The Role of the Ubiquitin Ligase Nedd4-1 in Skeletal Muscle Atrophy

by

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Abstract

Skeletal muscle (SM) atrophy complicates many illnesses, diminishing quality of life and increasing disease morbidity, health resource utilization and health care costs. In animal models of muscle atrophy, loss of SM mass results predominantly from ubiquitin-mediated proteolysis and ubiquitin ligases are the key enzymes that catalyze protein ubiquitination. We have previously shown that ubiquitin ligase Nedd4-1 is up-regulated in a rodent model of denervation-induced SM atrophy and the constitutive expression of Nedd4-1 is sufficient to induce myotube atrophy *in vitro,* suggesting an important role for Nedd4-1 in the regulation of muscle mass. In this study we generate a Nedd4-1 SM specific-knockout mouse and demonstrate that the loss of Nedd4-1 partially protects SM from denervation-induced atrophy confirming a regulatory role for Nedd4-1 in the maintenance of muscle mass *in vivo*. Nedd4-1 did not signal downstream through its known substrates Notch-1, MTMR4 or FGFR1, suggesting a novel substrate mediates Nedd4-1's induction of SM atrophy.

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List of Abbreviations

AIDS:	Acquired immune deficiency syndrome
ATP:	Adenosine triphosphate
AKT:	Thymoma viral oncogene homolog 1
AMPK:	Adenosine monophosphate activated protein kinase
bHLH:	basic helix-loop-helix
COPD:	Chronic obstructive pulmonary disease
CHF:	Chronic heart failure
CNS:	Central nervous system
dNedd4:	drosophila Nedd4
dNTP:	deoxynucleotide
eIF3-f:	elongation initiation factor 3 subunit 5
ENaC:	Amiloride-sensitive epithelial sodium channel
EGFR:	Epidermal growth factor receptor
ECL:	Enhanced chemiluminescence
ECM:	Extracellular matrix
FES:	Functional electrical stimulation
FoxO:	Forkhead O
FGFR1:	Fibroblast growth factor receptor 1
FITC:	Fluorescein Isothiocyanate

FYVE:	Fab1, YGL023, Vps27, EEA1
GSK-3β:	Glycogen synthase kinase 3 beta
Grb10:	Growth factor receptor-bound protein 10
HECT:	Homologous to E6-AP carboxy terminus
HRP:	Horseradish peroxidase
HA:	Hemagglutinin
HaCaT:	Human keratinocytes
IGF:	Insulin-like growth factor
Iκβα:	Nuclear factor of kappa light polypeptide gene enhancer in B cell Inhibitor, alpha
IKK:	Iκβ kinase
IGF-1R:	Insulin-like growth factor receptor 1
ISG-15:	Ubiquitin-like protein interferon stimulated gene 15
KO:	Knockout
LMP2A:	Latent membrane protein 2A
LAPTM:	Lysosome associated protein trans-membrane
MS:	Mass spectrometry
MudPIT:	Multidimensional protein identification technology
mTOR:	Mechanistic target of rapamycin
MDCK cells:	Madin-darby canine kidney cells

MEF2C:	Myocyte-specific enhancer factor 2C
Myf5:	Myogenic factor 5
MRF4:	Myogenic regulatory factor 4
MTM:	Myotubularin
MTMR4:	Myotubularin related protein 4
MMP:	Metalloproteases
MHC:	Myosin heavy chain
MyoD:	Myogenic differentiation antigen 1
MuRF1:	Muscle RING finger 1
NF-κB:	Nuclear factor kappa light chain enhancer of activated B cells
NHLBI:	National heart, lung, and blood institute
Nedd4:	Neural precursor cell expressed developmentally down-regulated 4
Ndfip:	Nedd4-WW domain binding protein
NMJ:	Neuromuscular junction
Notch1 FL:	Notch1 full length
Notch1 ICD:	Notch1 intracellular domain
PI3K:	Phosphatidylinositol 3 kinase
PTEN:	Phosphate and tensin homologue
PCR:	Polymerase chain reaction
PBS:	Phosphate buffered saline

Pax7:	Paired box 7
PTP:	Phosphatase
PI(3)P:	Phosphatidylinositol 3 phosphate
$PI(3,5)P_2$:	Phosphatidylinositol 3,5-bisphosphate
PAE cells:	Porcine aorta endothelial cells
P-Smad 2/3:	Phosphorylated Smad 2/3
qPCR:	Quantitative polymerase chain reaction
RING:	Really interesting new gene
ROS:	Reactive oxygen species
RT:	Room temperature
R-Smad:	Receptor-regulated Smad
RT-PCR:	Reverse transcription polymerase chain reaction
SCF:	Skp-1, cullin 1 and F-box protein complex
S6K:	RPS6-p70 protein kinase
SM:	Skeletal muscle
SMA:	Skeletal muscle atrophy
SMS:	Skeletal muscle specific
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SiRNA:	Short interfering RNA
SMA:	Smooth muscle actin

TNF:	Tumor necrosis factor
TRITC:	Tetramethylrhodamine-5-(and 6)-isothiocyanate
TGFβ1:	Transforming growth factor beta 1
Ub:	Ubiquitin
UPS:	Ubiquitin-proteasome system
VEGF-R2:	Vascular endothelial growth factor receptor 2
WT:	Wild type
WB:	Western blotting

Chapter 1

1 INTRODUCTION

1.1 SKELETAL MUSCLE

Skeletal muscles are voluntary muscles responsible for physical movement of the body. These muscles support the skeleton and account for up to 50% of total body weight (Angione et al., 2011). Skeletal muscle also participates in multiple regulatory metabolic processes and as such, serves as a reservoir for amino acid and energy stores for the body (Roach, 2002).

Skeletal muscle is made up of multiple muscle fibers held together by connective tissue. Muscle fibers are long, cylindrical and multinucleated cells formed from the fusion of terminally differentiated myocytes, and myogenic precursor cells known as myoblasts (Scott et al., 2001). Muscle fibers consist of myofibrillar proteins arranged in a repeating unit called a sarcomere (Figure 1.1). Sarcomeres are the basic functional unit of the muscle responsible for contraction, constituting approximately 55-60% of muscle protein by weight and are the most important proteins in terms of muscle power, muscle growth, and protein turnover (Schiaffino and Reggiani, 2011; Tskhovrebova and Trinick, 2012). There are over 15 myofibrillar/sarcomeric proteins; the predominant ones being, actin and myosin (Figure 1.1). Sarcoplasmic (cytosolic) proteins are involved in multiple muscle metabolic processes, (i.e. glycolysis, gluconeogenesis) and constitute only approximately 30-35% of total protein in skeletal muscle by weight (Goll et al., 2008).

Myosin is composed of heavy and light chains, and muscle fibers are divided into subtypes based on the kind of myosin heavy chain (MHC) present in them (Figure 1.1;

Hollingworth et al., 2011). Slow-twitch muscle (MHC type I) isoform fibers use oxidative metabolism, due to presence of a high number of mitochondria, and as a result are resistant to fatigue, are less pH sensitive, and are more suited for endurance-based activities. Fast-twitch (MHC type II) fibers use both oxidative and anaerobic metabolism and demonstrate faster force-generating capacity than type I, making them more efficient for short bursts of speed but also more prone to fatigue (Pette et al., 2000; 2001).

Regulation of MHC gene expression is controlled by a complex set of processes. Protein composition in skeletal muscle changes overtime and muscle fibers have tremendous capacity to adjust their molecular, metabolic, and functional properties in response to altered functional demands, mechanical loading, or changes in neuromuscular activity (Goll et al., 2008; Schiaffino and Reggiani, 2011). For instance, denervation of skeletal muscle induces a fiber type switch from type I to type II fibers (Finkelstein et al., 1993). Moreover, during fasting, denervation, exposure to glucocorticoids, as well as with pathological conditions associated with muscle wasting such as cancer cachexia or sepsis, type II muscle fibers demonstrate a greater loss of mass compared to type 1 oxidative fibers (Li and Goldberg, 1976; Lieber and Friden, 1988; Matsakas and Patel, 2009; Wallis et al., 1999).

1.2 MUSCLE ATROPHY

Atrophy is a decrease in cell size resulting from variable losses of cellular proteins, organelles and cytoplasm. Skeletal muscle atrophy is both a physiologic phenomenon that occurs as a natural consequence of muscle disuse, ageing and fasting and a pathophysiologic response to a multitude of insults including acute and chronic illnesses such as cancer, diabetes mellitus, chronic renal insufficiency, chronic obstructive pulmonary disease

(COPD) and denervation injury (Cooper et al., 2012; Hornberger et al., 2001; IJkema-Paassen et al., 2001, 2002; Johansen et al., 2003; Liu et al., 2000; Medina et al., 1995; Pereira et al., 2005; Rhoads et al., 2010; Stevenson et al., 2003, 2005; Testelmans et al., 2010). Extensive research in animal models has demonstrated that skeletal muscle atrophy results predominantly from a loss of sarcomeric proteins due to an imbalance between myocyte protein synthesis and protein degradation, whereby proteolysis overwhelms an inadequate synthetic response (Cao et al., 2005; Glass, 2010; Goldberg 1969; Hansen et al., 2006; Kandarian and Jackman, 2006). This net increase in protein catabolism results in smaller muscle fibers and decreased muscle mass which is associated with diminished force generating capacity.

Sarcopenia specifically refers to the natural age-related loss of muscle mass associated with reduced power and potential for recovery from injury (Hafer-Macko et al., 2008; Cooper et al., 2012; Giresi et al., 2005). Muscle mass and strength begins to decline after 40 years of age and more rapidly after 65, leading to the loss of approximately 30-40% of muscle mass (Hafer-Macko et al., 2008). This process is universal and is a major contributor to frailty, physical disability and injury in the elderly adult thereby necessitating placement in institutionalized care, and escalating home healthcare and hospital use, and increasing healthcare expenditures (Cooper et al., 2012). In 2000, direct health care costs attributable to sarcopenia-induced disability in the USA were $18.5 billion dollars (Janssen et al., 2004).

Cachexia is a multifactorial syndrome characterized by a severe reduction in muscle mass as a result of increased protein catabolism due to underlying chronic diseases such as heart and renal failure, COPD, cancer and diabetes mellitus (Gosker et al., 2003; Johansen et

al., 2003; Mostert et al., 2000; Rhoads et al., 2010; Volpato et al., 2002). Cachexia has a large negative impact on disease prognosis and increases complications in these illnesses. Muscle atrophy has been found to be the primary cause of weakness in dialysis patients leading to reduced physical performance, poor quality of life and increased mortality (Johansen et al., 2003). Similarly cachexia has a large negative impact on prognosis and quality of life and is associated with increased mortality in cancer patients (Argiles et al., 2004; Dewys et al., 1980; Warren, 1932). Mortality rates of chronic heart failure (CHF) patients with cachexia have been reported to be almost 3 times higher compared to non-cachexic patients (Anker et al., 1997). Skeletal muscle atrophy in COPD patients has been shown to be associated with diminished exercise capacity (Gosselink et al.,1996), quality of life (Mostert et al., 2000), increased health resource utilization and costs (Decramer et al., 1997) and is a powerful negative predictor of survival (Marquis et al., 2002; Schols et al., 1998). In 2004, The National Heart, Lung, and Blood Institute (NHLBI) estimated direct and indirect costs attributable to COPD in the USA to be $38.8 billion dollars (Foster et al., 2006), a component of which was directly attributable to cachexia induced by endstage COPD.

Muscle denervation resulting from peripheral nerve injury (e.g. traumatic, viral) leads to muscle fiber atrophy and functional impairment (Beehler et al., 2006; Rowan et al., 2012). There is an initial rapid loss of muscle mass (~70-85%) within weeks of denervation which is essentially completely reversible with excellent quality and timely muscle re-innervation (Bain et al., 2001; Finkelstein et al., 1993; Fu and Gordon, 1995). In contrast, prolonged denervation injury results in irreversible skeletal muscle fiber atrophy and fibrosis (Bain et al., 2001; Borisov et al., 2001; Fu and Gordon, 1995; Irintchev et al., 1990). As with

cachexia of chronic illness, muscle atrophy arising from denervation injury and poor quality re-innervation results in significant morbidity and increased costs arising from treatment, rehabilitation, lost workplace productivity and often the necessity for job retraining (Finkelstein et al., 1993; Fu and Gordon, 1995; Jaquet et al., 2001; Rosberg et al., 2005).

Current therapies for restoring muscle mass such as resistance exercise, anabolic steroids, and electrical stimulation are themselves inadequate to completely reverse muscle atrophy associated with chronic illnesses (Glass and Roubenoff, 2010). Resistance-based exercise is a potent stimulator of muscle protein synthesis and is sufficient to reverse atrophy in healthy muscle due to disuse and can partially protect against sarcopenia (Adams et al., 2007; Maddocks et al., 2011; Nicastro et al., 2011). Studies have demonstrated that resistance training also increases skeletal muscle mass and function in patients with cancer cachexia, CHF and COPD (Maddocks et al., 2011; Toth et al., 2012; Vogiatzis et al., 2010). Thus, resistance exercise is an effective therapy to treat muscle atrophy but in certain clinical situations, such as bed-ridden or casted patients, load bearing exercise is not an option.

Although less beneficial, functional electrical stimulation (FES) is an alternative treatment for patients unable to perform resistance exercise (Maddocks et al., 2011). FES therapy applies an electrical stimulus to produce a directed, controlled and comfortable contraction of the muscle undergoing therapy, with concurrent relaxation of the underlying muscles. FES stimulation in patients with acute denervation injuries and severe heart failure has been demonstrated to increase oxidative enzyme activity in skeletal muscle fibers to enhance muscle regeneration, and attenuate atrophy (Arena et al., 2010; Kern et al., 2002; Smart et al., 2012). However, the practical use of FES is limited to local application to focal muscle(s) and is not applicable to use in systemic disease. Furthermore, in those situations

where treatment of a limited number of target muscles is required, FES can induce the rapid onset of muscle fatigue (type II fibers are more easily stimulated than type I fibers), limiting its effectiveness (Gondin and Cozzone, 2011).

Sensory protection has provided targeted and partial protection in long term denervation injury, but is obviously a treatment modality restricted to patients with muscle atrophying due to interruption of innervation (Bain et al., 2001). Anabolic steroids have been used to prevent muscle wasting due to cancer, acquired immune deficiency syndrome (AIDS) and sarcopenia (Busquets et al., 2010; Cazares-Delgadillo et al., 2011; Von Roenn et al., 1994; Sattler et al., 2009). While steroids have the ability to increase muscle mass, they also show pleiotropic effects on multiple tissues with a resultant undesirable side effect profile (Garevik et al., 2012; Gruber et al., 2011). Therefore, understanding the molecular pathways that mediate skeletal muscle wasting provides the potential to develop interventions that inhibit muscle atrophy and diminish disease morbidity and the associated economic burden.

1.3 **PROTEIN DEGRADATION**

The rate of intracellular protein turnover in skeletal muscle, dictated by both protein synthesis and degradation, is constantly changing in response to physiological demands (Goldberg, 1969). Protein degradation plays an important role in the cell and helps to regulate various essential cellular processes such as cell cycle progression, transcription, signal transduction, and protein-quality control among many others (reviewed in Ciechnover, 2005). There are multiple proteolytic pathways in mammalian cells that participate in the development of skeletal muscle atrophy. These include the lysosome/autophagy, calcium-

dependent calpain, pro-apoptotic caspase, and the ubiquitin-proteasome, systems (Du et al., 2004; Goll et al., 2008; Lecker et al., 1999; Zhao et al., 2007).

1.3.1 **The Lysosome/autophagy System**

Lysosomes were originally identified as vacuolar structures in rat liver that contain hydrolytic enzymes and function optimally at an acidic pH (Gianetto and Viala, 1955). Lysosomal proteases such as cathepsins are involved in the digestion of extracellular proteins such as plasma proteins and hormones through receptor-mediated endocytosis, phagocytosis, and pinocytosis (Figure 1.2; Ciechanover, 2005). Following internalization of proteins, early endosomes fuse with multi-vesicular bodies that eventually fuse with lysosomes for destruction of these proteins. The use of inhibitors of lysosomal proteases has demonstrated that, while the lysosomal pathway plays a major role in degrading extracellular proteins, it is not involved to any great extent in the normal turnover of cytosolic myofibrillar proteins in rat skeletal muscle under basal conditions (Goll et al., 2008).

Autophagy involves the engulfment of intracellular proteins or organelles within vesicles (autophagosomes), followed by the delivery of the vesicles and fusion of their contents with lysosomes for subsequent degradation (Figure 1.2; Ciechnover, 2005). Several studies have demonstrated that autophagy is activated, and may play a positive role in the induction of muscle atrophy *in vivo* secondary to starvation, denervation or glucocorticoids (Mammucari et al., 2007; Mizushima et al., 2004; O'leary and Hood, 2009; Sandri, 2010; Wang et al., 2005; Zhao et al., 2007, 2008). For example, O'Leary and Hood demonstrated increased expression of multiple autophagy-promoting genes in the muscle of mice denervated for one and two weeks (O'Leary and Hood, 2009). However, Masiero et al. very

recently reported that deletion of atg-7, a gene essential to the autophagic process, in mice led to the inhibition of autophagy and unexpectedly, the subsequent accumulation of myofiber protein aggregates, abnormal mitochondria, induction of oxidative stress and the unfolded protein response, all of which induced myofiber degeneration and muscle weakness (Masiero et al., 2009). Thus, the data together suggest that the extent of autophagy is critical to the maintenance of a healthy, normal muscle mass; excessive autophagy plays a positive role in the induction of muscle atrophy while the absence of autophagy enables the accumulation of toxic products and muscle degeneration.

1.3.2 **The Caspase System**

Caspases are proteases involved in pro-apoptotic pathways (Goll et al., 2008). Several groups have demonstrated a positive role for caspase-3 in rodent models of muscle atrophy induced by diabetes mellitus/insulin resistance, chronic renal insufficiency, and denervation (Du et al., 2004; Plant et al., 2009; Siu et al., 2006; Wang et al., 2006). Caspase-3-deficient mice are partially protected against denervation-induced muscle atrophy due to suppression of apoptotic signaling (Plant et al., 2009). Caspase-3 also regulates muscle proteolysis by cleaving actinomyosin complexes, releasing myofibrils from the sarcomere for subsequent degradation by the proteasome (Du et al., 2004). Elevated caspase-3 activity is evident in the atrophic skeletal muscle of individuals with diabetes mellitus and chronic renal insufficiency (Du and Mitch, 2005; Workeneh et al., 2006).

1.3.3 **The Calpain System**

Calpains are calcium-dependent cysteine proteases that have been shown to regulate the turnover of myofibrillar proteins (Goll et al., 2008). There are approximately 14 distinct

calpains in mammals. Two ubiquitously expressed calpains, u-calpain and m-calpain, and their inhibitor calpastatin, are found in skeletal muscle (Goll et al., 2008). *In vitro* over-expression of a dominant negative m-calpain, or of an inhibitory domain of calpastatin in rat myoblasts decreases the rate of muscle proteolysis by 30% and 63%, respectively (Huang and Forsberg, 1998). Calpains have additionally been shown to mediate muscle atrophy *in vivo* in animals. Activation of μ- and m-calpains is reported to induce muscle proteolysis in models of aging-, denervation- and sepsis-induced muscle atrophy (Smith et al., 2008; Vinciguerra et al., 2010; Williams et al., 1999). Increased stimulation of calpain activity due to increases in intracellular calcium has been demonstrated in muscular dystrophy and other muscle pathologies associated with muscle atrophy (Alderton and Steinhardt, 2000). β-adrenergic agonists attenuate muscle proteolysis by stimulating calpastatin activity (Kretchmar et al., 1989).

1.3.4 **The Ubiquitin-Proteasome System (UPS)**

Although the above-mentioned proteolytic pathways are involved in the degradation of skeletal muscle, ubiquitin-proteasome mediated proteolysis appears to be the predominant mechanism leading to the development of skeletal muscle atrophy (Caron et al., 2011; Tawa and Goldberg, 1997; Lecker et al., 1999; Soloman and Goldberg, 1996; Wang et al., 2006; Wing et al., 1995). Ubiquitination is a reversible post-translational modification that involves the covalent attachment of ubiquitin moieties onto target proteins (Ciechnover, 1994). Ubiquitin (Ub) is a heat stable and evolutionary conserved protein that consists of 76 amino acids and seven lysine residues (Schwartz and Ciechnover, 1992). It is ubiquitously expressed in all eukaryotes and localizes to the cytoplasm, nucleus and several subcellular organelles. Ubiquitin modification of proteins regulates a variety of cell processes including

protein sub-cellular localization, cellular signaling and degradation of damaged and defective proteins (Kornitzer and Ciechnover, 2000; Mukhopadhyay et al., 2007). Protein fate in the cell relies on the pattern of ubiquitin modification present (Marmor and Yarden, 2004). Mono-ubiquitination involves the covalent attachment of a single ubiquitin moiety onto a lysine residue, while multi-ubiquitination involves the attachment of several single ubiquitin moieties onto the target protein. Mono- and multi-ubiquitination serve as signals for endocytosis/sorting and/or trafficking of proteins to lysosomes for degradation. In contrast, poly-ubiquitination involves the attachment of a poly-ubiquitin chain onto a single lysine residue, targeting the protein for degradation by the 26S proteasome (Hershko and Ciechnover, 1998; Mukhopadhyay et al., 2007; Tanahashi et al., 1999).

Three key enzymes, the E1, E2 and E3 ligases (Figure 1.3), each play an indispensable role in the ubiquitination of proteins (Hershko and Ciechnover, 1998; Kornitzer and Ciechnover, 2000; Mukhopadhyay et al., 2007). E1s are ubiquitin-activating enzymes that catalyze the adenosine triphosphate (ATP)-dependent formation of a high-energy thiol ester intermediate between the C-terminal glycine of ubiquitin and cysteine present in the side chain of E1 (Ciechnover, 1994). Once the ubiquitin is activated, E2 or ubiquitin-conjugating (UBC) enzymes transfer the activated ubiquitin to the cysteine residue on the active site of E2 through trans-esterification. While E2 enzymes can directly transfer ubiquitin to the target proteins in case of mono-ubiquitination, a third set of enzymes, the E3 or ubiquitin ligases, are essential for poly-ubiquitination and thus targeting of the protein for 26S proteasome mediated proteolysis.

The E3 ligases link ubiquitin to the target protein and confer specificity to the entire system by interacting with select substrates through specific protein:protein interaction

domains (Rotin and Kumar, 2009). The poly-ubiquitinated proteins are subsequently shuttled to the 26S proteasome, a barrel-shaped structure composed of a 20S subunit and two 19S regulatory complexes (Tanaka and Chiba, 1998; Tanaka, 1998; Tanahashi et al., 1999). The 20S subunit is cylindrical and has a catalytic core with a central cavity containing multiple protease sites. Both sides of the 20S subunit are capped with the 19S regulatory subunits forming a lid and a base. The lid contains deubiquitinating (DUB) enzymes that bind to the poly-ubiquitinated proteins while the base is composed of ATPases that unfold the proteins and use the energy of ATP to pull the substrates through the central pore of the proteasome. This results in the degradation of target proteins while ubiquitin moieties are released for recycling (Kornitzer and Ciechnover, 2000).

1.4 E3 LIGASES AND MUSCLE ATROPHY

While ubiquitin-proteasome mediated proteolysis has been known for decades to play a critical role in the induction of skeletal muscle atrophy (Tawa and Goldberg, 1997), it is only recently that key E3 enzymes involved in the process have been identified (Bodine et al., 2001; Gomes et al., 2001; Plant et al., 2009). There are two families of E3 ligases: HECT (homologous to E6-AP carboxy-terminus) domain and RING (Really Interesting New Gene) finger ubiquitin ligases (Huibregtse et al., 1995; Jackson et al., 2000). HECT-domain ligases first form a thioester bond with ubiquitin and then transfer it to the target protein while RING finger enzymes form multi-subunit Ub-ligase complexes to mediate the transfer of ubiquitin. There are currently approximately one thousand known E3 enzymes, the majority of which are RING finger ligases (Rotin and Kumar, 2009).

RING finger enzyme, E3α (yeast homologue UBR1) of the N-end rule pathway was the first E3 ligase to be implicated in muscle atrophy (Solomon et al., 1998). E3α/UBR1 recognizes primary motifs of N-end rule protein substrates and binds to basic or bulky-hydrophobic amino acids on the amino-terminus (Ciechnover, 1994). The mechanism of E3α/UBR1 is conserved but its role and substrates are still not fully known. E214k is an E2 enzyme that together with E3α/UBR1 accounts for most of the protein ubiquitination through N-end rule pathway in skeletal muscles of rat, rabbits and most mammals. The involvement of E3α/UBR1 in muscle atrophy was proposed with the demonstration of increased mRNA levels of E214k and UBR1 in atrophying muscles (Lecker et al., 1999). Subsequent studies however revealed that E214k (HR6B-/-) and E3α (UBR1-/-) knockout mice are not protected against fasting-induced skeletal muscle atrophy (Adegok et al., 2002; Kwon et al., 2001). A second isoform of UBR1, UBR2, was proposed to play a compensatory role, preventing the development of muscle atrophy (Kwon et al., 2001). Redundancy of these enzymes in N-end rule pathway and inability to delete all the isoforms together in an adult mouse (as they are embryonic lethal), makes it difficult to study the role of this pathway in muscle atrophy (Cao et al., 2005).

In constrast, in 2001 two muscle-specific RING finger E3 ligases, atrogin-1 and MuRF1 (muscle RING finger 1), were found to play a key role in the induction of skeletal muscle atrophy (Gomes et al., 2001; Bodine et al., 2001). Both ubiquitin ligases were identified by muscle transcript profiling and found to be up-regulated in rodent fasting and immobilization models of skeletal muscle atrophy. Mice lacking either the atrogin-1 or MuRF1 gene demonstrated partial protection against denervation-induced skeletal muscle atrophy (Bodine et al., 2001). Numerous studies have subsequently demonstrated increased

expression of both of these E3 ligases in virtually all *in vivo* models of skeletal muscle atrophy ranging from traumatic denervation injury and acute unloading to acute inflammatory states associated with cytokine (eg. TNFα) release and chronic metabolic illnesses including chronic renal insufficiency and diabetes mellitus (Bodine et al., 2001; Doucet et al., 2010; Gomes et al., 2001; Jagoe et al., 2002; Lecker et al., 2004; Stevenson et al., 2003).

Atrogin-1 is a member of Skp-1, Cullin1 and F-box (SCF) containing protein complex (Foletta et al., 2011). To date, atrogin-1 substrates have been determined to be proteins that positively regulate skeletal muscle development. For example, atrogin-1 has been shown to interact with and ubiquitinate MyoD (myogenic differentiation antigen 1), a positive regulator of myoblast proliferation, and eIF3-f (elongation initiation factor 3 subunit 5), an initiation factor known to induce muscle hypertrophy, in skeletal muscle (Lagirand-Cantaloube et al., 2008; Tintignac et al., 2005). Atrogin-1 has also been shown to ubiquitinate and stimulate the proteasomal degradation of myogenin, a transcription factor regulating myoblast differentiation, in a cell culture model of dexamethasone-induced myotube atrophy (Jogo et al., 2009). Interestingly, a recent study has identified myogenin as a transcriptional regulator of atrogin-1, suggesting its role in a feedback loop to control muscle size (Moresi et al., 2010).

MuRF1 preferentially interacts with and ubiquitinates structural proteins such as troponin 1, titin, myosin-binding protein C, and myosin light chain resulting in proteolysis of muscle fibers (thick filament) (Eddins et al., 2011; Kedar et al., 2004; Witt et al., 2005). MuRF1 has also been reported to interact with several proteins associated with glucose production and glycogen metabolism (Foletta et al., 2011). Research is ongoing to determine

the manner by which the targeting of the respective non-structural or non-contractile substrates by both atrogin-1 and MuRF1 induce skeletal muscle atrophy.

More recently we and others have reported a third ubiquitin ligase, Nedd4-1 (neural-precursor-cell-expressed developmentally down-regulated 4-1), to be up-regulated in rodent models of muscle atrophy associated with skeletal muscle inactivity (i.e denervation, unloading, and disuse) (Batt et al., 2006; Koncarevic et al., 2007; Plant et al., 2009). Interestingly, where increased expression of atrogin-1, MuRF1 and Nedd4-1 are all evident in rat gastrocnemius muscle that has been denervated for 1 month, only Nedd4-1 continues to demonstrate a sustained increase in expression long-term (3 months) when muscle atrophy is still progressing (Batt et al., 2006). Increased Nedd4-1 expression is also evident in the atrophied muscle of humans with end stage COPD (Plant et al., 2009). Controversy exists as to whether or not the constitutive over-expression of Nedd4-1 results in the induction of myocyte atrophy (Koncarevic et al., 2007; our unpublished data Figure 1.4) and will be discussed in detail in section 1.6.4 below. Whether Nedd4-1 plays a causative role in the development of skeletal muscle atrophy remains to be determined.

1.5 UPSTREAM SIGNALING AND RECRUITMENT OF ATROPHY-INDUCING NETWORKS

Cellular signaling pathways upstream of and involved in the recruitment of the UPS and other proteolytic systems engaged in the development of skeletal muscle atrophy are currently being delineated. NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling plays an important role in the development of skeletal muscle atrophy in response to inflammatory cytokines (e.g. TNFα), unloading and disuse and results, at least in

part, from NF-κB activation of ubiquitin-proteasome mediated proteolysis (Cai et al., 2004; Hunter et al., 2002; Rhoads et al., 2010). Inhibition of NF-κB signaling in models of disuse-mediated skeletal muscle atrophy and cancer cachexia partially protects against the loss of muscle mass (Hunter and Kandarian, 2004; Wyke and Tisdale, 2005).

There are 5 known mammalian NF-κB family members that exist as dimers in the cytosol; RelA, RelB, c-Rel, p50 and p52 and all are expressed in skeletal muscle (Glass, 2005). In an inactivated state, NF-κB is located in the cytosol in a complex with the inhibitory protein, IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha). Extracellular signals activate the enzyme IκB kinase (IKK) leading to phosphorylation, ubiquitination and proteasomal degradation of IκBα (Gammeren et al., 2009; Sandri, 2008). Degradation and dissociation of IκBα from NF-κB results in the translocation of NF-κB to the nucleus where it regulates gene transcription (Figure 1.5). Activation of IKK/NF-κB signaling has been demonstrated to induce increases in MuRF1, atrogin-1 and Nedd4-1, thus engaging the UPS and inducing muscle atrophy (Cai et al., 2004, Judge et al., 2007). Conversely, deletion of MuRF1 gene partially inhibits muscle wasting associated with activation of NF-κB signaling (Cai et al., 2004).

Transcriptional regulation of MuRF1 and atrogin-1 also occurs via the family of FoxO (Forkhead O) transcription factors 1, 3 and 4 (Sandri et al., 2004). Activation of FoxO proteins occurs via their dephosphorylation, which enables their translocation from the cytosol to the nucleus where they directly or indirectly lead to the up-regulation of atrogenes (Figure 1.6; Glass, 2005). FoxO3a directly binds to the atrogin-1 promoter in mouse skeletal muscle to activate its transcription (Sandri et al., 2004). FoxO4 is responsible for TNFα-

mediated increase in atrogin-1 expression in mouse myoblasts (Moylan et al., 2008). Interestingly, phosphorylation of FoxO proteins is maintained by AKT (thymoma viral oncogene homolog 1), a serine/threonine kinase of the IGF-1 (insulin-like growth factor)/PI3K (phosphatidylinositol 3 kinase)/AKT signaling network that is the primary network responsible for the induction of muscle hypertrophy, demonstrating a reciprocal link between signaling networks that regulate skeletal muscle atrophy and hypertrophy (Figure 1.6; Kandarian and Jackman, 2006). Stimulation of the IGF-1/PI3K/AKT pathway by ligand (i.e. IGF-1 or insulin) binding with the IGF-1 receptor activates PI3K which in turn phosphorylates and activates AKT (Figure 1.6). AKT subsequently phosphorylates several downstream substrates, two of which, the mTOR (mechanistic target of rapamycin)/S6K (RPS6-p70 protein kinase) and GSK-3β (glycogen synthase kinase 3 beta) pathways, are activated and inhibited by AKT respectively, to induce muscle protein synthesis (Glass, 2010; Dupont-Versteegden and Waters, 2011; Sandri, 2008; Stitt et al., 2004). The concurrent phosphorylation of the FoxO transcription factors by AKT maintains their retention in the cytosol, resulting in the inhibition of atrogin-1 and MuRF1 transcription (Sandri et al., 2004). Thus, the hypertrophy-inducing IGF1/PI3K/AKT pathway also dominantly inhibits the development of skeletal muscle atrophy. Conversely, low PI3K/AKT activity results in diminished FoxO phosphorylation, nuclear translocation and increased expression of MuRF1 and atrogin-1 with the ensuing consequence of increased UPS-mediated muscle proteolysis (Figure 1.6; Lee et al., 2004; Zhao et al., 2007; Stitt et al., 2004; Sandri et al., 2004). More recently, it has been determined that the FoxO proteins induce the transcription of genes that mediate autophagy (i.e. Beclin 1, LC3, Ulk2 and Gabarapl1) demonstrating yet another reciprocal link between cellular signaling networks regulating

skeletal muscle hypertrophy and atrophy (Figure 1.6; Lecker et al., 2004; Mammucari et al., 2007; Sandri, 2010; Zhao et al., 2007; Zhao et al., 2008).

Mitochondria are crucial organelles involved in the production of energy and in the regulation of intracellular signaling cascades. Damage to mitochondria has been shown to activate caspases that not only stimulate apoptosis but, as previously noted, also induce the dismantling of sarcomeric proteins for proteasome-mediated degradation contributing to muscle atrophy resulting from catabolic diseases (eg. chronic renal insufficiency) and denervation (Adhihetty et al., 2007; Du et al., 2004; Romanello and Sandri, 2010; Workeneh et al., 2006). Alternatively, expression of mitochondrial fission machinery has been shown to activate AMPK (adenosine monophosphate-activated protein kinase) which subsequently activates FoxO3 to promote protein breakdown via autophagy/lysosome and ubiquitin-proteasome pathways (Romanello et al., 2010). Moreover, mitochondria are the predominant source of reactive oxygen species (ROS) production in skeletal muscle (Barbieri and Sestili, 2012). Oxidative stress due to ROS has been shown to contribute to muscle loss in sarcopenia, denervation and immobilization-induced atrophy (Min et al., 2011; Muller et al., 2007; Singh and Hood, 2011). Studies suggest that ROS induce muscle proteolysis and decrease muscle fiber size by activation of both NF-κB and FoxO signaling pathways (Furukawa-Hibi et al., 2002; Li et al., 1998; Sandri et al., 2004). More recently others have shown that inactivity-induced oxidative stress leads to the activation of caspase-3 and calpains in skeletal muscle (Barbieri and Sestili, 2012; Min et al., 2011). Subsequent treatment with mitochondrial-targeted antioxidants attenuates inactivity-induced increases in mitochondrial ROS production and prevents oxidative stress, protease activation, and

myofiber atrophy. Thus, present studies provide clear evidence that mitochondrial energy depletion and ROS production plays an important role in the loss of muscle mass.

1.6 **Nedd4-1**

Nedd4-1 is a HECT domain ubiquitin ligase (Scheffner and Staub, 2007). It was originally identified as a highly-expressed gene in the early embryonic central nervous system (CNS) of the mouse that is later down-regulated during development (Kumar et al., 1992). Nedd4-1, originally thought to be expressed only in the CNS, is now known to be ubiquitously expressed in multiple tissues and cell types (Kumar et al., 1997; Staub et al., 1996). In mammals there are nine members in the Nedd4 family of proteins; Nedd4-1 (Nedd4), Nedd4-2 (Nedd4L), Smurf1 and 2, Itch, NEDL1 and 2 and WWP 1 and 2, of which Nedd4-1 and Nedd4-2 are the most closely related (Scheffner and Staub, 2007; Yang and Kumar, 2010). Nedd4-1 is the ancestral member while Nedd4-2 originated later in evolution perhaps due to gene duplication (DiAntonio, 2010). Nedd4-1 is closely related to the single member of the Nedd4 family in yeast *Sachharomyces cerevisiae*, Rsp5 (Dunn et al., 2004; Dunn and Hicke, 2001). Four Nedd4 proteins exist in Drosophila, including dNedd4, but there are no Nedd4-2 orthologues (Ing et al., 2007). All vertebrates have both Nedd4-1 and Nedd4-2 genes and although both isoforms are present in skeletal muscle, Nedd4-1 remains the predominant one (Kamynina et al., 2001).

1.6.1 **Structure of Nedd4-1 and Nedd4-2**

Nedd4-1 and Nedd4-2 share a similar domain structure comprised of an N-terminus C2 domain, 3 or 4 WW domains and a C-terminus catalytic HECT domain (Figure 1.7).

Human and zebrafish Nedd4-1 contain four WW domains while rat and mouse Nedd4-1 have only three, missing the WW3 domain (Henry et al., 2003; Kamynina et al., 2001).

The C2 domain of Nedd4-1 binds to membrane phospholipids in a calcium-dependent manner (Luo and Weinstein, 1993; Medkova and Cho, 1998) and has been shown to associate with annexin XIIIb to induce calcium-dependent redistribution of Nedd4-1 from the cytosol to the plasma membrane in Madin-Darby canine kidney (MDCK) cells (Plant et al., 1997; 2000). The HECT domain is the Nedd4-1 catalytic domain that has an evolutionary conserved cysteine residue that forms thioester bond with ubiquitin moieties (Rotin and Kumar, 2009). The WW domains are protein:protein interaction domains containing 2 highly conserved tryptophans and an invariant proline that binds to proline rich sequences (PY motifs, L/PPXY) in Nedd4-1 substrates (Staub and Rotin, 1996). There is a unique role for each WW domain and they share greater sequence similarity between 2 proteins than between domains of the same protein suggesting they have evolved to carry out distinct functions (Sudol et al., 1995). The WW3 and WW4 domains are the key Nedd4-1-PY motif binding domains, with WW3 exhibiting the highest affinity (Kanelis et al., 2006). Recently, it has been reported that the WW3 and C2 domains of human and zebrafish Nedd4-1 also bind to a novel non-canonical (non-PY motif) sequence, VL***PSR, in activated fibroblast growth factor receptor 1 (FGFR1; Persaud et al., 2011).

1.6.2 **Functions of Nedd4-1 and Nedd4-2**

Nedd4-1 and Nedd4-2 regulate a number of cellular processes including endocytosis, membrane protein sorting and trafficking, and protein degradation. While there is some redundancy of the signaling networks they engage, both proteins demonstrate distinct

preferences for substrate binding, and thus regulate distinct cellular functions (Yang and Kumar, 2010).

Regulation of voltage gated channels

The first and best characterized substrate of Nedd4 is the amiloride-sensitive epithelial sodium channel (ENaC; Abriel and Staub, 2005). Although initial studies suggested both Nedd4-1 and Nedd4-2 bind and ubiquitinate ENaC, it is now clear Nedd4-2 is the predominant E3 ligase that targets this channel (Henry et al., 2003; Kamynina et al., 2001). ENaC activity is down-regulated by Nedd4-mediated ubiquitination and degradation in response to increased intracellular sodium. Nedd4-2 interacts with the PY motifs of the β and γ subunits of ENaC through its WW3 and WW4 domains and mutation or deletion of any PY motif leads to Liddle's syndrome, an autosomal dominant form of hypertension (Harvey et al., 1999; Staub et al., 2000).

Growth factor receptor regulation

Nedd4-1 has been shown to be involved in the regulation of growth factor receptor stability and downstream signaling. These include insulin-like growth factor receptor 1 (IGF-1R), epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor-2 (VEGF-R2) (reviewed in Yang and Kumar, 2010). Conflicting views exist as to whether Nedd4-1 is a positive or negative regulator of IGF-1R signaling. IGF-1R interacts indirectly with Nedd4-1 via the adapter protein Grb10 (growth factor receptor-bound protein 10), but the consequence of this interaction is disputed. Some studies suggest the interaction attenuates IGF-1R signaling by inducing the endocytosis and degradation of IGF-1R

(Vecchione et al., 2003). In contrast, others demonstrate that Nedd4-1 stabilizes IGF-1R cell surface expression, promoting its downstream signaling (Cao et al., 2008). VEGF-R2 is ubiquitinated by Nedd4-1, targeting it for degradation, but interestingly Grb10 inhibits this degradation (Murdaca et al., 2004). Nedd4-1 indirectly regulates EGFR degradation through interaction, ubiquitination, and subsequent degradation of activated cdc42-associated tyrosine kinase (Lin et al., 2010).

Regulation of T-cell function

Nedd4-1 promotes T-cell activation and enhances the adaptive immune response by regulating the ubiquitination and degradation of cbl-b, a protein that disrupts T-cell receptor signaling. Nedd4-1 deficient mouse T-cells do not proliferate well and show decreased response to antigen (Yang et al., 2008).

Regulation of viral budding

Nedd4-1 has been shown to bind and ubiquitinate viral matrix proteins such as LMP2A (latent membrane protein 2A) of Epstein Barr virus to allow signal trafficking through the vesicular transport machinery of the host (Ikeda et al., 2000).

Possible regulation of tumorigenesis

Nedd4-1 has been shown to be up-regulated in mouse cancer models and multiple human cancer samples compared to nearby normal tissues and *in vitro* studies have shown that Nedd4-1 ubiquitinates and degrades PTEN (phosphatase and tensin homologue), a major tumour suppressor gene, suggesting a positive regulatory role for Nedd4-1 in tumorigenesis

(Trotman et al., 2007; Wang et al., 2007). In contrast, Nedd4-1 knockout and siRNA knockdown studies have failed to detect any changes in the levels of PTEN (Fouladkou et al., 2007). Therefore, the role of Nedd4-1 in tumorigenesis remains controversial.

Regulation of protein sorting

Nedd4-1 regulates proper sorting of LAPTM (lysosome associated protein trans-membrane) proteins to the lysosome in HEK293 cells. A fifty percent reduction was shown in the lysosomal localization of LAPTM4a in Nedd4-1-deficient mouse fibroblasts (Milkereit and Rotin, 2011). LAPTM4a and LAPTM4b are ubiquitously expressed and have been shown to play a role in multidrug resistance in yeast (Hogue et al., 1999). Over-expression of these proteins can cause increased proliferation of cells leading to cancer (Kasper et al., 2005). Therefore, proper sorting of these proteins by Nedd4-1 is crucial to the cell.

1.6.3 **Regulation of Nedd4-1 and Nedd4-2 Substrate Binding and Ubiquitination**

Multiple proteins regulate the interaction of the Nedd4 family of proteins with their substrates. Ndfip (Nedd4-WW domain binding protein) 1 and 2 are adaptor proteins that attenuate Nedd4 substrate binding by interacting with WW domains of Nedd4-1 and sequestering it from its substrates (Shearwin-Whyatt and Kumar, 2006). Over-expression of Ndfip2 in *Xenopus* oocytes leads to the inhibition of Nedd4-2-mediated regulation of ENaC (Konstas et al., 2002). 14-3-3 proteins are ubiquitously expressed in eukaryotes and regulate cellular signaling by providing scaffolds, causing protein conformational changes, or by binding and preventing protein:protein interactions (Bridges and Moorhead, 2005). 14-3-3 binds Nedd4-2 to prevent Nedd4-2 mediated ubiquitination and subsequent degradation of

ENaC (Ichimura et al., 2005). Similarly, AKT-mediated phosphorylation of Nedd4-2 WW domains prevents Nedd4-2 binding with ENaC (Snyder et al., 2002). ISG-15 (Ubiquitin-like protein interferon stimulated gene 15) binds to Nedd4-1 and interferes with Nedd4-1 and E2 enzyme interaction, thereby affecting the catalytic activity of Nedd4-1 (Malakhova and Zhang, 2008). This inhibition prevents Nedd4-1 from ubiquitinating viral matrix proteins and releasing virus particles (Kumar and Yang, 2010).

It has been recently demonstrated that Nedd4-2 can self-regulate its catalytic activity through a weak intra-molecular interaction between its WW2, 3 and 4 domains and PY motif within the HECT domain (Figure 1.7; Bruce et al., 2008). Upon substrate presentation, the inhibitory interaction between WW domains-HECT domain (PY motif) of Nedd4-2 is disrupted due to the higher affinity of the substrate PY motif for the Nedd4-2 WW domains. Once the substrate is ubiquitinated, dissociation of the Nedd4-2 HECT domain-PY motif and WW domains allows self-ubiquitination of Nedd4-2 leading to its de-stabilization. Therefore, it appears that inter- or intra-molecular WW-domain-HECT domain PY-motif interaction stabilizes Nedd4-2 by preventing its auto-ubiquitination.

1.6.4 **The Role of Nedd4-1 in Skeletal Muscle**

Although Nedd4-1 has been well studied in other tissues, little is known about the biological role of Nedd4-1 in skeletal muscle. Nedd4-1 null mice are embryonic/perinatal lethal (Liu et al., 2009, Cao et al., 2008, Kawabe et al., 2010) due to cardiac and vascular developmental abnormalities (Fouladkou et al., 2010). The embryos have however been assessed for a skeletal muscle phenotype and it appears that Nedd4-1 regulates muscle innervation and neuromuscular junction (NMJ) development (Liu et al., 2009; Kawabe et al.,

2010). Secondary intramuscular motor neurons are markedly defasciculated in the Nedd4-1 null embryos, leading to aberrant muscle innervation patterns and failure of phrenic nerve innervation of the ventral diaphragm, suggesting that Nedd4-1 plays a role in fine tuning the interaction between neuron and skeletal muscle (Liu et al., 2009). It is of interest that Nedd4-1 is not expressed in motor neurons, so that its influence must occur through non-cell autonomous mechanisms, regulated either by Nedd4-1 expression within skeletal muscle or the nerve Schwann cells, or both. Nedd4-1 null embryos are also reported to demonstrate skeletal muscle developmental hypoplasia (Liu et al., 2009). Since correct innervation and NMJ function are essential for normal muscle development, it is unclear if the skeletal muscle hypoplasia results from an intrinsic problem in myogenesis due to muscle Nedd4-1 deficiency, or alternatively, occurs secondarily as a result of the abnormalities of skeletal muscle innervation and the NMJ, or both. A caveat however, is necessary here. The Nedd4-1 null embryos are reported to be globally smaller than wildtype littermates but muscle size is not normalized to body length or weight so it is not possible to determine if muscle developmental hypoplasia is truly present, or simply a manifestation of a global decrease in animal size (Cao et al., 2008; Fouladkou et al., 2010).

Postnatal assessment of Nedd4-1 in skeletal muscle to date has focused on the potential involvement of Nedd4-1 as a positive regulator of muscle atrophy. Several groups have independently demonstrated an increase in the expression of Nedd4-1 in the muscles of rodents subjected to models of hind-limb unloading and short- and long-term models of denervation-induced muscle atrophy (Batt et al., 2006; Koncarevic et al., 2007; Plant et al., 2009). In contrast to atrogin-1 and MuRF1, which increase only in short-term denervated muscle in rats, Nedd4-1 expression was sustained long term, suggesting that it plays a more

encompassing role in the induction of skeletal muscle atrophy (Batt et al., 2006). Koncarevic et al. have demonstrated that in rodents, the increase in Nedd4-1 occurs exclusively in muscle atrophy associated with muscle inactivity, such as denervation, disuse and unweighting leading to speculation that Nedd4-1 is only relevant to atrophy induced by inactivity (i.e. denervation and unloading) (Koncarevic et al., 2007). In contrast, Plant et al. reported increased Nedd4-1 expression in the atrophied muscle of individuals with COPD, where muscle loading and innervation are intact, and therefore the proposal that Nedd4-1 expression only accompanies inactivity-induced skeletal muscle atrophy appears to be an oversimplification (Plant et al., 2009).

Koncarevic et al. have demonstrated that electro-transfer of a catalytically inactive dominant negative Nedd4-1 into rat soleus muscle did not protect the muscle from atrophy induced by seven days of unloading (Koncarevic et al., 2007). This group also reported that short-term overexpression via cDNA electro-transfer of Nedd4-1 into muscle did not induce atrophy over seven days in weight-bearing rats. In contrast to these findings, our laboratory has demonstrated that the constitutive over-expression of Nedd4-1 is sufficient to induce atrophy (i.e. a reduction in area) in mouse C2C12 myotubes *in vitro* (unpublished data Figure 1.4). These apparent discrepancies may result from the use of a dominant negative Nedd4-1 as opposed to a knockout/knockdown approach and/or inadequate levels or duration of Nedd4-1 expression *in vivo.* In addition, the early and single seven day time point of assessment in the rat *in vivo* model is inadequate to observe a prolonged or delayed effect of Nedd4-1, which remains up-regulated for many weeks following denervation while muscle atrophy progresses. This is particularly relevant when one considers that MuRF1 null mice do not demonstrate protection against denervation-induced skeletal muscle atrophy until

fourteen days post nerve transection (Bodine et al., 2001). Finally, while Nedd4-1 clearly induces myotube atrophy *in vitro*, this reported absence of an effect *in vivo* may reflect the presence of compensatory mechanisms *in vivo*, not evident in myotube cultures *in vitro*. Given the existing controversy in the literature, the role of Nedd4-1 in postnatal skeletal muscle, and specifically its role in the induction of muscle atrophy remain to be defined.

1.7 HYPOTHESIS AND OBJECTIVES

1.7.1 PART 1: Investigation of the role of Nedd4-1 in denervation-induced skeletal muscle atrophy

The ubiquitin ligase Nedd4-1 is increased in muscle atrophying as a result of inactivity (denervation, immobilization, unloading). We have shown that the constitutive over-expression of Nedd4-1 decreases the area of C2C12 mouse myocytes *in vitro*. We hypothesize that Nedd4-1 positively regulates the induction and maintenance of skeletal muscle atrophy *in vivo.* The objective of the current study is to assess the role of Nedd4-1 in the development of skeletal muscle atrophy in a genetic model *in vivo*. To this end, we generated a novel genetic tool, Nedd4-1 skeletal muscle specific knockout (Nedd4-1 SMS-KO) mice, for study.

Specific Aim 1: Confirm the specific deletion of Nedd4-1 from skeletal muscle exclusively, in Nedd4-1 SMS-KO mice.

Specific Aim 2: Determine the effect of the deletion of Nedd4-1 on the development of muscle atrophy. In order to achieve this aim, we will subject the Nedd4-1 SMS-KO and control littermate mice to the well validated denervation-induced model of SM atrophy. We will assess skeletal muscle mass by measuring muscle weights and morphometrics (fiber type specific cross sectional area).

1.7.2 **PART 2: Determine which downstream substrates of Nedd4-1 participate in the development of denervation-induced skeletal muscle atrophy**

We hypothesize that Nedd4-1 signals downstream through its known muscle substrate(s) Myotubularin related protein 4 (MTMR4), Notch1 and/or Fibroblast growth factor receptor 1 (FGFR1), to mediate denervation-induced skeletal muscle atrophy.

Specific Aim: Determine the protein levels of MTMR4, Notch1 and FGFR1 in denervated and control skeletal muscle in Nedd4-1 SMS-KO and littermate control mice. We reason that if Nedd4-1 signals downstream via MTMR4, FGFR1 or Notch-1 to induce muscle atrophy, then these protein(s) will be protected from degradation in the denervated muscle of Nedd4-1 SMS-KO mice.

1.7.3 **PART 3: Investigation of the role of the Nedd4-1 substrate MTMR4 in the development of skeletal muscle fibrosis**

Denervation of skeletal muscle induces both atrophy and fibrosis. The inositol phosphatase MTMR4, a known skeletal muscle substrate of Nedd4-1, has been recently demonstrated to attenuate TGFβ1 (Transforming Growth Factor beta-1) signaling by dephosphorylating Smad2 and Smad3. TGFβ1, signaling downstream via its substrates Smad2/3, induces fibrosis and inhibits reparative myogenesis in denervated muscle. In denervated skeletal muscle, MTMR4 protein levels decrease concomitant to the increase in Nedd4-1 protein levels. Hence, we hypothesize that MTMR4 negatively regulates TGFβ1-

induced muscle fibrosis by dephosphorylation of P-Smad2/3 and that the Nedd4-1-mediated decrease of MTMR4 in denervated muscle leads to muscle fibrosis by enhancing TGFβ1 signaling.

Specific Aim 1: Demonstrate that MTMR4 attenuates TGFβ1 signaling by interacting directly or indirectly with, and dephosphorylating, P-Smad2/3. In order to achieve this aim, we will confirm Smad2 and Smad3 phosphorylation upon TGFβ1 treatment in NIH-3T3 cells and will investigate whether MTMR4 co-immunoprecipitates with Smad2/3 in a TGFβ1-dependent manner.

Specific Aim 2: Determine if MTMR4 inhibits TGFβ1-induced fibrosis in C2C12 mouse myoblasts. To achieve this aim, we will constitutively express wildtype MTMR4, and a catalytically inactive MTMR4 mutant in C2C12 myoblasts, and assess the expression of markers of fibrosis in response to TGFβ1 treatment.

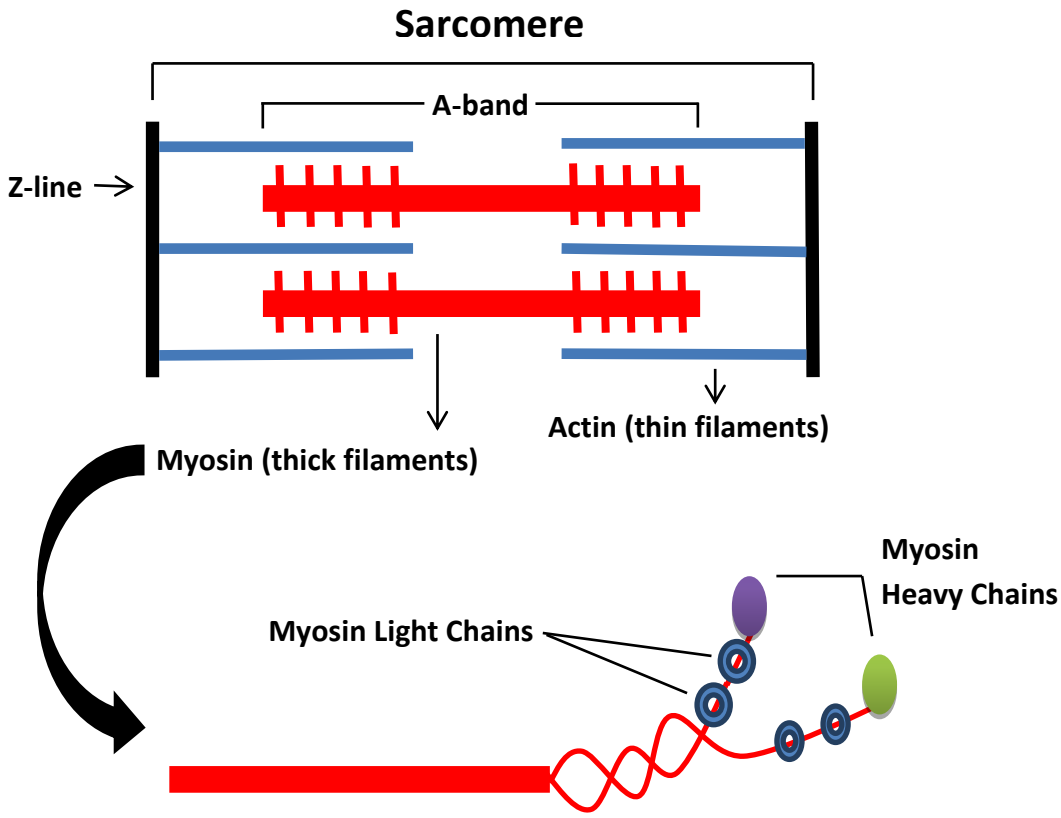


Figure 1.1. Schematic of a sarcomere. Each sarcomere is bounded by two Z lines. Within the sarcomere are the thick myosin filaments and thin actin filaments. The myosin filaments are in the center of the sarcomere within the A-band. In the resting state, the actin filaments overlap with the myosin filaments. Myosins are composed one or two heavy chains and four or more light chains. Each of the heavy chains has a tail region and a globular head region for adenosine triphosphate (ATP) hydrolysis and actin binding. Cyclical interaction between myosin and actin causes muscle to contract and this contraction is driven by the concominant hydrolysis of ATP.

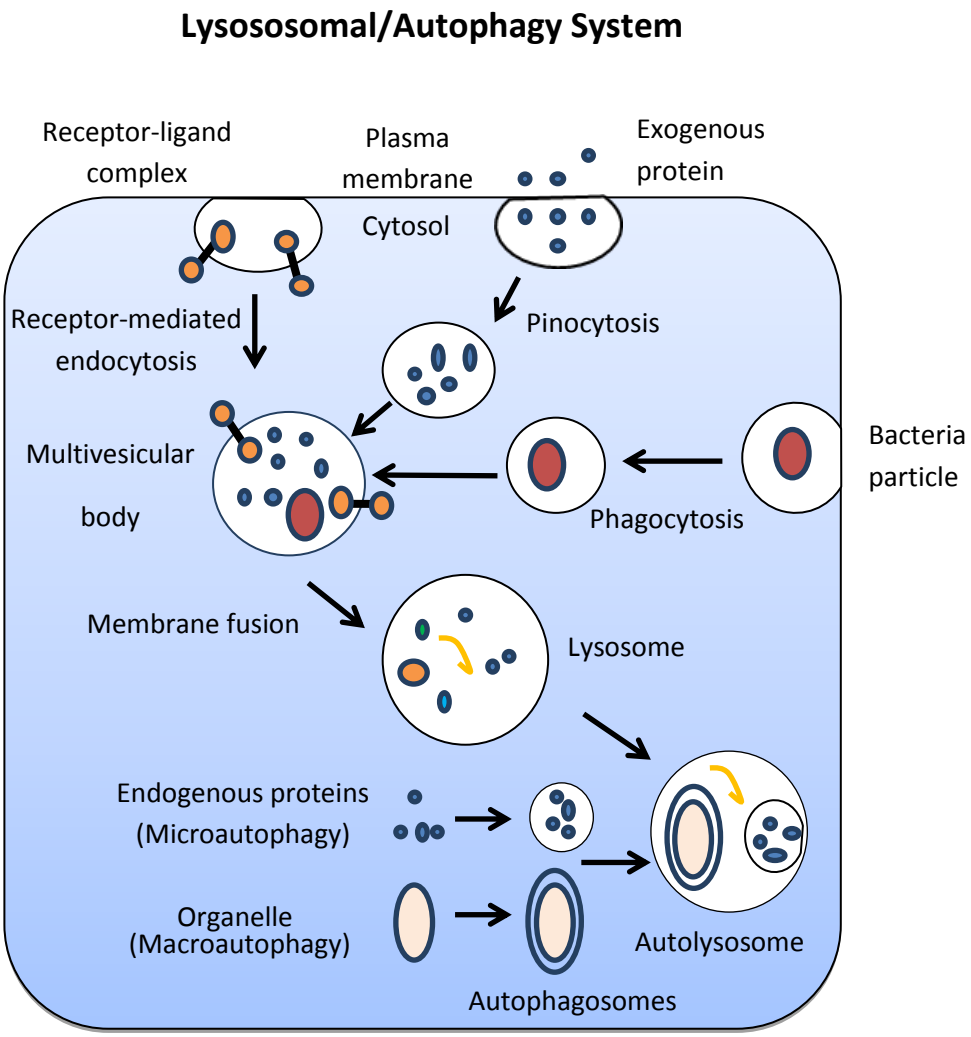


Figure 1.2. The lysosomal/autophagy system. The digestive processes mediated by the lysosome are specific receptor-mediated endocytosis, pinocytosis (the nonspecific engulfment of extracellular fluid), phagocytosis (the engulfment of extracellular particles) and autophagy [the engulfment of intracellular proteins (microautophagy) and organelles (macroautophagy)]. Early endosomes containing internalized proteins fuse to form multivesicular bodies which eventually fuse with lysosomes for degradation. Autophagosomes fuse with the lysosomes to form autolysosome for degradation of organelles and intracellular proteins.

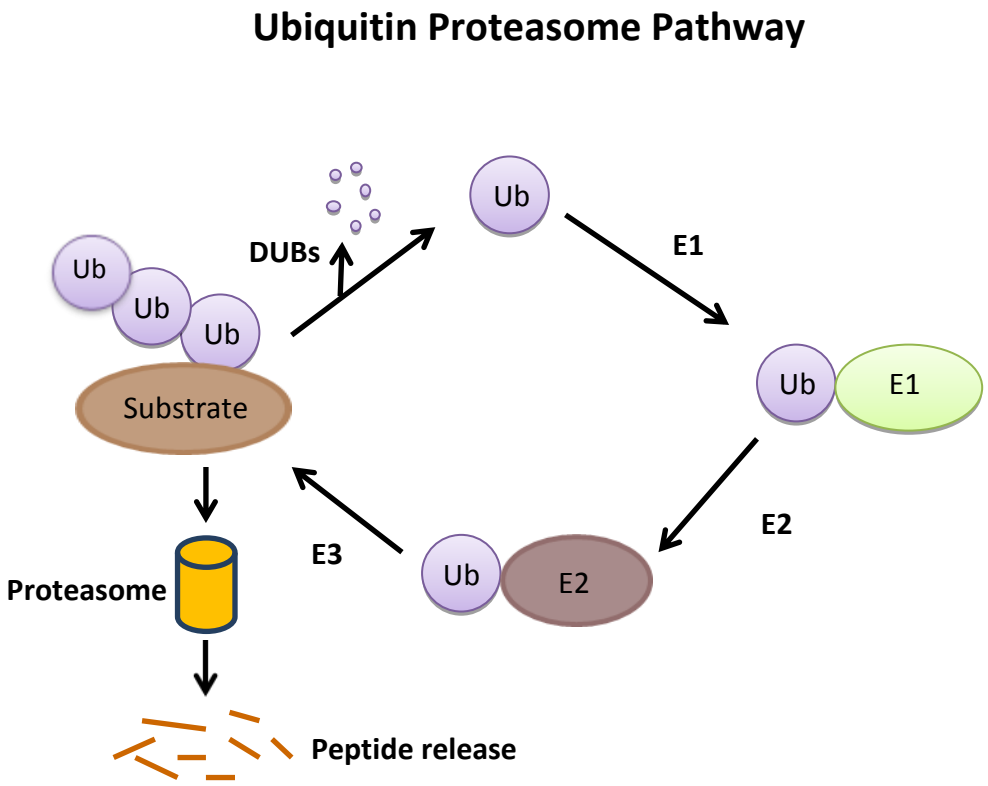


Figure 1.3. **The ubiquitin-proteasome pathway.** E1, ubiquitin activating enzyme, binds to ubiquitin to activate it. The ubiquitin-carrier enzyme E2 takes over the ubiquitin from E1. E2 transfers the ubiquitin to a protein substrate bound to the ubiquitin ligase, E3 or directly to E3. E3 ligase subsequently extends the ubiquitin chain and poly-ubiquinated proteins are targeted for degradation by the 26S proteasome. Each step of the extension can be reversed by de-ubiquitinating enzymes (DUBs). With degradation of the substrate, the ubiquitin molecules are released by deubiquitinating enzymes and peptides are released by the proteasome.

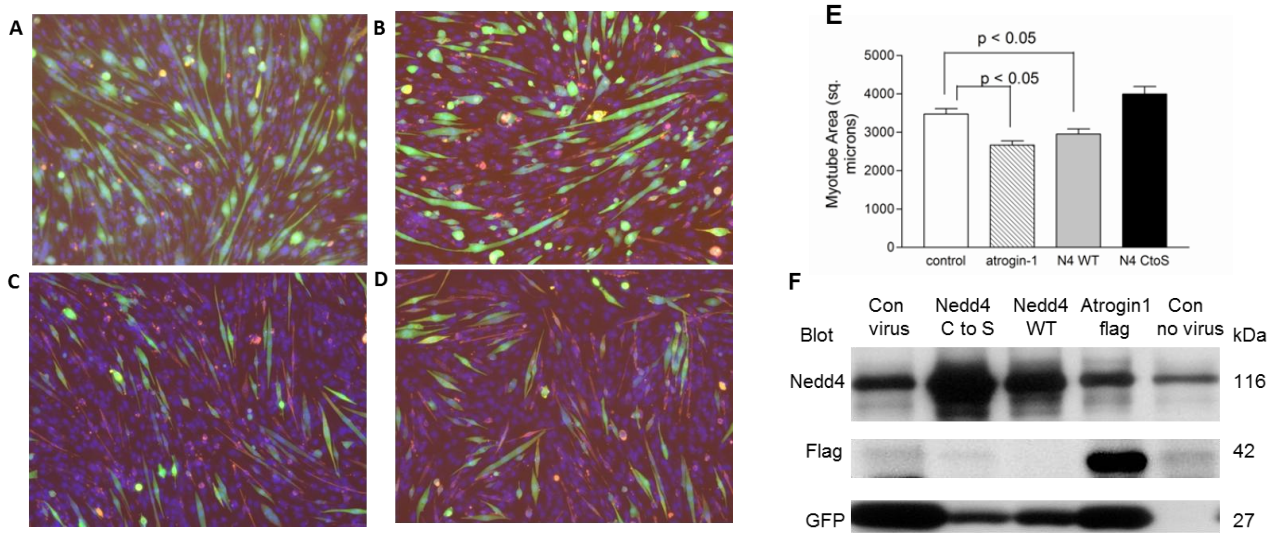


Figure 1.4. Nedd4-1 induces C2C12 myotube atrophy. C2C12 myotube cultures grown on Matrigel, differentiated with low serum, and transduced with Stratagene AdEasy adenovirus expressing (A) control virus, (B) catalytically inactive Nedd4-1 C to S, (C) flag-tagged atrogin-1 and (D) wild type (WT) Nedd4-1 for 96 hours were immunostained for myosin heavy chain (MF20 antibody, Developmental Hybridoma Bank & Alexa-Fluor 555 secondary Ab, orange), and the nucleus stained with Hoechst (blue). The virus has a bicistronic expression cassette that co-expresses GFP, so virus infected cells are green. (E) The area of successfully transduced myotubes (GFP positive, MHC positive multinucleated cells) was measured by a blinded reviewer with SigmaScan Pro (Sigma) image analysis software. Myotubes expressing Nedd4-1 (N4 WT) or atrogin-1 were significantly smaller than control ($p < 0.05$). There was no difference between control and Nedd4-1 C to S (N4CtoS) myotube area. At least 200 myotubes in at least 5 fields were measured for each condition. Data was analyzed with ANOVA followed by Tukey to compare multiple means. (F) SDS-PAGE and Western blotting of myotube protein lysates reveals GFP in virus transduced myotubes. Endogenous Nedd4-1 is present in all myotubes, with increased expression noted in myotubes transduced with WT or C to S Nedd4-1. Atrogin-1 expression is denoted by Flag immunoblotting.

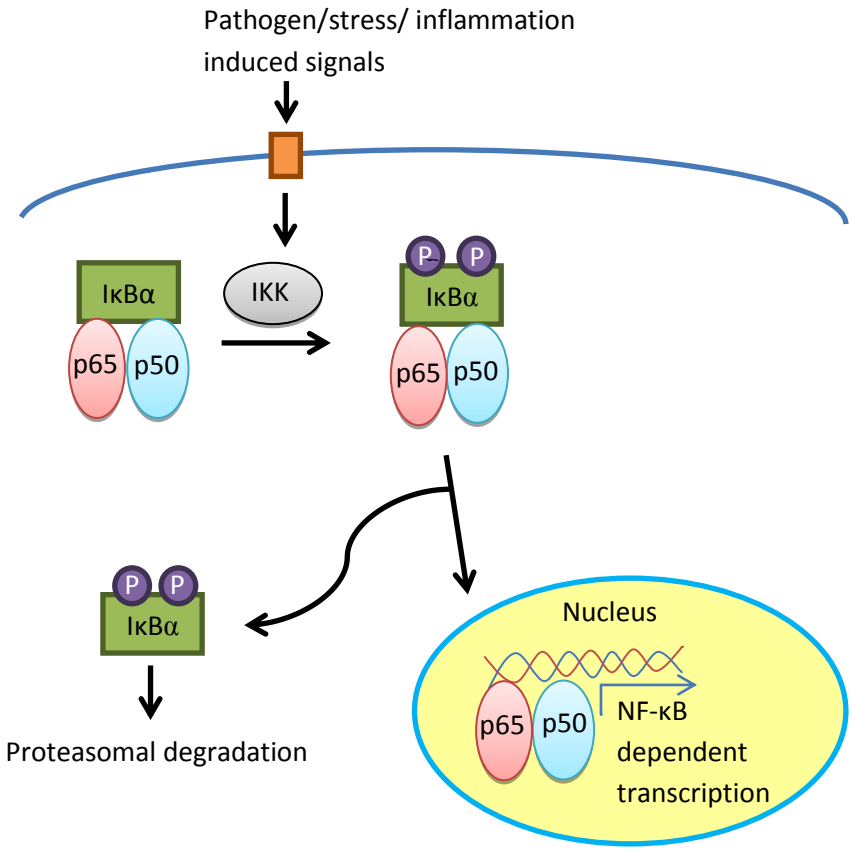


Figure 1.5. Mechanism of NF-κB action. In this figure, the NF-κB heterodimer between p65 and p50 proteins is used as an example. While in an inactivated state, NF-κB is located in the cytosol complexed with the inhibitory protein IκBα. Variety of extracellular signals can activate the enzyme IκB kinase (IKK) which, in turn, phosphorylates the IκBα protein, resulting in dissociation of IκBα from NF-κB, ubiquitination and eventual degradation of IκBα by the proteosome. The activated NF-κB is then translocated into the nucleus where it regulates the transcription of target genes.

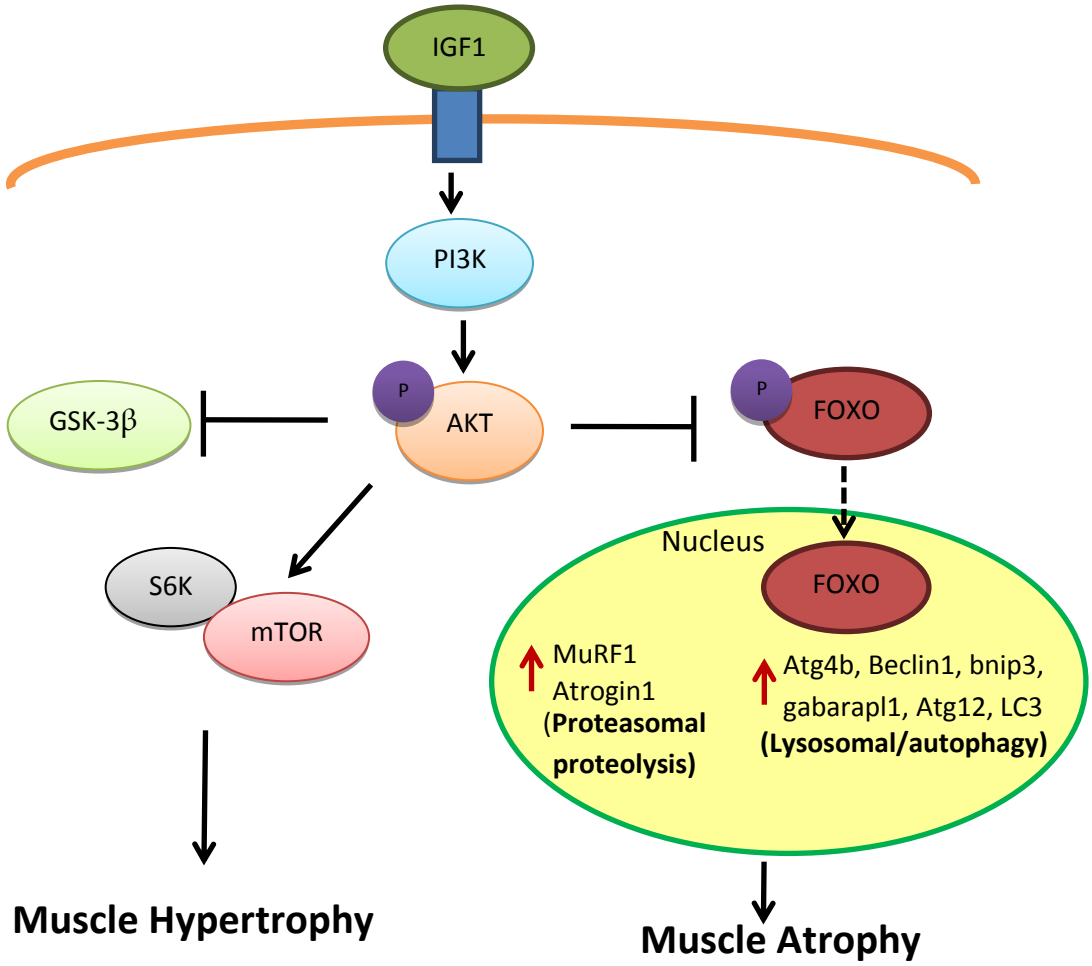


Figure 1.6. IGF-1/PI3K/AKT signaling and the balance between skeletal muscle hypertrophy and atrophy. IGF-1 or insulin binding with the IGF-1 receptor activates PI3K which in turn phosphorylates and activates AKT. AKT subsequently phosphorylates several downstream substrates, two of which, the mTOR/S6K and GSK-3β (pathways are activated and inhibited by AKT respectively to induce muscle protein synthesis). Activation of AKT/mTOR pathway concurrently phosphorylates FoxO transcription factors, inhibiting their nuclear translocation and subsequent transcription of MuRF1, atrogin-1, and autophagy inducing genes. Conversely low PI3K/AKT activity causes dephosphorylation of FoxO proteins leading to their translocation to the nucleus to induce the expression of autophagy-related genes and E3 ligases such as MuRF1 and atrogin-1 to induce muscle proteolysis and atrophy.

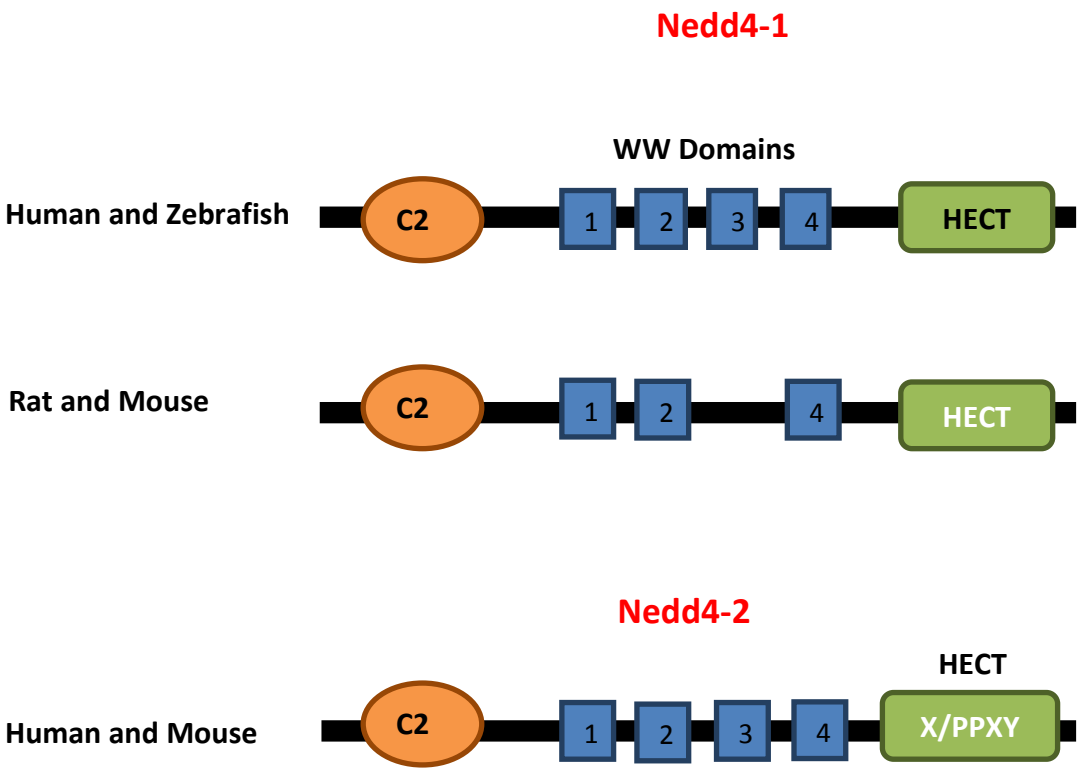


Figure 1.7. Schematic diagram of Nedd4-1 and Nedd4-2. Both proteins consist of the calcium/lipid binding C2 domain, 3 or 4 WW domains that interact with protein substrates and the catalytic HECT domain. Human and zebrafish Nedd4-1 contain 4 WW domains while rat and mouse Nedd4-1 only have three, missing the WW3 domain. Human and mouse Nedd4-2 contain 4 WW domains. The HECT domain of Nedd4-2 also has an internal PY motif (X/PPXY) for auto-ubiquitination.

Chapter 2

2 The ubiquitin ligase Nedd4-1 participates in the development of denervation-induced skeletal muscle atrophy

2.1 INTRODUCTION

Skeletal muscle atrophy is a phenomenon associated with muscle disuse, aging and various acute and chronic illnesses such as traumatic denervation injury, burns, diabetes mellitus, chronic uremia, chronic obstructive pulmonary disease (COPD) and cancer (Cooper et al., 2012; Hornberger et al., 2001; IJkema-Paassen et al., 2001, 2002; Johansen et al., 2003; Liu et al., 2000; Medina et al., 1995; Pereira et al., 2005; Rhoads et al., 2010; Stevenson et al., 2003, 2005; Testelmans et al., 2010). Muscle atrophy is associated with increased disease morbidity, mortality and poor quality of life resulting in significant health resource utilization and socio-economic costs (Celli, 2010; Decramer et al., 1997; DeOreo et al., 1997; Foster et al., 2006; Gosker et al., 2003; IJzerman et al., 2012; Janssen et al., 2004; Jaquet et al., 2001; Mostert et al., 2000; Rosberg et al., 2005; Tawney et al., 2003; Volpato et al., 2002).

Skeletal muscle atrophy results from an imbalance between protein synthesis and protein degradation and can be enhanced by inadequate muscle regeneration (Cao et al., 2005; Goldberg, 1969; Hansen et al., 2006; Kandarian and Jackman, 2006). Ubiquitin-proteasome mediated proteolysis is the predominant proteolytic pathway responsible for the development of skeletal muscle atrophy (Annabelle et al., 2011; Lecker et al., 1999; Soloman and Goldberg, 1996; Wang et al., 2006; Wing et al., 1995; Tawa and Goldberg, 1997). In the ubiquitination process, the coordinated action of several enzymes (E1, E2 and E3 ubiquitin

ligases) covalently attaches a single or branched ubiquitin moiety to lysine residue(s) of the target substrate protein, which serve as markers for regulated cellular degradation of the protein by the 26S proteosome or lysosome/autophagy (Hershko and Ciechnover, 1998; Kornitzer and Ciechnover, 2000; Mukhopadhyay and Riezman, 2007). E3 ligases are the key enzymes that confer specificity to the ubiquitin-proteasome system by binding and linking ubiquitin moieties to the substrate through protein:protein interaction domains. There are two families of E3 ligases: HECT (homologous to E6-AP carboxy terminus) domain and RING (Really Interesting New Gene) finger ubiquitin ligases (Huibregtse et al., 1995; Jackson et al., 2000).

Two muscle specific ubiquitin ligases, the RING containing MuRF1 and atrogin-1, are involved prominently in the development of skeletal muscle atrophy following metabolic insult as well as atrophy resulting from denervation and immobility/unloading (Bodine et al., 2001; Doucet et al., 2010; Gomes et al., 2001; Jagoe et al., 2002; Lecker et al., 2004; Stevenson et al., 2003). Mice lacking either the atrogin-1 or MuRF1 gene are partially protected from the development of skeletal muscle atrophy implying other E3 ligases may partake in the regulation of skeletal muscle mass (Bodine et al., 2001). We and others have demonstrated an increase in the HECT domain ubiquitin ligase, Nedd4-1, in the muscle of rodents subjected to models of hind-limb unloading and denervation-induced skeletal muscle atrophy (Batt et al., 2006; Koncarevic et al., 2007; Plant et al., 2009). In contrast to atrogin-1 and MuRF1, which increase only in short-term denervated muscle in rats, Nedd4-1 expression was sustained long term, suggesting that it plays a more encompassing role in the induction of skeletal muscle atrophy (Batt et al., 2006). Koncarevic et al. have demonstrated that in rodents, the increase in Nedd4-1 occurs exclusively in muscle atrophy associated with

muscle inactivity, such as denervation, disuse and unweighting (2007). We have also demonstrated that Nedd4-1 is increased in the atrophic muscles of individuals with severe COPD (Plant et al., 2009). Our laboratory has found that the constitutive over-expression of Nedd4-1 induces mouse C2C12 myotube atrophy (Figure 1.4). Thus, we speculate that Nedd4-1, like its RING E3 counterparts, is a critical ligase in the induction of inactivity induced-muscle proteolysis and that the absence of Nedd4-1 in skeletal muscle will spare its atrophy following insults associated with inactivity.

A second Nedd4 isoform, Nedd4-2 (also known as Nedd4L) is present in humans and mice (Henry et al., 2003; Kamynina et al., 2001). Despite their structural similarities, Nedd4-1 and Nedd4-2 for the most part, target distinct substrates and serve distinct functions (reviewed in Yang & Kumar, 2010). Nedd4-1 is the predominant skeletal muscle isoform and in *Drosophila,* Nedd4-1 (dNedd4) was shown to regulate neuromuscular synaptogenesis (Ing et al., 2007). Nedd4-1 germline deficient (complete null) mice are embryonic/perinatal lethal, exhibiting cardiac and vascular defects which are likely responsible for death (Fouladkou et al., 2010). Nedd4-1 null embryonic mice also demonstrate abnormalities in neuromuscular junction formation and possibly developmental muscle hypoplasia, although the relevance of this finding remains uncertain since the Nedd4-1 null embryos demonstrate a globally reduced body size, and muscle size was not normalized to body size (Liu et al., 2009). Others have reported muscle developmental delay as part of a global developmental delay in Nedd4-1 null embryonic mice (Cao et al., 2008).

2.2 MATERIALS AND METHODS

2.2.1 Generation of Nedd4-1 skeletal muscle specific knockout (Nedd4-1 SMS-KO) mice

Nedd4-1 SMS-KO mice were created in collaboration with Dr. Nils Brose (Max Planck Ins., Germany). Mice with a floxed Nedd4-1 allele (Nedd4-$1^{flox/flox}$) generated by the Brose laboratory (Kawabe et al., 2010) were crossed to mice in which cre-recombinase expression was controlled by the mouse *myogenin* promoter and the skeletal muscle specific enhancer of the mouse MEF2C (myocyte-specific enhancer factor 2C) gene (generously supplied by Dr. Eric Olson, University of Texas, South Western) and referred to as myo-cre (Myo^{Cre}) mice (Li et al., 2005). Both of these regulatory elements are active only in the skeletal muscle lineage from embryonic day (E) 8.5 to adulthood, thus allowing us to generate a mouse line in which Nedd4-1 was deleted specifically from skeletal muscle. Mice heterozygote for the floxed Nedd4-1 allele and heterozygote for the myo-cre transgene (Myo^{Cre}; Nedd4-$1^{flox/+}$) were bred to create Nedd4-1 SMS-KO mice (Myo^{Cre}/Nedd4-$1^{flox/flox}$) mice and control littermate (Myo^{Cre}; Nedd4-$1^{+/+}$, Nedd4-$1^{+/+}$ and Nedd4-$1^{flox/flox}$) mice.

2.2.2 Genotyping

Mice were genotyped using polymerase chain reaction (PCR) methodology to identify the myo-cre transgene and floxed Nedd4-1 allele. Tail genomic DNA was extracted by digesting tail clippings in lysis buffer (0.5% SDS, 50mM Tris pH 8.0, 5mg/ml NaCl, 2.5mM EDTA, 2.4ug/ml Proteinase K) for 3 hours at 63°C. Cold phenol chloroform solution (1:1 by volume) was added and tubes vortexed until the solution turned cloudy following which the tubes were centrifuged at 4°C for 5 minutes at 13000 ***g***. Cold 100% ethanol was

then added to the supernatant to precipitate the DNA and tubes were incubated at -20°C for 1 hour followed by centrifugation at 4°C for 15 minutes at 13000 ***g***. The DNA pellet was washed with 70% ethanol, dried at room temperature and re-suspended in water. DNA was quantified using a nanodrop spectrophotometer (Thermo Scientific, Ottawa, ON).

PCR reactions for the myo-cre transgene and the Nedd4-1 floxed allele were performed separately. Briefly, amplification of the myo-cre transgene was undertaken using 140ng DNA with 5U Taq polymerase (Invitrogen, Grand Island, NY), 0.2µM forward and reverse primers (Table 2.1) , 0.2mM deoxynucleotides (dNTP; Genedirex, Toronto, ON) and 1.5mM $MgCl_2$ (Invitrogen, Grand Island, NY). Amplification of the Nedd4-1 allele was undertaken with 300ng DNA, 1U HiFi Taq DNA polymerase (Invitrogen, Grand Island, NY), 0.2µM forward and reverse primers (Table 2.1), 0.2mM dNTP and 2mM $Mg(SO_4)_2$ (Invitrogen, Grand Island, NY). Negative controls for each reaction consisted of no template (water) and amplification cycling parameters are listed in Table 2.2. Amplification products were separated on 2% agarose gel, stained with ethidium bromide, to determine the presence of i) the myo-cre transgene & ii) the floxed and/or wild type (WT) Nedd4-1 gene.

2.2.3 **Experimental denervation model and muscle preparation**

Our collaborator, Dr. James Bain (McMaster University, Hamilton, Canada), performed the denervation experiments as previously described (Bain et al., 2001). The gastrocnemius muscle denervation model was employed and approved by the Research Ethics Board, Hamilton Health Sciences Corporation, McMaster University. Briefly, the right tibial nerve was transected under inhalational Halothane anaesthesia completely denervating the gastrocnemius muscle in Nedd4-1 SMS-KO mice and littermate Myo^{Cre}; Nedd4-$1^{+/+}$ control mice, 3 to 5 months of age. The proximal portion of the tibial nerve was

sutured to the superficial surface of the biceps femoris muscle to prevent errant re-innervation of the gastrocnemius muscle. The contralateral leg served as an internal control in each animal. Mice were maintained under conditions of routine care for 1 or 2 weeks. Subsequently the mice were sacrificed and the gastrocnemius muscles were harvested from the operated experimental limb and non-operated control limb. After rapid, atraumatic dissection and weighing, the muscle was split and fixed in 10% buffered formalin or snap frozen in liquid nitrogen. Fixed tissue was processed for immunohistochemistry and morphometric measurements. Frozen muscle was used for extraction of cellular protein.

2.2.4 **Western Blotting**

Total protein was extracted by homogenizing (Polytron PT 1200E, Kinematica, Lucerne, Switzerland) the muscle in muscle lysis buffer [5mM Tris-HCl pH 8.0, 1mM EDTA, 1mM EGTA, 1mM β-mercaptoethanol, 1% glycerol, PMSF (1mM), leupeptin, aprotinin (10ug/ml each)] for 3x 30seconds on ice and homogenates were centrifuged at 1600 ***g*** for 10 min at 4^{o}C. The supernatant was cleared by centrifuging further for 10 min at 4^{o}C, 10,000 ***g***. All fractions were quantified using the BCA protein assay kit (Pierce, Rockford, IL) and normalized for equal loading for SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and western blot analysis.

25 or 50 μg of muscle protein lysates were separated by SDS-PAGE, transferred to nitrocellulose, ponceauS stained and immunoblotted. The commercial primary antibodies used included anti-Nedd4-1 (#611480, BD-Biosciences, Mississauga, ON), -HPRT (ab109021, Abcam, Cambridge, MA), -Pax7 (DSHB, Iowa City, IA), -Nedd4-2 (#4013, Cell Signaling, Danvers, MA), -Ubiquitin (#MMS-257P, Covance, Princeton, NJ) and anti-GAPDH (#ab8245, Abcam, Cambridge, MA). HPRT was used as a loading control for all

western blots with denervated muscle lysates as it showed consistent expression as opposed to other housekeeping genes. Secondary antibodies were HRP (horseradish peroxidase)-linked anti-rabbit or anti-mouse and protein bands were detected with ECL (Enhanced Chemiluminescence; GE Healthcare, Baie d'Urfe, QC) or Immunstar Western C **(**Biorad Laboratories, Hercules, CA). The chemiluminescent signal was acquired and quantified with Bio-Rad Fluor S Max Acquisition System (Biorad Laboratories) and Image Lab software (Biorad Laboratories).

2.2.5 **Immunohistochemistry and muscle morphometrics**

Gastrocnemius muscles were fixed in 10% buffered formalin phosphate for 24 hours at room temperature, rinsed in ethanol, paraffin embedded and sectioned (10um thickness) on cross section. The sections were rehydrated in a series of xylene and ethanol washes and then endogenous peroxidases were quenched with 30 minute incubation in 0.3% H_2O_2. Antigen retrieval was performed by microwaving the sections in 10mM sodium citrate, pH 6.0, and 3x 5 minutes at 90% power (720 Watt oven). Sections were blocked with Protein Block – Serum Free (Dako/Agilent, Mississauga, Canada). Sections were rinsed and fibers were immunostained using either anti-skeletal muscle myosin, fast isoform antibody (MY-32, Sigma, St. Louis, MO) specific to fast-twitch muscle fibers, or anti-Pax-7 (Pax 7, Developmental Studies Hybridoma Bank, University of Iowa, USA), a marker of muscle satellite cells/myoblasts, followed by biotinylated secondary antibody and streptavidin-HRP/DAB (Vectastain ABC Elite Peroxidase kit, Vector Laboratories Inc., Burlingame, CA, USA). For a negative control, the primary antibody was omitted during immunostaining. Hematoxylin was used as a counterstain. Cross sectional area (CSA) of type I and type II fibers were determined using ImageJ software (NIH) by 3 independent reviewers blinded to

mouse genotype. A minimum of 100 fibers per muscle were measured.

2.2.6 **Primary Myoblast Isolation and Culture**

Primary myoblast cultures were generated as previously described (Conboy et al., 2002). Briefly, hindlimb muscles of Nedd4-1 SMS-KO and control littermate mice were dissected and incubated with dulbecco's modified eagle's medium (DMEM; Gibco) containing 3mM $CaCl_2$ and 2% collagenase II (Gibco) for 90 minutes at 37°C. Digested muscle was then washed in growth media (Ham F10 media; Gibco) with 20% fetal bovine serum (FBS), 5ng/ml basic fibroblast growth factor (Atlanta Biological) and 1% penicillin/streptomycin) followed by trituration to dissociate muscle into single myofibers. Myofibers were then allowed to sediment at 37°C for 30 minutes following which the debris and single cells were aspirated off. Myofibers were washed 4 times rigorously with phosphate buffered saline (PBS) containing 100mg/L calcium chloride and 100mg/L magnesium chloride (Gibco) to remove cells adherent to myofibers (satellite cells are present beneath the basal lamina). Satellite cells were liberated by further digesting the myofibers in 10 volumes of PBS, 0.5U/ml dispase (Invitrogen), 38U/ml collagenase II (US Biological) for 30 minutes at 37°C with agitation. FBS (1:10 by volume) was added to the digests and centrifuged at 500 ***g*** for 1 minute to settle debris. The supernatant was filtered using 50uM mesh and centrifuged at 1000 ***g*** for 5 minutes to pellet satellite cells. Satellite cells were then re-suspended in growth media and cultured on uncoated plates for 40 minutes before transferring to plates coated with 10ug/ml lamin (Gibco) and 0.002% collagen (sigma) and maintained in culture at 37°C in 5% CO_2.

2.2.7 **Immunocytology**

Myoblast cultures were fixed with 4% paraformaldehyde in PBS for 15 minutes, washed with PBS and paraformaldehyde was subsequently deactivated with 100mM glycine in PBS for 10 min. Cells were washed, and then permeabilized in buffer containing 0.1% Triton X-100 diluted in PBS for 20 minutes at room temperature (RT). Cells were washed with PBS and then blocked with the addition of 5% BSA in PBS for 1 hour at RT. Cells were labelled with rabbit anti-MyoD (Santa Cruz Biotechnologies, Santa Cruz CA, USA, sc-304) and mouse anti-Nedd4-1 (#611480, BD Biosciences, Mississauga, ON) antibodies for 1 hour at RT. The cells were then washed with PBS and labeled with Alexa Fluor -488 and -568 secondary antibodies (Molecular Probes, Eugene, OR, USA) and Hoechst nuclear stain in PBS for 1 hour. Samples were washed with PBS, mounted and stored at 4°C until imaged. Cellular fluorescence was visualized with an Olympus BX50 microscope (Olympus America, Center Valley, PA, USA) using 40x magnification. Images were captured using CellSens Software (Olympus America). TRITC (Tetramethylrhodamine-5-(and 6)-isothiocyanate) and FITC (Fluorescein Isothiocyanate) filter sets were used to visualize Nedd4-1 and MyoD expressing cells, respectively.

2.2.8 **Statistical Analyses**

Continuous data are reported as a mean and standard error. Two way analysis of variance (ANOVA) was used to compare normalized measures (muscle weights, fiber CSA, protein levels) between Nedd4-1 SMS-KO and myo^{Cre}; Nedd4-1$^{+/+}$ control mice at 1 and 2 weeks post denervation. Differences in absolute measures (muscle weights or fiber CSA) between Nedd4-1 SMS-KO and myo^{Cre}; Nedd4-1$^{+/+}$ control innervated and denervated gastrocnemius at 1 week or 2 weeks were assessed by one way ANOVA followed by post-

hoc Bonferroni's multiple comparison tests. Statistical significance was assumed in all cases if $p < 0.05$.

2.3 RESULTS

Generation of Nedd4-1 SMS-KO mice

To genotype the mice, PCR was performed to determine the presence of the myo-cre transgene and floxed and/or wild type Nedd4-1 gene. When the PCR reaction products are separated on an agarose gel, mice heterozygote or homozygote for the myo-cre transgene demonstrate an amplicon of approximately 250bp (Figure 2.1A). Mice homozygote for the Nedd4-1 floxed gene reveal a single amplicon of 260bp, while mice homozygote for the Nedd4-1 WT allele display a single amplicon of 230bp (Figure 2.1B). Mice heterozygotes for the floxed and wild type Nedd4-1 genes display both amplicons.

To confirm deletion of Nedd4-1 protein in Nedd4-1 SMS-KO skeletal muscle, we performed SDS-PAGE and western blotting (WB) analysis of gastrocnemius muscle lysates from Nedd4-1 SMS-KO mice and littermate Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ control mice (Figure 2.2). The results reveal the absence of Nedd4-1 in the Nedd4-1 SMS-KO mice while a GAPDH loading control remains unaltered (Figure 2.2A). When other tissues (heart, liver and kidney) were examined, the amount of Nedd4-1 protein in Nedd4-1 SMS-KO and littermate control mice was equivalent in all tissues (Figure 2.2B), demonstrating the skeletal muscle specificity of the Nedd4-1 knockout. Furthermore to ensure that the knock-out was specific for Nedd4-1, expression of Nedd4-2 was confirmed in the Nedd4-1 SMS-KO skeletal muscle

(Figure 2.2A). These data validate our genetic model and confirm the specificity of the null phenotype in skeletal muscle.

Denervation increases skeletal muscle Nedd4-1 protein expression and total protein ubiquitination

Previously, we and others have shown increased Nedd4-1 expression and increased levels of total protein ubiquitination in gastrocnemius muscles undergoing denervation and unweighting-induced atrophy (Batt et al., 2006; Koncarevic et al., 2007; Stevenson et al., 2003). To confirm this phenomenon in our genetic model, Nedd4-1 SMS-KO and littermate Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ control mice underwent right tibial nerve transection with the left hind limb serving as control. Gastrocnemius muscles were harvested 1 and 2 weeks post-denervation, weighed and lysed for western blotting. HPRT was used as a loading control in all western blots with denervated muscle lysates as it showed consistent expression as opposed to other housekeeping genes. Nedd4-1 expression in the denervated compared to the contralateral control muscle of littermate control mice was increased dramatically (Figure 2.3). Nedd4-1 is absent from the Nedd4-1 SMS-KO control gastrocnemius, but trace Nedd4-1 expression becomes evident with denervation. Ubiquitinated protein levels were increased significantly in the denervated gastrocnemius muscles of both Nedd4-1 SMS-KO mice and littermate control mice but there was no significant difference in the magnitude of the increase between the two cohorts of mice (Figure 2.4).

Proliferation of satellite cells post denervation injury results in trace expression of Nedd4-1 in denervated muscle of Nedd4-1 SMS-KO mice

To determine the source of the trace Nedd4-1 expression in the denervated gastrocnemius muscle, we assessed muscle satellite cells/myoblasts for Nedd4-1 protein

expression. Quiescent satellite cells/myoblasts activate to proliferating myoblasts in a reparative response to denervation injury, increasing from about 1%, to up to 10%, of all muscle cells (Jejurikar et al., 2002, 2006; Kuschel et al., 1999; McGeachie and Allbrook, 1978). Our knockout design does not delete Nedd4-1 from skeletal muscle satellite cells/myoblasts. During embryogenesis, expression of the transcription factors MyoD and myogenic factor 5 (Myf5) specify mesodermal precursor cells to the myogenic lineage, generating proliferative myoblasts (Charge and Rudnicki, 2004). Subsequent expression of myogenin and myogenic regulatory factor 4 (MRF4) induce terminal differentiation of myoblasts to mononucleated myocytes which then fuse together to form multinucleated muscle fibers. During skeletal muscle development, a subpopulation of myoblasts, known as satellite cells, fail to differentiate but remain associated with the myofibers and retain the capacity to proliferate and differentiate so as to regenerate skeletal muscle post-natally (Conboy et al., 2002; Holterman and Rudnicki, 2005). As myoblasts express myogenin, and differentiate to myocytes, Nedd4-1 expression is eliminated. Since cre-recombinase expression in our mice is under the control of the myogenin promoter, Nedd4-1 should still be expressed post-natally in the quiescent satellite cells and proliferating myoblasts of Nedd4-1 SMS-KO mice, because myogenin is not yet expressed in these progenitor cells. Indeed, we were able to demonstrate the co-expression of Nedd4-1 and MyoD in cultured myoblasts derived from the Nedd4-1 SMS-KO mice (Figure 2.5). We additionally demonstrated increased expression of the satellite cell/myoblast marker, paired box 7 (Pax7), in the denervated gastrocnemius muscle of both Nedd4-1 SMS-KO and littermate control mice (Figure 2.6), in keeping with the known activation of satellite cell/myoblast proliferation following denervation. These data together support the conclusion that trace

Nedd4-1 expression induced in the denervated gastrocnemius of Nedd4-1 SMS-KO mice likely results from associated satellite cell/myoblast proliferation.

Nedd4-1 SMS-KO mice are partially protected against denervation-induced atrophy

As a measure of muscle sparing, denervated gastrocnemius muscles were harvested 1 and 2 weeks post nerve transection, weighed, fixed, cut on cross-section and immunostained for fast twitch myosin to identify type I and type II fibers. At baseline, the Nedd4-1 SMS-KO mice are slightly smaller than, and the gastrocnemius muscle weighs less than, that of littermate control mice (Figure 2.7A). However, there is no difference in the muscle mass when normalized to body weight (Figure 2.7B). Denervated gastrocnemius muscle weights, normalized to the weight of the contralateral control muscle, were significantly greater in Nedd4-1 SMS-KO compared to littermate Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ control mice at both 1 and 2 weeks post tibial nerve transection demonstrating an attenuated atrophic response in the Nedd4-1 null muscle (Figure 2.7C). Similarly the type II fiber CSA normalized to the CSA in the contralateral control muscle was significantly larger in the denervated gastrocnemius muscle of Nedd4-1 SMS-KO compared to control Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ mice (Figure 2.8A, D). This is despite the fact that control innervated myo^{Cre}; $Nedd4\text{-}1^{+/+}$ gastrocnemius type II fiber CSA was larger than that of Nedd4-1 SMS-KO mice (Figure 2.8B, C). Denervation induces a fiber type switch from type I to type II fibers (Finkelstein et al., 1993) and as a result, an inadequate number of type I fibers were available in the denervated gastrocnemius muscle for measurement.

2.4 DISCUSSION

In the present study, we generated a novel genetic tool, the Nedd4-1 SMS-KO mouse, to assess the role of Nedd4-1 in the loss of skeletal muscle mass. We subjected the mice to a well-validated model of denervation-induced muscle atrophy. We show heavier weights and larger type II fiber cross-sectional area in denervated relative to control gastrocnemius muscle of the Nedd4-1 SMS-KO mice, demonstrating conclusively that the ubiquitin ligase Nedd4-1 participates in the development of denervation-induced skeletal muscle atrophy. This is in accord with the robust increase in the expression of Nedd4-1 in skeletal muscle atrophying as a result of inactivity (i.e. denervation, muscle unloading and immobility), that is sustained beyond that of the E3 ligases atrogin-1 and MuRF1 (Batt et al., 2006) and ubiquitin mediated proteolysis is known to play an essential role in the loss of muscle mass resulting from muscle inactivity (Lecker et al., 2006; Sandri, 2008; Solomon and Goldberg, 1996). In keeping with this fact, we noted a marked increase in the total levels of ubiquitinated proteins in the denervated muscle of both Nedd4-1 SMS-KO and littermate control mice concomitant to the increase in Nedd4-1. The fact that the loss of Nedd4-1 does not appear to diminish the extent of total ubiquitinated protein levels suggests that there is possibly up-regulation of compensatory pathways or alternatively, an assessment of overall ubiquitination is too insensitive to detect differences generated by substrate-specific ubiquitination between the Nedd4-1 SMS knockout and littermate animals.

Others have also interrogated the role of Nedd4-1 in muscle proteolysis and reported that short-term Nedd4-1 over-expression via cDNA electro-transfer into muscle did not induce myofiber atrophy at seven days in weight bearing rats, nor did over-expression of a catalytically inactive dominant negative Nedd4-1 rescue myofibers from atrophy induced by

seven days of unloading (Koncarevic et al., 2007). The reason for these findings, which are contradictory to our observations here, is not clear but we speculate that it may result from the use of a dominant negative, as opposed to a genetic or knockdown approach and/or inadequate levels or duration of Nedd4-1 expression *in vivo.* With exogenous expression, the early and single seven day time point of assessment may have been inadequate to observe the Nedd4-1 effect, which remains up-regulated in muscle for many weeks following denervation (Batt et al., 2006). The generation of our genetic mouse model here has provided us with a unique tool to bypass these limitations and demonstrate a positive role for Nedd4-1 in the mediation of denervation-induced skeletal muscle atrophy.

The contribution of Nedd4-1 to the regulation of denervation-induced muscle atrophy appears to be smaller than that provided by either atrogin-1 or MuRF1. Genetic deletion of these ligases under similar conditions resulted in the retention of a larger muscle fourteen days post denervation, when compared to our results here with Nedd4-1 (Bodine et al., 2001). Nedd4-1's influence on muscle mass may be more extensive than what is demonstrated at this time since Nedd4-1 is only deleted in the Nedd4-1 SMS-KO muscle when myogenin is expressed, which occurs with the differentiation of satellite cells/myoblasts to myocytes. Loss of Nedd4-1 may influence satellite cell/myoblast proliferation, but we were unable to assess this phenomenon in our genetic model. Nonetheless, given that the individual absence of each of Nedd4-1, atrogin-1 or MuRF1 was inadequate to provide complete protection against muscle atrophy, this implies that the co-ordinated activity of these three ligases is necessary to mediate the loss of muscle mass following denervation.

We found the deletion of Nedd4-1 from skeletal muscle provided protection from denervation-induced atrophy early post denervation (seven days), like atrogin-1 and in contrast to MuRF1 which demonstrates a delayed protective effect, being evident at only fourteen days post denervation (Bodine et al., 2001). The varied temporal effects of these E3 ligases may result from the different downstream substrates each engages. MuRF1 and atrogin-1 have distinct targets in skeletal muscle (Foletta et al., 2011). MuRF1 preferentially interacts with and ubiquitinates structural proteins such as titin, myosin-binding protein C and the myosin light chain resulting in proteolysis of muscle fibers (thick filament) (Eddins et al., 2011; Kedar et al., 2004; Witt et al., 2005). In contrast, it appears atrogin-1 ubiquitinates myogenic transcriptional regulators such as MyoD and myogenin inferring that the effect of atrogin-1 influences the development of muscle atrophy, at least in part, by regulating myogenesis (Jogo et al., 2009; Lagirand-Cantaloube et al., 2008; Moresi et al., 2010; Tintignac et al., 2005). We have not yet identified the substrates that Nedd4-1 targets to promote the induction of denervation-induced skeletal muscle atrophy.

In conclusion, using a novel and unique genetic tool, the Nedd4-1 SMS-KO mouse, we have demonstrated that the ubiquitin ligase Nedd4-1 participates in the positive regulation of denervation-induced skeletal muscle atrophy.

Table 2.1 Primer sequences for PCR amplification of the Myo-cre transgene and Nedd4-1 gene

Gene	Primers	Primer sequence
Myo-cre	Forward primer	5’ TCG ACC AGT TTA GTT ACC C 3’
	Reverse primer	5’ AGG TTC GTT CAC TCA TGG 3’
Nedd4-1	Forward Primer	5’ GTA CAT TTT AGT TCA TGG TTC TCA CAG 3’
	Reverse Primer	5’ CAG AGG TCA CAT GGC TGT GGG 3’

Table 2.2. Cycling parameters for Myo-cre and Nedd4-1 PCR reactions

Myo-cre PCR Reaction		
Initial Denaturation	95°C	4 minutes
30 Cycles	96°C	30 seconds
	55°C	30 seconds
	72°C	30 seconds
Final Extension	72°C	4 minutes
Hold	4°C	∞
Nedd4-1 PCR Reaction		
Initial Denaturation	95°C	3 minutes
34 Cycles	94°C	45 seconds
	57°C	30 seconds
	72°C	30 seconds
Final Extension	72°C	10 minutes
Hold	4°C	∞

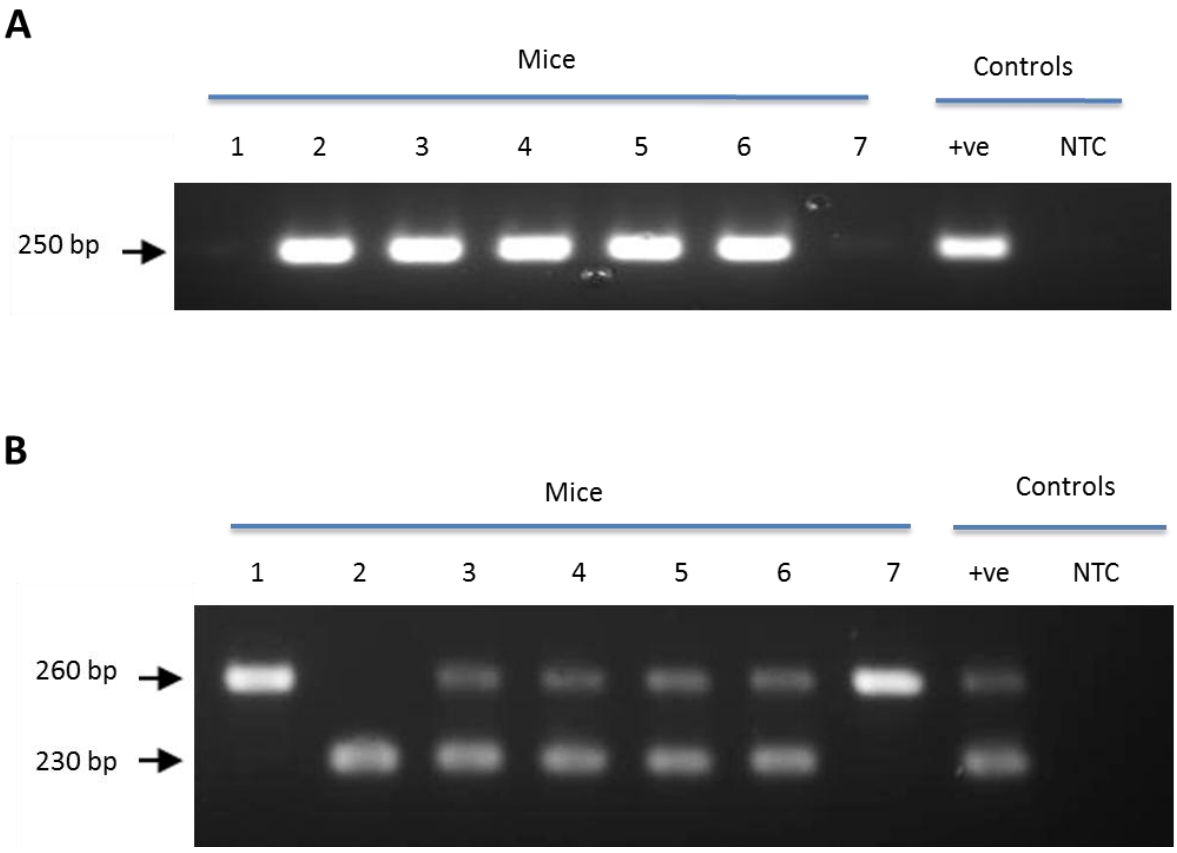


Figure 2.1. PCR genotyping of Nedd4-1 SMS-KO mice. (A) Representative agarose gel electrophoresis of the products of the PCR reaction for the myogenin-cre transgene: the Myo^{Cre} amplicon appears as a 250bp band. Mice 2, 3, 4, 5, and 6 contain the myogenin-cre transgene while mice 1 and 7 do not. (B) Representative agarose gel electrophoresis of products of the PCR reaction for the Nedd4-1 gene: the Nedd4-1 WT allele is 230bp and the floxed Nedd4-1 allele is 260bp. Mice 1 and 7 are homozygote for the floxed Nedd4-1 allele; mouse 2 contains only the Nedd4-1 WT allele and mice 3, 4, 5, and 6 are heterozygotes (contain both the Nedd4-1 floxed and WT alleles). (NTC = water; +ve control = Myo^{Cre}; Nedd4-$1^{flox/+}$ DNA).

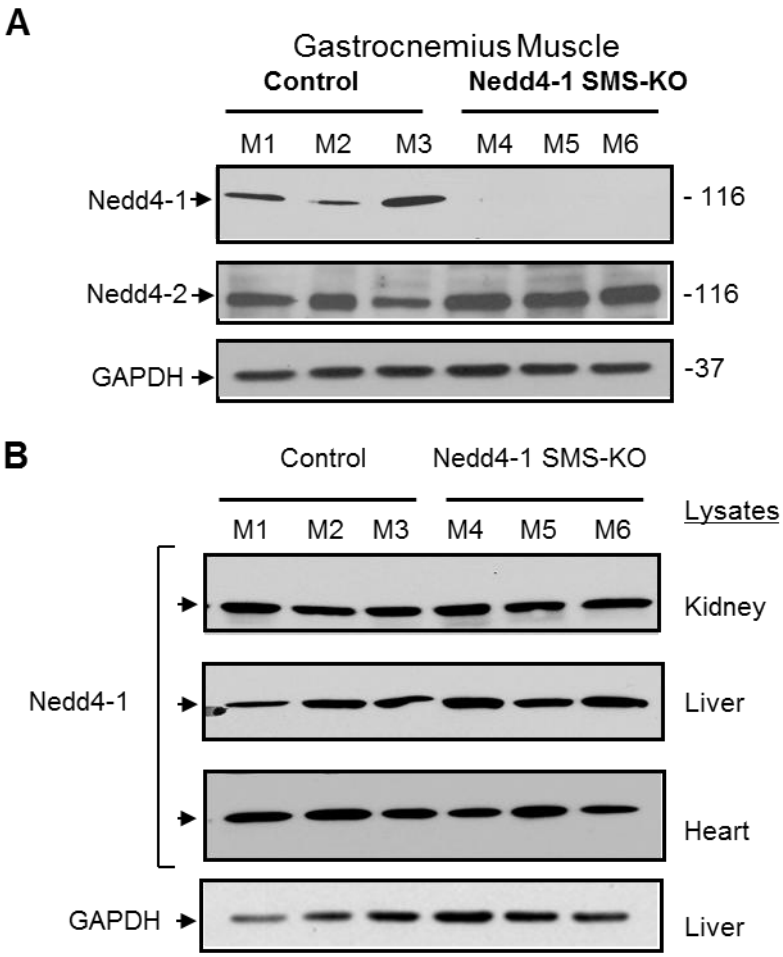


Figure 2.2. Nedd4-1 protein is absent from Nedd4-1 SMS-KO skeletal muscle. (A) SDS-PAGE and Nedd4-1 Western blotting (WB) of 50 μg of gastrocnemius muscle lysates from representative Nedd4-1 SMS-KO mice (M4, M5, M6) and littermate Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ control mice (M1, M2, M3) reveal the absence of Nedd4-1 in the Nedd4-1 SMS-KO muscle. Nedd4-2 protein remains equally expressed in the muscle of Nedd4-1 SMS-KO and Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ control mice. GAPDH served as a loading control. (B) WB of heart, liver and kidney protein lysates reveal equal Nedd4-1 protein in these tissues in the Nedd4-1 SMS-KO and littermate Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ control mice. GAPDH served as a loading control for all blots; liver lysate is shown as a representative blot.

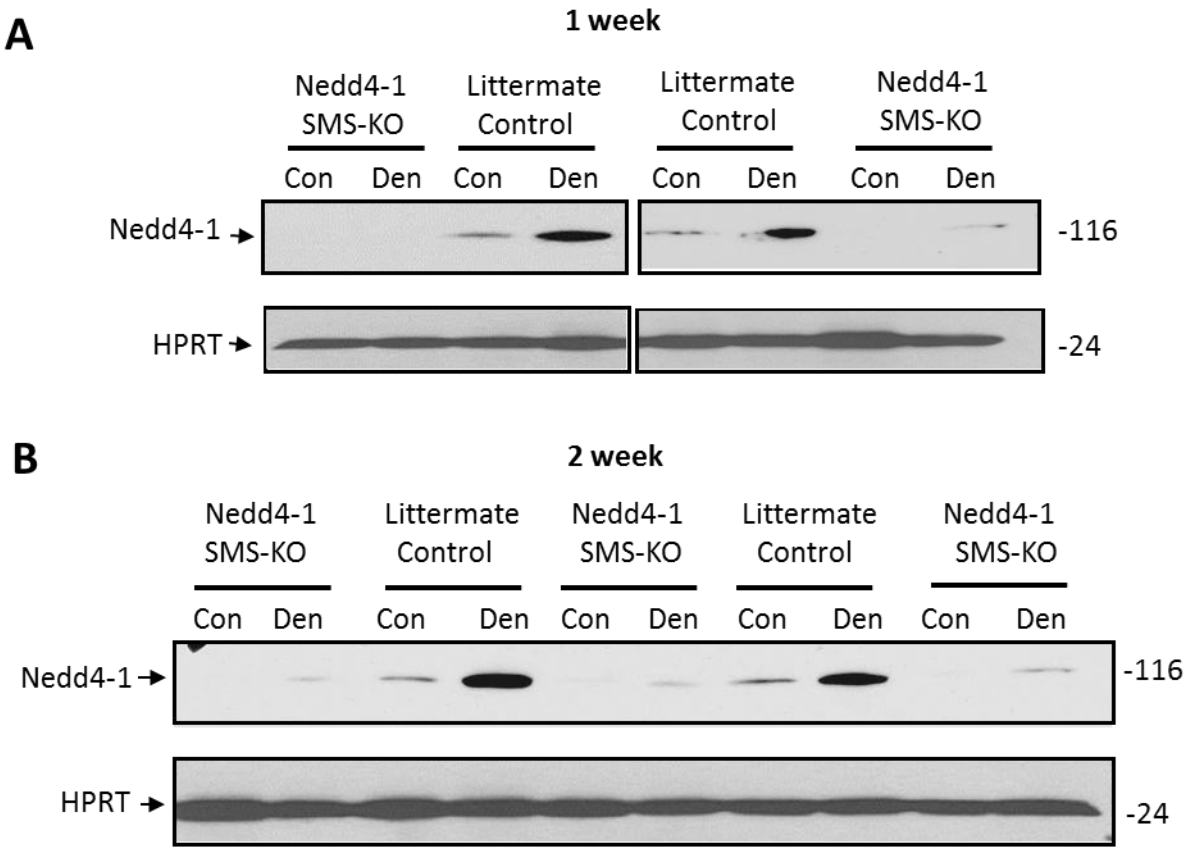


Figure 2.3. Denervation induces increased gastrocnemius muscle Nedd4-1 protein expression. Nedd4-1 SMS-KO and littermate Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ control mice underwent right tibial nerve transection with the left hind limb serving as control. Western blotting of gastrocnemius protein lysates denervated for (A) 1 week and (B) 2 weeks show increased Nedd4-1 protein in the denervated (Den) compared to the contralateral control (Con) muscle of Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ mice. Nedd4-1 is absent from the Nedd4-1 SMS-KO control gastrocnemius, but trace Nedd4-1 expression becomes evident with denervation. HPRT served as a loading control and representative blots are shown (n = 8 to 9 mice/cohort).

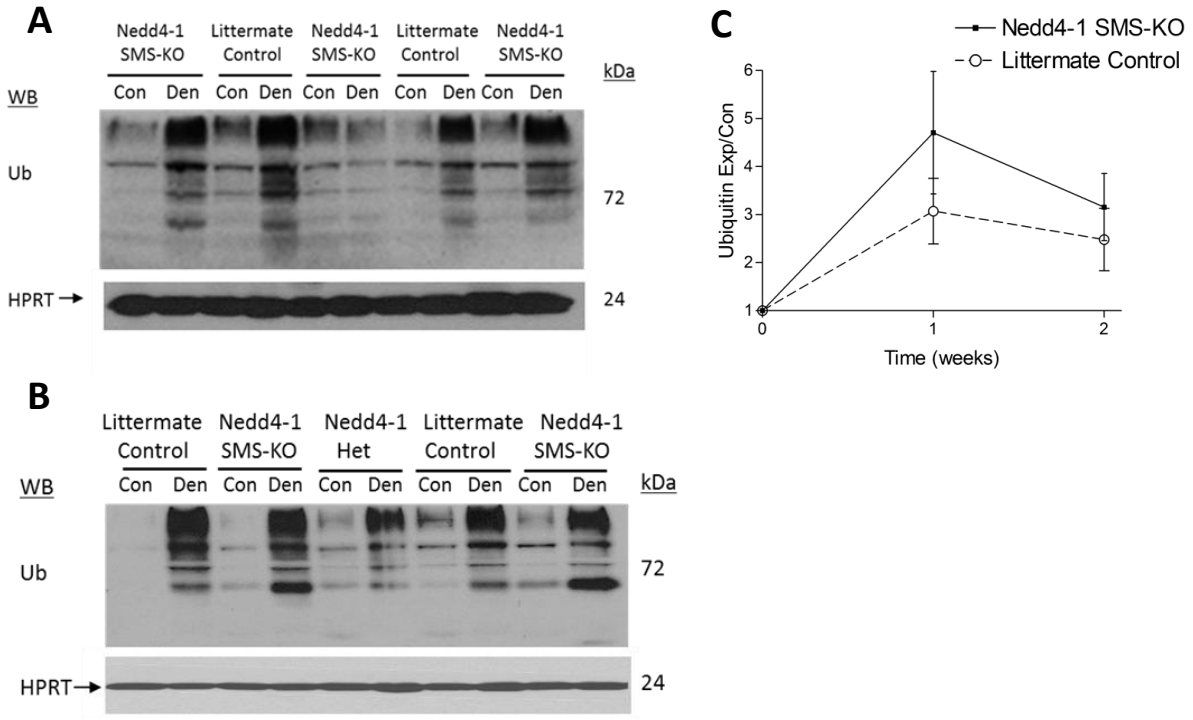


Figure 2.4. Denervation induces increased total protein ubiquitination in gastrocnemius muscle. Representative western blots (WB) of gastrocnemius muscle protein lysates demonstrate increased levels of ubiquitinated proteins in denervated (Den) compared to contralateral control (Con) muscle (A) 1 week and (B) 2 weeks post tibial nerve transection in both Nedd4-1 SMS-KO and littermate Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ control mice. Ubiquitinated products appear as a high molecular weight smear. HPRT served as a loading control and representative blots are shown. (C) The chemiluminescent signal was quantified and total ubiquitinated protein levels were normalized to the HPRT levels. Ubiquitinated protein levels in denervated (Exp) muscle are expressed as a fraction of the level in control (Con) muscle. Ubiquitinated protein levels are significantly increased in the denervated gastrocnemius muscle of both Nedd4-1 SMS-KO and Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ (dashed line) mice at both 1 and 2 weeks post-tibial nerve transection but there is no difference in the magnitude of the increase between the two cohorts of mice. Data are presented as the mean ± SEM and * $p < 0.05$, n = 8 mice/group).

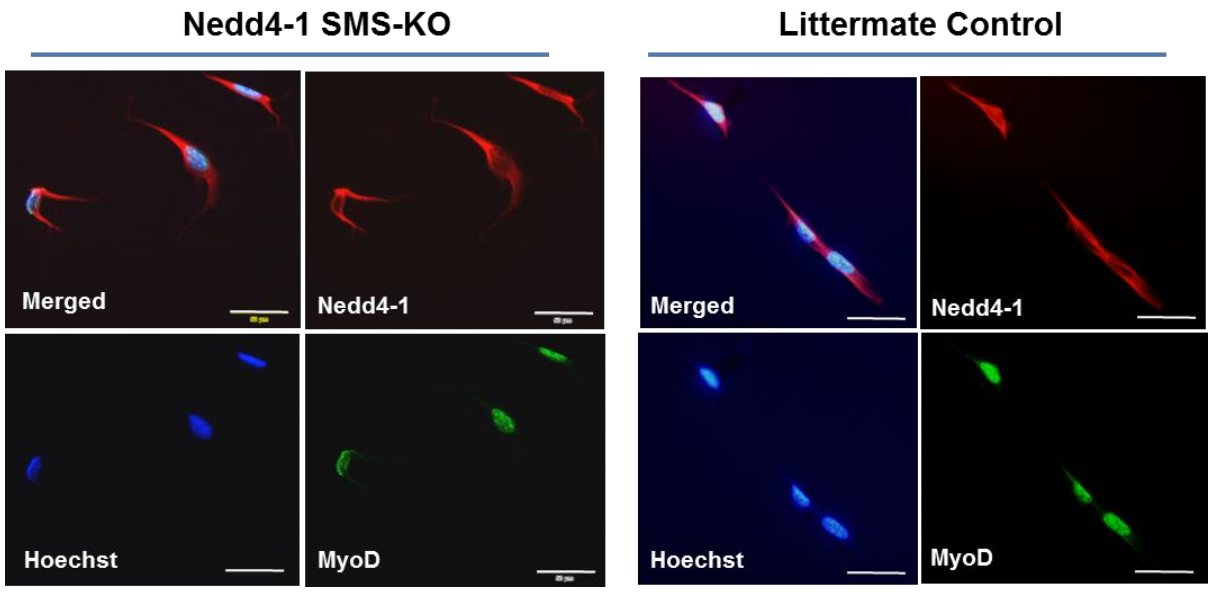


Figure 2.5. Satellite cells/myoblasts isolated from Nedd4-1 SMS-KO mice express Nedd4-1. Muscle satellite cells/myoblasts were isolated from Nedd4-1 SMS-KO mice and littermate Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ control mice and maintained in culture. Cultures were immunostained for Nedd4-1 (red) and MyoD (green) and nuclei were visualized with Hoechst (blue). MyoD, a specific marker of myoblasts, is expressed in nuclei; Nedd4-1 is predominantly cytoplasmic. Myoblasts derived from both Nedd4-1 SMS-KO and littermate control mice demonstrate co-expression of MyoD and Nedd4-1. Immunostaining with 2º antibodies alone served as a negative control and did not demonstrate non-specific staining (data not shown). Scale bar shown = 50 μm.

Figure 2.6. Satellite cell/myoblast population is increased in denervated gastrocnemius muscle. (A) Cross sections of gastrocnemius muscle from Nedd4-1 SMS-KO and littermate Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ mice were immunostained for the satellite cell/ myoblast marker Pax 7 (brown nuclei, indicated by white arrows). Myonuclei are counterstained purple with hematoxylin. There is an apparent increase in satellite cell number in the denervated gastrocnemius of both Nedd4-1 SMS-KO and littermate Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ control mice. (B) Similarly, representative Western blots (WB) of gastrocnemius protein lysates denervated for 1 week (left panel) and 2 weeks (right panel) show increased expression of Pax 7 in the denervated (Den) compared to the contralateral control (Con) muscle of Nedd4-1 SMS-KO and littermate Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ mice. HPRT served as loading control. (C) The chemiluminescent signal was quantified and Pax-7 protein levels were normalized to corresponding HPRT levels (lane matched). Normalized Pax-7 levels in denervated (Exp) muscle were expressed as a fraction of the normalized Pax-7 level in control (Con) muscle and these values are depicted in the graph. Pax-7 is significantly increased in the denervated gastrocnemius muscle of both Nedd4-1 SMS-KO and Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ mice at both 1 and 2 weeks post-tibial nerve transection ($n = 6$ mice/group, $p < 0.05$), but there is no difference in the magnitude of the increase between Nedd4-1 SMS-KO and Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ mice. Data are presented as the mean ± SEM.

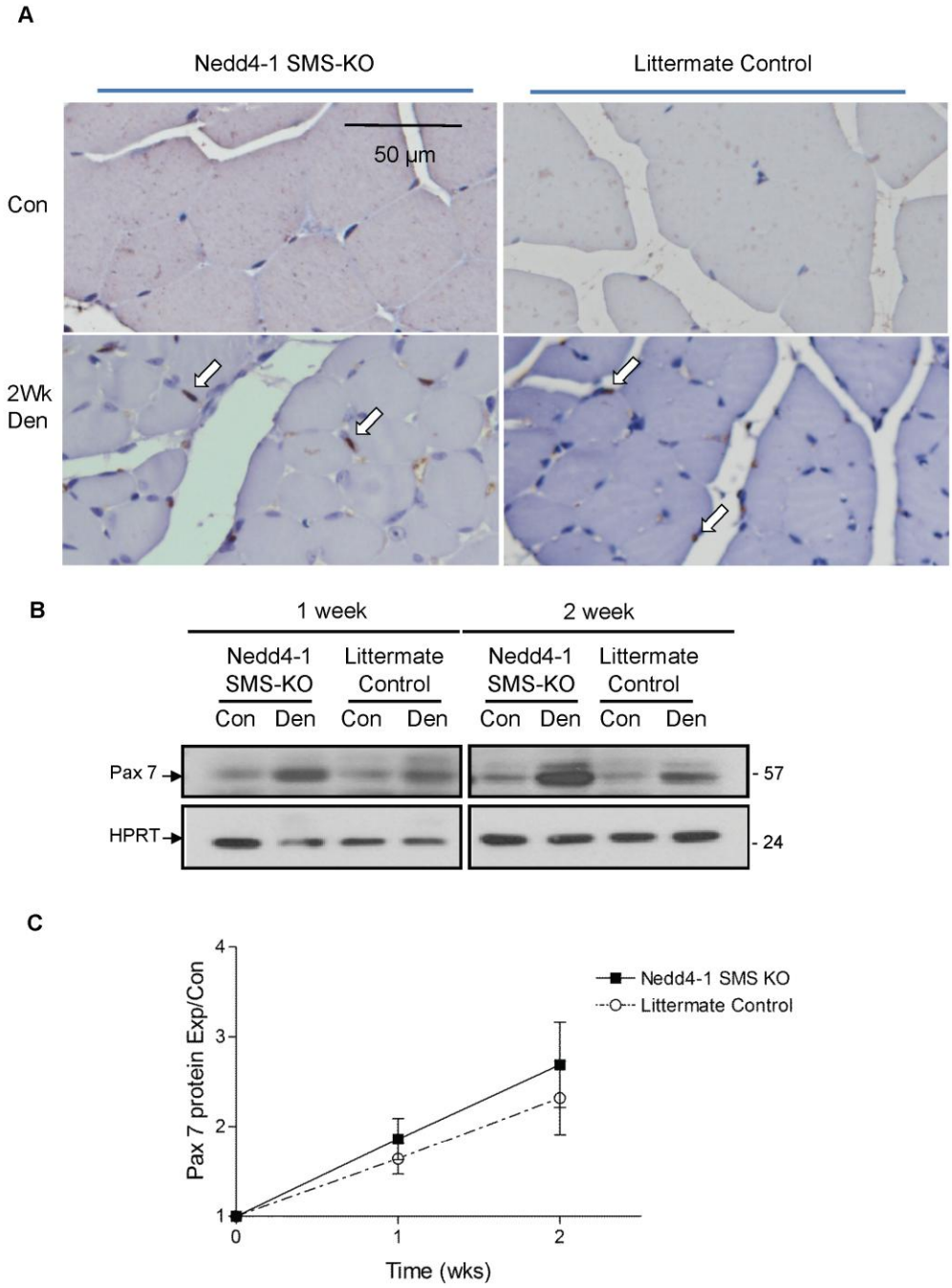
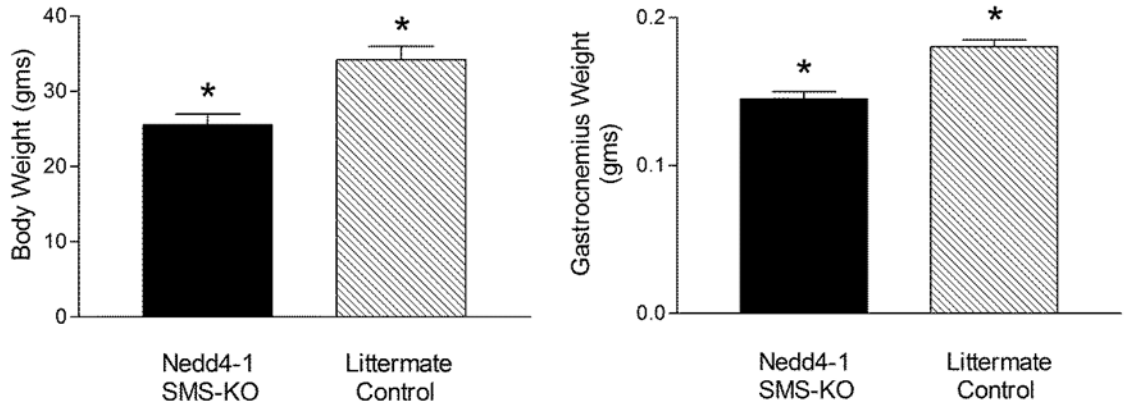
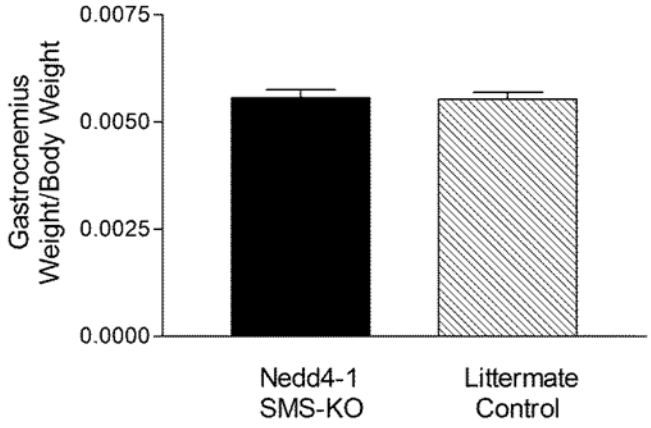

A
Nedd4-1 SMS-KO
Littermate Control
50 μm
Con
2Wk Den
B
1 week
2 week
Nedd4-1 SMS-KO
Littermate Control
Nedd4-1 SMS-KO
Littermate Control
Con Den Con Den Con Den Con Den
Pax 7
HPRT
- 57
- 24
C
Pax 7 protein Exp/Con
Time (wks)
Nedd4-1 SMS KO
Littermate Control
1
2
3
4
0
1
2

Figure 2.7. Nedd4-1 SMS-KO gastrocnemius muscle weights are partially spared from denervation-induced atrophy. (A) Total body weights (left panel) and gastrocnemius muscle weights (right panel) of Nedd4-1 SMS-KO and littermate Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ control mice. Nedd4-1 SMS-KO mice are slightly smaller, and their gastrocnemius muscle weigh less, than littermate controls ($n = 16$ pairs, * $p < 0.05$). (B) There is no difference in the basal gastrocnemius muscle mass between the Nedd4-1 SMS-KO and control mice when muscle weight is normalized to body weight ($p > 0.05$). (C) Nedd4-1 SMS-KO and littermate control mice underwent right tibial nerve transection, denervating the gastrocnemius muscle, with the intact left hind limb serving as internal control. Denervated gastrocnemius muscle weights are normalized to the weight of the contralateral control muscle. Denervated muscle of Nedd4-1 SMS-KO (solid line, closed squares) mice demonstrates an attenuated atrophic response weighing significantly more than in Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ (dashed line, open circles) mice at 1 and 2 weeks post denervation, (* $p < 0.05$, $n = 8$ or 9 mice/cohort and data are presented as the mean ± SEM).

A


Body Weight (gms)
40
30
20
10
0
*
*
Nedd4-1 SMS-KO
Littermate Control
Gastrocnemius Weight (gms)
0.2
0.1
0.0
*
*
Nedd4-1 SMS-KO
Littermate Control

B


Gastrocnemius Weight/Body Weight
0.0075
0.0050
0.0025
0.0000
Nedd4-1 SMS-KO
Littermate Control

C

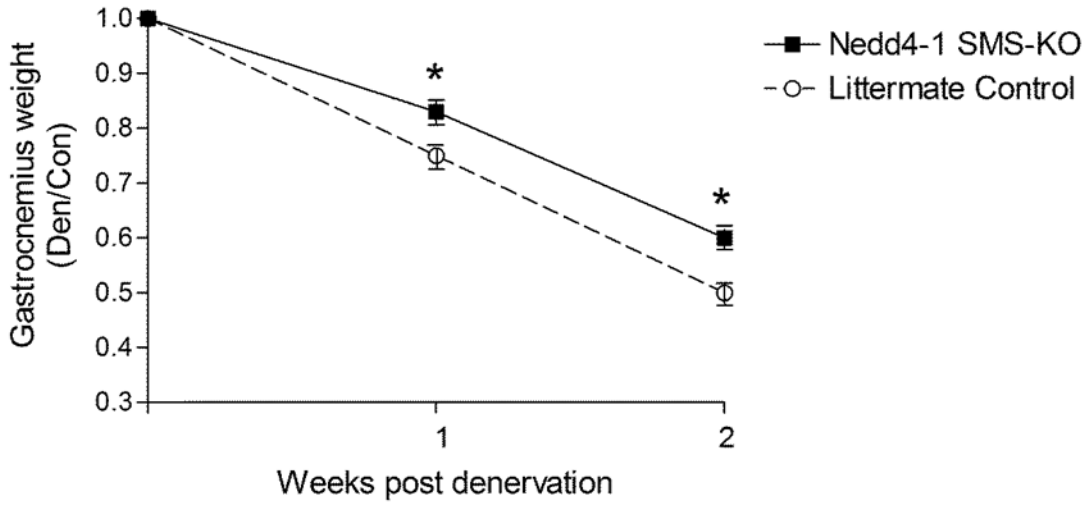
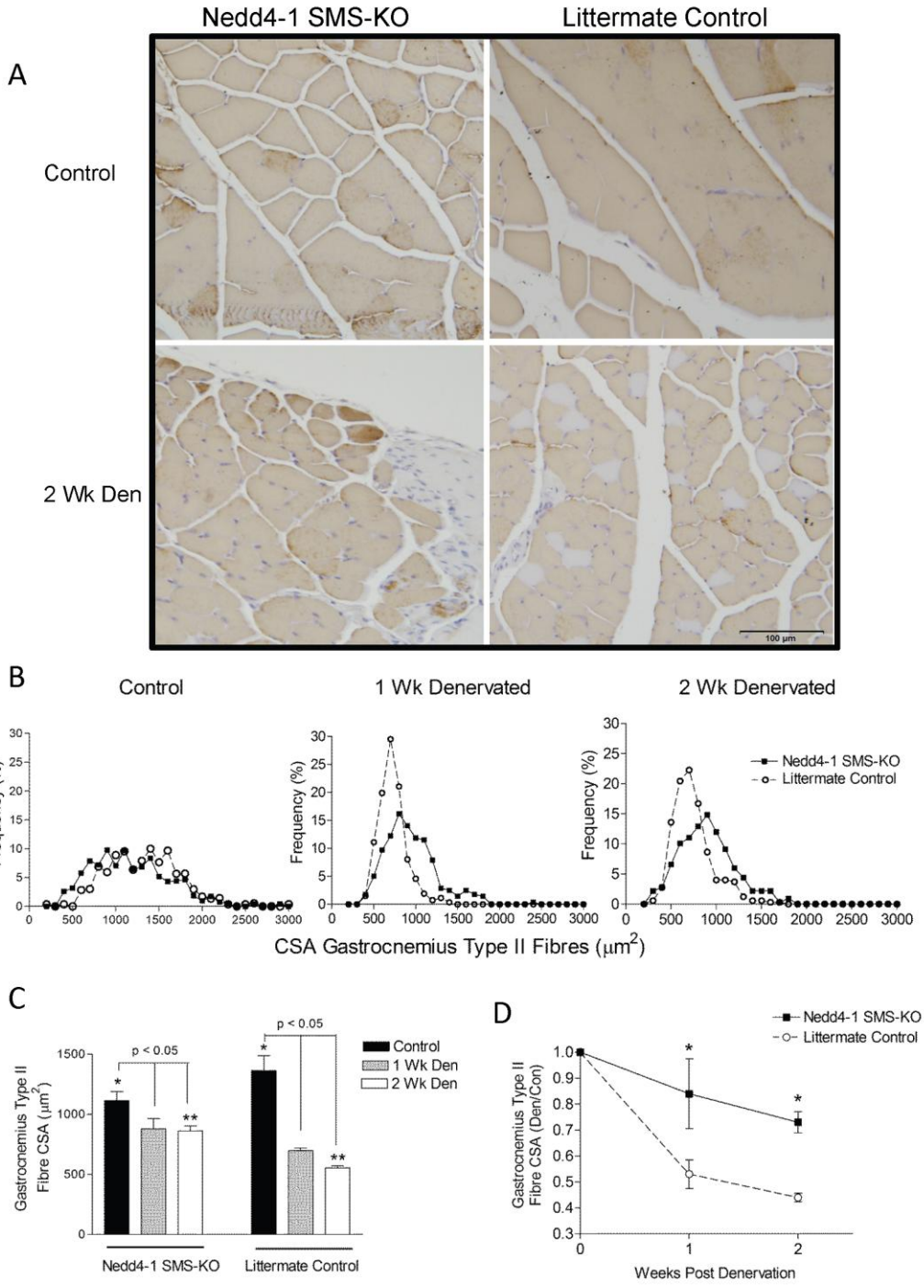

Gastrocnemius weight (Den/Con)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
*
*
1
2
Weeks post denervation
Nedd4-1 SMS-KO
Littermate Control

Figure 2.8. Nedd4-1 deletion attenuates denervation-induced decreases in gastrocnemius Type II fiber cross-sectional area. (A) Cross sections of denervated and control gastrocnemius muscle of Nedd4-1 SMS-KO and Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ (littermate control) mice were immunostained with an anti-fast twitch myosin antibody and counterstained with hematoxylin. Fast twitch type II fibers stain brown, and slow twitch type I fibers stain light purple. Representative cross sections from control and 2 week denervated gastrocnemius muscle from both Nedd4-1 SMS-KO and littermate control mice are shown. (B) Histograms of type II fiber cross sectional areas (CSA) for control innervated (left panel), 1 week denervated (middle panel) and 2 week denervated (right panel) gastrocnemius muscles in Nedd4-1 SMS-KO (solid line, closed squares) and littermate control (dashed line, open circles) mice are shown. (C) At baseline, Nedd4-1 SMS-KO gastrocnemius type II fiber CSA is smaller than that of Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ mice (*). Following denervation, there is a significant decrease in type II fiber CSA for both cohorts of mice. However the CSA of Nedd4-1 SMS-KO muscle fibers denervated for 2 weeks is significantly larger than fibers of Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ mice (**) denervated for 2 weeks. (D) Similarly, when Type II fiber CSA in denervated gastrocnemius muscle is normalized to CSA in the contralateral control muscle, type II fibers are significantly larger in Nedd4-1 SMS-KO (solid line, closed squares) mice compared to Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ (dashed line, open circles) mice at both 1 week and 2 weeks post denervation. A minimum of 300 myofibers/muscle was measured, (** and * $p < 0.05$, $n = 8$ or 9 mice/cohort and data are presented as the mean ± SEM).


A
Nedd4-1 SMS-KO
Littermate Control
Control
2 Wk Den
100 µm
B
Control
1 Wk Denervated
2 Wk Denervated
Frequency (%)
Nedd4-1 SMS-KO
Littermate Control
CSA Gastrocnemius Type II Fibres (µm²)
C
Gastrocnemius Type II Fibre CSA (µm²)
p < 0.05
Control
1 Wk Den
2 Wk Den
Nedd4-1 SMS-KO
Littermate Control
D
Gastrocnemius Type II Fibre CSA (Den/Con)
Weeks Post Denervation

Chapter 3

3 Substrate targets of Nedd4-1 in denervation-induced skeletal muscle atrophy

3.1 INTRODUCTION

We have confirmed Nedd4-1 as a participatory E3 ligase involved in the positive regulation of denervation-induced skeletal muscle atrophy. The mechanism by which Nedd4-1 elicits this effect is unknown, but several Nedd4-1 muscle substrates have been identified and could act as downstream effectors in the induction of skeletal muscle atrophy (Yang and Kumar, 2010). To identify potential Nedd4-1 substrates that regulate the loss of muscle mass, we focused on known Nedd4-1 substrates that have been reported to demonstrate decreased protein levels in denervated, unloaded or immobilized muscle, and identified potentially three; myotubularin related protein 4 (MTMR4), fibroblast growth factor receptor 1 (FGFR1) and Notch1 (Koncarevic et al., 2007; Persaud et al., 2011; Plant et al., 2009). We reasoned that, if Nedd4-1 signalled downstream via MTMR4, FGFR1 or Notch-1 to induce muscle atrophy, then these protein(s) would be ubiquitinated and subsequently degraded in denervated muscle. Similarly, we expect MTMR4, FGFR1 or Notch-1 to be protected from degradation in the denervated gastrocnemius muscle of Nedd4-1 SMS-KO mice.

MTMR4

MTMR4 belongs to the myotubularin (MTM) family of tyrosine/dual specificity phosphatases (PTP/DSP; Laporte et al., 2003; Lorenzo et al., 2006; Patterson et al., 2009).

These proteins contain a catalytic phosphatase (PTP) domain, phospholipid binding FYVE (Fab1, YGL023, Vps27, EEA1) domain and a PY (XPPXY) motif that binds WW 3 and 4 domains of Nedd4-1 (Figure 3.1; Plant et al., 2009). Myotubularins regulate cellular endocytosis and trafficking by dephosphorylating phosphatidylinositol 3-phosphate [PI(3)P] and phosphatidylinositol 3,5-bisphosphate [$PI(3,5)P_2$] to produce PIP and PI(5)P (Clague and Lorenzo, 2005; Robinson and Dixon, 2006; Zhao et al., 2001).

PI(3)P is highly enriched in early endosomes and internal vesicles of multi-vesicular endosomes and its depletion delays trafficking through early endosomes and impairs formation of intraluminal cargo vesicles (Stenmark and Gillooly, 2001). PI(3)P depletion has also been demonstrated to lead to inefficient sorting and trafficking of growth factor receptors to late endosomes (Patterson et al., 2009; Robinson and Dixon, 2006; Simonsen et al., 2005), influencing receptor signalling. MTMR4 has been shown to regulate trafficking and degradation of the epidermal growth factor receptor (EGFR) in the endosome/lysosome pathway by regulating PI(3)P levels (Lorenzo et al., 2006), thus influencing cell growth.

$PI(3,5)P_2$ localization/biology is not well-defined in mammalian cells but studies in yeast indicate its role in vacuolar homeostasis and membrane trafficking at late endosomes/ lysosomes (Robinson and Dixon, 2006). Depletion of $PI(3,5)P_2$ in yeast results in enlarged and poorly acidified vacuoles and impaired movement of endocytosed growth factor receptor to the lysosomes (Cao et al., 2008; Robinson and Dixon, 2006).

PI(5)P is a newly identified phosphoinositide and recent studies have reported its role in activating the PI3K/AKT signaling pathway (Carricaburu, 2003; Ramel et al., 2011). Production of PI(5)P by bacterial phosphatase, IpgD, has been reported to trigger EGFR

activation and accumulation in the early endosomes thereby preventing its degradation and promoting cell survival through increased PI3K/AKT signaling (Ramel et al., 2011).

The role of MTMR4 in skeletal muscle is unknown, but one can speculate that regulation of the expression levels of its substrate phospholipids might influence endocytosis and signaling of growth factor receptors, thus influencing muscle mass. Indeed loss of function mutations in other MTM family members lead to skeletal muscle myopathies with hypoplastic fibers (Berger et al., 2002; Buj-Bello et al., 2002; Laporte et al., 1996; Ma et al., 2008; Robinson and Dixon, 2006; Senderek et al., 2003). Mutations of the myotubularin family member MTM1 in humans are associated with an X-linked atrophic myotubular myopathy and MTM1-knockout mice demonstrate a progressive atrophic myopathy starting a few weeks after birth, suggesting MTM1 is required for muscle structural maintenance. Knockdown of *Caenorhabditis elegans* MTMR3, the closest relative of MTMR4, results in severe impairment of worm body movement, revealing a crucial role for MTMR3 in muscle function (Ma et al., 2008). Our laboratory reported that MTMR4 is ubiquitinated by Nedd4-1 and MTMR4 expression is decreased concomitant to increased Nedd4-1 expression in denervated skeletal muscle (Plant et al., 2009), suggesting this novel interaction may underlie the loss of muscle mass.

Notch1

Notch-1 is a trans-membrane receptor protein that is sequentially cleaved upon ligand binding, releasing its “activated” intracellular domain, which translocates from the cytosol to the nucleus to influence transcription (Figure 3.2; Zanotti and Canalis, 2010). In muscle satellite cells Notch-1 positively regulates cell proliferation, and decreased expression of

Notch-1 coincides with the onset of terminal differentiation to myocytes (Conboy et al., 2002, Luo et al., 2005). A role for Notch-1 in mature muscle fibers has not yet been elucidated.

The response of Notch-1 to denervation has been disputed. Koncarevic et al. reported that Nedd4-1 targets full length Notch1 (Notch1 FL) and its intracellular domain (Notch1 ICD) for ubiquitin-proteasome mediated degradation in denervated and unweighted skeletal muscle (2007). In contrast, Liu et al. have recently demonstrated an increase in nuclear Notch1 ICD 7, 35 and 56 days post denervation injury (2011). The reason for these discrepant findings is not immediately apparent. While Notch-1 appears to be a *bonafide* Nedd4-1 substrate, its involvement in the mediation of post-natal muscle atrophy, if any, remains unclear.

FGFR1

FGFR1 is a trans-membrane tyrosine kinase receptor and consists of an extracellular ligand-binding domain, a trans-membrane domain and a cytoplasmic catalytic kinase domain (Figure 3.3; Turner and Grose, 2010). FGFR1 is expressed in skeletal muscle and its expression has been shown to influence myogenic development (Scata et al., 1999; Mitchell and DiMario, 2010; Templeton and Hauschka, 1992). Post-natally, ectopic over-expression of FGFR1 in mouse gastrocnemius by electrocorporation significantly inhibited inactivity-induced atrophy, and FGFR1 is increased in myofibers that are partially resistant to inactivity-induced atrophy (Eash et al., 2007).

Human and zebrafish Nedd4-1 bind and ubiquitinate activated FGFR1, regulating its endocytosis and attenuating its downstream signaling (Persaud et el., 2011). This binding

takes place by a non-canonical mechanism, between a novel non-PY motif in the FGFR1 C-terminus, VL***PSR, and the WW3 or C2 domain of Nedd4-1. The strongest binding occurs between the WW3 domain and FGFR1. Interestingly, while human and zebrafish Nedd4-1 contain a WW3 domain, mouse and rat Nedd4-1 do not (Figure 1.7); rodent FGFR1 however, does contain the VL***PSR sequence. Although Persaud et al. were unable to detect binding between FGFR1 and rat Nedd4-1, they acknowledge that binding may still occur between the C2 domain of rodent Nedd4-1 and FGFR1, albeit with weaker affinity and thus below the detection limits of their assay. Binding to mouse Nedd4-1 specifically was not assessed. Thus, we speculated FGFR1 may be a candidate substrate by which Nedd4-1 signalled downstream to regulate muscle mass such that the loss of Nedd4-1 abolishes activated FGFR1 ubiquitination and degradation, thus enhancing the positive effect of FGFR1 on muscle protein synthesis and inhibition of atrophy.

3.2 MATERIALS AND METHODS

3.2.1 Western Blotting

Nedd4-1 SMS-KO and littermate Myo^{Cre}; $Nedd4\text{-}1^{+/+}$ control mice were subjected to tibial nerve transection, and their gastrocnemius muscle was harvested at 1 and 2 weeks post denervation, as described in Chapter 2. Gastrocnemius protein lysates were separated by SDS-PAGE, transferred to nitrocellulose, ponceauS stained and immunoblotted for proteins of interest. Primary antibodies included anti-HPRT (ab109021, Abcam, Cambridge, MA), -MTMR4 (generated in the Batt Laboratory), -Notch1 (#3608, Cell Signaling Technology, Danvers, MA), and -FGFR1 (#ab824, Abcam, Cambridge, MA) antibodies. Secondary antibodies were HRP (horseradish peroxidase)-linked anti-rabbit or anti-mouse and protein

bands were detected with ECL (Enhanced Chemiluminescence; GE Healthcare, Baie d'Urfe, QC). The chemiluminescent signal was acquired and quantified with Bio-Rad Fluor S Max Acquisition System (Biorad Laboratories, Hercules, CA) and Image Lab software (Biorad Laboratories).

3.2.2 **Generation of an anti-MTMR4 antibody**

The anti-MTMR4 antibody was generated in our laboratory. We created a glutathione S-transferase (GST) fusion protein of the C-terminal region of MTMR4 (RARELMSQQLKKPIATASS; aa 1172-1195, accession # CAQ52069), that was purified from bacteria on glutathione agarose beads, then subjected to thrombin cleavage to release the MTMR4 specific peptide (Zhao et al., 2001). As the peptide molecular weight is only approximately 3kDa, we were unable to visualize the final product on a coomasie stained gel. Thus, confirmation of peptide size was attained with mass spectrometry analysis. Rabbits were administered 100ug of purified peptide subcutaneously six times every 20 days. Serum was obtained pre and post immunization. Specificity of the anti-MTMR4 antibody was confirmed by western blotting protein lysates of 293 cells, transfected with HA-tagged MTMR4 cDNA, with pre- and post-immune serum.

3.2.3 **Statistical Analyses**

Data from the denervated muscle were normalized to data from the contralateral control limb within each mouse. Continuous data are reported as a mean and standard error and were compared using two-way analysis of variance. Statistical significance was assumed if $p < 0.050$.

3.3 RESULTS

Generation of an anti-MTMR4 antibody

In order to assess the role of MTMR4 in Nedd4-1 mediated skeletal muscle atrophy, western blot analysis of endogenous MTMR4 expression in muscle was necessary. However, the commercially available anti-MTMR4 antibody (AP17037c, Abgent, San Diego, CA) was unable to detect endogenous MTMR4 in skeletal muscle protein lysates. Therefore, we generated an anti-MTMR4 antibody using the peptide sequence earlier described by Zhao et al. (2001). Western blotting of 293 cell lysates expressing HA-tagged MTMR4 cDNA revealed several protein bands unique to the anti-MTMR4 post-immune serum, with one being at the appropriate molecular weight of 180kDa and identifying both the endogenous MTMR4 and over-expressed HA-MTMR4 protein (Figure 3.4).

Nedd4-1 substrates MTMR4, Notch1 and FGFR1 are not differentially expressed in the denervated gastrocnemius muscle of Nedd4-1 SMS-KO and littermate myo-cre/Nedd4-$1^{+/+}$ control mice.

MTMR4, FGFR1 and Notch1 are known Nedd4-1 substrates that demonstrate decreased expression in denervated, unloaded and immobilized muscle (Koncarevic et al., 2007; Persaud et al., 2011; Plant et al., 2009). We reasoned if Nedd4-1 signalled downstream, via binding and ubiquitination of these substrates to induce muscle atrophy, then these protein(s) would be protected from degradation in the denervated gastrocnemius muscle of Nedd4-1 SMS-KO mice.

We found that levels of both MTMR4 and FGFR1 proteins decreased in the denervated gastrocnemius muscle of control mice coincident to the increase in Nedd4-1

(Figure 3.5), but there was no attenuation of that decrease for either protein in the denervated gastrocnemius of Nedd4-1 SMS-KO mice, suggesting that neither MTMR4 nor FGFR1 are substrates involved in Nedd4-1's mediation of denervation-induced muscle atrophy. In contrast, cleaved Notch-1 demonstrated an increase in the 2 week denervated gastrocnemius compared to control muscle (Figure 3.5) and the extent of this increase was similar between Nedd4-1 SMS-KO and littermate control mice, again suggesting that Notch-1 does not serve as a substrate in Nedd4-1's induction of skeletal muscle atrophy. Notch-1 full length (FL) was not detectable in muscle lysates with several Notch-1 antibodies tested (data not shown).

3.4 **DISCUSSION**

We have demonstrated that Nedd4-1 positively regulates denervation-induced skeletal muscle atrophy, but the substrate(s) through which it mediates this effect are unknown. Therefore, we interrogated three known skeletal muscle substrates, MTMR4, which we identified, and Notch-1 and FGFR1, identified by others, as potential downstream targets in the induction of muscle atrophy (Koncarevic et al., 2007; Persaud et al., 2011; Plant et al., 2009). We demonstrate here that both MTMR4 and FGFR1 demonstrate decreased expression in denervated muscle relative to controls, concomitant with increased Nedd4-1 expression, suggesting that Nedd4-1 mediated ubiquitination of these target proteins may be causally associated with the denervation-induced loss of muscle mass. However, this does not appear to be the case as we found no attenuation of this decrease in MTMR4 and FGFR1 protein levels in the denervated gastrocnemius of Nedd4-1 SMS-KO mice.

In keeping with Liu's findings (Liu et al., 2011) and in contrast to Kandarian's report (Koncarevic et al., 2007), we demonstrate an increase in cleaved Notch-1 protein levels in the denervated gastrocnemius muscle of Nedd4-1 SMS-KO and littermate control mice and there is no difference in the magnitude of the increase between the two cohorts of mice. This increase in Notch-1 may reflect the increase in satellite cell proliferation that occurs in response to denervation as a self-limited attempt of the muscle at a reparative response (Jejurikar et al., 2002, 2006; Kuschel et al., 1999; McGeachie and Allbrook, 1978). We currently find no evidence of Nedd4-1 engaging Notch-1 to mediate denervation-induced skeletal muscle atrophy.

In conclusion, while we report alterations in MTMR4, Notch1 and FGFR1 protein levels in gastrocnemius muscle denervated for 1 and 2 weeks, this effect does not appear to be mediated by Nedd4-1. Hence the substrates and downstream signalling networks Nedd4-1 engages to mediate denervation-induced skeletal muscle atrophy remain unknown.

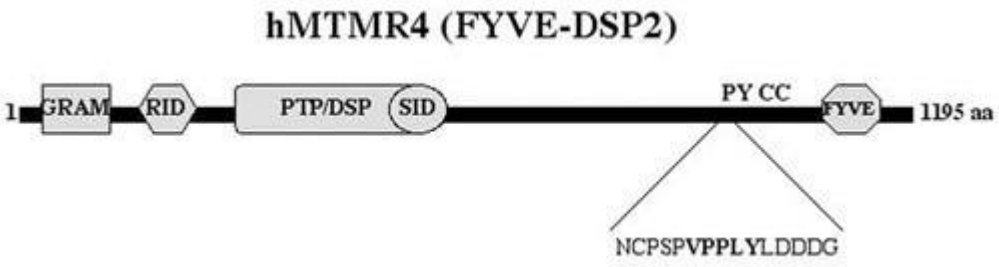


Figure 3.1. Schematic diagram of hMTMR4. hMTMR4 consists of GRAM (glucosyltransferase, Rab-like GTPase activator of myotubularins) domain, RID (Rac-induced recruitment domain), PTP/DSP domain, SID (SET-interacting domain), PY motif (XPPXY) that binds Nedd4-1, CC (coiled-coil) domain and FYVE domain.

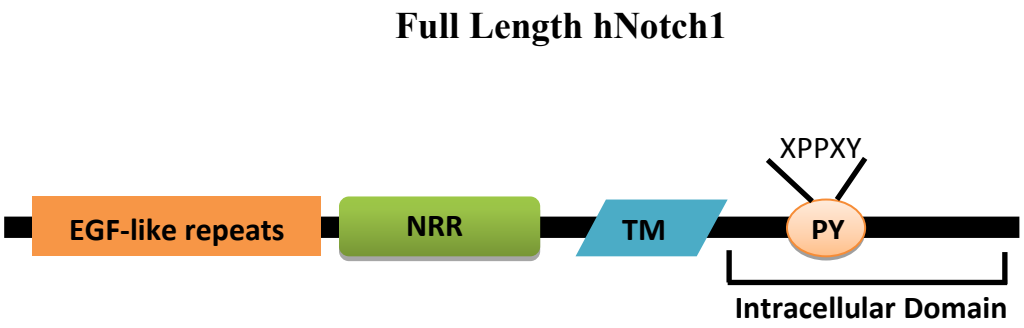


Figure 3.2. Schematic diagram of Full Length hNotch1. hNotch1 is comprised of N-terminal 36 EGF-like repeats, negatively regulatory regions (NRR) containing a hetero-dimerization domain and cleavage sites, a short trans-membrane domain and C-terminal intracellular domain (ICD) containing the PY (XPPXY) motif that binds Nedd4-1.

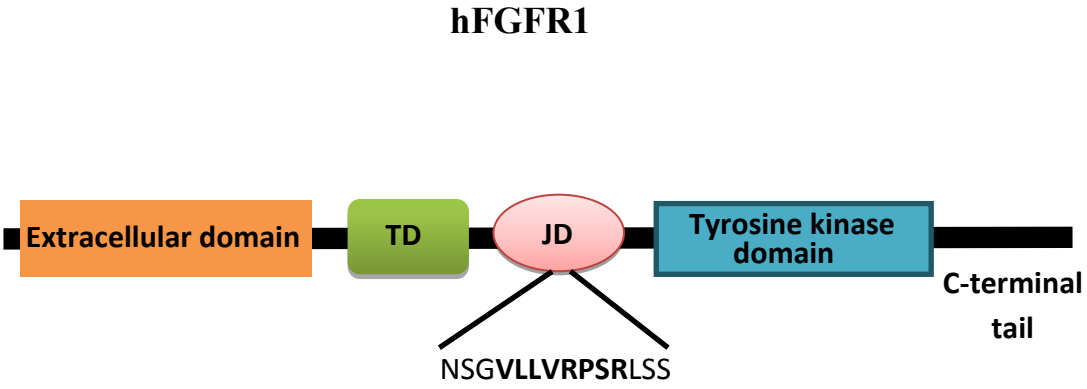


Figure 3.3. Schematic diagram of human FGFR1 (hFGFR1) protein. FGFR1 is a trans-membrane tyrosine kinase receptor and consists of an extracellular ligand-binding domain, trans-membrane domain (TD), juxtamembrane domain (JD) containing the non-canonical sequence VLLVRPSR that binds to Nedd4-1 and cytoplasmic catalytic kinase domain.

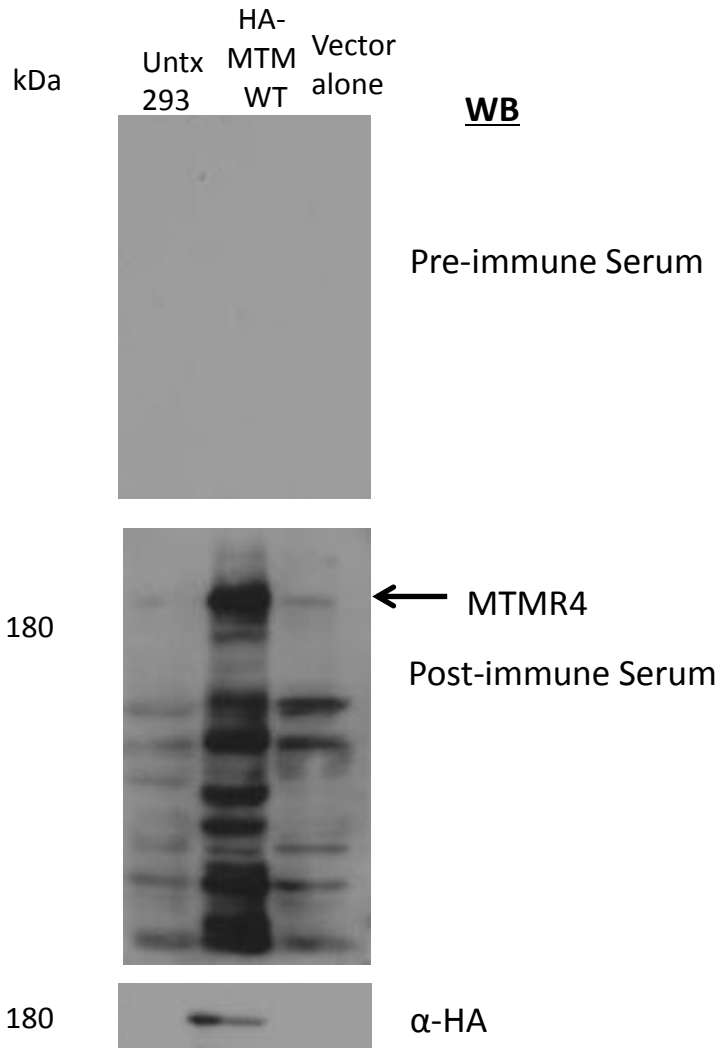
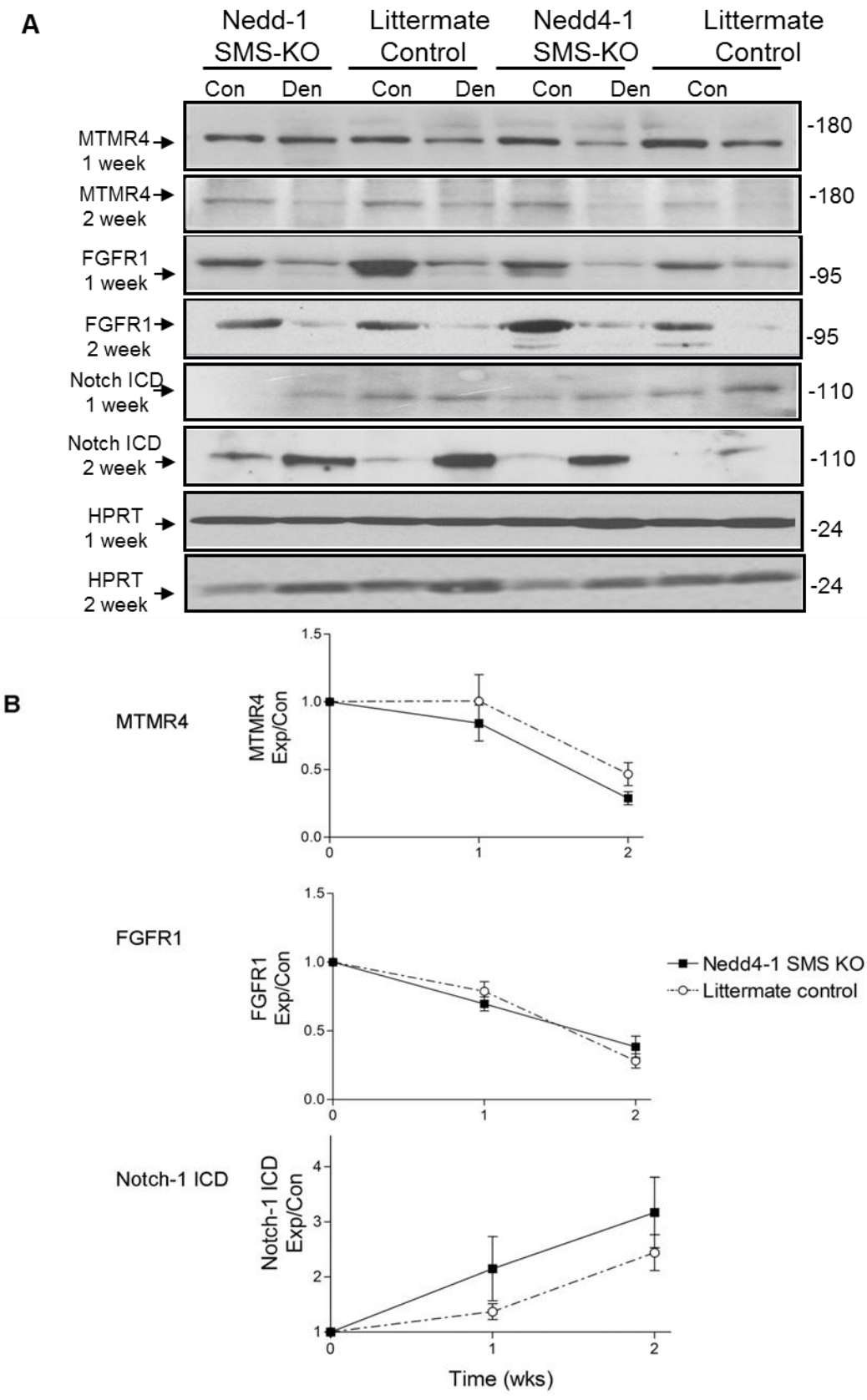


Figure 3.4. Anti-MTMR4 rabbit polyclonal antibody recognizes endogenous and exogenously expressed MTMR4. Western blot of 293 cells untransfected (Untx 293) and transfected with HA-tagged MTMR4 (HA-MTMR4 WT) or pcDNA3.1 (Vector alone) immunoblotted with pre-immune serum (upper panel), anti-MTMR4 post-immune serum (middle panel) and anti-HA antibodies (bottom panel). The anti-MTMR4 antibody recognizes both endogenous and exogenously expressed MTMR4.

Figure 3.5. Levels of MTMR4 and FGFR-1 are not maintained in the denervated gastrocnemius of Nedd4-1 SMS-KO mice, while cleaved Notch-1 expression is increased. (A) Representative Western blots of MTMR4, FGFR1, cleaved Notch-1 (Notch-1 ICD) and HPRT (as a loading control) in protein lysates from denervated gastrocnemius muscle (Den) or the contralateral control gastrocnemius (Con) muscle in Nedd4-1 SMS-KO and littermate Myo^{Cre}; Nedd4-$1^{+/+}$ control mice at 1 and 2 weeks post tibial nerve transection. (B) The chemiluminescent signal was quantified, MTMR4, FGFR1 and Notch-1 protein levels were normalized to the corresponding HPRT levels (lane matched) and numerical values are depicted in bar graphs. For each of MTMR4, FGFR1 and Notch-1, protein levels in denervated (Exp) muscle are expressed as a fraction of the protein level in control (Con) muscle. MTMR4 levels were significantly decreased in denervated compared to control gastrocnemius muscle in both Nedd4-1 SMS-KO ($n = 6$) and Myo^{Cre}; Nedd4-$1^{+/+}$ ($n = 6$) mice at 2 weeks ($p < 0.05$), but there was no difference in the magnitude of MTMR4 decrease between the Nedd4-1 SMS-KO compared to control mice. FGFR1 levels were similarly significantly decreased in denervated compared to control gastrocnemius muscle in both Nedd4-1 SMS-KO ($n = 8$) and littermate Myo^{Cre}; Nedd4-$1^{+/+}$ control ($n = 8$) mice at 1 week and 2 weeks ($p < 0.05$) post tibial nerve transection, and again there was no difference in the magnitude of the decrease between the 2 cohorts of mice at either timepoint. Cleaved Notch-1 levels (Notch ICD) were significantly increased in denervated compared to control gastrocnemius in both Nedd4-1 SMS-KO ($n = 6$) and Myo^{Cre}; Nedd4-$1^{+/+}$ control ($n = 6$) mice at 2 weeks ($p < 0.05$), but not at 1 week. There was no difference in the magnitude of the increase between the 2 cohorts of mice. Data are mean ± SEM.


A
Nedd-1 SMS-KO
Littermate Control
Nedd4-1 SMS-KO
Littermate Control
Con
Den
Con
Den
Con
Den
Con
MTMR4 1 week
MTMR4 2 week
FGFR1 1 week
FGFR1 2 week
Notch ICD 1 week
Notch ICD 2 week
HPRT 1 week
HPRT 2 week
-180
-180
-95
-95
-110
-110
-24
-24
B
MTMR4
MTMR4 Exp/Con
FGFR1
FGFR1 Exp/Con
Notch-1 ICD
Notch-1 ICD Exp/Con
Nedd4-1 SMS KO
Littermate control
Time (wks)

Chapter 4

4 Role of MTMR4 in Skeletal Muscle Fibrosis

4.1 INTRODUCTION

Phosphorylated Smad2 and Smad3 are MTMR4 protein substrates

The myotubularin (MTM) family of tyrosine/dual specificity phosphatases (PTP/DSP) dephosphorylate both lipid and protein (phospho-tyrosine and phospho-serine/threonine) substrates (Patterson et al., 2009; Robinson and Dixon, 2006). Until recently, only lipid substrates of MTMR4 [PI(3)P and PI(3,5)P_2] had been identified (Clague and Lorenzo, 2005; Robinson and Dickson 2006; Zhao et al., 2001). Yu and colleagues have recently reported that MTMR4 binds and dephosphorylates the proteins Smad2 and Smad3 to down-regulate transforming growth factor beta1 (TGFβ1) signaling, confirming the role of MTMR4 as a protein phosphatase (2010).

TGFβ1 is a cytokine involved in the regulation of several cellular processes including proliferation, differentiation, homeostasis, wound repair, chemotaxis and apoptosis (Leask and Abraham, 2004). TGFβ1 induces its effects by binding and activating the type I and type II TGFβ receptors (TβRI and TβRII respectively). TGFβ1 binding to TβRII, a constitutively active kinase, initiates TβRII/ TβRI hetero-dimerization (Figure 4.1). TβRII subsequently transphosphorylates TβRI, which activates its serine/threonine kinase activity and leads to the recruitment of TGFβ1-dependent signaling cascades. The activated TβRII/ TβRI receptor complex phosphorylates the cytoplasmic proteins, receptor-regulated Smad2 and Smad3 (R-Smads). Phosphorylated R-Smads form a trimeric complex with a common mediator Smad4

and then translocate to the nucleus where they regulate the transcription of TGFβ1 target genes (Figure 4.1; Cutraneo, 2007; Kollias and McDermott, 2008).

MTMR4 has been shown to suppress the transcriptional activity induced by TGFβ1 thereby negating a variety of TGFβ1-mediated effects such as promotion of hypertrophic cell growth and cell proliferation in porcine aorta endothelial (PAE) and HaCaT (human keratinocytes) cells (Yu et al., 2010). Yu et al. demonstrated that MTMR4 immunoprecipitates and dephosphorylates phospho-Smad2 (P-Smad2) and -Smad3 (P-Smad3), preventing their nuclear translocation and in this manner, attenuated TGFβ1 signaling. Investigations to determine whether the MTMR4 binding of Smad2/3 was direct, or indirect, were not however, reported.

The role of TGFβ1 in skeletal muscle

Recent studies have demonstrated that TGFβ1 induces skeletal muscle atrophy. When injected into muscles of healthy mice, TGFβ1 reduces muscle fiber size and force generating capacity by increasing atrogin-1 expression and stimulating ubiquitin-proteasome mediated muscle proteolysis (Mendias et al. 2012; Zugmaier et al., 1991). This occurs as a result of Smad2 and Smad3-dependent activation of the atrogin-1 promoter (Sartori et al., 2009). TGFβ1 also negatively regulates post-natal skeletal muscle mass by impairing muscle regeneration (Kollias and McDermott, 2008). TGFβ1 inhibits satellite cell proliferation and differentiation and induces satellite cell apoptosis (Kollias and McDermott, 2008; Zimowska et al., 2009) TGFβ1 mediates its effects on reparative myogenesis via activation of Smad2 and 3, which influence the expression of key myogenic transcription factors including myogenic differentiation antigen 1 (MyoD) and myocyte-specific enhancer factor 2C (MEF2C). In addition to causing muscle atrophy, TGFβ1 is also recognized as a major

inducer of fibrosis resulting in the trans-differentiation of myoblasts into myofibroblasts, again signaling downstream through R-Smads to achieve this effect (Cencetti et al., 2010; Gardner et al., 2010).

Muscle Fibrosis

Denervation of skeletal muscle induces both atrophy and fibrosis and profound changes in cellular composition and the extracellular matrix (ECM) of skeletal muscle. Fibrosis occurs when myofibers are replaced by progressive deposition of ECM proteins (Cencetti et al., 2010). Fibroblasts and other reparative cells migrate to the injury site and subsequently proliferate to generate new collagen matrix (Cutroneo, 2007). ECM of skeletal muscle predominantly contains collagen type I and III proteins (Salonen et al., 1985). In denervation atrophy, the fibrotic change of muscle tissue occurs due to an increase in collagen type I and III in the endomysium and perimysium of skeletal muscles (Kumar and Sharma, 2006; Savolainen et al., 1988; Sawai, 1982).

Fibrosis is regulated by a complex set of cytokines including TGFβ1 (Mann et al., 2011). TGFβ1 expressed within skeletal muscle post denervation has been demonstrated to mediate muscle fibrosis (Meng et al., 2011; Zhou et al., 2006). TGFβ1 induces trans-differentiation of myoblasts into myofibroblasts, as indicated by the concomitant decreased expression of myogenic proteins (myosin heavy chain (MHC), myogenin) and increased expression of protein associated with fibrosis (laminin, transgelin and alpha smooth muscle actin (α-SMA)) (Cencetti et al 2010; Gardner et al., 2011; Wu et al., 2007). TGFβ1 inhibits differentiation of myoblasts into myotubes by targeting the myogenic transcription factors MyoD and myogenin (Brennan et al., 1991; Martin et al., 1992; Vaidya et al., 1989).

Myogenic transcription factors share homology within a basic helix-loop-helix (bHLH) motif that binds to a conserved DNA sequence, CANNTG, known as E-Box of numerous muscle-specific genes to regulate their transcription (Arnold and Winter, 1998). TGFβ1 represses myogenesis by either blocking the expression of MyoD and myogenin or by supressing the expression of muscle-specific genes through a post-transational mechanism that targets the bHLH regions of myogenic regulators to silence their activity (Brennan et al., 1991; Martin et al., 1992). Following TGFβ1 treatment, Smad3 has been shown to bind to the bHLH region of MyoD interfering with the dimerization of MyoD and other bLHL containing proteins, E12 and E47 and thus subsequently inhibiting their binding to the E-Box DNA of muscle-specific genes (Liu et al., 2001). Smad3 also has the ability to interact with MEF2C to prevent the association of the MyoD/E47 dimerization with MEF2 resulting in the repression of muscle specific gene expression (Liu et al., 2004). The mechanism whereby Smad2 signaling mediates TGFβ1 repression of myoblast differentiation is currently unknown (Angelis et al., 1998). Therefore, TGFβ1 inhibits myogenesis and induces fibrosis through the regulation of R-Smads both *in vivo* and *in vitro* (Mendias et al., 2012).The molecular mechanisms underlying TGFβ1-mediated induction of fibrosis are well studied phenomena in multiple tissues, although assessment in muscle is sparse. TGFβ1 induces ECM matrix deposition via inducing expression of collagens, and suppressing the expression of genes that suppress fibrosis, such as the matrix metalloproteases (MMP). These effects results from Smad2/3 dependent influences on collagen and MMP promoters, although TGFβ1 activation of MAP kinases has also been shown to contribute to the fibrotic response in most tissues (Leask and Abraham, 2004).

Given the above, we hypothesize that MTMR4 inhibits skeletal muscle fibrosis by negatively regulating TGFβ1 signaling. The objectives of the current study were to i) demonstrate that MTMR4 attenuates TGFβ1 signaling by interacting directly or indirectly with, and dephosphorylating, Smad2/3 and ii) to determine whether MTMR4 inhibits TGFβ1-induced fibrosis in mouse C2C12 myoblasts.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture

NIH-3T3 (mouse embryonic fibroblast) and C2C12 (mouse myoblast) cells were maintained in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/l streptomycin at 37°C under 5% CO_2.

4.2.2 Western Blotting

Total protein was extracted from 3T3 and C2C12 cells in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1%Triton X-100, 10% glycerol, 1.5 mM MgCl2 and 1 mM EGTA) with protease inhibitor cocktail tablets (Cat # 11836170001, Roche), vortexed, and incubated on ice for 10 minutes. The lysate was then cleared by centrifugation at 12000 ***g*** for 10 min, and the protein concentrations in the supernatant were quantified using BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL). 40 or 50 µg of muscle protein lysates were separated by SDS-PAGE, transferred to nitrocellulose, ponceauS stained and immunoblotted. The primary antibodies used included anti-Myosin Heavy Chain (Developmental Studies Hybridoma Bank), -transgelin (#ab10135, Abcam, Cambridge, MA), -α-SMA (#ab5694, Abcam, Cambridge, MA), -GAPDH (#ab8245, Abcam, Cambridge, MA), -Smad2 (#5339,

Cell Signaling Technology, Danvers, MA), -Smad3 (# 9523, Cell Signaling Technology, Danvers, MA), -Smad2/3 (#3102, Cell Signaling Technology, Danvers, MA), phosphorylated Smad2 (P-Smad2 #3108, Cell Signaling Technology, Danvers, MA), phosphorylated Smad3 (P-Smad3 (#9520, Cell Signaling Technology, Danvers, MA) and anti-HA (#MMS101P, Covance, Princeton, NJ). Secondary antibodies were HRP (Horseradish peroxidase)-linked anti-rabbit or anti-mouse and protein bands were detected with ECL (Enhanced Chemiluminescence; GE Healthcare, Baie d'Urfe, QC). The chemiluminescent signal was acquired using a Versa-Doc CCD (charged-coupled device) camera (BioRad Laboratories, Hercules, CA) and the total signal quantified using Image Lab software (Biorad Laboratories, Hercules, CA) with volume analysis.

4.2.3 **Immunoprecipitation**

To investigate MTMR4-Smad2/3 interactions, NIH-3T3 cells were co-transfected with plasmids expressing wild type HA-tagged human MTMR4 (accession number NM_004687, a gift from Dr. Joe Zhao, Vanderbilt-Ingram Cancer Center, Nashville, TN, U.S.A.) (MTM WT), or a HA-tagged catalytically inactive PTP mutant (critical cysteine mutated to serine, MTM PTP, with flag-tagged Smad2 or myc-tagged Smad3 using Polyjet (SignaGen laboratories, Rockville, MD) as per manufacturer's instructions. Vector alone served as a negative control in this experiment to avoid confounding of results due to transfection-induced changes in protein expression. At 24 and 48 hours post transfection, cells were thoroughly washed with phosphate buffer saline (Gibco), and treated with 5ng/ml TGFβ1 (Gibco) for 1 hour at 37°C. Equal amounts of lysate from MTM WT, MTM PTP or pcDNA 3.1 (vector alone) transfected or untransfected 3T3 cells were incubated with 1ug of

anti-HA or 1:100 dilution of anti-Smad2 (#5339, Cell Signaling Technology, Danvers, MA) and -Smad3 (#9523, Cell Signaling Technology, Danvers, MA) antibodies overnight on ice at 4°C. 20 ul (50% slurry) of Protein A–Sepharose beads (Sigma, St. Louis, MO) was added to the lysate and incubated for 1 hour at 4°C while rotating. Lysate was also incubated with beads, in the absence of antibody, to serve as a negative control. Beads were then precipitated by centrifugation at 10,000 ***g*** for 1 min at 4℃ and washed twice in high-salt HNTG buffer (500 mM NaCl, 20 mM Hepes, pH 7.5, 10% glycerol and 0.1% Triton X-100) and three times in low-salt HNTG buffer (150 mM NaCl, 20 mM Hepes, pH 7.5, 10% glycerol and 0.1% Triton X-100). The beads were mixed with 30 ul of 1× Sample Buffer and boiled at 95℃ for 5 min to elute proteins. 250ug or 500ug of protein eluate was resolved by SDS/8% PAGE and transferred on to Protran 0.45 um pore-size nitrocellulose membranes (Biorad Laboratories, Hercules, CA) for Western blotting.

4.2.4 **Fibrosis Assay**

C2C12 myoblasts, seeded at a density 134000 cells per cm^2,were transfected with MTM WT, MTM PTP and pcDNA3.1 using lipofectamine 2000 (Invitrogen, Grand Island, NY) as per manufacturer's instructions or left untransfected. Cells were maintained 6 hours post transfection and then treated with 5ng/ml TGFβ1 (Gibco) for 24, 48 and 72 hours. Following TGFβ1 treatments, cells were lysed for protein extraction, separated by SDS PAGE and western blotting was performed.

4.2.5 **Statistical analysis**

Statistical significance was determined by Student's **t**-test or one-way analysis of variance followed by post-hoc TuKey analyses, where appropriate. $P < 0.05$ was accepted as significant.

4.3 **RESULTS**

TGFβ1 treatment of C2C12 myoblasts induces fibrosis and inhibits myogenesis.

To optimize an *in vitro* model of fibrosis, we treated C2C12 myoblasts with TGFβ1 for 24, 48 and 72 hours. Western blotting of these lysates for markers of myogenesis (MHC) and fibrosis (transgelin, α-SMA) revealed that TGFβ1 treatment increased transgelin expression and concomitantly decreased MHC protein levels, demonstrating trans-differentiation of the myoblasts (Figure 4.2; Cencetti et al., 2010). α-SMA levels did not change (Figure 4.2A,C).

MTMR4 does not diminish the pro-fibrotic effect of TGFβ1 on C2C12 myoblasts.

To determine whether MTMR4 negatively regulates TGFβ1 signaling in skeletal muscle, we treated C2C12 cells expressing MTM WT or MTM PTP with TGFβ1. Neither the constitutive over-expression of wild type MTMR4 nor the dominant-negative catalytically inactive MTMR4 mutant influenced the TGFβ1-induced increase in transgelin in C2C12 myoblasts (Figure 4.3A-C). Given the lack of effect of MTMR4 on the trans-differentiation of C2C12, we wanted to assess the levels of endogenous expression of MTMR4 protein. Our laboratory had previously demonstrated, using RT-PCR, that MTMR4 transcripts were expressed in C2C12 myoblasts and myotubes. Endogenous MTMR4 protein levels are so low in C2C12 cells, it is not detectable using the anti-MTMR4 antibody

generated in our laboratory (Figure 4.3D), although MTMR4 is readily visible in adult mouse skeletal muscle, suggesting C2C12 cells are not a good model for study.

MTMR4 and Smad2/3 do not interact in 3T3 fibroblasts

Transfection efficiency in C2C12 mouse myoblasts with standard lipid based methods is low, so to conduct immunoprecipitation experiments to confirm the interaction between MTMR4 and Smad2 and 3, we used NIH-3T3 cells, which are easily transfectable. As has been previously shown (Nakamura et al., 2011), we demonstrated that TGFβ1 treatment of NIH-3T3 cells induces phosphorylation and nuclear translocation of endogenous Smad2 and Smad3 (Figure 4.4), confirming that these cells are TGFβ1 responsive. MTMR4 has been shown to interact with Smad2 and Smad3 in endosomes of HEK-293 and HaCaT cells in a TGFβ1 dependent manner (Yu et al., 2010). To confirm this interaction in 3T3 cells, we over-expressed MTM WT or MTM PTP and assessed immunoprecipitation of endogenous phospho-Smad2 and phospho-Smad3 with MTMR4 on cytosolic 3T3 lysates (Figure 4.5). Neither phospho-Smad2 nor phospho-Smad3 immunoprecipitated wild type or the catalytically inactive mutant MTMR4 suggesting that MTMR4 does not interact with Smad2/3 in 3T3 cells.

MTMR4 overexpression does not attenuate TGFβ1 signaling in 3T3 cells

To further confirm that MTMR4 and Smad2 do not interact, we assessed the effect of MTMR4 expression on TGFβ1-induced Smad2/3 phosphorylation in 3T3 cells. We found no difference in TGFβ1-induced Smad2/3 phosphorylation in 3T3 cells expressing MTM WT, MTM PTP, or vector alone (Figure 4.6). Therefore, we confirmed that MTMR4 overexpression does not attenuate TGFβ1 signaling in 3T3 cells.

4.4 DISCUSSION

In the current study we demonstrate that MTMR4 does not bind and dephosphorylate Smad2 or Smad3 to down-regulate TGFβ1 signaling in contrast to previous reports, nor does MTMR4 appear to inhibit TGFβ1-induced fibrosis in C2C12 myoblasts. We show that TGFβ1 leads to trans-differentiation of C2C12 myoblasts into myofibroblasts. This pro-fibrotic effect of TGFβ1 has been previously demonstrated by assessing C2C12 expression of both myogenic and fibrotic markers (Cencetti et al., 2010; Gardner et al., 2010). Consistent with the literature, we demonstrate a TGFβ1-induced up-regulation of the fibrotic marker transgelin and down-regulation of myogenic marker MHC in C2C12 myoblasts. In contrast, increases in the fibrotic marker α-SMA have not been consistently reported in the literature in response to TGFβ1 treatment, and indeed our work here fails to demonstrate a TGFβ1 mediated increase in α-SMA (Cencetti et al., 2010).

Interestingly, MTMR4 over-expression in C2C12 myoblasts did not attenuate TGFβ1 signaling to prevent fibrosis. There are several possible explanations for this finding. Transfection efficiency of the C2C12 cells with a lipid based method is moderate to weak, and may have produced inadequate MTMR4 expression to see a biologic effect. Use of a virus transduction system would have provided much higher expression efficiency. We did not pursue this methodology however; as it became apparent that C2C12 skeletal muscle cell line is not an ideal system to work with. At the outset of the planning of these experiments we had determined the expression of MTMR4 in C2C12 cells using qPCR methodology. Confirmation at the protein level was not possible due to the absence of a commercially available antibody that was reliable. Following generation of our own anti-MTMR4 antibody we subsequently found that we were unable to detect MTMR4 is C2C12 cells, although it

was easily seen in mouse skeletal muscle protein lysates. Thus, a second possible reason we did not see MTMR4-induced attenuation of TGFβ1-mediated myofibrosis in C2C12 cells is that MTMR4 appears to be markedly down-regulated in this immortalized cell line, and thus cellular signaling networks in which it is involved may be marginally functional, or non-functional as opposed to skeletal whole muscle. The C2C12 line is not an optimal muscle cell for study, and use of a primary skeletal muscle cell, that retains MTMR4 expression, would be appropriate for further investigation.

A third possible reason that MTMR4 did not attenuate TGFβ1-mediated C2C12 myofibrosis is that MTMR4 does not bind and dephosphorylate Smad2 or Smad3. To confirm this newly reported interaction we choose to work in an easily transfectable, TGFβ1 responsive cell line, NIH-3T3. We were unable to identify any interaction between MTMR4 and Smad2 or Smad3 in 3T3 fibroblasts, nor did over-expression of MTMR4 attenuate TGFβ1-induced phosphorylation of Smad2 in 3T3 cells, suggesting that the MTMR4-Smad2/3 interaction is a cell type specific phenomenon. Given the cumulative negative findings, we decided to terminate this project.

In conclusion, we have demonstrated that MTMR4 does not interact with or dephosphorylate P-Smad2 or P-Smad3 in mouse NIH-3T3 fibroblasts, nor does it influence TGFβ1-induced C2C12 myofibrosis. Thus, it appears that we have not found the optimal model system to test the current hypothesis.

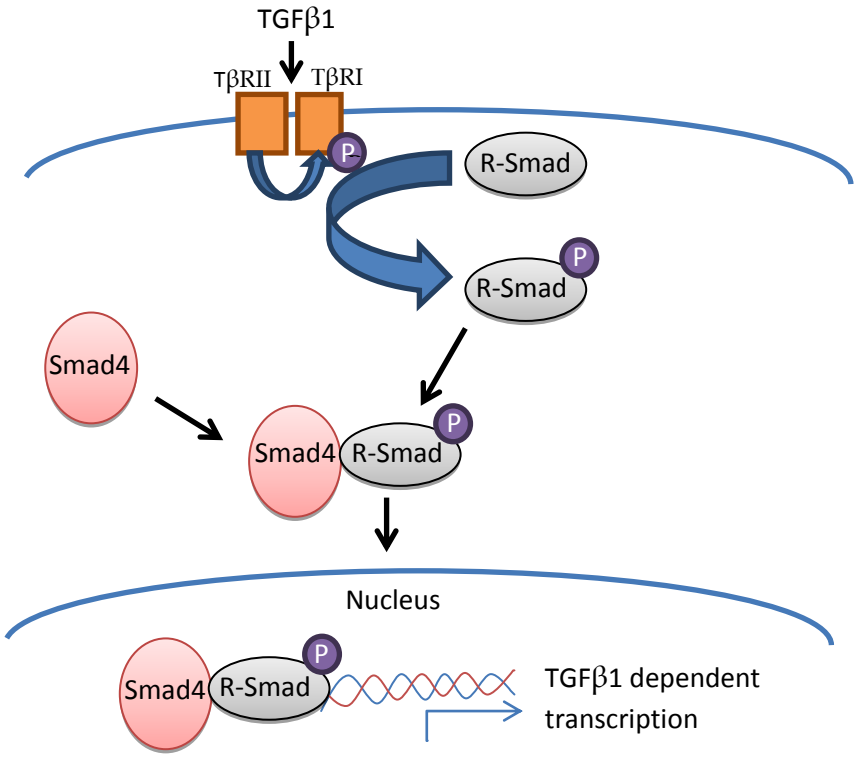


Figure 4.1. TGFβ1 signaling pathway. TGFβ1 binds to TβRII, a constitutively active kinase, to initiate its hetero-dimerization with TβRI. Following receptor dimerization, TβRII transphosphorylates TβRI. This activated receptor complex phosphorylates the intracellular signaling mediators, receptor-regulated Smad2/3 (R-Smads). Activated R-Smads form a trimeric complex with a common mediator Smad (Co-Smad, Smad4), and translocate to the nucleus to regulate transcription of TGFβ1 target genes.

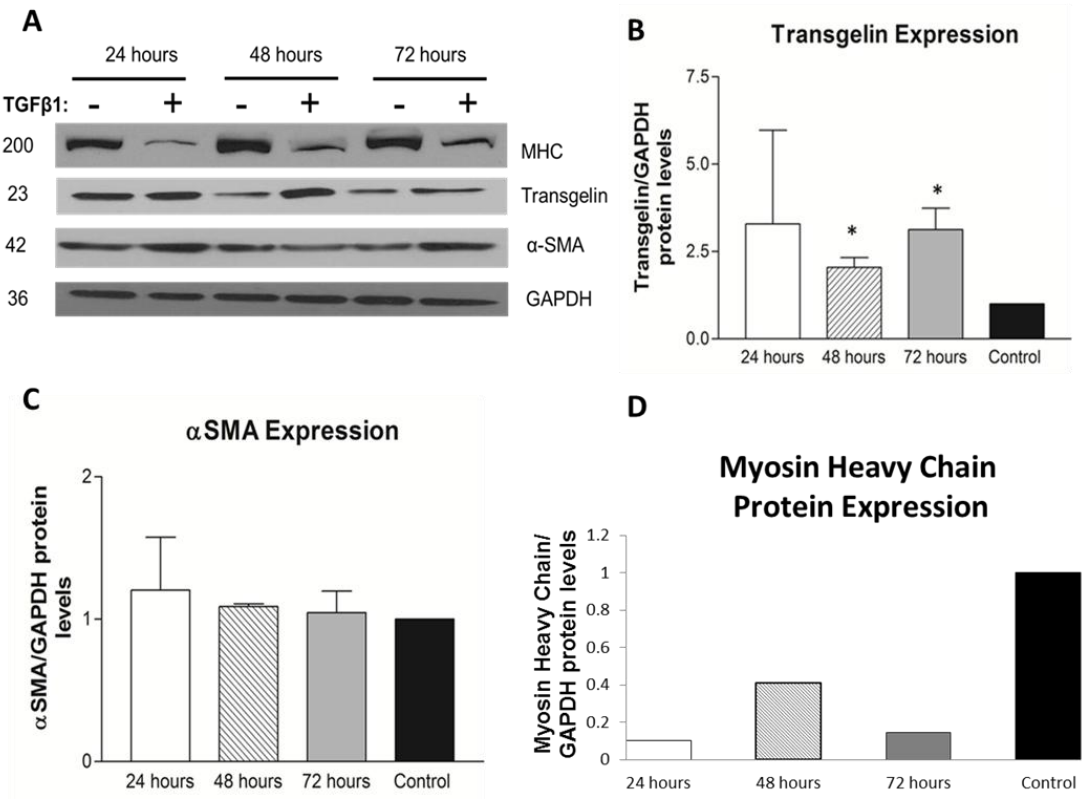


Figure 4.2. TGFβ1 treatment of C2C12 myoblasts induces fibrosis and inhibits myogenesis. (A) Western blots of Myosin Heavy Chain (MHC), transgelin, alpha smooth muscle actin (α-SMA) and GAPDH from TGFβ1 treated (5ng/ml) and untreated C2C12 cells at 24, 48 and 72 hours. (B-D) The chemiluminescent signal was quantified, total protein levels were normalized to the GAPDH levels and the numerical values are depicted in bar graphs. Protein levels of MHC, transgelin and α-SMA are expressed as a fraction of GAPDH protein levels. Western blot analysis demonstrates up-regulation of the fibrotic marker transgelin and no change in SMA ($n = 3$, * $p < 0.05$) and shows down-regulation of the myogenic marker MHC ($n = 2$). GAPDH serves as a loading control and representative blot is shown. Data are presented as the mean ± SEM.

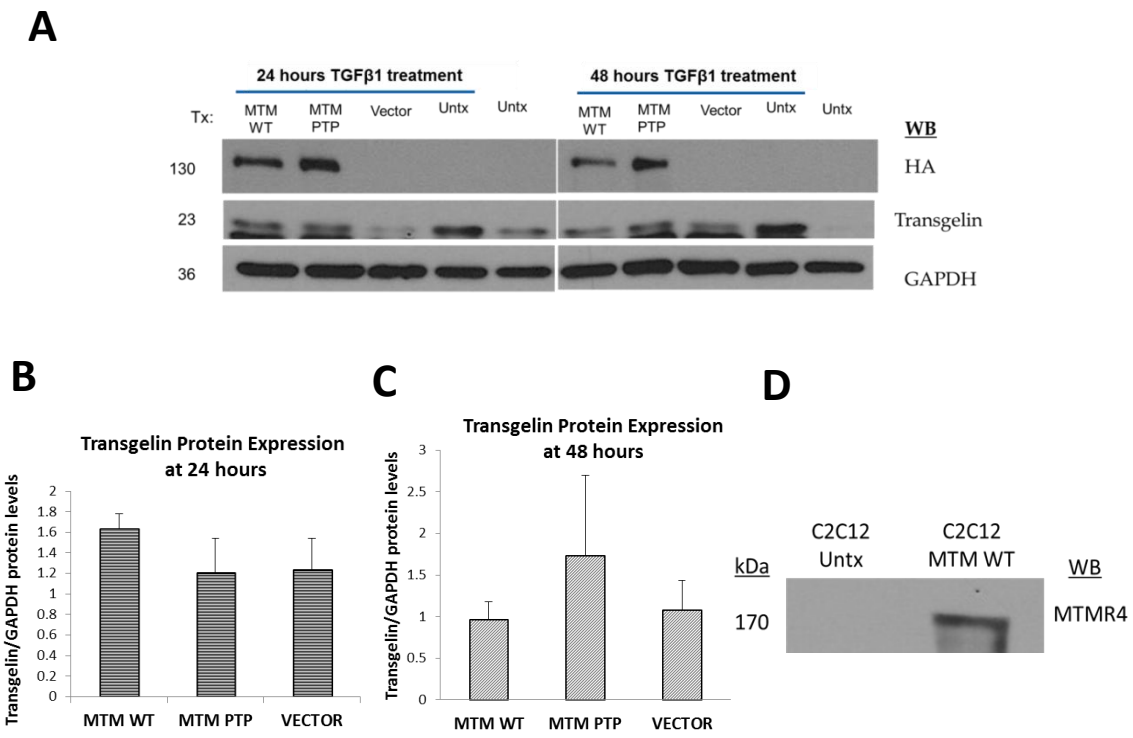


Figure 4.3. MTMR4 does not influence the pro-fibrotic effect of TGFβ1 on C2C12 myoblasts. (A) Representative western blots of MTMR4 and transgelin in protein lysates of C2C12 myoblasts untransfected (Untx) or transfected with HA-MTMR4 wild type (MTM WT), HA-MTMR4 catalytic mutant (MTM PTP), pcDNA 3.1 (vector) followed by TGFβ1 treatment for 24 or 48 hours. GAPDH served as a loading control and representative blot is shown. (B,C) The chemiluminescent signal was quantified, and transgelin levels were normalized to GAPDH levels. There is significant difference in TGFβ1-induced transgelin expression in cells expressing MTMR4 WT, the dominant negative MTMR4 catalytic mutant or vector alone. Experiments were repeated 3 times and data are presented as the mean ± SEM. (D) Western blot of C2C12 myoblasts untransfected (C2C12 Untx) or transfected with HA-MTMR4 WT (C2C12 MTM WT) were immunoblotted with anti-MTMR4. MTMR4 antibody only recognizes over-expressed MTMR4 in C2C12 cells and does not detect endogenous MTMR4 protein levels.

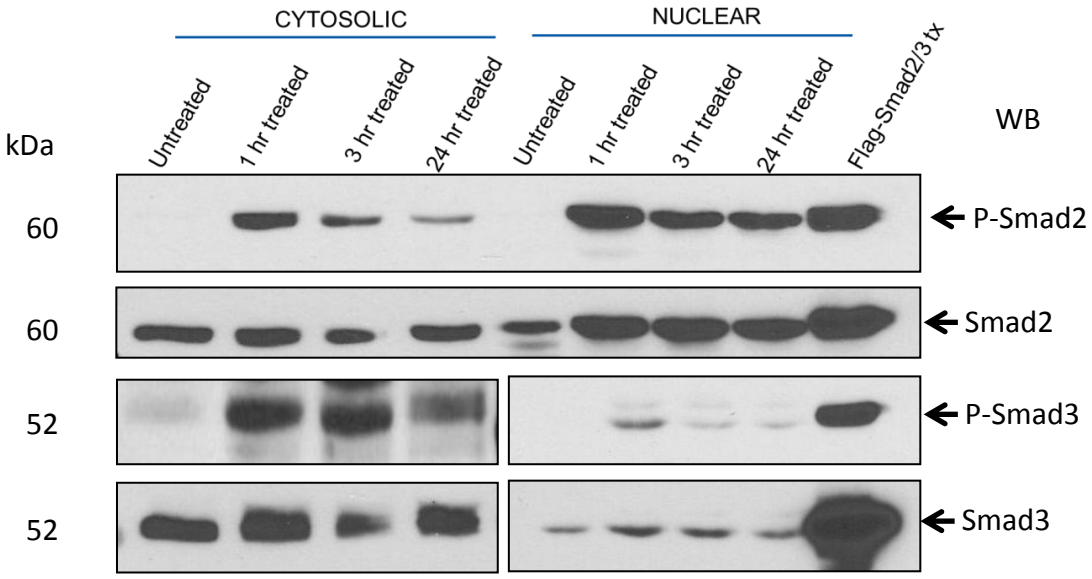


Figure 4.4. TGFβ1 induces Smad2 and Smad3 phosphorylation in mouse 3T3 fibroblasts. Representative western blots for Smad2, Smad3 and phosphorylated Smad 2 and 3 (P-Smad2; P-Smad3) in the cytosolic and nuclear fraction of protein lysates from NIH-3T3 cells treated with TGFβ1 (5ng/mL) for 1, 3, or 24 hours. Protein lysates from 3T3 cells transfected with flag-tagged Smad2 or Smad3 served as positive controls. TGFβ1 induces phosphorylation of Smad2 and Smad3 in 3T3 cells at all time-points assessed and is evident in both cytosolic and nuclear fractions. Endogenous Smad2 and Smad3 act as loading controls and show similar expression in untreated and treated lysates.

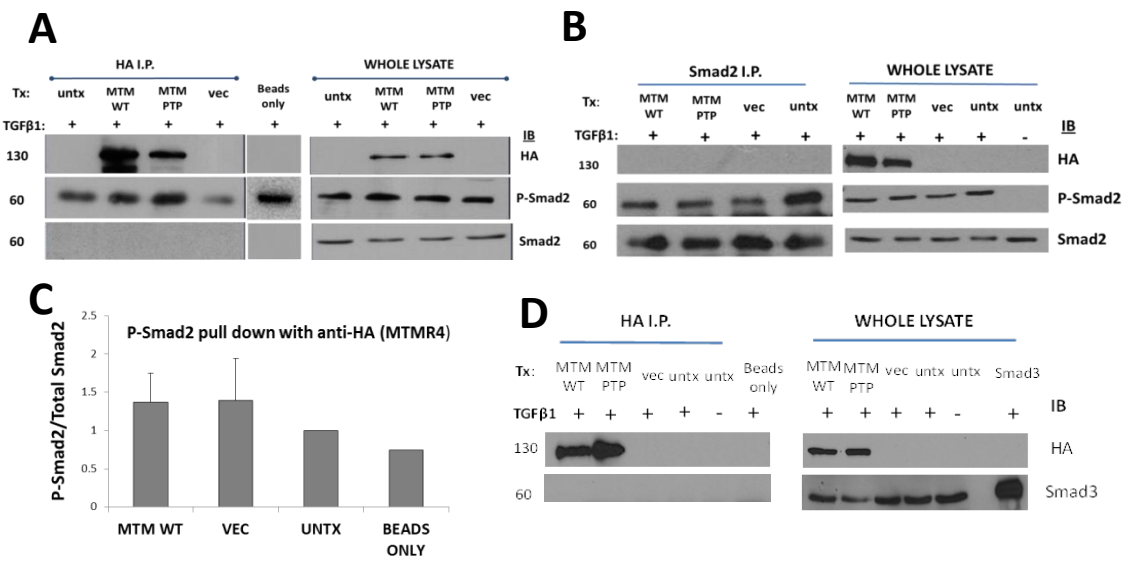


Figure 4.5. MTMR4 and P-Smad2 do not interact to attenuate TGFβ1 signaling in 3T3 fibroblasts. NIH-3T3 cells were transfected with HA–hMTMR4 wild type (MTM WT), HA-hMTMR4 catalytic mutant (MTM PTP), pcDNA 3.1 vector alone (vec), and untransfected (Untx) followed by TGFβ1 (5ng/ml) treatment or no treatment for 1 hour on day 3. Cytosolic lysates were immunoprecipitated (IP) using (A) anti-HA, (B) anti-Smad2 that specifically identifies non-phosphorylated Smad2, (C) anti-Smad3 antibodies and equal amounts of whole lysates were run as controls. (A,B,D) Immunoblotting (IB) was performed with anti-HA (monoclonal) antibodies followed by anti-P-Smad2/3 (polyclonal) antibodies and blots were stripped and re-probed with anti-Smad2/3 antibodies. (C) Western blot analysis of HA IP shows that there is no difference in the pull-down of P-Smad2 between MTM WT, MTM PTP and untx. There is non-specific binding of P-Smad2 to the beads due to which there is no differential pull down of P-Smad2 with MTM WT, MTM PTP and Untx. Therefore, MTM WT does not interact with P-Smad2. Molecular masses are indicated in kDa. Experiments were repeated 3 times and data are presented as the mean ± SEM.

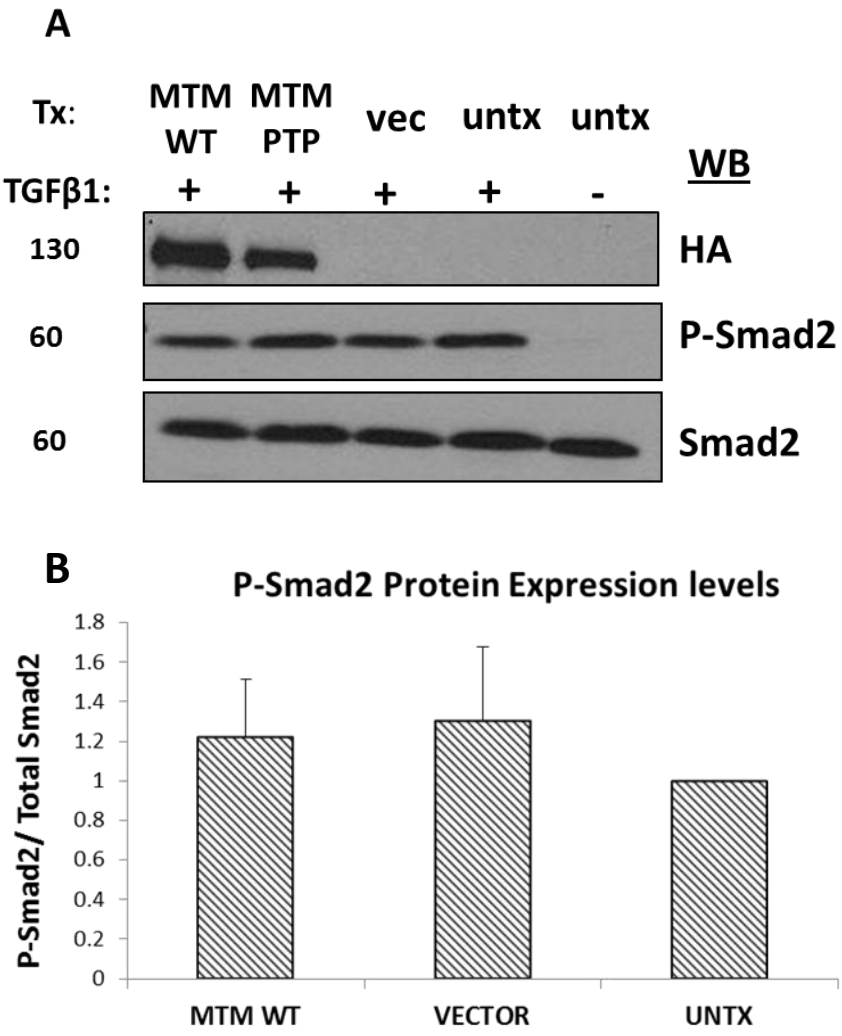


Figure 4.6. MTMR4 overexpression does not dephosphorylate P-Smad2 to attenuate TGFβ1 signaling. (A) NIH-3T3 cells were transfected with wild type HA–hMTMR4 (MTM WT), HA-hMTMR4 catalytic inactive mutant (MTM PTP), pcDNA 3.1 vector alone (vec or vector alone), or untransfected (Untx) followed by TGFβ1 (5ng/ml) treatment for 1 hour on day 3. (B) Western blotting analysis of nuclear 3T3 lysates shows that endogenous P-Smad2 levels remain unchanged when MTMR4 is overexpressed. Total endogenous protein levels of Smad2 served as loading control. Molecular masses are indicated in kDa. Experiments were repeated 3 times and data are presented as the mean ± SEM.

Chapter 5

5 Project Summary and Future Directions

5.1 SUMMARY

Skeletal muscle atrophy is a phenomenon resulting from aging, inactivity and multitude of acute and chronic illnesses (Cooper et al., 2012; Hornberger et al., 2001; IJkema-Paassen et al., 2001, 2002; Johansen et al., 2003; Liu et al., 2000; Medina et al., 1995; Pereira et al., 2005; Rhoads et al., 2010; Stevenson et al., 2003, 2005; Testelmans et al., 2010). In illness, muscle atrophy is associated with increased disease morbidity, mortality and poor quality of life resulting in significantly increased health resource utilization and socio-economic costs (Celli 2010; Decramer et al., 1997; DeOreo et al., 1997; Foster et al., 2006; Gosker et al., 2003; IJzerman et al., 2012; Janssen et al., 2004; Jaquet et al., 2001; Mostert et al., 2000; Rosberg et al., 2005; Tawney et al., 2003; Volpato et al., 2002). Current therapies for restoring muscle mass, such as resistance exercise, anabolic steroids, and electrical stimulation are themselves inadequate to completely reverse muscle atrophy associated with acute and chronic illnesses (Arena et al., 2010; Colson et al., 2010; Glass and Roubenoff, 2010; Smart et al., 2012; Toth et al., 2012).

Skeletal muscle atrophy results predominantly from the loss of sarcomeric proteins due to an imbalance between protein synthesis and protein degradation, whereby proteolysis overwhelms an inadequate synthetic response (Cao et al., 2005; Glass, 2010; Goldberg 1969; Hansen et al., 2006; Kandarian and Jackman, 2006). Among many proteolytic pathways present in skeletal muscle, the ubiquitin-mediated proteasome system is the predominant

pathway resulting in the development of skeletal muscle atrophy. E3s or ubiquitin ligases are the key enzymes that confer specificity to this system, targeting proteins for degradation. Two ubiquitin ligases (atrogin-1 and MuRF1) have been identified to be positive regulators of muscle atrophy resulting from metabolic insult and inactivity (denervation or immobility/unloading). However, their absence, induced by knockout *in vivo,* did not provide complete protection against the development of muscle atrophy implying that other ubiquitin ligases may be involved. Our laboratory and others demonstrated an increase in Nedd4-1 in the muscle of rodents subjected to models of hind-limb unloading and short and long-term models of denervation-induced skeletal muscle atrophy (Batt et al., 2006; Koncarevic et al., 2007; Plant et al., 2009). In order to study the role of Nedd4-1 in skeletal muscle atrophy, we generated a novel genetic tool, the skeletal muscle specific Nedd4-1 knockout mouse and subjected it to the well-validated model of denervation-induced skeletal muscle atrophy. We demonstrate here, that the absence of Nedd4-1 spares muscle wasting, conclusively identifying Nedd4-1 as a third ubiquitin ligase that participates in the induction of denervation-induced skeletal muscle atrophy.

In order to identify the target substrates through which Nedd4-1 regulates muscle mass, we assessed three known Nedd4-1 muscle substrates MTMR4, Notch1 and FGFR1, but found none of these substrates signal downstream of Nedd4-1 to mediate denervation-induced muscle atrophy. Hence, the downstream mechanism by which Nedd4-1 contributes to the induction of denervation-induced skeletal muscle atrophy remains unknown.

In a separate component of my thesis project, I planned to assess the manner by which Nedd4-1 targeting of its substrate MTMR4 for ubiquitin-mediated proteasomal

degradation might regulate denervation-induced skeletal muscle fibrosis. MTMR4 has been recently reported to negatively regulate TGFβ1 signaling by dephosphorylating P-Smad2 and P-Smad3. TGFβ1 is known to induce skeletal muscle fibrosis and inhibit myogenesis. Therefore, I hypothesized that the denervation-induced decrease in MTMR4 might enhance TGFβ1 signaling leading to muscle fibrosis. However, I was unable to confirm any interaction between MTMR4 and P-Smad2 or P-Smad3, nor did I find that MTMR4 influenced TGFβ1-induced trans-differentiation of C2C12 myoblasts into myofibroblasts. Thus, I was not able to find an optimal system to test this hypothesis and hence discontinued this line of investigation.

5.2 FUTURE DIRECTIONS

5.2.1 Identification of Nedd4-1 substrate(s) regulating skeletal muscle atrophy by Mass Spectrometry.

Since we were unable to identify the substrates of Nedd4-1 involved in the induction of muscle atrophy, it would be interesting to investigate other possible substrates of Nedd4-1 involved in the regulation of muscle mass through quantitative multidimensional protein identification technology (MudPIT) mass spectrometry (MS). This is a non-gel approach for the identification of proteins from complex mixtures. The technique consists of a two-dimensional chromatography separation, prior to electrospray mass spectrometry. By exploiting a peptide's unique physical properties of charge and hydrophobicity, complex mixtures can be separated prior to sequencing by tandem MS. Mass spectrometric analysis of denervated gastrocnemius muscle of wild type and Nedd4-1 muscle specific knockout mice could theoretically identify proteins that are differentially expressed in the Nedd4-1 deficient

muscle. Since Nedd4-1 is absent, its substrate protein should not be ubiquitinated, nor targeted for degradation, and therefore should be present in larger quantity in the denervated Nedd4-1 deficient muscle compared to control. Identified proteins that are differentially expressed would be verified with SDS PAGE and quantitative western blotting and interaction between Nedd4-1 and the protein of interest could be confirmed with immunoprecipitation experiments.

5.2.2 **Identification of other proteolytic pathways involved in Nedd4-1 mediated skeletal muscle atrophy.**

It would be interesting to use this genetic model to determine the proteolytic pathways, apart from ubiquitin-proteasome mediated proteolysis, in which Nedd4-1 engages to target proteins for degradation. Recent studies by O'Leary and Hood have demonstrated increased expression of autophagy-promoting genes, Beclin-1 and LC3-II, in the muscle of mice denervated for one and two weeks (2009). Hence, it would be interesting to assess the expression of Beclin-1 and LC3-II in denervated muscles of wild type and Nedd4-1 SMS-KO mice through western blotting. Western blotting of denervated gastrocnemius muscle of wild type and Nedd4-1 SMS-KO would allow us to assess differential expression of autophagy-promoting genes in the Nedd4-1 deficient muscle. If Nedd4-1 engages in protein degradation of denervated muscles through autophagy, then we should see decreased expression of Beclin-1 and LC3-II in Nedd4-1 deficient muscle.

Similarly, caspase-3 is activated by denervation and there is also an upregulation of 14kDa actin fragment in denervated muscle (Plant et al., 2009). Therefore, it would be interesting to assess the differential expression of activated caspase-3 and actin in denervated

muscles of wild type and Nedd4-1 SMS-KO mice. This can be performed by immunostaining the transverse sections of denervated gastrocnemius muscle for activated caspase-3 in wild type and Nedd4-1 SMS-KO mice. SDS PAGE and western blotting of 14kDa actin fragment in the insoluble portion of gastrocnemius muscle would allow us to assess the expression of actin in the two cohorts of mice. If Nedd4-1 is involved in the proteasomal degradation of actin fragments, then actin should be present in larger quantities in the denervated Nedd4-1 deficient muscle compared to control.

5.2.3 **Identification of compensatory pathways for protein degradation in Nedd4-1 deficient muscle.**

Nedd4-1 provided partial protection against denervation-induced muscle atrophy but the magnitude of protection is not as large as that provided by atrogin-1 or MuRF1 knockout mice despite of the fact that Nedd4-1 shows a sustained increase. This might occur due to compensatory up-regulation of atrogin-1 and MuRF1 in denervated limbs of Nedd4-1 SMS-KO mice. We did not assess the expression of atrogin-1 and MuRF1 in our genetic model due to lack of working antibodies for these proteins. In future, real-time RT-PCR can be used to assess expression levels of transcripts for the ubiquitin ligases atrogin-1 and MuRF1. These genes do not produce proteins that require post-translational modification for activity; therefore, determination of the level of expression of the transcripts is adequate. Since, atrogin-1 and MuRF1 expression returns to basal level in long-term denervated muscle (Batt et al., 2006); we should measure the weights of control and denervated gastrocnemius muscle post 21 or 30 days of denervation to better assess the protection against denervation atrophy in Nedd4-1 SMS-KO mice.

Since there is some redundancy in the function of two Nedd4 isoforms, it is possible that Nedd4-2 is up-regulated to compensate for the loss of Nedd4-1 in Nedd4-1 SMS-KO mice. We have performed SDS PAGE and western blotting of denervated gastrocnemius muscles of both wild type and Nedd4-1 SMS-KO mice for Nedd4-2 protein expression but it was not quantified. Hence, quantification of western blots can be done in the future to determine differential expression of Nedd4-2 in denervated muscles of wild type and Nedd4-1 SMS-KO mice.

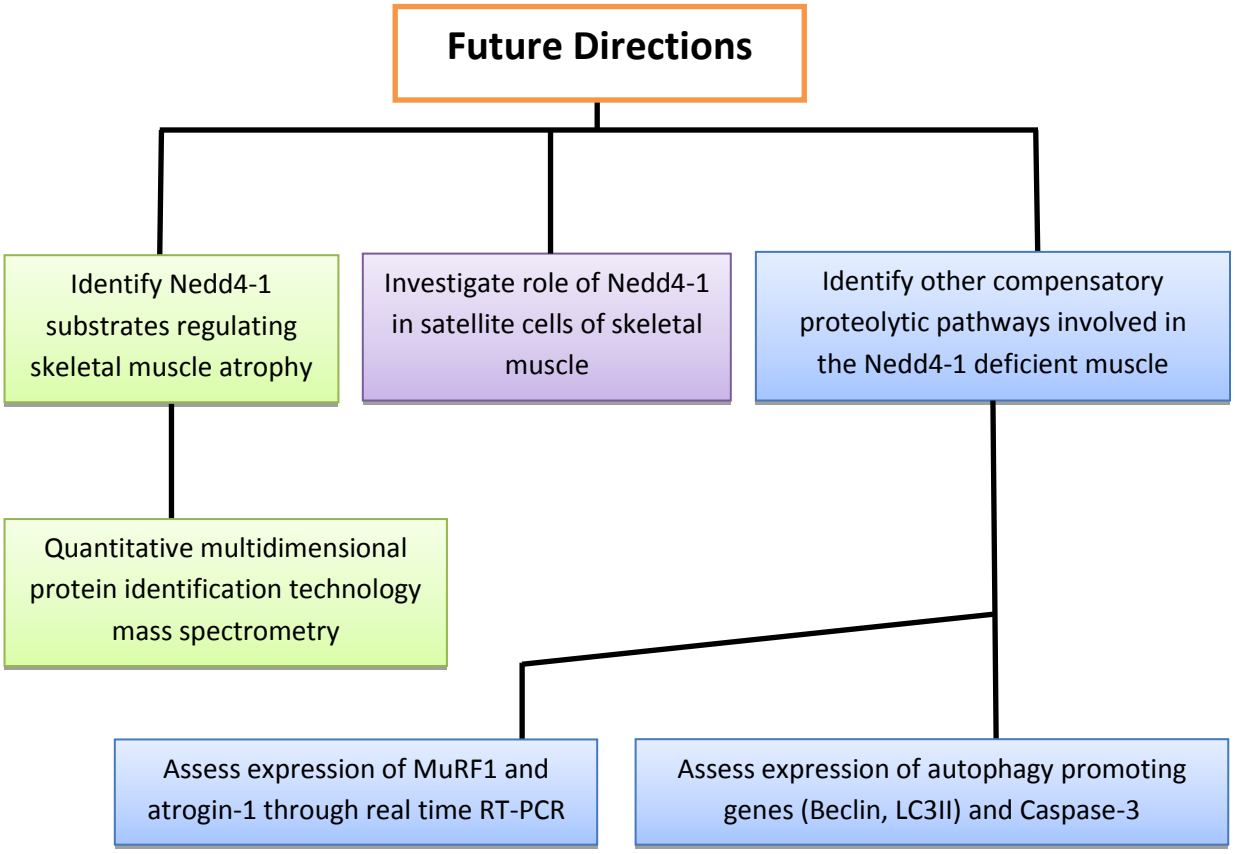
5.2.4 **Investigate the role of Nedd4-1 in satellite cells of skeletal muscle**

Our genetic model does not delete Nedd4-1 from satellite cells and this could have contributed to reduced protection from muscle atrophy in denervated limb of Nedd4-1 SMS-KO mice. Hence, we need to investigate the role of Nedd4-1 in satellite cells to clearly understand the repercussions of our genetic model. In order to achieve this, we would inject antibodies directed against Nedd4-1 into Nedd4-1 deficient muscle of Nedd4-1 SMS-KO mice. Once the antibody is injected, it will bind to Nedd4-1 and thereby prevent it from binding to its substrates and will negate its downstream signaling. Serum with no anti-Nedd4-1 antibody would serve as a negative control in this experiment and would be injected into the gastrocnemius muscle of another set of Nedd4-1 SMS-KO mice. Following antibody or serum injection, right tibial nerve of the mice would be transected to denervate the muscle and the contralateral leg would serve as an internal control. Both sets of mice with antibody or serum treatment would be weighed post 7 and 14 days to determine whether there is a difference in the magnitude of protection against denervation atrophy. If Nedd4-1 expression reduces the regenerative capacity of satellite cells post denervation injury, then the

denervated gastrocnemius muscle with anti-Nedd4-1 treatment would be heavier than the serum treated denervated muscle.

Nedd4-1 is an ubiquitin ligase involved in the induction and development of skeletal muscle atrophy. Hence, above mentioned studies would allow us to delineate mechanisms through which this phenomenon occurs and also allow us to generate pharmacological interventions to reverse this process of muscle atrophy.

Table 5.1. Future directions for Nedd4-1 mediated skeletal muscle atrophy project



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