α7β1 INTEGRIN REGULATION OF SKELETAL MUSCLE GROWTH IN RESPONSE TO MECHANICAL LOADING

BY

ALIF LAILA TISHA

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Kinesiology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2018

Urbana, Illinois

Master's Committee:

Associate Professor Marni Boppart
Assistant Professor Adam Konopka

ABSTRACT

The α7β1 integrin has been proposed to serve as a mechanosensor and essential regulator of myofiber remodeling given its localization at the membrane and primary role in adhering the outer extracellular matrix to the inner actin cytoskeleton. However, additional work is necessary to affirm a primary role for the α7β1 integrin subunit in the regulation of skeletal muscle mass in response to mechanical loading. The purpose of this study was to 1) to utilize an automated computer program (SMASH) to reassess myofiber cross-sectional area in previously collected samples, and 2) to determine the mechanism by which the α7β1 integrin can promote myofiber growth following a mechanical stimulus. Aim 1 used an automated computer program to re-analyze myofiber CSA and confirm a role for the α7β1 integrin in myofiber hypertrophy following chronic mechanical loading. Aim 2 assessed potential mechanisms for integrin-mediated growth in response to chronic mechanical load, including upregulation of Yes-associated protein (YAP) content in skeletal muscle. The results from this study suggest that 1) the use of an automated computed program (SMASH) provides results that are comparable to manual measurement, 2) chronic mechanical loading elicits an increase in fiber splitting that may result in an underestimation of myofiber CSA, and 3) overexpression of the α7 integrin subunit increases baseline YAP content in skeletal muscle, an event that may influence the rate of myofiber hypertrophy in response to load. Future studies will incorporate the SMASH program for assessment of myofiber size while continuing to probe the mechanistic basis for integrin-mediated myofiber growth in response to load.

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation for my advisor. Thank you Dr. Marni Boppart, for taking me under your wing and teaching me how to be a graduate student. Since my freshman year, I looked up to you not just as a genius scientist, but also as a strong woman of character. You lead by example, and your spirit of scholarship, brute determination to succeed and love for science never ceases to amaze me.

I would like to thank Dr. Adam Konopka for agreeing to be in my thesis committee and for providing additional insight to this project. I am glad the UIUC Department of Kinesiology and Community Health has a bright young mind to lead the way towards ground-breaking research initiatives. I am truly grateful for your support.

I would like to thank Julie Jenkins and Dr. Steven Petruzzello for supporting me throughout my graduate career. Your words of encouragement and support helped me more than you know. I would also like to thank Dr. Nicholas Burd and his lab for introducing me to the world of muscle physiology research at UIUC. Thank you for inspiring me with exciting research methods, and introducing me to the limitless realm of scientific research.

I would like to thank my lab-mates at the Molecular Muscle Physiology lab, one at a time, for each of them had a helping hand in who I am today. Thank you Ziad, for teaching me all the scientific techniques that led to my thesis. You were a great teacher. Thank you Slav, for getting the experimental supplies even at a moment's notice. Your enthusiasm to keep the lab running for

everyone will never be forgotten. Thank you Michael, for explaining in great detail the answers to my “hows” and “whys”. Your exemplary depth of knowledge sets the bar for any graduate student, and I know you will take the world by storm. Thank you Yu Fu, for moral support and giving me the opportunity to pass down some knowledge myself.

I would like to thank all the friends I have made here in the USA, for making me feel at home away from home. As Bhupen Hazarika wrote in his song Ami Ek Jajabor, “Pother manush apon hoyechhe, apon hoyechhe por.” (The people I meet on this journey are my own now, and my own are strangers.) This lyric quote resonates with me in some way every day, because of all of you. A special shout-out to Katherine, Rehab, Tani, Nina, Kali, Kanika, Yana, Lauren, and Kaitlin for being like sisters to me. You made all my birthdays and holidays so special with the surprise parties, that I didn’t have the chance to miss home. Thank you Illini Bhangra and Trikhala, for defining my experience as a student. You taught me how much I can accomplish with focus, and more importantly, passion. Thank you to the Bengali community here at UIUC, for giving me the chance to keep in touch with my roots and traditions. Thank you to the Manchanda family for always welcoming me with open arms to your holiday celebrations, and sending me to campus with bags of delicious food. Thank you to Eric and family, for treating me like one of your own. I love you all.

Last but not least, I would like to thank my parents and little sister for loving and supporting me from 14 time zones away. Thank you for letting me go and have adventures of my own, make mistakes of my own, and create a life of my own. I know you are always there if I need to fall, and that is all that matters.

To Ammu and Abbu, thank you for being my guardian angels from 9500 miles away. Your love transcends distance and your prayers keep me warm.

To Eric, my love, thank you for always anticipating my coffee needs. Words can't espresso how much you bean to me.

To Autumn and Winter, for being the fluffiest reasons to come home.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

The α7β1 integrin is a heterodimeric transmembrane protein that adheres to laminin in the extracellular matrix, representing a critical link that maintains skeletal muscle structure and integrity, particularly during the process of contraction. In addition to preventing eccentric exercise-induced skeletal muscle injury, the α7β1 integrin has been proposed to serve as an intrinsic mechanosensor, initiating cellular growth in response to mechanical strain. The early mechanisms leading to skeletal muscle remodeling post-exercise are complex and not completely understood, however, increased protein translation and abundance are required elements of myofiber growth.

α7β1 Integrin

The α7β1 integrin heterodimer includes isoform-specific α and β subunits, each comprised of 1) a large extracellular domain which binds proteins in the extracellular environment, 2) a single-membrane-spanning transmembrane domain, and 3) a short intracellular cytoplasmic tail domain, which links to cytoskeletal elements via cytoplasmic adaptor proteins [1]. Integrins specifically bind to extracellular matrix (ECM) glycoproteins, including a variety of laminins [1, 2]. In addition, integrins also play key roles in the assembly of the actin cytoskeleton, as well as modulation of signal transduction pathways that control biological and cellular functions, including cell adhesion, migration, proliferation, cell differentiation, and apoptosis [3]. Since their discovery approximately 20 years ago, significant progress has been made in the integrin biology field that has resulted in a greatly improved understanding of adhesion molecule structure and function [4].

In skeletal muscle, the α7β1 integrin has been proposed to serve as a mechanosensor and essential regulator of myofiber remodeling given its localization at the membrane and primary role in adhering the outer extracellular matrix to the inner actin cytoskeleton [5, 6]. The α7β1 integrin is highly expressed in skeletal muscle, and is localized primarily at the neuromuscular and myotendinous junctions [7]. The α7 subunit preferentially binds laminin in the basal lamina of the extracellular matrix, and the β1 subunit binds the actin cytoskeleton within the muscle fiber, resulting in the stabilization of Z-bands within each sarcomere [8, 9]. Early studies demonstrated that the absence of the α7β1 integrin in muscle provides the basis for a congenital myopathy in humans and elimination in a mouse model can result in loss of myofiber and neuromuscular junction integrity, an event particularly evident following contraction [7, 9, 10]. Endogenous α7β1 integrin mRNA and protein are upregulated in skeletal muscle in humans and mice lacking the dystroglycan/dystrophin complex in an attempt to compensate for the absence of transmembrane adhesion. Furthermore, transgenic overexpression of the α7BX2 integrin (MCK:α7BX2 integrin; α7Tg) can ameliorate disease pathology and extend the lifespan of mice lacking dystrophin [11].

Role in protecting against eccentric exercise-induced mechanical damage

In addition to protecting muscle from disease, there is substantial evidence to suggest an important role for the α7β1 integrin in the maintenance of myofiber and whole muscle structure and function following eccentric exercise. Boppart et al. (2008) provided the first evidence that an acute bout of eccentric exercise can promote a significant increase in the gene expression of the α7β1 integrin subunit in mouse skeletal muscle, including all known extracellular and intracellular isoforms [12]. The change in gene expression translates to a 70% increase in α7 integrin subunit

protein expression within 24 h post-exercise [12]. Similarly, DeLisio et al. (2015), provided the first evidence that α7 integrin subunit protein expression is increased nearly 4-fold in human skeletal muscle at 24 hours following an acute bout of eccentric exercise [13]. Upregulation of the integrin in response to eccentric exercise appears to protect against damage associated with mechanical strain, as evidenced by a reduction in sarcolemmal damage, prevention of a force deficit, suppression of immune cell infiltration, and reduction in MAPK signaling in α7Tg mice at 24 hr post-exercise [10, 14]. Although the results from these experiments provide unequivocal evidence of a role for the integrin in protection from exercise-induced damage, minimal information exists regarding a role for the integrin in load-induced myofiber remodeling and growth.

Mechanism by which the integrin can regulate growth in response to exercise

Leuders et al. (2010) substantiated the extent to which the α7β1 integrin affects muscle hypertrophy following eccentric exercise [14]. Within one week, a single bout of downhill running exercise enhanced mean myofiber CSA to a greater extent in transgenic (α7Tg) mice than in wild type (WT) controls. Satellite cell content and new fiber synthesis were also markedly enhanced in α7Tg mouse muscle post-exercise. The lack of enhancement in myonuclear content suggest that intrinsic mechanisms rather than a satellite cell fusion event may be responsible for the improvement in growth observed following the acute stimulus [14].

Zou et al. (2011) investigated the extent to which the α7β1 integrin regulates hypertrophic signaling (mTORC1) and muscle growth in response to 4 weeks of eccentric exercise training in the form of downhill running [6]. The myofiber CSA was markedly increased in α7Tg mice

compared with WT mice in both sedentary and post-eccentric training conditions. Specifically, a significant increase in the proportion of large caliber fibers (>3,000 μm) was observed in α7Tg mice compared to WT mice. In addition, myofibrillar protein content was significantly enhanced in α7Tg muscle compared to WT. Thus, this study provided the first evidence that the α7β1 integrin can promote myofiber and whole muscle enlargement following eccentric exercise training. Additionally, mTOR and p70S6K are intricately involved in the initiation of growth with mechanical strain [15]. Although mTORC1 signaling was found to be enhanced in α7Tg mice in this study, mTORC1 signaling was downregulated in α7Tg following an acute bout of eccentric exercise [10]. The discrepancy in signaling results suggested that additional studies are necessary to address the importance of mTORC1 in integrin-mediated growth, perhaps using traditional loading models at multiple time points.

Myofiber response to chronic mechanical overload

Elimination of the gastrocnemius and soleus muscles in a mouse model results in the placement of chronic mechanical load on the compensatory plantaris muscle. This surgical model of chronic mechanical loading, also known as synergist ablation, has been used as a traditional method for evaluation of cellular and molecular mechanisms that result in muscle remodeling and growth. With assistance from the Hornberger Lab (University of Wisconsin, Madison), we have been trained to perform a modified version of this technique on mice. Myotenectomy (MTE), a simple procedure of cutting the Achilles tendon at the gastrocnemius muscle, stimulates growth of the compensatory plantaris while minimizing fiber damage. Mahmassani et al. (Boppart laboratory, unpublished) recently demonstrated an increase in muscle weight in α7Tg muscle compared to WT muscle at 1 and 14 days post-MTE, yet no increase in myofiber cross-sectional area (CSA)

was observed. In the Mahmassani et al. study, a low number of samples were included in some treatment groups due to the labor intensive requirement of manually assessing hundreds of fibers in each muscle. Thus, we identified the need for incorporating an automated, unbiased approach to analyzing myofiber CSA. Semi-Automatic Muscle Analysis using Segmentation of Histology (SMASH), developed by Smith and Barton (2014), is an open source MATLAB script that serves to freely, efficiently and consistently analyze physiological properties of immunofluorescent muscle sections [16]. With regard to mechanism, Mahmassani et al. did not observe enhanced mTORC1 signaling in α7Tg mice compared to WT in response to MTE, yet these results were deemed unreliable given the lack of increase in CSA observed. Goodman et al. recently reported involvement of Yes-associated protein (YAP) content in fiber growth post-load, an event that did not require mTORC1 signaling [17]. The YAP signaling pathway may underlie integrin-mediated growth, yet confirmation of myofiber hypertrophy is necessary prior to any further mechanistic assessment, including both mTORC1 and YAP.

Conclusion

Prior results from our lab suggest an important role for the α7β1 integrin in load-induced myofiber growth. However, the primary mechanisms that underlie this observation remain unclear. This study will assist in our exploration of potential mechanisms and ultimately introduce new strategies to prevent anabolic resistance that can occur with disease and age.

Aims and Hypotheses

The mechanistic basis for α7β1 integrin-mediated muscle remodeling is not completely understood. Based on the above information, it was evident that further exploration is necessary. Thus, we set out to address the following aims:

Aim 1: To utilize an automated computer program (SMASH) to reassess myofiber cross-sectional area in previously collected samples.

Hypothesis: *The use of SMASH will provide accurate assessment of fiber CSA that will confirm the ability for the integrin to regulate myofiber growth following chronic mechanical loading associated with myotenectomy (MTE).*

Aim 2: To determine the mechanism by which the α7β1 integrin can promote myofiber growth following a mechanical stimulus.

Hypothesis*: The α7β1 integrin promotes an increase in Yes-associated protein (YAP), a co-transcriptional activator that can influence cellular growth.*

CHAPTER 2: METHODOLOGY

Animals

All mice were housed in the Beckman Institute animal facility at the University of Illinois in a temperature-controlled, pathogen-free animal room maintained on a 12:12 light-dark cycle. The animals were fed standard laboratory chow and water *ad libitum*. Protocols for animal use were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois at Urbana-Champaign. Mice overexpressing the rat α7β1 integrin subunit under the control of a muscle-specific promoter (MCK:α7BX2 integrin) and littermate controls were bred and maintained.

Myotenectomy

Young adult (6 month old for Mahmassani samples – 1 and 14 days post-MTE; 4 month old for the current study – 7 days post-MTE) Wild Type (WT) and α7 Transgenic (α7Tg) male mice were randomly assigned to receive myotenectomy (MTE) or sham (sham) surgery on both hind legs. Thus, the four treatment groups were Wild Type-Sham (WT-Sham), Transgenic-Sham (α7Tg-Sham), Wild Type-MTE (WT-MTE), and Transgenic-MTE (α7Tg-MTE). The number of animals assessed in each group is provided in the figure legends. Animals were weighed and then anesthetized with 2-3% isoflurane with 95-99% oxygen via inhalation. The hind legs were shaved using electric hair clippers, followed by disinfecting with betadine solution and alcohol wipes. For the MTE group, the Achilles tendon was severed at the base of the gastrocnemius muscle, yet the soleus and plantaris muscles remain intact. This allows for equal distribution of load across the plantaris and soleus muscles, thus reducing some of the inflammation and damage observed in the plantaris with the classic synergist ablation model. Sham surgery included the incision of the skin

and fascia surrounding the posterior muscle compartment. The incisions were closed using PDS sutures and Vetbond (3M, St. Paul, MN) and monitored for healing. The mice received a subcutaneous injection of buprenorphine (1 mg/kg) before surgery and every 12 hours for 72 hours.

Tissue collection and preservation

Animals were sacrificed 1, 7, and 14 days post-surgery for analysis. Mice were euthanized via carbon dioxide asphyxiation. One plantaris muscle and one soleus muscle were frozen in liquid nitrogen cooled isopentane for immunohistochemistry (IHC), and the plantaris and soleus from the other leg were snap frozen in liquid nitrogen for western blotting. Only the plantaris muscle was assessed for this thesis project.

Immunofluorescence assessment of tissue sections

Plantaris muscles were cut at the midline along the axial plane. The distal end was embedded in an optimum cutting temperature compound (Tissue-Tek; Fischer Scientific). Three transverse cryosections per sample (10 μm nonserial sections, each separated by a minimum of 40 μm) were cut for each histological assessment using a CM3050S cryostat (Lecia, Wezlar, Germany). Sections were placed on microscope slides (Superfrost; Fischer Scientific, Hanover Park, IL) and stored at -80°C before staining.

For fiber type-specific cross sectional area (CSA) analysis, plantaris sections were stained with anti-myosin heavy chain 1 (BA.D5), anti-myosin heavy chain 2A (SC.71), anti-myosin heavy chain 2B (BF.F3), and anti-myosin heavy chain 2X (6H1) antibodies obtained from Developmental Studies Hybridoma Bank (Iowa City, IA, USA). The sections were co-stained with

anti-dystrophin (Abcam) to outline the myofiber, necessary for measuring individual fiber CSA. Many of the fibers obtained from mice 1D post-MTE did not express dystrophin (damaged fibers). Therefore, these samples were stained for laminin and fiber type-specific CSA was not obtained. Briefly, frozen sections were fixed in ice-cold acetone for 10 minutes and blocked with PBS containing 70um/ml of AffiniPure anti-mouse fab fragments diluted in 0.5% BSA and 0.5% Triton-X for 60 minutes at room temperature. Primary antibodies were diluted to a concentration of 1:50 for BF.F3 and SC.71, and 1:20 for BA.D5 and 6H1, in PBS containing 0.5% BSA and 0.5% Triton-X and applied to the tissue sections for 60 minutes at room temperature. The secondary antibodies used were Alexa 350 conjugated anti-mouse IgG2b (diluted 1:100, Invitrogen Cat# A21140, UV-blue), Alexa 488 anti-mouse IgM μ chain specific (diluted 1:100, JacksonImmuno 115-545-075), Alexa Fluor 633 goat anti rabbit (diluted 1:100, Invitrogen), AMCA conjugated anti-mouse IgM u chain specific (diluted 1:100, Jackson Immunoresearch Cat#115-155-075, 1:100) and Alexa 488 conjugated anti-mouse IgG subclass 1 (diluted 1:100, Jackson Immunoresearch).

For assessment of collagen content, sections were co-stained with Collagen I antibody obtained from Abcam (diluted 1:100) and Dystrophin. This stain followed the same steps as the previously described above except for there was Tween-20 added in place of Triton-X. The secondary antibody were used was FITC goat anti-rabbit IgG (diluted 1:200).

For assessment of vascular growth, sections were co-stained with the CD31 antibody obtained from e-bioscience, clone 390. This stain followed the same steps as the previously described above except for there was Tween-20 added in place of Triton-X. Primary antibodies were diluted 1:100. The secondary antibodies used were Alexa Fluor 633 goat anti- rat IgG

(dilution 1:200) and Alexa Fluor 488 goat anti-rabbit IgG (dilution 1:100). 4,6-diamidino-2-phenylindole (DAPI) stain was added to the second to last wash step (1:20,000) (Sigma Aldrich).

Immunofluorescence quantitation

For all immunofluorescence assessments, images were captured at 10x and 20x magnification using Leica DMRXA2 microscope and Axiovision software (Zeiss, Thornwood, NY).

To assess myofiber CSA, the SMASH software was used to quantitate images acquired with a Zeiss AxioCam digital camera. The program uses a MATLAB code written by Smith and Barton [16]. The detailed protocol for cross-sectional area (CSA) quantification is attached in the Appendix. Co-stained images of dystrophin and type-specific fiber types were acquired at 20x magnification from each sample, then imported into the SMASH program where all fibers in the section were identified. This program automatically grabs the positively stained pixels, decreasing both subjectivity and interassessment error. The average number of fibers in the plantaris muscles of each group ranged from 316-728. All fibers within a section with a complete dystrophin or laminin outline were assessed for CSA analyses. The CSA for each fiber was recorded in a measurement log, and the results for each sample were then averaged using Microsoft Excel.

For assessment of collagen I content, 3 non-overlapping, representative images were captured from the best section at 20x magnification using Leica DMRXA2 microscope and Axiovision software (Zeiss, Thornwood, NY). Collagen content was quantified with a threshold intensity program from Image J. RGB channels were separated and then the red channel was thresholded to remove background in order to determine the percent of collagen observed within each imaged

muscle section. The mean percentage from the three representative images was calculated and recorded.

For assessment of vascular growth, 5 non-overlapping, representative images were captured from the best section at 20x magnification using Leica DMRXA2 microscope and Axiovision software (Zeiss, Thornwood, NY). $CD31^+$ stains were manually counted using ImageJ, and fiber CSA was also calculated via ImageJ. Capillary density was calculated as following: [total number of capillaries (CD31 dots)/total fiber CSA]; capillary to fiber ratio was calculated as following: [total number of capillaries/total number of fibers]. The mean quantity from the five representative images was calculated and recorded.

To assess the quantity of split fibers, 20x images of an entire muscle section (~10-15 images) from each animal were obtained using Leica DMRXA2 microscope and Axiovision software (Zeiss, Thornwood, NY). The number of fibers with evidence of splitting (fine lines within the boundaries of a single enlarged myofiber) in each section were counted using Adobe Photoshop and the total was recorded for each sample. The average number of split fibers were calculated for each group and recorded.

Protein extraction and western blotting

Plantaris muscles were placed directly in 500 uL of ice-cold lysis buffer containing HEPES (20 mM), EGTA (2 mM), β-glycerophosphate (50 mM), DTT (1 mM), Na_3VO_4 (1 mM), Triton X-100 (1%), glycerol (10%), leupeptin (10 μM), benzamidine (3 mM), pepstatin A (5 μM), aprotinin (10 μg/ml), PMSF (1 mM). The tissue was disrupted using a hand held homogenizer and

the homogenates were rotated at 4°C for 1 h and centrifuged at 14,000*g* for 5 minutes at 4°C. The supernatant was collected and the concentration of protein was determined with the Bradford protein assay using bovine serum albumin (BSA) for the standard curve. Muscle water content was indirectly determined after estimating the protein contribution to muscle weight from soluble and insoluble fractions (raw protein data from each fraction/plantaris weight X 100).

Equal amounts of protein (30 µg) were subjected to SDS-PAGE western blotting using 8% acrylamide gels, and transferred to nitrocellulose membranes. Equal protein loading was verified by PonceauS staining. Membranes for detection of phosphorylated (serine 127) and total Yes-associated protein (YAP) (Cell Signaling Technology, antibody numbers here) were blocked for 1 hour in Tris-buffered saline (pH 7.8) with Tween20, containing 5% Bovine Serum Albumin (BSA), followed by incubation in YAP antibody (1:1000) overnight. Appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were applied for 1 hour at 1:2000. Bands were detected using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL) and a Bio-Rad ChemiDoc XRS system (Bio-Rad). Quantification was completed using Quantity One software (Bio-Rad). All bands were normalized to PonceauS.

Statistical analysis

All averaged data are presented as means ± Standard Error (SE). To determine significance, comparisons between groups were evaluated by two-way ANOVA, followed by LSD post-hoc analysis when a significant interaction was detected. Data was considered significant at $p<0.05$. Statistical analyses were conducted using SPSS version 24.

CHAPTER 3: RESULTS

Reassessment of fiber CSA 1 day and 14 days following MTE

Assessment of CSA of Type I, Type IIa , Type IIx, and Type IIb myofibers by SMASH resulted in similar results as the manual measurements obtained via Adobe Photoshop, even when additional samples were added to the assessment (Figure 1). An Ablation main effect was noted for Type IIa CSA using the SMASH ($p<0.05$). However, no other main effects were noted. Removal of small caliber fibers from the analysis (<300 μm) did not alter these results.

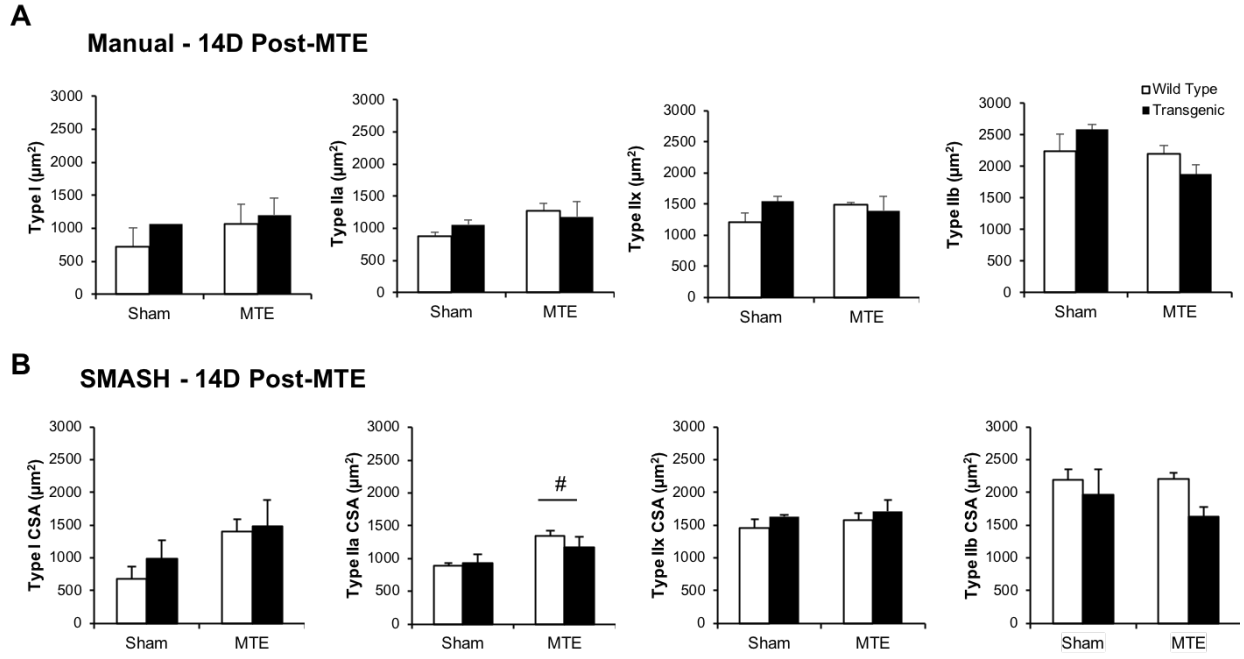


Figure 1. Manual vs SMASH CSA analyses of plantaris muscle 14 days post-MTE. 6 month old male C57BL/6 mice were subjected to 14 days of MTE and fiber type specific CSA was assessed. (A) Manual analyses of Type I, Type IIa, Type IIx, and IIb myofiber CSA. (B) SMASH analyses of Type I, Type IIa, Type IIx, and IIb myofiber CSA. Values are mean ± SE. n=4-6/group. #Ablation main effect, $p<0.05$.

The lack of increase in myofiber growth indicated that further investigation was necessary to understand the basis for the significant enhancement in muscle weight in α7Tg mice post-MTE as compared to the WT mice.

Water content is not enhanced in α7 integrin transgenic muscle post-MTE

Water content in each sample was indirectly assessed based on the estimation of protein content in each sample. As shown in Figure 2, no significant changes in estimated water content were observed as a result of ablation or transgene expression.

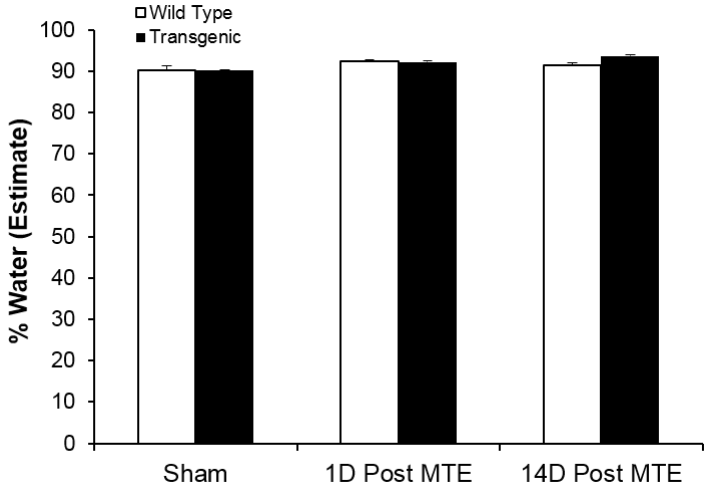


Figure 2. Estimated percentage of water in the plantaris muscle 1 and 14 days post-MTE. Values are mean ± SE. n= 3-6/group.

Collagen content is significantly increased in α7 integrin transgenic muscle post-MTE

Extracellular matrix remodeling is commonly observed in skeletal muscle following exercise and loading [18]. Although collagen accumulation would not be expected due to the high rate of turnover (synthesis = degradation), we evaluated collagen I expression since it could potentially contribute to total muscle weight. An ablation x transgene interaction was observed ($P<0.01$), such that less collagen was present in α7Tg muscle compared to WT in the sham condition, whereas an increase in collagen was noted in α7Tg muscle compared to WT 14D post-MTE (Figure 3).

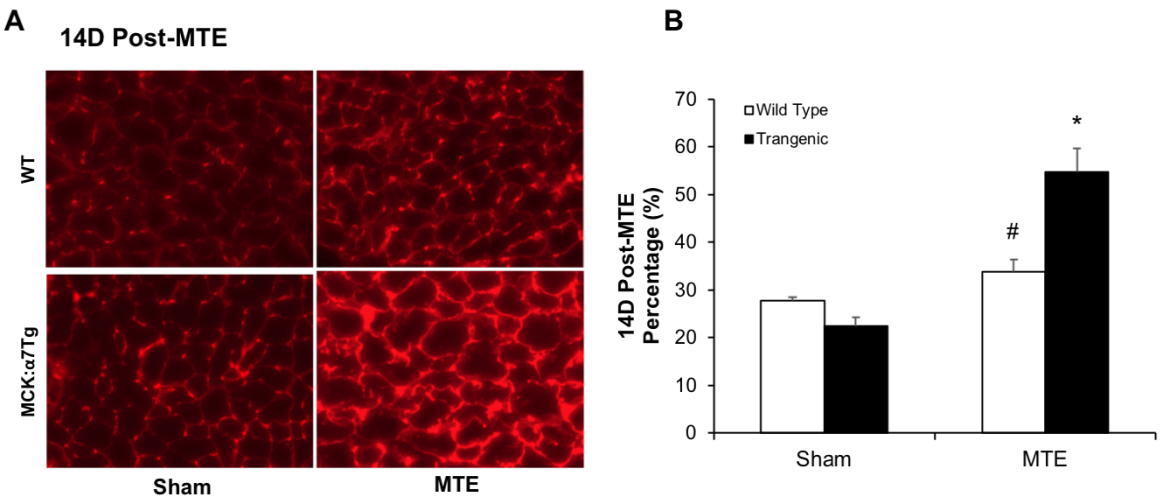


Figure 3. Collagen I content in the plantaris muscle 14 days post-MTE. (A) Representative images of collagen staining. (B) Percentage area of section covered with collagen staining. Values are mean ± SE. n=4-6/group. *p<0.05 vs. all groups; #p<0.05 vs. α7Tg-Sham and α7Tg-MTE groups.

Capillary density does not account for the increase in collagen content in α7 integrin transgenic muscle post-MTE.

Huntsman et al. previously reported a significant increase in arteriogenesis in α7Tg muscle following acute and chronic eccentric exercise [19]. We hypothesized that the increase in collagen in the α7Tg may occur as a natural response to support newly formed vessels. Changes in $CD31^+$ vessel quantity were noted (Figure 4). A main effect of ablation was observed for $CD31^+$ capillaries normalized to tissue area ($p<0.05$), and both genotype and ablation effects were observed for the average quantity of $CD31^+$ capillaries per fiber ($p<0.05$). The changes in vascularization did not correlate with collagen content. Thus, changes in vascularization may not account for enhancement of collagen content post-MTE (Figure 3).

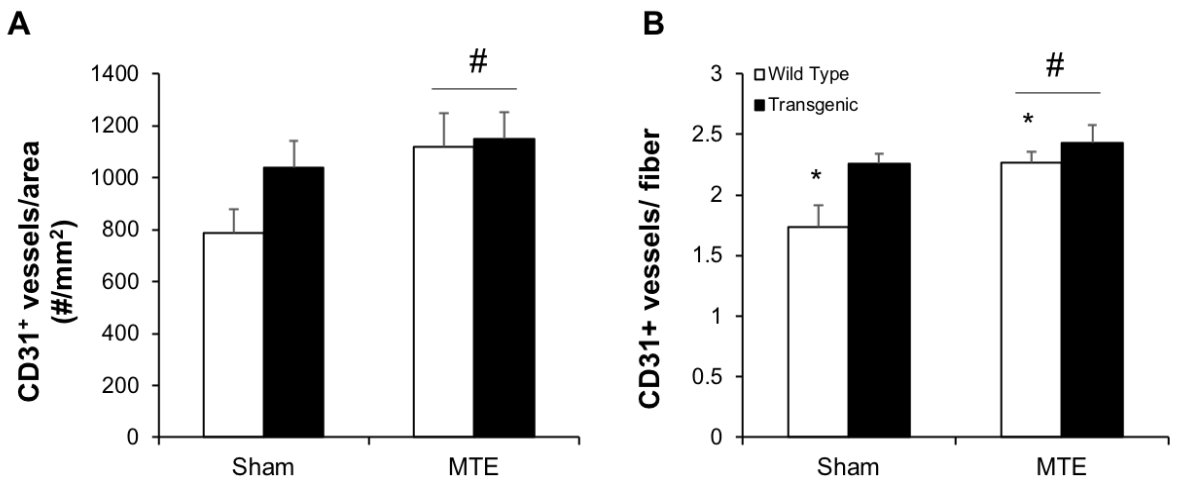


Figure 4. Capillary content in plantaris muscle 14 days post-MTE. (A) Capillary content normalized to total fiber area and (B) average number of capillaries supplying each myofiber. Values are mean ± SE. n= 3-4/group. *Transgene main effect, $p<0.05$; #Ablation main effect, $p<0.05$.

The quantity of split fibers is significantly enhanced in α7 integrin transgenic muscle post-MTE

Recent studies suggest that supraphysiological growth associated with chronic loading can initiate splitting of fibers in an effort to better support post-natal hypertrophic fibers with nutrients and oxygen [20]. We quantitated the number of split fibers in samples collected 1 day post-MTE, as well as newly collected 7 day post-MTE samples (Figure 5). An ablation x transgene interaction was observed 1 day post-MTE ($p<0.01$), such that more split fibers were present in α7Tg muscle compared to all other groups (Figure 5A). Similar results were noted at 7 days post-MTE (interaction, $p<0.05$) (Figure 5B).

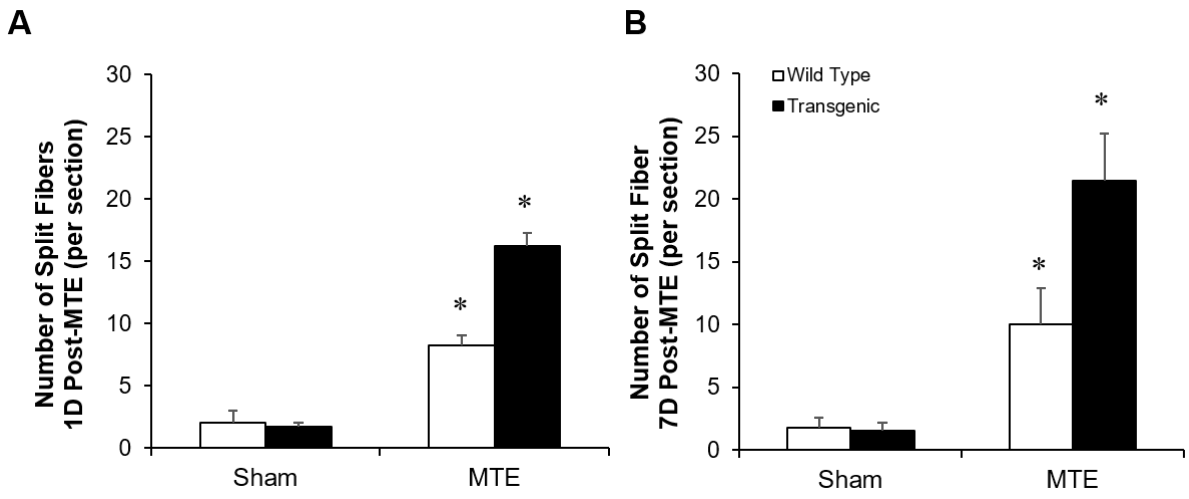


Figure 5. Number of split fibers in plantaris muscle 1 and 7 days post-MTE. (A) Number of split fibers 1 day post-MTE. (B) Number of split fibers 7 days post-MTE. Values are mean ± SE. n= 2-5/group (1 day), n=4/group (7 day). *p<0.05 vs. all groups.

Interestingly, the maximal number of split fibers (~40/section) was observed at 14D post-MTE, but no difference was noted between α7Tg and WT groups at this time point (Ablation main effect, $p<0.05$; data not presented). The fact that the SMASH program identifies each section of a split fiber as a unique fiber suggest that the actual fiber CSA is underestimated in the α7Tg group post-MTE.

YAP content is elevated in α7 integrin transgenic muscle in the sham condition, but not increased post-MTE

Goodman et al. recently demonstrated the capacity for chronic loading to increase Yes-associated protein (YAP) content and the involvement of YAP in initiating myofiber growth in an mTORC1-dependent manner [17]. YAP protein content increases in the context of chronic loading around 3 days after the initiation of the stimulus, peaks 3-7 days, and returns to baseline levels by 14 days post-load [17]. Previously, Mahmassani et al. detected an increase in total YAP content under baseline conditions in α7Tg muscle compared to WT, but no further increase was detected

1 day post-MTE. The 1 day time point may not be optimal for detection of an increase in YAP post-load. Therefore, new samples were collected at the 7D time point to address the impact of α7β1 integrin expression on regulation of YAP content. YAP phosphorylation on serine 112 can target YAP for degradation. Thus, YAP phosphorylation and the ratio of YAP phosphorylation to total YAP was evaluated (Figure 6). An ablation x transgene interaction was observed 7 days post-MTE ($p<0.01$), such that muscle weight was higher in α7Tg muscle compared to all other groups (Figure 6A). A main effect of ablation was detected for mean myofiber CSA ($p<0.05$) (Figure 6B). No significant changes were detected for total YAP, YAP phosphorylation, or the phospho-YAP to YAP ratio (Figures 6C-E).

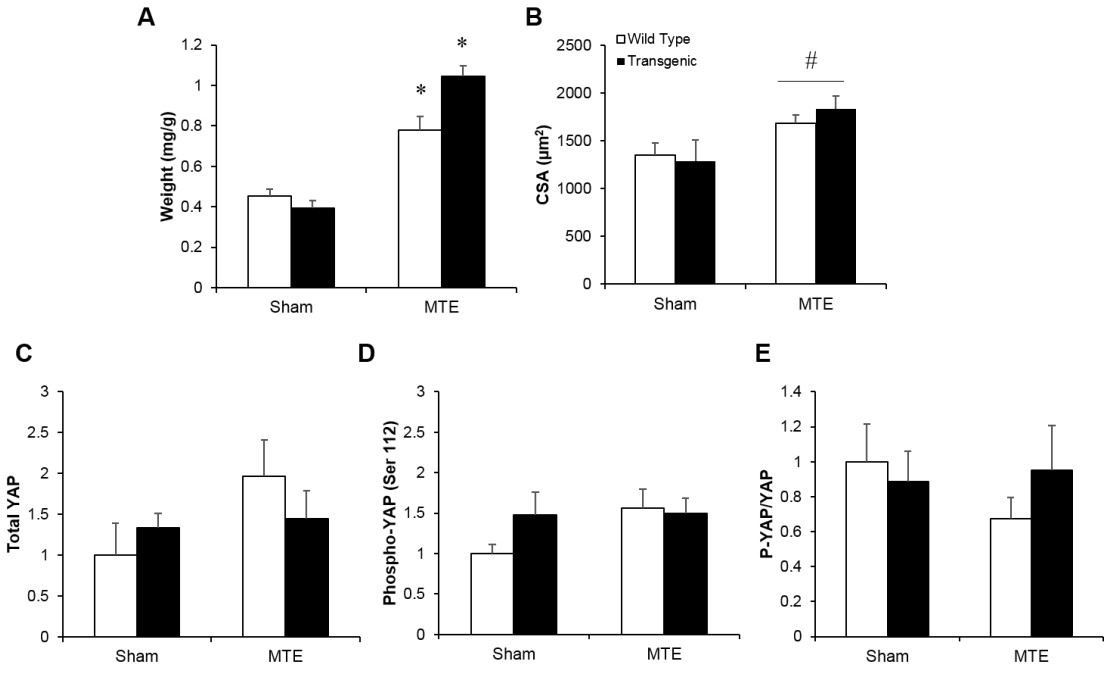


Figure 6. Evaluation of YAP phosphorylation and content in plantaris muscle 7 days post-MTE. (A) Verification of the increase in muscle weight (B) Mean fiber CSA (C) Total YAP (D) Phospho-YAP (E) ratio of phospho-YAP to total YAP. Values are mean ± SE. n= 4/group. *$p<0.05$ versus all groups; #Ablation main effect, $p<0.05$.

In an effort to discern YAP localization in α7Tg mice, muscle sections were stained for YAP using standard immunofluorescence methods (Figure 7). Consistent with previous 1 day

post-MTE results, YAP expression was higher in α7Tg mice compared to WT in the sham condition. YAP was predominantly localized inside the fiber, in the subsarcolemmal compartment, not necessarily associated with myonuclei (Figure 7).

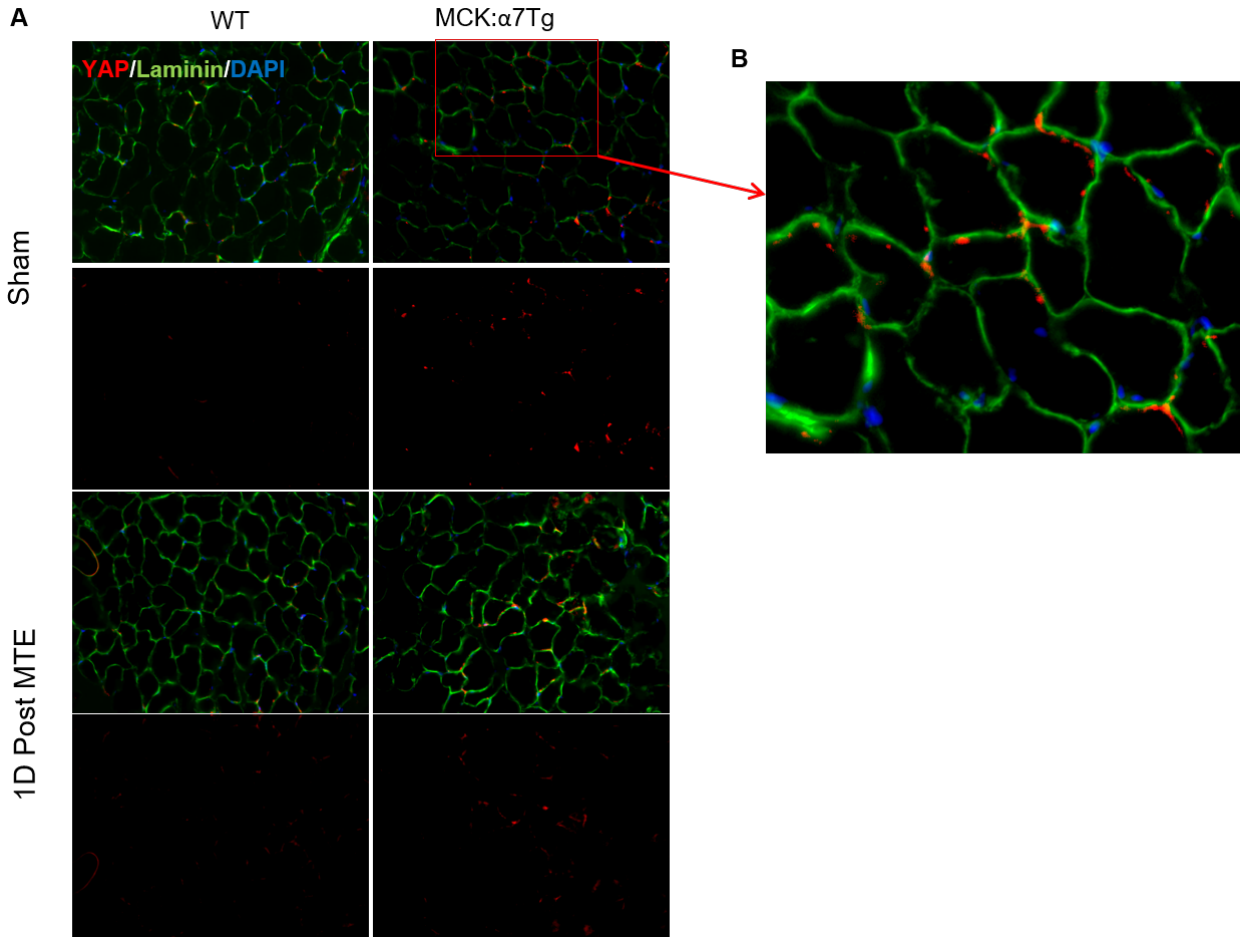


Figure 7. Immunohistochemistry images of YAP in plantaris muscle 1 day post-MTE. (A) Representative images of YAP (red), laminin (green) and DAPI (blue). The first row shows merged images and the second row shows just the YAP stain for the same images. (B) magnified image demonstrating YAP localization within the myofiber.

At 7 days post-MTE, no changes were noted in YAP expression or localization in either group. Overall, these data suggest that the α7 integrin can regulate baseline YAP content in skeletal muscle. How this observation may impact protection from damage or the growth response to loading is not clear.

Figure 8 shows representative images of YAP co-localization with active β1 (CD29) in α7Tg muscle at 7 days post-MTE.

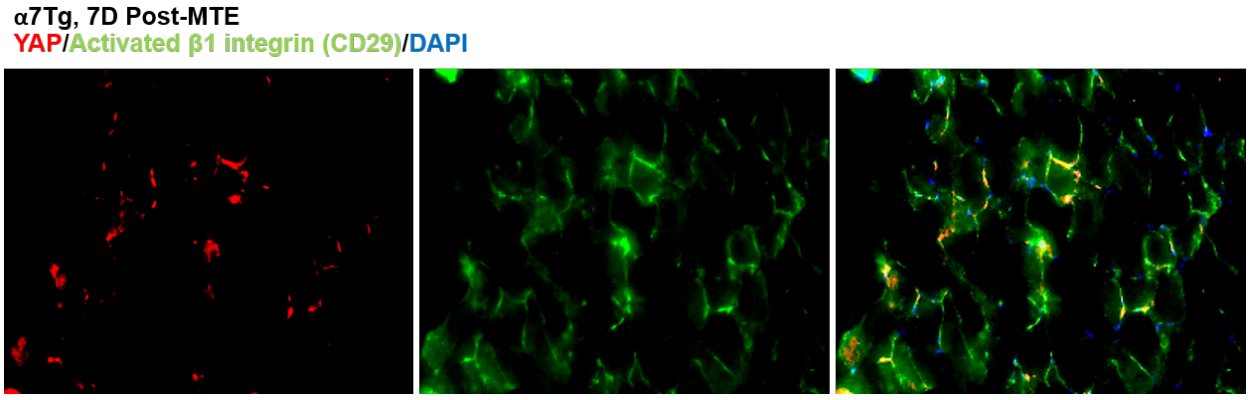


Figure 8. Immunohistochemistry images of YAP, activated beta 1 integrin (CD29) and DAPI in plantaris muscle 7 days post-MTE.

CHAPTER 4: DISCUSSION

Utilization of a standardized quantification method for assessment of myofiber CSA

Our lab has previously established that overexpression of the α7β1 integrin subunit in skeletal muscle can significantly enhance muscle fiber size in response to acute and multiple bouts of eccentric exercise [5, 6]. Despite these intriguing results, the ability for the integrin to skeletal muscle remodeling using the surgical model of synergist ablation and chronic loading had never been tested. Recent results from our lab demonstrate the ability for the integrin to facilitate an increase in plantaris weight with chronic overload with a myotenectomy procedure, yet no increase in fiber CSA was observed. Thus, the first aim of this project was to re-evaluate fiber CSA in previously collected samples using an automated computer program (SMASH). The results from this study suggest that manual counting and an automated computer program (SMASH) result in similar outcomes. Unfortunately, the results confirmed that myofiber CSA was not increased in α7Tg muscle, suggesting a discrepancy between muscle weight and fiber CSA. Thus, further analyses were conducted as a major part of this project to elucidate some of the mechanisms responsible for the preferential weight gain in α7β1 integrin transgenic mice post-MTE.

Contribution of collagen to enhanced weight in α7Tg muscle post-MTE

Collagen content was significantly increased in α7Tg mice 14 days post-MTE. It is unlikely that the observed increases in collagen were due to damage as Mahmassani et al.'s work confirmed protection from damage in α7Tg muscle compared to WT muscle post-MTE. Although vascularity was increased in muscle with chronic loading, the predominant increase occurred in the WT group, suggesting no correlation with collagen content. Collagen within the extracellular matrix provides

a mechanical scaffold for cells within tissues [21]. Thus, collagen may increase in α7Tg muscle in an effort to provide structural support to splitting fibers – or fibers preparing for this event.

Contribution of hyperplasia (fiber splitting) to enhanced weight in α7Tg muscle post-MTE

Kelly (1996) reviewed the impact of mechanical overload on fiber quantity in certain animal species [22]. Mechanical overload resulted in increases in muscle mass, muscle fiber area (hypertrophy) and muscle fiber number (hyperplasia). However, the increases in fiber area were twice as great as increases in fiber number [22]. It appears that hyperplasia in animals is greatest when certain types of mechanical overload, particularly stretch, are applied [22]. The results of this investigation are thus similar to this narrative review as they concluded that muscle fiber hyperplasia consistently occurs as a result of chronic stretch [22].

The processes involved in these changes include satellite cell proliferation and longitudinal fiber splitting [22]. In the current study, fiber splitting was significantly increased in α7Tg mice at 1 and 7 days post-MTE compared to WT. The fact that similar amounts of splitting was observed in both α7Tg and WT groups at a later point, 14 days post-MTE, suggest that splitting is not specific to muscle that overexpresses the integrin, but rather is a natural response that is simply accelerated as a result of the integrin presence. These data are striking and suggest that the integrin can initiate a rapid intrinsic growth process that ultimately results in splitting and the lack of ability to observe increases in myofiber CSA. These data also suggest that in vitro experiments or alternative assessments of growth should be incorporated into future in vivo studies (rate of protein synthesis) to accurately assess the ability for the integrin to regulate skeletal muscle remodeling.

Baseline Yes-associated protein content is enhanced in α7Tg muscle

The Hippo signaling pathway, first discovered as a suppressor of tissue overgrowth in genetic studies of Drosophila [23]. It has gained increasing attention over the past 20 years because of its critical role in organ size control, regeneration, development, and tumorigenesis. Phosphorylation of YAP on serine 112 in mouse muscle results in retention in the cytoplasm and subsequent degradation, whereas non-phosphorylated YAP can translocate to the nucleus to act as a co-transcription factor with TEAD1-4 [23]. Studies have demonstrated that cell attachment and actin stress fiber formation promotes YAP nuclear accumulation, while actin destabilization and detachment leads to phosphorylation and cytoplasmic retention via the Hippo pathway [23-25]. Mechanical stimulation can alter actin cytoskeleton tension in a manner that can regulate YAP. Thus, structural properties of myofibers and the extracellular matrix likely regulate cell growth via actin stabilization and subsequent regulation of YAP.

Mahmassani's unpublished work demonstrated that baseline YAP protein content is elevated in α7Tg muscle compared to WT and is maintained at 1D post-MTE. We collected additional samples at 7 days post-MTE to maximize detection of YAP content, as described by Goodman et al. in [17]. Our results were very consistent with Mahmassani, demonstrating an elevation of YAP content at baseline (sham conditions) and maintenance at 7D post-MTE. Based on the role for the integrin in stabilizing the actin cytoskeleton, as well as our preliminary results suggesting co-localization of YAP with the activated form of the β1 integrin, we speculate that the integrin stabilizes YAP in the cytoplasm and protects it from degradation. The extent to which this complex can subsequently facilitate an accelerated and/or enhanced growth response in α7Tg muscle is not known and requires further study.

CHAPTER 5: CONCLUSION

This study was designed to address two primary aims: 1) To utilize an automated system to reanalyze myofiber CSA in previously collected samples and 2) To further elucidate the mechanistic basis for α7β1 integrin regulated growth by adding a time point for observation (7 days post-MTE). Our results suggest that the use of SMASH is an efficient method to assess myofiber CSA. In addition, enhancement of YAP protein content under baseline (unstimulated) conditions in α7Tg muscle may prime skeletal muscle for an accelerated growth response to loading. Additional experiments are necessary to clarify a role for YAP in α7β1 integrin-mediated myofiber growth following a mechanical stimulus.

REFERENCES

[1] Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell*, *110*(6), 673-687.

[2] Plow, E. F., Haas, T. A., Zhang, L., Loftus, J., & Smith, J. W. (2000). Ligand binding to integrins. *Journal of Biological Chemistry*, *275*(29), 21785-21788.

[3] Schwartz, M. A., Schaller, M. D., & Ginsberg, M. H. (1995). Integrins: emerging paradigms of signal transduction. *Annual Review of Cell and Developmental Biology*, *11*(1), 549-599.

[4] Srichai, M. B., & Zent, R. (2010). Integrin structure and function. In *Cell-Extracellular Matrix Interactions in Cancer* (pp. 19-41). Springer, New York, NY.

[5] Lueders, T. N., Zou, K., Huntsman, H. D., Meador, B., Mahmassani, Z., Abel, M., ... & Boppart, M. D. (2010). The α7β1-integrin accelerates fiber hypertrophy and myogenesis following a single bout of eccentric exercise. *American Journal of Physiology-Cell Physiology*, *301*(4), C938-C946.

[6] Zou, K., Meador, B. M., Johnson, B., Huntsman, H. D., Mahmassani, Z., Valero, M. C., ... & Boppart, M. D. (2011). The α7β1-integrin increases muscle hypertrophy following multiple bouts of eccentric exercise. *Journal of Applied Physiology*, *111*(4), 1134-1141.

[7] Burkin, D. J., & Kaufman, S. J. (1999). The α7β1 integrin in muscle development and disease. *Cell and Tissue Research*, *296*(1), 183-190.

[8] Bao, Z. Z., Lakonishok, M., Kaufman, S., & Horwitz, A. F. (1993). Alpha 7 beta 1 integrin is a component of the myotendinous junction on skeletal muscle. *Journal of Cell Science*, *106*(2), 579-589.

[9] Boppart, M. D., Volker, S. E., Alexander, N., Burkin, D. J., & Kaufman, S. J. (2008). Exercise promotes α7 integrin gene transcription and protection of skeletal muscle. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *295*(5), R1623-R1630.

[10] Boppart, M. D., Burkin, D. J., & Kaufman, S. J. (2006). α7β1-Integrin regulates mechanotransduction and prevents skeletal muscle injury. *American Journal of Physiology-Cell Physiology*, *290*(6), C1660-C1665.

[11] Burkin, D. J., Wallace, G. Q., Nicol, K. J., Kaufman, D. J., & Kaufman, S. J. (2001). Enhanced expression of the α7β1 integrin reduces muscular dystrophy and restores viability in dystrophic mice. *The Journal of Cell Biology*, *152*(6), 1207-1218.

[12] Boppart, M. D., Volker, S. E., Alexander, N., Burkin, D. J., & Kaufman, S. J. (2008). Exercise promotes α7 integrin gene transcription and protection of skeletal muscle. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *295*(5), R1623-R1630.

[13] De Lisio, M., Farup, J., Sukiennik, R. A., Clevenger, N., Nallabelli, J., Nelson, B., ... & Boppart, M. D. (2015). The acute response of pericytes to muscle-damaging eccentric contraction and protein supplementation in human skeletal muscle. *Journal of Applied Physiology*, *119*(8), 900-907.

[14] Lueders, T. N., Zou, K., Huntsman, H. D., Meador, B., Mahmassani, Z., Abel, M., ... & Boppart, M. D. (2010). The α7β1-integrin accelerates fiber hypertrophy and myogenesis following a single bout of eccentric exercise. *American Journal of Physiology-Cell Physiology*, *301*(4), C938-C946.

[15] Goodman, C. A., Miu, M. H., Frey, J. W., Mabrey, D. M., Lincoln, H. C., Ge, Y., ... & Hornberger, T. A. (2010). A phosphatidylinositol 3-kinase/protein kinase B-independent activation of mammalian target of rapamycin signaling is sufficient to induce skeletal muscle hypertrophy. *Molecular Biology of the Cell*, *21*(18), 3258-3268.

[16] Smith, L. R., & Barton, E. R. (2014). SMASH–semi-automatic muscle analysis using segmentation of histology: a MATLAB application. *Skeletal Muscle*, *4*(1), 21.

[17] Goodman, C. A., Dietz, J. M., Jacobs, B. L., McNally, R. M., You, J. S., & Hornberger, T. A. (2015). Yes-Associated Protein is up-regulated by mechanical overload and is sufficient to induce skeletal muscle hypertrophy. *FEBS letters*, *589*(13), 1491-1497.

[18] Mendias, C. L., Schwartz, A. J., Grekin, J. A., Gumucio, J. P., & Sugg, K. B. (2016). Changes in muscle fiber contractility and extracellular matrix production during skeletal muscle hypertrophy. *Journal of Applied Physiology*, *122*(3), 571-579.

[19] Huntsman, H. D., Zachwieja, N., Zou, K., Ripchik, P., Valero, M. C., De Lisio, M., & Boppart, M. D. (2012). Mesenchymal stem cells contribute to vascular growth in skeletal muscle in response to eccentric exercise. *American Journal of Physiology-Heart and Circulatory Physiology*, *304*(1), H72-H81.

[20] Murach, K. A., White, S. H., Wen, Y., Ho, A., Dupont-Versteegden, E. E., McCarthy, J. J., & Peterson, C. A. (2017). Differential requirement for satellite cells during overload-induced muscle hypertrophy in growing versus mature mice. *Skeletal Muscle*, *7*(1), 14.

[21] Smith, L. R., & Barton, E. R. (2014). Collagen content does not alter the passive mechanical properties of fibrotic skeletal muscle in mdx mice. *American Journal of Physiology-Cell Physiology*, *306*(10), C889-C898.

[22] Kelley, G. (1996). Mechanical overload and skeletal muscle fiber hyperplasia: a meta-analysis. *Journal of Applied Physiology*, *81*(4), 1584-1588.

[23] Juan, W. C., & Hong, W. (2016). Targeting the Hippo signaling pathway for tissue regeneration and cancer therapy. *Genes*, *7*(9), 55.

[24] Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., ... & Elvassore, N. (2011). Role of YAP/TAZ in mechanotransduction. *Nature*, *474*(7350), 179.

[25] Zhao, B., Li, L., Wang, L., Wang, C. Y., Yu, J., & Guan, K. L. (2012). Cell detachment activates the Hippo pathway via cytoskeleton reorganization to induce anoikis. *Genes and Development*, *26*(1), 54-68.

APPENDIX A: EXPERIMENTAL PROTOCOLS

Table 1: List of Materials

Material	Company	Product Number
Anti-Dystrohpin	Abcam	AB15277
Anti-Laminin	R&D Systems	MAB4656
Anti-CD31	e-Bioscience	Clone 390
Anti-Type I MHC antibody	Developmental Studies Hybridoma Bank at the University of Iowa	(BA-D5- supernatent
Anti-Type 2X	Developmental Studies Hybridoma Bank at the University of Iowa	(6H1-supernatent
Anti-Type 2A	Developmental Studies Hybridoma Bank at the University of Iowa	Sc-71-supernatent
Anti-Type 2B	Developmental Studies Hybridoma Bank at the University of Iowa	BF-F3-concentrate
Anti-YAP	Cell Signaling Technology	#4912
Anti-phospho YAP (Ser 127)	Cell Signaling Technology	#4911S
Anti-Collagen Type I	Abcam	Ab34710
Anti CD29	BD Biosciences	BDB550531
AMCA conjugated anti-mouse IgM u chain specific	Jackson Immunoresearch	Cat#115-155-075
Alexa 488 conjugated anti-mouse IgG subclass 1	Jackson Immunoresearch	Cat#115-545-205
Alexa 350 conjugated anti-mouse IgG2b	Invitrogen	Cat# A21140
Alexa 488 anti-mouse IgM μ chain specific	Jackson Immunoresearch	Cat# 115-545-075
Alexa Fluor Goat anti Rabbit IgG	Invitrogen	A-11008
Alexa Fluor Goat anti Rat IgG	Invitrogen	A-21094
Alexa Fluor Goat anti Rabbit IgG 633	Invitrogen	A-21071

IHC for Laminin.

- Thaw slides for 5-10 mins.
- Fix with ice cold acetone at 4^{o}C for 5-10 mins.
- Allow to dry. Outline the sections with an immunopen.
- Wash section with 1X PBS.

  3x5mins _ _ _
- Block: 5% BSA + 0.05% Tween20 + Fab Fragment Goat anti-mouse (115-007-003) diluted 1:20.

  1 hour
- Rinse slides with wash buffer = 0.05% Tween 20 + 1% BSA in PBS

  3x5mins _ _ _
- Incubate sections in the warm room at 37^{o}C with primary antibody diluted in wash buffer = 0.05% Tween 20 + 1% BSA in PBS

  1 hour
- Rat anti-mouse Mouse Laminin alpha 1 (R&D Systems, MAB4656), dilution 1:100
- Wash slides 3X5 mins with wash buffer.

**** Light sensitive steps ahead*** Turn off the lights and proceed in dark****

- Dilute the secondary antibody in wash buffer = 0.05% Tween 20 + 1%BSA in PBS and incubate sections at room temperature (cover the slide chamber with foil).

  1 hour
- Alexa Fluor 633 goat anti rat (A-21094, 1:200)

- Wash sections in wash buffer.

  3x5mins _ _ _

- Wash sections in 1xPBS.

  1x5mins

- Dilute the DAPI solution in PBS 1:20,000. Add the diluted DAPI solution on the sections.

  1-2mins.

- Wash the sections with 1X PBS.

  3x3mins _ _ _

- Add a few drops of Vectashield, apply coverslip, seal with nail polish, store at 4^{o}C.

IHC for CD31 and dystrophin

- Thaw slides for 5-10 mins.
- Outline the sections with an immunopen.
- Fix with ice cold acetone at 4^0C for 5-10 mins.
- Wash section 3X for 5 mins with 1X PBS.
- Block for 1 hour in 5% BSA + 0.05% Tween20 + Fab Fragment Goat anti-mouse (115-007-003) diluted 1:20. Always add enough solution to cover the sections.
- Rinse slides with wash buffer = 0.05% Tween 20 + 1% BSA in PBS
- Incubate sections in the warm room at 37^0C for an hour with primary antibody diluted in wash buffer = 0.05% Tween 20 + 1% BSA in PBS
    - Rat anti-mouse CD31 (e-bioscience, clone 390), dilution 1:100
    - Rabbit anti-mouse Dystrophin (ab15277), dilution 1:100
- Wash slides 3X for 5 mins with wash buffer.

**** Light sensitive steps ahead**** Turn off the lights and proceed in dark****

- Dilute the secondary antibody in wash buffer = 0.05% Tween 20 + 1%BSA in PBS and incubate sections for 1 hour at room temperature (cover the slide chamber with foil). (Optional: Filter the secondary antibody mix through a ~0.2-0.8 micron pore size syringe filter).
    - Alexa Fluor 633 goat anti- rat IgG, dilution 1:200
    - Alexa Fluor 488 goat anti-rabbit IgG, dilution 1:100
- Wash sections 3X for 5 mins in wash buffer.
- Wash sections 1X for 5 mins in PBS.

- Dilute the DAPI solution in PBS 1:20,000. Add the diluted DAPI solution on the sections for 3mins.
- Wash the sections 3X for 3 mins with 1X PBS.
- Add a few drops of Vectashield, apply coverslip, seal with nail polish, store at 4^{o}C.

Myofiber Cross-sectional Area analyses via Semi-Automated muscle analysis using segmentation of histology (SMASH)

Protocol extracted from: Smith, Lucas R; R Barton, Elisabeth (2014): SMASH – semi-automatic muscle analysis using segmentation of histology: a MATLAB application. (https://doi.org/10.6084/m9.figshare.1247634.v5)

Running Muscle Histology Analysis and selecting File

1) Select the "SMASH_App" App from the App Menu.
    a. If running outside MATLAB run the SMASH_Stand_Alone.exe.
2) Locate and press the "Select File" button. (Figure 1A)
3) Navigate to and select the tiff image file you would like to analyze.
    a. Always select the original image file, not the mask file created by the program.
    b. The selected file name will appear on the screen (Fig 1A)
4) In the Select Function window select the analysis function to perform and press "Select Parameters." (Figure 1A).
    a. Initial Segmentation must be run before any other functions.
    b. It is also recommended that fiber filter is run prior to other analysis functions.

Initial Segmentation

1) Enter the parameters required for initial segmentation (Figure 2A).
    a. Fiber outline color: is the RGB channel which contains the fiber outlines.

b. Pixel size: on image (μm/pixel).

c. Segmentation Filter: Input the filter value you would like to use.

    i. Lower number is more segmented image.

2) Press the "Run Function" button

    a. If a mask for the file is already created you will be asked to overwrite the previous mask to continue.

3) Examine the image that is produced containing white lines over the fiber boarders and if it is satisfactory press the "Manual Edit" button, otherwise change the Segmentation Filter value and press "Run Function" again.

    a. The Zoom and drag button at the top toolbar allow the user to scan the image in detail.

4) Manually fix any undersegmentation using the Edit Segmentation menu tools (Figure 2C).

    a. Draw Line

        i. Once pressed any previously drawn line is saved.

        ii. Pan and zoom to the conjoined fiber.

        iii. Freehand draw a line to create a boarder to fully separate the fiber.

            1. Ensure that no tools are selected so that the freehand draw is active.

        iv. Repeat until all fibers are properly segmented.

    b. Undo: Press if the most recent fiber drawn was not drawn correctly.

    c. Done: Press when all fibers have been segmented.

        i. A new mask file will be saved.

Fiber Filter

1) Enter the parameters required for filtering fibers and press "Run Function" (Figure 4A).
    a. Pixel size on image (μm/pixel).
    b. The minimum area in that is counted as a fiber (μm^2).
    c. The maximum area in that is counted as a fiber (μm^2).
    d. The Maximum eccentricity a fiber is allowed to have.
        i. More eccentric is more linear and less circular.
    e. The minimum convexity a fiber is allowed to have.
        i. The area of the fiber as a fraction of the total area including concavities.
2) Inspect image that appears to determine if it is satisfactory.
    a. If filter parameters should be changed edit them in the window and repress "Run Function."
    b. If the filtering was satisfactory press "Manual Filter."
3) Navigate to any objects that are not fibers using pan and zoom and press any key
4) Click on all visible objects that are not fibers.
    a. A cross hair will be placed on the image where clicked.
    b. To remove most recent selection press "backspace" or "delete."
    c. To end selecting double-click on final selection, ctrl/alt click on final selection, or press "Enter."
5) The image will show the fibers removed and allow you to make a selection to confirm fiber removal (Figure 4C).

a. Yes: to delete selected fibers and continue to select fibers at step 3).
b. No: keep all fibers selected, but continue to select fibers at step 3).
c. Done: to delete selected fibers, save the modified mask file and proceed to further analysis.

Note: If Yes is pressed after the last fiber to be filtered place a new cross hair over a non-fiber area to bring up the menu.

Fiber properties

6) Enter the parameters required for fibers properties and press "Run Function". (Figure 5A).
   a. Pixel size on image (μm/pixel).
   b. Input the folder in which you would like to have the excel file output to.
7) Examine the histograms (Figure 5B) and choose if you would like to output the data to Excel by pressing "Yes" or "No."

Fiber Typing

8) Enter the parameters required for fiber typing and hit OK. (Figure 6A).
   a. Pixel size on image (μm/pixel).
   b. Fiber outline color: is the RGB channel which contains the fiber type stain.
   c. Input the folder in which you would like to have the excel file output to.

9) Examine the image (Figure 6C) with pan and zoom to determine if calls for fiber type are appropriate.

10) If you would like to change the automatic threshold for fiber types enter the new threshold value and press “Adjust”, otherwise press “Accept.”

    a. The histogram of average intensity is helpful in determining appropriate thresholds. (Figure 6C, bottom left).

11) Choose if you would like to output the data to Excel by pressing “Yes” or “No.”

Immunohistochemistry for Fiber Type 2A and 2B

- 10 μm sections from cryostat were fixed immediately with –30 °C acetone for total 10 min, and stored inside –80 °C freezer before staining.
- Let sections warm at room temperature for 5 min.
- Wash with 1 X PBS 3 times for 5 mins.
- Switch to incubation with 0.5% BSA and 0.5 % Triton-X plus Fab anti-mouse IgG(1:10) (AffiniPure Fab Fragment Goat Anti-Mouse IgG (H+L) Jackson #115- 007-003) –for 1hr at room temperature
- Wash with 3X5 min changes of PBS.
- Primary AB Incubation conditions.

  -Mouse IgM anti-type 2b MHC antibody (BF-F3-concentrate from Developmental Studies Hybridoma Bank at the University of Iowa (1:50, fridge))

  -Mouse IgG1 anti-type 2a MHC antibody (Sc-71-supernatent from Developmental Studies Hybridoma Bank at the University of Iowa (1:50, fridge)

  - Rabbit anti-mouse dystrophin (Abcam ab15277, 1:50)

  in PBS containing 0.5 % BSA and 0.5 % Triton-X for 1 hr room temperature
- Wash with 3X5 min changes of PBS.
- Secondary AB Incubate conditions:

  -AMCA conjugated anti-mouse IgM u chain specific (Jackson Immunoresearch Cat#115-155-075, 1:100, UV-blue, Freezer box#3),

  -Alexa 488 conjugated anti-mouse IgG subclass 1 (Jackson Immunoresearch Cat#115-545-205, 1:100, green).

- Alexa Fluor 633 goat anti rabbit (Invitrogen, 1:100)

  in PBS containing 0.5 % BSA and 0.5 % Triton-X for 1 hr room temperature.

- Rinse in PBS (3X5 min).
- Image with 4.0X0.7 magnification. Conversion factor = 5.1754

Immunohistochemistry for Fiber Types 2X and I

- 10 μm sections from cryostat were fixed immediately with –30 °C acetone for total 10 min, and stored inside –80 °C freezer before staining.
- Wash with 1 X PBS 3 times for 5 mins.
- Switch to incubation with 0.5% BSA and 0.5 % Triton-X plus Fab anti-mouse IgG(1:10) (AffiniPure Fab Fragment Goat Anti-Mouse IgG (H+L) Jackson #115- 007-003) for 1hr at room temperature
- Wash with 3X5 min changes of PBS.
- Primary AB Incubation conditions.

  -Mouse IgG2b anti-type I MHC antibody (BA-D5-supernatent from Developmental Studies Hybridoma Bank at the University of Iowa (1:20))

  -Mouse IgM anti-type 2x MHC antibody (6H1-supernatent from Developmental Studies Hybridoma Bank at the University of Iowa (1:20) (28μg/μL))

  - Rabbit anti-mouse dystrophin (Abcam ab15277, 1:100)

  in PBS containing 0.5 % BSA and 0.5 % Triton-X for 1 hr room temperature
- Wash with 3X5 min changes of PBS.
- Secondary AB Incubate conditions:

  - Alexa 350 conjugated anti-mouse IgG2b 1:100 (Invitrogen Cat# A21140, UV-blue)

  -Alexa 488 anti-mouse IgM μ chain specific 1:100 (JacksonImmuno 115-545-075),

  - Alexa Fluor 633 goat anti rabbit (Invitrogen, 1:100) in PBS containing 0.5 % BSA and 0.5 % Triton-X for 1 hr room temperature.
- Rinse in PBS (3X5 min).

Immunohistochemistry for Yes-Associated Protein, Laminin and DAPI

- 10 m sections from cryostat were fixed immediately with –30 C acetone for total 10 min, and stored inside –80 C freezer before staining.
- Allow to dry. Outline sections with immunopen
- Wash section with 1xPBS, 3x5mins
- Permeabilization: 0.1% Triton X in 1xPBS for 10 mins
- Wash section with 1xPBS, 3x5mins
- Block: 5% BSA, 0.05% Tween 20, Fab Fragment Goat Anti Mouse diluted to 1:20
  For 1 hour
- Rinse slides with wash buffer (0.05% Tween 20, 1%BSA in PBS), 3x5mins
- Incubate sections in the warm room at 37°C with primary antibody diluted in wash buffer for 1 hour
  Primary antibodies: Rat anti-mouse Laminin alpha 1, Rabbit anti-mouse YAP
- Dilute secondary antibody in wash buffer
  Secondary antibodies: Alexa Fluor 633 Goat anti rat (1:200 dilution), Alexa Fluor 488 goat anti-rabbit (1:200).
- Incubate sections at room temperature for 1 hour. Cover with foil.
- Wash sections in wash buffer, 3x5mins
- Wash sections in 1xPBS, 1x5mins
- Add DAPI solution (diluted 1:20,000) to sections for 1-2 mins.
- Wash sections in 1xPBS, 3x3mins
- Add a few drops of Vectashield, apply coverslip, store at 4°C.

Western Blotting General Protocol

Gel Casting:

1. Place the casting frame upright with the pressure cams in the open position and facing forward on a flat surface.
2. Make sure the glass plates are clean and dry and slide the two plates into the casting frame, keeping the short plate facing the front of the frame.
3. When the glass plates are in place and correctly aligned lock the pressure cams and secure the glass cassette sandwich in the casting frame.
4. Place the casting frame into the casting stand by positioning the casting frame onto the casting gasket while engaging the spring-loaded lever of the casting stand onto the spacer plate. (Get a good seal between the rubber gasket and the glass. If you don't the gel will leak out the bottom.)
5. Prepare the running and stack gel solutions (minus the APS and TEMED) according to the above table.
6. Add APS and TEMED to the running gel solution and gently swirl. Pour the running gel solution into the glass cassette using a plastic syringe. Immediately after pouring

the gel gently add approximately 200 µl of ethanol on top of the gel to avoid exposure to the air.

7. Leave the gel to polymerize (typically 15-20 min). A sharp line between the ethanol and gel layers indicates completion of polymerization.
8. When the polymerization of the running gel is complete, pour the layer of ethanol down the sink and absorb any excess with whatman paper.
9. Add APS and TEMED to the stacking gel solution and gently swirl. Pour the stacking gel solution into the glass cassette using a plastic syringe, and immediately insert the comb.
10. Leave the gel to polymerize. Note: Gel can be made up the night before; remove from casting frame, wrap in saran wrap and save in the refrigerator.

Preparing the Samples:

1. Thaw the samples on ice.
2. Determine the amount of sample to load. We usually load 50-100 µg (depends on the level of expression that you expect; e.g. low expression- load more protein) of protein per well in a total volume of 20-30 µl.
3. Prepare samples to be loaded in 1.5 ml eppendorf tubes. The total volume of protein, water and laemmli buffer should add up to the volume held by a single well in the Mini-PROTEAN comb.
4. *For reducing conditions add 31 mg of DTT to 500 µl of Laemmli Buffer, and prior to loading heat for 5 min at 100°C then centrifuge down.

Sample Loading and Electrophoresis:

1. Set the clamping frame to the open position on a clean flat surface.
2. Place the first gel cassette onto the gel supports with the short plate facing inward. Note that the gel will rest at a 30° angle, tilting away from the center of the clamping frame.
3. Place the second gel cassette onto the gel supports with the short plate facing inward, and with one hand gently pull both cassettes inward so that they rest firmly and squarely against the green gaskets.
4. While gently squeezing the cassettes slide the green arms of the clamping frame over the cassettes, locking them into place.
5. Fill the assembly with 1x running buffer and make sure there are no leaks.

   Note: When running 2 gels use the electrode assembly with the banana plugs. Also, if running an odd number of gels you must use the buffer dam.
6. Load samples into the gels with a loading pipette tip and with the assembly OUTSIDE of the Tetra tank. Make sure the assembly is on a flat surface and load slowly, allowing the samples to settle evenly on the bottom of each well.

   Note: For imaging purposes I like to load the gel in the reverse order that I have written in my lab notebook realizing that the front of the gel is facing way from me

when I load. This ensures that the samples are in the order I want them when I get to the imaging stage.

7. Carefully transfer the assemblies into the Tetra tank.
    - If running 2 gels place the electrode assembly in the back position of the tank, making sure the red (+) electrode matches the red marking on the top inside edge of the tank.
    - If running 4 gels place the electrode assembly in the back position and the companion module (no electrodes) in the front position. Make sure that the red (+) electrode matches the red marking on the top inside edge of the tank for both assemblies.
8. Fill the Tetra tank with 1x running buffer to the indicated level.
9. Place the lid on the Mini-Protean Tetra tank. When the lid is on correctly the raised tabs on each side of the tank will slide through the slots on the lid.

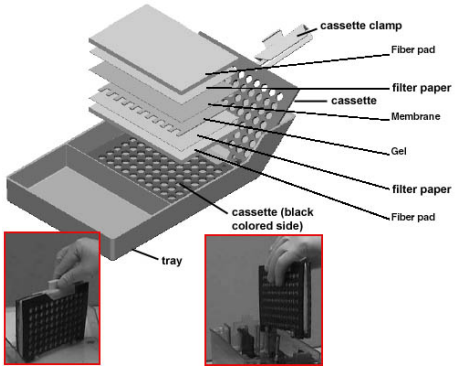
    Caution: When running 1-2 gels do not place the companion module in the tank. Doing so will cause excessive heat generation and will prevent electrophoretic separation.
10. We run our gels at a set voltage. The lower the voltage the better the resolution. A recommended voltage is 100 V for the first 10-15 min as the proteins migrate through the stacking gel, then 200 V for the remainder of the run. Run time will be determined based on protein molecular weight. The larger the protein the longer the

run. Also, it may be necessary to run the gel on ice or in a cold room to prevent melting of the gel depending on the total run time.

11. When the run is complete turn off the power supply and carefully lift out the electrode assemblies. Pour the running buffer down the sink and thoroughly rinse the tank with distilled H_2O after each use.
12. Open the arms of each assembly and remove the gel cassettes.

Protein Transfer:

1. Prepare the gel/bot assembly tray as shown in the image below. Pour the chilled 1x transfer buffer into both sides of the tray.



2. Carefully remove the short glass cassette and rinse the gel with 1x transfer buffer.
3. Carefully cut off stacking gel and upper left hand corner or gel (for orientation purposes) with a razor blade.
4. Place two layers of wet Whatman paper directly onto the gel and carefully peel the gel off of the glass.
5. Place the Whatman paper and gel onto the wet fiber pad in the transfer cassette, Whatman paper down, then place the wet membrane and two more layers of wet Whatman paper on top of the gel.

Caution: The nitrocellulose membrane is very fragile. Always handle the membrane with extreme care and always use the membrane forceps (never touch the membrane with your hands).

6. Use the roller to make sure there are no air bubbles between the gel and the membrane. Make sure the membrane and gel stay wet during this entire procedure. It is during this time that the gel will equilibrate in the 1x transfer buffer.

   Note: Two gels will fit in each transfer cassette.
7. Wet the second fiber pad and place it on top of the gel/membrane sandwich.
8. Close the cassette and clamp it in the locked position.
9. Place the cassette in the blotter tank making sure that the red side of the cassette is facing the red electrode.
10. If you ran 4 gels repeat steps 2-9.
11. Place the frozen ice block in the tank and add the remaining 1x transfer buffer to the fill level marked on the blotter tank.
12. **Note:** To further prevent the buffer from getting too hot we often put a magnetic stirrer in the tank and stir during transfer. Another level of protection would be to transfer on ice or in the cold room.
13. Place the lid on the tank and plug the cables into the power supply. Typical transfer conditions are 30 min at 100V, but this will vary depending on the molecular weight

of the protein. The larger the protein the longer the transfer (which also means greater potential for melting the gel- see note above).

14. After the transfer is complete carefully disassemble the transfer sandwich and remove the membrane for analysis.
15. Place the membrane in a square container for Ponceau S staining. Clean the tank, fiber pads, and cassettes with distilled H_2O. The gels and Whatman paper can be discarded.

Ponceau S:

1. Pour Ponceau S over the membrane and gently swirl for 30 sec to 1 min.
2. Pour the Ponceau S back into the falcon (this solution can be reused) and rinse the membrane 1-3 times with distilled H_2O to remove access Ponceau S staining.
3. Image the entire membrane with white light in the BioRad system. The filter on top of the BioRad system should be pushed away from you.
4. This step is important to show equal loading of protein in each well.
5. After you are done imaging, thoroughly rinse the membrane with dd H_2O to remove the majority of the Ponceau S stain.

Protein Detection:

1. Block the membrane to prevent non-specific binding.
    - Incubation time: 1 hour at room temperature on shaker

Blocking Solution

50 ml 1xTBS-Tween

50 x 0.05 = 2.5g non-fat milk or BSA

500 µl NaN_3

*This solution can be reused (keep 1 month @ 4°C)

- For some 1° Ab's (especially anti-phosphotyrosine Ab) it is better to use BSA
- Blocking overnight at 4°C is an option

2. Primary Antibody
    - Incubation time: Depends on the protein, typically overnight @ 4°C on shaker

        <u>Primary Ab Solution</u>

        1° Ab dilution depends on the Ab

        20 ml 1x TBS-Tween

        20 x 0.02 = 0.4 g non-fat milk or BSA

        200 µl NaN_3

        *This solution can be reused (1 year if stored @ 4°C)

    - Use the same protein solution as you used for blocking (milk-milk or BSA-BSA)
3. Wash Membrane
    - Wash with TBS-T 3 x 10 min at room temperature on the shaker
4. Secondary Antibody
    - Incubation time: 1 hour at room temperature on the shaker

        <u>Secondary Ab Solution</u>

        2° Ab dilution- we typically use 1:2000

        10 µl 2° Ab conjugated HRP

        20 ml 1x TBS-Tween

20 x 0.025 = 0.5 g milk

5. Wash Membrane
    - Wash with TBS-T 4 x 10 min at room temperature on the shaker
6. ECL Detection
    - Add 3 ml of each solution in the West Dura Extended Duration Substrate Kit to the small plastic box
    - Place one membrane at a time into the small plastic box and cover with foil
    - Incubate for 5 min
    - Remove the membrane from the substrate and place on the plastic folder inside the BioRad system
    - Make sure the Quantity One program is set to chemi high sensitivity
    - The filter on the top of the BioRad system should be pulled toward you
    - Open the iris all the way up
    - Start the imaging process with auto exposure to give you an approximate exposure time to start with, then adjust for optimal saturation
    - It may be necessary to cover the over saturated bands to improve the images of your band of interest.
7. Wash Membrane
    - Wash for 5-10 min in TBS-T on shaker
    - Save the membrane by sandwiching it in whatman paper then wrapping it in saran wrap and storing it @ -20°C
    - The membrane can be used for further probing and analysis.