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## Transcriptional Regulation of Neurogenic Atrophy-Induced Gene Expression by Muscle Ring Finger-1 and Myogenic Regulatory Factors

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TRANSCRIPTIONAL REGULATION OF NEUROGENIC ATROPHY-INDUCED  
GENE EXPRESSION BY MUSCLE RING FINGER-1 AND MYOGENIC  
REGULATORY FACTORS

By

Theodore Bjorn Olson II

A thesis submitted to the Department of Biology  
in partial fulfillment of the requirements for the degree of  
Master of Science in Biology  
University of North Florida

December 2013

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## Abstract

Skeletal muscle wasting is a consequence of numerous physiological conditions, including denervation, corticosteroid treatment, immobilization, and aging. The E3 ubiquitin ligases, MuRF1 and MAFbx, are induced under nearly all atrophy conditions and are believed to play a key role in protein degradation in atrophying muscle. However, the preliminary data described in this study provides new evidence that MuRF1 may also act as a transcriptional modulator of atrophy-induced gene activity, including the regulation of MAFbx and MuRF1 expression. To characterize the transcriptional regulation of MuRF1 and MAFbx, reporter gene constructs containing fragments of the proximal promoter regions of these genes were developed, transfected into C<sub>2</sub>C<sub>12</sub> cells with or without a MuRF1 expression plasmid and monitored for differences in reporter gene activity. The MuRF1 and MAFbx reporters each showed repressed activity in cells ectopically expressing MuRF1 compared to cells that did not overexpress MuRF1. Furthermore, ectopic expression of the myogenic regulatory factors (MRFs), MyoD1 and myogenin, caused significant activation of the MuRF1 and MAFbx reporter constructs. However, co-overexpression of MuRF1 with MyoD1 or myogenin resulted in reversal of MRF induction of reporter gene activity, and synergistic repression of a constructed E-box reporter system. To further characterize the role of the MuRF1 gene product in repression of MuRF1 expression, a MuRF1 RING domain mutant and a MuRF1 c-terminal mutant were created. The mutant constructs were then co-transfected along with MRF expression plasmids and the MuRF1 reporter construct into C<sub>2</sub>C<sub>12</sub> cells and reporter gene activity was assessed. The MuRF1 RING mutant failed to reverse MRF activation



of the reporter gene, while the c-terminal mutant successfully reversed activation of the reporter gene. These findings suggest that ubiquitin ligase activity is required for MuRF1 transcriptional regulatory effects. These data offer exciting evidence of a potential new function for MuRF1 as a transcriptional modulator of atrophy-induced changes in gene expression.

## Chapter 1: Overview of the Role of MuRF1 in Skeletal Muscle Atrophy

### Introduction

The goal of this thesis was to provide a better understanding of the transcriptional regulation of atrophy-induced genes, called atrogenes. Atrogenes are genes commonly regulated under models of skeletal muscle atrophy, which is caused by a number of physiological conditions including chronic disease, denervation, immobilization, and aging.<sup>1, 2</sup> Key components of the atrophy pathways have been identified; however, the role and regulation of these components are not yet fully understood. The Muscle RING (Really Interesting New Gene) Finger Protein-1 (MuRF1) gene has been identified as a major effector of skeletal muscle wasting.<sup>1</sup> MuRF1 has been classified as an E3-ubiquitin ligase that tags proteins for destruction by the proteasome.<sup>3</sup> MuRF1 is also transcriptionally up-regulated under virtually all atrophy conditions; however the mechanism of its regulation is currently incomplete.<sup>1</sup> Therefore, the goal of this research was to further explore the mechanisms by which MuRF1 is transcriptionally regulated and expand on the role of MuRF1 in the skeletal muscle atrophy signature.

The primary objective of this investigation was to further characterize the transcriptional regulation of MuRF1, as well as its potential downstream effects, including the possibility that MuRF1 coordinates a negative feedback mechanism to transcriptionally down-regulate a subset of atrophy-induced genes, including itself, and a second E3-ubiquitin ligase, Muscle Atrophy F-box (MAFbx). The data presented herein

demonstrates that the mechanism by which MuRF1 transcriptionally regulates a diverse array of atrogenes is mediated by direct and/or indirect interaction and modification of muscle-specific transcription factors, including myogenin and MyoD1. MyoD1 and myogenin sequentially and transiently associate with the promoters of a wide array of muscle-specific genes, and are necessary for the development of functional skeletal muscle and myogenic commitment, respectively.<sup>4, 5</sup> MuRF1 and MAFbx expression rise dramatically during most models of atrophy,<sup>1</sup> as does the expression of MyoD1 and myogenin.<sup>6</sup> Furthermore, MyoD1 and myogenin have been shown to be important regulators of neurogenic atrophy-induced gene expression (i.e. denervation), including the induction of MuRF1 and MAFbx.<sup>6</sup> Our work focused on the cooperative transcriptional regulation of atrogenes through the interaction of MuRF1 and myogenic regulatory factors (MRFs), such as MyoD1 and myogenin. Furthermore, the importance of specific domains of the MuRF1 protein in atrogene regulation was also explored. Specifically, the role of the RING finger domain and the acidic carboxyl-terminal domain of the MuRF1 protein in modulating MRF activity were investigated.

#### MuRF1 as an E3 Ligase In the Ubiquitin-Proteasome Pathway

The findings from this project reveal a potentially new functional role for MuRF1 in the skeletal muscle atrophy cascade. MuRF1 has long been known to be involved in mediation of muscle atrophy, presumably by targeting proteins for degradation via the well-characterized ubiquitin-proteasome system (UPS), which is the main mechanism for degradation of intracellular proteins.<sup>7</sup> Briefly, proteins are first marked by the covalent addition of ubiquitin, a 76 amino acid polypeptide. These tagged proteins are then

degraded into smaller peptides through proteolysis by the 26S proteasome complex (Figure 1).

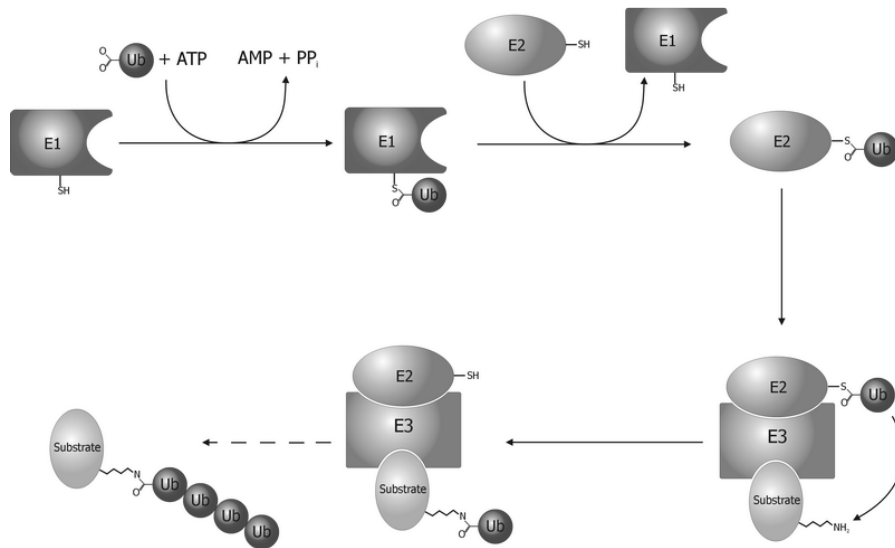


Figure 1. Schematic diagram of the ubiquitin-proteasome system.<sup>8</sup>

The mechanism by which proteins are tagged with ubiquitin involves a three step process. The E1 ubiquitin-activating proteins first establish a form of ubiquitin (Ub) which is highly reactive.<sup>9</sup> After activation, the ubiquitin is forwarded from an E1 to ubiquitin carriers known as E2 proteins.<sup>9</sup> E2 proteins typically exhibit a conserved core region of 16kDa.<sup>10</sup> This region contains cysteine, which is used in the thiol-ester linkage between the activated ubiquitin and the enzyme.<sup>10</sup> The main function of these E2 enzymes is to serve as ubiquitin couriers for E3 ubiquitin-protein ligases, such as MuRF1. The E3 ubiquitin ligases transfer the activated ubiquitin to a lysine residue on the final protein substrate, typically forming long ubiquitin chains which are then recognized by the 26S proteasome. It is likely that it is the E3 ligase which is responsible for the discrimination and accuracy of the process of ubiquitination.<sup>11</sup> The resulting ubiquitin-tagged protein

complex is then broken down into small peptides by the proteolytic activity of the 26S proteasome.<sup>12</sup> In skeletal muscle, this breakdown of protein may contribute to loss of muscle mass. MuRF1, which has been classified as an E3 ubiquitin ligase, has been well characterized as a general marker of skeletal muscle wasting.<sup>1</sup>

#### MuRF1 as a Marker of Skeletal Muscle Atrophy

MuRF1 expression has previously been shown to be induced under different atrophy conditions.<sup>1</sup> This work measured changes in mRNA levels over time in experimental subject mice following atrophy-inducing conditions, including denervation of the sciatic nerve, immobilization of the hind limbs, and hind-limb suspension.<sup>1</sup> Following transcript profiling, Northern blots were used to verify the expression patterns of potential genes of interest (Figure 2).<sup>1</sup> While many genes showed increased expression under one or two conditions, only two genes showed significant up-regulation under all three atrophy conditions. These two key genes are Muscle RING (Really Interesting New Gene) Finger-1 (MuRF1) and Muscle Atrophy F-box (MAFbx).

Image redacted, paper copy available upon request to home institution.

Figure 2. Northern Blot of MuRF1 and MAFbx expression. MuRF1 and MAFbx gene expression in mice are found to increase in expression over time following immobilization, denervation, and hind limb suspension. Numbers represent days post-denervation.<sup>1</sup>

Since MuRF1 is quickly and significantly up-regulated following atrophy-inducing conditions, it is believed to be a major player in the atrophic process via the mediation and subsequent proteolytic degradation of target proteins. Indeed, mice that are deficient in MuRF1 show significant resistance to muscle atrophy following denervation, immobilization, and hind limb suspension.<sup>1</sup> Surprisingly, in the dozen years since their discovery, very few targets of MuRF1 and MAFbx have been characterized leading to a reevaluation of the role of these genes in the atrophy cascade. To this end, data from a microarray comparing gene expression profiles in MuRF1-null and wild-type mice under neurogenic atrophy conditions was conducted and recently published.<sup>6</sup> The results of that investigation suggest a possible transcriptional regulatory role for MuRF1.<sup>6</sup> Therefore, it was the goal of this research to characterize the mechanism by which MuRF1 might act as a transcriptional modulator of atrophy-induced gene expression.

#### Additional Roles of MuRF1 in the Muscle Atrophy Cascade

There are aspects of both MuRF1 and MAFbx that suggest these genes may play additional roles in the atrophy processes. First, these E3 ligases have few known targets within skeletal muscle, and of the known targets, one is a muscle-specific transcription factor called MyoD1.<sup>13</sup> This muscle-specific transcription factor has been shown to be targeted for degradation specifically by MAFbx.<sup>13</sup> Because this tagging of MyoD1 may likely cause other downstream changes in gene expression, this finding supports the possibility that these E3 ligases may play a more global role in gene regulation than previously thought. Furthermore, MuRF1 has been hypothesized to monoubiquitinate some target substrates in cardiac muscle.<sup>14</sup> For example, it has recently been suggested

that MuRF1 monoubiquitinates the peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) and regulates its nuclear localization in cardiac muscle.<sup>14</sup> In contrast to polyubiquitination, monoubiquitination is not commonly believed to cause degradation by the proteasome. Monoubiquitination of proteins more commonly serves to change the protein's structure, function, cellular localization, or serves as a signal for the recruitment and binding of additional transcription factors.<sup>15</sup> The nuclear localization of PPAR $\alpha$  in response to modification by MuRF1 in cardiac muscle gives precedence that MuRF1 could act as a potential regulator of protein function beyond its assumed role of simply targeting proteins for degradation. Therefore, we hypothesized that MuRF1 may serve to tag proteins for further regulation in skeletal muscle in addition to tagging proteins for degradation by the proteasome.

#### Important Structural Characteristics of MuRF1

The first step to better understanding the function and regulation of MuRF1 was the exploration of the proposed three-dimensional structure of its gene product. The different functional domains of MuRF1 suggest that this E3 ligase may have distinct functions in muscle. MuRF1 contains a well-characterized RING domain needed for E3 ligase activity as well as additional domains of interest described below. A RING domain is a zinc finger with a consensus sequence of C-X<sub>2</sub>-C-X<sub>[9-39]</sub>-C-X<sub>[1-3]</sub>-H-X<sub>[2-3]</sub>-C-X<sub>2</sub>-C-X<sub>[4-48]</sub>-C-X<sub>2</sub>-C, in which C represents a conserved cysteine residue, H represents a conserved histidine, and X represents any amino acid. The cysteine and histidine residues are involved in interacting with zinc ions, and RING fingers frequently bind ubiquitination enzymes as well as target proteins in order to facilitate ubiquitination.<sup>16</sup> A

sequence alignment of the MuRF1 protein from mouse, rat, and human is shown in Figure 3.



Figure 3. Alignment of MuRF1 Protein. MuRF1 protein sequences from mouse, rat and human were downloaded from the PubMed database ([www.PubMed.org](http://www.PubMed.org)). The proteins were then aligned using sequence alignment tools (<http://www.ncbi.nlm.nih.gov>).

The RING domain is predicted to perform the catalytic action of MuRF1 as it participates in the ubiquitination processes. The B-box and B-box c-terminal (Bbc) domains are thought to form additional zinc fingers that may play roles in DNA and/or protein binding. The acidic c-terminus of MuRF1 is of special interest and has no known function; however acidic protein tails may have possible roles in the cellular location of proteins, including nuclear translocation.<sup>17, 18</sup>



## MuRF1 as a Transcriptional Regulatory Factor in Skeletal Muscle

Microarray data comparing differential gene expression profiles in MuRF1-null and wild-type mice under denervation conditions suggest that MuRF1 may act as a potential transcriptional regulatory factor in skeletal muscle.<sup>6</sup> The MuRF1 knock-out (KO) mouse was engineered via insertion of a  $\beta$ -galactosidase-encoding lacZ cassette within the MuRF1 gene.<sup>1</sup> Thus, under control of the endogenous MuRF1 promoter, the MuRF1 gene product was expressed in the wild-type mouse, while the  $\beta$ -galactosidase gene product was produced in the knockout mouse. This allowed for quantification of the different gene products (i.e.  $\beta$ -galactosidase vs MuRF1) under control of the endogenous MuRF1 promoter.

MuRF1 gene expression increased in the wild-type mice following denervation; however, it decreased back to baseline by 14 days following denervation (Figure 4A). In contrast, the levels of  $\beta$ -galactosidase increased and remained elevated in the MuRF1-null animals at 14 days post-denervation (Figure 4B).

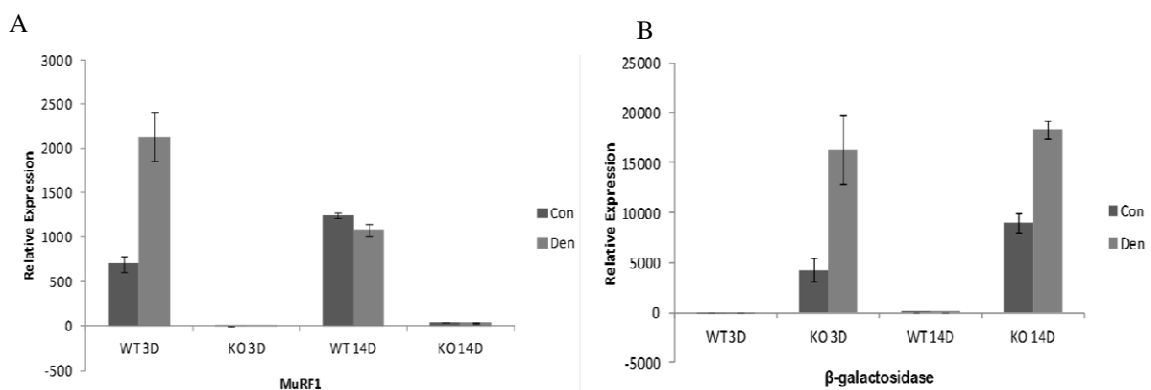


Figure 4. Microarray data showing MuRF1 and  $\beta$ -galactosidase expression in wild-type (WT) and MuRF1-null (KO) mice. (A) The MuRF1 gene expression rises following denervation but returns to baseline by day 14 in WT mice. (B) In contrast,  $\beta$ -galactosidase expression remains elevated at 14 days in KO mice. Whole genome expression analysis was conducted on gastrocnemius muscle from wild-type (WT) and MuRF1-null (KO) mice following 3 days (3D) and 14 days (14D) denervation. Each condition was conducted in triplicate and the expression is the average of three individual mice. Error bars represent  $\pm$  S.E.M of the average expression of the three individual animals from each condition.<sup>6</sup>

The significance of this data rests in the maintenance of elevated expression of  $\beta$ -galactosidase ( $\beta$ -gal) over time in the MuRF1 KO animals, as compared to the transient increase in expression of MuRF1 and the subsequent return to baseline by 14 days post-denervation in the wild-type mice. Because  $\beta$ -gal (in the KO mice) and MuRF1 (in the wild-type mice) are under the control of the same regulatory region, it is reasonable to hypothesize that MuRF1 may feedback and repress its own transcriptional activity.

Furthermore, MuRF1 also appears to be necessary for repression of MAFbx expression following denervation. MAFbx gene activity increases following denervation but returns to baseline by 14 days post-denervation in wild-type mice (Figure 5A). However, in the MuRF1 KO mice, MAFbx expression increases following denervation and remains elevated (Figure 5B).

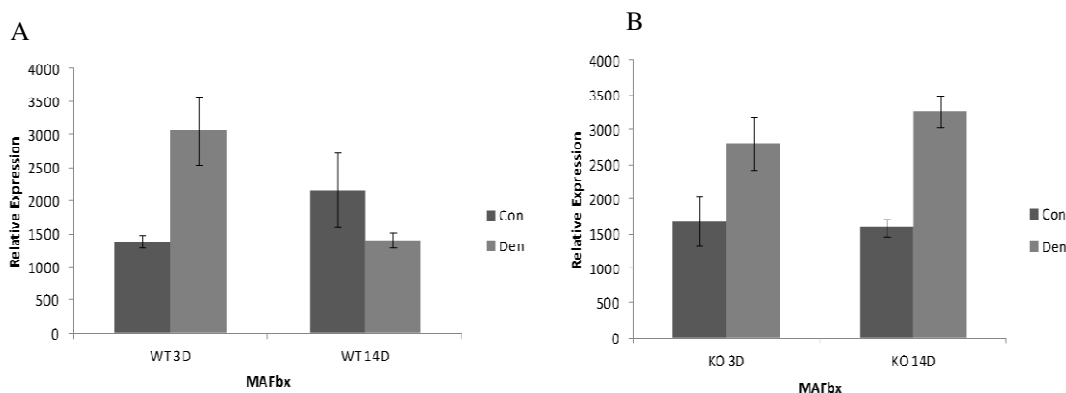


Figure 5. Microarray data showing MAFbx expression in wild-type (WT) MuRF1-null (KO) mice. (A) MAFbx gene expression rises following denervation but returns to baseline by day 14 in WT mice. (B) In contrast, MAFbx expression remains elevated at 14 days in KO mice at 14 days. Whole genome expression analysis was conducted on gastrocnemius muscle from wild-type (WT) and MuRF1-null (KO) mice following 3 days (3D) and 14 days (14D) denervation. Each condition was conducted in triplicate and the expression is the average of three individual mice. Error bars represent  $\pm$  S.E.M of the average expression of the three individual animals from each condition.<sup>6</sup>

In light of the above data, the work described in this thesis explores the potential transcriptional regulatory actions of MuRF1 in skeletal muscle tissue. Specifically, this

research examines the ability of MuRF1 to negatively transcriptionally regulate itself and other atrogenes, including MAFbx. Additionally, because MuRF1 codes for a protein found in skeletal muscle, the potential interaction of MuRF1 with myogenic regulatory factors (MRFs), which are also dramatically up-regulated in response to neurogenic atrophy (i.e. denervation), was also investigated.

### Myogenic Regulatory Factor Regulation of MuRF1 and MAFbx Expression

The transcriptional regulation of MuRF1 and MAFbx by myogenic regulatory factors (MRFs) has been previously analyzed.<sup>19</sup> These transcription factors ultimately act in concert with co-activators or co-repressors to mediate transcription of muscle-specific genes.<sup>20</sup>

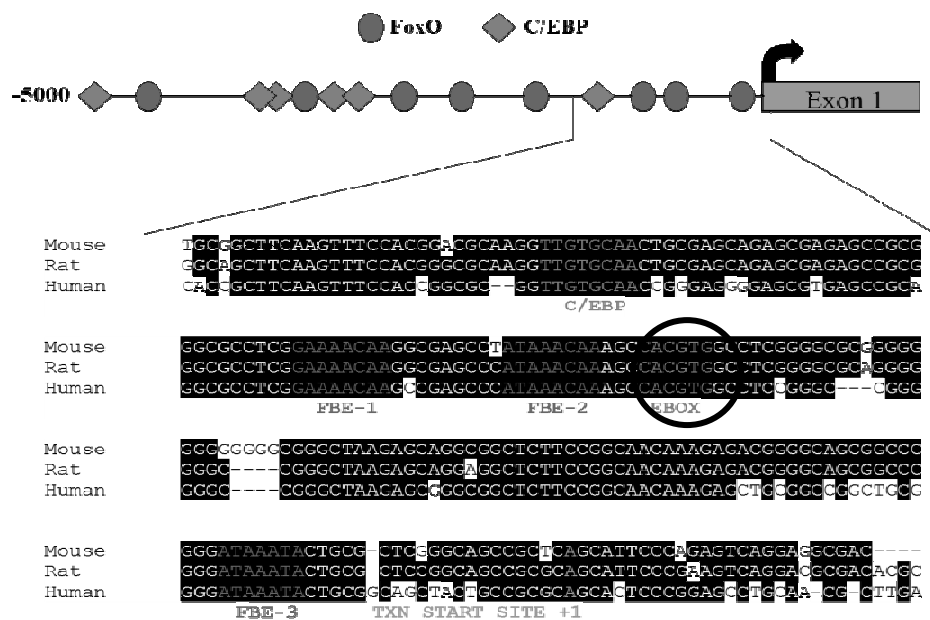


Figure 6. MAFbx Promoter Alignment. Promoter sequences from mouse, rat, and human MAFbx genes (5000 base pairs upstream of the transcription start site (+1) through the first exon) were downloaded from the Ensembl database ([www.ensembl.org](http://www.ensembl.org)) and aligned using the ClustalW algorithm. Identical sequences for the indicated regions are highlighted in black. Approximate positions of potential MRF binding sites are circled in the alignment : O class, or FoxO, Forkhead binding site (G/A)TAAA(T/C)AA (Ovals); C/EBP TT(G/T)NGNAA (Diamonds); Muscle specific E box CANGTG (MyoD, etc.) (Large circle)

Notably, the proximal promoters of both MuRF1 and MAFbx contain E-box consensus sequences (5'-CANNTG-3') which are known sites of MRF binding (Figure 6 and Figure 7).<sup>21</sup> The E-boxes in both the MuRF1 and MAFbx proximal promoters have previously been shown to interact with myogenic regulatory factors, including MyoD1 and myogenin.<sup>22</sup> Furthermore, MyoD1 and myogenin levels increase sharply in response to neurogenic atrophy of muscle and lead to the induction of MuRF1 and MAFbx expression (Figure 8).<sup>6</sup>



Figure 7. MuRF1 Promoter Alignment. Promoter sequences from mouse, rat, and human MuRF1 genes (5000 base pairs upstream of the transcription start site (+1) through the first exon) were downloaded from the Ensembl database ([www.ensembl.org](http://www.ensembl.org)) and aligned using the ClustalW algorithm. Identical sequences for the indicated regions are highlighted in black. Approximate positions of potential transcription factor binding sites are indicated in the schematics and highlighted in the alignments: FoxO, (G/A)TAAA(T/C)AA Ovals); C/EBP TT(G/T)NGNAA (Hexagons); Muscle specific E box CANGTG (MyoD1, etc.) (Large Circles)

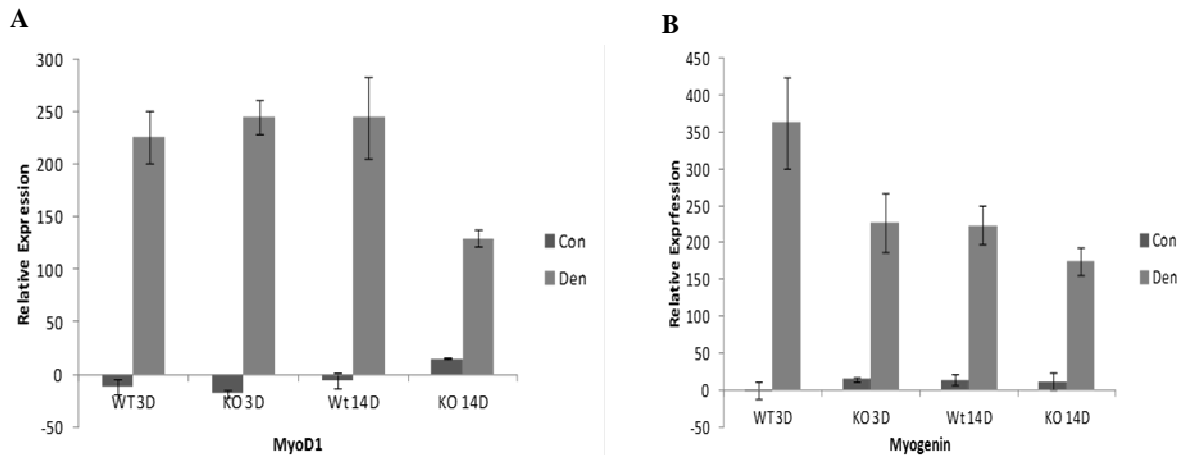


Figure 8. Microarray data showing MyoD1 and myogenin expression profiles under atrophy conditions in wild-type (WT) and MuRF1-null (KO) mice. (A) MyoD1 and (B) myogenin expression levels following 3 days (3D) and 14 days (14D) of denervation. Whole genome expression analysis was conducted on gastrocnemius muscle from wild-type (WT) and MuRF1-null (KO) mice following 3 days (3D) and 14 days (14D) of denervation. Each condition was conducted in triplicate and the expression is the average of three individual mice. Error bars represent +/- S.E.M of the average expression of the three individual animals from each condition.<sup>6</sup>

MyoD1 and myogenin are basic helix-loop-helix (bHLH) transcription factors.<sup>22</sup>

Basic helix-loop-helix transcription factors are characterized by a protein motif consisting of two  $\alpha$ -helices connected by a loop. DNA binding is facilitated by the basicity of the amino acid residues of one of the loops.<sup>23</sup> Myogenic regulatory factors such as MyoD1 and myogenin bind to the 5'-CANNTG-3' E-box consensus sequence, and often play roles in myogenic differentiation and proliferation.<sup>24</sup> For example, MyoD1 is an early marker of myogenic commitment to the skeletal muscle lineage and is known to halt proliferation of muscle cells by increasing transcription of p21, effectively removing cells from the cell cycle.<sup>25</sup> Interestingly, differentiated C<sub>2</sub>C<sub>12</sub> mouse myoblast cells undergoing MyoD1 knockdown have been shown to dedifferentiate and re-enter the cell cycle as proliferating myoblasts.<sup>26</sup> In contrast to MyoD1, myogenin is known to be necessary for proper development of skeletal muscle.<sup>27</sup> Mice without functional myogenin are unable to produce mature functional muscle fibers.<sup>28</sup> Myogenin has previously been shown to bind

to the MuRF1 and MAFbx promoters and mice without myogenin lose the ability to up-regulate MuRF1 and MAFbx following denervation.<sup>22</sup>

Current dogma states that MuRF1 and MAFbx regulate skeletal muscle dynamics by targeting proteins for degradation, but increasing evidence, including data presented in this thesis, suggests that MuRF1 may also function as a muscle-specific transcription factor. In light of the information described herein, the regulation of MuRF1 and MAFbx, as well as the role of MuRF1 as a transcriptional modulator of atrogene regulation was explored. The data in this thesis supports the hypothesis that MuRF1 may act as a transcriptional regulator of muscle-specific gene expression.

## Chapter 2: Transcriptional Repression of Atrogenes by MuRF1 and Myogenic Regulatory Factors

### Introduction

Skeletal muscle is highly organized, and is the most abundant tissue by mass found in the human body. Skeletal muscle tissue is dynamic and is able to readjust its size not only as a response to nutritional status, but also as a response to many other cues including stress, mechanical load, neural activity, hormones and growth factors. While the main function of skeletal muscle is involved in movement and force generation, skeletal muscle also plays a major role in global metabolism and the maintenance of energetic homeostasis. For example, during periods of low nutrition, skeletal muscle tissue is capable of releasing amino acids that are used in the liver to raise glucose levels via gluconeogenesis.<sup>29</sup> Skeletal muscle is necessary for normal metabolic processes, and recent evidence suggests that skeletal muscle may communicate with the rest of the body in order to maintain normal metabolic functions of other tissues and organs.<sup>30</sup>

Skeletal muscle atrophy, or loss of muscle mass, is characterized by a reduction in protein content and fiber diameter, and a concomitant decrease in force production. The E3-ubiquitin ligase, MuRF1 is hypothesized to be a major regulator of the atrophy process. MuRF1 is thought to function in the atrophy cascade since it is expressed

predominantly in skeletal muscle, is up-regulated after varied atrophic stressors, and deletion of the MuRF1 gene leads to resistance of muscle loss following denervation in mice.<sup>1, 22, 31</sup> MuRF1 is well characterized as a participant in the proteolytic pathways; however, there is evidence that it may play additional roles in muscle plasticity. The recently discovered nuclear localization of peroxisome proliferator activated receptor alpha (PPAR $\alpha$ ) via monoubiquitination by MuRF1 in cardiac muscle provides a precedence that the ubiquitin ligase activity of MuRF1 may serve to tag proteins for further regulation in skeletal muscle in addition to just simply tagging them for degradation by the proteasome.<sup>14</sup>

MuRF1 and MAFbx are both up-regulated under numerous conditions of muscle atrophy, but these genes have few known targets within skeletal muscle.<sup>1</sup> Of the known targets, at least one of them is a muscle-specific transcription factor called MyoD1, which has been shown to be targeted for degradation by MAFbx.<sup>13</sup> Because this may likely cause other downstream changes in gene expression, this supports the possibility that these atrophy-induced E3 ligases may also play a role in gene regulation under atrophic conditions.

Microarray data comparing wild-type and MuRF1-null mice under denervation conditions suggest that MuRF1 may be a potential transcriptional regulatory factor in skeletal muscle.<sup>6</sup> MuRF1 gene expression increases in wild-type mice following denervation; however, it decreases back to baseline expression levels by 14 days post-denervation. When the MuRF1 gene is disrupted via insertion of a LacZ cassette, the MuRF1 promoter remains active with no subsequent decline to baseline,<sup>6</sup> suggesting a possible negative-feedback loop in which the MuRF1 gene product causes repression of



its own promoter. In addition, MAFbx gene activity also increases following denervation and returns to baseline expression levels by 14 days in wild-type mice but remains elevated in the MuRF1 KO mice at 14 days post-denervation.<sup>6</sup> Therefore, it is reasonable to propose that MuRF1 expression may lead to the transcriptional regulation of other atrogenes, though the mechanism of this control remains unclear.

There are distinct functional domains of the MuRF1 gene product which might be implicated in the potential role of MuRF1 as a transcriptional regulatory factor. MuRF1 contains a well-characterized RING (really interesting new gene) domain needed for E3 ligase activity. RING fingers frequently bind ubiquitination enzymes as well as target proteins, and are thus predicted to perform the catalytic action of E3-ubiquitin ligase enzymes.<sup>32</sup> The B-box and B-box c-terminal (Bbc) domains of MuRF1 are thought to be zinc fingers that may play a role in DNA and/or protein binding.<sup>33</sup> The acidic c-terminus of MuRF1 is of special interest and has no known function; however acidic protein tails may have a role in cellular localization, including nuclear translocation.<sup>17, 18</sup> Mutations were introduced during our work to inactivate the RING domain or delete the c-terminal domain of MuRF1 to better explore the potential roles of these domains in transcriptional regulation by MuRF1.

The work presented in this thesis examines the potential transcriptional regulatory actions of MuRF1 in skeletal muscle atrophy. Specifically, the ability of MuRF1 to negatively transcriptionally regulate itself and other atrogenes, including MAFbx, was explored using reporter assays to measure MuRF1 and MAFbx expression under various conditions, including in response to overexpression of MuRF1. Additionally, because MuRF1 codes for a protein found in skeletal muscle, the potential

interaction of MuRF1 with myogenic regulatory factors (MRFs) was also explored. Finally, in order to determine if MuRF1 catalytic activity was necessary for the ability of MuRF1 to modulate transcription, mutations were introduced in order to inactivate the RING domain or delete the carboxyl-terminal domain. The data presented below provides an analysis of the role of MuRF1 as a transcriptional regulator of atrogenes. This thesis proposes a new function for MuRF1 in the skeletal muscle atrophy process beyond its classical role as a post-translational ubiquitinase of target proteins.

## Materials and Methods

### Cell Culture

C<sub>2</sub>C<sub>12</sub> mouse myoblast cells were obtained from a cryostored stock (American Type Culture Collection, Manassas, VA). The cells were thawed, and subsequently grown in a 10 cm cell culture dish in 10 mL of proliferation media (DMEM supplemented with 10% FBS, Pen/Strep, nonessential amino acids, and gentamycin) at 37°C in a 6% CO<sub>2</sub> humidified chamber.

### Transfections and SEAP Reporter Assays

C<sub>2</sub>C<sub>12</sub> cells were plated at a density of 50,000 cells/well into 12-well plates and cultured until an approximate confluency of 70-90% was reached. Prior to transfection, media was aspirated from cells, and 1 mL of fresh proliferation media was added. A total of 1 µg of DNA/well was transiently transfected into each well of the 12-well plates. The transfected DNA cocktail consisted of 250 ng/well of reporter construct, 125 ng/well of

pCMV- $\beta$ -Galactosidase and 125 ng/well of expression plasmids (i.e., pcDNA3.1-MuRF1, pcDNA3.1-MuRF1-RING-mut, pcDNA3.1-MuRF1-c-term-mut, pcDNA3.1-MyoD1, and/or pcDNA3.1-myogenin), and pBluescript as filler to bring to 1  $\mu$ g/well total DNA. The DNA mixtures were then added to TransIT-LT1 Transfection Reagent (MirusBio, Madison, WI) diluted in unsupplemented DMEM according to the manufacturers protocol. The DNA/LT-1 solutions were mixed gently, and incubated at room temperature for 30 min. Following the 30 minute incubation, 75  $\mu$ L of DNA/LT-1 mixture for each reaction condition was added to each well of C<sub>2</sub>C<sub>12</sub> cells in a 12-well plate. The C<sub>2</sub>C<sub>12</sub> cells were incubated at 37°C and 6% CO<sub>2</sub> for 24 hours, and then 1 mL of fresh differentiation media (DMEM with 2% FBS, Pen/Strep, nonessential amino acids and gentamycin) was added to each well to induce myoblast differentiation. Levels of secreted alkaline phosphatase (SEAP) were measured at 24, 48, and 72 hours post-media change using a commercial protocol from Clontech Laboratories according to the manufacturer's instructions (Mountain View, CA.). The C<sub>2</sub>C<sub>12</sub> myotubes were lysed at the conclusion of each experiment, and SEAP numbers were normalized to  $\beta$ -galactosidase activity to correct for variations in transfection efficiency. Each condition was done in triplicate and error reflects +/- standard deviation.

#### Promoter Cloning of MuRF1 and MAFbx

Cloning of the pSEAP-MuRF1-Pro500, pSEAP-MuRF1-Pro1000, pSEAP-MuRF1-Pro2000, pSEAP-MuRF1-Pro5000, pSEAP-MAFbx-Pro500, pSEAP-MAFbx-Pro1000, pSEAP-MAFbx-Pro2000, and pSEAP-MAFbx-Pro5000 plasmids has been previously described.<sup>34</sup> The 500 bp, 1000 bp, 2000 bp, and 5000 bp promoter fragments

were amplified by PCR using BAC clones purchased from CHORI (Oakland, CA). The resulting PCR product was cloned into the SEAP2-Basic reporter vector (Clontech Laboratories).

### MuRF1 cDNA Cloning

RNA was extracted from homogenized C<sub>2</sub>C<sub>12</sub> mouse muscle cells using RNeasy columns per the manufacturer's protocol (Qiagen, Valencia, CA). Mouse cDNA was reverse-transcribed from the mouse mRNA using the following conditions: oligo (dT) primers (500 µg/ml) and dNTP mixture (2.5 mM) were combined with mouse mRNA (1 ng) with nuclease-free sterile water. The mixture was incubated at 65°C for 5 minutes and then incubated on ice for 1 minute. The mixture was briefly centrifuged and First-Strand Buffer (Invitrogen Corporation, Carlsbad, CA), DTT (0.1 M), and Ribonuclease Inhibitor were added per manufacturer's instructions. The mixture was briefly mixed and incubated at 37°C for 2 minutes, followed by the addition of Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Invitrogen Corporation). The reaction was then incubated at 37°C for 50 minutes followed by heat inactivation at 70°C for 15 min. The MuRF1 cDNA was amplified via PCR using the C<sub>2</sub>C<sub>12</sub> and gene specific primers, cloned into the EcoRI/XbaI sites of pcDNA3 and sequenced to confirm the identity of the cDNA.

### Generation of MuRF1 Mutants

The MuRF1 DNA and protein sequences were downloaded from the PubMed database ([www.PubMed.org](http://www.PubMed.org)). The MuRF1 protein sequences for rat, mouse and human were then aligned using sequence alignment tools (<http://www.ncbi.nlm.nih.gov>). These alignments were then used to identify conserved amino acids for site-directed mutagenesis. Site-directed mutagenesis reactions were performed as instructed in the manufacturer's protocol (Stratagene, La Jolla, California). Site-directed mutagenesis primers were designed and used to introduce mutations in the RING domain or the acidic c-terminal tail region of the MuRF1 gene. The c-terminal MuRF1 mutant was created using the following primer sequences:

FWD: 3'-CTTTGGGACAGATTAGTTAGGAGGAGGAGGAGGAG-5',

REV: 3'-CTCCTCCTCCTCCTCCTACTAATCTGTCCCAAAG-5'. The MuRF1 RING mutant was generated using the following primer sequences:

FWD: 3'CCAACACCAACCTCAGCCGGAAGAGTGCCAACGACATC-5' and

REV: 3'-GATGTCGTTGGCACTCTTCCGGCTGAGGTTGTGTTGG-5'. The resulting clones were sequenced to confirm introduction of the correct mutation.

### Generation of E-box Mutations in the MuRF1 Promoter

The two E-boxes identified in the MuRF1 proximal promoter were mutated via site-directed mutagenesis. Site-directed mutagenesis was performed on the MuRF1-Pro500-SEAP reporter plasmid as instructed in the manufacturer's protocol (Stratagene).

The primer sequences used to mutate the E-box at position -156 in the MuRF1 promoter were:

FWD-5'-GGCCCTCTCAGATCCAGGCAGGGATG-3' and

REV-3'-GCTCATCCCTGCCTGGGACTGAGAGGGCC-5'.

The primer sequences used to mutate the E-box at position -57 in the MuRF1 promoter were FWD-5'-CCTCCTGGGGCTCCTGGGACAGAGGTGCAGC-5' and

REV-3'- GCTGCACCTCTGTCCCAGGAGCCCCAGGAGG-3'.

The resulting DNA was sequenced to confirm introduction of the correct mutation.

#### Construction of a Concatemerized 4X E-box Reporter

Four discrete E-boxes were cloned upstream of a minimal SV40 promoter fused to the SEAP reporter gene (Figure 9). Oligonucleotides with sequences of FWD-5'-CGCGCCTGCATGTGATAT-3' and REV-5'-CGCGAGATCACATGCAGG-3' were end-phosphorylated using T4 Polynucleotide Kinase and then annealed by mixing and heating to 95°C for two minutes and slowly cooling to 25°C for 45 minutes in a thermocycler. The annealed oligonucleotides were then ligated into the MluI site in front the SV40 minimal promoter of linearized pSEAP2-Promoter plasmid (Clontech) and sequenced to confirm insertion and orientation.

### Western Blotting

C<sub>2</sub>C<sub>12</sub> cells were transfected with either pcDNA-MuRF1 or pcDNA-MuRF1-RING mutant expression plasmids. The cells were harvested at 72 hours post-transfection and lysed using 500 µL of Universal Lysis Buffer (ULB) (50 mM Tris, 150 mM NaCl, 50 mM NaF, 0.5% Igepal, 1 mM PMSF, 1mM DTT, 10mM β-glycerophosphate , 2 mM Sodium Molybdate and a protease inhibitor cocktail) . The cells were then incubated on ice for 30 minutes and centrifuged at 4°C for 5 minutes at 18,000 × g. The supernatant was then transferred to new 1.5mL Eppendorf tubes and stored at -80°C. Protein concentrations were determined using a modified Bradford protein assay method according to the manufacturer's protocol (BioRad, Hercules, CA). Total protein (200µg) was separated on an SDS-PAGE gel, and then transferred overnight to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for one hour with a blocking solution of 5% milk + 0.05% Tween in Tris Buffered Saline (TTBS). The blocked membrane was then probed by a protein-specific primary antibody. Commercially available primary antibodies for MyoD1 (M-318, rabbit, Santa Cruz Biotechnology, Inc.,) and myogenin (F5D, mouse, Santa Cruz Biotechnology, Inc.,) were incubated with the membrane at a concentration of 1:500 for 1 hr with shaking. Following four washes with TTBS, the membrane was incubated with an appropriate secondary antibody at a 1:5000 concentration for 1 hour and then washed in TTBS. Signal development closely followed manufacturer's instructions for the Pierce ECL Western Blotting kit and imaged using the Typhoon Imager (Thermoscientific, Rockford, IL.).

## Results

### Transcriptional Repression of MAFbx Expression by MuRF1

The ability of MuRF1 to regulate the expression of MAFbx was first tested using a series of MAFbx promoter constructs, and the results revealed that the MAFbx reporter constructs are repressed by MuRF1 ectopic expression. A reporter plasmid containing a 500 bp MAFbx proximal promoter fragment fused to SEAP (MAFbx-Pro500) was cotransfected into C<sub>2</sub>C<sub>12</sub> cells with or without a MuRF1 expression plasmid. As shown in Figure 9, the MAFbx 500 bp promoter shows a significant decrease in activity in cells overexpressing MuRF1.

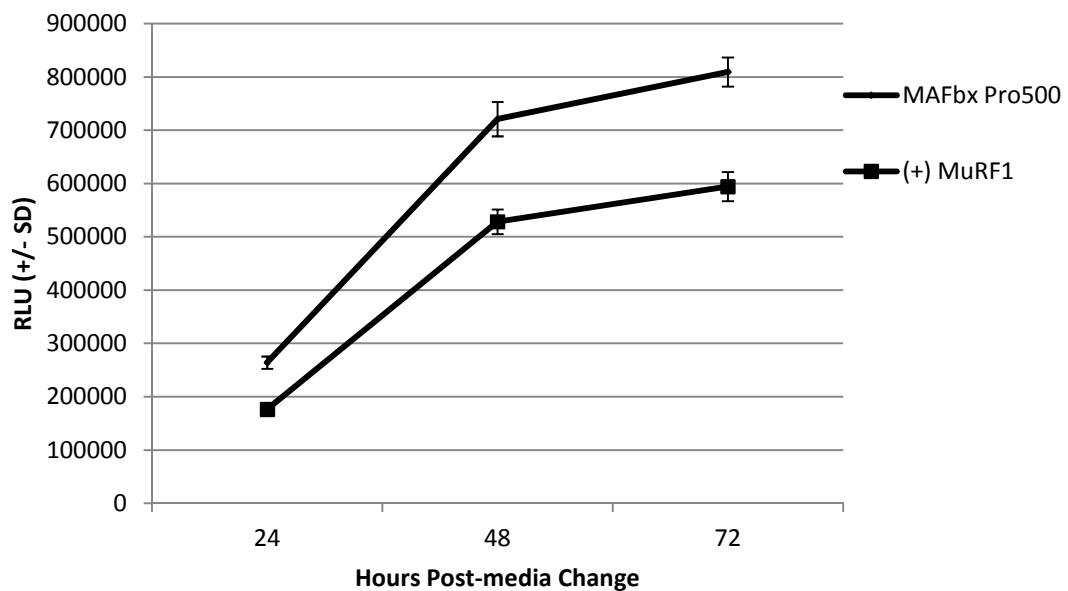


Figure 9. MAFbx-Pro500 reporter shows transcriptional repression in response to overexpression of MuRF1. C<sub>2</sub>C<sub>12</sub> myoblasts were transfected with a reporter construct consisting of the MAFbx-500bp promoter fragment fused to the SEAP2-Basic plasmid, a  $\beta$ -galactosidase expression plasmid, and an expression plasmid for MuRF1. The myoblasts were maintained in standard differentiating culture media. Samples of media were taken at 24 hr intervals and measured for SEAP activity. The samples were normalized with  $\beta$ -galactosidase activity to correct for variation in transfection efficiency. Each condition was done in triplicate and error bars represent standard deviation ( $\pm$  S.D.) of the mean.



## Cooperative Regulation of MAFbx Expression by MuRF1 and Myogenic Regulatory Factors

MuRF1 negatively regulates the transcriptional activity of MAFbx as shown in Figure 9 above. Since the MAFbx proximal promoter contains a functional E-box and has previously been shown to be up-regulated by the myogenic regulatory factors MyoD1 and myogenin,<sup>22</sup> we next tested if MuRF1 overexpression might modulate MRF transcriptional regulation of MAFbx. These experiments show that MyoD1 causes activation of the MAFbx-Pro500 reporter. However, co-overexpression with MuRF1 not only reversed MyoD1 activation, but also caused a cooperative repression of the MAFbx-Pro500 reporter (Figure 10A). In addition, the MuRF1/MRF combinatorial effect was also observed when the MAFbx-Pro500 reporter was transfected into cells along with MuRF1 and myogenin expression plasmids. Co-overexpression of MuRF1 with myogenin also resulted in significant repression of the MAFbx-Pro500 reporter activity (Figure 10B).

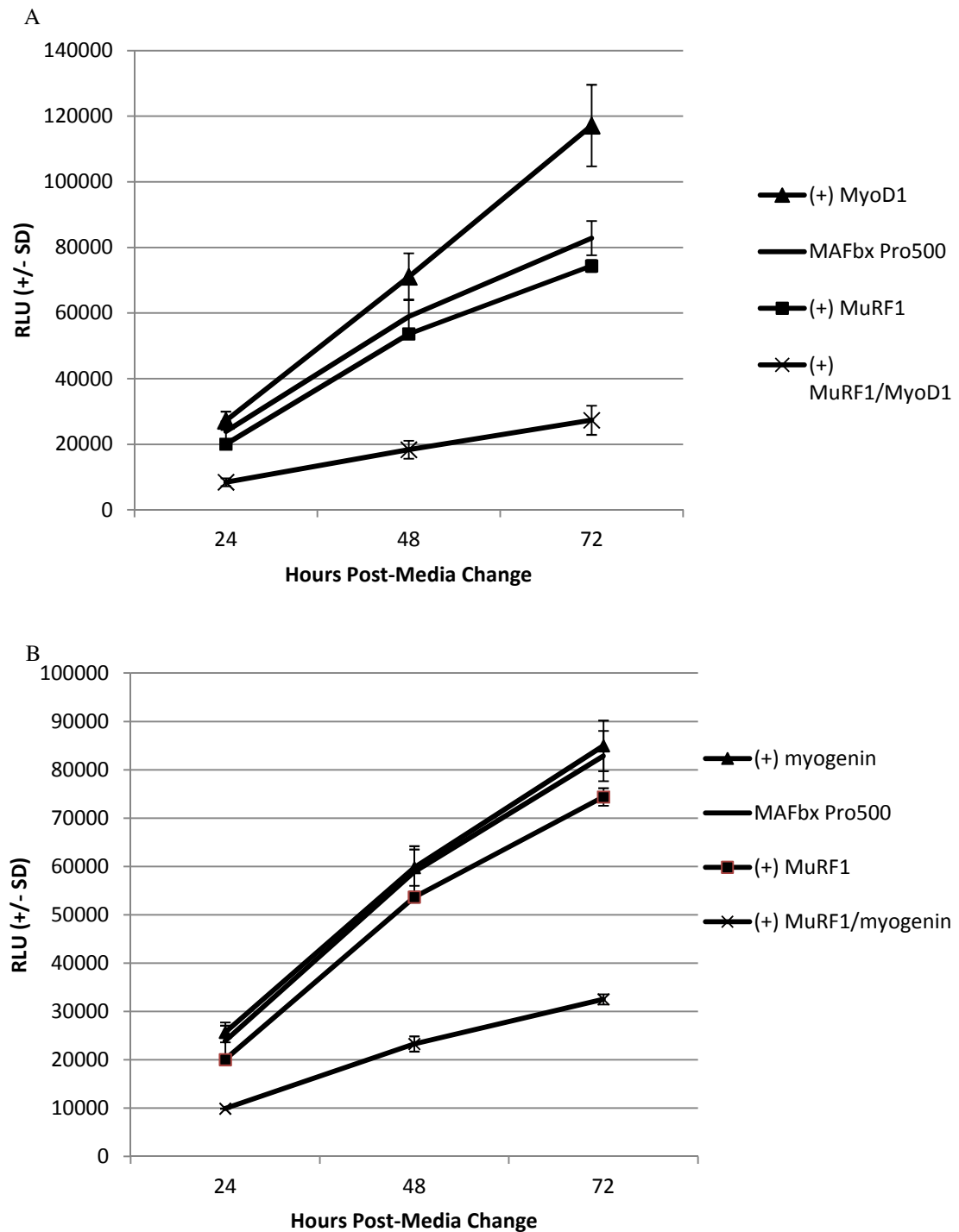


Figure 10. MuRF1 and MRFs Cooperatively Regulate MAFbx Reporter Activity. The MAFbx-Pro500 shows transcriptional repression in response to co-overexpression of MuRF1 and (A) MyoD1 and (B) myogenin. C<sub>2</sub>C<sub>12</sub> myoblasts were transfected with a reporter construct consisting of the MAFbx-500bp promoter cloned into the SEAP2-Basic plasmid, a  $\beta$ -galactosidase expression plasmid, and expression plasmids for MuRF1 alone or in combination with MyoD1 or myogenin. The myoblasts were maintained in standard differentiating culture media. Samples of media were taken at 24 hr intervals and measured for SEAP activity. The samples were normalized to  $\beta$ -galactosidase activity to correct for variation in transfection efficiency. Each condition was done in triplicate and the error bars represent standard deviation ( $\pm$  S.D.) of the mean.

The MuRF1/MRF repressive effect was also observed when larger regions (i.e. 1000 bp, 2000 bp and 5000 bp fragments) of the MAFbx proximal promoter were evaluated (Figure 11 and Figure 12). For all fragment sizes of the MAFbx promoter that were evaluated, overexpression of MuRF1 caused significant transcriptional repression. Furthermore, overexpression of MyoD1 or myogenin caused transcriptional activation of the 1000 bp MAFbx promoter but caused repression of the 2000 bp and 5000 bp MAFbx reporter constructs. In a pattern similar to that seen in the MAFbx 500 bp promoter, cooperative repression of the MAFbx 1000, 2000 and 5000 bp promoter fragments by MuRF1 in combination with MyoD1 (Figure 11) or myogenin (Figure 12) was observed. In all cases, the repressive trend on the MAFbx reporter constructs by the co-overexpression of MuRF1/MyoD1 or MuRF1/myogenin was significantly greater than that caused by any factor individually.

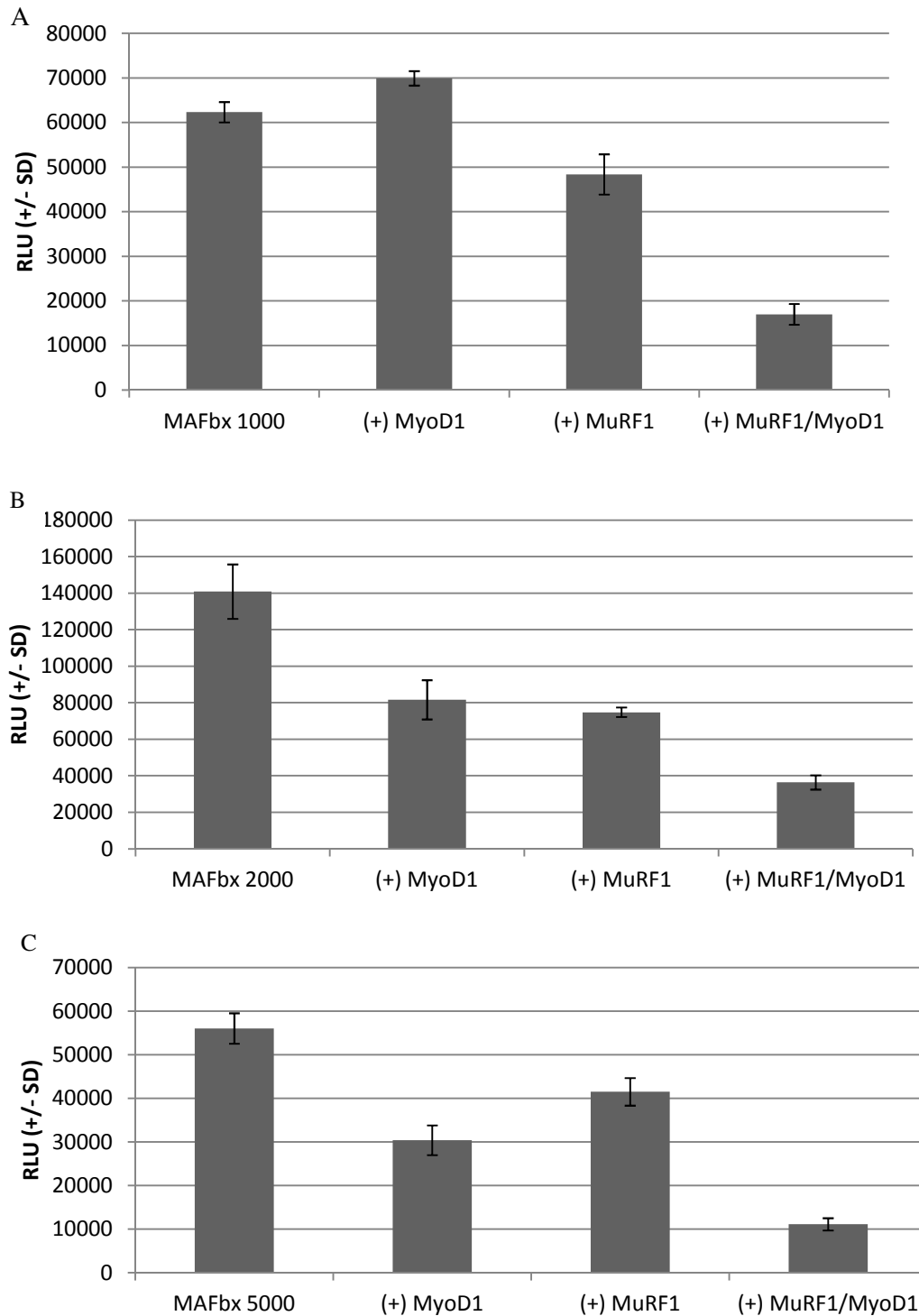


Figure 11. MuRF1 and MyoD1 cooperatively regulate larger MAFbx promoter fragments. The (A) 1000 bp, (B) 2000 bp, and (C) 5000 bp promoters of MAFbx show cooperative repression when MyoD1 is expressed in combination with MuRF1. C<sub>2</sub>C<sub>12</sub> myoblasts were transfected with a reporter construct consisting of either the MAFbx 1000 bp, 2000 bp, or 5000bp promoter cloned into the SEAP2-Basic plasmid, a  $\beta$ -galactosidase expression plasmid, and expression plasmids for MyoD1 and/or MuRF1. The myoblasts were maintained in standard culture media. Samples of media were taken at 72 hrs and measured for SEAP activity. The samples were normalized to  $\beta$ -galactosidase activity to correct for variation in transfection efficiency. Each condition was done in triplicate and the error bars represent standard deviation (-/+ S.D.) of the mean.

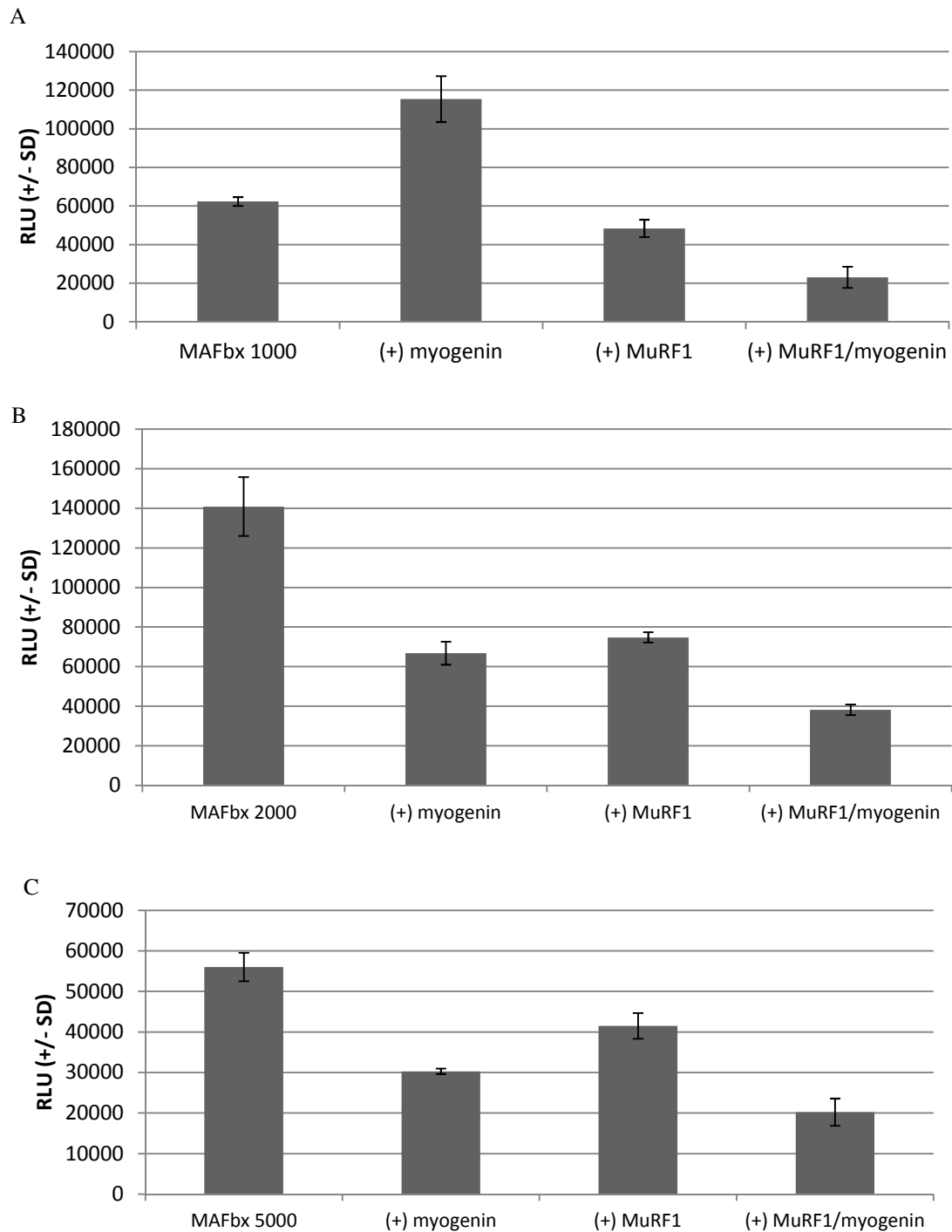


Figure 12. MuRF1 and myogenin cooperatively regulate larger MAFbx promoter fragments. The (A) 1000 bp, (B) 2000 bp, and (C) 5000 bp promoters of MAFbx show cooperative repression when myogenin is expressed in combination with MuRF1. C<sub>2</sub>C<sub>12</sub> myoblasts were transfected with a reporter construct consisting of either the MAFbx 1000 bp, 2000 bp, or 5000bp promoter clone into the SEAP2-Basic plasmid, a  $\beta$ -galactosidase expression plasmid, and expression plasmids for myogenin and/or MuRF1. The myoblasts were maintained in standard culture media. Samples of media were taken at 72 hrs and measured for SEAP activity. The samples were normalized to  $\beta$ -galactosidase activity to correct for variation in transfection efficiency. Each condition was done in triplicate and the error bars represent standard deviation ( $\pm$  S.D.) of the mean.

### Negative Transcriptional Regulation of MuRF1 Reporter Activity by MuRF1

Previous results suggest that MuRF1 may participate in its own transcriptional regulation.<sup>6</sup> Therefore, we tested the effects of ectopic expression of MuRF1 on MuRF1 reporter constructs in C<sub>2</sub>C<sub>12</sub> cells and observed transcriptional repression of a 500 bp promoter construct (Figure 13).

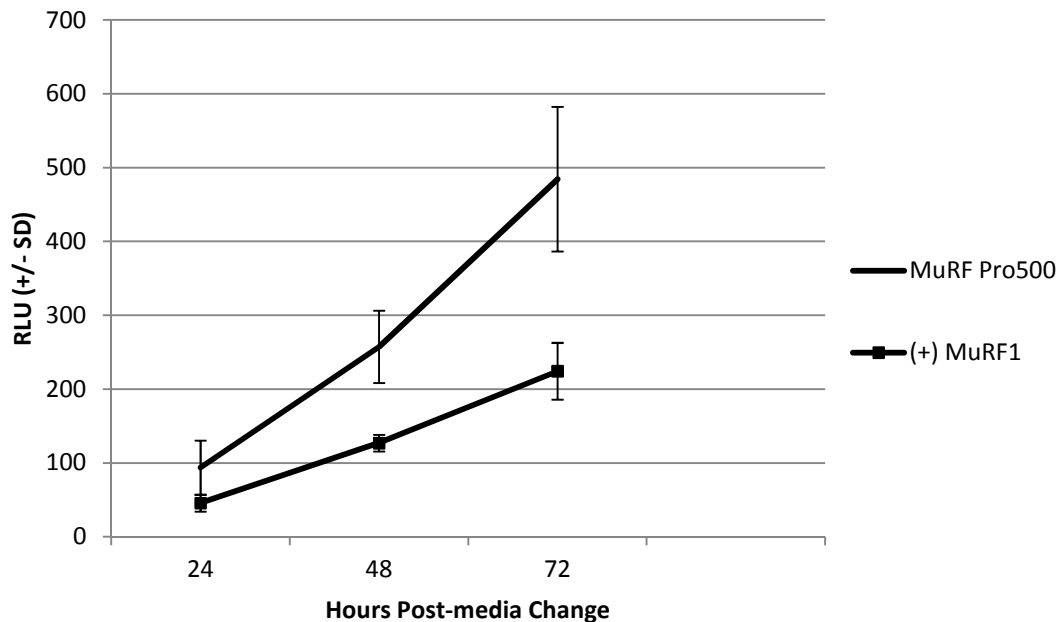


Figure 13. MuRF1 transcriptional regulation of MuRF1 reporter activity. The MuRF1-Pro500 reporter construct shows transcriptional repression in response to overexpression of MuRF1. C<sub>2</sub>C<sub>12</sub> myoblasts were transfected with a reporter construct consisting of the MuRF1 500bp promoter cloned into the SEAP2-Basic plasmid, a  $\beta$ -galactosidase expression plasmid, and an expression plasmid for MuRF1. The myoblasts were maintained in standard differentiating culture media. Samples of media were taken at 24 hr intervals and measured for SEAP activity. The samples were normalized to  $\beta$ -galactosidase activity to correct for variation in transfection efficiency. Each condition was done in triplicate and the error bars represent standard deviation ( $\pm$  S.D.) of the mean.

The MuRF1 proximal promoter has two functional E-boxes that have previously been shown to be necessary for proper MuRF1 expression, and have been shown to bind MyoD1 and myogenin.<sup>19, 22</sup> Therefore, we tested the ability of MyoD1 and myogenin to regulate MuRF1 reporter activity in conjunction with MuRF1 ectopic expression. The MuRF1 500 bp promoter showed significant activation by both myogenin and MyoD1 (Figure 14), with overexpression of myogenin causing a 10-fold increase in MuRF1

promoter activity, while MyoD1 overexpression led to a 50-fold increase in MuRF1 promoter activity. Furthermore, co-overexpression of MuRF1 and MyoD1 reduced MyoD1-induced MuRF1 promoter activity from 50-fold induction to approximately 10-fold induction (Figure 14A), while this MuRF1 reporter showed complete loss of activation in response to ectopic expression of MuRF1 in combination with myogenin overexpression (Figure 14B).

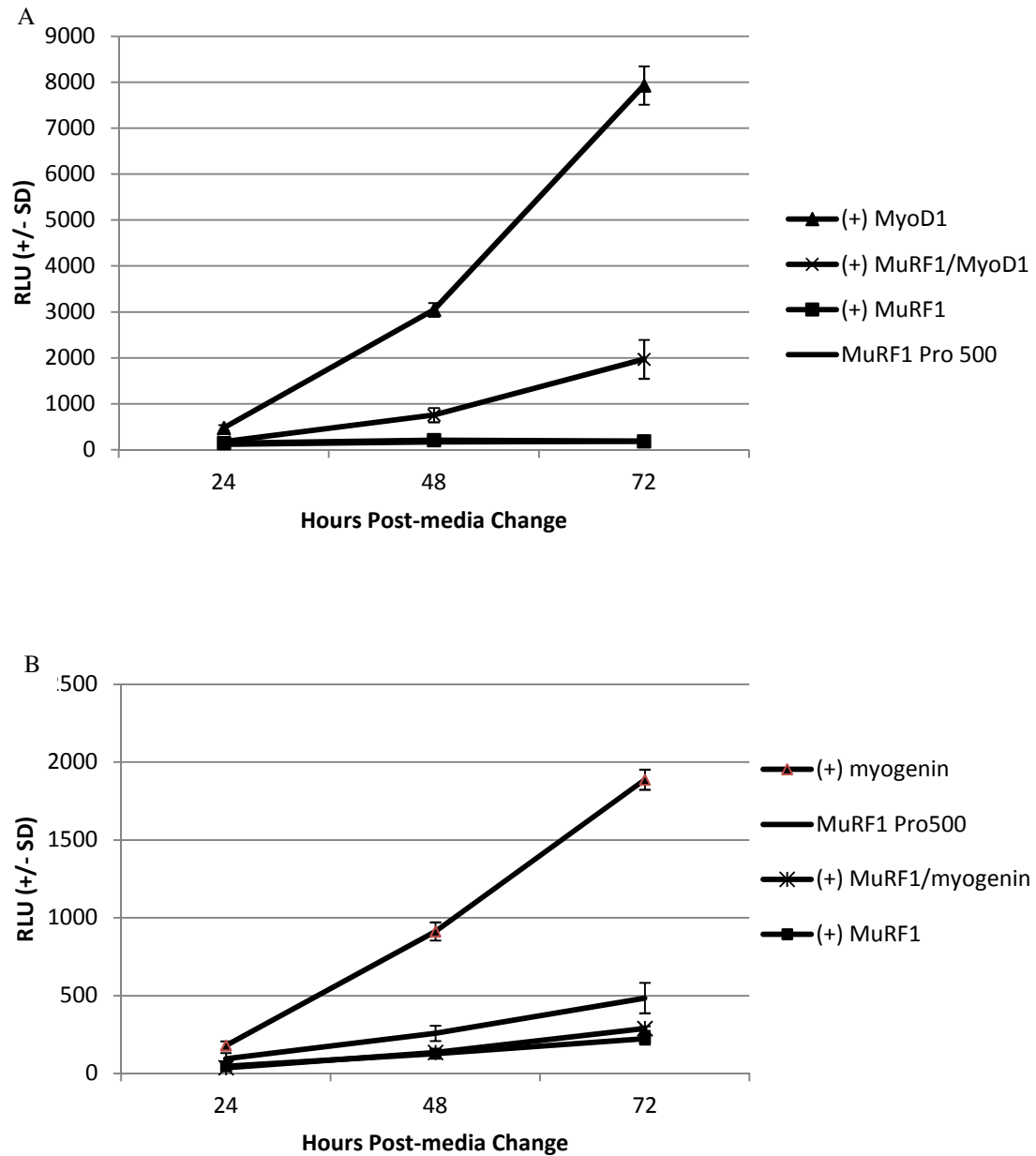


Figure 14. MuRF1 and MRFs cooperatively regulate MuRF1 reporter activity. The MuRF1-Pro500 reporter shows transcriptional repression in response to co-overexpression of MuRF1 and (A) MyoD1 or (B) myogenin. C<sub>2</sub>C<sub>12</sub> myoblasts were transfected with a reporter construct consisting of the MuRF1-500bp promoter cloned into the SEAP2-Basic plasmid, a  $\beta$ -galactosidase expression plasmid, and expression plasmids for MuRF1 alone or in combination with myogenin or MyoD1. The myoblasts were maintained in standard differentiating culture media. Samples of media were taken at 24 hr intervals and measured for SEAP activity. The samples were normalized to  $\beta$ -galactosidase activity to correct for variation in transfection efficiency. Each condition was done in triplicate and the error bars represent standard deviation ( $\pm$  S.D.) of the mean.



The MuRF1/MRF combinatorial repressive effect was also observed when larger regions (i.e. 1000 bp, 2000 bp and 5000 bp fragments) of the MuRF1 proximal promoter were evaluated. The effects of MyoD1 and myogenin either alone or in combination with MuRF1 on the activity of the 1000 bp, 2000 bp, and 5000 bp promoter regions of MuRF1 were tested, and while the larger sizes of the MuRF1 promoter showed increased general expression, they showed identical patterns of regulation compared to the 500 bp promoter. In each case, the MuRF1 promoter fragments showed induction by either MyoD1 or myogenin alone (Figure 15 and Figure 16, respectively), but this activation was greatly reduced or completely abolished when MyoD1 or myogenin was expressed in combination with MuRF1.

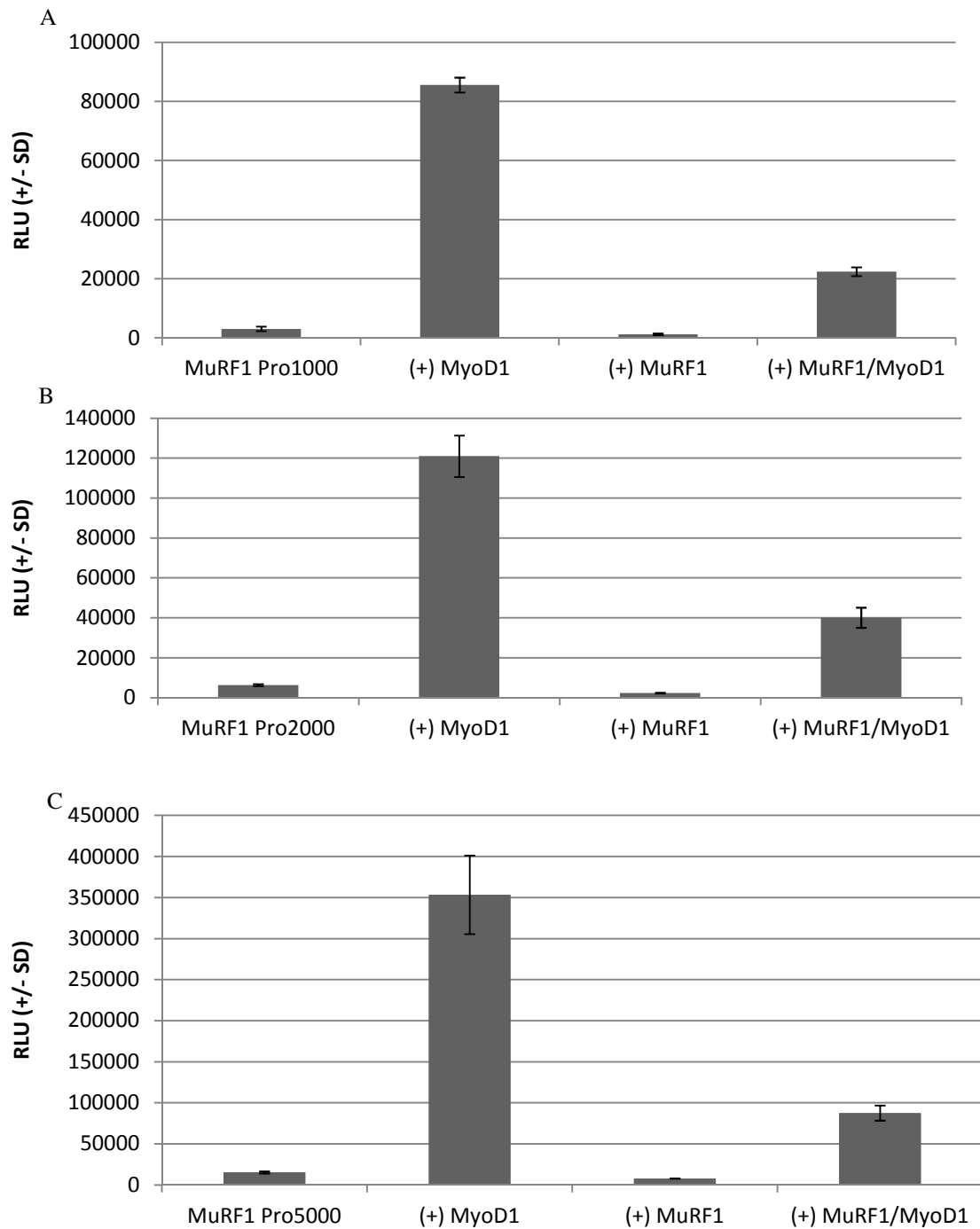


Figure 15. MuRF1 and MyoD1 cooperatively regulate larger MuRF1 promoter fragments. The (A) 1000, (B) 2000, and (C) 5000 bp promoters of MuRF1 show marked induction by MyoD1 in C<sub>2</sub>C<sub>12</sub> cells, which is largely abolished when MyoD1 is overexpressed in combination with MuRF1. C<sub>2</sub>C<sub>12</sub> myoblasts were transfected with a reporter construct consisting of either the MuRF1 1000, 2000, or 5000bp promoter cloned into the SEAP2-Basic plasmid, a  $\beta$ -galactosidase expression plasmid, and expression plasmids for MyoD1 and/or MuRF1. The myoblasts were maintained in standard culture media. Samples of media were taken at 72 hrs post-media change and measured for SEAP activity. The samples were normalized to  $\beta$ -galactosidase activity to correct for variation in transfection efficiency. Samples were done in triplicate and the error bars represent standard deviation ( $\pm$  S.D.) of the mean.

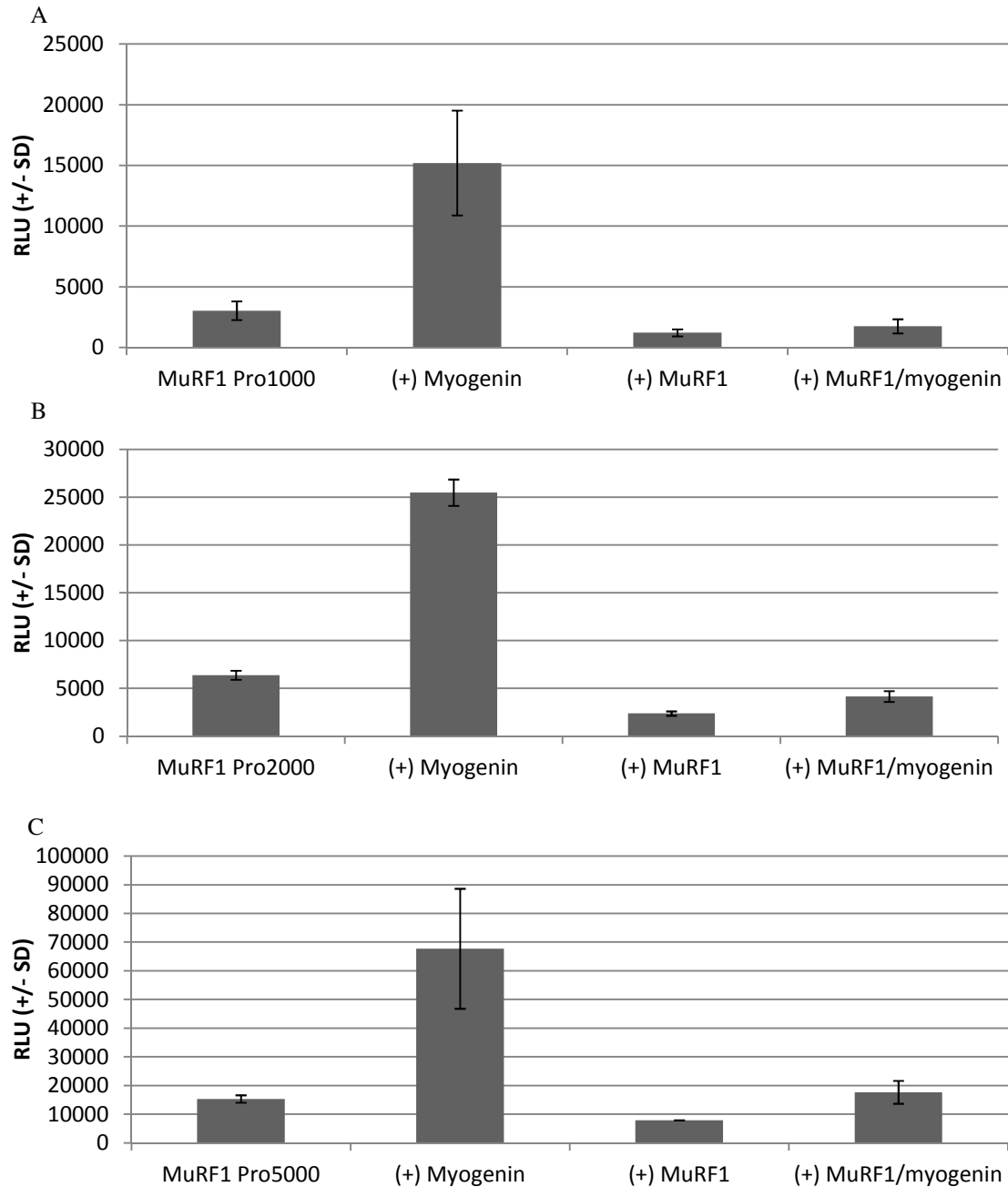


Figure 16. MuRF1 and myogenin cooperatively regulate larger MuRF1 promoter fragments. The (A) 1000, (B) 2000, and (C) 5000 bp promoters of MuRF1 show marked induction by myogenin in C<sub>2</sub>C<sub>12</sub> cells, which is completely abolished when myogenin is overexpressed in combination with MuRF1. C<sub>2</sub>C<sub>12</sub> myoblasts were transfected with a reporter construct consisting of either the MuRF1 1000, 2000, or 5000 bp promoter cloned into the SEAP2-Basic plasmid, a  $\beta$ -galactosidase expression plasmid, and expression plasmids for myogenin and/or MuRF1. The myoblasts were maintained in standard culture media. Samples of media were taken at 72 hrs post-media change and measured for SEAP activity. The samples were normalized to  $\beta$ -galactosidase activity to correct for variation in transfection efficiency. Samples were done in triplicate and the error bars represent standard deviation ( $\pm$  S.D.) of the mean.

### E-box Sequence is Sufficient for MuRF1/MRF Cooperative Transcriptional Repression

To further characterize the role of the E-boxes found in the MuRF1 500 bp promoter, a series of four concatemerized E-boxes corresponding to the E-box sequence at position -156 in the MuRF1 regulatory region were cloned in front of a minimal SV40 promoter that contains no enhancers (Figure 17). Co-overexpression of MuRF1 with either MyoD1 (Figure 18A) or myogenin (Figure 18B) caused a significant repression of the 4x-Ebox-SEAP reporter.

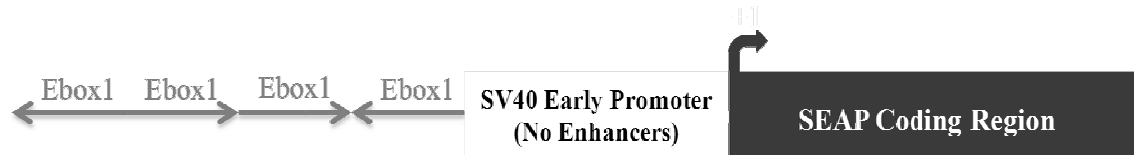


Figure 17. An illustration showing the reporter construct including the orientation of the concatemerization of 4 MuRF1 E-boxes cloned upstream of the SV40 Early Promoter and fused to the pSEAP2-Promoter plasmid. The directions of the arrows represent the 5' → 3' orientation of the E-boxes.

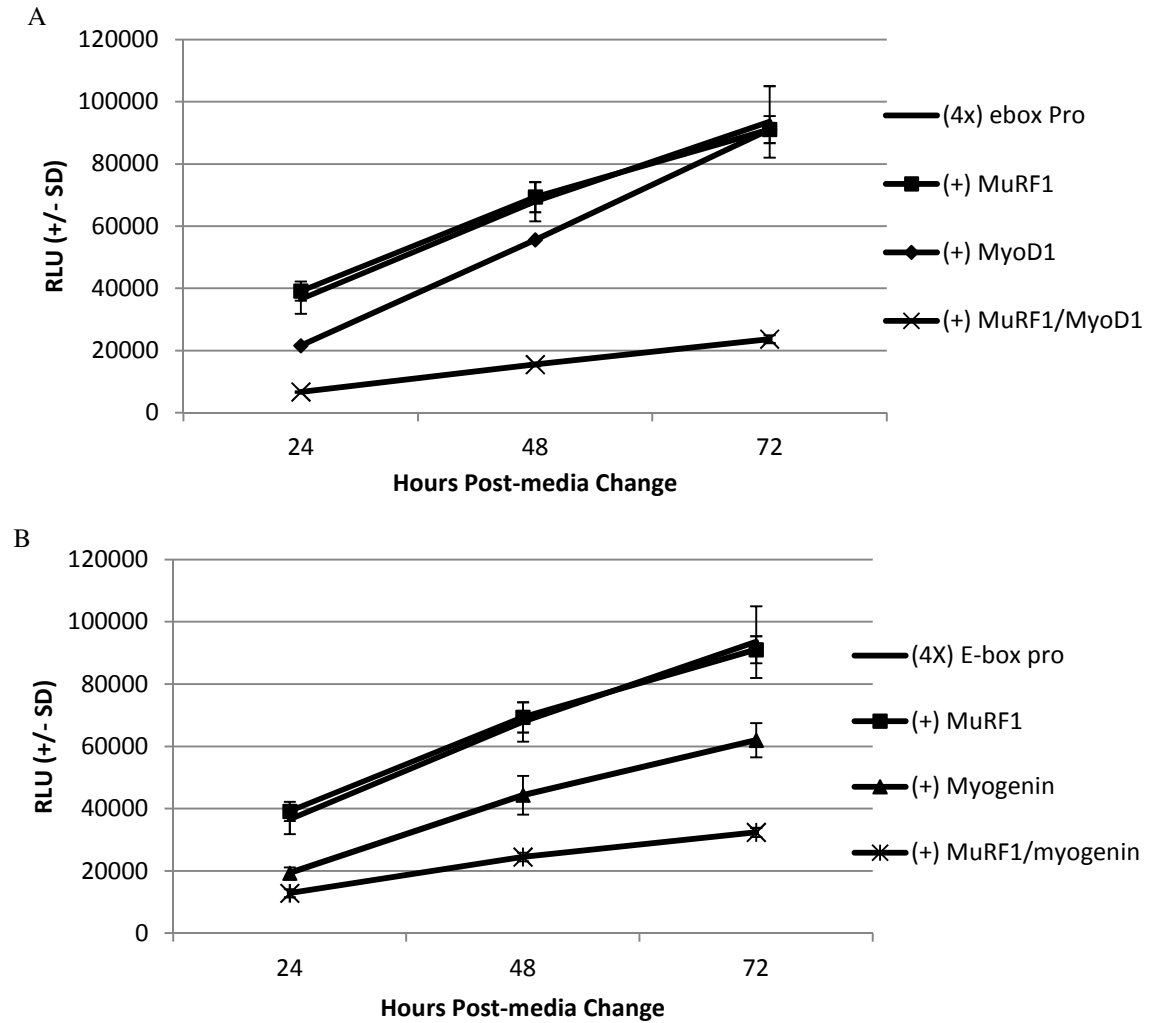


Figure 18. The concatemericized 4x-Ebox-SEAP reporter exhibits cooperative repression by MuRF1 and (A) MyoD1 or (B) myogenin. C<sub>2</sub>C<sub>12</sub> myoblasts were transfected with a reporter construct consisting of the concatemericized 4X-Ebox construct cloned into the SEAP2-Promoter plasmid, a  $\beta$ -galactosidase expression plasmid, and expression plasmids for MyoD1, myogenin, and/or MuRF1. The myoblasts were maintained in standard culture media. Samples of media were taken at 24 hr intervals and measured for SEAP activity. The samples were normalized to  $\beta$ -galactosidase activity to correct for variation in transfection efficiency. Each condition was done in triplicate and the error bars represent standard deviation ( $\pm$  S.D.) of the mean.

## MuRF1 Catalytic Activity is Necessary for MuRF1-mediated Reversal of MRF-induced Reporter Gene Activity

Overexpression of MuRF1 has consistently resulted in little to no repression of the transcriptional activity of the 4x-Ebox-SEAP reporter, but consistently represses this construct when co-overexpressed with MyoD1 or myogenin (Figure 18). However, the MuRF1/MyoD1 cooperative repression of the 4X-Ebox-SEAP reporter is largely abrogated when MyoD1 is co-overexpressed with a MuRF1-RING-mutant (Figure 19A). In addition, overexpression of myogenin alone caused a decrease in transcriptional activity of the 4X-Ebox-SEAP reporter construct, while co-overexpression of myogenin with MuRF1 resulted in a dramatic cooperative repression of the reporter (Figure 19B). Furthermore, the overexpression of myogenin in combination with the MuRF1-RING-mutant caused no repression of the 4X-Ebox-SEAP reporter (Figure 19B).

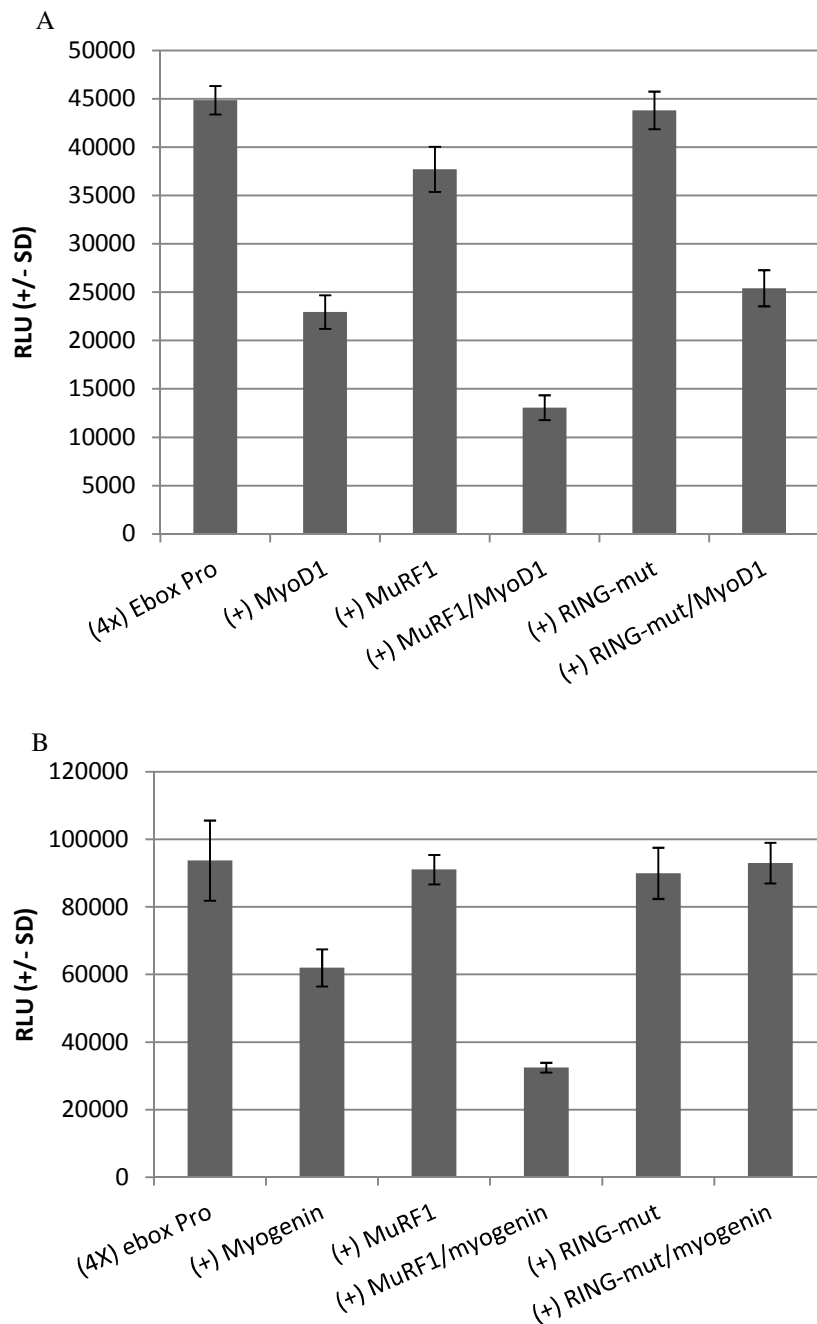


Figure 19. MuRF1 and MRF cooperative repression of the 4X-Ebox-SEAP reporter is abrogated when (A) MyoD1 or (B) myogenin is co-overexpressed with the MuRF1-RING-mutant. C<sub>2</sub>C<sub>12</sub> myoblasts were transfected with a reporter construct consisting of a concatemericized 4X-E-box cloned into the SEAP2-Promoter plasmid, a  $\beta$ -galactosidase expression plasmid, and expression plasmids for MuRF1, MuRF1-RING-mutant, MyoD1 and/or Myogenin. The myoblasts were maintained in standard culture media. Samples of media were taken at 72 hrs post-media change and measured for SEAP activity. The samples were normalized to  $\beta$ -galactosidase activity to correct for variation in transfection efficiency. Each condition was done in triplicate and the error bars represent standard deviation (-/+ S.D.) of the mean.

In order to determine if MuRF1 catalytic activity is also required for cooperative repression of MuRF1 reporter activity, cells were transfected with the MuRF1-Pro500 reporter construct and MyoD1 along with MuRF1 or the MuRF1-RING mutant. Overexpression of MyoD1 again caused induction of the promoter construct, which was partially abrogated by co-overexpression with MuRF1. This abrogation of activation was not observed when MyoD1 was co-overexpressed with the MuRF1-RING-mutant (Figure 20A). Furthermore, overexpression of myogenin alone caused a significant increase in the transcriptional activity of the MuRF1-Pro500 construct, which was completely reversed when myogenin was co-overexpressed in combination with MuRF1. In contrast, overexpression of myogenin in combination with the MuRF1-RING-mutant actually caused an increase in reporter transcriptional activity (Figure 20B).



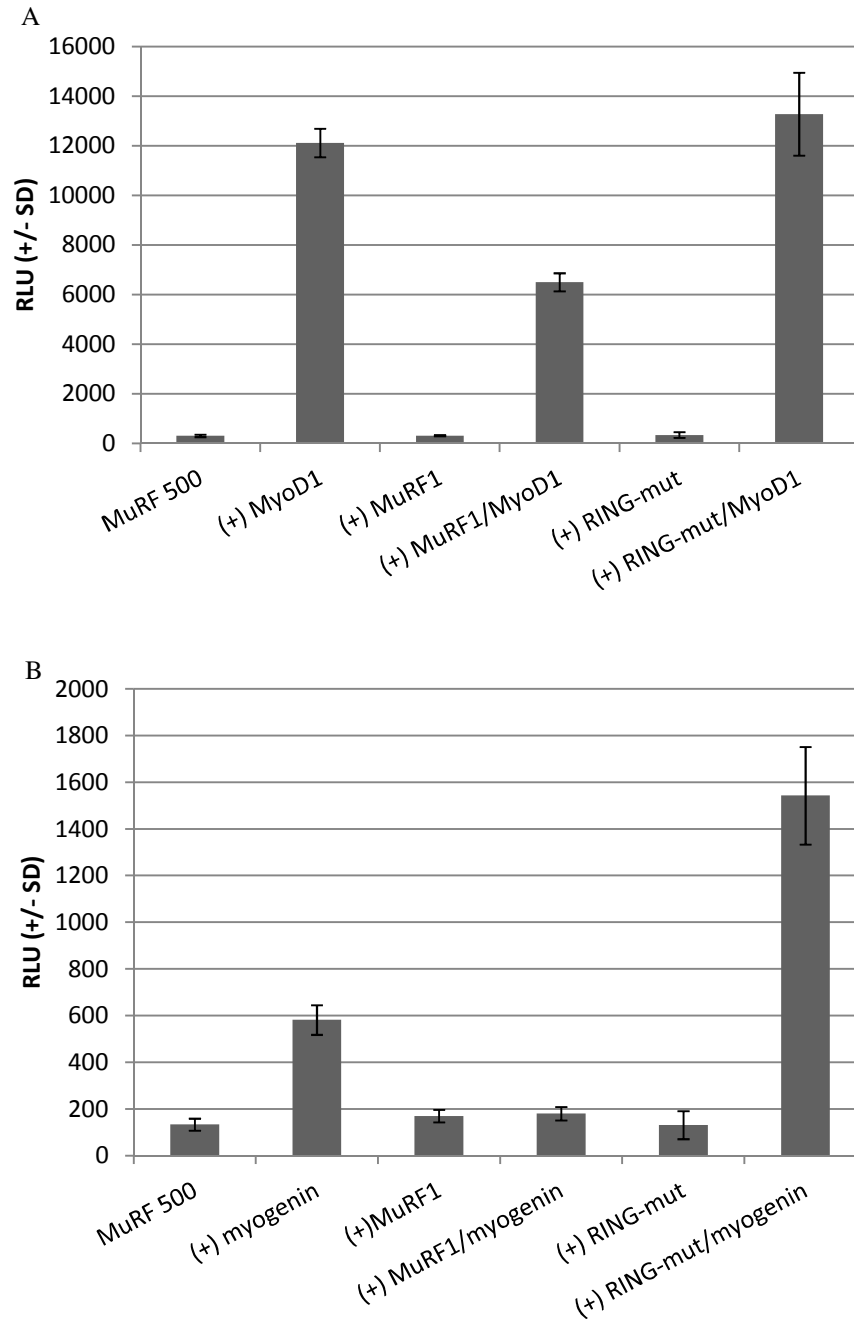


Figure 20. MuRF1 and MRF cooperative repression of the MuRF1-Pro500 reporter is abrogated when (A) MyoD1 or (B) myogenin is co-overexpressed with the MuRF1-RING-mutant. C<sub>2</sub>C<sub>12</sub> myoblasts were transfected with a reporter construct consisting of MuRF1 500 bp promoter fragment cloned into the SEAP2-Basic plasmid, a  $\beta$ -galactosidase expression plasmid, and expression plasmids for MuRF1, MuRF1-RING-mutant, MyoD1 and/or Myogenin. The myoblasts were maintained in standard culture media. Samples of media were taken at 72 hrs post-media change and measured for SEAP activity. The samples were normalized to  $\beta$ -galactosidase activity to correct for variation in transfection efficiency. Each condition was done in triplicate and the error bars represent standard deviation ( $\pm$  S.D.) of the mean.

## The MuRF1 Acidic Carboxyl-Terminal Tail is Not Required for MuRF1-mediated Reversal of MRF-induced Reporter Gene Activity

Truncation of the acidic c-terminus of the MuRF1 protein did not significantly alter the cooperative repression of the 4X-Ebox-SEAP reporter by MuRF1 and MyoD1. As shown previously, the transcriptional activity of this reporter is repressed in response to ectopic expression of MyoD1 alone, while this repression is further increased when MyoD1 was co-overexpressed in combination with MuRF1 (Figure 21). Furthermore, overexpression of MyoD1 in combination with the MuRF1-c-terminal mutant was indistinguishable from co-overexpression of MyoD1 with full-length MuRF1 (Figure 21).

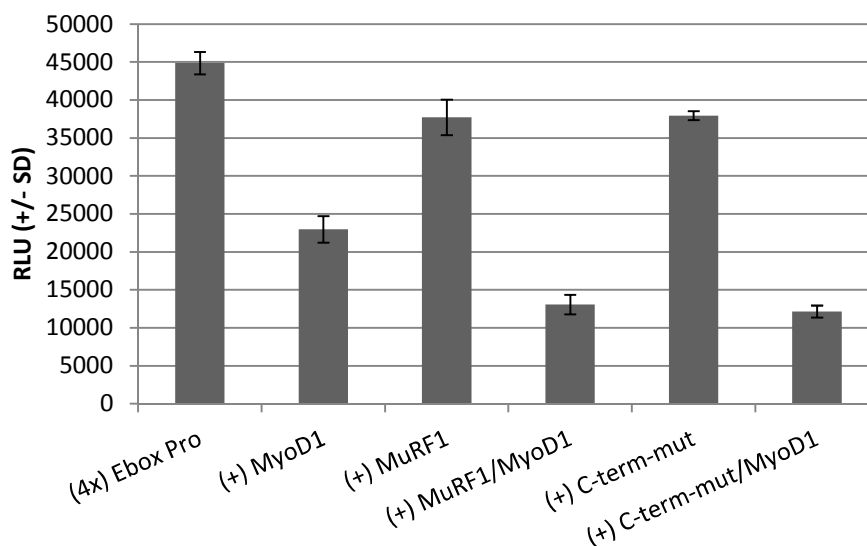


Figure 21. The MuRF1-c-terminal mutant in combination with MyoD1 cooperatively represses the 4X-Ebox-SEAP reporter. C<sub>2</sub>C<sub>12</sub> myoblasts were transfected with a reporter construct consisting of the concatemericized 4X-E-boxes cloned into the SEAP2-Promoter plasmid, a  $\beta$ -galactosidase expression plasmid, and expression plasmids for MuRF1, MuRF1-c-terminal-mutant, and/or MyoD1. The myoblasts were maintained in standard culture media. Samples of media were taken at 72 hr and measured for SEAP activity. The samples were normalized to  $\beta$ -galactosidase to correct for variation in transfection efficiency. Samples were done in triplicate and the error bars represent standard deviation ( $\pm$  S.D.) of the mean.

In order to further determine if the carboxyl-terminal region of MuRF1 is required for cooperative repression of the MuRF1 expression, cells were transfected with the MuRF1-Pro500 reporter construct and MyoD1 along with MuRF1 or the MuRF1-C-term

mutant. As shown previously, overexpression of MyoD1 caused a significant increase in transcriptional reporter activity, which was completely abolished when MyoD1 was co-overexpressed in combination with MuRF1. Furthermore, overexpression of the C-terminal MuRF1 mutant alone showed no appreciable difference compared to the effect of full-length MuRF1 on the transcriptional activity on the MuRF1-Pro500 reporter construct, while co-overexpression of the MuRF1 c-terminal mutant with MyoD1 also showed no significant differences on the MuRF1-Pro500 reporter when compared to MuRF1/MyoD1 co-overexpression (Figure 22).

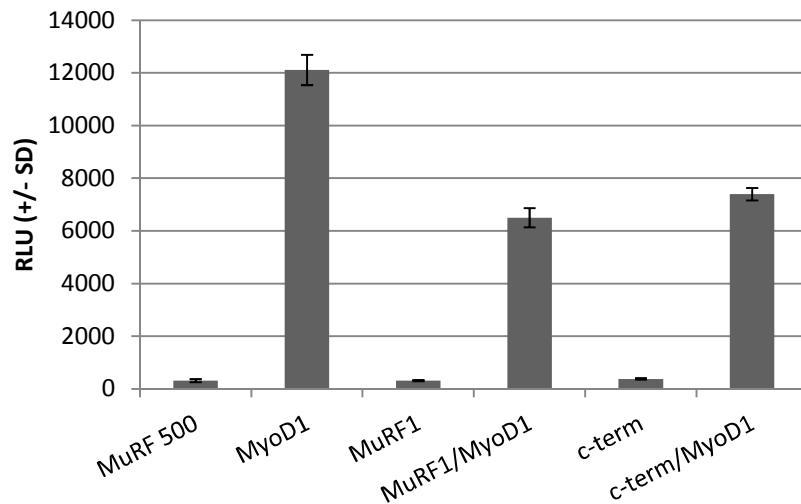


Figure 22. The MuRF1-c-terminal mutant in combination with MyoD1 cooperatively represses the MuRF1-Pro500 reporter. C<sub>2</sub>Cl<sub>2</sub> myoblasts were transfected with a reporter construct consisting of the MuRF1 500 bp promoter fragment cloned into the SEAP2-Basic plasmid, a  $\beta$ -galactosidase expression plasmid, and expression plasmids for MuRF1, MuRF1-c-terminal-mutant, and/or MyoD1. The myoblasts were maintained in standard culture media. Samples of media were taken at 72 hr and measured for SEAP activity. The samples were normalized to  $\beta$ -galactosidase to correct for variation in transfection efficiency. Samples were done in triplicate and the error bars represent standard deviation ( $\pm$  S.D.) of the mean.

### Ectopic Expression of MuRF1 Does Not Alter Endogenous Myogenin Protein Levels

Myogenin protein levels were unchanged in C<sub>2</sub>C<sub>12</sub> cells overexpressing either MuRF1 or the MuRF1-RING mutant. This data suggests that the mechanism by which MuRF1 modulates MRF activity is not simply due to destabilizing these myogenic regulatory factors (Figure 23).

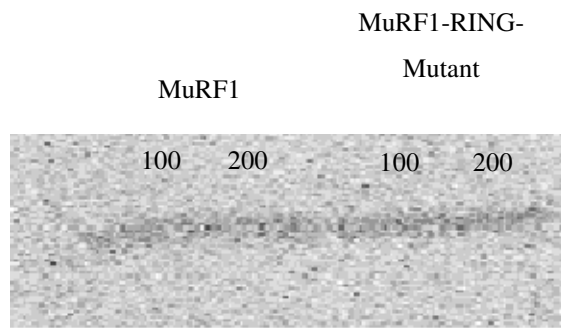


Figure 23. Similar levels of myogenin protein observed in C<sub>2</sub>C<sub>12</sub> cells over-expressing MuRF1 and in C<sub>2</sub>C<sub>12</sub> cells over-expressing the MuRF1-RING-mutant. C<sub>2</sub>C<sub>12</sub> myoblast cells were transfected with an expression plasmid containing either MuRF1, or the MuRF1-RING-mutant. The myoblasts were maintained in standard culture media and harvested at 72 hours. Western blotting was performed on 100µg and 200µg of total protein using antibodies against myogenin.

### Discussion

MuRF1 expression increases in response to an array of atrophy-inducing conditions;<sup>1</sup> however, the mechanism of the transcriptional regulation of this gene is still not completely understood. Furthermore, the results from this study suggest that MuRF1 may also play a role in the transcriptional regulation of a subset of atrogenes, including itself. MuRF1 has been shown through this research to be an inhibitive transcriptional regulator of the MAFbx and MuRF1 gene. These results are consistent with recent microarray data in which MuRF1-null animals showed differential gene expression compared to wild-type (WT) littermates.<sup>6</sup> MuRF1-null animals exhibited continued elevation of MuRF1 (evidenced by increased β-gal expression in the MuRF1-null mice)

and MAFbx expression following denervation, while the expression of these genes returned to baseline in the WT mice following denervation. MuRF1 has long been described as a marker for muscle atrophy, given that the expression of MuRF1 increases in response to numerous atrophic stimuli<sup>1</sup>; however, the present study expands the role of MuRF1 to include that of a regulator of atrophy-induced gene expression.

Furthermore, this thesis provides insight into the mechanisms by which MuRF1 inhibits atrophy-induced gene expression. Specifically, we show that MuRF1 acts, at least in part, via direct and/or indirect modulation of the MyoD-family of myogenic regulatory factors, including MyoD1 and myogenin. MyoD1 and myogenin are basic helix-loop-helix proteins that have been shown to bind E-boxes in the promoters of muscle specific-genes.<sup>22</sup> In addition, we have confirmed transcriptional activation of MuRF1 and MAFbx by MyoD1 and myogenin; however, we demonstrate for the first time here that MuRF1 cooperates with both MyoD1 and myogenin to repress MuRF1 and MAFbx reporter gene expression. These findings support the microarray data showing that both MAFbx and MuRF1 expression remain elevated in MuRF1-null mice, suggesting that the MuRF1 gene product is necessary for returning atrogene expression to baseline levels following a atrophic stimulus. Our findings suggest that MuRF1 acts as a modulator of myogenic regulatory factors, although the exact mechanism is still unclear. Intriguingly, MuRF1 has been previously shown to regulate the localization of PPAR $\alpha$  in cardiac muscle via monoubiquitination providing a possible mechanism by which MuRF1 might impact MRF function.<sup>14</sup>

While the exact mechanism of MuRF1 regulation of atrophy-responsive genes via modulation of MyoD1 and myogenin is not yet known, there are multiple possible

explanations. It is possible that MuRF1 serves to a) directly interact with MyoD1 and myogenin and alter their function, stability or cellular localization, b) ubiquitinate MyoD1 and myogenin as means to change their function, stability, or cellular localization, c) target recruitment of additional transcription factors to the promoters of atrophy-induced genes, or d) interact with or recruit additional proteins which themselves associate with the promoters of muscle-specific genes.<sup>5</sup>

The latter possibility is of special interest given that recent research has shown that many myogenic regulatory factors, including MyoD1 and myogenin, associate with proteins such as HEB (TF12) and the E2A gene products E12 and E47. It has been well established that members of the MyoD family of transcription factors, including MyoD1 and myogenin, function as heterodimers with more universally-expressed E proteins. Multiple myogenic regulatory factors have been shown to associate with E proteins in a sequential manner at numerous muscle-specific genes including muscle creatine kinase (Ckm), desmin (Des), fast-twitch skeletal muscle troponin I, type 2 (Tnni2), leimodin 2 (lmod2), and titin cap (Tcap).<sup>5</sup> While neither MuRF1 nor MAFbx were included in the above study, we predict that these genes may also be regulated in part by the ubiquitously expressed E proteins in association with MRFs, due to the presence of conserved E boxes in both the MuRF1 and MAFbx proximal promoter. Interestingly, MuRF1-null mice show differential gene expression in cardiac muscle, with a majority of the differentially regulated genes being E2F regulated.<sup>35</sup> The data presented in this research suggest that MuRF1 gene expression may modulate the activity, recruitment, or stability of myogenic regulatory factors and/or their known binding partners.

While the exact mechanism of MuRF1-mediated repression of muscle-specific genes through the potential modulation of MRFs remains less than complete, the data presented here suggests that the RING domain of MuRF1 may be important. Overexpression of the catalytically dead MuRF1 RING mutant in combination with either MyoD1 or myogenin failed to cause any significant repression of the MuRF1 reporter gene. This suggests that ubiquitin tagging of MRFs by MuRF1 might be a possible explanation for MuRF1-mediated regulation of MyoD1 and myogenin function in muscle cells. It is also possible that MyoD1 and myogenin may recruit MuRF1 to the promoters of target genes and allow for modification of other participants in the transcriptional regulatory process. This MuRF1-mediated tagging may cause a change in association and/or recruitment of the MRFs and/or their E protein binding partners to E-box elements within target gene regulatory regions. This possibility is supported by our data showing a complete loss of cooperative repression of the synthetic 4X-Ebox-SEAP reporter when the MuRF1-RING mutant is over-expressed in combination with MyoD1 or myogenin. Furthermore, it is unlikely that potential MRF ubiquitin tagging catalyzed by MuRF1 causes degradation of these transcription factors, as the level of myogenin protein in cells overexpressing MuRF1 closely mirrors that seen in cells over-expressing the MuRF1-RING mutant.

The catalytic RING domain of MuRF1 may participate in an array of functions in addition to the interaction between MuRF1 and myogenic regulatory factors. Thus, there are additional possible explanations for the data presented in this thesis. For example, the loss of cooperative repression of the MuRF1 reporter construct and of the 4X-Ebox reporter construct by MRFs and the MuRF1-RING mutant may also be explained by a

change in the cellular localization of the MuRF1 protein following mutation. Previous work has shown that the RING domain may function in nuclear localization of the MuRF1 protein, likely through interaction with SUMO-3 (small ubiquitin-related modifier-3,) which has itself been shown to participate in nuclear import.<sup>36, 37</sup> Since it is possible that mutation of the 3<sup>rd</sup> and 4<sup>th</sup> cysteine residues in the RING domain of MuRF1 may impact the localization of MuRF1 in the cell, future work to verify this possibility will involve tagging the MuRF1 protein and the MuRF1-RING mutant with a GFP tag in order to monitor cellular localization. Finally, in contrast to the RING domain, the acidic c-terminal domain of MuRF1 is not suggested by this research to be integral to the cooperation between MuRF1 and the myogenic regulatory factors in regulating gene activity. This is supported by the observation that the 4X-Ebox and the MuRF1 reporter constructs both show similar activity patterns in response to co-overexpression of the MuRF1 c-term mutant or full-length MuRF1 with MyoD1 or myogenin. In summary, the present study provides exciting new evidence supporting the hypothesis that MuRF1 may act as a major transcriptional regulator of atrophy-induced gene expression. Specifically, we demonstrate here that 1) MuRF1 acts as a transcriptional modulator of atrophy-regulated genes, including itself, 2) MuRF1 does so in part via either direct or indirect interactions with myogenic regulatory factors, and 3) specific domains of the MuRF1 gene product likely play an important roles in mediating this interaction.



## Conclusions and Future Directions

This thesis provides new evidence supporting the hypothesis that MuRF1 acts as a major regulator in skeletal atrophy. Future work on this project should include continued exploration of the potential interaction of MuRF1 with myogenic regulatory factors and E proteins. It is conceivable that the synergistic repression of the MuRF1 and MAFbx promoters by the combination of MuRF1 and MyoD1 or myogenin is mediated by a ubiquitination effect by MuRF1. The stability and ubiquitination status of MyoD1, myogenin, and associated E proteins could be evaluated via co-immunoprecipitation experiments in cells that are over-expressing MuRF1 or the MuRF1-RING mutant. Furthermore, immunoprecipitation of MyoD1 and myogenin from C<sub>2</sub>C<sub>12</sub> cells that ectopically express MuRF1 or MuRF1 RING mutant, followed by western blotting for ubiquitin would help determine if MuRF1 is actually tagging these proteins in the cell.

In addition, future work using Chromatin Immunoprecipitations (ChIPs) of the MuRF1 and MAFbx promoters with antibodies against MyoD1 and myogenin should be performed in to explore the level of MRF occupancy of these promoters in response to ectopic expression of MuRF1. Our preliminary research suggests that the degree of myogenic commitment of the myoblast cells may play a large role in the effects of MuRF1. Furthermore, MyoD1 and myogenin have recently been shown to transiently and sequentially bind the promoters of other muscle-specific genes,<sup>5</sup> suggesting that further work should including exploration of this possible regulation its potential contribution to cellular differentiation. Furthermore, MRF occupancy of the MuRF1 and MAFbx promoters should be evaluated in response to ectopic expression of MuRF1 mutants, as well as siRNA knockdown of MuRF1 in cells of varying stages of myogenic

commitment. Finally, because skeletal muscle atrophy is a complex process and is regulated on many levels, future work should extend beyond the exploration of the transcriptional regulation of atrogenes by MuRF1. The analysis of the regulation of MuRF1 on a post-transcriptional level would also likely yield important information regarding the pathways of muscle plasticity.

#### Implications of this research

This research has allowed for a better understanding of the role that MuRF1 plays in the regulation of muscle atrophy. A role of MuRF1 in the modulation of known muscle regulatory factors suggests that MuRF1 plays a more global role in muscle dynamics than has been previously thought. A better understanding of the regulation of MuRF1 has added crucial pieces to the puzzle of MuRF1's potential role in muscle metabolic control mechanisms. There are also numerous aspects of interest to the clinician as well as to the academic researcher, considering that MuRF1 has been implicated in numerous medical pathologies. In addition to the well-documented effects during denervation and cachexia, MuRF1 has also been recently implicated in muscle wasting associated with acute lung injury.<sup>38</sup> A better understanding of the regulation of MuRF1, and the regulation of other muscle specific genes by MuRF1, is therefore of high interest.

## Chapter 3: Post-Transcriptional Regulation of MuRF1 and MAFbx

### Overview

A continuation of the research described in this thesis will involve exploration of the regulation of MuRF1 and MAFbx expression at additional genetic levels, namely at the post-transcriptional level. Unlike the transcriptional regulation that directly activates or represses gene promoters, post-transcriptional regulation often occurs after the DNA has been transcribed by RNA Pol II into mRNA, but before the RNA has been translated into a protein. This process is often mediated by microRNAs (miRNAs), which are frequently localized to the untranslated region of mRNA at the 3' end of the transcript (3'UTR).<sup>39</sup> Future work in this area will include an investigation into the effect of extracellular signals such as Insulin-like Growth Factor-1 (IGF-1) and Transforming Growth Factor- $\beta$  (TGF- $\beta$ ) on the post-transcriptional regulation of MuRF1 and MAFbx via miRNA signaling, since these pathways have been implicated in both microRNA regulation and muscle tissue dynamics.<sup>40, 41</sup>

### MuRF1 and MAFbx Have Long 3' UTRs

Both MuRF1 and MAFbx have recently been revealed to have long and highly conserved 3'UTRs. Because the majority of microRNA-mediated post-transcriptional regulation occurs at the 3' region of target mRNA molecules, MuRF1 and MAFbx are

good candidates for such regulation. The basic structure of a eukaryotic gene is shown below in Figure 24.

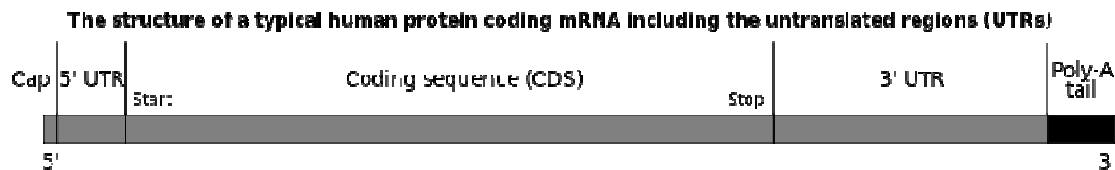


Figure 24. Schematic of the Basic Structure of a Eukaryotic Gene. (Open-access image)

In the figure shown above, the 3'UTR is illustrated at an approximately appropriate scale. The median length of a eukaryotic 3'UTR is roughly 700bp, and this region is known to affect the stability of the mRNA, translational efficiency, cellular localization, polyadenylation, and often contains binding sites for miRNA.<sup>42,43</sup>

The Ensembl genetic database ([www.ensembl.org](http://www.ensembl.org)) shows that MAFbx has an unusually long 3'UTR. If the MAFbx 3'UTR is indeed a conserved 5500 bp region of a transcript totaling 7000 bp, then it is likely that the MAFbx 3'UTR may play a significant regulatory role. Furthermore, the MuRF1 3'UTR is predicted to be approximately 750 bp in length. An analysis of the MuRF1 and MAFbx 3'UTRs by TargetScan, ([www.targetscan.org](http://www.targetscan.org)), reveals sequences that may act as potential seed sites of known miRNAs. The MuRF1 (Trim63) 3'UTR has potential binding sites for miR-23ab, miR-29abc, miR-101, and miR-144 (Figure 25), while the MAFbx (Fbxo32) 3'UTR has potential binding sites for miR-144, miR-30, miR-23ab, and let-7 (Figure 26).

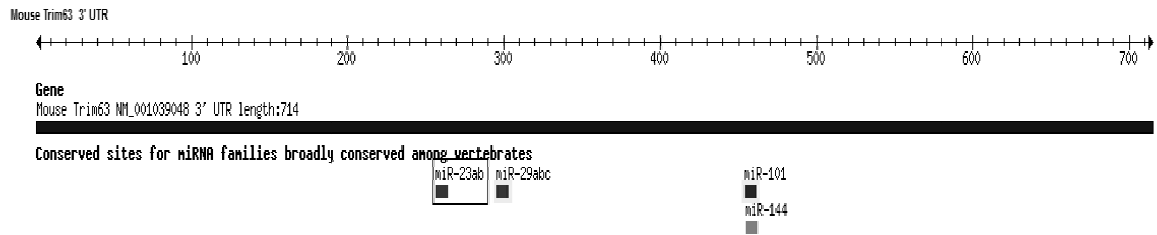


Figure 25. MuRF1 3' UTR with potential binding sites for known miRNAs. (image from [www.TargetScan.org](http://www.TargetScan.org))

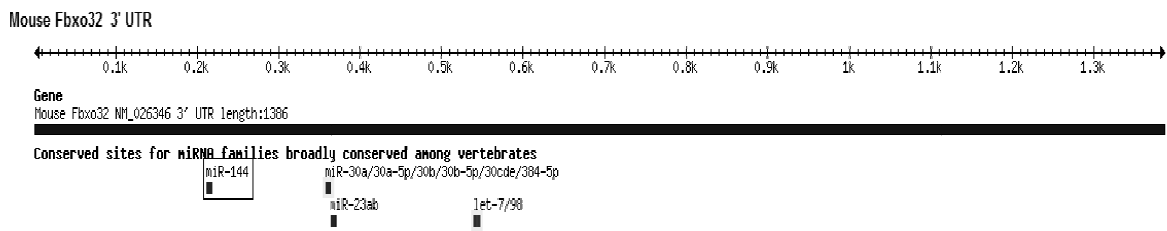


Figure 26. MAFbx 3' UTR with potential binding sites for known miRNAs. (image from [www.TargetScan.org](http://www.TargetScan.org))

### MicroRNA Structure and Processing

The existence of potential binding sites for multiple microRNAs (miRNAs) in the 3'UTRs of both MuRF1 and MAFbx supports the hypothesis that there might be post-transcriptional mechanisms of regulation for both MuRF1 and MAFbx. miRNAs are a focus of current interest in the field of molecular genetics, as they are highly prevalent, participate in nearly every cellular process, and are predicted by bioinformatic analyses to regulate roughly 60% of all protein-coding genes.<sup>44</sup> The discovery of miRNAs in unicellular organisms indicated that they have a much more ancient evolutionary origin than has been previously thought, and they have recently been shown to play a significant role in muscle atrophy.<sup>45,46</sup>

MicroRNAs are roughly 21 nucleotide-long RNA molecules that regulate gene expression. As seen below in Figure 27, miRNAs are initially transcribed as long pri-miRNA molecules.

Image redacted, paper copy available  
upon request to home institution.

Figure 27. The Biogenesis of MicroRNA molecules. (open-access image)

These large molecules are then processed in the nucleus by the RNase complex Drosha into smaller stem-loop miRNA precursors called pre-miRNA.<sup>47</sup> These pre-miRNA molecules are then exported from the nucleus by Exportin, and cleaved in the cytoplasm into mature miRNAs by the RNase complex Dicer.<sup>48</sup> One of the strands of double-stranded mature miRNA is incorporated into the RNA-Induced Silencing Complex (RISC) and the other strand is degraded.<sup>49</sup> This complex effectively targets complementary seed sequences in target mRNA molecules. The subsequent inhibition of the translation of mRNA to protein, or the de-polyadenylation and subsequent decay of mRNA both serve to negatively regulate target genes.<sup>50</sup>

The role of miRNAs in muscle remodeling is of increasing interest. Recent research has shown miRNAs, such as miR-23a, promote resistance to glucocorticoid-induced muscle atrophy, while concurrently repressing the translation of MuRF1 and MAFbx through their 3'UTRs.<sup>46</sup> Other research has shown miRNAs to be involved in the

processes of muscle myoblast differentiation by targeting muscle-specific transcription factors such as myogenin and MyoD1 activate genes that induce myoblasts to retreat from the cell cycle and ultimately fuse into multi-nucleated myotubes.<sup>51</sup> Additionally, TGF- $\beta$ , a known inducer of skeletal muscle atrophy, and IGF-1, a known inducer of muscle hypertrophy, both have been shown to affect miRNA expression in skeletal muscle.<sup>40</sup> Consequently, the role of TGF- $\beta$  and IGF-1 in miRNA-mediated regulation of MuRF1 and MAFbx is a potential avenue of continued exploration in our lab.

#### IGF-1 and TGF- $\beta$ Signaling Pathways Regulate Skeletal Muscle Dynamics

IGF-1 and TGF- $\beta$  play important regulatory roles in skeletal muscle hypertrophy, atrophy and fibrosis. Furthermore, these growth factors have been shown to impose their regulatory effects of muscle-specific gene expression at the post-transcriptional and transcriptional levels. For example, Insulin-like growth factor-1 (IGF-1) has been shown to induce muscle hypertrophy via the PI3K-Akt-FoxoO pathway leading to decreased MuRF1 and MAFbx expression (Figure 28).<sup>52</sup>

Image redacted, paper copy available upon  
request to home institution.

Figure 28. Schematic of the TGF- $\beta$  and IGF-1 Signaling Pathways.<sup>53</sup>

Furthermore, the overexpression of IGF-1 in mice causes an increase in muscle mass and cross-sectional area (CSA).<sup>54</sup> Conversely, the knock-down of the IGF-1 receptor causes a loss of muscle mass and CSA.<sup>55</sup> The binding of IGF-1 ligand to its corresponding receptor activates a cascade of events ultimately leading to protein synthesis, as shown in Figure 28.<sup>53</sup> In addition, IGF-1 and miRNAs reciprocally regulate each other in skeletal muscle, suggesting that IGF-1 may transcriptionally regulate miRNA expression, leading to downstream and indirect post-transcriptional regulation of muscle-specific genes.<sup>41</sup>

Like the muscle hypertrophy pathways, the muscle atrophy pathways are also highly regulated processes, which involve cross-talk with the synthesis pathway described above, providing another potential link to regulation of MuRF1 and MAFbx expression. A significant amount of research involves characterization of myostatin, a member of the TGF- $\beta$  superfamily, but recent research has shown that Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) may also play an important role in skeletal muscle function.



TGF- $\beta$ 1 is a well characterized cytokine that promotes the proliferation of fibroblast cells, causes fibrosis and inflammation, and is associated with many diseases of skeletal muscle.<sup>56-58</sup> TGF- $\beta$ 1 also regulates microRNAs that influence skeletal muscle differentiation.<sup>59</sup> Because TGF- $\beta$ 1 has been implicated in skeletal muscle wasting and fibrosis, this research also currently involves the exploration of the miRNA-mediated mechanism by which it may affect MuRF1 and MAFbx expression.

Interestingly, MAFbx expression levels and muscle protein ubiquitination have been demonstrated to increase quickly following TGF- $\beta$ 1 administration, but the molecular mechanisms behind the TGF- $\beta$ 1-mediated effects on MAFbx expression are not yet fully understood.<sup>60, 61</sup> TGF- $\beta$ 1 has also been previously shown to alter muscle plasticity via modulation of miR-29, and recently it has been determined that this effect is mediated, at least in part, by Smad3 inhibition of MyoD1 binding to the miR-29 promoter.<sup>62</sup> Future research will focus closely on the role of TGF- $\beta$ 1 in the post-transcriptional regulation of MuRF1 and MAFbx.

Finally, IGF-1 has also been shown to block upregulation of MuRF1 and MAFbx, in part, through the inhibition of myostatin.<sup>63</sup> Myostatin, a member of the TGF- $\beta$  superfamily of genes, phosphorylates and thus activates Smad3, which then regulate downstream genes, including MuRF1 and MAFbx.<sup>63</sup> The mediation of Smad3 via TGF- $\beta$ 1 and IGF-1 on MuRF1 and MAFbx expression has been explored further in our lab, with a focus on the role of miRNAs in this process.

## Materials and Methods

### Cloning of the 3'UTRs of MuRF1 and MAFbx

The MuRF1 and MAFbx 3'UTRs were analyzed using the Ensembl database (www.ensembl.org). The following primers were designed to amplify ~750 bp of the MuRF1 3'UTR and ~1300 bp of the MAFbx UTR:

MuRF1 FWD: 5'-GC-GAGCTC-AGA AGG AGA TGA GTG AGA CAC GC-3'

MuRF1 REV: 5'-GC-AAGCTT-GAG GCA GAG TCT CTC TAT GTA GC-3'

MAFbx FWD: 5'-GC-GAGCTC-AT AAT CCC AGC ACA CGA ACA CAC TTC AG-3'

MAFbx REV: 5'-GC-AAGCCT -GTT TGC CAA GAG CAT GCA TAG TGG C-3'

These primers were used in an RT-PCR reaction that consisted of PCR buffer, dNTP mixture, 1.5 mM MgCl<sub>2</sub>, forward and reverse primers, 2.0 µL of RT-reaction, and *Taq* polymerase diluted in nuclease-free water. The cycling conditions were 94°C for 2 min, followed by 30 cycles of 94°C for 45 sec, 55°C for 1 min, and 72°C for 1.5 minutes, a final extension at 72° for 10 min and then held at 10°C until the reactions were removed from the thermocycler. Following PCR, the products were electrophoresed on a 0.7% agarose gel, purified via a commercial purification protocol (Qiagen, Valencia, CA,) and cloned into the pMIR-Report luciferase reporter plasmid (Promega, Madison, WI).

### Construction of 3'UTR-SEAP-Reporter Plasmids

Pilot transfection experiments were performed in C<sub>2</sub>C<sub>12</sub> myoblast cells with little luciferase activity recorded, so an alternative approach was designed. The 1500 bp sequence of secreted alkaline phosphatase cDNA from the pSEAP2-Basic plasmid was amplified by PCR and cloned into the pMIR-Report reporter plasmid, lacking the luciferase coding region. Briefly, the 1500 bp sequence of the pSEAP2-Basic plasmid corresponding to the secretion of alkaline phosphatase was amplified via PCR using primers containing restriction enzyme sites for Bgl II in the forward primer and SpeI in the reverse primer. The PCR product was then run on a 0.7% gel and purified as previously described. The previously-designed pmir-Report plasmids with the MuRF1 and MAFbx 3'UTRs were digested with Bam HI and Spe I in order to remove the luciferase cDNA from the pMIR-Report plasmid. The 1500 bp SEAP fragment was then ligated into the linearized MuRF1-3'UTR-pMIR-Report and the MAFbx-3'UTR-pMIR-Report plasmids lacking the luciferase gene. This effectively formed two original plasmids with either the MuRF1 3'UTR or the MAFbx 3'UTR fused to the SEAP gene, (MuRF1-SEAP-Report and MAFbx-SEAP-Report, respectively) which are well suited for analysis in C<sub>2</sub>C<sub>12</sub> cells.

### SEAP Reporter Assays

The activities of the newly designed MuRF1-SEAP-Report and MAFbx-SEAP-Report plasmids were preliminarily tested in mouse myoblast cells. The MuRF1-SEAP-Report plasmid and the MAFbx-SEAP-Report plasmid was transfected into C<sub>2</sub>C<sub>12</sub> cells as described previously. Following a 24 hr incubation at 37°C at 6% CO<sub>2</sub>, the C<sub>2</sub>C<sub>12</sub> cells

were then treated with either 100 pM TGF- $\beta$ 1, 100 ng/ml IGF-1, 1  $\mu$ M Dexamethasone, or a control solution containing only DMEM media. The levels of secreted alkaline phosphatase and corresponding plasmid activities were measured at 24 hr intervals using a commercial SEAP protocol as described previously in Chapter 2.

### siRNA Knockdown

siRNA knockdown of Smad3 was performed to further illustrate the role this gene plays in the atrophy process. This was accomplished through the use of the pSuper-siRNA System (OligoEngine), which uses a mammalian expression vector that allows for the transcription of short RNA transcripts such as siRNAs.

Oligonucleotide sequences were designed to correspond exactly to identified nucleotide sequences identified in the mRNA transcript of Smad3. Two unique sequences for targeting Smad3 were designed, phosphate end-labeled with T4 Polynucleotide Kinase, annealed, cloned into the pSuper plasmid and sequenced to confirm lack of mutations. The sequences used were:

#### **Smad3 Set1**

```
5' GATCCCC ACT TTC TAC TGC CAC TTG G TTCAAGAGA C CAA GTG GCA GTA GAA AGT TTTT TC 3'
3' GGG TGA AAG ATG ACG GTG AAC C AAGTTCTCT G GTT CAC CGT CAT CTT TCA AAAA AGAGCT 5'
```

---

#### **Smad3 Set2**

```
5' GATCCCC GTT CTC CAG AGT TAA AAG C TTCAAGAGA G CTT TTA ACT CTG GAG AAC TTTT TC 3'
3' GGG CAA GAG GTC TCA ATT TTC G AAGTTCTCT C GAA AAT TGA GAC CTC TTG AAAA AGAGCT 5'
```

The resulting pSuper plasmids were transfected with the SEAP-MAFbx-Report plasmid or the SEAP-MuRF1-Report plasmid into C<sub>2</sub>C<sub>12</sub> cells as described previously. A pSuper-null plasmid, similarly designed but containing no inhibitory sequences, was used as a

negative control. The effects of Smad3 knockdown on the activity of the SEAP-MAFbx-Report and SEAP-MuRF1-Report plasmids were then analyzed using a commercial SEAP protocol as previously described.

### Preliminary Results

As seen below in Figure 29, the MAFbx-SEAP-Report and the MuRF1-SEAP-Report reporter constructs were successfully engineered and both showed quantifiable expression levels following transfection into C<sub>2</sub>C<sub>12</sub> cells. The activity of the constructed SEAP-Report plasmids containing either the MuRF1 3'UTR or MAFbx 3'UTR showed a marked increase in activity at 72 hrs when compared to the control SEAP-Report-null plasmid after treatment with TGF- $\beta$ 1, while IGF-1 had no effect on reporter activity.

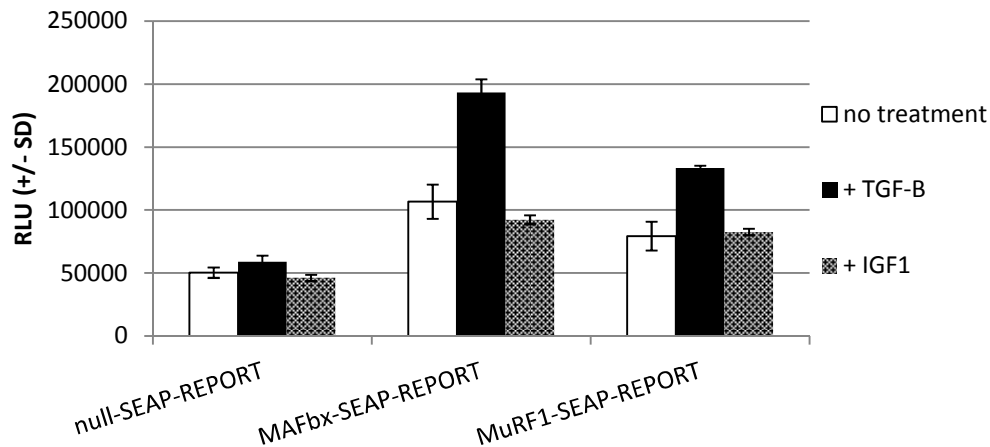


Figure 29. The MAFbx-SEAP-Report and MuRF1-SEAP-Report plasmids show an increase in expression in cells treated with TGF- $\beta$ 1. C<sub>2</sub>C<sub>2</sub> myoblasts were transfected with the MAFbx-SEAP-Report plasmid, the MuRF1-SEAP-Report plasmid, or a control plasmid consisting of only the empty SEAP-Report plasmid, and a  $\beta$ -galactosidase expression plasmid. The cells were treated with either TGF- $\beta$ 1, IGF-1, or DMEM as a negative control. The myoblasts were maintained in standard culture media. Samples of media were taken at 72 hrs and measured for SEAP activity. The samples were normalized to  $\beta$ -galactosidase activity to correct for variation in transfection efficiency. Each condition was done in triplicate and the error bars represent standard deviation ( $\pm$  S.D.) of the mean.

Furthermore, since dexamethasone is a synthetic glucocorticoid that is known to induce muscle atrophy, the activities of the MuRF1 and MAFbx 3'-UTR reporter constructs were analyzed in cells transfected with the glucocorticoid receptor and treated with dexamethasone. The data revealed no significant change in activity of the SEAP-null plasmid when treated with dexamethasone, however there was a marked increase in activity of both the SEAP-Report-MuRF1-3'UTR plasmid or the SEAP-Report-MAFbx-3'UTR after treatment with dexamethasone (Figure 30).

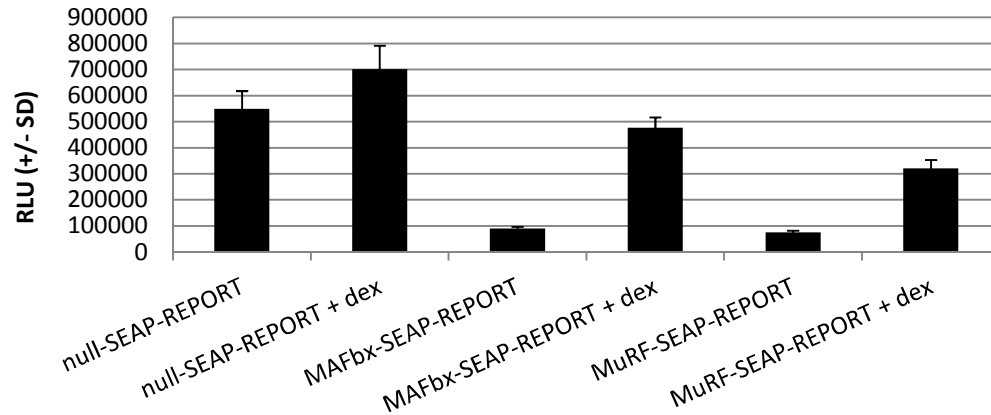


Figure 30. The MAFbx-SEAP-Report and MuRF1-SEAP-Report plasmids show increased expression when treated with Dexamethasone. C<sub>2</sub>C<sub>12</sub> myoblasts were transfected with the MAFbx-SEAP-Report reporter, the MuRF1-SEAP-Report reporter, or a control plasmid consisting of only the empty SEAP-Report plasmid, and a  $\beta$ -galactosidase expression plasmid. The cells were treated with either dexamethasone or DMEM as a negative control. The myoblasts were maintained in standard culture media. Samples of media were taken at 72 hrs and measured for SEAP activity. The samples were normalized to  $\beta$ -galactosidase activity to correct for variation in transfection efficiency. Each condition was done in triplicate and the error bars represent standard deviation ( $\pm$  S.D.) of the mean.

Finally, since Smad3 has been linked to several miRNA-mediated mechanisms of regulation, preliminary work has also involved investigating the effects of Smad3 knockdown on MuRF1 and MAFbx 3'UTR reporter plasmid activity. To test the role of Smad3 in MAFbx expression, activity of the MAFbx-SEAP-Report and MuRF1-SEAP-Report plasmids were compared to that of an empty control SEAP-Report plasmid with

no UTR, under conditions of Smad3 knockdown. Smad3 knockdown was accomplished by using either the Smad3-pSuper-siRNA expression plasmid or a control pSuper plasmid lacking an siRNA insert. The empty SEAP-Report-null plasmid showed no significant differences in activity in normal cells compared to Smad3-knockdown cells, while the MAFbx-3'UTR-SEAP-Report and MuRF1-3'UTR-SEAP-Report reporter construct showed a significant increase in activity in Smad3 knockdown cells compared to normal cells (Figure 31).

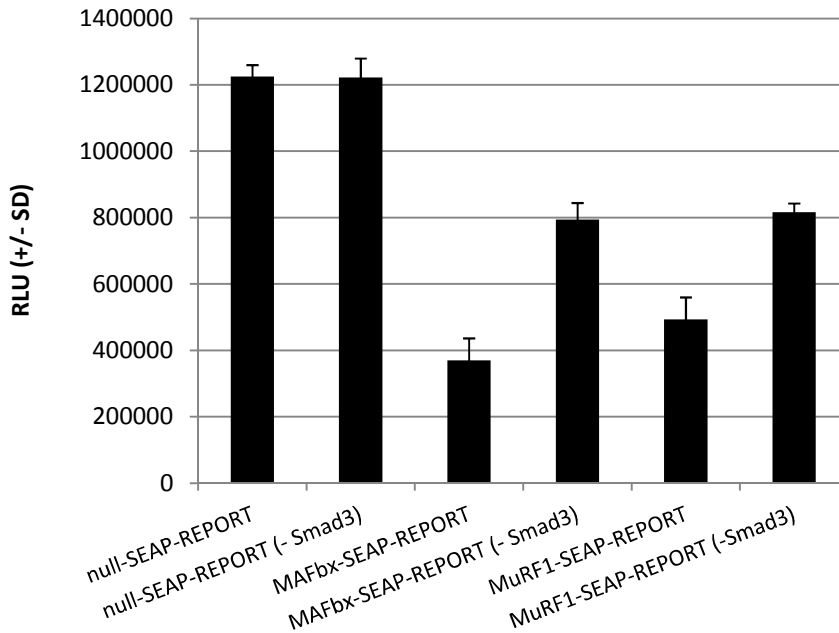


Figure 31. The MuRF1-SEAP-Report and MAFbx-SEAP-Report plasmids show an increase in expression in Smad3 knockdown cells. C<sub>2</sub>C<sub>12</sub> myoblasts were transfected with a reporter construct consisting of the MAFbx-SEAP-Report plasmid, MuRF1-SEAP-Report plasmid or a negative control plasmid consisting of only the empty SEAP-Report plasmid, and a  $\beta$ -galactosidase expression plasmid. The cells had previously been transfected with either pSuper-Smad3 siRNA, or an empty pSuper expression plasmid. The myoblasts were maintained in standard culture media. Samples of media were taken at 72 hrs and measured for SEAP activity. The samples were normalized to  $\beta$ -galactosidase activity to correct for variation in transfection efficiency. Each condition was done in triplicate and the error bars represent standard deviation ( $\pm$  S.D.) of the mean.

### Future Work:

In light of the above preliminary results, future work will involve continued exploration of the post-transcriptional regulation of MuRF1 and MAFbx with respect to Smad3-mediated miRNA activity. A bioinformatics approach was undertaken to further characterize the potential contribution of miRNA in the regulation of MuRF1 and MAFbx. The TargetScan database ([www.TargetScan.org](http://www.TargetScan.org)) was used to identify putative binding sites for miRNAs in the 3'UTRs of MuRF1 and MAFbx. miR-23 and mir-144 were identified as having binding sites present in the 3'UTRs of both MuRF1 and MAFbx, suggesting possible common patterns of regulation by these miRNAs. mir-23 has already been shown to play a role in muscle development, while miR-144 has recently been shown to be differentially expressed in aging muscle.<sup>64, 65</sup> Thus, future work on this project should focus on these miRNAs.

Reporter assays using the MuRF1-3'UTR-SEAP-Report or MAFbx-3'UTR-SEAP-Report plasmids should be performed with the simultaneous overexpression of miRNA-23 and/or miRNA-144 to determine if these microRNAs can alter the expression of the MuRF1 and MAFbx 3'-UTR reporter plasmids. Additionally, the introduction of anti-miRNA locked nucleic acids (LNAs) should be used to reverse these miRNA effects. LNAs are nucleic acid analogs in which the ribose ring is locked with a methylene bridge connecting the 2'-oxygen atom with the 4'-carbon within the ring. These LNAs bind to, and effectively inhibit, complementary microRNAs. The combination of experiments involving the overexpression of specific miRNAs with experiments involving specific miRNA inhibition, will allow us to determine with confidence that these microRNAs bind to the UTRs of MuRF1 and MAFbx.



Finally, in order to further examine post-transcriptional regulation of MuRF1 and MAFbx, this project will eventually compare levels of MuRF1 and MAFbx protein production following deletion of the 3'UTRs of these genes. If the levels of protein produced differ following deletion of the 3'UTR, then a post-transcriptional mechanism of regulation may be implicated. In order to address these questions, the cDNA molecules lacking the 3'UTRs of MuRF1 and MAFbx have been cloned. These genes have been tagged with myc, which is polypeptide protein tag, and cloned into expression plasmids. Future work will also involve cloning the MuRF1 and MAFbx cDNA with their 3'UTRs, tagging them with myc, and inserting them into an expression plasmid. These plasmids containing myc-tagged MuRF1 and MAFbx with or without their 3'UTRs will then be transfected into C<sub>2</sub>C<sub>12</sub> cells that will be subsequently treated with or without TGF- $\beta$ 1, IGF-1, or Dexamethasone. The cells will then be homogenized and proteins lysates will be used for Western Blotting to detect MuRF1 and MAFbx protein levels in order to determine in changes in protein production that may result due to changes in miRNA expression mediated by growth factor treatments.

## References

1. Bodine, S. Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science*, 1704-1708 (2001).
2. Frontera, W. R. et al. Aging of skeletal muscle: a 12-yr longitudinal study. *J. Appl. Physiol.* 88, 1321-1326 (2000).
3. Clarke, B. A. et al. The E3 Ligase MuRF1 degrades myosin heavy chain protein in dexamethasone-treated skeletal muscle. *Cell metabolism* 6, 376-385 (2007).
4. Berkes, C. A. & Tapscott, S. J. MyoD and the transcriptional control of myogenesis. *Semin. Cell Dev. Biol.* 16, 585-595 (2005).
5. Londhe, P. & Davie, J. K. Sequential association of myogenic regulatory factors and E proteins at muscle-specific genes. *Skeletal muscle* 1, 1-18 (2011).
6. Furlow, J. D. Altered Gene Expression Patterns in Muscle Ring Finger 1 Null Mice During Denervation and Dexamethasone Induced Muscle Atrophy. *Physiological Genomics* (2013).
7. Rock, K. L. et al. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* 78, 761-771 (1994).
8. Dodd, R. Ubiquitylation. Open-access Image. Attribution: Rogerdodd at the English language Wikipedia.
9. Hass, A. L. & Rose, I. A. The mechanism of ubiquitin activating enzyme. A kinetic and equilibrium analysis. *J. Biol. Chem.* 10329-10337 (1982).
10. Jentsch, S. The ubiquitin-conjugation system. *Annu. Rev. Genet.* 26, 179-207 (1992).

11. Deshaies, R. J. & Joazeiro, C. A. RING Domain E3 Ubiquitin Ligases. *Annu. Rev. Biochem.* 78, 399-434 (2009).
12. Peters, J., Cejka, Z., Harris, J. R., Kleinschmidt, J. A. & Baumeister, W. Structural features of the 26 S proteasome complex. *J. Mol. Biol.* 234, 932-937 (1993).
13. Tintignac, L. A. et al. Degradation of MyoD mediated by the SCF (MAFbx) ubiquitin ligase. *J. Biol. Chem.* 280, 2847-2856 (2005).
14. Liao, J. & Willis, M. The Ubiquitin Ligase MuRF1 Regulates Cardiac Metabolism via Mono-ubiquitination of PPAR $\alpha$  to Protect Heart Function. Department of Pathology & Laboratory Medicine, 2 McAllister Heart Institute University of North Carolina at Chapel Hill, Chapel Hill, NC (2011).
15. Hicke, L. Protein regulation by monoubiquitin. *Nature reviews molecular cell biology* 2, 195-201 (2001).
16. Freemont, P. S. The RING finger. *Ann. N. Y. Acad. Sci.* 684, 174-192 (1993).
17. Hohlfeld, S. et al. AC-terminal translocation signal is necessary, but not sufficient for type IV secretion of the *Helicobacter pylori* CagA protein. *Mol. Microbiol.* 59, 1624-1637 (2006).
18. McElhinny, A. S., Kakinuma, K., Sorimachi, H., Labeit, S. & Gregorio, C. C. Muscle-specific RING finger-1 interacts with titin to regulate sarcomeric M-line and thick filament structure and may have nuclear functions via its interaction with glucocorticoid modulatory element binding protein-1. *J. Cell Biol.* 157, 125-136 (2002).
19. Moresi, V. et al. Myogenin and class II HDACs control neurogenic muscle atrophy by inducing E3 ubiquitin ligases. *Cell* 143, 35-45 (2010).
20. Latchman, D. S. Transcription factors: an overview. *Int. J. Biochem. Cell Biol.* 29, 1305-1312 (1997).

21. Malik, S., Huang, C. & Schmidt, J. The Role of the CANNTG Promoter Element (E box) and the Myocyte-enhancer-binding-factor-2 (MEF-2) Site in the Transcriptional Regulation of the Chick Myogenin Gene. *European Journal of Biochemistry* 230, 88-96 (1995).
22. Moresi, V. et al. Myogenin and class II HDACs control neurogenic muscle atrophy by inducing E3 ubiquitin ligases. *Cell* 143, 35-45 (2010).
23. Atchley, W. R. & Fitch, W. M. A natural classification of the basic helix–loop–helix class of transcription factors. *Proceedings of the National Academy of Sciences* 94, 5172-5176 (1997).
24. Olson, E. N. MyoD family: a paradigm for development? *Genes Dev.* 4, 1454-1461 (1990).
25. Halevy, O. et al. Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* 267, 1018-1021 (1995).
26. Hecker, L., Jagirdar, R., Jin, T. & Thannickal, V. J. Reversible differentiation of myofibroblasts by MyoD. *Exp. Cell Res.* 317, 1914-1921 (2011).
27. Hasty, P. et al. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. (1993).
28. Nabeshima, Y. et al. Myogenin gene disruption results in perinatal lethality because of severe muscle defect. (1993).
29. Ruderman, N. B. Muscle amino acid metabolism and gluconeogenesis. *Annu. Rev. Med.* 26, 245-258 (1975).
30. Pedersen, B. K. The diseasome of physical inactivity—and the role of myokines in muscle–fat cross talk. *J. Physiol. (Lond. )* 587, 5559-5568 (2009).
31. Lecker, S. H. et al. Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. *The FASEB Journal* 18, 39-51 (2004).

32. Borden, K. L. & Freemont, P. S. The RING finger domain: a recent example of a sequence—structure family. *Curr. Opin. Struct. Biol.* 6, 395-401 (1996).
33. Short, K. M. & Cox, T. C. Subclassification of the RBCC/TRIM superfamily reveals a novel motif necessary for microtubule binding. *J. Biol. Chem.* 281, 8970-8980 (2006).
34. Waddell, D. S. et al. The glucocorticoid receptor and FOXO1 synergistically activate the skeletal muscle atrophy-associated MuRF1 gene. *American Journal of Physiology-Endocrinology And Metabolism* 295, E785-E797 (2008).
35. Willis, M. S. et al. Muscle ring finger 1 and muscle ring finger 2 are necessary but functionally redundant during developmental cardiac growth and regulate E2F1-mediated gene expression in vivo. *Cell Biochem. Funct.* (2013).
36. McElhinny, A. S., Kakinuma, K., Sorimachi, H., Labeit, S. & Gregorio, C. C. Muscle-specific RING finger-1 interacts with titin to regulate sarcomeric M-line and thick filament structure and may have nuclear functions via its interaction with glucocorticoid modulatory element binding protein-1. *J. Cell Biol.* 157, 125-136 (2002).
37. Dai, K. & Liew, C. A novel human striated muscle RING zinc finger protein, SMRZ, interacts with SMT3b via its RING domain. *J. Biol. Chem.* 276, 23992-23999 (2001).
38. Files, D. C. et al. A critical role for muscle ring finger-1 in acute lung injury–associated skeletal muscle wasting. *American journal of respiratory and critical care medicine* 185, 825 (2012).
39. Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281-297 (2004).
40. Sun, Q. et al. Transforming growth factor- $\beta$ -regulated miR-24 promotes skeletal muscle differentiation. *Nucleic Acids Res.* 36, 2690-2699 (2008).

41. Elia, L. et al. Reciprocal regulation of microRNA-1 and insulin-like growth factor-1 signal transduction cascade in cardiac and skeletal muscle in physiological and pathological conditions. *Circulation* 120, 2377-2385 (2009).
42. Pesole, G. et al. Structural and functional features of eukaryotic mRNA untranslated regions. *Gene* 276, 73-81 (2001).
43. He, L. & Hannon, G. J. MicroRNAs: small RNAs with a big role in gene regulation. *Nature Reviews Genetics* 5, 522-531 (2004).
44. Lewis, B. P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120, 15--20 (2005).
45. Zhao, T. A complex system of small RNAs in the unicellular green algae *Chlamydomonas reinhardtii*. *Genes Dev.*, 1190-1203 (2007).
46. Wada, S. et al. Translational suppression of atrophic regulators by microRNA-23a integrates resistance to skeletal muscle atrophy. *J. Biol. Chem.* 286, 38456-38465 (2011).
47. Denli, A. M., Tops, B. B., Plasterk, R. H., Ketting, R. F. & Hannon, G. J. Processing of primary microRNAs by the Microprocessor complex. *Nature* 432, 231-235 (2004).
48. Lund, E., Güttinger, S., Calado, A., Dahlberg, J. E. & Kutay, U. Nuclear export of microRNA precursors. *Science* 303, 95-98 (2004).
49. Kim, V. N. MicroRNA biogenesis: coordinated cropping and dicing. *Nature reviews Molecular cell biology* 6, 376-385 (2005).
50. Fabian, M. R., Sonenberg, N. & Filipowicz, W. Regulation of mRNA Translation and Stability by microRNAs. *Annu. Rev. Biochem.* 79, 351-379 (2010).

51. Rao, P. K., Kumar, R. M., Farkhondeh, M., Baskerville, S. & Lodish, H. F. Myogenic factors that regulate expression of muscle-specific microRNAs. *Proceedings of the National Academy of Sciences* 103, 8721-8726 (2006).
52. Sandri, M. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell*, 399-412 (2004).
53. Trendelenburg, A. U. et al. Myostatin reduces Akt/TORC1/p70S6K signaling, inhibiting myoblast differentiation and myotube size. *American Journal of Physiology-Cell Physiology* 296, C1258-C1270 (2009).
54. Musaro, A. Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. *Nature Genetics* 27, 195-200 (2001).
55. Mavalli, M. D. et al. Distinct growth hormone receptor signaling modes regulate skeletal muscle development and insulin sensitivity in mice. *J. Clin. Invest.* 120, 4007-4020 (2010).
56. LEASK, A. & ABRAHAM, D. J. TGF- $\beta$  signaling and the fibrotic response. *The FASEB Journal* 18, 816-827 (2004).
57. Roberts, A. B. et al. Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proceedings of the National Academy of Sciences* 83, 4167-4171 (1986).
58. Bogdanovich, S., Perkins, K. J., Krag, T. O. B. & Khurana, T. S. Therapeutics for Duchenne muscular dystrophy: current approaches and future directions. *Journal of Molecular Medicine* 82, 102-115 (2004).
59. Sun, Q. et al. Transforming growth factor- $\beta$ -regulated miR-24 promotes skeletal muscle differentiation. *Nucleic Acids Res.* 36, 2690-2699 (2008).
60. McFarlane, C. et al. Myostatin induces cachexia by activating the ubiquitin proteolytic system through an NF- $\kappa$ B-independent, FoxO1-dependent mechanism. *J. Cell. Physiol.* 209, 501-514 (2006).

61. Li, Y. P. et al. TNF- $\alpha$  acts via p38 MAPK to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle. *The FASEB Journal* 19, 362-370 (2005).
62. Zhou, L. et al. Inhibition of miR-29 by TGF-beta-Smad3 signaling through dual mechanisms promotes transdifferentiation of mouse myoblasts into myofibroblasts. *PLoS One* 7, e33766 (2012).
63. Morissette, M. R., Cook, S. A., Buranasombati, C., Rosenberg, M. A. & Rosenzweig, A. Myostatin inhibits IGF-I-induced myotube hypertrophy through Akt. *American Journal of Physiology-Cell Physiology* 297, 1124-1132 (2009).
64. Wang, L. et al. MiR-23a inhibits myogenic differentiation through down regulation of fast myosin heavy chain isoforms. *Exp. Cell Res.* (2012).
65. Mercken, E. M. et al. Age-associated miRNA Alterations in Skeletal Muscle from Rhesus Monkeys reversed by caloric restriction. *Aging (Albany NY)* 5, 692 (2013).