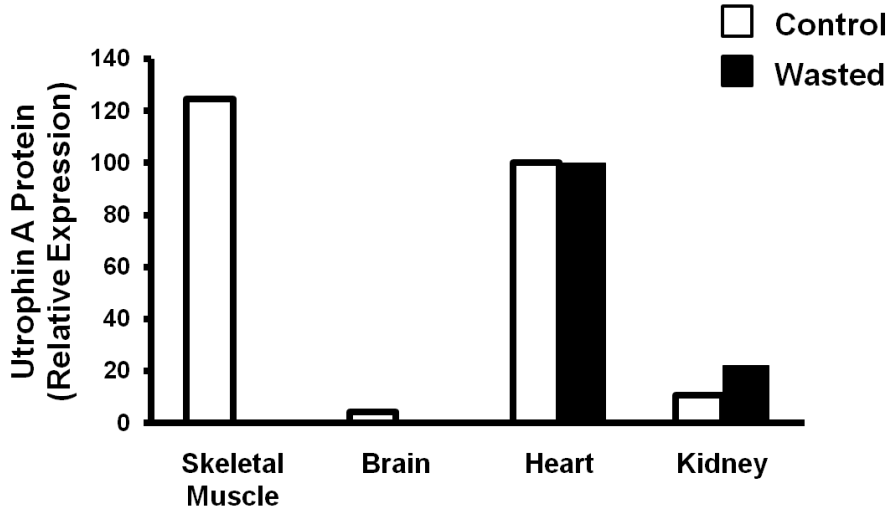
Figure 15. Utrophin A expression in tissues of 21 day old wasted mice.

Skeletal muscle, brain, heart and kidney were collected from 21 day old wasted and control mice. Total cell lysates were isolated and analyzed by western blot. A) Representative western blot analysis of utrophin A expression in tissues of control and wasted mice. β-actin was used as a loading control. B) Graphical representation of utrophin A protein levels in wasted and control mice. There is a decrease in utrophin A protein expression in skeletal muscle and brain of the wasted mice. (n=1) Densitometry values were normalized to β-actin, relative to heart control value. C) Total RNA was subsequently extracted and analyzed by qRT-PCR. No significant difference in utrophin A transcript levels was observed in all tissues examined. qRT-PCR values are normalized to 18S ribosomal RNA. All transcript levels in wasted tissues are relative to their respective control. Results represent one of two independent experiments, performed in duplicate. kD (kilo Dalton)

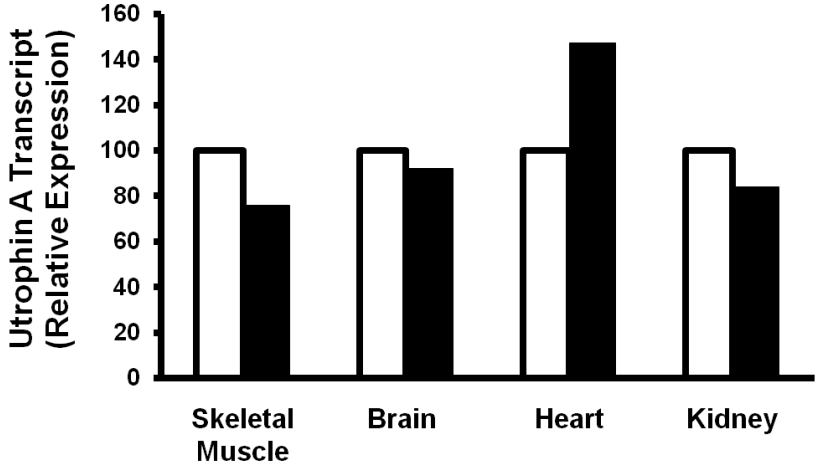
A)


Skeletal Muscle Control
Skeletal Muscle Wasted
Brain Control
Brain Wasted
Heart Control
Heart Wasted
Kidney Control
Kidney Wasted
Utrophin A
β-actin
395 kD
42 kD

B)


Control
Wasted
Utrophin A Protein
(Relative Expression)
140
120
100
80
60
40
20
0
Skeletal Muscle
Brain
Heart
Kidney

C)


Utrophin A Transcript
(Relative Expression)
160
140
120
100
80
60
40
20
0
Skeletal Muscle
Brain
Heart
Kidney

Localization of utrophin A in skeletal muscle of 21 day old wasted mice

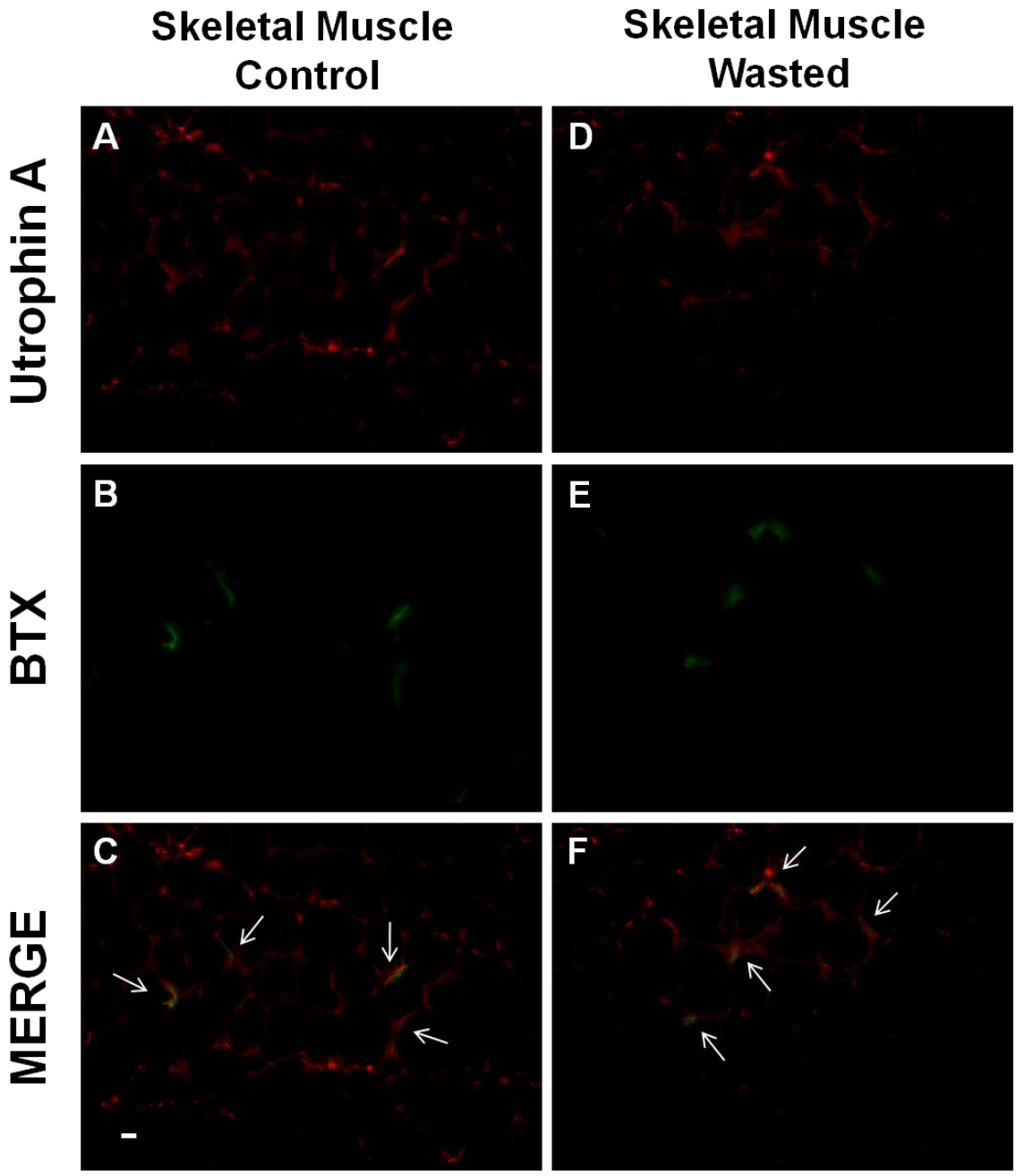
To determine utrophin A localization on the sarcolemma of wasted mice, utrophin A expression was analyzed by immunofluorescence. Skeletal muscle from wasted and control mice were sectioned and co-stained with utrophin A specific antibodies and α-bungarotoxin (BTX; AlexaFluor 488) to indicate the neuromuscular junctions (NMJ) (**Figure 16B,E**). We noticed a decrease in utrophin A staining in skeletal muscle of wasted mice that appear to occur predominantly in extra-synaptic regions (**Figure 16A,D**), since we could still co-localize utrophin A at the NMJs (**Figure 16C,F**; indicated by arrows). A negative staining with no utrophin A primary antibody was done to control for non-specific labeling at the sarcolemma and at the NMJs (data not shown). These observations indicate that in the absence of eEF1A2, not only is utrophin A protein expression decreased in skeletal muscle of wasted mice (**Figure 15**), but that this decrease is largely occurring in extra-synaptic regions.

In contrast to what we observed in immunofluorescence experiments (**Figure 16**), we could not detect utrophin A protein in skeletal muscle of wasted mice by western analysis (**Figure 15**). One explanation could be that detection of utrophin A protein in skeletal muscle of wasted mice was not within the basic linear range ($5X10^2$) of a typical autoradiography film (Heinicke et al., 1992). In order to prevent saturation of the band corresponding to the control skeletal muscle by prolonging the exposure time, we could use the charge coupled device (CCD) camera (dynamic range of 10^3-10^5). This approach enables the direct measurement of chemiluminescent light output and ultimately converting the signal into a quantifiable value (Boniszewski et al, 1990). Using this

technique would prevent saturation of the control signal at exposure levels where the ‘wasted’ signal is within a linear range of detection.

Figure 16. Decreased localization of utrophin A in extra-synaptic regions of skeletal muscle of wasted mice.

Skeletal muscle from wasted and control mice we immunolabeled with utrophin A specific antibodies (AlexaFluor 594). A,D) There is a decrease in utrophin A staining in skeletal muscle of wasted mice when compared to control. B,E) α-Bungarotoxin (BTX; AlexaFluor 488) staining was used to localize and label neuromuscular junctions (NMJ). C,F) Merged images of utrophin A and α-bungarotoxin staining to analyze co-localization. Utrophin A co-localizes with α-bungarotoxin at the NMJs in skeletal muscle of wasted and control mice, as indicated by the arrows. There is a decrease in immunostaining of utrophin A, more specifically in extra-synaptic regions. Scale bar, 50μm.


Skeletal Muscle
Control
Skeletal Muscle
Wasted
Utrophin A
BTX
MERGE
A
B
C
D
E
F

Discussion

Studies have shown that the 5'UTR of utrophin A contains an IRES which is active in regenerating muscle tissues and in glucocorticoid-treated cells (Miura et al., 2005;Miura et al., 2008). Also, transgenic mice harbouring a utrophin A 5'UTR bicistronic reporter was shown to direct IRES activity preferentially in skeletal muscle tissues (Miura et al., 2010). Furthermore, binding experiments led to the identification of a skeletal muscle-specific factor known as eEF1A2 that appears to interact with the 5'UTR of utrophin A, in addition to enhancing utrophin A IRES activity in rat fibroblast cells overexpressing eEF1A2 (Miura et al., 2010). These studies suggested that eEF1A2 could play a role in regulating utrophin A expression in skeletal muscle.

eEF1A2 directly interacts with endogenous utrophin A mRNA

There are two isoforms of eEF1A, eEF1A1 and eEF1A2, and they share 92% sequence identity. Both eEF1A isoforms play the same major role in translation elongation (Ann et al., 1991;Knudsen et al., 1993;Lund et al., 1996). Generally, when eEF1A isoforms are in their active GTP-bound form, they shuttle aminoacylated tRNAs to the ribosomal A site and enable continuation of protein synthesis (Browne and Proud, 2002;Merrick, 1992;Moldave, 1985). Several regulatory elements such as E-box, EGR and MEF2 binding sites were identified on the promoter region of eEF1A2, which explains the specific expression of eEF1A2 in terminally differentiated skeletal, cardiac and neuronal tissues (Bischoff et al., 2000). In contrary, eEF1A1 is expressed in almost all adult tissues (Lund et al., 1996). Although they both play the same major role in the cell, a number of non-canonical roles have been identified. For instance, several studies

have demonstrated that in terminally-differentiated myotubes, eEF1A1 is pro-apoptotic whereas eEF1A2 is anti-apoptotic (Ruest et al., 2002). Also, eEF1A2 is highly upregulated in breast, ovarian and lung cancer, further characterizing it as an inhibitor of apoptosis (Lee and Surh, 2009). Based on our observations, we attribute an additional non-canonical role for eEF1A2 as an RNA-binding protein, as demonstrated by the direct interaction of eEF1A2 with endogenous utrophin A mRNA.

eEF1A2 regulates utrophin A expression in skeletal muscle

The physiological importance of eEF1A2 in skeletal muscle tissues is well demonstrated in mice lacking functional eEF1A2, known as the 'wasted mouse' (Shultz et al., 1982). As mentioned previously, wasted mice exhibit premature death due to muscle wasting and motor neuron degeneration (Shultz et al., 1982). Given the canonical role of both eEF1A isoforms in translation elongation, the absence of protein synthesis of survival genes after day 21 could undoubtedly explains for the majority of the observed phenotype (Chambers et al., 1998;Khalyfa et al., 2001). Apart from its role in protein synthesis, analysis of the wasted mice led to the idea that eEF1A2 expression is essential for proper maintenance of motor neurons and NMJs (Abbott et al., 2009;Chambers et al., 1998;Lutsep and Rodriguez, 1989;Newbery et al., 2005;Woloschak et al., 1987;Woloschak et al., 1996). Thus, atrophying skeletal and cardiac muscle fibers, and degenerating neuromuscular synapses in wasted mice possibly account for respiratory and cardiac failure, further demonstrating the importance of eEF1A2 in survival.

Here, we examined the physiological role of eEF1A2 in regulating endogenous utrophin A expression in skeletal muscle of 21 day old wasted mice. Our findings further

suggest that eEF1A2 might play a role in regulating cap-independent translational activity of utrophin A *in vivo*. Since eEF1A2 is an important and crucial factor for protein synthesis, it becomes important to further investigate the expression of utrophin A at an earlier time point in development where the wasted mice expresses both eEF1A isoforms in their skeletal muscle, such as day 15 (Chambers et al., 1998). We could then confirm that the variation in utrophin A protein expression is solely associated with the absence of eEF1A2, and not because of the inhibition of protein synthesis, which is due to the lack of both necessary elongation factors in skeletal muscle. Nonetheless, we cannot rule out the possibility of additional mechanisms that could regulate protein stability of utrophin A in skeletal muscle.

In a similar fashion, it has been previously shown that knocking down ITAFs that normally enhance cellular IRESs results in a decrease in protein expression, without affecting the corresponding transcript levels. For instance, this was observed in HELA cells in which knockdown of the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), the trans-acting factor that normally activates the IRES-dependent translation of FGF2, caused a decrease in FGF2 protein levels, but not in its mRNA expression (Bonnal et al., 2005). The present study adds to the growing list of ITAFs thought to regulate the expression of cellular genes that contain IRESs in their 5'UTRs.

Potential role of eEF1A2 in regulating utrophin A expression in neuronal tissues

In contrast to eEF1A2, utrophin A is ubiquitously expressed in different organs and tissues (Baby et al., 2010;Weir et al., 2002). Regulatory *cis*-elements in the promoter region of utrophin A and an intronic enhancer located within the second intron of the

utrophin A RNA partially explains the broad distribution of utrophin A expression (Stocksley et al., 2005;Takahashi et al., 2005;Tanihata et al., 2008;Tokunaga et al., 2005). We previously suggested that eEF1A2 might be required for skeletal muscle-specific utrophin A IRES activity because of the higher ratio of eEF1A2 to eEF1A1 found in skeletal muscle compared with heart and brain (Miura et al., 2010). The elevated utrophin A, but not mRNA expression has been observed in the brain of *mdx* mice compared to control mice (Baby et al., 2010;Weir et al., 2002). Interestingly, utrophin A expression in the brain of the wasted mice was regulated in a similar manner as skeletal muscle. It was previously shown that the transgenic mice harboring a utrophin A 5'UTR bicistronic reporter did not display utrophin A IRES activity in the brain. We suggested that this occurs due to the fact that both eEF1A isoforms are expressed in the brain and that eEF1A1 might compete with eEF1A2 for binding to the utrophin A 5'UTR and thus oppose the positive effect that eEF1A2 has on IRES-mediated translation (Miura et al., 2010). In light of the present findings, we hypothesize that supplementary neuron-specific factors or protein modifications in the wasted mice, in addition to eEF1A2, are required to enhance cap-independent translational activity of utrophin A in neuronal tissues (**Figure 17A**).

Localization of utrophin A in skeletal muscle is regulated by eEF1A2

It is well established that utrophin A mRNA and protein are more enriched in slow-oxidative muscles, compared to fast-glycolytic muscles, and that the higher expression is in part be due to an enrichment in extra-synaptic regions (Gramolini et al., 2001b). Here, we provide evidence that eEF1A2 plays a role in regulating the localization of utrophin A to extra-synaptic regions of skeletal muscle. These preliminary

observations suggest that, in addition to the regulatory elements within the 3'UTR of utrophin A controlling the localization of utrophin A mRNA, more specifically to the cytoskeleton (Gramolini et al., 2001a), eEF1A2 could also play a role in regulating the cellular localization of utrophin A. Interestingly, eEF1A isoforms were shown to play an important role in the proper localization of the survival of motor neuron (SMN) protein in neurons by forming a complex with zing finger-containing transcription factor (ZPR1), which in turn binds to SMN protein. ZPR1 is more likely to bind GDP-associated proteins which leads to the presumption that eEF1A2 is the predominant complex-associating isoform (Abbott et al., 2009;Matera and Hebert, 2001;Mishra et al., 2007). Here we present a post-transcriptional event, which involves eEF1A2, important in regulating localization of utrophin A in extra-synaptic regions of skeletal muscles. However, further work is needed to confirm this supposition. For instance, it will be important to determine if eEF1A2 colocalizes with utrophin A in both synaptic and extra-synaptic regions of skeletal muscles.

A more thorough understanding of eEF1A2's role in IRES-dependent translation of utrophin A may lead to the identification of new muscle specific pharmacological targets. Our goal is to augment utrophin A in dystrophic muscles with the hopes of compensating for the lack of dystrophin in DMD patients. Understanding the regulation of utrophin A expression by eEF1A2 could also, in part, explain the devastating phenotype seen in the wasted mice.

Materials and Methods

Cell culture

C_2C_{12} myoblast cells were cultured under standard conditions (Miura et al., 2005). Cells were maintained in differentiation medium for 2-5 days and then used for RNA-ImmunoPrecipitation (IP) experiments.

Mouse Strains

All the animals used in this study were maintained and cared for at the Medical Research Council Mammalian Genetics Unit under the guidance issued by the Medical Research Council in *Responsibility in the Use of Animals for Medical Research* (1993) and Home Office Project License. The maintenance of the wasted mice was as described (Abbott et al., 1994).Control animals were age-matched animals from the appropriate background strain.

RNA-ImmunoPrecipitation

Differentiated C_2C_{12} myotubes were cross-linked with 1% formaldehyde in PBS for 10 minutes at room temperature. Cell lysates were then extracted with freshly made RIPA buffer (1M Tris-HCl pH 7.5, 5M NaCl, 10% NP40, 10% DOC, 10% SDS, 0.5M EDTA and a supplement of protease inhibitor) and then sonicated several times (until completely homogenized). Protein G beads were washed with RIPA buffer for 10 minutes at 4°C and then diluted in a 1:1 ratio with RIPA buffer. Cells lysates were incubated for one hour at 4°C with Protein G beads and yeast tRNA (35μg/μL). Cell lysates were then incubated with 10μL of antibodies directed to eEF1A1 and eEF1A2 (kindly provided by Dre. Catherine Abbott's laboratory) or control mouse IgG (Santa Cruz Biotechnology) overnight, at room temperature, with constant mixing. The cell

lysate-antibody solutions were then incubated with prewashed Protein G beads for one hour at 4°C with constant mixing. Protein G beads were then washed with RIPA buffer to elute bound mRNP complexes. Reverse cross-linking of cell lysates was done for two hours at 70°C in freshly made elution buffer (1M Tris-HCl Ph7.5, 0.5M EDTA, 1M DTT, 10% SDS). Approximately 10% of the immunoprecipitate product was boiled and used for western blot analysis. The remaining product was used for total RNA isolation. The mRNA was precipitated in the presence of 10 μg of RNase-free glycogen (GenHunter) and subsequently used for qRT-PCR analysis.

Western blotting

Protein expression was analyzed by western blotting as previously described (Miura et al., 2005). Tissues from wasted mice (21 day), along with its prospective control, were kindly giving to us by Dr. Catherine Abbott (University of Edinburgh, Edinburgh, UK) (Newbery et al., 2007). Samples were crushed on dry ice and then directly homogenized in RIPA buffer (Sigma-Aldrich), supplemented with protease inhibitor (Roche). A total of 30-120 μg of protein extracts, or the appropriate amount of cell lysates from the RNA-IP experiment, were resolved on either a 5% SDS-PAGE gel for utrophin A analysis, or a 12% SDS PAGE gel, for eEF1A1,eEF1A2 and β-actin analysis, with a 4% stacking gel. Proteins were separated at 100-200V for 3-5h and then transferred overnight at 4^{o}C onto either a nitrocellulose membrane (BIO-RAD, 0.45μm) for utrophin A western analysis or on a Immobilion-P polyvinylidene fluoride (PVDF) membrane (Milipore Corporation, Bedford, MA) for eEF1A1, eEF1A2, and β-actin analysis.

Membranes were then incubated overnight at 4°C with primary antibodies to utrophin A (1:500, Transduction Laboratories), eEF1A1 (1:2000, Newbery et al. 2007), eEF1A2 (1:2000, Newbery et al. 2007) and β-actin (1:10000) and suitable HRP-conjugated IgG secondary antibodies (Jackson ImmunoResearch). Protein detection was done by exposing the membranes using western lightning enhanced luminescence reagent (Perkin Elmer) and then by developing them with CL-XPosure film (Thermo Scientific) and a Kodak X-OMAT 2000A processor. Average band intensity was quantified via densitometry analysis (National Institutes of Health, ImageJ).

RNA isolation and qRT-PCR

RNA isolation and qRT-PCR were performed using a method as described previously (Miura et al., 2009). In brief, total RNA was isolated from RNA-IP cell lysates and tissues from wasted mice using TRIzol reagent (Invitrogen), as recommended by the manufacturer. Qreal-time RT-PCR was carried out with primers to amplify a portion of utrophin A, MyoD, AchR, eEF1A1 and eEF1A2 mRNAs (Miura et al., 2009;Miura et al., 2010). Additional primer sets used are as follows: MyoD5'-CCACTATGCTGGACAGGCAGT-3' and 5'-TGGCATGATGGATTACAGCG-3', AChR5'-TCGTCTCGCCGCATGGGTTTA-3' and 5'-GAGCCATCTCTCCAGCCCCTGTTT-3'. As a housekeeping gene, we used 18S ribosomal and GAPDH mRNA. Real-time PCR was performed on reverse transcribed RNA using QuantiTect SYBR green PCR kit (Qiagen) on a Stratagene MX3005p. To reduce variation, all reactions were performed in duplicate. The delta delta Ct method was used to quantify the expression of endogenous utrophin A, eEF1A1, eEF1A2, MyoD, and AchR mRNAs relative to 18S ribosomal RNA and GAPDH mRNA.

Immunofluorescence

Immunofluorescence experiments were performed as previously described (Chakkalakal et al., 2003). Briefly, muscles of wasted mice were cross-sectioned longitudinally at a 10μm thickness and placed on positively charged slides (Fisher). Sections were immediately blocked with 5% fetal bovine serum in PBS for 15 minutes at room temperature followed by incubation with antibody directed against utrophin A (1:200, NCL-DRP2, Novacastra) in blocking solution at 4°C overnight. Sections were then incubated with secondary antibody (1:500, Alexa Fluor 594 conjugated, Invitrogen) for 1 h at room temperature. Alpha-bungarotoxin was used to label the NMJs (1:500, Alexa Fluor 488-conjugated Jackson ImmunoResearch, West Grove, PA). Fluorescent images were digitally acquired (Sony) using Zeiss Axioscope 2 microscopes. Images were equally adjusted to enhance color and clarity (Photoshop CS, Adobe).

Acknowledgements

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Funding

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Chapter 4

General discussion

4.1. Summary

The main purpose of this work was to study the cap-independent translation of utrophin A using a transgenic mouse model expressing the utrophin A 5'UTR bicistronic reporter. In addition, we wanted to identify trans-acting factors that would bind to the 5'UTR of utrophin A and regulate utrophin A IRES activity. We found that utrophin A IRES activity is specifically expressed in the skeletal muscles of our utrophin A 5'UTR bicistronic reporter transgenic mice (Chapter 2). Also, we showed that eEF1A2 is a trans-acting factor that enhances utrophin A IRES activity and interacts with endogenous utrophin A mRNA (Chapter 2 and 3). Finally, we assessed the physiological role of eEF1A2 in skeletal muscle using a mouse model that is deficient in eEF1A2 (wasted mice) and found that it regulates utrophin A expression (Chapter 3).

4.2. Skeletal muscle-specific regulation of the utrophin A IRES

The molecular mechanisms regulating cellular IRESs are much more complex and less understood then those described in viral IRESs. Regardless, transgenic mice expressing specific cellular IRESs, such as the fibroblast growth factor 2 (FGF-II), the vascular endothelial growth factor-A (VEGF) and the c-myc proto-oncogene, were important in understanding various physiological conditions related to development, maturation, tissue specificity and disease-states (Audigier et al., 2008;Bornes et al., 2007;Creancier et al., 2000;Creancier et al., 2001;Gonzalez-Herrera et al., 2006b;Gonzalez-Herrera et al., 2006a). For instance, the FGF-II IRES is thought to be

developmentally regulated in the brain and may be important in synapse formation and maturation (Audigier et al., 2008). Our study contributes to the growing list of potential cellular IRESs that may serve as pharmacological targets to regulate the expression of proteins necessary to alleviate disease-related pathology, such as DMD.

Bicistronic reporter assays are by far the most common method used to assess IRES activity of viral and cellular mRNAs. However, there have been several reports regarding the misuse of the luciferase bicistronic reporter system. IRES activity measured using the pRF vector, which has the Renilla Luciferase as the upstream cistron and the Firefly Luciferase as the downstream cistron, resulted, to a certain extent, from splicing events occurring within the reporter system (Holcik et al., 2005;Kozak, 2005;Van Eden et al., 2004). Despite all the controversies regarding the usage of bicistronic vectors for IRES-based studies (Kozak, 2005), it should be noted that we provided numerous control experiments, both in this study and in previous studies, to ensure that the utrophin A IRES activity assessed by our bicistronic vector was not subjected to any splicing events that could result in false positive results (Miura et al., 2005;Miura et al., 2008).

4.3. Regulation of utrophin A expression by trans-acting factor eEF1A2

Most cellular IRESs require, in addition to the presence of specific sequences and structures, a specific subset of ITAFs to function efficiently (Mitchell et al., 2001;Mitchell et al., 2003). One well-described example is the apoptotic protease-activating factor protein 1 (APF-1) IRES which, in order to function appropriately, requires the correct structural conformation mediated by the pre-binding of the upstream of N-ras (unr) protein to the IRES structure. Consequently, the neuronal enhanced version

of PTB (nPTB) protein can bind to the three-dimensional structure and enhance IRES-dependent translation of APF-1 by allowing easy accessibility to the ribosomal subunits (Mitchell et al., 2003). Thus, it became important to search for ITAFs that could regulate the utrophin A IRES in skeletal muscles. We found, using several methodologies, that the eEF1A2 translation factor is one trans-acting factor suggested to play a role in mediating IRES-dependent translation of utrophin A, specifically in skeletal muscle and brain (Chapter 2 and 3). It is still unknown exactly how eEF1A2 mediates IRES-dependent translation of utrophin A.

As we have previously suggested in chapter 2, eEF1A2 might interact with tRNA-like IRES structures (given its ability to bind aminoacyl-tRNA) contained within viral mRNAs, such as the Cricket paralysis virus, the pestivirus and hepatitis C virus, and mediate their cap-independent translation initiation activity (Lancaster et al., 2006;Lyons and Robertson, 2003;Pestova and Hellen, 2003). Along these lines, future studies would consist on identifying specific structure(s) and/or consensus sequence(s) in the utrophin A 5'UTR that are required for eEF1A2 to appropriately bind and regulate IRES-dependent translation, which will provide further insight into how cellular and viral IRESes function. Given our preliminary results, we suggest an additional non-canonical role for eEF1A2 in the regulation of utrophin A expression in skeletal muscle and brain. We suggest the following possible roles to be 1) eEF1A2 binds the utrophin A IRES structure and unwinds the mRNA to allow for the ribosome and other initiation factors to bind and initiate protein synthesis, 2) eEF1A2 is part of a complex of muscle specific ITAFs required to recruit the ribosome to the 5'UTR of utrophin A and enhance internal ribosome initiation, or 3) eEF1A2 binds to muscle specific ITAFs (which is unlikely

since we show direct binding of eEF1A2 to utrophin A mRNA, Figures 11 & 13) that play a role in inhibiting IRES-dependent initiation of utrophin A (**Figure 17**).

As previously mentioned in the general introduction, utrophin A is more abundant in slow-oxidative muscles (soleus) compared with fast-glycolytic muscles (EDL) (Chakkalakal et al., 2003;Chakkalakal et al., 2008). In the contrary, we observed in our utrophin A 5'UTR transgenic mice that the EDL displayed higher utrophin A IRES activity compared to the soleus (Miura et al., 2010). These observations led us to believe that fiber-type specific trans-acting factors are responsible for the cap-independent activation of utrophin A in fast-glycolytic muscles.

4.4. Targeting of the utrophin A IRES as a therapy to treat DMD

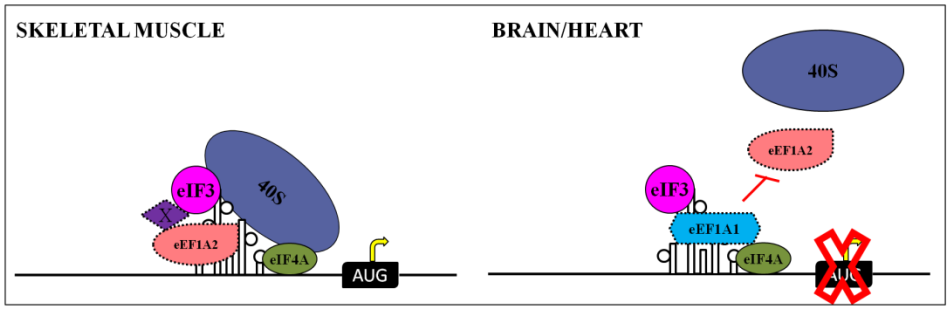
The utrophin A 5'UTR bicistronic reporter mice could be used to study the specific regulatory mechanisms involved in enhancing utrophin A IRES activity in regenerating skeletal muscles. In addition, our transgenic mice will be useful to study the possible involvement of IRES-dependent translation of utrophin A during development and aging, considering the previously identified discordance between utrophin protein and mRNA levels (Roma et al., 2004). It would be worthwhile to cross our utrophin A 5'UTR bicistronic reporter transgenic mice with the *mdx* mice, and then assess the importance of IRES-dependent protein synthesis of utrophin A in regenerating muscle tissues of *mdx* mice, as well as in other tissues of *mdx* mice that show discordant expression of utrophin protein and mRNA, more specifically in skeletal muscle, heart and brain (Baby et al., 2010;Weir et al., 2002).

It is well established that fast muscle fibers are the first subset of muscles to be affected in DMD patients (Webster et al., 1988). Thus, the upregulation of utrophin A expression and increased localization to extra-synaptic regions by pharmacologically targeting eEF1A2 to enhance utrophin A IRES activity may serve as a therapeutic strategy to alleviate the dystrophic pathology in fast-type muscles of DMD patients. Finally, the utrophin A 5'UTR bicistronic reporter mice could potentially be used as an animal model for testing compounds that would target the activity of the utrophin A IRES under various physiological conditions.

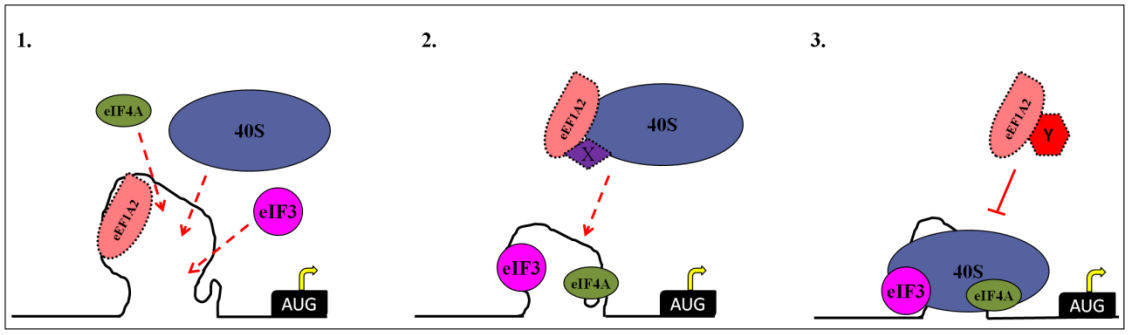
Figure 17. Potential roles of eEF1A2 in regulating IRES-dependent translation of utrophin A.

A) In skeletal muscle, eEF1A2 binds to the 5'UTR of utrophin A (within nucleotides 1-363, Figure 11), along with initiation factors, the 40S ribosomal subunit and other muscle-specific proteins, to further initiate cap-independent protein synthesis of utrophin A. In brain and heart tissues, IRES-dependent translation initiation of utrophin A is inhibited due to the presence of eEF1A1 (a highly similar isoform of eEF1A2), which in turn outcompetes the binding of eEF1A2 to the utrophin A 5'UTR. B) The possible mechanistic roles that eEF1A2 might play on IRES-dependent translation initiation of utrophin A are 1. eEF1A2 unwinds the secondary structure within the utrophin A 5'UTR to allow for the ribosome and other initiation factors to bind, 2. eEF1A2 is part of a complex of muscle specific ITAFs required to recruit the ribosome to the utrophin A 5'UTR and enhance internal ribosome initiation or, 3. eEF1A2 binds to muscle specific ITAFs that play a role in inhibiting IRES-dependent translation initiation of utrophin A (which is unlikely since we show direct binding of eEF1A2 to utrophin A mRNA, Figures 11 & 13). eIF, eukaryotic initiation factor; 40S, 40kDa subunit; eEF1A1 and eEF1A2, Eukaryotic initiation factors 1A1 and 1A2, respectively; X and Y, tissue-specific proteins that either enhance or inhibit IRES-dependent translation, respectively.

A)


SKELETAL MUSCLE
BRAIN/HEART
40S
eEF1A2
eIF3
40S
eEF1A2
eIF4A
AUG
eIF3
eEF1A1
eIF4A

B)


1.
2.
3.
eIF4A
40S
eEF1A2
eIF3
AUG
eEF1A2
40S
eIF3
eIF4A
AUG
eEF1A2
Y
40S
eIF3
eIF4A
AUG

Conclusion

The main goal of this study was to characterize the regulation of utrophin A IRES activity using a transgenic mouse model expressing the utrophin A 5'UTR bicistronic reporter. Moreover, we wanted to identify trans-acting factors that could mediate the activity of the utrophin A IRES and potentially regulate its expression *in vivo*. The findings of this study have led to 4 major conclusions: 1) Utrophin A IRES activity is specifically expressed in skeletal muscles; 2) The eEF1A2 protein is a muscle-specific trans-acting factor that can interact with utrophin A and mediate IRES-dependent translation of utrophin A; 3) Endogenous utrophin A protein levels, but not transcript levels, in skeletal muscle and brain are affected by the absence of eEF1A2 in wasted mice; 4) The expression of eEF1A2 in skeletal muscle plays an important role in the localization of endogenous utrophin A to extra-synaptic regions of muscle fibers. In addition to previous reports concerning the translational regulation of utrophin A, our observations indicate that the utrophin A IRES is specifically regulated in skeletal muscle and that eEF1A2 is important in mediating utrophin A IRES activity as well as the expression of utrophin A. Together, the analysis of mechanisms regulating the utrophin A IRES not only adds to the knowledge related to understanding all the regulatory elements controlling utrophin A expression, but also could serve as a therapeutic target to treat DMD patients by compensating for the lack of dystrophin.

Chapter 5

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