Identification of PDLIM7 as a Nedd4-1 substrate in the regulation of skeletal muscle mass

by

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Institute of Medical Science University of Toronto

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ABSTRACT

Identification of PDLIM7 as a Nedd4-1 substrate in the regulation of skeletal muscle mass

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Skeletal muscle atrophy occurs both as a natural consequence of muscle disuse and aging, and as a pathophysiological response to acute and chronic disease. The ubiquitin proteasome system is the predominant proteolytic machinery responsible for atrophy of skeletal muscle and Nedd4-1 is one of a series of E3 ubiquitin ligases known to mediate inactivity-induced muscle wasting. Muscle substrates of Nedd4-1, and mechanisms by which Nedd4-1 regulates muscle mass, are poorly understood. In the present study, I identified PDLIM7 as a novel target of Nedd4-1 in skeletal muscle. Nedd4-1 expression in muscle atrophied by denervation is coincident with a decrease in PDLIM7, and PDLIM7 protein levels are stabilized in denervated muscle of Nedd4-1 knockout mice. These results identify PDLIM7 as a *bona fide* skeletal muscle substrate of Nedd4-1 and suggest that this interaction may underlie the progression of skeletal muscle atrophy, offering a potential therapeutic target to attenuate its onset.
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CONTRIBUTIONS

Unless otherwise indicated, all experiments were performed by Robert D’Cruz.

Pamela J. Plant and Robert D’Cruz performed experiments in Figure 3.1.

Pamela J. Plant, Judy Correa, and Leslie A. Pablo performed experiments in Figure 3.2.

Joshua Chackowicz and Shouzhe Lin contributed to experiments in Figure 3.3 and Figure 3.4.

Caterina Di Ciano-Oliveira and Paul Taylor were consulted for their technical expertise.

Darquise Denis and Maribeth Mitri offered their technical assistance.

Jane A.E. Batt and Pamela J. Plant conceived and designed the study outlined in Chapter 3, James R. Bain performed all animal surgeries, and Robert D’Cruz, Jane A.E. Batt and Pamela J. Plant analyzed the data and co-wrote the manuscript.


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Jane A.E. Batt, Andras Kapus, Robert D’Cruz, and Michael Kofler conceived and designed the preliminary work outlined in Chapter 5: Future Directions.
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LIST OF ABBREVIATIONS

AKT: Thymoma viral oncogene homolog 1
ALK4/5: Type-1 activin receptor serine kinase
ALP: actin-associated LIM protein
AMPARs: α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors
AMPK: AMP-activated protein kinase
CMA: Chaperone-mediated autophagy
Comm: Commissureless
COPD: Chronic obstructive pulmonary disease
c-Src: Tyrosine-protein kinase CSK
DMM: Duchenne muscular dystrophy
DUBs: Deubiquitination enzymes
EGFR: Epithelial growth factor receptor
ENH: Enigma homology protein
FAK: Focal adhesion kinase
FGF: Fibroblast growth factor
FGFR1: Fibroblast growth factor receptor 1
FoxO: Forkhead box O
GFP: Green fluorescent protein
GPCRs: G-protein-coupled receptors
Grb10: Growth factor receptor-bound protein 10
GSK-3β: Glycogen synthase kinase 3 beta
GST: Gluthatione-S-transferase
HECT: Homologous to the E6AP carboxyl terminus
HEK: Human embryonic kidney
HGH: Hepatocyte growth factor
HPRT: Hypoxanthine-Guanine Phosphoribosyl Transferase
ICUAW: Intensive care unit-acquired weakness
ID-2: DNA-binding protein inhibitor
IGF1: Insulin-like growth factor 1
IGF-R1: Insulin growth factor receptor 1
InsR: Insulin Receptor
LAP: Leukemia associated protein
Lats1/2: Large tumor suppressor kinases 1 and 2
LC3: Microtubule-associated protein 1 light chain 3
MA: Megestrol acetate
MABp1: Monoclonal anti-interleukin-1α antibody
MAFbx: Muscle atrophy F-box
MHC: Myosin heavy chain
MRFs: Myogenic regulatory factors
Mrf4: Myogenic factor 6
MTMR4: Myotubularin-related protein 4
mTOR: Mechanistic target of rapamycin
Mst1/2: Mammalian STE20-like protein kinases 1 and 2
MudPIT: Multidimensional protein identification technology
MuRF1: Muscle RING finger 1
MUSA1: Muscle ubiquitin ligase of SCF complex in atrophy-1
MVB: Multi-vesicular bodies
MyBP-C: Myosin-binding protein C
MyLC1/2: Myosin light chain-1/2
Myf5: Myogenic factor 5
MyoD: Myogenic differentiation 1
Nedd4-1: Neural precursor cell expressed developmentally down-regulated protein 4
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
Pax3: Paired box 3
Pax7: Paired box 7
PBS: Phosphate buffered saline
PDLIM7: PDZ and LIM domain containing protein 7
PI3P: Phosphatidylinositol 3-phosphate
PI3,5P2: Phosphatidylinositol-3,5-bisphosphate
PKC: Protein kinase C
PGC-1α: Peroxisome proliferator gamma coactivator-1α
PHD: Plant homeodomain
PIP3: PtdIns(3,4,5)
PTEN: Phosphatase and tensin homolog
Ret: Rearranged during Transfection
Robo: Roundabout
ROS: Reactive oxidative species
RING: Really interesting new gene
SARMs: Selective androgen receptor modulators
Sav1: Salvador homolog 1
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHH: Sonic Hedgehog
SMS-KO: Skeletal muscle specific knockout
SP-C: Surfactant protein C
TAZ: WW domain-containing transcription regulator protein 1
Tbx4/5: T-box transcription factors 4/5
TENS: Transcutaneous electrical nerve stimulation
TFA: Trifluoroacetic acid
TGF-β: Transforming growth factor-β
TRAF6: TNF receptor adaptor protein 6
TRIM32: Tripartite motif containing 32
Tsp-1: Thrombospondin-1
UPS: Ubiquitin-proteosome system
USPs: Ubiquitin-specific proteases
VEGF-R2: Vascular endothelial growth factor receptor-2
W: Tryptophan
WT: Wild type
YAP: Yes-associated protein
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CHAPTER 1: LITERATURE REVIEW

1.1 SKELETAL MUSCLE – STRUCTURE AND FUNCTION

Skeletal muscle is a highly complex and specialized organ of the body responsible for multiple important functions including support, locomotion, thermoregulation, and is involved in metabolic processes such as maintenance of blood glucose levels and providing amino acid reservoirs. It is one of the three muscle types found in the body, the others being cardiac and smooth muscle. It is differentiated from these by the fact that it is striated, multi-nucleated, and controlled by the voluntary somatic nervous system (Table 1.1).

Table 1.1. Differences between skeletal, cardiac, and smooth muscle

<table>
<thead>
<tr>
<th>Skeletal</th>
<th>Cardiac</th>
<th>Smooth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found attached to skeleton by tendons</td>
<td>Found only in heart</td>
<td>Found in hair erectors, pupils, GI tract, bronchi, and blood vessels</td>
</tr>
<tr>
<td>Voluntary contraction</td>
<td>Involuntary contraction</td>
<td>Involuntary contraction</td>
</tr>
<tr>
<td>Striated</td>
<td>Striated</td>
<td>Non-striated</td>
</tr>
<tr>
<td>Sensitive to fatigue</td>
<td>Fatigue slowly</td>
<td>Fatigue slowly</td>
</tr>
</tbody>
</table>
Skeletal muscle cells (myocytes) fuse together to form multinucleated muscle fibers, which are bundled together by collagenous connective tissue. Three layers of connective tissue surround skeletal muscle: the endomysium which surrounds each individual muscle fiber, the perimysium which surrounds bundles of muscle fibers, and the epimysium which surrounds the entire muscle. These layers enable a contractile force generated by skeletal muscle fibers to be transmitted to bones through the connective tissue and attached tendons. (Figure 1.1)

Each skeletal muscle fiber is composed of long cylindrical strands of contractile proteins called myofibrils. These can be further divided into repeating segments called sarcomeres, which are the functional contractile units within skeletal muscle. The sarcomere is composed of the actin containing thin myofilaments and myosin containing thick myofilaments (Figure 1.2). Repeating sarcomeres are separated by Z-discs which act as platforms for attached thin filaments to extend centrally. Thick filaments are found in the center of the sarcomere interspaced between thin filaments. Following motor nerve activation and stimulation through an action potential, intracellular calcium is released within each myofiber from the sarcoplasmic reticulum, facilitating an interaction between myosin and actin filaments. Myosin thick filaments form cross-bridges with thin filaments, and pull them towards the center of the sarcomere. This causes a contraction as thin filaments and attached Z-discs move towards each other.

Human skeletal muscle, for the most part, contains mixtures of three subtypes of myofibers, differentiated by the class of myosin of which each is composed (Wilson, Loenneke et al. 2012). Type I myofibers contain myosin heavy chain (MHC) type I filaments, and are known as slow twitch oxidative fibers due to the fact that they contain a high density of mitochondria making them resistant to fatigue and are better suited for long-term aerobic activity. Type IIA and type IIB myofibers contain MHC type II filaments, and are known as fast...
Figure 1.1. Structural anatomy of skeletal muscle. Anatomical breakdown of skeletal muscle down to the microfibril and surrounding layers of connective tissue. The epimysium surrounds each muscle, the perimysium surrounds each fascicle, and the endomysium surrounds individual muscle fibers.
Figure 1.2. Comparison of a relaxed and contracted sarcomere. Microfibrils are composed of repeating units called sarcomeres separated by Z-disks. Each repeating sarcomere is composed of myosin containing thick filaments interspaced between actin containing thin filaments. Following stimulation, calcium binding to myosin-associated proteins allows for myosin and actin to form cross-bridges. Myosin thick filaments then pull actin thin filaments and attached Z-discs towards each other, causing contraction through sarcomere shortening.
twitch fibers. Type IIA myofibers also contain high densities of mitochondria and are moderately resistant to fatigue, whereas type IIB myofibers contain low densities of mitochondria making them prone to fatigue and better suited for short term anaerobic activity (Table 1.2) (Schiaffino, Gorza et al. 1989, Tu, Levin et al. 2016).

**Table 1.2. Differences between skeletal muscle fiber types**

<table>
<thead>
<tr>
<th>Type I (Slow twitch)</th>
<th>Type IIA (Fast twitch)</th>
<th>Type IIB (Fast twitch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow oxidative</td>
<td>Fast oxidative</td>
<td>Fast glycolytic</td>
</tr>
<tr>
<td>High resistance to fatigue</td>
<td>Intermediate resistance to fatigue</td>
<td>Low resistance to fatigue</td>
</tr>
<tr>
<td>Aerobic respiration</td>
<td>Aerobic respiration</td>
<td>Anaerobic respiration</td>
</tr>
<tr>
<td>MHC Type I filaments</td>
<td>MHC Type II filaments</td>
<td>MHC Type II filaments</td>
</tr>
</tbody>
</table>
1.2 SKELETAL MUSCLE IN HEALTH AND DISEASE

Myopathy is a clinical dysfunction of skeletal muscle affecting myofiber structure, contractility and metabolism. Its onset can be either acquired or genetically inherited, leading to metabolic abnormalities and weakness.

Genetic myopathies can be classified into the categories of muscular dystrophy and mitochondrial/metabolic myopathies. While devastating, genetic myopathies are rare, afflicting only a small proportion of the population. As an example, Duchenne muscular dystrophy (DMM), caused by a mutation in the dystrophin gene on the X chromosome, is the most studied genetic myopathy. It affects 1 in 3500 males causing intellectual disability and progressive deterioration of muscle, which ultimately leads to early mortality (Berardo, DiMauro et al. 2010). There is currently no cure for DMM; treatment consists primarily of measures to improve quality of life and manage symptoms.

Mitochondrial and metabolic myopathies are even rarer, and are classified by defects in genes affecting mitochondria and cellular energy metabolism (glycogen storage and fatty acid oxidation) (Berardo, DiMauro et al. 2010). In contrast to the rare genetic myopathies, acquired myopathy, and specifically muscle wasting, commonly occurs with chronic illness, traumatic injury and is a naturally occurring consequence of ageing, inactivity or fasting

Sarcopenia

Sarcopenia is defined as a progressive geriatric syndrome characterized by a decrease in muscle mass leading to a reduced functional capacity, contraction velocity, and regenerative
capacity (Hafer-Macko, Ryan et al. 2008, Brioche and Sophie 2016). The rate of muscle mass loss has been shown to progress from 8% per decade after the age of 50 years to approximately 15% per decade after the age of 70 years (Grimby and Saltin 1983). Muscle loss increases likelihood of falls, disability, and a loss of independence. In the United States, it is estimated that 1.5% of health care spending result from complications induced by sarcopenia, making it a significant component of health expenditure (Janssen, Shepard et al. 2004). Currently, we are unable to substantially alter the onset or progression of sarcopenia. Understanding the underlying biological mechanisms mediating sarcopenia is required to enable prevention and management strategies to combat its onset and progression, and mitigate associated costs.

**Inactivity and Fasting**

Inactivity and fasting are associated with alterations affecting protein metabolism leading to diminished skeletal muscle mass. For example, 14 days of bed rest causes loss of myofibrillar proteins through reducing rates of protein synthesis by 50% (Stuart, Shangraw et al. 1988, Biolo, Ciocchi et al. 2004, Biolo, Ciocchi et al. 2005). Both fasting and inactivity have been shown to induce muscle wasting in part by causing decreased levels of circulating amino acids and insulin-resistance, which combine to inhibit protein synthesis and promote turnover (Ferrando, Paddon-Jones et al. 2006). Upregulated muscle protein degradation also contributes to loss of muscle mass, regardless of the etiology of inactivity (ie unloading, casting, denervation). In contrast to sarcopenia, in the healthy individual the muscle loss associated with fasting or inactivity is essentially, or nearly completely, reversible with resumption of exercise/activity, and adequate caloric intake.
**Traumatic Denervation Injuries**

Peripheral nerve injury is common in heavy industry manufacturing and trauma, and results in muscle atrophy (Rowan, Rygiel et al. 2012). Animal studies suggest, and clinical experience demonstrates, that in circumstances of denervation, lost muscle mass can be substantially restored by good quality re-innervation at the earliest possible time post injury (Bain, Veltri et al. 2001). In its absence, the permanent muscle wasting that occurs with delayed and/or poor quality re-innervation, induces disability, work absenteeism and lost productivity in this typically young and previously healthy working population, resulting in significant opportunity costs and economic burden (Jaquet, Luijsterburg et al. 2001, Rosberg, Carlsson et al. 2005).

**Chronic Disease States**

Muscle wasting occurs in a number of acute and chronic disease states including, for example, diabetes mellitus, chronic obstructive pulmonary disease (COPD), renal failure, and critical illness (Batt, dos Santos et al. 2013, Drescher, Konishi et al. 2015, Malavaki, Sakkas et al. 2015, Berger, Blochlinger et al. 2016). In some of these conditions muscle wasting occurs as part of the cachexia syndrome; a complex multifactorial pathological syndrome characterized by a reduction in both adipose tissue and skeletal muscle mass, and accompanied by anorexia (Fearon, Glass et al. 2012, Taylor and Pendleton 2016). Regardless of the precipitating illness, the onset of muscle wasting in disease can complicate a patient’s treatment tolerance, and negatively impacts quality of life (Mostert, Goris et al. 2000). In patients with severe COPD for example, muscle wasting contributes significantly to reduced exercise capacity, which is already compromised as a result of impaired lung function (Wüst and Degens 2007). Diminished muscle
mass also increases disease morbidity and substantially increases health resource utilization and health care costs in the COPD patient (Decramer, Gosselink et al. 1997, Mostert, Goris et al. 2000, Saini, Faulkner et al. 2009). Rapid onset of severe muscle wasting, as occurs in the critically ill patient with ICU acquired weakness (ICUAW), is associated with increased hospital mortality (De Jonghe, Bastuji-Garin et al. 2007, Ali, O'Brien et al. 2008), and healthcare-related hospitalization costs are 30.5% higher in weaker patients following ICU discharge (Hermans and Van den Berghe 2015). 40% of those most seriously afflicted die within the first year after ICU discharge and the surviving patients demonstrate severe and lifelong physical functional dependency preventing return to work, and necessitating acquisition/engagement of a caregiver and significantly negatively impacting quality of life (Batt, dos Santos et al. 2013, Dos Santos, Hussain et al. 2016, Herridge, Chu et al. 2016). Currently we are unable to fully reverse, or substantially mitigate the loss of muscle and the subsequent negative consequences of muscle wasting in chronic disease. Identification of cellular and molecular biologic mechanisms that contribute to, and maintain muscle loss and dysfunction in chronic illness is critical to inform the development of prevention and treatment strategies and interventions.

1.2.1 Current Therapies for Muscle Wasting

The most effective therapy to combat or attenuate muscle wasting to date, is resistance exercise (Adams, Haddad et al. 2007, Maddocks, Murton et al. 2011, Nicastro, Zanchi et al. 2011). For example, in cachectic COPD patients, pulmonary rehabilitation, in which target muscle resistance training is a predominant and important component, is known to increase peripheral muscle remodeling and muscle fiber cross-sectional area, significantly improving functional capacities (Vogiatzis, Simoes et al. 2010). Low intensity exercise combined with other
therapies have been shown to prevent losses in muscle mass and strength in cancer cachexia (Pin, Busquets et al. 2015).

Although resistance-based exercise has potential benefits in the attenuation of muscle wasting its successful implementation can be difficult. Many patient and illness related factors, such as age-related limitations due to joint disease and balance difficulties, bed-ridden states or hemodynamic instability for example, prevent load bearing exercise from being utilized. In addition, even when effective resistance exercise can be undertaken, this does not necessarily reverse or even offer substantial protection from muscle wasting, as is evident in studies in the critically ill (Fan, Cheek et al. 2014). In these situations the atrophy stimuli appear to outweigh/overwhelm competing exercise stimuli. This makes the development of novel pharmacological therapies targeting pro-atrophic cellular networks in combination with low intensity exercise potentially the most effective and efficient treatment.

Chronic disease has been shown to induce a pro-inflammatory milieu with peripheral and central targets altering appetite (von Haehling and Anker 2015) in cachexia states. Megestrol acetate (MA), a synthetic derivative of progesterone, is a drug approved for treatment of anorexia-cachexia syndrome in the United States and some European countries in 1993 (Argiles, Anguera et al. 2013). Clinical studies have shown that administration of MA in patients with cancer cachexia increased appetite and subsequently nutritional intake, body weight, and grip strength and quality of life, (Wen, Li et al. 2012, Cuvelier, Baker et al. 2014).

Inflammatory processes are known to maintain muscle wasting in patients with cachexia. Treatment of cancer cachexia patients with anti-inflammatory mediators such as monoclonal anti-interleukin-1α antibody (MABp1) or Thalidomide has been shown to increase lean body
mass, improve appetite, and positively influence quality of life (Eriksson, Bjorkman et al. 2001, Hong, Hui et al. 2014). Despite these results, the ultimate implementation and efficacy of these therapies on a patient wide basis remains problematic due to the well-documented severe adverse effects associated with these drugs such as carcinogenicity and teratogenicity.

Anabolic steroids have been used effectively in the treatment of muscle wasting, however, their administration is limited as off target side effects such as prostate cancer in men, virilization in women, and cardiovascular complications worsen the risk-benefit balance. (Basaria, Coviello et al. 2010). A new therapy utilizing selective androgen receptor modulators (SARMs) offers the potential benefit of anabolic steroids without many of the adverse effects (Ebner, Steinbeck et al. 2014). Enobosarm is a specific SARM that improves lean body mass and physical function in pre-clinical studies and requires ongoing evaluation (Dalton, Barnette et al. 2011).

Transcutaneous electrical nerve stimulation (TENS) is a therapy utilized in the ICU that applies electrical current to generate targeted and controlled muscle contractions (Sluka and Walsh 2003). TENS has been shown to improve muscle structure and function following immobilization by preserving muscle protein synthesis (Poulsen, Moller et al. 2011, Maffiuletti, Roig et al. 2013). Despite these potential benefits, TENS requires direct application to muscle making it useful for targeted therapy in muscle wasting associated with traumatic nerve injury, however its use in systemic disease is not practical.

Resistance-based exercise, TENS, and therapies seeking to remedy muscle wasting through modulation of inflammation, growth stimulation with anabolic steroids, and appetite stimulation in the anorexic patient have shown some benefit in select groups, however,
inconsistent efficacy across studies and negative adverse drug effects have largely hindered their successful implementation into clinical practice. Gaps in current knowledge exist in the understanding of the biological and cellular mechanisms involved in the mediation of muscle wasting. Further understanding these molecular mechanisms yields the potential to develop novel therapeutic targets that can be used in combination with current clinical practice to attenuate or reverse muscle atrophy, thus improving patient outcomes, lessening demand for health services, and cutting health care costs.

1.3 REGULATION OF SKELETAL MUSCLE GROWTH AND DIFFERENTIATION

Skeletal muscle comprises approximately half of the total body mass in humans, making it the most abundant tissue within the body. It exhibits much adaptability and plasticity, responding to environmental and biological stimuli to elicit changes in development, metabolism and functional activity. These stimuli influence generation of myofibers and regulation of muscle size via the initiation of one of two responses: an increase in muscle mass through stimulation of hyperplasia and hypertrophy, or a decrease in muscle mass through induction of atrophy (Sandri 2014).

1.3.1 Myogenesis

Myogenesis, the process of generating muscle, begins in the early embryo from mesoderm derived embryonic progenitor cells which originate in the dermomyotome. These
progenitor cells migrate from the myotome to limb buds, and delaminate. The ability of these cells to undergo migration and survive is due to high expression of c-Met, a receptor tyrosine kinase on the progenitor cells which activates a range of signaling pathways following binding of hepatocyte growth factor (HGF) (Organ and Tsao 2011). Pax3, a paired box transcription factor associated with embryonic cell specification is required for successful progenitor cell migration, as it induces expression of c-Met (Wang and Rudnicki 2012).

As progenitor cells undergo extensive proliferation at their target sites, they begin to downregulate Pax3 and undergo fate specification towards a myoblast lineage. This change is associated with activation of basic helix-loop-helix myogenic regulatory factors (MRFs) such as Myogenic factor 5 (Myf5), Myogenic factor 6 (Myf6, Mrf4, Herculin), and Myogenic differentiation 1 (MyoD). Myoblasts will continue to proliferate until particular growth factors are depleted, stimulating them to leave the cell cycle and triggering commitment to a myocyte (Singh and Dilworth 2013). Further differentiation occurs as myocytes fuse together to form mature multi-nucleated myofibers, which are able to bundle together to form functional muscle. This is stimulated through sequential expression of Mrf4 and MyoD, as well as myogenin (Figure 1.3A).

Another division of cells are derived from the central dermomyotome, termed satellite cells (Fig 1.3B). This class of cells is thought of as muscle stem cells, contributing to muscle growth and regeneration in post-natal life. Satellite cells are found in mature muscle at the basal lamina in a quiescent and undifferentiated state (Relaix and Zammit 2012). This is largely due to high expression of the paired box transcription factor Pax7, which inhibits activation of MyoD and myogenin (Seale, Sabourin et al. 2000, Yablonka-Reuveni 2011, von Maltzahn, Jones et al. 2013). Following injury, satellite cells respond to both cellular and environmental cues to
Figure 1.3. Cellular markers involved in the regulation of myogenesis. (A) Skeletal muscle is derived from embryonic progenitor cells originating in the dermomyotome. In their undifferentiated state, they maintain their multipotency by expressing c-Met and Pax3. These factors become down-regulated with commitment to myoblast lineage and expression of transcription factors Myf5 and MyoD increases. As myoblasts continue to differentiate to mononucleated myocytes, they express Mrf4 and myogenin, as well as MyoD at reduced expression levels. Myocytes then fuse to form multi-nucleated myofibers which can undergo further differentiation and bundling. (B) Satellite cells are skeletal muscle precursors found in a quiescent state at the basal lamina expressing Pax7. Following mechanical strain or injury, they are activated to differentiate into myoblasts, then myocytes, which are then capable of fusing with existing myofibers or creating new ones.
become activated by a number of soluble regulatory factors. These regulatory factors can be released from vasculature, such as fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β), as well as secreted by adjacent myocytes, immune cells, and motor neurons (Ten Broek, Grefte et al. 2010, Wang and Rudnicki 2012). This complex regulatory network, which is currently not fully understood nor delineated, triggers migration and specification towards myoblast lineage through down-regulation of Pax7. Newly formed myoblasts are then able to commit towards myocyte lineage and either fuse with existing myofibers or fuse with each other, to initiate repair and regeneration (Bentzinger, Wang et al. 2013). Critical to satellite cell activation is the maintenance of the satellite cell pool. It has been proposed that this is accomplished through initial asymmetric division of satellite cells following activation, with one daughter cell remaining in a quiescent, Pax7 expressing state, and the other daughter cell undergoing myogenic differentiation (Motohashi and Asakura 2014, Dumont, Wang et al. 2015).

1.3.2 Muscle Hyperplasia and Hypertrophy

Increases in muscle mass can occur through hyperplasia and hypertrophy. Hyperplasia refers to the increase muscle mass evident due to regulation of myocyte turnover and rapid muscle regeneration, while hypertrophy refers to an increased muscle mass through a regulation of protein turnover (Costamagna, Costelli et al. 2015). Overall these responses act to increase muscle mass through increasing myocyte production and promoting an increase in the rates of muscle protein synthesis over degradation (Schiaffino, Dyar et al. 2013).

1.3.3 Muscle Atrophy

Skeletal muscle atrophy is characterized by a decrease in muscle mass due to a loss of cellular organelles, cytoplasm, and protein. These changes are a reflection of activation of
pathways regulating protein turnover, promoting a net increase in the rates of protein degradation relative to the rates of protein synthesis (Sartorelli and Fulco 2004). Our current understanding of the molecular regulation of skeletal muscle mass is derived from the use of multiple animal, predominantly rodent, models of induced-atrophy which demonstrate net increased protein degradation in the onset and persistence of skeletal muscle atrophy.

Experimental animal models used to mimic muscle disuse-induced atrophy include denervation, joint immobilization, tenotomy and tail suspension models. In denervation models, typically hindlimb muscles are experimentally denervated via transection of the sciatic nerve (Holecek 2012). Crushing or transection of the tibial nerve however, provides a model to induce targeted atrophy of the gastrocnemius, soleus, and plantaris muscles and reduces the associated animal morbidity (Batt and Bain 2013). Utilization of this procedure appears to be the most reliable model for assessing disuse-induced atrophy, as it provides the advantage of using innervated hindlimb muscles from the contralateral side, as a comparative control. Rodent tail suspension models of induced-atrophy are also used, but they appear to be more applicable to microgravity than a disuse state (Ohira, Yoshinaga et al. 2002). Joint immobilization models (casting, surgical joint fixation) are being less utilized over recent years due to the fact that they cause stress responses to animals, confounding results. Other atrophy induction models that have been used include neuromuscular blockade and fasting (Larsson 2007, Holecek 2012, Roseno, Davis et al. 2015), and models where metabolic syndromes with resultant muscle wasting are recapitulated, including, for example, the induction of diabetes mellitus with streptozocin (Nonaka, Une et al. 2014), and the use of corticosteroids (Gilson, Schakman et al. 2007, Zhao, Pan et al. 2008).
Although several proteolytic systems are known to be involved in mammalian protein catabolism such as the caspase-3 activation and oxidative stress responses, most recent studies indicate that calcium-dependent calpain, lysosome/autophagy, and the ubiquitin-proteasome systems (UPS) play predominate roles in the mediation of muscle atrophy (Bonaldo and Sandri 2013, Sandri 2013, Schiaffino, Dyar et al. 2013, Huang and Zhu 2016).

**The Calcium-Dependent Calpain System**

Calpains are a family of ubiquitously expressed calcium-dependent cysteine proteases, with the founding members being μ- and m-calpains. These enzymes are heterodimers composed of two subunits: an 80 kDa catalytic domain and a 30 kDa regulatory domain (Goll, Thompson et al. 2003). Calpain activation occurs in a calcium-dependent manner with calcium binding to the subunits, causing a conformational change exposing active sites and initiating proteolytic activity (Ono and Sorimachi 2012). Increased calcium uptake in muscle has been reported in chronic diseases known to be associated with muscle wasting such as sepsis (Benson, Hasselgren et al. 1989, Fischer, Sun et al. 2001), burn injury (Sayeed 2000), and cancer (Costelli, Bossola et al. 2002), providing the potential for calpain activation. Structural sarcomeric proteins, including titin and nebulin are known calpain substrates (Smith, Lecker et al. 2008). Evidence suggests that calcium-dependent proteolysis plays a role in the development of muscle wasting associated with sarcopenia and cachexia (Dargelos, Poussard et al. 2008, Brule, Dargelos et al. 2010), and μ-calpains knockout studies have shown attenuation of skeletal muscle protein proteolysis (Geesink, Kuchay et al. 2006).
**The Lysosome/Autophagy System**

Autophagy is an evolutionarily conserved pathway involved in the lysosomal-mediated degradation of damaged cellular proteins and organelles. The pathway involves the endosomal internalization of proteins, cytoplasm, or organelles through assembly of phagosomes, followed by their subsequent fusion with lysosomes. Assembly of the phagosome whereby intracellular components form an endosome can occur in one of three ways: chaperone-mediated autophagy (CMA), microautophagy, or macroautophagy (Levine, Mizushima et al. 2011). In CMA, chaperone proteins selectively target proteins with KFERQ-like sequences to form a complex which is able to be transported to lysosomes for degradation (Liu, Huang et al. 2015). Microautophagy is a less selective process whereby sections of the cytoplasm invaginate from membranes into vesicles (Sahu, Kaushik et al. 2011). Macroautophagy is a targeted isolation of large quantities of cytoplasmic components into a double-membrane autophagosome (Yang and Klionsky 2010). Following vesicle formation by one of these three methods, fusion with multivesicular bodies (MVB) and lysosomes occurs. This fusion allows for lysosomal hydrolases, which are hydrolytic enzymes functioning optimally at the acidic microenvironment found within the lysosome, to elicit their degradative effects on phagosome contents. The degradation products can then be recycled into the cytoplasm to be utilized for biosynthesis or ATP production (Eskelinen and Saftig 2009).

In recent years, the lysosome/autophagy system has been found to be involved in muscle wasting associated with cancer (Penna, Costamagna et al. 2013), fasting (Mizushima, Yamamoto et al. 2004), disuse (Brocca, Cannavino et al. 2012), COPD (Taivassalo and Hussain 2015), and denervation (O’Leary, Vainshtein et al. 2012). In many of these conditions activation of the lysosome/autophagy system is reported to be involved in the effective removal of accumulated
defective proteins and damaged organelles. Inhibition of the system causes these accumulated products to compound, compromising myofiber integrity (Masiero, Agatea et al. 2009). For example, muscle-specific deletion of the important autophagy promoting gene Atg7 in mice resulted in the development of muscle atrophy and diminished functional capacity due to accumulation of abnormal mitochondria and disorganization of sarcomeric components (Masiero, Agatea et al. 2009). Removal of dysfunctional mitochondria is essential to the maintenance of healthy muscle; the absence of which induces muscle wasting due to increased oxidative stresses. Over-expression of the mitochondrial autophagy transcriptional co-activator peroxisome proliferator gamma coactivator-1α (PGC-1α) resulted in protection from denervation-induced reduction in mitochondria and muscle mass (Vainshtein, Desjardins et al. 2015).

In contrast, increased autophagic activity is evident in wasted denervated muscle (Schiaffino and Hanzlikova 1972, O'Leary and Hood 2009, O'Leary, Vainshtein et al. 2012, Furuya, Ikeda et al. 2014) suggesting excessive autophagy in itself may induce muscle atrophy through activation of autophagic cell death pathways. Elevated levels of autophagy promoting proteins microtubule-associated protein 1 light chain 3 (LC3) and Beclin1 are reported in mice post-denervation at 7 and 14 days respectively (O'Leary and Hood 2009). These studies point towards a dynamic role of autophagy in muscle maintenance, with increased autophagy mediating atrophy via degradation of cytosol and contractile proteins and inadequate autophagy inducing atrophy through generation of aggregated damaged proteins leading to muscle degeneration. More studies are needed to further elucidate the regulation of this critical balance of autophagy in the mediation of muscle loss.
The ubiquitin-proteasome system is the principle mechanism involved in protein catabolism across mammalian cells types. Central to this pathway is ubiquitin, which is a 76 amino acid residue protein highly conserved across eukaryotes. The discovery and characterization of the function of ubiquitin earned Avram Hershko, Aaron Ciechanover and Irwin Rose the Nobel Prize for Chemistry in 2004 (Ciechanover and Iwai 2004, Ciechanover 2005, Hershko 2005, Rose 2005). This work identified that linkage of ubiquitin to target proteins within a cell served as a recognition tag for downstream processing. Ubiquitination is now known to serve as a modification involved in the regulation of several cell processes including, for example, cell proliferation, cell differentiation, signal transduction, protein trafficking, immune response, apoptosis and proteasome mediated proteolysis (Welchman, Gordon et al. 2005). This range of functions is determined by the nature of the targeted ubiquitin linkage. Proteins can undergo addition of a single ubiquitin moiety, termed monoubiquitination, or addition of a multi-ubiquitin chain, termed polyubiquitination (Figure 1.4). Addition of the initial ubiquitin unit occurs through a covalent bond between the ubiquitin carboxyl-terminal glycine residue, and a substrate lysine residue (Wang and Maldonado 2006). In polyubiquitination, subsequent ubiquitin molecules are conjugated to each other by one of seven internal lysine residues: K6, K11, K27, K29, K33, K48 and K63 (Ikeda and Dikic 2008). K48-linked and K11-linked polyubiquitination chains target proteins for proteasomal-mediated degradation, while monoubiquitination, multiubiquitination, branched/atypical polyubiquitination, and K63-linked ubiquitination generally target proteins for other cellular processes such as endocytosis, protein trafficking, viral budding, NF-κB signaling, and DNA repair mechanisms (Ikeda and Dikic 2008, Sadowski, Suryadinata et al. 2012).
Figure 1.4. Different ubiquitin linkage modifications and their functional roles. Protein substrates can undergo several different types of ubiquitin modification, each of which determine functional roles and sorting. Monoubiquitination has been implicated in viral budding, endocytosis, and DNA repair, while multiubiquitination causes solely endocytosis. K48 polyubiquitination is the most common pattern, and both K48 and K11 linkages lead to proteasomal degradation. K63 polyubiquitination has been shown to participate in NF-κB signaling, receptor endocytosis, and DNA repair. Other atypical and branched lysine polyubiquitination patterns elicit unknown responses (Ikeda and Dikic 2008, Sadowski, Suryadinata et al. 2012).
The ubiquitin-proteasome system is dependent on three key enzyme groups: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes/ubiquitin-carrier proteins (E2) and ubiquitin ligases (E3) (Glickman and Ciechanover 2002) (Figure 1.5). The first step in the ubiquitination cycle involves an ATP-dependent reaction whereby E1 enzymes activate and bind ubiquitin, forming a high-energy thiol ester intermediate (Hershko, Heller et al. 1983). The next step involves the transfer of the activated ubiquitin to E2 enzymes. E2 enzymes facilitate the covalent attachment of the activated ubiquitin moieties to either target proteins, or to E3 ubiquitin ligase intermediates. E3 ubiquitin ligases confer specificity to the system, by recognizing a narrow range of target substrates through precise protein interaction domains. There are an estimated 600 different E3 ubiquitin ligases in humans, each conferring specificity to particular target proteins (Nakayama and Nakayama 2006). Under most circumstances, E3 ligases act as scaffolds to stabilize a complex consisting of the E2-ubiquitin intermediate and a substrate protein, allowing for the transfer of ubiquitin from the E2 enzyme to the substrate, as is the case with really interesting new gene (RING) -finger E3 ubiquitin ligases. In some circumstances, the activated ubiquitin is transferred from the E2 enzyme to the E3 ligase as an intermediate. The E3 ligase then transfers the ubiquitin directly to the target substrate, giving it a more catalytic role, as is the case with homologous to the E6AP carboxyl terminus (HECT) domain family E3 ligases (Glickman and Ciechanover 2002).

Polyubiquitinated proteins are often shuttled to the 26S proteasome for their targeted degradation. The 26S proteasome consists of two subcomplexes: the 20S protease containing subunit and the 19S regulatory subunit (Zwickl, Voges et al. 1999). The 20S proteolytic subunit is a cylindrical structure composed of four rings, while the 19S subunit forms a lid structure involved in the recognition of ubiquitinated substrates. Upon substrate recognition by the 19S
Figure 1.5. The ubiquitin-proteasome system. The UPS is the major proteolytic mechanism within the cell. The first step involves ubiquitin activation through E1 enzymes which activate the carboxyl-terminal glycine residue of a ubiquitin moiety. The next step involves ubiquitin conjugation to cysteine residues of E2 enzymes. The final step involves ubiquitin ligation to target substrates. This can occur through two mechanisms depending on the classification of E3 ubiquitin ligase involved. RING finger E3s act as scaffolds to allow for direct transfer of ubiquitin from E2 enzyme to substrate, while HECT domain E3s act as intermediates facilitating the transfer of ubiquitin from E2 to E3 and then subsequently to substrate.
subunit, ubiquitin chains are removed and ATPases facilitate the unfolding of proteins to allow for degradation by the 20S subunit (Durairaj and Kaiser 2014). Removal of ubiquitin units from its conjugates is a crucial step in maintaining a cellular pool of ubiquitin moieties. This process is facilitated by deubiquitination enzymes (DUBs). DUBs also serve as a proofreading mechanism within the cell, deubiquitinating substrates to inhibit their degradation (Lam, Xu et al. 1997). Although much research has shown an involvement of the ubiquitin-proteasome system in muscle atrophy, little is known about the role of deubiquitination. The largest class of deubiquitinating enzymes are ubiquitin-specific proteases (USPs), and knockdown of USP19 in vitro resulted in increased myosin heavy chain protein degradation (Sundaram, Pang et al. 2009).

For years it has been appreciated that the UPS is a predominant and critical proteolytic process activated in, and mediating, skeletal muscle atrophy in acute and chronic disease states (Wing and Goldberg 1993, Mitch, Medina et al. 1994, Price, Bailey et al. 1996). Despite this long standing recognition, key regulatory E3 ligases in muscle remained unknown until 2001. At that time Atrogin-1/muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1), the first two muscle specific ubiquitin ligases identified, were cloned and definitively demonstrated to be positive regulators of skeletal muscle wasting (Bodine, Latres et al. 2001, Gomes, Lecker et al. 2001). Over the following decade, we and others have identified a handful of additional E3 ubiquitin ligases that mediate muscle wasting; these enzymes demonstrate differential temporal recruitment during the wasting of muscle, with some specificity in the upstream stimulus inducing atrophy; unloading/inactivity versus metabolic pathologic processes. Nedd4-1 is one of these E3 ligases.
1.3.4 *Upstream Regulatory Networks of Muscle Atrophy*

Muscle wasting is induced by a number of upstream stressors such as neural inactivity, mechanical unloading, inflammation, metabolic stress, and elevated glucocorticoids (Bonaldo and Sandri 2013) which result in a multiplicity of biologic phenomenon such as mitochondrial dysfunction, production of reactive oxidative species (ROS), and increased cytokine generation. These stressors engage intracellular signaling networks known to regulate protein degradation machinery, which ultimately affect muscle fiber size via control of protein turnover (Romanello and Sandri 2015). Although a number of signaling networks can be involved in the regulation of protein turnover, two signaling pathways, the insulin-like growth factor 1-thymoma viral oncogene homolog 1 (IGF1-Akt) network (Sandri, Barberi et al. 2013, Schiaffino, Dyar et al. 2013) and the Myostatin/TGFβ-Smad signaling pathway, play a predominant role in the regulation of skeletal muscle mass. These regulatory networks simultaneously act towards controlling opposing pathways of protein synthesis and protein degradation (Figure 1.6).

*IGF1/PI3K/-Akt Pathway*

Under catabolic conditions, up-regulation of UPS and autophagy promoting genes can be mediated by the Forkhead box O (FoxO) transcription factors. FoxO is normally negatively regulated in skeletal muscle by the Akt pathway (Schiaffino, Dyar et al. 2013). There are four members of the FoxO family of transcription factors, FoxO1, FoxO3, FoxO4, and FoxO6, however FoxO1, FoxO3, and FoxO4 are the most thoroughly studied with regards to the regulation of skeletal muscle mass (Bonaldo and Sandri 2013). FoxO1 mRNA skeletal muscle expression has been shown to be increased in muscle atrophied by denervation and inactivity (Lecker, Jagoe et al. 2004, Sacheck, Hyatt et al. 2007), and both FoxO3 and FoxO4 activation
Figure 1.6. Signaling pathways involved in the regulation of the atrophic response. Three major stimulants of the atrophic response in muscle that act to affect protein synthesis and degradation are IGF1, myostatin, and TNFα. IGF1 acts through binding to its receptor to mediate Akt phosphorylation. This leads to downstream mTOR activation and GSK3β inhibition to promote protein synthesis, and FoxO inhibition to prevent protein degradation. Myostatin negatively regulates muscle growth by binding to its receptor ActRIIB to promote Smad2/3 phosphorylation and inhibit Akt.
occurs with starvation or dexamethasone treatment (Moylan, Smith et al. 2008). Upon activation, FoxO transcription factors are able to regulate the expression of E3 ubiquitin ligases such as MuRF (Waddell, Baehr et al. 2008) and autophagy promoting genes, including, but not limited to cathepsin A (Lapierre, Kumsta et al. 2015), Beclin1, and LC3 (Kang, Yeo et al. 2016) by binding to their promoters and initiating transcription. In order for FoxO transcription factors to target promoters they must undergo nuclear localization. Nucleocytoplasmic shuttling is controlled by post-translational phosphorylation of the FoxO proteins. The main kinase involved in FoxO phosphorylation is Akt, which sequentially phosphorylates at S253, S316, and lastly T24 (Vogt, Jiang et al. 2005). This signals 14-3-3 chaperone proteins to bind to FoxO, blocking nuclear localization signals and transporting the transcription factors out of the nucleus (Huang and Tindall 2007).

The Akt pathway is activated in skeletal muscle by binding of insulin or insulin-like growth factor 1 (IGF-1) to its receptor. This binding causes Akt phosphorylation and activation, initiating its kinase activity and allowing it to phosphorylate downstream substrates. Two other substrates that undergo Akt-mediated phosphorylation and activation are glycogen synthase kinase 3 beta (GSK-3β) and mechanistic target of rapamycin (mTOR) (Manning and Cantley 2007). Upon phosphorylation, mTOR acts to promote protein synthesis while GSK-3β inhibits protein synthesis. Therefore, Akt signaling promotes muscle growth by activating protein synthesis through GSK-3β and mTOR signaling, while inhibiting protein degradation through preventing nuclear localization of FoxO (Peng, Xu et al. 2003), (Bodine, Stitt et al. 2001).

While the PI3K/AKT signaling network appears to be critically important in the nucleocytoplasmic shuttling of FoxO transcription factors in muscle, other signaling networks have also been demonstrated to directly affect FoxO activation, such as AMP-activated protein
kinase (AMPK) (Lee, Ochi et al. 2015), PGC-1α (Kang and Ji 2016), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (McCarthy and Esser 2010). Similarly, tyrosine or serine/threonine kinases other than PI3K, have been shown to activate Akt directly, in response to growth factors, inflammation or DNA damage. Akt can be activated in response to heat shock or increases in cellular Ca^{2+} concentration, via Ca^{2+}/Calmodulin-dependent protein kinase kinase (CAMKK) (Song, Salmena et al. 2012).

Myostatin/Smad

Myostatin is a protein expressed predominantly in skeletal muscle, belonging to the TGF-β family of proteins. It is a negative regulator of skeletal muscle growth and differentiation. Muscle myostatin levels are increased in a variety of catabolic states including, for example, ageing (Yarasheski, Bhasin et al. 2002), bed rest (Zachwieja, Smith et al. 1999, Reardon, Davis et al. 2001), AIDS (Gonzalez-Cadavid, Taylor et al. 1998), and heart failure (Gruson, Ahn et al. 2011). In mice, deletion of myostatin produces a phenotypic change resulting in increased size and numbers of skeletal muscle fibers (McPherron, Lawler et al. 1997). Also, a naturally occurring mutation in the myostatin gene of Blonde d'Aquitaine cattle results in a hypertrophic muscle phenotype (Bouyer, Forestier et al. 2014).

In skeletal muscle, myostatin binds to a propeptide which keeps the protein in an inactive state. Following activation by proteolysis or an increase in concentration of free radicals, myostatin is released allowing it to bind to the high-affinity type-2 activin receptor ActRIIB (Lee 2008). This binding results in activation of type-1 activin receptor serine kinase (ALK4/5) which phosphorylates Smads2/3 to change transcriptional activity leading to downstream atrophic responses (Han and Mitch 2011). Inhibition of the myostatin signaling pathway has been
evaluated as a treatment to increase muscle mass in preclinical trials; cynomolgus monkeys treated with FS344 (a myostatin-sequestering protein) caused an increase in muscle mass and strength (Kota, Handy et al. 2009). Utilization of myostatin inhibition therapies in muscle wasting are being evaluated as a potential treatment in humans.

1.4 E3 UBIQUITIN LIGASES AND ATROPHY

Following identification of E3 ubiquitin ligases Atrogin-1 and MuRF1 as novel regulators of muscle atrophy (Bodine, Latres et al. 2001, Gomes, Lecker et al. 2001), these genes were termed “atrogenes”, indicating they induce muscle atrophy. Two main classes of E3 ubiquitin ligases exist: HECT domain family, and RING and RING-related E3s. RING-related E3s designate the sub-family consisting of plant homeodomain (PHD) and leukemia associated protein (LAP) finger proteins, and members of the U-box family (Metzger, Hristova et al. 2012). Identification of other E3 ubiquitin ligase atrogenes and their specific substrate targets has been a major focus of research into the molecular regulation of skeletal muscle atrophy. This results from the fact that E3 ubiquitin ligases are the UPS enzymes that confer specificity to this proteolytic machinery, by binding their target substrates through very precise protein-protein interaction domains. Identification of E3 ligases which mediate atrophy thus provides potential novel therapeutic targets to inhibit the onset and attenuate persistence of skeletal muscle atrophy in acute and chronic illness.
The First Identified Muscle Specific Ubiquitin Ligases: Atrogin-1/MAFbx and MuRF1

Atrogin-1/MAFbx is a RING finger E3 ubiquitin ligase selectively expressed in skeletal and cardiac muscle. In preclinical models including *in vitro* overexpression studies, atrogin-1 promotes myotube atrophy and mice deficient in atrogin-1 are partially protected against denervation- and fasting-induced skeletal muscle atrophy (Bodine, Latres et al. 2001, Cong, Sun et al. 2011). Atrogin-1 has been shown to be upregulated under a variety of atrophy-inducing conditions in humans such as immobilization (Jones, Hill et al. 2004), mechanical ventilation (Levine, Nguyen et al. 2008), and COPD (Doucet, Russell et al. 2007). Atrogin-1 contains four conserved domains: an F-Box motif, a bipartite nuclear localization sequence, a cytochrome c facility heme-binding site signature, and a PDZ domain (Gomes 2001). Identification of cellular targets of atrogin-1 mediated ubiquitination has been a research area of substantial interest. The presence of two nuclear localization signals within the enzyme suggests involvement in the targeting of transcription factors. Thus far, two well established targets of atrogin-1 in skeletal muscle have been identified; the myogenic regulatory factor MyoD (Tintignac, Lagirand et al. 2005) and eukaryotic translation initiation factor 3 subunit f (eIF3-f) (Lagirand-Cantaloube, Offner et al. 2008). Both MyoD and eIF3-3 are required to initiate expression of muscle structural proteins and MyoD is required for satellite cell activation, proliferation and the transitioning to differentiation. Both have been shown to undergo ubiquitin-mediated degradation in the presence of atrogin-1, although further validation *in vivo* demonstrating this induces muscle atrophy is required (Bodine and Baehr 2014).

Muscle RING finger 1 (MuRF1), identified at the same time as atrogin-1, is the second muscle-specific RING finger E3 ubiquitin ligase shown to regulate atrophy. MuRF1-null mice subjected to hind limb denervation experience significantly less muscle atrophy than littermate
controls (Bodine, Latres et al. 2001), and an attenuated atrophic response in muscle following hind limb unloading (Labeit, Kohl et al. 2010) and glucocorticoid treatment (Baehr, Furlow et al. 2011). In humans, increased activity of MuRF1 has been reported following immobilization (de Boer, Selby et al. 2007), bed rest (Ogawa, Furochi et al. 2006), mechanical ventilation (Levine, Nguyen et al. 2008), COPD (Doucet, Russell et al. 2007), and ageing (Raue, Slivka et al. 2007). MuRF1 contains a canonical N-terminal RING domain followed by five potential binding regions: a MuRF family conserved region, a zinc-finger domain, a leucine-rich coiled-coil domain, and an acidic C terminal tail (Foletta, White et al. 2011). Reported substrate targets of MuRF1 are mainly structural proteins, suggesting a role in regulating muscle integrity and remodeling. For example, MuRF1 is shown to bind to titin at the M-line to regulate kinase activity, potentially mediating downstream signaling pathways (Centner, Yano et al. 2001). MuRF1 has also been shown to interact and mediate degradation of myosin binding proteins such myosin-binding protein C (MyBP-C) and myosin light chain-1/2 (MyLC1/2) following denervation or fasting (Cohen, Brault et al. 2009). This selective ubiquitination may be one of multiple mechanisms contributing to thick filament disassembly in atrophy. Despite these findings, inhibitor studies have failed to show an attenuation of atrophy under catabolic conditions, suggesting it is one of multiple degradation drivers in atrophy and inhibiting only its contribution is inadequate to elicit a substantial impact (Foletta, White et al. 2011).

Nedd4-1 and other E3 Ubiquitin Ligases Mediating Muscle atrophy

Since discovery of atrogin-1 and MuRF1, a small number of other E3 ubiquitin ligases shown to be associated with the regulation of skeletal muscle atrophy, have been identified. Muscle ubiquitin ligase of SCF complex in atrophy-1 (MUSA1) is an F-box protein shown to be upregulated upon denervation, and shRNA knockdown of MUSA1 protein partially prevented
denervation-induced atrophy in mice (Sartori, Schirwis et al. 2013). Tripartite motif containing 32 (TRIM32) is a ubiquitously expressed E3 ubiquitin ligase whose mutation has been linked with muscle dystrophy (Kudryashova, Kudryashov et al. 2005, Schoser, Frosk et al. 2005). Trim32-knockout mice are shown to have impaired skeletal muscle growth following hindlimb unloading, suggesting a possible role for the E3 ubiquitin ligase in regulating satellite cell proliferation and differentiation (Kudryashova, Wu et al. 2009). Recently, TNF receptor adaptor protein 6 (TRAF6), an adaptor protein possessing E3 ubiquitin ligase activity, has been shown to be upregulated in skeletal muscle following denervation (Paul, Gupta et al. 2010) and starvation (Paul, Bhatnagar et al. 2012).

Our lab has identified the HECT domain ubiquitin ligase neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4-1; reviewed in detail in the following section 1.5) as a positive regulator of skeletal muscle atrophy. We and others have reported an increase in the expression of Nedd4-1 in skeletal muscle post denervation (Batt, Bain et al. 2006, Koncarevic, Jackman et al. 2007, Nagpal, Plant et al. 2012), hind limb unloading (Koncarevic, Jackman et al. 2007), and in the wasted muscle of individuals with COPD (Plant, Brooks et al. 2010). Nedd4-1 muscle specific knockout mice are partially protected from inactivity induced (denervation, tenotomy) muscle atrophy. Interestingly, although atrogin-1 and MuRF1 transcript levels were elevated in rats after short term denervation (1 month), they returned to baseline expression with long term denervation (3 months). Nedd4-1 transcript and protein expression, however, exhibits sustained long term increases (Batt, Bain et al. 2006) suggesting a potential role in not only the initiation, but also in the maintenance, of an atrophic response making Nedd4-1 potentially more functionally relevant when considering methods by which to inhibit the development of muscle wasting.
1.5 NEDD4-1 - STRUCTURE AND FUNCTION

1.5.1 Nedd4 Family

Nedd4-1 is a member of an evolutionary conserved family of ubiquitously expressed HECT domain ubiquitin ligases. It is highly expressed in kidney, liver, muscle, brain, and heart (Trotman, Wang et al. 2007). In mammals, there are nine members of the Nedd4 family of proteins including NEDD4-1 (NEDD4), NEDD4-2 (NEDD4L), ITCH, SMURF1, SMURF2, WWP1, WWP2, NEDL1, and NEDL2 (Yang and Kumar 2010). Of this family of proteins, Nedd4-1 and Nedd4-2 are most closely related, with Nedd4-1 being the ancestral member and Nedd4-2 undergoing gene duplication later in evolution (Figure 1.7) (Fotia, Cook et al. 2006). Both Nedd4-1 and Nedd4-2 are expressed in skeletal muscle, but Nedd4-1 is the predominate isoform (Yang and Kumar 2010).

1.5.2 Nedd4-1 Structure

Nedd4-1 protein contains an N-terminal C2 domain, 3-4 WW domains, and a C-terminal catalytic HECT domain. Human, drosophila, and xenopus Nedd4-1 all contain 4 WW domains, while mouse and rat Nedd4-1 contains 3 WW domains (Yang and Kumar 2010) (Figure 1.7). The HECT domain of Nedd4-1 contains approximately 350 amino acid residues, functioning in the transfer of cysteine-bound activated ubiquitin to a lysine residue on a substrate protein (Ingham, Gish et al. 2004, Chen and Matesic 2007). The C2 domain is roughly 120 amino acids in length and acts as a calcium-binding domain. Calcium binding causes activation of the C2 domain, leading to plasma membrane localization through interactions with phosphatidylinerine, phosphatidylinositol, and phosphatidylcholine liposomes (Plant, Yeger et al. 1997). Interestingly,
Figure 1.7. Schematic diagram of Nedd4-1 and Nedd4-2 across species. All proteins consist of an N-terminal calcium/lipid binding C2 domain, 3 or 4 WW domains that interact with protein substrates, and a catalytic C-terminal HECT domain. Human Nedd4-1 (hNedd4-1; 900 aa), drosophila Nedd4-1 (dNedd4-1; 834 aa), xenopus Nedd4-1 (xNedd4-1; 971 aa), and human Nedd4-2 (hNedd4-2; 975 aa) all contain 4 WW domains while rat (rNedd4-1; 887 aa) and mouse (mNedd4-1; 957 aa) Nedd4-1 only have three, missing the corresponding WW3 domain.
within the Nedd4-1 protein, an auto-inhibition mechanism exists to negatively regulate catalytic activity through binding between the C2 domain and the HECT domain (Wiesner, Ogunjimi et al. 2007, Wang, Peng et al. 2010). This auto-inhibition loop is dependent on tyrosine phosphorylation sites present in both the C2 domain (Tyr43) and the HECT domain (Tyr585). Phosphorylation of these residues acts to dissociate the C2-HECT interaction, allowing for increased catalytic activity of the enzyme (Wang, Peng et al. 2010, Persaud, Alberts et al. 2014).

Nedd4 proteins are able to canonically interact with substrates via binding between their WW domains and the substrate PY (PPXY) motifs. WW domains are classic protein: protein interaction domains composed of 38-40 amino acid residues. Within these regions contain two conserved tryptophan (W) residues spaced 21 amino acids apart (Ingham, Gish et al. 2004). Structural analysis of the WW-substrate complex has shown that the second tryptophan is involved in substrate binding, while the first tryptophan is stabilizes the complex (Macias, Gervais et al. 2000, Zarrinpar and Lim 2000). WW domains are separated into various classes dependent upon their binding affinity to particular motifs. Human Nedd4-1 WW domains are characterized as Class 1 due to their recognition of a PY motif (Kay, Williamson et al. 2000). Each of the WW domains of human Nedd4-1 bind to PY motifs with different affinities; the third WW domain (WW3) displays strongest binding affinity, WW2 and WW4 display moderate binding affinity, and WW1 displays very weak binding affinity (Lott, Coddington-Lawson et al. 2002). Binding affinities are substrate specific, and some variation may exist dependent upon the Nedd4 target. Interestingly, an non PY novel binding motif (VLLVRPSRLSSS) for Nedd4-1 was recently indentified in FGFR1 (Persaud, Alberts et al. 2011). Whether this is a new class of motif that will be found in other Nedd4-1 substrates, or unique to FGFR1 remains to be determined.
1.5.3 Nedd4-1 Substrates and Functions

Although Nedd4-1 is closely related to Nedd4-2, each protein demonstrates several distinct and non redundant functions, due in part to the ability of each to associate with unique substrates. For example, Nedd4-2 is known to be involved in the regulation of action potentials by targeting the voltage-gated sodium channel Nav1.5 (Huang, Wang et al. 2016), anion transport through ubiquitination of protein kinase C (PKC) (Xu, Wang et al. 2016), and surfactant protein C (SP-C) trafficking by alveolar type-II epithelial cells (Conkright, Apsley et al. 2010). Nedd4-1 is unable to target these same substrates to elicit similar functions. In contrast, Nedd4-1 has demonstrated specificity for substrates in the regulation of cardiac, vascular and neuromuscular function. I provide a brief review of the breadth and scope of known Nedd4-1 substrates, and the cellular functions influenced, in the following section.

Growth Factor Receptor and Signalling Regulation

Nedd4-1 has been shown to be involved in both development and post natal growth processes via its negative regulation of a number of growth factor receptors. Nedd4-1 is reported to bind, and ubiquitinate FGFR1, leading to its endocytosis and termination of its downstream signaling, an interaction which if blocked leads to differentiation of neural stem cells (Persaud, Alberts et al. 2011). Epithelial growth factor receptor (EGFR) (Katz, Shtiegnan et al. 2002) and vascular endothelial growth factor receptor-1 (VEGF-R2) (Murdaca, Treins et al. 2004) have similarly been shown to be regulated by Nedd4-1 in vitro. While Nedd4-1 is able to directly bind and ubiquitinate VEGF-R2, targeting it for degradation, (Murdaca, Treins et al. 2004), it regulates EGFR through binding and ubiquitinating an adaptor protein of EGFR called Hgs to negatively influence EGFR endocytosis (Katz, Shtiegnan et al. 2002). In contrast, Nedd4-1
appears to positively regulate IGFR1, as analysis of Nedd4-1 null mice revealed reduced insulin and IGF-R1 signaling (Cao, Lill et al. 2008), which was associated with neonatal lethality and significant global growth retardation; Nedd4−/− newborns weighed on average 65% less than their wild-type littermates (Cao, Lill et al.). The mechanism by which Nedd4-1 regulates IGF-R1 is still unclear. It has been proposed that Nedd4-1 regulates IGF-R1 indirectly through growth factor receptor-bound protein 10 (Grb10), which is an adaptor protein that binds and inhibits IGF-R1. Nedd4-1 downregulates Grb10, which in turn promotes IGF-R1 growth signaling (Yang and Kumar 2010).

Neuromuscular Junction

Early studies done in Drosophila melanogaster have shown that dNedd4, the Drosophila orthologue to human Nedd4-1, regulates transmembrane proteins involved in the stability of the neuromuscular junction such as roundabout (Robo) receptor and Commissureless (Comm). dNedd4-1 knockdown causes defective Robo and Comm endocytosis, leading to increased accumulation of each of these proteins at the neuromuscular junction and innervations problems. (Myat, Henry et al. 2002, Ing, Shteiman-Kotler et al. 2007). Analysis of Nedd4-1 null mice generated by Liu and colleagues, revealed an embryonic/perinatal lethal phenotype with severe defects in neuromuscular junction formation. In these Nedd4-1 mutants, motorneuron numbers were reduced and fail to effectively facilitate an interaction between nerve and muscle (Liu, Oppenheim et al. 2009, Kawabe, Neeb et al. 2010). Nedd4-1-null neurons also exhibited impaired dendritogenesis through reduced dendrite extension, aborization, and synaptic transmission through ubiquitination of Rap2A, a regulator of neurite development (Kawabe, Neeb et al. 2010).
**Phosphatidylinositol Metabolism and Endosome Trafficking**

Our lab has shown a potential role for Nedd4-1 in the regulation of endosomal trafficking through interaction with myotubularin-related protein 4 (MTMR4). MTMR4 is a tyrosine/dual specificity phosphatase containing a catalytic phosphatase (PTP) domain, a phospholipid binding FYVE domain, and a PY motif which is able to bind to the third and fourth WW domains of Nedd4-1 (Patterson, Brummer et al. 2009, Plant P, Correa et al. 2009). The myotubularin family of proteins are able to regulate endosomal trafficking through dephosphorylation of phosphatidylinositol 3-phosphate (PI3P) and phosphatidylinositol-3,5-bisphosphate (PI3,5P2) (Nandurkar and Huysmans 2002). PI3P is highly expressed in early endosomes, and precise regulation of its expression is necessary for effective trafficking of growth factor receptors such as EGFR (Morino, Kato et al. 2004, Lorenzo, Urbe et al. 2006, Patterson, Brummer et al. 2009). PI3,5P2 is involved in late endosome trafficking as PI3,5P2 deficient cells exhibit damaged endolysosomes and trafficking defects in the late endocytic pathway (Dong, Shen et al. 2010). Our lab identified MTMR4 as a novel Nedd4-1 substrate, providing another indirect mechanism whereby Nedd4-1 may influence the endocytosis of membrane bound growth factor, and other receptor, proteins. (Plant P, Correa et al. 2009).

**Cancer and Tumorigenesis**

Recently, a role for the Nedd4-1 ubiquitin ligase has been implicated in cancer and tumorigenesis due to its overexpression across a variety of human cancers such as gastric (Sun, Yu et al. 2014), colorectal (Hong, Moon et al. 2014), breast (Jung, Li et al. 2013), and lung (Amodio, Scrima et al. 2010). A proposed mechanism by which Nedd4-1 is able to promote tumor growth is through facilitating the ubiquitination and subsequent degradation of the tumor
suppressor phosphatase and tensin homolog (PTEN). Mutations in PTEN have been identified in a number of human cancers such as prostate and liver (Song, Salmena et al. 2012, Dillon and Miller 2014). Loss of PTEN causes increased PtdIns (3,4,5) (PIP₃) accumulation at the plasma membrane, which is able to phosphorylate and activate Akt to promote cell survival (Song, Salmena et al. 2012). Although Wang et al showed that Nedd4-1 targets PTEN with polyubiquitnation tags (Wang, Trotman et al. 2007), studies in Nedd4-1 null mice revealed that PTEN activity was unaffected by Nedd4-1 deficiency (Fouladkou, Landry et al. 2008). These apparent discrepancies may be explained by the fact that Nedd4-1 is also able to monoubiquitinate PTEN at Lys13 and Lys289 to facilitate its nuclear translocation instead of its degradation (Trotman, Wang et al. 2007). This finding may suggest that Nedd4-1 can both positively, through monoubiquitination, and negatively, through polyubiquitination, regulate PTEN function. Further work is necessary to establish what specific conditions or regulators are involved in determining the nature and pattern of Nedd4-1 mediated ubiquitination of PTEN.

**Cytoskeletal Organization**

Nedd4-1 is closely related to the single member of the Nedd4 family in yeast Saccharomyces cerevisiae, Rsp5 (Fang, Chan et al. 2014), which has been implicated in cytoskeletal organization as actin structural arrangement was reported to be defective in a strain lacking Rsp5 (Kaminska, Spiess et al. 2011). Over-expression of human Nedd4-1 in yeast negatively influences the dynamic organization of the actin cytoskeleton through inhibition of polymerization (Stawiecka-Mirota, Kaminska et al. 2008).
**Synaptic Transmission**

In neurons within the brain, α-Amino-3-hydroxy-5-methyl-isoazole-4-propionic acid receptors (AMPARs) undergo ubiquitination to facilitate their endocytosis and reduced cell surface localization which mediates excitatory synaptic transmission. Nedd4-1 has been shown to ubiquitinate AMPAR, leading to reduced surface membrane expression and diminished excitatory synaptic transmission (Lin, Hou et al. 2011). Recently, stress and cognitive impairment have been shown to increase Nedd4-1 expression (Wei, Xiong et al. 2016) suggesting a potential mechanism whereby Nedd4-1 may regulate stress-induced responses.

**Heart Development**

A role for Nedd4-1 in heart development has been implied as Nedd4-1 knockout mice exhibited an embryonic lethality, surviving until mid gestation, with heart and vasculature defects (Fouladkou, Lu et al. 2010). Thrombospondin-1 (Tsp-1), an inhibitor of angiogenesis, was shown to be significantly upregulated in Nedd4-1 knockout embryos. Although these results show a role for Nedd4-1 in heart and vasculature development, the exact pathway involved in the Nedd4-1 mediated regulation of Tsp-1 remains unknown.

1.6 **NEDD4-1 IN SKELETAL MUSCLE**

As previously noted, our lab and others have identified Nedd4-1 as a positive regulator of skeletal muscle atrophy. Nedd4-1 increases in expression in rodent models of muscle atrophy including denervation, unloading/inactivity (Koncarevic, Jackman et al. 2007, Plant, Correa et al.
2009, Nagpal, Plant et al. 2012) and tenotomy (unpublished results), and is increased in the wasted muscle of individuals with chronic COPD (Plant, Brooks et al. 2010). In contrast to the other few E3 ligases known to date to contribute to the induction of muscle wasting, increased Nedd4-1 expression is robust and durable, maintained long-term throughout the period of muscle loss suggesting it plays a more critical role in development of muscle atrophy. Since knockout of Nedd4-1 in mice produces an embryonic and/or perinatal lethal phenotype, (the variability is apparently dependent upon the mouse strain used and design of the gene deletion), we developed a Nedd4-1 skeletal muscle specific knockout (SMS-KO) mouse. In this animal the knockout cassette is under the control of the myogenin promotor, such that Nedd4-1 is retained in the satellite cell and proliferating myoblast, but is lost upon terminal differentiation to muscle. While the majority of Nedd4-1 SMS KO mice die hours after birth, from an apparent hypoventilatory defect due to diaphragm dysfunction (unpublished results), those that survive demonstrate normal peripheral skeletal muscle size and structure at baseline. However, upon denervation (tibial nerve transection) (Nagpal, Plant et al. 2012) and tenotomy (unpublished results), Nedd4-1 SMS-KO mice exhibit heavier muscle weights as well as an attenuation of decreased gastrocnemius Type II fiber cross-sectional area compared to littermate controls (Nagpal, Plant et al. 2012), confirming its role as a positive mediator of skeletal muscle atrophy.

Interestingly, very recently and while I was completing my thesis, the transcription factor Pax7, the crucial regulator of muscle satellite cell specification and maintenance, was reported to be a target of Nedd4-1 ubiquitination (Bustos, de la Vega et al. 2015). The expression of Pax7 is required for satellite cell activation, proliferation and self-renewal, and its deficiency results in the loss of satellite cell “stemness”, with commitment to progression to a terminally differentiated myocyte. Nedd4-1 monoubiquinates Pax7, facilitating its degradation by the
nuclear UPS machinery. The decreased Pax7 levels halt myoblast proliferation and promote precocious muscle differentiation, suggesting that Nedd4-1 plays an anti proliferation, pro differentiation role in regulating muscle regeneration. Had we been able to delete Nedd4-1 from satellite cells in our muscle specific knockout mice, we speculate the protective effect against muscle wasting might have been even more profound, as satellite cells proliferation may have been left unchecked.

The mechanism(s) by which Nedd4-1 mediates muscle wasting remains unknown and the skeletal muscle substrates of the ubiquitin ligase remain poorly characterized. We sought to identify the downstream substrates Nedd4-1 engaged to begin to delineate the responsible mechanisms. We searched for known Nedd4-1 substrates that had been reported to demonstrate decreased levels in denervated, and/or unloaded or immobilized muscle and identified three – MTMR4, FGFR1 and Notch1 (Koncarevic, Jackman et al. 2007, Plant, Correa et al. 2009, Persaud, Alberts et al. 2011). We reasoned that these proteins, should they be causally involved in the atrophic response, would be decreased in expression coincident to Nedd4-1 increases in wasted muscle, due to Nedd4-1 mediated ubiquitination and targeting for degradation. Conversely, the proteins would demonstrate stability of expression in Nedd4-1 null mature muscle. In addition, we required that the candidate substrates had the potential to positively influence muscle mass. Ectopic over-expression of FGFR1 in mouse gastrocnemius by electroporation significantly inhibited inactivity-induced atrophy, and FGFR1 is increased in myofibres that are partially resistant to inactivity-induced atrophy (Eash, Olsen et al. 2007). MTMR4 could influence growth factor receptor signalling by regulating endocytosis, via PI metabolism. Notch1, although not known to have a role in mature muscle, had been shown to
positively regulate satellite cell proliferation, and decreased expression of Notch-1 correlates with the onset of terminal differentiation to myocytes (Luo, Renault et al. 2005).

We were able to confirm that all three Nedd4-1 substrates decreased in denervated muscle, temporally coincident to the increase in Nedd4-1. However, none demonstrated attenuation of this decrease in the denervated Nedd4-1 SMS KO muscle, suggesting that they are NOT substrates mediating the effect of Nedd4-1 on skeletal muscle mass. Thus, Nedd4-1 appears to play a predominate role in the onset and maintenance of muscle atrophy, however the downstream substrates engaged to elicit this response remain to date, unknown. Identification of its substrates and signaling networks recruited, will enhance our understanding of the cellular mechanisms underpinning muscle atrophy, and ultimately, provide potential targets for the development of therapeutic interventions.
CHAPTER 2:

HYPOTHESIS AND AIMS

2.1 INVESTIGATION OF PDLIM7 AS A NOVEL TARGET OF THE UBIQUITIN LIGASE NEDD4-1 IN SKELETAL MUSCLE

Increased ubiquitin proteasome mediated proteolysis has been shown to contribute to the net increase in protein catabolism evident in muscle atrophied by inactivity. Our lab has previously identified Nedd4-1 as an E3 ubiquitin ligase which increases in expression in muscle atrophied by inactivity, and Nedd4-1 skeletal muscle specific knockout (SMS-KO) mice exhibit partial protection against muscle wasting (Nagpal, Plant et al. 2012). Targets of Nedd4-1 mediated ubiquitination in muscle remain poorly identified. In this research project for my Masters thesis, I set out to identify Nedd4-1 substrates and downstream signaling networks it engages in muscle, to begin to delineate the mechanism by which Nedd4-1 mediates muscle atrophy. Using a mass spectrometry approach to identify Nedd4-1 muscle substrates, we subjected Nedd4-1 SMS-KO mice to the tibial nerve transection model of denervation-induced muscle atrophy, and pursued proteins that were differentially expressed in the gastrocnemius muscle of the knockout compared to control wild type mice, as potential Nedd4-1 substrates. PDLIM7 (Enigma), a PY motif containing scaffolding protein, was one of a number of proteins that was significantly increased in the Nedd4-1 knock-out muscle, suggesting the absence of
Nedd4-1 was stabilizing its expression. I hypothesize that PDLIM7 is a bonafide Nedd4-1 substrate and a downstream target of Nedd4-1 in the positive regulation of skeletal muscle wasting. To test this hypothesis, I will undertake the following three specific aims:

**Aim 1: Confirm the mass spectrometry results and evaluate the expression of Nedd4-1 and PDLIM7 proteins in muscle.**

To confirm the mass spectrometry results, I will perform Western blot analyses for PDLIM7 and Nedd4-1, in protein lysates from skeletal muscle of *Nedd4-1* SMS-KO and littermate control mice, at baseline, and in response to denervation. If Nedd4-1 is affecting PDLIM7 expression, it would be expected that i) denervation-induced increases in Nedd4-1 would be associated with decreased PDLIM7 levels and ii) PDLIM7 protein reduction will be attenuated in the muscle of *Nedd4-1* SMS-KO mice compared to that of littermate control mice.

**Aim 2: Determine if PDLIM7 is a Nedd4-1 substrate**

To determine whether PDLIM7 is a Nedd4-1 substrate, I will assess whether the two proteins are able bind one another and whether this interaction occurs in canonical fashion through Nedd4-1 WW domain(s) and a PDLIM7 PY motif, or via an alternative method. I will analyze if Nedd4-1 binding to PDLIM7 results in PDLIM7 ubiquitination, and whether mutation
of the domains mediating this protein:protein interaction inhibits Nedd4-1/PDLIM7 binding and the subsequent ubiquitination of PDLIM7.

**Aim 3: Begin to determine the biologic relevance of the interaction between PDLIM7 and Nedd4-1 in skeletal muscle.**

To achieve this aim I will evaluate the expression, localization and interaction of endogenous PDLIM7 and Nedd4-1 in the C2C12 mouse muscle cell line *in vitro*, as the cells undergo natural progression from myoblast to myocyte and mature, fused myotubes. Should the studies in the two previous aims demonstrate that PDLIM7 is a Nedd4-1 substrate as anticipated, I will begin to evaluate the consequences of the ubiquitination of PDLIM7 in C2C12 cells.
CHAPTER 3:

PDLIM7 IS A NOVEL TARGET OF THE UBIQUITIN LIGASE NEDD4-1 IN SKELETAL MUSCLE


3.1 INTRODUCTION

Cellular ubiquitination is a proteolytic pathway, whereby ubiquitin moieties are covalently attached to specific proteins, often directing them to degradation by the 26S proteasome (Kornitzer and Ciechanover 2000, Mukhopadhyay and Riezman 2007). Specific targets of ubiquitin mediated proteolysis are largely determined by the E3 ligase involved in the cascade, which selectively binds to substrate proteins via interaction domains and facilitates the attachment of ubiquitin. Two main classes of E3 ubiquitin ligases exist: HECT domain family, and RING and RING-related E3s. RING-related E3s consist of three sub-families: plant homeodomain (PHD), leukemia associated protein (LAP) finger proteins, and members of the U-box family (Metzger, Hristova et al. 2012). The ubiquitin-mediated proteolytic pathway plays a
predominant role in the process of skeletal muscle atrophy resulting from illness or muscle disuse (Wang, Hu et al. 2006, Caron, Haroun et al. 2011, Foletta, White et al. 2011, An, Ganio et al. 2013, Sandri 2013, Bodine and Baehr 2014) and the E3 ubiquitin ligase Nedd4-1 has been shown to increase in models of skeletal muscle atrophy (Koncarevic, Jackman et al. 2007, Nagpal, Plant et al. 2012). Nedd4-1 is a ubiquitously expressed HECT domain E3 ubiquitin ligase that regulates stability and organization of a variety of proteins through interactions between its WW domain(s) and PY motifs of its substrates (Staub, Dho et al. 1996, Bouamr, Melillo et al. 2003, Henry, Kanelis et al. 2003, Kanelis, Bruce et al. 2006).

Nedd4-1 has been previously shown to mediate skeletal muscle atrophy; Nedd4-1 increases in expression in muscle atrophied by inactivity (i.e. denervation, unloading) and Nedd4-1 skeletal muscle specific knockout (SMS-KO) mice exhibit partial protection against muscle wasting (Plant, Correa et al. 2009, Nagpal, Plant et al. 2012). The mechanisms by which Nedd4-1 mediates this process remain unknown; as such, we sought to identify novel protein substrates of Nedd4-1 in muscle. Using a mass spectrometry approach, we subjected Nedd4-1 SMS-KO and wild-type mice to the tibial nerve transection model of denervation-induced muscle atrophy, and pursued proteins that were differentially expressed in the gastrocnemius muscle of the knockout compared to control wild type mice. We identified a PY motif-containing protein PDLIM7 (Enigma), that was significantly increased in the Nedd4-1 knock-out muscle, suggesting the absence of Nedd4-1 was affecting (stabilizing) it’s expression.

PDLIM7 contains an amino-terminal PDZ domain, three carboxyl-terminal LIM domains and a canonical PY (PPXY) motif (Te Velthuis, Isogai et al. 2007), and belongs to the PDZ-LIM family of proteins which act as scaffolds, binding actin-associated proteins including tropomyosin via the PDZ domain, and a range of signaling proteins via the LIM domains (Guy,
Kenny et al. 1999). These interactions are diverse, and function to mediate signal transduction, cell migration, and differentiation (Dawid, Breen et al. 1998, Fanning and Anderson 1999, Kadrmas and Beckerle 2004, Krcmery, Camarata et al. 2010) PDLIM7 is heavily expressed in both cardiac and skeletal muscle and a role for PDLIM7 in the process of muscle development has been documented. In zebrafish; PDLIM7 deficiency induces disorganized tail muscle fibers and muscle contractile dysfunction (Ott, Sakalis et al. 2007, Camarata, Krcmery et al. 2010).

We found that PDLIM7 and Nedd4-1 co-immunoprecipitate, interact via a WW domain-PY motif interaction and co-localize in differentiated myotubes. We show that increased Nedd4-1 expression in atrophying muscle is coincident with a decrease of PDLIM7, that Nedd4-1 ubiquitinates PDLIM7 and mutation of its PY motif inhibits Nedd4-1 mediated ubiquitination, suggesting that PDLIM7 is a true Nedd4-1 muscle substrate. We propose PDLIM7 may play a role in the development of Nedd4-1 mediated skeletal muscle atrophy.

3.2 MATERIALS AND METHODS

Muscle atrophy model

Nedd4-1 SMS-KO (myo$^{Cre}$;Nedd4-1$^{flox/flox}$) and sibling wild type control mice (myo$^{Cre}$;Nedd4-1$^{+/+}$), generated in our laboratory (Nagpal, Plant et al. 2012), were subjected to the tibial nerve transection model of denervation induced skeletal muscle atrophy, as previously described (Batt and Bain 2013). All procedures involving animals were carried out in accordance
with the Canadian Council on Animal Care guidelines and were approved by the Animal Research Ethics Board of McMaster University. Briefly, the right tibial nerve was transected in Nedd4-1 SMS-KO and control mice pairs under inhalational Halothane anaesthesia, resulting in complete denervation of the gastrocnemius muscle. The contralateral leg served as an internal control in each animal. Mice were maintained under conditions of routine care for 7 days after which they were sacrificed and the gastrocnemius muscles were harvested from the operated experimental limb and non-operated contralateral control limb. After rapid, atraumatic dissection, the muscle was snap frozen in liquid nitrogen and stored at -80°C for subsequent protein extraction.

**Mass Spectrophotometry**

Gastrocnemius muscle (10 mg) was minced and solubilized in 0.5-1.0 mL lysis buffer (20mM HEPES pH 8.0, 8M Urea). The solution was sonicated, and centrifuged at 20,000 xg for 15 min. The supernatant was collected and reduced at 4.1 DTT mM at 60°C for 20 min, and cooled to room temperature (RT). The solution was alkylated by addition of 8.3mM iodoacetamide to the supernatant at RT in the dark for 15 min, diluted with 20mM HEPES to a concentration of 2mM urea, and subsequently digested with bovine trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK-trypsin) overnight at RT. Trifluoroacetic acid (TFA) was added to a final concentration of 1%, and the solution was left at RT for 10 min. The digested peptides were collected in the supernatant after centrifugation at 2000 xg for 5 min at room temperature.

The peptides were loaded on a 7 cm pre-column (150µm i.d.) containing a Kasil frit packed with 3.5 cm 5µm Magic C18 100 Å reversed-phase material (Michrom Bioresources) followed by 3.5 cm Luna 5µm SCX 100 Å strong cation exchange resin (Phenomenex, Torrance,
The samples were automatically loaded from a 96-well microplate autosampler using the EASY-nLC system (Thermo-Fisher Scientific, Odense, Denmark) at 3µL/min. The pre-column was connected to an 8 cm fused silica analytical column (75µm i.d.) via a microsplitter tee to which a distal 2.3 kV spray voltage was applied. The analytical column was pulled to a fine electrospray emitter using a laser puller. For the peptide separation on the analytical column, a water/acetonitrile gradient was applied at an effective flow rate of 400 nL/min, controlled by the EASY-nLC. Ammonium acetate salt bumps (8μL) were applied at ratios of 0%, 10%, 20% 30% 40% and 100% of 500 mM ammonium acetate, using the 96-well microplate autosampler at a flow-rate of 3 mL/min in a vented-column setup. The eluted peptides were electrosprayed directly into the MS. The MS operated in a cycle of one full-scan mass spectrum (400-1800m/z), followed by 6 data-dependent MS/MS spectra at 35% normalized collision energy, which was continuously repeated throughout the entire MudPIT separation. The MS functions and the HPLC solvent gradients were controlled by the Xcalibur data system (Thermo Fisher Scientific, San Jose, CA). The raw data files were searched using Sequest (Thermo-Fisher Scientific San Jose, Ca) using a parent ion accuracy of 5 ppm and a fragment accuracy of 0.5 Da. A fixed modification of carbamidomethyl cysteine and variable modification of oxidized methionine were included in the search. Exclusive unique peptide sequences from hits in the knock-out samples and not the wild-type samples were then entered into the NCBI BLAST algorithm to identify proteins.

**Cell Culture**

All cells were cultured at 37°C in 5% CO₂.
For biochemical characterization of the Nedd4-1/PDLIM7 interaction, plasmids expressing wild-type (human) hNedd4-1 (accession number NM006154), and HA-tagged hPDLIM7 (accession number AF265209 from the SIDNET MGC collection), wild-type or PY motif hPDLIM7 mutant (whereby the second invariant proline of the PPXY motif was mutated to alanine using Quik Change Mutagenesis Kit; Stratagene), were transfected into HEK 293 cells grown in DMEM, 10% FBS, Pen/Strep using standard CaCl$_2$ transfection. 48 hours post-transfection, cells were harvested and ruptured in lysis buffer (150 mM NaCl, 50 mM HEPES, 1% Triton X-100, 10% glycerol, 1 mM MgCl$_2$, 1 mM EGTA) with a protease inhibitor cocktail, vortexed for 10 sec. and incubated on ice for 10 min. Lysate was then cleared by centrifugation at 12,000 xg for 10 min. and the protein concentrations in the supernatant were quantitated using Pierce BCA protein assay.

Protein subcellular localization and characterization of the Nedd4-1/PDLIM7 interaction was determined in the mouse C2C12 skeletal muscle cell line. Differentiation of myoblasts into myotubes was achieved by growing C2C12 cells in DMEM, 10%FBS, Pen/Strep until confluency (48 hours), followed by treatment with differentiation media (2% Horse Serum, 1% Pen/Strep in DMEM) for 2 to 5 days. Cells were either immunostained, or whole cell protein lysates were harvested. At serial time points after plating myoblasts and differentiating into myotubes, cells were ruptured in lysis buffer (150 mM NaCl, 50 mM HEPES, 1% Triton X-100, 10% glycerol, 1 mM MgCl$_2$, 1 mM EGTA) with a protease inhibitor cocktail, vortexed for 10 sec. and incubated on ice for 10 min. Lysate was then cleared by centrifugation at 12,000 xg for 10 min. and the protein concentrations in the supernatant were quantitated using Pierce BCA protein assay. This produced the soluble fraction of whole cell lysates containing predominantly cytosolic proteins. Pellets were solubilized in 150 µL of a 1% solution of Triton-X-100,
sonicated, and quantified using Pierce BCA protein assay to provide the insoluble fraction of whole cell lysates, containing mostly cytoskeletal proteins, including actin.

To assess the ubiquitination of transfected or endogenous PDLIM7 protein in HEK 293 and C2C12 cells respectively, 25 µM of the 26S proteasome inhibitor MG132 (Sigma-Aldrich) was added to transfected HEK 293 cells, or C2C12 myoblasts and myotubes, 6 hours prior to harvesting lysates.

**Western blotting and Immunoprecipitation**

Gastrocnemius total protein was extracted by homogenizing (Polytron PT 1200E, Kinematica, Lucerne, Switzerland) the muscle in Muscle Lysis Buffer (5 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM EGTA, 1mM β-mercaptoethanol, 1% glycerol, PMSF (1 mM), leupeptin, aprotinin (10 ug/ml each) for 3 X 30sec. and homogenates were centrifuged at 1600 xg for 10 min at 4°C. The supernatant was cleared by centrifuging further for 10 min at 4°C, 10,000xg. Protein lysates were quantified using Pierce (Rockford, IL) BCA Protein Assay Kit and normalized for equal loading. 25 µg of the muscle lysate, 60 µg of HEK 293 transfected with various constructs, or 30 µg of C2C12 lysate was separated on 8% SDS-PAGE. Western blotting was performed using the following primary antibodies: monoclonal anti-Nedd4-1 antibodies (BD Biosciences) at a 1:500 dilution; polyclonal anti-PDLIM7 antibodies (Proteintech), 1:1,000 dilution; monoclonal anti-HA antibodies (Covance, Berkeley, CA), 1:1,000 dilution; monoclonal anti-ubiquitin antibodies (Covance), polyclonal anti-K48-linkage Specific Polyubiquitination antibodies (Cell Signaling)1:1,000 dilution and polyclonal anti-HPRT antibodies (Abcam, Cambridge, MA), 1:1,000 dilution. Protein bands were detected with Horse Radish Peroxidase-linked goat anti-rabbit or anti-mouse secondary antibodies (Cell Signaling Technology, Inc., Beverly, MA.) used at a 1:10,000 dilution. The chemiluminescent signal was acquired using a
CCD camera (BioRad VersaDoc) and the total signal quantified using Image Lab software (Biorad Laboratories, Hercules, CA) with volume analysis.

For co-immunoprecipitation of PDLIM7 and Nedd4-1, equal amounts of lysate from hNedd4-1 or HA-hPDLIM7 wild-type or PY mutant transfected or untransfected 293 cells were incubated with 25µL anti-HA-agarose beads (Abcam) for 6 hours at 4°C, or equal amounts of C2C12 myoblast and myotube lysates were incubated with 5 µL of anti-PDLIM7 antibodies or anti-Nedd4-1 antibodies overnight at 4°C. 25 µl of Protein-G agarose beads (50% slurry) was then added to the C2C12 lysates and incubated for 1 hr. at 4°C while spinning. Anti-HA-agarose beads or Protein-G agarose beads were then collected by centrifugation (10,000 xg, 1 min., 4°C) and washed 3X in high salt HNTG (500 mM NaCl, 20 mM HEPES pH 7.5, 10% glycerol, 0.1% Triton X-100) and 3X in low salt HNTG (same, with 150 mM NaCl) to remove non-specifically bound proteins. The beads were mixed with 35 µl 1X Sample Buffer and boiled at 95°C for 5 min. to elute proteins. Protein eluate was resolved on 8% SDS-PAGE and transferred onto Protran 0.2 µm nitrocellulose (PerkinElmer, Boston, USA) for Western blot analysis.

**In vitro binding assays**

Human Nedd4-1 WW domains in pQE-30 (Qiagen) were a gift from Daniela Rotin (Hospital for Sick Children, Toronto). The WW domains were cloned with the following boundaries: WW I 638-760 bp; WW II 1112-1231 bp; WW III 1318-1425 bp; WW IV 1487-1606 bp. 6X His-tagged proteins were produced in M15 (pREP4) bacteria and purified following manufacturer’s instructions (Qiagen). GST fusion proteins of hPDLIM7 were produced by PCR of the PY-motif containing region (286-407 bp) and a proline-rich region (415-615 bp; as a negative control), TA cloning into pCR2.1-TOPO (Invitrogen), subsequent cloning into pGEX-5X1 vector and expression in BL21 (DE3) pLys S bacteria according to manufacturer’s protocol.
GST fusion proteins were bound on glutathione agarose beads (50% slurry) by incubating 1 hr at 4\(^\circ\)C. Proteins were eluted with 30 mM reduced glutathione by incubating 1 hr at 4\(^\circ\)C. Eluted GST proteins and bound 6X-His proteins were quantitated and equal amounts of GST-PY (PY motif) and GST-Pro (proline-rich region) hPDLIM7 fusion proteins (300 uM) were incubated with equal amounts of 6X-His bound WW proteins (500 uM) for 2 hr at 4\(^\circ\)C. The beads and bound proteins were washed 3X with high salt HNTG (as above) and 2X with low salt HNTG. Beads were then mixed with 20 µl 1X sample buffer and boiled at 95\(^\circ\)C for 5 min. to elute proteins. Protein eluate was resolved on 15% SDS-PAGE and transferred onto Protran 0.2 um nitrocellulose and subjected to Western blotting with anti-Penta (5X) His (Qiagen) and anti-GST antibodies (Sigma).

**Immunostaining**

C2C12 cells were plated on glass coverslips and immunostained as myoblasts or (differentiated) myotubes. Coverslips were fixed in 3.7% paraformaldehyde, permeabilized in 0.2% Triton X-100, and blocked in 3% BSA in PBS. Coverslips were incubated with primary antibodies for 1 hr at room temperature, and then were washed in PBS. Secondary antibodies conjugated to fluorophores were added to the coverslips for 45 min. at room temperature, followed by Hoechst at a dilution of 1:12,000 for 2 min. at room temperature. Coverslips were washed in PBS and mounted on slides in Dako mounting medium (Dako, Glostrup, Denmark). Images were acquired using a point scanning confocal microscope (LSM 700; Zeiss Canada Ltd, Toronto, ON) and analyzed using ZEN software. Primary antibodies used were monoclonal anti-Nedd4-1 at a 1:50 dilution (Hatakeyama, Jensen et al. 1997), and polyclonal anti-PDLIM7 at a 1:50 dilution (Barrès 2006). Secondary antibodies used were goat anti-mouse-AlexaFlour568 at a 1:1,000 dilution and goat anti-rabbit-AlexaFlour488 at a 1:1,000 dilution (Molecular Probes), To
detect the actin cytoskeleton cells were immunostained with rhodamine phalloidin at a 1:1,000 dilution (Cytoskeleton, Denver, CO). For quantification of co-localization, images were analyzed with the FIJI coloc2 to determine the Manders correlation coefficients.

**Statistical Analyses**

Continuous data are reported as a mean and standard deviation and were compared using students T-test, or ANOVA followed by Tukey post-analysis, to compare multiple means when appropriate. Statistical significance was assumed if p < 0.05.

### 3.3 RESULTS

**PDLIM7 skeletal muscle expression is affected by Nedd4-1 in a murine model of denervation-induced muscle atrophy**

As increased Nedd4-1 expression is correlated with, and mediates the progression of atrophic processes in muscle cells, we sought to identify potential substrates that may be targeted by Nedd4-1 in muscle to elicit its effects. We hypothesized that any potential Nedd4-1 substrates would be protected from ubiquitin mediated degradation in *Nedd4-1* knockout muscle, and thus be present in higher levels than in wild-type muscle. Since Nedd4-1 muscle expression increases upon denervation, we subjected *Nedd4-1* SMS-KO (n=2) and control mice (WT) mice (n =2) to the tibial nerve transection model of muscle atrophy, and at 1 week post tibial nerve transection used mass spectrometry to identify proteins that were differentially expressed in the experimental samples. The predominant peptides identified in both *Nedd4-1* SMS-KO and WT
samples were from cytoskeletal associated proteins (such actin, myosin, tropomyosin) and some were found to be differentially expressed (increased) in the knock-out samples relative to WT. We chose to focus on peptides that were not associated with the cytoskeleton and were “lower abundance” peptides. Dozens of peptides fulfilling these criteria were enriched in the knock-out samples compared to the wild-type, and several of these enriched peptides corresponded to proteins that contained PY motifs. In particular, one set of spectra, had 10 times the number of peptides (14% sequence coverage) in the Nedd4-1 SMS-KO muscle compared to the wild-type samples. These enriched unique peptide sequences were used in a BLAST search and identified the PDZ, LIM domain and PY-motif containing protein, PDLIM7 (Fig. 3.1A) which was significantly higher (95% peptide threshold) in the Nedd4-1 SMS-KO denervated muscle compared to the control, suggesting its expression was influenced by Nedd4-1. mPDLIM7 is a family of proteins with several isoforms. The peptides identified in the mass spectrometry and used in our subsequent BLAST search correspond to the murine PDLIM7 “a” isoform, (which has the highest degree (94%) of identity to the human PDLIM7 isoform 1, used in our biochemical experiments below), and which contains a conserved PY motif in the linker region between the PDZ and LIM domains (Figure 3.1A).

To confirm the mass spectrometry results, we performed Western blot analysis of protein lysates from denervated and contralateral control gastrocnemius muscle of Nedd4-1 SMS-KO and littermate (wild-type) control mice (Figure 3.1B). PDLIM7 protein levels in denervated gastrocnemius muscle of control mice were significantly decreased compared to Nedd4-1 SMS-KO mice (0.51±0.08 vs 0.98±0.16 respectively; Figure 3.1C). The decreased levels of PDLIM7 occurred concomitant to increased levels of Nedd4-1 protein (Figure 3.1B), supporting the notion of PDLIM7 being a novel target of Nedd4-1-mediated degradation.
A

PDZ

PDLIM7

LIM

LIM

LIM

497aa

SAPAADPPRYTFAPSV  Human
LTPPADPPRYTFAPSA  Mouse
FTPPADPPRYTFAPSA  Rat

B

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Gel Image:

- α-PDLIM7
- α-Nedd4-1
- α-HPRT

C

Graph:

- PDLIM7 Protein Density
- Nedd4-1 SMS KO
- Littermate Control

*
Figure 3.1. PDLIM7 is a PY-motif containing protein whose expression is decreased in denervated atrophic muscle, and retained in the absence of Nedd4-1. (A) Schematic of the PDZ and LIM domain containing protein, PDLIM7, shows an N terminus PDZ domain, 3 terminal LIM domains in addition to a conserved PY motif, (PPXY), the canonical binding site for the Nedd4-1 WW domains. (B) Western blots of Nedd4-1, PDLIM7 and HPRT (loading control) in protein lysates from denervated gastrocnemius muscle (Den) and the contralateral control gastrocnemius (Con) muscle of Myo$^{Cre};Nedd4-1^{-/-}$ (Nedd4-1 SMS-KO, n=7) and Myo$^{Cre};Nedd4-1^{+/+}$ (littermate control, n=7) mice at 1 week post tibial nerve transection. Representative blots from 4 mice (2 Nedd4-1 SMS-KO and littermate control pairs) are shown. Lysate from HEK 293 cells transfected with PDLIM7 (293 Tx PDLIM7) and fully differentiated C2C12 myotubes (MT) serve as positive controls for PDLIM7 immunoblotting. Nedd4-1 is absent in the mature muscle of Nedd4-1 SMS-KO mice, but trace expression is evident upon denervation due to retained satellite cell Nedd4-1 expression (Nagpal, Plant et al. 2012). (C) PDLIM7 protein levels were normalized to the corresponding HPRT levels (lane matched) and PDLIM7 expression is reported as the ratio of protein in denervated (Den) gastrocnemius compared to contralateral control (Con) muscle. PDLIM7 levels were significantly decreased in denervated gastrocnemius muscle of littermate control mice, but not in Nedd4-1 SMS-KO mice. (Data are mean ± SEM; * p < 0.05) (293 Untx = HEK 293 cells untransfected).
Nedd4-1 and PDLIM7 interact via a WW domain-PY motif interaction.

PDLIM7 contains a PY- motif (PPRY) in its linker region (between the PDZ and LIM domains) that is conserved across mammalian species and adheres to the canonical WW domain binding motif sequence for Nedd4-1, suggesting that the interaction between the two proteins may occur in a similar manner. To verify that the PDLIM7/Nedd4-1 interaction occurs by WW domain-PY motif binding, we expressed 6X His-tagged versions of the four WW domains of human Nedd4-1 and incubated equal amounts of the fusion proteins (bound to Ni$^{2+}$ beads) with soluble, purified GST fusion proteins of regions of hPDLIM7 encompassing the PY motif (GST-PY) or a proline rich region, C-terminal to the PY motif (GST-Pro) as a negative control (Figure 3.2). We found that the GST-PY consistently bound to WWII and WW III of hNedd4-1, albeit the intensity of binding to WWIII appeared much greater. GST-Pro did not bind to any of the 6X His-WW fusion proteins. This data suggests that the interaction between the two proteins in vivo is mediated in canonical fashion through a PY-WW domain interaction.
Figure 3.2. hPDLIM7 and hNedd4-1 binding is mediated through a PY motif–WW domain interaction. (A) GST-fusion proteins of the area encompassing the PY motif of hPDLIM7 (GST-hPDLIM7 PY) and of a non-PY-containing proline-rich region of hPDLIM7 (GST-hPDLIM7 PRO) as control were generated. Samples of 0.1 μg soluble GST–PY or GST–Pro were incubated with 0.3 μg of His6-tagged hNedd4–WWI, –WWII, –WWIII or –WWIV immobilized on Ni²⁺-nitrilotriacetate beads (His-hNedd4 WW domain I–IV). Following thorough washes of the beads, bound proteins were eluted with sample buffer, separated by SDS/PAGE, and Western blotting was performed with anti-GST and anti-His antibodies, as indicated. Molecular masses are indicated in kDa. Binding of the PDLIM7 PY motif is evident with WWII and WWIII domains of Nedd4-1. (B) Sequence alignment of the 4 WW domains of hNedd4-1 and percentage sequence identities derived using NCBI Protein BLAST. A colon represents an identical match to WW domain III. The XP groove of WWIII is in bold.
Nedd4-1 mediated PDLIM7 ubiquitination is dependent on an intact PY motif

To verify that hNedd4-1 and hPDLIM7 binding is mediated by a PY-WW domain interaction in living cells and to determine whether this interaction results in ubiquitination of PDLIM7, cDNA for HA tagged hPDLIM7 or HA tagged hPDLIM7 PY motif mutant was co-transfected into HEK 293 cells along with hNedd4-1 cDNA. Lysates from these cells were subjected to immunoprecipitation with HA-agarose beads. Immunoprecipitates and cell lysates were separated on SDS-PAGE and probed with anti-PDLIM7, anti-ubiquitin, anti-Nedd4-1, and anti-HPRT (as a loading control) to detect expression of the respective proteins. Transfected wild-type hPDLIM7 was able to co-immunoprecipitate hNedd4-1, but transfected hPDLIM7 PY mutant was unable to do so, re-inforcing the notion that interaction between the two proteins is likely mediated by the WW domain-PY motif binding (Figure 3.3 A,B). Co-transfection with HA-hPDLIM7 and hNedd4-1 displayed a notable increase in ubiquitinated PDLIM7 compared to co-transfection with HA hPDLIM7 PY mutant and hNedd4-1, as evidenced by the high molecular mass smear present in the former and absent in the latter (Figure 3.3 A,C). Furthermore, the addition of the 26S proteasome inhibitor MG 132 results in a high molecular weight smear of the PDLIM7 band, in keeping with the accumulation of ubiquitinated forms of PDLIM7, when the wild type protein is co-expressed with Nedd4-1 and to a lesser extent, when expressed on its own (Figure 3.3D). This patterning is not evident with the PY mutant PDLIM7. These data together suggest that the interaction between Nedd4-1 and PDLIM7 results in ubiquitination of PDLIM7 and that PDLIM7 is a *bona fide* Nedd4-1 substrate. The smaller extent of apparent ubiquitinated PDLIM7 accumulation in the absence of transfected Nedd4-1 is likely due to the presence of other endogenous ubiquitin ligases, or possibly low levels of endogenous Nedd4-1.
### A

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<tr>
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**Blot:**
- α-Ubiquitin
- α-PDLIM7
- α-Nedd4-1

### B

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**Blot:**
- α-Nedd4-1
- α-PDLIM7
- α-HPRT

### C

**Ubiquitinated PDLIM7/ PDLIM7 protein level**

- hPDLIM7
- hPDLIM7 mPY
- hNedd4-1
- hPDLIM7 mPY/

* & #

### D

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**Blot:**
- α-PDLIM7
- α-HPRT
Figure 3.3. PDLIM7 ubiquitination is dependent on an intact PY motif. (A) HEK 293 cell lysate transfected with HA-tagged hPDLIM7, hNedd4-1, HA-tagged hPDLIM7 PY motif mutant (HA-PDLIM7 mPY), HA-tagged hPDLIM7 with Nedd4-1, HA-PDLIM7 mPY with hNedd4-1, or untransfected lysate were subjected to immunoprecipitation with anti-HA-tagged agarose beads. Beads were washed and separated by SDS/PAGE, then subjected to Western blotting with anti-ubiquitin and anti-PDLIM7 antibodies, and then stripped and re-probed with anti-Nedd4-1 antibodies. Ubiquitinated PDLIM7 is seen as a high-molecular-mass smear above 63 kDa in the HA-tagged hPDLIM7 and hNedd4-1 transfected lysate only. (B) Aliquots of whole-cell (WC) lysate were subjected to immunoblotting with anti-PDLIM7 and anti-Nedd4-1 antibodies, and reprobed with anti-HPRT as a loading control. (C) The ubiquitination signal obtained via CCD camera acquisition in figure 3A was quantified using ImageLab software (BioRad), and normalized to the immunoprecipitated PDLIM7 (lane matched). Co-transfection of hPDLIM7 and hNedd4-1 results in a significant increase in PDLIM7 ubiquitination. (D) Protein lysates of HEK 293 transfected and untransfected cells treated with or without 25 µM MG132 for 6 hours prior to harvesting, were subjected to immunoblotting with anti-PDLIM7 and anti-HPRT as a loading control. (Molecular masses are indicated in kDa; *, &c, # P <0.05)
**Nedd4-1 and PDLIM7 interaction and co-localization in C2C12 muscle cells occurs during myotube differentiation**

To verify that PDLIM7 is a Nedd4-1 substrate in muscle we assessed the subcellular co-localization of the endogenous proteins in C2C12 muscle cells, during differentiation from myoblast to fully differentiated myotubes, with immunocytology, and also evaluated protein binding with co-immunoprecipitation experiments. Using confocal microscopy, PDLIM7 was seen to localize both throughout the cytosol and (to a lesser degree) in association with the actin cytoskeleton in multi-nucleated, fully differentiated myotubes (Figure 3.4 A). Co-localization of PDLIM7 with Nedd4-1 was evident in the myotube cytoplasm upon overlay (Figure 3.4 B). In contrast, in undifferentiated myoblasts, PDLIM7 was predominantly associated with the actin cytoskeleton (Figure 3.4 C), with minimal co-localization with Nedd4-1, whose expression was largely restricted to the cytoplasm (Figure 3.4 D).

We subsequently performed immunoprecipitation experiments in C2C12 myoblasts differentiated into myotubes over a 7 day time-course (Figure 3.5), and similarly found that Nedd4-1 and PDLIM7 co-immunoprecipitated in fully differentiated myotubes, but not in undifferentiated myoblasts (Figure 3.6A). Furthermore, analysis revealed differential expression of PDLIM7 in the C2C12 muscle cell during the process of differentiation. While PDLIM7 was highly expressed in the soluble, cytosol-rich fraction of whole cell lysates of myotubes, only trace expression was evident in the myoblasts. In contrast PDLIM7 was evident in the actin-rich insoluble fractions of the cellular lysate in undifferentiated myoblasts, but not in the myotubes (Figure 3.6B). Together, these results confirm binding between endogenous PDLIM7 and Nedd4-1 in C2C12 cells, and suggest that this interaction may regulate the process of muscle differentiation as PDLIM7 translocates, in part, to the cytosol.
To determine whether PDLIM7 ubiquitination occurs in C2C12 cells and is regulated during differentiation, we subjected C2C12 lysates, differentiated over a 7 day time-course and treated with or without the proteasome inhibitor MG132, to immunoprecipitation with anti-PDLIM7 antibodies (Figure 3.6C). Substantially more PDLIM7 ubiquitination, as indicated by a high molecular mass smear on ubiquitin blotting, was evident in early differentiated myotubes (day 3 and day 5) treated with MG132 compared with levels in untreated myotubes. Most significantly, no ubiquitination was evident in undifferentiated myoblasts suggesting that the ubiquitination of PDLIM7 is associated with early stages of myotube development. To further elucidate the consequences of PDLIM7 ubiquitination in the early stages of myotube development, we subjected C2C12 lysates differentiated over a 7 day time-course to immunoprecipitation with anti-PDLIM7 antibodies and analyzed ubiquitination patterns using a K48-linkage specific polyubiquitination antibody (Figure 3.6D), which directs 26S proteasomal-mediated degradation. A K48 polyubiquitination linkage pattern was evident, as indicated by high molecular mass banding, in early myotubes (day 3 to day 7) but not undifferentiated myoblasts, suggesting that the consequence of PDLIM7 ubiquitination in early myotube development is indeed proteasomal degradation.
Figure 3.4. Nedd4-1 and PDLIM7 co-localize in muscle cells in vitro. Immunofluorescence viewed on point scanning confocal microscope of C2C12 differentiated multinucleated myotubes (A, B) or undifferentiated myoblasts (C, D), with DAPI (blue, to indicate nuclei), PDLIM7 (green) and Nedd4-1 or Rhodamine Phalloidin (red). Overlay shows merging of the images and co-localization of proteins is reported using the Mander’s correlation coefficient generated using FIJI coloc2 software. Scale bar = 20 um.
Figure 3.5. C2C12 mouse myoblasts subjected to 7 day time-course of differentiation. Representative phase contrast images taken using a Nikon Eclipse TS100 microscope of C2C12 mouse myoblast cells prior to lysate collection at 12 hours, 2 days, 3 days, 5 days, and 7 days post-plating. C2C12 myoblast cells were grown in DMEM, 10%FBS, Pen/Strep until confluency (2 days), followed by treatment with differentiation media (2% Horse Serum, 1% Pen/Strep in DMEM) for 1 to 5 days.
Figure 3.6. PDLIM7 ubiquitination occurs in muscle cells in vitro. (A) C2C12 cells, subjected to a time-course of differentiation, were lysed and the soluble portion of whole cell (W.C.) lysates was subjected to immunoprecipitation with anti-Nedd4-1 antibodies and Protein G agarose beads. Beads were washed, the immunoprecipitated proteins separated by SDS-PAGE, and immunoblotted with anti-PDLIM7 and anti-Nedd4-1 antibodies. Aliquots of soluble lysate were subjected to immunoblotting with anti-PDLIM7, anti-Nedd4-1 and anti-HPRT as a loading control. (B) The insoluble fractions of the same lysates were solubilized in 1% Triton-X-100, sonicated, and subjected to immunoblotting with anti-PDLIM7 and anti-actin as a loading control. (C) C2C12 cells were subjected to a time-course of differentiation and were treated with or without 25 µM MG132 for 6 hours prior to cell lysis. The soluble fraction collected from whole cell lysate was subjected to immunoprecipitation with anti-PDLIM7 antibody, and immunoblotted with anti-ubiquitin, and anti-PDLIM7. Soluble lysates were subjected to immunoblotting with anti-PDLIM7, anti-Nedd4-1, and anti-HPRT as a loading control. (D) C2C12 cells were subjected to a time-course of differentiation and soluble fractions collected from whole cell lysates were subjected to immunoprecipitation with anti-PDLIM7 antibody and immunoblotted with anti-K48-linkage specific polyubiquitination, and anti-PDLIM7. Molecular masses are indicated in kDa. (MB = myoblasts, MT = myotubes, D= days).
3.4 DISCUSSION

In this study we describe a novel interaction between the E3 ubiquitin ligase Nedd4-1 and the scaffolding protein PDLIM7. While an interaction between PDLIM7 and Nedd4-1 has not yet been described, it is not entirely unexpected, as both proteins are known to associate with, and influence the actin cytoskeleton. Over-expression of human Nedd4-1 in yeast has previously been shown to negatively influence the dynamic organization of the actin cytoskeleton through inhibition of actin polymerization, and has been suggested to target conserved actin-associated proteins (Stawiecka-Mirotta, Kaminska et al. 2008). PDLIM7 has also been previously identified to bind and localize to actin filaments in mammals via interaction with beta-tropomyosin, suggesting a putative role in cytoskeleton organization (Nakagawa, Hoshijima et al. 2000). Other members of the PDZ-LIM family of proteins such as PDLIM1, PDLIM3, and PDLIM5 are known to localize to integrins, mediating focal adhesion kinase (FAK) signaling cascades (Vallenius, Scharm et al. 2004, Loughran, Healy et al. 2005), which results in actin cytoskeleton modification as well.

Furthermore, we and others have previously shown a role for Nedd4-1 in skeletal muscle atrophy (Batt, Bain et al. 2006, Plant, Correa et al. 2009, Nagpal, Plant et al. 2012), and a role for PDZ-LIM proteins in skeletal muscle organization and maintenance has been well documented. Knock-down of Ldb3/Cypher, a member of the PDZ-LIM family in zebrafish, leads to abnormalities in somite compartmentalization and skeletal muscle organization (van der Meer, Marques et al. 2006). In mice, Ldb3 null mutations are embryonic or perinatal lethal, with mice dying from a myopathy characterized by disorganized and fragmented Z lines in skeletal and
cardiac muscle (Krcmery, Camarata et al. 2010). Upon knock-down of PDLIM7 in zebrafish by morpholino antisense oligonucleotides, Camarata et al. show severe defects in growth and tail muscle development, a phenomenon that was suppressed by co-injection with Pdlim7 mRNA (Camarata, Snyder et al. 2010). In mice, global loss of PDLIM7 via a gene trap approach resulted in neonatal lethality in the majority of Pdlim7−/− mice, purported to result from unexpected alterations of hemostatic function (Krcmery, Gupta et al. 2013). While the effect of PDLIM7 deficiency on skeletal muscle was not reported, surviving adult Pdlim7−/− mice exhibited a lower body weight compared to littermate controls despite similar birthweights, (Krcmery, Gupta et al. 2013) which may be contributed to by a reduced skeletal muscle mass.

We have shown differential localization of PDLIM7 in developing myotubes ex vivo, as the protein translocates, in part, from the actin cytoskeleton in C2C12 myoblasts to a diffuse cytoplasmic staining in multi-nucleated myotubes. This was further confirmed by Western blot analysis, revealing an increase in cytoplasmic PDLIM7 in myotubes compared to myoblasts. Interestingly, Guy et al. were unable to demonstrate differential protein expression of PDLIM7 (Enigma) in C2C12 myoblasts and myotubes differentiated for 5 days (Guy, Kenny et al. 1999). The reason for this discrepancy is unclear, but may be due to the analysis of a combined soluble and insoluble fraction of cell lysates, which was not clearly reported. Our experiments indicate a definitive increase in cytoplasmic PDLIM7 with myotube formation, suggesting that the translocation of the protein may be associated with the process of myotube development. This finding has not been previously reported, however, Enigma homolog 1 (ENH1/PDLIM5) is an anchoring protein homologous to PDLIM7, which has been shown to promote myogenic genes and initiate C2C12 myotube formation (Ito, Hashimoto et al. 2012).
Evidence of a dynamically localized PDLIM7 is well documented, as PDLIM7 has been shown to bind signaling molecules and direct their subcellular localization. PDLIM7 is known to bind to receptor tyrosine kinases such as Rearranged during Transfection (RET) and Insulin Receptor (InsR). Interactions with each of these receptor kinases is mediated by binding to LIM domains; RET binds to LIM domain 2 (Durick, Wu et al. 1996) and InsR binds to LIM domain 3 (Wu and Gill 1994). Interactions with PDLIM7 lead to receptor kinase transport to the actin cytoskeleton providing a possible mechanism for this scaffolding protein to elicit its effects, although the significance of its involvement in these signaling cascades is not fully understood (te Velthuis and Bagowski 2007, Krcmery, Camarata et al. 2010).

An interesting newly identified role for PDZ-LIM proteins is their involvement in binding key nuclear proteins, tethering them, and re-localizing them, to the actin cytoskeleton. PDLIM1 has been shown to tether the nuclear kinase Clik1 to actin stress fibers (Vallenius and Makela 2002) while PDLIM5 is known to bind to DNA-binding protein inhibitor (ID-2), targeting it to the cytoplasm, resulting in loss of its inhibitory effects on basic helix-loop-helix transcription factors (Kurooka and Yokota 2005, Lasorella and Iavarone 2006). PDLIM7 has been found to bind to T-box transcription factors 4/5 (Tbx4/5), sequestering them to the actin cytoskeleton and preventing their activation of target genes in the nucleus (Krause, Zacharias et al. 2004, Camarata, Bimber et al. 2006, Bimber, Dettman et al. 2007). It is possible then, that the differential subcellular localization of PDLIM7 influences myoblast proliferation, differentiation and/or myotube formation through initiation of signaling cascade pathways, or alternatively regulating transcriptional activity of myogenic factors and other proteins.

Our experiments suggest that PDLIM7 is a novel substrate to the E3 ubiquitin ligase Nedd4-1, interacting in mature myotubes and binding via its PY motif in canonical fashion to
Nedd4-1 WWII and WWIII domains, albeit the binding by WWIII appears to be much stronger. This is in keeping with previous reports of Nedd4-1 WWIII demonstrating the highest affinity binding to several other Nedd4-1 substrates including ENaC and Commissureless (Kanelis, Bruce et al. 2006, Bobby, Medini et al. 2013). This enhanced affinity appears to be due to a broad XP groove that exists in WWIII, facilitating contact between the substrate and the domain. The biological significance of this interaction in the context of muscle atrophy has not yet been identified, but can be speculated upon, as we have now shown that Nedd4-1 over-expression correlates with progression of atrophic processes in skeletal muscle, concomitant to decreases in PDLIM7 protein expression. Furthermore, the absence of Nedd4-1 in denervated muscle stabilizes PDLIM7 expression and partially inhibits muscle atrophy. Thus, PDLIM7 appears to be a likely target of K48-linkage specific Nedd4-1 mediated ubiquitination and subsequent degradation in atrophying muscle. This interaction may inhibit cellular processes initiated by PDLIM7 that are required for maintenance of muscle size, such as binding, translocation and possible recycling of growth factor receptors/signaling molecules to the actin cytoskeleton. Alternatively, PDLIM7 regulation of gene expression by binding and tethering transcription factors such as Tbx5/Tbx4 to the actin cytoskeleton, preventing their nuclear translocation and initiation of transcription, could provide an alternative mechanism for the regulation of muscle size (Camarata, Krcmery et al. 2010). Deletion of Tbx5 and Tbx4 in mice is known to lead to equivalent disruptions of normal skeletal muscle splitting patterns during development (Hasson, DeLaurier et al. 2010). Increasing levels of PDLIM7 result in decreased Tbx5 transcriptional activity, which influences zebrafish cardiogenesis. (Camarata, Krcmery et al. 2010). Further studies analyzing myogenic transcription factor expression, for example, in the context of PDLIM7 localization and interaction with Nedd4-1 can shed light into this possible mechanism.
In conclusion, we have identified a novel Nedd4-1 substrate in muscle, the scaffolding protein PDLIM7, which associates with the actin cytoskeleton and regulates muscle structure and function. The mechanism by which this interaction induces muscle loss remains unknown, yet, this work distinguishes PDLIM7 as a candidate for future studies examining the biological role of Nedd4-1 action in atrophying muscle, and as a potential therapeutic target for muscle weakness.
CHAPTER 4:

PROJECT SUMMARY AND GENERAL DISCUSSION

Skeletal muscle atrophy occurs both as a natural consequence of muscle disuse, ageing, and fasting, and as a pathophysiologic response to a number of acute and chronic diseases such as diabetes mellitus, COPD, cancer, critical illness and denervation injuries (Batt, dos Santos et al. 2013, Drescher, Konishi et al. 2015, Malavaki, Sakkas et al. 2015, Berger, Bloechlinger et al. 2016). In each of these conditions, onset of atrophy correlates with poor clinical outcomes, an increase in disease morbidity, a diminished quality of life, as well as an increase in health resource utilization and costs. The most successful therapy to date to mitigate muscle wasting is resistance-based exercise, but its use in many disease states is difficult due to severity of illness/frailty limiting capacity, poor uptake and adherence to exercise programs by individuals, and cost of providing appropriate institutional or in home support personnel and equipment. The development of pharmacological therapies, to be used with or without resistance based exercise, could potentially provide the most effective and efficient treatment for muscle wasting. Inconsistent efficacy and negative adverse drug effects have largely hindered the successful implementation of currently available pharmacologic interventions (i.e. appetite stimulants, anabolic steroids) to date into clinical practice. Gaps in our understanding of the biological and cellular mechanisms involved in the mediation of muscle wasting impede the development of effective therapies
Skeletal muscle atrophy is characterized by a decrease in muscle mass due to a loss of cellular organelles, cytoplasm, and protein. These changes are a reflection of activation of pathways regulating protein turnover, promoting a net increase in the rates of protein degradation relative to the rates of protein synthesis (Sartorelli and Fulco 2004, Sandri 2014). Increased protein degradation is well documented in the onset and persistence of skeletal muscle atrophy, and the primary pathway responsible for increased proteolytic activity in muscle in a variety of chronic states appears to the ubiquitin-proteasome system (Wing and Goldberg 1993, Mitch, Medina et al. 1994, Price, Bailey et al. 1996). E3 ubiquitin ligases confer specificity to UPS mediate muscle proteolysis and the identification of critical E3 ligases and their substrates provide potential therapeutic targets by which to inhibit or mitigate muscle wasting.

Our lab has previously identified Nedd4-1 as an E3 ubiquitin ligase which increases in expression in muscle atrophied by inactivity, and Nedd4-1 skeletal muscle specific knockout (SMS-KO) mice exhibit partial protection against muscle wasting (Nagpal, Plant et al. 2012). In this research project I have identified a novel Nedd4-1 skeletal muscle target, the scaffolding protein PDLIM7, a member of the PDZ–LIM family of proteins, which associates with the actin cytoskeleton and regulates muscle structure and function (D'Cruz, Plant et al. 2016). Nedd4-1 expression in muscle atrophied by denervation is coincident with a decrease in PDLIM7 and PDLIM7 protein levels are stabilized in denervated muscle of Nedd4-1 skeletal muscle-specific knockout mice. PDLIM7 and Nedd4-1 interact via canonical binding between the PY motif of PDLIM7 and the second and third WW domains of Nedd4-1. We show differential localization of PDLIM7 in developing C2C12 myotubes \textit{ex vivo}, as the protein translocates, in part, from the actin cytoskeleton in myoblasts to a diffuse non-nuclear staining in multi-nucleated myotubes. In the fully differentiated C2C12 myotubes, PDLIM7 and Nedd4-1 bind in the cytoplasm, and
PDLIM7 is ubiquitinated with a K48-linkage specific polyubiquitination pattern which targets the protein for proteasomal-mediated degradation.

These results identify PDLIM7 as a *bona fide* skeletal muscle substrate of Nedd4-1 and suggest that this interaction may underlie the progression of skeletal muscle atrophy. This offers a novel therapeutic target that could be potentially used to attenuate muscle atrophy.
PART I: ROLE OF THE NEDD4-1/PDLIM7 INTERACTION IN MUSCLE ATROPHY

While I have definitively established that PDLIM7 is a novel substrate of Nedd4-1, my data demonstrating that i) the absence of Nedd4-1 stabilizes PDLIM7 expression in vivo in muscle partially protected from denervation induced atrophy and ii) the two proteins interact in vitro in differentiated myocytes suggests, but does not prove, PDLIM7 is a downstream target of Nedd4-1 in its regulation of muscle mass. Further experimentation, beyond the scope of this Masters thesis, is required to solidify the premise that PDLIM7 is the Nedd4-1 substrate responsible for the induction of muscle atrophy. In that regard, three aims of investigations could be undertaken.

1) *Determine the fate of PDLIM7 ubiquitinated by Nedd4-1.*

I have shown that PDLIM7 undergoes K-48 linked ubiquitination in C2C12 cells, suggesting the protein is targeted to the 26S proteasome for degradation. Further evaluation with exogenously expressed Nedd4-1 and PDLIM7 in a cell line *in vitro,* can be performed with Ub linkage specific antibodies to determine all patterns of PDLIM7
ubiquitination mediated by Nedd4-1, which would indicate other concurrent possible fates. Targeting of PDLIM7 to the 26S proteasome can be evaluated in this system by determining the protein’s half life in the presence or absence of Nedd4-1 and proteasome inhibitors. Evaluation of the fate of endogenous PDLIM7 in response to Nedd4-1 can be undertaken in C2C12 myoblasts and myotubes, by knocking out/down Nedd4-1 and evaluating PDLIM7 ubiquitination and sub cellular localization. I anticipate this series of experiments will show Nedd4-1 targets PDLIM7 predominantly for proteasome mediated degradation.

2) **Further evaluation of PDLIM7/Nedd4-1 interaction in Nedd4-1 SMS KO mice in vivo.**

If Nedd4-1 mediated ubiquitination and degradation of PDLIM7 induces muscle atrophy, then one would expect a correlation between the extent of muscle atrophy and PDLIM7 ubiquitination. *Nedd4-1* SMS-KO mice subjected to tibial nerve transection, should demonstrate the absence, or at minimum attenuation, of PDLIM7 ubiquitination in the gastrocnemius muscle, relative to wild type mice. Furthermore rescue, by adeno or lentiviral transduction of PDLIM7 into gastrocnemius muscle of wild type mice, should be able to attenuate the atrophic response of the gastrocnemius to denervation injury. In the event the level of expression of transduced PDLIM7 is inadequate to overcome the denervation induced increase in Nedd4-1, or alternatively, should experiments in Aim 1 demonstrate that Nedd4-1 ubiquitination of PDLIM7 results in outcomes other than, or in addition to, proteasome mediated degradation, such as altered subcellular localization, then the transduction of the PY mutant PDLIM7 which Nedd4-1 is unable to bind, should attenuate the atrophic response.
3) **Evaluation of PDLIM7 knockout/knockdown in skeletal muscle in vivo and in vitro.**

If changes in PDLIM7 expression regulate muscle size, then its loss from muscle *in vivo*, or in muscle cell culture models would be expected to lead to muscle and myotube atrophy, respectfully. The caveat here is, that it depends upon the role PDLIM7 has in muscle – i.e. does it enhance muscle protein synthesis, or inhibit muscle proteolysis. If it somehow inhibits upregulation of proteolysis, then at baseline – i.e. in the absence of an atrophic stimulus, muscle mass may appear normal. Only once a proteolytic stimulus is applied, might the protective effect of PDLIM7 be notable. *PDLIM7* null mice are available, and could be requested for study and subjected to tibial nerve transection injury. One might expect an enhanced gastrocnemius atrophic response in the absence of PDLIM7, which could be reversed by viral mediated transduction of PDLIM7, if it plays a role in muscle wasting. Tissue culture studies would be more difficult, as an “inactivity” model is not available *in vitro*. Treatment of muscle cell culture with a corticosteroid is the standard *in vitro* model of myotube atrophy, but pharmacologic/metabolic induction of muscle wasting may not engage PDLIM7. Hence the majority of study of the influence of PDLIM7 on muscle wasting will likely require *in vivo* study, in the knockout mouse.

The studies outlined in Aims 1 to 3 should together, determine the specific outcome of Nedd4-1 mediated PDLIM7 ubiquitination, and whether this interaction results in skeletal muscle atrophy.
PART II: ROLE OF THE NEDD4-1/PDLIM7 INTERACTION IN MYOBLAST DIFFERENTIATION

We have reported a novel potential role for PDLIM7 in the regulation of myoblast differentiation. PDLIM7 localization changes from the actin cytoskeleton in myoblasts to a diffuse cytosolic localization in myotubes, where it interacts with Nedd4-1. PDLIM7 K48-linkage specific ubiquitination is evident in differentiated myotubes, but not myoblasts. A role for PDLIM7 and its ubiquitination by Nedd4-1 in myogenesis requires further investigation and can begin with the following series of studies.

Our analysis of differential PDLIM7 localization with myoblast differentiation, and co-localization with Nedd4-1, was performed using the C2C12 mouse myoblast cell line and should be reproduced in other muscle lines (i.e. rat L6 cells) and primary myoblast culture. If PDLIM7 expression and subcellular localization participates in the regulation of myoblast differentiation, it would be expected that its knockdown, or exogenous overexpression, in a muscle cell culture system would influence this process. Similarly, the generation of PDLIM7 mutant constructs, including for example, a PDZ deficient mutant which would presumably inhibit PDLIM7 association with the actin cytoskeleton, or the use of cytoskeletal stabilizers and destabilizers which would influence PDLIM7 localization, should regulate myoblast differentiation. Overexpression of a PDLIM7 construct with all lysine residues mutated would delineate the role of PDLIM7 ubiquitination in differentiation, and the role of Nedd4-1 in the process could be explored with the PY motif deficient PDLIM7. This would allow for delineation of the possible role for the scaffolding protein in the facilitation of myoblast differentiation.
PART III: IDENTIFICATION OF PDLIM7 INTERACTING TARGETS IN SKELETAL MUSCLE

Future studies are required to assess and identify downstream interacting targets of PDLIM7 in order to determine the mechanism by which the Nedd4-1/PDLIM7 interaction may contribute to atrophy and myogenesis. A number of PDLIM7 interacting proteins such as beta-tropomyosin (Nakagawa, Hoshijima et al. 2000), Ret (Durick, Wu et al. 1996), InsR (Wu and Gill 1994), and Tbx4/5 (Krause, Zacharias et al. 2004, Camarata, Bimber et al. 2006, Bimber, Dettman et al. 2007) have been previously identified, however, whether these interactions mediate an atrophic response or influence myogenesis remains unknown.

More interesting is a newly identified role for PDZ-LIM proteins is their involvement in binding key nuclear proteins, tethering them, and re-localizing them, to the actin cytoskeleton, thus regulating their ability to influence nuclear activities such as transcription. WW domain-containing transcription regulator protein 1 (TAZ) nuclear factor is emerging as a critical regulator of skeletal muscle development, inducing myoblast differentiation and undergoing nuclear translocation with myotube formation. However the regulation of TAZ nucleocytoplasmic shuttling remains poorly understood. Given this information, and my observations of the potential role of PDLIM7 in myoblast differentiation, I speculated that PDLIM7 may bind TAZ via a PY motif/WW domain interaction, “tethering” it to the actin cytoskeleton, thus influencing its nucleocytoplasmic shuttling and ultimately, myoblast differentiation. My exciting preliminary results identify TAZ as a binding partner of PDLIM7. I rationalize and present this early work briefly in the following sections.
TAZ/YAP

TAZ and the closely related Yes-associated protein (YAP) are the fundamental downstream effectors of the Hippo signal transduction network. Although they are associated with the Hippo pathway, other signaling networks including the Wnt/β-catenin pathway and sonic hedgehog pathway (Shh), can also regulate their activity. Both transcriptional regulators, TAZ and YAP and have been the focus of intense research over recent years due to their diverse and encompassing roles in progenitor cell proliferation, tumourigenesis, and organogenesis (Piccolo, Dupont et al. 2014). Although TAZ/YAP present potential therapeutic targets in regenerative medicine, many questions regarding their regulation, downstream targets, and nucleocytoplasmic transport remain unanswered. Both TAZ and YAP contain N-terminal Tead binding domains, 14-3-3 binding domains, 1-2 WW domains, a TAD transcriptional activation domain, and a C-terminal PDZ binding domain (Figure 5.1). The human YAP gene contains four isoforms; isoforms 1, 3, and 4 contain two WW domains, whereas isoform 2 contains only one WW domain. Only one isoform of TAZ exists, which is a 400 residue protein that shares 46% amino acid sequence identity to YAP isoform 3 (Hong and Guan 2012). Although TAZ and YAP are often considered together, they are not completely redundant, and each possesses some unique physiological functions.

TAZ/YAP Functions in Skeletal Muscle

Roles for TAZ/YAP have been demonstrated in skeletal muscle myogenesis, satellite cell activation, and the regulation of muscle size. In myoblasts YAP promotes proliferation and inhibits myotube differentiation (Watt, Judson et al. 2010). Overexpression of YAP in C2C12 myoblasts increases myoblast proliferation through binding to Myf5 and inducing its
Figure 5.1. Schematic of YAP and TAZ proteins. Schematic representation of YAP (isoform 3) and TAZ reveal that each contain N-terminal Tead binding domains, 14-3-3 binding domains, 1-2 WW domains, a TAD transcriptional activation domain, and a C-terminal PDZ binding domain. YAP is a 488 residue protein which shares 46% amino acid sequence identity to the 400 residue protein TAZ.
transcriptional activity (Watt, Judson et al. 2010). The exact upstream mechanism regulating YAP nuclear translocation and activation of Myf5 is unclear. While YAP is highly expressed in myoblasts and inhibits myotube formation, TAZ is highly expressed in differentiated muscle and promotes myotube formation. In C2C12 myocytes, TAZ becomes activated and induces myogenenic differentiation through directly associating with MyoD to promote its transcriptional activity (Jeong, Bae et al. 2010, Park, Jeong et al. 2014, Yang, Nakagawa et al. 2014).

The Hippo Signal Transduction Network

The Hippo pathway is a signal transduction pathway crucial to multiple biological processes including, but not limited to, development, stem cell specification, tissue regeneration, and organogenesis (Pan 2007, Tremblay and Camargo 2012, Yu and Guan 2013, Mo, Park et al. 2014). Overactivity of the Hippo pathway has also been implicated in the development of human disease, such as cancer (Pan 2010). The Hippo pathway is a kinase network that consists of two central proteins: mammalian STE20-like protein kinases 1 and 2 (Mst1/2) and large tumor suppressor kinases 1 and 2 (Lats1/2) (Figure 5.2). In this pathway, Mst1/2 first binds to Salvador homolog 1 (Sav1) through interaction between the WW domains of Sav1 and a PY-motif in Mst1/2 (Pires, Taha-Nejad et al. 2001). The exact mechanisms regulating this first step are complex and not fully understood; regardless the interaction activates Mst1/2 kinase activity, allowing it to phosphorylate threonine residues on Lats1/2 and Mob kinase activator 1 (Mob1α/β). Normally, Lats1/2 has autoinhibitory activity; however, upon phosphorylation of the threonine residue on Mob1α/β, it is able to bind to Lats1/2 to prevent this autoinhibitory activity (Wackerhage, Del Re et al. 2014). Following Lats1/2 phosphorylation and activation, its PY-motif binds to WW domains present in TAZ or YAP, allowing for their subsequent phosphorylation. When TAZ/YAP proteins are phosphorylated, this causes their sequestration to
**Figure 5.2. The central Hippo signaling pathway.** The Hippo pathway consists of the central proteins Mst1/2 and Lats1/2. By mechanisms which are complex and not fully understood, Mst1/2 is phosphorylated along with Sav which facilitates binding. This activates Mst1/2 kinase activity, allowing it to phosphorylate a threonine residue on Lats1/2. Mst1/2 is also able to phosphorylate Mob proteins, which are then able to bind to Lats1/2 to remove autoinhibitory functions. Following Lats1/2 phosphorylation and activation, its PY-motif binds to WW domains present in TAZ or YAP, allowing for their subsequent phosphorylation. This inhibits their nuclear shuttling as phosphorylation causes interaction and tethering to cytosolic 14-3-3 proteins. When TAZ/YAP becomes activated, however, they translocate into the nucleus to initiate gene expression by binding to transcription factors such as Tead1/4.
the cytoplasm via interactions with 14-3-3 proteins (Zhao, Wei et al. 2007, Ren, Zhang et al. 2010). In contrast, when TAZ/YAP becomes activated, they translocate into the nucleus to initiate gene expression via binding to and regulating transcription factors such as Tead1/4 (Goulev, Fauny et al. 2008, Zhao, Ye et al. 2008). The exact mechanism which releases TAZ/YAP into the nucleus remains poorly understood.

**TAZ/YAP Nucleocytoplasmic Shuttling**

As previously noted, TAZ and YAP phosphorylation by Lats1/2 is a key determinant in their nuclear accumulation. There are five serine residues on YAP and four serine residues on TAZ which undergo Lats1/2 phosphorylation. Phosphorylation of YAP S127, which corresponds to TAZ S89 (Piccolo, Dupont et al. 2014) enables binding to 14-3-3 proteins, leading to TAZ/YAP cytoplasmic retention. However, nuclear accumulation of S127-phosphorylated YAP and S890-phosphorylated TAZ has been reported, suggesting that another, as of yet unidentified regulatory mechanism, modulates still TAZ/YAP nucleocytoplasmic shuttling (Fischer, Rikeit et al. 2016). I speculated that PDLIM7, which is known to bind to nuclear factors tethering them to the cytoskeleton, may be involved in the regulation of TAZ and/or YAP nucleocytoplasmic transport. Although an interaction between PDLIM7 and TAZ/YAP has not been previously reported, the presence of WW domains on TAZ and YAP which are known to bind to PY (PPXY) motifs present in PDLIM7 supports this potential interaction. I set out to determine whether PDLIM7 is a binding partner of TAZ and/or YAP.

**TAZ is an Interacting Protein of PDLIM7**

My preliminary results show that upon co-transfection, PDLIM7 and TAZ co-immunoprecipitate in HEK 293 cells (Figure 5.3), indicating an interaction in living cells that has
Figure 5.3. PDLIM7 and TAZ co-immunoprecipitate in HEK293 cells. (A) HEK 293 cell lysate transfected with combinations of HA-tagged hPDLIM7, mCitrine-tagged YAP, mCitrine-tagged TAZ, mCitrine empty vector, or untransfected lysate were subjected to immunoblotting with anti-PDLIM7 and anti-GFP antibodies to confirm successful transfection. (B) Lysates were subjected to immunoprecipitation with GFP-TRAP agarose beads. Beads were washed and separated by SDS/PAGE, then subjected to Western blotting with anti-PDLIM7 and anti-GFP antibodies. mCitrine-tagged TAZ, but not mCitrine-tagged YAP or mCitrine empty vector was seen to co-immunoprecipitate with PDLIM7.
not been previously reported. As discussed, I have identified PDLIM7 as a protein which is differentially localized upon myotube formation; PDLIM7 localization changes from the actin cytoskeleton in myoblasts to a diffuse cytoplasmic localization in multi-nucleated mature myotubes (D'Cruz, Plant et al. 2016). Differential localization of nuclear factors TAZ and YAP is well documented to play a role in myotube formation (Jeong, Bae et al. 2010, Watt, Judson et al. 2010), as TAZ promotes differentiation whereas YAP promotes proliferation, however the mechanism of differential regulation remains unknown. Evidence of PDLIM7 being involved in sequestration of nuclear factors has been previously reported (Bimber, Dettman et al. 2007), potentially outlining a PDLIM7-mediated regulation of TAZ nucleocytoplasmic transport. This interaction may represent the upstream mechanism whereby TAZ nuclear transport is differentially regulated from YAP in myogenesis, which is an exciting finding that needs further evaluation.

TAZ contains an N-terminal Tead binding domain, a 14-3-3 binding domains, 1 WW domain, a TAD transcriptional activation domain, and a C-terminal PDZ binding domain (Hong and Guan 2012). The presence of a WW domain points towards a potential interaction with PDLIM7 via a WW domain-PY motif interaction. Further experiments analyzing the binding domains involved in the PDLIM7/TAZ interaction can elucidate whether WW domain-PY motif binding underlies this interaction. Also, future experiments can look into analyzing whether this interaction is biologically relevant to TAZ mediated myogenesis. If these experiments are conclusive, TAZ represents an interacting protein of PDLIM7 that potentially regulates muscle development.
REFERENCES


