2019

KLHL41 in skeletal muscle development

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

KLHL41 IN SKELETAL MUSCLE DEVELOPMENT

by

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B.S., Villanova University, 2017

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2019

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ACKNOWLEDGMENTS

I would like to thank Dr. Vandana A Gupta for her mentorship and guidance throughout this project, and for encouraging my growth as a scientist in the process. I would also like to thank Caroline Jirka for her contribution to Figure 5 of the thesis. Finally, I would like to thank Dr. Brigitte Ritter for reviewing this thesis and providing constructive feedback.

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ABSTRACT

Skeletal muscle consists of an extremely regular organization of myofibers that are specialized in contraction. Development and maintenance of skeletal muscle function depends on the precise organization of sarcomeric contractile proteins that consist the myofibrils. Impaired or delayed myofibrillogenesis has been identified as the primary pathological mechanism of many skeletal muscle myopathies. Several members of the Kelch family of proteins have been implicated in skeletal muscle development and diseases, and mutations in these proteins have resulted in perturbations in the ubiquitin proteasome system (UPS), which is the primary means of proteasomal degradation in eukaryotes. In particular, KLHL41 of the BTB-BACK Kelch family is primarily expressed in skeletal muscle and has been identified as a regulator of the skeletal muscle differentiation process that results in the normal development and functioning of mature skeletal muscles. KLHL41 acts as a substrate-specific adaptor for Cullin 3 (Cul3) E3 ubiquitin ligase, implicating the role/s of KLHL41 in proteasomal ubiquitination processes in skeletal muscle. Recent studies have determined that the degradation of nebulin-related anchoring protein (NRAP), which was found to interact with KLHL41, is a critical process in skeletal myofibril maturation that is caused by KLHL41-mediated ubiquitination of the NRAP protein. Through this study, it was further confirmed that KLHL41 changes in localization as maturation occurs, which may provide insight into the mechanism of its functions in myofibril maturation. In addition, the study found that

KLHL41 promotes the critical process of nebulin-related anchoring protein (NRAP) degradation. Lastly, mutations in the KLHL41, which are known to cause Nemaline Myopathy (NM) in patients, were modeled in murine C2C12 myoblasts to gain a greater understanding of how KLHL41 mutations may affect protein stability and Cul3 E3 ubiquitin ligase activity. Overall, the findings of this thesis support the critical role of KLHL41 in the formation of mature myofibrils, and provides insight into how deficiency of KLHL41 contributes to a disease state through regulation of the CUL3 protein complex.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BSA……...……Bovine serum albumin

COP9……..…….. Constitutive photomorphogenesis 9

CRL……...……Cullin-RING ubiquitin ligase

CSN……...……...……COP9-signalosome complex

CUL1……...……Cullin 1

CUL3……...…Cullin 3

CHX……..…….……Cycloheximide

DMEM……Dulbecco's Modified Eagle Medium

FBS…….……Fetal bovine serum

GAPDH.....Glyceraldehyde 3-phosphate dehydrogenase

HRP……..……Horseradish peroxidase

IFRD1…….……Interferon-related developmental regulator 1

KBTBD……...…Kelch repeat and BTB domain containing

KBTBD10…….....…Kelch repeat and BTB domain containing protein 10

KLHDC……..……...…Kelch domain containing

KLHL…….……...…Kelch-like

KLHL41……...…Kelch-like protein 41

KRP1……..……...…Kelch-related protein 1

LDS…….…….……Lithium dodecyl sulfate

MyBC……...……... Myosin binding protein C

Myf5…….……..…...……..….Myogenic factor 5

MYHC ..... Myosin heavy chain

MyoD ……..…….Myogenic determination protein

NEB……..……..……...…Nebulin

NEDD8…..…Neural precursor cell expressed, developmentally down-regulated protein 8

NM…….Nemaline myopathy

NP40…….……..nonionic polyoxyethylene 40

NRAP.…….……Nebulin-related anchoring protein

PBS……...……Phosphate-buffered saline

PVDF .....Polyvinylidene difluoride

PSG……..……Penicillin-streptomycin-glutamate

RBM4 …… RNA-binding motif protein 4

RIPA .....Radioimmunoprecipitation assay

SRS……..……...……Substrate-recognizing subunit

TBS……..……...……Tris-buffered saline

TBS-T……..……...……..…Tris-buffered saline with 1% Tween

UBTF……..…Upstream binding transcription factor

UPS…….……Ubiquitin proteasome system

WCL……..…….……Whole cell lysate

WT…….……Wild type

INTRODUCTION

Skeletal Muscle Development

Skeletal muscle consist of cells that are specialized in contraction. To achieve this contraction, each differentiated muscle cell contains a bundle of syncytial myofibrils, and each myofibril consists of repeated sarcomere units. The sarcomere is the smallest functional contractile unit of skeletal muscle myofibers. It is a highly regular, dynamic network of macromolecular proteins composed of myosin thick filaments, actin thin filaments, and many other sarcomeric proteins that are all involved in the structure and the force generating activity of the sarcomere (Clark et al., 2002). While the initial developmental stages of myogenesis, such as cell fate specification and determination, have been extensively studied, the terminal process of skeletal muscle cell differentiation which leads to mature myofibril formation is still not well understood (Boateng and Goldspink, 2008; Buckingham, 2017). Though many sarcomere proteins have been identified and some of their interactions have been established, how these proteins form into a complex assembly in sarcomere to form myofibrils, is not completely understood.

Skeletal muscle development begins in the embryonic stage of development at the dermomyotome in developing somites that differentiate from the presomitic mesoderm primarily by Notch, Wnt and FGF signaling pathways (Jiang et al., 2000; Hubaud and Pourquié 2014). The dorsal epithelial portion of the dermomyotome consists of multipotent progenitor cells that express the Pax3 transcription factor in mammals (Horst et al., 2006), and both Pax3 and Pax7 transcription factors in lower vertebrates (Otto et al., 2006). These transcription factors direct the expression of myogenic regulatory

factors myogenic factor 5 (Myf5) and myogenic determination protein (MyoD), causing differentiation of the progenitor cells into myoblasts (Relaix et al., 2006). The dermomyotome epithelium consisting of the $Pax3^{+}$ progenitor cells delaminates to form the primary myotome (Chal and Pourquié, 2017). From the primary myotome, primary myogenesis occurs as the mononucleated progenitor cells of the primary myotome that are sandwiched between the dermomyotome and the sclerotome fuse to form multinucleated primary myofibers. These primary myofibers then eventually fuse and constitute myofibers of the first trunk muscle that primarily express slow myosin heavy chain (MyHC) contractile proteins (Buckingham and Relaix, 2007). Some of these $Pax3^{+}$ progenitor cells also delaminate and migrate to the limb buds and body wall and begin to form primary myofibers. Primary myofibers act as scaffolds for fetal muscle growth.

As the embryo reaches the fetal stage of development, secondary myogenesis occurs as a subset of Pax3 progenitor cells from the central dermomyotome begin to express Pax7 and downregulate Pax3 (Bentzinger et al., 2012). These Pax7 progenitor cells delaminate and migrate to sites of epaxial (trunk) and hypaxial (limb and ventral body wall) myogenesis (Buckingham and Relaix, 2007; Sieiro-Mosti et al., 2014; Gros et al., 2009). These progenitor cells then either fuse with primary myofibers or proliferate to contribute more myonuclei for cell fusion and secondary myofiber formation (Zhang et al., 1995; White et al., 2010). Terminal differentiation of myoblasts into myocytes in secondary myogenesis requires the expression of myogenin in myoblasts as shown in myogenin-mutant mice that are born with severe muscle deficiency and die within minutes of birth (Venuti et al., 1995). In the early developmental stages, myogenin-

mutant mice exhibit similar myogenic development in comparison to control mice during primary myogenesis. In secondary myogenesis, however, myogenin-mutant mice exhibit impaired myoblast differentiation during development, thus restricting secondary myofiber formation (Hasty et al., 1993). Secondary myogenesis ends at the conclusion of neonatal myogenesis and is the source of the majority of skeletal muscle fibers present at birth (Tajbakhsh, 2009). The myofibers that form from primary and secondary myogenesis lay the groundwork for future adult myogenesis.

Some of the Pax7 progenitor cells from the central dermomyotome also become satellite cells (Kassar-Duchossoy et al., 2005; Gros et al., 2005). Satellite cells are the main myogenic progenitor cells of adult muscle, identified by their location between the basal lamina and sarcolemma of skeletal muscle fibers in adult skeletal muscle (Mauro, 1961). Satellite cells are mainly identified by their expression of the Pax7 transcription factor (Seale et al., 2000, Sambasivan et al., 2011). As secondary myogenesis occurs, satellite cell precursors migrate from the central dermomyotome and localize under the basal lamina of the secondary myofibers. Studies in mice have shown that satellite cells proliferate and contribute to myonuclei that fuse with existing myofibers to cause myofiber hypertrophy during the neonatal period (White et al., 2010). After the neonatal period, they remain quiescent and become activated and fuse with one another to form new adult skeletal muscle or with existing myofibers during muscle growth or in response to muscle damage (Yin et al., 2013). Upon activation of satellite cells into the myoblast state, transcription factor Pax3 is degraded, and Pax7 becomes dominant. Myogenic regulatory factors Myf5 and MyoD are upregulated in active satellite cells. As

these satellite cells become committed to differentiation, myogenin is expressed which downregulates Pax7. MRF4 is expressed for the fusion and hypertrophy of the new myofibers (Zammit et al., 2006; Boutet et al., 2007; Olguin et al., 2007; Le Grand and Rudnicki, 2007). Normal adult myogenesis mainly consists of myofiber hypertrophy by an increase in cytoplasmic volume by synthesis of sarcomeric proteins or by the fusion of satellite cell myonuclei (White et al., 2010). Satellite cells activate, proliferate and migrate to the damaged myofiber area to fuse with both existing damaged fibers and with each other to form new myofibers. This occurs in response to damage and stress conditions during daily wear or exercise in adult skeletal muscle and contribute to muscle mass. Satellite cells self-renew as they return back to a quiescent state (Collins et al., 2005; Sirabella et al., 2013).

Skeletal Myofibrillogenesis

Skeletal muscle progenitor cells differentiate into myoblasts during primary, secondary, and adult myogenesis and fuse to form mature myofibers. The basic structural units of skeletal muscle myofibers are repeating contractile protein units called sarcomeres (Clark et al., 2002). The four main structural components of sarcomeres are the Z-disks which flank a sarcomere, the thick myosin filament that are comprised by a bipolar complex of myosin II, the polar antiparallel thin actin filament, and titin, which acts as a molecular spring and permanently connects thick filaments to Z-disks and contributes to passive elasticity of skeletal muscle (de Winter and Ottenheijm, 2017; Weitkunat et al., 2017). Multiple sarcomere units consist the myofibril and several

myofibrils form a single myofiber surrounded by the sarcolemma. *In vitro* studies of cultured skeletal muscle cells and cardiomyocytes have led to the three-step model of myofibrillogenesis consisting of the premyofibril stage, nascent myofibril stage, and mature myofibril stage (Sanger et al., 2017; White et al., 2014; LoRusso et al. 1997). This model has also been supported by *in vivo* immunofluorescence of developing zebrafish embryos (Sanger et al., 2009), however, the *in vivo* relevance of such a model in mammalian muscle is unknown.

The premyofibril stage consists of minisarcomeres with Z-bodies enriched with muscle α-actinin marking the general borders between each unit. Non-muscle myosin IIB filaments interlock with F-actin filaments that contain troponins and tropomyosin. The fast-growing barbed ends of the actin filaments are at the Z-bodies of the premyofibrils. Subsequently, CapZ and nebulin appear at the developing thin filaments along the area that forms the I-band. During the nascent myofibril stage, the Z-bodies of adjacent rows of premyofibrils align in parallel and form beaded, discontinuous Z-bands. At this stage, molecules of titin and muscle myosin II filaments begin to appear. As the myofibril transitions to the mature myofibril stage, the beaded Z-bands become continuous Z-bands and nonmuscle myosin II proteins are replaced by muscle specific myosin isoforms. Myosin Binding Protein C (MyBC) bind to adjacent muscle myosin filaments to form the thick filament, and the M-line forms with the addition of myomesin (Sanger et al., 2005; White et al., 2014; Sanger et al. 2017).

However, it must be taken into account that *in vivo* muscle formation is significantly different from *in vitro* cell cultures of contractile skeletal muscle in that *in*

vivo, there is a set polarity of contraction direction and predetermination of myofiber muscle length due to the attachment of muscles to bone via tendons, allowing for specific range and directions of human body movement. *In vivo* studies of myofibrillogenesis have been mostly lacking except for recent studies in the invertebrate *Drosophila* model that have contributed towards an understanding of the overall dynamics of sarcomere assembly and structure that has been initially explored by *in vitro* studies (Weitkunat et al., 2014; Weitkunat et al., 2017).

The in vivo studies of skeletal myofibrillogenesis in *Drosophila* have shown that in addition to molecular events observed *in vitro*, two additional critical steps contribute to the formation of mature muscle. Attachment of myofibers to tendons and mechanical tension, as well as spontaneous Ca^{2+}-dependent twitching of immature myofibrils to form mature striated muscle are critical processes for *in vivo* myofibrillogenesis. Following initial attachment of immature myofibrils to tendons, mechanical tension is required for the simultaneous alignment of the irregular myofibrils that span the entire flight muscle, which eventually mature to form the regular myofibrillar architecture of *Drosphila* indirect flight muscles. This suggests a self-organization mechanism for sarcomeric proteins upon the initial attachment and buildup of muscle tension. The prevention of tendon attachment as well as the severing of tendon-muscle connections upon initial attachment both result in defective myofibrillogenesis (Weitkunat et al., 2014). Additional *in vivo* studies in *Drosophila* abdominal muscle showed that the mechanical tension of immature myofibrils precede the spontaneous Ca^{2+}-dependent contractions of immature myofibrils which is also required for the formation of mature, striated

myofibrils. Chemical inhibition of Ca^{2+}-dependent contractions led to striation muscle development defects (Weitkunat et al., 2017). Similarly, mice lacking ryanodine receptor types 1 and 3, which are involved in Ca^{2+} release in skeletal muscle, exhibit small muscles lacking muscle striations suggesting severe defects in myofibrillogenesis (Barone et al., 1998).

Recent gene mutations affecting human skeletal muscle development and causing myopathies have heightened the importance of studying myofibrillogenesis to gain better insights into disease pathology. Skeletal muscle is the main force-generating tissue of the human body, and from the embryonic developmental stage to the end of life, it undergoes dynamic changes in proliferation, hypertrophy, as well as degradation due to the natural wear and tear of the tissue during activities of daily living. Myoblast fusion and differentiation is critical for the formation of healthy, regular skeletal muscle during development and adult myogenesis. Impaired or delayed myofibrillogenesis is increasingly recognized as the primary pathological mechanism of many skeletal muscle diseases (Shi and Garry, 2006; Gupta et al., 2013; Garg et al., 2014; Crist et al., 2017; Papizan et al., 2017; Jirka et al., under review). Therefore, identification of molecular processes that regulate myofibrillogenesis is critical for the understanding of skeletal muscle disease pathology in human myopathies.

Ubiquitin-Proteasome System

The ubiquitin-mediated proteolytic pathway or the ubiquitin-proteasome system (UPS) is considered to be the major mechanism of protein degradation in eukaryotic cells

(Pickart and Eddins, 2004). This pathway involves the post-translational modification of a target protein via the covalent binding of a single ubiquitin protein or a polyubiquitin chain, which signals for the proteasomal degradation of the target protein (Pickart and Eddins, 2004). In addition to cellular protein homeostasis, the UPS is known to have a role in other proteolytic and nonproteolytic processes such as intracellular signaling, receptor internalization, protein complex assembly, protein localization, DNA repair, cell death, and transcriptional control of gene expression (Popovic et al., 2014). Protein degradation occurs as a normal function of cells to remove unwanted or toxic proteins and is critical for the regulation of many cellular processes in humans and other eukaryotic species (Pickart and Eddins, 2004). Ubiquitination also regulates the function of target proteins by altering their localization and activity (Pickart and Eddins, 2004).

The UPS consists of the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin-protein ligase. The E1 ubiquitin-activating enzyme uses 1 ATP molecule to bind ubiquitin via thiolester linkage thus activating the ubiquitin. The activated ubiquitin is then transferred to the E2 ubiquitin-conjugating enzyme. The E3 ubiquitin-protein ligase forms a complex in which the ubiquitinated E2 interacts with E3 ubiquitin-protein ligase, and E3 brings E2 and substrate together during the transfer of ubiquitin. Ubiquitin is transferred either directly from E2 to substrate, or from E2 to E3 and then the substrate (Bosu and Kipreos, 2008). One E1 enzyme, about 40 E2 enzymes, and greater than 600 E3 enzymes are present in mammals. This suggests that E3 ubiquitin ligases provide specificity and diversity to ubiquitination processes in

multicellular organisms as different proteins are targeted by different E3 ligases (Berndson and Wolberger, 2014).

Within E3 ubiquitin ligase family, Cullin-RING ubiquitin ligases (CRLs) are the largest known category of ubiquitin ligases. Cullin-RING ligases are complexes that include a cullin protein, a RING H2 finger protein, a substrate recognizing subunit (SRS), and an adaptor subunit that connects the SRS to the rest of the complex (with the exception of cullin 3 CRLs which do not have this adaptor). The CRL complex provides a scaffold for substrates such as E2 ubiquitin-conjugating enzymes, ubiquitin, and target proteins to interact with one another to allow for ubiquitination and subsequently cause proteolytic and nonproteolytic processes in the cell (Berndson and Wolberger, 2014). Neural precursor cell expressed, developmentally down-regulated protein 8 (NEDD8) and the constitutive photomorphogenesis 9 (COP9) signalosome complex (CSN) regulate the ubiquitination activity of CRLs by forming a reversible complex with the CRL (Wu et al., 2005; Saha and Deshaies, 2008; Petroski and Deshaies, 2005; Deshaies and Joazeiro, 2009).

Neddylation, which involves the conjugation of ubiquitin-like protein NEDD8 to the lysine of cullin proteins (Wu et al. 2005) is necessary for cullin-organized E3 CRL activities. However, NEDD8 conjugation of cullin not only activates CRL activities but also causes components of the CRL to become targets of proteasomal degradation, mediated by autoubiquitination by the E2 component and an unknown degradation pathway, potentially as a means to terminate E3 ligase activities (Wu et al., 2006). This is where CSN isopeptidase activity steps in, as a deneddylator of cullin 1 (Cul1) and cullin

3 (Cul3). CSN activity not only deactivates the CRL complex but also promotes the stabilization of neddylated cullins and promotes Cul1 & Cul3 E3 ligase activity *in vivo*. This cyclic activity of cullin neddylation and deneddylation helps to recycle cullins, freeing them upon the completion of one task and allowing them to be active in another task thus maintaining their multiple activities or availability to different proteins in the cell. A decrease in deneddylation activity was shown to cause a decrease in E3 activity further supporting the importance of the role of the CSN in CRL activity (Wee et al., 2005; Wu et al., 2006).

E3 ubiquitin ligases, with the aid of specific adaptor proteins that provide binding specificity, are implicated in the transition between skeletal muscle cell types in different points of development to the final mature myofiber stage. The inhibition of E3 CRLs prevents myotube formation (Blondelle et al., 2017). Inhibition of CRL neddylation via the MLN4924 compound (Soucy et al., 2009) or by siRNA against *NEDD8* in murine C2C12 myoblast cells as well as human satellite cells blocks myogenic differentiation (Blondelle et al., 2017). There are five cullin proteins that form E3 cullin-RING ligase complexes: cullin 1, cullin 2, cullin 3, cullin 4, and cullin 5 (Bosu and Kipreos, 2008). Of the five, the Cul3 E3 ligase complex has particularly been studied in skeletal muscle as Cul3 and many BTB-Kelch protein substrate adaptors have been shown as the critical regulators of muscle development and function (Shi and Garry, 2006; Gupta et al., 2013; Garg et al., 2014; Crist et al., 2017; Papizan et al., 2017; Jirka et al., under review). Skeletal muscle-specific knockout of *Cul3* in mice causes severely disorganized sarcomeres, reduction in skeletal muscle mass, physical features indicative of defective

excitation-contraction coupling, and neonatal lethality due to respiratory failure, implicating Cul3 in the skeletal muscle maturation process (Papizan et al., 2018).

Kelch Proteins

Cullin-RING E3 ubiquitin ligases do not interact directly with their substrates and require Kelch adapter proteins for substrate recognition (Deshaies and Joazero, 2009). Kelch proteins are members of the highly evolutionarily conserved Kelch family of proteins (Gupta and Beggs, 2014). The Kelch superfamily consists of 66 known proteins which all contain a Kelch-repeat domain of five to seven Kelch motifs ranging from 44 to 56 amino acids in length (Adams et al., 2000). The superfamily is further divided into three subgroups depending on the presence of different protein domains: the Kelch-like (KLHL), Kelch repeat and BTB domain containing (KBTBD), and Kelch domain containing (KLHDC) subgroups. The KLHL subgroup of proteins is identified by a BTB/POZ domain at the N-terminus, followed by a BACK domain, and a Kelch repeat domain at the C-terminus. The KBTBD subgroup of Kelch proteins only contain a BTB/POZ domain at the N-terminus and a Kelch repeat domain at the C-terminus. Lastly, the KLHDC subgroup of proteins either only contains the Kelch repeat domain or has domain(s) in addition to the Kelch repeat domain that are not BTB/POZ or BACK domains (Stogios and Privé, 2004; Gupta and Beggs, 2014).

Cul3 E3 ligase interacts with BTB-Kelch proteins through BTB/POZ domain of KLHL and KBTBD proteins (Sambuughin et al., 2012; Canning et al., 2013). Mutations in BTB domain-containing Kelch proteins have been implicated in skeletal muscle

diseases caused by mutations in *KLHL40*, *KLHL41*, *KLHL9*, and *KLHL31* of the KLHL subgroup, and *KBTBD13* of the KBTBD subgroup of Kelch proteins (Gupta and Beggs, 2014). This suggests that Cul3 E3 ubiquitin ligase may play critical roles in skeletal muscle disease through ubiquitination of specific target proteins by interacting with different Kelch proteins via the BTB domain.

KLHL41

Kelch like protein 41 (KLHL41), initially known as Kelch-related protein 1 (KRP1) or Kelch repeat and BTB domain containing protein 10 (KBTBD10), is a Kelch protein of the KLHL subgroup (Paxton et al., 2011). KLHl41is a substrate-specific adaptor of the Cul3 E3 ubiquitin ligase that interacts with the Cul3 ubiquitin ligase through the BTB/POZ domain (Zhang et al., 2005; Canning et al., 2013; Dhanoa et al., 2013). KLHL41 is present in striated muscle, with highest levels in adult skeletal muscle compared to cardiac muscle (Ramirez-Martinez et al., 2017). At E10.5 of the embryonic stage of development in mice, KLHL41 has been shown to be highly expressed in the myotome, and at later stages, in skeletal muscle throughout the embryonic development (Ramirez-Martinez et al., 2017). Colocalization studies via immunofluorescence of mouse and human skeletal muscle has suggested that KLHL41 is located in the I-band area in myofibers (Gupta et al., 2013).

KLHL41 has been implicated to have a critical role in myoblast differentiation. KLHL41 is upregulated in murine C2C12 skeletal muscle cells during differentiation (Paxton et al., 2011). This upregulation occurs upon expression of critical myogenic

transcription factor myogenin and precedes MyHC expression which is a marker for thick filament formation. (Paxton et al., 2011; Hong et al., 2016). *In vitro*, nonproteolytic polyubiquitination of KLHL41 stabilizes nebulin and prevents its aggregation (Ramirez-Martinez et al., 2017). An *in vivo* study of *Klhl41* knockout mice found that the absence of KLHL41 results in sarcomere disarray, abnormal protein depositions in muscle, Z-line streaming, and neonatal lethality due to difficulty in suckling and breathing (Ramirez-Martinez et al., 2017). These studies suggest that KLHL41 has a potential role in the stages of muscle development and differentiation processes that lead to the mature myofibril.

Mutations in the *KLHL41* gene have been implicated in the pathophysiology of the rare congenital muscle disease nemaline myopathy (NM), which is a clinically heterogenous skeletal muscle disease. Patients with missense mutations in the gene have a mild to intermediate presentation of NM, with muscle weakness, impaired or absent muscle reflexes and survival into late childhood/early adulthood (Wallgren-Petterson et al., 2011). Patients with null truncating mutations in *KLHL41* have presented with severe NM, with clinical phenotypes including fetal akinesia, lack of antigravity movement, arthrogryposis, dislocation of the hip and knees, and death within the first few months of life due to respiratory failure (Wallgren-Petterson et al., 2011; “Nemaline Myopathy”, National Organization of Rare Diseases; Gupta et al., 2013; Gupta and Beggs, 2014). *KLHL41* mutations in human patients, knockdown of both *klhl41a* and *klhl41b* transcripts in *Danio rerio*, and *Klhl41* knockout mice have all shown myofibrillar disorganization as well as nemaline bodies, abnormal protein aggregates in striated muscle which are a

hallmark of nemaline myopathy (Gupta et al., 2013; Ramierez-Martinez et al., 2017). To date, seven *KLHL41* mutations have been found in human patients (Figure 1).

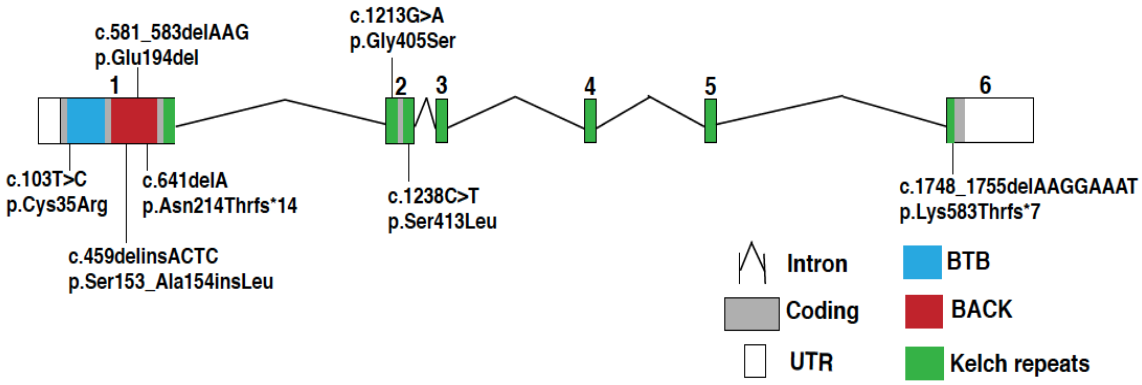

Figure 1: Human mutations in KLHL41. Seven unique missense and truncating mutations in the KLHL4l protein have been found in human patients. (Gupta et al., 2013; van Diemen et al., 2017).

KLHL41 directly interacts with nebulin (NEB), nebulin-related anchoring protein (NRAP), interferon-related developmental regulator 1 (IRFD1) and RNA-binding motif protein 4 (RBM4) as shown by yeast two-hybrid screening of the KLHL41 protein against human fetal and adult skeletal muscle cDNA libraries (Jirka et al., under review). Interestingly, these proteins are implicated in skeletal muscle differentiation and maintenance. Of particular interest, NRAP has been found to be expressed in differentiating skeletal myotubes at the onset of myoblast fusion (Lu et al., 2008).

Nebulin-related anchoring protein

NRAP is a 196 kDa thin filament-binding chaperone protein that has been shown to interact with the KLHL41 protein (Jirka et al., under review). The protein is proposed

to be involved in myofibril assembly. NRAP primarily associates with premyofibrillar structures containing actin and α-actinin in both developing skeletal muscle and cultured murine C2C12 myoblast cells/differentiated myotubes, and is not present in mature myofibrils (Lu et al., 2003; Lu et al., 2008; Manisastry et al., 2009). NRAP is also implicated in myofibrillar assembly in the fetal heart and regulates force transmission from the sarcomere to the extracellular matrix in the adult heart (Truszkowska et al., 2017).

Specific aims

The objective of this research study is to provide an understanding of the role of BTB-Kelch protein, KLHL41, in regulation of ubiquitination in skeletal muscle development. Specific aims of this research thesis include:

1. To investigate the localization of the KLHL41 protein in myoblasts.
2. To explore the role of KLHL41-mediated ubiquitination of thin-filament chaperone protein NRAP during muscle development.
3. To investigate the effect of wild-type and NM causing KLHL41 variants on protein stability and Cul3 E3 ligase activity.

These studies will help to gain a better understanding of the activities of KLHL41 as a substrate-specific adaptor of CUL3 E3 ubiquitin ligase in skeletal muscle development and function.

METHODS

Mammalian cell culture

C2C12 cells are adherent skeletal muscle myoblasts derived from *Mus musculus*. C2C12 cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) containing L-glutamine, phenol red, and sodium pyruvate. 20% fetal bovine serum (FBS) and 1% penicillin-streptomycin-glutamine (PSG) antibiotic solution consisting of 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 29.2 mg/mL of L-glutamine, were added to supplement the growth media. The C2C12 cells were grown at 37°C and 5% CO2 in a humidified cell culture incubator. The *Klhl41* knockout C2C12 cell line was previously made by the Gupta Lab via CRISPR/Cas9 gene knockout targeting exon1 of the *Mus musculus Klhl41* gene.

Transfection

C2C12 cells were trypsinized with 0.25% Trypsin-EDTA, phenol red (Gibco, NY) and then seeded onto a 6-well tissue culture-treated plate at a density of 2.0 x 10^5 cells per well in high glucose DMEM (20% FBS, 1% PSG) growth media. Cells were incubated for 16 hours at 37°C, 5% CO_2. Upon reaching approximately 70% confluency, the cells were transfected using Lipofectamine 3000 (Invitrogen, CA). C2C12 cells were transfected with 2.5 µg of plasmid DNA, using the plasmids listed in Table 1. Transfected cells were incubated at 37°C, 5% CO_2, and cells were harvested after 48 hours. Cell lysates were prepared in Pierce© radioimmunoprecipitation assay (RIPA) Buffer (Thermo Scientific, IL) for western blotting.

Expression vectors

The expression vector plasmids that were used in transfection of the C2C12 myoblast cells were previously constructed by the Gupta Lab and were used for studies described in this work.

Table 1. Expression vectors used for transfection of murine C2C12 muscle cells

Protein	DNA Mutation	Vector Backbone	Tag
KLHL41 (WT)		pEZY	FLAG
KLHL41 (Cys35Arg)	103T>C	pEZY	FLAG
KLHL41 (Ser153_Ala54insLeu)	457delinsACTC	pEZY	FLAG
KLHL41 (Gly405Ser)	1213G>A	pEZY	FLAG
KLHL41 (Ser413Leu)	1238G>A	pEZY	FLAG
NRAP (WT)		pcDNA3.1V5	V5

Cycloheximide Chase Assay

Control and *Klhl41* knockout murine C2C12 cells were plated in 35 mm tissue culture-treated plates at a seeding density of 5.0 x 10^5 cells/dish and incubated for 12 hours at 37°C, 5% CO_2. The growth medium was then replaced with differentiation medium consisting of high glucose DMEM, 1% FBS, and 1% PSG. Differentiation medium was replaced every 24 hours. After 2 days in differentiation medium, medium was replaced with fresh differentiation medium containing 100µg/ml cycloheximide (CHX). Cells were collected at different time points (0 hours, 8 hours, 16 hours, 24 hours) after cycloheximide addition. Cell lysates were prepared and analyzed by Western

blotting. *Klhl41* knockout C2C12 cells were created using CRISPR technology by the Gupta Lab and were made available for use.

Cell lysis

Growth medium was removed from 6-well or 35 mm plates and 75 µL of cell lysis buffer (consisting of 5 mL Pierce© RIPA Buffer (Thermo Scientific, IL) and 1 cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail tablet (Roche, Germany)) was placed into each well/plate. Plates were placed on ice for 5 minutes and swirled occasionally to distribute the lysis buffer. Cells were then scraped and transferred into Eppendorf tubes. Cell lysate samples underwent sonication at amplitude #3 for 1-5 seconds, avoiding foaming (550 Sonic Dismembrator, Fisher Scientific) and then were centrifuged at 13,200 rpm for 15 minutes at 4°C. The supernatants were stored at -80°C for further analysis.

Pierce BCA Protein Assay

The protein concentrations of each sample that was to be used in Western blotting were standardized using a Pierce BCA Protein Assay kit (Thermo Scientific, CA). Known concentrations of Bovine Serum Albumin (BSA) at 2000, 1500, 1000, 750, 500, 250, 125, 25 and 0 µg/mL were used to create a standard reference curve to determine the concentrations of the unknown samples. 10µL of standard samples as well as 2µL of unknown samples diluted with 8µL nuclease free H2O were added to 200 µL of working BCA reagent in separate tubes and incubated at 37°C for 30 minutes. The absorbance of

each known and unknown sample was measured at 562 nm using a Nanodrop 1000 spectrophotometer (Thermo Fisher, CA). The protein concentrations of the unknown samples were calculated using the standard curve created from the BSA standard samples.

Biochemical fractionation

C2C12 cells growing in 100mm tissue culture-treated plates (90% confluent) were trypsinized and centrifuged at 1000 rpm (Thermo Scientific ST 16R, MA) for 5 minutes at room temperature. Supernatant was discarded and the cell pellet was washed in ice-cold 1X phosphate buffered saline (PBS). The cells were then centrifuged at 7500 rpm (Eppendorf Centrifuge 5415D, NY) for 30 seconds at 4°C. 1X PBS was removed and cell pellet was suspended in ice-cold 900 µL 0.1% nonionic polyoxyethylene 40 (NP40) in 1X PBS. Cell suspension were triturated 5 times with P1000. 300 µL of the suspension was collected as the whole cell lysate (WCL) fraction; 100 µL of 4X lithium dodecyl sulfate (LDS) sample buffer (Invitrogen, CA) was added to the WCL fraction, briefly vortexed, and then placed on ice. The remaining 600 µL cell suspension was then spun at 13,200 rpm for 30 seconds at 4°C. 300 µL of the supernatant was collected as the cytoplasmic fraction; 100 µL of 4X LDS sample buffer was added to the cytoplasmic fraction, briefly vortexed, and then placed on ice. The remaining supernatant was then fully removed before resuspending the cell pellet in 1 mL of 0.1% NP40 in 1X PBS by trituration. The suspension was centrifuged at 13,200 rpm for 30 seconds at 4°C. The supernatant was removed, and the nuclear pellet was resuspended in 180 µL of 1X LDS

sample buffer, vortexed, then placed on ice. The WCL, cytoplasmic, and nuclear fraction samples were then sonicated 2 x 5 seconds at 20% amplitude. 6µL 10X Bolt reducing agent (Life Technologies, NY) was added to 54µL of each sample, and then samples were boiled at 95°C for 5 minutes, centrifuged at 14,000 rpm (Thermo Scientific ST 16R, MA) for 5 minutes at room temperature, and then used for western blotting.

Western blot

100µg-170µg of protein samples were analyzed by western blot. 15 µL of 4X Bolt LDS sample buffer and 6µL 10X Bolt reducing agent (Life Technologies, NY) were added to each sample. RIPA buffer was added to each sample to adjust the final volume to 60 µL. Samples were heated at 95°C for 5 minutes. Running buffer was prepared by diluting 20 mL of 20X Bolt MES SDS Running Buffer (Life Technologies, NY) in 380 mL of deionized water. Novex Bolt 4-12% Bis-Tris Plus 10-well pre-cast gels (Invitrogen, CA) were used in a Thermo Fisher Scientific Mini Gel Tank (Thermo Scientific, NC). Each well of the gel was loaded with 60 µL of sample. 10µL of SeeBlue Plus2 Pre-stained Protein Standard (Life Technologies, NY) was loaded as the protein size marker. Electrophoresis was carried out using a BioRad PowerPac Basic Power Supply (BioRad, CA) at 120V for 60-80 minutes, depending on the size of the protein of interest. The separated proteins were then transferred from the gel to an Immun-Blot polyvinylidene difluoride (PVDF) membrane (0.2µm pore size) (BioRad,CA) using the Mini Blot Module wet transfer system (Life Technologies, NY) in transfer buffer

consisting of 50mL 20X Bolt Transfer Buffer (Life Technologies, NY), 100mL methanol, and 850mL deionized water. The transfer was run at 30V for 90 minutes at 4°C.

Upon completing transferring, the PVDF membrane was taken out of the transfer system and blocked in blocking buffer solution consisting of 5% non-fat milk in 1X Tris Buffered Saline (Boston Bioproducts, MA) and 0.1% Tween 20 (Sigma Aldrich, PA) (TBS-T) on a shaker for 1 hour at room temperature. For analysis of the western blot membranes, the following primary antibodies were used for probing: V5 Antibody (Life Technologies, NY), Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam, MA), Anti-FLAG (Sigma Aldrich, MO), Anti-KLHL41 (Sigma Aldrich, MO), and Anti-Cul3 (Abcam, MA). The following corresponding secondary antibodies were used: Goat Anti-Mouse IgG (HL)-HRP (Biorad, CA), Goat Anti-Rabbit IgG (HL)-HRP (Biorad, CA). Antibodies were diluted in blocking buffer solution. Membranes were incubated in primary antibody overnight at 4°C on a shaker, washed 2 x 15 minutes in TBS-T at room temperature on a shaker, and then incubated in secondary antibody for 1 hour at room temperature on a shaker. After the secondary antibody incubation, the membrane was washed 2 x 15 minutes in TBS-T at room temperature on a shaker. The primary and secondary antibody dilutions that were used are listed in Table 2.

Table 2. Antibody pairings and associated dilutions used for western blot immunodetection

Primary Antibody	Secondary Antibody
Anti-V5 (1:1000)	Anti-Mouse (1:5000)
Anti-GAPDH (1:1000)	Anti-Mouse (1:10000)
Anti-KLHL41(1:500)	Anti-Rabbit (1:2500)
Anti-FLAG (1:500)	Anti-Mouse (1:2500)
Anti-UBTF (1:500)	Anti-Rabbit (1:2500)
Anti-LaminA (1:150)	Anti-Mouse (1:2500)
Anti-CUL3 (1:500)	Anti-Rabbit (1:1000)

The western blot membrane was then developed with 3mL of SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific, NC) for 5 minutes and subsequently developed on an x-ray film with a KODAK X-OMAT machine or electronically imaged using GeneSys (Syngene, MD).

In order to probe the same membrane with another primary antibody, the membrane was briefly washed in TBS-T and stripped using the Restore™ Western Blot Stripping Buffer (Thermo Scientific, NC) solution for 30-180 minutes at room temperature on a shaker. The membrane was then washed 2 x 5 minutes in TBS-T and blocked in preparation for another antibody probing, or wrapped in plastic wrap and stored at -20°C.

Band intensity quantification

The relative quantification of western blot protein bands was performed using ImageJ. The band densities of the proteins of interest were normalized against the GAPDH probing of the same samples.

RESULTS

KLHL41 subcellular localization studies in C2C12 proliferating myoblasts

KLHL41 is localized in the nucleus during myoblast proliferation. This sub-cellular localization changes to the cytoplasm during myoblast differentiation. In differentiated mouse FDB myofibers, KLHL41 is localized in the I-band area (Gupta et al., 2013). This raises the question of how the changes in KLHL41 localization regulate diverse functions in skeletal muscle differentiation. As previous localization studies were performed with immunofluorescence, in this study, biochemical fractionation was performed to validate KLHL41 localization in proliferating myoblasts. Biochemical fractionation for cytoplasmic and nuclear fractions was performed in proliferating murine C2C12 myoblast cells. Whole cell lysate (WCL) was used as the positive control in these studies. Western blot analysis of whole cell lysate, nuclear and cytoplasmic fractions indicated that in C2C12 myoblast cells, endogenous KLHL41 is present in the nuclear and WCL fractions but not in the cytoplasm (Figure 2). Upstream binding transcription factor (UBTF) was used as a marker for the nuclear fraction. Biochemical fractionation and western blot analysis of control C2C12 myoblast cells were repeated and different sub-cellular markers were used to validate the localization of KLHL41. LaminA antibody was used as a marker for the nuclear fraction, and GAPDH antibody was used as a marker for the cytoplasmic fraction. Western blot analysis on whole cell lysate and sub-cellular fractions validated that KLHL41 is present in the nuclear and WCL fractions (Figure 3).

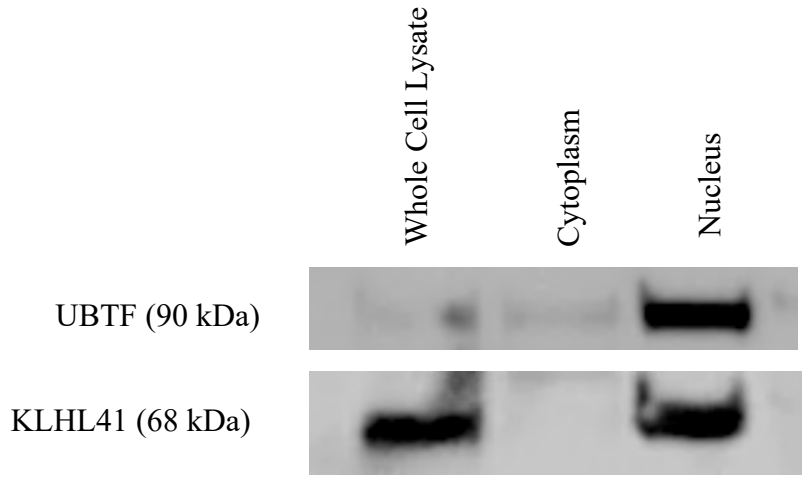

Figure 2. Biochemical fractionation of C2C12 control myoblast cells probed with nuclear fraction marker UBTF. Western blot analysis of fractionated samples. Fractions, antibodies used, and approximate protein size are labeled. KLHL41 is present in the nuclear fraction identified by the nuclear marker, UBTF.

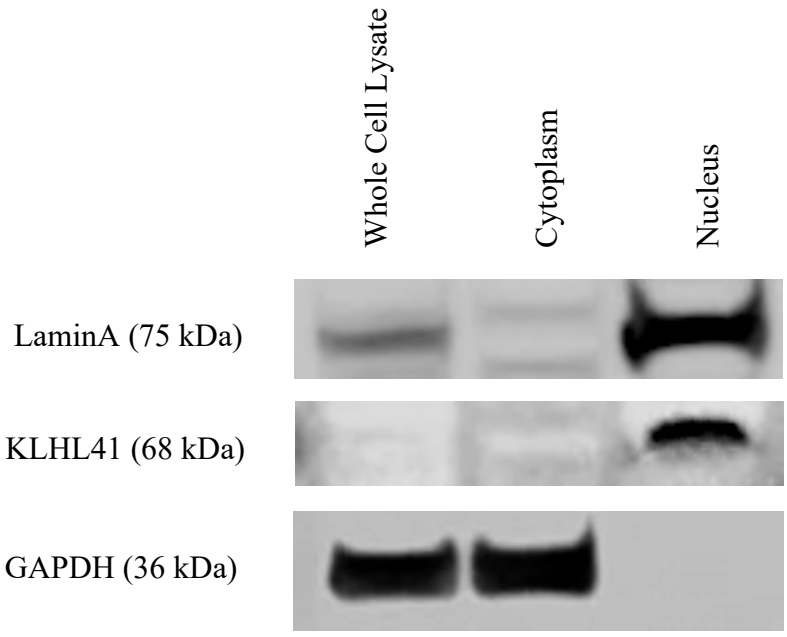

Figure 3. Biochemical fractionation of C2C12 control myoblast cells with nuclear fraction marker Lamin A and cytoplasmic fraction marker GAPDH. Western blot analysis of fractionated samples. Fractions, antibodies used, and approximate protein size are labeled. KLHL41 is present in the LaminA labeled nuclear fraction and absent in the GAPDH labeled cytoplasmic fraction.

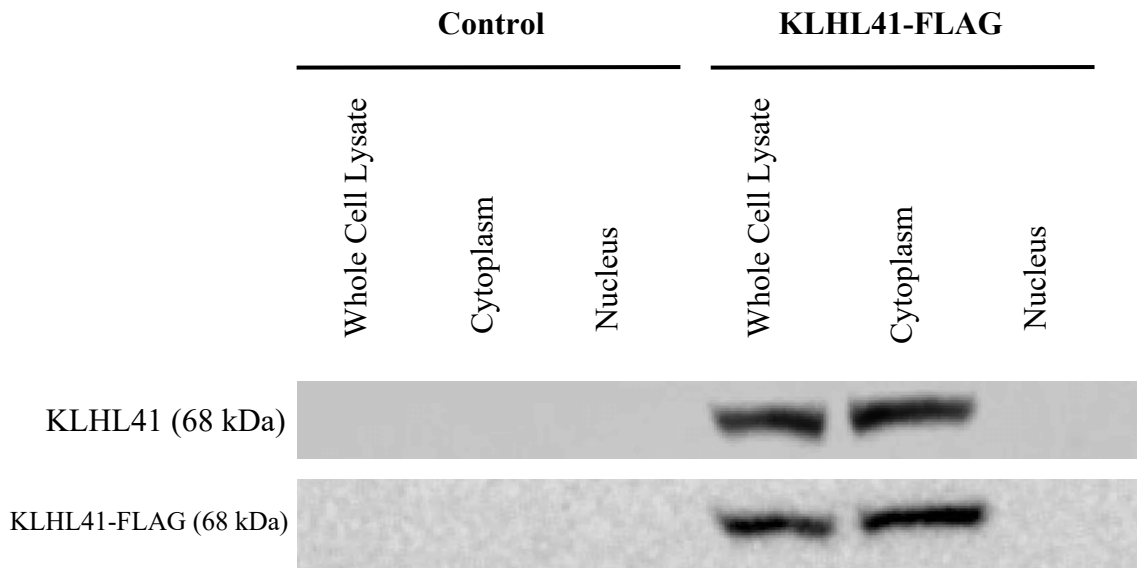

Figure 4. Biochemical fractionation of C2C12 control and KLHL41 (WT)-FLAG myoblast cells. Western blot analysis of fractionated samples. Sample type, fractions, antibodies used, and approximate protein size are labeled.

As these studies utilized antibodies targeting endogenous proteins, in order to rule out any non-specific binding of KLHL41 antibody for endogenous proteins, sub-cellular localization of KLHL41 was also determined by an alternate approach, by exogenous expression of KLHL41 WT tagged with FLAG for detection with an anti-FLAG antibody. Western blot analysis of different fractions from cells transfected with KLHL41-FLAG revealed that exogenously expressed human KLHL41-FLAG protein is present in the cytoplasmic and WCL fractions, and not in the nuclear fraction (Figure 4). These studies demonstrate that endogenously expressed KLHL41 is primarily localized in the nuclei of proliferating myoblasts.

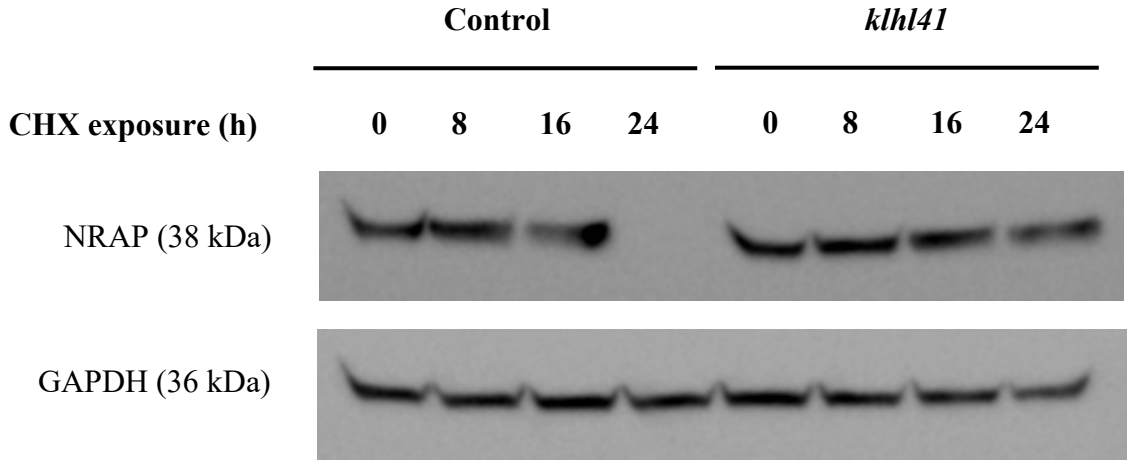

Figure 5. A) Cycloheximide chase assay of C2C12 control and C2C12 *Klhl41* knockout myotube cells. Western blot analysis of cell lysates collected at different hours of exposure to CHX. Sample type, antibodies used, and approximate protein size are labeled.

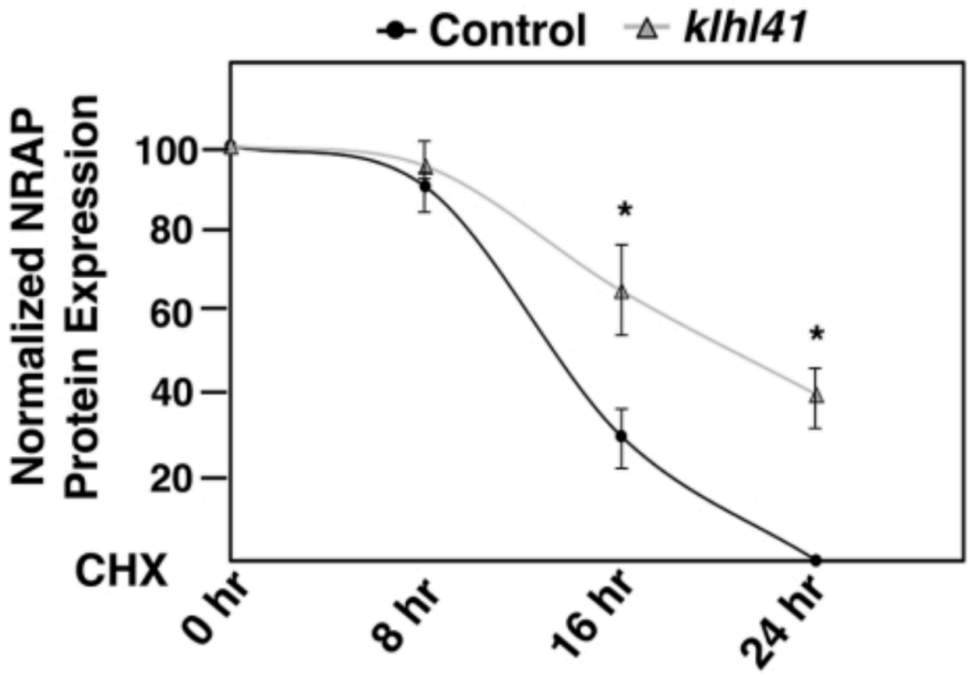

Figure 5. B) Cycloheximide chase assay of C2C12 control and C2C12 *Klhl41* knockout myotube cells. ImageJ quantification of NRAP. NRAP protein expression was normalized to GAPDH and then normalized to t=0h CHX exposure controls. *p<0.01

Effect of KLHL41 on NRAP regulation

Previous studies have shown that NRAP is critical for premyofibril assembly and disappears during myofibril maturation (Lu et al., 2008; Manisastry et al., 2009). As KLHL41 is known to interact with NRAP and regulate its stability through ubiquitination, we investigated the effect of KLHL41 on the stability of NRAP protein in differentiating myotubes (Jirka et al., under review).

To test the effect of KLHl41 on NRAP stability, cycloheximide chase assay was conducted in control C2C12 and *Klhl41*-knockout C2C12 differentiating myotubes. Control or Klhl41 knockout cells were treated with CHX and samples were collected at 0, 8,16, and 24 hours of CHX exposure. Cell lysates were prepared and analyzed by Western blot (Figure 5A). NRAP levels were quantified using ImageJ (Figure 5B). Band intensity of NRAP was normalized to GAPDH and then normalized to t=0h CHX exposure controls. Data are shown as mean±SD. Blots are representative of 3 different experiments (n=3). *p* values were calculated using a two-tailed Mann-Whitney *U*-test. In the absence of endogenous KLHL41, there is decreased protein degradation of NRAP in comparison to control C2C12 myotubes. At 24 hours of cycloheximide exposure, there is no NRAP in the control C2C12 myotubes whereas about 40% of NRAP remains in KLHL41 knockout C2C12 myotubes (Figure 5A). This suggests that KLHL41 contributes to reduced stability of NRAP in differentiating myotubes.

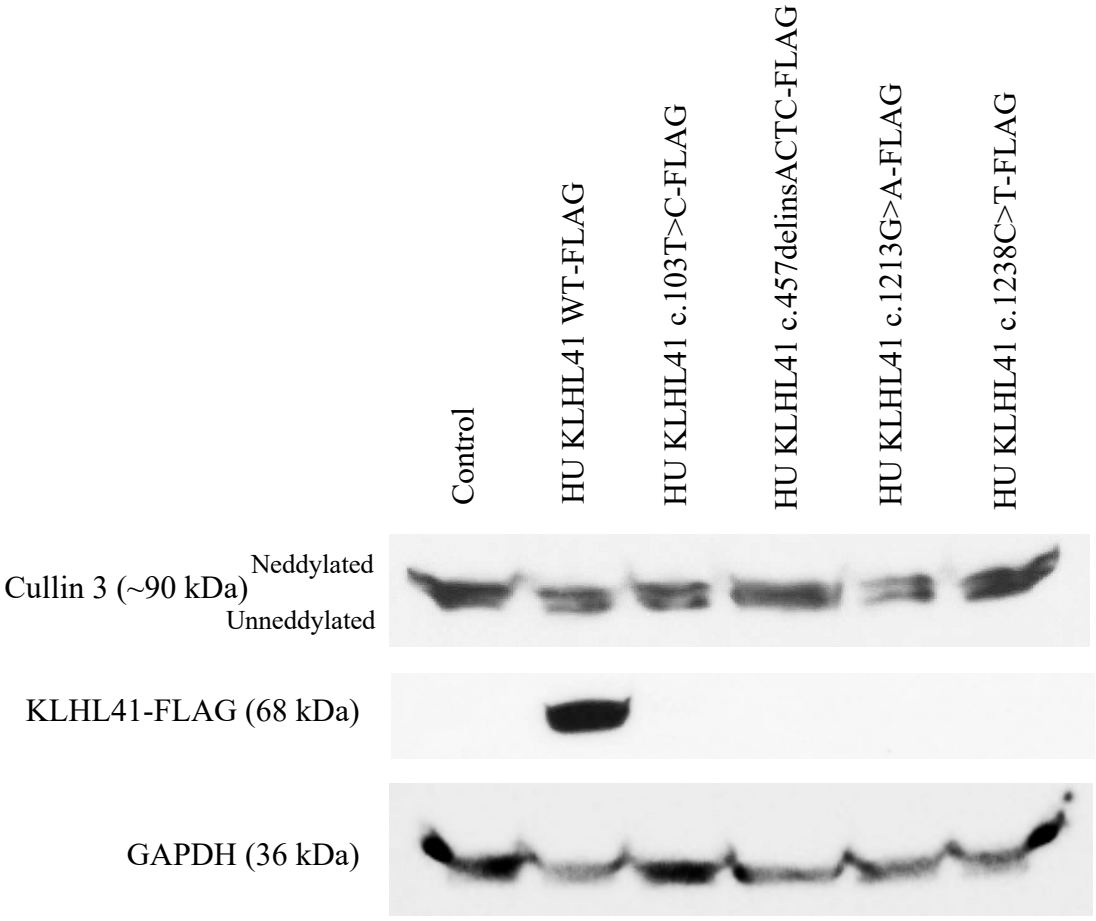

Figure 6. Western blot analysis of C2C12 control and human KLHL41 WT and missense mutation plasmid-transfected myoblasts. Sample type, antibodies used, and approximate protein sizes are labeled. All images were taken from a single western blot membrane. The sample of control C2C12 cells contained 130 μg of protein as detected by BSA protein quantification, whereas the samples of other five C2C12 transfected cells contained 170 μg of protein.

Effect of WT and nemaline myopathy-causing KLHL41 variants on Cul3 E3 ligase activation by neddylation

CUL3 E3 ubiquitin ligase activity is required for skeletal muscle function as skeletal muscle-specific knockout results in neonatal lethality and extensive hypotonia. The E3 ligase activity of cullins is allosterically activated by neddylation via NEDD8, a small ubiquitin-like protein, that is reversed by the COP9 signalosome complex (CSN). Downregulation of either of these protein complexes result in muscle differentiation defects (Ba et al., 2017; Blondelle et al., 2017). Dimerized Kelch adapter proteins such as KLHL41 bind to CUL3 E3 ligase and induce a conformational change that is a prerequisite for binding and activation by NEDD8 protein complex (Boh et al., 2011; Lydeard et al., 2013). We hypothesize that mutations in KLHL41 that abolish or reduce interactions with CUL3 will hinder the activation of CUL3 E3 ligase by neddylation and may cause a decrease in ubiquitination of KLHL41 substrates. To test this hypothesis and study the effect of NM-causing genetic mutations of KLHL41 on the overall stability of KLHL41 and its effect on the Cul3 E3 ligase, C2C12 myoblast cells were used as a model system. Cells were transfected with FLAG-tagged pEZY plasmids containing human *KLHL41* WT or *KLHL41* missense mutations identified in human NM patients. The study only included missense mutations as truncating mutations renders protein unstable (Gupta et al., 2013). Analysis of transfected cells via western blot indicate that the overexpression of WT *KLHL41*-pEZY results in expression of the KLHL41 protein. In contrast, no mutant proteins were detected by Western blot analysis under similar

conditions. This suggests that NM-causing KLHL41 mutations result in a disease phenotype caused by reduced protein levels, therefore a deficiency of KLHL41 proteins.

To further investigate the effect of KLHl41 mutations on Cul3 neddylation, the western blot was reprobed with Cul3 antibody to identify neddylated (active, high molecular weight band) and unmodified (inactive, low molecular weight band) Cul3 protein. Western blot revealed the presence of neddylated Cul3 E3 ligase as well as unmodified Cul3 E3 ligase in untransfected control cells (lower band). The effect of WT and human NM causing KLHL41 mutations was also examined on Cul3 protein levels and neddylation status. C2C12 myoblast cells were transfected with pEZY plasmids expressing FLAG-tagged human KLHL41 WT, KLHL41 c.103T>C, KLHL41 c.457delinsACTC, KLHL41 c.1213G>A, or KLHL41 c.1238C>T.

Control untransfected C2C12 myoblasts showed a higher amount of neddylated Cul3 in comparison to unmodified Cul3, indicating that more of the Cul3 is in an active state. KLHL41 WT-expressing C2C12 myoblasts exhibited equally dense bands of neddylated and unmodified Cul3 indicating that overexpression of KLHl41 results in a decrease in Cul3 neddylation. In addition, lower overall density of both neddylated and unmodified bands of Cul3 E3 ligase were seen in C2C12 myoblasts expressing mutant type KLHL41 except for KLHL41 c.1238C>T. This suggests that the presence of mutant KLHl41 at these positions results in reduced stability of Cul3 ligase.

Interestingly, C2C12 myoblasts transfected with KLHL41 c.457delins_ACTC showed a single broad band spanning the positions of the neddylated and unmodified bands on the SDS-PAGE instead of two distinct neddylated and unneddylated bands of

Cul3 as observed in the other conditions. Finally, C2C12 myoblasts expressing KLHL41 c.1238C>T appears to have a greater amount of the unneddylated form of Cul3 E3 ubiquitin ligase. GAPDH was used as a loading control in this study. In sum, these results demonstrate that different mutations in KLHL41 reduce the neddylation or stability of Cul3 E3 ligase. These studies indicate that reduced neddylation or stability of Cul3 E3 ligase is a likely disease mechanism in the pathogenesis of KLHL41-mediated nemaline myopathy.

DISCUSSION

Localization of KLHL41 in the Nuclear Fraction of C2C12 myoblasts

Previous studies showed that KLHL41 exhibits a dynamic subcellular localization during myoblast differentiation. KLHL41 is localized to the nuclear compartment of myoblast cells and this localization changes to the cytoplasmic fraction on differentiation in to myotubes. In differentiated myofibers, KLHL41 is localized at the I-band area of cultured murine FDB myofibers and human skeletal muscle as shown by immunofluorescence (Gupta et al., 2013). As these experiments were performed with KLHL41 antibodies to detect endogenous expression, we performed follow up studies to validate KLHL41 expression by biochemical fractionation of endogenously expressed KLHL41 proteins or exogenously expressed KLHL41-FLAG in C2C12 myoblasts.

The biochemical fractionation of C2C12 myoblasts conducted in this study confirmed these previous findings that KLHL41 is localized in the nuclear fraction and not in the cytoplasmic fraction. In contrast, exogenous human KLHL41 WT-FLAG expressed in C2C12 myoblasts showed localization in the cytoplasmic fraction but not in the nuclear fraction. As the FLAG tag is fused to the N-terminus of KLHL41, we hypothesize this may interfere with the nuclear localization signal and prevent the transport of KLHL41 into the nucleus. In the same experiment, no detection of endogenous KLHL41 was observed in the nuclear fraction, that could be due to the effect of exogenously expressed KLHL41 on the endogenous protein. KLHL41 proteins form homodimers and homodimerization of the endogenous KLHL41 with KLHL41-FLAG

may also prevent the localization of the endogenous KLHL41 protein to the nucleus. Future studies may be able to validate these insights into KLHL41 localization.

KLHL41 is critical in NRAP degradation

NRAP is required for the maturation of pre-myofibrils to mature myofibrils (Jirka et al., under review). NRAP is present on developing thin filaments during the premyofibril stage, but is absent at the mature myofibril stage (Lu et al., 2003). On the other hand, NRAP interactor proteins such as actin and α-actinin remain as primary components of the mature myofibril (Lu et al., 2008). NRAP also interacts with KLHL41 in developing myofibrils (Lu et al., 2003), however, the implication of this interaction on skeletal muscle development are not fully known (Manisastry et al., 2009; Jirka et al., under review).

Yeast two-hybrid screenings and homogenous time-resolved fluorescence (HTRF) assays showed that KLHL41 protein interacts with nebulin and NRAP protein (Jirka et al., under review). As NRAP protein disappears during myofibril formation, this suggests that the substrate adaptor KLHL41 may interact with NRAP to target the protein for ubiquitination and subsequent proteolysis via the Cul3 E3 ubiquitin ligase. Previous work in the Gupta lab supports this suggestion as the Klhl41 knockout myotubes exhibit a higher accumulation of NRAP protein in comparison to controls suggesting a defect in protein degradation in the absence of KLHL41 (Jirka et al., under review).

CHX, which is a protein synthesis inhibitor in eukaryotes (Schneider-Poetsch et al., 2010), was used in this study to determine the stability of NRAP in the presence of

KLHL41. The CHX chase assay done in this study also revealed that the absence of KLHL41 in C2C12 myotubes lead to reduced degradation of NRAP in comparison to that of control C2C12 control myotubes. This suggests that KLHL41 has a critical role in the degradation of NRAP in myotubes. As NRAP overexpression prevents mature myofibril formation, the degradation of NRAP by KLHL41-mediated proteasomal ubiquitination is a critical step that must be achieved in order for premyofibrils to mature. This data further helps to place NRAP into the narrative of myofibrillogenesis at the premyofibril stage, at which NRAP binds to the developing barbed ends of α-actinin polymers to establish the polarity of thin filaments and help align them to form Z-discs. Following premyofibril formation, NRAP fulfils its role as a scaffold by its ubiquitination and subsequent proteasomal degradation by Cul3 E3 ligase with the help of substrate-specific adaptor KLHL41, while KLHL41 simultaneously also helps nebulin to take the place of NRAP and start to assemble at the I-band and then along the thin filament. In the absence of KLHL41, the progression of premyofibrils to the mature myofibril gets disrupted as the NRAP protein accumulates and binds to other sarcomeric proteins such as nebulin, thus resulting in disorganized, unhealthy skeletal myofiber formation and sarcomeric protein aggregates (Gupta et al., 2013; Ramirez-Martinez et al., 2017).

Effect of Human KLHL41 WT and NM variants on KLHL41 Stability

The preliminary study of the overexpression of FLAG-tagged human KLHL41 WT and KLHL41 missense mutations found in human patients with nemaline myopathy showed expression of WT protein did not show expression mutant KLHL41 proteins,

potentially indicating that the mutations cause reduced stability of the KLHL41 protein. As many missense mutations make proteins susceptible for degradation by proteasomes, repeating this experiment in the presence of proteasome inhibitors (such as MG132) may stabilize the expression of mutant protein and reveal their effect on E3 mediated ubiquitination in skeletal muscle. Another alternative would be to increase the mutant plasmid expression by transfection with a higher amount of plasmid DNA to test if the amount of mutant proteins could be increased.

Effect of Human KLHL41 WT and Mutant Type Overexpression on Cul3 E3 Ligase Activity

In control C2C12 myoblasts, a denser higher molecular weight band of Cul3 is detected, indicating higher amount of neddylated Cul3 in comparison to unmodified Cul3 (lower molecular weight band). As neddylation is required for CRL activities (Wu et al., 2006), this result indicates that Cul3 E3 ligase activity is shifted to a more active state of ubiquitination activity in control C2C12 myoblasts. In KLHL41 WT overexpressed cells, it was intriguing to find that there is a decreased overall amount of Cul3 E3 ligase complexes in comparison to the control, as indicated by equally less dense neddylated and unmodified Cul3 bands in comparison to that of the control C2C12 myoblasts. This may be explained by the potential E3 ligase autoubiquitination activities that may occur to downregulate E3 ligase activity due to the shift to overactivity due to the overexpression of substrate adaptor KLHL41.

Co-immunoprecipitation studies have shown that of the three domains in Kelch-like proteins, the BTB domain is critical in its interaction with the CUL3 protein (Ramierez-Martinez et al., 2017). Of the four human KLHL41 mutations that were used to transfect C2C12 myoblasts, the KLHL41 mutation in c.103T>C is located at the BTB domain of KLHL41. The mutation in c.457delinsACTC is located in the BACK domain, and the mutations in c.1213G>A and c.1238C>T are located at the second Kelch repeat domain from the N-terminal. This makes the results of this study particularly interesting as the mutation in the BTB domain caused similar changes in neddylation of Cul3 as that of the mutation in the BACK domain as well as a mutation of the Kelch repeat. For all mutant KLHL41 transfected C2C12 myoblast samples excluding the KLHL41 c.1238C>T-transfected sample, there appears to be lower amounts of Cul3 E3 ubiquitin ligase, as shown by the less dense bands of neddylated and unmodified Cul3 in comparison to untransfected controls. This may be partially explained by the degradation of mutant type protein seen in Figure 6. Overexpression of WT KLHL41 and absence of KLHL41 mutant proteins lead to reduced CUL3 proteins in both unmodified and neddylated forms. This suggests that CUL3 activity is tightly regulated in cells and any perturbations due to changes in adapter proteins leads to overall reduction in active CUL3.

An exception to the effect of mutant type KLHL41 on Cul3 degradation is seen in the KLHL41 c.1238C>T-transfected sample. The Cul3 bands of this sample appear to be denser, indicating decreased Cul3 degradation in comparison to that of the other mutant KLHL41 samples. In addition, the unmodified band of Cul3 appears to be slightly denser

in comparison to the neddylated Cul3 band. This indicates that the Cul3 E3 ligase may be shifted more to an inactivated than activated state of ubiquitination. As the KLHL41 c.1213G>A mutation is located at the same Kelch repeat as KLHL41 c.1238C>T, the differences seen in Cul3 E3 ligase stability may not be general to a mutation in the Kelch repeat, but may be specific to the specific localization of the Kelch repeat mutation that not only affects the stability of the Cul3 E3 ligase, but also causes greater activity of the CSN in comparison to neddylation activity.

Interestingly, KLHL41 c.457delinsACTC mutation, located in the BACK domain, not only resulted in the possible degradation of Cul3 as indicated by the lower density of the band signal, but also resulted in the lack of two distinct bands of neddylated and unmodified murine Cul3 E3 ligase. This may indicate that there may be multiple neddylation sites on the Cul3 E3 ligase, and that this specific KLHL41 mutation interferes with the complete neddylation of the Cul3 E3 ligase. Intermediate forms of the neddylated Cul3 E3 ligase could lead to a smeared band of Cul3 E3 ligases with different amounts of NEDD8 conjugation. A future direction to take with this data would be to determine the number of neddylation sites of the murine Cul3 E3 ligase using a neddylation site predictor software program (Yavuz et al., 2015), and then calculate whether any of the intermediate neddylated forms would match the protein weights (~90kDa) within the Cul3 smear band. Another potential experiment would be to probe this western blot with NEDD8 antibody to determine whether the NEDD8 localizes throughout the smear band, which would indicate intermediate forms of neddylated Cul3 E3 ligase.

The study of skeletal myofibrillogenesis is critical for the greater understanding of skeletal muscle disease pathophysiology. This study may have elucidated the characteristics of a few protein interactions in the skeletal muscle, but it even more so displayed the great complexity and unknowns of the skeletal muscle development and regulation. However, by continuing to take on the challenging endeavor of determining how sarcomeric proteins properly align and develop healthy, regular striated muscle in early development and even in adult myofibrillogenesis, targeted therapeutics may someday be developed against the myopathies that directly interfere with this highly intricate process.

LIST OF JOURNAL ABBREVIATIONS

Am J Hum Genet	American Journal of Human Genetics
Am J Physiol	American Journal of Physiology
Anat Embryol (Berl)	Anatomy and Embryology (Berlin)
Anat Rec (Hoboken)	Anatomical Record (Hoboken)
Annu Rev Biochem	Annual Review of Biochemistry
Annu Rev Cell Dev Biol	Annual Review of Cell and Developmental Biology
Biochem Biophys Res Commun	Biochemical and Biophysical Research Communications
Biochim Biophys Acta - Mol Cell Res	Biochimica et Biophysica Acta - Molecular Cell Research
BMC Dev Biol	BMC Developmental Biology
BMC Pharmacol Toxicol	BioMed Central BMC Pharmacology and Toxicology
Cardiovasc Res	Cardiovascular Research
Cell Div	Cell Division
Cell Motil Cytoskeleton	Cell Motility and the Cytoskeleton
Curr Opin Cell Biol	Current Opinion in Cell Biology
Dev Dyn	Developmental Dynamics
EMBO Rep	European Molecular Biology Organization Reports
Exp Cell Res	Experimental Cell Research
Exp Mol Med	Experimental and Molecular Medicine
FEBS Lett	Federation of European Biochemical Societies Letters

Genes Dev	Genes and Development
Hum Genomics	Human Genomics
Int J Dev Biol	The International Journal of Developmental Biology
J Biol Chem	The Journal of Biological Chemistry
J Catechexia Sarcopenia Muscle	Journal of Cachexia, Sarcopenia and Muscle
J Cell Sci	Journal of Cell Science
J Clin Invest	The Journal of Clinical Investigation
J Intern Med	Journal of Internal Medicine
J Mol Biol	The Journal of Molecular Biology
J Muscle Res Cell Motil	Journal of Muscle Research and Cell Motility
J Neuromuscul Dis	Journal of Neuromuscular Diseases
J Pathol	The Journal of Pathology
Mol Cell	Molecular Cell
Nat Cell Biol	Nature Cell Biology
Nat Med	Nature Medicine
Nat Rev Mol Cell Biol	Nature Reviews. Molecular Cell Biology
Physiol Rev	Physiological Reviews
Proc Natl Acad Sci	Proceedings of the National Academy of Sciences
Sci Rep	Scientific Reports
Semin Pediatr Neurol	Seminars in Pediatric Neurology
Skelet Muscle	Skeletal Muscle

Trends Biochem Sci	Trends in Biochemical Sciences
Trends Cell Biol	Trends in Cell Biology

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