Investigating the Pattern of Coordination between the Sarcoplasmic Reticulum Ca^{2+} Handling Channels and Calcineurin/NFAT Signalling Pathway in Distinct Mouse Models and Approaches of Muscular Dystrophy Studies

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complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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ABSTRACT

Investigating the Pattern of Coordination between the Sarcoplasmic Reticulum Ca^{2+} Handling Channels and Calcineurin/NFAT Signalling Pathway in Distinct Mouse Models and Approaches of Muscular Dystrophy Studies

Mohammad Hadi Salah

Duchenne muscular dystrophy is a progressive neurodegenerative muscle disease that makes the patients wheelchair-dependent around the age of 12 and eventually causes death in the late twenties due to lung and/or heart failure. A functional dystrophin is absent in DMD patients which makes their muscle cells prone to physical damage by every single movement. A homolog of this fundamental protein is utrophin which is known to be able to partially compensate for the lack of dystrophin. Hence, up-regulation of this protein has been promising for years as a strategy to improve this disorder. It has been repetitively proved that this approach could be achievable via stimulation of calcineurin/NFAT signalling pathway which in turn promotes the oxidative capacity of the muscle. Also, pharmacological induction of the Adenosine monophosphate - activated protein kinase pathway can provoke the slow oxidative myogenic program. Nevertheless, recent evidence shows the capability of the Heat Shock Protein 70 to ameliorate the dystrophic phenotype by preserving the function of sarco/endoplasmic reticulum Ca^{2+} ATPase channels independent of utrophin. Thus in this study, we sought to shed light on the underlying mechanisms by which intracellular Ca^{2+} oscillations coordinate with calcineurin/NFAT signalling in these adaptive responses. Our data suggest an inhibitory effect of AMPK activators on calcineurin activity and further prove the independence of HSP70 regulation from the calcineurin /NFAT pathway. Moreover, we were able to represent a more detailed understanding of the way Ca^{2+} handling proteins determine the activity rate of calcineurin in response to altered cytosolic Ca^{2+} oscillations. Collectively, our results may bring the scientific community one step closer to finding a comprehensive approach to cure or improve the dystophic disorders in general and duchenne muscular dystrophy in particular.

PREAMBLE

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At last, I would like to thank my parents, my brother, and my friends for their support, encouragement, and amity during the past couple of years.

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LIST OF ABBREVIATIONS

AICAR: AMPK Activator 5-aminoimidazole-4-carboxamide-1-β-D-Ribofuranoside

Akt: Protein Kinase B

AMPK: Adenosine Monophosphate (AMP) - activated protein Kinase

Ca^{2+}: Calcium

$[Ca^{2+}]i$: Intracellular Calcium

CaM: Calmodulin

CaMBP: Calmodulin Binding Protein

CLN: Centrally Located Nuclei

Cn: Calcineurin

CnA*: Constitutively Active Cn

DAPC: Dystrophin-Associated Protein Complex

DKO: Double-knock out

DMD: Duchenne Muscular Dystrophy

EDL: Extensor Digitorum Longus

Gas: Gastrocnemius

GSK3β: Glycogen Synthase Kinase 3 beta

HSP70: Heat Shock Protein-70

MET: Metformin

NFAT: Nuclear Factor of Activated T-cells

NF-κB: Nuclear Factor-κB

p-Akt: Phosphorylated Protein Kinase B

p-GSK3β: Phosphorylated Glycogen Synthase Kinase 3 beta

PLN: Phospholamban

PV: parvalbumin

RCAN: Regulator of Calcineurin

RYR1: Ryanodin Rceptor-1

SERCA: Sarco/Endoplasmic Reticulum-Ca^{2+}-ATPase

SLN: Sarcolipin

Sol: Soleus

SR: Sarcoplasmic Reticulum

TA: Tibialis Anterior

TG: Transgenic

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1. INTRODUCTION

1.1 - *DUCHENNE MUSCULAR DYSTROPHY*

Duchenne muscular dystrophy (DMD) is a progressive neurodegenerative muscle disease caused by a recessive loss of function mutation in the dystrophin gene located on the short arm of X chromosome[1,2]. Each year, one in 3600-6000 newborn males are diagnosed for this disorder. Individuals with DMD begin to show symptoms earliest at the age of three, become wheelchair dependent at around the age of 12 and eventually die in their late twenties due to lung and/or heart failure[2,3]. Several redundant approaches to amend the severity of the disorder have been developed, yet no cure or effective treatment has been reported though.

1.1.1 - *DMD Etiology*

In DMD cases, the gene coding for the 427 kDa dystrophin protein is mutated resulting in either a truncated small non-functional protein or the deletion of the gene[4,5]. A full length skeletal muscle isoform of dystrophin protein is made up of four domains including an N-terminal actin binding domain, a rod domain, a cysteine-rich domain, and a carboxyl-terminal domain[6].

This large cytoskeletal protein associates with various proteins to form the dystrophin-associated protein complex (DAPC) which provides a link between the muscle membrane and the actin filaments (Fig. 1)[2]. In other words, it links the extracellular matrix to the intracellular microfilament network. This is crucial for the stability and integrity of the membrane during contraction and relaxation of muscle[7]. Accordingly, lack of dystrophin in DMD patients results in a leaky membrane that causes muscle cells to be replaced by adipose and scar tissue[8,9]. This along with degeneration of muscle fibres makes the muscle non-resistant to mechanical pressure produced by walking and as a result exacerbates the status of the disease as it progress[10-12].

In molecular medicine, there are three known markers for diagnosis of muscle damage. First, and the most common, is the increase of muscle creatine kinase levels in the blood (serum) of patients who are developing a muscle myopathy due to the efflux from the leaky sarcolemma[13-15]; secondly is central localization of muscle nuclei, and thirdly is the aberrant size of muscle fibres known as fibre-size variability [15,16].

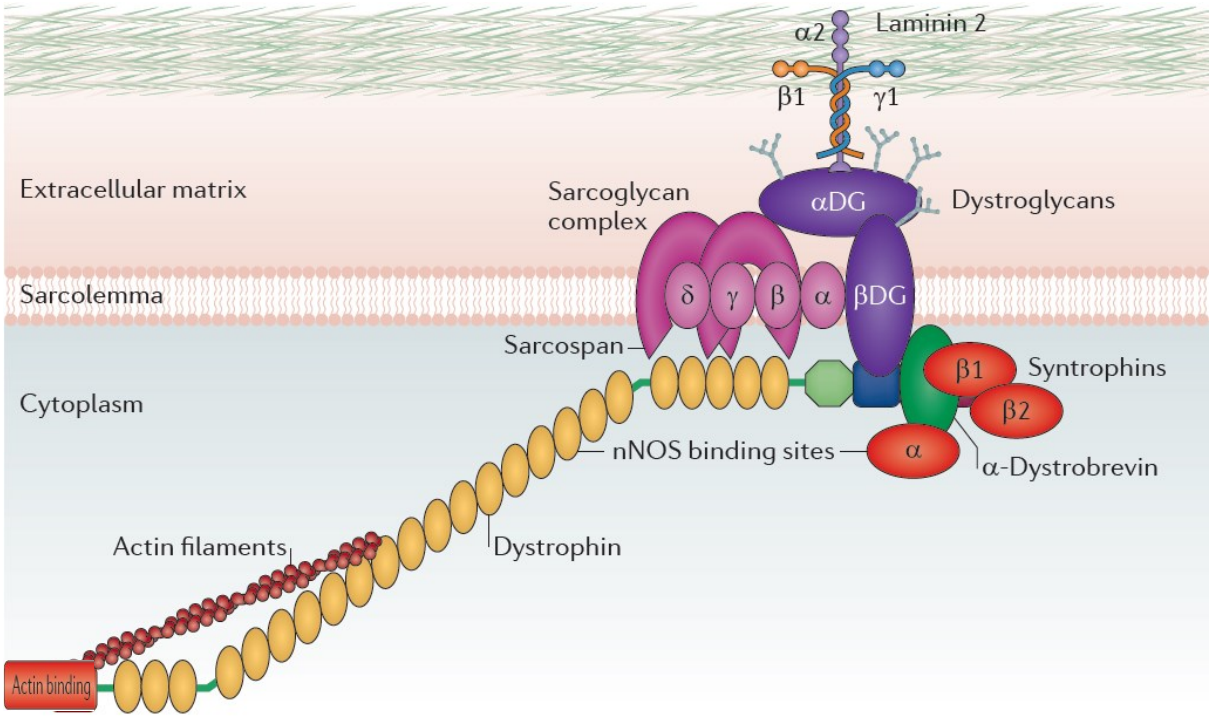

Fig. 1 The schematic structure of the dystrophin-associated protein complex (DAPC)- (adapted from[2]). Using its N-terminal actin binding domain, the large dystrophin protein links the actin filaments to the membrane.

1.1.2 - *The mouse model for DMD*

In 1983 a group of researchers announced that using enzyme assays they had found a spontaneous mutation arisen in their inbred C57BL/10 colony of mice after 5 generations. This mutation had caused a significant increase in serum levels of protein kinases such as creatine kinase and pyruvate kinase indicative of potential muscle damage[17]. This was confirmed by the presence of centrally localized nuclei showed by hematoxylin and eosin stained muscle cross sections. Finally, the mutated gene (i.e. dystrophin), was located on the X chromosome by positional cloning[17]. Later on, the sequence of dystrophin cDNA from normal and mutated mice revealed that a point mutation in mouse *mdx* gene resulted in a termination codon which prematurely stops the translation[18].

The mice from this model of DMD, which are named *mdx*, are histologically normal early after birth, but after 3-4 weeks of development they begin to develop cell and molecular abnormalities similar to those in DMD patients[19-21]. A study on *mdx* mice showed a reduction of their life span compared to wild-type mice, resembling the DMD cases regardless of the fact that *mdx* mice never lose ambulation[22].

1.1.3 - *DMD treatment strategies*

Glucocorticoids such as prednisone and deflazacort have been conventionally used to treat DMD for about 50 years[23] and remain clinically the most beneficial treatment for the patients due to their ability to improve muscle strength by delaying the rapid progression of muscle weakness and as a result rendering the patients more ambulatory up to approximately two years[24]. There is still no consensus about the exact mechanism of action of these steroids. Some researchers suggest that glucocorticoids bind to their receptors and trigger a cascade of pathways that eventually inhibit inflammatory factors such as NF-κB, therefore reducing fibrosis[25]. Other studies show that corticoids can induce myoblast proliferation by up-regulating insulin-like growth factors and therefore increase muscle fibre regeneration[26]. *In_vitro* studies have shown that utrophin, the homologue of dystrophin, can be either up-regulated or stabilized by corticoid treatment at both transcriptomic and preoteomic levels[27].

There is also the exon skipping technique that attempts to bring the out of frame dytrophin gene sequence in frame so that a smaller but functional dystrophin can be translated[28]. However, there are many clinical and theoretical issues with that, spanning from hurdles of the delivery route of the anti-sense oligonucleotides to the need of personalized and mutation-specific production of such oligonucleotides[2].

1.2 - *Utrophin up-regulation*

Utrophin is the autosomal homologue of Dystrophin which shares highly similar structure (i.e. 80% identical protein sequence) and function[29]. The N-terminal domain of utrophin contains an actin-binding site and therefore may compensate for the absence of dystrophin[30,31]. During the early stages of differentiation and development of an embryo, the two isoforms of utrophin are expressed prior to dystrophin, encompassing muscle cells membrane (utrophin-A) and blood vessels (utrophin-B). But as the embryo matures, utrophin-A is substituted by dystrophin and disappears or gets restricted to neuromuscular junctions[32]. DMD patients show higher levels of utrophin (Fig. 2)[4] and that was the start point for the scientists to focus on developing strategies aimed at up-regulating its expression.

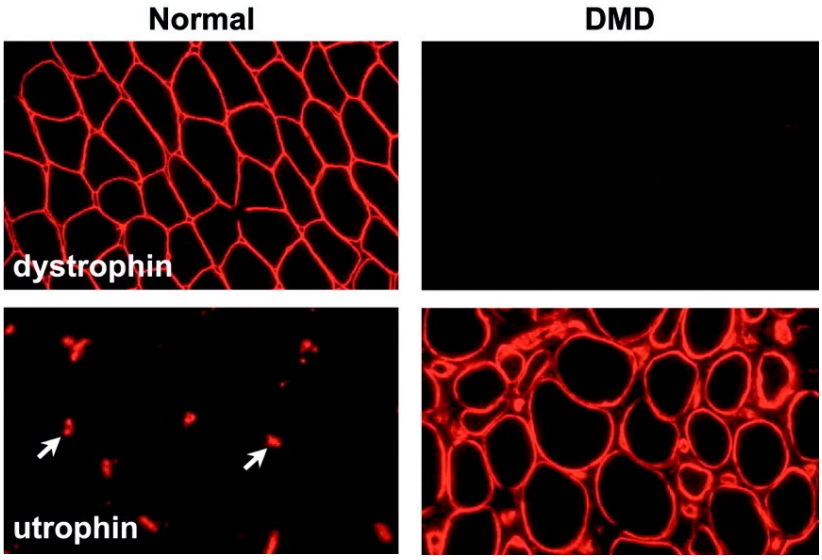

Fig. 2 The expression of utrophin in DMD vs normal- (adapted from[4]). Cross-sections of the muscles of DMD and normal human stained for dystrophin and utrophin shows significantly higher levels of utrophin in DMD. As it could be seen, when dytrophin is present (i.e. normal healthy) utrophin is expressed in very low levels.

1.2.1 - *Fibre-specific expression of utrophin*

Skeletal muscles are composed of four types of fibres among which three are type 2 fast glycolytic and one is type 1 slow oxidative[33]. Soleus muscle is predominantly made up of type 1 slow fibres ranging from 64 to 100 percent (the other being type 2a fibres)[34]. It is revealed that utrophin protein levels are 3 to 4 times higher in soleus compared to the fast extensor digitorium longus (EDL) muscle indicating that utrophin expression in skeletal muscles is specific to slower more oxidative fibres[35]. However, the level of utrophin transcripts in soleus is almost equal to that in EDL. The reason why soleus muscles tend to have more utrophin protein is that utrophin transcripts are more stable in oxidative fibres, ranging from minutes to days[35]. Since utrophin levels are also higher in DMD patients than healthy individuals, as mentioned previously, strategies that aim to up-regulate utrophin levels predominantly involve promoting the transition of muscle fibres from a faster more affected, to a slower, more resistant phenotype through pathways that regulate the oxidative myogenic program[36].

1.2.2 - *Calcineurin-NFAT signalling pathway*

Calcineurin (Cn)-mediated Nuclear Factor of Activated T-cell (NFAT) signalling is primarily known for its crucial role in immune system responses. As a result of high levels of intracellular calcium ([Ca^{2+}i]), calmodulin binds Ca^{2+} ions, undergoes conformational changes which let it interact with the phosphatase protein Cn (Fig. 3)[37,38]. As a result of this interaction, Cn is activated which in turn de-phosphorylates NFAT thereby causing it to translocate into the nucleus where it promotes the transcription of cytokines[37]. Certain kinases such as creatine kinase 1 and Glycogen Synthase Kinase 3 Beta (GSK3β) can reverse this translocation by further phosphorylating NFAT[39]. Cn can be inhibited by regulator of Cn (RCAN) in a negative feedback loop fashion[40].

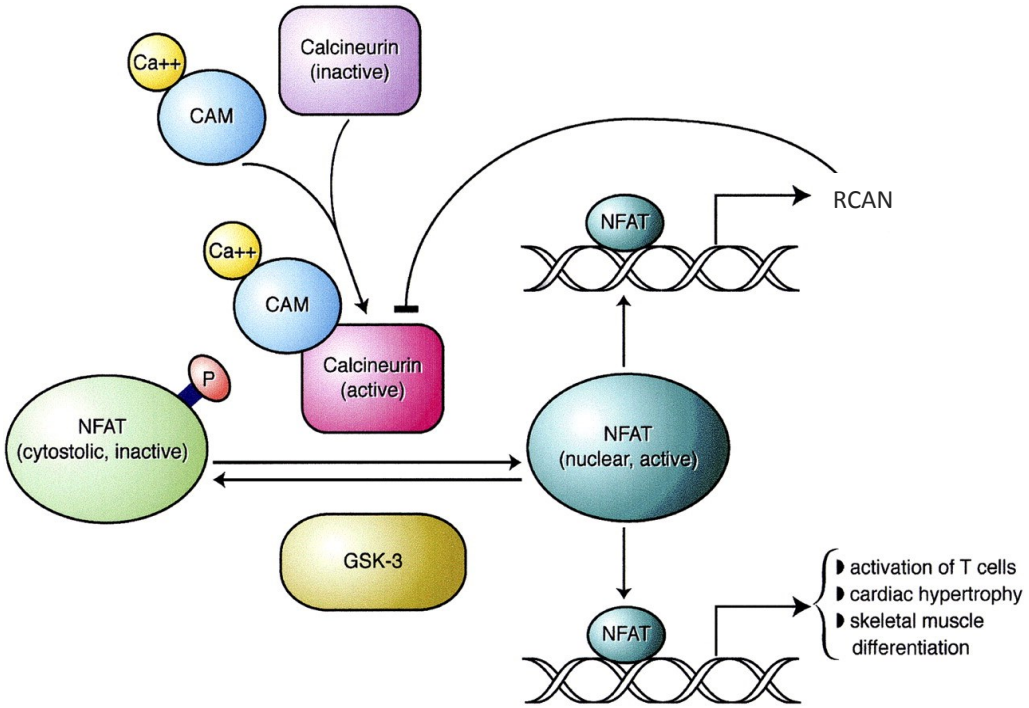

Fig. 3 An outline of the Cn signalling pathway- (adapted from[38]). Interaction of the Ca^{2+}-activated calmodulin with Cn, activates Cn and therefore de-phosphorylates NFAT causing it to migrate in to the nucleus where it can initiate the transcription of multiple of genes including RCAN. Activation of NFAT and Cn can be inhibited by GSK-3 and RCAN, respectively.

In skeletal muscle, the promoter of the genes that are part of the oxidative myogenic program possess binding sites for NFAT proteins indicating the involvement of Cn as a key contributor to fibre type specification[41]. In support of this, it was shown that treating mice

with pharmacological Cn blockers such as Cyclosporin-A (CsA) and FK506 could induce slow-to-fast fibre transformation[41].

Electrophoretic mobility shift assay followed by *supershift* assay experiments have revealed the existence of NFAT binding sites on the promoter of utrophin-A[42]. In light of that, introduction of an activated form of Cn (CnA*) to *mdx* mice increased the expression of utrophin-A introducing a novel method to strategies aimed for up-regulation of utrophin[43].

1.2.3 - *AMPK signalling pathway*

Adenosine monophosphate-activated protein kinase (AMPK) is one of the enzymes that can sense and restore the energy levels of the cell in response to energy homeostasis-related signals[44]. Many studies have shown that chronic stimulation of AMPK can trigger the expression of mitochondria biogenesis biomarkers (e.g. PGC-1α) indicative of the promotion of the slow myogenic program[45-47]. Since AMPK has such a potential, it wouldn't be improbable that utrophin expression eventually increase too. Accordingly, in 2011 it was shown that utrophin was up-regulated in parallel with the augmentation of slow myogenic markers when mice were chronically treated with the AMPK activator 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR)[48]. Additionally, *in_vitro* and *in_vivo* administration of the type 2 diabetes treatment drug Metformin (MET), have been shown to elevate utrophin-A levels (unpublished data) and promote the oxidative program through AMPK pathway[49].

1.3 - *Intracellular calcium ($[Ca^{2+}]i$) handling in DMD*

$[Ca^{2+}]i$ of skeletal muscle cells are known to be increased in DMD[50,51]. Sub-cellular Ca^{2+} localization studies on DMD have elucidated significant higher cytosolic Ca^{2+} concentration[52,53]. Further detailed studies on *mdx* mice revealed that this concentration of Ca^{2+} is predominantly restricted to the sub-sarcolemma region of myotube cells[54,55]. As mentioned previously, lack of dystrophin in DMD cases makes the muscle cell membrane susceptible to rupture during muscle contraction, leading to a leaky membrane. Accordingly, the elevation of $[Ca^{2+}]i$ is first and foremost attributed to this incident whereby Ca^{2+} leak channels are activated and consequently a large flow of Ca^{2+} is allowed to enter the cytosol[56]. This abnormal cytosolic concentration of $[Ca^{2+}]i$ leads to permanent but transient

cycles of Ca^{2+} uptake and release by Ca^{2+} handling channels of the sarcoplasmic reticulum (SR). Moreover, high concentrations of $[Ca^{2+}]i$ triggers the activation of a series of proteins that could be both beneficial (e.g. activation of Cn/NFAT signalling) and harmful (e.g. activation of certain proteinases) for the cell[57-59].

Ca^{2+} channels of SR consist of ryanodin receptor (RYR) Ca^{2+}-release channel and Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) Ca^{2+} uptake channel. These channels are normally inhibited by sarcolipin (SLN) and phospholamban (PLN)[60]. Whenever the calcium equilibrium is skewed, these proteins get phosphorylated by protein kinase A (PKA) and B (Akt), lose their inhibitory effect, and as a result lead to opening of the channels[61]. Some studies on *mdx* mice have shown the reduced ability of muscle cells to remove the excessive cytosolic Ca^{2+} introduced during contraction, indicating potential malfunction of SERCA channels as an important factor[62]. Accordingly, several recent studies aiming at amelioration of the dystrophic phenotype have drawn their attention towards promoting the function of these channels[63] and in general deciphering the complex mechanism of Ca^{2+} handling to re-stabilize the Ca^{2+} homeostasis[64,65].

1.3.1 - *Heat shock protein as a chaperon for SERCA*

As the name indicates, heat shock proteins (HSPs) are a group of proteins that are mainly regulated in response to environmental stress particularly heat shock[66]. Different isoforms of these proteins are usually named based on their polypeptide size ranging from 10 to ~ 100 kDa[67-69]. Among various functions that are reported for HSPs, they are best known for their chaperone activity[69-71]. This characteristic of HSPs has shown its capacity to interact with and ameliorate the function of cytoskeletal proteins such as actin[68,72] and the SR Ca^{2+} pump SERCA[63]. The latter finding is of a great importance since Ca^{+} homeostasis is disrupted in DMD. Accordingly, a group of scientists in Australia showed that induced expression of HSP70 either genetically or pharmacologically can improve and maintain the function of SERCA and consequently highly mitigate the dystrophic phenotype without the need for utrophin (Fig. 4)[63]. This is a breakthrough finding in DMD research for its independence from utrophin, the protein that has been a focal point of research in the field for more than 30 years. In addition to phenotypic improvements, augmentation of HSP70 decreased necrosis by shortening the time required for "overall repair of damaged fibres"[63].

Intact HSP70

Induced HSP70

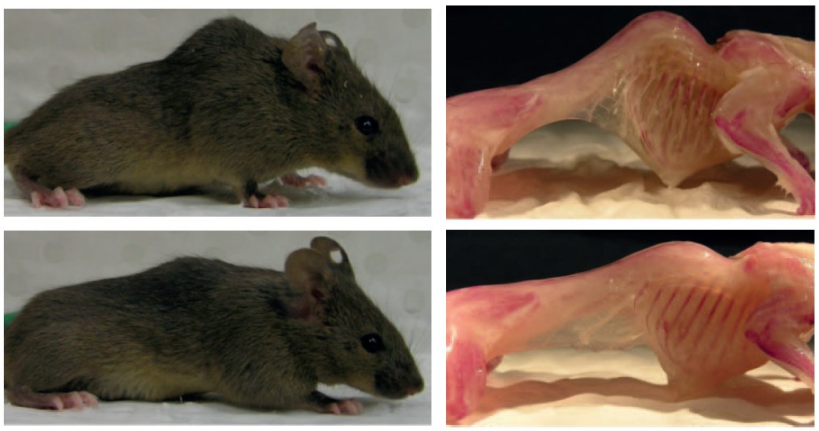

Fig. 4 Overexpression of HSP70 ameliorates the dystrophic phenotype (adapted from[63]). Pharmacological induction of HSP70 expression in utrophin-dystrophin double knock-out *mdx* mice (*mdx* DKO) significantly decrease the spinal curvature, the main phenoypical marker of DKO mice. This amelioration is independent of utrophin since DKO mice are null for this gene.

1.3.2 - *The calcium buffering protein Parvalbumin*

Parvalbumin (PV) is an intracellular high affinity Ca^{2+}-binding protein specifically expressed in fast fibres and found at higher levels in DMD fast fibers than normal[73]. It has the ability to buffer the sudden cytosolic Ca^{2+} level rise therefore bringing back the muscle to its relaxation state[74]. Slow fibre forced expression of PV in wild-type mice reduces the oxidative capacity of the muscle and is linked to changes in Cn/NFAT signalling pathway[75]. However, fibre type composition of the muscle is not changed indicating the possibility of involvement of other Ca^{2+} - dependent regulatory pathways[75]. To further investigate the role of PV in skeletal muscle, it is reasonable to also examine the alterations of Cn/NFAT in *mdx* mice overexpressing PV and comparing it to the mice that, in contrast, overexpress the constitutively active Cn (CnA*). Utrophin as a marker for Cn/NFAT signalling alterations and HSP70 as a chaperon for Ca^{2+} handling proteins can be good candidates for this comparison. Additionally, searching for probable Cn/NFAT signalling changes in *mdx* mice by administration of the drugs which are known to promote the oxidative capacity through AMPK pathway can also open a new window to our understanding of the molecular interplay mechanisms between Cn/NFAT signalling pathway and Ca^{2+} homeostasis.

2. HYPOTHESIS AND SCIENTIFIC RATIONAL

2.1- *Scientific rational of this project*

In order to get better insight into the intracellular mechanism by which Ca^{2+} homeostasis is regulated in coordination with the Cn/NFAT signalling pathway and in response to changes in oxidative capacity of the skeletal muscle fibres in dystrophic conditions, three concomitant approaches were adapted. To fulfill the requirements of such an experiment, *mdx* mice expressing either constitutively active Cn (CnA*) or slow-fibre force-directed Ca^{2+} buffering protein Parvalbumin (PV) were generated by crossing *mdx* mice with CnA* and PV transgenic mice. Promotion of the slow fibre program was also triggered in another group of *mdx* mice by pharmacological administration of AICAR and Metformin, which are known to provoke this triggering through stimulation of the AMPK pathway. Therefore, having three different experimental models that all share the substantial need for regulation of intracellular Ca^{2+} homeostasis in response to different triggers can potentially give us a more profound understanding of the coordination status between Cn/NFAT signalling and the Ca^{2+} handling channels of the sarcoplasmic reticulum.

2.2 - *Hypothesis*

The quantity and activity of SR calcium-handling proteins can alter the Cn/NFAT signalling and potentially other known or unknown Ca^{2+} dependent pathways in dystrophic myopathy.

3. Specific aims

3.1 - *Aim 1*

To examine how the treatment of *mdx* mice with AICAR and Metformin (two drugs that stimulate the AMPK signalling pathway) alter Cn/NFAT signalling in *mdx* mice.

3.2 - *Aim 2*

To impose a fast fibre-specific Ca^{2+} metabolism condition on slow fibres by directing the expression of PV into the slow fibres of *mdx* mice (i.e. generation of *mdx*/PV mice).

3.3 - *Aim 3*

To make the Cn/NFAT signalling constitutively active in fast fibres by directing the expression of CnA* into the fast fibres of *mdx* mice (i.e. generation of *mdx*/CnA*)

3.4 - *Aim 4*

To assess the regulation of Ca^{2+} handling proteins of SR in response to imposed Ca^{2+} homeostasis and Cn/NFAT signalling alterations in *mdx* mice (i.e. *mdx*/PV and *mdx* treated mice).

3.5 - *Aim 5*

To evaluate the expression status of the chaperon of SERCA channels (i.e. HSP70) in *mdx*, *mdx*/PV and *mdx*/CnA* mice.

4. METHODS

4.1 - *Animal care*

All animal care and handling was administrated according to the guidelines and protocols of the Canadian Council of Animal Care. These protocols had been approved by the University Animal Research Ethics Committee (UAREC). Male transgenic mice less than six month old expressing either PV or CnA* were crossbred with similarly aged male *mdx* mice. The presence of the transgenes was confirmed by Polymerase Chain Reaction (PCR) on the genomic DNA of the mice, which was extracted from their tail tissue. The identity of wild-type, PV, *mdx,* and *mdx*-cross-bred mice was also detected in the same fashion. The sleeping quarters of the mice were consecutively kept under a twelve-hour daylight cycle.

4.2 - *Mice genotyping*

Mice were genotyped as described previously by Chakkalakal, Chin, and Shin[75-77]. Briefly, ~ 5mm of the mice's tail tips were clipped and subjected to over-night incubation with 500 µl standard tail digestion buffer containing 4 µl proteinase K. The following morning, DNA was extracted with the conventional phenol-chloroform-based DNA isolation method. Appropriate primers were used to amplify each relevant loci by PCR (Table 1).

Amplicons were then electrophoresed on a 1.5 % agarose gel containing ethidium bromide and later exposed to UV-light to visualize stained DNA.

4.3 - *Animal surgeries and muscle extraction*

Mice of 10-12 weeks old were anesthetized and the soleus (Sol), gastrocnemius (Gas), tibialis anterior (TA), and extensor digitorum longus (EDL) muscles of both legs were harvested in the surgery room of Concordia University animal facility. For biochemical procedures, muscles were weighed and rapidly flash-frozen in liquid nitrogen. For histochemical procedures, muscles were weighed, embedded by commercial optimal cutting temperature (OCT) compound, frozen in a pool of cool melting isopentane surrounded by liquid nitrogen-frozen isopentane, and then dropped into the liquid nitrogen. These samples were then transferred into -80 C degree ultra-freezers until use.

4.4 - *AICAR and Metformin treatment of mice*

Drug treatments and corresponding mice tissue extractions were carried out in the lab of Dr. Bernard Jasmin with the approval of the University of Ottawa, and in accordance with Canadian Council of Animal Care guidelines. Protocol and treatment dosage are described previously by Ljubicic[48,78]. Briefly, mdx mice three to four weeks old were treated with AICAR (500 mg/kg/day) once a day by subcutaneous interscapular injections for 24-30 days, and with Metformin 5 mg/L in the drinking water for six weeks[48,79].

4.5 - *Protein extraction and Immunoblotting*

Frozen muscles were pulverized in liquid nitrogen using mortar and pestle. Pulverized tissue was homogenized in sufficient volumes (i.e. 12-15 µl x the weight of the muscle in mg) of a protein extraction buffer comprised of 75 mM Tris pH 6.8, 3.8% SDS, 4 M urea, 20% (vol/vol) glycerol, and Roche cOmplete Mini Protease Inhibitor cocktail for one hour on ice, along with short vortexes from time to time. Samples were then heated for two minutes at 95 ̊C degrees, followed by centrifugation at 2000 x *g* for 15 minutes. Afterwards, supernatants were collected and the protein concentration of each sample was measured using the Bradford assay (Bio-RAD). Either 20 or 25 µg amounts of proteins were then resolved by electrophoresis on either 5% Tris-Glycine polyacrylamide gels for high molecular weight (HMW) proteins at 130 V for 1.5-2 hours, or 7% Tris-Tricine

polyacrylamide gels for low molecular weight (LMW) proteins at 100 V for 1-1.5 hours. Once the run was complete, gels were transferred to nitrocellulose membranes overnight at low constant amperage (i.e. 50 mAmps for HMWs and 150 mAmps for LMWs). On the second day, transfer efficiency was checked by Ponceau and then after, membranes were blocked in either Blotto (i.e. 5% w/v non-fat milk, 0.1% tween (T) in phosphate-buffered saline (PBS) or in 1-3% Bovin Serum Albumin (BSA) in 0.1% T/PBS buffers for one hour at room temperature, followed by incubation with titrated concentration of proper primary antibodies (Table 2) in the blocking buffer overnight in a cold room (4 C degrees) on a Belly Dancer shaker. On the third day, membranes were washed 3 x 10 minutes each by 0.1% T/PBS, followed by incubation in horseradish peroxidase (HRP) - conjugated anti-mouse or anti-rabbit secondary antibodies for 1-1.5 hours at RT. After 3 x 10 minute washes with 0.1% T/PBS, membranes were incubated with enhanced chemiluminescence (ECL) reagent (EMD Millipore) for five minutes in the dark, then exposed using the Alpha Innotech FlourChem imaging system.

4.6 - *Quantification and statistical analysis*

The band intensity of each blot was quantified using either the FlourChem image analysis software or the NIH Image J program. Each target was normalized to its corresponding loading control (e.g. alpha-tubulin or Vinculin). The standard deviation of each group (n=3) was considered, and student's t-test was used to compare each two sets of data (e.g. mdx vs mdx/PV or WT vs PV).

Mouse of question	Forward primer	Reverse primer	Amplicon size (bp)
mdx	5'-GCGCGAAACTCATCAAATATGCGTGTTAGTGT-3'	*mutant*: 5'-CGGCCTGTCACTCAGATAGTTGAAGCCATTTTA-3	117
		WT: 5'-GATACGCTGCTTTAATGCCTTTAGTCACTCAGATAGTTGAAGCCATTTTG-3'	134
PV transgenic	5'-CCCACCAGCCCAGCTTTTCTA-3'	5'-TTAGGCGTAGTCGGGCACGTCATATGGGTAGCTTTGGCCAC-3'	450
CnA* transgenic	5'-CGATTCAAAGAACCACCTGCTTATGGG-3'	5'-CCCAAGCTTGGGTTTCTGATGACTTCCTTC-3'	600

Table 1. Detailed list of the primers that were used to genotype the transgenic animals.

Target protein	Supplier and product #	Antibody dilution	Detection size (kDa)
Utrophin	Novocastra leica #NCL-DRP2	1:500 in 1% blotto	395
NFATc1	Santa Cruz #sc-13033	1:500 in 1% BSA	90-140
Calcineurin	Cell signaling #2614	1:1000 in 5% blotto	60
p-GSK3β	Cell signaling #9322	1:3000 in 3% BSA	46
GSK3β	Cell signaling #9315	1:3000 in 3% BSA	46
p-Akt	Cell signaling #4051	1:1000 in 3% BSA	60
Akt	Cell signaling #9272	1:1000 in 3% BSA	60
Alpha-tubulin	Cell signaling #2125	1:2000 in 5% blotto	52
Vinculin	Sigma #V9131	1:5000 in 5% blotto	116
RCAN1	Sigma #D6694	1:5000 in 2% blotto	26 and 38
Ryanodin receptor 1 (RYR1)	Thermo #MA3-925	1:500 in 1% blotto	565
SERCA1	Thermo #MA3-912	1:7500 in 3% BSA	110
SERCA2	Thermo #MA3-919	1:1000 in 3% BSA	110
Phospholamban	Thermo #MA3-922	1:500 in 1% BSA	6 and 28
HSP70	Enzo #SPA-812	1:1000 in 1% blotto	70/72
Sarcolipin	EMD Millipore #ABT13	1:500 in 5% blotto	6

Table 2. Detailed list of antibodies and their dilution for immunoblotting experiments.

5. RESULTS

5.1 - *Administration of AMPK activator drugs, AICAR and Metformin, has an inhibitory effect on Cn/NFAT signalling*

Our collaborators (Bernard Jasmin's group, University of Ottawa) have shown that chronic treatment of *mdx* mice with AICAR up-regulates the markers of slow-oxidative myogenic program and utrophin, the compensator for the absence of dystrophin in DMD, through an AMPK pathway[45]. They have also managed to show similar effects for Metformin (unpublished data). However, they have not examined possible alterations in the Cn/NFAT signalling pathway. Therefore, we decided to analyze the muscle proteins of the same treated mice for Cn/NFAT signalling to see if this pathway also plays a role in their findings. EDL, the very fast muscle which was used for their study, was not available anymore. Therefore, we sufficed with gastrocnemius, which has a mixture of all possible fibres, including pure and hybrids. We first examined the phosphorylation status of GSK3β by

acquiring the ratio of phosphorylated to total GSK3β, and observed that it was lower under those treatments compared to the *mdx* vehicle mice (Fig. 5B).

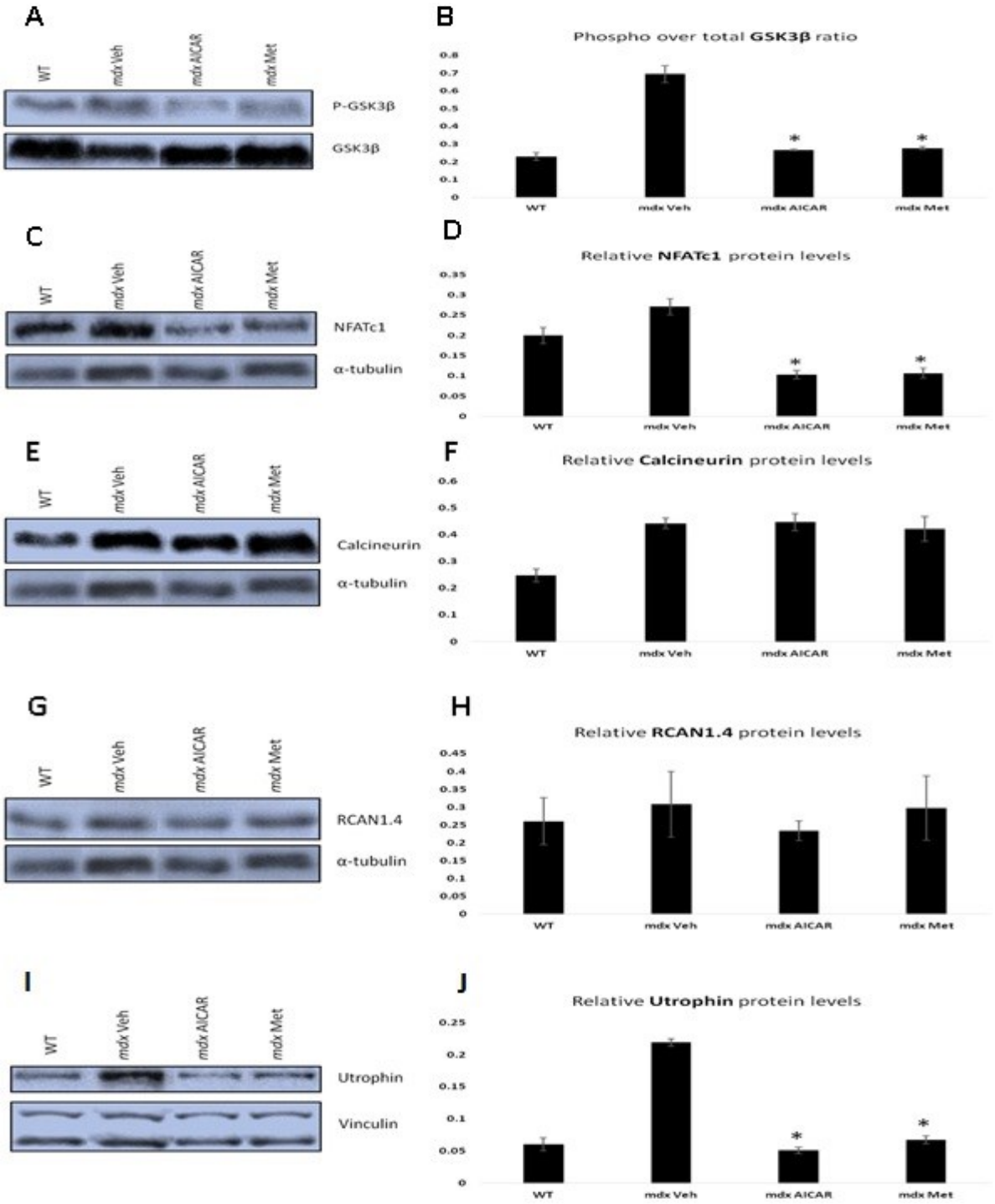

Fig. 5 Chronic activation of AMPK pathway decreases the markers of Cn activity. Representative immunoblot and quantification for A-B) phosphorylated GSK3β over total GSK3β, C-D) NFATc1 E-F) Cn, G-H) RCAN1.4, and I-J) utrophin protein levels in the Gas muscle of WT, *mdx* Veh, *mdx* AICAR and *mdx* Met mice. Relative quantities are normalized to either alpha-tubulin or vinculin (n=3; P<0.05). *compared to *mdx* Vehicle. ±STDEV are shown.

This means a less active form of GSK3β, which may happen when there are less NFAT to be shuttled to the nucleus. To examine this hypothesis, we probed for the main isoform of NFAT (i.e. NFATc1) and observed decreased expression of this protein in *mdx* mice treated with AICAR and Metformin compared to the *mdx* vehicle mice (Fig. 5D). These were the initial signs of decreased Cn/NFAT signalling. The next step was to investigate the expression of Cn and its inhibitor RCAN1.4 (the skeletal muscle isoform of RCAN). Interestingly, we observed no changes in RCAN1.4 and Cn (Fig. 5H), which could bring us one step closer to believe that Cn activity is reduced. To further prove this strong probability, we investigated the expression of utrophin, which directly undergoes significant alterations by the augmentation or inhibition of Cn activity. As expected, the utrophin content was decreased in both cases to a level similar to that in wild-type (Fig. 5J).

Intracellular Ca^{2+} kinetics are controlled by the Ca^{2+} release and uptake pumps of sarcoplasmic reticulum's lumen. More activity of uptake pumps or less activity of release pumps can determine the cytosolic levels of Ca^{2+}. Alterations in the activity of Cn correlates with the cytosolic Ca^{2+} concentration. Thus, we next examined the expression and activity of these Ca^{2+} channels in order to be able to justify the observed reduction of Cn/NFAT signalling. We did not see any changes in the expression of the fast and slow isoforms of SERCA (i.e. 1 and 2 respectively) uptake channels or the ryanodin receptor 1 release channels (Fig. 6B-D). However, sarcolipin, the inhibitor of SERCA channels, was highly reduced (Fig. 6E), indicating more activity in the uptake channels and less available cytosolic Ca^{2+} as a result. The phosphorylation of sarcolipin is predominantly done by Akt, and therefore, less expression of sarcolipin should correlate with a reduced ratio of the phosphorylated Akt over the total Akt. Our data confirms this fact (Fig. 6F).

5.2 - *Overexpression of the Ca^{2+} buffering protein, parvalbumin, leads to down-regulation of utrophin*

For years, our lab has repeatedly shown that changes in Cn/NFAT signalling directly correlates with changes in utrophin expression[7,42,43]. Here in this study, we sought to test the hypothesis that changes in the upstream of the Cn/NFAT pathway will also affect the expression of utrophin in response to correlated changes in the pathway. For this purpose,

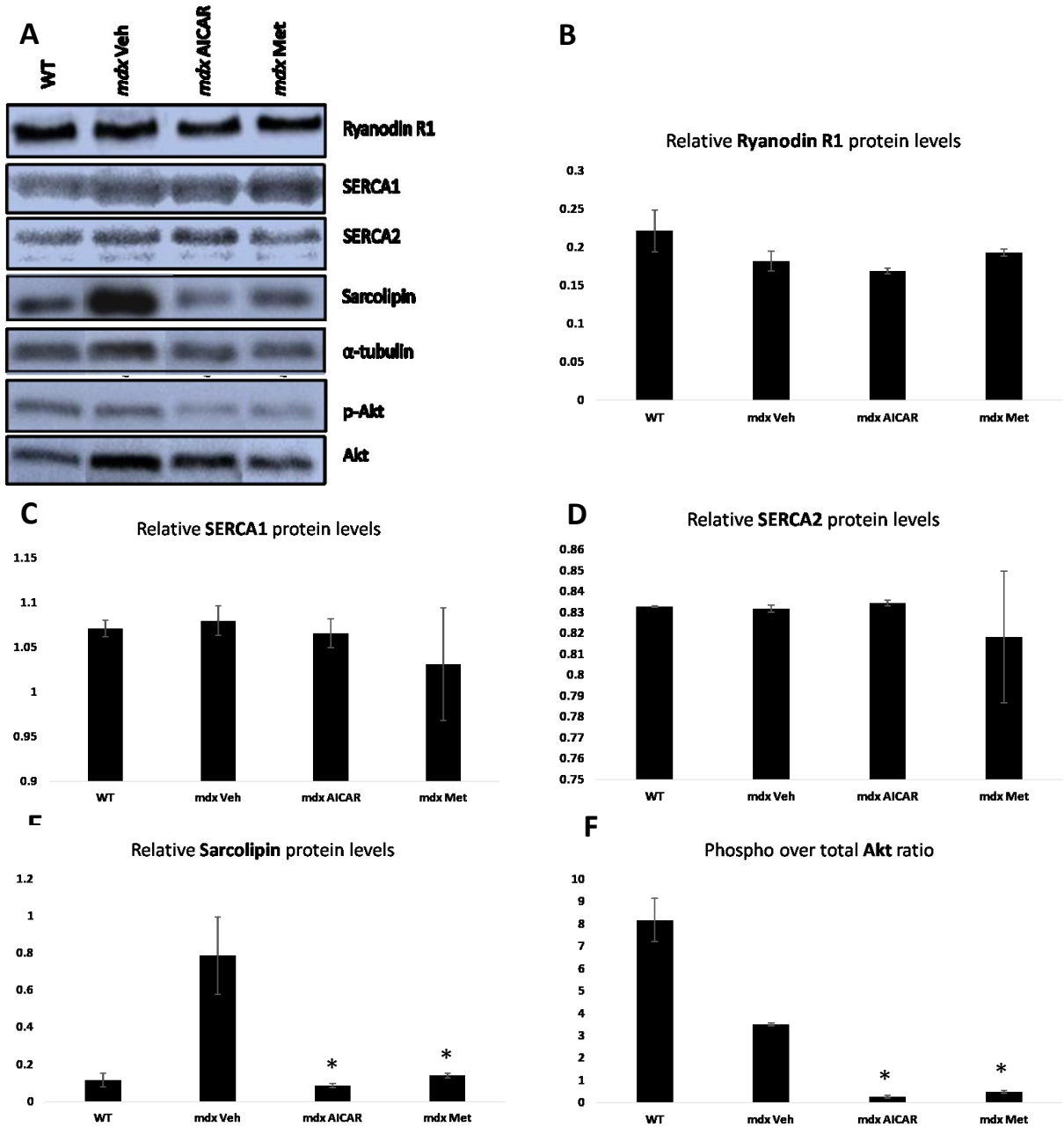

Fig. 6 Calcium uptake channels are less inhibited by chronic activation of AMPK pathway. Representative immunoblot (A) and quantification for B) RYR1, C) SERCA1, D) SERCA2, E) SLN, and F) phosphorylated Akt over total Akt protein levels in the Gas muscle of WT, *mdx* Veh, *mdx* AICAR and *mdx* Met mice. Relative quantities are normalized to either alpha-tubulin or vinculin (n=3; P<0.05). *compared to *mdx* Vehicle. ±STDEV are shown.

we compared the expression of utrophin in the slow muscle of wild-type and mdx mice carrying the fast-muscle-specific Ca^{2+} buffering protein parvalbumin, to that in wild-type and *mdx* non-transgenic ones. Our data shows that directed expression of PV in slow fibres significantly decreases the utrophin content of the muscle in PV and *mdx*/PV transgenic mice (Fig. 7B).

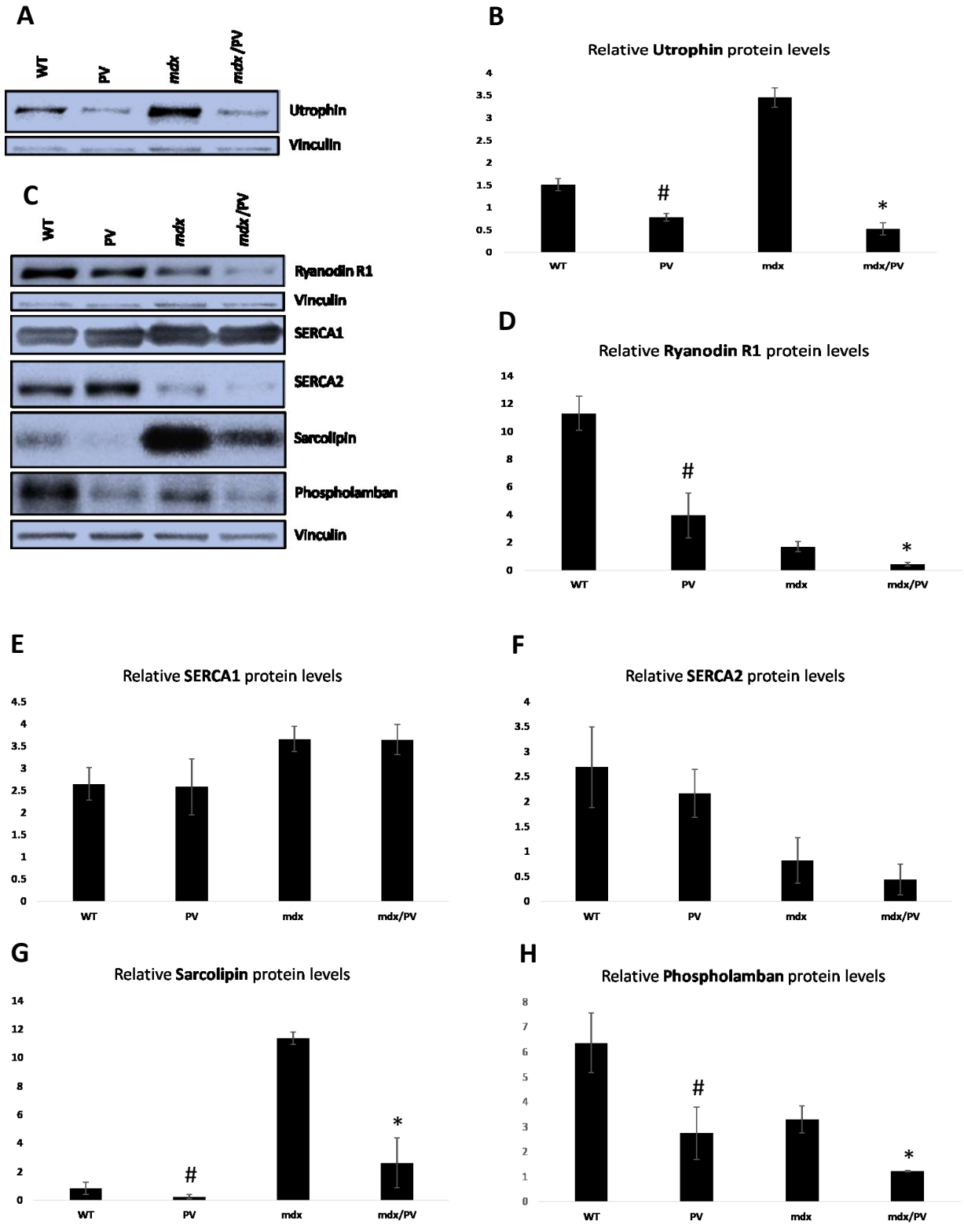

Fig. 7 Alterations of Ca^{2+} kinetics by forced expression of PV in slow fibres down-regulate utrophin through Cn/NFAT signalling. Representative immunoblot (A and C) and quantification for B) utrophin, D) RYR1, E) SERCA1, F) SERCA2, G) SLN, and H) PLN protein levels in the Sol muscle of WT, PV, *mdx*, and *mdx*/PV mice. Relative quantities are normalized to vinculin (n=3; P<0.05). *compared to *mdx*, #compared to WT. ±STDEV are shown.

In order to track the changes upstream of the pathway due to the introduction of PV, we sought to investigate the expression and activity of the SR Ca^{2+} handling proteins. Our results for this purpose suggest no change in the expression of either isoforms of SERCA (i.e. SERCA1 and 2) Ca^{2+} uptake channels, while the inhibitors of these channels, sarcolipin and phospholamban, were remarkably decreased (Fig. 7E-H). In contrast, the Ca^{2+} release channel, ryanodin receptor, was down-regulated (Fig. 7D).

5.3 - *Expression pattern of HSP70 under direct and indirect manipulation of Cn/NFAT signalling pathway*

For years, up-regulation of utrophin as a compensator for the absence of dystrophin has been the main area of the focus to improve the impaired pathology of the muscle in DMD. In our lab, this objective has been shown to be achievable through the promotion of the slow oxidative myogenic program via the Cn/NFAT pathway[7]. These adaptive changes in the slow phenotype regulation is known to be correlated with increasing levels of HSP70[80]. Nevertheless, a recent study gives strong evidence that the up-regulation of HSP70 alone ameliorates the dystrophic phenotype independent of utrophin[63]. Accordingly, we were curious to see if HSP70 is regulated via Cn/NFAT signalling. Hence, we probed for HSP70 in mice who overexpress either PV (i.e. upstream manipulation of any Ca^{2+}-dependent pathway) or CnA* (i.e. direct manipulation of Cn/NFAT signalling). This experimental strategy not only could reveal if the expression of HSP70 is directly influenced by Cn/NFAT signalling, but also It could give us the ability to see if HSP70 is in fact regulated via any mechanisms dependent on Ca^{2+} signalling in the first place. Moreover, it could give us the ability to further confirm that utrophin is regulated via Cn/NFAT signalling and as a result any alterations in Ca^{2+} homeostasis would affect its expression. Accordingly, our data showed down-regulation of HSP70 in parallel with utrophin in PV and *mdx*/PV crossbreds, despite no changes of it in *mdx*/CnA* transgenic mice where utrophin is up-regulated due to the constitutivly active Cn (Fig. 8C-F).

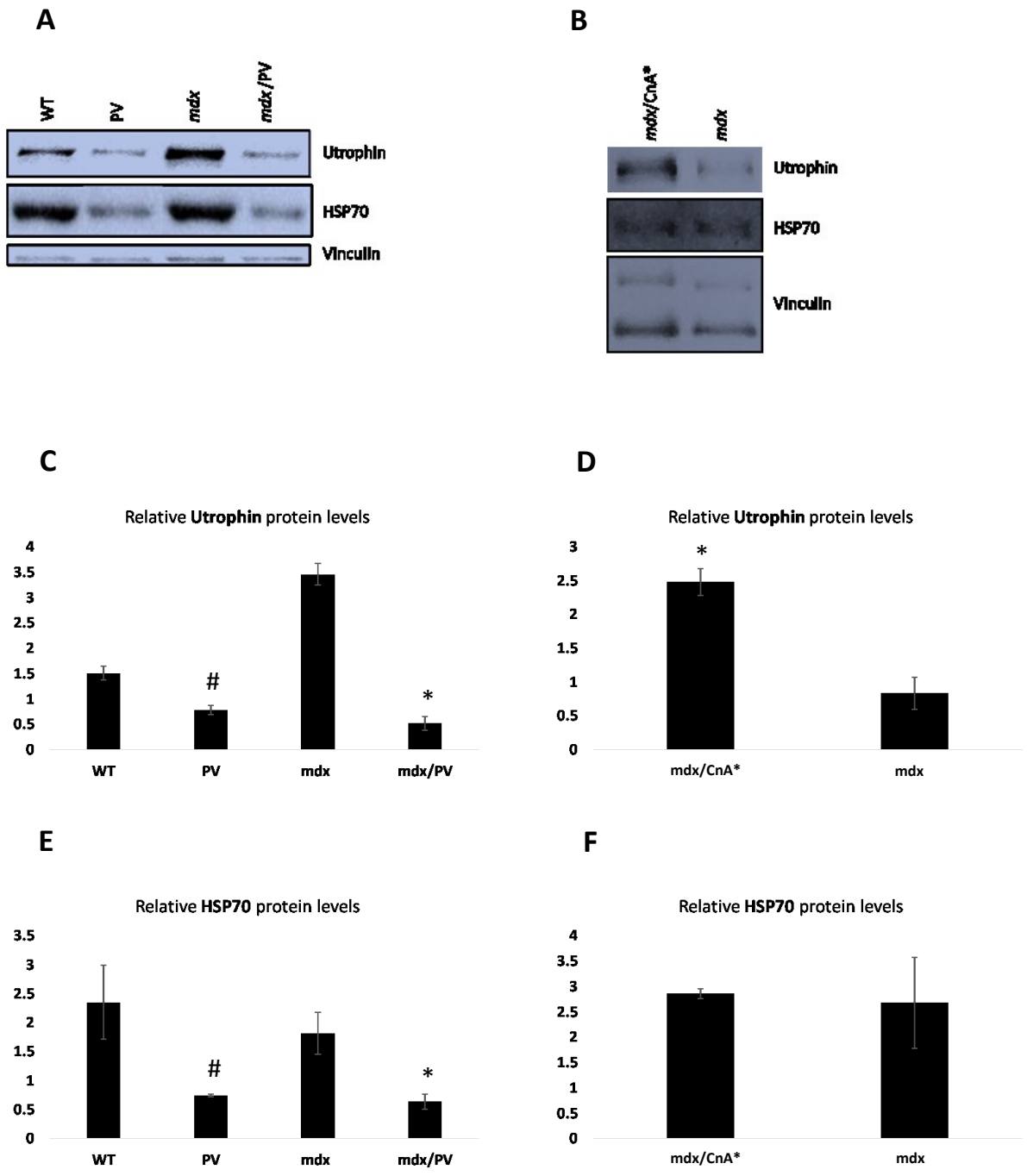

Fig. 8 Regulation of HSP70 is a Ca^{2+}-dependent process independent of Cn/NFAT signalling pathway. HSP70 is down-regulated in PV mice, while it is unchanged in mice carrying a constantly active form of Cn (i.e. CnA*). In contrast, utrophin undergoes significant changes in both cases. Representative immunoblot (A and B) and quantification for C-D) utrophin and E-F) HSP70 protein levels in WT, PV, *mdx*, *mdx*/PV, and *mdx*/CnA* mice. Sol and EDL muscles were used for the PV and the CnA* experiments, respectively. Relative quantities are normalized to vinculin (n=3; P<0.05). *compared to *mdx*, #compared to WT. ±STDEV are shown.

6. DISCUSSION

6.1 - *AICAR and Metformin drugs decrease the activity of calcineurin*

AMPK is one of the enzymes that can sense and restore the energy levels of the cell in response to energy homeostasis-related signals[44]. The AMPK signaling led by this enzyme can be pharmacologically promoted by administration of AICAR, the activator of AMPK pathway. Our collaborators in Ottawa have previously shown that treating mice with 500 mg/kg/day of AICAR for four weeks induces the expression of the markers for slow, oxidative myogenic program through the AMPK pathway[48]. As a result of this induction, the central localization of nuclei was decreased and the integrity of sarcolema was enhanced during contraction-induced damages[48]. This was also associated with a significant increase (i.e. up to two-fold) in utrophin levels of EDL muscle compared to wild-type[48]. However, this raised the question of whether these pathological adaptations were due to more utrophin content, or because of the increased expression of slow fibre markers. Therefore, a few years later, they compared utrohpin-dystrophin DKO mice with *mdx* under AICAR treatment, and observed that despite similar changes in the expression of slow fibre markers, DKO mice which lack utrophin were unable to improve the integrity of the sarcolema and showed more signs of damage compared to *mdx*-treated mice. This indicated the crucial role of utrophin in the improvement of dystrophic pathology following the pharmacological induction of the slow myogenic program[78]. On the other hand, our lab has shown that utrophin expression is regulated in part by Cn/NFAT signalling, meaning that the constant activation of Cn in fast fibres eventually leads to increased levels of utrophin in the muscle[43]. In light of this, we were curious to find out if the up-regulation of utrophin in the AICAR treatment study had anything to do with Cn/NFAT signalling. Accordingly, proteins from the gastrocnemious muscles of the same treated mice in that study were isolated and subjected to western blot analysis. Our immunoblot experiments showed a decreased expression of NFATc1, despite no changes in Cn and Cn inhibitor (i.e. RCAN1.4) levels (Fig. 5C-H). Aside from this, the down-regulation of NFATc1 was in accordance with a lower active (phosphorylated) form of GSK3β (Fig. 5A-B), the protein that reverses the nuclear translocation of NFATcs. This can suggest that the pharmacological promotion of a slow, oxidative program through the AMPK pathway is achieved in part by a reduction of the activity (not expression) of Cn.

Lower activity of Cn was associated with a decreased expression of utrophin (Fig. 5J), which is in agreement with our previous findings[77]. However, it is contrary to the findings of our collaborators, where induction of the AMPK pathway led to an increase in utrophin in relation with promoted slow myogenic program[78]. One possible explanation for this contradiction could be the differences between the muscles used in each study - in theirs, they analyzed the EDL muscle of mice, while we analyzed the Gas muscles. The fibre-type composition of these muscles are highly different - EDL is mainly composed of pure fast fibres (i.e. only ~5 percent Type 1 slow), while Gas muscles can have up to ~20 percent slow fibres supplemented with slower fast hybrid fibres[81]. Moreover, the transition of EDL's highly glycolytic fast fibres to a slower type of fibre under AICAR treatment may act in a muscle-specific manner and consequently affect the fibre type composition of other muscles (detailed studies for such a hypothesis is definitely required). Our results show the same pattern of expression of the Cn/NFAT signaling players for the Metformin-treated mice identical to those of the AICAR treatment, and therefore also contrary to the unpublished data of our collaborators where utrophin was up-regulated.

6.2 - *Slow fibre-directed expression of PV in mdx mice decreases the Cn/NFAT signalling*

Intracellular Ca^{2+} oscillations are not alike between different muscle types. This is due to the differences in the volume of Ca^{2+} influx in response to different patterns of contraction impulses in fast and slow fibres[82,83]. One of the main calcium buffering proteins specific to fast fibres is parvalbumin (PV)[84]. Forced expression of PV in slow fibres has been shown to reduce the oxidative capacity of the muscle in association with a significant decrease in Cn activity[75]. On the other hand, $[Ca^{2+}]i$ levels are higher in dystophic cases than normal[50,51]. In light of these facts, we hypothesized that directed expression of the fast-fibre-specific PV in the slow fibres of *mdx* will also lead to a similar reduction of Cn activity, and as a result, decrease the expression of utrophin, which will eventually exacerbate the dystrophic pathology. Signs of severe damage to muscle fibres have been confirmed in our lab by measuring the central localization of the nuclei (unpublished data). Our results confirm the aforementioned hypothesis and further approve that any fluctuations in the activity of Cn/NFAT, whether caused by direct targeting of the pathway's elements or indirectly through changes in the calcium homeostasis, eventually affect the expression of utrophin (Fig. 7B).

6.3 - *Cytosolic Ca^{2+} levels controlled by SR channels determine the activity rate of calcineurin*

Muscle contraction and relaxation are in direct concomitance with the Ca^{2+} levels of cytosol. When contraction is about to happen, extracellular Ca^{2+} entry to the cytosol triggers Ca^{2+} release from SR to cytosol through ryanodin receptor channels[85]. This leads to a sudden increase in the concentration of cytosolic calcium[85]. Once the contraction is over and the muscle goes back to its relaxed state, this concentrated cytosolic Ca^{2+} needs to be reuptake into the SR[85,86]. This necessity is fulfilled by the SERCA channels[86]. In DMD cases, cytosolic Ca^{2+} levels are naturally higher regardless of the contraction/relaxation state of the muscle, which although it leads to the upregulation of utrophin through Cn/NFAT signalling, can also have its own detrimental consequences (e.g. activation of necrotic proteases)[57,58]. Here in this study, by introducing a fast fibre-specific Ca^{2+}-buffering protein (PV) to the slow fibres of *mdx* mice, we have shown that utrophin is down-regulated, indicating that Cn/NFAT signalling is highly affected by cytosolic Ca^{2+} levels (Fig. 7B). On the other hand, the reduction of cytosolic Ca^{2+} is in coordination with the activity levels of SR calcium channels[86]. Accordingly, since the calcium oscillation of slow fibres in *mdx*/PV mice forcedly mimics that of fast fibres, we can see that despite the unchanged expression of SERCA channels, their activity is less inhibited due to the decreased expression of sarcolipin (SLN) and phospholamban (PLN), the inhibitors of SERCAs (Fig. 7E-H).

It is shown that the inhibition of the expression of ryanodin receptor 1 in fast fibres triggers a fast-to-slow fibre type transition[87]. Interestingly, our data shows a decrease in the expression of RYR1 in *mdx*/PV mice (See fig. 7D), while the fast fibre-specific SERCA1 expression was not changed. When both are together, this may indicate the attempt of the fibres to maintain their slow-oxidative capacity when PV, and as a result, a faster fibre type status, is imposed on them. Moreover, the decrease in SERCA2 is an *mdx* effect and PV independent, since its expression level is almost identical between *mdx* and *mdx*/PV. However, supporting this interpretation requires the assessment of the expression and activity of L-type Ca^{2+} channels of sarcolemma as well.

SLN and PLN, the inhibitors of SERCA channels, were down-regulated in *mdx*/PV compared to *mdx*, indicating higher activity of SERCA channels, and as a result, more prevention of the Cn/NFAT pathway from cytosolic Ca^{2+}.

Our results for the AICAR and Metformin-treated mice also show a great reduction in the expression of SLN despite no changes in the expression of SERCA and RYR1 channels (See fig. 6B-E) indicative of higher overall activity of SERCA. As a result, there is more reuptake of Ca^{2+}, leading to less cytosolic Ca^{2+}, and consequently, decreasing Cn/NFAT signalling. In addition to this, phosphorylated Akt over total Akt was also decreased, which has further resulted in less phosphorylated SLN, and consequently, less inhibition of SERCAs (Fig. 6F).

The data obtained by these two strategies (*MDX*/PV and mice treatments with the aforementioned drugs) suggests that $[Ca^{2+}]i$ homeostasis which is intrinsically correlated with the activity and expression of SR calcium handling proteins determines the rate of the activity (not the expression) of Cn in the very upstream part of the pathway, therefore affecting the expression of Cn/NFAT signalling-regulated proteins. Therapeutically, this could be of great importance for strategies aimed towards up-regulating utrophin in dystrophic myopathy studies.

6.4 - *HSP70 expression is affected by cytosolic Ca^{2+} levels independent of Cn/NFAT signalling*

The HSP family of proteins has been shown to revive some proteins of the cytoskeleton, such as actin and cytosolic membrane channels, as a result of mechanical damage to the fibres in dystophic conditions[63,72]. Furthermore, HSP70 is shown to be expressed more in the soleus where there are more type 1 slow oxidative fibres available than in the faster-glycolytic plantaris, suggesting a correlation between the oxidative capacity of the muscle and its regenerative power[80]. Recently, scientists could prove the protective role of HSP70 over SERCA channels, resulting in the amelioration of dystrophic phenotype independent of utrophin up-regulation[63]. Accordingly, it was in the interests of our lab to assess the pattern and relevance of the expression of utrophin and HSP70 in a Cn/NFAT signalling-oriented approach, so we examined their expression under directly and indirectly altered Cn/NFAT signalling conditions. Interestingly, we observed that the expression of HSP70 was decreased when cytosolic Ca^{2+} levels of slow fibres were decreased (i.e. PV and mdx/PV mice), and was not changed when Cn/NFAT signalling was constitutively activated (i.e. *mdx*/CnA* mice) (Fig. 8). This strongly suggests that the regulation of HSP70 is a Ca^{2+}-dependent procedure independent of Cn/NFAT signalling pathway.

7. CONCLUSION

In summary, we have managed to use three different approaches to add new findings to our current understanding of Cn/NFAT signalling activity alterations in association with intracellular Ca^{2+} homeostasis. This was achieved by immunoblot analysis of Cn/NFAT signalling-involved factors and SR Ca^{2+} handling channels in *mdx* mice over-expressing either a Ca^{2+} buffering protein, parvalbumin, or a constitutively active form of Cn (CnA*). Aside from this, the analysis of *mdx* mice treated with AMPK pathway activators, AICAR, and Metformin as a complementary approach not only helped in understanding the process, but also led to another novel finding. First and foremost, our results suggest that the upstream determiner of the activity alterations of Cn/NFAT signalling and potentially other Ca^{2+}-dependent pathways is the quantity and activity rate of the SR Ca^{2+} handling channels, since forced cytosolic calcium changes in *mdx*/PV could down-regulate utrophin similarly to our previous findings where direct interference with Cn/NFAT signalling by a calmodulin binding protein (CaMBP) also led to the down-regulation of utrophin[77]. Secondly, by comparing the expression of utrophin and HSP70 in *mdx*/PV and *mdx*/CnA*, we could infer that HSP70 is regulated by a Ca^{2+}-dependent pathway rather than Cn/NFAT, since its expression was changed in *mdx*/PV mice but remained intact in *mdx*/CnA* mice. Finally, we observed that the administration of AICAR and Metformin, which is known to activate the AMPK pathway, can reduce the activity of Cn. Whether this is a direct effect or a result of AMPK up-regulation, it needs further detailed analysis. Collectively, these results have a great potential for future elucidations of molecular therapeutic approaches toward myopathic disorders, particularly DMD.

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