UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of Cullin-RING Ligases During Myogenesis

A Thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

by

Paige Shapiro

Committee in Charge:

Professor Stephan Lange, Chair
Professor Deborah Yelon, Co-Chair
Professor Eric Bennett

2016

The Thesis of Paige Shapiro is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2016

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to acknowledge Professor Stephan Lange for his support as the chair of my committee. His guidance in writing and generally throughout the program have been instrumental in my journey in research.

I would also like to acknowledge Jordan Blondelle of the Lange Lab, for sharing his project with me. He challenged me to think critically and to become independent in the lab. His knowledge and support as a friend and mentor have been invaluable.

Lastly, I would like to thank all of the Lange Lab and Department of Cardiology for their assistance and encouragement in my project.

The data in this thesis is currently being prepared for submission for publication. Blondelle, Jordan; Shapiro, Paige; Lange, Stephan. The thesis author was the secondary investigator and author of this material.

ABSTRACT OF THE THESIS

The Role of Cullin-RING Ligases During Myogenesis

by

Paige Shapiro

Master of Science in Biology

University of California, San Diego, 2016

Professor Stephan Lange, Chair

Cullin-RING ligases (CRLs) are one of the main families of E3 ligases in the Ubiquitin-Proteasome degradation system, which is responsible for the degradation of ~80% of muscle proteins. CRL substrate adaptors have been established as players in muscle atrophy and nemaline myopathies. This led to us to investigate whether CRLs are important for muscle development. C2C12 muscle cells were treated with MLN4924, an inhibitor of CRL activation. Treatment at the start of myoblast differentiation showed a defect in myotube formation due to an absence of cell commitment to the differentiation program, indicating that CRLs are important for early myogenesis. We obtained similar results for satellite cells isolated from mouse skeletal muscles, confirming the absolute necessity of CRL activity for normal myogenesis. The inhibition of CRL activity also led

to a defect in acetylcholine receptor aggregation. Similarly, a receptor defect has also been observed in CRL-linked nemaline myopathies, again indicating a possible role for these E3-ligases in the disease etiology. Currently, MLN4924 is used as a cancer drug that is undergoing clinical trials. However, cancer patients typically experience cachexia, or muscle wasting, which would likely be further exacerbated by the use of MLN4924. We observed a rescue of the MLN4924 induced differentiation-blockage several days after removal of the inhibitor. We also found that treatment with 5'Aza'dC, a drug that stimulates Myogenin expression, alleviates the phenotype. Our data indicate significant roles for CRLs during muscle differentiation as well as negative side effects for the use of MLN4924 in patients.

INTRODUCTION

Muscle formation

Skeletal muscles are essential components of our body and represent 30-40% of the body weight (Janssen et al, 2000). They are required for both voluntary and involuntary movements such as walking and breathing (McComas, 1996). In order to form mature skeletal muscles, muscle stem cells (also called satellite cells) differentiate into myoblasts (mononucleated muscle cells) that subsequently fuse together to form multinucleated myotubes or myofibers (Constanzo, 2002). This whole process is regulated by the sequential activation of transcription factors CD34, Pax7, Myf5, MyoD, and Myogenin (Figure 1; Buckingham, 2001). Myotubes then start to express sarcomere-associated proteins, like cross-striated muscle specific actin and myosin that are required for the formation of sarcomeres, which then organize into myofibrils (Constanzo, 2002). In parallel to myogenesis, synaptogenesis takes place, where myofibers are stimulated by neural agrin, a proteoglycan secreted by motor neurons that stimulates acetylcholine receptor (AChR) clustering (Figure 1; Sanes and Lichtman, 1999). Skeletal muscles are in a constant state of equilibrium between hypertrophy (growth) and atrophy (wasting away), reflecting the balance between protein synthesis and degradation at the molecular level (Hoffman and Nader, 2004). Protein synthesis is required for the growth of muscle, or hypertrophy (Lecker et al., 1999). Because muscles are constantly damaged, for example in exercise, protein degradation is important for the removal of damaged proteins resulting in muscle atrophy (Lecker et al., 1999). Defects in protein degradation can lead to accumulation of misfolded proteins or premature degradation of necessary

proteins, leading to muscle diseases such as congenital myopathies (Schiaffino et al., 2013). Protein degradation systems are known to be important for homeostasis and are increasingly studied in the context of muscle formation, maintenance, and function (Stewart et al, 1999).

Protein Degradation Systems

Muscle cells contain two major systems for protein degradation: the autophagy-lysosome and the ubiquitin-proteasome systems (UPS), of which the UPS is responsible for the degradation of ~80% of muscle proteins (Attaix and Taillandier, 1998; Sandri, 2013). The UPS consists of 26S Proteasomes that recognize proteins tagged with K48-linked poly-ubiquitin chains resulting in their degradation. The tagging of protein substrates by ubiquitin occurs via a three-step enzymatic cascade, E1-E3 (Figure 2; Lecker et al, 1999). First, the E1-Ubiquitin Activating Enzyme (Uba1) forms a thio-ester bond with ubiquitin through an ATP-dependent reaction, which also activates the ubiquitin. Once activated, ubiquitin is transferred to the E2-Ubiquitin Conjugating Enzymes, of which there are about 40, who then carry the ubiquitin to the cellular E3-Ubquitin ligase enzymes. In the last step, one of the hundreds of E3-Ubiquitin ligase enzymes both recruits a specific substrate, and transfers the ubiquitin from the E2 enzyme to the substrate. Once poly-ubiquitinated, the substrate is targeted to the proteasome for degradation (Lecker et al, 1999). Each E3-ligase has typically one or more specific protein substrates, most of which remain unknown (Bonaldo and Sandri, 2013).

Cullin Proteins

Cullin-RING (*Really Interesting New Gene*) ligases (CRLs) are one of the largest E3-Ubiquitin families (Bosu and Kipreos, 2008). Zhou and colleagues described that the Cullin protein family, which consists of 7 independent and ubiquitously expressed members (Cullin1, 2, 3, 4a, 4b, 5 and 7), provides the structural backbone or scaffold for the functioning E3-ligase complex (Zhou et al, 2013; Sarikas et al, 2011). In addition to Cullin, the fully functional CRL also includes an adaptor protein that recruits substrates and a ligating enzyme (Rbx) that attaches to the E2 Conjugating enzymes. CRLs are also modified by Nedd8, a ubiquitin-like modifier, which is important for the activation of the complex.

The activation of Nedd8 itself is analogous to the UPS pathway. Nedd8 is first adenylated by ATP and the Nedd8 Activating Enzyme (NAE). Nedd8 is then transferred to a cysteine on NAE to form a NAE-Nedd8 thio-ester. Nedd8 is transthiolated to Ubc12, which acts as an E2 enzyme, before it is ultimately conjugated to the Cullin protein of the then functional CRL (Soucy et al, 2010). The small molecule inhibitor MLN4924 was discovered to bind to Nedd8 and block the activation by NAE (Brownell et al, 2010; Hakenjos et al, 2011). This subsequently inhibits activation of CRLs and provides a method for their study. This along with the finding that substrates of CRLs play a role in cell cycle progression (Soucy et al, 2009), and that Cyclin E is a CRL3 substrate that is required for the passage from the G_1 phase to the S phase of the cell cycle (Singer et al., 1999), has led to the intensive study of the role of Cullin E3-ligases in the context of cancer. In fact, MLN4924 is currently in clinical trials as a potential cancer treatment

(Bhatia et al, 2016). However, in the last decade, there has been an emerging role for CRLs in muscles.

Substrate adaptors and Muscle Disease

The role of CRLs in muscle is beginning to be defined by the discovery that CRL1 mediates muscle atrophy via its substrate adaptor MAFbx/Atrogin-1 (Bodine, 2001; Gomes et al, 2001). Despite these advancements in Cullin-RING Ligase characterization, little is known about their role in muscle development. While Cullin proteins are ubiquitously expressed, their substrate adaptor proteins that link CRLs to specific substrates are expressed in a tissue specific manner (Zhou et al, 2013). Especially for skeletal muscles it emerged recently that several putative substrate adaptors of Cullin-3 have been implicated in the development of myopathies (Cirak et al, 2010; Sambuughin et al, 2010). One such family of CRL substrate adaptors are the BTB/Kelch protein families (e.g. KLHL, KBTB, or KLHDC) that are characterized by their BTB/POZ-domain (Bric-a-brac, Tramtrac, and Broad complex; Poxvirus and Zinc finger) that specifically interact with the Cullin-3 E3-ubiquitin ligase (Geyer et al, 2003; Gupta and Beggs, 2014). However, we cannot say that all Kelch proteins are Cullin-3 interactors, since some proteins with these domains have been shown not to interact with Cullin-3 (Ji et al, 2015).

Neuromuscular Junction

Clustering of acetylcholine receptors (AChRs) is an important part of muscle development as they allow the myofiber to receive signals from motor neurons via the

neuromuscular junction (NMJ) (Vrbová, 1978). During myogenesis, agrin released from motor neurons binds to the receptor tyrosine kinase MuSK (Muscle Specific Kinase) on the membrane of the muscle cell, stimulating its phosphorylation. This signals rapsyn to stimulate clustering of AChRs into a midline also called the motor endplate (Sanes and Lichtman, 1999). C2C12 myotubes treated with agrin mimic the formation and clustering of AChRs, allowing *in vitro* investigations (Wallace, 1991). In the fully formed myofiber acetylcholine binds to AChRs on the muscle cell membrane to stimulate contraction (Constanzo, 2002).

Nemaline myopathy is a neuromuscular disease affecting skeletal muscles, in which symptoms include muscle atrophy and muscle weakness, which is also commonly seen in other muscle diseases (Ryan, 2001). One characteristic of this disease is that young patients are particularly susceptible to respiratory failure, implicating skeletal muscles that directly affect breathing: the diaphragm and intercostal muscles (Ryan, 2001). Interestingly, mutations in several putative substrate adaptors for Cullin-3, such as KLHL40, KLHL41, and KBTB13 were associated with nemaline myopathy (Ravenscroft et al, 2013; Gupta and Beggs, 2014). Importantly, the striking beneficial effect of the acetylcholinesterase inhibitor treatment in a patient affected by KLHL40-related nemaline myopathy, suggests that Cullin targeted protein turnover could be important for NMJ function (Benito et al, 2015).

Summary

In this thesis, we investigated the role of CRL activity on early phases of myogenesis by treating differentiating C2C12 myoblasts with MLN4924, an NAE inhibitor, which leads to a subsequent abrogation of CRL activity. We treated C2C12 cells at different stages of differentiation, and with various concentrations of MLN4924 to understand how CRL activity is important for muscle differentiation. These data were verified using primary cultures of mouse skeletal muscle stem cells. We also tested the effect of MLN4924 on the formation of acetylcholine receptors in C2C12 cells, as neuromuscular junction formation is an important component for muscle differentiation and contraction. Finally, we attempted to rescue defects in muscle differentiation upon MLN4924 treatment, in order to gain insight into the molecular mechanisms that could be disrupted by the loss of CRL activity during myogenesis. Ultimately, our data will provide insights into the important roles that CRLs play for myogenesis and muscle cell development.

MATERIALS AND METHODS

Cell Culture

Cell culture and reagents C2C12 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Corning Life Sciences, Tewksburry, MA) containing 4.5g/l glucose, supplemented with 15% fetal bovine serum (FBS, Gemini Bio-Products, Sacramento, CA) and penicillin/streptomycin (Cellgro). Cells were maintained at 37°C in a saturated humidity atmosphere containing 5% CO2. Differentiation of C2C12 myoblasts was triggered when cells reached 70% of confluency, by replacing FBS with 2% horse serum (Lonza BioWhittaker, Basel, Switzerland). The fusion index was calculated as the mean number of nuclei per myotube at day 5 of differentiation. Myotubes were defined as sarcomeric myosin-heavy chain or α-actinin positive cells containing at least 2 nuclei.

Mouse primary myoblasts were obtained by dissection of hindlimb muscles from 1 month-old mice. Muscles were digested in Collagenase B-Dispase II (Roche, USA) solution for 30 minutes at 37°C. Cell suspension was filtered through a 75µm cell strainer, resuspended in Ham's complete medium (Ham's F-10 media, (Corning Life Sciences, Tewksburry, MA), 20% FBS, 2% penicillin-streptomycin (Cellgro) and bFGF (5ng/mL; Invitrogen, Carlsbad, CA) and pre-plated on uncoated dish for 2 hours at 37°C with 5% CO2. Supernatant was collected and plated on 0.2% gelatin (Sigma, Saint-Louis, MO)-coated dish and cells were incubated at 37°C with 5% CO2. Ham's complete

medium was refreshed every 2 days. Differentiation of cells was achieved with 2% horse serum (Lonza BioWhittaker, Bazel, Switzerland).

MLN4924 compound was purchased from Millenium (Cambridge, MA), redissolved in DMSO and stock solution was stored at a concentration of 1mM at -20°C. 5-aza-dC (Aza) was purchased form Fisher Scientific and redissolved in DMSO. Working solutions were prepared by dilution of stock solution into DMSO culture media. Media with MLN4924 inhibitors was refreshed every 48 hours. Control cells were treated with a corresponding concentration of DMSO.

90KDa recombinant rat agrin compound was purchased from R&D Systems (Minneapolis, MN), reconstituted in sterile PBS containing 0,1% BSA. AChR clustering was induced by overnight incubation with 5ng/mL recombinant rat agrin.

Extraction of total RNA, RT-PCR and RT-qPCR analysis

Cells were washed with PBS, then lyzed using TRIzol reagent (Thermo Fisher). RNA was extracted through the phenol-chloroform technique according to the manufacturers instructions. Purity of RNAs was assessed by a ratio of absorbance at 260nm and 230nm >1.7. 200ng of RNA was used for reverse transcriptase reaction using the Maxima First Strand cDNA Synthesis kit (Fermentas). cDNA were amplified using Syber green (Quanta Biosciences).

Immunofluorescence and brightfield microscopy

Cells were rinsed one time with PBS, fixed for 15 minutes with 4% paraformaldehyde/PBS and rinsed 3 times with PBS. Cells were then permeabilized for 10 minutes in 1xPBS supplemented with -0.21% Triton X-100 (Sigma), washed 3 times with PBS and incubated with PBS-5% BSA (Sigma) in blocking solution (Gold Buffer [GB, 150mM NaCl, 20mM Tris pH7.4] supplemented with 1% BSA) for 1 hour at room temperature before incubation with primary antibodies. Primary against sarcomeric anti-sarcomeric Myosin heavy chain (clone 1025, DSHB, 1:400), α-actinin (clone EA-53, VWR), anti-Desmin (Santa Cruz Biotechnology, 1:500), diluted in blocking solution. Fluorescently labeled bungarotoxin (1/200) was either incubated for 2 hours at room temperature, or overnight at 4°C. Following incubation, cells were washed three times for 5 minutes with PBS, and incubated with secondary antibodies diluted into blocking solution for 1 hour at room temperature. Secondary antibody mixtures also contained DAPI (4', 6'diamidino-2-phenylindol) and/or fluorescently linked α-bungarotoxin (BGTX; Molecular Probes) when appropriate. After washing three times with PBS for 5 minutes, cells and revealed using secondary Alexa488-linked donkey anti-rabbit (1:200) and Cy3-linked donkey anti-mouse (2:200) antibodies. Slides were mounted using fluorescent mounting medium (Dako). Confocal microscopy was performed using an Olympus FV1000 confocal microscope equipped with a 20x air, and a 40x and 63x oil immersion objectives, and zoom rates between 1 and 3, and in sequential scanning mode (Waltham, MA).

For brightfield microscopy, fixed or unfixed cells were imaged using an Olympus IX70 inverted microscope, equipped with a digital camera and SPOT imaging software (Diagnostic Instruments, Inc, Sterling Heights, MI).

Staining and Quantification of AChR in C2C12 myotubes

After 5 days of differentiation, myotubes were treated with MLN4924 compound or DMSO for one day then incubated with recombinant rat agrin overnight at 37°C in 5% CO2. Treated cells with recombinant rat agrin were fixed in PFA 4% for 10 minutes. Quantification of AChR expressed at the myotubes surface was determined by incubating fixed cells with FITC-conjugated bungarotoxin for one hour at room temperature. Confocal microscopy was performed as described before. Area, number and size of AChR were assessed using ImageJ (1.48v) software.

Western blot analysis

Cells were washed in PBS and put in lysis buffer (150mM NaCl, 50mM Tris-HCl pH8, 0,1% Triton-100, 1x Complete EDTA-free protease inhibitor (Roche), 1x PhosStop phosphatase inhibitor (Roche)) for 20 minutes on ice. Lysates were sonicated and spun for 10 minutes at 10000g. After quantification of protein concentration using Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Whaltham, MA), 5 to 15µg of clarified lysates were separated by SDS-PAGE. Transfer was performed on nitrocellulose membranes (BioRad). Membranes were incubated with blocking solution (TBS-Tween containing 5% BSA) for 1 hour at room temperature, then incubated with antibodies against Cullin1 (Sigma Aldrich), Cullin3 (Sigma Aldrich, or gift from J. Singer), Nedd8

(Cell Signaling), Myosin heavy chain (clone 1025, DSHB), Myogenin (clone F5D, DSHB), or anti-GAPDH (Santa Cruz Biotechnologies) overnight at 4°C. After washing, anti-mouse or anti-rabbit secondary HRP-linked antibodies (DAKO or Cell Signaling) were applied for 1 hour at room temperature. After washing, antibody-bound proteins were visualized on X-ray films using Supersignal West Pico Chemiluminescent kit (Thermo Scientific) on X-ray films (Genesee). Quantification was performed using ImageJ software.

Statistical analysis

Significance was assessed using the two- tailed student's t-test in Excel. Data are expressed as mean ± standard error of the mean, and differences were considered statistically significant when P-value < 0.05.

RESULTS

We set out to investigate the role that Cullin-RING ligases (CRLs) play for muscle differentiation. In our immunoblot analyses of differentiating C2C12 muscle cells we observed a surprising increase in Nedd8 at 80kDa that corresponds to the molecular weight of neddylated Cullin-1 and Cullin-3 proteins, suggesting a potential role for CRLs during early myogenesis (Figure 3). To investigate the role of CRLs during muscle differentiation further, we utilized MLN4924, an inhibitor of the Nedd8 Activating Enzyme (NAE) that leads to subsequent CRL inaction. C2C12 cells treated with MLN4924 showed decreased myotube formation compared to controls (Figure 4A). We verified that Nedd8 (CRL activator) and CRL activity was decreased in cells treated MLN4924 by looking at the neddylation state of several Cullin proteins relevant for muscle biology, such as Cullin-1 and Cullin-3 (Figure 4E). Immunoblot analysis with a Nedd8 antibody revealed a decrease in a band corresponding to the molecular size of neddylated Cullin-1 and Cullin-3 proteins, confirming the efficacy of the inhibitor on Nedd8 activation. Also, Nedd8 mRNA levels decreased in treated cells as shown by RT-PCR (Figure 4F). Differentiating C2C12 cells showed decreased myotube formation when MLN4924 was added at earlier time points (day 1, compared to day 2 and day 3) of the differentiation program (Figure 4B). We quantified this defect by counting the number of nuclei per myotube (also called fusion index) (Figure 4C). Cells treated with MLN4924 showed a 75% decrease in the fusion index. We also looked at the distribution of myotubes with a set number of nuclei (Figure 4D). Treated cells showed an increase in the number of myotubes that contained very few nuclei per myotube (2-3 nuclei per

myotube). Generally, cells treated at later time points showed a slight alleviation in the effect of MLN4924 on differentiation: e.g. an increase in fusion index compared to those treated at the onset of differentiation (Figure 4B-4D).

In order to confirm the relevance of our data obtained in the C2C12 cell line for muscle development, we isolated satellite cells from 3 month-old mice. Satellite cells were then treated with MLN4924. Similar to C2C12 cells, treated primary cultures of satellite cells also showed almost no myotube formation compared to vehicle treated controls (Figure 5A). Immunoblot analysis shows that neddylation (upper band) of CRL1 is diminished in MLN4924 treated cells, which corresponds to markedly reduced expression levels of sarcomeric myosin heavy chain (MyHC; Figure 5B).

In order to verify a dose-dependent effect of the inhibitor, and to try and find a threshold for the effects observed with MLN4924, we performed a dose-response analysis. C2C12 cells were treated with 30nM, 130nM, 230nM or 330nM MLN4924 at the onset of differentiation. We observed an inverse correlation of decreased myotube formation in brightfield (Figure 6A) and immunofluorescence (Figure 6D) images of differentiated C2C12 cells with higher concentrations of MLN4924. Our data were quantified through fusion index analysis (Figure 6B). Analysis of Nedd8 and sarcomeric Myosin Heavy Chain (MyHC) protein expression levels decreased as the concentration of MLN4924 increased (Figure 6C), suggesting that decreased differentiation is attributed to decreases in CRL activity.

Defects in myotube formation can be attributed to deficiencies in multiple processes during myogenesis. An early issue could be an abnormal commitment of the cells into the muscle cell differentiation program. In order to test if cells entered normally into the myogenic program, we tested the expression of early expressed myogenic transcription factors, such as Myogenin, as well as mature myotube markers such as sarcomeric myosin heavy chain (MyHC) or α-actinin in the presence of MLN4924. Cells treated with 330nM MLN4924 showed decreased amounts of cells expressing MyHC and a-actinin in immunofluorescence (Figure 7A and 7B). There was also a decrease in MyHC at the mRNA level as seen by RT-PCR analysis (Figure 7E). Cells treated with MLN4924 also showed decreased amounts of the muscle specific transcription factor Myogenin at both protein and mRNA levels (Fig 7C and 7D).

Next, we tested the effect of MLN4924 on acetylcholine receptor formation and clustering. In order not to interfere with myogenesis, we treated myotubes with the inhibitor 5 days after the onset of differentiation. Upon treatment with agrin in conjunction with MLN4924 at day 5 of differentiation, myotubes showed a decrease in number and length of acetylcholine receptors per mm^2 (Figure 8A, 8E, 8F). However, cells did not show a decrease in the number of receptor clusters per myotube (Figure 8D). Both treated and control cells had about the same myotube length (Figure 8B) and number of nuclei per myotube (Figure 8C).

We wondered if the effects of MLN4924 treatment on differentiating C2C12 muscle cells were irreversible, or whether myoblasts exposed to the inhibitor regain their ability to differentiate after removal of the compound. In order to test the 'reversibility' of the defects observed during myogenesis, we removed the inhibitor 5 days after start of differentiation, and continued to culture C2C12 cells without MLN4924. 'Rescued' cells resumed their differentiation and formation of myotubes (Figure 9A). 'Rescued' C2C12 cells showed similar numbers of myotubes as controls at Day 5 of differentiation, and about half as many as controls at Day 10 (Figure 9B). Cells allowed to resume differentiation had an increase in MyHC and neddylation compared to cells at 5 days of MLN4924 treatment (Figure 9C). The MyHC expression levels were quantified to show relative percentage of MyHC. Cells that resumed growth without MLN4924 had about 80% expression levels compared to untreated controls (Figure 9D).

Expression of the muscle specific transcription factor Myogenin was found to be severely affected by the loss of CRL activity in our previous data (Figure 7C). We were interested to understand if the absence of Myogenin was partially responsible for the observed phenotype. Treatment of C2C12 cells with Azacitidine (5'Aza'dC), a DNA methyltransferase inhibitor, was shown to induce Myogenin expression in this myoblast cell line (Oikawa et al, 2011; Heidt et al, 2007). Hence, in order to attempt to rescue the phenotype of the cells treated with MLN4924, we administered 5'Aza'dC in parallel. C2C12 cells treated with 5'Aza'dC along with MLN4924 at the onset of differentiation showed a modest but significant increase in myotube formation compared to those treated with MLN4924 alone (Figure 10A). We quantified these data, revealing that cells treated

with 5'Aza'dC and MLN4924 showed a slight but significant increase in the measured fusion index (Figure 10B). We also analyzed the distribution of myotubes with a set number of nuclei in cells treated with 5'Aza'dC and MLN4924, and found a distribution between that of controls and cells treated only with MLN4924 (Figure 10C). Treatment of cells with 5'Aza'dC led to increased expression of MyHC without increasing the neddylation state of 80kDa proteins as expected (Fig 10D).

DISCUSSION

We found that Cullin-Ring E3-Ubquitin Ligase (CRL) activity is required for the differentiation of myoblasts. C2C12 skeletal muscle cells and primary cultures of mouse satellite cells with deficient CRL activity were unable to form myotubes. We verified that the effects were caused by MLN4924 by showing that the extent of myotube formation was dose-dependent. Treated C2C12 cells also showed a decrease in myogenic transcription factor Myogenin and markers of mature muscles like sarcomeric Myosin Heavy Chain (MyHC), and α-actinin, as well as defects in acetylcholine receptor (AChR) clustering. Furthermore, we showed that effects of MLN4924 can be reversed with removal of the inhibitor, or partially rescued by global DNA demethylation.

One of our key findings was that C2C12 cells treated with MLN4924 at early time points during differentiation showed less myotube formation than those treated at later time points. Moreover we noticed a strong decrease in the expression of myogenic factors like Myogenin. These data suggest that CRL activity is required for the commitment of cells into the myogenic program. Because MLN4924 inhibits all CRL activity, our data do not allow us to determine which Cullin-based complexes play a major role in early myogenesis. However, CRL1 and CRL3 were shown to be implicated in muscle health and formation via their substrate adaptors. CRL1 is the best characterized in muscles for its role in muscle atrophy mainly through its substrate adaptor MAFbx/Atrogin-1, though little is known about its role in myogenesis (Bodine, 2001; Gomes et al, 2001). Although little is known about the role of CRL3 for muscles, the Cullin-3 protein is highly

expressed in muscle tissues and putative substrate adaptors have been linked to muscle disease (Blondelle and Lange, 2015). The emerging role of several putative substrate adaptors for Cullin-3 in muscles lead us to hypothesize that the strong defect we observed may be partially due to loss of CRL3 activity, and guided our investigation of the link between CRL activity and adaptor proteins

The Kelch and BTB family of proteins are thought to be substrate adaptors of Cullin-3 and a few like KLHL11 and KCTD6 have been confirmed, but one instance has shown that this is not always the case (Ji et al, 2015). Even so, what is known about some of the Kelch proteins seems to mirror our findings that CRL activity is necessary for muscle development and function. For example, KLHL40 (KBTBD5), KLHL41, KLHDC1, and KLHDC2 have been shown to modulate differentiation of myoblasts (Gupta and Beggs, 2014), though none of these caused such a dramatic and early defects in muscle cells as we see with overall loss of CRL activity. Indeed overexpression or down-regulation of these proteins interferes with myogenesis, but it is not known what substrates may be affected, and if loss or gain in substrate selectivity is responsible for the phenotype. Here we show that it is the complete loss of CRL activity that leads to the blockage of differentiation and not only varying expression in substrate adaptor proteins as shown previously.

Further defects in the KLHL40 gene are also tied to the hereditary and congenital neuromuscular disease nemaline myopathy, whose symptoms include muscle weakness and hypoventilation (Ravenscroft et al, 2013; Ryan, 2001). Recently, there was a report

of an improvement of the clinical conditions of a patient affected by a KLHL40-related nemaline myopathy by treatment with an acetylcholinesterase inhibitor, which blocks the degradation of acetylcholine (Benito et al, 2015). This suggests an involvement of neuromuscular junctions (NMJs) in the pathogenesis. When we tested acetylcholine receptor (AChR) clustering in C2C12 cells treated with MLN4924 and agrin, which recapitulates one important aspect of NMJ formation, we noticed subtle but significant defects in cluster length and density (cluster/mm^2). However, there were no significant changes in myotube length or number of clusters per myotube. This indicates that MLN4924 treatment at this stage of differentiation results in defects in AChR cluster formation, but not in AChR expression, as we observed similar quantities of clusters per myotube. For example, receptors in control myotubes had increased aggregation/clustering of receptors in a row, whereas MLN4924 treated cells had receptors spaced all over the myotube. Because we see this clustering defect of receptors after only one day of treatment, this suggests that loss of CRL activity due to MLN4924 is swift and pronounced. These changes may be aggravated *in vivo*, where additional processes like AChR linkage to motor neuron endplates, or alignment of AChR along the myofibers contribute to the successful formation of functional NMJs (Sanes and Lichtman, 1999).

Despite advances in CRL research in muscle, CRLs have been mainly studied in the context of cell cycle progression and cancer (Soucy et al, 2009; Soucy et al, 2010) Recently, MLN4924 was used in clinical trials for the treatment of cancer in order to arrest the cell cycle (Bhatia et al, 2016). However, our data revealed a striking effect of

this very same inhibitor on muscle development. Of note, most cancer patients experience cachexia, or muscle wasting (Tan and Fearon, 2008), This cachexia has been linked to defects in muscle regeneration (Ametller, 2011), suggesting deficiencies in myogenesis in cancer patients. Because our use of MLN4924 leads to a strong defect in myotube formation, it is likely that muscle wasting in patients taking this drug would be further exacerbated. This led us to investigate whether this phenotype could be remedied. After treating C2C12 cells with MLN4924 for 5 days at the start of differentiation and then removing MLN4924 for another 5 days, we saw that myotubes began to reform. MLN4924 seemed to cause a state of 'hibernation', as myotube formation was consistent with control cells allowed to differentiate for 5 days. This indicates that MLN4924 does not kill the muscle stem cells, and that CRL activity may not be important for cell survival, while also suggesting that potential muscle side effects of patients using MLN4924 may be reversible.

We also noticed that MLN4924 treatment of differentiating C2C12 cells caused a dramatic reduction in Myogenin expression. This muscle-specific basic-helix-loop-helix transcription factor is characterized as one of the key myogenic factors, responsible for the expression of Adamts5, Alcam, CD24a, Hdac11, Itga4, Ptgis, Sema3D, Socs3, Ckmt2, Itgb1bp3, Mef2c, Spp1 (Meadows et al, 2008). The Myogenin promoter is sensitive to methylation, and increased methylation leads to decreased expression of Myogenin (Faralli and Dilworth, 2012). We reasoned that differentiation of myoblasts would be enhanced by adding 5'Aza'dC, a global DNA demethylator that would also affect the methylation status of the Myogenin promoter. A demethylated Myogenin

promoter would stimulate transcription of the Myogenin gene, and increase Myogenin protein levels. Myotube formation in cells treated with MLN4924 and 5'Aza'dC was slightly but significantly increased. This suggest that at least a part of the phenotype observed in absence of CRL activity may be directly due to the absence of Myogenin gene induction. While it further indicates that MLN4924 effects in muscle may be remedied *in vitro*, this is not an accurate estimate for use in *in vivo* models, as 5'Aza'dC would disrupt other cell processes via demethlyation and not just in myoblasts.

Although these data provide the earliest and strongest instances at which CRL inactivity leads to defects in myogenesis, there is still much to discern about the role of CRLs in muscle formation. More studies should be done looking at the roles of individual CRLs for muscle formation and function. Verifying substrate adaptors of CRLs and finding their specific substrates will also provide more characterization of CRL activity in muscle. We also suggest that patients using MLN4924 should be closely monitored, since cachexia has been linked to death in cancer patients (Evans et al, 2008).

Our own data and observations in the literature indicate that Cullin-3 and its adaptors play important roles in myogenesis and muscle maintenance. Further studies in our lab will investigate *in vivo* models for the loss of Cullin-3 in skeletal muscles, and identify substrates and substrate adaptors for Cullin-3.

The data in this thesis is currently being prepared for submission for publication. Blondelle, Jordan; Shapiro, Paige; Lange, Stephan. The thesis author was the secondary investigator and author of this material.

FIGURES

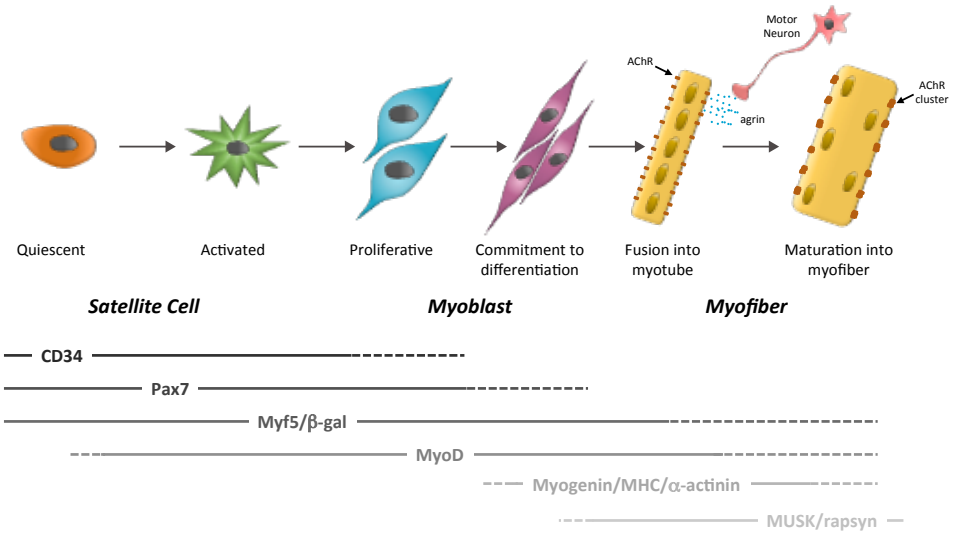

Figure 1. Myogenesis of Mammalian Muscle. Adapted from Zammet et al, 2006 and Sanes and Lichtman, 1999.

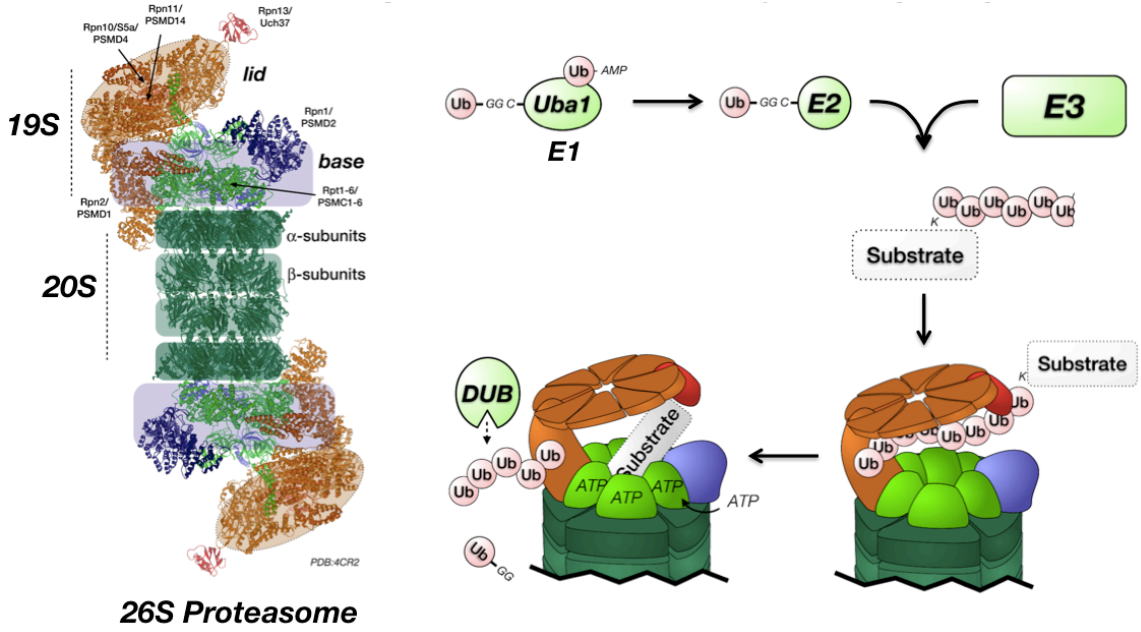

Figure 2. Ubiquitin-Proteasome System. Adapted from Blondelle and Lange, 2015.

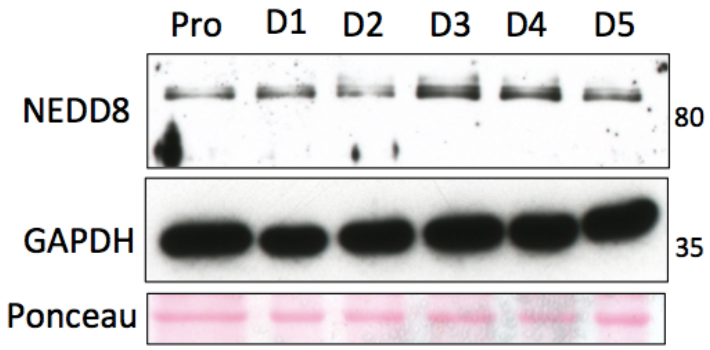

Figure 3. Nedd8 levels increase during differentiation of C2C12 cells.

Immunoblot analyses of Nedd8 expression levels of C2C12 cells in proliferation (Pro), after 1 day in differentiation medium (D1), 2 days in differentiation medium (D2), 3 days in differentiation medium (D3), 4 days in differentiation medium (D4), or 5 days in differentiation medium (D5). GAPDH and Ponceau were used as loading controls.

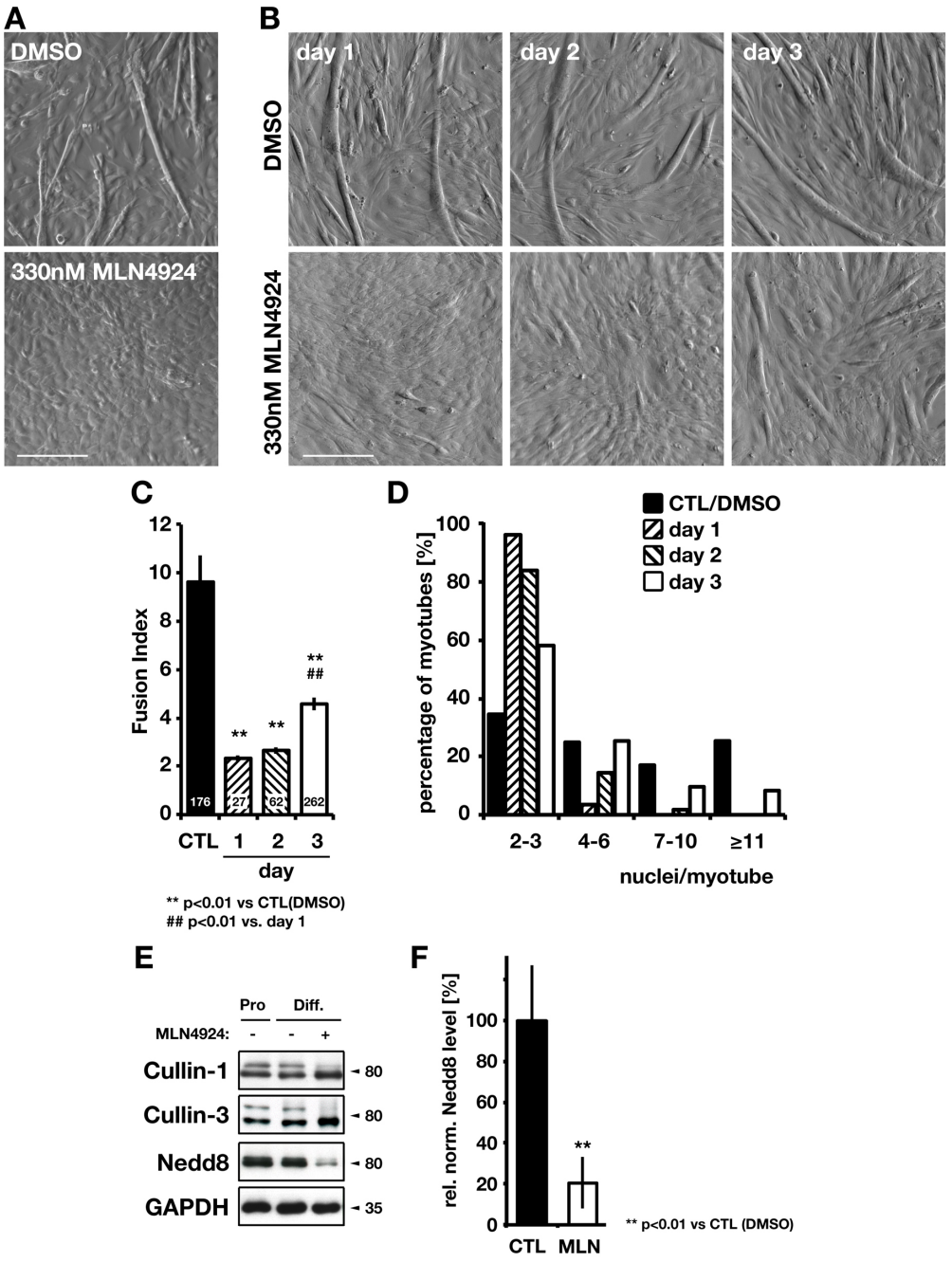

Figure 4. Cullin-RING ligase activity is required for myotube formation in C2C12 cells.

A & B) Brightfield images of C2C12 cells after 5 days in differentiation medium. Cells were treated with vehicle control (DMSO) or 330nM Nedd8 Activating Enzyme inhibitor

(MLN4924) from the onset of differentiation (A), or one, two or three days after start of differentiation (B). Scale bar = 40μm. C) Quantification of fusion index for C2C12 cells exposed after one, two or three days at start of differentiation with 330nM MLN4924, or vehicle control (DMSO). D) Percentage of myotubes with 2-3 nuclei, 4-6 nuclei, 7-10 nuclei, or more than 11 nuclei per myotube, from cultures exposed to 330nM MLN4924 after one, two or three days of differentiation, or vehicle control (DMSO). E) Immunoblot analyses of CRL-1 (Cullin-1), CRL-3 (Cullin-3), and Nedd8 expression levels of C2C12 cells in proliferation (Pro), or after 5 days in differentiation (Diff) medium with 300nM MLN4924 (+) or vehicle control (DMSO, -). Activated CRL complex is indicated by the upper band while inactive CRL is the lower band. GAPDH was used as loading control. F) RT-PCR of Nedd8 in MLN4924 treated C2C12 cells five days after differentiation compared to controls (CTL). P-values are indicated in figure. Sample size is three separate samples.

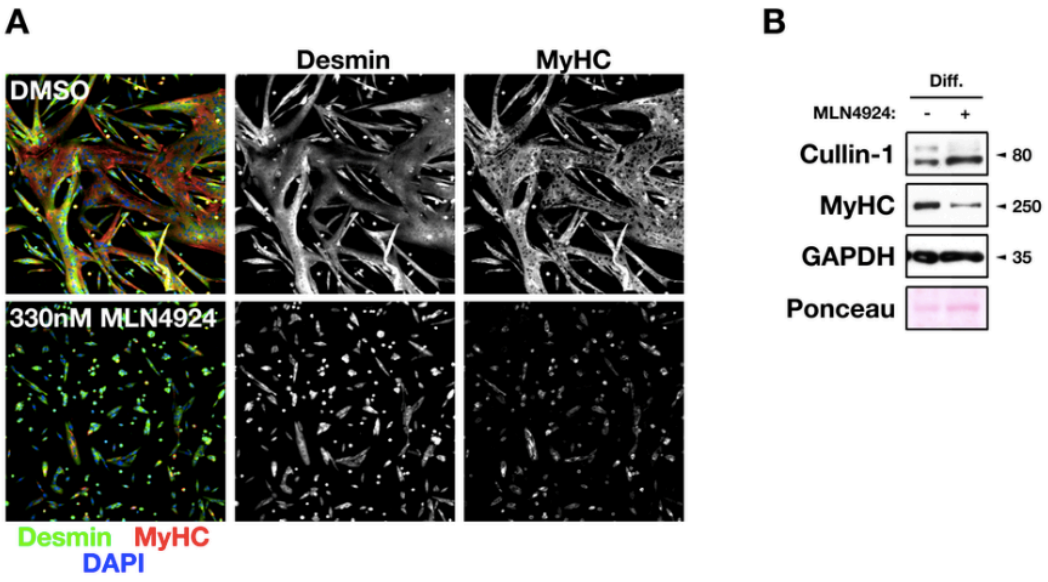

Figure 5. Cullin-RING Ligase activity is required for myotube formation of mouse muscle stem cells.

A) Immunofluorescence analysis of primary cultures of mouse muscle stem cells after 5 days of differentiation. Cells were differentiated in presence of 330nM MLN4924 or vehicle control (DMSO), and stained with antibodies against desmin and sarcomeric myosin heavy chain (MyHC). DAPI was used as counterstain. Scale bar = 100µm. B) Immunoblot analysis of cullin-1 and sarcomeric myosinheavy chain (MyHC) protein levels in total lysates of differentiated primary mouse muscle stem cell cultures, treated with 330nM MLN4924 (+) or vehicle control (DMSO, -). GAPDH and ponceau stained actin band were used as loading controls.

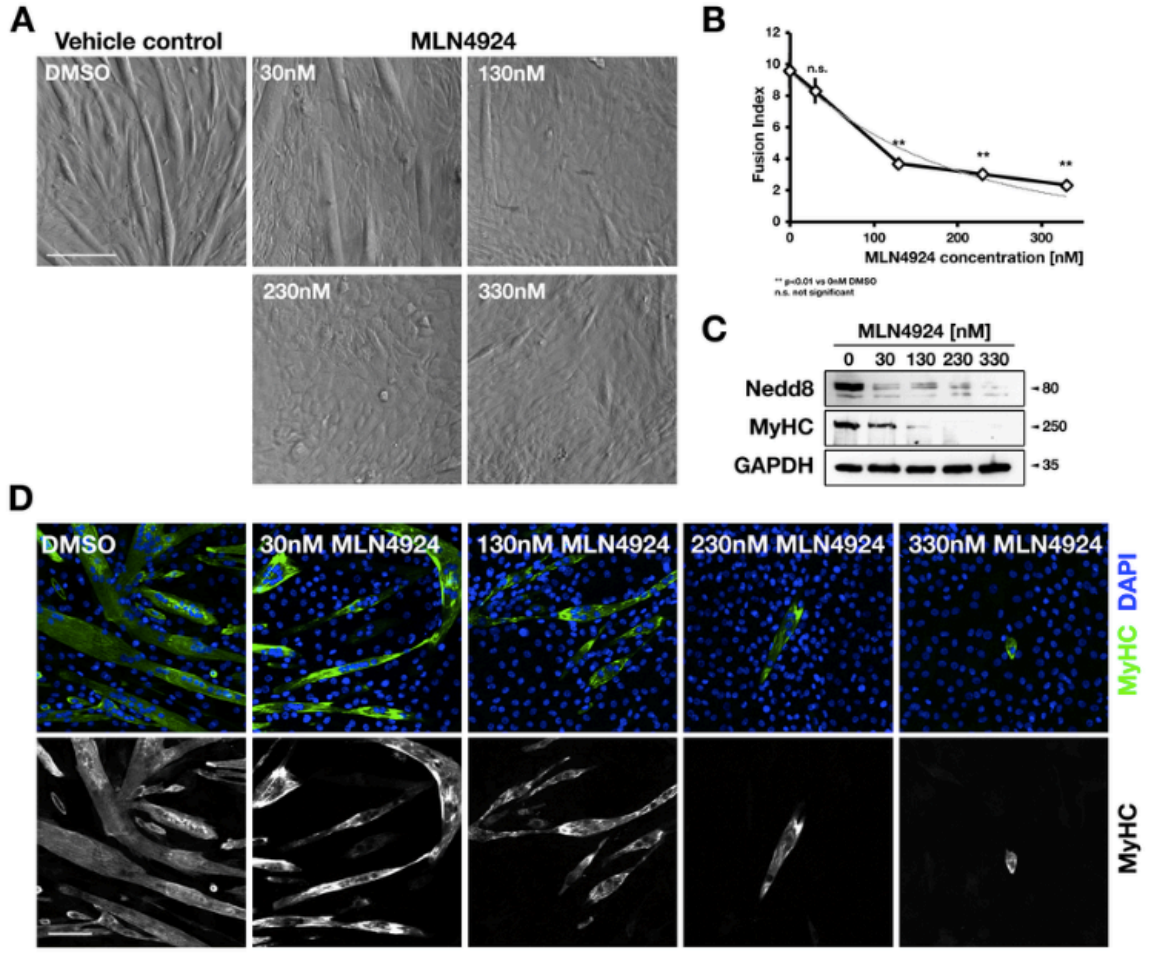

Figure 6. Dose-response curve of C2C12 fusion index with increasing MLN4924 concentrations.

A) Representative brightfield images of C2C12 cells five days after differentiation in presence of 30nM, 13065nM, 250nM 230nM or 330nM MLN4924, or vehicle control (DMSO). Scale bar = 40μm. B) Quantification of fusion index in response to increasing MLN4924 concentrations. Shown are average fusion indices, standard errors and p-values (indicated in figure). Grey line indicates fitted curve to determine IC50 value. C) Immunoblot analysis of Nedd8 and sarcomeric myosin heavy chain (MyHC) protein

levels in total protein samples of C2C12 cells differentiated for 5 days in presence of 30nM, 130nM, 230nM or 330nM MLN4924, or vehicle control (0nM MLN4924; DMSO). GAPDH was used as loading control. D) Immunofluorescence analysis of C2C12 cells differentiated for 5 days in presence of 30nM, 130nM, 230nM or 330nM MLN4924, or vehicle control (DMSO), with an antibody against sarcomeric myosin heavy chain (MyHC). DAPI was used as counterstain. Scale bar = 100μm.

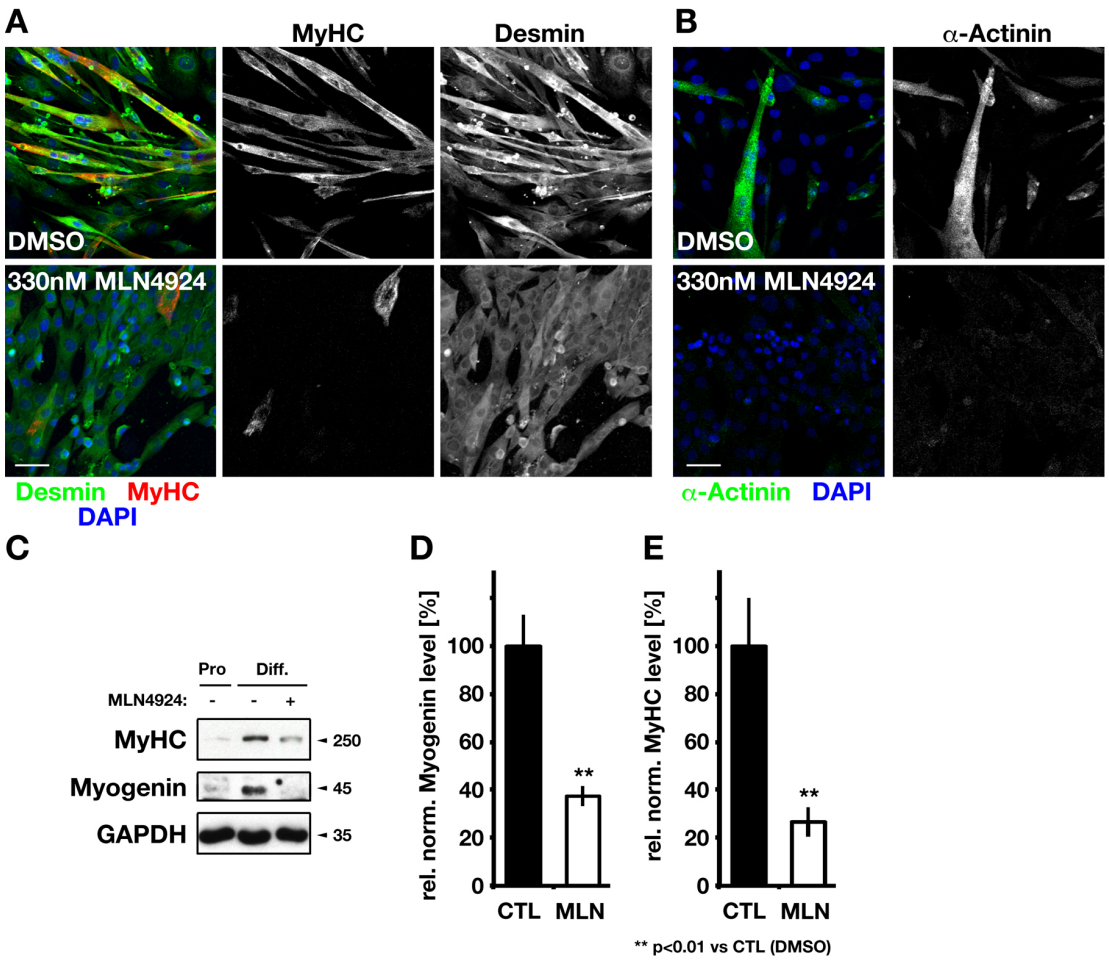

Figure 7. MLN4924 inhibits entry into the myogenic program.

A & B) Immunofluorescence analysis of C2C12 cells differentiated for 5 days in presence or absence of 330nM MLN4924, stained with antibodies against sarcomeric myosin heavy chain (MyHC) and desmin (A), or α-actinin (B). DAPI was used as counterstain in A and B. Scale bar = 50μm. C) Immunoblot analysis of sarcomeric myosin heavy chain (MyHC) and myogenin expression in whole cell lysates of C2C12 cells either in proliferation (Pro), or after 5 days in differentiation (Diff) medium with 300nM MLN4924 (+) or vehicle control (DMSO, -). GAPDH was used as loading

control. D-E). RT-PCR analysis of mRNA levels of Myogenin (D) and sarcomeric myosin heavy chain (MyHC, E) in MLN4924 (MLN) treated C2C12 cells five days after differentiation compared to controls (CTL). Three independent samples were run per group; p-values are indicated in the figure.

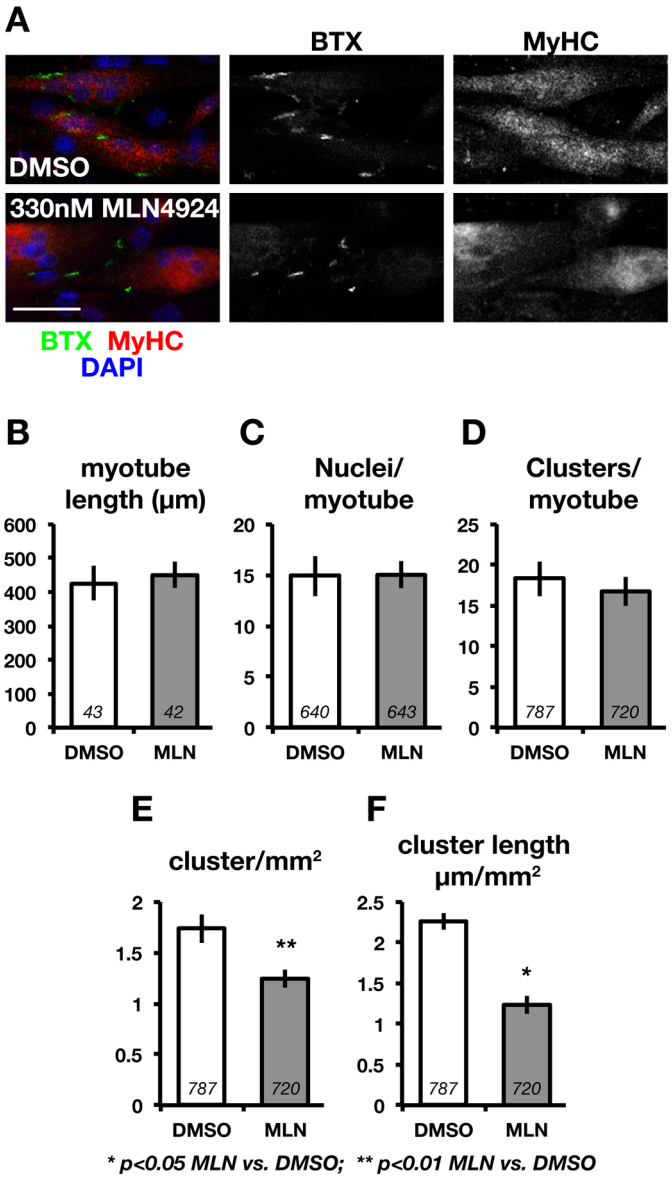

Figure 8. Effect of Cullin-RING Ligase inhibition on agrin-induced acetylcholine receptor clustering in C2C12 cells.

A) Immunofluorescence analysis of agrin treated C2C12 cells 5 days after differentiation in presence of 330nM MLN4924 or vehicle control (DMSO). Cells were stained with

antibodies against sarcomeric myosin heavy chain (MyHC), and alexa488-labeled α-bungarotoxin to visualize acetylcholine receptors. DAPI was used as counterstain. Scale bar = 50μm. B-F) Quantitative analysis of myotube length (B), fusion index (nuclei/myotube; C), number of acetylcholine receptor clusters per myotube (D), acetylcholine receptor cluster number per mm^2 (E) and cluster length per mm^2 (F). Shown are averages and standard errors. Sample size and p-values are indicated in the figure.

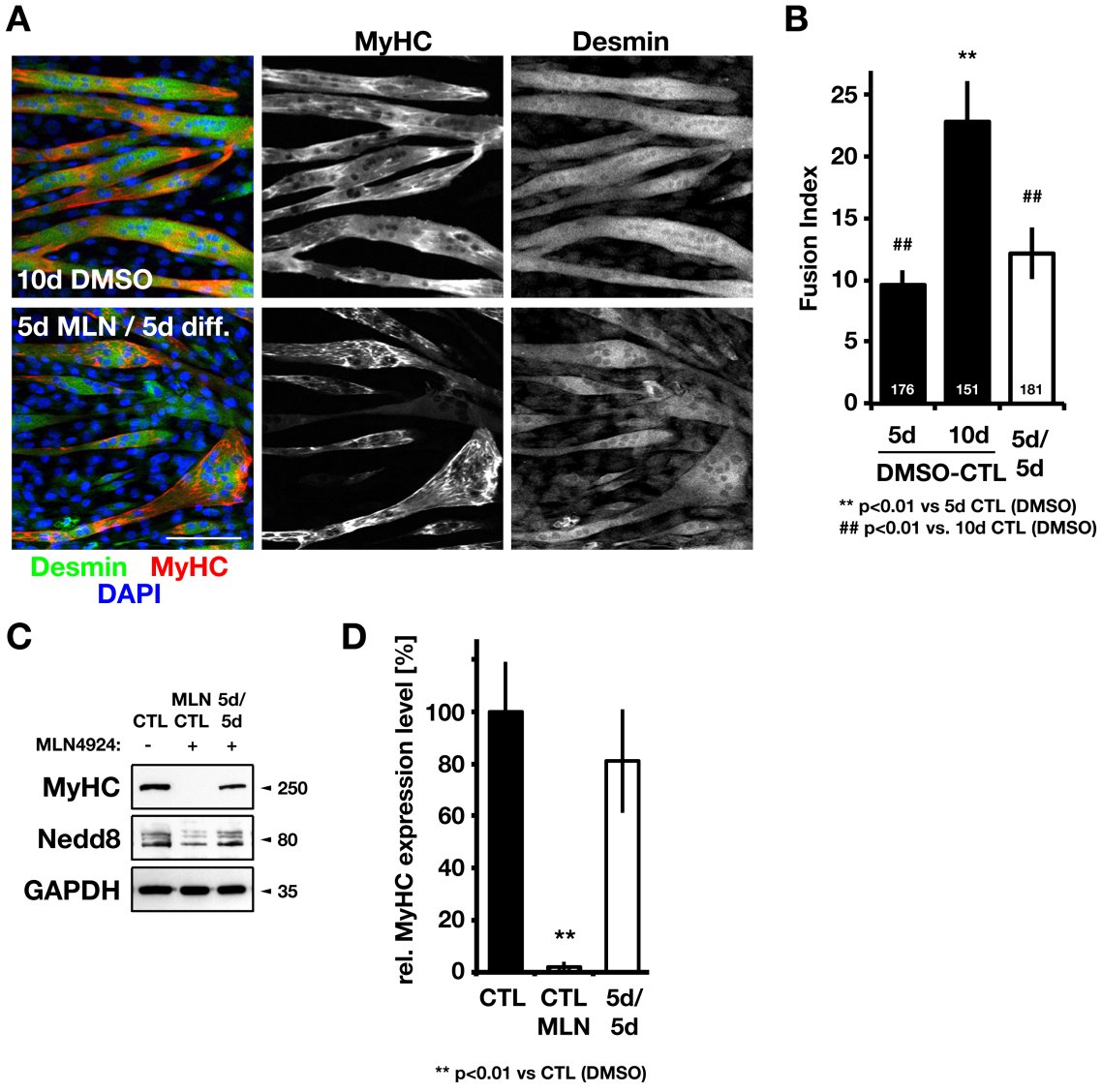

Figure 9. Effect of Cullin-RING Ligase inhibition on muscle differentiation are partially reversible.

A) Immunofluorescence analysis of C2C12 cells after ten days of differentiation in vehicle (10d DMSO) supplemented differentiation medium, compared to cells cultured for five days in differentiation medium supplemented with 300nM MLN4924 followed

by five days in differentiation medium (5d MLN / 5d diff.). Cells were stained with antibodies against sarcomeric myosin heavy chain (MyHC) and desmin. DAPI was used as counterstain. Scale bar =100μm. B) Quantitative analysis of fusion index of differentiated C2C12 cells as in (A), compared to average fusion index of differentiated C2C12 cells after five days in culture (5d DMSO-CTL). Shown are averages and standard errors. Sample sizes and p-values are indicated in the figure. C) Immunoblot analysis of whole cell lysates of C2C12 cells differentiated for 5 days with MLN4924, followed by 5 days differentiation medium (5d/5d), compared to MLN4924 treated (MLN) as well as vehicle treated control (CTL, DMSO) cells differentiated for 10 days. Shown are representative immunoblots stained with antibodies against sarcomeric myosin heavy chain (MyHC) and nedd8. GAPDH was used as loading control. D) Quantitative analysis of myosin heavy chain protein levels (as in C).

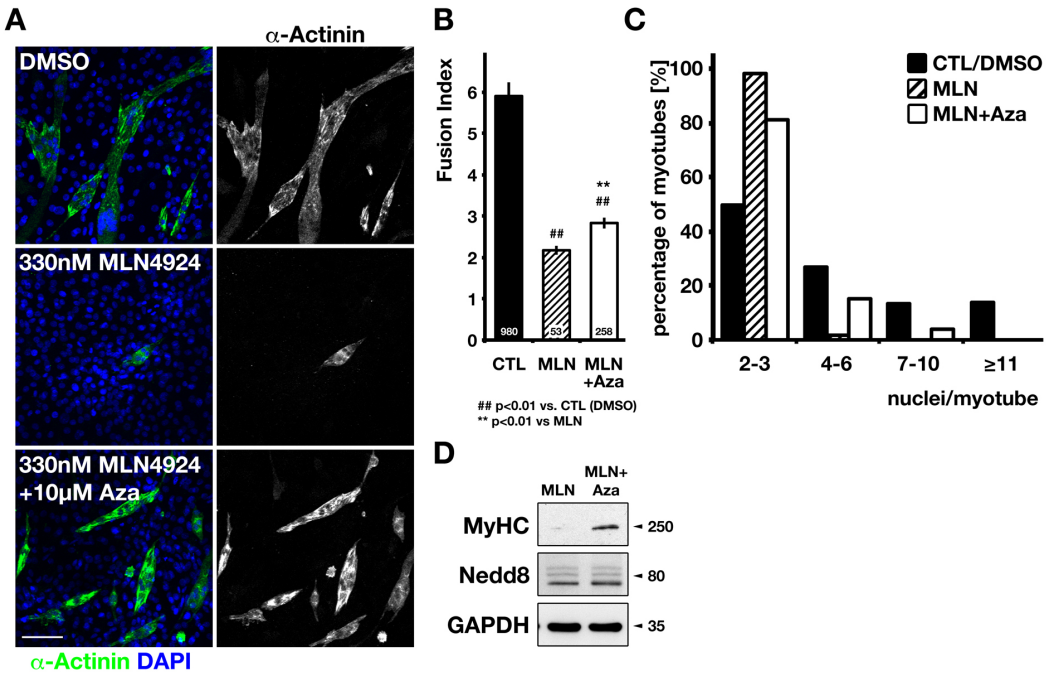

Figure 10. 5-aza-dC (Aza) partially alleviates the inhibitory effects of MLN4924 on C2C12 differentiation.

A) Immunofluorescence of C2C12 cells five days after differentiation in presence of 330nM MLN4924 alone, in combination with 10μM 5-aza-dC (Aza), or vehicle control (DMSO). Cells were stained with an antibody against α-actinin (green in overlay). DAPI (blue in overlay) was used as counterstain. Scale bar = 100μm. B, C) Fusion index (B) and cluster analysis of nuclei/myotube (C) of 330nM MLN4924 (MLN) treated, or 330nM MLN4924 and 10μM Aza treated C2C12 five days after differentiation, compared to vehicle control (CTL/DMSO). Sample size and p-values (in B) are indicated in the figure. D) Immunoblot analysis of sarcomeric myosin heavy chain (MyHC) and Nedd8 protein levels in differentiated C2C12 cells after five days in culture. Samples are

total protein lysates of cells treated with 330nM MLN4924 (MLN) alone, or in combination with 10μM Aza.

REFERENCES

Ametller, E., Busquets, S., Fuster, G., Figueras, M. T., Oliveira, C. C., Toledo, M., Korzeniewska K., Argilés J.M., López-Soriano, F. J. (2011). Effects of formoterol on protein metabolism in myotubes during hyperthermia. *Muscle & Nerve Muscle Nerve, 43*(2), 268-273. doi:10.1002/mus.21852

Attaix, D., & Taillandier, D. (1998). The Critical Role of the Ubiquitin-Proteasome Pathway in Muscle Wasting in Comparison to Lysosomal and Ca2 -Dependent Systems. *Intracellular Protein Decradation Advances in Molecular and Cell Biology,* 235-266. doi:10.1016/s1569-2558(08)60463-4

Benito, D. N., Nascimento, A., Abicht, A., Ortez, C., Jou, C., Müller, J. S., Evangelista T., Töpf A., Thompson R., Jimenez-Mallebrera C., Lochmüller, H. (2016). KLHL40-related nemaline myopathy with a sustained, positive response to treatment with acetylcholinesterase inhibitors. *Journal of Neurology J Neurol, 263*(3), 517-523. doi:10.1007/s00415-015-8015-x

Bhatia, S., Pavlick, A. C., Boasberg, P., Thompson, J. A., Mulligan, G., Pickard, M. D., Faessel H., Dezube B.J., Hamid, O. (2016). A phase I study of the investigational NEDD8-activating enzyme inhibitor pevonedistat (TAK-924/MLN4924) in patients with metastatic melanoma. *Invest New Drugs Investigational New Drugs*. doi:10.1007/s10637-016-0348-5

Blondelle, J., & Lange, S. (n.d.). Waste Disposal and Recycling in Cardiomyocytes. In E. Ehler (Ed.), *Cardiac cytoarchitecture: How to maintain a working heart*. Springer International Publishing Switzerland.

Bodine, S. C. (2001). Identification of Ubiquitin Ligases Required for Skeletal Muscle Atrophy. *Science, 294*(5547), 1704-1708. doi:10.1126/science.1065874

Bonaldo, P., & Sandri, M. (2012). Cellular and molecular mechanisms of muscle atrophy. *Disease Models & Mechanisms, 6*(1), 25-39. doi:10.1242/dmm.010389

Bosu, D. R., & Kipreos, E. T. (2008). Cullin-RING ubiquitin ligases: Global regulation and activation cycles. *Cell Div Cell Division, 3*(1), 7. doi:10.1186/1747-1028-3-7

Brownell, J. E., Sintchak, M. D., Gavin, J. M., Liao, H., Bruzzese, F. J., Bump, N. J., Soucy T.A., Milhollen M.A., Yang X., Burkhardt A.L., Ma J., Loke H.K., Lingaraj T., Wu D., Hamman K.B., Spelman J.J., Cullis C.A., Langston S.P., Vyskocil S., Sells T.B., Mallender W.D., Visiers I., Li P., Claiborne C.F., Rolfe M., Bolen J.B., Dick, L. R. (2010). Substrate-Assisted Inhibition of Ubiquitin-like Protein-Activating Enzymes: The NEDD8 E1 Inhibitor MLN4924 Forms a

NEDD8-AMP Mimetic In Situ. *Molecular Cell, 37*(1), 102-111. doi:10.1016/j.molcel.2009.12.024

Buckingham, M. (2001). Skeletal muscle formation in vertebrates. *Current Opinion in Genetics & Development, 11*(4), 440-448. doi:10.1016/s0959-437x(00)00215-x

Cirak, S., Deimling, F. V., Sachdev, S., Errington, W. J., Herrmann, R., Bonnemann, C., Brockmann K., Hinderlich S., Lindner TH., Steinbrecher A., Hoffmann K., Privé G.G., Hannink M., Nürnberg P., Voit, T. (2010). Kelch-like homologue 9 mutation is associated with an early onset autosomal dominant distal myopathy. *Brain, 133*(7), 2123-2135. doi:10.1093/brain/awq108

Costanzo, L. S. (2002). *Physiology*. Philadelphia, PA: Saunders/Elsevier.

Evans, W. J., Morley, J. E., Argilés, J., Bales, C., Baracos, V., Guttridge, D., Jatoi A., Kalantar-Zadeh K., Lochs H., Mantovani G., Marks D., Mitch W.E., Muscaritoli M., Najand A., Ponikowski P., Rossi Fanelli F., Schambelan M., Schols A., Schuster M., Thomas D., Wolfe R., Anker, S. D. (2008). Cachexia: A new definition. *Clinical Nutrition, 27*(6), 793-799. doi:10.1016/j.clnu.2008.06.013

Faralli, H., & Dilworth, F. J. (2012). Turning on Myogenin in Muscle: A Paradigm for Understanding Mechanisms of Tissue-Specific Gene Expression. *Comparative and Functional Genomics, 2012*, 1-10. doi:10.1155/2012/836374

Geyer, R., Wee, S., Anderson, S., Yates, J., & Wolf, D. A. (2003). BTB/POZ Domain Proteins Are Putative Substrate Adaptors for Cullin 3 Ubiquitin Ligases. *Molecular Cell, 12*(3), 783-790. doi:10.1016/s1097-2765(03)00341-1

Gomes, M. D., Lecker, S. H., Jagoe, R. T., Navon, A., & Goldberg, A. L. (2001). Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proceedings of the National Academy of Sciences, 98*(25), 14440-14445. doi:10.1073/pnas.251541198

Gupta, V. A., & Beggs, A. H. (2014). Kelch proteins: Emerging roles in skeletal muscle development and diseases. *Skeletal Muscle, 4*(1), 11. doi:10.1186/2044-5040-4-11

Hakenjos, J. P., Richter, R., Dohmann, E. M., Katsiarimpa, A., Isono, E., & Schwechheimer, C. (2011). MLN4924 Is an Efficient Inhibitor of NEDD8 Conjugation in Plants. *Plant Physiology, 156*(2), 527-536. doi:10.1104/pp.111.176677

Heidt, A. B., Rojas, A., Harris, I. S., & Black, B. L. (2007). Determinants of Myogenic Specificity within MyoD Are Required for Noncanonical E Box Binding. *Molecular and Cellular Biology, 27*(16), 5910-5920. doi:10.1128/mcb.01700-06

Hoffman, E. P., & Nader, G. A. (2004). Balancing muscle hypertrophy and atrophy. *Nature Medicine Nat Med, 10*(6), 584-585. doi:10.1038/nm0604-584

Janssen, I., Heymsfield, S. B., Wang, Z., & Ross, R. (2000). Skeletal muscle mass and distribution in 468 men and women aged 18–88 yr. *Journal of Applied Physiology, 89*(1), 81-88.

Ji, A. X., Chu, A., Nielsen, T. K., Benlekbir, S., Rubinstein, J. L., & Privé, G. G. (2016). Structural Insights into KCTD Protein Assembly and Cullin3 Recognition. *Journal of Molecular Biology, 428*(1), 92-107. doi:10.1016/j.jmb.2015.08.019

Lecker, S. H., Solomon, V., Price, S. R., Kwon, Y. T., Mitch, W. E., & Goldberg, A. L. (1999). Ubiquitin conjugation by the N-end rule pathway and mRNAs for its components increase in muscles of diabetic rats. *Journal of Clinical Investigation J. Clin. Invest., 104*(10), 1411-1420. doi:10.1172/jci7300

McComas, A. J. (1996). *Skeletal muscle: Form and function*. Champaign, IL: Human Kinetics.

Meadows, E., Cho, J., Flynn, J. M., & Klein, W. H. (2008). Myogenin regulates a distinct genetic program in adult muscle stem cells. *Developmental Biology, 322*(2), 406-414. doi:10.1016/j.ydbio.2008.07.024

Oikawa, Y., Omori, R., Nishii, T., Ishida, Y., Kawaichi, M., & Matsuda, E. (2011). The methyl-CpG-binding protein CIBZ suppresses myogenic differentiation by directly inhibiting myogenin expression. *Cell Res Cell Research, 21*(11), 1578-1590. doi:10.1038/cr.2011.90

Ravenscroft, G., Miyatake, S., Lehtokari, V., Todd, E., Vornanen, P., Yau, K., Hayashi Y.K., Miyake N., Tsurusaki Y., Doi H., Saitsu H., Osaka H., Yamashita S., Ohya T., Sakamoto Y., Koshimizu E., Imamura S., Yamashita M., Ogata K., Shiina M., Bryson-Richardson R.J., Vaz R., Ceyhan O., Brownstein C.A., Swanson L.C., Monnot S., Romero N.B., Amthor H., Kresoje N., Sivadorai P., Kiraly-Borri C., Haliloglu G., Talim B., Orhan D., Kale G., Charles AK., Fabian V.A., Davis M.R., Lammens M., Sewry C.A., Manzur A., Muntoni F., Clarke N.F., North K.N., Bertini E., Nevo Y., Willichowski E., Silberg I.E., Topaloglu H., Beggs A.H., Allcock R.J., Nishino I., Wallgren-Pettersson C., Matsumoto N., Laing, N. (2013). Mutations in KLHL40 Are a Frequent Cause of Severe Autosomal-Recessive Nemaline Myopathy. *The American Journal of Human Genetics, 93*(1), 6-18. doi:10.1016/j.ajhg.2013.05.004

Ryan, M. M. (2001). *Nemaline myopathy: A clinical, pathological and genetic study*.

Sambuughin, N., Yau, K. S., Olivé, M., Duff, R. M., Bayarsaikhan, M., Lu, S., Gonzalez-Mera L., Sivadorai P., Nowak K.J., Ravenscroft G., Mastaglia F.L., North K.N., Ilkovski B., Kremer H., Lammens M., van Engelen B.G., Fabian V., Lamont P.,

Davis M.R., Laing N.G., Goldfarb, L. G. (2011). Dominant Mutations in KBTBD13, a Member of the BTB/Kelch Family, Cause Nemaline Myopathy with Cores. *The American Journal of Human Genetics, 88*(1), 122. doi:10.1016/j.ajhg.2010.12.013

Sandri, M. (2013). Protein breakdown in muscle wasting: Role of autophagy-lysosome and ubiquitin-proteasome. *The International Journal of Biochemistry & Cell Biology, 45*(10), 2121-2129. doi:10.1016/j.biocel.2013.04.023

Sanes, J. R., & Lichtman, J. W. (1999). Development Of The Vertebrate Neuromuscular Junction. *Annu. Rev. Neurosci. Annual Review of Neuroscience, 22*(1), 389-442. doi:10.1146/annurev.neuro.22.1.389

Sarikas, A., Hartmann, T., & Pan, Z. (2011). The cullin protein family. *Genome Biol Genome Biology, 12*(4), 220. doi:10.1186/gb-2011-12-4-220

Schiaffino, S., Dyar, K. A., Ciciliot, S., Blaauw, B., & Sandri, M. (2013). Mechanisms regulating skeletal muscle growth and atrophy. *FEBS J FEBS Journal, 280*(17), 4294-4314. doi:10.1111/febs.12253

Singer, J. D., Gurian-West, M., Clurman, B., & Roberts, J. M. (1999). Cullin-3 targets cyclin E for ubiquitination and controls S phase in mammalian cells. *Genes & Development, 13*(18), 2375-2387. doi:10.1101/gad.13.18.2375

Soucy, T. A., Dick, L. R., Smith, P. G., Milhollen, M. A., & Brownell, J. E. (2010). The NEDD8 Conjugation Pathway and Its Relevance in Cancer Biology and Therapy. *Genes & Cancer, 1*(7), 708-716. doi:10.1177/1947601910382898

Soucy, T. A., Smith, P. G., & Rolfe, M. (2009). Targeting NEDD8-Activated Cullin-RING Ligases for the Treatment of Cancer. *Clinical Cancer Research, 15*(12), 3912-3916. doi:10.1158/1078-0432.ccr-09-0343

Stewart, S., Solomon, V., Mitch, W., & Goldberg, A. (1999). Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. *Journal of Nutrition,* (129), 227-237.

Tan, B. H., & Fearon, K. C. (2008). Cachexia: Prevalence and impact in medicine. *Current Opinion in Internal Medicine, 7*(5), 441-448. doi:10.1097/mci.0b013e328315510b

Vrbová, G., Gordon, T., & Jones, R. (1978). Development of the neuromuscular junction. *Nerve-Muscle Interaction,* 33-52. doi:10.1007/978-94-010-9541-9_3

Wallace, B. G., Zhican, Q., & Richard, H. L. (1991). Agrin induces phosphorylation of the nicotinic acetylcholine receptor. *Neuron, 6*(6), 869-878. doi:10.1016/0896-6273(91)90227-q

Zammit, P. S., Partridge, T. A., & Yablonka-Reuveni, Z. (2006). The Skeletal Muscle Satellite Cell: The Stem Cell That Came in From the Cold. *Journal of Histochemistry and Cytochemistry, 54*(11), 1177-1191. doi:10.1369/jhc.6r6995.2006

Zhou, W., Wei, W., & Sun, Y. (2013). Genetically engineered mouse models for functional studies of SKP1-CUL1-F-box-protein (SCF) E3 ubiquitin ligases. *Cell Res Cell Research, 23*(5), 599-619. doi:10.1038/cr.2013.44