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Transcriptional Regulation of Dual-Specificity Phosphatase 4 (Dusp4) by Muscle RING Finger 1 (MuRF1) and Myogenic Regulatory Factors

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Transcriptional Regulation of Dual-Specificity Phosphatase 4 (Dusp4) by Muscle RING Finger 1 (MuRF1) and Myogenic Regulatory Factors

By

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Abstract

Skeletal muscle atrophy can occur at any age and as a result of numerous physiological conditions and thus, it was necessary to better identify the molecular underpinnings of the atrophy cascade so that new therapeutic targets to treat muscle wasting might be identified. MuRF1 was first identified as a marker of skeletal muscle atrophy over a decade ago; however, recent work suggests that this E3 ubiquitin ligase may participate in muscle wasting by regulating the transcriptional activity of genes differentially expressed in response to muscle atrophy. Dusp4, a dual-specificity phosphatase and member of the MAPK cascade, is induced in response to neurogenic atrophy; however, this induction is significantly blunted in the MuRF1-null mice which are resistant to muscle atrophy. The research presented in this thesis aims to characterize the mechanism by which MuRF1 may transcriptionally regulate Dusp4 and characterizes the function of Dusp4 in skeletal muscle.

Chapter 1: Background of Skeletal Muscle Atrophy and the Role of MuRF1

Skeletal Muscle Physiology and Muscle Atrophy

The maintenance of healthy skeletal muscle is a dynamic process that requires the muscle tissue to sense and interpret signals from the surrounding environment and integrate those signals into action. This interpretation works to either increase protein turnover (atrophy) or protein synthesis (hypertrophy) depending on cues received in response to physiological and/or pathological conditions. Continuous restructuring occurs within the skeletal muscle tissue that depends on a careful balance between protein synthesis and protein degradation, a balance that may shift depending on the physiological needs of the individual [1]. Muscle hypertrophy, an increase in muscle fiber size, occurs when there is a shift towards protein synthesis that happens most commonly during development [2]. Skeletal muscle atrophy occurs when the individual myofibers experience increased protein turnover and decreased protein synthesis that results in a decrease in size, net force production, and elevated muscle fatigue [3]. Muscle atrophy may result from a variety of physiological conditions including limb immobilization, cancer (cachexia), aging (sarcopenia), corticosteroid use, and denervation [4]. The molecular mechanisms that regulate different types of muscle atrophy (disuse, malnutrition, or neurogenic pathologies) can vary widely and the cellular cascade of events, including gene regulation or induction of reactive oxygen species (ROS), all lead to increased protein degradation and a reduction in muscle fiber size [5, 6]. Although initial research in the field of skeletal muscle wasting focused on protein turnover, understanding the complete molecular signaling processes that leads to the phenotype of atrophy has been difficult to define completely.

Skeletal muscle wasting is observed in many conditions that plague human health including patients suffering from stroke, burns and arthritis; however, neurogenic atrophy which is the most severe form can be found in patients with Guillain-Barre syndrome, multiple sclerosis, and Lou Gehrig's disease [7]. There is no repair system in humans that facilitates the repair of damaged nerves which connect to muscles. In lower vertebrate organisms, such as mice, damaged neurons have a unique plasticity and can reestablish neural connections with skeletal muscle myofibers following a traumatic event due to the

presence of neural stem cells; this phenomenon does not exist in higher mammals, such as humans [8]. Identifying the pathways linking regenerative neural plasticity to skeletal muscle fibers would offer many new therapeutic opportunities for people suffering from severe muscle wasting. One potential regulatory mechanism that has the ability to alter the differentiated state of committed myotubes is through the influence of myogenic regulatory factors (MRF), myogenin and MyoD1, which are responsible for transcriptional regulation of muscle specific genes. In the murine model, down-regulation of myogenin reverses terminal muscle cell differentiation [9] and in myofibroblasts reduction of MyoD1 results in cells de-differentiating and re-entering the cell cycle [10]. Current research has focused on characterizing the molecular mechanisms of skeletal muscle atrophy in cell culture models, potentially leading to the identification of new drug targets and therapies.

Identification of Markers of Skeletal Muscle Wasting

The first step in unraveling the physiological mystery of muscle atrophy was to identify molecular mediators, particular genes with significant differential expression in response to a range of disparate conditions known to induce atrophy. To this end, a differential expression study was conducted using gastrocnemius muscle isolated from Sprague-Dawley rats that were subjected to hind limb suspension, limb immobilization, or sciatic nerve denervation in order to promote skeletal muscle wasting [11]. A total of 74 genes showed alterations in activity under one or two conditions but only two genes, muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx) showed increased gene expression under all conditions (Figure 1) [11]. These findings helped to identify MuRF1 and MAFbx as hallmark genetic markers in the overall process of skeletal muscle atrophy. Additional research has shown that MuRF1-null and MAFbx-null mice are resistant to most forms of skeletal muscle wasting compared to the wild-type mice further demonstrating the importance of these genes in the muscle atrophy signature [11].

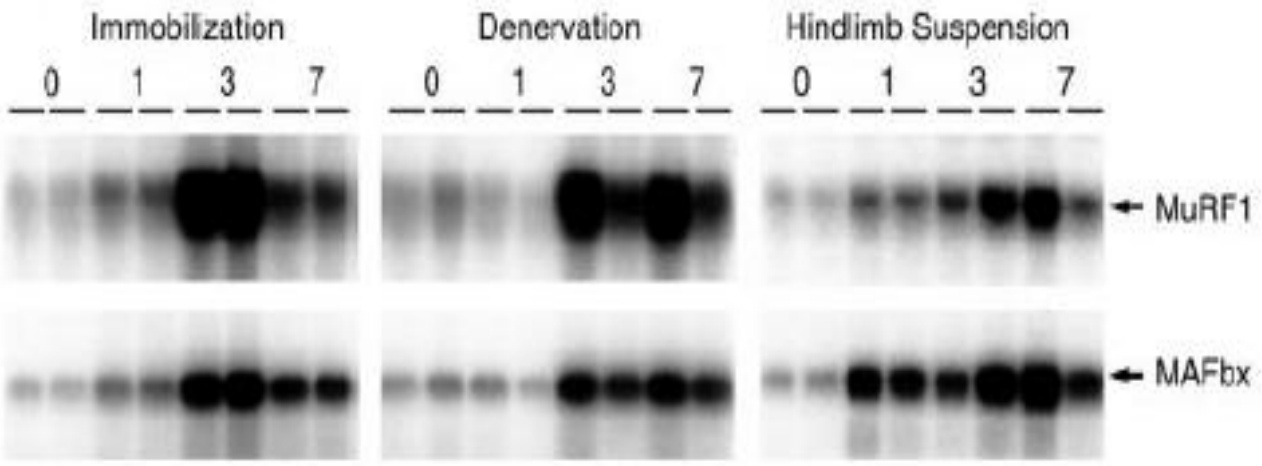


Figure 1. mRNA expression profiles of MuRF1 and MAFbx in Sprague-Dawley rat gastrocnemius muscle under muscle atrophy conditions. MuRF1 and MAFbx showed increased expression following limb immobilization, sciatic nerve denervation, and hind limb suspension by day 1 with maximum expression reached by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

MuRF1 and the Ubiquitin-Proteosome System (UPS)

MuRF1 and MAFbx were identified as markers of skeletal muscle atrophy following experimentation which evaluated conditions known to induce atrophy such as immobilization, sciatic nerve denervation, and hind limb unweighting [11]. The identification of two genes, both of which are E3 ubiquitin ligases, began the long journey to identify protein targets following an atrophy-inducing stimulus. The increased expression of MuRF1, which is believed to participate in the ubiquitin-proteasome system (UPS), provides an attractive model for how MuRF1 might function during muscle wasting and may help explain the decrease in muscle fiber size which is known to occur during skeletal muscle atrophy [12].

Muscle breakdown and protein turnover is coupled with increased activity of the ubiquitin-proteasome system [13]. The UPS is an ATP-dependent system composed of an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase that function in tandem to covalently tag proteins with ubiquitin and subsequently target them for breakdown by the 26S proteasome (Figure 2) [14-16]. The ubiquitin-proteasome system and the subsequent turnover of damaged or unneeded proteins occurs normally; however, it is the up-regulation of these processes during skeletal muscle atrophy that

has garnered interest and has pushed the research focus towards elucidating the molecular mechanisms of muscle wasting, including transcriptional regulation of genes and post-translational modification of proteins, that remain uncharacterized in the progression of muscle atrophy [17].

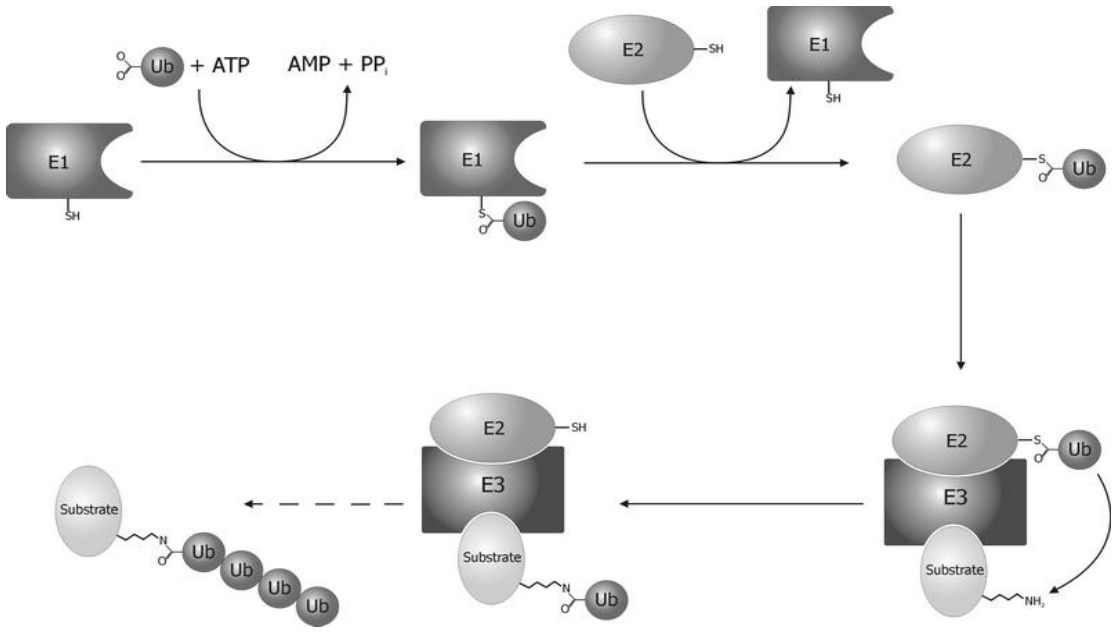


Figure 2. Schematic of the ubiquitin-proteasome system. Protein degradation by the proteasome involves collaboration of three enzymes; a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase (E3). (Open access image created by Roger Dodd)

Identification of Differentially Expressed Genes Following Neurogenic Atrophy

Given that muscle-specific E3 ligases show increased expression following neurogenic atrophy and that MuRF1 knock-out animals are resistant to muscle wasting, a microarray using gastrocnemius muscle obtained from wild-type (WT) and MuRF1-null (KO) mice was performed following sciatic nerve denervation in order to evaluate global changes in gene expression [18]. MuRF1 gene expression increased in the wild-type animals at 3 days post-denervation, but returned to baseline levels by 14 days post-denervation; however, β-galactosidase expression, that was controlled by the intact MuRF1 promoter, was up-regulated following denervation at 3 days and remained elevated at 14 days post-denervation (Figure 3) [18]. Numerous genes involved in cellular proliferation, differentiation, apoptosis, DNA packaging, muscle-specific transcription factors as well as the mitogen-activated protein (MAP)

kinase cascade showed differential expression following denervation; however, some of the observed changes in gene activity were altered in the animals lacking MuRF1 expression [18]. Whether MuRF1 is necessary for the direct transcriptional regulation of these genes or whether MuRF1 may modulate separate transcription factors important for gene activity has yet to be determined.

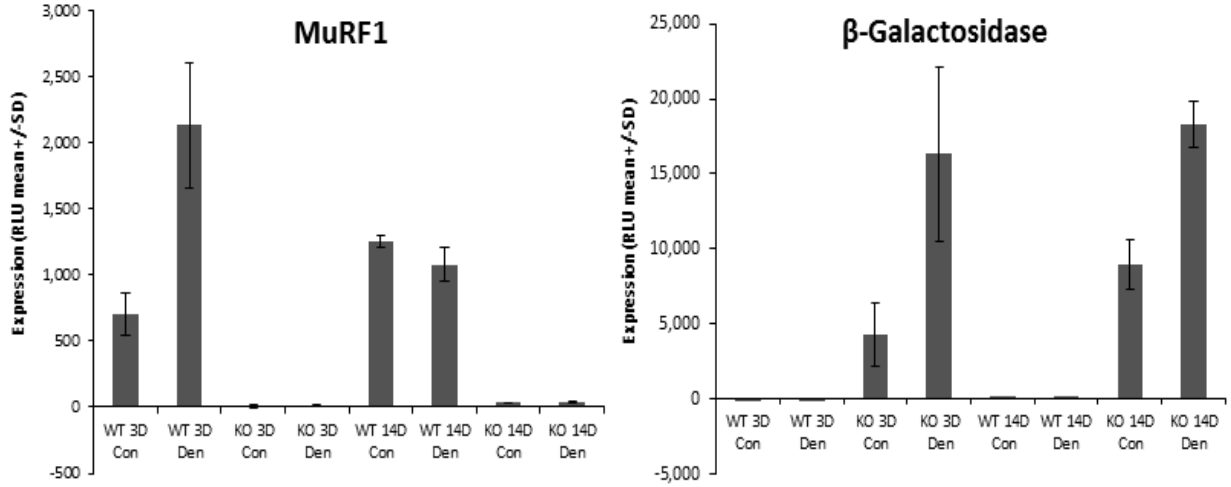


Figure 3. MuRF1 and β-galactosidase expression profiles in wild-type (WT) and MuRF1-null (KO) mice. (A) MuRF1 gene expression increased following denervation in WT animals at 3 days and returned to baseline levels by 14 days post-denervation. (B) β-galactosidase expression, which is under the control of the MuRF1 gene promoter, showed increased expression following 3 days of denervation and remained elevated at 14 days post-denervation. Error Bars represent standard deviation.

Myogenic Regulatory Factors Control Tissue Development

Myogenin and MyoD1 are muscle-specific transcription factors that are referred to as myogenic regulatory factors (MRF) (Figure 4) [19]. MRFs contain the basic helix-loop-helix (bHLH) motif consisting of two α-helices connected by a loop [20]. Myogenin is expressed early during the developmental stage and is required for viable embryonic myofiber formation; additionally, myogenin is upregulated following damage to the muscle and helps aid in regenerating damaged muscle tissue [13, 20, 21]. MyoD1 is also important for muscle cell regulation as it functions in the myogenic determination of myoblasts [22]. Interestingly, differentiated myofibroblasts subjected to MyoD1 knock-down were able to dedifferentiate and reenter the cell cycle mimicking the undifferentiated myoblast stage [10]. Down regulation of myogenin reverses terminal muscle cell differentiation [9], therefore we believed it was

important to this study to assess the effects that both myogenin and MyoD1 have on the regulation of Dusp4 transcriptional activity in skeletal muscle cells.

Figure 4. 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.)

Muscle tissue requires MRF expression in order to maintain the differentiated, multi-nucleated mature muscle fibers that are necessary for motor function and muscle contraction [23]. The microarray that was performed to evaluate global changes in gene expression following denervation-induced skeletal muscle atrophy shows that two myogenic regulatory factors, MyoD1 and myogenin, were markedly increased at both 3 days and 14 days post-denervation compared to non-denervated wild-type littermates (Figure 5). Although MyoD1 expression was increased at 14 days post-denervation, there was blunted induction at 14 days in the MuRF1-null animals. A similar trend was observed for myogenin which showed increased expression at 3 and 14 days post-denervation in both WT and KO animals; however, the increase in myogenin expression was blunted in the MuRF1-null animals (Figure 5). The increase in these two important transcription factors, which are necessary to maintain the differentiated status of skeletal muscle cells, shows that the presence of functional MuRF1 protein may be needed for full activation of MRFs after a denervation event.

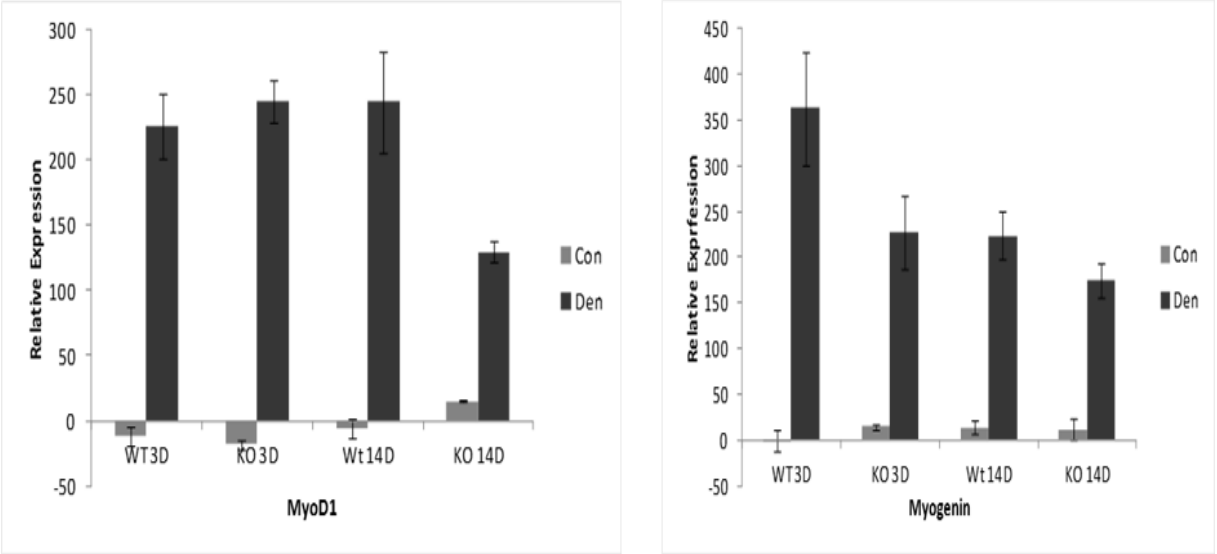


Figure 5. Myogenic regulatory factors show increased mRNA expression in response to sciatic nerve denervation. MyoD1 and myogenin expression increased at both 3 and 14 days following denervation in wild-type and MuRF1-null mice. Error bars represent ± standard deviation.

Dusp4 Expression Increases Following Neurogenic Muscle Atrophy

One gene from the microarray described above that was significantly up-regulated at both 3 and 14 days following sciatic nerve denervation was dual-specificity phosphatase 4 (Dusp4) (Figure 6). Dusp4 is a member of the mitogen activated protein (MAP) kinase cascade which functions to control numerous cellular processes ranging from proliferation, differentiation and apoptosis in the maintenance of cellular homeostasis [24]. Dusp4 has been the focus of cancer research due to its role in proliferation and apoptosis in lymphatic tissue as well as in the brain [25, 26]; however, little work has been performed to identify what role it may play in the formation of myotubes and the development of healthy skeletal muscle. Increased Dusp4 activity has previously been linked to the inflammatory response as well as to cell cycle signaling by controlling the G_2/M phase transition, but the role of Dusp4 in skeletal muscle has not been well characterized [26, 27].

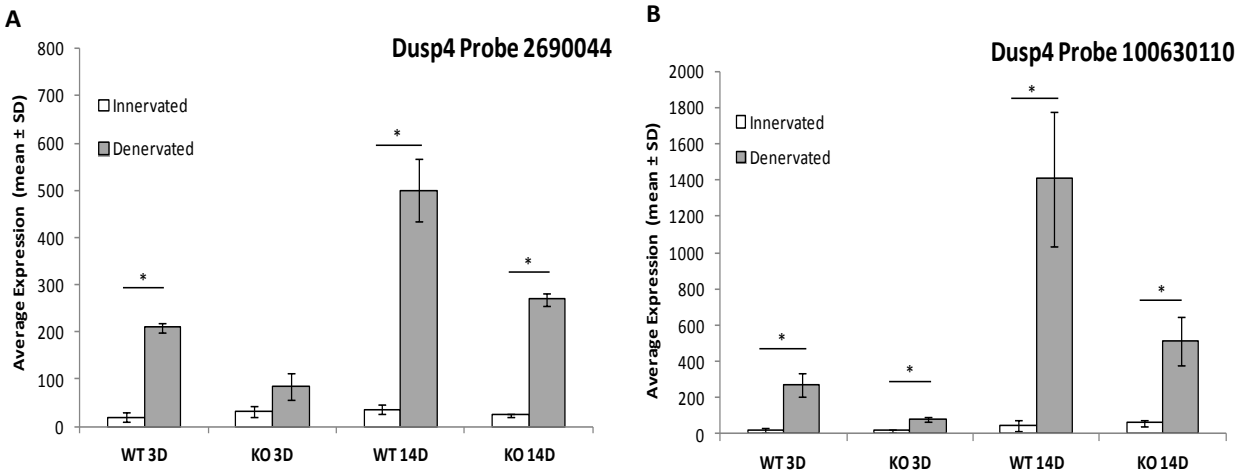


Figure 6. Dusp4 expression profiles in wild-type (WT) and MuRF1-null (KO) mice. Whole genome expression analysis was conducted on gastrocnemius muscle from WT and KO mice following 3 days (3D) and 14 days (14D) denervation. Each condition was conducted in triplicate and the expression is the average of three individual mice. The error bars represent ± standard deviation. The microarray had two separate Dusp4 probes and both probes listed above showed similar expression profiles. Dusp4 expression increased significantly following denervation in WT mice at both 3 and 14 days, however expression was significantly suppressed in the MuRF1-null mice following denervation when compared to the WT mice (* $p \leq 0.05$ Two tailed T-test).

Dusp4 is a dual-specificity phosphatase that exhibits the ability to dephosphorylate both threonine and tyrosine residues of phosphate-activated MAP kinases. The Dusp4 protein is composed of an N-terminal MAP kinase binding domain, two cdc25 homology domains that are found in all mitogen-activated protein kinase phosphatases (MKPs) which reflect their common evolutionary origin, a C-terminal catalytic phosphatase domain that has conserved aspartic acid (D), cysteine (C), and arginine (R) residues that form the catalytic phosphatase pocket, and an N-terminal nuclear localization signal (NLS) making Dusp4 a type-1 dual specificity phosphatase (Figure 7) [28-30].

Depending on the cell type, Dusp4 has the ability to inactivate MAPK's such as extra-cellular signal related kinase (ERK), c-jun related kinase (JNK), and p38 [31, 32]. The substrate specificity may be tissue specific, since previous research suggests that Dusp4 has the ability to dephosphorylate all three MAPK's while other research suggests its function is restricted to dephosphorylating only ERK [24, 31]. Furthermore, research suggests that after Dusp4 removes the activated phosphate group from ERK, the dephosphorylated ERK then translocates back to the cytoplasm to wait for an additional signaling response while Dusp4 remains located in the nucleus, due to its nuclear localization signal [33].

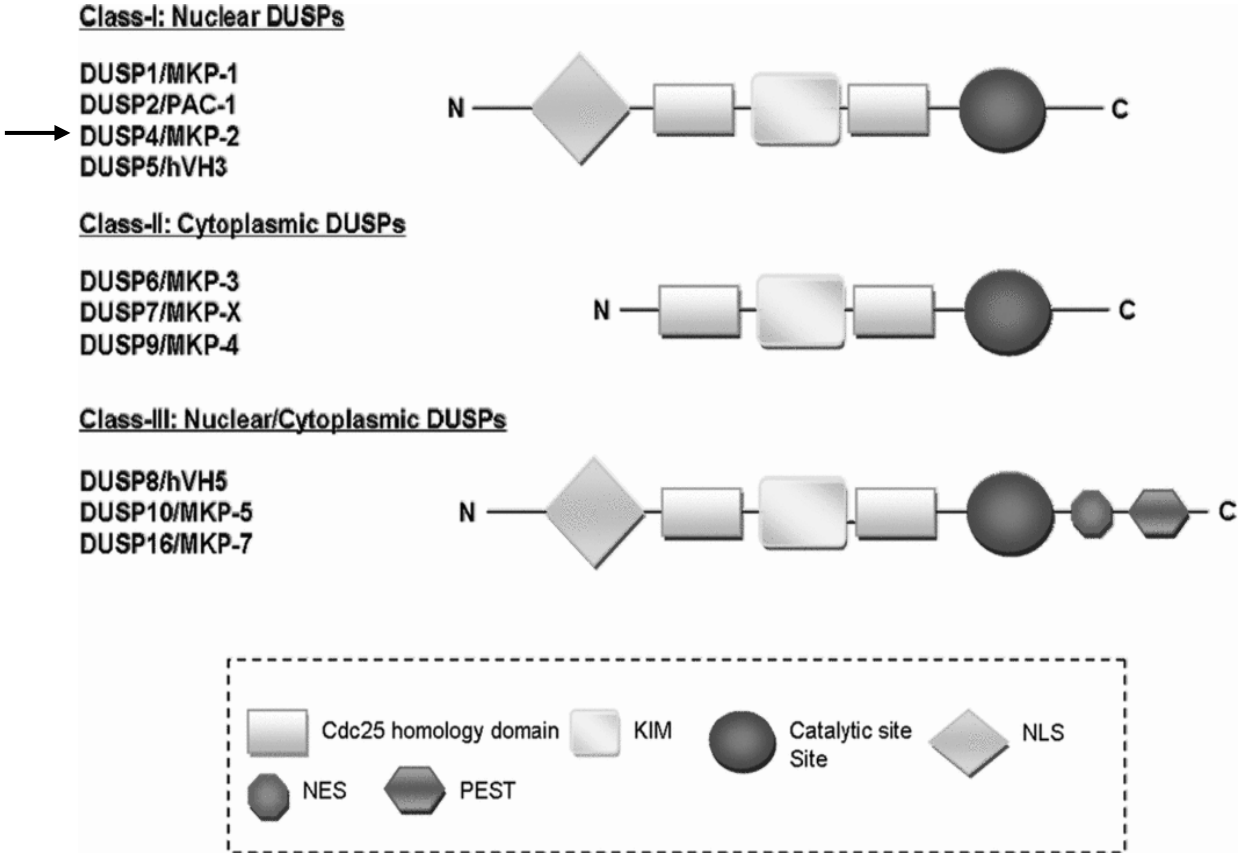


Figure 7. Schematic of the different classes of dual-specificity phosphatases. Dusp4 is a class 1 dual-specificity nuclear phosphatase which contains two cdc25 domains, an N-terminal MAP kinase interacting motif (KIM), an N-terminal nuclear localization signal (NLS), and a C-terminal catalytic phosphatase domain. (Adapted from Lawan, et al., 2011.)

The focus of this study was to ascertain the function of Dusp4 in skeletal muscle, since it was found to be up-regulated following sciatic nerve denervation and has not been previously characterized in muscle tissue (Figure 6) [18]. Developing a better understanding of how the Dusp4 gene is regulated by MuRF1, as well as MRFs, and characterizing the functional role of Dusp4 in skeletal muscle may help to clarify why increased Dusp4 expression is observed following denervation. The possible interaction between MuRF1 and the MRFs, which bind to E-box elements located in the Dusp4 promoter, is a potential way by which MuRF1 may alter Dusp4 gene expression (Figure 8). Additionally, the timing of Dusp4 protein expression in muscle was evaluated by western blot using C_2C_{12} cells and was found to be expressed in proliferating myoblasts, but not differentiated myotubes. Finally to confirm the sub-cellular location of Dusp4 in myoblasts, the cDNA of Dusp4 was engineered with a green fluorescent protein (GFP) tag and

visualized using confocal microscopy revealing that Dusp4 is localized exclusively to the nucleus. The information obtained in this study helps characterize the transcriptional regulation of Dusp4 and helps determine the function of Dusp4 in muscle in response to denervation and provides evidence for the potential physiological importance of this gene in skeletal muscle.

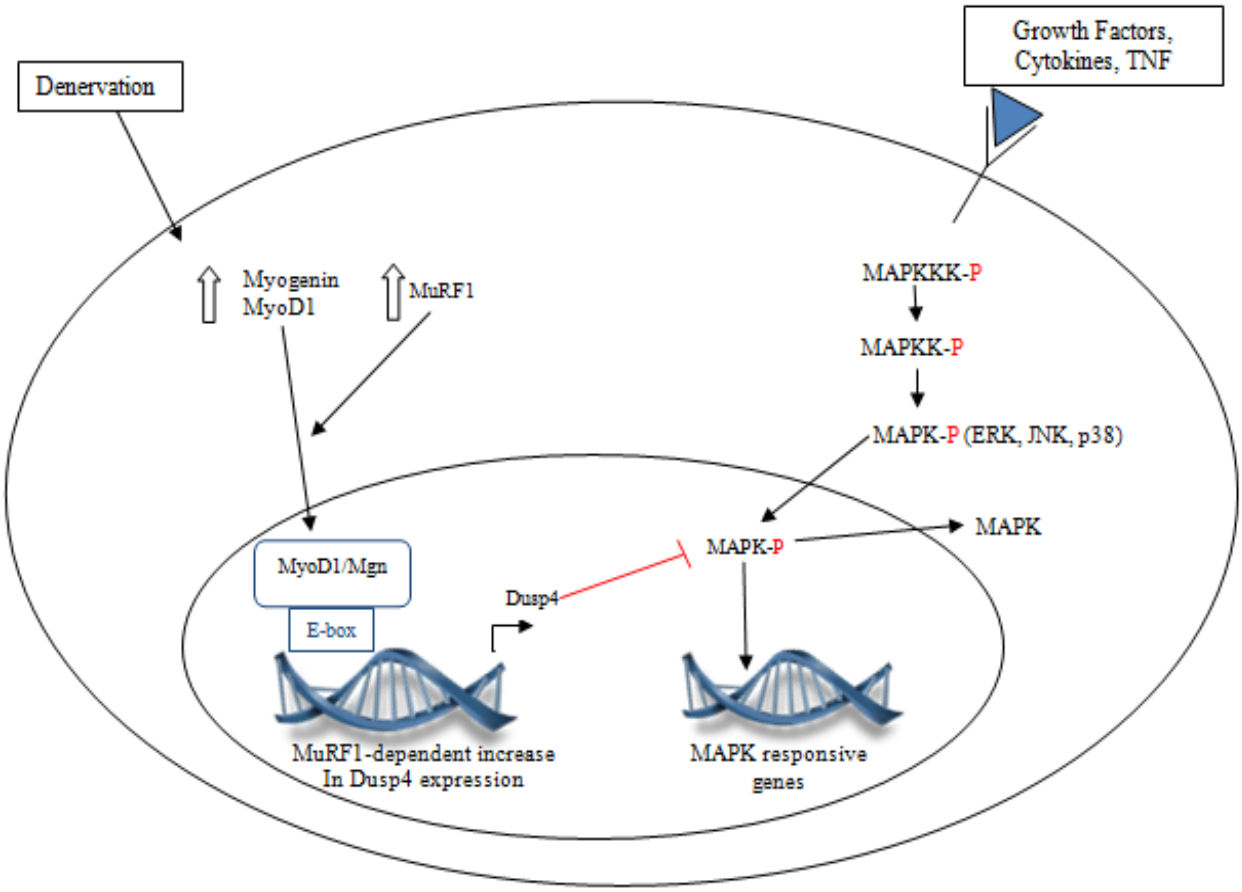


Figure 8. Schematic of the MAP kinase pathway. The diagram illustrates both the cytoplasmic and nuclear portions of this signaling pathway in response to denervation as well as cellular signaling. During normal cellular conditions Dusp4 dephosphorylates phosphate-activated nuclear MAP kinases (ERK, JNK, and p38) which results in the inactivation of MAPK responsive genes. Following neurogenic atrophy, MuRF1, myogenin, and MyoD1 are upregulated which results in Dusp4 repression. This reduction in Dusp4 activity could result in increased MAPK phosphorylation leading to the activation of MAPK responsive genes.

Chapter 2: Characterization of the Transcriptional Regulation and Function of Dusp4 in Skeletal Muscle

Overview

Protein phosphorylation represents a critical event in the activation and regulation of a variety of cellular pathways. The mitogen-activated protein (MAP) kinase cascade represents an important step that can alter gene expression profiles and change the behavior of cells. MAP kinase kinase kinases (MAPKKK) phosphorylate MAPKKs, which in turn phosphorylate and activate MAPKs [33]. In mammalian cells, there are four MAPK families: extra-cellular signal-related kinase 1/2 (ERK 1/2), ERK5, p38, and c-jun N-terminal kinase (JNK) [34]. MAP kinases such as ERK, p38, and JNK can be phosphorylated on threonine or tyrosine residues [35]. The MAP kinase contains a core Thr-X-Tyr (TXY) motif in which threonine or tyrosine residues may be phosphorylated although it may be necessary for both amino acids to be phosphorylated for full activity [31]. The central amino acid (X) may be either a glutamine, proline, or another tyrosine residue, in ERK, JNK, or p38 MAP kinases [28]. The particular amino acid that becomes phosphorylated may play a pivotal role in the function of the MAPK or may dictate other binding partners for further regulation [36]. Activated MAPKs have the ability to translocate from the cytoplasm to the nucleus where they can phosphorylate serine, threonine, or tyrosine residues of other proteins, namely transcription factors, that directly affect gene regulation [35]. Because of their role in ligand-activated signal transduction pathways, MAP kinases play important roles in stress response, cellular regeneration, differentiation, metabolic diseases, and cancer [37].

Due to the energy requirements for transcription and translation of a protein, it is important to ensure that cellular signals instructing protein synthesis are indeed necessary. At each step of the MAP kinase cascade, in addition to the kinases required for further activation of the pathway, there are also phosphatases that remove phosphate groups from proteins, thus inactivating the signal. The dual-specificity phosphatase (Dusp) family contains 25 members and has the ability to remove a phosphate from an activated MAP kinase located in the nucleus (type I), cytoplasm (type II), or may function in both

the nucleus and cytoplasm to dephosphorylate MAPKs (type III) [24, 36]. Dual-specificity phosphatases, unlike other enzymes with catalytic phosphatase activity, are unique in their ability to remove a phosphate from either serine, threonine, or tyrosine residues [26].

The founding member of the Dusp family (Dusp1/MKP-1) has been identified in a variety of cells and tissue types. Dusp1 is a type-1, nuclear dual-specificity phosphatase which preferentially inactivates p38, but maintains the ability to dephosphorylate ERK and JNK as well, and it is up-regulated in response to pro-inflammatory stimuli [37]. In 1995, MKP-2 (Dusp4) was identified and was also found to be expressed in a variety of tissue types; however, since its discovery very little work has been performed to ascertain the function of Dusp4 in skeletal muscle [38]. The majority of the research on Dusp4 has focused on its function as a tumor suppressor protein in an assortment of cancers, as well as its role in immune function [25, 26, 39, 40]. Interestingly, Dusp4 has been shown to be significantly upregulated in skeletal muscle tissue in response to neurogenic atrophy, while this induction is dramatically blunted in MuRF1-null animals that are resistant to muscle wasting. Therefore, the primary aims of the research presented in this thesis were to identify a function for Dusp4 in the skeletal muscle atrophy cascade and characterize the transcriptional regulation of Dusp4.

Materials and Methods

Cell Culture

C_2C_{12} mouse myoblast cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were stored in liquid nitrogen until needed. Myoblast cells were thawed at room temperature and subsequently grown in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (FBS) (GE Healthcare Hyclone Laboratories, Logan, UT), non-essential amino acids, Penicillin/Streptomycin, and Gentamicin (Life Technologies, Grand Island, NY). The cells were maintained in a 6% CO_2 humidified chamber at 37°C.

Cloning of the Dusp4 Gene Promoter

Fragments (500, 1000, and 2000 base pairs) of the Dusp4 regulatory region were amplified by PCR, sub-cloned into the pGemT-EZ vector (Promega), sequenced, and then moved into the pSEAP2-Basic (Clontech) reporter vector using the restriction enzymes Kpn1 and Nhe1. The primers used to amplify the Dusp4 promoter fragments are 2000-F 5′-GCG GTA CCC CGG TGA AAT CGG CCG AGA CTT GC-3′, 1000-F 5′-GCG GTA CCC CTG GAA AGA GGG ATG CGG AAA CTC G-3′, 500-F 5′-GCG GTA CCG CAC GAA GAA CGA GAT TAA GTC CGG C-3′, and reverse primer 5′-GCG CTA GCG CCG GCT CTC CCG GGG GAG GGA AGA G-3′. PCR reactions were performed with 1µg of genomic mouse DNA as well as 0.5µL Taq DNA polymerase according to the manufacture's protocol (Life technologies, Grand Island, NY). Thermocycler conditions for promoter amplification were one cycle at 94°C for 2 min followed by 30 cycles at 94°C for 45 sec, 55°C for 45 sec, and 72°C for 3 min.

Site-directed Mutagenesis

AP-2 Binding Element:

The Dusp4 promoter sequences were obtained from the Ensembl database (www.ensembl.org) and a putative activator protein 2 (AP-2) binding element located within the Dusp4 500bp regulatory region was mutated using the primer sequences: (F) 5′-GGT AGT AAT GAC TCT TCC GTG CCC CGG GAG AAG CAG GC -3′ and (R) 5′-GCC GGC TCT CCC GGG GCA CGG AAG AGT CAT TAC TAC C-3′. The mutagenesis reaction was performed using 100ng of the pSEAP2-Dusp4 Pro-500 reporter construct. Cycling conditions for mutagenesis were as follows: one cycle at 95°C for 30 sec followed by 18 cycles at 95°C for 30 sec, 55°C for 1 min, and 68°C for 12 min.

E-box Element:

The mouse Dusp4 promoter sequences were obtained from the Ensembl database (www.ensembl.org) and six consensus E-box enhancer elements were identified within the 2000bp promoter fragment of Dusp4; however, only one was conserved between human, rat, and mouse. Primers were designed to mutate the

single conserved E-box element: (F) 5′-CTT TTC CTT CGT ACC GAA CGT AGA CCT TAA CCG TC-3′ and (R) 5′-GAC GGT TAA GGT CTA CGT TCG GTA CGA AGG AAA AG-3′. The mutagenesis reaction was performed using 400ng of pSEAP2-Dusp4 Pro-2000 plasmid. Cycling conditions for mutagenesis were as follows: one cycle at 95°C for 30 sec followed by 18 cycles at 95°C for 30 sec, 55°C for 1 min, and 68°C for 16 min.

C-Terminal Catalytic Phosphatase Domain:

The C-terminal catalytic phosphatase domain of Dusp4 was mutated through site-directed mutagenesis according to the manufacture's protocol (Stratagene, La Jolla, CA). The amino acid that was altered was located within the conserved active site motif (HCxxGxxR) and primers were designed to alter a single nucleotide from a thymine to a guanine resulting in an amino acid change at position 284 from cysteine to glycine. The primers used were (F) 5′-GCT GGT TCA CGG CCA GGC CGG C-3′ and (R) 5′-GCC GGC CTG GCC GTG AAC CAG C-3′. The resulting Dusp4 clone was sequenced to confirm the correct mutation was introduced into the gene.

MuRF1 RING Domain:

The MuRF1 cDNA and protein sequences were downloaded from the PubMed database (www.Pubmed.org). The MuRF1 protein sequences for rat, mouse, and human were aligned using sequence alignment tools (http://www.ncbi.nlm.nih.gov). The aligned sequences were used to identify conserved amino acids for site-directed mutagenesis. Site-directed mutagenesis reactions were performed according to 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.

Amplification and Cloning of Dusp4 cDNA

Total RNA was isolated from homogenized C_2C_{12} mouse myoblast cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The purified total RNA (0.75-1.5 µg) was then reverse transcribed using Oligo-dT primers (500µg/mL), dNTP mixture (2.5mM), and nuclease-free sterile water. The mixture was incubated at 65°C for 5 minutes then incubated on ice for 5 minutes. The mixture was briefly centrifuged and First Strand Buffer (Invitrogen Corporation, Carlsbad, CA), DTT (0.1M), and Ribonuclease Inhibitor were added according to the 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 (Life Technologies, Grand Island, NY). The reaction was incubated at 37°C for 50 minutes followed by heat inactivation at 70°C for 15 minutes. The Dusp4 cDNA was then amplified via PCR using gene specific primers, cloned into the XhoI/BamHI restriction sites of pcDNA3.1-zeo plasmid and sequenced to confirm the absence of mutations. Primer sequences for Dusp4 cDNA amplification are: (F) GCC TCG AGG CTT ACT GCG TTT TGC CGG CGA CAT GG and (R) GCG GAT CCG GAT TTG GGG TCC AGA GAC GAG TCC.

Cloning the AP-1 Reporter Construct

The canonical sequence for the activator protein 1 (AP-1) response element 5′-TGACTCA-3′ was cloned into the pSEAP2-Promoter plasmid using the MluI restriction site following the manufacture's protocol (Sigma Aldrich, St. Louis, MO). Samples were sequenced to confirm insertion of the AP-1 response element.

Transfections

C_2C_{12} cells were plated at 50,000-75,000 cells per well in 12 well plates and grown to approximately 70% confluency prior to transfection. One hour prior to transfection the media was removed from the cells and fresh culture media was added to the wells. 1 µg of total DNA (1µg total DNA/well) was transiently transfected using Turbofect transfection reagent according to the manufacturer's instructions (Thermo

Scientific, Rockford, IL). The DNA mixture consisted of 250 ng/well of indicated reporter construct, 125 ng/well of TK-UAS-β-Galactosidase and 125-250 ng/well of expression plasmids (pcDNA3.1-Dusp4, pcDNA3.1-Dusp4-C284G mutant, pcDNA3.1-MuRF1, pcDNA3.1-MuRF1-RING-mutant, pcDNA3.1-MyoD1, and/or pcDNA3.1-myogenin), and pBluescript plasmid as filler DNA. At 24 hours post-transfection, media was aspirated and fresh media containing 2% FBS (differentiation media) was added to each well.

Reporter Gene Assays

Secreted alkaline phosphatase (SEAP) reporter gene assays were performed according to the manufacturer's protocol (Clontech, Mountain View, CA) 72 hours following the switch to low serum media (2% FBS). SEAP activity was measured in relative light units (RLU) and corresponds to the activity of the Dusp4 reporter gene. The resulting data values were normalized to β-galactosidase activity to correct for variations in transfection efficiency. Each condition was done in triplicate and error reflects the standard deviation from the mean.

Bioinformatics Analysis

The nucleotide sequence corresponding to the regulatory regions of mouse, rat, and human *Dusp4* (Ensembl Transcript ID: ENSMUSG00000031530) from -2000 through the first exon 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 Dusp4 were downloaded from the Ensembl database and aligned and shaded as described for the nucleotide sequences above.

Protein Purification and Western Blot Analysis

C_2C_{12} cells were plated and grown in 10 cm plates and harvested over a time course of 12 days, from proliferating myoblasts to differentiated myotubes. Following harvest, the cells were lysed using 500µL of Universal Lysis Buffer (ULB) [50mM Tris, 150mM NaCl, 50mM NaF, 1mM PMSF, 1mM DTT, 10mM β-glycerophosphate, 2mM Sodium Molybdate, and a protease inhibitor cocktail]. The cells were incubated on ice for 30 minutes and centrifuged at 4°C for 5 minutes at 18,000 × g. The homogenates were stored at -80°C until needed. Protein concentrations were determined using a Bradford protein assay method according to the manufacturer's protocol (BioRad, Hercules, CA). Total protein (100-150µg) was separated on a 9% SDS-PAGE gel and then transferred overnight to a polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Billerica, MA). The membrane was Ponceau S stained to ensure efficient protein transfer was achieved before probing. The membrane was allowed to block for one hour in a solution of 5% milk + 0.05% Tween in Tris Buffered Saline (TTBS). Following blocking, the membrane was probed with protein-specific antibodies for Dusp4, MyHC, and α-tubulin (S-18, rabbit polyclonal; F59, mouse monoclonal; and DM1A, mouse monoclonal, Santa Cruz Biotechnology, Inc.) at a 1:500 dilution for one hour with shaking at room temperature. The membrane was washed 3X with 1X TTBS, incubated with secondary antibody at a 1:5000 dilution for one hour at room temperature, and then washed 4X with 1X TTBS. The membrane was incubated for 2 minutes in Pierce ECL western blot substrate before imaging (Thermo Scientific, Rockford, IL). Imaging was carried out using X-ray film with Kodak developing and fixing solutions.

Human embryonic kidney 293T cells transfected with either pcDNA3.1-Dusp4 or pEGFP-C1-Dusp4 expression plasmids and then harvested upon confluency and lysed exactly as described for C_2C_{12} cells. Total protein (25µg-75µg) was separated on a 9% SDS-PAGE gel and analyzed as described above.

Confocal Fluorescence Microscopy

C_2C_{12} cells were plated onto glass cover slips in 6 cm plates. The cells were washed with 1X PBS and fresh media was added 1 hour prior to transfection. Cells were transfected with either the empty pEGFP-C1 vector or pEGFP-Dusp4 using Turbofect transfection reagent (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol. The cells were washed 2X with 1X PBS ~18-24 hours post-transfection, fixed with 4% paraformaldehyde in 0.1M sodium cacodylate, pH 7.6, stained with Draq5 far red fluorescent dye (Biostatus, Leicestershire, UK) and mounted with Vectashield Hardset Mounting Medium (Vector Laboratories, Burligame, CA). Images shown were taken with an Olympus Fluoview FV-1000 confocal Fluorescent microscope with either a Super Apochromat UPLSAPO 20X objective or a Super Apochromat UPLSAPO 60X-water objective. The GFP and Draq5 images were merged with open access ImageJ software with the Open Microscopy Environment Bio-formats software plugin tool.

Statistical Analysis

All conditions were performed in triplicate and data are presented as the mean ± the standard deviation. Statistical analysis was performed by using XL-STAT software program for either a one-way analysis of variance (ANOVA) for comparison of data to control with an REGW post-test or a two-way ANOVA to compare data between groups. Values were considered significant at a P-value ≤ 0.05.

Results

Characterization of the Dusp4 Proximal Promoter

Our first objective was to characterize the transcriptional activity and regulation of Dusp4 in skeletal muscle cells. To that end, segments of the Dusp4 proximal promoter region were cloned into the pSEAP2-Basic reporter plasmid. The activity, in relative light units (RLU), of the Dusp4 reporter constructs were then compared to the activity of the empty pSEAP2-Basic reporter, which contained no regulatory machinery and was used as a negative control. These experiments show that the Dusp4 promoter fragments are active in muscle cells and can be used to evaluate the regulation of this gene in

cell culture. The 500bp regulatory region was the most active in cell culture, but the 1000bp and 2000bp fragments also had significant activity as well (Figure 9). A one-way ANOVA was performed and a Ryan-Einot-Gabriel-Welsch Multiple Range (REGW) Test showed that there was significant difference between the activity of the Dusp4 promoter constructs and the pSEAP2-Basic reporter.

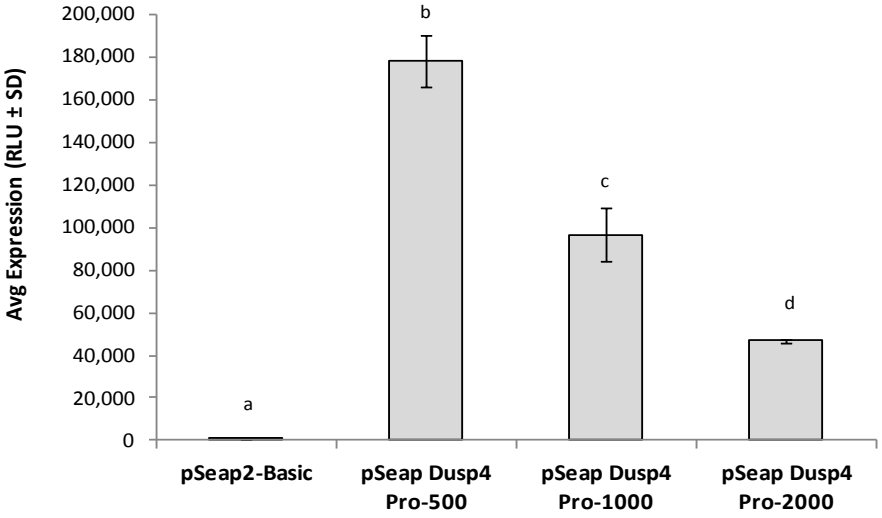


Figure 9. The activity of the Dusp4 promoter fragments compared to the empty pSEAP2-Basic plasmid in C_2C_{12} mouse myoblasts. C_2C_{12} cells transfected at 70-80% confluency with either the empty pSEAP2-Basic plasmid or the SEAP reporter plasmid fused to a 500bp, 1000bp, or 2000bp proximal promoter fragment of mouse Dusp4. Media was sampled 72 hours post- transfection and each condition was performed in triplicate. The samples were normalized with β-galactosidase activity to correct for variation in transfection efficiency. Error bars represent the standard deviation. An ANOVA was performed using corrected triplicate values. All pair-wise comparisons were significantly different (ANOVA; $F_{3,11}$=150.12; P<0.0001) (a) compared to pSEAP2-Basic, (b) compared to pSEAP-Dusp4 Pro-500, (c) compared to pSEAP-Dusp4 Pro-1000, (d) compared to pSEAP-Dusp4 Pro-2000.

MuRF1 Inhibits Dusp4 Reporter Gene Activity

The data obtained from comparing changes in gene expression between wild-type and MuRF1-null animals revealed differences in mRNA expression for Dusp4 following denervation-induced skeletal muscle atrophy (Figure 6). The blunted induction of Dusp4 mRNA in the MuRF1-null gastrocnemius tissue samples, at both 3 days and 14 days following sciatic nerve denervation, suggested that MuRF1 may be necessary for full activation of the gene subsequent to a traumatic neurogenic event. Therefore, we decided to look more closely at the regulation of Dusp4 in order to determine a mechanism by which MuRF1 might regulate Dusp4 expression.

In order to evaluate the transcriptional regulatory mechanisms governing the Dusp4 gene *in vitro*, 500bp, 1000bp, and 2000bp fragments of the proximal promoter were amplified from genomic mouse DNA and inserted into the pSEAP2 reporter plasmid. Recombinant Dusp4 reporter gene plasmids were then subjected to ectopic expression of MuRF1 and changes in reporter gene activity were evaluated. The Dusp4 500bp regulatory region showed significant repression in response to ectopic expression of MuRF1 (Figure 10A). A similar pattern of repression by MuRF1 was observed for the 1000bp and 2000bp Dusp4 reporter gene constructs (Figure 10B and C, respectively). The results using the Dusp4 reporter gene constructs suggest that MuRF1 is able to regulate the transcriptional activity of Dusp4 in C_2C_{12} cells.

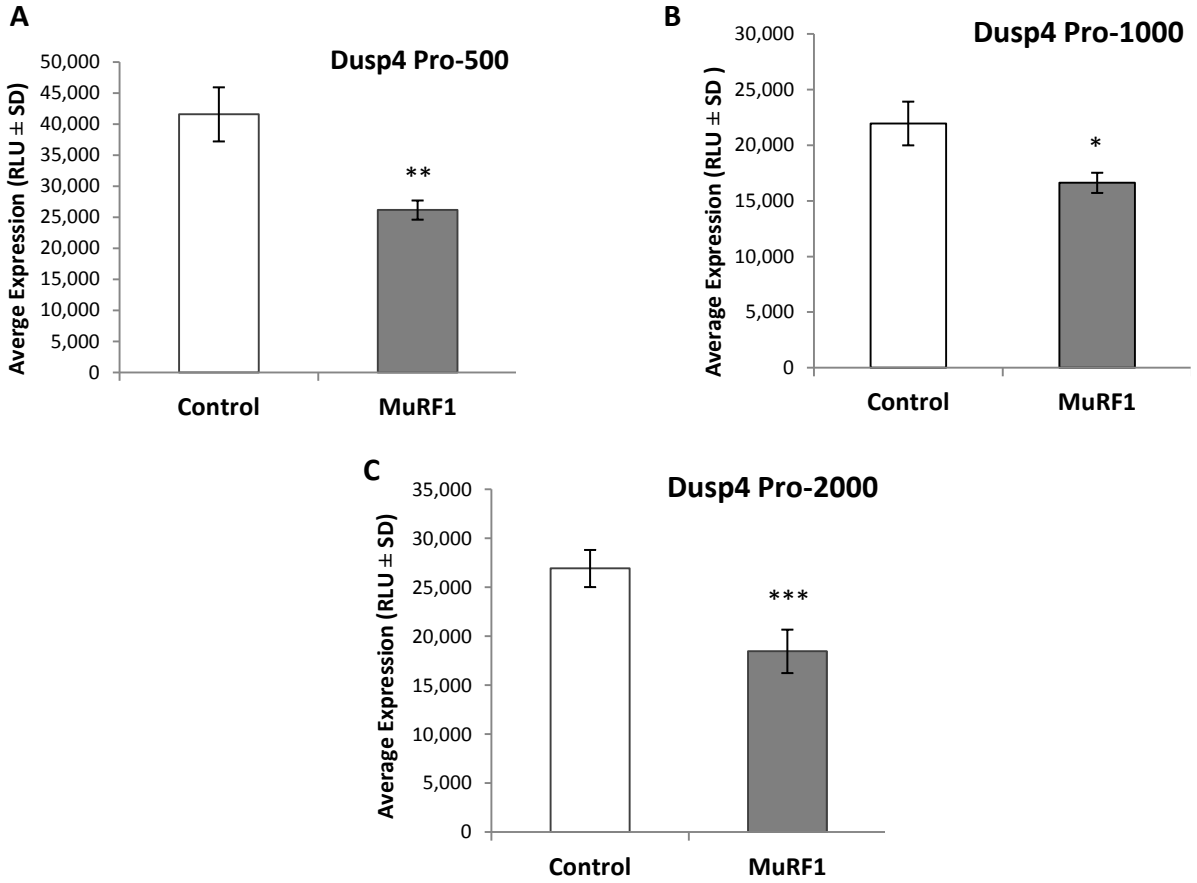


Figure 10. MuRF1 represses Dusp4 reporter gene activity. MuRF1 over-expression results in transcriptional repression of the (A) 500bp, (B) 1000bp, and (C) 2000bp Dusp4 reporter gene fragments at 72 hours post-transfection. C_2C_{12} cells were transfected at approximately 70-80% confluency with either Dusp4 500bp, 1000bp, or 2000bp promoter fragments fused to the pSEAP2-Basic reporter plasmid with or without a MuRF1 expression plasmid. The cells were changed to differentiation media (2% FBS) 24 hours following transfection. Samples of media were taken 72 hours after the switch to 2% media and measured for SEAP activity. The samples were normalized with β-galactosidase activity to correct for variation in transfection efficiency. Each condition was done in triplicate and error bars represent standard deviation (Two tailed T-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Mutagenesis of the Dusp4 AP-2 Binding Element Increases Dusp4 Reporter Gene Activity

There were differences regarding the effect of MuRF1 on Dusp4 between *in vitro* and *in vivo* experimentation models so it was necessary to take a closer look at the Dusp4 promoter region to locate potential transcription factor binding elements that may provide clarification about Dusp4 gene expression in response to MuRF1 over-expression in skeletal muscle. A particular region of interest located within the 500bp regulatory region of Dusp4 was the sequence 5′-CCCTCCCC-3′. This sequence corresponds to a canonical activator protein 2 (AP-2) binding region that may be important for neural development and restructuring in vertebrates [41], which provides Dusp4 with a potential function during neurogenic muscle atrophy. In addition to the AP-2 binding element, there were many additional areas of interest within the Dusp4 promoter region including CAATT box elements, a FoxO binding element, and multiple E-box elements (Figure 11).

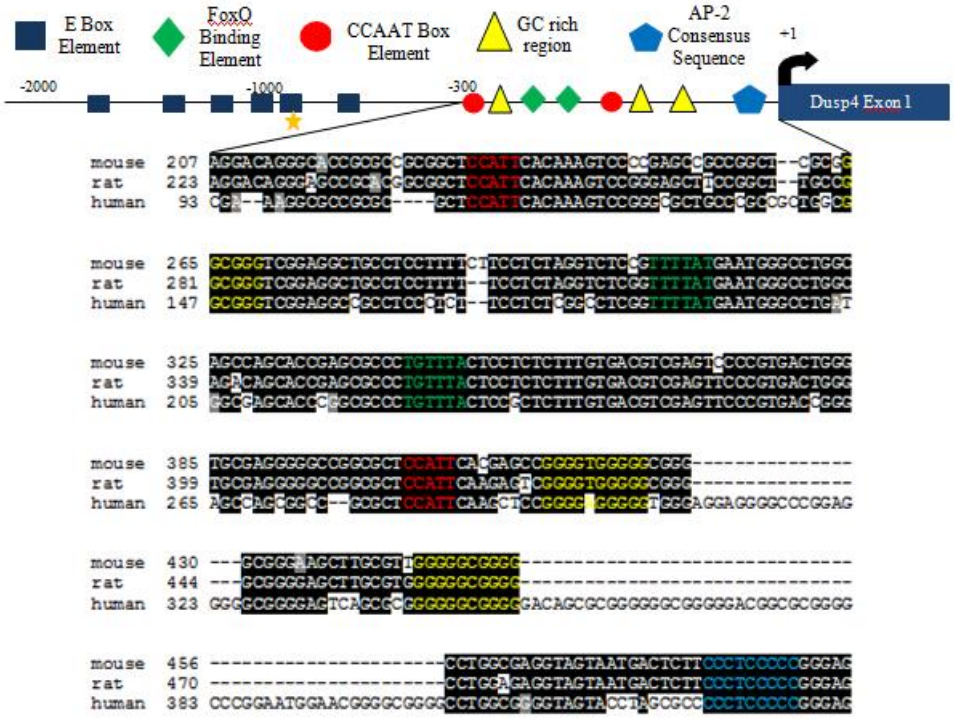


Figure 11. **Dusp4 proximal promoter alignment**. Promoter sequences from mouse, rat, and human Dusp4 genes (2000 base pairs upstream of the transcription start site (+1) through the first exon) were downloaded from the Ensembl database (www.ensembl.org) and aligned using the Clustal omega algorithm. Identical sequences for the indicated regions are highlighted in black. Approximate positions of potential transcription factor binding sites are indicated in the diagram and highlighted in the alignments: Muscle-specific E-box elements CANNTG (Dark blue square), CAATT box element (Red circle), GC rich region (yellow triangle), FoxO binding element TGTTTA (Green diamond), and an AP-2 element CCCTCCCCC (Blue pentagon).

Mutation of the AP-2 binding element resulted in increased Dusp4 Pro-500 reporter gene activity compared to the wild-type promoter. The increased Dusp4 activity observed in the AP-2 mutant was even more pronounced in late passage (P18) compared to earlier passage (P7) C_2C_{12} mouse muscle cells (Figure 12). The confluency of the cells was similar at the time of transfection so the increase in the pSEAP2-Dusp4 500bp promoter activity for late passage cells could be the result of differences in the cells as they age and will be investigated in future studies.

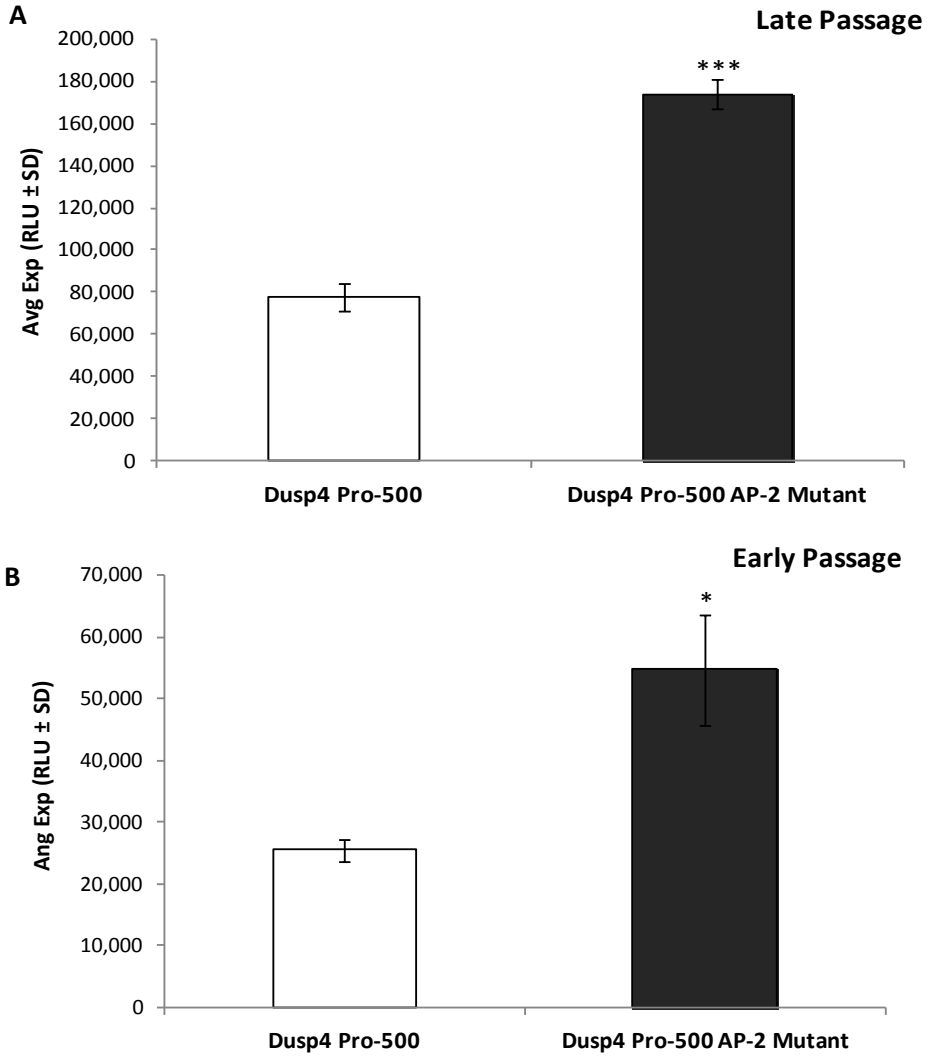


Figure 12. Dusp4 Pro-500 WT and Dusp4 Pro-500 AP-2 mutant gene reporter activity in C_2C_{12} mouse myoblasts. Overall activity for the two promoter fragments was greater in late passage cells (A) compared to early passage (B), while the AP-2 mutant had greater activity than the wild-type Dusp4 500bp promoter fragment. Cells were transfected at approximately 70-80% confluency and the SEAP assay was performed 72 hours post-transfection. The samples were normalized with β-galactosidase activity to correct for variation in transfection efficiency. Error bars represent standard deviation. A two tailed T-test was performed using the corrected triplicate data values (* $p < .05$, *** $p < .001$).

The effect of ectopic MuRF1 expression on the wild-type Dusp4 reporter was different than that observed for the cells transfected with the AP-2 mutant Dusp4 promoter. The AP-2 Dusp4 mutant reporter still appears to be regulated by MuRF1, however ectopic MuRF1 expression resulted in a 50.5% decrease in the Dusp4 Pro-500 AP-2 mutant promoter as compared to a 41.4% decrease in the WT Dusp4 (Figure 13). All pair-wise comparisons were statistically significant, with the exception of the wild-type pSEAP2-Dusp4 500bp reporter compared to the pSEAP2-Dusp4 AP-2 mutant Dusp4 500bp reporters in response to ectopic MuRF1 expression. The over-expression of MuRF1 in cells transfected with the AP-2 mutant Dusp4 promoter construct resulted in expression values back down to levels observed in the WT Dusp4 500bp control promoter series. This can be seen in Figure 13 however, this research study was not focused on comparing the differences between the WT untreated and the AP-2 mutant Dusp4 promoter in the presence of MuRF1 over-expression, although this could be evaluated in future studies.

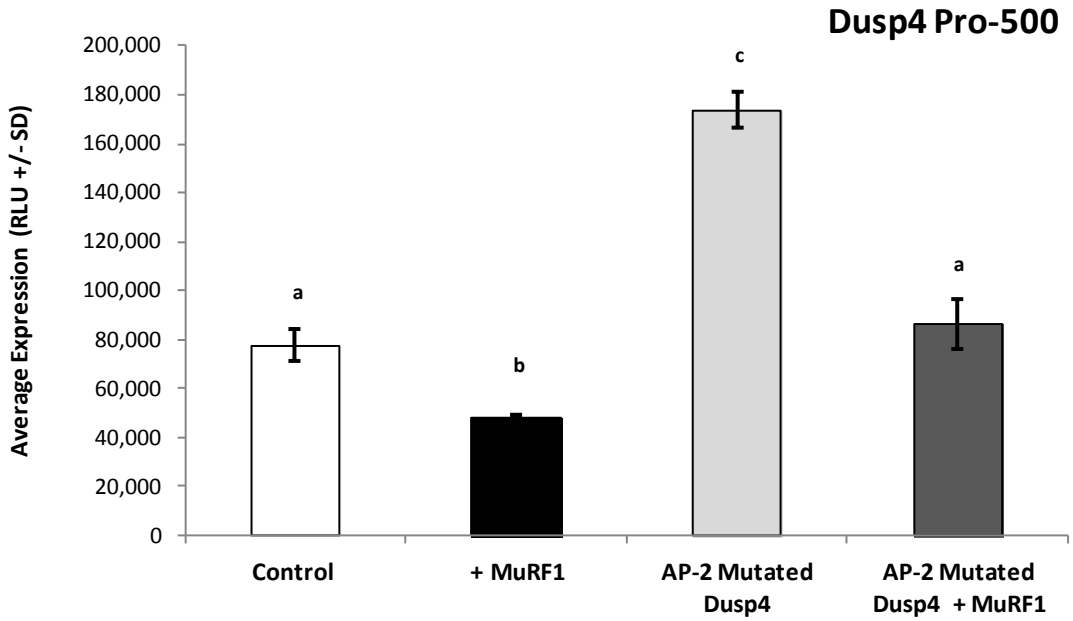


Figure 13. Dusp4 Pro-500 WT and Dusp4 Pro-500 AP-2 mutant gene reporter activity in response to ectopic MuRF1 expression in late passage (P18) C_2C_{12} mouse myoblasts. The WT-Dusp4 Pro-500 reporter and Dusp4 Pro-500 AP-2 mutant reporter were evaluated in response to ectopic MuRF1 expression. Both promoter fragments show repression in response to MuRF1 over-expression. Cells were transfected at approximately 70-80% confluency and the assay was performed 72 hours following transfection. The samples were normalized with β-galactosidase activity to correct for variation in transfection efficiency. Error bars represent standard deviation. A two-way ANOVA was performed using the corrected triplicate data values. All pair-wise comparisons were significantly different except Dusp4 Pro-500 control and AP-2 mutated Dusp4 Pro-500 + MuRF1 (ANOVA; $F_{3,11}$ Control=123.02; P<0.0001, ±MuRF1=146.80; P<0.0001, WT Dusp4/AP-2 Mutant Dusp4=190.65; P<0.0001, MuRF1/AP-2=31.59; P<0.005) (a) compared to pSEAP-Dusp4 Pro-500, (b) compared to pSEAP-Dusp4 Pro-500 + MuRF1, (c) compared to pSEAP-Dusp4 Pro-500 AP-2 mutant.

MuRF1 RING Domain Mutant Fails to Repress Dusp4 Reporter Gene Activity

As previously discussed, ectopic expression of MuRF1 represses Dusp4 reporter gene activity. Therefore, we next decided to determine if the E3 ubiquitin ligase activity is necessary for MuRF1-mediated repression of Dusp4 reporter gene activity. In addition to exposing myoblasts to wild-type MuRF1, a MuRF1 mutant was generated to try to identify the mechanism by which MuRF1 may act to alter the transcriptional machinery of certain genes. The MuRF1 protein contains a RING-Finger domain that is required for catalytic activity of this E3 ubiquitin ligase. RING domains contain a zinc-finger that has a consensus sequence of $C\text{-}X_2\text{-}C\text{-}X_{[9\text{-}39]}\text{-}C\text{-}X_{[1\text{-}3]}\text{-}H\text{-}X_{[2\text{-}3]}\text{-}C\text{-}X_2\text{-}C\text{-}X_{[4\text{-}48]}\text{-}C\text{-}X_2\text{-}C$, C stands for a conserved cysteine residue, H stands for a conserved histidine residue, and X may be any amino acid. Zinc ions are known to interact with cysteine and histidine residues forming the RING-Finger domain, which interacts with ubiquitination enzymes and may help facilitate the ubiquitination process [42]. Mutations were introduced into the RING domain to determine how MuRF1 may function to regulate transcription (Figure 14). A catalytically dead MuRF1 protein was generated by mutating two of the cysteine residues to serine (C39S and C44S) of the RING domain, which have been shown to be important for full catalytic activity [42].

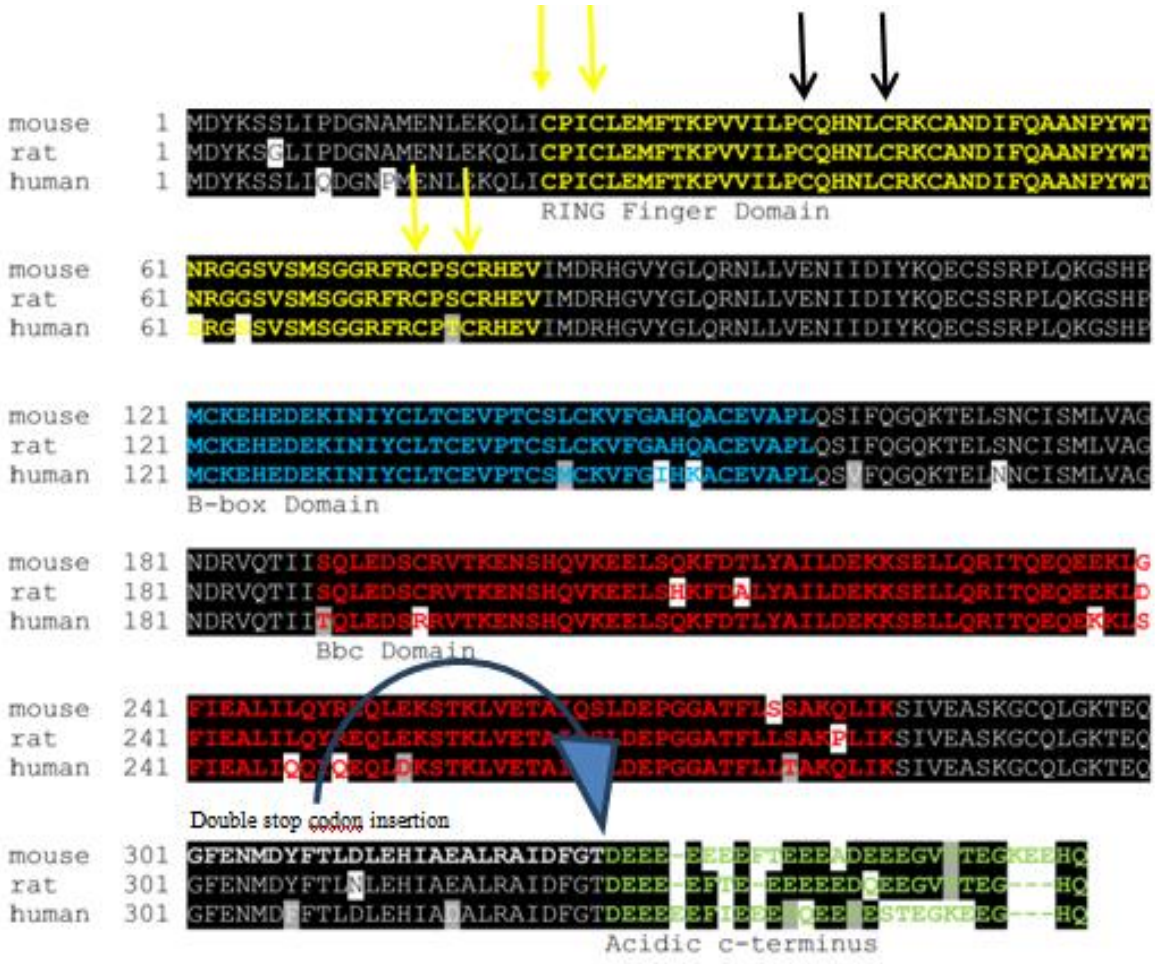


Figure 14. Alignment of the MuRF1 amino acid sequence. MuRF1 amino acid sequences from mouse, rat and human were obtained from the PubMed database (www.PubMed.org). The proteins were then aligned using sequence alignment tools (http://www.ncbi.nlm.nih.gov). Yellow letters represent the RING (Really Interesting New Gene) Finger domain, yellow and black arrows show the relevant cysteine residues located within the RING finger domain, blue letters represent the Bcb (B-box c-terminus) domain, and green letters represent the acidic c-terminal region. (The black arrows indicate the location of the mutated cysteine amino acids.)

Reporter assays were performed using wild-type MuRF1 or the MuRF1-RING mutant to assess their ability to regulate the 500bp, 1000bp, and 2000bp proximal promoter regions of Dusp4. The data obtained from the reporter gene assays show that WT-MuRF1 represses the Dusp4 promoters while the MuRF1-RING mutant lost the ability to fully repress the Dusp4 reporter constructs (Figure 15).

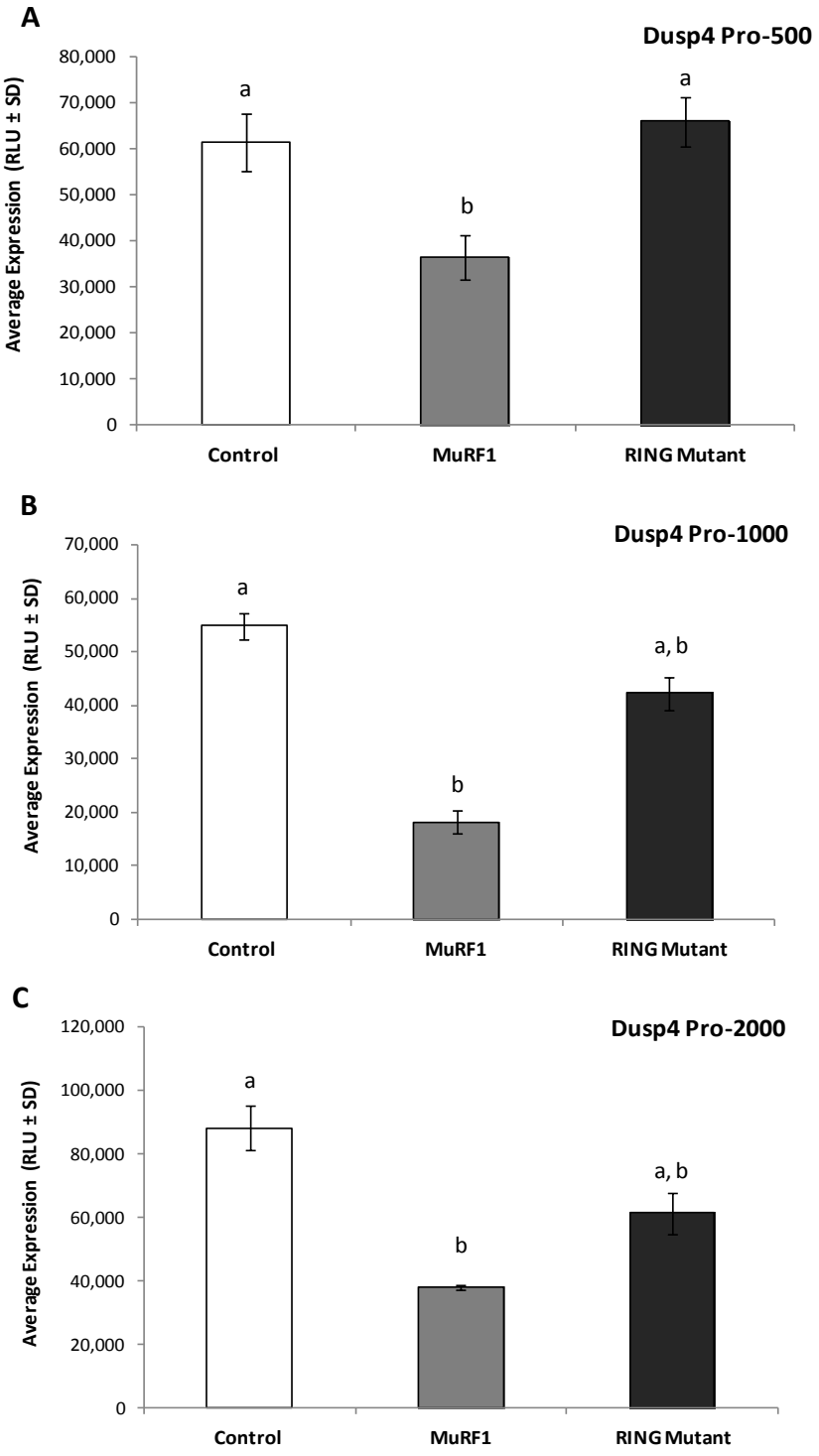
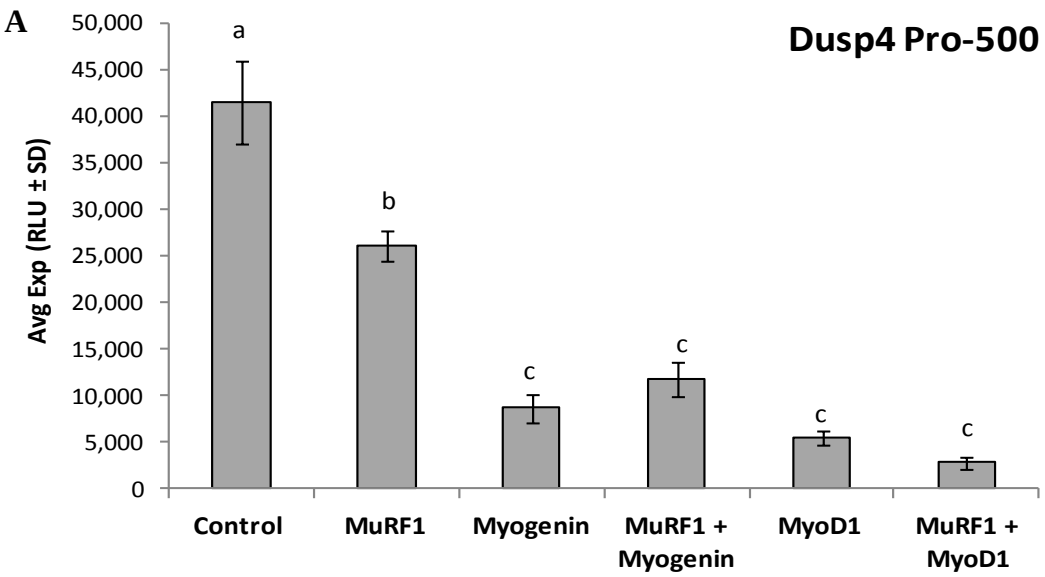


Figure 15. MuRF1, but not the MuRF1-RING mutant, represses Dusp4 reporter gene activity. The Dusp4 promoters were negatively regulated by the addition of MuRF1; however, there was a loss of repression observed with the MuRF1-RING mutant. (A) Dusp4 Pro-500, (B) Dusp4 Pro-1000, and (C) Dusp4 Pro-2000. C_2C_{12} cells were transfected at 70% confluency before being switched to low serum (2% FBS) media. Samples were taken 72 hours following media change and performed in triplicate. The samples were normalized with β-galactosidase activity to correct for variation in transfection efficiency. Graphs represent mean values ± SD. Statistical significance was evaluated using ANOVA and values were considered significant when $P \leq 0.05$ [a) compared to control, b) compared to WT-MuRF1].

Myogenic Regulatory Factors Inhibit Dusp4 Reporter Gene Activity

Located within the murine Dusp4 promoter are six putative canonical E-box elements that may be bound by myogenic regulatory factors (MRF), such as myogenin and MyoD1. MRFs are muscle-specific transcription factors that are dramatically upregulated in response to neurogenic atrophy. Considering that the Dusp4 proximal promoter has MRF binding elements, we next decided to determine if MRFs can modulate Dusp4 reporter gene activity in cultured muscle cells. In addition, we also assessed the effect of ectopic co-overexpression of MuRF1 and MRFs on Dusp4 reporter gene activity. The results show that myogenin and MyoD1 repress Dusp4 reporter gene activity when over expressed alone for all promoter fragments evaluated (Figure 16). Interestingly, the combination of MuRF1 and myogenin overexpression resulted in a loss of repression compared to overexpression of myogenin alone; however, over-expressing both MuRF1 and MyoD1 together resulted in a cooperative repressive effect that was greater than the addition of either MuRF1 or MyoD1 alone (Figure 16). An analysis of variance (ANOVA) was performed on the corrected triplicate values: Dusp4 Pro-500 ANOVA; $_{F5,\ 17}$ =145.96; P<0.0001, MuRF1=0.00; P=0.9754, myogenin=362.58; P<0.0001, MyoD1=581.37; P<0.0001, MuRF1+myogenin=55.51; P<0.0001, MuRF1+MyoD1=25.32; P=0.003. Dusp4 Pro-1000 ANOVA; $_{F5,\ 17}$ =101.10; P<0.0001, MuRF1=3.39; P=0.0904, myogenin=333.03; P<0.0001, MyoD1=349.77; P<0.0001, MuRF1+myogenin=23.81; P=0.0004, MuRF1+MyoD1=0.01; P=0.9116. Dusp4 Pro-2000 ANOVA; $_{F5,\ 17}$ =134.47; P<0.0001, MuRF1=0.50; P=0.4951, myogenin=332.07; P<0.0001, MyoD1=541.35; P<0.0001, MuRF1+myogenin=59.79; P<0.0001, MuRF1+MyoD1=8.20; P=0.0142. Statistical analysis revealed there were no significant differences between the pair-wise comparisons upon the addition of ectopic MuRF1 overexpression alone and the combination of ectopic MuRF1+MyoD1 co-overexpression for each Dusp4 promoter that was evaluated. This was not a direct focus of the study and may be due to the fact that upon addition of MuRF1 or MyoD1 alone, repression of the control promoter was observed. Interestingly, MuRF1 reversed the repression when co-transfected with myogenin, resulting in a null difference in the statistical analysis (Figure 16 A-C).


A
Dusp4 Pro-500
Avg Exp (RLU ± SD)
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15,000
10,000
5,000
0
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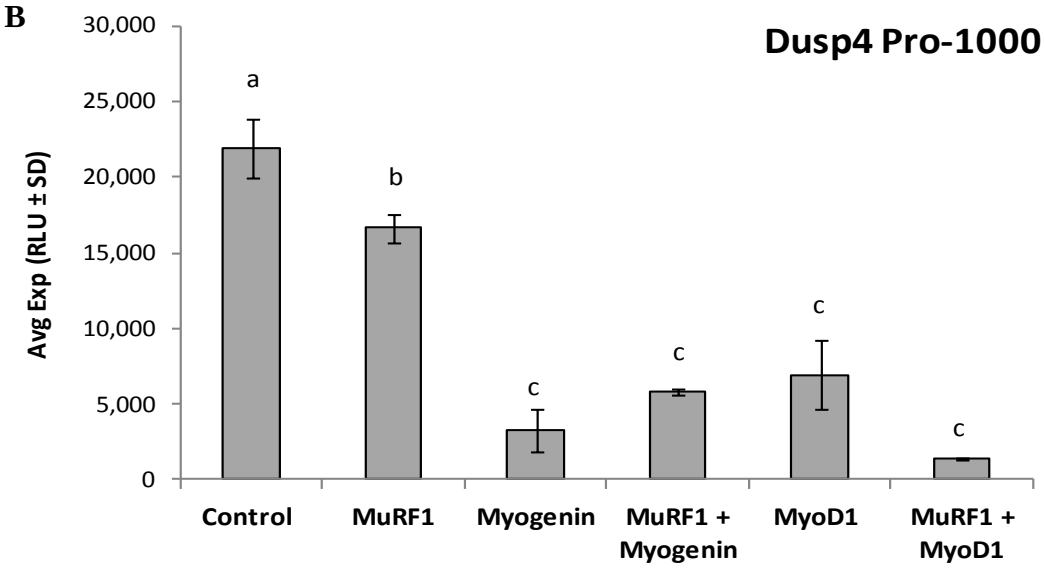
c
Control
MuRF1
Myogenin
MuRF1 + Myogenin
MyoD1
MuRF1 + MyoD1


B
Dusp4 Pro-1000
Avg Exp (RLU ± SD)
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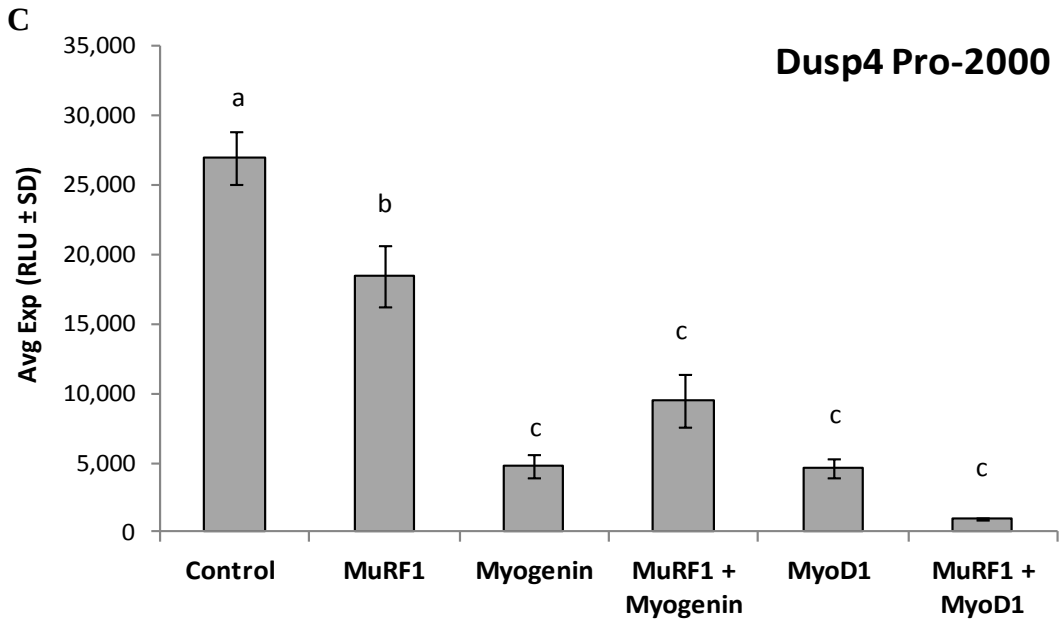

Figure 16. Myogenic regulatory factors inhibit Dusp4 reporter gene activity in C_2C_{12} mouse myoblasts. The (A) 500bp, (B) 1000bp, and (C) 2000bp fragments of the Dusp4 promoter were fused with the pSEAP2-Basic reporter. C_2C_{12} mouse myoblasts were transfected at approximately 70-80% confluency before being switched to low serum media (2% FBS). Samples were taken 72 hours following media change and performed in triplicate. The samples were normalized with β-galactosidase activity to correct for variation in transfection efficiency. Graphs represent mean values ± SD. Statistical significance was evaluated using an ANOVA test of the corrected triplicate values. All pair-wise comparisons were significantly different except Dusp4 Pro-500, Pro-1000, and Pro-2000 following the ectopic addition of MuRF1 alone or when MuRF1+MyoD1 treatment was added. Data was considered significant when $P \leq 0.05$ [a) compared to control, b) compared to MuRF1, c) different from control or MuRF1].

Conserved E-box in the Dusp4 Promoter is Necessary for Full Promoter Activity

In total there are six canonical E-box elements located throughout the murine Dusp4 2000bp promoter region (Figure 17); however, only one was conserved between mouse, rat, and human (Table 1). The presence of E-box elements located within the proximal promoter suggests Dusp4 may be important in muscle tissue. Experiments were performed to see how mutating the single conserved E-box (CAAATG→CGAACG) located within the Dusp4 promoter would alter Dusp4 reporter gene activity in C_2C_{12} cells.

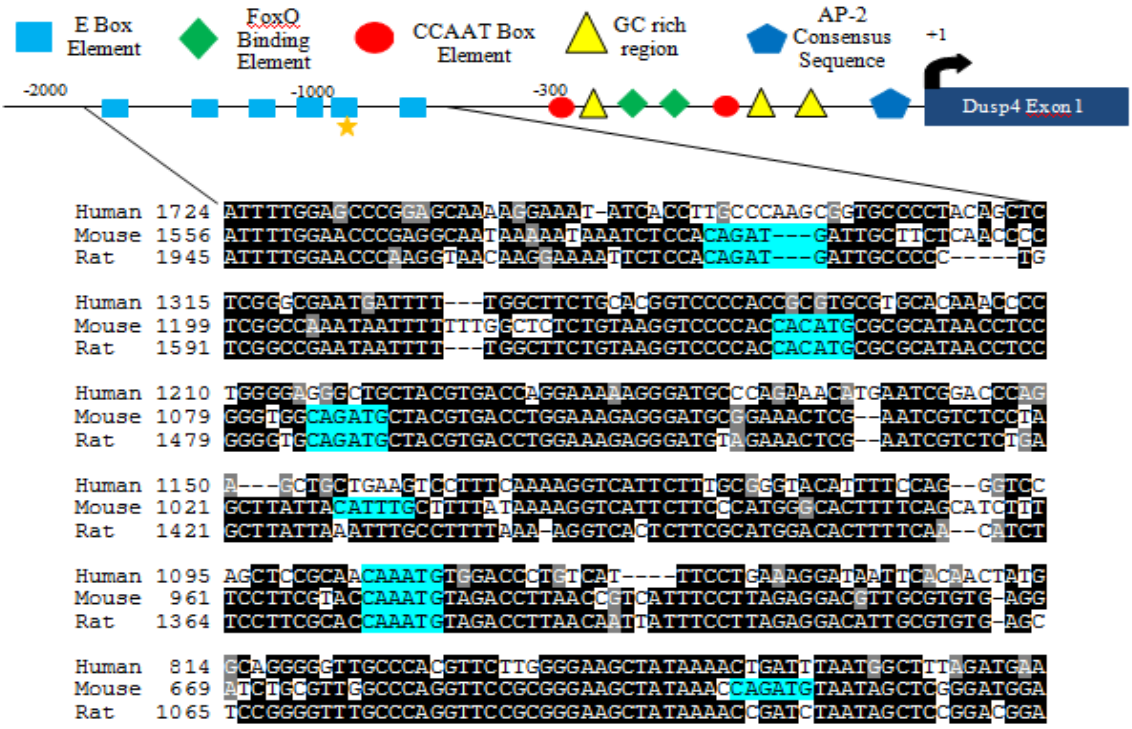

Figure 17. Dusp4 promoter sequence alignment comparing the location of the six E-box elements between human, mouse, and rat. The nucleotide sequence corresponding to the regulatory regions of mouse, rat, and human *Dusp4* (Ensemble Transcript ID: ENSMUSG00000031530) from -2000 through the first exon were downloaded from the Ensemble database (www.ensemble.org), aligned using ClustalW2 alignment tool on the EMBL website (http://www.ebi.ac.uk/Tools/msa/clustalw2/), followed by Boxshade analysis of the ClustalW2 alignment output data (http://www.ch.embnet.org/software/BOX_form.html).

E box Sequence	E box location
CAGATG	-1519
CACATG	-1158
CAGATG	-1072
CATTTG	-1012
CAAATG	-950
CAGATG	-631

Table 1. Location of the E-box elements within the 2000bp regulatory region of Dusp4. There are no known consensus E-box enhancer sequences within the 500bp region, 3 are located in the 1000bp region, and 6 total are located within the Dusp4 2000bp promoter. The highlighted red sequence corresponds to the conserved E-box located in the Dusp4 promoter.

Site-directed mutagenesis of the conserved E-box enhancer element within the Dusp4 2000bp regulatory region resulted in nearly a complete loss of promoter activity compared to the wild-type Dusp4 Pro-2000 reporter gene (Figure 18). There are 5 other E-boxes that remained intact located within this region, but it

is apparent that this conserved E-box sequence is necessary for the full functionality of the Dusp4 promoter in skeletal muscle cells. It is unknown if mutating the other E-box sequences would have a similar effect on gene activity, but it will be evaluated in future studies.

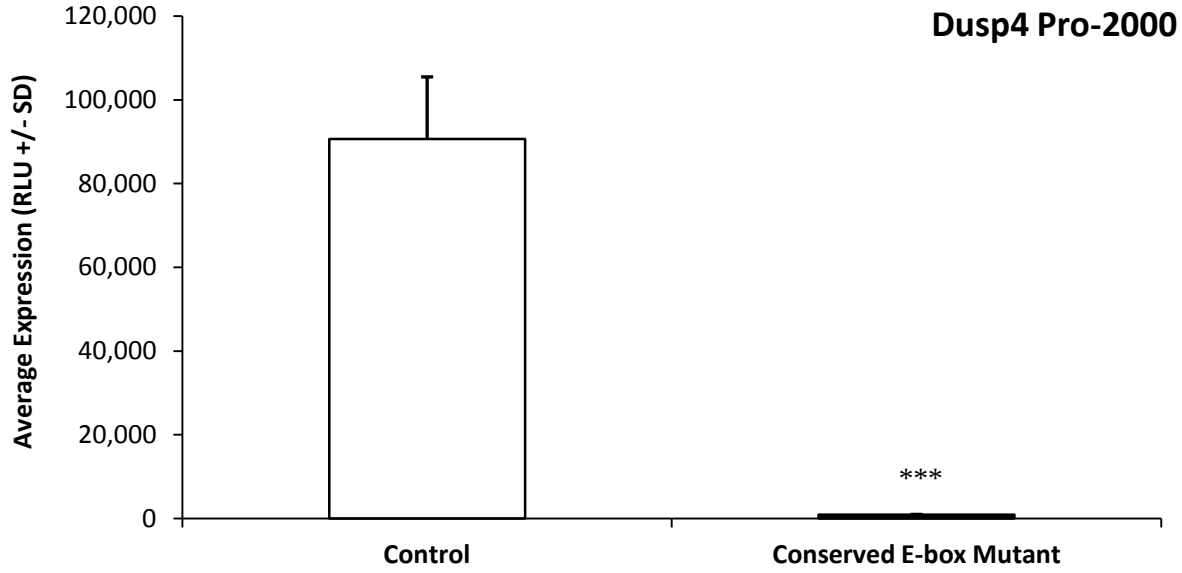

Figure 18. Conserved E-box mutagenesis abolished activity of the Dusp4 reporter gene in C_2C_{12} myoblasts. C_2C_{12} mouse myoblasts were transfected with either the WT pSEAP2-Dusp4 Pro-2000 or the pSEAP2-Dusp4 Pro-2000 E-box mutant reporter constructs. Cells were transfected at approximately 80% confluency and switched to low serum (2% FBS) media 24 hours post-transfection. SEAP analysis was performed 72 hours following media change. The samples were normalized with β-galactosidase activity to correct for variation in transfection efficiency. Conditions were conducted in triplicate and graphs represent mean values ± SD. Statistical significance was evaluated using a two-tailed T-test. Data was considered significant when $P \leq 0.05$ (*** $P \leq 0.001$).

Characterization of Dusp4 Protein Expression in Mouse Myoblasts

Experiments using the pSEAP reporter plasmid revealed that the Dusp4 promoter is active in C_2C_{12} mouse myoblasts. It was next important to determine at what point during the proliferation and differentiation phases that the Dusp4 protein was being expressed in C_2C_{12} cells. A western blot was performed using C_2C_{12} cell lysates harvested from proliferation through differentiation phases and 150µg of total protein lysate was separated on a SDS-PAGE gel and then probed for Dusp4. Dusp4 was only detectable during the proliferation phase in C_2C_{12} cells. Following a media change to induce differentiation, the signal for Dusp4 was reduced and was not observed in the developing and differentiated myotubes (Figure 19).

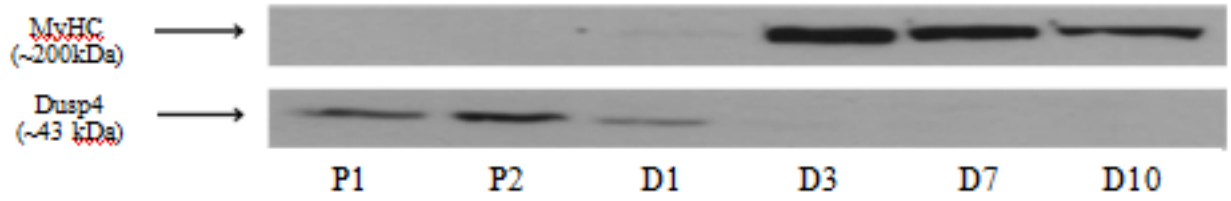

Figure 19. Western Blot for Dusp4 from C_2C_{12} protein isolates over a 12 day time course. Dusp4 was expressed at the protein level in C_2C_{12} cells. Western blot analysis was performed using an antibody against Dusp4 on protein homogenates from proliferating (P) and differentiated (D) C_2C_{12} cells harvested over a 12 day differentiation time course. Cells were maintained in proliferation media (10% serum) and harvested at 1 and 2 days post-plating and the remaining cells were then switched to differentiation media (2% serum) and harvested at 1, 3, 7 and 10 days post-media change. Myosin Heavy Chain (MyHC) was analyzed as a marker for skeletal muscle cell differentiation.

Identifying the Sub-cellular Localization of Dusp4 in C_2C_{12} Cells

Previous research has identified Dusp4 as a type-I dual-specificity phosphatase that has the functional ability to remove a phosphate from either threonine or tyrosine residues of MAP kinases. The type-I distinction qualifies this protein as having a nuclear sub-cellular localization; however, there has been very little work performed on Dusp4 in skeletal muscle tissue so we decided to determine where in muscle cells Dusp4 is localized. Since information from the western blot revealed that Dusp4 protein expression was greatest when cells were fully confluent, but had not yet begun to fuse and form myotubes, the cells were transfected with GFP-Dusp4 and imaged during the peak of proliferation. In order to confirm sub-cellular location, Draq5 was used to identify the nuclear region of the cells. The results agreed with information from previous research that Dusp4 was indeed a nuclear localized dual-specificity phosphatase as the fluorescence that was observed was exclusively nuclear (Figure 20).

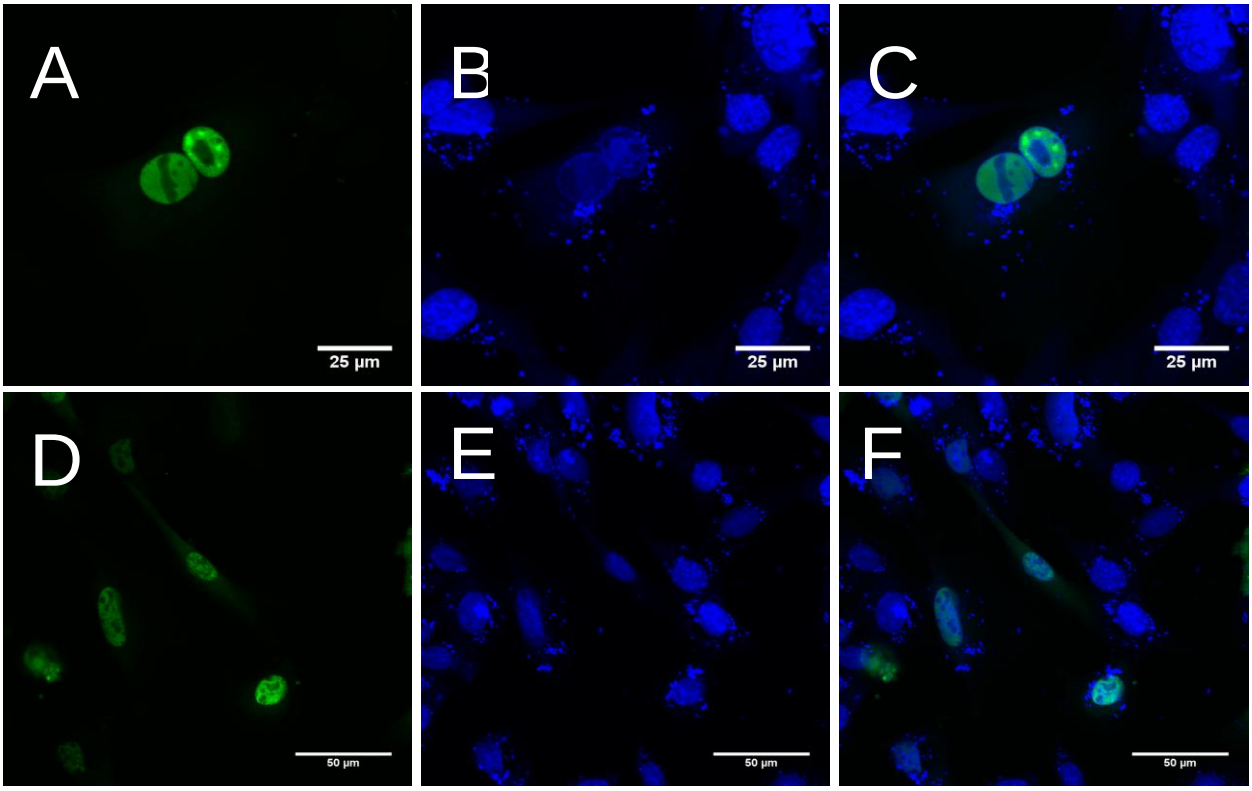

Figure 20. GFP-Dusp4 is localized to the nucleus of C_2C_{12} mouse myoblast cells. Dusp4 cDNA was fused with GFP and the plasmid was transfected into C_2C_{12} cells plated on glass cover slips in 6 cm culture dishes at approximately 60% confluency. GFP-tagged Dusp4 protein expression in C_2C_{12} cells (A and D), Draq5 was used to identify the nuclear region of the cells (B and E), and the merged images of GFP-Dusp4/Draq5 show nuclear localization of Dusp4 (C and F).

Dusp4 Negatively Regulates the MAPK Signaling Cascade in Skeletal Muscle Cells

Dusp4 is believed to dephosphorylate different MAPK's (p38, ERK, and JNK) depending on the cell type being analyzed. In skeletal muscle, there has been virtually no work done to identify the functional ability of Dusp4 to regulate the MAP kinase cascade. In order to verify that Dusp4 was able to inactivate the MAPK pathway, an AP-1 reporter system was created. AP-1 was chosen because it has been identified as an ERK-activated transcription factor. An AP-1 canonical consensus element was cloned into the pSEAP2-Promoter reporter plasmid and was tested to confirm that the reporter was active in C_2C_{12} cells by comparing its activity to that of the empty pSEAP2-Promoter plasmid (Figure 21). The experiment was conducted in both low serum (2%) and high serum (10%) media to determine the most efficacious

protocol for evaluating AP-1 reporter activity in mouse muscle cells. Similar trends were observed under both media conditions and the reporter plasmids were determined to be active in myoblasts (Figure 21).

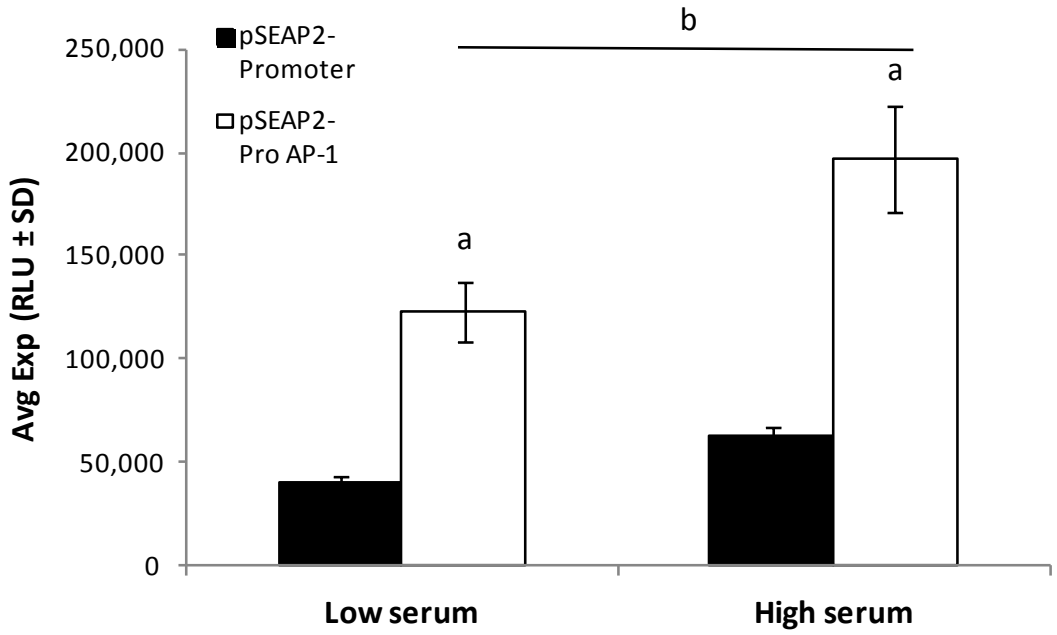

Figure 21. AP-1 reporter plasmid is active in C_2C_{12} cultured myoblasts. C_2C_{12} cells were transfected with either pSEAP2-Promoter or pSEAP2 AP-1 reporter plasmid at 70-80% confluency. Fresh media, either 10% FBS or 2% FBS was added to the cells 24 hours post-transfection. Samples were taken 48 hours following media change and performed in triplicate. The samples were normalized with β-galactosidase activity to correct for variation in transfection efficiency. Graphs represent mean values ± SD. Statistical significance was evaluated using an ANOVA and values were considered significant when $P \leq 0.05$ [a) compared within groups $P \leq 0.01$, b) compared between groups $P \leq 0.01$].

Since the AP-1 reporter system was determined to work in C_2C_{12} cells, the next step was to observe how the ERK-activated transcription factor would behave in response to the overexpression of Dusp4. Cells were transfected with the AP-1 reporter alone or in combination with an expression plasmid containing Dusp4 cDNA. Overexpression of Dusp4 resulted in reduced activity of the AP-1 reporter system by nearly 50%, but did not completely abolish activity (Figure 22).

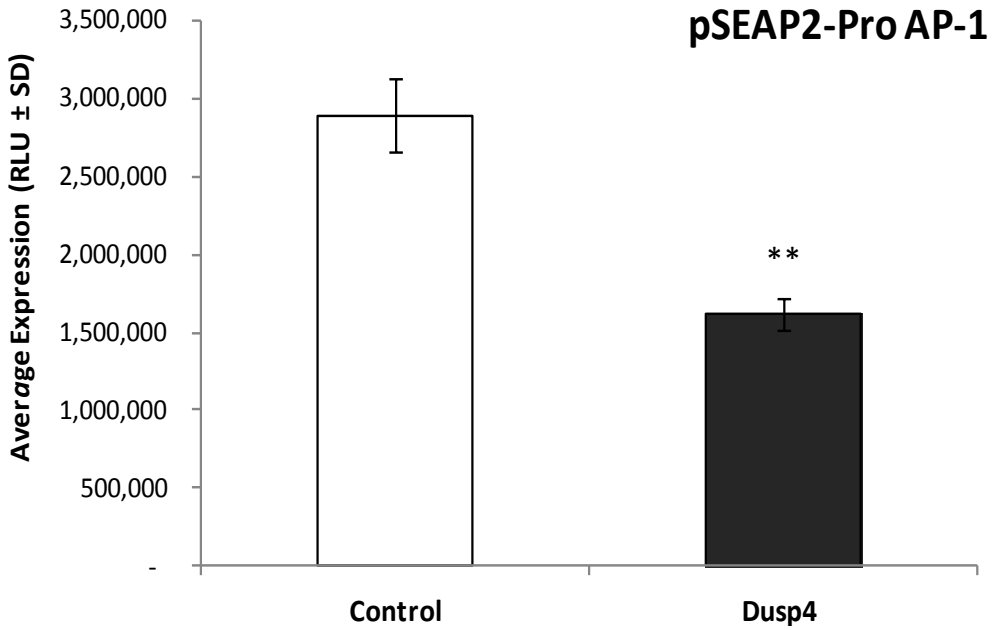

Figure 22. AP-1 reporter is repressed by ectopic Dusp4 expression. C_2C_{12} myoblasts were transfected at approximately 60% confluency with either the pSEAP2 AP-1 reporter alone or in conjunction with an expression plasmid containing Dusp4-cDNA. The media was changed 24 hours after plating to 2% FBS media and samples were taken 72 hours after media change and performed in triplicate. The samples were normalized with β-galactosidase activity to correct for variation in transfection efficiency. Graphs represent mean values ± SD. Statistical significance was evaluated using a two tailed T-test and values were considered significant when $P \leq 0.05$ (** $P \leq 0.01$).

Mutagenesis of the Dusp4 Phosphatase Domain and the MAPK Binding Domain

The results presented above show that addition of exogenous Dusp4 resulted in reduced AP-1 reporter activity *in vitro*, so a phosphatase domain mutant was generated and used to determine if AP-1 reporter repression requires Dusp4 phosphatase activity. The amino acid that was altered was located within the conserved active site motif (HCxxGxxR) and was generated by substituting a single nucleotide which resulted in an amino acid change from cysteine to glycine C→284→G at that residue (Figure 23).

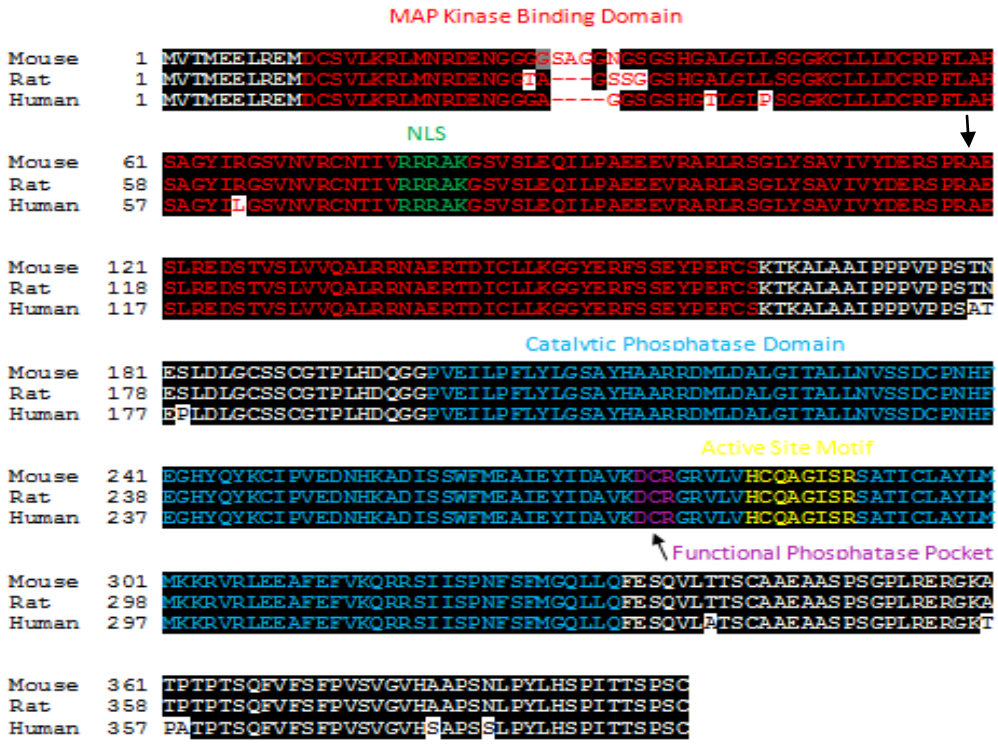

Figure 23. Alignment of Dusp4 Protein. Dusp4 protein sequences from mouse, rat and human were downloaded from the PubMed database (www.PubMed.org) and aligned using Clustal Omega sequence alignment tools (http://www.ebi.ac.uk/Tools/msa/clustalo/). The Dusp4 protein contains a MAP kinase binding domain (red), a nuclear localization signal (NLS) (green), and the catalytic phosphatase domain (blue). The highlighted yellow region indicates highly conserved amino acids of the active site of the phosphatase domain and the purple DCR motif shows the functional pocket in which dephosphorylation occurs.

After site-directed mutagenesis was performed on Dusp4, the mutant constructs were compared to the wild-type Dusp4 to evaluate how the loss of catalytic activity would affect the ability of Dusp4 to repress the AP-1 reporter system. Dusp4 mutants 2 and 4 contained only the expected mutation located within the catalytic domain and the over-expression of those mutants resulted in a loss of repression by Dusp4 on the pSEAP2 AP-1 reporter system (Figure 24). In addition to the C→G mutation within the C-terminal domain of Dusp4, a second mutation was introduced into Dusp4 mutant 3 which altered a single nucleotide (adenine→guanine) located within the N-terminal MAPK binding domain. This lone nucleotide change resulted in an amino acid switch at position 119 from an alanine (non-polar) to a serine (polar) residue. The result was that this Dusp4 mutant regained the ability to repress the AP-1 reporter system in a level similar to wild-type Dusp4 by likely acting in a dominant-negative fashion (Figure 24).

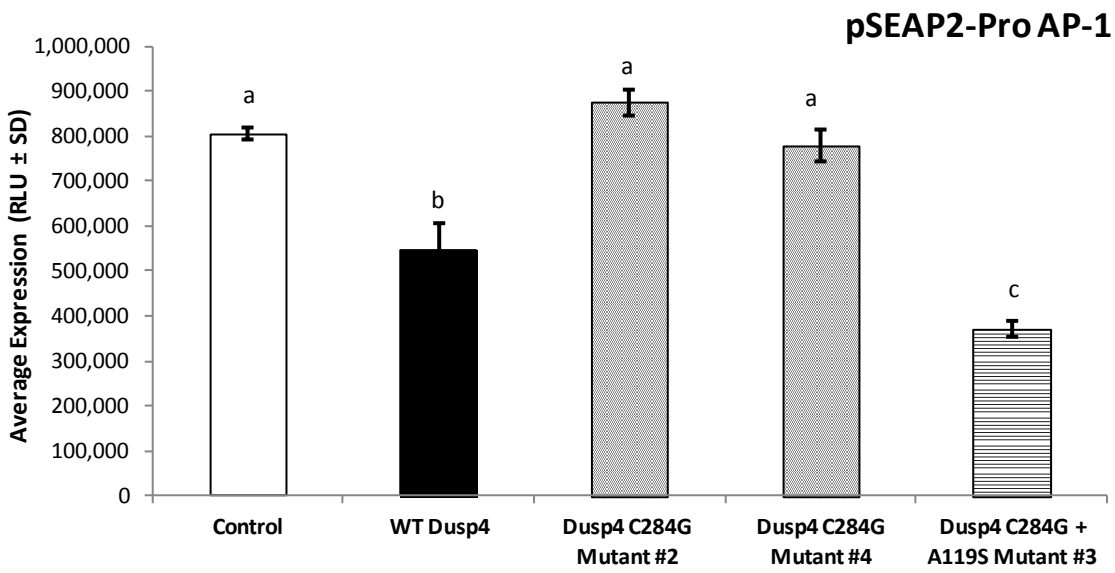

Figure 24. Dusp4 repression of an AP-1 reporter requires catalytic phosphatase activity. C_2C_{12} myoblasts were transfected at approximately 70% confluency with either the pSEAP2 AP-1 reporter alone or in conjunction with WT-Dusp4 or the Dusp4 C-terminal mutants. The media was changed approximately 24 hours after plating to 2% FBS media and samples were taken 72 hours after media change and performed in triplicate. The samples were normalized with β-galactosidase activity to correct for variation in transfection efficiency. Graphs represent mean values ± SD. Statistical significance was evaluated using an ANOVA and values were considered significant when $P \leq 0.05$ [a) compared to control $P \leq 0.05$, b) compared to WT-Dusp4 $P \leq 0.05$, c) compared to Dusp4-Mutant 3 $P \leq 0.001$). Dusp4 mutant constructs 2 and 4 contained only the mutation within the catalytic domain (C284G). Dusp4 mutant construct 3 also acquired a secondary mutation within the N-terminal MAPK binding domain (A119S).

We next decided to evaluate if the repression observed by mutant 3 (double mutant) could be enhanced in a dose-dependent manner. Wild-type Dusp4, Dusp4 mutant 4 (single mutant), and Dusp4 mutant 3 (double mutant) were analyzed at varying concentrations from 125ng to 500ng. The data show that increasing the concentration of expression plasmids for Dusp4 did result in greater repression at higher concentrations; however, the Dusp4 catalytic mutant was not able to repress AP-1 to the same extent as the wild-type, while the double mutant showed a similar repression to WT-Dusp4 at all concentrations (Figure 25).

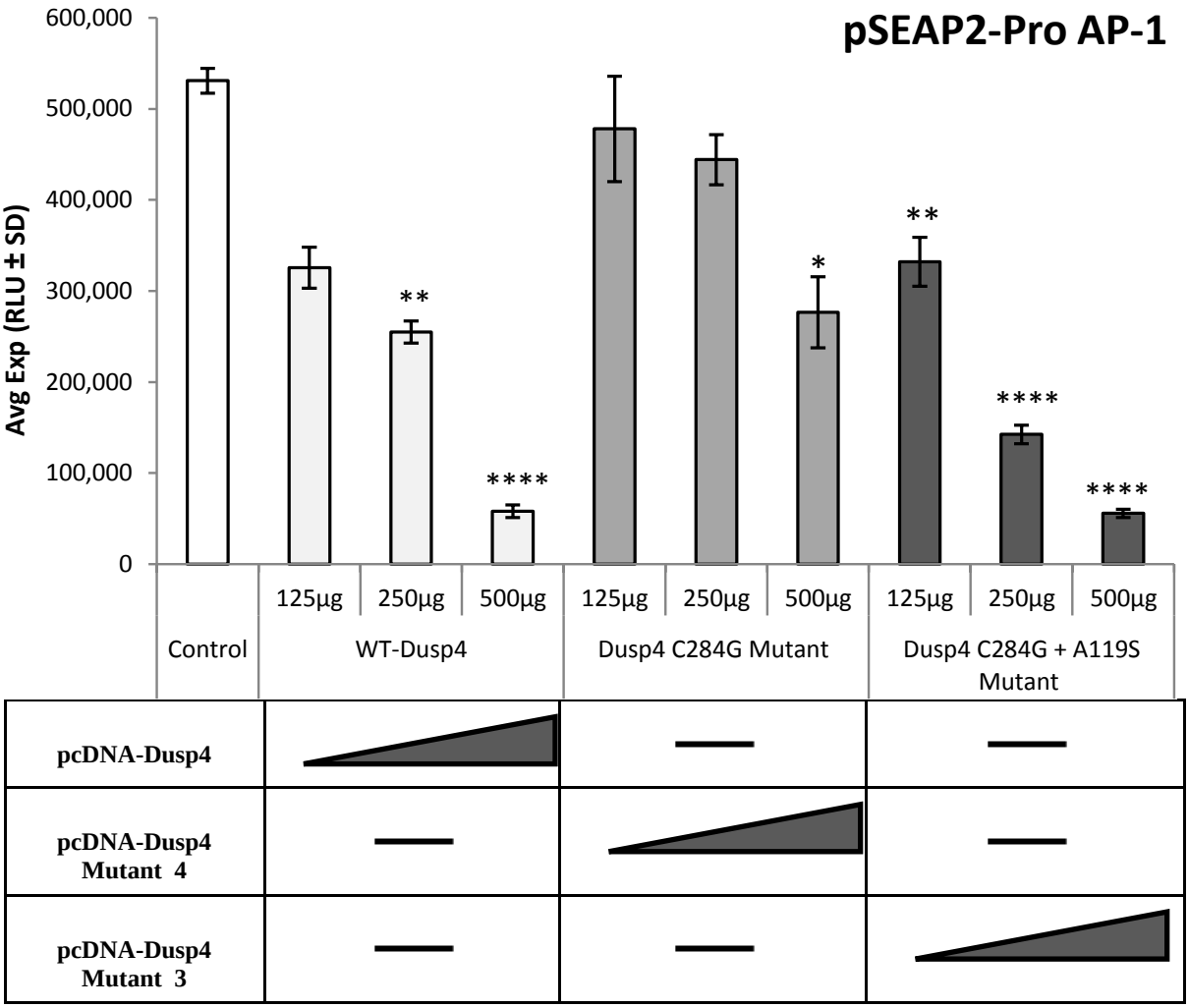

Figure 25. Dusp4 represses the pSEAP2 AP-1 reporter in a dose dependent manner. C_2C_{12} mouse myoblasts were transfected at 70-80% confluency with either the pSEAP2 AP-1 reporter alone or in conjunction with WT-Dusp4 cDNA or the cDNA of the Dusp4 C-terminal mutants (Dusp4 mutant 4-phosphatase domain only, Dusp4 mutant 3-phosphatase domain and MAPK binding domain) at incremental increases in concentration ranging from 125ng-500ng. The media was changed approximately 24 hours after plating to 2% FBS and samples were taken 72 hours after media change and performed in triplicate. The samples were normalized with β-galactosidase activity to correct for variation in transfection efficiency. Graphs represent mean values ± SD. Statistical significance was evaluated using an ANOVA and values were considered significant when $P \leq 0.05$ (* $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$).

Discussion

MuRF1 has long been characterized as a marker for skeletal muscle atrophy due to its increased expression in response to a variety of atrophy-inducing stimuli [11]. The research performed in this study suggests that MuRF1 may also be able to transcriptionally regulate expression of certain atrophy-induced genes, including Dusp4. Microarray data from MuRF1-null (KO) and wild-type (WT) littermates show

differential gene expression following denervation. In WT animals, MuRF1 expression returned to baseline levels by day 14 post-denervation, but in the KO-animals β-galactosidase expression remained up-regulated following denervation, which shows that the MuRF1 promoter was still active and responsive to denervation (Figure 3) [18]. Additionally, there was differential Dusp4 gene expression observed between the wild-type (WT) and MuRF1-null (KO) animals, at both 3 days and 14 days, under neurogenic atrophy conditions (Figure 6). It was the purpose of this study to establish a potential model to explain the how MuRF1 may transcriptionally regulate Dusp4 gene expression and to characterize a potential role of Dusp4 in skeletal muscle.

Dusp4 is part of an elaborate ligand-activated signal transduction cascade known as the MAP kinase pathway. Dusp4 is widely expressed in many tissues types such as the pancreas, stomach and prostate [43]. Furthermore, research has been conducted in many cell lines, other than skeletal muscle, to confirm that dual-specificity phosphatases are important regulators of cell proliferation, growth, and apoptosis [28, 36, 44]. While there is limited information on the direct role dual-specificity phosphatases (Dusp's) may play in the development of skeletal muscle tissue, type 1 Dusp's including Dusp1, Dusp2, and Dusp5 have been the focus of research involving cardiac muscle [45, 46]. The research conducted in those studies focused on the ability of nuclear Dusp's to regulate MAP kinase signaling in cardiac myopathies.

Similar findings regarding the regulation of MAPK kinase signaling and cell growth for type 1 nuclear localized Dusp's have been at the heart of immunology research for years. Dusp1 has been found to regulate glucose metabolism and maintain cellular homeostasis, depending on the signaling input from surrounding cells [47, 48]. Dusp2 (PAC-1) positively regulates the function of immune cells and helps to control the inflammatory response [49]. Additionally, Dusp5 has also been reported to play a role in the development and maintenance of T-cells through MAP kinase signaling [50]. Since Dusp4 is also a type 1 nuclear phosphatase, it is believed to also play a role in the regulation and maintenance of certain tissues, including that of skeletal muscle. While not a focus of this study, it was observed that when C_2C_{12} mouse

myoblasts were transfected with plasmids containing the cDNA for Dusp4 the individual cells appeared smaller and began to shift toward a differentiated state more quickly than non-transfected cells, suggesting that Dusp4 may regulate the growth and proliferation status of skeletal muscle cells as well (data not shown).

There is little information regarding the transcriptional regulation and downstream targets of Dusp4 in skeletal muscle. This thesis is focused on understanding how MuRF1 may regulate Dusp4 expression under neurogenic atrophy conditions. Dusp4 expression was induced at both 3 days and 14 days following denervation in wild-type mice, but there was blunted Dusp4 induction in the MuRF1-KO animals (Figure 6) [18]. The transcriptional regulation of Dusp4 was examined by fusing fragments of the promoter region to the pSEAP2 reporter gene and testing the response in the presence of ectopic MuRF1 expression. *In vitro* studies using C_2C_{12} myoblasts show that MuRF1 represses Dusp4 promoter activity, suggesting a possible transcriptional regulatory role for MuRF1 in Dusp4 expression.

Many of the genes that showed differential expression following denervation in MuRF1-KO and WT animals from the microarray, including Dusp4, contain important regulatory regions such as an activator protein 2 (AP-2) binding element and E-box enhancer sequences. AP-2 regulation has been most extensively studied for its importance in neural development and in neural restructuring in vertebrate organisms [41]. The presence of the AP-2 regulatory region in the Dusp4 regulatory region suggests a possible functional role for Dusp4 during neurogenic skeletal muscle atrophy. A possible function of Dusp4 during neurogenic atrophy could center on reorganization of the neuromuscular junctions following a denervation event in an attempt to reestablish neural connections between the muscle tissue and the nervous system in order to prevent muscle wasting. In the central nervous system, AP-2 proteins are necessary for proper embryonic development and are evolutionarily conserved in flies, amphibians, and birds [51]. Whether the AP-2 element within the Dusp4 promoter functions to control this gene in response to molecular signaling following denervation is still unclear, but it is possibly important in the

repair process following denervation in order to repair muscle damage and reduce muscle wasting. Site-directed mutagenesis of the AP-2 binding element within the Dusp4 promoter resulted in increased activity of the Dusp4 Pro-500 reporter gene compared to the WT-Dusp4 Pro-500 reporter gene (Figure 12); however, this work was conducted under non-denervation conditions. Further studies using tissue samples isolated from denervated skeletal muscle could provide more insight on whether this particular AP-2 sequence associated with Dusp4 is in fact necessary for the functionality of myoblast proliferation and differentiation following injury. The ability of MuRF1 to repress Dusp4 activity was not greatly affected by mutating the AP-2 binding element within the promoter. Exogenous MuRF1 expression resulted in a 50% decrease in the Dusp4 Pro-500 AP-2 mutant promoter as compared to a 38% decrease in the WT Dusp4 (Figure 13).

The addition of exogenous WT-MuRF1 resulted in significant repression on all of the Dusp4 reporter constructs (Figure 10). Experiments performed by exogenously expressing a catalytically dead MuRF1-RING mutant resulted in a complete loss of repression of the 500bp Dusp4 reporter construct and a slight loss of repression for the 1000bp and 2000bp Dusp4 reporter gene fragments (Figure 15). One major difference between the Dusp4 regulatory regions that were tested was the presence or absence of E-box enhancer elements. The Dusp4 Pro-500 construct contains no consensus E-boxes and there was a complete loss of repression in response to ectopic expression of the MuRF1-RING mutant. It appeared that there may be a connection between MuRF1's ability to transcriptionally regulate Dusp4 and the presence or absence of E-box elements located within the promoter. Since transfection experiments with MuRF1, myogenin, or MyoD1 alone showed repression of the Dusp4 promoter constructs, myogenin and MyoD1 were tested in combination with MuRF1 to determine if a cooperative regulation and further repression of the Dusp4 reporter could be achieved. Myogenin or MyoD1 alone were able to repress all Dusp4 promoter fragments, including the 500bp region that lacks E-box elements (Figure 16). Other research performed with promoter fragments for genes which code for acetylcholine receptor subunit genes, TGF-β, and TTC39C (tertatricopeptide repeat domain 39C) have shown that there may be a

cooperation between the myogenic regulatory factors and MuRF1 in transcriptional regulation; although the results have shown both cooperative repression and activation depending on the particular gene being studied (unpublished data). There is also a strong possibility that other transcription factors, such as E proteins (E12 and E47), HEB (human basic helix-loop-helix factor), myf5 (myogenic factor 5), and PBX-1 (Pre B cell leukemia homeobox 1), may participate in the regulatory effects observed following the addition of myogenin and MyoD1 *in vitro* [20]. Over-expressing both MuRF1 and myogenin resulted in a loss of Dusp4 repression, compared to ectopic expression of myogenin alone. However, when MuRF1 and MyoD1 were co-overexpressed there was a cooperative repressive effect resulting in greater repression than was observed for either factor alone (Figure 17). Myogenin and MyoD1 show increased expression following denervation in mice (Figure 5), so the results from these experiments suggest that Dusp4 expression may be reduced by MRFs during atrophy, which may lead to myoblast fusion and myotube development in an attempt to repair or replace some of the damaged tissue. Dusp4 was not the only gene for which this result has been observed. Unpublished data for Nip30 (NEFA interacting nuclear protein 30), Rspry1 (RING finger and SPRY domain containing 1), and TTC39C demonstrate these genes are also cooperatively regulated by MuRF1 and MRFs. These results indicate that while other transcription factors may be involved, there is a potential interaction between MRFs and MuRF1 that has not been previously identified in the transcriptional regulation of neurogenic atrophy-induced gene expression.

The presence of the putative E-box elements, which can be bound by muscle-specific transcription factors, in the Dusp4 promoter suggest that this particular gene is expressed in muscle and is important in skeletal muscle development. It is known that other muscle related genes contain E-box elements within their promoter regions including Musk (muscle creatine kinase) and desmin [21]; however, the conserved E-box elements may be even more important for the overall regulation of the gene than the number of E-box sequences. Research has not been conducted to determine if the number of E-box elements present within the promoter region of a gene directly correlates with gene regulation, as many genes retain

evolutionarily conserved regions that no longer function in the regulation of a particular gene. Site-directed mutagenesis on the single conserved E-box element located approximately 950bp upstream from the start of transcription resulted in a significant reduction in the activity of the Dusp4 Pro-2000 reporter gene (Figure 18); however, the E-box mutant promoter is still negatively regulated by myogenin and MyoD1, possibly due to the non-conserved functional E-box sequences still present in this promoter fragment.

In addition to trying to better elucidate a mechanism for the transcriptional regulation of Dusp4 by MuRF1, we also took a closer look at the Dusp4 gene product in order to better understand its function in skeletal muscle. Dusp4 has been studied in a variety of other cells and tissues for its role in apoptosis and as a tumor suppressor protein [25, 40], but very little work has centered on the function of Dusp4 in skeletal muscle. Work performed using human umbilical vein endothelial cells (HUVEC) showed that over expression of Dusp4 resulted in decreased JNK-dependent activation of pro-inflammatory cytokines and reduced apoptosis [40]. It has been determined that Dusp4 is silenced through epigenetic hypermethylation of the gene's promoter in malignant tumors of the nervous system called gliomas, resulting in significant development of additional glioblastomas [25]. These research findings suggest a role for Dusp4 in regulating cellular proliferation, but it is unclear if Dusp4 may have the ability to potentially initiate differentiation in committed cell lines.

Since it appeared that Dusp4 may function in an anti-proliferative role in other cell lines, we wanted to determine when Dusp4 is being expressed in C_2C_{12} muscle cells. Western blots of C_2C_{12} cell lysates over a 12 day time course revealed that the Dusp4 protein was only present under proliferating conditions (Figure 19). Data from the Western blot suggest that the function of Dusp4 in skeletal muscle may be to signal the end of proliferation and trigger myotube differentiation. In addition to identifying when the Dusp4 protein was expressed in skeletal muscle cells, we also wanted to confirm that the Dusp4 protein

was localized to the nucleus of C_2C_{12} myoblasts, which was not surprising because Dusp4 is a type-I dual-specificity phosphatase, meaning it possesses an N-terminal nuclear localization signal (Figure 20).

Determining a potential function of Dusp4 in skeletal muscle was accomplished by designing an AP-1 reporter system, which was used to assess MAP kinase signaling pathway activity in response to ectopic over expression of Dusp4. The AP-1 reporter was more active under proliferative conditions compared to differentiation conditions, which is to be expected since proliferating cells are maintained in a higher percentage of growth serum (Figure 21). AP-1 is an ERK-activated transcription factor and so this allowed us to determine that Dusp4 is able to negatively regulate the ERK signaling cascade in C_2C_{12} cells [52]. The data suggest that Dusp4 was able to dephosphorylate ERK in C_2C_{12} cells, indicated by a reduction in AP-1 reporter activity following over expression of Dusp4 (Figure 22). Since wild-type Dusp4 was able to inactivate an ERK-regulated transcription factor, site-directed mutagenesis was performed on the catalytic phosphatase domain of Dusp4 to abolish its ability to dephosphorylate MAP kinases. The catalytically dead Dusp4 mutants that were generated lost the ability to repress pSEAP2-Pro AP-1 reporter activity, suggesting that intact catalytic function was needed to dephosphorylate ERK in C_2C_{12} cells (Figure 24). However, the Dusp4 double-mutant (C-terminal catalytic and N-terminal MAPK binding domain mutant) regained its ability to repress AP-1 activity and showed greater repression than WT Dusp4, even though it lacks phosphatase activity. The repression of the AP-1 reporter in response to the Dusp4 double mutant is not the result of dephosphorylation but may be the result of the mutant Dusp4 interacting with the activated MAP kinase in a dominant-negative fashion. Further research into how the MAPK binding domain mutation affects the function of Dusp4 will need to be conducted to better understand the repression observed in the AP-1 reporter system.

Conclusions

In summary, this thesis work helps to contribute to a better understanding of the transcriptional regulation of Dusp4 and its activation following denervation-induced muscle atrophy. It is possible that Dusp4 may

be involved in controlling the proliferation status of mouse myoblasts and it may promote the cellular differentiation to myotubes through the negative regulation of the MAP kinase signaling pathway. The work presented here identifies MuRF1 as a potential transcriptional regulator as well as provides evidence that ERK might be a target of Dusp4 dephosphorylation in skeletal muscle.

Work with C_2C_{12} mouse myoblasts reveals that the Dusp4 promoter is transcriptionally active in muscle cells and is negatively regulated by exogenous MuRF1 expression. Dusp4 is activated in response to neurogenic atrophy in mice and the full activation appears to be dependent on the presence of MuRF1. These findings appear to conflict; however, there are many differences between *in vivo* and *in vitro* conditions, including vascular and neural signals as well as signaling from other transcription factors, including myogenin and MyoD1 that would be present following denervation and could account for these differences. Further work will be needed to elucidate the complete role of Dusp4 in skeletal muscle wasting.

Myogenic regulatory factors, including myogenin and MyoD1, were also found to repress Dusp4 reporter activity likely through interaction with one of the E-box enhancer elements located upstream from the start of Dusp4 transcription. It is possible that the repression observed due to MuRF1 may be in part through a mechanism involving interaction with these MRFs at the promoter. Reporter assays using a catalytically dead MuRF1 construct show that it is the catalytic ubiquitination function of MuRF1 that appears to be important for the repression of the Dusp4 reporter gene constructs, since the MuRF1-RING mutant was not able to negatively regulate the reporter genes in a fashion similar to WT-MuRF1. Additionally, the conserved E-box element within the Dusp4 promoter was determined to be necessary for the activation of this gene in skeletal muscle cells. The findings presented in this thesis provide support for MuRF1 as a potential transcriptional regulator of Dusp4 gene expression. There is still work that needs to be done in order to confirm the mechanism by which MuRF1 regulates gene expression in skeletal muscle; however, one possible mechanism may occur through an interaction between MRFs and

MuRF1 that ultimately alters MRF binding of E-box elements and future studies will be conducted in order to determine if this potential model is correct.

Future Directions

Site-directed mutagenesis of non-conserved E-box elements

In addition to the conserved E-box enhancer element that was mutated in the Dusp4 2000bp promoter region, there are five other non-conserved E–box elements that exist for the mouse Dusp4 gene. Four of the five E-box elements match the sequence determined to be most important for regulation by MRF's [20]. It is unclear if mutating any of the non-conserved E-boxes will have the same effect on Dusp4 promoter activity or if the repressive effects of myogenin and MyoD1 will be lost if certain E-box elements are no longer functional. Site-directed mutagenesis of the non-conserved E-box elements will be performed to determine their necessity and function in the transcriptional regulation of Dusp4.

Site-directed Mutagenesis of the Dusp4 NLS

The N-terminal nuclear localization signal (NLS) is what characterizes Dusp4 as a type-I dual-specificity phosphatase. The sequence has been identified as RRRAK [53]. The basic residues arginine (R) and lysine (K) have been identified as being necessary for nuclear translocation, while the spacer amino acid, alanine (A), appears to be important, but mutations in this region do not affect the ability of proteins to move into the nucleus [54]. Therefore, the final lysine (K) residue of the NLS, at position 82, will be mutated to glycine (G). By preventing Dusp4 from entering the nucleus, it will provide an opportunity to evaluate C_2C_{12} cell proliferation and differentiation rates which will improve our understanding of Dusp4 function during muscle cell proliferation and differentiation.

Determine Dusp4 target substrate specificity in C_2C_{12} cells

The AP-1 reporter system that was designed for this thesis project helped confirm the function of Dusp4 as a MAPK phosphatase in C_2C_{12} cells. Since AP-1 is an ERK-activated transcription factor, the

repression of the AP-1 reporter system that was observed following exogenous Dusp4 expression validated Dusp4 in the dephosphorylation and inactivation of the MAP kinase ERK signaling cascade. While this information is useful, it does not show whether Dusp4 has the ability to inactivate other MAP kinase pathways, such as JNK and p38, in skeletal muscle. In order to determine if Dusp4 can also regulate the activity of those MAPK's, reporter systems that are JNK-specific and p38-specific will need to be created so that a complete understanding of Dusp4 target proteins can be determined in skeletal muscle.

Site-directed Mutagenesis of the MAPK binding domain

The Dusp4 double mutant, containing the N-terminal MAPK binding domain and C-terminal catalytic domain mutations, showed differences from both the WT Dusp4 and Dusp4 C-terminal catalytic mutant in its ability to repress AP-1 reporter activity. In order to better determine the necessary amino acid residues that are important for Dusp4 functionality, a MAPK binding domain only mutant will be generated. This Dusp4 mutagenesis will only focus on altering the alanine to serine (A-119-S) residue located within the N-terminal MAPK binding domain to determine if that amino acid is necessary for the binding of Dusp4 to MAP kinases that enter the nucleus.

siRNA knockdown of Dusp4 in C_2C_{12} cells

Small-interfering RNA (siRNA) targeting Dusp4 will be engineered to evaluate how the C_2C_{12} cells will respond to reduced levels of the protein. In addition to monitoring the growth rates of the cells, western blot analysis will be performed to look for changes in markers of differentiation (i.e. MyHC). Several different oligo sequences will be used to analyze whether a specific siRNA sequence has a greater ability to reduce Dusp4 protein levels in skeletal muscle cells. Effective siRNA-mediated Dusp4 protein knockdown will be monitored by western blotting for Dusp4 in C_2C_{12} cells.

Transgenic Dusp4 and Dusp4-KO mouse models

A wealth of information can be determined from experimentation with C_2C_{12} cells and it can provide insight into molecular mechanisms by teasing out other factors; however, *in vitro* research can only advance so far the understanding of denervation-induced skeletal muscle atrophy and the role of Dusp4 in the process of muscle wasting, therefore transgenic Dusp4 mice and Dusp4-KO animals will be considered in the future. Work has already been performed using mouse embryonic fibroblasts (MEF's) derived from Dusp4-KO mice to determine that cell proliferation rates were markedly reduced [27]; however, muscle cells were not evaluated in that study. Dusp1 is also a type-1 dual-specificity phosphatase that may compensate for the reduction in Dusp4 protein expression [37]. Research performed using Dusp1/Dusp4 double-null mice shows unrestrained activation of the MAP kinase p38, resulting in the development of cardiomyopathy [55]. By over expressing the Dusp4 protein or removing it from the mouse genome, the role of this gene product and its connection to other physiological systems, which cannot be mimicked in cell culture studies, can be evaluated.

All of the future directions previously described will help to not only provide additional knowledge regarding the role and function of Dusp4 in skeletal muscle, but it will also contribute to our understanding of how this gene is induced following sciatic nerve denervation and the role MuRF1 may play both in that activation and as a transcriptional regulator in general. The more information that can be obtained regarding Dusp4, a crucial member of the MAPK signaling cascade involved in cellular proliferation, the better our understanding will be of the unseen events that occur during the process of skeletal muscle atrophy as well as the impact of MuRF1 on the transcriptional regulation of Dusp4.

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Curriculum vitae

Education

- **University of North Florida**
  M.S. in Biology, July 2015

- **University of North Florida**
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- **Florida State College at Jacksonville**
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Work Experience

Microbiology laboratory prep coordinator- University of North Florida (January 2014-current)

Research Experience and Presentations

Identifying the transcriptional regulatory mechanisms of dual-specificity phosphatase 4 (Dusp4) in mouse myoblasts. (Spring 2011-current) PI-Dr. David Waddell

Showcase of Osprey Advancements in Research and Scholarships (SOARS) UNF 2012- Poster presentation "Transcriptional regulation of Dusp4 by muscle-specific RING finger-1 (MuRF1)"

Florida Undergraduate Research Conference (FURC) Stetson University 2012- Poster presentation "Transcriptional regulation of Dusp4 by muscle-specific RING finger-1 (MuRF1)"

Showcase of Osprey Advancements in Research and Scholarships SOARS UNF 2013- Poster presentation "Transcriptional regulation of Dusp4 by muscle-specific RING finger-1 (MuRF1)"

Showcase of Osprey Advancements in Research and Scholarships SOARS UNF 2014- Oral presentation "MuRF1 and myogenic regulatory factors coordinate the transcriptional regulation of Dusp4"

Experimental Biology Conference (EB) San Diego, CA 2014- Poster presentation "Transcriptional regulation of Dusp4 by muscle RING finger-1 (MuRF1) and myogenic regulatory factors"

Showcase of Osprey Advancements in Research and Scholarships SOARS UNF 2015- Oral presentation "Muscle-specific RING finger 1 (MuRF1) Dependent Activation of Dual-specificity Phosphatase 4 (Dusp4) Under Neurogenic Atrophy Conditions"