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BASAL SIGNALING THROUGH DEATH RECEPTOR 5 AND CASPASE 3 ACTIVATES P38 KINASE TO REGULATE SERUM RESPONSE FACTOR – MEDIATED MYOD TRANSCRIPTION

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December 1999

Submitted in partial fulfillment of requirements for the degree

DOCTOR OF PHILOSOPHY IN REGULATORY BIOLOGY

at the

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December, 2020

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DEDICATION

I suppose this is the place to write something exceedingly clever and inspirational for anyone who deigns to peruse this manuscript. Being neither a clever nor particularly inspired person, I had a hard time finding the right words for this section (and as you continue, you will discover that the right words have still eluded me).

I've always found it difficult to qualify anything I've done as an "accomplishment". The concept of accomplishment implies that imagination and desire have been married and give birth to a goal. This goal is then made the object of focus, and is shaped into possibility by dedication and hard work. Finally, continued focus, dedication and hard work transform this possibility into reality.

However, my journey – with respect to science and otherwise - has been slightly less romantic. Simply put: sometimes I get to do what I want to do… the rest of the time I do what I have to do. Luckily for me, what I wanted to do and what I had to do were at last one in the same, and so I simply couldn't remove myself from the obligation to achieve what I truly wanted. And I believe that this inability to quit is my only contribution to the opportunity for me to write these words here. Somewhat luckier for me is the fact that other people around me also offered their focus, dedication and hard work toward my goal; and so, this achievement, such as it is, is theirs as much as mine.

Thus, this document is dedicated to those people, about whom I will speak briefly in the next section.

ACKNOWLEDGEMENTS

I am thankful first and foremost to my mother, who taught me every truly important thing that I know, and to the God to Whom she introduced me. I am thankful to all my friends for becoming my family, and to my goddaughters, who have become my reason for facing the day. I am also thankful to my mentor, advisor, advocate and friend Dr. Crystal Weyman, without whom I obviously wouldn't have finished this, but also after whom I've patterned my thoughts and attitude.

I've been blessed by all of these people and also by others: faculty who have helped me along the way, coworkers and lab-mates that made it all bearable, family who have moved on… there is simply not enough room here to list them all. I am eternally humbled by their faith in me, and cannot have been more fortunate to have known them all.

BASAL SIGNALING THROUGH DEATH RECEPTOR 5 AND CASPASE 3 ACTIVATES P38 KINASE TO REGULATE SERUM RESPONSE FACTOR – MEDIATED MYOD TRANSCRIPTION

JASON A. ROSS

ABSTRACT

The formation of functional skeletal muscle is the consequence of both the differentiation and apoptosis of skeletal myoblasts. Ex vivo culture of skeletal myoblasts provides a tractable model for the study of these two coordinately regulated processes. We have previously reported that 23A2 myoblasts stably expressing a dominant negative Death Receptor 5 (A2:dnDR5 myoblasts) exhibit decreased basal mRNA and protein expression of the master muscle regulatory transcription factor MyoD. This decrease at the mRNA level is not a consequence of altered stability. Binding of the transcription factor SRF to a non-canonical CArG box within a serum response element (SRE) in the distal regulatory region (DRR) of the MyoD gene is required for basal MyoD expression. Herein, we report that A2:dnDR5 myoblasts exhibit a decrease in the amount of SRF bound at this CArG box. Additionally, in A2:dnDR5 myoblasts, we observe a decrease in the phosphorylation indicative of activation of SRF as well as a decrease in the phosphorylation indicative of activation of the mitogen-activated protein kinase p38, which is known to activate SRF. Pharmacological inhibition of p38, or of caspase-3, in parental 23A2 myoblasts mimics the decreased activation of SRF and p38, the decreased binding of SRF to the MyoD CArG box, and the decreased levels of MyoD mRNA and protein detected in the A2:dnDR5 myoblasts. Taken together, these results suggest that

basal signaling through DR5 to caspase 3 leads to the activation of p38 and subsequently SRF to maintain basal expression of MyoD

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CHAPTER I

INTRODUCTION

1.1 Skeletal Myogenesis

Skeletal myogenesis is an important process for the formation of functional muscle both during development of the mammalian embryo and in response to muscle injury in the adult animal. Myogenesis for the purpose of muscle repair involves the activation of quiescent satellite cells, entry into the cell cycle, proliferation, commitment, withdrawal from the cell cycle, differentiation and the terminal fusion of myoblasts and formation of multinucleated myotubes (**Figure 1**). Defects in myogenesis can lead to a number of debilitating myopathies, such as facio-scapulo-humeral and Duchenne muscular dystrophies, which are characterized by excessive myoblast apoptosis.[1] Improving the efficiency of myogenesis would increase the efficacy of muscle repair needed as a consequence of injury in otherwise healthy individuals and the efficacy of stem cell-like myoblast transfer therapies in individuals with genetically compromised skeletal muscle. Thus, a greater understanding of the mechanisms underlying myoblast survival and differentiation is warranted.

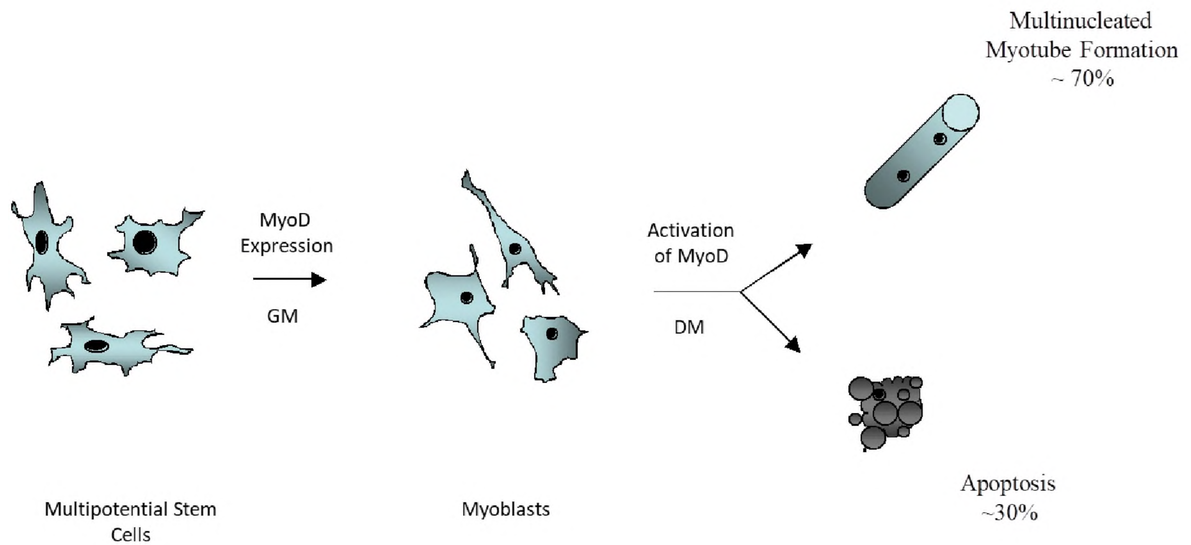

Figure 1. Skeletal Myogenesis Results in the Formation of Multinucleated Myotubes. Multipotential fibroblasts can differentiate into myoblasts upon the activation of muscle-specific factors such as MyoD. These myoblasts are maintained in a proliferative state by the presence of growth factors. When these growth factors are removed, a process which can be simulated by culture of the cells in media lacking fetal bovine serum (FBS), myoblasts move toward one of two mutually exclusive physiological endpoints; namely, the formation of multinucleated myotubes or apoptosis. Approximately 30% of a given population will undergo apoptosis while the rest undergo differentiation.

1.2 Regulation of Myogenesis by Transcription Factors

Investigation of the mechanisms and signaling pathways that control the myogenic program has revealed that the highly-conserved basic helix loop-helix (bHLH) muscle regulatory factor (MRF) family of transcription factors (consisting of Myf-5, MyoD, myogenin and MRF4) controls skeletal myogenesis. The first of these proteins to be identified was isolated in 1987 by a hallmark series of experiments[2] which began with treatment of murine C3H10T½ fibroblasts with 5-azacytidine, a potent histone deacetylase (HDAC) inhibitor, capable of facilitating promiscuous DNA transcription by altering chromatin structure in a way that allowed access to a variety of transcription factors previously limited in access to DNA promoter regions. These fibroblasts were specified into myoblasts at a frequency which indicated that only one or a few closely related regulatory loci controlled this transition. A myoblast-specific cDNA library was prepared from proliferating cells in order to compare with a differentiated myoblast-specific cDNA library for common factors. One of the three selected cDNA fragments, now referred to as MyoD, was capable of specification of fibroblasts - as well as a variety of other cell types - into myoblasts in a consistent fashion.[3,4,5] Further cDNA screening and other investigations over the subsequent three years revealed the remaining factors of the MRF family, which share homology with MyoD and are also capable of promoting progress of C3H10T½ fibroblasts to stable myoblasts, although it quickly became clear that the timing of the expression of these factors and the presence of serum in the growth media of transfected cells was critical to this process.[6]

The sequential expression of these MRFs control a highly orchestrated, multi-step process that begins with robust changes in protein expression in pleuripotent stem cells, largely controlled by transcriptional activation of muscle-specific genes. Myf-5 and MyoD are expressed early in the myogenic program, and control renewal of the satellite cell pool and progress toward cellular differentiation, respectively. Myogenin is activated at, or early after, the onset of differentiation and is indispensable for progress of fibroblasts to myocytes, while MRF4 is expressed primarily in terminally differentiated muscle fibers. These factors become activated and act in concert with a host of other factors and effectors to begin, sustain and complete the myogenic program. The process of myogenesis culminates in terminal differentiation and the formation of functional, multinucleated myotubes.

Activation of adult satellite cells during muscle repair is also characterized by the rapid upregulation of MyoD and Myf-5. MyoD -/- mice display a reduced regenerative capacity marked by the increase in activated satellite cell population (muscle precursor cells) and reduced myotube formation.[7] In addition, *ex vivo*, MyoD deficient myoblasts are delayed in differentiation upon receiving signals to initiate the process.[8] This data suggests that MyoD is required before the onset of differentiation to prime myoblasts for the process. Therefore, maintenance of MyoD levels and activity is critical for cells to complete the myogenic program.

MyoD and Myf-5 have partially overlapping and compensatory functions in activated precursor cells. While the absence of both MyoD and Myf-5 in developing embryos results in severely reduced muscle formation and embryonic lethality, deficiency in either factor without loss of the other results only in small and transient defects in muscle formation and muscle repair.[9] Developing embryos display extensive co-expression of both factors in some cells and also considerable predominance of one factor in other cells. However, there is evidence that not only do MyoD and Myf-5 activate discrete and distinct subsets of muscle-specific genes,[10] but also that all cells fated for myogenesis must progress through a MyoD-positive stage to complete myogenesis.[11] It is also known that both MyoD and Myf-5 have relatively short half-lives (<1 hr)[12,13] and that the expression of these factors is cell-cycle regulated,[14] and as such the mechanisms governing the relationship between these two factors during development remain elusive. However, it is widely accepted that while Myf-5 is redundant with MyoD in cell lineage specification, MyoD is the predominant MRF in differentiating satellite cells and is the final MRF to be robustly expressed prior to the onset of differentiation; consequently, culturing of adult satellite cells and MyoD-activated myoblasts provides a tractable model for the study of myogenesis in adult mammals.

During development, muscle precursor cells from the somites express Myf-5 and MyoD, which are sufficient for stem cell specification. Notably, these transcription factors share a target sequence (CANNTG) but bind in a stepwise manner to elicit histone acetylation of target genes (mediated by Myf-5) and recruitment of RNA Polymerase II (mediated by MyoD).[15] This target sequence, referred to as an E-box, is ubiquitously

present throughout the genome. Binding to these target sequences is most efficient when Myf-5 and MyoD heterodimerize with other bHLH proteins, termed E-proteins (e.g. E12 and E47, which are both splice variants of the pro-survival transcription factor gene E2a).[16] E-proteins are ubiquitously expressed, while MRF expression is limited to cells with myogenic capacity. These E-proteins comprise the Class I bHLH factor family, while MRFs are considered to be members of the Class II bHLH family. The Class II bHLH proteins, while capable of homodimerization, form weak homodimers in the presence of target DNA sequences, yet form strong heterodimers with Class I bHLH proteins under the same conditions.[17] Both MRFs and E-proteins have DNA binding ability, and DNA binding is most efficient when both proteins are present as a heterodimer; therefore, some muscle specificity of these transcription factors is conferred by the requirement of closely linked E-boxes in target DNA sequences.[18]

Further regulation of myogenesis is accomplished by the sequestration of E-proteins by Id proteins, a family of HLH proteins which hetero-oligomerize with E-proteins and prevent their association with MRFs. Id proteins may also form weak homodimers but form strong heterodimers with bHLH proteins, and bind preferentially to Class I bHLH factors.[19] Id proteins lack the basic region adjacent to the HLH region; since the basic region is required for DNA binding, these new oligomers are unable to efficiently initiate transcription. At the same time, formation of MRF/E-protein complexes is inhibited, resulting in an abrogation of MRF-mediated initiation of transcription.[20] Id is dramatically induced in the presence of growth factors and is rapidly degraded in the absence of serum,[21] and the loss of Id is associated highly with the formation of new

MRF/E-protein heterodimers; indeed, the down-regulation of Id proteins correlates highly with the onset of differentiation in a variety of cell types.[22] Upon serum withdrawal, such as that which occurs during development as multipotent cells migrate away from the notochord to differentiate, MRFs become free to bind E-proteins and initiate transcription of muscle specific genes.

Myogenesis can also be inhibited by Twist, a bHLH protein which can dimerize with E-proteins but possesses a different basic region that is incapable of association with DNA; therefore, these E-protein heterodimers are transcriptionally inert.[23] Association of Twist with E-proteins sequesters them from MRF proteins, thereby preventing the formation of MRF/e-protein heterodimers, reducing the affinity of MRFs for e-boxes, and inhibiting myoblast differentiation. This divergent basic region on the Twist molecule can, however, bind to the basic region of MRFs, preventing the association of MRFs with their target e-boxes in the promoters of muscle-specific genes. Twist also interacts directly with myocyte enhancer 2 (Mef2) proteins, inhibiting their ability to enhance transcription of genes involved in myogenesis.[24] Other factors, such as Mist-1, MyoR and Mdfi inhibit myogenesis by forming transcriptionally inactive heterodimers with MRFs or by occupying E-boxes and blocking transcription initiation at those sites.[25,26,27] Since Twist and other factors inhibit myogenic differentiation in a robust fashion via a number of related but distinct pathways, these factors must as a consequence be dramatically down-regulated at the onset of differentiation.

Myogenesis during skeletal muscle repair closely mirrors the developmental process. In quiescent cells, MRF proteins are undetectable; however, when signals from muscle injury are received, these cells exit the quiescent phase and begin to express muscle specific factors. MyoD is the first MRF to be expressed (usually within 12 hours post activation, and before any cellular division occurs),[28] followed by Myf-5, and subsequently these cells enter the cell cycle and begin to proliferate, at which point they are referred to as myoblasts. A subset of these cells returns to quiescence to replenish the resting progenitor pool.[29] Proliferating myoblasts are characterized by a striking increase in MyoD and Myf-5 expression, wherein MyoD upregulation is controlled by transcriptional activation.[30] Upregulation of MyoD marks the onset of myoblast differentiation. The beginning of the terminal differentiation program is thought to be controlled by the expression of myogenin, which itself is controlled by MyoD transcriptional activation of the myogenin gene, which is in turn regulated by the activity of paired box 7 (Pax7), an antagonist of MyoD transcriptional activity.[31]

In healthy adult skeletal muscle and during development of the embryo, satellite cells are characterized by the expression of a duo of proteins, paired box 3 (Pax3) and Pax7. These transcription factors are thought to regulate specification of satellite cells and to be largely responsible for maintaining cells in a state of quiescence (generally defined as cellular respiration and metabolism without cellular division or progress through the cell cycle).[32] While both Pax3 and Pax7 bind equivalent target DNA sequences, Pax7 is highly expressed in all muscle satellite cells while Pax3 is highly expressed in only a subset of these cell types (e.g. the diaphragm) and binds only a small percentage of Pax7 target

DNA motifs (6.4%).[33] Cells expressing Pax3/7 can either (1) become activated and subsequently begin to undergo myogenesis by directly activating the transcription of Myf5 and MyoD while simultaneously suppressing Pax3/7 expression, or (2) return to a quiescent state without expressing MRFs or downregulating Pax3/7 expression in order to maintain the satellite cell population.[34] There is evidence to suggest that Myf-5 cooperates with Pax7 to renew the satellite cell pool.[35] Pax7 has been reported to be responsible for both induction of Myf-5 transcription[36] and for negative regulation of MyoD induction of differentiation.[37] Long-term inhibition of Pax7 in muscle tissues results not only in a complete abrogation of muscle regenerative capacity in response to injury, but also in dramatic depletion of the satellite cell population and loss of heterochromatin condensation in the surviving cells, and it has been proposed that Pax3/7 interact with histone methyltransferases to regulate chromatin integrity and transcription factor access in the satellite cell population.[38]

Muscle specificity of MRFs is increased not only by the availability of E-boxes, but also by the coincident binding of members of the Mef2 family of transcription factors to adjacent sites in muscle specific genes. Mef2 proteins mediate and enhance the binding of MRFs to muscle-specific genes via association with A/T-rich DNA sequences near MRF/E-protein DNA binding sites.[39] Indeed, direct interaction between Mefs and MRF/E-protein heterodimers is required for myogenic specification of 10T½ fibroblasts.[40,41] Mef proteins are members of the MADS box family of transcription factors and contain a DNA binding domain (Mef domain) adjacent to the MADS domain which targets DNA sequences that may appear near closely associated e-boxes.[42]

Therefore, bHLH protein heterodimerization and the close positioning of two e-box and one MADS box DNA sequences are essential for the efficient binding of MRFs and maximum activation of muscle specific genes. There are four isoforms of MEF2(A-D) which are all localized exclusively to the nucleus and bind enhancer elements in the promoters of muscle creatine kinase (MCK), myosin light chain 1/3(MLC1/3) and other muscle specific genes.[43] MEF2 proteins preferentially bind to enhancers that contain multiple e-box elements, and is rapidly up-regulated when myoblasts are induced to differentiate.[44]

The alterations in gene expression profiles during myogenesis is facilitated by changes in chromatin architecture which dictate and restrict the binding of transcription factors at target sites. DNA exists in the cell *in vivo* as chromatin, or strands of DNA wound around large octameric proteins called histones, providing the “beads on a string” structure characteristic of DNA as viewed under a high-powered microscope. The acetylation status of histones associated with target promoters is a useful indicator of transcriptional activity. Histone acetyl transferases (HATs) can bind independently of, or can be recruited by, transcription factors; acetylation of the histones changes their conformation, allowing for a more relaxed structure in contrast to the tight, closed structure of chromatin characterized by deacetylated histones. This relaxed structure allows DNA promoter regions, which were previously unavailable due to the closed structure, to become available for transcription factor binding and transcriptional initiation. Histone deacetylases (HDACs) can shut down transcription by reversing this process, resulting in more compacted chromatin structure, limiting access to DNA promoters by transcription factors. Depending on any number of

extra regulatory elements and additional trans-acting factors, the same transcription factor may induce hyper-or hypo- acetylation of histones, further complicating the regulation of transcription of target genes.

Hyperacetylation of target promoter regions has been shown to occur just before or concomitant with MyoD binding to these regions.[45] Of particular interest is the observation that MyoD binds promoters in a temporally specific manner. Early genes induced by MyoD show hyperacetylation and MyoD binding (which is sufficient for induction), while the promoters of late genes such as desmin remain hypoacetylated until enrichment of MyoD is detected late in the differentiation process.[46]

1.3 Regulation of Myogenesis by p38 kinase

While some of the signaling pathways involved in the regulation of Pax7 activity have been revealed, the mechanisms surrounding the control of Pax7 expression have remained somewhat elusive. It is known that Pax7 is phosphorylated by casein kinase 2 (Ck2, highly conserved and implicated during development in cell proliferation and apoptosis[47]), an event that prevents its ubiquitin-mediated degradation.[48] Of note is the observation that signaling through the mitogen activated protein kinase (MAPK) p38 may negatively regulate Pax7 expression via activation, secretion and recognition of tumor necrosis factor alpha (TNFα),[49] a protein secreted by macrophages which triggers the extrinsic pathway of apoptosis via death receptor signaling, but is best known for its role in the inflammatory response. In this way, p38 may regulate myogenesis by altering the

ratio of Pax7/MyoD both directly (by downregulating Pax7) or indirectly (since Pax7 inhibits MyoD activity).

The p38 family of MAP kinases, consisting of four known isoforms (denoted α, β, γ and δ), can also activate or suppress transcriptional activity of target genes in stem cells in response to muscle injury.[50] These kinases are activated via direct phosphorylation by MAPK kinases, which are in turn activated by MAPKK kinases, which again can be activated by a wide array of cellular signals. Signals initiated by injury are transduced to the nucleus via p38-mediated TNFα signaling, resulting in enhanced transcriptional activity of a variety of pro-inflammatory genes, muscle specific genes and cytokines; p38α and p38β signaling not only promotes the recruitment and assembly of MyoD/e-protein complexes to target genes, but also the localization of SWI/SNF chromatin remodeling complexes[51] as well as Ash2L-containing mixed-lineage leukemia (MLL) methyltransferase complexes to these same loci.[52] In contrast, the p38γ isoform has been implicated in the inhibition of MyoD transcriptional activity by association with the histone 3- lysine 9 methyltransferase KMT1A and direct phosphorylation of MyoD, resulting in increased recruitment of MyoD to the myogenin promoter, but also in reduced transcription of the myogenin gene.[53]

The MAPK p38 is activated immediately and in a robust fashion in differentiating myoblasts, and remains activated at a high level throughout the differentiation process. Activation of p38 mediates and is essential for specification of fibroblasts to myoblasts and

MyoD-dependent expression of muscle-specific genes.[54] In addition, p38 activation in myoblasts occurs independently of growth factor signaling,[55] suggesting a pathway for activation that, in this context, is independent of differentiation-inducing signals. Two of the four p38 isoforms, α and β, also directly phosphorylate myocyte enhancer factor 2 (Mef2) proteins (only the Mef2A and Mef2C isoforms) at multiple sites,[56] and p38-mediated MEF2C phosphorylation at Thr293 is sufficient to result in enhanced transcriptional activation of MEF2C-targeted muscle-specific genes.[57] In rhabdomyosarcoma cells, a transformed cell line in which differentiation is abrogated while MyoD expression remains robust (presumably because in these cells MyoD is incapable of inducing expression of the cell cycle regulator p21 and cells are unable to exit the cell cycle to differentiate), it has been reported that constitutive p38 activation was able to rescue a differentiation-capable phenotype,[58] suggesting that p38 is not only involved in the regulation of MyoD expression, but also in MyoD activity. P38 is also required for the association of Mef-2 and MyoD to muscle-specific genes that are activated late in the differentiation program, possibly through a mechanism that involves chromatin remodeling, indicating that p38 activation is required throughout myogenesis.[59]

1.4 Regulation of MyoD

MyoD itself is highly regulated at the transcriptional level. The regulatory elements necessary for MyoD expression in muscle precursor cells are contained within a 6 kb DNA fragment located upstream of the transcription initiation site.[60] These three enhancers are thought to be activated relatively independently of one another and thus are association with different stages of development. The Proximal Regulatory Region (PRR) is a 275 bp

minimal enhancer which is functionally silent in the absence of activity at the Distal Regulatory Region (DRR).[61] The Core Enhancer (CE), a highly conserved 258 bp sequence located about 20 kb upstream of the transcriptional start site, is required for MyoD expression during embryonic development. Mutation of this core enhancer resulted in dramatic, although transient, delays in MyoD expression in the limb buds of the developing embryo, presumably due to cooperation of this element with the DRR. However, depletion of this element in satellite cells, differentiated myocytes or proliferating or activated muscle precursor cells does not significantly reduce MyoD protein expression.[62] The DRR is the most extensively studied of the three elements and contains a serum response element (SRE) that is required is for MyoD expression in proliferating and differentiating myoblasts.[63] It has been reported that two transcription factors, Serum Response Factor (SRF) and Mef2c, compete for binding this cis-element and that their activity may help control myogenesis.[64] SREs canonically contain CArG sequences (where "r" represents any pyrimidine) to which SRF and cofactors may bind to increase transcription. A CArG element located in the SRE at the MyoD DRR diverges in sequence from canonical CArG boxes in that its sequence (CCATTTATAG) is somewhat different at the 3′ end from the published SRF canonical sequence ($CC(A/T)_6GG$), resulting in more transient binding by SRF and Mef2 (**Figure 2**).

SRF binds to SREs when complexed with ets domain proteins such as SAP-1 or elk-1, which bind to an ets motif upstream the CArG box, and ets proteins can only do this when complexed with SRF.[65] SRF is a highly conserved, ubiquitously expressed and predominantly nuclear 67 kDa phosphoprotein, and is a founding member of the MADS

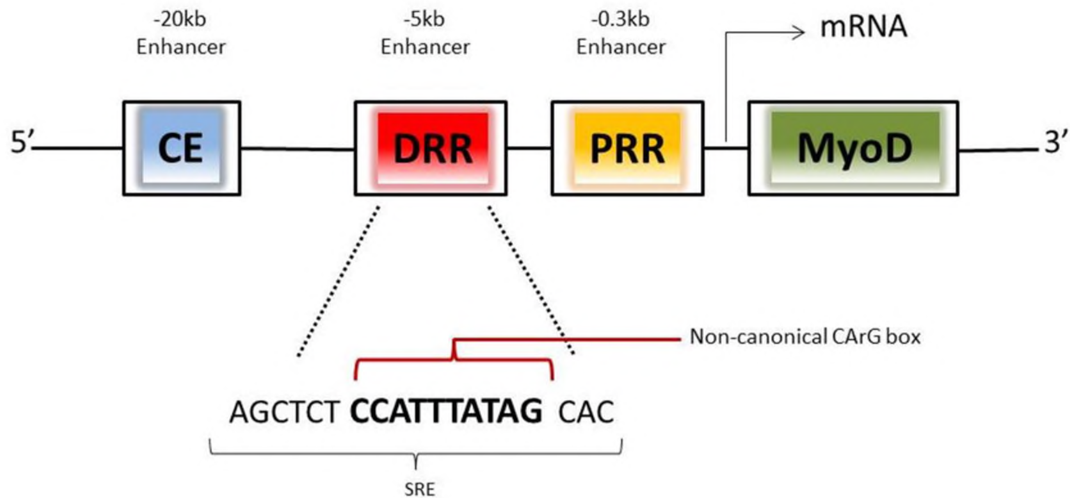

Figure 2. Transcriptional Control of MyoD Expression by a Non-Canonical CArG Box Located in the MyoD Distal Regulatory Region. The expression of MyoD is regulated by three enhancers contained in its 5'UTR: a core enhancer (CE) responsible largely for control of MyoD expression during development, a minimal promoter termed the proximal regulatory region (PRR) and a distal regulatory region (DRR). The DRR is required for MyoD expression during specification in the embryo and during activation of adult satellite cells during regeneration. The serum response factor (SRF) is known to associate with a non-canonical CArG element in the DRR of the MyoD promoter region. This cis-acting element is required for MyoD induction and myogenesis.

box family (MCM1, Agamous, Deficiens and SRF) of transcription factors. SRF binds as a homodimer - on its own or in a ternary complex with an ets domain protein as a binding partner - to SREs in the promoter regions of immediate early genes that are growth factor-stimulated, such as the proto-oncogene c-fos.[66] CArG box-dependent gene activation is a very early phenomenon during ex vivo myogenesis, occurring at the same time as MyoD gene activation in 10T½ myoblasts treated with 5-az (i.e. C2C12 myoblasts), and is required for the expression of many muscle-specific genes. In addition, SRF antisense RNA reduces MyoD and myogenin expression and delays differentiation, and blocking the association of SRF with SREs also blocks differentiation;[67] therefore, SRF activation of target genes is also required for efficient myogenesis and for the maintenance of MyoD protein levels prior to differentiation, in proliferating myoblasts.

It has been revealed, however, that while Mef2 transcriptional activation may be required for maximal expression of MyoD, it may not be necessary for the maintenance of basal levels. A Mef2c deletion mutant incapable of binding DNA was still able to cooperate with MRFs to induce myogenesis, but Mef2c-/- cells are still capable of muscle regeneration in response to injury.[68] Interestingly, Mef2 protein expression exists below the level of detection by Western blotting prior to the onset of differentiation,[69] suggesting that Mef2 activity is dispensable for the maintenance of muscle-specific protein levels. Therefore, DNA binding and transcriptional activation by Mef2c are not required for Mef2c transcriptional enhancement during myogenesis, and other Mef proteins may fill redundant roles in this process, highlighting SRF as the primary factor regulating MyoD expression levels in activated, proliferating myoblasts.

Maintenance of MyoD levels in activated, proliferating myoblasts is critical to keeping the cells primed for differentiation. Secreted growth factors such as some members of the FGF family increase MyoD's initiation of the differentiation program by activation of PI3K,[70] which induces the phosphorylation of phosphoinositides, leading to the activation and recruitment of SRF to SREs.[71] Through mechanisms that remain partially unclear, removal of growth factor stimulation results in changes in MyoD protein expression (up-regulated in C2C12 myoblasts and down-regulated from a comparatively high level in 23A2 myoblasts) and the differentiation program is initiated; however, studies have revealed that insulin-like growth factor 2 (Igf2) - a growth factor implicated in the differentiation of several cell types - negatively regulates the transcription of MyoD,[72] and that IGF-1 promotes recruitment of MyoD to – and hyperacetylation of – the promoters of muscle-specific genes.[73] An SRF mutant that is capable of binding DNA but that is transcriptionally inert, or incubation with purified anti-SRF antibodies, abolishes MyoD expression in proliferating myoblasts;[74] thus, basal signaling to SRF is required for the maintenance of MyoD levels in proliferating myoblasts.

1.5 Coordinate Regulation of Differentiation and Apoptosis

Our lab and others have demonstrated that the generation of functional skeletal muscle also results in the apoptosis of subset of activated, differentiating myoblasts.[75] The concurrent regulation of myoblast differentiation and apoptosis is required for proper muscle formation and tissue homeostasis. Apoptosis is the tightly controlled programmed

cell death that is responsible for the organized packaging and removal of damaged and irregular cells without evoking an immune response, and is canonically associated with the removal of excess and dangerous cells during neuronal and muscular development, as well as during immune cell maturation, oxidative stress and viral infection.[76] Apoptosis can be activated by a variety of stimuli, including DNA damage and the removal of growth factors, and generally occurs through two pathways that converge at the activation of caspases (cysteine aspartic acid-specific proteases). The intrinsic pathway of apoptosis is controlled by the interplay of pro- and anti-apoptotic members of the B cell lymphoma 2 (Bcl-2) family of proteins. Pro-apoptotic Bcl-2 family members (Bax, Bak and BH3-only proteins) are maintained in an inactive state by association with the anti-apoptotic Bcl-2 family members (e.g. Bcl-xL and Bcl-2). In response to apoptotic signals that originate inside the cell, BH3-only proteins are activated by a variety of pathways, including upregulation at the level of transcription (e.g. Bax, PUMA; however, in the case of 23A2 myoblasts, PUMA is the sole controlling Bcl-2 family member[77]) and the dephosphorylation of Bad. Bad is phosphorylated by the serine/threonine kinase Akt, which is activated by Phosphoinositide-3 kinase (PI3K) signaling in the presence of growth factors. When growth factor stimulation is removed, Bad translocates to the mitochondria and associates with Bcl-XL, resulting in the formation of pores on the outer membrane of the mitochondrion, and a reduction in mitochondrial membrane potential. Once the thresholds for an excess of pro-apoptotic protein activity and membrane potential have been reached, these proteins cause permeabilization of the outer mitochondrial membrane, releasing several mitochondrial proteins from the inner membrane space. The most critical of these with respect to the apoptotic process is the electron-transfer protein cytochrome c,

which then becomes free to associate with apoptotic peptidase activating factor 1 (Apaf-1). This complex oligomerizes and forms the apoptosome, a focus of proteins that can result in the activation of a highly conserved family of proteins, the cysteine-containing aspartic acid-specific proteases (caspases), which are indispensable for and initiators and effectors of apoptosis (**Figure 3**).

The extrinsic pathway involves the binding of so-called death ligands to cognate death receptors on the cell surface. In murine myoblasts, this process is mediated by the tumor necrosis factor superfamily (TNFSF) of secreted ligands, which bind molecules of the TNF receptor superfamily (TNFRSF) on the cell surface. These ligand/receptor interactions are involved in the regulation of development, tissue homeostasis and immunity. TNFSF molecules are generated as type II or type III transmembrane proteins, with soluble forms produced by alternative splicing or proteolysis. They contain a conserved hydrophobic domain termed the TNF homology domain (THD) which mediates trimerization of the molecule to form the active ligand.[78] The TNFRSF family is largely composed of type I transmembrane proteins, which homotrimerize upon ligand interaction and which contain a conserved, approximately 180 kda C-terminal cytoplasmic domain referred to as a death domain (DD). After ligand binding and receptor trimerization, conformational changes allow the recruitment of adaptor molecules to the DD domain; these adaptor proteins can be divided into two categories: (1) DD-linked proteins such as Fas associated DD (FADD) and TNF receptor-associated DD (TRADD) which lead to apoptosis, and (2) TNF receptor-associated factors (TRAFs) which can activate Nf-kB and

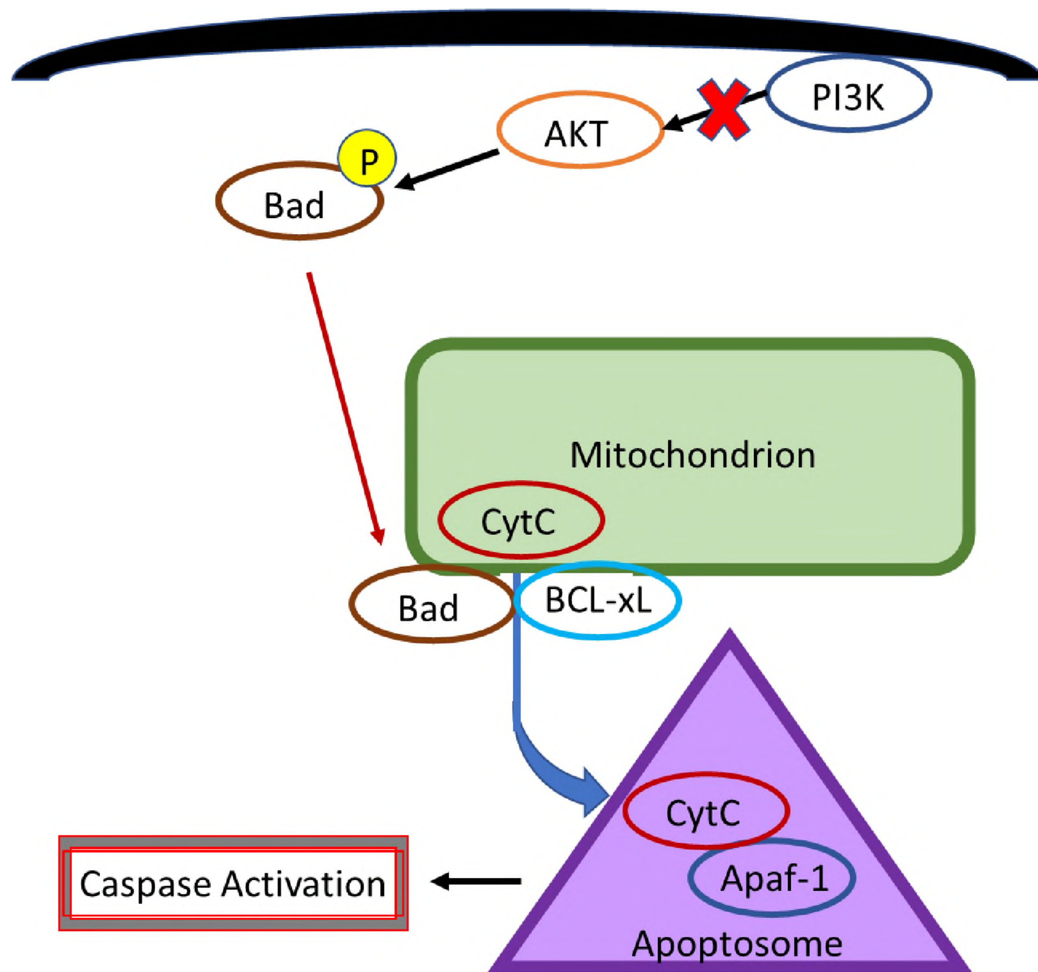

Figure 3. Simplified Schematic of the Intrinsic Pathway of Apoptosis. Under normal conditions Bad is phosphorylated by Akt, which is activated by PI3K signaling. When growth factor stimulation is removed, Bad translocates to the mitochondria and associates with Bcl-XL, resulting in the formation of pores on the outer membrane of the mitochondrion, and a reduction in mitochondrial membrane potential. This results in permeabilization of the outer mitochondrial membrane, releasing Cytochrome C (CytC) from the inner membrane space. CytC then becomes free to associate with Apaf-1. This complex oligomerizes and forms the apoptosome, that results in the activation of caspases, which mediate apoptosis.

JNK signaling to promote survival and differentiation.[78] In the case of murine myoblasts, death receptor 5 (DR5) is the only TNFRSF family member expressed and recruits adaptor proteins from the first category upon receiving TNFSF signaling; namely, the multiple molecules of the adaptor protein FADD are recruited to the receptor though its death domain (DD). This adaptor molecule also contains a death effector domains (DED), which allows it in turn to recruit multiple molecules which also contain the DED, such as the inactive initiator caspase zymogen pro-caspase 8. Pro-caspase 8 is cleaved and activated by proximity-induced proteolysis after oligomerizing with FADD; the activated initiator caspase 8 can then cleave and activate downstream effectors such as caspase 3 (**Figure 4**). Additionally, a link may exist between basal caspase signaling and p38 activation. In Jurkat T-cells, receptor-mediated apoptosis not only results in activation of the initiator caspase 8 but also in robust activation of p38. Similarly, chemical caspase inhibition not only suppresses receptor-mediated apoptosis but also inhibits p38 activation.[79]

Caspases are subdivided into two major categories: those involved in inflammation (1, 4, 5, 11 and 12) and those which mediate apoptosis (2, 3, 6, 7, 8, 9 and 10).[80] The apoptotic caspases are further subdivided into two groups: the initiator (2, 8, 9 and 10) and effector (3, 6 and 7) caspases. The extrinsic pathway results in the activation of caspase-8 (or -10, in some cases). In addition to catalyzing the cleavage and activation of caspase-3, caspase-8 can cleave and activate the pro-apoptotic Bcl2 family member Bid to its truncated form (tBid), which in turn can mediate MOMP. The role of caspases in apoptosis and inflammation has been well-characterized. They are expressed as inactive zymogens

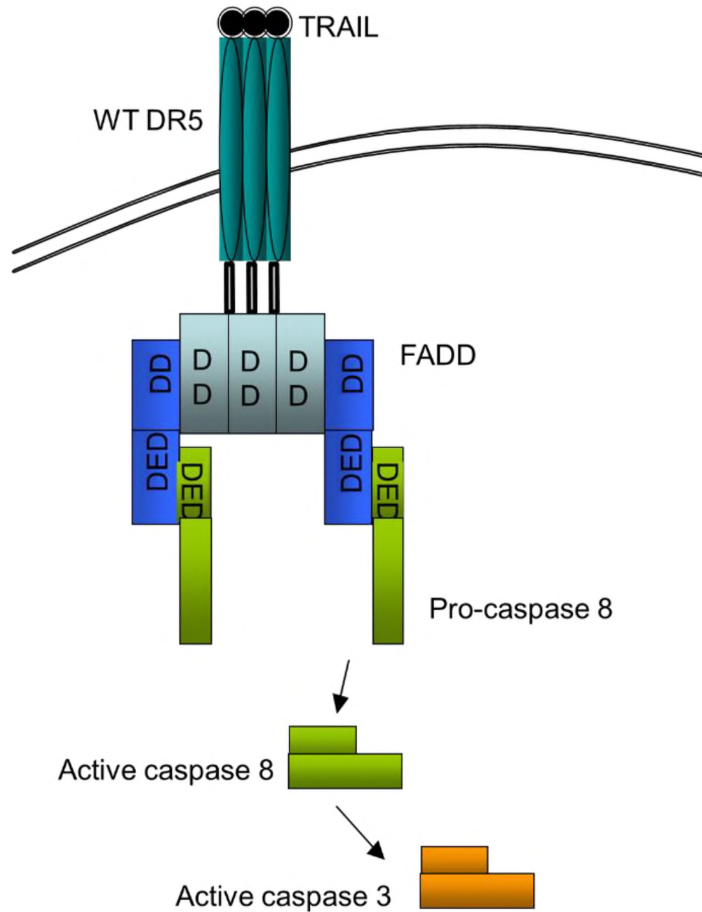

Figure 4. The Extrinsic Pathway of Apoptosis. Following the binding of the death ligand (TRAIL) to its receptor (DR5), the adaptor molecule FADD is recruited to the receptor through the interaction of Death Domains (DD) present on each molecule. In murine cells, Pro-caspase 8 is then recruited to the complex via interaction of Death Effector Domains (DED) on both the Procaspase 8 and FADD molecules, where it oligomerizes at the receptor and is subsequently cleaved and activated by proximity-induced proteolysis. Active caspase 8 can then activate downstream molecules such as caspase 3.

which are capable of forming homodimers, and the formation of procaspase homodimers causes a conformational change which allows some catalytic activity. These molecules may cleave and activate one another, forming a potent tetrameric protease complex. Caspases are the main effectors of cell death, cleaving and destroying certain targets (e.g. key pro-survival proteins) in addition to cleaving and activating other pro-apoptotic targets (e.g. caspase activated DNAse (CAD)).[81] Caspases also fill a well-defined role in signal transduction during the inflammatory response, either facilitating the inflammatory response by responding to lipopolysaccharide, or inhibition of the inflammatory response, such as in the case of caspase-12, which can antagonize caspase-1 activity in a protease-independent manner.[82] With respect to the apoptosis associated with differentiation, caspases are activated by both the intrinsic and extrinsic pathways. One result of this process is membrane blebbing, packaging of cellular materials and signaling to macrophages for the removal of the product of this process. Activated macrophages at the site of apoptotic muscle can also signal for the activation of adult satellite cells for muscle repair.[83]

The role for serine proteases in apoptosis has also been well-established, albeit not yet well-defined; these proteases are activated in response to traditional apoptotic cues, such as DNA damage, treatment with TRAIL and chronic ER stress. The finding that serine protease activity is also required for the differentiation of many cell types (e.g. keratinocytes and epithelial cells [84,85]) reinforces the concept that the differentiation and apoptotic processes in mammals are coordinately regulated. Our lab has discovered that when skeletal myoblasts are induced to differentiate in the presence of AEBSF, a general

serine protease inhibitor, not only is the associated apoptosis abrogated, but differentiation is inhibited as well. While the mechanism of AEBSF inhibition of serine protease activity involves inactivation of S1P and other serine proteases (by sulfonylating the serine at the active site of S1P), it does not affect caspase activation; TRAIL-induced apoptosis is not successfully inhibited with either AEBSF or the pan caspase inhibitor z-vad-fmk, but simultaneous treatment with these inhibitors blocks apoptosis. Surprisingly, treatment with either inhibitor blocks the apoptosis associated with differentiation, occurring as a result of a reduction in MyoD mRNA and protein expression, suggesting that caspase and serine protease activity is required to maintain basal transcription or to prevent degradation of the transcript. Further investigation into the role of serine proteases in the maintenance of MyoD expression is warranted, but is not the focus of this dissertation.

1.6 Project Goals

In order to study the contribution that the extrinsic pathway of apoptosis plays in differentiation-associated cell death, our lab has created myoblasts stably expressing a dominant negative form of the DR5 (dnDR5). Using these cell lines, we have demonstrated that the TRAIL/DR5 pathway is activated to initiate the extrinsic pathway (**Figure 5**). Further, we have discovered a non-canonical role for the TRAIL/DR5 pathway in the regulation of differentiation. Specifically, cells expressing dnDR5 display delayed differentiation as measured by the expression of Myosin Heavy Chain (MHC) protein, as well as reduced MyoD mRNA and protein levels. In addition, because the observed difference in MyoD mRNA levels is not due to altered mRNA stability, we inferred that regulation of MyoD mRNA occurs at the level of transcription. The purpose of this

dissertation will be to elucidate how basal signaling through DR5 results in the maintenance of MyoD levels in proliferating myoblasts.

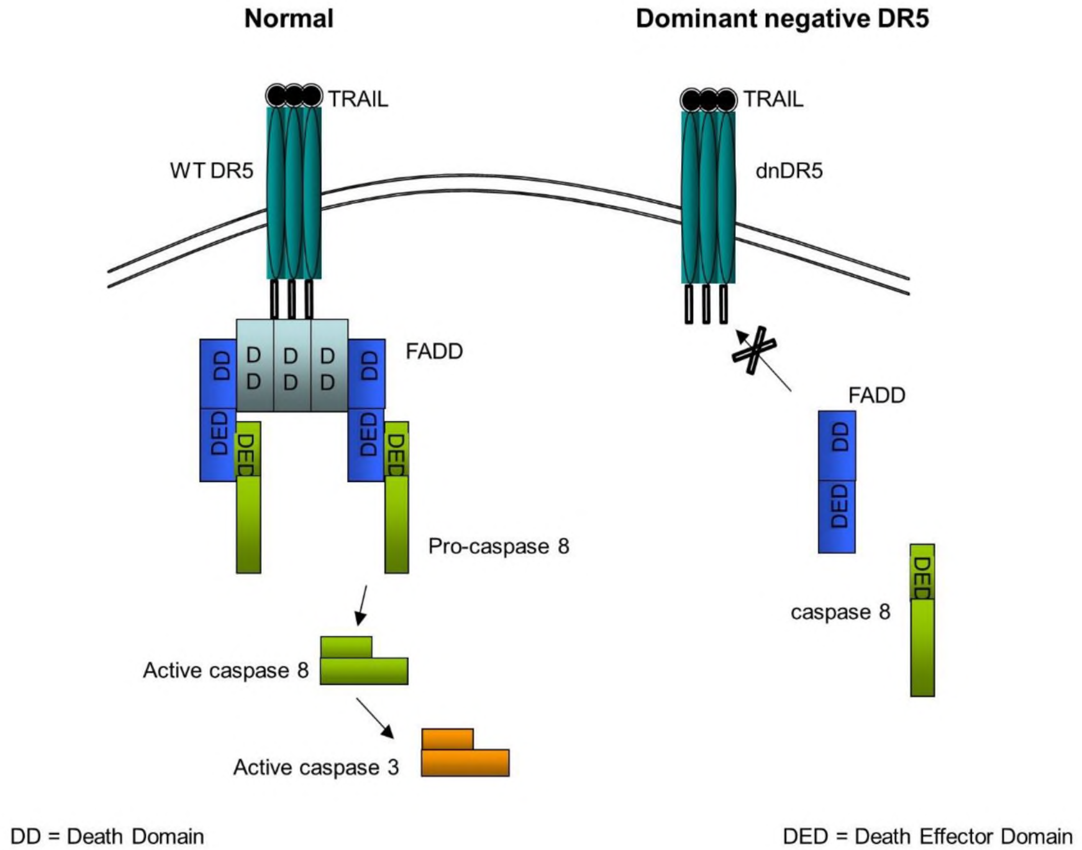

Figure 5. Schematic representation of the classical death ligand pathway of apoptosis and the effect of dnDR5 expression. Stable expression of a dominant negative of DR5 (dnDR5), which lacks a DD, prevents the recruitment of FADD to the receptor, abrogating apoptotic signaling through the DR5 pathway.

CHAPTER II

MATERIALS AND METHODS

2.1 Cells and cell culture

The growth 23A2 myoblasts and 23A2 myoblasts expressing dnDR5 have been reported previously [10]. The Z-DEVD-fmk caspase inhibitor (20 μM final treatment concentration; Calbiochem) and SB 203580 (3 μM treatment concentration; Sigma) were each dissolved in DMSO. Appropriate volumes of DMSO or methanol alone were added to control cultures and did not exceed 0.15% v/v.

2.2 Chromatin immunoprecipitation

ChIP was performed following the protocol provided in the EZ-ChIP™ kit (Millipore/Upstate) and as described in [13]. Cells were plated on 150 mm plates. The next day, cells were fixed in 0.5% formaldehyde for 10 minutes at room temperature. Formaldehyde was inactivated by the addition of .125 M glycine to the cells for 5 minutes at room temperature. Cells were then washed with ice cold PBS containing 5 mM Na Butyrate and 0.5 mM PMSF and pelleted by centrifugation at 1500 rpm for 5 minutes and then resuspended in 5 ml cold Cell Lysis Buffer (CLB: 60 mM KCl, 15 mM NaCl, 5 mM

MgCl, 10 mM Tris pH 7.4, 300 mM sucrose, 0.1 mM EGTA, 0.1% NP-40, 5 mM Na Butyrate, 0.5 mM PMSF). Cells were sonicated once for 10 sec to ensure lysis of the plasma membrane. Isolated nuclei were washed once in 30 ml of CLB and once in 1 ml of cold Nuclei Digestion Buffer (Cell Lysis Buffer without NP-40 and PMSF). For MNase digestion, intact nuclei were resuspended in 125 μl of Nuclei Lysis Buffer (prewarmed to 37°C), digested with MNase (50 units/ml) at 37°C for 5 minutes, and terminated by 5 mM EDTA. An aliquot from each sample was assessed for sufficient chromatin fragmentation (500–1000bp) by gel electrophoresis. Samples were sonicated twice to ensure lysis of the nuclei prior to immunoprecipitation. The remaining steps of the immunoprecipitation were performed using the EZ ChIP™ Chromatin Immunopreipitation Kit (Upstate) per manufacturer's instructions. Subsequently, anti-SRF (Santa Cruz) or appropriate IgG control (Sigma Aldrich) were added for immunoprecipitation. For each immunoprecipitation, 5 μg of the appropriate antibody was incubated with a precleared chromatin aliquot overnight at 4°C with rotation. The next day, protein A/G sepharose beads were added and incubated for 1 hour at 4° with rotation. The immunoprecipitates were pelleted, washed and the antibody-protein-DNA complex was eluted from bead by incubation in 100 mM NaHCO3 and 1%SDS. Following immunoprecipitation and elution, the eluent was treated with RNase A followed by reverse crosslinking by incubation at 65°C overnight. Protein was removed by addition of proteinase K and incubation at 45° for 2 hours. DNA was purified using mini columns provided by kit manufacturer. Purified DNA was amplified by specific primers and PCR was performed under the following conditions: 1 cycle at 95° for 15 min, 40 cycles of 94° 1 min, 58° 1 min, 72° 1 min; and a final extension step at 72° for 5 minutes followed by analysis of melting curve. Data was

normalized to the signal detected from the input of each sample. The fold enrichment of each target site was calculated as 2 to the power of the cycle threshold (cT) difference between input chromatin and ChIP samples.

2.3 Western Analysis

Lysates were prepared and 100 μg were denatured and electrophoresed through denaturing polyacrylamide gels (10%) followed by electrophoretic transfer as previously described. For assessment of phospho-SRF and phospho-p38 relative to their cognate total protein levels, the same lysate was run on separate gels due to the utilization of a separate membrane blocking protocol for anti-phospho antibodies relative to the blocking protocol used for all other antibodies. For the following antibodies, each diluted 1:1000: from Santa Cruz; anti-SRF, from Cell Signalling; anti-p38, and from Abcam; anti-MyoD, membranes were blocked for one hour in 1 × TBS/0.1%NP40 with 10% newborn calf serum and 5% dry milk. For the following antibodies, each diluted 1:1000: from Cell Signaling, anti-phospho-p38 (Thr180/Tyr182) and anti-phospho-SRF (ser103), membranes were blocked for one hour in 1 × TBS/0.1%NP40 with 5% BSA. Western analysis using anti-actin (Sigma) or anti-hsp70 (BD Biosciences) served as loading and transfer controls (each diluted 1:30,000). All primary antibodies were incubated overnight at 4°C. Appropriate HRP-conjugated secondary antibodies, diluted 1:1000, were incubated with the membranes for one hour. After each incubation with antibody and prior to the addition of chemiluminescent substrate, membranes were washed five times in 1 × TBS (Tris- buffered saline pH 7.4) with 1% Tween 20. Membranes were then incubated with (SuperSignal West Pico Chemiluminescent Substrate: Thermo Scientific: #34078) for 60 seconds and bands

were visualized using (Li-Cor Phospho-imager: Image Studio Ver. 2.1). Note that multiple Western analysis were run from the same set of samples to eliminate as much variablity as possible.

2.4 Quantitative RT-PCR

Myoblasts were plated at equal density and the next day cultured as indicated in the figure legend. For quantitative RT-PCR, total RNA was prepared using 1 mL of Trizol (Invitrogen) reagent per 100 mm plate for lysis and following the manufacturer's instructions. Five hundred ng of RNA was then used for a 20 µL SuperScript III RT (Invitrogen) reverse transcription reaction. Quantitative PCR (qPCR) was performed for MyoD as described using a Bio-Rad DNA Engine Opticon 3 Real-Time PCR System using SYBR® Green Master PCR Mix according to the manufacturer's instructions (Qiagen).

CHAPTER III

RESULTS

3.1 A Role for acetylation in the regulation of MyoD levels by dnDR5

Activated skeletal myoblasts are maintained in a proliferative state by the presence of mitogens. This is accomplished ex vivo by culturing cells in media supplemented with 10% Fetal Bovine Serum (FBS) as a mitogen source, referred to as growth media (GM). Others in our lab were successful in generating two stable myoblast cell lines expressing a dominant negative version of DR5 (a DR5 molecule lacking the DD required for signal propagation) denoted clone 3 and clone 5. Expression of this dominant negative mutant results not only in inhibition of TRAIL-induced apoptosis, but also the apoptosis associated with differentiation (as measured by DNA fragmentation and release of cytosolic nucleosomes).[86] Surprisingly, the dnDR5-expressing myoblasts were also defective for differentiation (as measured by the expression of Myosin Heavy Chain (MHC)) when these cells were induced to differentiate by the removal of FBS (i.e. culture of myoblasts in media lacking serum, termed differentiation media (DM)).

In dnDR5-expressing cells cultured in GM, we observed a reduction of MyoD protein and mRNA expression as compared with parental 23A2 myoblasts. When we investigated the cause of the observed decrease in the level of MyoD mRNA, we found that the half-life of the message was not reduced in dnDR5 myoblasts;[87] thus, the observed depletion of MyoD protein is likely due to a change at the level of transcription.

We next assessed the histone acetylation status of the MyoD promoter region by performing ChIP assay. We hypothesized that if histone acetylation status reflects transcriptional activation and if MyoD protein expression in dnDR5-expressing myoblasts is regulated at the transcriptional level, then the MyoD promoter region should display hypo-acetylation in dnDR5-expressing myoblasts relative to parental control myoblasts. To test this, 5 μg each of antibodies raised against acetylated histones H3 and H4 were used as probes during the immunoprecipitation step, since these two histones are the most highly modified of the five histone molecules. Primer sets representing a 20bp sequence spanning the MyoD promoter region and representing a 20bp sequence spanning the MyoD CArG box were used to amplify DNA precipitated along with acetylated H3 and H4. By comparing associated DNA levels, we found that histones associated with the MyoD promoter and the MyoD CArG region display dramatically reduced acetylation (approximately 60 to 80% reduction in H3 and 70 to 80% reduction in H4 at the MyoD promoter region and 90% reduction in H3 and 70 to 80% reduction in H4 at the MyoD CArG region). These findings indicate a closed chromatin structure and suggest a reduction in transcriptional activation.

To further corroborate our suspicions about transcriptional control of MyoD expression in dnDR5-expressing myoblasts via decreased acetylation of histones, we sought to restore MyoD protein expression by treatment of these dnDR5-expressing cells with trichostatin A (TSA), a potent, membrane-soluble HDAC inhibitor. MyoD protein expression was abrogated in dnDR5-expressing myoblasts for up to 72 hours of non-confluent growth in GM; however, MyoD protein expression could be elevated in the dnDR5-expressing myoblasts after 72 hours in growth media containing TSA (**Figure 1**). We also observed an increase in MyoD expression in parental 23A2 myoblasts after 72 hours of TSA treatment. The concentration of TSA (100 ng/mL) was empirically determined for treatment of dnDR5 myoblasts, and a comparable concentration of a vehicle control (DMSO) was used to treat parental myoblasts.

We next sought to determine the transcription factor(s) responsible for the acetylation status of histones associated with the CArG box. L'Honore et. al analyzed recruitment of trans-acting factors to the CArG region located in the non-canonical SRE at the MyoD DRR, and discovered SRF-containing, high molecular weight complexes bound to this region in GM, which decreased after 4 days in DM (i.e. terminally differentiated muscle fibers) in C2Cl2 myoblasts. We therefore set out to recapitulate the results observed by the Fernandez group, and to confirm them using our 23A2 myoblast model system, prior to assessing the effect of dnDR5 expression on SRF recruitment. When we examined SRF recruitment via ChIP analysis to the CArG box located in the MyoD DRR using the aforementioned CArG region primers, we discovered that SRF enrichment decreased in DM (approximately 40% reduction in recruitment) as compared with C2C12

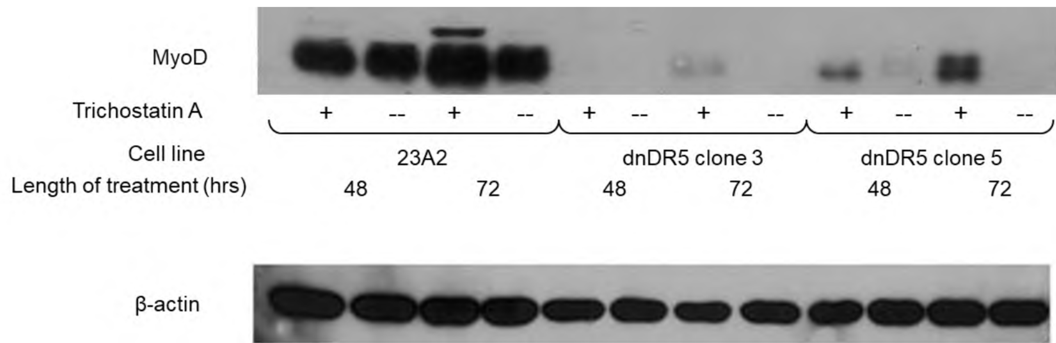

Figure 1. dnDR5 Regulates MyoD Expression at the Level of Transcription. TSA treatment partially restores MyoD expression in dnDR5 myoblasts. Cells were plated at $4x10^5$ and the next day treated with TSA, a histone deacetylase inhibitor. At the indicated times, whole cell protein lysates were prepared and subjected to SDS-PAGE. Western analysis was performed using anti-MyoD or anti-β-actin and visualized by autoradiography.

myoblasts cultured in GM (**Figure 2A**). Similarly, SRF recruitment in 23A2 myoblasts was reduced by approximately 35% in DM when compared to GM (**Figure 2B**).

Having confirmed our ability to successfully perform ChIP analysis using anti-SRF antibodies in our system, we compared the recruitment of SRF to the CArG element in the DRR in wild type 23A2 cells and 23A2 cells expressing dnDR5. Although an investigation into the mechanisms surrounding displacement of SRF at earlier timepoints during differentiation was intriguing, we chose to perform this analysis in GM because the inhibition of MyoD mRNA and protein expression in dnDR5 myoblasts was detected in proliferating cells. When we performed ChIP analysis using the SRF specific antibody in these cells, we observed an approximately 50% reduction in the recruitment of SRF the MyoD DRR CArG element in dnDR5-expressing 23A2 myoblasts as compared with parental myoblasts (**Figure 3A**). Our finding that expression of dnDR5 resulted in decreased recruitment of SRF to the CArG box in the MyoD promoter is consistent with SRF driving both MyoD expression in GM and the decreased level of MyoD detected in dnDR5-expressing myoblasts.

To determine whether the reduction in SRF recruitment to the CArG box was due to a change in SRF protein expression, we performed Western blotting analysis using lysates obtained from proliferating 23A2 and 23A2 dnDR5-expressing cells. The same antibody (Abcam) that was employed in our ChIP analysis is also suitable for

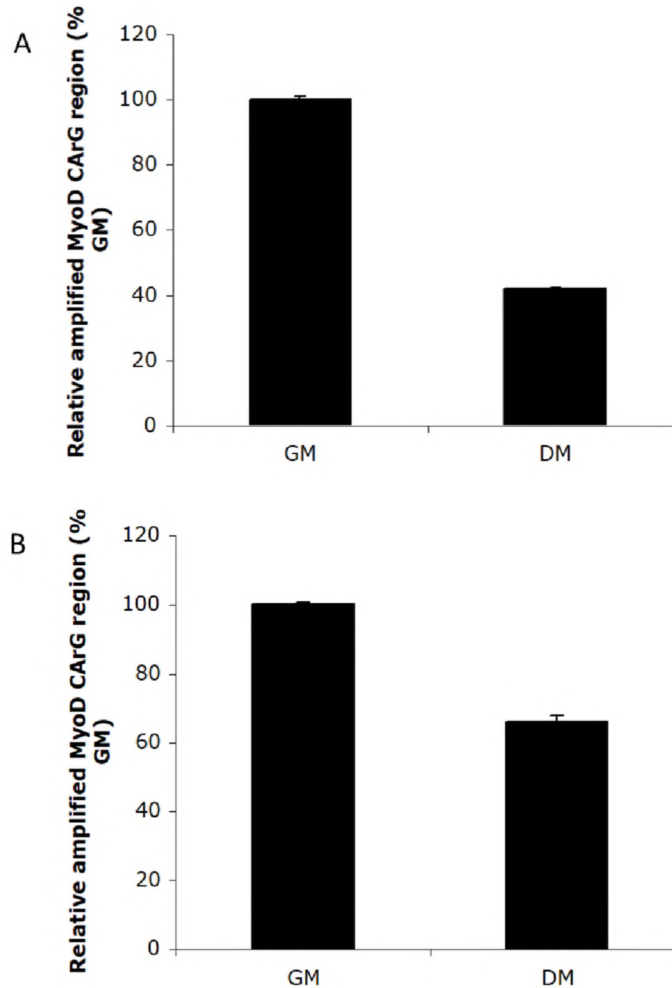

Figure 2A and 2B. SRF Enrichment at the MyoD CArG Region is Reduced in Myoblasts Cultured in DM as Compared with GM. In (A) and (B), chromatin from 1 $\times 10^7$ cells was cross-linked and digested with MNase to a length between 500–1000 bp. Chromatin Immunoprecipitation was performed on each cell sample using EZ ChIP™ Chromatin Immunoprecipitation Kit (Upstate) per manufacturer's instructions. Chromatin from 2 $\times 10^6$ cells was immunoprecipitated as described in Methods. Quantitative PCR was used to assay for the relative levels of SRF binding near MyoD CArG element. Data was normalized to the signal detected from the input of each sample and presented as a percent of the signal obtained from parental C2C12 (A) and 23A2 (B) myoblasts. Error bars represent mean +/– SEM of triplicates.

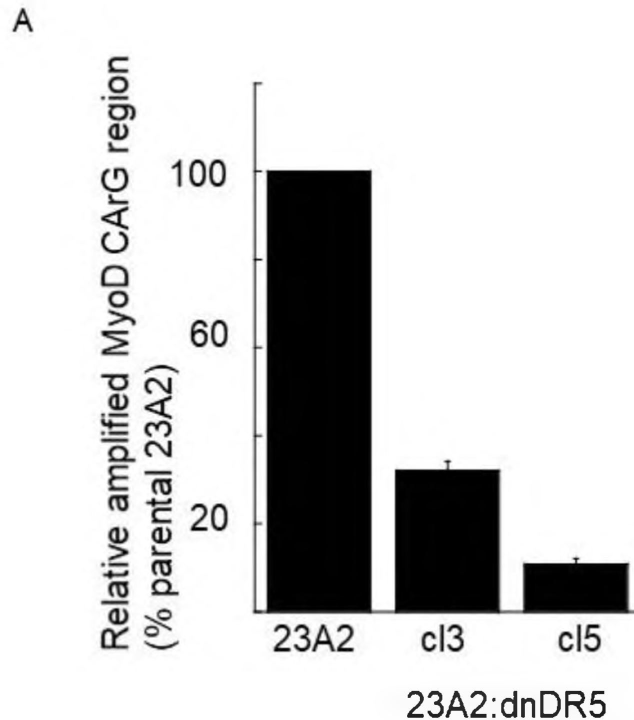

Figure 3A. dnDR5 Expression Decreases SRF Binding to the CArG Box in the DRR of the MyoD Enhancer. In (A) chromatin from 1×10^7 cells was cross-linked and digested with MNase to a length between 500–1000 bp. Chromatin Immunoprecipitation was performed on each cell sample using EZ ChIP™ Chromatin Immunoprecipitation Kit (Upstate) per manufacturer's instructions. Chromatin from 2×10^6 cells was immunoprecipitated as described in Methods. Quantitative PCR was used to assay for the relative levels of SRF binding near MyoD CArG element. Data was normalized to the signal detected from the input of each sample and presented as a percent of the signal obtained from parental 23A2 myoblasts. Error bars represent mean +/– SEM of triplicates.

immunoblotting and thus this antibody was also used to perform the Western blot. We observed no significant difference in SRF protein expression between 23A2:dnDR5 cells and parental myoblasts when normalized to a beta-actin loading control (**Figure 3B**).

SRF can induce canonical CArG box-dependent transcription in the presence of anisomycin and cyclohexamide, molecules which are inhibitors of new protein synthesis; this indicates not only that SRF activation is essential for its function as a transcriptional activator, but also that its activation requires posttranslational modification. It has indeed also been established that phosphorylation of SRF is a necessary prerequisite for SRE-dependent transcriptional activation.[88] SRF binds as dimer and in a ternary complex with Ets proteins; these phosphorylation events do not alter its ability to dimerize but directly alter the ability of SRF to bind DNA. SRF association with SREs can be completely ablated by deletion of its C-terminal region.[89] Studies have shown that there are five phosphorylation sites in the SRF N-terminal region (serines 77, 79, 83, 85, and 103). SRF phosphorylation at serine 103 is an indicator of SRF activation, and is required for its association with the promoters of many immediate early genes such as c-fos.[90] While the c-fos SRE is constitutively occupied by SRF (i.e. before, during and after induction), L'Honore et al have shown that recruitment of SRF to the non-canonical CArG box located in the MyoD DRR does not display the same pattern, in that SRF recruitment is vastly reduced in terminally differentiated myotubes. We have also observed dissociation of SRF

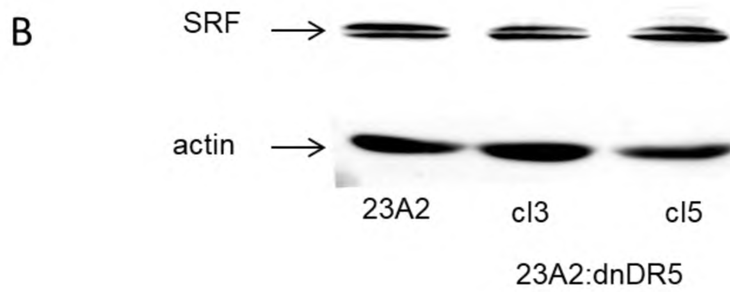

Figure 3B. dnDR5 Expression does not affect Total SRF Protein Expression. In (B), equal cell numbers were plated and the next day lysates were prepared and subjected to SDS-PAGE. Western analysis was performed using anti-SRF or anti-β-actin (loading and transfer control) and visualized as described in Methods. Shown are the results of one experiment that is representative of two independent experiments.

from this element after the onset of differentiation. In addition, the level of phosphorylation seems to be variable before, during and after induction of the c-fos SRE. Phosphorylation at Ser77 and Ser79 has been shown to have little to no effect on the ability of SRF to bind DNA, while phosphorylation at serines 83, 85 and 103 grants SRF high affinity for the c-fos SRE.[91] Ser83 and Ser85 phosphorylation determine the rate of binding in studies of the binding kinetics of SRF to the c-fos SRE; the original studies indicate that ser103 phosphorylation is of comparable significance to SRE association as all four other possible phosphorylation events combined.[92] Thus, phosphorylation at ser103 is a useful indicator of SRF activation leading to association and transcriptional activation of SREs.

We sought to measure SRF activation by measuring phosphorylation of SRF by Western analysis using a phospho-specific (ser103) SRF antibody (Cell Signaling). When 23A2dnDR5 cells were compared with 23A2 parental cells, we observed a reduction in phosphorylated SRF in dnDR5 cells when normalized to a beta-actin control (**Figure 4**), suggesting that activation of SRF in these cells was reduced, and suggesting a loss of transcriptional activation of the MyoD CArG box.

It has been reported that phosphorylation of SRF at serines 77, 79, 83 and 85 is accomplished by casein kinase II (CKII) in serum-activated cells.[93] Though our understanding of the kinases and signaling pathways involved in the phosphorylation of SRF is not yet complete, it has been well-established that SRF can be activated via

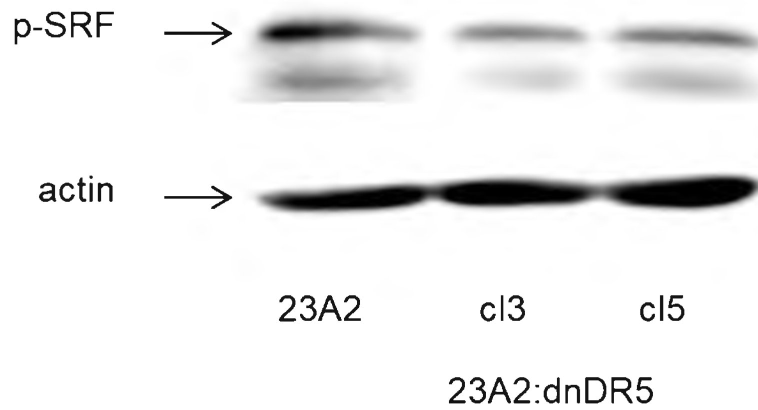

Figure 4. dnDR5 Expression Decreases Specific Phosphorylation of SRF. Equal cell numbers were plated and the next day lysates were prepared and subjected to SDS-PAGE. Western analysis was performed using anti-phospho SRF (ser103) or anti-β-actin (loading and transfer control) and visualized as described in Methods. Shown are the results of one experiment that is representative of three independent experiments.

phosphorylation by MAPKAP kinase 2 (MK2), which is primarily activated by p38 MAPK, but can also be activated by c-jun N-terminal kinase (JNK).[94] The MAPK p38 belongs to a family of serine/threonine kinases (including JNK) that are potently activated by cellular stress and growth factor signaling but weakly activated by mitogens. p38 is activated by phosphorylation at Thr-180 and Tyr-182 by the MAPKKs 3 and 6.[95] Since phosphorylation of p38 is widely considered to be indicative of its activation, and p38 activation could lead to phosphorylation and activation of SRF (and its subsequent enrichment at the MyoD DRR), we decided to assess the level of p38 phosphorylation in parental and dnDR5 myoblasts. Using a phospho-specific antibody (Tyr-182) which is specific for the p38 alpha and beta isoforms, we observed a reduction in the amount of phosphorylated p38 in dnDR5-expressing myoblasts as compared with parental myoblasts when normalized to a bet-actin control **(Figure 5A)**.

To confirm that this observed change in phosphorylation of p38 reflects a change in specific activity and is not due to a change in overall protein expression, we analyzed total p38 expression by Western blotting using an antibody specific for all four p38 isoforms. We were not able to observe any significant difference in total p38 expression between parental myoblasts and 23A2 dnDR5 myoblasts when normalized to a beta actin control **(Figure 5B)**.

In an attempt to confirm the role of p38 signaling in the maintenance of SRF activation, we elected to assess the levels of activated, phosphorylated SRF in parental

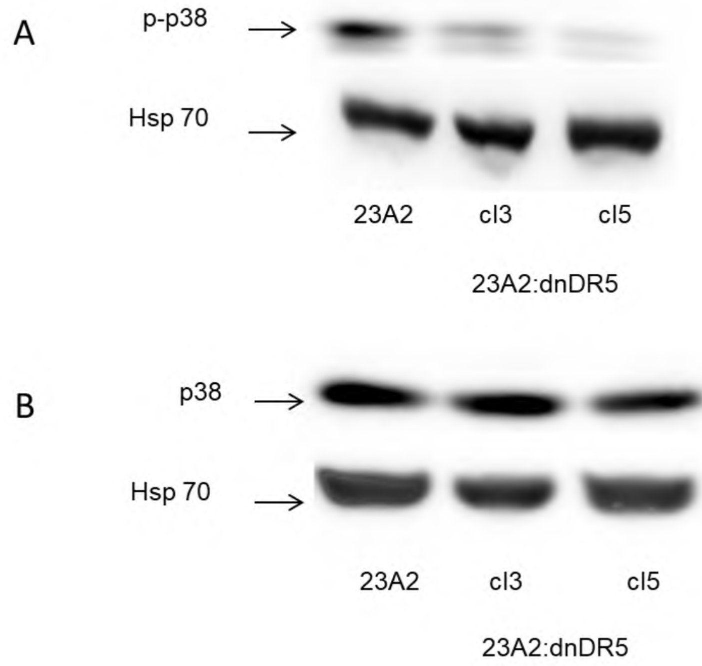

Figure 5A and 5B. dnDR5 Expressing Myoblasts Possess Decreased Levels of Specifically Phosphorylated p38. For each, equal cell numbers were plated and the next day lysates were prepared and subjected to SDS-PAGE. Western analysis was performed using anti-phospho p38 (Thr180/Tyr182) (A), anti-p38 (B), or anti-Hsp70 (loading and transfer control) and visualized as described in Methods. Shown are the results of one experiment that are representative of three independent experiments for (A) and two independent experiments for (B).

myoblasts treated with a pharmacological inhibitor of p38. 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole (SB203580) is a membrane-soluble pyridinyl imidazole that inhibits the activation of p38 alpha and beta by binding to its ATP binding pocket. SB203580 does not affect the threonine 180 or tyrosine 182 phosphorylation of p38 alpha or beta, and thus does not affect activation of these isoforms, but rather inhibits their ability to phosphorylate downstream substrates. SB203580 also does not suppress signaling through other MAPKs and is therefore an effective tool for the investigation of p38 and MK2 regulatory pathways. In order to confirm that p38 signaling controls SRF phosphorylation/activation in our system, 23A2 parental myoblasts were cultured in the presence of 6 μM SB203580 for 3 to 6 hours. We observed, at the minimum, a 40% decrease in SRF phosphorylation at ser103 as analyzed by Western blot analysis using lysates from these cultures and an antibody specific for SRF phosphorylation at ser103. This reduction mimicked the inhibition of SRF phosphorylation observed in 23A2 dnDR5 cells. We chose to treat parental myoblasts with SB203580 for 3 to 6 hours because others have shown that 3 to 6 hours of SB treatment is sufficient to abrogate p38 signaling and reduce injury in embryonic rat heart–derived cells and adult rat cardiocytes during simulated ischemia.[96] We normalized to HSP-70 loading control **(Figure 6A)**. This result was not due to an effect of SB on total SRF **(Figure 6B)**.

We next hypothesized that if p38 signaling controls SRF activation, then treatment of parental myoblasts with SB203580 should result in a reduction of MyoD protein and

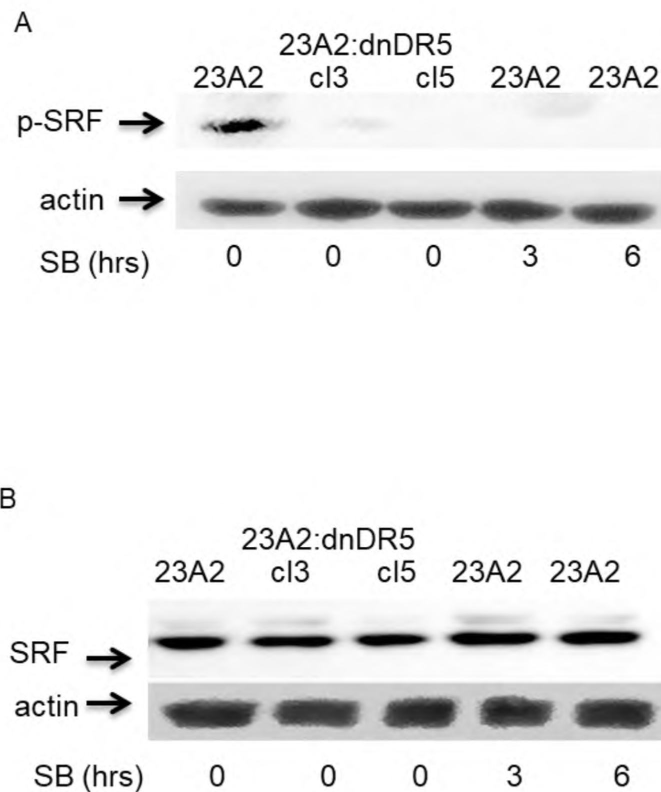

Figure 6A and 6B. Myoblasts Treated with a p38 Inhibitor Possess Decreased Levels of Specifically Phosphorylated SRF. For each, equal cell numbers were plated and the next day treated (or not) with SB 203580 for the indicated times. In (A) and (B), Western analysis was performed using anti-phospho SRF (ser103) or anti-SRF, respectively with anti-actin serving as the loading and transfer control. Bands were visualized as described in Methods. Shown are the results of one experiment that are representative of three independent experiments for (A) and two independent experiments for (B).

mRNA. To test this hypothesis, we collected total cellular RNA using a Trizol extraction protocol from proliferating parental myoblasts cultured in the presence or absence of SB203580 for 3 to 6 hours, followed by RT-PCR. We also collected total protein lysates from parallel culture plates under the same conditions. We observed a reduction of MyoD expression by Western blot under these conditions which was comparable to the level of impairment of MyoD protein expression in proliferating, untreated dnDR5-expressing myoblasts when these results were normalized to a beta-actin loading control (**Figure 7**). RT-PCR of Trizol extracted lysates revealed a corresponding decrease in MyoD mRNA in SB203580 treated cells as compared with untreated myoblasts (**Figure 8**).

Finally, we sought to show that the observed reduction MyoD expression and SRF activation in the presence of an inhibitor of p38 activity is a function of reduced enrichment of SRF at the non-canonical CArG box in the MyoD DRR. We treated parental myoblasts with SB203580 for 3 hours and performed ChIP analysis on treated and untreated cells, as well as dnDR5-expressing myoblasts for a more direct comparison. We found that SRF recruitment to the CArG in SB203580 treated parental cells is reduced at a level comparable to that seen in untreated dnDR5 myoblasts (**Figure 9**).

3.2 Pharmacological Inhibition of Caspase Signaling Mimics Expression of dnDR5

We next sought to prove that disruption of apoptotic signaling, such as that conferred by expression of dnDR5, was responsible for the observed decrease in p38

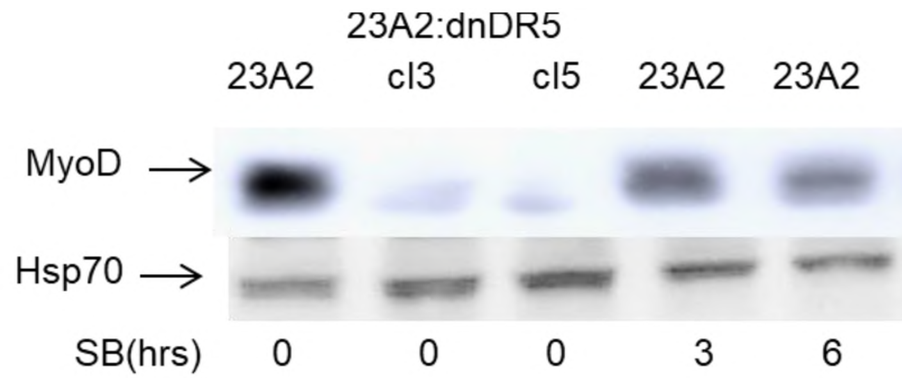

Figure 7. Myoblasts Treated with a p38 Inhibitor Possess Decreased Levels of MyoD Protein. Equal cell numbers were plated and the next day treated (or not) with SB 203580 for the indicated times. Western analysis was performed using anti-MyoD or anti-Hsp70 (loading and transfer control) and visualized as described in Methods. Shown are the results of one experiment that are representative of three independent experiments.

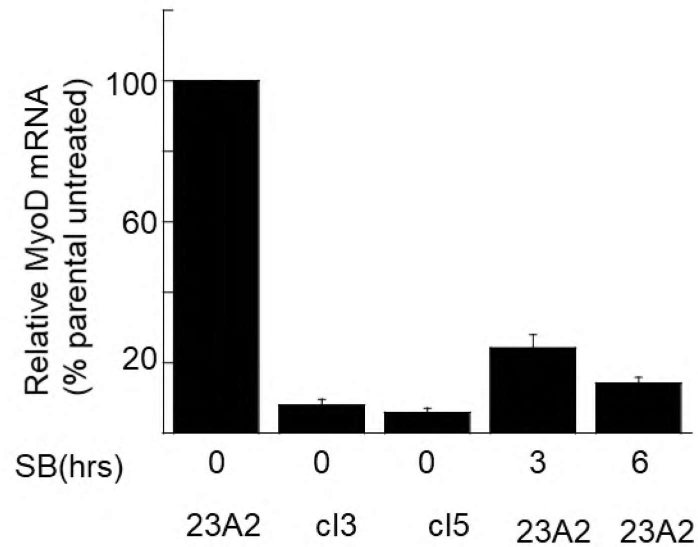

Figure 8. Myoblasts Treated with a p38 Inhibitor Possess Decreased Levels of MyoD mRNA. Quantitative RT-PCR was used to assay for the relative levels of MyoD mRNA in total RNA samples derived from the indicated cell cultures. The Ct values for the MyoD PCR product were normalized to the Ct values for a β-actin product, run in parallel, as described in Methods. Error bars represent mean +/– SEM from triplicates.

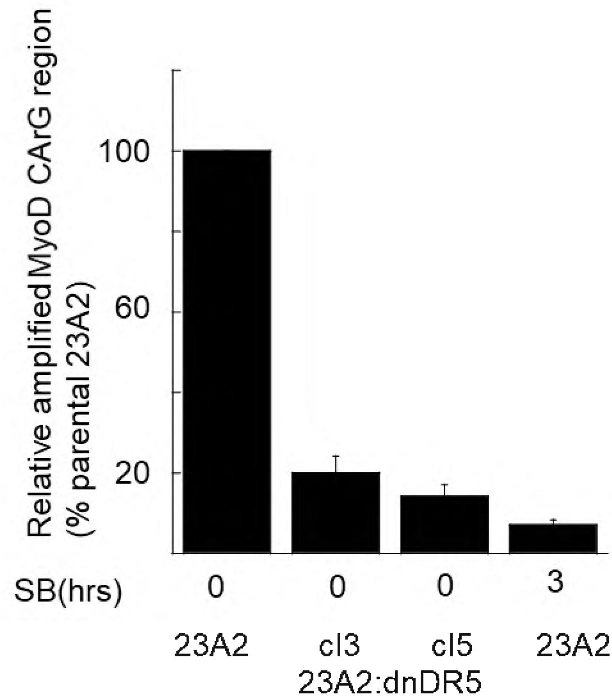

Figure 9. Myoblasts Treated with a p38 Inhibitor Possess Decreased Levels of Binding to the CArG Box in the DRR of the MyoD Enhancer. Chromatin from 1×10^7 cells was cross-linked and digested with MNase to a length between 500–1000 bp. Chromatin Immunoprecipitation was performed on each cell sample using EZ ChIP™ Chromatin Immunoprecipitation Kit (Upstate) per manufacturer's instructions. Chromatin from 2×10^6 cells was immunoprecipitated as described in Methods. Quantitative PCR was used to assay for the relative levels of SRF binding near MyoD CArG element. Data was normalized to the signal detected from the input of each sample and presented as a percent of the signal obtained from parental 23A2 myoblasts. Error bars represent mean +/– SEM of triplicates.

activation. We chose to employ Z-DEVD-fmk (DEVD), a cell-permeable inhibitor of caspase 3 and caspase 7. This inhibitor is so named because it binds covalently and irreversibly to the active site of caspases 3 and 7, which recognize as their substrate the four amino acids in the moniker of the inhibitor (DEVD). The inhibitor also contains a fluoromethyl ketone that cannot be hydrolyzed, effectively rendering the active site of these caspases inoperative. DEVD is commonly used in studies of apoptosis because it is a potent inhibitor with no measurable cytotoxic effects, even when used at relatively high doses.[97] Our goal was to mimic the effects of dnDR5 expression in parental myoblasts.

We cultured parental cells in the presence or absence of 2 μM DEVD for 3 to 6 hours, collected whole cell lysates of parental cells along with dnDR5-expressing myoblasts and subjected them to Western blotting. We found that treatment with DEVD produced a similar inhibitory effect on p38 phosphorylation as did dnDR5 expression (**Figure 10A and 10B**), and that this effect was not due to a change in overall p38 expression. We also discovered a corresponding decrease in expression of phosphorylated SRF as compared with untreated parental myoblasts, and a similar pattern in dnDR5 myoblasts under the same conditions (**Figure 11A and 11B**). When we assessed MyoD mRNA expression by Trizol extraction and RT-PCR, we found that caspase inhibition also reduced MyoD mRNA by an amount comparable to that observed in dnDR5 myoblasts (**Figure 12**). Cells were then cultured in the presence or absence of DEVD and lysates subjected to SDS-PAGE for the purposes of assessing MyoD protein levels for comparison with dnDR5-expressing cells. We discovered that caspase inhibition reduced MyoD

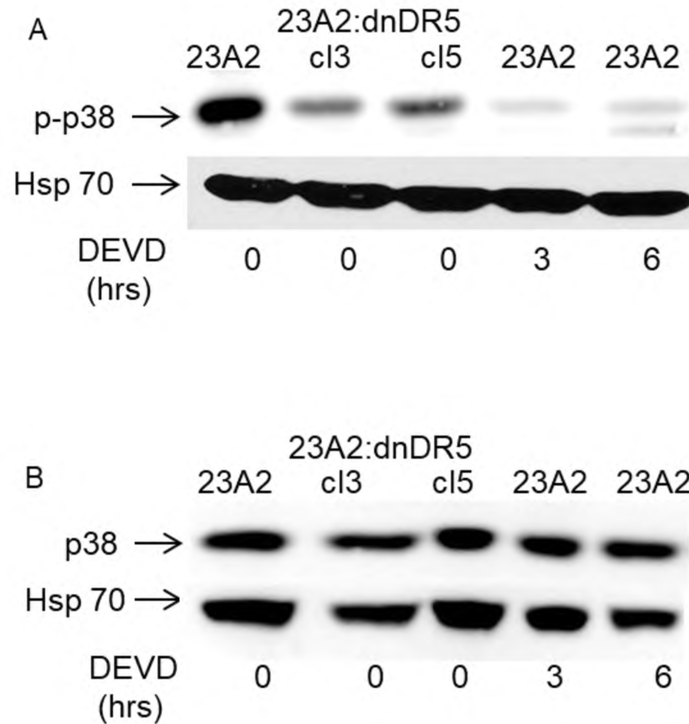

Figure 10A and 10B. Myoblasts Treated with a caspase 3 Inhibitor Possess Decreased Levels of Specifically Phosphorylated p38. For each, equal cell numbers were plated and the next day treated (or not) with DEVD-fmk as indicated in Methods. After the indicated times, lysates were prepared and subjected to SDS-PAGE. Western analysis was performed anti-phospho p38 (Thr180/Tyr182) (A) or anti-p38 (B). Western analysis using anti-Hsp70 served as the loading and transfer controls. Bands were visualized as described in Methods. Shown are the results of one experiment that are representative of three independent experiments for (A) and two independent experiments for (B).

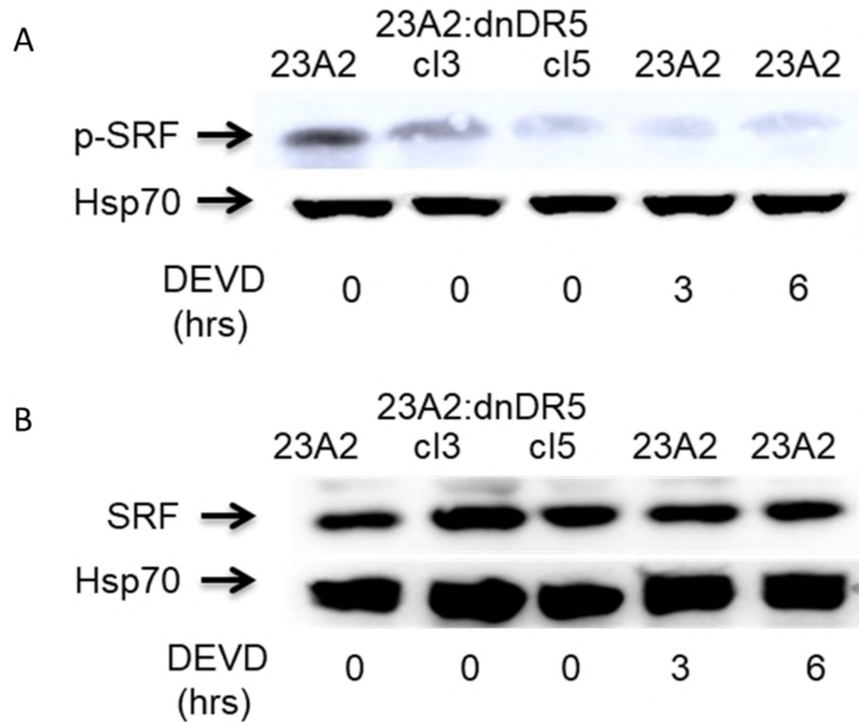

Figure 11A and 11B. Myoblasts Treated with a caspase 3 Inhibitor Possess Decreased Levels of Specifically Phosphorylated SRF. For each, equal cell numbers were plated and the next day treated (or not) with DEVD-fmk as indicated in Methods. In (A) and (B), Western analysis was performed using anti-phospho SRF (ser103) or anti-SRF, respectively with anti-Hsp70 serving as the loading and transfer control. Bands were visualized as described in Methods. Shown are the results of one experiment that are representative of three independent experiments for (A) and two independent experiments for (B).

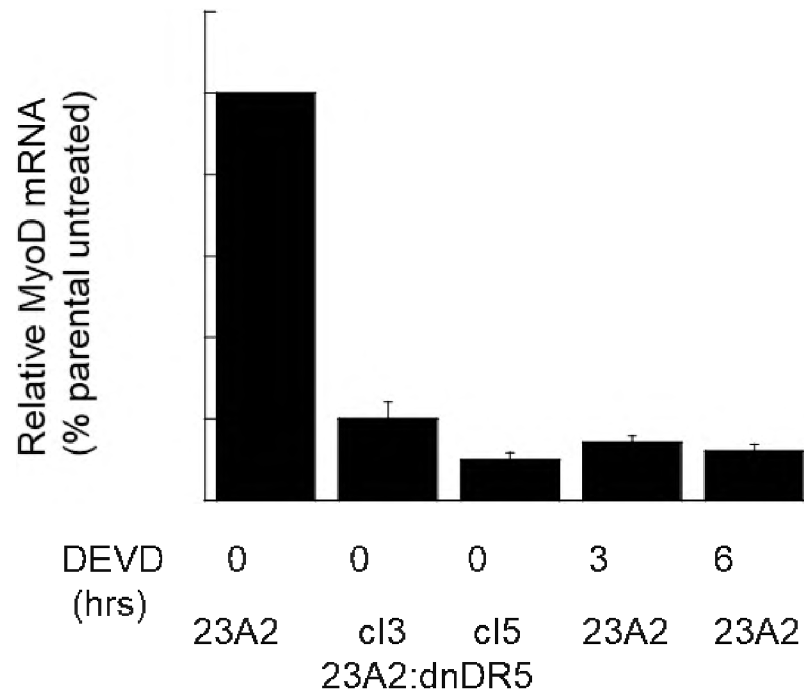

Figure 12. Myoblasts Treated with a caspase 3 Inhibitor Possess Decreased Levels of MyoD mRNA. Quantitative RT-PCR was used to assay for the relative levels of MyoD mRNA in total RNA samples derived from the indicated cell cultures. The Ct values for the MyoD PCR product were normalized to the Ct values for a β-actin product, run in parallel, as described in Methods. Error bars represent mean +/– SEM from triplicates.

protein expression in 23A2 myoblasts to a degree similar to cells stably expressing dnDR5 (**Figure 13**).

To confirm that basal caspase signaling regulated SRF recruitment to the MyoD CArG box, we treated parental myoblasts with DEVD for 3 hours and compared them with untreated parental cells and dnDR5 myoblasts by ChIP analysis. We observed reduced enrichment of SRF at the CArG box in dnDR5 cells as well as in DEVD-treated myoblasts as compared with untreated parental cells (**Figure 14**).

3.3 Investigation of an Alternative Molecular Link between dnDR5/caspase Signaling and MyoD Expression

A target of caspase activity which plays a role in p38 signaling is the serine/threonine kinase mammalian sterile 20-like kinase 1 (Mst1). This MAPKKKK was first identified in yeast and is involved in the mating pathway. Mst1 is a 55 kda enzyme that is cleaved by caspase-3-mediated proteolysis in response to staurosporine treatment, Fas ligand stimulation and other apoptotic stimuli.[98] The autophosphorylation of Mst1 is required for caspase 3-mediated cleavage; in fact, it has been shown that pharmacological caspase inhibition prevents the cleavage of Mst1.[99] Mst1 contains an N-terminal kinase domain and a C-terminal regulatory domain. Caspase-mediated cleavage of Mst1 removes the C-terminal regulatory domain, resulting in a 37 kda N-terminal cleavage fragment, and the activated peptide has been shown to activate many

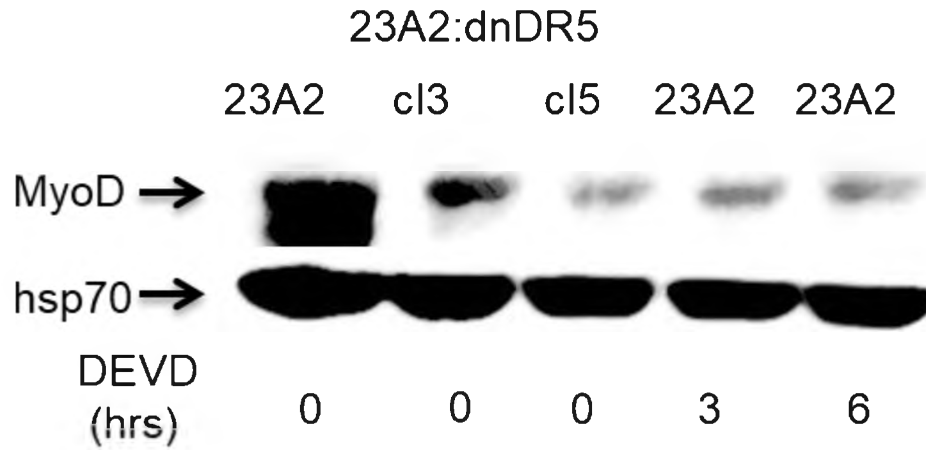

Figure 13. Myoblasts Treated with a caspase 3 Inhibitor Possess Decreased Levels of MyoD protein. Equal cell numbers were plated and the next day treated (or not) with DEVD-fmk for the indicated times. Western analysis was performed using anti-MyoD or anti-Hsp70 (loading and transfer control) and visualized as described in Methods. Shown are the results of one experiment that are representative of three independent experiments.

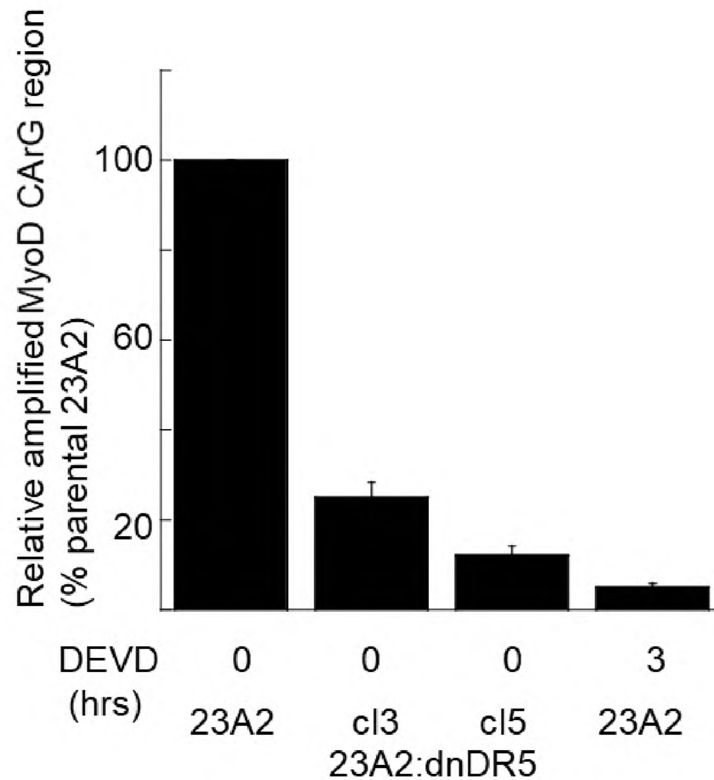

Figure 14. Myoblasts Treated with a caspase 3 Inhibitor Possess Decreased Levels of SRF Binding to the CArG Box in the DRR of the MyoD Enhancer. Chromatin from 1 × 10^7 cells was cross-linked and digested with MNase to a length between 500–1000 bp. Chromatin Immunoprecipitation was performed on each cell sample using EZ ChIP™ Chromatin Immunoprecipitation Kit (Upstate) per manufacturer's instructions. Chromatin from 2 × 10^6 cells was immunoprecipitated as described in Methods. Quantitative PCR was used to assay for the relative levels of SRF binding near MyoD CArG element. Data was normalized to the signal detected from the input of each sample and presented as a percent of the signal obtained from parental 23A2 myoblasts. Error bars represent mean +/– SEM of triplicates.

MAPK proteins, including the MAPK p38.[100] However, the full length Mst1 protein is almost exclusively cytoplasmic, suggesting that cleavage of Mst1 is required for the translocation of the active fragment to the nucleus.[101] Indeed, the 37 kda cleavage fragment has been reported to have almost unfettered nuclear access.[102]

We therefore supposed that if basal caspase signaling through Mst1 to p38 is responsible for p38-mediated maintenance of MyoD protein levels in proliferating myoblasts, then reduction of caspase signaling (i.e. expression of dnDR5) should result in reduction of a constitutively cleaved Mst1 C-terminal fragment and an increase in total full-length protein. To test this supposition, we subjected 23A2 myoblasts and dnDR5-expressing myoblasts to Western blot analysis using an antibody specific to an epitope on the N-terminal end of Mst1. We were unable to reproducibly detect the cleavage fragment but were able to observe a modest increase in full length protein in dnDR5 myoblasts as compared with parental myoblasts **(Figure 15)**.

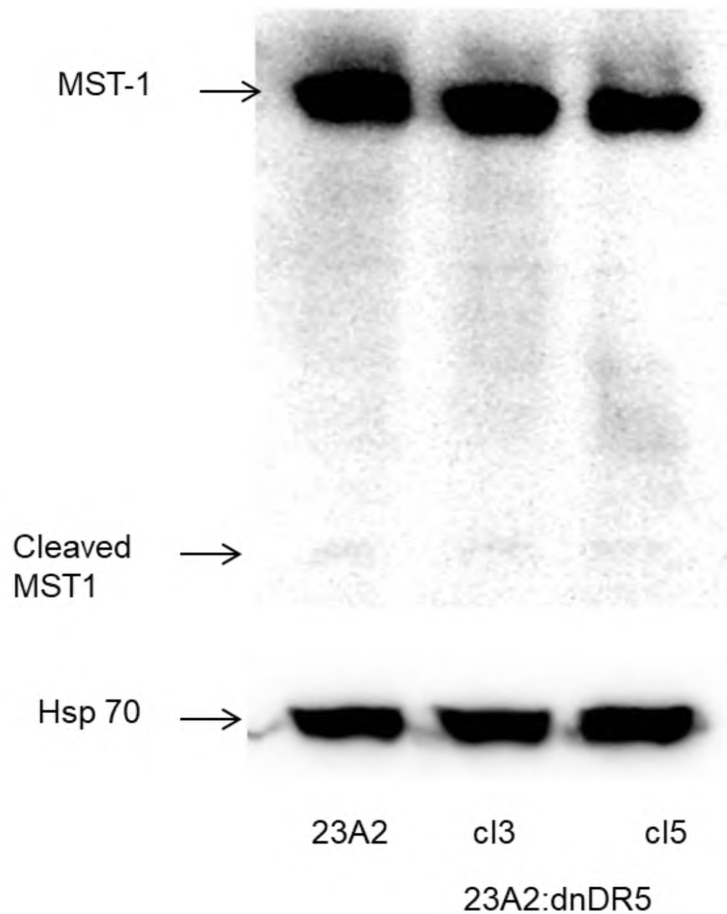

Figure 15. Effect of dnDR5 Expression on Cleavage Activation of MST1. Equal cell numbers were plated and the next day lysates were prepared and subjected to SDS-PAGE. Western analysis was performed using anti-MST1 or anti-HSP 70 (loading and transfer control) and visualized as described in Methods. Shown are the results of one experiment that is representative of two independent experiments.

CHAPTER IV

DISCUSSION

4.1 Myoblast transfer as a therapeutic approach

Adult skeletal muscle precursor cells exist as satellite cells in muscles, and are largely located on the periphery of myofibers, beneath the basal lamina and near the endomysium.[103] The regeneration of skeletal muscle initially depends upon the activation and proliferation of these satellite cells as myoblasts. Myoblasts existing in adult tissues are characterized by the expression of pax7 and MyoD, and may be isolated and proliferated ex vivo for the purposes of myoblast transfer therapies to treat a variety of muscular degenerative diseases. Of the muscular dystrophies, Duchenne Muscular Dystrophy (DMD) is the most common, arising from a mutation in the gene encoding dystrophin, a cytoplasmic protein which complexes with a number of transmembrane glycoproteins in the muscle fiber in order to connect the cytoskeleton with the extracellular matrix. Among other responsibilities involving transport and signal transduction, this complex also serves to protect the membrane of the mature muscle fiber from shearing

stress caused by multiple contractions.[104] Mutations in the reading frame of the dystrophin gene results in the loss of functional dystrophin protein that characterizes DMD. Other mutations giving rise to partial functionality of dystrophin result in a milder phenotype, termed Becker Muscular Dystrophy (BMD).[105]

Available treatment options for DMD are limited to a small number of inefficient strategies. Currently, the primary treatment is glucocorticosteroid therapy, which is accompanied by any number of well-established and severe side effects of consistent steroid usage (e.g. weight gain, reduced bone density, hormone irregularities resulting in secondary diseases, etc.), and offers only meager benefit.[106,107] Existing gene therapies using Adeno-Associated Virus (AAV) vehicles are limited, as all gene therapies currently are, by the potential of innate immune responses to the vectors. Other gene therapies such as exon skipping (which employs an antisense oligonucleotide specific for the mutated region, allowing for the mutated exon to be "skipped" during translation, restoring the reading frame, and resulting in the expression of an internally deleted but mostly functional protein) can only be effective in a subset of DMD patients (approximately 13%), and result in a more BMD phenotype.[108] Myoblast transfer therapies offer the differentiation and fusion of healthy myoblasts with dystrophic muscle fibers, which are then able to produce the full length, functional dystrophin protein, causing restoration of muscle strength and function. However, the induced immune response and rejection of myoblasts infused from culture limits the number of myoblasts available to undergo terminal differentiation and fusion. This problem is being addressed by the use of

immunosuppressants coupled with strategies to reduce the toxicity and pitfalls of long-term immunosuppression.[109]

4.2 Coordinate regulation of differentiation and apoptosis

Nonetheless, the main problem with myoblast transfer therapies remains poor survivability of transplanted cells, independent of host rejection.[110] We and others have reported that myoblast differentiation consistently results in the apoptosis of a predictable subset (30%) of the induced population. Thus, a better understanding of molecular mechanisms surrounding the apoptosis associated with differentiation could improve its potency.

An overwhelming amount of evidence has supported the idea that differentiation and the associated apoptosis are coordinately regulated processes. However, this coordinated regulation can be divided into two categories. Specifically, a category where apoptotic aspects are part of the differentiation process and a category where, while inclusive of the former, apoptosis and differentiation ultimately are mutually exclusive endpoints. As an example of the former, a major component of keratinocyte differentiation is the destruction and elimination of intracellular material, most notably DNA, mitochondria and endoplasmic reticulum.[111] This process has also been observed in the differentiation of embryonic lens fiber cells following serum withdrawal.[112] The breakdown and elimination of intracellular components is obviously the cornerstone of apoptosis.

Skeletal myoblasts are categorized as the latter, where apoptosis and differentiation are ultimately mutually exclusive endpoints. However, their differentiation does involve at least two aspects of apoptosis. The first of these is the fragmentation of DNA as noted above in keratinocytes. However, in myoblasts this fragmentation is early in the differentiation process to allow the expression of p21 and exit from the cell cycle. Specifically, caspase-activated DNase (CAD) plays a major role in apoptosis-related DNA fragmentation. CAD is maintained in an inactive state by direct interaction with the inhibitor of caspase-activated DNase (ICAD). In response to apoptotic signaling, ICAD is cleaved by caspase 3, releasing CAD to generate its characteristic fragmentation of DNA. CAD inhibition blocks differentiation in C2C12 myoblasts, and myoblasts expressing these CAD-associated DNA strand breaks show preferential cleavage of the p21 promoter during differentiation. This transient strand break formation is indispensable for p21 expression during differentiation, and in CAD shRNA-expressing myoblasts, p21 expression is inhibited.[113] Thus, caspase signaling is required for p21 upregulation during myogenesis.

The second example involves plasma membrane reorganization. During apoptosis, this process involves the exposure of phosphatidylserine residues into the extracellular space, signaling for the clearance of these cells by the immune system. However, during the last step of the differentiation of myoblasts, this process is critical for fusion into multinucleated myotubes.[114] Thus, while the breakdown of organelles and reorganization of cellular structure are processes classically recognized as hallmarks of

apoptosis in all somatic cells, these processes are also integral to the completion of the differentiation program in many progenitor cells.

4.3 Regulation of myoblast differentiation by the DR5 pathway

Investigations of the mechanisms controlling differentiation and the associated apoptosis have benefited from the examination of these processes in stable cell lines directly isolated from CH3 mice (such as C2C12 myoblasts) or derived from CH3-isolated multipotential 10T1/2 fibroblasts (23A2 myoblasts). Much of these queries, in turn, have been concerned with the signaling events regulating the induction and completion of terminal differentiation. Interestingly, differentiated myotubes are resistant to apoptosis by mitogen withdrawal while proliferating myoblasts are not. This indicates that the fate of skeletal myoblasts is determined early during the progression of differentiation, certainly prior to myotube formation, but likely prior to the signal to differentiate is even received.

Previous studies have established that MyoD expression is increased in response to mitogen withdrawal in proliferating myoblasts, resulting in the inhibition of proliferation and culminating in enhanced formation of multinucleated myotubes.[115] Other evidence indicates that the loss of MyoD expression in proliferating myoblasts maintains myoblasts in a proliferative state, and inhibits differentiation in response to growth factor deprivation.[116] We have reported that low (but nevertheless detectable) levels of MyoD expression in myoblasts result in delayed, but not abrogated, differentiation.[87] These findings clearly demonstrate that in order for extracellular signaling to culminate in the

induction of differentiation, certain molecular conditions must be previously established within the cell. Chief among these is the requirement of a certain steady state level of MyoD protein. The importance of the cell's maintenance of a tight threshold of MyoD protein expression is highlighted by the fact that new transcription is required for both differentiation and the apoptosis associated with differentiation in skeletal myoblasts. In proliferating myoblasts, MyoD is kept transcriptionally inactive by the presence of Inhibitor of Differentiation (Id), but must be available in sufficient quantity to exert its effect at the induction of differentiation, upon which Id is rapidly degraded. When MyoD levels are not sufficient to respond to differentiation signals, both differentiation and apoptosis are impaired, again reinforcing the importance of this transcription factor in controlling both processes.

It has been well-established that MyoD protein expression is driven in large part by SRF transcriptional activation. SRF is a ubiquitously expressed member of the MADS box family of transcription factors and is implicated in both myoblast differentiation and proliferation. Low affinity binding of SRF to the non-canonical CArG box located in the MyoD DRR SRE in C2C12 myoblasts and activation of MyoD transcription requires multi-protein complexes containing the histone acetyltransferase (HAT) CBP and/or other C/EBP family members.[63] While it has not yet been proven that histone acetylation is required for MyoD protein expression, it is broadly accepted that p300/CBP-mediated HAT activity is required for myogenesis, and it has been reported that deacetylation of histone H4 and the inaccessibility of CArG box DNA inhibits SRF binding to SREs in smooth muscle cells.[117] A decrease in SRF recruitment would lead to a reduction in

CBP activity at the DRR in 23A2 dnDR5 cells, which may account for our observed decrease in the acetylation of histones H3 and H4 at this element in GM. Furthermore, an examination of SRF protein levels in GM reveals little difference in SRF protein levels between parental and dnDR5-expressing myoblasts. These results suggest that basal signaling through the extrinsic TRAIL/DR5 pathway of apoptosis may be required to maintain SRF recruitment levels to the MyoD DRR and/or SRF activity - and, subsequently, MyoD expression - in proliferating myoblasts.

Studies have shown that SRF is required for MyoD gene expression in proliferating myoblasts. Myoblasts stably expressing antisense SRF mRNA, or in which dominant negative SRF proteins are introduced, showed abrogated MyoD expression, resulting in complete inhibition of differentiation.[118] As our lab and others have shown SRF exerts its control of MyoD gene expression by direct interaction with the MyoD-DRR CArG box in a signal-responsive manner. SRF by definition responds to growth factor deprivation, and this response is controlled proteins that are able to influence SRF activity by post-translational modification.

The Weyman lab has reported that constitutive expression of dnDR5 and thus constitutive blockade of the signal through DR5 blocks the apoptosis associated with differentiation in skeletal myoblasts.[87] However, for blockade of this pathway in this manner to be a target for therapeutic manipulation, constitutive blockage cannot interfere with differentiation. Herein, we demonstrate that constitutive blockade of signaling

through DR5, and thus blockage of basal TRAIL/DR5/caspase signaling in proliferating myoblasts, also abrogates MyoD protein levels. Specifically, basal caspase-dependent activation of p38 MAPK causes SRF activation through direct phosphorylation and phosphorylated SRF drives MyoD expression in proliferating myoblasts. An emerging body of evidence supports the idea that, similar to p38, basal caspase signaling is involved in a number of enterprises in myoblasts and acts at multiple stages of myogenesis. For example, in vivo, inhibition of caspase 3/7 blocks muscle regeneration and also blocks the activation of satellite cells, resulting in the accumulation of Pax7 protein.[119] This indicates that caspase activity is required for the downregulation of Pax7, which is a necessary event for satellite cell activation and proliferation in the presence of growth factors.

p38 MAPK is also involved in p21 control of cell cycle exit during myogenesis. TGF-B1 signaling is known to activate p38α (but not p38β). This event results in the phosphorylation of p21. P21 is phosphorylated at serine 130 by both p38α and JNK, but other kinases have been shown to target p21 at different sites. Depending on the site of phosphorylation these phosphorylation events could affect the p21 PCNA-binding domain, nuclear localization signal, CDK binding domain, or cyclin binding regions resulting in a range of functional outcomes including stabilization, destabilization or translocation.[120] The half-life of de-phosphorylated p21 is less than 30 minutes, subjecting this molecule to ubiquitin-mediated degradation at the proteasome.[121] Phosphorylation by p38α and/or JNK stabilizes the molecule, and since the rapid turnover of p21 prevents its initiation of cell cycle arrest, stabilized p21 is free to induce the cell to exit the cell cycle.[120] While

the induction of p21 is necessary for cell cycle exit for differentiation, a basal level of p21 (potentially maintained in part by constitutive p38α phosphorylation) may be required for the inductive response of p21 to growth factor withdrawal and subsequent differentiation. Taken together, these observations suggest that p38 MAPK acts in concert with basal caspase signaling to ensure the readiness of cells for myogenesis.

Our lab has reported that constitutive Ras signaling inhibits both differentiation and apoptosis in skeletal myoblasts. In 23A2 myoblasts, stable expression of G12V:H-Ras, a naturally occurring, constitutively active mutant Ras (A2:G12V:H-Ras myoblasts), results in a transformed, differentiation-defective phenotype.[122] These myoblasts exhibit elevated MAPK activity, and this effect is not due to increased expression of MAPK protein. However, treatment of A2:G12V:H-Ras cells with a highly selective pharmacological inhibitor of MEK1, while reverting their transformed phenotype to mimic that of parental 23A2 myoblasts, does not restore the ability of these cells to undergo differentiation in response to mitogen withdrawal.[123] This suggests that the effect of oncogenic Ras on differentiation and the associated apoptosis in these cells is accomplished via a MAPK-independent pathway. Of relevance to the current topic, oncogenic Ras expression inhibits MyoD protein and mRNA expression in proliferating myoblasts.[124] While a role for p38 in this oncogenic Ras-mediated phenotype was not explored, the Weyman lab has recently discovered that these A2:G12V:H-Ras myoblasts possess reduced levels of SRF and display reduced recruitment of SRF to the CArG box in the DRR of MyoD (Feeback/Weyman, in preparation). Thus, at least two signaling pathways that simultaneously inhibit both differentiation and apoptosis, Ras signaling and DR5

signaling, both target SRF-mediated regulation of MyoD expression, albeit through unique mechanisms.

4.4 Future work: Determine the link between basal caspase signaling and p38 activation

Phosphorylation and activation of p38 kinase can occur in response to a multitude of signaling events.[125] Thus, we would first focus specifically for a mechanism whereby a decrease in caspase activity, such as the inhibition detected upon expression of dn-DR5 in skeletal myoblasts,[87] could lead to a decrease in the phosphorylation of p38 kinase. To this end, we would focus on protein kinase N (PKN) and (MST-1), two kinases capable of phosphorylating and activating p38 kinase that are also activated by caspase-3 mediated cleavage.[126,127,128]

4.5 Other possible mechanisms controlling SRF recruitment to the CArG box in the DRR of MyoD

There are many other mechanisms that might affect SRF recruitment to the MyoD DRR. In addition to CBP/p300, other cofactors can potentiate SRF activity. For example, the major regulator of smooth muscle gene expression, myocardin, is known to bind to and enhance SRF binding and activation of cardiac and smooth muscle genes.[129] In skeletal myoblasts, myocardin-related transcription factors (MRTFs A and B) may perform an analogous role with respect to SRF-inducible genes.[130] In addition, enhancer of polycomb 1 (epcl) has been shown to induce skeletal myoblast differentiation by enhancing SRF-dependent activation of muscle-specific genes.[131] Epc1 is a chromatin protein

found to recruit both SRF and the histone acetyltransferase p300 to the proximal CArG box in the α-actin promoter in murine skeletal myoblasts. Epcl activity is therefore required for the binding of SRF to the α-actin SRE and for the acetylation of associated histones.[132] Moreover, epcl has been shown to potentiate MyoD protein expression induced by SRF by binding directly to SRF and facilitating its association with SREs.[105] However, a link between basal caspase signaling and MRTFs or epc1 has yet to be explored.

Further analysis of the roles of Epcl and SRF in α-actin promoter activity reveals that Ret finger protein (RFP), an oncogenic E3 ubiquitin ligase, interacts with Epc 1 and blocks skeletal muscle differentiation by interfering with the physical interaction between Epc 1 and SRF.[133] Future ChIP experiments comparing parental myoblasts to dnDR5 expressing myoblasts should reveal the influence of epc1 and MRTFs on SRF binding to the CArG element in the MyoD DRR, and any observed difference in epc1 binding may be further elucidated by ChIP assays for the presence of RFP.

Signaling through the Ras family member and small GTPase RhoA is required for the expression of MyoD, but not Myf-5, in proliferating myoblasts. RhoA signaling can lead to the phosphorylation and activation of PI3K, Protein Kinase N (PKN) and Rho Kinase (ROK). Treatment of C2Cl2 myoblasts with C3-transferase results in delayed differentiation and reduced MyoD protein levels.[134]

RhoA transcriptional activation of MyoD during differentiation requires SRF. RhoA regulates SRF activity by activation of PRK-2, which activates the MAPK p38.[135,136] If RhoA is regulated by basal caspase signaling in a manner that reduces SRF recruitment to SREs, then dnDR5-expressing cells may display a decrease in PRK-2 activity.

There is evidence that relative levels of site-specific phosphorylation (at ser-162) in the SRF MADS box is correlated with reduced activation of SRF target genes. This phosphorylation event, mediated by Protein Kinase C (PKC), prevented SRF association with target CArG box elements.[137] It is known that one method of PKC activation involves its cleavage and the subsequent generation of an active fragment by caspases, and it has been reported that TRAIL-induced proteolytic activation of PKC is mediated by caspase activity in smooth muscle cells.[138] Given that SRF association with the MyoD DRR is diminished in dnDR5-expressing myoblasts, it is possible that PKC-mediated phosphorylation of SRF, reduced by the attenuation of basal caspase signaling in dnDR5 cells, plays a role in MyoD protein expression.

The transcription factor and myogenic repressor Kruppel-like factor 4 (KLF4) has been shown to recruit HDAC4 to SRF target genes and block SRF association with methylated histones and with chromatin associated with CArG elements in smooth muscle.[139] Additionally, caspases have been shown to cleave HDAC4, and truncated HDAC4 can translocate to the nucleus and bind and repress Mef-2 activity.[140] If KLF4

is regulated by basal caspase signaling, then, ostensibly, an increase in KLF4 activity and subsequent increase in HDAC recruitment to SREs may help to explain the reduced recruitment of SRF to CArG elements in dnDR5 expressing cells. The resulting decrease in acetylation of CArG box-associated histones may in turn help to explain the reduction in the recruitment of Mef-2 protein to these CArG elements.

4.6 Techniques and Future Strategies

The use of pharmacological inhibition and the expansion and manipulation of stable cell lines provided effective tools for the investigation of the effect of DR5 signaling on MyoD expression and myoblast differentiation. Nevertheless, since this project began, a number of alternative approaches and advances in biotechnology and genomics have made it possible for researchers to more precisely examine the effects of single gene expression on phenotype.

4.6.1 RNA Interference as a Strategy

RNAi is a relatively inexpensive and highly accessible technique for targeted gene silencing, wherein 21-25 nucleotide dsRNA sequences (i.e. short interfering RNAs (siRNAs)) are introduced to cells to reduce protein expression post-transcriptionally. RNAi technology is derived from the observation that post-transcriptional silencing of some genes in C elegans can occur in the presence of double-stranded RNA (dsRNA) [141], and developed using the knowledge that siRNAs cause the silencing of genes in almost all eukaryotic cells by activating the degradation of complementary DNA sequences

[142]. While the therapeutic potential of RNAi has been somewhat limited due to a number of key obstacles such as unanticipated immune responses and nonspecific toxicity [143], the usefulness of RNAi as an investigative tool is undeniable.

By the most well-known pathway, once siRNAs are introduced into mammalian cells, they are bound by Argonaute (AGO) proteins to form the ribonucleoprotein complex termed the RNA-induced silencing complex (RISC). After the siRNA duplex is separated and the passenger strand is degraded, the "mature" RISC complex, comprised of AGO proteins and the guide strand, can associate with complementary mRNA. Catalytically active AGO proteins then cleave the target mRNA sequence [144]. Since this process requires near perfect complementarity, and the design of and production of individual siRNAs is now trivial (in addition to the fact that the RISC machinery exists in all mammalian cells), targeting specific mRNA for degradation using siRNA has become a powerful and high-throughput method for gene silencing at the post-transcriptional level.

The transient nature of siRNA transfection provides at least a few advantages over the stably transfected cell line studies described herein. For example, we may simply reproduce the observed effects of dnDR5 expression in proliferating myoblasts by using siRNA to knockdown p38α expression, ostensibly resulting in reduced SRF recruitment to the MyoD CArG element and in reduced MyoD expression, although questions regarding the specific activity of phosphorylated p38α would remain. RNAi may also be useful in screening for potential cofactors upstream in our system, such as epc1 or MRTFs, to

observe the effects of the transient loss of these molecules at the onset of differentiation in our system.

In yet another application, siRNA is frequently used as a control for other forms of gene silencing. For example, Gauthier-Rouviere et al. observed that reduction of SRF expression by SRF antisense oligonucleotides (ASO) results in inhibition of MyoD expression. This was observed in a C2C12-derived cell line (anti-6) stably transfected with a dexamethasone-inducible plasmid containing SRF ASO. However, the group reported that white dexamethasone treatment resulted in dramatically reduced SRF and MyoD expression in anti-6 cells, untreated cells showed a smaller, yet measurable, inhibition of both SRF and MyoD expression, attributed to a leaky promoter.[145] Using siRNA in C2C12 to knock down SRF expression in parental C2C12 would strengthen the conclusions drawn from these experiments, especially in light of the susceptibility of these single-stranded ASOs to endogenous nucleases.[146]

RNAi has limited effectiveness, however, as an aid in studying complete gene silencing. Off-target effects complicate data interpretation in a number of ways, including: miRNA-like silencing effects of siRNA produced by imperfect complementarity [147], displacement of miRNA from AGO proteins by siRNA, leading to a loss of miRNA-mediated gene regulation [148], and siRNA-induced cytotoxicity [149]. Also, the use of siRNA frequently results in incomplete silencing of the target gene, and while this may afford the ability to study the effect various levels of expression of the gene, the overall

effect of ablation of expression cannot be ascertained. In addition, although recent reports have provided evidence that nuclear RNA can be targeted by siRNA in mammalian cells [150], RNAi largely occurs in the cytoplasm, and thus nuclear transcripts are difficult to manipulate.

4.6.2 Direct Gene Editing as a Strategy

The concept of altering eukaryotic gene expression at the level of transcription was brought into fruition with the discovery of restriction enzymes and the recognition that cloning exogenous DNA into a vector and introducing it to cells may cause the exogenous DNA to be integrated into the host genome by homologous recombination (HR).[151] Later, it was discovered that the introduction of DNA double strand breaks (DSBs) by ionizing radiation or use of nucleases activates endogenous repair mechanisms that result in greatly improved integration of exogenous DNA.[152] One of these mechanisms, the aforementioned process of HR, requires long homologous templates to guide reconstruction of the DNA and depends heavily upon cell type and place in the cell cycle. However, the vast majority of DSBs in mammalian cells are repaired by the predominant of these mechanisms, non-homologous end joining (NHEJ). [153] Because it involves no homologous DNA to guide the repair process, NHEJ is prone to errors and tends to result in random DNA insertions and deletions at the site of the DSB (although researchers have taken advantage of this fact by recognizing that these insertions and deletions generally lead to transcriptionally inactive DNA). Researchers have therefore acknowledged that if DSBs could be focused on the gene of interest and minimized at other loci, NHEJ due to DSB could effectively silence genes at the level of transcription, overcoming some of the

limitations of RNAi. As a result, the recently developed ability to target DSBs more deliberately, and the use of highly specific nucleases to generate DSBs, have revolutionized our capacity to investigate the contribution of a single gene product on phenotype. This has been accomplished by the development of so-called "programmable nucleases" such as ZFNs, TALENs and CRISPR/Cas9 cassettes.

Zinc fingers are small protein motifs, stabilized by the presence of zinc ions, which are able to bind to distinct 3 bp DNA sequences. The adoption of zinc finger technology into gene editing protocols allows the targeting of nucleases to DNA sequences of interest in a highly specific manner when assembled with multiple zinc finger proteins, due to the fact that the assemblage of these multi-unit zinc finger complexes increases the specificity of DNA binding. Typically, 6 or 7 of these zinc finger motifs are complexed with the catalytic subunit of an endonuclease such as Fok1 to form zinc finger nucleases (ZFNs). [154] Since fok1 has no observable sequence specificity, ZNFs and similar programmable nucleases have become highly customizable tools for gene editing. ZFNs may be introduced into cells to generate mutations at the site of the DBS when the cleavage site is repaired by NHEJ. In contrast, when ZFNs are introduced into cells in conjunction with a donor DNA template, ZFN-mediated DSBs result in targeted gene replacement. [155]

The subsequent discovery of transcription activator-like effector (TALE) molecules in the proteobacterium Xanthomonas has led to an even tighter level of specificity of these programmable nucleases. TALEs, unlike zinc fingers which recognize 3 bp sequences,

specifically bind single base pairs. Thus, multiple TALE molecules specific for a target sequence, when complexed with Fok1 endonuclease, produce even higher affinity programmable TALE nucleases (TALENs). Nevertheless, while ZFNs and TALENs offer high frequency and high specificity cleavage of target DNA, there remain a number of drawbacks to these approaches to gene editing. For instance, there is a limit to the targetable sequences for zinc fingers and a redundancy in possible ZFN/DNA interactions, potentially resulting in a number of off-target DNA cleavage events. [156] Due to their higher specificity TALENs have low off-target efficiency and flexibility as compared with ZFNs but it is laborious to construct TALE modules and the endonuclease domain. Fortunately, some of these challenges have been circumvented by the discovery and implementation of CRISPR/Cas9 technologies.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) are small, palindromic repeats of DNA sequences which are integral components of some prokaryotic immune responses to invading viruses. Very simplistically, viral DNA is recognized by the bacterial immune system and inserted into the genome to form a CRISPR region termed a protospacer. The CRISPR region is then transcribed and processed to a mature crispr RNA (crRNA). In the most uncomplicated of the CRISPR pathways, the mature crRNA, with the help of another RNA molecule ("tracrRNA"), directs the nuclease CRISPR-associated protein 9 (Cas9) to foreign DNA sequences which are complementary to the protospacer. Cas9 can then very efficiently cleave the target dsDNA. [157] In the CRISPR/Cas9 gene editing system, programmable crRNA and fixed tracrRNA are combined to form a single guide RNA (sgRNA). This sgRNA guides Cas9 to the target site to create DSBs, which

can then be repaired by HR or NHEJ. The specificity of DNA binding by sgRNA allows CRISPR/Cas9 systems to very precisely edit the host genome and thereby produce a number of mutations, including complete knockout resulting from stop codon formation and reading frame mutations resulting from repair by NHEJ, complete deletion, replacement or inversion of genes upon the introduction of multiple DSBs, and to introduce or correct point mutations.

The latter of these may be of great interest to the topics discussed in this dissertation. Modern gene editing tools such as TALENs and CRISPR/Cas9 systems allow for very precise analysis of the effect of basal DR5 signaling in our system by using these gene editing tools to generate complete DR5 knockout clones, unfettered by the restrictions imposed by the stable expression of dnDR5. Alternatively, these gene editing tool may allow us to study the effect of the described phosphorylation events by phospho-mimetics. [158] For example, the SRF serine and p38α tyrosine phosphorylation events described herein may be further scrutinized by either mutation of SRF ser-103 or p38α tyr-182 to alanine to prevent potential phosphorylation, or by mutation of these residues to glutamic acid or aspartic acid to mimic constitutive phosphorylation. One potential obstacle produced when expressing phospho-mimetic mutants is that their expression belies true physiological conditions. In other words, under normal conditions, phosphorylation is often a transient event, and a single protein may require multiple phosphorylation events to achieve its "intended" function. In order to address these issues, at least in the case of SRF phosphorylation, gene editing tools would allow the editing of the DNA docking site (i.e. the MyoD CArG element) in order to confirm that phosphorylation of SRF is required

for recruitment. [159] The ablation of phosphorylation of these molecules and of the effects of phosphorylation in parental myoblasts may corroborate our findings by leading to reduced SRF enrichment at the MyoD CArG region and reduced MyoD protein expression.

Another intriguing application of these methods involves the analysis of histone modifications such as histone acetylation. Because CRISPR/Cas9 methods are useful for targeting multiple genes simultaneously, they allow the editing of multiple copy genes such as genes encoding for histones. This in turn can produce an unprecedented level of understanding of histone acetylation events, since the editing of specific histones using CRISPR/Cas9 would result the complete and identical modification of each copy of the gene. Histone acetylation is generally studied by mutation of the lysine of interest with a glutamine residue to mimic constitutively acetylated lysine or an arginine residue to mimic constitutively deacetylated lysine. [160] In our system, our observed decrease in the acetylation of histones H3 and H4 at the MyoD CArG element in proliferating dnDR5-expressing myoblasts could be simulated in parental myoblasts by the replacement of target lysine residues with arginine.

Recent advances in CRISPR/Cas9 technology has involved the use of catalytically inactive Cas9 proteins (dCas9) fused to enzymes involved in histone modifications. Thus, an alternative method to mimetic histone mutations might be recruitment of HATs or HDACs to target regions, resulting in locally acetylated histones. It has already been

shown that recruitment of P300 mediated by dCas9 fusion (dCas9-P300) results in an increase in levels of local H3K27 acetylation levels at enhancer elements, and that fusion of dCas9 to full-length HDAC3 effectively silences target genes in various cell types. [161] In this way, we might begin to elucidate the precise mechanisms by which phosphorylated SRF drives MyoD expression.

4.7 Summary

In summary, the Weyman lab has reported that constitutively blocking either signaling by PUMA or signaling through the DR5 pathway blocks the apoptosis associated with differentiation in skeletal myoblasts (**Figure 1**). However, for either pathway to be a target for therapeutic manipulation, their constitutive blockade cannot interfere with differentiation. My early work contributed to the discovery that constitutively blocking signaling through DR5 also blocked differentiation by blocking the expression of MyoD.[87] The bulk of this dissertation has focused on determining that the molecular mechanism involves the blockade of basal signaling from caspases to the activation of p38 followed by the activation of SRF to drive MyoD expression. Since the Weyman lab has recently reported that MyoD also plays a role in the apoptotic process through directly increased expression of PUMA, my work suggests that the regulation of apoptosis by DR5 signaling could be indirect through maintaining MyoD levels such that PUMA can be induced to drive apoptosis. Determination of the contribution of DR5 signaling that is indirect via regulation of MyoD expression and that contribution of DR5 signaling which is direct via abrogation of the elevated DR5 signaling detected during apoptosis awaits

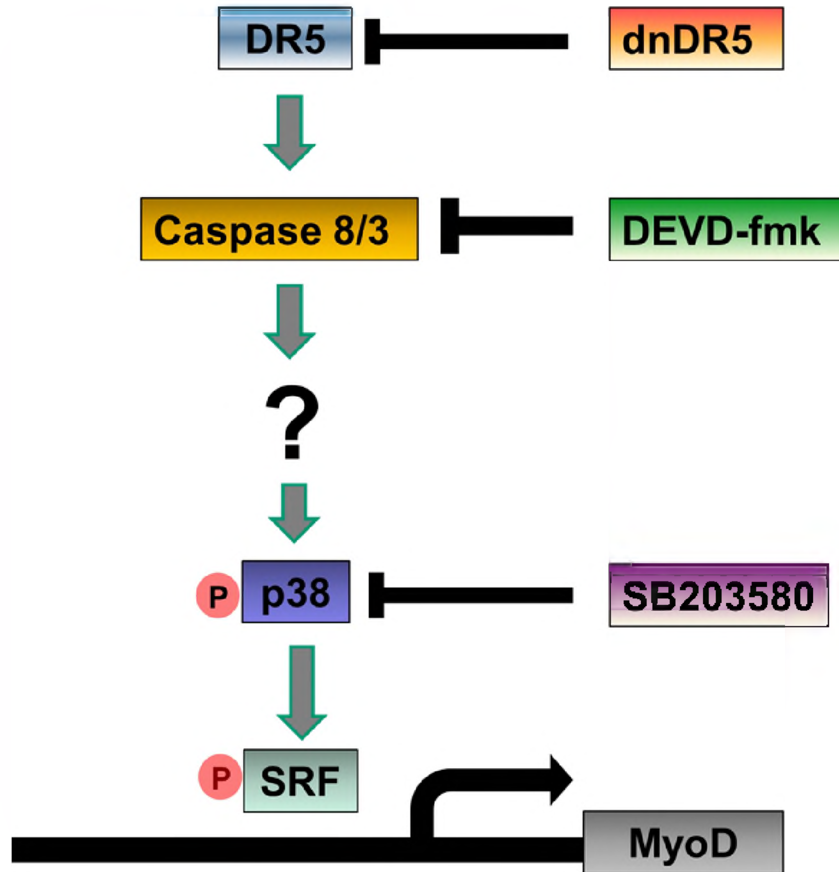

Figure 1. Regulation of MyoD Transcription by dnDR5 Expression. This schematic demonstrates the way in which it was determined that basal DR5 signaling regulates the expression of MyoD protein at the level of transcription. When dnDR5 is expressed, or when proliferating parental cells are treated with a caspase inhibitor or a p38 inhibitor, MyoD protein expression is reduced. This direction is caused by a reduction in activated SRF, and a resulting reduction in activated SRF enrichment at the MyoD CArG region.

investigation utilizing an inducible blockade to DR5 signaling that can either be invoked prior to, or concomitant with, the induction of differentiation and the associated apoptosis.

CHAPTER V

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