INVESTIGATING THE HOMOLOGUE OF THE MAMMALIAN GENE *MYOCYTE STRESS 1* IN *DROSOPHILA MELANOGASTER*

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Investigating the homologue of the mammalian gene *myocyte stress 1* in *Drosophila melanogaster.*

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Abstract

The stress responsive gene *myocyte stress 1* (*ms1*) has been implicated in the initiation of cardiac and skeletal muscle hypertrophy. Upregulation of *ms1* is observed shortly after stress induction in rat hearts before the development of left ventricular hypertrophy. The protein sequence of MS1 is highly conserved across many species including the fruit fly model organism *Drosophila melanogaster,* with high levels of homology found in the actin binding domain. Here, the potential *Drosophila* homologue of *ms1* (*dms1,* designated CG3630) has been investigated to determine the function and the level of functional homology to mammalian *ms1*. The role of this gene in the *Drosophila* heart and somatic muscle has been examined by manipulating the expression of *dms1*. Knockdown of *dms1* affects somatic muscle function, reducing the ability of flies to climb and decreasing their daily activity levels. Overexpression of *dms1* appears to increase climbing ability, but does not affect daily activity, suggesting that muscle strength is increased but energy is not. Expression of mouse MS1 in *Drosophila* muscle appears to have a similar effect to *dms1* on muscle function, but under starvation conditions may increase longevity, whereas *dms1* does not. This suggests a fundamental difference in function between mammalian and *Drosophila ms1* in metabolism. The results of this project revealed an important role for *dms1* in the function of *Drosophila* muscle, and have contributed to the development of a fruit fly model for research into this gene.

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Abbreviations

abdA – abdominal-A

ABLIM - actin binding LIM protein

ABRA - actin binding Rho activating protein

ACE – angiotensin converting enzyme

AHL - artificial haemolymph

AMP – adult muscle precursor

ANP – atrial natriuretic peptide

ASK1 - Apoptosis signal-regulating kinase 1

bap - bagpipe

bHLH – basic helix-loop-helix

BMAL1 – brain and muscle ARNT-like 1

BNP – brain/B-type natriuretic peptide

bp – base pairs

BSA - bovine serum albumin

Ca^{2+} - calcium ion

Cas – CRISPR-associated

cDNA - complementary deoxyribonucleic acid

CDS – coding sequence

CF2 – chorion factor 2

cGMP - cyclic guanosine monophosphate

CLOCK - circadian locomotor output cycles kaput (mouse)

CNS – central nervous system

CO_2 – carbon dioxide

CPT1α - carnitine palmitoyltransferase 1-α

CRISPR - clustered regularly interspaced short palindromic repeats

cry - cryptochrome

CyO - Curly of Oster (balancer chromosome)

DAM – *Drosophila* activity monitor

day – day active in yeast

Dcr – dicer

DD – constant darkness

DEPC - diethylpyrocarbonate

DMD – Duchenne Muscular Dystrophy

dms1 – Drosophila ms1

DNA – deoxyribonucleic acid

DNaseI - deoxyribonuclease I

dNTPs - deoxyribonucleotides

doc1 – dorsocross1

Dpp - decapentaplegic

EGF – epidermal growth factor

ERK - extracellular signal-regulated kinase

ERRα - oestrogen-related receptor-*α*

eve – evenskipped

FCM – fusion competent myoblast

FRT - flippase recognition target

FSS – fluid shear stress

GFP - green fluorescent protein

GMR – glass multiple reporter

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

H_2O - water

IGF - insulin-like growth factor

IGF-1R - insulin-like growth factor 1 receptor

IPTG - isopropyl β-D-1-thiogalactopyranoside

JNK - Jun NH2-terminal kinase

Jra – jun-related antigen

KCNQ - potassium channel, voltage-Gated, KQT-like subfamily

KD - knockdown

Kr - kruppel

LD - cycling light-dark conditions

LIM - Lin11, Isl-1, Mec-3

l'sc – lethal of scute

LVH – Left ventricular hypertrophy

M - molar

MADS-box – MCM1, agamous, deficiens, srf-box

MAPK – mitogen activated protein kinase

mef2 - myocyte enhancer factor 2

$MgCl_2$ – magnesium chloride

MHC – myosin heavy chain

MLK – mixed lineage kinase

MRTF – myocardin related transcription factor

ms1 - myocyte stress 1

$NaHCO_3$ – sodium bicarbonate

NaH_2PO_4 - sodium dihydrogen phosphate monohydrate

nau - nautilus

NFAT - nuclear factor of activated T-cells

nmr – neuromancer

O_2 - oxygen

OCT – optical coherence tomography

PAM - protospacer adjacent motif

pBS – BlueScript plasmid

PBS – phosphate buffered saline

PCR - polymerase chain reaction

PGC1-α - peroxisome proliferator-activated receptor gamma co-activator 1-*α*

PI3K - phosphoinositide 3-OH kinase

PKB – protein kinase B

PKG - cGMP-dependent protein kinase

pnr - pannier

pBS – bluescript plasmid

PBS - phosphate buffered saline

qPCR – quantitative real-time PCR

RAF - Rapidly Accelerated Fibrosarcoma

RGS - Regulator of G-protein signalling

RNA - ribonucleic acid

RNAi - RNA interference

rpm – revolutions per minute

RT – reverse transcriptase

SAP - shrimp alkaline phosphatase

sd - scalloped

SDS - sodium dodecyl sulphate

SNP – single nucleotide polymorphism

SRF – serum response factor

STARS - striated muscle activator of Rho signalling

svp - sevenup

TAK1 - TGF-beta Activated Kinase 1

TALENs – transcription activator-like effector nuclease

tbx – T-box

tim - timeless

tin - tinman

TM3 – third chromosome multiply inverted 3 (balancer)

TM6 - third chromosome multiply inverted 6 (balancer)

tup - tailup

twi – twist

Tx – Triton-X

UAS - upstream activation sequence

Ubx – ultrabithorax

UNG - uracil-N-glycosylase

ush – u-shaped

vg – vestigial

w – white

wg – wingless

whd - withered

Wnt – Wg/Int signalling pathway

X-Gal - 5-bromo-4-chloro-indolyl-β-D-galactopyranoside

ZFN – zinc finger nuclease

ZT – zeitgeber time

Table of Contents

Chapter 1: Introduction

1.1 Hypertrophy

The stress responsive gene *myocyte stress 1* (*ms1*) also known as *STARS* (striated muscle activator of Rho signalling) and *ABRA* (actin-binding Rho activating) plays a vital role in the cellular response to chemical and physical pressure, and upregulation of *ms1* results in many changes in gene expression. The *ms1* pathway is known to act in many ways, and has been shown to be involved in such diverse functions as apoptosis prevention, arteriogenesis and metabolism. However, *ms1* was originally discovered as an important regulator of hypertrophy, specifically left ventricular hypertrophy (LVH) (Mahadeva *et al.*, 2002), and has been shown to be a vital link between those pathways that sense physical stimuli and the mechanisms that allow growth of muscle cells leading to an increase in muscle mass.

Hypertrophy is characterised by an increase in tissue mass resulting from growth in the size of cells, in contrast to an increase in cell number (hyperplasia). There are two main types of hypertrophy; physiological, the healthy growth of muscle mass under conditions such as exercise and pregnancy, and pathological, in response to disease states (Olson & Schneider, 2003). Physiological hypertrophy is part of normal cardiac and skeletal muscle growth and a beneficial adaptive response of these tissues to such stimuli as exercise. In contrast, pathological hypertrophy occurs mostly in cardiac tissue in response to disease states, including hypertension, myocardial infarction, oxidative stress and ischaemia (Pokharel *et al.*, 2003). This type of hypertrophy can be beneficial in the short term, increasing the output of the heart under stress, but if prolonged can be detrimental to cardiac function and may eventually lead to heart failure (Rohini *et al.*, 2010).

1.1.1 Cardiac hypertrophy

As heart disease is one of the most common causes of mortality in the Western world research into the causes of cardiac hypertrophy is an extremely important area and investigation into the pathways regulating the hypertrophic response is necessary to fully understand the mechanisms behind cardiovascular disease. Cardiac hypertrophy, as with hypertrophy in general, can be physiological or pathological, depending on conditions leading to this response. Within these types of hypertrophy, two types of hypertrophic growth exist, concentric and eccentric. These are specific to hollow organs such as the heart and do not occur in skeletal muscle. Both concentric and eccentric growths are seen in physiological and pathological hypertrophy, but are associated with different types of stimuli (Lorell & Carabello, 2000). Concentric growth, which increases the thickness of the ventricular wall, occurs in response to pressure overload. Eccentric, which increases the chamber volume, occurs in response to volume overload (Sugden & Clerk, 1998). These types of growth are characterised by differences in the way in which cell size increases and sarcomeres are added, leading to changes in cardiac structure specific to the types of overload the heart is subjected to. Concentric growth results from an increase in the width of cardiomyocytes and addition of sarcomeres in parallel, increasing the cross-sectional area of the cardiac muscle and thereby increasing wall thickness. Eccentric growth is the result of lengthening of cardiomyocytes and addition of sarcomeres in series, increasing the relative length of the ventricular wall and in this way increasing the chamber diameter (Frey *et al.*, 2004).

1.1.1.1 Physiological cardiac hypertrophy

Physiological hypertrophy in the heart is a normal, beneficial adaptive response that allows the heart to increase its output to meet the increased demands of the body. Post-natal cardiac growth is the result of physiological hypertrophy. The heart is the first organ to form in many species, including vertebrates, and during embryogenesis growth of the vertebrate heart is due to hyperplasia of cardiomyocytes, whereas after birth this is due to hypertrophy (Olson, 2004). The differentiated cardiomyocytes cease division in the post-natal period and increase of heart mass is dependent on an increase in cell size.

Cardiac hypertrophy in pregnancy is necessary to increase the output of the heart due to the increased requirements of the body and developing foetus. This process is reversible and a decrease in cardiac muscle mass in the mother is seen after birth (Schannwell *et al.*, 2002). In this case, the hypertrophic growth seen is eccentric growth as pregnancy produces a volume overload rather than pressure overload (Dorn *et al.*, 2003). None of the markers for pathological hypertrophy are observed in pregnancy, and a hormonal component is thought to contribute to this response (Eghbali *et al.*, 2005).

Exercise can increase cardiac mass, and enlargement of the heart is seen in athletes due to remodelling, including LVH (Weiner & Baggish, 2012). Many studies have demonstrated enlargement of the heart in high level athletes (Pelliccia *et al.*, 1991; Sun *et al.*, 2007; Whyte *et al.*, 2004), and exercise trained mice have shown an increase in cardiac mass through physiological hypertrophy (Kemi *et al.*, 2002). Different types of exercise cause different types of hypertrophic growth depending on the requirements of the heart under different conditions. Exercise that requires strength and prolonged muscle contraction, such as weight-lifting, often results in

concentric cardiac hypertrophy, whereas eccentric growth is seen in response to more high energy sports such as long-distance running (Lorell & Carabello, 2000).

1.1.1.2 Pathological cardiac hypertrophy

While cardiac hypertrophy is observed as a beneficial response to states such as pregnancy and exercise, when an increase in cardiac output is required to meet the increased demands of the body, it is often used as a marker for cardiovascular disease, as LVH is the hearts response to stress conditions. Pressure or volume overload as a result of such conditions is recognised by the heart and causes the structural changes and increase in ventricular mass seen in LVH. The remodelling of the heart under these conditions, is at first a beneficial reaction, and patients with LVH can often appear asymptomatic for many years before developing any further indications of a heart condition, and methods such as echocardiography are the only way to determine an increase in LV muscle mass and cardiac output (Gradman & Alfayoumi, 2006). However, in later stages this may become damaging to cardiac function and can eventually lead to further excessive remodelling, ventricular dilation and a decline in function and output of the heart (Frey *et al.*, 2004). Figure 1.1 demonstrates changes in cardiac muscle mass during LVH, showing the increase in wall thickness associated with this condition. Along with hypertrophy, remodelling of the heart often includes cardiac fibrosis, apoptosis of cardiomyocytes, arterial thickening and inflammation (Sun, 2009), and these all contribute to the eventual failure of cardiac function, however, LVH is a powerful marker for potential cardiac dysfunction as it is usually one of the first responses of the heart to any kind of stress.

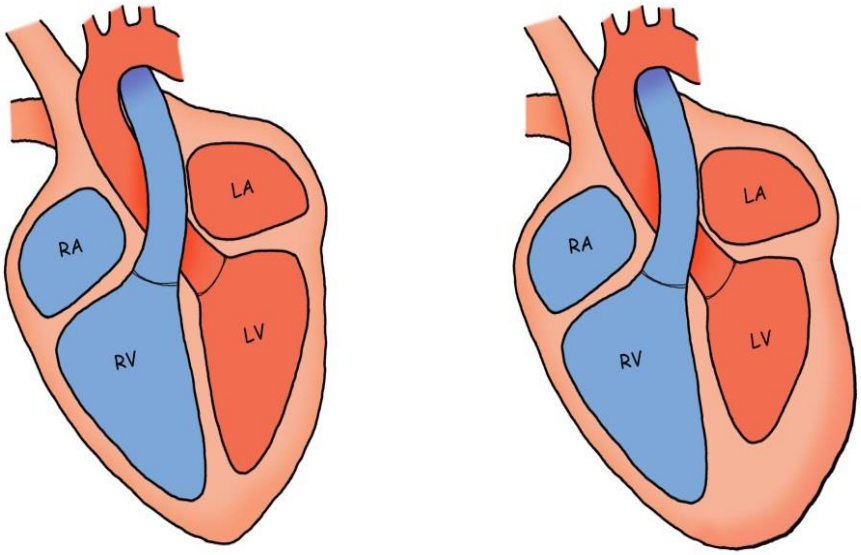

Figure 1.1: diagram showing left ventricular hypertrophy in the human heart. A: normal sized heart. B: heart with LVH, displaying thickening of the ventricular wall and reduced LV capacity. RA = right atrium, LA = left atrium, RV = right ventricle, LV = left ventricle.

1.1.1.3 Foetal gene reactivation in cardiac hypertrophy

The molecular pathways leading to LVH include the reactivation of foetal genes in order to induce cell growth. These genes are expressed in the developing heart and contribute to cardiac growth, but after birth are repressed and expression is not seen under normal conditions. However, in cardiac disease states, particularly those that induce LVH and pathological remodelling, many of these genes are reactivated (Kuwahara *et al.*, 2012). Included in this group are structural genes encoding contractile proteins such as β-myosin heavy chain and skeletal α-actin. The pre-natal developing heart expresses both these proteins at high levels which become silenced and are replaced by α-myosin heavy chain and cardiac α-actin after birth respectively (Kuwahara *et al.*, 2012). These genes have been shown to be re-expressed in adult hearts in pressure-overload induced hypertrophy by acidic and

basic fibroblast growth factors (Parker *et al.*, 1990). Smooth muscle α-actin, shown to be an important component in the developing heart, is also reactivated in response to stress conditions and contributes to remodelling (Black *et al.*, 1991). Atrial and brain/B-type natriuretic peptides (ANP and BNP) are hormones expressed by the heart and are important for growth of cardiomyocytes during development and for regulation of heart function in the adult heart, and while these genes are expressed in the adult heart, levels in the foetal heart are much higher (Cameron & Ellmers, 2003). Both these proteins are found to increase in concentration in hypertrophic cardiac tissue in a rat model, with levels becoming higher as LV mass increases (Kawakami *et al.*, 1996). This has also been observed in human cases of myocardial infarction and dilated cardiomyopathy, where an upregulation of both ANP and BNP is observed (Saito *et al.*, 1989; Hosoda *et al.*, 1991). These peptides are considered a potential avenue for treatment of MI, heart failure and hypertension due to their cardioprotective properties (Hayek & Nemer, 2011).

Ion channel protein-encoding genes are also reactivated during hypertrophy, and there are various types of ion channel that are expressed during cardiac development but this expression is silenced in the adult heart. These include calcium channel proteins, specifically T-type Ca^{2+} channels which are expressed in the ventricle of developing embryos but expression is highly restricted in the adult heart, where no expression is seen in contractile cells (Kuwahara *et al.*, 2012). However, when hypertrophy is stimulated expression is reactivated and in animal models blocking these channels increases survival and hearts display reduced arrhythmias and repolarization of resting membrane potential (Clozel *et al.*, 1999; Kinoshita *et al.*, 2009), demonstrating the contribution of these ion channels to the pathology in LVH.

1.1.2 Skeletal muscle hypertrophy

Hypertrophy is seen in skeletal muscle and is generally a method of increasing muscle mass and therefore strength. This type of muscular hypertrophy is seen during developmental growth of muscle size, and is often seen as a result of exercise. The stresses caused by exercise induce changes in gene expression leading to an increase in myocyte size. Muscles increase in cross-sectional area during normal growth and after exercise but do not increase in numbers of fibres after a certain point in development (Stewart & Rittweger, 2006). Skeletal muscle hypertrophy can be found both with and without exercise-induced muscle damage, usually seen after unaccustomed exercise, and while it has been suggested that this type of damage is necessary for hypertrophy, it is possible that there are other mechanisms that can lead to an increase in muscle size (Schoenfeld, 2012). Exercise is shown to increase skeletal muscle mass through hypertrophy in a mouse model in the same way as humans (Kemi *et al.*, 2002), so it is likely that the pathways regulating hypertrophic growth of skeletal muscle are conserved.

Many of the targets of hypertrophy-inducing pathways in skeletal muscle are, as in cardiac hypertrophy, structural proteins such as myosin heavy chain (MHC) and desmin. MHC is an integral component of muscle structure, and MHC type I has been shown to be reduced after immobilization of muscles and upregulated after reversal of this treatment, and these observations are consistent with the levels of muscular hypertrophy seen at each stage of treatment (Hortobagyi *et al.*, 2000). Desmin is an intermediate filament found in the Z-band of the sarcomere and knockout results in mice with reduced longevity and muscle weakness (Stewart & Rittweger, 2006). Desmin has been shown to be upregulated in hypertrophy after resistance exercise and levels appear to increase before those of MHC

(Woolstenhulme *et al.*, 2006). Microarray data from studies investigating gene expression changes after exercise in rats discovered a number of different processes to be affected by work overload in skeletal muscle. As well as those genes encoding structural proteins and transcriptional regulators, genes involved in processes such as autocrine signalling, extracellular matrix modification and immune response were upregulated (Carson *et al.*, 2002).

1.1.3 Pathways leading to hypertrophy

Many pathways are thought to be involved in the regulation and initiation of hypertrophy in response to various stimuli, including chemical and physical stresses. Different pathways may be essential for cellular growth, or may simply contribute to this process. There are also many examples of cross-talk between pathways, demonstrating the complex regulation of the hypertrophic response. The signalling of calcineurin, mitogen activated protein kinases (MAPKs) and AKT are some of the more important and well characterised pathways and are described in more detail here.

1.1.3.2 Mitogen activated protein kinases (MAPK)

The mitogen activated protein kinase (MAPK) signalling pathways are known to be involved in many cellular processes in response to receptor signalling, and have been shown to regulate the hypertrophic response. These signalling cascades consisting of three layers of phosphorylation involve a MAP3K, MAP2K and MAPK, and the specificity of these cascades varies with interactions in different cell and tissue types. There are three main subfamilies of MAPK involved in hypertrophic growth, including extracellular signal-regulated kinases (ERKs), c-Jun

NH2-terminal kinases (JNKs), p38 kinases, summarised in figure 1.2 (Rose *et al.*, 2010).

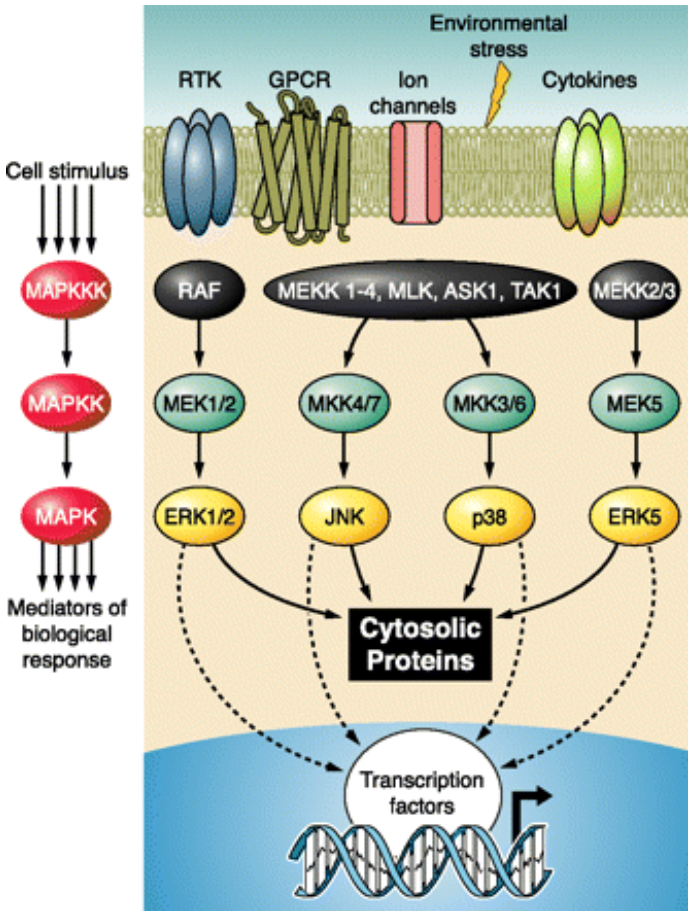

Figure 1.2: MAPK signalling pathways, showing three tiered kinase cascades and stimuli. Stimuli such as stress are detected at the cell surface and receptors activate MAPK cascades. ERK1/2 are activated by RAF (Rapidly Accelerated Fibrosarcoma)-MEK(MKK)1/2 signalling. JNK and p38 signalling is activated by MEK kinase (MEKK) 1-4, Mixed Lineage Kinase (MLK), Apoptosis signal-regulating kinase 1 (ASK1) and TGF-beta Activated Kinase 1 (TAK1), and these activate MKK4/7 and 3/6 respectively. MEKK2/3-MEK5 signalling activates ERK5. From Rose *et al.* (2010).

ERK 1 and 2 are primarily activated by growth factors such as epidermal growth factor (EGF) (Ramos, 2008). These proteins lie downstream of a signalling cascade comprising Raf-1 and its target MEK1/2, which then phosphorylates and activates

ERK1/2 (Muslin, 2008). There is much evidence for a role in hypertrophy for ERK signalling. Cardiac-specific overexpression of MEK1 in transgenic mice results in activation of ERK1/2 along with seemingly non-pathogenic cardiac growth through concentric hypertrophy (Bueno *et al.*, 2000), suggesting that this pathway is involved in physiological hypertrophy. This experiment also showed increased levels of foetal development genes such as those previously described. As well as this phenotype found with overexpression of MEK/ERK, reduction of ERK activation also has an effect on initiation of hypertrophy. Chemical inhibition of Raf-1 reduces hormone-induced cellular hypertrophy, sarcomeric organisation and associated protein upregulation (Yue *et al.*, 2000). Additionally, a dominant negative form of Raf has been shown to have the same effect, as mice expressing this form do not exhibit the normal levels of cardiac hypertrophy in response to aortic banding (Harris *et al.*, 2004). It is thought that this pathway is involved in the activation of GATA4, known to be important in regulation of hypertrophy, as ERK1/2 have been shown to directly phosphorylate GATA4 and inhibition of MEK1 reduces the transcriptional activity of GATA4 (Liang *et al.*, 2001). ERK1/2 signalling also has a role in skeletal muscle hypertrophy, as use of an ERK1/2 signal inhibitor has been shown to block IGF-induced hypertrophy in rat skeletal muscle (Haddad & Adams, 2004). Inhibition of this pathway using a MEK inhibitor results in a decrease in myotube size, showing a role for ERK1/2 in maintenance of muscle mass (Shi *et al.*, 2009).

ERK5 is another important subgroup of MAPKs and is considered to have separate functions to ERK1/2. ERK5 and its associated pathways have been shown to be essential for cardiac development and play a role in the hypertrophic response (Hayashi & Lee, 2004). It is also possible that ERK5 has some functions originally

thought to be those of ERK1/2 due to experiments carried out with inhibitors of ERK1/2 that also target ERK5 (Wang & Tournier, 2006). Cardiac-specific knockout of ERK5 in a mouse model has shown a reduction in hypertrophic growth and a decrease in expression levels of *mef2*, revealing *mef2* as a target for the ERK5 pathway (Kimura *et al.*, 2010).

JNK and p38 kinases are usually activated by stress including physiological stress such as pressure overload, chemical and oxidative stress and radiation (Rose *et al.*, 2010). Both these kinases are known to be upregulated in cardiac tissue during ischaemia/reperfusion, and this is thought, particularly in the case of p38 kinase, to be partly as a result of the production of reactive oxygen species under these conditions (Clerk *et al.*, 1998). JNK has been shown in thoracic banding experiments in mice to be the first MAPK to become fully activated within a few hours of treatment, with ERK and p38 activation occurring later and full levels not achieved for a few days (Esposito *et al.*, 2001). Heart-specific overexpression of a dominant negative form of JNK1 and 2 in mice results in increased hypertrophy with pressure overload from thoracic aortic banding (Liang *et al.*, 2003). Deletion of JNKs has a more complex phenotype, as deletion of JNK1 or 2 does not appear to affect the hypertrophic response after pressure overload, but mice with deletion of JNK1 display a reduction of left ventricular function and fractional shortening for some weeks after treatment before recovering to a normal state (Tachibana *et al.*, 2006). Activation of p38 proteins has demonstrated a role for this pathway in cardiac hypertrophy, as increasing levels of activation can induce cellular growth and expression of developmental genes such as ANP (Wang *et al.*, 1998). Knockout of p38α in the heart does not affect the hypertrophic response to stress, but knockout mice develop cardiac fibrosis and an increase in levels of apoptosis (Nishida *et al.*,

2004). These results suggest that both JNKs and p38 play a role but are not essential in the regulation of cardiac hypertrophy, but may be involved in maintaining the function of the heart and contribute to cell survival. In skeletal muscle hypertrophy in response to exercise, activation of JNKs has been shown to be upregulated along with ERKs around 48hrs after resistance training in humans (Thompson *et al.*, 2003) and both p38 and JNK signalling are increased during recovery from atrophy in rats subject to limb immobilisation (Childs *et al.*, 2003), suggesting that these pathways are involved in muscular recovery and growth in response to hypertrophy-inducing movement. Inhibition of JNKs and p38 does not appear to have an effect on myotube size during normal development and so may not be involved in maintenance of skeletal muscle (Shi *et al.*, 2009).

1.1.3.1 Calcineurin

Calcium signalling plays an important role in the initiation of the hypertrophic response. Increases in levels of intracellular Ca^{2+} occur in response to ventricular stress, leading to activation of calcineurin, a protein phosphatase known to be involved in hypertrophy induction (Houser & Molkentin, 2008). The action of calcineurin is to dephosphorylate nuclear factor of activated T-cells (NFAT), allowing this transcription factor to translocate to the nucleus and activate gene expression (Molkentin *et al.*, 1998; Wilkins & Molkentin, 2004). Figure 1.3 shows the calcium signalling pathway in which calcineurin is involved and how activation of NFAT leads to gene transcription. Levels of calcineurin have been shown to increase significantly in aortic banded rats (Molkentin *et al.*, 1998), and expression is required for skeletal muscle growth (Dunn *et al.*, 1999). Inhibition of calcineurin has, however, yielded varying and contradictory results, with studies showing evidence

for calcineurin as both an activator and repressor of hypertrophy. Mice treated with cyclosporine, an inhibitor of calcineurin have been shown to exhibit a reduction in cardiac hypertrophy in various studies (Taigen *et al.*, 2000; Lim *et al.*, 2000; De Windt *et al.*, 2001). Some other studies do not support this hypothesis and show the opposite result when calcineurin is inhibited, suggesting that loss of calcineurin does not affect hypertrophy or that it in fact increases the hypertrophic response (Zhang *et al.*, 1999; Ding *et al.*, 1999). This could reflect a complex effect of calcineurin in the pathways leading to hypertrophy, however, the amount of evidence available supports a role for activation of this pathway by calcineurin. This has also been demonstrated in skeletal muscle, as cyclosporine treatment in mouse skeletal muscle showed a reduction in muscle growth when overloaded (Dunn *et al.*, 1999).

In addition to regulation of hypertrophy, further functions for calcineurin signalling have been demonstrated. For example, calcineurin is required for differentiation of skeletal muscle, and overexpression of calcineurin or NFAT causes the production of more slow fibres, while inhibition of calcineurin results in upregulation of fast fibres, suggesting that the calcineurin pathway is responsible for the differentiation of slow muscle fibre (Delling *et al.*, 2000; Chin *et al.*, 1998). In cardiac tissue, studies have revealed a role for calcineurin signalling in regulation of apoptosis. In calcineurin-induced cardiac hypertrophy, cardiomyocytes appear to be protected and a lower level of apoptosis is seen (De Windt *et al.*, 2000). Dilated cardiomyopathy model mice show an increase in apoptosis when calcineurin is knocked out (Heineke *et al.*, 2010). These data show calcineurin may function in an anti-apoptotic manner in cases of hypertrophy.

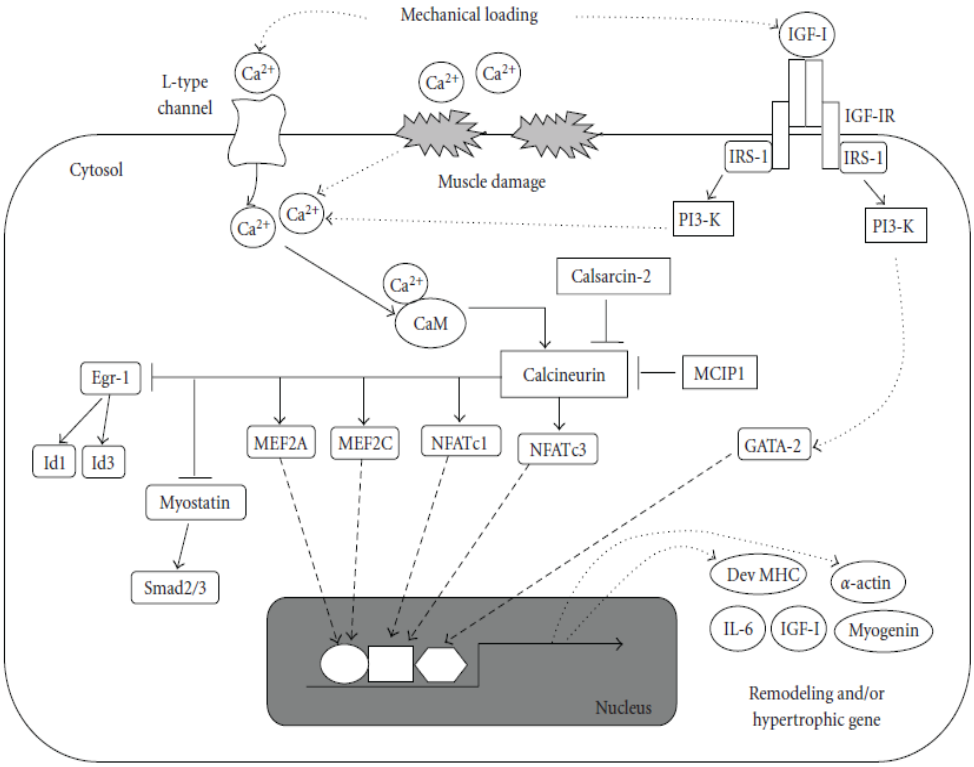

Figure 1.3: The role of calcineurin in the regulation of skeletal muscle hypertrophy and hypertrophic gene expression. Muscle damage induces changes in calcium ion channelling and intracellular Ca^{2+} levels. From Sakuma and Yamaguchi (2010).

This calcineurin-dependent pathway interacts with many other signalling pathways known to be involved in induction of hypertrophy. For instance, there are interactions between the MAPK pathway and that of calcineurin. Links between ERK and calcium signalling have been demonstrated in a mouse model in which the presence of a calcineurin transgene leads to an increase in the levels of phosphorylation of ERK and also of JNK, thereby activating these kinases in the heart (De Windt *et al.*, 2000). In contrast, ERK, JNK and p38 when overexpressed have all been shown to inhibit NFAT transcriptional activity through affecting nuclear localisation of the protein (Porter *et al.*, 2000). These results show the

interplay between these two pathways is complex and there is much that is not yet known about the way in which calcium or calcineurin levels affect MAPK signalling in the initiation of hypertrophy. As well as interactions with MAPK pathways, calcineurin/NFAT can also affect other hypertrophy-related proteins. In cardiac tissue, NFAT interacts with GATA4 in order to activate transcription of cardiac-specific genes, and acts as a link between calcium signalling and GATA4-dependent transcription in response to stress conditions (Molkentin *et al.*, 1998). Activation of calcineurin is dependent on an increase in Ca2+ concentration, and this occurs as a result of receptor signalling in response to stress. Increases of intracellular Ca2+ are observed under treatment with the hormones angiotensin II, endothelin-1 and aldosterone. These lead to the induction of hypertrophy, and this has been shown to be through the action of Ca2+ signalling and the calcineurin-NFAT pathway (Colella *et al.*, 2008). Calcineurin is also thought to lie downstream of the insulin-like growth factor (IGF) pathway, as indicated in figure 1.3. Insulin-like growth factor receptor I signalling (IGF-IR) and the insulin receptor have been shown in a mouse model to be necessary for exercise induced cardiac hypertrophy, and knockout of these receptors in cardiomyocytes leads to loss of hypertrophy in response to exercise training (Kim *et al.*, 2008). In skeletal muscle IGF is important for maintenance of muscle mass, and increases of IGF-1 levels are associated with hypertrophy-inducing exercise (Loughna *et al.*, 1992). However, in skeletal muscle cells knockdown of calcineurin completely inhibits growth of myotubes even with treatment with IGF (Semsarian *et al.*, 1999), suggesting that in order for IGF to effect its influence on muscle growth, calcineurin must be present as part of this pathway.

The calcineurin signalling pathway can be inhibited by such molecules as cyclic GMP (cGMP) and its effector, cGMP-dependent protein kinase (PKG). Reduction of cGMP signalling and knockout of PKG results in a more severe hypertrophic phenotype in response to pressure overload or to hypertrophy-inducing signals (Frantz *et al.*, 2011; Zhang *et al.*, 2010), and cGMP is thought to inhibit the calcineurin pathway upstream of calcineurin itself in response to nitric oxide, and thus reducing myocyte growth (Fiedler *et al.*, 2002). Not only this, but cGMP has also been implicated in G-protein signalling, specifically as an activator of RGS proteins (Regulator of G-protein signalling). These proteins inhibit G-protein signalling, thereby reducing the hypertrophic response (Takimoto, 2012). This suggests that cGMP and PKG work as negative regulators of hypertrophy and restrict hypertrophic growth under stress.

1.1.3.3 AKT

AKT, also known as protein kinase B, is involved in many cellular processes including cell growth, angiogenesis, apoptosis and is also thought to act during glucose metabolism (Chaanine & Hajjar, 2011). Activation of AKT occurs as a result of phosphoinositide 3-OH kinase (PI3K) signalling, and is required for hypertrophy of cardiomyocytes during post-natal growth of the heart (Shiojima & Walsh, 2006). There are three isoforms of AKT in mammals, AKT1, 2 and 3, also known as PKBα, β and γ, with different but overlapping functions. This has been demonstrated by knockout studies showing distinct phenotypes for disruption of each gene. Growth retardation is seen in mice with knockout of *akt1* along with an increase in levels of apoptosis and an increased sensitivity to radiation. These mice also have a shorter lifespan when exposed to radiation stresses (Chen *et al.*, 2001).

Severe atherosclerosis is also seen in *akt1* deficient mouse models (Fernandez-Hernando *et al.*, 2007). Growth defects are found in *akt2* knockout mice, but these are less severe than *akt1* knockout. More obviously, *akt2* knockout mice display a diabetes-like phenotype and insulin resistance (Cho *et al.*, 2001), and have been shown to suffer higher levels of reperfusion injury in the heart (DeBosch *et al.*, 2006). AKT3 knockout results in smaller brain size and atherosclerosis (Easton *et al.*, 2005; Ding *et al.*, 2012). Reduction of all *akt* forms however does result in viable mice despite these phenotypes, and only one functional copy of *akt1* is required for survival (Dummler *et al.*, 2006). It is apparent that AKTs have a wide range of functions, and these include a role in the initiation of hypertrophy.

The function of AKT in both physiological and pathological cardiac hypertrophy has been demonstrated in various studies. In mouse models reduction in AKT activity leads to the development of much smaller hearts as the physiological hypertrophy necessary for normal heart growth is impaired in these animals. This phenotype can be rescued by overexpression of *akt1* (Shiojima *et al.*, 2002). Short-term activation is sufficient to induce physiological hypertrophy in the heart, but if activation is prolonged this can induce pathological hypertrophy and remodelling (Chaanine & Hajjar, 2011). Forced long-term overexpression of AKT in mouse hearts leads to pathological hypertrophy and an increased risk of sudden death (Matsui *et al.*, 2002).

Similarly to that seen in the heart, the PI3K-AKT pathway is involved in skeletal muscle hypertrophy. Activation levels of AKT rise and fall with skeletal muscle hypertrophy and atrophy respectively, and forced activation of AKT can induce hypertrophy in animal models (Bodine *et al.*, 2001; Lai *et al.*, 2004). Mechanical stress such as stretching induces AKT activation and signalling in myotubes, and atrophy-inducing detraining results in lower levels of phosphorylated/active AKT

(Sasai *et al.*, 2010; Leger *et al.*, 2006). Links between AKT signalling and known regulators of hypertrophy have been found, such as myostatin, a growth differentiation factor and negative regulator of hypertrophy. This protein is thought to act through inhibition of AKT and p38 kinase to prevent myocyte growth (Morissette *et al.*, 2006). AKT is also known to inhibit downstream genes such as the FOXO family transcription factors, known to reduce the hypertrophic response. Phosphorylation of these proteins by AKT inhibits their action and reduces signalling by atrophy-inducing genes (Skurk *et al.*, 2005). Exercise is also known to lead to the activation of AKT in the heart and skeletal muscle, as activation of AKT increases dramatically as a result of exercise training (Kemi *et al.*, 2008).

PI3K signalling and activation of AKT is known to be induced by IGF and insulin which are also involved in regulation of hypertrophy. Reduction of AKT activity has been observed in mice with heart-specific knockout of the insulin receptor and activation through insulin receptor signalling is one way that AKT regulates physiological cell growth (Shiojima *et al.*, 2002). The importance of AKT in the insulin signalling pathway is demonstrated by the phenotype seen in *akt2* knockout mice, and has also been observed in some human cases of diabetes, with associations found between various different mutations in *akt2* and cases of diabetes and other metabolic conditions (Tan *et al.*, 2007). In engineered artificial skeletal muscle tissue AKT has been shown to become activated when myocytes are exposed to higher levels of IGF-1 (Sato *et al.*, 2011).

1.2 *Myocyte stress 1* (*ms1*)

1.2.1 Discovery of *ms1*

Myocyte stress 1 (*ms1*) was discovered in 2002 by two groups (Mahadeva *et al.*, 2002; Arai *et al.*, 2002). For Mahadeva *et al*. the discovery of *ms1* was the result of a molecular indexing approach (Mahadeva *et al.*, 1998) to identify genes that undergo changes in expression levels following pressure overload from aortic banding in rat hearts. A peak in transient *ms1* upregulation was found to occur approximately 4 hours after pressure overload which returned to basal after 24 hours, and further analysis found expression of *ms1* to be restricted to cardiac and skeletal muscle. Sequence analysis revealed a region which is highly conserved, with homologues in many species ranging from *C. elegans* and *Drosophila melanogaster* to rodents, zebrafish and humans. The conserved region lies at the C-terminus of mammalian homologues but at the N-terminus of species such as *Drosophila*.

Arai *et al.* (2002) identified MS1/STARS as an actin-binding protein with a role in Rho signalling and actin polymerisation in mouse. The function of MS1 was shown to be important in activation of serum response factor (*SRF*), with correlations found between MS1 activity and transcriptional activation by SRF. SRF is involved in the regulation of many genes in various processes, and has been found to play a role in the regulation of several factors thought to be upregulated as a result of hypertrophic stimuli, including reactivated foetal genes (Kuwahara *et al.*, 2012). The conserved region of MS1 is in the actin binding domain, with mouse and human protein sequences showing particularly high levels of homology. It was discovered, using a rat model, that this domain was able to activate downstream transcription without the presence of the N-terminal sequence, implying that this gene has a similar function in

many species. This study also found MS1 to localise to the sarcomere of cardiomyocytes, displaying highest levels in the I-band of the sarcomere.

1.2.2 Interactions of *ms1*

It has been shown that MS1 has high importance in a signalling pathway leading to gene activation by SRF. The main link between the action of MS1 and *SRF* upregulation was found to be a myocardin-related transcription factor (MRTF). Myocardin and MRTFs are important in regulating *SRF* transcription in muscle tissue (Wang & Olson, 2004; Cen *et al.*, 2004), and overexpression of MRTF-A is able to induce cardiac hypertrophy in rats (Liao *et al.*, 2011). MRTF, when inactive, localises to the cytoplasm, bound to G-actin monomers. Rho signalling affects this interaction and allows MRTF to translocate to the nucleus and activate transcription of *SRF* (Miralles *et al.*, 2003) and MS1 works alongside RhoA, a GTPase family protein, to release MRTF and facilitate its translocation into the nucleus. This occurs because the combined action of MS1 and RhoA causes the polymerisation of G-actin monomers into F-actin polymers, shown in figure 1.4, and loss of *ms1* causes a significant decrease in *SRF* activation via this pathway (Kuwahara *et al.*, 2005). Originally it was believed that MS1 worked upstream of RhoA as an activator (Arai *et al.*, 2002), but it is now thought to work alongside it to activate the *SRF* signalling pathway (Kuwahara *et al.*, 2005). The link between *ms1* and MRTFs has been further confirmed by evidence that MS1 overexpression leads to an increase in expression of MRTF/SRF-dependent genes (Koekemoer *et al.*, 2009).

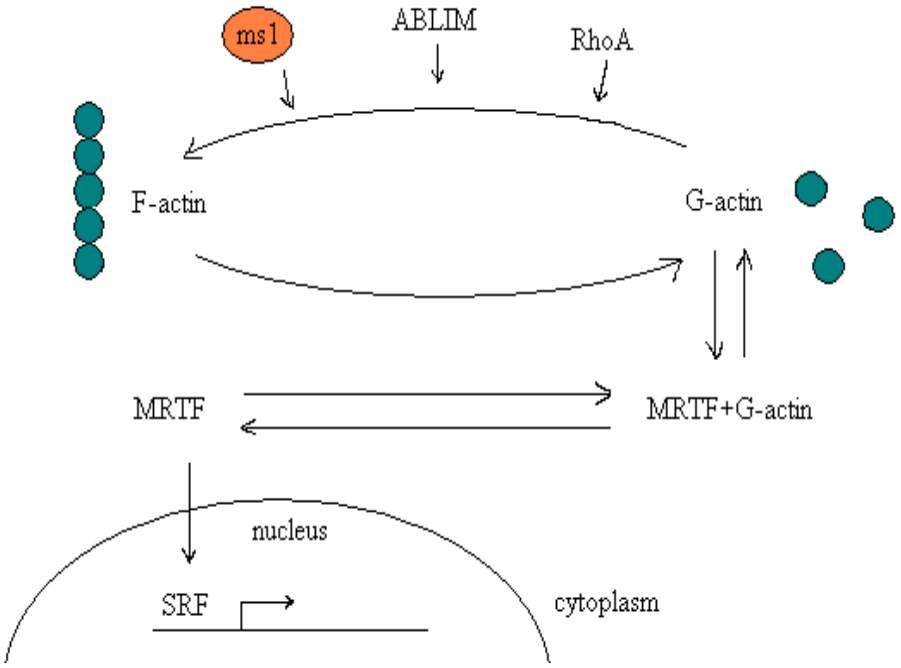

Figure 1.4: proposed action for *ms1* and interacting proteins resulting in upregulation of gene expression. Based on Barrientos *et al.* (2007).

The interaction of MS1 and RhoA is required to induce activation of *SRF* and its downstream targets, but other interactions are necessary to activate this pathway to sufficient levels. Actin-binding LIM (Lin11, Isl-1 and Mec-3) (Bach, 2000) proteins ABLIM2 and 3 associate with MS1 to initiate polymerisation of actin, and loss of these proteins can significantly reduce *SRF*-dependent transcription (Barrientos *et al.*, 2007). ABLIM2 is expressed as one of two splice variants and is known to be important for neuronal development (Klimov *et al.*, 2005) as well as being expressed in heart and skeletal muscle (Barrientos *et al.*, 2007), whereas ABLIM3 has a much wider expression range including liver, brain and lungs (Krupp *et al.*, 2006). This difference in expression could contribute to any tissue specificity seen in this pathway. Both proteins have been shown to interact with MS1, and both strongly

bind F-actin. The high level of homology between these proteins and those found in other species indicate their importance in this and other pathways in different tissues (Barrientos *et al.*, 2007).

1.2.3. The role of *ms1* in cardiac hypertrophy

The importance of *ms1* in regulation of left ventricular hypertrophy (LVH) in response to stress has been demonstrated in several studies. It was initially shown to be upregulated in the early stages of hypertrophy (Mahadeva *et al.*, 2002), suggesting a role in the initiation of the stress-response pathway. Some genes that appear to be upregulated after forced overexpression of *ms1* have previously been associated with hypertrophy, for example cardiac α-actin can be upregulated up to 2 fold after overexpression of *ms1* in cultured cells (Koekemoer *et al.*, 2009). Expression of *ms1* during embryonic stages of development suggests a role in early development of the heart in addition to a role as a stress response gene in later life (Mahadeva *et al.*, 2002; Arai *et al.*, 2002). Using a zebrafish model, it has been shown that *ms1* is expressed at early stages of cardiac development, and that knockdown of *ms1* results in morphological and functional abnormalities. This phenotype can be rescued by overexpression of *SRF*, suggesting that *SRF* lies downstream of *ms1* (Chong *et al.*, 2012).

1.2.4. The role of *ms1* in skeletal muscle hypertrophy

The action of *ms1* has not only been documented in the heart. The expression pattern of *ms1* is not restricted to cardiac muscle, but is also equally highly expressed in skeletal muscle (Mahadeva *et al.*, 2002) and appears to play a similar role in the induction of hypertrophy in these tissues as well as in the heart. Changes

in *ms1* expression have been observed during muscle cell differentiation, suggesting a role for *ms1* in this process (Ounzain *et al.*, 2008). Studies have been carried out investigating the effect of exercise on the expression of various genes, including *ms1*. Hypertrophy-stimulating exercise causes an upregulation of not only *ms1*, but also other genes in this pathway, including RhoA, MRTFs and SRF, as well as several SRF target genes involved in muscle growth (Lamon *et al.*, 2009). In addition to this, these increases in expression were reversed during atrophy of the muscles, suggesting that this pathway is important in growth of skeletal muscle as a result of exercise. This idea was supported by another study which showed eccentric exercise caused an upregulation of *ms1* in response to muscle tissue damage (MacNeil *et al.*, 2010). Eccentric exercise builds muscle by causing damage to the muscle fibre structure, which induces repair pathways leading to remodelling of the muscle (Clarkson & Hubal, 2002). *ms1* appears to be upregulated as a result of this and is likely to play a role in repair of the muscle tissue. This has led to the hypothesis that calcium signalling plays a role in regulation of *ms1* due to the involvement of this pathway in repair of muscle tissue after exercise (MacNeil *et al.*, 2010). The proposed pathway is shown in figure 1.5. There is also evidence for a role for *ms1* in response to endurance exercise, as *ms1* and downstream genes are upregulated under these conditions. Regulation of this pathway involves the transcriptional activators peroxisome proliferator-activated receptor gamma co-activator 1-*α* (PGC-1*α*) and oestrogen-related receptor-*α* (ERR*α*) (Wallace *et al.*, 2011), shown to be upregulated after endurance exercise and also to target *ms1* for upregulation. This study also identified a fat metabolism gene, carnitine palmitoyltransferase I (CPT1-α), as a downstream of the *ms1* pathway, suggesting a role for *ms1* in metabolism in skeletal muscle. The

importance of *ms1* in muscle development and growth is further demonstrated by the fact that it is expressed at embryonic stages in a pig model, an animal known to share many physiological similarities with humans (Peng *et al.*, 2008). Not only is this an important model for translation to research in humans, but *ms1* may affect the muscle structure and therefore the meat quality from pigs. This study also confirmed expression of *ms1* in smooth muscle cells from pigs, suggesting that the expression pattern of *ms1* is not restricted to cardiac and skeletal muscle.

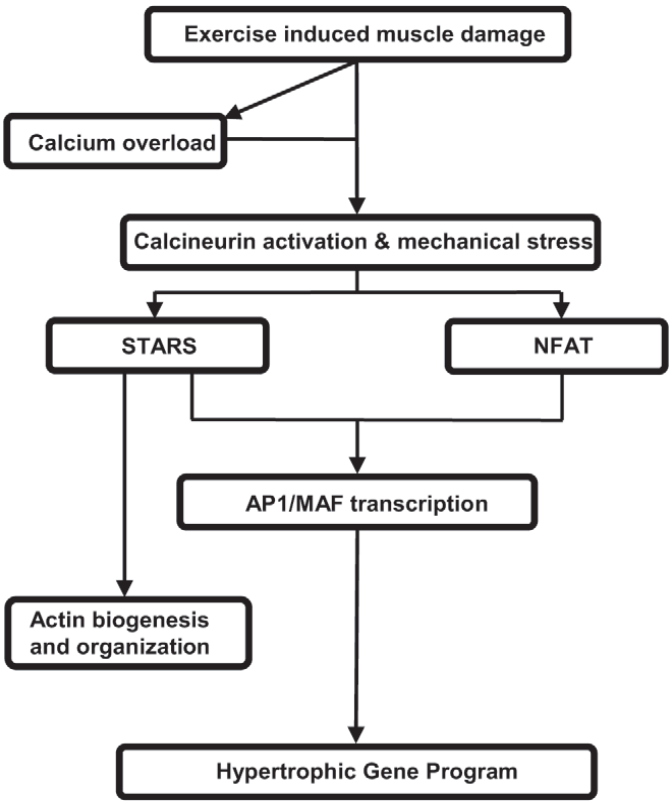

Figure 1.5: proposed role for STARS/MS1 in regulation of skeletal muscle hypertrophy after exercise. STARS/MS1 is involved in the reorganisation of actin and also interacts with targets of calcineurin signalling to initiate the transcriptional regulation of hypertrophic genes. From MacNeil *et al*. (2010).

1.2.5 Functions of *ms1* outside of hypertrophy

Accumulating evidence demonstrates that *ms1* has an important role in initiation of LVH and skeletal muscle growth, but further studies have indicated that the hypertrophic response is only one of many functions that this gene carries out. As mentioned above, a potential role for *ms1* in metabolism has been suggested, due to the upregulation of CPT1-α as a result of an increase in *ms1* expression (Wallace *et al.*, 2011), and this has been supported by studies into gene regulation in cases of diabetes and insulin resistance. Upregulation of *ms1* has been found in an insulin resistant type-2 diabetes mouse model and higher levels of *ms1* were found with lower insulin sensitivity, as well as an upregulation of the SRF pathway and downstream genes in cases of insulin resistance (Jin *et al.*, 2011). Insulin resistance in skeletal muscle is one of the main characteristics of type-2 diabetes and intensive insulin treatment in muscle leads to many changes in expression of genes involved in metabolism, as well as signalling, cell growth and many other functions (Sreekumar *et al.*, 2002), and it is likely that the SRF pathway is involved in this regulation. As a known regulator of the SRF pathway, *ms1* could also have a role in gene regulation in response to insulin. It has also been shown that oxidative phosphorylation genes that are known to be regulated by PGC1-α are downregulated in cases of diabetes (Mootha *et al.*, 2003). When considered with evidence that *ms1* expression is affected by PGC1-α (Wallace *et al.*, 2011), this strengthens the idea that the *ms1* pathway is involved in this process and has a role in insulin signalling.

Cardiovascular disease states that are known to induce the hypertrophic response often result in cardiac cell death by apoptosis and the loss of cells contributes to the pathology of these diseases and potentially can increase the risk of heart

failure (Lee & Gustafsson, 2009). It has been demonstrated that inhibition of apoptosis plays a role in preventing heart failure and acts in a cardioprotective manner. Many known apoptosis-repressing genes have been shown to be cardioprotective, and indeed some compounds used in treatment of cardiovascular disease have been shown to repress apoptotic pathways (Pollesello & Papp, 2007; Burger *et al.*, 2009; Lin *et al.*, 2011). There is evidence that MS1 is involved in repression of apoptosis and therefore in protection of cardiac tissue (Koekemoer *et al.*, 2009), as upregulation of *ms1* in cultured rat cardiac cells leads to an increase in expression of NOL-3/ARC, a Bcl family protein known to act as an apoptosis repressor by targeting and interacting with caspases (Koseki *et al.*, 1998). This suggests a cardioprotective role for *ms1* which, in addition to increasing the output of the heart through the hypertrophic response, may provide longer term protection of cardiac function due to prevention of cardiac cell loss.

Many of the functions of *ms1* are active in striated muscle tissue, either cardiac or skeletal, but roles have been suggested for *ms1* in smooth muscle, particularly in the vascular system and blood vessels (Troidl *et al.*, 2009). Rho signalling has been demonstrated to be necessary for arteriogenesis, the growth and increase in diameter of collateral blood vessels after damage (Eitenmuller *et al.*, 2006), suggesting that this may involve the *ms1* pathway in which RhoA plays a vital role. Fluid shear stress (FSS) can act as a trigger for arteriogenesis due to the forces exerted on the vessel walls (Rossitti *et al.*, 1995). This appears to be the stimulus for upregulation in *ms1* expression in vascular tissue, as vessels subjected to an increase in FSS show an increase in *ms1* expression, and *ms1* knockout mice display significantly reduced levels of arteriogenesis (Troidl *et al.*, 2009). This suggests another role for *ms1* in addition to those described in striated muscle. The

increasing number of described functions for *ms1* demonstrates the importance of this gene and its associated pathways in a wide range of biological processes.

1.2.6 Regulation of *ms1*

There is little information on the regulatory mechanisms controlling the expression of *ms1*, however, analysis of promoter sequence and manipulation of potential upstream genes have revealed widely conserved genes involved in regulation of *ms1*. The MADS-box transcription factor *mef2* is highly conserved across many species, including yeast, *C. elegans*, *Drosophila melanogaster*, and vertebrates, and is involved in many cellular processes in various cell types (Potthoff & Olson, 2007). There are four vertebrate *mef2* genes, A-D, expressed in different but overlapping patterns, with *mef2-C* being the earliest to be expressed in the early heart tube precursor cells in the early embryo (Edmondson *et al.*, 1994). All isoforms are expressed throughout human heart development, with alternative splicing observed at later stages rather than changes in expression level to alter the effect of *mef2* on the developing heart (Iida *et al.*, 1999). Differences in DNA binding between the isoforms and splice variants and the ability of *mef2* to homo- or heterodimerise allows differential gene activation in muscle precursors (Olson *et al.*, 1995). Expression of *mef2* is required for development of all muscle types from early stages and is necessary for myogenesis and muscle cell differentiation (Ornatsky *et al.*, 1997), and mutants for *mef2* fail to undergo myogenesis correctly. Regulation of *mef2* is thought to be under the control of pathways such as the MAPK cascades, demonstrated in many species (Han *et al.*, 1997; Vrailas-Mortimer *et al.*, 2011; Dodou & Treisman, 1997) and also through calcium signalling (Zarain-Herzberg *et al.*, 2011). These pathways have been implicated in

the initiation of cardiac hypertrophy (Molkentin *et al.*, 1998; Bogoyevitch, 2000) as well as in skeletal muscle (Olson & Williams, 2000), and *mef2* is thought to be an important component of pathways leading to the hypertrophic response. Indeed, overexpression of *mef2* itself has been shown in mice to increase heart enlargement and contributes to cardiomyopathy (Xu *et al.*, 2006). It is therefore sensible to predict involvement of *mef2* in the *ms1* pathway. A *mef2* binding site has been found in the regulatory regions upstream of *ms1* and changes in *mef2-C* expression affects levels of *ms1* (Kuwahara *et al.*, 2007). Mice mutant for *mef2-C* show reduced levels of *ms1*, and expression of *mef2-C* is able to activate the *ms1* promoter. As *ms1* is able to activate *srf* transcription, this evidence confirms *ms1* as a link between *mef2* and SRF-dependent gene transcription in the regulation of muscle development and the hypertrophic response. It has been shown that the DNA binding ability of *mef2* is reduced in diabetes mouse models (Thai *et al.*, 1998), and since *mef2* is known to upregulate *ms1*, this could be related to the fact that *ms1* expression is reduced in cases of type-2 diabetes, and could contribute to this downregulation.

As well as being essential for development of all muscle types, *mef2* is also known to be important in development of neuronal cells, as all isoforms of *mef2* are found in early neuronal differentiation (Li *et al.*, 2001), and may also be involved in the maintenance of circadian rhythms, due to the observation of an effect on daily rhythms in *mef2* mutant *Drosophila* (Blanchard *et al.*, 2010). A role has also been found in vertebrate bone development as *mef2-C* has been shown to initiate hypertrophy in chondrocytes, found in cartilage, and mutants display impaired growth of bones (Arnold *et al.*, 2007). Neural crest cell development is also dependent on correct expression of *mef2*, as neural crest cell-specific knockout of

mef2-C leads to lethality shortly after birth in mice (Verzi *et al.*, 2007). It is possible that *ms1* expression under the control of *mef2* contributes to the muscle specificity of *mef2* signalling, as *ms1* expression is restricted to muscle tissue. As *ms1* is expressed in different muscle types, it is likely that are tissue specific regulatory factors for *ms1* that control expression. *Mef2* is widely expressed and is involved in various pathways, but the transcription factor MyoD has been found to regulate *ms1* in a more muscle-specific manner. MyoD has been shown to bind the *ms1* promoter and is able to upregulate *ms1* transcription (Ounzain *et al.*, 2008). MyoD is an E-box binding bHLH factor and an important regulator of myogenesis and early muscle differentiation (Brand-Saberi, 2005). Forced expression in fibroblasts is sufficient to drive conversion to muscle cells (Davis *et al.*, 1987), and the DNA binding domain of MyoD has been demonstrated to be highly muscle specific (Davis *et al.*, 1990). A role has also been suggested for MyoD in motor axon and neuromuscular junction development, indicating that as well as muscle cell differentiation, MyoD has a wider role in muscular development (Wang *et al.*, 2003). The early expression of MyoD and its role in development is thought to be the reason for changes in expression of *ms1* early in muscle development. The proposed method by which MyoD is thought to regulate changes in *ms1* is via HDAC recruitment, binding a single site in the *ms1* promoter and causing repression of *ms1* at early stages before differentiation. At later stages MyoD interacts with proteins that cause changes to chromatin, opening up an additional MyoD binding site in the *ms1* promoter. Binding to this site activates *ms1* expression when cells are terminally differentiated, and ensures muscle specificity of *ms1* (Ounzain *et al.*, 2008). MyoD is also thought to be regulated by SRF (Carnac *et al.*, 1998), and this could indicate a feedback loop in which MyoD regulates its own transcription through the *ms1*

pathway and SRF. RhoA and factors known to regulate RhoA activity have been shown to affect MyoD expression (Gopinath *et al.*, 2007), and as RhoA and *ms1* are known to interact it is likely that *ms1* is involved in this regulation. In this way, early MyoD expression is able to regulate subsequent expression during development of muscle cells.

An important regulator of the hypertrophic response of the heart is GATA4, a transcription factor from the GATA family of proteins. It has been demonstrated in both cultured cells and *in vivo* that GATA4 is required for regulation of hypertrophy in cardiomyocytes, playing a protective role in hearts subject to pressure overload (Bisping *et al.*, 2006), so it sensible to suggest that GATA4 is involved with control of the *ms1* pathway. Binding sites for GATA4 have been found in the promoter region of *ms1* and binding to these sites has been observed in all stages of development using a mouse model (Ounzain *et al.*, 2012). GATA4 represses *ms1* expression through this promoter binding, and knockdown of GATA4 results in higher levels of *ms1*, with the opposite effect seen with overexpression of GATA4. This suggests that the protective effect of GATA4 acts through repression of *ms1*, restricting the hypertrophic response and therefore limiting the potentially harmful remodelling of the heart in disease states. Not only this, but GATA4 is thought to be an important factor in hyperglycaemia-induced cardiac failure, often as a consequence of diabetes, as GATA4 levels decrease in response to hyperglycaemia, reducing the cardioprotective effect (Kobayashi *et al.*, 2007). Levels of *ms1* are known to increase in skeletal muscle with cases of diabetes (Jin *et al.*, 2011), and it has been shown that this is also the case in the mouse heart (Ounzain *et al.*, 2012). It is likely that the reduction in GATA4 levels as a result of hyperglycaemia is at least

partly responsible for this change in *ms1* expression in cardiac muscle, as lower GATA4 levels reduce the repression of *ms1*.

The pathways leading to upregulation of *ms1* appear to be varied, as it has been shown to be involved in hypertrophy resulting from pressure overload in the heart, exercise, damage to muscle tissue, as well as modifications in various signalling pathways. The regulatory mechanisms for the *ms1* pathway appear to be specific in different types of hypertrophy, pathological hypertrophy resulting from disease states, and physiological, for example from exercise. *ms1* appears to be a common factor in these processes suggesting that there are either specific regulators of *ms1* expression under different conditions, or that other pathways initiated under the same conditions interact with genes downstream of *ms1* to stimulate different types of hypertrophy.

1.2.7 Circadian regulation and its links to *ms1*

An interesting observation is that both the transcription factors *mef2* and MyoD that have been demonstrated to regulate *ms1* expression have also been implicated in control of the circadian clock. There is much evidence that the circadian clock has a strong impact on heart function. This is shown particularly in the fact that heart attacks predominantly happen in the early morning (Feng & Tofler, 1995), suggesting that there is a change in protein levels around this time that somehow decreases the protection of the heart, or causes it to become temporarily weaker. Shift workers also show a higher incidence of heart disease, implying that the disruption to the circadian clock through drastically altering their sleep patterns is putting the heart under pressure and making them more susceptible to heart disease (Durgan & Young, 2010). Heart rate is known to fluctuate within a daily

cycle (Scheer *et al.*, 2004), with levels being higher during the day, and this may contribute to the increased levels of heart failure seen in mornings (Boudreau *et al.*, 2012). Levels of certain hormones, whose secretion is controlled by the circadian clock, are thought to have an impact on heart function. Particularly, the pineal hormone melatonin is known to have protective properties with regards to the heart, and is more highly expressed at night (Dominguez-Rodriguez *et al.*, 2010). It has been suggested that the reduction in melatonin levels, along with a morning increase in another hormone, cortisol, may contribute to the high occurrence of heart attack early in the morning (Boudreau *et al.*, 2011). Not only this, but central clock genes have also been implicated in cardiomyopathy, with mutants being more susceptible to heart failure and defects in development (Lefta *et al.*, 2012). Differences in the cardiac hypertrophic response and expression of involved genes have been observed in mouse models with mutations in the cardiomyocyte clock (Durgan *et al.*, 2011). Such observations have led to more research into the circadian aspects of heart disease and dysfunction, and there is the potential for this research to increase the effectiveness of certain treatment by offering them in a clock-dependent manner. As well as a circadian component in control of the heart, there is also evidence for this in skeletal muscle, with a daily peak in muscle performance and hypertrophy in response to exercise in the late afternoon (Hayes *et al.*, 2010; Sedliak *et al.*, 2009).

It has been shown that changes in *mef2* expression in clock neurons in Drosophila affect the rhythmicity of the clock in various ways (Blanchard *et al.*, 2010). A reduction in *mef2* expression caused flies to become arrhythmic, whereas an increase in expression caused them to develop complex rhythms and longer daylength in conditions of constant darkness. This suggests an important role for

MEF2 in the regulation of the circadian clock, and, as it is so widely expressed, it could be that MEF2 maintains this function in other tissues, including heart and muscle. As there is evidence that MEF2 regulates expression of *ms1*, this could potentially imply that ms1 has a circadian expression pattern, or is in some way involved in the clock in heart and muscle tissues.

The suggestion that *ms1* may have some circadian aspects to its regulation is further supported by the fact that *myoD* is thought to be a target of the central clock regulating transcription factors CLOCK and BMAL1. Studies using a mouse model have shown that these transcription factors are required for proper muscle development and maintenance through regulation of *myoD*, which has also been shown to cycle in a circadian pattern (Andrews *et al.*, 2010), and binding sites have been found within the MyoD promoter for CLOCK and BMAL1 (Zhang *et al.*, 2012). Knockout of BMAL1 and myoD, as well as a specific deletion of CLOCK give the same phenotype, disruption of myofibrils and skeletal muscle structure, along with decreased expression of genes essential to muscle fibres, such as actin and myosins. This study also showed decreased expression of PGC1α when CLOCK and BMAL1 were knocked out. PGC1α has been shown to affect expression levels of ms1 (Wallace *et al.*, 2011), and while *PGC1α* does not cycle as circadian genes do, this evidence may show another link between *ms1* and the circadian clock.

1.3 The *Drosophila* model system

The fruit fly *Drosophila melanogaster* is a widely used model organism for many different areas of research. Due to the relative ease of use and housing *Drosophila*, their short life cycle and well characterised genome, fruit flies have become an ideal

model for research into human diseases including neurological conditions, cancer, diabetes and heart disease. Despite the vast differences between mammalian and *Drosophila* physiology many genes are highly conserved and therefore research using *Drosophila* is translatable into mammalian models and even humans. In addition to this, there is a wide range of resources for information on *Drosophila* gene expression, gene structure, as well as mutant fly lines and GAL4/UAS lines available for purchase (see section 2.3.1 for details on the GAL4/UAS system). *Drosophila* heart and muscle development and structure share many similarities with those of mammalian models and therefore *Drosophila* are a good early-stage research model for this type of investigation.

1.3.1 *Drosophila* somatic muscle

In the adult fly, there are three distinct muscle types. These are somatic, cardiac and visceral muscle, somatic and visceral being the invertebrate equivalent of skeletal and smooth muscle respectively (Sink, 2006). These muscle types are further divided into specific muscle groups. Somatic muscle groups include thoracic muscles such as the indirect flight muscles that power wing movement, as well as tubular jump muscles and abdominal intersegmental muscles (Swank, 2012; Krzemien *et al.,* 2012). The somatic musculature of *Drosophila* is shown in figure 1.6. Cardiac muscle comprises the *Drosophila* heart and surrounding pericardial muscle layers (Tao & Schulz, 2007). Visceral muscle is associated with structures such as the gut, and forms a layer of muscle around these organs (Klapper, 2000).

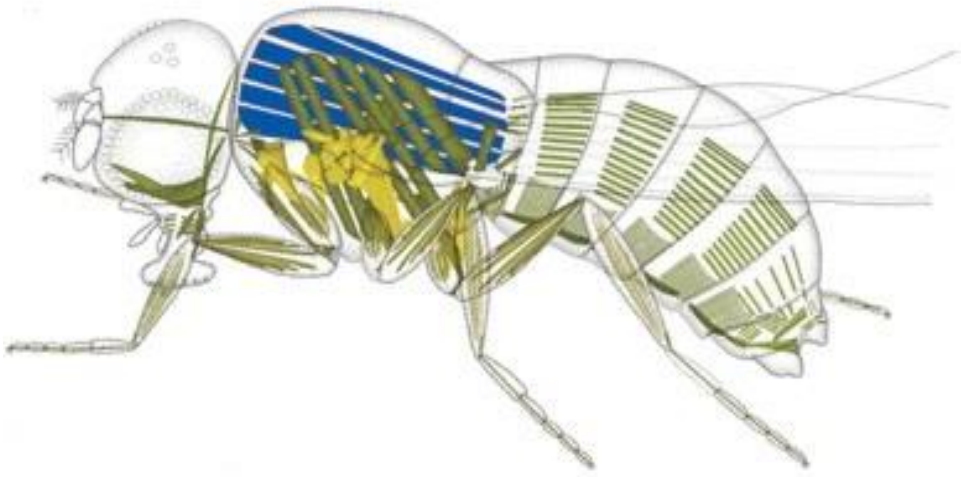

Figure 1.6: Somatic musculature of the adult *Drosophila*. Dorsal longitudinal muscles are shown in blue, dorsoventral, leg and abdominal intersegmental muscles in green, and direct flight muscles and muscles close to body wall associated with leg movement in yellow From Augustin *et al*. (2009).

Muscle specification begins after mesoderm formation in the early embryo with the establishment of competence domains, a group of cells that have undergone myogenic determination and have high levels of the early mesodermal transcription factors *twist* and *lethal of scute* (*l'sc*). Within these competence domains expression and lateral restriction of these genes result in the specification of muscle founder cells. The formation of each somatic muscle relies on the presence of a founder cell, the role of which is to recruit surrounding cells to form muscle fibres. In larval stages these founder cells fuse with neighbouring myoblasts, known as fusion-competent myoblasts (FCMs), and, depending on the genes being expressed therein, differentiate into specific muscle types, (Baylies *et al.*, 1997; Beckett & Baylies, 2006) as shown in figure 1.7. The differences in gene expression along the antero-posterior and dorso-ventral axes determine the pattern and type of muscles that develop in each segment of the body. These genes include many of the body-

patterning genes such as *even-skipped*, *Kruppel*, *slouch* and *vestigial*, as well as interactions with Hox genes and inhibitions from neighbouring cell groups which lead to the eventual fate of each group of progenitor cells (Tixier *et al.*, 2010). Each group of myoblasts develops into a myotube, and maturation into muscle involves attachment to tendon cells and interaction with motor neurons to from a neuromuscular junction, resulting in a fully formed larval muscle (de Joussineau *et al.*, 2012). Larval somatic musculature has a fairly regular pattern, with 30 muscle fibres repeated in each segment controlling movement of the larva (Haralalka & Abmayr, 2010) and these muscles are mostly broken down at metamorphosis to make way for adult structures. Adult somatic muscles develop from founder cells, and the pattern of muscle specification occurs during embryogenesis, even though adult muscle does not form until metamorphosis. Imaginal discs form from adult muscle precursors (AMPs) and lie dormant until pupal stages, when they are activated and develop into adult structures. AMPs are derived from the same cell types that also become founder cells, but while founder cells fuse with surrounding FCMs, AMPs proliferate to become muscle imaginal discs (de Joussineau *et al.*, 2012). While the somatic musculature is almost completely replaced during pupal stages, retaining only a template for the adult muscles, cardiac and visceral muscle are not broken down during metamorphosis, but are instead subject to remodelling to develop into adult structures (Curtis *et al.*, 1999; Klapper, 2000).

Figure 1.7: The role of founder cells in *Drosophila* somatic muscle development. Founder cells, shown in dark grey, recruit surrounding FCMs to form muscle fibres. The founder cells shown here specficially express the segment-specific genes *even-skipped* (*eve*), *vestigial* (*vg*) and *Kruppel* (*Kr*). From Baylies *et al*. (1997).

1.3.2.1 Genetic regulation of *Drosophila* somatic muscle development

Genes expressed in the early mesoderm are often essential for muscle specification, and affecting the expression of these genes, even after mesoderm formation, can lead to severe muscle phenotypes. One such gene is *twist*, a mesodermal transcription factor that has been shown to be essential for muscle specification (Leptin, 1991). *Twist* is a bHLH factor that is expressed at very early stages of embryogenesis, originally thought to be specifically required for gastrulation and specification of the dorso-ventral axis (Thisse *et al.*, 1987), but is retained in the mesoderm and high levels are essential for somatic muscle differentiation (Baylies & Bate, 1996). During formation of the mesoderm the maternal transcription factor *dorsal* activates *twist*, and as *dorsal* is present in a gradient across the embryo a high concentration is required to activate *twist*, leading to expression only in the most ventral cells of the embryo (Jiang *et al.*, 1991). Expression of *twist* in each segment of the embryo varies, with higher

levels present towards the posterior of the segment. These high levels give rise to somatic and cardiac muscle from the ventral and dorsal sections of each segment respectively, whereas lower levels at the anterior of each segment form visceral muscle, demonstrating the effect of levels of *twist* in muscle differentiation (Borkowski *et al.*, 1995). Subsequently, *twist* regulates other early-stage mesodermal specification factors such as *snail*, a zinc finger family protein involved in repression of dorsal-specific genes, and *twist* is also able to regulate itself in order to maintain its own transcription (Leptin, 1991). After mesoderm formation *twist* is able to regulate other muscle specific genes such as *mef2* and *tinman* (Yin *et al.*, 1997; Bryantsev & Cripps, 2009). Regulation of downstream genes by *twist* is dependent on dimerization either as a homodimer to activate expression, or as a heterodimer with *daughterless* (*da*) to repress downstream genes (Wong *et al.*, 2008). *Twist* continues to be expressed in AMPs in the late embryo after embryonic and larval muscles have been formed, and this expression is required for adult muscle development. These precursors are specified relatively early, and ablation of these cells affects adult muscle formation, with many groups of muscle missing in the adult fly (Broadie & Bate, 1991).

Drosophila mef2 is primarily considered to be muscle specific and is known to be essential for muscle formation in flies, both during embryogenesis and the development of larval muscle, and during metamorphosis and the formation of adult muscle (Black & Olson, 1998). *Drosophila* have just one *mef2* gene in contrast to the four found in mammals. In early larval development *mef2* is found to localise to the mesoderm in cells that give rise to in particular somatic and visceral muscle, and is regulated by genes such as *twist* and *snail* (Lilly *et al.*, 1994). It has been noted that while *mef2* is expressed throughout embryogenesis and muscle

differentiation, many of the genes under its control are expressed at different levels at various stages of development (Sandmann *et al.*, 2006). One way in which this occurs is through changes in *mef2* expression, as many of the genes regulated by *mef2* require certain levels before they can be expressed (Elgar *et al.*, 2008).

It has been shown that all three adult *Drosophila* muscle types are dependent on correct *mef2* expression, and mutations can lead to abnormal development of muscle tissue. These can range from defects in the development and patterning of somatic body wall muscle, to loss of certain myosin gene expression in the cardiac tube and gut malformation (Ranganayakulu *et al.*, 1995). However, early stage development of precursor cells for somatic muscle does not appear to be affected by mutations in *mef2*, as other muscle-determining factors are still present, such as *even-skipped* and *nautilus*, the *Drosophila* homologue of MyoD, and these allow differentiation to the myoblast stage, however, without *mef2* these are unable to further differentiate to form normal musculature (Bour *et al.*, 1995). After this differentiation stage, the importance of *mef2* appears to diminish, as temporally conditional knockdown of *mef2* in later adult myogenesis still allows muscles to develop and many muscle-specific genes are expressed, although muscles often lose efficiency if *mef2* is reduced between muscle fibre differentiation and eclosion (Bryantsev *et al.*, 2012). The expression pattern of adult *Drosophila mef2* is divided into early and late expression, representing the separate roles of *mef2* in muscle development and maintenance (Soler *et al.*, 2012).

Due to the wide expression pattern of *mef2*, there are many genes that are thought to regulate MEF2 in order to specify cell fates in the different muscle lineages. *Twist* is an early stage regulator of *mef2* expression, but later stage regulation, for example, in organ precursor cells, is dependent on tissue-specific factors (Gajewski

et al., 1997; Gajewski *et al.*, 2001). Many examples also exist for transcriptional cofactors working with *mef2* to activate tissue-specific genes. The zinc finger transcription factor chorion factor 2 (CF2) is thought to interact with *mef2*, particularly in activation of actin genes during muscle formation. Binding sites in the regulatory region of *Actin57B* have been found for both MEF2 and CF2, and while both are able to upregulate *Actin57B* individually, interaction between the two leads to higher expression and is able to maintain the level of expression needed for correct muscle development (Tanaka *et al.*, 2008). Interactions between *mef2* and the genes *scalloped* (*sd*) and *vg* have also been found, and are thought to contribute to the muscle-specific phenotypes associated with these genes (Deng *et al.*, 2009).

1.3.2 The *Drosophila* heart

The *Drosophila* heart is a relatively simple structure compared to that of mammals. Also known as the dorsal vessel, the adult fly heart is situated in the abdomen attached firmly to the abdominal cuticle by alary muscles (Medioni *et al.*, 2009). The function of the heart in *Drosophila* is to maintain the circulation of haemolymph within the body, and in this respect it is similar to a mammalian circulatory system. However, *Drosophila* do not have the complex network of blood vessels that mammals do, rather, they have an open circulatory system where haemolymph can freely move throughout the body. The heart is attached to a primitive aorta which extends into the thorax and carries haemolymph towards the head (Wu & Sato, 2008) as shown in figure 1.8. It has no chambers like those of a mammalian heart, instead being a muscular tube comprised of a single layer of cardioblasts arranged in a circular pattern forming the main vessel, surrounded by a

longitudinal muscle layer made up of pericardial cells (Tao & Schulz, 2007). Inlet valves known as ostia are present on the sides of the heart surrounded by ostium cells which open and close with the heartbeat to allow inflow of haemolymph (Bryantsev & Cripps, 2009). The anterior-posterior wave movement of the heart then moves this towards the anterior of the fly, the direction of the wave suggesting pacemaker cells are present in the posterior section of the heart (Medioni *et al.*, 2009). Surrounding the cardiac tube are pericardial cells which do not form part of the musculature of the cardiac region, but arise as a result of many different gene pathways and interactions (Ward & Skeath, 2000). Their function is believed to be involved with haemolymph detoxification, but there may be other, yet undiscovered functions for this diverse group of cells (Bryantsev & Cripps, 2009). Unlike the mammalian circulatory system, where the most important function is to supply the body with oxygenated blood, the *Drosophila* heart is not required for movement of oxygen around the body of the fly; rather, its primary function is to ensure the movement of nutrients around the body. Nutrients enter the haemolymph in the abdomen and a pump, the heart, is required to move this haemolymph through the body and particularly towards the head and brain. A completely separate system, the tracheal system, is responsible for the supply of oxygen in the fly, simply being a series of tubes and air sacs through which air enters and diffuses throughout the body. Despite these differences in structure and function between mammalian and *Drosophila* hearts, there are many similarities, particularly when comparing genes involved in development and regulation of heart function. As with mammals, the *Drosophila* heart is the one of the first organs to form and is present in embryonic and larval stages as well as in the adult fly (Monier *et al.*, 2007).

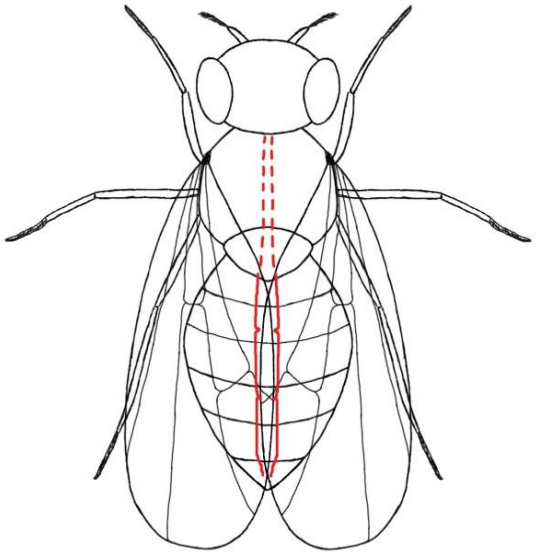

Figure 1.8: Position of the adult *Drosophila* heart. Heart is shown in solid red with aorta shown in dotted red..

1.3.1.1 Genetic regulation of *Drosophila* cardiac development

During development *Drosophila* heart formation is controlled by various genes that have homologues in mammals. The earliest signs of heart formation in flies are initiated by Hox gene expression, genes responsible for body plan organisation, specifically *ultrabithorax* (*Ubx*) and *abdominal-A* (*abdA*) (Lo *et al.*, 2002) as well as BMP-related factors such as *decapentaplegic* (*dpp*) (Frasch, 1995). These genes initiate pathways that result in migration of mesodermal cells forming internal lines of pre-cardiac cells from which the heart tube is derived (Bryantsev & Cripps, 2009). This parallels the early stages of mammalian heart formation, the development of which begins with formation of a heart tube from mesodermal cells (Olson, 2004).

One of the earliest cardiac development genes in *Drosophila* to be expressed is *tinman*, the *Drosophila* homologue of the *Nkx2* family of vertebrate transcription factors, the cardiac-specific homologue being *Nkx2-5* (Bartlett *et al.*, 2010). *Tinman*, as with many *Drosophila* genes, is named due to the mutant phenotype, with null mutations being embryonic lethal due to the failure to form a heart (Bodmer, 1993). A similar phenotype is seen in mice null for *Nkx2-5*, as mutations cause abnormal heart development and potential congenital heart disease phenotypes in humans (Bartlett *et al.*, 2010). *Drosophila* null mutants for *tinman* can be partially rescued by mouse *Nkx2-5*, showing at least some homology, although this is not a complete rescue, showing there is some divergence between the two (Ranganayakulu *et al.*, 1998). In flies, early *tinman* expression is seen in the blastoderm stage and is required for formation of the mesoderm. Expression at these early stages is more widespread, and has been seen to be present in the majority of mesodermal cells, although as development progresses *tinman* expression is increasingly restricted, eventually being expressed only in the cells that make up the heart tube and aorta (Bodmer *et al.*, 1990) as shown in figure 1.9. Indeed, it has been shown that loss of *tinman* is only embryonic lethal in the early mesoderm, but if expression is repressed at later stages after the heart tube has formed, flies can develop as far as adult stages, although various cardiac abnormalities are observed due to improper differentiation of cardiomyocytes (Zaffran *et al.*, 2006).

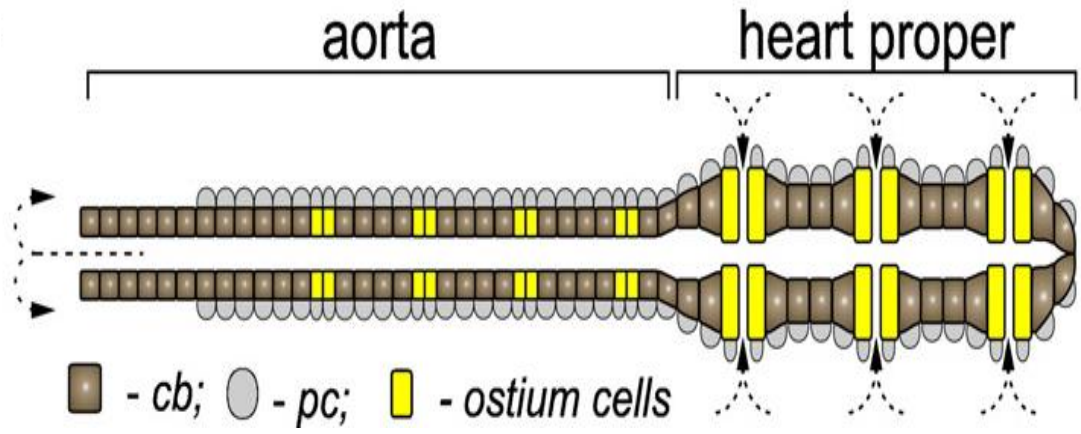

Figure 1.9: The embryonic/larval cardiac tube showing the 52 pairs of cells that make up the heart. *Tinman*-positive cardioblasts (cb) are shown in brown, *svp*-positive ostium (inlet valve) cells in yellow. Surrounding pericardial cells (pc) are shown in grey. Arrows indicate the direction of haemolymph as it enters the heart through ostia and is pumped towards the anterior of the animal, moving nutrient-rich haemolymph towards the head and brain. From Bryantsev and Cripps (2008).

Tinman expression is dependent on the activity of *Dpp*, as shown in figure 1.10, activating *tinman* through the Smad4 homologue Medea which, along with other factors including the Tinman protein itself, promote expression in cardiac cells (Xu *et al.*, 1998), while *wingless* (*Wg*) signalling is responsible for *tinman* repression in other cells types during development (Lee & Frasch, 2005). *Tinman* is a homeodomain-containing transcription factor which activates, among others (see figure 1.10), such downstream genes as *pannier* (*pnr*), a GATA factor homologue (Gajewski *et al.*, 2001), *Hand*, a basic helix-loop-helix transcription factor (Han & Olson, 2005), and *seven up* (*svp*), another early-stage regulator of mesodermal cell fate (Ryan *et al.*, 2007). Interestingly, *seven up* is known to regulate *tinman* as a repressor and is thought to regulate the differences between *svp*-positive and *tin*-positive cardioblasts in the adult *Drosophila* heart (Lo & Frasch, 2001; Gajewski *et al.*, 2000), with *svp* expressed in the cells surrounding the ostia where *tinman* is not

expressed in the mature heart tube (Bryantsev & Cripps, 2009) . Figure 1.9 shows the expression patterns of *tinman* and *svp* in the mature heart tube, and demonstrates the specificity of the final expression patterns of these genes. *Tinman* is also thought to regulate *even-skipped* (*eve*), a gene expressed in certain cells within the mesoderm that give rise to a subset of pericardial cells. *Eve* is required for correct formation of pericardial cells and *eve* mutants display defects in cardiac development and rhythmicity (Fujioka *et al.*, 2005). Binding sites for *tinman* have been found in an enhancer, the activity of which is necessary for *eve* activation (Knirr & Frasch, 2001), demonstrating that *tinman* expression is necessary for development of pericardial cells despite later being restricted to cardiomyocytes.

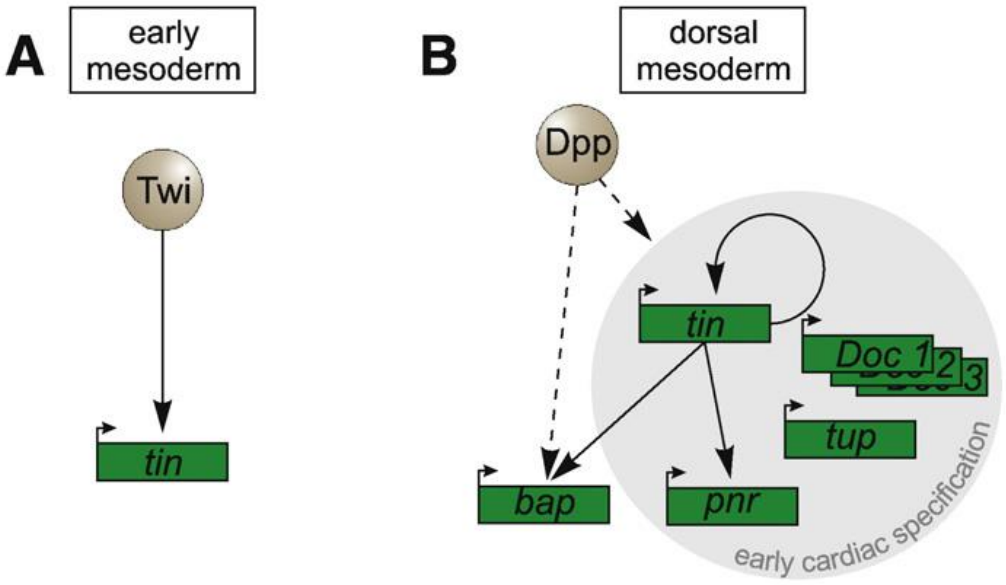

Figure 1.10: interactions between early mesodermal and cardiac specification factors in *Drosophila*. (A) In the early mesoderm *tinman* (*tin*) is activated by *twist* (*twi*), contributing to mesoderm specification. (B) In later stages *decapentaplegic* (*Dpp*) activates *tinman* in cardiac precursors, which activates and interacts with *pannier* (*pnr*) and *bagpipe* (*bap*). *Dorsocross* (*Doc1-3*) genes and *tailup* (*tup*) also contribute to cardiac development alongside *tinman*. From Bryantsev and Cripps (2008).

Alongside *tinman*, another important early-stage transcriptional regulator is *pannier* (*pnr*). This is the *Drosophila* homologue of GATA4, 5 and 6, the cardiac-specific GATA factors in mammals (Pikkarainen *et al.*, 2004). Another gene, *serpent*, is thought to be the homologue of GATA1, 2 and 3, expressed in hematopoietic cells (Han & Olson, 2005). GATA factors are zinc-finger transcription factors and *pannier* is expressed from early stages of cardiac development in *Drosophila* in the cardiogenic mesoderm. In mammals, overexpression of GATA4 can lead to cardiac hypertrophy (Pikkarainen *et al.*, 2004), showing a potential link between this signalling pathway and the hypertrophy-inducing pathway that includes *ms1*. It has been shown that *pannier* is required for cardiac cell development, and the importance of this gene is demonstrated by the fact that even a heterozygous mutant fly has impaired cardiac function and increased incidence of heart failure under electrical pacing (Qian & Bodmer, 2009). Mouse GATA4 can rescue the *pnr* mutant phenotype (Gajewski *et al.*, 1999) showing the high level of similarity between the two homologues. *tinman* is known to activate *pnr*, but also acts alongside *pnr* to regulate expression of cardiac specific genes (Gajewski *et al.*, 2001), and *pnr* may also function in regulation of *tinman* at early stages, working to initiate and maintain *tinman* expression in the mesoderm (Klinedinst & Bodmer, 2003). The binding partner of *pnr* is a zinc finger protein called *u-shaped* (*ush*), and this negatively regulates binding of *pnr* to DNA (Haenlin *et al.*, 1997). *Ush* is also required for activation of *tinman* but seems to only be essential in later stages, when *tinman* expression is restricted to the cardiac tube (Klinedinst & Bodmer, 2003). The interactions and dependency between *tinman* and *pnr* indicate that both genes are essential for correct heart formation in *Drosophila*, as the presence of both proteins appears to be necessary to induce expression of downstream genes such as

mef2 (Gajewski *et al.*, 2001) . The evidence for interaction between these two proteins is further demonstrated by the fact that *tinman* is unable to compensate for loss of *pnr* (Qian & Bodmer, 2009).

The transcription factors from the T-box gene family are involved in initiation of heart development in flies. *Dorsocross* (*Doc*) genes 1, 2 and 3 are expressed in early cardioblast specification and act alongside *tinman* in activation of *pannier* as shown in figure 1.10, and therefore are important in the *Dpp* signalling pathway to which these genes belong in cardiac cells (Reim & Frasch, 2005). In humans, the *Doc* homologue *Tbx5*, along with *Nkx2-5* and *GATA4*, is a gene in which mutations often cause congenital heart disease as a characteristic of Holt-Oram syndrome (Granados-Riveron *et al.*, 2012). The importance of *Doc* in early *Drosophila* development is demonstrated by the high expression in the dorsal mesoderm and that loss of all three *Doc* genes affects germ band retraction, but more specific cardiac expression is seen later in development and appears to be required for formation of the ostia (Reim & Frasch, 2005). However, *Doc* genes are not the only T-box genes involved in heart development. The human genes *Tbx1*, *2*, *5* and *20* are expressed during initial formation of the heart tube, and later *Tbx3* and *18* are also involved during formation of the multi-chambered heart structure (Greulich *et al.*, 2011). The *Drosophila* homologue of *Tbx20*, *neuromancer* (*nmr*), is yet another gene shown to interact with *tinman*, and mutants for *nmr* display increased arrhythmias and developmental defects comparable to those found in mammalian models and even in humans (Qian *et al.*, 2008).

1.3.1.2 Remodelling of the *Drosophila* heart during metamorphosis

In the larval stages the heart is free to move around the body cavity, only becoming affixed to the inner cuticle in adult stages (Vogler & Ocorr, 2009). The adult heart is formed during metamorphosis by modifications and remodelling of the larval heart tube, with the adult heart arising from the larval aorta through development of contractile function in cardiomyocytes and the formation of new ostia (Shah *et al.*, 2011). This process does not involve any increase in cell numbers and, unlike other tissues that are completely dissolved during pupal stages to make way for adult structures, much of the heart remains intact but is subject to extensive remodelling (Curtis *et al.*, 1999). Various changes in gene expression are required to carry out this remodelling, and the signal for these changes appears to come from a steroid, 20-hydroxyecdysone (also referred to as ecdysone), a hormone known to play a vital role in the initiation of metamorphosis (Monier *et al.*, 2005). The Hox genes *Ubx* and *AbdA*, whose expression is required during determination of cardiac positioning and regulation of early cardiac-specific genes in embryonic development, are thought to be involved in changes in gene expression during adult heart development. Specifically, *Ubx* appears to be repressed in those cells in the heart that express *tinman*, whereas *abdA* is required for formation of the posterior terminal segment (A5) of the heart tube from the original larval heart as shown in figure 1.11 (Monier *et al.*, 2005).

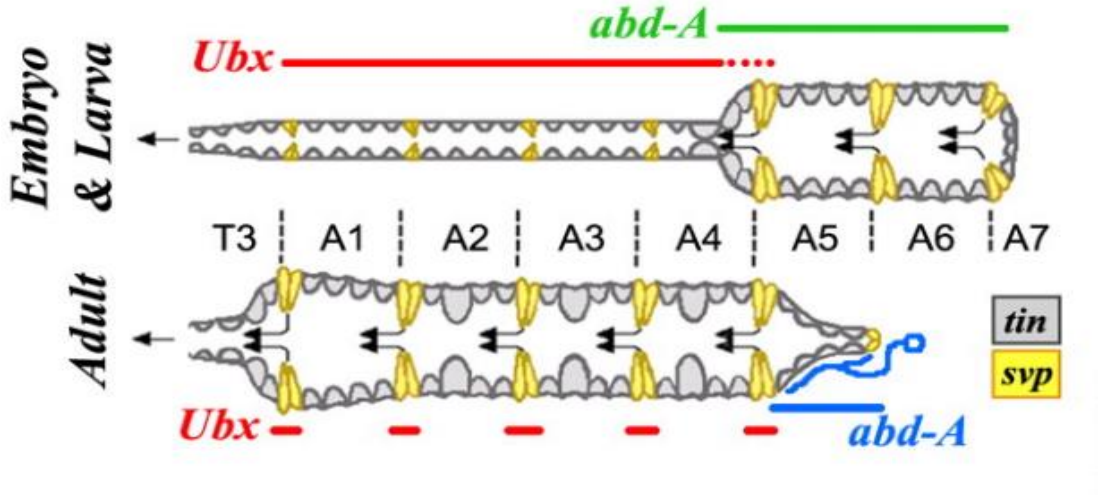

Figure 1.11: heart segment fate during metamorphosis comparing embryonic/larval heart with adult heart structure. Segment fate is initially determined by *ultrabithorax* (*Ubx*) and *abdominal A* (*abd-A*). *Tinman*- and *svp*-positive cells are indicated in grey and yellow respectively. From Monier *et al*. (2005).

Important signalling pathways have also been implicated control of adult heart development, including the *Wnt* signalling pathway (Zeitouni *et al.*, 2007). The *Wnt* pathway has functions in transcriptional regulation via β-catenin, and also in control of cell polarity and calcium signalling (Strutt, 2003), and in the case of the adult heart appears to be necessary for formation of the posterior terminal segment, as inhibition of this pathway causes more anterior segments of the heart to develop into A5-type segments, which is characterised by differences in myofibril orientation and diameter reduction of the heart tube itself (Zeitouni *et al.*, 2007). A role for *seven-up* has also been suggested during metamorphosis as a regulator of gene expression controlling formation of new ostia in the adult heart when those in the larval heart are lost as a result of remodelling (Shah *et al.*, 2011).

1.3.2.2 Muscle and cardiac research in *Drosophila*

Due to the homology between mammalian and *Drosophila* genes and the mechanism in which these act during muscle and heart development, *Drosophila* can make a good model for research in this area. This kind of work can help to elucidate the specific mechanisms behind correct development of muscle and cardiac tissue, and can uncover the mechanisms behind dysfunction related to disease or ageing. With regards to somatic/skeletal muscle this type of research can be used to investigate conditions in which muscle is affected. These include muscular dystrophies, which tend to affect all muscle types, and other more specific muscle diseases which only affect certain muscle groups and require further investigation into the reasons behind their specificity (Tixier *et al.*, 2010), The *Drosophila* model provides a relatively simple way in which to carry out the early stages of research into these conditions. An example of this is the discovery of the *Drosophila dystrophin* gene, which is essential for maintenance of muscle integrity. Human *dystrophin* is the longest known gene in the human genome and mutations are associated with many types of muscular dystrophy, including Duchenne Muscular Dystrophy (DMD), a debilitating and life-shortening X-linked disease characterised by progressive muscle weakness (Hoffman *et al.*, 1987). *Drosophila dystrophin* has been shown to be expressed in many different isoforms, each having its own specific function, and certain isoforms are known to be essential for muscle stability and arrangement of fibres (van der Plas *et al.*, 2007). Muscles with knockdown of these isoforms deteriorate and show progressively disorganised muscle fibres and increased amounts of cell death. A fly model has also been developed for the disease myotonic dystrophy, also known as Steinert disease. Different forms of this disease are associated with mutations and expansions in

different genes (Harley *et al.*, 1991; Day *et al.*, 2003), and the fly model has identified *muscleblind* as a factor that could be involved. Knockdown of *muscleblind* results in a phenotype which displays similarities to that seen in myotonic dystrophy (Machuca-Tzili *et al.*, 2006) demonstrating the importance of this protein in muscle maintenance and the role this pathway plays in the pathology of this disease, which can lead to possible avenues for research into treatment for this type of disease. Ageing has a dramatic effect on muscle function, and the genetic reasons behind the processes that lead to this decline can also be investigated using a *Drosophila* model. Genes found to have alterations in their expression levels in older people are also found to be affected in a similar way in other species including flies (Zahn *et al.*, 2006), suggesting conserved pathways are involved in the ageing process in muscle tissue. Evidence from *Drosophila* models has revealed an important role for apoptosis in muscle wasting and functional decline associated with age (Zheng *et al.*, 2005), and a decrease in mitochondrial function has also been implicated in contributing to muscle ageing (Ferguson *et al.*, 2005).

Much research into the cardiovascular system and heart development using *Drosophila* has been carried out in the last few years. Only relatively recently has this been developed as a model for this type of study, and many of the pathways and mechanisms controlling *Drosophila* heart function have been investigated. This has allowed the similarities between fly and mammalian systems to be discovered and has proven this model to be an effective and useful tool in cardiovascular research. Recently, a screen by Neely *et al.* (2010) was designed to identify genes involved in heart development and function by using RNAi to create lines of flies each with a suspected cardiac-involved gene knocked down. These lines were tested for

embryonic and adult lethality, then placed at a raised temperature to test their survival under stress conditions. Any knockdown that was found to affect survival was further investigated by visualisation of the heart and analysis of the heartbeat and contractility. This screen discovered various genes that appeared to play a role in the *Drosophila* heart, particularly the gene *not3*, which was further investigated in a mouse model. Mice with a heterozygous knockout of *not3* were viable but showed an increased stress response, with higher levels of hypertrophy and apoptotic cardiomyocytes observed compared to control mice. In addition to this, SNP analysis in humans revealed a potential link between a SNP found in the promoter of this gene and abnormal QT interval length. This study gives an excellent example of the power of the *Drosophila* model in cardiac research, and the translational capacity for findings using this model. Because of the relative ease in using a *Drosophila* model it has been possible to test large numbers of genes to maximise the chances of finding a gene of interest, whereas using a mammalian model this would not be possible in such large numbers.

More focused research has been carried out looking at particular genes involved in the *Drosophila* cardiovascular system. The genes already described in this section have been characterised and this has helped to elucidate the mechanisms behind development of the fly heart, and have not only confirmed homology between flies and mammals in the function of these genes, but have also suggested additional functions that were not previously known. Genes involved in heart function and maintenance of the heartbeat have been studied, including genes encoding ion channel proteins such as KCNQ, a potassium channel protein and the *Drosophila* homologue of the KCNQ1 family of genes found in mammals. In humans, mutations in KCNQ1 are known to cause arrhythmicity and long QT syndrome

(Robbins, 2001), and this is mirrored by *Drosophila* with mutations in KCNQ. Mutations in this gene cause the fly heart to suffer bouts of arrhythmia and, depending on the type of mutation, these can be extremely severe, causing some strains of mutant flies to have an almost entirely arrhythmic heartbeat by three weeks of age (Ocorr *et al.*, 2007). Typical M-modes associated with these mutations are shown in figure 1.12 compared against a wild type heartbeat.

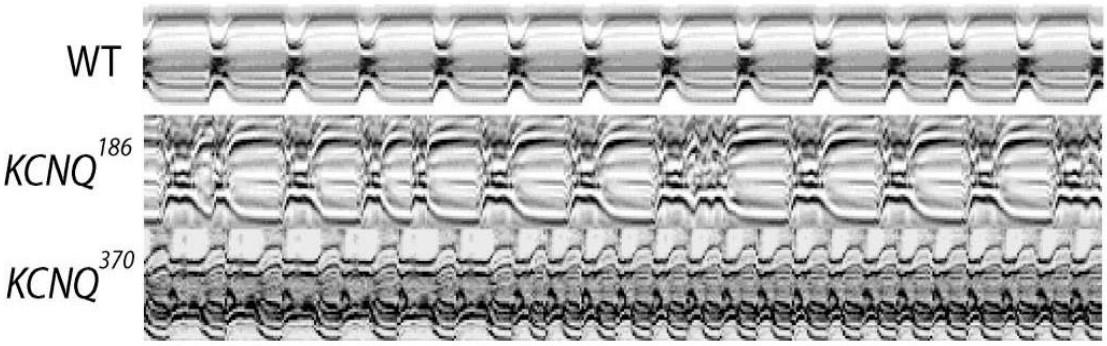

Figure 1.12: example of an M-mode used to analyse Drosophila heartbeat. M-modes from two different KCNQ mutants are shown compared against the wild-type phenotype. From Ocorr *et al.* (2007).

The effect of age has also been studied using *Drosophila*, as heart disease is more commonly observed in older people. Deterioration of the fly heart correlates with age, as the heart beats with increasing arrhythmicity as flies get older (Fink *et al.*, 2009). These effects and the similarity between fly heart function and that of mammals supports the use of *Drosophila* in cardiovascular research, as these and many other results have proven to be translatable to mammalian models.
While *Drosophila* are a good model for this kind of work, there are still many differences in the general physiology between flies and vertebrates. This means that many new techniques have had to be developed for analysis of the fly heart.

Methods have been developed for observation of the adult heart using semi-intact preparations which allow the heart to continue to beat autonomously after the fly has been dissected, enabling the natural heartbeat to be observed without influence from the brain and therefore making it easier to find defects in heart function (Vogler & Ocorr, 2009). Analysis of these preparations can be carried out using specially designed computer programs that use videos of the beating heart and movement detection algorithms to assess the function of the heart (Ocorr *et al.*, 2009). Other methods are available to image the heart in adult flies, for example using optical coherence tomography (OCT), which gives images of the beating heart and can be used to analyse heart function without the need for dissection or anaesthetic (Wolf *et al.*, 2006). Electrophysiological methods can also be used, both in intact adult *Drosophila* and in isolated hearts (Akasaka & Ocorr, 2009; Papaefthmiou & Theophilidis, 2001) to measure heartbeat and action potentials. Larvae can also be used, and various methods allow analysis of the heart in intact and dissected larvae, including simple visualisation of the heartbeat through the transparent skin of the larvae, and dissection to allow electrophysiological measurements and electrical pacing (Cooper *et al.*, 2009).

1.4 *Drosophila ms1*

The present project investigated the potential *Drosophila* homologue of *ms1* (*dms1*) and the role it plays in the *Drosophila* heart and muscle. Little is known about this gene and while the gene structure is listed in databases such as Flybase (flybase.org) and FlyAtlas (flyatlas.org), the role of this gene has not been studied, and there is no published data on the biological function of *dms1*. The alternative gene name for *dms1* is CG3630 and assigned Flybase gene number is

FBgn0023540, and FlyAtlas microarray data shows expression of *dms1* in the crop, heart and adult and larval carcass (Chintapalli *et al.,* 2007), suggesting high expression in somatic muscle, which is consistent with what is known about mammalian *ms1*, as expression is known to occur primarily in muscle tissue. Sequence homology between DMS1 and mammalian MS1 is restricted to the actin binding domain found at the N-terminus of DMS1 and the C-terminus of mammalian as shown by the alignment in figure 1.13 (Arai *et al.,* 2002). This means that both genes have unique sequence outside of this region.

As there is no information on the action of *dms1* it is difficult to tell what the functions of these unique sequences are and whether they give the different homologues additional functions specific to each organism. As *ms1* has been shown to play a role in a variety of physiological processes in mammals, it could be that mammalian *ms1* has gained functions over the course of evolution through changes in sequence outside of the actin binding domain, and therefore *dms1* could represent an ancestral version of *ms1*. Using a *Drosophila* model to investigate *ms1* will give an idea of any differences in function between these homologues, as well as providing a simple model for in-depth investigation into the conserved function and the processes in which this gene is involved.

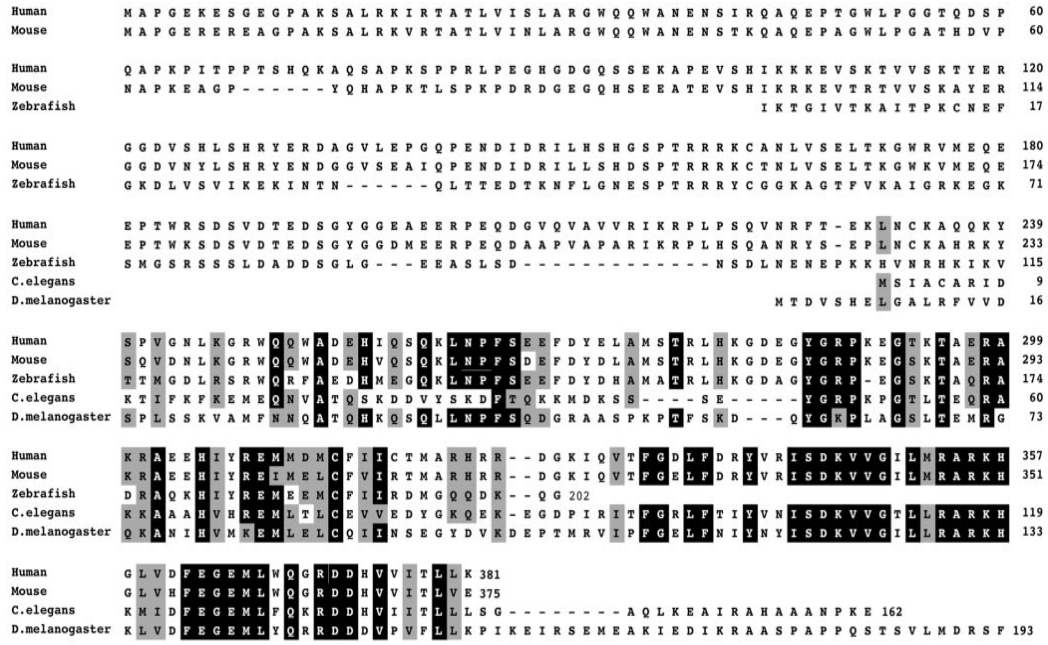

Figure 1.13: alignment of human, mouse, zebrafish and *Drosophila ms1* showing homology in the actin binding domain. From Arai *et al.* (2002).

The main aims of this project are:

- To determine *Drosophila ms1* gene structure and investigate the effect of *dms1* on potential upstream and downstream genes to discover if these pathways are conserved between species.
- To investigate the role of *dms1* in the *Drosophila* heart and its effect on development and function.
- To investigate the role of *dms1* on *Drosophila* somatic muscle and its effect on development and function.

Chapter 2: Materials and methods

2.1 Fly husbandry

Fly stocks were kept at 18°C in 25 x 95 mm clear plastic vials with approximately 2cm of maize food (7.20g maize meal, 7.93g glucose, 5g brewers' yeast, 0.85g agar, 0.3ml propionic acid and 1.35ml 20% Nipagin solution in 100ml water). Vials were sealed using cotton wool balls. Temperature controlled rooms subject flies to a daily cycle of 12 hours of light and 12 hours of dark (12:12 LD) to simulate an average day. Stocks were turned every 4 weeks into new vials.

2.2 Fly crosses

All crosses described here were carried out at 25°C in the same vials as used for stock keeping. Approximately 4-5 males and 4-5 virgin females of the appropriate genotypes were isolated from their stocks while anesthetised with CO_2 and transferred to a new vial. Crosses were left for 10 days when adult progeny were required, and less for other developmental stages. Details of specific crosses used can be found in the relevant results sections.

2.3 Fly stocks

Table 2.1: Fly stocks used in this project, listing genotypes, names used in this report and the source of the stock.

Genotype	Referred to as	Source
w; *tinman*CΔ4-GAL4; +	*tin*CΔ4-GAL4	Manfred Frasch at Universität Erlangen-Nürnberg*
UAS-Dcr2; *mef2*-GAL4; +	*mef2*-GAL4-UAS-Dcr	Bloomington Stock Center
yw; +; *mef2*-GAL4	*mef2*-GAL4(3^{rd})	Bloomington Stock Center
yw; UAS-RNAi(CG3630); + GD line	UAS-RNAi(GD)	Transformant ID: 21697 Vienna Stock Centre
w; UAS-RNAi(CG3630); + KK line	UAS-RNAi(KK)	transformant ID: 110512 Vienna Stock Centre
w; UAS-RNAi (calcineurin); +	UAS-RNAi(calcineurin)	transformant ID: 27270 TRiP stock centre
w; +;UAS-GFP	UAS-GFP	Bloomington Stock Center

*(Lo & Frasch, 2001)

2.3.1 GAL4-UAS system

The fly stocks listed in this section are driver (GAL4) or UAS lines which utilise the GAL4-UAS system to drive expression of specified genes in a driver-dependent pattern (Brand & Perrimon, 1993). GAL4 constructs consist of a promoter region placed upstream of the coding sequence for the GAL4 transcription factor, driving expression of GAL4 in a pattern specific to that of the promoter. UAS constructs consist of an upstream activating sequence (UAS) lying upstream of the gene to be expressed. When a GAL4 and UAS line are crossed together GAL4 is able to bind UAS and activate transcription of the gene of

interest in a pattern specific to the promoter sequence used, as demonstrated in figure 2.1.

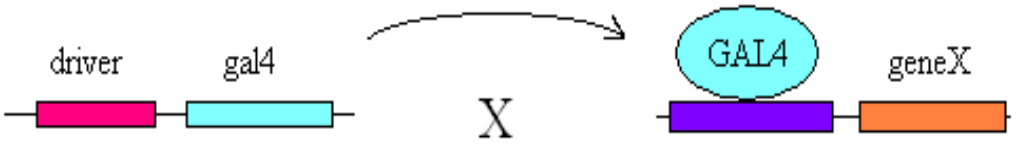

Figure 2.1: The GAL4-UAS system used to drive gene expression in Drosophila. A promoter driving expression in a specific pattern is used to drive expression of the transcription factor GAL4. This binds to UAS (upstream activating sequence causing activation of gene X (gene of interest). The GAL4 and UAS constructs are present in different fly strains, so the system is only activated when the two strains are crossed.

2.3.2. UAS-RNAi

UAS-RNAi lines are designed to allow expression of RNAi under the control of a selected GAL4 driver. This is achieved by creating an inverted repeat sequence complementary to the sequence of the gene of interest downstream of UAS which, when expressed, forms a hairpin RNA. This double-stranded RNA is broken down into small interfering RNAs. These bind the mRNA of the gene of interest form double stranded RNA, which is then recognised and broken down by the cell. This mechanism is described in figure 2.2. RNAi lines are available from the Vienna *Drosophila* RNAi Centre (VDRC) (Dietzl *et al.*, 2007).

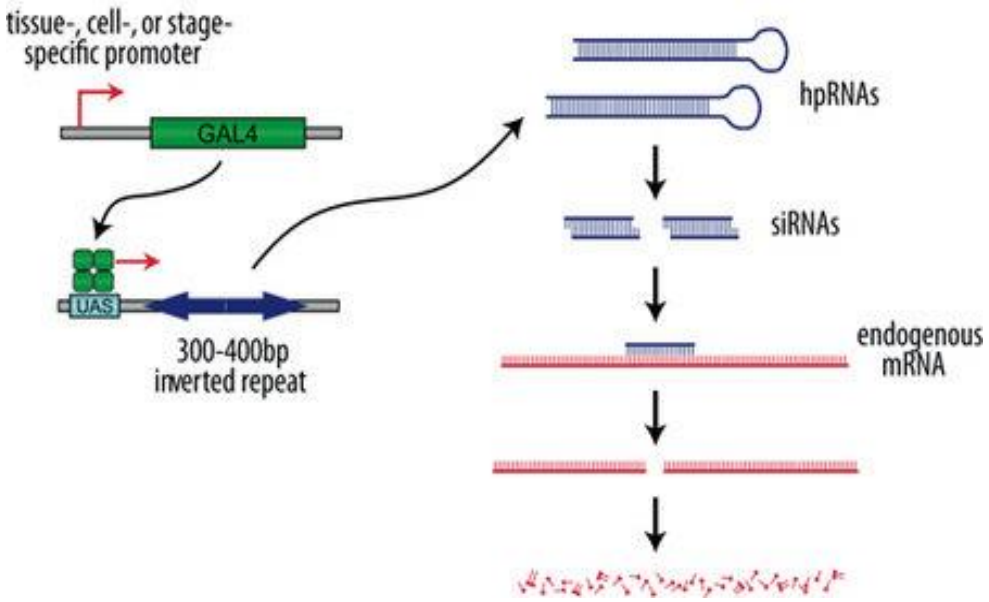

Figure 2.2: the mechanism of UAS-RNAi used to knock down expression of genes in *Drosophila*. An inverted repeat is expressed to form hairpin RNA, this is broken down and binds endogenous mRNA, resulting in the degradation of this mRNA, thereby effectively reducing its expression. From Dietzl *et al*. (2007).

2.4 Backcrossing of lines

All lines used in the experiments described here were backcrossed to laboratory strain w^{1118} to ensure the same genetic background for all lines. Virgin females from each line were crossed to males of w^{1118} and red eyed virgin female progeny collected. These were then crossed to males of w^{1118}. This process was repeated 6 times. After 6 backcrosses red eyed males and females were collected and crossed to each other. The eyes of the resulting progeny were examined for higher expression of the *white* gene (darker red eyes) to indicate the presence of two constructs (ie. homozygous for the construct). These selected progeny were used to create backcrossed stocks for each fly line. Stocks were examined after each generation to ensure homozygosity of the constructs.

2.5 Driver expression patterns

The expression pattern of each GAL4 line was tested by crossing males of the GAL4 lines to females of w; +; UAS-GFP. Progeny were dissected according to the protocol outlined in section 2.14, viewed under a Nikon SMZ800 fluorescence microscope and images taken using a Leica DFC365 FX digital camera.

2.6 Amplification of *dms1* to confirm gene structure

2.6.1 Trizol RNA extraction

RNA was extracted from whole flies using Trizol/chloroform. Flies were collected and immediately frozen in liquid nitrogen. Whole body tissue was homogenised in 1ml Trizol (Invitrogen) and extraction carried out as described by the manufacturer. Quality was checked using a 1% agarose gel.

2.6.2 cDNA synthesis for normal PCR

This protocol was used for synthesis of cDNA for use in normal PCR. An alternative protocol and kit were used to synthesise cDNA for use in quantitative Real-Time PCR.

RNA was first given DNase treatment using DNase1 (Invitrogen), then 1 µl was incubated with 1 µl oligo dT primers (Promega) and 3 µl H_2O for 5 minutes at 70°C, then 5 minutes on ice.

Reverse transcription was carried out as follows:

4.6 µl H_2O
4 µl RT buffer
2.4 µl $MgCl_2$
1 µl dNTPs
1 µl RNasin
Vortex reaction mixture here
1µl reverse transcriptase
Add oligo dT/RNA primer mix = 20µl reaction

The reaction mixture was covered with mineral oil and the following incubations carried out:

25°C for 5 min
42°C for 60 min
70°C for 15 min.

2.6.3 Polymerase Chain Reaction (PCR)

The PCR reaction mixture was as follows:

2 µl DNA
5 µl 5x buffer (containing $MgCl_2$ and dNTPs)
1 µl 10 mM primer1/primer2
0.25 µl KAPA Taq polymerase (KAPABiosystems)
15.75 µl H_2O

The reactions were placed in a thermocycler and the following program run:

92°C 2 min
35 cycles: 92°C 15 sec, 60°C 30 sec, 72°C 30 sec
72°C 30 sec
Incubate at 10°C

The PCR products were run on a 1% agarose gel using 6x loading buffer and appropriate size marker DNA ladder.

Primers were designed to amplify overlapping sections covering all intronic and flanking regions of dms1 to verify the position of each intron and identify any splice variants. PCR was carried out to amplify sections of *dms1* as shown in figure 2.3.

Table 2.2: Primer sequences used to amplify sections of *dms1*, amplified sections are shown in figure 2.1.

Primer name	Sequence
Set1_forward	ACGTGTGCATTTTGCATTGT
Set1_reverse	GGGGAGCACAAAAACAGAAA
Set2_ forward	ATCCGAATTCCGGTTCATCT
Set2_reverse	CCCAGTTCATGCGATACGTC
Set3_ forward	GGACCAGTCACCGCATTC
Set3_reverse	CCCAAAGTAGGGGTGTGTTG
Set 4_ forward	GTGAGGAGCCAGAACAAAGC
Set4_reverse	CCACTTCCCACAGCAGAAAT
Set5_ forward	CGGTCCGTGTGATATGAATG
Set5_reverse	TCCTGGGAAAATGGATTCAG
Set6_ forward	GCAGCTGCTGAATCCATTTT
Set6_reverse	GTAGACGGAGCTGGAACTGC
Set 7 primers	set1_F and set2_R
Set 9 primers	set1_F and set5_R

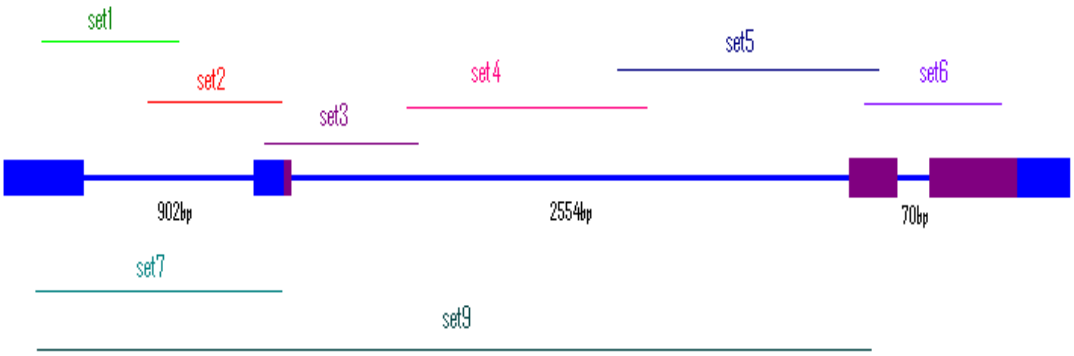

Figure 2.3: *dms1* and positions of PCR products amplified using different sets of primers. Purple area of the gene is coding sequence. Sizes of introns (bp) are indicated.

2.6.4 Verification of fragment sequence

PCR products were run on a 1% gel to separate the fragments. Each fragment was excised from the gel using a scalpel blade and extracted using EZNA gel extraction

kit (Omega Biotek). DNA from the different splice variants was cloned using TOPO-TA vectors and TOPO chemically competent cells (Invitrogen) according to the manufacturer's protocol. Bacteria were plated out on agar plates containing ampicillin and left overnight at 37°C, colonies picked and incubated overnight at 37°C with shaking at 200 rpm. Plasmids were isolated using EZNA miniprep kit (Omega Biotek).

2.6.4.1 Sequencing of gene fragments

Sequencing was carried out using BigDye Terminator v3.1 Cycle Sequencing reaction mix (Applied Biosystems). A "BigDye mix" was made up as follows:

1 µl BigDye reaction mix
3.5 µl sequencing buffer
3.5 µl H_2O

8 µl of this reaction was added to 1 µl DNA, 7 µl H_2O and 4 µl primer. Fragments were sequenced using set1_F primer in the forward direction and set5_R in the reverse direction to give coverage of the whole fragment. A sequencing program was run:

94°C 30 sec
25 cycles: 96°C 10 sec, 50°C 5 sec, 60°C 4 min

2 µl 2.2% sodium docedyl sulphate (SDS) was added to the reaction mixture and was incubated at 98°C for 5 minutes, then 25°C for 10 minutes.
To remove unincorporated nucleotides the reaction was passed through a Performa Spin column (EdgeBio). The resulting reaction mixture was sequenced by PNACL (University of Leicester).

2.7 Cloning of *Drosophila* and mouse *ms1*

The coding sequences of *Drosophila* and mouse *ms1* were cloned and tagged to create new fly lines for overexpression of these genes. This would be achieved by creating UAS-*ms1* lines for use with selected GAL4 lines to drive expression of *ms1* in a specific pattern. Four lines were created: *dms1* tagged with an HA-tag and untagged, and mouse *ms1* tagged with a c-myc tag and untagged.

2.7.1 PCR of *Drosophila* and mouse *ms1*

Drosophila ms1 was amplified using Phusion High Fidelity DNA polymerase (NEB) in the following reaction mixture:

31.5 µl H_2O
10 µl Phusion buffer
2.5 µl 10 mM forward/reverse primer
1 µl 10 mM dNTPs
2 µl DNA
0.5 µl Phusion polymerase

DNA used to amplify *Drosophila ms1* was Canton S cDNA. Mouse *ms1* DNA was obtained using a pBS vector carrying mouse *ms1* tagged with c-myc, provided by Nelson Chong and Andrea Koekemoer (University of Leicester).

Primers were designed to include *Eco*RI (forward primers) or *Xho*I (reverse primers) restriction sites, *Drosophila* Kozac sequence (CAAC, untagged forward primers only) and the first 18-20 bases of the gene. In the case of the mouse tagged primer this sequence is part of the c-myc tag. Sequences are shown in table 2.3.

Table 2.3: Primer sequences used to amplify *dms1* and mouse *ms1* including *Eco*RI restriction site (red), *Xho*I site (green, reverse primers only) and Kozac sequence (blue, forward primers only). *Dms1* tagged forward does not contain a Kozac sequence as it still remains to be tagged and CAAC is added at a later stage.

Primer name	**Sequence**
Dms1 untagged forward	GGGGAATTCCAACATGACGGACGTATCGCATG
Dms1 tagged forward	GGGGAATTCGCAGCCATGACGGACGTA
Dms1 reverse	GGGCTCGAGTCCTTCCTTCCCAGCTAACA
Mouse *ms1* untagged forward	GGGGAATTCCAACATGGCTCCAGGAGAAAGGGA
Mouse *ms1* tagged forward	GGGGAATTCCAACATGGAGCAGAAGCTCATCAGC
Mouse *ms1* reverse	GGGCTCGAGTTACTCAAGGAGAGTAATCA

PCR amplification was carried out as follows:

98°C 30 sec
35 cycles: 98°C 10 sec, 60°C 20 sec, 72°C 20 sec
72°C 6 min

Products were run on a 1% agarose gel and extracted using Zymogel Gel extraction kit (Zymo Research) according to the manufacturer's protocol. PCR products were sequenced using forward and reverse primers separately to ensure there were no errors in the coding sequence (as in section 2.6.4.1).

2.7.2 Cloning into pBS

10 µl pBS vector was digested using 2 µl blunt cutting enzyme *Eco*RV in a reaction mixture with 4 µl NEB buffer 3, 4 µl bovine serum albumin (BSA) and 20 µl H_2O for 3 hours at 37°C. The digested vector was treated with 2 µl shrimp alkaline phosphatase (SAP) for 15 minutes at 37°C, followed by SAP inactivation step (10 min at 65°C). Plasmids were isolated by gel extraction as before.

2.7.3 Ligation reactions

Ligation reactions were carried out between linearised pBS and PCR products of *ms1*. Replicates of the ligation reaction contained either 3:1 or 1:1 ratio of vector to plasmid in 10 µl reactions with 1 µl ligation buffer, 1µl ligase (NEB) and H_2O to 10 µl. Reactions were left for 2 hours at room temperature then overnight at 4°C.

2.7.4 Transformation of *E. coli*

DH5α electrically competent *E. coli* were transformed by introducing 5 µl pBS-ms1 plasmid into 50 µl bacteria. Cells were pipetted into an electroporation cuvette and given a 1.5K volt electric shock. 1 ml Luria broth was added and the cells shaken for 1 hour at 37°C to allow expression of antibiotic resistance genes. The transformed cells were then plated out onto agar plates containing ampicillin (100 µg/ ml), X-Gal (100µg/ml), and IPTG (100mM), allowing for blue white selection of transformants, and incubated overnight at 37°C. White colonies that indicate transformed bacteria were picked and cultured overnight at 37°C with shaking at 200 rpm. Plasmids were isolated from bacteria using EZNA miniprep kit (Omega Biotek).

2.7.5 Tagging of *dms1*

Digestions of a pBS vector carrying HA-*day* tagged gene and of pBS-*dms1* were carried out using restriction enzymes *Eco*RI and *Xho*I with 10 µl plasmid DNA, 4 µl buffer 4 (NEB), 1 µl each restriction enzyme, 20 µl H_2O and 4 µl BSA. The products of the digest were run on a 1% agarose gel. The smaller band from the pBS-*dms1* digest was extracted (containing *dms1* coding sequence only) using Zymoclean Gel DNA Recovery Kit (Zymo Research). As the pBS-HA vector was so similar in size to that of *day* the digestion products were also further digested with

MscI which cuts *day* but not the vector. This allowed the vector to be identified as the largest band on a gel and then extracted in the same way. The vector was then subjected to SAP treatment as in section 2.7.2.

The presence of *Eco*RI and *Xho*I restriction sites allowed *dms1* to be ligated to the pBS-HA vector in the correct orientation, with the HA tag at the beginning of the gene. Ligations and subsequent transformations into electrically competent cells were carried out as in section 2.7.3-4. Figure 2.4 shows the constructs for *dms1* and mouse *ms1* before insertion into pUAStattB.

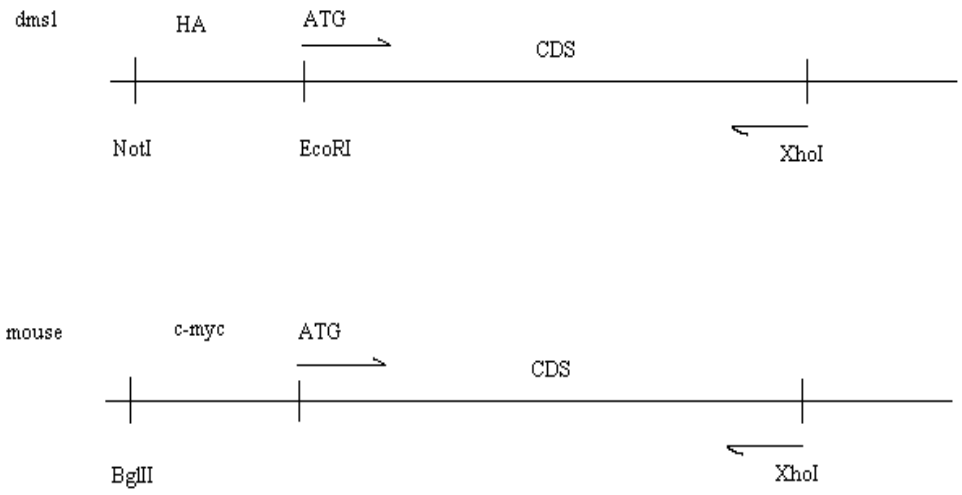

Figure 2.4: diagram of cloning scheme for tagged genes showing positions of gene coding sequence, tags and specific enzyme restriction sites. Restriction sites at the ends are used to insert the constructs into pUAStattB in the correct orientation. Untagged versions of each gene are the same but without the tag sequences, and contain the same restriction sites as the tagged versions shown here.

2.7.6 Cloning into pUASTattB

Primers were designed to include 18-20 bp of gene/tag sequence plus a primer extension specific to the pLeics74 vector, a pUASTattB vector containing 10xUAS provided by University of Leicester Protein Expression Laboratory (Protex), sequences shown in table 2.4. This method uses phiC31 integration loci to allow directed insertion of constructs in to the genome (Markstein *et al.*, 2008).

Table 2.4: Primer sequences used to amplify *dms1* and mouse *ms1* from pBS for cloning into pUASTattB, containing pUASTattB-specific sequences (red).

Primer name	**Sequence**
dms1 untagged forward (protex)	TCTGCTACCACAGCCGCGGATCCCAACATGACGGACGTATCGCATG
dms1 tagged forward (protex)	TCTGCTACCACAGCCGCGGATCCCAACATGTACCCTTATGATGTG
dms1 reverse (protex)	TCCTTCACAAAGATCCTCTAGATTAAGCGGGCGTCGACTCTG
mouse *ms1* untagged forward (protex)	TCTGCTACCACAGCCGCGGATCCCAACATGGCTCCAGGAGAAAGGGA
mouse *ms1* tagged forward (protex)	TCTGCTACCACAGCCGCGGATCCCAACATGGAGCAGAAGCTCATCAGC
mouse *ms1* reverse (protex)	TCCTTCACAAAGATCCTCTAGATTACTCAAGGAGAGTAATCA

Amplification of the constructs using these primers and insertion into the pUASTattB vector was carried out by Protex according to their own protocols.

2.7.7 Injection into fly embryos

Injection of the pUASTattB vector into fly embryos was carried out by the University of Cambridge Fly Facility Embryo Injection Service according to their own protocols. Vectors were injected into the following fly line for integration into a chosen site on the third chromosome:

y w P{y+t7.7=nosphiC31\int.NLS}X#12;;P{y+t7.7=CaryP}attP2

For further details of the University of Cambridge Fly Facility see:

http://www.gen.cam.ac.uk/department/flylab/flylabwelcome.html#embryoinjserv

2.8 Confirmation of expression of mouse *ms1*

RNA was extracted and cDNA synthesised as in section 2.6 from flies expressing mouse *ms1* under the control of a *mef2*-GAL4 driver. PCR was carried out as in section 2.6.3 to amplify a section of mouse *ms1* using cDNA as template.

Table 2.5: primer sequences for amplification of mouse *ms1* from transgenic *Drosophila* cDNA.

Primer name	**Sequence**
Mouse PCR_forward	GATATGGAGGAGAGGCCTGA
Mouse PCR_reverse	CTTGGCTGTATTTCCGATGG

2.9 Real-Time Quantitative PCR (qPCR)

An absolute quantification method was used here and an exogenous spike-in (*Aequorin*) was used for normalisation of results.

2.9.1 Synthesis of Spike-in RNA

The gene *Aequorin* was chosen as a spike-in gene as no known homologue is present in the *Drosophila* genome. The gene was amplified from a plasmid carrying the cDNA sequence of *Aequorin* using a forward primer containing a T3 RNA polymerase binding site and reverse primer containing a polyA tail, as shown in table 2.6. The PCR reaction mix and program used were as in section 2.6.3.

Table 2.6: Primer sequences for synthesis of spike-in RNA template. Forward primer contains T3 RNA polymerase binding site shown in green. Reverse primer contains polyA tail shown in orange.

Primer name	**Sequence**
Spike-in synthesis forward	ATTAACCCTCACTAAAGGGAACCCAAGATG GATTGGACGACACA
Spike-in synthesis reverse	TTTTTTTTTTTTTTTTTTTTTTCGCAAGCAGGA TCCATGGTGTA

The PCR product was run on a 1% agarose gel and extracted using Zymoclean Gel DNA Recovery Kit (Zymo Research).

RNA was synthesised using the Promega T3 RNA synthesis protocol. The following reaction mixture was made up:

20µl T3 transcription buffer 5x
10µl DTT 100mM
100U Recombinant RNasin ribonuclease inhibitor
20µl rNTP mix*
2µl DNA template (2-5µg/µl)
40U Phage RNA polymerase
DEPC H_2O to 100µl

*(2.5 mM rATP, 2.5mM rGTP, 2.5mM rUTP, 2.5mM rCTP in DEPC H_2O)

The reaction mixture was incubated for 2 hours at 37°C.

2.9.2 Serial dilution of standards

PCR was carried out using wild-type CantonS *Drosophila* cDNA (synthesised as in section 2.6) as template for the *Drosophila* standards, and plasmid containing *Aequorin* as template for the spike-in standards. The PCR reaction mix and program were the same as those in section 2.6.3. Primers used are listed below:

- *Aequorin* standard F: ACCCAAGATGGATTGGACGACACA
- *Aequorin* standard R:TTCGCAAGCAGGATCCATGGTGTA
- *Dms1* set3_forward and set5_reverse (see table 2.2)
- Standards for *srf, jra*, *actin*88F and *actin*79B genes were amplified using the qPCR primers listed in table 2.7.

The PCR product was run on a 1% agarose gel and extracted using Zymoclean Gel DNA Recovery Kit. The products were then serial diluted to give the following concentrations:

300,000 copies of product in 5µl
30,000 copies of product in 5µl
3,000 copies of product in 5µl
300 copies of product in 5µl
30 copies of product in 5µl

Calculations were made using an Excel spreadsheet designed by Eran Tauber.

2.9.3 Preparation of RNA

RNA was extracted from whole fly bodies using the Trizol extraction protocol described in Section 2.6. For each genotype 3 separate RNA extractions were carried out to give independent biological replicates. RNA was quantified using the

Nanodrop 8000 spectrophotometer. 22pg of spike-in RNA was added to 50µg of RNA.

10 µg RNA was diluted in 50µl H_2O and treated with Turbo DNA-free kit (Ambion). 5 µl DNase buffer and 1µl DNase were added and the mixture incubated for 1 hour at 37ºC. Another 1µl of DNase was added and a further incubation of 1 hour at 37ºC was carried out. Inactivation was carried out by adding 0.1x volume DNase Inactivation reagent and incubating for 5 minutes at room temperature, mixing occasionally. Reaction was then centrifuged for 1.5 minutes at 10,000g and RNA transferred to a new tube.

2.9.4 cDNA synthesis for qPCR

1µg DNase-treated RNA was used. cDNA was synthesised using Quantitect Reverse Transcription kit (Qiagen) with gDNA removal step included. RNA and gDNA Wipeout buffer were incubated for 2 minutes at 42ºC before addition of the following mix:

1 µl Quantiscript Reverse Transcriptase
4µl Quantiscript RT Buffer, 5x
1 µl RT Primer Mix

This was incubated for 15 minutes at 42ºC then 3 minutes at 95ºC.

2.9.5 qPCR protocol

The kit used for qPCR was QuantiTect SYBR Green PCR +UNG Kit (Qiagen). The following reaction mix was used:

12.5µl 2x QuantiTect SYBR Green PCR Master Mix
1.5µl each primer
0.25µl UNG
5 µl cDNA (diluted 1 in 20)
10.75µl RNase-free H_2O

Programme: 50°C, 2 min
95°C, 15 sec
94°C, 15 sec
60°C, 30 sec } x 40
72°C, 30 sec

Primers for each gene were designed to give products between 100 and 150bp and flank an intron to allow for identification of genomic contamination. For each genotype 3 technical replicates and 3 biological replicates were carried out. The reaction was carried out once with *dms1* primers and once with *Aequorin* primers to control for differences in the efficiency of cDNA synthesis.

Table 2.7: Primer sequences for use in qPCR.

Primer name	**Sequence**
Aequorin forward	TAACCTTGGAGCAACACCTGAGCA
Aequorin reverse	ATGCAGGCCAATCAGTTTCCACAC
dms1 forward	GACGTATCGCATGAACTGGG
dms1 reverse	AAAATGGATTCAGCAGCTGC
srf forward	GCGTCGCTACACGACCTTCT
srf reverse	CGCGTGGCAAATGTGTACAC
jra forward	GTGGCAGTGCAGAAAGCAGA
jra reverse	CTTAAGTTCGCAGCAGCGGA
whd forward	GGCCAATGTGATTTCCCTGC
whd reverse	GAAGGTACGTTCATACTGCC
*actin*88F forward	GTGGACATCCGCAAGGATCT
*actin*88F reverse	GATGGGGCCAGGGCAGTGAT
*actin*79B forward	CCAACAATGTGCTGTCTGGC
*actin*79B reverse	GATCCAGACGGAGTACTTGC

2.10 Longevity of *Drosophila*

The appropriate crosses were made and vials of 20 adult progeny per genotype were set up. Males and females were not separated so as to simulate normal life conditions, and 10 of each were placed in each vial. Flies were left at 18°C and moved to new vials every few days, when necessary. Live adult flies were counted and recorded on a weekly basis.

2.11 Longevity under hypoxic conditions

A hypoxia box was built using a 9L plastic box and rubber tube with holes in. CO_2 was pumped through the tube which diffused into the box, lowering the O_2 concentration to around 1%. The gas leaves the box through the gap between the box and the lid, as this is not airtight. The O_2 concentration remains constant as long as there is a constant supply of CO_2. The box was tested using CantonS flies to determine their response to different length of exposure to hypoxic conditions and their recovery rates. A diagram and photograph of the hypoxia box are shown in figures 2.5 and 2.6.

Vials of 20 flies per genotype were set up as in section 2.10 and subjected to 1 hour of hypoxia every day beginning at 1pm (ZT5). Live adult flies were counted and recorded daily.

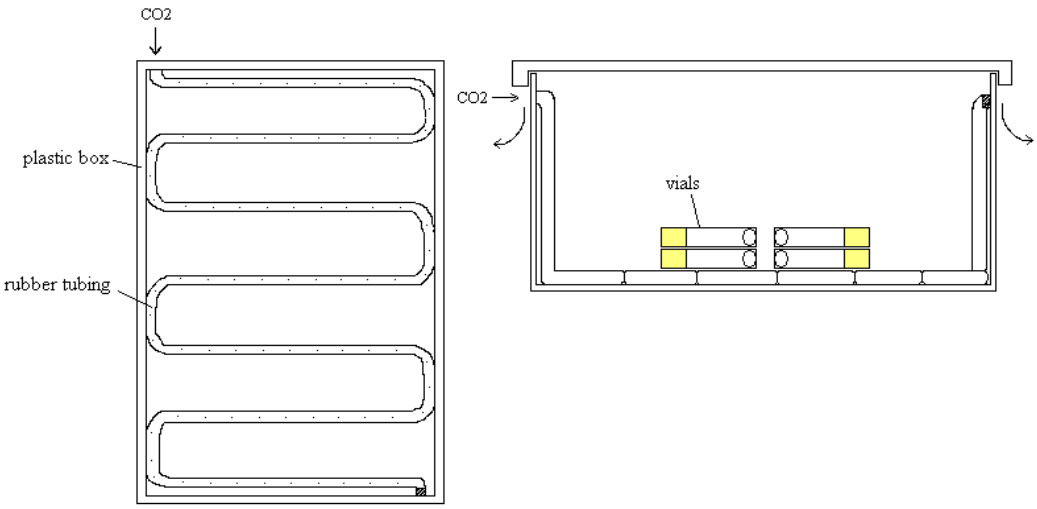

Figure 2.5: diagram of hypoxia box, showing movement of CO2 through the box via plastic tubing, and out under the lid.

Figure 2.6: Photo of hypoxia box showing holed plastic tubing through which CO_2 enters the box.

2.12 Climbing assay

Climbing experiments use the negative geotaxis response of *Drosophila*, as they will instinctively climb upwards when knocked down. Protocol was based on that

of Pendleton *et al*. (Pendleton *et al.*, 2002). Plastic vials were prepared by taping two vials with the open ends together to give the flies space to climb. A line was drawn at 8cm from the bottom of one vial.

Flies were crossed using the appropriate driver and UAS lines and progeny aged to 1 week before being collected. 10 flies were used per replicate. Flies were knocked to the bottom of the vial and given 10 seconds to climb. The number of flies that crossed the 8cm line in this time was recorded. Each group of flies was allowed to climb 10 times with a 1 minute rest between each climb and an average was taken of the results.

2.13 Activity assay

Glass tubes (5mm x 100mm) containing approximately 15mm sugar food (4.63g sucrose, 4.63g live yeast, 0.71g agar and 0.2g Nipagin in 100ml water) were prepared, allowed to dry for around 5 hours, and a rubber bung placed on the end of the tube containing food. 20 male flies of each genotype were used. Flies were anesthetised using CO_2 and a single fly placed in each tube. The end was then sealed using cotton wool. Tubes were placed in a *Drosophila* activity monitor (DAM2, Trikinetics) with the capacity for 32 tubes in each monitor, shown in figure 2.7. Tubes were secured using elastic bands and then placed into incubators set at 25°C and 12:12 LD. Monitors emit an infra-red beam that crosses the tube in each channel and records each time a fly crosses this beam. Each monitor was connected to a computer running Trikinetics DAM software (v3.03) which records each crossing. Flies were subjected either to 12:12 LD for up to 5 days, then constant darkness for 1 week, or were left in 12:12 LD conditions for the whole experiment.

Data from activity experiments was analysed using Trikinetics DAM FileScan software which groups the data into half-hour bins, and this was transferred to Microsoft Excel using the BeFly! (v7.23) macro (Edward Green, unpublished).

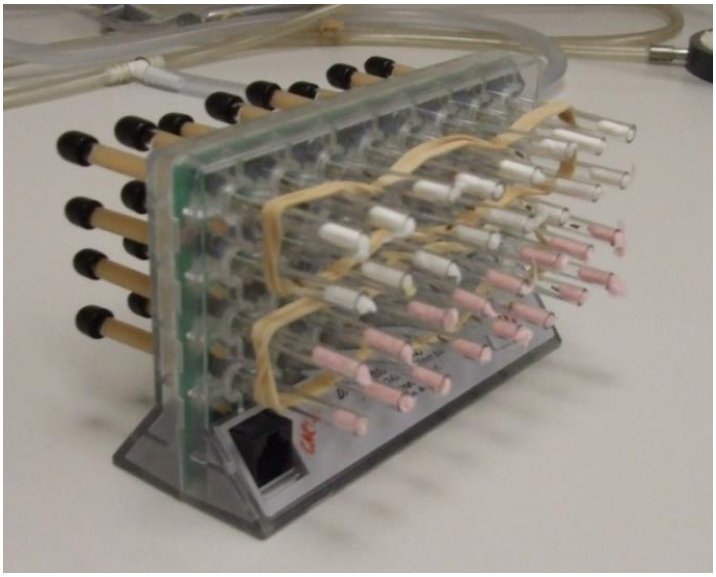

Figure 2.7: *Drosophila* activity monitor (DAM) from Trikinetics (www.trikinetics.com). Each tube contains a single male fly and sugar food.

2.14 Dissection using semi-intact preparation and heart viewing

A semi-intact preparation was used to visualise the beating heart of the Drosophila (Vogler & Ocorr, 2009). This protocol requires the animal to be submerged in oxygenated artificial haemolymph (AHL, oxygenated for 15 minutes). The flies are then affixed to a surface using petroleum jelly, and head and legs are removed. The ventral side of the abdomen is then cut open and the abdominal organs removed to reveal the beating heart which is attached to the dorsal cuticle. The heart will continue to beat fairly rhythmically for a few hours as long as fresh oxygenated AHL is added to the preparation every hour. This preparation allows

visualisation of the beating heart. The dissection protocol is demonstrated in figure 2.8.

Dissections for video purposes were carried out on a glass slide with a well to hold AHL.

AHL was made up using 108 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 8 mM $MgCl_2$, 1 mM NaH_2PO_4, 4 mM $NaHCO_3$, 5 mM HEPES at pH 7.1. Sucrose and trehalose solutions were made up separately and added just before use to 10 mM and 5 mM respectively.

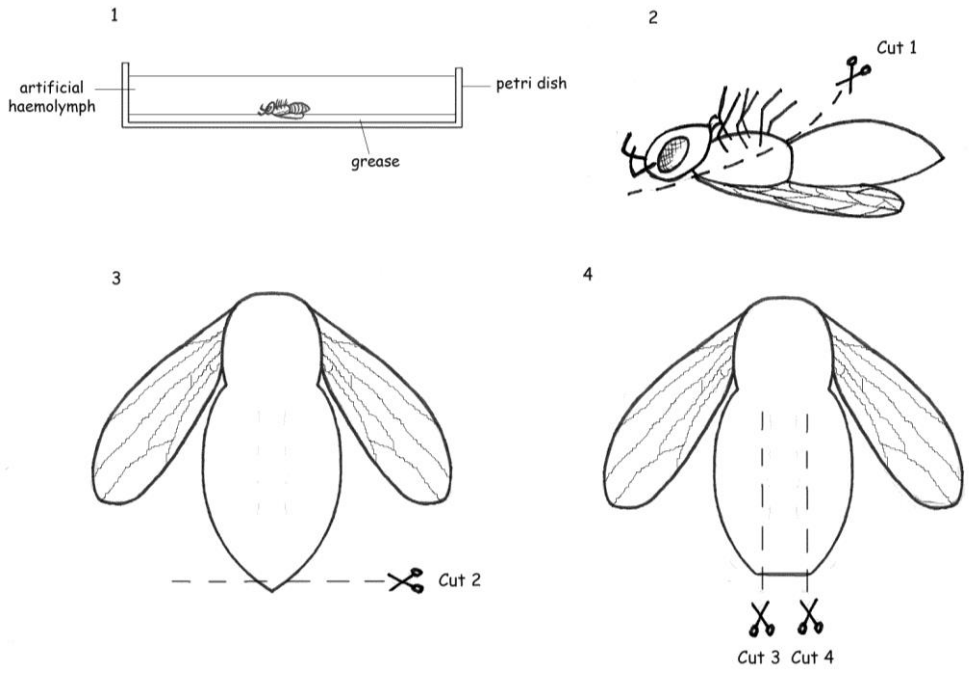

Figure 2.8: protocol for semi-intact preparation for visualisation of the Drosophila heart (Vogler & Ocorr, 2009).

2.15 Videos of *Drosophila* heartbeat

Dissections were carried out as in section 2.14 in custom-made glass slides with a dissection well created from a rubber O-ring. This allowed the preparations to be submerged. Slides were placed under high magnification using an Olympus BX61 upright with ScanView scanning platform (Applied Spectral imaging). 30 second videos were taken of beating hearts using the software Volocity (PerkinElmer).

2.16 Heart function under hypoxic stress (subjective analysis)

Semi intact preparations were carried out as described. Hearts were checked to ensure rhythmic beating, then oxygenated AHL was removed and replaced with AHL that had not been oxygenated. Hearts were observed every 15 minutes and characterised using a "scale of arrhythmicity" for up to 90 minutes.

Scale of arrhythmicity:
0 – totally rhythmic
1 – occasional ectopic beat
2 – mostly rhythmic with bouts of arrhythmia
3 – mostly arrhythmic with bouts of rhythmic beating
4 – totally arrhythmic
5 – stopped/ no definite beats

This was carried out with flies of ages 1 week to 4 weeks. No readings were carried out after 105 minutes as hearts appear to be constantly arrhythmic by this time.

2.17 Eclosion assay

Crosses were carried out between the appropriate driver and UAS lines. Pupae were collected at the late pupal stage and transferred to fresh vials. 20 pupae were placed in each vial per replicate. The number of adult flies that eclosed from the pupae was

recorded. 4 replicates were carried out and an average of eclosed flies per vial was calculated for analysis.

2.18 Dissection of *Drosophila* for visualisation of thoracic muscle

To allow visualisation of thoracic muscle, flies were anaesthetised using CO_2 and affixed to a petri dish using petroleum jelly. Dissections were carried out in AHL, as in section 2.14. Head and abdomen were removed using a scalpel blade. The thorax was cut down the centre starting between the right and left sets of legs, cutting from ventral to dorsal sides so each section is attached to three legs and one wing.

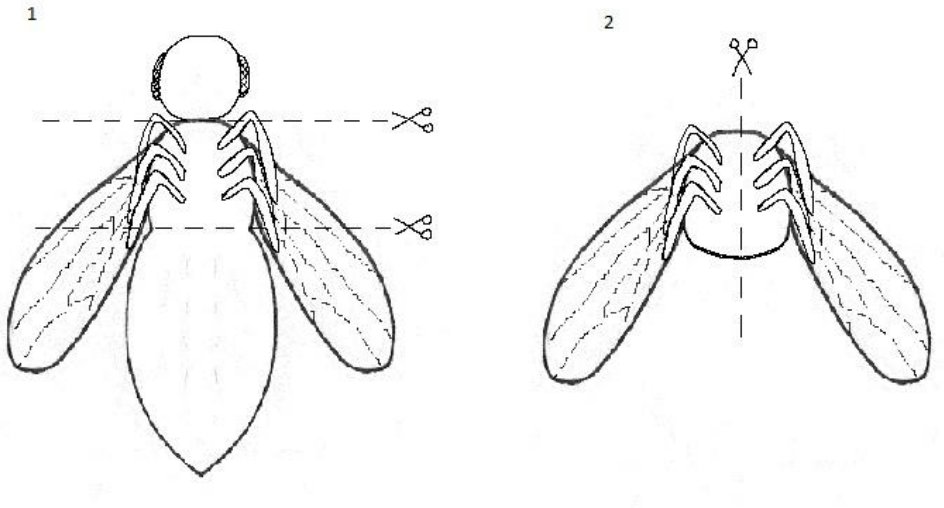

Figure 2.9: protocol for dissection to visualise thoracic musculature of *Drosophila*. 1: Cuts are made to remove head and abdomen. 2: Thorax is cut down the centre to reveal internal musculature.

2.19 Fluorescence microscopy

Staining of hearts was carried out based on the protocol by Alayari *et al.* (2009). *Drosophila* were dissected as in section 2.14 and hearts checked for rhythmicity.

The heartbeat was stopped by addition of relaxing buffer (AHL containing 10 mM EGTA). Hearts were fixed in 4% paraformaldhyde in 1x PBS and subsequently washed three times in PBSTx (1x PBS containing 0.1% Triton-X-100) for 10 minutes each time with gentle shaking. The edges of the cuticle were trimmed back and the abdomen separated from the thorax using scissors, and hearts were placed in 100 µl PBSTx containing Alexa594-phalloidin (Invitrogen, diluted 1:1000). This was incubated for 1 hour at room temperature with gentle shaking, then washed three times in PBSTx as before. A final 10 minute wash with PBS was carried out to remove Triton-X-100.

Fluorescent staining of *Drosophila* thoracic muscle was carried out using the dissection protocol described in section 2.18, and fixing and staining was carried out as with the heart staining.

Samples were mounted onto glass slides using mounting medium containing 3% propyl gallate. Two 18 x 18 mm coverslips were placed onto a glass slide 10-15 mm apart adhered by mounting medium. A drop of mounting medium was placed onto a third slide and samples placed onto the drop with hearts facing down. The coverslip was inverted and placed on the first two coverslips so that a bridge was formed. Samples could then be visualised using confocal microscopy (Olympus FV1000 confocal laser scanning microscope).

2.20 Exercising Drosophila

In order to exercise *Drosophila* the negative geotaxic response has been used to repeatedly induce climbing. The FlyGym was designed based on the Power Tower by Tinkerhess *et al*. which uses this principle to repeatedly knock flies to the bottom of vials to induce negative geotaxis (Tinkerhess *et al.*, 2012). The FlyGym

does this but on a much smaller scale due to availability of equipment. A rotor wheel was used to provide movement to the system, and a plastic flipper attached to the wheel pushes down onto a lever, the other end of which the vials of flies are attached to. As the wheel moves round the vials move upwards and are then released and hit the bench, knocking the flies to the bottom of their vials. This mechanism is shown in figure 2.10.

Exercise treatment was carried out on three replicates of groups of 30 wild-type Canton S flies. Flies were exercised 2 hours every day for 2 weeks at the same time every day (during the morning peak in activity).

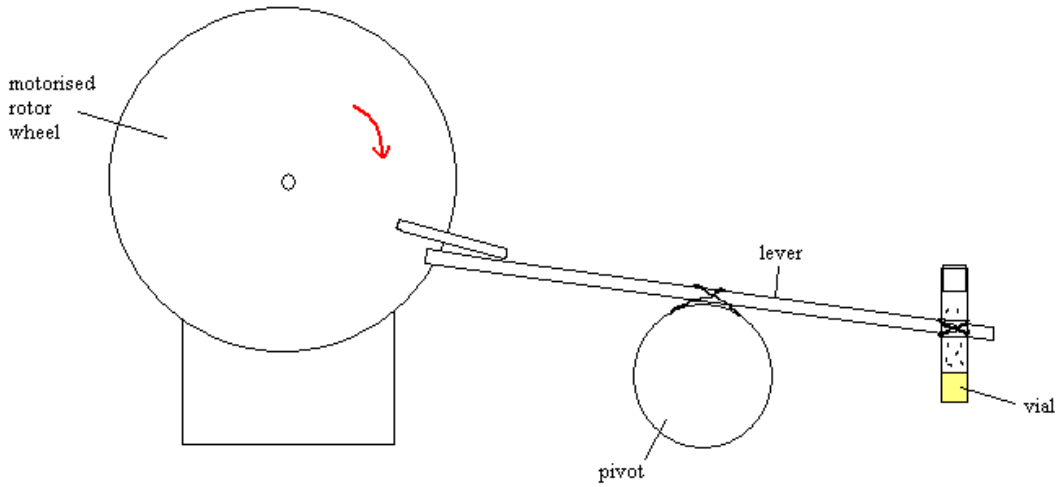

Figure 2.10: Diagram of the FlyGym showing how the motion of the rotor wheel moves the lever to lift vials of flies and then drop them onto the benchtop. Flies are then knocked to the bottom of the vials and are compelled to climb upwards.

Chapter 3: *Myocyte stress 1*, genes and pathways

3.1 Introduction

Myocyte stress 1 (*ms1*) was discovered in 2002 by two separate groups (Mahadeva *et al.*, 2002; Arai *et al.*, 2002). Mahadeva *et al*. found *ms1* to be an important regulator of LVH in a rat model and discovered a potential homologue in *Drosophila melanogaster*, while Arai *et al.* discovered the actin-binding properties of the protein and found *ms1* to be involved in regulation of the SRF pathway in mice. Since this discovery a number of studies have focused on *ms1* in mammalian models, primarily mice and rats, but also pigs (Peng *et al.*, 2008), and some work has been done looking at expression levels in human skeletal muscle (Lamon *et al.*, 2009; MacNeil *et al.*, 2010). However, the *Drosophila* homologue has not been examined, therefore the present project was designed to characterise the gene itself before further work was done with regards to manipulation of *Drosophila ms1* (*dms1*) and phenotypic effects. It was necessary to confirm *dms1* expression in the adult fly, and to check that information from Flybase (Flybase.org) was correct regarding exon/intron boundaries and presence of splice variants.

The functional pathways of *ms1* are of interest, as little is known about these processes in *Drosophila* or mammals, therefore part of this study was designed to look at potential upstream and downstream genes, the effect of upstream genes on *ms1* expression and the effect of changes in *ms1* levels on downstream gene expression. Candidate genes were chosen either from previous work done in mammals, or prediction of association with *ms1*. Downstream genes were chosen

from various different sources, as studies have been carried out that have identified many potential MS1-dependent genes. These are often MRTF-SRF dependent genes, as MS1 is involved in this pathway. Acute forced overexpression of MS1 in cultured rat myocytes found several genes that were upregulated (Koekemoer *et al.*, 2009). Two of these were chosen for this investigation as they have potential homologues in *Drosophila*. These were *jun-B* (*jra* in *Drosophila*) and α-actin (*actin88F* and *actin79B* were chosen to represent actin genes in *Drosophila*). Another gene chosen was *SRF*, known as *blistered* in *Drosophila*, as it is has been known for some time that *ms1* is able to regulate *SRF* expression (Arai *et al.*, 2002). The final gene chosen for this study was CPT1 (*withered* in *Drosophila*), as CPT1-β has been shown to be upregulated along with *ms1* after endurance exercise in human skeletal muscle (Wallace *et al.*, 2011). CPT1-β is also known to affect hypertrophy in mammalian cardiac tissue, increasing the hypertrophic response of the heart under pressure overload (He *et al.*, 2012).

Upstream genes were more difficult to select, as many of the genes of interest were important for a wide range of pathways, and changes in expression would lead to lethality. The candidate gene chosen was a *Drosophila* homologue of calcineurin, designated CG4209 (Flybase gene no. FBgn0010014). Overexpression of *ms1* in a mouse model does not have any significant effect on cardiac phenotype unless under stress conditions or if there is an increase in calcineurin expression (Kuwahara *et al.*, 2007). This could indicate that *ms1* is in some way linked to the calcineurin signalling pathway.

3.2 Results

3.2.1 Gene structure and splice variants

Database information shows *dms1* to be a 5, 467bp gene with 3 introns. The reported gene name is CG3630, showing that the functional significance of this gene is not currently known. Overlapping primers were designed along the length of the gene to confirm the introns and exons (figure 2.3).

When primers were used to amplify the gene from upstream of intron 1 and downstream of intron 2 (primer set9 shown in figure 2.3), two distinct products were found (figure 3.1). These products were extracted from the gel and sequenced. This revealed that the smaller product is missing exon 2 and could indicate the presence of a splice variant. This corresponds with the PCR products in figure 3.1 as the smaller can be seen to be approximately 150bp shorter than the larger, the size of this exon being 166bp in reality.

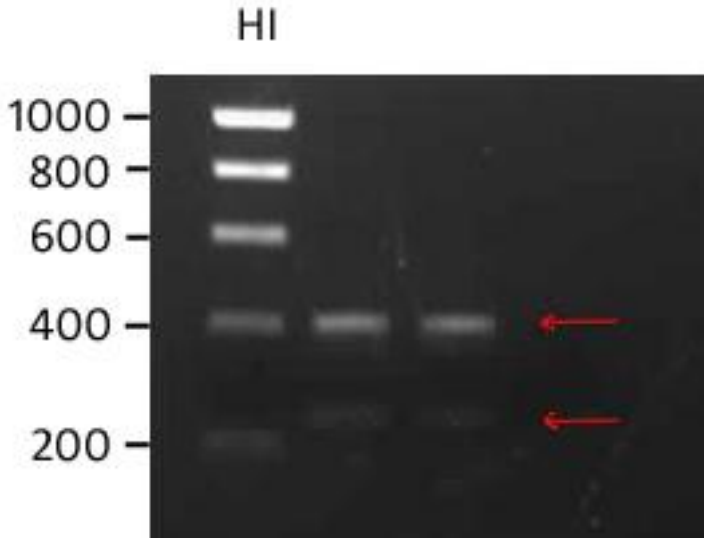

Figure 3.1: Gel showing two products (red arrows) of PCR using primer set 9 (see figure 2.3). Two replicates are shown in separate lanes. Marker DNA used is 100bp hyperladder (HI).

Data from Flybase shows that this splice variant has now been identified, designated CG3630-RB (figure 3.2). Exon 2 is spliced out in this variant. The PCR results in figure 3.1 show that this variant is expressed at a much lower level than the full length *dms1*, possibly this is a tissue-specific variant. Exon 2 contains a small amount of protein coding sequence, 45bp coding for 15 amino acids. Alignment of *dms1* with mammalian versions of *ms1* show that these 15 amino acids lie just upstream of the actin binding domain, the highly conserved region of *ms1*. This suggests that this variant still has actin-binding capabilities.

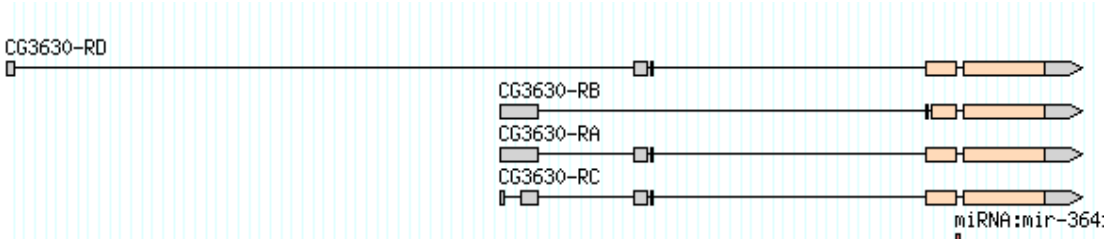

Figure 3.2: Flybase GBrowse data showing splice variants of *dms1* (CG3630) also known in Flybase as FBgn0023540. Splice variant CG3630-RB is missing exon 2, and is the variant identified in this section.

3.2.2 The effect of changes in expression of *dms1* on potential downstream genes

Several genes were tested for an effect on their expression of different levels of *dms1*. The expression levels of the genes *SRF, Jra, whd, actin88F* and *actin79B* were determined using quantitative real-time PCR (qPCR) with knockdown and overexpression of *dms1*, and also with expression of mouse *ms1*. All UAS-controlled constructs were driven using the 2nd chromosome mef2-GAL4 driver.

3.2.2.1 Confirmation of knockdown by qPCR

To confirm that *dms1* was being significantly knocked down qPCR was used to measure levels of *dms1* in whole flies when RNAi of *dms1* was driven in all muscles. Two different *mef2*-GAL4 drivers were used, on the second and third chromosomes, in order to determine which, if either, was the stronger. The crosses made were as follows:

w; *mef2*-GAL4; + x w; UAS-RNAi(KK); +
w; +; *mef2*-GAL4 x w; UAS-RNAi(KK); +
w; *mef2*-GAL4; + x w; +; +
w; +; *mef2*-GAL4 x w; +; +
w; +; + x w; UAS-RNAi(KK); +

Adult flies were collected 1 week after eclosion, whole body cDNA was prepared and qPCR carried out using *dms1* primers as described in section 2.9.5. Figures 3.3 and 3.4 show the results of qPCR measuring levels of *dms1* with knockdown using drivers on the 2nd and 3rd chromosome respectively.

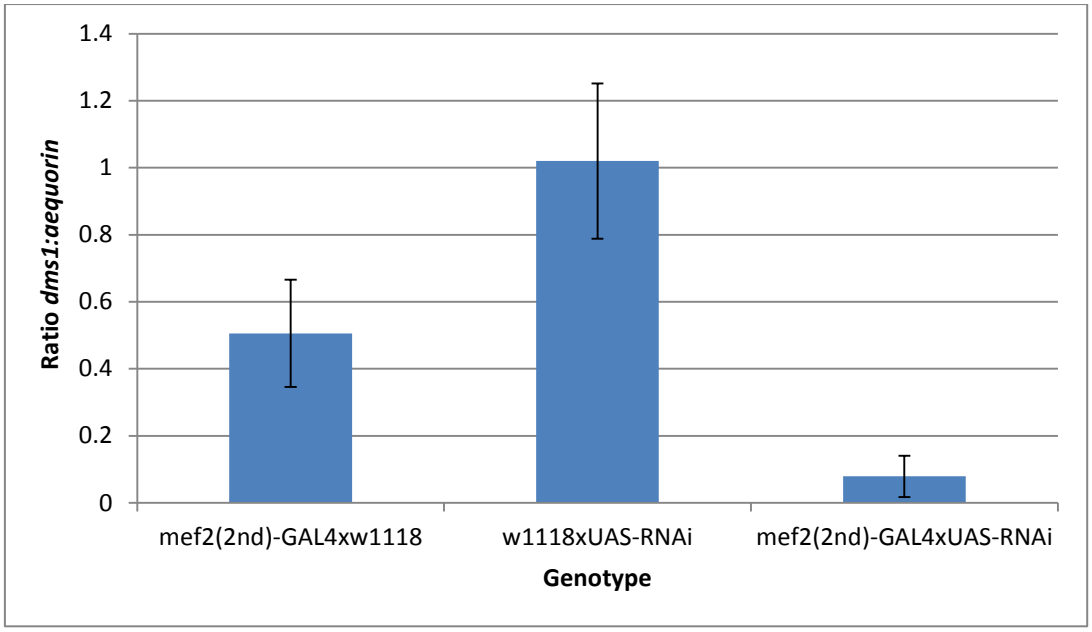

Figure 3.3: levels of ***dms1*** as determined by qPCR with knockdown of *dms1* by RNAi compared with controls ($p<0.005$ using one-way ANOVA, experimental line is significantly different from both controls using Tukey's HSD Post Hoc test at $p=0.05$ level). RNAi is driven by mef2-GAL4 driver positioned on the 2nd chromosome.

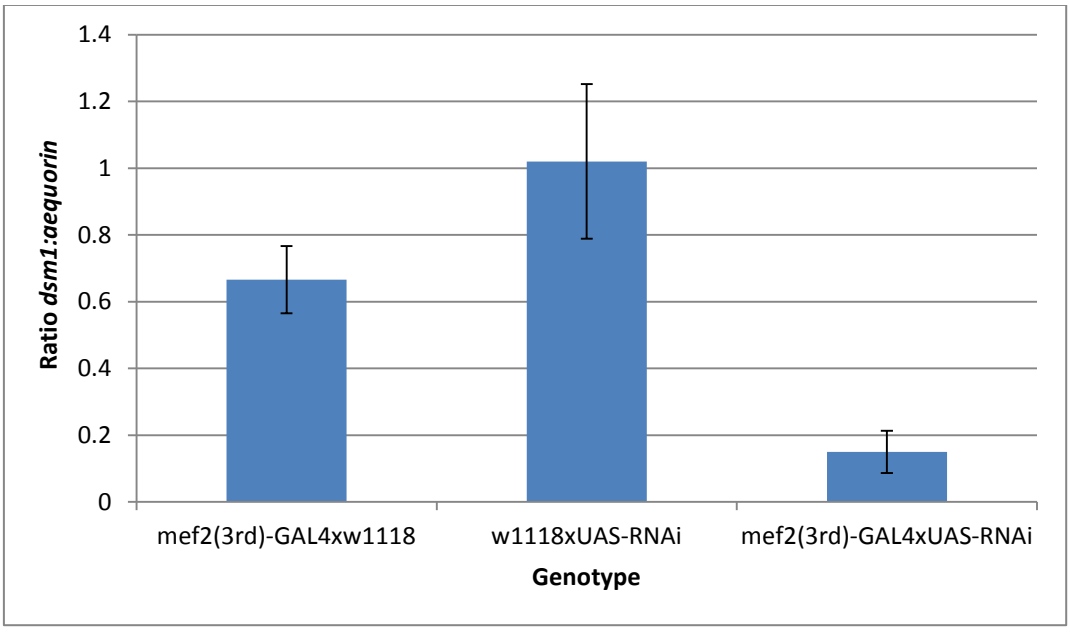

Figure 3.4: levels of ***dms1*** as determined by real-time qPCR ($p<0.001$ using one-way ANOVA, experimental line is significantly different from both controls using Tukey's HSD Post Hoc test at $p=0.05$ level). RNAi is driven by mef2-GAL4 driver positioned on the 3rd chromosome.

Both figures 3.3 and 3.4 show a significant reduction in *dms1* levels with knockdown using UAS-RNAi under the control of both *mef2* drivers. The levels of knockdown have been determined by comparing the value from experimental flies with that of the control with the lowest level of *dms1*. This gives the reduction of *dms1* that can be attributed to *mef2*-GAL4 driven RNAi expression. With *mef2*-GAL4 on the second chromosome knockdown is 84.4%, as seen in figure 3.3. With *mef2*-GAL4 on the third knockdown is 78.6%, shown in figure 3.4. These results show a greater knockdown using the driver on the 2nd chromosome, so this has been used for many of the subsequent experiments in preference to the 3rd chromosome driver.

Both figures show a large difference in *dms1* expression between the driver control and the UAS-RNAi control. This could be explained by the presence of GAL4 in the driver control that has nothing to bind to. GAL4 can be toxic to the animal if it is allowed to build up, and with no UAS to bind to in the driver control GAL4 levels will rise in all muscle cells and may have an effect on gene expression, including *dms1*.

3.2.2.2 Confirmation of overexpression of UAS-*dms1*

To confirm that *dms1* was being overexpressed using the UAS-*dms1* construct under the control of a mef2 driver qPCR was used to determine levels of *dms1* in overexpression flies compared to controls. The following crosses were carried out:

w; *mef2*-GAL4; + x w; +; UAS-*dms1*
w; +; + x w; +; UAS-*dms1*
w; *mef2*-GAL4; + x w; +; +

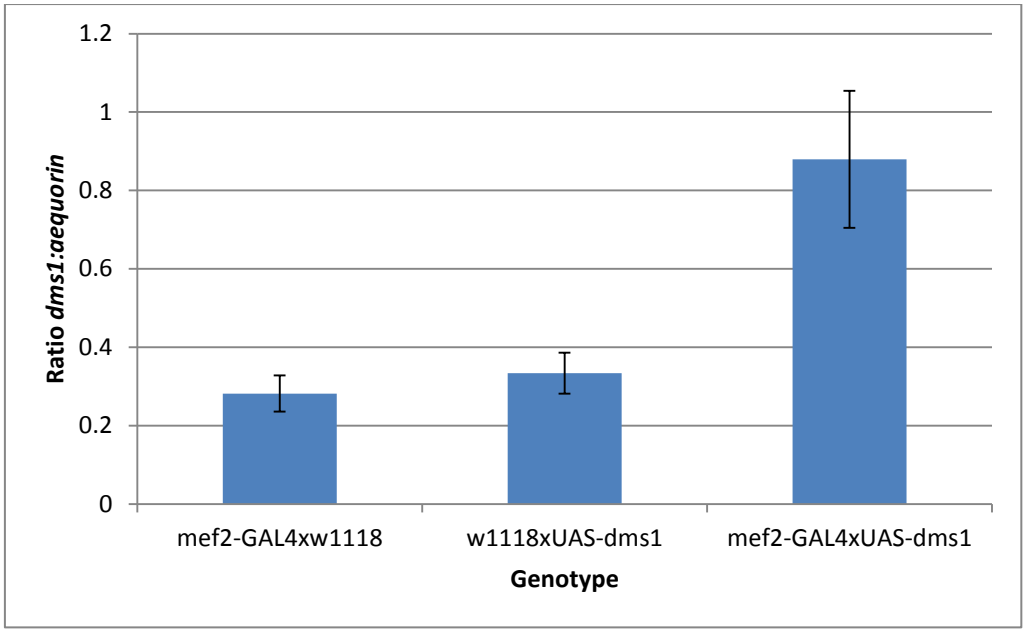

Figure 3.5: levels of ***dms1*** as determined by qPCR with muscle-specific overexpression of *dms1* by RNAi compared with controls ($p<0.001$ using one-way ANOVA, experimental line is significantly different from both controls using Tukey's HSD Post Hoc test at $p=0.01$ level). UAS-*dms1* is driven by mef2-GAL4 driver positioned on the 2nd chromosome.

Figure 3.5 shows the level of *dms1* expression is significantly higher than in either of the controls, which are not significantly different from each other, showing that the UAS-*dms1* construct is functioning as expected and is expressed at a high level under the control of the second chromosome *mef2*-GAL4 driver.

3.2.2.3 Overexpression of mouse *ms1*

A transgene for mouse *ms1* was created with the intention of testing whether the presence of mouse *ms1* has a similar effect to overexpression of *dms1*. If flies expressing mouse *ms1* behave in a similar way to those overexpressing *dms1* it is possible that the two genes have homologous functions, and that they have roles in the same pathway in different organisms. Figure 3.6 shows confirmation of expression of this mouse-*ms1* transgene in *Drosophila* under the control of a *mef2*-

GAL4 driver using mouse sequence-specific primers. No expression is seen in control flies, as expected, due to the absence of this gene within the *Drosophila* genome.

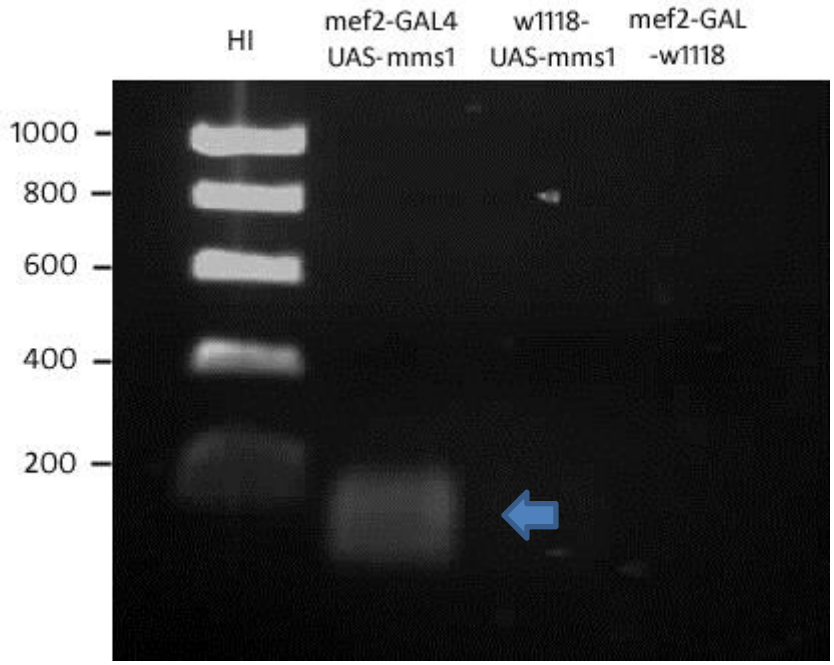

Figure 3.6: gel confirming expression of mouse *ms1* in all muscle types compared with controls indicated with blue arrow. Template DNA used is cDNA from transgenic flies. Marker DNA used is 100bp hyperladder (HI), sizes shown in bp.

3.2.2.4 The effect of changes in *ms1* on serum response factor (*SRF*)

Expression of the serum response factor (*SRF*) homologue *blistered* was tested under knockdown of *dms1* to see if there was any change in *SRF* expression, as this would imply that *SRF* is downstream of *dms1*. Results are shown in figure 3.7. In mammals *ms1* is known to have an integral role in one of the pathways that initiates *SRF* expression under stress conditions, having been shown to be able to activate regulators of *SRF* transcription, specifically MRTFs (Kuwahara *et al.*,

2005), and affect genes downstream of *SRF* (Arai *et al.*, 2002). A knockdown of *ms1* in cultured cardiac and skeletal muscle cells can also reduce *SRF* expression (Kuwahara *et al.*, 2005). If *SRF* expression is affected in a similar way in *Drosophila* then this suggests that mammalian *ms1* and *dms1* have similar function.

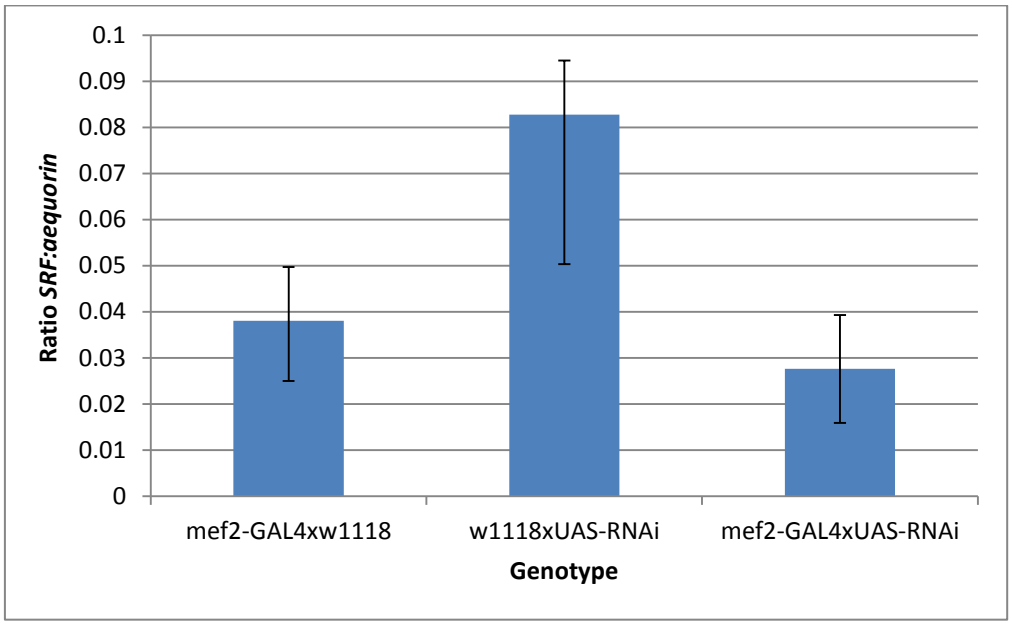

Figure 3.7: levels of expression of ***SRF*** with muscle-specific knockdown of *dms1* compared to controls as determined by qPCR. Differences are not significant ($p>0.05$ using one way ANOVA, difference between experimental and driver control is not shown to be significant using Tukey's HSD post Hoc test at $p=0.05$ level).

As figure 3.7 shows, there is no significant difference between *SRF* expression in the experimental line and that observed in the driver control line. The difference found between the experimental line and UAS control is significant but due to the driver control this cannot be considered a true result. Levels of *SRF* expression appear to follow the levels of *dms1* expression observed in figure 3.3, but further experiments would be required to ascertain whether this indicates any link between the two

genes. As no significance is found here however, no conclusions can be reached using the data in figure 3.7.

SRF expression was also tested with overexpression of *dms1*, with results shown in figure 3.8. As with knockdown of mammalian *ms1*, overexpression of *ms1* is known to affect genes downstream of *SRF*, including *SM22* and α-actin (Arai *et al.*, 2002). These genes have SRF binding sites in their promoters and have been shown to increase in expression after an increase in *ms1*.

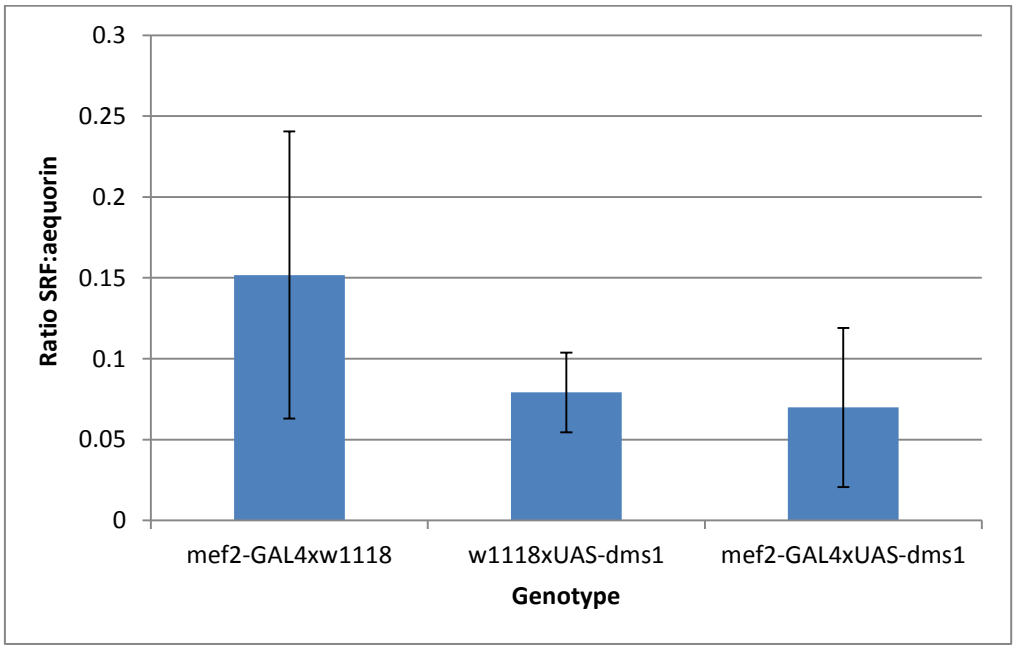

Figure 3.8: levels of expression of ***SRF*** with muscle-specific overexpression of *dms1* compared to controls as determined by qPCR. Differences are not significant (p=0.19 using one-way ANOVA).

Figure 3.8 shows no significant difference in *SRF* expression between experimental and control flies when *dms1* is overexpressed in muscle tissue. This suggests that

SRF is not upregulated when *dms1* is overexpressed, or that differences cannot be detected using this experimental procedure.

Testing the effect of the presence of mouse *ms1* in *Drosophila* allows comparison of the mammalian and *Drosophila* systems, not only the functions of *ms1* itself, but of the pathway in which it has its role. Levels of *SRF* have been tested when mouse *ms1* is present in all muscle cells to give an idea of whether the two homologues of *ms1* have a similar function, and also whether the downstream genes are similar enough to be recognised by different versions of *ms1*. Results are shown in figure 3.9.

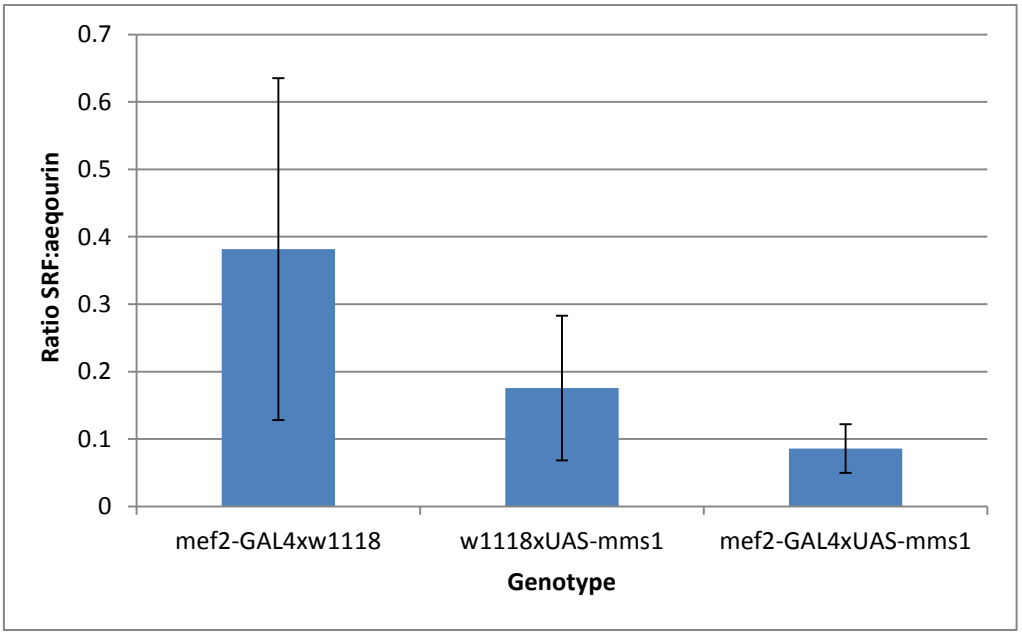

Figure 3.9: levels of expression of ***SRF*** with muscle-specific expression of mouse *ms1* compared to controls as determined by qPCR. Differences are not shown to be significant (p=0.09 using one-way ANOVA).

Figure 3.9 shows that, as with overexpression of *dms1*, overexpression of mouse *ms1* does not appear to result in any significant difference between *dms1* expression in experimental flies and controls.

Investigating the effect of changes in expression of *dms1* on *SRF* levels did not give any significant results for any of the experiments carried out. This is not what was expected as *SRF* is probably the most obvious choice for testing downstream genes of *dms1*, being the most likely gene to be affected by *dms1* levels considering the large amount of evidence from mammalian and cell culture systems found by other groups.

3.2.2.5 The effect of changes in *ms1* on *jun-related antigen* (*Jra*)

Overexpression of *ms1* in cultured cells leads to upregulation of *Jun-B*, a transcription factor and target of *SRF* (Koekemoer *et al.*, 2009). *Drosophila* do not have a gene that is specifically homologous to *Jun-B*, but have a gene that is thought to be the homologue for both *Jun-B* and *c-jun* known as *jun-related antigen* (*Jra*). Levels of *Jra* have been tested with knockdown of *dms1* to determine if this gene is dependent on levels of *dms1*, the results of which are shown in figure 3.10.

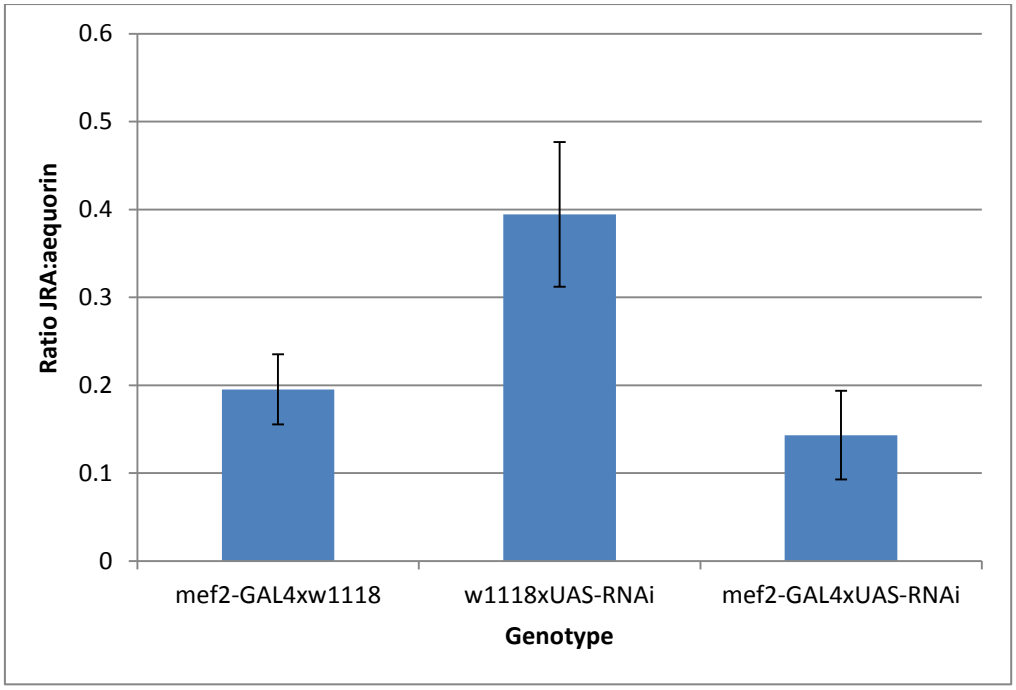

Figure 3.10: levels of expression of ***Jra*** with muscle-specific knockdown of *dms1* compared to controls as determined by qPCR. Differences are not significant ($p>0.05$ using one-way ANOVA, difference between experimental and driver control is not shown to be significant using Tukey's HSD post Hoc test at $p=0.05$ level).

The results in figure 3.10 show a similar results to that of *SRF* levels when *dms1* is knocked down. While levels appear to follow those of *dms1* after knockdown, there is no statistical significance, and so no conclusions can be reached from these results.

Studies have shown that under exercise conditions *ms1* levels increase, along with levels of many potential downstream genes (Lamon *et al.*, 2009; MacNeil *et al.*, 2010). One particular study has found that JUN-B levels increase under exercise conditions along with *ms1*, further confirming the link between these two genes (Wallace *et al.*, 2011). Figure 3.11 shows the results of an experiment that tests

whether overexpression of *dms1* leads to an increase in the potential JUN-B homologue *Jra*.

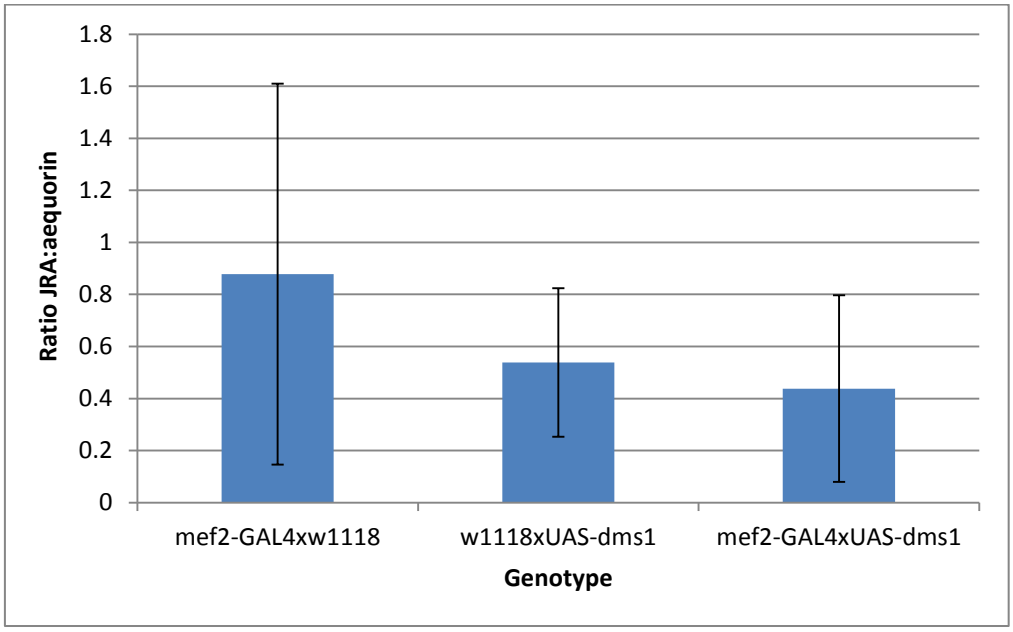

Figure 3.11: levels of expression of ***Jra*** with muscle-specific overexpression of *dms1* compared to controls as determined by qPCR. Differences are not significant (p=0.34 using one-way ANOVA).

Figure 3.11 shows there is no significant difference in levels of *Jra* between flies with knockdown of *dms1* and controls, indicating that there is either no link between these two genes, or that any difference cannot be detected using this experimental procedure.

As with *SRF*, *Jra* levels were tested with expression of mouse *ms1* in all muscle cells in order to indicate if this pathway is the same in *Drosophila* as in mice and if there is any homology between the two (figure 3.12).

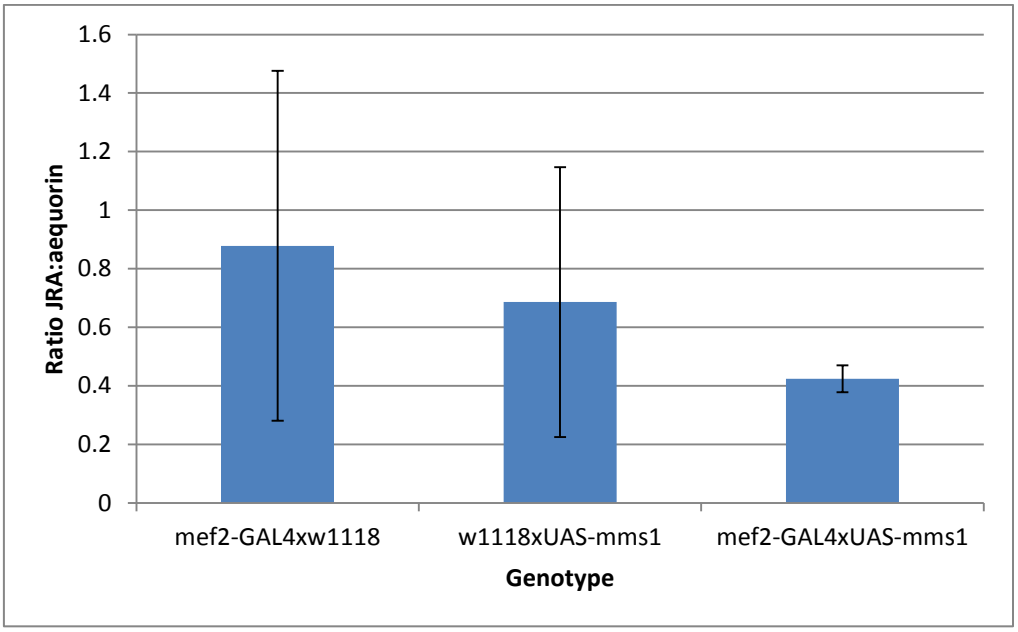

Figure 3.12: levels of expression of ***Jra*** with muscle-specific overexpression of mouse *ms1* compared to controls as determined by qPCR. Differences are not significant (p=0.39 using one-way ANOVA).

Expression of mouse *ms1* does not appear to affect levels of *Jra* in *Drosophila*. As figure 3.12 shows, there is no significant difference in levels between experimental flies and controls. The results of this investigation did not show any significant differences in *Jra* levels under different expression levels of *dms1* or mouse *ms1*, so although JUN-B, the potential homologue of *Jra* is known to increase and with *ms1* (Koekemoer *et al.*, 2009), this experiment suggests that this is not the case for *Jra*.

3.2.2.6 The effect of changes in *ms1* on *withered* (*whd*)

A study by Wallace *et al.* (2011) found that *ms1* expression was upregulated by an increase in peroxisome proliferator-activated receptor gamma co-activator 1-*α* (PGC-1α), and appears to be part of the pathway that regulates expression of

carnitine palmitoyltransferase-1*β* (CPT-1β), a known *SRF* target gene (Wallace *et al.*, 2011; Moore *et al.*, 2001). When *ms1* was reduced PGC1α is unable to increase CPT-1β, implying that in a mammalian system CPT-1β is downstream of *ms1*. *Drosophila* do not have a homologue of PGC-1α, however, they have a carnitine palmitoyltransferase gene known as *withered* (*whd*). While this cannot be activated by exactly the same pathway as in mammals due to the lack of PGC-1α, *whd* may still potentially be downstream of *dms1*. Here, levels of *whd* were tested with knockdown of *dms1* (figure 3.13).

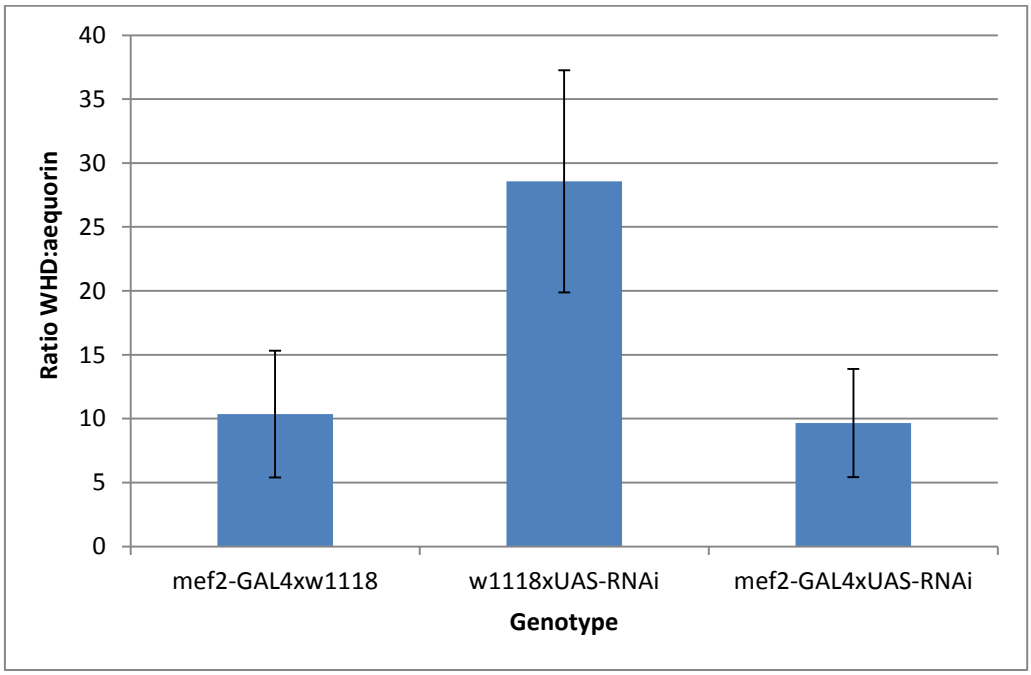

Figure 3.13: levels of expression of ***whd*** with muscle-specific knockdown of *dms1* compared to controls as determined by qPCR. Differences are not significant ($p>0.05$ using one-way ANOVA, difference between experimental and driver control is not shown to be significant using Tukey's HSD post Hoc test at $p=0.05$ level).

As with *SRF* and *Jra*, the expression levels of *whd* shown in figure 3.13 appear to follow the pattern of expression seen with *dms1* in these genotypes, but as no

statistical significance is found between the experimental and control lines, no conclusions can be drawn from these data. The difference between the experimental and UAS control is significant, but levels of *whd* expression in the driver control are very close to that of the experimental.

Overexpression of PGC-1α is also known to increase expression of CPT-1β (Wallace *et al.*, 2011), and this is likely to be through the *ms1* pathway as described earlier due to the observed link between these genes and *ms1*. Overexpression of *dms1* has been tested here to find any effect on *whd* expression and results are shown in figure 3.14.

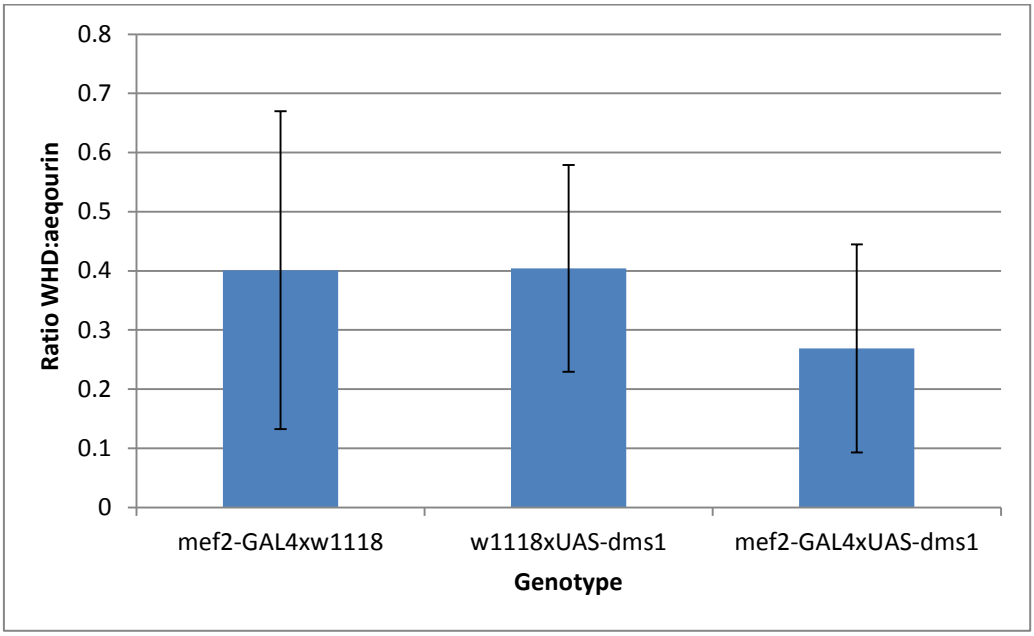

Figure 3.14: levels of expression of ***whd*** with muscle-specific overexpression of *dms1* compared to controls as determined by qPCR. Differences are not significant (p=0.61 using one-way ANOVA).

Levels of *whd* expression do not appear to be significantly different with knockdown of *dms1* when compared to controls, as shown in figure 3.14. This is similar to the results found when testing for *SRF* and *Jra* (figures 3.7 and 3.10) and suggests no link between *dms1* and *whd* expression.

The effect of expression of mouse *ms1* on *whd* has also been tested to investigate the homology between the two pathways. However, as results in figure 3.15 show, no significant difference was found between the experimental line and controls, suggesting that mouse *ms1* does not affect this pathway.

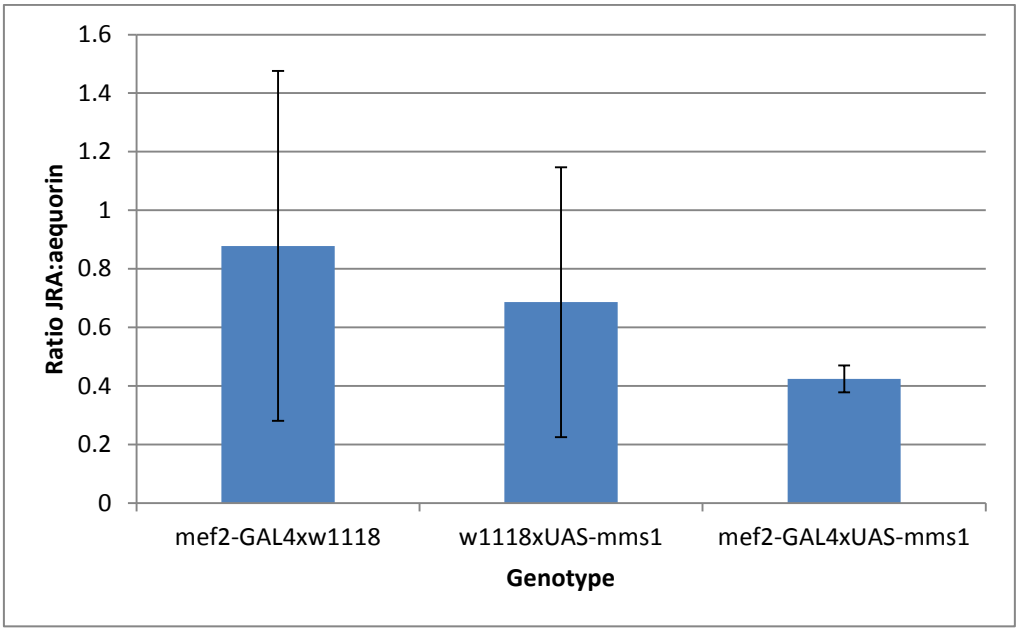

Figure 3.15: levels of expression of ***whd*** with muscle-specific expression of mouse *ms1* compared to controls as determined by qPCR. Differences are not significant (p=0.21 using one-way ANOVA).

Investigation into *whd* levels under changes in *dms1* expression revealed no significant results. Similarly to *SRF* and *Jra* the levels of *whd* appear to follow the

levels of *dms1* in each sample when *dms1* expression is knocked down to some extent, although no difference at all is seen between the experimental sample and driver control, as shown by figure 3.13. Therefore these results cannot be considered significant.

3.2.2.7 The effect of changes in *ms1* on *actin88F*

The pathway in which *ms1* plays a role results in an initiation of hypertrophic growth (Kuwahara *et al.*, 2007). This implies that genes required for cell enlargement would be upregulated as a result of this pathway, and therefore as a result of increase in *ms1*. Similarly, when skeletal muscle atrophy is observed, levels of *ms1* are lower than previously seen (Lamon *et al.*, 2009), indicating a decrease in genes required for an increase in cell size. Actin is an obvious candidate for a target gene of the *ms1* pathway as all cells require actin as part of their cytoskeleton, and actin is an essential component of muscle filaments. Cardiac α-actin is known to be affected by changes in *ms1* expression (Koekemoer *et al.*, 2009), and is also known to be a target of *SRF*, alongside which *Nkx2-5* can act to increase expression further, particularly during development (Chen *et al.*, 1996). However, there are many different types of actin, specific to different muscle types, and this is the case for both mammals and *Drosophila*. Here, *actin88F* has been chosen as this is expressed in thoracic muscle at extremely high levels, particularly the indirect flight muscles (Sanchez *et al.*, 1983; Gajewski & Schulz, 2010). Levels of *actin88F* were tested with knockdown of *dms1* (figure 3.16).

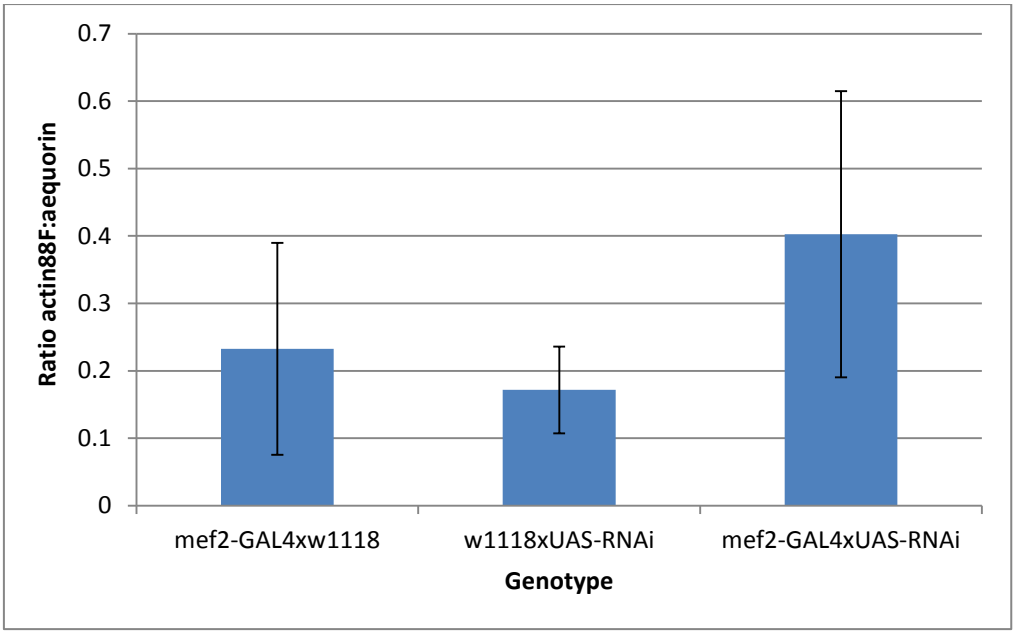

Figure 3.16: levels of expression of ***actin88F*** with muscle-specific knockdown of *dms1* compared to controls as determined by qPCR. Differences are not significant (p=0.18 using one-way ANOVA).

No effect of *dms1* knockdown on *actin88F* expression is observed in this experiment. As figure 3.16 shows, the differences in expression between experimental and controls are not significant, suggesting that *dms1* does not lie upstream of *actin88F*, or that a different procedure is required to detect any changes in gene expression.

Cardiac α-actin has been shown to be upregulated when *ms1* is overexpressed in cultured rat heart cells (Koekemoer *et al.*, 2009). This backs up the idea that *ms1* plays an important role in LVH, and that actin upregulation is necessary for the growth of cardiac myocytes after initiation of this pathway. If *dms1* and the pathway it has a role in are homologous to those in mammals and *actin88F* is a target of this pathway, there may be a change in *actin88F* expression when *dms1* is overexpressed (figure 3.17).

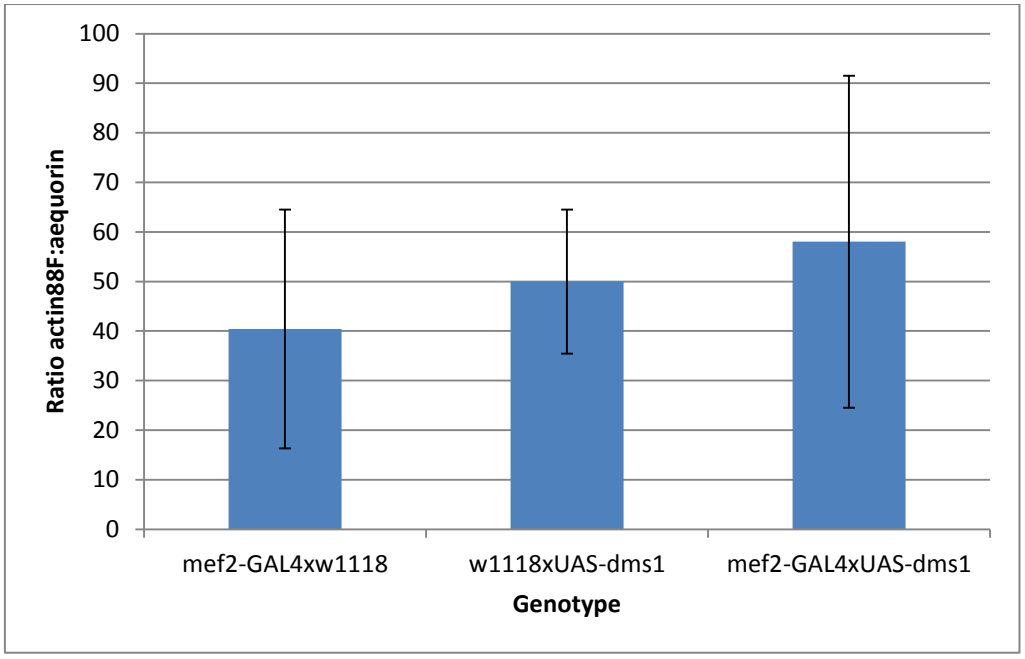

Figure 3.17: levels of expression of ***actin88F*** with muscle-specific overexpression of *dms1* compared to controls as determined by qPCR. Differences are not significant (p=0.64 using one-way ANOVA).

Figure 3.17 shows no significant difference in expression of *actin88F* between experimental and control lines. Levels of *actin88F* expression appear to be very similar across all genotypes, suggesting no change in expression with overexpression of *dms1*.

Actin88F expression has also been tested with expression of mouse *ms1* to determine if expression of this homologue is able to induce changes in potential downstream genes in *Drosophila*, which could possibly indicate homology between the two species. However, as shown in figure 3.18, no significant difference has been found between experimental and control lines, and therefore suggests either this pathway is not affected by changes in *ms1*, or that there is no link between the two genes.

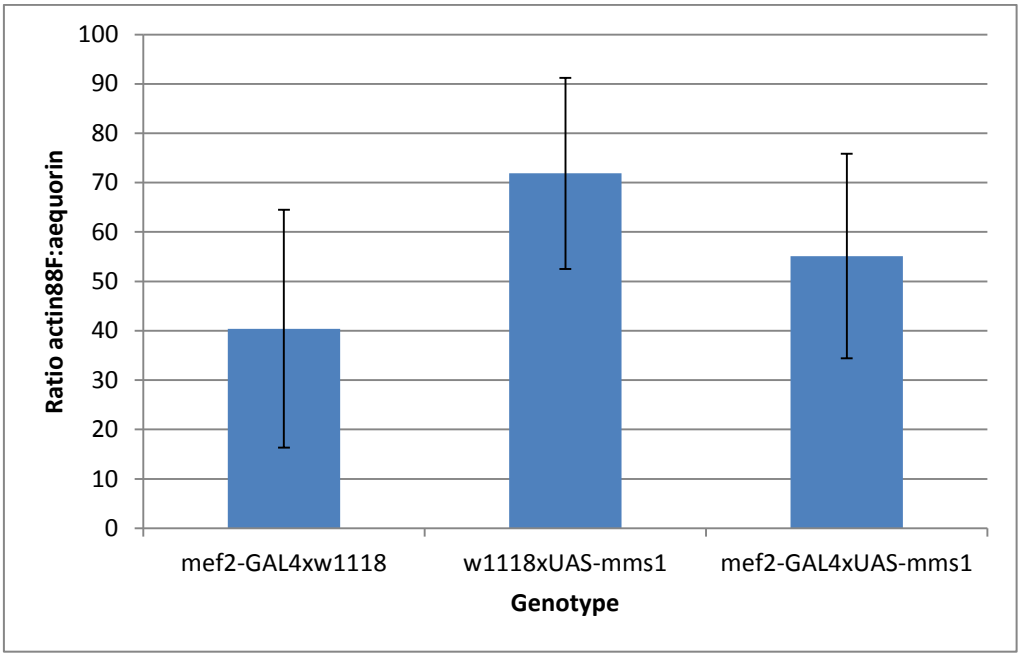

Figure 3.18: levels of expression of ***actin88F*** with muscle-specific expression of mouse *ms1* compared to controls as determined by qPCR. Differences are not significant (p=0.20 using one-way ANOVA).

3.2.2.8 The effect of changes in *ms1* on *actin79B*

Despite the reported high expression levels of *actin88F*, experiments described in chapter 2 demonstrated that changes in *ms1* expression affect the activity levels and climbing ability of adult flies (see section 5.2.2). Therefore another actin gene, *actin79B* was also chosen to investigate, due to the fact that it is known to be expressed in leg muscles, as well as flight muscles and supporting muscles in the thorax and abdomen (Courchesne-Smith & Tobin, 1989; Ohshima *et al.*, 1997).

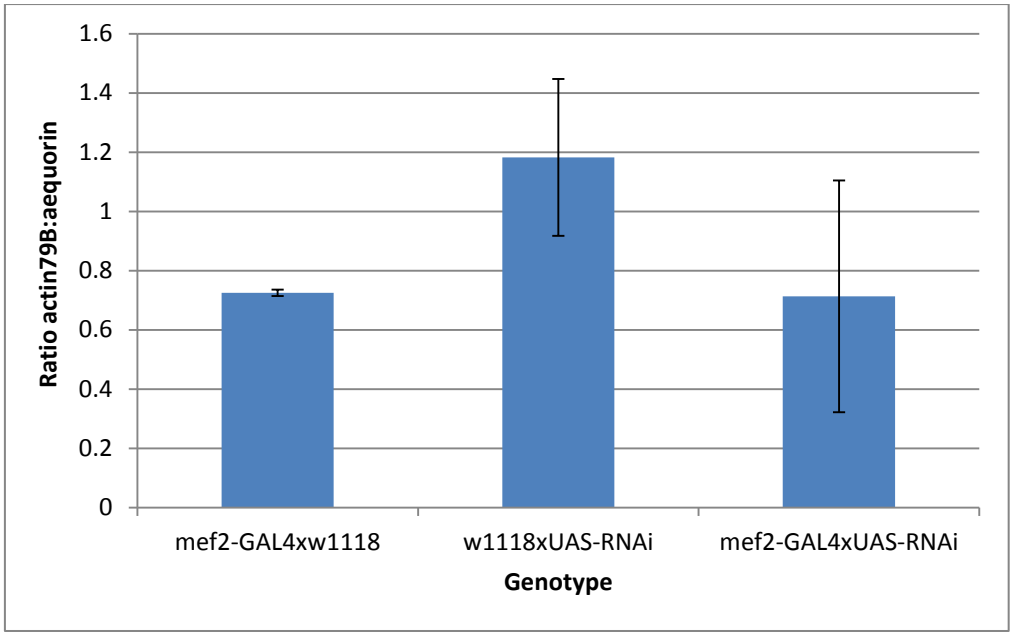

Figure 3.19: levels of expression of ***actin79B*** with muscle-specific knockdown of *dms1* compared to controls as determined by qPCR. Differences are not significant (p=0.14 using one-way ANOVA).

As shown in figure 3.19, there are no significant differences in *actin79B* expression between experimental flies and controls with knockdown of *dms1*. Experimental flies do not differ enough from controls for any conclusions to be drawn from these data, suggesting that *actin79B* is not affected by changes in *dms1* levels, or that changes cannot be detected using this experimental procedure.

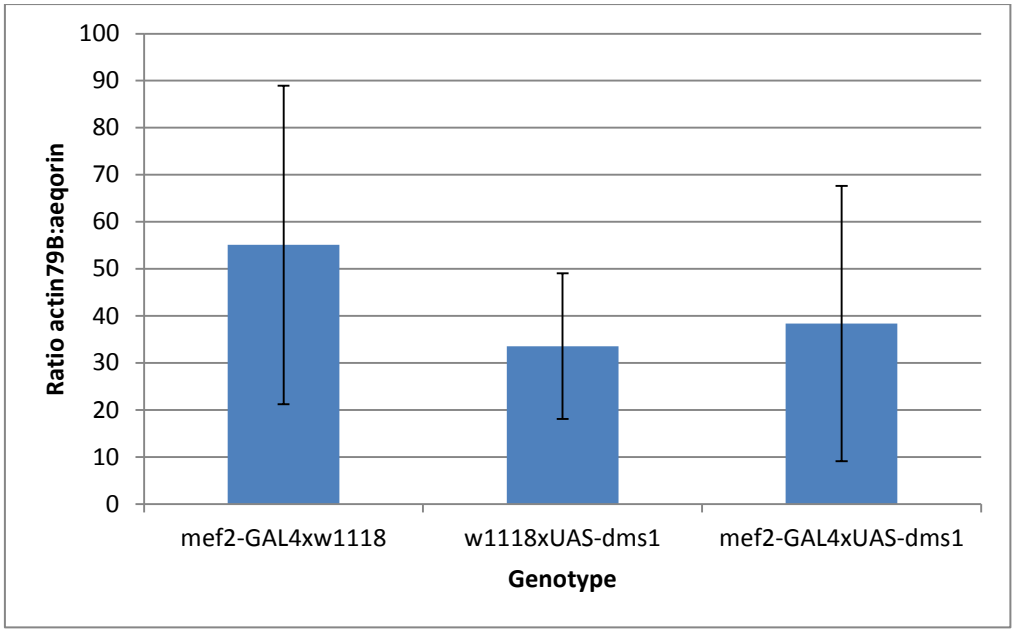

Figure 3.20: levels of expression of ***actin79B*** with muscle-specific overexpression of *dms1* compared to controls as determined by qPCR. Differences are not significant (p=0.54 using one-way ANOVA).

Levels of *actin79B* were also tested with overexpression of *dms1*, as shown in figure 3.20. These results did not show any significant differences in expression between experimental flies and controls, and all genotypes appear to have very similar levels of expression, indicating that there is no effect of *dms1* overexpression in this pathway.

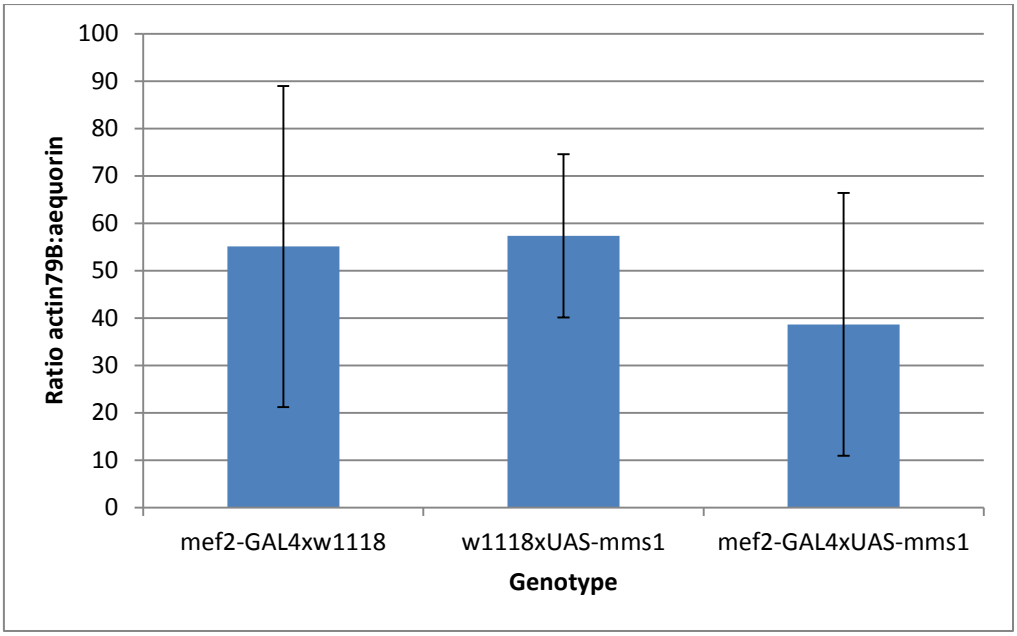

Figure 3.21: levels of expression of ***actin79B*** with muscle-specific expression of mouse *ms1* compared to controls as determined by qPCR. Differences are not significant (p=0.60 using one-way ANOVA).

Levels of *actin79B* were also tested with expression of mouse *ms1*, but no significant differences found between experimental flies and controls, as shown in figure 3.21.

Neither *actin88F* or *79B* are expressed during embryogenesis (Tobin *et al.*, 1990), and are found in adult muscle tissue, so these are the actin genes that are most likely to change in expression if *dms1* levels are affecting the activity and climbing ability of the adult flies (see section 5.2.2.3-4). However, no significant changes are seen here either when *dms1* is knocked down or overexpressed, and there are no changes when mouse *ms1* is expressed.

3.2.3 Investigating the effect of changes to potential upstream regulators on *dms1* expression

There are several genes that could be considered as potential upstream regulators of *dms1*. Looking at mammalian systems gives an idea of which genes could belong to the hypertrophic response pathway, including GATA4 (*pnr*), *mef2*, MyoD and others. It is difficult, however, to manipulate the expression of these genes in a fly model without having a serious effect on the viability of flies. Knockdown of many of these important regulatory genes causes embryonic lethality or at the least flies and larvae that are very unhealthy, causing many attempts to create knockdown flies through crossing to fail. Genes such as *mef2* are involved in many pathways in many tissues, acting as a regulator to genes involved in early-stage development, and changing the expression levels of these high level transcription factors affects too many processes for survival of the animal.

The gene chosen here is calcineurin, a protein phosphatase involved in calcium signalling which has been shown to play a role in regulation of cardiac hypertrophy (Frey *et al.*, 2004). Knockdown has been achieved using an UAS-RNAi line in the same way as was used to knock down *dms1* in previous experiments. The following crosses were carried out:

w; *mef2*-GAL4; + x w; UAS-RNAi(calcineurin); +
w; +; + x w; UAS-RNAi(calcineurin); +

RNA was extracted in duplicate from one week old flies. cDNA synthesised using the protocol for qPCR sample preparation and qPCR carried out using *dms1* primers as described in section 2.9.5.

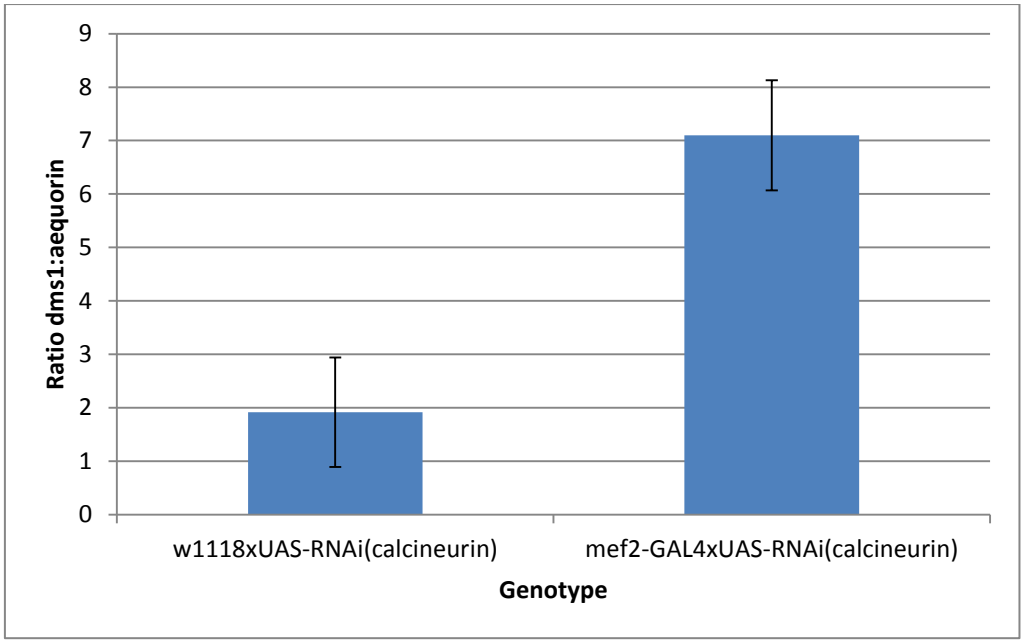

Figure 3.22: levels of expression of ***dms1*** with muscle-specific knockdown of calcineurin compared to control as determined by qPCR. Differences are shown to be significant (p=0.02 using unpaired T-test).

The data shown in figure 3.22 indicate a considerable increase in *dms1* expression when calcineurin is knocked down under the control of the *mef2* driver, potentially suggesting a link between calcineurin and *dms1*. Expression of *dms1* appears to be upregulated approximately 3 fold with knockdown of calcineurin, suggesting a possible repression of *dms1* by calcineurin.

3.3 Discussion

The aim of this chapter was to investigate the structure and function of *dms1*. This was done by using PCR to check existing data on the gene structure, in the process discovering a novel splice variant, and by using qPCR to investigate potential upstream regulators and downstream targets.

Analysis of PCR products while checking the gene structure revealed that specific amplifications gave more than one product, suggesting the presence of a smaller splice variant. At the time of this experiment no splice variants had been described, and sequence analysis showed this variant to be missing exon 2. This appears to leave the conserved actin binding domain intact, however, there is no data as to the expression patterns of this variant and whether it is tissue specific. It seems to be expressed at a much lower level than the full length *dms1*, as can be seen from figure 3.2, so may only be expressed in a small number of tissues.

The data gathered from qPCR analysis of potential downstream genes did not give any significant positive results. There could be several reasons for this. For many of the genes tested there was a large difference in expression between the two controls. It has been suggested that this is to do with the amount of GAL4 being expressed in the driver control, and this effect is particularly apparent when testing the knockdown samples. This could be masking any knockdown in the genes examined as they could also be unintentionally knocked down in the driver control as well. It is also possible that changes in *dms1* were not extreme enough; perhaps a complete knockout is required to see any significant change in downstream genes, or a greater overexpression of *dms1*. A change in driver line or collection of younger flies may also be of benefit. For instance, using newly emerged flies may increase the expression of the *mef2* driver, as *mef2* expression is higher during metamorphosis than in the adult fly (Soler *et al.*, 2012). It is certainly worth repeating this experiment, with changes to the experimental procedure. Chosen carefully, different controls may be able to reduce any effect GAL4 is having in the driver control, as certainly in the knockdown data, this control seems to have much lower levels of expression for most of the genes tested.

If differences still cannot be detected with experimental changes, it is possible that *dms1* and mammalian *ms1* do not have homologous functions in this context. The homology between mammalian *ms1* and *Drosophila ms1* is found in the actin binding domain, with an overlap seen in the sequence, such that the actin binding domain is at the C-terminal end of mammalian *ms1* and at the N-terminal end of *Drosophila ms1* (Mahadeva *et al.*, 2002). This raises the question of whether *dms1* is a true homologue and whether it has the same functions as the mammalian gene. It is certainly likely to be an actin binding protein due to sequence similarity to the actin binding domain, but it is possible that the N-terminal section of mammalian *ms1* gives it another function, either additional to that of *dms1*, or a different function entirely. *Drosophila ms1* could be closer to an ancestral version of this gene which throughout mammalian evolution has gained new functions, possibly in the process losing the old functions. This could be a reason for the results seen when looking for potential downstream target genes, as the pathways could have diverged so much during evolution that the *ms1* genes no longer have the same function in mammals and *Drosophila*.

There is significant homology between *Drosophila* and mammalian *SRF*, found mostly in a region of sequence containing a MADS-box which shows 93% homology with human *SRF* (Affolter *et al.*, 1994). While there are large regions of non-homologous sequence either side of the MADS-box sequence, *Drosophila SRF* has other shorter conserved regions in its coding sequence, and still has the same DNA binding capability and should be able to regulate transcription of any gene under the control of a serum response element (SRE) in *Drosophila*. Here, however, there is no significant evidence that *SRF* is regulated by the *ms1* pathway in *Drosophila*. With knockdown and overexpression of *dms1*, no

significant differences were found between experimental and control groups, and expression of mouse *ms1* did not appear to affect *SRF* levels. This indicates that there is either no homology in this pathway between mammals and *Drosophila*, or that this protocol cannot detect any changes in expression.

Investigation of levels of *Jra* did not show any significant differences with different levels of *dms1*. After the results from the investigation into *SRF* were analysed this was unsurprising, as JUN-B is known to be regulated by *SRF*, and no significant differences were found in levels of *SRF*. There is once again no significant difference in *Jra* levels when *dms1* is overexpressed, or with expression of mouse *ms1*. The lack of significant results could indicate that *Jra* is not the true homologue of JUN-B and is not a target of *SRF* in this context, or that *Jra* does not function in this pathway. It could also simply be that this pathway is different in flies and mammals and does not function as predicted. *Drosophila Jra* is known to share functional homology with the JUN genes in mammals, C-JUN and JUN-B. Genes in the JUN family form the heterodimeric transcription factor AP1 along with FOS-family and ATF proteins (Hess *et al.*, 2004), and *Drosophila Jra*, along with *fos-related antigen* (*Fra*), have the same function, forming the subunits of *Drosophila* AP1 (Perkins *et al.*, 1988). It has been suggested that AP1 is involved in muscle hypertrophy, and JUN-B overexpression has been shown to induce skeletal muscle hypertrophy, and vice versa (Raffaello *et al.*, 2010). It is unknown whether this is conserved between mammals and flies, and whether *Jra* is a factor in initiating muscle growth, but it may be worth reconsidering the experimental procedure to better test this theory. This is also the case for *whd*, as it may not have exactly the same properties as its mammalian counterpart, CPT1, the β form of which is known to be downstream of mammalian *ms1* (Wallace *et*

al., 2011) . The evidence here suggests that expression of the *Drosophila* homologue *whd* is not significantly altered with changes in *ms1* expression. It has been shown that *whd* is involved in protection against reactive oxygen species, and mutations cause sensitivity to oxidative stress and hyperoxia (Strub *et al.*, 2008) , and so appears to be a stress responsive gene, but whether it acts in response to the *ms1* pathway is unknown. The results here do not support the suggestion that *whd* is part of this pathway. This could be because of the differences between the pathways in mammals and *Drosophila*, as *Drosophila* do not have a homologue for PGC1-α, the gene known to initiate this pathway in mammals (Wallace *et al.*, 2011) . However, this gene is thought to be upstream of *ms1*, so this may not be an issue in *Drosophila*, and the reason for the lack of significant results could simply be that the two systems are not homologous. In the case of both *Jra* and *whd*, *Drosophila* only have one gene to cover the functions of many, so it is only to be expected that some of these roles may not exist in *Drosophila*, and may have been gained throughout evolution of higher animals, and a role in regulating muscle hypertrophy may be one of these functions. There is experimental evidence that *whd* undergoes alternative splicing to allow it to take on many different roles, with different splice variants able to interact in different ways in various pathways (Price *et al.*, 2010) , even so, it is unlikely that all the functions of the CPT1 genes could be covered by a single gene.

The investigation into the actin genes *88F* and *79B* showed no significant change with different levels of *dms1*. Both are known to be adult actin genes, expressed predominantly in adult muscle tissue (Tobin *et al.*, 1990), so were chosen because of the phenotype found in adult flies with knockdown of *dms1* (see chapter 5). As

well as the high expression levels in the indirect flight muscles, *actin88F* is also expressed in other muscle tissue, including the legs and uterine musculature (Nongthomba *et al.*, 2001). Experiments that took place prior to this suggested a significant change in muscle tissue, particularly in flies with knockdown of *dms1* due to their reduced climbing ability and general levels of activity (for further details see section 5.2.2). This was not supported by the qPCR data found here, as it was hypothesised that some change in actin levels would be seen for at least one of these genes. The results of testing *SRF* levels did not reveal any differences in expression with changes in *dms1*, and it has been shown that mammalian actin genes are upregulated in response to SRF (Koekemoer *et al.*, 2009) . It is possible that this is not the case in flies, however, if the *SRF* pathway is not associated with *dms1*, it does not explain the data collected from experiments testing the behaviour of flies particularly with knockdown of *dms1*, which show a significant decrease in activity, as well as climbing and eclosion ability. These imply that the muscles are lacking in some way, and possibly that actin levels decrease under these conditions, but there appears to be no evidence of this here. It is possible that levels of mRNA are not affected, and therefore there is no change to gene expression, but that there is a post-transcriptional change that affects the protein but not the mRNA. This change could be due to another protein that is upregulated by MS1 or its downstream pathway, which could be the reason for the change in phenotype when *ms1* expression is altered. Another explanation is that actin is not involved and not a target of the *dms1* pathway, and the phenotype seen is due to some sort of damage caused by lack of *dms1* and its true targets. Knockdown of *dms1* may be causing damage to the muscle tissue in some way, which causes the flies to have difficulty with locomotor activity, rather than

reducing the expression of actin. Alternative experiments may have to be designed to test this hypothesis, for instance looking at the structure of the muscle using an electron microscope may allow visualisation of the microstructure of the muscle and allow any defects to be seen. Due to the nature of *ms1* as a stress-responsive gene, it could be that changes in expression of these genes can only be detected under conditions of stress or perhaps exercise, and testing the levels of *actin79B* and *88F* under these conditions may give a different result. It is also possible that changes in actin are temporal, and may only be detected at other stages of development or even time of day, so testing at different times may also reveal an effect.

There were fundamental difficulties when examining upstream pathways. Genes such as *mef2*, *pannier*, and other crucial transcription factors have many diverse functions and even a small change in their expression can affect the viability of the flies. Here, calcineurin was chosen due to its potential interaction with the *ms1* pathway, since overexpression of *ms1* and calcineurin gives a significant cardiac phenotype (Kuwahara *et al.*, 2007). Calcineurin is an important factor in calcium signalling and knocking down in all muscle types was a risk since this may have not given viable offspring. It was noted that the cross of *mef2*-GAL4 driver to UAS-calcineurin resulted in only female offspring and perhaps males were not viable with this high level of knockdown. In mammalian systems an increase in calcineurin can lead to initiation of cardiac hypertrophy (Molkentin *et al.*, 1998), implying that the *ms1* pathway could be activated by the calcineurin pathway to induce the hypertrophic response. If that is the case here, the result expected would be the opposite of that observed, with levels of *dms1* reduced in the calcineurin knockdown compared to the control. The results here could indicate a

different involvement for calcineurin, or indeed a different role for *dms1* in the *Drosophila* model. However, while the results of this experiment appear to be significant, due to failed crosses and time constraints the protocol was incomplete, as only two biological replicates were carried out with only one control, the UAS control. There is no data for a driver control, which is important to include as the presence of GAL4 in *Drosophila* cells can have a toxic effect. This may affect the results by showing no significant difference between this control and the knockdown line. It was also noted that only female offspring of the experimental cross appeared to be viable, although the reasons for this are unknown. Repeating the crosses, perhaps with a different knockdown line may give better viability, and therefore better reliability of results. A third biological replicate would also add to the significance of any differences seen here, and will ensure that the effect seen is reliable. Completion of this experiment would allow the reliability of these data to be confirmed or refuted and would allow analysis of the pathway to which these genes belong. Another experiment that was planned to test upstream genes was an overexpression of *pnr*, but the cross required did not result in viable offspring, demonstrating the importance of *pnr* in development. This experiment would have particular importance as it has recently been shown that GATA4 is able to repress *ms1* directly at all stages of development (Ounzain *et al.*, 2012). Future experiments would need to be modified, possibly using different driver lines or crossing in different conditions, for instance at a lower temperature reducing stress during development.

In conclusion, using the fly crosses and experimental design here, no significant change in expression of putative downstream genes were found in *ms1* KD compared with control flies. However, some results appear to show an interesting

trend such as levels of *SRF*, *jra* and *whd* when *dms1* is knocked down, and the effect of calcineurin knockdown on *dms1*. Modifications to the experimental procedure would allow further analysis, and to see if the effect seen here is a true effect. Issues with controls, time constraints and lack of stress/exercise treatment have contributed to the variability of these results, and perhaps overcoming these problems will give more consistent data and reveal the links between the genes tested here. It is also conceivable that there is no effect here and that *dms1* is part of a pathway that is not homologous to the one found in mammals, in which case, the interesting question would be; what is the function of *ms1* in *Drosophila*? If *Drosophila ms1* has a novel function, it is possible that mammalian *ms1* also has this function, but that this has not yet been discovered.

Chapter 4: *Drosophila ms1* and cardiac biology

4.1 Introduction

Discovery of *ms1* by Mahadeva et al. (2002) was the result of supra-renal abdominal aortic banding experiments in rats. This gave a pressure-overload model which was used to screen for acute upregulated genes. *Ms1* mRNA levels were found to increase 4 hours after pressure overload, thus before left ventricular hypertrophy. This indicated that *ms1* may be an important regulator of cardiac hypertrophy and may be of significant importance in cardiac remodelling and disease states. *Ms1* appears to play a vital role in the pathway regulating LVH, as upregulation can affect expression of SRF (Arai *et al.*, 2002) and SRF-dependent genes (Koekemoer *et al.*, 2009). It is therefore an important aim of this project to identify a role for *ms1* in the *Drosophila* heart.

Heart research using *Drosophila melanogaster* is a relatively new area, with most work carried out in the last few years. There are, however, still a variety of techniques recently developed for analysis of the *Drosophila* heart, from dissection techniques for visualisation of the heart (Vogler & Ocorr, 2009), to video and movement capture tools to analyse the heartbeat (Ocorr *et al.*, 2009). Molecular tools have also been developed, for example, the heart-specific tinman-GAL4 driver (Lo & Frasch, 2001) used extensively in this chapter. *Drosophila* have been used in studies looking at a range of different aspects of cardiac research, including early cardiac development (Reim & Frasch, 2005), ion channel disruption (Ocorr *et al.*, 2007), and screens to identify genes involved in human heart disease (Neely *et al.*, 2010; Wolf *et al.*, 2006).

The aim here is to use *Drosophila* genetics to manipulate the levels of *ms1* specifically in cardiac and surrounding muscle cells and to characterise any resultant phenotype. If any effect can be found by changes in *ms1* expression with regards to heart function in *Drosophila*, then it is likely that *ms1* has a similar function in flies as it does in mammals. A selection of *Drosophila* heart analysis tools has been used in order to get a broad overview of, firstly, how the heart works in *Drosophila*, and secondly, to investigate any role *ms1* has in the heart and what effect changes in expression have on the development and function of the heart.

4.2 Results

4.2.1 Expression pattern of cardiac-specific *tinmanCΔ4*-GAL4 driver

The experiments in this chapter require a method of changing expression levels of *ms1* specifically in the heart of the fly. To do this, a *tinmanCΔ4*-GAL4 line was used to drive expression of UAS constructs in cardioblasts, the inner layer of muscle in the *Drosophila* heart. This driver was created by Lo *et al.* (2001) using a deletion of the *tinman* promoter that is expressed specifically in cardioblasts. In order to ensure that this driver has the expected expression pattern it was necessary to cross the driver line to a UAS-GFP line so expression of the driver could be visualised. The resulting pattern of GAL4 expressed under the *tinmanCΔ4* driver can be seen in figure 4.1.

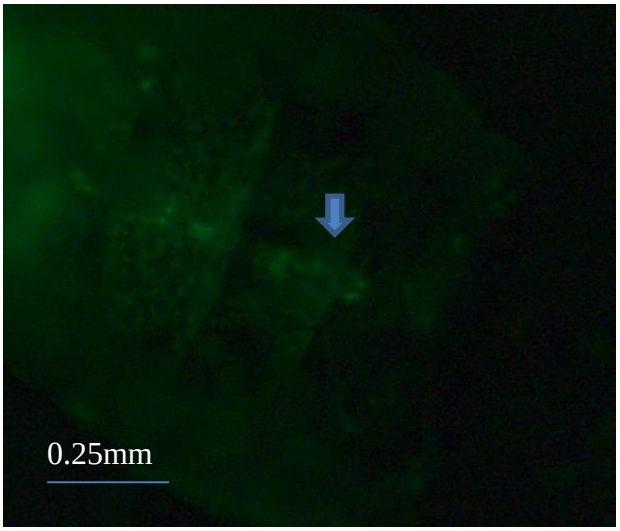

Figure 4.1: Expression pattern of *tinmanCΔ4*-GAL4 visualised using UAS-GFP. Picture shows only the abdomen of the fly with heart is indicated by the arrow. Fly is positioned anterior to the left and posterior to the right of the picture.

Figure 4.1 shows expression of GFP in a *tinmanCΔ4* pattern. The heart tube, indicated by the blue arrow, is brighter than the surrounding area, particularly at the ostia or inlet valves of the heart. This is because only the inner layer of muscle in the heart is expressing GFP here, and this layer can only clearly be seen where there are gaps in the outer longitudinal muscle layer, at the ostia.

4.2.2 Longevity with heart specific knockdown of *dms1*

In order to investigate any significant effects knockdown of *dms1* has on the viability of *Drosophila* a longevity experiment was carried out. This tests the survival of the flies under normal conditions and compares flies with tissue-specific knockdown of *dms1* to driver and UAS controls. This will allow any effect of lowering the expression of *dms1* on the overall health of the flies to be seen. Here, knockdown of *dms1* is specific to the inner layer of the heart tube.

4.2.2.1 Longevity of non-backcrossed flies with cardiac-specific knockdown of *dms1* using UAS-RNAi(GD)

Drosophila *ms1* (*dms1*) was knocked down specifically in cardioblasts using the *tinmanCΔ4*-GAL4 driver crossed to UAS-RNAi. Crosses carried out were as follows:

w; *tinCΔ4*-GAL4; + x w; UAS-RNAi(GD); +
w; *tinCΔ4*-GAL4; + x w^{1118}; +; +
w^{1118}; +; + x w; UAS-RNAi(GD); +

The experiment was carried out both before and after backcrossing of all fly lines to remove background effects (see section 2.4).

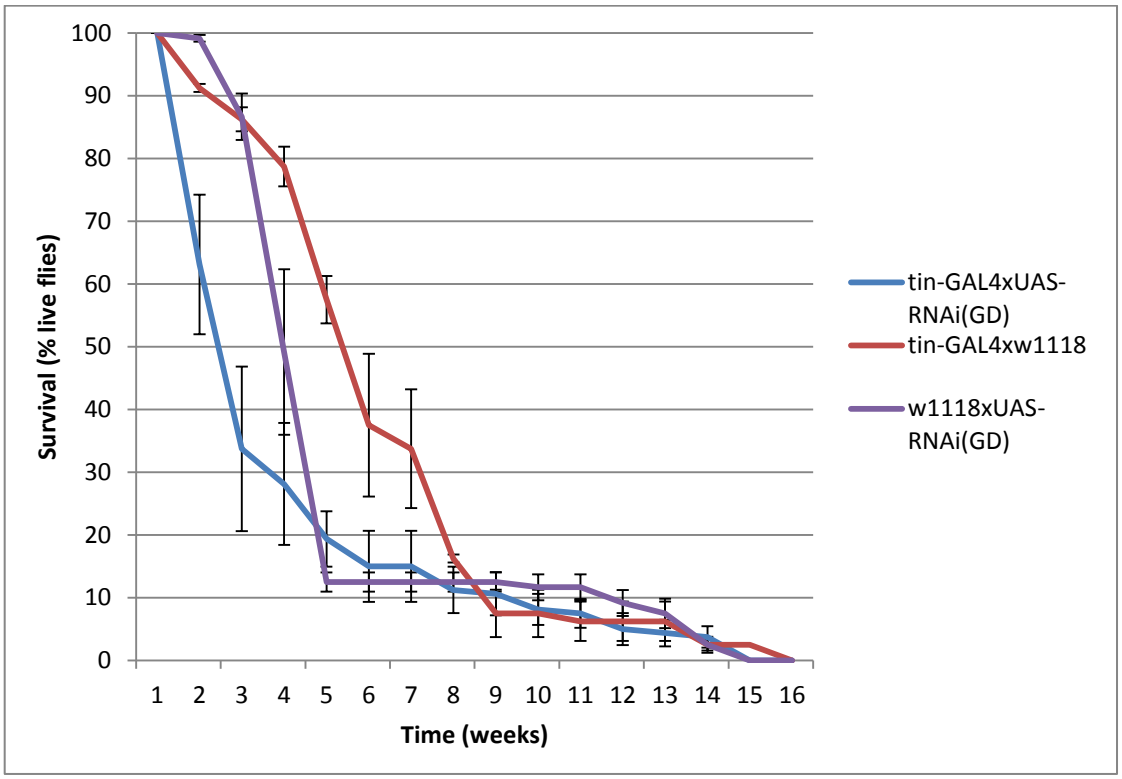

Figure 4.2: Longevity of non-backcrossed flies with cardioblast-specific knockdown of *dms1* (blue) compared to controls (red/purple) (n=40 flies). The RNAi line used here is UAS-RNAi(GD). Results are shown to be significant ($p<0.001$ using Mantel-Cox test and Gehan-Breslow-Wilcoxon test).

As figure 4.2 shows, cardiac-specific knockdown of *dms1* in non-backcrossed flies seems to have an effect on the flies longevity. In the early stages of the experiment, particularly the first 3-4 weeks, the flies with knockdown do not appear to survive as well as the controls. The effect is only seen in young adult stages, after this point, the levels of survival for all three genotypes become fairly similar.

4.2.2.2 Longevity of backcrossed flies with cardiac-specific knockdown of *dms1* using UAS-RNAi(GD)

Crosses were carried out as in the previous section but using backcrossed lines instead of non-backcrossed.

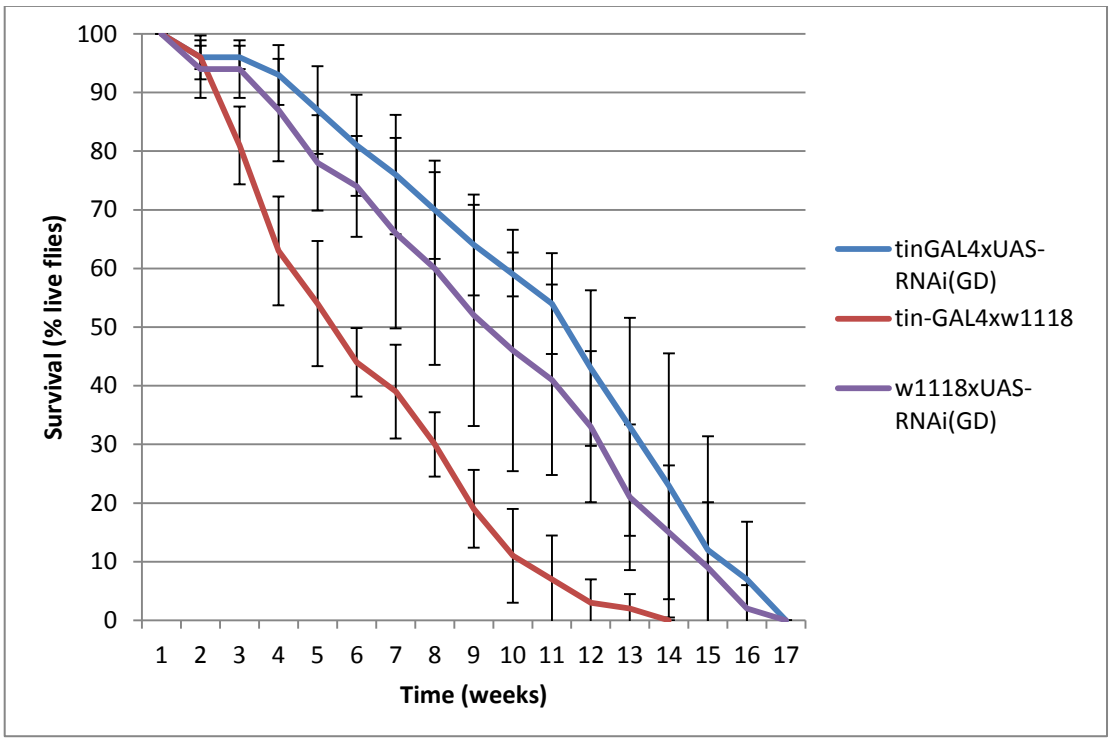

Figure 4.3: Longevity of backcrossed flies with cardioblast-specific knockdown of *dms1* (blue) compared to controls (red/purple). The RNAi line used here is UAS-RNAi(GD) line (n=40 flies). Differences between knockdown and UAS control are shown to be significant ($p<0.05$ using Mantel-Cox test and Gehan-Breslow-Wilcoxon test).

Results from the longevity experiment using backcrossed flies appear to show the opposite of the previous results with non-backcrossed. In figure 4.3, backcrossed flies with cardiac-specific knockdown of *dms1* seem to survive better than the controls throughout the experiment. Before backcrossing (figure 4.2), the fly lines all have different genetic backgrounds, and this may have been having an effect on their longevity. Backcrossing of the flies to give the same background may have removed any interaction *dms1* is having with background factors, thereby removing the effect on longevity that a decrease in *dms1* appears to have in figure 4.2. However, backcrossing has affected the results in an unexpected way, as survival of the driver control appears to be much lower than the other lines. The survival of the *tinmanCΔ4*-GAL4 driver control is similar to that of the non-backcrossed flies, meaning that backcrossing into the w^{1118} background has increased the lifespan of the experimental line and the other control, but not the driver control. This implies that under the original genetic background the UAS-RNAi line has lower fitness than under the *w* background.

4.2.2.3 Longevity of backcrossed flies with cardiac-specific knockdown of *dms1* using UAS-RNAi(KK)

The experiment was also carried out using another RNAi line (UAS-RNAi(KK). This line has directed insertion of the UAS-RNAi construct to ensure sufficient expression of the inverted repeat under the control of the appropriate GAL4 driver. Expression of the inverted repeat with a KK line is also known to be stronger than the GD line when using the same driver (VDRC, Dietzl *et al*. 2007). This experiment not only investigates the effect of lower levels of *dms1* than previously, but also allows a comparison of different levels of expression, to see if there is any

difference between the two, as an effect may only be seen if *dms1* expression drops below a certain level. The crosses carried out were as follows:

w; *tinCΔ4*-GAL4; + x w; UAS-RNAi(KK); +
w; *tinCΔ4*-GAL4; + x w^{1118}; +; +
w^{1118}; +; + x w; UAS-RNAi(KK); +

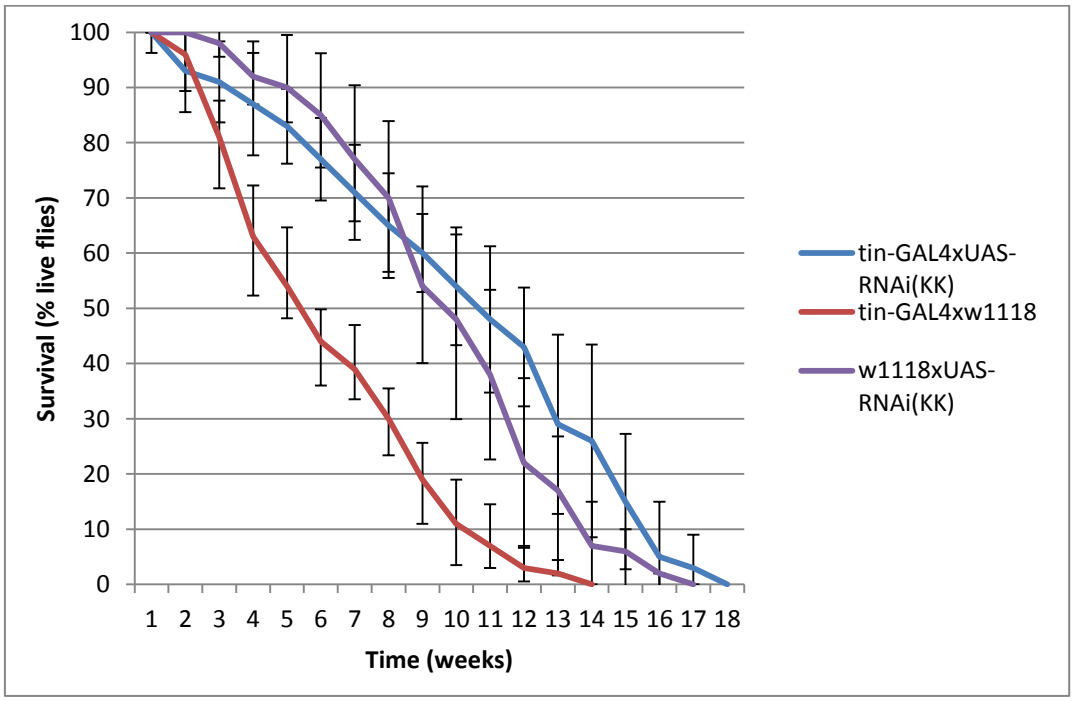

Figure 4.4: Longevity of backcrossed flies with cardioblast-specific knockdown of *dms1* (blue) compared to controls (red/purple). The RNAi line used here is UAS-RNAi(KK) (n=40 flies). Difference between knockdown and UAS control not shown to be significant (p=0.25 using Gehan-Breslow-Wilcoxon test).

As figure 4.4 shows, use of the RNAi(KK) line does not appear to affect longevity of knockdown flies compared to the w^{1118}xUAS-RNAi(KK) control. As with the previous experiment using the RNAi(GD) line, the tinCΔ4-GAL4xw^{1118} has significantly lower longevity compared to the other lines and cannot reliably be used for comparison. As before, this means these results do not give a reliable indication

of the effect of *dms1* knockdown, but may indicate that lower levels of knockdown with the GD line have more of an effect on longevity than higher levels with the KK line.

4.2.3 Longevity under daily hypoxic stress with heart specific knockdown of *dms1*

Longevity experiments were carried out with the addition of a daily 1 hour hypoxia treatment as described in section 2.11. The crosses carried out were the same as those described for experiments investigating longevity under normoxia in order to allow comparison of longevity under normal and hypoxic stress conditions. This would show any effect reduction of *dms1* is having under the different conditions with regards to survival. *ms1* is a stress response gene, and therefore is more likely to have an effect under conditions of stress than under normoxic conditions. This could either cause flies to die more quickly than controls, implying that *dms1* is necessary for the stress response in flies and that this response is required for survival, or increase longevity, suggesting that loss of *dms1* is giving flies some advantage, perhaps the loss of heart overload due to the stress response reduces the pressure on the heart. This experiment was designed to test the tolerance of flies with different levels of cardiac *dms1* to daily hypoxic stress.

4.2.3.1 Longevity under daily hypoxic stress of non-backcrossed flies with cardiac-specific knockdown of *dms1* using UAS-RNAi(GD)

Crosses were carried out as in section 4.2.2.1.

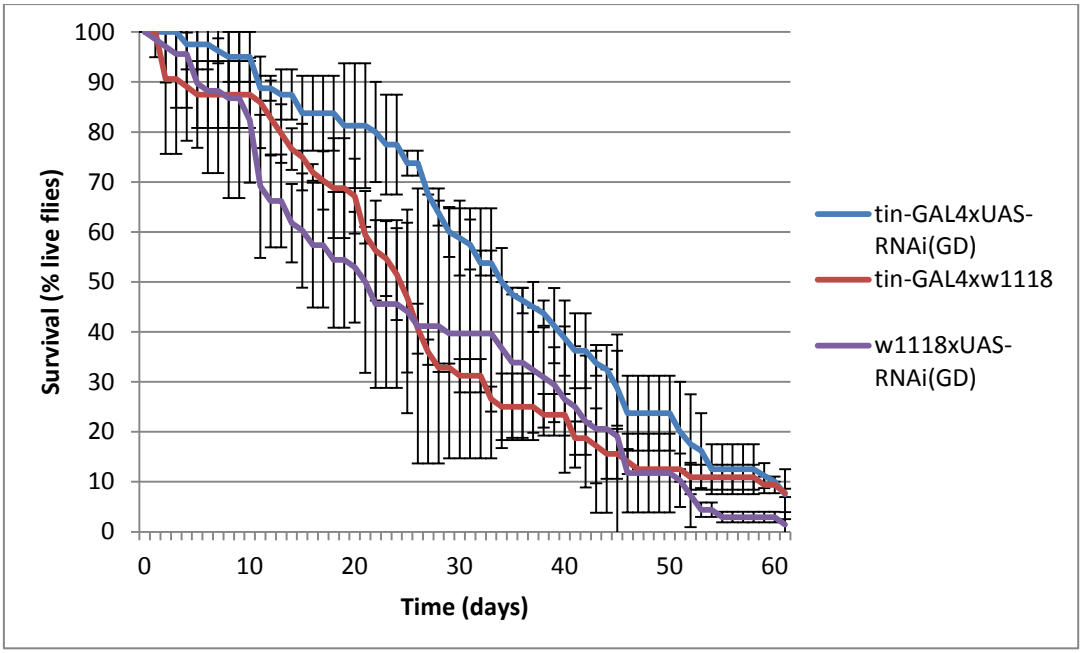

Figure 4.5: Graph showing longevity under daily 1 hour hypoxic stress of non-backcrossed flies with cardioblast-specific knockdown of *dms1* (blue) compared to controls (red/purple) using UAS-RNAi(GD) line (n=80 flies). Differences are shown to be significant ($p<0.05$ using Mantel-Cox test and Gehan-Breslow Wilcoxon test).

Figure 4.5 shows longevity under daily hypoxic stress of non-backcrossed flies. A significant difference is seen between those with cardiac-specific knockdown of *dms1* and controls. The survival of knockdown flies appears to be higher than controls, particularly after approximately 10 days of treatment, and remains higher for the remainder of the experiment.

4.2.3.2 Longevity under daily hypoxic stress of backcrossed flies with cardiac-specific knockdown of *dms1* using UAS-RNAi(GD)

The experiment was repeated using flies that had been backcrossed in to a w^{1118} background.

Crosses were carried out as in section 4.2.2.2.

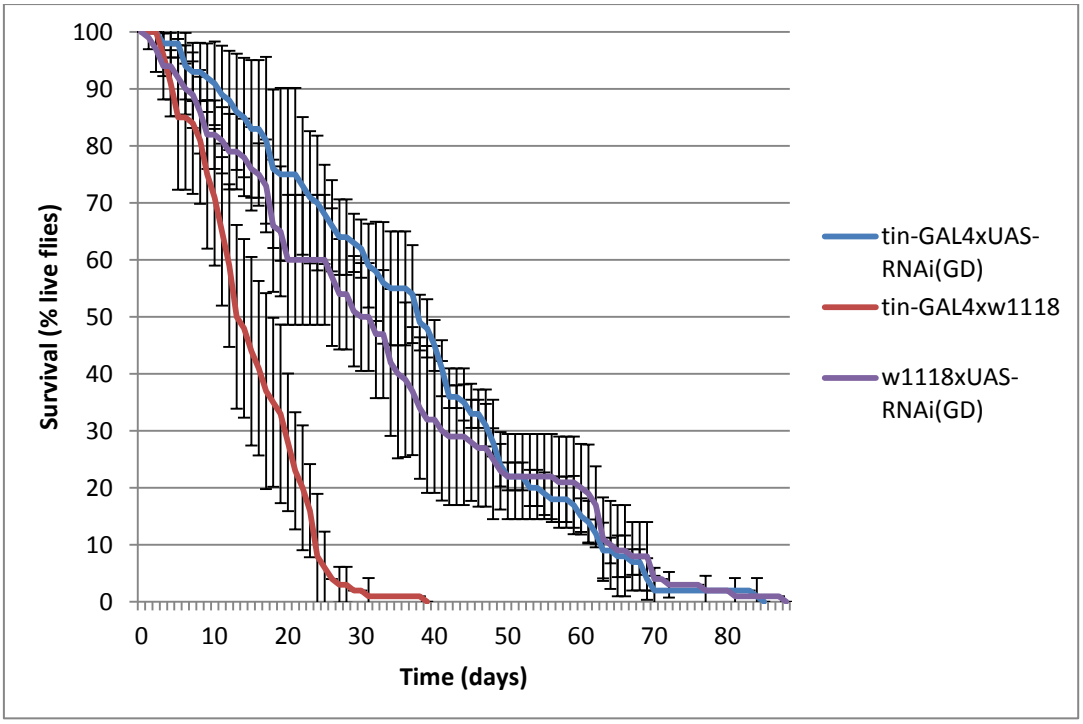

Figure 4.6: Graph showing longevity under daily 1 hour hypoxic stress of backcrossed flies with cardioblast-specific knockdown of *dms1* (blue) compared to controls (red/purple) using UAS-RNAi(GD) line (n=100 flies). Differences between knockdown and UAS control are not shown to be significant (p=0.61 using Mantel-Cox test, p=0.12 using Gehan-Breslow-Wilcoxon test).

Figure 4.6 shows the longevity of backcrossed flies with daily hypoxic stress. After backcrossing the fly lines into a w^{1118} background, the beneficial effect of *dms1* knockdown seen in figure 4.5 is lost and experimental flies are not significantly different from the UAS control. As before, the driver control is not as viable as the other lines and these flies die much quicker than experimental flies and UAS control, once again showing this is not a reliable result. However, the indication is that as backcrossing removes the effect seen in figure 4.5, this may be because of genetic background, and therefore there is no true effect of *dms1* knockdown under these conditions.

4.2.3.3 Longevity under daily hypoxic stress of backcrossed flies with cardiac-specific knockdown of *dms1* using UAS-RNAi(KK)

The experiment was repeated using a higher level of knockdown from a UAS-RNAi(KK) line. Crosses were carried out as in section 4.2.2.3.

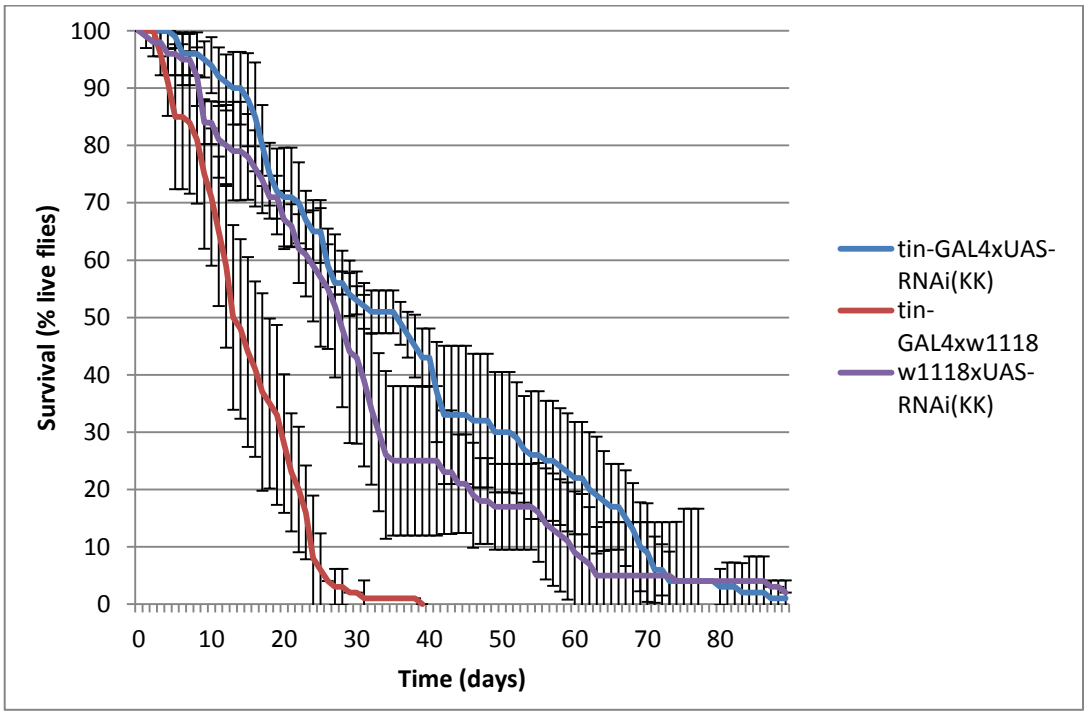

Figure 4.7: Graph showing longevity under daily 1 hour hypoxic stress of backcrossed flies with cardioblast-specific knockdown of *dms1* (blue) compared to controls (red/purple) using UAS-RNAi(KK) line (n=100 flies). Differences are shown to be significant ($p<0.05$ using Mantel-Cox test and Gehan-Breslow Wilcoxon test).

As figure 4.7 shows, survival with *dms1* knockdown is higher than for controls, but this difference varies throughout the course of the experiment. The difference is greater between day 26 and 70 (4-10 weeks) and continues until around day 70. As observed in previous experiments, the longevity is increased for experimental (blue) and UAS control (purple) after backcrossing, but no increase is seen in the driver control (red). If knockdown with the KK line is in fact stronger than the GD line, the

results shown in figure 4.6 and 4.7 imply that lower levels of *dms1* in cardiac tissue are more beneficial under hypoxic conditions.

4.2.4 Quantitative analysis of heartbeat videos with knockdown of *dms1*

It is known that *ms1* is an important gene involved in heart development and the response of the adult heart to stress in mammals. In order to investigate this in *Drosophila* a dissection technique developed by Vogler *et al.* (2009) was used to visualise the beating heart of 1 week old flies. This technique allows the heart to be videoed under high magnification, making analysis of the heartbeat easier than in real time. These experiments provide information on whether *dms1* is important in adult cardiac function and if a reduction in expression affects the heartbeat.

4.2.4.1 Analysis of heartbeat with heart-specific knockdown of *dms1*

Preparations were carried out with 1 week old flies under oxygenated AHL and 30 second videos were taken of hearts beating normally.

The following crosses were carried out:

w; *tinCΔ4*-GAL4; + x w; UAS-RNAi(KK); +
w; *tinCΔ4*-GAL4; + x w^{1118}; +; +
w^{1118}; +; + x w; UAS-RNAi(KK); +

Two types of analysis were used to determine heart function. Firstly, a count was made of the number of ectopic beats observed in 30 seconds. Secondly, bouts of arrhythmia lasting for longer than 2 ectopic beats were timed, also in 30 seconds. The results of these types of analysis are shown in figures 4.8 and 4.9 respectively. This gives an idea of the levels of arrhythmicity seen in the different genotypes and

also allows a comparison of different severity of arrhythmia, as occasional ectopic beats are considered less severe than bouts of arrhythmia that last several seconds.

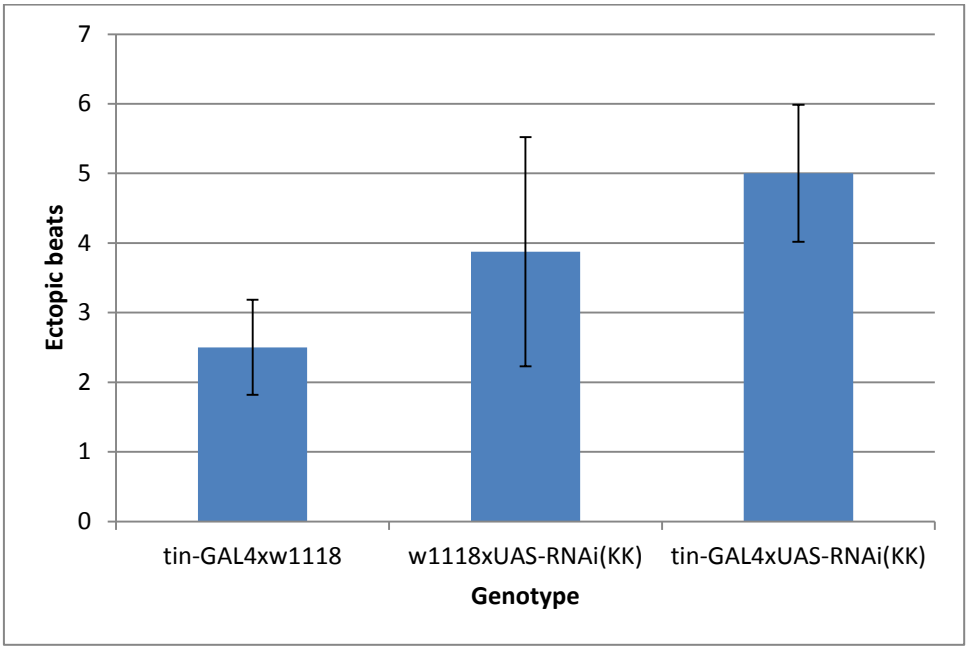

Figure 4.8: Average number of ectopic beats per 30 second video of 1 week old flies with cardioblast-specific knockdown of *dms1* compared with controls (n=12). Difference are not shown to be significant (p=0.01 using one-way ANOVA, difference between experimental and UAS control not significant using Tukey's HSD post hoc test).

As figure 4.8 shows, there appears to be no significant difference in the number of ectopic beats observed in hearts with knockdown of *dms1* in comparison to controls. It could be suggested that ectopic beats are slightly elevated in the experimental line, but this is not statistically significant, therefore it can be concluded that there is no effect of knockdown observed here.

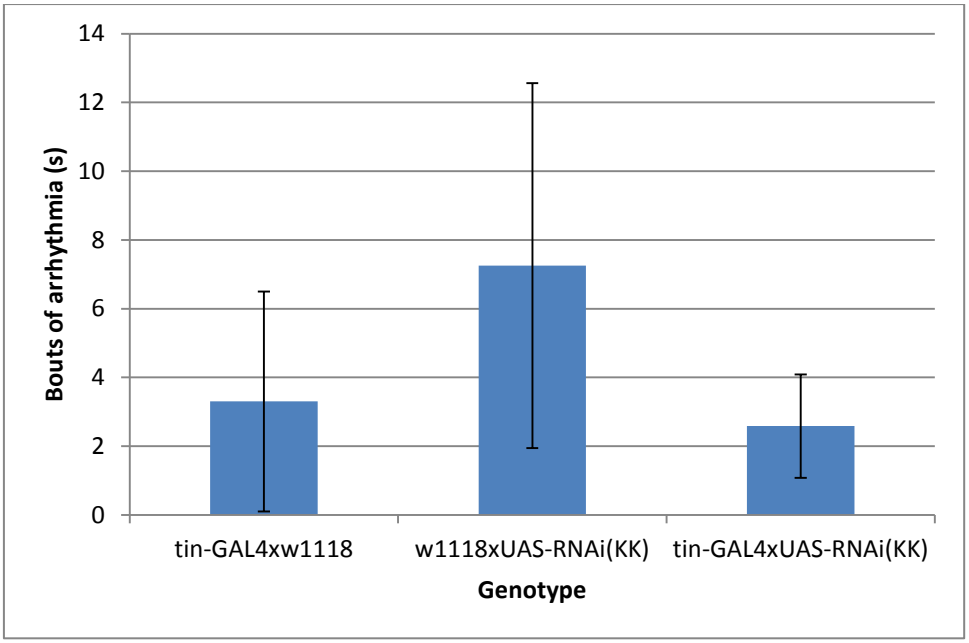

Figure 4.9: Average amount of time spent in bouts of arrhythmia per 30 second video by flies with cardioblast-specific knockdown of *dms1* compared to controls (n=12). Differences are not shown to be significant (p=0.46 using one-way ANOVA).

There is no significant effect of *dms1* knockdown on length of arrhythmic bouts compared to controls. As figure 4.9 shows, the amount of time spent in arrhythmia is low for all genotypes, and no significant differences are found, suggesting that knockdown does not cause the heart to become particularly arrhythmic.

4.2.4.2 Analysis of heartbeat with muscle-specific knockdown

The experiment was carried out in the same way as with cardiac-specific knockdown of *dms1* but using a muscle-specific *mef2*-GAL4 driver. This drives expression of the UAS-RNAi construct in all muscle tissue (see section 5.2.1 for expression pattern), driving knockdown not only in the inner layer of cardioblasts but also in the outer longitudinal muscle layer of the heart, thereby knocking down *dms1* in the entire cardiac region.

The following crosses were carried out:

w; *mef2*-GAL4; + x w; UAS-RNAi(KK); +

w; *mef2*-GAL4; + x w^{1118}; +; +

w^{1118}; +; + x w; UAS-RNAi(KK); +

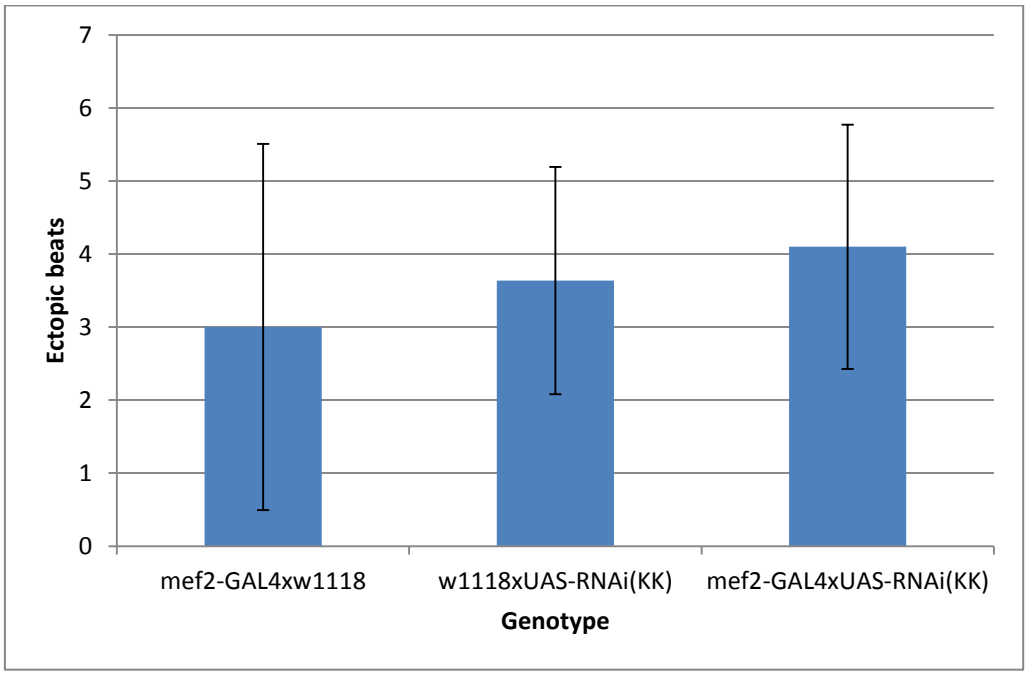

Figure 4.10: Average number of ectopic beats per 30 second video of 1 week old flies with muscle-specific knockdown of *dms1* compared with controls (n=10). Differences are not shown to be significant (p=0.73 using one-way ANOVA).

Figure 4.10 shows that the number of ectopic beats observed in hearts of each genotype are very similar, and there are no significant differences between experimental hearts and controls. This indicates that the higher level of knockdown using a *mef2*-GAL4 driver does not induce isolated ectopic beats.

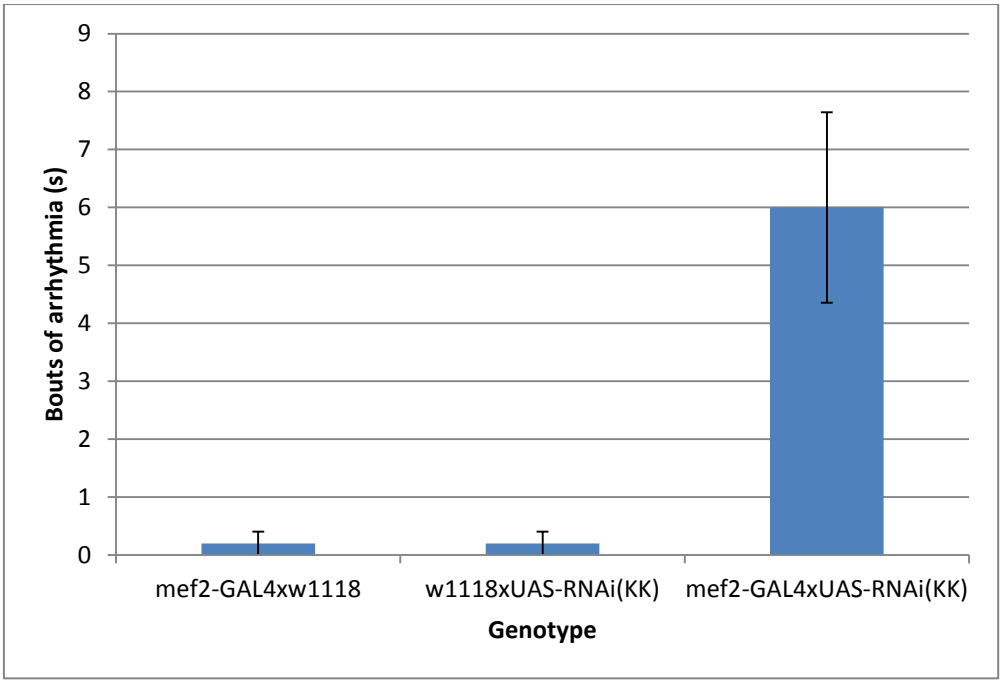

Figure 4.11: Average amount of time spent in bouts of arrhythmia per 30 second video by flies with muscle-specific knockdown of *dms1* compared to controls (n=5). Differences between knockdown and controls are significant ($p<0.002$ using one-way ANOVA, experimental line is significantly different from both controls using Tukey's HSD Post Hoc test at $p=0.05$ level).

As shown in figure 4.11, there appears to be a significantly higher level of bouts of arrhythmia with knockdown of *dms1* than in controls, which display a very low level of severe arrhythmia here. This is in contrast to the number of ectopic beats seen with muscle-specific knockdown (figure 4.10), and suggests that the higher level of knockdown under the *mef2*-GAL4 driver induces more severe arrhythmia, instead of the occasional ectopic beat. This level of arrhythmia is also higher compared to that observed with the cardiac-specific driver (figure 4.9), so it may be that a more widely expressed driver is required to induce this phenotype.

4.2.5 Contractility of hearts with knockdown of *dms1*

Contractility of the mammalian heart is known to be affected by hypertrophy, as LVH is known to be correlated with contractile defects with increasingly severe contractile issues associated with more severe LVH in disease states (Dahan *et al.*, 1997). Still images were taken from videos of beating hearts at their most contracted (systole) and most dilated (diastole) in order to get a measure of the contractility of the hearts, shown in figure 4.12. Crosses carried out were as described in section 4.2.4.1. Measurements were made and percentage fractional shortening (FS) calculated using the formula:

FS = (((Diastolic diameter - Systolic diameter)/Diastolic diameter) x 100)

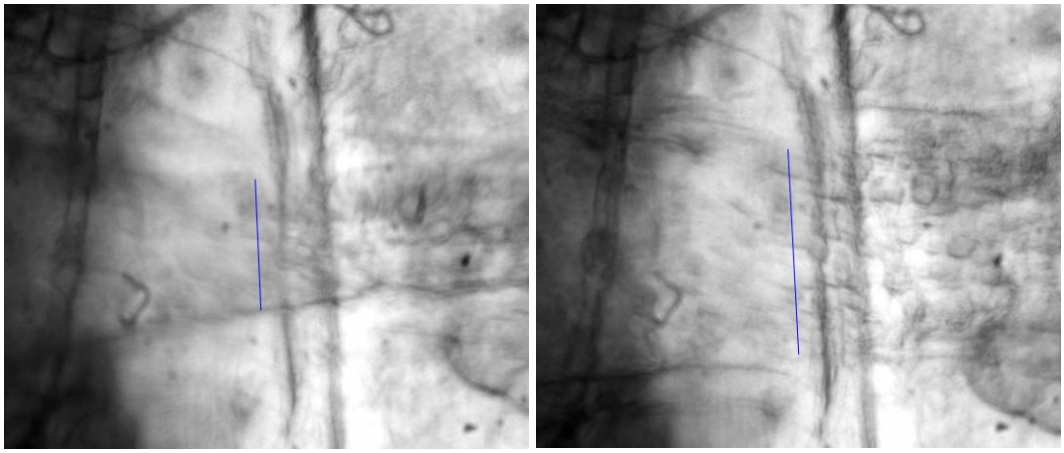

Figure 4.12: images of a *Drosophila* heart at its most contracted (left) and most dilated (right). Blue lines indicate the measurement taken of the heart diameter for calculation of fractional shortening.

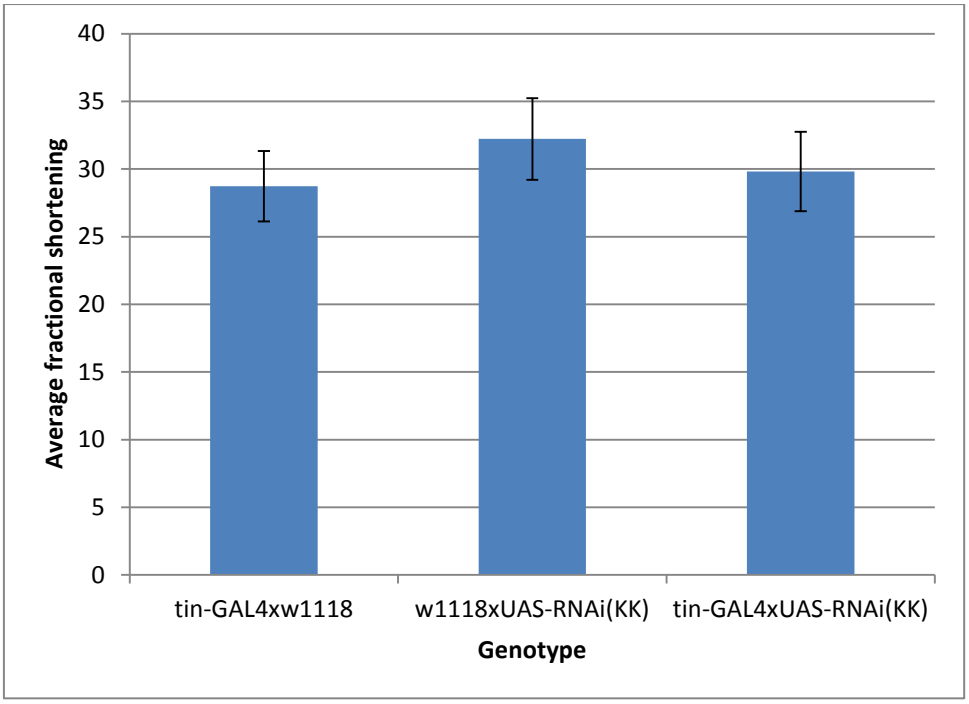

Figure 4.13: average fractional shortening of 1 week old hearts with cardioblast-specific knockdown of *dms1* compared to controls (n=7). Differences are not shown to be significant (p=0.75 using one-way ANOVA).

The results in figure 4.13 do not show any differences between genotypes, indicating that knockdown of *dms1* does not affect the contractile functioning of the heart tube.

4.2.6 Subjective analysis of arrhythmia under hypoxic stress

A preliminary investigation was carried out to assess the levels of arrhythmia of the *Drosophila* heart under hypoxic stress. The experiment was designed to take into account time spent under hypoxic conditions, the effect of hypoxia at different ages, and also the effect of cardiac-specific knockdown of *dms1* under hypoxic conditions. The dissection technique used to visualise the beating heart in *Drosophila* allows the heart to beat normally for several hours as long as the artificial haemolymph (AHL) is kept freshly oxygenated (Vogler & Ocorr, 2009).

Here, AHL was not oxygenated as the interest lay in how the hearts reacted to conditions of hypoxia. A subjective "scale of arrhythmicity" was used to characterise the observed heartbeat. Further experimental details are given in section 2.16.

Scale of arrhythmicity:
0 – totally rhythmic
1 – occasional ectopic beat
2 – mostly rhythmic with bouts of arrhythmia
3 – mostly arrhythmic with bouts of rhythmic beating
4 – totally arrhythmic
5 – stopped/ no definite beats

The following crosses were carried out and progeny reared to the appropriate age:

w; *tinCΔ4*-GAL4; + x w; UAS-RNAi(KK); +
w^{1118}; +; + x w; UAS-RNAi(KK); +

Flies of 1 week and 4 weeks old were tested to find if age changes the effect of *dms1* on heartbeat rhythmicity.

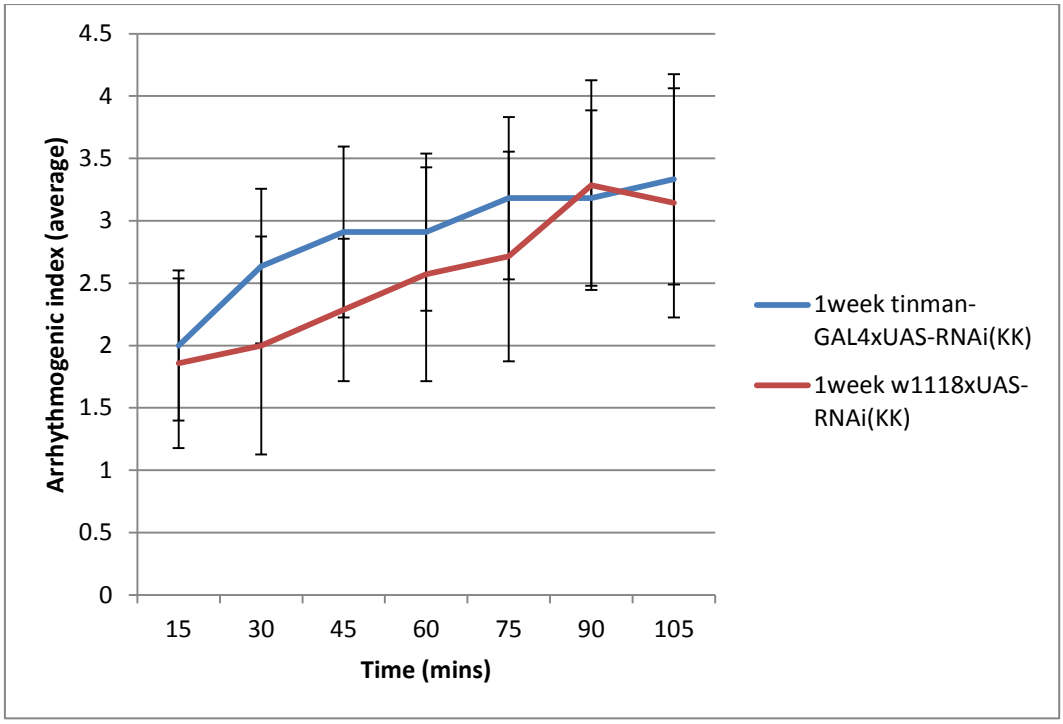

Figure 4.14: Arrhythmicity of 1 week old hearts over 105 minutes (n=11). Comparison of flies with knockdown of *dms1* in cardioblasts (blue) and control (red).

Figure 4.14 shows the average arrhythmicity of heartbeats at 1 week old. The decline over time can be seen in both genotypes, however, there is no significant difference between hearts with knockdown of *dms1* and control hearts. Unpaired students t-test shows p values between 0.22-0.85 for data points between 15 and 90 minutes. These data can be shown in a different way, further demonstrating the differences in a trend towards arrhythmicity in flies with knockdown of *dms1*. Figure 4.15 highlights the difference in rhythmicity between 30 and 60 minutes, as this is shown in figure 4.14 to be the range at which this difference is greatest.

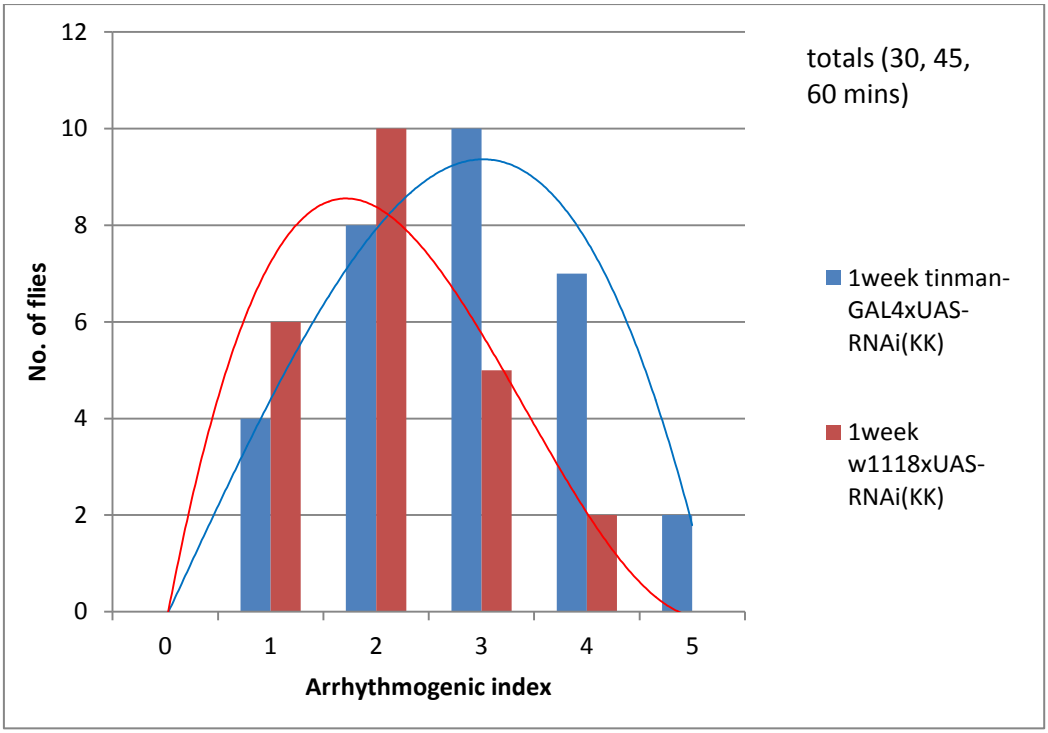

Figure 4.15: Bar graph showing arrhythmicity of 1 week old hearts between 30 and 60 minutes (n=11). Comparison of flies with knockdown of *dms1* in cardioblasts (blue) and control (red).

As figure 4.15 shows, at 1 week old there appears to be a trend towards higher levels of arrhythmicity with heart-specific knockdown of *dms1* compared to controls, although not significant. Flies with knockdown seem to become arrhythmic under hypoxia slightly faster than the controls, but both increase in arrhythmicity as time progresses. However, as there is a lack of statistical significance, no conclusions can be drawn from this data.

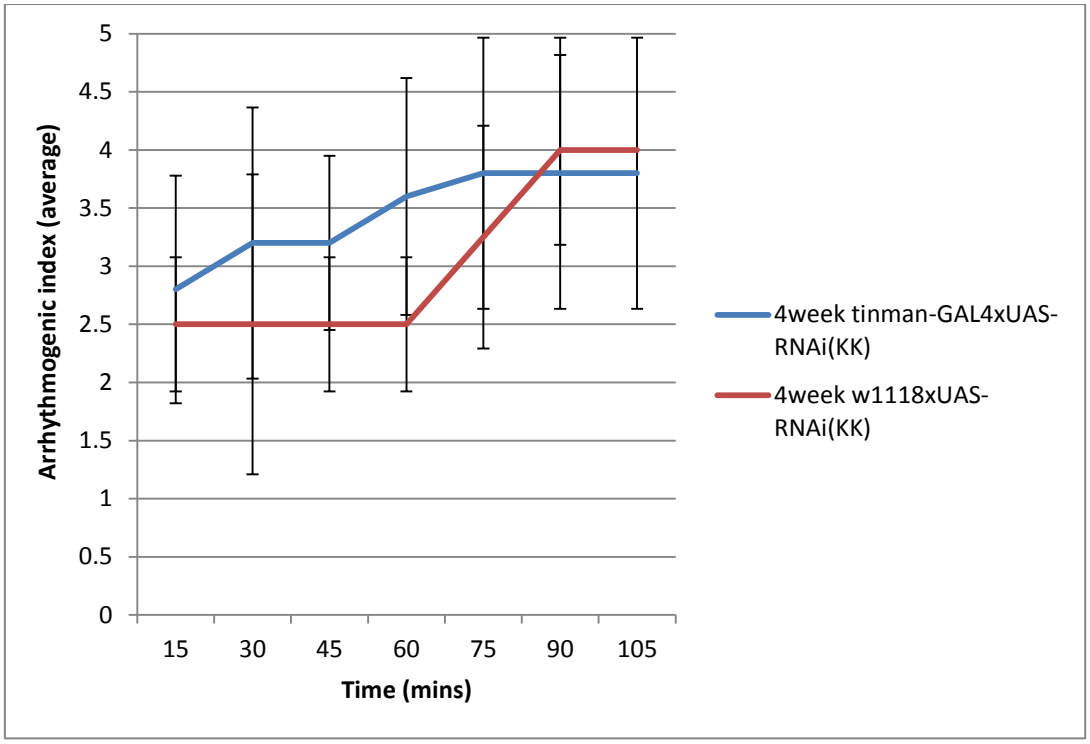

Figure 4.16: Arrhythmicity of 4 week old hearts over 105 minutes (n=5). Comparison of flies with knockdown of *dms1* in cardioblasts (blue) and control (red).

Knockdown hearts appear to be more arrhythmic, particularly between 30 and 75 minutes, as shown in figure 4.16. However, differences are not shown to be statistically significant (p values between 0.12-0.79 using unpaired T-test for data points between 15 and 90 minutes). As before, the difference in rhythmicity can be presented in an alternative way, highlighting the trend towards arrhythmicity in hearts with knockdown of *dms1* between 30 and 60 minutes, shown in figure 4.17.

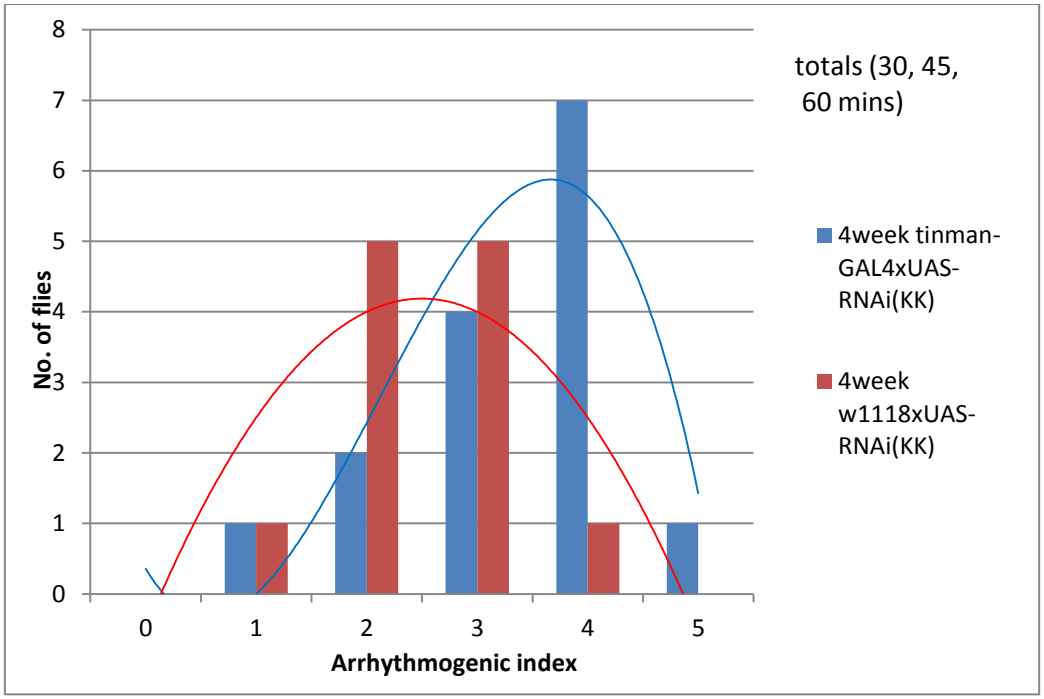

Figure 4.17: Bar graph showing arrhythmicity of 4 week old hearts between 30 and 60 minutes (n=5). Comparison of flies with knockdown of *dms1* in cardioblasts (blue) and control (red).

Figure 4.17 indicates that at 4 weeks old there is a similar effect to that seen at 1 week old, where hearts with knockdown of *dms1* appear to become more arrhythmic faster than the control line. The greatest difference is seen between 30 and 60 minutes. However, these data are not statistically significant, therefore no conclusions can be drawn at this time.

Hearts with knockdown of *dms1* at different ages were also compared against each other to demonstrate the effect of age on the rhythmicity of these hearts (figure 4.18).

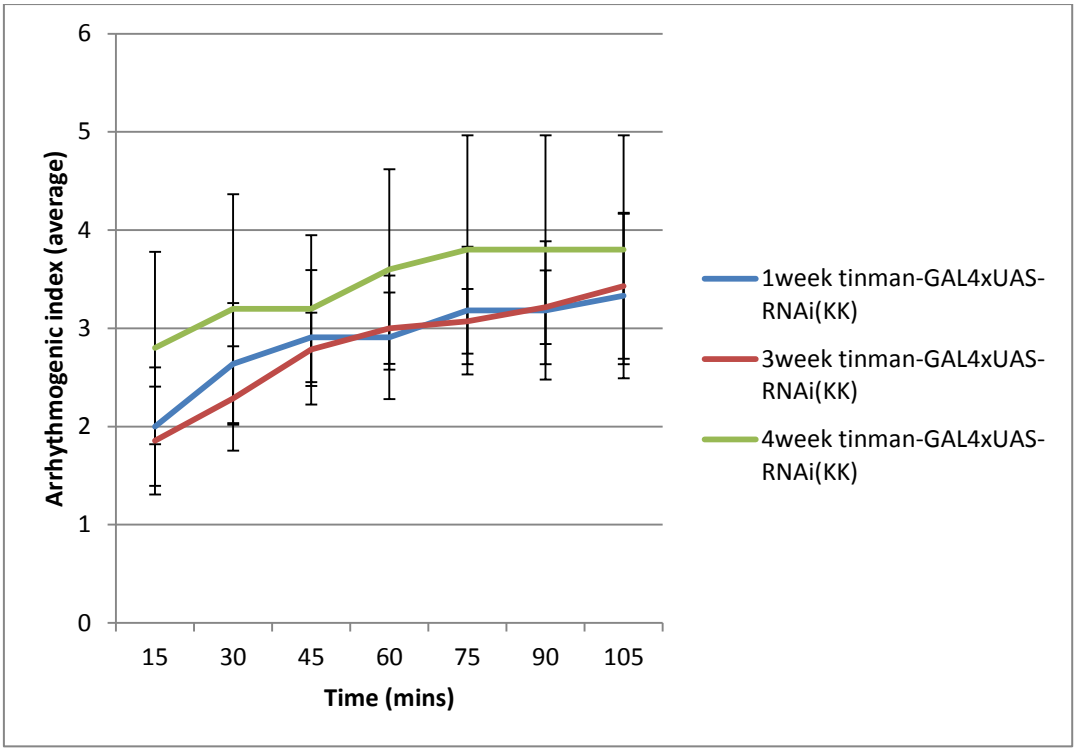

Figure 4.18: Arrhythmicity of hearts over 105 minutes at different ages with knockdown of *dms1*. Ages are 1 week (blue, n=11), 3 weeks (red n=14) and 4 weeks (green, n=5).

Overall, the data show that hearts become more arrhythmic the longer they spend in hypoxic conditions. In both 1 week old and 4 week old flies knockdown of *dms1* appears to increase arrhythmicity of the heartbeat, particularly earlier in the experiment. Both 1 week and 4 week old flies with knockdown of *dms1* tend to be more arrhythmic before 90 minutes, when the results converge with those of the control, however, in this case this has not been shown to be statistically significant. Figure 4.18 also demonstrates the effect age has on the rhythmicity of the heart, with the hearts at 1 week and 3 weeks old almost identical in rhythmicity, while at 4 weeks hearts appear more arrhythmic, although again not statistically significant.

4.2.7 Confocal imaging of heart structure

The Drosophila heart has a well characterised structure consisting of an inner layer of contractile myocytes and an outer longitudinal layer of muscle (Alayari *et al.*, 2009). If the pattern of heart muscle is disrupted due to damage or developmental problems this can affect the functioning of the heart and cause defects in heartbeat and efficiency. The heart structure can be visualised using a muscle-specific staining, Alexa594-phalloidin, which binds to actin and, due to the Alexa-Fluor dye (Invitrogen), causes all muscles to fluoresce under the appropriate light. If changes in *dms1* expression affect the structure of the heart, it can be visualised in this way.

4.2.7.1 Confocal imaging of heart structure with knockdown of *dms1*

Dissections were carried out on flies expressing RNAi for *dms1* to expose the heart in situ and stained for visualisation. A larger number of dissections than can be shown here were prepared, and examples of comparisons of hearts with and without *dms1* knockdown at different magnifications are shown in figures 4.19 - 4.21. Here, a *mef2*-GAL4 driver was used to knock down *dms1* expression in all muscle tissue, so expression is reduced in all heart tissue and any defects caused by knockdown will be more easily identified. The following crosses were carried out and progeny used for dissection:

w; *mef2*-GAL4; + x w; UAS-RNAi(KK); +
w; *mef2*-GAL4; + x w^{1118}; +; +
w^{1118}; +; + x w; UAS-RNAi(KK); +

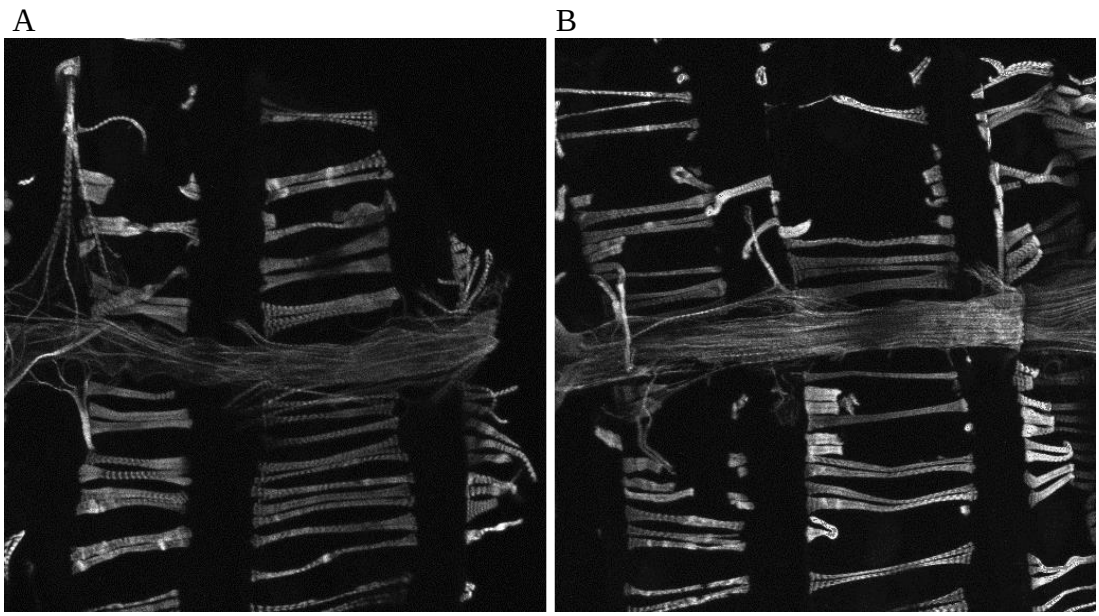

Figure 4.19: Confocal image of *Drosophila* hearts with muscle-specific knockdown of *dms1* (A) compared to driver control (B) at 10x magnification. Hearts are positioned with the anterior towards the right of each image.

Hearts visualised at 10x magnification are shown in figure 4.19. At this magnification the majority of the heart tube can be visualised and gives an idea of the structure as a whole and how organised in terms of alignment the muscle fibres look from a distance. In many of the images obtained at this magnification hearts with *dms1* knockdown appear to be slightly less organised than controls. Controls hearts have very obviously well aligned fibres, particularly in the outer longitudinal muscle layer, whereas hearts with lower levels of *dms1* seem to have fibres that are slightly less neatly arranged. This effect, while subtle and unlikely to have a significant effect on the ability of the heart to function adequately, is consistently observed in 3 images of hearts with knockdown, 5 of driver control hearts and 4 of UAS controls hearts at 10x magnification.

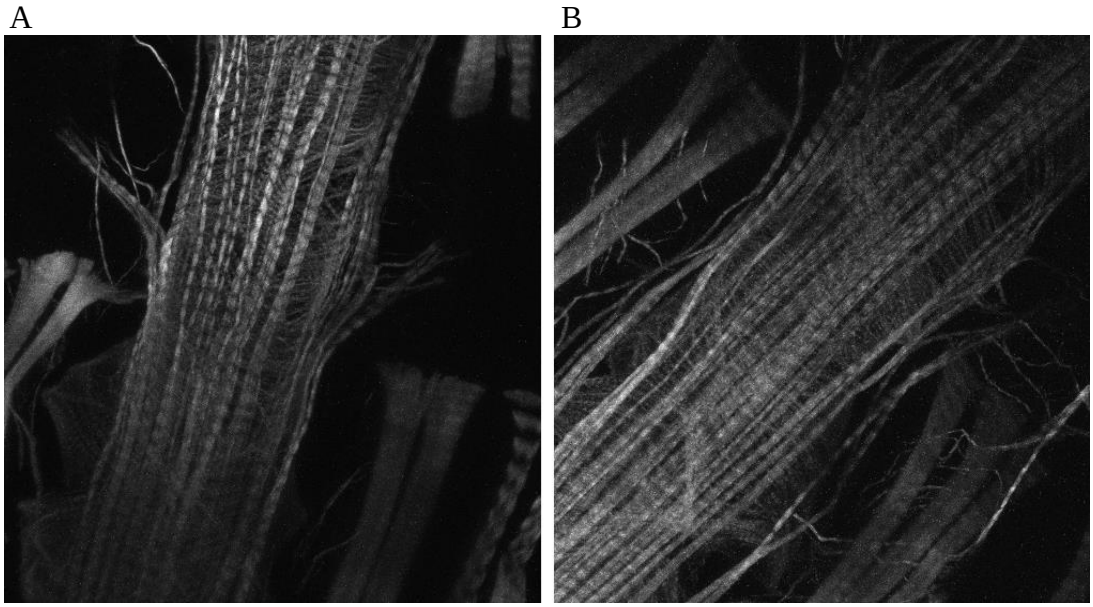

Figure 4.20: Confocal images of *Drosophila* hearts with muscle-specific knockdown of *dms1* (A) compared with driver control hearts (B) at 60x magnification.

Figure 4.20 shows hearts at 60x magnification, the highest available using this system, which allows visualisation of the heart structure in great detail. These images demonstrate that the alignment of muscle fibres appears to be regular in both hearts with knockdown of *dms1* and in controls. These images were taken of the middle area of the heart where the fibres are less dense. This enables heart tube defects to be more easily seen, as there is a tendency for hearts structure to become more disorganised further down the tube. In the images in figure 4.20 (A) there are possibly fewer fibres present in the heart with *dms1* knockdown, but this is not an obvious effect and there are no other images available to confirm that this is a true effect of reduction in *dms1* expression. Overall, at 60x magnification differences between hearts with knockdown of *dms1* and controls are not easily visualised and suggest that there is no significant effect on the alignment of heart muscle fibres.

4.2.7.2 Confocal imaging of heart structure with overexpression of *dms1*

Images were also taken of hearts overexpressing *dms1* in order to visualise any differences in structure. The following crosses were carried out:

w; *mef2*-GAL4; + x w; +; UAS-*dms1*
w; *mef2*-GAL4; + x w^{1118}; +; +
w^{1118}; +; + x w; +; UAS-*dms1*

Staining was carried out as before and hearts were magnified to 10x, shown in figure 4.21.

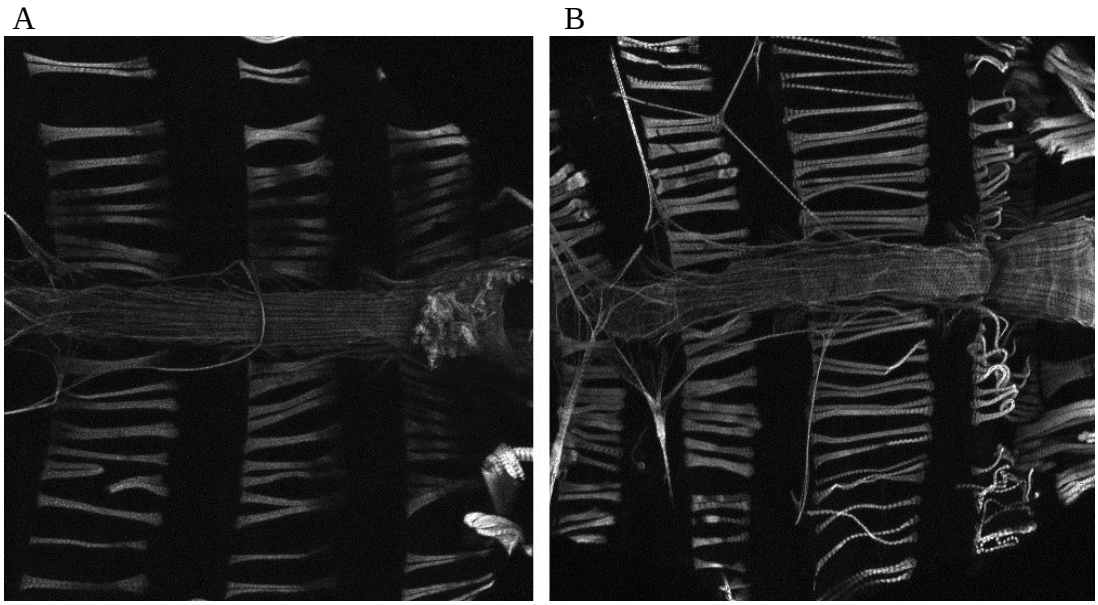

Figure 4.21: Confocal image of *Drosophila* hearts with muscle-specific overexpression of *dms1* (A) compared to UAS control (B) at 10x magnification. Hearts are positioned with the anterior towards the left of each picture.

When visualised at 10x, shown in figure 4.21, hearts with overexpression of *dms1* do not appear to have any significant differences from controls. This was consistent

over 6 hearts with *dms1* overexpression, 7 of driver control and 3 of UAS control. There also does not appear to be any difference in width of the heart tube or number of fibres between those with overexpression and controls, although more detailed images would be needed to confirm this.

4.2.7.3 Confocal imaging of heart structure with overexpression of mouse *ms1*

A further investigation was carried out into the effect of overexpressing a mouse *ms1* transgene in *Drosophila* to identify any changes in heart structure. This is to see if a homologue has the same function as *dms1*and to find any additional effects of the transgene. If any difference is seen between overexpression of *dms1* and mouse *ms1*, depending on the effect seen it could indicate a that the two genes are not true homologues or alternatively that one of the genes has an additional function that is not seen in the other, gained during evolution. The following crosses were carried out:

w; *mef2*-GAL4; + x w; +; UAS-*mms1*
w; *mef2*-GAL4; + x w^{1118}; +; +
w^{1118}; +; + x w; +; UAS-*mms1*

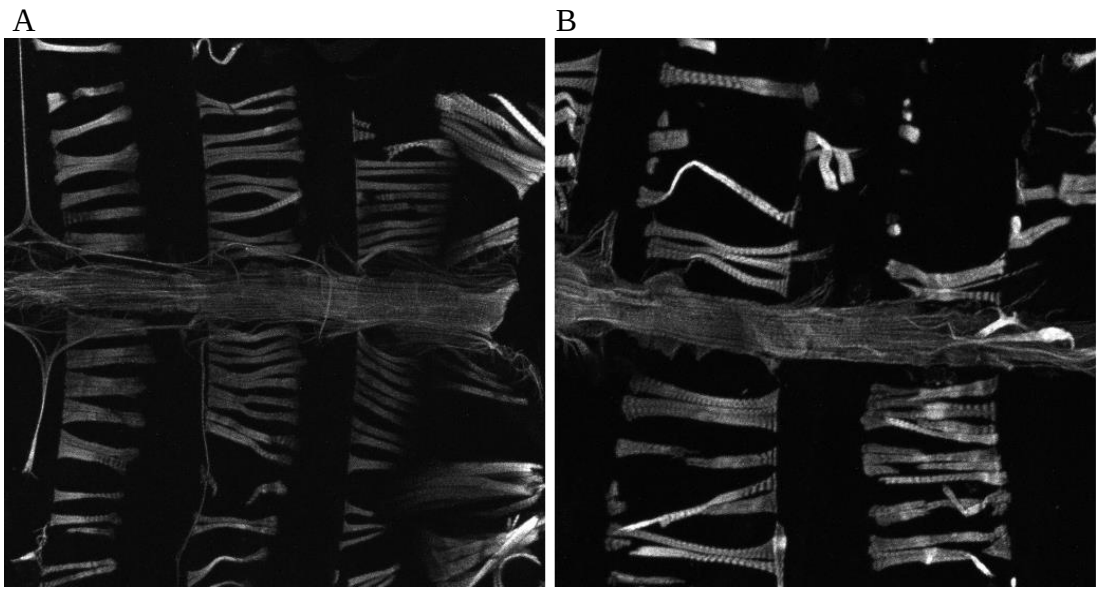

Figure 4.22: Confocal image of *Drosophila* hearts with muscle-specific overexpression of mouse *ms1* (A) compared to driver control (B) at 10x magnification. Hearts are positioned with the anterior towards the left of A and right of B.

As with overexpression of *dms1* there does not appear to be any effect of expression of mouse *ms1* on heart structure observed in figure 4.22. This effect is seen in 6 hearts with overexpression, 7 of driver control and 4 of UAS control. The overall heart structure and width is not affected and there are similar numbers of fibres observed in each dissection.

4.3 Discussion

The aim of this chapter was to establish an intact model system to investigate cardiac function in *Drosophila*, to get an overview of how the heart works, and to identify the effect of changes in *dms1* expression on the development and function of the heart. This was done by looking at various aspects of the overall health of the flies and more specific investigation into heart contractility and structure.

To test the overall health of flies with cardiac-specific knockdown of *dms1* a series of longevity experiments was carried out. This allowed an overview of how these flies were affected in terms of how long they were able to survive compared to controls, and if knockdown of *dms1* significantly altered survival, this would imply that this gene is important for the general health of the flies under normal conditions. The results presented here suggest that levels of *dms1* in the heart are required to maintain a normal level of health, as some significant differences were found between flies with cardiac-specific knockdown of *dms1* and controls. However, this was not consistent across the experiments, as differences are found between results from backcrossed and non-backcrossed flies, and also between the different RNAi lines used. Use of the UAS-RNAi(KK) line in particular resulted in no significant difference between flies with knockdown and controls. However, experiments 4.2.2.1 and 4.2.2.2 suggest that there is an effect of *dms1* reduction on longevity. There are effects on survival seen in early adult stages before backcrossing of the fly lines, as flies with knockdown of *dms1* appear to die quicker than controls, suggesting an interesting effect of low levels of *dms1* on longevity and health. One theory is that *dms1* is necessary for the hearts response to stress at early stages, so high mortality is seen in flies with lower levels. Prolonged *dms1* expression may put the heart under excessive pressure at older age, as in mammalian models of hypertrophy, so survival at these later stages may stabilise in flies with lower *dms1* expression as they would not have this pressure. However, backcrossing of the fly lines removed this effect, so it is possible that this is simply caused by genetic background. After backcrossing knockdown of *dms1* appears to have a beneficial effect on longevity, as flies with knockdown using the GD line survive better than controls. It could be that backcrossing has removed a background effect that had

some influence on the *dms1* pathway, thereby reducing longevity as seen in experiment 4.2.2.1.

The differences between use of the KK and GD lines may indicate a complex effect of reducing *dms1* expression. KK lines generally have a higher expression of RNAi than the GD lines due to the design of the construct, and therefore this will result in lower levels of *dms1* expression. If this is accurate, it is possible that lower levels of *dms1* knockdown are able to reduce the hearts stress response to a level at which it will not be overloaded, but so that it is still able to initiate this response to some extent if required. Higher levels of knockdown may reverse this benefit to the heart, and so the effect on longevity observed with use of the GD line is lost when higher levels of RNAi are used. If this is the case, it could be that full knockout is required to significantly reduce overall health, as no effect is seen with use of the KK line. However, it is possible that the result found with use of the KK line is accurate, in which case it could imply that *dms1* is not essential and the heart will still develop sufficiently without *dms1* to maintain general health. Alternatively, it could reflect the contribution of the heart to overall health. The heart is not as important in *Drosophila* as it is in mammals and while necessary to keep the flow of nutrients circulating, adult flies can still develop even with fairly severe heart defects, although these flies may have relatively poor health depending on the severity of the cardiac defect (Neely *et al.*, 2010). If the knockdown of *dms1* is causing functional or developmental defects in the heart, this may still not affect longevity.

Experiments were carried out with backcrossed and non-backcrossed flies using a GD RNAi line, allowing any background effects to be identified. A difference can clearly be seen between backcrossed and non-backcrossed flies, implying that there

is some factor in the background of the experimental lines in particular that is affecting longevity. Backcrossing increases the longevity of knockdown and UAS control flies, but not of the driver control although the reasons for this are unknown. The crosses that include the UAS-RNAi lines are the ones which have a shorter lifespan before backcrossing, suggesting there is something in the genetic background of these flies specifically that is affecting longevity. This demonstrates the importance of keeping all flies in the same background, in this case w^{1118}. This ensures that any differences seen are due to the experimental changes in gene expression, and not some unknown factor.

Longevity was also tested with hypoxia treatments of 1 hour per day. It is known that *ms1* is a stress responsive gene, upregulated when the heart needs to increase its output (Mahadeva *et al.*, 2002). Hypoxic stress could be a trigger for *dms1* upregulation, so to test this hypothesis flies were subjected to low levels of oxygen for 1 hour per day to see if hypoxic stress affects the survival of flies with *dms1* knockdown. The results show significant differences between flies with knockdown of *dms1* and controls, as flies with knockdown appear to survive better than controls. Backcrossing of the fly lines has the same effect as before, increasing the longevity of the experimental and UAS control flies, but not the driver control, once again suggesting the effect of the UAS background on longevity.

Non-backcrossed flies with cardiac-specific knockdown of *dms1* appear to survive better with daily hypoxia than controls throughout their lifespan, particularly after day 21. This effect is also seen in data from backcrossed flies, albeit less obvious. Flies are known to have increased cardiac arrhythmia after 3 weeks (Fink *et al.*, 2009) so this fits with the observed data shown here. After 3 weeks it could be

that reduction of *dms1* is reducing the flies stress response, therefore the heart does not become overloaded and can still beat effectively at later stages. It is known that prolonged hypertrophy is detrimental to cardiac function in human disease states (Rohini *et al.*, 2010), and it could be that the results here reflect this fact, since flies with a fully functioning stress-response gene have slightly reduced longevity. However, *Drosophila* are known to be extremely adaptive and have a molecular adaptive response to many types of stress conditions, especially those they may encounter regularly. Particularly, they are known to be able to tolerate low oxygen concentrations and have a high recovery rate from this type of stress (Haddad *et al.*, 1997). Therefore this could explain why the controls also seem to survive well under conditions of 1 hour hypoxia per day. Although longevity is reduced when comparing flies under normal conditions and with hypoxic treatment, this is understandable as hypoxia still places the flies under stress despite their mechanisms to cope with this.

As well as comparing longevity under hypoxia to that under normoxic conditions, this comparison can be extended to observations of the heart itself. Hypoxic stress and its effect on heart rhythmicity reflects the effect on reduction of *dms1* on stress response and allows visualisation of the heart-specific effect of hypoxia. No real effect was found by treating hearts to hypoxia, suggesting that the role of *dms1* in cardiomyocytes is negligible or that it cannot be tested in this way. A trend is seen in hearts under hypoxia with knockdown compared to control hearts, as knockdown appears to make hearts slightly more arrhythmic under hypoxic stress, although this is not statistically significant. Experiments carried out in this manner gave a good representation of the decline in rhythmicity under hypoxia, as the *Drosophila* heart goes through stages where the rhythmicity can be

characterised with the scale used in this experiment. However, a less subjective analysis would make these results more reliable and repeatable by others. Video analysis could be used here to achieve this. A video movement capture-based method of analysis was attempted using the videos taken for this project but could not be carried out due to software incompatibilities.

Several approaches were taken to look at the heart specifically under normoxic conditions and test whether knockdown of *dms1* has an effect on heart function and structure. Video analysis of heart function under normoxic conditions does not give any significant difference between hearts with knockdown of *dms1* and controls. There seem to be slightly more ectopic beats observed in hearts with knockdown, suggesting that lower levels of *dms1* may cause heart tubes to be susceptible to mild arrhythmia more than those with normal levels, but further analysis would be required to determine if this is a consistent effect. Data recording more severe arrhythmia, i.e. bouts of arrhythmia, do not show any differences, so it is more likely that cardiac-specific knockdown does not have a severe effect on heart function. With further levels of knockdown using a *mef2*-GAL4 driver, no significant differences are found in the number of ectopic beats but there is a large difference in bouts of arrhythmia. Longer bouts of arrhythmia are observed with knockdown of *dms1* in all muscle cells which, unlike the *tinmanCΔ4*-GAL4 driver, expresses RNAi not only in the inner circular muscle layer of the heart, but also in the outer longitudinal layer. When analysed along with the data obtained using cardiac-specific knockdown, it appears that lower levels of *dms1* lead to more severe arrhythmia and cardiac dysfunction. If *dms1* is involved in development of the heart then knockdown may be affecting the ability of the heart to beat rhythmically. This could be a result of defects in the musculature of the heart, through problems in the

pacemaker and electrical signalling leading to the heartbeat becoming more arrhythmic, or potentially a combination of the two if changes in heart structure affect how the electrical signal is conducted. It is possible that the microstructure of the heart or cardiac gene expression has been altered with *dms1* knockdown, but further experiments would be needed to confirm this.

Contractility does not appear to be affected by knockdown of *dms1*. The circular contractile myocytes are found only in the inner layer of the heart tube (Tao & Schulz, 2007) and the experiment described here looks at hearts with knockdown in the inner layer only using the *tinmanCΔ4*-GAL4 driver, so if contractility is affected by reduced levels of *dms1* the use of the *tinmanCΔ4*-GAL4 driver will be sufficient to reveal any associated phenotypes. As there is no effect on the percentage contractility it seems that reduction of *dms1* is not affecting the alignment or the strength of these cells, and therefore the ability to contract at the same efficiency as controls is maintained in hearts with *dms1* knockdown. Considering the results of the video analysis along with these data, it supports the idea that *tinmanCΔ4*-driven knockdown is not enough to cause significant defects in heart structure and function. In contrast to the video analysis, it is unlikely that further knockdown with a muscle-specific driver would cause contractility issues, as the contractile cells in the heart are those that express *tinman*. On the other hand, it may be that the organization of the outer muscle layer affects the contractile myocytes in a way that has not yet been characterised, so it may be too early to suggest that no effect would be seen.

Knockdown of *dms1* produced no significant cardiac phenotype. There are hints that there may be a subtle effect on the heart, but the reasons behind this are unclear. However, further experiments and analysis would be required to find if this is a true

effect. A method used here to look into this was confocal microscopy and high magnification of the heart structure, allowing visualisation of the heart fibres and any defects that may be present in the structure. Knockdown was driven using a muscle-specific driver as this was more likely to give a visible phenotype than cardiac-specific knockdown alone. More arrhythmia is seen with muscle-specific knockdown than cardiac-specific in section 4.2.4, so this supports the idea that any effect of reduction in *dms1* on heart structure would be more obvious using a *mef2*-GAL4 driver. However, there are no apparent changes to heart structure, therefore it is probable that *dms1* knockdown has no significant effect on heart structure or development. Hearts overexpressing *dms1* and mouse *ms1* also do not appear to differ from controls in this investigation in any way. Forced expression of *dms1* could cause the heart to respond as if it were under stress, and lead to enlargement of the heart tube, or changes during development. Evidence from this data suggests that there is no real effect of *dms1* overexpression as viewed at the magnifications used here, but a more powerful system could allow visualisation of changes at the cellular level. Additionally, comparison with hearts that have undergone stress with endogenous levels of *dms1* could reveal whether *dms1* is involved in the stress response in *Drosophila*. Dissections of hearts overexpressing mouse *ms1* also show no changes from controls, and this could indicate that there is no effect of overexpression of either of these genes but does not exclude the possibility of a shared function that cannot be seen from the results of this experiment.

In general, using the techniques described here, the data obtained do not support a critical role for *dms1* in cardiac function in *Drosophila*. The lack of significant results, particularly with cardiac-specific knockdown of *dms1*, suggests that if there is a phenotype associated with lower expression levels then it is perhaps a

subtle one. It is also possible that the methods used here are not sensitive enough to detect changes within the heart or are not appropriate in this case. Reduction of *dms1* consistently appears to produce a trend towards arrhythmicity of the heartbeat in normal and hypoxic conditions, but these results are also consistently statistically insignificant and extensive further experimentation would be required to verify this as a true effect. Knockdown of *dms1* in the heart tube and surrounding muscle has more of an effect, but this is still not enough to convincingly say that *dms1* is important in heart development and function. It is possible that knockdown of *dms1* is not enough and only full knockout would show an effect of heart function, or at least increase the subtle effect seen in these results. This is supported by confocal images, which show only no effect with changes in *dms1* or mouse *ms1*. To improve on the data shown here, further experiments along the same lines as those in this chapter could increase the significance of the potential subtle effect of *dms1* knockdown, and techniques such as electron microscopy and automated video analysis software (Ocorr *et al.*, 2009) could be employed to look in more detail for effects on rhythmicity and cellular structure. Overall, this chapter has demonstrated that, while a significant phenotype has not been found using these experiments, the data is encouraging and would be worth further investigation.

Chapter 5: Role of *Drosophila myocyte stress 1* in somatic muscle

5.1 Introduction

The role of *ms1* in muscle hypertrophy has recently been investigated as a potential regulator of muscle growth after exercise. It has been found that *ms1* is upregulated in response to exercise, particularly exercise designed to build muscle, such as resistance training (Lamon *et al.*, 2009). Unaccustomed eccentric exercise is also known to increase expression of *ms1*, potentially as part of a repair and regeneration pathway (MacNeil *et al.*, 2010). This implies that *ms1* is playing a role in skeletal muscle similar to that in the heart as part of a pathway that builds muscle in response to stress, in this case, the stress of exercise. This does, however, also suggest that *ms1* is not only involved in initiation of pathological hypertrophy as in conditions of heart disease, but also in physiological hypertrophy after exercise. This could be due to differences in regulatory factors in the different tissue types, but since the mechanisms behind pathological and physiological hypertrophy are not well characterised, this would be difficult to test.

As with heart research, *Drosophila* can be easily used to investigate muscle function. Climbing and activity assays can provide better understanding on how well the muscles are working, giving an insight on the flies ability and inclination to move. Dissection allows visualisation of the different muscle types within the body of the fly, not only showing any defects in muscle development and structure, but also any differences between muscle types.

Here, various techniques have been used to investigate the role of *ms1* in muscle function, and to test whether different levels of *ms1* have an effect on muscle

development, and the strength and activity of flies. The majority of this section of work has been done using adult flies, due to the higher level of similarity between adult fly muscle and that of mammals, in contrast with larval musculature which shares less structural similarity with mammalian muscle (Soler *et al.*, 2012). RNAi and overexpression vectors are driven using a *mef2*-GAL4 driver, expressed in all muscle types.

5.2 Results

5.2.1 Expression pattern of muscle-specific drivers

The majority of the experiments in this chapter use a mef2-GAL4 driver to drive expression of UAS-RNAi for knockdown or UAS-*dms1*/UAS-mouse *ms1* for overexpression/expression of mouse *ms1*. To ensure this driver expressed in the expected pattern, i.e., all muscle types including the heart and skeletal muscle, the driver line was first crossed to a UAS-GFP line. This results in a fly that expresses GFP where *mef2* is expressed, allowing visualisation of this pattern.

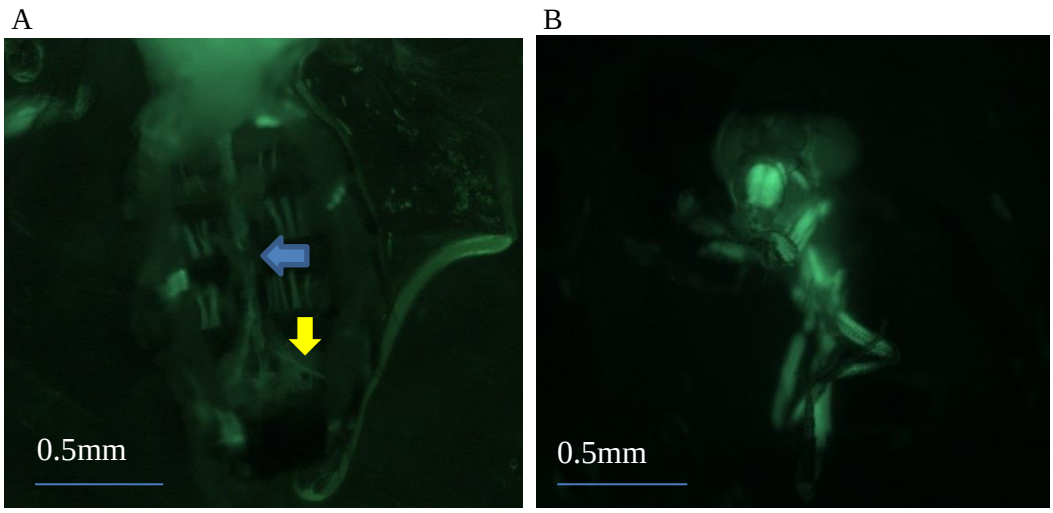

Figure 5.1: expression pattern of *mef2*-GAL4 driver in the adult fly visualised using UAS-GFP. Figure A shows only the abdomen of the fly, the heart tube is indicated by the blue arrow, alary muscles by the yellow arrow. Fly is positioned with the anterior towards the top of the picture. Figure B shows the head and front legs of the fly.

The expression of GFP shown in figure 5.1A demonstrates the wide range of expression of *mef2* while demonstrating it to be strictly muscle specific. Expression is seen in the heart as well as the muscle that attach the heart to the cuticle (alary muscles indicated by yellow arrow). Also seen in this example are the abdominal intersegmental muscles, and a section of gut can also be visualised here. Figure 5.1B shows the expression of *mef2* in the head and legs, and fluorescence can be clearly seen in the legs and into the thorax, confirming expression of this driver in thoracic and leg muscles, as well as in parts of the head.

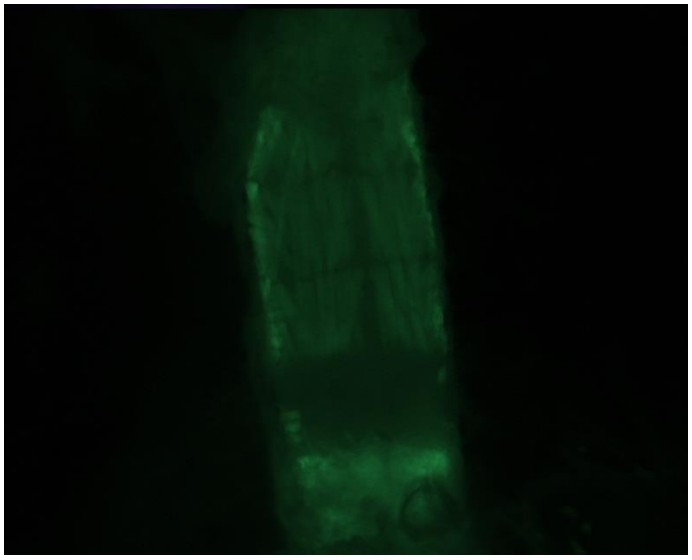

Figure 5.2: expression pattern of *mef2*-GAL4 driver in larvae, visualised using UAS-GFP. Head is positioned towards the top of the picture.

Figure 5.2 shows the expression pattern of GFP under the control of the *mef2*-GAL4 driver in larvae. Intersegmental muscles can be seen in sections in the anterior half of the body, and further muscles are seen towards the posterior. This confirms muscle-specific expression of the *mef2*-gal4 driver in larval stages as well as in the adult fly. Expression of *mef2* is known to occur at most stages of development, and is particularly high during myogenesis, with adult levels lower but constant (flybase.org). Therefore any gene expression that is under the control of this driver will occur throughout the life of the animal.

5.2.2 Knockdown of *dms1* in all muscle types

5.2.2.1 Longevity under normoxic conditions with knockdown of *dms1*

A measure of overall health can be obtained from longevity experiments, as survival indicates if there is anything that is making them die faster. Even a small detrimental

effect on health would cause a decline in longevity, so here the effect of *dms1* reduction on survival in normal conditions is investigated.

Longevity experiments were carried out as described in section 2.10. The experiments shown below used a mef2-GAL4 driver, driving muscle specific knockdown of *dms1*. Crosses were carried out using non-backcrossed flies, making available the use of a mef2-GAL4-UAS-Dicer line, the expression of which increases the effect of the RNAi by providing a higher level of Dicer enzyme to degrade the double-stranded RNA generated by the RNAi construct. The following crosses were carried out:

w; +; *mef2*-GAL4 x w; UAS-RNAi(GD); +
w; +; *mef2*-GAL4 x w^{1118}; +; +
w^{1118}; +; + x w; UAS-RNAi(GD); +
UAS-Dcr2; *mef2*-GAL4; + x w; UAS-RNAi(GD); +
UAS-Dcr2; *mef2*-GAL4; + x w^{1118}; +; +

Figures 5.3 and 5.4 show the results of testing the longevity of non-backcrossed flies with different levels of muscle-specific knockdown of *dms1*.

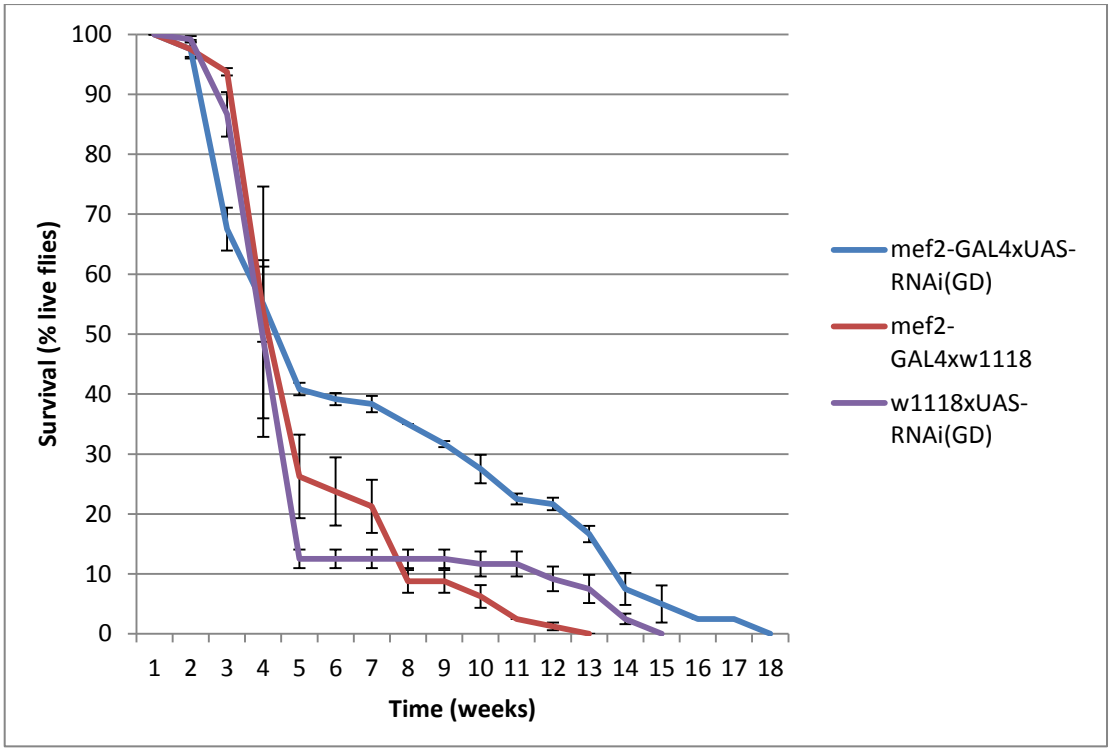

Figure 5.3: Longevity of non-backcrossed flies with muscle-specific knockdown of *dms1* (blue) compared to controls (red/purple). The RNAi line used here is UAS-RNAi(GD) (n=40 flies). Differences are shown to be significant ($p<0.05$ using Mantel-Cox test).

Figure 5.3 shows longevity with lower levels of knockdown, ie. the efficiency of the knockdown is not increased by the use of a UAS-Dcr construct. Survival is similar between all 3 genotypes for the first 4 weeks, but after this point the experimental line appears to survive better than both controls until the end of the experiment. These differences are significantly different, suggesting that this genotype has some advantage with regards to longevity.

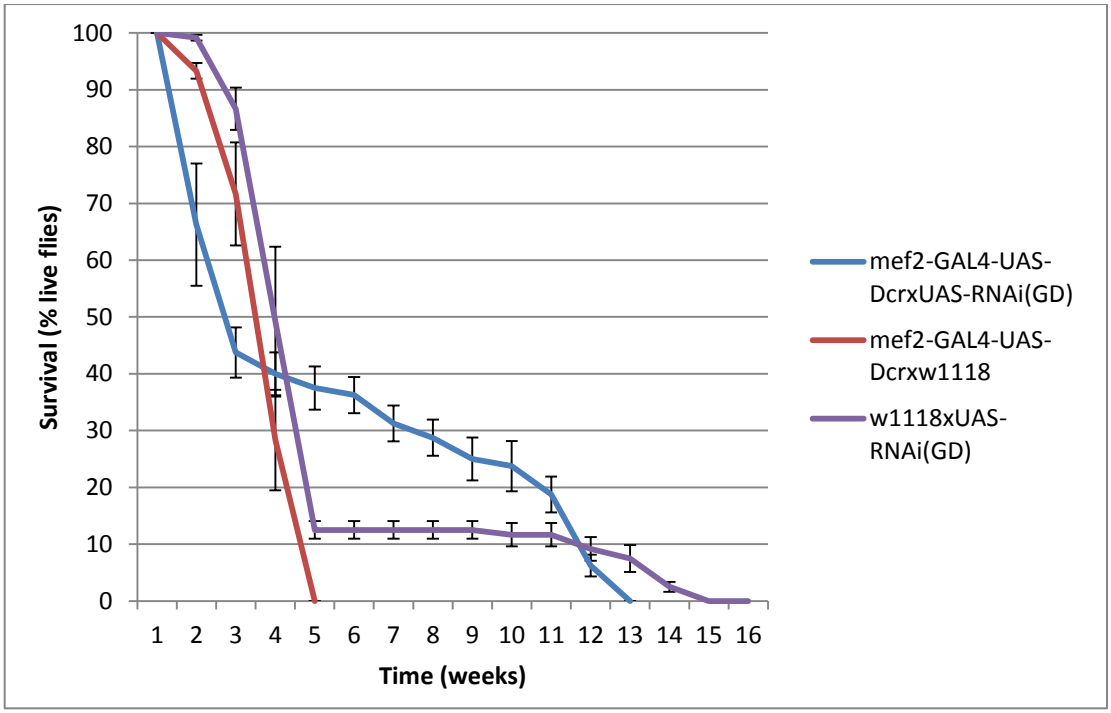

Figure 5.4: Longevity of non-backcrossed flies with muscle-specific knockdown of *dms1* (blue) compared to controls (red/purple). This experiment has the addition of a UAS-Dicer (Dcr) construct to increase the effect of RNAi. The RNAi line used here is UAS-RNAi(GD) (n=40 flies). Differences are shown to be significant ($p<0.01$ using Mantel-Cox test and Gehan-Breslow-Wilcoxon test).

When looking at the longevity of non-backcrossed flies, in both flies with and without a UAS-Dcr construct, the same effect is seen. Figure 5.4 indicates that survival is similar between all genotypes for the first 3-4 weeks then the controls both seem to die faster than the experimental line, which survives better until around 11 weeks without UAS-Dcr and 14 weeks with UAS-Dcr. The knockdown of *dms1* appears to give these flies an advantage around what could be considered 'middle age' as survival is better than the controls during this time. This effect is consistent with both levels of knockdown (figures 5.3 and 5.4).

The above experiment was also carried out using a UAS-RNAi(KK) line. The KK lines generally have a higher expression of RNAi, and this allows a comparison of

different levels of *dms1* knockdown on longevity. These flies are also backcrossed so as to remove any background effect on longevity. Unfortunately this also removes the opportunity to use a line with UAS-Dcr, so only one level of knockdown can be investigated here. The following crosses were carried out:

w; *mef2*-GAL4; + x w; UAS-RNAi(KK); +

w; *mef2*-GAL4; + x w^{1118}; +; +

w^{1118}; +; + x w; UAS-RNAi(KK); +

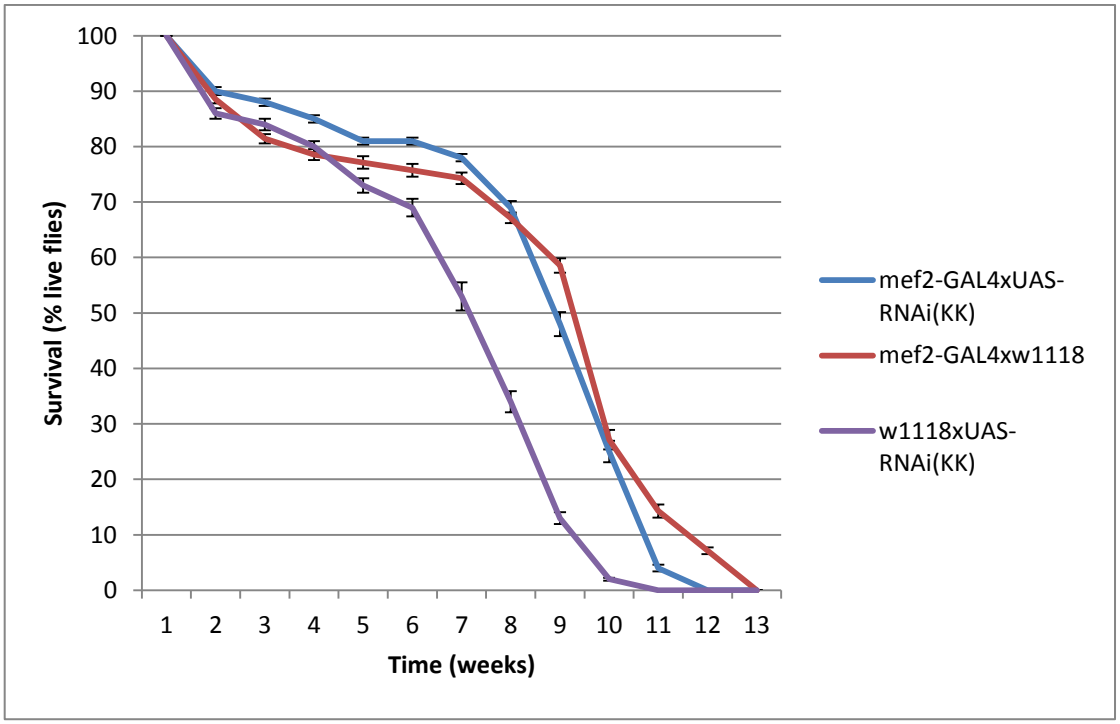

Figure 5.5: Longevity of backcrossed flies with muscle-specific knockdown of *dms1* (blue) compared to controls (red/purple). The RNAi line used here is UAS-RNAi(KK) (n=100 flies). Differences are shown to be significant ($p<0.01$ using Mantel-Cox test and Gehan-Breslow-Wilcoxon test).

Figure 5.5 shows that the effect seen with knockdown of *dms1* in non-backcrossed flies appears to disappear when flies are backcrossed to a w^{1118} background,

implying that before backcrossing the apparent effect of reducing *dms1* expression is merely a background effect. After backcrossing there is less of an effect, flies with knockdown of *dms1* differ in survival from controls in a different way, and survival levels of knockdown flies and controls are a lot closer here, particularly to the driver control. As in section 4.2.2, this demonstrates the importance of backcrossing to remove any effects that may be due to differences in genetic background rather than experimental differences.

5.2.2.2 Longevity under hypoxic conditions with knockdown of *dms1*

As *ms1* is a stress responsive gene, hypoxic stress may affect survival differently when there is a reduction of *ms1* than when expression is normal. If *ms1* expression is diminished then the pathway to which it belongs may not be able to respond to stress conditions and affect overall health. Here, flies with muscle-specific knockdown plus controls have been treated with 1 hour of hypoxia per day and survival recorded.

Crosses were carried out as in section 5.2.2.1. The results of testing different levels of knockdown (with and without a UAS-Dcr construct) are shown in figures 5.6 and 5.7.

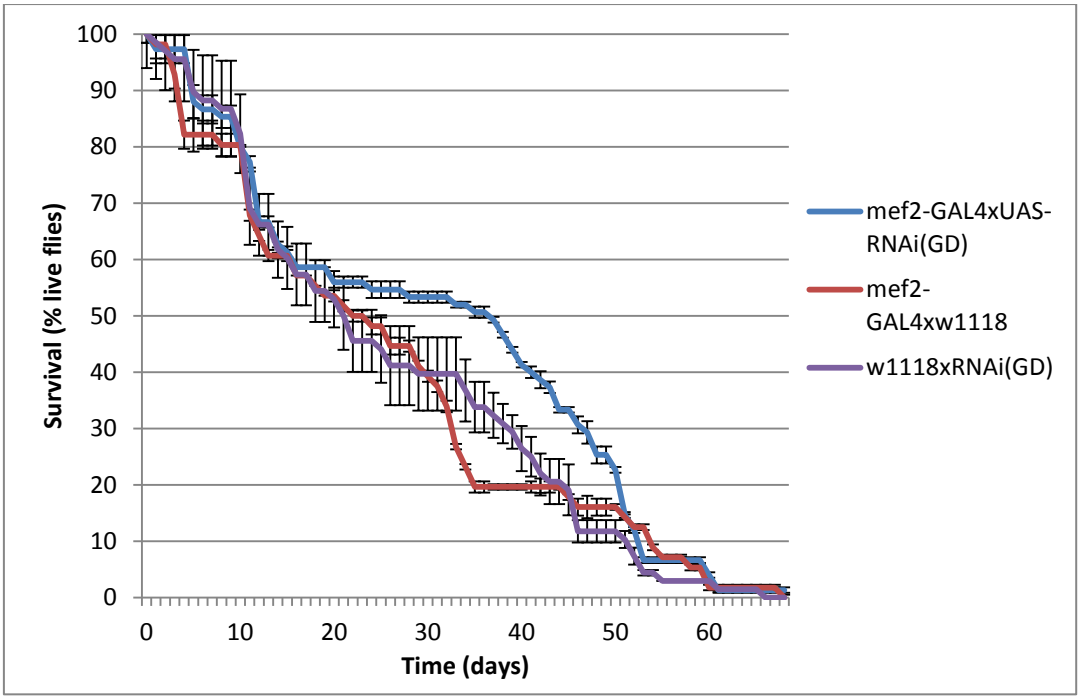

Figure 5.6: Graph showing longevity under daily 1 hour hypoxic stress of non-backcrossed flies with muscle-specific knockdown of *dms1* (blue) compared to controls (red/purple) using UAS-RNAi(GD) line (n=80 flies). Differences are not shown to be significant (p=0.09 using Mantel-Cox test and Gehan-Breslow-Wilcoxon test).

Figure 5.6 shows survival is similar for the first 15 days between all genotypes, after which the experimental line appears to survive better than both controls. This continues until around 50 days, when all lines have very low survival. The differences between the experimental line and controls have not been shown to be significant, therefore this suggests no effect of this level of knockdown on longevity with hypoxic treatment.

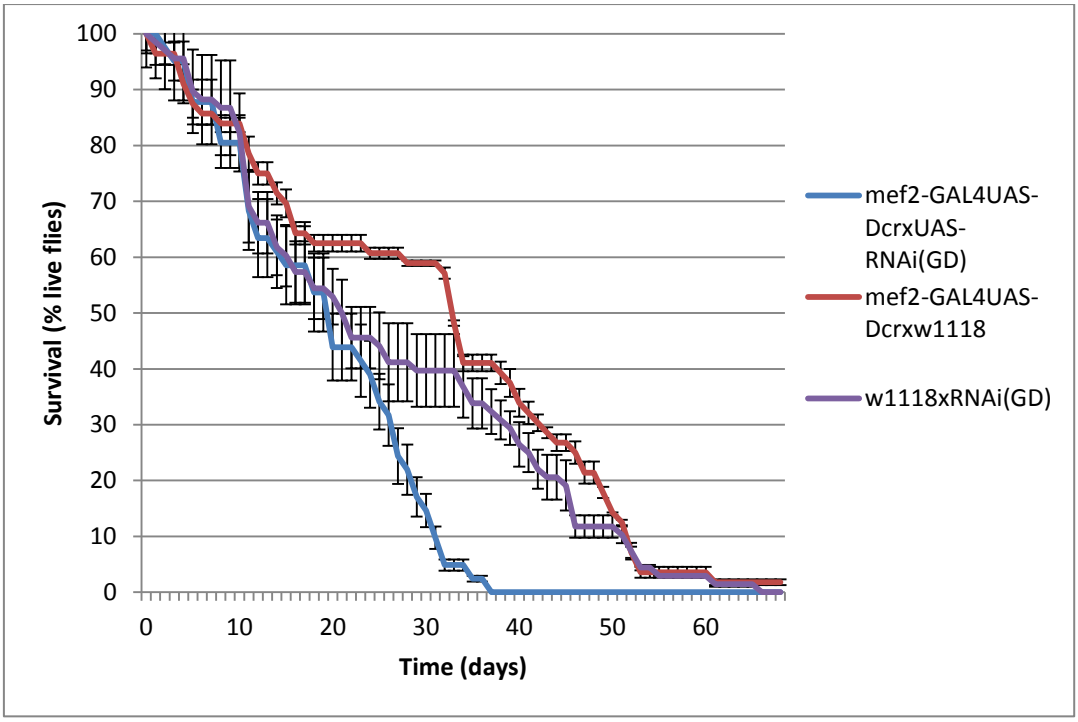

Figure 5.7: Graph showing longevity under daily 1 hour hypoxic stress of non-backcrossed flies with muscle-specific knockdown of *dms1* (blue) compared to controls (red/purple) using UAS-RNAi(GD) line (n=80 flies). This experiment has the addition of a UAS-Dicer (Dcr) construct to increase the effect of RNAi. Differences are shown to be significant ($p<0.002$ using Mantel-Cox test).

Figures 5.7 shows no apparent difference between all lines during early stages of the experiment, however, after around 20 days the survival of the experimental line decreases rapidly is comparison to controls. All experimental flies are dead by 40 days, whereas control flies are able to survive until 60 days.

Hypoxic stress appears to affect the longevity of non-backcrossed flies in different ways with different levels of *dms1* knockdown. Under the *mef2*-GAL4 driver alone (figure 5.6) no apparent difference is seen between experimental flies and controls. However, with the addition of UAS-Dcr flies appear to die much faster with knockdown of *dms1* after 3 weeks (figure 5.7). This indicates that there may be a threshold for reduction of *dms1* and a significant phenotype when looking at longevity. There is no data here for flies that have been backcrossed into a w^{1118}

background, so there could still be a background effect, and further investigation would confirm whether this is a true result.

5.2.2.3 Activity of adult flies with knockdown of *dms1*

MS1 is required for muscle growth and reduction of MS1 is observed in conditions of muscle atrophy (Lamon *et al.*, 2009). Since *ms1* is expressed during development and is known to be expressed in skeletal muscle (Mahadeva *et al.*, 2002; Arai *et al.*, 2002) it is likely that there is a role for *ms1* in development of muscle tissue, and that reduction of *ms1* would cause developmental defects in muscle. To test this hypothesis using *Drosophila*, *dms1* has been knocked down and the normal levels of activity were recorded to see if lowering *dms1* expression affects the ability of the flies to move normally. This would show any effect on how much the flies move, and in addition will indicate if there is any effect on the circadian rhythm of these flies. It is possible that changing expression of *ms1* would have some sort of circadian phenotype, since one of the regulators of *ms1* in muscle, MyoD, is known to be expressed in a circadian pattern and is regulated itself by known central circadian clock genes (Andrews *et al.*, 2010). Knockdown here is driven by a *mef2*-GAL4 driver as *mef2* is not only muscle-specific, but has also been shown to be expressed in clock neurons and is required to maintain the clock in *Drosophila* (Blanchard *et al.*, 2010). This means that this experiment will not only show any differences in activity due to changes within the muscle but will also show any effect on the circadian clock of *dms1* knockdown.

The following crosses were carried out using backcrossed flies:

w; *mef2*-GAL4; + x w; UAS-RNAi(KK); +

w; *mef2*-GAL4; + x w^{1118}; +; +

w^{1118}; +; + x w; UAS-RNAi(KK); +

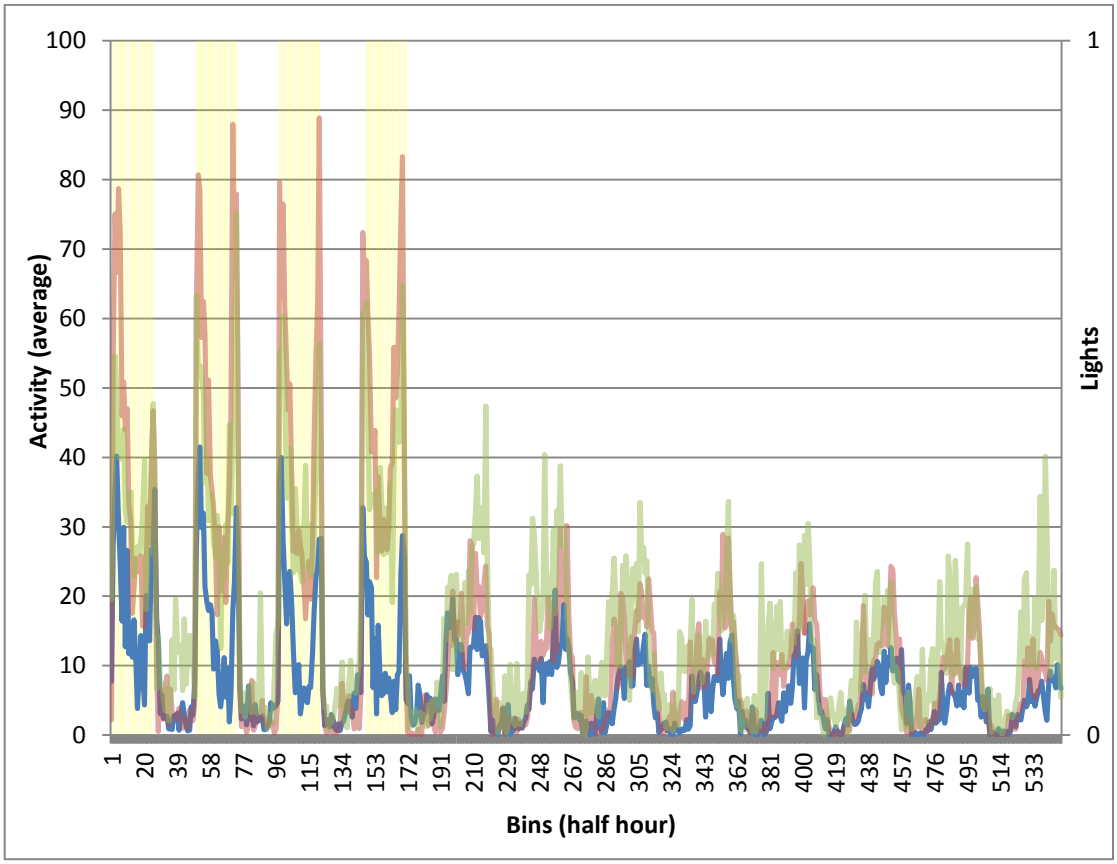

Figure 5.8: activity plot showing activity levels of flies with muscle specific knockdown of *dms1* (blue) compared with driver control (red) and RNAi construct control (green). Activity scale refers to number of times fly crosses infrared beam in activity monitor. Flies are kept in LD for 4 days and DD for the remainder of the time, lights are shown in yellow (1=on, 0=off) (n=18 flies). Statistical significance has been determined by comparing data from single timepoints and results are shown to vary in significance depending on the time of day.

Table 5.1: statistical significance of data determined by one-way ANOVA between all data sets. P</=0.05 marked in red. Timepoints chosen were those at both daily peaks of activity and the centre point of the afternoon siesta for each day during LD.

Timepoint	P value: one way ANOVA
Point5	0.08
Point17	0.1
Point25	0.63
Point56	0.02
Point63	0.21
Point70	0.03
Point99	<0.001
Point111	0.02
Point118	0.31
Point150	0.03
Point157	0.08
Point171	0.2

The activity plot in figure 5.8 shows an obvious effect of muscle-specific knockdown of *dms1* on the levels of activity of adult flies, as with knockdown they are much less active. This is more noticeable during the LD period when activity is prompted by the simulation of daylight. Flies with *dms1* knockdown appear to be unable or less willing to move as much as controls. Table 5.1 shows that statistical significance of this difference in activity varies throughout the course of the experiment, but there are several timepoints where differences are shown to be significant. While there is a change in activity levels, there is no change to circadian rhythms seen here, implying that reduction in *dms1* does not severely alter the clock.

An additional activity experiment was carried out testing levels of activity using a fly line that has a PiggyBac element inserted into the second intron of *dms1*. This insertion is not reported to affect *dms1* expression and has no reported phenotype, so this experiment was designed to test whether this insertion reduces *dms1* expression by looking at activity levels and comparing them to the known phenotype of *dms1*

knockdown. Any reduction in activity levels would indicate that the PiggyBac element is reducing *dms1* expression. However, this was not seen here, with no significant difference found between flies with the PiggyBac element and w^{1118} control flies. These results can be seen in figure 5.9 and show that both lines have highly similar levels of activity. This suggests that the presence of the PiggyBac element is not lowering *dms1* expression, or is not lowering it enough to show a phenotype similar to that seen with RNAi of *dms1*. As with *dms1* RNAi there is also no change to circadian rhythms with the presence of the PiggyBac insertion.

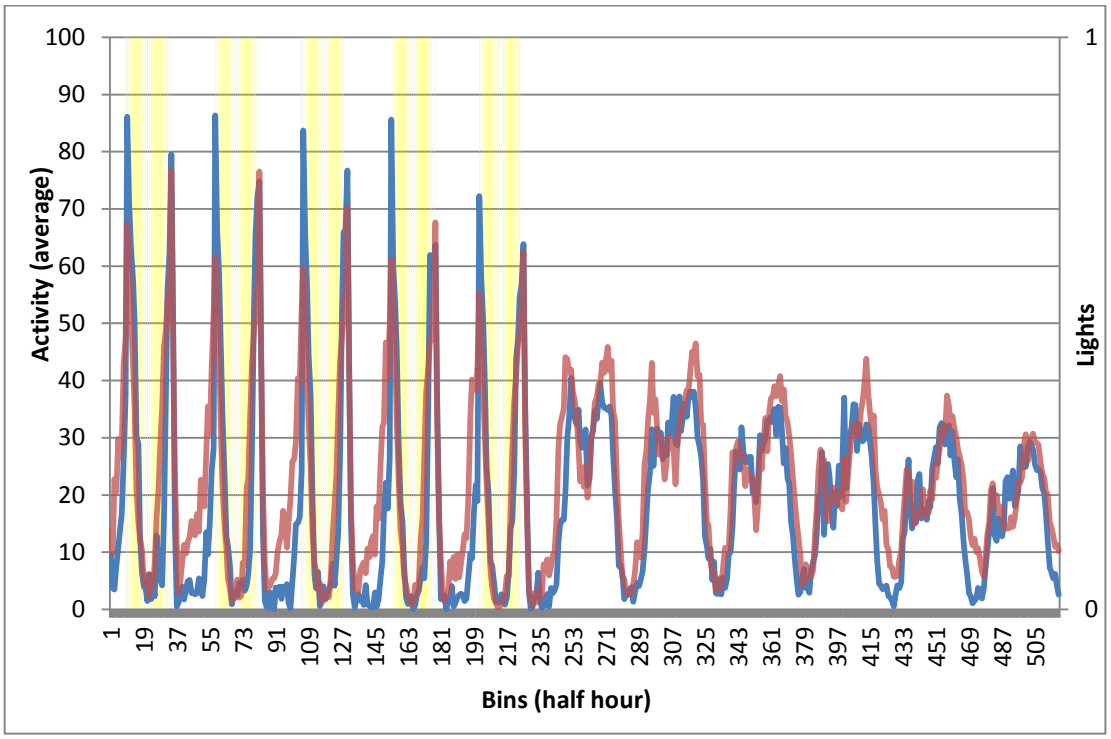

Figure 5.9: activity plot showing activity levels of flies with a PiggyBac element insertion in *dms1* (blue) compared to control (w^{1118}, red). Activity scale refers to number of times fly crosses infrared beam in activity monitor. Flies are kept in LD for 5 days and DD for the remainder of the time, lights are shown in yellow (1=on, 0=off) (n=20 flies). Levels of activity are not significantly different.

5.2.2.4 Climbing ability of adult flies with knockdown of *dms1*

A climbing assay was carried out to determine whether the ability to climb when prompted was affected in a similar way to their normal daily activity levels when *dms1* expression is reduced. This climbing assay uses the flies negative geotaxic response to elicit climbing when flies are knocked to the bottom of a tube. This will further elucidate the effect of *dms1* reduction on the muscles, as if climbing is reduced, it could indicate that there is damage to the muscles, rather than simply a general lack of energy, as flies may still climb when prompted with lower energy levels, but if the muscle is damaged they will not be able to climb as well.

Crosses were carried out as in section 5.2.2.3. Climbing assays used 30 flies in three groups of 10. Each group was allowed to climb 10 times, with a minute break between each climb, and the number of flies that climbed 8cm or more was recorded. Figure 5.10 shows the results of the climbing experiment using 1 week old flies.

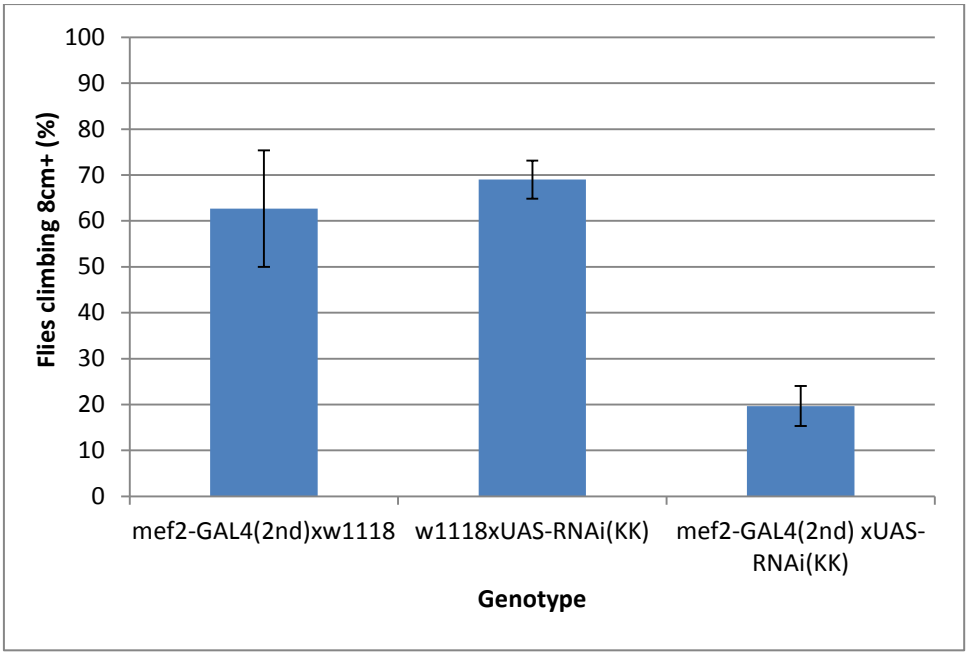

Figure 5.10: Climbing ability of adult flies with knockdown of *dms1*, represented by the percentage of flies able to climb >8cm in 10 seconds (n=30 flies, each group of 10 allowed to climb 10 times). Climbing ability is significantly reduced with knockdown of *dms1* ($p<0.001$ using one way ANOVA, experimental line is significantly different from both controls using Tukey's HSD Post Hoc test at $p=0.05$ level).

The results in figure 5.10 clearly show that climbing ability is significantly reduced with *dms1* knockdown. Climbing ability decreased by approximately 65% with knockdown of *dms1* compared to the closest control. This fits with the data found when looking at daily activity levels and shows that a decrease in *dms1* expression is impacting on the flies ability to move both under normal circumstances and when prompted.

5.2.2.5 Motility of larvae with knockdown of *dms1*

It is known that *ms1* is involved in development, and as *dms1* could potentially be involved in the formation of muscle during development it may be important at stages other than the adult stage of the *Drosophila* life cycle. This experiment was

designed to test the effect of knockdown of *dms1* at the larval stage, specifically the 3rd instar, which occurs before the wandering stage when larvae climb to find a place to pupate.

Crosses were carried out as in section 5.2.2.3 and two different types of analysis were carried out on the results. The total distance travelled and the number of turns taken by each larva was recorded, and these results are shown in figures 5.11 and 5.12 respectively.

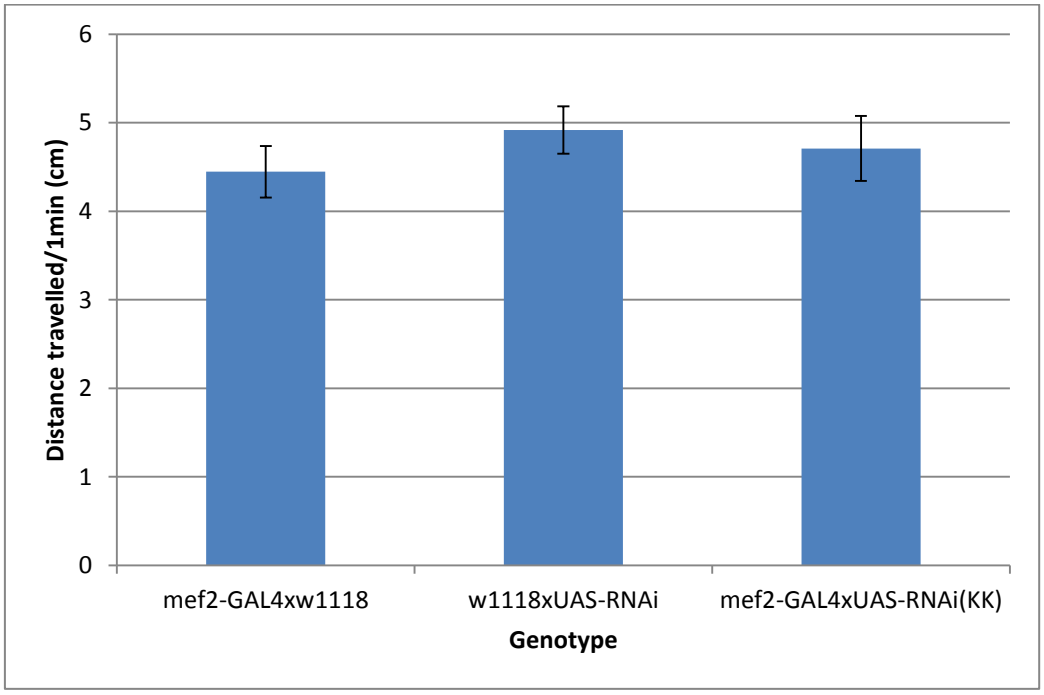

Figure 5.11: Distance travelled by 3rd instar larvae in 1 minute (cm), comparing larvae with muscle-specific knockdown of *dms1* with controls. Differences are not shown to be significant (p=0.10 using one-way ANOVA).

Knockdown of *dms1* does not appear to affect the ability of the larvae to move in a straight line, and larvae with knockdown are able to travel as far as the controls. Figure 5.11 shows no significant differences between experimental larvae and either

control, suggesting that those muscles and movements involved in moving the animal forward are not affected by changes to *dms1* expression.

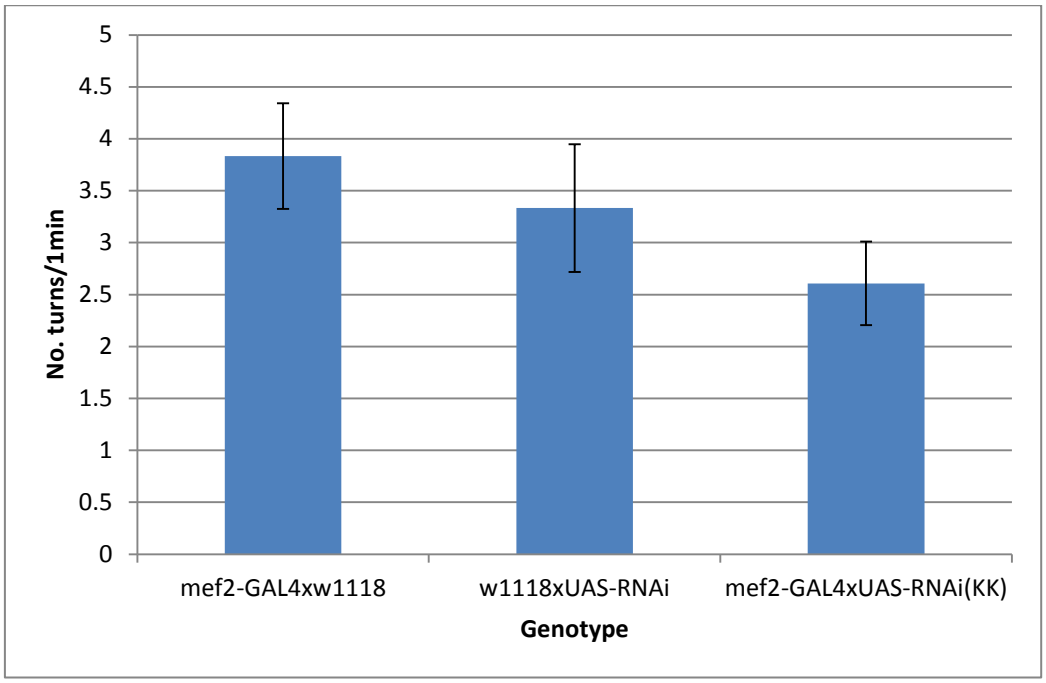

Figure 5.12: Number of turns made by 3rd instar larvae in 1 minute comparing larvae with knockdown of *dms1* with controls. A turn is defined as a noticeable head movement and change of direction of travel. Differences are shown to be significant between experimental and driver control using one way ANOVA ($p<0.005$), confirmed using Tukey's HSD Post Hoc test at $p=0.05$ level. Differences between experimental and UAS control were not shown to be significant using Tukey's HSD Post Hoc test at $p=0.05$ level.

As shown in figure 5.12, the turning ability of larvae with knockdown of *dms1* is somewhat reduced, as experimental larvae tend to travel more in a straight line than controls and prefer not to make noticeable changes in direction. The results shown in figure 5.12 show significant difference between experimental and driver control, but not UAS control. However, when a Tukey's HSD test is performed on these data, a value of 3.01 is found, very close to the $p=0.05$ level value of 3.40. It is possible that

further replicates may find this data to be significant. If this is the case, this could reflect a problem with the muscles responsible for head movement, as the larva moves its head to sense which direction to move in, and the head leads the change in direction. This could indicate that reduction of *dms1* is having some effect on larval mobility, possibly in only one type of muscle, although this phenotype does not seem to be as significant as the phenotype seen in adult flies with *dms1* knockdown. However, the data shown in figure 5.12 is not shown to be statistically significant, therefore this is merely speculation.

5.2.2.6 Eclosion of adult flies with knockdown of *dms1*

Data from Flybase (flybase.org) states that the peak of *dms1* expression is at the final stages of pupal development, shortly before eclosion. It has been demonstrated here that *dms1* is important for muscle function, and because of this, it may be that a reduction in *dms1* expression will impact on the flies ability to eclose from their pupae, as it requires muscular strength to push out from the pupal casing. It was noticed in previous experiments that when crosses were made between *mef2*-GAL4 and UAS-RNAi(KK) there was a larger number of pupae than usual that contained adult flies but did not eclose. Indeed, before backcrossing this cross did not result in any eclosion at all. Backcrossing of the lines partly removed this effect, resulted in more flies eclosing, but there were still many that did not. This experiment was carried out to quantify exactly by how much eclosion was reduced with knockdown of *dms1*.

Both *mef2*-GAL4 drivers (2nd and 3rd chromosomes) were used for eclosion experiments. The following crosses were carried out:

w; *mef2*-GAL4; + x w; UAS-RNAi(KK); +
w; *mef2*-GAL4; + x w^{1118}; +; +
w; +; *mef2*-GAL4 x w; UAS-RNAi(KK); +
w; +; *mef2*-GAL4 x w^{1118}; +; +
w^{1118}; +; + x w; UAS-RNAi(KK); +

Twenty pupae of each genotype were isolated for each replicate and the number of adult flies that emerged from the pupae was recorded. Four replicates were carried out. Figures 5.13 and 5.14 show the results of eclosion using drivers on the 2nd and 3rd chromosomes respectively.

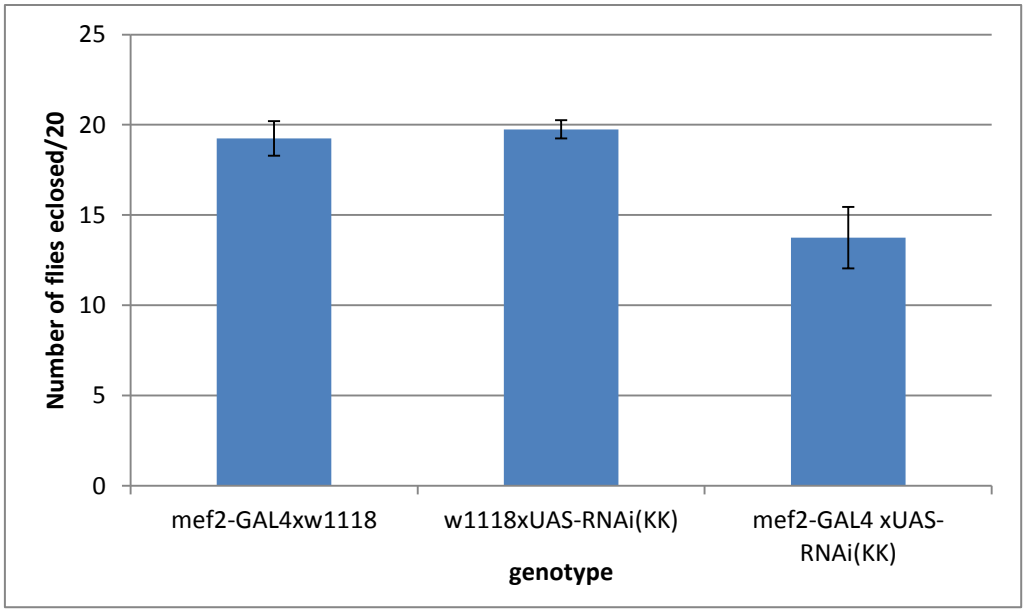

Figure 5.13: average eclosion of adult flies using 2nd chromosome *mef2*-GAL4 driver. Eclosion is significantly reduced in flies with knockdown of *dms1* ($p<0.001$ using one-way ANOVA, experimental line is significantly different from both controls using Tukey's HSD Post Hoc test at $p=0.05$ level).

With the 2nd chromosome driver knockdown of *dms1* reduces the ability of flies to emerge from the pupae by approximately 30% compared to the driver control. Eclosion rates are consistent across all 4 replicates, with very little variance, showing the reliability of these results. This low variance is also seen in the results obtained using the 3rd chromosome driver, although the level of eclosion in the experimental line is higher as shown in figure 5.14.

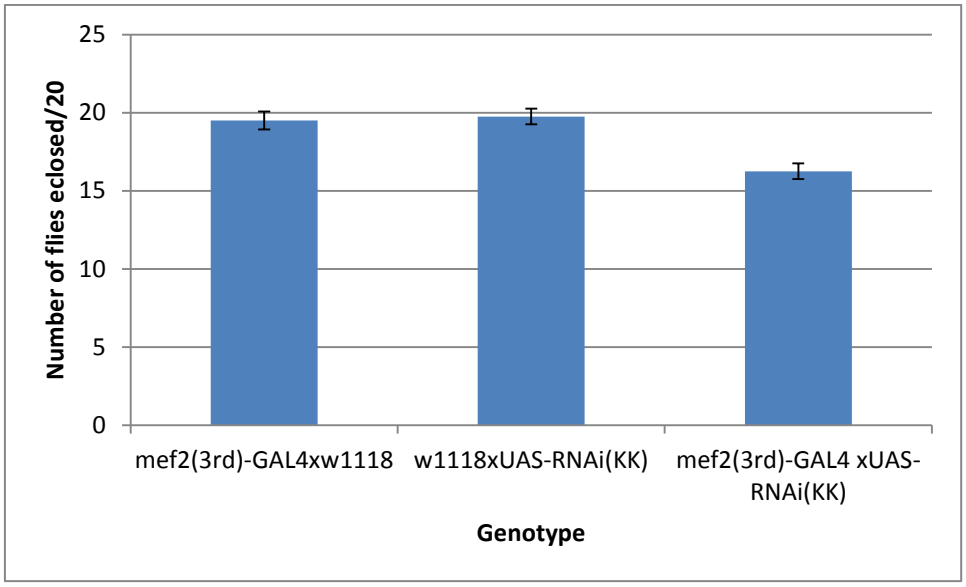

Figure 5.14: average eclosion of adult flies using 3rd chromosome *mef2*-GAL4 driver. Eclosion is significantly reduced in flies with knockdown of *dms1* ($p<0.001$ using one-way ANOVA, experimental line is significantly different from both controls using Tukey's HSD Post Hoc test at $p=0.05$ level).

As figure 5.14 shows, with the third chromosome driver eclosion is reduced by approximately 17%. This is a smaller reduction than with the second chromosome driver and is consistent with the qPCR data in section 3.2.2.1 which shows a higher level of reduction of *dms1* with the second chromosome driver. These data confirm

that eclosion is being significantly affected by knockdown of *dms1* and shows the extent to which this happens under these conditions.

5.2.2.7 Confocal microscopy of thoracic muscle with knockdown of *dms1*

There are around 80 muscles in the thorax of *Drosophila*, and considerable structural differences are found between the dorsal and ventral groups of muscle (Lawrence, 1982). Two important muscle types are the indirect flight muscles, which include the dorsal longitudinal and dorsoventral muscles (Fernandes *et al.*, 1991), and tubular muscles, which include the trochanter or jump muscles. The indirect flight muscles are more similar to mammalian cardiac muscle, whereas tubular muscles bear more resemblance to skeletal muscle (Swank, 2012).

Here, the musculature of flies with *dms1* knockdown has been examined for defects. As it has been demonstrated in previous experiments that *dms1* reduction can affect muscle function, it could be that there is a change in the structure of the muscle. Activity and climbing experiments indicate that muscles controlling the legs at least may be affected. It is unknown whether a decrease in *dms1* expression affects flight muscles or flying ability.

Crosses were carried out as in section 5.2.2.3 and flies were dissected to reveal thoracic muscle. Both types of muscle were examined and are shown in figure 5.15-16.

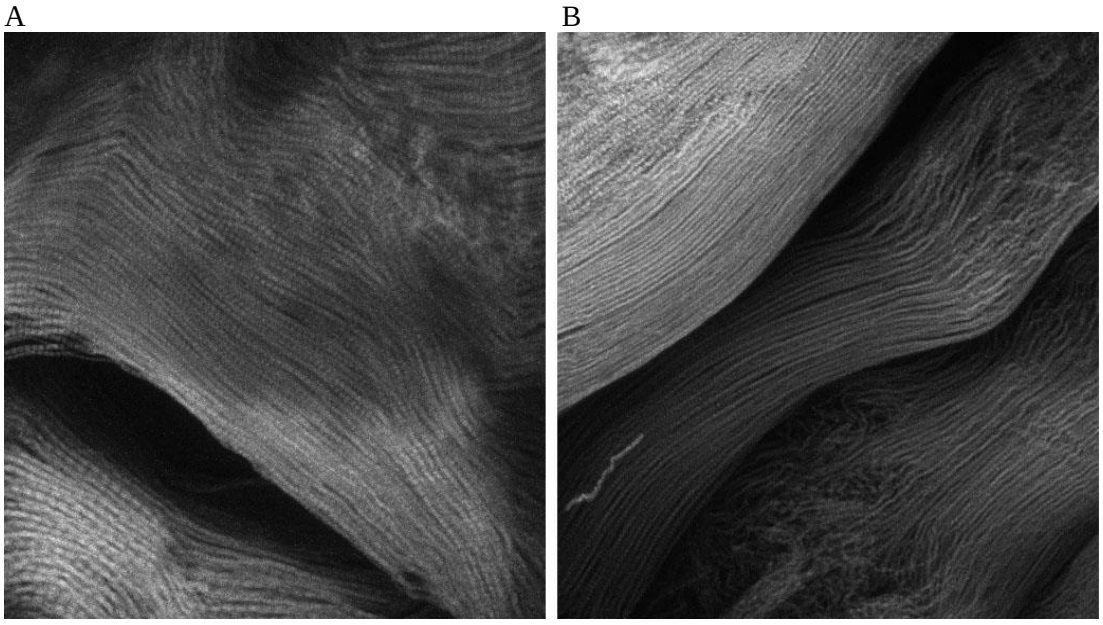

Figure 5.15: Confocal image of dorsal thoracic muscles with muscle-specific knockdown of *dms1* (A) compared with driver control (B) at 60x magnification.

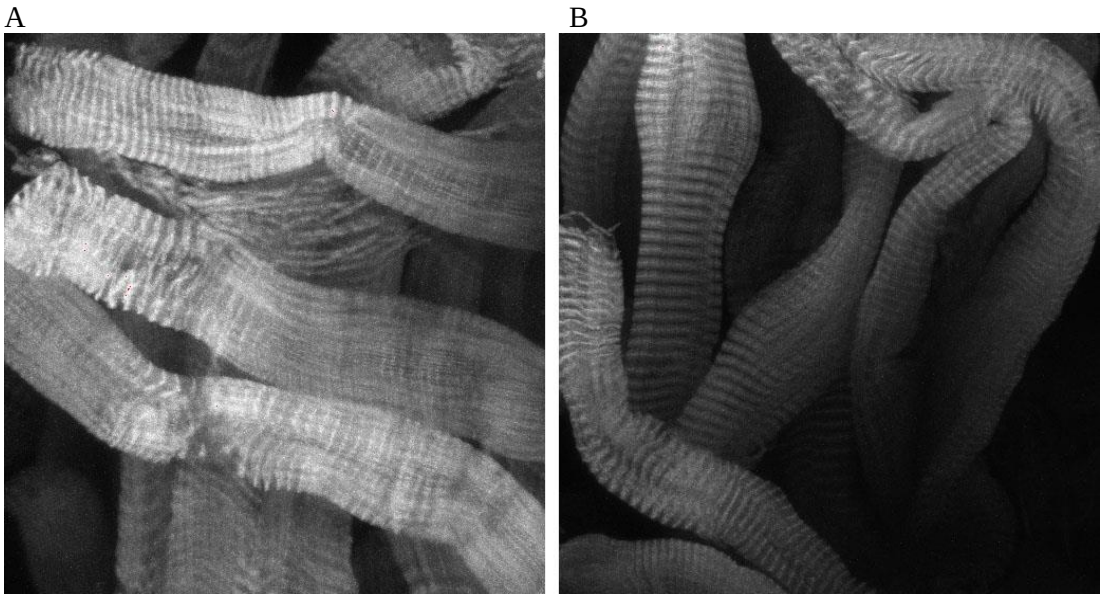

Figure 5.16: Confocal image of ventral tubular thoracic muscles with muscle-specific knockdown of *dms1* (A) compared with driver control (B) at 60x magnification.

Figures 5.15-16 show two distinct muscle types found in the thorax of *Drosophila*.

The muscles on the ventral side of the thorax, shown in figure 5.16, are found

adjacent to the legs of the fly and have a clear tubular structure in which striations can be seen. The dorsal muscles shown in figure 5.15 are associated with contraction of the thorax and are more fibrous in structure, with individual fibres lying parallel to each other. Examination of confocal images obtained in this investigation did not reveal any significant difference between muscle with knockdown of *dms1* and driver and UAS controls, although a larger sample size may reveal more (n=2 of each genotype in this experiment). Figure 5.16 may show a somewhat more disorganised structure in the tubular muscle, but this is likely to be due to issues during dissection and does not show any effect of *dms1* knockdown.

5.2.3 Overexpression of *dms1*

As RNAi targeting *dms1* has had a significant effect on the movement and muscular strength of *Drosophila*, many of the experiments used to find this data were repeated using an overexpression construct for *dms1*, a UAS-*dms1* fly line. This would reveal if overexpression of *dms1* has the opposite effect to that of the RNAi, i.e., increase activity and movement of the flies. If *dms1* is important for muscle development and maintenance then a modest increase in expression may cause muscles to be more efficient and give the flies more strength and ability.

5.2.3.1 Activity of adult flies with overexpression of *dms1*

Activity was measured as in section 5.2.2.3, with the exception that this experiment did not contain a DD component, as it was felt that this was not necessary due to the lack of a circadian phenotype associated with changes in *dms1* expression.

The following crosses were carried out:

w; *mef2*-GAL4; + x w; +; UAS-*dms1*

w; *mef2*-GAL4; + x w^{1118}; +; +

w^{1118}; +; + x w; +; UAS-*dms1*

Figure 5.17 shows the results of this activity experiment.

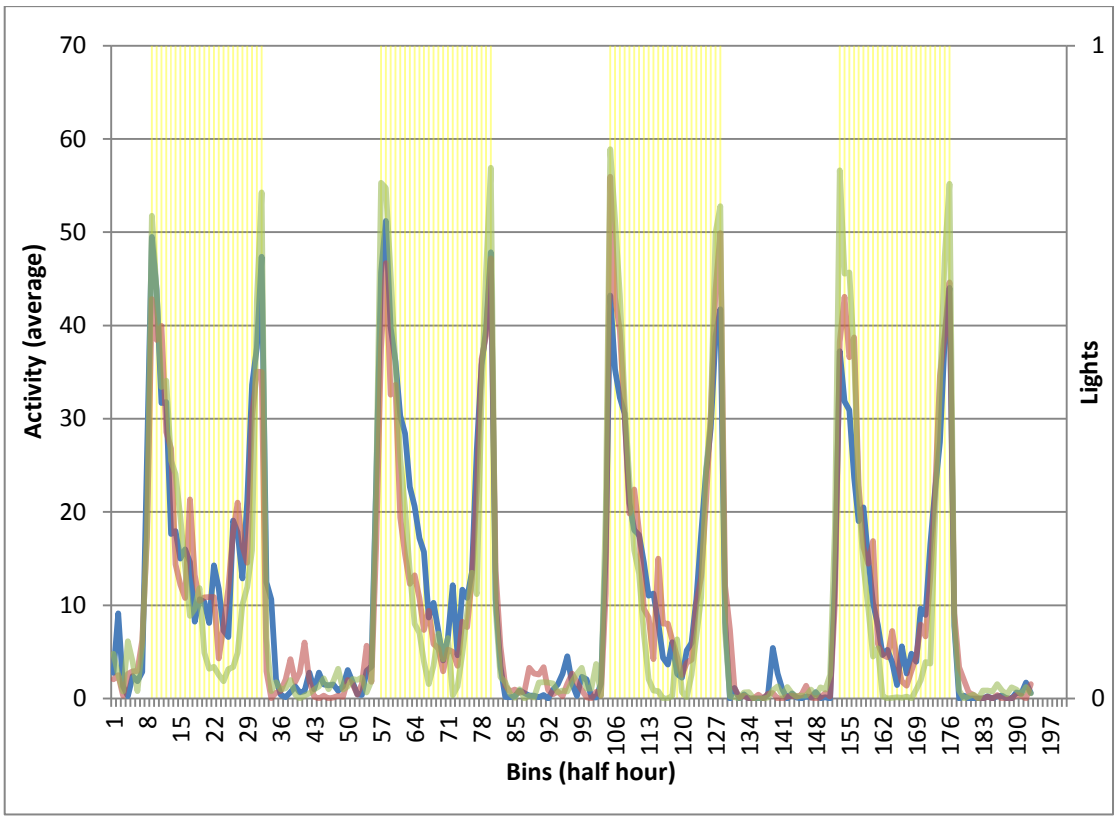

Figure 5.17: activity plot showing activity levels of flies with overexpression of *dms1* (blue) compared to UAS control (red) and driver control (green). Activity scale refers to average number of times fly crosses infrared beam in activity monitor (n=20 flies). Flies are kept in 12:12 LD, lights are shown in yellow. Levels of activity are not significantly different.

The results shown in figure 5.17 indicate that there is no significant difference in normal activity levels with overexpression of *dms1*. Movement of the flies is very

similar to that of controls and they have what is considered to be a normal daily cycle, suggesting that overexpression of *dms1* does not have an effect on the flies ability (or inclination) to move, or it is not sufficient to induce an increase in movement.

5.2.3.2 Climbing ability of adult flies

Climbing experiments were carried out as in section 5.2.2.4. As before, this experiment is designed to test the flies ability to climb when provoked, so unlike investigating normal activity levels the flies are being forced to move instead of being left to move at will. Crosses were carried out as in section 5.2.3.1 and flies were tested at 1 week old and 4 weeks old to find any effect of age on the results. As muscle function is known to deteriorate with age, it is possible that increased *dms1* expression may counteract this decline at later stages of adult life. Figures 5.18 and 5.19 show the results of climbing experiments using 1 and 4 week old flies respectively.

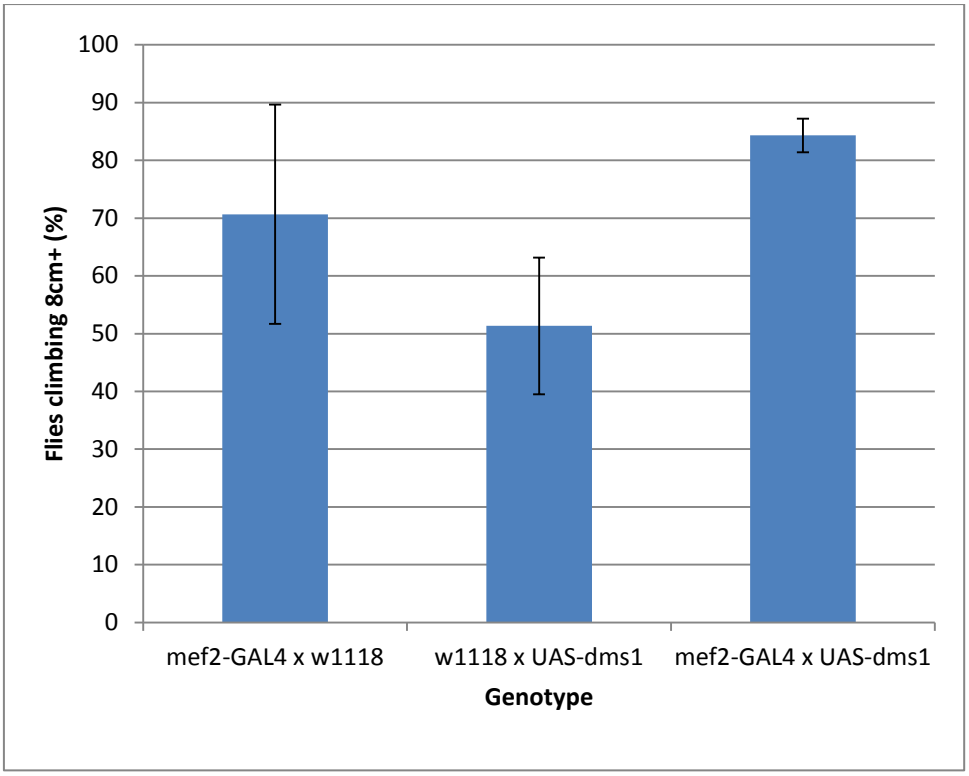

Figure 5.18: Climbing ability of 1 week old adult flies with overexpression of *dms1*, represented by the percentage of flies able to climb >8cm in 10 seconds (n=30 flies, each group of 10 allowed to climb 10 times). Differences are shown to be significant ($p<0.05$ using one-way ANOVA, experimental line is significantly different from both controls using Tukey's HSD Post Hoc test at $p=0.05$ level).

Figure 5.18 shows that there is a significant difference in climbing ability between 1 week old flies with overexpression of *dms1* and both driver and UAS controls. This suggests that an increase in *dms1* expression increases muscle function of these flies, allowing them to climb faster than controls. Flies with overexpression of *dms1* seem to have a consistently high climbing ability, and while the driver control flies also appear to be able to climb well, this is not as consistent and results have a higher level of variance. When carrying out this experiment with flies with overexpression it often results in 100% of flies being able to climb 8cm in 10 seconds, so a more sensitive test, perhaps with a longer distance to climb,

could be more useful in finding differences between these flies and the controls at 1 week old.

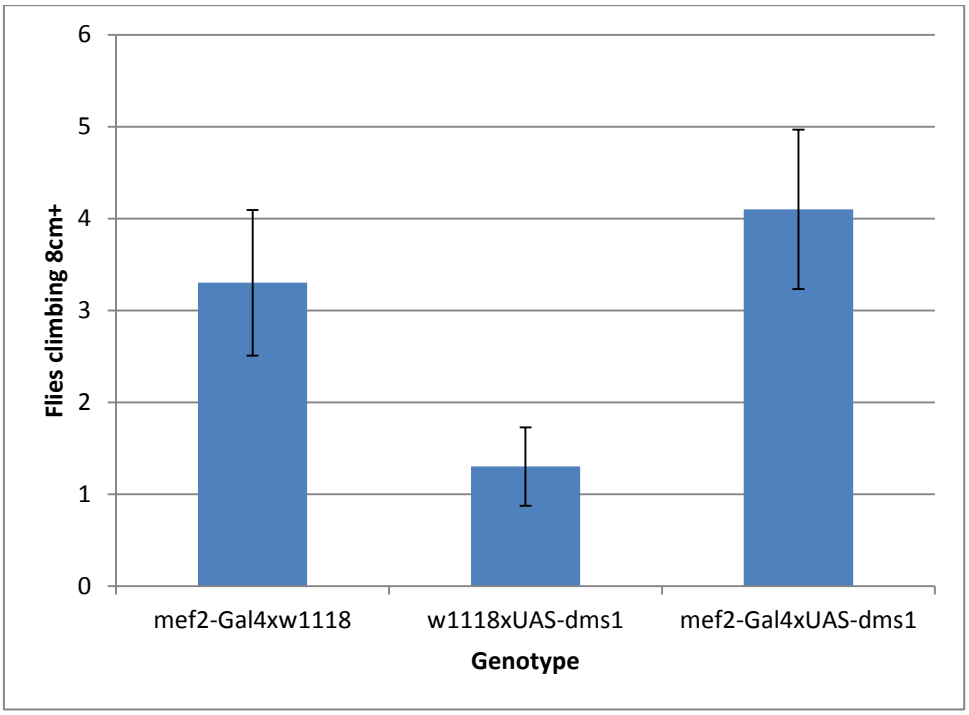

Figure 5.19: Climbing ability of 4 week old adult flies with overexpression of *dms1*, represented by the percentage of flies able to climb >4cm in 20 seconds (1 group, n=10 flies, allowed to climb 10 times) ($p<0.05$ using one way ANOVA, differences are not significant between experimental and driver control using Tukey's HSD Post Hoc test)

Figure 5.19 shows the climbing ability of 4 week old flies with overexpression of *dms1*. At 4 weeks there seems to be the same pattern of climbing ability seen at 1 week, however the difference between the experimental line and UAS control has been reduced and has not been shown to be statistically significant. At 4 weeks the test has had to be modified considerably, as flies have a lot of difficulty climbing, and so were given 20 seconds to climb 8cm.

In general, it appears that overexpression of *dms1* is having at least some effect on climbing ability, making flies more able to climb when prompted. This, along with the activity data from section 5.2.3.1, suggest that an increase in *dms1* enhances the ability of flies to move when under pressure to do so, but does not increase their inclination to move under normal conditions.

5.2.3.3 Activity levels under conditions of starvation with overexpression of *dms1*

Overexpression of *dms1* has been shown here to increase movement only when the flies are forced to move, as they appear to climb better when stimulated but do not move more under normal conditions. Flies may, however, be more inclined to increase their activity if they have to search for food. *Drosophila* exhibit increased activity under conditions of starvation, likely as a mechanism to increase their chances of finding food (Lee & Park, 2004). This experiment will investigate if *dms1* overexpression allows flies to increase this response further when no food is available. This will test whether overexpression of *dms1* allows the flies to increase their activity without stimulation, or if it is only when flies are directly forced to move that the effect of overexpression is seen. It will also give an indication of any role for *dms1* in metabolism as if longevity is affected by an increase in *dms1* levels then it is possible that this is an additional pathway that is connected with that of *dms1*. Studies have shown that upregulation of *ms1* in humans can result in the upregulation of genes involved in fat metabolism (Wallace *et al.*, 2011), and is also associated with insulin resistance, with lower levels of *ms1* found to increase sensitivity to insulin (Jin *et al.*, 2011) as well as the finding that *ms1* upregulation is seen in a diabetes mouse model (Ounzain *et*

al., 2012). This evidence implies a role for the *ms1* signalling pathway in metabolism, and the experiment outlined here attempts to uncover any role that *dms1* may have in response to food availability.

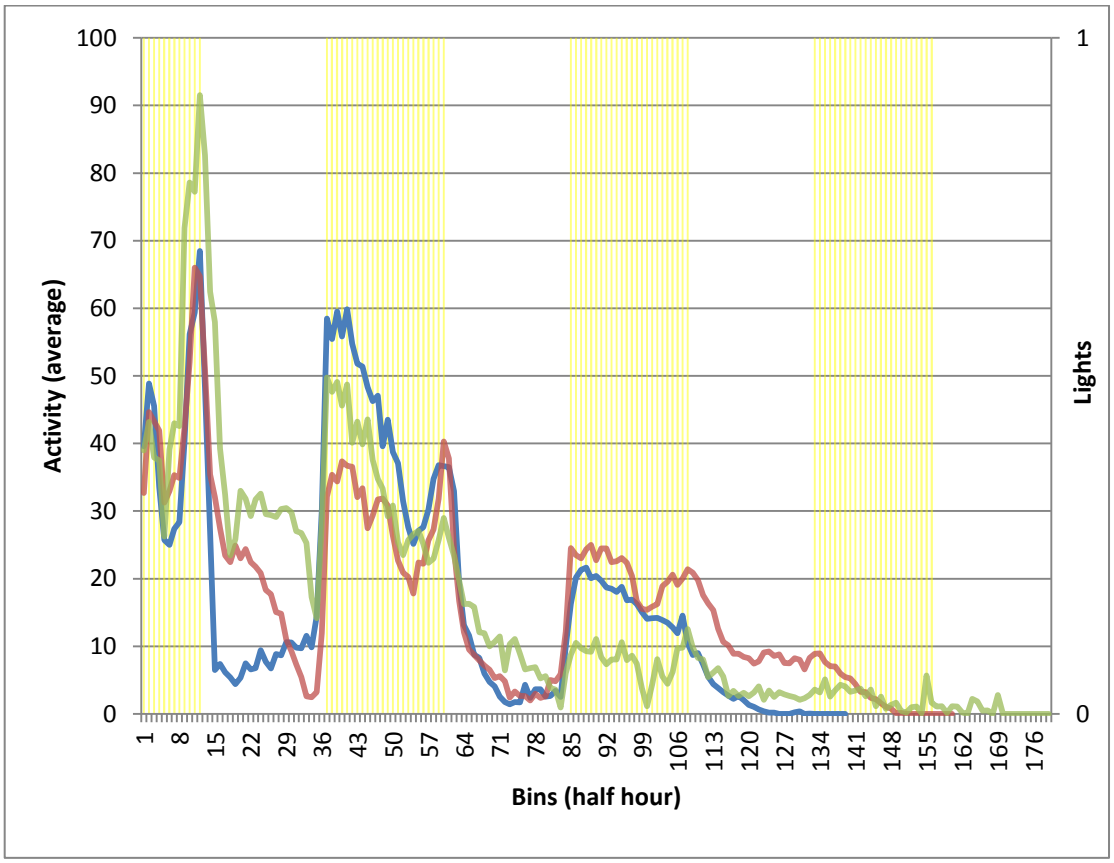

Figure 5.20: activity plot showing activity levels under starvation conditions of flies with overexpression of *dms1* (blue) compared to UAS control (red) and driver control (green). Activity scale refers to average number of times fly crosses infrared beam in activity monitor (n=20 flies). Flies are kept in 12:12 LD, lights are shown in yellow.

Figure 5.20 shows the overall longevity of flies under starvation conditions does not appear to change significantly with overexpression of *dms1* compared to controls. Here, the flies with overexpression have all died at an earlier point than

controls, but control survival is still quite low at this point, despite the fact that a few flies still survive for longer. All genotypes retain some rhythmicity, although this is more pronounced in the overexpression genotype for the first 24 hours shown in figure 5.20. The biggest difference in this is seen between timepoints 15 and 28, and differences are significant at timepoints 15 and 20, $p<0.05$ using one-way ANOVA. There appear to be differences in survival at some timepoints, which could be affecting the average results during this time and making the experimental line appear more active. The data has also been plotted as a survival curve (figure 5.21) to determine if there are any differences between flies with overexpression and controls. As figure 5.21 shows, there are no significant differences, indicating that changes in *dms1* levels do not affect survival under starvation conditions. As the activity and survival data show very few significant differences no real conclusions can be drawn from this experiment, and it is likely that there is no effect of *dms1* overexpression under these conditions.

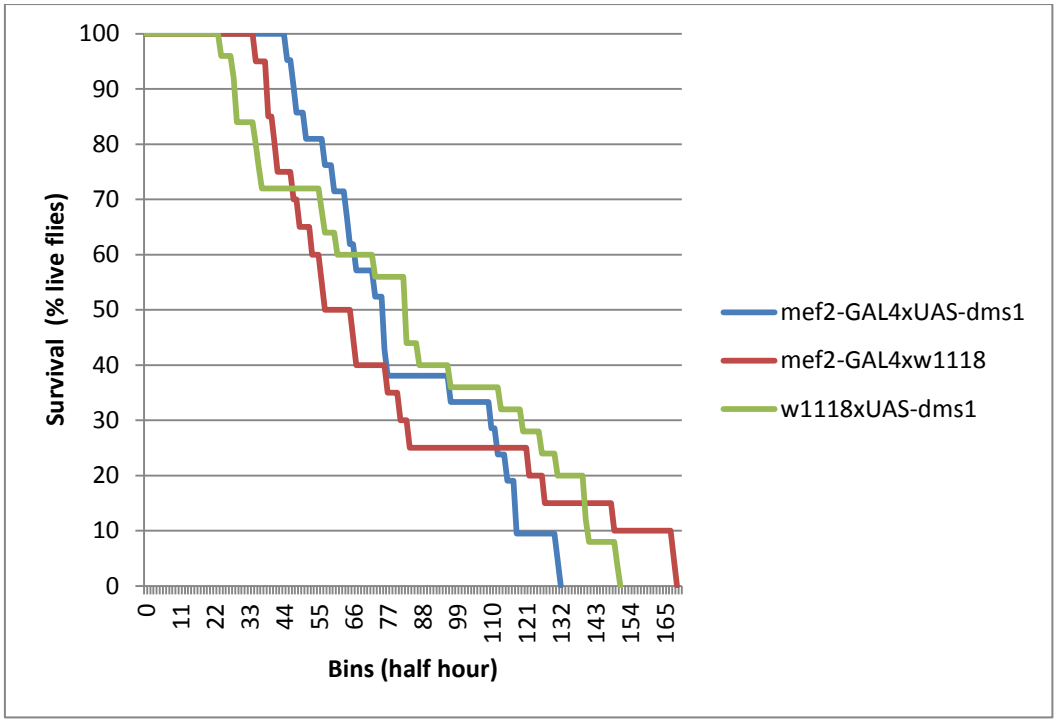

Figure 5.21: percentage survival of flies during activity assay under conditions of starvation. Flies overexpressing *dms1* are shown in blue compared with driver (green) and UAS (red) controls (n=20 flies). Differences are not shown to be significant (p=0.86 using Mantel-Cox test).

5.2.3.4 Confocal microscopy of skeletal muscle with overexpression of *dms1*

As in section 5.2.2.7, the thoracic musculature of flies with overexpression of *dms1* was examined under high magnification to identify any changes in structure from controls. This allows any enhancement of the general muscle structure that may be causing the behavioural changes seen previously to be visualised. Crosses were carried out as in section 5.2.3.1 and as before both ventral and dorsal muscles were identified and images taken.

A B

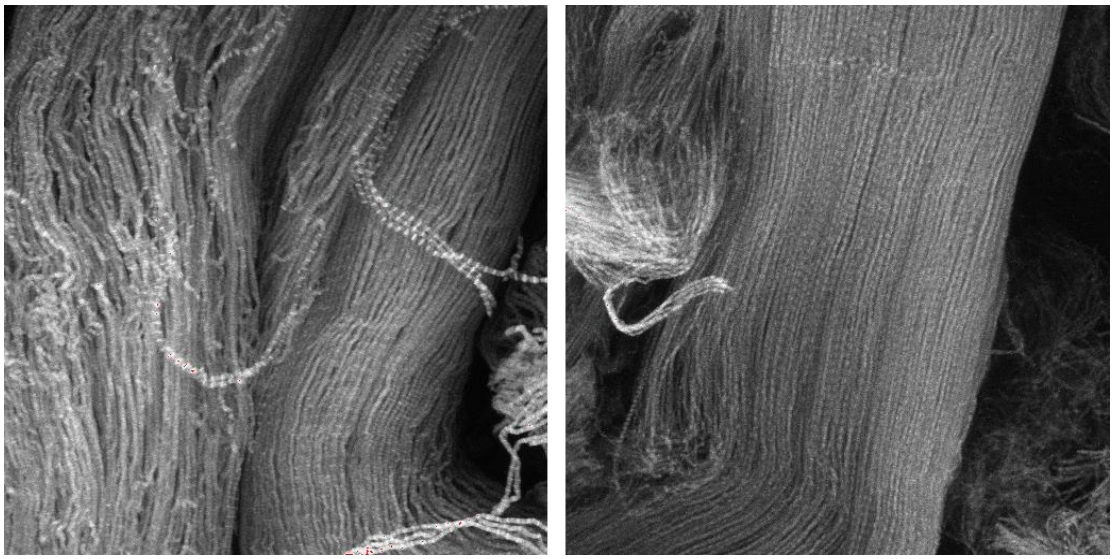

Figure 5.22: Confocal image of dorsal thoracic muscles with muscle-specific overexpression of *dms1* (A) compared with driver control (B) at 60x magnification.

A B

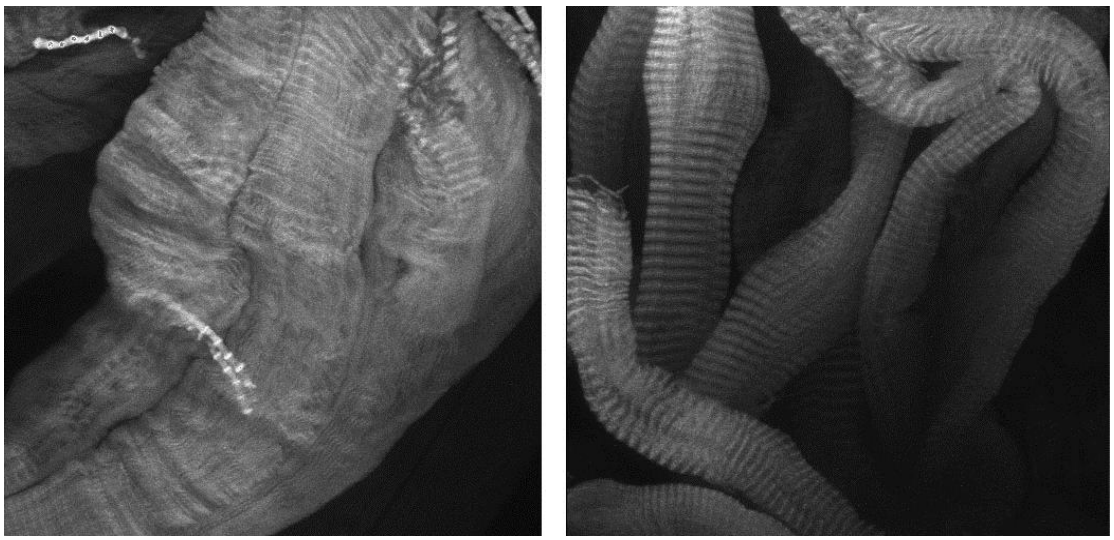

Figure 5.23: Confocal image of ventral tubular thoracic muscles with muscle-specific overexpression of *dms1* (A) compared with driver control (B) at 60x magnification. The driver control used here is the same as that used for comparison in figure 5.16B due to a lack of usable images.

Alignment of muscle fibres seen in figure 5.22 does not appear significantly different when comparing muscle with overexpression and controls. Unfortunately

due to issues during dissection, some fibres are damaged through experimental procedure and not as an effect of changes in *dms1*. Those fibres that are aligned are deeper into the image and therefore further from the thoracic cut made during dissection. These are more likely to be representative of how the fibres are normally and do not show any obvious difference from the controls (driver control shown in figure 5.22B), suggesting that *dms1* is not having a significant effect on the overall structure of the muscle. Examination of the tubular muscle shows the same, as there does not appear to be any disruption or obvious changes to the structure when *dms1* is overexpressed. The images shown in figure 5.23 do differ somewhat, but this is believed to be simply because a different type of muscle is shown in figures A and B, rather than any effect of *dms1*. A lack of useable images in this case prevented a direct comparison of tubular muscle, as is shown in section 5.2.2.7.

5.2.4 Overexpression of mouse *ms1*

The homology between mammalian *ms1* and *Drosophila ms1* has been disputed, as sequence homology is only found in the actin binding domain of the coding sequence of these genes and their proteins, and sequence to the N-terminus of mammalian and C-terminus of *Drosophila ms1* is different (Arai *et al.*, 2002; Mahadeva *et al.*, 2002). To test this, a UAS-*mms1* (mouse *ms1*) construct has been created in order to express mouse *ms1* in *Drosophila*. Here this has been used in the same way as overexpression of *dms1*, and experiments have been carried out to test activity under normal and starvation conditions, climbing ability and muscle structure to find any effect of mouse *ms1* on the phenotype seen in *Drosophila*. If a similar effect is seen to that of flies overexpressing *dms1* it could be that these two genes have a similar function.

5.2.4.1 Activity of adult flies with overexpression of mouse *ms1*

A measure of the flies activity will allow comparison with results found in section 5.2.3.1. Normal activity levels were recorded comparing flies with muscle-specific overexpression of mouse *ms1* to that of controls. Results are shown in figure 5.24.

The following crosses were carried out:

w; *mef2*-GAL4; + x w; +; UAS-*mms1*

w; *mef2*-GAL4; + x w^{1118}; +; +

w^{1118}; +; + x w; +; UAS-*mms1*

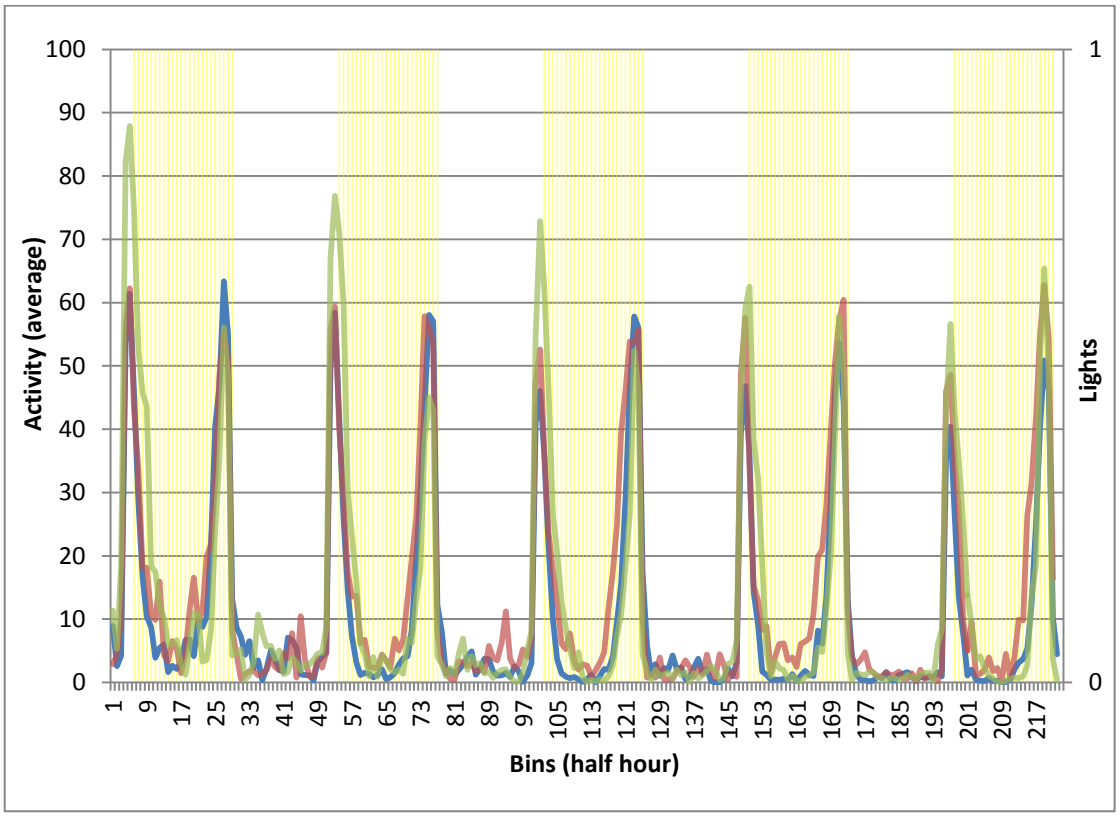

Figure 5.24: activity plot showing activity levels of flies with overexpression of mouse *ms1* (blue) compared to UAS control (red) and driver control (green). Activity scale refers to average number of times fly crosses infrared beam in activity monitor (n=20 flies). Flies are kept in 12:12 LD, lights are shown in yellow. Levels of activity are not significantly different.

The activity plot in figure 5.24 does not show any differences between the levels of activity of flies expressing mouse *ms1* and those of controls. All flies seem to be rhythmic and have normal daily rhythms. Activity is unchanged by expression of mouse *ms1*, and this is a similar result to that seen when overexpressing *dms1*. Therefore this neither confirms nor disputes the homology of these two genes.

5.2.4.2 Climbing ability of adult flies with overexpression of mouse *ms1*

Climbing assays were carried out as in section 5.2.2.4 with flies overexpressing mouse *ms1* compared to controls. This gives an idea of whether mouse *ms1* is able to increase climbing ability under pressure in a similar way as is seen with overexpression of *dms1*. An increase in *dms1* leads to an elevated climbing response when flies are knocked to the bottom of a tube compared to the climbing ability of controls. A similar result here would support the theory that mouse and *Drosophila ms1* are true homologues. Results are shown in figure 5.25.

Crosses were carried out as in section 5.2.4.1.

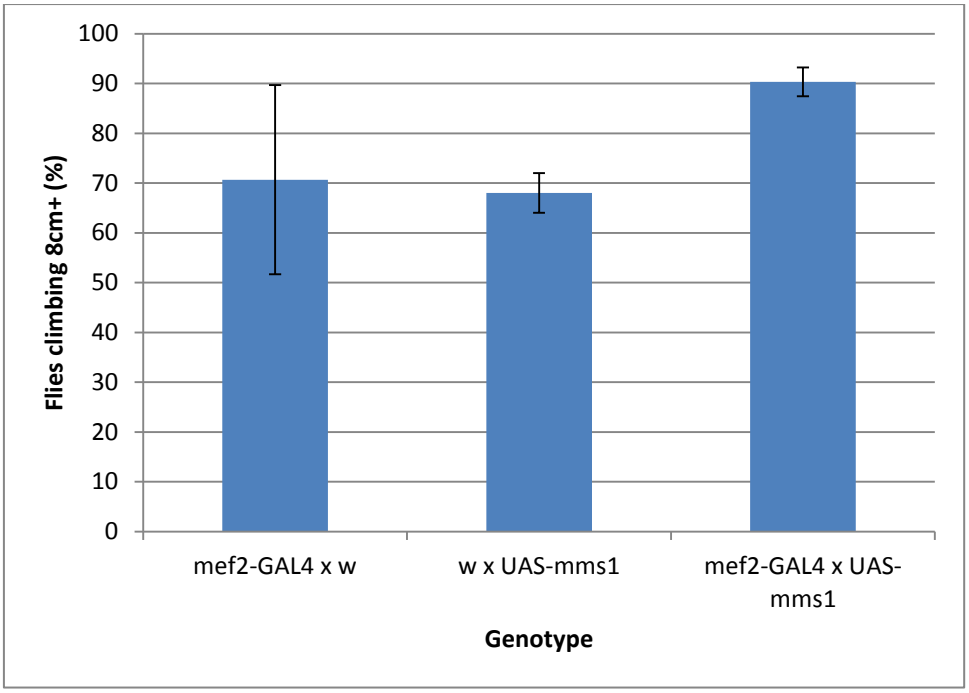

Figure 5.25: Climbing ability of 1 week old adult flies with overexpression of mouse *ms1*, represented by the percentage of flies able to climb >8cm in 10 seconds (n=30 flies, each group of 10 allowed to climb 10 times). Differences are shown to be significant ($p<0.05$ using one-way ANOVA, experimental line is significantly different from both controls using Tukey's HSD Post Hoc test at $p=0.05$ level).

The results shown in figure 5.25 are very similar to those found with overexpression of *dms1*, as climbing ability appears to increase with overexpression of mouse *ms1*. As with overexpression of *dms1*, the experimental line are more consistently able to climb than the driver control line, which once again seems to represent a wide range of climbing ability in contrast to the overexpression flies. This results, along with those from overexpression of *dms1* suggest a similar function for these genes in muscle function.

5.2.4.3 Activity levels under conditions of starvation with overexpression of mouse *ms1*

An activity assay was carried out under starvation conditions as in section 5.2.3.3 comparing flies with muscle-specific overexpression of mouse *ms1* to controls. Crosses were carried out as in section 5.2.4.1. and results are shown in figure 5.26.

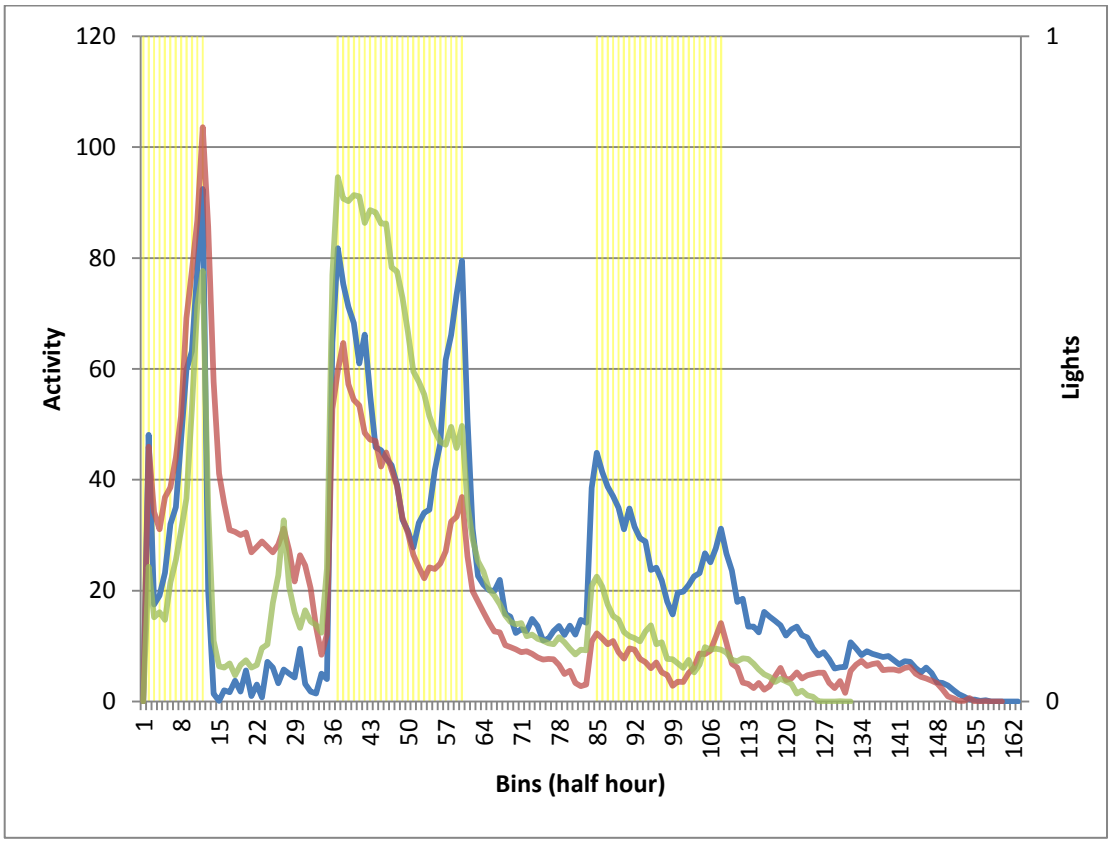

Figure 5.26: activity plot showing activity levels under starvation conditions of flies with overexpression of mouse *ms1* (blue) compared to UAS control (red) and driver control (green). Activity scale refers to average number of times fly crosses infrared beam in activity monitor (n=21 flies). Flies are kept in 12:12 LD, lights are shown in yellow.

Figure 5.26 shows that overexpression of mouse *ms1* in *Drosophila* appears to have a complex effect on activity levels under conditions of starvation. Activity appears to be higher, particularly towards the end of the experiment, however, this can be explained by the survival rate as 9 out of 20 flies with overexpression of *mms1* are dead at this point compared to 14 of the driver control and 16 of the UAS control. The differences in activity/survival between timepoints 85 and 108 varies in significance, with significance found at timepoints 87 and 90, $p<0.05$ using one-way ANOVA. The difference in survival could also explain why the experimental line appears to retain its rhythmicity better than controls, as the peak of activity seen at the end of the second "day", around point 60, could be down to a higher average for activity caused by higher survival in this line. This effect is not seen with overexpression of *Drosophila ms1*. Overall, all flies are dead by timepoint 160, and this does not differ significantly from the UAS control. However, flies with mouse *ms1* expression seem to survive better than flies with overexpression of *Drosophila ms1* throughout the experiment, particularly during the second half. Figure 5.27 shows the survival of the flies used in this experiment and differences are shown to be significant between the experimental line and both controls. This suggests a function for mouse *ms1* that is not observed with *dms1* in response to starvation conditions.

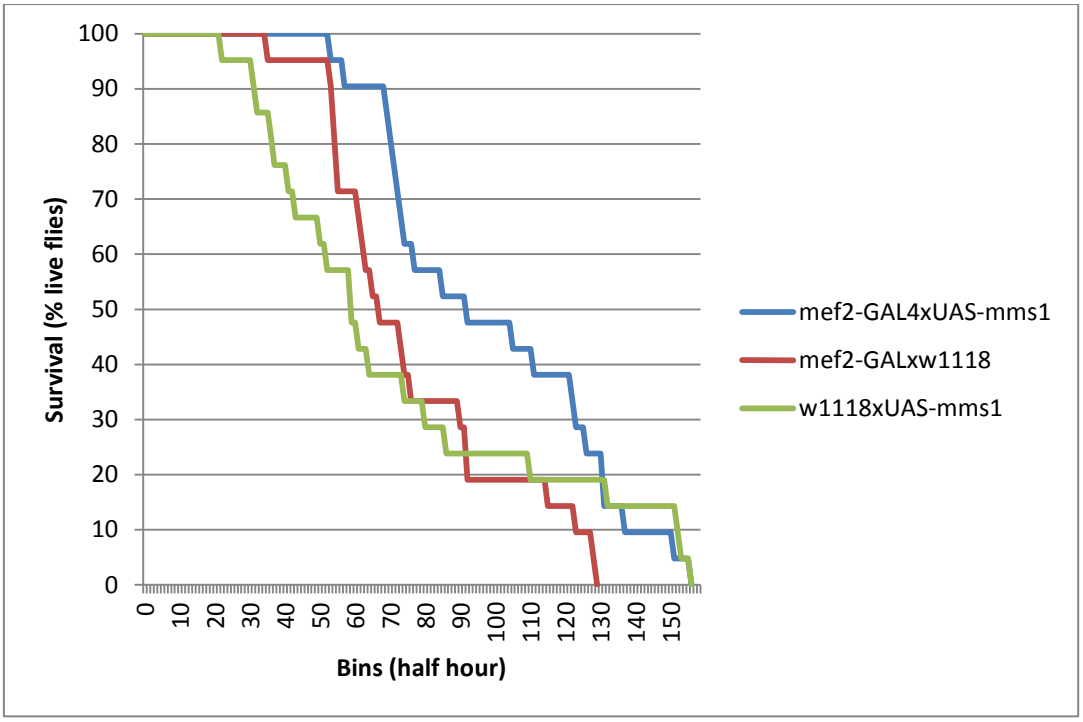

Figure 5.27: survival of flies during activity assay under conditions of starvation. Flies overexpressing mouse *ms1* are shown in blue compared with driver (green) and UAS (red) controls (n=21 flies). Differences are shown to be significant between experimental and UAS control ($p<0.05$ with Mantel-Cox test and Gehan-Breslow-Wilcoxon test) and between experimental and driver control when using Gehan-Breslow-Wilcoxon test ($p<0.01$) but not with Mantel-Cox test ($p=0.15$).

5.2.4.4 Confocal microscopy of muscle structure with overexpression of mouse *ms1*

The thoracic musculature has been examined here to identify any changes in structure and fibre alignment when mouse *ms1* is expressed in a muscle-specific pattern. This will allow comparison with controls and also with muscle with overexpression of *dms1* to find any similar effects that may indicate homology between the two genes. Crosses were carried out as in section 5.2.4.1 and images of dorsal and ventral muscle were taken at high magnification, shown in figures 5.28 and 5.29.

A B

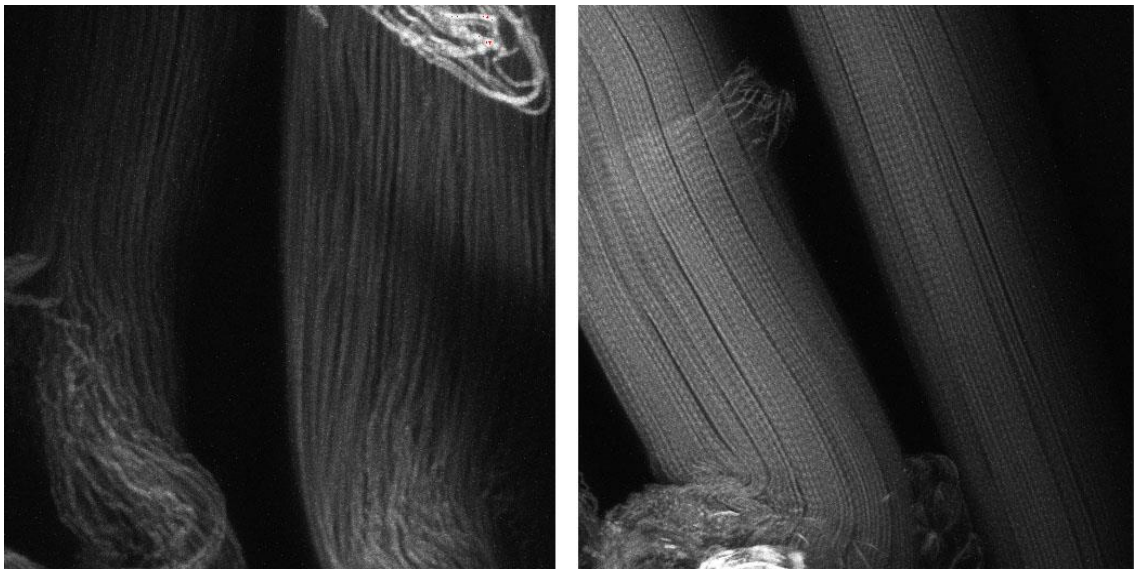

Figure 5.28: Confocal image of dorsal thoracic muscles with muscle-specific overexpression of mouse *ms1* (A) compared with driver control (B) at 60x magnification.

A B

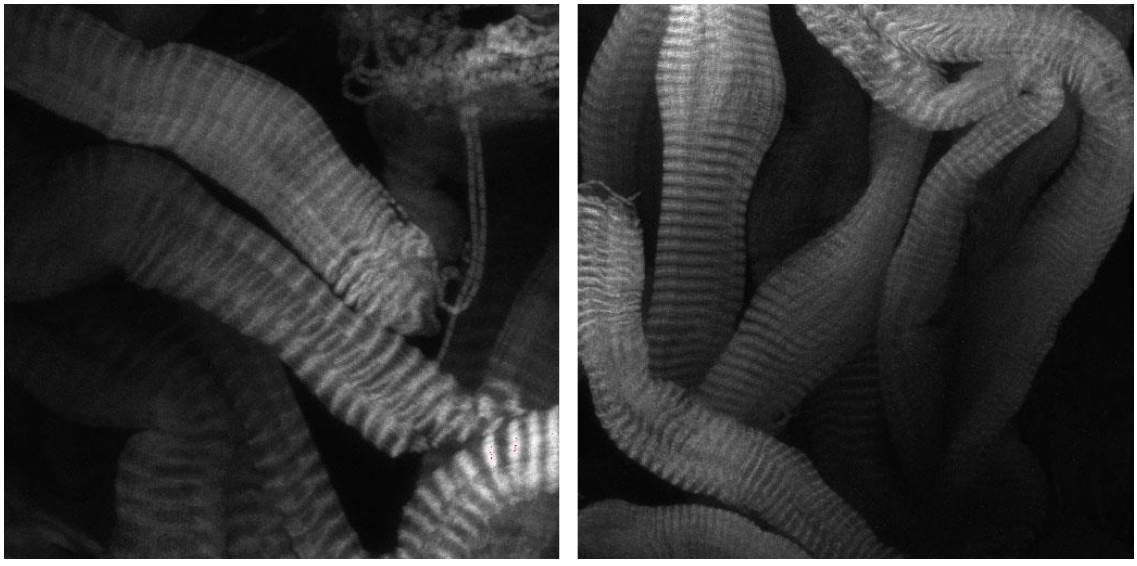

Figure 5.29: Confocal image of ventral tubular thoracic muscles with muscle-specific overexpression of mouse *ms1* (A) compared with driver control (B) at 60x magnification. The driver control used here is the same as that used for comparison in figure 5.16B due to a lack of usable images.

As with overexpression of *dms1*, overexpression of mouse *ms1* does not appear to have any effect on muscle structure. Alignment of fibres in the dorsal muscle in figure 5.28 is not disrupted significantly, and striations and overall structure in the ventral tubular muscles in figure 5.29 appears the same as that of controls. This is similar to the results found in flies with overexpression of *dms1* and so do not give any indication as whether *dms1* and mouse *ms1* are having a similar effect on muscle.

5.2.5 Levels of *ms1* under exercise conditions

In humans, levels of *ms1* have been shown to increase under exercise conditions (Lamon *et al.*, 2009; MacNeil *et al.*, 2010), resulting in an increase in muscle size and output. Taking this into consideration, and the fact that the results in this chapter could imply that *dms1* may have a similar function to that of mammalian *ms1*, it is possible that *dms1* may also increase if flies are exercised. This has been achieved here using the flies' negative geotaxic response, the instinct to climb upwards when knocked down. The FlyGym based on Piazza *et al.*'s Power Tower (Piazza *et al.*, 2009; Tinkerhess *et al.*, 2012) was designed to use this response and allow climbing to continue over long periods of time by repeatedly knocking the flies to the bottom of their vials.

Each biological replicate for qPCR consisted of four different treatments of Canton S wild-type flies, described in section 2.20. Quantitative Real-time PCR (qPCR) data for levels of *dms1* expression under each condition are shown in figure 5.30.

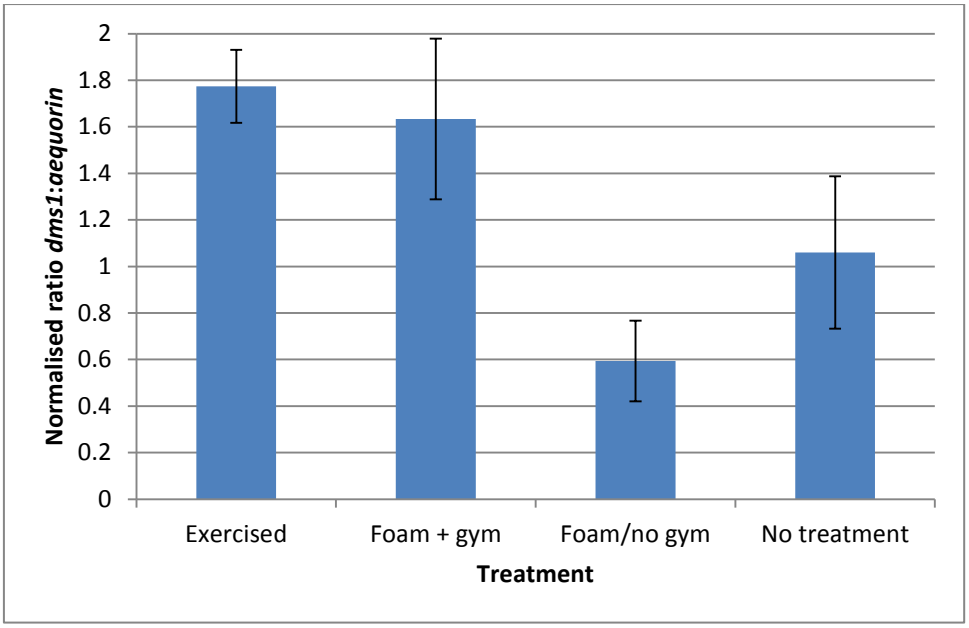

Figure 5.30: levels of *dms1* as determined by real-time qPCR, comparing exercised flies with controls (n=30 flies). Foam + gym control is placed on the FlyGym with a foam stopper to restrict climbing. Foam/no gym control also has foam stopper but is not placed on the FlyGym. The final control has no treatment.

The results in figure 5.30 show an upregulation in both groups of flies that were placed on the FlyGym compared to those that were not. There is also evidence that the foam stopper decreased *dms1* expression compared to conditions without the stopper. These data appear to show a correlation between *dms1* expression and pressure to move, as well as freedom to move. The difference between the fully exercised flies and both groups not placed on the FlyGym is significant ($p<0.005$ using one-way ANOVA, confirmed using Tukey's HSD Post Hoc test), as is the difference between the foam + gym and untreated control, but the difference between exercised flies and the foam + gym control is not.

5.3 Discussion

The aim of the experiments outlined in this chapter was to investigate the role of *dms1* in somatic muscle. This was done by assessing the effect of knockdown of *dms1* on flies, including longevity experiments to look at the overall health of these flies, activity and climbing assays to find effects on ability and inclination to move, measuring relative strength by examining the ability to eclose, and any disruption or change to muscle structure. The effect of overexpression of *dms1* was also examined in similar ways to find if this has the opposite effect of knockdown, thereby giving a better idea of phenotypes associated with changes in expression of *dms1*. The homology of *dms1* and mouse *ms1* has also been investigated by carrying out parallel experiments with overexpression of both. This gives some idea of whether these genes are true homologues. The results found in this chapter certainly suggest an important role for *dms1* in muscle development and maintenance, as changes in expression appear to have an effect on behaviour and muscle function.

Longevity is significantly affected by knockdown of *dms1*, although survival changes in different ways depending on genetic background. Before backcrossing of the lines flies are shown to survive as well as controls up until around 3 weeks, then better for the remainder of their lives. This is seen with both *mef2*-GAL4 driven knockdown with UAS-RNAi(GD) and with the addition of UAS-Dcr which increases the level of knockdown. This would appear to be an interesting result, as it could suggest that after this age knockdown becomes advantageous and increases the health of older flies. A reduction of *dms1* expression could be reducing any stress response that is seen in older flies, therefore the body does not have to maintain any phenotype associated with this response. As the *ms1* pathway is also associated with many other molecular mechanisms, such as a

potential role in metabolism (Wallace *et al.*, 2011; Jin *et al.*, 2011) or arteriogenesis (Troidl *et al.*, 2009), under normal conditions *dms1* may not be required and may be putting the body under more stress by being expressed normally, thus knocking down this expression gives an advantage to these flies. However, this effect on longevity is lost after backcrossing when using a UAS-RNAi(KK) line, suggesting that either this is simply a background effect or that the effect is lost with a higher level of knockdown using this particular RNAi line. If this is the case it is worth noting that the longevity of flies with knockdown by KK line seems to be reduced in comparison to the GD line, suggesting that their health is reduced to that of the controls by a higher level of *dms1* knockdown. This is similar to results found with cardiac-specific knockdown of *dms1*, as the beneficial effect of *dms1* knockdown with the GD line is lost with use of the KK line. Knockdown under hypoxic stress affects longevity significantly, especially with a higher level of *dms1* knockdown. With the addition of UAS-Dcr longevity decreases rapidly in knockdown flies after 3 weeks, around the same point where an increase in survival is seen under normal conditions, and also under hypoxic conditions without UAS-Dcr. While low levels of *dms1* reduction may be advantageous under normal conditions and even under hypoxia due to the high tolerance of *Drosophila* to low oxygen levels (Haddad *et al.*, 1997), a higher level of knockdown under hypoxia may be reducing the stress response of these flies to such an extent that they cannot survive as well as control flies, as well as affecting other mechanisms in which *dms1* has a role. This is more obvious after 3 weeks as flies are older and may be less healthy in general at this point. The experiment was not carried out after backcrossing and so the results could still be due to a background effect, and further experiments would prove this either way, but the

survival does seem significantly reduced under hypoxia with higher levels of *dms1* in contrast to results seen under normoxia, and so this could be a true effect and suggests that *dms1* knockdown in all muscles is reducing the flies response to low oxygen enough to affect longevity.

Muscle-specific knockdown of *dms1* leads to a reduction in daily activity in *Drosophila* compared to controls. Average levels of activity are reduced by at least 50% during 12:12 LD conditions, and this reduction persists into constant darkness (DD). Flies do not seem either able or willing to move as much as flies with normal levels of *dms1*, suggesting that reduction in expression is somehow causing a problem in muscle strength or energy levels. Rhythmicity is not affected and the circadian clock does not appear to be disrupted in any way, so the difference in activity seems to be purely the result of a problem within the muscle. The phenotype observed could be because flies are unable to move as much as the controls due to a lack of *dms1* during development, causing the muscles to develop incorrectly. It is likely that *dms1* has a role in development of adult *Drosophila* muscles due to the peak in expression during pupal stages (flybase.org), and a reduction may be affecting this process. Alternatively it could be a lack of *dms1* is affecting the muscles' ability to utilise energy and the flies are simply unwilling to move due to tiredness, rather than unable. These data were the first indication that *dms1* is important for muscle function and significantly affects the flies activity. Therefore, further experiments were designed to follow up on these results. However, no activity phenotype is seen when *dms1* is overexpressed in all muscle tissue. Activity levels are similar to that of both control lines and do not differ in rhythmicity or daily cycles. This could indicate that while *dms1* may be necessary to ensure correct development of muscles, as

implied by data from the knockdown flies, it does not force the flies to move more than they normally would, and that flies would have to be put under pressure to reveal any differences in ability or strength with overexpression of *dms1*. In mammalian hearts no phenotype is found when overexpression of *ms1* is forced in cardiac tissue, but when calcineurin is knocked down the hearts have an increased hypertrophic response to stress (Kuwahara *et al.*, 2007). If this is the case in *Drosophila* muscle, it is possible that further modifications to gene expression are required in order to see a phenotype when overexpressing *dms1*.

To further examine the effect of *dms1* knockdown on muscle function, climbing experiments were carried out to test the flies ability to climb when prompted. This used the negative geotaxic response of *Drosophila* to force a climbing response when flies were knocked to the bottom of a vial. The main difference between this and the activity experiment is that looking at locomotor activity shows the normal movement over the course of the day with no pressure put upon flies to move, whereas climbing assays forced flies to climb, so comparison of these two sets of results can give an idea of the way in which *dms1* knockdown is affecting muscle function, and whether it could be ability or just inclination to move that is being reduced. The results of the climbing assays show that ability is significantly reduced in flies with knockdown of *dms1*, suggesting that defects are impairing muscle function to the extent that flies are unable to climb as well as controls with normal levels of *dms1* expression.

Climbing assays were also done for flies with overexpression of *dms1*, and appeared to show the opposite of the results obtained when knocking down *dms1*. A higher expression level of *dms1* appears to increase the flies ability to climb when under pressure to do so, suggesting that higher *dms1* levels increase the strength or

function of muscle tissue. This result is a contrast from that found when activity levels of overexpression flies was measured, as no differences were found between these and controls, so it is likely that overexpression of *dms1* increases the flies ability to move but not their inclination, so they are able to climb better when forced to but do not feel compelled to move more under normal conditions, likely in order to conserve energy and thereby increase survival.

Results obtained from knocking down *dms1* were further supported by the observation that in the crosses carried out for activity and climbing assays many flies of the experimental genotype did not eclose from their pupae. An eclosion assay was designed to quantify the extent to which eclosion was reduced by reduction of *dms1* expression and it was found that there was a significant reduction in adult fly emergence compared to controls using both available *mef2*-GAL4 driver lines. A reduced ability to eclose could indicate an inability to break through the wall of the pupa in flies with a knockdown of *dms1*. This could be due to an underdevelopment of muscles meaning that the flies do not have the strength to break through the pupal wall. This fits with expression data from Flybase (flybase.org) which shows the highest levels of *dms1* are found just before eclosion. If this level is reduced it could affect the final stage of development of the adult fly muscles, resulting in weaker muscles and therefore affecting the ability of knockdown flies to eclose. Along with data from activity and climbing assays, this experiment supports the theory that *dms1* has an important role in muscle development and function. Specifically, the eclosion assay suggests again that *dms1* is required for muscles to develop to the expected strength, which would give a specific reason as to why flies with *dms1* knockdown are less able to climb. Eclosion assays were not carried out with overexpression flies as eclosion was close to 100% in most of the control replicates

and so any difference in strength due to *dms1* overexpression would not have been seen here.

Despite the phenotypes associated with knockdown and overexpression of *dms1*, there does not seem to be any obvious change in muscle structure or fibre alignment that can be seen using confocal microscopy. Any changes may only be visible at much higher magnification, or there may be differences in the arrangement of the sarcomere. It is known that *ms1* mainly localises to the I-band of the sarcomere in mammalian cardiac cells (Arai *et al.*, 2002), and if this is the same for *dms1* then any changes may be on a much smaller scale than can be seen with this technology. Much higher magnification, possibly with electron microscopy, would be required to examine this further.

All of the experiments investigating the effect of overexpression of *dms1* were run in parallel with a line of flies overexpressing a transgene for mouse *ms1*. This is to allow comparison of these results and find any similarities between phenotypes seen with overexpression of these genes in *Drosophila*. Activity and climbing assays were carried out, along with examination of muscle structure under high magnification and activity under conditions of starvation. Of these, many showed similar results with overexpression of *dms1* and mouse *ms1*. Activity under normal conditions in flies expressing mouse *ms1* does not differ from controls, as is seen with overexpression of *dms1*. Levels of activity are similar and rhythmicity is unchanged. This suggests either there is no effect of this gene on *Drosophila* activity, or that other factors are required to cause a phenotype to be seen, as described earlier. These results do not confirm any similarity between *dms1* and mouse *ms1*, but they do not rule it out either, as no significant difference was seen between experimental line and controls in either experiment.

However, results from the climbing assays appear to show an increase in climbing ability in flies with overexpression of *dms1* compared to controls, and this effect is seen again with overexpression of mouse *ms1*. Indeed, flies expressing mouse *ms1* actually appear to climb slightly better than those overexpressing *dms1*, and, as with *dms1*, their climbing ability is consistently better than controls. This result suggests that mouse *ms1* is able to increase muscle function and strength to the same extent as the endogenous *dms1*, and may in fact outperform *dms1*. A more sensitive test would show if this is the case as the climbing abilities of the two experimental groups are very similar. While showing that this mouse gene can function in *Drosophila*, the similarity of the results between overexpression of *Drosophila* and mouse *ms1* indicate that these genes do share homology, at least in the pathway controlling muscle development and function. Confocal microscopy, however, does not show any differences in muscle structure between flies with overexpression of mouse *ms1* and controls, similar to results seen previously in this chapter, with both knockdown and overexpression of *dms1*, and this could be due to defects only being visible at much higher magnification. Again, this gives no indication that *dms1* and mouse *ms1* are true homologues. One experiment that would give a good indication of homology between the two genes in muscle development would be to attempt to rescue knockdown of *dms1* with overexpression of mouse *ms1*. The phenotype of flies with knockdown of *dms1* is particularly obvious, and if mouse *ms1* was able to rescue this phenotype and restore full mobility to these flies it would be easily seen. If mouse *ms1* was able to allow flies with knockdown of *dms1* to move as well as flies with normal levels of *dms1*, or was at least able to improve the performance of these flies, it would indicate that mouse *ms1* performs a similar function to *dms1* and can allow

muscle to develop normally. However, this experiment could not be carried out here due to difficulties in creating cross schemes to ensure flies would carry all required GAL-4 and UAS constructs.

The only differences between data from flies overexpressing *dms1* and mouse *ms1* are found when flies are subjected to starvation. Overexpression of *dms1* does not appear to affect activity or survival under these conditions. Only two timepoints show significant differences during the first night, where experimental flies have lower activity than controls. This could indicate that higher levels of *dms1* allow the flies to retain their rhythmicity for longer, however, the statistical significance is low and would require further investigation to determine if this is a true effect. Data from activity assays under starvation conditions with flies overexpressing mouse *ms1* show somewhat different results. Activity levels are similar to those of controls for the first 24 hours, then average activity appears to increase in the experimental line, but this is almost certainly due to the differences in survival seen after this point. Flies with overexpression of *ms1* appear more able to survive at later stages of starvation than controls, although the maximum longevity does not change. The differences between the results found with overexpression of these two genes could be indicative of additional functions gained during evolution. As the mouse homologue has unique N-terminal sequence (Arai *et al.*, 2002), this may give it additional functions that the *Drosophila* homologue does not possess, and the same can be said for the unique C-terminal sequence found in *dms1*. This experiment was designed to examine the effect of overexpression on the flies ability to move more in order to find food, so activity levels under pressure to find an effect on muscle function, but also to look at survival and any potential effect of these genes on tolerance to starvation. This could indicate a role

in metabolism as has been demonstrated in previous studies (Wallace *et al.*, 2011). As survival seems to be increased with overexpression of mouse *ms1* and not with *dms1* this could show a difference in function between the two, and suggests that mouse *ms1* has evolved a function in metabolism where *Drosophila ms1* has not, despite the apparent homologous functions in muscle development and maintenance. If this is the case, then it could be that the homologous actin-binding domains of mouse and *Drosophila ms1* play a role in muscle development and the unique N-terminus of mouse *ms1* gives this gene other functions in addition to this.

As well as investigating the effect of changes in *dms1* and mouse *ms1* expression on the behavioural and structural phenotype of flies, it was hypothesised that *dms1* upregulation could be induced by exercise. It has been shown that exercise in human causes a significant upregulation of *ms1* and it is likely that *ms1* plays an important role in muscle building and repair after exercise (Lamon *et al.*, 2009; MacNeil *et al.*, 2010). A method of exercising flies was developed based on that of Piazza *et al.* (2009) utilising the negative geotaxis response of *Drosophila* to induce repeated climbing and levels of *dms1* were measured after 3 weeks of daily exercise. The most obvious result from the FlyGym data is the difference in *dms1* expression between those flies that were placed on the machine and those that were not. Higher expression is seen with both groups of flies that were put on the machine than those that did not have this treatment. It could be said that the effect of the machine puts the flies under stress that causes upregulation of *dms1*. This could be through damage to the muscles as a result of impact of the vial on the benchtop each time the flies are knocked down, and this damage could be initiating repair pathways of which *dms1* may be a part. Another suggestion is that

the foam control is not fully immobilising the flies, but merely restricting their ability to climb the walls of the vial. They are still able to walk freely around the base of the vial, and this movement is further prompted by the action of the FlyGym knocking the flies around. The lack of significance in the difference between this control and the exercised flies can be explained by this, as the foam control flies may still be able to move enough to cause *dms1* to be upregulated to a point where the difference between this and flies that can move freely cannot be determined. However, the presence of the foam stopper may be restricting movement just enough that these flies have a low enough, or inconsistent enough, upregulation of *dms1* that the difference between these and untreated flies is also insignificant. Attempts to fully immobilise the flies has proved unsuccessful without causing harm to the flies or severely affecting the exercised flies ability to climb when ensuring consistent conditions for all treatment groups. Results comparable to these can be found in the study that inspired the FlyGym by Piazza *et al.* (2009) with their Power Tower, where results from comparison between flies on the Tower and controls kept off the Tower showed greater difference than the difference between exercised and non-exercised (foam + Tower) control (Piazza *et al.*, 2009) . However, there does still appear to be a trend in the results from the FlyGym experiment, as *dms1* seems to correlate with climbing induction and freedom to move, suggesting that *dms1* expression can indeed be induced by exercise in *Drosophila*.

Overall, the results from this chapter strongly suggest a role for *dms1* in muscle tissue. Normal activity levels, eclosion and climbing ability are all reduced along with reduction in *dms1* expression, and climbing is increased with overexpression, as well as an increase in climbing ability in flies with overexpression of mouse *ms1*,

indicating homology between these genes. Upregulation of *dms1* may also be induced by exercise. Further experiments could increase understanding of the mechanisms behind the phenotypes seen here, including putting flies under stress with different levels of *dms1* expression, and also measuring *dms1* expression in response conditions such as hypoxia or oxidative stress. A *dms1* knockout line would also be interesting to look into, as this may reduce muscle function even further, and would also make any rescue experiments easier to carry out. Comparing the results from this chapter with those of chapter 4, it appears that the role of *dms1* in muscle is more important than its role in the heart, but this could be due to the importance of the heart itself in *Drosophila*. While *ms1* is known to be important in mammalian cardiac regulation, this importance does not appear to be demonstrated in *Drosophila*. This leads to the suggestion that it may be more fruitful to focus future research on the role of *dms1* in muscle, with the potential to use this system for research into muscle-specific disease.

Chapter 6: General discussion

This project was designed to explore the functions of the stress-responsive gene *ms1* using the fruit fly model organism *Drosophila melanogaster*. It is known that *ms1* is upregulated within a few hours of pressure overload in the heart (Mahadeva *et al.*, 2002) and is an important factor in the development of left ventricular hypertrophy. Upregulation of *ms1* is also found in skeletal muscle in response to exercise and is involved in hypertrophy under these conditions (Lamon *et al.*, 2009; MacNeil *et al.*, 2010). Here, the function of *Drosophila ms1* (*dms1*) has been investigated to determine whether this gene, designated CG3630, is the true homologue of mammalian *ms1*, and what role it plays in the heart and somatic muscle. It was found that changes to the expression of *dms1* have a significant effect on the function of somatic muscles, particularly those used for walking and climbing. Knockdown of *dms1* caused flies to become less mobile and exhibit reduced strength in comparison to controls. Changes to expression levels in cardiomyocytes did not give any significant results, although a possible trend towards increased arrhythmicity was identified in hearts with knockdown of *dms1*. However, findings from investigation into the molecular reasons for these results were inconclusive, and homology between the associated pathways in flies and mammals could not be confirmed.

6.1 Issues with experimental procedure.

A recurring problem throughout this project is the difference between the two controls used. In many experiments, there was a significant difference in results from the two control lines. This was observed primarily in the qPCR data

measuring levels of *dms1* with knockdown (section 3.2), where the driver control was shown to have lower *dms1* expression than the UAS control. This may be the reason for a similar effect observed in subsequent experiments such as climbing, where results appear to follow the pattern of those from qPCR. Despite this, the experimental result is considered to be reliable, as *dms1* levels are still lower than both controls.

A reason for the differences between controls could be the effect of GAL4 on the organism. In the driver control GAL4 has no UAS binding site, and therefore may accumulate to toxic levels, having a detrimental effect on gene expression and on the behaviour of the animal itself. Various studies have investigated this effect and, while not widely observed, it is more likely to be seen with strong drivers, such as the *mef2* driver used here. One study in particular has shown that expression of an eye-specific GAL4 construct, *glass multiple receptor* (*GMR*)-GAL4, can lead to defects within the eye structure when expressed without the presence of a UAS (Kramer & Staveley, 2003). This effect is dose-dependent as homozygotes display a more severe phenotype than heterozygotes, and higher temperatures also increase the levels of defects. High levels of apoptosis are observed, particularly in homozygotes and when a widely expressed driver is used. The full mechanism behind this apparent toxicity of GAL4 is unclear. It could be that high levels are blocking translocation into the nucleus by binding its nuclear import protein *members only* (*mbo*) (Uv *et al.*, 2000), thereby preventing other proteins from being imported. Alternatively it may be affecting other proteins directly, interfering with their function or expression, as demonstrated by Liu and Lehmann. (2008). This study found several endogenous genes that were upregulated in response to GAL4 when a heat-shock promoter was used, without

the presence of a UAS. This included genes involved in apoptosis pathways. The use of widely expressed promoters again appeared to be necessary for this effect. To overcome this problem it may be necessary to reconsider the controls used, perhaps to give GAL4 a binding site that will not affect gene expression, so that accumulation does not occur. The use of a UAS-GFP construct has been considered, as this would allow the excess GAL4 to bind a UAS and would therefore not accumulate to toxic levels. GFP has low toxicity and is widely used as a reporter for visualisation of cellular structures. However, studies suggest that the use of GFP may significantly affect climbing ability and ageing of the flies when expressed in the whole body (Mawhinney & Staveley, 2011). Under a muscle-specific driver there would be high levels of GFP expression, and as climbing and survival are investigated in this project use of a GFP construct would not be advisable. Potentially a different reported gene could be used, such as *lacZ*, as this GFP study reported that this effect was not observed with a *lacZ* reporter construct. This would enable the binding of GAL4 but would be less likely to affect the outcome of experiments in the way GFP does.

Another solution could be to use temperature sensitive (ts) methods of driving gene expression. This could involve the use of ts promoters that are only expressed at certain stages of development, although it may be a challenge to find a specific promoter that fits the requirements of the current project. Alternatively, ts GAL4 mutants have also been isolated and can be expressed under the control of a promoter of choice (Mondal *et al.*, 2007). In this case GAL4 is only expressed at the permissive temperature and expression of a UAS-controlled gene can be delayed and timed for a chosen stage in development. This would allow knockdown to be restricted, perhaps to pupal stages of development, and would

prevent excessive accumulation of GAL4 throughout the life of the animal. As well as delaying GAL4 expression using temperature, methods for controlling expression with drugs exist (Osterwalder *et al.*, 2001; Roman *et al.*, 2001). However, the delivery method for this system is through food, and may be difficult to quantify the dose or to ensure that all flies get the same dose, whereas temperature is likely to be easier to control. The GAL4-UAS system has been adapted from an endogenous yeast system (Brand & Perrimon, 1993), and there are further regulatory mechanisms that have also been adapted in the same way, such as the GAL80 regulatory protein. GAL80 represses GAL4 activity, preventing it from activating transcription by binding its transcriptional activation domain (Suster *et al.*, 2004). When ts GAL80 variants are used, this provides a method of inhibiting GAL4 activity using temperature (Zeidler *et al.*, 2004). Inactivating ts GAL80 at the required stage in development will allow the GAL4-UAS system to function.

The use of the *mef2*-GAL4 driver may be contributing to the toxic effect of GAL4, due to its widespread expression. However, a high level of expression is required in order to ensure the best possible knockdown/overexpression of *ms1*, and making sure that any effects of these changes in expression are observed. A useful tool in this case would be a *dms1*-GAL4 line, as this is likely to have a more restricted expression pattern than that of *mef2*-GAL4, which may reduce any toxic effect. If a line such as this could be created, it would allow expression to be restricted only to those cells where it is required, namely those cells where *dms1* is expressed normally. This would also ensure high expression in these cells throughout adult stages, as data from Flybase indicates that *dms1* is expressed at higher levels than *mef2* in adult flies (flybase.org).

There are alternatives to the transgenic approach used here, which would remove any problems with drivers or with controls. A knockout line would be a useful tool in this case, particularly as the RNAi is a knockdown and may have a different phenotype to a complete knockout. There are deficiency stocks available by various groups which have been generated to delete a section of chromosome, and these are easily ordered from stock centres. For example, the Drosdel lines are generated by using flippase recognition target (FRT) recombination to create a genome-wide collection of deficiencies, in which can be found a deletion of any gene (Ryder *et al.*, 2007). Similarly, the Exelixis collection offers the same, but created using P element excision as well as FRT recombination (Thibault *et al.*, 2004; Parks *et al.*, 2004). While these lines are useful, unfortunately each deficiency deletes more than one gene, and there are no lines that remove only the gene of interest. This leaves the problem that if any phenotype is found using these lines, it is not possible to rule out the other deleted genes as a cause.

Other methods of creating a knockout line are available. These include the use of zinc finger nucleases (ZFNs) designed to target a specific gene, which recognise the gene of interest and cleave the DNA. This break is repaired by non-homologous end joining which often results in an imperfect repair, and therefore a null mutation in that gene (Santiago *et al.*, 2008). These nucleases are highly specific and rarely have off-target effects, with a permanent change to genetic information, in contrast to RNAi. They also have a relatively high success rate compared to previously used methods of knockout (Wilson, 2008), so this is an effective way of creating a knockout line. Transcription activator-like effector nucleases (TALENs) work in a similar way, causing double strand breaks and thereby inducing endogenous mechanisms to imperfectly repair the break. The DNA binding domain of these

nucleases is derived from bacterial DNA binding proteins and, like ZFNs, can be customised to specifically target chosen genes (Joung & Sander, 2013). They are also easy to design and have a success rate similar to that of ZFNs in many organisms. Recent studies, however, suggest that TALENs may be more effective than ZFNs in *Drosophila* (Beumer *et al.*, 2013), and so may be a better option for the project described here.

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) method of gene knockout has been adapted from a bacterial defence system in which Cas endonucleases recognise foreign DNA such as viruses or conjugative plasmids, and base pair to a target sequence by use of an RNA component. The target DNA is then cut, resulting in double strand breaks (Richter *et al.*, 2013).

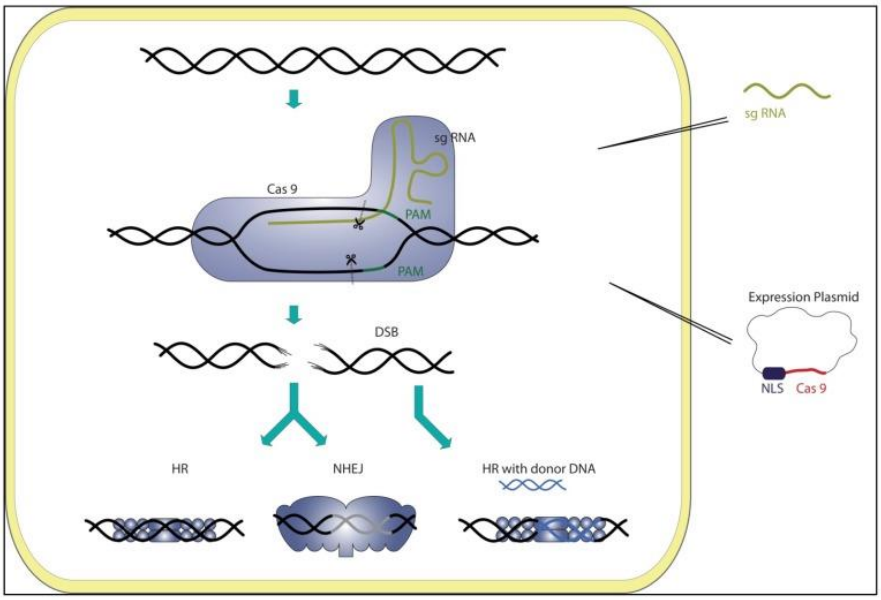

Figure 6.1: The mechanism of the CRISPR/Cas method of gene knockout. The Cas9 endonuclease and RNA component are introduced into the organism at the embryonic stage. These recognise specific sequences within the target DNA and cause a double strand break upstream of the protospacer adjacent motif (PAM), which is then repaired by the cell. Taken from Richter *et al.* (2013).

This system has been adapted for use in gene modification, using the RNA component to target chosen genes, and as with the other methods described, gene knockout is a result of endogenous repair mechanisms as shown in figure 6.1. However, the choice of target sites is restricted by the requirement of a protospacer adjacent motif (PAM) for DNA binding and destabilisation of the helix (Richter *et al.*, 2013), so currently not all genes can be targeted by this system. Despite this, it has been proven to be highly efficient and has been shown to have a high success rate, particularly in *Drosophila* (Bassett *et al.*, 2013) and so may also be a useful tool in this project.

6.2 Implications for future research

The results of this project have confirmed a role for *dms1* in the muscular function of *Drosophila*, showing that *dms1* may have homology to mammalian *ms1* with regards to the pathways controlling muscle growth and maintenance. This suggests that in this context *Drosophila* could be an excellent model for further investigation into the function of *ms1*, interacting proteins and downstream transcriptional changes, as well as the activators of the *dms1* pathway. As much is yet to be discovered about the pathways that control muscle function, this would provide a good early stage model for research in this area. There are results here that suggest *Drosophila ms1* may not share all the functions of mammalian *ms1*, particularly those involved in metabolism, but data from experiments looking at muscle function appear to be significant, so in this context at least, the fly model may be a valuable tool.
While the initial question of the function of *dms1* has at least partially been answered by the work done here, there are many areas that are still open for investigation. The lack of significant results when looking at downstream genes

raises the question of alternative pathways that may be activated by *dms1*. It may be possible to confirm that *dms1* activates *SRF* transcription and subsequently *SRF*-dependent pathways, but there may be other pathways that are also activated, and further investigation would reveal additional functions for *dms1*. Microarrays could be used as a method to identify any genes that change in expression levels in response to up- or downregulation of *dms1*. There are candidates for upstream activators of *dms1* in addition to calcineurin which was tested here. Signalling pathways such as those of MAPKs, PI3K and GATA4/*pnr* could be investigated to find any effect on *dms1* levels, and any downstream genes that have been identified could also be tested to see if these pathways are activating *dms1*-dependent transcription. It may also be interesting to test any associated phenotypes when altering the activation of these signalling pathways. The difficulty in looking at upstream genes is that it is problematic to alter their expression as it may have deleterious effects on viability and survival, to the extent that no offspring may be produced. It may therefore be necessary to develop a range of different techniques to those used in this project, including the use of conditional expression systems. Temporal or temperature-sensitive promoters could be used to drive expression after the flies have reached the adult stage to prevent early-stage lethality.

Proteins that interact with DMS1 could include *Drosophila* homologues of ABLIM or RhoA, as these are known in mammals to work alongside MS1 in the polymerisation of actin. ABLIM2 and 3 have been shown to interact with the conserved region of MS1 using the yeast-2-hybrid system, so if *Drosophila* ABLIM has the same function as in mammals, these may interact. *Drosophila* have one ABLIM homologue instead of the 3 genes found in mammals, and this is known as *Unc-115*, however, analysis of this gene shows that its function lies

within the central nervous system, so it is possible that it does not share the functions of mammalian ABLIM2 and 3 (Garcia *et al.*, 2007). The *Drosophila* homologue of *RhoA* is known as *Rho1*, and is only one of the many GTPases found in fruit flies. *Rho1* is widely expressed and has similar functions to mammalian *RhoA* (Hariharan *et al.*, 1995; Sasamura *et al.*, 1997). ABLIM and RhoA are both required with MS1 to achieve a high enough level of dissociation of MRTFs from actin and transcriptional activation of *SRF* (Kuwahara *et al.*, 2007; Barrientos *et al.*, 2007), and similar experiments as have been used in the original investigations into this pathway can be used in *Drosophila* to identify any conservation in this mechanism. These include investigation of downstream genes expression with reduction of RhoA, MS1 and ABLIM, as well as binding assays between these proteins and binding affinity with actin.

Another aspect of *dms1* expression that was not possible to cover during this project was localisation of the protein, both in terms of the animal, as in which tissues are expressing *dms1*, and in terms of sub-cellular localisation. When cloning of *ms1* was carried out to create transgenic fly lines, tagged versions of both *Drosophila* and mammalian *ms1* were also created. These could be used to follow the pattern of localisation of these genes within the tissue to determine where it localises, using antibodies that recognise the tag sequences used here. This may also give some insight into the mechanism of *dms1* and could help to explain the phenotype observed when *dms1* is knocked down. This may also reveal any differences in localisation pattern between mouse and *Drosophila ms1*, and could help to determine how conserved the function of these two genes is. Antibodies to *dms1* itself could also be created to follow the localisation pattern of untagged *dms1*.

The work done here and any immediate future experiments that can be carried out will help to determine if *Drosophila* are a good model for investigation into *ms1*. Once this has been demonstrated, research can move on to potentially look at disease states and the effect that the *ms1* pathway has on disease phenotypes. When discussing muscle wasting, there are various conditions such as muscular dystrophies and neuromuscular disease that are characterised by atrophy of muscular tissue, with muscular atrophy observed as a side effect in many other conditions that restrict movement of an individual, not to mention as an effect of ageing. From the results found here, the most obvious phenotype was that of the somatic muscle. Therefore it will be an important step to develop a model of muscle wasting and atrophy in *Drosophila* and carry out investigations into the role of the *ms1* pathways in this context. *Drosophila* models of muscular atrophy have been developed, including a model of spinal muscular atrophy (Praveen *et al.*, 2012), and models of age-related muscle deterioration (Augustin & Partridge, 2009), so this may prove to be a useful model when looking for ameliorators of atrophy within the *ms1* pathway, and potential treatment through an *ms1*-related mechanism. With regards to the heart and the role of *dms1* in cardiac tissue, it will depend on whether a phenotype can be observed using the *Drosophila* model, possibly using chemical stress or exercise. However, even if a suitable cardiac phenotype cannot be found for research into *dms1*, there are many similarities between the pathways regulating skeletal and cardiac muscle, and it is possible that any research on the role of *dms1* in somatic muscle may also contribute to cardiac research.

In general, there is a lot of further investigation into the expression, function and homology of *ms1* and its role in disease and muscular decline that can be done using

Drosophila, but in the long term results found here will only be an early stage of research in this field. The work done using this model will subsequently be used to guide research in models in higher animals such as mice and rats. If the *Drosophila* model has revealed any mechanisms by which *dms1* can be utilised to ameliorate muscle atrophy or disease-related muscle wasting phenotypes, this data can be transferred to a mammalian system which will give a better idea of whether these findings could potentially lead to some manner of treatment for human disease. Ultimately, the goal of research into *ms1* and its associated pathways and mechanisms is to develop treatment for human conditions related to the role of *ms1*, and the use of a *Drosophila* model, as in many areas of research, is a good starting point, providing a relatively simple way to determine the basics, before moving into a higher model system.

In conclusion, this project has had some success in achieving its objectives. This work will help to establish *Drosophila* as a model for *ms1* research and other muscle-enriched factors, and may prove valuable in studies into treatment for cardiac and skeletal muscle defects in humans.

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