# Investigating the Potential Effectiveness of Two Therapeutic Targets, Utrophin and HSP70, in the Treatment of Duchenne Muscular Dystrophy

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## ABSTRACT

**Investigating the Potential Effectiveness of Two Therapeutic Targets, Utrophin and HSP70, in the Treatment of Duchenne Muscular Dystrophy**

Daniel Spensieri

Duchenne muscular dystrophy is an incurable, genetic neuromuscular disorder that causes progressive, severe muscle wasting in young men and eventually leads to early death. The disease is caused by a mutation in the gene encoding the cytoskeletal protein dystrophin, which leads to stress induced sarcolemmal tearing. Abnormally high levels of intracellular $Ca^{2+}$ in muscle cells is a main feature of the disorder as it triggers many secondary effects that are detrimental to the proper functioning of skeletal muscle. Both utrophin and HSP70 have been suggested to have potential therapeutic potential as a result of their preferential expression in the relatively damage-resistant slow, oxidative fibres. In my studies, I found that by forcing transgenic expression of the $Ca^{2+}$ chelator parvalbumin in slow fibres, utrophin expression in slow fibres was blunted without altering HSP70 expression. In this case, the slow fibres showed increased signs of stress and damage therefore suggesting that endogenous HSP70 alone may not spare dystrophin deficient muscle fibres. These results indicate that utrophin (but not HSP70) is a potential therapeutic target that will prevent muscle degeneration in patients with DMD.

## PREAMBLE

The work in this thesis was produced while working in the laboratory of Dr. Robin Michel, who funded and directed the experiments for most of my M.Sc. For the final months of my thesis, as the PI had to go on leave, I requested that Drs. Darlington and Courtemanche help supervise the final months of my work, so I could finish on the timetable agreed upon in the last committee meeting. They then became my supervisors for the last few months. Dr. Mohamed Elbakry was also kind enough to provide advice in the data analysis and writing as an added committee member.

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A special thanks must go to Dr. Manal Al Zein who worked very closely with me throughout the course of this project. We spent many late hours trying to solve the very many technical issues that we encountered in this project and I can truly say that I may not have come this far without her guidance. Special thanks must also go to Dr. Elbakry for always taking the time to sit down with me and share his wisdom and experience and pushing me to find ways to improve.

Second, I would like to thank my committee members Dr. Brett, and Dr. Elbakry as well as my co-supervisor Dr. Courtemanche and supervisor Dr. Darlington for their involvement in the completion of this thesis. Each member went above and beyond what I ever expected or could ever ask for.

Finally, I would like to thank my parents and my girlfriend for their never ending support and patience.

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## LIST OF ABBREVIATIONS

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

CaM: Calmodulin

CaMBP: Calmodulin Binding Protein

CLN: Centrally Located Nuclei

Cn: Calcineurin

CnA*: Activated Calcineurin-A

DAPC: Dystrophin-Associated Protein Complex

DMD: Duchenne Muscular Dystrophy

EDL: Extensor Digitorum Longus

f-actin: filamentous-actin

FSV: Fibre-Size-Variability

H&E: Hematoxylin and Eosin

HSP70: Heat Shock Protein-70

IF: Immunofluorescence

MCK: Muscle Creatine Kinase

MyHC: Myosin Heavy Chain

NFAT: Nuclear Factor of Activated T-cells

NF-κB: Nuclear Factor-κB

NMJ: Neuromuscular Junction

Pv: parvalbumin

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

TG: Transgene

TnIS: Troponin I Slow

TNF-α: Tumour Necrosis Factor-α

WT: Wildtype

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# 1. INTRODUCTION

## 1.1 – *DUCHENNE MUSCULAR DYSTROPHY*

Duchenne muscular dystrophy (DMD) is the most prevalent genetic neuromuscular disorder in the world (1). It is an X-linked disorder affecting 1 in 3600-6000 males (2). Symptoms begin at an early age when affected children experience difficulties in walking or stair-climbing. Patients are wheelchair bound by their teenage years. Death of DMD patients occurs in the late twenties, usually as a result of cardiac or respiratory failure (1, 2). Although modern medicine has extended the life expectancy and has made it easier to live with the disorder, there is still no known cure.

### 1.1.1 – *DMD Etiology*

A mutation in the gene encoding dystrophin is the cause of DMD (3). Dystrophin is a 427-kDa cytoskeletal protein predominantly expressed in skeletal and cardiac muscle with smaller amounts also found in the brain (3-5). The 2.5 Mb dystrophin gene is located on the X chromosome and is composed of 79 exons, making it the largest gene in the human genome (3, 6). The mutation either results in a non-functional protein in DMD; or a truncated, yet still functional form of dystrophin in Beckers muscular dystrophy; a much less devastating disorder (3, 7).

Dystrophin is a member of the dystrophin-associated protein complex (DAPC) (8). The DAPC is an extensive protein scaffolding complex localized throughout the entire sarcolemma and is important in maintaining the structural integrity of the entire muscle fibre (9). The DAPC links extracellular laminin protein to the intercellular filamentous actin protein (f-actin) (fig1) (10). In DMD and the murine-model equivalent (mdx mouse), muscles are susceptible to stretch and contraction-induced damage (7, 11). Healthy muscles have a fairly high regenerative capacity to repair damaged tissue (12), however dystrophin-deficient muscle tissue undergoes continuous cycles of degeneration and regeneration which eventually exhausts the muscle progenitor pool of satellite cells responsible for repair (13). This exhaustion leads to muscle failure as contractile tissue is replaced with inert scar tissue and fatty adipose deposits (14). Degeneration-regeneration cycles can be phenotypically identified histologically by an increase in centrally located nuclei (CLN) and fibre-size variability (FSV), as well as increases in serum levels of creatine kinase (15, 16).

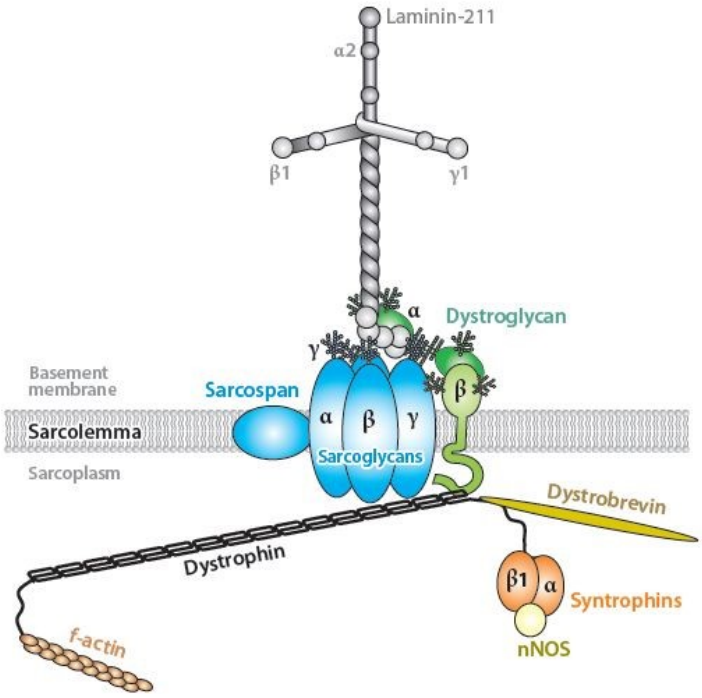


**Figure 1 Dystrophin-Associated Protein Complex -** adapted from (17)

Dystrophin binds to cytoskeletal f-actin at its NH2 terminus. At its COOH terminus, dystrophin is associated with dystroglycan which then associates with extracellular laminin via the sarcoglycans. Mutations leading to loss of functional dystrophin lead to membrane weakness in DMD or a truncated protein at the central rod-domain leads to smaller, but still functional dystrophin protein in the less devastating Becker muscular dystrophy.

### **1.1.2** – *Mdx mouse model*

The mdx mouse model of this disease was first discovered when a group of inbred C57BL/10 mice demonstrated three-fold higher levels of creatine kinase and pyruvate kinase (18). In mdx mice, a point mutation in exon 23 on the X chromosome causes a premature stop codon resulting in a truncated, non-functional dystrophin protein (18, 19). Contrary to DMD, the lifespan of mdx mice is not significantly reduced (20) and they do not display any signs of obvious weakness (21). Muscles from mdx mice do however display significant decreases in normalized force and power outputs per unit of muscle mass (21). Hindlimb muscles are considered to be less affected in mdx mice since they display relatively little fibrotic activity and never display adipose deposition (22). Regenerating fibres hallmarked by CLN are mainly seen between 2-4 months of age (22). Skeletal muscles are composed of four different fibre type ranging from fast and glycolytic type 2 fibres, to the slow and oxidative type 1 fibres (23). Biopsies taken from DMD patients, display that fast muscle fibre types are preferentially affected (24) and the

same effect is observed when comparing the fast extensor digitorum longus (EDL) muscle to the slow soleus muscle in mdx mice (25). The pathophysiological differences between human DMD and mdx mice are not fully understood however the mdx mouse remains an important model in DMD research.

### **1.1.3** –*DMD Treatment*

The glucocorticoid therapies used to treat DMD provide limited results and do not prevent early death. Glucocorticoids do not decrease membrane fragility but rather decrease secondary inflammation caused by damaged muscle fibres (26). More specific therapeutic strategies which prevent fibrosis and loss of function of dystrophin-deficient muscles are currently being explored. Several gene therapy studies are underway to replace the mutated dystrophin gene with a functional one however major hurdles still need to be overcome before this can be achieved (27-29). Large-scale gene therapy treatment for DMD may not be clinically available for several years therefore a more promising shorter-term avenue is to increase endogenous expression of the dystrophin-related protein utrophin, as utrophin is homologous to dystrophin in both structure and function (30, 31).

## **1.2** - *UTROPHIN-MEDIATED RESCUE*

Utrophin has a spectrin-like central rod domain and binds f-actin at one end and interacts with DAPC members at its other end in the same way as dystrophin (31). The main difference between dystrophin and utrophin is its localization within the muscle fibre. In healthy muscle fibres, dystrophin is located along the length of the sarcolemma while utrophin is restricted to the neuromuscular junction (NMJ) and myotendinous junctions (10, 32, 33). In some dystrophin deficient muscle fibres, total utrophin expression is increased and its localization is extended beyond the NMJ and myotendinous junction, and throughout the sarcolemma (34, 35).

### **1.2.1** - *Extrajunctional utrophin is found in slower fibre-types*

A connection between utrophin and slow muscle fibre types was first discovered by investigating dystrophic extraocular muscles (36). The primarily slow, extraocular muscles, show relatively little signs of damage in mdx mice, however in the absence of both utrophin and dystrophin, these muscles are severely affected (36). Further investigation via immunofluorence (IF) and in situ hybridization experiments in wildtype (WT) soleus (slow) and EDL (fast) muscles revealed extrajunctional utrophin localization is specific to the slower type I and IIa fibres (37). An increased utrophin level in type 1 fibres is attributed to an increase in mRNA stability in slow muscles (37). The findings of

utrophin's selective extrajunctional presence in slow fibres in conjunction with the fact that slow fibres are more resistant to the dystrophic pathology spawned the theory that promoting the slower, more oxidative myogene program could attenuate the dystrophic phenotype. Identification of signaling pathways which promote this program then became a focal point of DMD research. The calcineurin (Cn) signaling pathway is known to promote the slower fibre myogene program (38).

**1.2.2** – *Calcineurin/NFAT signaling pathway*

Calcineurin is a $Ca^{2+}$/Calmodulin (CaM) regulated serine/threonine protein phosphatase (39). Cn enzymatic activity requires a catalytic (CnA) and a regulatory (CnB) subunit (40). The CnA subunit includes protein domains conferring catalytic activity, CnB interaction and a CaM-binding site (41). It also includes a C-terminal autoinhibitory domain, which blocks the catalytic site and is removed in response to $Ca^{+}$ release (41). Constitutively active CnA (CnA*) -lacks the autoinhibitory domain and CaM-binding sites- (42) is commonly used to study the role of CnA activity *in vitro* and *in vivo*.

Cn-dependant signaling mechanisms were first characterized extensively in the activation of cytokine gene expression in T and B lymphocytes where the binding of $Ca^{2+}$ to a CaM/Cn complex stimulates serine/threonine phosphatase activity of Cn of which dephosphorylates many downstream targets (43). The family of Nuclear Factor of Activated T-cells (NFATc1-NFATc4) transcription factors represent the major targets of CaM/Cn signaling (fig2) (44). Dephosphorylation of NFAT binding sites by Cn removes phosphate groups that mask its nuclear localization signal thereby enabling translocation from the cytoplasm to the nucleus, where it binds to, and stimulates transcription of target genes (43-45). Cn/NFAT activity responds preferentially to sustained low-amplitude intracellular elevations of $Ca^{2+}$ and is insensitive to transient, high-amplitude intracellular oscillations in $Ca^{2+}$ (43, 46). Similarities between Intracellular $Ca^{2+}$ oscillations found in T and B lymphocytes and those found in skeletal muscle upon neuromuscular stimulation pointed to a potential role for Cn/NFAT signaling in skeletal muscle.

In skeletal muscle, signals from motor neurons trigger the rapid release and uptake of intracellular $Ca^{2+}$ during muscle contraction (47). Tonic motor-nerve activity found in slow muscle fibres is characterized at 10-15Hz (48), resulting in sustained, low-amplitude elevations in intracellular calcium concentrations $[Ca^{2+}]_i$ (49) similar to those found in T lymphocytes. The sustained levels of intracellular $Ca^{2+}$ found in slow fibres is sufficient enough to activate CnA/NFAT signaling (38, 50, 51). Conversely, CnA/NFAT signaling does not occur with short bursts of 50Hz stimulation, consistent with those found in fast fibres (48, 50). Once activated, CnA/NFAT signaling plays an important role in the determination of

skeletal muscle fibre type by promoting the slower, more oxidative myogene program (38, 52, 53). CnA* treatment of skeletal myocytes targets expression of the slow fibre gene program promoters, troponin I slow (TnIs) and myoglobin, but not the fast muscle creatine kinase (MCK) promoter (38, 54) whereas treatment of mice with the CnA blockers, Cyclosporin-A and FK506, induces slow to fast fibre type conversions

**1.2.3** - *Transgenic alterations of CnA/NFAT signaling*

As a result of its vast range of signaling targets, several transgenic models of mice have been created to alter Cn activity in skeletal muscle in order to better investigate its role in skeletal muscle. One model of increased Cn activity, uses CnA* and links it to the MCK promoter (fig2). MCK is preferentially expressed in type 2 muscle fibres, this ensures that Cn activity is now present in all skeletal muscle myofibrils (52, 55). In order to inhibit Cn activity, another transgenic mouse was created which links calmodulin binding peptide (CaMBP) to the TnIS promoter (fig2) (53). CaMBP binds CaM thereby inhibiting its activation of Cn. TnIS is specific to type 1 fibres therefore making the transgene highly active in these fibres and ensuring a complete silencing of skeletal muscles CnA activity.

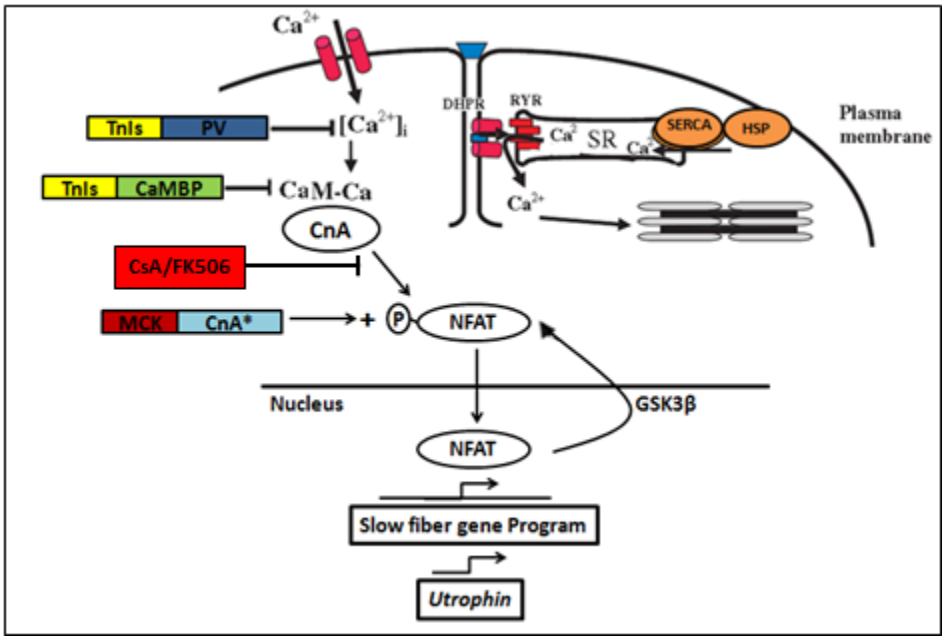


**Figure 2 Transgenic models used to study CnA Signaling -** Adapted from (56)

1) The CnA* mice express a constitutively active form of Cn in fast fibres thereby increasing CnA/NFAT signaling. 2) In CaMBP mice, CnA/NFAT signalling is inhibited by expressing Calmodulin binding peptide in slow fibres. 3) PV mice express $Ca^{2+}$ chelator parvalbumin in slow fibres which prevents $Ca^{2+}$-mediated activation of the CnA/NFAT signaling pathway.

**1.2.4** - *CnA/NFAT promotes utrophin-mediated mdx rescuing*

As a result of CnA's implication in the slow fibre gene program, expression of utrophin transcripts in mice having altered CnA activity was studied and revealed that increased CnA/NFAT activity upregulates extrajunctional utrophin expression (fig. 3) (57). Furthermore, electrophoretic mobility and supershift assays revealed the presence of a NFATc1 binding site on the utrophin promoter and either mutation of the NFAT binding site or CsA/FK506 treatment decreases utrophin expression (57, 58). It is therefore theoretically possible that promoting CnA/NFAT activity could stimulate extrajunctional utrophin expression and restore sarcolemmal integrity in dystrophin deficient muscles.

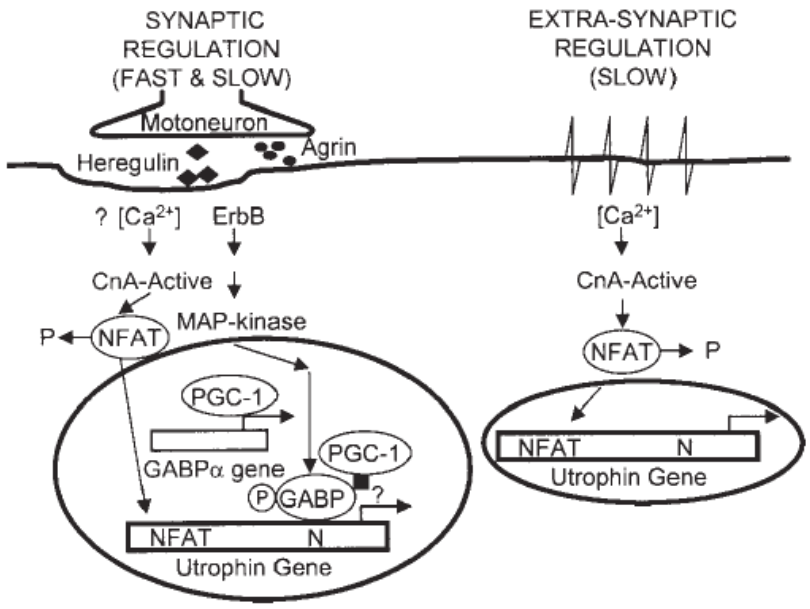


**Figure 3 Schematic model regulatory events controlling utrophin gene expression** adapted from (58)

Synaptic regions of fast and slow fibres express utrophin. In slow muscle fibres, tonic electrical activity triggers specific patterns of $Ca^{2+}$ flux thereby activating the CnA/NFAT signaling pathway. Once dephosphorylated, NFAT is free to travel to myonuclei to transactivate the utrophin-A promoter in extrasynaptic (extrajunctional) compartments. This pathway is not active in fast muscle fibres, thus explaining the absence of utrophin in their extrasynaptic compartments.

By crossing mdx mice with the mck-CnA* mice, our lab has previously shown that promoting a slow fibre gene program was able to rescue the dystrophic pathology as a result of increased CnA/NFAT-mediated utrophin expression (57, 59). In contrast, the soleus from mdx mice crossed with TnIS-CaMBP mice have decreased CnA signalling, resulting in decreased extrajunctional utrophin expression and an exacerbated dystrophic phenotype as measured by an increase in CLN, FSV and collagen infiltration (60).

These studies showed not only the therapeutic potential of increased CnA/NFAT signaling but also emphasized the role that CnA/NFAT plays in limiting slow fibre damage.

### **1.3** – *RESTORING $CA^{2+}$ HOMEOSTASIS HAS THERAPEUTIC POTENTIAL*

An important feature of dystrophin deficient muscles is higher levels of $[Ca^{2+}]_i$ (61, 62). Calcium homeostasis is critical for the normal functioning of many aspects of muscle function (63). Though the genetic defect underlying the disorder is not directly related to $Ca^{2+}$ activity, disturbed intracellular $Ca^{2+}$ signaling is a significant contributor to the pathology of DMD. The sustained, influx of $[Ca^{2+}]_i$ in mdx does increase CnA/NFAT activity in slow fibres however it also triggers other unwanted effects such as increased inflammatory response (64, 65), increased proteolysis (66) and mitochondrial dysfunction (67). The increase in $[Ca^{2+}]_i$ is attributed to a combination of three pathomechanisms occurring in dystrophin deficient tissue; 1) hyperactive $Ca^{2+}$ channels (68-70) 2) transient sarcolemmal tearing (11, 63) and decrease function of $Ca^{2+}$ handling proteins (71, 72). For this reason, another therapeutic avenue of research, independent of utrophin-related research, has developed in recent years which moves to restore $Ca^{2+}$ homeostasis in dystrophic tissue (71, 73-75).

#### **1.3.1** – *HSP70-mediated rescue*

A recent publication by the Lynch group has shown that mdx muscles with forced Heat Shock Protein-70 (HSP70) activity either by transgenic mutation or by the pharmaceutical agent BGP-15 show less damage (74). They speculated that this occurs as a result of HSP70's protective role on Sarco/Endoplasmic Reticulum-$Ca^{2+}$-ATPase (SERCA) (74, 76). SERCA is responsible for pumping out large amounts of $Ca^{2+}$ out of the sarcoplasm after muscle contraction (77). As high $[Ca^{2+}]_i$ caused by leaky $Ca^{2+}$ channels is a feature of dystrophin-deficient muscles, preserving SERCA function could theoretically correct the $[Ca^{2+}]_i$ (74). Interestingly the Lynch group showed that HSP70-mediated rescuing occurred independent from utrophin as dystrophin/utrophin dKO mice also showed less signs of damage (74).

#### **1.3.2** - *HSP70 preferentially expressed in type I and IIa fibres*

Heat shock proteins are a set of proteins that are rapidly synthesized in cells in response to protein-damaging stresses (78). They are most commonly associated with having a protective role in response to heat-related stress (78). Although normally found at lower levels in unstressed skeletal muscle fibres, the inducible isoform of the HSP70 family is constitutively expressed in slow fibres (79, 80). The exact reasoning for this is not fully understood, however it is postulated that the constitutive

expression of HSP70 in slow fibres is related to the higher activity levels of these fibre types. Fast to slow fibre type remodelling of the rat plantaris muscle induced by functional overload increases CnA/NFAT activity as well as HSP70 expression (52, 79, 81). The strong evidence of HSP70's localization in slow fibres may be a potential explanation for the findings by the Lynch group since slow fibres are already known to be resistant to dystrophic phenotype. Furthermore, it could be argued that the improvements observed when HSP70 was pharmacologically introduced into dystrophin/utrophin dKO mice were not as pathophysiologically significant as other studies aimed at increasing extrajunctional utrophin. No changes in contraction-mediated damage, no decrease in inflammatory markers, or functional improvements were found upon increased HSP70 expression. Finally, although significant increase in the time to death was found in dystrophin/utrophin dKO mice, premature death was still not prevented by the pharmacological induction of HSP70 (74). The role for HSP70 in regulating $Ca^{2+}$ homeostasis and rescuing the dystrophic phenotype in mdx mice still remains unclear.

## 2. HYPOTHESIS AND SCIENTIFIC RATIONALE

**2.1** – Scientific r*ationale*

In order to get a better understanding of the relationship that utrophin and HSP70 may have in the rescuing of slow fibres with altered $Ca^{2+}$ activity, a transgenic line of mdx mice was developed which overexpress the $Ca^{2+}$ buffering protein parvalbumin (PV) in slow type 1 fibres via the TnIS promoter (fig. 2). Parvalbumin is a high affinity $Ca^{2+}$-binding protein endogenously expressed in fast type 2 fibres (82). Its high affinity to $Ca^{2+}$ allows for accelerated removal of $Ca^{2+}$ from the sarcoplasm (83). Altered PV function in fast fibres leads to higher intracellular $Ca^{2+}$ levels and prolonged time required to attain peak twitch tension (83). PV overexpression in slow fibres attenuates CnA/NFAT signalling, and leads to altered contractile properties of slow twitch muscle, decreased oxidative and glycolytic capacities and increased expression of genes related to the fast phenotype without actually altering the MyHC composition of the fibres (84). The insertion of the pv TG in dystrophic slow fibres facilitated the study of the behaviour of HSP70 in an environment of altered $Ca^{2+}$ activity as well as decreased CnA/NFAT activity along with its downstream target, utrophin. Under these conditions, it was possible to observe HSP70's therapeutic role in protecting dystrophin and utrophin null fibres.

**2.2** - *Hypothesis*

Altered $Ca^{2+}$ homeostasis in slow muscle fibres will exacerbate the dystrophic pathology as a result of decreased calcineurin signaling and will not be reversed by endogenous levels of HSP70.

## 3. SPECIFIC AIMS

### 3.1 Aim 1

To decrease Cn signaling by altering $Ca^{2+}$ oscillatory patterns in slow muscle fibres (mdx/pv model).

### 3.2 Aim 2

To measure the volume of damaged fibres as a result of the absence of extrajunctional utrophin in mdx/pv mice

### 3.3 Aim 3

To observe if utrophin deficient dystrophic fibres can be rescued by endogenous levels of HSP70.

## 4. METHODS

### **4.1** - *Animal Care Protocols*

All animal protocols were carried out in accordance with the guidelines of the Canadian Council for Animal Care. These procedures were approved by the University Animal Research Ethics Committee (UAREC) of Concordia University. Transgenic mice expressing either PV-HA, CnA*, CaMBP were crossbred with mdx mice. Transgenic mice were identified by PCR screening of genomic DNA isolated from tails. All mice were housed in a room with a twelve hour light cycle.

### **4.2** - *Generation and identification of mdx/pv mice*

To assess the role of $[Ca^{2+}]_i$ kinetics in a dystrophin deficient background, mdx mice expressing a PV transgene specifically in slow muscle fibers was generated. Previous findings have determined that mdx/pv mice are healthy and capable of breeding. PCR-based screening was used to identify animals expressing PV-HA transgene as well as mdx mice carrying a spontaneous nonsense mutation in exon 23 of the dystrophin gene. Immunoblotting analyses of soleus and the primarily fast EDL muscles from WT, pv, mdx and mdx/pv mice (n=3) demonstrated the presence of endogenous pv in both soleus and EDL muscles of all mice and the expression of PV-HA solely in soleus muscles of PV and mdx/PV mice with no detectable levels in EDL muscles (unpublished results).

### **4.3** - *Mice Genotyping*

Genomic DNA was isolated from 5mm clippings of 6 week old mouse tails. Briefly, tail clippings were left to digest overnight at 55$^{o}$C in a mix of lysis buffer and proteinase K solution. DNA was then purified using a phenol/chloroform isolation method. Amplification was performed by polymerase chain reaction as described previously for the following targets: mdx (85), PV-HA (84), CnA* (59) and CaMBP (60). Genotyping primers are listed in table 1.

### **4.4 -** ***Animal surgeries and tissue extraction***

Tissues from mdx mice were extracted at 10-14 weeks of age. All surgical procedures were performed under aseptic conditions on animals anesthetized by intramuscular injection

(1.2 µl/g) of 100 mg/ml ketamine hydrochloride (Ketalean, CDMV, Canada) and 10 mg/ml xylazine (rompun, CDMV, Canada) in a volume ratio of 1.6:1.0. Bilateral soleus and GAS samples were excised and embedded in frozen tissue mounting medium (Neg-50, Thermo Scientific, Canada) and frozen in a pool of melting isopentane cooled in liquid nitrogen. The mice were then euthanized via cervical dislocation. Tissues were stored at -80$^{O}$C until processed.

**4.5** - *Assessment of NFATc1 Nuclear Localization*

Assessment of NFATc1 nuclear localization was performed as previously described (59). Briefly, soleus muscle cross-sections (10µm thick) were fixed with 4% paraformaldehyde (PFA), blocked and permeabilized with 2% goat serum and 0.2% Triton X-100, then washed with 1X phosphate buffer saline (PBS) and incubated with rabbit anti-NFATc1 (sc-13033, Santa Cruz, USA) at 4$^{o}$ overnight. After washing, sections were incubated with goat anti-rabbit IgG Alexa-546 (Invitrogen, Canada) for one hour, then washed and mounted with Vectashield containing 1.43nM 4',6-diamidino-2-phenylindole (DAPI) (Vector laboratories, Canada) for nuclear staining. Quantification of NFATc1 nuclear localization was performed by counting the number of myonuclei positively stained for NFATc1 in cross sectional views of myofibers and calculating the percentage of nuclear NFATc1 (n=3).

**4.6** - *Assessment of Central Nucleation and Muscle Fiber Size Variability*

Degeneration and regeneration of soleus muscles from mdx and mdx/pv mice was assessed by examining the percentage of fibers displaying central nucleation using the Hematoxylin and Eosin (H&E) stain-counterstain method. Hematoxylin stains the nucleus purple, whereas eosin stains the cytoplasm pink. 10µm cross sections from soleus muscles were stained for 5mins in a Hematoxylin solution and then counterstained for 3mins in an Eosin solution. Sections were then dehydrated in a series of ethanol solutions, cleared with xylene and mounted using permount mounting medium (Fisher Scientific, Canada). The sections were visualized using a standard halogen light microscope, where 2-3 10x images per soleus section were captured so as to represent the entire area of the soleus without acquiring overlapping areas. A percentage of fibres displaying central nucleation (CLN) was then quantified using the

following formula; $Percentage\ of\ CLN = \frac{Total\ CLN\ Fibres}{Total\ Fibres}$ (n=3). Fibre size variability was assessed as previously described (86) by using the variance coefficient between fibres of a given section using the following formula; $variance\ coefficient = \frac{Standard\ deviation\ of\ muscle\ fibre\ area}{mean\ muscle\ fibre\ area}$ (n=3).

**4.7** - *Immunofluorescence Experiments*

Serial cryosections (9µm) of soleus and GAS midbellies were captured onto superfrost plus slides (VWR, Canada) and stored in a humid chamber until usage. Sections were fixed in cold acetone for 10mins and then washed 2x5 mins in 25mM PBS. Sections were then blocked in either 5% goat serum or mouse on mouse blocking reagent (M.O.M staining standard kit, Vector Laboratories, Canada) for 1h. Sections were washed 2x2 mins in PBS or M.O.M diluent followed by 30 min primary antibody incubation with either rabbit anto-HSP70: SPA-812 (Enzo Life Sciences, USA) 1:50 or mouse anti-utrophin: DRP3/20C5 (Leica biosystems, Canada) 1:200. Sections were then washed 3x5 mins in PBS followed by secondary antibody incubation for 10mins with either biotinylated anti-mouse IgG or biotinylated anti-rabbit IgG (Vector Laboratories). This was followed by another PBS wash and 5 minute incubation with streptavidin-Dylight 594 (Vector laboratories, Canada).

Separate serial sections were also incubated overnight with the following primary antibodies: mouse anti-Serca1: MA3-912 (Thermo Scientific, Canada) 1:200; mouse anti-Serca2: MA3-919 (Thermo Scientific, Canada) 1:200; mouse anti-MyHC I: A4.840, (DSHB, USA) 1:25; mouse anti-MyHC IIa: SC71 (DSHB, USA) 1:10 or mouse anti-MyHC IIb: BF-F3 (DSHB, USA) 1:10. Sections were then washed 6x5 mins followed by secondary antibody incubation for 2 hours with either goat anti-mouse IgG Alexa-488 or goat anti-mouse IgM Alexa-546 (Invitrogen, Canada) 1:500 for Serca1/2 or 1:25 for the MyHC's.

All slides were then washed extensively in PBS and air dried for 30mins. Coverslips were then mounted with Vectashield mounting medium containing 1.43nM DAPI (Vector Laboratories, Canada). Coverslips were sealed with nail polish and slides were stored at 4$^{o}$C.

Control experiments omitting primary antibodies revealed absent or very low-level background staining (fig. S1). Antibody specificity of the polyclonal HSP70 was also verified with two other monoclonal antibodies (fig. S1).

**4.8** - *Immunofluorescent Image Analysis*

Images were acquired on an Olympus BX-60 fluorescent microscope (Olympus) using the same exposure settings between sets. All analyses were performed using Image-pro 6.2 software (Olympus, Canada).

For all analyses in soleus muscles, three randomly selected areas per set of either mdx or mdx/pv were analyzed for protein expression of MyHC I, MyHC IIa, and utrophin as well as localization of nuclear DAPI staining (n=3). For all analyses in GAS muscles, three areas per set of either mdx, mdx/pv, mdx/CnA* or mdx/CaMBP were selected only under the condition that they have at least one representative fibre of each fibre type. Areas were then analyzed for protein expression of MyHC I, MyHC IIa, MyHC IIb, HSP70, utrophin, as well localization of nuclear DAPI staining (n=2). Serca1 and Serca2 were also assessed in mdx and mdx/pv samples (n=1).

**4.9** - *Statistical analysis*

Statistical analyses were performed using the SPSS software program version 17.0. (IBM SPSS, USA). Results were expressed as means ± SEM. Independent samples T tests assuming equal variances were used to compare means ($P<0.05$).

| Transgene | Forward | Reverse | Product size (bp) |
|---|---|---|---|
| Mdx | 5’-GCGCGAAACTCATCAAATATGCGTGTTAGTGT-3’ | mutant: 5’-CGGCCTGTCACTCAGATAGTTGAAGCCATTTTA-3 | 117 |
| | | WT: 5’-GATACGCTGCTTTAATGCCTTTAGTCACTCAGATAGTTGAAGCCATTTTG-3’ | 134 |
| PV-HA | 5’-CCCACCAGCCCAGCTTTTCTA-3’ | 5’-TTAGGCGTAGTCGGGCACGTCATATGGGTAGCTTTGGCCAC-3’ | 450 |
| CnA* | 5'-CGATTCAAAGAACCACCTGCTTATGGG-3' | 5'-CCCAAGCTTGGGTTTCTGATGACTTCCTTC-3' | 600 |
| CaMBP | 5'-CCTTTACTTCTAGGCCTGTACG-3' | 5'-AGCATTTTTTTCACTGCATTCTAGTTGT-3' | 450 |

**Table 1: Genotyping primers**

## 5. RESULTS

### **5.1** - *Mdx/pv mice have decreased CnA/NFAT signaling and increased dystrophic phenotype*

Dystrophin-deficient mdx mice were inserted with a transgene that preferentially expresses pv in type 1 fibres via the TnIS promoter (fig. 2), in order to assess the role of calcium kinetics. In WT mice, the TG displayed Cn activity levels that were 36% of their WT counterparts (84). Although in the current study, calcineurin phosphatase activity was not directly assayed, it was indirectly measured via IF experiments of the downstream target NFATc1 (fig. 4A). For this purpose, the primarily slow mdx and mdx/pv soleus muscles were studied, as this is where the TG is known to be most active based on the high levels of TnIS. Since dephosphorylated NFATc1 is shuttled to the nucleus, the quantification of how many nuclei expressing NFATc1 is a reliable indication of NFATc1 activity. As predicted, nuclei from mdx/pv soleus muscles had 34% of activated NFATc1 compared to mdx mice (fig. 4A) (87).

The next step was to confirm whether this decrease in CnA activity exacerbated the dystrophic phenotype. The number of centrally located nuclei in mdx soleus is reported to be just over 60% between 8-13 weeks of age (20). Similar results were found in this study, with 63% of fibres from the mdx soleus displaying CLN. As hypothesized, a significantly larger number of fibres with CLN were found in the mdx/pv soleus (81%) (fig. 4B).

Another histological indicator of damage in dystrophin deficient mice is the fibre-size variability in the cross-sectional area of fibres. Generally, mdx muscles have a variance coefficient double that of their WT counterparts (86). Surprisingly, no significant difference in the FSV was observed in the presence of the pv TG (4C).

Overall, the results suggest that the pv TG altered the $Ca^{2+}$ kinetics of mdx mice and decreased CnA signaling activity enough to exacerbate the dystrophic pathology, however, the histological phenotype was not as severe as found in dystrophin/utrophin dKO mice (88).

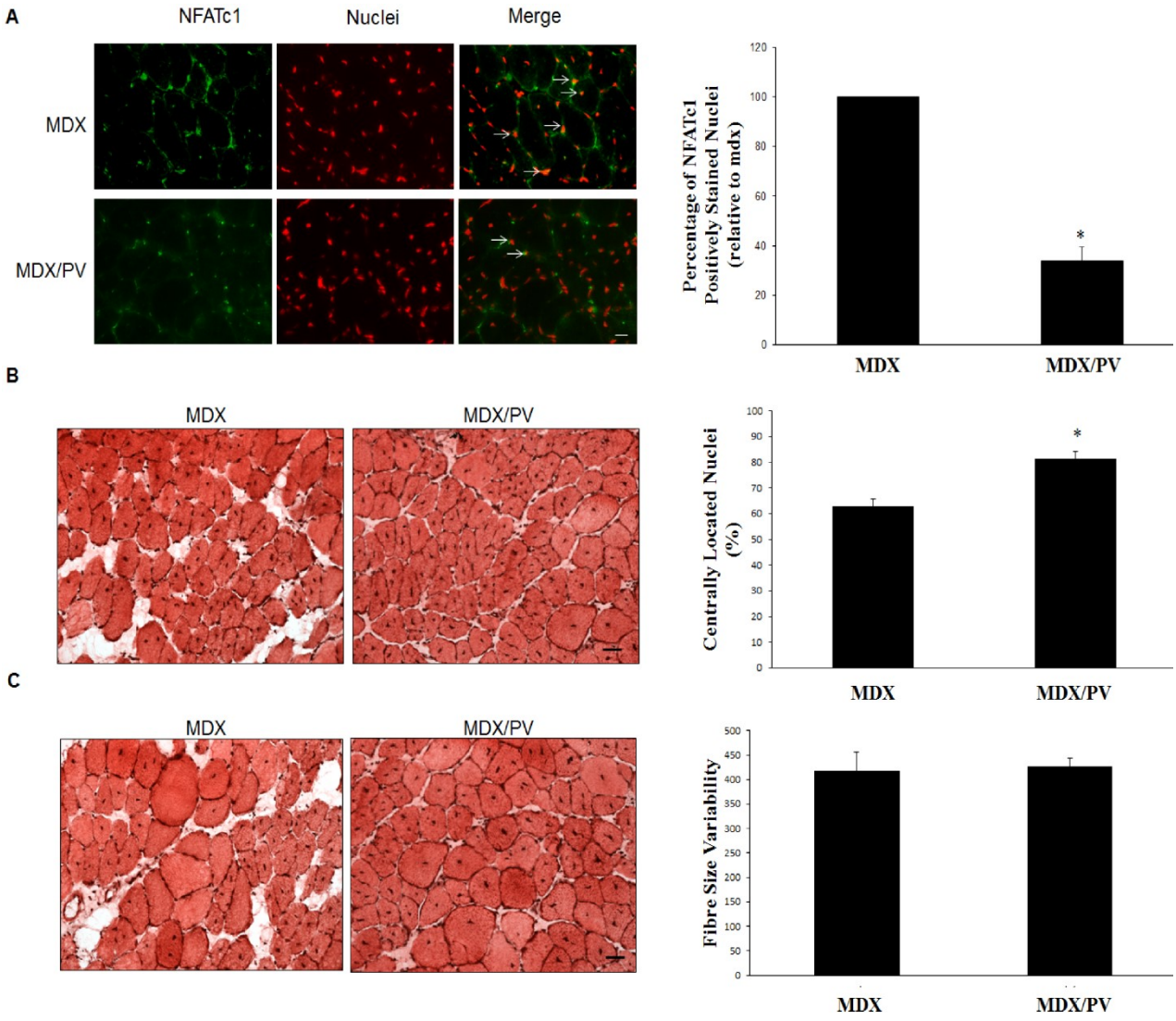


**Figure 4. Characterization of mdx/pv model**

Forced expression of the pv transgene in slow muscle fibres results in decreased CnA/NFAT signaling and increased markers in damage. (A) Representative photomicrographs showing a decrease in NFATc1 nuclear localization. Arrows indicate nuclei positively co-staining with NFATc1. Panel on the right shows the quantification (performed by Dr. Al Zein) of the percentage of nuclei staining positive for NFATc1 (relative to mdx). Scale bar, 20µm. (B) Representative photomicrographs and quantification of H&E stains showing an increase in the percentage of fibres displaying centrally located nuclei. (C) Representative photomicrographs and quantification of H&E stains showing no significant difference in the fibre size variability between mdx and mdx/pv mice. H&E scale bars, 50µm. Means ± SEM are shown. (*) indicates statistical significance (n=3, $p<0.05$).

**5.2** - Mdx*/PV soleus have decreased utrophin and more damage*

Immunofluorescence stains were then performed in serial cross-sections of soleus muscles to confirm that the pv TG also affects utrophin levels of mdx/pv mice. The soleus was studied as it is comprised of approximately 50% type I fibres (89), therefore making the TG highly active in this muscle. In addition to its high proportion of slow fibres, the soleus' function as a weight bearing muscle increases the risk of stress-induced damage when compared to other slow muscles such as the extra-ocular muscles. All fibres within a randomly selected area were first analyzed for their presence of either MyHC I or MyHC IIa (fig. 5). Next, serial sections of the fibres were analyzed for the presence of extrajunctional utrophin. As predicted, a qualitative difference in the amount of extrajunctional utrophin seen in the mdx soleus compared to their transgenic counterparts with the latter displaying less (fig. 5). Furthermore, an increase in the number of type I fibres displaying CLN was observed in mdx/pv mice (fig. 5).

These results confirm previous findings that dystrophin deficient mice with decreased CnA/NFAT signaling display decreased levels of utrophin and show increased signs of damage (59, 60).

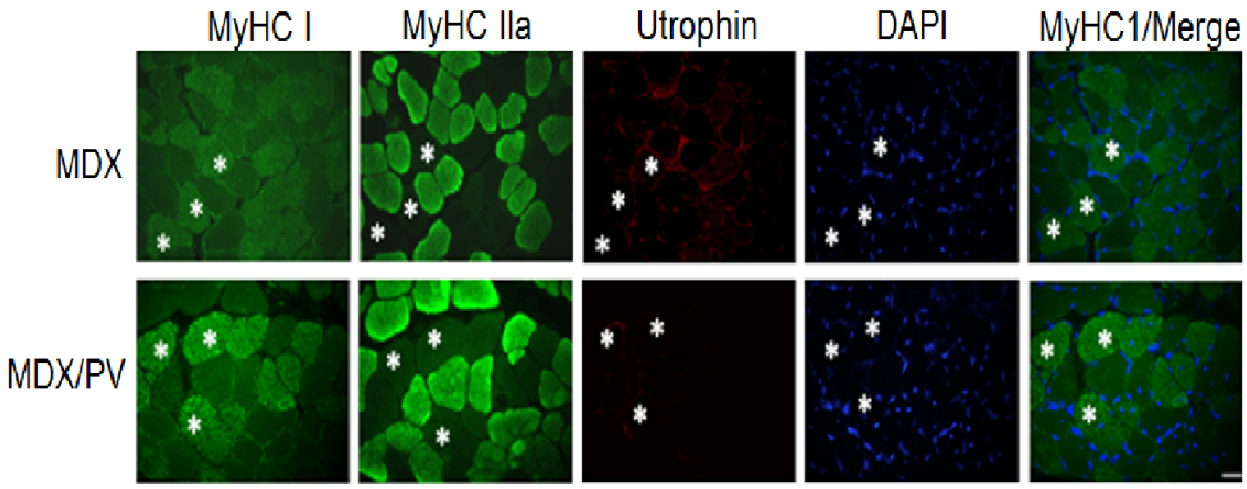


**Figure 5. Decreased utrophin expression in mdx/pv mice results in more damage**

Representative photomicrographs of serial crossections from mdx and mdx/pv soleus muscles. In the mdx soleus, the majority of type I fibres display extrajunctional utrophin and are resistant to damage hallmarked by a lack of centrally located nuclei as demonstrated by (*). Mdx/pv soleus type I fibres do not display extrajunctional utrophin resulting in an increased number of centrally located nuclei as indicated by (*). n=3 (performed in collaboration with Dr. Al Zein).

**5.3** – *Mdx fibres are still damaged in the presence of HSP70 but not utrophin*

It was then determined if HSP70 could rescue dystrophic fibres in the absence of utrophin. To verify this, the "mixed-fibre" GAS muscle was used. Since it is already known that HSP70 is present in the slower type I and IIa fibres but not the faster IIb fibres, it was then possible to assess whether there was a difference in the number of damaged utrophin-deficient fibres in the presence or absence of HSP70. The fast IIb fibres served as an internal negative control for both utrophin and HSP70 staining as well as a positive control for damaged fibres in both mdx and mx/pv mice. For this analysis, type I and IIa fibres were grouped together and termed as type "IIb-negative" fibres, as has been previously performed (59).

All fibres within a given capture-frame were analyzed for their presence of either MyHC I, MyHC IIa or MyHC IIb with any unstained fibres assumed as IIx fibres. To ensure that no other

compensatory mechanisms to restore normal $Ca^{2+}$ levels in slow fibres had occurred, fibres were also assessed for the presence of either SERCA1 or SERCA2. No difference in SERCA isoforms was observed between mdx and mdx/pv mice, with type I fibres displaying SERCA2 expression and type II fibres displaying SERCA1 expression (fig. 6). As expected, the majority of type IIb fibres were positive for damage in both mdx and mdx/pv mice with no noticeable difference between the two groups. As previously shown in the mdx GAS (57), extrajunctional utrophin was present in all MyHC IIb-negative fibres (fig. 6A). Similar to the soleus muscle, forced expression of pv in slow fibres of the GAS muscle also resulted in a decrease in extrajunctional utrophin expression (fig. 6B). Finally HSP70 localization was observed and revealed no difference in mdx/pv compared to their mdx counterparts, with all IIb-negative fibres staining positive for HSP70 (fig. 6).

Finally, all IIb-negative fibres were assessed for the presence of CLN. In the mdx GAS, IIb-negative fibres displayed both extrajunctional utrophin and HSP70 and were resistant to damage. Alternatively, the utrophin-deficient IIb-negative fibres found in mdx/pv mice, showed relatively more damage despite the presence of HSP70.

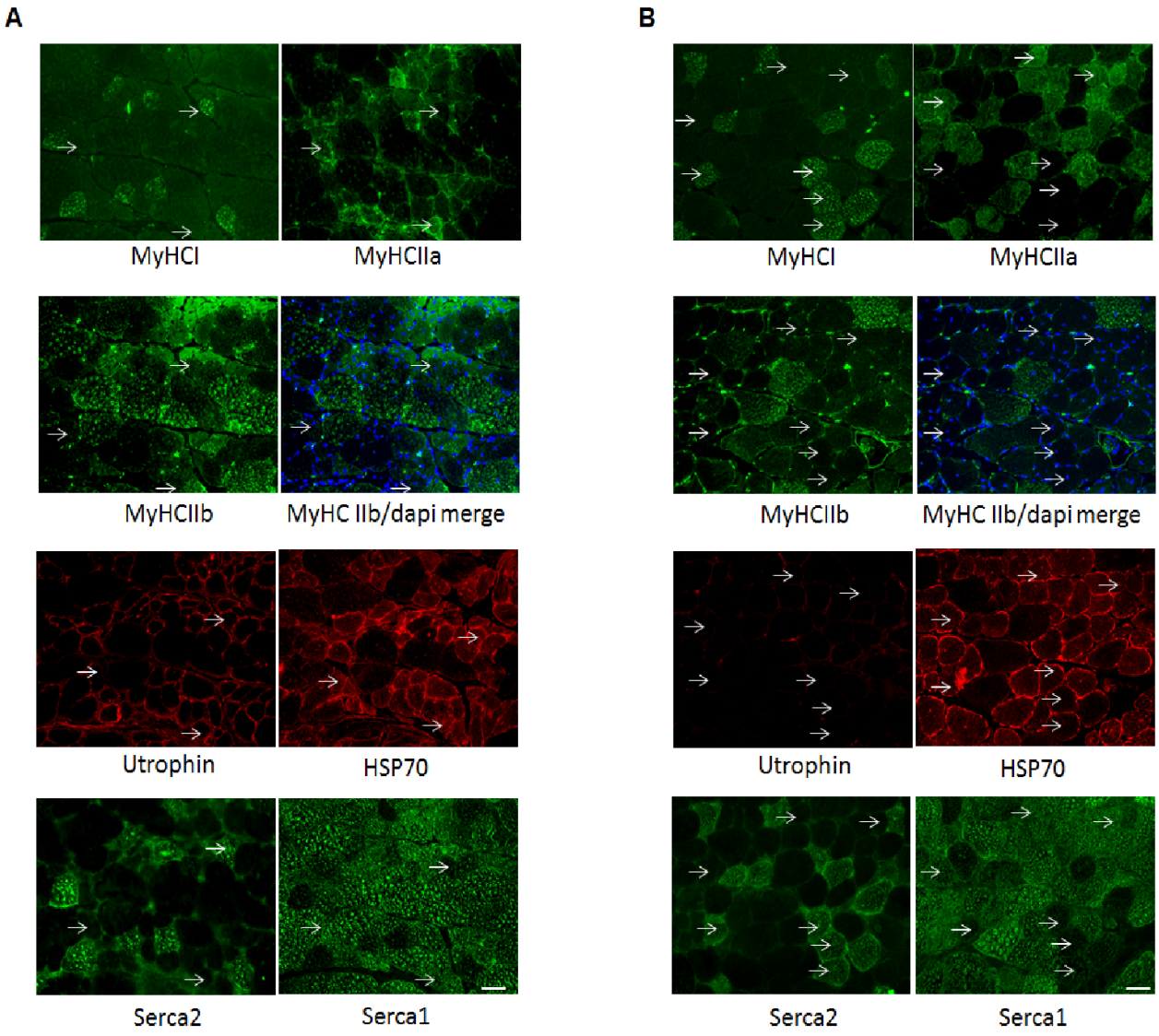


**Figure 6. IIb-negative fibres are not rescued by HSP70 in the Absence of Utrophin**

Representative photomicrographs of serial sections of mdx (A) and mdx/pv (B) "mixed-fibre" GAS muscle. The top four panels display the MyHC isoform of each fibre in which unstained fibres can be assumed to be IIx fibres. The MyHCIIb/DAPI merge shows that the majority of type IIb fibres have centrally located nuclei in both mdx and mdx/pv mice. In mdx mice, a relatively small portion of MyHC IIb-negative fibres display centrally located nuclei however in mdx/pv mice, a larger portion have centrally located nuclei present (indicated by arrows). All IIb-negative fibres are also positive for both utrophin and HSP70 in mdx mice however in mdx/pv mice, IIb-negative fibres are positive for HSP70 but have no extrajunctional utrophin. Bottom two panels show no difference in SERCA distribution in which Serca2 is found only in type I fibres whereas Serca1 is found in all type II fibres. Mdx: n=3 (n=1 for SERCA IFs); mdx/pv: n=1.

**5.4** – *Extrajunctional Utrophin Rescues IIb Fibres Despite the Absence of HSP70*

$Ca^{2+}$ has a wide variety of signaling targets in skeletal muscle therefore it was important to confirm the findings from the mdx/pv crosses by also performing similar IF stains in other well-known models of altered CnA/NFAT signaling. The two models used in this study target CnA/NFAT signaling more directly without affecting the $Ca^{2+}$ oscillatory patterns.

The first model used was the mdx/CaMBP crossbreeds, which are already known to decrease extrajunctional utrophin expression and exacerbate the dystrophic phenotype (60). The CaMBP TG blunts CnA/NFAT signaling in slow fibres more effectively than the pv TG by binding to, and inhibiting the catalytic domain of CnA thereby preventing dephosphorylation of NFATc (fig. 2). As previously described (60), the CaMBP TG decreased extrajunctional utrophin expression in IIb-negative fibres (fig. 7C). Similar to mdx/pv mice, the IIb-negative fibres of mdx/CaMBP mice also displayed increased damage compared to mdx mice despite expressing high levels of HSP70 (fig. 7).

The second model used was the mdx/CnA* crossbreeds, which are already known to increase extrajunctional utrophin expression and ameliorate the dystrophic phenotype. The CnA* TG is active in all type II fibres therefore CnA/NFAT signaling is activated in all four fibre-types (fig. 2). Studying this model provided access to dystrophic fibres that displayed extrajunctional utrophin but relatively little HSP70 for the purpose of assessing damage. As predicted, an increase in extrajunctional utrophin was observed in IIb fibres of mdx/CnA* mice however HSP70 remained localized in IIb-negative fibres (fig. 7B). This resulted in a decrease in the number of damaged IIb fibres despite the absence of HSP70 (fig. 7).

All together these results confirm that the exacerbation of the dystrophic phenotype observed in mdx/pv mice is a result of decreased CnA/NFAT signaling and not likely from another $Ca^{2+}$ signaling mechanism. It also indicates that endogenous levels of HSP70 do not have protective role in utrophin deficient dystrophic fibres.

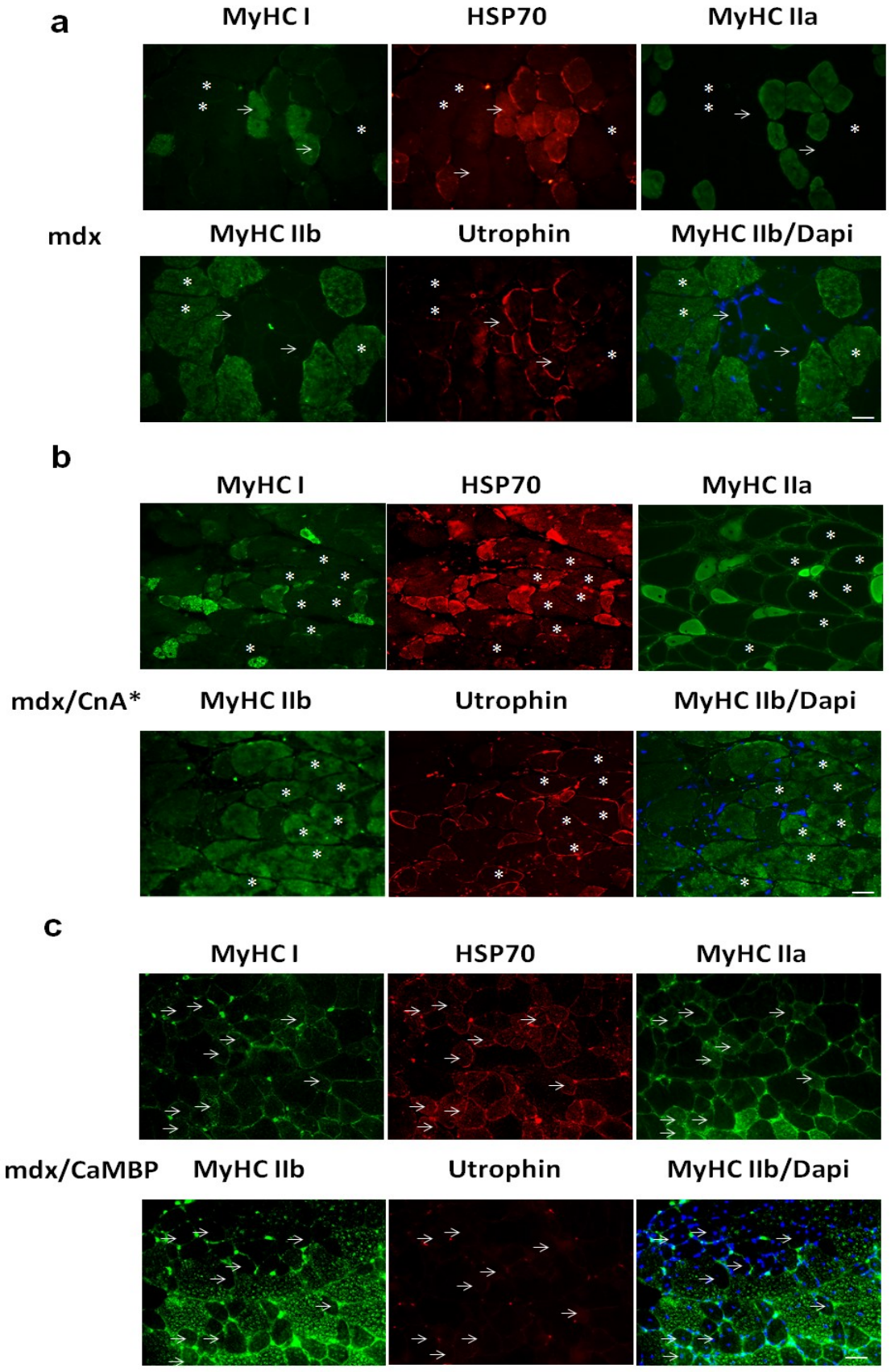
a
MyHC I
HSP70
MyHC IIa
mdx
MyHC IIb
Utrophin
MyHC IIb/Dapi
b
MyHC I
HSP70
MyHC IIa
mdx/CnA*
MyHC IIb
Utrophin
MyHC IIb/Dapi
c
MyHC I
HSP70
MyHC IIa
mdx/CaMBP
MyHC IIb
Utrophin
MyHC IIb/Dapi

**Figure 7. Directly Targeting Cn/NFAT Signaling Shows Similar Results**

Representative photomicrographs of serial sections from the "mixed-fibre" GAS of mdx (A) and two previously characterized models of altered Cn/NFAT signaling. The mdx/CnA* model (B), expresses activated CnA in fast fibres resulting in a larger number of type IIb fibres to express extrajunctional utrophin with no changes in HSP70 expression. An increased number of type IIb fibres show no signs of damage as indicated by (*). The mdx/CaMBP model (C) directly inhibits CnA activity in slow fibres without altering $Ca^{2+}$ oscillations. MyHC IIb-negative fibres do not display extrajunctional utrophin but still express HSP70. There is an increase in the number of IIb-negative fibres with centrally located nuclei as indicated by arrows. mdx: n=3; mdx/CnA* and mdx/CaMBP: n=2.

## 6. DISCUSSION

### **6.1** – *Forced PV in Slow Muscle Fibres Exacerbates the Dystrophic Phenotype*

The maintenance of $Ca^{2+}$ homeostasis is essential for the proper functioning of skeletal muscles however it is disrupted in dystrophin deficient muscles (63, 90). Restoring normal $Ca^{2+}$ transients in dystrophic muscles can protect muscle fibres from stress induced damage (71, 73, 90). Although excessive $Ca^{2+}$ entry can have adverse effects in muscle fibres (63), dystrophic slow fibres are particularly resistant to this effect, in part, due to the activation of the $Ca^{2+}$ sensitive CnA/NFAT pathway under these conditions (37, 38). The forced expression of pv in WT slow muscle fibres effectively disrupts the slow and sustained $Ca^{2+}$ oscillatory patterns that are characteristic of slow muscle fibres and subsequently decreases CnA activity by over 60% (84). In this study, CnA/NFAT activity was indirectly measured in mdx/pv mice and a similar result was observed (fig. 4). Measuring dephosphorylated, nuclear NFAT levels is not as accurate as directly assaying CnA activity since other factors may influence levels of NFAT activity (44). The reduced NFATc1 nuclear localization in mdx/PV soleus muscles could, in part, be explained via activation of MAPK-JNK1 pathway. Altered $Ca^{2+}$ levels can also activate other transcriptional factors, including stress-activated MAPK-JNK1 pathway (91, 92). Increased JNK1 activity increases interactions with NFATc1 and results in its dislocation from the nucleus (93). Overall, the decrease in nuclear NFAT observed in mdx/pv mice was similar to WT mice with the transgene (84). Therefore the decrease in nuclear NFAT was likely a result of decreased CnA activity.

As hypothesized, altering $Ca^{2+}$ homeostasis in dystrophic slow muscles increased the number of damaged fibres (fig. 4). It did not however, increase the fibre size variability, another phenotypic histological indicator of damage in mdx muscles. This can be explained by two possible reasons; 1) CnA activity in mdx/pv mice is not completely blocked therefore the measurement of CLN may be more sensitive than FSV in these mice and 2) the age of the mice used in the study. Although the progression of the disease in mdx mice is slower in the soleus, it is still progressive and certain stress markers such as FSV are already plateaued at 12 weeks of age (20). It is possible that the fibre size variability may have progressed and plateaued earlier

in the life cycle of the mdx/pv mice however at three months of age, as studied here, both mdx and mdx/pv mice had similar levels of fibre size variability.

It is widely accepted that extrajunctional utrophin expression can prevent stress-induced damage in slow fibres (30, 36, 37). In the current study, co-staining between MyHC I, MyHC IIa and utrophin confirmed that only the type I fibres display extrajunctional utrophin in the mdx soleus. Furthermore, significantly less fibres displayed CLN when extrajunctional utrophin was expressed compared to when utrophin expression was limited to the NMJ thereby confirming previous results (37, 59). In agreement with the decreased NFATc1 nuclear localization found in mdx/pv mice, a decrease in extrajunctional utrophin was observed not only in IIa fibres but also type I fibres. This resulted in an increase in the number of type I fibres displaying CLN in the mdx/pv soleus compared to the mdx soleus. Taken together, this suggests that disrupted $Ca^{2+}$ oscillatory patterns in slow muscle fibres results in an increased risk of stress-induced damage, largely as a result of impaired CnA/NFAT signaling and its downstream target, utrophin.

Altered $Ca^{2+}$ homeostasis can also have effect on other signaling pathways including the $Ca^{2+}$ sensitive protease calpain (63, 94). Calpain selectively degrades key proteins in the $Ca^{2+}$ cycle and is involved in several muscle diseases including limb-girdle muscular dystrophy type 2A (95). In this context it is theoretically possible that altered calpain activity as a result of the pv TG in mdx mice could also account for the increase in damage found in both soleus and GAS tissues. Although no measurement of calpain activity was conducted in this study, two other well characterized models of altered CnA/NFAT signaling were looked at in mdx mice, neither of which alter $Ca^{2+}$ homeostasis and by association, calpain activity. In these models, results similar to the mdx/pv data were observed (fig. 7); therefore confirming that the increase in damage found in mdx/pv mice was likely a result of decreased CnA/NFAT activity.

**6.2** – *HSP70's Therapeutic Role is Dependant on Extrajunctional Utrophin Expression*

It has been recently suggested that the pharmacological induction of HSP70 can ameliorate the dystrophic phenotype (74) however no evidence was found in the current investigation that supports this claim. Results from IF experiments, in agreement with previous

data, show that type IIb-negative fibres are more resistant to the dystrophic phenotype in the mixed-fibre GAS. Furthermore, HSP70 was consistently found in IIb-negative fibres, confirming previously found data on the location of HSP70 expression in skeletal muscle tissue (81, 96). Since both HSP70 and utrophin are present in the rescued IIb-negative fibres, a comparison was performed to identify whether rescued fibres are more closely associated with the presence of HSP70 or extrajunctional utrophin. As hypothesized, the absence of utrophin resulted in a large proportion of damaged fibres however no obvious increase in the number damaged fibres was observed in the absence of HSP70. This can partly be explained by the proposed therapeutic roles that each utrophin and HSP70 are suggested to assume. While utrophin functionally replaces dystrophin in the DAPC therefore restoring sarcolemmal strength, HSP70 is suggested to limit secondary complications found as a result of stress-induced membrane tearing (30, 74). Transgenic overexpression of HSP70 in mdx mice does not improve muscle fibre structural integrity (74). The fact that more fibres with CLN are found in the mdx/pv mice despite sustained HSP70 expression may indicate that dystrophin deficient fibres have a stronger necessity for the restoration of membrane integrity (via extrajunctional utrophin expression) rather than a limitation of secondary effects caused by membrane tearing as is suggested of HSP70's therapeutic role. Overall, the preliminary IF data suggests that the potential therapeutic effects of HSP70 in muscular dystrophy may only be a small member of a collaborative effort of the slower, more oxidative muscles fibres.

HSP70 may still have a therapeutic role in ameliorating the dystrophic phenotype since it also acts as a molecular chaperone protein that inhibits inflammatory mediators including the aforementioned p-JNK (97) as well as the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and nuclear factor-κB (NF-κB) (98). Although TG overexpression of HSP70 in mdx mice does not decrease p-JNK or NF-κB activity, it does decrease mRNA expression of macrophage markers CD68 and F4/80 as well as expression of TNF-α (74). Since inflammation promotes degradation in mdx mice (64), it is possible that HSP70 can be used in combination with other therapeutic remedies to ameliorate muscle phenotype in DMD however current glucocorticoid treatments are more effective at decreasing inflammation and are already approved for treatment of people with DMD (26).

HSP70 was also suggested to improve SERCA function as it is known to bind SERCA and prevent functional deactivation under cellular stress (74, 76). Improved SERCA function regulates $Ca^{2+}$ homeostasis and therefore prevents degeneration of dystrophic muscle (71). The current study shows no difference in the expression of SERCA isoforms between mdx and mdx/pv mice as shown by preliminary immunofluorescent stains (fig. 6) as well as by western blotting (unpublished results). Although no difference in SERCA protein expression was found, the limitations of the techniques used to measure expression are not sensitive to SERCA activity levels. In mdx mice, SERCA activity decreases compared to WT mice, largely due to post-translational modifications such as nitrosylation, which decrease maximal SERCA activity as a result of changes in $Ca^{2+}$ binding and ATP-binding domains (76). It is possible that there is a difference in max SERCA activity in mdx/pv mice despite having similar expression patterns to mdx mice. In this case it would be important to further investigate whether HSP70 helps to preserve SERCA function in the absence of utrophin.

## 7. CONCLUSION

Collectively, the current results emphasize a need to restore skeletal muscle membrane integrity in DMD. Although the restoration of $Ca^{2+}$ homeostasis does improve dystrophin-deficient muscle function, it does not prevent membrane tearing caused by sarcolemmal structural weakness. Dystrophin-related protein utrophin upregulation continues to be a primary therapeutic candidate. Unfortunately, utrophin upregulation via the CnA/NFAT signaling pathway is not pharmacologically possible as a result of calcineurin's important functions elsewhere in the body, namely in the heart and immune system. It is therefore more important to pursue other avenues which increase CnA/NFAT signaling in muscle indirectly by promoting a fast to slow fibre-type conversion. Various other targets have been shown to promote the fast to slow fibres type conversion and ameliorate the dystrophic phenotype in mdx mice such as PGC1-α/PPAR (58), AMPK (99), and Sirt1 (100).

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## SUPPLEMENTARY FIGURES

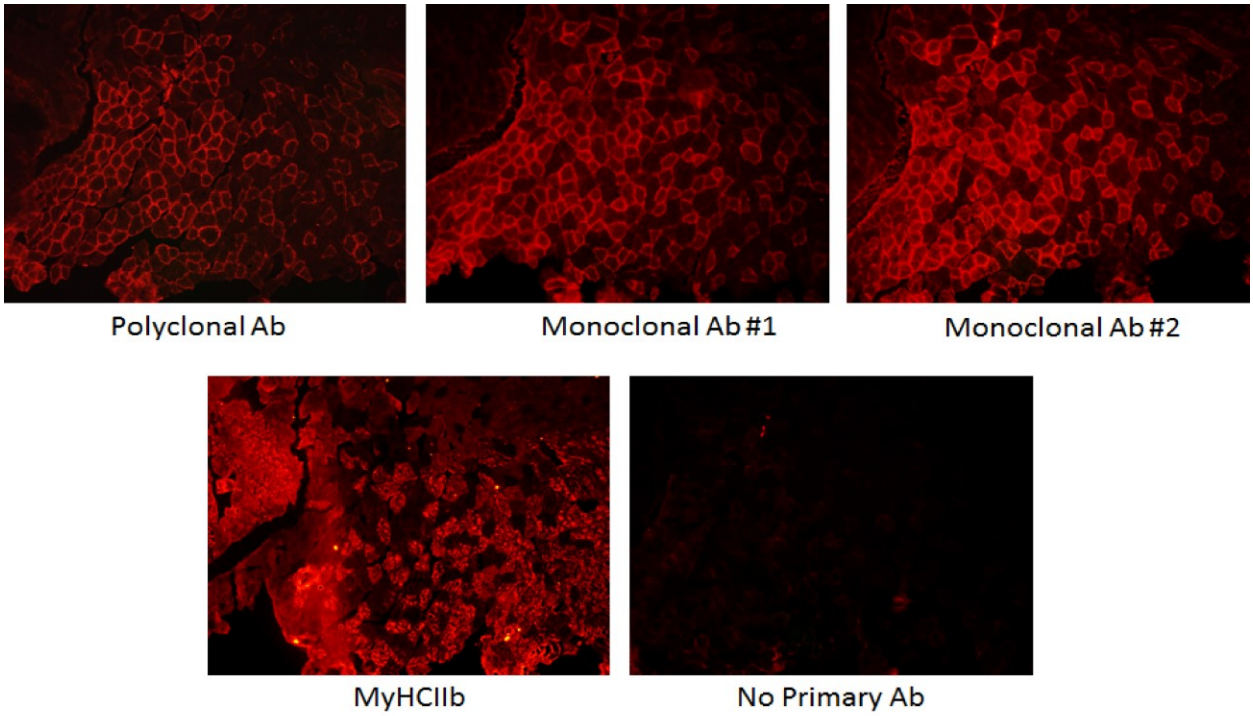


**Figure S1. Polyclonal Antibody is Specifically Found in 2b –negative fibres**

Representative photomicrographs taken with a 10X objective lens of serial sections from the "mixed-fibre" GAS of WT mice testing the specificity of the polyclonal AB used in these experiments. The polyclonal Ab was tested alongside two different monoclonal Ab's and displayed similar staining patterns in which each Ab co-stained with 2b-negative fibres. Separate serial sections were also captured and followed the same staining procedure with no primary Ab and displayed no specific staining pattern. n=2.