

Heart function in mouse models of muscular dystrophy

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Abstract

Duchenne muscular dystrophy (DMD) is an X-linked recessive disease caused, in most cases, by the complete absence of the 427 kD cytoskeletal protein, dystrophin. Without dystrophin, the dystrophin-associated protein complex (DAPC) does not form and the plasma membrane is destabilised. There is no effective treatment and affected individuals die from respiratory failure and cardiomyopathy by age 30. This thesis describes experiments in which in vivo cardiac function was measured using non-invasive magnetic resonance imaging in a number of mouse models relevant to muscular dystrophy. As syncoilin forms a link from the DAPC to the cytoskeleton, it was postulated in Chapter 3 that the syncoilin knockout mouse would have cardiac defects similar to those caused by the loss of dystrophin. However, the loss of syncoilin did not alter the protein levels of its binding partners, measured by western blotting, and caused no defect in heart function or structure, measured using histological staining. Similarly, in Chapter 4, a mouse with a mutation in the transient receptor potential channel canonical type 3 (TRPC3), a receptor/stretch-activated cation channel thought to be involved in the pathogenesis of DMD, was found to have no functional or morphological cardiac defect. In the *mdx* mouse, a mouse model of DMD that lacks dystrophin, cardiomyopathy was prevented by either increasing levels of the dystrophin related protein, utrophin, or of dystrophin, in the diaphragm, which thereby restored diaphragm function. In Chapter 5 it was found that in a transgenic *mdx* mouse in which utrophin was over-expressed in skeletal muscle and diaphragm, but not in the heart, cardiac function was restored to wild-type levels. However, histologically the transgenic heart showed more fibrosis and immune cell infiltration than that of untreated *mdx* controls. In Chapter 6 it was found that in *mdx* mice treated with a peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO), that resulted in high dystrophin restoration in skeletal muscle and diaphragm only, cardiac function was also restored to wild-type levels. In Chapter 6 it was also found that in dystrophin/utrophin-deficient double-knockout (*dKO*) mice, a more severely affected animal model of DMD, treatment with a PPMO again produced high levels of dystrophin only in skeletal muscle and diaphragm, and once more restored cardiac function to wild-type levels. In the *dKO* mouse, there was no difference in heart function between treatment of the diaphragm plus the heart and treatment of the diaphragm alone. Restoration of diaphragm and other respiratory muscle function, irrespective of the method used, was sufficient to prevent cardiomyopathy in dystrophic mice. The novel mechanism of treating respiratory muscles to prevent cardiomyopathy in dystrophic mice has implications for the study of heart function in the current DMD mouse models and suggests a new approach to treatment.

Declaration

The work presented in this thesis was carried out in the MRC Functional Genomics Unit, Department of Physiology, Anatomy and Genetics. Except for Chapter 6, the work reported in this thesis is my own and has not been submitted for any degree in this or any other University or Institute of Learning.

In Chapter 6, experiments on PPMO-treated mice were in collaboration with Dr Aurélie Goyenvallé (Davies Laboratory; P007-PMO experiments) and Dr HaiFang Yin (Wood Laboratory; all other PPMO experiments). Treated mice were supplied by collaborators, imaged by me and then returned alive to the collaborators. Subsequently the mice were killed and western blots in Sections 6.3.2.1, 6.3.2.2, 6.3.2.3, 6.3.3.2 and 6.3.3.3, immunostaining in Section 6.3.3.2 and immunohistochemistry and quantification of dystrophin positive fibres in Sections 6.3.2.2 and 6.3.2.3 were performed by collaborators. I produced all cine-MRI data in this thesis, except for the data from age-matched *C57/Bl10* wild-type controls in Section 6.3.2.1, which was supplied by Dr Dan Stuckey (Clarke Laboratory).

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Abbreviations

ACE	angiotensin I converting enzyme
ALS	Amyotrophic lateral sclerosis
AOs	antisense oligonucleotides
AT1	angiotensin II type 1 receptor
BM	body mass
BMD	Becker muscular dystrophy
BNP	B-type natriuretic peptide
CaMK	Ca ²⁺ /calmodulin-dependent kinase
cGMP	cyclic Guanosine Monophosphate
CK	creatine kinase
CO	cardiac output
DAG	diacylglycerol
DAPC	dystrophin-associated protein complex
<i>des</i> -/-	desmin deficient mouse
<i>dKO</i>	dystrophin/utrophin-deficient double-knockout mouse
DMD	Duchenne muscular dystrophy
<i>dtna</i> -/-	α -dystrobrevin deficient mouse
E/A ratio	ratio between early (E) and late (atrial - A) ventricular filling velocity
ECG	electrocardiogram
ECL	enzymatic chemiluminescence
ECMs	extracellular matrix components
EDB	extensor digitorum brevis
EDL	end diastolic volume

EF	ejection fraction
ENU	N-ethyl-N-nitrosourea
ESE	exonic splicing enhancer
ESL	end systolic volume
ESS	exonic splicing silencer
H&E	Haematoxylin and Eosin
HRP	horseradish peroxidase
HAS	human skeletal actin
IF	intermediate filament
IL	interleukin
iNOS	inducible nitric oxide synthase
IV	intravenous
IP	intraperitoneal
IP ₃	inositol trisphosphate
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
kD	kilodaltons
KO	knockout
LV	left ventricle
MD	muscular dystrophy
MEF2	myocyte-specific enhancer factor 2
MHC	myosin heavy chain
MRI	magnetic resonance imaging
MTJ	myotendinous junction
MW	<i>Moonwalker</i>

NAD(P)H	nicotinamide adenine dinucleotide phosphate
NCX1	$\text{Na}^+/\text{Ca}^{2+}$ exchanger
NFAT	nuclear factor of activated T cells
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NMJ	neuromuscular junction
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOX	NAD(P)H oxidase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PER	peak ejection rate
PFR	peak filling rate
PKC	protein kinase C
PLC	phospholipase C
PMO	phosphorodiamidate morpholino oligomer
PPMOs	cell-penetrating peptide conjugated PMOs
QPCR	quantitative PCR
RACC	receptor activated cation channel
ROS	reactive oxygen species
RPM	revolutions per minute
RT-PCR	reverse transcription PCR
RV	right ventricle
SAC	stretch-activated channels
SEM	standard error of the mean
SERCA	sarco/endoplasmic reticulum Ca^{2+} -ATPase

SR	sarcoplasmic reticulum
SV	stroke volume
<i>sync</i> -/-	syncoilin deficient mouse
TA	tibialis anterior
TBST	tris-buffered saline and Tween
TGF β	transforming growth factor β
TNF- α	tumour necrosis factor α
TRPC	transient receptor potential channel canonical type
<i>utrn</i> -/-	utrophin deficient mouse
WT	wild-type

Chapter 1. Introduction

1.1 Duchenne muscular dystrophy, *dystrophin* and the dystrophin associated protein complex

Duchenne muscular dystrophy (DMD) is an X-linked recessive disease caused, in most cases, by the complete absence of the 427 kD cytoskeletal protein, dystrophin. It has an incidence of around 1 in 3,500 boys (though girls can be affected through non-random X-inactivation (Boyd *et al.*, 1986; Giacalone and Francke, 1992; Jacobs *et al.*, 1981; Yoshioka *et al.*, 1998)), and as such is the most prevalent genetic form of muscular dystrophy. DMD can be inherited from a carrier mother or occur due to a spontaneous mutation; the majority of cases (approximately 2/3^{ths} of cases) used to be inherited but due to the increase in genetic screening more cases are now spontaneous as carriers choose not to have children. Its pathology is characterised by rapid muscle degeneration and regeneration of myofibres, with elevated serum creatine kinase (CK) levels, progressive fibrosis and progressive failure of regeneration, and scoliosis (curvature of the spine). Patients are typically wheelchair bound by age 12 and develop heart problems sometime in their early teens, with around 95% of patients exhibiting clinically relevant cardiomyopathy before death (Eagle *et al.*, 2007). Death occurs by age 30 of respiratory or heart failure, with a mean age of death of 19. As yet there is no cure.

Cardiomyopathy in DMD begins with extensive replacement of myocardial fibres by connective tissue, initially at the posterobasal and lateral walls of the left ventricle (LV) (Perloff *et al.*, 1966), and electrocardiogram (ECG) changes (Manning and Cropp, 1958;

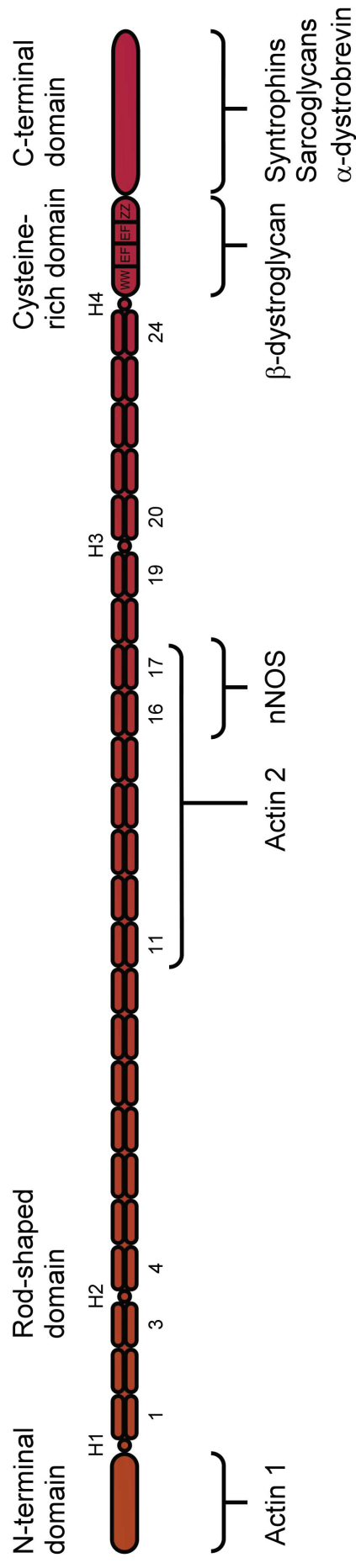
Rubin, 1952). This leads to a compensatory hypertrophy of the heart, with LV wall-motion abnormalities (Goldberg *et al.*, 1982). At death, as much as 50% of the myocardium may have been replaced with fibrosis or fat (Frankel and Rosser, 1976; Nishimura *et al.*, 2001). At the ultrastructural level, the DMD heart has disordered myofilaments and sarcoplasmic reticulum (SR) dilation, and a change in the number, location and structure of mitochondria (Sanyal *et al.*, 1978; Wakai *et al.*, 1988). The DMD heart also has an abnormal response to stress with a lower response to dobutamine seen in both the LV and right ventricle (RV) (Bosser *et al.*, 2004). The extent of involvement of the RV is unclear. Manning *et al* reports that fibrosis was especially bad in the RV (Manning and Cropp, 1958), and pulmonary hypertension in DMD patients has been shown to increase right atrial pressure, suggesting the presence of RV failure (Yotsukura *et al.*, 1988). There are also some reports of an increase in RV end-diastolic volume (EDL) and a decrease in RV peak ejection rate (PER) (Melacini *et al.*, 1996b) and a deficient response to stress in the RV (Bosser *et al.*, 2004). However most studies focus on LV dysfunction, due to the relative ease with which it can be measured.

The *dystrophin* gene is one of the largest genes in the genome, at 2.4 Mb long. It is comprised of 79 exons, taking 16 hours to transcribe and resulting in a mature mRNA 14 kb long, coding for 3,685 amino acids (Koenig *et al.*, 1988; Tennyson *et al.*, 1995). The main type of DMD causing mutation is partial gene deletion (65%), with the majority of mutations occurring in just 2 regions of the gene (Koenig *et al.*, 1987). A further 7% of DMD is caused by duplications (Hu *et al.*, 1990) and the remnant due to point mutations in both the coding sequence and promoter region. Dystrophin is a 427 kD cytoplasmic protein comprised of four domains: a 240 amino acid N-terminal domain, conserved with the actin-binding domain of α -actinin; a large, rod-shaped domain consisting of 24 triple-

helical segments, approximately 2700 amino acids long; a cysteine-rich domain; and a 420 amino acid C-terminal domain (Koenig *et al.*, 1988) (Figure 1.1). Dystrophin is a member of the spectrin superfamily of actin-binding proteins, but unlike other members it exists as a monomer (Chan and Kunkel, 1997; Kahana *et al.*, 1997). It is localised to the sarcolemma (Zubrzycka-Gaarn *et al.*, 1988) and the neuromuscular and myotendinous junctions (NMJ and MTJ) (Miike *et al.*, 1989; Samitt and Bonilla, 1990; Shimizu, 1989), where the N-terminal domain (Levine *et al.*, 1990) and rod domain (Amann *et al.*, 1998; Rybakova *et al.*, 1996) bind to F-actin and the cysteine rich and C-terminal domains bind to the integral membrane glycoproteins of the dystrophin-associated protein complex (DAPC) (Campbell and Kahl, 1989; Suzuki *et al.*, 1992). Despite being present at only small amounts in muscle ($\sim 0.002\%$ total protein (Hoffman *et al.*, 1987)) dystrophin plays a vitally important role connecting the actin cytoskeleton of the muscle fibre to the cell membrane embedded DAPC and through that to the extracellular matrix.

The DAPC is comprised of a number of peripheral and integral membrane proteins and provides a structural link from the actin cytoskeleton (via dystrophin) to the extracellular matrix (via laminin) (Campbell and Kahl, 1989; Ervasti *et al.*, 1990; Ervasti and Campbell, 1991; Ervasti *et al.*, 1991; Ibraghimov-Beskrovnaya *et al.*, 1992; Ohlendieck *et al.*, 1991b; Yoshida and Ozawa, 1990). The DAPC also has a signalling role through binding partners, but is believed its structural role is most important in muscular dystrophy. It can be considered to be comprised of three parts: a) the cytoskeletal protein dystrophin and the syntrophins bound to it; b) α - and β -dystroglycans; c) the sarcoglycans (α , β , γ , δ (and ϵ)) and sarcospan bound to them (Figure 1.2). There are a number of other proteins bound to them that were not included in the original papers, and may or may not be considered as part of the DAPC, such as α -dystrobrevin (Blake *et al.*, 1996; Sadoulet-Puccio *et al.*,

Dystrophin 427 kD 3,685 amino acids



Utrophin 395 kD 3,433 amino acids

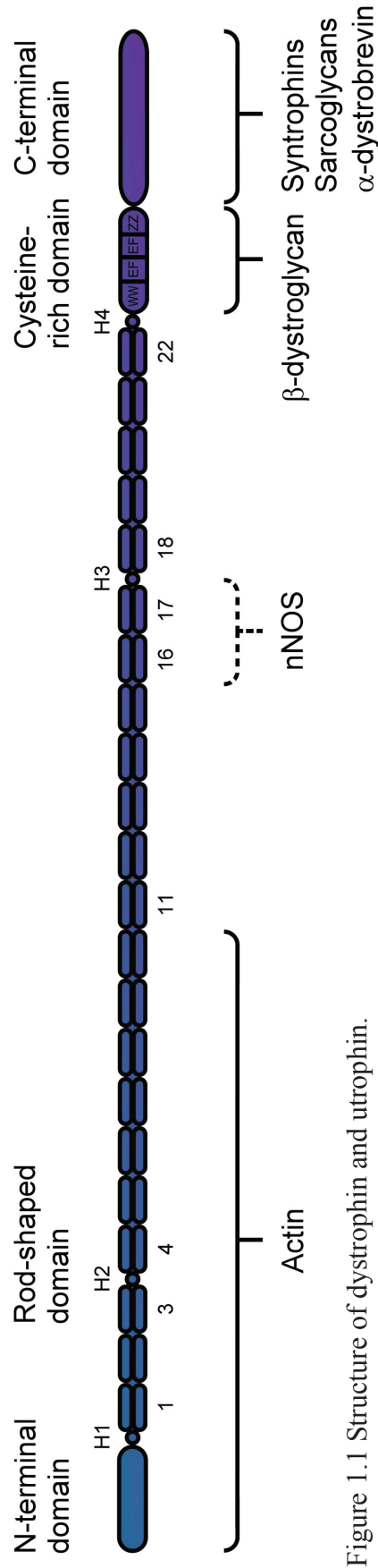


Figure 1.1 Structure of dystrophin and utrophin. Structure of dystrophin and utrophin proteins, showing domain structure and location of protein binding sites. H signifies dystrophin/utrophin hinge region. Possible nNOS binding site is signified by dotted line.

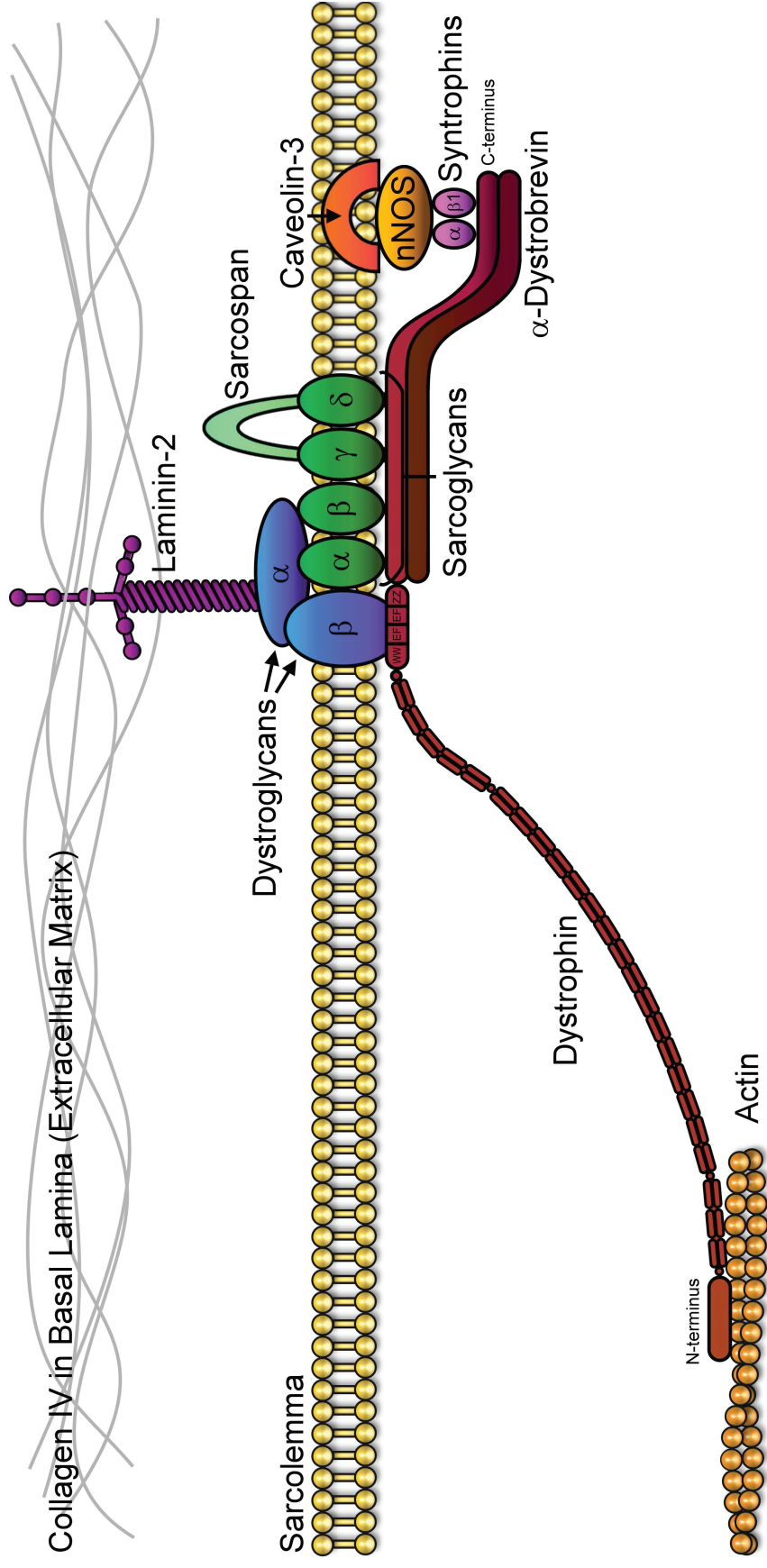


Figure 1.2 Organisation of the DAPC. The DAPC is comprised of dystrophin and a number of peripheral and integral membrane proteins; and provides a structural link from the actin cytoskeleton (via dystrophin) to the extracellular matrix (via laminin). In DMD the loss of dystrophin prevents the DAPC from forming. As a result the sarcolemma is less stable and tears in it form during muscle contractions. This allows the efflux of large amounts of creatine kinase and other muscle enzymes and the influx of large amounts of Ca^{2+} , which leads to the muscle degeneration characteristic of DMD.

1997b), nNOS (Brenman *et al.*, 1995; Brenman *et al.*, 1996) and caveolin 3 (Crosbie *et al.*, 1998; Song *et al.*, 1996).

The syntrophins are a family of five 59 kD proteins (α , β 1, β 2, γ 1, γ 2), that act as adapter proteins to localize a variety of cytoplasmic signalling proteins to specific locations. α -syntrophin is the predominate isoform but the β -syntrophins and γ 2-syntrophin are also found in skeletal muscle. Dystrophin primarily interacts with α - and β 1-syntrophin (Peters *et al.*, 1997). Dystrophin has a single syntrophin binding site in exon 74 (Ahn and Kunkel, 1995; Suzuki *et al.*, 1995; Yang *et al.*, 1995), but stoichiometry shows two syntrophins for every dystrophin (Ervasti and Campbell, 1991; Yoshida and Ozawa, 1990). This is due to the presence of a second syntrophin binding site on α -dystrobrevin (Ahn and Kunkel, 1995; Sadoulet-Puccio *et al.*, 1997a). α -dystrobrevin is a 87 kD cytoplasmic protein that binds to dystrophin through a coiled-coil motif, with the C-terminus of dystrobrevin binding specifically to the C-terminus of dystrophin (Sadoulet-Puccio *et al.*, 1997b). α -dystroglycan is a 156 kD extracellular protein that binds to laminin in the basement membrane, connecting the DAPC to the extracellular matrix (Ibraghimov-Beskrovnaya *et al.*, 1992). β -dystroglycan is a 43 kD transmembrane protein that binds to α -dystroglycan extracellularly and to dystrophin intracellularly, the latter via its cysteine rich domain (Chung and Campanelli, 1999; Ibraghimov-Beskrovnaya *et al.*, 1992). The sarcoglycans are a family of six transmembrane proteins – α - sarcoglycan (also called adhalin), which is 50 kD (Roberds *et al.*, 1993); β - sarcoglycan which is 43 kD; γ - sarcoglycan which is 35 kD; δ - sarcoglycan which is also 35 kD; ϵ - sarcoglycan which is 43 kD and, in contrast to the others, is highly expressed in non-muscle cells as well as muscle cells; and ζ -sarcoglycan (Ettinger *et al.*, 1997; McNally *et al.*, 1998). The sarcoglycans bind to dystrophin at the C-terminal domain (Albrecht and Froehner, 2002) and interact with

sarcospan (especially γ (Crosbie *et al.*, 2000)). Sarcospan is a 25 kD transmembrane protein containing four transmembrane spanning helices with both N- and C-terminal domains located intracellularly (Crosbie *et al.*, 1997). nNOS (neuronal nitric oxide synthase) is a cytoplasmic enzyme that catalyses the formation of nitric oxide from L-arginine. Nitric oxide is an important signalling molecule. It binds to α -syntrophin (Hillier *et al.*, 1999; Kameya *et al.*, 1999; Tochio *et al.*, 1999) and dystrophin (Lai *et al.*, 2009). Caveolin 3 is a ~20 kD transmembrane protein, that interacts with NOS and inhibits its activity (Ju *et al.*, 1997; Venema *et al.*, 1997) and also directly interacts with β -dystroglycan, in a manner that may inhibit its binding to dystrophin (Sotgia *et al.*, 2000). Without dystrophin the components of the DAPC no longer localise to the sarcolemma (Ervasti *et al.*, 1990; Ibraghimov-Beskrovnaya *et al.*, 1992; Ohlendieck and Campbell, 1991; Ohlendieck *et al.*, 1993).

Mutations in other components of the DAPC are found in other types of muscular dystrophy (Davies and Nowak, 2006) and in cardiomyopathies, for example mutations in α , β , γ or δ (but not ϵ) sarcoglycans genes are responsible for limb girdle muscular dystrophy type 2D (Ljunggren *et al.*, 1995; Piccolo *et al.*, 1995; Roberds *et al.*, 1994), 2E (Bonnemann *et al.*, 1995; Lim *et al.*, 1995), 2C (Ben Othmane *et al.*, 1992; Piccolo *et al.*, 1996) and 2F (Nigro *et al.*, 1996; Passos-Bueno *et al.*, 1996), respectively; and mutations in β and δ sarcoglycans genes are found in both familial and sporadic dilated cardiomyopathy (Sylvius *et al.*, 2003; Tsubata *et al.*, 2000).

1.2 Animal models of DMD

1.2.1 *mdx* mouse

The *mdx* mouse has a stop-codon mutation in the *dystrophin* gene resulting in the premature termination of translation in exon 23 (Sicinski *et al.*, 1989). This is a naturally occurring mutation in inbred *C57/Bl10* mice (Bulfield *et al.*, 1984). This would produce a severely truncated dystrophin protein (27% full-length), lacking 2/3rds of the central repeat domain and, more importantly, the functionally necessary cysteine-rich and the C-terminal domains making the protein non-functional. However no protein is detected (Hoffman *et al.*, 1987), and mRNA levels have been shown to be reduced to about 16% of wild-type (Chamberlain *et al.*, 1988). The *mdx* mouse has a slightly reduced life span (Chamberlain *et al.*, 2007), with slightly reduced fertility, elevated serum CK levels and histological lesions in limb muscles from 3 weeks (Bulfield *et al.*, 1984). At this point muscles show a marked atrophy and the beginning of regeneration, as indicated by central nuclei. Most limb muscle consists of regenerated fibres by 9 weeks (Bulfield *et al.*, 1984). There is a reduction in muscle strength at around 3-4 weeks, at the beginning of the degenerative phase, with diaphragm strength at around 65% of normal at 3 months (Cox *et al.*, 1993), but in adult mice regeneration has restored the limb muscles to normal (Anderson *et al.*, 1988a; Coulton *et al.*, 1988; Dangain and Vrbova, 1984; DiMario *et al.*, 1989). However regeneration becomes impaired with age (Morrison *et al.*, 2000; Pastoret and Seville, 1995), with fibrosis having a positive feedback effect on itself (Alexakis *et al.*, 2007). Unlike limb muscles, in which regeneration largely restores structure, the *mdx* mouse diaphragm undergoes progressive degeneration. By 16 months, the diaphragm has undergone extensive fibre loss and fibrosis, with collagen density seven times that seen in

control diaphragm and ten times that seen in *mdx* hind limbs (Stedman *et al.*, 1991). By 24 months, the diaphragm has only 13.5% the strength of control diaphragm (Stedman *et al.*, 1991). It is difficult to say whether the *mdx* mouse is a good model for all aspects of DMD, but the more severe changes seen in the diaphragm, as opposed to other muscles, may explain why DMD patients usually die of respiratory failure. The mouse shows no overt phenotype before 12 months, when the mouse may be uncoordinated and have a mild tremor (Bulfield *et al.*, 1984).

In the *mdx* mouse heart, there is progressive fibrosis from 3 months, with a three-fold increase in collagen density from 6-18 months (Van Erp *et al.*, 2010). While fibrosis may eventually replace as much as 50% of the DMD myocardium (Frankel and Rosser, 1976; Nishimura *et al.*, 2001) even in 17 month old *mdx* mice only 8% is replaced (Quinlan *et al.*, 2004). By 6 months the *mdx* mouse has abnormal ECGs (Bia *et al.*, 1999), and by 11 months has LV dysfunction (Quinlan *et al.*, 2004). RV dysfunction precedes the LV dysfunction, with a significant reduction in RV ejection fraction apparent at 8 months, as well as a reduced LV peak filling rate (Zhang *et al.*, 2008b). Cardiac hypertrophy is also present in the *mdx* mouse by 11 months (Jearawiriyapaisarn *et al.*, 2010; Quinlan *et al.*, 2004). The *mdx* mouse heart has an abnormal response to stress, with a reduced response to *in vivo* high dose dobutamine (a β 1-adrenergic agonist) stress (Quinlan *et al.*, 2004) but a 60-75% mortality rate from this treatment (Bostick *et al.*, 2009; Wu *et al.*, 2008). Pressure overload, induced by aortic banding, caused focal fibrotic lesions in *mdx* mouse heart but no increase in mortality (Kamogawa *et al.*, 2001) and increased exercise also caused fibrosis (Townsend *et al.*, 2008). *Ex vivo*, *mdx* mouse hearts show a decreased ability to tolerate mechanical stress, showing sarcolemmal damage and decreased

contractility (Danialou *et al.*, 2001). This correlates with the deficient response to stress found in DMD hearts (Bosser *et al.*, 2004).

Overall, this is a much less severe phenotype than that of DMD patients, despite the shared genetic cause. This reduction in severity is partially due to upregulation of a homologue of dystrophin, called utrophin (Figure 1.1). In normal tissue utrophin is ubiquitously expressed at low levels but in the absence of dystrophin is found at elevated levels at the sarcolemma (Nguyen *et al.*, 1991), NMJ and MTJ (Khurana *et al.*, 1991). Here, utrophin associates with the DAPC, partially replacing dystrophin (Matsumura *et al.*, 1992). More detail on utrophin can be found below in 1.4.1 (Possible treatments for DMD) and in Chapter 5.

1.2.2 *dKO* mouse

To create a more severe mouse model of DMD the *mdx* mouse was crossed with the utrophin knockout mouse to create the dystrophin/utrophin-deficient double-knockout mouse (*dKO*). Two separate groups have created *dKO* mice, one knocking out only utrophin A (Deconinck *et al.*, 1997a), the other knocking out all isoforms of utrophin (Grady *et al.*, 1997b). For this study the former was used. The utrophin null mouse displays little phenotype apart from a small NMJ defect (Grady *et al.*, 1997a), so it is assumed that the phenotype of the *dKO* mouse is more severe than that of the *mdx* mouse because lacking utrophin, it cannot compensate for the loss of dystrophin. The untreated *dKO* mouse shows progressive muscle weakness, with marked kyphosis (curvature of the spine), weight loss and an average lifespan of 8-10 weeks (Deconinck *et al.*, 1997a). Degeneration and regeneration occurs at similar levels to the *mdx* mouse and the skeletal

and cardiac morphology of the *dKO* mouse is not drastically worse than the *mdx* mouse to 8 weeks (Deconinck *et al.*, 1997a). However there is an increase in cardiac inflammation and fibrosis (Hainsey *et al.*, 2003), as well as a worsening of cardiac contractile function and a reduced response to stress at 10 weeks (Janssen *et al.*, 2005). ECG changes were observed in 14 week old *dKO* mice (Bia *et al.*, 1999). The principle difference between the *mdx* and *dKO* mice is in the time-course of the disease, while necrosis in muscle fibres and connective tissue is first seen in the diaphragm of 6 day old *dKO* mice, with regeneration also occurring by 2 weeks of age (Deconinck *et al.*, 1997a), in *mdx* mice necrosis does not occur until 3 weeks.

1.3 Molecular mechanisms

1.3.1 Molecular mechanisms of degeneration

The DAPC has two separate, but related, mechanical functions: transduction of force from the myofibres to the extracellular matrix, which has already been mentioned, and maintenance of membrane stability. The anchoring of the sarcolemma to the cytoskeleton and extracellular matrix is believed to contribute to its mechanical strength and enable it to resist the stress of repeated deformation during contraction. In the absence of dystrophin, and resulting loss of the DAPC, muscle fibres sustain transient tears in the sarcolemma (Menke and Jockusch, 1991; Mokri and Engel, 1975; Rowland, 1980), which allow the efflux of large amounts of creatine kinase and other muscle enzymes and the influx of large amounts of Ca^{2+} (Bertorini *et al.*, 1982; Bertorini *et al.*, 1984; Bodensteiner and Engel, 1978; Jackson *et al.*, 1991; Morandi *et al.*, 1990). Ca^{2+} may also enter the fibre

through stretch-activated channels (SAC), such as members of the TRPC (Transient receptor potential channel canonical type) family (Allen *et al.*, 2010), which are active during stress-induced damage. This type of damage has been shown to be more severe in dystrophin deficient muscle than normal muscle (Head *et al.*, 1992; Petrof *et al.*, 1993). The balance between these two entry methods is unclear, but this influx of Ca^{2+} starts degeneration in dystrophin deficient fibres. Degeneration is believed to occur via a number of different pathways (Figure 1.3). Increased Ca^{2+} levels activate calpain, a proteolytic enzyme, which degrades a variety of skeletal muscle proteins (Goll *et al.*, 2003; Zaidi and Narahara, 1989) and has been shown to have greater activity in *mdx* muscles during regeneration and degeneration (Spencer *et al.*, 1995). Inhibition of calpain has been shown to substantially reduce muscle degeneration and necrosis in the *mdx* mouse (Badalamente and Stracher, 2000). A second possible pathway is generation of reactive oxygen species (ROS). *Mdx* muscle has been shown to have greater sensitivity to ROS (Rando *et al.*, 1998). There is an induction of expression of genes encoding antioxidant enzymes in *mdx* muscle before the onset of degeneration and there is increased lipid peroxidation in *mdx* muscle, suggesting ROS production is occurring (Disatnik *et al.*, 1998). ROS have also been implicated in the activation of the transcription factor NF- κ B (Kumar and Boriek, 2003). NF- κ B regulates the expression of pro-inflammatory cytokines, such as TNF- α and interleukin (IL)-1 β , which were found to be increased before the onset of degeneration (Kumar and Boriek, 2003). Inhibition of NF- κ B reduced necrosis and enhanced regeneration (Tang *et al.*, 2010) and inhibition of TNF- α reduced necrosis and inflammatory cell infiltration (Grounds and Torrisi, 2004). nNOS can scavenge ROS (Tidball, 2005), but it is downregulated in DMD (Brenman *et al.*, 1995). Upregulation of nNOS in *mdx* muscle produced a reduction in muscle damage and plasma CK levels (Wehling *et al.*, 2001).

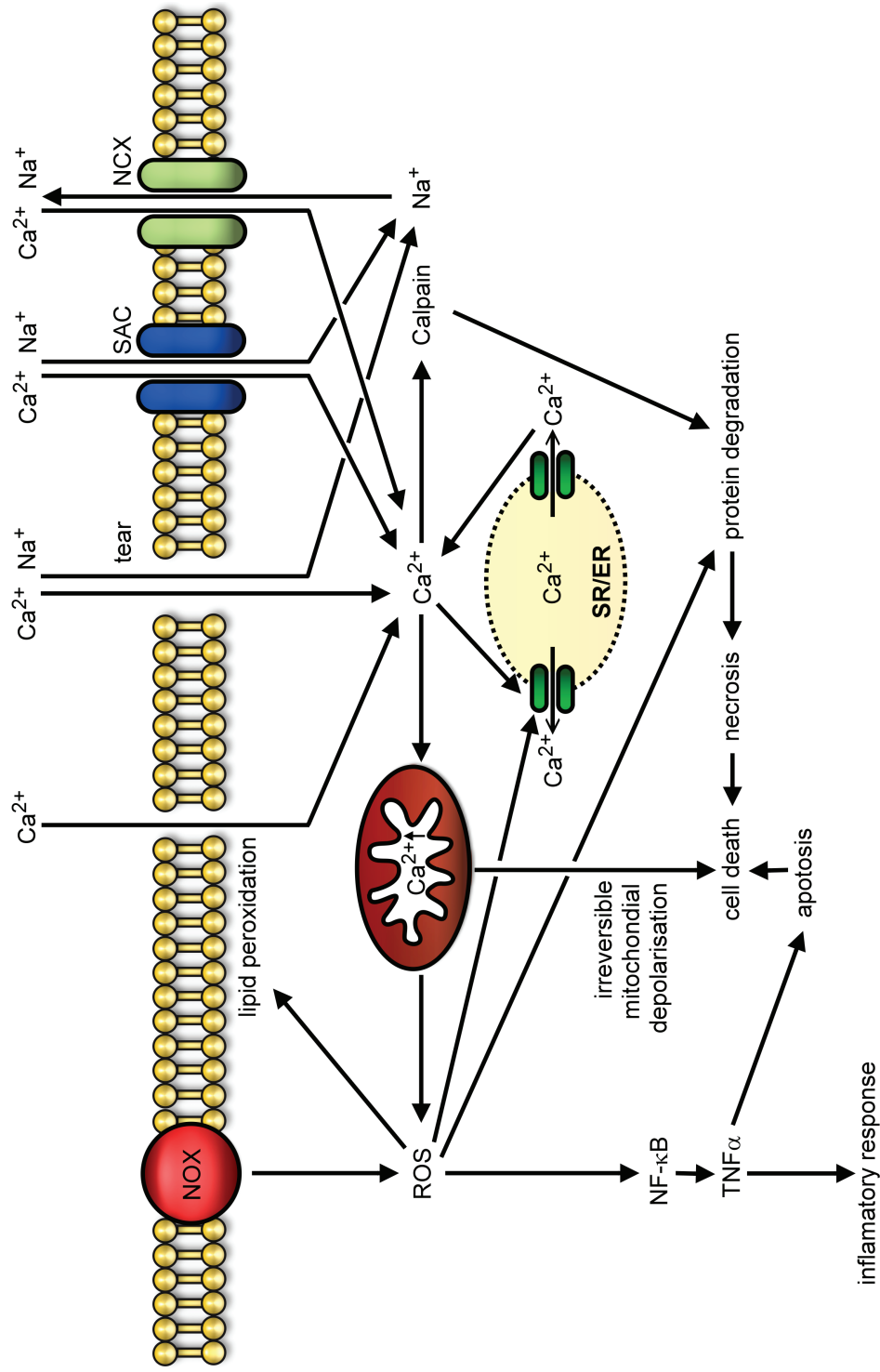


Figure 1.3 Molecular mechanisms of degeneration in cardiac muscle

Ca^{2+} enters the myocyte through membrane tears, SACs and the NCX channel. Ca^{2+} activates calpain, which causes protein degradation, and accumulates in the mitochondria, causing the production of ROS, which are also produced by NOX. The accumulation of Ca^{2+} in the mitochondria causes irreversible mitochondrial depolarisation, leading to cell death. ROS cause membrane damage, protein degradation and activate transcription factor NF- κ B, causing the release of pro-inflammatory cytokines, such as TNF- α . ROS also cause more Ca^{2+} release from the SR, as does the increasing Ca^{2+} level in the myocyte. TNF- α activates the cell inflammatory response and causes apoptosis.

In skeletal muscle dystrophin expression at the sarcolemma is localised to costameres, which align with the Z-disk (Porter *et al.*, 1992), but in cardiac muscle dystrophin expression is more evenly spread along the sarcolemma (Stevenson *et al.*, 1997). This may explain why the *mdx* mouse heart does not show degeneration as early as skeletal muscle. However there is evidence to show that tears in the sarcolemma also occur in cardiomyocytes (Yasuda *et al.*, 2005). Ca^{2+} has been shown to enter cardiomyocytes via both the routes outlined above, membrane rupture and SACs, but also through $\text{Na}^+/\text{Ca}^{2+}$ exchange via the NCX1 channel, with the necessary Na^+ entering via the other two routes (Fanchaouy *et al.*, 2009) (Figure 1.3). These pathways combine to raise the intracellular Ca^{2+} level sufficiently to trigger Ca^{2+} release from the sarcoplasmic reticulum (SR). This SR Ca^{2+} release can also be triggered by the rapid generation of ROS by, among others, NAD(P)H oxidase (NOX) (Jung *et al.*, 2008), which is overexpressed in the dystrophic heart (Williams and Allen, 2007). Excessive cytosolic Ca^{2+} is taken up by mitochondria, themselves a source of ROS (Brookes *et al.*, 2004), where it can lead to irreversible mitochondrial depolarisation (Huser and Blatter, 1999) and ultimately cause apoptotic or necrotic cell death (Brookes *et al.*, 2004; Jung *et al.*, 2008). It is likely that calpain also plays a role in the heart, as calpain inhibitors have been shown to protect the heart from ischaemia–reperfusion injury (Yoshikawa *et al.*, 2005). Cardiac hypertrophy is frequently reported in aged *mdx* mice (11 months old) (Jearawiriyapaisarn *et al.*, 2010; Quinlan *et al.*, 2004). Calcineurin activation by increased intracellular Ca^{2+} levels has been shown to be sufficient to cause hypertrophy (Molkentin *et al.*, 1998), suggesting that hypertrophy, when occurring, may also be induced by this route. The NCX1 channel and calpain likely also play roles in degeneration in skeletal muscle. Differences between degeneration in skeletal muscle and cardiac muscle are likely due to its mechanical role, which is well

established in skeletal muscle but uncertain in cardiac muscle, and due to its role in signalling, particularly via nNOS. nNOS is co-localised with dystrophin at the sarcolemma in skeletal muscle, but in cardiomyocytes is found at the SR, where it impairs both Ca^{2+} release and uptake (Meszaros *et al.*, 1996; Xu *et al.*, 1999).

1.3.2 Molecular mechanisms of regeneration and fibrosis

Occurring simultaneously with the processes of degeneration, and in response to them, are the processes of regeneration. Injury to adult muscle causes the release of cytokines and growth factors from the injured cells and infiltrating inflammatory cells. Cytokines promote the migration, proliferation and survival of regenerating cells, while inflammatory cells phagocytose damaged proteins. Satellite cells (stem cells) from beneath the basal lamina are activated and proliferate and differentiate to form myoblasts along the edge of the injury. New myoblasts fuse with the damaged myofibres to form new myofibres. The basement membrane of the necrotic fibres is used to guide the new myofibres to roughly the same position. The new myofibres can be distinguished from old ones by reduced cross sectional area and central nucleation, as the nucleus of old myofibres is located at the edge. Simultaneously fibroblasts proliferate to produce new extracellular matrix components (ECMs).

In DMD the continual muscle degeneration interferes with this system. Inflammatory cells remain in the muscle and phagocytose the basement membrane of the necrotic fibres before it can be used to position new myofibres, resulting in the abnormal fibre organisation seen in dystrophic muscle (Wakai *et al.*, 1988). Furthermore the continuing degeneration and presence of inflammatory cells results in an excess of cytokines and

growth factors, causing an excess of fibroblasts, this leads to an excess of ECM components (including collagen) and the substitution of muscle by fibrous tissue (fibrosis) (Frankel and Rosser, 1976; Nishimura *et al.*, 2001; Perloff *et al.*, 1966; Stedman *et al.*, 1991; Van Erp *et al.*, 2010). In dystrophic muscle regeneration is impaired with age (Morrison *et al.*, 2000; Pastoret and Sebillé, 1995). This process seems to be due to alteration in the mechanisms of control of production of the ECM components, with an impairment in the production of collagen type I, leading a positive feedback loop of production of fibrous tissue (Alexakis *et al.*, 2007).

1.3.3 Molecular mechanisms of cardiac dysfunction

1.3.3.1 Impaired ECG and wall-motion abnormalities

In DMD patients (Manning and Cropp, 1958; Rubin, 1952) and *mdx* and *dKO* mice (Bia *et al.*, 1999) abnormal ECGs are observed. There are several possible mechanisms for these changes. Firstly, and most simply, the continuing replacement of muscle by fibrous tissue results in a change of conduction properties. This in particular is likely to contribute to the wall-motion abnormalities seen in the dystrophic heart (Goldberg *et al.*, 1982), as the electrical impulse to contract is slowed by passing through connective tissue and reaches different parts of the heart at different times.

Secondly, there is a loss of nNOS protein from the sarcolemma in *mdx* mice and DMD patients (Brenman *et al.*, 1995) and an increase in iNOS (inducible NOS) in response to cytokines such as TNF α (Bia *et al.*, 1999; Nathan, 1997); a change that is also observed during cardiomyopathy (de Belder *et al.*, 1997; Drexler *et al.*, 1998). There are no changes

in eNOS (Endothelial NOS) levels in *mdx* mice (Bia *et al.*, 1999). NO has different effects in different parts of the heart. This is regulated by localisation of the different NOS isoforms to their targets (Barouch *et al.*, 2002; Paton *et al.*, 2002). The activity of nNOS throughout the *mdx* mouse heart is reduced by ~80%, this may be due a wide-spread defect in regulation, rather than just its loss from the DAPC/sarcolemma (Bia *et al.*, 1999). In the heart nNOS is also located in the sarcoplasmic reticulum (SR), where it is believed to regulate Ca^{2+} release from the SR, playing a role in the regulation of ordinary contractions and having a large effect in the response to β -adrenergic stimulation (Ashley *et al.*, 2002; Meszaros *et al.*, 1996; Xu *et al.*, 1999). L-type Ca^{2+} channel are spatial located with junctional SR, so NO produced by nNOS may also effect the L-type channel (Gathercole *et al.*, 2000; Sears *et al.*, 2003). The loss of nNOS may affect the ECG by these mechanisms, particularly as the significant prolongation of the QT interval in the *mdx* ECG suggests that Ca^{2+} channel abnormalities play a role in the observed changes. Further evidence for the role of nNOS in the ECG abnormalities found in the *mdx* mouse heart is found in a transgenic *mdx* with nNOS upregulated in the heart (admittedly to above normal levels) in which fibrosis and the ECG abnormalities were abolished (Wehling-Henricks *et al.*, 2005). *Dystrophin* is also expressed in neurons, so its loss may have an effect on neuronal control of the heart beat. Changes in levels of iNOS are not seen until 12 months of age in *mdx* mice, so these are unlikely to be involved in the changes in ECG seen from 6 months (Bia *et al.*, 1999).

1.3.3.2 Impaired diastolic function

The relaxation rate of the *mdx* mouse myocardium (Sapp *et al.*, 1996) and the DMD ventricles (Goldberg *et al.*, 1980; Kovick *et al.*, 1975) is slowed, causing impaired diastolic function, as seen in the *mdx* mouse heart (Zhang *et al.*, 2008b). Relaxation of myocytes is due to the removal of intracellular Ca^{2+} to the SR, a process believed to be dominated by SERCA (Bers, 1991). This process would also act to reduce the effect of the degenerative processes outlined above and it has been shown that overexpression of SERCA1a in the *mdx* mouse diaphragm reduces susceptibility to contraction induced damage (Morine *et al.*, 2010). However the increased intracellular Ca^{2+} level found in DMD patient myocytes (Turner *et al.*, 1988) and a possible deficit in SR Ca^{2+} uptake, as evidenced by slower SR Ca^{2+} uptake in DMD patient muscles (Samaha and Gergely, 1969; Takagi *et al.*, 1973), overwhelms the mechanisms, contributing to the observed reduced relaxation rate.

1.3.3.3 Impaired stress response

Mdx mice show a reduced inotropic ability during both treatment with dobutamine (Bostick *et al.*, 2009; Quinlan *et al.*, 2004; Wu *et al.*, 2008) and pressure overload, induced by aortic banding (Kamogawa *et al.*, 2001). The *mdx* mouse heart also shows a reduced inotropic ability *ex vivo* in a decreased ability to tolerate mechanical stress (Danialou *et al.*, 2001). This is also seen in the LV and RV of DMD patient hearts (Bosser *et al.*, 2004). There are several possible reasons for this. First the relaxation rate of the *mdx* myocardium (Sapp *et al.*, 1996) and the DMD ventricles (Goldberg *et al.*, 1980; Kovick *et al.*, 1975) is slowed and the passive compliance (tolerance to passive length extension) of *mdx* myocytes is significantly reduced from wild-type (Townsend *et al.*, 2007; Townsend *et al.*,

2008; Yasuda *et al.*, 2005). This increased stiffness causes an inability to respond to stress, as the heart is limited in the amount it can change its output. Furthermore, there is a loss of nNOS protein from the sarcolemma in *mdx* mice and DMD patients (Brenman *et al.*, 1995) and an increase in iNOS expression (Bia *et al.*, 1999). In the heart nNOS stimulates SR Ca^{2+} release and reuptake, facilitating Ca^{2+} -induced Ca^{2+} release (Barouch *et al.*, 2002) and, as such, the nNOS knockout mouse has a reduced inotropic response to dobutamine (Dawson *et al.*, 2005). Upregulation of iNOS is also associated with a reduced inotropic response (Drexler *et al.*, 1998), though changes in levels of iNOS are not seen until 12 months of age in *mdx* mice, so these are unlikely to be important in studies of heart function in young mice (Bia *et al.*, 1999).

As well as the reduced inotropic response, a high mortality rate is seen following dobutamine administration (Bostick *et al.*, 2009; Wu *et al.*, 2008). The mechanism of cardiomyocyte death is likely to be Ca^{2+} overload. When isolated cardiomyocytes are stretched too far they become unstable, suffering large membrane tears, resulting in increased intracellular Ca^{2+} fluctuation, eventual calcium overload and subsequent cardiomyocyte death (Yasuda *et al.*, 2005). This is also seen *in vivo* where, during a 30-minute dobutamine infusion, *mdx* hearts showed progressive decline in peak LV systolic pressure and eventual failure (Townsend *et al.*, 2007). Increased damage is also caused by other stressors, such as isoproterenol and forced running (Carter *et al.*, 1995; Danialou *et al.*, 2001; Townsend *et al.*, 2008). In general *mdx* mice seem to suffer greater levels of sarcolemmal injury and consequent contractile dysfunction in response to mechanical stress than wild-type mice (Danialou *et al.*, 2001). This suggests that dystrophin may have a mechanical role in the transduction of force in the cardiomyocyte (as in skeletal muscle) and be required for sarcolemmal stability. An alternative possibility is that the lack of

dystrophin results in a defective NO/cGMP (cyclic guanosine monophosphate) pathway. Evidence for this comes from transgenic *mdx* mice overexpressing, in a cardiomyocyte-specific manner, guanylyl cyclase (which increases cytoplasmic production of cGMP). These mice had improved sarcolemmal integrity and an increased ability to sustain an increased heart rate during β -adrenergic stimulation *in vivo* (Khairallah *et al.*, 2008). Inhibition of cGMP breakdown in a non-transgenic *mdx* had a similar effect.

1.4 Possible Treatments for DMD

1.4.1 Utrophin upregulation (see Chapter 5)

As discussed above the relatively mild phenotype of the *mdx* mouse is partially due to upregulation of utrophin. Utrophin is highly homologous to dystrophin, with particularly high homology at the actin binding domain (85%) and carboxy terminus (83%) but low in the central rod domain (30%) (Love *et al.*, 1989; Tinsley *et al.*, 1992). It binds to most of the same proteins as dystrophin (Figure 1.1) and can replace dystrophin in connecting the DAPC to the actin cytoskeleton (Matsumura *et al.*, 1992; Pons *et al.*, 1994). In the absence of dystrophin utrophin is found at elevated levels at the sarcolemma (Nguyen *et al.*, 1991), NMJ and MTJ (Khurana *et al.*, 1991). Studies have shown that artificially upregulating utrophin expression via use of a utrophin transgene (Squire *et al.*, 2002; Tinsley *et al.*, 1998; Tinsley *et al.*, 1996), or natural (Krag *et al.*, 2004) or artificial (Mattei *et al.*, 2007) transcription factors for the endogenous utrophin can completely ameliorate the condition of the *mdx* mouse, in a dose-dependent manner.

1.4.2 Exon-skipping (see Chapter 6)

Although the *mdx* mouse is dystrophin null, the occasional revertant, dystrophin-positive, myofibres are found (Hoffman *et al.*, 1990; Lu *et al.*, 2000). The *mdx* mutation creates a stop codon in exon 23 (Sicinski *et al.*, 1989), but in these fibres the cellular splicing machinery has ‘skipped’ this exon, and many others, producing a truncated, but functional, dystrophin protein (Lu *et al.*, 2000). This has led to the notion of skipping mutation-containing exons in DMD patients by interfering with the splicing machinery of the cell. Here, antisense oligonucleotides (AOs) bind to required splicing sites in the pre-mRNA, blocking the binding of the spliceosome, and preventing inclusion of the target exon in the mature mRNA. This exon skipping could frequently correct point mutations, by removing the exon they are in, however the exons of *dystrophin* are not all in-frame relative to each other, and the skipping of an exon may result in all subsequent exons being out of frame. This problem can be avoided by skipping multiple exons so that the reading frame is preserved (Figure 1.4). This works because large parts of *dystrophin* are, mostly, unnecessary for its function, and in fact at least 46% of the gene can be deleted (a large part of the rod domain) and the gene still be largely functional (England *et al.*, 1990). Exon-skipping could also frequently correct frame-shift errors introduced by deletions of parts of the gene, by skipping enough exons to restore the normal reading-frame. It is estimated that, were the appropriate AOs developed, exon-skipping could give up to 92% of DMD patients a partially functional dystrophin protein (Aartsma-Rus *et al.*, 2004). Various types of AO have been shown to be able to restore dystrophin with high-efficiency in both the *mdx* and *dKO* mouse models of DMD and, by doing so, to restore function to various muscles (Alter *et al.*, 2006; Lu *et al.*, 2005; Wu *et al.*, 2008; Yin *et al.*, 2008; Yin *et al.*, 2009; Yin *et al.*, 2010).

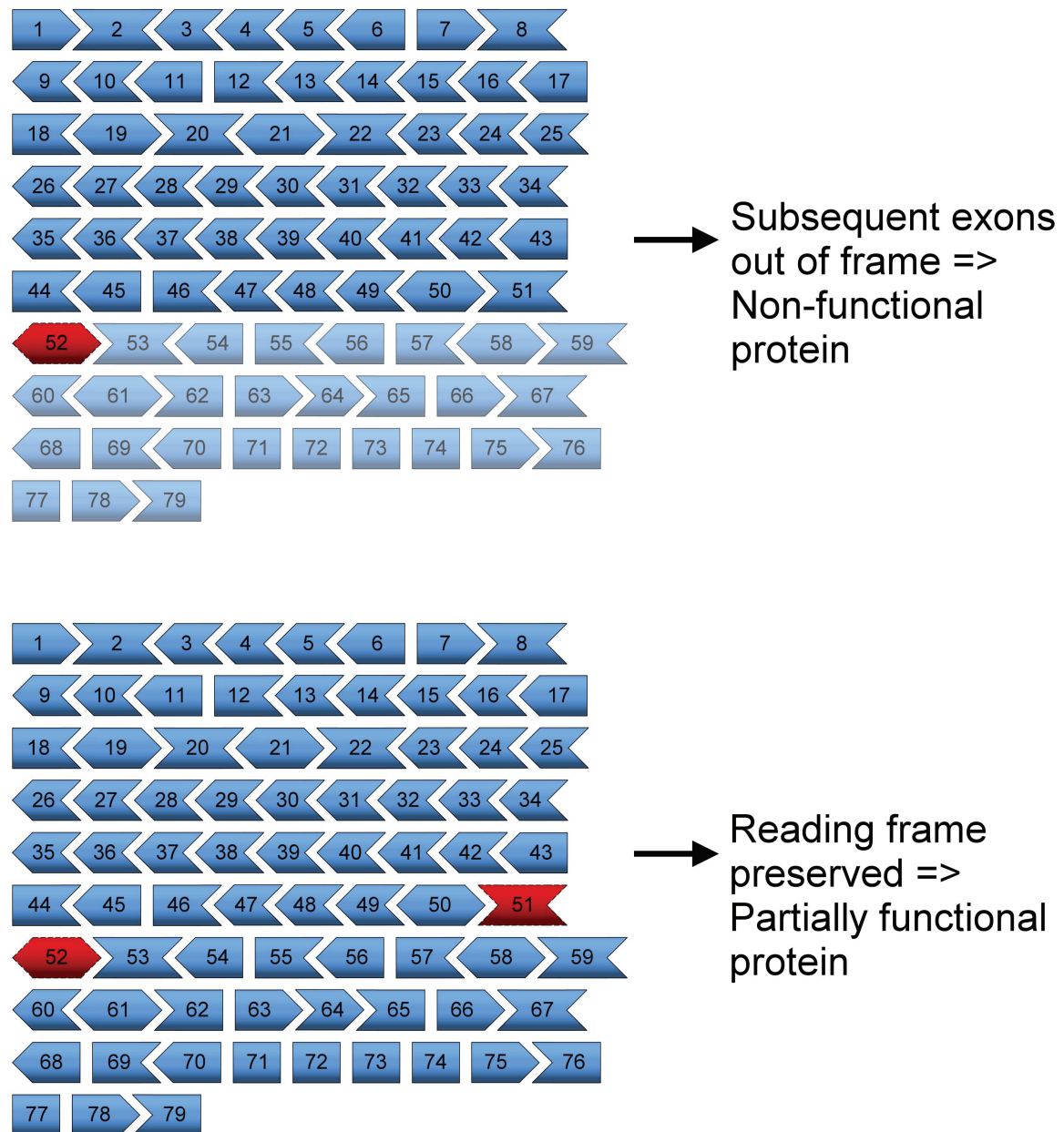


Figure 1.4 Exon structure of *dystrophin* gene.

Dystrophin is comprised of 79 exons. Removing exon 52 (as shown in red) means all subsequent exons will be out of frame, as indicated by the jigsaw like edges on each exon whereby exon 51 and exon 53 do not join up, resulting in a severely truncated, non-functional protein. Removing both exons 51 and 52 preserves the reading frame, as indicated by the jigsaw like edges showing exons 50 and 53 can connect, resulting in a partially functional protein.

1.4.3 Other drug treatments

1.4.3.1 Steroid treatment

Glucocorticoid corticosteroid therapy is the standard of treatment for DMD, it can improve muscle strength and function for six months to two years, delaying the progression of the disease (Brooke *et al.*, 1987; DeSilva *et al.*, 1987; Manzur *et al.*, 2008). It can have significant side effects such as weight gain, cushingoid appearance, and excessive hair growth (Griggs *et al.*, 1991).

1.4.3.2 Gene therapy

Gene therapy to treat DMD has gone down two routes: the expression of a *de novo* full-length or truncated *dystrophin/utrophin* gene, and correction of the fault in the existing *dystrophin* gene. The expression of a *de novo* full-length or truncated *utrophin* gene is covered above and in Chapter 5 so no more will be said here. Due to *dystrophin*'s extreme length it will not fit in most viral vectors. As such research initially focussed on the use of a truncated *dystrophin* gene missing exons 17-48, coding for repeats 4-19 of the central rod domain (between the H2 and H3 regions), which was shown to prevent the *mdx* mouse pathology (Dunckley *et al.*, 1993; Phelps *et al.*, 1995; Wells *et al.*, 1992; Wells *et al.*, 1995). Full-length *dystrophin* transgenes have also been used, which were cloned into plasmids to circumvent the viral vector size limitations, with similar results (Cox *et al.*, 1993; Phelps *et al.*, 1995; Wells *et al.*, 1995). A problem with this approach is that the use of any method involving viral delivery will likely need the patient to be immunosuppressed.

The correction of the existing deficit can be carried out in several ways, either by use of AOs, as mentioned in section 1.4.2, or by use of meganucleases, which induce double-stranded breaks in DNA, allowing the insertion of a few base pairs and the restoration of normal reading-frame or the deletion of a harmful point mutation (Chapdelaine *et al.*, 2010). The use of meganucleases might avoid the need for immunosuppression. Another problem for gene therapy is that there are approximately 640 muscles in the human body and, while treatment of some may be unnecessary (extraocular muscles appear spared in DMD (Khurana *et al.*, 1995)), direct treatment of each muscle is obviously impractical. This is a problem for many treatments discussed in this thesis and ultimately systemic delivery will be necessary, be it by subcutaneous injection, intravenous injection or orally.

1.4.3.3 Read through of stop codons

Around 15% of DMD is caused by the creation of a *de novo* stop codon in the *dystrophin* gene, as is the case in the *mdx* mouse (Barton-Davis *et al.*, 1999). Aminoglycoside antibiotics can suppress stop codons and as such restore dystrophin (Barton-Davis *et al.*, 1999). In the *mdx* mouse gentamicin can restore ~15% of normal levels of dystrophin and significantly reduce CK levels (Malik *et al.*, 2010). This has also been shown with a novel drug, ataluren (PTC124) (Welch *et al.*, 2007), however it has so far proved ineffectual in humans (PTC Therapeutics, 2010).

1.4.3.4 Anti-inflammatories

By blocking inflammatory cytokines, like TNF α , the aim is to prevent the inflammatory response and the induction of necrosis and apoptosis. A number of anti-inflammatory

drugs have been shown to reduce necrosis and CK levels in *mdx* mice (Hodgetts *et al.*, 2006; Radley *et al.*, 2008).

1.4.3.5 Protease inhibitors

Protease inhibitors prevent the degradation of muscle by proteases, such as calpain.

Inhibition of calpain has been shown to substantially reduce muscle degeneration and necrosis in the *mdx* mouse (Badalamente and Stracher, 2000).

1.4.3.6 Membrane sealants

As detailed above, one of the principle causes of muscle degeneration in dystrophin-lacking muscle is the emergence of tears in the sarcolemma. Treatment of isolated *mdx* cardiac myocytes with a membrane sealant improved function and prevented dobutamine-induced cell death (Yasuda *et al.*, 2005). However relatively little is known about the effects and safety of membrane sealants *in vivo*, so the practicality of their use remains uncertain.

1.4.3.7 Antioxidants

By reducing ROS, antioxidants can inhibit the inflammatory response, protein degradation and reduce membrane damage (Figure 1.3), thereby emulating the effects of a number of other classes of drug listed here. Antioxidants have been shown to improve heart function, reduce fibrosis and prevent dobutamine-induced cell death in *mdx* mice (Buyse *et al.*, 2009) and to improve the survival of dystrophic muscle cells in culture (Dorchies *et al.*, 2009).

1.4.3.8 Stem cells

Injection of normal muscle precursor cells into irradiated *mdx* muscle has shown the potential for the creation of *de novo* dystrophin-positive fibres (Morgan *et al.*, 1993). Similarly injection of normal mesoangioblasts in the dystrophic dog has been shown to increase dystrophin expression and improve muscle function (Sampaolesi *et al.*, 2006). In subsequent work the patients' own stem cells have been obtained and transduced using AOs in viral vectors. Cells so treated and injected into immunosuppressed *mdx* mice have been shown to improve muscle function (Benchouir *et al.*, 2007). The principle objection to this type of treatment is the necessity of immunosuppression and/or destruction of the patients' stem cells, however the use of stem cells obtained from the patient would avoid the need for immunosuppression.

1.4.3.9 Inhibition of ACE (angiotensin I converting enzyme) pathway

ACE produces angiotensin II, and this pathway inhibits TGF β signalling, which affects muscle regeneration by inhibiting myoblast differentiation and promoting fibrosis. *Mdx* mice diaphragm, the most severely affected muscle in *mdx* mice (Stedman *et al.*, 1991), has elevated levels of TGF β (Gosselin *et al.*, 2004). ACE inhibitors have been shown to delay the onset and progression of LV dysfunction in DMD (Duboc *et al.*, 2005), while angiotensin II type 1 receptor (AT1) inhibition can decrease fibrosis and increase regenerative capacity in *mdx* mice leading to an increase in muscle mass (Chamberlain, 2007).

1.4.3.10 β 2-adrenergic agonists

β 2-adrenergic agonists can improve muscle strength in *mdx* mice and healthy humans (Martineau *et al.*, 1992; Rothwell and Stock, 1985), but have no positive effect on disease progression and they increase muscle fatigability (Dupont-Versteegden *et al.*, 1995).

1.5 Aims of this project

The exact mechanism leading to cardiomyopathy in the *mdx* mouse remains unclear. The first aim of this project was to determine whether mouse models having mutations in proteins associated with the pathology of DMD display a heart dysfunction similar to that seen in the *mdx* mouse and so reveal more of the cellular mechanisms underlying the dysfunction.

In Chapter 3, the *Syncoilin knockout* mouse was studied because, like dystrophin, syncoilin forms a link between the DAPC and the cytoskeleton (Poon *et al.*, 2002). Furthermore, knockouts of both of syncoilin's binding partners, desmin and α -dystrobrevin, display cardiac dysfunction (Grady *et al.*, 1999; Milner *et al.*, 1996; Milner *et al.*, 1999; Thornell *et al.*, 1997), suggesting that knockout of syncoilin may also cause cardiac dysfunction.

In Chapter 4, the *Moonwalker* mouse was studied. In the *Moonwalker* mouse, the transient receptor potential channel canonical type 3 (TRPC3) cation channel is mutated. As it is a candidate to be a SAC (stretch-activated channel), it may allow the influx of Ca^{2+} into cardiomyocytes, as seen during degeneration in DMD and in *mdx* mice (see Section 1.3.1). The Moonwalker mutation is believed to make the channel more active (Becker *et al.*,

2009), so the *Moonwalker* mouse might display a cardiomyopathy similar to that of the *mdx* mouse.

In Chapter 5, the *Fiona* mouse, a transgenic *mdx* mouse with utrophin upregulated (see Section 1.4.1) throughout the skeletal muscle, including the diaphragm, but not the heart, was studied. The *mdx* mouse has a severely affected diaphragm at 6 months (Stedman *et al.*, 1991) and LV dysfunction by 11 months (Quinlan *et al.*, 2004). RV dysfunction precedes the LV dysfunction, with a significant reduction in RV ejection fraction at 8 months (Zhang *et al.*, 2008b). It was hypothesized that this RV dysfunction might be due to the severely affected diaphragm, and not the heart itself, and therefore that it would be possible to improve cardiac function by improving diaphragm function, without directly restoring or compensating for dystrophin loss in the heart. The proposed mechanism is that severe respiratory muscle weakness (especially that of the diaphragm) causes hypoventilation, in turn causing hypoxemia. This leads to pulmonary arterial hypertension, which causes increased afterload on the right ventricle, leading to a reduced RV stroke volume (due to Starling's law of the heart). Over time this change is pathological and leads in turn to RV dysfunction. Thus, the *Fiona* mouse is the perfect model in which to test this hypothesis and see if restoration of diaphragm function alone can restore heart function. Pulmonary hypertension has been shown to occur in DMD patients, at least some of the time without concurrent LV dysfunction, supporting the hypothesis that it is pulmonary arterial hypertension, not pulmonary venous hypertension (Melacini *et al.*, 1996b; Yotsukura *et al.*, 1988).

The second aim of this project was to study the effects of treatment on heart function in existing mouse models of DMD. In Chapter 6, the effects on heart function of *de novo*

exon-skipping drugs (see section 1.4.2) was studied. The purpose of this was two-fold. Firstly, most exon-skipping drugs tested to this point have functioned poorly in the heart, but there is a new class of drugs specifically designed to target the heart. It is important to know if they can improve heart function. Secondly this study allowed further testing of the hypothesis from Chapter 5, that restoration of diaphragm function alone can restore heart function. As dystrophin was not restored to the heart in the controls used for these drugs, it is possible to see whether another method for improving diaphragm function can improve heart function, and to directly compare methods that treat the diaphragm and the heart to methods that only treat the diaphragm.

Finally Chapter 7 presents a summary of the key findings from the work in this thesis and attempts to put them in perspective and explain their importance to the field.

Chapter 2: Materials and methods

The methods in this thesis were largely identical from chapter to chapter, so the general methods will be described in this chapter and then any variations from the general method that were used in different chapters will be detailed in the specific chapters. Methods specific to chapters will be detailed in those chapters; in particular those methods used by collaborators in Chapter 6 are detailed there. All of the experiments were carried out in the Animal units of the Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK according to procedures authorized by the UK Home Office.

2.1 Animals

Mice were kept in the animal units of the Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK. These consist of both ‘clean’ and ‘dirty’ units. The majority of mice were kept in the ‘dirty’ units, but the mice used in Chapter 5 (whose results were reused in Chapter 6) were kept in the ‘clean’ unit. This occurred because a new, ‘clean’ animal facility was constructed during my DPhil and we were supposed to transfer to it. This was problematic because mice that had been in a ‘dirty’ unit could not be transferred to a ‘clean’ unit. As a result the stocks of mice were run down over this period and obtaining sufficiently large numbers of mice for experiments was difficult. This is why, in Chapter 4, there is a shortage of young *Moonwalker* mice and, in Chapter 6, contributed to the difficulty in obtaining *dKO* mice. Most mice were bred in the units but the *C57/Bl10* mice used in Chapter 5 (whose results were reused in Chapter 6)

were obtained from Harlan at an age of 8 weeks. A further difficulty was the very poor conditions in the 'dirty' units. At one point an infestation of mites affected the *Moonwalker* and *C3H* mice. While other strains of mice seemed relatively unaffected by the infestation the *C3H* derived strains seemed unable to cope with the itching and literally bit their own skins off, so had to be euthanised. Treatment with Ivermectin, an anti-parasitic that activates glutamate-gated chloride channels, enhancing inhibitory neurotransmission, killed the infestation. Data from these infected mice were highly variable and are not shown in this thesis. Details of the strain, derivation and number of mice used in each chapter are given in the chapters in which the mice were used.

2.2 Western Blots

Mice were killed by cervical dislocation and their hearts removed and placed in PBS. They were gently squeezed a few times to pump out any blood they contain then dried and flash-frozen in liquid nitrogen. They were stored at -80°C until needed. The subsequent method is the most variable of all the methods used as it depends upon the antibodies being used.

In general whole hearts were homogenised in a solution of 1 mL of cold Newcastle Buffer and 10 ml of Protease inhibitors (a concentration of 1/100). The homogenate was then centrifuged at 14,000 RPM/ 4°C for 10 min and the supernatant kept to remove any remaining solid tissue. A protein DC assay was performed to determine the concentration of the resultant mixture. PAGE was then performed with 8% running gels and 5% stacking gels. Gels were run at 120V for approximately 1½ hours and then transferred to nitrocellulose membranes overnight (~16 hours) at 35V. The membranes were

subsequently blocked for 1 hour in 5% milk, then blotted in primary antibody in 5% milk for 3 hours. The membranes were washed for 10 minutes, 3 times in TBST (1% Tween). The membranes were then washed in HRP-conjugated secondary antibodies, of the species relevant to the primary, at 1:2000 in 5% Milk (except for those requiring anti-goat, which was used at 1:160000) for 1 hour. The membranes were then washed as before. Subsequently membranes were dried and treated with 3 mL per gel of ECL blotting reagents. These were left in the dark for 5 mins and the membranes are blotted dry. The membranes were then exposed to films for 10 s, 30 s, 3 min and 10 min.

Details of antibodies are contained in table 2.1.

Table 2.1: Antibodies.

Protein for:	Name and supplier:	Concentration used:	Secondary used:
Syncoilin	2991, produced by Eurogen tech	1:500	anti-rabbit
Desmin	sc-7559, Santa Cruz	1:100	anti-goat
α -Dystrobrevin	α 1-CT-FP, as in Blake <i>et al.</i> , 1998	1:1000	anti-rabbit
α -Actinin	N-19, Santa Cruz	1:200	anti-goat
TRPC3	ACC-016, Alomone	1:200	anti-rabbit
Utrophin	Mancho 3, as in Nguyen <i>et al.</i> , 1991	1:50	anti-mouse

Details of the antibodies used for western blotting in this thesis.

2.3 Cine-MRI

The three principle methods of measuring heart function *in vivo* in mice are cardiac catheterisation, transthoracic echocardiography and MRI. Catheterisation can measure both pressure and volume, while echocardiography and MRI are best at measuring volumes, though estimates of pressure can be obtained by measuring blood velocity. Cardiac catheterisation is an invasive procedure requiring that the mice it is used on be killed after the procedure. This means large numbers of mice are necessary and longitudinal studies cannot be done. As this work planned to look at changes over time this would clearly have been an undesirable method. Generally speaking MRI has a higher temporal resolution than echocardiography, leading to greater reproducibility (Stuckey *et al.*, 2008), however high-frequency echocardiography may be as good as MRI (Amundsen *et al.*, 2011). MRI has the added advantage of allowing image capture in a completely arbitrary plane, while echocardiography is limited by the shape of the mouse. This results in it being difficult to image the RV using echocardiography, while it is no more difficult to image than the LV using MRI. The RV can be imaged using transesophageal echocardiography, but this is relatively invasive so not suitable for anything but the shortest longitudinal study. Given one of the main objectives for this thesis was to investigate RV function, MRI was determined to be the best method.

Anaesthesia affects heart function. The anaesthetic used here, isoflurane, acts as a vasodilator, reducing blood pressure. This causes a reduction in end diastolic volume and so cardiac output and ejection fraction. However this effect is minimal and isoflurane is more molecularly stable than other inhalational anaesthetics, like halothane. This reduces its metabolism and possible toxicity. Various studies have found the inhalational

anaesthetics have less effect on heart rate and function than injected anaesthetics (Roth *et al.*, 2002; Takuma *et al.*, 2001). Of the three alternatives listed only echocardiography can be performed in awake mice but, as discussed above, not while measuring RV function and not for the high-frequency echocardiography that is comparable in accuracy to MRI. Different mice strains require different degrees of anaesthesia. This was noted in this study where the mice with a *C3H* background used in Chapter 4 required a lower level of anaesthesia than those on *C57* backgrounds used in the other chapters. This would affect any comparisons made between the mice.

Under physiologic conditions mice are usually horizontal, but the mice in this MRI scanner were positioned vertically. Obviously this has an effect on their blood pressure due to the new distribution of blood. Wiesmann *et al* has shown, using cardiac catheterisation, that when anaesthetised mice are placed vertically there is a transient decrease of LV systolic pressure to 96% of starting levels which is restored to physiological levels within six minutes, subsequently being stable for at least an hour (Wiesmann *et al.*, 2001).

Experiments on the same magnet used for this thesis found that cardiac function parameters agreed well with those previously obtained in a horizontal bore magnet and were stable for at least an hour, with a reasonable degree of stability to three hours (Schneider *et al.*, 2003; Schneider *et al.*, 2004). The change from vertical to horizontal is fully compensated for by a normal orthostatic response in anaesthetised mice allowing the measurement of heart function under physiological conditions.

There is no published SoP for *mdx* assessment using MRI yet available from TREAT-NMD so the standard method for the Clarke lab was used instead.

Mice were anaesthetised using 4% isoflurane at a rate of 4 l/min. When a mouse showed no hind limb response it was transferred to the MRI cradle and the isoflurane was lowered to 2-3% at a rate of 2 l/min. The cradle was designed for positioning mice vertically in the magnet and was comprised of an in-built nose cone, lines for the delivery and scavenging of anaesthetic gases, temperature control, and electrocardiogram (ECG) and respiratory monitoring (Figure 2.1). Temperature was maintained using a blanket that was heated by air that had flowed through a home-built heat exchanger placed within the animal holder and above the coil in the magnet. An ECG and respiratory gating device was used for monitoring heart and respiration signals, derived from two needles subcutaneously inserted in the front paws. Respiratory signals could also be derived from a loop loosely fitted on the chest and abdomen of the animals. Mice were secured within the holder using surgical tape, without compressing their abdomen or chest regions.

The cradle was then lowered into an 11.7 T (500 MHz) vertical-bore magnet with a 1H-imaging probe. Long-axis and short-axis scout images were acquired so that true short-axis images could be planned using a segmented, cardiac-triggered FLASH sequence. The coil was then tuned and matched, followed by slice-selective shimming.

Seven to 12 contiguous slices (slice thickness = 1 mm) were then acquired in short-axis orientation covering the entire heart (Figure 2.2).

The imaging parameters were FOV = 25.6 mm², matrix size = 256 × 256, TE/TR = 1.43/4.6 ms, 15° Gaussian excitation pulse, NAE = 2. The sequence was ECG triggered and respiratory gated. Between 19 and 28 frames per heart cycle and two to eight PE steps per respiration cycle were acquired in a segmented fashion, resulting in a total experimental time of approximately one hour in the vertical position (including experimental preparation).

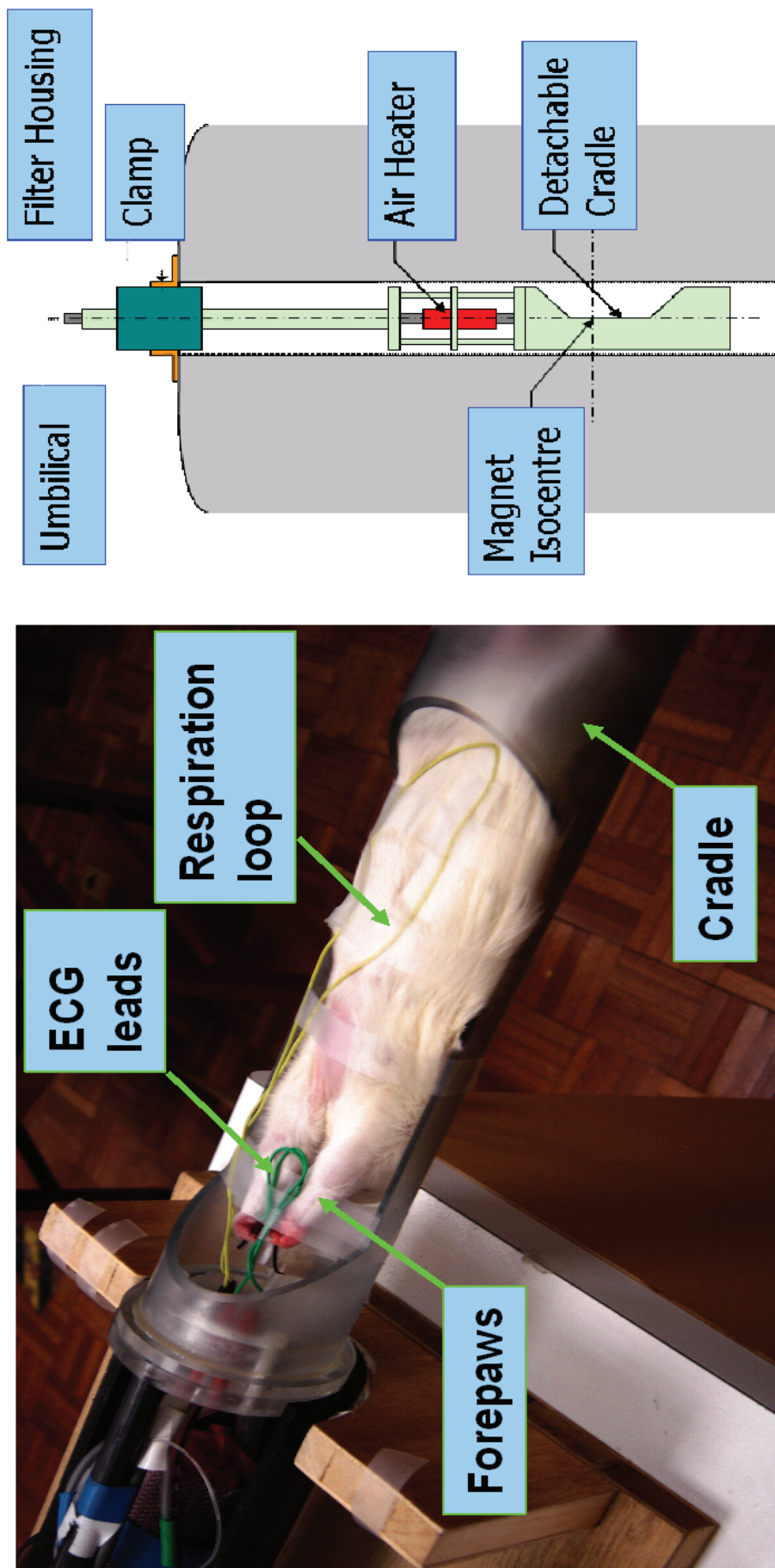


Figure 2.1 Positioning of animal in MRI.

The anaesthetised animal (in this picture a rat) is placed in the cradle. ECG leads are inserted subcutaneously in each paw and the animal is taped down. A further wire (the respiratory loop) is taped to the chest. In the magnetic field of the MRI the movement of the chest (and wire) induces a current in the wire allowing monitoring of the respiratory rate. Air is passed down the umbilical to the cradle, passing over an air heater, to maintain the body temperature of the animal. The cradle is then inserted down the centre of the magnet, leaving the animal placed head-up vertically in the magnet.

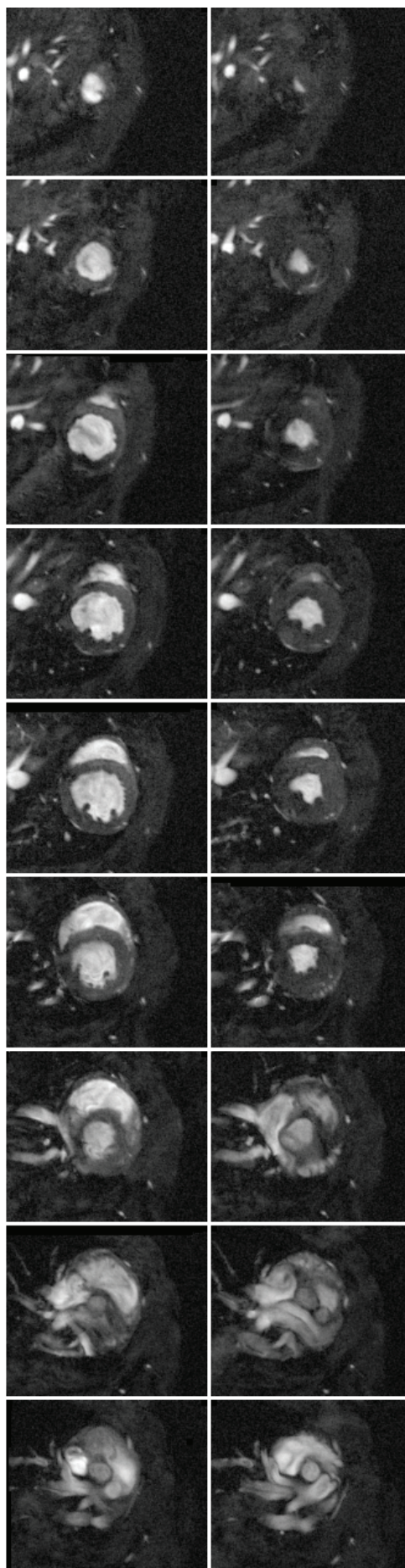


Figure 2.2 Cine-MRI measurement of cardiac function. End-diastolic (top row) and end-systolic (bottom row) short axis images in 1mm slices of the heart of a 6 month old Fiona mouse, imaged using cine-MRI. Images are displayed from the apex of the heart (left) to the base (right).

The epicardial and endocardial borders were outlined in end diastolic and end systolic frames using the free hand drawing function of ImageJ. The number of voxels in each compartment multiplied by the pixel size of 0.015mm^3 yielded the respective volumes. Finally, global end diastolic volume (EDV), end systolic volume (ESV), stroke volume ($SV = EDV - ESV$), ejection fraction ($EF = SV/EDV$) and cardiac output ($CO = SV \times \text{heart rate}$) were calculated. Peak ejection and filling rates were calculated from the difference in cavity volume over time from a single cine-MR image acquired at the mid-papillary level at reduced spatial but increased temporal resolution (matrix size = 128×128 , TE/TR = minimum/2.4 ms).

To test the intra-rater reliability of the ImageJ measurements, data from the same mouse was processed five times. This gave an average variation across all measurements of 3.7%, with the LV data having an average variation of 3.3% and the RV of 4.5%. This difference may reflect the fact that RV is typically vertically shorter than the LV so covers 1-3 less slices of the heart. Multiplying this variation by the average values obtained from the genotype of the tested mouse gives values on the order of the standard error of the mean (SEM) values for the genotype. That suggests the variability due to the processing is of the order of the inter-mouse variance, so it should not effect the measurements of significance. To test the inter-rater reliability of the ImageJ measurements data from five different mice was processed by three different people. This gave an average variation across all measurements of 5.9%, with the LV data having an average variation of 5.4% and the RV of 6.8%. This is on the order of 1.5 times the variability given by the SEM for the genotype these mice were from.

2.4 Dobutamine Stress Test

The two main drugs used to induce cardiac stress in mice are dobutamine and isoproterenol. Both are β -adrenergic agonists, however isoproterenol is non selective while dobutamine is predominantly a β_1 -agonist, with only weak β_2 activity. Both have been used widely to study heart function in *mdx* mice, with similar results. However given that the β_1 activity is the desired activity and that the majority of the studies looking at heart function in mice treated by restoring dystrophin used dobutamine, dobutamine stress was chosen for this study. However, given the high death rate reported in *mdx* mice treated with dobutamine (up to 75% (Bostick *et al.*, 2009)) it was subsequently decided that the animal license would prevent the use of dobutamine on *mdx* mice and it would only be used on *Syncoilin knockout* and *Moonwalker* mice and their controls.

Following a standard cine-MRI (as described above in section 2.3) mice were intraperitoneally injected with 5 μ g dobutamine/g body mass in 1M NaCl. Heart rates were allowed to stabilise for approximately 10 minutes then a mid-papillary slice, an apical slice and a basal slice were acquired, using modified parameters to keep the frame rate as close as possible to the pre-dobutamine scans. This was done by reducing TE/TR to minimum / 4.1 ms. A diastolic scan was then acquired as above. As the dobutamine only has a short-term effect there is only sufficient time to capture the three systolic slices and one diastolic slice. As such for comparison with the pre-dobutamine systolic parameters only the corresponding three slices from the seven to 12 systolic pre-dobutamine scans are used. This results in lower figures for volumes being given in the dobutamine stress test sections throughout this thesis.

2.5 Histology

Histology in this thesis was intended to look at three things: general morphology of the heart, levels of infiltrating immune cells and fibrosis. On this basis two stains were chosen - Haematoxylin and Eosin (H&E) and Sirius red.

2.5.1 Haematoxylin and Eosin (H&E)

Haematoxylin stains the nuclei blue, while the eosin stains the cytoplasm and some other proteins pink to red. This allows one to see the general morphology of the heart as well as infiltrating immune cells, which appear as lighter patches with many blue dots (nuclei) in them. The method used was as follows:

Mice were killed by cervical dislocation, their hearts removed and embedded in OCT on liquid nitrogen-cooled isopentane and stored at -80°C until needed. 8µm sections were cut from these using a cryostat and placed on slides and stored at -80°C until needed. Slides were placed in haematoxylin for 8-15 minutes (depending on how fresh it was). Then rinsed in distilled water. Then placed in 0.3% acid alcohol (700ml 100% ethanol, 300ml distilled water, 3ml hydrochloric acid) for 10 seconds. Then washed in running tap water till it turns blue (15-20 seconds). Then stained with eosin for 2 minutes. Then rinsed in distilled water. Then dehydrated by immersing 30x each in three increasing concentrations of alcohol (60%, 75%, 100%). Subsequently they were placed in Histoclear for 2 minutes and finally a drop of Histomount and then a cover-slip place on top.

2.5.2 Sirius red

Sirius red stains the cytoplasm yellow and collagen red, the brighter the red the more collagen. This allows the visualisation of fibrosis. The method used was as follows:

Obtaining of hearts and sectioning proceeded as for the H&E stain. The slides were then placed in picro-Sirius red (0.5g Sirius red F3B, 500ml saturated aqueous solution of picric acid) for 25 minutes. They were then washed in 0.3% acid water (1000ml distilled water, 3ml hydrochloric acid). Then dehydrated by immersing 30x each in three increasing concentrations of alcohol (60%, 75%, 100%). Subsequently they were placed in HistoClear for 2 minutes and finally a drop of Histomount and then a cover-slip place on top.

2.6 Statistical analysis

All cine-MRI data are reported as mean values $\pm$ SEM. Statistical differences between test groups and control groups were evaluated by two-tailed student's t test.

Chapter 3: Heart function in the *Syncoilin* knockout mouse

3.1 Introduction

This study set out to measure heart function in the *syncoilin* knockout mouse to test the hypothesis that it would display a cardiac dysfunction similar to that found in mice lacking other components of the DAPC, e.g. in *α -dystrobrevin* knockout mice (Grady *et al.*, 1999) and *desmin* knockout mice (Milner *et al.*, 1996; Milner *et al.*, 1999; Thornell *et al.*, 1997), especially as, like dystrophin, it forms a link from the DAPC to the cytoskeleton (Poon *et al.*, 2002). It was hoped that understanding any dysfunction resulting from the knockout of syncoilin would add in understanding of the mechanisms of heart failure in muscular dystrophy.

Syncoilin is a 64 kDa intermediate filament (IF)-like protein. However it does not form filaments by homo- or heteropolymerising with other IF proteins like the majority of IFs (Poon *et al.*, 2002). It consists of a central rod domain, comprised of four coiled-coil α -helical regions, a N-terminal head domain and a C-terminal tail domain (Newey *et al.*, 2001). It was initially identified as a binding partner of α -dystrobrevin, part of the DAPC (Newey *et al.*, 2001). Subsequently it was determined to also bind to the desmin IF network (Poon *et al.*, 2002), providing a potentially important link from the DAPC to the cytoskeletal IF network (Figure 3.1). As the loss of the other well known link between the DAPC and the cytoskeleton (dystrophin) has such a huge effect this suggested that syncoilin might be important for the structural integrity of muscle.

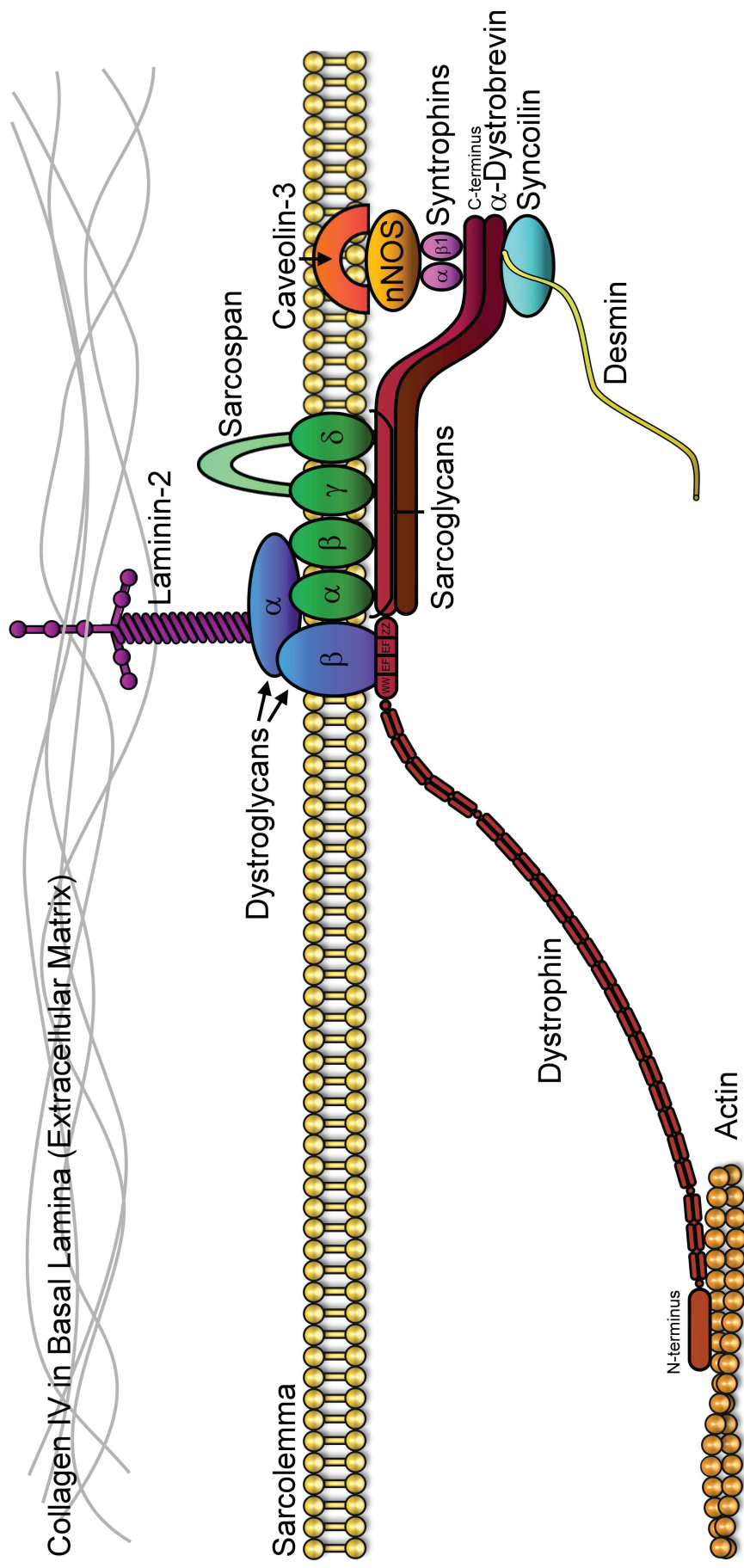


Figure 3.1 Syncoilin and the DAPC
 Syncoilin interacts with the DAPC (via α -dystrobrevin) and the desmin IF network, forming a connection between the cytoskeletal IF network and the extracellular matrix.

Syncoilin is highly expressed in skeletal and cardiac muscle (Newey *et al.*, 2001). Its expression pattern is concentrated at the sarcolemma, Z-lines, perinuclear space, MTJ, and NMJ. Its localisation is lost in *desmin knockout* (*des* ^{-/-}) but not *α -dystrobrevin knockout* (*dtna* ^{-/-}) mice (McCullagh *et al.*, 2007), however in the latter case syncoilin expression is upregulated. Syncoilin is also upregulated in a number of different neuromuscular disorders: DMD, congenital MD and desmin-related myopathy (Brown *et al.*, 2005; Howman *et al.*, 2003) and in the *mdx* and *dKO* animal models of DMD (Newey *et al.*, 2001). Changes are not seen in the *utrophin knockout* (*utrn* ^{-/-}) mouse, suggesting that the syncoilin upregulation is either a compensation for or contributory to muscle damage. Immunohistochemistry shows higher syncoilin expression in newly regenerated fibres suggesting it may play a role in regeneration (McCullagh *et al.*, 2007; Newey *et al.*, 2001). This suggests the *syncoilin knockout* mouse might show greater susceptibility to damage.

The desmin IF network forms a scaffold around the Z-disk of the sarcomere, connecting Z-disks together, and connecting them to the sarcolemma via the DAPC (Bar *et al.*, 2004; Paulin and Li, 2004). Desmin is particularly important in the heart with the myocardium containing more than skeletal muscles, 2% versus 0.35% (Price, 1984). In the *desmin knockout* (*des* ^{-/-}) mouse macroscopic lesions are observed in the heart from 2 weeks old, initially in the intraventricular septum and RV wall and degenerating myofibres seen from 5 days (Thornell *et al.*, 1997). The myofibres and the mitochondria are disorganised (Milner *et al.*, 1996), as seen in DMD patients (Sanyal *et al.*, 1978; Wakai *et al.*, 1988). Cardiac hypertrophy is observed from 1 month old, with dilation of both LV and RV chambers and LV systolic dysfunction from 13 months (Milner *et al.*, 1999). Enhanced

degeneration appears to occur in a similar manner to that in the *mdx* mouse (Agbulut *et al.*, 2001). Desmin mutations can cause myopathies (Goebel, 1995; Goldfarb *et al.*, 2004), which frequently include cardiac symptoms, including cardiac conduction blocks, arrhythmias, progressive fibrosis and restrictive heart failure (Goldfarb *et al.*, 1998). Mutations have also been found in familial idiopathic dilated cardiomyopathy (DCM) (Li *et al.*, 1999) and non-familial DCM (Taylor *et al.*, 2007), with a frequency of between 1% and 6% (Taylor *et al.*, 2007; Tesson *et al.*, 2000).

α -Dystrobrevin is a cytoplasmic protein that binds to dystrophin and the syntrophins. A large percentage of *α -dystrobrevin knockout* (*dtna* *-/-*) mice show myopathic changes in the heart from 1 month, with degenerating myocytes, immune cell infiltration and fibrosis across both ventricles. However the hearts do not appear significantly dilated or hypertrophic (Grady *et al.*, 1999). Evidence suggests these changes are largely due to α -dystrobrevin's interactions with the DAPC. Mutations in α -dystrobrevin have been found in non-compaction cardiomyopathy (Ichida *et al.*, 2001), though this disease is genetically heterogeneous, with mutations in many different genes capable of causing it (Moric-Janiszewska and Markiewicz-Loskot, 2008). Non-compaction cardiomyopathy is associated with a number of muscular dystrophies, including Becker muscular dystrophy and Emery-Dreifuss muscular dystrophy, as well as other diseases causing muscle weakness such as Barth syndrome (Moric-Janiszewska and Markiewicz-Loskot, 2008).

The *Syncoilin knockout* mouse was derived in-house (McCullagh *et al.*, 2008). A targeting construct was generated containing genomic regions around exon 2 of the *syncoilin* gene and a *loxP* site. The construct was then electroporated into embryonic stem cells. Southern blotting was used to screen the cells and identify those that had correctly taken up the

construct. Two affected cells were injected into *C57/Bl6* blastocysts to produce chimeric mice. These were then bred with transgenic mice ubiquitously expressing Cre recombinase, under the control of the CMV promoter, causing Cre-lox recombination and the removal of *syncoilin* exon 2 from half the chromosomes. As exon 2 encodes roughly 85% of the amino acid sequence this effectively removed the gene. The resulting heterozygous *Syncoilin* +/- mice were then crossbred to produce homozygous *Syncoilin* -/- mice. Studies of this mice found maximum isometric force generation was significantly reduced in skeletal muscle and there was a significant increase in serum CK levels following forced running (McCullagh *et al.*, 2008). However there were no signs of increased regeneration or in forced running ability.

As both *des* -/- mice (Milner *et al.*, 1996; Milner *et al.*, 1999; Thornell *et al.*, 1997) and *dtna* -/- mice (Grady *et al.*, 1999) display cardiac dysfunction and both desmin and α -dystrobrevin are mutated in various cardiomyopathies, it was hypothesised that syncoilin null mice would also display cardiac dysfunction, as syncoilin acts as a link between both these proteins. To test this hypothesis 1-year old *sync* -/-, *des* -/- and wild-type mice were killed, their hearts removed and histology performed (for details of method see Chapter 2). Both Haematoxylin and Eosin (H&E) and Sirius red stains were used. *Des* -/- mouse hearts were studied as a positive control as they have been previously reported to show lesions from 2 weeks (Thornell *et al.*, 1997). 6-month old *sync* -/- and wild-type mice were killed and their hearts removed for western blots. Anti-syncoilin, anti-desmin, anti- α -dystrobrevin and anti- α -actinin antibodies were used to see if loss of syncoilin expression had an effect on expression of desmin and α -dystrobrevin. Finally a longitudinal time course of cine MRIs was performed with the same *sync* -/- and wild-type mice being imaged at 6,12,18 and 24 months of age. *Des* -/- mice were also imaged at 12 months, as a

positive control. At the 24-month time point the *sync* $-/-$ and wild-type mice were treated with dobutamine to look at stressed heart function.

3.2 Methods

3.2.1 Animals

Six-month old male *Syncoilin* knockout (*sync* $-/-$) and age-matched male *C57/Bl6* control mice were used for the longitudinal *Syncoilin* knockout mouse heart function experiments ($n = 6$ in each group), with 12-month old male *Desmin* knockout (*des* $-/-$) mice used as a positive control at this age ($n = 4$). Further 6-month old *sync* $-/-$ and wild-type mice were killed and their hearts removed for western blots ($n = 4$) and 1-year old *sync* $-/-$, *des* $-/-$ and wild-type mice killed and their hearts removed for histology ($n = 4$). The *Syncoilin* knockout mouse was derived in-house, as described in McCullagh *et al* (McCullagh *et al.*, 2008). Briefly, a targeting construct was generated from genomic regions around exon 2 of the *Syncoilin* gene and electroporated into embryonic stem cells. Two affected cells were injected into *C57/Bl6* blastocysts, which allowed the generation of heterozygous *Syncoilin* $-/+$ mice, which were finally crossed to produce homozygous *Syncoilin* $-/-$ mice. The *Desmin* knockout mice were originally supplied by Denise Paulin (Université Paris, Paris, France), though have been bred in-house for several years. They were generated in much the same manner, with a targeting construct targeting exons 1-6 of the *Desmin* gene, as described in Thornell *et al* (Thornell *et al.*, 1997).

3.2.2 Western Blot

The general method was followed (as described in section 2.2) except for when using the anti-utrophin antibody, where the method was slightly altered. Total muscle extracts were prepared by homogenization in a different extraction buffer (125 mM Tris, pH 8, 4% SDS, 40% glycerol, 0.05% PMSF, 0.1M DTT), then heating at 95 °C for 5 min. Samples were loaded onto 6% polyacrylamide gels and run overnight at 50V, transferred to nitrocellulose overnight at 50V. Utrophin expression was detected using a 1:50 dilution of MANCHO3 (Nguyen *et al.*, 1991) and made visible with anti-mouse IgG linked to horseradish peroxidase and the ECL Western Blotting Analysis system (Amersham Pharmacia Biosciences, UK). As a loading control α -actinin was used and detected using monoclonal antibody with a 1:200 dilution (Sigma, UK).

3.3 Results

3.3.1 Histology

Sections from one-year-old *sync* *-/-*, *des* *-/-* and wild-type mice hearts were stained with Haematoxylin and Eosin (H&E), to look at general morphology and infiltrating inflammatory cells, and Sirius red, to show cardiac fibrosis (Figure 3.2). *Des* *-/-* mouse hearts show infiltrates and focal fibrotic regions mostly in the RV, as previously reported (Thornell *et al.*, 1997), as well as some in the LV. Both *sync* *-/-* and wild-type mice hearts show no infiltrates or regions.

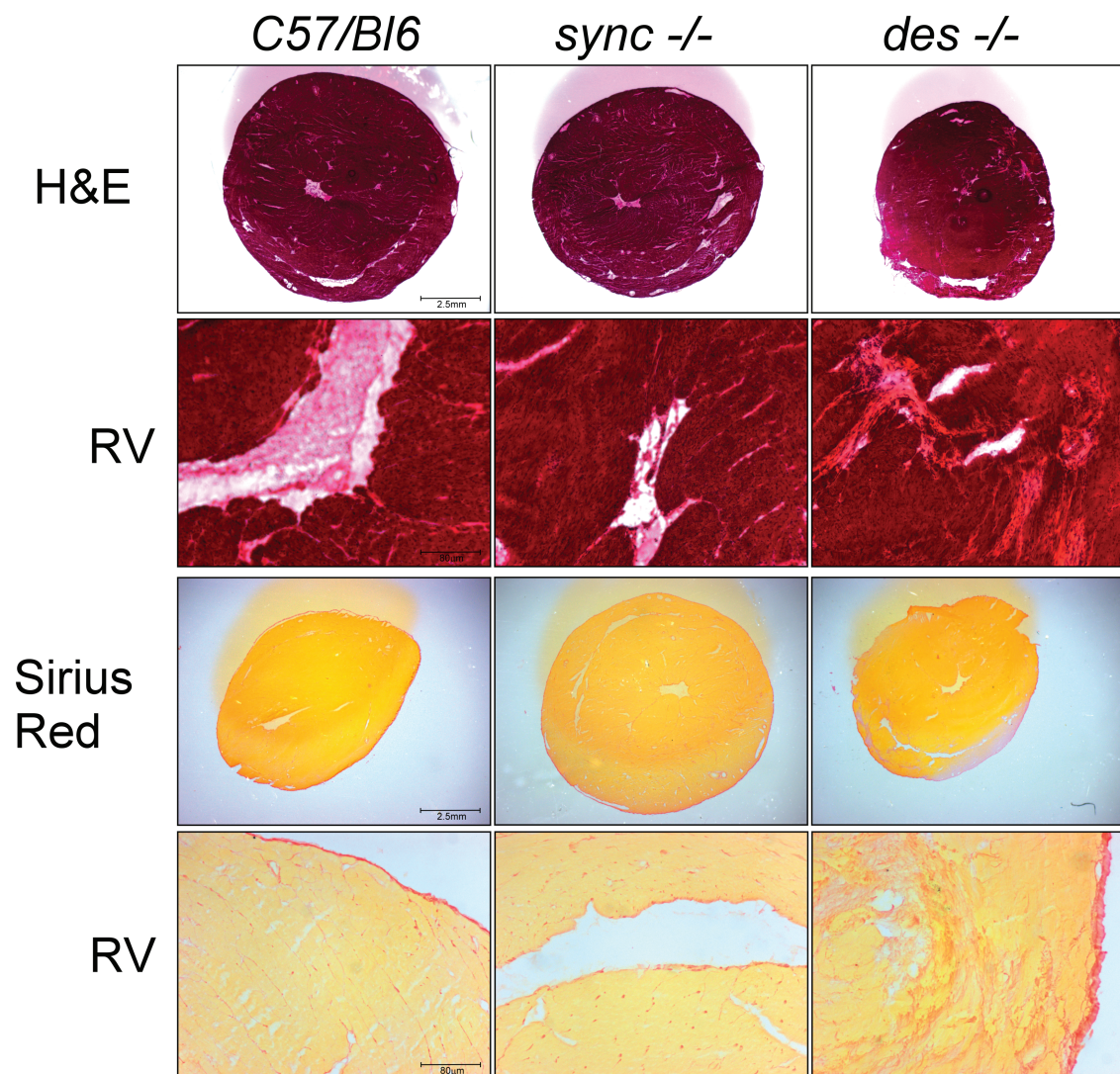


Figure 3.2 Histology

Sections from one-year-old wild-type, *sync -/-* and *desmin -/-* mice hearts stained with H&E and Sirius red stains.

3.3.2 Western blots

Western blots performed on whole heart homogenates (as described in Chapter 2) from six-month old mice showed no difference in desmin and α -dystrobrevin expression between *sync* $-/-$ and wild-type mice (Figure 3.3). This demonstrated that syncoilin expression is not essential for desmin and α -dystrobrevin expression in the heart, and that their absence could not be the reason for any cardiac phenotype found in the *sync* $-/-$ mouse.

3.3.3 Time course of MRIs

Cine-MRIs were performed on the same *sync* $-/-$ and wild-type mice at 6,12,18 and 24 months of age and a number of LV and RV parameters measured (as described in Chapter 2) (Tables 3.1-3.4, located at the end of the chapter). At 6 and 18 months old *sync* $-/-$ mice had a significantly smaller EDL than age-matched *C57/Bl6* mice. There were no other significant differences in measured parameters at any time points. As a positive control *des* $-/-$ mice were imaged at 12 months (Table 3.2). LV dysfunction has been previously been reported from 13 months (Milner *et al.*, 1999) but here RV dysfunction was observed preceding the LV dysfunction, with a significantly reduced RV ejection fraction compared to both wild-type and *sync* $-/-$ mice. A number of other parameters, such as RV stroke volume, were close to being significantly reduced. The experiment may have too little power to detect the significance of these differences.

Body mass was measured, at first weekly and then fortnightly, prior to the MRIs, and then as part of the MRI procedure. This showed that, up to two years, the body mass of the wild-type and *sync* $-/-$ mice follows a nearly identical course (Figure 3.4). These data

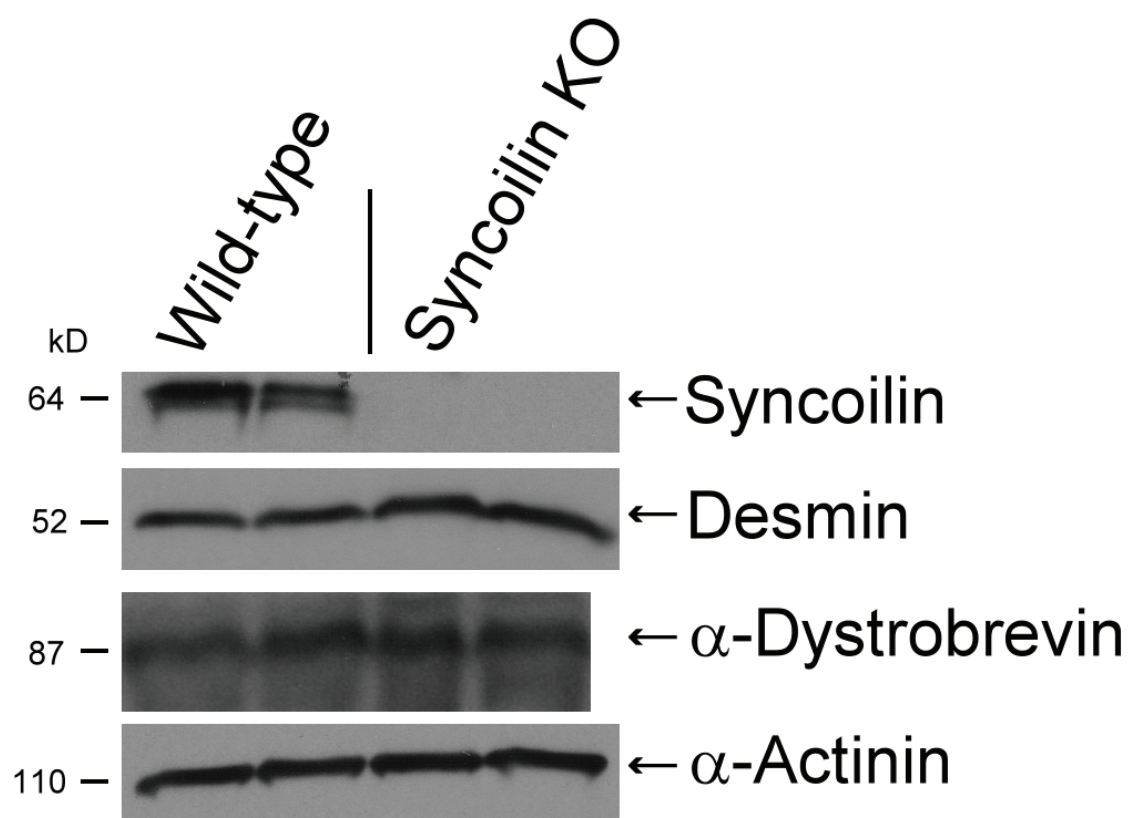


Figure 3.3 Protein levels in *Syncoilin* KO heart
Western blot for syncoilin, desmin and α -dystrobrevin expression in *sync* $-/-$ and wild-type mice. α -actinin was used as the loading control.

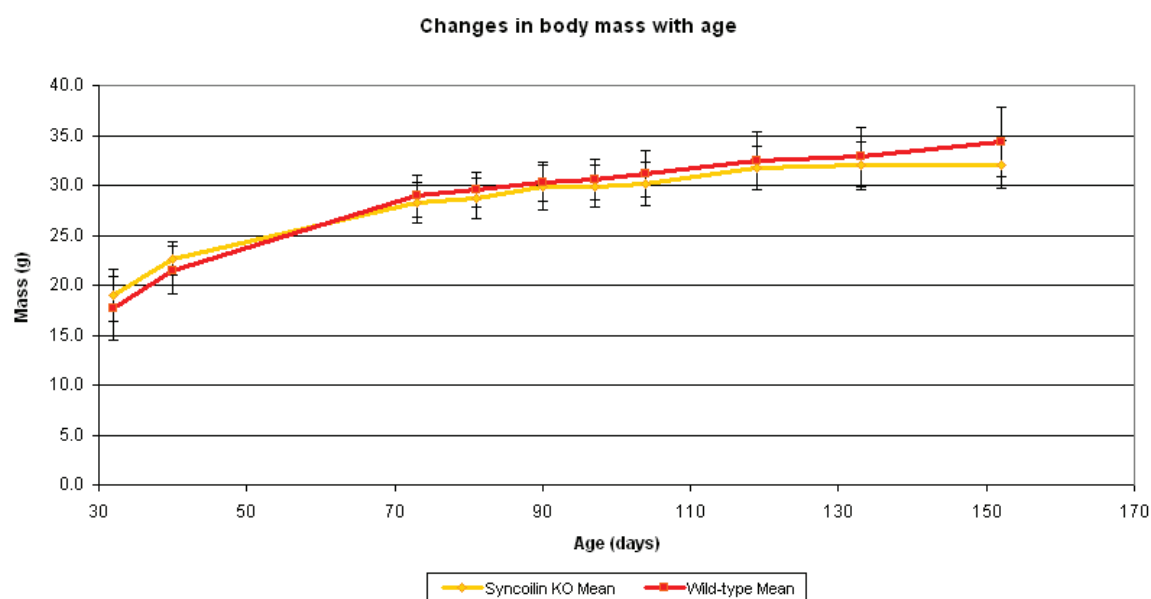


Figure 3.4 Changes in body mass with age in *sync* $-/-$ and wild-type mice.

showed there is little functional difference between the *sync* $-/-$ and wild-type heart and that, like the *mdx* mouse heart, the *des* $-/-$ mouse heart displayed loss of RV prior to loss of LV function.

This fits with the histological data, where there was no difference between the *sync* $-/-$ and wild-type mice, but the *des* $-/-$ mice displayed infiltrates and focal fibrotic lesions mostly in the RV.

3.3.4 Dobutamine stress

To test the response of the *sync* $-/-$ heart to stress at the 24-month time point used above, a low dose of dobutamine was administered to the mice, following the standard MRI. Only the LV was studied (Table 3.5, located at the end of the chapter). Note that results for volumes here appear inconsistent with those from the time-course, this is because only three slices through the heart are used for these calculations, rather than the seven to 12 used in the time-course. Dobutamine stress caused a significant reduction in ESL in the wild-type and *sync* $-/-$ heart and a significant reduction in EDL in the *sync* $-/-$ heart. There were no other significant changes in heart function as a result of the dobutamine stress, probably due to a combination of the low n number and the low-dose of dobutamine used. There were also no significant differences in response to dobutamine between the two genotypes.

3.4 Discussion

This work, and other work on the phenotype of the *sync* ^{-/-} mouse has found almost no significant differences in heart or skeletal muscle function (McCullagh *et al.*, 2008; Zhang *et al.*, 2008a) and no differences in morphology or fibrosis, or in response to dobutamine stress between the *sync* ^{-/-} and wild-type mouse. This is likely due to the several other proteins that also link the DAPC to desmin, namely synemin (Bhosle *et al.*, 2006), plectin (Hijikata *et al.*, 2003; Reznicek *et al.*, 2007), dysbindin (Benson *et al.*, 2001) and myospryn (Kouloumenta *et al.*, 2007). At both 6 and 18 months old the *sync* ^{-/-} mice had a significantly smaller LV EDL than age-matched *C57/Bl6* mice. The changes in EDL correlate well with changes in stroke volume, as would be expected, but also with changes in ESL – which is why there are no significant differences in stroke volume. These differences in EDL may indicate an underlying pathology, for instance an increase in venous compliance due to the loss of syncoilin in the vasculature. However *sync* ^{-/-} was not studied so this remains conjecture. Furthermore the changes in EDL were only seen in two of the time points and changes in EDL in response to dobutamine were not significantly different between the *sync* ^{-/-} and wild-type mouse so they are inconsistent.

The skeletal muscle phenotype that the *sync* ^{-/-} mouse does display is a reduction in maximum force capacity in extensor digitorum longus muscle (McCullagh *et al.*, 2008; Zhang *et al.*, 2008a) and an increase in serum CK levels after forced running (McCullagh *et al.*, 2008). However, it is believed that these differences may be at least partially due to a neuronal deficit, rather than due to changes in the muscle. In neurons syncoilin co-localises and interacts with peripherin (a neuron-specific IF protein) (Clarke *et al.*, 2010). The *sync* ^{-/-} mouse has normal sensory neuron function but a significant deficit in motor

neuron function compared to wild-type mice (Clarke *et al.*, 2010). This provides a possible link to motor neuron diseases such as ALS.

The *des* ^{-/-} mouse MRI shows that, as in the *mdx* mouse, heart dysfunction begins with RV dysfunction, closely followed by LV dysfunction. The pattern of fibrosis and inflammatory cell infiltration is also mirrored, with initial lesions largely found in the RV. Unfortunately the *des* ^{-/-} mice died before the next MRI at 18 months old, so the progression of the heart dysfunction could not be studied. In humans desmin-related myopathy has been shown to cause RV failure (Otten *et al.*, 2010) suggesting the *des* ^{-/-} mouse is a good model of the human cardiomyopathy.

In conclusion the *sync* ^{-/-} mouse does not display a strong muscular dystrophy phenotype, with no differences in heart function from wild-type mice. As yet no mutations in syncoilin have been discovered in muscular dystrophy, however syncoilin may have a role in motor neuron function and mutations may be discovered in motor neuron disease such as ALS.

Table 3.1: Heart function in 6 month old *sync* ^{-/-} mice

6 month old		WT	<i>sync</i> ^{-/-}
Number of mice		6	6
Body Mass	g	35.8 ± 1.5	34.4 ± 0.7
Heart Rate	bpm	441 ± 33	490 ± 37
LV			
Ejection Fraction	%	63.2 ± 4.0	69.7 ± 2.1
Cardiac Output	ml/min	21.7 ± 1.6	23.6 ± 1.7
Heart Mass as % BM	%	0.40 ± 0.02	0.41 ± 0.01
Muscle Volume	μl	138.2 ± 5.8	134.0 ± 3.9
End Diastolic Lumen	μl	78.7 ± 4.5	65.0 ± 4.7 *
End Systolic Lumen	μl	29.3 ± 4.4	19.8 ± 2.6
Stroke Volume	μl	49.3 ± 2.6	45.2 ± 2.7
RV			
Ejection Fraction	%	65.7 ± 4.7	59.8 ± 5.6
Cardiac Output	ml/min	16.6 ± 1.5	20.3 ± 3.2
End Diastolic Lumen	μl	58.4 ± 5.8	66.4 ± 11.2
End Systolic Lumen	μl	20.3 ± 3.7	27.5 ± 6.9
Stroke Volume	μl	38.0 ± 3.7	38.8 ± 5.8

Data obtained by *in vivo* cardiac cine-MRI of *C57/Bl6* and *sync* ^{-/-} mice at 6 months of age. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

* indicates measurements significantly different to wild-type (P>0.05)

** indicates measurements highly significantly different to wild-type (P>0.005)

Table 3.2: Heart function in 12 month old *sync* ^{-/-} and *des* ^{-/-} mice

12 month old		WT	<i>sync</i> ^{-/-}	Desmin KO
Number of mice		6	6	4
Body Mass	g	45.3 ± 3.5	48.0 ± 3.6	25.0 ± 1.8 *†
Heart Rate	bpm	383 ± 26	430 ± 45	439 ± 21
LV				
Ejection Fraction	%	55.6 ± 4.2	65.2 ± 3.5	56.3 ± 2.8
Cardiac Output	ml/min	19.5 ± 0.8	24.6 ± 3.6	16.2 ± 1.1
Heart Mass as % BM	%	0.33 ± 0.02	0.34 ± 0.04	0.44 ± 0.04
Muscle Volume	μl	145.0 ± 1.6	154.5 ± 9.7	104.3 ± 9.3 *†
End Diastolic Lumen	μl	92.2 ± 1.9	88.7 ± 15.0	62.7 ± 8.7 *
End Systolic Lumen	μl	41.0 ± 4.8	31.2 ± 7.1	27.6 ± 5.4
Stroke Volume	μl	51.1 ± 2.8	57.5 ± 8.5	35.1 ± 3.7 *
RV				
Ejection Fraction	%	58.9 ± 0.6	60.6 ± 2.0	49.9 ± 0.5 *††
Cardiac Output	ml/min	24.6 ± 2.3	24.5 ± 1.6	17.5 ± 0.4 *
End Diastolic Lumen	μl	98.4 ± 12.1	112.3 ± 6.8	75.7 ± 2.8 *
End Systolic Lumen	μl	40.3 ± 4.4	48.0 ± 6.6	37.9 ± 1.8
Stroke Volume	μl	58.1 ± 7.8	64.7 ± 8.6	37.7 ± 1.0

Data obtained by *in vivo* cardiac cine-MRI of *C57/Bl6*, *sync* ^{-/-} and *des* ^{-/-} mice at 12 months of age. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

- * indicates measurements significantly different to wild-type (P>0.05)
- ** indicates measurements highly significantly different to wild-type (P>0.005)
- † indicates measurements significantly different to Syncoilin KO (P>0.05)
- †† indicates measurements significantly different to Syncoilin KO (P>0.005)

Table 3.3: Heart function in 18 month old *sync* ^{-/-} mice

18 month old		WT	<i>sync</i> ^{-/-}
Number of mice		6	6
Body Mass	g	39.0 ± 2.1	43.6 ± 3.2
Heart Rate	bpm	451 ± 40	393 ± 24
LV			
Ejection Fraction	%	65.6 ± 5.9	58.2 ± 2.8
Cardiac Output	ml/min	19.5 ± 1.9	19.7 ± 1.6
Heart Mass as % BM	%	0.35 ± 0.02	0.35 ± 0.02
Muscle Volume	μl	148.2 ± 3.9	132.3 ± 4.9
End Diastolic Lumen	μl	86.5 ± 9.1	70.5 ± 7.1 *
End Systolic Lumen	μl	35.8 ± 2.1	24.7 ± 6.9
Stroke Volume	μl	50.7 ± 7.7	45.7 ± 0.7
RV			
Ejection Fraction	%	57.4 ± 5.0	55.2 ± 2.8
Cardiac Output	ml/min	25.5 ± 3.7	23.4 ± 2.9
End Diastolic Lumen	μl	109 ± 16	105 ± 14
End Systolic Lumen	μl	49.2 ± 9.7	45.7 ± 11.1
Stroke Volume	μl	59.8 ± 8.0	59.5 ± 4.4

Data obtained by *in vivo* cardiac cine-MRI of *C57/Bl6* and *sync* ^{-/-} mice at 18 months of age. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

* indicates measurements significantly different to wild-type (P>0.05)

** indicates measurements highly significantly different to wild-type (P>0.005)

Table 3.4: Heart function in 24 month old *sync* ^{-/-} mice

24 month old		WT	<i>sync</i> ^{-/-}
Number of mice		5	5
Body Mass	g	37.7 ± 0.7	41.5 ± 13.1
Heart Rate	bpm	418 ± 22	454 ± 28
LV			
Ejection Fraction	%	64.6 ± 7.1	67.9 ± 0.9
Cardiac Output	ml/min	17.8 ± 1.1	20.2 ± 0.0
Heart Mass as % BM	%	0.43 ± 0.03	0.44 ± 0.05
Muscle Volume	μl	157 ± 12	172 ± 34
End Diastolic Lumen	μl	81 ± 10	65.5 ± 5.1
End Systolic Lumen	μl	38 ± 10	21.0 ± 2.2
Stroke Volume	μl	43.0 ± 3.5	44.5 ± 2.8
RV			
Ejection Fraction	%	71.9 ± 4.5	60.8 ± 14.1
Cardiac Output	ml/min	17.6 ± 2.5	22.2 ± 6.6
End Diastolic Lumen	μl	58.9 ± 8.4	79.7 ± 0.0
End Systolic Lumen	μl	16.1 ± 2.6	31.2 ± 11.1
Stroke Volume	μl	42.8 ± 7.1	48.5 ± 11.2

Data obtained by *in vivo* cardiac cine-MRI of *C57/Bl6* and *sync* ^{-/-} mice at 24 months of age. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

* indicates measurements significantly different to wild-type (P>0.05)

** indicates measurements highly significantly different to wild-type (P>0.005)

Table 3.5: Dobutamine stress in 24 month old *sync*^{-/-} mice

24 month old	WT				sync +/-	
Number of mice	5				5	
Body Mass	g	37.7 ± 0.7			41.5 ± 13.1	
	Pre-DB	Post-DB	Changes		Pre-DB	Post-DB
LV						Changes
Heart Rate	bpm	438 ± 22	508 ± 25	70 ± 26 *	454 ± 28	510 ± 28
Ejection Fraction	%	59.8 ± 9.4	76.3 ± 3.3	16.5 ± 30.1	63.2 ± 1.1	73.7 ± 5.6
Cardiac Output	ml/min	8.82 ± 1.2	10.0 ± 1.5	1.3 ± 6.6	9.09 ± 0.4	7.86 ± 2.2
End Diastolic Lumen	μl	33.3 ± 2.4	21.4 ± 0.5	-11.3 ± 7.4 *	31.6 ± 0.1	22.8 ± 6.2
End Systolic Lumen	μl	13.2 ± 2.1	5.05 ± 0.5	-8.2 ± 6.5 *	11.6 ± 0.4	5.83 ± 0.3
Stroke Volume	μl	20.0 ± 4.6	16.3 ± 1.1	-3.6 ± 13	20.0 ± 0.2	17.0 ± 5.8

Data obtained by *in vivo* cardiac cine-MRI of C57/Bl6 and *sync*^{-/-} mice at 24 months of age. For each genotype the columns show the value before dobutamine injection, after dobutamine injection and the difference between the two. The difference (the Changes column) is in black if positive and red if negative. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

- * indicates measurements significantly different after dobutamine treatment (P>0.05)
- ** indicates measurements highly significantly different after dobutamine treatment (P>0.005)

Chapter 4: Heart function in the *Moonwalker* mouse

4.1 Introduction

This study measured the function of the *Moonwalker* mouse heart to test the hypothesis that it might show similar dysfunction to the *mdx* mouse heart, specifically that it would be hypertrophied and have LV dysfunction. Evidence suggested that the ion channel mutated in the *Moonwalker* mouse (TRPC3) may cause part of the increase in intracellular Ca^{2+} seen in the *mdx* mouse and that the *Moonwalker* mutation would cause the channel to become more active, resulting in an increase in intracellular Ca^{2+} that mirrored the *mdx* mouse phenotype (Becker *et al.*, 2009). Similar heart dysfunction would support the hypothesis that TRPC3 was involved in the induction of the *mdx* mouse pathology.

The *Moonwalker* mouse is an ENU mouse mutant produced at the MRC Mammalian Genetics Unit, Harwell. It displays an ataxic phenotype as a result of a Threonine to Alanine mutation in the S4-S5 Linker region of the *TRPC3* (Transient receptor potential channel canonical type 3) gene (Becker *et al.*, 2009) (Figure 4.1). This particular amino acid (and region) is highly conserved across *TRPC3*, 6 and 7 in mammals (Table 4.1, located at the end of the chapter). The region is also partially conserved in *TRPC2*, 4 and 5. *TRPC3* is ubiquitously expressed in the mouse, but is expressed most highly in the cerebellum, especially in purkinje cells, where it causes cell death from 4 months. The ataxic phenotype is displayed long before the onset of purkinje cell death and as such it is believed that the mutation has an effect on the arborisation of purkinje cell dendritic trees during development. Electrophysiology shows that the *Moonwalker* TRPC3 channel acts

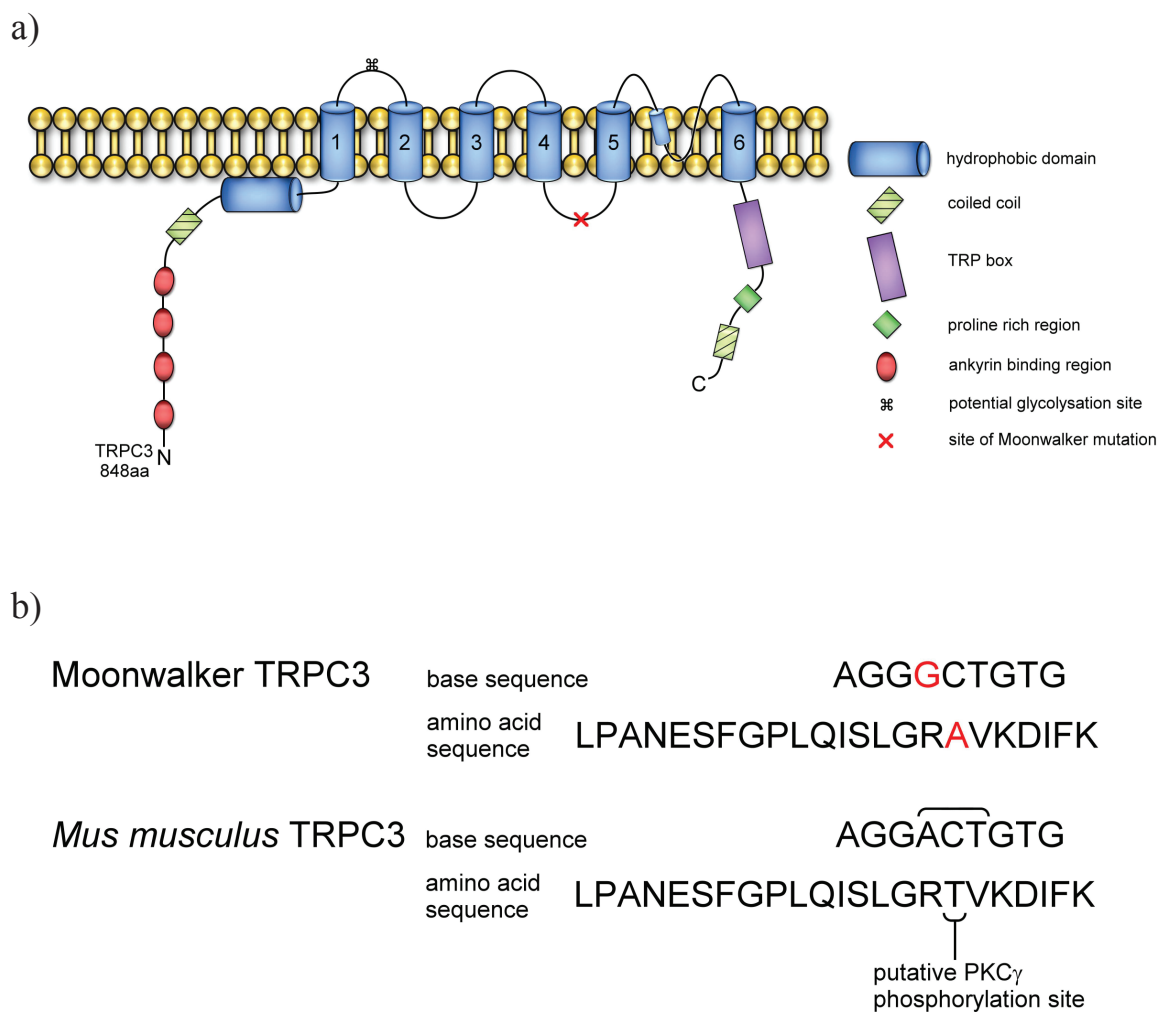


Figure 4.1 Structure of TRPC3.

(a) Domain structure of TRPC3 in the plasma membrane, showing the site of the moonwalker mutation. (b) Base sequence and amino acid sequence of moonwalker and wild-type TRPC3, showing the site of the moonwalker mutation (in red), and the elimination of the putative PKC γ phosphorylation site.

the same as wild-type at high agonist concentrations but is more active than wild-type at low concentrations. Analysis of the S4/S5 linker region of TRPC3 suggests that the mutation may eliminate a PKC γ phosphorylation site (Figure 4.1b). PKC γ has been shown to inhibit TRPC3 channel activity (Trebak *et al.*, 2005; Venkatachalam *et al.*, 2003) so the elimination of this phosphorylation site may explain the observed increase in activity.

Transient receptor potential channels were first identified in *Drosophila* (Montell and Rubin, 1989). Seven mammalian TRPC channels have been identified. The channels are comprised of six transmembrane domains and the number of members is highly conserved from *C. elegans* to *D. melanogaster* to *H. sapiens*. They act as receptor activated cation channels (RACCs), linked to agonists by the PLC-signalling pathway (Hofmann *et al.*, 1999; Nishida *et al.*, 2006) and in the heart are involved in hypertrophy induced by receptor stimulation or pressure overload. Five groups have independently shown that TRPC channels are involved in NFAT-dependent hypertrophy; though they have separately suggested every member is responsible except TRPC2 – TRPC1 (Ohba *et al.*, 2007), TRPC3 (Bush *et al.*, 2006; Nakayama *et al.*, 2006; Onohara *et al.*, 2006), TRPC6 (Onohara *et al.*, 2006), TRPC4&5 (Seth *et al.*, 2004), TRPC7 (Sato *et al.*, 2007).

In the heart TRPC3 may form a heterodimer with TRPC6 (Onohara *et al.*, 2006), where they have been shown to activate the NFAT/calcineurin signalling pathway in cardiomyocytes (Nakayama *et al.*, 2006), leading to the expression of hypertrophy inducing genes such as BNP (Brenner and Dolmetsch, 2007). This pathway is activated in a PLC-dependent manner by angiotensin II (Onohara *et al.*, 2006), as depicted in figure 4.2; though there is good evidence to suggest that this pathway can also be activated by other agonists, such as endothelin-1 (Adebiyi *et al.*, 2010; Peppiatt-Wildman *et al.*, 2007)

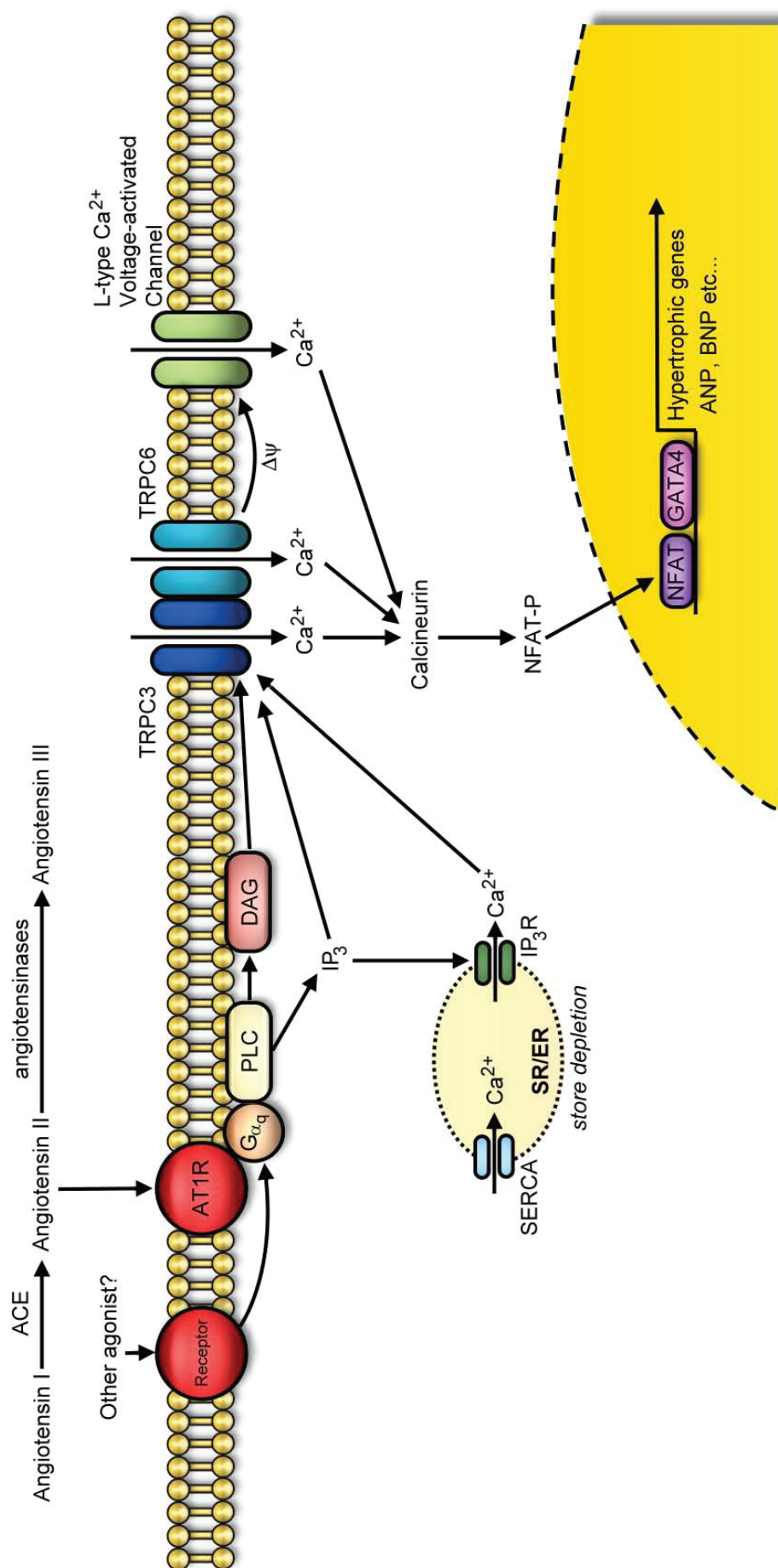


Figure 4.2 TRPC3 activation.

Proposed model of the development of cardiac hypertrophy via the TRPC/calcineurin/NFAT pathway. Neurohumoral factors upregulate the production of DAG and IP₃. DAG and IP₃ activate TRPC3 directly; IP₃ also activates TRPC3 via the release of Ca²⁺ from the SR/ER. The Ca²⁺ entry from TRPC3 causes a change in membrane potential (Δψ), activating voltage-activated channels (such as the L-type channel) and further increasing intracellular Ca²⁺. These Ca²⁺ entry channels induce a prolonged, low-amplitude Ca²⁺ rise. The sustained Ca²⁺ rise activates the calcineurin-NFAT pathway. One of the downstream targets of the TRPC/calcineurin-NFAT pathway is the BNP gene.

and $\alpha 1$ -adrenoceptor agonists (Bush *et al.*, 2006; Hill *et al.*, 2006; Pigozzi *et al.*, 2006; Thebault *et al.*, 2005). TRPC3 has also been shown to be activated by IP₃ (Lockwich *et al.*, 2008; Woodard *et al.*, 2010; Xi *et al.*, 2008; Zhang *et al.*, 2001; Zhu and Tang, 2004) but there is some debate over which mode of activation predominates and IP₃ activation is claimed to be unimportant in the development of cardiac hypertrophy (Onohara *et al.*, 2006). TRPC3 has been shown to act as a non-specific cation channel, allowing both Na⁺ and Ca²⁺ ions to pass through (Lintschinger *et al.*, 2000; Thyagarajan *et al.*, 2001; Zitt *et al.*, 1997), though there has been some question to the importance of direct Ca²⁺ influx via TRPC3 channels. Irrespective of whether this plays an important role Na⁺ influx via TRPC3 channels has been shown to induce Ca²⁺ influx both via Na⁺/Ca²⁺ exchange via the NCX1 channel (Rosker *et al.*, 2004) and due to the inward current from the Na⁺ or Ca²⁺ influx activating L-type voltage activated channels (Onohara *et al.*, 2006), which carry Ca²⁺ into the cell.

Recently Millay *et al* (Millay *et al.*, 2009) found that, skeletal muscle-specific, overexpression of TRPC3 induced muscular dystrophy at 2 months, as measured by an increase in central nucleation, reduced myofiber size, increased fibrosis and macrophage infiltration. Moreover they demonstrated that inhibition of TRPC activity in the *mdx* mouse reduced central nucleation, number of small fibres, fibrosis and CK levels. Muscle degeneration and regeneration occurs in the *mdx* mouse from about 3 weeks of age (Bulfield *et al.*, 1984) and is driven by an increase in intracellular Ca²⁺ due to entry via membrane tears (Bertorini *et al.*, 1982; Bertorini *et al.*, 1984; Bodensteiner and Engel, 1978; Jackson *et al.*, 1991; Morandi *et al.*, 1990) and via stretch-activated channels (SAC) (Allen *et al.*, 2010) (for more details see Chapter 1, section 1.3.1). TRPC channels are a good candidate for SACs, with research into TRPC1 showing that it may act in this role

(Gervasio *et al.*, 2008). The results of Millay *et al* (Millay *et al.*, 2009) suggest that TRPC3 may also act as a SAC. Similarly, a transgenic mouse with heart-specific overexpression of TRPC3 was found to have increased calcium influx in the heart, inducing hypertrophy and leading to death by 12 months (Nakayama *et al.*, 2006). The mice showed significant LV dilation and a loss of ventricular performance. Studies have shown that cardiac TRPC3 expression is elevated in rats subjected to pressure overload, in the failing heart of a rat model of hypertension and heart failure and in the LV of rats treated with an α -adrenergic receptor agonist (Bush *et al.*, 2006). These results, combined with the finding that the *Moonwalker* mutation leads to increased channel activation at low agonist concentrations suggested the *Moonwalker* mouse heart might show similar dysfunction to the *mdx* mouse heart and the hypothesis that the *Moonwalker* mouse heart would be hypertrophied and have LV dysfunction.

12-month old *Moonwalker* and wild-type mice were killed and their hearts, cerebella and cortex's extracted. Half of each heart was processed to extract RNA and QPCR performed to determine the relative levels of TRPC3 RNA in each (for details of method see Chapter 2). The other half of each heart was used for western blotting with anti-TRPC3 and anti- α -actinin antibodies. 18-month old *Moonwalker* and wild-type mice were killed and their hearts extracted and used for western blotting with the same antibodies. Subsequently a longitudinal time course of cine MRIs was performed on the same *Moonwalker* and wild-type mice at 3,6,9,12 and 18 months of age. As the *Moonwalker* mice are on a *C3H* background rather than the *C57/Bl6* background of the *Sync* $-/-$ mice the wild-type heart function is different from that seen in the previous chapter and from that in subsequent chapters. At the 12-month time-point, following the standard MRI, mice were treated with dobutamine to look at stressed heart function. Finally at the end of the time-course, the

mice were killed and histology was performed on sections from the hearts. Both Haematoxylin and Eosin (H&E) and Sirius red stains were used.

4.2 Methods

4.2.1 Animals

Three-month old *Moonwalker* males and females and age- and sex-matched *C3H* control mice were used in the longitudinal *Moonwalker* mouse heart function study (initially $n = 8$ for males and female wild-type and $n = 4$ for *Moonwalker* females, but expanded to $n = 8$ for all groups from 9 months). Further 12-month old *Moonwalker* and wild-type mice were killed and their tissues used for QPCR and western blots ($n = 4$) and 18-month old *Moonwalker* and wild-type mice were killed and their hearts used western blots ($n = 4$). The *Moonwalker* line was derived at MRC Harwell using the mutagen ENU (N-Ethyl-N-nitrosourea), which causes point mutations. Three weekly doses of 100 mg/kg of ENU were injected intraperitoneally into *C3H* male mice from 10 weeks of age. These mice were then mated to *C3H/HeH* females to produce various lines. The *Moonwalker* mouse was initially identified as ataxic due to its unusual gait, which causes it to walk backwards on smooth surfaces. Subsequent investigations in the Davies lab identified the mutation-causing gene as TRPC3 (Transient receptor potential channel canonical type 3) (Becker *et al.*, 2009).

4.2.2 Western Blot

The procedure was largely the same as the general method (as described in section 2.2) but hearts were homogenised in RIPA lysis buffer (0.05M Tris pH8, 0.15M NaCl, 1% NP40, 5% Sodium Deoxycholate, 0.1% SDS), as TRPC3 is membrane bound. Using Newcastle buffer does not extract TRPC3 protein. The TRPC3 antibody and its secondary were used in 3% BSA with 0.5% Tween, rather than in 5% milk and the primary blotting was done overnight, rather than just for 3 hours.

4.2.3 QPCR

Mice were killed by cervical dislocation and their hearts removed and placed in PBS. They were gently squeezed a few times to pump out any blood they contain then dried and flash-frozen in liquid Nitrogen. They were stored at -80°C until needed. To extract RNA half a heart was ground in a pestle and mortar in liquid nitrogen. When completely homogenised it was placed into 1 mL of TRIzol. The TRIzol method (Acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 2006)) of RNA extraction was then followed. The quality and concentration of the resultant RNA was then determined using a Nanodrop Spectrophotometer. A reverse transcriptase reaction was then carried out on the RNA to produce cDNA using the SuperScriptII Reverse Transcriptase Kit. The cDNA was added to primers and SYBR Green Mix and then put into a 96-well plate. Each sample had three replicates The QPCR reaction was then performed with an Applied Biosystems StepOnePlus Instrument and the results are analysed with the Applied Biosystems StepOne software. This gave relative quantitation results using the $\Delta\Delta\text{CT}$ method.

Primers used:

Target		Sequence
TRPC3	Forward	AAAGAAAACGATGAGGTGAATGAAG
	Reverse	CATAACGAAGGCTGGAGATATCCT
DAG1	Forward	CCAAGGAGCAGATCATAGGGC
	Reverse	AGAGCATTTGGAGAAGGCAGG

Primers were obtained from Esther Becker and had already been validated in the *Moonwalker* mouse (Becker *et al.*, 2009).

4.3 Results

4.3.1 Histology

Sections from 2-year-old *Moonwalker* and wild-type hearts were stained with Haematoxylin and Eosin (H&E), to look at general morphology, and Sirius red, to show cardiac fibrosis (Figure 4.3). Haematoxylin and Eosin staining showed normal morphology of the *Moonwalker* heart and Sirius red staining showed no difference in collagen fibres between *Moonwalker* and wild-type mice (Figure 4.3). This is in complete contrast to *mdx* mice where infiltrates and fibrosis are seen in the heart from three months (Van Erp *et al.*, 2010) and by 17 months hearts are around 8% fibrotic (Quinlan *et al.*, 2004).

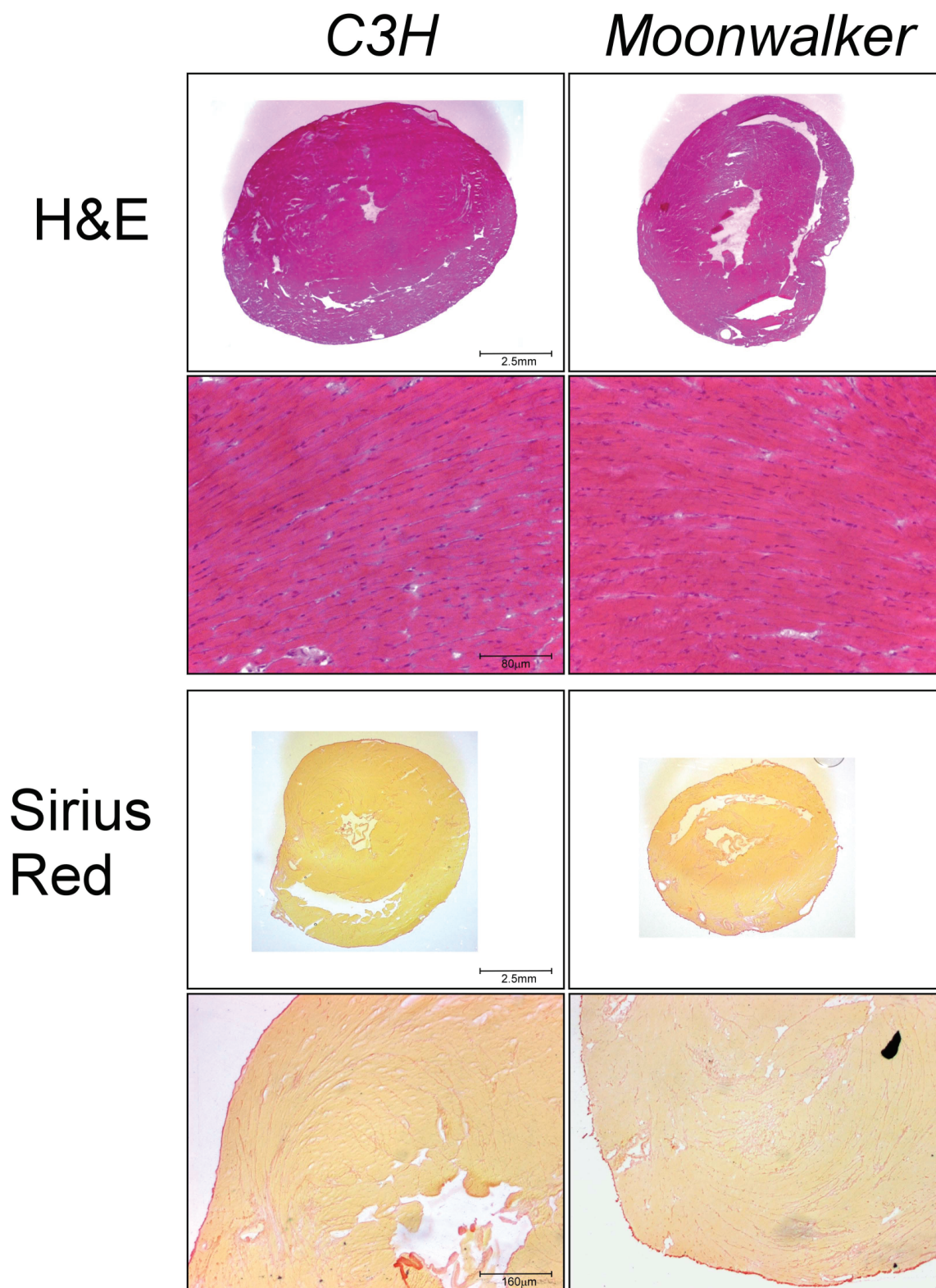


Figure 4.3 Histology.

Sections from 2-year-old wild-type and *Moonwalker* mice hearts stained with H&E and Sirius red stains.

4.3.2 TRPC3 RNA and protein levels

QPCR of whole organ homogenates of cerebellum, cortex and heart from two *Moonwalker* and two wild-type 12 month old mice showed TRPC3 RNA levels were highest in wild-type cerebellum but about 1/3rd of this in *Moonwalker* cerebellum (Figure 4.4), likely due to the large-scale purkinje cell loss by this age (Becker *et al.*, 2009). Remaining TRPC3 RNA may be mainly due to expression in the granule cell layer (Li *et al.*, 2005). In the other two organs there was no significant difference between *Moonwalker* and wild-type. TRPC3 RNA levels in the cortex were about 1/5th of those in the wild-type cerebellum and RNA levels in the heart about 1/80th of those in the wild-type cerebellum.

Western blots performed at 12 and 18 months (Figure 4.5) showed no difference in TRPC3 protein levels between that found in wild-type mice and *Moonwalker* mice and, in the 18 month old mice, no difference between expression levels in male and female mice, thus demonstrating that the *Moonwalker* mutation had no effect on TRPC3 RNA or protein levels.

4.3.3 Time course of MRIs

4.3.3.1 Heart function in age-matched mice

Cine-MRIs were performed on the same mice at 3,6,9,12 and 18 months of age and a number of LV parameters measured. In 3-month-old mice (Table 4.2, located at the end of the chapter) the *Moonwalker* males were significantly smaller than age-matched wild-type mice and had significantly lower cardiac outputs, muscle volumes, EDLs and stroke volumes. However due to the significant difference in body mass those heart function

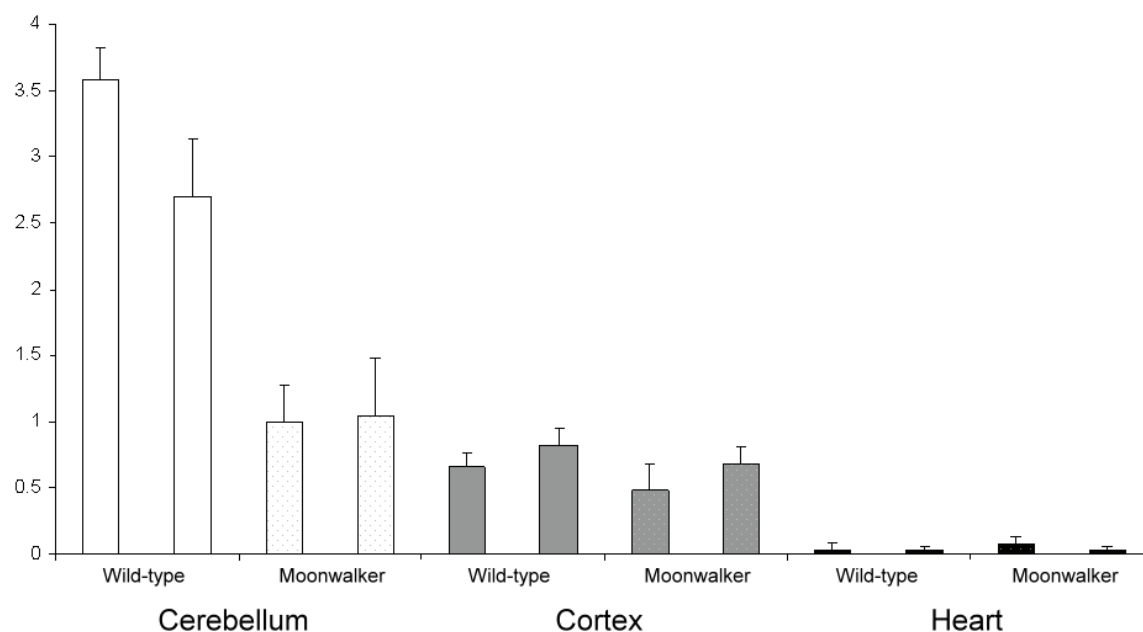


Figure 4.4 TRPC3 RNA levels in brain and heart.

TRPC3 RNA levels in cerebellum, cortex and heart from *Moonwalker* and wild-type mice as measured by QPCR. DAG1 was used as the control.

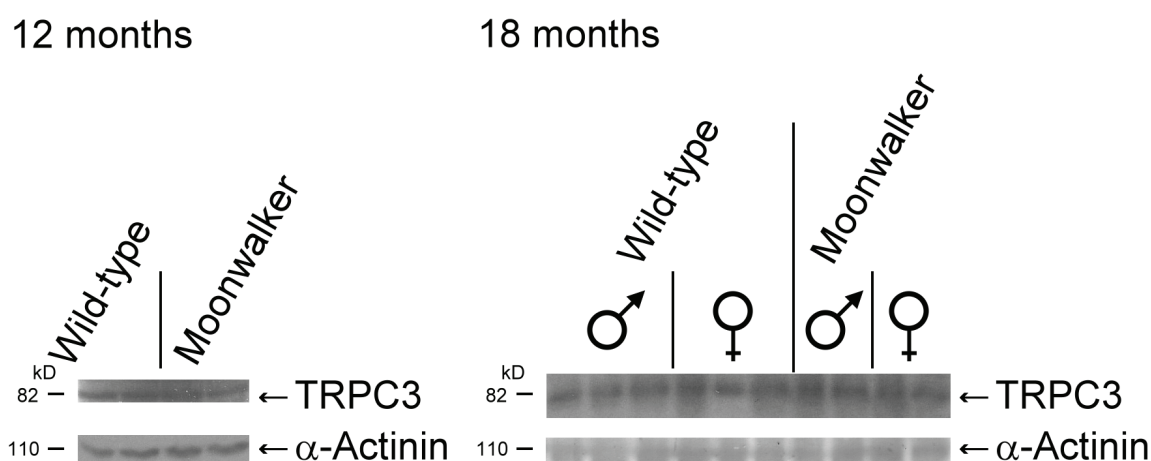


Figure 4.5 TRPC3 protein levels in heart.

Western blot for TRPC3 expression in wild-type and *Moonwalker* mice hearts at 12 and 18 months. α -actinin was used as the loading control.

parameters affected by size (muscle volume, cardiac output, EDL, ESL and stroke volume) cannot be meaningfully compared in this way. To allow comparisons between these parameters it is necessary to normalise the data by dividing by body mass. Subsequently all of these differences were found to be no longer significant when divided by body mass. In the age matched females the *Moonwalkers* were also significantly smaller and had significantly smaller EDLs and ESLs. Again these differences were no longer significant when dividing by body mass. Between the sexes, in wild-type mice the males were significantly larger than the females with significantly larger muscle volumes and EDLs; in the *Moonwalker* mice the males had larger ESLs. These sex-based differences were all non-significant when dividing by body mass.

In 6-month-old mice (Table 4.3, located at the end of the chapter) the *Moonwalker* males were significantly smaller than age-matched wild-type mice and had significantly lower EDLs, ESLs and E/A ratio and a significantly larger left ventricular mass as a percentage of body mass ratios (a measure of hypertrophy). These EDL and ESL differences were not abolished when dividing by body mass, instead they reversed – the EDL/mass and EDL/mass figures are significantly higher in the *Moonwalker* mice. There was also a significantly higher stroke volume/mass in the *Moonwalker* mice. In females the *Moonwalkers* were significantly smaller than age-matched wild-type controls and had a significantly lower ejection fraction, cardiac output, stroke volume and E/A ratio and a significantly higher A filling rate. The cardiac output and stroke volume were no longer significantly smaller when dividing by body mass. Between the sexes there were no significant differences in the *Moonwalker* mice but the wild-type males were significantly larger, with a significantly larger muscle volume, EDL and ESL and a significantly smaller ejection fraction than age-matched females. When divided by body mass these differences

were no longer significant but the wild-type females did have significantly larger cardiac output/mass and stroke volume/mass.

In 9-month-old mice (Table 4.4, located at the end of the chapter) the *Moonwalker* males were significantly smaller than age-matched wild-type mice and had a significantly lower muscle volume. This difference remained significant when divided by body mass, though again reversing sign and the cardiac output/mass, EDL/mass, ESL/mass and stroke volume/mass were all significantly larger in the *Moonwalker* males. In females the *Moonwalkers* were significantly smaller than age-matched wild-type controls and had a significantly lower cardiac output, muscle volume, EDL, stroke volume, E peak filling rate and E/A ratio. Cardiac output, muscle volume, EDL and stroke volume were not significantly lower when divided by body mass. Between the sexes wild-type male mice were significantly larger than female mice, with a significantly larger muscle volume, EDL and ESL. When dividing by mass these differences were abolished and instead the female mice had a significantly larger stroke volume/mass. The *Moonwalker* males had a significantly larger EDL and ESL than the females, a difference that was also significant when dividing by mass.

In 12-month-old mice (Table 4.5, located at the end of the chapter) the *Moonwalker* males were significantly smaller than age-matched wild-type mice and had a significantly lower cardiac output, muscle volume, EDL and stroke volume. These differences remain significant when divided by body mass, again reversing sign so that cardiac output/mass, EDL/mass, stroke volume/mass and ESL/mass as well were all significantly larger in the *Moonwalker* males. In females the *Moonwalker* mice were significantly smaller than age-matched wild-type controls and had a significantly lower cardiac output, muscle volume,

EDL, stroke volume and peak ejection rate. When dividing by body mass none of these differences were significant. Between the sexes male wild-type mice were significantly larger than female mice with a significantly larger muscle volume, EDL and ESL. These differences were no longer significant when dividing by mass and instead the females had a significantly larger cardiac output/mass and stroke volume/mass. Between the *Moonwalker* mice the males had a significantly larger muscle volume, EDL, ESL stroke volume and peak ejection rate. Of these EDL, ESL and stroke volume were still significantly larger when divided by mass.

In 18-month-old mice both LV and RV function was measured (Table 4.6, located at the end of the chapter). The *Moonwalker* males were significantly smaller than age-matched wild-type mice and in the LV had a significantly lower cardiac output, muscle volume, EDL and stroke volume. None of these differences were significant when divided by body mass. The *Moonwalker* females had a significantly smaller muscle volume than age-matched wild-type controls but this was not significant when dividing by body mass. Between the sexes male wild-type mice were significantly larger than female mice with a significantly larger cardiac output, muscle volume and stroke volume. There were no significant differences between the *Moonwalker* sexes. In the RV *Moonwalker* males had a significantly smaller EDL and stroke volume, though these were not significant when divided by mass. There were no significant differences between the wild-type and *Moonwalker* females. Between the sexes the male wild-type mice were significantly larger than female mice with a significantly larger EDL and stroke volume, though these differences were not significant when dividing by mass. In the *Moonwalker* mice the males had a significantly EDL/mass.

These results show that, while an initial reading might suggest that the *Moonwalker* mice have worse heart function than wild-type mice (with, for example, a reduced cardiac output at 3 and 12 months), as hypothesised, if one normalises for body mass the differences are reversed (e.g. increases in EDL/mass and ESL/mass, and consequently stroke volume and cardiac output at 6, 9 and 12 months), suggesting hypertrophy of the *Moonwalker* mouse heart. However by 18 months the differences have been abolished. As the *Moonwalker* mice are on a *C3H* background rather than the *C57/B16* background of the *Sync* $-/-$ mice the wild-type heart function is different from that seen in the previous chapter.

4.3.3.2 Heart function in mass-matched mice

At (almost) all time points there were significant differences in body mass between wild-type and *Moonwalker* mice in both sexes. To allow comparisons between heart parameters that would be affected by size – muscle volume, cardiac output, EDL, ESL and stroke volume I normalised by dividing by body mass. But is this legitimate? If the heart mass as a percentage body mass is plotted against body mass (Figure 4.6) it is found that in larger wild-type mice of either sex heart mass as a % body mass is lower than in smaller mice i.e. as a mice grows its heart becomes smaller as a percentage of its body mass. This suggests that, at least for these parameters, we can only compare mice of the same mass and that the above finding of possible hypertrophy in the *Moonwalker* mouse heart may be wrong.

Choosing data sets as close in age as possible, to limit the effects of aging, this limits us to one set of comparisons per sex: for females we can compare 3 month old wild-type mice with 6 month old *Moonwalker* mice, while for males we can compare 3 month old wild-type mice with 12 month old *Moonwalker* mice (Table 4.7, located at the end of the

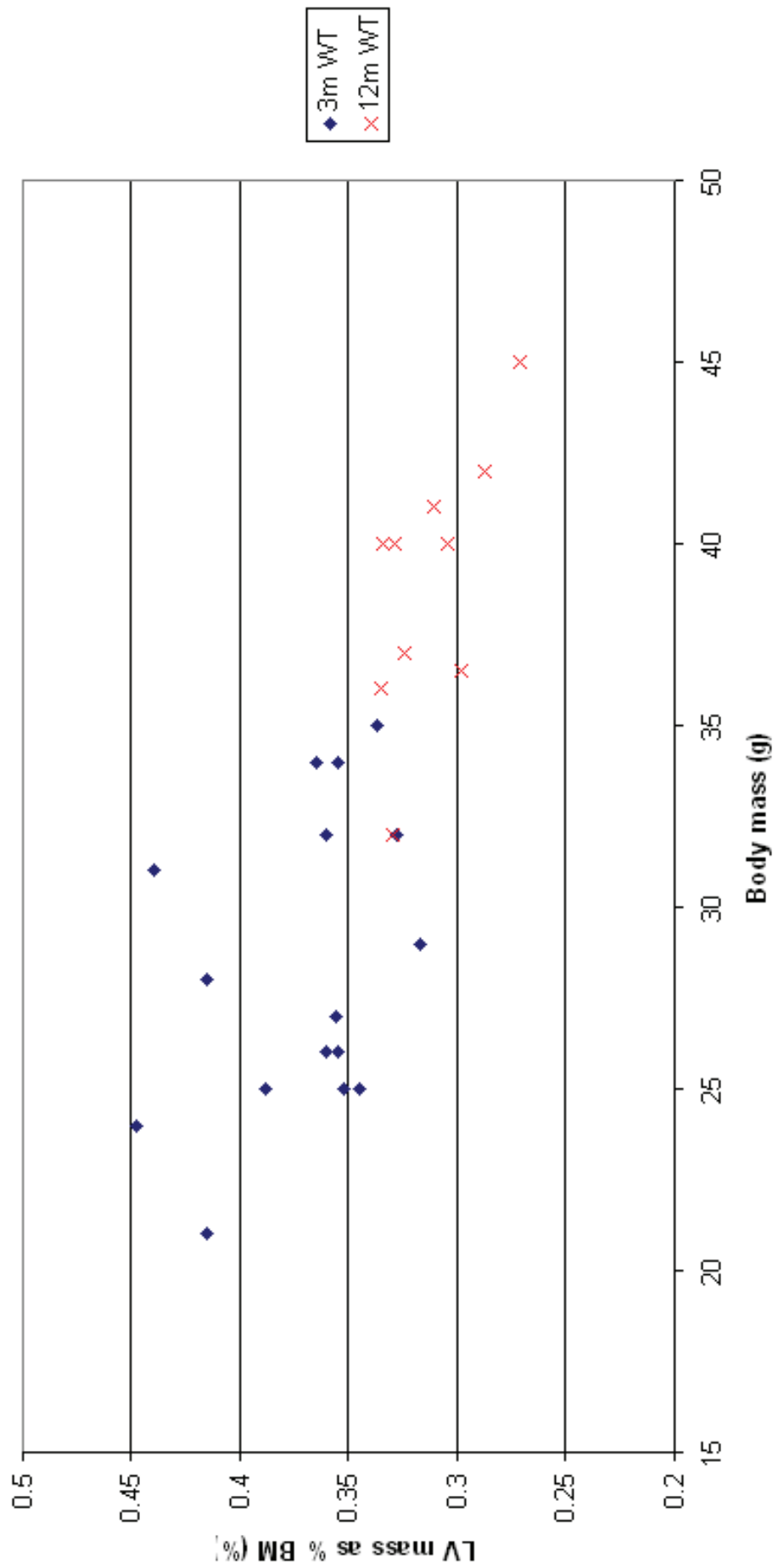


Figure 4.6 LV mass as a percentage of body mass against body mass.
LV mass as a percentage of body mass against body mass for 3 month old and 12 month old wild-type mice. LV volume was measured by *in vivo* cine-MRI and multiplied by average density to determine mass. The same mice were imaged at 3 months and 12 months.

chapter). There are still significant differences between the body masses of the male mice – the *Moonwalker* mice are significantly smaller, but this cannot be helped. For the 3-month old male wild-type versus 12-month old *Moonwalker* mice, other than the significant difference in mass noted above there is only one significant difference - the heart mass as a % body mass of the *Moonwalker* mice is significantly higher than that of the wild-type mice. For the 3-month old female wild-type versus 6-month old *Moonwalker* mice there are no significant differences. These results fit with the age-matched data, where there were no significant differences between wild-type and *Moonwalker* mice when normalising for body mass at 18 months.

4.3.3.3 Normalisation by heart mass

While 3 and 6 month old mice are not hugely dissimilar 3 and 12-month old mice are developmentally hugely different. This makes the above results for the male mice uncertain. Due to this the age-matched data for the parameters that would be affected by size has also been normalised by heart mass as well as the body mass normalisation used above. This allows one to directly see if the hearts are functioning better for their size rather than for the size of the animal. Body mass may also be a poor value to normalise to because it can vary depending on body fat percentage.

This normalisation gives the following results: At 3 months (Table 4.2) there were no significant differences between wild-type and *Moonwalker* mice for either sex and no differences between the sexes, just as when normalised by body mass. At 6 months (Table 4.3) the observed differences between males when normalised to body mass are all abolished. In females the stroke volume/heart mass is significantly smaller in the *Moonwalker* mice, where previously when normalised to body mass it was not. There are

no significant differences between the *Moonwalker* sexes but in the wild-type mice the males have significantly larger ESL/heart mass (unlike when dividing by body mass) and significantly smaller stroke volume/heart mass (the same as when dividing by body mass). At 9 months (Table 4.4) there are no significant differences between wild-type and *Moonwalker* mice for either sex and no differences between the sexes, unlike when normalising by body mass where there were many differences in the males. At 12 months (Table 4.5) the male *Moonwalker* mice had a significantly larger EDL/heart mass and ESL/heart mass, mirroring the results when dividing by body mass (though other parameters were also significantly different). The female *Moonwalker* mice have a significantly smaller cardiac output/heart mass, unlike when dividing by body mass where there were no significant differences. Between the sexes the female wild-type mice had a significantly larger cardiac output/body mass, as when dividing by body mass. The male *Moonwalker* mice had a significantly larger EDL/heart mass and ESL/heart mass, the same as when dividing by body mass. At 18 months in the LV and RV (Table 4.6) there were no significant differences between wild-type and *Moonwalker* mice for either sex when dividing by heart mass and no differences between the sexes; broadly the same as when dividing by body mass. Taking the mass-matched data shown above and further normalising it for heart mass found no significant differences in the male 3 and 12-month old mice. In the 3 and 6 month old female mice the *Moonwalker* females had a significantly smaller stroke volume/heart mass. These results reverse the trend seen in the age-matched mice normalised for body mass, where the *Moonwalker* mice had better heart function than wild-type mice at a number of intermediate time points, and now, when normalising for heart mass, they have worse heart function at those same time points. By unit body mass the *Moonwalker* mice have better function at those points, but by unit heart

mass they have worse function. However by 18 months the differences have again been abolished.

4.3.3.4 Trends in heart function over time

Finally if you look at the changes in the various parameters over time, at all time points *Moonwalker* males are significantly smaller than wild-type males, while *Moonwalker* females are significantly smaller up till 18 months old (Figure 4.7). In *Moonwalker* females, but not males, there is a trend for a smaller LV ejection fraction than wild-type from 3 months, but by 18 months the ejection fraction is higher in *Moonwalker* (Figure 4.8). This is also seen in mass-matched males and females. There is a trend of lower E/A ratio in *Moonwalker* mice from 6 months old, compared with wild-type, than is largely abolished in 18 month old mice (Figure 4.9). It is hard to determine if *Moonwalker* mice hearts are hypertrophied when compared to wild-type mice due to the difficulties discussed above (Figure 4.6) so instead male and female *Moonwalker* mice were directly compared, as they are never significantly different in size (Figure 4.7). There is a gradual reduction in LV mass as a percentage of body mass with time up to 9 months old when the males start to increase, while the females carry on decreasing up to 12 months, when they increase slightly, but the difference between males and females increases from 9 months onwards suggesting an induction of hypertrophy in males only at this point, rather than from 6 months as was suggested by the original body mass corrected data (Figure 4.10). Again most trends are abolished in 18-month-old mice, leaving the probable induction of hypertrophy the only difference in heart function between the *Moonwalker* and wild-type mice, and only in male *Moonwalker* mice at that.

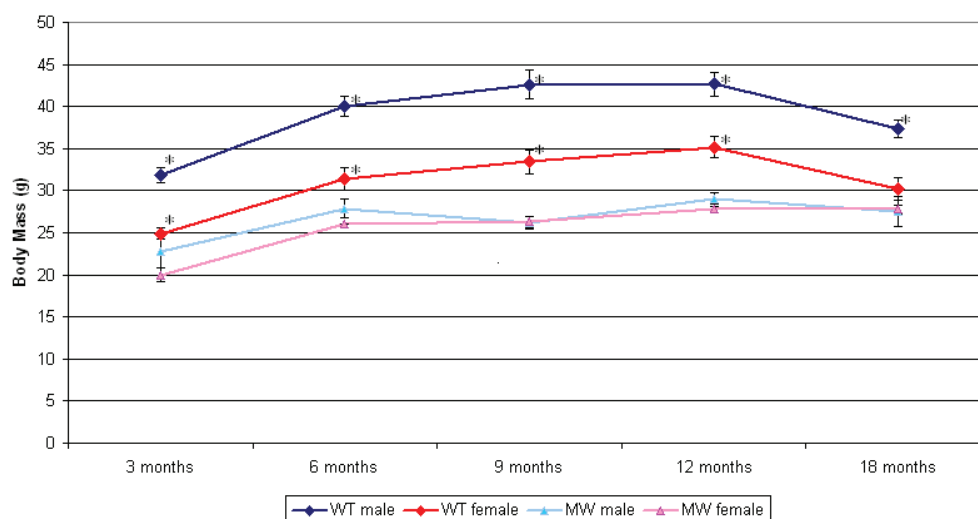


Figure 4.7 Changes in body mass with time.

Changes in body mass with time for wild-type and *Moonwalker* mice. The same mice were weighed at 3, 6, 9, 12 and 18 months.

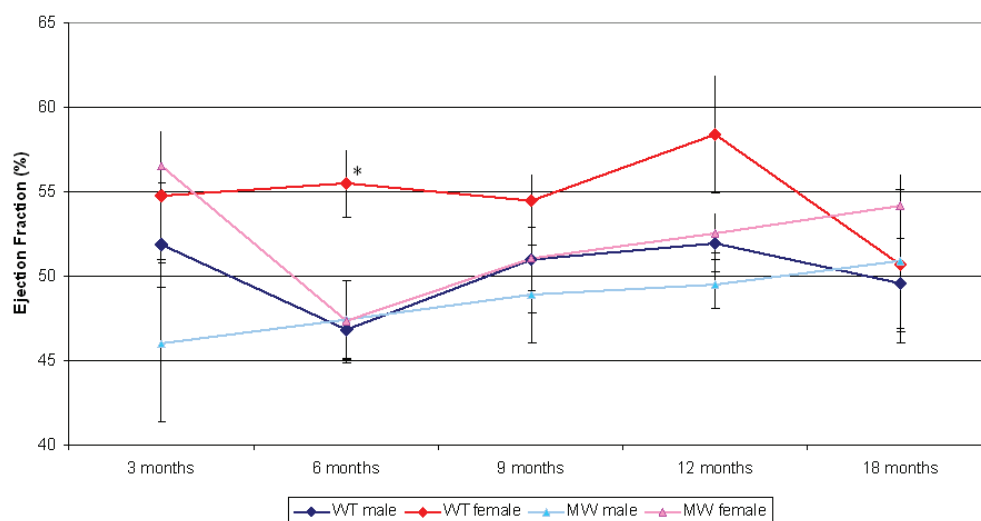


Figure 4.8 Changes in ejection fraction with time.

Changes in ejection fraction with time for wild-type and *Moonwalker* mice. Ejection fraction was measured by *in vivo* cine-MRI. The same mice were imaged at 3, 6, 9, 12 and 18 months.

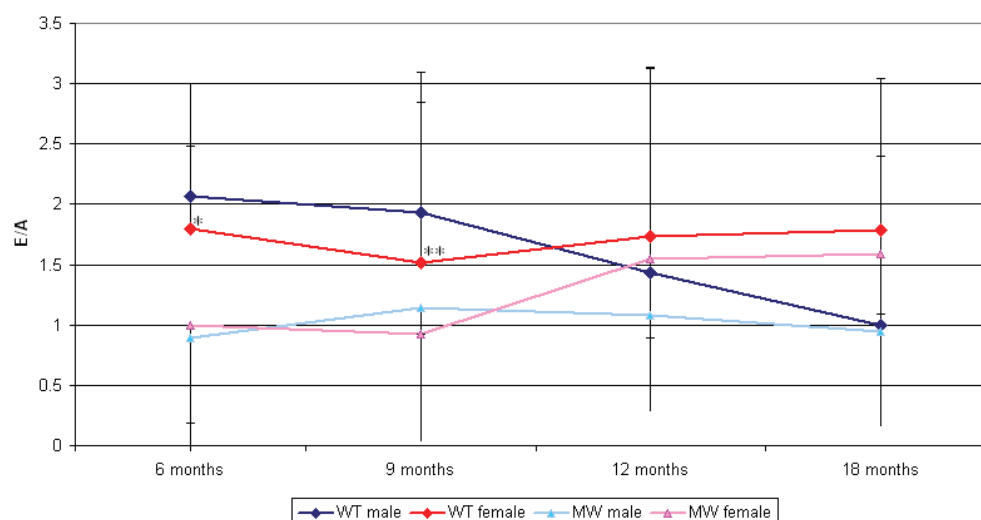


Figure 4.9 Changes in E/A ratio with time.

Changes in E/A ratio with time for wild-type and *Moonwalker* mice. E/A was measured by *in vivo* cine-MRI. The same mice were imaged at 3, 6, 9, 12 and 18 months.

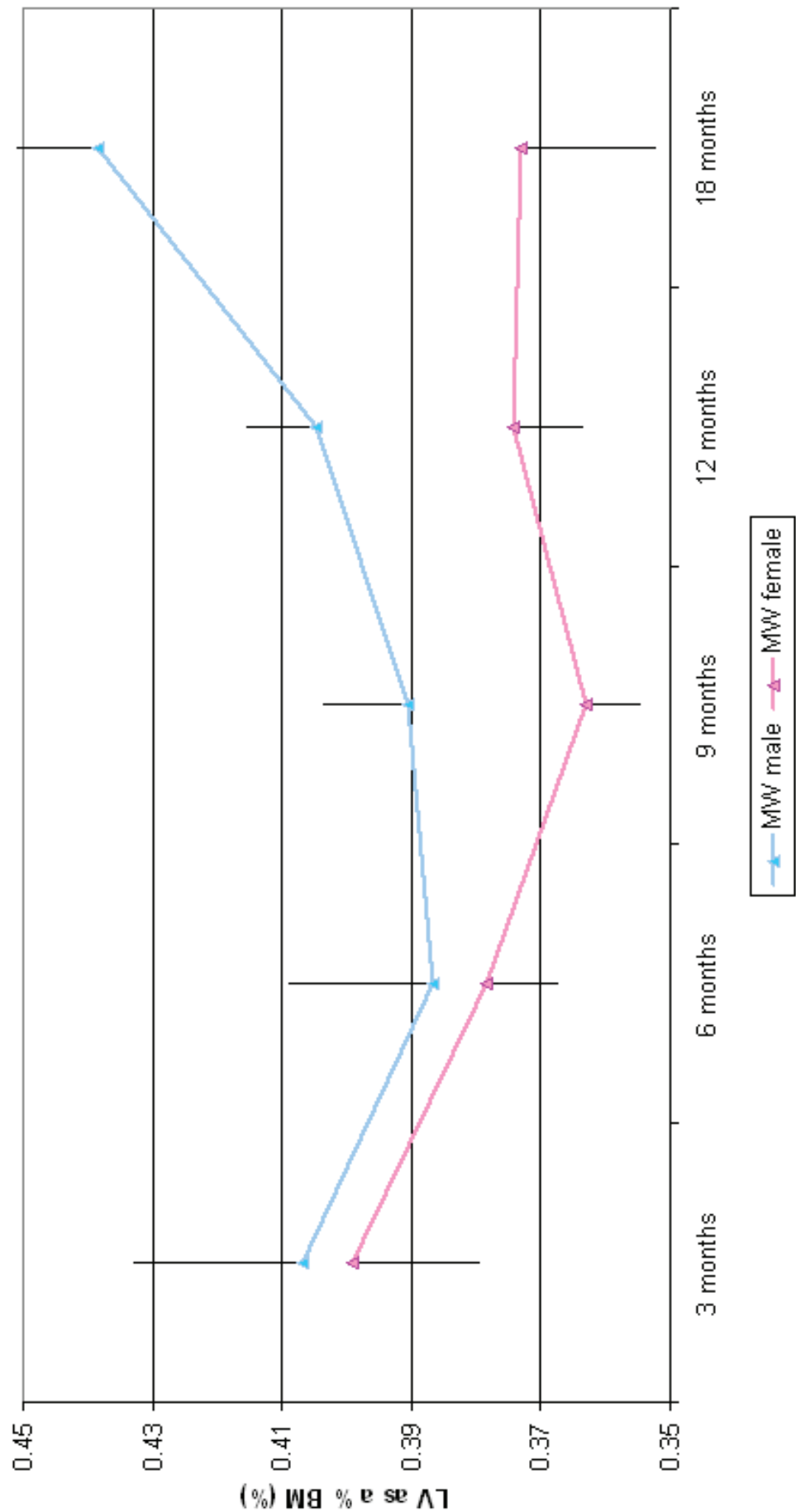


Figure 4.10 Changes in LV mass as a percentage of body mass with time in *Moonwalker* mice. Changes in LV mass as a percentage of body mass with time for *Moonwalker* mice. LV volume was measured by *in vivo* cine-MRI and multiplied by average density to determine mass. The same mice were imaged at 3, 6, 9, 12 and 18 months.

4.3.4 Dobutamine stress

The *mdx* heart shows abnormal response to dobutamine stress, with a reduced response to in vivo high dose dobutamine stress (Quinlan *et al.*, 2004) and a 60-75% mortality rate from this treatment (Bostick *et al.*, 2009; Wu *et al.*, 2008). To test the response of the *Moonwalker* heart to stress at the 12-month time point used above, following the standard MRI, low dose dobutamine stress was used on the mice. Only the LV was studied (Table 4.8, located at the end of the chapter). In both *Moonwalker* and wild-type males dobutamine stress caused a significant increase in ejection fraction and cardiac output, and significant decrease in EDL and ESL; all as would be expected. There was no significant difference in the changes due to the dobutamine stress between *Moonwalker* and wild-type mice. Likewise in both *Moonwalker* and wild-type females dobutamine stress caused a significant increase in ejection fraction and significant decrease in EDL and ESL. Here the change in PFR in *Moonwalker* females was significantly smaller than in wild-type females – it decreased in *Moonwalker*, but increase in wild-type. There were no other significant difference in the changes due to the dobutamine stress between *Moonwalker* and wild-type mice, so the significance of this change is uncertain, however the PFR is significantly smaller in 9 month old (but not 12 month old) *Moonwalker* females, compared to wild-type, suggesting that this change may be indicative of some underlying deficit in *Moonwalker* female diastolic function. Therefore the *Moonwalker* mice do not display the same deficient response to dobutamine stress seen in the *mdx* mouse.

4.4 Discussion

In this study it is demonstrated that overall the *Moonwalker* mutation has little effect on heart function. Initially the results appeared to show a growing heart dysfunction in both male and female mice, with a growing number of significant differences seen at the intermediate time points, however this transpires to be largely due to the significant difference in size between wild-type and *Moonwalker* mice, and by 18 months, when there was no longer a size difference in females, most differences were no longer significant.

There is some evidence of the predicted induction of hypertrophy. The male *Moonwalker* heart initially appears to be hypertrophied when compared to wild-type, with significant differences in left ventricular mass as a percentage of body mass at 6,9 and 12 months; but, as can be seen from (Figure 4.6), as a mice grows its heart becomes smaller as a percentage of its body mass and so this direct comparison is useless. However the male *Moonwalker* heart can be compared with the female *Moonwalker* heart, as the mice are never significantly different in size, and this (Figure 4.10) appears to show that the male *Moonwalker* heart is hypertrophied from 9 months. Instead the main effect of the mutation appears to be a delay in growth, with significantly smaller *Moonwalker* mice of both sexes up to 12 months of age, whereupon the females finally catch up and male heart function catches up when normalising for the still significant body mass difference.

This mouse was studied because it was hypothesised that it would display similar heart function to the *mdx* mouse. In the *mdx* mouse hypertrophy is seen from about 11 months (Jearawiriyapaisarn *et al.*, 2010; Quinlan *et al.*, 2004) with changes in LV systolic function from 11 months (Quinlan *et al.*, 2004) and changes in RV function and LV diastolic

function from 8 months (Zhang *et al.*, 2008b). It also displays an abnormally small reaction to dobutamine administration. As discussed above there appears to be some hypertrophy occurring in male *Moonwalker* mice from 9 months, so this is similar. There are some significant changes in systolic function seen in *Moonwalker* mice, and in *Moonwalker* females, but not males, there is a trend for a smaller LV ejection fraction than wild-type from 3 months; but by 18 months there are next to no significant differences in females, and no significant differences in males when correcting for body mass. RV function was only measured at 18 months but again there are no significant differences when correcting for body mass. Lastly looking at LV systolic function there are a few significant differences at various time points and there is a trend of lower E/A ratio in *Moonwalker* mice from 6 months old, compared with wild-type, again largely gone at 18 months. In the *mdx* mouse, there is significant heart dysfunction by this age, with LV dysfunction from 11 months (Quinlan *et al.*, 2004) and RV dysfunction from 8 months (Zhang *et al.*, 2008b) (LV from 9 months and RV from 6 months in Chapters 5 and 6), neither of which is seen in *Moonwalker* mice even by 18 months. The *Moonwalker* mouse also shows next to no difference from wild-type in response to dobutamine stress, with the only difference likely being a diastolic function deficit rather than a response to stress deficit. This shows that the *Moonwalker* mouse is a poor model for *mdx* heart failure but does not eliminate the possibility that TRPC3 may act as a SAC in the *mdx* mouse.

In both previous papers where TRPC3 was found to have dramatic effects it was highly overexpressed (Millay *et al.*, 2009; Nakayama *et al.*, 2006). While it is ubiquitously expressed the QPCR data (Figure 4.4) shows its expression in the heart is only 6% of that in the cortex and only about 1% of that in the cerebellum, suggesting that at endogenous levels, its expression in the heart is just not high enough to have a large effect there.

Furthermore it has been suggested that the mechanism of activation of TRPC3 may depend upon its level of expression, with PLC activation occurring at high levels of expression and IP3 activation occurring at low levels of expression (Vazquez *et al.*, 2003). If this is true then the effects observed by Millay *et al* and Nakayama *et al* (Millay *et al.*, 2009; Nakayama *et al.*, 2006) may well not be relevant, as in vivo TRPC3 would be activated by an entirely different mechanism.

Future experiments could cross the *Moonwalker* mouse and the *mdx* mouse. In the introduction it was hypothesised that the effect of the *Moonwalker* mutation would be to increase intracellular Ca^{2+} in a manner that might induce a muscular dystrophy like phenotype. By adding this putative effect to the known increase in intracellular Ca^{2+} found in the *mdx* mouse and increased sensitivity to stress-induced damage by for example, reactive oxygen species, an additive effect might be seen (Head *et al.*, 1992; Petrof *et al.*, 1993; Rando *et al.*, 1998). It is possible that a cross between *Moonwalker* mice and *mdx* mice would reveal differences in heart (and muscle) function that are not seen with the *Moonwalker* mutation alone.

Table 4.1: TRP channel amino acid sequence is conserved across species and channels

TRPC3, 6 & 7 (and TRPM2)

Moonwalker TRPC3	LPANESFGPLQISLGRAVVDIFK
<i>Mus musculus</i> TRPC3	LPANESFGPLQISLGRTVVDIFK
<i>Mus musculus</i> TRPC6	LPANESFGPLQISLGRTVVDIFK
<i>Mus musculus</i> TRPC7	LPANESFGPLQISLGRTVVDIFK
<i>Homo sapiens</i> TRPC3	LPANESFGPLQISLGRTVVDIFK
<i>Homo sapiens</i> TRPC6	LPANESFGPLQISLGRTVVDIFK
<i>Homo sapiens</i> TRPC7	LPANESFGPLQISLGRTVVDIFK
<i>Rattus norvegicus</i> TRPC3	LPANESFGPLQISLGRTV EDIFQ
<i>Rattus norvegicus</i> TRPC6	LPANESFGPLQISLGRTVVDIFK
<i>Bos taurus</i> TRPC3	LPANESFGPLQISLGRTVVDIFK
<i>Canis lupus familiaris</i> *TRPC3	LPANESFGPLQISLGRTVVDIFK
<i>Cavia porcellus</i> TRPC3	LPANESFGPLQISLGRTVVDIFK
<i>Equus caballus</i> *TRPC3	LPANESFGPLQISLGRTVVDIFK
<i>Gallus gallus</i> TRPC6 & 7	LPANESFGPLQISLGRTVVDIFK
<i>Macaca mulatta</i> TRPC6	LPANESFGPLQISLGRTVVDIFK
<i>Macaca mulatta</i> TRPM2	LPANESFGPLQISLGRTVVDIFK
<i>Monodelphis domestica</i> *TRPC3	LPANESFGPLQISLGRTVVDIFK
<i>Ornithorhynchus anatinus</i> *TRPC3	LPANESFGPLQISLGRTVVDIFK
<i>Pan troglodytes</i> TRPC3	LPANESFGPLQISLGRTVVDIFK
<i>Danio rerio</i> *TRPC3	LPANESFGPLQISLGRTVVDIFK

TRPC2, 4 & 5

<i>Mus musculus</i> TRPC2	LPAN HESL GT LQISIGKMIDD MIR
<i>Mus musculus</i> TRPC4	AN SHL GPLQISLGR MLLD ILK
<i>Mus musculus</i> TRPC5	AN SHL GPLQISLGR MLLD ILK
<i>Homo sapiens</i> TRPC4	AN SHL GPLQISLGR MLLD ILK
<i>Homo sapiens</i> TRPC5	AN SHL GPLQISLGR MLLD ILK
<i>Rattus norvegicus</i> TRPC2	LPAN HESL GT LQISIGKMIDD MIR
<i>Rattus norvegicus</i> TRPC4	AN SHL GPLQISLGR MLLD ILK
<i>Rattus norvegicus</i> TRPC5	AN SHL GPLQISLGR MLLD ILK

* Protein similar to

Amino acid sequences of TRPC and TRPM proteins showing the point mutation in the Moonwalker mouse (**bold**) and changes in sequence in other species/proteins (**red**).

Table 4.2 Heart function in 3 month old *Moonwalker* mice

3 month old		Males		Females	
		Wild-type	<i>Moonwalker</i>	Wild-type	<i>Moonwalker</i>
Number of mice		8	6	8	4
Body Mass	g	31.8 ± 0.9	22.8 ± 1.9 **	24.8 ± 0.6 ††	20.0 ± 0.8 **
Heart Rate	bpm	448 ± 11	433 ± 23	457 ± 13	485 ± 27
LV					
Ejection Fraction	%	51.8 ± 2.5	46.0 ± 4.6	54.7 ± 3.8	56.5 ± 1.0
Cardiac Output	ml/min	16.3 ± 0.8	10.7 ± 1.6 *	14.7 ± 1.2	12.5 ± 0.8
Heart Mass as % BM	%	0.36 ± 0.01	0.40 ± 0.02	0.37 ± 0.01	0.39 ± 0.01
Muscle Volume	μl	110 ± 5	87 ± 6 *	89 ± 2 ††	76 ± 5
End Diastolic Lumen	μl	70.8 ± 3.0	53.8 ± 4.0 **	58.9 ± 1.8 ††	45.8 ± 3.7 *
End Systolic Lumen	μl	34.3 ± 3.0	29.3 ± 3.9	26.8 ± 2.8	19.9 ± 1.5 *†
Stroke Volume	μl	36.4 ± 1.3	24.5 ± 2.7 **	32.0 ± 1.8	25.9 ± 2.3
CO by Body Mass	ml/min/g	0.51 ± 0.02	0.48 ± 0.07	0.59 ± 0.04	0.62 ± 0.02
EDL by Body Mass	ul/g	2.23 ± 0.10	2.41 ± 0.20	2.38 ± 0.12	2.29 ± 0.12
ESL by Body Mass	ul/g	1.08 ± 0.09	1.29 ± 0.14	1.09 ± 0.14	0.99 ± 0.05
SV by Body Mass	ul/g	1.14 ± 0.05	1.11 ± 0.17	1.29 ± 0.08	1.29 ± 0.08
CO by Heart Mass	ml/min/mg	0.14 ± 0.00	0.11 ± 0.01	0.15 ± 0.00	0.15 ± 0.01
EDL by Heart Mass	ul/mg	0.61 ± 0.01	0.58 ± 0.01	0.63 ± 0.02	0.57 ± 0.04
ESL by Heart Mass	ul/mg	0.29 ± 0.02	0.31 ± 0.02	0.29 ± 0.03	0.25 ± 0.02
SV by Heart Mass	ul/mg	0.31 ± 0.01	0.27 ± 0.03	0.34 ± 0.01	0.32 ± 0.02

Data obtained by *in vivo* cardiac cine-MRI of *C3H* and *Moonwalker* mice at 3 months of age. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

- * indicates measurements significantly different to sex-matched WT (P>0.05)
- ** indicates measurements highly significantly different to sex-matched WT (P>0.005)
- † indicates measurements significantly different to genotype-matched other sex (P>0.05)
- †† indicates measurements highly significantly different to genotype-matched other sex (P>0.005)

Table 4.3 Heart function in 6 month old *Moonwalker* mice

6 month old		Males		Females	
		Wild-type	<i>Moonwalker</i>	Wild-type	<i>Moonwalker</i>
Number of mice		8	6	8	4
Body Mass	g	40 ± 1	27.8 ± 1.1 **	31.3 ± 1.3 ††	26 ± 0 **
Heart Rate	bpm	460 ± 11	454 ± 31	463 ± 11	482 ± 17
LV					
Ejection Fraction	%	46.8 ± 2.0	47.3 ± 2.3	55.5 ± 1.9 †	47.3 ± 2.2 *
Cardiac Output	ml/min	17.7 ± 1.0	15.3 ± 2.1	17.5 ± 0.8	14.6 ± 0.1 *
Heart Mass as % BM	%	0.31 ± 0.01	0.38 ± 0.02 *	0.34 ± 0.02	0.37 ± 0.01
Muscle Volume	μl	118 ± 3	102 ± 8	102 ± 4 ††	93 ± 3
End Diastolic Lumen	μl	82.3 ± 2.0	69.4 ± 4.8 *	68.0 ± 1.4 ††	61.9 ± 4.1
End Systolic Lumen	μl	43.8 ± 2.1	36.2 ± 2.0 *	30.3 ± 1.5 ††	32.7 ± 3.5
Stroke Volume	μl	38.5 ± 1.8	33.1 ± 3.4	37.7 ± 1.4	29.1 ± 1.0 **
PER	μl/s	296 ± 18	251 ± 35	292 ± 8.9	250 ± 27
PFR	μl/s	491 ± 57	456 ± 48	440 ± 11	442 ± 10
E	μl/s	491 ± 57	317 ± 73	440 ± 11	414 ± 37
A	μl/s	259 ± 30	392 ± 52	268 ± 30	416 ± 17 **
E/A		2.07 ± 0.40	0.89 ± 0.20	1.80 ± 0.20	1.00 ± 0.10 *
CO by Body Mass	ml/min/g	0.44 ± 0.03	0.54 ± 0.06	0.56 ± 0.03 †	0.56 ± 0.00
EDL by Body Mass	ul/g	2.06 ± 0.07	2.48 ± 0.10 *	2.19 ± 0.10	2.38 ± 0.16
ESL by Body Mass	ul/g	1.10 ± 0.06	1.30 ± 0.06 *	0.97 ± 0.04	1.26 ± 0.13
SV by Body Mass	ul/g	0.96 ± 0.05	1.18 ± 0.08 *	1.22 ± 0.08 †	1.12 ± 0.04
CO by Heart Mass	ml/min/mg	0.14 ± 0.00	0.14 ± 0.01	0.16 ± 0.00	0.14 ± 0.00
EDL by Heart Mass	ul/mg	0.66 ± 0.00	0.64 ± 0.02	0.63 ± 0.02	0.62 ± 0.03
ESL by Heart Mass	ul/mg	0.35 ± 0.01	0.34 ± 0.01	0.28 ± 0.01 †	0.33 ± 0.03
SV by Heart Mass	ul/mg	0.30 ± 0.01	0.30 ± 0.01	0.35 ± 0.01 †	0.29 ± 0.01 *

Data obtained by *in vivo* cardiac cine-MRI of C3H and *Moonwalker* mice at 6 months of age.

Figures are in bold where significantly different from sex-matched wild-type (WT). Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

- * indicates measurements significantly different to sex-matched WT (P>0.05)
- ** indicates measurements highly significantly different to sex-matched WT (P>0.005)
- † indicates measurements significantly different to genotype-matched other sex (P>0.05)
- †† indicates measurements highly significantly different to genotype-matched other sex (P>0.005)

Table 4.4 Heart function in 9 month old *Moonwalker* mice

9 month old		Males		Females	
		Wild-type	<i>Moonwalker</i>	Wild-type	<i>Moonwalker</i>
Number of mice		7	7	8	7
Body Mass	g	42.6 ± 1.7	26.1 ± 0.8 **	33.4 ± 1.3 ††	26.2 ± 0.6 **
Heart Rate	bpm	455 ± 15	458 ± 10	449 ± 14	461 ± 13
LV					
Ejection Fraction	%	50.9 ± 1.8	48.9 ± 2.9	54.4 ± 1.5	51.0 ± 3.2
Cardiac Output	ml/min	18.5 ± 1.1	15.7 ± 1.9	16.7 ± 0.7	13.4 ± 1.0 *
Heart Mass as % BM	%	0.31 ± 0.01	0.39 ± 0.01 **	0.33 ± 0.02	0.36 ± 0.00
Muscle Volume	μl	125 ± 4	97 ± 4 **	106 ± 5 ††	90 ± 2 *
End Diastolic Lumen	μl	79.5 ± 2.5	69.7 ± 5.1	68.3 ± 1.0 ††	57.0 ± 1.1 ***†
End Systolic Lumen	μl	39.0 ± 1.9	35.5 ± 3.0	31.1 ± 1.2 ††	27.8 ± 1.5 †
Stroke Volume	μl	40.5 ± 1.9	34.1 ± 3.7	37.1 ± 1.0	29.2 ± 2.2 *
PER	μl/s	244 ± 31	243 ± 45	304 ± 15	266 ± 16
PFR	μl/s	380 ± 61	330 ± 64	415 ± 30	300 ± 34 *
E	μl/s	354 ± 53	288 ± 60	415 ± 30	248 ± 25 **
A	μl/s	238 ± 70	277 ± 67	279 ± 22	275 ± 32
E/A		1.93 ± 0.34	1.17 ± 0.21	1.52 ± 0.11	0.92 ± 0.01 **
CO by Body Mass	ml/min/g	0.43 ± 0.03	0.59 ± 0.05 *	0.51 ± 0.04	0.51 ± 0.03
EDL by Body Mass	ul/g	1.87 ± 0.07	2.64 ± 0.12 **	2.07 ± 0.11	2.17 ± 0.05 ††
ESL by Body Mass	ul/g	0.91 ± 0.04	1.35 ± 0.09 **	0.94 ± 0.06	1.06 ± 0.07 †
SV by Body Mass	ul/g	0.95 ± 0.05	1.29 ± 0.11 *	1.12 ± 0.06 †	1.11 ± 0.07
CO by Heart Mass	ml/min/mg	0.14 ± 0.00	0.15 ± 0.02	0.15 ± 0.00	0.14 ± 0.01
EDL by Heart Mass	ul/mg	0.60 ± 0.02	0.68 ± 0.04	0.61 ± 0.02	0.60 ± 0.01
ESL by Heart Mass	ul/mg	0.29 ± 0.01	0.34 ± 0.02	0.28 ± 0.01	0.29 ± 0.01
SV by Heart Mass	ul/mg	0.30 ± 0.00	0.33 ± 0.04	0.33 ± 0.01	0.30 ± 0.02

Data obtained by *in vivo* cardiac cine-MRI of C3H and *Moonwalker* mice at 9 months of age.

Figures are in bold where significantly different from sex-matched wild-type (WT). Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

- * indicates measurements significantly different to sex-matched WT (P>0.05)
- ** indicates measurements highly significantly different to sex-matched WT (P>0.005)
- † indicates measurements significantly different to genotype-matched other sex (P>0.05)
- †† indicates measurements highly significantly different to genotype-matched other sex (P>0.005)

Table 4.5 Heart function in 12 month old *Moonwalker* mice

12 month old		Males		Females	
		Wild-type	<i>Moonwalker</i>	Wild-type	<i>Moonwalker</i>
Number of mice		8	9	7	7
Body Mass	g	42.6 ± 1.4	28.9 ± 0.7 **	35.1 ± 1.2 ††	27.8 ± 0.4 **
Heart Rate	bpm	431 ± 11	425 ± 15	447 ± 14	423 ± 21
LV					
Ejection Fraction	%	51.9 ± 1.6	49.5 ± 1.4	58.3 ± 3.4	52.5 ± 1.1
Cardiac Output	ml/min	18.3 ± 0.5	14.8 ± 0.7 **	17.9 ± 0.8	12.7 ± 0.7 **
Heart Mass as % BM	%	0.34 ± 0.01	0.40 ± 0.01 **	0.33 ± 0.01	0.37 ± 0.01
Muscle Volume	μl	138 ± 5	111 ± 4 **	113 ± 6 ††	99 ± 2 *†
End Diastolic Lumen	μl	82.4 ± 3.0	70.6 ± 3.0 **	69.3 ± 2.2 ††	57.0 ± 1.1 ***††
End Systolic Lumen	μl	39.8 ± 2.4	35.8 ± 2.2	29.0 ± 2.9 †	27.0 ± 0.8 ††
Stroke Volume	μl	42.6 ± 1.4	34.8 ± 1.2 **	40.2 ± 2.1	29.9 ± 0.9 ***††
PER	μl/s	293 ± 28	265 ± 15	311 ± 30	209 ± 19 *†
PFR	μl/s	476 ± 60	485 ± 41	407 ± 32	399 ± 52
E	μl/s	419 ± 44	370 ± 41	396 ± 35	376 ± 18
A	μl/s	351 ± 69	398 ± 51	261 ± 41	309 ± 73
E/A		1.43 ± 0.21	1.08 ± 0.22	1.73 ± 0.23	1.55 ± 0.31
CO by Body Mass	ml/min/g	0.43 ± 0.01	0.51 ± 0.02 *	0.51 ± 0.01 †	0.45 ± 0.02
EDL by Body Mass	ul/g	1.94 ± 0.09	2.44 ± 0.09 **	1.98 ± 0.08	2.04 ± 0.04 ††
ESL by Body Mass	ul/g	0.93 ± 0.06	1.24 ± 0.07 *	0.83 ± 0.09	0.97 ± 0.02 ††
SV by Body Mass	ul/g	1.00 ± 0.04	1.20 ± 0.04 **	1.14 ± 0.04 †	1.07 ± 0.03 †
CO by Heart Mass	ml/min/mg	0.12 ± 0.00	0.12 ± 0.00	0.15 ± 0.00 †	0.12 ± 0.00 *
EDL by Heart Mass	ul/mg	0.56 ± 0.01	0.60 ± 0.01 *	0.58 ± 0.03	0.54 ± 0.01 †
ESL by Heart Mass	ul/mg	0.27 ± 0.00	0.30 ± 0.01 *	0.24 ± 0.02	0.26 ± 0.00 †
SV by Heart Mass	ul/mg	0.29 ± 0.01	0.29 ± 0.00	0.34 ± 0.02	0.28 ± 0.01

Data obtained by *in vivo* cardiac cine-MRI of C3H and *Moonwalker* mice at 12 months of age.

Figures are in bold where significantly different from sex-matched wild-type (WT). Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

- * indicates measurements significantly different to sex-matched WT (P>0.05)
- ** indicates measurements highly significantly different to sex-matched WT (P>0.005)
- † indicates measurements significantly different to genotype-matched other sex (P>0.05)
- †† indicates measurements highly significantly different to genotype-matched other sex (P>0.005)

Table 4.6 Heart function in 18 month old *Moonwalker* mice

18 month old		Males		Females	
		Wild-type	<i>Moonwalker</i>	Wild-type	<i>Moonwalker</i>
Number of mice		6	6	5	5
Body Mass	g	37.3 ± 1.0	27.5 ± 1.8 **	30.2 ± 1.3	27.8 ± 0.5
Heart Rate	bpm	427 ± 15	400 ± 7	417 ± 19	399 ± 7
LV					
Ejection Fraction	%	49.5 ± 2.6	50.9 ± 4.2	50.6 ± 4.6	54.1 ± 1.8
Cardiac Output	ml/min	20.6 ± 1.1	13.6 ± 0.9 **	15.4 ± 1.0	13.4 ± 0.4
Heart Mass as % BM	%	0.47 ± 0.04	0.43 ± 0.02	0.42 ± 0.01	0.37 ± 0.02
Muscle Volume	μl	166 ± 12	113 ± 8 **	122 ± 7	98 ± 4 *
End Diastolic Lumen	μl	100 ± 10	68.2 ± 5.6 *	74.7 ± 6.1	62.1 ± 1.9
End Systolic Lumen	μl	51.5 ± 8.0	34.2 ± 5.2	37.5 ± 5.9	28.5 ± 1.8
Stroke Volume	μl	48.6 ± 3.0	33.9 ± 1.8 **	37.1 ± 2.1	33.5 ± 1.0
PER	μl/s	337 ± 25	366 ± 58	300 ± 24	269 ± 21
PFR	μl/s	438 ± 52	316 ± 38	425 ± 12	238 ± 15
E	μl/s	370 ± 65	266 ± 11		238 ± 15
A	μl/s	406 ± 38	301 ± 44		152 ± 21
E/A		1.00 ± 0.23	0.94 ± 0.12		1.59 ± 0.11
CO by Body Mass	ml/min/g	0.55 ± 0.04	0.50 ± 0.04	0.59 ± 0.04	0.62 ± 0.02
EDL by Body Mass	ul/g	2.70 ± 0.33	2.49 ± 0.16	2.38 ± 0.12	2.29 ± 0.12
ESL by Body Mass	ul/g	1.38 ± 0.23	1.22 ± 0.15	1.09 ± 0.14	0.99 ± 0.05
SV by Body Mass	ul/g	1.31 ± 0.11	1.26 ± 0.11	1.29 ± 0.08	1.29 ± 0.08
RV					
Ejection Fraction	%	52.2 ± 1.7	56.9 ± 4.6	53.0 ± 4.8	58.2 ± 2.8
Cardiac Output	ml/min	18.1 ± 3.8	17.2 ± 0.9	14.7 ± 1.2	14.3 ± 1.8
End Diastolic Lumen	μl	95.7 ± 4.0	77.2 ± 5.4 *	69.2 ± 7.6	61.2 ± 6.9
End Systolic Lumen	μl	45.8 ± 3.1	34.0 ± 5.9	33.3 ± 6.4	25.4 ± 3.2
Stroke Volume	μl	49.8 ± 1.7	43.1 ± 2.2 *	35.8 ± 2.8	35.7 ± 4.4
CO by Body Mass	ml/min/g	0.49 ± 0.10	0.64 ± 0.05	0.59 ± 0.04	0.62 ± 0.02
EDL by Body Mass	ul/g	2.57 ± 0.12	2.82 ± 0.16	2.38 ± 0.12	2.29 ± 0.12 †
ESL by Body Mass	ul/g	1.23 ± 0.09	1.22 ± 0.17	1.09 ± 0.14	0.99 ± 0.05
SV by Body Mass	ul/g	1.33 ± 0.04	1.60 ± 0.16	1.29 ± 0.08	1.29 ± 0.08
LV					
CO by Heart Mass	ml/min/mg	0.12 ± 0.00	0.11 ± 0.00	0.12 ± 0.00	0.13 ± 0.00
EDL by Heart Mass	ul/mg	0.57 ± 0.02	0.56 ± 0.02	0.58 ± 0.02	0.60 ± 0.01
ESL by Heart Mass	ul/mg	0.29 ± 0.02	0.28 ± 0.03	0.28 ± 0.03	0.27 ± 0.01
SV by Heart Mass	ul/mg	0.28 ± 0.00	0.28 ± 0.02	0.29 ± 0.02	0.32 ± 0.01
RV					
CO by Heart Mass	ml/min/mg	0.10 ± 0.02	0.14 ± 0.01	0.11 ± 0.00	0.13 ± 0.01
EDL by Heart Mass	ul/mg	0.55 ± 0.03	0.65 ± 0.06	0.53 ± 0.05	0.59 ± 0.05
ESL by Heart Mass	ul/mg	0.26 ± 0.01	0.28 ± 0.05	0.25 ± 0.05	0.24 ± 0.02
SV by Heart Mass	ul/mg	0.29 ± 0.02	0.37 ± 0.03	0.28 ± 0.02	0.34 ± 0.03

Data obtained by *in vivo* cardiac cine-MRI of C3H and *Moonwalker* mice at 18 months of age.

Figures are in bold where significantly different from sex-matched wild-type (WT).

Where:

* indicates measurements significantly different to sex-matched WT (P>0.05)

** indicates measurements highly significantly different to sex-matched WT (P>0.005)

† indicates measurements significantly different to genotype-matched other sex (P>0.05)

Table 4.7 Heart function in mass-matched *Moonwalker* mice

		Males		Females	
		3 month old	12 month old	3 month old	6 month old
		Wild-type	<i>Moonwalker</i>	Wild-type	<i>Moonwalker</i>
Number of mice		8	9	8	4
Body Mass	g	31.8 ± 0.9	28.9 ± 0.7 *	24.8 ± 0.6	26 ± 0
Heart Rate	bpm	448 ± 11	425 ± 15	457 ± 13	482 ± 17
LV					
Ejection Fraction	%	51.8 ± 2.5	49.5 ± 1.4	54.7 ± 3.8	47.3 ± 2.2
Cardiac Output	ml/min	16.3 ± 0.8	14.8 ± 0.7	14.7 ± 1.2	14.6 ± 0.1
Heart Mass as % BM	%	0.36 ± 0.01	0.40 ± 0.01 *	0.37 ± 0.01	0.37 ± 0.01
Muscle Volume	μl	110 ± 5	111 ± 4	89 ± 2	93 ± 3
End Diastolic Lumen	μl	70.8 ± 3.0	70.6 ± 3.0	58.9 ± 1.8	61.9 ± 4.1
End Systolic Lumen	μl	34.3 ± 3.0	35.8 ± 2.2	26.8 ± 2.8	32.7 ± 3.5
Stroke Volume	μl	36.4 ± 1.3	34.8 ± 1.2	32.0 ± 1.8	29.1 ± 1.0
PER	μl/s		265 ± 15		250 ± 27
PFR	μl/s		485 ± 41		442 ± 10
E	μl/s		370 ± 41		414 ± 37
A	μl/s		398 ± 51		416 ± 17
E/A			1.08 ± 0.22		1.00 ± 0.10
CO by Body Mass	ml/min/g	0.51 ± 0.02	0.51 ± 0.02	0.59 ± 0.04	0.56 ± 0.00
EDL by Body Mass	ul/g	2.23 ± 0.10	2.44 ± 0.09	2.38 ± 0.12	2.38 ± 0.16
ESL by Body Mass	ul/g	1.08 ± 0.09	1.24 ± 0.07	1.09 ± 0.14	1.26 ± 0.13
SV by Body Mass	ul/g	1.14 ± 0.05	1.20 ± 0.04	1.29 ± 0.08	1.12 ± 0.04
CO by Heart Mass	ml/min/mg	0.14 ± 0.00	0.12 ± 0.00	0.15 ± 0.00	0.14 ± 0.00
EDL by Heart Mass	ul/mg	0.61 ± 0.01	0.60 ± 0.01	0.63 ± 0.02	0.62 ± 0.03
ESL by Heart Mass	ul/mg	0.29 ± 0.02	0.30 ± 0.01	0.29 ± 0.03	0.33 ± 0.03
SV by Heart Mass	ul/mg	0.31 ± 0.01	0.29 ± 0.00	0.34 ± 0.01	0.29 ± 0.01 *

Data obtained by *in vivo* cardiac cine-MRI of C3H and *Moonwalker* mice. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

* indicates measurements significantly different to sex-matched WT (P>0.05)

** indicates measurements highly significantly different to sex-matched WT (P>0.005)

12 month old	Wild-type	Moonwalker
	Females	
N	7	7
LV	Pre-DB	Post-DB
Heart Rate	447 ± 14	515 ± 22
Ejection Fraction	50.1 ± 2.9	72.0 ± 3.1
Cardiac Output	8.32 ± 0.3	10.9 ± 1.0
End Diastolic Lumen	39.4 ± 1.3	29.0 ± 1.9
End Systolic Lumen	19.7 ± 1.4	8.20 ± 1.1
Stroke Volume	19.7 ± 1.0	20.8 ± 1.4
PER	306 ± 35	380 ± 61
PFR	526 ± 66	452 ± 95
E	446 ± 53	327 ± 26
A	371 ± 90	401 ± 13
E/A	1.54 ± 0.33	1.26 ± 0.33
	Changes	Post-DB
	68 ± 22 *	423 ± 21
	21.8 ± 6.3 **	47.4 ± 2.4
	2.61 ± 2.0 *	6.94 ± 0.4
	-10.1 ± 2.3 **	34.3 ± 1.2
	-11.3 ± 1.8 **	18.1 ± 1.2
	1.15 ± 3.2	16.1 ± 0.6
	52 ± 81	265 ± 16
	-53 ± 11	485 ± 44
	-13 ± 10	370 ± 44
	-35 ± 21	398 ± 54
	-0.5 ± 0.40	1.08 ± 0.21
		1.13 ± 0.11
		0.04 ± 0.20
	Changes	Post-DB
	51 ± 16 *	474 ± 16
	17.1 ± 2.7 **	64.6 ± 1.3
	1.84 ± 0.5 *	8.79 ± 0.5
	-6.8 ± 1.2 **	27.4 ± 0.6
	-8.4 ± 1.4 **	9.74 ± 0.5
	1.53 ± 0.7 *	17.7 ± 0.3
	20 ± 10	286 ± 20
	-17 ± 66 **	308 ± 22
	-10 ± 47 *	264 ± 25
	-14 ± 79 *	251 ± 21
		1.13 ± 0.11
		0.04 ± 0.20

Data obtained by *in vivo* cardiac cine-MRI of C3H and Moonwalker mice at 12 months of age. For each genotype the columns show the value before dobutamine injection, after dobutamine injection and the difference between the two. The difference (the Changes column) is in black if positive and red if negative. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

- * indicates measurements significantly different after dobutamine treatment ($P > 0.05$)
- ** indicates measurements highly significantly different after dobutamine treatment ($P > 0.005$)

Chapter 5: Heart function in the *Fiona* mouse

5.1 Introduction

This study measured the heart function of the *Fiona* mouse to look at the mechanisms of early cardiac dysfunction in the *mdx* mouse and test the hypothesis that upregulation of utrophin in the diaphragm would be sufficient to prevent the onset of RV dysfunction. The *Fiona* mouse is ideal for determining this as it has completely restored diaphragm function but an untreated heart, as such any improvement in heart function compared to the *mdx* mouse would have to come from the improvement in diaphragm function.

The *Fiona* mouse is a transgenic *mdx* mouse where utrophin is expressed, in a skeletal muscle specific manner, under the control of the human skeletal actin (HSA) promoter (Tinsley *et al.*, 1998). Utrophin is highly homologous to dystrophin, with particularly high homology at the N terminus (85%) and C terminus (83%) but low in the central rod domain (30%) (Love *et al.*, 1989; Tinsley *et al.*, 1992). Due to the high homology at the termini utrophin binds to many of the same proteins as dystrophin, and can replace dystrophin in connecting the DAPC to the actin cytoskeleton (Figure 5.1) (Matsumura *et al.*, 1992; Pons *et al.*, 1994). Unlike dystrophin it is ubiquitously expressed throughout the body at high levels during embryonic development but expression drops in adult skeletal muscle and become limited to the neuromuscular and myotendinous junctions (NMJ and MTJ) (Clerk *et al.*, 1993; Khurana *et al.*, 1991; Ohlendieck *et al.*, 1991a), though it remains expressed in other organs.

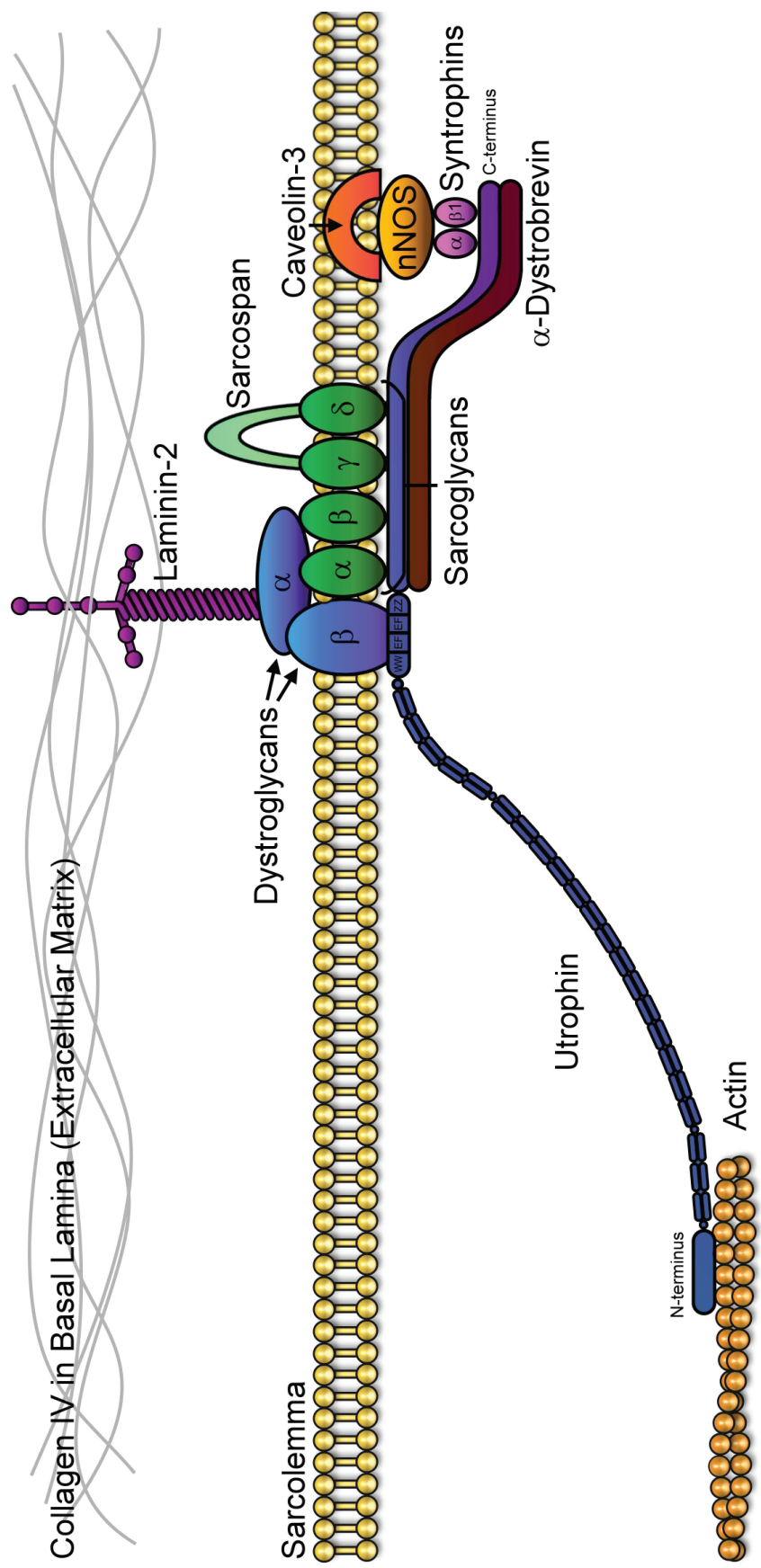
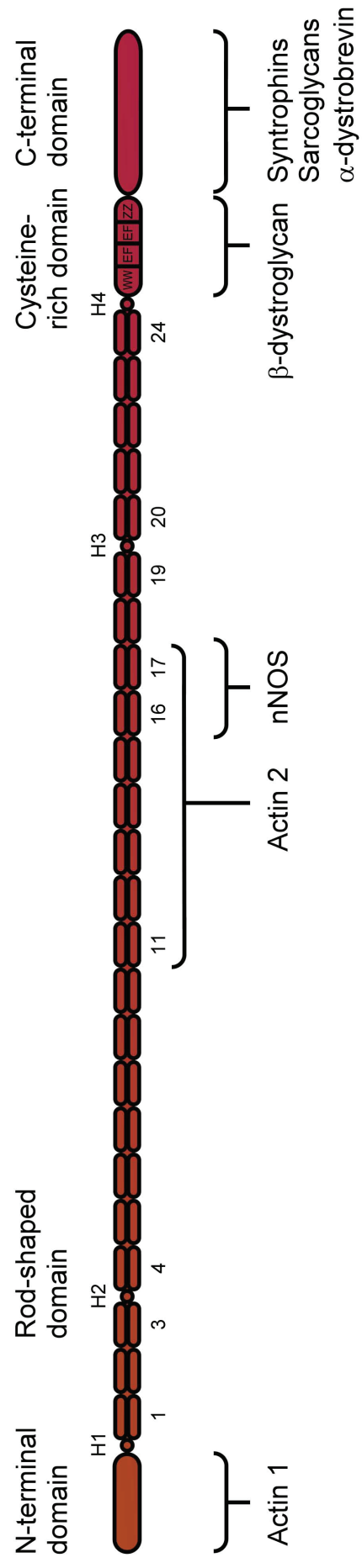


Figure 5.1 Utrophin and the DAPC. The DAPC is normally comprised of dystrophin and a number of peripheral and integral membrane proteins; and provides a structural link from the actin cytoskeleton (via dystrophin) to the extracellular matrix (via laminin). Dystrophin may be substituted for by utrophin, which has high sequence similarity at both termini and most of the same binding partners.

Utrophin is a much smaller gene than *dystrophin*, only 900 kb long (versus 2.4 Mb), though with a similar number of exons – 74 (Li *et al.*, 2007). It has two isoforms, utrophin A and B, with A primarily expressed in mature skeletal muscle and B primarily expressed in endothelial cells (Burton *et al.*, 1999; Chakkalakal *et al.*, 2003). Subsequently, unless otherwise stated, utrophin should be taken to mean utrophin A. As implied above, utrophin has the same structural domains as dystrophin, but is slightly smaller than dystrophin, at 395 kD, as it lacks two of the repeats in the central rod domain (Tinsley *et al.*, 1992) (Figure 5.2). While the homology between dystrophin and utrophin is very low in the central rod domain, this has been shown to be relatively unimportant, as only 4 of the 24 repeats found in dystrophin are necessary to largely retain function (Harper *et al.*, 2002). *Utrophin knockout* mice are viable and display very little phenotype apart from a small NMJ defect (Grady *et al.*, 1997a), suggesting utrophin is unnecessary in healthy mice. There are no known mutations in *utrophin* linked to muscular dystrophies or cardiomyopathies (Hilton-Jones and Squier, 1993) but mutations resulting in a truncated, inactive *utrophin* have been seen in a number of cancers, where it has also been found to be frequently downregulated (Li *et al.*, 2007). Overexpression of a wild-type utrophin in breast cancer cells has been shown to suppress tumour growth (Li *et al.*, 2007), suggesting it is a good candidate gene for tumour suppression.

In DMD patients (Nguyen *et al.*, 1991) utrophin is expressed at elevated levels at the sarcolemma, NMJ and MTJ, i.e. where dystrophin would normally be expressed. The same is seen in the *mdx* mouse model of DMD, where utrophin is also expressed at high levels in regenerating fibres (Khurana *et al.*, 1991; Law *et al.*, 1994). Studies have shown that utrophin's expression increases with age (Taylor *et al.*, 1997), however it does not reach high enough levels to rescue the heart (Fanin *et al.*, 1999) even in 21 month old *mdx* mice

Dystrophin 427 kD 3,685 amino acids



Utrophin 395 kD 3,433 amino acids

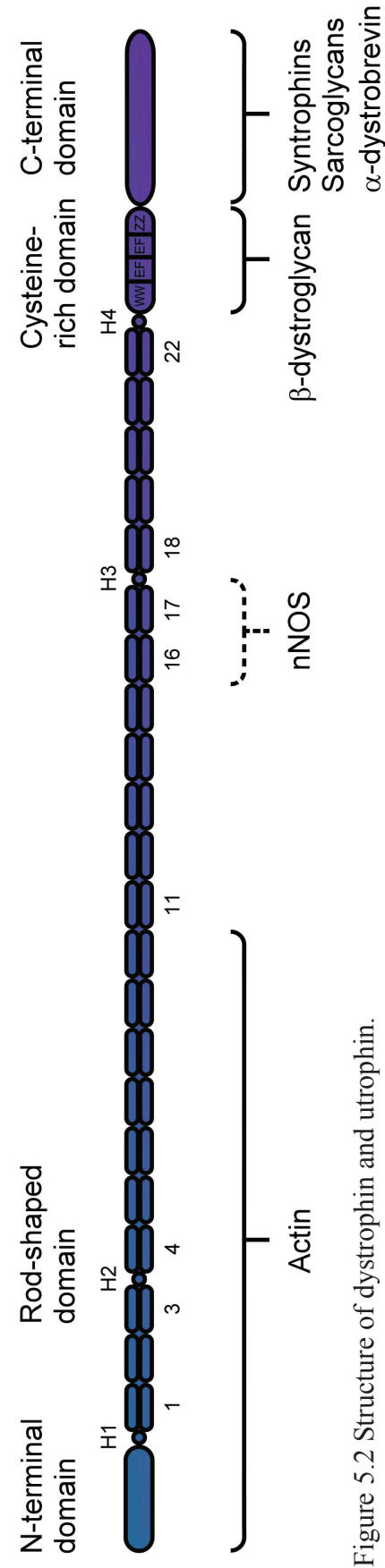


Figure 5.2 Structure of dystrophin and utrophin. Structure of dystrophin and utrophin proteins, showing domain structure and location of protein binding sites. H signifies dystrophin/utrophin hinge region. Possible nNOS binding site is signified by dotted line.

(Bostick *et al.*, 2008). This upregulation happens on a myocyte by myocyte basis, as can be seen in a transgenic *mdx* mouse with mosaic dystrophin expression (Bostick *et al.*, 2008). In dystrophin-null areas, utrophin was upregulated, while in dystrophin-positive areas it was not (Bostick *et al.*, 2008). These changes in utrophin expression are related to the differences in fibre type seen in DMD and dystrophic mice. There are three types of muscle fibre: Type I (or slow oxidative), Type IIa (or fast oxidative) and Type IIb (or fast glycolytic), which vary in the form of myosin heavy chain (MHC) they express. Muscles are a mix of the three types, the proportion depending on the function of the muscle. In *mdx* mice there is a progressive increase in the proportion of type I fibres, with twice as many type I fibres found in the *mdx* soleus at 7 months than in the wild-type (Anderson *et al.*, 1988b; Carnwath and Shotton, 1987). Similarly, *mdx* extensor digitorum longus shows a change from Type IIb to type IIa fibres (Anderson *et al.*, 1988b). This change is not due to preferential degeneration of certain fibre types (Geissinger *et al.*, 1990; Pastoret and Sebillé, 1993). There is also an increase in the expression of intermediate fibre types, co-expressing more than one MHC, providing further evidence of a fibre-type switch (Pastoret and Sebillé, 1993).

Calcium signalling via the Ca^{2+} /calmodulin-dependent phosphatase calcineurin and the Ca^{2+} /calmodulin-dependent kinases CaMKI and CaMKII directs this switch, in response to sustained changes in intracellular Ca^{2+} concentration (this pathway is also involved in cardiac hypertrophy (Molkentin *et al.*, 1998)). The intracellular Ca^{2+} concentration is raised in *mdx* mice as a consequence of muscle degeneration (see Chapter 1). Calcineurin activates NFAT (nuclear factor of activated T cells) (Liu *et al.*, 2001) and MEF2 (myocyte-specific enhancer factor 2) pathways, which result in the activation of downstream genes that encode for slower, more oxidative-type muscle proteins

(Chakkalakal *et al.*, 2003; Oh *et al.*, 2005). One of the activated proteins is utrophin, which as such is expressed more highly in type I muscle fibres (Chakkalakal *et al.*, 2003). Calcineurin/NFAT dependant fibre-type switch and utrophin expression has been shown to attenuate dystrophic pathology when upregulated (Chakkalakal *et al.*, 2004), and to exacerbate the phenotype when inhibited (Chakkalakal *et al.*, 2006). As a negative feedback mechanism, utrophin mRNA is less stable in the presence of type II muscle fibre proteins (Chakkalakal *et al.*, 2008). The exact cause and effect of this switch is uncertain - whether the purpose of the fibre-type switch is to increase utrophin expression or if the fibre-type switch has some other purpose, possibly related to metabolic changes, is uncertain. This fibre type switch is seen in DMD patients (Marini *et al.*, 1991), but also, to a greater extent than seen in the *mdx* mouse, in the utrophin-deficient *dKO* mouse (Deconinck *et al.*, 1997a; Rafael *et al.*, 2000). This may suggest another role for the switch or simply be pathological. Restoration of a mechanically non-functional dystrophin (lacking the cysteine-rich domain it cannot restore the dystro- or sarcoglycans) in the *dKO* mouse restored the fibre type ratios to *mdx* levels (Rafael *et al.*, 2000), possibly by stabilising the membrane and hence reducing intracellular Ca^{2+} concentration in a non-DAPC-dependent manner. The switch in fibre type is a glycolytic to oxidative switch, which is believed to cause muscle fatigue and the very high levels of fibre type switching seen in the *dKO* mouse may contribute to its very short lifespan (8-12 weeks).

Despite their similarities utrophin and dystrophin have different binding properties for a number of proteins - β -dystroglycan, the syntrophins, nNOS and actin. Utrophin shows a two-fold lower affinity for β -dystroglycan (Ishikawa-Sakurai *et al.*, 2004), but it remains capable of binding it at physiological conditions. While dystrophin primarily interacts with α - and β 1-syntrophin, utrophin primarily interacts with β 1- and β 2-syntrophin, however

utrophin has been shown to be able to recruit α -syntrophin when highly expressed (Peters *et al.*, 1997). nNOS binds to the repeat segments in the central rod domain of dystrophin (Lai *et al.*, 2009), while these particular repeats are conserved in utrophin their sequence is not and utrophin may not bind nNOS, and does not restore its localisation to the sarcolemma in *mdx* mice (Li *et al.*, 2010) (Figure 5.2). This is unrelated to the differential binding of the syntrophins, which bind nNOS. Dystrophin binds actin via two different sites (Amann *et al.*, 1998; Levine *et al.*, 1990; Rybakova *et al.*, 1996) but utrophin interacts with actin through only a single site (Amann *et al.*, 1999; Moores and Kendrick-Jones, 2000) (Figure 5.2). This site is much larger than the first site in dystrophin, involving the first 10 repeats in the rod domain, as well as the N-terminus (Rybakova and Ervasti, 2005) (N-terminus alone in dystrophin (Levine *et al.*, 1990)) and their binding sites on actin also vary (Rybakova *et al.*, 2006). This results in a lower stoichiometry for actin binding, reduced from 24:1 (actin:dystrophin) (Rybakova *et al.*, 1996) to 14:1 (actin:utrophin) (Rybakova *et al.*, 2002).

It is suspected that the normal role of utrophin in the heart is to maintain intracellular associations but, in the dystrophin-null heart, its upregulation is a natural functional compensation for the loss of dystrophin. Utrophin has been shown to be able to functionally compensate for dystrophin loss when upregulated in the *mdx* mouse (Fisher *et al.*, 2001; Krag *et al.*, 2004; Mattei *et al.*, 2007; Squire *et al.*, 2002; Tinsley *et al.*, 1998; Tinsley *et al.*, 1996), *dKO* mouse (Deconinck *et al.*, 1997b; Wakefield *et al.*, 2000) and dystrophic dog (Cerletti *et al.*, 2003).

Initial studies used a truncated utrophin transgene, missing repeats 4-17 of the central rod domain (everything between H2 and H3) (Tinsley *et al.*, 1996) (Figure 5.2), under the

control of the HSA promoter; as a similarly truncated dystrophin had been previously shown to be functional (Dunckley *et al.*, 1993; Phelps *et al.*, 1995; Wells *et al.*, 1992; Wells *et al.*, 1995). While this eliminates part of the actin binding region, mice showed improvement with a reduction in serum CK levels and central nucleation. Immunostaining showed utrophin was found at the sarcolemma, where it relocalised the components of the DAPC. Subsequent studies showed that the use of a full-length utrophin could restore mice to wild-type condition (Squire *et al.*, 2002; Tinsley *et al.*, 1998), as could upregulation of endogenous utrophin by a natural (Krag *et al.*, 2004) or artificial transcription factor (Mattei *et al.*, 2007). However, as would be expected, restoration was dose-dependent; the less utrophin present, the closer the condition of the mouse was to *mdx*.

In the *Fiona* mouse utrophin is upregulated to 3-4 times normal levels in the hind limb muscles and approximately twice the normal level in the diaphragm, but not upregulated in the heart (Tinsley *et al.*, 1998). It shows restored localization of DAPC components, a reduction in the numbers of centrally nucleated fibers and normalized force generation in both hind limb muscles and diaphragm (Tinsley *et al.*, 1998). This suggests that 3-4 fold upregulation of utrophin is sufficient to rescue the *mdx* phenotype. This level has been shown to be non-toxic in non-muscle tissue, which would allow non-targeted utrophin upregulation (Fisher *et al.*, 2001). Previous studies have shown that the *mdx* mouse has severely affected diaphragm at 6 months (Stedman *et al.*, 1991) and LV dysfunction by 11 months (Quinlan *et al.*, 2004). It has been shown that there is RV dysfunction preceding the LV dysfunction, with a significant reduction in RV ejection fraction apparent at 8 months (Zhang *et al.*, 2008b). It was hypothesized that this RV dysfunction is due to the severely affected diaphragm and not the heart itself and therefore that it would be possible to improve cardiac function by improving diaphragm function, without directly restoring

or compensating for dystrophin loss in the heart. As the *Fiona* mouse has completely restored diaphragm function but an untreated heart study of its heart function should prove or disprove this hypothesis. The proposed mechanism is that severe respiratory muscle weakness (especially that of the diaphragm) causes hypoventilation, in turn causing hypoxemia. This leads to pulmonary arterial hypertension, which causes increased afterload on the right ventricle, leading to a reduced RV stroke volume (due to Starling's law of the heart). Over time this change is pathological and leads in turn to RV dysfunction.

A longitudinal time course of cine MRIs were performed on the same *C57/Bl10, mdx* and *Fiona* mice at 6 and 9 months of age (as described in Chapter 2). At the end of the final scan these mice were killed and their hearts, diaphragms and quadriceps removed. Half of the hearts were imbedded in OCT on liquid nitrogen-cooled isopentane and used for histological sections, while the other half, and the diaphragms and quadriceps were snap-frozen in liquid nitrogen and used for western blotting. Both Haematoxylin and Eosin (H&E) and Sirius red stains were used for histology. The antibodies used for western blotting were anti-utrophin and anti- α -actinin.

5.2 Methods

5.2.1 Animals

Six-month old male *Fiona*, *mdx* and *C57/Bl10* mice were used for the longitudinal mouse heart function experiments (n = 9 in each group). These mice were killed and their tissues used for western blots and histology. The *Fiona* mouse was derived in-house as described in Tinsley *et al* (Tinsley *et al.*, 1998). The cDNA for *Utrophin* was cloned into a vector containing the human skeletal α -actin promoter. This was then injected into the nucleus of *C57/Bl6* mice oocytes. This process generated a number of transgenic lines, of which the *Fiona* line was one.

5.2.2 Western Blot

The procedure was as for anti-utrophin antibody in Chapter 3 (as described in section 3.2.2). The intensity of the bands obtained from *Fiona*, *mdx* and *C57/Bl10* muscles was measured by Image J software; the quantification is based on band intensity and area.

5.3 Results

5.3.1 Histology

Sections from 9-month-old *Fiona*, *mdx* and *C57/Bl10* hearts were stained with Haematoxylin and Eosin (H&E), to look at general morphology and infiltrating inflammatory cells (Figure 5.3), and Sirius red, to show cardiac fibrosis (Figure 5.4). H&E staining of *mdx* mice hearts showed infiltrates mostly in the RV, with occasional infiltrates in the LV. Staining of *Fiona* mice hearts showed extensive infiltrates throughout both the LV and RV. Wild-type hearts had no infiltrates. Sirius red staining of *mdx* mice hearts showed focal fibrotic lesions largely around the RV, but with occasional lesions in LV muscle. Staining of *Fiona* mice hearts showed extensive lesions in both LV and RV muscle. Wild-type hearts showed no lesions. From this one would expect to find RV dysfunction in the *mdx* mouse, as has been previously reported (Zhang *et al.*, 2008b), and both LV and RV dysfunction in the *Fiona* mouse.

5.3.2 Western Blot

In the *Fiona* mouse utrophin is upregulated to approximately twice the normal level in the diaphragm resulting in normalized force generation (Tinsley *et al.*, 1998). To confirm that the same levels of upregulation are still seen 9-month old *Fiona*, *mdx* and *C57/Bl10* mice were sacrificed. Western blot analysis of the quadriceps, diaphragm and heart found similar levels of upregulation in the quadriceps and diaphragm but no upregulation in the heart (Figure 5.5a). By proxy one can say that there is still full restoration of diaphragm function.

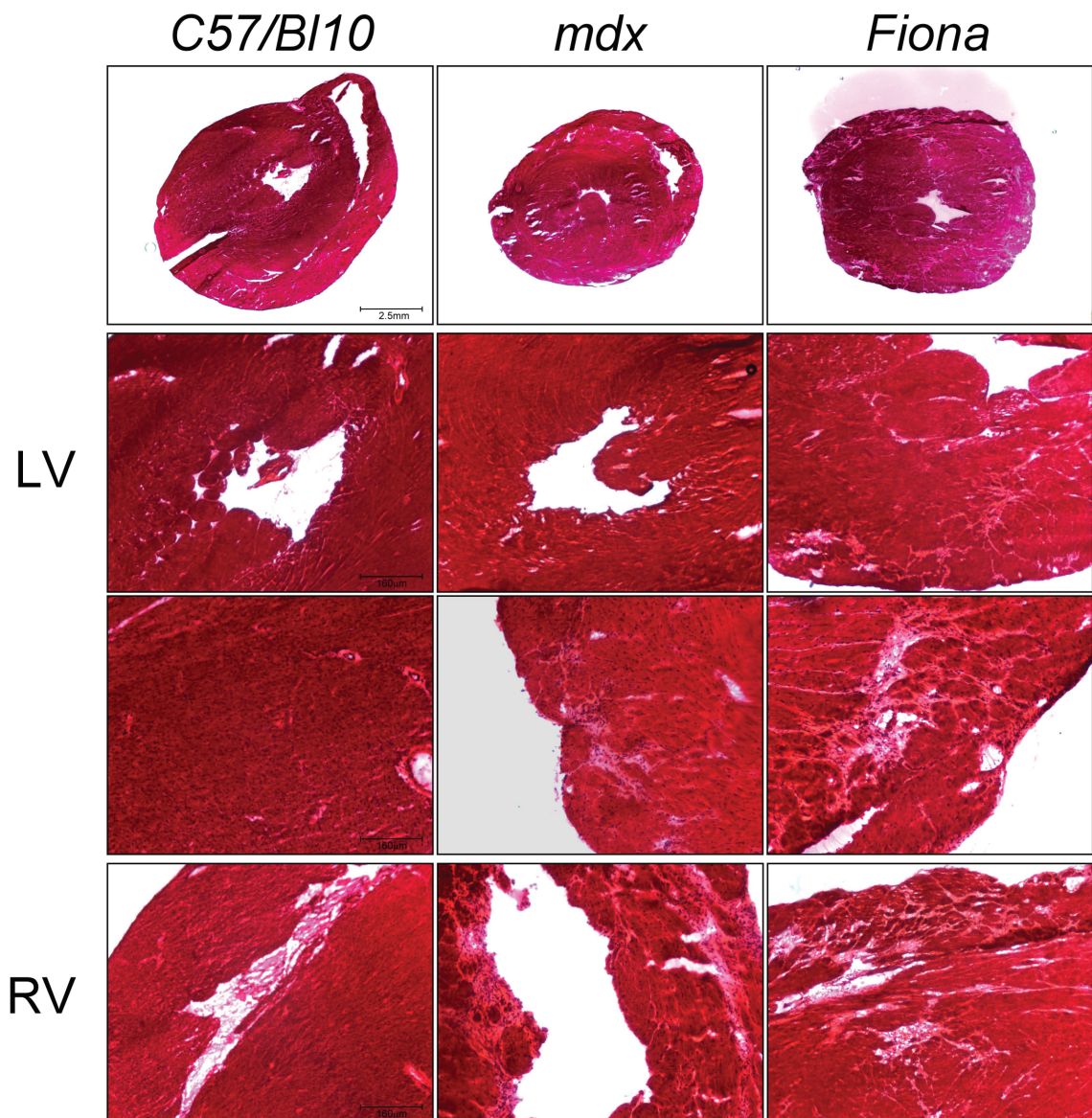


Figure 5.3 H&E staining.

H&E staining of sections from 9 month old *C57/Bl10*, *mdx* and *Fiona* mice hearts. *Mdx* mice hearts show infiltrates mostly in the RV, with occasional infiltrates in the LV. *Fiona* mice hearts show extensive infiltrates throughout both the LV and RV.

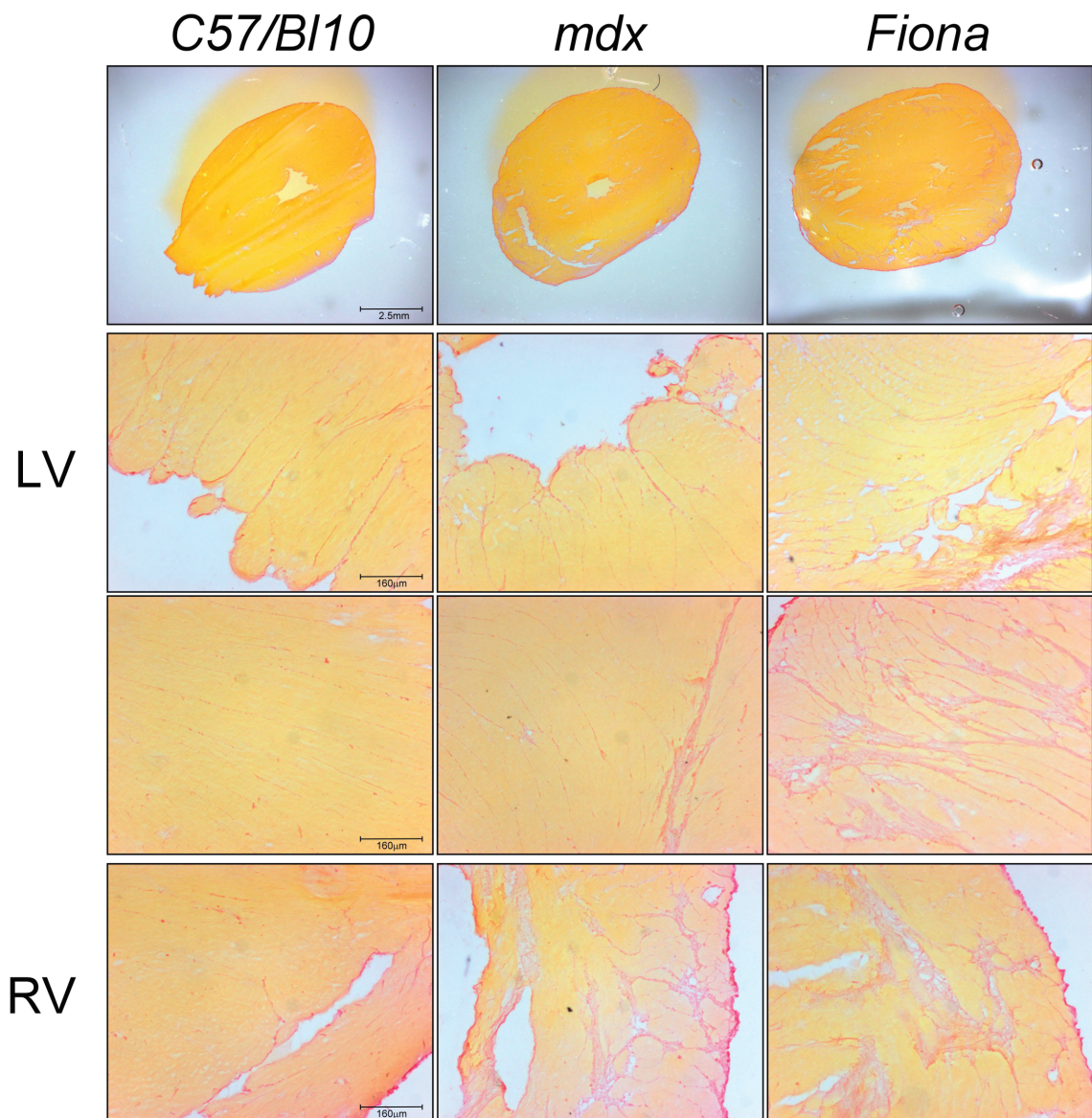


Figure 5.4 Sirius Red staining

Sirius red staining of sections from 9 month old *C57/Bl10*, *mdx* and *Fiona* mice hearts. *Mdx* mice hearts show fibrosis mostly in the RV, with occasional fibrosis in the LV. *Fiona* mice hearts show extensive fibrosis throughout both the LV and RV.

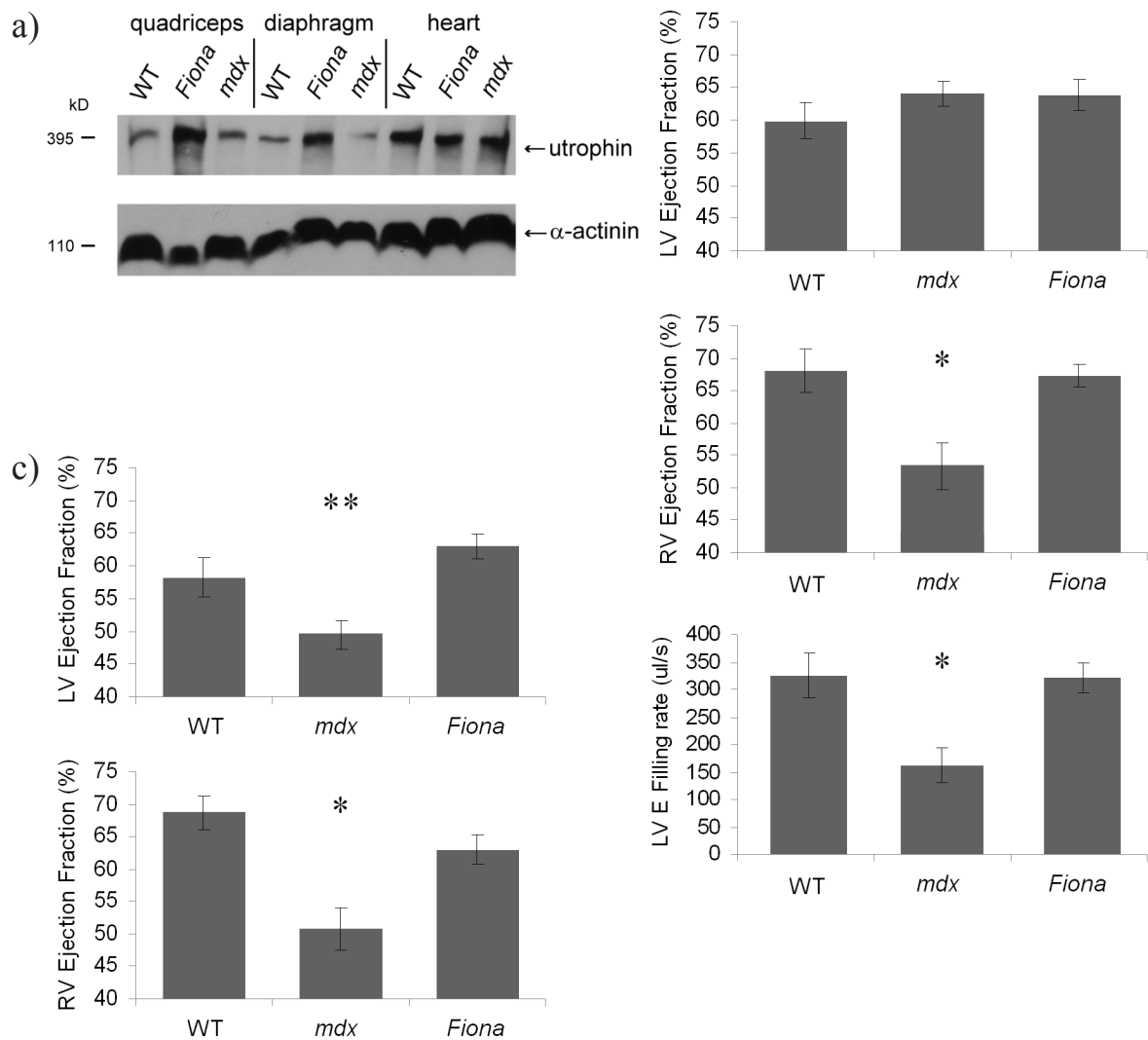


Figure 5.5 Utrophin upregulation and cardiac function improvement in *Fiona* mice.

(a) Western blot for utrophin expression in heart, diaphragm and quadriceps of *Fiona*, *mdx* and *C57/Bl10* mice. α -actinin was used as the loading control. (b) *In vivo* cardiac cine-MRI of *Fiona* mice at 6 months. At 6 months the *mdx* mouse had a significant decrease in RV ejection fraction and LV E peak filling rate compared with age-matched wild-type *C57/Bl10* mice ($P < 0.05$), but no difference in LV ejection fraction. In age-matched *Fiona* mice heart function was restored to normal levels. * indicates measurements significantly different to WT ($p < 0.05$). (c) *In vivo* cardiac cine-MRI of *Fiona* mice at 9 months. At 9 months the *mdx* mouse had a highly significant reduction in LV ejection fraction ($P < 0.005$) significant decrease in RV ejection fraction ($P < 0.05$) compared with age-matched *C57/Bl10* mice. In age-matched *Fiona* mice heart function was restored to normal levels. * indicates measurements significantly different to WT ($p < 0.05$) and ** indicates measurements highly significant different to WT ($p < 0.005$).

5.3.3 Measurement of heart function by cine-MRI

To determine if restoring normal function to the diaphragm was sufficient to correct heart function *Fiona* mice were imaged using cine-MRI and their cardiac function compared with age-matched *mdx* and *C57/Bl10* control mice at both 6 and 9 months (Table 5.1 and 5.2). At 6 months the *mdx* mouse has a significant reduction in RV ejection fraction, and LV peak ejection rate (PER) and E peak filling rate, compared to age-matched wild-type *C57/Bl10* mice. This mirrors the findings of Zhang *et al* (Zhang *et al.*, 2008b). In age-matched *Fiona* mice both the RV systolic deficit and the LV diastolic deficit were corrected and heart function was restored to near wild-type levels (Figure 5.5b). At 9 months the *mdx* mouse was found to have a significant reduction in LV and RV ejection fraction and LV PER compared to age-matched *C57/Bl10* mice. This was again corrected in the age-matched *Fiona* mouse (Figure 5.5c). These results showed that utrophin upregulation in the diaphragm, at a level that had previously been shown to be sufficient to restore full function (Tinsley *et al.*, 1998), is sufficient to restore normal cardiac function to the *mdx* mouse.

At 6 months the *Fiona* and *mdx* mice showed a significant reduction in LV end diastolic volume (EDL) compared to wild-type mice and the *Fiona* mice showed a significant reduction in RV EDL compared to both wild-type and *mdx* mice (Figure 5.6a). Similarly at 9 months the *Fiona* mouse showed a significant reduction in LV muscle volume, LV EDL and end systolic volume (ESL) and RV EDL compared to both wild-type and *mdx* mice (Figure 5.6b). It was hypothesised this was due to differences in diet between the *Fiona/mdx* mice, which were bred in-house, and the wild-type controls, which were

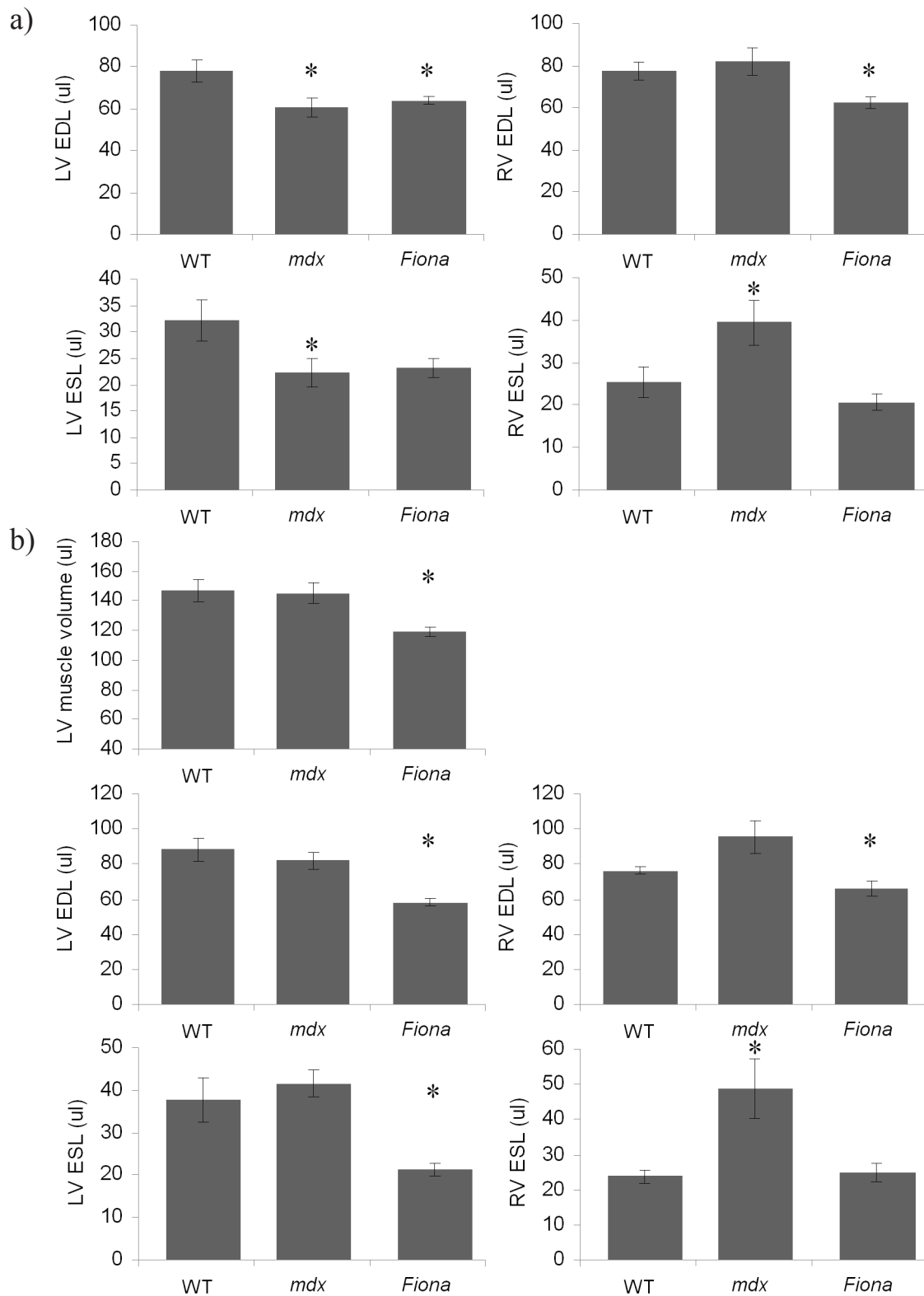


Figure 5.6 Differences in heart size in *Fiona*, *mdx* and wild-type mice.

(a) *In vivo* cardiac cine-MRI of *Fiona* mice at 6 months. At 6 months the *mdx* mouse has a significantly smaller LV EDL and ESL and significantly larger RV ESL compared with age-matched wild-type *C57/B110* mice ($P < 0.05$). The *Fiona* mouse has a significantly smaller LV and RV EDL compared with age-matched wild-type *C57/B110* mice ($P < 0.05$). * indicates measurements significantly different to WT ($p < 0.05$). (b) *In vivo* cardiac cine-MRI of *Fiona* mice at 9 months. At 9 months the *mdx* mouse has a significantly larger RV ESL compared with age-matched wild-type *C57/B110* mice ($P < 0.05$). The *Fiona* mouse has a significantly smaller LV muscle volume and LV EDL and ESL and RV EDL compared with age-matched wild-type *C57/B110* mice ($P < 0.05$). * indicates measurements significantly different to WT ($p < 0.05$).

sourced from an external supplier (Harlan). Notionally this dietary difference would cause the *Fiona/mdx* mice to have smaller LV muscle volumes and LV and RV EDLs and ESLs than the wild-type mice at both time-points but the induction of cardiac hypertrophy in the *mdx* mice, which is known to happen from 6 months (Bia, 1999), increases EDL and ESL and LV muscle volume progressively first in the RV then the LV so that the *mdx* heart size reaches that of the wild-type mice. Evidence for this hypothesis can be seen in the significantly increased RV ESL and increased RV EDL found in *mdx* mice compared to both *Fiona* and wild-type mice at both 6 and 9 months (Figure 5.6).

While the *mdx* mouse heart function data fits with the histological data, despite the infiltrates and extensive lesions observed in both the LV and RV with histological staining, the *Fiona* mouse shows no reduction in heart function from wild-type, allowing for the volume changes discussed above.

5.4 Discussion

This study shows that complete rescue of the diaphragm by utrophin upregulation was sufficient to restore the *mdx* heart to wild-type levels of cardiac function to at least 9 months of age, as measured by significant improvements in RV systolic function and LV diastolic function at 6 months and both LV and RV systolic function and LV diastolic function at 9 months. While the *Fiona* mouse has utrophin upregulated in both limb muscle and the diaphragm, and restored function in both (Tinsley *et al.*, 1998), the observed improvements in heart function are most likely just due to the improvement in diaphragm function because there is a well established mechanism for compromised lung

function to affect RV function (Simmons *et al.*, 1961) and it has been shown in DMD patients that pulmonary hypertension is occurring and that it increases right atrial pressure, suggesting that RV dysfunction may be present in DMD (Yotsukura *et al.*, 1988). This pulmonary hypertension has also been shown to occur both with and without LV involvement, proving that, at least some of the time, it is not pulmonary venous hypertension (Melacini *et al.*, 1996b). This supports the proposed mechanism but is not to say that the heart lacks a defect as well. A possible explanation for the induction of LV failure by the RV failure is that the reduced RV function at 6 months results in decreased blood delivery to the LV which, due to Starling's law of the heart, causes reduced LV stroke volume (which is seen in Table 5.1). As in the RV this change is pathological over time, causing LV dysfunction. The biggest argument against this hypothesis is that diaphragm function was not actually measured. However diaphragm function has previously been measured for *Fiona* mice and found to be improved in them, compared to *mdx* mice, and western blots have shown that the levels of utrophin upregulation in the *Fiona* mice used here were the same as those in the original mice (Tinsley *et al.*, 1998). As such it is not unreasonable to conclude that diaphragm function in the *Fiona* mice used here is the same as when it was originally measured.

Previous work has suggested that functional improvement in skeletal muscles due to dystrophin restoration may be harmful to the heart (Townsend *et al.*, 2008), due to an increase in exercise capacity putting greater stress on the heart. The increased inflammatory cell infiltration and fibrosis found in this study seems to bear this out. While full-function has been restored to the heart it shows even more infiltrates and fibrosis than an untreated *mdx* mouse suggesting that improving diaphragm function may only be a short term fix, and that ultimately the heart will need treatment. However it is possible that

this could just be a side effect of the use of utrophin upregulation. It is known that a very high level of utrophin upregulation (50-100 times) in the *mdx* heart can reduce levels of necrosis in the heart (Bia, 1999), but, as detailed above (Section 5.1), there are differences in dystrophin / utrophin binding, most notably to nNOS. Recent work has shown that a difference in muscle function can be seen between *Fiona* and wild-type mice if they are subjected to a 8 week forced running protocol (Li *et al.*, 2010), and claimed that this was specifically due to the lack of nNOS binding. This suggests that utrophin upregulation cannot completely rescue the *mdx* phenotype. In general studies of humans have failed to find a correlation between utrophin levels and disease severity (Helliwell *et al.*, 1992), though some have (Kleopa *et al.*, 2006) However naturally occurring upregulation of utrophin is at very low levels, and it is possible that a greater level of utrophin upregulation is needed than in the *Fiona* mouse so the issue requires further study.

This leads into the issue of the effect of stress on the *mdx* heart. It is well established that the *mdx* mouse heart cannot cope as well with various types of stress as the wild-type heart (Bostick *et al.*, 2009; Danialou *et al.*, 2001; Kamogawa *et al.*, 2001; Quinlan *et al.*, 2004; Wu *et al.*, 2008). *Fiona* mice are usually considered to have fully rescued skeletal muscle function, but the above forced running study (Li *et al.*, 2010) showed that under stress they do not. While the results presented in this chapter found a full restoration of heart function it seems likely that if the *Fiona* mice had been put on the same forced running protocol a full restoration would not have been seen, especially given the increase in damage seen in the *Fiona* mouse heart, compared to the *mdx* mouse heart. This may provide a way round the difficulty, when treating the diaphragm and the heart, of determining which organ is improving heart function. Obviously the issue could be skirted by only treating the heart but then one would need multiple drugs. A second possibility, made more likely by the

observed poor condition of the heart muscle, is that if one continued to age the mice differences in heart function between the wild-type and *Fiona* mice would emerge that might allow one to judge if a treatment was actually having an effect on the heart. Unfortunately due to time constraints I was unable to test mice older than a year old in this study. Only being able to test treatments in mice over a year old would be hugely detrimental to research. If one can see changes in heart function, that are independent of the condition of the diaphragm, by stressing the heart, perhaps by forced running or by high-dose dobutamine stress, then these two burdensome alternatives could be avoided. It is already established that a difference in heart function between high-dose dobutamine-stressed *mdx* and wild-type mice can be seen in mice as young as 4 months (Wu *et al.*, 2008), the question is whether improving diaphragm function alone has no effect on this, some effect on this or completely removes this deficit. As such this warrants further study.

This study raises important questions for the methodology of studies into heart function in *mdx* mice. It is estimated that around 95% of DMD patients exhibit clinically relevant cardiomyopathy before death (Eagle *et al.*, 2007) so the treatment of heart failure in *mdx* mice is an important issue. Many studies that look at this in mice treat both the heart and the diaphragm but nonetheless conclude that their treatment has directly improved heart function. The results presented in this thesis demonstrate that in fact they may just have improved diaphragm function.

Another question raised by this chapter is: is improved diaphragm function just maintaining heart function or can improving diaphragm function improve heart function? Or put another way: If utrophin were upregulated in the diaphragm of six month old *mdx* mice, that were already displaying RV deficits, could their RV function be restored to

wild-type? In the *Fiona* mouse utrophin is upregulated from birth so it cannot be used to answer this question. Previous studies in *mdx* mice using an inducible transgenic expression system for utrophin (Squire *et al.*, 2002) have found that the onset of utrophin upregulation had a large effect on the resulting phenotype of the mice. With upregulation occurring before 10 days of age (before the onset of degeneration) improvements were observed, but with upregulation occurring at 30 days of age (after the onset of degeneration) the phenotype was largely unchanged. However the study did not look at the heart, and it is notable that the diaphragm showed the most improvement in central nucleation in the latter group (later onset of upregulation), with respect to other muscles, almost to the level seen in the group where treatment started earlier. Studying the heart function of these mice would be a good step towards answering this question.

In summary this study shows that *mdx* heart function improvement can be achieved without directly treating the heart, but instead improving diaphragm function by utrophin upregulation. This shows that the initial heart defect in the *mdx* mouse, in the RV and then LV, is not due to the lack of dystrophin in the heart, though it is possible that later defects, and the deficient response of the *mdx* heart to stress are. However, while heart function was improved, histologically the heart was in worse condition than if treatment had not occurred. Given this finding the testing methodology for heart failure treatments in *mdx* mice must be altered to ensure genuine heart effects of the treatment are seen and not background effects due to improved diaphragm function.

Table 5.1: Heart function in 6 month old *C57/Bl10*, *mdx* and *Fiona* mice.

6 month old		<i>C57/Bl10</i>	<i>mdx</i>	<i>Fiona</i>
Number of mice		9	9	9
Body Mass	g	29.7 ± 0.9	35.4 ± 1.4 **	31.1 ± 0.7 †
Heart Rate	bpm	427 ± 13	426 ± 18	407 ± 14
LV				
Ejection Fraction	%	59.8 ± 2.8	64.0 ± 1.7	63.7 ± 2.3
Cardiac Output	ml/min	20.0 ± 1.2	16.5 ± 1.3	16.4 ± 0.4 *
Heart Mass as % BM	%	0.44 ± 0.01	0.38 ± 0.01 *	0.39 ± 0.01 *
Muscle Volume	μl	127 ± 8	129 ± 6	115 ± 3
End Diastolic Lumen	μl	78.3 ± 5.2	60.5 ± 4.4 *	63.8 ± 1.8 *
End Systolic Lumen	μl	32.2 ± 3.9	22.2 ± 2.7 *	23.2 ± 1.9
Stroke Volume	μl	46.1 ± 1.9	38.3 ± 1.9 *	40.5 ± 1.3 *
PER	μl/s	304 ± 21	201 ± 39 *	448 ± 161
PFR	μl/s	325 ± 41	320 ± 93	322 ± 27
E	μl/s	325 ± 41	161 ± 32 *	322 ± 27 †
A	μl/s	256 ± 42	320 ± 93	317 ± 56
E/A		1.51 ± 0.20	0.78 ± 0.12	1.25 ± 0.23
RV				
Ejection Fraction	%	68.1 ± 3.3	53.4 ± 3.6 *	67.3 ± 1.7 ††
Cardiac Output	ml/min	22.7 ± 1.5	18.4 ± 1.1 *	16.9 ± 1.2 *
End Diastolic Lumen	μl	77.4 ± 4.8	81.9 ± 6.6	62.4 ± 3.3 * †
End Systolic Lumen	μl	25.3 ± 3.7	39.5 ± 5.4 *	20.7 ± 2.0 †
Stroke Volume	μl	52.1 ± 2.6	42.4 ± 2.2 *	41.7 ± 1.7 *

Data obtained by *in vivo* cardiac cine-MRI of *C57/Bl10*, *mdx* and *Fiona* mice at 6 months of age. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

* indicates measurements significantly different to WT (P>0.05)

** indicates measurements highly significantly different to WT (P>0.005)

† indicates measurements significantly different to *mdx* (P>0.05)

†† indicates measurements highly significantly different to *mdx* (P>0.005)

Table 5.2: Heart function in 9 month old *C57/Bl10*, *mdx* and *Fiona* mice.

9 month old		<i>C57/Bl10</i>	<i>mdx</i>	<i>Fiona</i>
Number of mice		8	9	9
Body Mass	g	33.3 ± 0.6	38.6 ± 1.1 **	32.7 ± 0.7 ††
Heart Rate	bpm	447 ± 10	464 ± 13	454 ± 13
LV				
Ejection Fraction	%	58.2 ± 3.1	49.5 ± 2.2 *	63.0 ± 1.9 ††
Cardiac Output	ml/min	22.5 ± 0.9	18.8 ± 1.1 *	16.6 ± 0.6 *
Heart Mass as % BM	%	0.46 ± 0.01	0.39 ± 0.01 *	0.38 ± 0.00 *
Muscle Volume	μl	147 ± 7	145 ± 7	119 ± 4 * ††
End Diastolic Lumen	μl	88.7 ± 7.1	82.6 ± 5.1	58.5 ± 2.3 * ††
End Systolic Lumen	μl	38.0 ± 5.3	41.8 ± 3.2	21.6 ± 1.5 * ††
Stroke Volume	μl	50.7 ± 2.9	40.8 ± 3.0 *	36.8 ± 1.7 *
PER	μl/s	390 ± 27	274 ± 20 *	291 ± 22
PFR	μl/s	438 ± 72	323 ± 51	266 ± 20
E	μl/s	261 ± 17	292 ± 57	186 ± 15
A	μl/s	286 ± 13	250 ± 32	238 ± 25
E/A		0.92 ± 0.11	1.16 ± 0.10	0.86 ± 0.12
RV				
Ejection Fraction	%	68.7 ± 2.5	50.7 ± 3.2 **	63.0 ± 2.4 †
Cardiac Output	ml/min	17.9 ± 4.0	21.6 ± 1.1	18.1 ± 0.7 †
End Diastolic Lumen	μl	76.0 ± 2.3	95.3 ± 9.4	66.0 ± 4.2 * †
End Systolic Lumen	μl	23.7 ± 2.0	48.6 ± 8.5 *	24.7 ± 2.7 †
Stroke Volume	μl	52.2 ± 2.4	46.6 ± 2.3	41.2 ± 2.3 *

Data obtained by *in vivo* cardiac cine-MRI of *C57/Bl10*, *mdx* and *Fiona* mice at 9 months of age. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

- * indicates measurements significantly different to WT (P>0.05)
- ** indicates measurements highly significantly different to WT (P>0.005)
- † indicates measurements significantly different to *mdx* (P>0.05)
- †† indicates measurements highly significantly different to *mdx* (P>0.005)

Chapter 6: Heart function in dystrophic mice treated with exon-skipping compounds

6.1 Introduction

This study measured the heart function of dystrophic mice treated with exon-skipping compounds for two reasons; firstly to test the effectiveness of *de novo* compounds specifically designed to target the heart at improving heart function and secondly, given the findings of the previous chapter, to test whether restoring diaphragm function via a different method would restore heart function and whether restoring dystrophin to both the heart and the diaphragm would give different results that just restoring dystrophin to the diaphragm.

DMD is caused by mutations in the *dystrophin* gene, however some mutations cause a less severe form of muscular dystrophy, called Becker muscular dystrophy (BMD). The aim of exon-skipping treatments is to change DMD into BMD. In BMD a partially functional dystrophin protein is produced, as opposed to the complete lack of dystrophin in DMD. BMD has a huge range of severity depending on the exact mutation and resulting amount and function of dystrophin protein produced. It occurs in around 1 in 14-18,000 males (about 1/5th of the incidence of DMD) (Bushby *et al.*, 2003; Emery, 1991), though it is suspected that mild cases are frequently misdiagnosed. Phenotypically, the difference between DMD and BMD is defined by losing the ability to walk at an age greater than 16. The main affected areas are the proximal limb muscles and the heart, with BMD sufferers typically losing the ability to walk from 40-60, though occasionally as early as 20-30

(Ringel *et al.*, 1977), and with >70% of patients in heart failure by age 40 (Hsu, 2010; Kirchmann *et al.*, 2005; Melacini *et al.*, 1996a; Nigro *et al.*, 1995). While DMD patients usually die by 30, BMD patients rarely die before 40 and can have nearly normal life spans.

The severity of the disease resulting from a particular mutation in the dystrophin gene does not depend on the size of the mutation – in one reported case of BMD there was a deletion of 46% of the gene but the patient was still ambulant at 61 years of age (England *et al.*, 1990); but instead on the type of mutation, with DMD being predominately caused by frame-shift mutations (Monaco *et al.*, 1988) (Figure 6.1). In the *mdx* mouse the dystrophin mutation causes premature termination of translation within exon 23 (Sicinski *et al.*, 1989). This should prevent synthesis of a functional protein, but occasional, albeit rare, dystrophin-positive (revertant) fibres are found in the *mdx* mouse (Hoffman *et al.*, 1990; Lu *et al.*, 2000) (and DMD patients (Nicholson *et al.*, 1989)) suggesting that a certain amount of natural exon-skipping is occurring (Klein *et al.*, 1992; Wallgren-Pettersson *et al.*, 1993). This has led to a novel method of treatment whereby the exon in which the harmful mutation is found is spliced out during transcription, removing the mutation. This results in a truncated version of the dystrophin protein, but can be sufficient to turn DMD into BMD. Pre-mRNA splicing to remove introns requires 5' and 3' splice site sequences at each end of the intron, the locations and sequences of which are highly conserved (Green, 1986). Splicing can be regulated by the binding of splicing enhancer or repressor proteins to sequences in either the intron itself (intronic splicing enhancers, ISE / intronic splicing silencers, ISS) or neighbouring exons (exonic splicing enhancers, ESE / exonic splicing silencers, ESS)(Tanaka *et al.*, 1994). Exon-skipping compounds work by binding to these sites. It has been shown that targeting of the 3' splice site (Wilton *et al.*, 1999) and

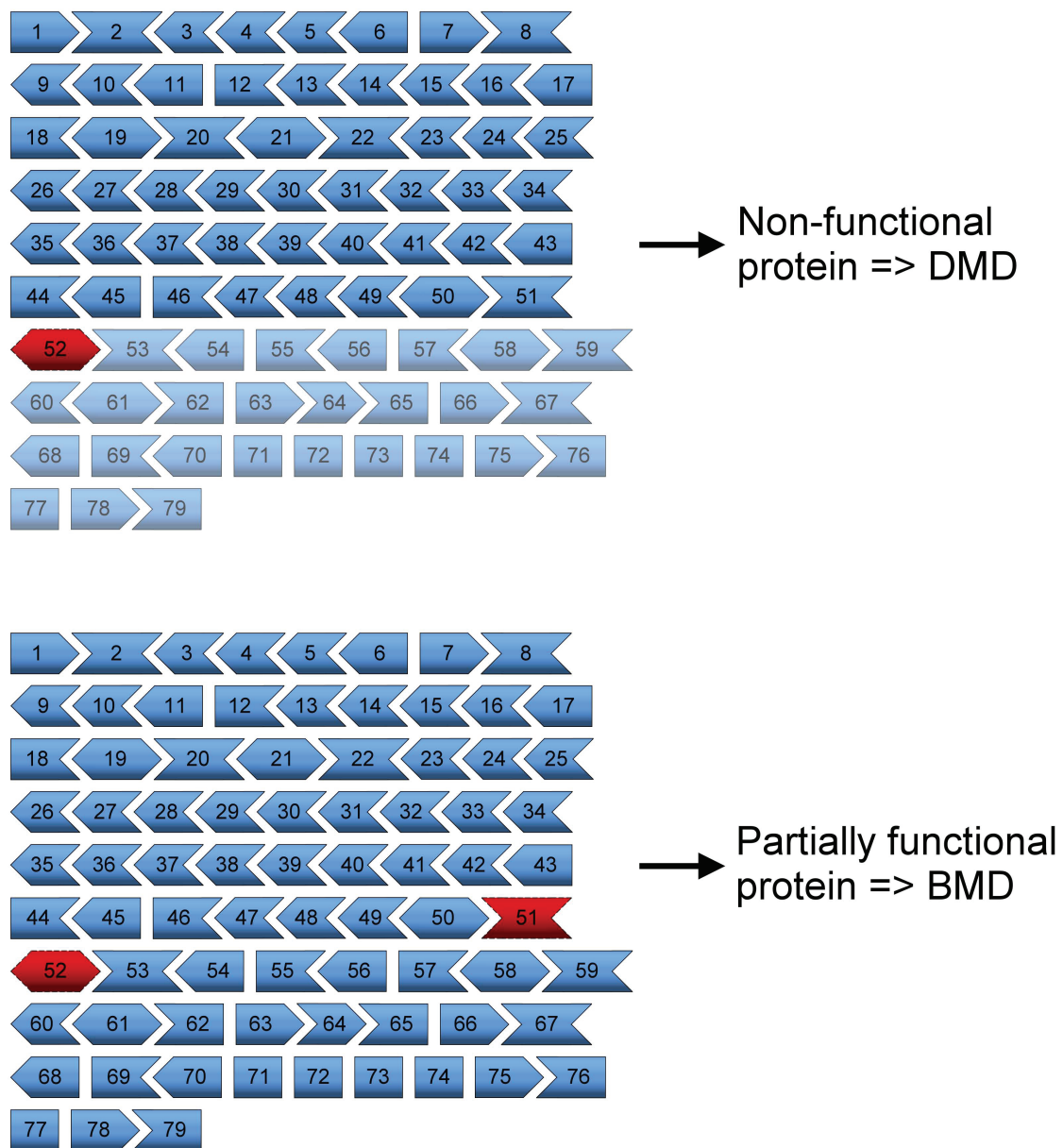


Figure 6.1 Exon structure of *dystrophin* gene.

Dystrophin is comprised of 79 exons. In humans lacking exon 52 all subsequent exons will be out of frame, as indicated by the jigsaw like edges on each exon whereby exon 51 and exon 53 do not join up, resulting in a severely truncated, non-functional protein and DMD. In humans lacking exons 51 and 52 the reading frame is preserved, as indicated by the jigsaw like edges showing exons 50 and 53 can connect, resulting in a partially functional protein and BMD.

the 5' splice site (Barabino *et al.*, 1992; Dominski and Kole, 1993; Wilton *et al.*, 1999) and ESEs (Takeshima *et al.*, 1995) by antisense oligonucleotides (AOs) can modulate splicing by preventing the binding of spliceosome components (Figure 6.2). Splicing modification by blocking of an ESE has been shown to occur naturally in a 52bp deletion of part of Dystrophin exon 19, which results in the splicing out of the entire of exon 19. An antisense 31-mer 2'-O-methyl ribonucleotide complementary to the 5' half of the deleted sequence induced skipping of exon 19 in wild-type pre-mRNA (Takeshima *et al.*, 1995). It has been shown in *mdx* mice that restoration of only 20-30% endogenous dystrophin levels in skeletal muscle by expression of a full-length *dystrophin* or a mini-*dystrophin* cloned from the above BMD patient with the very large deletion is sufficient to correct the primary myopathic deficits, particularly central nucleation and serum CK levels (Wells *et al.*, 1995).

The situation is made more complicated because the skipping of a particular exon may cause a frame shift, as happens with deletions in many cases of DMD. Therefore it may be necessary to skip multiple exons. For instance skipping exon 52 would still cause DMD because everything post 52 would be out of frame and the resulting product would be non-functional, whereas skipping exons 51 and 52 causes BMD, due to a restoration of reading frame. This is not a problem in the *mdx* model, as the removal of exon 23 does not disrupt the reading frame.

The situation is also complicated by dystrophin having 79 exons, meaning with single exon-skipping there are a large number of possible drugs. The greatest number of patients that could be treated by skipping a single exon is 16% by skipping exon 51 (Beroud *et al.*, 2007), which would restore the reading frame in patients carrying a deletion of either

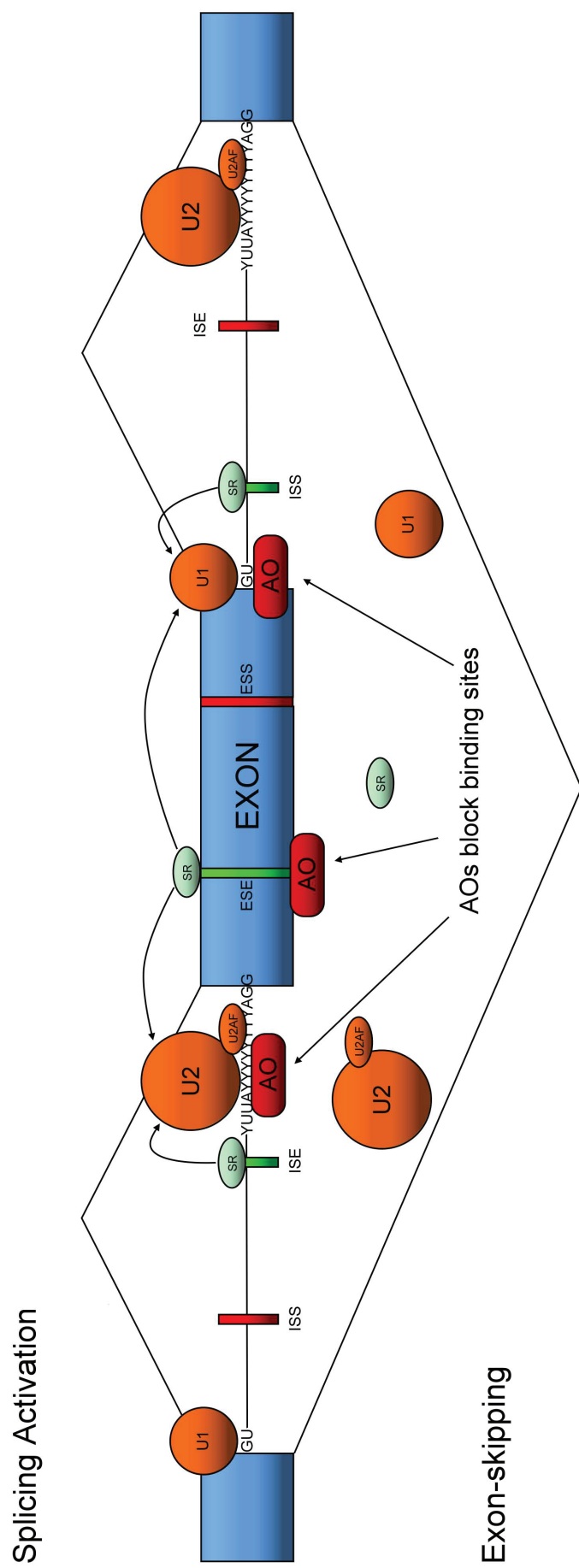


Figure 6.2 Modulation of Pre-mRNA splicing naturally and by exon-skipping compounds.

Pre-mRNA splicing to remove introns requires 5' and 3' splice site sequences at each end of the intron, the locations and sequences of which are highly conserved. The U1 and U2/U2AF snRNPs of the spliceosome bind to these respectively. Alternative splicing is regulated by the binding of splicing enhancer or repressor proteins to sequences in either the intron itself (intrinsic splicing enhancers, ISE / intrinsic splicing silencers, ISS) or neighbouring exons (exonic splicing enhancers, ESE / exonic splicing silencers, ESS). Most activator proteins binding to ISEs/ESEs are members of the SR protein family, while most repressor proteins binding to ISSs/ESSs are heterogeneous nuclear ribonucleoproteins (hnRNPs). Exon-skipping compounds (AOs) work by binding to the 3' splice site, the 5' splice site or ESEs and preventing the binding of spliceosome components or splicing activators.

exons 45–50, 47–50, 48–50, 49–50, 50, 52, or 52–63 (Aartsma-Rus *et al.*, 2002). As a drug for each exon would have to be developed and put through clinical trials separately this would be prohibitively expensive and for the rarer mutations there would not be enough patients to perform trials. The solution to these problems is creation of a multi exon-skipping drug. Creating a drug that would skip exons 45–55 would create a functioning dystrophin protein and be capable of treating 63% of patients (Beroud *et al.*, 2007). It is estimated that mono exon-skipping could treat >75% of all patients, using multiple drugs, while multi exon-skipping could treat up to 92%, i.e. excluding those mutations that affect the functionally critical domains of the N- and C-terminal domains, the promoter region and the first exon, or translocations (Aartsma-Rus *et al.*, 2004).

Rather elegantly AOs do not require targeting to muscle. The lack of dystrophin prevents the DAPC from forming and this in turn leads to the formation of holes in the sarcolemma (Menke and Jockusch, 1991; Mokri and Engel, 1975; Rowland, 1980). The AOs, which are not otherwise membrane permeable, pass through these holes and so only reach the muscle. This allows systemic delivery rather than requiring intramuscular injections, something that would be impractical in humans (Alter *et al.*, 2006; Fletcher *et al.*, 2006; Lu *et al.*, 2005). However this does produce patchy restoration of dystrophin as the holes in the sarcolemma are not evenly distributed. This is not necessarily a problem, as a transgenic *mdx* mouse with mosaic dystrophin expression showed functional improvements (Bostick *et al.*, 2008).

There are several different types of chemistry available for exon-skipping (Goyenvallé *et al.*, 2004; Maier *et al.*, 1995; Schmajuk *et al.*, 1999). In the experiments presented in this thesis phosphorodiamidate morpholino oligomers (PMOs) (Summerton and Weller, 1997)

were used. Structurally the difference between PMOs and RNA is that PMO bases are bound to morpholine rings instead of ribose rings, connected through phosphorodiamidate groups instead of phosphodiester groups (Figure 6.3). PMOs have high specificity (sequence dependent) and are highly stable because they are not degraded by cells or serum due to their unnatural backbone (Hudziak *et al.*, 1996; Youngblood *et al.*, 2007). They are also non-toxic as they do not activate innate immune responses and are not known to alter DNA methylation (Iversen, 2001; Summerton, 1999).

PMOs have been shown to restore dystrophin levels at high efficiency in the *mdx* and *dKO* mouse, and the dystrophic dog after systemic delivery (Alter *et al.*, 2006; Lu *et al.*, 2005; Wu *et al.*, 2008; Yin *et al.*, 2008; Yin *et al.*, 2009; Yin *et al.*, 2010). In humans, treatment with an exon 51 skipping PMO, via intramuscular injection to the extensor digitorum brevis (EDB), resulted in a maximum of 44-79% of myofibres being dystrophin positive, with 22-32% of normal dystrophin level per fibre (Kinali *et al.*, 2009), meaning that the levels shown to be necessary for improvements in function were reached (Wells *et al.*, 1995). The main problem with PMOs is that during systemic delivery they do not effectively restore dystrophin in the heart (Alter *et al.*, 2006; Fletcher *et al.*, 2006; Lu *et al.*, 2005). As around 95% of DMD patients exhibit clinically relevant cardiomyopathy (Eagle *et al.*, 2007), it is necessary for any therapy to restore, or compensate for, the function of dystrophin in the heart. Dystrophin restoration with PMOs can also be very patchy, as they only enter the muscle where there are holes in the sarcolemma, which are not evenly distributed. To provide targeting for the PMOs in order to allow them to reach the heart and to obtain more even dystrophin restoration they have been conjugated with cell-penetrating peptides (PPMOs), which have been shown to induce exon-skipping, restoring functional dystrophin with high efficiency (Wu *et al.*, 2008; Yin *et al.*, 2008; Yin

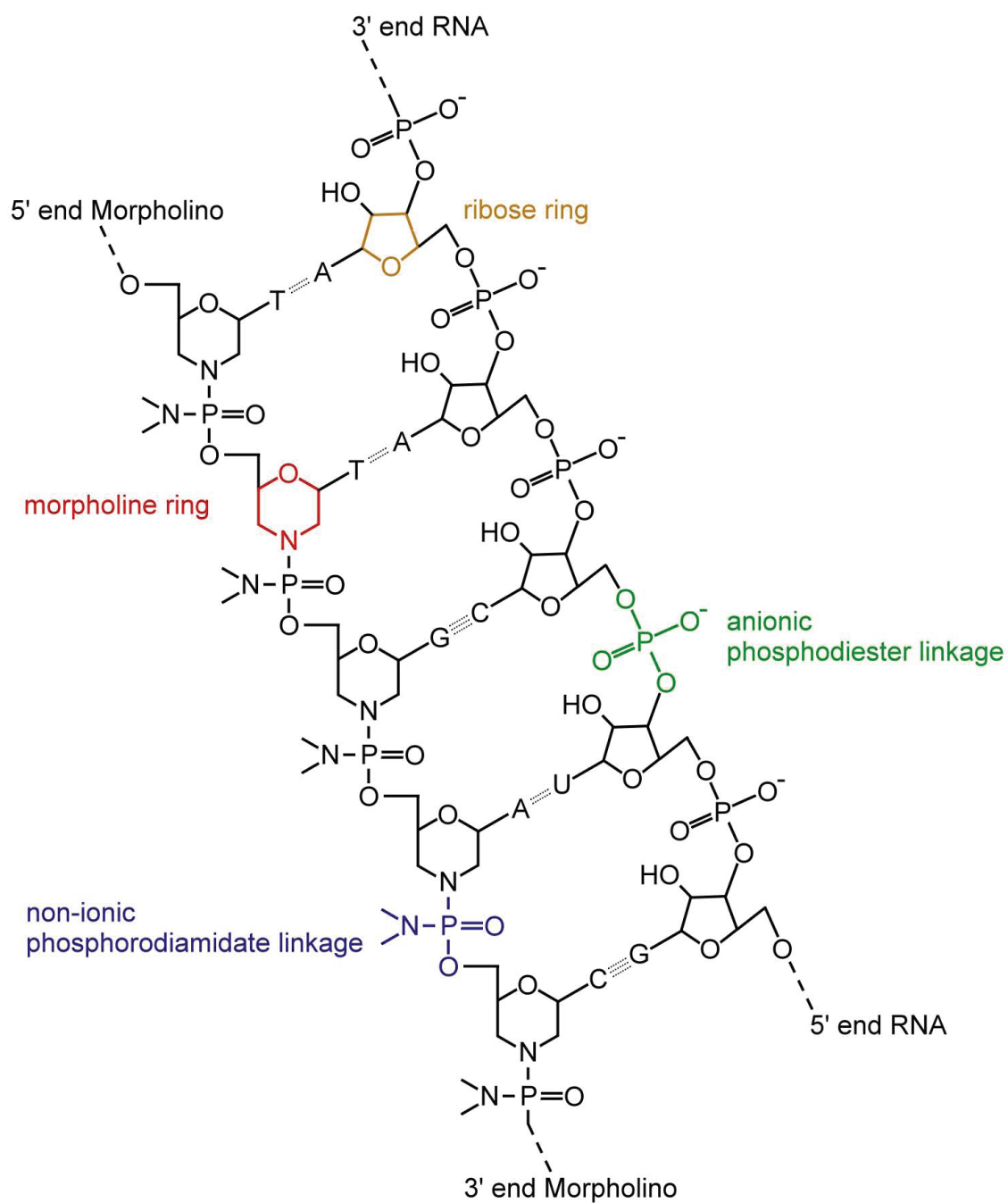


Figure 6.3. Structure of phosphorodiamidate morpholino oligomer (PMO) bound to RNA. PMO bases are bound to morpholine rings (red) instead of ribose rings (yellow) connected through phosphorodiamidate groups (blue) rather than phosphodiester groups (green).

et al., 2009). The use of PPMOs allows treatment at lower doses (Fletcher *et al.*, 2007). However the cell-penetrating peptides can be toxic (Amantana *et al.*, 2007; Wu *et al.*, 2007), including causing lethargy, weight loss and tubular degeneration in kidney. The toxicity varies between species with monkeys more sensitive to the PPMO-related toxicity than mice. Off-target effects can also contribute to the toxicity (Coffman *et al.*, 2004).

The aim of this study was to assess the heart function of *mdx* and *dKO* mice treated with exon-skipping compounds to assess the efficiency of *de novo* PPMOs targeting the heart at improving heart function compared to PPMOs that do not target the heart. Previous work (Jearawiriyapaisarn *et al.*, 2010; Wu *et al.*, 2008) has suggested that PPMO-induced cardiac dystrophin restoration in *mdx* mice can improve LV function. However, given the findings of the previous chapter (Chapter 5) It was thought that the previously observed improvements in heart function were a result of diaphragm dystrophin restoration and not directly due to cardiac dystrophin restoration in the heart itself. This study also aims to assess the degree to which restoration of dystrophin in the heart is actually necessary to improve heart function.

Several different PPMOs were used in this study, differing in their peptide or mode of conjugation (Table 6.1, located at the end of the chapter). This, and variations in route of administration and dose, resulted in differences in levels of dystrophin restoration in the skeletal muscles, diaphragm and heart. These PPMOs were used in different combinations, at different timepoints and in different mice models so displaying exactly what was done in this chapter is best done via a table (Table 6.2).

Table 6.2: Summary of injections given to dystrophic mice in chapter 6.

PPMO	Model	Age	Dose	Administration route	Restoration in		
					Skeletal muscle	Diaphragm	Heart
B-PMO 1	<i>mdx</i>	3 months	18.75mg/kg	Intravenous (IV)	Yes	Yes	No
pip5e-PMO	<i>mdx</i>	3 months	18.75mg/kg	IV	Yes	Yes	Yes
B-PMO 1	<i>mdx</i>	6 months	18.75mg/kg	IV	Yes	Yes	No
pip5e-PMO	<i>mdx</i>	6 months	18.75mg/kg	IV	Yes	Yes	Yes
RXB-PMO	<i>mdx</i>	9 months	18.75mg/kg	IV	Yes	Yes	No
pip5e-PMO	<i>mdx</i>	9 months	18.75mg/kg	IV	Yes	Yes	Yes
B-PMO 2	<i>dKO</i>	from 10 days	25 mg/kg/week	IV	Yes	Yes	Yes
B-PMO 2	<i>dKO</i>	from 10 days	25 mg/kg/week	Intraperitoneal (IP)	Yes	Yes	No
P007-PMO	<i>dKO</i>	from 10 days	25 mg/kg/week then per month	IP	Yes	Yes	No

Summary of injections given to dystrophic mice in chapter 6, showing drug (PPMO), age of mice, dosage, route of administration and restoration pattern of dystrophin.

As well as these treatments, that all took place on different sets of mice, there were a series of controls that were shared, insofar as it was possible and include both the *mdx* and *C57/Bl10* mice from chapter 5. The first section of the results deals with the difficulty of selecting the proper controls for each experiment (see section 6.3.1). Many of the experiments took place concurrently and the controls were not generally available for imaging at the same time as the treated mice (for reasons due to the availability of mice discussed in chapter 2 and below in section 6.2.1).

In the first experiment 3-month old *mdx* mice were IV injected with a single dose of pip5e-PMO or B-PMO1 at 18.75mg/kg. Their heart function was assessed by cine-MRI (as described in Chapter 2) and compared with untreated *mdx* littermates and data from age-matched *C57/Bl10* wild-type controls supplied by Dr Dan Stuckey (Clarke Lab). Treated mice were then killed by cervical dislocation, their hearts extracted and used for western blotting with anti-dystrophin and anti- α -actinin. In the second experiment 6-month old *mdx* mice were IV injected with a single dose of pip5e-PMO or B-PMO1 at 18.75mg/kg. Their heart function was assessed by cine-MRI and compared with untreated *mdx* littermates and age-matched *C57/Bl10* mice (those used as controls in chapter 5). Treated mice were then killed by cervical dislocation, their hearts extracted and used for western blotting, with anti-dystrophin and anti- α -actinin, and immunostaining with dystrophin. In the third experiment 9-month old *mdx* mice were IV injected with a single dose of pip5e-PMO at 18.75mg/kg, their heart function assessed by cine-MRI and compared with untreated *mdx* littermates and *C57/Bl6* controls. Treated mice were then killed by cervical dislocation, their hearts extracted and used for western blotting, with anti-dystrophin and anti- α -actinin, and immunostaining with dystrophin. In the fourth experiment 8-week old *dKO* and *C57/Bl6* control mice were imaged by cine-MRI and their heart function assessed. In

the fifth experiment 10-day old *dKO* mice were injected either intravenously or intraperitoneally with B-PMO2 at a 25mg/kg dose weekly for 23 weeks. At 6-months old their heart function was assessed by cine-MRI and compared with age-matched *mdx* and *C57/Bl6* mice. Treated mice were then killed by cervical dislocation, their hearts extracted and used for western blotting, with anti-dystrophin and anti- α -actinin, and immunostaining with dystrophin, components of the DAPC and early myosin isoform antibody. In the sixth experiment 10-day old *dKO* mice were injected intraperitoneally with P007-PMO at a 25mg/kg dose weekly for 6 weeks, then subsequently reinjected monthly at the same dose. At 6 and 9 months their heart function was assessed by cine-MRI and compared with age-matched *mdx* and *C57/Bl6* mice. Two were then killed by cervical dislocation, their hearts extracted and used for western blotting with anti-dystrophin and anti- α -actinin. The remaining two were left alive to see to what age they would live.

6.2 Methods

6.2.1 Animals

Six-month old *mdx* mice with their *mdx* littermates and *C57/Bl10* mice as controls were used in the single-dose B-PMO1 intravenous administration experiments at 18.75 mg/kg dose (n = 6 for both *mdx* groups and n = 8 for wild-type controls).

Eight-week old *dKO* and *C57/Bl6* mice as controls were used in the experiment to look at heart function in untreated *dKO* mice (n = 7 for the *dKO* mice and n = 11 for the controls).

Ten-day old *dKO* mice and *mdx* littermates as controls were used in the long-term B-PMO2 administration experiments (n = 3 in the test groups and n = 6 in control groups) after genotyping. For systemic intravenous and intraperitoneal injections B-PMO2 in 80 μ l saline buffer was injected into *dKO* mice at the final dose of 25 mg/kg.

Ten-day old *dKO* mice were used in the long-term P007 administration experiments (n = 4). They were injected intraperitoneally 25 mg/kg/week for 6 weeks, as previously described (Goyenvallé *et al.*, 2010), then subsequently re-injected monthly. For controls, age-matched *C57/Bl6* mice (n = 8) and the, age-matched, *mdx* mice from the Chapter 5 experiments were used

Injected mice and their littermates, where used, were supplied by collaborators.

The *dKO* mouse was derived by breeding female *mdx* mice (on a *C57/Bl10* background) with male *utrophin null* mice (on a *C57/Bl6* background) (Deconinck *et al.*, 1997a). This produces F1 progeny heterozygous for utrophin (*utrn*) and either heterozygous for the *mdx* mutation (females) or hemizygous for the *mdx* mutation (males). There are three possible crossings from these mice to produce *dKO* mice:

- 1) Male F1 (*utrn*+/-, *mdx*) x female (*utrn*+/+, *mdx/mdx*) → F2 mice which are all *mdx* with either *utrn*+/+ or *utrn*+/-

Male F2 (*utrn*+/-, *mdx*) x female F2 (*utrn*+/-, *mdx/mdx*) → 25% *dKO*

- 2) Male (*utrn*-/-, +/+) x female F1 (*utrn*+/-, *mdx*+) → 25% *dKO*

3) Male F1 (*utrn*^{+/-}, *mdx*) x female F1 (*utrn*^{+/-}, *mdx*/+) → 12.5% *dKO*

The complicated nature of these crossings makes obtaining large numbers of *dKO* mice difficult. As such mixed sex groups were chosen for the experiment to look at heart function in untreated *dKO* mice and in the long-term P007 administration experiments.

6.2.2 Collaborators Methods

6.2.2.1 Western Blot

For the exon skipping experiments with B-PMO1&2 - protein extraction and western blot were carried out as previously described (Yin *et al.*, 2008) and with p007-PMO - protein extraction and western blot were carried out as previously described (Goyenvallé *et al.*, 2010). Various amounts of protein from normal *C57/BL6* mice were used as a positive control and corresponding amounts of protein from muscles of treated or untreated mice were used. The membrane was probed with DYS1 (monoclonal antibody against dystrophin R8 repeat, 1:200, NovoCastra, UK) for the detection of dystrophin protein. The bound primary antibody was detected by horseradish peroxidase-conjugated rabbit anti-mouse IgGs and the ECL Western Blotting Analysis system (Amersham Pharmacia Biosciences, UK).

6.2.2.2 RNA extraction and nested RT-PCR analysis

Total RNA was extracted with Trizol (Invitrogen, UK) and 200ng of RNA template was used for 20µl RT-PCR with OneStep RT-PCR kit (Qiagen, UK). The primer sequences were used as previously reported (Yin *et al.*, 2008). The products were examined by electrophoresis on a 2% agarose gel.

6.2.2.3 Immunohistochemistry and histology

8µm sections were examined for dystrophin expression and dystrophin-associated protein complex (DAPC) proteins with a series of polyclonal antibodies and monoclonal antibodies as described (Yin *et al.*, 2008). For detecting regenerating muscle fibres, mouse fetal myosin antibody was used, which was a gift from Professor Neal A. Rubinstein (University of Pennsylvania, USA) and goat-anti-mouse IgGs Alexa 594 secondary antibody (Invitrogen) was applied.

6.3 Results

6.3.1 Variability in controls

In undertaking these experiments there were a number of difficulties to overcome in the choice of which wild-type and *mdx* controls to use. The mice for these experiments were spread across three animal houses with two different cleanliness levels. Also, the *dKO* has a mixed background – while its *mdx* component is on *C57/Bl10* the utrophin knockout is on *C57/Bl6*. Furthermore, as the experiments in this chapter were in collaboration, with Dr Aurelie Goyenvallé (P007-PMO experiments) and Dr HaiFang Yin (all other PPMO experiments), there was little control over the sex of the mice supplied. Therefore it was necessary to establish to what extent these variables would affect heart function. The following experiments studied animals at 4 time points – 8 weeks old, 3 months old, 6 months old and 9 months old. Using cine-MRI, the heart function of a number of different groups of wild-type and *mdx* mice was assessed, as detailed below in table 6.3.

Table 6.3: Different genotypes and sexes of mice used in chapter 6.

Age / genotype (animal house)	Male	Female
	n	n
8 weeks old		
<i>C57/Bl6</i> (dirty)	4	7
3 months old		
<i>mdx</i> (dirty)	6	6
6 months old		
<i>C57/Bl10</i> (clean)	8	-
<i>C57/Bl6</i> (dirty)	8	8
<i>mdx</i> (clean)	9	-
<i>mdx</i> (dirty)	6	-
9 months old		
<i>C57/Bl10</i> (clean)	8	-
<i>C57/Bl6</i> (dirty)	6	7
<i>mdx</i> (clean)	9	-
<i>mdx</i> (dirty)	-	4

The number of mice of different genotypes and sexes of mice used in chapter 6, divided into age groups.

At 8 weeks old there were no significant differences between male and female *C57/Bl6* mice from the 'dirty' animal house (Table 6.4).

At 3 months old female *mdx* from the 'dirty' animal house were significantly smaller than male littermates, with a significantly smaller muscle volume, LV cardiac output and stroke volume and RV cardiac output, EDL and ESL. When normalising for body mass these differences were no longer significant. (Table 6.5)

At 6 months old there were no significant differences between *C57/Bl10* males from the 'clean' animal house and *C57/Bl6* males from the 'dirty' animal house (Table 6.6). *C57/Bl6* females from the 'dirty' animal house were significantly smaller than *C57/Bl6* males from the 'dirty' animal house (Table 6.7). In the LV they had a significantly smaller cardiac output, average muscle volume, EDL, ESL, stroke volume and PER. When normalised for body mass, these differences were not significant. In the RV the females had a significantly smaller cardiac output, EDL and stroke volume. These were still significantly smaller when normalising for body mass.

Also at 6 months old comparing *mdx* males from the 'dirty' and 'clean' animal houses showed that the dirty males had a significantly larger LV ejection fraction (Table 6.8). There were no other significant differences between the mice. However the LV cardiac output and RV ejection fraction were lower in the 'clean' mice, while the LV and RV EDL and ESL were higher, suggesting that on the whole the 'clean' mice were in worse condition than the 'dirty' mice contrary to expectations. This is likely due to differences in diet between the mice. In the 'clean' animal house the mice were fed a diet with 1/3rd more fat in it than those in the 'dirty' animal house.

At 9 months old there were no significant differences between *C57/Bl10* males from the ‘clean’ animal house and *C57/Bl6* males from the ‘dirty’ animal house (Table 6.9).

C57/Bl6 females from the ‘dirty’ animal house were significantly smaller than *C57/Bl6* males from the ‘dirty’ animal house (Table 6.10). They had a significantly smaller LV cardiac output, muscle volume, EDL, ESL and stroke volume and a significantly smaller RV EDL, ESL and stroke volume. However none of these differences were significant when normalised for body mass.

Also at 9 months old comparing *mdx* males from the ‘dirty’ and ‘clean’ animal houses showed that the dirty males were significantly smaller (Table 6.11), with a significantly larger LV ejection fraction. In the LV they also had a significantly smaller average muscle volume, EDL and ESL, but muscle volume/body mass, EDL/body mass and ESL/body mass were not significantly different. Stroke volume/body mass was significantly larger in the ‘dirty’ mice. In the RV they also had a significantly smaller cardiac output, EDL, ESL and stroke volume. When normalised for body mass only ESL was still significantly smaller. This suggests the ‘dirty’ mice were unusually well, as the reductions in LV and RV ejection fraction normally seen at this age were absent. They also had significantly improved RV ejection fractions compared to 6 month old *mdx* mice from the same rack in the same animal house and higher RV ejection fractions than 8 month old *mdx* mice in the published literature (Zhang *et al.*, 2008b).

Overall for wild-type mice there were no significant differences between *C57/Bl10* males and *C57/Bl6* males, despite the difference in health condition of their housing. There were significant differences between *C57/Bl6* males and females, but these were mostly

abolished by normalisation for body mass. This showed that when choosing wild-type controls for the exon-skipping experiments genotype was the least important factor, but one should try to sex-match, though normalisation by body mass should be sufficient. As *dKO*s have a B16 / B110 mixed background either could have been used, but as most of the *dKO*s were female, and I lacked B110 female data, all *dKO*s are compared with age-matched *C57/B16* controls.

Overall for *mdx* mice the situation was much less clear. At 3 months old female *mdx* from the ‘dirty’ animal house were significantly smaller than male littermates, but normalising for this abolished all differences in heart function. At 6 months old male *mdx* mice from the ‘clean’ and ‘dirty’ house had statistically indistinguishable heart function except for a significant increase in LV ejection fraction in the ‘dirty’ mice. At 9 months old male *mdx* mice from the ‘clean’ and ‘dirty’ house had many significant differences in heart function, with the ‘dirty’ mice seeming to be abnormally well. Differences increased with age, with the ‘dirty’ mice seeming to have better heart function than the ‘clean’ mice, likely due to the differences in diet outlined above. This means care must be taken when choosing untreated *mdx* controls, with matching the animal house condition the most important criteria.

The following experiments were done in collaboration with Dr Aurélie Goyenvalle (Davies Laboratory) (P007-PMO experiments) and Dr HaiFang Yin (Wood Laboratory) (all other PPMO experiments). Mice treated with PPMOs were supplied by collaborators, imaged by me and then returned to the collaborators still alive. Subsequently the mice were killed and western blots in sections 6.3.2.1, 6.3.2.2, 6.3.2.3, 6.3.3.2 and 6.3.3.3, immunostaining in section 6.3.3.2 and immunohistochemistry and quantification of dystrophin positive fibres in sections 6.3.2.2 and 6.3.2.3 were performed by collaborators. In the results section, it is explicitly stated at the end of a sentence about an experimental result if a collaborator performed the experiment.

6.3.2 Exon-skipping in *mdx* mice

6.3.2.1 Heart function in *mdx* mice treated with B-PMO1 and pip5e-PMO at 3 months old

There are large numbers of exon skipping compounds available so to speed up screening for the compounds having the largest effect on heart function it would be best to study mice as young as possible. Accordingly heart function was first studied at 3 months of age. Three-month old *mdx* mice were treated with a single intravenous (IV) injection of pip5e-PMO or B-PMO1 at 18.75mg/kg. Pip5e-PMO is a new peptide designed specifically to get high levels of dystrophin restoration in the heart, while B-PMO1 is a commercially produced PPMO, that has previously been shown to restore dystrophin effectively in *mdx* mice (Alter *et al.*, 2006; Jearawiriyapaisarn *et al.*, 2008; Jearawiriyapaisarn *et al.*, 2010; Lu *et al.*, 2005). B-PMO2, which is very similar to B-PMO1 - differing only in the method of conjugation, has been previously shown to induce cardiac dystrophin restoration, but this

was at much higher single doses (30mg/kg IV) or repeated daily doses (two cycles of four once daily IV injections at 12mg/kg/day)(Jearawiriyapaisarn *et al.*, 2010; Wu *et al.*, 2008). At the dose used here B-PMO1 mostly only induces skeletal muscle and diaphragm dystrophin restoration. Western blot analysis subsequent to the heart function testing confirmed this; while in pip5e-PMO treated mice dystrophin was restored in the skeletal muscle, diaphragm and heart (Western blot performed by Dr HaiFang Yin) (Figure 6.4).

Previous studies had shown that the highest degree of restoration occurred 2 weeks post injection (personal communication from Dr HaiFang Yin). At this point their heart function was assessed using cine-MRI and compared with that of *mdx* littermates and with data from age-matched *C57/Bl10* wild-type controls supplied by Dr Dan Stuckey (Clarke Lab) (Table 6.12). The untreated *mdx* mice had a significantly smaller LV ESL and RV cardiac output and a significantly larger muscle volume compared with age-matched *C57/Bl10* mice. The B-PMO1 treated mice had a significantly smaller LV and RV cardiac output than wild-type mice and a significantly smaller muscle volume and significantly larger LV A peak filling rate than their untreated *mdx* littermates. Similarly the pip5e-PMO treated mice had a significantly smaller LV and RV cardiac output and LV EDL and ESL than wild-type mice and a significantly smaller muscle volume than their untreated *mdx* littermates. They were also significantly smaller than wild-type and untreated *mdx* controls.

Overall there was no clear pattern to the results, with only minor significant differences between the wild-type and untreated *mdx* mice, suggesting it is necessary to study older mice to see any drug-related effect on heart function.

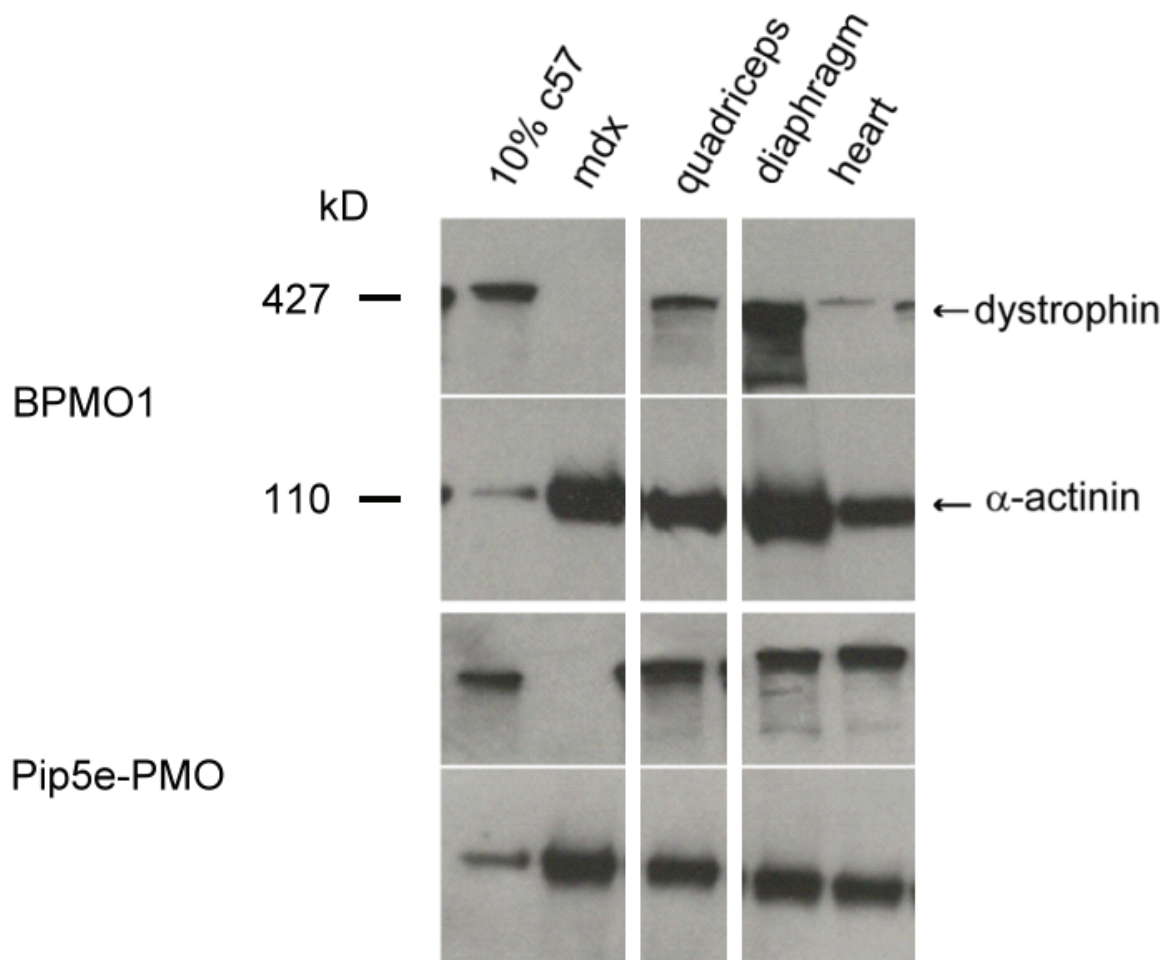


Figure 6.4 Dystrophin restoration in 3 month old *mdx* mice treated with B-PMO1 and pip5e-PMO injected intraperitoneally at 18.75mg/kg single dose.

Western blot for dystrophin expression in B-PMO1 and pip5e-PMO treated *mdx* mice. α -actinin was used as the loading control.

6.3.2.2 Heart function in *mdx* mice treated with B-PMO1 and pip5e-PMO at 6 months old

Having seen no clear pattern of differences at 3 months, the experiment was repeated at 6 months on different mice. 6-month old *mdx* mice were treated with a single IV dose of 18.75mg/kg of B-PMO1 or pip5e-PMO. Subsequent Western blot analysis showed that, in B-PMO1 treated mice effective dystrophin restoration was observed in the skeletal muscle and diaphragm, but not the heart, while in pip5e-PMO treated mice dystrophin was restored in the skeletal muscle, diaphragm and heart (Western blot performed by Dr Haifang Yin) (Figure 6.5a).

Two weeks after injection mice were imaged using cine-MRI and cardiac function compared with untreated *mdx* littermates and age-matched *C57/Bl10* mice. (Table 6.13). The untreated *mdx* were significantly larger than age and sex-matched *C57/Bl10* mice and had a significantly smaller LV cardiac output, heart mass as a percentage of body mass, EDL, ESL, stroke volume, PFR and E peak filling rate and RV ejection fraction, cardiac output and stroke volume. This all fits with the expected results, but they also had a significantly larger LV ejection fraction than wild-type controls, this is notable as being the only significant difference between them and the 6 month old untreated *mdx* mice used in the previous chapter (as discussed above), so can probably be dismissed.

The B-PMO1 treated *mdx* mice showed a significant increase in RV ejection fraction, LV and RV cardiac output and LV E peak filling rate compared to their untreated littermates. The treatment restored cardiac function to wild-type levels (Figure 6.5b). However the pip5e-PMO treated mice were more complicated. Both sets of treated mice were mixed sex, but while the B-PMO1 treated mice all had similar results the pip5e-PMO treated

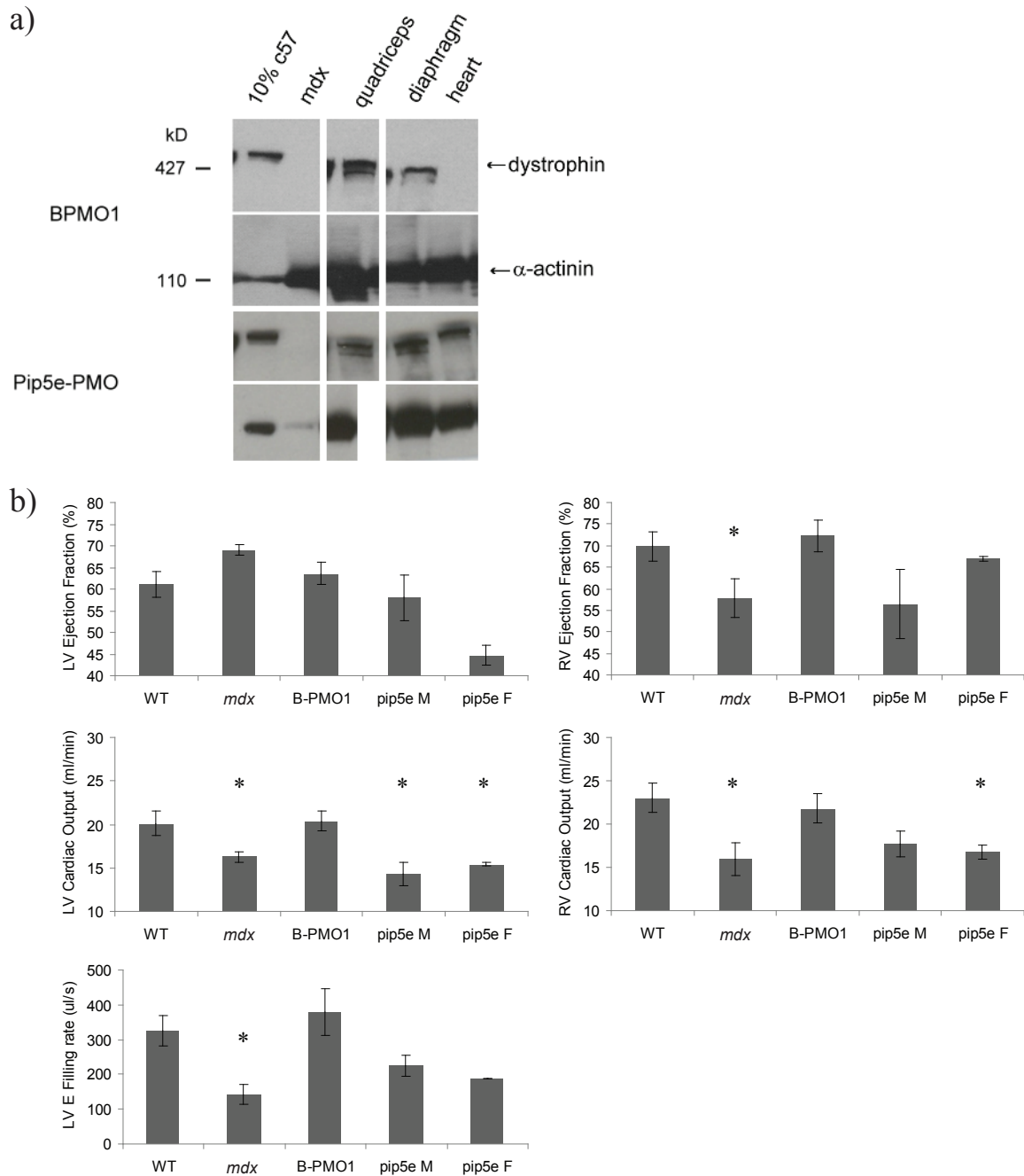


Figure 6.5 Dystrophin restoration and cardiac function improvement in 6 month old *mdx* mice treated with B-PMO1 and pip5e-PMO injected IP at 18.75mg/kg single dose. (a) Western blot for dystrophin expression in B-PMO1 and pip5e-PMO treated *mdx* mice. α -actinin was used as the loading control. (b) *In vivo* cardiac cine-MRI of *mdx* mice treated with B-PMO1 and pip5e-PMO. A significant decrease in LV E peak filling rate, RV ejection fraction and LV and RV cardiac output was found in untreated *mdx* mice compared with age-matched *C57/Bl10* mice ($P < 0.05$). Heart function was restored to normal levels in B-PMO1 treated mice. In male pip5e-PMO treated mice heart function was not significantly different from untreated *mdx* mice, though their LV ejection fraction was lower. In female pip5e-PMO treated mice heart function was also not significantly different from untreated *mdx* mice, though their RV ejection fraction was higher and their LV ejection fraction was much lower. * indicates measurements significantly different to WT ($p < 0.05$).

c)

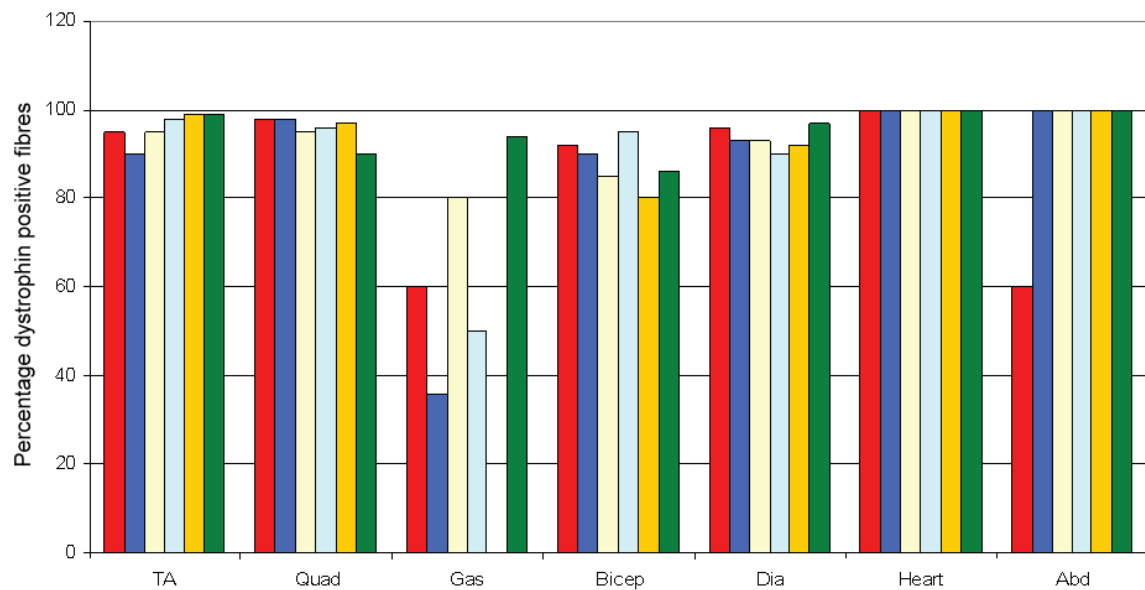


Figure 6.5 Dystrophin restoration 6 month old *mdx* mice treated with pip5e-PMO injected intraperitoneally at 18.75mg/kg single dose.

(c) Quantification of percentage of dystrophin positive fibres seen in immunohistochemistry following pip5e-PMO treatment. Each colour is a different mouse. Percentage of dystrophin fibres was calculated by dividing the number of fibres positive for dystrophin by the total number of visible fibres.

mice segregated into two groups by sex. The male pip5e-PMO treated mice had broadly similar heart function to their untreated *mdx* littermates (with only a significantly reduced LV PER separating them) suggesting the drug had very little effect on them. The female pip5e-PMO treated mice also had broadly similar heart function to their untreated *mdx* littermates, but with several important differences. They had a larger (but not significantly so) RV ejection fraction, restored to about wild-type levels, suggesting the drug had worked, but a much smaller (but not significantly so) LV ejection fraction, suggesting it had seriously impacted LV function in doing so.

The B-PMO1 results support the hypothesis that diaphragm dystrophin restoration is sufficient to restore heart function to wild-type levels and corroborate the results found with the Fiona mice, despite the different method used for restoring diaphragm function. The pip5e-PMO results are difficult to interpret. Unfortunately my collaborators had not kept track of which mice were which sex when processing samples so it was impossible to directly compare heart function to percentage dystrophin restoration on an individual basis, but all mice showed 90-100% dystrophin positive fibres in diaphragm and 100% dystrophin positive fibres heart (Figure 6.5c) showing that the unchanged heart function seen in the males was not due to the drug not working. The evidence suggests that cardiac dystrophin restoration does not equate to an improvement in cardiac function and that, given the very low LV ejection function in the female pip5e-PMO treated mice, the drug may be toxic.

6.3.2.3 Heart function in *mdx* mice treated with pip5e-PMO at 9 months old

Due to the confusion over the results from the pip5e-PMO treated mice, the experiment was repeated at 9 months on different mice. A different batch of pip5e-PMO was used in case the previous result had been due to problems occurring during production. 9-month old *mdx* mice were treated with a single IV dose of 18.75mg/kg pip5e-PMO. Western blot analysis showed that dystrophin was restored in the skeletal muscle, diaphragm and heart (Western blot performed by Dr HaiFang Yin) (Figure 6.6a).

Two weeks post-injection mice were imaged using cine-MRI and their heart function compared with untreated *mdx* littermates and *C57/Bl6* controls (Table 6.14). There were no significant differences between the untreated *mdx* mice and *C57/Bl6* controls, *Bl6* females being used in this instance as all of the untreated *mdx* mice and most of the treated mice were female. This suggests the untreated *mdx* mice were unusually well. The reductions in LV and RV ejection fraction seen in the 9 month old *mdx* mice used in the dystrophin upregulation experiments were absent. They also had significantly improved RV ejection fractions compared to 6 month old *mdx* mice from the same rack in the same animal house and higher RV ejection fractions than 8 month old *mdx* mice in the published literature (Zhang *et al.*, 2008b). Consequently, as there are no differences between wild-type and *mdx* mice, one would not expect to see any improvements in the treated mice. In the pip5e-PMO treated mice, where both mice were female, the LV stroke volume was significantly decreased compared to untreated *mdx* mice and the RV ejection fraction was decreased, though not significantly, compared to untreated *mdx* mice. The pip5e-PMO treated mice also had significantly decreased LV cardiac output and stroke volume and RV ejection fraction and stroke volume compared to age-matched wild-type controls. Due to the unusually healthy condition of the untreated *mdx* mice it is not clear how reliable this

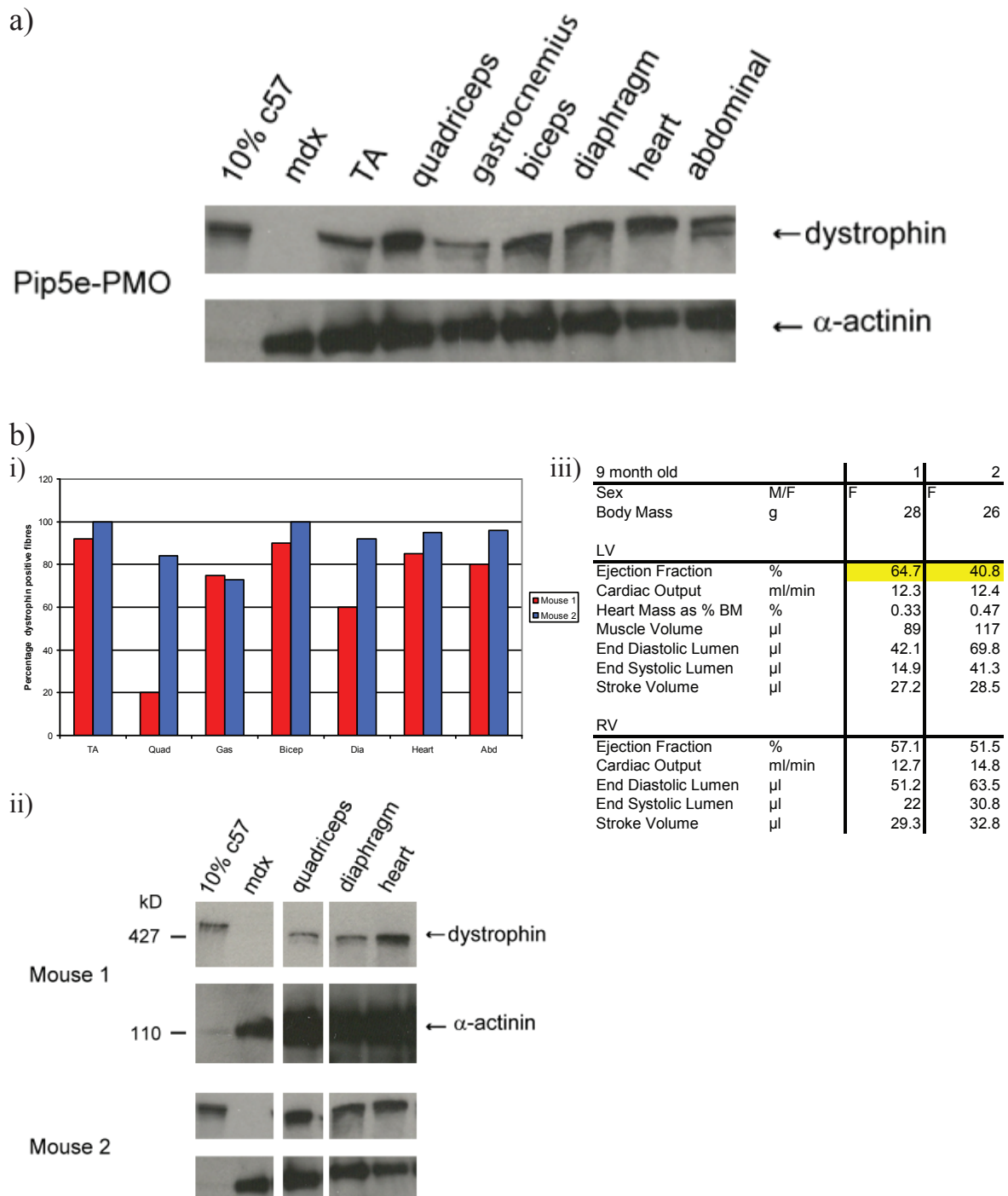


Figure 6.6 Dystrophin restoration and cardiac function improvement in 9 month old *mdx* mice treated with pip5e-PMO injected intraperitoneally at 18.75mg/kg single dose. (a) Western blot for dystrophin expression in pip5e-PMO treated *mdx* mice. α -actinin was used as the loading control. (b) (i) Quantification of percentage of dystrophin positive fibres seen in immunohistochemistry following pip5e-PMO treatment. Percentage of dystrophin fibres was calculated by dividing the number of fibres positive for dystrophin by the total number of visible fibres.;(ii) western blot for dystrophin expression in pip5e-PMO treated *mdx* mice (same conditions as (a)) and (iii) table showing cardiac function in each mouse, measured by cine-MRI. Mouse 1 has less dystrophin positive fibres and a lower level of dystrophin restoration (notably in the diaphragm) but higher heart function, as notable from the very different LV ejection fractions (highlighted in yellow).

data is, however the drugs appear to suppress heart function slightly, particularly in pip5e-PMO treated mice. This correlates with the 6 month old results where pip5e-PMO also lowered stroke volume and LV ejection fraction in female mice and did very little in male mice (the 9 month old pip5e-PMO treated *mdx* mice are female). Taking each pip5e-PMO treated mouse individually (Table 6.15) one can see that there were some quite large differences between them, particularly in LV ejection fraction (41% vs. 65%). Correlating this with the western blotting for this experiment found that in the mouse with the worse heart function the dystrophin restoration was much higher (Figure 6.6b). This again suggests that pip5e may be toxic, especially in females.

6.2.3 Exon skipping in *dKO* mice

6.3.3.1 Differences in heart function between 8 week old *dKO* mice and wild-type controls

The dystrophin / utrophin-deficient double-knockout mouse (*dKO*) recapitulates the severity and progression of DMD more closely in the skeletal muscles and heart. The untreated *dKO* mouse has an average lifespan of 8-10 weeks (Deconinck *et al.*, 1997a), with an increase in inflammation and fibrosis in the heart at 10 weeks (Hainsey *et al.*, 2003). The exact cause of death for these mice is uncertain, but believed to be due to respiratory failure because of myopathy and kyphosis compromising respiration. The inability to eat and drink properly due to weak tongue muscles and difficulty in moving to food and water may also play a part but cardiomyopathy is not believed to.

Testing PPMOs in the *dKO* mouse could give a better idea of whether they would work in DMD patients, however there has been very little study of *dKO* hearts to date due to their extremely short lifespan (8-12 weeks). It has been published that treatment with exon-skipping compounds can increase their lifespan to 16 weeks (Goyenvallé *et al.*, 2010) and, in a continuation of that work, to at least 18 months (personal communication from Dr Aurélie Goyenvallé), however under these circumstances one cannot use age-matched untreated *dKO* controls. Therefore it was important to determine if there were sufficient significant differences in heart function at 8 weeks of age to allow the study of the effect of exon-skipping drugs on heart function at this age. The dramatic life extension in this case is believed to be due to improvements in skeletal muscle function (as the drug used does not restore dystrophin in the heart), restoring respiratory function. This finding also raised the question of whether dystrophin restoration in the heart was really necessary.

Due to the limited fecundity of *dKO* mice and the complicated crossings required to produce them from *mdx* and *utrophin null* mice (see Chapter 2) they are extremely limited in number. As it had established that there were, almost, no significant differences between male and female *C57/Bl6* mice at this age (Table 6.4) a mixed sex cohort was used for both *dKO* mice and wild-type controls. The heart function of 1 male and 6 female 8-week-old *dKO* mice was assessed using cine-MRI and compared to age-matched *C57/Bl6* wild-type controls (4 males, 7 females) (Table 6.16). The *dKO* mice were smaller than age-matched controls, though not significantly so. They did have a significant smaller LV cardiac output, muscle volume, EDL, stroke volume, PER, PFR, E and A filling rate and a significantly smaller RV stroke volume.

While there were significant differences between the *dKO* mice and wild-type controls they were fairly limited in scope, meaning it might be hard to show that a treated mouse

had heart function closer to wild-type than to *dKO*. Furthermore, while it was not significant, the *dKO* mice were smaller than wild-type and if the differences are normalised to body mass they mostly disappear. Due to this and the difficulty in obtaining significant numbers of *dKO* mice to treat and use as controls it was decided to study older treated *dKO* mice and use untreated *mdx* mice as controls. It is likely that older untreated *dKO* mice would have a more severe phenotype than age-matched *mdx* mice as the *dKO* does have a worse phenotype until its death (Deconinck *et al.*, 1997a) therefore any significant differences seen compared to untreated *mdx* mice would have greater significance when compare to untreated *dKO* mice.

6.3.3.2 Cardiac function following long-term intraperitoneal and intravenous delivery of B-PMO2 to *dKO* mice

Having established that there would be unlikely to be significant differences in heart function at 8 weeks of age it was decided to test heart function in 6 month old treated *dKO* mice, as it had already been established that one could see significant differences in the *mdx* heart at this age (see above). For this experiment *dKO* mice were injected with B-PMO2 (which differs from B-PMO1 only in the method of conjugation) either intravenously or intraperitoneally. It was found that treatment of *dKO* mice with B-PMO2 via IV injection restored dystrophin throughout all skeletal muscles, diaphragm and the heart, while treatment via intraperitoneal (IP) injection only restored dystrophin in the skeletal muscles and diaphragm (Western blot performed by Dr Haifang Yin) (Figure 6.7a). As such this would allow the direct assessment of the contribution that restoration of cardiac dystrophin would have on cardiac function.

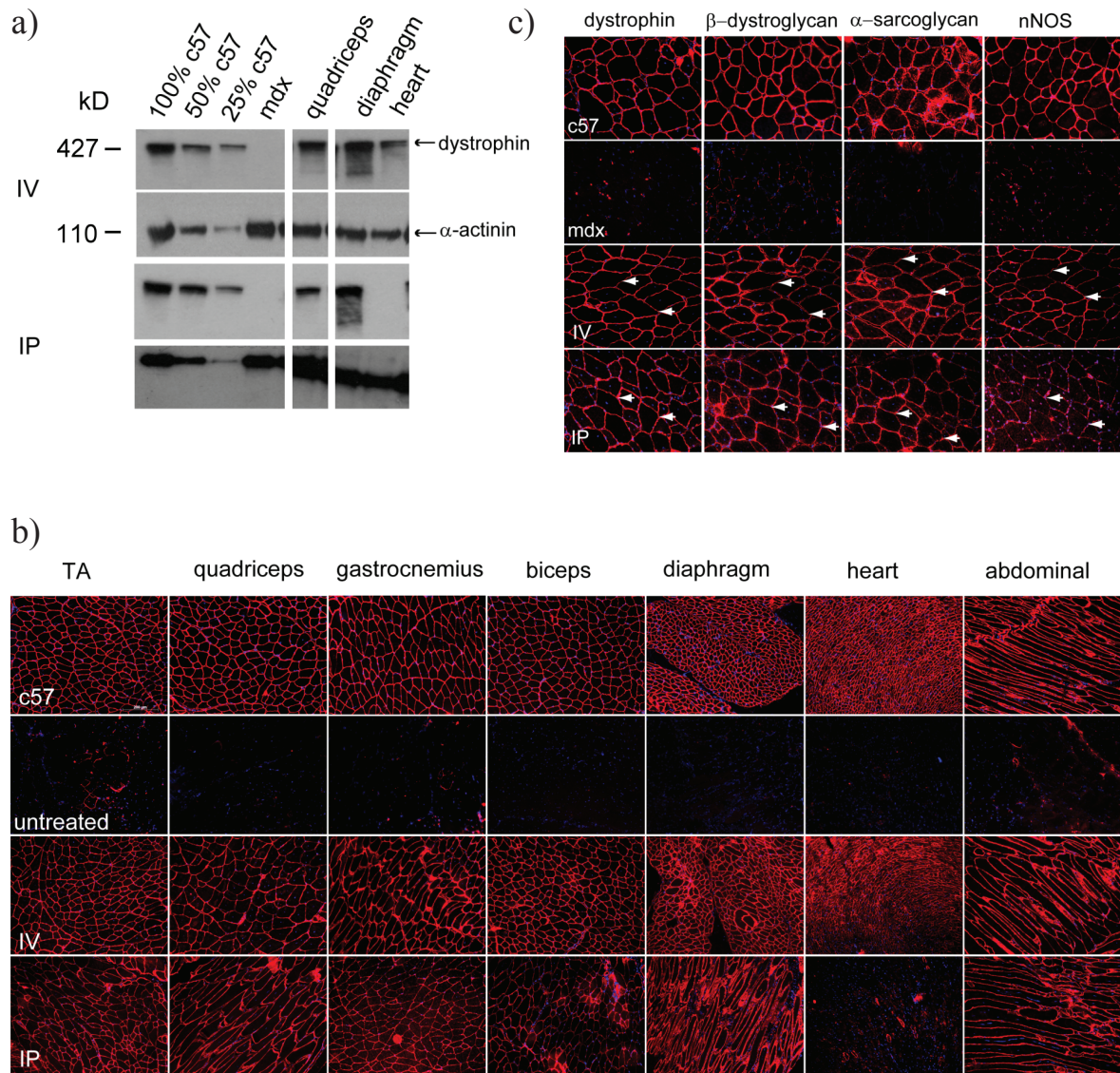


Figure 6.7 Dystrophin restoration and cardiac function improvement following repeated systemic administration of B-PMO2 to *dKO* mice. *dKO* mice were treated with 23 repeated weekly injections of B-PMO2 at 25mg/kg dose either intravenously (IV) or intraperitoneally (IP).

(a) Western blot for dystrophin expression in IV and IP treated *dKO* mice. α -actinin was used as the loading control. (b) Immunostaining of muscle tissue cross-sections to detect dystrophin protein expression and localisation in C57/BL6 normal control (top panel), untreated *dKO* mouse (second panel), IV treated (third panel) and IP treated *dKO* mice (bottom panel). Muscle tissues analysed were from tibialis anterior, gastrocnemius, quadriceps, biceps, diaphragm, heart and abdominal wall muscles (scale bar=200 μ m). (c) Re-localization of the dystrophin-associated protein complex (DAPC) in *dKO* mice treated with B-PMO2 at 25mg/kg was studied to assess dystrophin function and recovery of normal myoarchitecture. DAPC protein components β -dystroglycan, α -sarcoglycan and nNOS were detected by immunostaining in cross sections of tibialis anterior muscles from treated *dKO* mice compared with C57BL/6 normal mice and untreated *dKO* control mice. All detected DAPC components are found to be successfully re-localized to sarcolemma after treatment (scale bar=200 μ m).

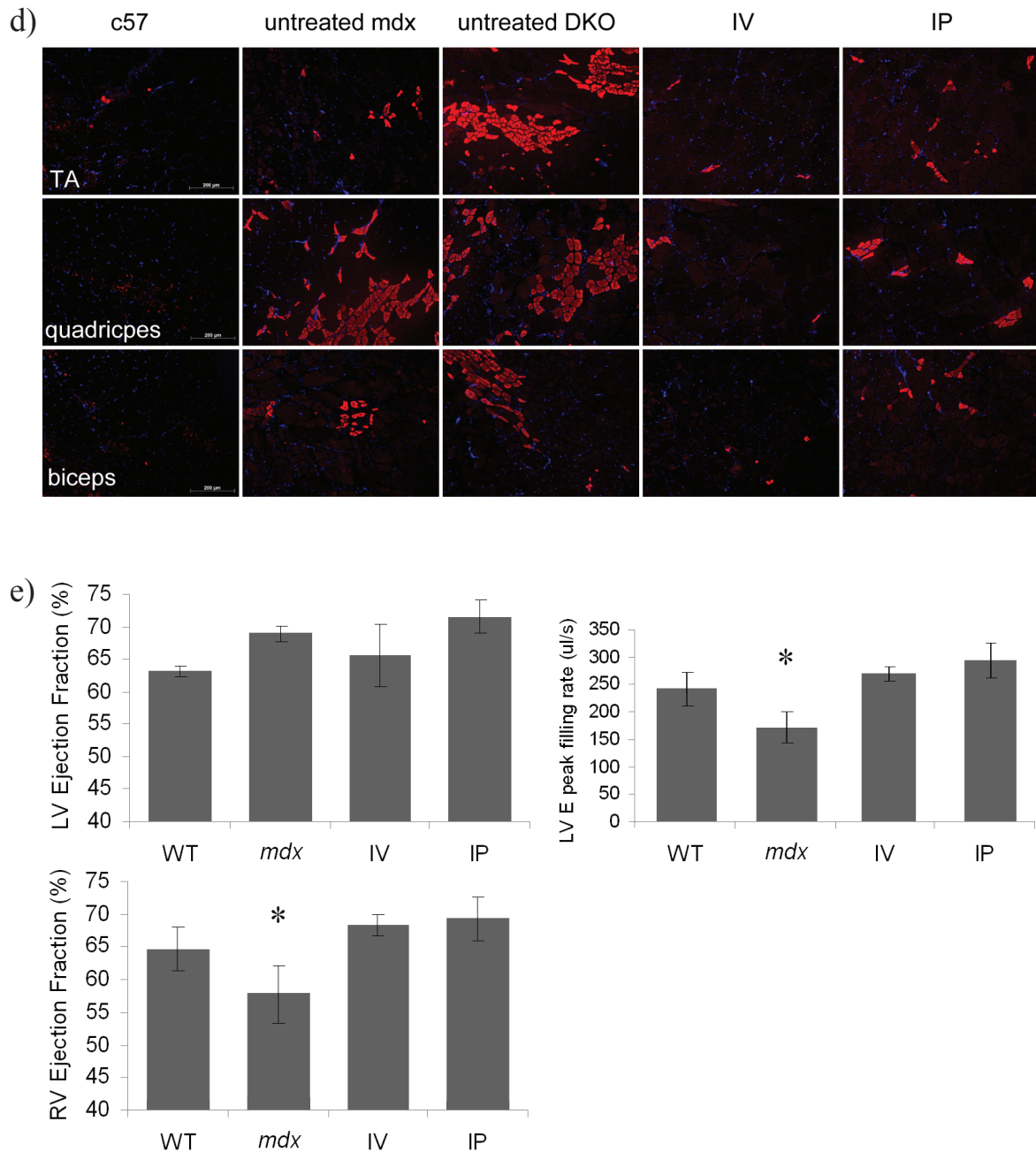


Figure 6.7 Dystrophin restoration and cardiac function improvement following repeated systemic administration of B-PMO2 to *dko* mice. *dko* mice were treated with 23 repeated weekly injections of B-PMO2 at 25mg/kg dose either intravenously (IV) or intraperitoneally (IP).

(d) Assessment of regenerating fibres in treated tibialis anterior, quadriceps and biceps muscles. Data shows the decrease of regenerating fibres in treated *dko* samples detected by early myosin isoform antibody (scale-bar=200µm). (e) *In vivo* cardiac cine-MRI of *dko* mice treated with B-PMO2 at 25mg/kg repeated doses. A significant decrease in RV ejection fraction and LV E peak filling rate was found in untreated *mdx* mice compared with age-matched wild-type *C57/Bl6* mice ($P<0.05$), but no difference in LV ejection fraction. Heart function was restored to normal levels in *dko* mice treated with B-PMO2 both intravenously and intraperitoneally. * indicates measurements significantly different to wild-type ($p<0.05$).

10-day old *dKO* mice were injected either intravenously or intraperitoneally with B-PMO2 at a 25mg/kg dose weekly for 23 weeks and sacrificed 2 weeks after the last injection.

Both survival rate and health conditions were significantly improved following treatment and all treated *dKO* mice were observed to be as healthy as their *mdx* littermate controls to the end- point of the experiment (at 6-months of age; data not shown).

Immunohistochemical staining showed that in IV and IP injected mice a near normal level of dystrophin expression was present in all the tested skeletal muscles and in IV injected mice dystrophin restoration was also present in the heart (Western blot performed by Dr Haifang Yin) (Figure 6.7b). Western blotting showed approximately 100% of normal levels of dystrophin were restored in all skeletal muscles and even in heart tissue about 50% dystrophin could be detected following IV administration of B-PMO2. In the samples treated via IP injection 100% dystrophin restoration could only be seen in diaphragm with less dystrophin (about 30-50%) restored in other skeletal muscles and no dystrophin detectable in heart (Western blot performed by Dr Haifang Yin) (Figure 6.7a).

Both administration routes resulted in the successful relocalization of multiple DAPC component proteins as shown by serial immunostaining (Immunostaining performed by Dr Haifang Yin) (Figure 6.7c) and significant force recovery when compared with age-matched untreated *mdx* controls, as shown by grip strength tests (Grip strength tests performed by Dr Haifang Yin, data not shown). Centrally-nucleated myofibres counts showed a significant reduction in both IV and IP injected mice compared with untreated age-matched *mdx* littermate controls, a finding which is corroborated by a significant decrease in the percentage of regenerating fibres detectable in treated muscles (Immunostaining performed by Dr Haifang Yin) (Figure 6.7d). Other measurements, for example of serum CK (performed by Dr Haifang Yin, data not shown) also indicated phenotypic improvement in treated *dKO* mice.

Treated *dKO* mice were imaged using cine-MRI immediately before sacrifice (at 6 months) and their heart function compared with age-matched *mdx* and wild-type C57BL6 mice (Table 6.17). A significant increase in RV ejection fraction and LV E peak filling rate was found in *dKO* mice treated via either route compared with untreated age-matched *mdx* controls (Figure 6.7e), indicating that cardiac function was improved irrespective of treatment route. Cardiac function for both groups of *dKO* mice was statistically indistinguishable from that of the age-matched wild-type C57BL6 mice controls. No additional benefit was observed in heart function in treated *dKO* mice with dystrophin restored in both diaphragm and heart compare to restoration in the diaphragm alone. These data further confirmed the hypothesis that diaphragmatic rescue is sufficient to restore heart function in dystrophic mice.

6.3.3.3 Cardiac function following long-term intraperitoneal delivery of p007-PMO to *dKO* mice

As mentioned above it has recently been demonstrated that PPMO treatment could prevent the onset of dystrophic pathology in, and increase the lifespan of, the *dKO* mouse (Goyenvalle *et al.*, 2010). Interestingly, whilst these *dKO* mice appeared almost completely rescued, there was no dystrophin restoration in the heart, making it possible that they would develop cardiomyopathy as they aged. The mice showed relocation of DAPC components, reduction in serum CK levels and normalized force generation in both fore- and hindlimb muscles. In collaboration with Aurelie Goyenvalle, the heart function of these mice was studied to see how it changed with age.

Ten-day old *dKO* mice were injected intraperitoneally with 25 mg/kg/week of P007-PMO for 6 weeks, as previously described (Goyenvallé *et al.*, 2010), then subsequently re-injected monthly at the same dose. This treatment resulted in an improvement of their dystrophic pathology and an increase in lifespan. Of four mice studied two were sacrificed at 13 months to confirm levels of dystrophin restoration in heart, diaphragm and other muscles, and two remain alive at 18 months. RNA and protein analysis of the treated muscles at 13 months confirmed the previous results obtained at 13 weeks (Goyenvallé *et al.*, 2010). Dystrophin was present in all the tested muscles except heart, with particularly high levels of restoration in the diaphragm (Western blot performed by Dr Aurélie Goyenvallé) (Figure 6.8a), consistent with the immunohistochemical staining (data not shown).

Due to the limited availability of *dKO* mice the mice in this study had variable ages. When the decision was taken to study their heart function it was too late to image them all at 6 months. Initially two mice were imaged using cine-MRI at 9 months and two mice were imaged at 6 months (to be reimaged when they had reached 9 months). Cardiac function was compared with age-matched *mdx* and wild-type *C57/BL6* mice. At 6 months the two p007-treated *dKO* mice were significantly smaller than age-matched untreated *mdx* controls and significantly larger than age and sex matched wild-type controls (Table 6.18). They had a significantly smaller LV ejection fraction and cardiac output than the untreated *mdx* mice and, unlike in previous experiments, their RV ejection fraction was not significantly increased. At 9 months the two p007-treated *dKO* mice had a significantly larger LV and RV ejection fraction than age-matched *mdx* mice (Table 6.19). This discrepancy may have been due to the length of time from the monthly reinjection till the MRI to determine cardiac function. While the 9 month old mice were imaged 7 days after

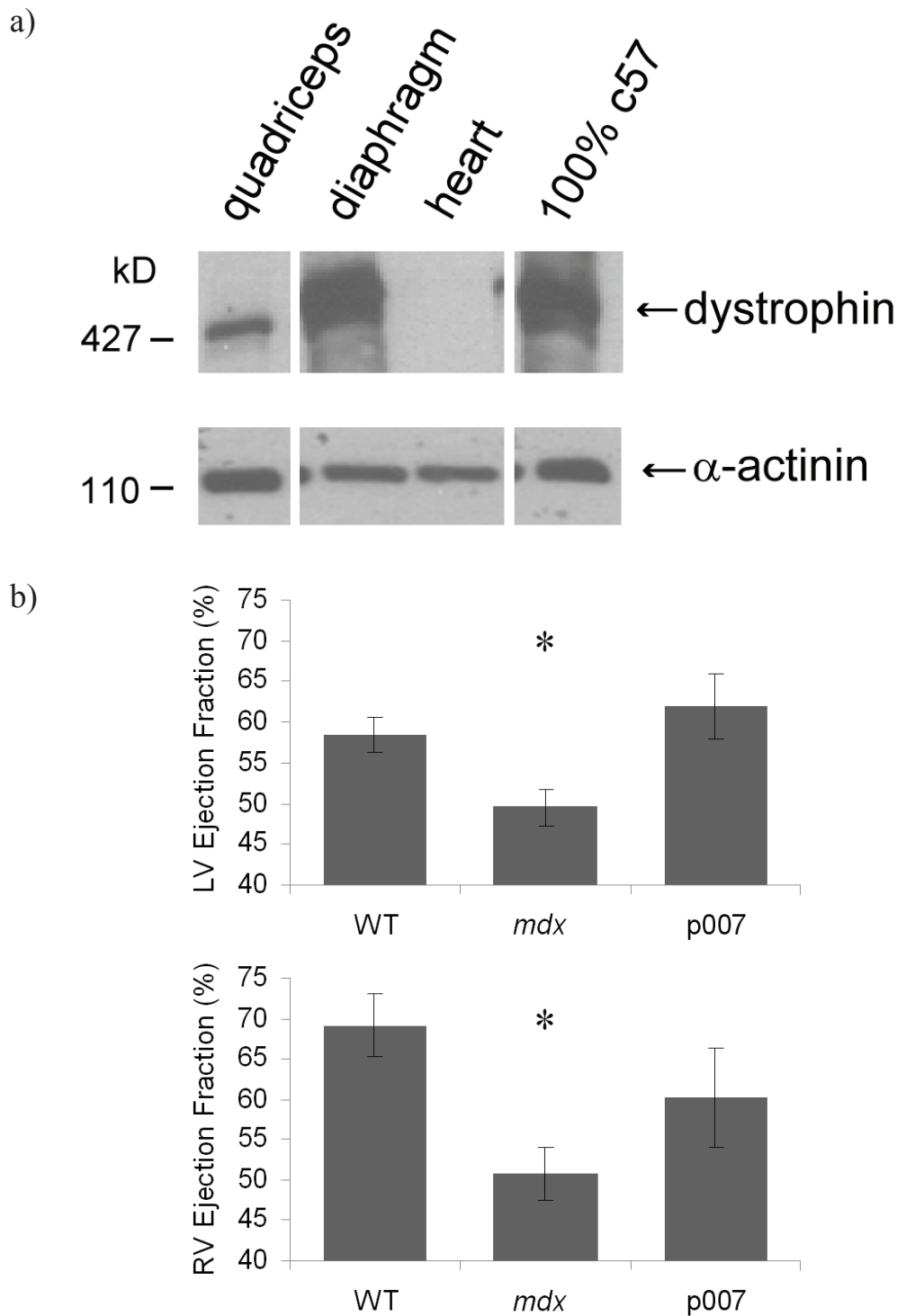


Figure 6.8 Dystrophin restoration and cardiac function improvement in *dKO* mice treated with P007-PMO at 25mg/kg monthly repeated doses intraperitoneally.

(a) Western blot for dystrophin expression in P007-PMO treated *dKO* mice. α -actinin was used as the loading control. (b) *In vivo* cardiac cine-MRI of *dKO* mice treated with P007-PMO at 25mg/kg repeated doses. A significant decrease in LV and RV ejection fraction was found in untreated *mdx* mice compared with age-matched *C57/Bl6* mice ($P < 0.05$). In treated *dKO* mice LV ejection fraction was restored to normal levels and RV ejection fraction restored to near-normal levels. * indicates measurements significantly different to WT ($p < 0.05$).

reinjection the 6 month old mice were not imaged until 20 days after reinjection. As such when the 6 month old mice were re-imaged at 9 months old it was done at approximately 7 days after reinjection.

When all four mice had been imaged at 9 months their cardiac function was compared with age-matched *mdx* and wild-type *C57/BL6* mice (Table 6.20). A significant increase in LV ejection fraction (to wild-type levels) and an increase in RV ejection fraction (to near wild-type levels) were found compared with untreated age-matched *mdx* controls (Figure 6.8b), as well as significant reductions in muscle volume and LV EDL and ESL to wild-type levels.

This data again supports the hypothesis that diaphragm dystrophin function alone is sufficient to restore heart function but, importantly, it also suggests that PPMO treatment can improve heart function rather than just prevent its deterioration. Between the 6 month scan, where the two younger *dKO* mice had no improvement in function compared to untreated *mdx* controls, and the 9 month scan, where they did, LV and RV ejection fraction increased by ~4%. Over the same period *mdx* heart function declined, with LV ejection fraction falling by ~20% and RV ejection fraction falling by ~7%. This suggests that the heart function in these *dKO* mice varies sinusoidally with time, first increasing after the monthly reinjection, peaking, and then falling again over the month. This would be undesirable in humans but could likely be avoided by varying the dose and administration route.

6.4 Discussion

AO-mediated exon skipping is a promising therapeutic candidate for the treatment of DMD. Work on skeletal muscle treatment has been ongoing for some time, but studies have recently started to focus on rescuing the heart. In the study presented here, using *mdx* and *dKO* animal models and evaluating both therapeutic approaches, it is demonstrated that compensation for or restoration of diaphragmatic dystrophin is sufficient to rescue cardiac function without direct treatment of the heart itself.

Recently exon-skipping AOs have been studied in clinical trials in the Netherlands (van Deutekom *et al.*, 2007) and United Kingdom (Kinali *et al.*, 2009). However based on the data from pre-clinical animal models, high doses of unmodified AOs are likely to be required for correction of dystrophin expression in skeletal muscles and the amount of dystrophin restoration in the heart muscle remains very low (Alter *et al.*, 2006; Lu *et al.*, 2005). Conjugation of cell-penetrating peptides to PMOs has resulted in remarkable and sustained dystrophin restoration in skeletal muscles and also, at lower levels, in heart (Jearawiriyapaisarn *et al.*, 2008; Jearawiriyapaisarn *et al.*, 2010; Wu *et al.*, 2008; Yin *et al.*, 2008). A number of these studies (Jearawiriyapaisarn *et al.*, 2010; Wu *et al.*, 2008) have shown improvement in cardiac function following treatment of the *mdx* mouse with these PPMOs. The above results show that following single injections of B-PMO1 in *mdx* mice, leading to restoration of high levels of dystrophin in the diaphragm, but none in the heart, normal levels of cardiac function are found. This suggests that the previous studies may have incorrectly attributed the observed improvements in cardiac function to dystrophin restoration in the heart.

In the more severely affected *dKO* model it was found that long-term IP administration of P007-PMO restored high levels of dystrophin to the skeletal muscles and diaphragm but not the heart, resulting in a greatly increased life-span, with treated mice continuing to live beyond 18 months. These mice showed essentially normal cardiac function at 9 months, further supporting the result in the *mdx* mice. Furthermore in *dKO* mice treated with B-PMO2, despite the difference in restoration of dystrophin expression in the heart between IP and IV administration routes, there was no significant difference in terms of phenotypic, functional and morphological parameters and both groups displayed normalised cardiac function at 6 months. This suggests that, at least up to 9 months of age, cardiac function in dystrophic mice can be normalised without directly treating the heart. This data supports the results found following utrophin upregulation in the diaphragm (see Chapter 5) and suggests that in the *mdx* mouse restoration of normal diaphragm function, irrespective of method, is sufficient to restore heart function to wild-type levels.

The biggest argument against this hypothesis is that diaphragm function was not actually measured, either here or in chapter 5. With respect to chapter 5, diaphragm function has previously been measured for *Fiona* mice and found to be improved in them, compared to *mdx* mice, and western blots have shown that the levels of utrophin upregulation in the *Fiona* mice used here were the same as those in the original mice. As such it is not unreasonable to conclude that diaphragm function in the *Fiona* mice used here is the same as when originally measured. However diaphragm function was never measured for the mice used here. This is not to say that no measurements of force generation were ever made. For both sets of *dKO* mice improvements in force generation were demonstrated following treatment, for the B-PMO2 treated mice grip strength was significantly improved, while for the P007-PMO treated mice the original study found an improvement

in grip strength and hind limb muscle function, with a significant increase in peak force generation (Goyenvalle *et al.*, 2010). Grip strength has also been shown to be significantly improved in *mdx* mice treated with B-PMO2 (Wu *et al.*, 2008). From this it seemed likely that restoration of dystrophin at the levels found in this chapter was sufficient to restore function to skeletal muscle, including the diaphragm.

Findings from a number of previous papers also support the hypothesis that cardiac function in dystrophic mice can be normalised without directly treating the heart. Bostick *et al* (Bostick *et al.*, 2009) found that heart-specific expression of a mini-*dystrophin* gene, that was sufficient to restore skeletal muscle function, could not restore full heart function in 20 month old *mdx* mice. The above findings suggest that this was because of the poor diaphragm function in *mdx* mice this old (Stedman *et al.*, 1991). Jearawiriyapaisarn *et al* (Jearawiriyapaisarn *et al.*, 2010) treated 8 week old *mdx* mice with a PPMO, which restored dystrophin in the skeletal muscle and the heart, and found long-term improvements in heart function (normal to 11 months) despite short term cardiac dystrophin restoration (<1% present at 7 months). This is likely due to long-term diaphragm dystrophin restoration.

Previous work has suggested that functional improvement in skeletal muscles due to dystrophin restoration may be harmful to the heart (Townsend *et al.*, 2008), due to an increase in exercise capacity putting greater stress on the heart. However there is a well established mechanism whereby compromised lung function affects RV function (Simmons *et al.*, 1961), and it has been shown in DMD patients that pulmonary hypertension is occurring and that it increases right atrial pressure, suggesting that RV dysfunction may be present in DMD (Yotsukura *et al.*, 1988). This pulmonary

hypertension has also been shown to occur both with and without LV involvement, proving that, at least some of the time, it is not pulmonary venous hypertension (Melacini *et al.*, 1996b). It is likely that utrophin upregulation or dystrophin restoration in the respiratory muscles, most importantly in the diaphragm, prevents a decline in lung function; leading to the assertion that it is the improvement in diaphragm function that is responsible for the improvement in heart function.

There is very little published work on the RV in DMD, other than Yotsukura *et al.*, which showed an increase in right atrial pressure (Yotsukura *et al.*, 1988), however there is evidence that RV dysfunction precedes LV dysfunction in BMD (Melacini *et al.*, 1996a), which strongly suggests this is also the case in DMD. In BMD the incidence of cardiomyopathy is highest in patients with few to no skeletal symptoms (Melacini *et al.*, 1996a). This is believed to be because, unaware of their dystrophinopathy, they may exercise heavily putting stress on a heart that cannot handle it, as has been shown in the *mdx* mouse (Townsend *et al.*, 2008). In DMD patients typically develop cardiac symptoms early in their teens but, even those that do die of heart failure ($\sim 1/3^{\text{rd}}$), usually do not die for another 10 years. This is believed to be because they are typically wheelchair bound so they put very little stress on their hearts. This evidence suggests that the course of cardiomyopathy in DMD/BMD is similar to that in *mdx* mice and so an improvement in respiratory function would improve heart function. This likely already occurs, as many treatments for DMD improve respiratory function. There is also a heart dysfunction independent of respiratory function as evidenced by the extremely poor response of the dystrophic heart to stress and so heart specific treatment remains necessary.

Some studies in *mdx* mice hearts indicate that decreasing afterload can improve heart function, which is taken to be evidence of a heart defect (Adamo *et al.*, 2010; Spurney *et al.*, 2011). This is demonstrated by using drugs that reduce hypertension (Sildenafil – a cGMP-specific PDE5 inhibitor and Losartan - an angiotensin II receptor antagonist respectively). As the proposed mechanism for the diaphragm damage to affect the heart is via pulmonary arterial hypertension increasing afterload on the RV then these drugs would also be treating this; in fact Sildenafil is specifically used for this. Both drugs have been shown to improve heart function in various other models of heart dysfunction in animals (Pourjabbar *et al.*, 2005; Salloum *et al.*, 2008; Takimoto *et al.*, 2005) and also in humans (Dahlof *et al.*, 2002; Konstam *et al.*, 2000), so it remains possible that in *mdx* mice they also treat a heart specific defect by reducing afterload or by acting specifically on cardiomyocytes, however these effects cannot be distinguished from these experiments.

The data establishes that, to see the effects of exon-skipping compounds on unstressed *mdx* heart function, it is necessary to wait for at least 6 months of age, or at least 9 months of age to see LV systolic changes; though this age limitation may be overcome in the stressed heart (see Discussion Chapter 5). The same effect can be seen in the more seriously affected *dKO* heart, though, having not looked at 3 months, one can only say that *dKO* mice must be older than 8 weeks to see the effects of exon-skipping compounds on unstressed heart function. However, given the up to 60% mortality rate seen in dobutamine stressed *mdx* mice at 4 months (Wu *et al.*, 2008), it seems likely that stressing the *dKO* heart would prove universally fatal. The data also suggests that Pip5e-PMO is possibly toxic. This is the first evidence of cardiac toxicity in PPMO treatment and, importantly, the observed reduction in heart function is occurring despite very high levels of dystrophin restoration. This means that high levels of dystrophin restoration do not automatically

confer functional benefits and that it is important to always measure function in PPMO-treated muscles. Finally the data hints at a possible answer to one of the questions raised in the Discussion of the previous chapter: Is improved diaphragm function just maintaining heart function or can improving diaphragm function improve heart function? In the P007-PMO treated *dKO* mice heart function was varying other the month between reinjections and was shown to increase between mice imaged 20 days after reinjection and the same mice imaged ~3 months later, 7 days after reinjection, suggesting that, improving diaphragm function later in life may be able to improve heart function.

In summary this study indicates that, in both the *mdx* and *dKO* models of DMD, treatment of the heart is not necessary in order to improve heart function. Restoration of diaphragm (and other respiratory muscle) function, irrespective of method and AO chemistry, is sufficient to restore cardiac function to near normal levels in mice up to 9 months of age. This suggests that, in dystrophic mice, the use of PPMOs with heart activity may be unnecessary. Further work is necessary to establish whether normal diaphragm function remains sufficient to preserve cardiac function in older mice and to determine whether stressing the heart, for example by forced running or high-dose dobutamine infusion, may give a measure of cardiac function that is independent of diaphragm function. These observations provide a novel mechanism for dystrophic cardiomyopathy and its correction and suggest that treatments that maintain or restore diaphragmatic function are likely to have consequent benefits for heart function in DMD patients.

Table 6.1: Oligonucleotide and peptide nomenclature and sequences

Name	Sequence	Abbreviation	Length
M23D	5'-GGCCAAACCTCGGCTTACCTGAAAT-3'	PMO	25
P007	N- RXRRXRRXRRXRXB -C	(RXR) ₄ XB	14
B peptide	N- RXRRBRRXRRBRXB -C	(RXRRBR) ₂ XB	14
pip5e	N- RXRRBRRXRILFQYRXRBRXRB -C	-	22

Sequence of the exon 23 boundary targeting PMO and the cell-penetrating peptides conjugated to it to produce PPMOs.

Where R=L-arginine, X=6-aminohexanoic acid, B=β-alanine, I=isoleucine, L=leucine, F=phenylalanine, Q=glutamine, Y=tyrosine; B-PMO1 and B-PMO2 were from different suppliers. B-PMO1 and pip5e-PMO were provided by Gait's laboratory (Medical Research Council, Laboratory of Molecular Biology, Cambridge, UK) using the synthesis and conjugation protocols as reported (Ivanova et al., 2008; H.Yin and A.Saleh, manuscript submitted). B-PMO2 and p007-PMO were from AVI Biopharma (Oregon, USA) using published methods (Wu et al., 2007).

Table 6.4: Heart function in 8 week old male and female C57/Bl6 mice.

8 week old C57/Bl6		M	F
Number of mice		4	7
Body Mass	g	18.2 ± 1.5	17.8 ± 0.3
Heart Rate	bpm	485 ± 14	497 ± 31
LV			
Ejection Fraction	%	70.4 ± 2.3	68.0 ± 2.4
Cardiac Output	ml/min	14.5 ± 1.4	15.3 ± 1.2
Heart Mass as % BM	%	0.42 ± 0.01	0.42 ± 0.02
Muscle Volume	μl	74 ± 8.9	72 ± 4.7
End Diastolic Lumen	μl	42.7 ± 4.8	45.4 ± 2.5
End Systolic Lumen	μl	12.8 ± 2.5	14.6 ± 1.5
Stroke Volume	μl	29.8 ± 2.3	30.8 ± 1.7
PER	μl/s	477 ± 20	329 ± 56
PFR	μl/s	296 ± 70	354 ± 27
E	μl/s		323 ± 19
A	μl/s		284 ± 42
E/A			1.28 ± 0.2
RV			
Ejection Fraction	%	71.6 ± 3.7	74.6 ± 1.7
Cardiac Output	ml/min	10.3 ± 3.5	13.3 ± 2.3
End Diastolic Lumen	μl	42.4 ± 5.9	41.9 ± 2.4
End Systolic Lumen	μl	12.5 ± 3.5	10.7 ± 1.1
Stroke Volume	μl	29.9 ± 2.4	31.2 ± 1.5

Data obtained by *in vivo* cardiac cine-MRI of male and female C57/Bl6 mice at 8 weeks of age. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

* indicates measurements significantly different to left hand column (P>0.05)

** indicates measurements highly significantly different to left hand column (P>0.005)

Table 6.5: Heart function in 3 month old male *mdx* and female *mdx* mice.

6 month old <i>mdx</i>		M	F
Number of mice		6	6
Body Mass	g	28.3 ± 0.7	22.5 ± 0.4 **
Heart Rate	bpm	455 ± 32	402 ± 12
LV			
Ejection Fraction	%	70.3 ± 2.2	69.1 ± 3.3
Cardiac Output	ml/min	18.7 ± 1.6	13.5 ± 0.6 *
Heart Mass as % BM	%	0.38 ± 0.01	0.36 ± 0.02
Muscle Volume	μl	102 ± 2	78.1 ± 4.6 **
End Diastolic Lumen	μl	58.7 ± 3.9	49.1 ± 2.4
End Systolic Lumen	μl	17.5 ± 2.1	15.3 ± 2.1
Stroke Volume	μl	41.2 ± 2.5	33.8 ± 1.7 *
PER	μl/s	316 ± 60	211 ± 17
PFR	μl/s	267 ± 29	305 ± 59
E	μl/s	266 ± 28	267 ± 49
A	μl/s	200 ± 25	271 ± 50
E/A		1.36 ± 0.11	1.01 ± 0.13
CO by Body Mass	ml/min/g	0.65 ± 0.04	0.60 ± 0.02
EDL by Body Mass	ul/g	2.06 ± 0.09	2.18 ± 0.11
ESL by Body Mass	ul/g	0.61 ± 0.06	0.68 ± 0.09
SV by Body Mass	ul/g	1.45 ± 0.06	1.50 ± 0.06
RV			
Ejection Fraction	%	64.2 ± 3.9	72.9 ± 2.1
Cardiac Output	ml/min	16.5 ± 1.2	13.3 ± 0.8 *
End Diastolic Lumen	μl	58.3 ± 1.7	45.3 ± 2.2 **
End Systolic Lumen	μl	20.8 ± 2.4	12.2 ± 1.0 *
Stroke Volume	μl	37.4 ± 2.3	33.1 ± 2.0
CO by Body Mass	ml/min/g	0.58 ± 0.03	0.58 ± 0.02
EDL by Body Mass	ul/g	2.06 ± 0.08	2.01 ± 0.08
ESL by Body Mass	ul/g	0.74 ± 0.10	0.54 ± 0.04
SV by Body Mass	ul/g	1.31 ± 0.06	1.46 ± 0.06

Data obtained by *in vivo* cardiac cine-MRI of male and female *mdx* mice at 3 months of age. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

* indicates measurements significantly different to left hand column (P>0.05)

** indicates measurements highly significantly different to left hand column (P>0.005)

Table 6.6: Heart function in 6 month old male *C57/Bl10* and male *C57/Bl6* mice.

6 month old		'Clean' <i>C57/Bl10</i>	'Dirty' <i>C57/Bl6</i>
Number of mice		9	8
Body Mass	g	29.7 ± 0.9	29.3 ± 1.0
Heart Rate	bpm	427 ± 13	434 ± 20
LV			
Ejection Fraction	%	59.8 ± 2.8	61.0 ± 2.9
Cardiac Output	ml/min	20.0 ± 1.2	20.1 ± 1.4
Heart Mass as % BM	%	0.44 ± 0.01	0.43 ± 0.01
Muscle Volume	μl	127 ± 8	123 ± 8.1
End Diastolic Lumen	μl	78.3 ± 5.2	76.9 ± 5.7
End Systolic Lumen	μl	32.2 ± 3.9	30.6 ± 4.1
Stroke Volume	μl	46.1 ± 1.9	46.2 ± 2.2
PER	μl/s	304 ± 21	313 ± 22
PFR	μl/s	325 ± 41	325 ± 44
E	μl/s	325 ± 41	325 ± 44
A	μl/s	256 ± 42	256 ± 45
E/A		1.51 ± 0.20	1.51 ± 0.2
RV			
Ejection Fraction	%	68.1 ± 3.3	69.8 ± 3.3
Cardiac Output	ml/min	22.7 ± 1.5	23.0 ± 1.7
End Diastolic Lumen	μl	77.4 ± 4.8	76.8 ± 5.4
End Systolic Lumen	μl	25.3 ± 3.7	23.8 ± 3.8
Stroke Volume	μl	52.1 ± 2.6	52.9 ± 2.8

Data obtained by *in vivo* cardiac cine-MRI of male *C57/Bl10* and *C57/Bl6* mice at 6 months of age. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

* indicates measurements significantly different to left hand column (P>0.05)

** indicates measurements highly significantly different to left hand column (P>0.005)

Table 6.7: Heart function in 6 month old male C57/Bl6 and female C57/Bl6 mice.

6 month old C57/Bl6		M	F
Number of mice		8	8
Body Mass	g	29.3 ± 1.0	23.1 ± 0.8 **
Heart Rate	bpm	434 ± 20	454 ± 11
LV			
Ejection Fraction	%	61.0 ± 2.9	63.1 ± 0.7
Cardiac Output	ml/min	20.1 ± 1.4	15.6 ± 1.1 *
Heart Mass as % BM	%	0.43 ± 0.01	0.42 ± 0.01
Muscle Volume	μl	123 ± 8.1	92 ± 5.5 *
End Diastolic Lumen	μl	76.9 ± 5.7	54.0 ± 2.4 **
End Systolic Lumen	μl	30.6 ± 4.1	19.8 ± 0.7 *
Stroke Volume	μl	46.2 ± 2.2	34.1 ± 1.7 **
PER	μl/s	313 ± 22	254 ± 18 *
PFR	μl/s	325 ± 44	361 ± 46
E	μl/s	325 ± 44	242 ± 30
A	μl/s	256 ± 45	283 ± 59
E/A		1.51 ± 0.2	1.19 ± 0.3
CO by Body Mass	ml/min/g	0.68 ± 0.03	0.67 ± 0.04
EDL by Body Mass	ul/g	2.60 ± 0.13	2.33 ± 0.08
ESL by Body Mass	ul/g	1.03 ± 0.11	0.86 ± 0.03
SV by Body Mass	ul/g	1.57 ± 0.06	1.47 ± 0.06
RV			
Ejection Fraction	%	69.8 ± 3.3	64.6 ± 3.3
Cardiac Output	ml/min	23.0 ± 1.7	14.6 ± 1.0 **
End Diastolic Lumen	μl	76.8 ± 5.4	51.1 ± 3.4 **
End Systolic Lumen	μl	23.8 ± 3.8	18.5 ± 2.9
Stroke Volume	μl	52.9 ± 2.8	32.6 ± 1.7 **
CO by Body Mass	ml/min/g	0.78 ± 0.04	0.63 ± 0.03 *
EDL by Body Mass	ul/g	2.60 ± 0.13	2.21 ± 0.13 *
ESL by Body Mass	ul/g	0.80 ± 0.11	0.80 ± 0.12
SV by Body Mass	ul/g	1.80 ± 0.09	1.41 ± 0.06 **

Data obtained by *in vivo* cardiac cine-MRI of male and female C57/Bl6 mice at 6 months of age. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

* indicates measurements significantly different to left hand column (P>0.05)

** indicates measurements highly significantly different to left hand column (P>0.005)

Table 6.8: Heart function in 6 month old male *mdx* mice from the ‘clean’ and ‘dirty’ animal houses.

6 month old <i>mdx</i>		clean	dirty
Number of mice		9	6
Body Mass	g	35.4 ± 1.4	32.7 ± 0.8
Heart Rate	bpm	426 ± 18	463 ± 16
LV			
Ejection Fraction	%	64.0 ± 1.7	69 ± 1.2 *
Cardiac Output	ml/min	16.5 ± 1.3	16.3 ± 0.6
Heart Mass as % BM	%	0.38 ± 0.01	0.38 ± 0.01
Muscle Volume	μl	129 ± 5.9	119 ± 5.2
End Diastolic Lumen	μl	60.5 ± 4.4	51.2 ± 2.8
End Systolic Lumen	μl	22.2 ± 2.7	16.0 ± 1.4
Stroke Volume	μl	38.3 ± 1.9	35.2 ± 1.5
PER	μl/s	201 ± 39	290 ± 24
PFR	μl/s	320 ± 93	185 ± 20
E	μl/s	161 ± 32	142 ± 28
A	μl/s	320 ± 93	185 ± 20
E/A		0.78 ± 0.1	0.98 ± 0.2
RV			
Ejection Fraction	%	53.4 ± 3.6	57.8 ± 4.4
Cardiac Output	ml/min	18.4 ± 1.1	16.0 ± 1.9
End Diastolic Lumen	μl	81.9 ± 6.6	63.1 ± 6.6
End Systolic Lumen	μl	39.5 ± 5.4	27.3 ± 5.1
Stroke Volume	μl	42.4 ± 2.2	35.8 ± 2.8

Data obtained by *in vivo* cardiac cine-MRI of male *mdx* mice from the ‘clean’ and ‘dirty’ animal houses at 6 months of age. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

* indicates measurements significantly different to left hand column (P>0.05)

** indicates measurements highly significantly different to left hand column (P>0.005)

Table 6.9: Heart function in 9 month old male *C57/Bl10* and male *C57/Bl6* mice.

9 month old		'Clean' <i>C57/Bl10</i>	'Dirty' <i>C57/Bl6</i>
Number of mice		8	6
Body Mass	g	33.3 ± 0.6	32.6 ± 1.4
Heart Rate	bpm	447 ± 10	488 ± 18
LV			
Ejection Fraction	%	58.2 ± 3.1	54.4 ± 1.6
Cardiac Output	ml/min	22.5 ± 0.9	21.7 ± 1.6
Heart Mass as % BM	%	0.46 ± 0.01	0.46 ± 0.01
Muscle Volume	μl	147 ± 7.3	143 ± 5.8
End Diastolic Lumen	μl	88.7 ± 7.1	81.9 ± 5.4
End Systolic Lumen	μl	38.0 ± 5.3	37.5 ± 3.3
Stroke Volume	μl	50.7 ± 2.9	44.4 ± 2.5
PER	μl/s	390 ± 27	324 ± 40
PFR	μl/s	438 ± 72	398 ± 63
E	μl/s	261 ± 17	275 ± 13
A	μl/s	286 ± 13	251 ± 30
E/A		0.92 ± 0.1	1.12 ± 0.0
RV			
Ejection Fraction	%	68.7 ± 2.5	62.7 ± 3.6
Cardiac Output	ml/min	17.9 ± 4.0	9.26 ± 5.3
End Diastolic Lumen	μl	76.0 ± 2.3	81.2 ± 6.5
End Systolic Lumen	μl	23.7 ± 2.0	31.0 ± 5.5
Stroke Volume	μl	52.2 ± 2.4	50.2 ± 2.6

Data obtained by *in vivo* cardiac cine-MRI of male *C57/Bl10* and *C57/Bl6* mice at 9 months of age. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

* indicates measurements significantly different to left hand column (P>0.05)

** indicates measurements highly significantly different to left hand column (P>0.005)

Table 6.10: Heart function in 9 month old male C57/Bl6 and female C57/Bl6 mice.

9 month old C57/Bl6		M	F
Number of mice		6	7
Body Mass	g	32.6 ± 1.4	24.4 ± 0.7 **
Heart Rate	bpm	488 ± 18	444 ± 14
LV			
Ejection Fraction	%	54.4 ± 1.6	58.5 ± 2.2
Cardiac Output	ml/min	21.7 ± 1.6	15.4 ± 0.8 *
Heart Mass as % BM	%	0.46 ± 0.01	0.46 ± 0.01
Muscle Volume	μl	143 ± 5.8	108 ± 3.2 **
End Diastolic Lumen	μl	81.9 ± 5.4	60.6 ± 3.0 *
End Systolic Lumen	μl	37.5 ± 3.3	25.2 ± 2.0 *
Stroke Volume	μl	44.4 ± 2.5	35.4 ± 2.0 *
PER	μl/s	324 ± 40	249 ± 15
PFR	μl/s	398 ± 63	372 ± 32
E	μl/s	275 ± 13	279 ± 35
A	μl/s	251 ± 30	368 ± 31
E/A		1.12 ± 0.0	0.75 ± 0.0
CO by Body Mass	ml/min/g	0.67 ± 0.05	0.63 ± 0.02
EDL by Body Mass	ul/g	2.51 ± 0.14	2.47 ± 0.06
ESL by Body Mass	ul/g	1.15 ± 0.09	1.02 ± 0.06
SV by Body Mass	ul/g	1.36 ± 0.07	1.44 ± 0.06
RV			
Ejection Fraction	%	62.7 ± 3.6	69.1 ± 3.9
Cardiac Output	ml/min	9.26 ± 5.3	15.3 ± 2.9
End Diastolic Lumen	μl	81.2 ± 6.5	57.8 ± 2.8 *
End Systolic Lumen	μl	31.0 ± 5.5	17.7 ± 2.3 *
Stroke Volume	μl	50.2 ± 2.6	40.1 ± 3.3 *
CO by Body Mass	ml/min/g	0.28 ± 0.16	0.62 ± 0.11
EDL by Body Mass	ul/g	2.49 ± 0.19	2.37 ± 0.10
ESL by Body Mass	ul/g	0.95 ± 0.16	0.73 ± 0.11
SV by Body Mass	ul/g	1.54 ± 0.07	1.63 ± 0.11

Data obtained by *in vivo* cardiac cine-MRI of male and female C57/Bl6 mice at 9 months of age. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

* indicates measurements significantly different to left hand column (P>0.05)

** indicates measurements highly significantly different to left hand column (P>0.005)

Table 6.11: Heart function in 9 month old male *mdx* mice from the ‘clean’ and ‘dirty’ animal houses.

9 month old <i>mdx</i>		clean	dirty
Number of mice		9	4
Body Mass	g	38.6 ± 1.1	26.2 ± 1.5 **
Heart Rate	bpm	464 ± 13	410 ± 52
LV			
Ejection Fraction	%	49.5 ± 2.2	62.2 ± 4.3 *
Cardiac Output	ml/min	18.8 ± 1.1	15.2 ± 2.2
Heart Mass as % BM	%	0.39 ± 0.01	0.40 ± 0.03
Muscle Volume	μl	145 ± 7.0	101 ± 10 *
End Diastolic Lumen	μl	82.6 ± 5.1	60.2 ± 4.6 *
End Systolic Lumen	μl	41.8 ± 3.2	23.1 ± 4.2 *
Stroke Volume	μl	40.8 ± 3.0	37.0 ± 1.2
PER	μl/s	274 ± 20	230 ± 11
PFR	μl/s	323 ± 51	411 ± 13
E	μl/s	292 ± 57	222 ± 22
A	μl/s	250 ± 32	352 ± 17
E/A		1.16 ± 0.1	1.10 ± 0.6
CO by Body Mass	ml/min/g	0.48 ± 0.03	0.57 ± 0.07
EDL by Body Mass	ul/g	2.13 ± 0.11	2.28 ± 0.04
ESL by Body Mass	ul/g	1.07 ± 0.06	0.86 ± 0.11
SV by Body Mass	ul/g	1.05 ± 0.07	1.42 ± 0.07 *
RV			
Ejection Fraction	%	50.7 ± 3.2	65.8 ± 4.6
Cardiac Output	ml/min	21.6 ± 1.1	11.0 ± 4.0 *
End Diastolic Lumen	μl	95.3 ± 9.4	54.1 ± 2.0 **
End Systolic Lumen	μl	48.6 ± 8.5	18.6 ± 3.1 *
Stroke Volume	μl	46.6 ± 2.3	35.4 ± 1.5 **
CO by Body Mass	ml/min/g	0.56 ± 0.03	0.43 ± 0.15
EDL by Body Mass	ul/g	2.45 ± 0.20	2.07 ± 0.07
ESL by Body Mass	ul/g	1.24 ± 0.19	0.70 ± 0.08 *
SV by Body Mass	ul/g	1.20 ± 0.05	1.36 ± 0.13

Data obtained by *in vivo* cardiac cine-MRI of male *mdx* mice from the ‘clean’ and ‘dirty’ animal houses at 9 months of age. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

* indicates measurements significantly different to left hand column (P>0.05)

** indicates measurements highly significantly different to left hand column (P>0.005)

Table 6.12: Heart function in 3 month old *mdx* mice treated with B-PMO1 or pip5e-PMO at 18.75mg/kg single dose.

3 month old		<i>C57/Bl10</i>	<i>mdx</i> littermates	B-PMO1 treated <i>mdx</i>	pip5e-PMO treated <i>mdx</i>
Number of mice		11	6	3	7
Sex	M/F	M	M	M	M
Body Mass	g	28.7 ± 0.6	28.3 ± 0.7	27.3 ± 1.4	26 ± 0.8 *†
Heart Rate	bpm	427 ± 17	455 ± 32	414 ± 19	385 ± 34
LV					
Ejection Fraction	%	70.0 ± 1.6	70.3 ± 2.2	69.2 ± 5.8	70.9 ± 1.0
Cardiac Output	ml/min	20.6 ± 1.0	18.7 ± 1.6	16.6 ± 1.0 *	15.2 ± 1.3 *
Heart Mass as % BM	%	0.34 ± 0.01	0.38 ± 0.01	0.34 ± 0.03	0.35 ± 0.01
Muscle Volume	μl	94.0 ± 2.7	102 ± 2.3 *	88.7 ± 4.3 †	86 ± 3.4 †
End Diastolic Lumen	μl	65.3 ± 2.4	58.7 ± 3.9	58.6 ± 5.1	55.9 ± 1.4 *
End Systolic Lumen	μl	26.0 ± 1.3	17.5 ± 2.1 *	18.3 ± 4.6	16.2 ± 0.7 **
Stroke Volume	μl	39.2 ± 1.8	41.2 ± 2.5	40.3 ± 2.6	39.6 ± 1.1
PER	μl/s		316 ± 60	247 ± 2.8	237 ± 19
PFR	μl/s		267 ± 29	363 ± 30	265 ± 33
E	μl/s		266 ± 28	219 ± 11	247 ± 36
A	μl/s		200 ± 25	350 ± 27 †	211 ± 25
E/A			1.36 ± 0.1	0.65 ± 0.3	1.21 ± 0.1
RV					
Ejection Fraction	%	67.8 ± 2.3	64.2 ± 3.9	70.9 ± 2.9	66.7 ± 4.1
Cardiac Output	ml/min	21.3 ± 1.2	16.5 ± 1.2 *	17.1 ± 1.2 *	15.0 ± 1.6 *
End Diastolic Lumen	μl	59.5 ± 2.1	58.3 ± 1.7	58.3 ± 3.7	57.9 ± 2.4
End Systolic Lumen	μl	19.2 ± 1.5	20.8 ± 2.4	17.1 ± 2.7	19.2 ± 2.3
Stroke Volume	μl	40.3 ± 1.9	37.4 ± 2.3	41.2 ± 1.4	38.7 ± 2.9

Data obtained by *in vivo* cardiac cine-MRI of *C57/Bl10*, untreated *mdx*, B-PMO1 treated *mdx* and pip5e-PMO treated *mdx* mice at 3 months of age. Treated mice were injected intravenously with a single dose of 18.75mg/kg PMO. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

- * indicates measurements significantly different to WT ($P > 0.05$)
- ** indicates measurements highly significantly different to WT ($P > 0.005$)
- † indicates measurements significantly different to *mdx* ($P > 0.05$)
- †† indicates measurements highly significantly different to *mdx* ($P > 0.005$)
- § indicates measurements significantly different to treatment with other drug ($P > 0.05$)
- § § indicates measurements highly significantly different to treatment with other drug ($P > 0.005$)

Table 6.13: Heart function in 6 month old *mdx* mice treated with B-PMO1 or pip5e-PMO at 18.75mg/kg single dose.

6 month old		<i>C57/Bl10</i>	<i>mdx</i> littermates	B-PMO1 treated <i>mdx</i>	pip5e-PMO treated <i>mdx</i>	pip5e-PMO treated <i>mdx</i>
Number of mice		8	6	6	4	2
Sex	M/F	M	M	2/4	M	F
Body Mass	g	29.3 ± 1.0	32.7 ± 0.8 *	30.0 ± 2.2	35.7 ± 0.9 *§	25.5 ± 0.3 *††
Heart Rate	bpm	434 ± 20	463 ± 16	494 ± 15	437 ± 20	469 ± 28
LV						
Ejection Fraction	%	61.0 ± 2.9	69 ± 1.2 *	63.6 ± 2.6	58.0 ± 5.3	44.7 ± 2.4
Cardiac Output	ml/min	20.1 ± 1.4	16.3 ± 0.6 *	20.4 ± 1.1 †	14.3 ± 1.3 *§	15.4 ± 0.2 *§
Heart Mass as % BM	%	0.43 ± 0.01	0.38 ± 0.01 *	0.38 ± 0.03	0.38 ± 0.01 *	0.47 ± 0.00 †§
Muscle Volume	μl	123 ± 8	119 ± 5	108 ± 9	132 ± 4.8	115 ± 3.0
End Diastolic Lumen	μl	76.9 ± 5.7	51.2 ± 2.8 **	64.8 ± 7.1	59.1 ± 3.6 *	74.3 ± 1.6 †
End Systolic Lumen	μl	30.6 ± 4.1	16.0 ± 1.4 *	24.3 ± 4.2	24.8 ± 3.5	40.9 ± 0.9 *†§
Stroke Volume	μl	46.2 ± 2.2	35.2 ± 1.5 **	40.4 ± 2.9	34.2 ± 3.8	33.3 ± 2.5
PER	μl/s	313 ± 22	290 ± 24	317 ± 19	176 ± 21 *†§	233 ± 31
PFR	μl/s	325 ± 44	185 ± 20 *	379 ± 66	225 ± 30 *	272 ± 3 †
E	μl/s	325 ± 44	142 ± 28 *	379 ± 66 †	225 ± 30	188
A	μl/s	256 ± 45	185 ± 20	166 ± 31	187 ± 33	277
E/A		1.51 ± 0.24	0.98 ± 0.22	2.68 ± 0.61	1.27 ± 0.1	0.68
RV						
Ejection Fraction	%	69.8 ± 3.3	57.8 ± 4.4 *	72.3 ± 3.7 †	56.4 ± 8.1	67.0 ± 0.6
Cardiac Output	ml/min	23.0 ± 1.7	16.0 ± 1.9 *	21.8 ± 1.7 †	17.7 ± 1.5	16.8 ± 0.8 *
End Diastolic Lumen	μl	76.8 ± 5.4	63.1 ± 6.6	60.4 ± 8.7	78.6 ± 9.8	53.3 ± 0.7 *
End Systolic Lumen	μl	23.8 ± 3.8	27.3 ± 5.1	17.9 ± 4.7	36.2 ± 9.7	17.5 ± 0.0
Stroke Volume	μl	52.9 ± 2.8	35.8 ± 2.8 **	42.5 ± 4.4	42.3 ± 5.1	35.8 ± 0.8 *

Data obtained by *in vivo* cardiac cine-MRI of *C57/Bl10*, untreated *mdx*, B-PMO1 treated *mdx* and pip5e-PMO treated *mdx* mice at 6 months of age. Treated mice were injected intravenously with a single dose of 18.75mg/kg PMO. Yellow highlighted figures are very different from expectations, thought not significantly so due to a low number of mice. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

- * indicates measurements significantly different to WT (P>0.05)
- ** indicates measurements highly significantly different to WT (P>0.005)
- † indicates measurements significantly different to *mdx* (P>0.05)
- †† indicates measurements highly significantly different to *mdx* (P>0.005)
- § indicates measurements significantly different to treatment with other drug (P>0.05)
- § § indicates measurements highly significantly different to treatment with other drug (P>0.005)

Table 6.14: Heart function in 9 month old *mdx* mice treated with pip5e-PMO at 18.75mg/kg single dose.

9 month old		<i>C57/Bl6</i>	<i>mdx</i> littermates	Pip5e-PMO treated <i>mdx</i>
Number of mice		7	4	2
Sex	M/F	F	F	F
Body Mass	g	24.4 ± 0.7	26.2 ± 1.5	27 ± 1.4
Heart Rate	bpm	444 ± 14	410 ± 52	443 ± 13
LV				
Ejection Fraction	%	58.5 ± 2.2	62.2 ± 4.3	52.7 ± 16
Cardiac Output	ml/min	15.4 ± 0.8	15.2 ± 2.2	12.3 ± 0.0 *
Heart Mass as % BM	%	0.46 ± 0.01	0.40 ± 0.03	0.40 ± 0.09
Muscle Volume	μl	108 ± 3.2	101 ± 10	103 ± 19
End Diastolic Lumen	μl	60.6 ± 3.0	60.2 ± 4.6	55.9 ± 19
End Systolic Lumen	μl	25.2 ± 2.0	23.1 ± 4.2	28.1 ± 18
Stroke Volume	μl	35.4 ± 2.0	37.0 ± 1.2	27.8 ± 0.9 *†§
PER	μl/s	249 ± 15	230 ± 11	
PFR	μl/s	372 ± 32	411 ± 13	
E	μl/s	279 ± 35	222 ± 22	
A	μl/s	368 ± 31	352 ± 17	
E/A		0.75 ± 0.0	1.10 ± 0.6	
RV				
Ejection Fraction	%	69.1 ± 3.9	65.8 ± 4.6	54.3 ± 3.9 *
Cardiac Output	ml/min	15.3 ± 2.9	11.0 ± 4.0	13.7 ± 1.4
End Diastolic Lumen	μl	57.8 ± 2.8	54.1 ± 2.0	57.3 ± 8.7
End Systolic Lumen	μl	17.7 ± 2.3	18.6 ± 3.1	26.3 ± 6.2
Stroke Volume	μl	40.1 ± 3.3	35.4 ± 1.5	31.0 ± 2.4 *

Data obtained by *in vivo* cardiac cine-MRI of *C57/Bl6*, untreated *mdx* and pip5e-PMO treated *mdx* mice at 9 months of age. Treated mice were injected intravenously with a single dose of 18.75mg/kg PMO. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

- * indicates measurements significantly different to WT ($P > 0.05$)
- ** indicates measurements highly significantly different to WT ($P > 0.005$)
- † indicates measurements significantly different to *mdx* ($P > 0.05$)
- †† indicates measurements highly significantly different to *mdx* ($P > 0.005$)
- § indicates measurements significantly different to treatment with other drug ($P > 0.05$)
- § § indicates measurements highly significantly different to treatment with other drug ($P > 0.005$)

Table 6.15: Heart function in each 9 month old pip5e-PMO treated *mdx* mouse.

9 month old		1	2
Sex	M/F	F	F
Body Mass	g	28	26
Heart Rate	bpm	434	453
LV			
Ejection Fraction	%	64.7	40.8
Cardiac Output	ml/min	12.3	12.4
Heart Mass as % BM	%	0.33	0.47
Muscle Volume	μ l	89	117
End Diastolic Lumen	μ l	42.1	69.8
End Systolic Lumen	μ l	14.9	41.3
Stroke Volume	μ l	27.2	28.5
RV			
Ejection Fraction	%	57.1	51.5
Cardiac Output	ml/min	12.7	14.8
End Diastolic Lumen	μ l	51.2	63.5
End Systolic Lumen	μ l	22.0	30.8
Stroke Volume	μ l	29.3	32.8

Data obtained by *in vivo* cardiac cine-MRI of pip5e-PMO treated *mdx* mice at 9 months of age. Mice were injected intravenously with a single dose of 18.75mg/kg PMO. This is a per mouse breakdown of the pip5e-PMO treated *mdx* mice data found in table 6.12. The yellow highlighted LV ejection fraction figures are very different from each other. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume $\times$ heart rate.

Table 6.16: Heart function in 8 week old *C57/Bl6* and *dKO* mice.

8 week old		<i>C57/Bl6</i>	<i>dKO</i>
Number of mice		11	7
Sex	M/F	11 (4/7)	7 (1/6)
Body Mass	g	18 ± 0.7	14.7 ± 2.5
Heart Rate	bpm	493 ± 19	448 ± 22
LV			
Ejection Fraction	%	68.9 ± 2.2	67.1 ± 2.9
Cardiac Output	ml/min	15.0 ± 1.1	8.77 ± 1.8 **
Heart Mass as % BM	%	0.42 ± 0.02	0.41 ± 0.03
Muscle Volume	μl	73 ± 5.3	57 ± 7.9 *
End Diastolic Lumen	μl	44.4 ± 2.9	29.3 ± 6.1 *
End Systolic Lumen	μl	14.0 ± 1.7	9.89 ± 2.6
Stroke Volume	μl	30.4 ± 1.7	19.4 ± 3.5 **
PER	μl/s	373 ± 93	170 ± 31 *
PFR	μl/s	337 ± 36	168 ± 28 **
E	μl/s	323 ± 20	194 ± 9 **
A	μl/s	284 ± 44	83 ± 4 **
E/A		1.28 ± 0.2	2.35 ± 0.2
RV			
Ejection Fraction	%	73.5 ± 2.2	70.9 ± 3.4
Cardiac Output	ml/min	12.2 ± 2.5	10.5 ± 1.8
End Diastolic Lumen	μl	42.1 ± 3.1	32.5 ± 6.4
End Systolic Lumen	μl	11.4 ± 1.7	9.77 ± 2.7
Stroke Volume	μl	30.7 ± 1.6	22.8 ± 3.8 *

Data obtained by *in vivo* cardiac cine-MRI of *C57/Bl6* and *dKO* mice at 8 weeks of age. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

* indicates measurements significantly different to left hand column (P>0.05)

** indicates measurements highly significantly different to left hand column (P>0.005)

Table 6.17: Heart function in 6 month-old *dKO* mice treated with B-PMO2 at 25mg/kg dose either intravenously (IV) or intraperitoneally (IP).

6 month old		<i>C57/Bl6</i>	<i>mdx</i>	IV treated <i>dKO</i>	IP treated <i>dKO</i>
Number of mice		8	6	3	2
Sex	M/F	F	M	F	F
Body Mass	g	23.1 ± 0.8	32.7 ± 0.8 *	28 ± 1.9	27.5 ± 4.9
Heart Rate	bpm	454 ± 11	463 ± 16	400 ± 39	398 ± 35
LV					
Ejection Fraction	%	63.2 ± 0.7	69 ± 1.2	65.6 ± 4.8	71.6 ± 2.6
Cardiac Output	ml/min	15.6 ± 1.1	16.3 ± 0.6	14.7 ± 1.7	15.8 ± 5.7
Heart Mass as % BM	%	0.42 ± 0.01	0.38 ± 0.01 *	0.40 ± 0.01	0.40 ± 0.05
Muscle Volume	μl	92.9 ± 5.5	119 ± 5 *	108 ± 1	105 ± 6
End Diastolic Lumen	μl	54.0 ± 2.4	51.2 ± 2.8	57.1 ± 10	53 ± 0.9
End Systolic Lumen	μl	19.8 ± 0.8	16.0 ± 1.4	20.2 ± 6.7	16.0 ± 1.6
Stroke Volume	μl	34.2 ± 1.8	35.2 ± 1.5	36.7 ± 3.6	37.9 ± 0.7
PER	μl/s	254 ± 18	290 ± 24	335 ± 20 *	444 ± 282
PFR	μl/s	283 ± 59	185 ± 20	300 ± 44	294 ± 31
E	μl/s	242 ± 30	142 ± 28 *	269 ± 14 †	294 ± 31 †
A	μl/s	283 ± 59	185 ± 20	300 ± 44	225 ± 19
E/A		1.20 ± 0.30	0.98 ± 0.22	0.91 ± 0.11	1.30 ± 0.01
RV					
Ejection Fraction	%	64.7 ± 3.3	57.8 ± 4.4 *	68.3 ± 1.5 †	69.3 ± 3.4 †
Cardiac Output	ml/min	14.7 ± 1.0	16.0 ± 1.9	16.5 ± 2.5	16.0 ± 2.9
End Diastolic Lumen	μl	51.1 ± 3.4	63.1 ± 6.6	60.5 ± 8.1	55.2 ± 5.2
End Systolic Lumen	μl	18.5 ± 2.9	27.3 ± 5.1	19.3 ± 3.5	16.9 ± 0.3
Stroke Volume	μl	32.6 ± 1.7	35.8 ± 2.8	41.1 ± 4.5	38.3 ± 5.5

Data obtained by *in vivo* cardiac cine-MRI of *C57/Bl6*, untreated *mdx* and B-PMO2 treated *dKO* mice at 6 months of age. Treated mice were injected either intravenously (IV) or intraperitoneally (IP) with weekly repeated doses of 25mg/kg PMO. For full dosing details see section 6.2.3.2. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

- * indicates measurements significantly different to WT ($P > 0.05$)
- ** indicates measurements highly significantly different to WT ($P > 0.005$)
- † indicates measurements significantly different to *mdx* ($P > 0.05$)
- †† indicates measurements highly significantly different to *mdx* ($P > 0.005$)
- § indicates measurements significantly different to treatment with other drug ($P > 0.05$)
- § § indicates measurements highly significantly different to treatment with other drug ($P > 0.005$)

Table 6.18: Heart function in 6 month old *dKO* mice treated with P007-PMO at 25mg/kg monthly repeated doses intraperitoneally.

6 month old		<i>C57/Bl6</i>	<i>mdx</i>	P007-PMO treated <i>dKO</i>
Number of mice		7	6	2
Sex	M/F	F	M	F
Body Mass	g	23.1 ± 0.8	32.7 ± 0.8 *	28.5 ± 0.7 **†
Heart Rate	bpm	454 ± 11	463 ± 16	409 ± 42
LV				
Ejection Fraction	%	63.2 ± 0.7	69 ± 1.2	58.9 ± 1.8 †
Cardiac Output	ml/min	15.6 ± 1.1	16.3 ± 0.6	13.4 ± 0.8 †
Heart Mass as % BM	%	0.42 ± 0.01	0.38 ± 0.01 *	0.45 ± 0.08
Muscle Volume	μl	92.9 ± 5.5	119 ± 5.2 *	122 ± 26
End Diastolic Lumen	μl	54.0 ± 2.4	51.2 ± 2.8	56.5 ± 11.4
End Systolic Lumen	μl	19.8 ± 0.8	16.0 ± 1.4	23.3 ± 5.7
Stroke Volume	μl	34.2 ± 1.8	35.2 ± 1.5	33.2 ± 5.6
PER	μl/s	254 ± 18	290 ± 24	284 ± 10
PFR	μl/s	283 ± 59	185 ± 20	250 ± 17
E	μl/s	242 ± 30	142 ± 28	372
A	μl/s	283 ± 59	185 ± 20	222
E/A		1.20 ± 0.3	0.98 ± 0.2	1.67
RV				
Ejection Fraction	%	64.7 ± 3.3	57.8 ± 4.4 *	59.5 ± 2.9
Cardiac Output	ml/min	14.7 ± 1.0	16.0 ± 1.9	15.4 ± 1.6
End Diastolic Lumen	μl	51.1 ± 3.4	63.1 ± 6.6	62.8 ± 14.3
End Systolic Lumen	μl	18.5 ± 2.9	27.3 ± 5.1	25.6 ± 7.6
Stroke Volume	μl	32.6 ± 1.7	35.8 ± 2.8	37.2 ± 6.7

Data obtained by *in vivo* cardiac cine-MRI of *C57/Bl6*, untreated *mdx* and P007-PMO treated *dKO* mice at 6 months of age. Treated mice were injected intraperitoneally with monthly repeated doses of 25mg/kg PMO. For full dosing details see section 6.2.3.3. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

- * indicates measurements significantly different to WT ($P > 0.05$)
- ** indicates measurements highly significantly different to WT ($P > 0.005$)
- † indicates measurements significantly different to *mdx* ($P > 0.05$)
- †† indicates measurements highly significantly different to *mdx* ($P > 0.005$)

Table 6.19: Heart function in first two 9 month old *dKO* mice treated with P007-PMO at 25mg/kg monthly repeated doses intraperitoneally.

9 month old		<i>C57/Bl6</i>	<i>mdx</i>	P007-PMO treated <i>dKO</i>
Number of mice		7	8	2
Sex	M/F	F	M	2 (1/1)
Body Mass	g	24.4 ± 0.7	38.6 ± 1.1 *	29 ± 4.2
Heart Rate	bpm	444 ± 14	464 ± 13	465 ± 46
LV				
Ejection Fraction	%	58.5 ± 2.2	49.5 ± 2.2 *	64.0 ± 3.3 †
Cardiac Output	ml/min	15.4 ± 0.8	18.8 ± 1.1 *	12.8 ± 0.5 *††
Heart Mass as % BM	%	0.46 ± 0.01	0.39 ± 0.01 *	0.35 ± 0.04
Muscle Volume	μl	108 ± 3	145 ± 7 *	96 ± 3 *††
End Diastolic Lumen	μl	60.6 ± 3.0	82.6 ± 5.1 *	43.2 ± 4.7 *††
End Systolic Lumen	μl	25.2 ± 2.0	41.8 ± 3.2 *	15.6 ± 3.1††
Stroke Volume	μl	35.4 ± 2.0	40.8 ± 3.0	27.6 ± 1.5 *††
PER	μl/s	249 ± 15	274 ± 20	272
PFR	μl/s	372 ± 32	323 ± 51	561
E	μl/s	279 ± 35	292 ± 57	
A	μl/s	368 ± 31	250 ± 32	
E/A		0.75 ± 0.01	1.16 ± 0.10	
RV				
Ejection Fraction	%	69.1 ± 3.9	50.7 ± 3.2 *	64.9 ± 2.6 ††
Cardiac Output	ml/min	15.3 ± 2.9	21.6 ± 1.1	13.3 ± 2.7
End Diastolic Lumen	μl	57.8 ± 2.8	95.3 ± 9.4 *	44.4 ± 11.0 †
End Systolic Lumen	μl	17.7 ± 2.3	48.6 ± 8.5 *	15.4 ± 2.9 ††
Stroke Volume	μl	40.1 ± 3.3	46.6 ± 2.3	29.0 ± 8.6

Data obtained by *in vivo* cardiac cine-MRI of *C57/Bl6*, untreated *mdx* and P007-PMO treated *dKO* mice at 9 months of age. Treated mice were injected intraperitoneally with monthly repeated doses of 25mg/kg PMO. For full dosing details see section 6.2.3.3. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

- * indicates measurements significantly different to WT ($P > 0.05$)
- ** indicates measurements highly significantly different to WT ($P > 0.005$)
- † indicates measurements significantly different to *mdx* ($P > 0.05$)
- †† indicates measurements highly significantly different to *mdx* ($P > 0.005$)

Table 6.20: Heart function in 9 month old *dKO* mice treated with P007-PMO at 25mg/kg monthly repeated doses intraperitoneally.

9 month old		<i>C57/Bl6</i>	<i>mdx</i>	P007-PMO treated <i>dKO</i>
Number of mice		7	8	4
Sex	M/F	F	M	4 (1/3)
Body Mass	g	24.4 ± 0.7	38.6 ± 1.1 *	29.2 ± 1.4 * †
Heart Rate	bpm	444 ± 14	464 ± 13	460 ± 17
LV				
Ejection Fraction	%	58.5 ± 2.2	49.5 ± 2.2 *	61.9 ± 4.0 †
Cardiac Output	ml/min	15.4 ± 0.8	18.8 ± 1.1 *	13.4 ± 1.0 †
Heart Mass as % BM	%	0.46 ± 0.01	0.39 ± 0.01 *	0.40 ± 0.04
Muscle Volume	μl	108 ± 3	145 ± 7 *	111 ± 13 †
End Diastolic Lumen	μl	60.6 ± 3.0	82.6 ± 5.1 *	48.2 ± 7.0 †
End Systolic Lumen	μl	25.2 ± 2.0	41.8 ± 3.2 *	19.0 ± 5.0 †
Stroke Volume	μl	35.4 ± 2.0	40.8 ± 3.0	29.2 ± 2.0 * †
PER	μl/s	249 ± 15	274 ± 20	
PFR	μl/s	372 ± 32	323 ± 51	
E	μl/s	279 ± 35	292 ± 57	
A	μl/s	368 ± 31	250 ± 32	
E/A		0.75 ± 0.01	1.16 ± 0.10	
RV				
Ejection Fraction	%	69.1 ± 3.9	50.7 ± 3.2 *	60.2 ± 6.2
Cardiac Output	ml/min	15.3 ± 2.9	21.6 ± 1.1	15.3 ± 2.0 †
End Diastolic Lumen	μl	57.8 ± 2.8	95.3 ± 9.4 *	58.5 ± 14
End Systolic Lumen	μl	17.7 ± 2.3	48.6 ± 8.5 *	25.1 ± 10
Stroke Volume	μl	40.1 ± 3.3	46.6 ± 2.3	33.4 ± 4.6 †

Data obtained by *in vivo* cardiac cine-MRI of *C57/Bl6*, untreated *mdx* and P007-PMO treated *dKO* mice at 9 months of age. Treated mice were injected intraperitoneally with monthly repeated doses of 25mg/kg PMO. For full dosing details see section 6.2.3.3. This is the data in table 6.17 with the addition of a further two P007-PMO treated *dKO* mice. Data are means ± SEM. Stroke volume = end diastolic volume (EDL) – end systolic volume (ESL), ejection fraction = stroke volume / EDL, cardiac output = stroke volume × heart rate.

Where:

- * indicates measurements significantly different to WT ($P > 0.05$)
- ** indicates measurements highly significantly different to WT ($P > 0.005$)
- † indicates measurements significantly different to *mdx* ($P > 0.05$)
- †† indicates measurements highly significantly different to *mdx* ($P > 0.005$)

Chapter 7. Discussion

7.1 Summary of findings

The studies in this thesis have gone down two routes: the study of several mouse models that might have relevance to muscular dystrophy and the study of the effects of treatment on the heart function of existing mouse models of DMD.

The *sync* $-/-$ mouse was a potential model of muscular dystrophy due to syncoilin's role in connecting the DAPC to the cytoskeletal desmin IF network. This study found the mouse had no heart defect, confirming the findings of other studies (Zhang *et al.*, 2008a). This study also found that the *des* $-/-$ mouse cardiomyopathy initially presents with RV dysfunction and fibrosis prior to the previously reported LV dysfunction. The *Moonwalker* mouse was of relevance to muscular dystrophy due to the possibility of the TRPC3 channel being a stretch activated channel, involved in muscle degeneration. This study found no gross defects in heart function, but an increase in cardiac hypertrophy in the male *Moonwalker* mice compared to female *Moonwalker* mice. Rather than affecting heart function the mutation instead seemed to delay the growth of the *Moonwalker* mice, with females finally reaching wild-type size by 18 months.

Treatment of *mdx* mice, by utrophin upregulation and restoration of dystrophin by exon-skipping, showed that the initial RV heart function deficit is caused by reduced diaphragm function and not by the absence of dystrophin in the heart. This study demonstrated that utrophin upregulation in the *Fiona* mouse diaphragm could restore heart function to wild-

type levels, despite an increase in fibrosis and inflammatory cell infiltration compared to the *mdx* heart. PPMOs restoring dystrophin in the heart could similarly restore heart function to wild-type levels and there was no difference in heart function between *dKO* mice with dystrophin restored in the diaphragm and the heart and mice in which dystrophin was restored in the diaphragm alone. The exon-skipping study also presented the first evidence of cardiac toxicity in a PPMO, with reduced LV function in 6 month old female pip5e-PMO treated *mdx* mice. The fact that restoration of heart function following restoration of respiratory function was observed across a number of independent experiments, in several different strains of mice and that it was always observed in these experiments makes the finding more reliable.

Direct comparison of this data to data on *mdx* mouse heart function from other studies is limited to those studies using echocardiography (or MRI) as they also measure volumes. It is also necessary to use data collected at the same timepoints used in this study (6 and 9 months). Much data is gathered either in young mice (~3 months old), middle aged (~12 months) or very old mice (~22 month old), limiting that available for comparison. There are also variations in the wild-type control used. While most *mdx* strains are on a *C57/Bl10* background many studies use *C57/Bl6* mice as controls. While this study has demonstrated that their heart function is comparable (see section 6.3.1) this does introduce another variable. Heart function in the 9 month old *C57/Bl10* and *mdx* mice used in chapter 5 was compared with data from the only other paper to look at the RV, which used MRI to study heart function, and from the data given in the TREAT-NMD standard operating procedure (SOP) for non-invasive echocardiographic assessment of cardiac function in the *mdx* mouse model (Spurney *et al.*, 2008; Zhang *et al.*, 2008b). The timepoint was not exactly the same for these papers, Zhang *et al* looked at 8 month old mice and the SOP used 9-10

month old mice, but one would expect the data to be roughly comparable. The SOP data also used *C57/Bl6* mice as controls. Looking at the LV (all that can be done in all three papers) there is a correlation between the age of the mice studied and the values for ejection fraction between the papers, and to a lesser extent for stroke volume and cardiac output (Figure 7.1). There is a reduction in LV ejection fraction from wild-type to *mdx* mice in all three papers, though it appears bigger in the echocardiogram data. However the variance is also much larger in this paper so this difference will not be significant. For LV stroke volume and cardiac output both Zhang *et al* and the data from this study show a decrease from wild-type to *mdx* mice, while the echocardiogram data shows the opposite. However again there is a large variance for the echocardiogram data. The values roughly correlate across the papers, though the change in direction is a concern. It may be that the mice used in Spurney *et al* were smaller than in the other studies, explaining their lower stroke volume and the *mdx* mice had hypertrophied hearts, explaining why their stroke volume was above wild-type. In fact the paper makes mention of a trend towards an increase in LV mass of the *mdx* mice. Induction of hypertrophy is reported to occur somewhere between 9 and 11 months, though it was not present in the Zhang *et al* mice, or the mice from this study (Jearawiriyapaisarn *et al.*, 2010; Quinlan *et al.*, 2004). Comparing those RV parameters reported to be significantly different in Zhang *et al* (ejection fraction, EDL and ESL) found near identical values for all (Figure 7.1), the same is true for all other LV and RV parameters.

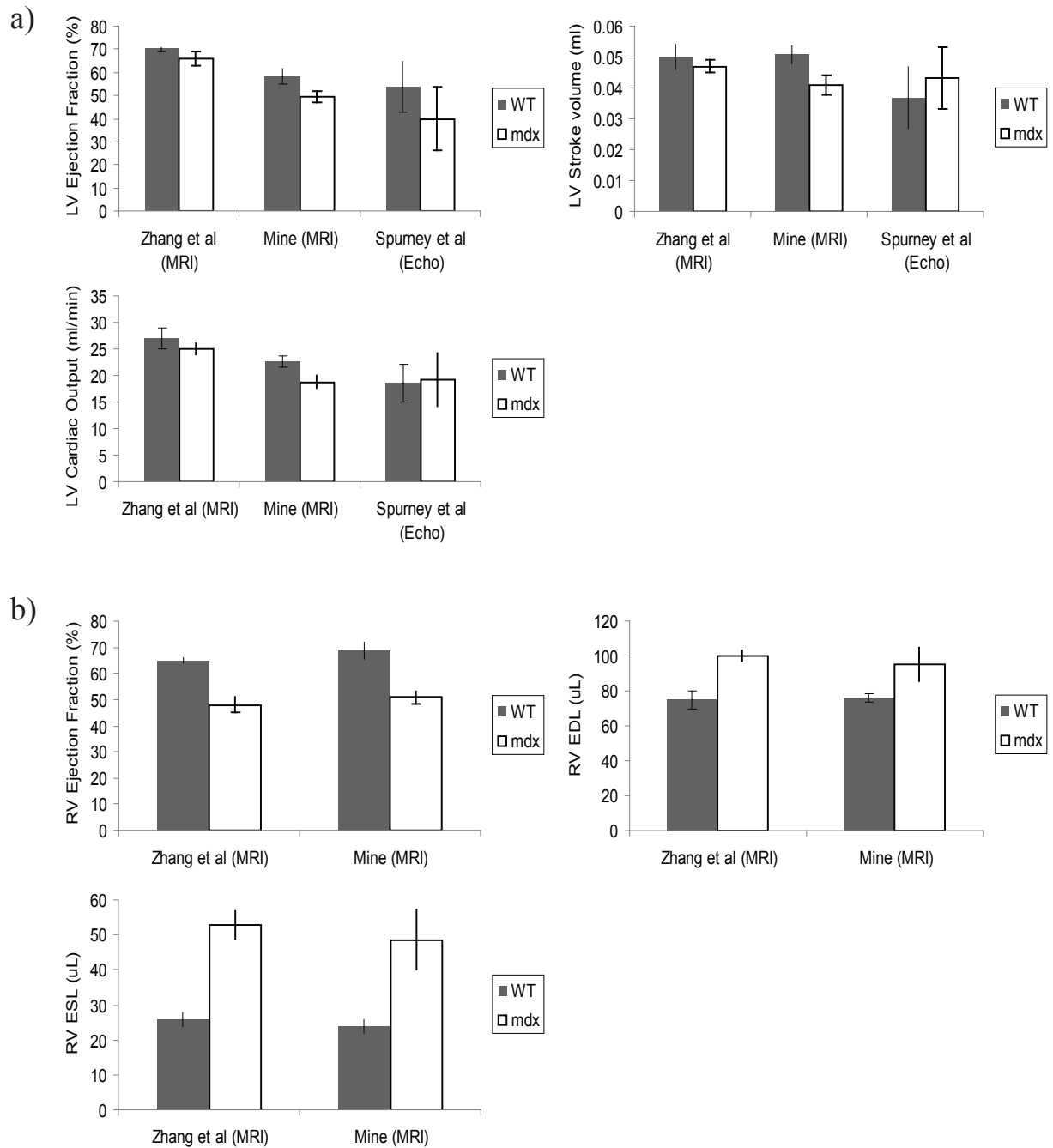


Figure 7.1 Comparison of heart function data obtained in this study (using cine-MRI) with that obtained by Zhang *et al* (using cine-MRI) and Spurney *et al* (using echocardiography).

Mice studied in Zhang *et al* were 8 months old, mice from this thesis 9 months old and mice from Spurney *et al* were 9-10 months old. Data from wild-type mice is shown in dark grey bars, that from *mdx* mice in white bars. Error bars are $\pm$ SEM. (a) Data for LV ejection fraction, stroke volume and cardiac output. (b) Data for RV ejection fraction, end diastolic volume and end systolic volume.

7.2 Limitations of findings

The biggest limitation of these findings is that restoration of diaphragm function was not actually measured. However diaphragm function has previously been measured for *Fiona* mice and found to be improved in them, compared to *mdx* mice, and western blots have shown that the levels of utrophin upregulation in the *Fiona* mice used here were the same as those in the original mice (Tinsley *et al.*, 1998). As such it is not unreasonable to conclude that diaphragm function in the *Fiona* mice used here is the same as when it was originally measured. The same is not true for the mice in the exon-skipping experiments. This is not to say that no measurements of force generation were ever made. For both sets of *dKO* mice improvements in force generation were demonstrated following treatment, for the B-PMO2 treated mice grip strength was significantly improved, while for the P007-PMO treated mice the original study found an improvement in grip strength and hind limb muscle function, with a significant increase in peak force generation (Goyenvallé *et al.*, 2010). Grip strength has also been shown to be significantly improved in *mdx* mice treated with B-PMO2 (Wu *et al.*, 2008). From this it seemed likely that restoration of dystrophin at the levels found in this chapter was sufficient to restore function to skeletal muscle, including the diaphragm.

Another limitation of these results is that the *Fiona* mice and those used in the exon-skipping study were only studied up to a year of age, as time limitations prevented longer studies. While this study concluded that the dystrophic mouse cardiomyopathy was due to impaired respiratory function, it seems likely that there is also a separate heart-specific defect. This may have manifested itself if the mice had been allowed to age further. This

study also did not apply any kind of stress to the dystrophic mouse heart – e.g. dobutamine or force running. Similarly this might have revealed a separate heart-specific defect.

A final limitation is the use of MRI to measure heart function. Changes in blood pressure in the heart are frequently observed before changes in volume of chambers. Changes in ejection fraction, used as a primary measure of heart function in this study, typically occur after the heart has tried to compensate for impairment and are downstream of other more immediate changes, like changes in blood pressure. By not measuring blood pressures this study may have missed out on pathological changes that had not yet affected heart volume. In particular this may mean that restoring diaphragm function has not in fact fully restored heart function in dystrophic mice. As discussed in chapter 2, blood pressures in the heart cannot be measure non-invasively in mice so there was good reason to do this but this is something that should be addressed by future work.

7.3 Importance of results

7.3.1 Importance to research direction

7.3.1.1 Do we need to treat the heart in the dystrophic mouse?

The most important finding of this work was that the initial RV deficit in the *mdx* mouse heart is due to reduced diaphragm function and not the absence of dystrophin in the heart; and that the deficit could be corrected by restoring diaphragm function, either by utrophin upregulation or dystrophin restoration. This raises the question, is the direct treatment of the heart in the *mdx* mouse necessary; is there a separate heart-specific defect that also

needs fixing? Despite the restoration to near wild-type function the *Fiona* mouse heart had greater levels of muscle degeneration than the untreated *mdx* mouse heart. As well as this, the *mdx* mouse heart has an increased susceptibility to stress, particularly dobutamine-induced stress, but also in response to pressure overload and forced running (Carter *et al.*, 1995; Danialou *et al.*, 2001; Kamogawa *et al.*, 2001; Quinlan *et al.*, 2004). It is unclear whether this deficiency can be repaired by diaphragm function restoration, as this study did not stress heart function. Studies have found impaired myocardial contractivity in the *mdx* heart, which may well be part of the reason why the *mdx* heart has a poor response to stress (see section 1.3.3.3) (Goldberg *et al.*, 1980; Janssen *et al.*, 2005; Kovick *et al.*, 1975; Sapp *et al.*, 1996). These factors suggest that there is a heart-specific defect in the *mdx* mouse. However, the *mdx* mouse has a largely normal life-span, as its pathology is not that severe. It is entirely possible that, despite a heart-specific defect, an unstressed *mdx* mouse might have fully restored heart function, due to restored diaphragm function, for its entire lifespan.

7.3.1.2 Is the *mdx* mouse a ‘good’ model of DMD heart function?

The evidence suggests that, at least in the early stages, *mdx* cardiac dysfunction is predominately right ventricular, with fibrosis from 3 months old and RV dysfunction occurring by 6 months old. Some studies have shown reduced LV systolic pressures in mice as young as 6 months old, but changes are not always reported this young and this could still be related to the RV failure as this study only places the onset between 3 and 6 months (Quinlan *et al.*, 2004; Van Erp *et al.*, 2010). Similarly ECG changes are reported from 6 months, likely related to fibrosis (Bia *et al.*, 1999). LV diastolic dysfunction has also been reported at this age (both in this thesis and Zhang *et al.*, 2008b). LV systolic dysfunction follows somewhere around 9-10 months, apparently induced by the RV

dysfunction/pulmonary hypertension rather than by a heart-specific defect (Spurney *et al.*, 2008; Zhang *et al.*, 2008b). Cardiac hypertrophy is also present in the *mdx* mouse by 11 months (Jearawiriyapaisarn *et al.*, 2010; Quinlan *et al.*, 2004). By 21-months-old the cardiomyopathy in *mdx* mice resembles LV dilated cardiomyopathy (Bostick *et al.*, 2008). These different studies variously collect data on heart function using cardiac catheterisation, echocardiogram and MRI. The absolute values given by these methods vary, for instance comparison of data from chapter 5 of this thesis with that given by Spurney *et al* shows only moderate correlation on ejection fraction, stroke volume and cardiac output (Section 7.1, Figure 7.1), but the timelines established by the various methods fit well within plus or minus a month. The variation in absolute values reflect the different sensitivities of the methods and the way they calculate the values; and likely also reflect differences in the precise genotypes used and the health condition of the mice.

In DMD ECG changes are found in 59% of patients between 6 and 10 years of age (Manning and Cropp, 1958; Nigro *et al.*, 1990; Rubin, 1952). Fibrosis begins at the posterobasal and lateral walls of the LV, followed by LV wall motion abnormalities and LV dilation so the cardiomyopathy is characterised as a LV dilated cardiomyopathy. It is uncertain to what extent the RV is involved in DMD cardiomyopathy (Goldberg *et al.*, 1982; Perloff *et al.*, 1966). RV dysfunction is present in BMD (Melacini *et al.*, 1996a) and Manning *et al* reported that fibrosis was especially bad in the RV (Manning and Cropp, 1958). As well as this pulmonary hypertension is reported in DMD patients, with an accompanying increase in right atrial pressure, meaning RV dysfunction may be present at least some of the time (Yotsukura *et al.*, 1988), which means it is possible that RV dysfunction may be occurring via the mechanism outlined in this thesis.

This evidence suggests that the course of cardiomyopathy in DMD/BMD is similar to that in *mdx* mice. The biggest difference between the pathology of the *mdx* mouse and the DMD patient is the severity of the condition. It may be there are two mechanisms for heart failure in the dystrophin-lacking heart, one due to pulmonary arterial hypertension (as outlined in this thesis) and the other due to a heart-specific defect, likely related to the greater susceptibility to stress seen in both DMD patients and *mdx* mice hearts. In the *mdx* mouse the balance (at least in young adults) is towards the first mechanism, whereas the much greater severity of the condition in the DMD patient means the balance shifts towards the second mechanism. In this model utrophin can compensate better for the lack of dystrophin in the heart than it can in the diaphragm. This is not unreasonable as the diaphragm is known to be particularly severely effected in *mdx* mice (Stedman *et al.*, 1991). The two mechanisms are not completely separate and the greater susceptibility to stress of the heart likely also contributes to the observed RV dysfunction, resulting in more damage than would otherwise be caused by the pulmonary hypertension. In this two mechanism system the *mdx* mouse can still be a useful model of DMD heart function, as long as one accounts for the proportionally greater importance of the respiratory system in *mdx* heart failure. A study of heart function during the entire lifespan of *mdx* mice in which the diaphragm has been rescued is needed to properly assess the role of a heart specific defect in the *mdx* mouse.

7.3.1.3 Can fixing the diaphragm later in life improve heart function?

A previous study of utrophin upregulation in *mdx* mice suggested that muscle function can only be restored if utrophin is upregulated prior to the onset of degeneration (Squire *et al.*, 2002), but this study did not look at heart function. One exon-skipping experiment presented here suggested that this may not be true in the diaphragm, at least for functional

improvement by dystrophin restoration (section 6.3.3.3). Here both LV and RV function was higher in treated *dKO* mice following a three month period over which untreated *mdx* mouse heart function declined. There are caveats to this, it was a small n number and the heart function seemed to be sinusoidally varying, but this improvement occurred well after the onset of degeneration in both the *mdx* and *dKO* heart (about 3 months and 10 days respectively).

7.3.1.4 Cardiac toxicity in PPMOs

It is important to know that PPMOs can cause cardiac toxicity, but more important than this is the observed sex-based difference, with females showing a reduced LV ejection fraction compared to males and untreated controls. If this truly is a sex-based difference and not just an anomaly then it would have consequences for the use of PPMOs to treat other diseases. AOs have been investigated for the treatment of thalassemias (Schmajuk *et al.*, 1999), chronic myelogenous leukemia, acute leukaemia and lymphoma and use as anticoagulants (Gewirtz *et al.*, 1998). In diseases other than DMD the ease of delivery of untargeted AOs, whereby they freely diffuse into myocytes through tears in the sarcolemma, would not exist so an obvious alternative for systemic delivery in these diseases would be targeted PPMOs. Whereas in DMD the vast majority of patients are male, so a drug that worked in males but was toxic in females would not be such an issue, in the other diseases listed above the proportions of males and females are more even, so female-specific toxicity of the cell-targeting peptide of the PPMO is a concern. This warrants further investigation, first to determine if the sex-based difference really exists, and second to determine its cause.

7.3.2 Importance to research methodology

7.3.2.1 Determining the cause of an improvement in heart function

Again, the greatest importance of this work to research methodology is that the improvement of diaphragm function in the *mdx* and *dKO* mouse can improve heart function. As previously stated, it is estimated that around 95% of DMD patients exhibit clinically relevant cardiomyopathy before death, so a great deal of work is ongoing into treating cardiomyopathy in mouse models of DMD. However isolating observed improvements in heart function due to improvements in diaphragm function from improvements due to an actual effect of the treatment in the heart is non-trivial. The most obvious solution is to use a treatment that only affects the heart but, as previously stated, this would involve the use of multiple drugs and is, in itself non-trivial. It can be done with a transgene using a cardiac specific promoter, but this approach is of no use to many treatments. The cell-penetrating peptide of the PPMO is notionally ‘heart-specific’ but, as can be seen from the western blots in Chapter 6, there are a great deal of off-target effects. A second approach involves aging the mice, and assuming that at a future time-point restoration of diaphragm function alone will be insufficient to fully restore heart function, at which point a difference could be seen between treatments affected the diaphragm and the heart and treatments affected the diaphragm alone. It is likely that this time-point does exist for the reasons discussed in section 7.3.1.1. In DMD patients a gradual improvement in the treatment of lung function over the years has increased life expectancy, but also the proportion of patients dying from heart failure, suggesting in humans the time-point is reached sometime in the late second decade. However aging mice to the degree that this would require is both expensive, and inconvenient. Another approach is to stress the *mdx* mouse heart. It is well established that the *mdx* mouse heart has an abnormal response to

stress (which also suggests the time-point exists), and it is probable, though in need of testing, that this abnormal response will not be completely ameliorated by only improving diaphragm function.

7.3.2.2 Proxies for the measurement of heart function

It is not unusual for studies treating the *mdx* mouse to use proxy measurements for muscle function, such as serum CK levels, central nucleation and myofibre size (as are used at several points in this thesis), however this may be a bad idea when it comes to heart function. This thesis establishes that cardiac fibrosis and inflammatory cell infiltration, as measured by histological stains, are poor measures of heart function. In the *Fiona* mouse, near full restoration of heart function to wild-type levels was seen at 9 months, but histology showed the hearts had higher levels of both cardiac fibrosis and inflammatory cell infiltration than the *mdx* heart. This in turn has significantly higher levels of both cardiac fibrosis and inflammatory cell infiltration than wild-type but significantly worse heart function (section 5.3). The increased levels of degeneration may indicate a reduced future function, but they did not correlate with current heart function. Much the same was seen in 8 week old untreated *dKO* mice heart. Previous studies have shown an increase in inflammation and fibrosis in the heart at about this age (Hainsey *et al.*, 2003), but here no reduction in heart function was seen. Similarly, in the exon-skipping experiments, a near complete level of dystrophin restoration could be seen in the 6 month old pip5e-PMO treated *mdx* mouse hearts (section 6.3.2.2) but their heart function was lowered, or at least not improved, compared to untreated *mdx* mice; showing that levels of dystrophin restoration also do not necessarily correlate with heart function. This highlights the

importance of actually measuring heart function, rather than using proxies for it when studying the treatment of dystrophic mice.

This study used the proxy of percentage dystrophin protein restored in the diaphragm for diaphragm function. In light of the above this may seem foolhardy. However there are reasons to think that the skeletal muscle function was improved in the mice and, in the case of the *Fiona* mice, diaphragm function was previously measured (discussed in section 7.2). Furthermore there are many studies looking at functional improvement in skeletal muscle of *mdx* mice treated with exon-skipping compounds and no reported examples of high levels of dystrophin restoration without high levels of functional improvement. By comparison there are only a handful of studies that have looked at heart function following treatment with exon-skipping compounds and these use a small number of different compounds (Jearawiriyapaisarn *et al.*, 2008; Jearawiriyapaisarn *et al.*, 2010; Wu *et al.*, 2008; Wu *et al.*, 2010).

7.4 Conclusions

There is a great deal of ongoing study into the treatment of heart failure in the *mdx* mouse. This thesis establishes that there is an urgent need for study of the mechanisms of this heart failure, in order to determine whether restoring diaphragm function in the *mdx* mouse can restore heart function to wild-type levels for the entire of its life, irrespective of induced stress; whether restoring diaphragm function later in life can improve heart function; and whether the *mdx* mouse is a useful model of heart failure in DMD.

Bibliography

Aartsma-Rus, A., Bremmer-Bout, M., Janson, A.A., den Dunnen, J.T., van Ommen, G.J., and van Deutekom, J.C. (2002). Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy. *Neuromuscul. Disord.* *12 Suppl 1*, S71-7.

Aartsma-Rus, A., Janson, A.A., Kaman, W.E., Bremmer-Bout, M., van Ommen, G.J., den Dunnen, J.T., and van Deutekom, J.C. (2004). Antisense-induced multiexon skipping for Duchenne muscular dystrophy makes more sense. *Am. J. Hum. Genet.* *74*, 83-92.

Adamo, C.M., Dai, D.F., Percival, J.M., Minami, E., Willis, M.S., Patrucco, E., Froehner, S.C., and Beavo, J.A. (2010). Sildenafil reverses cardiac dysfunction in the mdx mouse model of Duchenne muscular dystrophy. *Proc. Natl. Acad. Sci. U. S. A.* *107*, 19079-19083.

Adebiyi, A., Zhao, G., Narayanan, D., Thomas-Gatewood, C.M., Bannister, J.P., and Jaggar, J.H. (2010). Isoform-selective physical coupling of TRPC3 channels to IP3 receptors in smooth muscle cells regulates arterial contractility. *Circ. Res.* *106*, 1603-1612.

Agbulut, O., Li, Z., Perie, S., Ludosky, M.A., Paulin, D., Cartaud, J., and Butler-Browne, G. (2001). Lack of desmin results in abortive muscle regeneration and modifications in synaptic structure. *Cell Motil. Cytoskeleton* *49*, 51-66.

Ahn, A.H., and Kunkel, L.M. (1995). Syntrophin binds to an alternatively spliced exon of dystrophin. *J. Cell Biol.* *128*, 363-371.

Albrecht, D.E., and Froehner, S.C. (2002). Syntrophins and dystrobrevins: defining the dystrophin scaffold at synapses. *Neurosignals* 11, 123-129.

Alexakis, C., Partridge, T., and Bou-Gharios, G. (2007). Implication of the satellite cell in dystrophic muscle fibrosis: a self-perpetuating mechanism of collagen overproduction. *Am. J. Physiol. Cell. Physiol.* 293, C661-9.

Allen, D.G., Gervasio, O.L., Yeung, E.W., and Whitehead, N.P. (2010). Calcium and the damage pathways in muscular dystrophy. *Can. J. Physiol. Pharmacol.* 88, 83-91.

Alter, J., Lou, F., Rabinowitz, A., Yin, H., Rosenfeld, J., Wilton, S.D., Partridge, T.A., and Lu, Q.L. (2006). Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology *Nat. Med.* 12, 175-177.

Amann, K.J., Guo, A.W., and Ervasti, J.M. (1999). Utrophin lacks the rod domain actin binding activity of dystrophin. *J. Biol. Chem.* 274, 35375-35380.

Amann, K.J., Renley, B.A., and Ervasti, J.M. (1998). A cluster of basic repeats in the dystrophin rod domain binds F-actin through an electrostatic interaction. *J. Biol. Chem.* 273, 28419-28423.

Amantana, A., Moulton, H.M., Cate, M.L., Reddy, M.T., Whitehead, T., Hassinger, J.N., Youngblood, D.S., and Iversen, P.L. (2007). Pharmacokinetics, biodistribution, stability and toxicity of a cell-penetrating peptide-morpholino oligomer conjugate. *Bioconjug. Chem.* 18, 1325-1331.

Amundsen, B.H., Ericsson, M., Seland, J.G., Pavlin, T., Ellingsen, O., and Brekken, C. (2011). A comparison of retrospectively self-gated magnetic resonance imaging and high-frequency echocardiography for characterization of left ventricular function in mice. *Lab. Anim.* 45, 31-37.

Anderson, J.E., Bressler, B.H., and Ovalle, W.K. (1988a). Functional regeneration in the hindlimb skeletal muscle of the mdx mouse. *J. Muscle Res. Cell. Motil.* 9, 499-515.

Anderson, J.E., Bressler, B.H., and Ovalle, W.K. (1988b). Functional regeneration in the hindlimb skeletal muscle of the mdx mouse. *J. Muscle Res. Cell. Motil.* 9, 499-515.

Ashley, E.A., Sears, C.E., Bryant, S.M., Watkins, H.C., and Casadei, B. (2002). Cardiac nitric oxide synthase 1 regulates basal and beta-adrenergic contractility in murine ventricular myocytes *Circulation* 105, 3011-3016.

Badalamente, M.A., and Stracher, A. (2000). Delay of muscle degeneration and necrosis in mdx mice by calpain inhibition. *Muscle Nerve* 23, 106-111.

Bar, H., Strelkov, S.V., Sjoberg, G., Aebi, U., and Herrmann, H. (2004). The biology of desmin filaments: how do mutations affect their structure, assembly, and organisation? *J. Struct. Biol.* 148, 137-152.

Barabino, S.M., Sproat, B.S., and Lamond, A.I. (1992). Antisense probes targeted to an internal domain in U2 snRNP specifically inhibit the second step of pre-mRNA splicing. *Nucleic Acids Res.* 20, 4457-4464.

Barouch, L.A., Harrison, R.W., Skaf, M.W., Rosas, G.O., Cappola, T.P., Kobeissi, Z.A., Hobai, I.A., Lemmon, C.A., Burnett, A.L., O'Rourke, B., *et al.* (2002). Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. *Nature* *416*, 337-339.

Barton-Davis, E.R., Cordier, L., Shoturma, D.I., Leland, S.E., and Sweeney, H.L. (1999). Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of mdx mice. *J. Clin. Invest.* *104*, 375-381.

Becker, E.B., Oliver, P.L., Glitsch, M.D., Banks, G.T., Achilli, F., Hardy, A., Nolan, P.M., Fisher, E.M., and Davies, K.E. (2009). A point mutation in TRPC3 causes abnormal Purkinje cell development and cerebellar ataxia in moonwalker mice. *Proc. Natl. Acad. Sci. U. S. A.*

Ben Othmane, K., Ben Hamida, M., Pericak-Vance, M.A., Ben Hamida, C., Blel, S., Carter, S.C., Bowcock, A.M., Petrukhin, K., Gilliam, T.C., and Roses, A.D. (1992). Linkage of Tunisian autosomal recessive Duchenne-like muscular dystrophy to the pericentromeric region of chromosome 13q. *Nat. Genet.* *2*, 315-317.

Benchouir, R., Meregalli, M., Farini, A., D'Antona, G., Belicchi, M., Goyenvalle, A., Battistelli, M., Bresolin, N., Bottinelli, R., Garcia, L., and Torrente, Y. (2007). Restoration of human dystrophin following transplantation of exon-skipping-engineered DMD patient stem cells into dystrophic mice. *Cell. Stem Cell.* *1*, 646-657.

Benson, M.A., Newey, S.E., Martin-Rendon, E., Hawkes, R., and Blake, D.J. (2001).

Dysbindin, a novel coiled-coil-containing protein that interacts with the dystrobrevins in muscle and brain. *J. Biol. Chem.* 276, 24232-24241.

Beroud, C., Tuffery-Giraud, S., Matsuo, M., Hamroun, D., Humbertclaude, V., Monnier, N., Moizard, M.P., Voelckel, M.A., Calemard, L.M., Boisseau, P., *et al.* (2007). Multiexon skipping leading to an artificial DMD protein lacking amino acids from exons 45 through 55 could rescue up to 63% of patients with Duchenne muscular dystrophy. *Hum. Mutat.* 28, 196-202.

Bers, D.M. (1991). Ca regulation in cardiac muscle. *Med. Sci. Sports Exerc.* 23, 1157-1162.

Bertorini, T.E., Bhattacharya, S.K., Palmieri, G.M., Chesney, C.M., Pifer, D., and Baker, B. (1982). Muscle calcium and magnesium content in Duchenne muscular dystrophy. *Neurology* 32, 1088-1092.

Bertorini, T.E., Cornelio, F., Bhattacharya, S.K., Palmieri, G.M., Dones, I., Dworzak, F., and Brambati, B. (1984). Calcium and magnesium content in fetuses at risk and preneurotic Duchenne muscular dystrophy. *Neurology* 34, 1436-1440.

Bhosle, R.C., Michele, D.E., Campbell, K.P., Li, Z., and Robson, R.M. (2006).

Interactions of intermediate filament protein synemin with dystrophin and utrophin.

Biochem. Biophys. Res. Commun. 346, 768-777.

Bia, B.L. (1999). Cardiomyopathy in mouse models of Duchenne muscular dystrophy.

Bia, B.L., Cassidy, P.J., Young, M.E., Rafael, J.A., Leighton, B., Davies, K.E., Radda, G.K., and Clarke, K. (1999). Decreased myocardial nNOS, increased iNOS and abnormal ECGs in mouse models of Duchenne muscular dystrophy *J. Mol. Cell. Cardiol.* *31*, 1857-1862.

Blake, D.J., Nawrotzki, R., Loh, N.Y., Gorecki, D.C., and Davies, K.E. (1998). Beta-Dystrobrevin, a Member of the Dystrophin-Related Protein Family. *Proc. Natl. Acad. Sci. U. S. A.* *95*, 241-246.

Blake, D.J., Nawrotzki, R., Peters, M.F., Froehner, S.C., and Davies, K.E. (1996). Isoform diversity of dystrobrevin, the murine 87-kDa postsynaptic protein. *J. Biol. Chem.* *271*, 7802-7810.

Bodensteiner, J.B., and Engel, A.G. (1978). Intracellular calcium accumulation in Duchenne dystrophy and other myopathies: a study of 567,000 muscle fibers in 114 biopsies. *Neurology* *28*, 439-446.

Bonnemann, C.G., Modi, R., Noguchi, S., Mizuno, Y., Yoshida, M., Gussoni, E., McNally, E.M., Duggan, D.J., Angelini, C., and Hoffman, E.P. (1995). Beta-sarcoglycan (A3b) mutations cause autosomal recessive muscular dystrophy with loss of the sarcoglycan complex. *Nat. Genet.* *11*, 266-273.

Bosser, G., Lucron, H., Lethor, J.P., Burger, G., Beltramo, F., Marie, P.Y., and Marcon, F. (2004). Evidence of early impairments in both right and left ventricular inotropic reserves in children with Duchenne's muscular dystrophy. *Am. J. Cardiol.* 93, 724-727.

Bostick, B., Yue, Y., Long, C., and Duan, D. (2008). Prevention of dystrophin-deficient cardiomyopathy in twenty-one-month-old carrier mice by mosaic dystrophin expression or complementary dystrophin/utrophin expression. *Circ. Res.* 102, 121-130.

Bostick, B., Yue, Y., Long, C., Marschalk, N., Fine, D.M., Chen, J., and Duan, D. (2009). Cardiac expression of a mini-dystrophin that normalizes skeletal muscle force only partially restores heart function in aged Mdx mice. *Mol. Ther.* 17, 253-261.

Boyd, Y., Buckle, V., Holt, S., Munro, E., Hunter, D., and Craig, I. (1986). Muscular dystrophy in girls with X;autosome translocations. *J. Med. Genet.* 23, 484-490.

Brenman, J.E., Chao, D.S., Gee, S.H., McGee, A.W., Craven, S.E., Santillano, D.R., Wu, Z., Huang, F., Xia, H., Peters, M.F., Froehner, S.C., and Brecht, D.S. (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. *Cell* 84, 757-767.

Brenman, J.E., Chao, D.S., Xia, H., Aldape, K., and Brecht, D.S. (1995). Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell* 82, 743-752.

Brenner, J.S., and Dolmetsch, R.E. (2007). TrpC3 regulates hypertrophy-associated gene expression without affecting myocyte beating or cell size. *PLoS ONE* 2, e802.

Brooke, M.H., Fenichel, G.M., Griggs, R.C., Mendell, J.R., Moxley, R.T., 3rd, Miller, J.P., Kaiser, K.K., Florence, J.M., Pandya, S., and Signore, L. (1987). Clinical investigation of Duchenne muscular dystrophy. Interesting results in a trial of prednisone. *Arch. Neurol.* 44, 812-817.

Brookes, P.S., Yoon, Y., Robotham, J.L., Anders, M.W., and Sheu, S.S. (2004). Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am. J. Physiol. Cell. Physiol.* 287, C817-33.

Brown, S.C., Torelli, S., Ugo, I., De Biasia, F., Howman, E.V., Poon, E., Britton, J., Davies, K.E., and Muntoni, F. (2005). Syncoilin upregulation in muscle of patients with neuromuscular disease. *Muscle Nerve* 32, 715-725.

Bulfield, G., Siller, W.G., Wight, P.A., and Moore, K.J. (1984). X chromosome-linked muscular dystrophy (mdx) in the mouse. *Proc. Natl. Acad. Sci. U. S. A.* 81, 1189-1192.

Burton, E.A., Tinsley, J.M., Holzfeind, P.J., Rodrigues, N.R., and Davies, K.E. (1999). A second promoter provides an alternative target for therapeutic up-regulation of utrophin in Duchenne muscular dystrophy. *Proc. Natl. Acad. Sci. U. S. A.* 96, 14025-14030.

Bush, E.W., Hood, D.B., Papst, P.J., Chapo, J.A., Minobe, W., Bristow, M.R., Olson, E.N., and McKinsey, T.A. (2006). Canonical transient receptor potential channels promote

cardiomyocyte hypertrophy through activation of calcineurin signaling. *J. Biol. Chem.* 281, 33487-33496.

Bushby, K., Muntoni, F., and Bourke, J.P. (2003). 107th ENMC international workshop: the management of cardiac involvement in muscular dystrophy and myotonic dystrophy. 7th-9th June 2002, Naarden, the Netherlands. *Neuromuscul. Disord.* 13, 166-172.

Buyse, G.M., Van der Mieren, G., Erb, M., D'hooge, J., Herijgers, P., Verbeken, E., Jara, A., Van Den Bergh, A., Mertens, L., Courdier-Fruh, I., Barzaghi, P., and Meier, T. (2009). Long-term blinded placebo-controlled study of SNT-MC17/idebenone in the dystrophin deficient mdx mouse: cardiac protection and improved exercise performance. *Eur. Heart J.* 30, 116-124.

Campbell, K.P., and Kahl, S.D. (1989). Association of dystrophin and an integral membrane glycoprotein. *Nature* 338, 259-262.

Carnwath, J.W., and Shotton, D.M. (1987). Muscular dystrophy in the mdx mouse: histopathology of the soleus and extensor digitorum longus muscles. *J. Neurol. Sci.* 80, 39-54.

Carter, G.T., Wineinger, M.A., Walsh, S.A., Horasek, S.J., Abresch, R.T., and Fowler, W.M., Jr. (1995). Effect of voluntary wheel-running exercise on muscles of the mdx mouse. *Neuromuscul. Disord.* 5, 323-332.

Cerletti, M., Negri, T., Cozzi, F., Colpo, R., Andreetta, F., Croci, D., Davies, K.E., Cornelio, F., Pozza, O., Karpati, G., Gilbert, R., and Mora, M. (2003). Dystrophic phenotype of canine X-linked muscular dystrophy is mitigated by adenovirus-mediated utrophin gene transfer. *Gene Ther.* *10*, 750-757.

Chakkalakal, J.V., Harrison, M.A., Carbonetto, S., Chin, E., Michel, R.N., and Jasmin, B.J. (2004). Stimulation of calcineurin signaling attenuates the dystrophic pathology in mdx mice. *Hum. Mol. Genet.* *13*, 379-388.

Chakkalakal, J.V., Michel, S.A., Chin, E.R., Michel, R.N., and Jasmin, B.J. (2006). Targeted inhibition of Ca^{2+} /calmodulin signaling exacerbates the dystrophic phenotype in mdx mouse muscle. *Hum. Mol. Genet.* *15*, 1423-1435.

Chakkalakal, J.V., Miura, P., Belanger, G., Michel, R.N., and Jasmin, B.J. (2008). Modulation of utrophin A mRNA stability in fast versus slow muscles via an AU-rich element and calcineurin signaling. *Nucleic Acids Res.* *36*, 826-838.

Chakkalakal, J.V., Stocksley, M.A., Harrison, M.A., Angus, L.M., Deschenes-Furry, J., St-Pierre, S., Megeney, L.A., Chin, E.R., Michel, R.N., and Jasmin, B.J. (2003). Expression of utrophin A mRNA correlates with the oxidative capacity of skeletal muscle fiber types and is regulated by calcineurin/NFAT signaling. *Proc. Natl. Acad. Sci. U. S. A.* *100*, 7791-7796.

Chamberlain, J.S. (2007). ACE inhibitor bulks up muscle. *Nat. Med.* *13*, 125-126.

- Chamberlain, J.S., Metzger, J., Reyes, M., Townsend, D., and Faulkner, J.A. (2007). Dystrophin-deficient mdx mice display a reduced life span and are susceptible to spontaneous rhabdomyosarcoma. *FASEB J.* *21*, 2195-2204.
- Chamberlain, J.S., Pearlman, J.A., Muzny, D.M., Gibbs, R.A., Ranier, J.E., Caskey, C.T., and Reeves, A.A. (1988). Expression of the murine Duchenne muscular dystrophy gene in muscle and brain. *Science* *239*, 1416-1418.
- Chan, Y., and Kunkel, L.M. (1997). In vitro expressed dystrophin fragments do not associate with each other. *FEBS Lett.* *410*, 153-159.
- Chapdelaine, P., Pichavant, C., Rousseau, J., Paques, F., and Tremblay, J.P. (2010). Meganucleases can restore the reading frame of a mutated dystrophin. *Gene Ther.* *17*, 846-858.
- Chomczynski, P., and Sacchi, N. (2006). The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. *Nat. Protoc.* *1*, 581-585.
- Chung, W., and Campanelli, J.T. (1999). WW and EF hand domains of dystrophin-family proteins mediate dystroglycan binding. *Mol. Cell Biol. Res. Commun.* *2*, 162-171.
- Clarke, W.T., Edwards, B., McCullagh, K.J., Kemp, M.W., Moorwood, C., Sherman, D.L., Burgess, M., and Davies, K.E. (2010). Syncoilin modulates peripherin filament networks and is necessary for large-calibre motor neurons. *J. Cell. Sci.*

Clerk, A., Morris, G.E., Dubowitz, V., Davies, K.E., and Sewry, C.A. (1993). Dystrophin-related protein, utrophin, in normal and dystrophic human fetal skeletal muscle.

Histochem. J. 25, 554-561.

Coffman, J.A., Dickey-Sims, C., Haug, J.S., McCarthy, J.J., and Robertson, A.J. (2004).

Evaluation of developmental phenotypes produced by morpholino antisense targeting of a sea urchin Runx gene. BMC Biol. 2, 6.

Coulton, G.R., Curtin, N.A., Morgan, J.E., and Partridge, T.A. (1988). The mdx mouse skeletal muscle myopathy: II. Contractile properties. Neuropathol. Appl. Neurobiol. 14, 299-314.

Cox, G.A., Cole, N.M., Matsumura, K., Phelps, S.F., Hauschka, S.D., Campbell, K.P., Faulkner, J.A., and Chamberlain, J.S. (1993). Overexpression of dystrophin in transgenic mdx mice eliminates dystrophic symptoms without toxicity. Nature 364, 725-729.

Crosbie, R.H., Heighway, J., Venzke, D.P., Lee, J.C., and Campbell, K.P. (1997).

Sarcospan, the 25-kDa transmembrane component of the dystrophin-glycoprotein complex. J. Biol. Chem. 272, 31221-31224.

Crosbie, R.H., Lim, L.E., Moore, S.A., Hirano, M., Hays, A.P., Maybaum, S.W., Collin, H., Dovico, S.A., Stolle, C.A., Fardeau, M., Tome, F.M., and Campbell, K.P. (2000).

Molecular and genetic characterization of sarcospan: insights into sarcoglycan-sarcospan interactions. Hum. Mol. Genet. 9, 2019-2027.

Crosbie, R.H., Yamada, H., Venzke, D.P., Lisanti, M.P., and Campbell, K.P. (1998). Caveolin-3 is not an integral component of the dystrophin glycoprotein complex. *FEBS Lett.* 427, 279-282.

Dahlof, B., Devereux, R.B., Kjeldsen, S.E., Julius, S., Beevers, G., de Faire, U., Fyhrquist, F., Ibsen, H., Kristiansson, K., Lederballe-Pedersen, O., *et al.* (2002). Cardiovascular morbidity and mortality in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. *Lancet* 359, 995-1003.

Dangain, J., and Vrbova, G. (1984). Muscle development in mdx mutant mice. *Muscle Nerve* 7, 700-704.

Danialou, G., Comtois, A.S., Dudley, R., Karpati, G., Vincent, G., Des Rosiers, C., and Petrof, B.J. (2001). Dystrophin-deficient cardiomyocytes are abnormally vulnerable to mechanical stress-induced contractile failure and injury. *FASEB J.* 15, 1655-1657.

Davies, K.E., and Nowak, K.J. (2006). Molecular mechanisms of muscular dystrophies: old and new players. *Nat. Rev. Mol. Cell Biol.* 7, 762-773.

Dawson, D., Lygate, C.A., Zhang, M.H., Hulbert, K., Neubauer, S., and Casadei, B. (2005). nNOS gene deletion exacerbates pathological left ventricular remodeling and functional deterioration after myocardial infarction. *Circulation* 112, 3729-3737.

- de Belder, A., Robinson, N., Richardson, P., Martin, J., and Moncada, S. (1997). Expression of inducible nitric oxide synthase in human heart failure. *Circulation* 95, 1672-1673.
- Deconinck, A.E., Rafael, J.A., Skinner, J.A., Brown, S.C., Potter, A.C., Metzinger, L., Watt, D.J., Dickson, J.G., Tinsley, J.M., and Davies, K.E. (1997a). Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy *Cell* 90, 717-727.
- Deconinck, N., Tinsley, J., De Backer, F., Fisher, R., Kahn, D., Phelps, S., Davies, K., and Gillis, J.M. (1997b). Expression of truncated utrophin leads to major functional improvements in dystrophin-deficient muscles of mice. *Nat. Med.* 3, 1216-1221.
- DeSilva, S., Drachman, D.B., Mellits, D., and Kuncel, R.W. (1987). Prednisone treatment in Duchenne muscular dystrophy. Long-term benefit. *Arch. Neurol.* 44, 818-822.
- DiMario, J., Buffinger, N., Yamada, S., and Strohman, R.C. (1989). Fibroblast growth factor in the extracellular matrix of dystrophic (mdx) mouse muscle. *Science* 244, 688-690.
- Disatnik, M.H., Dhawan, J., Yu, Y., Beal, M.F., Whirl, M.M., Franco, A.A., and Rando, T.A. (1998). Evidence of oxidative stress in mdx mouse muscle: studies of the pre-necrotic state *J. Neurol. Sci.* 161, 77-84.
- Dominski, Z., and Kole, R. (1993). Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. *Proc. Natl. Acad. Sci. U. S. A.* 90, 8673-8677.

Dorchies, O.M., Wagner, S., Buetler, T.M., and Ruegg, U.T. (2009). Protection of dystrophic muscle cells with polyphenols from green tea correlates with improved glutathione balance and increased expression of 67LR, a receptor for (-)-epigallocatechin gallate. *Biofactors* 35, 279-294.

Drexler, H., Kastner, S., Strobel, A., Studer, R., Brodde, O.E., and Hasenfuss, G. (1998). Expression, activity and functional significance of inducible nitric oxide synthase in the failing human heart. *J. Am. Coll. Cardiol.* 32, 955-963.

Duboc, D., Meune, C., Lerebours, G., Devaux, J.Y., Vaksman, G., and Becane, H.M. (2005). Effect of perindopril on the onset and progression of left ventricular dysfunction in Duchenne muscular dystrophy. *J. Am. Coll. Cardiol.* 45, 855-857.

Dunckley, M.G., Wells, D.J., Walsh, F.S., and Dickson, G. (1993). Direct retroviral-mediated transfer of a dystrophin minigene into mdx mouse muscle in vivo. *Hum. Mol. Genet.* 2, 717-723.

Dupont-Versteegden, E.E., Katz, M.S., and McCarter, R.J. (1995). Beneficial versus adverse effects of long-term use of clenbuterol in mdx mice. *Muscle Nerve* 18, 1447-1459.

Eagle, M., Bourke, J., Bullock, R., Gibson, M., Mehta, J., Giddings, D., Straub, V., and Bushby, K. (2007). Managing Duchenne muscular dystrophy--the additive effect of spinal surgery and home nocturnal ventilation in improving survival. *Neuromuscul. Disord.* 17, 470-475.

Emery, A.E. (1991). Population frequencies of inherited neuromuscular diseases--a world survey. *Neuromuscul. Disord.* 1, 19-29.

England, S.B., Nicholson, L.V., Johnson, M.A., Forrest, S.M., Love, D.R., Zubrzycka-Gaarn, E.E., Bulman, D.E., Harris, J.B., and Davies, K.E. (1990). Very mild muscular dystrophy associated with the deletion of 46% of dystrophin. *Nature* 343, 180-182.

Ervasti, J.M., and Campbell, K.P. (1991). Membrane organization of the dystrophin-glycoprotein complex. *Cell* 66, 1121-1131.

Ervasti, J.M., Kahl, S.D., and Campbell, K.P. (1991). Purification of dystrophin from skeletal muscle. *J. Biol. Chem.* 266, 9161-9165.

Ervasti, J.M., Ohlendieck, K., Kahl, S.D., Gaver, M.G., and Campbell, K.P. (1990). Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. *Nature* 345, 315-319.

Ettinger, A.J., Feng, G., and Sanes, J.R. (1997). epsilon-Sarcoglycan, a broadly expressed homologue of the gene mutated in limb-girdle muscular dystrophy 2D. *J. Biol. Chem.* 272, 32534-32538.

Fanchaouy, M., Polakova, E., Jung, C., Ogrodnik, J., Shirokova, N., and Niggli, E. (2009). Pathways of abnormal stress-induced Ca²⁺ influx into dystrophic mdx cardiomyocytes. *Cell Calcium* 46, 114-121.

Fanin, M., Melacini, P., Angelini, C., and Danieli, G.A. (1999). Could utrophin rescue the myocardium of patients with dystrophin gene mutations? *J. Mol. Cell. Cardiol.* *31*, 1501-1508.

Fisher, R., Tinsley, J.M., Phelps, S.R., Squire, S.E., Townsend, E.R., Martin, J.E., and Davies, K.E. (2001). Non-toxic ubiquitous over-expression of utrophin in the mdx mouse. *Neuromuscul. Disord.* *11*, 713-721.

Fletcher, S., Honeyman, K., Fall, A.M., Harding, P.L., Johnsen, R.D., Steinhaus, J.P., Moulton, H.M., Iversen, P.L., and Wilton, S.D. (2007). Morpholino oligomer-mediated exon skipping averts the onset of dystrophic pathology in the mdx mouse. *Mol. Ther.* *15*, 1587-1592.

Fletcher, S., Honeyman, K., Fall, A.M., Harding, P.L., Johnsen, R.D., and Wilton, S.D. (2006). Dystrophin expression in the mdx mouse after localised and systemic administration of a morpholino antisense oligonucleotide. *J. Gene Med.* *8*, 207-216.

Frankel, K.A., and Rosser, R.J. (1976). The pathology of the heart in progressive muscular dystrophy: epimyocardial fibrosis. *Hum. Pathol.* *7*, 375-386.

Gathercole, D.V., Colling, D.J., Skepper, J.N., Takagishi, Y., Levi, A.J., and Severs, N.J. (2000). Immunogold-labeled L-type calcium channels are clustered in the surface plasma membrane overlying junctional sarcoplasmic reticulum in guinea-pig myocytes-implications for excitation-contraction coupling in cardiac muscle. *J. Mol. Cell. Cardiol.* *32*, 1981-1994.

Geissinger, H.D., Rao, P.V., and McDonald-Taylor, C.K. (1990). "Mdx" Mouse Myopathy: Histopathological, Morphometric and Histochemical Observations on Young Mice. *J. Comp. Pathol.* *102*, 249-263.

Gervasio, O.L., Whitehead, N.P., Yeung, E.W., Phillips, W.D., and Allen, D.G. (2008). TRPC1 binds to caveolin-3 and is regulated by Src kinase - role in Duchenne muscular dystrophy. *J. Cell. Sci.* *121*, 2246-2255.

Gewirtz, A.M., Sokol, D.L., and Ratajczak, M.Z. (1998). Nucleic acid therapeutics: state of the art and future prospects. *Blood* *92*, 712-736.

Giacalone, J.P., and Francke, U. (1992). Common sequence motifs at the rearrangement sites of a constitutional X/autosome translocation and associated deletion. *Am. J. Hum. Genet.* *50*, 725-741.

Goebel, H.H. (1995). Desmin-related neuromuscular disorders. *Muscle Nerve* *18*, 1306-1320.

Goldberg, S.J., Feldman, L., Reinecke, C., Stern, L.Z., Sahn, D.J., and Allen, H.D. (1980). Echocardiographic determination of contraction and relaxation measurements of the left ventricular wall in normal subjects and patients with muscular dystrophy. *Circulation* *62*, 1061-1069.

Goldberg, S.J., Stern, L.Z., Feldman, L., Allen, H.D., Sahn, D.J., and Valdes-Cruz, L.M. (1982). Serial two-dimensional echocardiography in Duchenne muscular dystrophy. *Neurology* 32, 1101-1105.

Goldfarb, L.G., Park, K.Y., Cervenakova, L., Gorokhova, S., Lee, H.S., Vasconcelos, O., Nagle, J.W., Semino-Mora, C., Sivakumar, K., and Dalakas, M.C. (1998). Missense mutations in desmin associated with familial cardiac and skeletal myopathy. *Nat. Genet.* 19, 402-403.

Goldfarb, L.G., Vicart, P., Goebel, H.H., and Dalakas, M.C. (2004). Desmin myopathy. *Brain* 127, 723-734.

Goll, D.E., Thompson, V.F., Li, H., Wei, W., and Cong, J. (2003). The calpain system. *Physiol. Rev.* 83, 731-801.

Gosselin, L.E., Williams, J.E., Deering, M., Brazeau, D., Koury, S., and Martinez, D.A. (2004). Localization and early time course of TGF-beta 1 mRNA expression in dystrophic muscle. *Muscle Nerve* 30, 645-653.

Goyenvallé, A., Vulin, A., Fougère, F., Leturcq, F., Kaplan, J.C., Garcia, L., and Danos, O. (2004). Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping. *Science* 306, 1796-1799.

Goyenvallé, A., Babbs, A., Powell, D., Kole, R., Fletcher, S., Wilton, S.D., and Davies, K.E. (2010). Prevention of Dystrophic Pathology in Severely Affected

Dystrophin/Utrophin-deficient Mice by Morpholino-oligomer-mediated Exon-skipping
Molecular Therapy 18, 198-205.

Grady, R.M., Grange, R.W., Lau, K.S., Maimone, M.M., Nichol, M.C., Stull, J.T., and Sanes, J.R. (1999). Role for alpha-dystrobrevin in the pathogenesis of dystrophin-dependent muscular dystrophies. *Nat. Cell Biol.* 1, 215-220.

Grady, R.M., Merlie, J.P., and Sanes, J.R. (1997a). Subtle neuromuscular defects in utrophin-deficient mice. *J. Cell Biol.* 136, 871-882.

Grady, R.M., Teng, H., Nichol, M.C., Cunningham, J.C., Wilkinson, R.S., and Sanes, J.R. (1997b). Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: a model for Duchenne muscular dystrophy. *Cell* 90, 729-738.

Green, M.R. (1986). Pre-mRNA splicing. *Annu. Rev. Genet.* 20, 671-708.

Griggs, R.C., Moxley, R.T., 3rd, Mendell, J.R., Fenichel, G.M., Brooke, M.H., Pestronk, A., and Miller, J.P. (1991). Prednisone in Duchenne dystrophy. A randomized, controlled trial defining the time course and dose response. Clinical Investigation of Duchenne Dystrophy Group. *Arch. Neurol.* 48, 383-388.

Grounds, M.D., and Torrisi, J. (2004). Anti-TNFalpha (Remicade) therapy protects dystrophic skeletal muscle from necrosis *FASEB J.* 18, 676-682.

Hainsey, T.A., Senapati, S., Kuhn, D.E., and Rafael, J.A. (2003). Cardiomyopathic features associated with muscular dystrophy are independent of dystrophin absence in cardiovascular Neuromuscul. Disord. 13, 294-302.

Harper, S.Q., Hauser, M.A., DelloRusso, C., Duan, D., Crawford, R.W., Phelps, S.F., Harper, H.A., Robinson, A.S., Engelhardt, J.F., Brooks, S.V., and Chamberlain, J.S. (2002). Modular flexibility of dystrophin: implications for gene therapy of Duchenne muscular dystrophy. Nat. Med. 8, 253-261.

Head, S.I., Williams, D.A., and Stephenson, D.G. (1992). Abnormalities in structure and function of limb skeletal muscle fibres of dystrophic mdx mice. Proc. Biol. Sci. 248, 163-169.

Helliwell, T.R., Man, N.T., Morris, G.E., and Davies, K.E. (1992). The dystrophin-related protein, utrophin, is expressed on the sarcolemma of regenerating human skeletal muscle fibres in dystrophies and inflammatory myopathies. Neuromuscul. Disord. 2, 177-184.

Hijikata, T., Murakami, T., Ishikawa, H., and Yorifuji, H. (2003). Plectin tethers desmin intermediate filaments onto subsarcolemmal dense plaques containing dystrophin and vinculin. Histochem. Cell Biol. 119, 109-123.

Hill, A.J., Hinton, J.M., Cheng, H., Gao, Z., Bates, D.O., Hancox, J.C., Langton, P.D., and James, A.F. (2006). A TRPC-like non-selective cation current activated by alpha 1-adrenoceptors in rat mesenteric artery smooth muscle cells. Cell Calcium 40, 29-40.

- Hillier, B.J., Christopherson, K.S., Prehoda, K.E., Bredt, D.S., and Lim, W.A. (1999). Unexpected modes of PDZ domain scaffolding revealed by structure of nNOS-syntrophin complex. *Science* 284, 812-815.
- Hilton-Jones, D., and Squier, M.V. (1993). Dystrophin-associated protein complex: clinical implications. *Lancet* 341, 528-529.
- Hodgetts, S., Radley, H., Davies, M., and Grounds, M.D. (2006). Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFalpha function with Etanercept in mdx mice. *Neuromuscul. Disord.* 16, 591-602.
- Hoffman, E.P., Brown, R.H., Jr, and Kunkel, L.M. (1987). Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* 51, 919-928.
- Hoffman, E.P., Morgan, J.E., Watkins, S.C., and Partridge, T.A. (1990). Somatic reversion/suppression of the mouse mdx phenotype in vivo. *J. Neurol. Sci.* 99, 9-25.
- Hofmann, T., Obukhov, A.G., Schaefer, M., Harteneck, C., Gudermann, T., and Schultz, G. (1999). Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. *Nature* 397, 259-263.
- Howman, E.V., Sullivan, N., Poon, E.P., Britton, J.E., Hilton-Jones, D., and Davies, K.E. (2003). Syncoilin accumulation in two patients with desmin-related myopathy. *Neuromuscul. Disord.* 13, 42-48.

Hsu, D.T. (2010). Cardiac manifestations of neuromuscular disorders in children. *Paediatr. Respir. Rev.* *11*, 35-38.

Hu, X.Y., Ray, P.N., Murphy, E.G., Thompson, M.W., and Worton, R.G. (1990). Duplicational mutation at the Duchenne muscular dystrophy locus: its frequency, distribution, origin, and phenotype-genotype correlation. *Am. J. Hum. Genet.* *46*, 682-695.

Hudziak, R.M., Barofsky, E., Barofsky, D.F., Weller, D.L., Huang, S.B., and Weller, D.D. (1996). Resistance of morpholino phosphorodiamidate oligomers to enzymatic degradation. *Antisense Nucleic Acid Drug Dev.* *6*, 267-272.

Huser, J., and Blatter, L.A. (1999). Fluctuations in mitochondrial membrane potential caused by repetitive gating of the permeability transition pore. *Biochem. J.* *343 Pt 2*, 311-317.

Ibraghimov-Beskrovnaya, O., Ervasti, J.M., Leveille, C.J., Slaughter, C.A., Sernett, S.W., and Campbell, K.P. (1992). Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature* *355*, 696-702.

Ichida, F., Tsubata, S., Bowles, K.R., Haneda, N., Uese, K., Miyawaki, T., Dreyer, W.J., Messina, J., Li, H., Bowles, N.E., and Towbin, J.A. (2001). Novel gene mutations in patients with left ventricular noncompaction or Barth syndrome. *Circulation* *103*, 1256-1263.

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

Iversen, P.L. (2001). Phosphorodiamidate morpholino oligomers: favorable properties for sequence-specific gene inactivation. *Curr. Opin. Mol. Ther.* 3, 235-238.

Jackson, M.J., Brooke, M.H., Kaiser, K., and Edwards, R.H. (1991). Creatine kinase and prostaglandin E2 release from isolated Duchenne muscle. *Neurology* 41, 101-104.

Jacobs, P.A., Hunt, P.A., Mayer, M., and Bart, R.D. (1981). Duchenne muscular dystrophy (DMD) in a female with an X/autosome translocation: further evidence that the DMD locus is at Xp21. *Am. J. Hum. Genet.* 33, 513-518.

Janssen, P.M., Hiranandani, N., Mays, T.A., and Rafael-Fortney, J.A. (2005). Utrophin deficiency worsens cardiac contractile dysfunction present in dystrophin-deficient mdx mice *Am. J. Physiol. Heart Circ. Physiol.* 289, H2373-8.

Jearawiriyapaisarn, N., Moulton, H.M., Buckley, B., Roberts, J., Sazani, P., Fucharoen, S., Iversen, P.L., and Kole, R. (2008). Sustained dystrophin expression induced by peptide-conjugated morpholino oligomers in the muscles of mdx mice *Mol. Ther.* 16, 1624-1629.

Jearawiriyapaisarn, N., Moulton, H.M., Sazani, P., Kole, R., and Willis, M.S. (2010). Long-term improvement in mdx cardiomyopathy after therapy with peptide-conjugated morpholino oligomers *Cardiovasc. Res.* 85, 444-453.

Ju, H., Zou, R., Venema, V.J., and Venema, R.C. (1997). Direct interaction of endothelial nitric-oxide synthase and caveolin-1 inhibits synthase activity. *J. Biol. Chem.* 272, 18522-18525.

Jung, C., Martins, A.S., Niggli, E., and Shirokova, N. (2008). Dystrophic cardiomyopathy: amplification of cellular damage by Ca²⁺ signalling and reactive oxygen species-generating pathways. *Cardiovasc. Res.* 77, 766-773.

Kahana, E., Flood, G., and Gratzer, W.B. (1997). Physical properties of dystrophin rod domain. *Cell Motil. Cytoskeleton* 36, 246-252.

Kameya, S., Miyagoe, Y., Nonaka, I., Ikemoto, T., Endo, M., Hanaoka, K., Nabeshima, Y., and Takeda, S. (1999). Alpha1-Syntrophin Gene Disruption Results in the Absence of Neuronal-Type Nitric-Oxide Synthase at the Sarcolemma but does Not Induce Muscle Degeneration. *J. Biol. Chem.* 274, 2193-2200.

Kamogawa, Y., Biro, S., Maeda, M., Setoguchi, M., Hirakawa, T., Yoshida, H., and Tei, C. (2001). Dystrophin-deficient myocardium is vulnerable to pressure overload in vivo. *Cardiovasc. Res.* 50, 509-515.

Khairallah, M., Khairallah, R.J., Young, M.E., Allen, B.G., Gillis, M.A., Danialou, G., Deschepper, C.F., Petrof, B.J., and Des Rosiers, C. (2008). Sildenafil and cardiomyocyte-specific cGMP signaling prevent cardiomyopathic changes associated with dystrophin deficiency. *Proc. Natl. Acad. Sci. U. S. A.* 105, 7028-7033.

Khurana, T.S., Prendergast, R.A., Alameddine, H.S., Tome, F.M., Fardeau, M., Arahata, K., Sugita, H., and Kunkel, L.M. (1995). Absence of extraocular muscle pathology in Duchenne's muscular dystrophy: role for calcium homeostasis in extraocular muscle sparing. *J. Exp. Med.* 182, 467-475.

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

Kinali, M., Arechavala-Gomez, V., Feng, L., Cirak, S., Hunt, D., Adkin, C., Guglieri, M., Ashton, E., Abbs, S., Nihoyannopoulos, P., *et al.* (2009). Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study *Lancet Neurol.* 8, 918-928.

Kirchmann, C., Kececioglu, D., Korinthenberg, R., and Dittrich, S. (2005). Echocardiographic and electrocardiographic findings of cardiomyopathy in Duchenne and Becker-Kiener muscular dystrophies. *Pediatr. Cardiol.* 26, 66-72.

Klein, C.J., Coover, D.D., Bulman, D.E., Ray, P.N., Mendell, J.R., and Burghes, A.H. (1992). Somatic reversion/suppression in Duchenne muscular dystrophy (DMD): evidence supporting a frame-restoring mechanism in rare dystrophin-positive fibers. *Am. J. Hum. Genet.* 50, 950-959.

Kleopa, K.A., Drousiotou, A., Mavrikiou, E., Ormiston, A., and Kyriakides, T. (2006). Naturally occurring utrophin correlates with disease severity in Duchenne muscular dystrophy. *Hum. Mol. Genet.* 15, 1623-1628.

Koenig, M., Hoffman, E.P., Bertelson, C.J., Monaco, A.P., Feener, C., and Kunkel, L.M. (1987). Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell* 50, 509-517.

Koenig, M., Monaco, A.P., and Kunkel, L.M. (1988). The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell* 53, 219-228.

Konstam, M.A., Patten, R.D., Thomas, I., Ramahi, T., La Bresh, K., Goldman, S., Lewis, W., Gradman, A., Self, K.S., Bittner, V., *et al.* (2000). Effects of losartan and captopril on left ventricular volumes in elderly patients with heart failure: results of the ELITE ventricular function substudy. *Am. Heart J.* 139, 1081-1087.

Kouloumenta, A., Mavroidis, M., and Capetanaki, Y. (2007). Proper perinuclear localization of the TRIM-like protein myospryn requires its binding partner desmin. *J. Biol. Chem.* 282, 35211-35221.

Kovick, R.B., Fogelman, A.M., Abbasi, A.D., Peter, J.B., and Pearce, M.L. (1975). Echocardiographic evaluation of posterior left ventricular wall motion in muscular dystrophy. *Circulation* 52, 447-454.

Krag, T.O., Bogdanovich, S., Jensen, C.J., Fischer, M.D., Hansen-Schwartz, J., Javazon, E.H., Flake, A.W., Edvinsson, L., and Khurana, T.S. (2004). Heregulin ameliorates the dystrophic phenotype in mdx mice. *Proc. Natl. Acad. Sci. U. S. A.* *101*, 13856-13860.

Kumar, A., and Boriek, A.M. (2003). Mechanical stress activates the nuclear factor-kappaB pathway in skeletal muscle fibers: a possible role in Duchenne muscular dystrophy *FASEB J.* *17*, 386-396.

Lai, Y., Thomas, G.D., Yue, Y., Yang, H.T., Li, D., Long, C., Judge, L., Bostick, B., Chamberlain, J.S., Terjung, R.L., and Duan, D. (2009). Dystrophins carrying spectrin-like repeats 16 and 17 anchor nNOS to the sarcolemma and enhance exercise performance in a mouse model of muscular dystrophy. *J. Clin. Invest.* *119*, 624-635.

Law, D.J., Allen, D.L., and Tidball, J.G. (1994). Talin, vinculin and DRP (utrophin) concentrations are increased at mdx myotendinous junctions following onset of necrosis. *J. Cell. Sci.* *107 (Pt 6)*, 1477-1483.

Levine, B.A., Moir, A.J., Patchell, V.B., and Perry, S.V. (1990). The interaction of actin with dystrophin. *FEBS Lett.* *263*, 159-162.

Li, D., Bareja, A., Judge, L., Yue, Y., Lai, Y., Fairclough, R., Davies, K.E., Chamberlain, J.S., and Duan, D. (2010). Sarcolemmal nNOS anchoring reveals a qualitative difference between dystrophin and utrophin. *J. Cell. Sci.* *123*, 2008-2013.

Li, D., Tapscoft, T., Gonzalez, O., Burch, P.E., Quinones, M.A., Zoghbi, W.A., Hill, R., Bachinski, L.L., Mann, D.L., and Roberts, R. (1999). Desmin mutation responsible for idiopathic dilated cardiomyopathy. *Circulation* *100*, 461-464.

Li, Y., Huang, J., Zhao, Y.L., He, J., Wang, W., Davies, K.E., Nose, V., and Xiao, S. (2007). UTRN on chromosome 6q24 is mutated in multiple tumors. *Oncogene* *26*, 6220-6228.

Li, Y., Jia, Y.C., Cui, K., Li, N., Zheng, Z.Y., Wang, Y.Z., and Yuan, X.B. (2005). Essential role of TRPC channels in the guidance of nerve growth cones by brain-derived neurotrophic factor. *Nature* *434*, 894-898.

Lim, L.E., Duclos, F., Broux, O., Bourg, N., Sunada, Y., Allamand, V., Meyer, J., Richard, I., Moomaw, C., and Slaughter, C. (1995). Beta-sarcoglycan: characterization and role in limb-girdle muscular dystrophy linked to 4q12. *Nat. Genet.* *11*, 257-265.

Lintschinger, B., Balzer-Geldsetzer, M., Baskaran, T., Graier, W.F., Romanin, C., Zhu, M.X., and Groschner, K. (2000). Coassembly of Trp1 and Trp3 proteins generates diacylglycerol- and Ca²⁺-sensitive cation channels. *J. Biol. Chem.* *275*, 27799-27805.

Liu, Y., Cseresnyes, Z., Randall, W.R., and Schneider, M.F. (2001). Activity-dependent nuclear translocation and intranuclear distribution of NFATc in adult skeletal muscle fibers. *J. Cell Biol.* *155*, 27-39.

Ljunggren, A., Duggan, D., McNally, E., Boylan, K.B., Gama, C.H., Kunkel, L.M., and Hoffman, E.P. (1995). Primary adhalin deficiency as a cause of muscular dystrophy in patients with normal dystrophin. *Ann. Neurol.* 38, 367-372.

Lockwich, T., Pant, J., Makusky, A., Jankowska-Stephens, E., Kowalak, J.A., Markey, S.P., and Ambudkar, I.S. (2008). Analysis of TRPC3-interacting proteins by tandem mass spectrometry. *J. Proteome Res.* 7, 979-989.

Love, D.R., Hill, D.F., Dickson, G., Spurr, N.K., Byth, B.C., Marsden, R.F., Walsh, F.S., Edwards, Y.H., and Davies, K.E. (1989). An autosomal transcript in skeletal muscle with homology to dystrophin. *Nature* 339, 55-58.

Lu, Q.L., Morris, G.E., Wilton, S.D., Ly, T., Artem'yeva, O.V., Strong, P., and Partridge, T.A. (2000). Massive idiosyncratic exon skipping corrects the nonsense mutation in dystrophic mouse muscle and produces functional revertant fibers by clonal expansion. *J. Cell Biol.* 148, 985-996.

Lu, Q.L., Rabinowitz, A., Chen, Y.C., Yokota, T., Yin, H., Alter, J., Jadoon, A., Bou-Gharios, G., and Partridge, T. (2005). Systemic delivery of antisense oligoribonucleotide restores dystrophin expression in body-wide skeletal muscles *Proc. Natl. Acad. Sci. U. S. A.* 102, 198-203.

Maier, M., Bleicher, K., Kalthoff, H., and Bayer, E. (1995). Enzymatic degradation of various antisense oligonucleotides: monitoring and fragment identification by MECC and ES-MS. *Biomed. Pept. Proteins Nucleic Acids* 1, 235-242.

Malik, V., Rodino-Klapac, L.R., Viollet, L., Wall, C., King, W., Al-Dahhak, R., Lewis, S., Shilling, C.J., Kota, J., Serrano-Munuera, C., *et al.* (2010). Gentamicin-induced readthrough of stop codons in Duchenne muscular dystrophy. *Ann. Neurol.* 67, 771-780.

Manning, G.W., and Cropp, G.J. (1958). The electrocardiogram in progressive muscular dystrophy. *Br. Heart J.* 20, 416-420.

Manzur, A.Y., Kuntzer, T., Pike, M., and Swan, A. (2008). Glucocorticoid corticosteroids for Duchenne muscular dystrophy. *Cochrane Database Syst. Rev.* (1), CD003725.

Marini, J.F., Pons, F., Leger, J., Loffreda, N., Anoal, M., Chevallay, M., Fardeau, M., and Leger, J.J. (1991). Expression of myosin heavy chain isoforms in Duchenne muscular dystrophy patients and carriers. *Neuromuscul. Disord.* 1, 397-409.

Martineau, L., Horan, M.A., Rothwell, N.J., and Little, R.A. (1992). Salbutamol, a beta 2-adrenoceptor agonist, increases skeletal muscle strength in young men. *Clin. Sci. (Lond)* 83, 615-621.

Matsumura, K., Ervasti, J.M., Ohlendieck, K., Kahl, S.D., and Campbell, K.P. (1992). Association of dystrophin-related protein with dystrophin-associated proteins in mdx mouse muscle. *Nature* 360, 588-591.

Mattei, E., Corbi, N., Di Certo, M.G., Strimpakos, G., Severini, C., Onori, A., Desantis, A., Libri, V., Buontempo, S., Floridi, A., *et al.* (2007). Utrophin up-regulation by an artificial transcription factor in transgenic mice. *PLoS One* 2, e774.

McCullagh, K.J., Edwards, B., Kemp, M.W., Giles, L.C., Burgess, M., and Davies, K.E. (2008). Analysis of skeletal muscle function in the C57BL6/SV129 syncoilin knockout mouse. *Mamm. Genome* 19, 339-351.

McCullagh, K.J., Edwards, B., Poon, E., Lovering, R.M., Paulin, D., and Davies, K.E. (2007). Intermediate filament-like protein syncoilin in normal and myopathic striated muscle. *Neuromuscul. Disord.*

McNally, E.M., Ly, C.T., and Kunkel, L.M. (1998). Human epsilon-sarcoglycan is highly related to alpha-sarcoglycan (adhalin), the limb girdle muscular dystrophy 2D gene. *FEBS Lett.* 422, 27-32.

Melacini, P., Fanin, M., Danieli, G.A., Villanova, C., Martinello, F., Miorin, M., Freda, M.P., Miorelli, M., Mostacciuolo, M.L., Fasoli, G., Angelini, C., and Dalla Volta, S. (1996a). Myocardial involvement is very frequent among patients affected with subclinical Becker's muscular dystrophy. *Circulation* 94, 3168-3175.

Melacini, P., Vianello, A., Villanova, C., Fanin, M., Miorin, M., Angelini, C., and Dalla Volta, S. (1996b). Cardiac and respiratory involvement in advanced stage Duchenne muscular dystrophy. *Neuromuscul. Disord.* 6, 367-376.

Menke, A., and Jockusch, H. (1991). Decreased osmotic stability of dystrophin-less muscle cells from the mdx mouse. *Nature* 349, 69-71.

Meszaros, L.G., Minarovic, I., and Zahradnikova, A. (1996). Inhibition of the skeletal muscle ryanodine receptor calcium release channel by nitric oxide. *FEBS Lett.* *380*, 49-52.

Miike, T., Miyatake, M., Zhao, J., Yoshioka, K., and Uchino, M. (1989). Immunohistochemical dystrophin reaction in synaptic regions. *Brain Dev.* *11*, 344-346.

Millay, D.P., Goonasekera, S.A., Sargent, M.A., Maillet, M., Aronow, B.J., and Molkentin, J.D. (2009). Calcium influx is sufficient to induce muscular dystrophy through a TRPC-dependent mechanism. *Proc. Natl. Acad. Sci. U. S. A.* *106*, 19023-19028.

Milner, D.J., Taffet, G.E., Wang, X., Pham, T., Tamura, T., Hartley, C., Gerdes, A.M., and Capetanaki, Y. (1999). The absence of desmin leads to cardiomyocyte hypertrophy and cardiac dilation with compromised systolic function. *J. Mol. Cell. Cardiol.* *31*, 2063-2076.

Milner, D.J., Weitzer, G., Tran, D., Bradley, A., and Capetanaki, Y. (1996). Disruption of muscle architecture and myocardial degeneration in mice lacking desmin. *J. Cell Biol.* *134*, 1255-1270.

Mokri, B., and Engel, A.G. (1975). Duchenne dystrophy: electron microscopic findings pointing to a basic or early abnormality in the plasma membrane of the muscle fiber. *Neurology* *25*, 1111-1120.

Molkentin, J.D., Lu, J.R., Antos, C.L., Markham, B., Richardson, J., Robbins, J., Grant, S.R., and Olson, E.N. (1998). A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* *93*, 215-228.

Monaco, A.P., Bertelson, C.J., Liechti-Gallati, S., Moser, H., and Kunkel, L.M. (1988). An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. *Genomics* 2, 90-95.

Montell, C., and Rubin, G.M. (1989). Molecular characterization of the *Drosophila* trp locus: a putative integral membrane protein required for phototransduction. *Neuron* 2, 1313-1323.

Moores, C.A., and Kendrick-Jones, J. (2000). Biochemical characterisation of the actin-binding properties of utrophin. *Cell Motil. Cytoskeleton* 46, 116-128.

Morandi, L., Mora, M., Gussoni, E., Tedeschi, S., and Cornelio, F. (1990). Dystrophin analysis in Duchenne and Becker muscular dystrophy carriers: correlation with intracellular calcium and albumin. *Ann. Neurol.* 28, 674-679.

Morgan, J.E., Pagel, C.N., Sherratt, T., and Partridge, T.A. (1993). Long-term persistence and migration of myogenic cells injected into pre-irradiated muscles of mdx mice. *J. Neurol. Sci.* 115, 191-200.

Moric-Janiszewska, E., and Markiewicz-Loskot, G. (2008). Genetic heterogeneity of left-ventricular noncompaction cardiomyopathy. *Clin. Cardiol.* 31, 201-204.

Morine, K., Sleeper, M.M., Barton, E.R., and Sweeney, L. (2010). Overexpression of SERCA1a in the mdx diaphragm reduces susceptibility to contraction induced damage. *Hum. Gene Ther.*

- Morrison, J., Lu, Q.L., Pastoret, C., Partridge, T., and Bou-Gharios, G. (2000). T-cell-dependent fibrosis in the mdx dystrophic mouse. *Lab. Invest.* *80*, 881-891.
- Nakayama, H., Wilkin, B.J., Bodi, I., and Molkenin, J.D. (2006). Calcineurin-dependent cardiomyopathy is activated by TRPC in the adult mouse heart. *FASEB J.* *20*, 1660-1670.
- Nathan, C. (1997). Inducible nitric oxide synthase: what difference does it make? *J. Clin. Invest.* *100*, 2417-2423.
- Newey, S.E., Howman, E.V., Ponting, C.P., Benson, M.A., Nawroztzki, R., Loh, N.Y., Davies, K.E., and Blake, D.J. (2001). Syncoilin, a novel member of the intermediate filament superfamily that interacts with alpha-dystrobrevin in skeletal muscle. *J. Biol. Chem.* *276*, 6645-6655.
- Nguyen, T.M., Ellis, J.M., Love, D.R., Davies, K.E., Gatter, K.C., Dickson, G., and Morris, G.E. (1991). Localization of the DMDL gene-encoded dystrophin-related protein using a panel of nineteen monoclonal antibodies: presence at neuromuscular junctions, in the sarcolemma of dystrophic skeletal muscle, in vascular and other smooth muscles, and in proliferating brain cell lines *J. Cell Biol.* *115*, 1695-1700.
- Nicholson, L.V., Davison, K., Johnson, M.A., Slater, C.R., Young, C., Bhattacharya, S., Gardner-Medwin, D., and Harris, J.B. (1989). Dystrophin in skeletal muscle. II. Immunoreactivity in patients with Xp21 muscular dystrophy. *J. Neurol. Sci.* *94*, 137-146.

Nigro, G., Comi, L.I., Politano, L., and Bain, R.J. (1990). The incidence and evolution of cardiomyopathy in Duchenne muscular dystrophy. *Int. J. Cardiol.* 26, 271-277.

Nigro, G., Comi, L.I., Politano, L., Limongelli, F.M., Nigro, V., De Rimini, M.L., Giugliano, M.A., Petretta, V.R., Passamano, L., and Restucci, B. (1995). Evaluation of the cardiomyopathy in Becker muscular dystrophy. *Muscle Nerve* 18, 283-291.

Nigro, V., de Sa Moreira, E., Piluso, G., Vainzof, M., Belsito, A., Politano, L., Puca, A.A., Passos-Bueno, M.R., and Zatz, M. (1996). Autosomal recessive limb-girdle muscular dystrophy, LGMD2F, is caused by a mutation in the delta-sarcoglycan gene. *Nat. Genet.* 14, 195-198.

Nishida, M., Hara, Y., Yoshida, T., Inoue, R., and Mori, Y. (2006). TRP channels: molecular diversity and physiological function. *Microcirculation* 13, 535-550.

Nishimura, T., Yanagisawa, A., Sakata, H., Sakata, K., Shimoyama, K., Ishihara, T., Yoshino, H., and Ishikawa, K. (2001). Thallium-201 single photon emission computed tomography (SPECT) in patients with duchenne's progressive muscular dystrophy: a histopathologic correlation study. *Jpn. Circ. J.* 65, 99-105.

Oh, M., Rybkin, I.I., Copeland, V., Czubryt, M.P., Shelton, J.M., van Rooij, E., Richardson, J.A., Hill, J.A., De Windt, L.J., Bassel-Duby, R., Olson, E.N., and Rothermel, B.A. (2005). Calcineurin is necessary for the maintenance but not embryonic development of slow muscle fibers. *Mol. Cell. Biol.* 25, 6629-6638.

Ohba, T., Watanabe, H., Murakami, M., Takahashi, Y., Iino, K., Kuromitsu, S., Mori, Y., Ono, K., Iijima, T., and Ito, H. (2007). Upregulation of TRPC1 in the development of cardiac hypertrophy. *J. Mol. Cell. Cardiol.* 42, 498-507.

Ohlendieck, K., and Campbell, K.P. (1991). Dystrophin-associated proteins are greatly reduced in skeletal muscle from mdx mice. *J. Cell Biol.* 115, 1685-1694.

Ohlendieck, K., Ervasti, J.M., Matsumura, K., Kahl, S.D., Leveille, C.J., and Campbell, K.P. (1991a). Dystrophin-related protein is localized to neuromuscular junctions of adult skeletal muscle. *Neuron* 7, 499-508.

Ohlendieck, K., Ervasti, J.M., Snook, J.B., and Campbell, K.P. (1991b). Dystrophin-glycoprotein complex is highly enriched in isolated skeletal muscle sarcolemma. *J. Cell Biol.* 112, 135-148.

Ohlendieck, K., Matsumura, K., Ionasescu, V.V., Towbin, J.A., Bosch, E.P., Weinstein, S.L., Sernett, S.W., and Campbell, K.P. (1993). Duchenne muscular dystrophy: deficiency of dystrophin-associated proteins in the sarcolemma. *Neurology* 43, 795-800.

Onohara, N., Nishida, M., Inoue, R., Kobayashi, H., Sumimoto, H., Sato, Y., Mori, Y., Nagao, T., and Kurose, H. (2006). TRPC3 and TRPC6 are essential for angiotensin II-induced cardiac hypertrophy. *EMBO J.* 25, 5305-5316.

Otten, E., Asimaki, A., Maass, A., van Langen, I.M., van der Wal, A., de Jonge, N., van den Berg, M.P., Saffitz, J.E., Wilde, A.A., Jongbloed, J.D., and van Tintelen, J.P. (2010).

Desmin mutations as a cause of right ventricular heart failure affect the intercalated disks. *Heart Rhythm* 7, 1058-1064.

Passos-Bueno, M.R., Moreira, E.S., Vainzof, M., Marie, S.K., and Zatz, M. (1996). Linkage analysis in autosomal recessive limb-girdle muscular dystrophy (AR LGMD) maps a sixth form to 5q33-34 (LGMD2F) and indicates that there is at least one more subtype of AR LGMD. *Hum. Mol. Genet.* 5, 815-820.

Pastoret, C., and Sebille, A. (1995). Age-related differences in regeneration of dystrophic (mdx) and normal muscle in the mouse. *Muscle Nerve* 18, 1147-1154.

Pastoret, C., and Sebille, A. (1993). Fibres of intermediate type 1C and 2C are found continuously in mdx soleus muscle up to 52 weeks. *Histochemistry* 100, 271-276.

Paton, J.F., Kasparov, S., and Paterson, D.J. (2002). Nitric oxide and autonomic control of heart rate: a question of specificity. *Trends Neurosci.* 25, 626-631.

Paulin, D., and Li, Z. (2004). Desmin: a major intermediate filament protein essential for the structural integrity and function of muscle. *Exp. Cell Res.* 301, 1-7.

Peppiatt-Wildman, C.M., Albert, A.P., Saleh, S.N., and Large, W.A. (2007). Endothelin-1 activates a Ca²⁺-permeable cation channel with TRPC3 and TRPC7 properties in rabbit coronary artery myocytes. *J. Physiol.* 580, 755-764.

Perloff, J.K., de Leon, A.C., Jr, and O'Doherty, D. (1966). The cardiomyopathy of progressive muscular dystrophy. *Circulation* 33, 625-648.

Peters, M.F., Adams, M.E., and Froehner, S.C. (1997). Differential association of syntrophin pairs with the dystrophin complex. *J. Cell Biol.* 138, 81-93.

Petrof, B.J., Shrager, J.B., Stedman, H.H., Kelly, A.M., and Sweeney, H.L. (1993). Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc. Natl. Acad. Sci. U. S. A.* 90, 3710-3714.

Phelps, S.F., Hauser, M.A., Cole, N.M., Rafael, J.A., Hinkle, R.T., Faulkner, J.A., and Chamberlain, J.S. (1995). Expression of full-length and truncated dystrophin mini-genes in transgenic mdx mice. *Hum. Mol. Genet.* 4, 1251-1258.

Piccolo, F., Jeanpierre, M., Leturcq, F., Dode, C., Azibi, K., Toutain, A., Merlini, L., Jarre, L., Navarro, C., Krishnamoorthy, R., *et al.* (1996). A founder mutation in the gamma-sarcoglycan gene of gypsies possibly predating their migration out of India. *Hum. Mol. Genet.* 5, 2019-2022.

Piccolo, F., Roberds, S.L., Jeanpierre, M., Leturcq, F., Azibi, K., Beldjord, C., Carrie, A., Recan, D., Chaouch, M., and Reghis, A. (1995). Primary adhalinopathy: a common cause of autosomal recessive muscular dystrophy of variable severity. *Nat. Genet.* 10, 243-245.

Pigozzi, D., Ducret, T., Tajeddine, N., Gala, J.L., Tombal, B., and Gailly, P. (2006). Calcium store contents control the expression of TRPC1, TRPC3 and TRPV6 proteins in LNCaP prostate cancer cell line. *Cell Calcium* 39, 401-415.

Pons, F., Robert, A., Fabbrizio, E., Hugon, G., Califano, J.C., Fehrentz, J.A., Martinez, J., and Mornet, D. (1994). Utrophin localization in normal and dystrophin-deficient heart. *Circulation* 90, 369-374.

Poon, E., Howman, E.V., Newey, S.E., and Davies, K.E. (2002). Association of syncoilin and desmin: linking intermediate filament proteins to the dystrophin-associated protein complex. *J. Biol. Chem.* 277, 3433-3439.

Porter, G.A., Dmytrenko, G.M., Winkelmann, J.C., and Bloch, R.J. (1992). Dystrophin colocalizes with beta-spectrin in distinct subsarcolemmal domains in mammalian skeletal muscle. *J. Cell Biol.* 117, 997-1005.

Pourdjabbar, A., Parker, T.G., Nguyen, Q.T., Desjardins, J.F., Lapointe, N., Tsoporis, J.N., and Rouleau, J.L. (2005). Effects of pre-, peri-, and postmyocardial infarction treatment with losartan in rats: effect of dose on survival, ventricular arrhythmias, function, and remodeling. *Am. J. Physiol. Heart Circ. Physiol.* 288, H1997-2005.

Price, M.G. (1984). Molecular analysis of intermediate filament cytoskeleton--a putative load-bearing structure. *Am. J. Physiol.* 246, H566-72.

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 ANNOUNCE PRELIMINARY RESULTS FROM THE PHASE 2B CLINICAL TRIAL
 OF ATALUREN FOR NONSENSE MUTATION DUCHENNE/BECKER MUSCULAR
 DYSTROPHY. 2010,
<http://ptct.client.shareholder.com/releasedetail.cfm?ReleaseID=448803>

Quinlan, J.G., Hahn, H.S., Wong, B.L., Lorenz, J.N., Wenisch, A.S., and Levin, L.S.
 (2004). Evolution of the mdx mouse cardiomyopathy: physiological and morphological
 findings *Neuromuscul. Disord.* 14, 491-496.

Radley, H.G., Davies, M.J., and Grounds, M.D. (2008). Reduced muscle necrosis and
 long-term benefits in dystrophic mdx mice after cV1q (blockade of TNF) treatment.
Neuromuscul. Disord. 18, 227-238.

Rafael, J.A., Townsend, E.R., Squire, S.E., Potter, A.C., Chamberlain, J.S., and Davies,
 K.E. (2000). Dystrophin and utrophin influence fiber type composition and post-synaptic
 membrane structure. *Hum. Mol. Genet.* 9, 1357-1367.

Rando, T.A., Disatnik, M.H., Yu, Y., and Franco, A. (1998). Muscle cells from mdx mice
 have an increased susceptibility to oxidative stress. *Neuromuscul. Disord.* 8, 14-21.

Rezniczek, G.A., Konieczny, P., Nikolic, B., Reipert, S., Schneller, D., Abrahamsberg, C.,
 Davies, K.E., Winder, S.J., and Wiche, G. (2007). Plectin 1f scaffolding at the sarcolemma
 of dystrophic (mdx) muscle fibers through multiple interactions with beta-dystroglycan. *J.*
Cell Biol. 176, 965-977.

- Ringel, S.P., Carroll, J.E., and Schold, S.C. (1977). The spectrum of mild X-linked recessive muscular dystrophy. *Arch. Neurol.* 34, 408-416.
- Roberds, S.L., Anderson, R.D., Ibraghimov-Beskrovnaya, O., and Campbell, K.P. (1993). Primary structure and muscle-specific expression of the 50-kDa dystrophin-associated glycoprotein (adhalin). *J. Biol. Chem.* 268, 23739-23742.
- Roberds, S.L., Leturcq, F., Allamand, V., Piccolo, F., Jeanpierre, M., Anderson, R.D., Lim, L.E., Lee, J.C., Tome, F.M., and Romero, N.B. (1994). Missense mutations in the adhalin gene linked to autosomal recessive muscular dystrophy. *Cell* 78, 625-633.
- Rosker, C., Graziani, A., Lukas, M., Eder, P., Zhu, M.X., Romanin, C., and Groschner, K. (2004). Ca^{2+} signaling by TRPC3 involves Na^{+} entry and local coupling to the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger. *J. Biol. Chem.* 279, 13696-13704.
- Roth, D.M., Swaney, J.S., Dalton, N.D., Gilpin, E.A., and Ross, J., Jr. (2002). Impact of anesthesia on cardiac function during echocardiography in mice. *Am. J. Physiol. Heart Circ. Physiol.* 282, H2134-40.
- Rothwell, N.J., and Stock, M.J. (1985). Modification of body composition by clenbuterol in normal and dystrophic (mdx) mice. *Biosci. Rep.* 5, 755-760.
- Rowland, L.P. (1980). Biochemistry of muscle membranes in Duchenne muscular dystrophy. *Muscle Nerve* 3, 3-20.

Rubin, I.L. (1952). The heart in progressive muscular dystrophy. *Am. Heart J.* *43*, 161-169.

Rybakova, I.N., Amann, K.J., and Ervasti, J.M. (1996). A new model for the interaction of dystrophin with F-actin. *J. Cell Biol.* *135*, 661-672.

Rybakova, I.N., and Ervasti, J.M. (2005). Identification of spectrin-like repeats required for high affinity utrophin-actin interaction. *J. Biol. Chem.* *280*, 23018-23023.

Rybakova, I.N., Humston, J.L., Sonnemann, K.J., and Ervasti, J.M. (2006). Dystrophin and utrophin bind actin through distinct modes of contact. *J. Biol. Chem.* *281*, 9996-10001.

Rybakova, I.N., Patel, J.R., Davies, K.E., Yurchenco, P.D., and Ervasti, J.M. (2002). Utrophin binds laterally along actin filaments and can couple costameric actin with sarcolemma when overexpressed in dystrophin-deficient muscle. *Mol. Biol. Cell* *13*, 1512-1521.

Sadoulet-Puccio, H.M., Feener, C.A., Schaid, D.J., Thibodeau, S.N., Michels, V.V., and Kunkel, L.M. (1997a). The genomic organization of human dystrobrevin. *Neurogenetics* *1*, 37-42.

Sadoulet-Puccio, H.M., Rajala, M., and Kunkel, L.M. (1997b). Dystrobrevin and dystrophin: an interaction through coiled-coil motifs. *Proc. Natl. Acad. Sci. U. S. A.* *94*, 12413-12418.

Salloum, F.N., Abbate, A., Das, A., Houser, J.E., Mudrick, C.A., Qureshi, I.Z., Hoke, N.N., Roy, S.K., Brown, W.R., Prabhakar, S., and Kukreja, R.C. (2008). Sildenafil (Viagra) attenuates ischemic cardiomyopathy and improves left ventricular function in mice. *Am. J. Physiol. Heart Circ. Physiol.* 294, H1398-406.

Samaha, F.J., and Gergely, J. (1969). Biochemical abnormalities of the sarcoplasmic reticulum in muscular dystrophy. *N. Engl. J. Med.* 280, 184-188.

Samitt, C.E., and Bonilla, E. (1990). Immunocytochemical study of dystrophin at the myotendinous junction. *Muscle Nerve* 13, 493-500.

Sampaolesi, M., Blot, S., D'Antona, G., Granger, N., Tonlorenzi, R., Innocenzi, A., Mognol, P., Thibaud, J.L., Galvez, B.G., Barthelemy, I., *et al.* (2006). Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. *Nature* 444, 574-579.

Sanyal, S.K., Johnson, W.W., Thapar, M.K., and Pitner, S.E. (1978). An ultrastructural basis for electrocardiographic alterations associated with Duchenne's progressive muscular dystrophy. *Circulation* 57, 1122-1129.

Sapp, J.L., Bobet, J., and Howlett, S.E. (1996). Contractile properties of myocardium are altered in dystrophin-deficient mdx mice. *J. Neurol. Sci.* 142, 17-24.

Satoh, S., Tanaka, H., Ueda, Y., Oyama, J., Sugano, M., Sumimoto, H., Mori, Y., and Makino, N. (2007). Transient receptor potential (TRP) protein 7 acts as a G protein-

activated Ca²⁺ channel mediating angiotensin II-induced myocardial apoptosis. *Mol. Cell. Biochem.* 294, 205-215.

Schmajuk, G., Sierakowska, H., and Kole, R. (1999). Antisense oligonucleotides with different backbones. Modification of splicing pathways and efficacy of uptake. *J. Biol. Chem.* 274, 21783-21789.

Schneider, J.E., Cassidy, P.J., Lygate, C., Tyler, D.J., Wiesmann, F., Grieve, S.M., Hulbert, K., Clarke, K., and Neubauer, S. (2003). Fast, high-resolution in vivo cine magnetic resonance imaging in normal and failing mouse hearts on a vertical 11.7 T system. *J. Magn. Reson. Imaging* 18, 691-701.

Schneider, J.E., Hulbert, K.J., Lygate, C.A., Ten Hove, M., Cassidy, P.J., Clarke, K., and Neubauer, S. (2004). Long-term stability of cardiac function in normal and chronically failing mouse hearts in a vertical-bore MR system. *MAGMA* 17, 162-169.

Sears, C.E., Bryant, S.M., Ashley, E.A., Lygate, C.A., Rakovic, S., Wallis, H.L., Neubauer, S., Terrar, D.A., and Casadei, B. (2003). Cardiac neuronal nitric oxide synthase isoform regulates myocardial contraction and calcium handling. *Circ. Res.* 92, e52-9.

Seth, M., Sumbilla, C., Mullen, S.P., Lewis, D., Klein, M.G., Hussain, A., Soboloff, J., Gill, D.L., and Inesi, G. (2004). Sarco(endo)plasmic reticulum Ca²⁺ ATPase (SERCA) gene silencing and remodeling of the Ca²⁺ signaling mechanism in cardiac myocytes. *Proc. Natl. Acad. Sci. U. S. A.* 101, 16683-16688.

Shimizu, . (1989). Dense immunostainings on both neuromuscular and myotendon junctions with an anti-dystrophin monoclonal antibody. *Biomedical Research* 10, 405.

Sicinski, P., Geng, Y., Ryder-Cook, A.S., Barnard, E.A., Darlison, M.G., and Barnard, P.J. (1989). The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science* 244, 1578-1580.

Simmons, D., Linde, L., Miller, J., and O'Reilly, R. (1961). Relation Between Lung Volume and Pulmonary Vascular Resistance *Circ. Res.* 9, 465-471.

Song, K.S., Scherer, P.E., Tang, Z., Okamoto, T., Li, S., Chafel, M., Chu, C., Kohtz, D.S., and Lisanti, M.P. (1996). Expression of caveolin-3 in skeletal, cardiac, and smooth muscle cells. Caveolin-3 is a component of the sarcolemma and co-fractionates with dystrophin and dystrophin-associated glycoproteins. *J. Biol. Chem.* 271, 15160-15165.

Sotgia, F., Lee, J.K., Das, K., Bedford, M., Petrucci, T.C., Macioce, P., Sargiacomo, M., Bricarelli, F.D., Minetti, C., Sudol, M., and Lisanti, M.P. (2000). Caveolin-3 directly interacts with the C-terminal tail of beta -dystroglycan. Identification of a central WW-like domain within caveolin family members. *J. Biol. Chem.* 275, 38048-38058.

Spencer, M.J., Croall, D.E., and Tidball, J.G. (1995). Calpains are activated in necrotic fibers from mdx dystrophic mice. *J. Biol. Chem.* 270, 10909-10914.

Spurney, C.F., Knobloch, S., Pistilli, E.E., Nagaraju, K., Martin, G.R., and Hoffman, E.P. (2008). Dystrophin-deficient cardiomyopathy in mouse: expression of Nox4 and Lox are

associated with fibrosis and altered functional parameters in the heart. *Neuromuscul. Disord.* *18*, 371-381.

Spurney, C.F., Sali, A., Guerron, A.D., Iantorno, M., Yu, Q., Gordish-Dressman, H., Rayavarapu, S., van der Meulen, J., Hoffman, E.P., and Nagaraju, K. (2011). Losartan decreases cardiac muscle fibrosis and improves cardiac function in dystrophin-deficient mdx mice. *J. Cardiovasc. Pharmacol. Ther.* *16*, 87-95.

Squire, S., Raymackers, J.M., Vandebrouck, C., Potter, A., Tinsley, J., Fisher, R., Gillis, J.M., and Davies, K.E. (2002). Prevention of pathology in mdx mice by expression of utrophin: analysis using an inducible transgenic expression system. *Hum. Mol. Genet.* *11*, 3333-3344.

Stedman, H.H., Sweeney, H.L., Shrager, J.B., Maguire, H.C., Panettieri, R.A., Petrof, B., Narusawa, M., Leferovich, J.M., Sladky, J.T., and Kelly, A.M. (1991). The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy *Nature* *352*, 536-539.

Stevenson, S., Rothery, S., Cullen, M.J., and Severs, N.J. (1997). Dystrophin is not a specific component of the cardiac costamere. *Circ. Res.* *80*, 269-280.

Stuckey, D.J., Carr, C.A., Tyler, D.J., and Clarke, K. (2008). Cine-MRI versus two-dimensional echocardiography to measure in vivo left ventricular function in rat heart. *NMR Biomed.* *21*, 765-772.

Summerton, J. (1999). Morpholino antisense oligomers: the case for an RNase H-independent structural type. *Biochim. Biophys. Acta* 1489, 141-158.

Summerton, J., and Weller, D. (1997). Morpholino antisense oligomers: design, preparation, and properties. *Antisense Nucleic Acid Drug Dev.* 7, 187-195.

Suzuki, A., Yoshida, M., and Ozawa, E. (1995). Mammalian alpha 1- and beta 1-syntrophin bind to the alternative splice-prone region of the dystrophin COOH terminus. *J. Cell Biol.* 128, 373-381.

Suzuki, A., Yoshida, M., Yamamoto, H., and Ozawa, E. (1992). Glycoprotein-binding site of dystrophin is confined to the cysteine-rich domain and the first half of the carboxy-terminal domain. *FEBS Lett.* 308, 154-160.

Sylvius, N., Duboscq-Bidot, L., Bouchier, C., Charron, P., Benaiche, A., Sebillon, P., Komajda, M., and Villard, E. (2003). Mutational analysis of the beta- and delta-sarcoglycan genes in a large number of patients with familial and sporadic dilated cardiomyopathy. *Am. J. Med. Genet. A.* 120A, 8-12.

Takagi, A., Schotland, D.L., and Rowland, L.P. (1973). Sarcoplasmic reticulum in Duchenne muscular dystrophy. *Arch. Neurol.* 28, 380-384.

Takeshima, Y., Nishio, H., Sakamoto, H., Nakamura, H., and Matsuo, M. (1995). Modulation of in vitro splicing of the upstream intron by modifying an intra-exon

sequence which is deleted from the dystrophin gene in dystrophin Kobe. *J. Clin. Invest.* 95, 515-520.

Takimoto, E., Champion, H.C., Li, M., Belardi, D., Ren, S., Rodriguez, E.R., Bedja, D., Gabrielson, K.L., Wang, Y., and Kass, D.A. (2005). Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat. Med.* 11, 214-222.

Takuma, S., Suehiro, K., Cardinale, C., Hozumi, T., Yano, H., Shimizu, J., Mullis-Jansson, S., Sciacca, R., Wang, J., Burkhoff, D., Di Tullio, M.R., and Homma, S. (2001). Anesthetic inhibition in ischemic and nonischemic murine heart: comparison with conscious echocardiographic approach. *Am. J. Physiol. Heart Circ. Physiol.* 280, H2364-70.

Tanaka, K., Watakabe, A., and Shimura, Y. (1994). Polypurine sequences within a downstream exon function as a splicing enhancer. *Mol. Cell. Biol.* 14, 1347-1354.

Tang, Y., Reay, D.P., Salay, M.N., Mi, M.Y., Clemens, P.R., Guttridge, D.C., Robbins, P.D., Huard, J., and Wang, B. (2010). Inhibition of the IKK/NF-kappaB pathway by AAV gene transfer improves muscle regeneration in older mdx mice. *Gene Ther.*

Taylor, J., Muntoni, F., Dubowitz, V., and Sewry, C.A. (1997). The abnormal expression of utrophin in Duchenne and Becker muscular dystrophy is age related. *Neuropathol. Appl. Neurobiol.* 23, 399-405.

Taylor, M.R., Slavov, D., Ku, L., Di Lenarda, A., Sinagra, G., Carniel, E., Haubold, K., Boucek, M.M., Ferguson, D., Graw, S.L., *et al.* (2007). Prevalence of desmin mutations in dilated cardiomyopathy. *Circulation* 115, 1244-1251.

Tennyson, C.N., Klamut, H.J., and Worton, R.G. (1995). The human dystrophin gene requires 16 hours to be transcribed and is cotranscriptionally spliced. *Nat. Genet.* 9, 184-190.

Tesson, F., Sylvius, N., Pilotto, A., Dubosq-Bidot, L., Peuchmaurd, M., Bouchier, C., Benaiche, A., Mangin, L., Charron, P., Gavazzi, A., *et al.* (2000). Epidemiology of desmin and cardiac actin gene mutations in a european population of dilated cardiomyopathy. *Eur. Heart J.* 21, 1872-1876.

Thebault, S., Zholos, A., Enfissi, A., Slomianny, C., Dewailly, E., Roudbaraki, M., Parys, J., and Prevarskaya, N. (2005). Receptor-operated Ca^{2+} entry mediated by TRPC3/TRPC6 proteins in rat prostate smooth muscle (PS1) cell line. *J. Cell. Physiol.* 204, 320-328.

Thornell, L., Carlsson, L., Li, Z., Mericskay, M., and Paulin, D. (1997). Null mutation in the desmin gene gives rise to a cardiomyopathy. *J. Mol. Cell. Cardiol.* 29, 2107-2124.

Thyagarajan, B., Poteser, M., Romanin, C., Kahr, H., Zhu, M.X., and Groschner, K. (2001). Expression of Trp3 determines sensitivity of capacitative Ca^{2+} entry to nitric oxide and mitochondrial Ca^{2+} handling: evidence for a role of Trp3 as a subunit of capacitative Ca^{2+} entry channels. *J. Biol. Chem.* 276, 48149-48158.

- Tidball, J.G. (2005). Inflammatory processes in muscle injury and repair *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 288, R345-53.
- Tinsley, J., Deconinck, N., Fisher, R., Kahn, D., Phelps, S., Gillis, J.M., and Davies, K. (1998). Expression of full-length utrophin prevents muscular dystrophy in mdx mice *Nat. Med.* 4, 1441-1444.
- Tinsley, J.M., Blake, D.J., Roche, A., Fairbrother, U., Riss, J., Byth, B.C., Knight, A.E., Kendrick-Jones, J., Suthers, G.K., and Love, D.R. (1992). Primary structure of dystrophin-related protein. *Nature* 360, 591-593.
- Tinsley, J.M., Potter, A.C., Phelps, S.R., Fisher, R., Trickett, J.I., and Davies, K.E. (1996). Amelioration of the dystrophic phenotype of mdx mice using a truncated utrophin transgene. *Nature* 384, 349-353.
- Tochio, H., Zhang, Q., Mandal, P., Li, M., and Zhang, M. (1999). Solution structure of the extended neuronal nitric oxide synthase PDZ domain complexed with an associated peptide. *Nat. Struct. Biol.* 6, 417-421.
- Townsend, D., Blankinship, M.J., Allen, J.M., Gregorevic, P., Chamberlain, J.S., and Metzger, J.M. (2007). Systemic administration of micro-dystrophin restores cardiac geometry and prevents dobutamine-induced cardiac pump failure. *Mol. Ther.* 15, 1086-1092.

Townsend, D., Yasuda, S., Li, S., Chamberlain, J.S., and Metzger, J.M. (2008). Emergent dilated cardiomyopathy caused by targeted repair of dystrophic skeletal muscle. *Mol. Ther.* *16*, 832-835.

Trebak, M., Hempel, N., Wedel, B.J., Smyth, J.T., Bird, G.S., and Putney, J.W., Jr. (2005). Negative regulation of TRPC3 channels by protein kinase C-mediated phosphorylation of serine 712. *Mol. Pharmacol.* *67*, 558-563.

Tsubata, S., Bowles, K.R., Vatta, M., Zintz, C., Titus, J., Muhonen, L., Bowles, N.E., and Towbin, J.A. (2000). Mutations in the human delta-sarcoglycan gene in familial and sporadic dilated cardiomyopathy. *J. Clin. Invest.* *106*, 655-662.

Turner, P.R., Westwood, T., Regen, C.M., and Steinhardt, R.A. (1988). Increased protein degradation results from elevated free calcium levels found in muscle from mdx mice. *Nature* *335*, 735-738.

Van Deutekom, J.C., Janson, A.A., Ginjaar, I.B., Frankhuizen, W.S., Aartsma-Rus, A., Bremmer-Bout, M., den Dunnen, J.T., Koop, K., van der Kooi, A.J., Goemans, N.M., *et al.* (2007). Local dystrophin restoration with antisense oligonucleotide PRO051 N. *Engl. J. Med.* *357*, 2677-2686.

Van Erp, C., Loch, D., Laws, N., Trebbin, A., and Hoey, A.J. (2010). Timeline of cardiac dystrophy in 3-18-month-old MDX mice. *Muscle Nerve*

- Vazquez, G., Wedel, B.J., Trebak, M., St John Bird, G., and Putney, J.W., Jr. (2003). Expression level of the canonical transient receptor potential 3 (TRPC3) channel determines its mechanism of activation. *J. Biol. Chem.* 278, 21649-21654.
- Venema, V.J., Zou, R., Ju, H., Marrero, M.B., and Venema, R.C. (1997). Caveolin-1 detergent solubility and association with endothelial nitric oxide synthase is modulated by tyrosine phosphorylation. *Biochem. Biophys. Res. Commun.* 236, 155-161.
- Venkatachalam, K., Zheng, F., and Gill, D.L. (2003). Regulation of canonical transient receptor potential (TRPC) channel function by diacylglycerol and protein kinase C. *J. Biol. Chem.* 278, 29031-29040.
- Wakai, S., Minami, R., Kameda, K., Okabe, M., Nagaoka, M., Annaka, S., Higashidate, Y., Tomita, H., and Tachi, N. (1988). Electron microscopic study of the biopsied cardiac muscle in Duchenne muscular dystrophy. *J. Neurol. Sci.* 84, 167-175.
- Wakefield, P.M., Tinsley, J.M., Wood, M.J., Gilbert, R., Karpati, G., and Davies, K.E. (2000). Prevention of the dystrophic phenotype in dystrophin/utrophin-deficient muscle following adenovirus-mediated transfer of a utrophin minigene. *Gene Ther.* 7, 201-204.
- Wallgren-Pettersson, C., Jasani, B., Rosser, L.G., Lazarou, L.P., Nicholson, L.V., and Clarke, A. (1993). Immunohistological evidence for second or somatic mutations as the underlying cause of dystrophin expression by isolated fibres in Xp21 muscular dystrophy of Duchenne-type severity. *J. Neurol. Sci.* 118, 56-63.

Wehling, M., Spencer, M.J., and Tidball, J.G. (2001). A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice *J. Cell Biol.* *155*, 123-131.

Wehling-Henricks, M., Jordan, M.C., Roos, K.P., Deng, B., and Tidball, J.G. (2005). Cardiomyopathy in dystrophin-deficient hearts is prevented by expression of a neuronal nitric oxide synthase transgene in the myocardium. *Hum. Mol. Genet.* *14*, 1921-1933.

Welch, E.M., Barton, E.R., Zhuo, J., Tomizawa, Y., Friesen, W.J., Trifillis, P., Paushkin, S., Patel, M., Trotta, C.R., Hwang, S., *et al.* (2007). PTC124 targets genetic disorders caused by nonsense mutations. *Nature* *447*, 87-91.

Wells, D.J., Wells, K.E., Asante, E.A., Turner, G., Sunada, Y., Campbell, K.P., Walsh, F.S., and Dickson, G. (1995). Expression of human full-length and minidystrophin in transgenic mdx mice: implications for gene therapy of Duchenne muscular dystrophy. *Hum. Mol. Genet.* *4*, 1245-1250.

Wells, D.J., Wells, K.E., Walsh, F.S., Davies, K.E., Goldspink, G., Love, D.R., Chan-Thomas, P., Dunckley, M.G., Piper, T., and Dickson, G. (1992). Human dystrophin expression corrects the myopathic phenotype in transgenic mdx mice. *Hum. Mol. Genet.* *1*, 35-40.

Wiesmann, F., Neubauer, S., Haase, A., and Hein, L. (2001). Can we use vertical bore magnetic resonance scanners for murine cardiovascular phenotype characterization? Influence of upright body position on left ventricular hemodynamics in mice. *J. Cardiovasc. Magn. Reson.* *3*, 311-315.

Williams, I.A., and Allen, D.G. (2007). The role of reactive oxygen species in the hearts of dystrophin-deficient mdx mice. *Am. J. Physiol. Heart Circ. Physiol.* 293, H1969-77.

Wilton, S.D., Lloyd, F., Carville, K., Fletcher, S., Honeyman, K., Agrawal, S., and Kole, R. (1999). Specific removal of the nonsense mutation from the mdx dystrophin mRNA using antisense oligonucleotides *Neuromuscul. Disord.* 9, 330-338.

Woodard, G.E., Lopez, J.J., Jardin, I., Salido, G.M., and Rosado, J.A. (2010). TRPC3 regulates agonist-stimulated Ca²⁺ mobilization by mediating the interaction between type I inositol 1,4,5-trisphosphate receptor, RACK1, and Orai1. *J. Biol. Chem.* 285, 8045-8053.

Wu, B., Moulton, H.M., Iversen, P.L., Jiang, J., Li, J., Li, J., Spurney, C.F., Sali, A., Guerron, A.D., Nagaraju, K., *et al.* (2008). Effective rescue of dystrophin improves cardiac function in dystrophin-deficient mice by a modified morpholino oligomer *Proc. Natl. Acad. Sci. U. S. A.* 105, 14814-14819.

Wu, B., Xiao, B., Cloer, C., Shaban, M., Sali, A., Lu, P., Li, J., Nagaraju, K., Xiao, X., and Lu, Q.L. (2010). One-year Treatment of Morpholino Antisense Oligomer Improves Skeletal and Cardiac Muscle Functions in Dystrophic mdx Mice. *Mol. Ther.*

Wu, R.P., Youngblood, D.S., Hassinger, J.N., Lovejoy, C.E., Nelson, M.H., Iversen, P.L., and Moulton, H.M. (2007). Cell-penetrating peptides as transporters for morpholino oligomers: effects of amino acid composition on intracellular delivery and cytotoxicity *Nucleic Acids Res.* 35, 5182-5191.

Xi, Q., Adebiyi, A., Zhao, G., Chapman, K.E., Waters, C.M., Hassid, A., and Jaggar, J.H. (2008). IP3 constricts cerebral arteries via IP3 receptor-mediated TRPC3 channel activation and independently of sarcoplasmic reticulum Ca²⁺ release. *Circ. Res.* *102*, 1118-1126.

Xu, K.Y., Huso, D.L., Dawson, T.M., Bredt, D.S., and Becker, L.C. (1999). Nitric oxide synthase in cardiac sarcoplasmic reticulum. *Proc. Natl. Acad. Sci. U. S. A.* *96*, 657-662.

Yang, B., Jung, D., Rafael, J.A., Chamberlain, J.S., and Campbell, K.P. (1995). Identification of alpha-syntrophin binding to syntrophin triplet, dystrophin, and utrophin. *J. Biol. Chem.* *270*, 4975-4978.

Yasuda, S., Townsend, D., Michele, D.E., Favre, E.G., Day, S.M., and Metzger, J.M. (2005). Dystrophic heart failure blocked by membrane sealant poloxamer. *Nature* *436*, 1025-1029.

Yin, H., Moulton, H.M., Betts, C., Seow, Y., Boutilier, J., Iverson, P.L., and Wood, M.J. (2009). A fusion peptide directs enhanced systemic dystrophin exon skipping and functional restoration in dystrophin-deficient mdx mice *Hum. Mol. Genet.* *18*, 4405-4414.

Yin, H., Moulton, H.M., Seow, Y., Boyd, C., Boutilier, J., Iverson, P., and Wood, M.J. (2008). Cell-penetrating peptide-conjugated antisense oligonucleotides restore systemic muscle and cardiac dystrophin expression and function *Hum. Mol. Genet.* *17*, 3909-3918.

Yin, H., Betts, C., Saleh, A.F., Ivanova, G.D., Lee, H., Seow, Y., Kim, D., Gait, M.J., and Wood, M.J. (2010). Optimization of Peptide Nucleic Acid Antisense Oligonucleotides for Local and Systemic Dystrophin Splice Correction in the mdx Mouse Molecular Therapy

Yoshida, M., and Ozawa, E. (1990). Glycoprotein complex anchoring dystrophin to sarcolemma. *J. Biochem.* 108, 748-752.

Yoshikawa, Y., Hagihara, H., Ohga, Y., Nakajima-Takenaka, C., Murata, K.Y., Taniguchi, S., and Takaki, M. (2005). Calpain inhibitor-1 protects the rat heart from ischemia-reperfusion injury: analysis by mechanical work and energetics. *Am. J. Physiol. Heart Circ. Physiol.* 288, H1690-8.

Yoshioka, M., Yorifuji, T., and Mituyoshi, I. (1998). Skewed X inactivation in manifesting carriers of Duchenne muscular dystrophy. *Clin. Genet.* 53, 102-107.

Yotsukura, M., Miyagawa, M., Tsuya, T., Ishihara, T., and Ishikawa, K. (1988). Pulmonary hypertension in progressive muscular dystrophy of the Duchenne type. *Jpn. Circ. J.* 52, 321-326.

Youngblood, D.S., Hatlevig, S.A., Hassinger, J.N., Iversen, P.L., and Moulton, H.M. (2007). Stability of cell-penetrating peptide-morpholino oligomer conjugates in human serum and in cells. *Bioconjug. Chem.* 18, 50-60.

Zaidi, S.I., and Narahara, H.T. (1989). Degradation of skeletal muscle plasma membrane proteins by calpain. *J. Membr. Biol.* 110, 209-216.

Zhang, J., Bang, M.L., Gokhin, D.S., Lu, Y., Cui, L., Li, X., Gu, Y., Dalton, N.D., Scimia, M.C., Peterson, K.L., Lieber, R.L., and Chen, J. (2008a). Syncoilin is required for generating maximum isometric stress in skeletal muscle but dispensable for muscle cytoarchitecture. *Am. J. Physiol. Cell. Physiol.* *294*, C1175-82.

Zhang, W., ten Hove, M., Schneider, J.E., Stuckey, D.J., Sebag-Montefiore, L., Bia, B.L., Radda, G.K., Davies, K.E., Neubauer, S., and Clarke, K. (2008b). Abnormal cardiac morphology, function and energy metabolism in the dystrophic mdx mouse: an MRI and MRS study *J. Mol. Cell. Cardiol.* *45*, 754-760.

Zhang, Z., Tang, J., Tikunova, S., Johnson, J.D., Chen, Z., Qin, N., Dietrich, A., Stefani, E., Birnbaumer, L., and Zhu, M.X. (2001). Activation of Trp3 by inositol 1,4,5-trisphosphate receptors through displacement of inhibitory calmodulin from a common binding domain. *Proc. Natl. Acad. Sci. U. S. A.* *98*, 3168-3173.

Zhu, M.X., and Tang, J. (2004). TRPC channel interactions with calmodulin and IP3 receptors. *Novartis found. Symp.* *258*, 44-58; discussion 58-62, 98-102, 263-6.

Zitt, C., Obukhov, A.G., Strubing, C., Zobel, A., Kalkbrenner, F., Luckhoff, A., and Schultz, G. (1997). Expression of TRPC3 in Chinese hamster ovary cells results in calcium-activated cation currents not related to store depletion. *J. Cell Biol.* *138*, 1333-1341.

Zubrzycka-Gaarn, E.E., Bulman, D.E., Karpati, G., Burghes, A.H., Belfall, B., Klamut, H.J., Talbot, J., Hodges, R.S., Ray, P.N., and Worton, R.G. (1988). The Duchenne

muscular dystrophy gene product is localized in sarcolemma of human skeletal muscle.

Nature 333, 466-469.