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

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

By

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Certificate of Approval

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Abstract

Skeletal muscle wasting occurs as a corollary of numerous physiological conditions, including denervation, 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. Data in this thesis provides interesting new evidence that MuRF1 may also act as a transcriptional modulator of atrophy-induced genes or atrogenes. The transcriptional regulation of MuRF1 and MAFbx were characterized using a reporter gene system and exhibited repressed activity in C_2C_{12} cells overexpressing MuRF1. Furthermore, ectopic expression of the myogenic regulatory factors (MRFs), MyoD and myogenin, caused significant activation of the MuRF1 and MAFbx reporter gene constructs, while co-overexpression of MuRF1 with MRFs resulted in reversal of MRF induction of reporter gene activity. Interestingly, ectopic expression of a catalytically dead MuRF1 RING mutant failed to reverse MRF activation of the reporters, suggesting that ubiquitin ligase activity may be necessary for MuRF1 transcriptional regulation. To further investigate a potential mechanism of MuRF1 regulation of MRF activity, Western blot analysis was performed to analyze MRF protein levels in C_2C_{12} cells overexpressing MuRF1 and MuRF1 RING mutant. Cells with ectopic MuRF1 or MuRF1 RING mutant showed repressed levels of myogenin. Additionally, cells overexpressing MuRF1 and MuRF1 RING mutant treated with MG132 showed only a partial rescue of myogenin protein levels. Finally, chromatin immunoprecipitation was performed to analyze occupancy of MRFs at the MuRF1 promoter. Overexpression of MRFs resulted in increased MuRF1 promoter immunoprecipitation (IP) and amplification, while co-overexpression of MuRF1 with MRFs resulted in a reversal of promoter IP and amplification. These findings suggest that MuRF1 may regulate MRF transcriptional activity in a non-canonical fashion giving insight into a potentially new mechanism by which MuRF1 may act to transcriptionally regulate atrophy-induced gene expression.

Chapter 1: Overview of the Involvement of MuRF1 in Skeletal Muscle Atrophy

Skeletal Muscle Atrophy

Skeletal muscle is a dynamic tissue distinguished by its ability to receive and interpret external cues and integrate them into a physiological response. The maintenance of this tissue depends partially on a balance between protein synthesis and degradation that is regulated in response to the physiological needs of the individual [1]. This balance can often be disrupted due to a myriad of physiological conditions, which cause the rate of protein degradation to outpace the rate of protein synthesis. This is characterized as muscle atrophy, a loss of proteins that are essential for muscle integrity and function [2, 3]. Some conditions that often give rise to skeletal atrophy include sarcopenia, cancer, corticosteroid use, joint immobilization, disuse, and denervation [1,4]. While skeletal muscle atrophy and many parts of the atrophy pathway have been well characterized, numerous elements in the pathway and their roles have been challenging to define. The characterization and future treatment of neurogenic muscle atrophy depends on research that will provide a better understanding of the molecular events occurring during this process.

The Ubiquitin Proteasome System and the Role of E3 Ubiquitin Ligases

Protein degradation in muscle atrophy is primarily handled by the 26S ubiquitin proteasome system (UPS). This system operates through ATP-dependent mechanisms driving the proteasome to degrade damaged or unneeded proteins by hydrolyzing peptide bonds [5]. Proteins destined for degradation through the UPS are tagged with a ubiquitin molecule by E3 ubiquitin ligases such as Muscle RING Finger-1 (MuRF1).

E3 ligase-mediated ubiquitination occurs via a covalent modification of a lysine residue on a target protein. This process requires a combination of three different enzymes: E1, E2 and E3. The E1 enzyme is responsible for hydrolyzing an ATP molecule and adding adenylate to a ubiquitin

molecule [5, 6]. After this step is completed, the adenylated ubiquitin is transferred to a cysteine residue of the E2, called the ubiquitin-conjugating enzyme [5]. Finally, the E3 ubiquitin ligase recognizes the protein that needs to undergo degradation and catalytically transfers the ubiquitin to the target protein (Figure 1) [5]. The E3 enzyme is the most selective component of this system, as it delegates substrate specificity for the UPS [6].

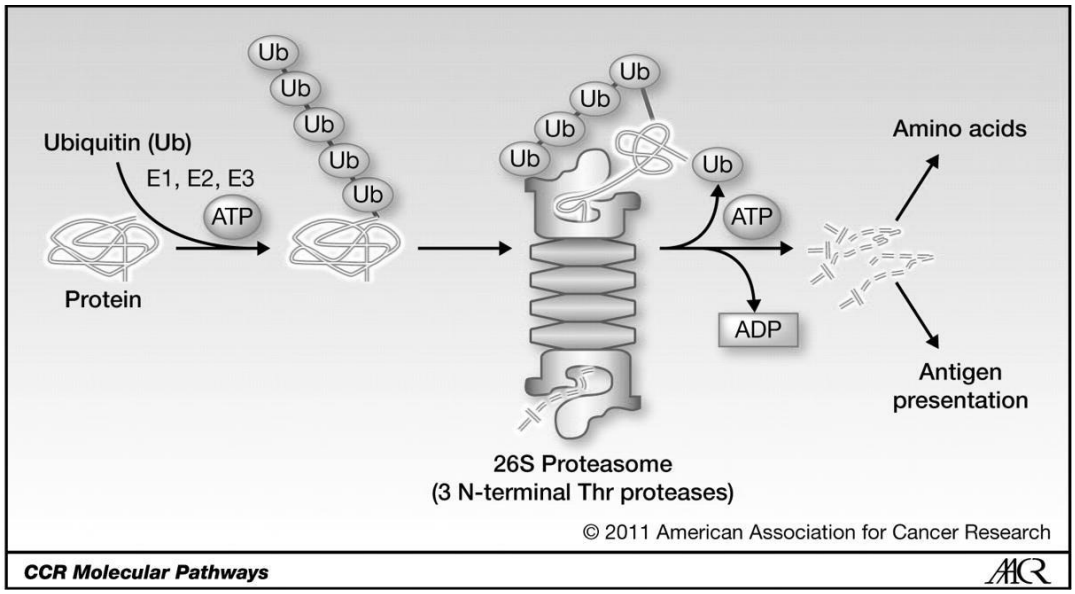

Figure 1. Schematic of the ubiquitin proteasome system. Protein degradation by the 26S proteasome involves collaboration of three enzymes; a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase (E3) (Adapted from Molineaux 2012).

MuRF1 as a Marker of Skeletal Muscle Atrophy

Previous research was conducted aiming to identify specific genetic markers expressed at high levels under atrophy conditions [4]. Two markers of interest were up-regulated in response to atrophic stimuli: the E3 ubiquitin ligases MuRF1 and Muscle Atrophy F-box (MAFbx) [4]. Bodine et al. identified MuRF1 and MAFbx in 2001 using differential expression analysis of muscle isolated from rats that had been subjected to immobilization, denervation, or hind limb suspension. Interestingly, numerous genes showed altered expression under one or two conditions, but only

MuRF1 and MAFbx exhibited induction in response to virtually all conditions of atrophy (Figure 2) [4]. Furthermore, mice lacking MuRF1 or MAFbx expression were resistant to skeletal muscle atrophy compared to their wild-type littermates, suggesting that MuRF1 and MAFbx are important mediators of muscle wasting [4, 7, 8, 9]. However, the mechanism by which MuRF1 and MAFbx regulate atrophy is still not fully understood. In the years since the discovery of these hallmark indicators of muscle atrophy very few targets of these E3 ubiquitin ligases have been identified.

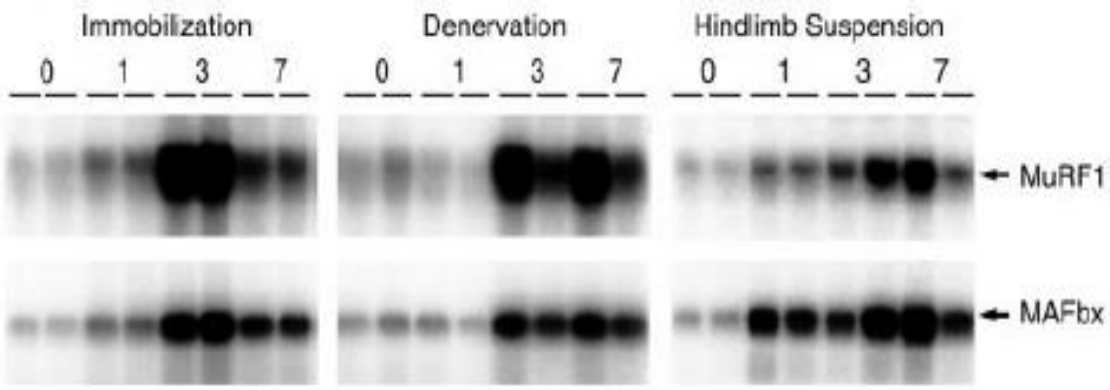

Figure 2. mRNA expression profiles of MuRF1 and MAFbx in Sprague-Dawley rat gastrocnemius muscle under muscle atrophy conditions. MuRF1 and MAFbx showed increased expression following limb immobilization, sciatic nerve denervation, and hind limb suspension by day 1 with maximum expression reached by 3 days. Numbers represent days post-denervation. (Adapted from Bodine, et al., 2001.)

MuRF1 is an E3 ubiquitin ligase induced under virtually all atrophy conditions, suggesting that it plays a large role in the destabilization of proteins through the UPS during muscle wasting [4, 10, 11, 12]. MuRF1 has three different functional domains that suggest its potential function(s) in muscle atrophy (Figure 3). The Really Interesting New Gene (RING) domain is predicted to perform the catalytic action of MuRF1's participation in ubiquitination and is needed for E3 ligase activity [13, 14, 15]. There are also B-box and B-box c-terminal (Bbc) domains that possibly form additional zinc finger structures that aid in binding to DNA and/or other proteins [15]. The function of the acidic c-terminus of MuRF1 currently has no known function; however, acidic protein termini are thought to have a possible role in the subcellular localization of proteins [16].

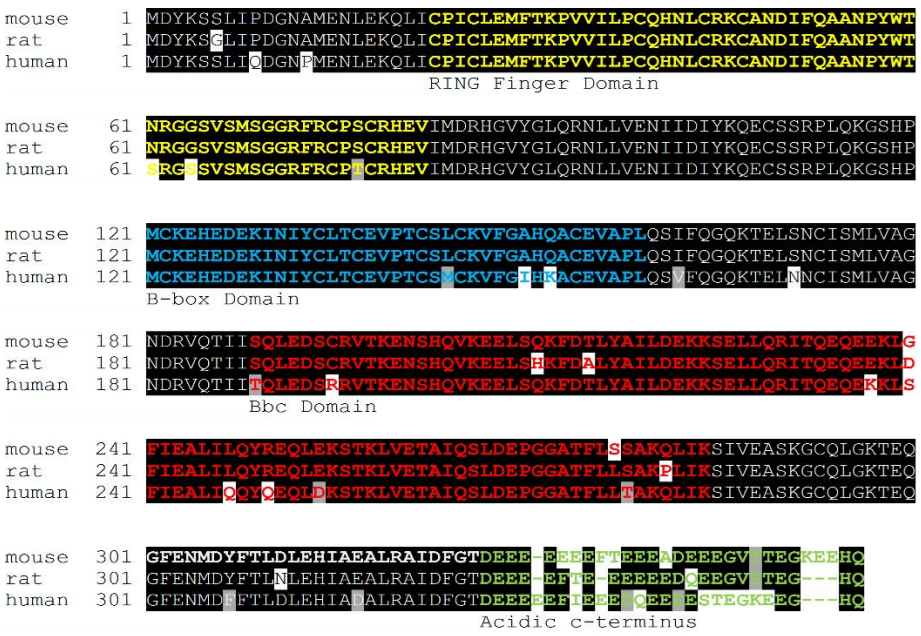

Figure 3. Alignment schematic of the MuRF1 Protein. MuRF1 protein sequences for mouse, rat and human were downloaded from the Ensembl database (www.ensembl.org). The sequences were then aligned using the Clustal Omega alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalo/) and shaded using the Boxshade tool available on the ExPASy Bioinformatics Resource Portal (http://www.expasy.org/).

MuRF1 as a Transcriptional Regulator of Muscle Atrophy

MuRF1 knock-out (KO) mice were created to better understand the targets of MuRF1 under atrophy conditions. A β-galactosidase-encoding lacZ cassette was inserted within the coding region of the MuRF1 gene. In theory, MuRF1 wild-type (WT) mice produce functional MuRF1 gene product while KO mice containing the lacZ cassette produce β-galactosidase under control of the MuRF1 endogenous promoter. Microarray analysis was performed on intact and denervated gastrocnemius tissue that was isolated from MuRF1 WT and KO mice [13]. MuRF1 gene expression increased in WT mice following denervation and decreased back to baseline levels by 14 days post-denervation. However, in the MuRF1-null mice, levels of β-galactosidase increased and remained elevated at 14 days after denervation. The significance of these data lies within the

observation that the levels of MuRF1 returned to baseline at two weeks post-denervation, whereas the levels of β-galactosidase remained elevated at 14 days post-denervation. Both β-galactosidase and MuRF1 are under the control of the same endogenous MuRF1 promoter, thus it is reasonable to hypothesize that in wild-type mice MuRF1 may participate in a feedback loop to negatively regulate its own transcriptional activity (Figure 4) [13].

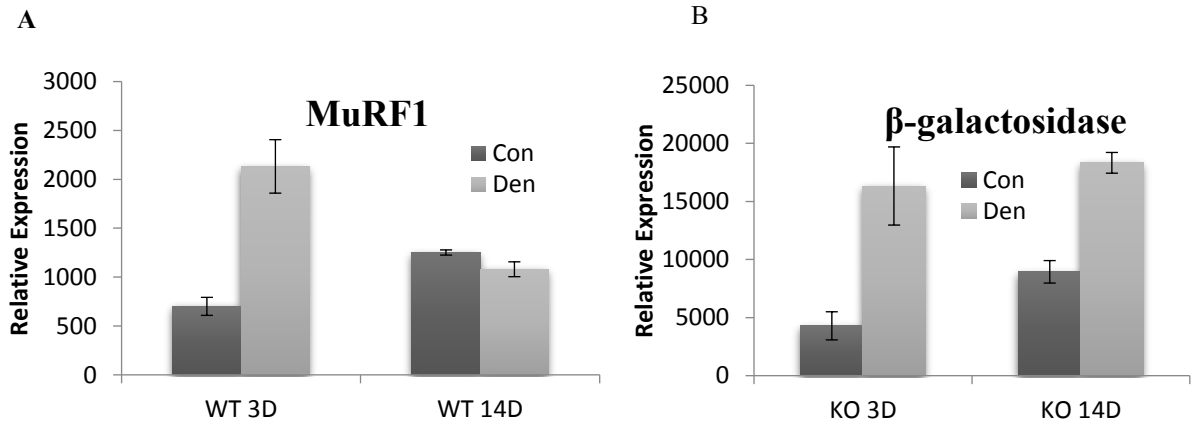

Figure 4. Transcriptional regulation of the MuRF1 gene locus is altered in MuRF1-null mice following denervation. Whole genome expression analysis was conducted on gastrocnemius muscle from (A) wild-type (WT) and (B) MuRF1-null (KO) mice following 3 days (3D) and 14 days (14D) post-denervation. MuRF1 expression was elevated at 3 days post-denervation but returned to baseline expression levels by 14 days post-denervation in WT mice (A). In contrast, β-galactosidase, which is inserted into the MuRF1 locus in the MuRF1-null mice and is under the control of the endogenous MuRF1 regulatory region, increased at 3 days post-denervation, but remained elevated at 14 days post-denervation in KO mice (B). Each condition was conducted in triplicate and the expression is the average of three individual mice and error reflects +/- SEM.

Data from the microarray also suggests that MuRF1 might be necessary for the transcriptional regulation of MAFbx expression under denervation conditions. Expression levels of MAFbx showed the same trend that was observed with MuRF1: in WT mice MAFbx expression increased after 3 days of denervation but returned to baseline by 14 days post-denervation. However, in the MuRF1-KO mice MAFbx expression increased following 3 days of denervation, but also remained elevated at 14 days post-denervation (Figure 5).

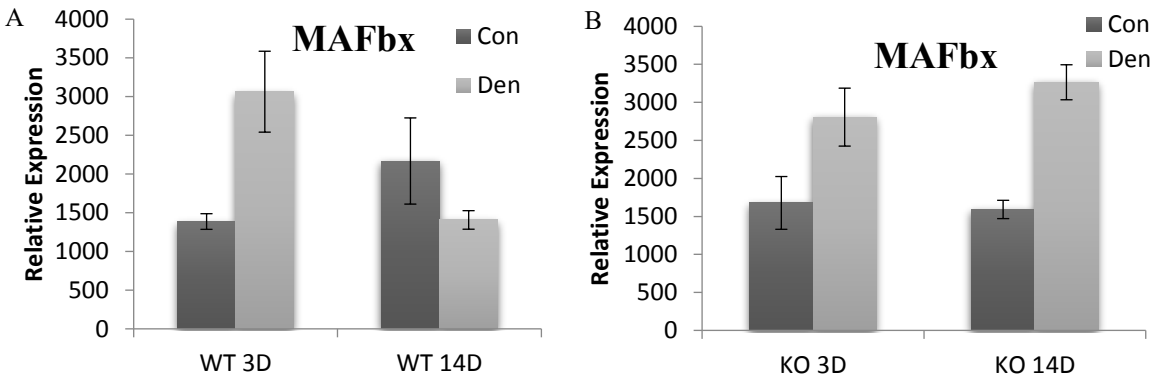

Figure 5. MAFbx expression is altered in MuRF1-null mice following denervation. Whole genome expression analysis was conducted on gastrocnemius muscle from (A) wild-type (WT) and (B) MuRF1-null (KO) mice following 3 days (3D) and 14 days (14D) post-denervation. MAFbx expression was elevated at 3 days post-denervation but returned to baseline expression levels by 14 days post-denervation in WT mice (A). In contrast, MAFbx expression increased at 3 days post-denervation, but remained elevated at 14 days post-denervation in KO mice (B). Each condition was conducted in triplicate and the expression is the average of three individual mice and error reflects +/- SEM.

As previously mentioned, very few targets of MuRF1 have been identified despite its establishment as an E3 ubiquitin ligase. Interestingly, outside of this function E3 ligases have been characterized to be capable of mono-ubiquitination and multi-ubiquitination [17]. These ubiquitination events do not flag proteins for degradation by the 26S proteasome but instead change the protein's structure, cellular localization, function, or serves as a recruitment or binding signal for additional transcription factors [17]. It is hypothesized that MuRF1 may function to transcriptionally regulate gene expression in skeletal muscle through ubiquitination events resulting in the ability to coordinate with myogenic regulatory factors to control gene activity. The ability of MuRF1 to act as a transcriptional regulator would demonstrate that E3 ligases such as MuRF1 may have a larger role in atrophy than previously thought.

Myogenic Regulatory Factors

Myogenic regulatory factors (MRFs) are crucial to stimulating and regulating the formation of muscle tissue (Figure 6). These transcription factors act in conjunction with co-activators or co-

repressors to facilitate transcription of muscle-specific genes. MRFs are characterized by a standard helix-loop-helix motif, which allows them to bind to the canonical Ebox consensus sequence of 5'-CANNTG-3' [18]. These Ebox sequences are found in the promoter regions of most muscle-specific genes, including MuRF1 and MAFbx, and function as binding sites for MRFs to modulate transcriptional activity. MyoD and myogenin sequentially and transitorily associate with promoter regions of a variety of muscle-specific genes. These factors are necessary for the development of functional skeletal muscle and myogenic commitment, respectively [4, 5, 19].

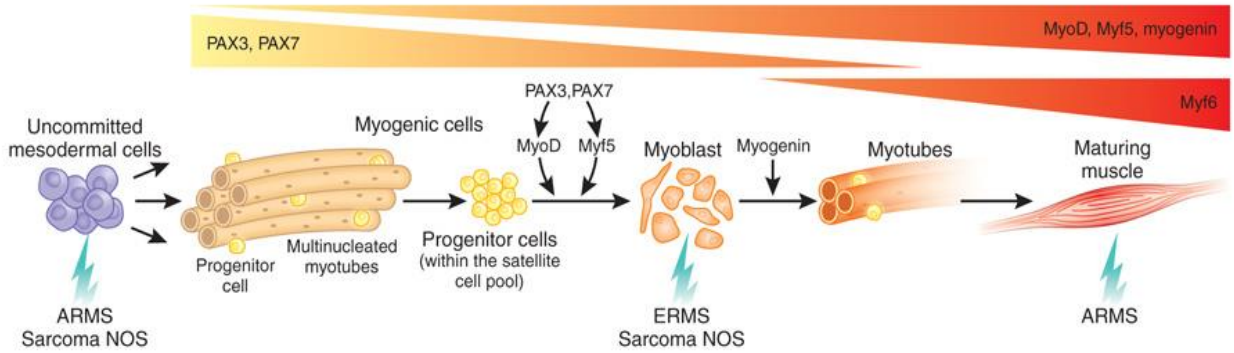

Figure 6. Model of skeletal muscle myogenesis. The growth of uncommitted mesodermal cells into committed myotubes depends on cellular signaling from transcription factors including Pax3, Pax7, MyoD1, and Myogenin. MyoD1 aids in the commitment from satellite cells to un-differentiated muscle myoblast cells whereas myogenin functions to regulate the formation of differentiated myotubes (Image adapted from Hettmer and Wagers, 2010).

The microarray data demonstrated that MuRF1 also has a role in regulating the transcription of the myogenic regulatory factors myogenin and MyoD, which are often upregulated in atrophy conditions [13]. Microarray analysis showed that myogenin and MyoD are significantly upregulated under denervation. The expression levels of MyoD in MuRF1-KO mice showed decreased levels at 14 days post-denervation compared to WT mice, whereas expression of myogenin showed a significant decrease at 3 days post-denervation in MuRF1-KO mice compared to WT mice (Figure 7). These data together further suggest that MuRF1 may act as a regulator of

muscle-specific genes and that myogenin and MyoD may play a role in the atrophy pathway involving MuRF1.

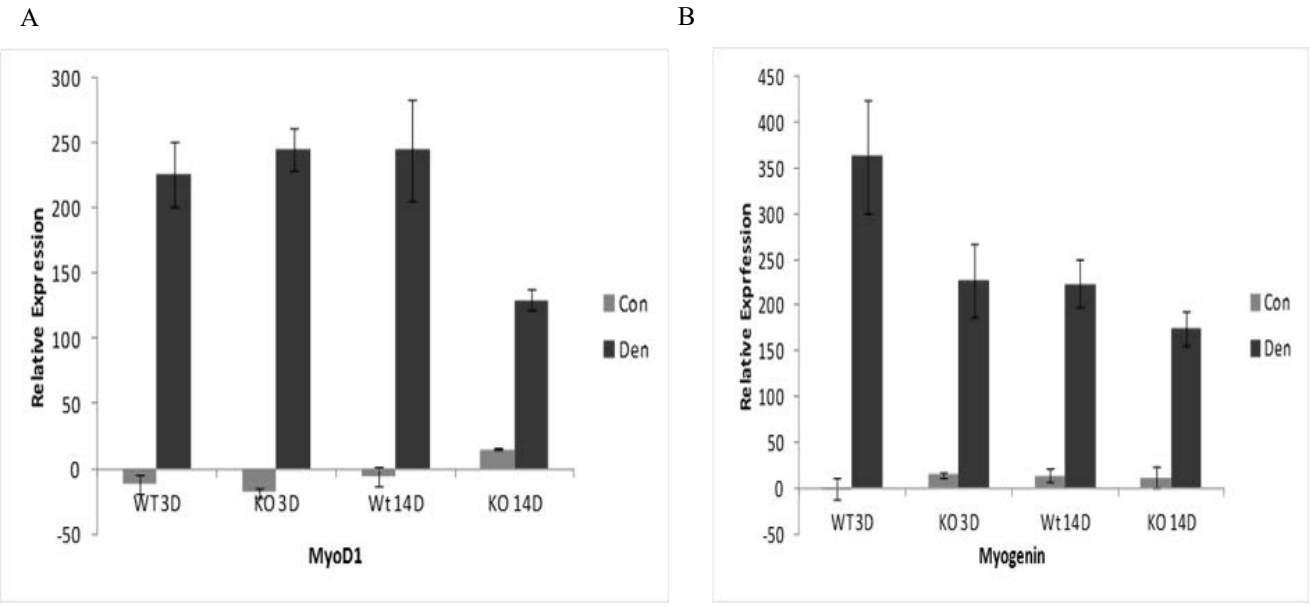

Figure 7. MRF expression is altered in MuRF1-null mice following denervation. Whole genome expression analysis was conducted on gastrocnemius muscle from wild-type (WT) and MuRF1-null (KO) mice following 3 days (3D) and 14 days (14D) post-denervation. A) MyoD1 and B) myogenin expression increased at both 3 and 14 days following denervation in wild-type and MuRF1-null mice. Each condition was conducted in triplicate and the expression is the average of three individual mice and error reflects +/- SEM.

Myogenin is established as an agent of myoblast commitment to differentiation and is required for cell viability to ensure the proper formation of embryonic myofibers [18, 20, 21, 22]. Interestingly, it was previously found that the deletion of myogenin resulted in diminished expression of MuRF1 in skeletal muscle and that when these mice experienced neurogenic atrophy (i.e. denervation) were resistant to muscle wasting [18]. MyoD acts as an early marker of myogenic commitment to the skeletal muscle lineage [18]. Furthermore, MyoD and myogenin have been characterized as important regulators of neurogenic atrophy-induced gene expression, including induction of MuRF1 and MAFbx [10, 16].

Myogenic Regulatory Factors as Regulators of MuRF1 and MAFbx

The transcriptional regulation of MuRF1 and MAFbx by MRFs has been investigated in previous unpublished literature which shows that these transcription factors act as co-activators or co-repressors to mediate transcription of muscle-specific genes [23,24]. Again, the proximal promoters of both MuRF1 and MAFbx contain canonical E-box sequences which are known binding sites of MRFs (Figure 8, Figure 9) [25]. These conserved E-box sequences have been characterized to interact with myogenic regulatory factors, specifically including MyoD and myogenin [26]. It is proposed that these muscle specific factors are working cooperatively with MuRF1 through conserved Ebox sequences in the promoter region of atrophy-related genes to suppress or activate expression under neurogenic atrophy conditions.

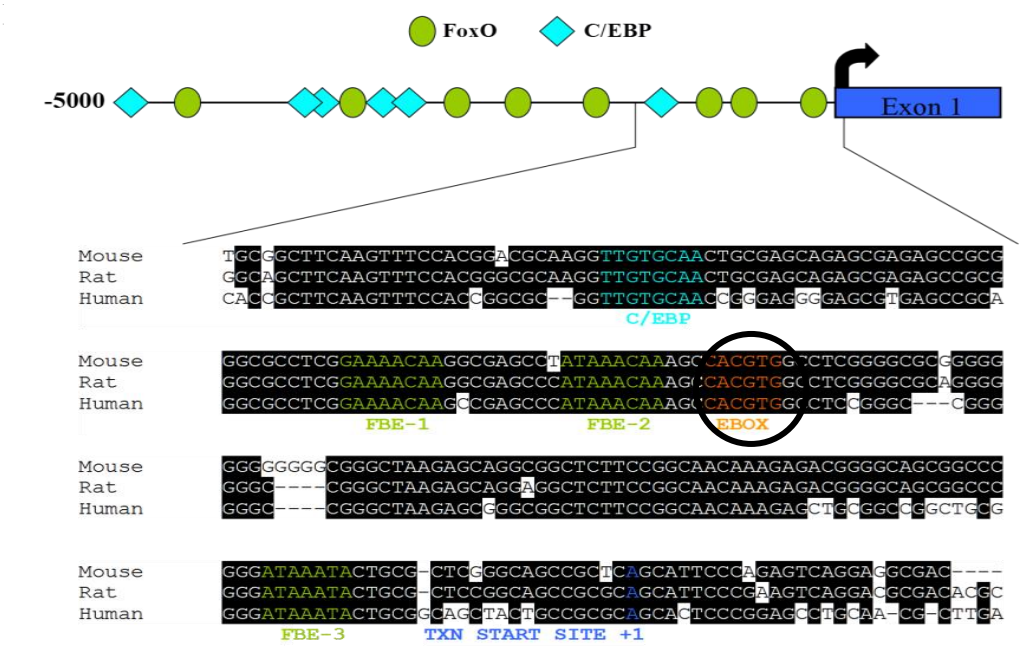

Figure 8. MAFbx Promoter Alignment. Promoter sequences from mouse, rat, and human MAFbx genes (5000 base pairs upstream of the transcription start site (+1) through the first exon) were downloaded from the Ensembl database (www.ensembl.org) and aligned using the ClustalW algorithm. Identical sequences for the indicated regions are highlighted in black. Approximate positions of potential MRF transcription factor 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 CANNTG (MyoD, etc.) (Large circle).

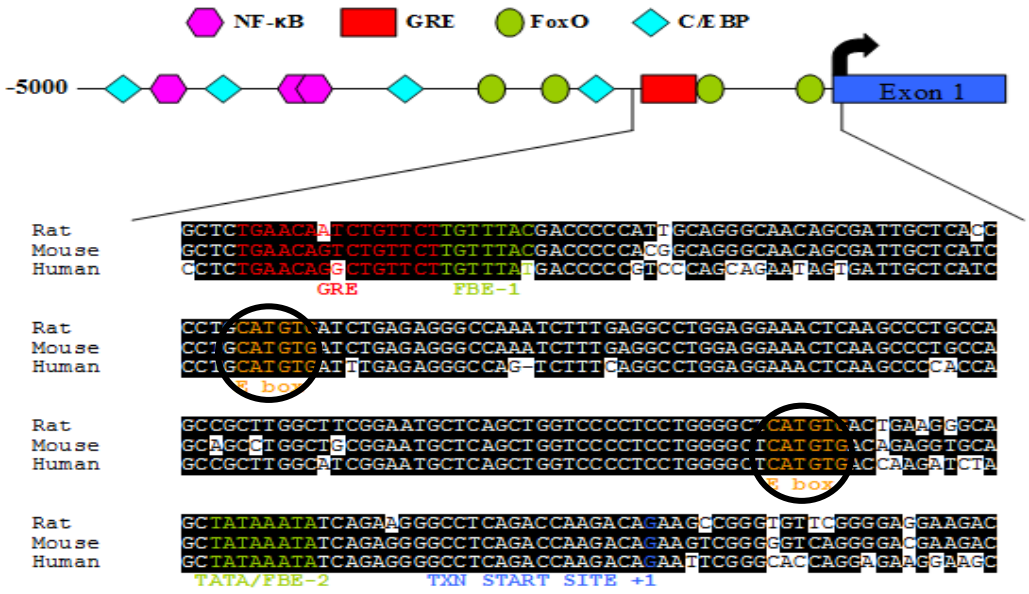

Figure 9. MuRF1 Promoter Alignment. Promoter sequences from mouse, rat, and human MuRF1 genes (5000 base pairs upstream of the transcription start site (+1) through the first exon) were downloaded from the Ensembl database (www.ensembl.org) and aligned using the ClustalW algorithm. Identical sequences 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 (Diamonds); GRE (Square); Muscle specific E box CANNTG (MyoD, etc.) (Large Circles).

Previous Characterization of MuRF1 and Myogenic Regulatory Factors in Transcriptional Regulation: MuRF1 as a Transcriptional Regulator of MuRF1 and MAFbx

Previous unpublished data showed the role of MuRF1 and the MRFs in the transcriptional regulation of atrogenes. It was demonstrated that MuRF1 acts, at least in part, by direct and/or indirect modulation of the MyoD-family of myogenic regulatory factors. The transcriptional activation of MuRF1 and MAFbx by myogenin and MyoD was confirmed and for the first time it was demonstrated that MuRF1 cooperates with both MyoD and myogenin to repress MuRF1 and MAFbx reporter gene expression (Figures 10 and 11, respectively) [23]. These findings provide support for 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 expression to baseline levels following neurogenic atrophy. These findings also suggest that

MuRF1 may act as a modulator of myogenic regulatory factors, although the exact mechanism has been unclear.

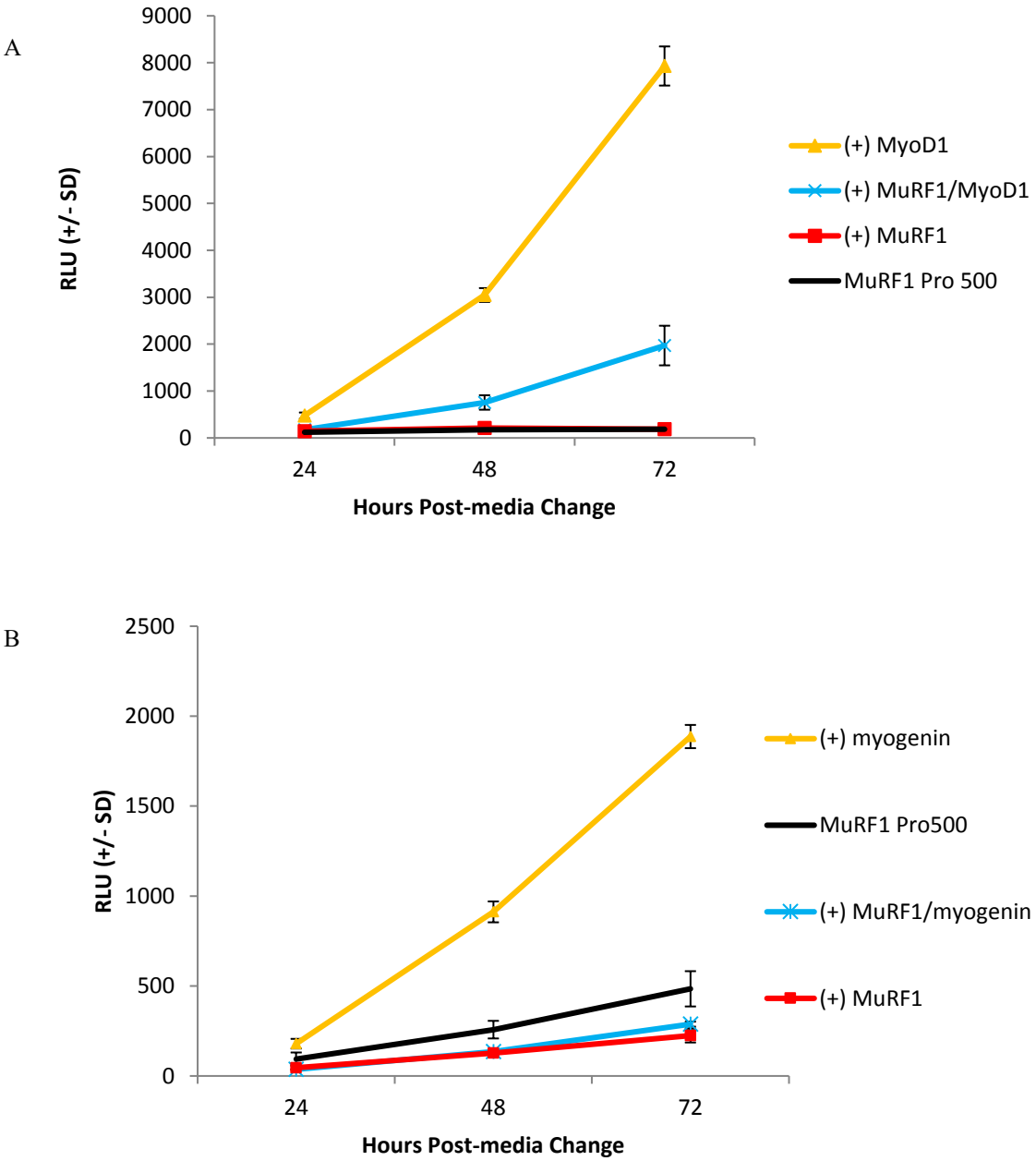

Figure 10. MuRF1 negatively regulates MRF induction of MuRF1 promoter reporter activity. The MuRF1 500 base pair promoter reporter shows transcriptional repression in response to co-overexpression of MuRF1 and (A) MyoD or (B) myogenin. C_2C_{12} myoblasts were transfected with a reporter construct consisting of the MuRF1-500bp promoter cloned into the SEAP2-Basic plasmid, a β-galactosidase expression plasmid, and expression plasmids for MuRF1 alone or in combination with myogenin or MyoD. The myoblasts were maintained in standard differentiating culture media. Samples of media were taken at 24 hour intervals and measured for SEAP activity. The samples were normalized to β-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 [23].

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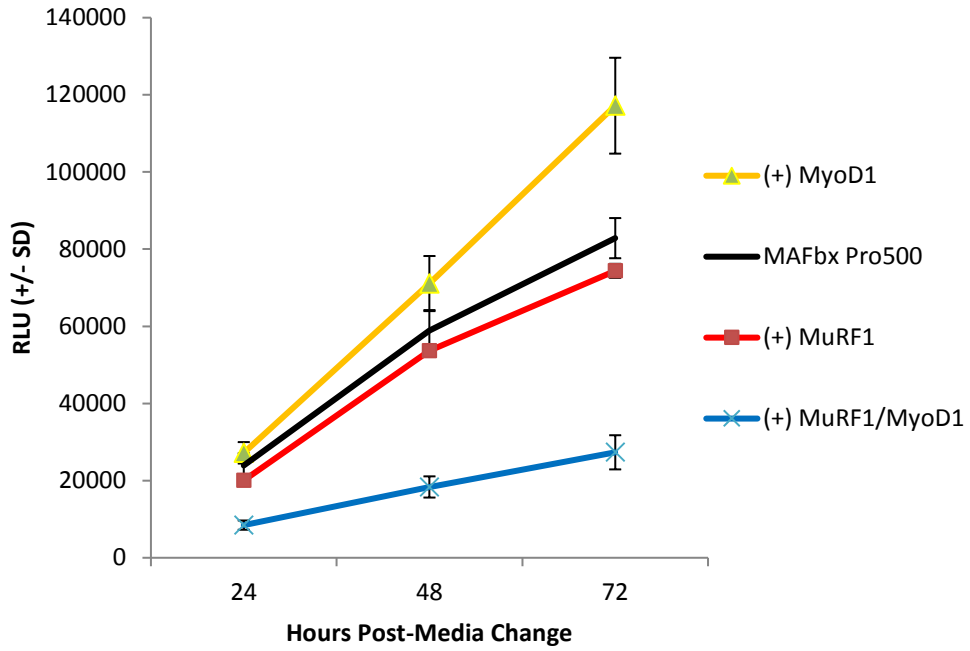

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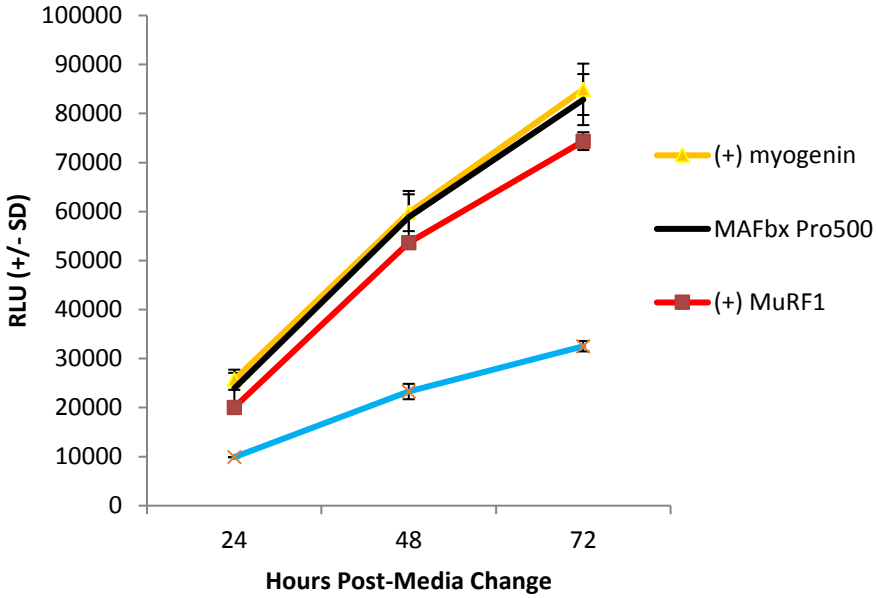

Figure 11. MuRF1 negatively regulates MRF induction of MAFbx promoter reporter activity. The MAFbx 500 base pair promoter reporter shows transcriptional repression in response to co-overexpression of MuRF1 and (A) MyoD and (B) myogenin**.** C_2C_{12} myoblasts were transfected with a reporter construct consisting of the MAFbx-500bp promoter cloned into the SEAP2-Basic plasmid, a β-galactosidase expression plasmid, and expression plasmids for MuRF1 alone or in combination with MyoD or myogenin. The myoblasts were maintained in standard differentiating culture media. Samples of media were taken at 24 hour intervals and measured for SEAP activity. The samples were normalized to β-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 [23].

Overview

MuRF1 and MAFbx are widely accepted as key regulators in skeletal muscle dynamics with roles in targeting proteins for degradation. Increasing evidence, including data herein, suggests that MuRF1 functions as a transcriptional regulator of muscle-specific gene. The details of MuRF1 and its role as a transcriptional regulator of downstream genes has not been thoroughly investigated to date. The purpose of this research was to build on preliminary data aiming to analyze the mechanism by which MuRF1 acts as a transcriptional regulator, specifically aiming to characterize the coordination of MuRF1 and the myogenic regulatory factors, myogenin and MyoD. The data herein suggests that MuRF1 regulates an array of atrogenes by indirect/direct modification and interaction with transcription factors such as myogenin and MyoD through the binding of the canonical Ebox sequences found in the promoter regions of atrophy-induced genes such as MuRF1 and MAFbx. The first aim of this thesis was to investigate the regulation of myogenin and MyoD by MuRF1. We hypothesize that MuRF1 may be acting as a regulator of myogenin and MyoD by one of two mechanisms. One possible way this may be occurring is at the post-translational level, through the 26S proteasome; MuRF1 may be acting as an E3 ligase to ubiquitinate the MRFs and degrade them through the ubiquitin proteasome system (Figure 12). An alternative mechanism is that MuRF1 may also be acting through a feedback mechanism that targets the transcriptional activation of the MRFs (Figure 13). We hypothesize that MuRF1 may be negatively regulating levels of MyoD and myogenin. A decrease in expression of myogenin and MyoD may cause differential expression of many muscle specific genes, as they are necessary regulatory factors for muscle-specific gene expression.

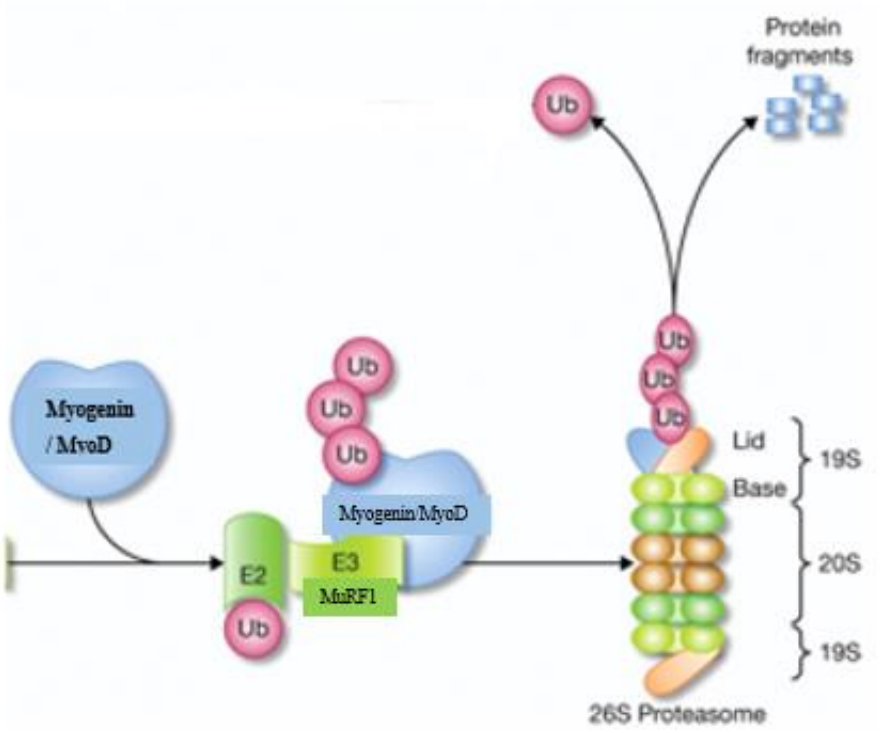

Figure 12. Proteasome Model: MuRF1 regulates myogenin and MyoD through the 26S proteasome. MuRF1 acts as an E3 ligase to poly-ubiquitinate myogenin and MyoD and flag them for degradation by the ubiquitin proteasome system, down regulating their expression levels and in turn differentially regulating the transcription of muscle-specific genes in neurogenic atrophy.

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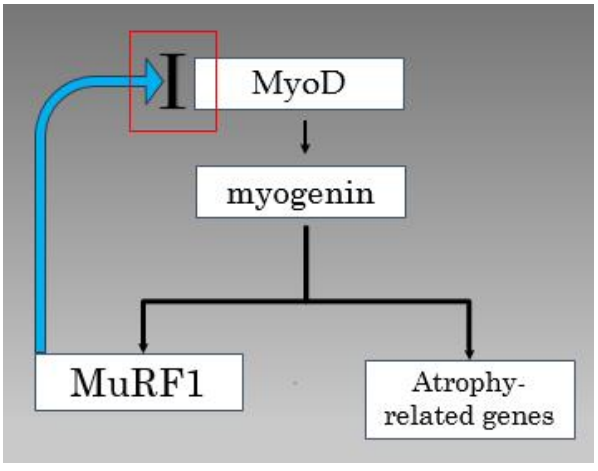

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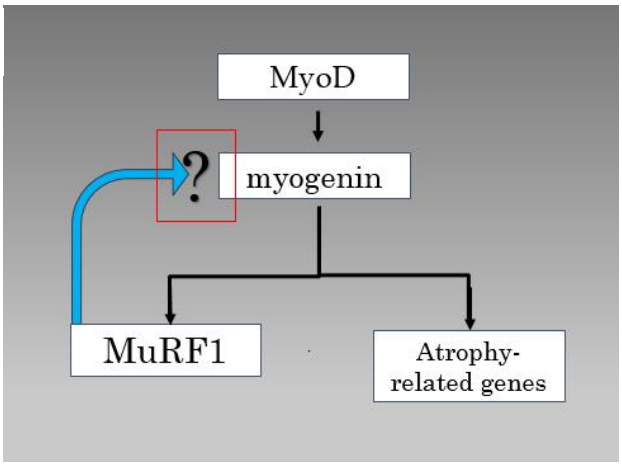

Figure 13. Transcription Model: MuRF1 transcriptionally regulates myogenin and MyoD expression. MuRF1 may feedback to negatively regulate the transcriptional activity of myogenin or MyoD. Transcriptional regulation of atrophy-related genes may be occurring through a) negative regulation of MyoD b) negative regulation of myogenin (possibly through modification of MyoD, a known transcriptional regulator of myogenin).

The second aim of this thesis was to investigate the mechanism by which MuRF1 is coordinating with the MRFs, specifically investigating the occupancy of the MuRF1 promoter region by myogenin and MyoD. We hypothesized that the MRFs are occupying the Ebox binding domain of the MuRF1 promoter at high levels under neurogenic atrophy conditions (i.e. high expression levels of the MRFs and MuRF1). We also hypothesized that co-overexpressing MuRF1 with the MRFs would result in MuRF1-medicated reversal of this occupancy, based on previously research suggesting that MuRF1 is able to transcriptionally regulate its own activity [23]. The data herein serves to characterize MuRF1 as a transcriptional regulator of atrophy induced genes through the regulation of myogenin and MyoD in addition to characterizing the molecular mechanism by which this is occurring.

Chapter 2: Characterization of the Molecular Interaction between MRFs and MuRF1

Experimental Design

Endogenous expression of myogenin and MyoD

The first objective of this research was to effectively identify and characterize the endogenous expression of both myogenin and MyoD in skeletal muscle cells. C_2C_{12} myoblast cells were plated and harvested at day 1 (U1) and day 2 (U2) of proliferation and differentiation day 1 (D1), differentiation day 3 (D3), differentiation day 7 (D7) and differentiation day 9 (D9). These time points were chosen to be representative of the differentiation process of C_2C_{12} mouse myoblast cells. The protein was extracted and Western blot analysis was performed to analyze protein levels over the time course using antibodies against myogenin or MyoD.

MuRF1 regulation of myogenin and MyoD

The next objective was to explore if MuRF1 has a role in regulating the expression levels of myogenin and MyoD and how this is occurring. We hypothesized that this regulation may be occurring post-translationally, through the ability of MuRF1 to catalytically tag proteins with ubiquitin molecules, in turn tagging them for degradation by the proteasome. The role of both the catalytic RING domain, which is responsible for ubiquitinating proteins, and the 26S proteasome were investigated as follows:

Role of the Catalytic RING Domain

In order to analyze the catalytic activity of MuRF1, site directed mutagenesis was performed to create a catalytically dead MuRF1 construct. In theory, this MuRF1 RING mutant is unable to ubiquitinate proteins and flag them for degradation by the 26S proteasome. C_2C_{12} cells were transfected with ectopic MuRF1 or MuRF1-RING mutant and harvested at differentiation day 2 (D2) and differentiation day 9 (D9). Western blot analysis was performed using antibodies against myogenin and MyoD.

Role of the 26S Proteasome

To evaluate the role of the 26S proteasome in this regulation, C_2C_{12} cells were transfected with MuRF1 and then harvested at differentiation day 2 (D2) and differentiation day 9 (D9). Four hours prior to harvesting the cells, the cells were treated with MG132 [12.5µg/µL], a general 26S proteasome inhibitor that acts as a peptide aldehyde to effectively block the proteolytic activity of the proteasome complex. Subsequently, proteins were extracted, purified and Western blot analysis was performed using antibodies against myogenin and MyoD.

MuRF1 promoter occupancy by myogenin and MyoD

MuRF1 promoter occupancy by endogenous myogenin and MyoD

To further characterize MuRF1 regulation of the myogenic regulatory factors, Chromatin Immunoprecipitation (ChIP) was performed to analyze the occupancy of myogenin and MyoD at the proximal promoter of MuRF1. C_2C_{12} cells were harvested at proliferation day 2 (PD2), differentiation day 2 (DD2), and differentiation day 9 (DD9). Chromatin immunoprecipitation was performed using antibodies to isolate myogenin or MyoD. DNA was isolated from the protein complexes and primers amplifying the conserved Ebox region of the MuRF1 promoter were used in qPCR, allowing us to quantify myogenin or MyoD occupation of the Ebox enhancer sequence in the promoter region of MuRF1.

Overexpression of MRFs

An additional experiment was performed to analyze the amount of association between the MuRF1 Ebox and MRFs when myogenin and MyoD were overexpressed in the cell line, which mimics their expression in neurogenic atrophy. C_2C_{12} cells were transfected to overexpress myogenin or MyoD and were harvested at 24 hours post-transfection. Chromatin immunoprecipitation was performed using antibodies to pull down myogenin or MyoD. DNA was isolated from the protein complexes and primers amplifying the conserved Ebox regions of the MuRF1 promoter were used in qPCR, allowing us to quantify myogenin or MyoD occupation of the Ebox enhancer sequence in the promoter region of MuRF1.

Overexpression of MuRF1 + MRFs

The next aim of this research was to assess the ability of MuRF1 to drive down myogenin and/or MyoD occupancy of the MuRF1 proximal promoter. C_2C_{12} cells overexpressing myogenin or MyoD +/- MuRF1 were harvested at 24 hours post-transfection, again mimicking expression levels

that are seen under neurogenic atrophy. Chromatin immunoprecipitation was performed using antibodies to pull down myogenin or MyoD. DNA was isolated from the protein complexes and primers amplifying the conserved Ebox regions of the MuRF1 promoter were used in qPCR, allowing us to quantify myogenin or MyoD occupation of the Ebox enhancer sequence in the promoter region of MuRF1.

Materials and Methods

Cell Culture

C_2C_{12} mouse myoblast cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in 10 cm cell culture dishes in 10 mL of media consisting of DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (GE Healthcare Hyclone Laboratories, Logan, UT), Pen/Strep, nonessential amino acids, and gentamycin (Life Technologies, Grand Island, NY) at 37°C in a 6% CO_2 humidified chamber.

Protein Purification and Western Blotting

C_2C_{12} mouse myoblast cells were plated into 10 cm culture dishes at a density of approximately 500,000 cells/mL Cells were harvested over a time course of 12 days, from proliferating myoblasts to differentiated myotubes. Cells being transfected to ectopically express MuRF1 or MuRF1-RING mutant were treated as follows: one hour prior to transfection the media was removed from the cells and 10 mL of fresh proliferation media was added to each plate. 10µg of total DNA per plate was transiently transfected using Thermo Scientific Turbofect reagent per the manufacturer's protocol. The DNA mixture consisted of a total of 10ug of indicated expressions constructs for pcDNA3.1-MuRF1 or pcDNA3.1-MuRF1 RING mutant. At approximately 24 hours post-transfection the cells were harvested, centrifuged at 5,000 x g for 5 minutes at 4°C, and stored at -

80˚C. Protein homogenates were prepared by resuspending the cells in a protein lysis buffer [50 mM Tris, 150 mM NaCl, 50 mM NaF, .5% NP-40] supplemented with a protease inhibitor cocktail, 1 mM PMSF, 1 mM DTT, 10mM β-glycarophosphate, 1mM Sodium Molybdate, incubated for 30 minutes on ice and centrifuged at 4°C for five minutes at 18,000 x g. The homogenate was aliquoted and stored at -80˚C. Protein concentrations were quantified using a modified Bradford reagent protein assay according to manufacturer's protocol (Bio-Rad, Hercules, CA). A total of 150µg of protein was loaded onto an SDS-PAGE gel, separated and then transferred to a PVDF membrane. The membrane was Ponceau-S stained to check for consistent protein loading and efficient transfer, washed, and then blocked for one hour in a 5% milk solution (5% dry milk weight/volume dissolved in .05% Tween-20 in Tris Buffered Saline). The membrane was washed for fifteen minutes with 1x TTBS and incubated in commercially available primary antibodies for one hour at room temperature (RT) (MyoD [M-318, rabbit, Santa Cruz Biotechnology, Inc. or 5.8A, mouse, Santa Cruz Biotechnology, Inc.]; myogenin [F5D, mouse, Santa Cruz Biotechnology, Inc.]; at a concentration of 1:500 myogenin, MyoD. The membranes were washed for fifteen minutes, and then incubated in secondary antibody (1:5000) for one hour. Signal development followed the manufacturer's protocol for the Pierce ECL Western Blotting Kit and the blots were imaged on x-ray film.

Site-Directed Mutagenesis of MuRF1-RING Finger Domain

The MuRF1 cDNA sequences for mouse, rat and human were downloaded from the Ensembl database (www.ensembl.org). The sequences were then aligned using the Clustal Omega alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalo/). The aligned sequences were used to identify conserved amino acids for site-directed mutagenesis. Site-directed mutagenesis reactions

were performed per the manufacture's protocol (Stratagene, La Jolla, CA). Mutations in the RING domain of the MuRF1 gene were accomplished by using the following primers: (F) 5′-CTACAGCAACCGTGAGAAGGCCGACTCCAACCACAACC-3′ and (R) 5′-GGTTGTGTTGGAGTCGGCCTTCTCACGGTTGCTGTAG-3′. The resulting MuRF1 clone was sequenced to confirm the correct mutation was introduced into the gene.

Chromatin Immunoprecipitation

C_2C_{12} cells were plated into 10 cm culture dishes at a density of approximately 500,000 cells/mL and grown to approximately 75% confluency. One hour prior to transfection the media was removed from the cells and 10 mL of fresh proliferation media was added to each plate. Total DNA (10µg per well) was transiently transfected using Thermo Scientific Turbofect reagent per the manufacturer's protocol. The DNA mixture consisted of a total of 10ug of indicated expression constructs for pcDNA3.1-MuRF1, pcDNA3.1-TCF3, pcDNA3.1-TCF12, pcDNA3.1-MyoD, and pcDNA3.1-Myogenin. Each plate was treated with 1% formaldehyde and incubated at 37˚ for ten minutes. The cells were treated with .125M glycine, incubated for five minutes, and washed with PBS containing protease inhibitor cocktail, 10mM β-glycerophosphate, and 0.5 M Na_3VO_4. Cells were harvested in 1 ml Collection Buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA), spun down at 4°C for five minutes at 3,000 x g, and stored at -80˚C. Cells were resuspended in 400µl lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, PIC, 10 mM β-glycerophosphate, 1 mM Na_3VO_4). The cell lysates were sonicated using a QSonica Ultrasonic Liquid Processor at the following optimized settings: 50% amplification; pulses of 20 seconds on, 20 seconds off x 30 cycles. The samples underwent centrifugation at full speed for 10 minutes and supernatant was removed and diluted to 10 mL with ChIP Dilution Solution (0.01% SDS, 1.1% Triton X-100, 1.2

mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, PIC, 10 mM β-glycerophosphate, 1 mM Na_3VO_4). The chromatin was pre-cleared using 60µl of a slurry of salmon sperm DNA/protein A agarose (Millipore, Temicula, CA) and rotated for 30 minutes at 4°C, beads were then pelleted by centrifugation for 2 minutes x 1000g at 4°C. The chromatin was combined with 1 µl of ChIP-grade antibody (c-myc [9E10, mouse, Santa Cruz Biotechnology, Inc.], myogenin [F5D, mouse, Santa Cruz Biotechnology, Inc.], MyoD [5.8A, mouse, Santa Cruz Biotechnology]) and incubated overnight with rotation at 4°C. The samples were combined with 50µl of washed 50% slurry of Dilution Solution and Protein A/salmon sperm and incubated with tumbling at 4°C for two hours, followed by centrifugation at 10,000 x g for 2 minutes. The beads were then washed 3x with 1 mL of each of the following buffers: TSEI (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), TSEII (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), TSEIII (1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 1% NP-40, 1% sodium deoxycholate, 0.25 M LiCl), Wash Buffer IV (10 mM Tris [pH 8.0], 1 mM EDTA). The beads were pelleted by centrifugation at 3,000 x g for 2 minutes, followed by addition of 100µl 10% Chelex and incubation at 95°C for ten minutes to reverse DNA-protein crosslinks. After incubation, the samples were treated with 2µl Proteinase K (Qiagen Sciences, MD) and incubated at 55°C for 30 minutes, followed by incubation at 95°C for ten minutes. The samples were centrifuged at full speed for two minutes and the supernatant was collected. The remaining beads were combined with 100 µl nuclease free water, vortexed, and centrifuged for 2 minutes a full speed. The resulting supernatant was collected and combined with respective supernatant from the previous step. The collected DNA aliquots were used for qPCR analysis.

qPCR

The qPCR reaction was prepared using 2 µL of chromatin from the ChIP assay, 1 µL of forward primer (500nM), 1 µL of reverse primer (500nM), 5 µL iTaq Universal SYBR Green Supermix (BioRad) in a total volume of 10 µL. The PCR reaction was cycled at the following parameters: 95˚C for two minutes, 95˚C for thirty seconds, 57˚C for thirty seconds, and 72˚C for one minute x 40 cycles. The primer sequences used to amplify the MuRF1 promoter were: forward ProEbox 5'-CGGCAGGGCAACAGCGATTT-3', reverse ProEbox 5'- GTCTTGGTCTGAGGCCCCTC-3'. These qPCR reactions were quantified by using a standard curve of amplification of serial dilutions of the MuRF1 promoter plasmid at 1,000 pM, 100 pM, and 10 pM in concentration. Each condition was performed in triplicate and standard deviation was calculated.

Bioinformatics Analysis

The nucleotide sequence corresponding to the regulatory regions of mouse, rat, and human MuRF1 and MAFbx promoters were downloaded from the Ensembl database (www.ensembl.org), aligned using ClustalW2 alignment tool on the EMBL website (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and shaded using Boxshade analysis of the ClustalW2 alignment output data (http://www.ch.embnet.org/software/BOX_form.html). The amino acid sequences for mouse, rat, and human MuRF1 were downloaded from Ensembl and aligned and shaded as described above.

Results

Characterization of the ectopic expression of myogenin and MyoD in C_2C_{12} myoblasts

To effectively characterize the endogenous expression of both MyoD and myogenin in skeletal muscle cells, C_2C_{12} cells and Western blot analysis was performed to analyze protein levels over the course of differentiation. MyoD showed an increase in expression by day 2 of proliferation and decreased in expression by day 7 of differentiation (Figure 14). Myogenin increased by day 3 of differentiation and decreased in expressed late in differentiation (Figure 15).

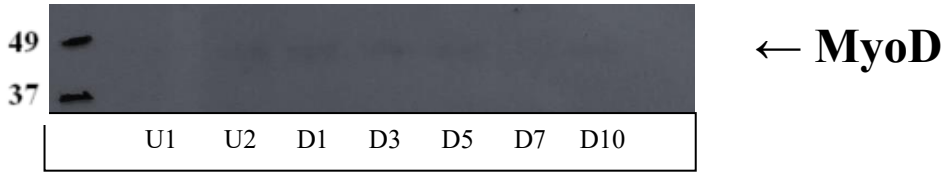

Figure 14. Protein expression profile of MyoD (~45 kDa) in C_2C_{12} mouse myoblasts increases in expression late in proliferation and decreases early in differentiation. C_2C_{12} myoblast cells were plated and harvested at day 1 (U1) and day 2 (U2) of proliferation and day 1 (D1), day 3 (D3), days 7 (D7) and day 9 (D9) after the switch to differentiation media (2% serum). Western blotting was performed using 150µg of total protein and probed using an antibody against MyoD.

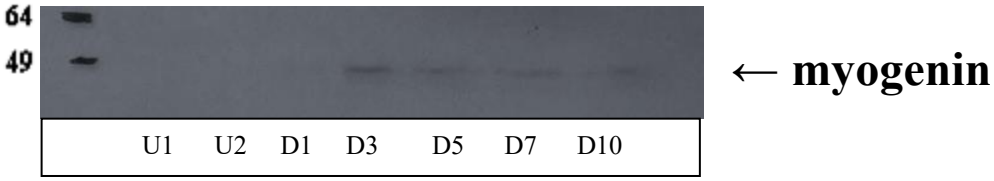

Figure 15. Protein expression profile of myogenin (~34 kDa) in C_2C_{12} mouse myoblasts increases in expression early in differentiation and decreases late in differentiation. C_2C_{12} myoblast cells were plated and harvested at day 1 (U1) and day 2 (U2) of proliferation and day 1 (D1), day 3 (D3), days 7 (D7) and day 9 (D9) after the switch to differentiation media (2% serum). Western blotting was performed using 150µg of total protein and probed using an antibody against MyoD.

Ectopic expression of MuRF1 and MuRF1-RING mutant downregulates myogenin but not MyoD in C_2C_{12} myoblasts

To explore if MuRF1 had a role in regulating the expression levels of myogenin and MyoD, Western blot analysis was performed on C_2C_{12} mouse myoblasts overexpressing MuRF1 or the

catalytically dead MuRF1 RING mutant at two time points of differentiation. Western blot analysis showed decreased myogenin expression when MuRF1 or MuRF1-RING mutant were overexpressed (Figure 16). Western blot analysis probing for MyoD did not show differences in expression when MuRF1 or MuRF1-RING mutant were overexpressed (Figure 17).

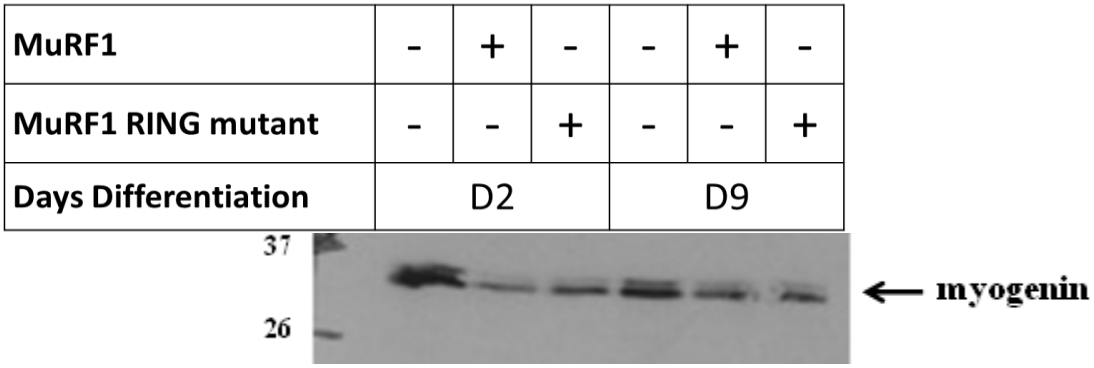

Figure 16. Protein expression profile of myogenin (~34 kDa) in C_2C_{12} mouse myoblasts is decreased upon overexpression of MuRF1 or MuRF1-RING mutant. C_2C_{12} myoblast cells were transfected with control expression plasmid alone (-/-) or expression plasmids for either MuRF1 or MuRF1 RING mutant and harvested at day 2 (D2) and day 9 (D9) after the switch to differentiation media (2% serum). Western blotting was performed using 150µg of total protein and probed using an antibody against myogenin (biological duplicate shown Figure S1).

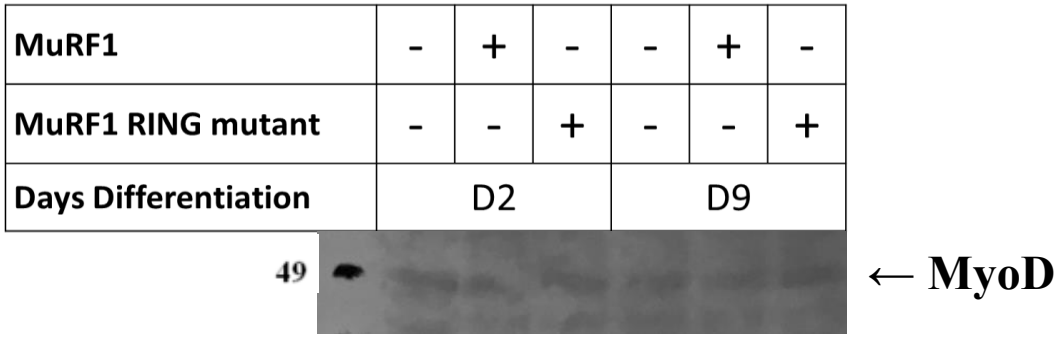

Figure 17. Protein expression profile of MyoD (~45 kDa) in C_2C_{12} mouse myoblasts remains unaltered upon overexpression of MuRF1 or MuRF1-RING mutant. C_2C_{12} myoblast cells were transfected with control expression plasmid alone (-/-) or expression plasmids for either MuRF1 or MuRF1 RING mutant and harvested at day 2 (D2) and day 9 (D9) after the switch to differentiation media (2% serum). Western blotting was performed using 150µg of total protein and probed using an antibody against MyoD (biological duplicate shown figure S2).

Inhibition of the 26S proteasome blunts MuRF1-mediated downregulation of myogenin

To evaluate how the role of the 26S proteasome in MuRF1 regulation of myogenin and MyoD, C_2C_{12} cells overexpressing MuRF1 were treated with MG132 [12.5µg/µL], a general 26S

proteasome inhibitor [27]. Western blot analysis showed partially increased levels of myogenin and MyoD in MG132 treated cells, however ectopic MuRF1 was still capable of blunting the expression levels of myogenin without the function of the 26S proteasome (Figure 18, 19).

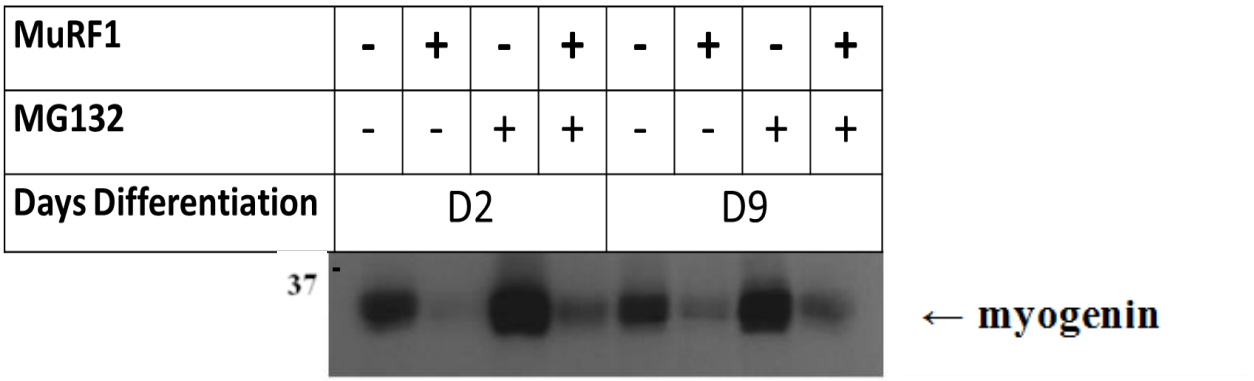

Figure 18. Protein expression profile of myogenin (~34kDa) in C_2C_{12} mouse myoblasts is increased upon cell treatment with MG132 (12.5µg/µL) but is still blunted by ectopic MuRF1. MuRF1-mediated repression of myogenin expression levels are partially reversed in the presence of MG132 but are not completely rescued from the blunting effect by ectopic MuRF1. Myoblasts were transfected with control expression plasmid alone (-/-) or an expression plasmid for MuRF1 and harvested at day 2 (D2) and day 9 (D9) after the switch to differentiation media (2% serum). Western blotting was performed using 150µg of total protein probed using an antibody against myogenin (biological duplicate shown Figure S3).

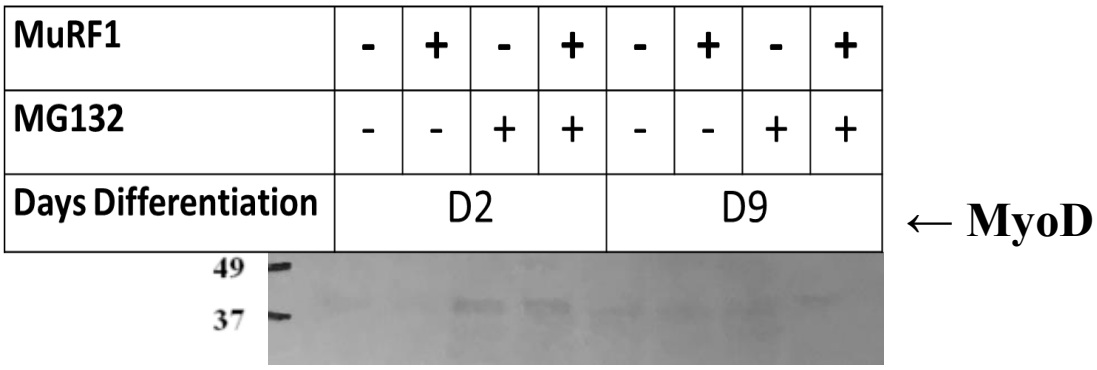

Figure 19. Protein expression profile of MyoD (~45kDa) in C_2C_{12} mouse myoblasts is increased upon cell treatment with MG132 (12.5µg/µL). MyoD expression levels are partially reversed in the presence of MG132, but no differences are seen upon expression of ectopic MuRF1. Myoblasts were transfected with control expression plasmid alone (-/-) or an expression plasmid for MuRF1 and harvested at day 2 (D2) and day 9 (D9) after the switch to differentiation media (2% serum). Western blotting was performed using 150µg of total protein probed using an antibody against MyoD (biological duplicate shown Figure S3).

Myogenic regulatory factors bind to the proximal promoter regions of MuRF1 and MAFbx

Previously, reporter assays showed that MuRF1 coordinates with myogenin and MyoD to regulate the promoter activity of atrophy-related genes, such as MuRF1 and MAFbx. Chromatin immunoprecipitation was performed to analyze the occupancy of endogenous myogenin and MyoD at the proximal promoter of MuRF1. Endogenous MuRF1 promoter binding appeared to be highest for myogenin early in differentiation, while MuRF1 promoter binding for MyoD appeared to be highest early in proliferation and did not show much of a response late in differentiation (Figure 20).

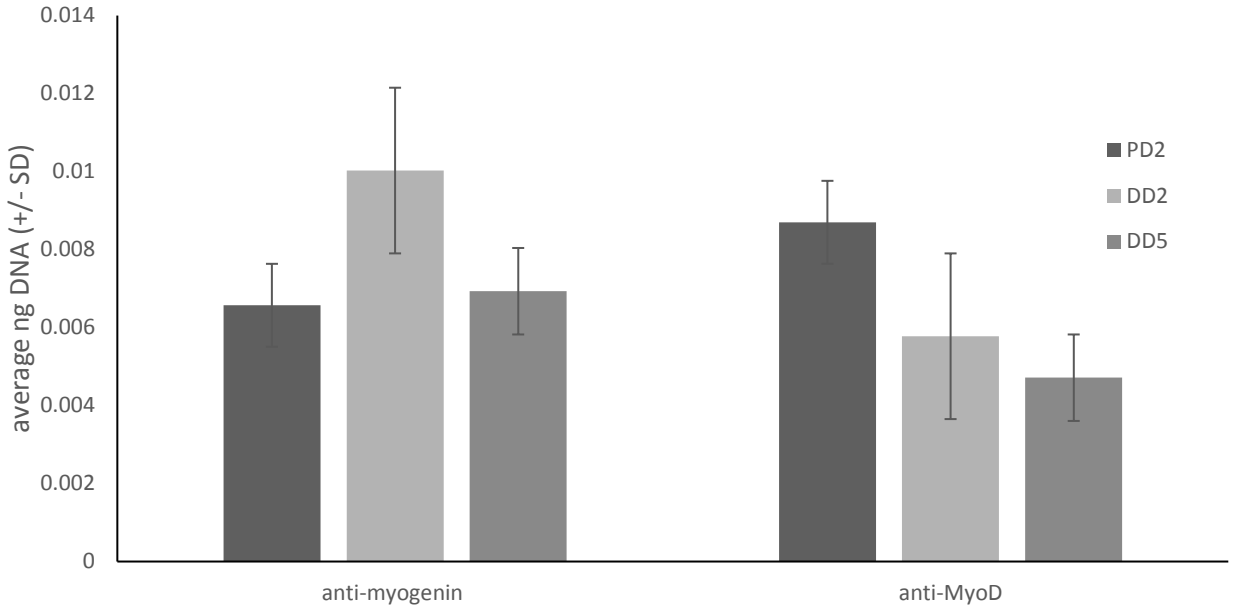

Figure 20. Endogenous myogenin and MyoD occupy the MuRF1 Ebox promoter region. The MuRF1 Ebox promoter is highly occupied by myogenin early in differentiation (DD2) and by MyoD early in proliferation (PD2). C_2C_{12} myoblast cells were harvested at day 2 post-plating (PD2) and day 2 (DD2) and day 5 (DD5) after the switch to differentiation media (2% serum). The myoblasts were maintained in standard culture media. ChIP was performed and DNA-protein complexes were pulled down with antibodies for myogenin (anti-myogenin) and MyoD (anti-MyoD). Each qPCR condition was run in triplicate and the error bars represent standard deviation (-/+ S.D.) of the mean.

Additionally, the occupancy of the MuRF1 promoter by MRFs was analyzed when myogenin or MyoD were overexpressed in the cell line. The qPCR analysis of cells overexpressing these MRFs demonstrated induced occupancy of the MuRF1 promoter region. (Figure 21).

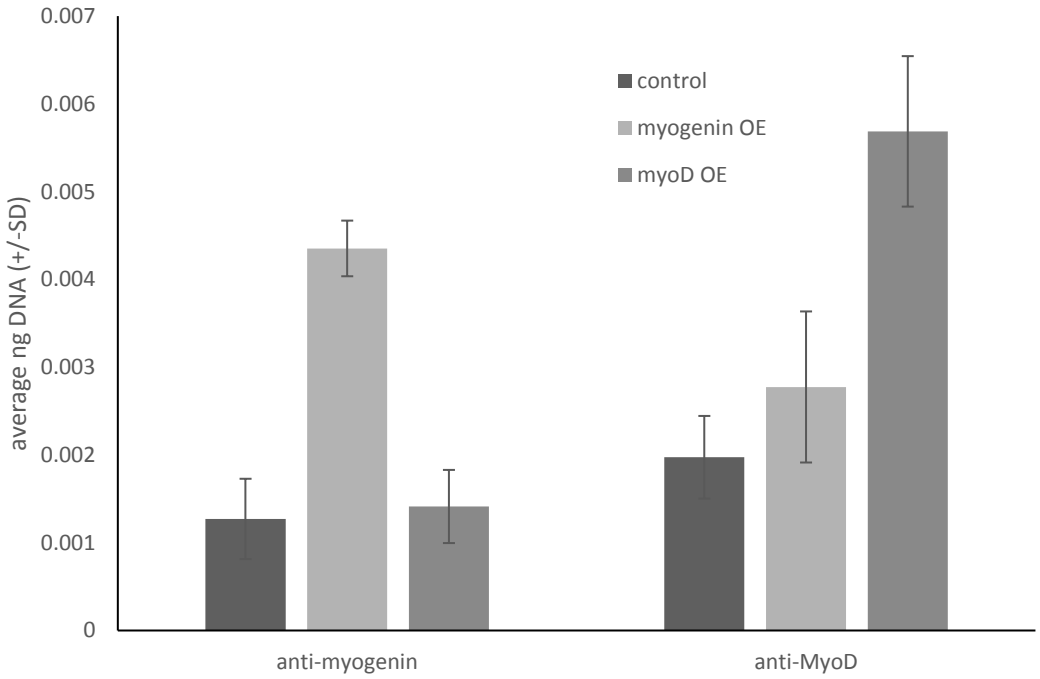

Figure 21. MRF overexpression induces MuRF1 Ebox promoter occupancy. MuRF1 Ebox promoter was highly occupied by myogenin in cells overexpressing myogenin, and with MyoD in cells overexpressing MyoD. C_2C_{12} myoblast cells were transfected one day after plating with pcDNA3 expression plasmids for either myogenin or MyoD and harvested at day 2 post-plating (PD2). The myoblasts were maintained in standard culture media. ChIP was performed and DNA-protein complexes were pulled down with antibodies for myogenin (anti-myogenin) and MyoD (anti-MyoD). Each qPCR condition was run in triplicate and the error bars represent standard deviation (-/+ S.D.) of the mean.

Ectopic expression of MuRF1 reverses MRF occupancy of the MuRF1 promoter region

Overexpressing ectopic MyoD and myogenin seemed to show induced levels of amplification of the MuRF1 promoter. In order to assess the ability of MuRF1 to drive down myogenin and/or MyoD occupancy of the MuRF1 proximal promoter cells overexpressing myogenin or MyoD +/- MuRF1 were used for ChIP assays as described above. The qPCR analysis showed the previously observed induction in occupancy of the MuRF1 promoter by myogenin and MyoD. More importantly, the overexpression of MuRF1 concurrently with either myogenin or MyoD

resulted in MuRF1-mediated reversal of the MRF occupancy of the MuRF1 proximal promoter (Figure 22).

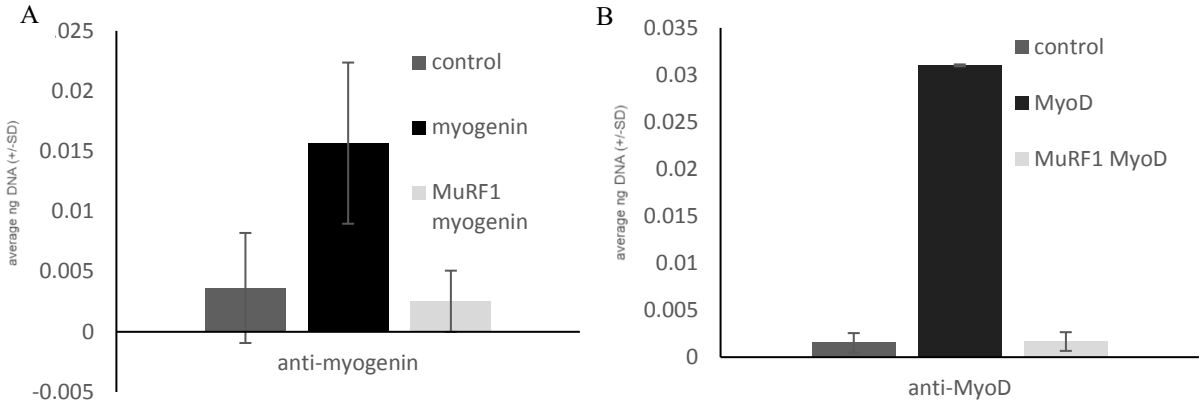

Figure 22. MuRF1-mediated reversal of MRF-induced MuRF1 Ebox promoter occupancy. MuRF1 Ebox promoter occupancy in cells overexpressing myogenin and MyoD was reversed when MuRF1 was overexpressed. C_2C_{12} myoblast cells were transfected one day after plating with pcDNA3 expression plasmids for either MuRF1 +/- myogenin or MyoD and harvested at day 2 post-plating (PD2). The myoblasts were maintained in standard culture media. ChIP was performed and DNA-protein complexes were pulled down with antibodies for a) myogenin (anti-myogenin) and b) MyoD (anti-MyoD). Each qPCR condition was run in triplicate and the error bars represent standard deviation (-/+ S.D.) of the mean.

Discussion

MuRF1 has been widely accepted as a marker and key regulator of skeletal muscle atrophy; however, very few mechanistic targets have been identified to date. Previously, it was found that MuRF1 expression showed a significant upregulation under denervation conditions, but returned to baseline levels by 14 days post-denervation in wild-type (WT) animals, while the MuRF1 locus (i.e. β-galactosidase) activity failed to return to baseline levels in MuRF1 knock-out (KO) animals. Furthermore, microarray analysis showed that myogenic regulatory factors such as myogenin and MyoD are significantly upregulated in response to denervation. These findings were among the first to suggest that MuRF1 may act as a transcriptional regulator in muscle atrophy.

Previous unpublished data from our lab supports the role of MuRF1 as a transcriptional regulator. MuRF1 and MAFbx reporter plasmid activity was driven down by overexpressing

MuRF1, suggesting that MuRF1 can transcriptionally regulate itself, MAFbx and other muscle-specific genes. Furthermore, when MRFs were overexpressed they induced MuRF1 and MAFbx promoter activity, while MuRF1 and MRF co-overexpression reversed MRF induction of the MuRF1 and MAFbx promoters. This was the first study to suggest that MuRF1 may modulate MRF transcriptional activity. This thesis served to characterize this previously unidentified role of MuRF1 as a transcriptional regulator in skeletal muscle through its regulation of myogenin and MyoD. These findings together support the hypothesis that MuRF1 is likely acting through the transcriptional model to alter the expression of atrophy related genes in muscle cells.

MyoD and myogenin show sequential expression in differentiating myoblasts

As mentioned, the MyoD-family of transcription factors controls muscle cell determination and differentiation [28]. MyoD has a previously determined role in myogenic determination and myogenin promotes the commitment of myoblasts to terminal differentiation [18]. Myoblast fusion is a highly-regulated process and previous studies suggest that MyoD acts as a "pioneer" to initiate a cascade of events triggering the expression of muscle-specific genes. It has been hypothesized that these events then allow the sequential binding and activity of myogenin. Myogenin and MyoD expression in C_2C_{12} muscle myoblasts showed that MyoD increased in expression early in proliferation and decreased by day 5 of differentiation (Figure 15). Myogenin expression increases early in differentiation, only after there is an increase in MyoD expression (Figure 14). This is consistent with a published study showing a similar trend of myogenin and MyoD expression in C_2C_{12} cell lines over a time course of differentiation [18]. These data confirmed and supported the characterization of the sequential expression of myogenin and MyoD in muscle cell differentiation.

MuRF1 and the catalytically dead MuRF1 RING mutant regulate myogenin but not MyoD protein expression

Previous unpublished data from reporter assays performed in our lab showed that MuRF1 had an ability to coordinate with myogenin and MyoD to regulate promoter activity of muscle-specific genes (Figure 10, Figure 11). Western blot analysis was performed on cells ectopically overexpressing myogenin and MyoD with and without overexpression of MuRF1 and the MuRF1-RING mutant. Ectopic expression of MuRF1 blunted the expression of myogenin. Additionally, the catalytically MuRF1-RING mutant, incapable of ubiquitinating proteins, was still able to blunt levels of myogenin (Figure 16). These data suggest that MuRF1 can effectively modulate levels of myogenin, even without a fully functional catalytic domain. These data were the first to suggest that this regulation was not occurring through catalytic activity of the RING Finger domain. Interestingly, when Western blot analysis was performed and MyoD was isolated there was no difference seen in MyoD protein expression upon over-expression of MuRF1 or the MuRF1-RING mutant (Figure 17). These findings support that MuRF1 is able to regulate the levels of myogenin, but not MyoD, and that this regulation is through a previously uncharacterized function by MuRF1.

In previous literature, the E3 ligase MAFbx has been characterized to be an F-box protein with a role in the ubiquitination of myogenin, which has a MAFbx- recognition motif [29]. Additionally, MAFbx has been characterized to regulate the expression of MyoD by means of ubiquitination [30]. While we know that MAFbx can regulate the expression of both myogenin and MyoD, this ability of MuRF1 to do so has not been previously investigated to our knowledge. Again, these data show that MuRF1 can alter the expression of myogenin but not MyoD. Previous research has suggested that even though MuRF1 and MAFbx are both

upregulated in atrophy conditions, the targets of these two E3 ligases may be completely different [31]. Thus, it is not entirely surprising we see effects on myogenin but not MyoD.

Inhibition of the 26S proteasome does not rescue myogenin protein levels in response to MuRF1 overexpression

To further investigate the mechanism by which MuRF1 is regulating myogenin, the role of the 26S proteasome was also investigated. Western blotting analysis was performed after treating cells with MG132, a general 26S proteasome inhibitor. The results showed that while there was an increase in the amount of myogenin recovered, proteasome inhibition was not enough to fully rescue myogenin protein levels, as myogenin expression was still blunted when MuRF1 was overexpressed in the cells (Figure 18). This further confirmed that MuRF1 is not regulating myogenin through the proteasome system. While we found that MuRF1 was not regulating myogenin through ubiquitination, these data further elucidate that the hypothesized proteasome model of MRF regulation is invalid. While it is widely accepted that MuRF1 polyubiquitinates proteins for degradation through the UPS, these data suggest that MuRF1 may be involved in a feedback mechanism responsible for transcriptionally regulating atrophy-related genes through the transcriptional regulation of myogenic regulatory factors such as myogenin.

No effect was seen on MyoD when the catalytic function of the MuRF1 RING domain was destroyed (Figure 17) or when the proteasome was inactivated (Figure 19). Conversely, MuRF1 was able to down-regulate myogenin levels in both of these cases. Previous literature has established MyoD as a transcriptional regulator of myogenin [38], therefore we have reason to believe that MuRF1 may be acting to regulate myogenin by negatively regulating MyoD, thus resulting in a down-regulating the expression levels of myogenin.

Endogenous myogenin and MyoD associate with the MuRF1 conserved Ebox sequence

Previous data suggests that MuRF1 regulates myogenin and/or MyoD to suppress MuRF1 and MAFbx reporter gene activity. To further investigate the mechanistic coordination of MuRF1 with the MRFs, chromatin immunoprecipitation (ChIP) was implemented to examine if the MuRF1 promoter was being occupied by endogenous levels of either myogenin or MyoD. The qPCR analysis using primers spanning the MuRF1 Ebox enhancer region suggested that MuRF1 was occupied the most by MyoD early in proliferation and myogenin early in differentiation (Figure 20).

These results suggest that myogenin and MyoD sequentially associate with Ebox enhancer sequences in the promoter region of MuRF1 in mouse myoblasts, with MyoD seemingly occupying the MuRF1 promoter early in proliferation and myogenin early in differentiation. This is not unexpected, as we know that MyoD is expressed at higher levels earlier in differentiation, while myogenin is expressed in higher levels later in differentiation. These findings support expression patterns that were observed with western blot analysis of endogenous MyoD and myogenin expression in the cell line. This may explain the possible pattern of the induced association between these MRFs and the MuRF1 Ebox region, especially if MuRF1 is being induced to regulate muscle-specific genes downstream in the atrophy cascade. Previous literature has confirmed that histone deacetylases such as Hdac4 upregulate the expression of MRFs which in turn upregulate MuRF1 and MAFbx, but a direct mechanism for the upregulation of MuRF1 has not been identified to our knowledge [21]. While previous unpublished data has characterized that atrogenes containing these conserved Ebox regions in the promoter can be

regulated by MRF overexpression, this thesis provides the first investigation of the mechanism by which MRFs directly bind to transcriptionally regulate the activity of these E3 ligases.

Co-overexpressing MRFs with MuRF1 shows MuRF1-mediated reversal of MRF-induced occupancy of the MuRF1 promoter

Myogenin and MyoD were overexpressed in C_2C_{12} cells and ChIP was performed to investigate the effect of overexpression on MRF occupancy of the MuRF1 promoter in atrophy-like conditions (i.e. induced MRF levels). It was predicted that high levels of MuRF1 promoter occupancy when myogenin and MyoD were overexpressed and pulled down, as overexpression of myogenin and MyoD in reporter assays induced reporter activity of MuRF1 and MAFbx. The results confirmed that occupancy of the MuRF1promoter was induced when overexpressing MRFs (Figure 21).

In previous research co-overexpression of MuRF1 with MRFs drove down the MRF-mediated induction of the MuRF1 promoter in reporter assays. Therefore, it was expected that MuRF1 overexpression would be able to drive down the amplification of MuRF1 promoter association of both myogenin and MyoD. The results of this experiment confirmed that co-overexpressing MuRF1 with MRFs resulted in a MuRF1-mediated reversal of myogenin and MyoD occupancy with the MuRF1 promoter (Figure 22). Additionally, it seems that there is more occupancy of the MuRF1 promoter by MyoD, which further supports our hypothesis that MuRF1 is regulating myogenin levels through somehow negatively regulating MyoD.

These data also signified that there is a possible feedback loop occurring in which MuRF1 is regulating its own transcriptional regulation. This is supported by the microarray analysis showing that MuRF1 promoter activity did not return to baseline levels in MuRF1 KO mice at 14

days, which was the first hint at MuRF1 acting as a transcriptional regulator of itself [13]. It also builds on preliminary research showing that overexpression of MuRF1 down-regulated MuRF1 reporter gene activity. These data strongly suggest that MuRF1 can, in fact, regulate its own transcriptional activity. It is apparent that myogenin and MyoD are associating at the Ebox region of the MuRF1 promoter. Additionally, when MuRF1 was overexpressed in C_2C_{12} cells there were lower levels of MRF occupancy at the MuRF1 promoter. This is the first study that hints at a mechanism by which MuRF1 acts as a transcriptional regulator indirectly through the regulation of myogenin, possibly by negatively regulating MyoD.

Transcriptional regulation of atrophy-related genes may be regulated by a potentially intricate set of feedback mechanisms between E3 ligases and MRFs. For example, myogenin and MyoD are upregulated in response to atrophy [36] and we know that MyoD must be present to activate myogenin recruitment [37]. In turn, myogenin acts as a transcriptional regulator of E3 ligases such as MuRF1 and MAFbx [38]. The data in this thesis supports the characterization of MuRF1 as a transcriptional regulator of not only itself, but other atrophy-related genes such as myogenin and MyoD. Thus, it is reasonable to propose that MuRF1 participates in a feedback mechanism that regulates its own expression and regulates the binding of myogenin and MyoD to Ebox enhancer regions, as they also play a key in the transcriptional regulation of muscle specific genes.

While this thesis confirms that MuRF1 is not regulating MRFs via the UPS or through the catalytic activity of the MuRF1 RING Finger domain, MuRF1 may be responsible in regulating 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. 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. MuRF1 could also be involved in targeting recruitment of additional transcription factors to the promoters of atrophy-induced genes, or interact with or recruit additional proteins which themselves associate with the promoters of muscle-specific genes.

High levels of expression of MuRF1 may play an additional role in preventing these MRFs from sitting on the Ebox sequence of promoter regions. It is thought that the primary interaction of the MRFs is to dimerize before they associate with a promoter region [39]. These transcription factors must either homodimerize or heterodimerize with factors such as E proteins to form a functional dimer that can sit down on the consensus E-box sequences [39, 40]. MuRF1 may have a role in regulating MyoD or the binding partners of MyoD, thus preventing them from dimerizing with their appropriate binding partners to effectively transcriptionally regulate myogenin. This may ultimately sequester these MRFs from effectively sitting down on conserved Ebox promoter regions.

We propose a novel function of MuRF1 in targeting downstream atrogenes through transcriptional regulation, mainly through the negative regulation of myogenin and MyoD at the conserved Ebox sequences in the promoter regions of muscle-specific genes. Further research on how MuRF1 is mechanistically modulating MRFs to ultimately transcriptionally regulate atrogenes will need be conducted, but this study serves as the first evidence of the coordination of MuRF1 and MRFs in the transcriptional regulation of muscle specific genes during skeletal muscle atrophy.

Conclusions

In summary, the data herein elucidates a previously uncharacterized function for MuRF1 as a transcriptional regulator of atrogenes through the transcriptional regulation of the myogenic regulatory factors, myogenin and MyoD. MuRF1 is largely recognized in literature as an E3 ubiquitin ligase that is expressed at high levels under virtually all atrophy conditions; however, these data suggest that MuRF1 is also working outside of the ubiquitin proteasome system to transcriptionally regulate itself and other muscle specific genes through a feedback loop that controls MRF-mediated regulation of gene expression in muscle atrophy.

Previously, it was shown that MuRF1 can regulate the promoter activity of itself by mediating the activity of MyoD and myogenin. We have now determined that MuRF1 modulates myogenin and MyoD occupation of the conserved Ebox sequence found in the promoter region of MuRF1. Interestingly, when MuRF1 was overexpressed concurrently with myogenin or MyoD we saw a decrease in the occupancy of the MRFs at the Ebox promoter region of MuRF1. Western blot analysis also indicated that MuRF1 regulates myogenin but not MyoD, suggesting that it has specific downstream targets that differ from other E3 ligases such as MAFbx. Furthermore, MuRF1 was still capable of decreasing the amount of myogenin protein expression without both the catalytic RING domain and without the function of the 26S proteasome. This refutes that MuRF1 is regulating the MRFs through its previously characterized function of an E3 ligase working through the UPS (Figure 12) and supports our previously mentioned transcription model (Figure 13); MuRF1 may be acting as a transcriptional regulator of myogenin and other atrophy-related genes, possibly via the negative regulation of MyoD.

Additional research is still needed to further characterize the exact mechanism by which MuRF1 is acting as a transcriptional regulator. The involvement of E proteins, which are the ubiquitously

expressed binding partners of myogenin and MyoD, should also be investigated to better understand how MuRF1 can modulate the MRFs. MRF occupancy of the MuRF1 promoter should also be evaluated in response to ectopic expression of MuRF1 mutants, as well as siRNA knockdown of MuRF1. The preferential regulation of myogenin over MyoD should also be further investigated, as this could elucidate the function of MuRF1 in regulating transcriptional activity of atrophy-related genes. Finally, MuRF1 might also be investigated beyond its capabilities as a transcriptional regulator; the nature of skeletal muscle atrophy is intricate and regulation occurs on many levels, therefore the analysis of MuRF1 post-transcriptionally would also likely yield pertinent information regarding the molecular mechanisms of skeletal muscle atrophy.

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Supplemental Figures

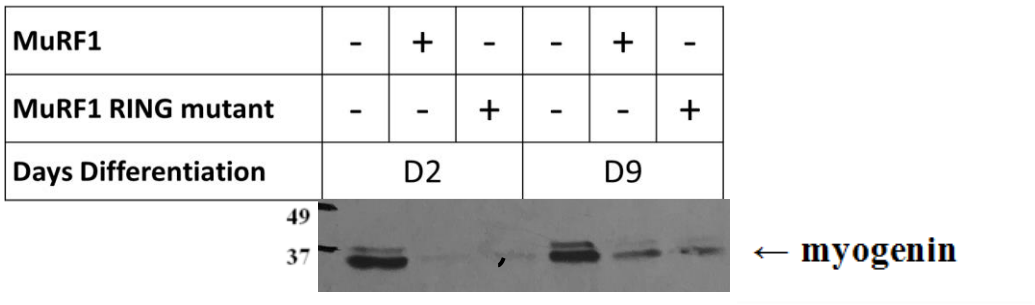

Figure S1. Protein expression profile of myogenin (~34 kDa) in C_2C_{12} mouse myoblasts is decreased upon overexpression of MuRF1 or MuRF1-RING mutant. C_2C_{12} myoblast cells were transfected with control expression plasmid alone (-/-) or expression plasmids for either MuRF1 or MuRF1 RING mutant and harvested at day 2 (D2) and day 9 (D9) after the switch to differentiation media (2% serum). Western blotting was performed using 150µg of total protein and probed using an antibody against myogenin.

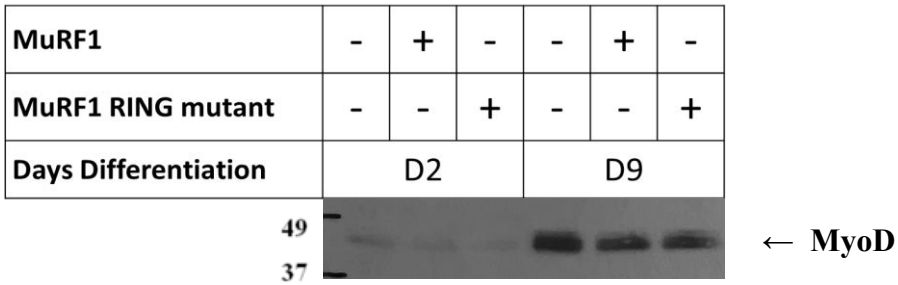

Figure S2. Protein expression profile of MyoD (~45 kDa) in C_2C_{12} mouse myoblasts remains unaltered upon overexpression of MuRF1 or MuRF1-RING mutant. C_2C_{12} myoblast cells were transfected with control expression plasmid alone (-/-) or expression plasmids for either MuRF1 or MuRF1 RING mutant and harvested at day 2 (D2) and day 9 (D9) after the switch to differentiation media (2% serum). Western blotting was performed using 150µg of total protein and probed using an antibody against MyoD.

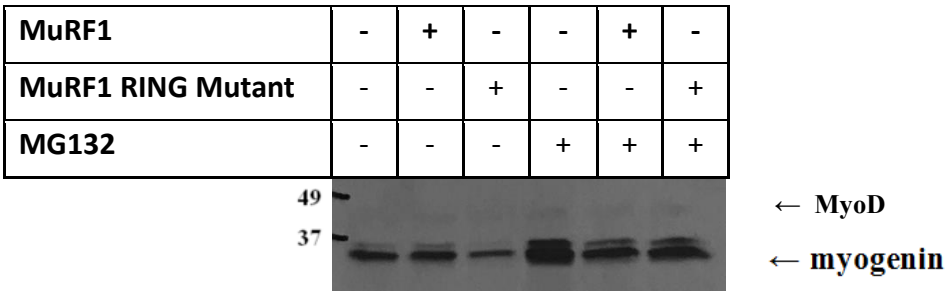

Figure S3. Protein expression profile of myogenin (~34kDa) and MyoD (~45 kDa) in C_2C_{12} mouse myoblasts treated with MG132 (12.5µg/µL) and ectopically expressing MuRF1 or the catalytically dead MuRF1 RING mutant. MuRF1-mediated repression of myogenin expression levels are partially reversed in the presence of MG132 but are not completely rescued from the blunting effect by ectopic MuRF1 or MuRF1 RING mutant. MyoD expression levels are partially reversed in the presence of MG132, but no differences are seen upon expression of ectopic MuRF1 or MuRF1 RING mutant. Myoblasts were transfected with control expression plasmid alone (-/-) or an expression plasmid for MuRF1 and harvested at day 2 (D2) and day 9 (D9) after the switch to differentiation media (2% serum). Western blotting was performed using 150µg of total protein probed using an antibody against myogenin or MyoD.