Combinatorial Utrophin A Activation in Muscle as a Therapeutic Strategy to Treat Duchenne Muscular Dystrophy

By
Aatika Ahmed

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies
University of Ottawa

In partial fulfillment of the requirements for the degree of Master of Science in Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine

Faculty of Medicine

University of Ottawa

ABSTRACT

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive neuromuscular disorder caused by mutations or deletions in the dystrophin gene. Utrophin up-regulation therapy is among the various therapeutic strategies that are being investigated to treat DMD. In this strategy utrophin, a dystrophin homologue, is up-regulated along the entire length of the sarcolemma to replace the absent dystrophin protein. Previous studies have revealed that utrophin A expression can be controlled by various transcriptional, post-transcriptional and translational mechanisms and pharmacological modulation of these pathways can stimulate its expression in muscle (Miura et al, 2009; Ljubicic et al, 2011; Chakalakkal et al, 2008; Miura et al, 2010). In the present study we screened several FDA approved and natural pharmacological compounds that can potentially activate utrophin A expression in muscle. We found that AICAR (AMPK activator) and heparin (p38 activator) were most effective in stimulating utrophin A expression in our C2C12 muscle cell system. Next, we analyzed the effect of combining these activators on utrophin A expression in muscle cells and preclinical mdx mouse model of DMD. Our findings revealed that combinatorial treatment of AICAR and heparin instigated an additive effect on utrophin A expression both in C2C12 muscle cells and mdx mice. Further characterization of treated mdx mice revealed that combinatorial treatment of AICAR and heparin caused improvements in the dystrophic phenotype as indicated by decreased central nucleation, decreased fiber size variability and improved sarcolemmal integrity in dystrophic muscle. Together these findings established that combinatorial treatment of AICAR and heparin ameliorates the dystrophic phenotype in mdx mice and may serve as an effective therapeutic strategy for DMD.

TABLE OF CONTENTS:

LIST OF FIGURES AND TABLES:

LIST OF ABBREVIATIONS

AAV- Adeno-associated viral

AChR- Acetylcholine Receptors

AICAR- 5-amino-4-imidazolecarboxamide riboside

AONs- Antisense oligonucleotides

ARE- AU-rich element

ARMD- ARE-mediated decay

ATBR- Anti-thrombin binding region

Ca^{2+}- Calcium

CN- Calcineurin

DAPC- Dystrophin-associated protein complex

DMD- Duchenne muscular dystrophy

ECM- Extracellular matrix

GAPB- GA binding protein

GLUT4- Glucose transporter type 4

GRMD- Golden retriever muscular dystrophy

IgM- Immunoglobin M

1RES- Internal ribosome entry site

KSRP- K-homology Splicing Regulatory Protein

LMWH- Low molecular weight heparin

MAPK- Mitogen- Activated Protein Kinase

mdx- X-linked muscular dystrophy

MEF2- Myocyte Enhancer Factor-2

MHC- Myosin heavy chain

NFAT- Nuclear factor of activated T cells

NMJ- Neuromuscular junction

NO- Nitric oxide

NOS- Nitric oxide synthase

PGC-1α- Peroxisome proliferator-activated receptor ϒ coactivator 1α

PPAR- Peroxisome proliferator-activated receptor

PPRE- PPAR- response element

PTC- Premature Termination Codon

RIP140- Receptor-Interacting Protein 140

RT-qPCR- Reverse transcription quantitative polymerase chain reaction

SIRT- Sirtuin (silent mating type information regulation 2 homolog)

TA- Tibialis anterior

TLR- Toll-like receptor

UCP2- Uncoupling protein 2

UFH- Unfractionated Heparin

A+ H- AICAR and Heparin combinatorial treatment

β-DG- β-dystroglycan

β-GPA- β-Guanidinopropionic Acid

ACKNOWLEDGEMENTS:

Conducting my Master's research project at University of Ottawa's Faculty of Medicine has been an excellent learning and enriching experience. Firstly, I would like to extend my deepest gratitude to my supervisor, Dr. Bernard Jasmin, for his invaluable support, encouragement and guidance. I would also like to thank all past and present members of the Jasmin lab for their help, expertise and friendship. I feel privileged to have worked alongside such an amazing group of researchers. A special thank you to Dr. Lucas Bronicki for his mentorship and support throughout my project. Thank you to John, Guy and Christine for their technical assistance and laboratory management. I am grateful to Dr. Vladimir Ljubicic, Dr. Olivier Joassard, Dr. Aymeric Ravel-Chapuis and Dr. Adel Amirouche for their expertise and insightful discussions. Tara has been a great friend and support in the lab and I thank her for that. Thank you to Emma, Hasanen, Amanda and Jonathan for their advice and friendship.

I would like to thank my committee members, Dr. Jean-Marc Renaud and Dr. Alexandre Blais for their guidance, assistance and direction. I am grateful to the Ontario Graduate Scholarship (OGS) and Queen Elizabeth II Graduate Scholarship (QEII-GSST) for funding this project. My gratitude also extends to The Canadian Institutes of Health Research (CIHR), the Muscular Dystrophy Association (MDA) and Jesse's Journey for their generous funding.

Thank you to all my friends and family who supported me and were always there for me. I am extremely grateful to my parents for their constant love, reassurance and inspiration. Thank you to Ayla and Aeyon for being the best siblings anyone could ask for.

1) INTRODUCTION

1.1 Duchenne Muscular Dystrophy (DMD)

The term Muscular Dystrophy (MD) encompasses a group of genetic muscle disorders that are characterized by severe muscle wasting and muscle weakness (Emery, 2002). Duchenne Muscular Dystrophy (DMD) is the most common form of muscular dystrophy that afflicts 1 in 3500 male live births (Emery, 1991). Clinical symptoms start to appear before age 5, where patients display proximal muscle weakness and have difficulty walking, running and climbing stairs. Other clinical symptoms include pseudohypertrophy of calf muscles, Gower's sign (weakness of lower proximal muscles) and abnormal gait (Gowers et al, 1892, Fairclough et al, 2011). Due to progressive muscle wasting, these patients become wheelchair bound by early teens and death ensues in their second or third decade of life as a result of respiratory or heart failure (Anderson and Kunkel 1992; Baxter, 2006). Cardiomyopathy is present in about 90 % of DMD patients and accounts for 10-40 % of DMD deaths (Eagle et al, 2007; Baxter, 2006). Another major reason of death in DMD patients is respiratory failure, caused mainly due to the loss of diaphragm muscle, accounting for 75 % of deaths in these patients (Finsterer and Stollberger, 2003).

1.2 Dystrophin gene and genetic defects in DMD:

DMD is an X-linked recessive disorder caused by mutations/deletions in the dystrophin gene that prevents the production of functional dystrophin protein in skeletal

muscle fibers (Worton and Thomson, 1988). The 2.5 million bp dystrophin gene, located on chromosome Xp21.1, is one of the largest known human genes corresponding to about 0.1% of human genome (Blake et al, 2002). Dystrophin gene is regulated by 7 independent promoters and consists of 79 exons. The 14kb mRNA transcript of the dystrophin gene is mainly expressed in skeletal and heart muscle while small amounts are also expressed in the brain (Muntoni, 2003). Most mutations that result in DMD disrupt the open reading frame of the dystrophin gene generating a premature stop codon, which leads to the absence of functional dystrophin protein at the sarcolemma of skeletal muscle fibers (Koenig et al, 1989; Goyenvalle et al, 2011). Intragenic deletions of the dystrophin gene are the most common type of mutations accounting for approximately 65% of dystrophin mutations. Other common mutations include point mutations and duplications that account for 20-30 % and 5-15 % of dystrophin gene mutations respectively (Muntoni et al, 2003).

1.3 Dystrophin and Dystrophin Associated Protein Complex (DAPC):

Dystrophin is a 427kDa cytoskeletal protein that is expressed mainly at the sarcolemma of skeletal muscle fibers (Koenig et al, 1988). Dystrophin is also vastly expressed at the myotendinous and neuromuscular junctions of the muscle fibers (Khurana et al, 1991). Dystrophin protein consists of four main domains: 1) an N-terminal domain, 2) a central rod domain 3) a cysteine rich domain 4) and a C-terminal domain (Koenig, 1988). The N-terminal domain of dystrophin binds to the cytoskeletal actin filaments (F-actin) via calponin homology domains (Korenbaum and Rivero, 2002). The central rod domain is comprised of 26 tandem repeats of spectrin and it protects muscle fibers from contraction-induced damage by providing elasticity and flexibility to the muscle (Grum et al, 1999). The

cysteine rich residue and C-terminal domain of dystrophin interact with a multifunctional signaling complex at the sarcolemma called the dystrophin associated protein complex (DAPC). The DAPC is composed of a group of transmembrane proteins including dystroglycans (α, β), α-dystrobrevins, syntrophins, sarcospan and sarcoglycans (Ehmsen et al, 2002; Yang et al, 1995).

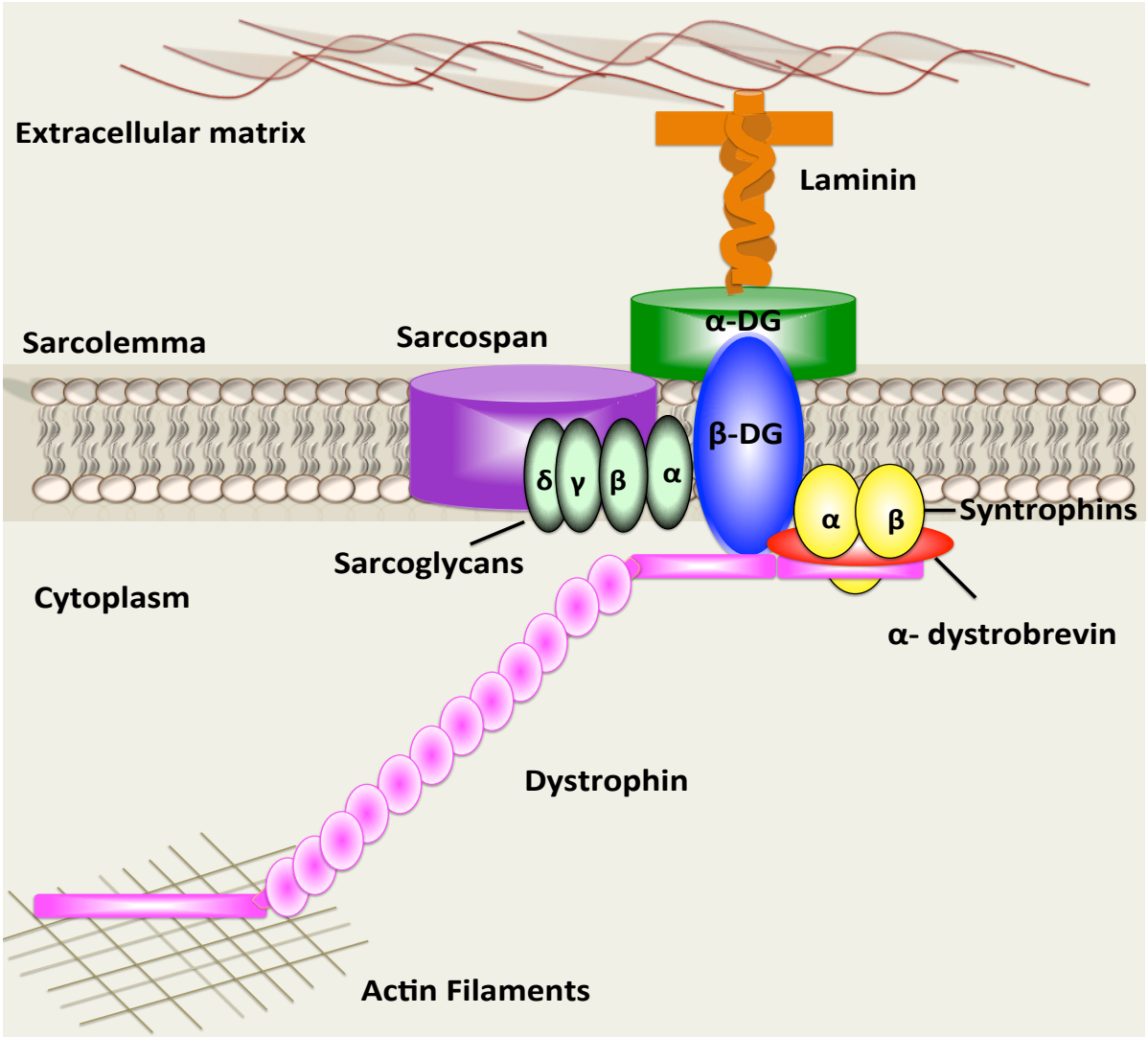
The Dystroglycan complex forms the core of DAPC and is composed of two proteins α-dystroglycan and β-dystroglycan (Ehmsen et al, 2002). β-dystroglycan interacts with cysteine rich domain of dystrophin at one end and to α-dystroglycan at the other end that links to proteins of the extracellular matrix such as laminin-2 (Ervasti and Campbell, 1993).

The sarcolgycan complex, composed of five transmembrane proteins, is important in anchoring DAPC to the sarcolemmal membrane. Studies have shown that loss of one or more of these sarcoglycan transmembrane proteins can lead to dissociation of the sarcoglycan complex and cause limb girdle dystrophies (Ehmsen et al, 2002). Syntrophins, a group of 4 transmembrane proteins, also help secure the DAPC at the sarcolemma (Crosbie et al, 1999; Ehmsen et al, 2002). In addition, syntrophins have been shown to recruit and interact with key signaling molecules such as nitric oxide synthase (nNOS) (Adams et al, 2000; Rando et al, 2001; Kameya et al, 1999; Stamler and Meissner, 2001). nNOS is an enzyme that catalyzes the production of NO (nitric oxide) from L-arginine and thus serves an important role in vasoconstriction (Miura and Jasmin, 2006). The abnormal blood vessel vasoconstriction and functional ischemia in DMD patients is linked to absence of nNOS at the sarcolemma (Sander et al, 2000; Ehmsen et al, 2002).

Other key components of the DAPC are α-dystrobrevins and syncolins. Out of the five α-dystrobrevin isoforms, α-dystrobrevin-2 is the most common isoform at the sarcolemma. α-dystrobrevins have been shown to interact with dystophin, sarcospan/sarcoglycan complex and syncoilins (Ehmsen et al, 2002). Syncoilins, intermediate filaments in muscle, are involved in forming a network between the DAPC and the intermediate filaments via their interactions with desmin protein (Ehmsen et al, 2002).

Through the aforementioned key interactions, dystrophin and DAPC form a strong mechanical and signaling link between the external sarcolemma and the internal cytoskeleton (Fairclough et al, 2011). In absence of functional dystrophin, the sarcolemma loses its integrity and the DAPC is disrupted. Sarcolemmal instability causes an increase in serum creatine kinase levels and a build up of Ca^{2+} within the cells (Florence et al, 1985; Bodensteiner and Engel, 1978). Influx of Ca^{2+} through leak channels disrupts Ca^{2+} homeostasis in muscle and activates calcium-mediated proteolysis that eventually leads to muscle degeneration (Alderton and Steinhardt, 2000; Tidball et al, 2005; Costelli et al, 2005). During initial stages of the disease, degenerating muscle undergoes repetitive cycles of regeneration. However, eventually the regenerative potential of muscle diminishes resulting in severe muscle wasting and muscle is replaced by fibrotic tissue (Menke and Jockusch, 1991;Weller et al., 1990; Allikian and McNally, 2007, Morrison et al, 2000). **Figure 1.1** illustrates key interactions of dystrophin with members of the DAPC and cytoskeletal actin filaments.

A



B

Figure 1.1: The interactions of dystrophin protein with cytoskeletal actin filaments and multifunctional signaling complex DAPC in normal (healthy) fibers. A) Dystrophin provides stability to the sarcolemmal membrane by binding to cytoskeletal actin filaments at its N-terminus and B-dystroglycan of the DAPC complex at its C terminus. B) Full-length dystrophin protein is shown containing four main domains i) The N-terminal domain ii) Central rod domain containing spectrin like repeats iii) Cysteine rich domain and iv) C terminal domain.

1.4 Mdx mouse: preclinical mouse model of DMD:

The mdx mouse, a naturally occurring mutant mouse, is the most commonly studied model for DMD and has been extensively characterized to develop an effective therapeutic strategy for the disease. The mdx mouse, first described by Bulfield et al, lacks dystrophin protein in muscle fibers (Bulfield et al, 1984). Molecular analysis of the defect revealed that mdx mice exhibit a point mutation in exon 23 of the dystrophin gene that disrupts the open reading frame by introduction of a Premature Termination Codon (PTC) and leads to absence of dystrophin protein (Fairclough et al, 2011). It is interesting to note that about 30% of DMD patients display a similar defect in which an early PTC is introduced resulting in absence of dystrophin protein (Bulfield et al, 1984; Sicinski et al, 1989). In comparison to the DMD patients, mdx mice have a less severe pathology and display a relatively normal life span. Despite the difference in severity of disease, mdx mice represent closely the DMD condition displaying similar myonecrosis, biochemical features such as enzyme leakage and variance in muscle fiber diameter (Coulton et al, 1988). Further histological characterization of the mdx mouse has revealed that the dystrophic muscle fibers display increased central nucleation and smaller diameter as compared to healthy/wild-type fibers. Further, mdx mice exhibit increased serum creatine kinase and pyruvate kinase levels (Bulfield et al, 1984). The mdx mice also have higher variations in muscle fiber size diameters as compared to normal wild-type mice (Torres et al, 1987).

1.5 Treatments for DMD:

Despite the detection of underlying molecular defect in DMD for decades and extensive characterization of the disease in various DMD models, there is no effective treatment for this devastating disease. However, several short-term pharmacological therapies and potential therapies are being explored.

1.5.1 Short-term pharmacological therapies:

Some drugs can delay the symptoms of DMD by targeting the secondary effects for instance glucocorticoids including predinisone, deflazacort and predinisolone (Bushby et al, 2010; Moxley et al, 2005; Manzur et al, 2008). However, these drugs only provide short-term benefits and have various detrimental side effects such as weight gain, growth and immune system suppression (Manzur et al, 2008). Nonetheless, these drugs have been shown to extend the amount of time patients are ambulatory and provide benefits in muscle strength, pulmonary function and delay the onset of cardiomyopathy (Balaban et al., 2005; Manzur et al., 2008; Monaco et al., 1988; Bushby et al, 2010).

1.5.2 Potential therapies for DMD:

Several therapies are being explored that can target the genetic defect or replace the absent dystrophin protein with a surrogate protein to compensate for the loss of dystrophin in muscle. These therapies include gene-based, cell-based and utrophin based therapies.

1.5.2.1 Gene- based therapies:

In recent years, gene based therapies are being examined to replace the absent dystrophin protein in DMD muscle. Given its large size, it is not feasible to replace the entire dystrophin gene (2.5 Mb). However, numerous studies have used dystrophin mini or micro genes that can produce a truncated but functional form of dytrophin protein (Wang et al, 2000; Goyenvalle et al, 2011; Pichavant et al, 2010). The concept of these strategies was derived from patients with a milder form of muscular dystrophy called Becker Muscular Dystrophy (BMD). BMD is caused due to mutations in the rod domain of the dystrophin gene resulting in production of a truncated but functional form of dystrophin protein lacking the spectrin repeats (Hoffman et al, 1988) (as shown in **Figure 1.1**). These patients have longer life spans and slower onset of symptoms compared to DMD patients (Hoffman et al, 1988). Mini-dystrophin genes lack portions of the rod domain and can be delivered systemically to the host by using recombinant Adeno Associated Virus (AAV) viruses (Wang et al, 2000). Systemic delivery of these micro-dystrophin genes has shown remarkable improvements in mdx mice and dogs (Gregorevic et al, 2008; Pichavant et al, 2010). While this strategy has attained success in several studies, recent studies have revealed that these approaches face major limitations such as insufficient virus production and immune response by host cells (Fairclough et al, 2011). Further, human clinical trials employing rAAV to deliver mini-dystrophin gene to DMD patients did not exhibit successful dystrophin restoration and it was revealed that these mini-dystrophin genes may evoke a T-cell response in treated patients (Mendell et al, 2010).

Alternative therapeutic approaches target dystrophin mRNA transcripts during the transcription process. One such approach is the suppression of a premature stop codon. In this approach, aminoglycoside antibiotics such as gentamicin are used to prevent the ribosome transcription machinery from recognizing premature stop codons in the mutated dystrophin gene (Manuvakhova et al, 2000). As a result, full-length mRNA transcripts are formed which can be translated into functional full-length dystrophin protein with a substituted amino acid in place of the premature stop codon (Manuvakhova et al, 2000). Despite delivering impressive results in early human clinical trials, this approach faces major hurdles including immune responses to dystrophin and toxic effects of drugs (Welch et al, 2007; Fairclough et al, 2011). PTC124 can also promote the read-through of premature termination codons and has been shown to increase dystrophin expression in mdx mice and improve the dystrophic phenotype (Welch et al, 2007). However, only about 13 % of DMD patients have been shown to have mutations involving premature stop codons. Therefore, this strategy is not applicable to the entire DMD population (Fairclough et al, 2011).

Exon skipping is another RNA based approach that targets dystrophin mRNA transcripts and induces skipping of exons that are not essential for the production of a functional dystrophin protein (for instance the exons that encode the spectrin repeat region of the dystrophin protein) (Goyenvalle et al, 2011). This approach relies on antisense oligonucleotides (AONs) that can block the splicing machinery from recognizing these exons, resulting in a shorter mRNA transcript that can produce a truncated but functional dystrophin protein (Fairclough et al, 2011; Foster et al, 2012). This strategy serves a large population of DMD patients and could target about 83 % of DMD mutations (Fairclough et

al, 2011). However, some major limitations associated with this strategy include poor uptake of AONs by the cells, variable efficiency in different muscles such as low efficiency in the heart muscle and rapid clearance from the body (Goyenvalle and Davies, 2011). Furthermore, since this strategy targets the underlying genetic mutation, specific AOs are required to target the specific genetic defect in DMD patients. To address this issue, recent studies have emphasized alternative strategies such as multiple exons skipping in which a mutation hot spot region in the dystrophin gene is targeted (Goyenvalle and Davies, 2011).

1.5.2.2 Cell-based therapies:

Cell based therapies involve the delivery of myoblasts or stem cells, that are capable of differentiating and forming new muscle, to the diseased area. Myoblasts, muscle precursor cells, were first to be employed in this approach where they were injected in mdx mice to fuse with dystrophin-negative fibers and induce dystrophin production (Partridge et al, 1989; Law et al, 1993). Although initially promising, myoblast transplantation did not exhibit encouraging results in human clinical trials as only 1 out of 12 DMD patients showed a 10 % increase in dystrophin positive fibers (Fairclough et al, 2011). The low effectiveness of this strategy was attributed to technical limitations such as immunosuppression and low distribution of cells to the diseased muscle (Mendell et al, 1995; Fairclough et al, 2011).

On the other hand, stem cell transplantation ensures a systemic delivery of cells and overcomes the delivery issue associated with myoblast transplantation (Gussoni et al, 1999; Meregalli et al, 2010). In this strategy various types of stem cells such as muscle-derived

CD133 + progenitor cells, non-muscle mesenchymal and mesoangioblast cells are delivered to the dystrophic muscle to form new muscle (Torrente et al, 2004; Sampaolesi et al, 2006; Fairclough et al, 2011). However, stem cell therapy has been associated with limitations such as implant rejection (Benchaouir et al, 2007; Mendell et al, 1995; Gussoni et al, 1992). To overcome this limitation, some laboratories have used genetically engineered stem cells that have a lentivirus transduced in them to induce exon skipping of the mutated exon (Benchaouir et al, 2007). However, this strategy of combining stem cell approach with genetic approach has an inherent risk of tumor development in clinical trial as it involves the injection of a provirus (Benchaouir et al, 2007).

1.5.2.3 Utrophin-based therapies:

Utrophin is a 395kDa cytoskeletal protein encoded by chromosome 6 in humans (Tinsley et al, 1992). It is an autosomal homologue of dystrophin, which has been shown to share high sequence and structural similarity with dystrophin (Blake et al, 2002; Love et al, 1989). The primary structure and sequence of utrophin is very similar to dystrophin, with N-terminal, cysteine rich and C-terminal domain sharing about 80 % similarity (Tinsley et al, 1992; Perkins and Davies, 2002). Further, utrophin has been shown to associate with members of DAPC such as α-dystrobrevin-1 and β-dystroglycan at its C-terminus (Peters et al, 1998; Ishikawa-Sakurai et al, 2004). In addition, the N-terminal domain of utrophin binds to the cytoskeletal F-actin filaments (Perkins and Davies, 2002; Winder et al, 1995). Figure 1.2 highlights a few of these key interactions.

Despite the structural and functional similarities, utrophin and dystrophin display differences in their expression patterns. Unlike dystrophin, which is expressed along the entire length of the sarcolemma in adult muscle fibers, utrophin expression is mainly restricted to the neuromuscular and myotendinous junction (Khurana et al, 1991; Ohlendieck et al, 1991). Interestingly, utrophin has been shown to be a developmentally regulated protein, which is expressed along the entire length of the sarcolemma in fetal and developing muscle fibers (Clerk et al; 1993). Further, the utrophin A expression is up-regulated in the dystrophic muscle of the mdx mice and DMD patients. However, this up-regulation is not enough to compensate for the loss of dystrophin protein in DMD condition (Kleopa et al, 2006; De la Porte et al, 1999; Mizuno et al, 1993). Several studies have demonstrated that transgenic overexpression of utrophin in skeletal muscle of mdx mice alleviates the dystrophic phenotype (Squire et al, 2002, Rafael et al, 1998; Perkins et al, 2001; Krag et al, 2004; Tinsley et al, 1996). Strategies such as utrophin gene transfer and several pharmacological intervention therapies can accomplish utrophin up-regulation in muscle.

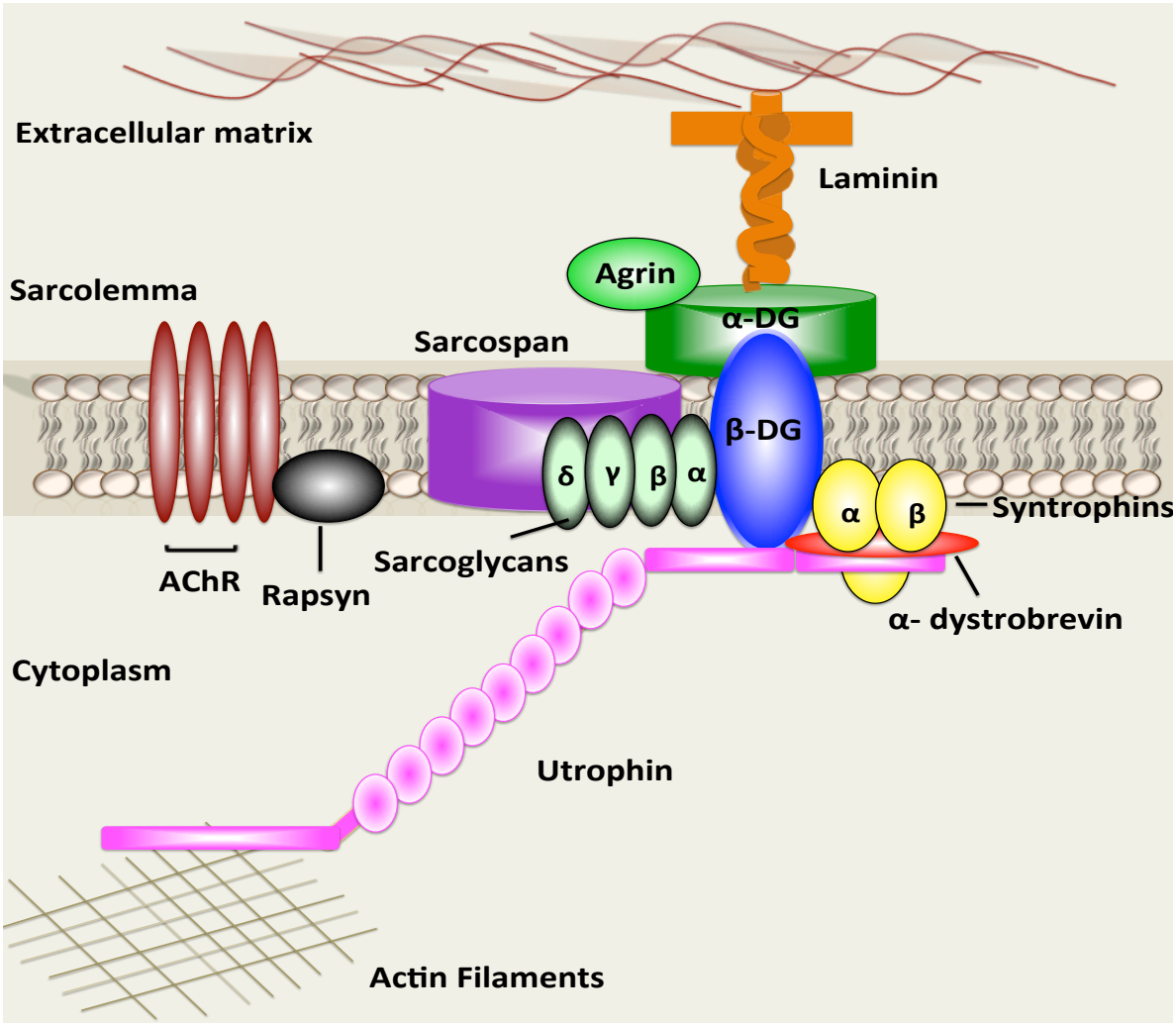
During utrophin- gene transfer techniques, full-length or mini utrophin genes are shuttled to DMD muscle using AAV vectors to stimulate its expression (Odom et al, 2008; Deol et al, 2007). Such strategies have been demonstrated to stimulate utrophin A expression and improve the dystrophic phenotype in mdx mice (Odom et al, 2008; Deol et al, 2007). One major advantage of using utrophin gene therapies in comparison to dystrophin gene therapies is that utrophin gene delivery minimizes the risk of immune reaction since utrophin is naturally expressed in the dystrophic muscle. However, utrophin gene transfer approach faces a few limitations involving poor cellular uptake, variable efficiency in different tissues

and rapid clearance from the body (Fairclough et al, 2011).

Pharmacological intervention is another appealing approach that has been shown to up-regulate utrophin expression in muscle. One of the main advantages of pharmacological up-regulation is that it could be delivered systemically since utrophin overexpression in non-muscle tissue is not associated with any deleterious effects (Fisher et al, 2001). Further, pharmacological intervention is effective for all DMD patients regardless of the specific genetic defect of the dystrophin gene (Fairclough et al, 2011). In this context, several pharmacological compounds have been used to up-regulate utrophin A expression in muscle including heregulin, L-arginine, SMT C1100 and activators of the slow myogenic program. Heregulin is a nerve derived trophic factor that has been shown to stimulate utrophin A transcription in muscle (Khurana et al, 1999; Gramolini et al, 1999). Further, heregulin treatment in mdx mice has been revealed to up-regulate utrophin expression and ameliorate the dystrophic phenotype (Krag et al, 2004). Another important drug in this context is L-arginine that stimulates utrophin A expression in muscle by increasing the production of nNOS (Voisin et al, 2005). Recent studies have shown that SMT C1100, a synthetic compound, increases utrophin A expression in muscle, increases overall muscle strength and ameliorates the dystrophic phenotype in mdx mice (Tinsley et al, 2011). SMT C1100 is now being tested in clinical trials and it was reported to be the first utrophin modulator to enter clinical trials (Tinsley et al, 2014).

In addition to the aforementioned pharmacological agents, the agonists of the slow myogenic program in muscle can also up-regulate utrophin. **(Section 1.6)**

A



B

Figure 1.2: Key interactions of utrophin protein with cytoskeletal actin filaments and members of DAPC. A) Like dystrophin protein, Utrophin A interacts with the actin filaments of the cytoskeleton through its N-terminus and associates with members of the DAPC such as β-dystroglycan, α-dystroglycan, sarcospan and sarcoglycan complex and α-dystrobrevin. Since utrophin A is predominantly present at the NMJ or myotendinous junction in adult fibers, the AChR are highlighted in the figure. B) Organization of utrophin protein and its key structural domains are shown.

1.6 Slow oxidative myogenic program and its role in regulation of utrophin A expression:

Skeletal muscle fibers can be classified into different subtypes based on the specific myosin isoform they contain. The slow twitch fibers Type I are composed of Myosin Heavy Chain I (MHC I) and rely on oxidative metabolism. In contrast, the fast twitch fibers are composed of Myosin Heavy Chain II (MHC II) and can be classified as Type IIa (oxidative, glycolytic), IIx and IIb (glycolytic) (Rivero et al, 1999). In addition, studies on mdx mice and DMD patients have demonstrated that these slow oxidative muscle fibers are less susceptible to damage as compared to their faster counterparts (Webster et al, 1988; Moens et al, 1993).

The molecular mechanisms underlying the resistance to dystrophic phenotype in slow oxidative muscle fibers versus fast glycolytic muscle fibers are not precisely known. However, there is mounting evidence that suggests that a higher utrophin expression in these muscle fibers might protect against the dystrophic phenotype (Gramolini et al, 2001; Chakalakkal et al, 2003). In this context, our laboratory was first to demonstrate that slower oxidative fibers have a higher expression of utrophin in extasynaptic regions of the muscle as compared to fast glycolytic fibers (Gramolini et al, 2001) Further, our laboratory has shown that when the slow, oxidative myogenic program is stimulated in mice through functional overload, utrophin A expression increases (Chakalakkal et al, 2003). Several studies by our laboratories and others have shown that the transcriptional mechanism that promote the slow myogenic program in muscle can also regulate utrophin A expression by

stimulating various transcriptional mechanisms described in more detail in the next section (Miura et al, 2009; Ljubicic et al, 2011; Gordon et al, 2013).

1.7 Transcriptional regulation of the utrophin A promoter:

There are two main isoforms of utrophin: utrophin A and utrophin B, that are regulated by two independent promoters (Burton et al, 1999; Perkins et al, 2001). Utrophin A is predominantly expressed in skeletal muscle fibers whereas utrophin B is expressed in vascular endothelial cells (Weir et al, 2002).

Utrophin A promoter contains an N-box motif (TTCCGG) that regulates utrophin A expression and has been shown to be important in post-synaptic expression of utrophin A (Dennis et al, 1996; Gramolini et al, 1997). Transcription factors such as GA-binding protein α and β (GABPα and GABPβ) can bind to this motif and stimulate utrophin transcription. Activation of GABPs is mediated by an extracellular signal-related kinase (ERK) pathway through a nerve-derived growth factor called heregulin (Khurana et al, 1999; Gramolini et al, 1999). Other upstream motifs of the utrophin promoter include a conserved E box, that serves as a binding site for myogenic regulatory factors such as helix-loop-helix proteins, myogenin and MyoD. These regulatory factors can enhance utrophin A expression by stimulating the utrophin A promoter (Gramolini and Jasmin, 1999).

The utrophin A promoter also contains an NFAT (Nuclear Factor of activated T cells) binding site which specifically binds NFAT transcription factor and regulates utrophin

expression (Dennis et al, 1996). Further, the utrophin A promoter contains recognition sites for transcription factors Sp1 and Sp3 that interact with GABP to stimulate utrophin A transcription (Galvagni et al, 2001; Miura and Jasmin, 2006). Studies have shown that Sp1 plays an important role in utrophin expression. In this context, okadaic acid has been shown to regulate utrophin A transcription via the Sp1 factor (Rodova et al, 2004). In addition, PGC-1α has been revealed to stimulate utrophin A transcription through activation of transcription of GABP α and β (Angus et al, 2005). Further, a PPAR- β/δ agonist, GW501516, has been shown to up-regulate utrophin A transcription via a PPRE site (PPAR responsive element) within the utrophin A promoter (Miura et al, 2009).

1.8 Key modulators of slow myogenic program in skeletal muscle fibers and their role in transcriptional regulation of utrophin A expression:

Previous studies have identified the following important mechanisms and pathways that can induce the slow, oxidative myogenic program and regulate utrophin A expression at the transcriptional level.

1.8.1. Calcineurin/NFAT signaling:

Calcineurin, a Ca^{2+} dependent phosphatase, is a key regulator of the slow myogenic program (Olson and Williams, 2000). A study from our laboratory by Chakalakkal et al. first demonstrated that activation of calcineurin could increase utrophin-A mRNA levels in slow oxidative muscle fibers of transgenic mice overexpressing calcineurin (Chakkalakal et al, 2003). This study also revealed that the effect of calcineurin on utrophin A expression is

mediated by NFAT (Nuclear Factor of Activated T cells) which binds to a specific site in the utrophin A promoter now called the NFAT binding site (Chakalakkal et al, 2003). These findings implied that stimulation of calcineurin/NFAT signaling could up-regulate utrophin A expression in muscle through activation of the slow myogenic program.

1.8.2. PPAR-β/δ:

PPARs (Peroxisome proliferator-activated receptors) are ligand dependent nuclear receptors involved in lipid metabolism and homeostasis (Ehrenborg and Krook, 2009). PPAR-β/δ is the most common isoform of PPARs in skeletal muscle and it mediates its effect on genes by forming a heterodimer with retinoid x receptor (RXR) and binding to peroxisomal proliferator response element (PPRE) in the promoter region of a gene (Ehrenborg and Krook, 2009; Ljubicic et al, 2013). PPAR-β/δ receptors have four functional domains: 1) N-terminal A/B domain which contains a ligand independent transcriptional activation domain 2) C-domain that binds to the DNA sequence 3) D-domain that is comprised of a hinge region important for interaction with cofactors such as PGC1α 4) E/F domain, which is highly conserved and consists of a ligand binding domain (Ehrenborg and Krook, 2009). PPAR-β/δ has several ligands including long-chain fatty acids, retinoic acid and synthetic ligands such as bezafibrate (Xu et al, 1999; Fruchart et al, 1999; Ehrenborg and Krook, 2009).

PPAR-β/δ has been shown to play a key role in regulation of skeletal muscle fiber types. Further, slow oxidative muscle fibers exhibit a higher expression of PPAR-β/δ as compared with the fast glycolytic muscle fibers (Ehrenborg and Krook, 2009). It has been

demonstrated through studies in transgenic mice that an increase in expression of PPAR-β/δ or its co-activator PGC-1α or a reduction in its co-repressor RIP140 (Receptor Interacting Protein) can induce the slow myogenic program (Wang et al, 2004; Gaudel et al, 2008; Ehrenborg and Krook, 2009). Further, studies in mice have shown that when calcineurin activity is inhibited in skeletal muscle by cyclosporine A administrations, PPAR-β/δ mediated changes in fiber type are repressed. This suggests that calcineurin and PPAR-β/δ pathway might be interconnected (Gaudel et al, 2008). Earlier work in our laboratory has demonstrated that administration of GW501516, PPAR-β/δ specific agonist, results in a switch from fast to slow fiber type leading to subsequent increase in utrophin A expression and rescue of muscle function in mdx mice (Miura et al, 2009). Further, it has been shown that this stimulation of utrophin A expression is mediated by a PPRE site in the utrophin A promoter (Miura et al, 2009).

1.8.3. PGC-1α:

PGC-1α (Peroxisome proliferator-activated receptor-gamma coactivator) is a master regulator of mitochondrial metabolism and biogenesis and was first identified by Spiegelman and colleagues (Ljubicic et al, 2013). The PPARγ coactivator-1 family consists of 3 main isoforms: PGC-1α, PGC-1β and PRC. These PGC-1 proteins have several key molecular and structural features: 1) Protein surfaces for interactions with factors such as PPAR and GABP, 2) Conserved DHDY motif that binds HCF enabling interactions with GABP, 3) Transcriptional activation domain and 4) sites for post-transcriptional modifications (Hock and Kralli, 2009). Specifically PGC-1α activity has been shown to be regulated by 3 important post-transcriptional events including phosphorylation by AMPK,

deacetylation by Sirtuin1 (SIRT1) and arginine methylation by PRMT1 methyl transferase (Hock and Kralli, 2009; Teyssier et al, 2005). PGC-1α has been shown to play a vital role in promoting the slow oxidative myogenic program in muscle. The evidence for this role of PGC-1α first came from studies by Lin et al, 2002 where the overexpression of PGC-1α in transgenic mice was shown to promote a shift towards a slower oxidative phenotype (Type II → Type IIa fibers) (Lin et al, 2002). Later, a study by Handschin and colleagues further established that knocking out PGC-1α leads to a shift in fiber type from slower fibers (Type I and IIa) to faster fibers (IIb and IIx) (Handschin et al, 2007). Earlier work from our laboratory has revealed that PGC-1α can stimulate utrophin A transcription in skeletal muscles through GABPα interaction at the utrophin A promoter (Angus et al, 2005). This study also demonstrated that PGC-1α can drive the formation of slow twitch muscle fibers and co-activate calcineurin signaling in skeletal muscle fibers (Angus et al, 2005). Together these studies indicate that modulators of PGC-1α activity can play a key role in utrophin-A up-regulation.

1.8.4. AMPK:

AMPK, a heterotrimeric protein, functions as an energy sensor and is activated in energy-deprived cells (Long and Zierath, 2006). AMPK plays a vital role in regulating skeletal muscle plasticity by targeting factors such as PGC-1, Histone Deacetylases (HDAC), SIRT1, MEF2 and p53 (Lira et al, 2010; Canto and Auwerx, 2010). Various studies have shown that AMPK activation in mice leads to stimulation of the slow myogenic program as indicated by studies where AMPK activation in transgenic mice stimulates a shift in fiber types from Type IIb fibers to IIa/X fibers, stimulates PGC-1α expression,

activates mitochondrial biogenesis and increases glycogen stores (Rockl et al, 2007; Garcia et al, 2008). More specifically, earlier work from our laboratory has established that AMPK synthetic agonist, 5-Aminoimidazole-4-carboxamide-1-b-c-ribofuranoside (AICAR), induces slow oxidative myogenic program and triggers utrophin A up-regulation in dystrophin-deficient mdx mice (Ljubicic et al, 2011). Further, there is evidence of cross talk between the pathways through which AMPK, PPAR-β/δ and PGC-1α induce the slow myogenic program. When PPAR-β/δ is overexpressed in AD293 cells, co-immunoprecipitation of PPAR-β/δ and AMPK subunits (α1 or α2) was observed indicating a physical interaction between PPAR-β/δ and AMPK (Narkar et al, 2008). However, the precise mechanisms through which PPAR-β/δ and AMPK interact with each other and with PPAR co-activator PGC-1α are still not entirely understood.

1.8.5. SIRT1:

SIRT1 belongs to a family of enzymes called sirtuins that differ from each other based on their activity, localization and nature of substrates (Lagouge et al, 2006; Baur et al, 2012). SIRT1 is a NAD+ dependent deacetylase that is activated when the cells are deprived of nutrients. Pharmacological activation of AMPK by AICAR in C2C12 myotubes has been shown to stimulate SIRT1 activity (Nemoto et al, 2004). More specifically SIRT1 has been shown to activate the slow myogenic program by deacetylation of PGC-1α transcriptional factor (Gerhart-Hines et al, 2007; Hock and Kralli, 2009). Additional studies in this context showed that when SIRT 1 was knocked out, PGC-1α activity decreased (Amat et al, 2009; Ljubicic et al, 2013), Since PGC-1 α can stimulate utrophin A expression by activating the slow myogenic program in muscle, SIRT1 also seems to play a crucial role in regulation of

utrophin A expression. In fact, recent studies by our laboratory and others have shown that resveratrol administration, a SIRT1 agonist, stimulates SIRT1, PGC-1α and utrophin A expression in mdx mice (Gordon et al, 2013, Ljubicic et al, 2014). **Figure 1.3** depicts the action of the slow myogenic program modulators and their agonists in stimulating utrophin A transcription.

1.9 Additional transcriptional activators of utrophin A expression:

In addition to the modulators of the slow myogenic program, other mechanisms such as the Nitric Oxide Pathway and Histone Deacetylase Inhibiton (HDACi) pathway are also important in regulating utrophin A expression in skeletal muscle.

As described earlier, the multi-signaling complex of the sarcolemma DAPC can interact with nNOS, a protein that catalyzes the formation of Nitric Oxide (NO) (Stamler and Meissner, 2001). NO is a key signaling molecule and exhibits anti-inflammatory properties (Stamler and Meissner, 2001). Previous studies have revealed that the dystrophic fibers have reduced expression of nNOS as compared to healthy fibers as a result of disruption of DAPC (Brenman et al, 1995). Studies where nNOS activity was stimulated or an nNOS transgene was introduced in mdx mice, reported that the dystrophic phenotype was improved (Barton et al, 2005; Voisin et al, 2005; Tidball and Wehling-Henricks et al, 2004). Additional studies have reported that L-arginine, an important substrate for nNOS, increases utrophin A expression in mdx mice (Chaubourt et al, 1999; Barton et al, 2005; Voisin et al, 2005). The precise mechanisms through which L-arginine exerts its effect on utrophin are not clear.

However, L-arginine was recently demonstrated to stimulate the utrophin A promoter activity in muscle cells indicating that the effect of L-arginine on utrophin A expression may be transcriptional to some extent (Moorwood et al, 2011).

There is mounting evidence that suggests that the inhibition of histone deacetylases (HDAC) could serve as an effective strategy to treat DMD. In multiple studies, treatment of mdx mice with Histone Deacetylase Inhibitors (HDACi) such as Trichostatin A (TSA), Valproic acid and phenylbutyrate was shown to ameliorate the dystrophic phenotype independent of utrophin A up-regulation. These studies reported an increase in cross-sectional Area (CSA) of myofibers, decrease in inflammation and necrotic scars (Minetti et al, 2006). However, recent studies by Vianello et al. in skeletal muscle cells and mdx mice have shown that HDACi such as TSA and Valproic acid stimulate utrophin A expression in DMD skeletal muscle cells (Vianello et al, 2013; Vianello et al, 2014). Further, a study by Moorwood et al. showed that TSA stimulates the utrophin A promoter activity (Moorwood et al, 2011). These results suggest that the protective effect of HDACi on the dystrophic phenotype is in part regulated by utrophin expression. The precise mechanisms through which HDACi acts on utrophin A expression are not entirely known (Miura and Jasmin, 2006). However, one of the postulations is that Myocyte Enhancer Factor 2 (MEF2) plays a crucial role in this regulation. MEF2 has been shown to stimulate PGC-1α expression by activating a positive feedback mechanism (Handschin et al, 2003). MEF2 activity is inhibited by de-acetylation by HDAC, which results in decreased PGC-1α expression. Therefore, the use of HDACi can increase PGC-1α expression and possibly utrophin A expression in muscle (Miura and Jasmin, 2006; Handschin et al, 2003).

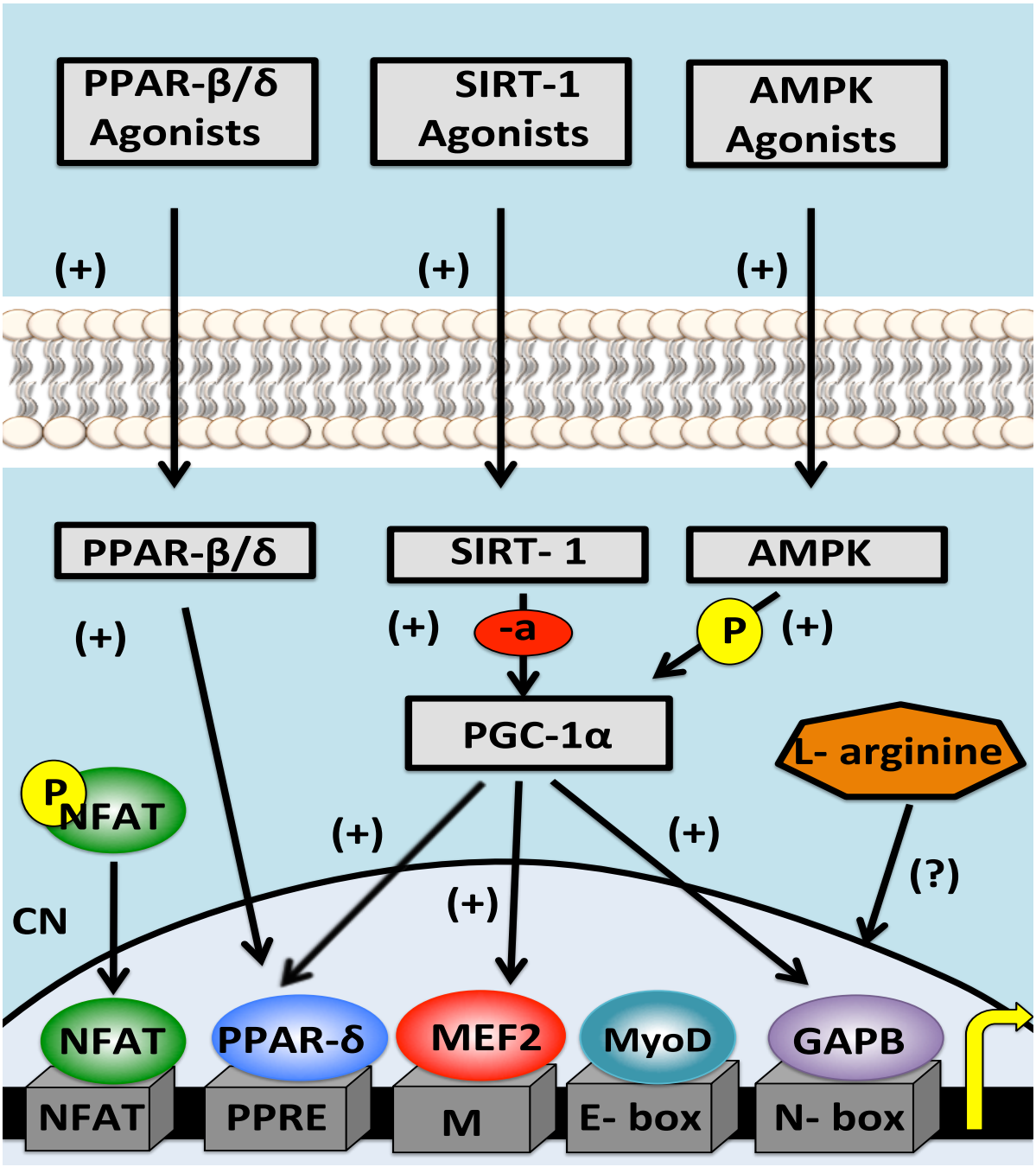


Figure 1.3 – Transcriptional regulation of utrophin A expression by pharmacological activation of key phenotypic modifiers. AMPK, PPARβ/δ and SIRT-1 agonists can activate utrophin A transcription in muscle. These phenotypic modifiers can act directly at the utrophin A promoter (i.e. PPARβ/δ → PPRE) and/or activate PGC-1α expression. PGC-1α can activate the transcription of factors GABP α and β that bind to the N-box region of the utrophin A promoter to activate its transcription. SIRT-1 can activate PGC-1α through deacetylation (-a) while AMPK activates PGC-1α through phosphorylation (P). Other activators of utrophin A transcription include NFAT, L-arginine, MEF2 and MyoD. (CN→ Calcineurin, M→ MEF2 binding site)

1.10 Post-transcriptional regulation of utrophin A expression:

The transcriptional regulation of utrophin A expression has been studied extensively (Khurana et al, 1999; Gramolini et al, 1999; Galvagni et al, 2001; Perkins and Davies, 2001; Miura et al, 2009). However, recent advances in this field have emphasized that the post-transcriptional regulation of utrophin A plays a crucial role in its expression. The evidence for possible post-transcriptional control of utrophin A expression initially came from studies where up-regulation of utrophin A protein was not accompanied by an increase in utrophin A mRNA levels. A study by Gramolini et al, 1999 showed that skeletal muscles of DMD patients had higher levels of utrophin A protein as compared to healthy subjects while utrophin A mRNA levels remained unchanged in the two groups (Gramolini et al, 1999). Another study reported that utrophin A protein is up-regulated in mdx mice but no increase in utrophin A mRNA levels was observed (Weir et al 2002). In this context, the 3'UTR and 5'UTR of utrophin have been shown to play a key role in the post-transcriptional regulation of utrophin. The 5'UTR of utrophin contains an Internal Ribosomal Entry Site (IRES) that can stimulate utrophin A expression in muscle during muscle regeneration and glucocorticoid therapies (Miura et al, 2005; Miura et al 2008). On the other hand, the 3'UTR of utrophin has been shown to contain important regions that can affect utrophin A expression. These regions are discussed in detail in the next section.

1.10.1 Post-transcriptional regulation of utrophin A via the 3'UTR and AREs:

Bioinformatic analysis studies have revealed that the 3'UTR of utrophin is about 2.4kb in length and contains important sites including microRNA binding sites and numerous cis elements (Basu et al, 2011). MicroRNAs can bind to specific sites within the 3'UTR and inhibit the synthesis of utrophin A expression. Several microRNAs have been revealed to regulate utrophin A expression such as miR-206, miR133b, let-7-c, miR150, miR-196b, miR 296 (Basu et al, 2011; Rosenberg et al 2006). In addition, the cis elements within the 3'UTR can also play a key role in post-transcriptional regulation of gene expression (Bakheet et al, 2003; Apponi et al, 2011).

ARE (AU-rich elements) are the most prevalent type of cis elements present in about 5-8% of human mRNAs (Bakheet et al, 2003; Apponi et al, 2011). As their name implies these motifs are rich in adenine and uridine bases (Gingerich et al, 2004). These AREs have been shown to mediate their effects on protein expression by controlling mRNA decay and steady-state levels (Gingerich et al, 2004;Gramolini et al, 2001; Chakalakkal et al, 2008). More specifically, the higher expression of utrophin A in slow oxidative muscle fibers versus the faster counterparts has been linked to the interaction between these AREs and calcineurin signaling (Chakalakkal et al, 2008).

The regulation of mRNA stability by AREs is mediated by their interaction with intracellular proteins called AUBPs (Chen et al, 2001; Gingerich et al, 2004). Depending on the type of AUBP that binds to the AREs, the interaction leads to either mRNA stabilization

and translation enhancement or mRNA destabilization and repression (Gingerich et al, 2004; Chen et al, 2001). For instance AUBP such as AUF1 (AU-rich element binding protein 1) has been shown to promote destabization of mRNA while HuR (Human Antigen R) has been shown to stabilize the mRNA transcripts (Gingerich et al, 2004).

A recent study from our laboratory demonstrated that an RNA binding protein KSRP specifically binds to the 3'UTR of utrophin A and regulates its expression in muscle (Amirouche et al, 2013). KSRP is an intracellular protein that interacts with AREs in the 3'UTR of target transcripts and mediates their decay often referred to as ARE-mediated decay (ARMD). KSRP is composed of four main domains including KH1, KH2, KH3, KH4 (Gherzi et al, 2004). The precise mechanism through which KSRP regulates utrophin A expression is not entirely understood. However, previous research has shown that the KH1 region of KSRP can be regulated by phosphorylation that promotes its binding to the protein 14-3-3 (Matoulkova et al, 2012) Other studies in this context have reported that p38 activation in muscle cells phosphorylates KSRP and inhibit its binding to AREs in the 3'UTR and therefore fails to promote mRNA decay (Briata et al, 2005) More specifically, previous work from our laboratory provided evidence that p38 activation plays a central role in utrophin A expression in muscle. In this study p38 activation was shown to down-regulate KSRP and stimulate utrophin A expression in muscle cells and mdx mice (Amirouche et al, 2013). This study further demonstrated that activation of p38 promotes sequestration of KSRP by 14-3-3 (Amirouche et al, 2013). **Figure 1.4** describes this post-transcriptional regulation of utrophin A expression by p38 activators and KSRP.

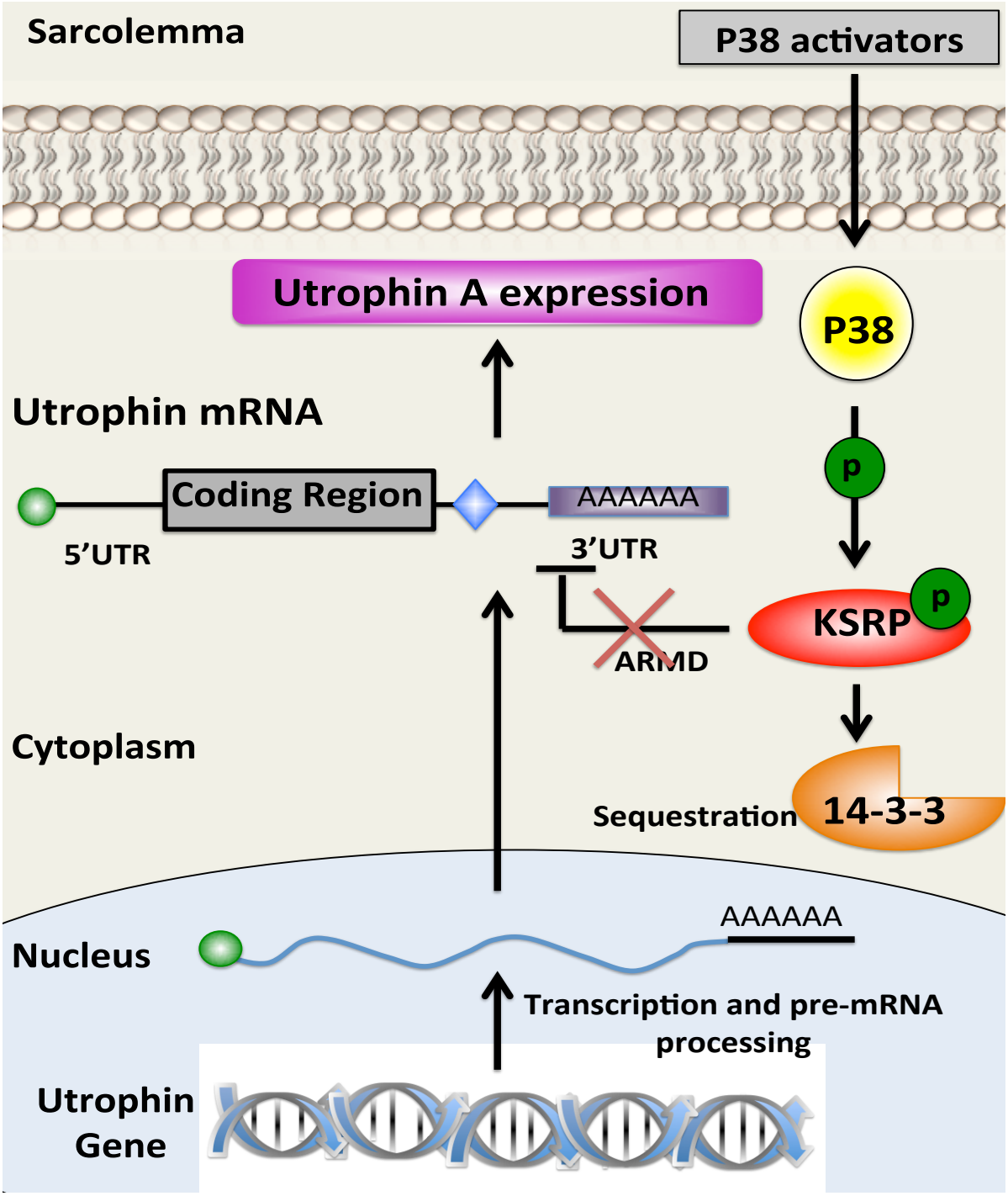


Figure 1.4 – Post-transcriptional regulation of utrophin A expression in muscle by activation of p38 signaling and down-regulation of KSRP. p38 activation can phosphorylate KSRP, an RNA binding protein that is thought to be involved in ARE mediated decay (ARMD) of utrophin transcripts. Phosphorylation (P) of KSRP promotes its sequestration by a regulatory protein 14-3-3. As a result; the inhibitory effect of KSRP on utrophin A expression is eliminated.

1.11 Statement of Problem:

Duchenne Muscular Dystrophy (DMD) is the most prevalent inherited neuromuscular disorder (Emery, 1991; Bushby et al, 2010). This disease is extremely severe as children become wheelchair-bound by early adolescence and death occurs in their second or third decade of life. At present there is no cure or effective treatment for this devastating disease, however, several therapeutic strategies are being examined around the world (Fairclough et al, 2011). One such strategy involves up regulation of utrophin, an autosomal homologue of dystrophin (Blake et al, 2002).

Utrophin A expression can be controlled at various transcriptional, post-transcriptional and translational mechanisms (Miura et al, 2009; Miura et al, 2010; Moorwood et al, 2011; Moorwood et al, 2013). In this context, our laboratory and others have demonstrated that pharmacological activation of phenotypic modifiers such as PPARβ/δ, AMPK, SIRT-1 can up-regulate utrophin A expression in muscle and these effects are mediated, at least in part, at the transcriptional level (Miura et al, 2009; Ljubicic et al, 2011; Ljubicic et al, 2014, Gordon et al, 2013). More recently, a study by Amirouche et al. demonstrated that an FDA approved drug, heparin, up-regulates utrophin A expression in muscle cells and mdx mice by down-regulation of an RNA binding protein, KSRP, mediated by transport protein 14-3-3 (Amirouche et al, 2013). Although several transcriptional and post-transcriptional activators of utrophin A have been identified to date, the combinatorial effect of these activators on utrophin A up-regulation remains elusive. Considering the individual beneficial effects of these activators on dystrophic phenotype and

utrophin A expression, it is imperative to determine if a combination of these activators will provide a greater effect on utrophin A expression and the dystrophic phenotype.

The purpose of our study is to screen various FDA approved and natural pharmacological compounds for their role in regulating utrophin A expression and subsequently determine the combinatorial effect of effective activators on utrophin A expression in muscle.

1.12 Hypothesis:

We hypothesize that combination of a transcriptional activator of utrophin A with its post-transcriptional activator will instigate an additive effect on utrophin A expression in muscle. Further, we predict that the combinatorial treatment of these activators will ameliorate the dystrophic phenotype in mdx mice.

1.13 Objectives:

I. Determine whether various pharmacological agents acting at the transcriptional and post-transcriptional level stimulate utrophin A expression in vitro.
II. Determine whether there is an additive effect of these activators on utrophin A expression in vitro.
III. Investigate in vivo the effect of combinatorial treatment of two effective pharmacological agents (determined from Objective I and II) on utrophin A expression and the dystrophic phenotype.

2. MATERIALS AND METHODS:

2.1 Cell culture:

Mouse C2C12 cells (American Type Culture Collection, Manassas, VA, USA) were plated on 6-well culture dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Wisent, St-Bruno, QC, Canada), 1% L-glutamine and 1 % penicillin/streptomycin as described previously (Amirouche et al, 2013). The cells were incubated at 37ºC with 5% CO_2 in a humidified chamber.

2.2 Transfections:

Transient transfections were performed using transfection reagent Lipofectamine (Invitrogen) by following manufacturer's instructions. C2C12 cells were transiently transfected at 50-60% confluency with a mixture of DNA (1ug)/Lipofectamine for 4 hours and incubates at 37ºC in a humidified chamber supplied with 5 % CO_2. After 4-hour transfection, cells were treated for 24 hours with various drugs as listed in table 1. The concentrations of the drugs were based on literature. Cells were harvested after 24-hour treatment for further analysis.

2.3 Generation of pGL4.14/1.3kb or 2.3 kb human utrophin-A promoter construct:

The 1.3 kb fragment of human utrophin A promoter was subcloned in pGL4.14 backbone vector upstream of the Luciferase reporter gene as described previously (Miura et

al, 2009). The 2.3 kb human utrophin A promoter fragment was amplified by PCR from Jurkat human genomic DNA (EMBL accession no. AL024474), using the primers 5'-TCAAACACTCCAATGTGGCCTTATTATCTA-3' and 5'-TAAAGCTTGGA-GAAGCAGACACGAAC-3' (Moorwood et al, 2011). The PCR product was TA-cloned into the pCR2.1-TOPO vector (Invitrogen) and subcloned into the multiple cloning site of pGL4.14 (Promega) using the restriction enzymes KpnI and EcoRV to generate the construct pGL4.14/utrophin-A promoter.

2.4 Treatments of C2C12 myoblasts:

Concentration of pharmacological compounds was based on previous studies or earlier work from our laboratory as mentioned below.

Treatment	Desired Conc.	Control	Reference
1) GW501516	1uM	DMSO	(Miura et al, 2009)
2) AICAR	1mM	H_2O	(Ljubicic et al, 2011)
3) Metformin	2mM	H_2O	(Suwa et al, 2006)
4) Resveratrol	50uM	DMSO	(Unpublished work from our laboratory)
5) β-GPA	1mM	H_2O	(Ohira et al, 2011)
6) Linoleic Acid	100uM	100% Ethanol	(Suh et al, 2008)
7) Bezafibrate	500uM	DMSO	(Cabrero et al, 2000)
8) Heparin	2.5IU	H_2O	(Amirouche et al, 2013)
9) Dalteparin	2.5IU	H_2O	(Same as heparin)

1. **Heparin treatment:**

Mouse C2C12 cells were plated on 6-well culture dishes, and treated with heparin (LEO Pharma) at 2.5 UI/ml or with saline for 24 h.

2. **AICAR treatment:**

Mouse C2C12 cells were plated on six-well culture dishes and transfected with the 1.3kb pGL4.14/utrophin A promoter as mentioned earlier. After 4 hours of transfection, the cells were treated with 1mM AICAR or control (sterile water) for 24 hours.

3. **GW501516 treatment:**

Mouse C2C12 cells were plated on six-well culture dishes and transfected with the 1.3kb pGL4.14/utrophin A promoter as mentioned earlier. After 4 hours of transfection, the cells were treated with 1uM of GW501516 or control (DMSO) for 24 hours.

4. **Resveratrol Treatment:**

Mouse C2C12 cells were plated on six-well culture dishes and transfected with the 1.3kb or 2.3kb pGL4.14/utrophin A promoter as mentioned earlier. After 4 hours of transfection, the cells were treated with 50uM Resveratrol or control (DMSO) for 24 hours.

5. **Metformin Treatment:**

Mouse C2C12 cells were plated on six-well culture dishes and transfected with the 1.3kb pGL4.14/utrophin A promoter as mentioned earlier. After 4 hours of transfection, the cells were treated with 2mM Metformin or control (sterile water) for 24 hours.

6. **B-GPA Treatment:**

Mouse C2C12 cells were plated on six-well culture dishes and transfected with the 1.3kb pGL4.14/utrophin A promoter as mentioned earlier. After 4 hours of transfection, the cells were treated with 1mM B-GPA or control (sterile water) for 24 hours.

7. **Bezafibrate Treatment:**

Mouse C2C12 cells were plated on six-well culture dishes and transfected with the 1.3kb pGL4.14/utrophin A promoter as mentioned earlier. After 4 hours of transfection, the cells were treated with 500uM of Bezafibrate or control (DMSO) for 24 hours.

8. **Linoleic Acid Treatment:**

Mouse C2C12 cells were plated on six-well culture dishes and transfected with 1.3kb pGL4.14/utrophin A promoter as mentioned earlier. After 4 hours of transfection, the cells were treated with 0.1 M Linoleic acid or control (Ethanol) for 24 hours.

9. **Dalteparin (LMWH) Treatment:**

Mouse C2C12 cells were plated on six-well culture dishes, and treated with Dalteparin (Fragmin Pfizer) at 2.5 UI/ml or with saline for 24 h.

2.5 RNA extraction and qRT-PCR:

Total RNA was extracted from muscle and C2C12 cells using TRIzol reagent (Invitrogen) as recommended by the manufacturer. TRIzol extracted RNA was treated for 1 h with DNAse I (Invitrogen) to eliminate possible DNA contamination. Reverse transcription (RT) was carried out using an RT reaction mixture containing 5 mM $MgCl_2$, 1× PCR buffer, 1 mM dNTP, 1 U/ml RNase inhibitor, 5 U/ml Moloney murine leukemia virus reverse transcriptase and 2.5mM random hexamers (Applied Biosystems, CA, USA).

A real-time quantitative PCR was performed on an MX3005p real-time PCR system (Stratagene, La Jolla, CA, USA) using a QuantiTect SYBR Green PCR kit (QIAGEN, Valencia, CA, USA). For these experiments, amplification of the 18S ribosomal subunit, GAPDH, Utrophin A, KSRP and PGC-1α was performed in duplicates with the following primer sequences:

1) **Utrophin A** → forward 5′ -ATCTTGTCGGGCTTTCCAC-3′ and reverse 5′ -ATCCAAAGGCTTTCCCAGAT-3′

2) **18S Ribosomal** → forward 5′-CGCCGCTAG AGGTGAAATC-3′ and reverse 5′ -CCAGTCGGCATCGT TTATGG-3’

3) **GAPDH** → forward 5′ -GGGTGTGAACCAC GAGAAAT-3′ and reverse 5′ -CCTTCCACAATGCCAAAGTT-3′.

4) **PGC-1α** → Forward 5’- TACGCAGGTCGAACGAAACT and reverse 5’- GAAGCAGGGTCAAAATCGTC -3’

5) **KSRP** → Forward 5’- TTATCGGGGACCCATACAAA- 3’ and reverse 5’- ACTCCGGCCAATGACTACAC- 3’

6) UCP2 → Forward- 5'- GTTCCTCTGTCTCGTCTTGC-3’ and Reverse 5'- GGCCTTGAAACCAACCA-3’

2.6. Western Blots:

Frozen muscle sections from dissected mice were ground to powder with a BioPulverizer on dry ice. Muscle samples were suspended in 300uL of Urea extraction buffer (7 M UREA, 2 M Thiourea, 4 M CHAPS, 100 mM DTT, 125 mM Tris-HCl pH 6.8)

and supplemented with complete Mini Protease Inhibitor Cocktail and phosphatase inhibitor PhosSTOP (Roche, Laval, Canada). The samples were vortexed for 30 minutes at room temperature and then centrifuged at 20000 g for 15 minutes. The supernatant was collected and stored at -80ºC. The protein concentrations were determined using CB-X Protein Assay Kit and bovine serum albumin was used as a standard.

10ug of extracted protein were run on a sodium dodecyl sulfate polyacrylamide gel (6 – 8% polyacrylamide) at 80-100V for 2-3 hours and electroblotted to nitrocellulose membranes (Bio-Rad, Mississauga, Canada). For utrophin western blots, SDS-gels were incubated in 20 % Glycerol solution for 1 hour with gentle rocking prior to the transfer. After transfer, membranes were stained with Ponceau S (Sigma-Aldrich) to confirm equal loading between lanes. Membranes were subsequently washed 4 times with 1× PBST (1X-PBS, 0.2% Tween) and blocked for 1 hour with a 5% skim milk in PBS-T solution. Blots were then incubated in blocking solution with an antibody directed against Utrophin (Novocastra; 1:500), PGC-1α (Abcam ab72230; 1:2000), KSRP (Bethyl Laboratories; 1:5000) overnight at 4ºC with gentle rocking. The blots were incubated in corresponding Horse Radish Peroxidase conjugated secondary antibodies for 1 hour at room temperature in blocking solution and washed 4 times with 1xPBS-T. The Chemiluminscent detection of proteins was performed using ECL reagent (Perkin Elmer). The films were scanned, developed and quantified using ImageJ (NIH version 1.0) and/or Image Lab.

2.7. In- vivo treatments:

Six week old mdx mice (The Jackson Laboratory, Bar Harbor, USA) were maintained in Animal Care and Veterinary Service of University of Ottawa under a constant 12 –hour light-dark cycle with full access to water and food. The experimental protocols were approved by University of Ottawa's Animal Care Committee and were in accordance with the Canadian Council of Animal Care Guidelines. 6 week old mdx mice were treated daily with vehicle, AICAR (500 mg/kg/day), heparin (500IU/mL) or AICAR + heparin (concentrations as mentioned earlier) by subcutaneous injections for 4 weeks.

2.8. Immunostaining:

Muscle sections were stained using utrophin primary antibody (1:200; Novocastra NCL-DRP2 Newcastle upon Tyne, UK). IgM staining was performed using IgM anti-mouse secondary antibody (Sigma-Aldrich, Oakville). The sections were washed in PBS 3 times for 10-15 mins to remove the secondary antibody. Next, the slides were mounted by Vectashield mounting medium (Vector Laboratories, Burlington). Finally, the secondary antibody was removed by washing 3X10 minutes with PBS and the slide was mounted using Vectashield mounting medium (Vector Laboratories, Burlington, Canada) and a cover slip. The slides were visualized with a fluorescent microscope and images were acquired. Immunofluorescence analysis was performed using a Zeiss Axioshop-2- microscope at 20X magnification.

2.9. Hematoxylin and Eosin Staining:

Muscle cross-sections (10um) of Tibialis Anterior and Diaphragm muscles were stained with hematoxylin and eosin dyes, dehydrated using a series of ethanol solutions at different dilutions (70%, 90%, 100 %) and subsequently washed with xylene. The slides were then mounted using Permount and covered with a coverslip. The slides were analyzed using a fluorescence microscope.

The percentage of central nucleation was determined by manually counting the total number of muscle fibers and the number of muscle fibers with central nucleation from 4-6 cross-sectional views by using the Northern Eclipse Software (NES, Expix Imaging, Mississauga, Ontario, Canada). Cross-sectional Area (CSA) of each fiber was measured using NES. The variance coefficient was calculated based on the CSA of muscle fibers using the formula "variance coefficient Z = 1000 x standard deviation of muscle fiber minimal diameters/mean muscle fiber minimal diameter."

2.10. Statistical Analysis:

The data were analyzed using paired and unpaired student's t-test and ANOVA (Analysis of Variance) and post- hoc tests as appropriate (GraphPad Prism). As illustrated in the graphs, statistical analysis was performed on raw data prior to conversion to fold difference (compared to control). Significance was accepted at $p < 0.05$.

3.0 RESULTS:

The overall goal of this study is to establish if a combination of two activators of utrophin A instigates an additive effect on its expression in muscle. To achieve this we first screened several FDA approved and natural pharmacological compounds in C2C12 muscle cells and selected two drugs that were most effective in stimulating utrophin A expression. Next, we assessed the effect of combining these drugs on utrophin A expression in C2C12 muscle cells and mdx mice. Lastly, we analyzed several pathological parameters in mdx mouse model to determine if the combinatorial therapy caused improvements in the dystrophic phenotype.

3.1 Post-transcriptional regulation of utrophin A expression by heparin and its depolymerized derivative, dalteparin:

Heparin, a naturally occurring polysaccharide, is one of the oldest drugs used as an anticoagulant for treatment of thrombosis (Gray et al, 2008). Heparin has been shown to activate p38 MAPK activity in skeletal muscle of wild-type mice (Zbinden-Foncea et al, 2012). A recent study by Amirouche et al, was first to demonstrate the effect of heparin on utrophin A expression in muscle (Amirouche et al, 2013). This study revealed that pharmacological activation of p38 by heparin stimulates utrophin A expression in C2C12 muscle cells and mdx mice through a post-transcriptional mechanism involving inhibition of an RNA binding protein, KSRP (Amirouche et al, 2013). In the present study, we set out to first confirm the effect of heparin on utrophin A expression in muscle cells and subsequently determine if a low-molecular weight derivative of heparin, dalteparin, has similar effects on

utrophin-A up-expression.

In the following set of experiments C2C12 myoblasts were transfected with the luciferase reporter construct containing the 3'UTR of utrophin A mRNA to assess if the tested compound mediates its effect on utrophin A expression via the 3'UTR. Further, the utrophin A mRNA and protein levels were measured in untransfected C2C12 myoblasts following a 24 hour treatment.

3.1.1. Heparin stimulates utrophin A expression in C2C12 muscle cells*:*

As shown in **Figure 3.1 (A)**, the activity of luciferase-reporter construct (containing the full-length 3'UTR of utrophin A mRNA) was stimulated in C2C12 cells treated with heparin by 1.6 fold ($p<0.05$). These results indicate that heparin treatment stimulates the 3'UTR of utrophin A. Next, we assessed the mRNA and protein levels of utrophin A expression in C2C12 myoblasts after a 24-hour heparin treatment or control (saline). Our results demonstrated that, heparin treatment stimulated endogenous utrophin A mRNA levels by 2 fold in treated C2C12 myoblasts when compared to untreated control as illustrated in **Figure 3.1 (B)** ($p<0.05$). Further, the protein levels of utrophin A were increased by ~ 2 fold following a 24-hour heparin treatment (Figure **3.1 (C) and (D)).** Our results are in accordance with previous studies (Amirouche et al, 2013) and emphasize the post-transcriptional effect of heparin on utrophin A expression.

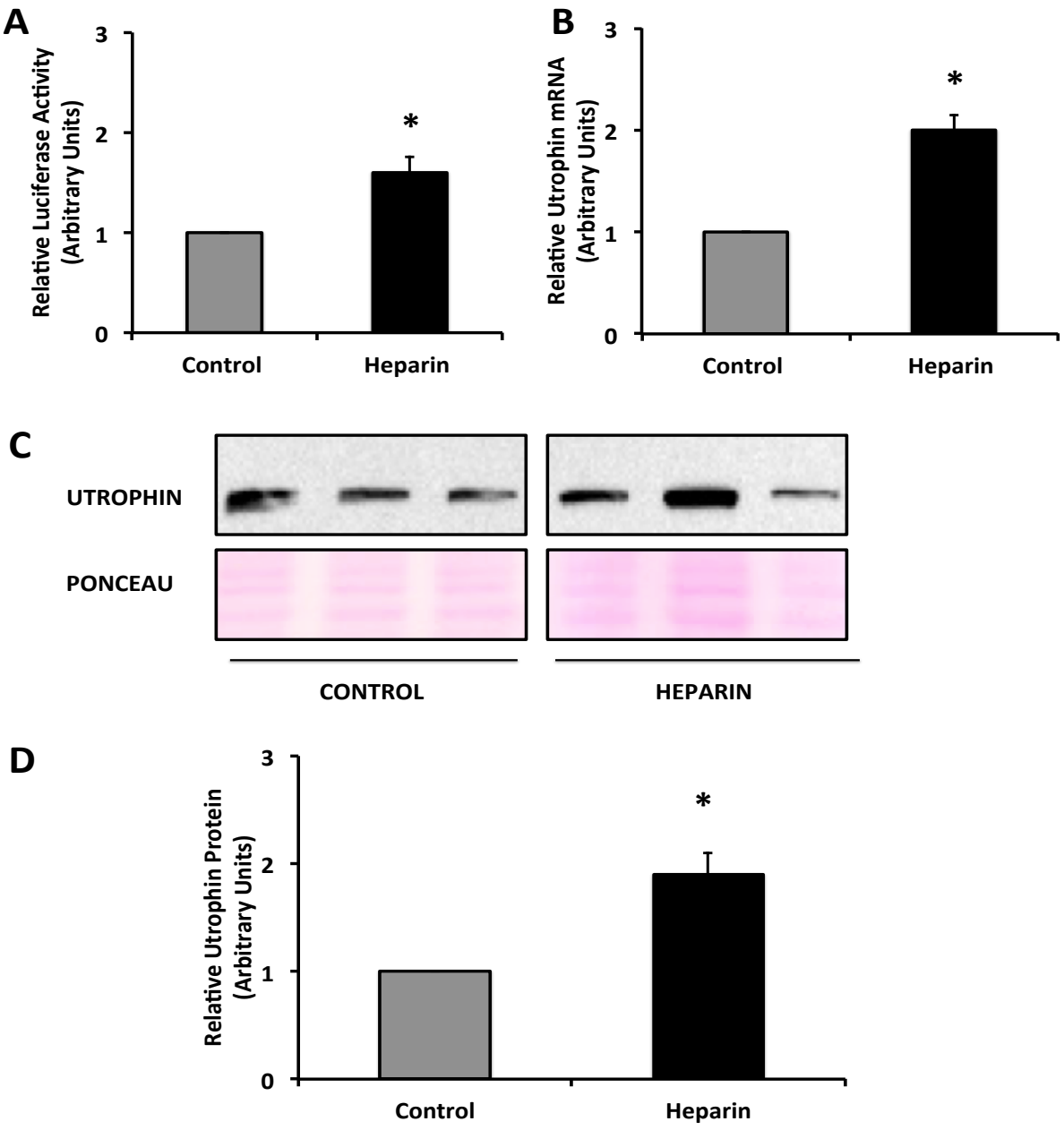


Figure 3.1. Heparin (a p38 activator) increases utrophin A expression in C2C12 muscle cells. (A) The activity of Luciferase reporter construct containing the full-length 3'UTR of utrophin A mRNA in C2C12 myoblasts treated with either control (saline) or heparin (2.5IU/ml) for 24 hours. B) Utrophin A mRNA levels as measured by qRT-PCR in C2C12 myoblasts treated with either control (saline) or heparin (2.5IU) for 24 hours. The values are normalized to 18S mRNA levels. C) Representative western blot of utrophin A and ponceau in C2C12 myoblasts treated with control (saline) or heparin (2.5IU) for 24 hours. D) Quantification of utrophin A protein levels as shown in (C). Values are expressed as means + SE (n = 3, 3 replicates each). ∗ indicates significant difference compared to control (saline) values ($p < 0.05$).

3.1.2. Dalteparin, a low-molecular weight heparin, does not stimulate utrophin A expression in C2C12 muscle cells:

After confirming the effect of heparin on utrophin A expression, we determined whether a low molecular weight heparin (LMWH), dalteparin, up-regulates utrophin A expression in C2C12 muscle cells. LMWH are depolymerized derivatives of heparin, with improved pharmacokinetic profiles for instance longer half-lives and a greater ease of administration compared to unfractionated heparin (Gray et al, 2008). As shown in **Figure 3.2 (A)**, dalteparin treatment in C2C12 myoblasts had no significant effect on luciferase activity in cells transfected with the luciferase-reporter construct containing the 3'UTR of utrophin A ($p > 0.05$). In addition, utrophin A mRNA and protein levels did not change in response to dalteparin treatment (**Figure 3.2 (B), (C) and (D)**, $p > 0.05$)).

Together these data suggest that while heparin stimulates utrophin A expression in C2C12 muscle cells, the depolymerized derivative, dalteparin, has no significant effect on utrophin A expression.

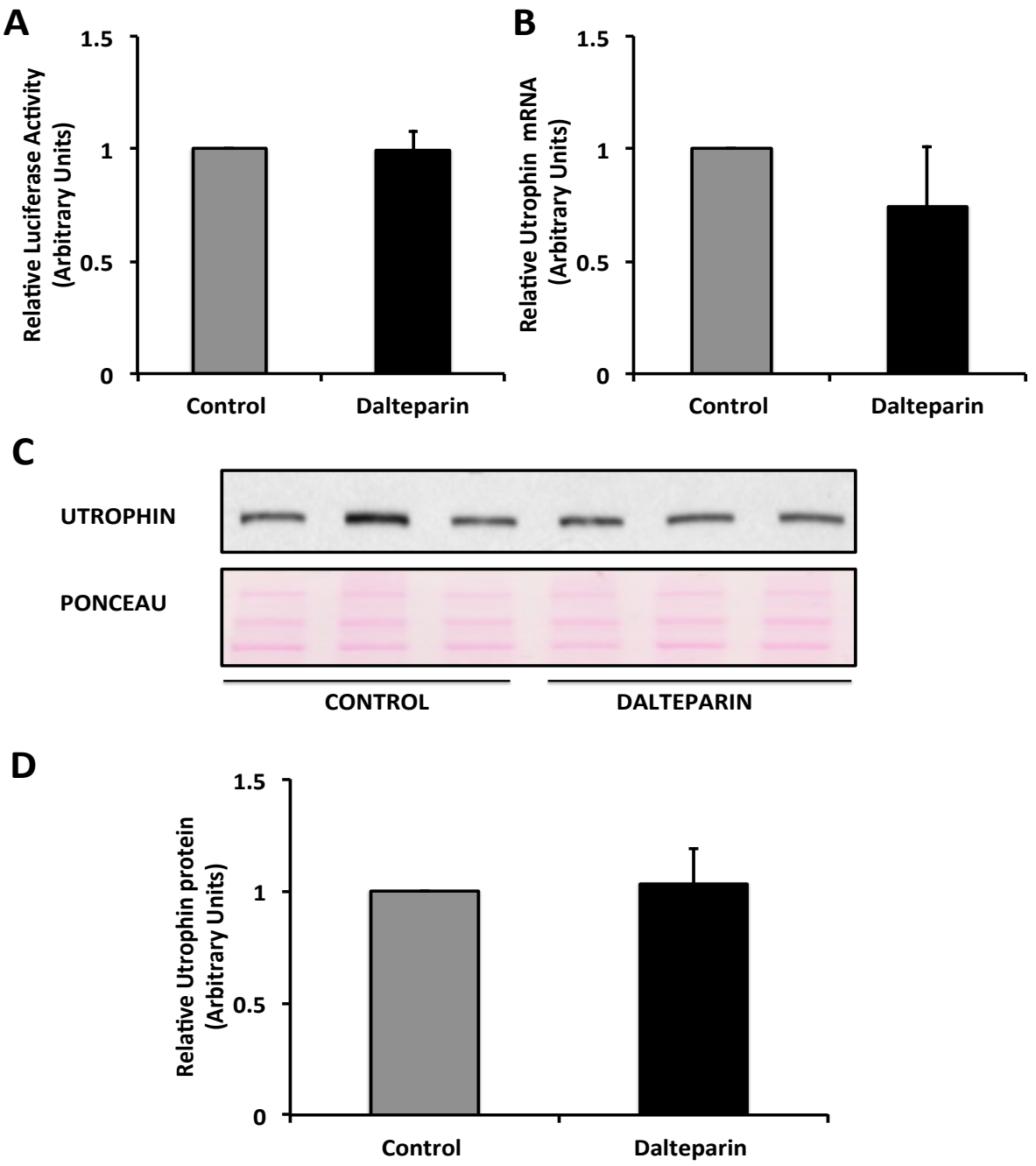


Figure 3.2. Dalteparin, a low molecular weight heparin, does not increase utrophin A expression in C2C12 muscle cells. (A) The activity of Luciferase reporter construct containing the full-length 3'UTR of utrophin A mRNA in C2C12 myoblasts treated with either control (saline) or Dalteparin (2.5IU) for 24 hours. B) Utrophin A mRNA levels as measured by qRT-PCR in C2C12 myoblasts treated with either control (saline) or dalteparin (2.5IU) for 24 hours. The values are normalized to 18S mRNA levels. C) Representative western blot of utrophin A and ponceau in C2C12 myoblasts treated with control (saline) or dalteparin (2.5IU) for 24 hours. D) Quantification of utrophin protein levels in treated or control C2C12 cells. Values are expressed as means + SE (n = 3, 3 replicates each)

3.2 Transcriptional regulation of utrophin A expression by various pharmacological agents:

There is mounting evidence that activators of phenotypic modifiers such as AMPK, PPAR- β /δ and SIRT1 are important transcriptional regulators of utrophin A expression in muscle (Miura et al, 2009; Moorwood et al, 2011; Ljubicic et al, 2013). In addition to possible direct effects of these modifiers on utrophin A transcription, these factors have been shown to activate PGC-1α expression by various translational and post-translational modifications (Wenz, 2009; Scarpulla et al, 2008; Scarpulla et al, 2012, Hofer et al, 2013). PGC-1α is a master regulator of the slow-oxidative myogenic program in muscle and has been shown to stimulate utrophin A transcription via increased transcription of GAPB α and β (Lin et al, 2002; Lira et al, 2010; Angus et al, 2005). Therefore, PGC-1α is an important therapeutic target in utrophin up-regulation therapies. Although, direct pharmacological activators of PGC-1α have not been identified, the agonists of PPAR β/δ, AMPK and SIRT1 have been shown to stimulate PGC-1α expression in muscle (Miura et al, 2009; Ljubicic et al, 2011; Jahnke et al, 2012; Gordon et al, 2013; Ljubicic et al, 2014).

In the present study we screened several established and novel potential activators of PPARβ/δ, AMPK and SIRT1 to investigate if these pharmacological agents stimulate the utrophin A promoter activity, utrophin A expression and any downstream targets (if known). To achieve this in the following experiments, we transfected C2C12 myoblasts with a 1.3kb human utrophin A promoter-reporter construct prior to the treatment. The 1.3kb fragment of the utrophin A promoter has been reported to contain all the essential elements involved in

regulation of utrophin A expression (Stocksley et al, 2005). After treatment of transfected cells with the compounds for 24 hours, we assessed the mRNA levels of the luciferase reporter (Firefly) to evaluate if these drugs directly induce the utrophin A promoter, endogenous utrophin A and a positive control/ downstream target to validate the therapeutic effectiveness of the drug being tested.

3.2.1 AMPK activators:

3.2.1.1 AICAR stimulates utrophin A expression in C2C12 muscle cells:

AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide), a synthetic agonist of AMPK, has been shown to stimulate AMPK activity in skeletal muscle (Merrill et al, 1997). Further, AICAR has been demonstrated to induce a shift towards slower oxidative fibers, promote mitochondrial biogenesis, improve performance during exercise and promote the expression of factors such as PGC-1α, PPAR-β/δ and GLUT-4 (Fillmore et al, 2010; Suwa et al, 2006, Winder, 2008; Narkar et al, 2008; Ljubicic et al, 2011). More specifically, previous work from our laboratory has demonstrated that AICAR treatment in mdx mice induces the slow oxidative myogenic program through AMPK activation and results in an up-regulation of utrophin A expression (Ljubicic et al, 2011). However, the direct transcriptional effects of AICAR on utrophin A promoter have not yet been reported.

In the present study, we investigated whether the effect of AICAR on utrophin A expression is a result of direct transcriptional induction of the promoter. To achieve this we

treated transfected C2C12 cells (as described previously) with 1mM AICAR for 24 hours. As depicted in **Figure 3.3 (A)**, AICAR treatment in C2C12 myoblasts stimulates luciferase reporter (Firefly) mRNA levels by 1.5 fold ($p<0.05$). Since AMPK activation has been shown to activate PGC-1α expression (Hock and Kralli, 2009), we also measured the levels of this transcriptional co-activator as a positive control. According to our data, PGC-1α mRNA levels were stimulated by 2 fold (**Figure 3.3 (B),** $p<0.05$). The endogenous utrophin A mRNA levels of were stimulated by 1.5 fold (**Figure 3.3 (C),** $p<0.05$). These findings are in line with previous studies (Ljubicic et al, 2011; Jahnke et al, 2012), and provide evidence that the effect of AICAR is mediated by direct transcriptional induction of the utrophin A promoter.

3.2.1.2 Metformin does not stimulate utrophin A expression in C2C12 muscle cells:

Metformin, an anti-hyperglycemic drug, is an activator of AMPK. It has been demonstrated that this activation is mediated through inhibition of AMP deaminase (Ouyang et al, 2011). Further, it has been shown that metformin treatment increases the expression of PGC-1α through AMPK mediated phosphorylation in skeletal muscle of rats (Suwa et al, 2006). However, the effect of metformin on utrophin A expression is largely unknown.

In the current study we investigated the effect of metformin on utrophin A promoter activity and mRNA levels of endogenous utrophin A. To achieve this, we treated transfected C2C12 myoblasts with 2mM Metformin for 24 hours. According to our study, metformin had no significant effect on luciferase reporter mRNA levels (**Figure 3.3 (A),** $p>0.05$) or utrophin A mRNA levels (**Figure 3.3 (C),** $p> 0.05$). However, PGC-1α mRNA levels were

significantly stimulated by about 1.5 fold (**Figure 3.3 (B)**, $p<0.05$). The results for PGC-1α expression are in accordance with previous studies (Suwa et al, 2006).

3.2.1.3: β -GPA, an AMPK activator, does not stimulate utrophin A expression in C2C12 muscle cells:

β-guanadinopropionic acid (β-GPA), a creatine analog, has been established to be a potent AMPK activator (Williams et al, 2009). Previous studies have demonstrated that β-GPA evokes a shift in slower oxidative fibers in rats, induce mitochondrial biogenesis and activate AMPK in muscle (Ren et al, 1995; Bergeron et al, 2001; Williams et al, 2009; Zong et al, 2002).

To determine the effect of β-GPA on utrophin A promoter and its expression, we treated transfected muscle cells with 1mM β-GPA or control for 24 hours. The mRNA levels of luciferase reporter (Firefly), PGC-1α and endogenous utrophin A remained unchanged in response to B-GPA treatment (**Figure 3 A-C** respectively, $p>0.05$). Since β-GPA has been shown to activate AMPK by previous studies (Williams et al, 2009), it is possible that the dose or experimental setup (cell type etc) was not be optimal for β-GPA to exert its effect on AMPK and possibly PGC-1α.

Together these data suggest that AICAR is the most effective drug among the various tested AMPK activators in stimulating utrophin A promoter and expression in growing C2C12 muscle cells.

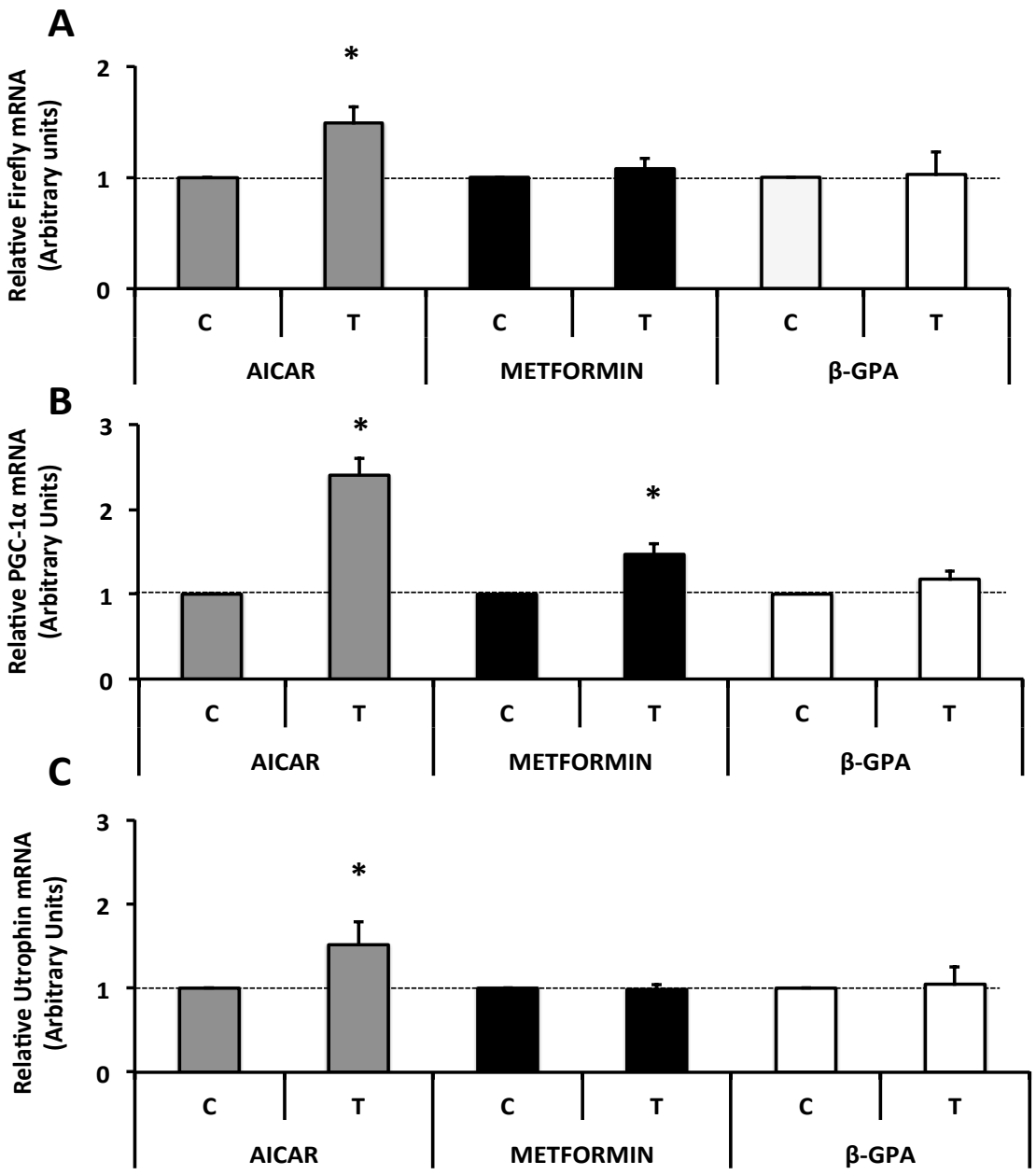


Figure 3.3: The effect of AMPK activators AICAR, Metformin and β-GPA on utrophin A expression in C2C12 muscle cells. C2C12 myoblasts transfected with 1.3kb fragment of human utrophin A promoter- luciferase reporter construct were treated for 24 hours with 1mM AICAR, Control (H_2O); 2 mM Metformin, Control (H_2O); or 1mM B-GPA, Control (H_2O). The mRNA levels of A) Firefly (Luciferase reporter), B) PGC-1α and C) utrophin A are dislayed as measured by quantitative RT-PCR. Values are standardized to 18S mRNA levels (n=4, 3 replicates each, * indicates significant difference compared to control values ($p<0.05$). Mean ± S.E.M are shown**.**

3.2.2. PPAR-β/δ activators:

3.2.2.1 GW501516 stimulates utrophin A promoter and UCP2 expression in C2C12 muscle cells:

Earlier work from our research group has demonstrated that administration of GW501516, a small molecule PPAR-β/δ agonist, results in a switch from fast to slow fiber type leading to subsequent increase in utrophin A expression and rescue of muscle function in mdx mice (Miura et al, 2009). Further, our laboratory has established that this stimulation of utrophin A expression is mediated through a PPRE half site located at the 5' end of utrophin A promoter (Miura et al, 2009). In addition, some studies have highlighted the effects of GW501516 on the slow myogenic program. For instance, GW501516 has been shown to increase the number of slow oxidative fibers in wild-type mice and enhance running endurance synergistically with exercise training (Narkar et al, 2008, Miura et al, 2009).

In the present study, we first evaluated whether GW501516 stimulates utrophin A expression via direct transcriptional induction of the utrophin A promoter as shown previously by our laboratory (Miura et al, 2009). Next, we assessed the effects of other PPAR-β/δ activators on utrophin A promoter and expression in C2C12 muscle cells (described later).

Transfected C2C12 cells were treated with 50μM GW501516 for 24 hours. Our results indicate that luciferase reporter (Firefly) mRNA levels driven by the utrophin A

promoter were stimulated by 1.5 fold in response to GW501516 treatment (**Figure 3.4 (A)**, $p<0.05$). Further, GW501516 treatment also stimulated the mRNA levels of UCP2, a downstream target of PPARβ/δ and a positive control, by 7 fold (**Figure 3.4 (B)**, $p<0.05$). These results corroborate earlier findings from Miura et al. (Miura et al, 2009). In our study, the levels of utrophin A mRNA were not significantly changed as a result of GW501516 treatment in transfected cells (**Figure 3.4 (C)**, $p> 0.05$). However untransfected cells showed about a 1.4-fold increase in utrophin A mRNA (n=1, Appendix Fig 7.1). These results are in accordance with earlier study by Miura et al, 2009 where GW501516 was shown to stimulate utrophin A and UCP2 mRNA levels in untransfected C2C12 muscle cells (Miura et al, 2009).

3.2.2.2 Bezafibrate, a pan-PPAR agonist, does not stimulate the utrophin A promoter or expression in C2C12 muscle cells:

Bezafibrate belongs to a class of drugs called fibrates. These drugs are amphipathic carboxylic acids that are used for treatment of atherosclerosis. Bezafibrate is a pan-PPAR agonist and has been shown to bind to all 3 isoforms of PPAR (α, β/δ, γ) at comparable affinities (Tennebaum and Fisman, 2012). Further, bezafibrate is the only clinically available pan-PPAR balanced ligand (Tennebaum and Fisman, 2012). Previous studies have established that bezaifbrate treatment increases PGC-1α expression and mitochondrial biogenesis in a mitochondrial myopathy mouse model ΔCOX10 (Wenz et al, 2008). In addition, bezafibrate has been shown to induce a series of genes involved in PGC-1α signaling pathway such as all 3 PPAR isoforms (PPAR α, β/ δ, γ) (Hofer et al, 2013).

However, to our best knowledge the effect of bezafibrate on utrophin expression has not been reported.

Therefore, in this study we aimed at determining if bezafibrate had an effect on utrophin A expression in C2C12 muscle cells. After 24-hour treatment of transfected C2C12 cells with 500μM bezafibrate, we analyzed the mRNA levels of luciferase-reporter, utrophin A and the positive control UCP2. As shown in **Figure 3.4 (A) and 3.4 (C)**, bezafibrate caused no significant effect on utrophin A promoter activity or mRNA levels ($p>0.05$). UCP2 mRNA levels were stimulated by ~5 fold as a result of bezafibrate treatment as shown in **Figure 3.4 (B)** ($p<0.05$). The stimulation of UCP2 expression by bezafibrate has been reported earlier in rat adipocytes (Cabrero et al, 2000).

3.2.2.4 Linoleic Acid does not stimulate utrophin A expression in C2C12 muscle cells:

Long chain fatty acids are important ligands for PPARs (Ehrenborg and Krook, 2009). Linoleic acid, ω-6 dietary lipid, has been shown to interact with PPARs to induce atherogenic effects in vascular cells (Fei et al, 2012). Further, earlier studies have demonstrated that linoleic acid increases the expression of PPAR-δ in chicken hepatocytes (Suh et al, 2008).

In this study, we aimed to assess the effect of linoleic acid on utrophin A promoter and utrophin A expression possibly via PPAR-β/δ activation. We treated transfected C2C12 muscle cells with linoleic acid or vehicle. As shown in **Figure 3.4**, linoleic acid treatment in

C2C12 myoblasts caused no significant changes in luciferase reporter, UCP2 or utrophin A mRNA levels (**Figure 3.4 (A), (B) and (C)** respectively; $p>0.05$).

Together these findings suggest that overall PPARβ/δ activators were not very effective in up-regulating utrophin A expression in growing C2C12 muscle cells. Since some of these drugs such as GW510516 have been shown to stimulate utrophin A expression in muscle cells and mdx mice (Miura et al, 2009), it is speculated that differences in cell types, cellular responses to drugs and doses can impact the effectiveness of drugs (Hofer et al, 2013). This speculation can be supported by the observation that within our own experimental set-up, variations were observed from trial to trial.

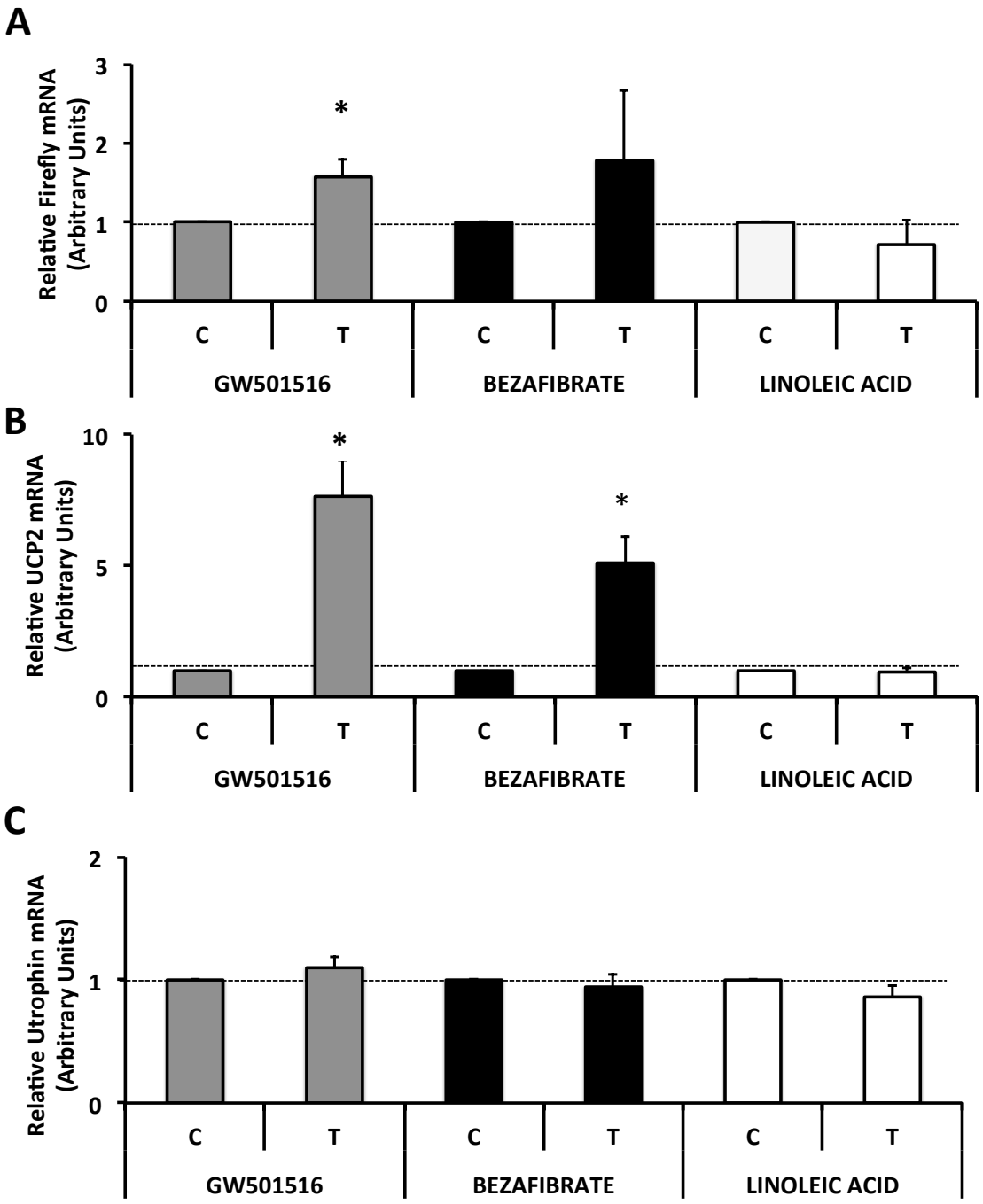


Figure 3.4: The effect of PPAR-β/δ activators GW501516, Bezafibrate and Linoleic Acid on utrophin A expression in C2C12 muscle cells. C2C12 myoblasts transfected with 1.3kb fragment of human utrophin A promoter- luciferase reporter construct were treated for 24 hours with 1uM GW501516, Control (DMSO); 500uM Bezafibrate, Control (DMSO) or 0.1M Linoleic Acid, Control (Ethanol). Relative mRNA levels of A) Firefly B) UCP2 and C) Utrophin A are shown with the corresponding treatment as measured by quantitative RT-qPCR. Values are standardized to 18S mRNA levels (n=4, * indicates significant difference compared to control values, $p<0.05$). Mean ± S.E.M are shown.

3.2.3. SIRT-1 activators:

3.2.3.1 Resveratrol stimulates the utrophin A promoter but has no significant effect on utrophin A expression in C2C12 muscle cells:

Resveratrol is a polyphenolic compound that is naturally present in fruits and vegetables (Wenzel et al, 2005). Previous studies have shown that resveratrol stimulates mitochondrial biogenesis in muscle and promotes the slow oxidative myogenic program (Lagouge, 2006). In addition, it has been revealed that resveratrol augments PGC-1α activity indirectly via activation of AMPK or SIRT1 (Canto et al, 2010). In this context a study by Gordon et al, 2013 demonstrated that resveratrol treatment in mdx mice resulted in a significant increase in PGC-1α, utrophin A and SIRT-1 mRNA levels (Gordon et al, 2013). More recently, a study from our laboratory by Ljubicic et al. demonstrated that resveratrol treatment can stimulate the slow oxidative myogenic program and enhances the expression of PGC-1α and SIRT-1 expression (Ljubicic et al, 2014).

To confirm the earlier findings and determine if the effect of resveratrol is mediated by transcriptional induction of the utrophin A promoter in our experimental set-up, we treated transfected C2C12 cells with 50μM resveratrol for 24 hours. As illustrated in **figure 3.5 (A),** resveratrol stimulated the utrophin A promoter by 2-fold ($p<0.05$). These results corroborate earlier work by Moorwood et al. where resveratrol treatment in C2C12 cells stably expressing a 2.3kb human utrophin A promoter-reporter construct, caused a dose-dependent increase in utrophin A promoter activity (Moorwood et al, 2011). In these set of

experiments, we concomitantly tested the effect of resveratrol on a larger 2.3kb fragment of human utrophin A promoter-reporter construct as designed by Moorwood et al (Moorwood et al, 2011). Our results revealed that resveratrol induced both 1.3kb and 2.3kb fragments of utrophin A promoter with no significant differences (Appendix Figure 7.2).

In our experimental set-up resveratrol treatment did not cause significant changes in PGC-1α or utrophin A mRNA levels (**Figure 3.5 (B) and (C)** respectively, $p>0.05$). Since earlier studies on resveratrol treatment in mdx mice by our laboratory and others have reported otherwise (Ljubicic et al, 2014; Gordon et al, 2013), we speculate that this discrepancy could be attributed to the specific experimental conditions such as the cell type, doses of drugs and cellular responses to drugs.

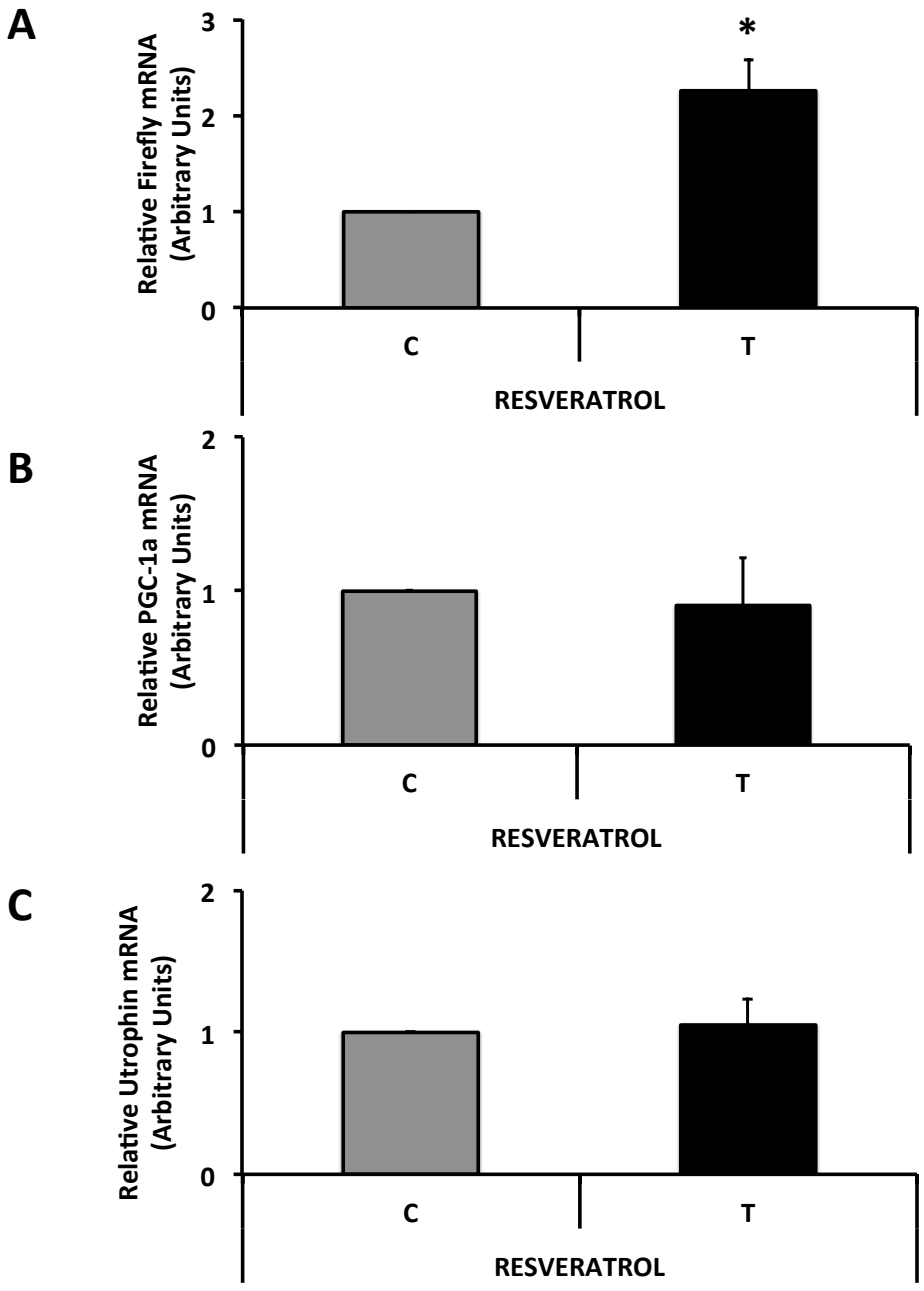


Figure 3.5: The effect of SIRT-1 activator Resveratrol on utrophin A expression in C2C12 muscle cells. C2C12 myoblasts transfected with a 1.3kb fragment of human utrophin A promoter- luciferase reporter construct were treated for 24 hours with 50uM resveratrol or Control (DMSO). Relative mRNA levels of A) Firefly B) PGC-1α and C) Utrophin A mRNA levels as measured by quantitative RT-PCR are shown. Values are standardized to 18S mRNA levels (n=4, * indicates significant difference compared to control values (DMSO), $p<0.05$). Mean ± S.E.M are shown.

3.2.4. Additional transcriptional activators of utrophin A expression:

Recent studies have revealed that activators of the HDAC inhibition pathway and Nitric Oxide Pathway such as Arginine butyrate can also regulate utrophin A expression in skeletal muscle (Vianello et al, 2013).

3.2.4.1 Arginine butyrate stimulates the utrophin A promoter but has no effect on utrophin A mRNA levels in C2C12 muscle cells:

Arginine butyrate is the butyric salt of the amino acid arginine. Previous studies have shown that it can stimulate the Nitric Oxide Pathway (via its L-arginine component) and Histone Deacetylase Inhibition (HDACi) pathway (via its butyrate component). Recently, Arginine butyrate has been shown to stimulate utrophin A expression in human myotubes and in mdx mice (Vianello et al, 2013). To evaluate the effect of Arginine butyrate utrophin A expression in C2C12 myoblasts, we treated transfected cells with 1mM Arginine butyrate for 24 hours. As depicted in **Figure 3.6 (A)**, Arginine butyrate stimulated the firefly (luciferase reporter) mRNA levels by 2.7 fold ($p<0.05$). However, arginine butyrate had no significant increase in utrophin A mRNA levels as shown in **Figure 3.6 (B).** Since Vianello et al, have reported an increase in utrophin protein levels in response to arginine butyrate treatment in human DMD myotubes (Vianello et al, 2013), it is speculated that cell-type differences between human DMD myotubes and C2C12 myoblasts may require a higher/different dose to stimulate a response in C2C12 myoblasts.

Together our data from the aforementioned experiments indicates that AICAR is the most effective pharmacological compound among the transcriptional activators in stimulating utrophin A promoter activity and expression in growing C2C12 muscle cells.

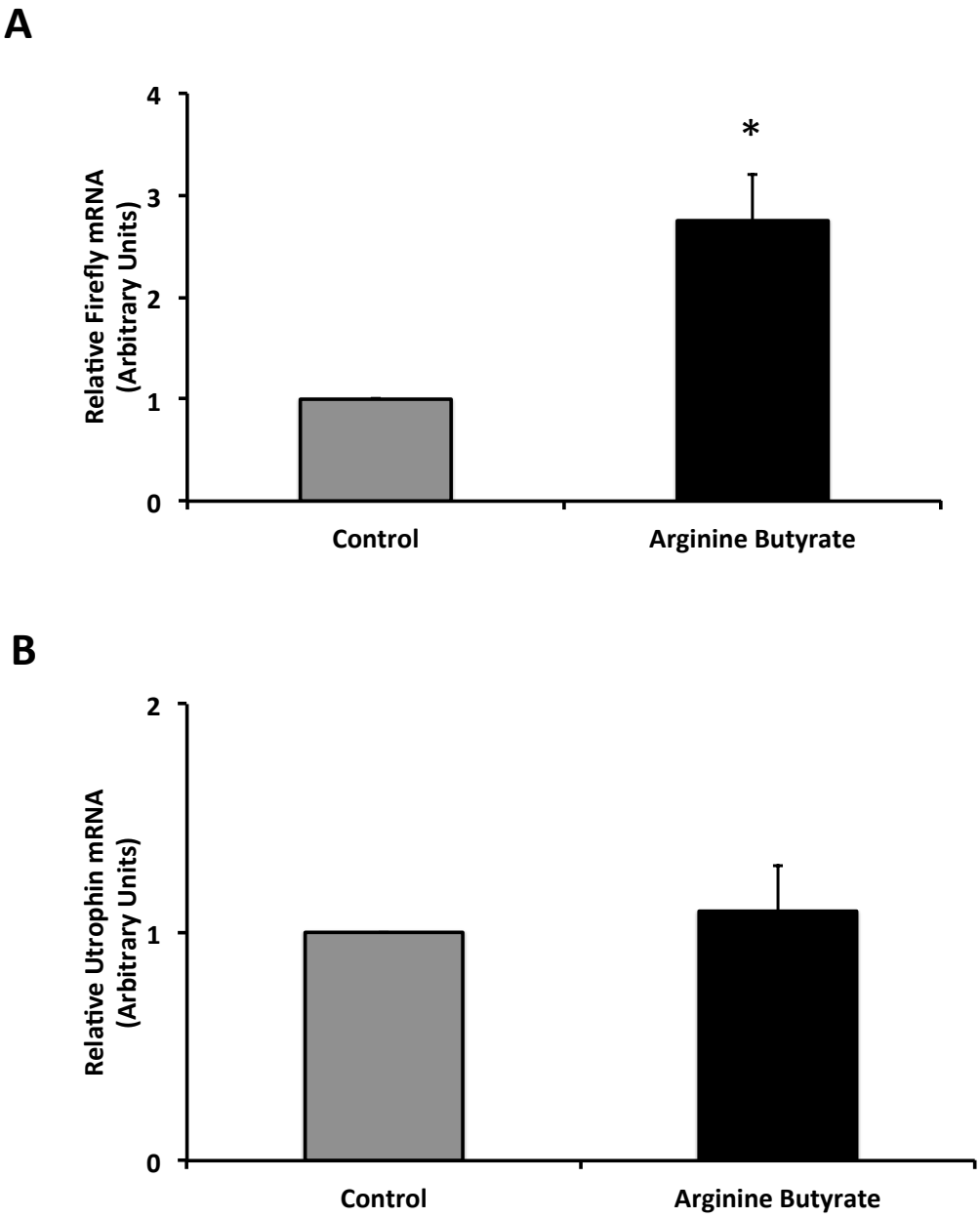


Figure 3.6: Arginine butyrate stimulates the utrophin A promoter activity but has no effect on utrophin A mRNA levels in C2C12 muscle cells. C2C12 myoblasts transfected with 1.3kb fragment of human utrophin A promoter- luciferase reporter construct were treated for 24 hours with 1mM Arginine butyrate or Control (H_2O). Relative mRNA levels of A) Firefly (luciferase reporter) and B) Utrophin A mRNA levels are shown as measured by quantitative RT-PCR. Values are standardized to 18S mRNA levels (n=4, * indicates significant difference when compared to control values (H_2O), $p<0.05$). Mean ± S.E.M are shown.

3.3 Combinatorial effect of AICAR and heparin treatment on utrophin A expression in-vitro and in-vivo

After establishing the individual effects of AICAR and heparin on utrophin A expression, we aimed to test our hypothesis that a combinatorial treatment of two utrophin A activators will result in an additive effect on its expression. Since AICAR and heparin worked best in our experimental set-up to promote utrophin A expression, we chose these two drugs to test our proposed hypothesis. To achieve this, we treated C2C12 muscle cells and mdx mice with a combinatorial therapy of AICAR and heparin and observed the effects on utrophin A expression and the dystrophic phenotype.

3.3.1. Combinatorial treatment of AICAR and heparin in C2C12 myoblasts produces an additive effect on utrophin A expression and stimulates PGC-1α expression:

To assess the outcome of combinatorial treatment of AICAR and heparin in-vitro, C2C12 myoblasts were treated with control (saline), AICAR, heparin or AICAR + heparin (A + H) for 24 hours. As shown in **figure 3.7 (A)**, the combinatorial treatment of AICAR and heparin triggered an additive effect on utrophin A mRNA levels as there was a 2.5 fold increase in utrophin A mRNA levels that was significantly greater than individual AICAR or heparin treatment ($p<0.05$). The individual treatments of AICAR and heparin also caused significant increase in utrophin A mRNA levels of about 1.3 and 1.8 fold respectively **(Figure 3.7 (A), $p<0.05$).**

Further, the mRNA levels of PGC-1α were also increased in response to AICAR and A + H combinatorial treatment by about 1.5 fold **(Figure 3.7 (B), $p<0.05$).** As expected, Heparin treatment had no significant effect on PGC-1α mRNA levels **(Figure 3.7 (B), $p>0.05$).** The individual treatment results of AICAR and heparin are in line with earlier studies from our laboratory and other (Ljubicic et al, 2011; Amirouche et al, 2013; Jahnke et al, 2012). The mRNA levels of KSRP had no significant change upon AICAR, heparin or AICAR + heparin treatment **(Figure 3.7 (C), $p>0.05$).**

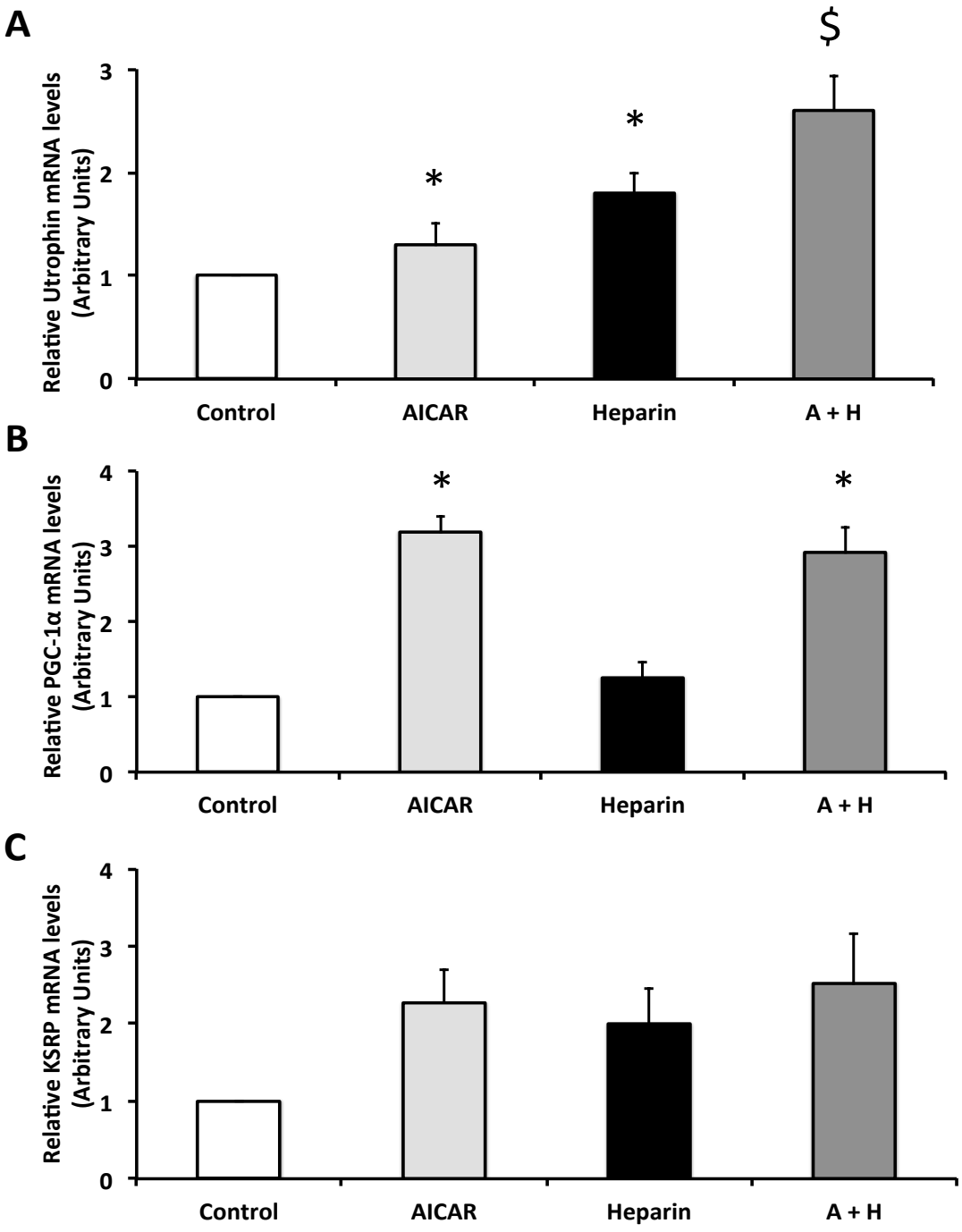


Figure 3.7: Combinatorial treatment of AICAR and heparin in C2C12 myoblasts results in a significant increase in Utrophin A and PGC-1α mRNA levels. (A-C) Results of qRT – PCR analyses for A) Utrophin A B) PGC-1α and C) KSRP mRNA levels in C2C12 cells treated with either saline, AICAR (1mM), heparin (2.5IU) or AICAR + heparin for 24 hours. Values are standardized to 18S mRNA levels (n=4, 3 replicates each, * indicates significant difference compared to control (saline), $ indicates significant difference compared to all treatments and control values, ($p<0.05$). Mean ± S.E.M are shown.

3.3.2. Combinatorial treatment of AICAR and heparin in mdx mice causes a significant increase in utrophin A and β-dystroglycan expression:

C2C12 cells serve as a practical model for initial screening of compounds since it translates with reasonable success in the DMD context. However, given the differences in utrophin A expression levels and localization in C2C12 muscle cells versus the in-vivo model, it was necessary to test the combinatorial treatment in-vivo. To achieve this, we treated 6 -week old mdx mice with subcutaneous injections of Saline (control), AICAR (500mg/kg body weight), Heparin (500IU/kg) or AICAR + heparin for a duration of 4 weeks. The treatment protocols were based on earlier studies from our laboratory (Ljubicic et al, 2011; Amirouche et al, 2013).

As seen in-vitro, the combinatorial treatment of AICAR and heparin resulted in an additive effect on utrophin A expression in the diaphragm muscles of treated mdx mice. As shown in **Figure 3.8 (B) and (C),** the combinatorial treatment of AICAR and heparin caused a 2.9 fold increase in utrophin A expression in the diaphragm muscle which was significantly higher than AICAR or heparin treatment alone. These results are very promising since the mdx diaphragm muscle displays a more severe pathology that is representative of the human DMD condition (Grounds et al, 2009). Although the combinatorial treatment caused ~ 2.3 fold up-regulation of utrophin A protein in TA muscle, no additive effect was observed in this muscle. This can be attributed to large variations between the samples and it is speculated that increasing the number of mice in the experiment could help overcome this issue **(Figure 3.9 (B) and (C).**

We also measured the mRNA levels of utrophin in response to all three treatments and our results showed that overall AICAR + heparin combinatorial treatment seems to stimulate utrophin A mRNA levels in both the TA and diaphragm muscle. However, due to large variations in samples some of these values did not reach significance (Figure 3.8(A), Figure 3.9 (A)).

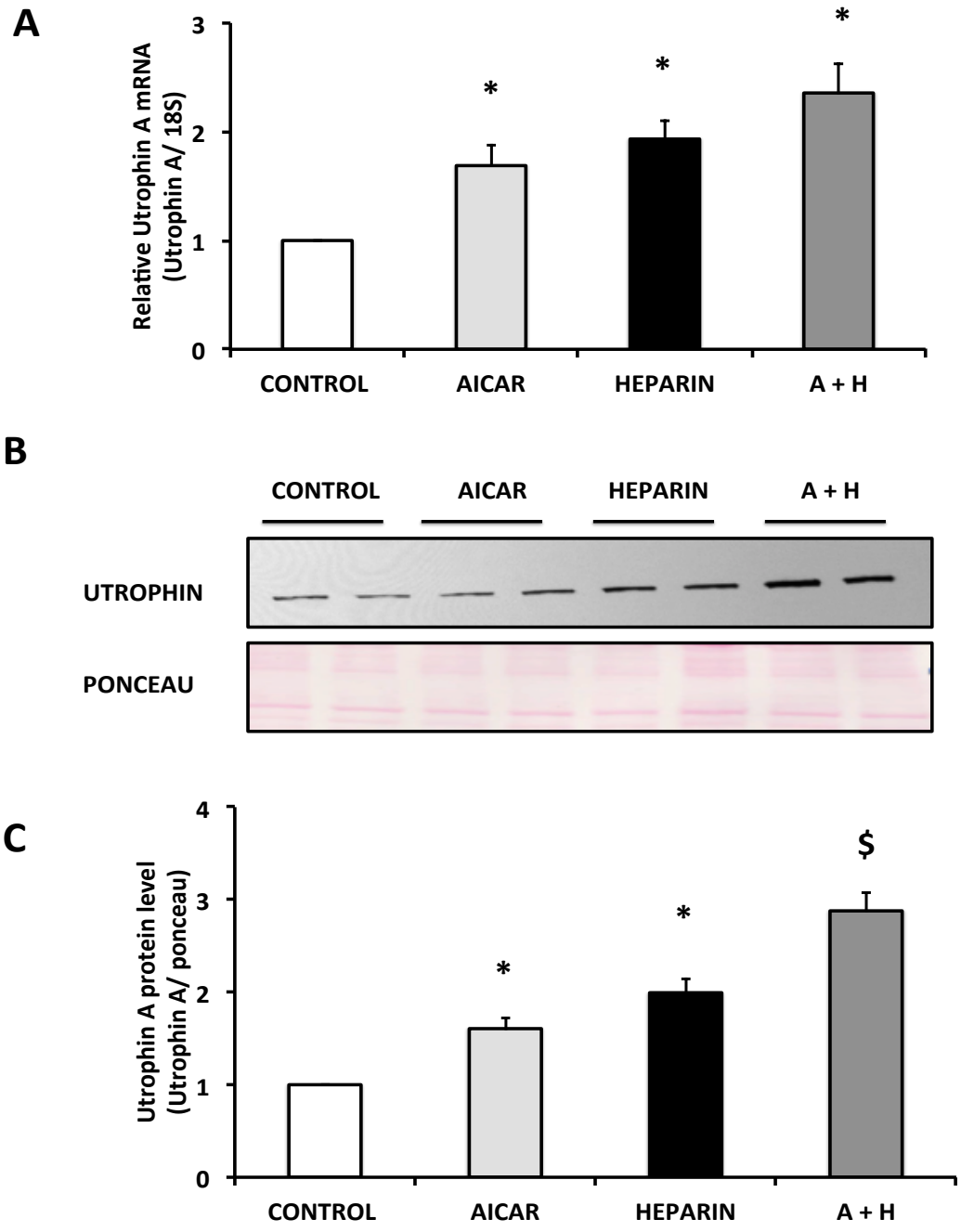


Figure 3.8: Treatment of mdx mice with a combination of AICAR and heparin causes a significant increase in utrophin A mRNA and protein levels in the diaphragm muscle. A) The mRNA expression of utrophin A in mdx mice treated with AICAR (500mg), Heparin (500IU), AICAR + heparin or control (saline) for 4 weeks as measured by qRT-PCR. Values are normalized to 18S mRNA levels B) Representative Western blots of utrophin A expression as a result of AICAR, heparin or AICAR + heparin treatment. C) Represents quantification of utrophin A protein levels normalized to ponceau staining shown in (A). Values are means ± SE (n=4), * indicates significant difference compared to control, $ signifies significant difference compared to all groups (treated and control), $p<0.05$.

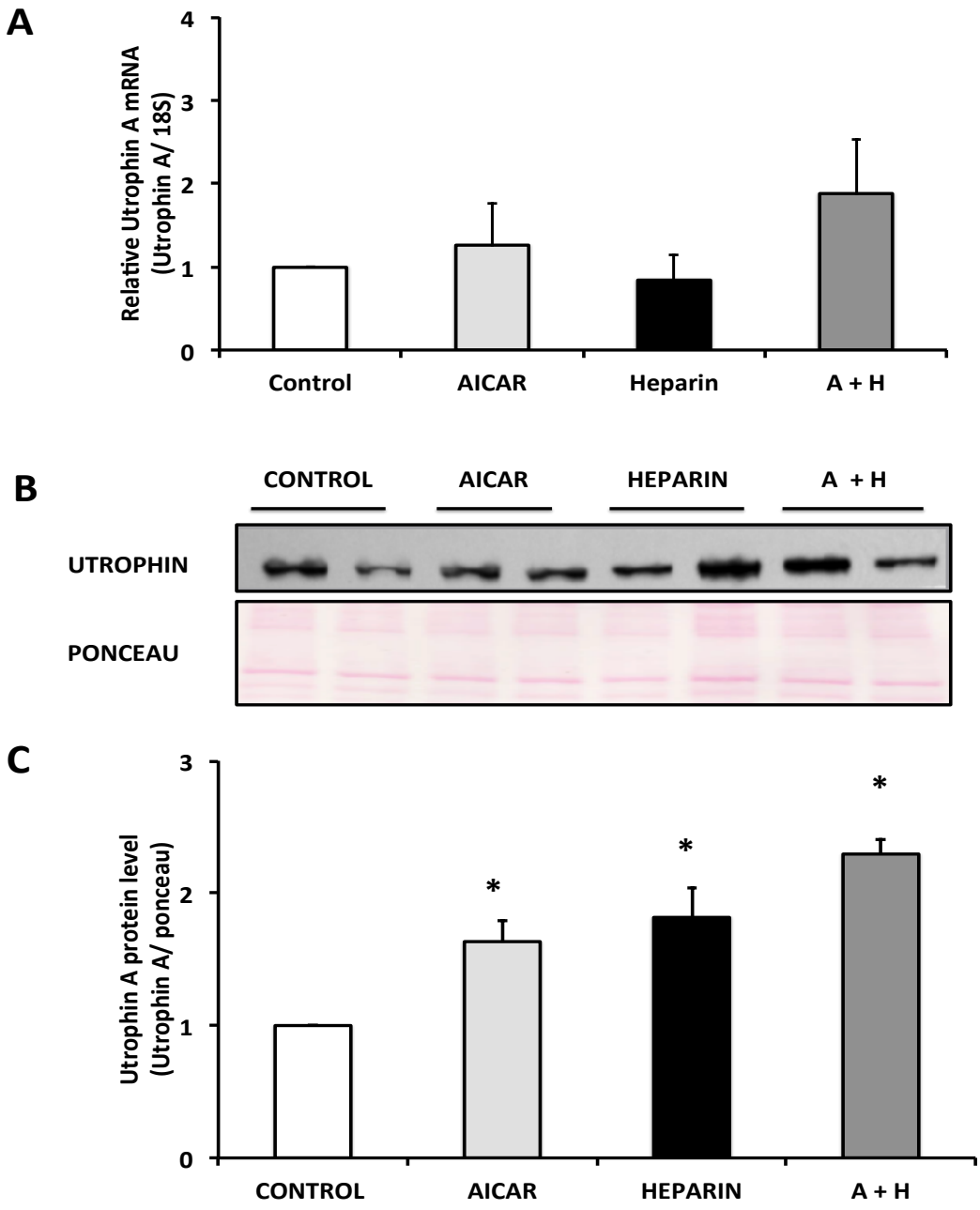


Figure 3.9: Treatment of mdx mice with a combination of AICAR and heparin causes a significant increase in utrophin A protein levels in the Tibialis Anterior (TA) muscle of mdx mice. A) The mRNA expression of utrophin A in mdx mice treated with AICAR (500mg), heparin (500IU), AICAR + heparin or control for 4 weeks as measured by qRT-PCR. Values are normalized to 18S mRNA levels B) Representative Western blots of utrophin A expression as a result of AICAR, Heparin or AICAR + heparin treatment in TA muscles of mdx mice. C) Represents quantification of utrophin A protein levels normalized to ponceau staining shown in (A). Values are means ± SE (n=4), * represents significant difference compared to control values, $p<0.05$.

3.3.3 Treatment of mdx mice with a combination of AICAR and heparin causes significant increase in utrophin A localization and β-dystroglycan expression in the diaphragm and TA muscles of mdx mice:

For utrophin up-regulation therapy to be effective, it is important that the utrophin expression is increased along the entire length of the sarcolemma so that it can replace the absent dystrophin protein (Blake et al, 2002). To determine the localization of increased utrophin A expression in treated mdx mouse muscle, we performed immunostaining on diaphragm and TA muscle cryosections. As shown in **Figure 3.10 (A) and (B) and Figure 3.11 (A) and (B)** utrophin immunofluorescence experiments further revealed an increase in utrophin A expression at the sarcolemma of muscles fibers in response to the combinatorial and individual treatments

Next, we evaluated whether the increased utrophin A expression in response to the treatments has the ability to recruit the members of the DAPC such as β-dystroglycan. As shown in **Figure 3.12,** qualitative assessment of β-dystroglycan staining shows that all 3 groups of treated mice (AICAR, heparin or AICAR + heparin) had a higher expression of β-dystroglycan at the sarcolemmal membrane as compared to the control (untreated) mdx mice suggesting effective recruitment of β-dystroglycan in response to the treatments.

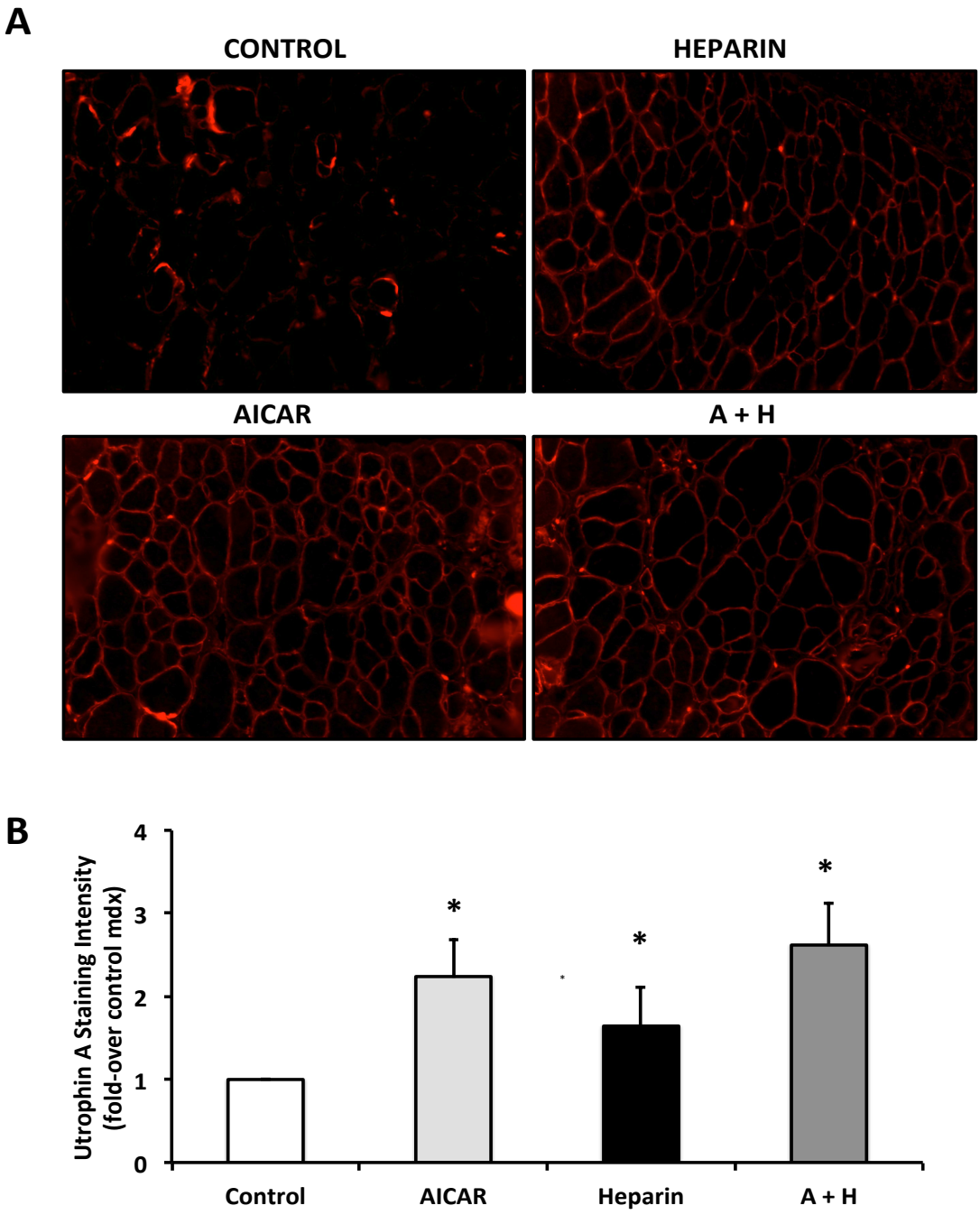


Figure 3.10: Treatment of mdx mice with a combination of AICAR and heparin causes a significant increase in utrophin A localization in the diaphragm muscle. A) Micrographs of utrophin A immunostaining in diaphragm muscle of mdx mice treated with AICAR (500mg), heparin (500IU), AICAR + heparin (A + H) or control for 4 weeks. c) Represents quantification of utrophin A immunostaining fluorescence in the diaphragm muscle of control and treated mdx mice. Values are means ± SE (n=4), * represents significant difference compared to control values (untreated), $p<0.05$)

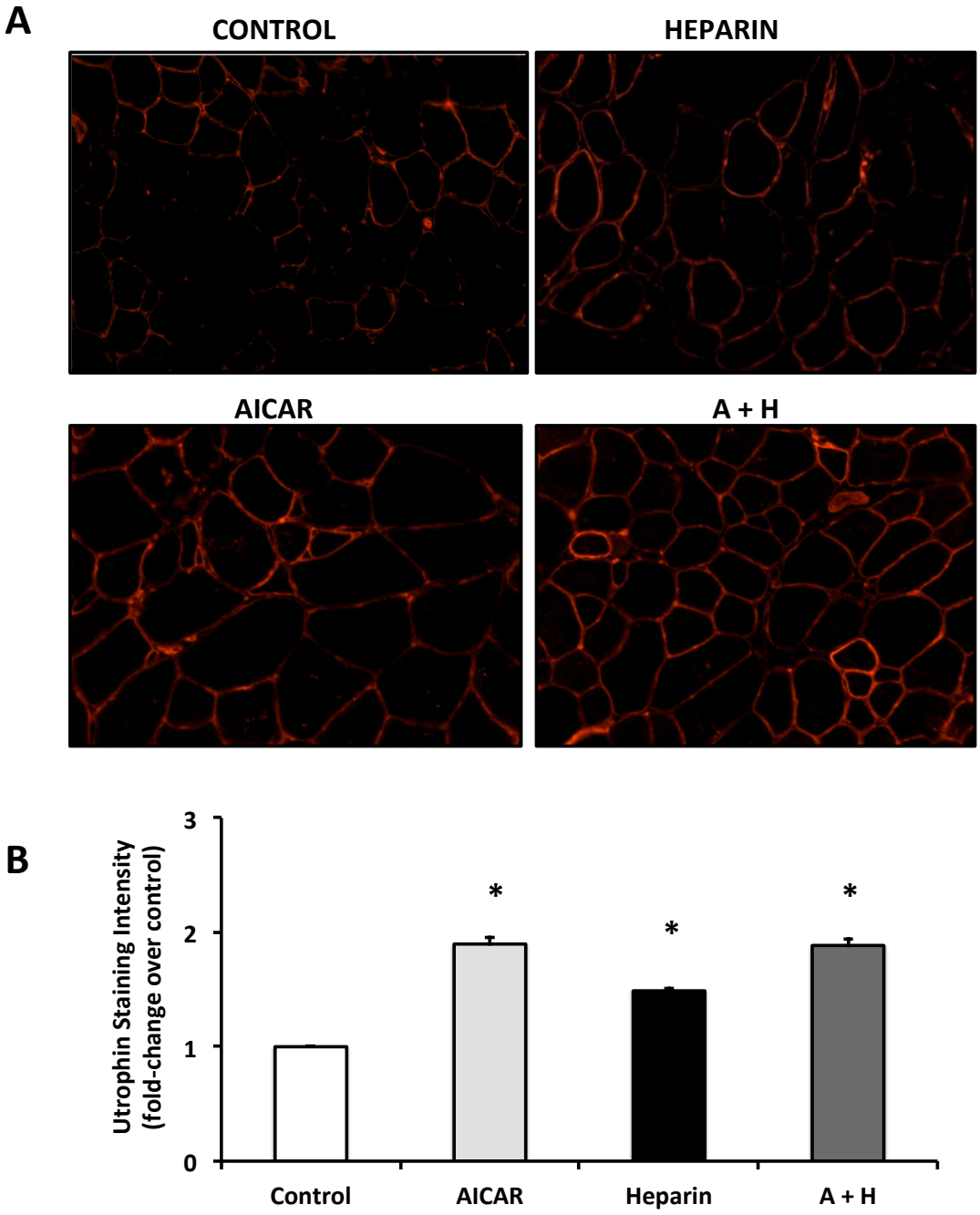


Figure 3.11: Treatment of mdx mice with a combination of AICAR and heparin causes a significant increase in utrophin A localization in the Tibialis Anterior (TA) muscle of mdx mice. A) Micrographs of utrophin A immunostaining in the TA muscle of mdx mice treated with AICAR (500mg), heparin (500IU), AICAR + heparin (A + H) or control for 4 weeks. c) Represents quantification of utrophin A immunostaining fluorescence in the TA muscle of control and treated mdx mice. Values are means ± SE (n=4), * indicates significant difference when compared to control values (untreated samples), $p<0.05$.

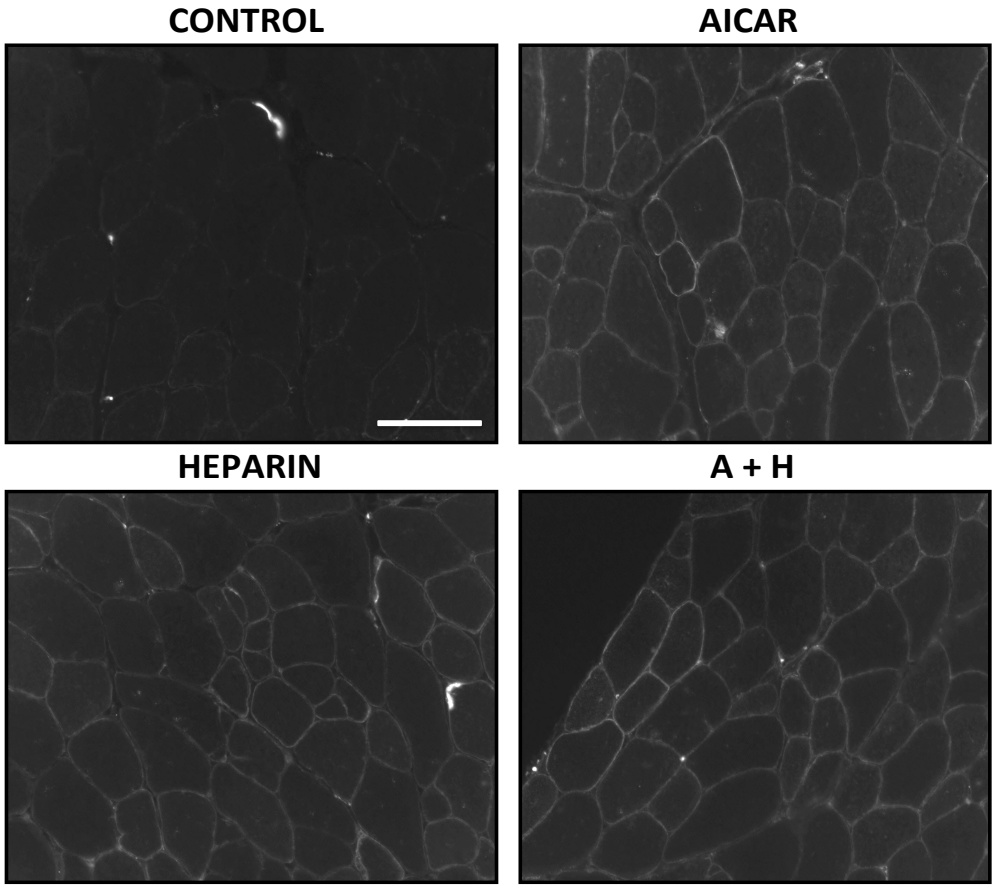


Figure 3.12: β-Dystroglycan localization and expression in Tibialis Anterior (TA) muscles of treated mdx mice. Representative micrographs of β-Dystroglycan immunostaining in TA muscles of mice treated with Control (saline), AICAR (500mg/Kg), heparin (500IU) or AICAR + heparin (A + H) for 4 weeks. Scale bar = 50uM. (n=4). Immunostaining performed by Christine Péladeau.

3.3.4. AICAR treatment induces PGC-1α expression in mdx mice:

PGC-1α, a master regulator of the slow myogenic program plays an important role in controlling utrophin A expression in skeletal muscle (Lin et al, 2002). Earlier work from our laboratory has established that AICAR treatment in mdx mice leads to an increase in PGC-1α protein levels (Ljubicic et al, 2011). Thus, we evaluated the levels of PGC-1α protein expression in the diaphragm muscle of mdx mice treated with AICAR, heparin and AICAR + heparin. As shown in **Figure 3.13 (B) and (C)**, PGC-1α protein levels were significantly increased by AICAR and AICAR + heparin combinatorial treatment (A + H) by 1.2 and 1.3 fold respectively ($p<0.05$). As expected, heparin treatment did not cause significant changes in PGC-1α protein levels **($p>0.05$, Figure 3.13 (C).** The mRNA levels of PGC-1α also showed increases in response to combinatorial treatment of AICAR and heparin but these changes did not reach significance **(Figure 3.13 (A)).**

3.3.5 Treatment of mdx mice with heparin decreases KSRP expression in mdx mice:

Our laboratory has earlier shown that expression of KSRP (K homology splicing regulator protein), an important negative regulator of utrophin A expression, is decreased in response to heparin treatment in C2C12 muscle cells and mdx mice (Amirouche et al, 2013). According to our data from the diaphragm muscle, heparin treatment resulted in a significant decrease of about 2 fold, while AICAR treatment had no significant effects on KSRP protein levels (Figure 15 B and C, $p<0.05$) in the diaphragm muscle. As shown in **Figure 3.14 (A),** the mRNA levels of KSRP did not show significant changes in treated vs control mdx mice.

Similarly, in the TA muscle **Figure 3.14 (B) and (C**,) Heparin treatment caused a significant decrease in KSRP protein levels by about 2.5-fold ($p<0.05$) while AICAR or AICAR+ heparin treatment had no significant effect on KSRP down-regulation ($p> 0.05$). As seen in the diaphragm muscle, KSRP mRNA levels were not significantly changed in the TA muscles in response to the treatments as compared to the control (**Figure 3.15 (A), p>0.05**).

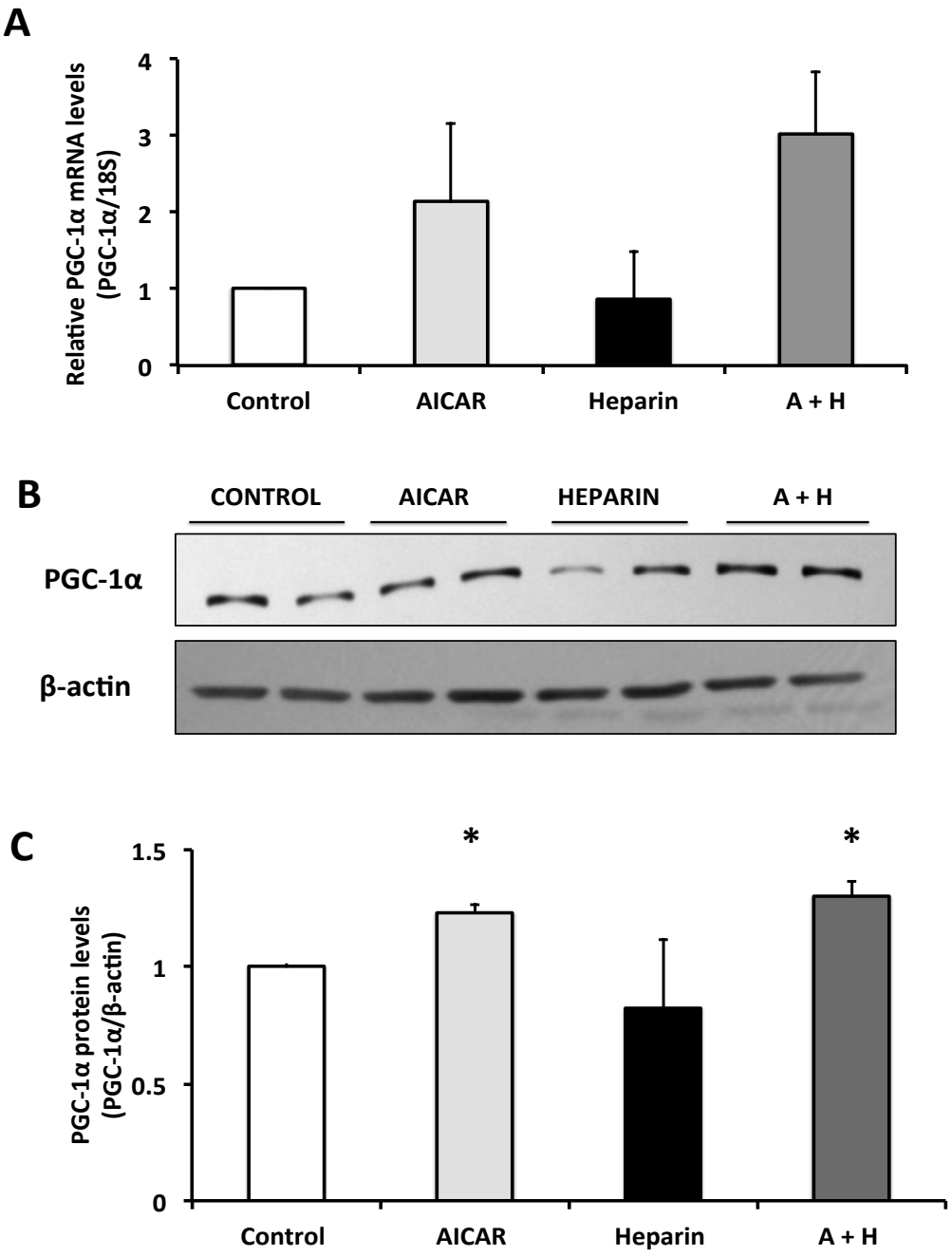


Figure 3.13: PGC-1α expression is enhanced in diaphragm muscle of mdx mice treated with AICAR. A) The mRNA expression of PGC-1α in diaphragm muscle of mdx mice treated with AICAR (500mg), Heparin (500IU), AICAR + heparin or control for 4 weeks. Values are normalized to 18S mRNA levels (B) Representative Western blot of PGC-1α and β-actin (loading control) levels in 6 week old mdx mice treated with AICAR (500mg), heparin (500IU), AICAR + heparin or control for 4 week in the diaphragm muscle. (C) Represents quantification of PGC-1α protein levels normalized to b-actin levels shown in (B). Values are means ± SE (n=4), * Indicates significant difference as compared to control (untreated) values ($P < 0.05$).

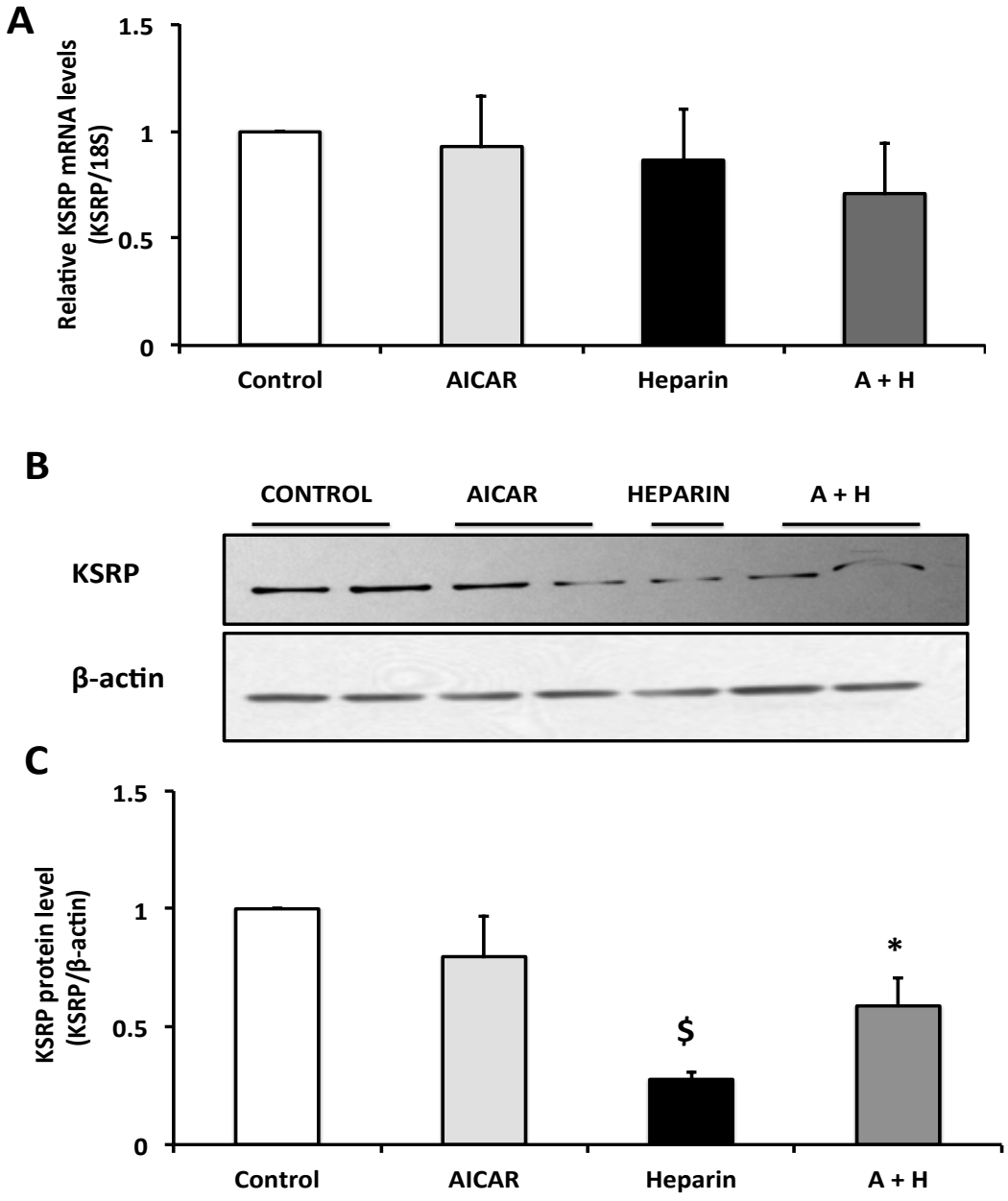


Figure 3.14: Treatment of mdx mice with heparin causes significant decrease in KSRP expression in diaphragm muscle of mdx mice treated with AICAR + heparin combinatorial treatment. A) The mRNA expression of KSRP in diaphragm muscle of mdx mice treated with AICAR (500mg), Heparin (500IU), AICAR + heparin or control (mdx) for 4 weeks normalized to 18S mRNA levels (B) Representative Western blot of KSRP and b-actin (loading control) levels in 6 - week old mdx mice treated with AICAR, heparin, A + H or control in the diaphragm muscle respectively. (C) Represents quantification of KSRP protein levels normalized to β-actin protein levels shown in (B). Values are means ± SE (n=4), * indicates significant difference compared to control values (mdx untreated), $ signifies significant difference compared to all groups (treated and control), $p<0.05$.

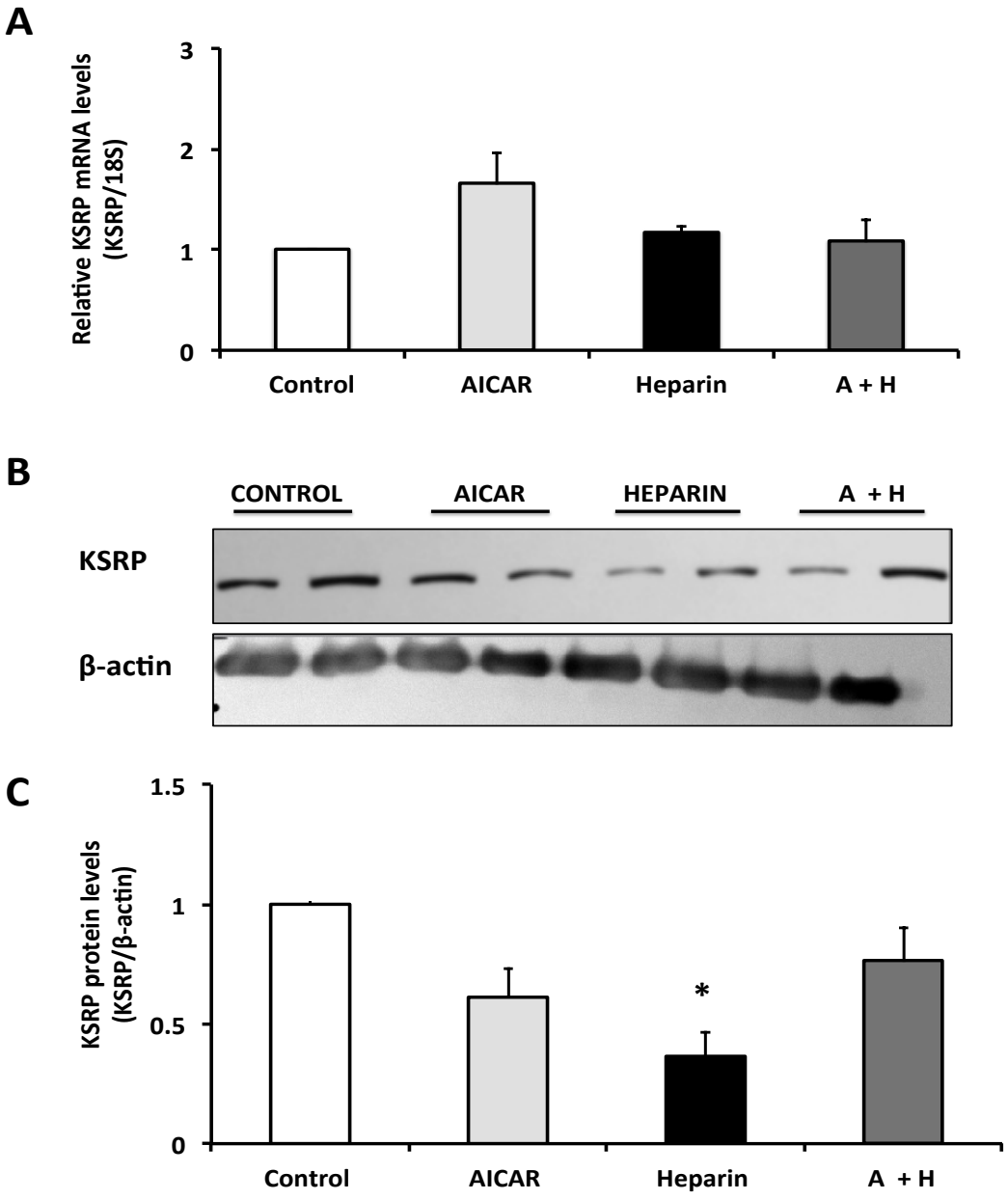


Figure 3.15: Treatment of mdx mice with heparin causes significant decrease in KSRP expression in Tibialis Anterior (TA) muscle of mdx mice treated with AICAR + heparin combinatorial treatment. A) The mRNA expression of KSRP in the TA muscle of mdx mice treated with AICAR (500mg), Heparin (500IU), AICAR + Heparin or control (mdx) for 4 weeks. Values normalized to 18S mRNA levels (B) Representative Western blot of KSRP and β-actin (loading control) levels in 6 - week old mdx mice treated with AICAR, Heparin, A + H or control in the TA muscle respectively. (C) Represents quantification of KSRP protein levels normalized to β-actin protein levels shown in (B). Values are means ± SE (n=4), * indicates significant difference compared to control values (mdx untreated), $p<0.05$.

3.3.6. The combinatorial treatment with AICAR and Heparin reduces central nucleation in dystrophic fibers and enhances sarcolemmal integrity:

Additional characterization of the treated mdx mice revealed that both AICAR and AICAR and Heparin (A + H) combinatorial treatment resulted in a significant decrease in percentage of central nucleation in muscle fibers. Central nucleation in muscle fibers serves as a good indicator of muscle damage and regeneration (Karpati et al, 1989). Our results showed that there was about a 40 % decrease in central nucleation in response to AICAR and A+ H combinatorial treatment in the diaphragm and TA muscle of treated mdx mice **(Figure 3.16 A and B, Figure 3.17 A and B, $p<0.05$)**. The heparin treatment did not show a significant decrease in central nucleation of muscle fibers in the diaphragm muscle or TA muscle **(Figure 3.16 A and B, Figure 3.17 A and B, $p>0.05$).**

To assess if the reassembly of DAPC in treated mice restores sarcolemmal integrity, we performed IgM (immunoglobulin) staining on muscle sections from treated and control mdx mice. IgM, an extracellular protein, can serve as a good indicator of sarcolemmal integrity as it penetrates within the muscle fiber when the sarcolemmal integrity is compromised (Straub et al, 1997; Chakalakkal et al, 2004). Qualitative assessment of relative IgM staining show that IgM staining was reduced in all 3 groups of treated mdx mice (AICAR, heparin, A + H) when compared to the control (untreated) mdx mice suggesting an improvement in sarcolemmal integrity in treated mdx mice **(Figure 3.18).**

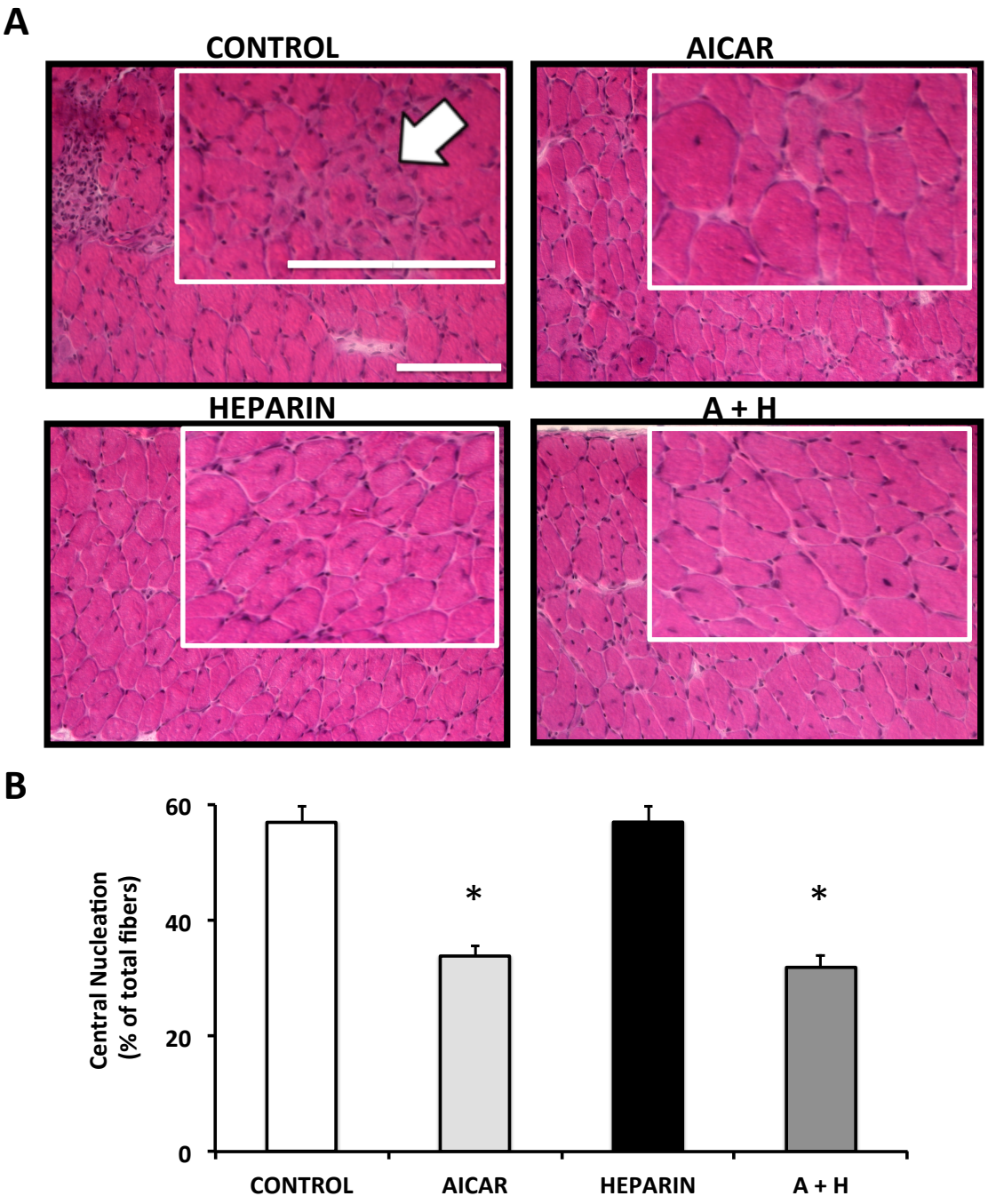


Figure 3.16: Central nucleation in the diaphragm muscles of AICAR, heparin or AICAR + heparin (A + H) treated mdx mice. A) Representative micrographs of H and E stained fibers in diaphragm muscles of mdx mice. (B) Graphical analysis of extent of central nucleation indicated by the number of fibers with central nucleation in the diaphragm muscle of control, AICAR, Heparin or AICAR + Heparin treated mdx mice $n = 4$. * Indicates significant difference as compared to control (untreated) ($P < 0.05$). Scale bar = 50uM. The white boxes represent magnified regions of H and E images. The white arrow points at centrally nucleated fibers. Scale bar = 50uM

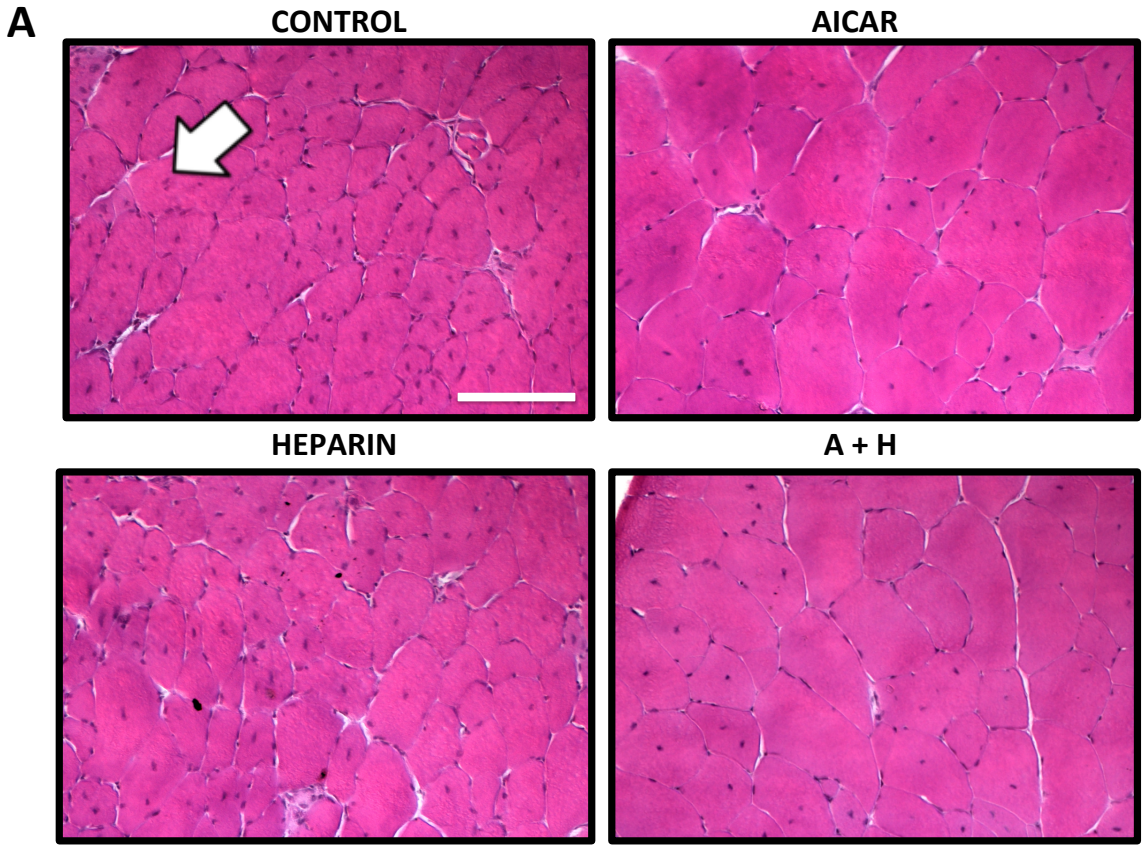


Figure 3.17: Central nucleation in Tibialis Anterior (TA) muscles of AICAR, heparin or AICAR + heparin treated mdx mice. A) Representative micrographs of H and E stained fibers in TA muscles of mdx mice. (B) Graphical analysis of extent of central nucleation indicated by the number of fibers with no central nucleation in the TA muscle of control, AICAR, heparin or AICAR + heparin treated mdx mice $n = 4$. * Indicates significant difference as compared to control (untreated) ($P < 0.05$). The white arrow points at centrally nucleated fibers. Scale bar = 50uM.

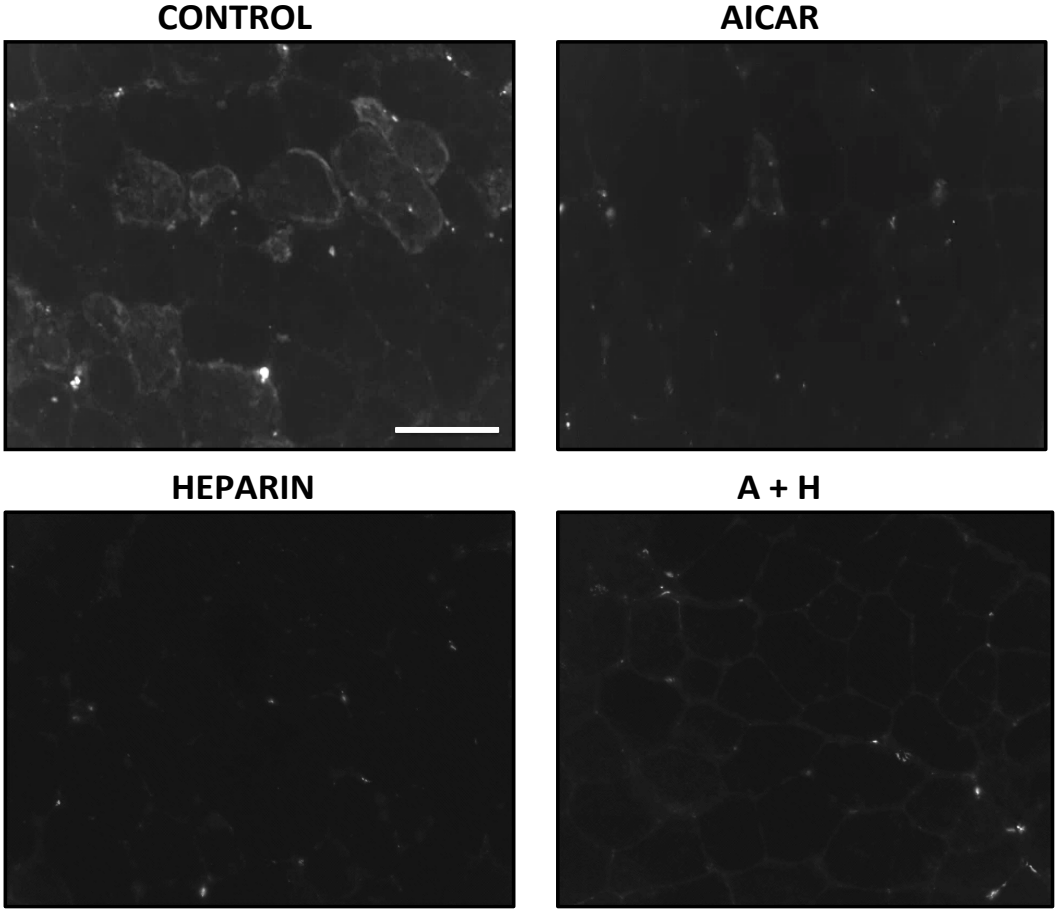


Figure 3.18. IgM intramyocellular protein localization in Tibialis Anterior (TA) muscles of treated mdx mice. Representative micrographs of fibers stained with IgM antibody in Tibialis Anterior muscle of mdx mice treated with Control (saline), AICAR (500mg/kg), heparin (500IU) and AICAR + heparin (A+H) for 4 weeks. n=4. Scale bar = 50 μm. Immunostaining performed by Christine Péladeau.

3.3.7 Combinatorial treatment of AICAR and heparin decreases variability in dystrophic fibers in mdx mice:

Histopathological studies have shown that abnormal fiber size distribution is characteristic of dystrophic muscle (Briguet et al, 2004). Mdx mice usually display an abnormal proportion of small and large muscle fibers that is indicative of the dystrophic phenotype (Briguet et al, 2004). To assess the effect of combinatorial treatment of AICAR and heparin on fiber size distribution, we measured the cross-sectional area (CSA) of individual fibers from the diaphragm and TA muscles of mdx mice. Our results indicate that the CSA profile of untreated mice displays large differences when compared to healthy wild-type mice. These could be caused by the continuous regeneration and generation cycles that these dystrophic muscle fibers go through. As shown in **Figure 3.19 and 3.20 (A-C**) all 3 treatments cause a shift in these frequency distribution profiles towards Wild-type values suggesting an improvement in muscle morphology in the treated mdx mice.

The mdx mouse undergoes cycles of degeneration followed by regeneration (Briguet et al, 2004). Research has shown that due to these continuous cycles, the mdx muscle fibers become more heterogeneous and have a higher amount of variability as compared to healthy fibers (Briguet et al, 2004, Torres et al, 1987). To further evaluate the protective effect of these treatments on muscle morphology, we measured the variance coefficient of muscle fibers in treated, untreated mdx mice and WT mice. Our results corroborated earlier findings and mdx mice were shown to have a significantly higher variance coefficient as compared to wild-type mice in the diaphragm and TA muscles **(Figure 3.21 A and B, $p<0.05$).** Further,

all three treatments significantly reduced the variance coefficient in treated mdx mice. Together these findings suggest that the combinatorial treatment of AICAR and heparin as well as the individual treatments are important in improving muscle morphology and variability in mdx mice

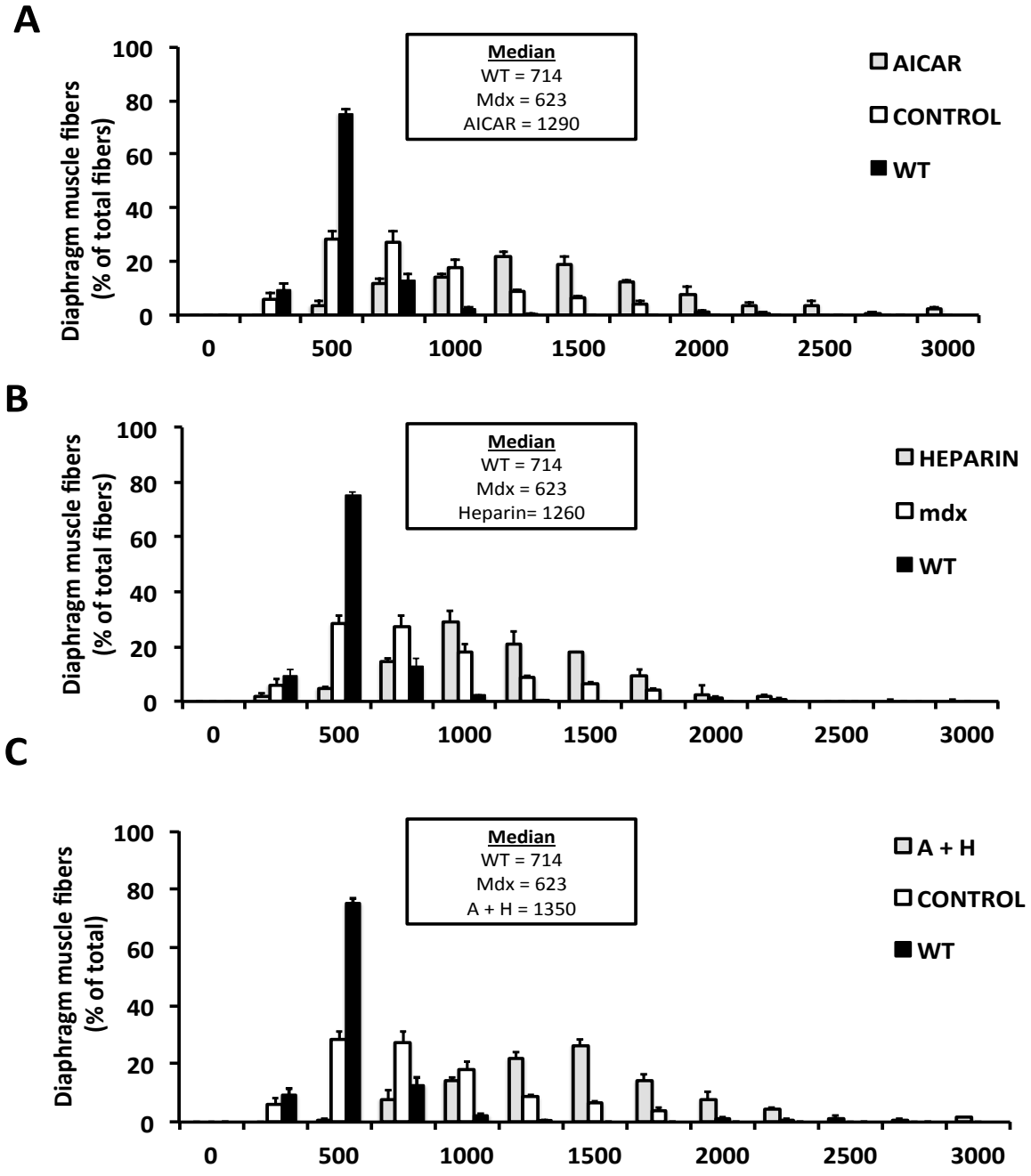


Figure 3.19. Myofiber Cross- sectional Area (CSA) in the diaphragm muscles of mdx mice treated with AICAR, heparin or AICAR + heparin (A + H) for 4 weeks. A), B) and C) Graphical representation of frequency of myofiber CSA expressed as a percentage of the total number of fibers in the diaphragm muscle from mice treated with AICAR, heparin and AICAR + heparin respectively as compared to the control mdx mice (n=4).

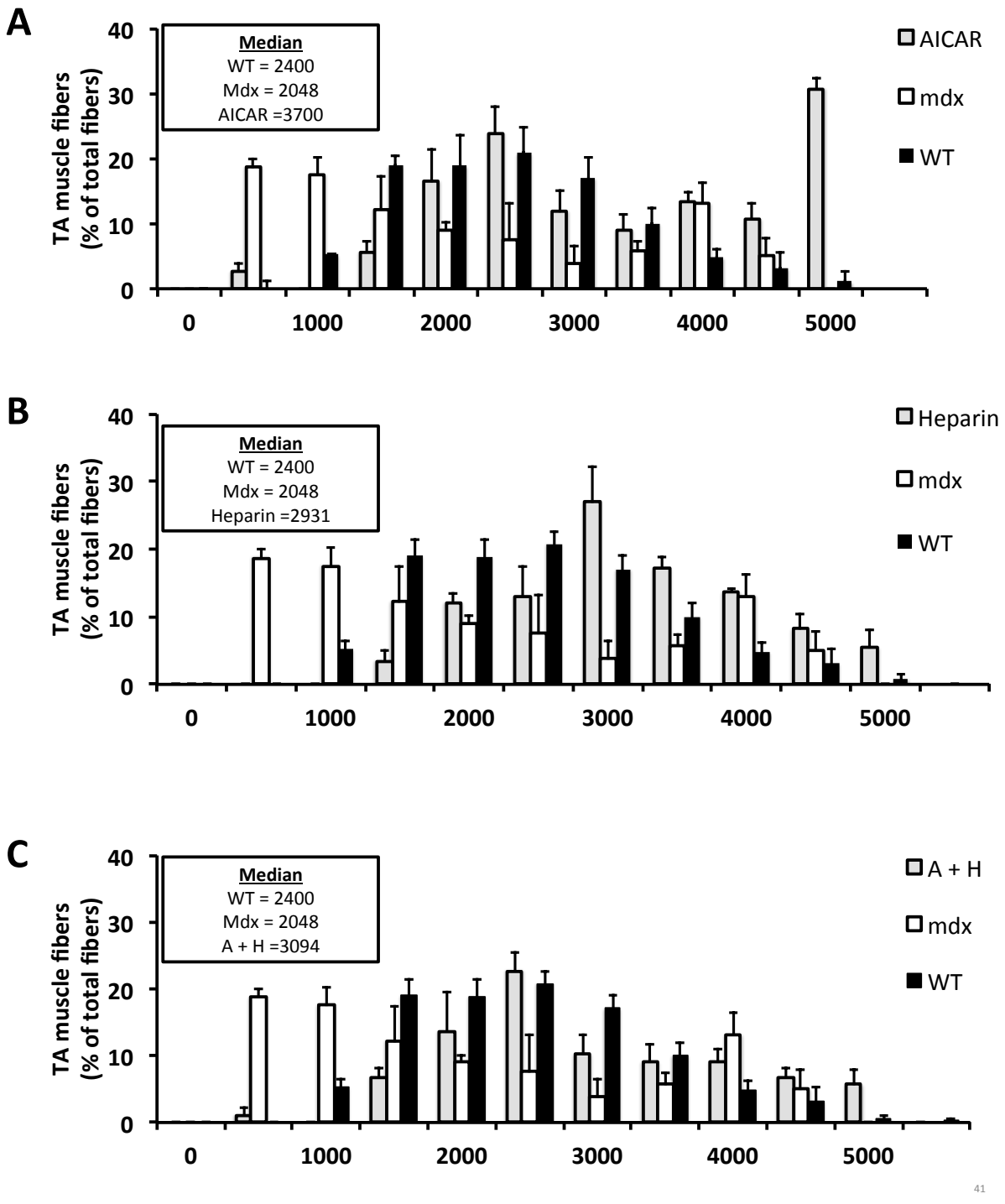


Figure 3.20. Myofiber Cross-sectional Area (CSA) in the Tibialis Anterior (TA) muscles of mdx mice treated with AICAR, heparin or AICAR + heparin for 4 weeks. A), B) and C) Graphical representation of frequency of myofiber CSA expressed as a percentage of the total number of fibers in the TA muscle from mice treated with AICAR, heparin and AICAR + heparin respectively as compared to the control mdx mice (n=4).

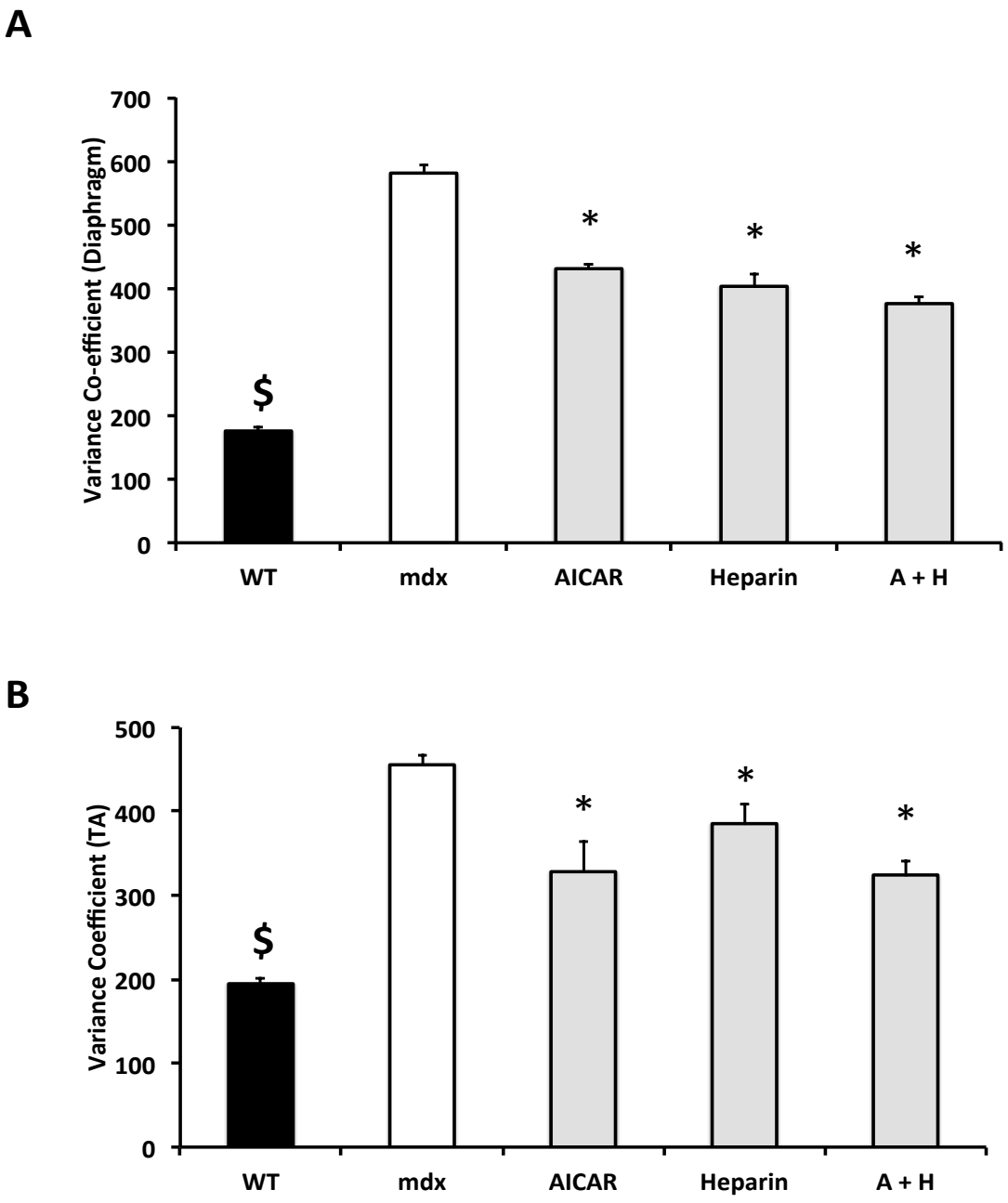


Figure 3.21. The variance coefficient of muscle fiber size determined by using the cross-sectional area method. A) Graphical representation of variance coefficient of muscle fiber sizes in diaphragm muscles of mice treated with control (mdx), AICAR(500mg/kg), heparin (500IU) and AICAR + Heparin (A + H). B) Graphical representation of variance coefficient of muscle fiber sizes in Tibialis Anterior (TA) muscles of mice treated with control (mdx), AICAR, heparin and AICAR + heparin. Mean ± SEM are shown; n=4 mice; * indicates difference compared to mdx (untreated) control mouse, $ represents significant difference compared to all treated and control groups, $P<0.05$)

4.0 DISCUSSION:

The findings reported in this study reveal that a combinatorial treatment of transcriptional and post-transcriptional activators of utrophin A, AICAR and heparin, instigates an additive effect on its expression in muscle and improves numerous pathological features in the preclinical mdx mouse model of DMD. The combinatorial treatment increased utrophin A expression and localization at the sarcolemma, improved sarcolemmal integrity, promoted reassembly of the DAPC and improved muscle morphology in mdx mice. Taken together, these results suggest that combinatorial treatment of AICAR and heparin could serve as an effective therapeutic strategy to treat DMD.

4.1: Dalteparin, a low-molecular weight heparin, does not stimulate utrophin A expression in muscle cells:

The transcriptional regulation of utrophin A promoter has been studied extensively by our laboratory and others (Gramolini et al, 1999; Khurana et al, 1999; Galvagni et al, 2001; Miura et al, 2009). However more recently, post-transcriptional mechanisms have been revealed to play a fundamental role in utrophin A regulation in muscle.

Initial evidence of possible post-transcriptional regulation of utrophin A came from studies where an increase in utrophin A protein levels in muscle was not accompanied by an increase in utrophin mRNA levels (Gramolini et al, 1999; Weir et al, 2002; Pasquini et al, 1995). Additional evidence of post-transcriptional regulation of utrophin A emanated from studies that highlighted the importance of the 5' and 3' UTR of utrophin A mRNA in

regulating its expression in muscle (Chakalakkal et al, 2008; Gramolini et al, 2001; Miura et al, 2008; Miura et al, 2010; Moorwood et al, 2013). In this context, our laboratory has demonstrated that specific ARE sites within the 3'UTR of utrophin A mRNA play a key role in its expression in muscle (Gramolini et al, 2001; Chakalakkal et al, 2008). More recently, our laboratory demonstrated that KSRP, an RNA binding protein, binds specifically to AREs with the 3'UTR of utrophin A and negatively regulates its expression in muscle cells and mdx mice (Amirouche et al, 2013). Further, this study demonstrated that pharmacological activation of p38 by heparin leads to down-regulation of KSRP, a subsequent increase in utrophin A expression in mdx muscle and amelioration of the dystrophic phenotype (Amirouche et al, 2013).

Heparin or unfractionated heparin (UFH), an FDA approved drug, is a naturally occurring sulfated polysaccharide that is used as an anticoagulant in treatment of thrombosis (Gray et al, 2008). Heparin has been shown to activate p38 MAPK activity in skeletal muscle of wild-type mice through Toll-like receptors (TLR) TLR2 and TLR4 (Zbinden-Foncea et al, 2012). Structural analysis of heparin has revealed that it is a large heterogenous molecule with several key regions such as repeating subunits of trisulfated disaccharides (Gray et al, 2008). The pentasaccharide sequence in the heparin molecule is responsible for its anti-coagulant activity and binds to anti-thrombin protein to inhibit blood coagulation cascades (Choay et al, 1983; Gray et al, 2008; Conrad, 1998).

In the present study we first confirmed the effects of heparin on utrophin A expression in muscle cells. Our results revealed that heparin was effective in stimulating the utrophin A 3'UTR, utrophin A mRNA and protein levels in C2C12 muscle cells. These

results corroborate previous work from our laboratory by Amirouche et al. (Amirouche et al, 2013). Next, we assessed the effects of dalteparin, a low-molecular weight heparin (LMWH), on utrophin A expression. LMWH are depolymerized derivatives of heparin, with improved pharmacokinetic profiles such as a longer half-life and a greater ease of administration compared to the unfractionated heparin (UFH) (Hirsh et al, 2001; Gray et al, 2008; Buyue et al, 2012). LMWH are synthesized through various chemical modifications including deaminative cleavage, β-elimination and oxidative phosphorylation. They range in size from about 3000-8000 kDa (less than half of the MW of heparin). Although LMWH range in size and development method, they all retain the anti-thrombin binding pentasaccharide region important for their anti-coagulation activity (Casu et al, 2014; Gray et al, 2008).

Our results revealed that dalteparin had no significant effects on utrophin A 3'UTR activity, utrophin A mRNA or protein levels in C2C12 muscle cells. To our best knowledge, our study is the first to report the effect of a LMWH on utrophin A expression in muscle. Our results suggest that specific regions in the full-length heparin molecule that are important for its role in regulating utrophin A expression are absent from the shorter dalteparin structure. The precise structure of heparin molecule that is involved in p38 activation remains elusive. However, there is evidence that the pentasaccharide sequence of heparin, that binds to anti-thrombin has no specific binding to other heparin binding proteins such as the Fibroblast Growth Factors, cytochrome c etc. (Conrad, 1998). These studies can explain why the shorter dalteparin molecule, that mainly retains the pentasaccharide region of heparin, has no effect on utrophin A expression.

4.2: AICAR stimulates utrophin A expression in muscle cells by direct transcriptional induction of the utrophin A promoter:

It is well documented that AMPK activation causes important phenotypic changes in skeletal muscle such as promotion of the slow oxidative myogenic program, stimulation of mitochondrial biogenesis, augmentation of important signaling proteins including PGC-1α and PPAR- β/δ and increase in GLUT 4 expression (Narkar et al, 2008; Jorgensen et al, 2007; Winder et al, 2000; Fillmore et al, 2010). More specifically, a previous study from our laboratory by Ljubicic et al., has demonstrated that pharmacological activation of AMPK by AICAR, stimulates the slow oxidative myogenic program and increases utrophin A expression in muscle cells and mdx mice (Ljubicic et al, 2011). In addition, this study demonstrated that AICAR's effect on utrophin A expression is at least in part regulated by PGC-1α as the expression of this signaling molecule was augmented in AICAR treated muscle cells and mice (Ljubicic et al, 2011). Additional studies have reported similar stimulation of PGC-1α expression in response to AMPK activation (Jager et al, 2007). Considering the key role of PGC-1α in transcriptional regulation of utrophin A expression (Lin et al, 2002; Angus et al, 2005), it was imperative to investigate whether the effect of AICAR on utrophin A expression is caused by the direct transcriptional induction of the utrophin A promoter. Our results revealed that AICAR stimulated the utrophin A promoter activity directly suggesting that AICAR's effect on utrophin is regulated, at least in part, at the transcriptional level. Further, the expression levels of utrophin A and PGC-1α were also significantly increased. These results are in accordance with earlier work from our laboratory (Ljubicic et al, 2011).

In addition to AICAR, we screened various other FDA approved drugs and natural compounds to evaluate their effects on utrophin A expression in muscle cells. These drugs included several activators of AMPK (β-GPA, Metformin), PPAR β/δ (GW501516, Bezafibrate, Linoleic acid), SIRT-1 (Resveratrol) and HDACi/NO pathway (Arginine butyrate). These pharmacological compounds were selected based on earlier studies that reported that pharmacological activation of AMPK, PPAR-β/δ, SIRT-1 and HDACi/NO pathways stimulates utrophin A expression in muscle (Ljubicic et al, 2011; Miura et al, 2009; Gordon et al, 2013; Ljubicic et al, 2014; Vianello et al, 2013). Our results demonstrated that while some of these drugs were effective at stimulating the utrophin A promoter such as GW501516, Resveratrol, Arginine butyrate, overall these agonists had no significant effect on utrophin A expression in growing C2C12 muscle cells. Since previous studies have reported successful up-regulation of utrophin A expression in response to some of these drugs (Miura et al, 2009, Ljubicic et al, 2013; Vianello et al, 2013), it is speculated that changes in experimental setup, cellular content, cell types and doses of drugs could be responsible for these discrepancies. Such discrepancies have been reported earlier for instance a study by Hofer et al. reported that the effect of several drugs on PGC-1α signaling cascade depends largely on the cell-type and cellular responses to the specific drug (Hofer et al, 2013).

Nevertheless, our findings established that AICAR effectively stimulates utrophin A expression through direct transcriptional induction of the promoter in C2C12 muscle cells and thus was selected as the drug of choice for the combinatorial treatment discussed in the next section.

4.3: Combinatorial treatment of AICAR and heparin stimulates an additive effect on utrophin A expression in muscle cells:

Combinatorial treatment approach to ameliorate the dystrophic phenotype is an emerging field of therapy for DMD. In this context, a study by Narkar et al. was among the first to demonstrate the combinatorial effect of exercise training with PPAR-β/δ agonist GW501516. This study revealed that GW501516 acts synergistically with exercise training to increase running endurance and the number of oxidative fibers in wild-type mice (Narkar et al, 2008). More recently, a study by Jahnke et al., demonstrated the effects of combinatorial treatment of AICAR and GW501516 in mdx mice. They reported that although combinatorial treatment of GW501516 and AICAR had no synergistic effect, all three treatments resulted in improvements in dystrophic phenotype, muscle function and behavioral activity in mdx mice (Jahnke et al, 2012). Another study in this context by Junior et al. combined AICAR and GW501516 treatment with exercise training in mdx mice (Junior et al, 2012). They reported synergistic effects in some aspects of muscle function and oxidative metabolism (Junior et al, 2012). Bruckbauer et al. recently observed the effects of combining three AMPK activators resveratrol, metformin and hydroxymethylbutyrate on insulin sensitivity in a diabetic mouse model (db/db) (Bruckbauer et al, 2013). This study revealed that a mixture of these pharmacological compounds produced additive effects on fatty acid oxidation and expression levels of key signaling molecules such as AMPK and SIRT1 levels in muscle cells (Bruckbauer et al, 2013).

There is mounting evidence that suggests that the drugs used in the aforementioned combinatorial treatments such as GW501516, AICAR, Metformin and resveratrol mediate their effects on utrophin A expression at the transcriptional level by activating key regulatory factors such as PPAR β/δ, AMPK, SIRT1 and PGC-1α. For instance, GW510516 has been demonstrated to directly activate the utrophin A promoter via a PPRE site in the promoter (Miura et al, 2009). Further AMPK activators, AICAR and Metformin, and SIRT-1 activator, Resveratrol, have been shown to activate PGC-1α expression in skeletal muscle (Suwa et al, 2006; Ljubicic et al, 2011; Jahnke et al, 2012; Gordon et al, 2013; Ljubicic et al, 2013). As mentioned previously, PGC-1α has been shown to induce utrophin A transcription by increasing the transcription of GABP α and β (Angus et al, 2005). Our current work on AICAR provides additional evidence that AICAR's effect is mediated by direct transcriptional induction of the promoter. Since the combinatorial treatments involving these putative transcriptional activators such as GW501516 and AICAR did not induce any synergistic or additive effect on utrophin A expression (Jahnke et al, 2012), we speculate that this may be due to saturation of binding sites of common downstream targets of these drugs such as PGC-1α. Based on these findings we anticipated that activators of utrophin A acting through distinct, yet complementary, pathways may overcome this issue and mediate additive effects on its expression in muscle.

Previous work from our laboratory has suggested that the effect of heparin on utrophin A expression is, at least in part, regulated at the post-transcriptional level. Thus, we examined the effect of combining heparin with a putative transcriptional activator of utrophin A, AICAR in muscle cells. Our results demonstrated that treatment of C2C12

muscle cells with a combination of AICAR and Heparin stimulated an additive effect on utrophin A mRNA levels. In addition, the mRNA levels of PGC-1α significantly increased in response to the combinatorial treatment and individual AICAR treatment. These results are in accordance with earlier results from our laboratory and others that have illustrated that AMPK activation results in augmentation of PGC-1α expression in muscle (Jager et al, 2007; Ljubicic et al, 2011; Jahnke et al, 2012; Gordon et al, 2013). Further, we noted that heparin treatment had no significant effect on PGC-1α mRNA levels suggesting that these pharmacological agents act through distinct mechanisms in regulating utrophin A expression in muscle.

We therefore propose a model **(Figure 4.1)** by which AICAR stimulates utrophin A expression through activation of AMPK and PGC-1α signaling pathways. In contrast, heparin mainly acts by activating the MAPK p38 that subsequently phosphorylates KSRP and promotes its sequestration by 14-3-3 proteins. The sequestration of KSRP inhibits ARE mediated decay of utrophin A transcripts and results in a higher utrophin A expression. Lastly, a combination of these pathways regulated by AICAR and heparin can have complementary effects on utrophin A expression.

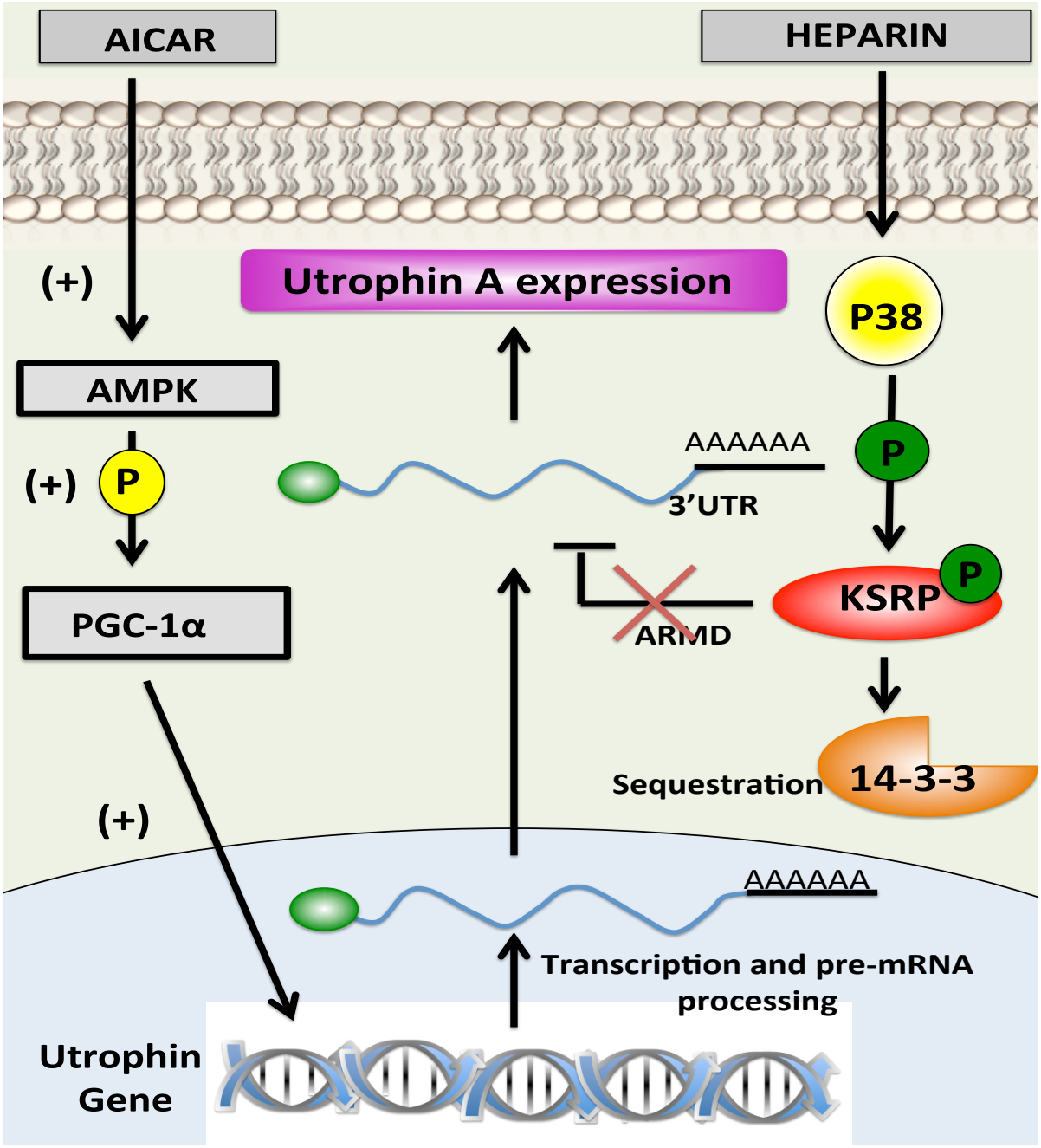


Figure 4.1: Model describing the combinatorial effect of AICAR and heparin on utrophin A expression in muscle. AMPK activation by AICAR activates PGC-1α expression in muscle. Upon activation, PGC-1α stimulates utrophin A transcription. P38 activation by heparin mediates phosphorylation of an RNA binding protein, KSRP, that is involved in ARE mediated decay (ARMD) of utrophin A transcripts. Phosphorylation of KSRP promotes its sequestration by a regulatory protein 14-3-3 and the inhibitory effect of KSRP on utrophin A expression is eliminated. A combination of these pathways leads to an additive effect on utrophin A expression in muscle.

4.4: Combinatorial treatment of AICAR and heparin stimulates utrophin A expression and improves several pathological features in the preclinical mdx mouse model of DMD

Our final goal in the current study was to confirm the combinatorial effect of AICAR and heparin on utrophin A expression in mdx mouse model of DMD and determine if the treatment causes improvements in the dystrophic phenotype. As seen in our in vitro experiments, our in vivo study demonstrated that AICAR and heparin triggered an additive effect on utrophin A expression in diaphragm muscle of treated mdx mice. The utrophin A protein levels were increased by ~ 3 fold in response to AICAR and heparin combinatorial treatment which is particularly encouraging since previous studies have reported that a 2-fold increase in utrophin protein levels in muscle is sufficient to ameliorate the dystrophic phenotype in mdx mice (Tinsley et al, 1998). Further, since diaphragm muscle in mdx mice shows a severe pathology as the disease progresses and is the most clinically relevant muscle, our findings are very promising (Grounds et al, 2009). Similar effects were seen in the Tibialis Anterior (TA) muscle, where the combinatorial treatment of AICAR and heparin caused a greater effect than individual treatments (not significant, $p>0.05$). The individual treatments also caused significant increases in utrophin A expression and these results corroborated the results from previous studies from our laboratory (Ljubicic et al, 2011; Amirouche et al, 2013).

To gain an insight into the signaling cascades through which the combinatorial treatment exerts its effects on utrophin A expression, we evaluated the expression levels of

key factors PGC-1α and KSRP (Table 4.1). In our study, treatment of mdx mice with combination of AICAR and Heparin or individual AICAR treatment resulted in significant escalations in PGC-1α protein levels in the diaphragm muscle. These results corroborate our in vitro data on PGC-1α mRNA levels. Further these results are in accordance with previous studies from our lab and other (Ljubicic et al, 2011, Jahnke et al, 2012). Next, we evaluated the expression of KSRP in mdx mice. Our findings revealed that heparin significantly decreased KSRP protein levels in both the diaphragm and TA muscles of mdx mice. These results confirm earlier work by Amirouche et al. on heparin and further highlight the distinct mechanisms of action of AICAR and heparin on utrophin A up-regulation (Amirouche et al, 2013).

As mentioned in the introduction section, utrophin A expression is confined to the neuromuscular and myotendinous junctions in healthy muscle fibers. For utrophin up-regulation therapy to be effective it is important that utrophin A expression is increased along the entire length of the sarcolemma to replace the absent dystrophin protein (Blake et al, 2002; Perkins and Davies, 2002). To assess the localization of utrophin A in muscle, we performed immunofluorescence studies. Our results demonstrated that all three treatments increased utrophin A localization at the sarcolemma. The results of individual treatments (AICAR or heparin alone) validated the results from previous studies from our laboratory (Ljubicic et al, 2011; Amirouche et al, 2013).

When expressed at the sarcolemma, utrophin A can bind to several members of the DAPC and recruit them to the sarcolemma. Previous studies have shown that utrophin and

dystrophin have similar binding domains and utrophin A effectively binds to members of DAPC such as β-dystroglycan and α-dystrobrevin (Ishikawa-Sakurai et al, 2004; Peters et al, 1998). To evaluate the ability of utrophin to recruit DAPC members at the sarcolemma, we qualitatively assessed β-dystroglycan expression in treated versus untreated mice. As expected, β-dystroglycan expression was improved at the sarcolemma of muscle fibers treated with AICAR, heparin or combinatorial treatment of AICAR and heparin.

To further establish the protective effect of the combinatorial treatment on the dystrophic phenotype, we assessed the degree of central nucleation in muscle fibers. Central nucleation in muscles is an indicator of muscle damage and is present in muscular dystrophy models including the mdx mice (Narita et al, 1999). Our results show that the combinatorial treatment (A + H) significantly reduced central nucleation in both the diaphragm and the TA muscles of treated mdx mice by similar levels (40%) as shown in **Table 4.2.** We also assessed IgM levels in the treated and control mice to confirm the improvement in sarcolemmal integrity in response to the combinatorial treatment. Increased IgM levels in muscle serve as an indicator of disrupted sarcolemmal membrane and muscle damage (Straub et al, 1997; Miura et al, 2009; Ljubicic et al, 2011). Our results demonstrated that the untreated mdx mice had a much higher leakage of IgM as compared to all three treated mice indicating protective effect of the treatments on sarcolemmal integrity. These studies support previous work from our laboratory (Ljubicic et al, 2011).

The mdx mouse muscle fibers undergo cycles of degeneration followed by regeneration in response to muscle damage (Briguet et al, 2004). As a result of these

continuous cycles, the mdx muscle fibers are more heterogeneous in size and display higher variability in size distributions compared to healthy wild-type fibers (Briguet et al, 2004, Torres et al, 1987). To establish the variability in fiber size, we analyzed the variance coefficient in treated versus untreated mice and also compared these values to the wild type mice. Our results show that as expected the wild-type mice had the lowest variability in fiber sizes compared to mdx and treated mdx mice. Further, all three treatments decreased the variability co-efficient in both the diaphragm and TA muscles of mdx mice.

Most of the pathological parameters measured in this study had no significant differences between the individual treatments and combinatorial treatments (except central nucleation which was not affected by heparin) **(Table 4.2).** It is possible that this is because the effect of increasing utrophin A expression on the dystrophic phenotype in mdx mice has already reached its maximum capacity and further increases in utrophin A expression do not provide greater benefits. In light of this, as mentioned earlier, studies have shown that ~ 2 fold increase in utrophin protein is sufficient to ameliorate the dystrophic phenotype in mdx mice (Tinsley et al, 1998). This might explain why no greater improvements in dystrophic phenotype are seen when utrophin expression is further increased. However, since the human DMD is much more severe compared to the mdx mice, the combinatorial treatment in human DMD patients might be more beneficial than individual treatments.

Table 4.1: Expression levels of various proteins in treated mdx mice

PROTEIN EXPRESSION	TREATMENTS	EFFECTS	
		DIAPHRAGM	**TA**
1) UTROPHIN A	**AICAR**	1.6 fold increase (*)	1.6 fold increase (*)
	HEPARIN	2 fold increase (*)	2 fold increase (*)
	A + H	3 fold increase ($)	2.3 fold increase (*)
2) PGC-1α	**AICAR**	1.2 fold increase (*)	-
	HEPARIN	N/S increase	-
	A + H	1.3 fold increase (*)	-
3) KSRP	**AICAR**	N/S decrease	N/S decrease
	HEPARIN	2 fold decrease ($)	2.3 fold decrease (*)
	A + H	1.6 fold decrease (*)	N/S decrease

- N/S = No significant difference compared to control (untreated mdx)
- Significant difference compared to control (untreated mdx), $ significant compared to all groups

Table 4.2: Pathological parameters after treatment in muscles of mdx mice

PROTEIN EXPRESSION	TREATMENTS	EFFECTS	
		DIAPHRAGM	TA
1) Central Nucleation	AICAR	40% decrease	40 % decrease
	HEPARIN	N/S decrease	N/S decrease
	A + H	40 % decrease	40 % decrease
2) Utrophin Immunostaining	AICAR	2.2 fold increase (*)	1.9 fold increase (*)
	HEPARIN	1.6 fold increase (*)	1.5 fold increase (*)
	A + H	2.6 fold increase (*)	1.9 fold increase (*)
3) β-dystroglycan Immunostaining	AICAR	Increase	Increase
	HEPARIN	Increase	Increase
	A + H	Increase	Increase
4) IgM staining	AICAR	Decrease	Decrease
	HEPARIN	Decrease	Decrease
	A + H	Decrease	Decrease
5) Fiber size Variability	AICAR	Decrease (*)	Decrease (*)
	HEPARIN	Decrease (*)	Decrease (*)
	A + H	Decrease (*)	Decrease (*)

4.5 Combinatorial treatment of AICAR and heparin: an effective therapy for DMD patients?

One of the central highlights of this study is the identification and exploitation of FDA approved drugs in DMD context. This strategy, termed as drug repositioning, exploits the off-target effects of various clinically approved drugs and offers many advantages such as quicker development times and reduced health risks (Ashburn and Thor, 2004). Successful examples of drug repositioning include anti-depressants like duloxetine. Initially developed to stimulate serotonin levels and relieve depression, duloxetine was later developed to treat both the SUI (Stress Urinary Inconsistency) and depression based on its off- target effects on the urinary sphincter (Ashburn and Thor, 2008). In light of this, previous studies from our laboratory and others have exploited the off-target effects of various drugs in stimulation of utrophin A expression in muscle (Miura et al, 2009; Gordon et al, 2013; Ljubicic et al, 2011; Ljubicic et al, 2014; Amirouche et al, 2013).

More specifically, our current study established that a combinatorial therapy of AICAR and heparin might serve as an effective therapeutic strategy to treat DMD. Heparin, an FDA approved drug, is being used to treat and prevent thrombosis for years (Gray et al, 2008). Further, studies have reported that since heparin is a naturally occurring compound in the human body, it is relatively safe to use with less severe side effects (Gray et al, 2008). On the other hand, AICAR is also being tested extensively in clinical trials for treatment of other diseases such as Type II diabetes (Boon et al, 2008; Babraj et al, 2009; Leick et al, 2010). Since heparin and AICAR have individually been tested in clinical trials on human

subjects, the combinatorial treatment of these drugs might progress faster through the drug development stages for use in DMD patients.

Despite the beneficial effects of the combinatorial treatment of AICAR and heparin on utrophin A expression and the dystrophic pathology in mice, it is important to be mindful of the limitations of this strategy in long-term therapies. Like any other drug, AICAR and heparin are associated with various side effects. Heparin has been shown to cause excessive bleeding, Heparin Induced Thrombocytopenia (HIT), skin necrosis and osteoporosis in treated patients. In contrast, AICAR is associated with liver and heart hypetrophy (Buhl et al, 2002). The combinatorial treatment of AICAR and heparin might increase the prevalence of the aforementioned side effects. Careful redevelopment of these pharmacological agents can help overcome some of these side effects. For instance, previous studies have suggested that chemical removal or blockage of the Anti-thrombin Binding Region (ATBR) of heparin might inhibit the anticoagulant activity and prevent excessive bleeding (Casu et al, 2014).

5.0 CONCLUSION AND FUTURE PERSPECTIVES:

In summary, our findings demonstrate that a combinatorial therapy involving two activators of utrophin A, AICAR and heparin, instigates an additive effect on its expression in muscle and ameliorates several pathological features in the preclinical mdx mouse model of DMD. Our results are particularly encouraging considering the fact that both AICAR and heparin are clinically approved drugs. This could help speed up the process of attaining clinical approval for their use in DMD patients.

Our findings established that the combinatorial treatment of AICAR and heparin resulted in ~2-3 fold induction in utrophin A protein levels in mdx mice. The effect of combinatorial treatment on utrophin A expression was significantly higher compared to individual AICAR or heparin treatments in the diaphragm muscle. Although not significant, a similar trend was observed in the TA muscles. These findings are promising since previous studies have reported that a 2-fold increase in utrophin A protein levels in mdx mice can ameliorate the dystrophic phenotype (Tinsley et al, 1998). The amount of utrophin A up-regulation that is sufficient to ameliorate the pathology in DMD patients remains elusive. However, clinical studies in DMD patients have demonstrated a positive correlation between modest increases in utrophin A expression and age at which these patients become wheelchair bound (Kleopa et al, 2006; D'Arcy et al, 2014). Therefore, the up-regulation of utrophin A expression as a result of the combinatorial treatment of AICAR and heparin can have beneficial effects in DMD patients. Another key finding of our study is the effective up-regulation of utrophin A expression in the diaphragm muscle in response to the combinatorial therapy. Diaphragm muscle in mdx mice is thought to be the most clinically

relevant muscle, as it closely resembles the severe pathology in human DMD patients. Studies have shown that as the disease progresses in mdx mice, the diaphragm muscle undergoes greater amount of degeneration and necrosis compared to the limb muscles (Grounds et al, 2009). Therefore, our current findings suggest that the combinatorial treatment of AICAR and heparin may be beneficial in the severe human DMD condition.

Finally, our study encourages the testing of other clinically approved drugs in the DMD context. Recent studies have reported that Celecoxib, a COX-2 inhibitor and anti-inflammatory drug, has been shown to activate p38 signaling in several cancer and muscle cell lines (Hsiao et al, 2007; Steffel et al, 2006). More recently, a study by Farooq et al. demonstrated that celcecoxib provided beneficial effects in Spinal Muscle Atrophy (SMA) mouse model. Further this study demonstrated that the effect of celecoxib is mediated through an RNA binding protein, HuR that binds to specific AREs in the target mRNAs and stabilizes them (Farooq et al, 2013). Since p38 activation by heparin has been linked with utrophin A expression by a previous study (Amirouche et al, 2013) from our laboratory, p38 activation by celecoxib may have a similar effect on utrophin A expression and thus it will be worthwhile to test this drug in the DMD condition. Another important drug in this context is calcitriol, a form of vitamin D, which has been shown to activate the p38 MAPK in C2C12 muscle cells (Buitrago et al, 2012). It will be worth researching the effects of the aforementioned drugs on utrophin A expression individually and in combination with other known activators of utrophin A expression in muscle to develop an effective therapeutic strategy for DMD.

6.0 REFERENCES:

Adams, M. E., Kramarcy, N., Krall, S. P., Rossi, S. G., Rotundo, R. L., Sealock, R. and Froehner, S. C. 2000. Absence of alpha-syntrophin leads to structurally aberrant neuromuscular synapses deficient in utrophin A. J. Cell Biol. 150: 1385 -1398.

Alderton JM, Steinhardt RA. How calcium influx through calcium leak channels is responsible for the elevated levels of calcium-dependent proteolysis in dystrophic myotubes. Trends Cardiovasc Med. 2000 Aug;10(6):268-72. Review.

Allikian MJ, McNally EM. Processing and assembly of the dystrophin glycoprotein complex. Traffic. 2007 Mar;8(3):177-83. Review.

Amat R, Planavila A, Chen SL, Iglesias R, Giralt M, Villarroya F. SIRT1 controls the transcription of the peroxisome proliferator-activated receptor-gamma Co-activator-1alpha (PGC-1alpha) gene in skeletal muscle through the PGC-1alpha autoregulatory loop and interaction with MyoD. J Biol Chem. 2009 Aug 14;284(33)

Amirouche A, Tadesse H, Lunde JA, Bélanger G, Côté J, Jasmin BJ. Activation of p38 signaling increases utrophin A expression in skeletal muscle via the RNA-binding protein KSRP and inhibition of AU-rich element-mediated mRNA decay: implications for novel DMD therapeutics. Hum Mol Genet. 2013 Aug 1;22(15)

Anderson MS, Kunkel LM. The molecular and biochemical basis of Duchenne muscular dystrophy. Trends Biochem Sci. 1992 Aug;17(8):289-92. Review.

Angus LM, Chakkalakal JV, Méjat A, Eibl JK, Bélanger G, Megeney LA, Chin ER, Schaeffer L, Michel RN, Jasmin BJ. Calcineurin-NFAT signaling, together with GABP and peroxisome PGC-1{alpha}, drives utrophin gene expression at the neuromuscular junction. Am J Physiol Cell Physiol. 2005 Oct;289(4)

Apponi LH, Corbett AH, Pavlath GK. RNA-binding proteins and gene regulation in myogenesis. Trends Pharmacol Sci. 2011 Nov;32(11):652-8.

Ashburn TT, Thor KB. Drug repositioning: identifying and developing new uses for existing drugs. Nat Rev Drug Discov. 2004 Aug;3(8):673-83. Review.

Babraj JA, Mustard K, Sutherland C, Towler MC, Chen S, Smith K, Green K, Leese G, Hardie DG, Rennie MJ, Cuthbertson DJ. Blunting of AICAR-induced human skeletal muscle glucose uptake in type 2 diabetes is dependent on age rather than diabetic status. Am J Physiol Endocrinol Metab. 2009 May;296(5)

Bakheet T, Williams BR, Khabar KS. ARED 2.0: an update of AU-rich element mRNA database. Nucleic Acids Res. 2003 Jan 1;31(1):421-3.

Balaban B, Matthews DJ, Clayton GH, Carry T. Corticosteroid treatment and functional improvement in Duchenne muscular dystrophy: long-term effect. Am J Phys Med Rehabil. 2005 Nov;84(11):843-50.

Barton ER, Morris L, Kawana M, Bish LT, Toursel T. Systemic administration of L-arginine benefits mdx skeletal muscle function. Muscle Nerve. 2005 Dec;32(6):751-60.

Basu U, Lozynska O, Moorwood C, Patel G, Wilton SD, Khurana TS. Translational regulation of utrophin by miRNAs. PLoS One. 2011;6(12)

Baur JA, Ungvari Z, Minor RK, Le Couteur DG, de Cabo R. Are sirtuins viable targets for improving healthspan and lifespan? Nat Rev Drug Discov. 2012 Jun 1;11(6):443-61.

Baxter P. Treatment of the heart in Duchenne muscular dystrophy. Dev Med Child Neurol. 2006 Mar;48(3):163.

Benchaouir R, Meregalli M, Farini A, D'Antona G, Belicchi M, Goyenvalle A, Battistelli M, Bresolin N, Bottinelli R, Garcia L, Torrente Y. Restoration of human dystrophin following transplantation of exon-skipping-engineered DMD patient stem cells into dystrophic mice. Cell Stem Cell. 2007 Dec 13;1(6):646-57. doi: 10.1016/j.stem.2007.09.016.

Bergeron R, Ren JM, Cadman KS, Moore IK, Perret P, Pypaert M, Young LH, Semenkovich CF, Shulman GI. Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. Am J Physiol Endocrinol Metab. 2001 Dec;281(6):E1340-6.

Bevilacqua A, Ceriani MC, Capaccioli S, Nicolin A. Post-transcriptional regulation of gene expression by degradation of messenger RNAs. J Cell Physiol. 2003 Jun;195(3):356-72. Review.

Blake DJ, Weir A, Newey SE, Davies KE. Function and genetics of dystrophin and dystrophin-related proteins in muscle. Physiol Rev. 2002 Apr;82(2):291-329. Review.

Bodensteiner JB, Engel AG. Intracellular calcium accumulation in Duchenne dystrophy and other myopathies: a study of 567,000 muscle fibers in 114 biopsies. Neurology. 1978 May;28(5):439-46.

Boon H, Bosselaar M, Praet SF, Blaak EE, Saris WH, Wagenmakers AJ, McGee SL, Tack CJ, Smits P, Hargreaves M, van Loon LJ. Intravenous AICAR administration reduces hepatic glucose output and inhibits whole body lipolysis in type 2 diabetic patients. Diabetologia. 2008 Oct;51(10):1893-900.

Brenman JE, Chao DS, Xia H, Aldape K, Bredt DS. Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. Cell. 1995 Sep 8;82(5):743-52.

Briata P, Forcales SV, Ponassi M, Corte G, Chen CY, Karin M, Puri PL, Gherzi R. p38-dependent phosphorylation of the mRNA decay-promoting factor KSRP controls the stability of select myogenic transcripts. Mol Cell. 2005 Dec 22;20(6):891-903.

Briguet A, Courdier-Fruh I, Foster M, Meier T, Magyar JP. Histological parameters for the quantitative assessment of muscular dystrophy in the mdx-mouse. Neuromuscul Disord. 2004 Oct;14(10):675-82.

Bruckbauer A, Zemel MB. Synergistic effects of metformin, resveratrol, and hydroxymethylbutyrate on insulin sensitivity. Diabetes Metab Syndr Obes. 2013;6:93-102.

Bueno Júnior CR, Pantaleão LC, Voltarelli VA, Bozi LH, Brum PC, Zatz M. Combined effect of AMPK/PPAR agonists and exercise training in mdx mice functional performance. PLoS One. 2012;7(9).

Buhl ES, Jessen N, Pold R, Ledet T, Flyvbjerg A, Pedersen SB, Pedersen O, Schmitz O, Lund S. Long-term AICAR administration reduces metabolic disturbances and lowers blood pressure in rats displaying features of the insulin resistance syndrome. Diabetes. 2002 Jul;51(7):2199-206.

Buitrago CG, Arango NS, Boland RL. 1α,25(OH)2D3-dependent modulation of Akt in proliferating and differentiating C2C12 skeletal muscle cells. J Cell Biochem. 2012 Apr;113(4):1170-81.

Bulfield G, Siller WG, Wight PA, Moore KJ. X chromosome-linked muscular dystrophy (mdx) in the mouse. Proc Natl Acad Sci U S A. 1984 Feb;81(4):1189-92.

Burton EA, Tinsley JM, Holzfeind PJ, Rodrigues NR, Davies KE. A second promoter provides an alternative target for therapeutic up-regulation of utrophin in Duchenne muscular dystrophy. Proc Natl Acad Sci U S A. 1999 Nov 23;96(24):14025-30.

Bushby K, Finkel R, Birnkrant DJ, Case LE, Clemens PR, Cripe L, Kaul A, Kinnett K, McDonald C, Pandya S, Poysky J, Shapiro F, Tomezsko J, Constantin C; DMD Care Considerations Working Group. Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. Lancet Neurol. 2010 Jan;9(1):77-93.

Bushby K, Finkel R, Birnkrant DJ, Case LE, Clemens PR, Cripe L, Kaul A, Kinnett K, McDonald C, Pandya S, Poysky J, Shapiro F, Tomezsko J, Constantin C; DMD Care Considerations Working Group. Diagnosis and management of Duchenne muscular dystrophy, part 2: implementation of multidisciplinary care. Lancet Neurol. 2010 Feb;9(2):177-89.

Buyue Y, Misenheimer TM, Sheehan JP. Low molecular weight heparin inhibits plasma thrombin generation via direct targeting of factor IXa: contribution of the serpin-independent mechanism. J Thromb Haemost. 2012 Oct;10(10):2086-98.

Cabrero A, Alegret M, Sánchez RM, Adzet T, Laguna JC, Vázquez M. Down-regulation of uncoupling protein-3 and -2 by thiazolidinediones in C2C12 myotubes. FEBS Lett. 2000 Oct 27;484(1):37-42.

Cantó C, Auwerx J. AMP-activated protein kinase and its downstream transcriptional pathways. Cell Mol Life Sci. 2010 Oct;67(20):3407-23.

Cantó C, Jiang LQ, Deshmukh AS, Mataki C, Coste A, Lagouge M, Zierath JR, Auwerx J. Interdependence of AMPK and SIRT1 for metabolic adaptation to fasting and exercise in skeletal muscle. Cell Metab. 2010 Mar 3;11(3):213-9.

Casu B, Naggi A, Torri G. Re-visiting the structure of heparin. Carbohydr Res. 2014 Jul 3.

Chakkalakal JV, Harrison MA, Carbonetto S, Chin E, Michel RN, Jasmin BJ. Stimulation of calcineurin signaling attenuates the dystrophic pathology in mdx mice. Hum Mol Genet. 2004 Feb 15;13(4):379-88.

Chakkalakal JV, Miura P, Bélanger G, Michel RN, Jasmin BJ. Modulation of utrophin A mRNA stability in fast versus slow muscles via an AU-rich element and calcineurin signaling. Nucleic Acids Res. 2008 Feb;36(3):826-38.

Chakkalakal JV, Stocksley MA, Harrison MA, Angus LM, Deschenes-Furry J, St-Pierre S, Megeney LA, Chin ER, Michel RN, Jasmin BJ. Expression of utrophin A mRNA correlates with the oxidative capacity of skeletal muscle fiber types and is regulated by calcineurin/NFAT signaling. Proc Natl Acad Sci U S A. 2003 Jun 24;100(13):7791-6.

Chargé SB, Rudnicki MA. Cellular and molecular regulation of muscle regeneration. Physiol Rev. 2004 Jan;84(1):209-38. Review.

Chaubourt E, Fossier P, Baux G, Leprince C, Israël M, De La Porte S. Nitric oxide and l-arginine cause an accumulation of utrophin at the sarcolemma: a possible compensation for dystrophin loss in Duchenne muscular dystrophy. Neurobiol Dis. 1999 Dec;6(6):499-507.

Chen CY, Gherzi R, Ong SE, Chan EL, Raijmakers R, Pruijn GJ, Stoecklin G, Moroni C, Mann M, Karin M. AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. Cell. 2001 Nov 16;107(4):451-64.

Choay J, Petitou M, Lormeau JC, Sinaÿ P, Casu B, Gatti G. Structure-activity relationship in heparin: a synthetic pentasaccharide with high affinity for antithrombin

III and eliciting high anti-factor Xa activity. Biochem Biophys Res Commun. 1983 Oct 31;116(2):492-9.

Clerk A, Morris GE, Dubowitz V, Davies KE, Sewry CA. Dystrophin-related protein, utrophin, in normal and dystrophic human fetal skeletal muscle. Histochem J. 1993 Aug;25(8):554-61.

Conrad, H. E. (1998) *Heparin-Binding Proteins* (Academic, San Diego).

Coulton GR, Morgan JE, Partridge TA, Sloper JC. The mdx mouse skeletal muscle myopathy: I. A histological, morphometric and biochemical investigation. Neuropathol Appl Neurobiol. 1988 Jan-Feb;14(1):53-70.

Costelli P, Reffo P, Penna F, Autelli R, Bonelli G, Baccino FM. Ca(2+)-dependent proteolysis in muscle wasting. Int J Biochem Cell Biol. 2005 Oct;37(10):2134-46. Review.

Crosbie RH, Lebakken CS, Holt KH, Venzke DP, Straub V, Lee JC, Grady RM, Chamberlain JS, Sanes JR, Campbell KP. Membrane targeting and stabilization of sarcospan is mediated by the sarcoglycan subcomplex. J Cell Biol. 1999 Apr 5;145(1):153-65.

D'Arcy CE, Feeney SJ, McLean CA, Gehrig SM, Lynch GS, Smith JE, Cowling BS, Mitchell CA, McGrath MJ. Identification of FHL1 as a therapeutic target for Duchenne muscular dystrophy. Hum Mol Genet. 2014 Feb 1;23(3):618-36.

Davies KE, Nowak KJ. Molecular mechanisms of muscular dystrophies: old and new players. Nat Rev Mol Cell Biol. 2006 Oct;7(10):762-73.

De la Porte S, Morin S, Koenig J. Characteristics of skeletal muscle in mdx mutant mice. Int Rev Cytol. 1999;191:99-148. Review.

Dennis CL, Tinsley JM, Deconinck AE, Davies KE. Molecular and functional analysis of the utrophin promoter. Nucleic Acids Res. 1996 May 1;24(9):1646-52.

Deol JR, Danialou G, Larochelle N, Bourget M, Moon JS, Liu AB, Gilbert R, Petrof BJ, Nalbantoglu J, Karpati G. Successful compensation for dystrophin deficiency by a helper-dependent adenovirus expressing full-length utrophin. Mol Ther. 2007 Oct;15(10):1767-74.

Eagle M, Bourke J, Bullock R, Gibson M, Mehta J, Giddings D, Straub V, Bushby K. Managing Duchenne muscular dystrophy--the additive effect of spinal surgery and home nocturnal ventilation in improving survival. Neuromuscul Disord. 2007 Jun;17(6):470-5.

Ehmsen J, Poon E, Davies K. The dystrophin-associated protein complex. J Cell Sci. 2002 Jul 15;115(Pt 14):2801-3. Review.

Ehrenborg E, Krook A. Regulation of skeletal muscle physiology and metabolism by peroxisome proliferator-activated receptor delta. Pharmacol Rev. 2009 Sep;61(3):373-93. doi: 10.1124/pr.109.001560. Review

Emery AE. Population frequencies of inherited neuromuscular diseases--a world survey. Neuromuscul Disord. 1991;1(1):19-29. Review.

Emery AE. The muscular dystrophies. Lancet. 2002 Feb 23;359(9307):687-95. Review.

Emery AEH & Muntoni F (2003). *Duchenne Muscular Dystrophy*. Oxford University Press, Oxford.

Ervasti JM, Campbell KP. A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. J Cell Biol. 1993 Aug;122(4):809-23.

Fairclough RJ, Bareja A, Davies KE. Progress in therapy for Duchenne muscular dystrophy. Exp Physiol. 2011 Nov;96(11):1101-13.

Farooq F, Abadía-Molina F, MacKenzie D, Hadwen J, Shamim F, O'Reilly S, Holcik M, MacKenzie A. Celecoxib increases SMN and survival in a severe spinal muscular atrophy mouse model via p38 pathway activation. Hum Mol Genet. 2013 Sep 1;22(17):3415-24.

Fei J, Cook C, Santanam N. ω-6 lipids regulate PPAR turnover via reciprocal switch between PGC-1 alpha and ubiquitination. Atherosclerosis. 2012 Jun;222(2):395-401.

Fillmore N, Jacobs DL, Mills DB, Winder WW, Hancock CR. Chronic AMP-activated protein kinase activation and a high-fat diet have an additive effect on mitochondria in rat skeletal muscle. J Appl Physiol (1985). 2010 Aug;109(2):511-20.

Finsterer J, Stöllberger C. The heart in human dystrophinopathies. Cardiology. 2003;99(1):1-19. Review.

Fisher R, Tinsley JM, Phelps SR, Squire SE, Townsend ER, Martin JE, Davies KE. Non-toxic ubiquitous over-expression of utrophin in the mdx mouse. Neuromuscul Disord. 2001 Nov;11(8):713-21.

Florence JM, Fox PT, Planer GJ, Brooke MH. Activity, creatine kinase, and myoglobin in Duchenne muscular dystrophy: a clue to etiology? Neurology. 1985 May;35(5):758-61.

Foster H, Popplewell L, Dickson G. Genetic therapeutic approaches for Duchenne muscular dystrophy. Hum Gene Ther. 2012 Jul;23(7):676-87.

Fruchart JC, Duriez P, Staels B. [Molecular mechanism of action of the fibrates]. J Soc Biol. 1999;193(1):67-75. Review. French.

Galvagni F, Capo S, Oliviero S. Sp1 and Sp3 physically interact and co-operate with GABP for the activation of the utrophin promoter. J Mol Biol. 2001 Mar 9;306(5):985-96.

Garcia-Roves PM, Osler ME, Holmström MH, Zierath JR. Gain-of-function R225Q mutation in AMP-activated protein kinase gamma3 subunit increases mitochondrial biogenesis in glycolytic skeletal muscle. J Biol Chem. 2008 Dec 19;283(51):35724-34.

Gaudel C, Schwartz C, Giordano C, Abumrad NA, Grimaldi PA. Pharmacological activation of PPARbeta promotes rapid and calcineurin-dependent fiber remodeling and angiogenesis in mouse skeletal muscle. Am J Physiol Endocrinol Metab. 2008 Aug;295(2):E297-304. doi: 10.1152/ajpendo.00581.2007.

Gerhart-Hines Z, Rodgers JT, Bare O, Lerin C, Kim SH, Mostoslavsky R, Alt FW, Wu Z, Puigserver P. Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha. EMBO J. 2007 Apr 4;26(7):1913-23.

Gherzi R, Lee KY, Briata P, Wegmüller D, Moroni C, Karin M, Chen CY. A KH domain RNA binding protein, KSRP, promotes ARE-directed mRNA turnover by recruiting the degradation machinery. Mol Cell. 2004 Jun 4;14(5):571-83.

Gingerich TJ, Feige JJ, LaMarre J. AU-rich elements and the control of gene expression through regulated mRNA stability. Anim Health Res Rev. 2004 Jun;5(1):49-63. Review.

Gordon BS, Delgado Díaz DC, Kostek MC. Resveratrol decreases inflammation and increases utrophin gene expression in the mdx mouse model of Duchenne muscular dystrophy. Clin Nutr. 2013 Feb;32(1):104-11.

Gowers WR (1892). *A manual of diseases of the nervous system*. J & A Churchill, London.

Goyenvalle A, Seto JT, Davies KE, Chamberlain J. Therapeutic approaches to muscular dystrophy. Hum Mol Genet. 2011 Apr 15;20(R1):R69-78.

Gramolini AO, Angus LM, Schaeffer L, Burton EA, Tinsley JM, Davies KE, Changeux JP, Jasmin BJ. Induction of utrophin gene expression by heregulin in skeletal muscle cells: role of the N-box motif and GA binding protein. Proc Natl Acad Sci U S A. 1999 Mar 16;96(6):3223-7.

Gramolini AO, Bélanger G, Thompson JM, Chakkalakal JV, Jasmin BJ. Increased expression of utrophin in a slow vs. a fast muscle involves posttranscriptional events. Am J Physiol Cell Physiol. 2001 Oct;281(4):C1300-9.

Gramolini AO, Dennis CL, Tinsley JM, Robertson GS, Cartaud J, Davies KE, Jasmin BJ. Local transcriptional control of utrophin expression at the neuromuscular synapse. J Biol Chem. 1997 Mar 28;272(13):8117-20.

Gramolini AO, Jasmin BJ. Expression of the utrophin gene during myogenic differentiation. Nucleic Acids Res. 1999 Sep 1;27(17):3603-9.

Gray E, Mulloy B, Barrowcliffe TW. Heparin and low-molecular-weight heparin. Thromb Haemost. 2008 May;99(5):807-18. doi: 10.1160/TH08-01-0032. Review.

Gregorevic P, Blankinship MJ, Allen JM, Chamberlain JS. Systemic microdystrophin gene delivery improves skeletal muscle structure and function in old dystrophic mdx mice. Mol Ther. 2008 Apr;16(4):657-64.

Griggs RC, Herr BE, Reha A, Elfring G, Atkinson L, Cwik V, McColl E, Tawil R, Pandya S, McDermott MP, Bushby K. Corticosteroids in Duchenne muscular dystrophy: major variations in practice. Muscle Nerve. 2013 Jul;48(1):27-31.

Grounds MD, Radley HG, Lynch GS, Nagaraju K, De Luca A. Towards developing standard operating procedures for pre-clinical testing in the mdx mouse model of Duchenne muscular dystrophy. Neurobiol Dis. 2008 Jul;31(1):1-19.

Grum VL, Li D, MacDonald RI, Mondragón A. Structures of two repeats of spectrin suggest models of flexibility. Cell. 1999 Aug 20;98(4):523-35.

Güell MR, Avendano M, Fraser J, Goldstein R. [Pulmonary and nonpulmonary alterations in Duchenne muscular dystrophy]. Arch Bronconeumol. 2007 Oct;43(10):557-61. Spanish.

Gussoni E, Pavlath GK, Lanctot AM, Sharma KR, Miller RG, Steinman L & Blau HM. 1992. Normal dystrophin transcripts detected in Duchenne muscular dystrophy patients after myoblast transplantation. Nature. 356: 435–438.

Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, Kunkel LM, Mulligan RC. Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature. 1999 Sep 23;401(6751):390-4.

Handschin C, Kobayashi YM, Chin S, Seale P, Campbell KP, Spiegelman BM. PGC-1alpha regulates the neuromuscular junction program and ameliorates Duchenne muscular dystrophy. Genes Dev. 2007 Apr 1;21(7):770-83.

Handschin C, Rhee J, Lin J, Tarr PT, Spiegelman BM. An autoregulatory loop controls peroxisome proliferator-activated receptor gamma coactivator 1alpha expression in muscle. Proc Natl Acad Sci U S A. 2003 Jun 10;100(12):7111-6.

Hirsh J, Warkentin TE, Shaughnessy SG, Anand SS, Halperin JL, Raschke R, Granger C, Ohman EM, Dalen JE. Heparin and low-molecular-weight heparin: mechanisms of action, pharmacokinetics, dosing, monitoring, efficacy, and safety. Chest. 2001 Jan;119(1 Suppl):64S-94S. Review.

Hock MB, Kralli A. Transcriptional control of mitochondrial biogenesis and function. Annu Rev Physiol. 2009;71:177-203. doi: 10.1146/annurev.physiol.010908.163119. Review.

Hofer A, Noe N, Tischner C, Kladt N, Lellek V, Schauß A, Wenz T. Defining the action spectrum of potential PGC-1α activators on a mitochondrial and cellular level in vivo. Hum Mol Genet. 2014 May 1;23(9):2400-15. doi: 10.1093/hmg/ddt631.

Hoffman EP, Fischbeck KH, Brown RH, Johnson M, Medori R, Loike JD, Harris JB, Waterston R, Brooke M, Specht L, et al. Characterization of dystrophin in muscle-biopsy specimens from patients with Duchenne's or Becker's muscular dystrophy. N Engl J Med. 1988 May 26;318(21):1363-8.

Hsiao PW, Chang CC, Liu HF, Tsai CM, Chiu TH, Chao JI. Activation of p38 mitogen-activated protein kinase by celecoxib oppositely regulates survivin and gamma-H2AX in human colorectal cancer cells. Toxicol Appl Pharmacol. 2007 Jul 1;222(1):97-104.

Ishikawa-Sakurai M, Yoshida M, Imamura M, Davies KE, Ozawa E. ZZ domain is essentially required for the physiological binding of dystrophin and utrophin to beta-dystroglycan. Hum Mol Genet. 2004 Apr 1;13(7):693-702.

Jäger S, Handschin C, St-Pierre J, Spiegelman BM. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. Proc Natl Acad Sci U S A. 2007 Jul 17;104(29):12017-22.

Jahnke VE, Van Der Meulen JH, Johnston HK, Ghimbovschi S, Partridge T, Hoffman EP, Nagaraju K. Metabolic remodeling agents show beneficial effects in the dystrophin-deficient mdx mouse model. Skelet Muscle. 2012 Aug 21;2(1):16.

Jørgensen SB, Jensen TE, Richter EA. Role of AMPK in skeletal muscle gene adaptation in relation to exercise. Appl Physiol Nutr Metab. 2007 Oct;32(5):904-11. Review.

Jung D, Yang B, Meyer J, Chamberlain JS, Campbell KP. Identification and characterization of the dystrophin anchoring site on beta-dystroglycan. J Biol Chem. 1995 Nov 10;270(45):27305-10.

Kameya, S., Miyagoe, Y., Nonaka, I., Ikemoto, T., Endo, M., Hanaoka, K., Nabeshima, Y. and Takeda, S. 1999. Alpha1-syntrophin gene disruption results in the absence of neuronal-type nitric-oxide synthase at the sarcolemma but does not induce muscle degeneration. J. Biol. Chem. 274: 2193 -2200.

Karpati G, Pouliot Y, Zubrzycka-Gaarn E, Carpenter S, Ray PN, Worton RG, Holland P. Dystrophin is expressed in mdx skeletal muscle fibers after normal myoblast implantation. Am J Pathol. 1989 Jul;135(1):27-32.

Khurana T.S., Watkins S.C., Chafey P., Chelly J., Tomé F.M., Fardeau M., Kaplan J.C., Kunkel L.M. 1991. Immunolocalization and developmental expression of dystrophin related protein in skeletal muscle. Neuromuscul. Disord. 1: 185-194.

Khurana TS, Rosmarin AG, Shang J, Krag TO, Das S, Gammeltoft S. Activation of utrophin promoter by heregulin via the ets-related transcription factor complex GA-binding protein alpha/beta. Mol Biol Cell. 1999 Jun;10(6):2075-86.

Kleopa KA, Drousiotou A, Mavrikiou E, Ormiston A, Kyriakides T. Naturally occurring utrophin correlates with disease severity in Duchenne muscular dystrophy. Hum Mol Genet. 2006 May 15;15(10):1623-8.

Koenig M, Beggs AH, Moyer M, Scherpf S, Heindrich K, Bettecken T, Meng G, Müller CR, Lindlöf M, Kaariainen H, et al. The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion. Am J Hum Genet. 1989 Oct;45(4):498-506.

Koenig M, Monaco AP, Kunkel LM. The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. Cell. 1988 Apr 22;53(2):219-28.

Korenbaum E, Rivero F. Calponin homology domains at a glance. J Cell Sci. 2002 Sep 15;115(Pt 18):3543-5. Review. Erratum in: J Cell Sci. 2002 Nov 15;115(pt 22):4387.

Krag TO, Bogdanovich S, Jensen CJ, Fischer MD, Hansen-Schwartz J, Javazon EH, Flake AW, Edvinsson L, Khurana TS. Heregulin ameliorates the dystrophic phenotype in mdx mice. Proc Natl Acad Sci U S A. 2004 Sep 21;101(38):13856-60.

Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, Messadeq N, Milne J, Lambert P, Elliott P, Geny B, Laakso M, Puigserver P, Auwerx J. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell. 2006 Dec 15;127(6):1109-22.

Law PK, Goodwin TG, Fang Q, Deering MB, Duggirala V, Larkin C, Florendo JA, Kirby DS, Li HJ, Chen M, et al. Cell transplantation as an experimental treatment for Duchenne muscular dystrophy. Cell Transplant. 1993 Nov-Dec;2(6):485-505.

Leick L., Fentz J., Biensø R.S., Knudsen J.G., Jeppesen J., Kiens B., Wojtaszewski J.F., Pilegaard H. 2010. PGC-1α is required for AICAR-induced expression of GLUT4 and mitochondrial proteins in mouse skeletal muscle. Am. J. Physiol. Endocrinol. Metab. 299: E456-E465.

Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R, Spiegelman BM. Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. Nature. 2002 Aug 15;418(6899):797-801.

Lira VA, Benton CR, Yan Z, Bonen A. PGC-1alpha regulation by exercise training and its influences on muscle function and insulin sensitivity. Am J Physiol Endocrinol Metab. 2010 Aug;299(2):E145-61.

Ljubicic V, Burt M, Jasmin BJ. The therapeutic potential of skeletal muscle plasticity in Duchenne muscular dystrophy: phenotypic modifiers as pharmacologic targets. FASEB J. 2014 Feb;28(2):548-68.

Ljubicic V, Burt M, Lunde JA, Jasmin BJ. Resveratrol induces expression of the slow, oxidative phenotype in mdx mouse muscle together with enhanced activity of the SIRT1-PGC-1α axis. Am J Physiol Cell Physiol. 2014 Jul 1;307(1):C66-82.

Ljubicic V, Jasmin BJ. AMP-activated protein kinase at the nexus of therapeutic skeletal muscle plasticity in Duchenne muscular dystrophy. Trends Mol Med. 2013 Oct;19(10):614-24.

Ljubicic V, Khogali S, Renaud JM, Jasmin BJ. Chronic AMPK stimulation attenuates adaptive signaling in dystrophic skeletal muscle. Am J Physiol Cell Physiol. 2012 Jan 1;302(1):C110-21.

Ljubicic V, Miura P, Burt M, Boudreault L, Khogali S, Lunde JA, Renaud JM, Jasmin BJ. Chronic AMPK activation evokes the slow, oxidative myogenic program and triggers beneficial adaptations in mdx mouse skeletal muscle. Hum Mol Genet. 2011 Sep 1;20(17):3478-93.

Long YC, Zierath JR. AMP-activated protein kinase signaling in metabolic regulation. J Clin Invest. 2006 Jul;116(7):1776-83. Review.

Love DR, Forrest SM, Smith TJ, England S, Flint T, Davies KE, Speer A. Molecular analysis of Duchenne and Becker muscular dystrophies. Br Med Bull. 1989 Jul;45(3):659-80. Review.

Manzur AY, Kuntzer T, Pike M, Swan A. Glucocorticoid corticosteroids for Duchenne muscular dystrophy. Cochrane Database Syst Rev. 2008 Jan 23;(1):CD003725.

Manuvakhova M, Keeling K & Bedwell DM. 2000. Aminoglycoside antibiotics mediate context-dependent suppression of termination codons in a mammalian translation system. RNA: 6: 1044–1055.

Matoulkova E, Michalova E, Vojtesek B, Hrstka R. The role of the 3' untranslated region in post-transcriptional regulation of protein expression in mammalian cells. RNA Biol. 2012 May;9(5):563-76.

Mendell JR, Kissel JT, Amato AA, King W, Signore L, Prior TW, Sahenk Z, Benson S, McAndrew PE, Rice R, Nagaraja H, Stephens R, Lantry L, Morris GE & Burghes AHM. 1995. Myoblast transfer in the treatment of Duchenne's muscular dystrophy. N Engl J Med. 333: 832–838.

Mendell JR, Campbell K, Rodino-Klapac L, Sahenk Z, Shilling C, Lewis S, Bowles D, Gray S, Li C, Galloway G, Malik V, Coley B, Clark KR, Li J, Xiao X, Samulski J, McPhee SW, Samulski RJ, Walker CM. Dystrophin immunity in Duchenne's muscular dystrophy. N Engl J Med. 2010 Oct 7;363(15):1429-37.

Menke A, Jockusch H. Decreased osmotic stability of dystrophin-less muscle cells from the mdx mouse. Nature. 1991 Jan 3;349(6304):69-71.

Meregalli M, Farini A, Parolini D, Maciotta S, Torrente Y. Stem cell therapies to treat muscular dystrophy: progress to date. BioDrugs. 2010 Aug 1;24(4):237-47.

Merrill GF, Kurth EJ, Hardie DG, Winder WW. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am J Physiol. 1997 Dec;273(6 Pt 1):E1107-12.

Minetti GC, Colussi C, Adami R, Serra C, Mozzetta C, Parente V, Fortuni S, Straino S, Sampaolesi M, Di Padova M, Illi B, Gallinari P, Steinkühler C, Capogrossi MC, Sartorelli V, Bottinelli R, Gaetano C, Puri PL. Functional and morphological recovery of dystrophic muscles in mice treated with deacetylase inhibitors. Nat Med. 2006 Oct;12(10):1147-50.

Miura P., Chakkalakal J. V., Boudreault L., Bélanger G., Hébert R. L., Renaud J.-M., Jasmin B. J. (2009) Pharmacological activation of PPARbeta/delta stimulates utrophin A expression in skeletal muscle fibers and restores sarcolemmal integrity in mature mdx mice. Hum. Mol. Genet. 18, 4640–4649.

Miura P, Coriati A, Bélanger G, De Repentigny Y, Lee J, Kothary R, Holcik M, Jasmin BJ. The utrophin A 5'-UTR drives cap-independent translation exclusively in skeletal muscles of transgenic mice and interacts with eEF1A2. Hum Mol Genet. 2010 Apr 1;19(7):1211-20.

Miura P, Jasmin BJ. Utrophin upregulation for treating Duchenne or Becker muscular dystrophy: how close are we? Trends Mol Med. 2006 Mar;12(3):122-9.

Miura P, Thompson J, Chakkalakal JV, Holcik M, Jasmin BJ. The utrophin A 5'-untranslated region confers internal ribosome entry site-mediated translational control during regeneration of skeletal muscle fibers. J Biol Chem. 2005 Sep 23;280(38):32997-3005.

Mizuno Y, Nonaka I, Hirai S, Ozawa E. Reciprocal expression of dystrophin and utrophin in muscles of Duchenne muscular dystrophy patients, female DMD-carriers and control subjects. J Neurol Sci. 1993 Oct;119(1):43-52.

Moens P, Baatsen PH, Maréchal G. Increased susceptibility of EDL muscles from mdx mice to damage induced by contractions with stretch. J Muscle Res Cell Motil. 1993 Aug;14(4):446-51.

Monaco AP, Bertelson CJ, Liechti-Gallati S, Moser H, Kunkel LM. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. Genomics. 1988 Jan;2(1):90-5.

Moorwood C, Khurana TS. Duchenne muscular dystrophy drug discovery - the application of utrophin promoter activation screening. Expert Opin Drug Discov. 2013 May;8(5):569-81.

Moorwood C, Lozynska O, Suri N, Napper AD, Diamond SL, Khurana TS. Drug discovery for Duchenne muscular dystrophy via utrophin promoter activation screening. PLoS One. 2011;6(10):e26169.

Moorwood C, Soni N, Patel G, Wilton SD, Khurana TS. A cell-based high-throughput screening assay for posttranscriptional utrophin upregulation. J Biomol Screen. 2013 Apr;18(4):400-6.

Morrison J, Lu QL, Pastoret C, Partridge T, Bou-Gharios G. T-cell-dependent fibrosis in the mdx dystrophic mouse. Lab Invest. 2000 Jun;80(6):881-91.

Moxley RT 3rd, Ashwal S, Pandya S, Connolly A, Florence J, Mathews K, Baumbach L, McDonald C, Sussman M, Wade C; Quality Standards Subcommittee of the American Academy of Neurology; Practice Committee of the Child Neurology Society. Practice parameter: corticosteroid treatment of Duchenne dystrophy: report of the Quality Standards Subcommittee of the American Academy of Neurology and the Practice Committee of the Child Neurology Society. Neurology. 2005 Jan 11;64(1):13-20.

Muntoni F, Torelli S, Ferlini A. Dystrophin and mutations: one gene, several proteins, multiple phenotypes. Lancet Neurol. 2003 Dec;2(12):731-40. Review.

Narita S, Yorifuji H. Centrally nucleated fibers (CNFs) compensate the fragility of myofibers in mdx mouse. Neuroreport. 1999 Oct 19;10(15):3233-5.

Narkar VA, Downes M, Yu RT, Embler E, Wang YX, Banayo E, Mihaylova MM, Nelson MC, Zou Y, Juguilon H, Kang H, Shaw RJ, Evans RM. AMPK and PPARdelta agonists are exercise mimetics. Cell. 2008 Aug 8;134(3):405-15.

Nemoto S, Fergusson MM, Finkel T. Nutrient availability regulates SIRT1 through a forkhead-dependent pathway. Science. 2004 Dec 17;306(5704):2105-8.

Odom GL, Gregorevic P, Allen JM, Finn E, Chamberlain JS. Microutrophin delivery through rAAV6 increases lifespan and improves muscle function in dystrophic dystrophin/utrophin-deficient mice. Mol Ther. 2008 Sep;16(9):1539-45.

Ohira Y, Matsuoka Y, Kawano F, Ogura A, Higo Y, Ohira T, Terada M, Oke Y, Nakai N. Effects of creatine and its analog, β-guanidinopropionic acid, on the differentiation of and nucleoli in myoblasts. Biosci Biotechnol Biochem. 2011;75(6):1085-9.

Olson EN, Williams RS. Calcineurin signaling and muscle remodeling. Cell. 2000 Jun 23;101(7):689-92. Review.

Ouyang J, Parakhia RA, Ochs RS. Metformin activates AMP kinase through inhibition of AMP deaminase. J Biol Chem. 2011 Jan 7;286(1):1-11.

Partridge TA, Morgan JE, Coulton GR, Hoffman EP, Kunkel LM. Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. Nature. 1989 Jan 12;337(6203):176-9.

Partridge TA. Invited review: myoblast transfer: a possible therapy for inherited myopathies? Muscle Nerve. 1991 Mar;14(3):197-212. Review.

Pasquini F, Guerin C, Blake D, Davies K, Karpati G, Holland P. The effect of glucocorticoids on the accumulation of utrophin by cultured normal and dystrophic human skeletal muscle satellite cells. Neuromuscul Disord. 1995 Mar;5(2):105-14.

Perkins KJ, Burton EA, Davies KE. The role of basal and myogenic factors in the transcriptional activation of utrophin promoter A: implications for therapeutic up-regulation in Duchenne muscular dystrophy. Nucleic Acids Res. 2001 Dec 1;29(23):4843-50.

Perkins KJ, Davies KE. The role of utrophin in the potential therapy of Duchenne muscular dystrophy. Neuromuscul Disord. 2002 Oct;12 Suppl 1:S78-89. Review.

Peters MF, Sadoulet-Puccio HM, Grady MR, Kramarcy NR, Kunkel LM, Sanes JR, Sealock R, Froehner SC. Differential membrane localization and intermolecular associations of alpha-dystrobrevin isoforms in skeletal muscle. J Cell Biol. 1998 Sep 7;142(5):1269-78.

Pichavant C, Chapdelaine P, Cerri DG, Bizario JC, Tremblay JP. Electrotransfer of the full-length dog dystrophin into mouse and dystrophic dog muscles. Hum Gene Ther. 2010 Nov;21(11):1591-601. doi: 10.1089/hum.2010.024.

Rafael JA, Tinsley JM, Potter AC, Deconinck AE, Davies KE. Skeletal muscle-specific expression of a utrophin transgene rescues utrophin-dystrophin deficient mice. Nat Genet. 1998 May;19(1):79-82.

Ren JM, Ohira Y, Holloszy JO, Hämäläinen N, Traub I, Pette D. Effects of beta-guanidinopropionic acid-feeding on the patterns of myosin isoforms in rat fast-twitch muscle. Pflugers Arch. 1995 Jul;430(3):389-93.

Rivero JL, Serrano AL. Skeletal myosin heavy chain composition and carriage training. Equine Vet J Suppl. 1999 Jul;(30):318-23.

Röckl KS, Hirshman MF, Brandauer J, Fujii N, Witters LA, Goodyear LJ. Skeletal muscle adaptation to exercise training: AMP-activated protein kinase mediates muscle fiber type shift. Diabetes. 2007 Aug;56(8):2062-9.

Rodova M, Brownback K, Werle MJ. Okadaic acid augments utrophin in myogenic cells. Neurosci Lett. 2004 Jun 10;363(2):163-7.

Rosenberg MI, Georges SA, Asawachaicharn A, Analau E, Tapscott SJ. MyoD inhibits Fstl1 and Utrn expression by inducing transcription of miR-206. J Cell Biol. 2006 Oct 9;175(1):77-85.

Ross J. mRNA stability in mammalian cells. Microbiol Rev. 1995 Sep;59(3):423-50. Review.

Sampaolesi M, Blot S, D'Antona G, Granger N, Tonlorenzi R, Innocenzi A, Mognol P, Thibaud JL, Galvez BG, Barthélémy I, Perani L, Mantero S, Guttinger M, Pansarasa O, Rinaldi C, Cusella De Angelis MG, Torrente Y, Bordignon C, Bottinelli R, Cossu G. Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. Nature. 2006 Nov 30;444(7119):574-9..

Sander M, Chavoshan B, Harris SA, Iannaccone ST, Stull JT, Thomas GD, Victor RG. Functional muscle ischemia in neuronal nitric oxide synthase-deficient skeletal muscle of children with Duchenne muscular dystrophy. Proc Natl Acad Sci U S A. 2000 Dec 5;97(25):13818-23.

Scarpulla RC. Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiol Rev. 2008 Apr;88(2):611-38.

Scarpulla RC, Vega RB, Kelly DP. Transcriptional integration of mitochondrial biogenesis. Trends Endocrinol Metab. 2012 Sep;23(9):459-66.

Shriver Z, Sundaram M, Venkataraman G, Fareed J, Linhardt R, Biemann K, Sasisekharan R. Cleavage of the antithrombin III binding site in heparin by heparinases and its implication in the generation of low molecular weight heparin. Proc Natl Acad Sci U S A. 2000 Sep 12;97(19):10365-70.

Sicinski P, Geng Y, Ryder-Cook AS, Barnard EA, Darlison MG, Barnard PJ. The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. Science. 1989 Jun 30;244(4912):1578-80.

Squire S, Raymackers JM, Vandebrouck C, Potter A, Tinsley J, Fisher R, Gillis JM, Davies KE. Prevention of pathology in mdx mice by expression of utrophin: analysis

using an inducible transgenic expression system. Hum Mol Genet. 2002 Dec 15;11(26):3333-44.

Stamler JS, Meissner G. Physiology of nitric oxide in skeletal muscle. Physiol Rev. 2001 Jan;81(1):209-237.

Steffel J, Akhmedov A, Fähndrich C, Ruschitzka F, Lüscher TF, Tanner FC. Differential effect of celecoxib on tissue factor expression in human endothelial and vascular smooth muscle cells. Biochem Biophys Res Commun. 2006 Oct 20;349(2):597-603.

Stocksley MA, Chakkalakal JV, Bradford A, Miura P, De Repentigny Y, Kothary R, Jasmin BJ. A 1.3 kb promoter fragment confers spatial and temporal expression of utrophin A mRNA in mouse skeletal muscle fibers. Neuromuscul Disord. 2005 Jun;15(6):437-49.

Straub V, Rafael JA, Chamberlain JS, Campbell KP. Animal models for muscular dystrophy show different patterns of sarcolemmal disruption. J Cell Biol. 1997 Oct 20;139(2):375-85.

Suh HN, Huong HT, Song CH, Lee JH, Han HJ. Linoleic acid stimulates gluconeogenesis via Ca2+/PLC, cPLA2, and PPAR pathways through GPR40 in primary cultured chicken hepatocytes. Am J Physiol Cell Physiol. 2008 Dec;295(6):C1518-27.

Suwa M, Egashira T, Nakano H, Sasaki H, Kumagai S. Metformin increases the PGC-1alpha protein and oxidative enzyme activities possibly via AMPK phosphorylation in skeletal muscle in vivo. J Appl Physiol (1985). 2006 Dec;101(6):1685-92.

T.A. Rando. 2001. The dystrophin–glycoprotein complex, cellular signaling, and the regulation of cell survival in the muscular dystrophies. Muscle and Nerve. 24: 1575–1594.

Tenenbaum A, Fisman EZ. Balanced pan-PPAR activator bezafibrate in combination with statin: comprehensive lipids control and diabetes prevention? Cardiovasc Diabetol. 2012 Nov 14;11:140. doi: 10.1186/1475-2840-11-140. Review.

Teyssier C, Ma H, Emter R, Kralli A, Stallcup MR. Activation of nuclear receptor coactivator PGC-1alpha by arginine methylation. Genes Dev. 2005 Jun 15;19(12):1466-73.

Tidball JG1, Wehling-Henricks M. 2005. Damage and inflammation in muscular dystrophy: potential implications and relationships with autoimmune myositis. Curr Opin Rheumatol. 17(6): 707-13.

Tidball JG, Wehling-Henricks M. Expression of a NOS transgene in dystrophin-deficient muscle reduces muscle membrane damage without increasing the expression of membrane-associated cytoskeletal proteins. Mol Genet Metab. 2004 Aug;82(4):312-20.

Tinsley J, Deconinck N, Fisher R, Kahn D, Phelps S, Gillis JM, Davies K. Expression of full-length utrophin prevents muscular dystrophy in mdx mice. Nat Med. 1998 Dec;4(12):1441-4.

Tinsley JM, Blake DJ, Roche A, Fairbrother U, Riss J, Byth BC, Knight AE, Kendrick-Jones J, Suthers GK, Love DR, et al. Primary structure of dystrophin-related protein. Nature. 1992 Dec 10;360(6404):591-3.

Tinsley JM, Fairclough RJ, Storer R, Wilkes FJ, Potter AC, Squire SE, Powell DS, Cozzoli A, Capogrosso RF, Lambert A, Wilson FX, Wren SP, De Luca A, Davies KE. Daily treatment with SMTC1100, a novel small molecule utrophin upregulator, dramatically reduces the dystrophic symptoms in the mdx mouse. PLoS One. 2011 May 6;6(5).

Tinsley JM, Potter AC, Phelps SR, Fisher R, Trickett JI, Davies KE. Amelioration of the dystrophic phenotype of mdx mice using a truncated utrophin transgene. Nature. 1996 Nov 28;384(6607):349-53.

Torrente Y, Belicchi M, Sampaolesi M, Pisati F, Meregalli M, D'Antona G, Tonlorenzi R, Porretti L, Gavina M, Mamchaoui K, Pellegrino MA, Furling D, Mouly V, Butler-Browne GS, Bottinelli R, Cossu G, Bresolin N. Human circulating AC133(+) stem cells restore dystrophin expression and ameliorate function in dystrophic skeletal muscle. J Clin Invest. 2004 Jul;114(2):182-95.

Torres LF, Duchen LW. The mutant mdx: inherited myopathy in the mouse. Morphological studies of nerves, muscles and end-plates. Brain. 1987 Apr;110 (Pt 2):269-99.

Trabucchi M, Briata P, Garcia-Mayoral M, Haase AD, Filipowicz W, Ramos A, Gherzi R, Rosenfeld MG. The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. Nature. 2009 Jun 18;459(7249):1010-4.

Vianello S, Bouyon S, Benoit E, Sebrié C, Boerio D, Herbin M, Roulot M, Fromes Y, de la Porte S. Arginine butyrate per os protects mdx mice against cardiomyopathy, kyphosis and changes in axonal excitability. Neurobiol Dis. 2014 Aug 26;71C:325-333.

Vianello S, Consolaro F, Bich C, Cancela JM, Roulot M, Lanchec E, Touboul D, Brunelle A, Israël M, Benoit E, de la Porte S. Low doses of arginine butyrate derivatives improve dystrophic phenotype and restore membrane integrity in DMD models. FASEB J. 2014 Jun;28(6):2603-19.

Vianello S, Yu H, Voisin V, Haddad H, He X, Foutz AS, Sebrié C, Gillet B, Roulot M, Fougerousse F, Perronnet C, Vaillend C, Matecki S, Escolar D, Bossi L, Israël M, de la Porte S. Arginine butyrate: a therapeutic candidate for Duchenne muscular dystrophy. FASEB J. 2013 Jun;27(6):2256-69.

Voisin V, Sébrié C, Matecki S, Yu H, Gillet B, Ramonatxo M, Israël M, De la Porte S. L-arginine improves dystrophic phenotype in mdx mice. Neurobiol Dis. 2005 Oct;20(1):123-30.

Wang, B., J. Li, and X. Xiao, Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in mdx mouse model. Proc Natl Acad Sci U S A, 2000. **97**(25): p. 13714-9.

Wang YX, Zhang CL, Yu RT, Cho HK, Nelson MC, Bayuga-Ocampo CR, Ham J, Kang H, Evans RM. Regulation of muscle fiber type and running endurance by PPARdelta. PLoS Biol. 2004 Oct;2(10):e294.

Webster C, Silberstein L, Hays AP, Blau HM. Fast muscle fibers are preferentially affected in Duchenne muscular dystrophy. Cell. 1988 Feb 26;52(4):503-13.

Weir AP, Burton EA, Harrod G, Davies KE. A- and B-utrophin have different expression patterns and are differentially up-regulated in mdx muscle. J Biol Chem. 2002 Nov 22;277(47):45285-90..

Welch EM, Barton ER, Zhuo J, Tomizawa Y, Friesen WJ, Trifillis P, Paushkin S, Patel M, Trotta CR, Hwang S, Wilde RG, Karp G, Takasugi J, Chen G, Jones S, Ren H, Moon YC, Corson D, Turpoff AA, Campbell JA, Conn MM, Khan A, Almstead NG, Hedrick J, Mollin A, Risher N, Weetall M, Yeh S, Branstrom AA, Colacino JM, Babiak J, Ju WD, Hirawat S, Northcutt VJ, Miller LL, Spatrick P, He F, Kawana M, Feng H, Jacobson A, Peltz SW, Sweeney HL. PTC124 targets genetic disorders caused by nonsense mutations. Nature. 2007 May 3;447(7140):87-91.

Weller B, Karpati G, Carpenter S. Dystrophin-deficient mdx muscle fibers are preferentially vulnerable to necrosis induced by experimental lengthening contractions. J Neurol Sci. 1990 Dec;100(1-2):9-13.

Wenz T, Diaz F, Spiegelman BM, Moraes CT. Activation of the PPAR/PGC-1alpha pathway prevents a bioenergetic deficit and effectively improves a mitochondrial myopathy phenotype. Cell Metab. 2008 Sep;8(3):249-56.

Wenz T. PGC-1alpha activation as a therapeutic approach in mitochondrial disease. IUBMB Life. 2009 Nov;61(11):1051-62.

Wenzel E, Somoza V. Metabolism and bioavailability of trans-resveratrol. Mol Nutr Food Res. 2005 May;49(5):472-81. Review.

Williams DB, Sutherland LN, Bomhof MR, Basaraba SA, Thrush AB, Dyck DJ, Field CJ, Wright DC. Muscle-specific differences in the response of mitochondrial proteins to beta-GPA feeding: an evaluation of potential mechanisms. Am J Physiol Endocrinol Metab. 2009 Jun;296(6).

Winder SJ, Hemmings L, Maciver SK, Bolton SJ, Tinsley JM, Davies KE, Critchley DR, Kendrick-Jones J. Utrophin actin binding domain: analysis of actin binding and cellular targeting. J Cell Sci. 1995 Jan;108 (Pt 1):63-71.

Winder WW, Holmes BF, Rubink DS, Jensen EB, Chen M, Holloszy JO. Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle. J Appl Physiol (1985). 2000 Jun;88(6):2219-26.

Winder, W.W., Can patients with type 2 diabetes be treated with 5'-AMP-activated protein kinase activators? Diabetologia, 2008. **51**(10): p. 1761-4.

Worton RG, Thompson MW. Genetics of Duchenne muscular dystrophy. Annu Rev Genet. 1988;22:601-29. Review.

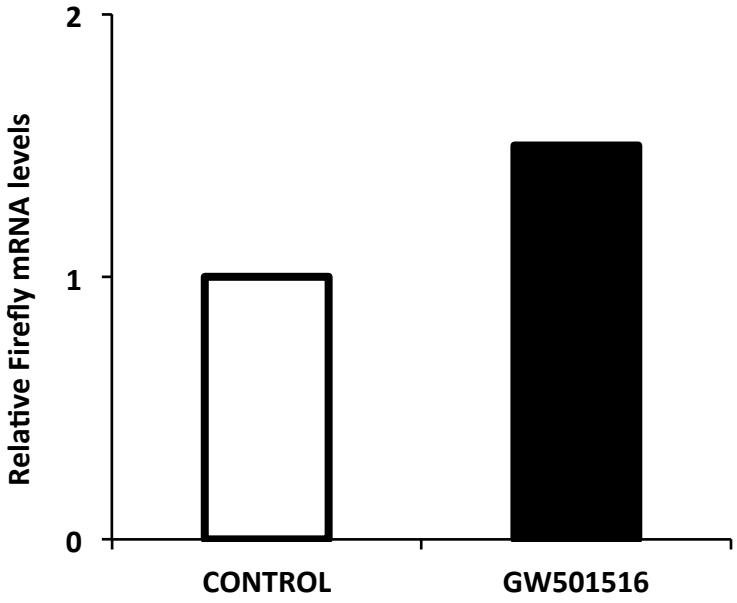
Xu HE, Lambert MH, Montana VG, Parks DJ, Blanchard SG, Brown PJ, Sternbach DD, Lehmann JM, Wisely GB, Willson TM, Kliewer SA, Milburn MV. Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. Mol Cell. 1999 Mar;3(3):397-403.

Yang B, Jung D, Rafael JA, Chamberlain JS, Campbell KP. Identification of alpha-syntrophin binding to syntrophin triplet, dystrophin, and utrophin. J Biol Chem. 1995 Mar 10;270(10):4975-8.

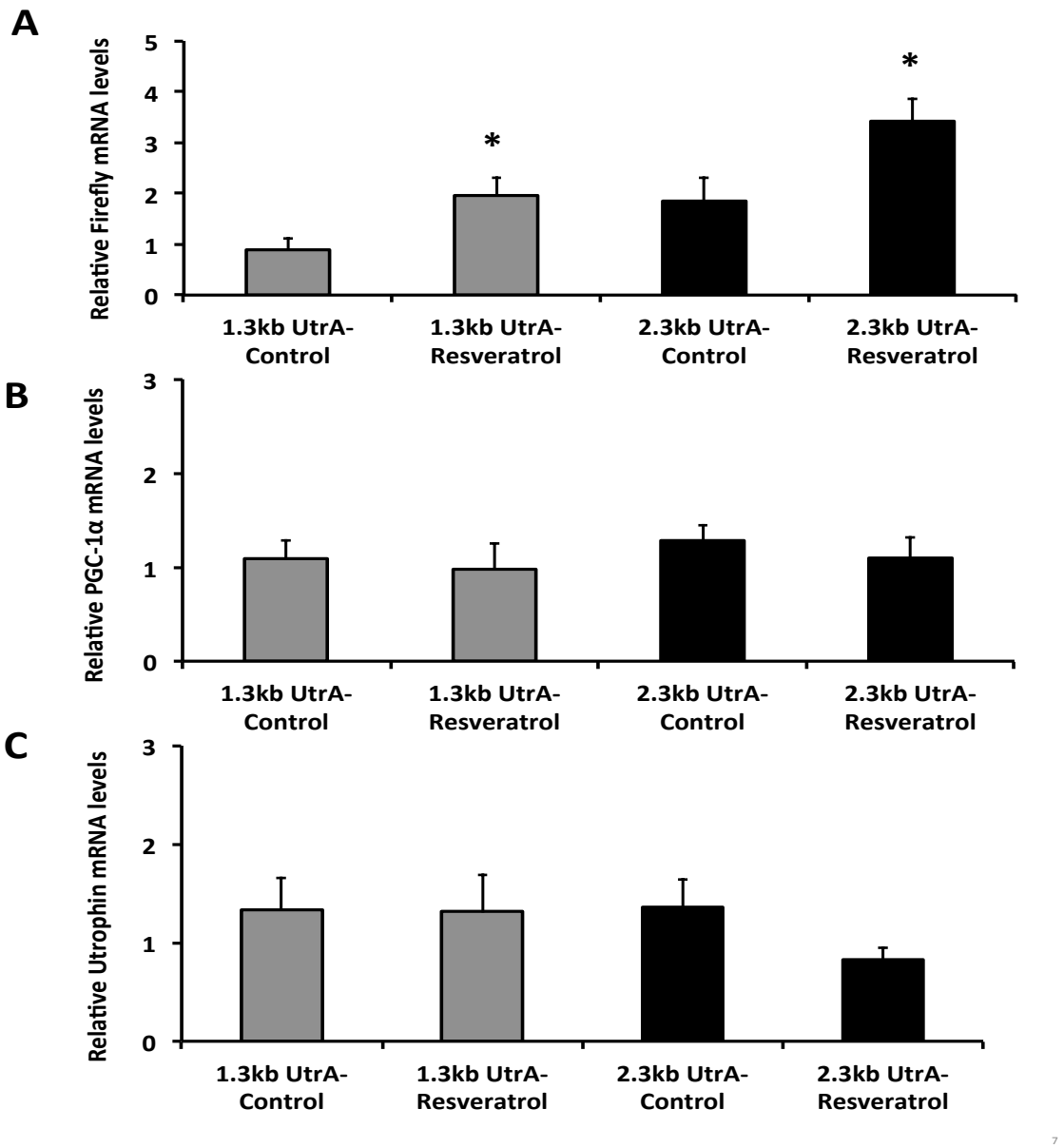
Zbinden-Foncea H, Deldicque L, Pierre N, Francaux M, Raymackers JM. TLR2 and TLR4 activation induces p38 MAPK-dependent phosphorylation of S6 kinase 1 in C2C12 myotubes. Cell Biol Int. 2012;36(12):1107-13.

Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum MJ, Shulman GI. AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. Proc Natl Acad Sci U S A. 2002 Dec 10;99(25):15983-7

7.0 APPENDICES:



Appendix Figure 7.1: The effect of GW501516 on utrophin A expression in untransfected C2C12 muscle cells. When untransfected C2C12 cells are treated with 1uM GW501516 for 24 hours there is ~ 1.5 fold increase in utrophin A promoter activity as indicated by the luciferase reporter levels (Firefly). These results are in accordance with earlier by Miura et al, 2009. The Firefly mRNA levels were measured by quantitative RT-PCR. Values are standardized to 18S mRNA levels (n=1, 3 replicates each). Refer to Page 51 for details.



Appendix Figure 7.2: The effect of SIRT-1 activator, resveratrol, on utrophin A expression in C2C12 muscle cells transfected with the 2.3kb or 1.3kb human utrophin A promoter fragment. C2C12 myoblasts transfected with a 1.3kb or 2.3kb fragment of human utrophin A promoter- luciferase reporter construct were treated for 24 hours with 50uM resveratrol or Control (DMSO). Resveratrol treatment resulted in an increase in A) Firefly (reporter) mRNA levels and had no significant effect on B) PGC-1α mRNA or C) Utrophin A mRNA levels as measured by quantitative RT-PCR. Values are standardized to 18S mRNA levels (n=4, * = $p<0.05$). Mean ± S.E.M are shown. Refer to page 56 for details.