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β-CATENIN REGULATION OF ADULT SKELETAL MUSCLE PLASTICITY

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β-CATENIN REGULATION OF ADULT SKELETAL MUSCLE PLASTICITY

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

Yuan Wen

Lexington, Kentucky

Director: Dr. John J. McCarthy, Associate Professor of Physiology

2018

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ABSTRACT OF DISSERTATION

β-CATENIN REGULATION OF ADULT SKELETAL MUSCLE PLASTICITY

Adult skeletal muscle is highly plastic and responds readily to environmental stimuli. One of the most commonly utilized methods to study skeletal muscle adaptations is immunofluorescence microscopy. By analyzing images of adult muscle cells, also known as myofibers, one can quantify changes in skeletal muscle structure and function (e.g. hypertrophy and fiber type). Skeletal muscle samples are typically cut in transverse or cross sections, and antibodies against sarcolemmal or basal lamina proteins are used to label the myofiber boundaries.

The quantification of hundreds to thousands of myofibers per sample is accomplished either manually or semi-automatically using generalized pathology software, and such approaches become exceedingly tedious. In the first study, I developed MyoVision, a robust, fully automated software that is dedicated to skeletal muscle immunohistological image analysis. The software has been made freely available to muscle biologists to alleviate the burden of routine image analyses. To date, more than 60 technicians, students, postdoctoral fellows, faculty members, and others have requested this software.

Using MyoVision, I was able to accurately quantify the effects of β-catenin knockout on myofiber hypertrophy. In the second study, I tested the hypothesis that myofiber hypertrophy requires β-catenin to activate c-myc transcription and promote ribosome biogenesis. Recent evidence in both mice and human suggests a close association between ribosome biogenesis and skeletal muscle hypertrophy. Using an inducible mouse model of skeletal myofiber-specific genetic knockout, I obtained evidence that β-catenin is important for myofiber hypertrophy, although its role in ribosome biogenesis appears to be dispensable for mechanical overload induced muscle growth. Instead, β-catenin may be necessary for promoting the translation of growth related genes through activation of ribosomal protein S6.
Unexpectedly, we detected a novel, enhancing effect of myofiber β-catenin knockout on the resident muscle stem cells, or satellite cells. In the absence of myofiber β-catenin, satellite cells activate and proliferate earlier in response to mechanical overload. Consistent with the role of satellite cells in muscle repair, the enhanced recruitment of satellite cells led to a significantly improved regeneration response after chemical injury. The novelty of these findings resides in the fact that the genetic perturbation was extrinsic to the satellite cells, and this is even more surprising because the current literature focuses heavily on intrinsic mechanisms within satellite cells. As such, this model of myofiber β-catenin knockout may significantly contribute to better understanding of the mechanisms of satellite cell priming, with implications for regenerative medicine.

KEYWORDS: skeletal muscle, image analysis, ribosome biogenesis, β-catenin, plasticity

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Chapter 1. Introduction

1.1 Adult Skeletal Muscle Plasticity

Muscle robustly changes its size, composition and metabolic profile in order to optimally adapt to environmental and physiological demands [1]. In response to increased mechanical loading, muscle progressively accumulates protein mass, leading to hypertrophy of individual myofibers [2]. Conversely, under chronic conditions of energy deprivation, unloading, and/or disease, muscle undergoes a relatively rapid atrophy resulting from a loss in myofiber cross sectional area [3, 4]. Additionally, adult skeletal muscle can regenerate after extensive loss of tissue due to injury. This high capacity for regeneration is entirely dependent on the resident stem cells, called satellite cells, which are rapidly recruited to replicate, differentiate into myoblasts, and fuse with each other to form new myofibers [5].

1.2 Ribosome Biogenesis

A critical determinant of muscle growth is the balance between protein synthesis and degradation. In order for skeletal muscle to grow, the rate of protein synthesis must be higher than protein breakdown. The rate of protein synthesis depends on the cell’s translational machinery, the ribosome. A cell’s capacity for translation is reflected in the total amount of ribosomes available to translate messenger RNAs into protein. The estimated stability of mature ribosomes is roughly 4-12 days [6, 7], suggesting that once made, ribosomes are relatively stable. It is believed that cell’s total translational capacity is primarily determined by the rate of ribosome biogenesis, which is highly regulated in response to environmental factors [8].
The functionally mature ribosome (80S) is a 4.3 megadalton ribonucleoprotein complex composed of two subunits. The large (60S) subunit contains three (28S, 5S, and 5.8S) ribosomal RNAs (rRNAs) and 47 ribosomal proteins (RPLs). The small (40S) subunit contains one (18S) rRNA molecule and 33 ribosomal proteins (RPSs). Three of the four rRNA molecules (28S, 5.8S, and 18S) are transcribed together as a single precursor rRNA molecule called the pre-47S rRNA. The pre-47S rRNA is transcribed by the dedicated RNA polymerase I (Pol I) from ribosomal DNA sequences (rDNA). The genome, both human and mouse, contains hundreds of copies of tandem repeats of rDNA sequences distributed across multiple chromosomes. The rDNA clusters are organized into characteristic regions within the nucleus known as the nucleolus [9]. Transcription of the pre-47S rRNA is regulated by numerous factors, collectively known as the “Pol I regulon”. Factors such as myc (see section 1.3 below), Polr1b, Rrn3 (TIF-1A), Polr1e (PAF53), Ttf1, Ubft (upstream-binding factor, UBF), Taf1b (selectivity factor, SL1, or TIF1B), and Taf1c have all been shown to localize to the rRNA promoter and contribute to the pre-initiation complex (PIC) [10]. SL1 is critical for PIC assembly and promotes the recruitment of UBF [11, 12]. SL1 and UBF interact with Poll to release RRN3 after PIC assembly to subsequently allow promoter escape and transcript initiation, which is the highly-regulated rate-limiting step in rRNA gene expression [13, 14]. Cleavage of the pre-47S rRNA by processing factors and small nucleolar ribonucleoproteins (snoRNPs) leads to the formation of the 28S, 5.8S, and 18S rRNA molecules required for small and large subunit assembly. The only rRNA molecule not transcribed in the nucleolus is the 5S rRNA, which is transcribed by
RNA polymerase III (Pol III). 5S rRNA molecules are transported into the nucleolus after synthesis in order to be assembled with the 28S and the 5.8S rRNAs to form the large subunit. Pol III is also responsible for transcribing transfer RNAs (tRNAs), which are transported out of the nucleus, loaded with amino acids, and used for polypeptide elongation [9].

The various ribosomal proteins are encoded by messenger RNAs (mRNAs), which are transcribed by RNA polymerase II (Pol II). Ribosomal protein mRNAs are transported out of the nucleus and translated into protein in the cytoplasm. mRNAs encoding assembly factors and transport proteins are also transcribed by Pol II and transported into the cytoplasm for translation. Ribosomal proteins and protein-processing factors are subsequently imported back into the nucleolus to be assembled onto the small and large subunits. Small subunit ribosomal proteins (RPSs) assemble onto the 18S rRNA scaffold and large subunit ribosomal proteins (RPLs) assemble onto the combined 28S, 5S, and 5.8S rRNA scaffold. Following assembly, the 40S and 60S subunits are exported out into the cytoplasm, where they bind their respective co-factors. The 40S and 60S subunits do not combine and mature into the functional 80S ribosome until translation initiation occurs on mRNA molecules [9].

1.3 C-myc Regulation of Ribosome Biogenesis

Ribosome biogenesis is a highly complex process with numerous points of regulation. Despite the complexity, a number of studies have collectively provided evidence that the proto-oncogene c-myc (c-myelocytomatosis oncogene) has a
central role in the control of ribosome biogenesis [15]. During Drosophila larval development, MYC has been shown to be both necessary and sufficient to regulate ribosome biogenesis through rRNA transcription [16]. In mammals, the set of c-myc target genes, called the myc core signature (MCS), is significantly enriched for functions in ribosome and ribonucleoprotein biogenesis [17]. After analyzing thousands of microarrays representing hundreds of cell types, the authors found that the MCS is independent of tissue type and may represent a primordial mechanism in the regulation of biomass accumulation [17]. The ability of c-myc to potently drive ribosome biogenesis is believed to be the cause of c-myc's remarkable oncogenic potential [18, 19].

MYC functions in multiple ways to stimulate rRNA transcription, including promoting the expression of three key factors in the “Pol I regulon”. MYC has been shown to bind to the promoters of SL1, UBF, and RRN3 to activate Pol II-mediated transcription in non-proliferating cells, suggesting a mechanism independent of cell cycle progression [20, 21]. In addition to the indirect control of the Pol I regulon gene expression, MYC also activates rRNA transcription through direct interactions within the nucleolus [22, 23]. MYC physically associates with SL1 to enhance UBF binding at the rRNA promoter and dramatically increase pre-47S transcription [23]. MYC binding at the rRNA promoter is also associated with changes in chromatin structure, including increased histone acetylation [23, 24] and hypomethylated DNA loop structures that’s thought to allow enhanced re-initiation of PolI transcription during periods of rapid growth [22, 25]. In all of these
studies, knocking out of c-myc completely blocks or severely reduces mitogen activated increases in rRNA transcription and ribosome biogenesis.

1.4 Ribosome Biogenesis in Skeletal Muscle Hypertrophy

1.4.1 Cell and Animal Models

Total RNA can be used as a measure of ribosome content given that ~85% of the RNA in the cell is rRNA; thus, an increase in RNA content is considered to be indicative of an increase in ribosome biogenesis [26]. One of the first studies to really investigate the importance of ribosome biogenesis and skeletal muscle hypertrophy was a study by Nader and colleagues [27]. Using an *in vitro* model of high serum stimulated myotube hypertrophy, the authors showed a ~70% increase in rRNA 48 hours post-serum stimulation that was associated with increased UBF availability [27]. Moving into the mouse, the Nader laboratory next investigated Pol1 regulon expression during the initial phase of hypertrophy induced by synergist ablation, a surgical model of hindlimb muscle hypertrophy [28]. After three days of synergist ablation, there was a 2-fold increase in total RNA concentration as the result of a 3-fold increase in pre-rRNA expression. The increase in rRNA expression was paralleled by ~2.5- to 6-fold increase in the transcript levels of Pol I regulon components such as *Polr1b*, *Rrn3* (TIF-1A), *Polr1e* (PAF53), *Ttf1* and *Taf1c* as well as UBF mRNA and protein. Consistent with the up-regulation of the Pol I regulon, chromatin immunoprecipitation assay showed enhanced binding of Pol I and UBF at the rDNA promoter.

The change in total RNA content of the muscle is also positively correlated with the magnitude of muscle growth [29-31]. Nakada and coworkers modified the
synergist ablation model in such a way as to produce four different levels of hypertrophy [32]. After five days of synergist ablation, plantaris muscle weight increased by 8%, 22%, 32% and 45% with rRNA content increasing by 1.8-fold, 2.2-fold and 2.5-fold in only the top three groups, respectively. Further, the increase in translational capacity observed at five days was strongly correlated \((r = 0.98)\) to muscle weight after 14 days of synergist ablation.

The findings from these studies provide clear evidence that in response to a hypertrophic stimulus, there is a dramatic increase in Pol 1-dependent transcription of rDNA, which results in increases in translational capacity that is highly correlated with the amount of muscle growth. This correlation exists for skeletal muscle with a reduced ability to grow following a hypertrophic stimulus. Muscle’s ability to mount a robust hypertrophic response reduces with old age. Consistent with the idea that effective ribosome biogenesis is necessary for muscle hypertrophy, the reduced growth response in aged muscle is accompanied by an impaired ability to produce new ribosomes [31]. Following two weeks of synergist ablation, the plantaris muscle of 24-month-old mice showed a significantly blunted hypertrophic response compared to 5-month-old mice, and the 24-month-old response was associated with a 50% increase (compared to a 250% increase in young mice) in the RNA content of the muscle [31]. The increase in rDNA transcription during hypertrophy was significantly less in old muscle compared to young muscle; after 3 days of synergist ablation, pre-rRNA expression increased 3-fold in the plantaris muscle of young mice and only 1.7-fold in old mice [31]. Furthermore, following seven days of synergist ablation, pre-rRNA expression continued to be elevated
3-fold in the plantaris muscle of young mice but returned to baseline levels in old mice [31].

1.4.2 Human Resistance Exercise (RE)

Nader and colleagues conducted a study to determine whether the change in muscle gene expression following an acute bout of RE (elbow flexion) was altered after 12 weeks of RE training [33]. These investigators found that both c-myc and pre-rRNA expression were significantly elevated in the biceps brachii in response to an acute RE bout regardless of training status [33].

Building on the findings of Nader and coworkers, Figueiredo and colleagues performed the first detailed human study investigating the regulation of ribosome biogenesis in response to RE, both acutely and following eight weeks of RE training [33, 34]. The total RNA concentration following training tended toward an increase (1.3-fold) but did not achieve statistical significance because of variability between subjects; however, the increase in total RNA from pre-training to post-training was significantly correlated ($r = 0.72$) to the percent change in the cross sectional area of the muscle [34]. Consistent with this trend, pre-rRNA expression in resting muscle was increased by 2-fold after training and reflected in a 2- to 4-fold increase in mature 18S, 5.8S, and 28S rRNAs [34]. Although UBF phosphorylation changed following exercise, UBF Ser$^{388}$ phosphorylation was ~5-fold higher in the rested state following eight weeks of RE training which the authors speculated was necessary for the increased translational capacity observed in trained muscle.
In a recent follow-up study, Figueiredo and colleagues investigated the impact of different recovery strategies on ribosome biogenesis following an acute bout of resistance exercise [35]. To provide an important temporal component to the study, muscle biopsies were collected at 2, 24 and 48 hours post-exercise, and pre-rRNA, and c-myc expression were significantly increased at both the 24 hours and 48 hours post-exercise time point [35]. Collectively, these data provide convincing evidence that ribosome biogenesis is activated in response to a single bout of RE in humans.

Although the studies by Figueiredo and coworkers clearly showed that ribosome biogenesis occurs following RE, their findings do not speak to whether or not ribosome biogenesis is necessary for skeletal muscle hypertrophy in humans. A study by the Bamman laboratory reported that in older individuals (age 60-75 y) only extreme responders (to 4 weeks of RE) showed a significant increase in total RNA (+26%) and rRNA content (+40%) which was associated with a ~350% increase in c-myc protein [36]. These findings were not completely unexpected given this group’s earlier study showing blunted ribosome biogenesis in older individuals following a bout of RE, in agreement with Kirby and colleagues study comparing the hypertrophic response of young and old mice [31, 37]. Although these findings do not definitively demonstrate that skeletal muscle hypertrophy requires ribosome biogenesis they do show that the hypertrophic response is enhanced by increases in translational capacity.

1.5 Dual Roles of β-Catenin in Skeletal Muscle

1.5.1 Transcriptional Activation of c-myc
β-catenin is most well known as the mediator of the canonical Wnt signaling pathway. When the Wnt signaling pathway is inactive, GSK-3β (glycogen synthase kinase-3β), the main kinase responsible for the phosphorylation of cytoplasmic β-catenin, labels cytoplasmic β-catenin for degradation by the destruction complex [38]. When Wnt ligands bind Frizzled (Fzd) receptor and other co-receptors, Disheveled (Dvl) is phosphorylated, which inactivates GSK-3β, thereby releasing its inhibitory regulation of β-catenin. Intact β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it dimerizes with TCF/LEF (T cell factor/lymphocyte enhancement factor) transcription factors to increase the transcription of c-myc [39-42].

In the mouse model of skeletal muscle hypertrophy, both the cytoplasmic and nuclear β-catenin protein levels increase, and this is accompanied by increased c-myc transcript abundance as well as c-myc protein nuclear translocation [43]. In a follow-up study, Armstrong and colleagues used adenovirus to genetically delete β-catenin in a small portion of muscle tissue (<20% of myofibers) [44]. Following two weeks of synergist ablation, the authors showed that myofibers infected with the virus were smaller than myofibers without the virus, suggesting that β-catenin is necessary for myofiber hypertrophy [44]. However, the low infection rate and lack of cell type specificity of the adenovirus make it difficult to assess whether β-catenin acted through c-myc to regulate ribosome biogenesis.

1.5.2 Cytoskeletal Anchor for Satellite Cells

In addition to its role as a mediator of canonical Wnt signaling and transcription factor, β-catenin also functions in cell-cell adhesion. β-catenin is an indispensable
component of the adherens junction protein complex [45]. The adherens junction is centered around calcium-dependent transmembrane proteins called cadherins, which homodimerize extracellularly with cadherins on neighboring cells [46]. Intracellularly, the C-terminus of cadherin binds to α-, β-, and γ-catenins as well as p120, which anchors to the cell cytoskeleton such as actin filaments [47, 48]. Skeletal muscle expresses N-cadherin (Cdh2) throughout development and during regeneration, but in the adult muscle, M-cadherin (Cdh15) expression is the most prominent [49, 50]. In mature muscle, M-cadherin is expressed in both myofibers and satellite cells, localized on the membranes at the interface between the myofibers and the satellite cells [51]. β-catenin and its interaction with microtubules have been shown to be important for the restructuring of the cytoskeleton during myoblast fusion and myotube formation in culture [52]. Inactivation of β-catenin in satellite cells significantly diminishes satellite cell activation and proliferation, while stabilization of β-catenin induces satellite cells to enter a pathologically activated state that is detrimental to regeneration [53]. Membrane junctions may also mediate mechanical signals between myofiber and satellite cells [54].

1.6 Hypothesis and Objectives

The overall hypothesis of the dissertation is that β-catenin activation of c-myc transcription is necessary for skeletal muscle hypertrophy by driving ribosome biogenesis.

1.6.1 Develop an automated image analysis program to quantify phenotypic adaptation of skeletal muscle cells (Chapter 2)
Hypothesis: Accurate and fast quantification of skeletal muscle images can be automated by a computer program to save time, increase productivity, and contribute to scientific rigor and reproducibility.

Rationale: A large number of images can be acquired relatively easily and quickly, but analysis and quantification are time-consuming. Numerous algorithms are reported in the literature, but none of them are readily usable. Developing a program will enable fast and reproducible analyses to test subsequent hypotheses.

1.6.2 Determine whether β-catenin is necessary for skeletal myofiber hypertrophy (Chapter 3)

Hypothesis: When β-catenin is deleted in skeletal muscle, ribosome biogenesis will be limited and skeletal muscle hypertrophy will be blunted as a consequence of decreased c-myc transcript levels.

Rationale: β-catenin is known to mediate Wnt signaling and drive c-myc expression. MYC as a transcription factor has broad activating effects on ribosome biogenesis, which appear to be vital for maximal skeletal muscle hypertrophy.
Chapter 2. MyoVision: Software for Automated High-Content Analysis of Skeletal Muscle Immunohistochemistry

2.1 Abstract

Analysis of skeletal muscle cross sections is an important experimental technique in muscle biology. Many aspects of immunohistochemistry and fluorescence microscopy can now be automated but most image quantification techniques still require extensive human input, slowing progress and introducing the possibility of user-bias. MyoVision is a new software package that was developed to overcome these limitations. The software improves upon previously reported automatic techniques and analyzes images without requiring significant human input and correction. When compared to data derived by manual quantification, MyoVision achieves an accuracy of ≥94% for basic measurements such as fiber number, fiber type distribution, fiber cross sectional area, and myonuclear number. Scientists can download the software free from www.MyoVision.org and use it to automate the analysis of their own experimental data. This will improve the efficiency and consistency of the analysis of muscle cross sections and help to reduce the burden of routine image quantification in muscle biology.

2.2 Introduction

Skeletal muscle is primarily composed of elongated, multinucleated cells, called fibers, which adapt to external stimuli and changing functional demands. Hormones, exercise, physical inactivity, spaceflight, denervation, aging, acute and chronic diseases/infections, genetic defects, and metabolic disorders all modulate
skeletal muscle fibers in ways that are visually discernable using immunofluorescence microscopy [3, 4, 36, 55-58]. Specifically, fiber cross sectional area (CSA), fiber type proportion (i.e. percent slow-twitch, fast-twitch, and hybrid fibers, based on myosin heavy chain (MyHC) expression), myonuclear number, and muscle stem cell (i.e. satellite cell) number can change profoundly in the aforementioned conditions. As a result, numerous research groups seek to measure tissue and cellular-level morphology as part of their analyses.

Image quantification is often the most time-consuming part of this process. In muscle biology labs at the University of Kentucky, samples are typically sectioned and stained in batches. The experimental protocols (including lengthy immunohistochemical procedures) are normally completed within a few days. In contrast, before the development of MyoVision, the software described in this manuscript, quantification of experimental datasets typically required weeks to months of intensive human effort.

Several groups, including the current authors, have described theoretical frameworks for automating the analysis of muscle cross sections without making software available for general use [59-65]. Other groups have published their computer code. For example, Bergmeister et al. shared a plug-in for ImageJ that can measure fiber number, size, and type [66]. SMASH is software written by Smith and Barton that can semi-automatically expedite analyses, mainly as a free alternative to expensive commercial software packages [67]. These programs have attractive features but require extensive user interaction and/or optimization. For example, in SMASH, the user has to set a segmentation filter interactively for
each image in order for the software to produce useful results. This makes it difficult to automate the analysis and introduces the potential for user-bias. MyoVision overcomes most of these limitations. MyoVision provides high content quantification of muscle features, including fiber number, CSA, minimum Feret diameter, myonuclear number, and fiber type distribution, without requiring human supervision. Data presented on the following pages show that it is also more reliable and more accurate than available alternative techniques and packages. It is available for free download from www.MyoVision.org.

2.3 Materials and Methods

2.3.1 Animals and Tissue preparation

All of the images used for estimating MyoVision accuracy were obtained during previous studies of mouse plantaris tissue [68, 69]. No mice were sacrificed specifically for the development and validation of the software. Each of the images showed a complete plantaris cross section and was formed by stitching together multiple fields of view (see Image Acquisition). One cross section per mouse was analyzed in all studies. The cell detection and counting comparisons used images from 6 plantaris cross sections labeled with anti-laminin antibody (total of 5,800 fibers). The CSA analyses included >14,000 muscle fibers from cross sections labeled with anti-dystrophin antibody (4 groups of N=4 mice per group subjected to sham, 3-day, 7-day, or 14-day synergist ablation surgery). 3,300 muscle fibers from 6 cross sections labeled with anti-laminin and isoform-specific anti-Myosin Heavy Chain (MyHC) antibodies were analyzed for the fiber type distribution
comparisons. Finally, the myonuclear counting analysis was based on 3,800 fibers from six plantaris cross sections stained with anti-dystrophin antibodies and DAPI.

2.3.2 Immunohistochemistry

Immunohistochemical procedures were carried out as previously described by Fry et al. [70]. A detailed, recommended protocol for both mouse and human muscle cross sections is included in the Appendix. In summary, for fiber typing, unfixed sections (7 µm) were incubated for 90 minutes at room temperature with antibodies to MyHC types I, IIA and IIB (1:100, Cat#BA.D5, SC.71 and BF.F3, respectively, University of Iowa Developmental Studies Hybridoma Bank, Iowa City, IA, USA) in addition to rabbit anti-laminin IgG (1:100, L9393, Sigma-Aldrich, St. Louis, MO, USA). MyHC type IIX expression was inferred from unstained fibers. Fluorescence-conjugated secondary antibodies to different mouse immunoglobulin subtypes were applied for 1 hour to visualize MyHC expression and laminin. Sections were post-fixed in absolute methanol before mounting.

For dystrophin identification, muscle sections were rehydrated with phosphate buffered saline and blocked in Mouse-On-Mouse Blocking Reagent (Vector Laboratories, Burlingame, CA, USA). After washing, incubation with anti-dystrophin antibody (1:50, Cat#VPD505 Vector) overnight was followed by incubation for 75 minutes with goat anti-mouse biotinylated secondary antibody (1:1000, 115-065-205, Jackson ImmunoResearch, West Grove, PA, USA). Sections were washed again, incubated 30 minutes in SA-FITC (1:150, # SA-5001, Vector) and post-fixed in 4% paraformaldehyde before mounting using Vectashield fluorescent mounting medium with DAPI (Vector).
2.3.3 Image Acquisition and Quantification

All images were acquired using an upright microscope at 20x magnifications (AxioImager M1, Zen 2.3 Imaging Software; Zeiss, Göttingen, Germany), which automatically acquires consecutive fields in multiple channels. These fields were then optionally stitched together into a single mosaic image.

Manual Analysis of Fiber Type Distribution: Manual analyses were performed first by a blinded operator using ImageJ followed by visual confirmation by a second researcher [71]. Briefly, multiple channels were background subtracted, normalized for their intensities, false-colored and merged into a single image using Zen 2 Lite or Pro. The different fiber types were visually identified based on color differences in the merged image using the cell counter tool. Fibers were sequentially counted as Type I (Cy5, pink), Type IIA (FITC, green), and Type IIB (TRITC, red). Fibers that were counted as negative under all three channels were classified as Type IIX. Classification of fiber type required roughly one hour per cross section.

Semi-Manual Analysis using Zen: Muscle fiber CSA was determined by a single operator using Zen 2 Pro (Zeiss) image processing software. The image analysis macro was designed using pre-existing tools within the Zen 2 Pro software. Images were pre-processed to optimize contrast for the membrane immunofluorescence. In the image analysis macro, the DAPI filter was selected with the entire cross section of muscle being used as the region of interest (ROI). The input image was smoothed with a Gaussian filter (sigma 1.5) without sharpening and a minimum 485-500 and maximum >1000 intensity threshold was used for the watershed
separation of fibers. A pre-determined minimum CSA exclusion of 150 µm² and maximum 5000 µm² was employed (representing the smallest and largest fibers found in a mouse plantaris cross section) [70]. After the initial automatic segmentation, the manual cutting and merging tools were utilized to correct segmentation errors. For an 800-1000 fiber cross section of whole muscle, this process generally took 1.5-2 hours. Fiber identification (ID) and CSA were selected as the output variables so that fiber number and average CSA could be determined. Visual verification of appropriate fiber sizes was then carried out, and all merged fibers that were not manually separated (generally <2% of fibers) were excluded from the analysis.

**SMASH.** Detailed descriptions of the SMASH software have been published [67]. When using the SMASH software for the analyses described in this study, the initial segmentation was performed on all laminin images with parameters set to blue (fiber outline color), 0.323 (pixel size – µm/pixel), and 8 (segmentation filter – low is more segmentation). The segmentation filter was selected by empirically testing values from 5 to 12 and visually inspecting results for the first image. Subsequent fiber filter parameters were set to default values. Although SMASH was designed to be semi-automatic, no manual correction was performed for either the initial segmentation or the fiber filter in order to compare to the fully automatic analyses of MyoVision.

**ImageJ plug-in.** The ImageJ plug-in was downloaded according to instructions described by Bergmeister et al. [66], and the intensity threshold for each laminin image was set to 600, which was within the range set by the manual analysis of
the same images. The ROI was set to the entire muscle cross section. The same CSA filters (a minimum of 150 \( \mu m^2 \) and a maximum 5000 \( \mu m^2 \)) were applied to all analyzed images.

### 2.3.4 MyoVision software implementation

For a detailed description of the algorithm rationale and implementation, please see the Appendix. Figure 2.1A shows an example of a muscle cross section stained for dystrophin. Figure 2.1B shows the same image after processing in the MyoVision software. The algorithm workflow is also diagrammed in Figure 2.2. Briefly, skeletal muscle cross sections labeled with anti-laminin or -dystrophin immunofluorescence were filtered and enhanced for line and edge structures. The enhanced image was segregated into membrane and cytoplasmic regions using Euclidian Distance K-means clustering. Each separated cytoplasmic region was designated as a seed region, which belongs to one of three classifications: (1) multiple connected cells, (2) a single cell, or (3) interstitial space (Figure 2.3A – red regions). Using shape descriptors (Figure 2.3B-F), seed regions more likely to be connected cells were selected for watershed separation. The contour of each processed seed region was fine-tuned by spline-evolution until convergence. Contour evolution was carried out over an energy field calculated using the Chan Vese – Vector Field Convolution. For fiber type and myonuclear analyses, fiber outlines generated from the laminin/dystrophin reference image were combined and converted into a mask overlay. This mask was subsequently applied to each additional single channel immunofluorescence image indicating nuclei or a particular MyHC isoform.
2.3.5 Accuracy Measurements

Accuracy of fiber and myonuclei counting and for analyses of cross sectional areas was defined by Equation (1).

\[
(1) \quad \text{Accuracy} = \left( 1 - \frac{|\text{MyoVision Value} - \text{Reference Value}|}{\text{Reference Value}} \right) \times 100\%
\]

The reference value was defined as the value obtained by trained muscle researchers using semi-automated or fully manual methods (Figure 2.4A – solid line) except for the case of cross sectional area. Because manual techniques typically underestimated fiber cross sectional area (see Figure 2.5), the reference cross sectional areas were calculated as \(A_0 \times \text{(Manual Value)} + A_1\) where \(A_0\) was the slope and \(A_1\) was the y-intercept of a regression line fitted to the algorithm values (Figure 2.6A – dashed lines).

Accuracy for analysis of fiber type distributions was defined by Equation (2) to prevent division by zero errors when muscle cross sections did not contain any Type I fibers.

\[
(2) \quad \text{Accuracy} = (1 - |\text{MyoVision Value (\%) - Manual Value (\%)|}) \times 100\%
\]

2.3.6 Statistics

Reported values represent mean ± SE. Statistical analyses were performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. P values less than 0.05 were considered to be statistically significant. One-way ANOVA followed by Tukey multiple comparisons test was performed for fiber counting accuracy measurements.
Paired, two-tail students’ t-tests were performed for accuracy measurements of fiber CSA and myonuclear counting. Repeated measures two-way ANOVA followed by Bonferroni multiple comparisons tests were performed for CSA changes with mechanical overload and for fiber typing accuracy measurements.

2.4 Results

2.4.1 Fiber Counting

The individual fibers are identified and outlined in yellow. One of the key features of MyoVision is that seed regions are pre-filtered using shape descriptors before the conventional watershed transformation is applied. Such a pre-filtering approach minimizes over-segmentation and improves the accuracy of fiber counts. Figure 2.4 shows that the number of fibers counted by MyoVision is 101.4±1.0% of the number counted by manual analysis, which corresponds to an accuracy of 98.2±0.9% (see Equation 1) if manual counts are accepted as true values. In contrast, SMASH (a program that implements metric-based filtering after the watershed transformation) over-estimates the number of fibers (113.8±3.0%) and had an accuracy of only 86.2±3.0%. The plug-in for ImageJ described by Bergmeister et al. over-segments (151.4±4.8%) the fibers (Figure 2.4F) and had an accuracy of only 48.6±4.8%. This plug-in was not considered further in this work because of its poor performance in these initial tests.

2.4.2 Fiber Cross Section Area

A direct assessment of the cytoplasmic outlines from MyoVision and semi-manual analyses demonstrates that MyoVision outlines lie closer to the membrane than
the semi-manual outlines (Figure 2.5). When comparing the CSAs calculated by the MyoVision and SMASH algorithms to the reference values obtained by semi-manual analysis, MyoVision produces higher CSA measurements than the semi-manual method (Figure 2.6A). This is because users typically choose a more conservative threshold (smaller cytoplasmic regions) in the semi-manual method in order to reduce the number of manual corrections needed to separate connected cells. Figure 2.6B shows that the areas calculated by the MyoVision software are more accurate than those produced by the SMASH algorithm (p<0.001).

Figure 2.6C shows that MyoVision-derived estimates for the relative increases in fiber CSA in response to a hypertrophic stimulus are indistinguishable from those determined by manual analysis (p>0.05). This contrasts with the relative growths calculated by SMASH which differ at 7 and 14 days of mechanical muscle overload from manual measurements and from MyoVision-derived values (all p-values are less than 0.05). These statistical tests show that MyoVision-based estimates of muscle fiber hypertrophy are more consistent with manual measurements than those obtained using SMASH.

### 2.4.3 Fiber Type Classification

Figure 2.7A shows a representative image of muscle fibers immunofluorescently labeled for various MyHC isoforms and delineated by the basal lamina. Staining is shown in arbitrary false color: blue for laminin, magenta for Type I, green for Type IIA, and red for Type IIB. Type IIX is inferred from a lack of MyHC immunofluorescence. MyoVision uses the laminin channel to detect and outline the muscle fibers and then classifies fiber types based on the intensities detected
for the different MyHC antibodies. Figure 2.7B shows an overlay of the MyoVision fiber type classification results. MyoVision results are linearly and positively correlated to manual counts (Pearson, R = 0.97), and a 2-way ANOVA shows no statistical difference between the proportions of each fiber type measured by hand and by MyoVision. The accuracy of MyoVision fiber type analysis is estimated to be 96.5±0.6% compared to manual analysis.

2.4.4 Myonuclear Number

Myonuclei are differentiated from interstitial nuclei because of their location under the sarcolemma. Automatic counting of myonuclei thus requires accurate delineation of the sarcolemma. Figure 2.8A shows dystrophin immunofluorescence demarcating the sarcolemma in red and DAPI-stained nuclear DNA in blue. Figure 2.8B shows the results from MyoVision myonuclear detection, where the sarcolemma is outlined in yellow dotted lines and the myonuclei (defined as any nuclear region having its centroid and greater than 50% of its area inside the sarcolemma) are marked with yellow crosses. MyoVision myonuclear counts are linearly correlated with manual counts (Pearson, R = 0.96), and a student’s t-test shows no statistical difference between the manual and MyoVision myonuclear counts. MyoVision myonuclear counting accuracy is estimated to be 94.9±1.7% relative to manual analysis.

2.4.5 Software and Interface

The software (including source code) along with instructions and demonstration videos are freely available at www.MyoVision.org.
2.5 Discussion

High-content microscopy is a vital tool for muscle biology research. Routine image-based techniques require significant manual optimization and continuous human supervision. ImageJ plug-ins can differ significantly among laboratories and without standardization, indiscriminate usage of ImageJ plug-ins may exacerbate problems with reproducibility. SMASH is a well-designed software package that acts as a free version of commercially available image analysis systems, but it requires varying amounts of manual correction to ensure accuracy. The primary goal for MyoVision was to decrease the demand for human guidance without sacrificing analytical accuracy and precision.

MyoVision incorporates several algorithms that have been used in previous analysis programs [59, 60, 62-65, 67, 72-80] but streamlines the workflow to enhance computational efficiency and robustness. In particular, most previous algorithms used one of two options: the watershed transformation or the active contour model (snake algorithm). In contrast, MyoVision invokes both algorithms in sequence. The hybrid “watersnake” approach first separates connected muscle fibers using the efficient watershed transformation and then fine-tunes the cytoplasmic boundaries using the active contour method.

The data presented in this work show that MyoVision measures fiber number, CSA, fiber type, and myonuclear number with high accuracy (98.2%, 96.9%, 96.5%, and 94.9%, respectively). While these measurements can be performed by carefully trained investigators, manual analysis is often subjective and time-consuming, especially for myonuclear counting. As described in Methods,
completing all of the above measurements using manual techniques might require hours of sustained human effort per cross section. MyoVision requires a few minutes to produce statistically equivalent results. It also eliminates inter-individual variation which may facilitate data sharing among labs throughout the muscle research community.

The biggest limitation for MyoVision is its sensitivity to the quality of the input image. Because fiber detection and outlining are performed entirely on the reference image, degraded fluorescence signals can detrimentally impact the software’s accuracy. Patches of weak staining within a cross section will prevent accurate fiber detection. Irregular noise levels, especially high-intensity punctate noise and/or holes within the fiber myofibrillar regions, also cause problems for fiber detection. Currently, incorrect outlines can be eliminated through the user-interface by defining regions of interest. However, the software does not yet allow for manual addition of fiber outlines and/or modification of fiber boundaries. High quality input images are therefore required for accurate results. Detailed protocols for immunohistochemical processing of both mouse and human skeletal muscle are provided in the Appendix for reference. These protocols should help investigators to produce high quality images that can be processed quickly and accurately using MyoVision.

2.6 Appendix

2.6.1 Part I.

A detailed description of the rationale for algorithm design and software implementation. MyoVision draws from multiple previously reported algorithms
from as early as 1998. During the early stages of development, it became apparent very quickly that different groups may develop similar algorithms to analyze skeletal muscle, but default parameters tend to differ, and pre- and post-processing methods vary as well. The following sections provide a brief review of the literature, software design rationale, and detailed information of how MyoVision analyzes immunofluorescence images of skeletal muscle cross sections.

Program Development Considerations. Several published automatic algorithms for muscle fiber segmentation utilized the active contour model (GVF snake) and demonstrated very high levels of accuracy and consistency [59, 60, 62, 64, 77]. In 1998, Klemenčič et al. reported for the first time reasonable success using a spline-based active contour model to detect fiber boundaries and measure cross sectional area [60]. However, their implementation was not fully automatic because it involved a manual initiation step in which a trained researcher selected the center point for each fiber region to be analyzed. Brox et al. and Kim et al. improved upon the classic edge-based active contour approach by extending it with region-based intensity and texture components and implementing an automatic initialization procedure [59, 72]. They showed accuracy of greater than 98% for fiber counting and cross sectional area measurements; however, the algorithm was developed for H&E stained images. Recognizing the advantages of the automated active contour model, Sertel et al. further developed the algorithm to automatically analyze ATPase stained muscle cross sections and for the first time demonstrated an 89% accuracy for metabolic fiber typing [81]. Mula et al. subsequently implemented a slightly different automatic initialization protocol and applied the
active contour model to muscle cross sections enhanced by immunofluorescent labeling in 2013 [64]. A common theme among these approaches was the inclusion of a robust line detection algorithm, known as multiscale vessel enhancement filtering [74]. Despite improvements afforded by the gradient vector flow method of energy calculation, noise and concavities can still present problems to the GVF snake. Improvements in the form of the vector field convolution (VFC) method dramatically increased the capture range and noise resistance of the parametric snake [82]. As demonstrated by Brox et al. and Kim et al., incorporating region-based information significantly improves accuracy. The most widely used region-based active contour model is the classic active contour without edges first described by Chan and Vese in 2001 [73]. A recent study where the VFC-based energy calculation is combined with a modified version of the Chan-Vese algorithm, named VFCCV, highlighted the benefits of using both line- and region-based information [79].

Historically, one of the major weaknesses of the active contour model was its high computational demand. In order to avoid such computational costs, several other automatic implementations for the segmentation of skeletal muscle fiber cross sections employed the more computationally efficient watershed transformation [66, 78]. This was the same fundamental approach to muscle fiber segmentation used by the semi-automatic programs as well [67, 83]. One of the known drawbacks of the watershed transformation is over-segmentation, where single regions are erroneously separated into multiple smaller ones [84]. As such, local minima suppression and marker-controlled approaches were introduced in order
to minimize over-segmentation, but boundary accuracy remained an area of concern [85]. Precision is vital for consistently applying the rules necessary for classifying a myonucleus because even slight overestimations of the myofiber boundaries can result in significant variations in myonuclear counts [86]. Additionally, muscle tissue from different species can vary in the degrees of membrane thicknesses and inter-fiber networks, which made it imperative that automated myofiber segmentation programs take the local changes in membrane staining into strict consideration [83].

In 2012, Sáez et al. directly benchmarked the watershed transformation against the active contour model in immunofluorescently stained skeletal muscle cross sections and showed that the watershed transformation is up to 6-times faster than the active contour model with little difference in accuracy [78]. However, Sáez et al. did not report any optimization in terms of their active contour initialization and evolution conditions. This can be a significant step because, in other applications such as live cell tracking, the active contour model demonstrates higher accuracy than the watershed transformation, and given improved initialization conditions, the active contour model is not significantly slower than the watershed transformation [87]. For MyoVision, using VFC instead of GVF for the external energy calculation improves upon the capture range, which indirectly optimizes contour initialization by allowing faster convergence.

On the one hand, the active contour (snake) algorithm is very good at using local image information to evolve contours to the edges of the cells. On the other hand, the watershed transformation is extremely efficient in separating connected
objects. It became reasonably obvious that these two algorithms can complement each other, and implementing one algorithm does not preclude the implementation of the second. Therefore, MyoVision was developed as a hybrid “watersnake” algorithm. It uses a watershed transformation to separate connected myofibers and then optimizes the boundaries of these fibers using an active contour algorithm.

**Myofiber Detection.** The analytical workflow is shown in Figure 2.2. Briefly, a single channel image of a muscle cross section was immunofluorescently labeled for laminin (Figure 2.2A). The membrane reference image labeled with laminin or dystrophin immunofluorescence is processed using a 3x3 median spatial filter (Figure 2.2B). Edges are subsequently enhanced by calculating the multiscale Hessian-based edge probability map as described in [74] using scales from $\sigma_{\text{min}} = 1$ to $\sigma_{\text{max}} = 4$ because Sertel et al. empirically determined these scales produced the best edge enhancement results for skeletal muscle cross sections [81]. Each pixel of the edge probability map is classified using an automatic K-means clustering algorithm where the number of clusters is determined based on minimizing the average pixel to cluster center distances. The lowest cluster is considered background/cytoplasm, therefore creating a binary image where the membrane is white and everything else is black (Figure 2.2C). After clearing away regions touching the edge of the image, shape descriptors (extent, convexity, solidity, and area) of each cytoplasmic seed region are calculated. The extent of a region is defined as the ratio of the area of the region to the area of the smallest-area rectangle containing the region. Convexity is
defined as the ratio of the perimeter of the region to the perimeter of the convex hull of the region, where the convex hull is defined as the smallest convex polygon that can contain the region. Solidity is defined as the ratio between the area of the region and the area of the convex hull of the region.

In order to determine the optimal threshold for shape descriptors, a preliminary calibration study was performed. 6781 seed regions were generated from dystrophin labeled images of skeletal muscle cross sections and manually classified as ‘connected fibers’, ‘single cell’, or “non-cellular space’ (Figure 2.3A). Regions where multiple fibers were inadequately separated represented less than 8% of all generated seeds, whereas interstitial spaces represented almost 35% of all the seeds. This suggested that typical algorithms applied watershed segmentation over 100% of all seed regions whereas only 8% of the regions actually required watershed separation.

Seed region area (expressed as a percentage of the maximum area of all seed regions) was the best predictor of non-cellular or interstitial space (Figure 2.3B). There appeared to be very little distinction between connected and unconnected seed regions in terms of eccentricity, therefore this measure is not included as a differentiation factor (Figure 2.3F). Seed regions with extent values below 0.5, solidity less than 0.85, and convexity greater than 1.25 (Figure 2.3C-E) were identified as potentially connected myofibers and selected for further processing via a single watershed segmentation. Distances greater than 5 μm were suppressed during this process to further minimize over-segmentation. MyoVision thus restricted the watershed analysis to the regions that were most likely to be
connected fibers based on the described combinations of shape descriptor variables. The utilization of shape descriptors helped to minimize the problems typically associated with indiscriminate use of watershed methods. The contours of the seed regions were subsequently refined using the parametric spline-based active contour model. Image forces were calculated by convolving a vector field kernel (width = 64 pixels, type = power, $\alpha = 2.6$) with the gradient magnitude of the multiscale enhanced edge map generated in the second step (for a detailed description of vector field convolution see [82]). Additionally, image region information was incorporated as the difference between the average fluorescence intensities inside and outside of the evolving contour, similar to the algorithm described in the VFCCV approach [79]. Contour evolution was set to stop upon convergence (when the area stopped changing per iteration).

**Myofiber Type Classification and Myonuclear Counting.** For fiber typing analyses, MyoVision used K-means clustering to classify each pixel as foreground (myosin heavy chain staining) or background based on fluorescence intensity. Then, using the myofiber outline as detected in the reference (laminin/dystrophin) image, MyoVision classified the myofibers with greater than 25% foreground area as positive for the particular myosin heavy chain isoform. For myonuclear counting analyses, all nuclei were detected using the fluorescence channel stained for DNA content (DAPI for our images). Initial binarization was achieved using the automatic threshold algorithm described by Otsu [76]. The detected nuclear regions were subsequently subjected to a marker-controlled watershed segmentation in order to separate connected nuclei. Nuclei were classified as
myonuclear if both the center of mass for the nucleus and at least 50% of the total area of that nucleus fell within the myofiber as outlined using the reference image in accordance with previously published counting methods [62, 88].

2.6.2 Part II.

A detailed protocol for skeletal muscle immunohistochemistry that generates high quality images for MyoVision analysis.

Tissue Processing

Mouse
1. After sacrifice, coat the muscle liberally with Tissue-Tek OCT (Sakura Finetek, Torrance, CA) and freeze muscle in liquid-nitrogen cooled isopentane.
   - Muscle should be completely covered in OCT, as insufficient coverage can affect the quality of tissue preservation during freezing, and also makes it more challenging to obtain complete muscle cross sections when sectioning.
   - When the isopentane begins to freeze around the periphery of the freezing vessel, it is at the correct temperature to freeze the muscle.
   - Muscle should be pinned to a cork covered in aluminum foil at resting length. The muscle can be frozen directly on the aluminum, and after the isopentane has evaporated (~5 minutes on dry ice), can be easily transferred to the storage container.
   - For small muscles such as the plantaris and soleus, ~15-20 seconds is sufficient to fully freeze the muscle, whereas larger muscles such as the gastrocnemius may take longer (~30 seconds).
2. Remove enough muscle with a blade or scalpel so that cutting is initiated at or near mid-muscle belly, then mount the tissue upright in a bolus of OCT using freeze-spray.
   - Use the freeze-spray to ensure that the warm OCT does not cause freeze damage, and all manipulations should be performed in the cryostat.
3. Let the tissue equilibrate to the temperature of the cryostat (23-24°C) for 20-30 minutes, then cut 7µm thick sections from the mounted tissue onto charged slides.

Human
1. Following the biopsy procedure, identify a portion of muscle (~50mg) where fiber orientation can be determined. Mount the muscle on a small piece of cork
with the base coated in tragacanth gum. The muscle biopsy is mounted upright on the cork such that the fibers are perpendicular to the surface of the cork.

- Properly orienting the muscle biopsy in regards to fiber direction is needed for cross sectional assessment of the sample.
- A 'base' of tragacanth gum can be created on the cork so that one end of the muscle sample can be partially submerged in the tragacanth gum to stand the sample upright on the cork. This will prevent the muscle sample from folding over during the freezing process.
- The same isopentane preparation for the freezing of mouse muscle is applied to the human sample. Once the isopentane is sufficiently cold, the muscle can be placed 'muscle-side down' in the isopentane by holding the cork with forceps.
- The human muscle should remain in the chilled isopentane for ~15-20 seconds to ensure that the entire sample is frozen.

2. Human muscle samples frozen in tragacanth gum can be cut from their cork base and then mounted upright using OCT and freeze spray in a similar fashion to mouse muscle.

3. As with mouse muscle, the mounted human muscle will need to equilibrate to the temperature of the cryostat (23-24°C) for 20-30 minutes prior to cutting 7µm thick sections from the mounted tissue onto charged slides.

**Mouse and Human slide processing**

4. Let frozen cross sections air-dry at room temperature for 1-6 hours.
   - It is important that the sections are completely dry before moving them to -20°C for storage or beginning a staining protocol. Moisture in the tissue will cause bubbling of the sections, resulting in poor staining quality.
   - Sections can be stored in a sealed box at -20°C for several months prior to staining.
   - If using sections from the -20°C, allow to air dry for at least 5 minutes on the bench.
   - **For best results**, begin the staining protocol on the day of sectioning (at least an hour after the sections have been drying at room temperature). Staining quality declines the longer the samples are stored at -20°C.

5. Prior to initiating the staining protocol, draw a circle around the section with a PAP pen (Vector, #H-4000, Burlingame, CA).

**Mouse staining**

6. Re-hydrate the sections in PBS for 5 minutes.

7. Block the sections for 1 hour with Mouse-on-Mouse blocking kit (1 drop per 1 ml PBS) (Vector, MKB-2213, Burlingame, CA).
   a. This blocking step allows for the use of mouse primary antibodies on mouse tissue.
8. Wash 3 x 5 minutes in PBS.

9. Incubate the sections in 1°Ab for 90 minutes at room temperature (RT) or rocking overnight at 4°C (produces comparable results). Concentrates are diluted into supernatants:
   a. **MyHC Type 1 fibers**: BA.D5 IgG2b concentrate (1:100) (BA.D5, from Developmental Studies Hybridoma Bank (DHSB), Iowa City, IA)
   b. **MyHC Type 2a fibers**: SC.71 IgG1 supernatant (DHSB)
   c. **MyHC Type 2b fibers**: BF.F3 IgM supernatant OR **Type 2x fibers**: 6H1 IgM supernatant (DHSB)
      i. In our experience, BF.F3 (MyHC 2b) generally produces better results than 6H1 (MyHC 2x) in mouse tissue.
   d. **Laminin (fiber borders)**: Rabbit anti-Laminin (1:150) (Sigma, L9393, St Louis, MO)
      i. SC.71 and BF.F3 supernatants are combined in a 1:1 ratio with BA.D5 and laminin concentrate added at a 1:100 and 1:150 dilution, respectively i.e. for 100 µl of antibody, combine 50 µl SC.71 with 50 µl 6H1 and add 1 µl BA.D5 and 0.75 µl Laminin.

10. Wash 3 x 5 minutes in PBS.

11. Incubate the sections in 2°Ab for 60 minutes at RT, diluted in PBS.
   - **MyHC Type 1 fibers**: Goat anti-Mouse IgG2b, Alexa Fluor 647 conjugated 2°Ab (1:250) (Invitrogen, A21242, Carlsbad, CA). Type 1 fibers appear pink.
   - **MyHC Type 2a fibers**: Goat anti-Mouse IgG1, Alexa Fluor 488 conjugated 2°Ab (1:500) (Invitrogen, A21121). Type 2a fibers appear green.
   - **MyHC Type 2x fibers**: Goat anti-Mouse IgM, Alexa Flour 555 conjugated 2°Ab (1:250) (Invitrogen, A21426). Type 2x fibers appear red.
   - **Fiber borders**: Goat anti-Rabbit IgG, AMCA conjugated 2°Ab (1:150) (Vector, Cl-1000). Laminin appears blue.

12. Mount with Vectashield mounting media (Vector, H-1000).

**Human staining**

6. Re-hydrate the sections in PBS for 5 minutes.

7. Incubate the sections in 1°Ab for 90 minutes at room temperature (RT) or rocking overnight at 4°C (produces comparable results). Concentrates are diluted into supernatants:
   - **MyHC Type 1 fibers**: BA.D5 IgG2b concentrate (1:100) (BA.D5, from Developmental Studies Hybridoma Bank (DHSB), Iowa City, IA)
   - **MyHC Type 2a fibers**: SC.71 IgG1 supernatant (DHSB)
   - **Type 2x fibers**: 6H1 IgM supernatant (DHSB)
      i. 6H1 (MyHC 2x) works well in human muscle sections.
• **Laminin (fiber borders):** Rabbit anti-Laminin (1:150) (Sigma, L9393, St Louis, MO)
  
i. SC.71 and 6H1 supernatants are combined in a 1:1 ratio with BA.D5 and laminin concentrate added at a 1:100 and 1:150 dilution, respectively i.e. for 100 µl of antibody, combine 50 µl SC.71 with 50 µl 6H1 and add 1 µl BA.D5 and 0.75 µl Laminin.

8. Wash 3 x 5 minutes in PBS.

9. Incubate the sections in 2°Ab for 60 minutes at RT, diluted in PBS.

• **MyHC Type 1 fibers:** Goat anti-Mouse IgG2b, Alexa Fluor 647 conjugated 2°Ab (1:250) (Invitrogen, A21242). Type 1 fibers appear pink

• **MyHC Type 2a fibers:** Goat anti-Mouse IgG1, Alexa Fluor 488 conjugated 2°Ab (1:500) (Invitrogen, A21121). Type 2a fibers appear green

• **MyHC Type 2x fibers:** Goat anti-Mouse IgM, Alexa Flour 555 conjugated 2°Ab (1:250) (Invitrogen, A21426). Type 2x fibers appear red

• **Laminin (fiber borders):** Goat anti-Rabbit IgG, AMCA conjugated 2°Ab (1:150) (Vector Laboratories, CI-1000). Laminin appears blue

10. Wash 3x5 minutes in PBS.

11. Mount with Vectashield mounting media (Vector, H-1000).

**Dystrophin Staining**

Dystrophin, as opposed to laminin, can also be used for muscle fiber CSA determination. One dystrophin antibody that we use in our laboratory is a mouse IgG1, which means MyHC 2a fibers (SC.71) cannot be stained simultaneously. If excluding MyHC 2a fibers, all other fiber types can be co-stained with dystrophin using the protocols above (substituting dystrophin for laminin). Another dystrophin that works well is raised in rabbit and is compatible with all fiber typing antibodies. Total muscle fiber CSA can be determined (with or without fiber type) using dystrophin or laminin.

Dystrophin 1° Ab: **Mouse anti-Dystrophin IgG1** (1:100) (Vector, VP-D505) (Mouse-on-Mouse block for 1 hour prior to 1° Ab if staining mouse sections) or **Rabbit anti-Dystrophin** (1:200) (Santa Cruz, SC-15376)

2° Ab: **Goat anti-Mouse IgG1**, Alexa Fluor 488 conjugated 2° Ab (1:500) (Invitrogen, A21121) or **Goat anti-Rabbit IgG**, AMCA conjugated 2°Ab (1:150) (Vector, CI-1000)

**Dystrophin and DAPI Staining for Myonuclear Counts**

Utilizing the same procedures as above:

Dystrophin 1° Ab: **Mouse anti-Dystrophin IgG1** (1:100) (Vector, VP-D505) (Mouse-on-Mouse block for 1 hour prior to 1° Ab if staining mouse sections) or **Rabbit anti-Dystrophin** (1:200) (Santa Cruz, SC-15376) in PBS for 90 minutes at RT or ON rocking at 4°C.
2° Ab: **Goat anti-Mouse IgG1**, Alexa Fluor 488 conjugated 2° Ab (1:500) (Invitrogen, A21121) or **Goat anti-Rabbit IgG** Alexa Fluor 555 (Invitrogen, A-21429) for 60 minutes in PBS at RT (Dystrophin appears green or red, respectively).

Nuclei can be identified via DAPI staining prior to applying coverslips: Incubate 5 minutes in **DAPI** (1:10,000 diluted in PBS at RT) (Invitrogen, D35471), then coverslip with Vectashield mounting media (Vector, H-1000). Alternatively, slides can be cover-slipped using Vectashield with DAPI (Vector, H-1200).
2.7 Figures

Figure 2.1. MyoVision Fiber Outline.

A. Dystrophin-labeled immunofluorescence intensity image of a mouse plantaris muscle cross section false-colored in green. B. Same image as in A with MyoVision cell outline dotted in yellow. Scale bar = 25 μm.
Figure 2.2. MyoVision Workflow.

1. Standard Median Filter

2. Multiscale Frangi Enhancement

3. K-Means Binarization

4. Fill in Holes

5. Filter out extracellular regions

6. Identify potentially connected cells

7. Separated potentially connected cells

8. Finalize seed regions for contour evolution

Final MyoVision Fiber Outline
Figure 2.2. MyoVision Workflow.

Major steps in the MyoVision cell detection and outlining algorithm. For detailed descriptions, please see the Appendix. Steps 1-2: filtering to prepare for initial segmentation. Step 3-4: convert the image to foreground and background and then perform morphological operations. Steps 5-6: calculate shape descriptors for each seed region and identify potentially connected seeds. Step 7: distance-based watershed transformation to separate the potentially connected fibers. Step 8: combine separated fibers with single fibers and generate parametric splines to prepare for contour evolution. Scale bar = 25 μm.
Figure 2.3. Empirically Determined Thresholds for Shape Descriptors.

A. Representative images of seed regions (red), dystrophin overlay (green) and their respective manual classifications. Shape descriptors are calculated for a total of 6781 seed regions (520 connected, 3939 single cells, and 2322 interstitial spaces). B-F. Shape descriptors and their respective frequency distributions (black dotted line represents the threshold selected for the MyoVision algorithm).
Figure 2.4. Fiber Detection and Counting.

A: Graph showing the number of myofibers detected by software compared to the number counted manually.

B: Bar chart comparing the estimated accuracy of MyoVision, SMASH, and ImageJ.

C and D: Images showing original and SMASH processed images.

E and F: Images showing MyoVision and ImageJ script processed images.
A. Comparison of MyoVision (blue), SMASH (red), and ImageJ plug-in (green) with manual fiber counting for 6 mouse plantaris muscles. MyoVision counts lie directly on the line of identity (black). Markers above the line of identity represent over-segmentation. B. The estimated accuracy of each method is expressed as percent difference from manual counts (*** denotes p < 0.001). C-F. Representative images of the segmentation for each algorithm compared to the original laminin-labeled (blue) image. Significant over-segmentation (a single fiber is broken up into multiple fibers) can be readily observed for the ImageJ plug-in. Scale bar = 25 μm.
**Figure 2.5. MyoVision Vs Semi-Manual Outlines.**

A. MyoVision (yellow) and manual (green) cell outlines for the same dystrophin-labeled image. B. Zoomed-in view of the region from A boxed in purple dotted line. White arrows highlight the difference between the MyoVision and the manual outlines. MyoVision outlines are closer to the dystrophin staining, therefore producing fiber CAS measurements that are consistently larger than the manual measurements (Figure 6A). Scale bar = 25 μm.
Figure 2.6. Average Fiber Cross Sectional Area.
Figure 2.6. Average Fiber Cross Sectional Area.

A. Comparison of MyoVision (blue) and SMASH (red) cell size measurements with manual measurements for 16 mouse plantaris cross sections with and without mechanical overload. The line of identity is shown in black. MyoVision measurements are bigger than semi-manual measurements for every sample. Dashed line denotes linear regression line. B. Estimated accuracy for MyoVision and SMASH measurements of fiber CSA (*** denotes p < 0.001). C. The relative increase in fiber CSA (hypertrophy) over the mechanical overload time course. SMASH demonstrates significant underestimation of fiber CSA as growth increases (* and ** denote p < 0.05 and 0.01, respectively).
Figure 2.7. Fiber Type Classification.

A. Plantaris cross section image immunofluorescently stained for three different myosin heavy chain subtypes and labeled for laminin. B. MyoVision outline and classification of fiber types in A. C. Comparison of MyoVision fiber type distribution results with manual counts for 6 murine plantaris muscles. The line of identity is shown in black. D. Comparison of average fiber type distribution for all mice shows no significant difference between MyoVision and manual analyses. Scale bar = 25 μm.
Figure 2.8. Myonuclear Number.

A. Plantaris cross section image immunofluorescently labeled with DAPI for nuclei and co-labeled for dystrophin. B. MyoVision outlines (dotted yellow lines) and classification of the myonuclei (yellow plus signs) in A. C. Comparison of MyoVision myonuclear counting results with human counts for 6 mouse plantaris muscles. The line of identity is shown in black. D. Comparison of average myonuclei per fiber for all mice shows no significant difference between MyoVision and human analysis. Scale bar = 25 μm.
Chapter 3. β-Catenin Regulation of Ribosome Biogenesis During Skeletal Muscle Hypertrophy

3.1 Abstract

Cytoplasmic free β-catenin is tightly regulated as a downstream effector in the canonical Wnt signaling cascade, which is capable of implementing a cellular growth program during development and regeneration. Previous studies suggest that Wnt signaling is intimately involved in the regulation myogenesis and muscle repair, and that β-catenin may be a key contributor to hypertrophic growth in adult skeletal muscle through the regulation of c-myc and ribosome biogenesis. We generated an adult muscle specific mouse model of inducible β-catenin inactivation. Using synergist ablation as a surgical model of skeletal muscle mechanical overload, we demonstrate that β-catenin is necessary for effective myofiber hypertrophy. After 7 days of overload, average myofiber cross sectional area increases significantly for the plantaris muscle with intact β-catenin but not for muscle with β-catenin deletion. Although pre-47S transcripts are significantly decreased with β-catenin knockout, c-myc transcript and total RNA do not differ, indicating no significant effect of β-catenin knockout on the accumulation of ribosomal RNA during the first week of overload. Interestingly, β-catenin deletion results in significantly reduced RPS6 activation after 3 days of overload with no difference in mTOR and p70S6K phosphorylation, suggesting a previously unstudied relationship between β-catenin and translational efficiency during the first week of mechanical overload that is independent of mTOR. Surprisingly, myofiber β-catenin depletion leads to an enhanced proliferation of satellite cells
during mechanical overload and enhances regeneration after chemically induced muscle injury. β-catenin is known to localize to the sarcolemma, and together with M-cadherins, contribute to the myofiber-satellite cell niche. These findings suggest that myofiber-specific perturbation of the M-cadherin/β-catenin junctional complex is sufficient to modify satellite cell quiescence and may represent a favorable therapeutic strategy to accelerate patient recovery from anticipated injuries.

3.2 Introduction

β-catenin is a transcription factor that functions in regulating cell proliferation and differentiation. It is known to be regulated through the canonical Wnt signaling pathway. Activation of Frizzled receptors by Wnt ligands causes Disheveled to inhibit GSK-3β, thereby relieving inhibition of β-catenin [38]. Cytoplasmic β-catenin accumulates, allowing it to translocate into the nucleus and drive expression of growth-related genes such as c-myc [39-41]. c-Myc is a transcription factor that promote Pol I-mediated transcription of ribosomal RNA by binding to the promoter of ribosome DNA [23, 24]. There is also evidence for c-myc regulation of ribosomal protein genes [89, 90]. During skeletal muscle hypertrophy in response to mechanical overload, β-catenin levels in the cytoplasm and the nucleus increase significantly, and this is accompanied by increased c-myc protein in nuclei [43]. A follow up study using adenoviral delivery of Cre recombinase into skeletal muscle of a floxed β-catenin mouse demonstrated that myofibers expressing the viral Cre recombinase are smaller in size compared to myofibers without Cre [44]. This finding suggests that β-catenin is necessary for myofiber hypertrophy, and its role is possibly related to the induction of c-myc mediated ribosome biogenesis.
Given the Wnt/β-catenin/c-myc regulation of ribosome biogenesis and the need for increased ribosome production prior to cell growth, we used an inducible, adult skeletal muscle-specific β-catenin knockout mouse to test the hypothesis that β-catenin regulates ribosome biogenesis through c-myc, and β-catenin is necessary for skeletal muscle cellular hypertrophy during mechanical overload.

3.3 Materials and Methods

3.3.1 Animals

All animal procedures were carried out in accordance with institutional guidelines for the care and use of laboratory animals, as approved by the Institutional Animal Care and Use Committee of the University of Kentucky. Mice were housed in a temperature- and humidity-controlled room with a 14:10 light-dark cycle. Food and water were provided ad libitum. The Cre driver mouse expressing mERCreER (mCm) under the control of the human skeletal actin (HSA) promoter (described in [91]) was crossed with a second mouse strain, containing the Ctnnb1 gene flanked (Exon 2-6) by loxP sequences (Ctnnb1fl/fl, [53, 92]), generating HSA-mCm X Ctnnb1fl/fl (Figure 3.1A). The HSA-mCm mouse allows the transcription of Cre recombinase to be regulated by the adult skeletal muscle-specific HSA promoter. This promoter has been demonstrated to drive high levels of gene expression only in mature myofibers [91]. The Cre recombinase is attached to the estrogen ligand binding domain which has been mutated so that it will no longer bind estrogen but will bind tamoxifen with high affinity [93]. Although mCm protein is expressed highly in adult skeletal muscle, in the absence of 4-hydroxy tamoxifen, heat shock protein 90 (HSP90) sequesters the mCm protein in the cytoplasm. When mice are treated
with tamoxifen, tamoxifen binds to mCm, releasing it from HSP90, and allowing mCm translocation into the nucleus, thereby allowing mCm to approach the genomic DNA. mCm is then able to recognize and act on the flanking loxP sites located before exon 2 and after exon 6 of the Ctnnb1 locus in the genome. These mice were used to specifically deplete β-catenin with tamoxifen administration (knockout; KO) and they are compared to control mice which are administered a sunflower seed oil vehicle (wild-type; WT).

A total of 18 animals were subjected to sham surgeries (9 WT and 9 KO); 8 animals were subjected to synergist ablation surgeries, injected with EdU, and sacrificed after 3 days of mechanical overload (3 WT and 5 KO); 10 animals were subjected to synergist ablation surgeries and sacrificed after 7 days of mechanical overload (5 WT and 5 KO); 9 animals were subjected to PBS/BaCl2 injections and sacrificed after 7 days of regeneration (4 WT and 5 KO). During tissue collection, one WT sham plantaris muscle was damaged, therefore the sample size is 8 for quantitative PCR analyses instead of 9 for cross section analyses.

### 3.3.2 Synergist Ablation Surgery

Following a three-week washout period after vehicle or tamoxifen treatment, mice were subjected to either sham or synergist ablation surgery (Figure 3.1B). Surgical removal of synergist muscles (gastrocnemius and soleus) bilaterally was performed as described by McCarthy et al. (2011) [94]. Mice were anesthetized using isoflurane under sterile conditions, and a longitudinal incision on the dorsal aspect of the hindlimb exposed the Achilles tendon. The tendon of the gastrocnemius muscle was isolated and used to guide the excision of this muscle;
the soleus muscle was then carefully removed without disturbing the nerve, blood supply, and plantaris muscle.

### 3.3.3 EdU Labeling

For labeling of proliferating satellite cells, mice were given 1 mg of EdU dissolved in sterile PBS injected i.p. starting immediately after synergist ablation surgery. The same dose was given every 24 hours for a total of 3 injections. Only animals subjected to three days of mechanical overload were injected with EdU.

### 3.3.4 BaCl₂-Induced Muscle Injury

Mice were anesthetized with isoflurane and the tibialis anterior (TA) were injected with either 50 µl of 1.2% BaCl₂ solution or sterile PBS [94]. After seven days, TA muscles were collected for analysis.

### 3.3.5 Tissue Preparation

During the synergist ablation surgery, the excised piece of gastrocnemius muscle from one hindlimb was flash frozen in liquid nitrogen and used for extraction of genomic DNA as well as total RNA. Genomic DNA was used to determine whether or not vehicle and tamoxifen administration induced the desired recombination of the β-catenin gene (Figure 3.1C). Total RNA was used to synthesize cDNA in order to assess the effect of recombination on β-catenin transcript levels under resting conditions (Figure 3.1D).

Plantaris muscle from sham and overloaded animals were collected bilaterally, with one muscle flash frozen in liquid nitrogen for biochemical analyses. The other muscle was pinned at resting length to a cork covered in aluminum foil and covered
in optimal cutting temperature (OCT) compound (Tissue Tek, Sakura Finetek, Torrance, CA, USA) in preparation for immunohistochemistry (IHC) analyses. OCT covered muscles were frozen in liquid nitrogen-cooled isopentane and stored at -80°C. At the time of sectioning, muscles were trimmed to mid-belly using a razor blade and oriented upright in OCT, which was then frozen using freeze spray in order to secure the muscle sample in place. Mid-belly plantaris muscle sections were cut on a cryostat at -20°C. Frozen muscle sections (7 µm) were air-dried for ≥2 hours and stored at -20°C.

3.3.6 Immunohistochemistry (IHC)

For quantification of myofiber cross sectional area (CSA) in Figure 3.2, muscles were fixed in 4% paraformaldehyde (PFA) for 7 minutes, incubated with rabbit anti-dystrophin IgG antibody overnight (1:100, ab15277, Abcam, Cambridge, MA, USA), washed 3 times 5 minutes each in PBS, and then incubated with Alexa Fluor 488 goat anti-rabbit IgG (1:250, A11034, Invitrogen, Carlsbad, CA, USA) for 1 hour.

For Pax7/Laminin/Nuclei IHC, muscles were fixed in 4% PFA for 7 minutes, incubated with rabbit anti-laminin IgG antibody overnight (1:100, L9393, Sigma-Aldrich, St. Louis, MO, USA), then incubated with Alexa Fluor 488 goat anti-rabbit IgG (1:250, A11034, Invitrogen, Carlsbad, CA, USA) for 1 hour. Following epitope retrieval in sodium citrate (10 mM, pH 6.5) for 20 minutes at 92°C, endogenous peroxidases were blocked for 7 minutes with 3% hydrogen peroxide in PBS, followed by 1 hour with 1% Tyramide Signal Amplification (TSA) blocking reagent (TSA kit, T20935, Invitrogen) supplemented with Mouse-on-Mouse (MoM) IgG
blocking reagent (Vector Laboratories, Burlingame, CA, USA). Sections were washed in PBS and incubated overnight with mouse anti-Pax7 IgG1 antibody (1:100, Developmental Studies Hybridoma Bank [DHSB], Iowa City, IA, USA) diluted in 1% TSA blocking reagent. The next day, sections were washed with PBS 4 times 5 minutes each time, incubated for 70 minutes in goat anti-mouse IgG1 biotinylated secondary antibody (1:1000, 115-065-205, Jackson ImmunoResearch, West Grove, PA, USA), washed in PBS, incubate for one hour in streptavidin-horseradish peroxidase (1:500, S-911, Invitrogen) diluted in PBS, washed again in PBS, then incubated for 15 minutes in TSA Alexa Fluor 594 (1:100, TSA kit, Invitrogen) in the supplied amplification diluents. Sections were stained with 4′,6-diamidino-2-phenylindole (DAPI) (1:10,000 in PBS, D35471, Invitrogen) for 5 minutes and mounted with VectaShield fluorescent mounting media (Vector).

For EdU detection, sections were first stained for laminin and Pax7 and then subjected to Click-iT® chemistry. Before counterstaining with DAPI, sections were incubated in a solution containing Tris base (100 mM), copper(II) sulfate (4 mM), biotin-conjugated azide (100 μm; Jena Biosciences), and ascorbic acid (100 mM) for 30 minutes at room temperature and washed, and this procedure was repeated. Sections were then incubated in Texas red–streptavidin (1:150; Vector Labs, Burlingame, CA) for 60 minutes at room temperature. Sections were post-fixed in 4% PFA and counterstained with DAPI.

To quantify embryonic myosin heavy chain (eMyHC) and CSA, sections were incubated for 1 hour with MoM blocking reagent (Vector), then incubated overnight
with Myh3 anti-eMyHC IgG1 (Neat, F1.652, DHSB) and anti-laminin (1:100, Sigma Aldrich). The following day, sections were washed and incubated with goat anti-
mouse Alexa Fluor 488 IgG1 (1:250, A-21121, Invitrogen) and Alexa Fluor 647 donkey anti-rabbit IgG (1:100, A31573, Invitrogen) for 1 hour, stained with DAPI (Invitrogen), then mounted with VectaShield (Vector). Dystrophin IHC was used to visualize fiber borders following the same protocol described above.

### 3.3.7 Image Quantification

Image quantification was performed using the scripted version of MyoVision. Basic analyses including average myofiber CSA and MyHC fiber typing were performed as described above in chapter 2 and in [95]. One muscle cross section per mouse was analyzed, representing 600-1200 myofibers per plantaris cross section and ~2000-3500 myofibers per TA cross section. Central nuclei counting was performed using a modified myonuclear counting algorithm with the addition of a new rule: the center of mass for the myonucleus must be at least one minimum Feret radius away from the closest point on the membrane as delineated by MyoVision using the dystrophin labeled image as a reference.

Satellite cells and dividing cells were counted manually. Nuclei defined by DAPI with more than 80% area labeled with either Pax7 or EdU was considered positively stained. Pax7-positive nuclei within the laminin border were considered satellite cell nuclei.

### 3.3.8 Genotyping and Recombination PCR
Genomic DNA was isolated from 20mg of tissue (liver, heart, and gastrocnemius muscle) using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany) and screened for the presence of the Ctnnb1 floxed (223 nt) and recombined (~500 nt) bands by PCR using the following primers [53, 92]:

- floxed forward, 5′- AAGGTAGAGTGATGAAAGTTGTT-3’
- recombined forward, 5′- TACACTATTGAATCACAGGGACTT-3’
- reverse 5′- CACCATGTCTCTGTCTATTC-3’

Genotyping reactions were PCR amplified through 30 cycles (primers 23F and 460R) (94°C for 30 seconds, 53°C for 30 seconds and 72°C for 1 minute).

### 3.3.9 RNA isolation

Total RNA was isolated from gastrocnemius muscle previously frozen in liquid nitrogen. Samples were homogenized in a tissue homogenizer (Bullet Blender, Next Advance Inc., Averill Park, New York, USA) using TRIzol, according to the manufacturer’s instructions except that chloroform was replaced by the less toxic 1-bromo-3-chloro-pentane (BCP) to achieve a tighter interphase and improve phase separation. Following isolation, RNA samples were treated with TURBO DNase (Ambion® | Thermo Fisher Scientific, Waltham, Massachusetts, USA). RNA concentration and quality was assessed using the NanoDrop spectrophotometer (Thermo Scientific) and measuring the ratio between absorbance values at 260 nm and 280 nm (~2.0 for good quality RNA) [96].

### 3.3.10 cDNA synthesis
cDNA was synthesized from 0.1 to 0.5 μg of total RNA using the SuperScript® VILO™ (ThermoFisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions.

### 3.3.11 RT-qPCR

*Ctnnb1* (Mm00483029_g1), *Myc* (Mm00487804_m1), *Rn7sk* (Mm03456444_s1), and *Gapdh* (Mm99999915_g1) gene expression were analyzed using a TaqMan gene expression assay (ThermoFisher Scientific, Waltham, Massachusetts, USA), TaqMan Gene expression Master Mix (2x) (ThermoFisher Scientific, Waltham, Massachusetts, USA), and a 20-fold dilution of cDNA in a 20µl final volume. Quantitative PCR for the *Pre-47S* gene was performed using KiCqStart SYBR Green qPCR ReadyMix (Sigma-Aldrich, St. Louis, MO) with the following cycle conditions: 95°C for 3 minutes, 40 cycles at 95°C for 30 seconds, and at 60°C for 60 seconds. All reactions were performed in duplicates and all transcripts were normalized to the geometric mean of *Gapdh* and *Rn7sk*.

Primer sequences for *Pre-47S*, *Rn7sk*, and *Gapdh* were described previously [68, 97].

- *Pre-47S* forward 5'-CTGACACGCTGTCCTTTCCC-3'
- *Pre-47S* reverse 5'-GTGAGCCGAAATAAGGTGGC-3'
- *Rn7sk* forward 5'-CCTGCTAGAACCTCCAAACAA-3'
- *Rn7sk* reverse 5'-TCTGGAGTCTTGGAAGCTTGA-3'
- *Gapdh* forward 5'-ACCCCTTCATTGACCTCAACTACATGG-3'
- *Gapdh* reverse 5'-ATTTGATGTTAGTGGGGTCTCGCTCCTCCT-3'
SYBR Green qPCR efficiency was calculated by linear regression from fluorescence increase in the exponential phase in the LinRegPCR software v11.1 as described in [98]. Quantification of relative fold change was calculated after correcting for amplification efficiency using the ΔΔCT method [99].

3.3.12 Protein preparation

Frozen muscles were cut into small pieces (~10 mg) on dry ice and homogenized in 30 volumes of RIPA buffer with Halt™ protease and phosphatase inhibitor cocktail (78429, ThermoFisher Scientific, Waltham, Massachusetts, USA) using a Bullet Blender (Next Advance, Inc, Averill Park, NY) with one scoop of 1 mm Zirconium Oxide beads at speed 10 for 3 minutes two times. Insoluble materials and beads are removed after centrifugation at 10,000g for 10 minutes at 4°C. Protein lysates were quantified using the BioRad DC protein assay and prepared for SDS-PAGE by boiling for 5 minutes in the presence of 2% SDS, 1% β-mercaptoethanol, 6% glycerol, 50 mM Tris-HCL (pH 6.8 at room temperature), and 0.004% bromophenol blue.

3.3.13 Western blot analysis

Protein lysates were subjected to SDS-PAGE. 20 µg of protein were loaded in each lane of a 1.5 mm 4-15% gradient gel and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membrane and blocked for 1 hour at room temperature using 5% bovine serum albumin (BSA) in Tris buffer saline with 0.1% Tween®-20 (TBST). Primary antibodies were diluted 1:1000 and secondary antibodies were diluted 1:10,000 in 5% BSA in TBST. Membranes were incubated
with gentle shaking in primary antibodies overnight at 4°C and in secondary antibodies for 30 minutes at room temperature. Primary antibodies against total mTOR (#2983), phospho-mTOR (Ser2448, #2971), total p70S6K (#9202), phospho-p70S6K (Thr389, #9206), total RPS6 (#2217), and phospho-RPS6 (Ser240/244, #5364) were obtained from Cell Signaling Technology (Danvers, MA, USA). Protein bands were visualized using goat anti-rabbit IgG Alexa Fluor 680 (A-21109) and goat anti-mouse IgG Alexa Fluor 790 (A11357) secondary antibodies obtained from ThermoFisher Scientific and images were taken using the Odyssey® imaging system (LI-COR Biosciences, Lincoln, NE, USA). Band quantification was performed using the ImageJ gel analysis tool.

### 3.3.14 Statistics

Reported values represent mean ± standard error (SE). Statistical analyses were performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. P values less than 0.05 were considered to be statistically significant. Two-sample two-tailed student’s t-test was performed for (1) Ctnnb1, myc, and pre-47S transcript analyses from gastrocnemius muscles prior to overload; (2) mTOR, p70S6K, and RPS6 phosphorylation; and (3) dividing satellite cell count for 3-day overload and regeneration eMyHC CSA analysis. Two-way ANOVA were performed for all other data, with β-catenin knockout as one factor and either duration of overload (sham, 3, and 7 days) or BaCl₂-injury as the second independent factor. Post-hoc analyses were performed only when one or both main effects were significant and post-hoc tests were selected to minimize number of comparisons and maximize
the power to detect a significant difference given the sample sizes. For the overload CSA data, a two-family two-comparison (4 total comparisons) Dunnett’s test was performed and all overload groups compared to their respective shams to detect significant increases in average myofiber CSA. For total RNA and c-myc transcript with overload, one-family five-comparison (5 total comparisons) Dunnett’s tests were performed and all groups were compared to WT Sham because there was no significant main effect of β-catenin knockout. For the pre-47S expression and satellite cell count data, one-family three-comparison Sidak’s tests were performed comparing KO to WT to detect the point during the first week of overload at which differences reached significance. For all TA injury data, one-family two-comparison Sidak’s tests were performed comparing KO to WT to detect whether the regeneration responses differed significantly.

3.4 Results

3.4.1 Skeletal muscle-specific deletion of β-catenin leads to decreased c-myc and pre-47S transcript levels in resting muscle

As previously described in [91], the HSA-mCm driven recombination is specific to skeletal muscle and only in the presence of tamoxifen, as illustrated graphically in Figure 3.1A. Mice were treated with vehicle or tamoxifen and muscle tissue was harvested three weeks after the end of treatment (treatment regimen diagrammed in Figure 3.1B). No Cre-mediated recombination was detected in non-skeletal muscle tissue with or without tamoxifen, and in skeletal muscle, only in the presence of tamoxifen was the β-catenin recombined band detected, indicative of deletion of exons 2-6 (Figure 3.1C). In order to quantify the level of ablation, β-
catenin transcript abundance in the gastrocnemius muscle from ambulatory animals was measured using qPCR. Three weeks following the end of vehicle or tamoxifen administration (Figure 3.1.B), β-catenin transcript abundance was significantly lower (p<0.001) by approximately 80% in KO animals compared to WT (Figure 3.1D). This reduction in β-catenin mRNA expression was accompanied by significantly (p<0.05 and p<0.01, respectively) decrease of approximately 40% in c-myc and pre-47S gene expression in the gastrocnemius muscle (Figure 3.1D). These data suggest that in resting muscle, myofiber β-catenin controls basal transcriptional activity of c-myc and pre-47S genes.

3.4.2 β-Catenin is required for efficient skeletal muscle myofiber hypertrophy

After vehicle or tamoxifen administration, synergist ablation was used to induce hypertrophy in both KO and WT mouse plantaris muscles (illustrated in Figure 3.2A). Mice were sacrificed three and seven days after overload and plantaris muscle was collected for analysis. Figure 3.2C-F show representative images of dystrophin-labeled muscle cross sections from both sham and overloaded plantaris after seven days of mechanical overload. Overloaded WT myofibers (Figure 3.2D) exhibited visibly larger areas compared to WT sham (Figure 3.2C), which is the definition of myofiber hypertrophy. This difference was not visually discernable for KO overload (Figure 3.2F) compared to KO sham (Figure 3.2E). Quantification of myofiber CSAs at both 3 and 7 days showed that mean myofiber CSAs from WT mice was significantly (p<0.01) larger compared to control (WT sham) muscle only after seven days of overload (Figure 3.2B). In contrast, mean
myofiber CSAs for KO overload were not significantly different from those of KO sham after three or seven days. Notably, there was a significant effect of β-catenin KO independent of overload on mean myofiber CSAs (two-way ANOVA, p<0.05), and the mean myofiber CSAs for KO plantaris is lower than WT CSA for all groups.

3.4.3 β-catenin’s effect on myofiber hypertrophy cannot be explained by its hypothesized role in ribosome biogenesis

At the beginning of the study, we hypothesized that β-catenin drives c-myc transcription which in turn increases pre-47S transcript levels to increase muscle ribosome biogenesis. Because rRNA contributes to the majority of total cellular RNA, we used total RNA as an indicator of ribosome content during overload. Figure 3.3 shows that although we detected significant differences in pre-47S (p<0.05), c-myc (p<0.0001), and total RNA (p<0.0001) between sham and overloaded muscle, only pre-47S transcript levels were significantly affected by β-catenin KO (p<0.05). Transcript levels for pre-47S were highest after three days of overload (Figure 3.3A). Consistent with pre-47S expression, c-myc transcript abundance was significantly higher after three days of overload (Figure 3.3B). Both pre-47S and c-myc expression changes preceded the significant increases in total RNA, which occurred after seven days of synergist ablation (Figure 3.3C). Gene expression for pre-47S resembles the average myofiber CSA data in the sense that the mean values are lower for KO muscle compared to WT muscle for all groups. More importantly, β-catenin KO had no significant effect on c-myc expression and total RNA accumulation.
Given that translational capacity was not reduced by β-catenin inactivation, yet the hypertrophic response was diminished in the KO, we hypothesized that translational efficiency was affected by the loss of β-catenin because translational efficiency is significantly increased during the early stages of skeletal muscle overload [33, 100, 101]. To test this hypothesis, western blots were performed to quantify the activation state of key regulators of translational efficiency (representative blots for mTOR, S6 kinase 1, and RPS6 are show in Figure 3.4A). We did not detect significant differences between WT and KO muscle in mTOR (Figure 3.4B) or S6 kinase 1 (p70S6K, Figure 3.4C) activation after three days of overload. However, RPS6 phosphorylation was significantly (p<0.05) lower in KO compared to WT muscle after three days of overload (Figures 3.4A and D).

**3.4.4 β-catenin knockout enhances overload-associated satellite cell proliferation**

It is well documented that mechanical overload activates resident muscle stem cells, or satellite cells. Satellite cell number, as quantified by the number of Pax7+ nuclei on cross section (Figures 3.5A-B, pink arrows), were higher with functional overload [102, 103]. Consistent with previous reports, the satellite cell pool expands significantly (p<0.0001) within the first week of overload, and β-catenin KO significantly (p<0.01) affected satellite cell number independent of overload (interaction p>0.05). Post-hoc test comparing KO to WT revealed significant (p<0.05) differences in satellite cell number only after seven days of overload (Figure 3.5C). Although Pax7+ nuclei did not differ after three days of overload, satellite cell EdU incorporation was significantly (p<0.05) greater in the β-catenin
KO muscle compared to WT by day three (Figure 3.5D). Because there was no detectable difference between WT and KO satellite cell number without overload, we interpret these data to suggest that lack of β-catenin within myofibers allows resident satellite cells to proliferate faster in response to a hypertrophic stimulus.

3.4.5 Myofiber β-catenin knockout enhances satellite cell response to injury

Given the results reported above, we hypothesized that depletion of myofiber β-catenin would enhance satellite cell response to an injury stimulus and improve muscle regeneration. To test this idea, tibialis anterior (TA) muscle of WT and KO mice, were subjected to BaCl₂ injections to induce muscle damage. Each contralateral TA muscle was injected with equal volumes of PBS to serve as a control. The TA muscles were allowed to regenerate for seven days under ambulatory conditions. TA mass and mean myofiber CSA were significantly (p<0.0001) lower for both WT and KO muscles following BaCl₂ compared to PBS injection, and KO significantly (p<0.01) affected both TA mass and mean fiber CSA independent of the effects of injury (interaction p>0.05). Post-hoc tests indicate that TA masses did not differ significantly between WT and KO muscles injected with PBS; however, one week after BaCl₂ injections, KO muscles had significantly (p<0.05) more mass than WT muscles (Figure 3.6A). Mean myofiber CSA in the KO TA were roughly 16.6% bigger (p<0.01) than those in the WT TA after PBS injections, but do not differ significantly between WT and KO TA after injury (Figure 3.6B). Notably, KO did not have the same effect on total myofiber number per TA cross section for injured and control muscles (interaction p<0.05). Figure 3.6C shows that injury caused a decrease in cell count for WT but an increase for KO
TA muscle on cross section, with a trending difference between KO and WT cell count after seven days of regeneration ($p=0.052$).

β-catenin KO injured muscle exhibited significantly ($p<0.001$) more embryonic myosin heavy chain positive (eMyHC+) myofibers than WT muscle (Figure 3.6D). In addition to having more eMyHC fibers in regenerating KO TA muscle, the average eMyHC+ myofiber CSA for KO injured muscles were 31.2% larger than those of WT injured muscles ($p<0.05$, Figure 4.6E). KO TA also exhibited a 24.2% higher number of central nucleated myofibers ($p<0.05$, Figure 3.6F), suggesting that more satellite cells had fused into each myofiber.

3.5 Discussion

Skeletal muscle under mechanical overload accumulates protein mass and increases myofiber size. This hypertrophic response, at the molecular level, is associated with significant increases in β-catenin and c-myc transcription, translation, and translocation into the nucleus [43, 44]. Because of the integral role of c-myc in regulating ribosome biogenesis and the importance of having enough ribosomes for protein translation during cellular growth, we hypothesized that in the absence of β-catenin, an activator of c-myc transcription, skeletal muscle would lose its ability to induce robust increases in c-myc expression and ribosomal RNA production. Our results showed that despite the ablation of myofiber β-catenin, the acute induction of c-myc expression was significant and no different compared to WT after three days of synergist ablation. In agreement with the c-myc response, pre-47S rRNA transcript abundance exhibited no significant interaction between overload and KO. Total RNA levels, as a readout of muscle ribosome content,
increased in both WT and KO in an identical fashion during the first week of overload, suggesting that β-catenin is dispensable for both overload-induced increases in c-myc and ribosome biogenesis. It’s important to keep in mind that synergist ablation is an extreme model that maximally stimulates plantaris growth and likely saturates any and all mechanisms capable of contributing to cell mass gain. Still, the absence of β-catenin resulted in a slight but reduced level of myofiber hypertrophy because average CSA, compared to sham control, were significantly higher in WT muscle after seven days of overload, but KO muscle failed to reach statistical significance compared to KO sham control. This relatively small effect of β-catenin deletion on muscle hypertrophy can be better interpreted outside the context of such an overwhelming stimulus as synergist ablation. In the absence of a growth stimulus, deleting myofiber β-catenin significantly reduced resting muscle c-myc and pre-47S transcript abundance. This effect was well correlated with the lower average CSA of KO relative to WT muscle for sham control as well as both overload time points. Taken together with the lack of significant interaction between β-catenin status and mechanical overload, these data suggest that β-catenin may be an important contributor to mass maintenance at rest but becomes less important during hypertrophic growth. These findings are consistent with the idea that the β-catenin/c-myc axis is an important regulator of ribosome biogenesis in resting muscle size. Interestingly, although β-catenin KO had no significant effect on ribosome biogenesis during overload, it reduced the hypertrophic response within the first week of overload. The effect was small and unlikely to be a consequence of
changes in translational capacity. This suggested that mechanisms regulating the efficiency of protein synthesis may be affected by β-catenin KO. Fu and colleagues reported in 2015 that in dendritic cells, genetic activation and ablation of β-catenin led to respectively increased and decreased activation of mTORC1, p70S6K, 4E-BP1, and RPS6 [104]. In our skeletal muscle KO model, RPS6 activation in three-day overload KO muscle was significantly reduced compared to WT. Contrary to the finding of Fu et al., the reduction of RPS6 activation was unlikely the result of changes in canonical mTORC1 signaling because the phosphorylation of upstream activators, mTOR and p70S6K1, were not significantly decreased with KO. It is unclear how β-catenin, a transcription factor with no kinase or phosphatase activity, can affect protein phosphorylation and activation. However, p70S6K1 has a splice isoform, p85S6K1, which retains the same kinase activity for phosphorylating RPS6 and contains a constitutive N-terminal nuclear localization sequence [105]. Future studies are needed to determine whether β-catenin interacts with nuclear p85S6K1 to influence RPS6 activation during skeletal muscle hypertrophy.

The most unexpected finding was that β-catenin depletion in myofibers resulted in enhanced proliferation of satellite cells in response to mechanical overload. To determine the functional consequence of this enhanced satellite cell proliferation, we evaluated the regenerative potential of β-catenin KO muscle in response to chemical injury. We hypothesized that skeletal muscle regeneration would be enhanced after KO and average KO myofiber CSA would be higher seven days after injury because reports of enhanced regenerative response in the literature
often show bigger myofiber size [106]. Consistent with our hypothesis, the average CSA of regenerating myofibers was significantly higher in KO compared to WT and the average number of central nuclei per myofiber was also significantly higher in KO muscle one week after injury. However, these effects were modest compared to the much higher number of regenerating myofibers per cross section in the injured KO muscles. Typically, myofibers are lost due to injury and satellite cells activate, proliferate, differentiate, and fuse to form new myofibers in order to repair the tissue damage. In the WT TA muscle, this myofiber loss was still apparent after seven days of regeneration, but the myofiber number in the injured KO TA was higher than the PBS controls. The increase in myofiber number is consistent with the significant amount of muscle mass regained after a single week of recovery. We interpret the higher myofiber count per cross section as an indication of increased de novo formation of myofibers. Thus, this improved mass gain can be explained by a more responsive satellite cell pool after β-catenin KO, which resulted in faster satellite cell proliferation after the chemical injury stimulus.

Although average myofiber CSA for regenerating muscle show no difference between WT and KO, it is important to interpret this lack of difference in light of the method of analysis. Mean myofiber CSA is calculated for every single cell on the entire cross section, which represents 2000-4000 cells. The chemical injury does not affect all regions of the TA muscle evenly; therefore, average cell size for the entire cross section may underestimate the effects of β-catenin knockout. The myofibers expressing eMyHC, which are presumed to be the ones regenerating
after injury, show significantly larger average cross sectional area compared to the WT controls.

Surprisingly, PBS injections result in significantly (p<0.01) increased average myofiber CSA in the KO TA (16.6% bigger), but BaCl₂ injections did not have the same effect (Figure 3.6B). PBS injections are typically mild perturbations for skeletal muscle and cause little to no effect, which makes this finding all the more intriguing because such a mild stimulus causes a significant hypertrophic response in a short period of time. The data presented in this study cannot fully explain this unanticipated finding, but one likely explanation is that β-catenin depletion in myofibers primes satellite cells to activate and fuse into the existing myofibers even in response to typically mild physiological stimuli such as saline injection.

It is possible that knockout of myofiber β-catenin simply allows satellite cells to enter one round of replication quickly before going into an otherwise normal regeneration response. This idea is consistent with recent reports showing that satellite cells isolated during the “G_{alert}” phase have a much shorter time to first division compared to their quiescent counterparts, but show no difference in growth rates afterward [106]. Future studies will be required to test whether myofiber β-catenin depletion indeed transitions satellite cells from G₀ to “G_{alert}” phase by assessing markers of mTORC1 activation.
3.6 Figures

Figure 3.1. Effective deletion of β-catenin in resting skeletal muscle significantly reduced rRNA and myc transcript levels.

**A.** Diagram of knockout strategy. The Human Skeletal Actin (HSA) promoter drove the expression of Cre recombinase in post-mitotic mature skeletal myofibers. The two modified estrogen receptor (mER) subunits, when inactive, keep CRE in the cytoplasm due to sequestration by HSP90. In the presence of tamoxifen (4-OHT), the CRE translocates into the nucleus to delete the segment of genomic DNA flanked by loxP sites, which have been inserted into the intron before exon 2 and after exon 6 of the β-catenin gene (Ctnnb1flo/flo).

**B.** Tamoxifen or vehicle is injected intraperitoneally once a day for five days and the animals are given three weeks of washout before the gastrocnemius muscles were collected for analysis.

**C.** CRE-mediated recombination was tissue-specific and inducible. The recombined band,

![DIagram](image-url)
indicating deletion of exons 2-6 of the β-catenin gene, is only present in skeletal muscle in the presence of tamoxifen. D. After three weeks of washout, there was a significant decrease in β-catenin transcript levels, which was accompanied by a concomitant decrease in c-myc and pre-47S transcripts. (n = 3 for WT and 5 for KO; *, **, and *** denote p<0.05, 0.01, and 0.001, respectively).
Figure 3.2. Myofiber β-catenin is necessary for effective hypertrophy.

A. Experiment strategy for β-catenin knockout and mechanical overload. Tamoxifen was injected intraperitoneally once a day for five consecutive days, and after at least three weeks of washout, synergist ablation or sham surgeries were performed to overload the plantaris muscle for 3 or 7 days before the muscle was collected for analysis. B. Average myofiber cross sectional area (CSA) for the plantaris muscle is measured on histological cross sections. 2way ANOVA indicates both overload and knockout explain significant portions of data variability, but post-hoc analysis indicates only WT CSA at 7 days is significantly higher than sham. (n=9 for sham, n=3 for WT 3-day overload, n=5 for KO 3-day overload, n=5 for 7-day overload; ** denotes p<0.01). C-F. False-colored representative immunofluorescence images of plantaris cross sections where the sarcolemma is labeled with anti-dystrophin antibodies. Scale bars represent 25 µm.
Figure 3.3. β-catenin deletion does not block increase in total RNA during mechanical overload.
Figure 3.3. β-catenin deletion does not block increase in total RNA during mechanical overload.

**A.** The pre-47S transcript is the precursor to the 5.8S, 18S, and 28S ribosomal RNA and represents a readout of RNA Polymerase I transcription activity. Both overload and β-catenin knockout contribute to significant \((p<0.05)\) pre-47S transcript data variability. However, pre-47S abundance does not differ between WT and KO for any group. **B.** The c-myc gene is a transcriptional target of β-catenin; however there is no significant effect of KO on myc expression during overload. **C.** Total RNA increased significantly with overload regardless of β-catenin knockout. \((n=9\) for sham, \(n=3\) and \(n=5\) for WT and KO 3-day overload, respectively, and \(n=5\) for 7-day overload; ***, and **** denote \(p<0.01, 0.001,\) and 0.0001, respectively).
Figure 3.4. RPS6 activation is reduced by β-catenin deletion.

A. Western blot analysis of mTOR, p70S6K1, and RPS6 activation. B-D. Quantification of band cumulative intensities for phosphorylated (activated) protein expressed as a ratio against total protein abundance for (B) mTOR (Ser2448), (C) p70S6K1 (Thr389), and (D) RPS6 (Ser240/244). Protein samples represent lysates from 3-day overloaded plantaris muscles (n=3 for WT and n=5 for KO; * denotes p<0.05).
Figure 3.5. Satellite cell activation during mechanical overload.

A-B. Immunofluorescence images of a portion of the plantaris cross section labeled for the basal lamina (laminin – green), nuclei (DAPI – blue), satellite cells (Pax7 – red), and proliferating cells (EdU – white). Overlap of Pax7 and DAPI signifies satellite cells (magenta arrows). Overlap of EdU and DAPI signifies nuclei that underwent DNA replication within the first three days of overload (white arrows). Overlap of EdU, Pax7, and DAPI signifies satellite cells that proliferated within the first three days of overload (yellow arrows). C. Quantification of all satellite cells for sham (n=4 WT; n=4 KO), 3-day (n=3 WT; n=5 KO), and 7-day (n=4 WT; n=4 KO) overload muscles. Data are expressed as percentage of satellite cell number over total nuclear content per cross section. D. Quantification of proliferating satellite cells within the first three days of overload. Data are expressed as percentage of dividing satellite cells (yellow arrows) over total satellite cells (magenta arrows) per cross section. (*) denotes p<0.05 and scale bars represent 25 µm.)
Figure 3.6. Myofiber β-catenin deletion enhances muscle regeneration

A. Normalized muscle mass. B-F. Quantification of IHC images. G-H. Representative IHC images. B. Average myofiber cross sectional area. C. Number of myofibers per cross section. D. Percent of myofibers per cross section expression embryonic myosin heavy chain. E. Average cell size of embryonic myosin heavy chain positive myofibers. F. Average number of central nuclei per myofiber. *, **, and *** denote p<0.05, 0.01, 0.001 and scale bars represent 50 µm.
Chapter 4. Summary and Future Directions

The main goal of this dissertation was to test the hypothesis that β-catenin is necessary for skeletal muscle hypertrophy through the regulation of ribosome biogenesis. In order to test this hypothesis, we generated a mouse model of inducible, skeletal muscle specific genetic ablation of β-catenin. We took advantage of the Cre-lox and the modified estrogen receptor systems to achieve temporal and tissue specific genetic manipulation in vivo. We successfully induced deletion of exons 2 through 6 of the β-catenin gene in skeletal muscle cells in the presence of tamoxifen. This was confirmed both qualitatively and quantitatively using PCR. We qualitatively assayed for the recombination event in the presence and absence of tamoxifen and in different tissues using 30 cycles of amplification with primers targeting the deleted segment of the genomic DNA. Subsequently, we quantitatively assessed the extent of genetic ablation in skeletal muscle using real-time PCR with the ΔΔCT method and we determined that our inducible knockout model exhibited roughly 80% reduction of β-catenin mRNA.

We hypothesized that β-catenin is responsible for promoting c-myc transcription, and as expected, c-myc mRNA levels decreased significantly in resting muscle. We further hypothesized that because c-myc is a potent driver of ribosomal RNA transcription, β-catenin knockout and reduced c-myc would decrease pre-47S transcription. Indeed, pre-47S mRNA levels were significantly reduced in resting muscle after β-catenin gene knockout. It seemed reasonable to conclude from these finding that β-catenin in fact contributes to rRNA transcription in resting
muscle. This interpretation is consistent with the literature in which various studies linked β-catenin to c-myc, ribosome biogenesis, and cell growth [42, 107, 108]. However, the crux of our hypothesis rested upon the necessity of β-catenin for muscle cellular hypertrophy as assessed by cross sectional analysis of myofiber area. We used a surgical model of mechanical overload to induce rapid hypertrophy in the mouse plantaris muscle and asked whether genetic knockout of β-catenin significantly blocked myofiber hypertrophy. Both the surgery and β-catenin knockout explained significant changes in average myofiber CSA over the course of a week. There was no statistically significant interaction between the two independent variables, which suggested that the effect of knockout was the same regardless of the level of overload. This argued against our original hypothesis that β-catenin is required for myofiber hypertrophy because if β-catenin were required for the growth response, we would expect the inhibitory effect of genetic knockout to become more pronounced as the level of growth increased. Despite β-catenin being dispensible for overload-induced increases in ribosome biogenesis, the overall level of myofiber hypertrophy in the KO muscles was attenuated. WT myofibers exhibited significantly higher average CSA relative to sham controls after seven days of overload while KO myofibers failed to do so. This is interesting in light of our findings regarding β-catenin knockout having no effect on ribosome biogenesis and accumulation within the first week of overload. One likely explanation is that the growth response to mechanical overload within the first week is a combination of multiple mechanisms, including significant changes in translational efficiency [109]. One of the major pathways regulating
protein translation during muscle growth acts through the activation of mTOR and its downstream effectors including p70S6K1, 4E-BP1, and RPS6 [110, 111]. Interestingly, there is evidence that genetic manipulation of β-catenin levels directly and significantly modify mTOR activation [104]. In light of our results, we hypothesized that KO of β-catenin reduced mTOR signaling to affect translational efficiency in the first week of muscle growth. Using Western blot analysis to assess the relative levels of phosphorylation, we did not detect significant differences between WT and KO muscle for either mTOR or p70S6K1 activation. However, RPS6 activation was significantly reduced in KO overloaded muscle despite the lack of change in threonine 389 phosphorylation of p70S6K1. Although T389 phosphorylation is well correlated with p70S6K1 activity in vivo, full activation of this kinase require additional phosphorylation in its autoinhibitory domain [112]. Our observed disconnect between p70S6K1 and the hypophosphorylation of RPS6 is reminiscent of changes in the brain of the ground squirrel to reduce reperfusion injury, where T389 phosphorylation increases on p70S6K1 while S240/244 phosphorylation on RPS6 decreases [113]. Additionally, the much less studied splice isoform of p70S6K1, known as p85S6K1, localizes in the nucleus and retains the same S6 kinase domains as p70S6K1 [105]. It is unclear why p70S6K1 would require a different version that goes into the nucleus and whether that has anything to do with nuclear β-catenin. The mechanism through which β-catenin contributes to RPS6 activation is novel and unexplored, and whether β-catenin acts through its nuclear, cytoplasmic, or membranous partners remain to be determined.
During the course of testing the above hypothesis, I ran into the difficult and time-consuming obstacle of quantifying cell size and other phenotypic adaptations of skeletal muscle. Realizing the shortcomings of current methodologies, I took a detour in my studies and developed an automated program, MyoVision, for skeletal muscle image quantification. MyoVision subsequently enabled me to accurately and rapidly assess changes in myofiber morphology, such as cross sectional area, embryonic myosin heavy chain expression, and central nucleation. I have made MyoVision available online for others to use, but it has been instrumental in facilitating my own efforts in elucidating the effects of skeletal myofiber β-catenin depletion on satellite cells.

Although β-catenin is most well-known and studied as the mediator of canonical Wnt signaling, it is by no means the only pathway involving the regulation or the functional outputs of β-catenin (see diagram in Figure 4.1). β-catenin cytoplasmic levels can be directly regulated by E-cadherin availability [114]. The tyrosine kinase receptor, c-Met, when activated by its ligand, the hepatocyte growth factor (HGF), can directly stabilize β-catenin through phosphorylation of Tyr654 and Tyr670 and induce β-catenin to detach from E-cadherin and translocate into the nucleus independent of Wnt [115-117]. The HGF/c-Met regulation of β-catenin transcription can be specifically inhibited by the small molecule PHA665752 [118, 119].

In the context of skeletal muscle stem cells, or satellite cells, β-catenin signaling appears to be integral to the transition out of quiescence and into an activated and proliferative state. When β-catenin is genetically ablated in satellite cells, the
muscle’s regenerative capacity is severely blunted; however, when satellite cell β-catenin is stabilized through the deletion of exon 3, the increased cytoplasmic β-catenin causes premature activation and differentiation, and also impairs muscle regeneration [53]. Numerous other perturbations in satellite cells also cause similar premature activation and depletion of the competent satellite cell pool [120-123].

Our data suggest that regeneration is not impaired after β-catenin knockout in myofibers, therefore it is unlikely to be explained by a mechanism that causes pathological activation of satellite cells. Interestingly, Goel et al. show that depletion of M-cadherin in satellite cells had little to no effect, but deletion of both N-cadherin and M-cadherin in satellite cells induced an expansion of the pool of satellite cells that retained their regenerative capacity [124]. Interestingly, the authors report a partially activated state after N- and M-cadherin double knockout that involves the low level expression of MyoD or Myf5, two myogenic markers of satellite cell activation and transition to the myoblast stage. It is important to note that the double knockout mice showed the significantly higher number of satellite cells in the absence of injury or stimulus, and the effects of partial activation are eliminated with satellite cell-specific β-catenin haploinsufficiency.

An important difference to highlight is that satellite cell number does not increase in ambulatory animals after β-catenin knockout in myofibers. Only after a stimulus such as mechanical overload or injury is applied does the satellite cell pool significantly increase. Rodgers et al. in 2014 demonstrated that an injury occurred far away from a skeletal muscle can subsequently prime that muscle’s satellite cells to regenerate faster in response to muscle injury [106]. This “Galert” phase, as
the authors called it, requires the cMet receptor. Furthermore, systemic administration of the proteolytic activator of cMet (HGFA) is sufficient to induce “Galert” in stem cells throughout the body [125]. Interestingly, cMet is also known to be associated with the β-catenin/E-cadherin complex and c-Met can directly activate β-catenin signaling [118, 119, 126]. Notably, satellite cells in the “Galert” do not express detectable levels of myogenic markers such as MyoD and Myog, which suggests that “Galert” may be an earlier intermediate phase than the partial activation stage described by Goel and colleagues [124].

Given the existence of multiple intermediate states between complete quiescence and full activation, β-catenin, c-Met, and N/M-cadherin may function in sequential roles. Alternatively, it is possible that the “little to no effect” observed after deleting M-cadherin in satellite cells may be due to the lack of a proper stimulus. Because N-cadherin is not expressed highly in adult myofibers, it is reasonable to hypothesize that myofiber β-catenin knockout causes satellite cells to enter the “Galert” phase and this effect is delivered through M-cadherin conformational changes to the satellite cells. To test the hypothesis, administering the cMet/β-catenin small molecule inhibitor, PHA665752, should eliminate the effect of myofiber β-catenin knockout because activation of cMet is required for entry into “Galert”. Future work from a therapeutic perspective may include testing soluble recombinant antibody fragments specific to the extracellular regions of M-cadherin [127]. A competitive inhibitor of extracellular M-cadherin interactions may induce satellite cell entry into “Galert” without affecting other stem cell populations. This may be of high interest to people at risk to repetitive strain injuries.
4.1 Figure

Figure 4.1. Diagram of Working Hypothesis.

Myofibers signals to satellite cells through β-catenin/M-cadherin conformational changes that activate c-Met and induces "Galert".
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**Abstracts**

