THE ROLE OF AMPK IN THE EXPRESSION OF THE DAPC

THE ROLE OF AMPK IN THE EXPRESSION OF THE DYSTROPHIN-ASSOCIATED PROTEIN COMPLEX IN SKELETAL MUSCLE

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Lay Abstract

The dystrophin-associated protein complex (DAPC) connects the interior and exterior of muscle cells. Activation of AMP-activated protein kinase (AMPK) increases the expression of the DAPC in skeletal muscle. We sought to determine whether AMPK was necessary for DAPC expression in skeletal muscle. Fast and slow muscles from normal mice, as well as from those deficient in skeletal muscle AMPK (MKO) were analyzed. We found DAPC levels and localization were similar between both groups, with the exception of nNOS, which was enriched at the muscle membrane in MKO muscles. Regulators of the DAPC were also not affected by the loss of AMPK. However, genes important for the production of muscle were significantly diminished in MKO muscles. Furthermore, we observed decrements in utrophin at the muscle membrane selectively in slow MKO muscles. Our work indicates that AMPK is not essential for the DAPC expression in skeletal muscle, however it is required for preserving utrophin levels in slow, oxidative muscles.

Abstract

The dystrophin-associated protein complex (DAPC) provides a mechanical link between the intracellular cytoskeleton and extracellular matrix, serving as a mechanosensor and signal transducer across the sarcolemma. Pharmacological stimulation of AMP-activated protein kinase (AMPK) induces the expression of DAPC components in skeletal muscle, whereas physiological reductions in AMPK are associated with DAPC dysfunction. We sought to determine whether AMPK was necessary for the maintenance of DAPC expression in skeletal muscle. Fast glycolytic extensor digitorum longus (EDL) and slow oxidative soleus (SOL) muscles from wild-type (WT) mice, as well as from littermates deficient in both isoforms of the AMPK-β subunit in skeletal muscle (MKO) were analyzed. DAPC mRNA levels, as well as protein expression and localization were similar between genotypes, with the exception of nNOS, which displayed a compensatory sarcolemmal enrichment in MKO muscles. The content of transcriptional and post-transcriptional regulators of the DAPC, such as PGC-1α and KSRP, were also not affected by the loss of AMPK. However, MyoD and myogenin expression was significantly diminished in MKO muscles, which is consistent with previous reports of myopathy in these animals. Furthermore, we observed decrements in extrasynaptic utrophin expression selectively in MKO SOL muscles, despite an adaptive accumulation of PGC-1α at the sarcolemmal

compartment. Collectively the evidence indicates that AMPK is sufficient, but not essential for the maintenance of DAPC expression in skeletal muscle. However, AMPK is required for preserving extrasynaptic utrophin levels in slow, oxidative muscles, which underscores the role of AMPK in the gene expression of this disease modifying protein.

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TABLE OF CONTENTS

Acknowledgements v

List of Figures vii

List of Abbreviations viii

Declaration of Academic Achievement xi

Review of the Literature 1

1. Introduction to Skeletal Muscle 2
    i) Structure and function 2
    ii) Skeletal muscle fiber types 2
    iii) Benefits of the slow, oxidative myofiber program 3
2. The Dystrophin-Associated Protein Complex. 4
    i) Structure and function of the DAPC 6
    ii) Expression patterns of the DAPC in different fiber types 8
    iii) Protection of slow fibers in neuromuscular disorders 8
3. AMPK in Metabolic Regulation 11
    i) AMPK subunit expression 11
    ii) AMPK structure and regulation 12
    iii) AMPK function 13
4. AMPK in Skeletal Muscle Remodelling 16
    i) AMPK in regeneration 16
    ii) AMPK drives the slow, oxidative myofiber program 17

5. Regulation of the DAPC 24

i) Transcriptional Regulation of the DAPC 24

ii) Post-transcriptional regulation of the DAPC 28

iii) Translational and post-translational regulation of the DAPC 32

6. Study Objectives 35

7. References 37

Manuscript 66

1. Abstract 68

2. Introduction 64

3. Methods 71

4. Results 79

5. Discussion 86

6. References 96

7. Figure Legends 107

8. Figures 111

List of Figures/Tables

Figures from Review of the Literature

Figure 1. The dystrophin-associated protein complex 5

Figure 2. Potential AMPK-mediated regulation of the DAPC 22

Figures from Manuscript

Figure 1. Subcellular localization of AMP-activated protein kinase in skeletal muscle 111

Figure 2.. DAPC transcript levels in skeletal muscle 112

Figure 3. DAPC protein content in skeletal muscle**.** 113

Figure 4. DAPC localization in skeletal muscle**.** 114

Figure 5. Utrophin localization in skeletal muscle**.** 115

Figure 6. Regulatory factors that control DAPC expression in skeletal muscle**.** 116

Figure 7.. Subcellular localization in skeletal muscle 117

Figure 8. RNA-binding proteins involved in DAPC mRNA stability**.** 118

List of Abbreviations

ACC – acetyl-CoA carboxylase

AICAR – 5'-aminoimidazole-4-carboxamide-1-β-D ribofuranoside

AMPK – 5' adenosine monophosphate-activated protein kinase

ARE – AU-rich element

AUF1 – AU-rich binding factor 1

Ca^{2+} – calcium

CD36 – cluster of differentiation 36

CTX – cardiotoxin

CUG-binding protein 1 – CUGBP1

DAPC – dystrophin-associated protein complex

DG – dystroglycan

dKO – double knockout

DM1 – myotonic dystrophy type 1

DM2 – myotonic dystrophy type 2

DMD – Duchenne muscular dystrophy

E-box – enhancer box

EDL – extensor digitorum longus

F-actin – filamentous actin

GABP – GA-binding protein

GLUT4 – glucose transporter type 4

HDAC – histone deacetylase

HuR – human antigen R

IRES – internal ribosomal entry site

kDa – kilodalton

KSRP – KH-type splicing regulatory protein

LKB1 – liver kinase B1

MBNL – muscleblind-like protein

MEF – myocyte enhancer factor

MHC – myosin heavy chain

MRF – myogenic regulatory factor

mTOR – mammalian target of rapamycin

Na^+-K^+-ATPase – sodium-potassium-ATPase

NMJ – neuromuscular junction

nNOS – neuronal nitric oxide synthase

NO – nitric oxide

PDZ - postsynaptic density-95/discs large/zona occludens-1 homology

PGC-1α – peroxisome proliferator activated receptor gamma coactivator 1-alpha

RBP – RNA-binding protein

RSV – resveratrol

SG – sarcoglycan

SIRT1 – silent mating type information regulator 2 homolog 1

SOL – soleus

TBC1D - Tre-2/BUB2/cdc 1 domain

UTR – untranslated region

Declaration of Academic Achievement

AGD was the principal contributor. JSL, ALB and GRS provided the MKO tissues. PR assisted with some analyses. VL assisted with conceiving and designing the study, as well as with writing the manuscript.

Review of the Literature

1. Introduction to Skeletal Muscle

i) Structure and function

Skeletal muscle is one of the largest organ systems in our body, and constitutes approximately 40% of human body mass[1]. Muscle is responsible for posture and locomotion, as well as other important functions in health and disease, such as glucose and fat metabolism[2]. This organ is composed of multinucleated cells called myofibers or myocytes. Within the myofibers are sarcomeres, the basic functional units in the cell, which provide the apparatus for contraction. Sarcomeres consist of the contractile filaments actin and myosin, which are interwoven most densely towards the center and less densely towards the ends of each sarcomere, giving the appearance of dark and light striations, respectively. The border at each end of the sarcomere is called the z-line, and contains structural intermediate filaments that run perpendicular to the contractile filaments to provide stability for the unit. The intermediate filaments extend beyond the sarcomere towards the sarcolemma in a chain of proteins containing desmin, filamentous actin (F-actin), and dystrophin, finally terminating at the dystrophin- and integrin-associated protein complexes. These dynamic structural chains create a means of lateral force transmission and stability for the myofiber[3],

ii) Skeletal muscle fiber types

There are two major versions of the myosin heavy chain (MHC) isoform contained in myofibers, which are characterized by their speed of contractility.

They are the slow-twitch type I, and the fast-twitch type II. Fast-twitch fibers can further be classified into type IIA and IIX fibers in humans, and type IIA, IIX and IIB in rodents[2]. There are many additional components that contribute to the unique characteristics of each fiber type. Type I fibers have an oxidative profile, possessing high mitochondrial content, capillary density and sensitivity to insulin[4,5]. Type IIA fibers are fast-twitch, but contain a similar oxidative profile to type I fibers. Meanwhile, IIX/B fibers are fast-twitch and contain a glycolytic profile, with less mitochondrial content and capillary density. Skeletal muscles possess varying proportions of these fiber types. For example, in mice the distribution of type I, IIA, IIX and IIB in the extensor digitorum longus (EDL) muscle is < 1% type I, ~8% IIA, ~22% IIX and ~66% IIB, while the soleus (SOL) muscle is ~37% I, ~56% IIA, ~6% IIX and 0% IIB[6].

iii) Benefits of the slow, oxidative myofiber program

Skeletal muscle fiber type can influence the resistance to certain neuromuscular diseases and myodegenerative disorders. For example, patients with Duchenne muscular dystrophy (DMD) exhibit quicker myofiber degeneration and death in type II fibers, while type I fibers are more resistant to the dystrophic pathology[7,8]. Similarly, myotonic dystrophy type 2 (DM2) patients demonstrate exacerbated atrophy in type II fibers[9]. Likewise, the loss of skeletal muscle mass and strength due to aging is characterized by greater atrophy of type II muscle fibers[10]. In contrast, patients with myotonic dystrophy type I (DM1)

display greater myofiber atrophy in type I, as compared to type II muscle fibers[9]. Additionally, muscle disuse in humans has been reported to cause more pronounced muscle wasting in type I muscle fibers[11,12]. The precise mechanism that creates resilience to the dystrophic pathology in slower, more oxidative muscles is unknown. However, numerous factors that are disparate between fiber types, such as the differences in intracellular calcium dynamics, sarcolemmal protein composition, contractile apparatus, molecular signalling infrastructure, as well as oxygen utilization and redox characteristics could all contribute to this physiological paradigm[13–17].

2. The Dystrophin-Associated Protein Complex

The dystrophin-associated protein complex (DAPC) is a scaffold of proteins along the sarcolemma of muscle cells. The DAPC links the intracellular cytoskeleton with the extracellular matrix, thus making the complex integral to structural stability and integrity, signalling and mechanotransduction, and force transmission[18,19]. The DAPC can be subdivided into multiple subcomplexes and components on the subsarcolemmal, transmembrane and extramembrane aspects of skeletal muscle.

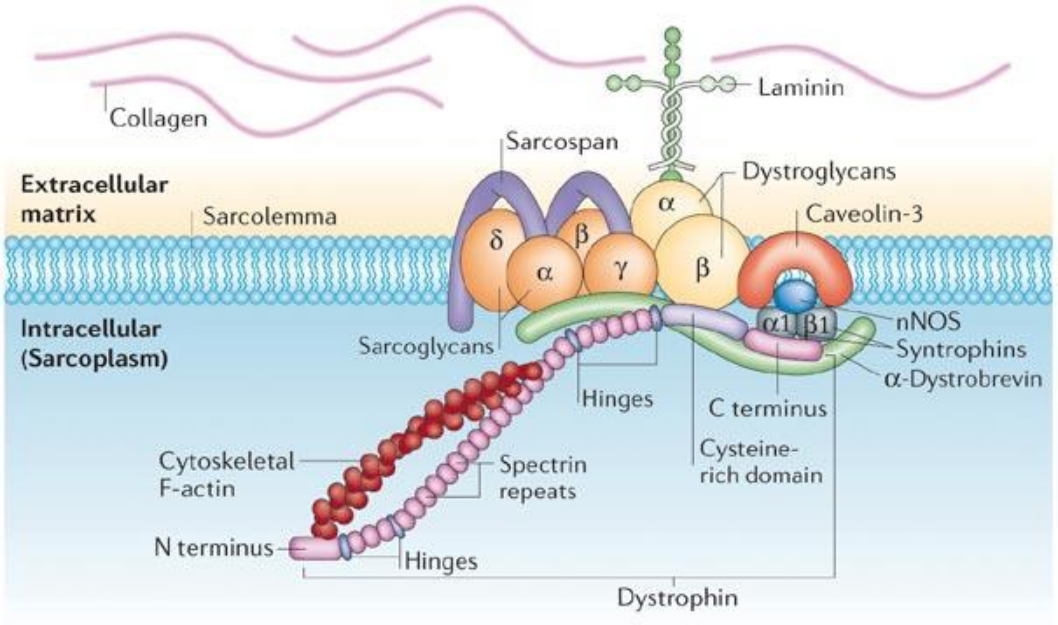

Figure 1. The dystrophin-associated protein complex (DAPC). Dystrophin binds to the dystrophin-associated protein complex (DAPC) at the sarcolemma through its C terminus. The DAPC is comprised of sarcoplasmic transmembrane and extracellular proteins. The N terminus of dystrophin binds to the cytoskeleton through F-actin. Therefore, the DAPC provides a strong mechanical link between the intracellular cytoskeleton and the extracellular matrix, acting also as a mechanosensor and signal transducer across the sarcolemma. Figure adapted from Davies K. E. & Nowak K. J. *Nat. Rev. Mol. Cell Bio.* **(7),** 762-773 (2006)

i) *Structure and function of the DAPC*

Dystrophin is a cytoskeletal protein transcribed by the DMD gene, which is defective in Duchenne Muscular Dystrophy (DMD). Dystrophin has several tissue specific promoters, including in the brain , muscle , and purkinje cells, reflecting the tissue distribution of dystrophin expression[20]. The muscle promoter drives high levels of expression of the largest dystrophin isoform (427 kDa) in striated and cardiac muscle[21]. Dystrophin contains a N-terminal acting binding domain, adjacent to a central rod domain containing spectrin-like repeats, and a cysteine rich C-terminus that allows for assembly of the DAPC[22]. Interestingly, most of the 24 rod-like repeat regions appear to be dispensable, as a dystrophin molecule with as little as 8 repeats maintains normal function[23]. The structure of these repeat regions in concert with the N-terminal actin binding sites allow dystrophin to act as a "molecular shock abosorber"[24] and is responsible for properly transmitting forces between the cytoskeleton and the sarcolemma during muscle contraction[3,25].

Along with dystrophin, the core of the DAPC is made up by the heavily glycosylated dystroglycans (DG). Together, the transmembrane β-DG and extrasarcolemmal α-DG are able to link dystrophin to laminin-2 (α2,β1,γ1), and thus connect the complex to the basement membrane. Both DGs are produced from the same post-translationally modified peptide, the removal of which is

embryonically-lethal[26]. Indeed, the lack of any naturally occurring mutations suggests that DG function is indispensable for survival.

The sarcoglycan (SG) subcomplex is adjacent to the dystroglycans and consists of five members: α-, β-, γ-, δ-, and ϵ-SG. γ- and δ-SG appear to be linked most closely to dystrophin and β-DG, respectively[27]. Mutations causing removal of one sarcoglycan causes loss of the others and results in a dystrophic phenotype known as limb girdle muscular dystrophy (LGMD). The four types of LGMD correspond to mutations of one of α-, β-, γ-, or δ-SG[28,29].

Also on the subsarcolemmal aspect of the DAPC is neuronal nitric oxide synthase (nNOS). nNOS exists in 4 protein variants, nNOSα , nNOSβ, nNOSγ, and nNOSμ, in non-vascular tissue[30]. nNOSα and nNOSμ contain a PDZ (postsynaptic density-95/discs large/zona occludens-1 homology) domain which allows binding of the enzyme to the cell membrane, allowing for quick activation due to calcium influx. These two variants exhibit the highest catalytic activity. However nNOSμ is the predominantly expressed form in differentiated skeletal muscle and is bound to the DAPC[18,31,32]. Meanwhile, nNOSβ and nNOSγ, lacking a PDZ domain, are cytosolic enzymes and are less enzymatically active variants[33]. nNOS produces nitric oxide (NO) at the muscle membrane, thereby allowing local vasodilation in skeletal muscle during contractile activity. nNOS is joined to dystrophin through its adjacent connection with α1 and β1 syntrophin. Each of the syntrophins contain two pleckstrin homology domains, which are found in many

signalling proteins. The syntrophins act as modular adaptors that recruit signalling proteins to the DAPC, including nNOS, Na^+-K^+-ATPase channels, and serine/threonine kinases[34] such as stress-activated protein kinase-3[35]. Thus, these proteins exhibit the bimodal role of the DAPC as both a structural scaffold and signalling apparatus vital to maintaining healthy myofibers. There are other components of the DAPC such as sarcospan, α-dystrobrevins, syncoilins, and calveolin-3. However, the structure and function of these molecules are beyond the scope of this review and have been detailed elsewhere[18,19,36].

ii) *Expression patterns of the DAPC in different fiber types*

For years it has been known that slower, more oxidative muscle fibers in DMD patients are more resistant to the dystrophic pathology than faster, glycolytic fibers[7]. Presumably, the elevated expression of DAPC in slow oxidative muscles confers this protective benefit to muscles exhibiting more slow-twitch, oxidative fiber[37]. This pattern remains true in the *mdx* mouse model of DMD[38]. The exact regulatory mechanisms which promote elevated DAPC expression are unknown, yet are presumably linked to the oxidative phenotype and/or those that maintain characteristics of continuous/tonic contractile activity [13–16].

iii) *Protection of slow fibers in neuromuscular disorders*

In addition to elevated DAPC levels, it is hypothesized that the primary cause of the heightened protection in slower, more oxidative muscles is their

enhanced utrophin expression, which demonstrates a greater extrasynaptic abundance along the sarcolemma[39–41]. Utrophin is an endogenous structural and functional homologue of dystrophin[24]. The main difference between utrophin and dystrophin is their contrasting expression pattern. While dystrophin is expressed along the length of the sarcolemma, utrophin A is confined to the myotendinous and neuromuscular junctions[42]. Its transgenic overexpression in muscle rescues dystrophin-null mice[43], while dystrophin-utrophin double knockout (dKO) mice present a much more severe phenotype and limited lifespan[44,45]. Interestingly, pharmacological activation of slower, more oxidative characteristics, includes increased utrophin expression and enhanced protection against contraction-induced damage[46,47]. Indeed, AMPK activation to dKO animals caused the slow, oxidative phenotype transition in the absence of any functional improvements[47]. These studies strongly suggest that utrophin is an important component to the protection afforded dystrophic skeletal muscle in the slower, more oxidative myocellular environment.

Although there is currently no cure for the DAPC deficiency observed in numerous muscular dystrophies and myopathies, recent pre-clinical work has attempted to leverage the benefits of the slow, oxidative myofiber phenotype through genetic or pharmacological interventions. PGC-1α is a powerful mediator of muscle plasticity and a promoter of the slow, oxidative myofiber program in skeletal muscle[48,49]. Elevated expression of PGC-1α in skeletal muscle of mdx

mice resulted in a greater proportion of slower fiber types, an improved histological profile, and elevated mitochondrial biogenesis in addition to augmented utrophin expression levels[50,51]. Another pharmacological simulator of the slow, oxidative muscle phenotype is the naturally occurring polyphenol resveratrol (RSV). RSV elicits the slow, oxidative myogenic program partly by stimulating silent mating type information regulator 2 homolog 1 (SIRT1)[52,53]. Chronic administration of RSV has been shown to improve skeletal muscle histology, increase the mRNA levels of utrophin, SIRT1, and PGC-1a, as well as the expression of slow MHC isoforms in *mdx* mice[54,55]. Together these studies are important proof-of-principle that the slow, oxidative myofiber program can confer dystrophic resistance to skeletal muscles, and this protective benefit can be induced by therapeutically available compounds.

5' AMP-activated protein kinase (AMPK) is a critical regulator of muscle metabolism and phenotype. AMPK plays an important role in shifting towards a slower oxidative phenotype, through phosphorylation-mediated changes in the expression and/or activity of proteins associated with slower, more oxidative muscle fibers. Recent evidence points towards the beneficial effects of AMPK activation in dystrophic muscle, including elevation of DAPC components and utrophin, concomitant with a shift towards the slow oxidative phenotype[16,46,47,56,57]. Together, these investigations reveal the numerous benefits

of the slow oxidative muscle phenotype, and the potential therapeutic benefit of promoting this myofiber program in neuromuscular disorders, such as DMD.

3. AMPK in Metabolic Regulation

i) *AMPK subunit expression*

Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric molecule, possessing catalytic α, scaffolding β, and regulatory γ subunits. Each AMPK subunit has multiple isoforms (i.e., α1, α2, β1, β2, γ1, γ2 and γ3) leading to a variety of possible αβγ heterotrimers. This complexity of AMPK expression is compounded by the fact that genes for AMPK isoforms exist on 5 separate chromosomes[58–60]. AMPK subunits also show differential expression in different tissues. α1 is uniformly expressed through multiple tissues including heart, liver, kidney, spleen and skeletal muscle. α2 is highly abundant in skeletal muscle and to a lesser degree, heart and liver, with only small amounts being detectable in other tissues[58,61,62]. β1 subunits show similar ubiquity in expression as α1 subunits, but are preferentially expressed in skeletal muscle and heart[62]. Similar to α1 and β1, γ1 is ubiquitously expressed. γ2 expression is highest in the brain, and is expressed to a lesser extent in skeletal muscle. γ3 has the most restricted expression, being largely confined to skeletal muscle[63]. Some evidence suggests that γ3 is expressed only in type IIb fast-twitch fibers, with no expression in the slow oxidative soleus which expresses type I fibers[64]. Type IIa

fast-twitch fibers display intermediate levels of γ3[65]. In skeletal muscle, the α2,β2,γ1 AMPK complex is the most common, comprising ~70% of AMPK expression. Moreover, the isoforms composing the α2,β2,γ1 complex make up the vast majority of AMPK subunits in muscle human and rodent tissue[59].

ii) *AMPK structure and regulation*

The α subunit of AMPK includes a N-terminal kinase domain that is imperative for the catalytic function of an enzyme. The β subunit contains a glycogen binding domain, as well as a motif known as the αγ subunit interacting domain (αγ-SID). The αγ-SID is responsible for binding both the α and γ subunits. For this reason the β subunit is imperative to the formation of functional αβγ heterotrimers[58–60]. During times of energy stress, cellular adenosine diphosphate (ADP) and adenosine monophosphate (AMP) concentrations rise. ADP and AMP replace sites on the γ subunit that normally bind ATP. Replacement of ATP by ADP/AMP at these sites promotes phosphorylation of the threonine-172 (Thr172) residue of the α subunit via upstream kinases, thereby increasing AMPK activity by almost 100-fold[66]. The major upstream kinase pathways in mammals are the liver kinase B1 – STE20 related adaptor – Mouse protein 25 (LKB1-STRAD-MO25) complex[67–69] and Ca^{2+}/calmodulin activated protein kinase kinase β (CaMKKβ)[70–72]. The LKB1-STRAD-MO25 complex provides high amounts of phosphorylation at threonine-172 (Thr172) in response to elevated AMP in the cell[73], while the latter pathway triggers activation of AMPK in response to

increases in Ca^{2+} levels and activates AMPK independent of cellular AMP levels[67,74].

iii) *AMPK function*

Inhibition of anabolic metabolism. Via its phosphorylation of downstream targets, AMPK is known as a master regulator of cellular energy homeostasis. Indeed, the canonical function of AMPK is two-fold in response to cellular energy stress. First, when the energy status of the cell is low, AMPK attenuates cellular anabolic processes such as the synthesis of fatty acids, proteins and gluconeogenic substrates. Secondly, AMPK initiates catabolic pathways in order to ensure proper energy provision and utilization. AMPK inhibits fatty acid synthesis by affecting the activity of acetyl-CoA carboxylase (ACC). AMPK is able to regulate the function of ACC1/2 via phosphorylation, thereby inhibiting its normal production of malonyl-CoA, a building block for the synthesis of fatty acids[75]. Likewise, AMPK activation has been shown to repress protein synthesis pathways. Originally it was observed that this was due to phosphorylation of tuberous sclerosis (TSC) 2 and downstream inhibition of mammalian target of rapamycin (mTOR) complex 1[76]. However, later investigations revealed a more complex regulatory relationship where AMPK phosphorylates mTOR binding partner raptor, exhibiting another point of AMPK influence on cellular growth control pathways[77]. Thirdly, AMPK is able to regulate expression of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase by

phosphorylation of CREB-regulated transcription co-activator 2[78]. Together, these studies indicate that AMPK elicits a multifactorial response to energy stress, and is able to elicit inhibition of the synthesis of all major mammalian macronutrients.

Stimulation of energy production. In a synergy with inhibitory effects on anabolism, AMPK initiates a myriad of catabolic events, such as energy substrate breakdown, as well as pro-autophagic signaling[79,80]. Inhibition of ACC by AMPK has been implicated in β-oxidation of fatty acids, as evidenced by the increase in fatty acid oxidation in skeletal muscle from transgenic mice expressing an AMPKγ3 gain-of-function mutation[64]. However, it has been argued that fatty acid oxidation is not completely dependent on the AMPK/ACC pathway[81]. Therefore, a second pathway involving the storage vesicles Tre-2/BUB2/cdc 1 domain (TBC1D) 1 has been proposed for AMPK-mediated fatty acid oxidation. TBC1D1 phosphorylation by AMPK promotes translocation of the fatty acid transporter cluster of differentiation 36 (CD36) to the muscle membrane[82] thereby facilitating fatty acid utilization[83]. In support of this, AMPK-deficient muscles lack phosphorylation of TBC1D1 at serine 237, potentially explaining the observed defect in fatty acid uptake in response to contraction[84]. AMPK is also able to promote cellular glucose uptake, by promoting the translocation of glucose transporter type 4 (GLUT4) to the plasma membrane. Normally, GLUT4 is bound to TBC1D1/4. Insulin activated kinase AKT phosphorylates TBC1D4, triggering its dissociation from GLUT4 vesicle[85]. AMPK-mediated phosphorylation of

TBC1D1 exhibits similar effects[86,87]. The necessity of this AMPK-mediated pathway for glucose uptake has been questioned, as AMPKα knockout mouse models have displayed mixed effects on glucose uptake following AMPK activation or contractile activity[88]. In contrast, recent studies with mice lacking both AMPKβ subunits in skeletal muscle have demonstrated the importance of AMPK to contraction-induced glucose uptake. In addition to blunted muscle glucose uptake, these mice were resistant to exercise, demonstrated by dramatically reduced running speed and endurance[89].

Initiation of autophagy. Finally, AMPK is able to induce autophagy, the process by which organelles are digested by coordinated function of autophagosomes and lysosomes. Activation of autophagy ensures optimal energy utilization for maintaining cellular energy homeostasis as well as the efficient turnover of damaged organelles. AMPK mediates autophagy via two points of interaction. Firstly, AMPK is able to activate the autophagy flagship protein, unc-like kinase 1 (ULK1) directly via phosphorylation. Secondly, AMPK indirectly activates autophagy through phosphorylation of TSC2, causing downstream inhibition of mTOR and its dissociation with ULK1[80].

Multifaceted effects of AMPK on several energy systems can elicit responses that quickly and effectively modulate substrate use in the case of compromised cellular energy status. However, additional functions exist for the molecule that link to long-term adaptations in cellular homeostasis. In fact,

chronic AMPK activity serves to remodel skeletal muscle phenotype, thus impacting health and disease.

4. AMPK in Skeletal Muscle Remodelling

While the acute metabolic effects of AMPK are achieved through phosphorylation of key regulatory proteins, its longer-term phenotypic consequences are instigated through chronic phosphorylation of transcription factors and coregulators,[90–92]. As discussed below, AMPK is able to induce skeletal muscle remodelling by promoting regeneration, and mitigate neuromuscular pathology through activation of the slow oxidative myofiber program.

i) *AMPK in regeneration*

AMPK is able to stimulate restorative changes in skeletal muscle through promotion of regeneration. The regeneration of myofibers relies on the skewing of pro-inflammatory M1 macrophages towards their anti-inflammatory M2 counterpart[93]. AMPK plays a role in mediating macrophage phenotype during skeletal muscle regeneration. Mounier *et al.*[94] displayed aberrant macrophage skewing in the absence of AMPK through pharmacological inhibition. In AMPKα1 null mice, the authors observed accumulation of necrotic tissue following muscle injury via cardiotoxin (CTX), suggesting inadequate cellular debris removal by M1 macrophages. Additionally, further investigation displayed downregulation of signals that initiate the production of M2, which promote

regeneration through the induction of myogenesis. The role of AMPK in myogenesis is highlighted by the ablation of LKB1, an upstream AMPK kinase. LKB1 nullification in satellite cells and subsequent lack of AMPK activation causes dysregulation of the AMPK/mTOR pathway. This perturbation causes cells to enter a proliferative state, unable to maintain long-term self-renewal[95]. Further work showed the induction of Warburg-like glycolysis following AMPK activation signals satellite cell activation and regeneration[96]. Attenuated activation of AMPK has also been linked to impaired regeneration in obesity, as shown by Fu *et al*[97]. Indeed, impaired regeneration was rescued by AICAR administration, yet AICAR administration failed to improve muscle regeneration with AMPKα1 KO satellite cells[97]. Further, acute administration of the AMPK-activiating diabetic drug metformin was able to prompt cytoprotective effects in response to CTX injury attributed to the reduction in Ca^{2+} influx following muscle damage[98], as well as alleviating certain myopathic phenotypes through the promotion of muscle regeneration[99]. Currently, clinical trials are underway to determine the efficacy of AMPK activators in promoting regeneration in healthy elderly populations[100].

ii) *AMPK drives the slow, oxidative myofiber program*

Muscle fibers exhibit different structural and functional characteristics in order to accommodate their variable functions, including, but not limited to, supporting body weight during standing to performing explosive movements

during intense exercise. The predominant form of myofiber categorization is based on four MHC types: type I, type IIA, type IIX and type IIB, as mentioned above (see *Introduction to Skeletal Muscle*). In general, muscles with faster contractile properties exhibit a more glycolytic profile (i.e. fast glycolytic, FG) and are able to handle larger fluxes of cellular Ca^{2+}. In contrast, slower, more oxidative (i.e. slow oxidative, SO) muscles are purposed for slower, and/or prolonged contractions with smaller magnitudes of Ca^{2+} influx per contraction. AMPK isoforms demonstrate a unique expression pattern in each fiber type of human and rodent skeletal muscle[101]. The α2,β2,γ1 heterotrimer is the predominant combination in human quadriceps muscle[102], as well as in mouse muscles of differing fiber types[103]. Chronic endurance-type exercise training stimulates AMPK, and may also result in a muscle fiber type shift from fast, glycolytic characteristics to a slower, more oxidative phenotype, which depends, in part, on the intensity, duration, and volume of exercise[104,105]. This suggests that AMPK plays a role in fiber type determination. Along these lines, AMPK-knockout models more clearly display the importance of AMPK in maintaining the oxidative profile of muscles. Generally, animals lacking skeletal muscle AMPK are intolerant to exercise, and display lower levels of mitochondrial content independent of fiber type changes[89]. Additionally, these muscles display a myopathy that includes elevated amounts of centrally nucleated and necrotic myofibers, with a decrease in myofiber capillarization[106]. Interestingly, these

conditions were much more exaggerated in mixed fiber type tibialis anterior muscles, as compared to the soleus muscle, which strongly suggests a resilience of slower, more oxidative myofibers in the absence of AMPK. Nevertheless, the role of AMPK in mediating fiber type transformation remains ambiguous, as AMPKβ1,β2-deficient mice display normal fiber type distribution[89], while AMPKα1,α2-deficient mice display an upregulation of type I fibers, perhaps as a compensatory adaptation[88]. Transgenic and pharmacological models of chronic AMPK activation lend further credence to the importance of the kinase in driving characteristics indicative of the slow, oxidative myogenic program. Indeed, a constitutively active AMPK mutant demonstrates elevated mitochondrial biogenesis that is exaggerated in glycolytic muscle[107]. Pharmacological activation of AMPK through administration of 5'-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) promotes the slow oxidative muscle phenotype, including a shift towards slower MHC expression[108–110]. More recently discovered AMPK activator R419 has also been shown to elicit benefits of the slow oxidative phenotype, including enhanced insulin tolerance, GLUT4 and mitochondrial content[111]. Thus, on balance, transgenic knockout and overexpression studies, as well as data from pharmacological interventions, indicate that AMPK activation mediates a shift in muscle fiber-type composition, yet its necessity in maintaining fiber type characteristics remains to be determined.

The mechanism(s) by which AMPK governs skeletal muscle plasticity has not been completely elucidated. The evidence suggests that the kinase exerts its effects in this context, in part, by stimulating the transcriptional coactivator PGC-1α[112]. PGC-1α is a master regulator of skeletal muscle phenotype maintenance and remodelling by driving the expression of the slow, oxidative myogenic program[48,49]. Indeed, AMPK exerts direct phosphorylation-mediated stimulation of PGC-1α activity[112], as well as indirectly maintaining PGC-1α gene expression via phosphorylation of histone deacetylase 5 (HDAC5), which relieves its repression of myocyte enhancer factor 2 (MEF2), thereby inducing PGC-1α transcription[113,114]. Genetic gain- or loss-of function mouse models further underscore the relationship between AMPK and PGC-1α. Muscles which lack the AMPKα2 subunit are intolerant to exercise, and display lower PGC-1α levels and mitochondrial enzyme content[104,115]. Conversely, muscle-specific AMPK activation through mutation of the γ1 subunit caused elevated PGC-1α content, glycogen stores, and exercise capacity[104,116].

In summary, the physiological effects of AMPK in skeletal muscle extend well beyond energy homeostasis. The kinase maintains several roles involved in important cellular processed for modifying the long term phenotype of muscle, including regeneration, and determination of muscle oxidative capacity and phenotype. However, the exact mechanisms by which AMPK governs muscle

phenotype are still not clear, and its role in regulating skeletal muscle genes warrants further investigation.

Figure 2

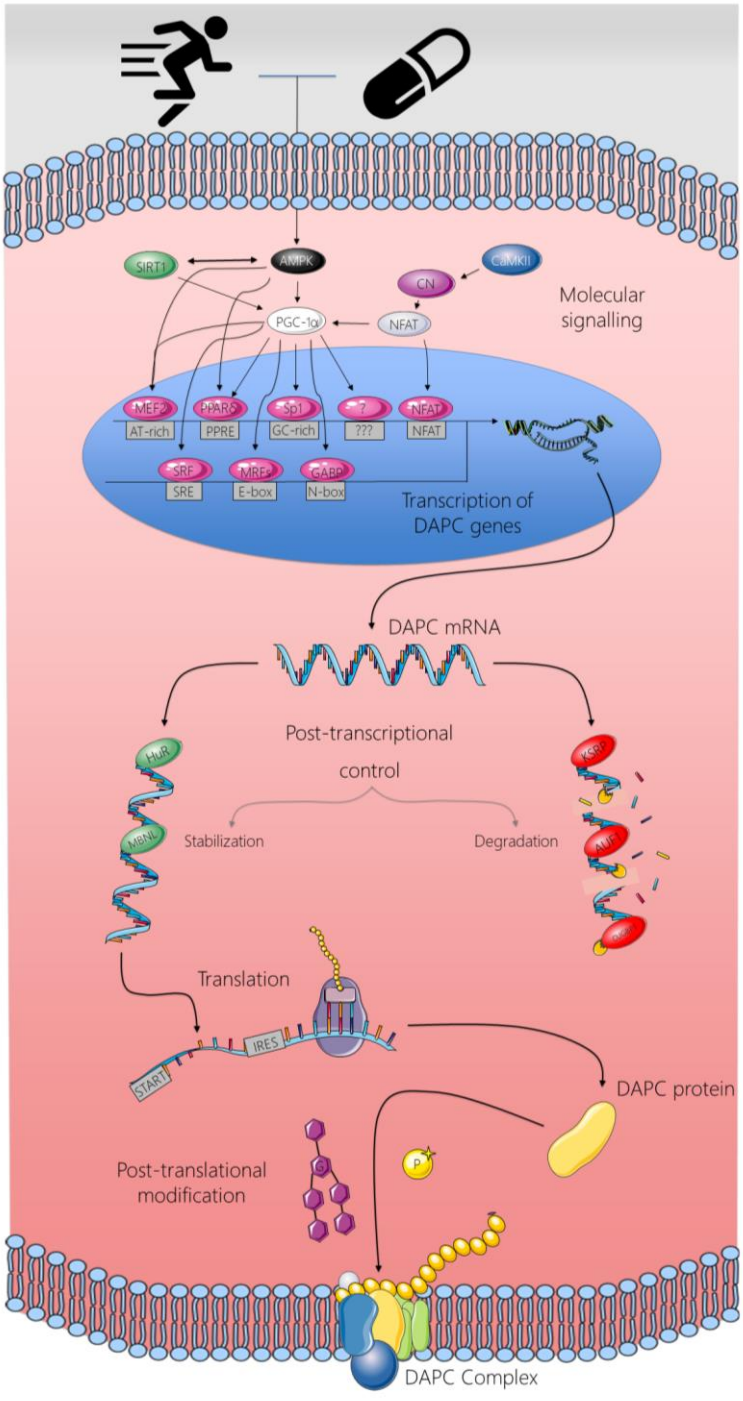
SIRT1
AMPK
CaMKII
CN
NFAT
PGC-1α
Molecular signalling
MEF2
AT-rich
PPARδ
PPRE
Sp1
GC-rich
???
NFAT
SRF
SRE
MRF2
E-box
GABP
N-box
Transcription of DAPC genes
DAPC mRNA
Post-transcriptional control
HuR
MBNL
Stabilization
Degradation
KSRP
AUF1
Translation
START
IRES
DAPC protein
Post-translational modification
P
DAPC Complex

Figure 2. AMPK-mediated signaling cascades that regulate expression of the DAPC in skeletal muscle. Exercise and several pharmacological activators stimulate AMPK activity in skeletal muscle. AMPK induces the function of several transcriptional activators and signaling molecules, such as MEF2, PPARδ, SIRT1, and PGC-1α. For example, AMPK targets the transcriptional co-activator PGC-1α for phosphorylation, thereby stimulating PGC-1α function. These transcriptional activators are capable of altering skeletal muscle phenotype, including stimulating DAPC gene expression. Post-transcriptional control of DAPC transcripts, via stabilization or degradation by RNABPs, affects mRNA content. Translation of DAPC transcripts occur via canonical, or in some cases, internal ribosome entry site (IRES)-mediated mechanisms. Finally, some post-translational modifications of DAPC proteins, such as phosphorylation (P) or glycosylation (G), are required for proper integration and assembly into the mature complex at the sarcolemma. Silent mating type information regulator 2 homolog 1 (SIRT1), 5' adenosine monophosphate-activated protein kinase (AMPK), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), calcineurin (CN), nuclear factor of activated T cells (NFAT), peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC-1α), myocyte enhancer factor 2 (MEF2), peroxisome proliferator activated receptor beta/delta (PPARβ/δ), specificity protein 1 (Sp1), serum response factor (SRF), myogenic regulatory factors (MRFs), GA-binding protein (GABP), human antigen R (HuR), muscleblind-like protein (MBNL), KH-type splicing regulatory protein (KSRP), AU-rich binding factor 1 (AUF1), CUG-binding protein 1 (CUGBP1).

5. Regulation of the DAPC

The expression of the DAPC involves various regulatory pathways and mechanisms converging onto the common endpoint of DAPC assembly. The diverse functions of individual DAPC components are highlighted by the variety of muscular dystrophies often brought on by the absence or dysfunction of individual DAPC members. In order to expand our knowledge of the DAPC, it is imperative to first understand the individual regulatory mechanisms involved in DAPC assembly, expression and function.

i) *Transcriptional regulation of the DAPC.*

Dystrophin. Dystrophin is encoded by the *DMD* gene, which is defective in Duchenne muscular dystrophy. The transcriptional regulation of dystrophin is complex, containing tissue specific promoters for brain, muscle and purkinje tissues, as well as four internal promoters[19,20]. Splicing at the first exon gives rise to truncated isoforms that are 260 kDa, 140 kDa, 116 kDa, and 71 kDa, as compared to the full length 427 kDa product[117–119]. Analysis of the upstream dystrophin promoter in skeletal muscle cells has revealed multiple regulatory sequences commonly involved in the transcription of skeletal muscle genes, including A/T-rich sites, and MEF-1 binding sites[120,121]. Also, four E-boxes are located in a distal enhancer region. Site-directed mutagenic analyses revealed the necessity for three of these sites for enhancer activity[122]. Studies also revealed putative binding sites for myocyte enhancer factor-2 (MEF-2) located within the

enhancer. Together, the combination of regulatory elements controlling dystrophin transcription points towards the preferential production for the protein in muscle cells.

Utrophin. While the structural and functional characteristics are similar to dystrophin, the 376 kDa utrophin protein varies mainly in its localization. Expression of utrophin is under control of two different promoters, utrophin-A and -B. Utrophin-B protein is restricted to the vascular endothelium, while utrophin-A protein shows expression in different tissues including the peripheral nerves, vascular smooth muscle, as well as at the neuromuscular and myotendinous junctions in skeletal muscle[20,123,124]. Analysis of the upstream utrophin-A promoter revealed an N-box motif that is necessary for synaptic induction of utrophin at the neuromuscular junction (NMJ) through binding of GA-binding protein (GABP)[124]. This subsynaptic expression of utrophin is heavily reliant on nerve-evoked electrical activity as shown by Grammolini *et al.*[124]. In this study, the authors elegantly displayed the ability of the nerve-derived trophic factor heregulin to transactivate GABP and stimulate its binding at the N-box, which in turn drove utrophin transcriptional activation. Additional transcription factors were found to bind within the utrophin-A promoter to induce transcription, including Sp1, Sp3 and Ap2[123,125]. An important second upstream E-box modulates myogenic induction of utrophin through interactions of myogenic

regulatory factors (MRF), showing a similar regulatory mechanism as seen in other muscle specific genes[126–129].

The dystroglycan complex. The *Dag1* gene simultaneously transcribes both members of the DG complex as a single precursor propeptide, which is proteolytically cleaved into α- and β-DG[130–132]. The *Dag1* promoter has three Sp1 recognition motifs and a more distal E-box important for maximum *Dag1* expression, as evidenced by promoter-reporter truncations assays[133]. Interestingly, the *Dag1* promoter, with multiple Sp1 sites and high content of GC- rich sequences, is typical of housekeeper genes, consistent with the ubiquitous expression of the DG complex.

The sarcoglycan complex. The SG complex contains four members: α-, β-, γ-, and δ-SG. Each possesses a unique promoter. SG promoter activities are correlated with myogenic differentiation. For example, α- and γ-SG show marked increases in transcriptional activity following the onset of myoblast differentiation as shown by Wakabayashi-Takai and colleagues[134]. Analysis of the γ-SG promoter by these authors identified A/T-rich and E-box elements, both of which were essential for transcriptional activation. Additionally, the myogenic transcription factors MyoD, myogenin, and MEF-2 were able to bind the upstream enhancer region of γ-SG. Furthermore, transfection of MyoD, but not myogenin was able to activate the γ-SG promoter in fibroblasts. Conversely, other studies have shown that MyoD negatively regulates α-SG promoter activity through one

of its two E-box motifs[135]. Interestingly, these results show that E-box motifs, besides acting as transcriptional enhancers, are able to fine-tune muscle-specific promoter expression through selective MRF activity.

Neuronal nitric oxide synthase (nNOS). nNOS exhibits the most complex transcriptional regulation of the established types of the NOS enzyme. The use of various alternative promoters appears to be a major factor driving its complex expression patterns. Nine distinct first exons have been revealed in humans, known as exons 1a-1i. These and their corresponding 5' flanking regions are spliced to a common exon 2[136,137]. The profound diversity of transcripts and their promoters may allow for allowing for differential regulation of nNOS, including differences in transcript localization and transcriptional efficiency. Exons 1a, 1b, and 1c, are all enriched in muscle[137,138]. Analysis of exon 1c shows prominent reactions with the Sp family of transcription factors[137]. Meanwhile, exon 1a was found to be translationally enhanced in differentiated muscle cells[138], suggesting possible differentiation-dependent translational *cis*-RNA elements within exon 1a. The full length NOS gene encodes 4 versions nNOS enzyme[139], which include α, β, γ, and μ. nNOS-α and nNOS-μ contain a PDZ domain, allowing binding of the enzyme to the cell membrane[33,140]. nNOS-μ is predominantly expressed in skeletal muscle and possesses a 102bp insertion between exons 16 and 17 that is unique to other variants[31]. β and γ variants lack the PDZ domain and are therefore cytosolic enzymes, with lower activity levels than α and μ[140,141].

In summary, while much work has been done to characterize its regulation, a complete understanding of DAPC transcription is still lacking. It is increasingly clear that production of the DAPC is closely managed by DNA elements and transcription factors related to the myogenic program, suggesting the process of myogenesis and the development of the DAPC are very closely related.

ii) *Post-transcriptional regulation of the DAPC.*

In eukaryotes, transcription and translation are physically separated processes, occurring in the nucleus and cytoplasm, respectively. This allows eukaryotes to carry out extensive post-transcriptional processing of pre-mRNAs after transcription, thereby providing an additional layer of gene expression regulation. Skeletal muscle development, repair, and function, are dependent on the highly coordinated expression of many genes, including those of the DAPC. Indeed, many MRFs that determine the myogenic identity of cells also directly and/or indirectly promote the transcription of the DAPC, as mentioned above. Therefore, similar post-transcriptional mechanisms are likely to regulate the DAPC as well as the broader myogenic program. One such mechanism is the regulation of mRNA processing and stability by RNA-binding proteins (RBPs). The focus of this section is to review the effects of RBPs on skeletal muscle mRNA metabolism, highlighting the roles of RBPs that are best characterized in skeletal muscle.

Human antigen R (HuR). HuR is an RBP that has multiple roles in mRNA metabolism including splicing, polyadenylation, localization, and stabilization of transcripts[142]. HuR binds to U- and AU-rich elements (AREs) in the 3'-untranslated regions (UTRs) of specific target mRNAs, providing stabilization of target transcripts. AREs are responsible for mRNA decay regulation, as well as maintaining a precise level of short-lived transcripts such as transcription factors and cell cycle regulators[143,144]. For instance, MyoD and myogenin ARE regions are sufficient to promote mRNA decay[145]. HuR binding to these AREs results in stabilization and increased levels of MyoD and myogenin mRNAs and subsequent increased levels of the respective proteins, thus promoting myogenesis[146,147].

KH-type splicing regulatory protein (KSRP). KSRP also binds to ARE-containing mRNAs and recruits partners that assist in the rapid decay of target transcripts[148]. KSRP competes with HuR for binding on AREs, and their binding to such transcripts is mutually exclusive. Due to these reasons, the given steady-state level of transcripts at have been attributed to the KSRP:HuR ratio of the cell[149]. During proliferation, KSRP binds ARE-containing mRNAs such as MyoD and myogenin and promotes their decay. During differentiation, phosphorylation of KSRP by the kinase p38 leads to reductions in binding to these transcripts. Loss of this decay-promoting function from KSRP, coincident with HuR activity, stabilizes MyoD and myogenin mRNAs, thereby promoting myogenesis[145]. Additionally, inactivation of KSRP through p38 phosphorylation or microRNA

mediated gene suppression has also been shown to increase utrophin transcript levels due to relief of KSRP-mediated decay[150,151].

CUG-binding protein 1 (CUGBP1). CUGBP1 binds a variety of 3'-UTR regulatory elements including GC/GU-rich elements (GRE), and AREs[152,153] that affect the alternative splicing as well as stability of several factors with key roles in myogenesis[154]. While some target transcripts of CUGBP1 display upregulated translation[154], its binding to most ARE-containing mRNAs causes transcript destabilization[153,155–157]. High-throughput assays of GRE-containing mRNAs, such as MyoD[158,159], are generally labile transcripts, and are therefore rapidly destabilized when bound by CUGBP1[160].

Muscleblind-like protein 1 (MBNL1). MBNL1 is a member of the MBNL family comprising the vast majority of MBNL expression in skeletal muscle versus MBNL2 or MBNL3 [161]. MBNL1 regulates alternative splicing in concert with CUGBP1, promoting transition towards splicing of adult transcripts during postnatal development[162–164]. In addition to splicing regulation, MBNL and CUGBP1 have been found to preferentially bind the 3'-UTR of mRNAs encoding transcription factors that can regulate cell development[165]. During muscle differentiation, the nuclear concentration of MBNL1 increases, while that of CUGBP1 decreases, suggesting that CUGBP1 may affect the transcripts of undifferentiated cells and MBNL1 may take over this role after

differentiation[162,164]. Together these reports suggest that this pair of RBPs may serve as a finely tuned regulator of skeletal muscle development.

AU-rich binding factor 1 (AUF1). Finally, (AUF1) is generally considered to promote the decay of target mRNAs containing AREs[166,167]. However, AUF1 also binds to the promoter and 3'-UTR of MEF2C mRNA, enhancing its transcription and translation, respectively[168]. These data suggest that AUF1 may partly regulate muscle differentiation through transcriptional and post-transcriptional interactions with MRFs.

RBPs in different fiber types and during exercise. The evidence clearly indicates a role for RBPs in the post-transcriptional regulation of muscle cell differentiation and development. Reports of RBPs in skeletal muscle suggest that some, such as AUF1, KSRP and HUR, are more highly expressed in slow-twitch skeletal muscle. Moreover, the ratio of stabilizing and destabilizing RBPs affects mRNA stability in muscles of different fiber type compositions. For example, D'Souza *et al.*[169] report the stabilizing HuR and destabilizing AUF1 to both be elevated in slow-twitch and cardiac muscle. However, simultaneous examination of AUF1 and HuR revealed that the AUF1:HuR ratio was elevated in cardiac muscle, coinciding with the reduced stability of target transcripts in that tissue type. Prolonged exercise training in humans, a stimulus for chronic AMPK activation, is shown to induce PGC-1α expression and thus mitochondrial biogenesis in the presence of elevated destabilizing RBPs CUGBP1 and AUF1[170].

Thus, it is possible that environments of decreased mRNA stability after exercise may also also promote cellular remodelling required for metabolic adaptations.

It should be noted that there are other means of post-transcriptional regulation not included here. These include, but are not limited to, regulation by microRNAs, which have been detailed elsewhere[171]. However, RBPs represent a dynamic network of post-transcriptional regulation that can modulate the stability of mRNAs and thus the functional availability of the transcript for translation of its protein product. With regards to the DAPC, unique 3'-UTR regions in dystrophin mRNA have been shown to affect its abundance[172], while interactions with KSRP and have been directly observed in utrophin mRNAs[150,151]. Furthermore, preliminary bioinformatic analyses have identified numerous putative RBP binding sites in multiple DAPC mRNAs (Dial *et al.*, unpublished data). Additionally, the interactions of RBPs with MRFs have been well documented, suggesting that the post-transcriptional control of these factors may modulate the tightly coordinated gene expression involved in skeletal muscle development, including the production of the DAPC.

iii) *Translational and post-translational regulation of the DAPC*

Internal ribosomal entry sites. While the majority of mutations in the dystrophin gene generally cause DMD, premature stop codon mutations in exon 1 and 2 do not result in the same severity of pathology. Interestingly, patients with these mutations exhibit very mild clinical phenotypes, with affected individuals

retaining the ability to walk well into adulthood[173]. It was found by Wein *et al.*[174] that mutations in the earlier exons cause alternative translation at AUG codons in exon 6 due to upstream internal ribosomal entry sites (IRESs)[175], allowing for a truncated, functional protein to be produced. Likewise, in response to regeneration and pharmacological interventions[176,177], the expression of utrophin protein content has been observed to robustly increase with only modest corresponding increases in mRNA transcript levels, which suggests this to be a translationally regulated increase in utrophin expression. Indeed, presence of an IRES in the utrophin 5'-UTR was associated with physiological and pharmacological inductions in utrophin translation. As modulating IRES function could have potential therapeutic applications for genetic conditions like DMD, these findings have prompted interest in IRES expression in other clinically relevant genes[173].

Glycosylation of the DAPC. The DGs are post-translationally cleaved at amino acid 653, resulting in α and β subunits[178,179]. DGs are heavily glycosylated at the membrane. However this process is not necessary for the cleavage of DGs, suggesting that the glycosylation occurs at the endoplasmic reticulum before DGs are trafficked to the membrane[180]. DGs undergo both N- and O-linked glycosylation. Treatment of the complex with N-glycanases suggests that N-linked glycosylation is not necessary for laminin or integrin binding at the plasma membrane. However, full chemical deglycosylation of DG results in the complete loss of ligand binding, including monocolonal antibody IIH6[181]. IIH6, inhibits

laminin binding to α-dystroglycan, suggesting that O-linked glycosylation of DGs is necessary for DAPC binding to laminin[181]. The incomplete glycosylation of DGs is observed in aging[182], and can lead to a variety of clinical symptoms including muscular dystrophy and central nervous system abnormalities[180]. Normal presence of the SG complex is also necessary for interaction of the DGs with the DAPC, as SG-null mutations lead to separation of the DG complex from the DAPC. Variations of limb girdle muscular dystrophies (LGMD), often associated with sarcoglycanopathy, have been linked to abnormal glycosylation[28]. Mutations in one sarcoglycan may affect the assembly of the others at the membrane. For example, in LGMD2F, absence of δ-SG results in the inability to assemble α, β and γ, leading to their rapid degradation[183,184].

Effects of phosphorylation on DAPC assembly. The signalling functions of the DAPC are also highlighted by the various phosphorylation sites within DAPC components. Indeed, dystrophin is the target of many kinases including calmodulin-dependent protein kinase II, $p34^{cdc2}$ kinase and casein kinase[185–187], and there are many putative phosphorylation sites at the c-terminal end of the protein[188]. The phosphorylation of dystrophin has been shown to affect its affinity for binding actin and syntrophin[189,190] *in vitro*, while phosphorylation of cysteine-rich region of dystrophin enhances its binding to β-DG[191]. Additionally, β-DG participates in adhesion-dependent phosphorylation, which mediates its binding to utrophin[192]. This signalling cascade has been shown to promote cell survival[193,194],

and involves β-DG through interaction with the signalling molecule Grb2[195]. Similar adhesion-dependent phosphorylation occurs at α- and γ-SG[196]. While the functional significance of these events is not yet known, it suggests at SG adhesion to the plasma membrane may also promote cell survival in a similar manner as β-DG.

In summary, the expression of the DAPC is reliant on a complex network of regulatory mechanisms. The transcription factors that regulate myogenesis appear to promote the common elevation of DAPC production. However, a myriad of other factors modulate the various stages of mRNA and protein processing, which affect the expression and function of DAPC components. While it has been the subject of intense research, many of the events that regulate the DAPC still remain to be elucidated. Since the modulation of DAPC content has several therapeutic benefits, the further investigation of DAPC regulators is a promising avenue of future research.

6. Study Objectives

Recent investigations have revealed that AMPK plays an important role in regulating the expression and function of proteins at the sarcolemma, including the Na^+/K^+-ATPase and nNOS[197–199]. Pharmacological activation of AMPK in dystrophic muscle leads to a rescue of DAPC expression and function, as well as to increased levels of the dystrophin homologue utrophin in skeletal muscle[13,46,106,199–203]. Meanwhile, aging-associated declines in AMPK are

concomitant with diminished expression and function of the DAPC[3,182,204,205]. However, whether AMPK is required for the basal expression and function of the DAPC in skeletal muscle is unknown. Thus, the purposes of this study were to 1) investigate the role of AMPK in the basal expression of the DAPC in skeletal muscle, and 2) expand our knowledge of differences in DAPC expression between muscle fiber types. We hypothesize that DAPC levels will be diminished in muscles lacking AMPK. Furthermore, we posit that DAPC expression will be higher in slower, more oxidative muscles.

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The role of AMPK in the expression of the dystrophin-associated protein complex in skeletal muscle

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Abstract

The dystrophin-associated protein complex (DAPC) provides a mechanical link between the intracellular cytoskeleton and extracellular matrix, serving as a mechanosensor and signal transducer across the sarcolemma. Pharmacological stimulation of AMP-activated protein kinase (AMPK) induces the expression of DAPC components in skeletal muscle, whereas physiological reductions in AMPK are associated with DAPC dysfunction. We sought to determine whether AMPK was necessary for the maintenance of DAPC expression in skeletal muscle. Fast glycolytic extensor digitorum longus (EDL) and slow oxidative soleus (SOL) muscles from wild-type (WT) mice, as well as from littermates deficient in both isoforms of the AMPK-β subunit in skeletal muscle (MKO) were analyzed. DAPC mRNA levels, as well as protein expression and localization were similar between genotypes, with the exception of nNOS, which displayed a compensatory sarcolemmal enrichment in MKO muscles. The content of transcriptional and post-transcriptional regulators of the DAPC, such as PGC-1α and KSRP, were also not affected by the loss of AMPK. However, MyoD and myogenin expression was significantly diminished in MKO muscles, which is consistent with previous reports of myopathy in these animals. Furthermore, we observed decrements in extrasynaptic utrophin expression selectively in MKO SOL muscles, despite an adaptive accumulation of PGC-1α at the sarcolemmal compartment. Collectively the evidence indicates that AMPK is

sufficient, but not essential for the maintenance of DAPC expression in skeletal muscle. However, AMPK is required for preserving extrasynaptic utrophin levels in slow, oxidative muscles, which underscores the role of AMPK in the gene expression of this disease modifying protein.

Introduction

Skeletal muscle is a dynamic tissue that constantly endures significant levels of mechanical strain and cellular stress with each contraction. Myofibers must be able to physically contract and generate forces necessary for movement, while preventing mechanical injury of the cell. The discovery of the cytoskeletal protein dystrophin in 1987[1] stimulated further research into the adjacent glycoprotein complex localized to the sarcolemma, now known as the dystrophin-associated protein complex (DAPC). The complex, which is generally accepted to be comprised of ~13 proteins, includes dystrophin, dystroglycans, sarcoglycans, neuronal nitric oxide synthase (nNOS), syntrophins, and dystrobrevin[2]. The DAPC serves multiple functions, such as providing a structural linkage from the sarcolemma to filamentous actin, as well as acting as a signal transduction apparatus between the extracellular matrix and interior of the cell[2,3]. Loss of the DAPC is one of the early pathogenic markers of various myopathies[4,5] and muscular dystrophies[6,7], while increased expression of the DAPC provides a protective benefit against dystrophic pathologies[8–12]. Indeed, recent investigations

have focused on promoting DAPC expression in skeletal muscle in an effort to mitigate Duchenne muscular dystrophy (DMD)[13,8,14,9,11,15–17].

Although the molecular biology of the DAPC has been investigated in earnest for over 30 years, the regulation of DAPC expression in skeletal muscle is still not fully understood. Earlier studies have suggested that within a given muscle, slow, oxidative myofibers display a higher expression of various constituents of the DAPC, as compared to their faster, more glycolytic counterparts[18,19]. The underlying cause for this discrepancy in DAPC expression between fiber types may be attributed, in part, to differences in the transcriptional control of DAPC genes. The regulatory regions of DAPC genes contain several DNA binding motifs that are targeted by transcription factors and transcriptional coregulators responsible for the maintenance and remodelling of skeletal muscle phenotype. For example, dystrophin expression is driven by the transcription factor MyoD[20,21], while γ-sarcoglycan transcription is promoted by myogenin[22,23]. Upstream molecules that are directly or indirectly responsible for the regulation of these transcription factors, and others that participate in DAPC expression, include calcineurin (CN), peroxisome proliferator-activated receptor (PPAR) γ coactivator-1α (PGC-1α), PPARβ/δ, silent information regulator two ortholog 1 (SIRT1), as well as AMP-activated protein kinase (AMPK)[24,9,15,25]. These upstream proteins display differential levels of fiber type-specific expression and activity[12]. Thus, it is reasonable to postulate that powerful phenotype-modifying

proteins such as PGC-1α or AMPK would impact DAPC expression in skeletal muscle.

AMPK is a critical regulator of skeletal muscle metabolism, transcription, and phenotype. It is activated by a shift in the AMP/ADP:ATP ratio which arises during times of metabolic stress, such as during the contractile activity elicited by exercise[26]. The kinase is a heterotrimer composed of a catalytic α subunit, a scaffolding β subunit and a regulatory γ subunit. Each subunit exists in multiple isoforms (i.e., α1, α2, β1, β2, γ1, γ2, and γ3) that are expressed in varying proportions throughout different tissues to produce several versions of the AMPK enzyme[27]. The most common AMPK heterotrimer in skeletal muscle is the α_2, β_2, γ_1[28]. During aging, attenuation of skeletal muscle AMPK activity[29] is associated with alterations in the sarcolemmal environment, including reduced DAPC protein content[30–34]. AMPK is a potent regulator of skeletal muscle phenotype primarily through phosphorylation-mediated changes in the expression and activity of downstream proteins capable of regulating phenotype maintenance and remodelling. These downstream targets include the transcription factors nuclear respiratory factor 1 (NRF-1) and NRF-2/GA-binding protein (GABP), myocyte enhancer factor 2 (MEF2), cAMP response element binding protein (CREB), as well as via stimulation of the transcriptional co-activator PGC-1α[12]. AMPK also mediates the content and function of proteins localized to the sarcolemma, including for example, the Na^+-K^+-ATPase[35]. Along these lines, an emerging area

of research has focussed on the role of AMPK in regulating sarcolemmal DAPC expression and function. Recent investigations have revealed that pharmacological activation of AMPK in models of DAPC deficiency leads to a rescue of DAPC expression and function, as well as to increased levels of the dystrophin homologue utrophin in skeletal muscle[13,9,4,11,12,15,36,37]. These adaptations occur concomitant with a shift towards characteristics indicative of the slow, oxidative myogenic program. These studies are supported by observations of reduced nNOS levels in skeletal muscle lacking AMPK[4]. However, the role of AMPK in the basal expression and function of the DAPC in skeletal muscle is otherwise unknown. Thus, the purpose of this study is to investigate the role of AMPK in the basal expression of the DAPC in skeletal muscle. We hypothesize that DAPC levels will be diminished in muscles lacking AMPK.

Methods

Animals. Mice with skeletal muscle-specific deletion of the AMPK β1 and β2 subunits (AMPKβ1β2M-KO; MKO) were generated as described previously[38]. WT littermates were used for all comparisons. Extensor digitorum longus (EDL) and soleus (SOL) muscles from 2 month-old animals were harvested and immediately snap frozen in liquid nitrogen. The contralateral EDL and SOL muscles were immersed in OCT compound (VWR, Mississauga, ON, Canada),

and frozen in melting isopentane cooled to the temperature of liquid nitrogen. All frozen samples were stored at -80 °C until subsequent processing and analyses.

RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR). We evaluated the mRNA expression of multiple DAPC components in order to understand the effect of skeletal muscle AMPK on DAPC gene expression. Total RNA was isolated from EDL and SOL muscles of MKO and WT mice in order to make fiber type and genotype comparisons, respectively. Muscles were homogenized in Trizol reagent (Thermo Fisher Scientific, Burlington, ON, Canada) followed by purification and elution with the E.Z.N.A. RNA Isolation Kit (VWR, Mississauga, ON, Canada). Reverse transcription was performed as per the manufacturer's protocol provided with the cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Burlington, ON, Canada). Endogenous mRNAs were measured by qPCR (Eppendorf. Mississauga, ON, Canada) and the delta delta CT method[39] was used to quantify expression of DAPC components relative to ribosomal protein S11 (RPS11). The primers used were:

dystrophin forward (F)- TTCACCTCTAGCTGGTCCGA. reverse (R)-
AGTCTTTGGGTGGCTGAGTG; utrophin forward (F)-
CCGGAGCTAAACACCACTGT, reverse (R)-
ATTCAGCTGAGCGAGCATGT; β-DG forward (F) –
CGTGGTGCTGGGTGAGTG, reverse (R)- TCTGCACAGCTGTTCCATCC; α-

SG forward (F)- GGACGGCTGAAGAGAGACAT, reverse (R)-
AGGATAAGAGGCATCTGTGCG; β-SG forward (F)-
CACATGGGAGGAGATGTGGAG, reverse (R)-
CAGCCCATGTTGTGACCTGT, γ-SG forward (F)-
CAGTCAACCCAGAACGTGACA, reverse (R)-
AGTGCTGGCTCTGGACTTCTA; nNOS forward (F)-
AGTGCTGGCTCTGGACTTCTA, reverse (R)-
GGCTCAACCGAATACAGGCT; PGC-1α forward (F)-
GGCTCAACCGAATACAGGCT, reverse (R)-
TCTTCATCCACGGGGAGACT; laminin forward (F)-
GAAATACTCCGGCTGCCTCA, reverse (R)-
ACAAAACCAGGCTTGGGGAA; biglycan forward (F)-
GGAGCCTGAGTTTTCTGCCTA, reverse (R)-
TTGATGCCCACCTTGGTGAT; RPS11 forward (F)-
CGTGACGAAGATGAAGATGC, reverse (R)-
GCACATTGAATCGCACAGTC.

Protein extraction and Western blot analysis. Muscles were initially ground into a fine powder using a CellCrusher tissue pulveriser (Cell Crusher Ltd., Portland, Oregon, USA). The powder was added to a sample tube with a pre-determined volume of RIPA buffer (20 µl of RIPA per 1 mg muscle weight; Sigma-Aldrich, Oakville, ON, Canada), supplemented with a protease and

phosphatase inhibitor cocktail (Roche, Mississauga, ON, Canada). The solution was further homogenized on ice using sonication (Thermo Fisher Scientific, Burlington, ON, Canada) at 50% power for 5 bouts of 2 seconds, with 30 seconds in between each bout. Samples were spun, and the resulting supernates were collected. A bicinchoninic acid assay (Thermo Fisher Scientific, Burlington, ON, Canada) was performed in order to determine protein concentrations of samples. Twenty μg of protein was loaded into each lane of 6% or 10% gels and subjected to SDS-PAGE, and then transferred to a nitrocellulose membrane. Ponceau S solution (G00040, Sigma-Aldrich, Oakville, ON, Canada) was used to verify equal loading across all lanes[40]. Ponceau solution was washed off with Tris-buffered saline with 1% Tween-20 (TBS-T). Membranes were then blocked with 5% milk for 60 minutes and subsequently washed 3 x 5 minutes with (TBS-T). Primary antibody dilutions were prepared in 5% milk or bovine serum albumin (BSA). Antibodies against phosphorylated AMPKα (Cell Signalling, Beverly, MA, USA) dystrophin (ab3149, Abcam, Toronto, ON, Canada), utrophin (NCL-DRP2, Leica Biosystems, Concord, Ontario, Canada), β-dystroglycan (6H1-s, University of Iowa Hybridoma, Iowa City, IA, USA), γ-sarcoglycan (NCL-G-SARC-CE, Leica Biosystems, Concord, Ontario, Canada), laminin (ab11575, Abcam, Toronto, ON, Canada), nNOS (372800, Thermo Fisher Scientific, Burlington, ON, Canada), were used to assay the expression of multiple components of the DAPC. Antibodies against PGC-1α (AB3242, EMD Millipore,

Etobicoke, ON, Canada), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; 33628, Cell Signalling, Beverly, MA, USA), MyoD (sc-304, Santa Cruz, Dallas, TX, USA), myogenin (F5D, University of Iowa Hybridoma, Iowa City, IA, USA), CUG-binding protein 1 (CUGBP1; sc-20003, Santa Cruz, Dallas, TX, USA), Muscleblind-like protein 1 (MBNL1; sc-47740; Santa Cruz, Dallas, TX, USA), KH-type splicing regulatory protein (KSRP; A302-021A; Bethyl Laboratories, Burlington, ON, Canada), human antigen R (HuR; sc-5261; Santa Cruz, Dallas, TX, USA), and AU-Rich Binding Factor 1 (AUF1; 07-260; EMD Millipore, Etobicoke, ON, Canada) were also employed. Primary antibodies were applied overnight at 4 °C with gentle shaking and washed off the following morning with 3 x 5 minute washes in TBS-T Appropriate horseradish peroxidase (HRP) linked secondary antibodies (Cell Signalling, Beverly, MA, USA) were applied for 2 hours at room temperature followed by 3 x 5 minute washes in TBS-T. Finally, enhanced chemiluminescence substrate (1705061, Bio-Rad, Mississauga, ON, Canada) was applied in order to detect target proteins. Images were captured with Alpha Innotech imaging equipment (Alpha Innotech, San Jose, CA, USA) and ImageJ[41] was employed for densitometry.

Immunofluorescence microscopy. The immunostaining procedure was carried out as described previously (6). EDL and SOL muscles stored in OCT from MKO and WT mice were sectioned on a cryostat (Thermo Fisher Scientific, Burlington, ON, Canada) into 5 μm slices. Slides were fixed with 4%

paraformaldehyde (PFA) for 10 minutes. Following PFA incubation, slides were washed in 1% PBS with Tween-20 (PBS-T) for 3 x 5 minutes. Slides were then incubated in a blocking solution of 10% goat serum in 1% BSA for 90 minutes. Following another 3 x 5 minute wash in PBS-T slides were incubated in primary antibodies. Protein expression and localization of the DAPC were examined by probing for dystrophin, β-dystroglycan, γ-sarcoglycan, and nNOS (antibodies listed above). All primary antibodies were incubated at a dilution of 1:1,000 overnight at 4 °C. After primary antibody incubation, slides were washed for 3 x 5 minutes in PBS-T. Alexa-conjugated secondary antibodies (Thermo Fisher Scientific, Burlington, ON, Canada) were applied to samples for 2 hours at room temperature (RT), followed by another 3 x 5 minute wash in PBS-T. 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI; D1306, Thermo Fisher Scientific, Burlington, ON, Canada) was incubated for 10 minutes on slides to label myonuclei. Slides were then washed for 5 minutes in PBS-T, followed by a final wash for 5 minutes in PBS. After slides were dried, fluorescent mounting media (Agilent Technologies, Mississauga, ON, Canada) was applied and the slide was mounted with a cover slip. A utrophin-specific primary antibody (mentioned above) was used to measure protein expression and localization. The primary antibody was incubated at 1:100 for 30 minutes at RT. Following 3 x 5 minute washes in PBS-T, Alexa-conjugated secondary antibody was incubated for 2 hours at RT, followed by another 3 x 5 minute wash in PBS-T. Slides were then

incubated with Alexa-conjugated α-bungarotoxin (α-BTX; Thermo Fisher Scientific, Burlington, ON, Canada) for 2 hours at 37°C in order to visualize acetylcholine receptors (AChRs). Following incubation, slides were washed, stained with DAPI, dried and mounted. Protein expression and localization of AMPK and PGC-1α were examined by probing for antibodies against specific primary antibodies (antibodies mentioned above). Primary antibody was incubated at 1:500 overnight at 4°C. Following primary antibody incubation, slides were washed for 3 x 5 minutes in PBS-T, then incubated in Alexa-conjugated secondary antibody at 1:500 for 2 hours at RT. Slides were then fixed in 4% PFA for 5 minutes, followed by 3 x 5 minute wash in PBS-T. Slides were then stained with laminin antibody at 1:500 for 15 minutes at RT, in order to visualize the skeletal muscle cell membrane. Following incubation, slides were washed, stained with DAPI, dried and mounted.

Slides were viewed with the Nikon Eclipse *Ti* Microscope (Nikon Instruments, Mississauga, ON, Canada), equipped with a high-resolution Photometrics CoolSNAP HQ2 fluorescent camera. Images were captured and analysed using the Nikon NIS Elements AR 3.2 software. All images were obtained with the 20× objective. The operator was blinded with respect to the experimental group of each sample. Images were taken of the entire 5 μm section. Five square regions of interest (ROIs) were created, each representing 10% of the area of the section, thereby representing half of the total cross-sectional area of the

muscle sample. A threshold was applied in order to create a binary layer to remove background fluorescence. For DAPC localization analysis, protein expression was determined by fluorescence density and was measured by Sum Intensity/ROI Area using NIS Elements. For utrophin analysis, AChRs were considered positive controls for utrophin localization and thus represented 100% of fluorescence intensity. 30% was considered the lower limit for thresholding. Three square ROIs were created (as mentioned above), each containing at least one neuromuscular junction, thereby representing 30% of the total cross-sectional area of the sample. Sarcolemmal utrophin was determined by fluorescence density (as mentioned above). Special consideration was made to exclude regions overlaid with AChRs or myonuclei, as well as extramyocellular punctate structures indicative of epithelial utrophin[42]. AMPK localization was determined as the percentage of AMPK fluorescence, measured by Sum Intensity, overlaid with laminin (i.e., membrane) or DAPI (i.e., myonuclear). The remaining AMPK fluorescence was considered cytosolic.

Statistical analyses. Differences in expression level between fiber types within a respective genotype (SOL vs EDL) and between genotypes of the same fiber type (WT vs MKO) were analyzed by one-way ANOVA and Bonferroni post hoc analysis. Statistical differences were considered significant if $p < 0.05$. All statistical analyses were performed on the raw data sets prior to

transformation to –fold differences as displayed in the graphical summaries. Data are presented as means ± SEM.

Results

Subcellular localization of AMPK in skeletal muscle. In order to elucidate the importance of AMPK in the expression of DAPC proteins, we first sought to investigate the myocellular localization of AMPK through the use of immunofluorescence imaging. This analysis was performed using EDL and SOL muscles from WT mice, as well as muscles from MKO animals (Fig. 1A, B). AMPK colocalization with laminin was employed to identify the presence of the kinase at the muscle membrane, while AMPK and DAPI colocalization was used to mark AMPK in the myonuclear compartment (Fig. 1B, C). AMPK staining within the myofiber but outside of myonuclei was considered cytosolic. In both EDL and SOL muscles of WT animals, AMPK localization was significantly greater (~10-12-fold) in the cytosol, as compared to the myonuclei (Fig. 1B, C). The content of membrane-localized AMPK was also 3-6-fold higher ($p < 0.05$) versus myonuclear AMPK expression. In turn, cytosolic AMPK abundance was also significantly greater (2-2.2-fold) than sarcolemmal AMPK levels. The pattern of cellular AMPK localization was similar between muscle types.

DAPC transcript levels in skeletal muscle. We next examined the mRNA expression of DAPC components in skeletal muscles of disparate fiber type

composition. In the mouse, the fiber type composition of the EDL muscle is < 1% type I, ~8% IIA, ~22% IIX and ~66% IIB, while the SOL muscle is ~37% I, ~56% IIA, ~6% IIX and 0% IIB[43]. In WT mice, dystrophin transcript levels were similar between the fast, glycolytic EDL muscle and the slower, more oxidative SOL muscle (Fig. 2A). Utrophin transcripts displayed ~46% higher ($p < 0.05$) expression in the SOL muscle, as compared to the EDL muscle (Fig. 2B). β-DG mRNA levels, along with all three SGs measured, were similar between muscle types in WT animals (Fig. 2C-F). nNOS mRNA content was 65% lower ($p < 0.05$) in the SOL relative to the EDL muscle (Fig. 2G). The mRNA expression of laminin and biglycan were 64% ($p < 0.05$) and 57% ($p < 0.1$) higher, respectively, in SOL muscle, as compared to EDL muscle (Fig. 2H, I).

In general, DAPC transcripts in MKO animals displayed similar muscle-specific expression patterns as in their WT counterparts. There was significantly higher dystrophin (+26%) and utrophin (+68%) mRNA levels in the SOL muscle, as compared to the EDL muscle (Fig. 2A, B). β-DG, α-SG, and β-SG transcript content were similar between muscle types in MKO mice (Fig. 2C-E). γ-SG levels were 30% higher ($p < 0.05$) in the SOL muscle versus the EDL muscle (Fig. 2F), whereas nNOS expression was significantly lower (-40%) in the SOL relative to the EDL muscle (Fig. 2G). Laminin and biglycan displayed 59% ($p < 0.05$) and 127% higher ($p < 0.1$) expression, respectively, in SOL muscle, as compared to the EDL muscle (Fig. 2H, 2I).

There were no differences in mRNA transcript expression between WT and MKO muscles of the same fiber type, with the exception of nNOS, which was 40% lower ($p < 0.05$) in the EDL muscle of MKO animals versus WT mice (Fig. 2G).

Skeletal muscle DAPC protein content. Next, we examined the protein expression of DAPC constituents using immunoblotting techniques. In the WT group, dystrophin, γ-SG, and laminin protein levels were similar between EDL and SOL muscles (Fig. 3A, B, E, G). Utrophin, β-DG, and nNOS expression was 55-100% greater ($p < 0.05$) in the SOL muscle relative to the EDL (Fig. 3A, C, D, F). Dystrophin, β-DG, γ-SG, and laminin content were similar between muscles in the MKO animals, whereas utrophin levels were significantly higher (+69%) in the SOL, as compared to the EDL muscle. nNOS levels were 41% higher in MKO SOL as compared to MKO EDL, albeit this difference approached statistical significance ($p = 0.07$). DAPC protein expression was similar between genotypes, with the exception of nNOS, which was 25% lower ($p < 0.05$) in SOL of MKO animals as compared to WT counterparts.

DAPC localization in the skeletal muscle of WT and MKO mice. We next investigated the expression and localization of key DAPC components through the use of immunofluorescence microscopy. In WT animals, β-DG expression was significantly higher (+115%) in SOL compared to EDL muscles (Fig. 4A, 4C). In contrast, dystrophin, γ-SG, nNOS, and laminin levels were similar

between muscle types (Fig. 4A, B, D-F). In MKO mice, nNOS expression was significantly higher in the SOL muscle, as compared to the EDL. The levels of other DAPC components were similar between EDL and SOL muscles, although statistical trends suggest that dystrophin ($p = 0.13$), β-DG ($p = 0.10$) and γ-SG ($p = 0.06$) expression were ~30-50% higher in the SOL muscle. nNOS levels were also higher in the SOL muscle of MKO mice versus their WT counterparts.

In WT animals, extrasynaptic sarcolemmal utrophin expression was significantly higher (148%) in the slow, oxidative SOL muscle compared to the fast, glycolytic EDL muscle (Fig. 5A-C). Utrophin levels were 52% lower ($p < 0.05$) in SOL muscles from MKO mice, as compared to SOL muscles from their WT littermates.

Expression of regulatory factors that control DAPC levels in muscle. To continue to comprehensively investigate the role of AMPK on DAPC gene expression, we examined the levels of regulatory molecules that participate in the transcriptional activation, as well as function, of the DAPC in skeletal muscle. PGC-1α mRNA content was ~2-2.5-fold higher ($p < 0.1$) in the SOL muscles relative to the EDL muscles in WT mice, as well as in MKO animals (Fig. 6A). CaMKII transcript levels were also modestly higher (+25%) in the SOL muscles compared to EDL muscles of WT mice, as well as in MKO animals (Fig. 6B). However, these data did not reach statistical significance. PGC-1α and CaMKII transcripts were similar between genotypes.

At the protein level, PGC-1α content was significantly greater (+100%) in the SOL muscles versus the EDL muscles of both WT and MKO animals (Fig. 6C, D). A pan CaMKII antibody, which identifies both α and β isoforms of the kinase, was employed to examine the protein content of the enzyme. In both WT and MKO groups, CaMKIIα levels were ~60% lower ($p < 0.05$) in the SOL muscles, as compared to the EDL muscles (Fig. 6C, E). In contrast, CaMKIIβ protein expression was significantly greater (+2.5-4-fold) in the SOL muscles versus the EDL muscles of WT animals, as well as in MKO mice (Fig. 6C, F). PGC-1α, CaMKIIα, and CaMKIIβ protein levels were similar between genotypes. The expression of the myogenic regulatory factors (MRFs) MyoD and myogenin were similar between muscle types in both WT and MKO animals (Fig. 6C, G, H). However, MyoD levels were 55-60% lower ($p < 0.05$) in the muscles from MKO mice, as compared to WT animals, while myogenin protein content was significantly lower (~50%) in the EDL muscles of MKO animals compared to their WT counterparts.

Subcellular localization of PGC-1α in skeletal muscle. To further investigate PGC-1α biology in relation to the role of AMPK in maintaining DAPC expression, we measured the abundance and subcellular localization of the transcriptional coactivator by employing immunofluorescence imaging. Analyses were performed in WT and MKO muscles, as well as muscles lacking PGC-1α, which served as a negative control, similar to the MKO samples in Figure 1B.

PGC-1α expression was 2.5-3-fold greater ($p < 0.05$) in SOL, as compared to EDL muscles of both genotypes (Fig. 7A, C), similar to our observations using immunoblotting assays (Fig. 6C, D). Statistical trends suggest that PGC-1α content was lower in the EDL ($p = 0.09$) and SOL ($p = 0.1$) muscles of MKO animals versus WT mice, albeit not significantly different (Fig. 7C). PGC-1α colocalization with laminin was employed to identify its presence at the muscle membrane, while PGC-1α and DAPI overlays were used to mark PGC-1α in the myonuclear compartment (Fig. 7A, B). PGC-1α staining within the myofiber but outside of myonuclei was considered cytosolic. In WT animals, SOL muscles displayed significantly lower expression of nuclear PGC-1α compared to EDL muscles, while cytosolic and membrane-associated PGC-1α were similar between muscle types (Fig. 7A, D). In MKO mice, SOL muscles contained a 4% lower ($p < 0.05$) expression of cytosolic PGC-1α versus EDL muscles, which was accompanied by a concomitant and significant 4% increase in PGC-1α abundance at the sarcolemma in SOL, as compared to EDL muscles (Fig. 7A, D). Additional strong statistical trends in the data suggest greater PGC-1α content in the membrane ($p = 0.05$) and myonuclear ($p = 0.06$) compartments of MKO SOL muscles, coincident with reduced amounts in the cytosol ($p = 0.1$), as compared to their WT counterparts (Fig. 7A, D).

Expression of mRNA stability regulators in skeletal muscle. The levels of DAPC components localized to the sarcolemma can be affected at various stages

of gene expression, including by the stability of their mRNAs prior to translation. In order to assess the impact of AMPK on factors that regulate DAPC post-transcriptional processing, we first executed a bioinformatic survey of the 3'-untranslated region (3'-UTR) of multiple DAPC components to assess the prevalence of possible binding sites for RNA-binding proteins (RBPs) that either enhance or reduce mRNA stability. We performed this analysis using UCSC Genome Browser[44] and RBPMap[45]. A number of RBP binding sites were identified in DAPC and utrophin 3'-UTRs, including consensus sequences for CUG triplet repeat RNA binding protein 1 (CUGBP1), Muscleblind-like protein 1 (MBNL1), KH-type splicing regulatory protein (KSRP), human antigen R (HuR), and AU-Rich binding factor 1 (AUF1). We then assessed the protein content of these RBPs in the muscles of WT and MKO mice. In the WT group, the data reveal higher levels ($p = 0.05$) of MBNL1 in the EDL compared to the SOL muscle (Fig. 8A, C), while the other RBPs displayed similar protein content between muscle types. In MKO mice, MBNL1 and CUGBP1 expression were 82% higher ($p < 0.05$) and 51% higher ($p = 0.09$), respectively, in the slow, oxidative SOL muscle, as compared to its faster, more glycolytic EDL counterpart (Fig. 8A-C). The abundance of all other RBPs examined was similar between muscle types in the MKO group. Finally, there was no difference in RBP levels between genotypes.

Discussion

In the current study we examined the role of AMPK in muscle DAPC biology. Confirming and extending earlier work[18,19,46] , our data demonstrate that slow, oxidative muscle has a higher expression of the DAPC and utrophin, as compared to faster, more glycolytic muscle. Generally, skeletal muscle AMPK was not essential for DAPC expression. However, the lack of AMPK resulted in altered levels of nNOS and extrasynaptic utrophin content, with the latter molecule exhibiting a significant downregulation in the SOL muscle. This decrease in utrophin occurred despite the upregulation of PGC-1α at the local membrane compartment. The maintenance of DAPC expression in MKO animals was not accompanied by compensatory increases in the content of a number of proteins that mediate the transcriptional or post-transcriptional control of DAPC levels. Given that chronic pharmacological AMPK activation augments DAPC content in skeletal muscle[9,10], our data therefore suggests that AMPK is sufficient, but not required, to impact DAPC levels. Our results also strongly support the hypothesis that AMPK is integral to the regulation of utrophin expression in muscle, particularly along the sarcolemma. This lends further credence to the examination of AMPK-mediated utrophin induction as a therapeutic modality in clinical situations where sarcolemmal utrophin upregulation would be therapeutically beneficial, such as in DMD.

We observed that AMPK preferentially accumulated in the cytosol, but also exhibited enrichment at the sarcolemma, with a small fraction remaining in nuclei. A significant portion of myocellular AMPK resides in close proximity to the muscle membrane, which suggests that the kinase executes important functions at the sarcolemma. Indeed, recent investigations have revealed that pharmacological AMPK activation promotes translocation of the Na^+/K^+-ATPase α_1 subunit to the sarcolemma, and increases activity of the ion pump[35]. Furthermore, Garbincius and Michele demonstrated that stretch-induced production of nitric oxide in cardiomyocytes was dependent on AMPK signalling following mechanotransduction from the DAPC[37]. Thus, our findings support the emergence of AMPK as an important component of the structure and function of the sarcolemmal compartment.

The aging-induced attenuation of skeletal muscle AMPK activity[29] is associated with alterations in the sarcolemmal environment, including reduced DAPC protein content[30–34]. Conversely, chronic pharmacological AMPK stimulation increases DAPC expression in skeletal muscle[9]. Moreover, the presence and function of the kinase at the sarcolemma has been recently established[35,37,47]. Based on this evidence, we therefore hypothesized that removal of muscle AMPK would decrease DAPC gene expression, and by extension reduce its membrane localization. We now report that DAPC transcript and protein levels, as well as its integrated sarcolemmal abundance, remain largely

unaffected in response to the genetic ablation of skeletal muscle AMPK. The exceptions were nNOS and utrophin, whose expression was altered in the muscles of MKO animals. nNOS mRNA was significantly lower in MKO muscles of both fiber types, while Western blotting detected diminished protein levels in only SOL muscles. Conversely, immunofluorescence measurements of nNOS detected significant elevations of the protein specifically at the sarcolemma. nNOS exists in four protein variants, including nNOSα, -β, -γ, and -μ[48]. nNOSα and -μ contain a postsynaptic density-95/discs large/zona occludens-1 homology (PDZ) domain, which allows binding of the enzymes to the cell membrane. In contrast, nNOSβ and -γ, both lacking the PDZ domain, are localized to the cytosol. nNOSμ, which contains the PDZ sequence, is predominantly expressed in skeletal muscle and is bound to the DAPC[49,50]. In the current study, we employed the use of reagents to detect pan-nNOS gene expression at the mRNA and protein levels, regardless of variant identity. Thus, the discordant nNOS mRNA and protein expression detected via complementary analyses is likely due, in part, to the existence of nNOS variants in skeletal muscle. Additionally, unlike the Western blotting assay that assesses nNOS levels in a muscle homogenate, our immunofluorescence measurements account specifically for sarcolemmal nNOS. Therefore, this metric is most reflective of nNOSμ expression, which maintains its PDZ domain adjacent to the sarcolemmal DAPC. The data reveal a selective upregulation of sarcolemmal nNOS in the SOL muscles of MKO animals, as compared to their

WT counterparts. It is reasonable to speculate that the enrichment of sarcolemmal nNOS content in these muscles is a compensatory upregulation in response to the absence of AMPK. This adaptation would likely occur in order to maintain higher basal levels of perfusion in slower, more oxidative muscles[51,52], thereby mitigating the loss of capillarization in MKO SOL muscles observed in a previous study[4].

It is well known that both whole muscle and extrasynaptic utrophin are more highly expressed in slow, oxidative muscles versus faster, more glycolytic tissues[19,25,53], and results from SOL versus EDL muscles in the present study affirm this relationship. The removal of skeletal muscle AMPK selectively attenuated sarcolemmal utrophin expression in the slow, oxidative SOL muscles. While we were unable to recognize this perturbation from the immunoblotting results, targeted immunofluorescence assessment revealed the specific reduction in extrasynaptic utrophin. Molecules such as PPARβ/δ, as well as CN/nuclear factor of activated T-cells (NFAT) signalling confer sarcolemmal expression to utrophin[11,25]. For example, chronic pharmacological PPARβ/δ activation in mdx mice increases the frequency of slow, oxidative myofibers, as well as upregulates sarcolemmal utrophin content[11]. As previous studies have identified binding between PPARβ/δ and AMPK that was associated with the stimulation of gene expression indicative of slower, more oxidative characteristics[54,55], a lack of interaction between these molecules may explain, at least in part, why ablation of

AMPK may lead to the downregulation of extrasynaptic utrophin in the SOL muscle. It is important to note that enriched sarcolemmal utrophin is clinically beneficial for DMD, as expansion beyond the typical subsynaptic expression of the protein serves to compensate for dystrophin loss along the muscle membrane[56]. AMPK gain-of function studies[14,9,10,57,58], and now our loss-of-function investigation, underscore the critical role of AMPK in regulating the therapeutically relevant expression and localization of utrophin in skeletal muscle.

We suspected that the maintenance of DAPC content and localization in MKO muscles might be the result of the upregulation of alternative signalling and regulatory factors that mediate DAPC biology. To this end, we examined the expression of CaMKII, PGC-1α, as well as the MRFs MyoD and myogenin, which participate, at various levels, to the transcriptional activation of DAPC components[59,22,20]. CaMKII and PGC-1α protein content were similar between muscles from WT and MKO animals. In addition, SIRT1 levels were also similar between genotypes (data not shown). These data suggest that AMPK is not required for homeostatic protein expression of these molecules in skeletal muscle, and that basal CaMKII and PGC-1α levels are sufficient to maintain, or contribute to the maintenance of, DAPC content in the absence of AMPK. In marked contrast, MyoD and myogenin protein levels were significantly reduced in EDL and SOL muscles of MKO mice compared to their WT counterparts. Recent work has demonstrated a critical role for AMPK in muscle regeneration, as removal of

AMPKα1 results in delayed and incomplete muscle repair in response to cytotoxic injury[60–62] . The dysregulated myogenic program in AMPKα1 mice was associated with the attenuated expression of MRFs, including MyoD and myogenin, as compared to their WT counterparts[63]. The myopathy previously observed in the MKO animals utilized in the current study involves the consistent presence of split fibers[4], which can arise from defects in muscle regeneration[64]. However, the deficit in MRF expression observed here was not accompanied by a corresponding loss of DAPC expression. Dumont et al. (2015) recently observed that β-DG and dystrophin are necessary for the proper association of cell polarity regulators and the ability for satellite cells to asymmetrically divide[65]. This function of the DAPC in the formation of myogenic progenitors suggests that its presence at the sarcolemma precedes the formation of mature satellite cells. Therefore, with the emergence of AMPK as a governor of muscle regeneration, it will be important for future studies to further clarify the roles of AMPK and the DAPC during execution of the myogenic program.

Within skeletal muscle, PGC-1α protein content, as well as its subcellular distribution, are critical variables when considering the function of the coactivator[66–68]. For example, the synaptic localization of PGC-1α is associated with enhanced expression of NMJ genes, including utrophin and AChR subunits[69]. Although the majority of the enzyme was situated in the cytosolic compartment, we observed a modest, but significant shift in PGC-1α

accumulation from the cytosol to the sarcolemma in SOL muscles from MKO animals compared to WT mice. This translocation suggests an enhanced activity of the enzyme at the membrane compartment. Considering the selective attenuation of extrasynaptic utrophin in the SOL muscles of MKO animals, as well as the significant mitochondrial defects in MKO mice[38], it is likely that this translocation of PGC-1α to the membrane represents a cellular adjustment in an effort to maintain both sarcolemmal utrophin levels and subsarcolemmal mitochondrial gene expression. Additionally, PGC-1α has the ability to modulate other aspects of the sarcolemmal environment, including inducing critical components of the satellite cell niche fibronectin and tenascin C[70]. Thus, enhanced, local PGC-1α activity may compensate for the lack of AMPK in administering the sarcolemmal compartment, however more work is required to confirm this assumption.

Post-transcriptional processing of mRNAs is an important regulatory step in the gene expression pathway. Numerous RBPs, by sequestering, folding, or chaperoning transcripts, have the capacity to influence critical events in gene expression such as the nuclear export, subcellular localization, degradation or translation of mRNAs[71,72]. Within skeletal muscle, gene expression is regulated post-transcriptionally by various RBPs, including HuR, AUF1, KSRP, MBNL1 and CUGBP1. Generally, HuR enhances mRNA stability, AUF1 and KSRP act to destabilize transcripts, while MBNL1 and CUGBP1 mediate alternative splicing

of pre-mRNAs[73,74]. Since the DAPC was largely unaffected by the removal of AMPK in skeletal muscle, we complemented our investigation of alternative transcriptional mechanisms by examining whether adaptations in RBP content contributed at the post-transcriptional level to the maintenance of the DAPC. However, similar to the DAPC, we detected no significant differences between genotypes in RBP expression levels. While these data suggest that AMPK has no influence on RBP content in skeletal muscle, they also imply that these RBPs do not contribute to the perturbations in utrophin, nNOS, or MRF expression in MKO muscles. Nevertheless, it is possible that the activity of RBPs may be altered in the absence of AMPK-dependent functional modifications. In support of this, work by Wang and colleagues demonstrated that transfection of constitutively active AMPK results in reduced cytoplasmic accumulation of HuR and destabilization of several HuR target transcripts in colorectal cancer cells[75], suggesting that AMPK activity can regulate RBPs and the subsequent stability of target transcripts. Indeed, phosphorylation of RBPs is a known modulator of their function. For example, phosphorylation of KSRP by the mitogen-activated protein kinase p38 results in its decreased cytoplasmic accumulation and function[76]. This is particularly relevant here since KSRP regulates skeletal muscle utrophin expression[77]. Although speculative, the possibility remains therefore, that adaptations to RBP function may be contributing to the maintenance of the DAPC in the absence of AMPK.

In summary, our data show that skeletal muscle-specific ablation of AMPK left the DAPC unaffected, with the exception of nNOS upregulation. Thus, when previous evidence demonstrating DAPC induction in response to chronic pharmacological AMPK stimulation[14,9,10,78] are considered with these data from an AMPK loss-of-function model, we conclude that AMPK is sufficient, but not necessary for DAPC expression in skeletal muscle. The diminished expression of MRFs MyoD and myogenin in muscles from MKO animals suggest impairment in regenerative capacity, corroborating previous findings of myopathy in AMPK KO animals. Furthermore, we observed decrements in extrasynaptic utrophin expression despite the adaptive accumulation of PGC-1α at the plasma membrane. These data indicate that AMPK is required to maintain sarcolemmal utrophin in slow, oxidative fibers. Further study is therefore warranted in order to determine the role of AMPK in the expression of utrophin and the utrophin-associated protein complex at the NMJ.

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Figure Legends

Figure 1. Subcellular localization of AMP-activated protein kinase in skeletal muscle. *A,* Representative Western blot of phosphorylated AMP-activated protein kinase (p-AMPK) in extensor digitorum longus (EDL) and soleus (SOL) muscles from wild-type (WT) mice and those with skeletal muscle-specific deletion of the AMPK β1 and β2 subunits (MKO). A typical ponceau S stain is displayed to demonstrate equal loading. *B,* Representative immunofluorescence images of laminin (Lam), AMPK, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) to identify myonuclei, and overlay, in WT EDL and SOL muscles, as well as in muscle from MKO mice. Note the absence of AMPK in the MKO samples. *C,* Graphical summary of AMPK subcellular localization in cytosolic (CYT), nuclear (NUC), and membrane (MEM) compartments of WT EDL and SOL muscles. n = 8; *, $p < 0.05$ vs. NUC; #, $p < 0.05$ vs. MEM.

Figure 2. DAPC transcript levels in skeletal muscle. Expression levels of dystrophin (*A*), utrophin (*B*), β-dystroglycan (*C*), α-sarcoglycan (*D*), β-sarcoglycan (*E*), γ- sarcoglycan (*F*), neuronal nitric oxide synthase (nNOS; *G*), laminin (*H*) and biglycan (*I*) mRNA content in EDL and SOL muscles from WT and MKO animals. All values are relative to WT EDL. n = 8; *, $p < 0.05$ vs. EDL within genotype; #, $p < 0.05$ vs. corresponding muscle of WT.

Figure 3. DAPC protein content in skeletal muscle. *A*, Representative Western blots of dystrophin, utrophin, β-dystroglycan (β-DG), γ-sarcoglycan (γ-SG), nNOS, and laminin, as well as a typical ponceau S stain. Graphical summaries of dystrophin (*B*), utrophin (*C*), β-DG (*D*), γ-SG (*E*), nNOS (*F*) and laminin (*G*) protein content in EDL and SOL muscles from WT and MKO mice. All values are relative to WT EDL. Dashed line in representative blots indicates rearrangement of non-contiguous lanes within the same image. $n = 8$; *, $p < 0.05$ vs. EDL within genotype; #, $p < 0.05$ vs. corresponding muscle in WT.

Figure 4. DAPC localization in skeletal muscle. *A*, Representative immunofluorescence images of dystrophin (Dys), β-DG, γ-SG, nNOS, and Lam. Graphical summaries of dystrophin (*B*), β-DG (*C*), γ-SG (*D*), nNOS (*E*), and laminin (*F*) levels in EDL and SOL muscles from WT and MKO mice. All values are relative to WT EDL. $n = 8$; *, $p < 0.05$ vs. EDL within genotype; #, $p < 0.05$ vs. corresponding muscle in WT.

Figure 5. Utrophin localization in skeletal muscle. *A*, Representative immunofluorescence images of utrophin (UTR), α-bungarotoxin (α-BTX) to identify acetylcholine receptors at neuromuscular junctions, myonuclei (DAPI), and overlay in EDL and SOL muscles from WT and MKO mice. *B*, Higher magnification images of UTR (*i*), α-BTX (*ii*), DAPI (*iii*) and overlay (*iv*), which

identifies both synaptic and extrasynaptic utrophin content within myofibers. Arrows depict areas of sarcolemmal utrophin expression. *C,* Graphical summary of extrasynaptic skeletal muscle utrophin expression, with values relative to WT EDL. $n = 8$. *, $p < 0.05$ vs. EDL within genotype. #, $p < 0.05$ vs. corresponding muscle in WT.

Figure 6. Regulatory factors that control DAPC expression in skeletal muscle. mRNA content of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α; *A*) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; *B*) in EDL and SOL muscles from WT and MKO mice. *C,* Representative Western blots of PGC-1α, CaMKIIα, CaMKIIβ, MyoD, and myogenin, as well as a typical Ponceau S stain. Graphical summaries of PGC-1α (*D*), CaMKIIα (*E*), CaMKIIβ (*F*), MyoD (*G*), and myogenin (*H*) protein expression levels. All values are relative to WT EDL. $n = 8$.*, $p < 0.05$ vs EDL within genotype; #, $p < 0.05$ vs corresponding muscle in WT.

Figure 7. Subcellular localization of PGC-1α in skeletal muscle. *A,* Representative immunofluorescence images of PGC-1α, Lam, myonuclei (DAPI) and overlay in EDL and SOL muscles from WT and MKO mice. *B,* Higher magnification images of PGC-1α (*i*), Lam (*ii*), DAPI (*iii*) and overlay (*iv*), which display perinuclear sarcolemmal PGC-1α accumulation. Arrows depict areas of

concentrated PGC-1α expression at the sarcolemma. *C,* Graphical summary of whole muscle PGC-1α expression. *D,* Graphical summary of PGC-1α subcellular localization in cytosolic (CYT), nuclear (NUC), and membrane (MEM) compartments of EDL and SOL muscles from WT and MKO mice. *Inset,* Magnified view of NUC PGC-1α localization. All values are relative to WT EDL. n = 8.*, $p < 0.05$ vs EDL within genotype.

Figure 8. RNA-binding proteins involved in DAPC mRNA stability. *A,* Typical Western blots of CUG triplet repeat RNA binding protein 1 (CUGBP1), muscleblind-like protein 1 (MBNL1), human antigen R (HuR), KH-type splicing regulatory protein (KSRP), AU-rich binding factor 1 (AUF1), and representative Ponceau S stain. Graphical summaries of CUGBP1 (*B*), MBNL1 (*C*), HuR (*D*), KSRP (*E*), and AUF1 (*F*) protein levels. All values are relative to WT EDL. n = 4-8.*, $p < 0.05$ vs EDL within genotype.

Figure 1

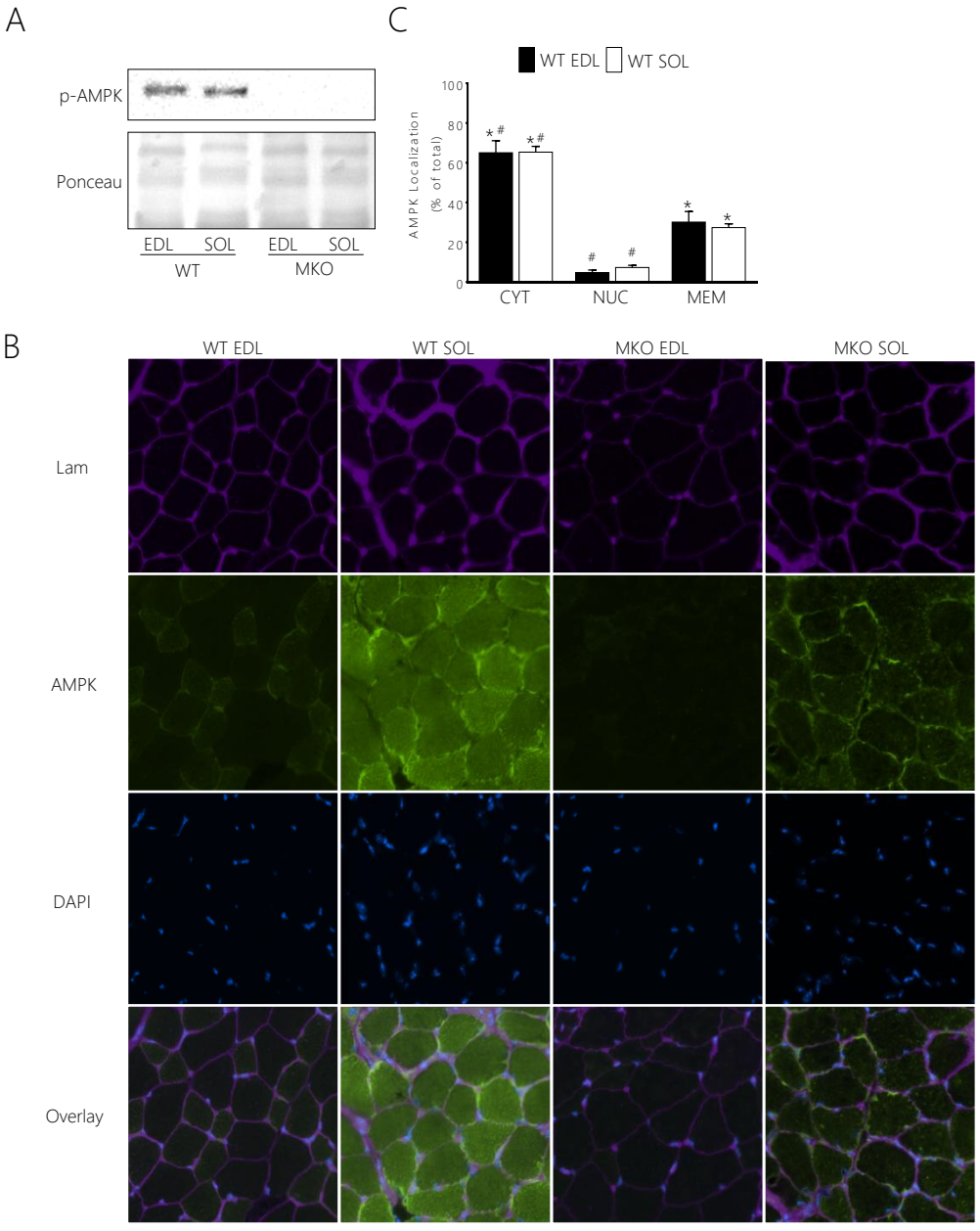

A
p-AMPK
Ponceau
EDL
SOL
EDL
SOL
WT
MKO
C
WT EDL
WT SOL
AMPK Localization (% of total)
100
80
60
40
20
0
CYT
NUC
MEM
B
WT EDL
WT SOL
MKO EDL
MKO SOL
Lam
AMPK
DAPI
Overlay

Figure 2

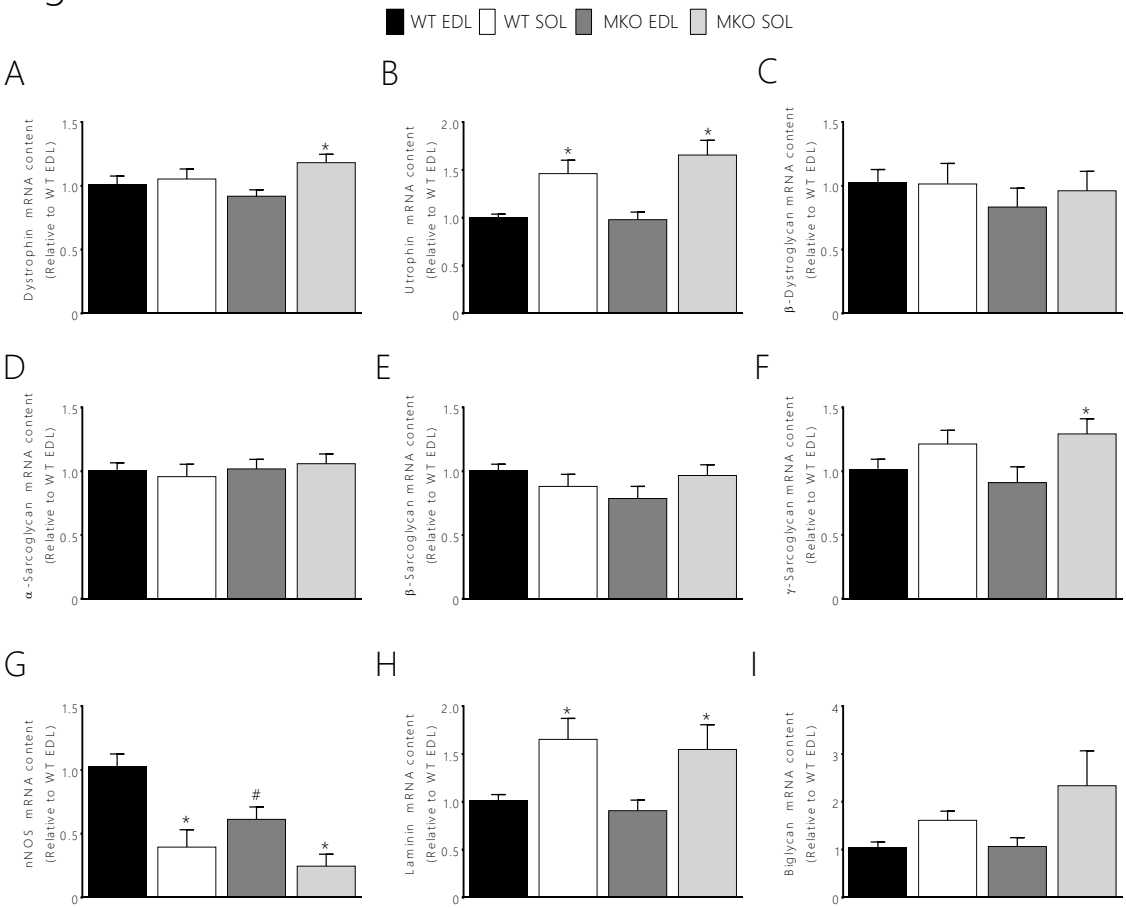

WT EDL
WT SOL
MKO EDL
MKO SOL
A
Dystrophin mRNA content (Relative to WT EDL)
B
Utrophin mRNA content (Relative to WT EDL)
C
β-Dystroglycan mRNA content (Relative to WT EDL)
D
α-Sarcoglycan mRNA content (Relative to WT EDL)
E
β-Sarcoglycan mRNA content (Relative to WT EDL)
F
γ-Sarcoglycan mRNA content (Relative to WT EDL)
G
nNOS mRNA content (Relative to WT EDL)
H
Laminin mRNA content (Relative to WT EDL)
I
Biglycan mRNA content (Relative to WT EDL)

Figure 3

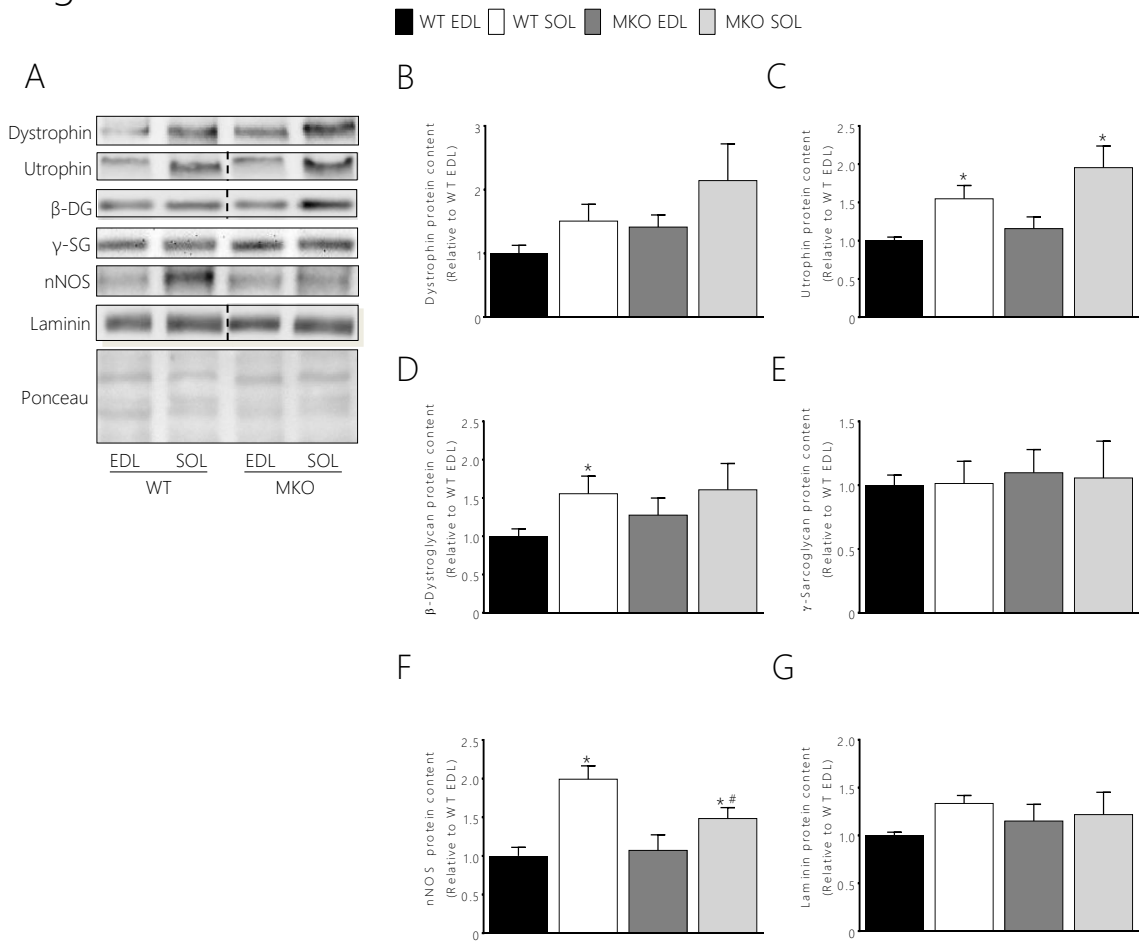

WT EDL
WT SOL
MKO EDL
MKO SOL
A
Dystrophin
Utrophin
β-DG
γ-SG
nNOS
Laminin
Ponceau
EDL
SOL
EDL
SOL
WT
MKO
B
Dystrophin protein content (Relative to WT EDL)
C
Utrophin protein content (Relative to WT EDL)
D
β-Dystroglycan protein content (Relative to WT EDL)
E
γ-Sarcoglycan protein content (Relative to WT EDL)
F
nNOS protein content (Relative to WT EDL)
G
Laminin protein content (Relative to WT EDL)

Figure 4

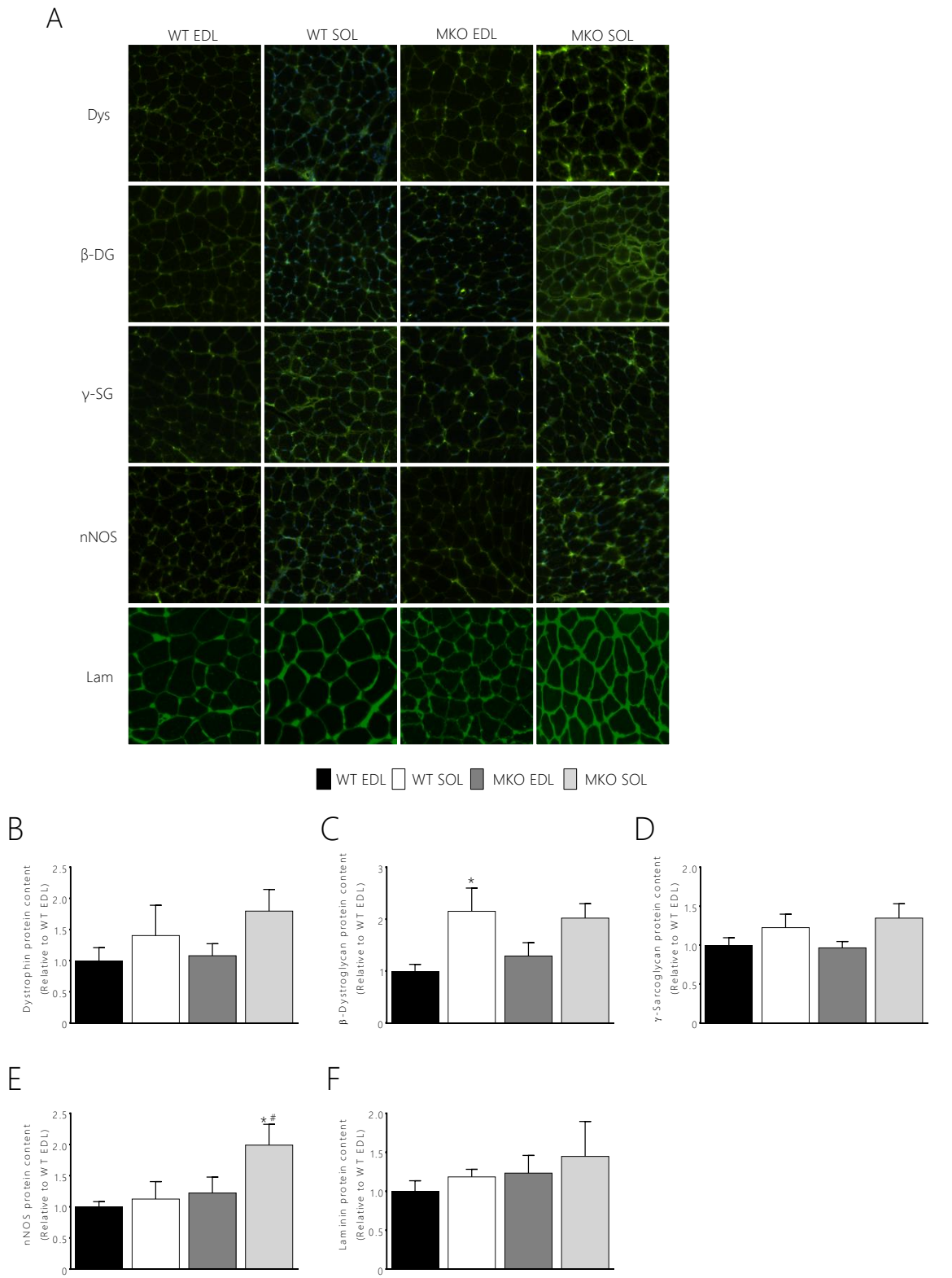

A
WT EDL
WT SOL
MKO EDL
MKO SOL
Dys
β-DG
γ-SG
nNOS
Lam
WT EDL
WT SOL
MKO EDL
MKO SOL
B
Dystrophin protein content (Relative to WT EDL)
C
β-Dystroglycan protein content (Relative to WT EDL)
D
γ-Sarcoglycan protein content (Relative to WT EDL)
E
nNOS protein content (Relative to WT EDL)
F
Laminin protein content (Relative to WT EDL)

Figure 5

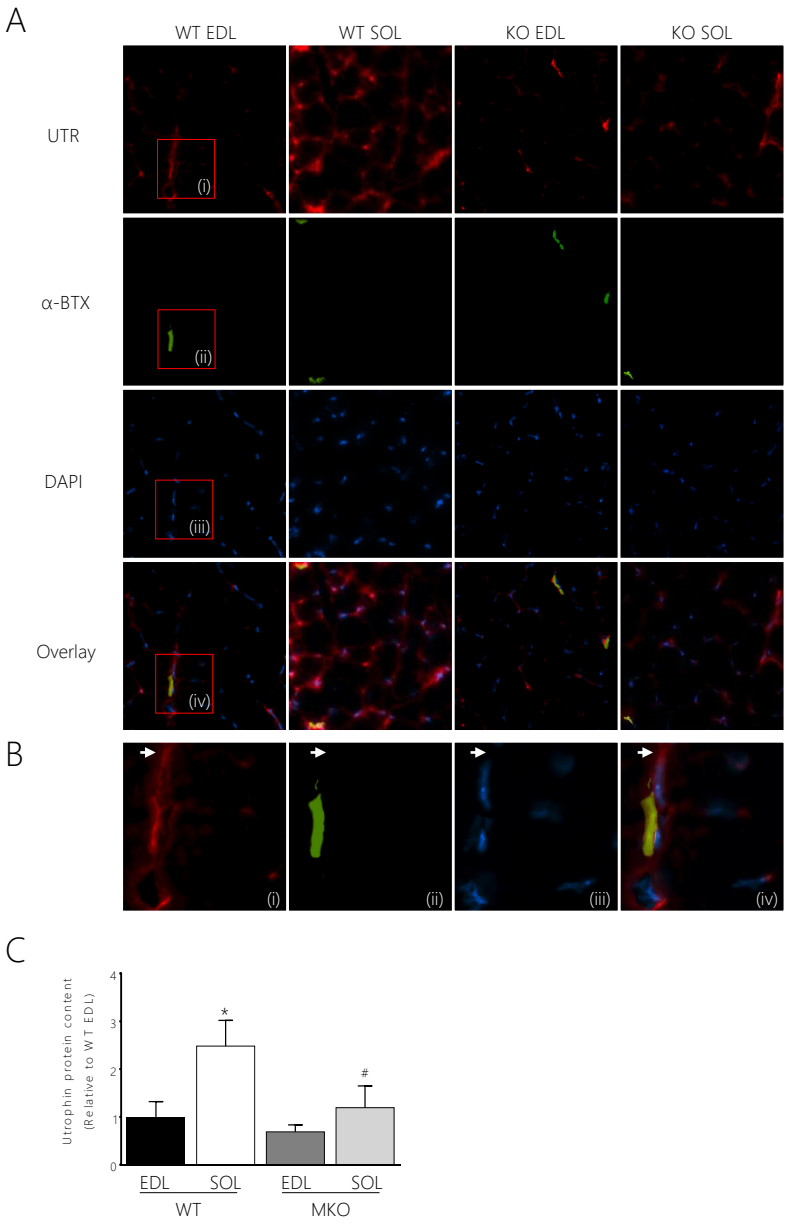

A
WT EDL
WT SOL
KO EDL
KO SOL
UTR
α-BTX
DAPI
Overlay
(i)
(ii)
(iii)
(iv)
B
C
Utrophin protein content
(Relative to WT EDL)
4
3
2
1
0
*
#
EDL
SOL
EDL
SOL
WT
MKO

Figure 6

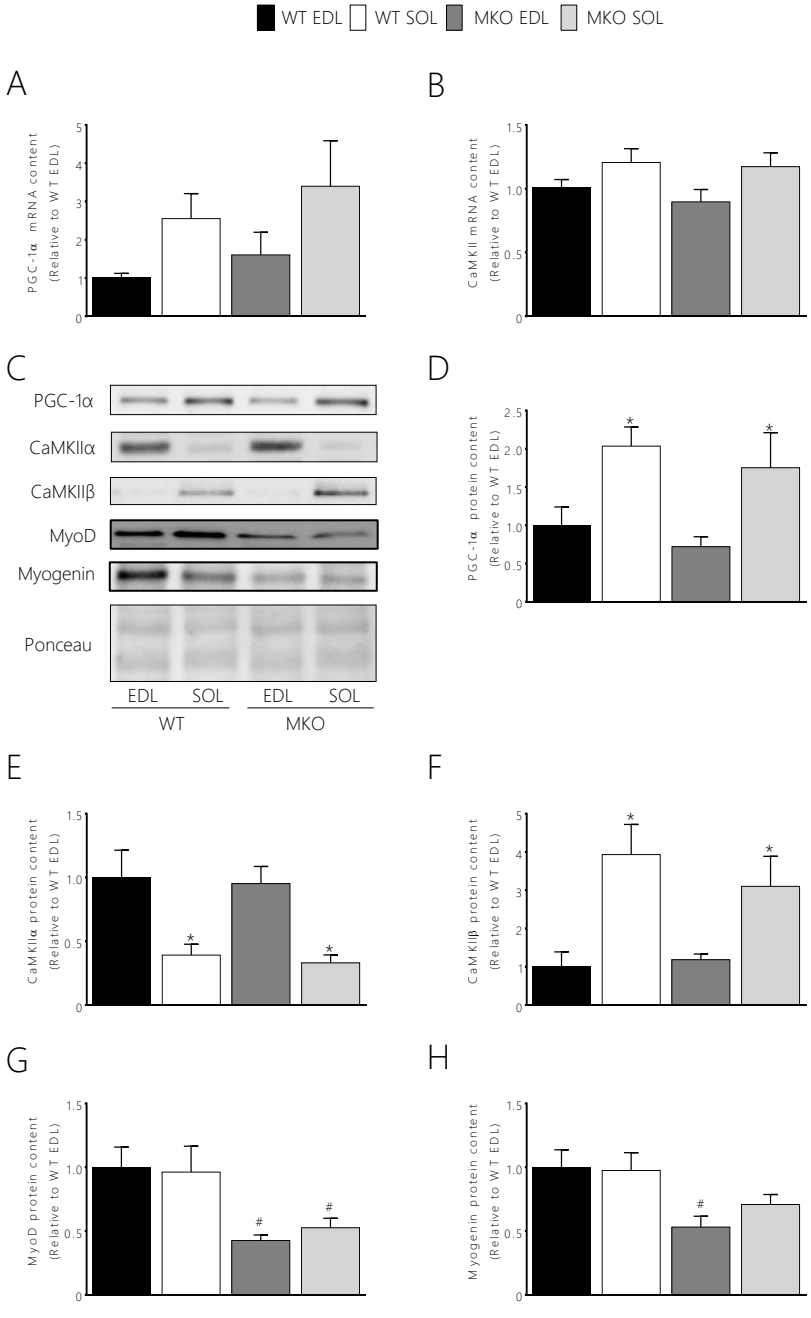

WT EDL
WT SOL
MKO EDL
MKO SOL
A
PGC-1α mRNA content (Relative to WT EDL)
B
CaMKII mRNA content (Relative to WT EDL)
C
PGC-1α
CaMKIIα
CaMKIIβ
MyoD
Myogenin
Ponceau
EDL
SOL
EDL
SOL
WT
MKO
D
PGC-1α protein content (Relative to WT EDL)
E
CaMKIIα protein content (Relative to WT EDL)
F
CaMKIIβ protein content (Relative to WT EDL)
G
MyoD protein content (Relative to WT EDL)
H
Myogenin protein content (Relative to WT EDL)

Figure 7

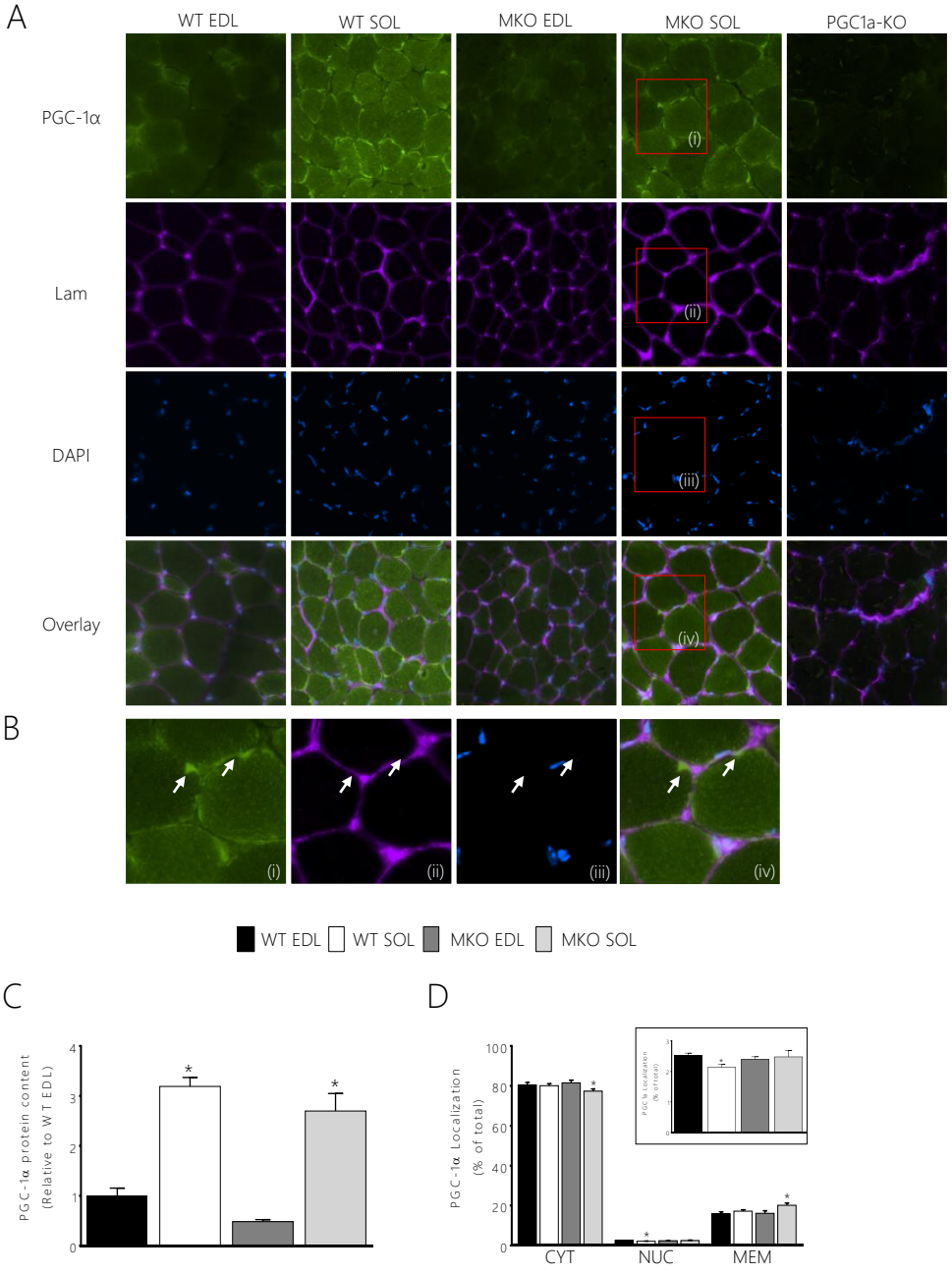
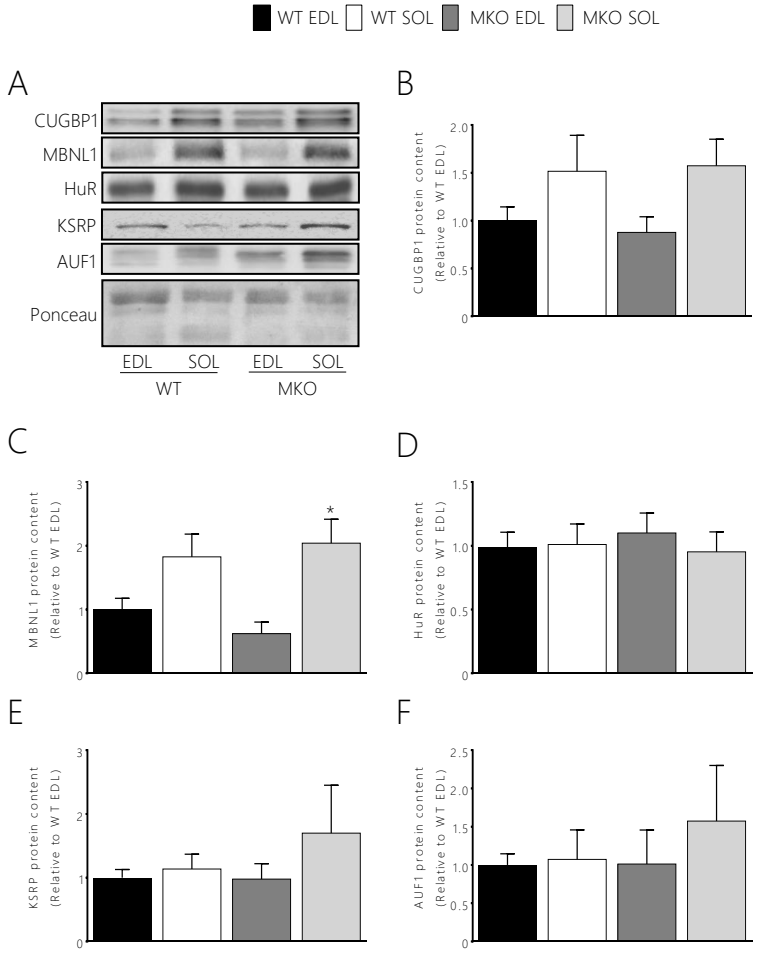

A
WT EDL
WT SOL
MKO EDL
MKO SOL
PGC1a-KO
PGC-1α
Lam
DAPI
Overlay
(i)
(ii)
(iii)
(iv)
B
(i)
(ii)
(iii)
(iv)
WT EDL
WT SOL
MKO EDL
MKO SOL
C
PGC-1α protein content
(Relative to WT EDL)
0
1
2
3
4
*
*
D
PGC-1α Localization
(% of total)
0
20
40
60
80
100
*
*
*
CYT
NUC
MEM

Figure 8


WT EDL
WT SOL
MKO EDL
MKO SOL
A
CUGBP1
MBNL1
HuR
KSRP
AUF1
Ponceau
EDL
SOL
EDL
SOL
WT
MKO
B
CUGBP1 protein content (Relative to WT EDL)
C
MBNL1 protein content (Relative to WT EDL)
*
D
HuR protein content (Relative to WT EDL)
E
KSRP protein content (Relative to WT EDL)
F
AUF1 protein content (Relative to WT EDL)