# ALL THE SARCOPLASM IS A STAGE; AND ALL THE ANABOLIC AND CATABOLIC COMPLEXES MERELY PLAYERS; THEY HAVE THEIR EXITS AND ENTRANCES

## AN ANALYSIS OF HOW PROTEOLYTIC PATHWAYS IMPACT PROTEIN SYNTHESIS CAPACITY IN C2C12 MYOTUBES

A Dissertation

by

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Submitted to the Graduate and Professional School of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2021

Major Subject: Kinesiology

ABSTRACT

The control of anabolism and catabolism in muscle, termed protein turnover, is a heavily studied research area. It is known that steps in anabolism involve the mechanistic Target of Rapamycin (mTOR) and can have a direct impact on catabolic pathways. While we acknowledge that catabolism within the cell provides an intracellular source of amino acids (AA) to support anabolism, lesser known is whether catabolic pathways within skeletal muscle have a direct impact on anabolic capacity. To better understand how catabolic pathways impact anabolism, these studies used key anabolic and/or catabolic inhibitors to determine their role on 24-h fractional protein synthesis rates (FSR) and anabolic signaling in cultured C2C12 myotubes. The underlying hypothesis for the present study was catabolic processes directly affect the anabolic capacity by affecting anabolic signal transduction. To complete these studies, specialized inhibitors designed to block the autophagic (NSC 185058) and/or proteasomal (MG-262) catabolic pathways and/or anabolic signaling through mTOR were utilized to delineate contributions of the respective catabolic pathways on mTOR activity and FSR. Application of MG-262 had no statistical impact on FSR. However, incubation of cells with NSC 185058 yielded blunted FSR ($p=0.0146$), similar to that of complete mTOR inhibition using Torin 1 ($p=0.0012$). When Torin 1 and NSC 185058 were combined, FSR was lowered beyond single inhibitor application levels (vs Control; $p<0.0001$). Western blot analyses revealed that NSC 185058 had a differential effect on the activation of two mTOR targets, showing a decrease in activation of p70S6K (involved with ribosomal biogenesis) with no change in eIF4EBP (involved with CAP-

dependent mRNA translation). This effect persisted when NSC 185058 was combined with either Rapamycin, MG-262, or Torin 1. Both p70S6K and eIF4EBP1 responded as expected to incubation with Torin 1, regardless of combination with either catabolic inhibitor. These results collectively suggest that there is a direct contribution of autophagic processes on normal anabolism in the cell affecting FSR, which could be mediated through disrupted mTOR signal transduction through a yet to be defined mechanism affecting ribosomal biogenesis. Additionally, the data indicate that autophagic contribution to cellular protein turnover outweighs the proteasomal contribution to intracellular AA utilized in anabolism.

# DEDICATION

I dedicate this dissertation to my grandparents, Olan and Ariewanna Meador and Pat and Elvira Cardin. They provided me so much love and support while they were alive.

ACKNOWLEDGEMENTS

These two words express gratitude, humility, understanding, as well as acknowledgement. I am here with you: my family, friends, peers, and mentors because I need to say that and much more. The colloquialism "it takes a village to raise a child" applies to getting through the PhD process. The village behind me and my dissertation is you: current and former MBL lab members.

Thank you to the College and HLKN Department for the opportunity to complete this PhD. I would like to highlight my committee members.

Dr. Shea-

I have always appreciated your teaching capabilities. You are exceptionally good at explaining difficult topics in your classes. I always appreciated your good natured personality and always looked forward to attending your class. I have always been envious of your sneaker game. Thank you.

Dr. Riechman-

I have always enjoyed chatting with you over the years. It was always nice to get the latest update on your kids. You have always brought a unique perspective with your inquisitive line in thinking that you demonstrated in the classroom and outside during our informal conversations. Thank you. Dr. Lightfoot-

I have always enjoyed taking your classes. They provide important instruction on the "soft skills" that aren't necessarily involved in a doctorate program. You have always been kind and generous in your time. Thank you.

Dr. Fluckey-

Thank you for taking me under your wing. You are a peerless teacher, boss, leader, and friend. You have provided endless opportunities beyond the traditional coursework and wet lab experience. Along with the unrivaled mentorship on all aspects of the doctoral process you have helped me to develop a deeper sense of compassion and empathy. Throughout my time in your lab, you have always treated your graduate students as people first. T am a better person and researcher because of the time spent in your lab. Please accept my heartfelt gratitude for your time, support and patience. Thank you.

Evan –

I am deeply appreciative for your support during the last several years. Thank you for your constant love and compassion. Thank you for your understanding, your never ending encouragement and for always being there for me. Your support has meant the world to me. I am a better person because of you. Thank you for loving me. I love you so very much.

My Parents –

I want to extend my thanks to my parents. Everything I am today and everything I may be tomorrow is all due to the sacrifices my parents have made for me. A big thank you to my parents.

Blue -

Blue passed away last week. He was my best friend for almost 15 years. He gave the best cuddles and was always by my side. Thank you for putting up with me for the last 15 years. His absence has saturated our home. Thank you for being the best dog.

# CONTRIBUTORS AND FUNDING SOURCES

## Contributors

This work was supervised by a dissertation committee consisting of Drs. Fluckey, Shea, Riechman, and Lightfoot of the Department of Health and Kinesiology.

The Western Blots gels were run by Patrick J. Ryan. All other work conducted for this dissertation was completed by the student independently.

## Funding Sources

Graduate study was supported by teaching assistantships from Texas A&M University.

This work was made possible in part by grants received from the Huffines Institution and from the College of Education and Human Development. Its contents are the sole responsibility of the authors and do not necessarily represent the official views of the Huffines Institute or the College of Education and Human Development.

# NOMENCLATURE

| | |
|---|---|
| AA | Amino Acid |
| GCMS | Gas Chromatogram Mass Spectrometry |
| eIF4EBP1 | Eukaryotic Initiation Factor 4 E Binding Protein |
| FSR | Fractional Synthesis Rates |
| mTOR | Mechanistic Target of Rapamycin |
| Phe | Phenylalanine |
| p70S6K | Ribosomal protein S6 kinase beta -1 |
| T2D | Type 2 Diabetes |

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# 1. INTRODUCTION

Accounting for ~40% of body weight of normal healthy individuals, skeletal muscle has the potential to impact whole body metabolism in a forceful manner. Maintenance of quality of life has been tied to quality and quantity of skeletal muscle mass (26, 216, 220). Certain pathophysiologies such as cancer (6, 142, 215), Type 2 Diabetes (T2D) (99, 173, 174), and bed rest (57, 83, 125, 126) have been associated with loss of muscle mass. Patients with lower muscle mass typically have lower "patient success" rates (12, 47, 82, 181). T2D has become one of the most common diagnoses in adults in the United States. Twenty-seven million Americans now live with T2D (27). In 2017 the indirect and direct costs associated with T2D within the United States alone was $327 billion (27). While there is a large effort to derive treatments to aid in the management of T2D, the etiology remains elusive. A significant portion of the research associated with T2D, cancer or bed rest has focused on understanding why patients have developed sarcopenic obesity, cachexia or atrophy (105, 123, 156). The previous efforts on understanding how muscle is lost, regardless of pathophysiology, has focused on the delineation of the amount of catabolism through either the proteasome or lysosome pathway. As these are the two main systems through which intracellular substrates can be degraded, understanding the timeline of the dysregulation of proteolytic mechanisms could provide an avenue to develop pharmaceutical interventions to alleviate muscle mass loss. As skeletal muscle is heavily dependent on glucose as an energy source (52), preserving muscle mass could alleviate many of the issues associated with the high blood glucose levels associated with T2D. A large cadre of the research community

indicates that autophagy is related to the onset of T2D (118, 145, 169). However, there are significant concerns with the ability of the current methods employed to account for the impact of catabolism on anabolic function. There is also a paucity of research that investigates the dual routes of intracellular degradation concomitantly with anabolic outcomes.

The anabolic machinery that allows protein synthesis to occur has been thoroughly documented over the last several decades. The first work on whole-body protein turnover was published in 1942 (203). It was a decade later in 1953 that the lysosome was discovered and that mammalian cells were first described as having the capacity to degrade intracellular proteins and that this process required ATP (192). In 1960, it was first demonstrated that proteolysis can also occur outside the lysosome via the Ca2+ dependent protease calpain (78). Over the next twenty years, researchers discovered the ATP- and ubiquitin-dependent proteasome (76), followed by the discovery of the 20S and 26S subunits in the 1980's (13, 112, 115). The past several decades have substantiated the recognition of the impact of protein degradation on the fields of physiology, genetics, medicine, and microbiology. This recognition began with the 1974 Nobel Prize being awarded to Christian de Duve for his work on the discovery of the lysosome. Thirty years later the Nobel Prize was awarded to the research trio of Aaron Ciechanover, Irwin Rose, and Avram Hershko for their discovery of the ubiquitin regulated protein degradation. The next Nobel Prize went to Yoshinori Ohsumi in 2016 for his work in autophagy. These awards show that the scientific community understands the significance of intracellular protein degradation.

The goal of this laboratory has been to expound on the known and unknown mechanisms that impact muscle mass. Skeletal muscle is a tissue that demonstrates a robust response to outside stimuli by entering either an anabolic or catabolic state. Skeletal muscle mass is one of the few tissues that runs on glucose and under starvation or certain disease conditions, skeletal muscle can catabolize itself and provide a source of nutrients. The adaptability of this tissue is of importance as it allows the body to adapt to the environment or stress. Modifications in protein metabolism are needed to meet the stages that cells go through of growth, maintenance, and atrophy. It is important to note that the change can occur through modulating either the rate of protein synthesis or protein degradation. As a cell enters the state of growth, synthesis rates will outpace degradation rates. As atrophy predominates the cell cycle, degradation rates will eclipse synthesis rates. Consequently, it is of importance that both degredation and synthesis rates are quantified. But the impact of catabolic end products has rarely been examined with anabolic endpoints such as fractional synthesis rate (FSR). The connectedness of catabolism and anabolism is usually only thought of as anabolism is anti-catabolic, with little work done to investigate how catabolism can impact anabolism. The first quantification of catabolism in skeletal muscle was in 1969 when Goldberg et al. noticed that skeletal muscle cells that were undergoing rapid growth had a slower loss of radioactively labelled protein (74). As time has progressed, the interest and capability to measure protein synthesis and degradation has grown. Our lab has pioneered a technique to measure protein synthesis using deuterium oxide. The advantage of this model is that we allow individual cells to manufacture their own precursor pool so that

labeling of proteins within the cell is relatively uniform over time. Antecedent work from this lab show that while Fatty Zucker rats with T2D have lower quantities of muscle mass, they maintain or have augmented levels of protein synthesis (163). Even when resistance exercise was introduced, the Fatty Zucker rats did not respond. It would be expected that resistance exercise should have activated the mTOR pathway which would ultimately lead to an anabolic response. Interestingly, the data suggest that proteolysis (through either the proteasome or lysosome) should be inhibited as TORC1 is presumed to be anti-catabolic(11, 124). mTORC1 activity is upregulated in the diabetic phenotype(162). Thus, while mTORC1 activation may serve to reduce proteolysis, it is plausible that the purported upregulation of catabolic responses in diabetic models service the elevated protein synthesis in the face of diminishing muscle mass. Conversely, the methodologies to quantify and isolate the contributions of each of the proteolytic pathways to the global degradation rate has been limited (21). Utilization of inhibitors to isolate each pathway are heavily utilized throughout the field, but these inhibitors typically have broader targets than the intended component within the proteolytic pathway of interest. It is imperative that inhibitors be rigorously screened to ensure off-target interactions are limited, and that it will not impact the other proteolytic pathway(s). Current literature only gives approximate delineations for the contributions of the major proteolytic pathways, with the lysosomal pathway accounting for 20-30% and the non-lysosomal (proteasome and cytosolic) accounting for 70-80% (39, 72, 73, 76, 86, 88, 224). Yet other work postulates that these rates are reversed (54, 131). Both of the catabolic pathways have been implicated with the onset of T2D. Autophagy has

been purported to contribute to the onset of T2D, as autophagic markers and rate have been noted to be aberrant in diabetic skeletal muscle(14, 51, 91, 130, 160, 231). However, researchers who are focused on the 26s proteasome have delineated dysregulation within this catabolic pathway that would lead to the development of the sarcopenic obesity phenotype (4, 10, 62, 134, 151, 154, 175). It is the mindset of this lab that there are alterations in catabolic rate that appears with the onset of T2D, which leads to sarcopenic obesity. Restoration of muscle mass could alleviate the maladies associated with T2D and allow for patients to return to a healthful state. Understanding the contrivances (and the gambit of catabolism that they are responsible for) that contribute to protein turnover in a whole cell view, rather than as independent and separate mechanisms, will aid in the identification of therapeutic targets for the amelioration of muscle mass loss.

Muscles cells throughout the body have a unique physical attribute that due to the contractile nature of these cells, the proteins responsible for the action are packed tightly and leave a limited amount of space within the sarcoplasm. Due to this morphology, the aggregation or degradation of proteins and organelles have a more demonstrative impact on the size of a muscle fiber, than in a typical cell where the quantity of intracellular proteins or organelles is not a determinant of size. When skeletal muscle fibers are exercised, the intracellular proteins and organelles increase in quantity, leading to hypertrophy of the muscle fiber. When the amount of intracellular content decreases, this leads to atrophy. A net catabolic state is typically achieved when protein degradation rate overcomes the rate of protein synthesis. Several disease states such as

cancer, diabetes, or inactivity/disuse can cause the balance of muscle mass to "tip" to an atrophic phase. The regulators of the protein synthesis and degradation rates have varied geneses ranging from skeletal muscle derived processes to extracellular hormones. These effectors can influence either the ubiquitin-proteasome or lysosomal systems to modulate rate of degradation. The degradation system, as a whole, will ultimately generate free amino acids that can be reincorporated into new proteins for the muscle fiber. By understanding if, and to what extent, these two systems impact each other by utilizing next generation catabolic inhibitors, this study aimed to delineate the interplay between anabolic and catabolic systems and how that relationship impacts protein synthesis. The following section will detail specific aspects of catabolic and anabolic processes and their role in protein turnover.

### 1.1. Hypothesis

Our group's overarching goal has been to elucidate the nuances of the anabolic framework within skeletal muscle (66, 68, 69, 162, 163, 204). However, to date, there has been no attempt to understand how catabolic signaling and ultimately the end-products of catabolism, impact anabolic capacity. With the understanding that intracellular amino acids can be derived from catabolic events of either an autophagic or proteasomal origin, it is imperative to quantify the extent that autophagy or the 26s proteasome play in skeletal muscle protein synthesis. To accomplish this, specific and highly targeted inhibitors of autophagy (NSC 185058) and 26s proteasome (MG-262), along with the anabolic inhibitors of Rapamycin (mTORC1 inhibitor) and Torin 1 (mTORC1 and mTORC2 inhibitor) were applied to C2C12 myotubes. We hypothesized

that inhibition of either the 26s proteasome or autophagy would impact FSR by limiting intracellular amino acid availability. In addition, we hypothesized that application of the catabolic inhibitors, NSC 185058 or MG-262, may cause disruption in mTOR related signal transduction. To test those hypotheses, we explored two specific aims. The first aim was designed to delineate the contribution of proteasomal-dependent and autophagic-dependent intracellular amino acid generation on FSR with the application of deuterium oxide and specific inhibitors that disabled cellular machinery within the proteasomal and autophagic degradation pathways. The second aim of this study was designed to analyze the impact of MG-262 and NSC 185058 application on the direct downstream targets of mTORC1, p70S6K and eIF4EBP1 using Western Blot analysis. Measurement of these key signaling gatekeepers informed on how catabolic processes interpose on anabolic capacity by affecting signal transduction within either ribosomal machinery recruitment (p70S6K) or CAP-dependent mRNA translation (eIF4EBP1). Together, these aims are expected to result in the identification of how the flux of protein degradation impacts myofibrillar protein synthesis and can help identify how much intracellular protein degradation is attributed to each of the main pathways (26s proteasome or autophagy) responsible for protein degradation. The implications of this study may identify mechanisms underlying a connection between catabolic and anabolic pathways that have been previously gone uncharacterized.

# 2. INTRACELLULAR DEGRADATION SYSTEMS

## 2.1. 26s Proteasome

It has been estimated that the Ub-Proteasome system handles ~80% of intracellular degradation across all cell types (39). Proteasomes can account up to 1% of soluble protein within cells (85, 208). The cellular proteins are digested to short peptides, with the majority having a size range of two – ten residues (110). Proteasomes can exist within cells for 40-200 hours (244). Proteasomes have a molecular weight of 700-750 kDa, a diameter of ≈12 nm and a length of ≈17 nm (40, 115). The oligopeptides are then released into the cytosol and are then hydrolyzed in an almost instantaneous fashion by cytosolic aminopeptidases. The resulting amino acids are then free to be sequestered by the cell for "recycling". Proteolysis within mammalian cells is a processive and irreversible activity that can have potent consequences if proteolysis becomes dysregulated. There appears to be high quantities of unengaged proteasomes that when needed (nutrient deprivation or prolonged starvation) can be "called upon" to increase degradation rates. To determine if a protein will be degraded two main steps must be accomplished. The first being an Ub-chain attaching to the substrate, and the second step is another binding-step that is highly dependent on structure and ATP hydrolysis. To accomplish attaching a Ub-chain to the substrate, the Ub is activated the E1 enzyme. There is only one form of E1 in mammalian cells (34). The E1 forms an active Ub-thioester and is then the activated Ub is transferred to an E2. E2's are Ub-carrier proteins. The final enzyme is called E3 and this attaches the Ub to the lysine residue (specifically the ε-amino group) of the substrate. E3's can also attach Ub's to

other Ub's to form a Ub-chain (33) and (71). E2's and E3's can spare certain proteins as these enzymes have the ability to ubiquitinate specific proteins at higher rates (34). Within mammalian cells, there are 10-15 E2's and more than 12 E3's (34, 35). By having a wide range of these enzymes, the cell can be very selective in its ability to degrade proteins (187). The final category of enzymes are the deubiquitinating enzymes (DUB). DUB enzymes are cysteine proteases that remove the Ub from substrate. The removal of Ub serve to disallow improper protein degradation, allowing the cell to recycle Ub-chains to future substrates and preclude Ub-chains from building up within the 20s and causing a decrease in functional efficiency (94).

The 26s proteasome is comprised of 3 separate parts. There are two 19s regulatory particles that act as gateways into the proteasome. The 19s particles bookend the central portion of the 26s proteasome which is the 20s core particle. The 20s particle has two rings that make a barrel-type structure. Each ring has seven subunits, β1-β7. There are 3 sites within each ring that have proteolytic capabilities, β1, β2, and β5. These catalytic sites face inwards and are able to sever after any amino acid. While the catalytic sites can excise after any amino acid, the active sites appear to have preferences. These active sites within the proteasome are threonine proteases (111). The active sites use the side-chain of the N-terminal threonine residue to cleave peptide bonds. The β1 subunit demonstrates a caspase-like activity and cuts after aspartic acid residues. β2 subunit has trypsin-like behavior and cleaves after basic amino acid residues. The final active subunit, β5, shows chymotrypsin-like behavior and appears to be the most important in rate of substrate degradation (188). The active sites within the

core unit appear to have an allosteric regulation that regulates the activity of the other active sites. If a substrate that is targeted for degradation has sites that can be cleaved by the β5 sites, the β1 and β2 sites become activated at an elevated rate. If there is a predominant β1 target substrate, the β2 and β5 sites are inhibited. As the β5 site is the rate limiting proteolytic site within the 26s proteasome, this would lead to a decrease in overall protein degradation rate. Research has proposed that allosteric inhibition or activation of the active sites within the 26s proteasome allow for a "bite and chew" mechanism (108). The "bite" being the activation of the β5 sites and the "chew" referring to the breaking of the remaining scissile bonds within the substrate by the additional active sites. Earlier work from Akopian et al (1997) showed that not all active sites are required for degradation of targets, but the most efficient degradation is achieved when all active sites are uninhibited (3).

The 19s cap/lid has 6 subunits, Rpt1-6. These subunits consume ATP to stimulate proteolytic function, unfold the substrate, and open the α-ring gate. The unfolded substrate is then translocated to the 20s for degradation. The 19s also contains subunits that are not involved with the ATP consumption steps which are called Rpn10 and Rpn13. These subunits help with substrate recognition and association between the lid and the base (102). DUB's are also present on the caps.

The structure of the substrate can determine the amount of time that the substrate and 26s structure have to interact and complete the degradation process. If a substrate is multi-ubquitylated, the dwell time can be increased when compared to the same substrate with a single Ub-chain (136). In contrast, when Ub-chains become forked

chains (Ub-chains are formed on contiguous lysines) the attached substrate will not be degraded (103). Proteasomes can degrade substrates with branched chains (lysines are separated by the donor Ub) with normal efficiency (153). When a substrate has a highly repetitive sequence (polyglutamines and polyalanines), the ability to translocate to the 26s (117) is negatively impacted and possibly causes aggregation of these potentially toxic sequences in diseases such as ALS, muscular dystrophies, and other neurodegenerative diseases (222). The rate of removal of the Ub chain is a primary element of dwell time of the substrate within the 26s proteasome. Substrates with more (or longer) Ub-chains would have a higher dwell time than substrates with fewer (or shorter) Ub-chains (136). The ubiquitin chain position and size could be an additional layer of control for degradation as the degree of branching and length of ubiquitin chain(s) have an inverse relationship with the affinity of the substrate to the 26s proteasome. The position of the first ubiquitinated lysine is also an important factor for degradation rate. If the first ubiquitinated lysine is located near the N-terminus of a substrate, that substrate has a faster degradation rate (136).

Once the substrate is bound to the 26s, ATP hydrolysis is activated. Degradation through the Ub-Proteasome system is an ATP-dependent process. There are 6 ATPases units within the 19s subunit. When the substrate is more tightly bound or has a higher degree of branching in the ubiquitin-chain(s), the energy expended and time to degrade the substrate increases 2-3 fold (178). The rate of degradation of ubiquitinated substrates, is directly related to the rate of ATP hydrolysis. Degradation of substrates is an extremely ATP-intensive, but rapidly occurring process. To degrade a ubiquitinated

DHFR molecule, a 26s proteasome requires ~50-80 ATP and ~ 23 seconds (178). As the size of the substrate increases, the ATP requirements and time to degrade will increase in proportion. This increase in time is due to the 26s to unwind and degrade each domain (191).

The products of 26s or 20s degradation range from 2-24 amino acids in length (195). In 1994, the "molecular ruler" was proposed by Wenzel et al. (1994). Their work postulates that proteasomes generate peptide fragments within a small range of sizes. Specifically, all peptide fragments would be within 7-9 amino acid residues (233). However, this work has been refuted (109, 110). The main concerns with the "molecular ruler" hypothesis stems from the experimental design and the lack of repeatability from other laboratories. The peptide fragments were collected after an extended incubation with 20s proteasomes. These core unit proteasomes only degrade unfolded proteins and could continually degrade protein fragments if kept in close proximity for extended periods of time. The work from Wenzel et al. also did not quantify the amount of peptide fragments within each subset of peptide length (233). The proteins that were utilized were human insulin and hemoglobin. These proteins do not represent a wide variety of proteins that are typically found within the intracellular environment. Kisselev, et al.'s work states that while the majority of products are 6-10 amino acid residues, there is a much larger range of peptides generated(109, 110). Kisselev and colleagues utilized proteins that ranged from 70-471 residues. There are two methods to determine the size of peptide fragments. The first is the "Two Rates" method and the second is the "Acid Hydrolysis" method (109). The work from Kisselev (1997)

described the number of peptide incisions per minute increase as the molecule size decreases (3). Conversely, the substrate increases in the length the number of cuts by the proteasome increases as well.

The oligopeptides generated from the 26s proteasome are then degraded by cytosolic peptidases. The products of this degradation are then further degraded by aminopeptidases. Botbol and Scornik have demonstrated that treatment with aminopeptidase bestatin, which targets aminopeptidases B and N, allows for the accretion of small peptides that range in length from 2-5 residues (22, 23, 199). This yields free amino acids that will be rapidly used by the cell for synthesis of new proteins, especially under starvation conditions. If these peptides were to accumulate within the cytosol, the concentration of olipeptides could become toxic (209).

Tripeptidyl peptidase II (TPP-II) is an oligopeptidase that removes N-terminal tripeptides in a stepwise fashion from oligopeptides (183). A large molecular weight enzyme was identified that is present in humans, rats and rabbits (107). The enzyme was found within several tissues that included liver, kindey, brain and skeletal muscle. TPP-II generates peptide fragments 7-9 residues from peptides longer than 15 residues (184). Reits et al. also produced data that would indicate that aminopeptidases are the primary digesters of olipeptides that are smaller than 15 residues (184). Work performed by Botbol and Scornik (20) report that application of aminopeptidase inhibitors culminated in accumulation of low weight oligopeptides with fewer than 5 amino acid residues (22).

The initial action to signal for the degradation of muscle mass is the activation of caspase-3, regardless of pathophysiology (55). When L6 muscle cells were incubated

with staurosporine, caspase-3 activation increased and protein degradation through the Ub-proteasome pathway was elevated. The degradation through the 26s proteasome was quantified by measuring the 14kDa actin fragments from the cell lysates. When L6 cells were serum deprived, L6 cells exhibit increased cleavage of actin filaments that are subsequently degraded. Du et al. also established evidence that calpain is not required for the accumulation of actin filaments post-serum deprivation. This finding was supported when calpain inhibitor ALLN was added and no further actin fragments were found in the samples (49).

Muscle RING finger protein 1 (MuRF1) is an E3 ubiquitin ligase that is required for degradation of myosin heavy chain (MHC). MuRF1 localizes to the sarcomere, specifically to titin. This protein is known to stabilize the M-line region within the sarcomere (147). MuRF1 is required for degradation of MHC in C2C12 myotubes. Therefore, the inhibition of MURF1, decreases the loss of MHC (36). Actin levels were found to remain unchanged during treatment. The levels of all but one of MHC isoforms were found to be unchanged leading investigators to believe that the alterations in protein levels were due to posttranscriptional events. When the investigators examined if MuRF1 expression was altered by proteasome inhibition by MG132 and epoxomicin, the expression remained unchanged.

Tripartite motif-containing protein 32 (Trim32) ubiquitinates and targets desmin, z-bands and thin filaments (37). Trim32 is an E3 ligase that is expressed throughout the body and mutations within this protein can cause myopathy, certain muscular dystrophies (61) and lower numbers in neurofilaments (120). Trim32 increases

proteolysis during fasting periods; however protein and mRNA levels of Trim32 do not increase during starvation. The destabilization of desmin appears to be the first step to allow Trim32 to further penetrate the Z-bands and its attached thin filaments. This action of Trim 32 will cause a destabilization of the Z-bands and the thin filaments. Actin is Trim32's preferential target but Trim32 also is responsible for disassembly and destruction of desmin filaments. When Trim32 function is diminished by either shRNA or a dominant-negative inhibitor noticeably rescued the decrease the loss of thin filaments and fiber diameter (38).

The degradation of the myofibril during atrophic conditions requires PAX4, a transcription factor, and desmin depolymerization. The desmin cytoskeleton must be phosphorylated and ubiquitinated before depolymerization begins. PAX4 is a transcription factor that induces the gene AAA-ATPase p97/VCP. This depolymerization causes an expedited route for myofibril degradation. Increases in phosphorylation and Trim32-dependent ubiquitination of the desmin filaments were noted shortly after denervation (119). The desmin filaments were then gradually degraded. When Trim-32 was inhibited, desmin filaments and myofibrillar proteins had decreased rates of degradation (196). Down-regulation of PAX4 or of p97/VCP also resulted in reduced myofibril breakdown (226). Fourteen days post denervation, the myofibril degradation is obligated to utilize desmin filament disassociation and PAX4 gene product creation (226).

Tribbles 3 (TRB3) protein is also a key regulator in skeletal muscle turnover and differentiation (30). Akt is negatively impacted by TRB3 by acting downstream of Akt,

but specific action sites have not been researched. Transgenic TRB3 mice had poor muscle function (46% decrease in running distance) and lower muscle mass (23% decrease in muscle mass). Decreased mTOR/s6K1 signalling and a concomitant increase in atrogene production, specifically Fox01 and Fox03a, caused these phenotypic differences (31). Along with the above mentioned differences, atrogin-1 and MuRF-1 transcripts were higher in the transgenic mice. As atrogin-1 and MuRF-1 are E-3 ligases, any elevation could cause an increase in myofibril degradation. The elevation of atrogin-1 and MuRF-1, alterations in mTOR/S6K1 pathway, and decreases in muscle mass and function when TRB3 is genetically modified, demonstrate that TRB3 plays an important role in skeletal muscle metabolism as it decreases the signaling that would cause protein synthesis and also upregulates the signaling that would cause degradation (29, 30).

The velocity of protein degradation is tightly linked to the rate of target-protein ubiquitination (135). Investigators have also demonstrated that the aptitude of the 26s proteasome to degrade is linked to the cAMP-PKA levels. Increasing cAMP levels, through either rolipram (for slow/gradual elevation) or forskolin (for rapid elevation), increases rates of degradation of short-lived proteins (both normal and aberrant) but long-lived proteins were not impacted by higher intracellular levels of cAMP (224). Myotubes harvested post rolipram administration demonstrated a doubling in chymotrypsin-like, trypsin-like, and caspase-like activities. Proteasomal phosphorylation was measured and found to have increased the capability to hydrolyze ubiquitinated proteins and ATP. The rolipram also increased levels of overall 26s

proteasomes. The elevated levels of cAMP phosphorylates Rpn6 two-fold higher than control myotubes at serine 14 but not Rpt6 (135).

### 2.2. Autophagy

Autophagy is a degradative process that is utilized by cells to balance sources of energy, amino acids, and other building blocks in response to a stressor. This process also helps the cell to rid itself of inefficient organelles (peroxisomes, endoplasmic reticulum, and mitochondria), misfolded proteins. Autophagy can be involved in a local immune response (41). There are three types of autophagy; macroautophagy, microautophagy, and selective autophagy. When the term autophagy is used in this manuscript, we will be referring to macroautophagy. Macroautophagy occurs when regions are the cell are encircled by a double membraned structure. Lysosomal based degradation is thought to account for ~20-30% of total intracellular degradation during basal metabolism (39, 84, 113).

#### 2.2.1. Induction

The process of autophagy begins with the induction of the phagophore. Autophagy-related proteins (ATG's) are relocated to specific subcellular locations. This location is referred to as the phagophore assembly site (PAS) and it resides along the phagophore membrane (46). This membrane is typically procured from either the endoplasmic reticulum or the trans-Golgi and endosomes (70). The phagophore begins as a u-shaped membrane. As the phagophore extends, the morphology of the phagophore changes into a sphere-like shape.

#### 2.2.2. Elongation

The elongation of the phagophore is accomplished in part due to the transportation of lipids. The elongation of the phagophore is accomplished by the transmembrane protein Atg 9 (132). Atg9 is activated by the Ulk1 complex. Two other Atg systems are present that mimic ubiquitin conjugation systems that contribute to the regulation of elongation. Atg12-Atg5 complex is required for the elongation and exaggeration of the curvature of the phagophore (70). Atg7 acts like an E1 enzyme and activates Atg12. Upon activation, Atg12 is transferred to Atg5. This transfer is orchestrated by Atg10. This function of Atg10 mimics an E2 enzyme activity. Atg5 then interacts with Atg16L. After this interaction is complete, the Atg-12-Atg5-Atg16L complex is formed. This complex will bind to the external membrane of the phagophore (1). This complex will disassociate once the phagophore is completely formed.

The second Atg system is the Atg8 orthologs (232). Within the Atg8 family, there are two subcategories. The first is the protein microtubule-associated protein light chain 3 (MAP1-LC3). The MAP1-LC3 family includes LC3a, LC3b and LC3c. The second is the γ-aminobutryic acid receptor-associated (GABARAP) proteins. This family encompasses Gabarap, Gabarap 11, Gate16/Gabarap12, and Gabarap13. While the exact roles of these two subfamilies currently requires elucidation, both are essential for the formation of the phagophore. Gabaraps are presumed to aid in the sealing of the phagophore as the Atg-12-Atg5-Atg16L complex is punted from the phagophore membrane (232). LC3's aid in the elongation from the U shape to the sphere shape. Both families play a role with the shepherd protein of p62/SQSTM1 (172).

The life cycle of the LC3 proteins is as follows. The brand new proteins are termed Pro-LC3. The cysteine protease Atg 4 then cleaves Pro-LC3 and the cytosolic form of LC3-1 is produced. As time proceeds, Atg7 acts like an E1 enzyme and Atg3 acts like an E2 enzyme phosphatidylethanolamine (PE) and becomes LC3-II. This form of the protein from the LC3 family is associated with only the autophagosome. The LC3-II are scattered within the inner and outer membranes of the autophagosome. Once the autophagosome is fused with the the lysosome the LC3-II molecules within the autophagosome are digested and the outer LC3-II molecules are are disservered and recycled for future use (Ravikumar et al 2010, autophagy book). Gabarap proteins are treated similarly during autophagic flux. Gabarap-II localizes alongside LC3-II on autophagosomes (106). As the phagophore extends, it will envelope misfolded/damaged/defunct proteins, cytoplasm and certain inoperative organelles within the double-membraned of the now fully formed autophagosome.

### 2.2.3. Maturation and Fusion

The maturation to a fully functional autophagolysosome or autolysosome requires the autophagosome to go through several progressions. After the ends of the phagophore membranes seal together endosomes attach to the structure (137, 152). The endosome is a structure that provides an environment that intracellular substrates can be housed and sorted before being trafficked to a degradative body (150). The new structure is termed an amphisome (16). The endosome aids in maturation by furnishing the amphisome with the required accouterment to attach to the lysosome (214). The authors suggested that the fusion of the autophagsome with the endosome and ultimately

with the lysosome shared procedural similarities with the process employed with the fusion of synaptic vesicles with the presynaptic membrane. The fusion with the lysosome is the final step before the amphisome can achieve functionality.

Once the aforementioned steps have been completed, the autophagolysosome becomes a fully functional degradatory body that the cell can utilize. The contents within the autophagolysosome are now subject to degradation with the wide variety of proteolytic hydrolases (155, 201). The hydrolases found within an autophagolysosome are varied to help ensure degradation of the large assortment of potential proteins and structures than can be enveloped by the autophagic process. Along with the cytosolic cargo within the autophagolysosome, the inner membrane is dissolved. Work from Schroder et al. detailed over 50 subcategories that include " proteinases, peptidases, nucleases, glycosidases, sulphatases, lipases, etc." (197). These hydrolases require an acidic environment that the lysosome and autophagolysosome maintain at approximately 4.5-5 pH. Cathepsins, specifically B and D, are the main degraders of the cytosolic cargo within the autophagic process. Of particular interest to this dissertation, these two cathepsins are also reported to be major players within the degradation of myofibrillar proteins (198). Cathepsin L is the primary hydrolase for degradation of the inner autophagic membrane (207). Inhibition of cathepsin L leads to lowered degradation of the inner membrane and its associated markers (LC3 and GABARAP) (217). The rates of degradation vary based on physiological conditions, cargo content, and rate of malformed proteins (32).

### 2.2.4. How Intracellular Degradation Systems Provide Amino Acids for Anabolism

Both autophagy and the proteasome generate oligopeptides that once released into the sarcoplasm, can be further degraded by oligopeptidases. These cytosolic peptidases trim the oligopeptides down to di- and tri-peptides which are further cleaved by aminopeptidases resulting in free amino acids. The free amino acids from the cleaved di- and tri-peptides can either be punted out of the sarcoplasm or used as building blocks for protein synthesis. Currently, the accepted hypothesis is that autophagy is only a contributing source of amino acids during times of extra- or intracellular stress such as amino acid starvation (161), lack of growth factors (84, 228), oxidative stress (58, 101, 127), endoplasmic reticulum stress (182, 238) or infection (17, 43, 44). There is a paucity of work that has been done with cells that are not in a pathophysiological state.

The response of the proteasome has been studied in numerous cellular stresses such atrophy (223, 234), infection (159), oxidative damage (121, 164), and several neural disorders (89, 95, 122, 148). Typically, the measurement of 26s function relies on synthetic proteins that have a flourogenic tag. These tagged proteins are then introduced into tubes containing purified 20s and 26s proteasomes from the tissue of interest. If the target proteins are degraded by the 20s or 26s proteasome, then the flourogenic tag can be read on a spectrophotometer. Nonetheless, the contributions of the proteasome are not quantified with in a “free-living” condition. The flourogenic substrates have also been documented to augment the rate of degradation within the proteasome (108), thus obscuring the ability to fully extrapolate findings from these experiments to an intact cell.

Extended periods of intracellular degradation, by either catabolic machinery, leads to elevated intracellular levels of amino acids (149, 206, 218, 243). As the amino acid levels rise, this could shift the cell towards an anabolic response. However, the impact of the 26s proteasome and autophagic contribution to intracellular amino acid production have notable drawbacks within the methodology to ascertain the extent of the role of each of these catabolic systems during basal metabolic function in healthy non-stressed cells. Individually, each catabolic system (26s proteasome or autophagy) has been studied with an improvident attitude to the whole system of protein metabolism. Researchers have investigated the impact of the mTORC1 and mTORC2 signaling on both systems but have limited insight on how catabolic machinery can interplay with anabolic signaling to allow for protein synthesis. By utilizing deuterium oxide as a tracer, this removes any issue with interpretation of end products from either catabolic pathway. These experiments aim to identify if there is a level of intercalation between each system and to what extent the role of autophagy or the 26s proteasome plays into protein synthesis within healthy skeletal muscle cells.

# 3. SIGNALING

## 3.1. mTOR

Target of rapamycin, TOR, is a protein kinase that lies at the center for many important cellular functions. The discovery of rapamycin in 1964 from the island of Rapa Nui (225), was the start of the scholarly interest in this signaling cascade. The target of rapamycin was not fully understood until 1994 when researchers demonstrated that rapamycin formed a complex with a large kinase (24, 193, 194). TOR is a large serine/threonine protein kinase that is approximately 300kDa and is a main integrator of nutritional status, especially amino acid levels, to the rest of the cell. As amino acid starvation is one of the most potent up-regulators of catabolic flux, it is critical to understand the relationship between catabolic systems and the mTORC complexes. Based on what the anti-catabolic nature of mTORC complexes and the catabolic pathways, the relationship would be counter-regulatory in nature. In this way, as mTORC activity increases, catabolic activities would decrease and vice versa. There have been attempts to delineate the relationship between mTORC complexes and the catabolic machinery (11, 128, 139), but with a focus on specific signaling molecules instead of whole system outcomes.

mTOR has multiple separate and distinct complexes that are more or less resolved, but the present study will focus on the two more widely known complexes called mTORC1(RAPTOR) and mTORC2 (RICTOR). mTORC1 responds to variety of environmental factors such as stress, oxygen, energy, nutrients, and growth factors.

After receiving these inputs from the cell, mTORC1 will effect autophagy, protein synthesis, lipid and nucleotide synthesis, and glucose metabolism. mTORC2 also responds to intracellular growth factors. mTORC2 mediates glucose metabolism, survival and proliferation, and arrangement of the cytoskeleton. Both of the subunits of mTOR play a large role in the regulation of protein metabolim. The impact of each subunit will be discussed below.

### 3.1.1. mTORC1

mTORC1 is comprised of the following subunits: raptor, GβL/mLst8, PRAS 40, and DEPTOR. As amino acid levels deplete within the cell, mTORC1 activity is decreased, allowing the process of autophagy to proceed. The ability to detect amino acid levels is executed through a system that includes RAG GTPases, Ragulator and v-ATPases (96). This is a vital transition as the process of autophagy can supply the cell with much needed amino acids during times of depletion (210).

mTORC1 also is a significant player in the cell's response to either cellular stress or growth factor signals. In regards to growth factors, the mTORC1 complex is up-regulated through the insulin/IFG1 pathway that will then signal through PDK1 and Rheb, and will cause a decrease in autophagic flux. Insulin signaling can also act upon PRAS40 as it is a substrate of AKT (116, 221). If mTORC1 is inhibited through the PTEN and TSC2 cascade, this will allow autophagy to proceed (140). Free radicals that belong to either radical oxygen species (ROS) or radical nitrogen species (RNS) are sensed by 5'AMP-activated kinase (171). AMPK can then either upregulate autophagy directly through ULK1 phosphorylation or inhibit mTOR (104). Hypoxia can also

activate AMPK (53). Another signaling mechanism that can be activated by hypoxic conditions is REDD1 (45). REDD1 negatively regulates mTOR by the TSC1-TSC2 complex.

#### 3.1.1.1. mTORC1 and Autophagy

mTORC1 can also directly inhibit formation of the autophagic vesicles in the beginning stages of autophagy. mTORC1 negatively regulates Atg1/ULK. Atg1/ULK is one of the most important "master-regulators" of the envagination of autophagic vesicles (146). Blocking the activity of this serine/threonine kinase would disallow any ability of autophagy to proceed. Atg1/ULK has been shown to be upregulated if cells were treated with rapamycin or starved (98). ULK1 binds with Atg13 and FIP200 to create a complex. mTORC1 also associates with the complex that is formed between ULK1 by connection with the Raptor subunit and ULK1 (90). Data has demonstrated that Rictor has no binding capacity for the ULK1-Atg13-FIP200 complex. When there is a surplus of nutrients, especially amino acids, mTORC1 will translocate to the ULK1-Atg13-FIP200 complex and phosphorylate Atg13 (63, 90, 97) and ULK1 on inhibitory sites (28, 90, 97). Once these sites have been phosphorylated, autophagy induction will be halted. ULK1-dependent autophagy proceeds when mTORC1 is inhibited by rapamycin (236). It is important to note that rapamycin has been shown to incompletely inhibit mTORC1 (213).

mTORC1 regulates autophagy by preventing transport of TFEB. This blockage of TFEB transport allows mTORC1 to exhibit control of autophagy at another pivotal transcriptional level (143). Inhibition of mTORC1 activity allows for lysosomes to be

active. Translocation of mTORC1 to lysosomal membranes does not correlate to the functionality of the lysosome. When the authors inhibited mTORC1 with rapamycin or PP242, mTORC1 attachment on the lysosomal membrane increased. This increase in attachment did not have a concomitant increase in cathepsin B activity.

Codongo and Poüs (citation) showed that lysosomal positioning plays a role in the relationship between mTORC1 and autophagy. In normal conditions the lysosome is anchored near the edge of the cell. When nutrient conditions become suboptimal, the lysosome has an increase in the intra-lysosomal pH. This increase disallows the lysosome to be held at the edge of the cell. This allows the autophagosomes that are developed in the perinuclear area to fuse with the lysosome and advance the autophagic process (180).

#### 3.1.1.2. mTORC2 and Autophagy

During starvation, mammalian target of rapamycin (mTOR) is inhibited as it is a nutritionally-sensitive kinase. Once mTOR inhibition is in effect, autophagic signaling commences. This allows the double membrane structures utilized by autophagy to be built, chaperone proteins that will shuttle degradative targets to the autophagosomes/autophagolysosome. It was previously demonstrated that mTOR would remain inhibited throughout extended starvation. However, it has been confirmed that mTOR is reactivated 6 hours post-starvation in cell culture by measuring phosphorylation levels of S6-kinase and 4E-BP1. These markers were negatively impacted 2-hours post starvation. The authors also demonstrated that the reactivation of mTOR is required for autophagic lysosome reformation (ALR). This was accomplished

by adding the mTOR inhibitor, rapamycin and the level of mTOR substrate phosphorylated declined and the rate of ALR was diminished. This impact lasted beyond 12 hours of starvation. The authors suggested that a small fraction of mTOR colocalizes with LC3 after the double-membrane structures have been formed (241).

Studies have shown that TORC2 is active during autophagic induction in cardiac myocytes by the phosphorylation of the downstream kinase of AKT at Ser 473 (79). TORC2 has been shown to be vital to the formation and biogenesis of production of autophagic bodies. When Rictor was knocked down, the phosphorylation of PKCα/β was decreased (79). Both Rictor and PKCα/β are positive regulators of the actin cytoskeleton. Without this activation, the formation of autophagic vesicles was negatively impacted (185).

TORC2 inhibits autophagy by promoting the amount of AKT which in turn increases the phosphorylation of FOX03. This increase in phosphorylation of FOX03 disallows the molecule to promote autophagic flux (138). As mTORC2 is an activator of AKT which then positively regulates mTORC1, the inhibitory effect of mTORC2 on autophagy is not direct. Another path for inhibition is through AKT as a negative regulator of FOX03. FOX03 is a transcription factor that will translocate to the nucleus upon activation and upregulate genes associated with catabolic activity.

mTORC2 has been shown to inhibit chaperone mediated autophagic flux in fibroblasts. The inhibition of chaperone mediate autophagy (CMA) is mediated by AKT. The authors suggest that the substrates of mTORC2 are the component that determines the directionality of its impact on autophagy (8). Inhibition of both

mTORC1 and mTORC2 by the inhibitor AZD8055, lead to autophagic induction in lung cancer cells (205). Concurrent inhibition of both mTORC complexes with OSI-027 in acute myoleogenous leukemia cells also showed induction of autophagic flux (5). OSI-027 demonstrated similar results in chronic myologenous leukemia cells (219). AZD-2014 blocked both mTORC complexes and showed an increase in autophagic cell death in colorectal cancer cells (92). Dramatic suppression of colorectal cancer cell growth by the dual mTORC1 and mTORC2 inhibitor AZD-2014. (92). AZD-2014 demonstrated autophagic induction in heptacullular carcinoma cells (133). When mTORC2 is inhibited by genetic knockouts of Rictor, the mitophagy rates increase in. *C elegans* (9).

Researchers have focused their efforts on examining the interaction between lysosomal mTORC2, PH domain and leucine rich repeat protein phosphatase (PHLPP1) and AKT. Their data show that during nutrient starvation, PHLPP1 (negative regulator of mTORC2) is recruited to the lysosome as mTORC2 activation is not high. However, 15-20 hours into starvation, mTORC2 increases activity. At this juncture, PHLPP1 is brought to the lysosomal membrane to check the anti-autophagic activity of mTORC2 (7). Inhibition of mTORC2 allowed for an increase in autophagic degradation in skeletal muscle cells (138). FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. This pro-autophagic state following the inhibition of mTORC2 is arbitrated by FOX03, a pro-autophagic molecule (144).

The scientific community is not in agreement regarding the impact of mTORC2 on autophagic flux. This lack of agreement could be due, in part, to a lack of systemic

method of measuring autophagic flux. Researchers also have divergent methodologies for measuring mTORC activation levels (of either mTORC1 or mTORC2). The current methods employ "autophagic" inhibitors that have broader targets than just the autophagic signaling cascade. Researchers need a unified and robust methodology to quantitate the extent of autophagic degradation within cells. Once this is achieved, then elucidating the impact of the mTOR complexes on autophagic rate, and the impact of autophagy on anabolism can be accomplished.

#### 3.1.1.3. p62 and mTOR

p62 has three structural domains. Those domains are the C-terminal ubiquitin-associated (UBA) domain, zinc finger domain (Zinc), and N-terminal Phox and Bem1 (PB1) domain (93). Previous work (114, 158, 202), has discussed the possibility of p62 interacting with LC3 directly. This would mean that p62 would be "pulled" to the autophagosome by the binding to LC3. After the p62/LC3 complex is within the autophagosome, it will be degraded alongside the contents of the autophagosome compartments. Ichimura et al. found that there are two hydrophobic pockets and N-terminal basic residues on LC3 that can act as docking regions for p62 (93). This mechanism allows the cells to have a "sorting" mechanism for the content that is destined for the autophagosome. The authors also show that the cells attempt to maintain a constant cytosolic level of p62 (93).

p62 has also been shown to influence mTORC1. It has been demonstrated that raptor is the protein that interacts with p62. mTORC1 and p62 have exhibited a positive feed forward mechanism wherein the higher the level of p62, the higher the level of

mTORC1. The elevated levels of mTORC1 then creates higher levels of p62 (157). The increased levels of mTORC1 has led to tumor promotion in several different cell types. p62 appears to be sensitive to amino acid levels, but not insulin levels. The level of intracellular amino acids will determine if p62 upregulates the anabolic machinery via mTORC1 activation or shift the cellular machinery towards a catabolic preset with an increase in autophagic degradation. The relationship between p62 and mTORC1 positions p62 as an intracellular rheostat that will determine the cells' response to intracellular amino acids.

#### 3.1.1.4. mTORCs and the 26s Proteasome

Determining the directionality of this relationship, and in different metabolic states, has led to a limited amount of papers. The 26s proteasome is the site of degradation of the mTORC1 subunit and its main inhibitor, DEPTOR (64, 176, 247). The ubiquitination of DEPTOR by SCFβTrCP, an E3 ligase, will cause the degradation of DEPTOR, thus allowing for mTORC1 to be active. However, if mTOR is ubiquitinated by SCFFBXW7, a different E3 ligase, the 26s proteasome will degrade the protein (141). This would decrease the ability of the cell to assemble mTORC1, thus decreasing its intracellular activity. SCFβTrCP can also ubiquitinate REDD1, calling for its destruction by the proteasome (100, 246). As REDD1 is a negative upstream regulator of mTORC1, its removal would allow for mTORC1 to be active.

Ablation of mTORC1 has been shown to decrease muscle mass across species and tissues (18, 166, 237, 240). The potential for interaction of mTORC1 and the 26s proteasome can occur on different strata. The first being that the individual components

of the 26s proteasome are themselves proteins. The 19s regulatory caps and the 20s core particle must first be synthesized by the cell before assembly into the final 26s complex. The rate of production of these particles is mediated by mTORC1 in a NRF1 dependent manner (25). mTORC1 could also upregulate mRNA translation of upstream proteasome inhibitors (211). However, other researchers have shown that mTORC1 inhibition increases 26s proteasomal function by noting an increase of a specific subunit of the 20s core particle (129), enhancing rate of protein ubiquitination (with no change in 26s content) (245). Conversely, other researchers have found mTORC1 activation increases rates of degradation by increasing 26s content to supply amino acids for sustained protein synthesis (242).

# 4. INHIBITORS

## 4.1. Catabolic Inhibitors

### 4.1.1. 26s Inhibitor – MG-262

MG-262 is a peptidyl boronate that while it requires binding, does not hydrolyze ATP to initiate inhibition (60). By having a peptide and boronic moiety, MG-262 inhibits the chymotryptic activity of the 20S proteasome. The 20S proteasome is responsible for total degradation after the protein has been tagged with ubiquitins by the ubiquitin-protein ligase, E3 (87). By inhibiting at the 20S stage of degradation, it will disallow any processing of the protein into smaller peptides.

### 4.1.2. Autophagic Inhibitor – NSC 185058

NSC 185058 is a recently discovered autophagic inhibitor. This compound effectively inhibits ATG4B activity in osteosarcoma tumors. More importantly, NSC 185058 demonstrated no effect on the mTOR or PI3K signaling cascades. The inhibition of ATG4B disallows autophagy, a specific proteolytic pathway that uses the lysosome in the final stage (2).

## 4.2. Anabolic Inhibitors

There are two prominent anabolic inhibitors that are widely used in the literature; Rapamycin and Torin1. Rapamycin is a naturally occurring substance that inhibitirs mTORC1, initially isolated from Streptomyces hygroscopius (77). Rapamycin inhibits the signaling functionality of the mTORC1 complex (56, 229). Rapamycin binds to its intracellular receptor FKBP12 which then allows for inhibition of mTORC1 (212).

Rapamycin has also been shown to reversibly inhibit 20s proteasome function by altering the conformation of the proteasomal gate, thereby slowing down degradation of ubiquitinated targets (168). However, mTORC2 appears to be resistant to the effects of Rapamycin (56, 212, 229). Torin1 is a man-made substance that is an ATP-competitive inhibitor that has been shown to inhibit both mTORC1 and mTORC2 complexes (77, 177).

## 5. DEUTERIUM OXIDE AND THE MEASURE OF PROTEIN SYNTHESIS

Mensuration of anabolic and catabolic rates has been achieved using labeled amino acids as tracers (227). These protocols employ a primed continuous infusion, continuous infusion, or flooding dose infusions (186). The choice of application will depend on subject type (human, animal, or cell culture). The radioactive label is either injected or infused with the isotope of choice into the circulatory system and a measurement is taken over a time period to quantify the rate of incorporation of the label into tissue protein. A tracer (labeled amino acid) and tracee (same amino acid lacking a label) are introduced to the subject. The researchers within the field of protein synthesis, have favored using a radiolabeled carbon atom ([14C]) or tritium ([3H]) on an amino acid(179). Amino acid that receives the radiolabel is typically either leucine or phenylalanine. These amino acids have yielded similar results, in terms of rates of synthesis. Phenylalanine has generated a preference amongst researchers as it is not oxidized in skeletal muscle. The choice of phenylalanine could lead to issues with calculation of protein synthesis measurements (75, 165).

The delivery of the radiolabeled and unlabeled amino acid cocktail can be accomplished in a variety of timing protocols. The continuous infusion is accomplished by providing the same amount of tracer:tracee over the time period of the study. The primed-constant infusion is essentially a two part protocol. The first part being that the subject is given a large bolus dose of the cocktail. A constant infusion of the cocktail is then provided for the remaining time period. The final application method, flooding dose, is achieved by administering a large, supraphysiological dose, in a short period of

time (seconds to minutes). Protein synthesis values from these studies are ~ 2% (in humans) (235) and 3-20+% (in rats) (65). These methodologies have notable concerns in regards to subjects eating, cost of procedure, and increased effort to handle the radiolabeled waste. Subjects can consume food before the initial infusion and potentially dilute the precursor pool. Consumption of food is an issue as it will confound the measurement of protein synthesis. To eliminate this problem, the study cannot be achieved in a free-living state. Subjects must be housed and monitored throughout the entirety of the study. Housing subjects for the entirety of the study raises the direct cost of the study, as researchers must account for the bed space, personnel, and subject compensation for time of the study. Once the samples are collected, the time, effort and money to properly dispose of the radioactive material is important to consider.

Fortunately, there is an alternative to radiolabel tracers. Developed in 1941, Hans Ussing developed a methodology that allows for the measurement of protein synthesis utilizing deuterium oxide. Once inside the body, 2H atoms rapidly equilibrate and attach to amino acids prior to new synthesized proteins (50). Application of deuterium oxide also is much easier than radio-labeled tracer methods, as subjects can orally imbibe the deuterium oxide. It is important to note, that small quantities should be consumed at a time to allow the inner ear of the subject to acclimate to the deuterium oxide. Deuterium oxide achieves labeling of amino acids by transfer of the H2 with H on amino acids during transamination (167). Deuterium oxide is relatively inexpensive and has a long half-life in body water. This allows deuterium oxide to be an ideal candidate for long term protein synthesis research endeavors.

Our research group focuses on alanine for quantification, as the structure of alanine allows for only one exchange which results in a simple and straightforward calculation for protein synthesis. The label remains attached during incorporation within a protein. Deuterium oxide labeling is not impacted by feeding (59). The lack of interference from feeding enables researchers to have project participants in a free-living state. As subjects do not need to be housed and monitored throughout the study, the utilization of deuterium oxide also decreases cost associated with the study. To our knowledge, there is no literature regarding the utilization of deuterium oxide to measure the impact of catabolism on protein synthesis.

# 6. MATERIALS AND METHODS

## 6.1. Approach Used

All methodologies used for the current study were in compliance and approved by Texas A&M University Biosafety committee. C2/C12 murine myotubes were cultured and assessed based on 24 hour exposure to inhibitors and deuterium oxide. Primary analyses included protein synthesis and signaling activity. A sample of the C2/C12 cell culture was collected and prepared for quantification of total label incorporation using gas chromatogram- mass spectrometry (GCMS). All cells were prepped for GCMS and immunoblotting after administration of deuterium oxide. Fractional Synthesis Rates (FSR) were quantified to determine protein rates

## 6.2. Cell Culture

Fifth passage C2/C12 myoblasts were grown on 10cm plates and passed until they reached the seventh passage. The growth media that was utilized was 1% Penicillin/Streptomycin, 10% Fetal Bovine Serum (FBS), with DMEM with sodium pyruvate serving as a base. At approximately 70% confluency, the myoblasts were given differentiation media to help shift the myoblasts into myotubes. The Differentiation Media was comprised of 2.5% horse serum, 1% Penicillin/Streptomycin and .04% insulin-transferrin-selenium. Myoblasts began to differentiate after 48 hours on Differentiation Media. Myotubes were fed for 6 days before applying inhibitor cocktail and deuterium oxide.

## 6.3. Protein Synthesis Rates

Protein synthesis was quantified using rates of accumulation of labeled peptides found in the cytosolic and myofibrillar compartment. This protein synthesis measurement spanned 24 hours to determine impact of catabolic (NSC 185058 at 100 uM and MG-262 at .01 uM) and anabolic (Torin1 25 uM and Rapamycin 50 uM) inhibitors over time. FSR will be assessed using deuterium oxide incorporation of (2H) into the myotubes and the cell culture media. Deuterium oxide is added at 4% for 24 hours prior to harvest. The cell culture media served as the precursor pool. The harvest technique began with aspiration of the media to be reserved for later preparation. The cells were then rinsed with two cycles of phosphate-buffered saline (PBS) and then the cells were removed from the plate. The cells were then spun at 150 g for 5 minutes to further remove any lingering PBS. Cell pellets were sonicated in 0.3mL Norris Buffer ([25 mM Hepes, 25 mM benzamidine, 10mM MgCl2, 5 mM-glycerophosphate, 4 mM EDTA, 2 mM PMSF, 0.2 mM ATP, 0.5% protease inhibitor cocktail P8340 (v/v; Sigma-Aldrich, St. Louis, MO), 0.1% Triton X-100 (v/v), 10 mM activated Na3VO4, and 100 mM NaF, pH 7.4]. Samples were left on ice for one hour. Samples were spun at 30,000 g for 30 minutes at 4°C. Post spin, the cytosolic fraction was decanted into a separate tube and reserved for either immunoblot analysis (see below for methods).

The myofibrillar pellet underwent another homogenization step, but in 10% (w/v) TCA. Samples were moved to the centrifuge at 5,000 g and spun for 15 minutes at 4°C. Post-spin the supernatant was decanted and discarded. This supernatant contains unbound free amino acids that are not desired for the FSR calculation. Another volume of TCA was added to the sample, pellet is re-suspended and spun at the above

specifications. The process of decanting the supernatant was performed for a total of three times for each sample. The samples were then placed on heat (100°C) for 24 hours with 0.4 mL of 6N HCl. 50 µL of sample was taken and placed in a fresh conical tube and dried down. 50 µL of a 3:2:1 (v/v/v) solution of methyl-8 (N,N-dimethylformamide dimethyl acetal) (Thermo Fisher Scientific,Waltham, MA), methanol, and acetonitrile for 1 h at 70°C to determine 2H-labeling of alanine on its methyl-8 derivative. The media that was reserved from the cell plates had an aliquot removed that was introduced to 10N NaOH and a 5% acetone in acetonitrile solution for 24 hours. Samples were extracted by adding 0.6mL of n-Hexane. All samples were enumerated in duplicate using an Agilent 789A GC coupled with an Agilent 5975C VL MSD (Agilent Technologies, Santa Clara, CA, USA), as described previously (67, 162, 163).

FSR was calculated using the following equation:

$$\mathrm{FSR} = \frac{[MPE_{myo}]}{3.7 * [MPE_{plasma}] * T}$$

Where $MPE_{MYO}$ represents the amount of protein bound $^{2}H$-alanine (mole percent excess), $MPE_{PLASMA}$ is the quantity of $^{2}H_2O$ in the media on the cells, T is the total time of incorporation and 3.7 represents the exchange of $^{2}H$ between body water and free alanine (66, 69).

**6.4. Immunoblot Analyses**

To quantify gatekeeper proteins within the anabolic signaling cascade, matching quantities of proteins obtained from C2C12 skeletal muscle samples were placed onto SDS-PAGE gels. Samples were placed into a 4X Laemmli buffer. Samples were

randomized to each gel. The gels separated the proteins based on their molecular weight. Then the transfer from the gel to the blotting membrane will occur, as described in Otter et al., (170). The gels will run for 20 minutes at 20 mA and then 40 minutes at 40 mA in a standard electrode buffer. Gels are maintained at 4°C for the entirety of the electrophoresis. This biphasic setup allows the proteins to slowly exit the well of the gel and smoothly transfer onto the body of gel. To transfer the proteins, a semi-dry method will be utilized. The transfer takes 55 minutes and the proteins will be transferred to either a nitrocellulose membrane. The membrane will be soaked in Otter buffer for 30 minutes prior to transfer (170).

After the transfer is complete, membranes will be exposed to Ponceau S. An image of the membrane will be captured and used to determine equal loading and transfer across the membrane. Membranes used for immunoblotting will be transferred to a sealed bag with 5% (w/v) nonfat dry milk in Tris-buffered saline (TBS) for 1 hour. The membrane is removed from the blocking buffer and is transferred to the primary antibody (1:1,000) at 4°C. Primary antibodies cover the signaling pathway downstream of mTOR. Membranes are rinsed in 1 X TBS and then incubated in a secondary antibody (1:2,000) on a rocker to provide gentle agitation at room temperature for 1 hour. 1 X TBS was placed on the membrane again to wash it. Enhanced chemiluminescense will be added to the membrane for a total of 5-10 minutes. Detection was accomplished by LAS 3000 coupled with Image Reader LAS-4000. Cell samples will be expressed relative to the control sample.

**6.5. Statistical Analyses**

All analyses used GraphPad Prism. One-way ANOVA was used to compare groups, and when significant F ratios are present, Tukey's post-hoc procedure will be used to test the differences between the group means.

# 7. RESULTS

## 7.1. Fractional Synthesis Rates

To date, few studies exist that have been designed to examine the impact of proteolysis on anabolic outcomes, and assessments were limited to the understanding of nitrogen balance (230). They determined that periods of elevated proteolysis, as evidenced by net negative nitrogen balance, was a key modulator of consequent protein synthesis, that approach was not sufficient to assess the impact on specific proteolytic mechanisms (81). Although much can be learned from these antecedent studies, the present study focused on the contribution of two major proteolytic systems (autophagic and proteosomal) with the utilization of the next-generation inhibitors in murine skeletal muscle. The two catabolic systems of autophagy and the 26s proteasome are largely believed to operate as individual pathways, and the majority of research in this area has focused on how the mTOR pathway affects the autophagic or 26s systems. However, there is a paucity of information concerning the opposite directionality of the catabolic pathways impacting global protein synthesis or mTOR signaling. To assess the impact of specific proteolytic systems on mTOR-mediated anabolism, we chose to use NSC 185058, which inhibits ATG4B and is a regulator of autophagosome maturation. To assess autophagy-independent proteolysis, we used MG 262, a boronic peptide acid, which inhibits the chymotryptic activity of the proteasome. Further, to assess the impact of how each or both proteolytic inhibitors affected the mTOR pathways, we used rapamycin, a naturally occurring toxin that inhibits the TORC1 complex, and/or Torin1, which inactivates both TORC1 and TORC2 complexes. By utilizing these specific

inhibitors, it allowed us to elucidate the impact of specific catabolic machinery, and ultimately their end products, on the anabolic function of murine myotubes.

### 7.1.1. Single Inhibitor

*NSC 185058 Lowers Protein Synthesis Rates Similarly as Torin 1*

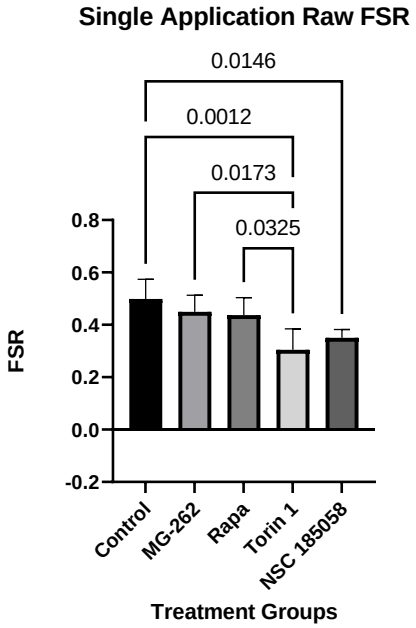


**Figure 1 Single Inhibitor Application Myofibrillar Fractional Synthesis Rates. Deuterium oxide tracer methods were used to quantify 24 hour cumulative myofibrillar FSR in C2C12 myotubes, n=5. Values are means with ± SE. Bars displaying a p-value are statistically different from the bar on the other side of the comparison.**

The first series of studies implemented the aforementioned inhibitors to determine if a particular component of either the catabolic pathway impacted anabolic function when compared to the well characterized effect of Rapamycin or Torin 1. Our results indicated that Rapamycin and MG-262 had no impact on FSR when compared to DMSO control cells. As expected, complete inhibition of mTOR complexes with Torin 1 significantly lowered FSR when compared to Control (p=0.0012), Rapamycin

(p=0.0325) and MG 262 (p=0.0173). Surprisingly, NSC 185058, an autophagic inhibitor, lowered myofibrillar FSR in murine myotubes (vs Control; p=0.0040).

### 7.1.2. Double Inhibitor

*Combination of NSC 185058 and Torin 1 has Additive Effect to Achieve Maximally Decreased FSR*

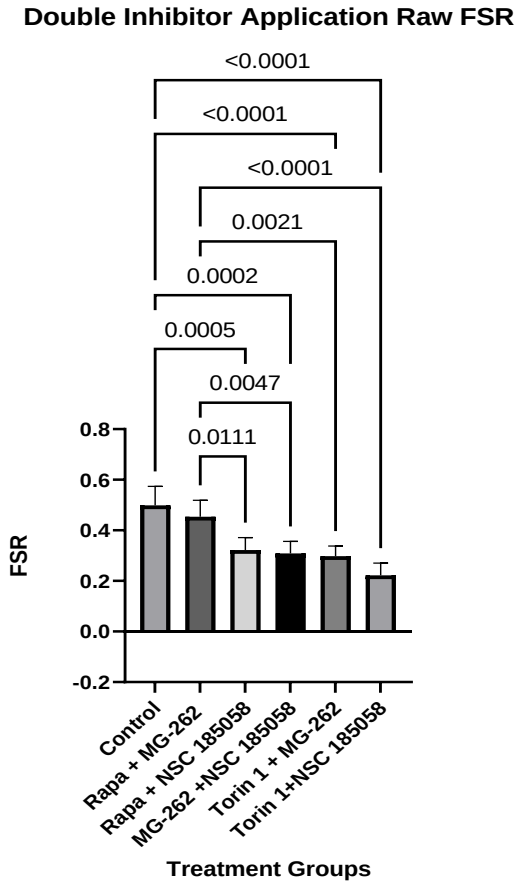


**Figure 2 Double Inhibitor Application Myofibrillar Fractional Synthesis Rates. Deuterium oxide tracer methods were used to quantify 24 hour cumulative myofibrillar FSR in C2C12 myotubes n=5. Values are means with ± SE. Bars displaying a p-value are statistically different from the bar on the other side of the comparison**

The next series of studies expanded on our initial inhibitor work by assessing specific combinations of two inhibitors on murine myobtube FSR. Treatment with

Rapamycin + MG-262 numerically lowered FSR, but was not significant relative to control. Rapamycin + NSC 185058 lowered FSR beyond the Rapamycin + MG-262 treatment ($p=0.0111$). When both catabolic inhibitors, MG-262 and NSC 185058, were combined, FSR was lowered significantly (vs Control; $p = 0.0002$). As anticipated, cells incubated with the anabolic inhibitor, Torin 1, had on of the lowest FSRs. Torin1 + NSC 185058 resulted in the lowest FSR ($p<0.0001$), and that rate was lower than any experiments from the single inhibitor studies. Of particular interest is that inclusion of NSC 185058 had lower FSR when compared to Control regardless of the other inhibitor, and Rapamycin + NSC 185058 was not different than Torin 1 + NSC 185058 ($p=0.0839$). These results could signify that autophagic degradation plays an important and sizeable role in anabolic capacity of myotubes.

### 7.1.3. Triple Inhibitor

*Triple Inhibitor Application Does Not Hinder FSR Beyond Previous Inhibitor Combinations*

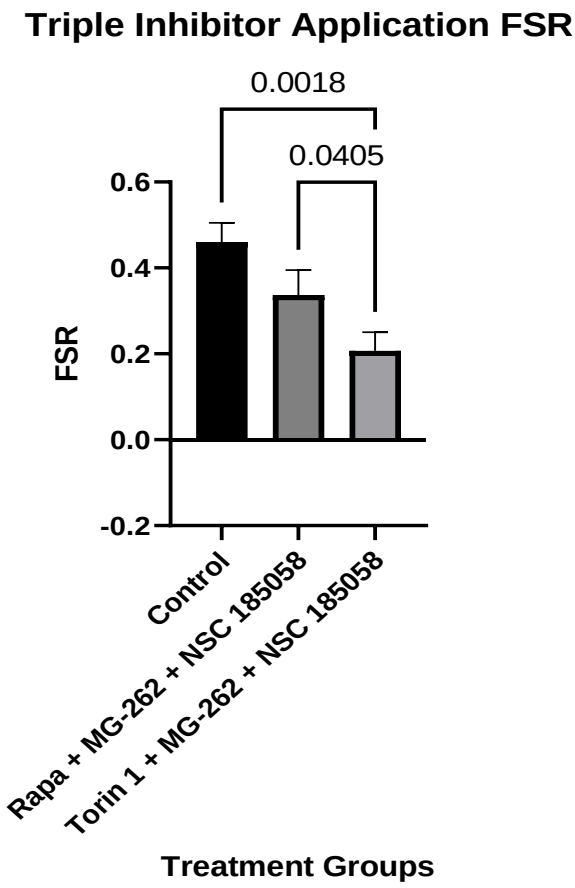


**Figure 3 Triple Inhibitor Application Myofibrillar Fractional Synthesis Rates. Deuterium oxide tracer methods were used to quantify 24 hour cumulative myofibrillar FSR in C2C12 myotubes, n=3. Values are means with ± SE. Bars displaying a p-value are statistically different from the bar on the other side of the comparison**

This cohort of murine myotubes was exposed to both MG-262 and NSC 185058 and one anabolic inhibitor (either Torin 1 or Rapamycin) in order to characterize the impact of general proteolytic inhibition on protein synthesis (DMSO) and when one or both mTOR complexes were inhibited. As anticipated, the Torin 1 group had the lowest FSR (p=0.0018). As Torin 1 inhibits two TORC (1 and 2) complexes, it follows that this group should have the largest decrement in FSR. Interestingly, inclusion of Rapamycin with the two proteolytic inhibitors resulted in a lower FSR when compared to controls

(p=0.0505), but that group was different than the Torin 1 group with both proteolytic inhibitors (p=0.0405).

### 7.2. Western Blotting

While it appears that autophagic inhibition impacts FSR, results from the above work cannot determine if the resulting inhibition was due to a potentially rate-limiting availability of amino acids with autophagic inhibition, or a more direct influence of autophagy on the mTOR pathway. To elucidate the activity of mTOR and its consequent FSR in these murine myotubes, we examined the effects of the anabolic and catabolic inhibitors on the expression and phosphorylation of hallmark mTOR targets that control several key aspects of protein synthesis. To establish the activity of the mTOR pathway, we assessed the downstream targets of p70S6K and eIF4EBP1. p70S6k is a Ser/Thr protein kinase that is responsible for phosphorylation of ribosomal protein s6 (rps6). eIF4EBP1 is a potent binding protein that prevents its binding partner (eIF4E) from participating in mTOR-mediated anabolism. Together, these two specific mTOR signaling indicators provide excellent insight as to events occurring in the anabolic cascade.

#### 7.2.1. p70S6K

##### 7.2.1.1. Single Inhibitor

*NSC 185058 Lowers Phospho:Total p70S6K Ratio in a Similar Fashion to Torin1*

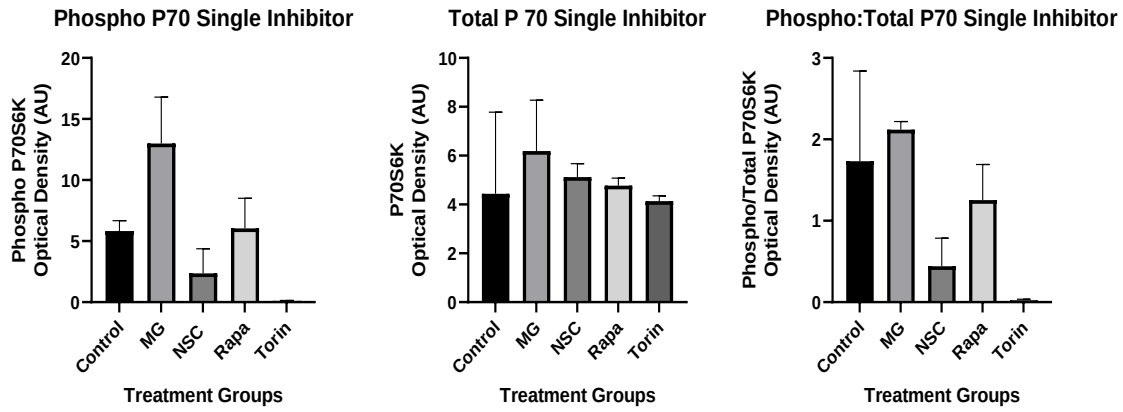


**Figure 4 Expression of p70S6K with Single Inhibitor Application. Immunoblot of the anabolic protein p70S6K in C2C12 myotubes. Assessed as phosphorylated protein (left), total protein (center) and phosphorylated protein to total (right). Values are means ± SE.**

Proteasomal inhibitor MG-262 exhibited a hyperphosphorylated p70S6K relative to any other single inhibitor treatment. MG-262 also had the highest level of Total p7026K which provided the highest total ratio of phospho/total ($p70S6K^{thr389}$/p70S6K) ratio. This data supports the FSR of the MG-262 cohort, lower levels of phospho p70S6K, relative to the other catabolic inhibitor, which was not statistically different from control. In contrast, the NSC 185058 treatment had lower phospho/total ($p70S6K^{thr389}$/p70S6K) ratio. The lower phospho/total ratio supports the lowered FSR of the NSC 185058 cohort. Both of the anabolic inhibitors decreased phospho p70S6K levels, with Torin 1 having the greatest effect. Torin 1 application lowered the phospho/total ($p70S6K^{thr389}$/p70S6K) ratio to the lowest of any single inhibitor.

### 7.2.1.2. Double Inhibitor

*NSC 185058 Acts in an mTORC1 Independent Manner to Lower Activation of p70S6K*

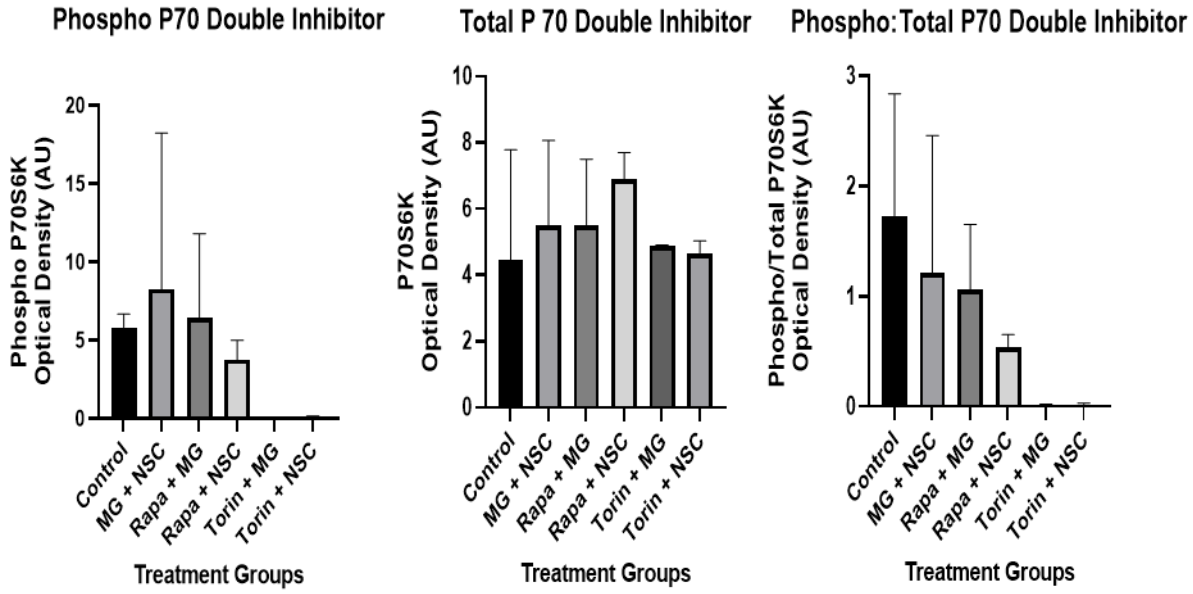


**Figure 5 Expression of p70S6K with Double Inhibitor Application. Immunoblot of the anabolic protein p70S6K in C2C12 myotubes. Assessed as phosphorylated protein (left), total protein (center) and phosphorylated protein to total (right). Values are means ± SE.**

Application of solely catabolic inhibitors (MG-262 and NSC 185058) increased phospho p70S6K levels above control values, with a similar but smaller impact on total p70S6K values. When Rapamycin was paired with MG-262 or NSC 185058 both phospho and total levels of p70S6K decreased. The treatment of NSC 185058 with Rapamycin lowered the phospho:total ratio of ($p70S6K^{thr389}$/p70S6K). This could indicate an mTORC1 independent (as Rapamycin is already inhibiting mTORC1), autophagic reliant mechanism that allows for crosstalk between both the catabolic and anabolic pathways within the cytosol. This mechanism could be related to the discontinued contribution of autophagy to the production of intracellular amino acids, which the myotube may prefer to extracellular amino acids, but it is yet undefined. NSC 185058 could also impact factors relating to the translation of p70S6k. All groups with

NSC 185058 inhibitor showed decreased levels of phospho/total p70S6K. Again, Torin 1 + NSC 185058 decreased the numerical value of the phospho p70S6K levels the most, leading to the lowest phospho/total ($p70S6K^{thr389}$/p70S6K) ratio. This is to be expected with both mTORC1 and mTORC2 being inhibited by Torin 1.

### 7.2.1.3. Triple Inhibitor

*Inhibition through Torin1 with addition of NSC 185058 Lowers Phospho:Total P70S6K Beyond Inhibition of mTORC1*

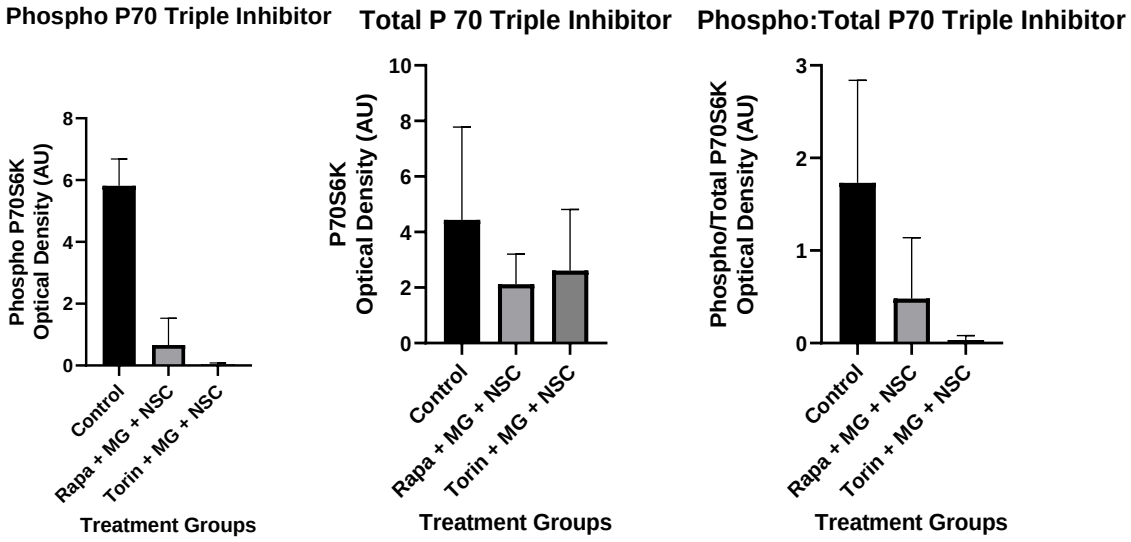


**Figure 6 Expression of p70S6K with Triple Inhibitor Application. Immunoblot of the anabolic protein p70S6K in C2C12 myotubes. Assessed as phosphorylated protein (left), total protein (center) and phosphorylated protein to total (right). Values are means ± SE.**

Each anabolic inhibitor was applied with both catabolic inhibitors. This set of treatments would yield information regarding the impact of both proteolytic systems when either mTORC1 (Rapamycin) or mTORC1 and mTORC2 (Torin 1) is inhibited. Both combinations yielded lower levels of phospho p70S6K compared to control. There was no difference within treatment groups for phospho p70S6K levels. The trend of

Rapamycin + MG-262 + NSC 185058 and Torin 1 + MG-262 + NSC 185058 having numerically lower values continued for total p70S6K levels, with the Torin1 combination generating the lowest value. Consequently, Rapamycin + MG 262 + NSC 185058 had decreased the phospho/total ($p70S6K^{thr389}$/p70S6K) ratio from control, while Torin 1 + MG-262 + NSC 185058 treatment had the lowest phospho/total ($p70S6K^{thr389}$/p70S6K) ratio. As Torin 1 inhibits mTORC1 and mTORC2 it follows that this combination had the lowest values compared to only inhibiting mTORC1.

### 7.2.2. eIF4EBP1

#### 7.2.2.1. Single Inhibitor

*Torin 1 Inhibition Decreases Phospho:Total eIF4EBP1*

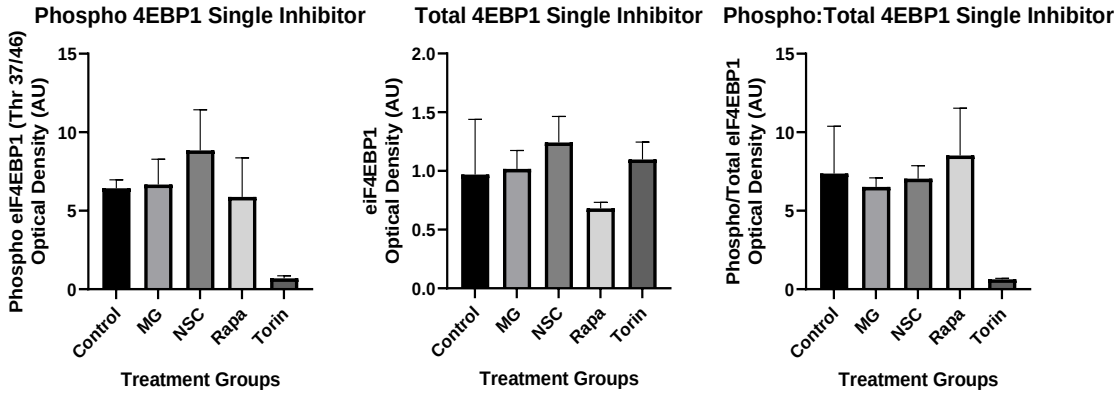


**Figure 7 Expression of eIF4EBP1 with Single Inhibitor Application. Immunoblot of the anabolic protein eIF4EBP1 in C2C12 myotubes. Assessed as phosphorylated protein (left), total protein (center) and phosphorylated protein to total (right). Values are means ± SE.**

NSC 185058 treatment elevated phospho eIF4EBP1 levels relative to Control ($p=0.8086$). Whereas MG-262 and Rapamycin had little effect on $eiF4EBP1^{thr37/46}$ relative to control. Torin 1 lowered $eiF4EBP1^{thr37/46}$ to the greatest degree, to 10% of Control values. Total eIF4EBP1 levels were relatively conserved across treatments with

no group being different within the cohort. The ratio of phospho/total ratios of eiF4EBP1 were relatively conserved, except for Torin1 lowering the ratio the most. Torin 1 lowered Phospho:Total ratios of eIF4EBP1 when compared to Rapamycin application.

### 7.2.2.2. Double Inhibitor

*eIF4EBP1 Responds to mTOR Inhibitors*

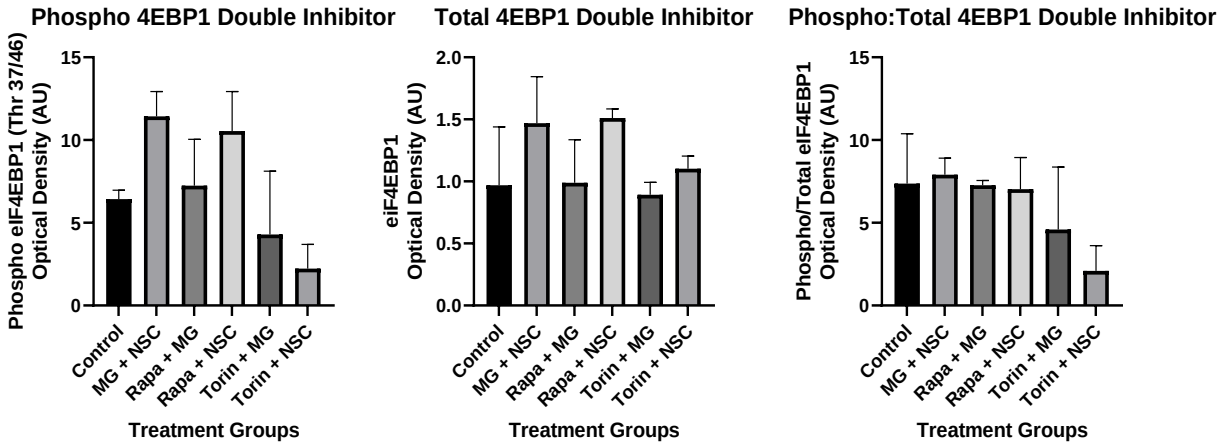


**Figure 8 Expression of eIF4EBP1 with Double Inhibitor Application. Immunoblot of the anabolic protein eIF4EBP1 in C2C12 myotubes. Assessed as phosphorylated protein (left), total protein (center) and phosphorylated protein to total (right). Values are means ± SE.**

Notably, application of MG-262 and NSC 185058 appears to have an additive effect when applied together and increase phosphorylated eiF4EBP1 levels beyond control and the respective single inhibitor application. However, when either catabolic inhibitor is separated and paired with Torin1, phosphorylated eiF4EBP1$^{thr37/46}$ levels decline. Phospho-4EBP1 was 31% lower in Torin 1 + MG-262 and 65% lower in Torin 1 + NSC 185058. This suggests that NSC 185058 application is not impacting mTORC signal transduction to eIF4EBP1. The mechanism through which NSC 185058 is

achieving lower FSRs may be accomplished through a target downstream from mTORC1 that disallows ribosomal machinery to be upregulated for protein synthesis when autophagic processes are inhibited.

### 7.2.2.3. Triple Inhibitor

*Triple Inhibitor Combinations Do Not Lower Phospho:Total eIF4EBP1 Beyond Levels Obtained with Double Inhibitors*

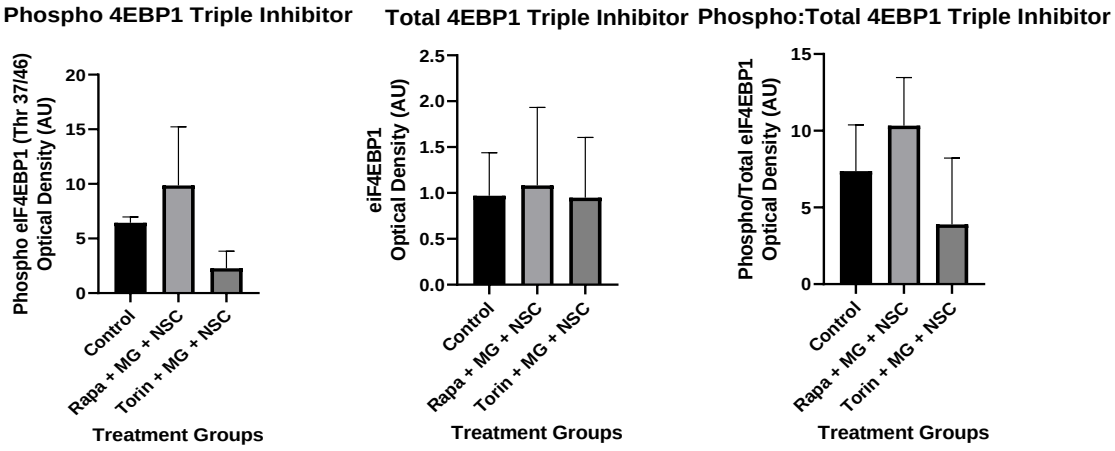


**Figure 9 Expression of eIF4EBP1 with Triple Inhibitor Application. Immunoblot of the anabolic protein eIF4EBP1 in C2C12 myotubes. Assessed as phosphorylated protein (left), total protein (center) and phosphorylated protein to total (right). Values are means ± SE.**

The treatment of Torin 1 + MG-262 + NSC 185058 lowered the phospho eiF4EBP1 $^{thr37/46}$ by 63%. Rapamycin + MG-262 + NSC 185058 had an augmented phospho eiF4EBP1 $^{thr37/46}$ response, relative to control and the Torin 1 + MG-262 + NSC 185058 group. No phenotypic differences of Total eIF4EBP1 levels across groups was observed. Ultimately, the Torin 1 + MG-262 + NSC 185058 had the lowest phospho/total ratios of eiF4EBP1 but did not lower beyond the Torin 1 + NSC 185058 combination in the previous cohort.

### 7.3. Summary

Together, the present data suggest that the decrement in FSR and key anabolic signaling events is primarily attributed to inhibition of mTORC1 and mTORC2 with the surprising finding that inhibition of the autophagic machinery can impact anabolic outcomes by disrupting mTOR signal transduction. Further, the lack of diminished response from inhibition of the 26s proteasome delineates a separation of roles within the catabolic pathways for contribution to intracellular amino acid content in healthy, metabolically unchallenged myotubes.

# 8. DISCUSSIONS

Previous research has strongly focused on mTOR signaling and its influence on skeletal muscle synthesis and as it relates to protein degradation in an inhibitory manner. Although prior studies have alluded to potential contributions of catabolic end products serving as building blocks during times of metabolic stress, no studies yet exist that have performed experiments on metabolically stable muscle cells. To our knowledge, this is the first set of experiments to quantify the impact of both autophagy and the 26s proteasome on FSR, specifically in skeletal muscle. The central hypothesis was that both catabolic pathways could impact protein synthesis as these systems provide amino acids within metabolically stable skeletal muscle. The major finding of this study, contrary to our hypothesis, is autophagy was the only proteolytic system to impact FSR. The suppression of protein synthesis through the catabolic autophagic-specific inhibitor NSC 185058 is of notable interest as this inhibitor blocks the formation of the autophagosome at the pre-sequestration stage. Previous versions of autophagic inhibitors target the lysosome, which is at the endpoint of autophagic degradation. NSC 185058 is an upstream inhibitor that targets ATG4B that has no known interactions within the anabolic signaling cascade. Therefore, it is very likely that the suppression of FSR with NSC 185058 treatment was mediated through an mTOR independent mechanism.

FSR in skeletal muscle, and most tissues, is mainly driven by mTOR through the respective complexes of mTORC1 and mTORC2. Rapamycin inhibits only mTORC1, thus suppresses FSR minimally in comparison to Torin 1 which inhibits both mTORC1

and mTORC2. The present results illustrate and support previous findings (18, 42, 48, 80, 237, 248) that mTORC1 and mTORC2 are required for full anabolic potential.

While we recognize autophagy and the 26s proteasome are important, the orchestration of catabolic machinery that allows for robust protein synthesis must be elucidated. Thus, a series of experiments with a combination of anabolic and catabolic inhibitors to allow for separation between catabolic pathways were executed. The application of the 26s inhibitor, MG-262, had minimal impact on FSR, suggesting that the magnitude of amino acids arising from the proteasome does not impose any rate-limiting adjustments to the amino acids necessary for FSR or proteosomal degradation. If so, the present data strongly suggests that proteosomal degradation is not the likely avenue utilized by stable, healthy murine muscle fibers for intracellular amino acid recycling. This could be due to that the overwhelming majority of oligopeptides that form major histocompatibility complex 1 originate from the 26s proteasome (187, 189, 190, 239). Theses oligopeptides could be preferentially trafficked away to disallow incorporation into new proteins. As the ubiquitin tagging and actual degradation by the 26s proteasome is costly, in terms of ATP, cells may have a mechanism to "protect their investment" and allow the oligopeptides from the autophagic system (zero to low ATP cost) to be utilized as building blocks for new proteins. The 26s proteasome is an ATP-dependent process that scales with the size of the target of degradation. To conserve ATP for anabolic activity, it would be the smartest route to utilize autophagy for intracellular amino acid recycling. If ATP conservation is of utmost importance, to allow protein synthesis to continue unimpaired, autophagic machinery can also degrade

ubiquitinated targets. By utilizing this route of intracellular degradation cells can reserve ATP for building proteins as autophagy has minimal to non-existent ATP costs. Consequently, when the protein synthesis machinery is in need of amino acid building blocks, and ATP is limited overall, autophagic recycling of aged/exhausted proteins would be the preferred catabolic route.

In an attempt to further expound on the mechanisms that drove the decrease in FSR with inhibition of the autophagosome versus the proteasome, expression of the anabolic signaling molecule p70S6K was measured. A direct target of mTORC1, p70S6k has been shown to be tightly linked to the regulation of protein synthesis through its ability to upregulate ribosomal activity. When phosphorylated P70S6k is decreased, skeletal muscle protein synthesis declines. This report corroborates the findings in the literature that a decrease of phospho:Total p70S6k resulting from mTOR inhibition demonstrated lower FSR compared to control cells. Of particular interest for the present study is that the addition of NSC 185058 resulted in a profound reduction of the phospho:total ratio of p70S6k, and the effects were additive to the effects of Rapamycin or Torin1 (see Figures 4-6). While it is well-known that the activation of mTOR impacts autophagic processes, our data are the first to support the notion that the formation of the autophagosome directly impacts mTOR signal transduction. Unfortunately, the present study was not designed to address this potential cross-talk, but will be directly assessed in our future work. Future studies should aim to characterize how the NSC 185058 molecule could accomplish these interactions and offer a better

explanation as to how autophagy integrates within the anabolic framework to allow for maximal anabolic capacity.

eIF4EBP1 is another signaling molecule within the anabolic network that is a direct target of mTORC1 phosphorylation at two sites (T37 and T46). When eiF4EBP1 is phosphorylated, its inhibitory effect is alleviated and allows eIF4F to complex with eIF4G to allow cap-dependent translation to continue. It is important to note that the majority of these inhibitor combinations did not yield notable differences (see Figures 7-9). As eIF4EBP1 was primarily only impacted by Torin 1, which is an anticipated response, this means that the potential mechanism through which NSC 185058 is impacting anabolic signaling is not through mTORC1 directly. If NSC 185058 did directly alter mTORC1, then both of its downstream targets (p70S6K and eIF4EBP1) would have decrements in phospho:total ratios. As the NSC 185058 drug binds to the active sites of ATG4B, Cys74, Asp278, and His280, mTORC1 does not have similar amino acids at the same locations. Thus, NSC 185058 would be unable to bind and attach to mTORC1. As the target sequence of NSC 185058 does not have a matching sequence on mTORC1, this points to NSC 185058 targeting downstream of mTORC1 that would disallow the elevation of ribosomal machinery needed for protein synthesis.

With the decrement of fractional synthesis occurring due to the application of NSC 185058, the preference of amino acids for protein synthesis could be intracellular (and only generated through autophagic processes) is in direct contrast to the canonical belief that extracellular amino acids drive protein synthesis (15, 19, 20, 200). Those studies utilized radiolabeled amino acids, such as $D_{2,5}$-Phe, [$^{15}$N]Phe, or [1-$^{13}$C]Leucine

as their tracers. By utilizing an extracellular tracer, it confounded the ability to distinguish any impact of amino acid origin on protein synthesis. Using those methodologies, presentation of the fixed label tracer to the cell was from an extracellular source (via the blood or media) which disallowed any transparency about how intracellular amino acids arising from proteolysis impacted synthesis rates. With the common understanding that the three ways to increase free amino acid levels within skeletal muscle by either degrading muscle protein, uptake from extracellular fluids, or conversion from other amino acids or intermediates, those tracer studies can only measure the uptake from the extracellular fluid. The methodology failed to address the contribution of either the degradation of muscle protein or conversion from other substrates/amino acids to the pool of available amino acids.

The utilization of deuterium oxide methodologies in the current study, wherein the label is attached via the cell's own transamination reaction, sidesteps the aforementioned experimental design issue and allows for a better investigation of the amino acid source/preference for protein synthesis. It is interesting to note that only the inhibition of autophagy, but not the proteasomal machinery, lead to a decrement in FSR. The predominant thought of autophagy is that it is only a source of amino acids during starvation or physiological stressful environment, yet the media that the cells were cultured in provided every nutritional and physiological requirement. Therefore, given our results, the prevalent thought that autophagy is only a source of amino acids during starvation or physiological stressful environment appears to be largely overstated. Our

results suggest that the role of catabolism, specifically autophagy, during a normal physiological state, is tightly linked with the anabolic potential of murine myotubes.

In summary, we provide evidence that protein synthesis of metabolically stable C2C12 myotubes relies on autophagic flux to a much greater extent than previously thought. Although the proteasomal system is thought to be the major degradation system in skeletal muscle, the application of the MG-262 had little effect on FSR or either downstream target of mTOR. Whether this is due to the oligopeptides generated from the proteasome having a pre-decided fate that is separate from the anabolic apparatus is not known; either inhibition of the proteasome is meaningless for FSR, or the 26s proteasome is not as big of a contributor to degradation for unchallenged skeletal muscle cells as previously thought. The autophagic-dependent mechanism that we've described appears to be mediated through an mTORC1 independent pathway that has yet to be elucidated. Our data strongly suggest that phosphorylation of p70S6K is directly impacted by application of NSC 185058 while phosphorylation of 4EBP1 remains unfettered. Rationale for that conclusion is based on the observation that if mTORC1 was directly impacted by NSC 185058, then both of the downstream targets would be impacted equally. To our knowledge, this is the first study to demonstrate that autophagy is permissive for full anabolic potential. While at present we cannot provide the underlying mechanism(s) that achieve this cross-talk between autophagy and the mTOR pathway, we can infer that the steps are related to disallowing recruitment of ribosomal machinery needed for protein synthesis. Taken together, these findings

underscore the importance of the autophagic flux for anabolic capacity and point towards a unique mechanism through which degradation and synthesis are tightly linked.

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