MEF2 and HDAC Proteins Regulate Striated Muscle Development and Remodeling

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To

My Wife Manda, My Daughter Emma, and My Family

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MEF2 AND HDAC PROTEINS REGULATE STRIATED MUSCLE DEVELOPMENT AND REMODELING

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The establishment of different tissues during embryogenesis requires coupling of upstream signal transduction pathways with networks of transcription factors that govern cell differentiation and morphogenesis. The myocyte enhancer factor 2 (MEF2) transcription factor acts as a lynchpin in the transcriptional circuits that control differentiation of diverse cell types including skeletal, cardiac and smooth muscle cells, neurons, chondrocytes, lymphocytes, endothelial cells and neural crest cells. Class II histone deacetylase (HDAC) proteins bind to MEF2 and regulate MEF2 activity in response to various signaling cascades.

To understand the role of MEF2 and class II HDAC proteins in skeletal muscle development and remodeling, we analyzed individual MEF2 knockout mice, HDAC knockout mice, and compound mutant mice. We discovered that skeletal muscle-specific deletion of *Mef2c* in mice results in disorganized myofibers and perinatal lethality. In contrast, neither *Mef2a* nor *Mef2d* are required for normal skeletal muscle development *in vivo*. Skeletal muscle deficient in *Mef2c* differentiates and forms normal myofibers during embryogenesis, but myofibers rapidly deteriorate after birth due to disorganized sarcomeres and a loss of integrity of the M-line. We discovered that MEF2C directly regulates important structural genes required for the maintenance of sarcomere integrity and postnatal maturation of skeletal muscle.

To address the function of class II HDACs and MEF2 proteins in adult skeletal muscle remodeling, we discovered that class II HDAC proteins, which function as transcriptional repressors of the MEF2 transcription factor, fail to accumulate in the soleus, a slow-twitch muscle, compared to fast-twitch muscles (*e.g.*, white vastus lateralis). Using gain- and loss-of-function approaches in mice, we discovered that class II HDAC proteins suppress slow, oxidative myofiber identity through the repression of MEF2 activity. Conversely, expression of a hyperactive form of MEF2 in skeletal muscle of transgenic mice promotes the slow fiber phenotype and enhances running endurance, enabling mice to run almost twice the distance of wild type littermates. Thus, the selective degradation of class II HDACs in slow skeletal muscle provides a mechanism for enhancing physical performance and resistance to fatigue by augmenting the transcriptional activity of MEF2.

To understand the functions of class I HDACs in cardiac development and remodeling, we generated cardiac-specific HDAC1 and HDAC3 transgenic mice. Overexpression of HDAC1 resulted in a dilated cardiomyopathy, while overexpression of HDAC3 produced a stress-induced cardiac phenotype. We establish an important role for these proteins in cardiac remodeling and provide potential mechanisms regulating these enzymes *in vivo*.

Taken together, these studies demonstrate an important role for MEF2 and HDAC proteins in muscle development and function. Moreover, these results provide important mechanistic insights into the regulation of MEF2 and HDAC proteins *in vivo*.

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CaMK	calcium/calmodulin-dependent protein kinase
HDAC	histone deacetylase
MEF2	myocyte enhancer factor 2
MHC	myosin heavy chain
MuRF	muscle RING (‘really interesting new gene’) finger-1 protein
NFAT	nuclear factor of activated T cells
PGC-1	peroxisome–proliferators-activated receptor-gamma coactivator-1
PKD	protein kinase D
PPAR	peroxisome-proliferator-activated receptor
bHLH	basic helix-loop-helix
cDNA	complementary DNA
DMEM	Dulbecco’s Modified Eagle’s Medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IP	immunoprecipitation
MADS box	MCM1, Agamous, Deficiens, and SRF box
NLS	nuclear localization signal
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
TAD	transcription activation domain
Tg	transgenic
WT	wild-type
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
β-gal	beta-galactosidase
TSA	trichostatin acid
SMRT	silencing mediator for retinoic acid and thyroid hormone receptors
N-CoR	nuclear receptor co-repressor
NuRD	nucleosome remodeling and deacetylating
VPA	valproic acid

Chapter I

Transcriptional Regulation of Skeletal Muscle Development, Remodeling, and Regeneration

Skeletal Muscle Development

The specification and differentiation of cells during embryogenesis requires the activation of tissue-specific transcription factors to establish a gene expression network that defines a particular cell type. In vertebrates, skeletal muscle arises from pre-somitic paraxial mesoderm which eventually segments to form the somites, located at either side of the notochord and neural tube (Buckingham et al., 2003; Christ and Ordahl, 1995). Different areas of the somite can be divided in epaxial and hypaxial zones which give rise to the sclerotome and dermomyotome (dermatome and myotome) (Buckingham, 2001; Buckingham et al., 2003). The epaxial dermomyotome gives rise to back muscles, whereas the hypaxial dermomyotome gives rise to the remaining muscles in the body including the limb muscles (Tajbakhsh and Buckingham, 2000). Muscle progenitor cells from the hypaxial dermomyotome delaminate and migrate into the limb field and position to give rise to the limb muscles (Christ and Ordahl, 1995). This migration requires positional cues from surrounding tissues to signal muscle precursor cells to migrate and express specific genes including Pax-3, c-met, HGF, and Lbx-1. The importance of these factors and their role in delamination and migration are described elsewhere (Buckingham et al., 2003).

Once muscle progenitor cells have migrated to the appropriate area, they become myoblasts by proliferating and expressing transcription factors for specification. The major factors that specify the muscle lineage are the MyoD family of bHLH transcription factors, Myf5 and MyoD. Subsequently, additional bHLH transcription factors myogenin and Mrf4 are expressed to signal differentiation, myoblast fusion into myotubes, and myofiber maturation. Members of the MyoD family of bHLH transcription factors:

Myf5, MyoD, myogenin, and MRF4, are expressed specifically in skeletal muscle and are each capable of activating the muscle gene program when expressed in non-muscle cells (reviewed in (Arnold and Winter, 1998; Olson, 1990; Rudnicki and Jaenisch, 1995; Tapscott, 2005)). Loss-of-function studies have shown that that *MyoD*$^{-/-}$*; Myf5*$^{-/-}$ double knockout mice fail to develop skeletal muscle (Rudnicki et al., 1993), reflecting redundant roles of these genes in the establishment of the skeletal muscle lineage. *Mrf4* has also been implicated in specification of muscle cell fate (Kassar-Duchossoy et al., 2004), whereas *myogenin* is required for skeletal muscle terminal differentiation (Hasty et al., 1993; Nabeshima et al., 1993).

Members of the MyoD family of transcription factors interact with MEF2 factors to cooperatively activate muscle-specific genes (Black and Olson, 1998). MEF2 factors alone do not possess myogenic activity, but potentiate the activity of bHLH factors (Molkentin et al., 1995). The role of MEF2 proteins in various tissues, including muscle, is reviewed in Chapter II.

Skeletal Muscle Remodeling

Elucidating the signaling pathways that regulate skeletal muscle remodeling is pertinent to understanding the pathology of myopathic diseases, such as muscle dystrophy, metabolic disorders, myositis and muscle atrophy. Although histologically skeletal muscle appears uniform, it is actually composed of a heterogeneous population of myofibers which differ in their metabolic and contractile properties. Myofibers are classified based on their expression of myosin heavy chain (MHC) genes (Schiaffino and Reggiani, 1996). Type I fibers, also termed slow-twitch fibers, express MHC type I/beta,

exert a slow contraction, are rich in mitochondria and myoglobin, exhibit an oxidative metabolism, and have high resistance to fatigue. Type II fibers, termed fast-twitch fibers, express MHC type II, fatigue rapidly, exert quick contractions, and can exhibit glycolytic or oxidative metabolism. Myofibers can alter their gene expression in response to environmental demands through activation of signal transduction pathways which ultimately promote adaptive changes in cytoarchitecture and protein composition. The physiological signals regulating skeletal muscle remodeling have been known for decades but the molecular mechanisms regulating these processes are now beginning to be elucidated. Here I discuss recent advances in identifying signaling pathways regulating myofiber remodeling and their relationship to muscle development, growth, adaptation and disease.

Plasticity of skeletal muscle

Primary myofibers expressing adult and embryonic myosins are established early in development, and adult myofibers are established postnatally with adult myosins replacing the embryonic ones (Garry et al., 1996). While primary myofibers are patterned and specified according to developmental cues, adult myofibers exhibit a high degree of plasticity and can phenotypically "remodel" or "switch fiber-types" in response to specific environmental and physiological cues including contractile load, hormonal milieu, and systemic diseases (Baldwin and Haddad, 2001; Sieck and Regnier, 2001). For example, exercise training results in transformation of pre-existing fast fibers to an oxidative phenotype (MHC type II to MHC type I), and conversely, decreased neuromuscular activity causes a slow-to-fast myofiber conversion (MHC type I to MHC

type II) (Talmadge, 2000). This remarkable ability of myofibers to respond to environmental demands occurs through activation of signal transduction pathways which ultimately remodel the myofiber through alterations in gene expression. Defining the factors in the signaling pathways in myofibers provides potential drug targets to therapeutically remodel skeletal muscle.

Calcineurin-NFAT signaling pathway regulating myofiber remodeling

Specific nerve impulses from muscle innervated motor neurons signal myofibers to change phenotypically. Tonic, low frequency motor neuron activity promotes the slow phenotype (type I) and phasic, high frequency firing promotes the fast phenotype (type II) (Olson and Williams, 2000). In slow fibers, these nerve impulses direct sustained, tonic contractile events and signal high intracellular calcium concentrations (100-300 nM) (Chin and Allen, 1996; Hennig and Lomo, 1985). Fast-twitch, glycolytic fibers, on the other hand, exhibit high-amplitude calcium waves and lower ambient calcium levels (<50 nM) (Westerblad and Allen, 1991). Calcineurin, a heterodimeric protein phosphatase, is specifically activated by sustained, low-amplitude calcium waves (Dolmetsch et al., 1998; Hennig and Lomo, 1985). When activated, calcineurin dephosphorylates nuclear factor of activated T cells (NFAT), translocating NFAT from the cytoplasm to the nucleus which allows NFAT to target and activate muscle remodeling genes (Fig. 1.1). The importance of calcineurin activity in establishing and maintaining slow myofibers is highlighted *in vivo* by the dramatic increase of slow (type I) fibers in mice overexpressing calcineurin and by the absence of slow fibers in mice overexpressing RCAN1 (previously known as MCIP-1), an inhibitor of calcineurin (Oh et al., 2005). A potential therapeutic

use for activated calcineurin in skeletal muscle disease is seen in the amelioration of muscle injury when calcineurin is overexpressed in skeletal muscle of *mdx* dystrophic mice (Stupka et al., 2007).

MEF2-HDAC signaling pathway regulating myofiber remodeling

Myocyte enhancer factor 2 (MEF2) transcription factors are key regulators of skeletal muscle development (Black and Olson, 1998; Molkentin and Olson, 1996). Although MEF2 is expressed and bound to DNA in muscle, its activity is repressed by association with class II histone deacetylases (HDACs) (Chang et al., 2004; McKinsey et al., 2000; McKinsey et al., 2002a). In response to specific phosphorylation signals, class II HDACs are shuttled from the nucleus to the cytoplasm, resulting in activation of MEF2-dependent differentiation genes (McKinsey et al., 2000; McKinsey et al., 2001) (Fig. 1.1). By defining the MEF2-HDAC signaling pathway, drugs can be designed to target factors in the pathway that may activate MEF2 and provide a therapeutic approach to ameliorate myopathies.

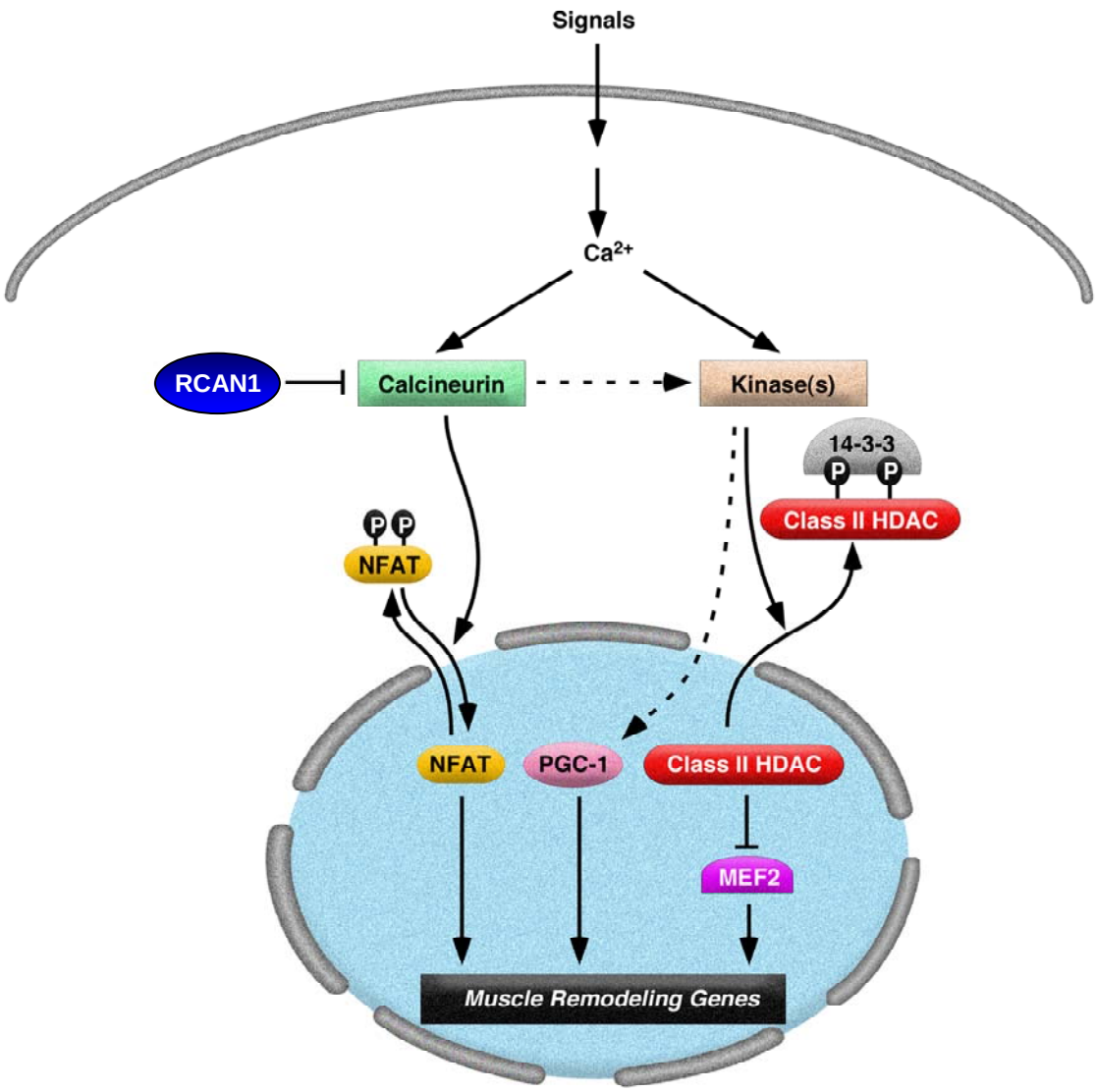

Figure 1.1. Signaling pathways activate skeletal muscle remodeling genes. In response to physiological demands, intracellular calcium concentration is elevated, activating the calcineurin-NFAT and MEF2-HDAC signaling pathways.

While MEF2 is important for skeletal muscle development, several lines of evidence have suggested a role for MEF2 proteins in myofiber remodeling. A valuable tool to measure MEF2 activity is a MEF2 reporter mouse (DesMEF lacZ). When MEF2 is activated, the tissues of this transgenic mouse stain blue due to the transgene containing three tandem MEF2 binding sites which drive the lacZ (beta-galactosidase)

gene (Naya et al., 1999). The use of MEF2 reporter mice revealed that MEF2 is activated in slow postural muscles. After subjected to running the MEF2 reporter mice demonstrated increased MEF2 activity in exercised muscles, suggesting the involvement of activated MEF2 in muscles transforming to an oxidative state (Wu et al., 2001). Interestingly, administration of a calcineurin inhibitor ablates the exercised-induced MEF2 activity (Wu et al., 2001), linking the calcineurin/NFAT and MEF2-HDAC signaling pathways during skeletal muscle remodeling.

Further evidence establishing the role of HDAC-MEF2 pathway in skeletal muscle remodeling and fiber type is seen using genetically altered mice models that are lacking HDAC and/or MEF2 in skeletal muscle (Potthoff et al., 2007). Genetic removal of any four alleles of class II HDACs results in a dramatic increase in the number of slow fibers, and consistent with this finding, loss of MEF2 proteins results in a significant decrease in slow fiber composition. Conversely, using mouse models that overexpress HDAC in skeletal muscle is sufficient to block exercised induced fiber-type switching, and as predicted, overexpression of a constitutively active form of MEF2 (MEF2-VP16) in skeletal muscles drives slow fiber formation in muscles normally composed of fast fibers and stimulates expression of oxidative and metabolic factors. Most remarkably, MEF2-VP16 transgenic mice display an increase in exercise endurance, running almost twice as long as wild-type littermates. These studies demonstrate that activation of MEF2 is sufficient to enhance skeletal muscle oxidative capacity and diminish muscle fatigability (Potthoff et al., 2007). The results of crossing MEF2-VP16 mice (endurance mice) with *mdx* (muscular dystrophy) mice will determine if activation of MEF2 in

dystrophic myofibers is a valid therapeutic approach to ameliorate skeletal muscle disorders.

Another approach to activate MEF2 is to activate the proteins that phosphorylate class II HDACs. Multiple kinases which phosphorylate class II HDACs have been identified and result in nuclear to cytoplasmic shuttling and activation of MEF2 (Berdeaux et al., 2007; McKinsey et al., 2000; Vega et al., 2004a). As anticipated, overexpression of class II HDAC kinases in skeletal muscle is sufficient to increase MEF2 activity and drive slow fiber formation *in vivo* (Wu et al., 2002). Recently, another class II HDAC kinase, salt inducible kinase (SIK1), was reported (Berdeaux et al., 2007; van der Linden et al., 2007). SIK1 is a direct target of CREB and provides a potential mechanism for regulating the myogenic program through activation of MEF2 (Berdeaux et al., 2007; Chen et al., 2005). Using a transgenic mouse that encodes an inhibitor of CREB activity (M-ACREB) and that exhibits muscle dystrophy, it was shown that SIK1 disrupts class II HDAC repression of MEF2, allowing myocyte survival (Berdeaux et al., 2007). Whether SIK1 plays a role in myofiber remodeling remains to be determined. Taken together these studies propose using HDAC kinases as a strategy to activate MEF2 in myofibers, potentially providing an approach to combat skeletal myopathies.

Metabolic influences on myofiber identity

Several metabolic transcription factors play a role in regulating myofiber identity including peroxisome-proliferator-activated receptor gamma coactivator-1α (PGC-1α), -1β (PGC-1β), and peroxisome-proliferator-activated receptor delta (PPARδ). Altering

the expression pattern of these metabolic transcription factors may provide therapeutic approach to remodel myofibers. PGC-1α, a transcriptional regulator of mitochondrial biogenesis, oxidative metabolism, and skeletal muscle biology (Handschin and Spiegelman, 2006), is preferentially expressed in slow fibers. Encouragingly, overexpression of PGC-1α is sufficient to drive slow fiber formation *in vivo* (Lin et al., 2002). However, PGC-1α is not required for slow fiber formation since mice lacking PGC-1α display normal distribution of slow fibers (Arany et al., 2005).

PGC-1α is linked to the MEF2-HDAC signaling pathway since PGC-1α expression is directly regulated by MEF2, and repression of MEF2 causes down-regulation of PGC-1α and other metabolic genes (Czubryt et al., 2003). In addition, PGC-1α and MEF2 cooperatively activate slow-fiber specific promoters (Lin et al., 2002). Recently, PGC-1α was shown to increase the expression of neuromuscular junction genes (Handschin et al., 2007). Whether this function of PGC-1α is involved with its role in slow fiber remodeling has not been determined.

While PGC-1α drives type I myofiber formation, PGC-1β is involved with type II fiber formation. Overexpression of PGC-1β is sufficient to increase expression of type II fibers, but not type I fibers (Arany et al., 2007). Interestingly, the effect of PGC-1β on type II promoters is almost exclusively due to cooperation with MEF2D. However, the skeletal muscles of mice lacking MEF2D display a decrease of only type I myofibers and not type II fibers (Potthoff et al., 2007). Nevertheless, it is interesting to note that activation of different metabolic pathways in different fiber types may be involved in the establishment or maintenance of myofiber identity.

PPARδ is the highest expressed PPAR isotype in skeletal muscle, and is up-regulated upon short or long-term exercise training. Similar to overexpression of MEF2-VP16, PGC-1α, and CaMK, skeletal muscle-specific overexpression of PPARδ is sufficient to drive slow and oxidative myofiber expression *in vivo* (Luquet et al., 2003; Wang et al., 2004a). Conversely, skeletal muscle specific deletion of PPARδ results in a reduction of oxidative fibers, which becomes gradually more severe with age (Schuler et al., 2006). Treatment of mice with a PPARδ agonist improves insulin sensitivity (Lee et al., 2006) and increases oxidative markers in muscle (Wang et al., 2003b), but its ability to drive slow or oxidative fibers is less understood. Together these results demonstrate an important role for PPARδ and other metabolic transcription factors in myofiber metabolism and remodeling.

Factors regulating fast myofiber formation

The mechanism(s) regulating the slow-to-fast fiber-type switch is less understood. Whether transcription factors regulate fast fiber formation, or whether the fast fiber is the default state is a subject of controversy. Six1 and Eya1 are two transcription factors proposed to specifically drive fast and glycolytic fiber formation (Grifone et al., 2004). Six1 and its partner Eya1 bind to the MEF3 control elements of target promoters, and when overexpressed, they can drive the transformation of slow, oxidative fibers to fast, glycolytic fibers. However, mice lacking Eya1 (Xu et al., 1999) or Six1 (Laclef et al., 2003) die as neonates, precluding loss-of-function analyses of adult fiber-type composition.

Signaling pathways regulating hypertrophy and atrophy

Skeletal muscle size is determined by the relationship between anabolic processes, generating hypertrophy, and catabolic process, producing atrophy. As exemplified by the physique of a bodybuilder, skeletal muscle can adapt to workload by increasing myofiber size. Studies show that activation of the IGF/Akt/mammalian target of rapamycin (mTOR) signaling pathway stimulates hypertrophy and anatagonizes the loss of muscle mass. Overexpression of IGF in skeletal muscle of mice activates the Akt/mTOR pathway and was observed to prevent age-related muscle loss as well as enhance skeletal muscle regeneration after muscle injury or nerve damage. Transgenic mice overexpressing constitutively active Akt, specifically in skeletal muscle, showed an increase in muscle mass owing to an increase in muscle fiber size (Lai et al., 2004) and are referred to as "mighty mouse". Although many of the initial studies were preformed in rodents, subsequent studies have shown that in humans an increase in activated Akt, mTOR, and GSK-3β proteins were observed following resistance training inducing muscle hypertrophy (Leger et al., 2006).

mTOR is a kinase that is activated by Akt and regulates protein synthesis on the basis of nutrient availability. Akt/mTOR signaling plays a critical role in the regulation of postnatal muscle size, especially under conditions of increased external loading (Bodine, 2006). Of clinical significance, rapamycin, a drug that is commonly used as an immunosuppressant drug for organ transplantation, inhibits mTOR activity and blocks cell growth. Muscles from rats subjected to muscle overload and treated with rapamycin, show activation of Akt in response to increased workload but do not show any change in myofiber size or weight. This demonstrates that activation of mTOR is necessary for

skeletal muscle hypertrophy. Although rapamycin is therapeutically used effectively as an immunosuppressant, it may result in a decrease in muscle mass, even in patients that are exercising.

Skeletal muscle atrophy, commonly known as muscle wasting, is seen with aging, immobilization, spaceflight, musculoskeletal trauma, in many catabolic diseases, such as cancer and diabetes, and neuromuscular disorders, such as Duchenne muscular dystrophy. Muscle mass loss occurs primarily through enhanced protein breakdown mediated by the ubiquitin proteasome pathway. Microarray analysis of atrophic skeletal muscle showed up-regulation of two genes involved in the ubiquitin proteasome pathway: muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx/atrogin-1) (Bodine et al., 2001; Gomes et al., 2001). Notably, FOXO transcription factors, substrates of Akt, have been shown to induce atrogin-1/MAFbx expression. In the presence of IGF-1, Akt is activated and phosphorylates FOXO, preventing it from entering the nucleus and activating atrophy-related genes. These findings highlight a network of factors, IGF/Akt/mTOR/FOXO, that determine muscle mass and are involved in both the hypertrophy and atrophy signaling pathways.

Current studies in multiple laboratories are involved in determining the targets of MuRF and MAFbx/atrogin-1 during skeletal muscle atrophy and in designing approaches to inhibit these proteins in the anticipation of inhibiting muscle atrophy. Muscles of mice lacking MuRF1 and MuRF3 show an accumulation of myosin heavy chain, indicating that myosin heavy chain is one of the substrates of MuRF. However, most importantly, mice lacking MuRF1 and MuRF3 display a distinctive myopathy reminiscent of myosin

storage disease in humans, cautioning against the inhibition of MuRF as a therapeutic approach to combat muscle wasting.

There is increasing evidence that prolonged inflammation, as observed during aging and chronic disorders, causes muscle loss. In mice, muscles depleted of NF-κB, a transcription factor activating the immune and inflammatory response, exhibit an increase in muscle strength, maintain normal muscle physiology, block protein degradation under atrophy conditions, and display enhanced muscle regeneration in response to injury (Mourkioti et al., 2006). These results suggest that therapies designed to control inflammatory pathways may provide clinical intervention of muscle atrophy and wasting.

New approaches to define signaling pathways in skeletal muscle remodeling

Many of the signaling pathways in skeletal muscle remodeling were determined by examining single genes or proteins in cell culture studies and validating the findings in transgenic and/or knockout mouse lines. The availability of microarray and proteomics technologies provides opportunities to generate global gene and protein expression profiles of skeletal muscle during remodeling (Hittel et al., 2007; Reecy et al., 2006).

Several groups have performed whole-genome gene expression analysis of skeletal muscles to assess skeletal myofiber diversity at the transcriptional level. Following a running exercise protocol in mice (Wu et al., 2003) that triggers transformation of glycolytic fibers into oxidative ones, or after an exhaustive bout of high-intensity cycling in humans (Mahoney et al., 2005), the gene expression profile of skeletal muscle is profoundly altered by the up-regulation of genes involved in various aspects of metabolism, mitochondrial biogenesis, oxidant stress management and

signaling, electrolyte handling, cell stress and damage, proteolysis, and cell growth and death, as captured by microarray analysis. The power of using microarray analysis is that it provides an unbiased approach to generate novel hypotheses regarding the transcriptional programs that participate in exercise, as well as those genes that contribute to the adaptations occurring after a period of training.

Microarray analysis has been used to identify genes and signaling pathways that are regulated during pathological states such as immobilization in humans (Urso et al., 2006) and muscle recovery after nerve injury in mice. In addition, microarray analysis has helped to identify changes in skeletal muscle gene expression in response to pharmacological compounds that are known to alter skeletal muscle metabolism, such as statins, which have a side effect of myositits and rhabdomyolysis (Morikawa et al., 2005) and epinephrine which interestingly had a profound effect on genes involved in the immunity and inflammatory response (Viguerie et al., 2004).

Clinical assessment may be combined with microarray analysis to determine the response of skeletal muscle to therapy. Muscle biopsies were obtained before and after therapy from patients with dermatomyositis (DM) who improved and patients with sporadic inclusion body myositis (sIBM) who did not improve after three monthly intravenous immunoglobulin (IVIg) infusions (Raju and Dalakas, 2005). Patients that did not improve with IVIg showed very high expression of inflammatory genes in their muscles, at levels higher than those seen in the muscles of patients with DM who responded to therapy. In addition, microarray analysis may be used to reveal distinct gene expression clusters associated with disease. Muscle biopsies were obtained from patients with polymyositis (PM) and dermatomyositis (DM) to obtain a global view of

genes participating in pathogenesis (Zhou et al., 2004). Further investigations along these lines may point to genes that are biomarkers in the immunopathogenesis of inflammatory myopathies, identify genes associated with response to therapy, and indicate the prognosis of a patient in response to therapy.

Although it is tantalizing to propose that microarray analysis of muscle biopsies could be used routinely to evaluate the status of patients' skeletal muscle and to define new signaling pathways, there are limitations to microarray analysis. A major consideration is the heterogeneous property of skeletal muscle and how the site of the biopsy will impact the gene profile results. Also, muscle biopsies include other cells types such as endothelial cells, fibroblasts, and resident macrophages which will contribute to the gene expression profile. Sophisticated software for data mining and cluster analysis is required for handling the voluminous data generated by a genome-wide approach. Ultimately microarray results must be validated by biochemical and molecular biology techniques, such as reverse transcription quantitative PCR and immunohistochemistry.

Protein expression profiles have been generated for skeletal muscle using a proteomics approach. Comparison of slow/oxidative fibers or fast/glycolytic fibers using two-dimensional protein maps and mass spectroscopy has provided a reference map of proteins to assess differences in protein distribution between functionally dissimilar muscle groups (Gelfi et al., 2006; Le Bihan et al., 2006; Okumura et al., 2005). Proteomics has also been used to identify differentially expressed proteins in skeletal muscle during exercise (Hittel et al., 2007) and in pathological and aged skeletal muscle fibers (Doran et al., 2007). Of note, proteomic profiling exposed low abundant muscle

proteins as potential biomarkers of muscular dystrophy, illustrating the increased sensitivity of mass spectrometric techniques. Proteomic analysis performed on muscle biopsies from patients with sIBM and compared to protein profiles of patients with inflammatory myopathy without clinicopathologic features of sIBM, identified sIBM-specific proteins (Li et al., 2006). These data not only enable differentiation of functionally distinct fiber types, but they also provide tools for investigating muscle remodeling in response to physiological and environmental conditions, such as hypertrophy and aging, and pathological conditions, such as idiopathic inflammatory myopathies and neuromuscular disease.

Emerging regulators in skeletal muscle remodeling

MicroRNAs (miRNAs), 19-22 nucleotide RNAs, are gaining universal recognition as powerful regulators of gene expression in plants, animals, and viruses (Krutzfeldt and Stoffel, 2006). miRNAs regulate genes posttranscriptionally by base pairing with complementary sequences in the untranslated regions and acting as negative regulators of target gene expression. Hundreds of miRNAs have been identified, and it has been proposed that the average animal miRNA has hundreds of target genes, so that up to 30% of all genes are miRNA targets. In skeletal muscle, miRNAs have been implicated in proliferation, differentiation, hypertrophy, regeneration, and disease. miRNA-1 and miRNA-133 are transcribed in a muscle-specific manner during development from a common polycistronic gene (Chen et al., 2006). miRNA-1 has been shown to promote myogenesis by targeting HDAC4, a transcriptional repressor of muscle gene expression (discussed in MEF2-HDAC signaling pathway). miRNA-133 enhances myoblast

proliferation by repressing the serum response factor. To add to the complexity of gene regulation and autoregulation, serum response factor and MyoD are transcriptional regulators of miRNA-1. Recent studies indicate that miRNA-133 directly down-regulates a splicing factor during muscle development, establishing a role for miRNAs in the control of a developmentally dynamic splicing program. Studies performed to determine whether skeletal muscle hypertrophy affects miRNAs expression showed that miRNA-1 and miRNA-133 were decreased, suggesting that these muscle-specific miRNAs may contribute to regulating the initial response of skeletal muscle to functional overload (McCarthy and Esser, 2007).

miRNA-206 is shown to be induced during *in vitro* myoblasts differentiation, and a target gene of miRNA-206 has been shown to be a subunit of DNA polymerase. This suggests a role for miRNA-206 in down-regulating a DNA polymerase gene resulting in inhibition of DNA synthesis, an important component of the differentiation program (Kim et al., 2006).

In vivo there is distinct miRNA expression within the functionally distinct muscle groups. miRNA-206 expression is higher in the soleus (slow/oxidative fibers) than in the plantaris (fast/glycolytic fibers) hinting at the possibility that miRNA-206 may contribute to establishing a type I phenotype by repressing the type II phenotype (McCarthy and Esser, 2007). Further studies are needed to determine the role of miRNA in establishing fiber type.

Studies to evaluate the role of miRNAs in muscular dystrophy showed a dramatic increase in miRNA-206 expression in the diaphragm of the *mdx* mouse. This suggests that increased miRNA-206 expression contributes to the chronic pathology observed in

the *mdx* diaphragm by repressing expression of genes that otherwise would serve a compensatory function. This finding therefore implicates miRNAs as a factor in the pathophysiology of muscular dystrophy (McCarthy et al., 2007).

Interestingly, a spontaneous mutation creating an illegitimate microRNA target site in the myostatin gene affects muscularity in sheep (Clop et al., 2006). In the myostatin allele of Texel sheep there is a mutation that creates a target site for miRNA-1 and miRNA-206, causing translational inhibition of the myostatin gene and contributing to the muscular hypertrophy associated with Texel sheep. This serendipitous mutation demonstrates that mutations creating or destroying putative miRNA target sites are abundant and might be important effectors of phenotypic variation.

Therapeutic strategies are being designed to target miRNAs in anticipation of blocking repression of gene expression that promotes pathology. Antagomirs are a novel class of chemically engineered oligonucleotides that efficiently and specifically silence endogenous miRNAs (Krutzfeldt et al., 2005). Studies performed in mice showed that intravenous administration of antagomirs against specific mRNAs result in a marked reduction of corresponding miRNA levels in specific tissues. These findings demonstrate that antagomirs silence specific miRNAs *in vivo* and may represent a future generation of drugs to combat myopathies.

Summary and Future Directions

Defining the signaling pathways that regulate skeletal muscle remodeling is pertinent to developing new drugs and therapies to ameliorate skeletal muscle disease. Great strides have been made in animal models to understand the signaling pathways involved in

muscle remodeling and currently these signaling pathways are being validated in humans. Furthermore, unbiased, global-wide approaches are being used on human tissue samples to reveal new factors and new biomarkers involved in skeletal muscle remodeling and pathology. While progress has been made in identifying factors involved in skeletal muscle remodeling, a major hurdle in somatic cell delivery is to direct the drug exclusively to skeletal muscle. It is well documented that while activation of signaling pathways produces positive effects on skeletal muscle, it causes deleterious effects on the heart. The discovery of microRNAs as regulators of gene expression and the selective expression of certain microRNAs in skeletal muscle hint at the possibilities of using these small RNAs are drugs to treat skeletal muscle myopathies.

Chapter II

MEF2: A Common Regulator of Diverse Developmental Programs

Introduction

The formation of specialized cell types and their integration into different tissues and organs during development requires the activation of cascades of regulatory and structural genes by combinations of widely expressed and cell type-restricted transcription factors, and the interpretation of extracellular signals by components of the transcriptional apparatus. Members of the MEF2 family of transcription factors play central roles in the transmission of extracellular signals to the genome and in the activation of diverse programs of gene expression that control cell differentiation, proliferation, survival and apoptosis. Here I review the mechanisms that regulate MEF2 activity and discuss commonalities in its functions as a regulator of differentiation of diverse tissues.

The MEF2 family

MEF2 proteins belong to the evolutionarily ancient MADS (MCM1, agamous, deficiens, SRF) family of transcription factors (Martin et al., 1993; Pollock and Treisman, 1991). *Saccharomyces cerevisiae*, *Drosophila*, and *C. elegans* possess a single MEF2 gene, while vertebrates have four *Mef2* genes, *Mef2a*, -*b*, -*c*, and –*d*. The MADS-box is a 57 amino acid motif located at the N-terminus of MEF2 factors, immediately adjacent to a 29 amino acid motif termed the MEF2 domain. Together, these domains mediate dimerization, DNA binding, and cofactor interactions (Black and Olson, 1998; McKinsey et al., 2002a). The C-terminal regions of MEF2 proteins possess transcriptional activation domains, which are subject to complex patterns of alternative splicing (Martin et al., 1994) (Fig. 2.1).

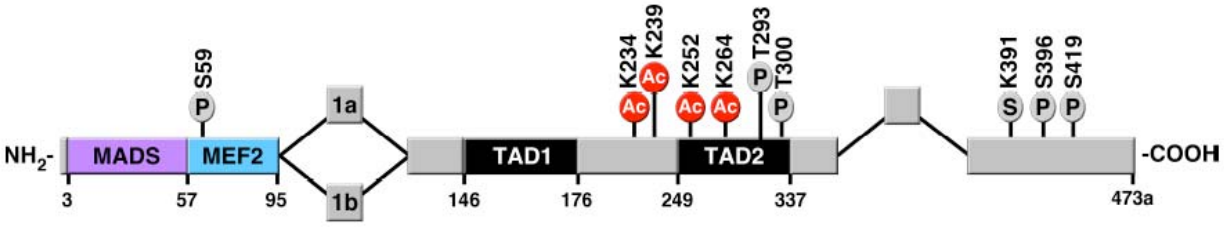

Figure 2.1. Structure of MEF2.
Domain structure and known post-translational modifications of human MEF2C. MEF2C contains an N-terminal MADS-box and MEF2 domain, which together mediate DNA-binding and co-factor interactions. The C-terminal region of MEF2C contains the transactivation domains (TAD) and this region is subject to alternative splicing. Ac, Acetyl; P, phosphate; S, sumo.

MEF2 proteins bind to the consensus sequence CTA(A/T)$_4$TAG as homo- or heterodimers (Gosset et al 1989)(Andres et al., 1995; Fickett, 1996; Molkentin and Olson, 1996, Yu, 1992 #4015; Pollock and Treisman, 1991; Yu et al., 1992). MEF2 is a relatively weak transcriptional activator and associates with other transcription factors to drive expression of its target genes. Complex transcriptional, translational and post-translational mechanisms (Fig. 2.1 and 2.2) exist to govern the activity and functions of MEF2.

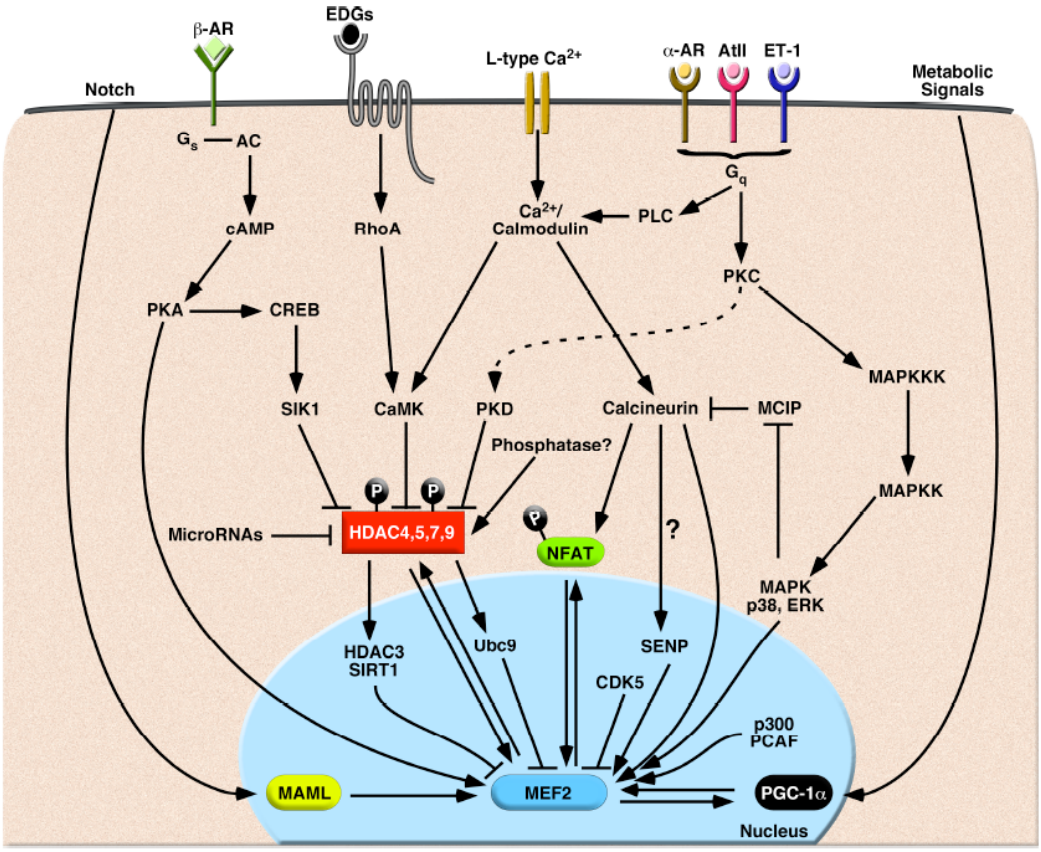

Figure 2.2. Signaling pathways regulating MEF2 activity.
Schematic diagram of signaling pathways that positively (arrows) or negatively (perpendicular lines) regulate MEF2 activity.

In vertebrates, the temporal and spatial expression patterns of *Mef2* genes are distinct, but overlapping, with highest expression in striated muscles and brain (Edmondson et al., 1994). However, MEF2 expression has also been observed in lymphocytes, neural crest, smooth muscle, endothelium, and bone, (Arnold et al., 2007; Edmondson et al., 1994) and several reports claim MEF2 proteins are ubiquitous (Black et al., 1997; Martin et al., 1993; McDermott et al., 1993; Pollock and Treisman, 1991; Yu et al., 1992). Expression of MEF2 proteins in many cell types including neurons, chondrocytes, and muscle (cardiac, skeletal, and smooth) appears concomitant with activation of differentiation programs. In adult tissues, MEF2 proteins act as a nodal

point for stress response and remodeling programs (Potthoff et al., 2007; Zhang et al., 2002). MEF2 proteins have also been implicated in cell survival, apoptosis, and proliferation. In each of these settings, the spectrum of target genes activated by MEF2 depends on specific post-translational modifications and interaction with co-factors.

Regulation of MEF2 activity by post-translational modifications

MEF2 factors are subject to a variety of post-translational modifications including phosphorylation, sumoylation, and acetylation (Fig. 2.1), which affect MEF2 activity by regulating DNA binding (Mao and Wiedmann, 1999; Molkentin et al., 1996b; Wang et al., 2005), MEF2 stability (Li et al., 2001; Okamoto et al., 2002), and interaction with transcriptional coactivators and corepressors (McKinsey et al., 2002a; Sartorelli et al., 1997; Sparrow et al., 1999).

Phosphorylation of MEF2 is associated with activation or repression of MEF2 target genes, depending on the residue(s) modified. Casein kinase II (CKII) and protein kinase A (PKA) phosphorylate MEF2 in the MADS-box, which promotes DNA binding (Molkentin et al., 1996b; Wang et al., 2005). Phosphorylation by these kinases does not appear to be a regulatory, but rather a constitutive modification (Black and Olson, 1998). ERK5 (Kato et al., 1997) and p38 (Han et al., 1997), on the other hand, phosphorylate MEF2 in the transactivation domain in response to specific signals, which enhances MEF2 transcriptional activity without affecting DNA binding. Another kinase, Cdk5, phosphorylates MEF2 in the transactivation domain (Ser444 in MEF2D) and inhibits MEF2 activity (Gregoire et al., 2006). Conversely, calcineurin can directly

dephosphorylate MEF2 and stimulate MEF2-dependent transcription (Chin et al., 1998; Gregoire et al., 2006; Liu et al., 1997; Wu et al., 2001).

MEF2 proteins also undergo sumoylation on conserved lysine residues (Gregoire and Yang, 2005; Kang et al., 2006; Riquelme et al., 2006; Shalizi et al., 2006; Zhao et al., 2005a). Like ubiquitination, sumoylation involves the covalent attachment of SUMO (small ubiquitin-related modifier) to the ε-amino group of target proteins (reviewed in (Seeler and Dejean, 2003)). Sumoylation of lysine 391 (Lys391) in MEF2C and lysine 439 (Lys439) in MEF2D inhibits transcriptional activity (Gregoire and Yang, 2005). Interestingly, class II histone deacetylases (HDACs), which act as MEF2 co-repressors, are also sumoylated, and their interaction with MEF2 increases MEF2 sumoylation by recruiting sumoylation machinery (Gregoire and Yang, 2005; Zhao et al., 2005a). This function of class II HDACs appears to be independent of the deacetylase domain (Gregoire and Yang, 2005). Subsequently, it was discovered that phosphorylation of Ser444 in MEF2D by Cdk5 is required for sumoylation and MEF2 inhibition (Gregoire et al., 2006). Conversely, dephosphorylation by calcineurin promotes desumoylation of MEF2 by SENP proteases (*e.g.*, SENP3) and MEF2 transcriptional activation (Gregoire et al., 2006).

The histone acetyltransferases (HATs) p300, PCAF, and CBP acetylate MEF2 proteins on conserved residues (K234, 239, 252, and 264 in MEF2C) in the transactivation domain (Ma et al., 2005; Shalizi et al., 2006; Zhao et al., 2005a). Acetylation of these residues reduces DNA binding, presumably through a conformational change, and weakens MEF2 activation. Histone deacetylase 3 (HDAC3), a class I HDAC, is recruited by class II HDACs (Fischle et al., 2002) and deacetylates

MEF2 (Gregoire et al., 2007). HDAC3 can also interact with MEF2 directly through the MADS-box, and represses MEF2 by deacetylating both MEF2 and associated HATs (Gregoire et al., 2007). However, treatment of cells with trichostatin A (TSA), an HDAC inhibitor, has produced conflicting results. One group reports that TSA does not alter MEF2 acetylation (Zhao et al., 2005a), while another reports a significant increase in MEF2 acetylation following TSA treatment (Gregoire et al., 2007). Both studies use TSA in MEF2D transfected HEK293 cells. The discrepancy in this result remains unclear.

Interestingly, class III HDACs, which are NAD^+-dependent and insensitive to TSA, also deacetylate MEF2. Treatment of cells with nicotinamide, a class III HDAC inhibitor, increases MEF2 acetylation (Gregoire et al., 2007; Zhao et al., 2005a), and SIRT1, a class III HDAC can deacetylate MEF2 through recruitment by HDAC4 (Zhao et al., 2005a). Thus, class II HDACs can recruit both class I and class III HDACs to deacetylate MEF2 and regulate its activity.

Modulation of MEF2 activity by class II HDACs

Phosphorylation cascades also affect MEF2 activity by modifying interactions with co-repressors. Class II HDACs, for example, are well known transcriptional repressors of MEF2 (reviewed in (McKinsey et al., 2001; McKinsey et al., 2002a; McKinsey et al., 2002b). Phosphorylation of class II HDACs on conserved serine residues in response to developmental and pathological signaling promotes nuclear to cytoplasmic shuttling, and subsequent activation of MEF2 (McKinsey and Olson, 2005; Zhang et al., 2002). In skeletal muscle, for example, class II HDACs are phosphorylated by multiple kinases

(*e.g.*, PKD, CaMK, Mark2)(Chang et al., 2005; McKinsey et al., 2000; Vega et al., 2004a), which regulate myocyte differentiation through MEF2 (McKinsey et al., 2000).

Loss-of-function mutations of MEF2 in Drosophila

The critical role of MEF2 in muscle development is perhaps best illustrated by loss-of-function studies of the single Mef2 gene in *Drosophila* (Bour et al., 1995; Lilly et al., 1995; Ranganayakulu et al., 1995). In Mef2 mutant embryos, skeletal muscle cells are appropriately specified and patterned, as shown by the expression of nautilus, an ortholog of MyoD, and other markers of the skeletal muscle lineage. However, there is a complete block to myoblast fusion and expression of markers of muscle differentiation. Similarly, loss of Mef2 prevents cardiomyocyte differentiation and expression of cardiac contractile proteins, but Mef2 mutant hearts continue to express early markers of the cardiac lineage, such as the cardiac homeobox gene tinman (Lilly et al., 1995). These findings indicate that Mef2 does not specify muscle cell fates, but instead acts to drive committed myoblasts down the differentiation pathway by activating the expression of muscle structural genes.

To identify MEF2 target genes in *Drosophila*, chromatin immunoprecipitation (ChIP) followed by microarray analysis (ChIP on chip) was performed to generate a temporal map of MEF2 activity during *Drosophila* embryogenesis. Interestingly, MEF2 exhibits three temporal patterns of enhancer binding. Though MEF2 is present at high levels early in development, it does not bind the enhancers of muscle differentiation genes until later in development, suggesting regulation of MEF2 DNA binding (Sandmann et al., 2007). In addition, consistent with previous studies (Blais et al., 2005),

the authors reported that MEF2 binds the promoters of many genes associated with neuromuscular junction (NMJ) formation. From this study, the authors conclude that MEF2 is a global muscle transcription factor and serves to amplify the robustness of the myogenic program (Sandmann et al., 2007).

While studies analyzing MEF2 function in *Drosophila* have provided valuable information, elucidation of the functions of mammalian *Mef2* genes has been comparably more difficult due to the existence of four related genes with overlapping expression patterns. In vertebrates, loss-of-function mutations frequently reveal only a subset of MEF2 functions in tissues in which the genes do not function redundantly. By generating conditional alleles of the different *Mef2* genes, we are now beginning to ascertain the importance of specific MEF2 proteins in various tissues through combinatorial deletion. Alternatively, overexpression of the chimeric MEF2 fusion proteins, such as the superactive MEF2-VP16 or super-repressive MEF2-Engrailed, has been implemented to elucidate MEF2 function in different tissues while bypassing functional redundancy (Arnold et al., 2007; Karamboulas et al., 2006; Potthoff et al., 2007).

Regulation of skeletal muscle differentiation by MEF2

Skeletal muscle differentiation is regulated by cooperative interactions of myogenic bHLH transcription factors with MEF2. MEF2 factors alone do not possess myogenic activity, but in combination with bHLH transcription factors, drive and amplify the differentiation program (Molkentin et al., 1995; Wang et al., 2001). In addition to regulating numerous muscle structural genes, MEF2 proteins regulate the expression of myogenic bHLH genes and themselves, providing a positive feedback loop to stabilize

the decision to differentiate (Molkentin and Olson, 1996). MEF2 not only positively regulates its own expression through cis-regulatory sequences, but also negatively regulates itself by inducing expression of HDAC9 through a negative feedback loop (Haberland et al., 2007)(Fig. 2.3). Signaling to class II HDACs, which dampens the negative feedback loop, provides a further mechanism for maintaining the precise balance of MEF2 activity in response to extracellular cues.

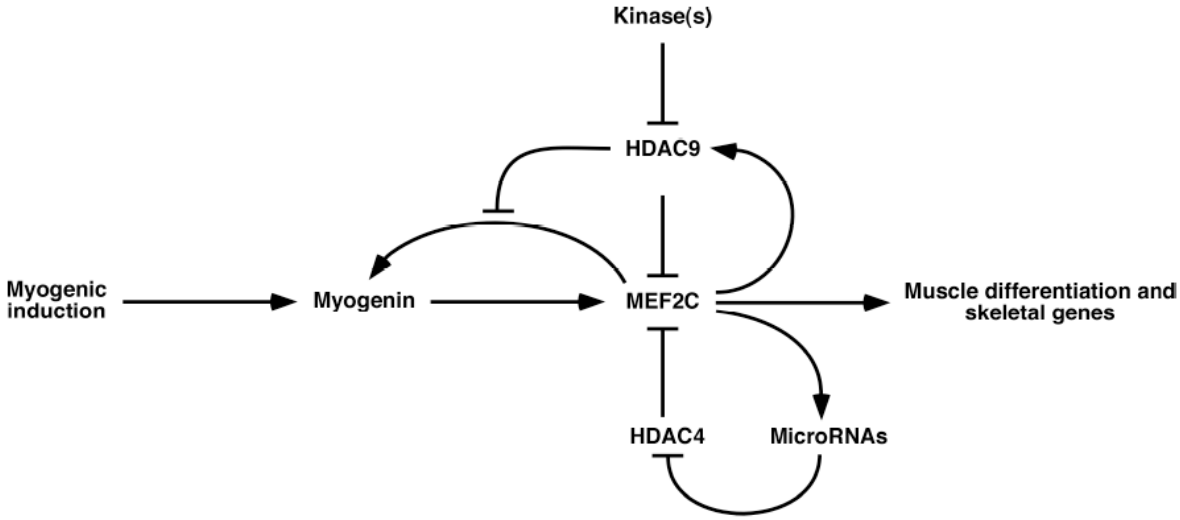

Figure 2.3. Positive and negative feedback loops regulating MEF2.
Diagram of regulatory pathways that precisely maintain MEF2 expression. Myogenic signals activate the differentiation program by stimulating expression of myogenin which subsequently drives MEF2 expression. MEF2 potentiates the differentiation program by amplifying bHLH transcription factor expression and by driving muscle differentiation and structural genes. MEF2 also drives the expression of HDAC9, which acts in a negative feedback loop to repress MEF2 activity. HDAC9 mediated repression can be alleviated by stress-dependent kinases which phosphorylate and shuttle HDAC9 from the nucleus to the cytoplasm. MEF2 also activates the expression of muscle-specific microRNAs which target HDAC4 and diminish its repression.

MEF2 establishes an additional level of myogenic regulation by regulating the expression of several microRNAs in skeletal muscle (Sokol and Ambros, 2005; Zhao et al., 2005b). MicroRNAs are approximately 22 nucleotide regulatory RNAs that post-

transcriptionally repress gene expression by binding the 3' untranslated region of mRNA targets and disrupting mRNA translation and stability (He and Hannon, 2004). Recently, several microRNAs were identified that affect skeletal muscle differentiation and proliferation (Boutz et al., 2007; Chen et al., 2006; Kim et al., 2006; Rao et al., 2006). Interestingly, several microRNAs target class II HDACs (Chen et al., 2006), establishing a positive feed forward mechanism for MEF2 activation and skeletal muscle differentiation (Fig. 2.3).

Several groups have attempted to identify global MEF2 target genes through microarray analysis (Blais et al., 2005; Junion et al., 2005; Nakagawa et al., 2005; Paris et al., 2004). These studies not only provide a broad understanding of MEF2 regulated processes, but have also identified novel MEF2 target genes. For example, Srpk3/Stk23, a novel muscle specific protein kinase gene and a direct target of MEF2C, was shown to be essential for normal skeletal muscle growth and homeostasis. Genetic deletion of Srpk3 results in a centronuclear myopathy and overexpression causes myofiber degeneration (Nakagawa et al., 2005). Together, these studies and others have led to the identification of an array of MEF2 target genes and have generated the framework to characterize the multitude of MEF2 regulated processes.

Despite extensive studies of MEF2 in skeletal muscle *in vitro*, relatively little is known about the roles of MEF2 proteins in vertebrate skeletal muscle *in vivo*. During mouse embryogenesis, *Mef2c* is the first MEF2 protein to be expressed in the somite myotome (~E9.0), with *Mef2a* and *Mef2d* expressed around a day later (Edmondson et al., 1994). Global deletion of *Mef2a* or *Mef2d* has little or no effect on skeletal muscle development (Potthoff et al., 2007). Since *Mef2c* null mice die around E9.5 (Lin et al.,

1997), its role in skeletal muscle was not examined until recently. Skeletal muscle deficient in *Mef2c* differentiates and forms normal myofibers during embryogenesis, but myofibers rapidly deteriorate after birth due to disorganized sarcomeres and a loss of integrity of the M-line (MJ Potthoff and ENO, unpublished data). Interestingly, similar results were observed in zebrafish after knockdown of *mef2c* and *mef2d* (Hinits and Hughes, 2007). Muscle specific overexpression of a super-active MEF2 protein, however, was not sufficient to drive skeletal muscle differentiation (Potthoff et al., 2007), consistent with previous *in vitro* studies (Molkentin et al., 1995). These results reveal a key role for *Mef2c* in maintenance of sarcomere integrity and postnatal maturation of skeletal muscle.

Recently, MEF2 was implicated in regulating skeletal myocyte survival through a CREB-dependent pathway. Berdeaux *et al.* reported that SIK1 disrupts class II HDAC repression of MEF2, allowing myocyte survival (Berdeaux et al., 2007). Disruption of HDAC activity using TSA in M-ACREB transgenic mice rescued the dystrophic phenotype (Berdeaux et al., 2007). Given that class II HDACs do not possess deacetylase activity (Fischle et al., 2002; Kao et al., 2000) and that class II HDAC deacetylase domain is not necessary for MEF2 sumoylation (Gregoire and Yang, 2005) and repression (Zhang et al., 2001), the molecular basis for this observation remains unclear.

Regulation of skeletal muscle fiber type by MEF2

Several lines of evidence have suggested a role for MEF2 proteins in myofiber remodeling and establishment of the slow skeletal muscle fiber type. Initially, when the

responsive elements of slow fiber specific promoters (SURE element) were analyzed, a consensus MEF2 binding site was discovered (Calvo et al., 1999; Nakayama et al., 1996), which was shown to be required for muscle specific gene expression (Calvo et al., 1999; Esser et al., 1999). Wu *et al.* subsequently discovered that MEF2 is directly activated by calcineurin signaling, which promotes the formation of slow fibers (Wu et al., 2000; Wu et al., 2001). Overexpression of class II HDAC kinases in skeletal muscle is also sufficient to increase MEF2 activity and drive slow fiber formation *in vivo* (Wu et al., 2002).

Using gain- and loss-of-function analyses for both class II HDACs and MEF2, we determined that class II HDACs determine slow, oxidative myofiber identity through the repression of MEF2 activity (Potthoff et al., 2007). Class II HDACs act redundantly in skeletal muscle, such that removal of any four alleles of class II HDACs results in a dramatic increase in the number of slow fibers, and conversely, overexpression of HDAC5 is sufficient to block exercise-induced fiber-type switching (Potthoff et al., 2007). Notably, treatment of mice with valproic acid (VPA), an HDAC inhibitor, did not alter fiber-type composition, suggesting that deacetylase activity is not required for myofiber remodeling (MJ Potthoff and EO unpublished results).

Loss of MEF2 proteins, on the other hand, results in a significant decrease in slow fiber composition (*Mef2c* and *Mef2d*, but not *Mef2a*). Conversely, overexpression of a super-activated form of MEF2C (MEF2C-VP16) in skeletal muscles drives slow fiber formation in muscles normally composed of fast fibers, and stimulates expression of oxidative and metabolic factors (Potthoff et al., 2007). Most remarkably, MEF2C-VP16 transgenic mice display an increase in exercise endurance, running almost twice as long

as wild-type littermates, which demonstrates that activation of MEF2 is sufficient to enhance skeletal muscle oxidative capacity and diminish muscle fatigability. Increased oxidative capacity and reduce fatigability is achieved, at least in part, through upregulation of metabolic transcription factors like PGC-1α which are sufficient to drive slow fiber formation (Potthoff et al., 2007). MEF2 also serves as a co-regulator of PGC-1 (Moore et al., 2003) and PPAR family members (Baldan et al., 2004), which also promotes slow fiber formation (Luquet et al., 2003; Wang et al., 2004a). Whether super-activation of MEF2 is sufficient to rescue dystrophic myofibers (*mdx* fibers) and ameliorate skeletal muscle disorders, is currently under investigation.

Since MEF2 remodels muscle in response to increased exercise and functional overload (Potthoff et al., 2007), MEF2 may also play a role in muscle regeneration in response to injury. Moreover, it is tempting to speculate that perturbations in signaling pathways that activate MEF2 may somehow contribute to muscle pathologies. Understanding the functions of MEF2 in skeletal muscle and the signals that regulate its activity may provide new therapeutic approaches to combat skeletal muscle disorders.

Control of heart development by MEF2

MEF2 regulates the expression of numerous cardiac structural and contractile proteins, and in mice, early, cardiac-specific overexpression of a MEF2C-Engrailed fusion protein with the Nkx2-5 enhancer (~E7.5) is sufficient to inhibit cardiomyocyte differentiation *in vitro* and *in vivo*. Moreover, overexpression of MEF2C-Engrailed causes misexpression of Gata and Nkx proteins (Karamboulas et al., 2006), confirming the role of MEF2 acting

in a cis-regulatory loop with core cardiac transcription factors and directly regulating cardiomyocyte differentiation.

Notably, cardiomyocyte development can still occur with loss of individual MEF2 proteins. In the mouse and chick, *Mef2c* is the first MEF2 factor to be expressed, appearing initially in mesodermal precursors which give rise to the heart (Edmondson et al., 1994). Shortly thereafter, the other *Mef2* transcripts are expressed. *Mef2c* null mice die around E9.5 due to cardiac looping defects (Lin et al., 1997), and *Mef2a* and *Mef2d* null mice exhibit perinatal lethality from an array of cardiovascular defects (Naya et al., 2002) and viability (Arnold et al., 2007), respectively. Though *Mef2c* null mice exhibit early embryonic lethality, cardiomyocytes are still able to differentiate prior to looping defects (Lin et al., 1997). Interestingly, mice with a cardiac specific deletion of *Mef2c* around E9.5 (*αMyHC*-Cre) are viable (Vong et al., 2005), which demonstrates that *Mef2c* is dispensible in the heart after cardiac looping, although some compensation by other MEF2 factors may exist. Together, these results demonstrate an important role for MEF2 proteins in cardiac differentiation, looping, and expression of structural and contractile proteins.

Control of pathological cardiac remodeling by MEF2

In response to specific extrinsic and intrinsic signals, the adult heart undergoes cardiac hypertrophy, characterized by an increase in cardiomyocyte size but not number. Cardiomyocytes cannot divide after birth, so to compensate for increased workload from exercise or cardiovascular disorders, individual myocytes increase their size to assemble additional sarcomere units in order to generate maximal force. While these effects are

beneficial for short intervals (*e.g.*, exercise), prolonged hypertrophy from pathological signals perturbs calcium handling and cardiac contractility, eventually leading to heart failure and arrhythmias (Antos et al., 2003).

MEF2 proteins and class II HDAC proteins are important regulators of cardiac hypertrophy (reviewed in (Frey and Olson, 2003; McKinsey and Olson, 2004; McKinsey and Olson, 2005; McKinsey et al., 2002a). Under basal conditions, class II HDACs repress MEF2 activity. In response to stress signals from calcium-dependent signaling pathways, class II HDACs are phosphorylated on conserved serine residues and shuttled from the nucleus to the cytoplasm by 14-3-3, alleviating MEF2 repression (McKinsey et al., 2000). Activation of MEF2 then increases expression of fetal cardiac genes, which are well-known markers of cardiac growth and hypertrophy (Chien et al., 1991). Loss-of function experiments of class II HDACs provides the most striking evidence for the involvement of MEF2-HDAC proteins in cardiac hypertrophy. Deletion of *Hdac9* (Zhang et al., 2002) or *Hdac5* (Chang et al., 2004) renders mice hypersensitive to pathological stress signals, and stress signaling in *Hdac9* null mice results in super-activation of MEF2 (Zhang et al., 2002). Cardiac specific overexpression of MEF2C or MEF2A *in vivo* induces dilated cardiomyopathy and lethality (Xu et al., 2006), but not hypertrophy, which may indicate that MEF2 drives late aspects of cardiac remodeling or high level transgenic over-expression of MEF2 is such a powerful pathological stimulus that it drives the heart directly to failure rather than through the typically gradual and graded response in which hypertrophic growth precedes dilative remodeling.

Regulation of cardiac conduction and metabolism by MEF2

MEF2 has also been shown to play important roles in the control of cardiac metabolism and conduction. *Mef2a* null mice, for example, are deficient in mitochondria, develop conduction disturbances, and are prone to sudden death (Naya et al., 2002). Moreover, human mutations in MEF2A are associated with coronary heart disease and myocardial infarction (Bhagavatula et al., 2004; Wang et al., 2003a). Consistent with this, cardiac specific overexpression of MEF2A or MEF2C alters the expression of structural, ion handling, and metabolic genes (Xu et al., 2006), and conversely, inducible, cardiac-specific overexpression of HDAC5 reduces expression of metabolic transcription factors and enzymes, which also results in sudden death (Czubryt et al., 2003). Therefore, MEF2 proteins are essential for regulating cardiac energetics and conduction (Czubryt and Olson, 2004).

Neuronal survival and differentiation by MEF2

MEF2 proteins are highly enriched in neurons and exhibit distinct patterns of expression in different regions of the brain with highest levels in the cerebellum, cerebral cortex, and hippocampus (Ikeshima et al., 1995; Leifer et al., 1993; Lin et al., 1996; Lyons et al., 1995). MEF2 proteins have been shown to be important in neuron cell survival and differentiation (Heidenreich and Linseman, 2004). In neurons, MEF2 protects cells from apoptotic cell death (Mao et al., 1999; Mao and Wiedmann, 1999; Okamoto et al., 2000), which is opposite to its function in thymocytes where MEF2 stimulates apoptosis (Woronicz et al., 1995). The ability of MEF2 to regulate different programs in neurons versus other cell types may occur by DNA-binding site selection. MEF2 from neurons

shows DNA-binding constraints for the flanking regions around the MEF2 site which is not observed with MEF2 factors from other cell types (Andres et al., 1995).

Recently it was shown that MEF2 proteins regulate dendrite morphogenesis, differentiation of post-synaptic structures (Shalizi et al., 2006), and excitatory synapse number (Flavell et al., 2006). Similarly to skeletal muscle, sumoylation of MEF2 in neurons represses MEF2 activity. Sumoylation of MEF2A on Lys403 promotes post-synaptic differentiation by repressing expression of Nur77 (Shalizi et al., 2006), a negative regulator of dendritic differentiation (Scheschonka et al., 2007). In addition, dephosphorylation of MEF2 by calcineurin regulates the expression of activity-regulated cytoskeletal associated protein (ARC) and synaptic RAS GTPase-activating protein (synGAP) (Flavell et al., 2006). ARC and synGAP play important roles in synaptic disassembly by promoting internalization of glutamate receptors (Flavell et al., 2006) and inhibiting Ras-mitogen-activated kinase signaling (Vazquez et al., 2004), respectively. In response to activity-dependent calcium signaling, calcineurin dephosphorylates MEF2 at Ser408, signaling a switch of Lys403 from sumoylation to acetylation, which restricts synapse number (Flavell et al., 2006) and inhibits dendritic claw differentiation (Shalizi et al., 2006) through activation of Nur77, ARC, and synGAP.

Thus, specific signaling events modulate gene expression by post-translationally modifying transcription factors, like MEF2, to control synapse development and plasticity. The functions of individual MEF2 proteins and their roles in synaptic differentiation and disassembly *in vivo* have yet to be examined. Based on recent literature, however, MEF2 appears to play a role in synapse plasticity and suggests an important role for these proteins in learning and memory.

MEF2 regulates chondrocyte hypertrophy and endochondral ossification

During embryonic development, bones develop through intramembranous or endochondral ossification. Endochondral ossification involves a cartilaginous intermediate, whereas intramembranous ossification occurs through the direct conversion of mesenchymal tissue into bone (Hall and Miyake, 1995). During endochondral ossification, mesenchymal precursor cells become committed to cartilage cells, forming a template for future bone. These committed mesenchymal cells differentiate into chondrocytes, proliferate rapidly to form a template for osteoblasts (committed bone precursor cells), secrete a cartilage specific extracellular matrix, and then stop dividing and become hypertrophic chondrocytes (Bruder and Caplan, 1989). Chondrocyte hypertrophy is necessary for bone vascularization, osteoblast differentiation and endochondral ossification.

Recently, MEF2 proteins were shown to be necessary and sufficient to drive chondrocyte hypertrophy (Arnold et al., 2007). Interestingly, this function of MEF2 is tightly regulated by the opposing function of HDAC4 (Arnold et al., 2007; Vega et al., 2004b). Genetic deletion of *Hdac4* (Vega et al., 2004b) or chondrocyte specific overexpression of a MEF2C-VP16 fusion protein is sufficient to drive premature bone formation (Arnold et al., 2007). Conversely, genetic deletion of *Mef2c* or overexpression of a MEF2-engrailed repressor in chondrocytes prevents chondrocyte hypertrophy and endochondral ossification (Arnold et al., 2007). MEF2 functions, at least in part, by directly activating Collagen 10a1 expression, a specific marker of chondrocyte hypertrophy, and Runx2, a transcription factor necessary for chondrocyte hypertrophy

(Arnold et al., 2007). Together, MEF2 and Runx2 drive the hypertrophic program in chondrocytes.

MEF2 in neural crest

Neural crest cells are multipotent, migratory cells that originate between the dorsal neural tube and epidermis of the embryo. In response to specific signaling cues, neural crest cells undergo an epithelial-to-mesenchymal transition, and then migrate to different parts of the embryo to give rise to a variety of cell types including neurons, skeletal and smooth muscle, chondrocytes, osteocytes, melanocytes, hormone producing cells, and many more. Recently, MEF2 was shown to be necessary for differentiation of neural crest derived cells. Loss of *Mef2c* in neural crest cells results in craniofacial defects and neonatal lethality due to upper airway obstruction (Verzi et al., 2007). MEF2C drives expression of the homeodomain transcription factors Dlx5 and Dlx6, and acts synergistically with these factors to direct craniofacial development (Verzi et al., 2007). However, as mentioned in the previous section, MEF2C regulates chondrocyte hypertrophy and it is uncertain whether the craniofacial defects in *Mef2c* neural crest cells mutants is a neural crest defect or a defect in the neural-crest derived cell (*i.e.*, chondrocytes). It will be interesting to determine whether MEF2 proteins also play a role in the development and differentiation of other neural-crest derived tissues.

Maintenance of vascular integrity by MEF2

The development of the vasculature occurs through two stages termed vasculogenesis and angiogenesis. Vasculogenesis is the de novo formation of blood vessels from

mesodermal progenitor cells, and angiogenesis is the expansion of a capillary plexus by forming additional branches from pre-existing vessels (Patan, 2000). MEF2 proteins are expressed in developing endothelial and smooth muscle cells (Lin et al., 1998), and are required for vascular development and maintenance of vascular integrity. The promoter of the *Mef2c* gene contains a conserved endothelial-specific enhancer that drives expression as early as E8.5 in all endothelial cells of the embryo and yolk sac (De Val et al., 2004). Endothelial cells are specified and differentiated in *Mef2c* null mice, but they are unable to organize properly (Lin et al., 1998).

MEF2 proteins have been implicated in maintaining vascular integrity through endothelial cell survival. As mentioned earlier, MEF2 proteins are phosphorylated and activated by ERK5 through a cascade of MAPK kinase signaling. ERK5 (also called BMK1) is necessary for endothelial cell survival and proliferation, such that conditional deletion of ERK5 from endothelial cells results in vascular demise and embryonic lethality (at E9.5-10.5) (Hayashi et al., 2004). Specifically, ERK5 deficient endothelial cells fail to proliferate and display increased apoptosis. Interestingly, this function of ERK5 in endothelial cell survival occurs, at least in part, through MEF2 activation (Hayashi et al., 2004). Removal of ERK5 from endothelial cells eliminates serum stimulated activation of MEF2 in endothelial cells, and constitutive activation of MEF2 in ERK5 deficient endothelial cells is sufficient to partially protect cells from apoptosis (reviewed in (Olson, 2004)).

Recently, we demonstrated an unexpected role for MEF2-HDAC signaling in vascular remodeling through regulation of enzymes that control cell-cell adhesion. Histone deacetylase 7 (HDAC7) is expressed specifically in endothelial cells during

development, and global deletion of *Hdac7* results in embryonic lethality due to blood vessel rupture from defects in cell-cell adhesion, a phenotype which is recapitulated by endothelial-specific deletion of *Hdac7* (Chang et al., 2006). Knockdown of HDAC7 in endothelial cells resulted in upregulation of matrix metalloproteinase 10 (MMP10), a secreted endoproteinase that degrades the extracellular matrix, and down-regulation of its inhibitor, tissue inhibitor of metalloproteinase 1 (TIMP1). MEF2 proteins directly activate expression of MMP10, and HDAC7 is sufficient to potently repress this activation (Chang et al., 2006). As mentioned earlier, MEF2A mutations have been associated with coronary disease (Bhagavatula et al., 2004; Wang et al., 2003a). Therefore, class II HDACs and MEF2 are uniquely involved in vascular integrity and demonstrate an important role for their interaction in vascular remodeling.

The vascular endothelium is a dynamic system subject to multiple stresses and if mishandled, can lead to a variety of cardiovascular disorders (*e.g.*, atherosclerosis). As demonstrated in other tissues, MEF2 is actively involved in survival signaling (*e.g.*, neurons) and stress response (*e.g.*, cardiac and skeletal muscle). During development, MEF2 may be involved in angiogenesis by promoting cell survival and sprouting, pruning and remodeling of blood vessels. In response to stress signals, MEF2 activation may actually promote vascular remodeling at the site of injury. In this regard, ERK5 has been demonstrated to be atheroprotective by responding to oxidative stress and displaying increased activation by fluid shear stress (Pi et al., 2004). Therefore, if MEF2 becomes activated at local sites of injury, which results in blood vessel remodeling, then removal of MEF2 regulation by deletion of *Hdac7* may explain the global vascular rupture observed in *Hdac7* null embryos.

In addition to endothelial cells, MEF2 has important functions in smooth muscle cells. While endothelial cells differentiate in *Mef2c* null embryos, smooth muscle cells fail to properly differentiate (Lin et al., 1998). This function of MEF2 occurs, at least partly, through myocardin, an SRF and MEF2 transcriptional coactivator that is necessary for smooth muscle differentiation (Wang et al., 2004b; Wang et al., 2003c). We recently showed that myocardin is a direct target of MEF2, and that myocardin also interacts with MEF2 to coactivate its own promoter in a positive feedback loop (Creemers et al., 2006). Interestingly, MEF2 is upregulated in activated smooth muscle cells (Firulli et al., 1996), which suggests MEF2 may function in the smooth muscle stress response after injury or pathological states (*e.g.*, arthrosclerosis).

MEF2 regulates T-cell development

The development and activation of thymocytes (T-cells) is a highly regulated process requiring multiple signaling cascades to direct changes in gene expression that alter T-cell state or fate. Calcium signaling pathways play important roles in T-cell selection during development and in T-cell receptor (TCR) induced apoptosis (Woronicz et al., 1995). The orphan nuclear receptor Nur77 is a crucial mediator of TCR-induced apoptosis, and TCR-induced expression of Nur77 is mediated through two MEF2 sites in the Nur77 promoter (Youn et al., 1999). In unstimulated T-cells, MEF2 is associated with transcriptional co-repressors, like HDAC7 and Cabin-1, which inhibit Nur77 expression (Dequiedt et al., 2003; Youn and Liu, 2000; Youn et al., 1999).

Following TCR activation, HDAC7 becomes dissociated from MEF2 through nucleocytoplasmic shuttling. Phosphorylation of HDAC7 by PKD1 on three conserved

serine residues (S155, S318, S448) recruits 14-3-3 and translocates HDAC7 to the cytoplasm, allowing activation of MEF2 (Parra et al., 2005). Conversely, HDAC7 is dephosphorylated by protein phosphatase 1β (PP1β) and myosin phosphatase targeting subunit 1 (MYPT1), components of the myosin phosphatase complex, which promote HDAC7 nuclear localization and repression of Nur77 expression (Parra et al., 2007). Therefore, regulation of MEF2 activity by association with transcriptional repressors is highly regulated in T-cells, and demonstrates the importance of MEF2-HDAC signaling in T-cell development, differentiation, and thymocyte selection (Kasler and Verdin, 2007).

Summary and Future Questions

The past decade has produced significant insights into the functions of MEF2 proteins in various tissues and the discovery of complex regulatory mechanisms that tightly control MEF2 activity. While MEF2 proteins perform diverse functions, seemingly unrelated tissues share a common role for MEF2 as a nodal point regulating transcriptional programs. During development, MEF2 interacts with cell-specific transcription factors to amplify and drive the differentiation program. In adults, MEF2 acts as a switchboard to stress signals and environmental demands by activating transcriptional programs to counteract stress and maintain homeostasis.

Recent discoveries have demonstrated the importance of MEF2 in an array of tissues. MEF2 may possess some ancestral function required by cells to differentiate and remodel in response to stress. However, why MEF2 functions in some cells and not others is not known. Moreover, why some cells require these functions of MEF2 and

other cells do not is poorly understood. Answers to these questions and others may provide insights into basic cellular processes and further demonstrate the importance of MEF2 in development and homeostasis.

Chapter III

Modulation of Myofiber Identity and Function by Histone Deacetylase Degradation and MEF2 Activation

Introduction

Skeletal muscle fibers of adult vertebrates differ markedly with respect to their contractile and metabolic properties, which reflect different patterns of gene expression (Williams and Neufer, 1996). Slow twitch or type I myofibers exhibit an oxidative metabolism, are rich in mitochondria, heavily vascularized, and resistant to fatigue. In contrast, fast twitch or type II fibers can exhibit glycolytic metabolism, are involved in rapid bursts of contraction, and fatigue rapidly. Myofibers can be further classified as either type I, IIa, IIx/d, or IIb depending on the type of myosin heavy chain (MHC) isoform expressed (Pette and Staron, 2000). The heterogeneity of skeletal myofibers is reflected at the molecular level in that almost every protein involved in contraction (MHC, myosin light chain, troponin I, -T, -C, actinin, *etc*.) has at least two isoforms expressed discretely in slow (type I) and fast (type II) fibers (Schiaffino and Reggiani, 1996). In adult animals, specialized myofiber phenotypes remain plastic and vary in response to contractile load, hormonal milieu, and systemic diseases (Baldwin and Haddad, 2001). Functional overload or exercise training results in transformation of pre-existing fast fibers to a slow-twitch, oxidative phenotype (Sugiura et al., 1993). Conversely, decreased neuromuscular activity induced by spinal cord injury, limb immobilization, space flight or blockade of action potential conduction causes a slow-to-fast myofiber conversion (Talmadge, 2000).

Functional demands modulate skeletal muscle phenotypes by activating signaling pathways that modify the gene expression profile of the myofiber. The signaling pathways involved in myofiber remodeling are of particular interest because of their relevance to several human disorders, including muscle dystrophy, metabolic disorders,

and muscle atrophy (Bassel-Duby and Olson, 2006). Increasing the abundance of slow, oxidative fibers in the *mdx* mouse model of Duchenne muscular dystrophy, for example, reduces the severity of the dystrophic phenotype (Chakkalakal et al., 2004; Stupka et al., 2006). Skeletal muscles also play an important role in whole-body metabolism, such that increasing the number of type I fibers enhances insulin-mediated glucose uptake and protects against glucose intolerance (Ryder et al., 2003), which could have important therapeutic implications for diabetes and other metabolic diseases.

The MEF2 transcription factor, a key regulator of muscle development, is preferentially activated in slow, oxidative myofibers (Wu et al., 2000) and responds to calcium-dependent signaling pathways that promote the transformation of fast, glycolytic fibers into slow, oxidative fibers (Wu et al., 2001). The transcriptional activity of MEF2 is repressed by class II histone deacetylases (HDACs) (Chang et al., 2004; McKinsey et al., 2000; McKinsey et al., 2002a). However, the potential involvement of MEF2 and class II HDACs in regulating myofiber identity *in vivo* has not been explored.

Recently, our work showed that class II HDACs are selectively degraded by the proteasome in slow, oxidative myofibers, enabling MEF2 to activate the slow myofiber gene program. Consistent with these conclusions, forced expression of class II HDACs in skeletal muscle or genetic deletion of *Mef2c* or *Mef2d* blocks activity-dependent fast-to-slow fiber transformation, whereas expression of a hyper-active MEF2 protein promotes the slow fiber phenotype, enhancing endurance and enabling mice to run almost twice the distance of wild type littermates. These findings provide new insights into the molecular basis of skeletal muscle performance and have important implications for

possible therapeutic manipulation of muscle function for amelioration of muscular diseases.

Results

Reduction of class II HDAC proteins in soleus muscle

We speculated that variations in MEF2 activity patterns seen among different types of myofibers (Wu et al., 2001) might arise from differences in the extent of MEF2 repression by class II HDACs. To begin to explore this possibility, we determined the expression patterns of HDAC proteins in several skeletal muscles containing different proportions of fast and slow myofibers by Western blot analysis (Fig. 3.1A).

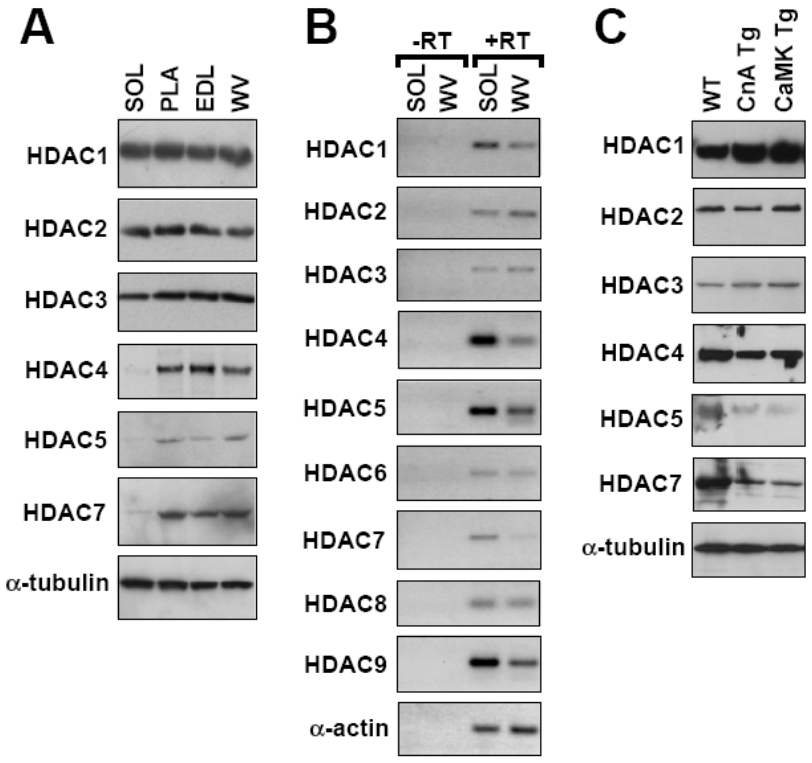

Figure 3.1. Post-transcriptional down-regulation of class II HDACs in soleus muscle.
Soleus (SOL), plantaris (PLA), extensor digitorum longus (EDL), and white vastus lateralis (WV) muscles were dissected form the hindlimbs of adult wild-

type mice (8-10 weeks of age). A. Protein expression of HDACs was assayed using antibodies specific for individual HDAC proteins. α-Tubulin level indicated equal loading. B. RNA expression of HDACs in SOL versus WV was analyzed by RT-PCR in the presence (+) or absence (-) of reverse transcriptase (RT). Skeletal α-actin primers were used to show equivalent cDNA input. C. Immunoblots of HDACs using WV muscle extracts from wild type (WT) and two transgenic mouse models (10 weeks old) overexpressing active calcineurin A (CnA Tg) or CaMKIV (CaMK Tg).

Soleus muscle is composed primarily of slow and fast, oxidative fibers with only a few fast, glycolytic fibers (Burkholder et al., 1994). This fiber type composition fits the physiological functions of soleus muscle, which is used almost continuously to maintain posture and antagonize gravity. Three other skeletal muscles, plantaris (PLA), extensor digitorum longus (EDL), and superficial white vastus lateralis (WV), contain very few slow fibers (Burkholder et al., 1994). As seen in Fig. 3.1A, class I HDACs (HDAC 1, 2 and 3) were expressed at comparable levels in different muscle groups. In contrast, the class II HDACs (HDAC 4, 5 and 7) were expressed preferentially in the fast fiber-dominant PLA, EDL and WV muscles, with relatively little expression in the slow fiber-enriched soleus (Fig. 3.1A). MEF2 protein expression levels did not differ between muscle types (data not shown). The relatively low level of class II HDAC protein expression in soleus appeared to reflect a post-transcriptional mechanism since mRNA transcripts encoding class II HDACs were more abundant in soleus than in WV muscles, as revealed by both RT-PCR and Northern blot analyses (Fig. 3.1B and data not shown).

Down-regulation of class II HDACs in transgenic skeletal muscles transformed toward a slow, oxidative phenotype

Forced expression of constitutively active calcineurin or CaM kinase IV in adult fast, glycolytic fibers of transgenic mice results in an increase in the number of slow fibers (Naya et al., 2000; Wu et al., 2002). We used these transgenic mouse models to determine whether fast-to-slow fiber transformation correlated with a down-regulation of class II HDACs, as might be expected if class II HDACs are involved in the fiber type switch. The levels of class I HDACs (1, 2, and 3) were similar in WV muscles from wild type and transgenic mice, whereas the transformation of WV muscles toward a slow myofiber identity was associated with diminished expression of class II HDAC (4, 5, and 7) proteins (Fig. 3.1C), consistent with the possibility that class II HDACs repress the expression of slow fiber genes in fast myofibers.

Class II HDACs redundantly regulate slow, oxidative fiber expression

To directly examine the potential role of class II HDACs in regulating fiber-type identity, we analyzed adult skeletal muscles from mutant mice lacking one or more class II HDACs. *Hdac5*$^{-/-}$ and *Hdac9*$^{-/-}$ mice are viable (Chang et al., 2004; Zhang et al., 2002), so we were able to analyze the fiber type composition of these homozygous mutants with global *Hdac* gene deletion in all tissues. However, because *Hdac4*$^{-/-}$ mice die at birth from skeletal defects (Vega et al., 2004b), and *Hdac7*$^{-/-}$ mice die during embryogenesis from vascular defects (Chang et al., 2006), we used floxed alleles to delete these genes specifically in skeletal muscle using a skeletal muscle-specific (SM-KO) Cre recombinase transgene (Myo-Cre), thereby avoiding lethality (Li et al., 2005). Mice lacking individual class II HDACs did not display abnormalities in fiber-type switching or skeletal muscle development (Fig. 3.2A). In contrast, soleus muscles from *Hdac5*$^{-/-}$;

Hdac9$^{-/-}$ and *Hdac4*$^{fl/-;Myo\text{-}cre}$; *Hdac5*$^{-/-}$ double knockout mice showed an increase in the percentage of slow myofibers from 48% +/- 2.6 to 70% +/- 3 ($p < 0.003$) and 63% +/- 6.6 ($p < 0.05$), respectively (Fig. 3.2A and B). The fiber type composition of the soleus of *Hdac5*$^{+/-}$; *Hdac9*$^{-/-}$ mice and *Hdac4*$^{+/-}$; *Hdac5*$^{-/-}$ mice was identical to wild type (data not shown), whereas *Hdac4*$^{+/-}$; *Hdac5*$^{-/-}$; *Hdac9*$^{+/-}$ mutant mice showed an increase in slow fibers comparable to that of double mutant mice (Fig. 3.2A), suggesting that deletion of any combination of four alleles of *Hdac4*, *5* or *9* results in enhanced slow fiber gene expression.

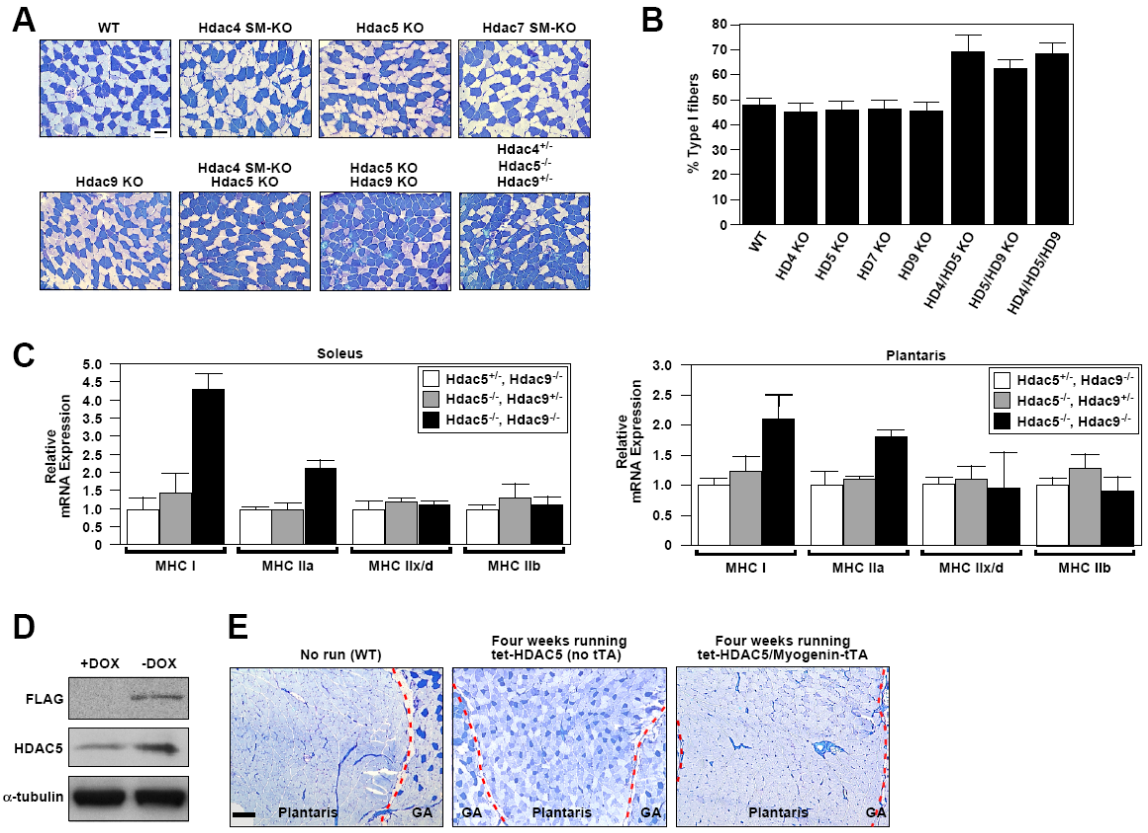

Figure 3.2. Class II HDACs redundantly regulate slow, oxidative fiber expression.

A. Soleus muscles (A, 10X magnification, size bar = 100 μm) from wild-type, *Hdac5*$^{-/-}$ (*Hdac5* KO), *Hdac9*$^{-/-}$ (*Hdac9* KO), *Hdac4*$^{fl/-;Myo\text{-}Cre}$ (*Hdac4* SM-KO), *Hdac7*$^{fl/-;Myo\text{-}Cre}$ (*Hdac7* SM-KO) and class II HDAC compound mutant mice were analyzed by metachromatic ATPase staining. Type I fibers stain dark blue. Type

II fibers stain light blue. B. Quantification of fiber type distribution based on fiber type analysis in A. C. Transcripts of MHC isoforms were determined in soleus and plantaris muscles from mice of the indicated genotypes by quantitative real-time PCR. D. Using anti-FLAG M2 antibody on a Western blot analysis of proteins isolated from gastrocnemius-plantaris muscles of four-week old myogenin-tTA/tet-HDAC5 mice treated with DOX or 10 days after removal of DOX. Tubulin served as a loading control. E. Metachromatic ATPase staining of gastrocnemius-plantaris muscles harvested from unexercised wild-type, four-week exercised control (tet-HDAC5 (no tTA)), and four-week exercised HDAC5 transgenic (Myogenin-tTA/tet-HDAC5) mice. (4X magnification, size bar = 300 μm; dashed red lines delineate gastrocnemius (GA) muscle from plantaris).

Analysis of the expression of transcripts encoding the individual MHC isoforms revealed an increase in expression of oxidative genes (MHC I and IIa) in soleus and plantaris muscles of *Hdac5*$^{-/-}$; *Hdac9*$^{-/-}$ mutant mice (Fig. 3.2C) compared to *Hdac5*$^{+/-}$; *Hdac9*$^{-/-}$ or *Hdac5*$^{-/-}$; *Hdac9*$^{+/-}$ littermates. These results suggested that a reduction in expression of class II HDAC proteins below a specific threshold results in an increase in slow and oxidative fibers.

Forced expression of HDAC5 blocks fiber-type switching

Exercise training transforms pre-existing fast fibers to an oxidative phenotype (Sugiura et al., 1993). To determine whether class II HDACs modulated fiber type switching in response to exercise, we constructed an inducible skeletal muscle-specific transgenic system using the myogenin promoter/MEF2 enhancer to drive expression of the tetracycline transactivater (tTA), which acts in trans to activate a tetracycline-responsive transgene. In this system, the transgene is not expressed in the presence of doxycycline (DOX), but is induced when the drug is removed. To verify the spatial expression of tTA in the myogenin-tTA transgenic mice, we generated transgenic mice harboring a lacZ reporter gene cloned behind the tet-responsive expression cassette (tet-lacZ). When the

myogenin-tTA transgenic mice were crossed to the tet-lacZ reporter mice in the absence of DOX, lacZ expression was observed specifically in skeletal muscle without preference for adult slow or fast fibers (data not shown).

The myogenin-tTA transgenic mice were bred to responder mice bearing a tet-responsive transgene encoding a signal-resistant FLAG-tagged human HDAC5 mutant protein ($HDAC5^{S/A}$) (Czubryt et al., 2003). Expression of the FLAG-tagged HDAC5 protein was detected by Western blot analysis with an anti-FLAG antibody using total protein extracts from gastrocnemius and plantaris (GP) muscles of four-week old tet-HDAC5/myogenin-tTA transgenic mice that had DOX removed ten days earlier (Fig. 3.2D). Overexpression of HDAC5 in transgenic mice was confirmed by probing with an HDAC5 antibody (Fig. 3.2D). FLAG-HDAC5 was not detected in the presence of DOX (Fig. 3.2D).

To analyze the influence of HDAC5 on fiber-type switching, DOX was removed from six-week old tet-HDAC5/myogenin-tTA double transgenic mice, and these mice were provided free access to a running wheel for four weeks, a time period shown previously to allow the transformation of fast, glycolytic fibers to oxidative fibers (Wu et al., 2001). Tet-HDAC5/myogenin-tTA transgenic mice and control mice ran voluntarily at comparable intensities (data not shown).

Metachromatic ATPase staining of skeletal muscles showed a pronounced increase in Type I and IIa fibers within GP muscles of exercised tet-HDAC5 mice (without tTA) compared to unexercised mice (Fig. 3.2E). In contrast, GP muscles from exercised tet-HDAC5/myogenin-tTA double transgenic mice without DOX did not show an increase in Type I and Type IIa fibers compared to sedentary mice (Fig. 3.2E).

Quantification of slow fibers revealed an approximate 10 fold reduction in the number of slow fibers from exercised HDAC5 transgenic mice compared to exercised wild-type mice (data not shown). Unexercised tet-HDAC5/Myogenin-tTA double transgenic mice with and without DOX displayed normal fiber-type distributions (data not shown). We conclude that continuous repression of class II HDAC target genes in adult skeletal muscle is sufficient to inhibit exercise-induced fiber-type switching.

Requirement of MEF2 for establishing slow, oxidative myofiber identity

To examine whether class II HDAC regulation of fiber-type switching occurs through repression of MEF2 activity, we analyzed the skeletal muscles from individual MEF2 knockout mice. Conditional alleles of *Mef2c* and *Mef2d* (Arnold et al., 2007; Haberland et al., 2006) were deleted specifically in skeletal muscle using transgenic mice that express Cre recombinase under the control of the myogenin promoter/MEF2 enhancer (Myo-Cre) (Li et al., 2005), which is active in both fast and slow fibers. As shown in Fig. 3.3A, skeletal muscle-specific deletion (SM-KO) of *Mef2c* or *Mef2d* using Myo-Cre resulted in a reduction in slow fibers within the soleus, whereas the abundance of slow fibers was unaltered in $Mef2a^{-/-}$ mice (Fig. 3.3A) or $Mef2c^{+/-}$ and $Mef2d^{+/-}$ mice (data not shown).

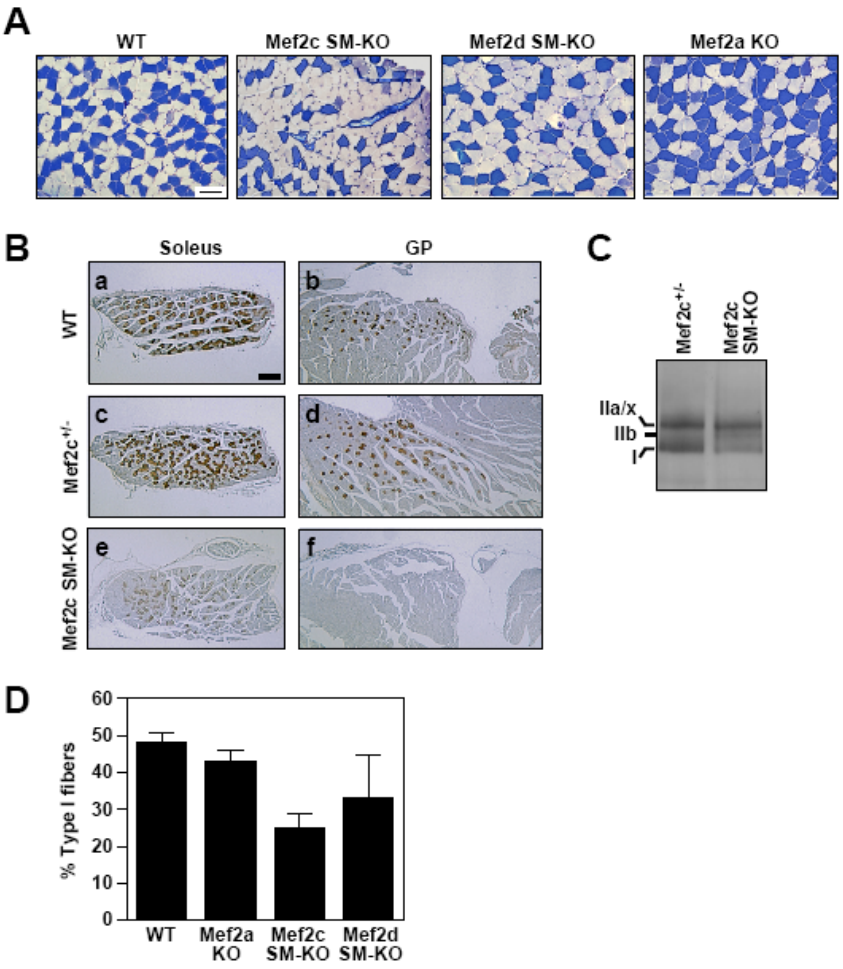

Figure 3.3. Requirement of MEF2 for establishing slow, oxidative myofiber identity.
Muscles from individual MEF2 knockout mice: *Mef2a*$^{-/-}$, and *Mef2c* SM-KO (*Mef2c*$^{fl/-;Myo\text{-}Cre}$) and *Mef2d* SM-KO (*Mef2d*$^{fl/fl;Myo\text{-}Cre}$) skeletal muscle conditional knockout mice were analyzed for fiber-type composition. A. Metachromatic ATPase staining of soleus muscle. Type I fibers stain dark blue. Type II fibers stain light blue. (10X Magnification, size bar = 100 μm). B. Immunohistochemistry of soleus and gastrocnemius, plantaris (GP) muscles of *Mef2c* SM-KO and wild-type littermates using a MHC type I specific antibody (2.5X magnification, size bar = 300 μm). C. Glycerol gradient silver-staining of protein extracts from soleus of wild-type and *Mef2c* SM-KO mice. MHC type I, IIa/d/x, and IIb isoforms are indicated. D. Quantification of fiber type distribution based on metachromatic ATPase staining of MEF2 knockout mice.

To further validate the reduction of slow fibers following skeletal muscle-specific deletion of *Mef2c*, we performed immunohistochemistry for type I fibers using a MHC

type I specific antibody. As shown in Fig. 3.3B, skeletal muscle lacking *Mef2c* displayed a loss of type I fibers in the GP and a reduction in number (and intensity) of type I fibers in the soleus. Moreover, using glycerol gradient silver staining of MHC isoforms from the *Mef2c* SM-KO soleus muscle, we discovered that these muscles display a reduction in MHC type-I (Fig. 3.3C). Specifically, a decrease in the percentage of slow myofibers from 48% +/- 2.6 to 25% +/- 3.6 ($p < 0.002$) and 33% +/- 11.4 ($p < 0.001$) was observed in the soleus of the *Mef2c* SM-KO and *Mef2d* SM-KO mice, respectively (Fig. 3.3D). These findings demonstrate that MEF2C and MEF2D activate slow fiber genes, and that repression of fiber type switching by class II HDACs is mediated, at least in part, through their repressive influence on MEF2C and MEF2D.

Activated MEF2 is sufficient to increase slow fiber gene expression muscle performance

To determine whether MEF2 proteins were not only necessary, but also sufficient, for properly establishing slow, oxidative myofiber distribution, we tested whether expression of a hyper-active MEF2C-VP16 chimera, which is insensitive to HDAC repression, was sufficient to increase slow, oxidative fiber expression. Indeed, skeletal muscle expression of MEF2C-VP16 was sufficient to increase the number of slow fibers in the plantaris, which is normally composed primarily of fast fibers (Fig. 3.4B, and data not shown). This increase in slow fiber abundance was remarkably similar to the fiber-type increase observed in wild-type mice after exercise training (compare to Fig. 3.2F). Analysis of muscle fiber markers and mitochondrial proteins by Western blot analysis revealed an increase in the slow fiber-specific contractile protein, Troponin I, and the type I fiber

oxidative proteins myoglobin and cytochrome c (Fig. 3.4A) in Myo-MEF2C-VP16 transgenic mice, confirming the results of metachromatic ATPase staining. In addition, an increase in other metabolic genes and important metabolic transcription factors, like PGC-1α, was observed in Myo-MEF2C-VP16 transgenic skeletal muscles (data not shown). These findings demonstrate that MEF2 is sufficient to drive fast-to-slow fiber transformation, mimicking the effect of exercise *in vivo*.

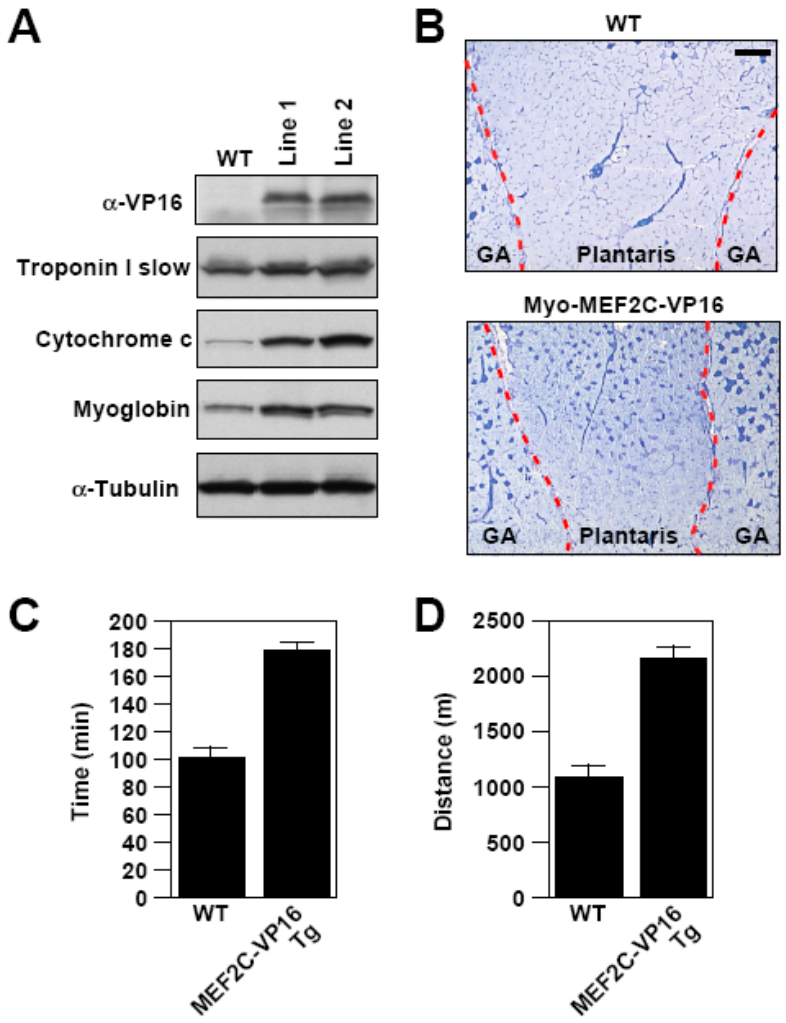

Figure 3.4. Activated MEF2 is sufficient to increase slow fiber expression. A. Western blot analysis of Myo-MEF2C-VP16 transgene expression using an anti-VP16 antibody. Expression of the slow fiber specific troponin I, and oxidative markers myoglobin and cytochrome c in protein extract of gastrocnemius and plantaris muscles of Myo-MEF2C-VP16 transgenic mice. B. Metachromatic

ATPase staining of gastrocnemius (GA) and plantaris muscles of wild-type and Myo-MEF2C-VP16 transgenic mice. (4X magnification, size bar = 300 μm; dashed red lines delineate gastrocnemius (GA) muscle from plantaris). C and D. Exercise endurance and muscle performance (total time running (min) and total distance ran (m), C and D, respectively) of Myo-MEF2-VP16 transgenic muscles were analyzed by forced treadmill exercise. Eight-week old Myo-MEF2C-VP16 transgenic and wild-type male mice with similar body weights were subjected to forced treadmill exercise (n = 5 for each group) on a 10% incline.

To examine the functional consequences of the increase in slow fibers and oxidative capacity of Myo-MEF2C-VP16 transgenic muscles, we measured the endurance of these mice by forced treadmill exercise on a 10% incline. As shown in Fig. 3.4C and 3.4D, MEF2C-VP16 transgenic mice displayed a 75% increase in running time and a 94% increase in distance, respectively, compared to wild-type mice. Thus, activation of MEF2 is sufficient to enhance skeletal muscle oxidative capacity and mitochondrial content, thereby diminishing muscle fatigability and augmenting endurance.

Class II HDACs are ubiquitinated and degraded by the proteasome

To begin to understand the mechanistic basis for the lack of accumulation of class II HDAC proteins in slow skeletal muscle fibers, we examined the half-life of HDAC5 *in vitro* using a stable C2C12 muscle cell line that constitutively expressed FLAG-tagged HDAC5. Inhibition of protein synthesis with cycloheximide for four hours resulted in a precipitous decrease in the level of HDAC5 protein in skeletal myocytes (Fig. 3.5A). By contrast, α-tubulin was stable over this time period. The proteasome inhibitor MG132 blocked the degradation of HDAC5 (Fig. 3.5B), suggesting that HDAC5 is degraded by the proteasome pathway.

Since ubiquitination is a prerequisite for degradation by the proteasome (Pickart, 2001), we examined whether HDAC5 was ubiquitinated. Indeed, ubiquitinated HDAC5 was readily detectable when HA-tagged ubiquitin was expressed in the HDAC5-expressing cell line in the presence of MG132 (Fig. 3.5C). The signal-resistant HDAC5$^{S/A}$ mutant (McKinsey et al., 2000), lacking the regulatory phosphorylation sites (serines 259 and 498), was ubiquitinated to the same extent as the wild type HDAC5 protein (Fig. 3.5C), indicating that phosphorylation of HDAC5 on these residues is not a prerequisite for ubiquitination. FLAG-HDAC4, -7, and MITR (a splice variant of HDAC9) were also ubiquitinated in C2C12 cells (data not shown).

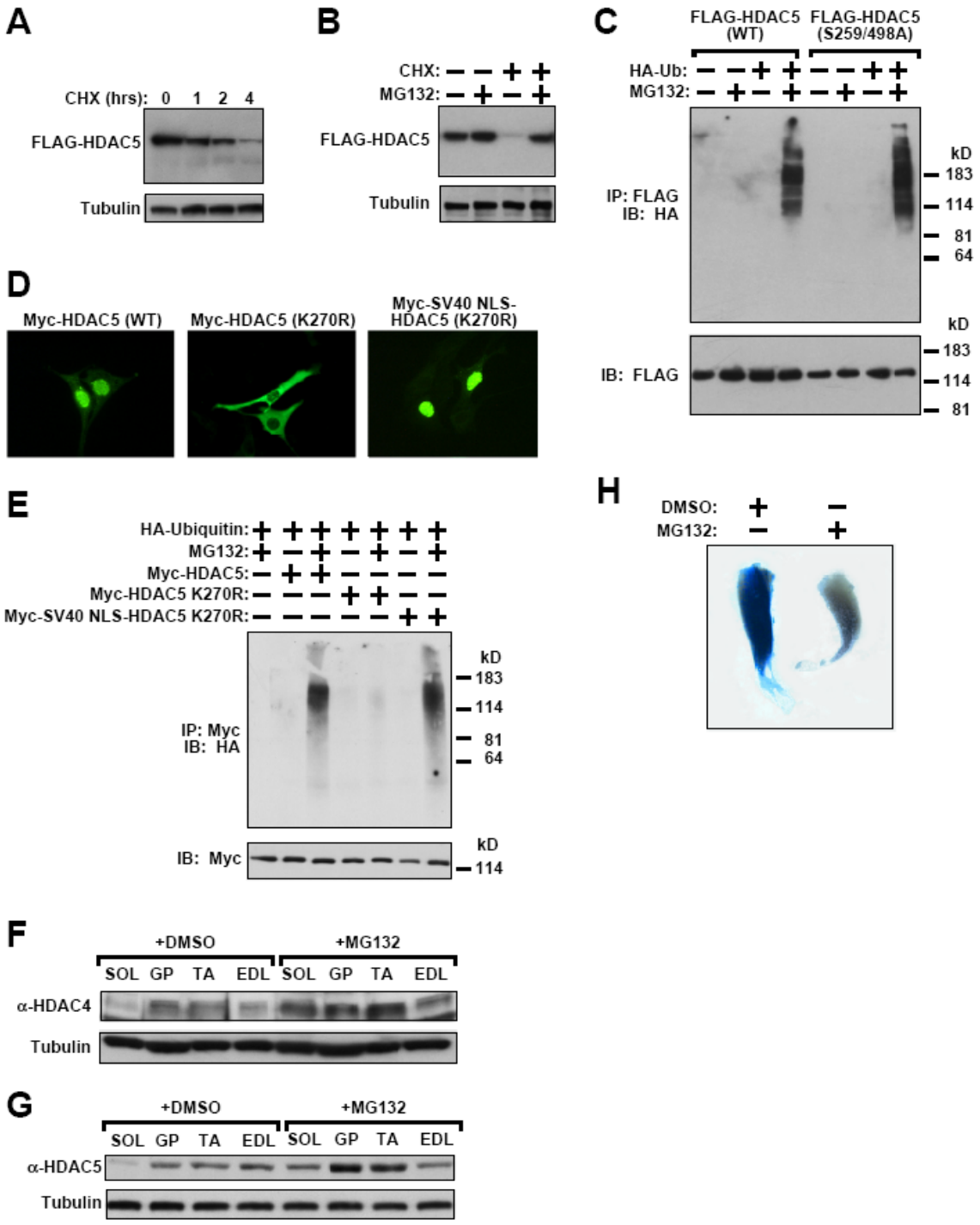

Figure 3.5. Ubiquitination and degradation of class II HDACs *in vitro* and *in vivo*.

A. C2C12 cells stably expressing FLAG-tagged HDAC5 (C2C12-HDAC5) were treated with cycloheximide (CHX, 25 μM) for 0, 1, 2, or 4 hours before cells were lysed and FLAG-HDAC5 expression was measured by Western blot analysis using anti-FLAG M2 antibody. Tubulin immunoblot showed equivalent loading of

each lane. B. C2C12-HDAC5 cells were treated with cycloheximide and MG132, singly or in combination for 4 hours, and FLAG-HDAC5 expression was analyzed. C. C2C12-HDAC5 and C2C12 cells stably expressing CaMK-resistant HDAC5 (C2C12-HDAC5$^{S/A}$) were transfected with or without HA-tagged ubiquitin, treated with or without MG-132 (25 μM) for four hours, and the ubiquitination status of wild-type and mutant HDAC5 was analyzed. FLAG expression in inputs shows equal loading. D. Subcellular localization of Myc-HDAC5(wt), Myc-HDAC5(K270R), or Myc-SV40 NLS-HDAC5(K270R) in C2C12 cells. F. The ubiquitination status of cytoplasmic HDAC5 (Myc-HDAC5(K270R)) or nuclear HDAC5 (Myc-SV40 NLS-HDAC5(K270R)) was analyzed. F and G. Wild-type C57BL/6 males (8 weeks old) were IP injected with DMSO or MG132 for 6 hours. Mice were then euthanized and soleus (SOL), gastrocnemius-plantaris (GP), tibialis anterior (TA), and extensor-digitorum skeletal muscles (EDL) muscles were dissected and total proteins were isolated for each muscle, run on a polyacrylamide gel, and probed with antibody recognizing (F) HDAC4 or (G) HDAC5. Tubulin shows equal loading. H. Treatment with MG132 decreases MEF2 activation. Sedentary MEF2 reporter mice (DesMEF-lacZ) do not express lacZ in skeletal muscle, but exercised mice do. Six hours after DesMEF-lacZ mice were injected with DMSO or MG132, mice were run for ~3 hours using forced treadmill exercise. Skeletal muscles were then isolated from DMSO and MG132 treated DesMEF mice and analyzed for lacZ expression. LacZ expression was reduced in MG132 treated muscles (where class II HDAC expression is increased).

To determine whether ubiquitination of HDAC5 occurs in the nucleus or cytoplasm, we compared the ubiquitination of wild-type HDAC5, which is expressed primarily in the nucleus (Fig. 3.5D), and an HDAC5 mutant with a mutation in the nuclear localization signal, HDAC5 K270R (Fig. 3.5D). As shown in Fig. 3.5E, the K270R mutant was not ubiquitinated, whereas fusion of an SV40-NLS to the HDAC5 K270R protein restored nuclear localization and ubiquitination. Together these results demonstrate that HDAC5 ubiquitination occurs in the nucleus.

Blockade to HDAC degradation *in vivo* by MG132

To determine whether class II HDACs are degraded via the proteasome pathway in slow, oxidative myofibers *in vivo*, eight-week old, wild-type C57BL/6 male mice were injected

with either DMSO or MG132 and expression of HDAC4 and 5 was examined in the soleus, GP, TA, and EDL skeletal muscles after six hours, a time period shown previously to provide proteasome inhibition *in vivo* (Luker et al., 2003). Treatment with MG132 *in vivo* increased the level of HDAC4 and HDAC5 protein expression in the soleus to that of the EDL (Fig. 3.5F and 3.5G, respectively), consistent with the *in vitro* results and demonstrating that class II HDAC proteins are specifically degraded by the proteasome in slow and oxidative myofibers.

Finally, since treatment with MG132 results in an increase in class II HDAC protein, we examined its effect on MEF2 activity *in vivo* using a transgene reporter mouse line (DesMEF lacZ) that expresses lacZ under control of three tandem MEF2 sites. Prior studies showed that the expression of lacZ in these mice provides a faithful measure of MEF2 activity (Wu et al., 2001). Sedentary MEF2 reporter mice (DesMEF lacZ) do not express lacZ in skeletal muscle. However, lacZ expression is observed when DesMEF mice are exercised (Wu et al., 2001). Therefore, we injected DesMEF lacZ transgenic mice with either DMSO or MG132, and after 6 hours ran the mice for ~3 hours using forced treadmill exercise. Skeletal muscles were then isolated from DMSO and MG132 treated DesMEF lacZ mice and analyzed for lacZ expression. Skeletal muscles (soleus and gastrocnemius-plantaris) from exercised, DMSO treated DesMEF lacZ mice expressed lacZ, while lacZ expression in exercised, MG132 treated DesMEF lacZ muscles was significantly reduced (Fig. 3.5H). These results demonstrate that inhibition of the proteasome *in vivo*, which prevents HDAC degradation, results as expected in activation of MEF2 and slow, oxidative fiber gene expression.

Discussion

The results of this study show that slow and oxidative myofiber identity and muscle performance are governed by the balance between positive and negative signaling by MEF2 and class II HDACs, respectively. Degradation of class II HDAC proteins in response to "slow" signals allows sustained activation of MEF2, which promotes the establishment of slow and oxidative myofibers and, strikingly, enhances muscle endurance and fatigue resistance (Fig. 3.6).

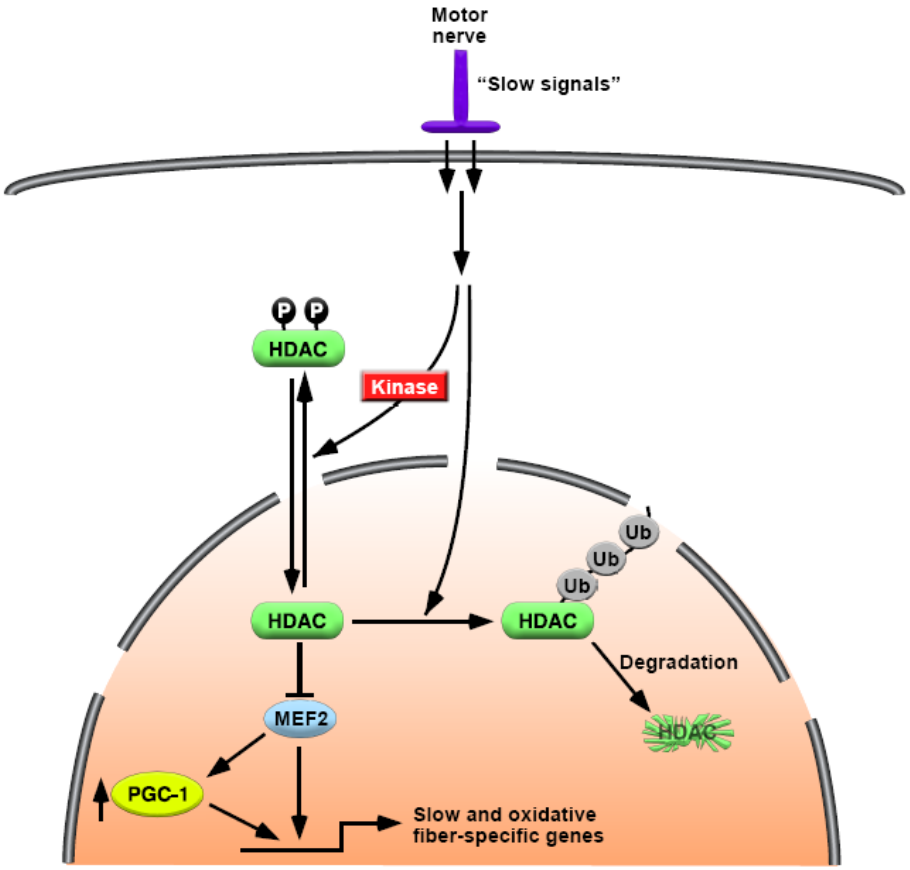

Figure 3.6. A model for the control of slow and oxidative fibers by MEF2 and class II HDACs.
Schematic model for the control of slow and oxidative fiber-specific genes. Motor nerve activity regulates MEF2 activity and myofiber identity through ubiquitination (Ub) and degradation of class II HDACs.

The contractile properties of skeletal myofibers reflect a combination of developmental and extrinsic inputs. During embryogenesis, fast and slow twitch fibers are patterned by specific developmental cues (Schiaffino and Reggiani, 1996). After birth, the pattern of motor innervation plays a key role in influencing muscle fiber type. Phasic motor neuron firing at high frequency (100-150 Hz) promotes the formation of fast fibers, which display brief, high-amplitude calcium transients and low ambient calcium levels (< 50 nM), whereas tonic motor neuron stimulation (10-20 Hz) favors the formation of slow fibers, which maintain higher intracellular calcium levels (100-300 nM) (Olson and Williams, 2000). Calcineurin and CaMK have been implicated in the transduction of calcium-dependent signals that up-regulate the expression of oxidative, slow fiber-specific genes in skeletal muscle (Freyssenet et al., 1999), (Chin et al., 1998; Naya et al., 2000; Wu et al., 2002). However, the precise mechanisms whereby these signaling pathways modulate the slow fiber phenotype have not been defined (Chin et al., 1998).

Our results show that skeletal muscle of transgenic mice that has undergone a fast-to-slow myofiber transformation in response to activated calcineurin and CaMK displays a reduction in abundance of class II HDAC proteins, suggesting that these calcium-dependent signaling pathways act, at least in part, by enhancing degradation of class II HDAC proteins. The calcineurin and CaMK signaling pathways have also been shown to promote the phosphorylation of class II HDACs on a series of conserved serine residues, which mediate signal-dependent nuclear export and de-repression of MEF2 target genes (McKinsey et al., 2000; McKinsey et al., 2002a). Thus, it seems likely that

some combination of the two mechanisms – proteolysis and regulated nuclear export - regulates class II HDACs and thereby MEF2 activity and myofiber identity.

NFAT transcription factors, which serve as transcriptional mediators of calcineurin signaling, have also been implicated in the control of slow fiber gene expression (Chin et al., 1998; Delling et al., 2000), as has the nuclear receptor peroxisome proliferator-activated receptor δ (PPARδ)(Wang et al., 2004a) and peroxisome proliferators-activated receptor-gamma coactivator-1α (PGC-1α)(Lin et al., 2002). Recently, peroxisome proliferators-activated receptor-gamma coactivator-1β (PGC-1β)(Arany et al., 2007) was implicated in regulating type IIx/d fiber formation. MEF2 interacts with NFAT (Blaeser et al., 2000) and PGC-1α (Moore et al., 2003) and also regulates PGC-1α expression (Czubryt et al., 2003). Indeed, the MEF2-VP16 superactivator upregulates PGC-1α, but not PGC-1β, expression in skeletal muscle (data not shown). Thus, MEF2 serves as a nodal point for the control of multiple downstream transcriptional regulators of the slow fiber phenotype and can potentially confer calcium sensitivity to other factors via its signal-dependent interaction with class II HDACs.

Our results show that ubiquitination of class II HDACs occurs in the nucleus. Nuclear ubiquitination of transcriptional activators has also been described as a mechanism to regulate the extent and duration of activation of transcriptional activators (Kodadek et al., 2006). The ubiquitination of class II HDACs provides another mechanism by which transcriptional activators may become activated. Class II HDACs and MEF2 are also sumoylated, which enhances the repressive activity of class II HDACs (Gregoire and Yang, 2005; Zhao et al., 2005a). Whether sumoylation might be regulated during myofiber specification *in vivo* has not been addressed.

The mechanism that directs ubiquitination and degradation of class II HDACs in response to calcium signaling remains to be determined. It is tempting to speculate that phosphorylation of HDACs serves as a signal for ubiquitination by recruiting specific E3 ligases. Identification of the E3 ligase(s) for class II HDACs and the signals regulating their degradation are currently under investigation.

Our results show that adult skeletal muscle phenotypes are dictated by the extent of repression of MEF2 by class II HDACs and that the fast fiber phenotype results from the absence of MEF2 activity. The fact that four *Hdac* alleles needed to be deleted to observe an increase in slow fiber gene expression suggests that there is substantial functional redundancy among different HDACs with respect to repression of the slow fiber gene program. Forced expression of a signal-resistant mutant of HDAC5 is sufficient to suppress the slow fiber phenotype, whereas expression of a hyper-active MEF2-VP16 chimera is sufficient to over-ride the repressive influence of endogenous class II HDACs and drive the slow fiber phenotype. These findings suggest that the fast fiber phenotype represents a "default" gene program resulting from the absence of MEF2 activity.

Therapeutic potential

The ability of activated MEF2 to enhance oxidative capacity and endurance of skeletal muscle suggests opportunities for therapeutically enhancing muscle performance by stimulating MEF2 activity. Increasing the number of slow fibers in skeletal muscle via MEF2 also represents a potential method for treating metabolic and muscular diseases (Chakkalakal et al., 2004; Stupka et al., 2006). One could imagine augmenting MEF2

activity by interfering with the repressive activity of class II HDACs by modulating the signaling pathways that control HDAC phosphorylation, subcellular localization or degradation. In this regard, HDAC inhibitors have recently been shown to suppress muscle pathology associated with muscular dystrophy (Avila et al., 2007; Minetti et al., 2006) in mice.

In addition to regulating skeletal muscle gene expression and function, MEF2 signaling has been shown to drive pathological cardiac growth and remodeling, which result from signal-dependent phosphorylation and nuclear export of class II HDACs in cardiac myocytes (Zhang et al., 2002). These adverse consequences of MEF2 activation pose interesting challenges to the goal of enhancing MEF2 activity in skeletal muscle, while avoiding possible cardiotoxicity of such strategies and, conversely, to pharmacologically preventing cardiac dysfunction without diminishing skeletal muscle function.

Methods

Plasmid constructs, tissue culture and cell transfection: The expression vector encoding HA-ubiquitin was described previously (Hakak and Martin, 1999). Myc-NLS-HDAC5 was a kind gift from Dr. Tim McKinsey. The K270R point mutation was generated by site-directed mutagenesis (Stratagene). Myogenin-tTA and Myo-MEF2C-VP16 transgenic constructs were generated by cloning the tetracycline transactivator (tTA) cassette from the pUDH 15-1 vector (Resnitzky et al., 1994), and fusing a VP16 activation domain in frame to the C- terminus of full length MEF2C, respectively, and cloning them into the HindIII site of a pBSIISK(+) vector containing a

hGHpolyA tail. The Myogenin promoter/MEF2C enhancer (Li et al., 2005) was then cloned upstream of the cassettes into KpnI/XhoI sites.

C2C12 myoblasts were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum and antibiotics (100 U of penicillin and 100 μg of streptomycin per ml). For transient transfection assays, cells were plated and transfected 12 hours later using Fugene (Roche) following the manufacturer's instructions. Cells were harvested 24-48 hours after transfection. C2C12 stable lines expressing FLAG-tagged HDAC5 or FLAG-tagged HDAC5$^{S/A}$ were established by G418 selection of C2C12 clones transfected with plasmids encoding wild type HDAC5 or a CaMK resistant HDAC5 mutant, in which Ser259 and Ser498 were mutated into alanines (McKinsey et al., 2000). Cells were treated with cycloheximide (Sigma) and/or MG132 (CalBiochem) at indicated concentrations.

Generation of transgenic mice and knockout mice: Transgenic mice that express constitutively active forms of calcineurin or CaMKIV under the control of a muscle-specific enhancer from the muscle creatine kinase (MCK) gene are described elsewhere (Naya et al., 2000; Wu et al., 2002). Tet-HDAC5$^{S/A}$ transgenic mice are described elsewhere (Czubryt et al., 2003). Myogenin-tTA, tet-lacZ, and Myo-MEF2C-VP16 transgenic mice were generated by injecting linearized constructs into the pronuclei of fertilized oocytes as previously described (Cheng et al., 1993).

Hdac5$^{-/-}$ (Chang et al., 2004), *Hdac9*$^{-/-}$(Zhang et al., 2002), and *Hdac7* conditional knockout mice have been generated previously (Chang et al., 2006). *Hdac4* conditional mice were generated by flanking exon 6 with loxP sites, which results in an out of frame

mutant (to be described in detail elsewhere). *Mef2a*$^{-/-}$ and *Mef2c* and *Mef2d* conditional knockout mice have been described previously (Arnold et al., 2007; Naya et al., 2002). Correct gene targeting was confirmed by Southern blot analysis, genomic sequencing and RT-PCR.

Immunoprecipitation and immunoblotting: Immunoprecipitations were performed as previously described (Vega et al., 2004b). Antibodies against HA (1:1000, Sigma), FLAG M2 (1:4000, Sigma), Myc (1:1000 Santa Cruz), α-tubulin (1:5000, Sigma), HDAC1, 2, 3 (1:1000 for all, Sigma), HDAC4 (1:500, Santa Cruz), HDAC5 (1:1000, Upstate), HDAC7 (1:1000, Cell Signaling Technology), MEF2A (1:1000, Upstate), MEF2C (1:1000, Santa Cruz), MEF2D (1:2500, BD Sciences), troponin I slow (1:2500, Santa Cruz), cytochrome c (1:2500, Pharmingen), and myoglobin (1:3000, Daco) were used for immunoblot analyses. For analyzing endogenous class II HDAC proteins, the ECL Advance Western Blotting Detection Kit (Amersham) was used.

***In vivo* pharmacological studies:** Myogenin-tTA and tet-HDAC5$^{S/A}$ transgenic mice were bred while receiving doxycycline (DOX) (200μg/ml) in water as previously described (Czubryt et al., 2003). Myogenin-tTA\tet-HDAC5$^{S/A}$ transgenic mice were maintained on DOX as needed. Voluntarily wheel running experiments were performed and measured as previously described (Wu et al., 2001). Animals were allowed to acclimate to running cages for four days prior to running recordings. DOX was removed from the mice for the days of acclimation to begin expressing the transgene. After four days, wheel-running activity was measured continuously for four weeks.

Forced treadmill exercise experiments for analyzing MEF2C-VP16 transgenic mice were performed as follows. Prior to exercise, mice were accustomed to the treadmill (Columbus Instruments) with a 5-min run at 7 m/min once per day for 2 d. The exercise test was performed on a 10% incline for 10 m/min for the first 60 min, followed by 1 m/min increment increases for three 15-min intervals, then 45 min at 13 m/min, followed by 1 m/min increment increases at 15-min intervals until exhaustion. Forced exercise ended when mice were unable to avoid repeated electric shocks. MEF2 reporter mice (DesMEF lacZ) were ran for ~3 hours at 9 m/min.

In vivo proteasome inhibition experiments were performed by intraperitoneally delivering DMSO or MG132 as previously described for 6 hours (Luker et al., 2003), except 30 μmol/kg body weight MG132 was used for injections. All experiments involving animals were reviewed and approved by the Institutional Animal Care and Research Advisory Committee.

RNA isolation and analysis: Total RNA was prepared from mouse tissues using Trizol (Invitrogen) following the manufacturer's instructions. 1.5 μg of total RNA was converted to cDNA using oligo dT primer and Superscript II reverse transcriptase (Invitrogen). For PCR reactions, 2% of the cDNA pool was amplified. PCR cycles were optimized for each set of primers. Sequences for HDAC PCR primers have been described previously (Wu and Olson, 2002). Quantitative real-time PCR was performed for indicated MHC isoforms using SYBR Green (Applied Biosystems). Northern blots were performed with 20 μg of total RNA in each lane and probed in Ultrahyb (Ambion) with labeled HDAC4, 5, or β-actin cDNA.

Fiber-type and immunohistological analysis: Soleus and gastrocnemius-plantaris (GP) muscles were harvested from mice and flash frozen in embedding medium or fixed in 4% paraformaldehyde as previously described (Oh et al., 2005). Fiber-type analysis using a metachromatic ATPase staining (Ogilvie and Feeback, 1990) and glycerol gradient silver staining were performed as previously described (Oh et al., 2005). Paraffin sections were stained with a MHC type-I antibody, followed by treatment with DAB (Vector Labs).

Chapter IV

Requirement of *Mef2c* for Assembly of Skeletal Muscle Sarcomeres and Postnatal Muscle Function

Introduction

The formation of skeletal muscle involves the specification of myogenic progenitor cells within the somites followed by the activation of a large array of muscle-specific genes through the synergistic activities of the MyoD and myocyte enhancer factor 2 (MEF2) families of transcription factors. Members of the MyoD family of transcription factors interact with MEF2 factors to cooperatively activate muscle-specific genes (Black and Olson, 1998). MEF2 factors alone do not possess myogenic activity, but potentiate the activity of bHLH factors (Molkentin et al., 1995). The MEF2 proteins: MEF2A, -B, -C, and –D contain a conserved N-terminal MADS (MCM1, agamous, deficiens, SRF) domain and an adjacent MEF2-specific domain which, together, are necessary and sufficient for dimerization, cofactor interactions, and binding to the DNA consensus sequence $CTA(A/T)_4TAG$ (Andres et al., 1995; Molkentin et al., 1996a; Pollock and Treisman, 1991; Yu et al., 1992).

Based on their expression patterns *in vivo* and activities *in vitro*, MEF2 factors are believed to function downstream of the bHLH transcription factors in the pathway for skeletal muscle development (Dodou et al., 2003; Martin et al., 1993; Molkentin and Olson, 1996; Wang et al., 2001). However, the promoters of the *myogenin* (Buchberger et al., 1994; Cheng et al., 1993; Edmondson et al., 1992; Yee and Rigby, 1993) and *Mrf4* (Black et al., 1995; Naidu et al., 1995) genes contain MEF2 binding sites that provide a mechanism for amplifying and maintaining their expression and stabilizing the muscle phenotype (Molkentin and Olson, 1996). The *Mef2c* gene also serves as a direct target of myogenic bHLH and MEF2 factors, which serves to further reinforce the decision of myoblasts to differentiate (Wang et al., 2001). Thus, the expression and activities of

these two classes of myogenic transcription factors are intimately integrated through multiple regulatory mechanisms (Molkentin and Olson, 1996; Wang et al., 2001).

During mouse embryogenesis, MEF2 proteins display distinct, but overlapping expression patterns in the skeletal muscle lineage, but unlike the myogenic bHLH transcription factors, MEF2 proteins are also expressed in other cell types such as neurons, cardiomyocytes, neural crest cells, chondrocytes, smooth muscle cells, and endothelial cells (Arnold et al., 2007; Chang et al., 2006; Edmondson et al., 1994). *Mef2c* is the first member of the MEF2 family to be expressed in the myotome (at ~E9.0), and its appearance lags approximately 18 hours behind *Myf5*, the first bHLH myogenic regulator to be expressed (Edmondson et al., 1994) (Fig. 4.1). *Mef2a* and *Mef2d* are expressed after *Mef2c* (Edmondson et al., 1994) (Fig. 4.1).

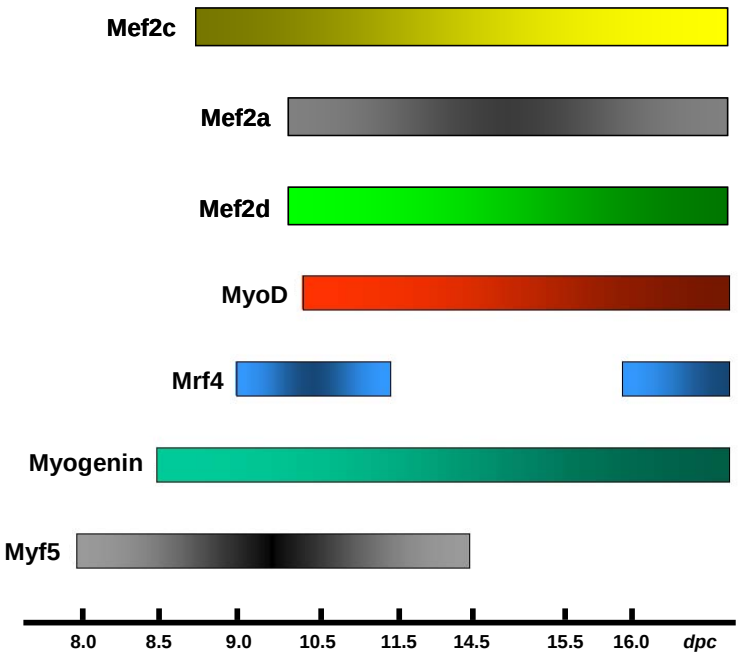

Figure 4.1. Expression pattern of bHLH and MEF2 proteins during skeletal myogenesis.
The myogenic bHLH transcription factors *Myf5* and *Myogenin* are the first differentiation transcription factors expressed in the myogenic lineage. *Mef2c* is the first MEF2 transcription factor expressed in skeletal muscle and its

expression immediately follows myogenin. The expression of the remaining bHLH and Mef2 proteins are indicated.

The importance of MEF2 genes *in vivo* is illustrated by loss-of-function studies in *Drosophila* and mice. Loss-of-function of the single *Drosophila* Mef2 gene results in a block to differentiation of all muscle cell types (Bour et al., 1995; Lilly et al., 1995; Ranganayakulu et al., 1995). Mice that lack *Mef2a* display an array of cardiovascular defects which cause most mice to die suddenly within the first week of life (Naya et al., 2002). Mice with homozygous mutations in *Mef2d* are viable (Arnold et al., 2007), whereas mice lacking *Mef2c* die at E9.5 from cardiovascular defects (Lin et al., 1997). The early lethality caused by the *Mef2c* loss-of-function mutation has therefore precluded analysis of its role in skeletal muscle at later developmental stages.

In addition to its role in muscle development, MEF2 has been implicated in establishing the slow myofiber phenotype by serving as a target for calcium dependent signaling to drive oxidative and slow-fiber specific genes (Chin et al., 1998; Wu et al., 2000). Recently, we showed that MEF2 proteins are necessary and sufficient to drive slow fiber formation (Chapter 3). Skeletal muscle-specific deletion of *Mef2c* in a mixed genetic background results in a substantial reduction of slow fiber formation, while overexpression of a super-active form of MEF2C (MEF2C-VP16) promotes the slow fiber phenotype and enhances endurance exercise (Potthoff et al., 2007).

To further explore the functions of *Mef2c* in developing skeletal muscle, we conditionally deleted a floxed *Mef2c* allele in skeletal muscle using two Cre recombinase transgenes that allow early versus late deletion of *Mef2c* in skeletal muscle. Here we show that early deletion of *Mef2c* results in neonatal lethality at postnatal day 1 (P1),

while mice with a later deletion of *Mef2c* are viable. In mice with early deletion of *Mef2c*, skeletal muscle differentiates to form myofibers with abnormally assembled sarcomeres and weakened M-lines. Microarray analysis revealed misregulation of sarcomere and stress response genes, including those encoding the M-line specific proteins myomesin and M-protein. Accordingly, we show that *Mef2c* directly regulates *myomesin* transcription. These results reveal an essential role for *Mef2c* in myofiber maturation and function, and demonstrate an important role for MEF2 proteins in terminal differentiation through maintenance of muscle integrity.

Materials and Methods

Plasmid Constructs, Tissue Culture and Cell Transfection: Wild-type *myomesin 1* and *2* (*M-protein*) promoters were cloned into TOPO TA (Invitrogen), and MEF2 and E-box sites were mutated by PCR (*myomesin 1* MEF2 (CTATATTTAT to CTGGGTTTAT) and E-box (CATGTG to TCTGTG) (-1035 bp to +88); *myomesin 2* MEF2 CTAAATATAG to CTAGGGATAG) (-1058 bp to +6 bp)). Wild-type and mutant promoters were then cloned into pGL3 and pGHlacZ vectors, for luciferase reporter and *in vivo* expression, respectively.

COS cells and C2C12 myoblasts were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum and antibiotics as described previously (Potthoff et al., 2007). For transient transfection assays, cells were plated and transfected 12 hours later using Fugene (Roche) following the manufacturer's instructions.

Generation of Mutant and Transgenic Mice: Mice with a *Mef2c* allele flanked by loxP sites have been described (Arnold et al., 2007). Skeletal muscle specific transgenic mice expressing cre recombinase under the *myogenin* promoter (Li et al., 2005) or *MCK* promoter (Bruning et al., 1998) have been described previously. Transgenic mice were generated as previously described (Cheng et al., 1993). Staining of embryos for β-galactosidase was performed as previously described (Cheng et al., 1993).

RNA in situ hybridization, Histology and Electron Microscopy: Embryos and tissue for histology were isolated in PBS, fixed in 4% paraformaldye overnight, processed and sectioned for hematoxylin and eosin (H&E) staining using standard procedures (Shelton et al., 2000). 0.1% DEPC PBS was used for *in situ* hybridization samples. ^{35}S-labeled RNA probes for Myf5, MyoD (Lu et al., 2002) and Myogenin were generated using a MAXISCRIPT Kit (Amersham).

Electron microscopy was performed as previously described (Li et al., 2005). Briefly, skeletal muscle from P1 pups was fixed overnight in 2% glutaraldehyde in PBS at 4°C, then postfixed in 1% OsO_4, and dehydrated in an ethanol series. Samples were then embedded in Spurr resin (Ted Pella, Inc., Redding, CA), stained with uranyl acetate and lead citrate, and sectioned at 80 nm.

RT-PCR, Quantitative Real-time PCR and Microarray Analysis: Total RNA was extracted from wild-type and mutant skeletal muscle with Trizol reagent (Invitrogen). Four micrograms of RNA from each sample was used to generate cDNA using SuperScript II First-Strand Synthesis kit (Invitrogen). RT-PCR for the deleted

region of *Mef2c* was performed using the following primer pair: FWD 5'-GATGAAGAAGGCTTATGAGCTGAGCGTGCTGTGCGACTGTGAG-3'; REV 5' - CTGTTATGGCTGGACACTGGGATGGTAACTGGCATCTCAAAG - 3'.

Quantitative real-time PCR was performed using TaqMan OneStep chemistry or SYBR Green on an ABI PRISM 7000 sequence detection system (Applied Biosystems). Predesigned intron-spanning primers were purchased from Applied Biosystems for Taqman (myogenin, MyoD, Acta1, Myh7, myozenin 2, Myl4, Myl7, MEF2A, MEF2D, TnnI1, TnnI2, TnnT3, and GAPDH). Primer sequences for SYBR Green are available upon request.

For microarray analysis, total RNA was extracted from wild-type or *Mef2c* SKM KO E18.5 hindlimbs using Trizol reagent (Invitrogen). Microarray analysis was performed using the Mouse Genome 430 2.0 array (Affymetrix) and results were analyzed by Genesifter.

Reporter Assays: The *myomesin 1*- and *2*-luciferase constructs contain a DNA fragment extending from -1035 bp to +88 bp and -706 bp to +6 bp from the *myomesin* and *M-protein* genes, respectively. The Myc-tagged MEF2C expression vector was described previously (Phan et al., 2005). COS cells in 24-well plates were transfected with 100 ng of reporter plasmids in the presence or absence of MEF2C (5-100 ng) and/or HDAC5. The reporter assays were performed as previously described (Chang et al., 2005).

Gel Mobility Shift Assays: Oligonucleotides corresponding to the conserved MEF2-binding site in the *myomesin* and *M-protein* promoters, a mutated site, and a bona fide MEF2 site from the *MCK* enhancer were synthesized (Integrated DNA Technologies), annealed, labeled with ^{32}P-dCTP using Klenow, and purified with G25 columns (Roche). The following sequences were used: for the *myomesin 1* wild-type, 5' – GGGATGTGCTGCTATATTTATCTGCCTT – 3'; *myomesin 1* ΔMEF2, 5' – GGGATGTGCTGCTACCGGTATCTGCCTT – 3'; *muscle creatine kinase* (*MCK*), 5' – GGGGATCGCTCTAAAAATAACCCTGTCG – 3'; *myomesin 2* wild-type; 5' – GGGCCCTTGCCTAAATATAGCACCTCCT – 3'; *myomesin 2* ΔMEF2; 5' – GGGCCCTTGCCTACCGGTAGCACCTCCT – 3'. Cell extracts were isolated from COS cells transfected with a myc-tagged MEF2C expression plasmid or empty vector. Reaction conditions were performed as previously described (Chang et al., 2001; Phan et al., 2005). DNA-protein complexes were resolved on 5% polyacrylamide native gels and visualized by phosphoimaging.

Results

Early embryonic deletion of *Mef2c* causes perinatal lethality.

Mice with a homozygous null mutation of *Mef2c* exhibit early lethality at E9.5 due to cardiovascular defects (Lin et al., 1997). To determine the function of *Mef2c* in skeletal muscle at later developmental stages, we deleted a floxed *Mef2c* allele (Arnold et al., 2007) specifically in skeletal muscle using the myogenin-Cre (Myo-Cre) transgene, which consists of a Cre recombinase expression cassette controlled by the *myogenin*

promoter and the skeletal muscle-specific enhancer of the *Mef2c* gene. This transgene is expressed specifically in skeletal muscle beginning at E8.5 (Li et al., 2005).

Myogenin-cre mediated, skeletal muscle-specific deletion of *Mef2c* (*Mef2c* SKM KO) resulted in lethality at postnatal day 1 (P1). This postnatal lethality was affected by genetic background. In a C57Bl6/129SV mixed genetic background, 100% lethality was observed. However, other backgrounds (*e.g.*, 129SV) produced some viable *Mef2c* SKM KO mice, which display a fiber-type switching phenotype described previously (Potthoff et al., 2007)(Chapter 3). *Mef2c* SKM KO pups in the C57Bl6/129SV mixed genetic background were slightly smaller than wild-type littermates (Fig. 4.2A) and died several hours after birth, always before postnatal day 2 (P2). At P1, *Mef2c* SKM KO pups were mobile but lethargic compared to wild-type littermates and did not feed (Fig. 4.2A). In contrast to the early perinatal lethality resulting from *Mef2c* deletion with Myo-Cre, mice with a later deletion of *Mef2c* beginning at ~E18.5 using a Cre transgene controlled by the *muscle creatine kinase* promoter (MCK-Cre) (Bruning et al., 1998) were viable.

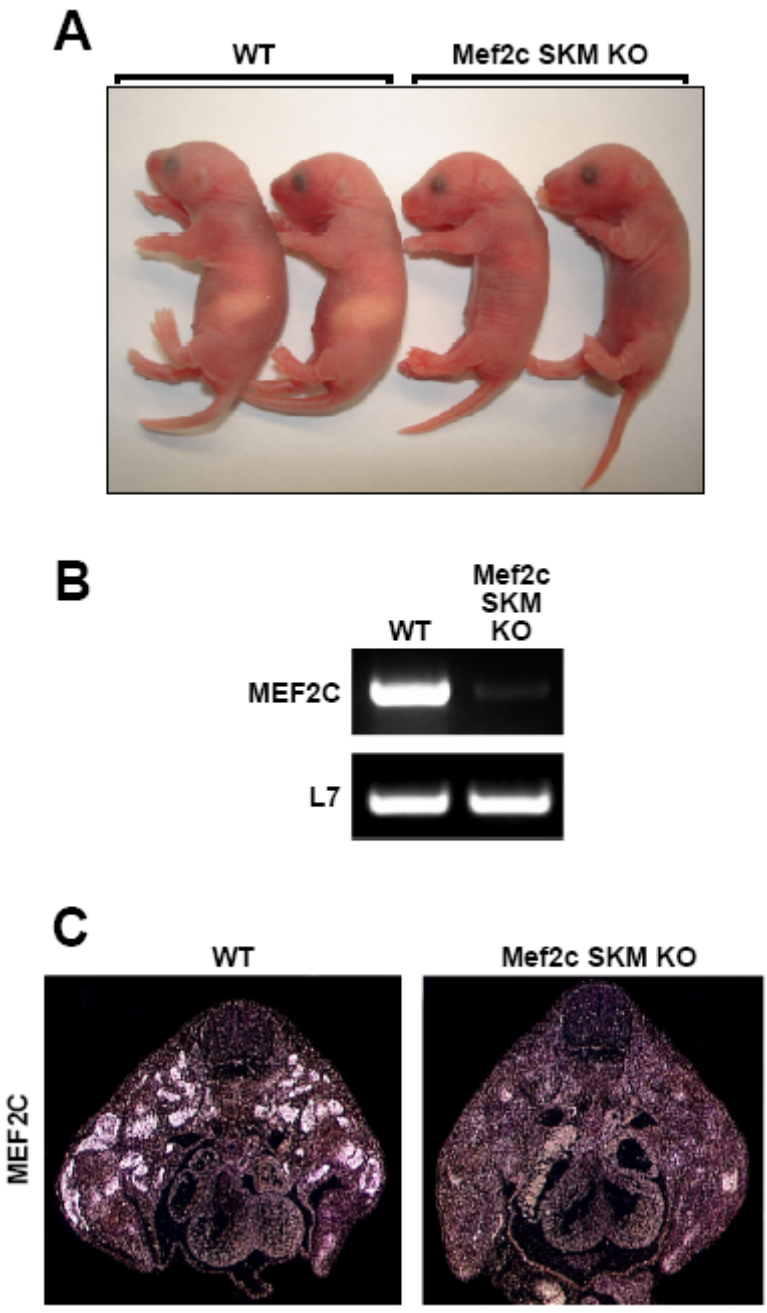

Figure 4.2. Early embryonic deletion of *Mef2c* causes perinatal lethality. A. *Mef2c* SKM KO mice (*Mef2c*$^{fl/-;myogenin\text{-}cre}$) are smaller than wild-type (WT) littermates and do not feed (note absence of milk in stomach). Analysis of takeout efficiency in *Mef2c* SKM KO mice in postnatal (B) and embryonic (C) muscles. B. Total RNA was isolated from hindlimb muscles of wild-type and *Mef2c* SKM KO mice at post-natal day 1 (P1) and analyzed by semiquantitative RT-PCR for MEF2C. L7 serves as a loading control. C. *In situ* hybridization for MEF2C in transverse sections of E12.5 WT and *Mef2c* SKM KO embryos.

To verify the efficiency of *Mef2c* gene deletion by Myo-Cre, we performed RT-PCR and *in situ* hybridization for the deleted region of *Mef2c* in wild-type and *Mef2c* SKM KO skeletal muscles. As shown in Fig. 4.2B and 4.2C, *Mef2c* transcripts were efficiently deleted in muscles from embryos and neonates, respectively.

Notably, *Mef2a* or *Mef2d* homozygous mutant mice did not display skeletal muscle developmental defects. We conclude that embryonic expression of *Mef2c* is specifically required for skeletal muscle development.

Loss of *Mef2c* results in myofiber disarray.

Histological analysis showed that the hindlimb muscles from *Mef2c* SKM KO pups at P1 were severely disorganized and fragmented (Fig. 4.3A). The diaphragm of *Mef2c* SKM KO mice was especially thin and lacking in well-developed myofibers, which is likely to be the cause of death (Fig. 4.3B). Notably, mutant myocytes were able to differentiate and fuse into myofibers (Fig. 4.3A), unlike muscles from *myogenin* knockout mice, which do not completely differentiate and form very few myofibers *in vivo* (Hasty et al., 1993). Skeletal muscle deletion of *Mef2c* with *MCK*-Cre did not disrupt myofiber organization (data not shown).

To define the time of onset of muscle defects in *Mef2c* SKM KO animals, we analyzed muscle at sequential developmental stages. Muscles appeared normal at E12.5, 14.5 and E16.5 (data not shown), whereas disorganization was apparent by E18.5 and became more severe by P1 (Fig. 4.3C and D).

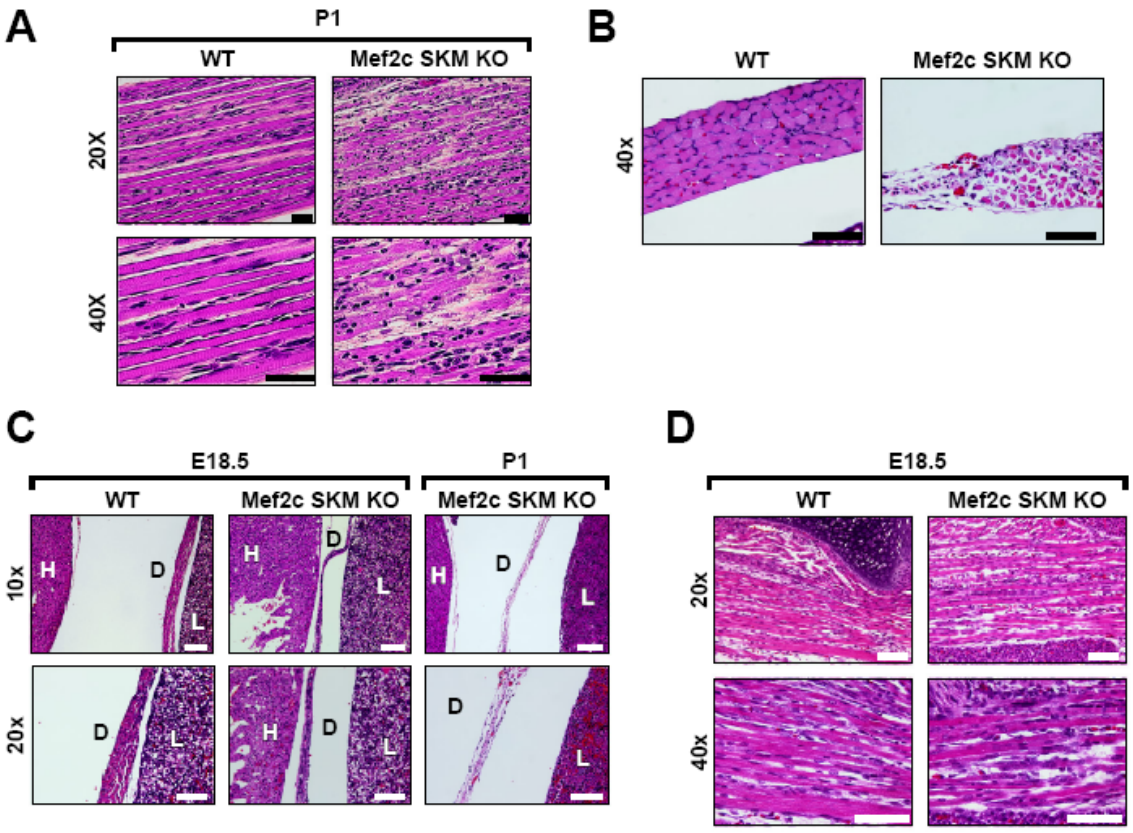

Figure 4.3. Myofiber disarray in *Mef2c* deficient muscles.
Hindlimb muscle and diaphragm of wild-type (WT) and *Mef2c* SKM KO (*Mef2c*$^{fl/-;myogenin\text{-}cre}$) mice. A. H&E staining of histological sections of representative hindlimb muscle from P1 WT and *Mef2c* SKM KO mice (20X and 40X mag.; scale bars: 300 nm). B. Thinned diaphragm and degenerated fibers in *Mef2c* SKM KO mice compare to WT littermates (40X mag.; scale bars: 100 nm). C. Diaphragm from E18.5 *Mef2c* SKM KO mice are thinner than diaphragms from wild-type controls, but not as severe as *Mef2c* SKM KO P1 diaphragms (H, heart; D, diaphragm; L, liver; scale bars equal 300 and 200 nm for 10X and 20X mag., respectively). D. H&E staining of histological sections of representative hindlimb muscle from E18.5 WT and *Mef2c* SKM KO mice (10X and 20X mag.; scale bars: 300 nm.)

Ultrastructural analysis showed that sarcomeres of skeletal muscle from *Mef2c* SKM KO mice at P1 were disorganized and degenerated compared to wild-type littermates (Fig. 4.4A). Fragmented myofibers along the M-line regions were especially

apparent in the mutant (Fig. 4.4B), suggesting a weakening of the M-line structure, which is essential for maintenance of sarcomere integrity.

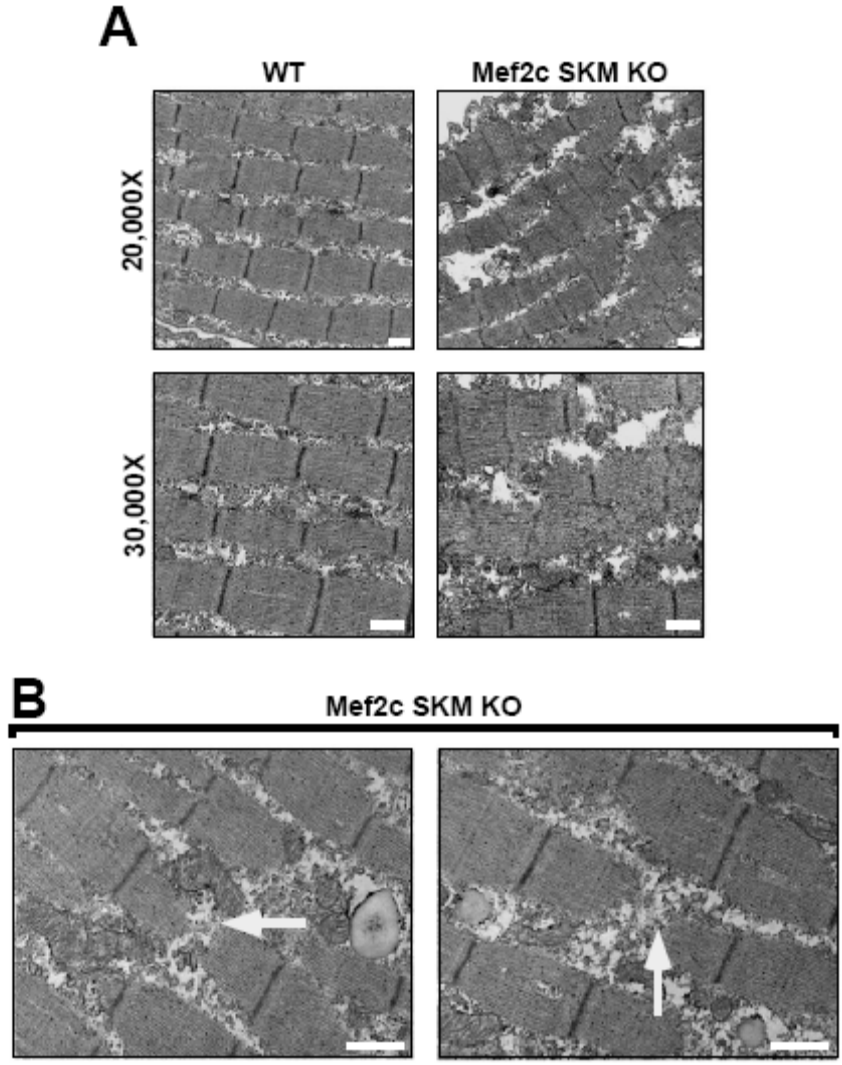

Figure 4.4. Disorganized sarcomeres and weaked M-line in *Mef2c* SKM KO muscles.
A. Hindlimb muscle from P1 wild-type (WT) and *Mef2c* SKM KO (*Mef2c*$^{fl/-;myogenin\text{-}cre}$) mice were analyzed by electron microscopy. The sarcomeres in *Mef2c* mutant mice are disorganized and fragmented compared to WT littermates. Magnifications are shown on left (scale bar: 500 nm). B. Higher magnification regions reveals severed M-lines as indicated by arrows (~40,000 mag.; scale bar: 500 nm).

Abnormalities of muscle gene expression in *Mef2c* mutants.

To determine whether the absence of *Mef2c* resulted in diminished expression of myogenic bHLH transcription factors, which might cause the skeletal muscle abnormalities in SKM KO mice, we performed *in situ* hybridization for myogenin, Myf-5 and MyoD. Myogenic bHLH transcription factor expression appeared unaltered in *Mef2c* SKM KO muscles at E9.5, E12.5 (Fig. 4.5A) and E16.5 (data not shown).

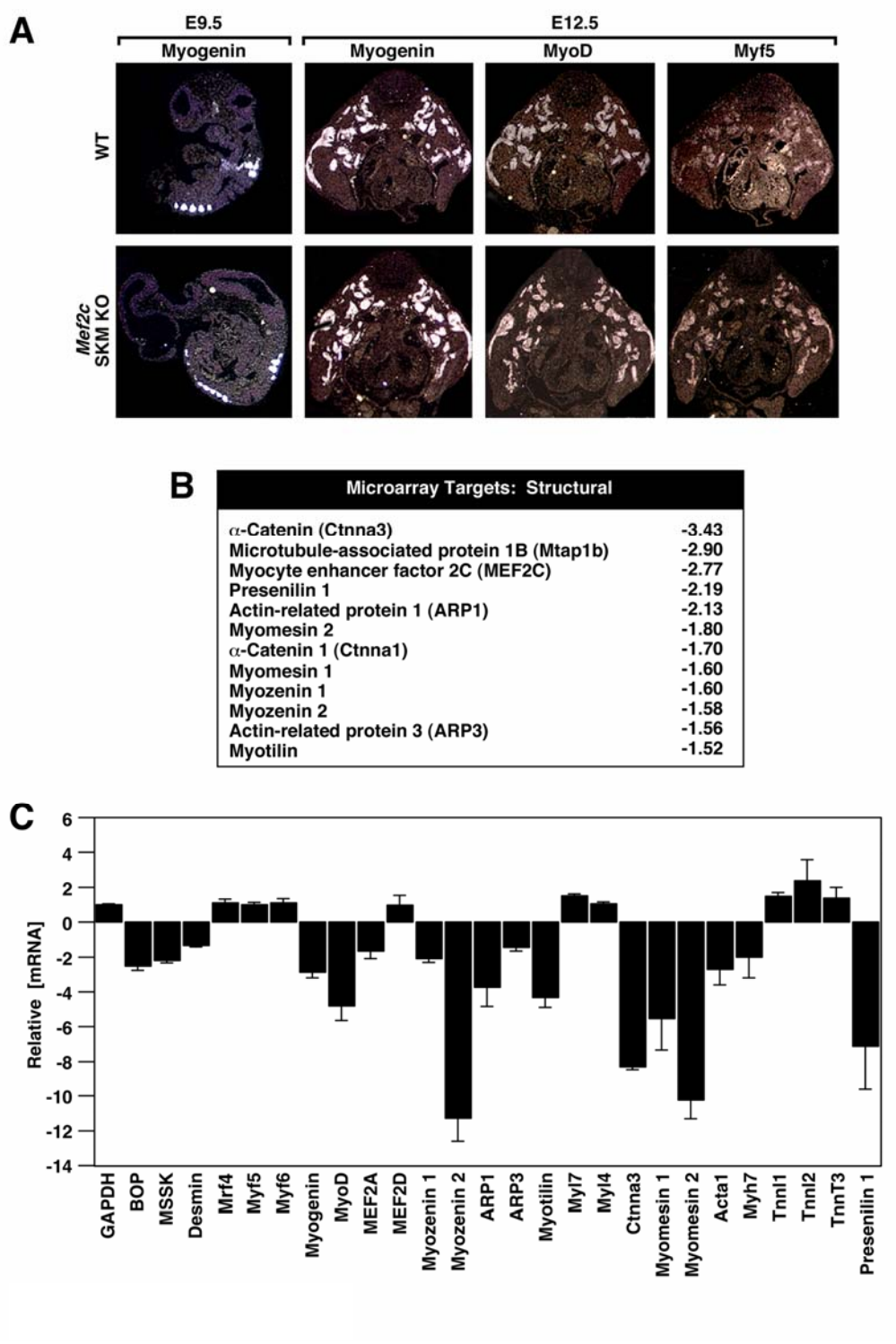

Figure 4.5. Analysis of muscle gene expression in *Mef2c* mutants.
A. Expression of Myogenin, MyoD, and Myf5 in wild-type (WT) and *Mef2c* SKM KO (*Mef2c*$^{fl/-;myogenin\text{-}cre}$) embryos (E9.5 and E12.5). B. Microarray analysis shows misregulated structural and sarcomere associated genes in *Mef2c* SKM

KO E18.5 hindlimbs. C. Validation of microarray data and analysis of gene expression of structural candidate genes by quantitative real-time PCR.

Microarray analysis of skeletal muscle from hindlimbs of wild type and SKM KO mice at E18.5, revealed numerous genes that were down-regulated in the mutant, including many genes encoding structural, cell-adhesion, and stress responsive proteins (Fig. 4.5B). Among the most dramatically down-regulated genes were *myomesin 1* and *myomesin 2* (also referred to as *M-protein*), which encode muscle-specific structural proteins that stabilize the sarcomere along the M-line by forming an elastic (Bertoncini et al., 2005), lattice structure that interacts with titin and myosin (Agarkova and Perriard, 2005; Obermann et al., 1997; Obermann et al., 1998). This "elastic web" stabilizes muscles by diminishing thick filament displacement and by returning the sarcomere to its original state after contraction (Agarkova et al., 2003; Agarkova and Perriard, 2005). Myomesin proteins are expressed in all types of vertebrate striated muscle and their importance is supported by their observed fixed expression ratio with myosin (Agarkova and Perriard, 2005; Agarkova et al., 2004). Moreover, myomesin proteins and the M-line are crucial for sarcomere stability since loss of M-line protein interaction with titin results in progressive sarcomere damage and lethality (Peng et al., 2005).

In addition to the *myomesin* genes, several additional sarcomere and stress responsive genes were misregulated in *Mef2c* SKM KO muscles including *myozenin 1* and *2*, *actin*, *myosin*, and *myotilin* (Fig. 4.5B and C). MEF2 proteins were previously shown to be important for expression of thick filament proteins *in vivo* (Hinits and Hughes, 2007), but these genes were only slightly downregulated in *Mef2c* SKM KO muscles. Myozenin 1 and 2, also called calsarcin 2 and 1, respectively, are Z-line

interacting proteins that are important stress sensors that link calcineurin with the sarcomere (Frey et al., 2000). Interestingly, MEF2 has previously been shown to regulate additional stress response genes in muscle (Blais et al., 2005), several which were confirmed in *Mef2c* SKM KO muscles (data not shown). Additionally, *myotilin*, which encodes an actin cross-linking necessary for sarcomere assembly (Salmikangas et al., 2003), was downregulated in *Mef2c* SKM KO muscles.

Transcripts encoding the bHLH transcription factors *MyoD* and *myogenin* were slightly downregulated, as detected by quantitative real-time PCR (qRT-PCR), while *Myf5* transcript levels were unchanged. Known MEF2 target genes (*e.g.*, *Bop*, *Srpk3 (MSSK)*, *desmin*) were also slightly downregulated in *Mef2c* deficient muscles (Fig. 4.5C).

The *myomesin 1* and *2* genes are direct targets of *Mef2c*.

Myomesin 1 and 2 play a crucial role in maintaining sarcomere organization (Agarkova et al., 2003), suggesting that their downregulation could be causal in the *Mef2c* SKM KO phenotype. We therefore searched the *myomesin* promoters for conserved MEF2 sites that might control their expression in skeletal muscle. As shown in Fig. 4.6A, conserved consensus MEF2 sites are located immediately upstream of both genes. In addition, the *myomesin 1* promoter contains a conserved MEF2 site directly adjacent to an E-box and this region confers transcriptional regulation to the *myomesin 1* gene (Steiner et al., 1999).

The first ~1000 bp and 700 bp of the *myomesin 1* and *2* promoters, respectively, were cloned into a luciferase reporter and found to be responsive to MEF2 when co-

transfected into COS cells (Fig. 4.6B). Mutation of the MEF2 sites in both promoters abolished responsiveness to MEF2 (Fig. 4.6B). In addition, when C2C12 cells were transfected with these luciferase constructs and allowed to differentiate, luciferase activity was observed with the wild-type *myomesin* promoter, but was significantly reduced with the promoters containing a mutated MEF2 site (data not shown). Mutagenesis of the E-box in the *myomesin 1* promoter reduced, but did not eliminate luciferase activity (Fig. 4.6B).

DNA binding assays using extracts from COS cells transfected with a Myc-MEF2C expression plasmid confirmed that the MEF2 sites in the *myomesin 1* and *2* promoters were bona fide MEF2 sites (Fig. 4.6C). The MEF2-consensus sequences from the *myomesin 1* and *2* promoters bound MEF2C comparably to the canonical MEF2 site from the *MCK* enhancer (Gossett et al., 1989) (Fig. 4.6C). This DNA-protein complex was abolished in the presence of excess competitor (unlabeled cognate DNA sequence) and was supershifted by an anti-Myc antibody, whereas a sequence containing a mutated MEF2 site was unable to compete for MEF2C binding (Fig. 4.6C).

To analyze the importance of the MEF2 site in the *myomesin 1* promoter *in vivo*, we generated transgenic mice with a lacZ reporter gene linked to the *myomesin 1* promoter. As seen in Fig. 4.6D, the wild-type *myomesin 1* promoter directed expression in the somites, limb muscles, and heart (Fig. 4.6D) at E11.5. Expression in all of these cell types was abolished by a mutation in the MEF2 site (Fig. 4.6D). These findings support the conclusion that MEF2C directly activates myomesin gene transcription *in vitro* and *in vivo*.

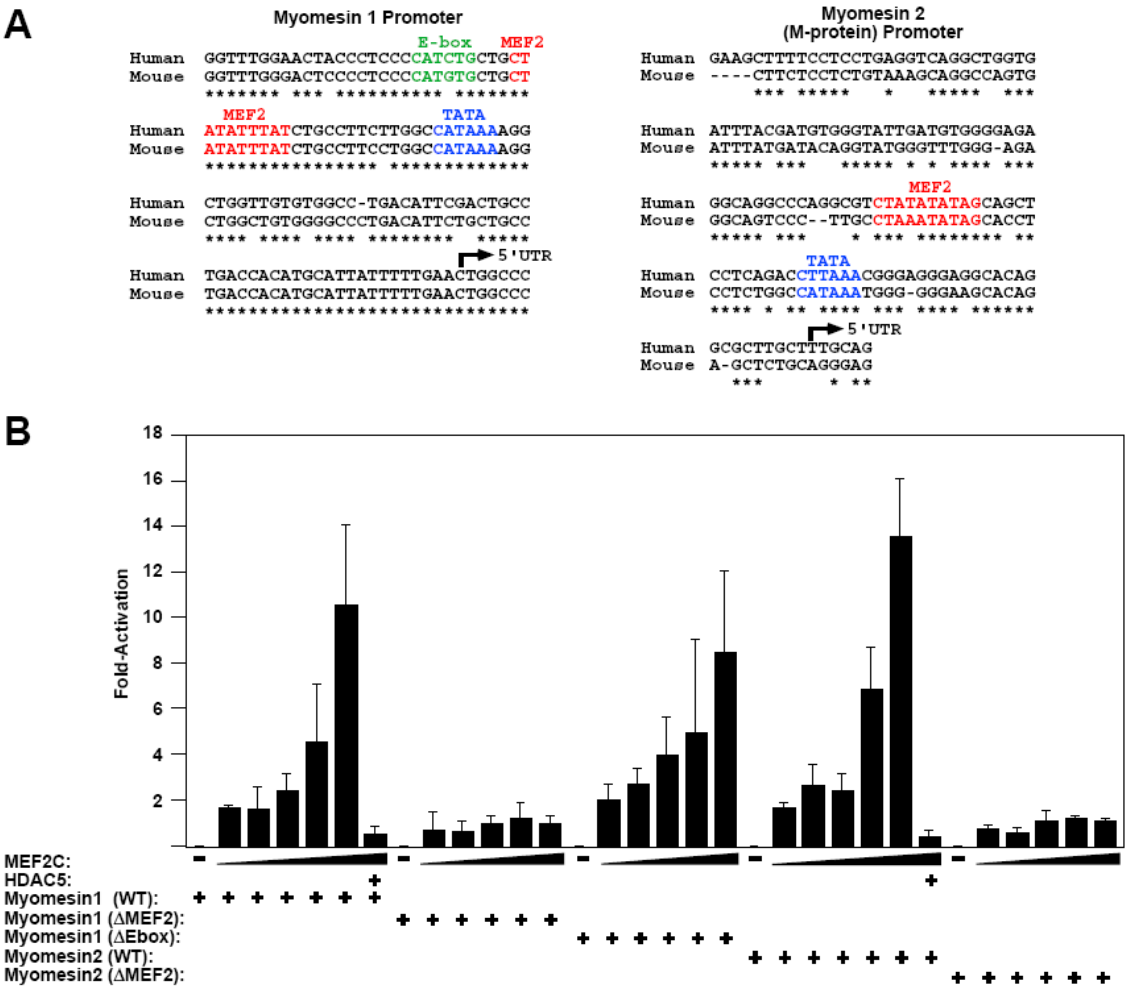
A
Myomesin 1 Promoter
E-box
MEF2
Human GGTTTGGAACTACCCTCCCCATCTGCTGCT
Mouse GGTTTGGGACTCCCCTCCCCATGTGCTGCT
MEF2
TATA
Human ATATTTATCTGCCTTCTTGGCCATAAAAGG
Mouse ATATTTATCTGCCTTCCTGGCCATAAAAGG
5'UTR
Human TGACCACATGCATTATTTTTGAACTGGCCC
Mouse TGACCACATGCATTATTTTTGAACTGGCCC
Myomesin 2
(M-protein) Promoter
Human GAAGCTTTTCCTCCTGAGGTCAGGCTGGTG
Mouse ----CTTCTCCTCTGTAAAGCAGGCCAGTG
Human ATTTACGATGTGGGTATTGATGTGGGGAGA
Mouse ATTTATGATACAGGTATGGGTTTGGG-AGA
MEF2
Human GGCAGGCCCAGGCGTCTATATATAGCAGCT
Mouse GGCAGTCCC--TTGCCTAAATATAGCACCT
TATA
Human CCTCAGACCTTAAACGGGAGGGAGGCACAG
Mouse CCTCTGGCCATAAATGGG-GGGAAGCACAG
5'UTR
Human GCGCTTGCTTTGCAG
Mouse A-GCTCTGCAGGGAG
B
Fold-Activation
18
16
14
12
10
8
6
4
2
MEF2C:
HDAC5:
Myomesin1 (WT):
Myomesin1 (ΔMEF2):
Myomesin1 (ΔEbox):
Myomesin2 (WT):
Myomesin2 (ΔMEF2):
C
Myc-MEF2C
Empty vector
α-Myc
Comp.
Myom1 WT
Myom2 WT
MCK WT
Probe
Myom1 WT
Myom1 Mut
Myom2 WT
Myom2 Mut
MCK WT
Supershift
MEF2C
Probe
D
Myom 1 (WT)
Promoter LacZ
Myom 1 (ΔMEF2)
Promoter LacZ
(5/6)
(0/5)

Figure 4.6. Myomesin 1 and 2 are direct targets of MEF2C.
A. Alignment of mouse and human *myomesin 1* and *2* promoters using ECR browser and ClustalW identifies conserved MEF2 binding sites in close proximity to transcription start site (MEF2 site (red), E-box (green), and previously identified TATA box (blue) are labeled; asterisks mark conserved residues). B. MEF2C transactivates the myomesin promoters. Cotransfection of the MEF2C expression plasmid with wild-type (WT) *myomesin 1* or *2* luciferase reporter constructs results in significant activation of the reporter, which is not seen with the reporter containing a mutated MEF2 site (MEF2). Activation of the myomesin promoters is inhibited by HDAC5. Mutation of the E-box (E-box) in the *myomesin 1* promoter does not abolish responsiveness to MEF2. C. Gel mobility shift assays were performed with labeled probes corresponding to the *myomesin 1*, *2*, or *MCK* MEF2 site, and extracts from COS cells overexpressing Myc-MEF2C. The DNA-protein complex was supershifted in the presence of an anti-Myc antibody, and was competed by excess unlabeled probe competitor. D. MEF2 regulates myomesin *in vivo*. Transgenic reporter mice harboring a lacZ cassette behind the wild-type myomesin promoter show -galactosidase expression in somites, heart, and facial and limb muscle. LacZ expression is abolished in transgenic mice when the conserved MEF2 site is mutated (MEF2).

Discussion

The results of this study show that skeletal muscle specific deletion of *Mef2c* results in hypoplastic myofibers, disorganized sarcomeres and defects of the M-line that cause perinatal death. *Mef2c* deficient muscles showed reduced expression of genes encoding important structural proteins, including myomesin and M-protein, which are localized to the M-line and maintain sarcomere integrity. Moreover, our results demonstrate that MEF2C directly regulates *myomesin* gene transcription. We conclude that *Mef2c* plays an essential role in the perinatal regulation of genes necessary for proper sarcomere assembly and maintenance of myofiber integrity.

Distinct functions of MEF2 factors in skeletal muscle.

Given the early and specific expression of *Mef2c* in the skeletal muscle lineage, and the ability of MEF2C to synergistically activate myofiber genes with members of the MyoD family, it is surprising that *Mef2c* deficient myocytes undergo early steps of differentiation including myofiber formation. It seems likely that other MEF2 proteins compensate for the loss of *Mef2c* during early stages of embryonic and fetal myogenesis. However, we should point out that we have also found that skeletal muscle deletion of both *Mef2c* and *Mef2d* does not exacerbate the *Mef2c* SKM KO phenotype (data not shown), raising the possibility that residual levels of *Mef2a* or *Mef2b* are adequate to support initial steps in muscle development.

Mef2c deficient muscles appear normal prior to birth, but rapidly degenerate immediately after birth. The timing of the phenotype in mutant mice may reflect the relative lack of contractility or weight-bearing stress on skeletal muscle before birth.

That is, since many mis-regulated genes in *Mef2c* SKM KO muscles are associated with stress responsiveness and sarcomere assembly, a defect may not be observed until sarcomeres begin to function or are subjected to stress.

It is puzzling that the absence of *Mef2c* results in such a specific diminution of *myomesin* expression, despite the continued expression of *Mef2a* and *Mef2d*. We suggest two potential explanations for this finding. 1) The *myomesin* genes might be exquisitely sensitive to the level of MEF2 expression, irrespective of the isoform, such that residual *Mef2a* and *d* cannot drive expression of these genes in the absence of *Mef2c*. 2) *Mef2c* may be specifically required for *myomesin* expression because of a function not shared with the other MEF2 isoforms, perhaps mediated by a unique structural domain.

Recently, knockdown of *mef2c* and *mef2d* in zebrafish was reported to disrupt muscle function and sarcomere assembly as a result of diminished expression of thick filament proteins (Hinits and Hughes, 2007). In contrast, our results demonstrate that loss of *Mef2c* alone is sufficient to disrupt sarcomere assembly. The differences between the zebrafish and mouse phenotypes may reflect species-specific differences in MEF2C function or the lack of complete takeout of *mef2c* protein by morpholino knockdown in zebrafish. Consistent with the zebrafish study, muscles lacking *Mef2c* undergo early steps of muscle differentiation but do not properly terminally differentiate. Interestingly, Hinits *et al.* report that muscles from *mef2c/d* double knockdown zebrafish display a phenotype similar to mice lacking titin's M-line region (Hinits and Hughes, 2007). We show that *Mef2c* SKM KO muscles display M-line defects and MEF2C directly regulates myomesin expression, which could explain the zebrafish phenotype.

Influence of genetic modifiers on the functions of *Mef2c* in skeletal muscle.

Recently, we reported that mice with a skeletal muscle deletion of *Mef2c* in a 129SvEv genetic background were viable and showed a reduction in slow fibers (Potthoff et al., 2007). In contrast, skeletal muscle deletion of *Mef2c* in a C57Bl6 mixed background shown here results in perinatal lethality with complete penetrance. These findings suggest that the activity of *Mef2c* in skeletal muscle development is highly sensitive to genetic modifiers, which are capable of modulating the degree of sarcomere damage. Notably, the cyto-architectural and mitochondrial defects observed in *Mef2a* deficient mice are also highly sensitive to genetic modifiers (Naya et al., 2002), suggesting MEF2 proteins are significantly impacted by these genetic components.

MEF2 factors as multifunctional regulators of muscle gene expression.

Considered together with other studies, it is now apparent that MEF2 factors play important roles in numerous steps of muscle development, including the control of myoblast differentiation and fusion (Molkentin and Olson, 1996; Ornatsky et al., 1997), maintenance of myofiber integrity as shown in the present study, and regulation of mitochondrial biogenesis and fiber type specification (Potthoff et al., 2007; Wu et al., 2002). In each of these settings, MEF2 regulates distinct sets of downstream target genes, which likely reflects its ability to associate combinatorially with other coactivators and corepressors and to respond to upstream signaling pathways that vary in response to developmental, physiological and pathological cues. Understanding the molecular basis

of target gene recognition and activation by different MEF2 isoforms in these various processes represents a fascinating and important problem for the future.

Chapter V

The Role of Class I HDACs in Cardiac Development and Remodeling

Cardiogenesis and Hypertrophy

The heart is the first organ to develop during embryogenesis and cardiac development is governed by a core network of well-characterized transcription factors (Olson, 2006). Heart development requires cardiac cell specification and morphogenesis of a heart tube into a three-dimensional, four-chambered mechanical pump. Unlike most organs, the heart must continue to operate during morphogenesis. While the morphology and structure of the heart is not conserved, the transcriptional networks that regulate cardiac development are well conserved. These core transcription factors include members from the NK2, MEF2, GATA, Tbx, and Hand family of transcription factors (Olson, 2006)(Fig. 5.1A). Of these, MEF2 has been extensively studied and is required for cardiac development and adult remodeling (Fig. 5.1B). Though MEF2 is a relatively weak transcriptional activator, MEF2 associates with the core transcription factors to synergize and drive cardiac muscle structural genes through cis-regulatory sequences.

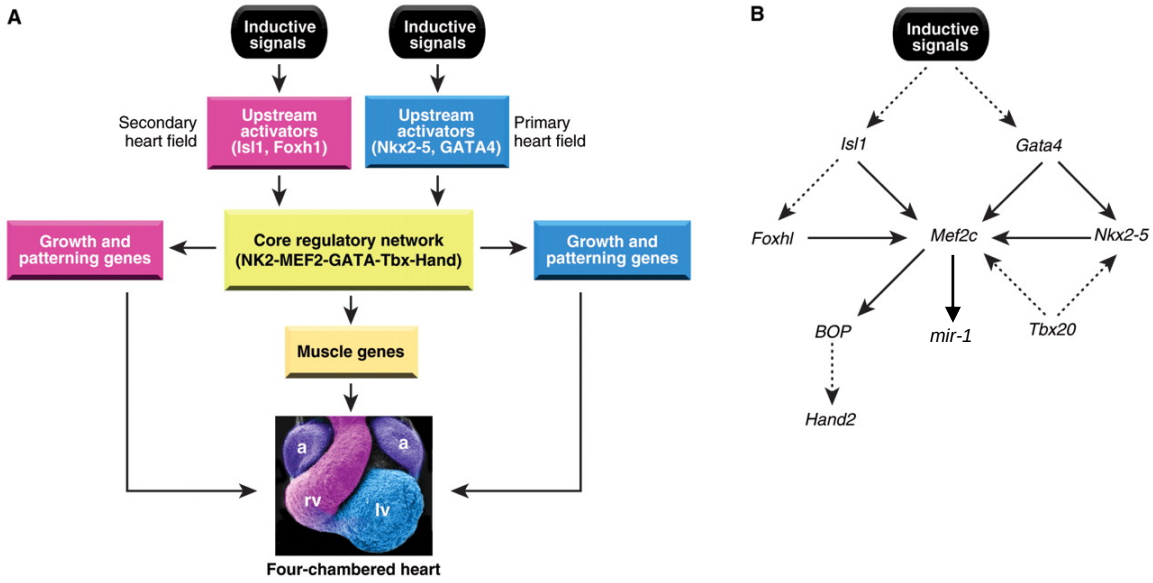

Figure 5.1. Transcriptional networks in the developing heart.
Schematic diagram of transcriptional networks involved in mammalian heart development. A. Inductive signals activate a cascade of upstream transcriptional

activators that induce expression of a core regulatory network (NK2-MEF2-GATA-Tbx-Hand) of transcription factors. The core network genes auto- and crossactivate additional genes which regulate the expression of muscle-specific genes and genes controlling cardiac growth and patterning. B. Regulation of cardiac transcription factors in the secondary heart field. Solid lines indicate known transcriptional connections with a direct interaction (Adapted from (Olson, 2006)).

MEF2 is known to regulate the expression of numerous cardiac structural and contractile proteins, and the importance of MEF2 in the heart and its role in cardiac hypertrophy is discussed in Chapter II.

In response to specific extrinsic and intrinsic signals, the adult heart undergoes cardiac hypertrophy (Fig. 5.2), a process defined as an increase in cell size but not cell number. Cardiomyocytes cannot divide after birth, so to compensate for increased pressure workload from exercise or cardiovascular disorders, individual myocytes increase their size to assemble additional sarcomere units in order to generate maximal force. While these effects are beneficial for short intervals (*e.g.*, exercise), prolonged hypertrophy from pathological signals perturbs calcium handling and cardiac contractility, eventually leading to heart failure from dilated cardiomyopathies and arrhythmias (Antos et al., 2003) (Fig. 5.2).

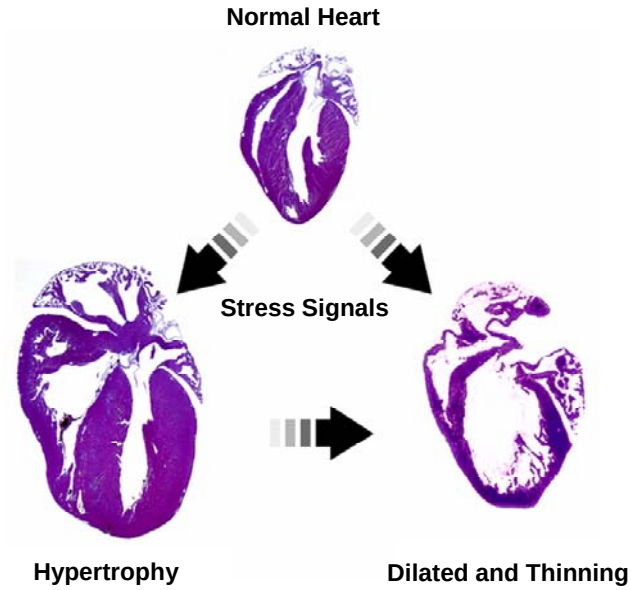

Figure 5.2. Abnormal growth of the adult heart.
The adult heart undergoes growth through hypertrophy, which can progress to a dilated myopathy in response to external or internal stress signals (Adapted from (Olson and Schneider, 2003)).

Class I HDAC Introduction

The viability, structure, and maintenance of cells require the transcriptional activation of cell-specific and housekeeping genes. Transcriptional activators and repressors regulate the expression of genes in response to internal and external signaling pathways. In eukaryotic cells, this transcriptional regulation is largely influenced by DNA accessibility. Eukaryotic DNA is packaged into nucleosomes, which consists of 146 bp of double-stranded DNA wrapped around a core of eight histones. Nucleasomes interact with each other to form a higher order structure which consolidates DNA into a smaller area and prevents transcription factor binding to DNA (Strahl and Allis, 2000). The local architecture of DNA is now known to be important in regulating gene expression, and numerous types of post-translational modifications to histones generate a "histone code,"

which allows chromatin condensation, relaxation, or accessibility depending on the modification or combination of modifications (Strahl and Allis, 2000).

During transcriptional activation of genes, post-translational modifications occur on the tails of histones, which allow chromatin relaxation and DNA binding proteins accessibility to DNA (Ito et al., 2000). The different types of reversible histone post-translational modifications include phosphorylation, methylation, acetylation, ubiquitination, sumoylation, and ribosylation. Perhaps the most-studied and best understood histone post-translational modification is acetylation. Histone acetylation occurs on the ε amino group of conserved lysine residues on the N-terminus of histone tails. All core histones are acetylated *in vivo*, but the modifications of H3 and H4 are the best characterized (Ito et al., 2000). Histone tail acetylation offsets the positive charge of lysine residues and thereby destabilizes the internucleosomal interactions and histone-DNA interactions (Cress and Seto, 2000). Therefore, acetylation relaxes DNA organization, allowing DNA accessibility and interaction with transcription factors (Grozinger and Schreiber, 2002; Marks et al., 2001).

The steady-state level of histone acetylation is regulated by the opposing activities of histone acetyltransferases (HATs) (Marmorstein and Roth, 2001; Roth and Allis, 1996) and histone deacetylases (HDACs) (de Ruijter et al., 2003). Hyperacetylation of histones by HATs results in transcriptional activation, while histone hypoacetylation by HDACs results in transcriptional repression. Both HATs and HDACs form multi-protein complexes that activate or repress transcription, respectively.

There are three classes of histone deacetylases (HDACs): class I HDACs (HDAC1, -2, -3, and -8), class II HDACs (HDAC4, -5, -6, -7, and -9), and class III

HDACs or sirtuin family (Sirt proteins). The class I HDACs are expressed ubiquitously, while the class II HDACs are expressed in a tissue enriched manner (reviewed in (McKinsey et al., 2002b)). The class I HDACs are smaller than then class II HDACs and are composed almost entirely of an HDAC domain (Fig. 5.3). HDAC1 (initially termed HD1) was the first histone deacetylase to be identified and cloned. HDAC1 was purified by affinity chromatography using a modified HDAC inhibitor for affinity purification (Taunton et al., 1996). Shortly thereafter, HDAC2 was identified as an YY1 interacting protein (Yang et al., 1996). HDAC3 was subsequently cloned by three independent groups through sequence comparison (Dangond et al., 1998; Emiliani et al., 1998; Yang et al., 1997). Of the class I HDACs, HDAC1 and HDAC2 share the highest homology with 75% identical DNA sequence and 85% identical protein sequence. HDAC3, however, shares only 50% DNA sequence and 53% protein sequence identity to HDAC1 (Cress and Seto, 2000).

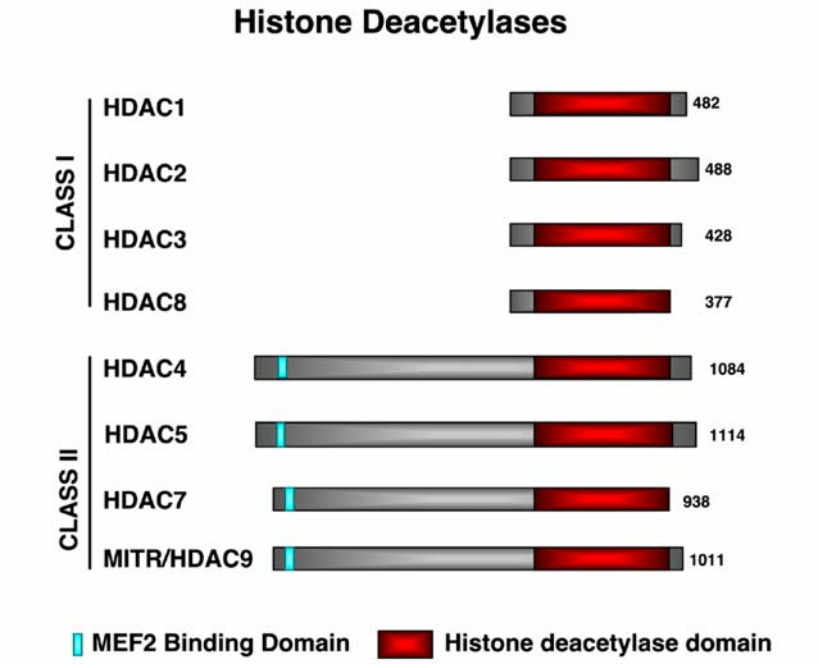

Figure 5.3. Class I and II histone deacetylase (HDAC) proteins.

Class I and II HDAC family domain structure. Class I HDACs are almost completely comprised of a deacetylase domain (red), while class II HDACs possess an N-terminal extension containing a MEF2 binding domain (blue).

Class I and class II HDAC proteins possess a conserved HDAC domain. However, class I HDACs possess deacetylase activity, while class II HDACs do not (Fischle et al., 2001; Fischle et al., 2002). Nevertheless, class II HDACs can recruit class I HDACs, and thus deacetylase activity, through their deacetylase domain (Fischle et al., 2002). The catalytic HDAC domain is ~390 amino acids and the deacetylase activity occurs in a small conserved pocket which removes acetyl groups through a charge-relay system consisting of two adjacent histidines, two aspartic acid residues, and a tyrosine residue (Finnin et al., 1999; Hassig et al., 1997). This charge-relay system is absolutely dependent on the presence of a zinc ion in the pocket of the deacetylase domain. Deacetylase inhibitors function by displacing this zinc ion and thereby disrupting the charge-relay system. The HDAC inhibitor trichostatin A (TSA), for example, fits into the active site of HDAC proteins and interacts with many of the same critical residues as the zinc atom (Finnin et al., 1999).

Based on sequence identity, it is not surprising that HDAC1 and HDAC2 interact with many of the same proteins, while HDAC3 appears to interact with and form unique transcriptional repression complexes. It is well documented that HDAC enzymes seldom act alone. HDAC proteins alone do not possess HDAC activity, but require post-translational modifications and interacting proteins to possess activity. Since HDAC proteins cannot bind DNA, they require other proteins for recruitment to appropriate target genes (de Ruijter et al., 2003).

HDAC1 and HDAC2 can homo- and hetero-dimerize (Hassig et al., 1998), and can participate in several different repression complexes including the Sin3, NuRD (nucleosome remodeling and deacetylating), and Co-REST complexes (reviewed in (Cress and Seto, 2000; de Ruijter et al., 2003; Grozinger and Schreiber, 2002)). HDAC3, on the other hand, interacts with SMRT (silencing mediator for retinoic acid and thyroid hormone receptors) and N-CoR (nuclear receptor co-repressor) to repress gene expression (Guenther et al., 2001).

While transcription co-repressors are necessary for HDAC activity, post-transcriptional regulation of class I HDACs are also necessary for their function. In the case of HDAC1 and HDAC2, both activity and complex formation is regulated by phosphorylation. Hyper-phosphorylation results in increased HDAC activity, with decreased complex formation, and vice versa, in order to maintain optimal HDAC activity levels. In the case of HDAC1, Ser^{421} and Ser^{423} are sites required for phosphorylation for HDAC activity (Cai et al., 2001; Galasinski et al., 2002; Pflum et al., 2001). HDAC3 is not only regulated by phosphorylation (Zhang et al., 2005), but also by heat shock proteins for proper folding, and interaction with SMRT for protein stability and deacetylase activity (Guenther et al., 2001; Guenther et al., 2002). In the absence of SMRT, HDAC3 is inactive.

While the molecular function and activity of class I HDACs is beginning to be understood, the biological and physiological function of these proteins, however, in different tissues and different developmental time points, remains to be determined. In *S. cerevisiae*, null alleles of the *RPD3* gene (HDAC1 orthologue) are viable but display a number of phenotypes (Dora et al., 1999; Kadosh and Struhl, 1997; Vidal and Gaber,

1991). However, global deletion of HDAC1 in all other species examined results in lethality. Potential insight into HDAC1 function came from *Drosophila* studies in which mutation of HDAC1 resulted in disruption of suppression of variegation (*Su(var)*), demonstrating a role for HDAC1 in maintaining heterochromatin (Mottus et al., 2000). Additionally, dominant negative mutations of HDAC1 resulted in more severe phenotypes, which may result from the "poisoning" of the deacetylase complex and prevents potential proteins like HDAC2 from compensating for loss of HDAC1 (Mottus et al., 2000). While the Mottus *et al.* studied suggested a role for HDAC1 as a global transcriptional regulator, microarray analysis of HDAC1 function in *Drosophila* S2 cells suggests HDAC1 regulates a specific subset of genes involved in metabolism, development, and protein biosynthesis (Cho et al., 2005).

In *C. elegans*, a catalytically inactive HDAC1 mutant impairs cell invasion (Whetstine et al., 2005). By performing microarray analyses, it was determined that HDAC1 regulates tissue specific and extracellular matrix genes. Surprisingly, despite being considered a global transcriptional repressor, HDAC1 appears to affect a limited number of genes (~2.2% in *C. elegans*) (Whetstine et al., 2005). This is supported by yeast (Bernstein et al., 2000) and human cell lines studies (Van Lint et al., 1996) which report that loss of HDAC1 and/or class I HDAC activity results in mis-expression of a relatively small percentage of genes (~6% and 2%, respectively).

In zebrafish, HDAC1 mutants display an array of abnormalities including hindbrain development, oligodendrocyte specification, cardiovascular defects, and embryonic lethality (Cunliffe, 2004; Cunliffe and Casaccia-Bonnefil, 2006; Golling et al.,

2002). These cardiovascular defects include pericardial oedema, no heart valve, and a weakly beating heart (Cunliffe, 2004).

In mice, loss of *Hdac1* results in embryonic lethality before embryonic day (E) 10.5 due to proliferation defects from increased levels of the cyclin dependent kinase inhibitors p21 and p27 (Lagger et al., 2002). In addition, loss of *Hdac1* resulted in upregulation of other class I HDACs (*Hdac2* and *Hdac3*), which were unable to compensate for loss of *Hdac1* (Lagger et al., 2002). While HDAC1 and HDAC2 may not be completely redundant, tissue specific deletion of *Hdac1* in all tissues examined (heart, brain, muscle, neural crest, bone, endothelial cells), using a floxed *Hdac1* allele, is viable, whereas deletion of both *Hdac1* and *Hdac2* in these tissues results in lethality. Interestingly, one copy of *Hdac1* or *Hdac2* appears to be sufficient for viability (Montgomery et al., 2007). In contrast, other studies have attributed non-redundant functions for HDAC1 and HDAC2, demonstrating roles for HDAC2 independent of HDAC1 (Huang et al., 2005). However, these latter studies were performed *in vitro*, not *in vivo*.

The roles of HDAC2 and HDAC3 *in vivo* are much less characterized compared to HDAC1. One group reports that *Hdac2* null mice are largely viable and are blunted to hypertrophy (Trivedi et al., 2007). However, we found that *Hdac2* null mice are born, breathe, sometimes feed, but are always lethal at post-natal day 1 (P1) (Montgomery et al., 2007). Additionally, we found that conditional deletion of *Hdac2* specifically in cardiomyocytes does not blunt cardiac hypertrophy in response to hypertrophic signals (Montgomery et al., 2007). While the discrepancies in these results remain unclear, Trivedi *et al.* used a LacZ insertion line, which frequently display variable phenotypes

due to alternative splicing within the mutant locus (Voss et al., 1998). Thus, it is conceivable that the mutation described by Trivedi *et al.* is a hypomorphic allele and not a true null, which provides sufficient levels of *Hdac2* for viability. The role of *Hdac3 in vivo* has not been explored.

Class I HDACs in Cardiac Hypertrophy

Transcriptional activators and repressors regulate gene expression in response to internal and external signals. In eukaryotic cells, this transcriptional regulation is largely influenced by DNA accessibility. During transcriptional activation of genes, histone tails are hyperacetylated by histone acetyltransferases (HATs). Acetylation offsets the positive charge of lysine residues, which destabilizes internucleosomal interactions and allows DNA accessibility and interaction with transcription factors (Grozinger and Schreiber, 2002; Marks et al., 2001). Conversely, histone deacetylases (HDACs) remove acetyl groups from histone tails, causing chromatin condensation and transcriptional repression (Grozinger and Schreiber, 2002).

Class I HDACs are well-known for their role in the progression of various cancers (Insinga et al., 2005a; Insinga et al., 2005b). HDAC inhibitors are currently in clinical trials for treatment of an array of cancers (Drummond et al., 2005), and have also been shown to ameliorate a number of pathologies including muscle dystrophy (Minetti et al., 2006), atrophy (Tsai et al., 2006), mental disorders, and many more. Recently, class I HDACs have been proposed to play a role in cardiac hypertrophy, which is an increase in myocyte size, not myocyte number. Interestingly, pathways that regulate cell

proliferation have been proposed to regulate the control of cardiac hypertrophy (Hoshijima and Chien, 2002).

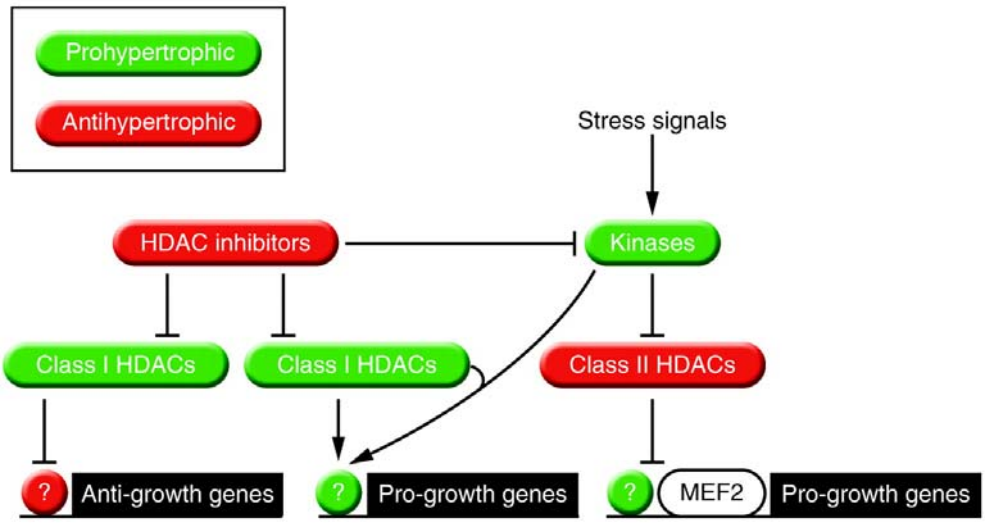

Figure 5.4. Model for class I HDAC regulation of cardiac hypertrophy. Potential roles of class I and II HDACs in cardiac myocytes. Class II HDACs repress hypertrophy, and are repressed by signal-induced phosphorylation by specific kinases. Class I HDACs appear to be prohypertrophic based on the ability of HDAC inhibitors to prevent hypertrophy *in vitro* and blunting of hypertrophy *in vivo*. Class I HDACs could act by inhibiting prohypertrophic signaling pathways, by inhibiting the expression of growth inhibitory genes or by activating the expression of pro-growth genes (McKinsey and Olson, 2005).

Since class II HDACs are involved in repressing cardiac hypertrophy, studies were performed to analyze the effect of total HDAC inhibition on cardiomyocyte growth and hypertrophy. Surprisingly, HDAC inhibition resulted in a complete blockade to signal induced hypertrophy in cardiomyocytes *in vitro* (Antos et al., 2003). Further support for class I HDAC involvement in cardiac hypertrophy came from additional studies demonstrating that HDAC inhibition *in vivo*, with an HDAC1 and HDAC2 specific inhibitor, could blunt hypertrophy (Kook et al., 2003)(Kee et al., 2006; Kong et al., 2006). Moreover, *Hdac2* mutant mice were reported to blunt cardiac hypertrophy

(Trivedi et al., 2007). However, we recently showed that cardiac-specific deletion of *Hdac1* or *Hdac2* alone does not blunt cardiac hypertrophy *in vivo*, and that *Hdac1* and *Hdac2* are functionally redundant in the heart (Montgomery et al., 2007). Together, these studies suggest that class I HDACs are prohypertrophic and HDAC inhibitors antagonize this function (Fig. 5.4).

Since inhibition of class I HDACs blunts cardiac hypertrophy, then overexpression of class I HDACs would be predicted to induce or promote cardiac hypertrophy. To address this question, we generated transgenic mice that overexpress HDAC1 or HDAC3 specifically in the heart, using the *alpha myosin heavy chain* (*αMHC*) promoter. Here we show that *αMHC*-HDAC1 transgenic mice display significant cardiac abnormalities which were not observed in *αMHC*-HDAC3 transgenic mice. *αMHC*-HDAC1 transgenics display both right and left ventricular failure from myocardial defects, which results in dilated cardiomyopathy and sudden death. *αMHC*-HDAC3 transgenic mice, on the other hand, display a less severe, stress-induced cardiomyopathy. Interestingly, overexpression of deacetylase dead HDAC (H140,141A) mutants mimic the phenotype of wild-type transgenic mice, which demonstrates deacetylase activity is not required for the phenotypes. Combined with previous studies, these results demonstrate an important role for class I HDACs in regulating normal cardiac function and disease progression.

Results

Cardiac-specific overexpression of HDAC1 results in lethality due to sudden cardiac death

Previous studies have demonstrated class I HDAC expression in the adult heart (Dangond et al., 1998; Mahlknecht et al., 1999), and we also detected class I HDAC expression embryonically by *in situ* hybridization (data not shown). To examine whether overexpression of a class I HDACs was sufficient to drive cardiac hypertrophy, we generated heart-specific HDAC1 and HDAC3 transgenic mice by cloning C-terminal Myc-tagged hHDAC1 and N-terminally Myc-tagged hHDAC3 under the control of the *α-myosin heavy chain* (αMHC) promoter. This promoter was previously shown to be expressed in differentiated cardiomyocytes pre- and postnatally (Agah et al., 1997). HDAC2 transgenic mice have been described previously (Trivedi et al., 2007).

Multiple *αMHC*-HDAC1 and *αMHC*-HDAC3 transgenic lines that overexpress HDAC1 or HDAC3 specifically in the heart were obtained and analyzed. Cardiac-specific overexpression of HDAC1 resulted in early lethality from sudden cardiac death at different time points, which was dependent on transgene expression level. *αMHC*-HDAC3 transgenic mice, however, did not display sudden cardiac death. *αMHC*-HDAC1 transgenic founders (F0) often perished prior to breeding from cardiac defects, so obtaining and maintaining stable lines was difficult. Two *αMHC*-HDAC1 transgenic stable lines that were able to be generated are described in detail in Fig. 5. Offspring (F1) from transgenic line 1 (T1) died at 33-37 days of age, while offspring from transgenic line 2 (T2) died at 15-18 days (Fig. 5.5A). HDAC1 transgenic mice from T2 exhibited a higher transgene expression level (Fig. 5.5B) than T1, which suggests the severity of the phenotype is dose-dependent. Mice from T1 displayed tachypnea, or rapid, shallow breathing prior to heart failure, while mice from T2 died immediately from sudden cardiac death.

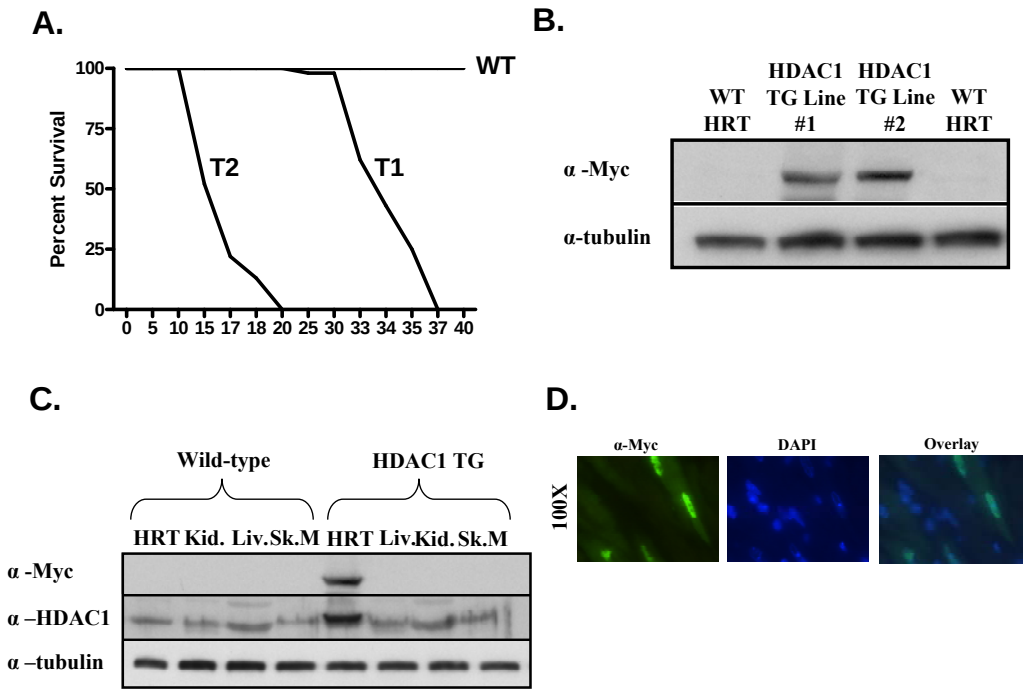

Figure 5.5. Generation of αMHC-HDAC1 transgenic mice.
A. Survival curves for two αMHC-HDAC1 transgenic lines. X-axis indicates number of days survived. Increased HDAC1 expression decreases survival time. B. Relative transgene expression between transgenic lines 1 and 2 using an α-Myc antibody. Tubulin antibody indicates equal loading. C. Heart-specific transgene expression as revealed by anti-Myc and –HDAC1 antibodies. D. Immunohistochemistry of αMHC-HDAC1 transgenic hearts with an anti-Myc antibody demonstrates nuclear localization.

As shown in Fig. 1C, transgene expression was observed specifically in the heart approximately eight fold higher than wild-type mice as measured by densitometry using an HDAC1 antibody. In addition, using an anti-Myc antibody HDAC1 expression was observed specifically in the nucleus (Fig. 5.5D).

*α*MHC-HDAC1 transgenic hearts from both T1 and T2 show gross morphological cardiac defects including enlarged atria, thrombosi, and ventricular malformations, the latter which were more apparent in line T1 (Fig. 5.6A) and in numerous F0 mice (data not

shown). Four-chamber histological analysis revealed extremely dilated ventricles, and massively dilated atria with thrombosi (Fig. 5.6B). However, these hearts did not appear hypertrophic. We therefore analyzed the expression of hypertrophic markers (BNP, alpha skeletal actin), markers of heart failure (αMHC to βMHC), and putative HDAC1 target genes (*e.g.*, p21). As shown in Fig. 2C, p21 levels are significantly reduced in *αMHC*-HDAC1 transgenic hearts, while hypertrophic markers were not increased in *αMHC*-HDAC1 transgenic hearts (Fig. 5.6C). However, p21 has been implicated as a repressor of hypertrophy (Li and Brooks, 1997; Tamamori et al., 1998), so reduced p21 levels may be involved in the myopathic phenotype.

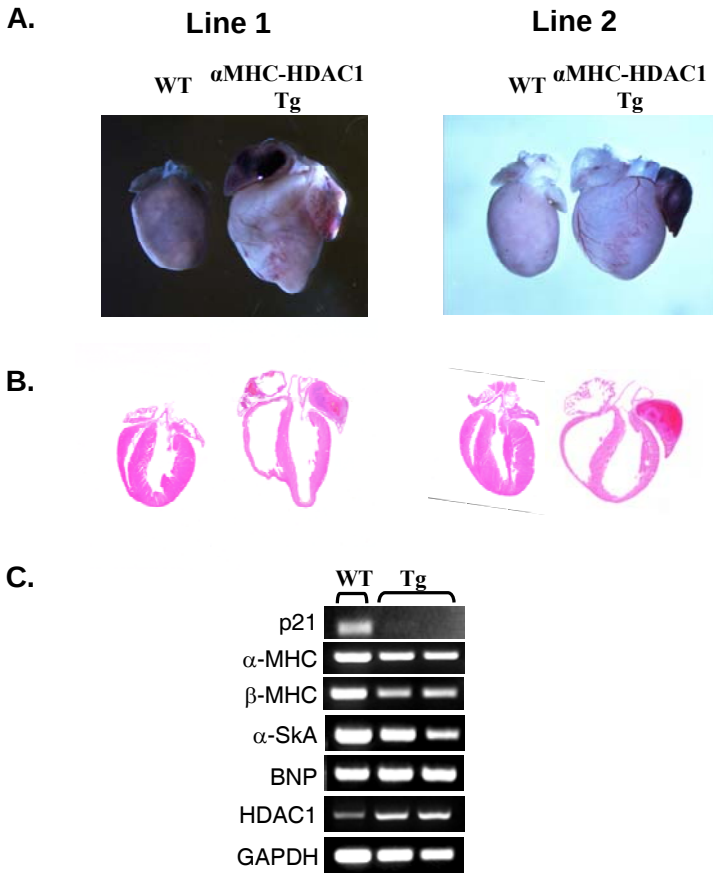

Figure 5.6. Cardiac-specific overexpression of HDAC1 causes a dilated cardiomyopathy.

A. □*MHC*-HDAC1 transgenic hearts appear larger, dilated, and thrombosed compared to wild-type littermates. B. H&E sections of hearts in A, reveals significantly dilated ventricles. C. RT-PCR for hypertrophic markers and putative HDAC1 targets reveals decreased levels p21 in HDAC1 transgenic hearts, but no increase in hypertrophic markers.

Higher magnification of H&E sections revealed myocytes of multiple sizes and shapes in *αMHC*-HDAC1 transgenic mice compared to wild-type controls (Fig. 5.7A). However, trichrome staining revealed no fibrosis in *αMHC*-HDAC1 transgenic hearts (Fig. 5.7A). Notably, many *αMHC*-HDAC1 transgenic myocytes possess nuclear inclusions (Fig. 5.7B), which are not seen in HDAC2 or HDAC3 transgenic hearts. *αMHC*-HDAC1 transgenic mice also show signs of right and left ventricular failure, as demonstrated by liver and lung histology, which revealed cell apoptosis and hemorraging, and edema with macrophage infiltration, respectively (Fig. 5.7C).

Since *αMHC*-HDAC1 transgenic mice die at a relatively early age, we were unable to perform echocardiogram analysis of these hearts. However, to determine whether the *αMHC*-HDAC1 transgenic phenotype is caused by arrhythmias, electro-cardiograms (ECG) were performed on *αMHC*-HDAC1 transgenic and age matched littermates. As shown in Fig. 5.7D, *αMHC*-HDAC1 transgenic hearts do not possess arrhythmias.

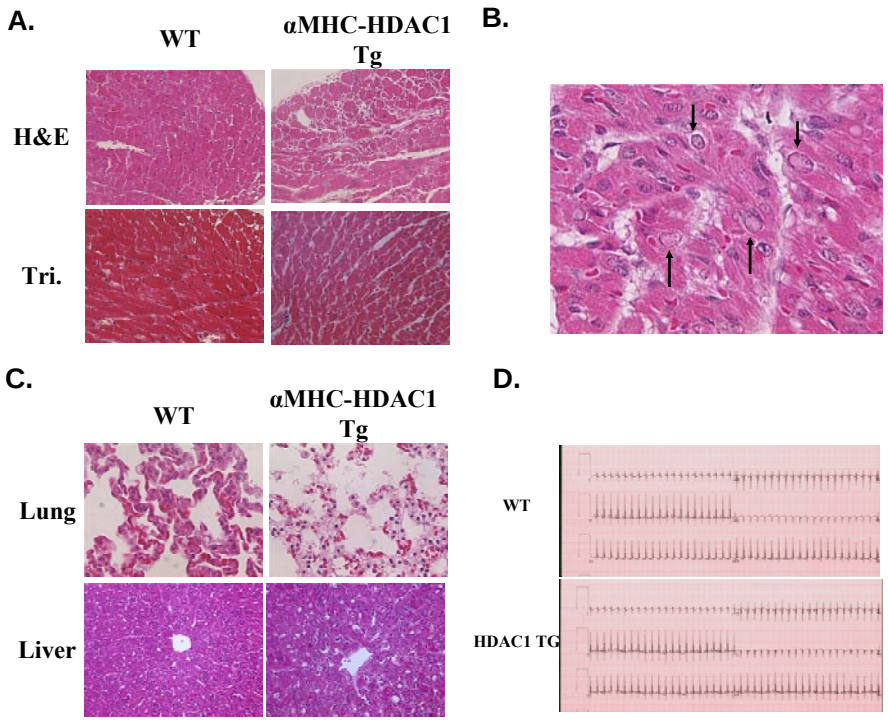

Figure 5.7. Characterization of HDAC1 transgenic mice.
A. H&E and trichrome staining of histological sections from wildtype (WT) and HDAC1 transgenic hearts (20X mag.). B. Arrows indicate nuclear inclusions in HDAC1 transgenic hearts. C. Lung and liver histology from □*MHC*-HDAC1 transgenic mice indicate left and right ventricular failure, respectively. D. Electrocardiograms from WT and HDAC1 transgenic mice.

Since HDAC1 was previously shown to regulate extracellular matrix genes (Whetstine et al., 2005), we wanted to examine whether misregulation of collagen proteins could be the cause of lethality, since changes in collagen ratios have been associated with dilated cardiomyopathy (Pauschinger et al., 1999). While trichrome staining did not reveal significant fibrosis (Fig. 5.7A), picrosirius staining revealed a significant upregulation of collagen proteins in *αMHC*-HDAC1 transgenic right and left ventricles (Fig. 5.8A). This increase in collagen proteins could result in cardiac rigidity, impairing cardiac contractility, and explain the right and left ventricular failure observed in these mice. Real-time analysis of collagen and extracellular matrix regulatory

enzymes revealed an overall increase in collagen proteins with an increase in collagen I and IV mRNA and a decrease in matrix metalloproteinases (MMPs) (Fig. 5.8B).

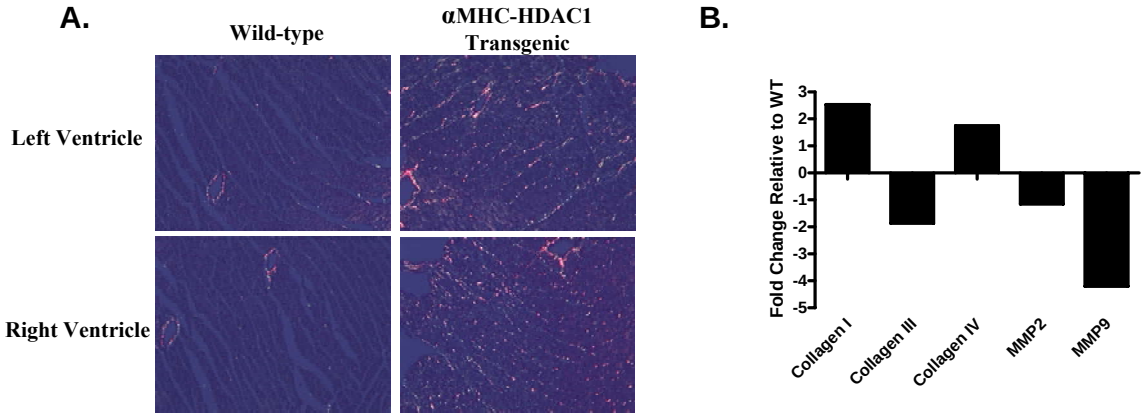

Figure 5.8. Increased collagen deposition in *αMHC*-HDAC1 transgenic hearts.
A. Picrosirius red staining of histological sections from wild-type and *αMHC*-HDAC1 transgenic hearts (20X mag.) B. Real-time PCR analyses of collagen and matrix metalloproteinase proteins expression in wild-type and HDAC1 transgenic hearts reveals in overall increase in collagen deposition.

To determine whether the *αMHC*-HDAC1 transgenic phenotype is dependent on deacetylase activity, we generated "deacetylase dead" HDAC1 transgenic mice (Fig. 5.9A). HDAC1 deacetylase activity was abolished by mutating 2 key histidine residues, necessary for the deacetylase charge-relay system, to alanines (H140,141A). Mutations of these residues was shown previously to abolish HDAC1 deacetylase activity without disrupting interaction with important co-repressor proteins (Hassig et al., 1998). Surprisingly, *αMHC*-HDAC1 deacetylase dead transgenic hearts mimic the wild-type HDAC1 transgenic phenotype, including dilated left atria, abnormal histology, and lethality at a similar time (Fig. 5.9B and data not shown). Moreover, consistent with *αMHC*-HDAC1 transgenic mice, deacetylase dead mice display a dilated heart, not a

hypertrophic heart. Therefore, we conclude that HDAC activity is not required for the observed phenotype of *αMHC*-HDAC1 transgenic mice.

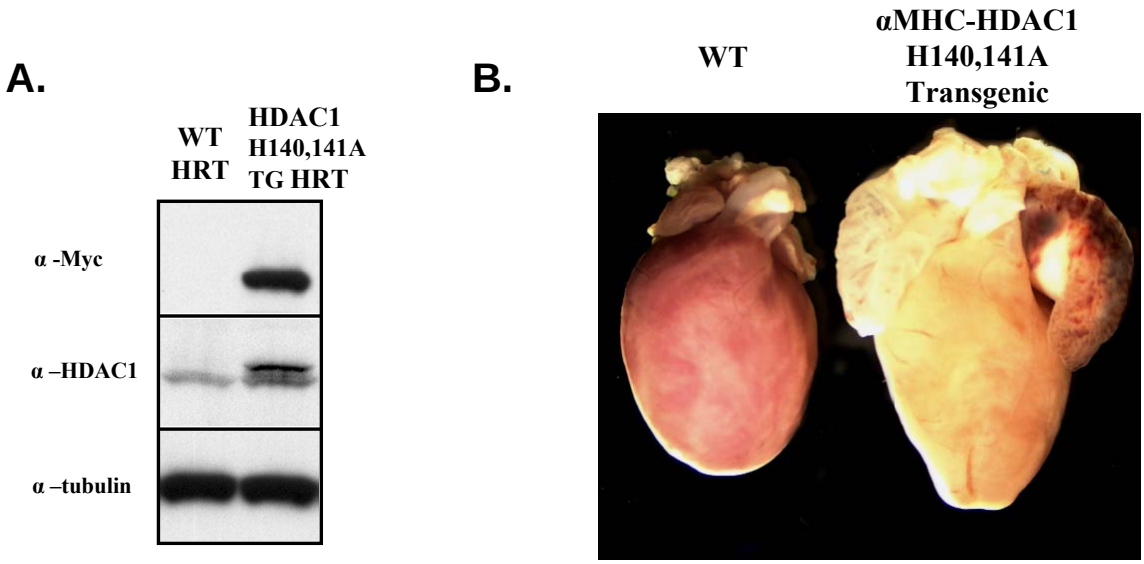

Figure 5.9. HDAC1 deacetylase activity is not required for cardiac defects. A. Transgene expression in □*MHC*-HDAC1 deacetylase dead (H140,141A) transgenic mice as assayed with □-Myc and -HDAC1 antibodies. Tubulin demonstrates equal loading. B. Picture of a HDAC1 deacetylase dead transgenic heart compared to a wild-type (WT) littermate.

Cardiac-specific overexpression of HDAC3 results in stress-induced cardiomyopathy

Histone deacetylase 3 (HDAC3) is a transcriptional repressor that is associated with the repression of nuclear hormone receptors. To examine the role of HDAC3 in the heart and to examine whether it is sufficient to drive cardiac hypertrophy, we generated *αMHC*-Myc-HDAC3 transgenic mice. Expression of the HDAC3 transgene was observed specifically in the heart (Fig. 5.10A), and in both males and females (Fig. 5.10B). In addition, analysis of HDAC3 localization in *αMHC*-HDAC3 transgenic hearts with an anti-Myc antibody revealed nuclear specific staining (Fig. 5.10C).

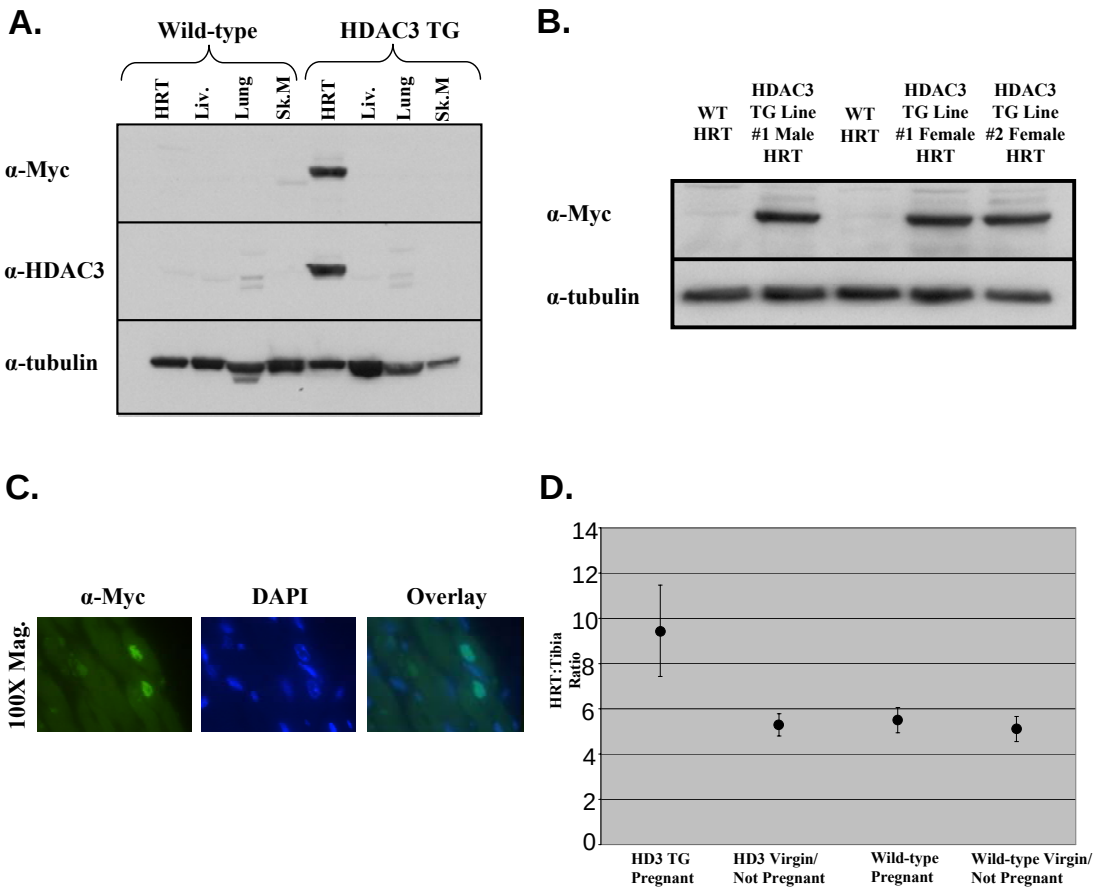

Figure 5.10. Generation of -*MHC*-HDAC3 transgenic mice.
A. HDAC3 transgene expression was observed specifically in the heart using an -Myc and –HDAC3 antibody. HDAC3 transgene expression in both males and females of multiple transgenic lines. C. Nuclear localization of Myc-HDAC3 in HDAC3 transgenic hearts. D. Hearts from pregnant HDAC3 transgenic mice show a significant heart:tibia ratio, but not virgin HDAC3, wild-type pregnant, and wild-type virgin females.

Interestingly, HDAC3 transgenic females die from cardiac defects and display signs of heart failure (Fig. 5.10D and 5.11D), but males appear unaffected. In fact, HDAC3 transgenic males and HDAC3 transgenic virgin females live and behave exactly like wild-type littermates, but pregnant HDAC3 transgenic females display cardiomegdaly and subsequent lethality (Fig. 5.11A and B). After two pregnancies, the majority of HDAC3 transgenic females die. Heart to body ratios for wild-type virgin,

wild-type pregnant, HDAC3 transgenic virgin, and HDAC3 transgenic pregnant females are listed in Fig. 5.10D. Unexpectedly, pregnant HDAC3 transgenic females possess dilated hearts (Fig. 5.11B), but HDAC3 transgenic females that have given birth display hypertrophic left ventricles with severely thrombosed atria (Fig. 5.11C). Histological analysis of HDAC3 transgenic females revealed normal sized myocytes, and trichrome staining showed significant fibrosis of both the right and left atria, suggesting a progressive defect (data not shown). Notably, phospho-H3 and Tunel staining revealed no alteration in myocyte proliferation or apoptosis (data not shown).

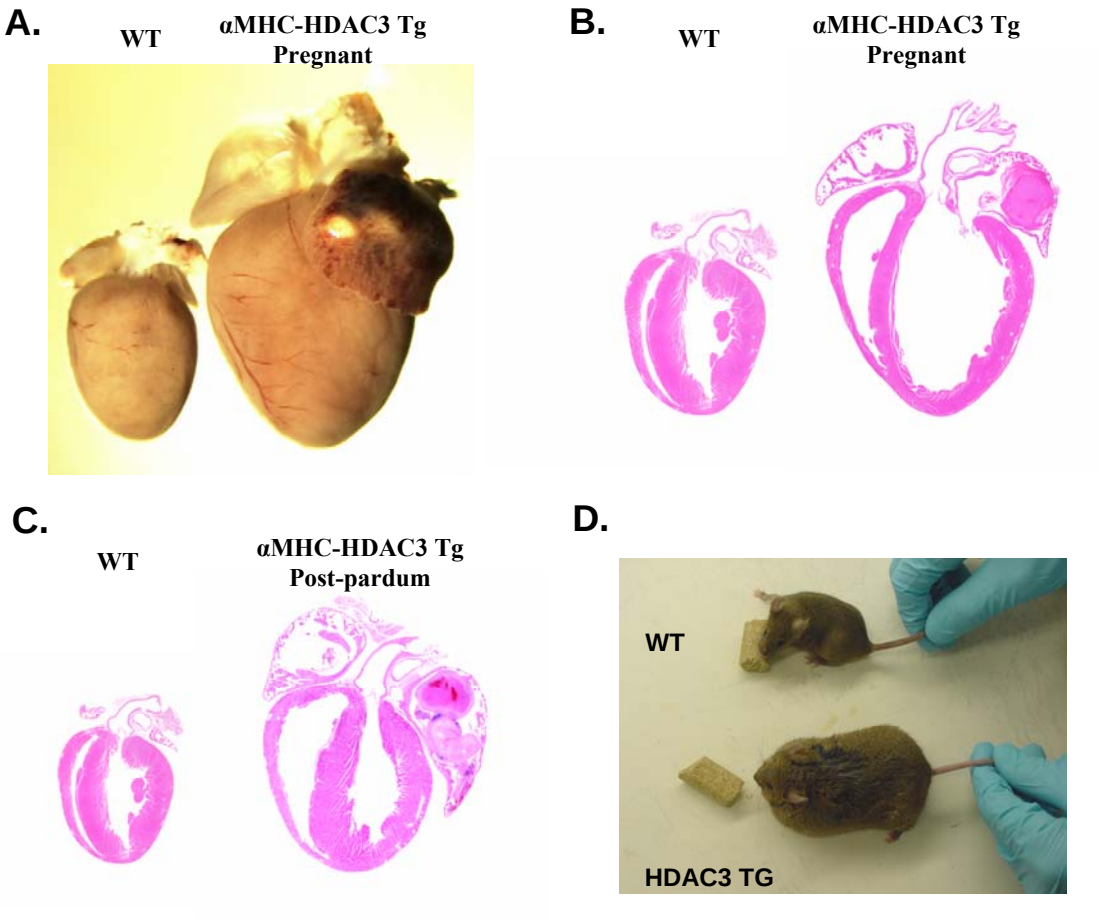

Figure 5.11. Cardiomegdaly in αMHC-HDAC3 transgenic mice.
A. Gross pictures of αMHC-HDAC3 transgenic hearts from pregnant females. B. H&E section of hearts in A. C. H&E section of αMHC-HDAC3 transgenic hearts

from post-partum females. D. Heart failure observed in a HDAC3 transgenic female after being pregnant.

Since HDAC3 transgenic females display a heart phenotype and males appear normal, we wanted to examine whether this phenotype may be due to disruption of estrogen signaling, especially since estrogen has been shown to be cardio-protective (Murphy and Steenbergen, 2007; Nikolic et al., 2007) and HDAC3 represses estrogen signaling (Liu and Bagchi, 2004). Analysis of estrogen receptor alpha (ERα) and beta (ERβ), as well as estrogen receptor target genes (Cox7, TERT, pS2, EFP) displayed normal expression in HDAC3 transgenic virgin and pregnant females compared to wild-type littermates (data not shown).

An alternative is that HDAC3 transgenic hearts are susceptible to stress and that pregnancy-induced stress results in dilation, explaining the observed gender-specific phenotype. To test this hypothesis, we stressed HDAC3 transgenic males by thoracic aortic banding (TAB) to analyze whether they display cardiomegdaly after stress. As shown in Fig. 5.12, several HDAC3 transgenic males that were subjected to TAB displayed increased heart to body ratios and a phenotype remarkably similar to HDAC3 transgenic pregnant females. Thus, *αMHC*-HDAC3 transgenic hearts are susceptible to physiological stress.

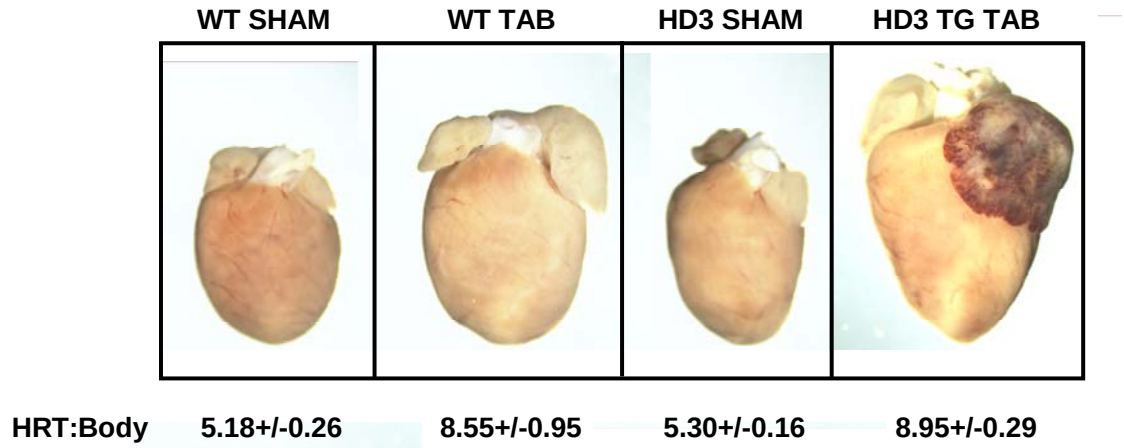

Figure 5.12. HDAC3 transgenic males are sensitized to stress.
Wild-type and αMHC-HDAC3 transgenic males were subjected to thoracic aorta banding (TAB). HDAC3 transgenic hearts do not become hypertrophic, but rather dilated and display a characteristic dilated and thrombosed left atria.

To determine whether the observed phenotype was dependent on histone deacetylase activity, we generated "deacetylase dead" αMHC-HDAC3 transgenic mice (Fig. 5.14A). As described earlier, deacetylase activity was abolished by mutating two key histidine residues necessary for deacetylase activity. As in the case of HDAC1, HDAC3 deacetylase dead transgenic mice mimicked the phenotype of wild-type HDAC3 transgenic mice (Fig. 5.12). These results reveal that deacetylase activity is not required for the HDAC3 gain-of-function phenotype and demonstrate an important role for HDAC3 in cardiac growth and remodeling.

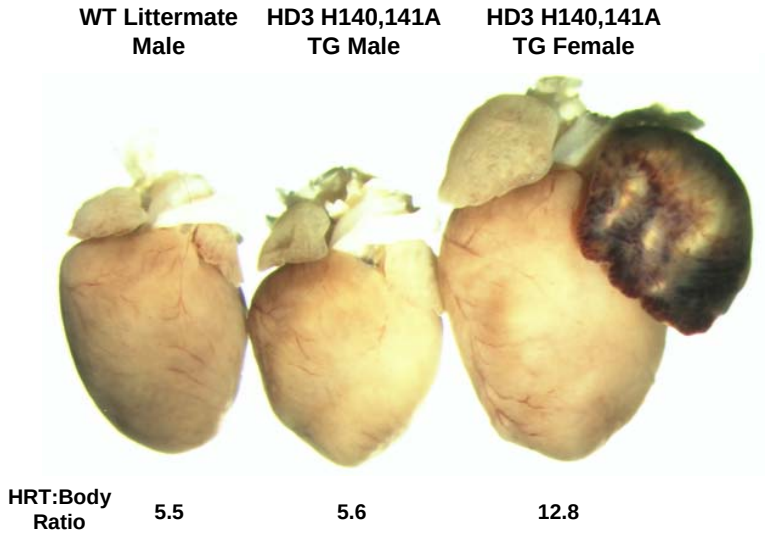

Figure 5.13. HDAC3 deacetylase activity is not required for cardiac defects. HDAC3 deacetylase dead transgenic hearts mimic the phenotype of wild-type HDAC3 transgenic hearts. □*MHC*-HDAC3 transgenic females display an enlarged heart with dilated and thrombosed left atria (when pregnant), while males appear unaffected.

Discussion

Transcriptional events regulated by histone deacetylases (HDACs) are important components involved in pathological cardiac remodeling. While class II HDACs are well-established stress-induced regulators of hypertrophy, recent studies have demonstrated the importance of class I HDACs in blunting hypertrophy. Here we show that overexpression of different class I HDACs, HDAC1 and HDAC3, which are predicted to develop unique repression complexes, results in distinct cardiac phenotypes. Cardiac specific overexpression of HDAC1 results in a dilated cardiomyopathy, followed by heart failure due to an increase in rigidity from increased collagen expression. HDAC3 transgenic hearts, however, do not display cardiac defects until stressed. Pregnancy induced cardiac stress results in dilation, followed by post-partum cardiac

hypertrophy. Interestingly, deacetylase activity is not required for either cardiac defect. These studies demonstrate the importance of class I HDACs in cardiac remodeling and establish functional differences between these two repression complexes on cardiac handling.

Contrary to our hypothesis, overexpression of class I HDACs fails to promote cardiac hypertrophy, but rather led to cardiac dilation and failure. A possible explanation for these observations may be similar to the rationale for MEF2 transgenic mice. As discussed in Chapter II, MEF2 transgenic mice do not display cardiac hypertrophy, despite extensive research demonstrating their role in this process. Perhaps overexpression of MEF2 or class I HDACs is such a powerful pathological stimulus that it drives the heart directly to failure, rather than through the typically gradual and graded response in which hypertrophic growth precedes dilative remodeling.

Histone deacetylase activity was previously reported to be necessary for HDAC1 repression in the Sin3a complex (Hassig et al., 1997). Our results, however, demonstrate that HDAC1 and HDAC3 do not require histone deacetylase activity for target gene repression. Since HDAC1 and HDAC2 are redundant in the heart (Montgomery et al., 2007), and HDAC1 and HDAC2 exist in the same repression complexes, it is conceivable that HDAC2 could compensate for loss of HDAC1 deacetylase activity, thereby providing a gain-of-function phenotype. However, HDAC3 does not have a redundant histone deacetylase, and loss of HDAC3 deacetylase activity does not alter the cardiac phenotype. Therefore, it is likely that overexpression of wild-type or deacetylase dead class I HDACs, results in an increase in repression complexes, which together with other transcriptional repressors, are sufficient to repress target genes.

While class I HDAC proteins play a critical role in transcriptional regulation, it has become clear that HDAC proteins regulate many additional cellular processes by deacetylating non-histone proteins. For example, deacetylation of Rb, p53, and many other proteins has been shown to regulate cell-cycle progression. A list of acetylated proteins and the functional consequence of this modification is described elsewhere (Drummond et al., 2005). Additionally, a recent study reported that over 195 proteins were acetylated on over 388 sites (Kim et al., 2006b). A very interesting conclusion from our study is that the observed phenotype is transcriptionally regulated, not the result of a non-histone protein modification. Since the deacetylase dead HDAC transgenics mimic the wild-type transgenic phenotypes, and since these HDAC mutants can still form transcriptional repression complexes (Hassig et al., 1998) but not deacetylate non-histone proteins, this demonstrates that loss of deacetylation of non-histone proteins does not contribute to the observed phenotypes.

Numerous studies involving HDAC inhibitors and class I HDAC knockout mice have demonstrated the importance of these proteins in cardiac pathology. Interestingly, an inhibitor of HDAC1 and HDAC2 is able to blunt hypertrophy (Kee et al., 2006; Kong et al., 2006; Kook et al., 2003), but genetic deletion of *Hdac1* or *Hdac2* alone in the heart does not blunt hypertrophy (Montgomery et al., 2007). In addition, cardiac-specific deletion of both *Hdac1* and *Hdac2* results in severe cardiac abnormalities, tachardia, and lethality by P14 (Montgomery et al., 2007). Therefore, inhibition of HDAC activity does not constitute a loss of HDAC function and demonstrates that HDACs still possess functions independent of HDAC activity. Elucidating the mechanisms and targets of

HDAC inhibitors presents an important challenge for generating new and specific targets to treat pathological cardiac diseases.

Materials and Methods

Generation of Transgenic Mice: Cardiac-specific transgenic mice were generated by cloning C-terminal Myc-tagged hHDAC1 and N-terminally Myc-tagged hHDAC3 under the control of the *α-myosin heavy chain* (αMHC) promoter (Gulick et al., 1991). Transgenic mice were generated as previously described (Cheng et al., 1993).

RT-PCR and Quantitative Real-time PCR: Total RNA was extracted from wild-type and transgenic hearts with Trizol reagent (Invitrogen). Four micrograms of RNA from each sample was used to generate cDNA using SuperScript II First-Strand Synthesis kit (Invitrogen). RT-PCR was performed using intron spanning primers for the indicated target. Primer sequences are available upon request. Quantitative real-time PCR was performed for indicated genes using SYBR Green (Applied Biosystems).

Histology, Immunohistological Analysis, and Staining: Tissues for histology were isolated in PBS, fixed in 4% paraformaldye overnight, processed and sectioned for hematoxylin and eosin (H&E), trichrome, or picrosirius red staining using standard procedures (Shelton et al., 2000). For immunohistological analyses, paraffin sections were stained with an anti-Myc antibody, followed by a FITC-labeled goat-anti-rabbit secondary antibody.

Thoracic Aorta Banding: Six- to 8-week-old male mice either underwent a sham operation or were subjected to pressure overload induced by thoracic aorta banding (TAB) as described (Hill et al., 2000).

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VITAE

Matthew Potthoff was born in Carroll, Iowa, son of Douglas Potthoff and Theresa Potthoff, and elder brother to Bryan Potthoff. After completing his study at Pauls Valley High School in 1998, he attended the University of Oklahoma in Norman, Oklahoma. In June 2000, he joined the laboratory of Dr. James Thompson, jr. in the Department of Zoology for his honors thesis study. The title of his thesis was, "Patterns of locomotion activity during hypergravity in larval *Drosophila melanogaster*." He received his Bachelor of Science with highest honors, *Summa cum laude*, with a major in Zoology/Biology from the University of Oklahoma in May, 2003. On June 7th, 2003, he married Manda Jensen. In August of 2003, he entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas. He joined the laboratory of Dr. Eric Olson in Jan. 2004, where he received his molecular biology training. He graduated with a Ph.D degree in August 2007.