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The Role of AMPK in the Regulation of Skeletal Muscle Proteostasis During Cancer Cachexia

Dennis K. Fix

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by

Dennis K. Fix

Bachelor of Science
University of North Carolina at Pembroke, 2011

Master of Science
University of South Carolina, 2014

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Accepted by:

Mark A. Sarzynski, Major Professor

James A. Carson, Committee Member

Ashley J. Smuder, Committee Member

Ho-Jin Koh, Committee Member

Cheryl L. Addy, Vice Provost and Dean of the Graduate School

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ABSTRACT

Cancer-induced cachexia is a debilitating disease that diminishes quality of life due to severe loss of skeletal muscle mass and function. Skeletal muscle proteostasis fluctuates diurnally depending on different fasting and feeding durations as well as in response to activity and exercise stimuli. Nutrient availability is key in the net loss and gain of skeletal muscle protein. The adenosine monophosphate protein kinase (AMPK) is a nutrient sensitive kinase that has long been established as a critical regulator of both protein synthesis and protein degradation. AMPK is capable of being activated by many different stimuli as well as diseases such as cancer cachexia. During severe cancer cachexia, skeletal muscle AMPK is dysregulated which could have dire consequences regarding muscle proteostasis. When activated, AMPK will phosphorylate tuberous sclerosis protein 2 (TSC2) and Raptor which inhibit mTORC1 activity. In addition to suppressing protein synthesis, AMPK controls degradation through the activation of its direct downstream targets ULK-1 and FOXO3a. While the chronic activation of AMPK and its potential role in anabolic suppression has been examined in detail, the role of this signaling axis in the regulation of protein turnover (synthesis and degradation through UPS and autophagy) is poorly understood in the skeletal muscle of cachectic mice. Moreover, given the sensitivity of this pathway to nutrient availability, further investigation is warranted to elucidate if the physiological fasting and feeding regulation of AMPK signaling is disrupted in the cancer environment. The purpose of this study was to determine if cachexia associated aberrant AMPK signaling is responsive to

fasting, feeding, and increased wheel running activity to regulate skeletal muscle proteostasis. We hypothesized that cancer cachexia induces the disruption mitochondrial quality control which disrupts the regulation of AMPK by fasting and feeding leading to muscle mass loss. We also hypothesized that increased physical activity will improve mitochondrial quality control and thus improve the regulation of AMPK to fasting and feeding thus improving protein turnover and attenuating wasting. Our results suggest that skeletal muscle AMPK is necessary for the regulation of protein turnover during cancer cachexia. Furthermore, cachectic mice exhibit an accelerated AMPK signaling response to fasting, which may serve to exacerbate muscle wasting. We also report that cachectic Min mice exhibit an increased sensitivity to nutrients or feeding following a short term fast, suggesting that fasting increases skeletal muscle sensitivity to feeding. Finally, we demonstrate that increased physical activity via wheel running is capable of attenuating or improving the cachectic skeletal muscle AMPK response to fasting.

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α	Alpha
±	Plus or Minus
β	Beta
%	Percent
κ	Kappa

LIST OF ABBREVIATIONS

ACC ..... Acetyl CoA Carboxylase

ADP ..... Adenosine Disphosphate

AKT ..... Protein Kinase B

AMP ..... Adenosine Monophosphate

AMPK ..... AMP-activated Protein Kinase

ANOVA ..... Analysis of Variance

APC ..... Adenomatous Polyposis Coli

ATP ..... Adenosine Triphosphate

BW ..... Body Weight

C-26 ..... Colon-26 Carcinoma

COPD ..... Chronic Obstruction Pulmonary Disease

DNA ..... Deoxyribonucleic acid

DRP ..... Dynamin-Related Protein

EDL ..... Extensor Digitorum Longus

Erk 1/2 ..... Extracellular signal-related Kinases

Fis ..... Fission Protein

FOXO ..... Forkhead Box O

GAPDH ..... Glyceraldehyde 3-phosphate dehydrogenase

gp130 ..... Glycoprotein 130

GTP ..... Guanosine Triphosphate

HSA ..... Human alpha-skeletal actin

IFN ..... Interferon

Km ..... Kilometer

IL-6. ..... Interleukin-6

KO ..... Knockout

LC3 ..... Microtubule-associated proteins 1A/1B light chain 3B

LIF ..... Leukemia Inhibitory Factor

LLC ..... Lewis Lung Carcinoma

LPS ..... Lipo-polysaccharide

MAPK ..... Mitogen Activated Protein Kinases

MFN ..... Mitofusion

mg ..... Milligrams

MHC ..... Myosin Heavy Chain

Min ..... $Apc^{Min/+}$

MLC ..... Myosin Light Chain

mRNA ..... Messenger Ribonucleic Acid

mTORC1 ..... Mechanistic Target of Rapamycin Complex 1

NF-κB ..... Nuclear Factor κ B

NRF ..... Nuclear Response Factor

OPA ..... Optic Atrophy Protein

p ..... Phosphorylated

PGC-1 ..... Peroxisome-Proliferator Gamma-Activated Receptor Coactivator

ROS ..... Reactive Oxygen Species

SUNsET .....Surface Sensing of Translation Technique

TSC2.....Tuberous Sclerosis Protein 2

ULK-1 ..... Unc-51 like kinase 1

CHAPTER 1

REVIEW OF LITERATURE

1.1 Cancer Cachexia

Cachexia is defined as a complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass(Baracos, Martin, Korc, Guttridge, & Fearon, 2018; Evans et al., 2008). Cancer cachexia is a debilitating wasting syndrome that occurs in approximately 80% of all cancer patients and is responsible for approximately 30% of all cancer related deaths (Argiles et al., 2010; Baracos, 2006a, 2006b, 2011; Evans et al., 2008; K. Fearon et al., 2011; K. C. Fearon & Baracos, 2010). Cachexia is infrequently identified or diagnosed and rarely treated(Baracos, 2011, 2013; Blum et al., 2010; Bruggeman et al., 2016). Cachexia development is not associated with all cancers, but the progression of cachexia is directly associated with cancer patient morbidity and mortality(Baracos, 2006a; Sadeghi et al., 2018; Solheim et al., 2011; Solheim et al., 2018). While cachexia has been reported to account for a significant number of cancer deaths successful treatment of the cachectic cancer patient remains elusive (Sadeghi et al., 2018; Solheim et al., 2018). There are currently no approved therapeutic treatments for cachexia, and while it has been a widely investigated syndrome the mechanisms that underlie cancer cachexia have yet to be fully elucidated.

Paradoxically, in some cases muscle protein synthesis has also been reported to be elevated due to an abundance of amino acids from accelerated muscle protein degradation(Dworzak, Ferrari, Gavazzi, Maiorana, & Bozzetti, 1998; Emery et al., 1984; Whitehouse, Smith, Drake, & Tisdale, 2001). Muscle wasting is an important pathophysiological characteristic of cachexia and a major cause of fatigue and decreased quality of life in cancer patients (Baracos, 2001; Dodson et al., 2011; K. Fearon et al., 2011). Skeletal muscle quality has also become a current method of classifying muscle, and is directly related to a muscle's metabolic capacity and ability to utilize substrates (Chomentowski, Coen, Radikova, Goodpaster, & Toledo, 2011; B. N. VanderVeen, Hardee, Fix, & Carson, 2017; White, Baltgalvis, et al., 2011). Disruption of these processes by cancer cachexia could impact health and survival and also regulate the muscles microenvironment. Muscle quality can also be expanded to include the regulation of muscle anabolic and catabolic processes which can also regulate the muscle proteostasis or balance between synthesis and degradation (Emery et al., 1984; Romanello & Sandri, 2015; Suliman & Piantadosi, 2016; Whitehouse et al., 2001).

Our laboratory as well as others have demonstrated a suppression of anabolic signaling mechanistic target of rapamycin complex 1 (mTORC1) and the induction of muscle protein degradation in pre-clinical animal models of cancer cachexia (White, Baynes, et al., 2011a; White, Puppa, Gao, et al., 2013). Interestingly coinciding with the suppression of the mTORC1 signaling pathway is the potent induction of adenosine monophosphate protein kinase signaling (AMPK)(White, Puppa, Gao, et al., 2013). AMPK has been deemed a critical regulator of skeletal muscle protein degradation through the regulation of multiple signaling pathways such as forkhead box O (FOXO)

and the autophagy lysosomal proteasome system (Koh, Brandauer, & Goodyear, 2008; Long & Zierath, 2006; Sanchez, Csibi, et al., 2012; White, Puppa, Gao, et al., 2013). AMPK has been deemed a target of multiple stimuli ranging from inflammatory cytokines such as IL-6, to acute exercise, and nutrient deprivation or fasting/starvation(Narsale & Carson, 2014; Sanchez, Csibi, et al., 2012; White, Puppa, Gao, et al., 2013). AMPK has also been deemed as a master regulatory switch that contributes the regulation of muscle mitochondria through the stimulation of mitochondrial biogenesis and its negative regulation of energy consuming pathways such as mTORC1(Carson, Hardee, & VanderVeen, 2016). Numerous pre-clinical models of cancer cachexia have demonstrated elevated AMPK in skeletal muscle that is concomitant with elevations in skeletal muscle protein degradation and decreased anabolic signaling (Mangum et al., 2016; Puppa, Gao, Narsale, & Carson, 2014; Puppa et al., 2012). The following review of literature will discuss cancer cachexia in detail, potential mechanisms, and regulators of skeletal muscle mass. Additionally, the mTORC1 anabolic signaling pathway and protein synthesis will be discussed and how cancer cachexia affects this signaling axis. The AMPK signaling pathway will also be discussed in detail and will encompass its regulatory roles in protein synthesis and degradation of skeletal muscle while highlighting current gaps in our understanding of this signaling pathway during cancer cachexia.

Weight Loss

Cachexia is a common modality that affects numerous cancer patients and patients with chronic diseases such as HIV, cancer, and chronic kidney disease (Argiles et al., 2010). While not all types of cancer are susceptible to cachexia many that do

present with the disease are often un-diagnosed or diagnosed far too late to have any chance of an intervention (Baracos, 2013; Baracos et al., 2018). Much of this initial problem was due to a lack of a consensus definition among clinicians and researchers as to what cachexia is (Blum et al., 2011; Bruggeman et al., 2016; Hemming & Maher, 2005). Further exacerbating this lack of consensus is the poorly understood mechanisms that underlie this disease (Baracos, 2006a, 2013, 2018; K. C. Fearon, Voss, Hustead, & Cancer Cachexia Study, 2006; Hemming & Maher, 2005; N. MacDonald, Easson, Mazurak, Dunn, & Baracos, 2003). Weight loss is a common and powerful variable that predicts mortality and morbidity in cancer patients (Balstad, Solheim, Strasser, Kaasa, & Bye, 2014; Bruggeman et al., 2016; Hemming & Maher, 2005; Roeland et al., 2017). In HIV patients, body weight loss as little as 3% increased morbidity and mortality, and the risk of death increases with the magnitude of weight loss (Evans et al., 2008; Sadeghi et al., 2018). However, therapies that slow or prevent weight loss are not always beneficial and have proven difficult to interpret in a clinical setting (K. Fearon et al., 2015; K. C. Fearon & Baracos, 2010; Johns et al., 2017; Khan et al., 2003; Martin et al., 2013). Some researchers argue that weight loss alone does not always encompass the full effect of cachexia on physical function and should not be used as the sole prognostic variable(Bruggeman et al., 2016; K. Fearon, Arends, & Baracos, 2013; Johns et al., 2017). However, it is clear that body weight changers can be accurately assessed, and the evidence demonstrates that it is highly predictive of morbidity and mortality in cachectic patients (Baracos, 2006a; Blum et al., 2014; Carson et al., 2016; Evans et al., 2008; Solheim et al., 2011; Solheim et al., 2018).

The Importance of Skeletal Muscle Mass and Cancer Cachexia

The loss of skeletal muscle mass is often used as a key feature of cancer cachexia and is often a major cause of fatigue in cancer patients (Baracos, 2001; Blum et al., 2011; Dodson et al., 2011). Regardless of body mass index (BMI), skeletal muscle depletion is often considered an important prognostic factor during cancer(Burckart, Beca, Urban, & Sheffield-Moore, 2010). The accelerated loss of muscle mass also has the ability to diminish quality of life and negatively effects the responsiveness to chemotherapy and survival (Aoyagi, Terracina, Raza, Matsubara, & Takabe, 2015; Martin, 2016; Sandri, 2016; Yoshida & Delafontaine, 2015). Additionally, shorter time to tumor progression and poorer surgical outcome are also reported in cachectic cancer patients(Penet & Bhujwalla, 2015). The molecular mechanisms that underlie cancer related muscle wasting have not been fully elucidated (Penna, Bonelli, Baccino, & Costelli, 2013; Petruzzelli & Wagner, 2016; Sakuma, Aoi, & Yamaguchi, 2017; Tan et al., 2014; Whitehouse et al., 2001; Yoshida & Delafontaine, 2015). Evidence suggests that skeletal muscle mass loss during cachexia is thought to be due to an acceleration of protein degradation or muscle breakdown(Baracos, 2000; Johns, Stephens, & Preston, 2012; Sandri, 2016; Whitehouse et al., 2001). Although it is important to state that impaired muscle protein synthesis and defective myogenesis may also be major contributors as well. The prevalence of muscle mass loss in cancer patients has been reported between 20% and 70% depending on the tumor type and criteria that were used for the assessment (Aversa, Costelli, & Muscaritoli, 2017). In lymphoma patients not receiving any type of exercise or preventative measure for muscle mass loss, a decrease of up to 14% in muscle strength was reported (Aversa et al., 2017). The loss of muscle mass and strength can

result in increased fatigue and lead to a decrease in physical activity which can potentially further exacerbate muscle mass loss (Drescher, Konishi, Ebner, & Springer, 2016). A more recent study of patients with cancer of the gastrointestinal tract also demonstrated a substantial loss of mass and strength regardless of daily nutritional intake (Drescher et al., 2016). Even though the mechanism of skeletal muscle mass loss during cancer cachexia have been intensely studied over recent years, the identification of key processes and therapeutic targets have proven to be challenging due to the large numbers of mediators and signaling pathways that are involved in this multi-modal syndrome. However, there is evidence of potential complex tissue interactions in this systemic syndrome mediated through inflammatory cytokines, tumor-derived factors, and even some hormones (Ali & Garcia, 2014; Argiles, Busquets, Stemmler, & Lopez-Soriano, 2015; Baracos, 2006a; Ebner, Elsner, Springer, & von Haehling, 2014; K. Fearon et al., 2013; Grabiec, Burchert, Milewska, Blaszczyk, & Grzelkowska-Kowalczyk, 2013; Mendes, Pimentel, Costa, & Carvalheira, 2015; "Molecular mechanisms of cancer cachexia," 2016; Petruzzelli & Wagner, 2016; Tan et al., 2014).

1.2 Pre-Clinical Models of Cancer Cachexia

Tumor Implants

Despite the gravity of cachexia as a disease, few large scale human trials have been conducted to evaluate compounds and establish potential treatments. Due to the heterogeneity of these trials and the underlying diseases, well characterized and established animal models have become the norm for studying cancer cachexia. Pre-clinical rodent models of cancer cachexia have become the standard for basic science

research to elucidate and study molecular mechanisms(Ballaro, Costelli, & Penna, 2016; Bennani-Baiti & Walsh, 2011; Cannon et al., 2007). Models can vary depending on tumor type and disease progression much like what is seen in the clinic by physicians. Initial models utilized implantable tumor cell lines such as the Lewis Lung Carcinoma (LLC), colorectal tumors (C26), and syngeneic sarcomas (Ballaro et al., 2016; Baracos, 2018; Bennani-Baiti & Walsh, 2011; Romanick, Thompson, & Brown-Borg, 2013; Stewart Coats, 2018). These cell lines are implanted subcutaneously and allowed to proliferate and grow to a particular burden that causes the symptoms of cachexia (Bonetto, Rupert, Barreto, & Zimmers, 2016). Many of these implanted tumor cell lines have been very useful in establishing inflammations role in the process of muscle wasting during cachexia as they often secret multiple inflammatory cytokines. These models are extremely beneficial in studying cancer cachexia however, they do have some drawbacks as they are often viewed as quick models, with death occurring anywhere from 14 to 35 days (Blackwell et al., 2018; Bonetto et al., 2016). This small window can make studying compounds or interventions difficult as the disease progresses extremely fast. Additionally, a non-clinical tumor burden can often result in these models as well with some LLC tumors accounting for nearly 10-30% of total bodyweight of the mouse (Bennani-Baiti & Walsh, 2011). This presents a problem when translating the findings to the human condition as cancer patients very rarely ever have a tumor of such magnitude.

A newer trend in cancer cachexia genetic mouse models are exploiting the potency of pancreatic cancer. A recently developed model known as the pancreatic ductal adenocarcinoma (PDAC), among all forms of malignancy, PDAC is among the most highly associated with cachexia (Michaelis et al., 2017). It is estimated that

approximately 83% of patients suffering from the condition present with cachexia clinically (Michaelis et al., 2017). This particular allograft model utilizes cells isolated from the PDAC lesion of a C57BL/6 mouse which is then genetically modified to produce oncogenic KRAS (KPC) mutation generating a highly malignant tumor cell line (K. C. Fearon et al., 1999; Henderson, Makhijani, & Mace, 2018). These cells are then cultured and injected into mice either intraperitoneally or sub-cutaneous. KPC injected mice exhibit gastrocnemius muscle mass loss at 7 days with death occurring at approximately 12-14 days post inoculation (Gopinathan, Morton, Jodrell, & Sansom, 2015). This model also exhibits a pronounced pro-inflammatory response with elevations in IL-6 gene expression in skeletal muscle as well as in white adipose tissue(Gopinathan et al., 2015; Martignoni et al., 2009; Martignoni et al., 2005). This model also produces significant fat loss as well as loss of cardiac muscle mass (Holecek, 2012). Most importantly, in line with other cachexia models, this model induces a pronounced effect in catabolism particularly in the mixed fiber type skeletal muscles of the hind-limb (gastrocnemius, quadriceps, and tibialis anterior)(Holecek, 2012). Overall, this model is an effective an efficient method to study regulatory mechanism of cancer cachexia.

Genetic Mouse Models

Genetic mouse models of cancer cachexia are also common and useful in establishing mechanisms and therapeutic targets. The *adenomatous polyposis coli* (*Apc*) gene is mutated in a large percentage of human colon cancer cases, this is a common model for studying environmental factors that influence the genetic predisposition for colorectal cancer (Baltgalvis, Berger, Pena, Davis, & Carson, 2008; Baltgalvis et al., 2010; Blum et al., 2011; White, Puppa, Narsale, & Carson, 2013). The $Apc^{Min/+}$ (Min)

mouse is heterozygous for a mutation in the APC gene and will spontaneously develop intestinal adenomas (Baltgalvis et al., 2010). Invasive cancers are reported in older mice but without metastases. Most Min mice will not live past 4-5 months due to the development of severe cachexia and anemia (Baltgalvis et al., 2008; Baltgalvis et al., 2010). However, this time-span allows for the study of cachexia as a progression and not simply just an endpoint measurement. Final tumor burden for these mice is typically set around 12 weeks of age, and by 15 weeks of age the mice will begin to loss body weight and initiate cachexia with severe cachexia occurring around 18-20 weeks of age (Baltgalvis et al., 2008). The advantage to using the Min mouse model is that these mice have little to no anorexia, are chronically inflamed, and have a slow rate of wasting which makes them ideal for interventions and closely mimics the human progression of cancer cachexia (Baltgalvis et al., 2008; Baltgalvis et al., 2010; Narsale et al., 2016b; White, Baynes, et al., 2011a; White, Puppa, Narsale, et al., 2013). Our laboratory has characterized the Min mice as having muscle weakness and fatigue that worsens as the disease progresses and will decrease volitional activity and pronounced elevated interleukin 6 (IL-6) levels (B. N. VanderVeen, Hardee, et al., 2017). Min mice respond to exercise in a normal manner pre-cachexia but do have decreased running performance as cachexia sets in (Baltgalvis et al., 2008; Baltgalvis et al., 2010). Additionally, our laboratory has demonstrated that the elevated circulating IL-6 found in cachectic Min mice suppresses muscle protein synthesis and can inhibit the response to muscle contraction. Furthermore, previous work from our laboratory has also demonstrated that cachectic Min mice also exhibit elevated adenosine monophosphate protein kinase (AMPK) signaling following a standard 5 hour fasting paradigm that is used to measure

protein synthesis (White, Puppa, Gao, et al., 2013). AMPK is a known and established target of inflammatory cytokines such as interleukin 6 (IL-6) and directly inhibits mechanistic target of rapamycin (mTORC1) signaling (White, Puppa, Gao, et al., 2013). Additionally, accelerated degradation is also a hallmark of the Min mouse model which AMPK also plays a role in (White, Baynes, et al., 2011a). Given the slow progression of cachexia the Min model is ideal for use with interventions such as exercise or therapeutic drug treatments. Our laboratory has utilized exercise, IL-6 receptor antibody treatments, and anti-oxidants in the Min mouse model (Puppa et al., 2012; White et al., 2012). All of these interventions have demonstrated promising benefits and have led to potential mechanisms of regulation and therapeutic targets for cancer cachexia.

Keeping in line with the previously mentioned newer trend in cachexia modeling is the Kras genetic mouse model of pancreatic cancer. Recent advances in genetic editing techniques and have provided new and exciting mouse models of PDAC (Gopinathan et al., 2015; Michaelis et al., 2017). In particular is the KPC genetically modified mouse model of cancer cachexia. This model is highly metastatic and aggressive and mimics the human condition often found in PDAC patients(Gopinathan et al., 2015; Holecek, 2012; Michaelis et al., 2017). The KPC mouse contains a conditional point mutation in the transformation related protein 53 and a point mutation in the KRAS gene (Wyart et al., 2018). Various iterations of this model have been genetically created and tested with varying degrees of tumor burden and progression (Wyart et al., 2018). Consistently across all genetic models of PDAC are the development of severe cachexia (Gopinathan et al., 2015). While the injectable or allograft model discussed previously leads to death in very rapid time frame, the genetic variation of PDAC cancer cachexia is

much slower with the mean survival occurring at 5 months of age which closely mimics the Min model (Gopinathan et al., 2015). While this model is still being characterized mechanistically, it is apparent that the skeletal muscle of these mice exhibits extreme muscle mass loss. The acceleration of degradation through the activation of muscle E3 ligases has been reported and characterized (Jones-Bolin & Ruggeri, 2007). Early studies of PDAC genetic mouse models suggest that much like the Min model there is an imbalance in protein turnover, with accelerated degradation and high inflammation(Jones-Bolin & Ruggeri, 2007). However, the question of protein synthesis signaling still remains to be fully elucidated in this model.

1.3 Potential Mechanisms of Muscle Mass Loss in Cachexia

Inflammation

Inflammatory signaling has recently become a very active area of investigation in cancer cachexia over the past two decades (Narsale & Carson, 2014; Onesti & Guttridge, 2014; Zimmers, Fishel, & Bonetto, 2016). Chronic inflammation has become a hallmark of cancer cachexia in both clinical patients and pre-clinical rodent models. Numerous pre-clinical mouse models of cachexia have demonstrated elevated circulating inflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) (Narsale & Carson, 2014; Onesti & Guttridge, 2014; Zimmers et al., 2016). IL-6 is pleiotropic cytokine that has emerged as a critical regulator of skeletal muscle mass and fat wasting during cancer cachexia (Garbers, Aparicio-Siegmund, & Rose-John, 2015). IL-6 signals through the ubiquitously expressed glycoprotein 130 (gp130)(Cron, Allen, & Febbraio, 2016). IL-6 has been extensively investigated in recent years by our laboratory

and has been demonstrated by others l to play a role in the regulation of both muscle protein synthesis and degradation(J. L. Chen et al., 2016; Hardee et al., 2018; Pettersen et al., 2017; Puppa, Gao, et al., 2014; Puppa et al., 2012; White, Baltgalvis, et al., 2011; White, Puppa, Gao, et al., 2013; White et al., 2012). Elevated circulating IL-6 can be observed in cachectic cancer patients and is strongly correlated to body weight and muscle mass loss (Kuroda et al., 2007).

Increased circulating IL-6 has been clearly demonstrated to activate immediate downstream target signal transducer and activator of transcription 3 (STAT3) as well as extracellular regulated signaling kinase (ERK), and AMPK signaling (Bonetto et al., 2012; Garbers et al., 2015; Hardee et al., 2018). The activation of these signaling pathways by IL-6 in tumor bearing mice has been demonstrated to induce muscle mass loss through the suppression of muscle protein synthesis (mTORC1) signaling and induction of muscle protein breakdown in the Min mouse model of cancer cachexia (White, Puppa, Gao, et al., 2013). This imbalance disrupts muscle proteostasis (balance between synthesis and degradation) and leads to not only decreased muscle mass but also a suppression of skeletal muscle function and metabolism(Guo, Wang, Wang, Qiao, & Tang, 2017; Narsale & Carson, 2014; Pettersen et al., 2017; Shyh-Chang, 2017; White, Puppa, Gao, et al., 2013). Over-expression of IL-6 accelerates cancer cachexia induced skeletal muscle wasting in Min mice(White, Puppa, Gao, et al., 2013; White et al., 2012). Additionally, the elevated IL-6 has also been demonstrated to suppress basal protein synthesis signaling in tumor and non-tumor-bearing mice(Hardee et al., 2018). Interestingly, the suppression of protein synthesis in non-tumor bearing mice occurred independent to muscle mass loss (Hardee et al., 2018).

Pharmacological inhibition targeting IL-6 and its downstream signaling targets STAT3 and ERK has demonstrated an attenuation of skeletal muscle mass loss; however, it was not sufficient to completely block cachexia (Au, Desai, Koniaris, & Zimmers, 2016; Bonetto et al., 2012; Zimmers et al., 2016). Recently, a role for the IL-6 signaling pathway has also been described in the regulation of muscle oxidative metabolism and mitochondrial quality control during cancer cachexia (B. N. VanderVeen, Fix D.K., Carson J.A., 2017; White et al., 2012). IL-6 has been demonstrated to disrupt mitochondrial remodeling and suppress mitochondrial biogenesis in tumor and non-tumor bearing mice (Puppa et al., 2012; White et al., 2012). Our laboratory has also collected preliminary data demonstrating that elevated circulating plasma IL-6 is capable of suppressing mitochondrial respiration at Complex I. These findings suggest that there may be a link between muscle protein turnover and muscle oxidative metabolism in the net regulation of muscle mass. It is interesting to speculate if the disrupted mitochondrial function is a consequence of decreased skeletal muscle protein synthesis or a driver of this process.

Furthermore, in human cancer cachexia studies, elevated serum IL-6 is consistently associated with weight loss and a reduced survival rate in various types of cancer patients, especially in those presenting with pancreatic and colorectal cancer. Clinical trials of IL-6 antibody therapies in cancer patients have yielded some fairly promising results (Mueller, Bachmann, Prokopchuk, Friess, & Martignoni, 2016b). A clinical trial of ALD518 in patients with non-small cell lung cancer showed that this treatment has the potential to improve anemia, reduce cachexia related fatigue all while having minimal adverse effects (Bayliss, Smith, Schuster, Dragnev, & Rigas, 2011;

Maccio, Madeddu, & Mantovani, 2012). Additionally, others IL-6 targeted therapies such as Selumetinib have also been shown to be well tolerated while improving fatigue and attenuating the loss of lean body mass (C. M. M. Prado et al., 2012). While IL-6 has been studied extensively as a potential driver of cancer cachexia, there appears to be no definitive answer as to how it elicits such a detrimental effect on skeletal muscle. Further research is warranted to examine the IL-6 signaling pathway to further establish a concrete mechanism of action.

TNF-α is another pro-inflammatory cytokine that is also elevated in several pre-clinical models of cancer cachexia and pharmacological inhibition of this cytokine demonstrated a reduction in weight loss due to cancer (Catalano et al., 2003). However, it is of note to state that the TNF-α (like IL-6) appears to only be a major player in certain pre-clinical models such as the Yoshida hepatoma and the Lewis Lung Carcinoma (LLC) model of cancer cachexia, which are commonly viewed as non-IL-6 models(Baracos, 2000). In humans, several studies have demonstrated correlations of TNF-α serum levels with cachexia(Karayiannakis et al., 2001). Pancreatic cancer patients often exhibit elevated serum TNF-α and are inversely correlated with BMI, hematocrit, hemoglobin, and serum albumin levels(J. Nakashima et al., 1998; Pfitzenmaier et al., 2003). Additionally, it has also been shown that the expression of TNF-α gene was elevated in pancreatic cancer patients and was normalized following surgical resection (Ariapart et al., 2002). Recently, TNF-α and ubiquitin were also shown to be upregulated in the skeletal muscle of 102 gastric cancer patients and was strongly correlated to disease stage and degree of weight loss. These findings suggest that the TNF-α is a potent induces of

weight and muscle mass loss in both pre-clinical models and human patients during cancer cachexia (Sun, Ye, Qian, Xu, & Hu, 2012).

The transforming growth factor β (TGF- β) family of cytokines is a super family that consists of 34 different proteins that are capable of regulating multiple signaling pathways and cellular processes (Greco et al., 2015; Guttridge, 2015). Activin A, TNF like weak inducer of apoptosis (TWEAK) and myostatin are all members of this family and bind to either type I or type II activin receptors in skeletal muscle (Mathew, 2011). Upon binding these cytokines can activate the SMA, mothers against decapentaplegic (SMAD) signaling proteins (Dschietzig, 2014). These proteins are capable of regulating multiple catabolic signaling processes such the activation of the ubiquitin proteasome system and suppression of Protein Kinase B (AKT) (Guttridge, 2015). Activin A in particular has even been demonstrated to induce muscle wasting in non-tumor bearing mice (J. L. Chen et al., 2014). Treatment of mice with a specific Activin A antibody was able to prevent cachexia and death (Mathew, 2011). While the evidence for TGF-β signaling as a regulator of muscle mass in mouse models has shown great promise, studies that have examined this cytokine in human cancer patients are scarce.

1.4 Accelerated Degradation in Cachexia

The Ubiquitin Proteasome System:

A hallmark feature of cancer cachexia is the net loss of skeletal muscle protein through an imbalance of synthesis and degradation. As mentioned above, nearly all pre-clinical mouse models of cachexia as well as human cancer patients exhibit elevated muscle protein degradation with a suppression of muscle protein synthesis. This

disruption of muscle proteostasis leads to a net loss of skeletal muscle protein leading to mass loss. While cancer cachexia may be a multifactorial condition, clear evidence suggest that the ubiquitin proteasome system is a major contributor muscle mass regulation. The following section will discuss the ubiquitin proteasome system of degradation in detail highlighting important studies that have examined this system and its regulation.

The ubiquitin proteasome system (UPS) is an essential ATP dependent process that mediates the degradation of misfolded and mutated proteins as well as many proteins involved in the regulation of development, differentiation, cell proliferation, signal transduction, and apoptosis (Tisdale, 2009). The UPS has an established regulatory role in skeletal muscle mass and has been widely investigated in numerous catabolic conditions as an essential part of skeletal muscle quality(Attaix, Combaret, Tilignac, & Taillandier, 1999b; Fanzani, Conraads, Penna, & Martinet, 2012; Lazarus et al., 1999b). Failure of this system can lead to catastrophic metabolic dysfunction. A hallmark feature of the UPS is the ability to target specific proteins for degradation in very highly regulated manner (Cohen et al., 2009). This complex and tightly regulated process occurs through hierarchical ordered series of enzymes, including the activation enzymes (E1), ubiquitin- conjugating enzyme (E2), and finally the heavily studied ubiquitin (E3) ligases (Bilodeau, Coyne, & Wing, 2016b). The E3 ligases allow for targeted proteins to be recognized by the 26S proteasome and ensure specificity of the UPS and additional adaptor proteins(Murton, Constantin, & Greenhaff, 2008). It is important to note that the conjugation of ubiquitin to a substrate protein is repeated until a minimum of four ubiquitin molecules have been attached (Murton et al., 2008). While only one E1 has

been characterized, there are several dozen E2s and now hundreds of E3s as research has progressed over recent years (Attaix et al., 2005). In skeletal muscle this particular system has many different regulatory points (Murton et al., 2008; Yuan et al., 2015). In the past two decades a number of genes have been implicated in the skeletal muscle wasting through the UPS (Attaix et al., 1999b; Bodine, Latres, et al., 2001). These genes are part of a group known as "atrogenes" that mediate muscle mass through degradation and inhibitory regulation of synthesis (Lecker et al., 2004).

The muscle specific E3 ligases muscle-specific RING-finger 1 (MuRF1) and MAFbx (Atrogin-1) were initially discovered to be elevated in various catabolic conditions such as fasting, diabetes, cancer, immobilization, inactivity, and denervation (Bodine, Latres, et al., 2001; Lecker et al., 2004). Conditions of decreased muscle use were a major part of characterizing muscle proteolytic activity and accelerated degradation through the induction of E3 ligases and the UPS (Murton et al., 2008). Disuse has been deemed a potent inducer of muscle MuRF1 and Atrogin-1, often being activated in as little as 3 days post-disuse (Bodine, 2013). These specific ligases have been extensively studied over the years and have been demonstrated to regulate muscle protein breakdown through both loss and gain of function studies both *in-vivo* and *in-vitro* (Bodine, 2013; Bodine, Latres, et al., 2001). Mice lacking MuRF-1 and Atrogin-1 appeared to be resistant to denervation induced muscle atrophy as well as hindlimb unloading and immobilization (Bodine & Baehr, 2014; Phillips, Glover, & Rennie, 2009). Often coinciding with the activation of E3 ligases is the suppression of AKT, induction of AMPK and the subsequent muscle protein synthesis signaling through mTOR (Kwak et al., 2004; Phillips et al., 2009; Sandri, 2016; White, Baynes, et al., 2011a). Interestingly,

the targeted of substrates by Atrogin-1 are often regulatory proteins involved in protein synthesis and regeneration such as myogenin, while MuRF-1 has been shown to target contractile and structural proteins such as myosin heavy chain (Bilodeau, Coyne, & Wing, 2016a). These processes are tightly regulated and controlled by various upstream signaling pathways, while some of these have been characterized extensively others are still being discovered and elucidated.

A critical regulator of MuRF-1 and Atrogin-1 is the process of muscle protein synthesis that occurs through the canonical Insulin like growth factor-1 (IGF-1)/AKT signaling pathway which will be discussed in greater detail later in this review. Briefly, the activation of AKT will then promote the activation of mTOR leading to protein synthesis (Glass, 2010). In addition to this process, AKT also is a regulator of protein degradation (Glass, 2010). The induction of AKT through stimuli such as feeding, or exercise promotes the phosphorylation and inactivation of FOXO (Sandri et al., 2004). If un-phosphorylated by AKT such as during starvation or in a diseased state such as cachexia, FOXO will translocate to the nucleus where it transcriptionally controls a host of catabolic genes and processes such as the E3 ligases (Sandri, 2016; Sandri et al., 2004; White, Puppa, Gao, et al., 2013). Thus, the activation of AKT and sub-sequent inhibition of FOXO has been a target of interest when studying the regulation of MuRF-1 and Atrogin-1 (Brunet et al., 1999; Pallafacchina, Calabria, Serrano, Kalhovde, & Schiaffino, 2002; Stitt et al., 2004). The catabolic pathways of skeletal muscle are initiated through various stimuli as briefly mentioned above. Metabolic stressors such as reactive oxygen species, inflammatory cytokines brought on by tumors or even acute exercise, are all capable of inducing MuRF-1 and Atrogin-1 (Dohm, Kasperek, Tapscott, & Beecher,

1980; Foletta, White, Larsen, Leger, & Russell, 2011; Forbes, Little, & Candow, 2012; Frost, Nystrom, Jefferson, & Lang, 2007; Romanello et al., 2010; Romanello & Sandri, 2015). These ligases are among the most widely studied under various atrophic conditions and are believed to be key regulators of muscle mass.

Initial evidence suggesting the UPS is a major contributor to muscle wasting during came from studies that observed inhibition of calpains and lysosomal proteases only accounted for about 10-20% reduction in total proteolysis (Medina, Wing, Haas, & Goldberg, 1991). Since the UPS is an ATP dependent system, ATP depletion studies produced much higher degrees of protein breakdown inhibition (Muscaritoli M., 2006). Upregulation of the UPS pathway has been reported in many experimental models of cancer cachexia (Attaix, Combaret, Tilignac, & Taillandier, 1999a; Busquets et al., 2000; Combaret, Ralliere, Taillandier, Tanaka, & Attaix, 1999; Costelli et al., 1995; Costelli et al., 2006; Khal, Wyke, Russell, Hine, & Tisdale, 2005; Lazarus et al., 1999a; White, Baynes, et al., 2011b). The degree of activation of the UPS may vary from model and tumor type, however activation of the system remains constant across nearly all pre-clinical models of cancer cachexia. Early cachexia studies using hepatoma-bearing rats demonstrated the robust activation of proteolytic activity through gastrocnemius mRNA expression as well as cleavage of fluorogenic substrates (Temparis et al., 1994). Additionally, our laboratory has demonstrated that the UPS is robustly induced in cachectic Min mice as well as the LLC model of cancer cachexia (Puppa, Gao, et al., 2014; White, Baynes, et al., 2011b; White, Puppa, Gao, et al., 2013; White, Puppa, Narsale, et al., 2013). Treatments using inflammatory cytokine inhibitors have demonstrated some effectiveness and have further provided evidence that, at least in part,

the activation of the UPS may be driven by inflammation during cancer cachexia (White, Baltgalvis, et al., 2011; White et al., 2012). However, it is important to state that while these animals did indeed show improved muscle mass, very few studies have examined functional outcomes and how inhibiting the UPS could alter muscle function and quality. In recent years, more attention has been given to the E3 ligases in the pathogenesis of muscle atrophy during cachexia (Romanello & Sandri, 2015; Sandri, 2016; Sandri et al., 2004). Over-expression of these ligases has been shown to induce atrophy independent of disease (Romanello & Sandri, 2015; Sandri, 2016; Sandri et al., 2004). Treatments utilizing new small molecule inhibitors of MuRF-1 have been recently shown to be effective in counter-acting muscle atrophy in cancer cachexia (Bowen et al., 2017). With all the evidence suggesting that the UPS is a major contributor to muscle atrophy during cancer cachexia, the degrees and mechanisms of activation appear to vary depending on the severity of cachexia and type of tumor.

It remains mostly unclear how tumors initiate the signal to stimulate muscle protein degradation and cachexia. The often anatomically distant location between tumors and the wasting muscles are suggestive of tumor derived factors such as cytokines into circulation to initiate signaling processes that cause muscle wasting. As mentioned above, such factors such as IL-6 and TNFα are often elevated in pre-clinical models of cancer cachexia and are thought to be main drivers of chronic inflammation (Bye et al., 2016; Han, Meng, Shen, & Wu, 2018; Narsale & Carson, 2014; Onesti & Guttridge, 2014). Recent evidence has also demonstrated that chronic inflammation is capable of disrupting metabolic homeostasis and producing reactive oxygen species (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017; Narsale et al., 2016b; B. N. VanderVeen, Fix

D.K., Carson J.A., 2017). The activation of numerous transcription factors by tumor derived factors such as inflammatory cytokines and reactive oxygen species has become an interesting target of investigation in cancer cachexia (B. N. VanderVeen, Fix, & Carson, 2017). As mentioned previously, the FOXO family of transcription factors is the most widely studied, as they have been shown to directly control the expression of E3 ligases MuRF-1 and Atrogin-1 (Judge et al., 2014; Sandri et al., 2004). Furthermore, the activation of the Smad2/3 signaling pathway also has been demonstrated to increase the expression of these proteins via FOXO (Winbanks et al., 2016). Our laboratory has previously demonstrated that over-expression of IL-6 in Min mice induced Atrogin-1 independent to MuRF-1 via the STAT3 signaling pathway (White, Baynes, et al., 2011b). Reactive oxygen species produced from dysfunctional mitochondria have also been reported to activate FOXO and induce atrophy both in tumor and non-tumor bearing mice (Abrigo et al., 2018; Powers, Morton, Ahn, & Smuder, 2016). While these E3 ligases may have similar effects (muscle degradation) their regulation and protein expression levels appear to be mediated by a variety of up-stream signaling pathways.

When compared to the clear-cut results and literature from pre-clinical animal models, studies of human skeletal muscle have so far produced ambivalent results in regard to the UPS system in cancer cachexia. Increased activity of the UPS has been clearly shown to correlate with disease severity in the skeletal muscle of gastric cancer patients, even before the clinical onset of cachexia (Bossola et al., 2001; Bossola et al., 2003; DeJong et al., 2005). In addition, it was also observed that systemic inflammation markers (IL-6) correlated with increased total ubiquitin of biopsies obtained from cachectic patients with pancreatic cancer (DeJong et al., 2005). Furthermore, this

increase also correlated with the degree of weight loss (severity of cachexia) (DeJong et al., 2005). Muscle mRNA of the proteasome subunits were also elevated in colorectal cancer patients and where returned to normal control levels following tumor resection (J. P. Williams et al., 2012). However, a study on pancreatic cancer patients only reported elevations in the UPS in subjects that presented with greater than 10% bodyweight loss (DeJong et al., 2005). Interestingly, studies that have examined lung cancer patients did not find any alteration in the skeletal muscle UPS system (Schmitt et al., 2007). Furthering the spread of ambivalent data, other studies reported no alteration in muscle MuRF-1 or Atrogin-1 protein expression of gastric and colorectal cancer patients (Schmitt et al., 2007).

It is clear that the UPS system is a major player in the disruption of muscle proteostasis during cancer cachexia. However, the exact role it plays in the regulation of skeletal muscle mass during cachexia is still often debated. Due to inconsistent results between animal and human studies, there remains much to be determined in the exact role the UPS has in cancer cachexia. Studies have demonstrated some promise in attempting to treat the hyper-activity of the UPS during cancer, however some have also reported no beneficial effects (Tisdale, 2005).

The Lysosomal Proteasome System: Autophagy

In addition to the UPS discussed above is another critical non-ATP driven system of protein degradation known as the lysosomal proteasome (cathepsins) system (Bechet, Tassa, Taillandier, Combaret, & Attaix, 2005; Sandri, 2011). Commonly, this system of cellular process of degradation is referred to as autophagy (Vainshtein & Hood, 2015).

Autophagy plays a critical role in the turnover of cell components in response to various stimuli, such as cellular stress, nutrient deprivation, exercise, and cytokines (Vainshtein & Hood, 2015). Three separate types of autophagy have been characterized for delivery of autophagic cargo to the lysosome (Glick, Barth, & Macleod, 2010). Macro autophagy (general autophagy), chaperone-mediated autophagy, and micro autophagy (Glick et al., 2010). The majority of research regarding autophagy in skeletal muscle pertains to macro autophagy (Glick et al., 2010). This process of macro autophagy (hereafter referred to as autophagy) is a highly conserved homeostatic mechanism used for degradation and recycling, through the lysosomal machinery, of long-lived proteins and organelles (Sandri, 2010). The turnover of proteins via autophagy has recently become an area of high interest in cancer cachexia as the upstream regulators of autophagy are often activated during the disease progression (Penna, Baccino, & Costelli, 2014). Interestingly, recent studies have begun to elucidate cross-talk or overlap between autophagy and the UPS suggesting that both systems respond to similar stimuli and are capable of regulating one another to induce protein degradation (Lilienbaum, 2013). The following section will encompass the process of autophagy as well as its newly established role in cancer cachexia.

Under normal physiological conditions autophagy plays a balanced role in maintaining cellular homeostasis through the careful degradation of cellular debris and dysfunctional intracellular organelles (Sandri, 2010; Yu, Chen, & Tooze, 2018). Autophagy consists of a small portion of the cytoplasm that includes organelles or proteins being sequestered by a phagophore to form an autophagosome (Sandri, 2010; Yu et al., 2018). The autophagosome will then fuse with the lysosome which then degrades

the cellular cargo contained within it (Sandri, 2011). These processes require a family of proteins known as the autophagy related genes (Atg) which control the major steps of autophagy including initiation, nucleation, and lysosomal function/degradation (Yu et al., 2018). Autophagy can be either a non-selective (e.g., starvation) or selective process that degrades specific organelles such as mitochondria (mitophagy) (Mammucari, Schiaffino, & Sandri, 2008; Schwalm et al., 2015). Overall, general non-selective autophagy is initiated classically through the induction of AMPK which serves as the cells energy sensor (Mammucari et al., 2008; Schwalm et al., 2015).

When a metabolic stressor such as nutrient deprivation or acute exercise occurs, AMPK is induced and will suppress the metabolically expensive process of protein synthesis in order to induce energy generating pathways such as autophagy (Koh et al., 2008; Sanchez, Csibi, et al., 2012). AMPK will phosphorylate downstream target Unc-51 like kinase (ULK-1) which then serves to initiate the formation of the phagophore through the complex of ATG13 and FIP2000 (Koh et al., 2008). Studies have demonstrated the importance of AMPK in the initiation of autophagy, even suggesting AMPK is required for the initiation of autophagy during fasting conditions (Y. Kim, Triolo, & Hood, 2017; Sanchez, Csibi, et al., 2012). Once activated, ULK-1 will activate Beclin-1 and microtubule-associated proteins 1A/1B light chain 3A (LC3) which is responsible for the development of the autophagosome (Sandri, 2010; Schwalm et al., 2015; Yu et al., 2018). Damaged proteins and organelles are then targeted to the developing autophagosome by sequestosome 1 (p62) which is a poly-ubiquitin tag (Komatsu & Ichimura, 2010; Sandri, 2010; Schwalm et al., 2015; Yu et al., 2018). Following formation of the autophagosome, fusion with a lysosome will complete the

degradation process (Glick et al., 2010). An important key step in this process is the conversion of cytosolic LC3 I to LC3 II by conjugation to phosphatidylethanolamine (Komatsu & Ichimura, 2010; Sandri, 2010; Schwalm et al., 2015; Yu et al., 2018). Upon fusion of the autophagosome with the lysosome LC3 II and p62 are degraded which makes them the most widely investigated targets in determining autophagy capacity and flux (Glick et al., 2010). The process of autophagy has recently gained a great deal of attention in numerous disease and atrophic conditions.

Autophagy in Cachexia: A New Target?

Numerous pre-clinical models of muscular dystrophy report a chronic suppression of autophagy that exacerbates the dystrophic phenotype (De Palma et al., 2012; Grumati, Coletto, Sandri, & Bonaldo, 2011). Mice that have a deletion of Atg 7 actually display a phenotype similar to muscular dystrophy due to the accumulation of protein aggregates and dysfunctional organelles (Masiero et al., 2009). Disorders in which autophagic vacuoles are seen in the skeletal muscle are generally referred to as autophagic vacuolar myopathies such as Pompei and Danon disease (Sandri, 2010). Cancer cachexia and skeletal muscle autophagy has become a very intriguing area of investigation (Penna et al., 2014). Numerous mouse models of cancer cachexia have demonstrated elevations in LC3 as well as p62 (Penna, Costamagna, et al., 2013; Tessitore, Costelli, & Baccino, 1994; Tessitore, Costelli, Bonetti, & Baccino, 1993). The gastrocnemius muscle of C26 and LLC tumor bearing mice exhibited robust inductions of Beclin-1, LC3 II, and p62 at 11 and 14 days which coincided with a reduction in cathepsin activity(Pettersen et al., 2017; Talbert, Metzger, He, & Guttridge, 2014; White, Baltgalvis, et al., 2011). Our laboratory has also demonstrated that LC3 II and Beclin-1 are also induced in the Min

mouse model of cancer cachexia (White, Baynes, et al., 2011a). Interestingly, some researchers argue that the elevation in LC3 and p62 is indicative of dysfunctional clearance of the autophagosome by the lysosome. The induction of skeletal muscle autophagy in tumor bearing animals is typically demonstrated by increased LC3 II levels, which are further induced in the presence of a lysosomal inhibitor such as colchicine (a recently employed technique to determine true autophagy flux(Ju, Varadhachary, Miller, & Weihl, 2010). The common link in pre-clinical models of cachexia appears to be LC3 II and p62, which are often reported as accumulated in cachectic mice (Pigna et al., 2016b).

In an effort to further examine the autophagy blockade and improve autophagic clearance studies have employed exercise treatments. Exercise has often been deemed a potent regulator of autophagy, stimulating it acutely and actually improving efficiency and clearance with chronic training (restoring normal levels of autophagy) (Pigna et al., 2016b; Schwalm et al., 2015; Vainshtein & Hood, 2015; Yu et al., 2018). Exercise training has been proposed as a method to counteract cachexia (Pigna et al., 2016b; Schwalm et al., 2015; Vainshtein & Hood, 2015; Yu et al., 2018). Furthermore, electrical stimulation of muscle contraction has been shown to reduce muscle wasting in tumor bearing mice by both our laboratory and others (Hardee et al., 2018; Hardee et al., 2016). Voluntary wheel running in C26 tumor bearing mice improved indices of cachexia by suppressing LC3II and attenuating P62 protein accumulation. These results suggest that exercise training has the capacity to improve or lower the chronic activation of autophagy during cancer cachexia (Pigna et al., 2016b). It is intriguing to speculate if the use of exercise training to lower chronically activated AMPK and suppress the upstream

regulators of autophagy are a potential mechanism of action during cancer cachexia. This could have important ramifications as a therapy in the treatment of cachexia and regulation of muscle mass.

As mentioned previously, IL-6 has been deemed a potent regulator of cancer cachexia and systemic inflammation, a recent study demonstrated a role for IL-6 trans (non-classical) signaling in the induction of autophagy during cancer cachexia (Pettersen et al., 2017). Serum from cachectic cancer patients containing elevated IL-6 was incubated on C2C12 myotubes, both p62 and LC3 were robustly induced (Pettersen et al., 2017). Additionally, blockage of lysosomal activity using Bafilomycin demonstrated a true increase in autophagy flux in these IL-6 treated C2C12 myotubes (Pettersen et al., 2017). Our laboratory also demonstrated that the administration of an IL-6 receptor antibody to Min mice after the initiation of cachexia was able to lower chronic AMPK activation and suppress downstream markers of skeletal muscle autophagy (White, Puppa, Gao, et al., 2013; White et al., 2012). Furthermore, skeletal muscle biopsies taken from cachectic cancer patients exhibited elevated mRNA for Beclin-1(Aversa, Pin, Lucia, Penna, Verzaro, Fazi, Colasante, Tirone, Rossi Fanelli, et al., 2016; Chacon-Cabrera et al., 2014). Protein expression of LC3 II and P62 were also elevated in cachectic cancer patients again suggesting either increased overall autophagy or impaired clearance of the autophagosome. It is interesting to suggest also that the accumulation of p62 without changes in LC3 II have also been reported (Aversa, Pin, Lucia, Penna, Verzaro, Fazi, Colasante, Tirone, Fanelli, et al., 2016). These results suggest a possible role for p62 accumulation in the regulation of lysosomal function. Another very interesting research study showed that while no differences in proteasome, calpain and caspase activities were

detected in skeletal muscle of esophageal cancer patients, activities are increased (Anandavadivelan & Lagergren, 2016; Schersten & Lundholm, 1972). These studies clearly demonstrate that multiple signaling pathways pertaining to degradation are activated with cancer and cachexia. However, these processes appear to differ greatly depending on the type of tumor/cancer present in these patients and pre-clinical models.

Some researchers have suggested the induction of autophagy in cancer hosts is likely associated with a negative energy balance that arises either from reduced food intake, mitochondrial damage, or dysfunctional metabolism (Abrigo et al., 2018; Aversa, Pin, Lucia, Penna, Verzaro, Fazi, Colasante, Tirone, Fanelli, et al., 2016; Penna et al., 2014; Sanchez, Csibi, et al., 2012). It is apparent from a recent influx of evidence that autophagy is a major contributor to skeletal muscle wasting during cancer cachexia. However, the cancer cachexia field suffers from a lack of mechanistic investigation into the question of autophagy. Studies that examine upstream regulators and cellular signaling are desperately needed to provide molecular details that could lead to new and innovative therapeutic targets in the regulation of skeletal muscle mass during cancer cachexia.

1.5 -Suppressed Protein Synthesis in Cachexia

Often debated in cancer cachexia and muscle mass regulation as a whole is the concept of muscle protein synthesis. Researchers have often argued that degradation being accelerated is only one side of muscle mass regulation. It has long been postulated that muscle atrophy in cancer cachexia is also due to decreased protein synthesis not just an increase in degradation(Eley & Tisdale, 2007; Sakuma & Yamaguchi, 2012). Skeletal

muscle responds to both systemic and local environmental stimuli. Inflammation, insulin resistance, and hypogonadism are systemic perturbations associated with cancer that can disrupt metabolism and proteostasis during cancer cachexia (Carson et al., 2016; Narsale & Carson, 2014; B. N. VanderVeen, Fix D.K., Carson J.A., 2017; White, Puppa, Narsale, et al., 2013). While cancer induced suppression of basal protein synthesis has been reported in patients and pre-clinical cachexia models, this suppression does not appear to fully account for severe muscle wasting observed with cachexia (Mueller, Bachmann, Prokopchuk, Friess, & Martignoni, 2016a).

Translational Capacity

The regulation of basal protein synthesis in skeletal muscle is tightly regulated through translational efficiency (polypeptide synthesis per ribosome) and capacity (total number of ribosomes) (Hershey, Sonenberg, & Mathews, 2012). Translational Efficiency is regulated at initiation, elongation, and termination steps(Hershey et al., 2012; Hinnebusch & Lorsch, 2012). Recent advances have improved our understanding of the regulation of initiation in skeletal muscle. Translation initiation includes binding of the GTP-bound form of eukaryotic initiation factor 2 (eIF2) and initiator methionyl-tRNA (met-tRNA) complex to the small (40S) ribosomal subunit to form the 43S preinitiation complex (Hershey et al., 2012; Hinnebusch & Lorsch, 2012). Secondly, this 43S complex will bind to the 7-methyguanosine cap to the 5' end of mRNA (Kimball, Horetsky, Jagus, & Jefferson, 1998). Finally, the 43S complex will scan the mRNA for the AUG initiation codon (Hershey et al., 2012). Upon codon recognition, the large 60S ribosomal subunit will join with the 40S subunit to form the 80S initiation complex capable of promoting elongation (Hershey et al., 2012). Several different eukaryotic

initiation factors and binding proteins play critical roles in this process and are capable of being regulated by various stimuli such as stress or nutrient availability (Hershey et al., 2012; Kimball, 1999). However, eIF2 is believed to be the rate limiting step for translation initiation and can be influenced by multiple post-translational modifications such as phosphorylation (Wek, Jiang, & Anthony, 2006). Several nutrient and energy sensitive kinases regulate eIF2 phosphorylation in response to atrophic stimuli (Wek et al., 2006). Overall, translation is a critical step in the regulation of skeletal muscle protein synthesis and is tightly linked to cellular stress and nutrient availability.

Mechanistic Target of Rapamycin-A Brief Overview

The IGF-1/AKT signaling pathway is the primary signaling cascade that activates protein synthesis. Stimuli such as feeding (insulin spike) or muscle contraction (exercise) will induce PI3K to phosphorylate AKT primarily at T308 (Bodine, Stitt, et al., 2001). The activation of AKT will then signal downstream to phosphorylate tuberous sclerosis complex 1/2 and release Ras homologue enriched in brain (Rheb) allowing for the activation of the mechanistic target of rapamycin. The mechanistic target of rapamycin is a serine/threonine kinase that interacts with several different intracellular signaling proteins and kinases to form two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Laplante & Sabatini, 2009). mTORC1 has been widely investigated for role in the regulation of anabolic signaling process related to protein synthesis (Yoon, 2017). Moreover, mTORC2 is still being examined and is thought to be essential for cellular survival, cytoskeletal organization, and metabolism (Laplante & Sabatini, 2009). mTORC1 serves as a critical regulatory point for anabolic stimuli such as growth factors, nutrients, and mechanical signaling or loading (Schiaffino &

Mammucari, 2011). Briefly, upon activation mTORC1 will directly phosphorylate eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and P70 S6 kinase 1 to promote protein synthesis (Schiaffino & Mammucari, 2011). This signaling has been well established in multiple rodent models of both health and disease and is believed to be a critical aspect of regulating basal skeletal muscle protein synthesis (Schiaffino & Mammucari, 2011; Tessitore et al., 1993; White, Baynes, et al., 2011a). The phosphorylation of 4E-BP1 prevents binding to eIF4E and the formation of the complex between these two proteins, resulting in the assembly of eIF4F complex and translation initiation (Kimball, 1999; Sonenberg & Hinnebusch, 2009). Recent evidence has also suggested a role for mTORC1 in cap-dependent translation, elongation, and ribosomal biogenesis (Kressler, Hurt, & Bassler, 2010a). Related to ribosomal capacity, mTORC1 has also been demonstrated to regulate translational machinery through the synthesis of mRNAs encoding ribosomal proteins and the transcription of ribosomal RNA (Kressler et al., 2010a). In summary, mTORC1 is a critical regulatory point that serves as a crossroads for growth and metabolic alterations that make it a target of interest in muscle wasting conditions.

Suppressed Protein Synthesis in Cachexia:

The suppression of skeletal muscle protein synthesis in pre-clinical animal models of cachexia has been investigated and reported upon extensively (Hardee et al., 2018; Johns et al., 2012; Tessitore et al., 1993; White, Puppa, Gao, et al., 2013). However, an exact mechanism as to how the suppression of protein synthesis occurs during cancer cachexia is still open to debate (Baracos, 2000). Some have postulated that that dysfunctional oxidative metabolism is to blame due to a lack of sufficient ATP

production and the chronic activation of AMPK (Koh et al., 2008; White, Puppa, Gao, et al., 2013). Others have suggested that a lack of sensitivity to nutrients (anabolic resistance) is to blame for the simple fact that nutrition appears to have a minimal effect on protein synthesis during cachexia (Burd, Gorissen, & van Loon, 2013; Cuthbertson et al., 2005). Furthermore, some have begun to argue that a lack of activity or increased disuse during cancer cachexia contributes to the suppression of protein synthesis (Argiles, Busquets, Lopez-Soriano, Costelli, & Penna, 2012). Inflammation has also been shown to regulate protein synthesis during cancer cachexia in Min mice (Hardee et al., 2018; White, Puppa, Gao, et al., 2013; Zimmers et al., 2016). Overall, there are multiple lines of evidence each demonstrating an effect on muscle protein synthesis during cancer cachexia. However, there appears to be no clear answer as to what causes these conditions as cachexia is a multifactorial syndrome further mechanistic investigation is warranted to more closely examine these mechanisms of regulation.

Dysfunctional Oxidative Metabolism and Protein Synthesis

Recently a role for oxidative metabolism in the regulation of protein synthesis during cancer cachexia has been suggested (Carson, Hardee, & VanderVeen, 2015). Mitochondrial respiration and ATP synthesis are linked tightly to cellular energy utilization (Buttgereit & Brand, 1995). Thus, it is not surprising that mitochondrial function/oxidative metabolism exert regulation over processes related to protein synthesis. Protein synthesis is a high energy demanding process accounting for approximately 20-30% of mammalian ATP consumption (Rolfe & Brown, 1997). Additionally, many non-ribosomal kinases are required for the synthesis and maintenance of ribosomes (Kressler, Hurt, & Bassler, 2010b). As briefly mentioned previously, the

adenosine monophosphate protein kinase AMPK regulates many different metabolic processes including protein synthesis and degradation (Koh et al., 2008). AMPK serves as a cellular energy sensor and is activated in times of low ATP or high AMP, and has a direct role in the regulation of mTORC1 signaling through the phosphorylation of TSC2 and Raptor (Shaw, 2009). TSC2 phosphorylation inhibits mTORC1 signaling through the Rheb keeping it in its GDP-bound inactive state (Shaw, 2009).

Mitochondrial dysfunction has recently been shown to precede the loss of muscle mass in LLC tumor bearing mice (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017). It is possible that the dysfunctional ATP production produce reactive oxygen species that stimulate AMPK and suppress protein synthesis in these mice. Dysfunctional mitochondrial quality control has also been implicated in the activation of AMPK (Romanello & Sandri, 2015). Altered mitochondrial dynamics and lack of mitophagy contribute to the buildup of damaged organelles alerting functional oxidative metabolism and producing metabolic stress (Suliman & Piantadosi, 2016). The Min model of cancer cachexia has been shown to induce mitochondrial remodeling and reduce mitochondrial biogenesis (White, Baltgalvis, et al., 2011). These alterations begin to appear in mildly cachectic animals and are exacerbated as cachexia reaches the sever stage. Through this signaling cascade it has been demonstrated that dysfunctional mitochondria and ATP production activate AMPK and suppress the metabolically expensive process of protein synthesis through the inhibition of mTORC1(Romanello & Sandri, 2015; White, Baltgalvis, et al., 2011).

Anabolic Resistance

Of recent interest in cancer cachexia and protein synthesis is the new concept of anabolic resistance (A. M. H. Horstman, Damink, Schols, & van Loon, 2016). Many cancer patients experience weight loss at the time of diagnosis and as mentioned previously, muscle loss is indicative of poor prognosis in many cancers (Baracos, 2006a, 2011; Baracos et al., 2018; Romanello & Sandri, 2015). An intriguing question among researchers is whether cachectic muscle retains anabolic plasticity. This could prove beneficial for clinicians looking to provide treatment and improve muscle mass and function. While circadian fluctuations in protein turnover are the established norm in muscle mass regulation, it has become evident that protein synthesis is highly responsive to environmental stimuli. Nutrients or feeding and exercise are extremely potent stimulators of skeletal muscle protein synthesis in basal healthy conditions; however, numerous wasting conditions have demonstrated reduced sensitivity to these anabolic signals (Argiles, Lopez-Soriano, & Busquets, 2015; Bodine, 2013; Bowen, Schuler, & Adams, 2015). Even the smallest decrement in daily protein synthesis could significantly impact muscle maintenance in wasting conditions, it is logical to suggest that anabolic resistance could also contribute to muscle loss during cancer cachexia (Cuthbertson et al., 2005).

It is established that feeding can stimulate whole body and skeletal muscle protein synthesis when compared to fasted healthy individuals (Rennie et al., 1982). Furthermore, ingestion of essential amino acids, especially leucine, can directly stimulate protein synthesis in young individuals (Paddon-Jones et al., 2004). This activation of protein synthesis is directly due to the activation of mTORC1 signaling. Upon

stimulation from amino acids, the Ragualtor-Rag complex directly targets mTORC1 to the lysosome where it will become activated by Rheb (Liu, Jahn, Wei, Long, & Barrett, 2002). Initial studies examining anabolic resistance occurred in aged individuals who exhibit a blunted response to both food and essential amino acids such as leucine (Sancak et al., 2010). The ability to stimulate mTORC1 and protein synthesis in these individuals remains intact but required larger doses than that of younger subjects (Paddon-Jones et al., 2004). These results suggested that while the machinery for the activation of protein synthesis remains intact a certain threshold of activation must be reached to achieve the desired result. Overall, these studies characterize the reduced sensitivity of skeletal muscle to feeding in both the aged and diseased state.

Since cancer cachexia cannot be observed by conventional nutritional supplementation, it is necessary to further our understanding of anabolic resistance. Growing evidence is supporting the disruption of protein synthesis during cancer cachexia; however, very few studies have examined if nutrients or feeding can stimulate anabolic signaling. Cancer patients are still capable of inducing protein synthesis in response to protein ingestion however, the degree of activation appears to be severely impaired (Dillon et al., 2012). A newer approach using specially formulated medical food that is high in protein and leucine has shown promise in cancer patients with involuntary weight loss (Deutz et al., 2011). Surprisingly, the cancer cachexia field is actually lacking in the study of pre-clinical model studies examining anabolic resistance. However, our laboratory has demonstrated blunted mTORC1 activation in response to glucose administration in cachectic skeletal muscle (White, Puppa, Gao, et al., 2013).

Inactivity-A Role for Exercise Training

Many cancer patients suffer from chronic fatigue, either from the disease itself or its treatment, which is often a confounder that limits regular exercise practice (Grande, Silva, & Maddocks, 2015). This lack of physical activity or exercise has been demonstrated to occur in both the clinic and pre-clinical models (Grande et al., 2015; Roberts, Frye, Ahn, Ferreira, & Judge, 2013). Pre-clinical models have demonstrated that voluntary activity actually precedes weight loss further establishing its importance in the progression of the condition (Baltgalvis et al., 2010). Voluntary muscle strength and exercise capacity are also found to be reduced in pre-clinical models and in human cancer patients (Glaus, 1998; Stewart, Skipworth, & Fearon, 2006). Furthermore, muscle contractile properties related to fatigue resistance are also disrupted during experimental cancer cachexia (B. N. VanderVeen, Hardee, et al., 2017). However, our mechanistic understanding of the role of exercise training to counter-act these deficits and stimulate protein synthesis are not well described.

The field of exercise oncology is limited by a lack of studies that examine cachectic cancer patients. However, initial progress has been made to improve our understanding of exercise adaptations during the development and progression of cachexia in pre-clinical models(Grande et al., 2014; Hardee et al., 2016; Mehl et al., 2005). Aerobic treadmill training either alone or with nutritional supplementation has been demonstrated to reduce tumor growth and improve muscle mass (Baltgalvis et al., 2008). Our laboratory has also demonstrated a role for treadmill training during systemic IL-6 overexpression to enhance mTORC1 signaling and improve mitochondrial quality control in tumor bearing mice (Puppa et al., 2012). The improvement of mitochondrial

quality control can lead to a reduction in the activation of AMPK and improve the ability to activate mTORC1 and stimulate protein synthesis. Resistance exercise using overload hypertrophy models and electrical stimulation have also demonstrated attenuated or preserved muscle mass (Hardee et al., 2018; Hardee et al., 2016). Additionally, our laboratory has also demonstrated that repeated high frequency electrical stimulation is capable of inducing myofiber hypertrophy in cachectic skeletal muscle (Hardee et al., 2016). Furthermore, this eccentric contraction training paradigm suppressed the cachexia induction of AMPK and robustly stimulated mTORC1 signaling and protein synthesis when compared to the contralateral control leg (Hardee et al., 2016). These findings were also found to coincide with improved muscle oxidative capacity in tumor-bearing mice (Hardee et al., 2016). Whether these alterations could improve basal protein synthesis long-term remains to be further investigated. Collectively, these studies suggest a role for exercise training to improve skeletal muscle mass during cancer cachexia. Further studies are required to determine exact mechanisms and if an interaction between feeding and exercise could provide beneficial effects on protein synthesis and aid in the treatment of cachectic cancer patients.

1.6 AMPK-Muscle Mass and Metabolism Regulation

AMPK an Overview

Skeletal muscle is a highly dynamic tissue often characterized by dramatic changes in energy turnover in response to various environmental stimuli (Koh et al., 2008). AMPK is a sensor of intracellular energy status that maintains energy stores by regulating anabolic and catabolic pathways (Kjobsted et al., 2018). AMPK's role as an

energy sensor is critical in tissues that display high turnover such as skeletal muscle (Mounier, Theret, Lantier, Foretz, & Viollet, 2015). Recent advances have highlighted the relevance of AMPK both as a multi-task gatekeeper and as an energy regulator in skeletal muscle (Kjobsted et al., 2018) . In this section AMPK's function as well as up and downstream regulators will be highlighted. Furthermore, cancer cachexia and AMPK will be discussed and its potential role in regulating muscle mass.

The adenosine monophosphate protein kinase (AMPK) exists as a heterotrimeric complex comprised of an α catalytic subunit, a scaffolding β subunit, and a regulatory γ subunit. In mammals each of these subunits have multiple isoforms (α1 α2, β1, β2, γ1, γ2, and γ3) (Kemp, Oakhill, & Scott, 2007). These subunits are all encoded by separate genes, which enable the formation of diverse combinations of the heterotrimeric complex (Kemp et al., 2007). AMPK activation involves two steps; reversible phosphorylation at threonine 172 (T172) in the α subunit and allosteric binding of AMP with the C-terminal region in the γ subunit (Witczak, Sharoff, & Goodyear, 2008). The β subunit is responsible for carbohydrate binding and has actually been demonstrated to inhibit AMPK activity (Kemp et al., 2007; Witczak et al., 2008). In skeletal muscle the major activator of AMPK is upstream liver kinase B1 (LKB1) and primarily is responsible for phosphorylating AMPK at T172 inducing its activation (Koh et al., 2008). This activation can be further enhanced by conformational changes caused by the allosteric binding of AMP and/or ADP to the γ subunit which promotes phosphorylation and protects AMPK from protein phosphatases (Kemp et al., 2007). The combined effect of phosphorylation at T172 and allosteric regulation can alter kinase activity >1000 fold making AMPK extremely sensitive to very subtle changes in energy status (Kemp et al.,

2007). It is also worth noting that Ca^{2+} has also been demonstrated to induce AMPK phosphorylation through calmodulin-dependent kinase kinase beta (CaMKKβ) independent of any changes in AMP: ATP or ADP:ATP ratio (Jensen et al., 2007) . This mechanism has been investigated and characterized in response to mild tetanic muscle contractions.

As briefly mentioned above the most commonly studied upstream activator of AMPK is LKB1(Sakamoto, Goransson, Hardie, & Alessi, 2004; Sakamoto et al., 2005). LKB1 is what is known as a tumor suppressor that is commonly mutated in a variety of cancers. However, physiologically LKB1 possess many different cellular functions in the regulation of skeletal muscle metabolism and systemic metabolism (Sakamoto et al., 2004; Taylor et al., 2004; Thomson et al., 2007). A little over a decade ago, researchers established that LKB1 was the missing kinase that phosphorylates and activates AMPK to induce its downstream signaling effects (Koh et al., 2006). The importance of LKB1 in this process has been demonstrated through loss and gain of function studies as muscle specific LKB1 knockout mice exhibit ablated AMPKα^2 activity (Koh et al., 2006; Sakamoto et al., 2005). These results further established LKB1 as the major AMPK kinase regulator in skeletal muscle. AMPK is also activated in response to various cellular energy stressors and pathways regulating insulin sensitivity and glucose transport (Kjobsted et al., 2018). Such stimuli include exercise, hypoxia, leptin and adiponectin. Furthermore, inflammation through the IL-6 signaling pathway has been demonstrated to induce AMPK in cultured C2C12 myotubes (White, Puppa, Gao, et al., 2013). Treatment of myotubes with an IL-6 inhibitor restored mTORC1 signaling and suppressed AMPK.

Another common regulator of AMPK is the alteration of energy status or energy availability most commonly seen and studied with nutrient deprivation or fasting. A fast will alter the metabolism of skeletal muscle leading to an increased flux of free fatty acids and alters the AMP:ATP ratio thus inducing the activation of AMPK (de Lange et al., 2007). AMPK will then induce Acetyl CoA carboxylase (ACC) to stimulate the breakdown and uptake of the fatty acids for oxidation in the mitochondria (Galic et al., 2018). Interestingly, studies have demonstrated that LKB1 is not sensitive to AMP and actually remains constitutively active (Lizcano et al., 2004). The binding of AMP to AMPK causes it to become a better substrate and increases the affinity of LKB1 to AMPK (Hawley et al., 2003) . Furthermore, multiple studies have examined acute exercise (a potent metabolic stressor) in rats and mice as a very potent inducer of AMPK (Friedrichsen, Mortensen, Pehmoller, Birk, & Wojtaszewski, 2013). The duration and intensity of exercise also directly has an effect on the degree of AMPK activity in skeletal muscle (Friedrichsen et al., 2013; Sakamoto et al., 2004). Acute exercise stimulation of AMPK also appears to regulate the same genes related to a fast (Park et al., 2002; Terada et al., 2002). Interestingly, in mice lacking the α^2 subunit the AMPK activation of was blunted following an acute exercise bout (Jorgensen et al., 2005). However, the genes related to exercise that were investigated were not found to be altered in either α^1 or α^2 muscle specific knockout mice (Jorgensen et al., 2005). Conversely to acute exercise, far less is understood regarding exercise training (Taylor et al., 2004). Aerobic exercise training has been demonstrated to induce LKB1 rather than AMPK protein expression as well as mitochondrial biogenesis markers in rat skeletal muscle (Taylor et al., 2004; Taylor et al., 2005). Surprisingly, these changes occurred despite a decrease in AMPK

kinase activity (Taylor et al., 2005). In human skeletal muscle exercise training has been demonstrated to suppress AMPK activity following a prolonged bout of exercise suggesting that training improves the skeletal muscle capacity to handle metabolic stress, possibly through improved mitochondrial function (McConell et al., 2005; Taylor et al., 2005). However, similar studies have also reported either no significant change in AMPK or an induction of activity (Z. P. Chen et al., 2000). These results are ambivalent and the examination of exercise trainings effects on AMPK require far more investigation to be considered conclusive. Muscle specific LKB1 knockout mice have demonstrated that LKB1 is actually an important regulator of exercise capacity and mitochondrial function (Taylor et al., 2004). Contrasting to these results the training induction of GLUT4, hexokinase II, and mitochondrial content markers were all intact (Sakamoto et al., 2005). These results suggest that LKB1-AMPK signaling is an important factor in skeletal muscle physiology, but the contradicting results suggest that other pathways regulate the response to exercise training.

AMPK- Regulation of Metabolic Downstream Targets

The activation of AMPK results in the phosphorylation of multiple downstream substrates who overall effects are to suppress ATP consuming pathways such as protein synthesis and induce ATP generating pathways and events such as fatty acid oxidation, glucose transport, and autophagy. The activation or suppression of these various downstream kinases have metabolic consequences that can affect the metabolism and cellular environment systemically in addition to skeletal muscle.

Fat and Glucose Metabolism-Metabolic Enzymes

AMPK was initially characterized as the upstream kinase regulating metabolic enzymes such as ACC and HMG-CoA reductase, these serve as the rate limiting steps for fatty acid control and sterol synthesis(Mihaylova & Shaw, 2011). In tissues that are metabolically active such as skeletal muscle, AMPK controls the uptake of glucose through AS160 and also is a key regulator of GLUT4 trafficking following exercise and insulin stimulation(Mihaylova & Shaw, 2011). ACC is responsible for generating malonyl coA, an allosteric inhibitor of CPT1, which is responsible for the uptake fatty acids into the mitochondria for β oxidation (Mihaylova & Shaw, 2011; Mills, Foster, & McGarry, 1983; Watt et al., 2006). The phosphorylation of ACC by AMPK inactivates the enzyme leading to decreased lipid synthesis rates and alleviates CPT1 inhibition (Hardie & Pan, 2002). The alleviation of CPT1 then increases fatty acid flux into the mitochondria where it is oxidized to generate ATP (Hardie & Pan, 2002). AMPK also increases the phosphorylation of hormone sensitive lipase (HSL) which serves to break down fat stores into free fatty acids to be oxidized for energy (Watt et al., 2006). In addition to activating HSL to induce lipolysis, AMPK will also induce the activation adipocyte triglyceride lipase (ATGL) (Hardie & Pan, 2002). The regulation of ATGL by AMPK has recently been demonstrated to be an important factor in pre-clinical models of cancer cachexia.

Acute AMPK activation is also associated with the suppression of glycogen synthesis rates. AMPK has been reported to directly phosphorylate glycogen synthase at Ser7, thus inhibiting its activity. This mechanism is of interest as it is generally coupled to an increase in glycolytic flux through the activation of 6-phosphofructo-2-kinase

(PFK-2) which is the rate limiting step of glycolysis (Carling & Hardie, 1989). The activation of PFK-2 by AMPK induces the synthesis of fructose 2,6 bisphosphate which is considered an extremely potent stimulator of glycolysis (Carling & Hardie, 1989; Marsin et al., 2000). This mechanism of activation suggests that the rapid acute activation of AMPK initiates this signaling cascade to induce the mobilization of glucose into ATP.

Regulation of Transcription Factors

AMPK is also able to regulate metabolism through the regulation of numerous transcription factors. These downstream targets have recently come to light through various lines of investigation. Notably, AMPK has recently been demonstrated to phosphorylate and regulate acetyltransferase p300, which is a member of the histone deacetylase family. AMPK has also been reported to actually regulate histones themselves in some cases (Suter et al., 2006). AMPK has also been deemed a master controller of mitochondrial biogenesis and content (Suwa, Nakano, & Kumagai, 2003). Sirtuin 1 (SIRT1) is a NAD^+ dependent deacetylase that is proposed to be required for the AMPK activation of mitochondrial biogenesis (Canto et al., 2010). Studies utilizing AMPK activating compounds have reported increased mitochondrial biogenesis in skeletal muscle and this biogenesis was not present with the loss of SIRT1 (Suwa et al., 2003). Similarly, these AMPK activating agents have little to no effect in mouse models with mutated or suppressed AMPK activity, suggesting that AMPK is a critical regulator of mitochondrial biogenesis in skeletal muscle (Mercurio, 2002). These results were further explored through numerous gain and loss of functions studies utilizing transgenic mouse models (Mercurio, 2002; Viollet et al., 2009). Overexpression of a kinase dead α^2

subunit in muscle demonstrated less running activity and blunted endurance performance when compared to wild type mice (Mu, Brozinick, Valladares, Bucan, & Birnbaum, 2001). Furthermore, these results were also replicated in a whole body systemic knockout of the α^2 subunit (Jorgensen et al., 2005). Contrasting to these results were different manipulations to activate AMPK activity and demonstrate increased mitochondrial gene markers.

The effects of AMPK on different mitochondrial genes are achieved through the regulation of multiple transcription factors and cofactors. AMPK is a master regulator of peroxisome proliferator gamma co-activator 1α (PGC-1α) (Jager, Handschin, St-Pierre, & Spiegelman, 2007; Viollet et al., 2009). PGC-1α controls and coordinates a variety of other transcription factors related to mitochondrial function, biogenesis, and content (Handschin & Spiegelman, 2006). AMPK is proposed to directly phosphorylate PGC-1α at Thr177 and Ser538 and induce its translocation to the nucleus where its transcriptional activity takes place (Jager et al., 2007). The establishment of AMPK and PGC-1α was demonstrated when AMPK activating compound AICAR was unable to induce mitochondrial biogenesis in the muscles of mice lacking PGC-1α (Jager et al., 2007). AMPK is also capable of activating PGC-1α through deacetylation (Canto et al., 2010). Interestingly, AMPK trimers containing the γ^3 subunit are actually responsible for the deacetylation of PGC-1α during fasting conditions or exercise (Canto et al., 2010). This is of particular interest as γ^3 subunits are only present in glycolytic muscles and are more sensitive to exercise induced stressed in mouse skeletal muscle (Jensen, Wojtaszewski, & Richter, 2009). Interestingly, the results from this study demonstrated that the activation

of AMPK following exercise was dependent primarily on the α^2 isoform (Jorgensen et al., 2004). It appears however that no exercise genes were negatively affected by the loss AMPK and actually suggest loss of one sub-unit leads to an increased activation of activity of the other to compensate for the loss (Jorgensen et al., 2004).

Another interesting transcription factor that is a known target of AMPK is the FOXO family of transcription factors. Studies examining FOXO have suggested an important role for lifespan extension and are commonly associated with oxidative stress defense in skeletal muscle (K. Nakashima & Yakabe, 2007). However, the primary two pathways that are transcriptionally controlled by the FOXO family are the ubiquitin proteasome and the autophagy lysosomal proteasome. FOXO is present in multiple isoforms in skeletal muscle and the majority of research has examined FOXO1 and FOXO3 which are controlled upstream by both AKT and AMPK (Greer et al., 2007). Classically, phosphorylation by the latter is believed to drive FOXO3 and FOXO1 into the nucleus inducing its transcriptional activity and often activating the atrophy program of skeletal muscle (Stitt et al., 2004). However, FOXO3 is capable of being phosphorylated at six different residues each of which elicit a different transcriptional response during glucose deprivation or fasting (Greer et al., 2007). Surprisingly, it is worth noting that recent evidence suggests that FOXO3 phosphorylation by AMPK does not necessarily affect its localization but rather its activity (Greer et al., 2007). In addition to phosphorylation, FOXO is also capable of being regulated by acetylation/deacetylation which again occurs through AMPK and SIRT1 (Fulco et al., 2008). The fact that AMPK tightly controls the transcriptional regulation of a number of genes has been known for some time. However, recent evidence has also demonstrated

that some of these transcription factors are immediate targets for phosphorylation by AMPK as well. An interesting challenge that has come to light and warrants further investigation is how AMPK itself shuttles in and out of the nucleus to target promoters.

AMPK Regulation of mTORC1 and Protein Synthesis

In conditions where nutrients are unavailable or scarce, AMPK serves as the metabolic checkpoint to inhibit growth (Guo, Cloughesy, Radu, & Mischel, 2010). The most commonly described mechanism of this regulation is through the mTORC1 signaling pathway (Guo et al., 2010). As mentioned briefly above, AMPK is activated or switched on during starvation to up-regulate energy conservation. More evidence has come to light that demonstrates the critical role of AMPK and mTORC1 as antagonistic forces that regulate muscles adaptations to nutrition, exercise, and overall growth stimulation (Shaw, 2009).

AMPK negatively regulates the mTORC1 signaling complex through the phosphorylation of tuberous sclerosis complex 1/2 (TSC1/2) (Corradetti, Inoki, Bardeesy, DePinho, & Guan, 2004). While this is the most commonly studied mechanism, AMPK has also been proven to phosphorylate mTORC1 directly at Thr 2446 (Shaw, 2009). Finally, AMPK has also been shown to phosphorylate raptor, an mTORC1 binding partner that is essential for its activity (Kwon & Querfurth, 2015). The phosphorylation of raptor leads to sequestration of raptor by 14-3-3 proteins (Corradetti et al., 2004; J. Kim, Kundu, Viollet, & Guan, 2011). Phosphorylation of these sites suppresses mTORC1 signaling and inhibits protein synthesis. In addition to its actions on mTORC1, AMPK also regulates protein synthesis through the inhibition of eukaryotic elongation

factor 2 (eEF2). Phosphorylation of eEF2 at Thr 56 inhibits binding to the ribosome thus slowing elongation rate (Thomson, Fick, & Gordon, 2008). AMPK accomplishes this phosphorylation by interacting with eEF2 kinase and inhibiting it (Thomson et al., 2008) . Inhibiting the upstream kinase leads to the inhibition of eEF2 itself. Classically, translation initiation is often considered the rate limiting step in synthesis however, inhibition of elongation can also be critical in the synthetic rate.

In many models that have impaired anabolic signaling such as cancer cachexia, there is often an induction of AMPK activation (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017; Puppa, Gao, et al., 2014; White, Puppa, Gao, et al., 2013). These results suggest that mTORC1 is an essential part of normal ATP production and its disruption induces energetic stress and AMPK activity (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017). The suppression of AMPK in P70S6 kinase deficient myotubes has been demonstrated to rescue myotube size and protein synthesis signaling further demonstrating a role for AMPK in the regulation of mTORC1 dependent signaling processes (White, Puppa, Gao, et al., 2013). Studies have also demonstrated that the constant infusion of AICAR suppresses mTORC1 and P70S6 kinase activation leading to suppressed synthesis rates (Suwa et al., 2003). This suppression is directly correlated to the chronic activation of AMPK (Suwa et al., 2003). Conversely, activation of mTORC1 in AMPK deficient myotubes has been demonstrated to robustly induce synthesis rate and lead to a 1.5 fold increase in myotube size (White, Puppa, Gao, et al., 2013).

Recent advances in genetic mouse models have allowed researchers to further examine the role of AMPK in-vivo. Mice that have a skeletal muscle specific knockout of AMPKα^1 demonstrated greater overload induced hypertrophy than that of their wild

type littermates (Mounier et al., 2009). Elevated P70S6 kinase and 4E-BP1 were reported in overload induced hypertrophy AMPKα^1 knockout mice (Mounier et al., 2009). However, it is worth noting that a knockout of AMPKα^2 actually suppressed the overload induced hypertrophy response (Mounier et al., 2009). These results suggest that AMPKα^2 may be necessary for the increase in muscle size following an overload stimulus. More recent work has built upon these initial findings through the generation of double $\alpha^1\alpha^2$ muscle knockout mice. Soleus muscles from these mice were larger by mass and fiber diameter when compared to wild type littermates. However, loss of the $\beta^1\beta^2$ were not different in mass or fiber diameter when compared to wild type controls (O'Neill et al., 2011). It is interesting to speculate however, if a difference in the cre promoter could have contributed to this. The β knockout used a muscle creatine kinase (MCK) promoter which is specific to cardiac and skeletal muscle, whereas the researchers using the α catalytic subunit utilized the human skeletal actin (HSA) promoter which is specific to just skeletal muscle and believed to be more efficient.

While these results clearly demonstrate a role for AMPK in the regulation of mTORC1 during overload and exercise induced protein synthesis, less is understood about its regulation of these processes during fasting or starvation induced conditions. Furthermore, these genetic knockout rodents have yet to be utilized in disease models such as cancer cachexia or other catabolic conditions that exhibit chronic AMPK activation. The use of these knockout models in a catabolic state could provide new insight and potential therapeutic targets to increase muscle mass and protein synthesis rates during disease.

AMPK- A Critical Regulator of Autophagy

As described previously above the process of autophagy includes the degradation of cellular content and organelles under low energy conditions such as fasting or exercise. The process of autophagy involves several signaling events that lead to engulfment of the targeted cargo and subsequent degradation by the lysosomal proteasome (Glick et al., 2010; Yu et al., 2018). The key regulatory kinase of autophagy is ULK-1 that serves as the link between mTORC1 and AMPK (J. Kim et al., 2011). During low energy conditions ULK-1 is phosphorylated at Ser 555 by AMPK inducing this signaling process to provide nutrients and amino acids from the degraded cargo (J. Kim et al., 2011). During periods of high energy availability such as a meal, mTORC1 will actually phosphorylate ULK-1 and inhibit the induction of autophagy (J. Kim et al., 2011).

AMPK has long been known to be a key regulator of autophagy (Kjobsted et al., 2018). While some initial studies reported conflicting results on AMPK's role in autophagy, it was later determined that these results were dependent on cell type and conditions used. Under normal basal conditions both mTORC1 and AMPK are associated with ULK-1 (J. Kim et al., 2011). Classically, a fast or nutrient deprivation will lead to AMPK activation and suppress mTORC1 which promotes autophagy through the direct phosphorylation of ULK-1 (Bagherniya, Butler, Barreto, & Sahebkar, 2018). However, it is now known that starvation is not the only stimulus capable of initiating autophagy. This phosphorylation up-regulates the ULK-1 kinase activity and disrupts the association of ULK-1 with mTORC1 (J. Kim et al., 2011). Before this was discovered it was believed that AMPK controlled autophagy solely through the regulation of mTORC1 itself (J. Kim et al., 2011). ULK-1 then phosphorylates Atg 13 and FIP2000 to induce the

initiation of autophagy (J. Kim et al., 2011; Masiero et al., 2009; Yu et al., 2018). AMPK also phosphorylates Beclin-1 which activates the pro-autophagy Vps34 complex. These initial signaling events lead to autophagosome formation through the activation and lipidation of LC3 and subsequent tagging of cargo for degradation by p62 (J. Kim et al., 2011; Masiero et al., 2009; Yu et al., 2018). Studies recently examining the loss of the AMPK $\beta^1\beta^2$ demonstrated a blunted autophagy response to fasting and aging in skeletal muscle (Bujak et al., 2015; Zhao & Klionsky, 2011). Loss of these AMPK subunits diminished AMPK phosphorylation and downstream ULK-1 activity mirrored AMPK activity perfectly (Bujak et al., 2015; Zhao & Klionsky, 2011). This study was instrumental in further establishing AMPK's role in fasting induced autophagy as it was one of the first studies to examine these outcomes using a genetic muscle specific knockout. Furthermore, a similar study using a kinase dead AMPK α^2 actually yielded higher LC3 lipidation content than its vector treated litter mates (Zong et al., 2002). While the authors were unable to provide an explanation for this finding, they did suggest that a loss of α^2 alone is not sufficient to suppress autophagy protein expression following a fast. These results further establish that simply deleting one of that α catalytic subunits results in the up-regulation of activity of the remaining subunit as a compensatory mechanism and may actually lead to accelerated autophagy.

The activation of AMPK by acute exercise has been recently reported to be a critical factor in the activation of ULK-1 and the initiation of autophagy as well as mitophagy processes (Laker et al., 2017). Utilizing both a dominant negative AMPK as well as a constitutively active AMPK the researchers were able to demonstrate that the exercise activation of ULK-1 is dependent on AMPK (Zhao & Klionsky, 2011).

Additionally, the researchers also were able to show that exercise induced metabolic adaptations required ULK-1 (Laker et al., 2017). These findings provide direct evidence of exercise-induced autophagy/mitophagy and demonstrate the importance of AMPK-ULK-1 signaling in skeletal muscle.

In addition to its regulation of ULK-1, AMPK is also capable of inducing FOXO transcriptional activity as discussed previously in this review (Sanchez, Csibi, et al., 2012). Both FOXO1 and FOXO3 have been implicated in the transcriptional regulation of both autophagy related genes and lysosomal activity (Sanchez, Csibi, et al., 2012). However, FOXO is also capable of regulating a host of atrophy related genes both in the autophagy lysosomal system and the ubiquitin proteasome. Since FOXO is capable of regulating multiple different processes downstream, this mechanism of autophagy is often considered indirect when compared to the direct interaction AMPK has with ULK-1 and mTORC1.

AMPK in Cachexia – A Lack of Investigation

AMPK has been very well characterized for its roles in exercise and metabolism (K. Nakashima & Yakabe, 2007; Sanchez, Csibi, et al., 2012) . However, only a few researchers have examined AMPK's role in cancer induced muscle wasting. These studies have provided putative mechanisms of action and provided the initial evidence that place AMPK at the center of muscle wasting. Few studies have mechanistically tried to investigate the role it plays in regulating protein synthesis and degradation during the progression of the disease (Hall et al., 2018; Rohm et al., 2016; White, Puppa, Gao, et al., 2013). AMPK as described above is a master energy sensor and regulator of skeletal

muscle metabolism and is capable of being activated by numerous stimuli. Downstream it regulates a host of signaling processes related to lipid metabolism, glucose metabolism, protein synthesis, degradation, and mitochondrial biogenesis which are all disrupted in cancer cachexia (Argiles, Busquets, et al., 2015; Carson et al., 2016; Sandri, 2016). Our laboratory has previously demonstrated a role for AMPK in the regulation of skeletal muscle protein synthesis signaling through mTORC1 during cancer cachexia (White, Baynes, et al., 2011a; White, Puppa, Gao, et al., 2013). Utilizing an IL-6 receptor antibody, our research group was able to establish a link between cachectic inflammation and the AMPK suppression of protein synthesis (White, Baynes, et al., 2011a; White, Puppa, Gao, et al., 2013). Furthermore, we have also established a potential role for mitochondrial quality control in the activation of AMPK during cachexia. Utilizing a potent anti-oxidant our laboratory demonstrated attenuated AMPK activation and improved indices of mitochondrial biogenesis and dynamics (Miao et al., 2017; Narsale et al., 2016a). However, an exact mechanism of action has yet to be established.

There are conflicting ideologies about AMPK in cancer cachexia, while many studies examining pre-clinical models have reported an elevation of skeletal muscle AMPK, very few have gone on to actually examine the ramifications of this activation (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017; White, Puppa, Gao, et al., 2013). Some researchers argue that stimulation of AMPK during cachexia is needed and can actually attenuate fat mass loss however, these findings may have pertained solely to white adipose tissue(Hall et al., 2018; Rohm et al., 2016). Our laboratory has extensively characterized the $Apc^{Min/+}$ and LLC mouse models of cachexia and demonstrated that AMPK is induced as the severity of cachexia progresses following a standard 5 hour

fasting paradigm used to measure mTORC1 signaling and protein synthesis (Baltgalvis et al., 2010; Hardee, Montalvo, & Carson, 2017; Puppa, Gao, et al., 2014; White, Baynes, et al., 2011b). Accompanying this induction is the suppression of anabolic signaling and increased degradation through both the ubiquitin proteasome system and autophagy lysosomal system. The disruption and chronic activation of AMPK is also accompanied by mitochondrial loss and decreased biogenesis, suggesting that normal AMPK regulation of these processes is no longer intact during cachexia (White, Baltgalvis, et al., 2011). Through the manipulation of IL-6 using a receptor antibody, our laboratory was able to demonstrate that elevated circulating IL-6 contributes to the chronic activation of AMPK and suppressing this signaling attenuates the AMPK mediated suppression of mTORC1(White, Baynes, et al., 2011b). Additionally, inhibition of myotube AMPK using Compound C during LLC treatment improved mTORC1 signaling and protein synthesis (White, Puppa, Gao, et al., 2013).

AMPK - Cachexia and Exercise

While it is well established that acute exercise activates AMPK our laboratory demonstrated that exercise training was able to attenuate the cachexia induced chronic activation of this pathway and improve muscle mass loss (Hardee et al., 2016; Puppa et al., 2012). These results suggest that exercise training may improve mitochondrial function during cancer cachexia and alleviate the chronic deficit of energy (ATP generation) thus lowering AMPK's activity. Similar results were also demonstrated using high frequency electrical stimulation in cachectic muscle (Hardee et al., 2016). Seven repeated bouts of high frequency stimulation were able to lower AMPK phosphorylation in cachectic $Apc^{Min/+}$ and stimulate muscle protein synthesis 3 hours post

the final treatment (Hardee et al., 2016). These results demonstrate that exercise training in the cachectic state has the ability to improve skeletal muscle AMPK signaling and alleviate the AMPK mediated inhibition of mTORC1 signaling and protein synthesis.

While both exercise and cytokine treatments have been demonstrated to improve AMPK signaling during cancer cachexia, less is understood about the role of fasting and feeding in these signaling processes. As mentioned previously, numerous models of cancer cachexia have reported both elevated AMPK and indices of autophagy (Pigna et al., 2016a; White, Baltgalvis, et al., 2011). While many pre-clinical models have reported food intake as being similar between cachectic animals and healthy controls, there are very few studies that have examined defined fasting and feeding during cancer cachexia. The majority of protein synthesis literature in cancer cachexia utilizes a fast (usually 5 hours) to account for acute effects of feeding. As previously described, our laboratory has always noted an increase in AMPK following this time period. This proposal will focus more on the role of skeletal muscle AMPK in the fasting and feeding response of cachectic skeletal muscle. Additionally, the role of exercise training in the regulation of these processes during cancer cachexia will also be explored. Key preliminary data from our laboratory suggest that cachectic Min mice are not necessarily anabolic resistant but rather hyper sensitive to a fast due to the chronic activation of AMPK that occurs in the fed state. This chronic activation is exacerbated further through an acute 12-hour fast and may suppress protein synthesis and induce autophagy when compared to normal healthy controls.

CHAPTER 2

THE ROLE OF AMPK SIGNALING IN SKELETAL MUSCLE PROTEIN TURNOVER DURING CANCER CACHEXIA

2.1 Abstract.

Cancer cachexia is a debilitating condition that is characterized by the progressive loss of muscle mass. The loss of muscle mass during cachexia is often attributed to altered protein turnover. The adenosine monophosphate kinase (AMPK) signaling pathway exerts regulatory control over skeletal muscle protein synthesis (mTORC1 activity), degradation, and mitochondrial quality control. Our laboratory and others have demonstrated that AMPK is chronically elevated in cachectic skeletal muscle. The elevation of AMPK coincides with suppressed mTORC1, increased degradation, and disrupted mitochondrial quality control. While these findings suggest a role for AMPK very little is understood about its downstream signaling and the regulation of protein turnover during cachexia. Therefore, the purpose of this study was to determine the role of AMPK signaling in skeletal muscle protein turnover during cancer cachexia. Male C57BL/6 (B6, N=8), AMPK$\alpha^1\alpha^2$ skeletal muscle specific knockout (KO), *Apc*$^{Min/+}$ (Min, N=10), and AMPK$\alpha^1\alpha^2$ *Apc*$^{Min/+}$ (Min KO, N=6) were tracked until ~19 weeks of age and fasted for a duration of 12 hours during the light cycle. Following completion of each treatment, hindlimb muscles were harvested and the gastrocnemius homogenized for protein analysis. Loss of AMPK in Min mice attenuated fasting induce bodyweight loss

and gastrocnemius mass. AMPK (T172) phosphorylation was induced in Min mice and suppressed by Min KO. TSC2 phosphorylation was induce in Min mice and rescued in Min KO mice. There was also a main effect of Min to induce Raptor and ULK-1 while also a main effect of KO to suppress Raptor and ULK-1 phosphorylation. E3 ligases MuRF-1 and Atrogin-1 were both induced in Min mice, but were rescued by Min KO. Furthermore, autophagy markers P62 and LC3B were also elevated in Min mice and rescued by Min KO. There was a main effect of KO and Min to suppress PGC-1α, MFN-1 was suppressed in Min mice but restored by Min KO. DRP-1 was induced in Min mice and this induction was attenuated in Min KO mice. Our results demonstrate that AMPK is required for the cachexia induction of E3 ligases and autophagy. Furthermore, our results also suggest that the cachexia regulation of mitochondrial dynamics proteins DRP-1 and MFN-1 occur through AMPK.

2.2 Introduction

Cancer cachexia is the unintentional loss of bodyweight loss which also includes skeletal muscle mass (Baracos, 2006a, 2011; Baracos et al., 2018). Perturbations in protein synthesis and protein degradation disrupt muscle proteostasis and metabolism (Bruggeman et al., 2016; Dodesini, Benedini, Terruzzi, Sereni, & Luzi, 2007; Duval, Jeanneret, Santoro, & Dormond, 2018; Johns et al., 2012). The underlying causes of these alterations in protein turnover are poorly understood and may depend a multitude of factors including inflammation, oxidative metabolism, type of cancer, and severity of cachexia (Hardee et al., 2017; Hatakeyama et al., 2016; Hetzler, Hardee, LaVoie, Murphy, & Carson, 2017; A. M. Horstman, Olde Damink, Schols, & van Loon, 2016; Mattox, 2017; Porporato, 2016). In human cancer patients, fasted protein synthesis rates

are suppressed in severe cachexia (Baracos, 2006a; A. M. Horstman et al., 2016; Lindsey & Piper, 1985; Wheelwright et al., 2016). Furthermore, our laboratory and others have demonstrated similar findings in pre-clinical models of cancer cachexia (Baltgalvis et al., 2009; Carson et al., 2015; Hardee et al., 2017; Narsale & Carson, 2014; White, Baltgalvis, et al., 2011; White, Baynes, et al., 2011a; White, Puppa, Gao, et al., 2013). While the suppression of protein synthesis has been established, many cancer cachexia clinical studies and pre-clinical models also report an acceleration of protein degradation (Attaix et al., 1999b; Aversa, Pin, Lucia, Penna, Verzaro, Fazi, Colasante, Tirone, Rossi Fanelli, et al., 2016; Baracos, 2006a; Bennani-Baiti & Walsh, 2011). This imbalance of synthesis and degradation leads to a net loss of muscle mass and can directly influence the response to interventions such as chemotherapy and exercise (Dillon et al., 2012; Kimura et al., 2015; Narsale & Carson, 2014; C. M. Prado et al., 2013; Ryan et al., 2016; Sadeghi et al., 2018).

The adenosine monophosphate protein kinase (AMPK) is a nutrient sensitive kinase that has the unique ability of regulating skeletal muscle protein synthesis, degradation, and mitochondrial quality control (Bujak et al., 2015; Z. P. Chen et al., 2000; Guo et al., 2010; Gwinn et al., 2008; Kemp et al., 2007; J. Kim et al., 2011; Kjobsted et al., 2018; Mounier et al., 2015). Our laboratory has previously reported that the degree of activation of AMPK progressively increases with the severity of cachexia in the $Apc^{Min/+}$ mouse model of cachexia and is tightly associated with muscle mass loss (White, Baynes, et al., 2011b). AMPK activation in normal healthy skeletal muscle will suppress mTORC1 activity through phosphorylation of TSC2 and Raptor (Sengupta, Peterson, & Sabatini, 2010; Shaw, 2009). In particular, the phosphorylation of Raptor is

especially important as it has been required for the suppression of mTORC1 activity (Gwinn et al., 2008). We have previously demonstrated that Raptor and TSC2 are induced in cachectic skeletal muscle while AMPK is robustly activated (White, Baynes, et al., 2011a; White, Puppa, Gao, et al., 2013). Coinciding with the induction of TSC2 and Raptor, AMPK also regulates the Forkhead Box O family of transcription factors (FOXO3a) increasing its transcriptional activity and E3 ligases Atrogin-1 and MuRF-1 (Hulmi, Silvennoinen, Lehti, Kivela, & Kainulainen, 2012; Jorgensen et al., 2005; K. Nakashima & Yakabe, 2007). The use of AMPK activator AICAR in-vitro and in-vivo has demonstrated that chronically elevating AMPK will induce FOXO3a and the expression of Atrogin-1 and MuRF-1 leading atrophy (Greer et al., 2007; K. Nakashima & Yakabe, 2007). Additionally, AMPK inhibitors have also been utilized to demonstrate a suppression of E3 ligases both in-vivo and in-vitro, further establishing the regulatory role of AMPK (Viollet et al., 2009). FOXO3a has been demonstrated to be robustly activated in various pre-clinical models of cancer cachexia, acute exercise, and fasting(Greer et al., 2007; Reed, Sandesara, Senf, & Judge, 2012; White, Baynes, et al., 2011b). Our laboratory and other research groups have previously reported elevated FOXO3a, Atrogin-1 and MuRF-1 signaling in both cachectic $Apc^{Min/+}$ and LLC mice (Puppa, Gao, et al., 2014; White, Baynes, et al., 2011a). AMPK also controls the autophagy lysosomal system through the phosphorylation of Unc-51 like kinase (ULK-1) at serine 555 (Bujak et al., 2015; Laker et al., 2017; Sanchez, Csibi, et al., 2012; Zhao & Klionsky, 2011). The activation of ULK-1 by AMPK is required for the initiation of autophagy (Wong, Puente, Ganley, & Jiang, 2013; Zachari & Ganley, 2017). Furthermore, recent evidence also suggest that this initiation of autophagy also requires

the phosphorylation of Raptor by AMPK in order to suppress mTORC1 and alleviate the hold mTORC1 exerts on ULK-1 (Gwinn et al., 2008; Hindupur, Gonzalez, & Hall, 2015). Autophagy has recently become a highly debated topic in cachexia and is reported elevated in many pre-clinical models and human cancer patients(Penna et al., 2014; Penna, Costamagna, et al., 2013). Previous research from our laboratory has demonstrated that both ubiquitin proteasome activity (ATP dependent) and autophagy lysosomal activity (ATP independent) both increase as the severity of cachexia and AMPK also increase (White, Baynes, et al., 2011a).

The final pathway of interest that AMPK directly regulates is the process of mitochondrial quality control (Laker et al., 2017; Mishra & Chan, 2016; O'Neill et al., 2011; Romanello et al., 2010; Romanello & Sandri, 2015). AMPK is activated during energy deficiency (fasting and exercise) and will induce the activation of peroxisome proliferator gamma co-activator 1α (PGC-1α) (Jager et al., 2007; Jorgensen et al., 2005). PGC-1α is induced by AMPK to compensate for a lack of energy availability and increase the amount of ATP production by the generation of new mitochondria (Canto & Auwerx, 2009; Jager et al., 2007). Studies utilizing AMPK activators have demonstrated that PGC-1α is required for AMPK mediated mitochondrial biogenesis(Canto et al., 2010; Jager et al., 2007; Terada et al., 2002). Interestingly, despite the chronic elevation of AMPK in cachectic skeletal muscle, PGC-1α is actually suppressed demonstrating a disconnect between the two proteins(Puppa, Gao, et al., 2014; Puppa et al., 2012; White, Baltgalvis, et al., 2011). The reason for this dysregulation remains to be elucidated but some have speculated that chronic inflammation through IL-6 as well as disrupted sirtuin activity may play a role in the elevation of AMPK and suppression of PGC-1α(Puppa,

Gao, et al., 2014; Puppa et al., 2012; White, Baltgalvis, et al., 2011). Another key component of mitochondrial quality control is the process of mitochondrial dynamics or the constant remodeling of the mitochondrial network through fission and fusion. Mitochondrial dynamics have been implicated in cancer cachexia and have also been demonstrated to induce muscle atrophy (Marzetti et al., 2017; Mishra & Chan, 2016; Romanello & Sandri, 2015; Suliman & Piantadosi, 2016; Vitorino, Moreira-Goncalves, & Ferreira, 2015). Dynamin related protein (DRP-1) and mitofusion 1 (MFN-1) are two key regulators of fission and fusion and have emerged as interesting targets in cancer cachexia and inflammation (Suliman & Piantadosi, 2016). A recent publication by our laboratory has demonstrated a role for inflammatory signaling through gp130/ERK1/2 signaling to induce DRP-1 and increased fission (Fix, VanderVeen, Counts, & Carson, 2019). Chronic mitochondrial fission has been demonstrated by our laboratory and others in severe cancer cachexia(Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017; Puppa, Gao, et al., 2014; White et al., 2012). Furthermore, mitochondrial fusion appears to be an early target of cachexia as suppression of MFN-1 worsens as the degree of cachexia increases(White, Baltgalvis, et al., 2011; White et al., 2012). Dysregulation of mitochondrial dynamics has been directly shown to influence muscle atrophy through FOXO3a signaling and the E3 ligases in non-tumor bearing mice (Romanello et al., 2010; Romanello & Sandri, 2015; Sandri et al., 2004). Recent evidence has suggested that these processes (especially mitochondrial fission), are AMPK sensitive targets and that the chronic elevation of AMPK can disrupt mitochondrial quality further contributing to energetic stress and muscle mass loss (Toyama et al., 2016). The chronic elevation of fission and suppression of fusion coupled with a lack of mitochondrial biogenesis through

PGC-1α suggest a lack of sufficient mitochondrial ATP generation to handle the metabolically expensive process of protein synthesis. The concept of oxidative health and metabolism regulating protein synthesis has been suggested by our laboratory before and evidence of mitochondrial function and quality regulating mTORC1 activity in skeletal muscle has become a new investigative target of cancer cachexia (Hardee et al., 2017; Suliman & Piantadosi, 2016).

While AMPK is an established regulator of muscle mass and metabolism, very little has been done to examine this signaling kinase in cachectic skeletal muscle. The chronic elevation of AMPK in the cachectic environment suggest a chronic energy deficit that disrupts protein turnover and contributes to the loss of muscle mass through an induction of protein degradation and suppression of metabolically expensive protein synthesis. While our laboratory has suggested a role for AMPK in these processes, very little has been done to determine if AMPK is required for the cachexia disruption of protein turnover. Therefore, the role of AMPK signaling in skeletal muscle protein turnover during cancer cachexia**.** We hypothesize that loss of AMPK in cachectic skeletal muscle will improve protein synthesis and degradation signaling. We also hypothesize that AMPK loss in cachectic skeletal muscle will restore mitochondrial dynamics to basal levels.

2.3 Methods

Animals

The *Apc*$^{Min/+}$ mouse is a genetic model of colorectal cancer and cachexia [83]. These mice harbor a heterozygous mutation in the adenomas polyposis coli (APC) gene,

which promotes the development of intestinal tumors beginning as early as 4 weeks of age. Mice develop an IL-6-dependent cancer cachexia phenotype between 3 and 6 months of age. Due to the slow onset and progression of body weight loss this model is advantageous as treatments can be started after the initiation of cancer cachexia. Male $Apc^{Min/+}$ mice on a C57BL/6 background were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred at the University of South Carolina's Animal Resource Facility. All mice used in the current study were obtained from the investigators breeding colony within the Center for Colon Cancer Research Mouse Core. At 3-wk of age mice were genotyped as previously described [298]. Mice were individually housed, kept on a 12:12-h light-dark cycle, and had access to standard rodent chow (#8604 Rodent Diet; Harlan Teklad, Madison, WI) and water ad libitum. 12 hours prior to sacrifice all mice had food removed from cage. Body weight measurements were taken weekly and the percentage body weight loss from peak body weight (~12-20 weeks of age) was calculated. Mice lacking the $Apc^{Min/+}$ mutation (C57BL/6) served as controls for all experiments. The University of South Carolina's Institutional Animal Care and Use Committee approved all animal experimentation in this study.

Skeletal Muscle Deletion of AMPK$\alpha^1\alpha^2$

To generate a skeletal muscle specific knockout of AMPK$\alpha^{1}\alpha^{2}$, mice that contained individually floxed alleles for AMPK$\alpha^{1}$and AMPKα^{2} were a kind gift from Dr. Hoh-Jin Koh at the University of South Carolina. These mice were then crossed together to generate a double floxed AMPK$\alpha^{1}\alpha^{2}$mouse. The AMPKa1a2 was then crossed with $Apc^{Min/+}$ mice to generate an AMPK$\alpha^{1}\alpha^{2}$ $Apc^{Min/+}$ mouse. Tamoxifen inducible Mer Cre

Mer driven by a Human Skeletal Actin promoter (HSA) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA).These mice were then crossed with our AMPK$\alpha^{1}\alpha^{2}$ to generate AMPK$\alpha^{1}\alpha^{2}$ HSA Cre mice. Finally, the AMPK$\alpha^{1}\alpha^{2}$ HSA Cre mice were crossed with AMPK$\alpha^{1}\alpha^{2}$ $Apc^{Min/+}$ mice giving a 50% chance of the offspring producing an AMPK$\alpha^{1}\alpha^{2}$ $Apc^{Min/+}$ HSA Cre mouse. Tamoxifen injection via intraperitoneal cavity deletes skeletal muscle AMPKa1a2 in the $Apc^{Min/+}$ mouse (Min KO).

Tissue Collection

Mice were anesthetized via a subcutaneous injection of ketamine/xylazine/acepromazine cocktail (1.4 ml/kg body weight) prior to sacrifice. Hind limb muscles were rapidly excised, cleared of excess connective tissue, rinsed in PBS, weighed, and snap frozen in liquid nitrogen. The gastrocnemius muscle was cut at the mid-belly, and the proximal portion was split laterally and medially for protein. The left and right soleus muscles were weighed and processed for protein analysis. Blood was collected prior to muscle collection via retro-orbital eye bleed with heparinized capillary tubes, placed on ice, and centrifuged (10,000 x g for 10 min at 4°C). The supernatant was removed and stored for plasma IL-6 analysis. Plasma and tissue samples were stored at -80°C until further analysis.

Western Blotting

Western blot analysis was performed as previously described. Frozen gastrocnemius muscle was homogenized in ice-cold Mueller buffer and protein concentration was determined by the Bradford method. Crude muscle homogenates were

fractionated on 6 – 15% SDS polyacrylamide gels and transferred to PVDF membranes overnight. Membranes were stained with Ponceau red to verify equal loading and transfer for each gel. Membranes were blocked at room temperature for 1 – 2 hrs. in 5% Tris-buffered saline with 0.1% Tween-20 (TBST) milk. Primary antibodies for p-AKT (T308), AKT, puromycin, p-FOXO3(S253), FOXO3, p-ULK-1 (S555), ULK-1, P62, LC3II/I, MuRF-1, Atrogin-1 (ECM Biosciences), p-AMPK (T172), AMPK, p-ACC (S79) , ACC, p-TSC2 (S1387), TSC2, p-raptor (S792), raptor, DRP-1, FIS-1, MFN-1 (Abcam),PGC-1a (Abcam), and GAPDH will be diluted 1:5000 – 1:2000 in 5% TBST-milk followed by a 4-8-hr incubation with membranes at 4 degrees. Anti-rabbit or anti-mouse IgG horseradish peroxidase conjugated secondary antibody was incubated with the membranes at 1:2000 dilutions for 1 hour in 5% TBST-milk at room temperature. Enhanced chemiluminescence (ECL) (GE Healthcare Life Sciences, Piscataway, NJ) was used to visualize the antibody-antigen interactions. Images were digitally scanned and quantified by densitometry using imaging software (Image J;NIH). All antibodies were from Cell Signaling unless otherwise stated.

Plasma IL-6 concentrations

Plasma IL-6 concentrations were determined as previously described [300]. A commercially available IL-6 enzyme-linked immunosorbent assay kit was obtained from BD Biosciences (San Diego, CA, USA) and the manufacturer's protocol was followed. Briefly, a Costar clear 96-well plate (Corning, NY, USA) was coated with IL-6 capture antibody and allowed to incubate overnight. The next morning, the plate was blocked with assay diluent buffer, washed, and IL-6 standards and plasma samples were added in duplicate to the plate. The plate was again washed and sAV-HRP reagent was added to

each well. After several washes, TMB substrate was added and the reaction was developed for 20 minutes. The reaction was stopped with sulfuric acid and absorbance was read in a Bio-Rad iMark plate reader (Hercules, CA, USA) at 450 nm.

Statistical Analysis

Results are reported as the means ± standard error. A Two-Way ANOVA or students pre-planned t-test was utilized to analyze the data where appropriate . Post-hoc analyses were performed with Tukey method when appropriate. The accepted level of significance was set at $p<0.05$ for all analysis. Statistical analysis was performed using Prism GraphPad 7 (GraphPad Software Inc., La Jolla, CA).

2.4 Results

Animal Characteristics of AMPK KO Mice

AMPK KO loss had no effect on gastrocnemius mass, bodyweight loss, stomach weight, spleen weight, seminal vesicles, testes or epididymal fat (Table 2.1). There was a significant age difference between Min mice and Min KO mice (Table 2.1). Bodyweight loss was similar prior to fasting in Mins and Min KO mice. Bodyweight loss after a 12 hour fast was attenuated in Min KO mice when compared to Mins (Table 2.1). Gastrocnemius mass was also rescued in Min KO mice when compared to Min mice. Stomach, liver, and spleen mass was also reduced in Min KO compared to Min mice (Table 2.1). No differences in tibia length were detected in any group suggesting all mice were approximately the same size (Table 2.1).

Skeletal Muscle AMPK$\alpha^1\alpha^2$ Loss

In order to determine AMPK's role in the regulation of protein turnover during cachexia we first verified our knockout model in WT and Min mice (Figure 2.1). Treatment with tamoxifen for 5 days followed by a 1 week washout period was sufficient to induce human skeletal actin (HSA) cre deletion AMPKα^1 and AMPKα^2 from skeletal muscle in both WT and Min mice (Figure 2.1). As expected, the loss of AMPK suppressed the induction of AMPK phosphorylation (T172) in Min mice (Figure 2.1). In order to further verify our knockout model, we examined acetyl co-a carboxylase (ACC) phosphorylation (S79) (Figure 2.1). There was an interaction of Min ACC phosphorylation to be different than all groups. These results demonstrate that the use of a tamoxifen inducible HSA cre was sufficient to generate a skeletal muscle specific knockout of AMPK$\alpha^1\alpha^2$ (Figure 2.1).

AMPK Regulation of Immediate Downstream Targets During Cancer Cachexia

After verification of our knockout model was complete we next examined the role of AMPK in the regulation of immediate downstream targets regulating mTORC1 activity and degradation (Figure 2.2). There was an interaction of TSC2 phosphorylation (S1387) to be induced in Min mice which was different to all groups (Figure 2.2). There was also a main effect of Min to induce Raptor phosphorylation (S729) and a main effect of KO to suppress raptor phosphorylation (Figure 2.2). We next examined ULK-1 and FOXO3a phosphorylation which are known regulators of both autophagy and E3 ligase expression respectively (Figure 2.2). In line with our other immediate AMPK downstream targets, there was a main effect of Min to induce ULK-1 phosphorylation

(S555) and a main effect of KO to suppress ULK-1 phosphorylation (Figure 2.2). There was an interaction of FOXO3a phosphorylation in Min and Min KO mice to be different than all other groups.

AMPK Regulation of Protein Turnover During Cancer Cachexia

Given the results from the immediate AMPK downstream targets we examined protein expression for E3 ligases, autophagy, and protein synthesis (puromycin incorporation) (Figure 2.3). There was an interaction of MuRF-1 to be significantly elevated in Min mice when compared to all groups (Figure 2.3). Interestingly, Atrogin-1 was induced in Min mice and was only different to WT Flox mice and this induction was suppressed by Min KO (Figure 2.3). Similar to Atrogin-1, P62 which is a ubiquitin tag used to mark cargo for degradation by the lysosome was only elevated in Min mice compared to WT Flox and this induction was suppressed by Min KO (Figure 2.3). There was an interaction of LC3B (II/I) ratio to be induced in Min mice and this induction was different to all groups (Figure 2.3). We next examined puromycin incorporation as an indices of protein synthesis through utilization of the Surface Sensing of Translation technique (SUnSET) . There was a main effect of KO to induce puromycin incorporation and a main effect of Min to suppress puromycin incorporation (Figure 2.3). Collectively these results demonstrate that the cachexia disruption of skeletal muscle protein turnover requires AMPK.

AMPK Regulation of Mitochondrial Quality Control During Cancer Cachexia

We lastly examined the role of AMPK in the regulation of mitochondrial quality control. We first examined PGC-1α as an index of mitochondrial biogenesis and content.

There was a main effect of Min and KO to suppress PGC-1α which corresponds to previously published literature demonstrating similar results with AMPK suppression (Figure 2.4). Interestingly, there was an interaction for MFN-1 to be elevated compared to Min Flox mice (Figure 2.4). There was also an interaction for DRP-1 to be elevated in Min Flox mice compared to WT Flox mice (Figure 2.4). Furthermore, Min KO suppressed DRP-1 protein expression compared to Min Flox. Collectively these results demonstrate that AMPK is not required for the cachexia suppression of PGC-1α (Figure 2.4). Additionally, These results also suggest that MFN-1 and DRP-1 are AMPK sensitive targets and loss of AMPK restores these proteins to WT levels.

2.5 Discussion

The disruption of protein turnover has been widely investigated in various catabolic conditions including cancer cachexia (Sandri, 2016; Suzuki, Asakawa, Amitani, Nakamura, & Inui, 2013; Tisdale, 2005; White, Puppa, Gao, et al., 2013; Yoshida & Delafontaine, 2015). Many researchers advocate that the net loss of muscle mass during cancer cachexia occurs primarily through an acceleration of degradation signaling via the ubiquitin proteasome and autophagy lysosomal system (Penna et al., 2014; Sandri, 2016; Schersten & Lundholm, 1972). Conversely, while degradation signaling is often elevated skeletal muscle protein synthesis is often suppressed (Jeevanandam, Horowitz, Lowry, & Brennan, 1984; Johns et al., 2012; A. J. MacDonald et al., 2015; Pettersen et al., 2017; Pisters & Pearlstone, 1993). While this has been studied and characterized in cancer cachexia, very little has been done to formally elucidate a clear mechanism of regulation that drives this proteostasis disruption. However, a constant finding in severely cachectic muscle is the chronic elevation of AMPK (Hardee et al., 2017; Sadeghi et al., 2018;

Sanchez, Csibi, et al., 2012; Sandri, 2016; White, Baynes, et al., 2011b; White, Puppa, Gao, et al., 2013). The elevation of AMPK in cachectic skeletal muscle suggest an energy deficit that may suppress the ability of skeletal muscle to induce muscle protein synthesis in response to various stimuli such as contraction or feeding. We have previously suggested that IL-6 in the Min model of cachexia may be a potential driver of AMPK activation and muscle mass loss (White, Baynes, et al., 2011a; White, Puppa, Gao, et al., 2013). In line with previous our laboratories previous studies we also report elevated AMPK in fasted Min mice (White, Baynes, et al., 2011a; White, Puppa, Gao, et al., 2013). In the current study we report that IL-6 levels were similar in Min KO mice when compared to Mins. We report the novel finding that loss of AMPK in Min mice attenuated gastrocnemius mass loss, spleen weight and interestingly circulating IL-6 levels. We also report the novel findings that AMPK is required for the cachexia induction of TSC2 and Raptor which serves to inhibit mTORC1 activity and suppress muscle protein synthesis. Furthermore, we also report the novel finding that the cachexia induction of ULK-1 and FOXO3a mediated degradation requires AMPK in Min mice. Furthermore, we also report that mitochondrial dynamics proteins MFN-1 and DRP-1 are regulated by AMPK in the cachectic environment. Taken together, these results suggest that the cachexia disruption of skeletal muscle protein turnover occurs through AMPK mediated downstream signaling.

AMPK is an energy sensitive kinase and has been referred to as a cellular fuel gauge. The purpose of AMPK activation in healthy skeletal muscle is to suppress energy consuming pathways and activate energy generating pathways (Thomson, 2018; Thomson et al., 2008; Viollet et al., 2009). Previous studies have demonstrated through

the infusion of AMP mimetic AICAR that chronic elevation of AMPK induces muscle atrophy (K. Nakashima & Yakabe, 2007; Suwa et al., 2003). Furthermore, recent evidence suggests that loss of AMPK in normal healthy skeletal muscle is capable of inducing mTORC1 anabolic signaling (Thomson, 2018; Thomson et al., 2008). In the cachectic environment the degree of AMPK activity is tightly associated with the severity of cachexia or degree of bodyweight loss (White, Baynes, et al., 2011a, 2011b). In the current study, AMPK was induced following a short term 12-hour fast in Min mice and this induction was suppressed by loss of AMPK. Immediate downstream target ACC was also suppressed, corroborating the loss of AMPK activity. AMPK activation classically serves to suppress the metabolically expensive process of protein synthesis through phosphorylation of TSC2 and Raptor (Gwinn et al., 2008; Inoki, Zhu, & Guan, 2003). Recent evidence suggest that the phosphorylation of Raptor by AMPK is actually required for the suppression of mTORC1 (Gwinn et al., 2008). In line with previous research from our laboratory Raptor and TSC2 phosphorylation were both induced in Min mice while puromycin incorporation was suppressed. However, in the current study we demonstrate that the loss of AMPK in Min mice suppresses the cachexia activation of these proteins and restores puromycin incorporation which is indicative of protein synthesis. It is interesting to speculate whether the activation of Raptor or TSC2 is vital for the cachexia suppression of protein synthesis or if this process requires both proteins to be phosphorylated by AMPK.

The AMPK regulation of skeletal muscle degradation has been widely investigated in many different conditions and models of disease (Bujak et al., 2015; Di Magno et al., 2016; K. Nakashima & Yakabe, 2007; Sanchez, Candau, et al., 2012;

Sanchez, Csibi, et al., 2012; Thomson, 2018). They elevation of skeletal muscle protein degradation in cancer cachexia has been established and investigated by our research group and numerous others (Hardee et al., 2017; White, Baynes, et al., 2011a; White, Puppa, Gao, et al., 2013). The focus of skeletal muscle protein degradation in cancer cachexia has been primarily related to the classical FOXO3a control of E3 ligases Atrogin-1 and MuRF-1(Blackwell et al., 2018; Reed et al., 2012; White, Baynes, et al., 2011a). In the current study we demonstrate the cachexia induction of FOXO3a mediated skeletal muscle degradation requires AMPK and that loss of AMPK inhibited the cachexia induction of MuRF-1 and Atrogin-1. AMPK phosphorylates FOXO3a at S413 to increase its transcriptional activity (Kjobsted et al., 2018). We have previously demonstrated that AKT phosphorylation of FOXO3a is suppressed in cachectic skeletal muscle which is suggestive of its translocation to the nucleus. The current data further builds on these findings by adding the AMPK mediated phosphorylation site that demonstrates increased activity of FOXO3a which is reflected by the downstream suppression of E3 ligases Atrogin-1 and MuRF-1. Another component of degradation that is often ignored or poorly investigated in cancer cachexia is the autophagy lysosomal system. The process of autophagy is the selective degradation of cellular cargo, organelles, and protein aggregates that are removed to improve the quality and metabolic health of the tissue (Sandri, 2010). AMPK is a known regulator of autophagy and has been shown to be critical in the initiation of the process through its phosphorylation of ULK-1 at S555 (Bujak et al., 2015; Mihaylova & Shaw, 2011; Sanchez, Csibi, et al., 2012; Zhao & Klionsky, 2011). AMPK and mTORC1 both regulate ULK-1 and have competing effects through phosphorylation events (J. Kim et al., 2011). Autophagy has

become a relatively new target in cancer cachexia and has been linked to cancer induced inflammation through the IL-6/gp130 signaling pathway in pre-clinical models and human cancer patients (Aversa, Pin, Lucia, Penna, Verzaro, Fazi, Colasante, Tirone, Rossi Fanelli, et al., 2016; Penna, Costamagna, et al., 2013; Pettersen et al., 2017). Recent studies in C26 tumor bearing mice demonstrate elevated autophagy and this is dependent on AMPK signaling (Penna, Costamagna, et al., 2013). Furthermore, data from our laboratory has established that Beclin-1 and LC3B is elevated in severely cachectic Min mice (White et al., 2012). Additionally, the more severe body weight loss in cachexia the higher the actual activity of the autophagy lysosome system (White, Baynes, et al., 2011a). In the current study we demonstrate that the cachexia induction of autophagy following a short term fast requires the AMPK phosphorylation of ULK-1. Loss of AMPK in Min mice suppressed ULK-1 and downstream targets LC3B (II/I) ratio and P62. When taken collectively the loss of skeletal muscle AMPK in cachectic skeletal muscle appears to suppress protein degradation and improve muscle protein synthesis through regulation of immediate downstream targets and the processes these targets control.

The concept of mitochondrial quality control involves the processes of mitochondrial biogenesis and dynamics (Mishra & Chan, 2016). AMPK has recently emerged as a critical regulator of these processes. The AMPK regulation of PGC-1α has been widely investigated has been deemed the primary method of inducing mitochondrial biogenesis during energetic stress such as fasting or exercise (Canto & Auwerx, 2009). However, in cachectic skeletal muscle the chronic elevation of AMPK is dissociated from PGC-1α. Numerous studies have demonstrated elevated AMPK and suppressed PGC-1α

protein and mRNA expression in a variety of pre-clinical cachexia models. Not surprisingly, our data demonstrates that AMPK is not required for the suppression of PGC-1α in cachectic skeletal muscle (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017; Brown, Rosa-Caldwell, Lee, Brown, et al., 2017; Puppa, Gao, et al., 2014; Puppa et al., 2012). These results and previous studies suggest an alternative mechanism of regulation for PGC-1α. Interestingly, a recent publication suggest that mitochondrial loss may occur actually prior to the initiation of cachexia (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017). The loss of mitochondria early and a lack of biogenesis in severe cachexia may be a powerful driver of metabolic dysfunction and muscle mass loss in cachexia. Further research investigating the role of SIRT-1 and acetylation/deacetylation of PGC-1α in cachectic skeletal muscle are warranted in order to elucidate the mechanism that governs mitochondrial biogenesis.

The process of mitochondrial dynamics involves the constant fission and fusion of mitochondria (Dahlmans, Houzelle, Schrauwen, & Hoeks, 2016; Mishra & Chan, 2016). AMPK has been identified as a powerful regulator of these processes, in particular a recently published study demonstrated that AMPK when activated targets mitochondrial fission downstream to induce remodeling (Toyama et al., 2016). Mitochondrial dynamics in cachexia have been deemed an important hallmark of the disease, although an exact mechanism of regulation has yet to be elucidated (B. N. VanderVeen, Fix, et al., 2017). A recent investigation by our laboratory suggest a role for IL-6/gp130/ERK1/2 inflammatory signaling axis in the regulation of mitochondrial fission proteins DRP-1 and FIS-1 (Fix et al., 2019). In cachectic skeletal muscle FIS-1 is induced and MFN-1 is suppressed suggesting mitochondrial remodeling is imbalanced (White et al., 2012).

Accelerated mitochondrial fission has been investigated in conjunction with AMPK/FOXO3a signaling and is capable of driving muscle atrophy (Romanello et al., 2010). Interestingly, much less is understood about the regulation of fusion in regard to the AMPK signaling pathway. We provide evidence that AMPK negatively regulates mitochondrial fusion protein MFN-1 in cachexia. Loss of AMPK in Min mice restored MFN-1 to wildtype levels. Furthermore, we also establish fission protein DRP-1 as an AMPK sensitive target in cachectic skeletal muscle. DRP-1 protein expression was induced in Min mice but was suppressed to basal levels by the loss of AMPK. Collectively these results suggest that the cachexia suppression of PGC-1α does not require AMPK. Furthermore, we identify mitochondrial dynamics proteins DRP-1 and MFN-1 as AMPK sensitive targets in cachectic muscle. The chronic elevation of fission and suppression of fusion when coupled with a lack of biogenesis could be a contributing factor to the disruption of proteostasis due to dysfunctional oxidative metabolic capacity.

The disruption of protein turnover that favors skeletal muscle degradation over synthesis has become a hallmark of cancer cachexia (Pisters & Pearlstone, 1993; Sandri, 2016; White, Baynes, et al., 2011b). Identifying a mechanism of regulation that governs these processes are critical to combating muscle mass lass and improving quality of life in cancer patients. In the current study we provide evidence that the cachexia suppression of protein synthesis and acceleration of degradation occurs through AMPK. Interestingly, we also identify that AMPK regulates skeletal muscle mitochondrial dynamics in cachectic skeletal muscle. The AMPK regulation of mitochondrial quality control further establishes the kinase as a critical regulator of muscle metabolism in tumor bearing mice. In particular, the current data identifies MFN-1 as a potential

AMPK sensitive target in cachexia. Our results demonstrate a clear role for AMPK in the regulation of both skeletal muscle protein synthesis and degradation. Taken collectively, these results identify the AMPK signaling pathway as a critical checkpoint regulating muscle mass and mitochondrial quality control during cancer cachexia.

Table 2.1. Characteristics of Skeletal Muscle AMPK Loss in Wildtype and *Apc*$^{Min/+}$ Mice.

	WT	AMPK KO	Min	Min KO
Sex	Male	Male	Male	Male
Age (weeks)	16.1	15.9 ± 0.1	21.2 ± 1.0	19.0 ± 1.3 *
N	8	8	10	10
Max BW (g)	23.5 ± 0.5	23.7 ± 0.5	23.8 ± 0.4	23.1 ± 0.7
BW @ Sac	22.5 ± 0.5	22.3 ± 0.6	21.2 ± 0.8	22.7 ± 0.6
% BWL After Fast	3.0 ± 1.0	4.0 ± 0.1	9.4 ± 3.0	4.0 ± 1.0*
Sac Glucose (mg/dL)	140.7 ± 7.3	125.7 ± 7.1	112.7 ± 5.3	132.8 ± 10.7 *
Food (g/day)	3.4 ± 0.2	3.2 ± 0.1	2.9 ± 0.3	3.1 ± 0.2
IL-6 (pg/ML)	0.0 ± 0.0	0.0 ± 0.0	15.6 ± 4.6	17.7 ± 3.8
Total Polyps	0.0 ± 0.1	0.0 ± 0.1	36.3 ± 6.7	36.9 ± 10.2
Gastrocnemius (mg)	120.2 ± 3.0	118.8 ± 2.1	99.9 ± 6.9	119.8 ± 3.6 *
Gastrocnemius/BW ratio (mg/g)	5.3 ± 0.1	5.4 ± 0.1	4.6 ± 0.2	5.1 ± 0.1 *
Stomach (mg)	250.7 ± 22.7	257.6 ± 9.8	423.9 ± 38.1	283.3 ± 21.7*
Stomach/BW ratio (mg/g)	11.4 ± 1.1	11.9 ± 0.5	20.1 ± 1.8	12.7 ± 0.8 *
Liver (mg)	1007 ± 40.7	970.5 ± 26.9	1287.3 ± 56.7	1039.7 ± 104.9 *
Liver/BW ratio (mg/g)	44.5 ± 1.2	44.8 ± 0.9	60.7 ± 2.3	46.4 ± 3.1 *
Spleen (mg)	60.7 ± 2.2	83.9 ± 16.9	344 ± 39.2	150.7 ± 29.8 *
Spleen/BW ratio (mg/g)	2.7 ± 0.1	3.4 ± 0.8	16.3 ± 1.9	16.3 ± 1.10 *
Sem Vesicles (mg)	176.4 ± 11.6	155.0 ± 14.4	131.3 ± 24.0	166.0 ± 18.5
Sem Vesicles/BW ratio (mg/g)	7.8 ± 0.5	7.2 ± 0.9	5.9 ± 0.9	7.2 ± 0.9
Testes (mg)	165.8 ± 10.5	168.9± 1.9	168.7 ± 11.4	170.5 ± 3.1
Testes/BW ratio (mg/g)	7.3 ± 0.4	7.8 ± 0.1	7.8 ± 0.4	7.3 ± 0.2
Epdidymal Fat (mg	237.3 ± 30.3	237.0 ± 22.3	99.1 ± 39.3	202.3 ± 15.0 *
Epididymal Fat/BW ratio (mg/g)	8.4 ± 2.3	10.9 ± 0.9	4.2 ± 1.6	8.7± 0.8 *
Tibia Length	16.9 ± 0.1	17.1 ± 0.1	16.8 ± 0.1	16.9 ± 0.1

Values are means ± SEM. Age given in weeks (wks). Body weights given in grams (g). % BWL determined from peak weight to weight prior to sacrifice, %BWL after fast determined from peak weight to sacrifice weight. All tissue weights expressed in milligrams (mg). AMPK floxed controls (Flox). *Apc*$^{Min/+}$ mice (Min). AMPK floxed HSA Cre/+ *Apc*$^{Min/+}$ (MIN KO). *Significantly different from Flox. $p<0.05$ (students pre-planned t-test within genotype).). All mice were fasted 12 hours during the light cycle and sacrificed at 7PM.

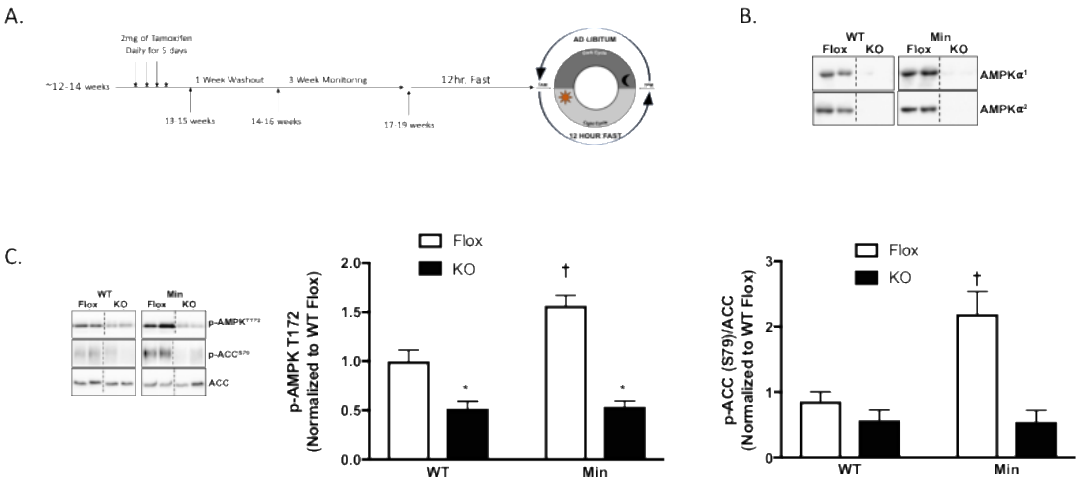

Figure 2.1. Verification of Skeletal Muscle AMPK Loss .A) Experimental design of AMPK inducible knockout and fasting paradigm. B) Representative western blot of AMPK$\alpha^{1}\alpha^{2}$ in gastrocnemius of WT and Min mice. C) Representative western blot and quantification of p-AMPK (T172), p-ACC (S79), and ACC. WT is wildtype AMPK flox control, Min is $Apc^{Min/+}$, KO is AMPK$\alpha^{1}\alpha^{2}$ knockout. † denotes different to all groups. * denotes different to WT Flox. Significance was set at $p<0.05$. Two-Way ANOVA.

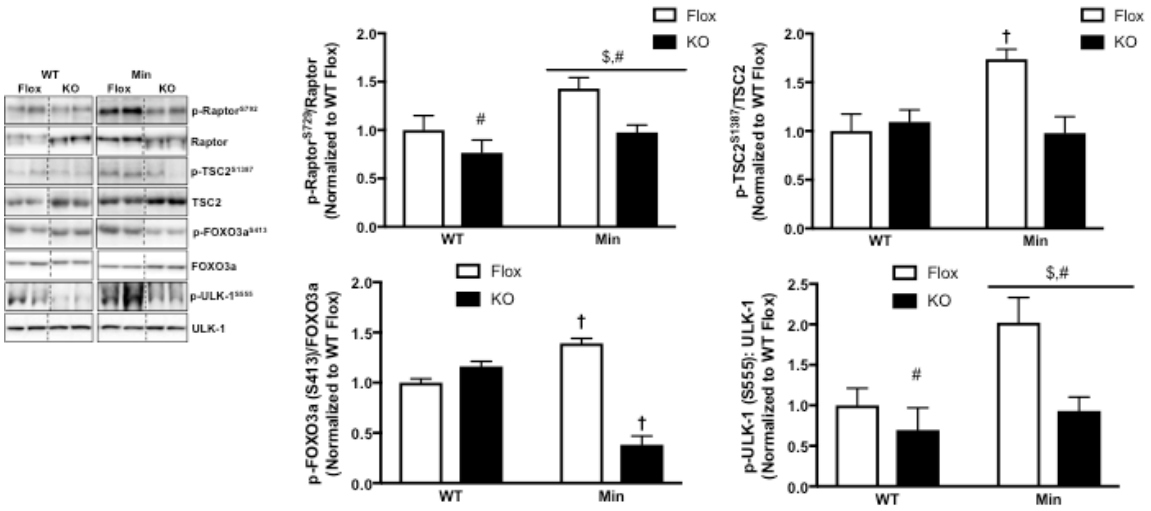

Figure 2.2. AMPK Regulation of Immediate Downstream Targets in Cachexia. Left: Representative western blots of p-Raptor (S792), Raptor, p-TSC2 (S1387), TSC2, p-FOXO3a (S413), FOXO3a, p-ULK-1 (S555), and ULK-1 in gastrocnemius of WT and Min mice. Right: Quantification of Western Blots. WT is wildtype AMPK flox control, Min is *Apc*$^{Min/+}$, Flox AMPK$\alpha^{1}\alpha^{2}$ intact controls. KO is AMPK$\alpha^{1}\alpha^{2}$ knockout. † denotes different to all groups. # denotes main effect of KO. $ denotes main effect of Min. Significance was set at $p<0.05$. Two-Way ANOVA.

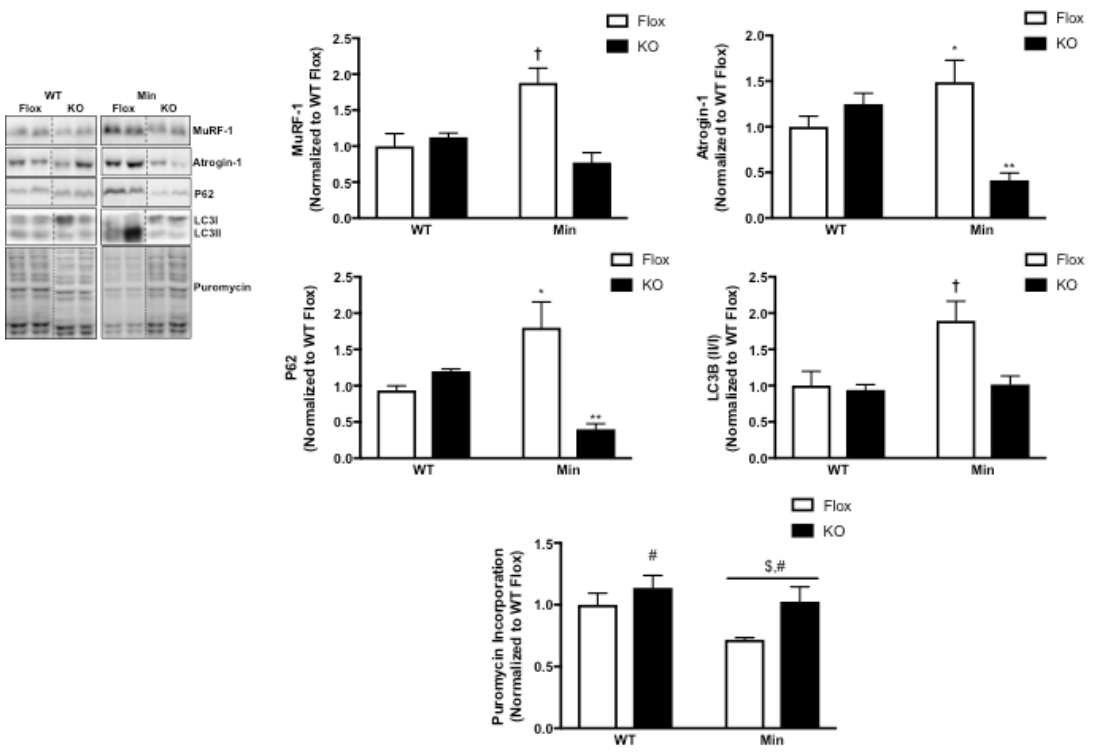

Figure 2.3. AMPK Regulation of Protein Turnover in Cachectic Skeletal Muscle. Left: Representative western blots of MuRF-1, Atrogin-1, P62, LC3 I/II, and Puromycin in gastrocnemius of WT and Min mice. Right: Quantification of Western Blots. WT is wildtype AMPK flox control, Min is *Apc*$^{Min/+}$, Flox AMPK$\alpha^1\alpha^2$ intact controls. KO is AMPK$\alpha^1\alpha^2$ knockout. † denotes different to all groups. # denotes main effect of KO. $ denotes main effect of Min. * denotes different to WT Flox. ** denotes different to Min Flox. Significance was set at $p<0.05$. Two-Way ANOVA.

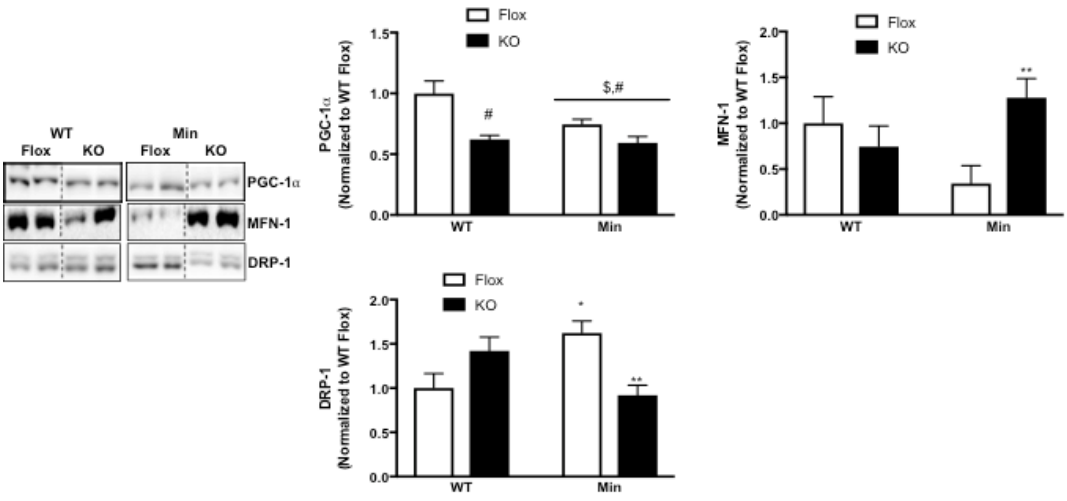

Figure 2.4. AMPK Regulation of Mitochondrial Quality Control During Cachexia. Left: Representative western blots of PGC-1α, MFN-1, and DRP-1 in the gastrocnemius of WT and Min mice. Right: Quantification of Western Blots. WT is wildtype AMPK flox control, Min is $Apc^{Min/+}$, Flox AMPK$\alpha^{1}\alpha^{2}$ intact controls. KO is AMPK$\alpha^{1}\alpha^{2}$ knockout. † denotes different to all groups. # denotes main effect of KO. $ denotes main effect of Min. * denotes different to WT Flox. ** denotes different to Min Flox. Significance was set at $p<0.05$. Two-Way ANOVA.

CHAPTER 3

CANCER CACHEXIA ACCELERATES PHYSIOLOGICAL AMPK SIGNALING BY FASTING AND FEEDING

3.1 Abstract

Cancer cachexia is a characterized by accelerated skeletal muscle protein turnover that contributes to muscle mass loss. While the basal regulation of muscle protein turnover during cancer cachexia has been widely investigated, there is far less understood about the dynamic regulation of skeletal muscle turnover with feeding, fasting, and physical activity. Skeletal muscle's physiological response to fasting involves a diverse network of metabolic pathways that serve to maintain metabolic homeostasis, and adenosine monophosphate protein kinase (AMPK) is a critical regulator and effector of this response. The cancer environment's effect on skeletal muscle fasting and feeding regulation of protein turnover warrants further investigation. Therefore, the purpose of this study was to examine the effect of the cancer environment on skeletal muscle's physiological response to fasting and feeding. Male C57BL/6 (B6, N=23) and ApcMin/+ (Min, N=25) mice were subjected to either ad libitum feeding, a 12 hour fast, or a 12 hour fast followed by a 1 ad libitum re-feed. Following the completion of each treatment, hindlimb muscles were harvested and the gastrocnemius homogenized for protein analysis. Min mice exhibited body weight loss and reduced gastrocnemius mass when compared to B6 mice. In the ad libitum fed state muscle AMPK phosphorylation (T172) was induced in the Min compared to B6. Fasting did not induce AMPK, Raptor (S792),

TSC2 (S1347), ULK-1 (S555), and FOXO3a (S416) phosphorylation in B6 mice; however, AMPK, Raptor (S792), TSC2 (S1347), ULK-1 (S555), and FOXO3a (S416) phosphorylation were induced in Min mice following a fast and were suppressed by re-feeding. PGC-1α was induced in fasted B6 mice only and was not altered by re-feeding. MFN-1 and DRP-1 were not altered by fasting, but were robustly induced by re-feeding in both B6 mice. DRP-1 was induced in fasted Min mice only and was not altered by re-feeding. Additionally, P62, LC3 II/I, Atrogin-1, and MuRF-1 protein expression were only induced in fasted Min mice and were also suppressed by re-feeding. Our results suggest that cancer increases skeletal muscle sensitivity to a short-term fast, which could serve to accelerate wasting with cancer. Furthermore, our results also demonstrate that a short-term fast sensitizes cachectic skeletal muscle to re-feeding.

3.2 Introduction

Cancer-induced cachexia is a wasting syndrome that occurs in approximately 80% of all cancer patients and results in 30% of deaths (Argiles et al., 2010; Narsale & Carson, 2014; Puppa et al., 2012). Cancer-induced cachexia is a debilitating disease that diminishes quality of life due to severe loss of skeletal muscle mass and function (Baracos, 2011, 2013; Baracos et al., 2018). There are currently no approved therapeutic approaches for cancer-induced cachexia, therefore it is necessary to elucidate key underlying factors and processes as potential therapeutic targets. Skeletal muscle wasting during cancer cachexia occurs through altered protein turnover (suppressed anabolic signaling/increased degradation) (Aversa, Pin, Lucia, Penna, Verzaro, Fazi, Colasante, Tirone, Fanelli, et al., 2016; Baracos, 2000). Our laboratory and others have demonstrated suppressed anabolic signaling through mechanistic target of rapamycin

(mTORC1) and the induction of muscle protein degradation in pre-clinical models of cancer cachexia (White, Puppa, Gao, et al., 2013). Coinciding with the suppression of mTORC1 signaling is the chronic activation of the adenosine monophosphate protein kinase (AMPK) (White, Puppa, Gao, et al., 2013). AMPK has been deemed a critical regulator of skeletal muscle degradation through the regulation E3 ligases and the process of autophagy, defined as the removal of organelles and protein aggregates through the lysosomal proteasome system (Lira et al., 2013). While altered protein turnover has been well characterized in the cachectic skeletal muscle of tumor bearing mice, less is understood about the role of AMPK in this process.

Skeletal muscle proteostasis fluctuates diurnally depending on different fasting and feeding durations as well as in response to activity and exercise stimuli. Adequate nutrient availability is key in the net loss and gain of skeletal muscle protein. AMPK is a nutrient sensitive energy sensor and is essential for skeletal muscle metabolic homeostasis (Romanello et al., 2010; Romanello & Sandri, 2015). AMPK is capable of being activated by many different stimuli as well as diseases such as cancer cachexia. During severe cancer cachexia, skeletal muscle AMPK is dysregulated and chronically activated which could have dire consequences regarding muscle proteostasis. The most widely investigated activators of skeletal muscle AMPK is the process of prolonged energy deficiency (fasting). While fasting has been proposed to have beneficial effects in regard to aging and some cancers, less is understood about its role in cancer cachexia and the regulation of muscle proteostasis. AMPK has long been established as a critical regulator of both protein synthesis and protein degradation. AMPK exerts its regulation the process of muscle protein synthesis through two inhibitory phosphorylation events.

When activated, AMPK will phosphorylate tuberous sclerosis protein 2 (TSC2) and Raptor which inhibit mTORC1 activity. Inhibition of mTORC1 activates 4EBP1 and inhibits P70S6K, thus inhibiting the initiation of translation and ribosomal proteins. The activation of degradation targets by AMPK induces both the ATP-independent autophagy lysosomal proteasome and the ATP-dependent ubiquitin proteasome respectively (FOXO3a is capable of regulating both degradation systems). The degradation of proteins through the ubiquitin proteasome is a highly coordinated process and in skeletal muscle utilizes the muscle specific E3 ligases Atrogin-1 and MuRF-1 which are controlled upstream by FOXO3a and were discovered initially through the studies examining starvation and fasting conditions. These E3 ligases allow for targeted proteins to be recognized by the 26S proteasome and ensure specificity of the UPS and additional adaptor proteins(Murton et al., 2008). The process of autophagy is induced through the AMPK activation of ULK-1 and has been extensively investigated with numerous catabolic conditions including fasting (Yan, Lira, & Greene, 2012). Autophagy is a highly conserved cellular degradation pathway that can be initiated selectively and non-selectively by sequestering cytosolic substrates within a phagophore which is then conjugated with ubiquitination-linked proteins and degraded by a lysosome (Sanchez, Csibi, et al., 2012; Vainshtein & Hood, 2015).

Conversely, a common suppressor of AMPK is nutrient stimulation through the consumption of glucose, a mixed meal (feeding), or amino acids (Koh et al., 2008; Long & Zierath, 2006). Feeding has been widely investigated as a regulator of mTORC1 and AMPK. The consumption of glucose or a mixed meal will induce a glucose and insulin response activating the IGF-1/AKT signaling cascade (Sengupta et al., 2010). AKT will

then phosphorylate TSC2 releasing its inhibition of mTORC1(Sengupta et al., 2010). Both AMPK and mTORC1 are energy sensing kinases and regulate one another through pathway cross talk. AMPK and mTORC1 negatively regulate one another, for example during energy demand AMPK is activated thereby suppressing energy consuming pathways (mTORC1) and stimulating energy generating pathways (ULK-1, FOXO3a) (Koh et al., 2008; Long & Zierath, 2006; Sanchez, Csibi, et al., 2012). Muscle mTORC1 signaling is activated with both exercise and feeding and will phosphorylate ULK-1 at Ser 757 and inhibit the ULK-1 activation of downstream autophagy signaling (Laker et al., 2017; Sanchez, Csibi, et al., 2012). To date, our lab and others have demonstrated that the cachectic environment elevates skeletal muscle AMPK following a 5 hour fast, showing that a very short period of fasting is capable of inducing AMPK in the cancer environment, suggestive of metabolic dysregulation (Penna et al., 2010; White, Baynes, et al., 2011a; White, Puppa, Gao, et al., 2013). Concomitant with the induction of AMPK, our laboratory has demonstrated mTORC1 suppression during cancer cachexia, signifying a role for AMPK in the suppression of anabolic signaling (Hardee et al., 2018; Hardee et al., 2016; White, Puppa, Gao, et al., 2013).

While the chronic activation of AMPK and its potential role in anabolic suppression has been examined previously examined during cachexia, the role of this signaling axis in the regulation of protein turnover in response to fasting and feeding is poorly understood in the skeletal muscle of cachectic mice. Therefore, the purpose of this study was to determine if cancer cachexia disrupts the physiological regulation of AMPK signaling by fasting and feeding in skeletal muscle.

3.3 Methods

Male C57BL/6 (B6) and *Apc*$^{Min/+}$ (Min) were purchased from Jackson Laboratories and were bred at the University of South Carolina's Animal Resources Facility. All animals were group housed and kept on a 12:12-h light-dark cycle. Body weights were measured weekly, and animals were monitored for signs of distress. Animals were given food and water *ad libitum* throughout the duration of the study. All animals were fasted 12 hours or were allowed ad libitum access to food prior to tissue collection. Mice were anesthetized with a ketamine-xylazine-acepromazine cocktail, and hindlimb muscles and select organs were carefully dissected and snap frozen in liquid nitrogen and stored at -80^{0}C until further analysis. All animal experiments were approved by the University of South Carolina's Institutional Animal Care and Use Committee. experimentation outline in this proposal.

Fasting and Re-Feeding

Mice were separated into either ad libitum fed (n=8 B6, N=8 Mins), 12-hour fast (n=8 B6, n=10 Mins), or 12-hour fast followed by a 1 hour refeed (n=7 B6, N=8 Mins). Ad libitum fed mice were sacrificed at 7 AM following the dark cycle to ensure that food was consumed, and they were in the fed state. 12 hour fasted mice had food removed during the 12-hour light cycle to ensure that a true fast had occurred and were sacrificed at 7 PM. The re-feed mice were fasted for 12-hours during the light cycle but were allowed 1 hour ad libitum access to a food pellet following the fast and were sacrificed at immediately upon completion of the 1 hour time limit.

Western Blotting

Western blot analysis was performed as previously described (Fix et al., 2018). Briefly, frozen gastrocnemius muscle was homogenized in ice-cold Mueller buffer and protein concentration was determined by the Bradford method. Crude muscle homogenates were fractionated on 6 – 15% SDS-polyacrylamide gels and transferred to PVDF membranes overnight. Membranes were stained with Ponceau red to verify equal loading and transfer for each gel. Membranes were blocked at room temperature for 1 – 2 hrs. in 5% Tris-buffered saline with 0.1% Tween-20 (TBST) milk. Primary antibodies for puromycin, p-FOXO3a (S416), FOXO3a, p-ULK-1 (S555) (S757), ULK-1, P62, LC3II/I, MuRF-1, Atrogin-1 (ECM Biosciences), p-AMPK (T172), AMPK, p-ACC (S79) , ACC, p-TSC2 (S1387), TSC2, p-raptor (S792), raptor, DRP-1, MFN-1 (Abcam), PGC-1α (Abcam), and GAPDH were diluted 1:5000 – 1:2000 in 5% TBST-milk followed by a 4-8-hr incubation with membranes at 4 degrees. Anti-rabbit or anti-mouse IgG horseradish-peroxidase conjugated secondary antibody was incubated with the membranes at 1:4000 dilutions for 1 hour in 5% TBST-milk at room temperature. Enhanced chemiluminescence (ECL) (GE Healthcare Life Sciences, Piscataway, NJ) was used to visualize the antibody-antigen interactions. Images were digitally scanned and quantified by densitometry using imaging software (Image J; NIH). All antibodies were from Cell Signaling unless otherwise stated.

Plasma IL-6 concentrations

Plasma IL-6 concentrations were determined as previously described (Hardee et al., 2016). A commercially available IL-6 enzyme-linked immunosorbent assay kit was obtained from BD Biosciences (San Diego, CA, USA) and the manufacturer's protocol was followed. Briefly, a Costar clear 96-well plate (Corning, NY, USA) was coated with IL-6 capture antibody and allowed to incubate overnight. The next morning, the plate was blocked with assay diluent buffer, washed, and IL-6 standards and plasma samples were added in duplicate to the plate. The plate was again washed and sAV-HRP reagent was added to each well. After several washes, TMB substrate was added and the reaction was developed for 20 minutes. The reaction was stopped with sulfuric acid and absorbance was read in a Bio-Rad iMark plate reader (Hercules, CA, USA) at 450 nm.

Statistical Analysis

Results are reported as the means ± standard error. An unpaired student's pre-planned t-test or Two-way ANOVA was employed when appropriate. Post-hoc analyses were performed with Student-Newman-Keuls methods when appropriate. The accepted level of significance was set at $p<0.05$ for all analysis. Statistical analysis was performed using Prism GraphPad 7 (GraphPad Software Inc., La Jolla, CA).

3.4 Results

Animal Characteristics Fed vs. Fast

There were no significant differences between B6 fed and fasted mice in terms of age, max bodyweight, sac glucose or circulating IL-6 (Table 3.1). Body weight at sac was lower in B6 fasted mice when compared to fed (Table 3.1). Interestingly, Min fasted

mice had lower circulating plasma IL-6 when compared to min fed mice (Table 3.1). Bodyweight loss percentage was not different between fed and fasted Min mice. Furthermore, B6 mice demonstrated decreased stomach and liver size following the fast and this finding was not present in Min mice.

AMPK Activation During Fasting

In order to examine the AMPK signaling pathway in fed and fasted B6 and Min mice we examined the phosphorylation of AMPK at T172 in the gastrocnemius (Figure 3.1A-B). We next examined if AMPK phosphorylation was altered by a short term fast in B6 and Min mice (Figure 3.1). AMPK phosphorylation was not altered by fasting in B6 mice, however Min mice demonstrated robust activation of AMPK T172 and was significantly different to all groups (Figure 3.1A-B). In order to further verify the activation of AMPK we also examined the phosphorylation of Acyl CoA Carboxylase (ACC) at S79 (Figure 3.1A-B). Interestingly, ACC was induced with fasting in B6 mice despite AMPK not being activated upstream (Figure 3.1B). Min mice also exhibited an increase in the phosphorylation of ACC in the fasted state (Figure 3.1B).

AMPK Downstream Signaling During Fasting

In order to examine if the cancer environment alters the fasting regulation of mTORC1 signaling we next examined further downstream of AMPK to determine the activation of its immediate downstream targets TSC2 (S1347) and Raptor (S792) (Figure 3.2) . Both TSC2 and Raptor are key regulators of mTORC1 activity. AMPK has been demonstrated to phosphorylate these targets and negatively regulate mTORC1 in various catabolic conditions. As expected there were no significant alterations to TSC2 or Raptor

phosphorylation in fasted B6 mice (Figure 3.2A). However, Min mice demonstrated a robust induction of TSC2 (S1347) and Raptor (S792) phosphorylation in the fasted condition (Figure 3.2B). We next examined the phosphorylation of ULK-1 (S555) and FOXO3a (S413). These proteins are responsible for the regulation of both autophagy and E3 ligases and are immediate AMPK downstream targets. The phosphorylation of ULK-1 was induced in fasted Min mice only (Figure 3.2B). However, FOXO3a was induced in both B6 and Min mice with fasting (Figure 3.2A-B). These results suggest that the cancer environment plus a short term fast is capable of inducing the AMPK activation of TSC2, Raptor, FOXO3a, and ULK-1 which serve to suppress the activation of mTORC1 signaling and increase muscle degradation.

AMPK Mediated Protein Turnover During Fasting

In order to establish that the cancer environment is altering the fasting regulation of protein turnover We next examined the protein expression of E3 ligases MuRF-1 and Atrogin-1 and autophagy proteins P62 and LC3B II/I . E3 ligase protein expression of Atrogin-1 and MuRF-1 were both induced in fasted Min mice only (Figure 3.3B). B6 mice exhibited no significant difference in the expression of these proteins in the fasted condition (Figure 3.3A). Interestingly, P62 was suppressed in fasted Min mice while LC3B II/I was induced robustly (Figure 3.3B). These results are suggestive of increased clearance of the autophagosome by the lysosome during a fast. To further examine protein turnover, we next examined puromycin incorporation using the SunSET technique as previously described (Goodman & Hornberger, 2013). A short term fast was sufficient to suppress puromycin incorporation in Min mice only which is indicative of suppressed muscle protein synthesis (Figure 3.3B).

AMPK and Mitochondrial Quality Control in the Fasted State

AMPK has recently come to light as an important regulator of mitochondrial quality control or the processes of biogenesis and dynamics (fission and fusion). We next examined if immediate downstream target PGC-1α was altered by the cancer environment and fasting (Figure 3.4 A-B). Despite a lack of AMPK activation, PGC-1α was induced in fasted B6 mice (Figure 3.4A). Interestingly, despite the elevated AMPK activation upstream, PGC-1α was not induced in fasted Min mice when compared to fed Min mice (Figure 3.4B). These results are similar with previous reports from our laboratory that have demonstrated elevated AMPK signaling with suppressed or un-altered PGC-1α expression downstream. We next examined mitochondrial fission marker DRP-1 and mitochondrial fusion marker MFN-1. DRP-1 protein expression was only induced in fasted Min mice (Figure 3.4B). MFN-1 was not altered with either fasting or genotype.

Animal Characteristics Fast vs. Re-Feed

We next examined if the cancer environment alters the response to nutrients or feeding (re-feeding). B6 re-feed mice were significantly older in age (Table 3.2). B6 re-feed mice also had smaller spleen weight when compared to their fasted counterparts (Table 3.2). Min re-feed mice exhibited increased sac glucose and decreased circulating plasma IL-6 when compared to the fasted state. Gastrocnemius, testes, and epididymal fat to bodyweight ratio were all reduced in Min re-feed mice compared to fasted.

AMPK Activation During Re-Feeding

In order to examine the AMPK signaling pathway in fasted and re-feed B6 and Min mice we examined the phosphorylation of AMPK at T172 in the gastrocnemius. AMPK T172 phosphorylation was not altered by re-feeding in B6 mice when compared with the fasted state (Figure 3.5A). However, Min mice demonstrated robust suppression of AMPK T172 and when compared with the fasted state (Figure 3.5B). In order to further verify the suppression of AMPK we next examined the phosphorylation of Acyl CoA Carboxylase (ACC) at S79. As expected, ACC was not altered in B6 re-feed mice. In line with our AMPK phosphorylation Min re-feed mice also exhibited a suppression of ACC phosphorylation compared to the fasted state (Figure 3.5B).

AMPK Regulation of Immediate Downstream Signaling During Fasting and Re-Feeding

Keeping in line with our fasting analysis targets, we moved to examine immediate downstream signaling targets of AMPK. The phosphorylation of Raptor was no affected by re-feeding in B6 mice when compared to fasting (Figure 3.6A). However, Raptor phosphorylation was suppressed by re-feeding in Min mice when compared to the fasted condition (Figure 3.6B). Despite no alteration to Raptor phosphorylation in B6 mice, TSC2 phosphorylation was suppressed by re-feeding compared to the fasted state in both B6 and Min mice when compared to the fasted state (Figure 3.6A-B). In order to determine muscle protein turnover, we examined the same markers as utilized above in the fasting condition. Phosphorylation of immediate AMPK downstream targets FOXO3a and ULK-1 demonstrated a suppression following re-feeding in Min mice only as the B6 remained un-altered when compared to fasting (Figure 3.6A-B).

AMPK Mediated Protein Turnover During Fasting and Re-Feeding

We next examined indices of protein turnover during re-feeing. Protein expression of muscle specific E3 ligases Atrogin-1 and MuRF-1 were both suppressed by re-feeding in B6 mice when compared to the fasted state (Figure 3.7A). Only MuRF-1 was suppressed in re-feed Min mice when compared to fasting (Figure 3.7B). We also examined autophagy proteins P62 and LC3B (II/I). P62 was suppressed in both B6 and Min mice by re-feeding compared to fasting (Figure 3.7B). Interestingly, despite a reduction of P62 by re-feeding in both genotypes, LC3 II/I was only suppressed in Min mice which suggest an attenuation of autophagy initiation and clearance when compared to the fasted condition (Figure 3.7B). In order to examine if the changes in Raptor and TSC2 are reflected downstream by muscle protein synthesis we next examined puromycin incorporation. Re-feeding induced puromycin incorporation in both B6 and Min mice when compared to the fasted state (Figure 3.7B). Collectively these results demonstrate that fasting during cancer cachexia sensitizes skeletal muscle to re-feeding.

AMPK and Mitochondrial Quality Control During Re-Feeding

We next examined if immediate downstream target PGC-1α was altered by the cancer environment and re-feeding. PGC-1α was not altered by re-feeding in B6 or Min mice when compared with fasting (Figure 3.8A-B). We next examined mitochondrial fission marker DRP-1 and mitochondrial fusion marker MFN-1. DRP-1 protein expression was induced B6 re-feed mice and was not altered by re-feeding in Min mice (Figure 3.8A-B). Shockingly, MFN-1 was robustly induced by re-feeing in both B6 and Min mice when compared to the fasted state (Figure 3.8A-B).

3.5 Discussion

Cancer cachexia is a debilitating disease that is associated with poor response to cancer treatment and increased mortality (Baracos, 2006a; Baracos et al., 2018). The loss of skeletal muscle mass can have dire consequence on metabolic health and quality of life (Bachmann et al., 2008; Baracos, 2018). Often associated with cachexia are changes in appetite and nutrient status (Ali, Chen, & Garcia, 2013; Ali & Garcia, 2014). Recent evidence has suggested that short term fasting is capable of improving the response to chemotherapy and other therapeutics (Brandhorst & Longo, 2016). However, the majority of these studies have only investigated fasting in non-cachectic cancer patients and even less has been done to examine if fasting and the cancer environment alters the response to feeding. The AMPK signaling pathway has been well established as a potent metabolic regulator and controller of muscle mass (Kemp et al., 2007; Koh et al., 2008; Mihaylova & Shaw, 2011; White, Puppa, Gao, et al., 2013). AMPK is critical for maintaining energy status and metabolic homeostasis, in times of energy deficiency or stress (fasting) AMPK will activate energy generating pathways such as the FOXO3a controlled E3 ligases or the ULK-1 mediated autophagy lysosomal system (Bujak et al., 2015; Mihaylova & Shaw, 2011; Sanchez, Csibi, et al., 2012). Furthermore, AMPK will suppress the metabolically expensive process of protein synthesis through the phosphorylation of TSC2 and Raptor (Corradetti et al., 2004; Gwinn et al., 2008; Shaw, 2009). This signaling axis is critical to maintain metabolic homeostasis and disruption of these normal physiological signaling events can drastically influence muscle mass and metabolism(Kemp et al., 2007; Koh et al., 2008; Mihaylova & Shaw, 2011; Mounier et al., 2015; K. Nakashima & Yakabe, 2007). Our laboratory and others have previously

established that AMPK is chronically induced in severe cachexia and this induction may be responsible for the suppression of mTORC1 activity and muscle protein synthesis (White, Baynes, et al., 2011b; White, Puppa, Gao, et al., 2013). However, many of these earlier studies failed to consider fasting or feeding and the influence of these stimuli on the AMPK signaling pathway in cachectic skeletal muscle. In line with previous literature from our laboratory, we report that AMPK is elevated in cachectic skeletal muscle (Hardee et al., 2018; Hardee et al., 2016; White, Baynes, et al., 2011b; White, Puppa, Gao, et al., 2013). However, we report the novel finding that fasting accelerates the activation of AMPK and its downstream targets controlling muscle protein turnover. Furthermore, we also report that a short term fast followed by re-feeding is capable of suppressing the fasting induced activation of AMPK signaling and restoring protein turnover to basal levels in cachectic skeletal muscle. Collectively we demonstrate that the cancer environment accelerates the fasting and feeding regulation of the AMPK signaling pathway and protein turnover.

The chronic activation of AMPK during severe cancer cachexia is not a new phenomenon and has been reported by many research groups (Gould, Lahart, Carmichael, Koutedakis, & Metsios, 2013; Pigna et al., 2016b; Sandri, 2016; White, Puppa, Gao, et al., 2013). However, the underlying cause of this activation remains un-elucidated. Some evidence has suggested a role for IL-6 driven inflammation inducing AMPK, thus activating the signaling cascade downstream and suppressing muscle protein synthesis through the negative regulation of mTOR(Haddad, Zaldivar, Cooper, & Adams, 2005; Hardee et al., 2018; Hardee et al., 2016; Puppa et al., 2012; White, Baynes, et al., 2011b; White, Puppa, Gao, et al., 2013). However, a common activator of AMPK is energy

deficiency, or a lack of nutrients commonly referred to as fasting, the fasting activation of AMPK in normal physiological conditions typically does not occur in rodent skeletal muscle until anywhere from 24-48 hours of continual fasting has been achieved (Bujak et al., 2015; Canto et al., 2010; Galic et al., 2018). Furthermore, with this activation of AMPK, it has been recently demonstrated that the downstream activation of ACC is required for fatty acid metabolism to maintain metabolic homeostasis (Galic et al., 2018). In the current study, AMPK and ACC were elevated in fed Min mice when compared to fed B6 mice. However, a short term fast of only 12 hours was sufficient to induce AMPK and ACC phosphorylation even further in Min mice only when compared to their fed state counterparts. The activation of AMPK in B6 mice remained un-altered following a 12 hour fast as expected. Interestingly, ACC was actually induced in fasted B6 mice when compared to fasting and is suggestive of metabolic substrate transition to fatty acids. Furthermore, the fasting activation of AMPK and ACC phosphorylation was blocked by a 1 hour re-feeding period Min mice only. These results suggest that the cancer environment accelerates the physiological regulation of AMPK by fasting. However, our results also demonstrate that the acceleration of AMPK by fasting sensitizes skeletal muscle to re-feeding, suggesting a hyper-metabolic phenotype during cancer cachexia.

The AMPK regulation of mTORC1 has been well established and studied in various atrophy and disease models (Gwinn et al., 2008; J. Kim et al., 2011; Mounier et al., 2009; Sandri, 2016; Thomson et al., 2008). The induction of AMPK by stressors such as disease and acute exercise serve to induce the phosphorylation of TSC2 and Raptor (Corradetti et al., 2004; Gwinn et al., 2008; Thomson, 2018). During energy

deficiency, AMPK will phosphorylate TSC2 and inhibit rheb activity which suppresses mTORC1 activation (Hardee et al., 2017; Inoki et al., 2003). However, recent evidence has demonstrated that TSC2 deficient cells are still responsive to energy stress thus suggesting that another signaling molecule is required for the AMPK suppression of mTORC1 during energy deficiency(Gwinn et al., 2008). In line with this finding, previous research has demonstrated that the phosphorylation of Raptor by AMPK is required for the inhibition of mTORC1 and cell cycle arrest that is induced by energy stress (Gwinn et al., 2008). Our laboratory has previously demonstrated that Raptor phosphorylation by AMPK is induced during severe cachexia and may also be an IL-6 sensitive target (White, Puppa, Gao, et al., 2013). In the current study TSC2 phosphorylation by AMPK was induced by fasting in both B6 and Min mice. Additionally, this fasting induction of TSC2 was inhibited by re-feeding. In line with previous reports suggesting that Raptor phosphorylation is required for AMPK suppression of mTOR we demonstrate that only fasted Min mice exhibited an increase in Raptor. Further establishing the importance of this event, only Min mice demonstrated a suppression of puromycin incorporation following a short term fast which is indicative of suppressed protein synthesis. This suggest that the cancer environment accelerates the AMPK suppression of mTORC1 following a short term fast and that the phosphorylation of Raptor is a key event in this process. However, despite the suppression of TSC2 and Raptor by re-feeding, there was no significant alteration to puromycin incorporation. We recognize that a limitation of our analysis is reflected by the inability of re-feeding to induce protein synthesis. This may potentially be explained as an issue of timing following the re-feeding paradigm as the mice were only allowed access to food for a

duration of 1 hour before collection. Furthermore, it is also possible that these mice may be anabolic resistant or incapable of stimulating protein synthesis in response to a meal. The concept of anabolic resistance has become a very interesting field of investigation during cancer cachexia.

The regulation of protein degradation is a tightly controlled process that involves multiple mechanisms of action (Baracos, 2000; Dohm et al., 1980; Johns et al., 2012; Komatsu & Ichimura, 2010; Sandri, 2016; Sandri et al., 2013; White, Baynes, et al., 2011b; Whitehouse et al., 2001). AMPK has been demonstrated to exert regulation over two key downstream targets that regulate both the E3 ligases and the autophagy lysosomal system (Bujak et al., 2015; K. Nakashima & Yakabe, 2007; Sanchez, Csibi, et al., 2012; Thomson, 2018). Expression of atrophy related genes Atrogin-1 and MuRF-1 are regulated through the forkhead box O (FOXO) family of transcription factors, in particular FOXO3a (Greer et al., 2007). Catabolic stimuli such as fasting, inflammation, cachexia, and oxidative stress increase MuRF-1 and Atrogin-1 in skeletal muscle (Sandri, 2016; White, Baynes, et al., 2011b; Whitehouse et al., 2001; A. Williams, Sun, Fischer, & Hasselgren, 1999). AMPK stimulates FOXO activity, activation of AMPK using AICAR in-vivo and in-vitro has been demonstrated to induce both FOXO1 and FOXO3a expression which induces both Atrogin-1 and MuRF-1 gene and protein expression(Suwa et al., 2003; Tong, Yan, Zhu, & Du, 2009). Recent evidence has demonstrated that AMPK will phosphorylate FOXO3a at serine 413 inducing its activity(Sanchez, Csibi, et al., 2012). However, it should be noted that this event does not necessarily affect the localization of FOXO to the nucleus but rather its transcriptional activity(Sanchez, Candau, et al., 2012). In the current study fasting induced FOXO3a phosphorylation by

AMPK in B6 and Min mice (Jorgensen et al., 2005). Interestingly, Atrogin-1 and MuRF-1 were only induced in fasted Min mice. These results suggest that despite AMPK phosphorylating FOXO3a in B6 mice, FOXO3a was only in the nucleus actively transcribing these genes in Min mice only. Re-feeding was sufficient to suppress the fasting induction FOXO3a in B6 and Min mice however, only MuRF-1 in was suppressed in Min mice suggesting that this may be a more sensitive target to feeding stimuli. To our surprise, both Atrogin-1 and MuRF-1 were suppressed in B6 re-feed mice when compared to the fasted condition and could be attributed to an acute increased sensitivity to nutrient stimuli following a fast.

Another target controlled by AMPK that regulates protein degradation is Unc-51 like kinase 1 (ULK-1) (Bujak et al., 2015; Zhao & Klionsky, 2011). ULK-1 is crucial for the initiation of autophagy or the selective degradation of damaged and dysfunctional proteins. ULK-1 is capable of being controlled by both mTORC1 (during anabolic stimuli such as feeding) and AMPK (during catabolic stimuli such as fasting) (J. Kim et al., 2011). Phosphorylation of ULK-1 by AMPK at serine 555 during catabolic conditions will initiate the activation of downstream autophagy related signaling such as LC3 and P62 (Laker et al., 2017; Sanchez, Csibi, et al., 2012; Zhao & Klionsky, 2011). Under energy abundance, mTORC1 will phosphorylate ULK-1 at serine 757 and suppress the initiation of autophagy signaling and formation of the autophagosome (J. Kim et al., 2011). Interestingly, autophagy has become a recent target of cancer cachexia research has been demonstrated to be elevated in both human and pre-clinical rodent models of cachexia although the exact mechanism behind this activation remains largely unknown (Penna et al., 2014; Pettersen et al., 2017; Pigna et al., 2016b). It is reasonable

to suggest that the chronic activation of AMPK often reported in various pre-clinical models of cachexia is likely responsible for the activation of autophagy, however many studies fail to report the duration of fasting or feeding in their animal models and this could directly affect these signaling events. In the current study fasting robustly induced ULK-1 phosphorylation by AMPK in Min mice. Furthermore, we report that P62 was suppressed by fasting and LC3B was induced in Min mice which is suggestive of increased clearance of the autophagosome by the lysosome. Re-feeding however induced the mTORC1 phosphorylation of serine 757 on ULK-1 which is indicative of a suppression of autophagy initiation. Indeed, we noted a strong reduction in both P62 and LC3B (II/I ratio) in the Min re-feed mice when compared to fasting. P62 was also reduced in B6 re-feed mice, however no significant alteration occurred in LC3B II/I.

Collectively these results demonstrate that the cancer environment appears to disrupt the physiological regulation of AMPK signaling in response to fasting and re-feeding stimuli. Min mice exhibit a heightened sensitivity to a short term fast that is not present in their wild type B6 counterparts. This increase in AMPK activity and downstream signaling may contribute to the acceleration of body and muscle mass loss. Furthermore, we demonstrate that the increased sensitivity to fasting is also accompanied by an accelerated response to feeding. The fasting induction of AMPK was suppressed by re-feeding attenuating the suppression of protein synthesis and the acceleration of degradation. While our fasting and re-feeding paradigm was short in duration we note that the hyper-metabolism present in Min mice appears highly sensitive to these stimuli.

Table 3.1. Animal Characteristics of Fed and Fasted B6 and Min Mice.

	B6		Min	
	Ad Libitum Fed	Fast	Ad Libitum Fed	Fast
Sex	Male	Male	Male	Male
Age (weeks)	12 ± 0.0	12 ± 0.1	19 ± 0.4	21.2 ± 1.0
N	8	8	8	10
Peak BW (g)	25.1 ± 0.3	23.8 ± 0.5	23.2 ±0.7	23.9 ± 0.4
BW (g)	25.2 ± 0.2	23.4 ± 0.5	21.8 ±0.5	23.2 ± 0.4
Pre Fast BW (g)	-	23.5 ± 0.5	-	23.1 ± 0.4
BW @ Sac (g)	25.3 ± 0.3	23.6 ± 0.4*	20.6 ± 0.8	21.2 ± 0.8
BW Change % (BW-Peak BW/Peak BW)	0.0 ± 0.0	2.0 ± 0.5	5.7 ± 0.01	3.7 ± 0.01
Fasting BW Change % (Pre Fast BW-BW @ Sac/Pre Fast BW)	-	1.0 ± 0.01	-	9.8 ± 0.02
Sac Glucose (mg/dL)	122.3 ± 5.2	128.4 ± 5.2	120.1 ± 5.0	112.7 ± 5.3
IL-6 (pg/ml)	15.1 ± 15.0	2.9 ± 3.0	41.7 ± 32.4	15.6 ± 4.6
Total Polyps	0.0 ± 0.0	0.0 ± 0.0	60.0 ± 9.0	36.3 ± 6.7*
Gastrocnemius (mg)	122.6 ± 1.7	121.5 ± 1.7	89.1 ± 5.7	99.9 ± 6.9
Stomach (mg)	557.5 ± 43.5	258.7 ± 22.7*	555.5 ± 69.1	423.9 ± 38.1
Liver (mg)	1156.4 ± 45.0	1043 ± 13.6*	1454.1 ± 149.1	1287.3 ± 56.7
Spleen (mg)	158.8 ± 23.1	142.2 ± 14.7	386.5 ± 24.7	344 ± 39.2
Sem Vesicles (mg)	189.5 ± 13.5	209.2 ± 9.1	85 ± 17.5	131.3 ± 24.0
Testes (mg)	187.8 ± 4.3	184.6 ± 6.4	142.7 ± 20.2	168.7 ± 11.4
Epdidymal Fat (mg	277.3 ± 21.4	290.4 ± 26.3	49.3 ± 24.6	99.1 ± 39.3
Tibia Length	16.8 ± 0.1	16.7 ± 0.1	16.8 ± 0.1	16.8 ± 0.1

Min Fast group are the same Min mice from specific Aim 1. Values are means ± SEM. Age given in weeks (wks). Body weights given in grams (g). BW Change is determined from BW-Peak BW/Peak BW, Fasting BW Change determined from Pre-Fast BW-BW @ Sac/Pre-Fast BW. All tissue weights expressed in milligrams (mg). Ad libitum Fed denotes ad libitum food access during the dark cycle and sacrificed at 7AM. Fast denotes 12-hour fast during the 12-hour light cycle and sacrificed at 7PM. Min denotes *Apc*$^{Min/+}$ mice . B6 denotes C57BL/6 mice *Significantly different from Fed state within genotype. $p<0.05$ (students pre-planned t-test within genotype).

Table 3.2. Animal Characteristics of Fasted and Re-Feed Mice.

	B6		Min	
	Fast	Re-Feed	Fast	Re-Feed
Sex	Male	Male	Male	Male
Age (weeks)	12.0 ± 0.0	19.6 ± 1.5*	21.2 ± 1.0	20.0 ± 0.5
N	8	7	10	8
Peak BW (g)	23.8 ± 0.5	25.0 ± 0.8*	23.9 ± 0.4	23.6 ± 0.2
BW (g)	23.4 ± 0.5	24.4 ± 0.7	23.2 ± 0.4	20.5 ± 0.3*
Pre Fast BW (g)	23.5 ± 0.5	24.5 ± 0.7	23.1 ± 0.4	20.4 ± 1.1*
BW @ Sac (g)	23.6 ± 0.4	24.2 ± 0.6	21.2 ± 0.8	20.6 ± 1.1
BW Change % (BW-Peak BW/Peak BW)	2.0 ± 0.5	1.0 ± 0.01	3.7 ± 0.01	8.0 ± 0.3*
Fasting BW Change % (Pre Fast BW-BW @ Sac/Pre Fast BW)	1.0 ± 0.01	-	9.8 ± 0.02	-
Sac Glucose (mg/dL)	128.4 ± 5.2	126.0 ± 6.2	112.7 ± 5.3	143.7 ± 3.4*
IL-6 (pg/ml)	2.9 ± 3.0	0.0 ± 0.0	15.6 ± 4.6	13.7 ± 3.7
Total Polyps	0.0 ± 0.0	0.0 ± 0.0	36.3 ± 6.7	25.8 ± 11.6
Gastrocnemius (mg)	121.5 ± 1.7	119.0 ± 4.2	99.9 ± 6.9	84.4 ± 8.3
Food Consumed During 1hr Re-Feed	-	0.5 ± 0.3	-	0.7 ± 0.5
Stomach (mg)	258.7 ± 22.7	324.3 ± 27.5	423.9 ± 38.1	444.0 ± 61.0
Liver (mg)	1043 ± 13.6	1143 ± 49.9	1287.3 ± 56.7	1332.6 ± 78.4
Spleen (mg)	142.2 ± 14.7	69.4 ± 1.2*	344 ± 39.2	425.6 ± 42.3
Sem Vesicles (mg)	209.2 ± 9.1	230.4 ± 17.5	131.3 ± 24.0	100.5 ± 26.5
Testes (mg)	184.63 ± 6.4	193.1 ± 6.3	168.7 ± 11.4	127.4 ± 18.6*
Epdidymal Fat (mg	290.4 ± 26.3	323.4 ± 24.2	99.1 ± 39.3	88.3 ± 58.9
Tibia Length	16.7 ± 0.1	16.9 ± 0.1	16.8 ± 0.1	16.6 ± 0.1*

B6 Fast and Min Fast groups are the same mice as depicted in table 3.1. Values are means ± SEM. Age given in weeks (wks). Body weights given in grams (g). BW Change is determined from BW-Peak BW/Peak BW, Fasting BW Change determined from Pre-Fast BW-BW @ Sac/Pre-Fast BW. All tissue weights expressed in milligrams (mg). Fast denotes 12-hour fast during the 12-hour light cycle and sacrificed at 7PM. Re-Feed denotes mice fasted for 12 hour fast during the 12-hour light cycle and allowed access to a food pellet for 1 hour and sacrificed at 8PM. Min denotes $Apc^{Min/+}$ mice. B6 denotes C57BL/6 mice *Significantly different from Fed state within genotype. $p<0.05$ (students pre-planned t-test within genotypee).

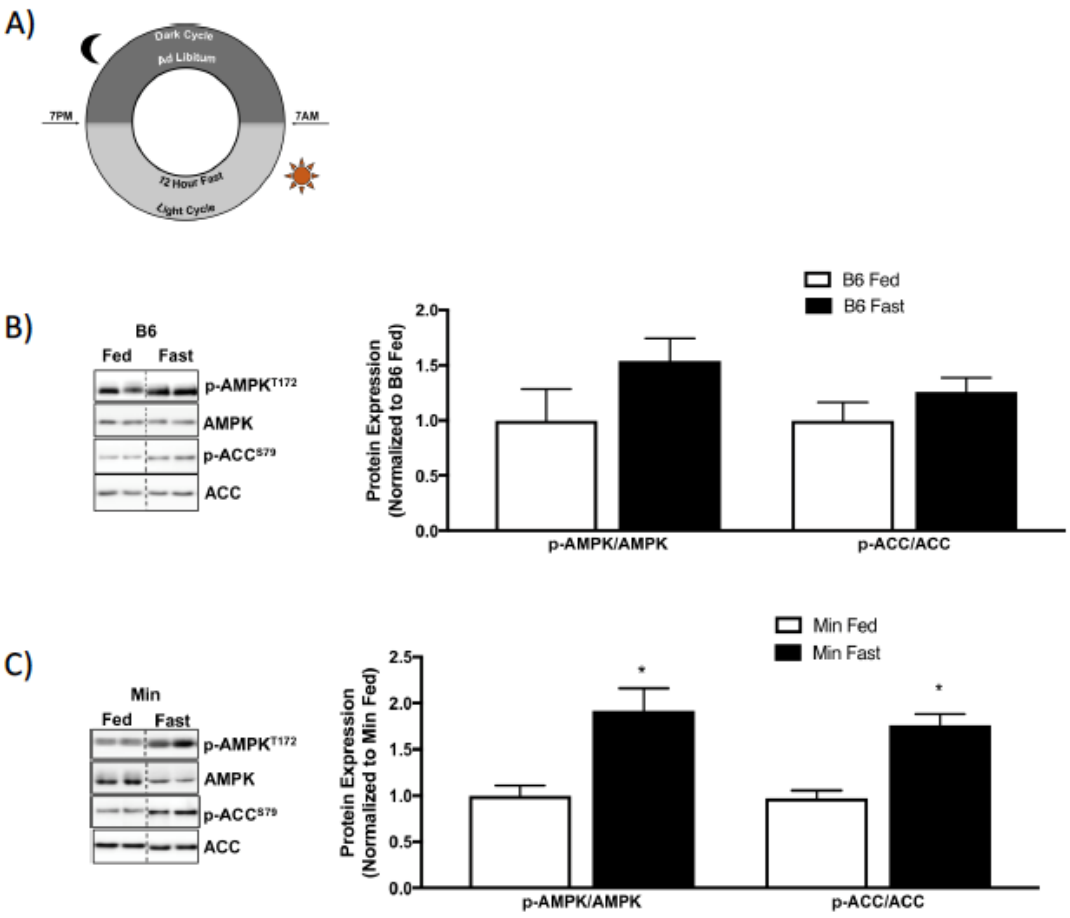

Figure 3.1. AMPK Activation During Short Term Fasting In Cachectic Muscle. A) Experimental design figure depicting 12-hour light cycle fasting protocol. B) Representative western blots of p-AMPK (T172), p-ACC (S79), ACC, and quantification of blots in B6 Fed and Fasted gastrocnemius muscle. C) Representative western blots of p-AMPK (T172), p-ACC (S79), ACC, and quantification of blots in Min Fed and Fasted gastrocnemius muscle. * denotes different to Fed state. Significance was set at $p<0.05$. Students pre-planned t-test.

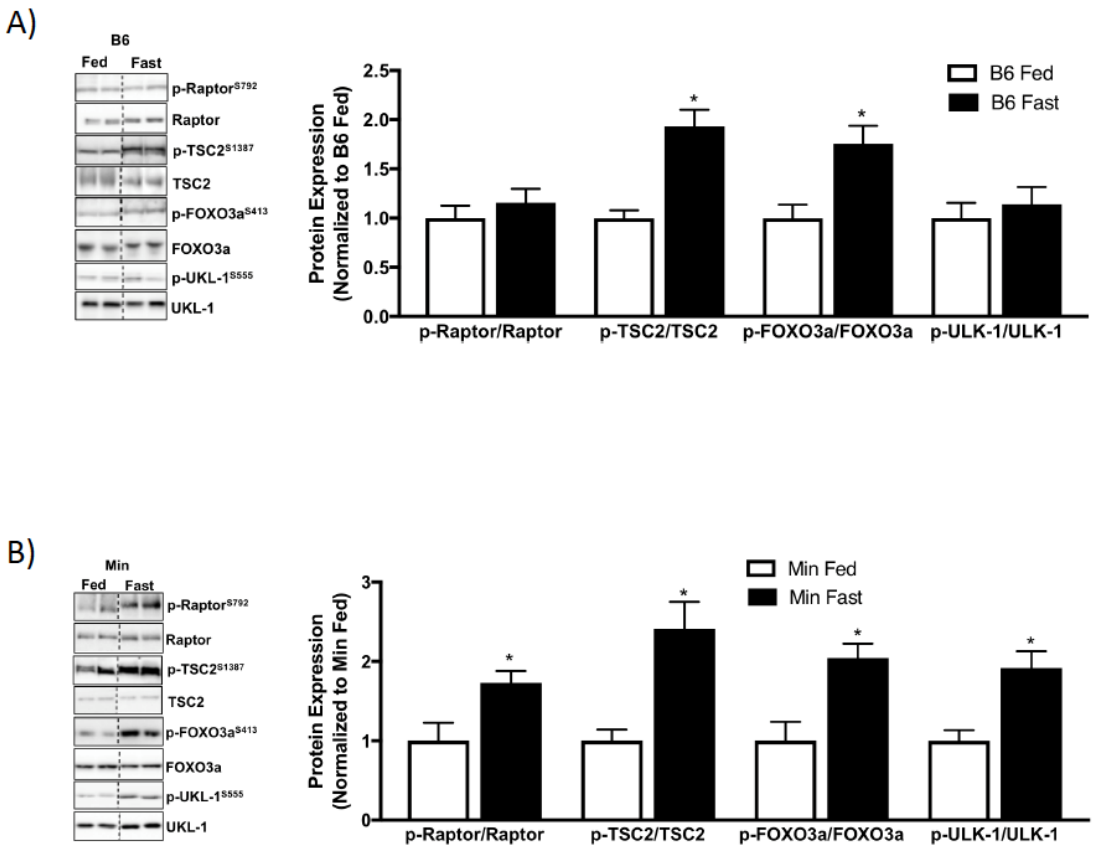

Figure 3.2. AMPK Immediate Downstream Targets in Fasted Cachectic Skeletal Muscle. A) Representative western blots of p-Raptor (S792), Raptor, p-TSC2 (S1387), TSC2, p-FOXO3a (S413), FOXO3a, p-ULK-1 (S555), and ULK-1and quantification of blots in gastrocnemius of in B6 Fed and Fasted gastrocnemius muscle. B) Representative western blots of p-Raptor (S792), Raptor, p-TSC2 (S1387), TSC2, p-FOXO3a (S413), FOXO3a, p-ULK-1 (S555), and ULK-1and quantification of blots in gastrocnemius of and quantification of blots in Min Fed and Fasted gastrocnemius muscle. * denotes different to Fed state. Significance was set at $p<0.05$. Students pre-planned t-test.

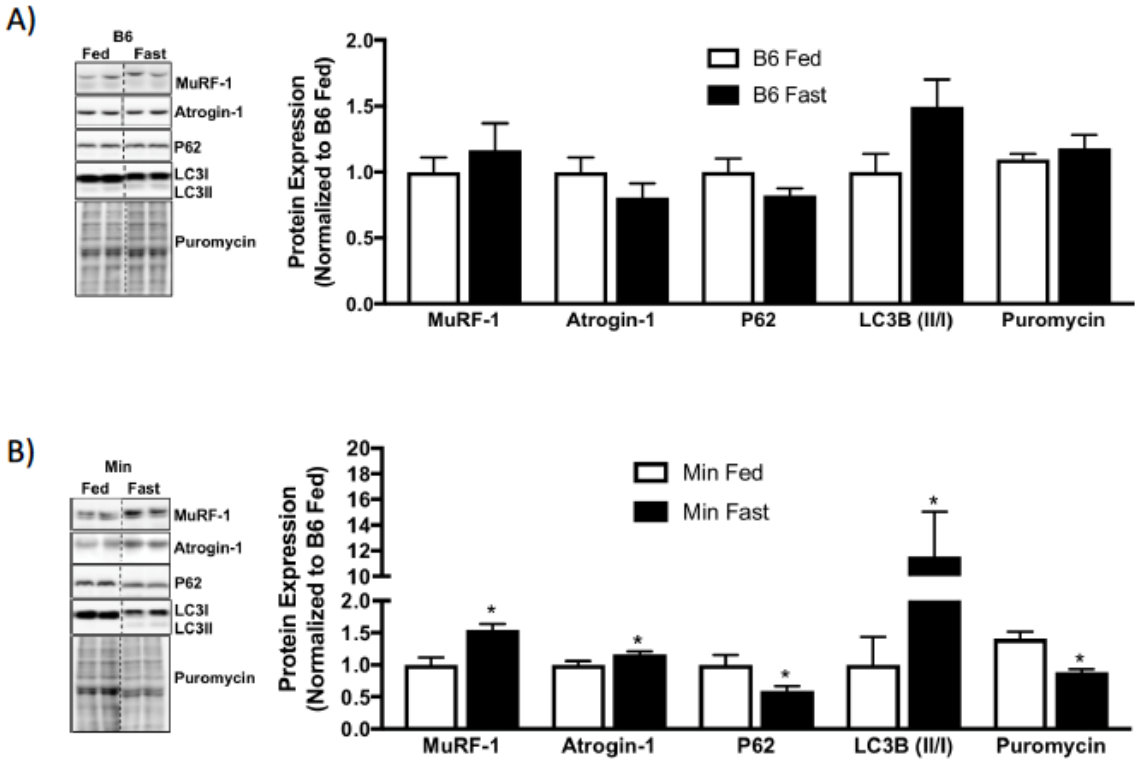

Figure 3.3. Protein Turnover in Fasted Cachectic Skeletal Muscle. A) Representative western blots of MuRF-1, Atrogin-1, P62, LC3 I/II, Puromycin and quantification of blots in gastrocnemius of in B6 Fed and Fasted gastrocnemius muscle. B) Representative western blots of MuRF-1, Atrogin-1, P62, LC3 I/II, Puromycin and quantification of blots in gastrocnemius of and quantification of blots in Min Fed and Fasted gastrocnemius muscle.* denotes different to Fed state. Significance was set at $p<0.05$. Students pre-planned t-test.

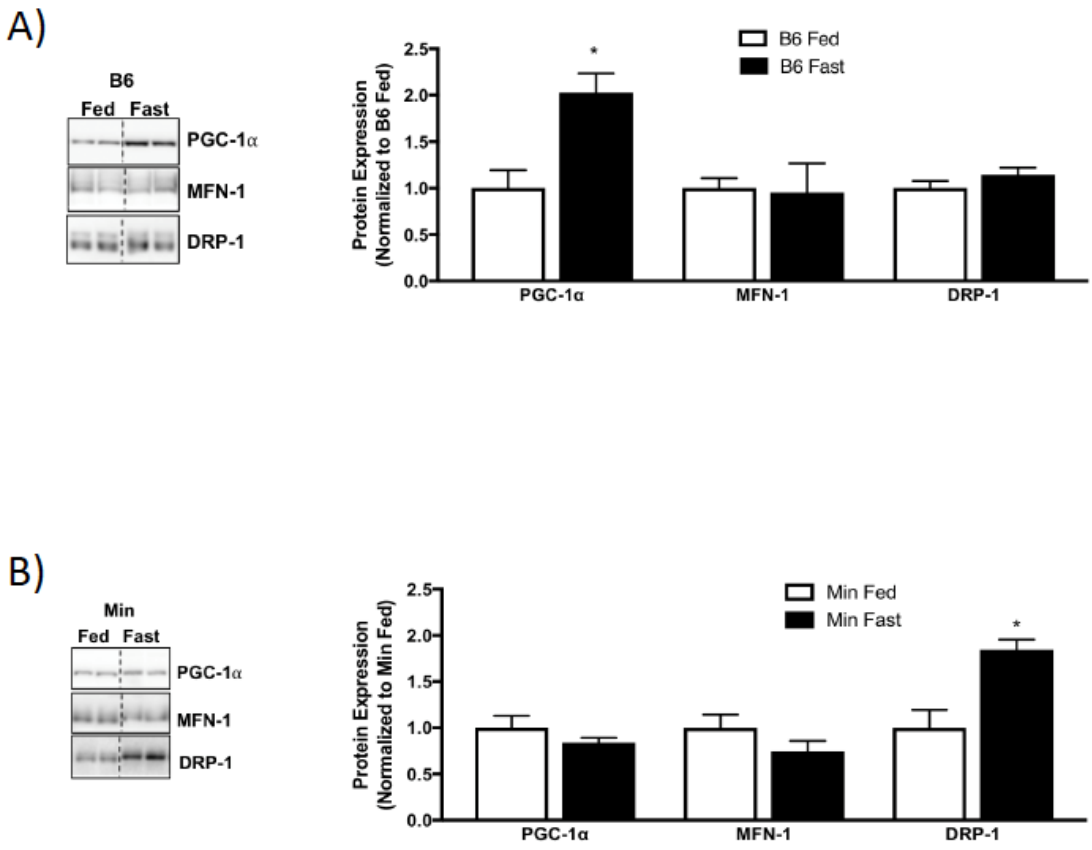

Figure 3.4. Mitochondrial Quality Control in Fed vs. Fasted Cachectic Skeletal Muscle. A) Representative western blots of PGC-1α, MFN-1, DRP-1 and quantification of blots in gastrocnemius of in B6 Fed and Fasted gastrocnemius muscle. B) Representative western blots of PGC-1α, MFN-1, DRP-1 and quantification of blots in gastrocnemius of and quantification of blots in Min Fed and Fasted gastrocnemius muscle.* denotes different to Fed state. Significance was set at $p<0.05$. Students pre-planned t-test.

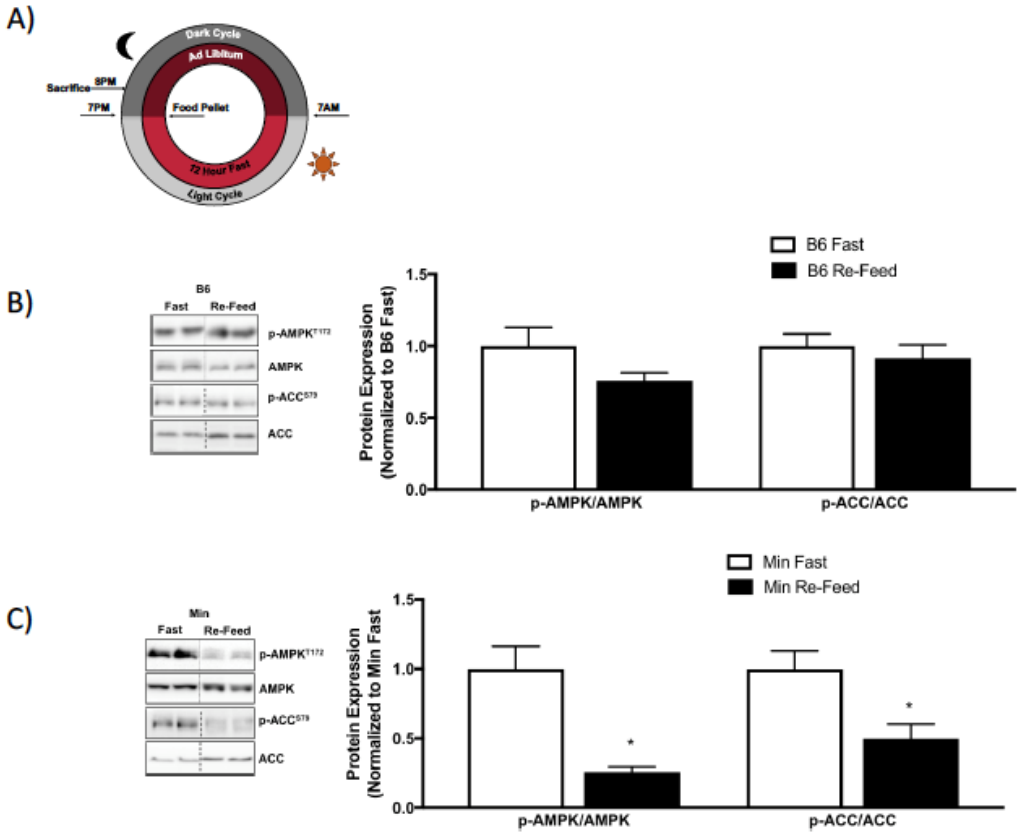

Figure 3.5. AMPK Activation During Re-Feeding. A) Experimental design figure depicting 12-hour light cycle fasting protocol and 1-hour re-feeding paradigm. B) Representative western blots of p-AMPK (T172), p-ACC (S79), ACC, and quantification of blots in B6 Fasted and Re-Feed gastrocnemius muscle. C) Representative western blots of p-AMPK (T172), p-ACC (S79), ACC, and quantification of blots in Min Fasted and Re-Feed gastrocnemius muscle. * denotes different to Fasted state. Significance was set at $p<0.05$. Students pre-planned t-test.

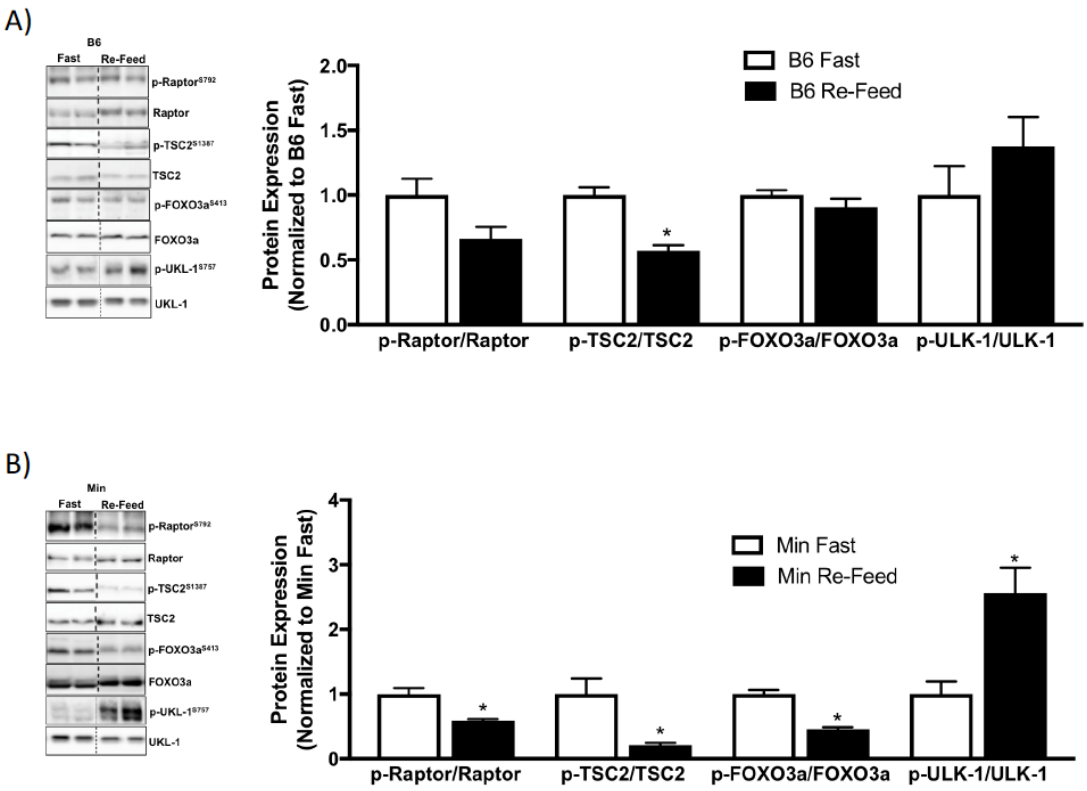

Figure 3.6. AMPK Downstream Signaling in Fasted and Re-Fed Cachectic Skeletal Muscle. A) Representative western blots of p-Raptor (S792), Raptor, p-TSC2 (S1387), TSC2, p-FOXO3a (S413), FOXO3a, p-ULK-1 (S555), ULK-1, and quantification of blots in B6 Fasted and Re-Feed gastrocnemius muscle. C) Representative western blots of p-Raptor (S792), Raptor, p-TSC2 (S1387), TSC2, p-FOXO3a (S413), FOXO3a, p-ULK-1 (S555), ULK-1, and quantification of blots in Min Fasted and Re-Feed gastrocnemius muscle. * denotes different to Fasted state. Significance was set at $p<0.05$. Students pre-planned t-test.

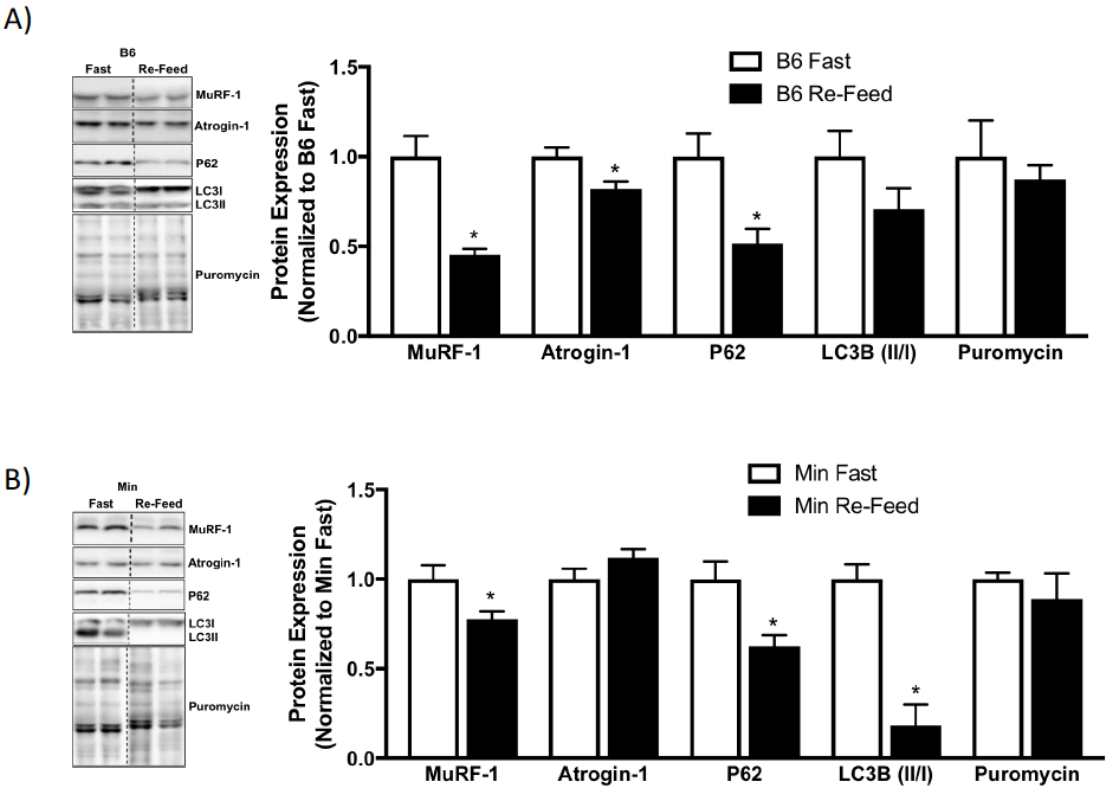

Figure 3.7. Protein Turnover in Fasted and Re-Fed Cachectic Skeletal Muscle. A) Representative western blots of MuRF-1, Atrogin-1, P62, LC3 I/II, Puromycin, and quantification of blots in B6 Fasted and Re-Feed gastrocnemius muscle. C) Representative western blots of MuRF-1, Atrogin-1, P62, LC3 I/II, Puromycin, and quantification of blots in Min Fasted and Re-Feed gastrocnemius muscle. * denotes different to Fasted state. Significance was set at $p<0.05$. Students pre-planned t-test.

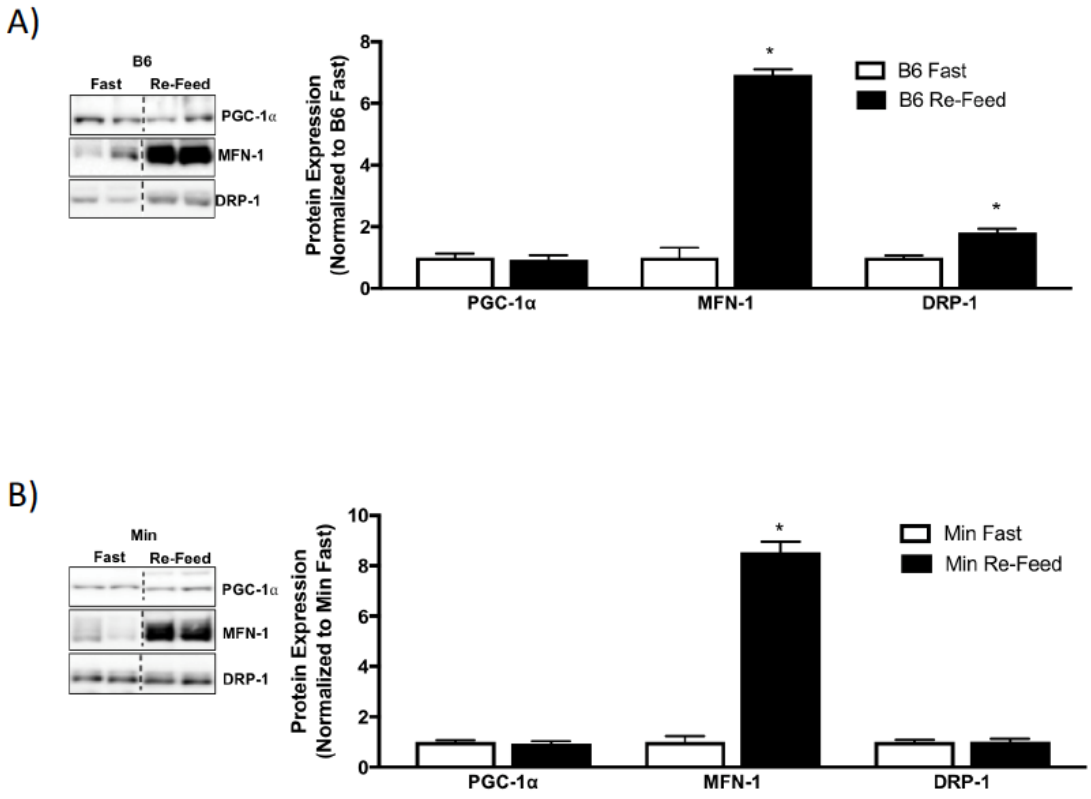

Figure 3.8. Mitochondrial Quality Control During Re-Feeding. A) Representative western blots of PGC-1α, MFN-1, DRP-1 and quantification of blots in B6 Fasted and Re-Feed gastrocnemius muscle. C) Representative western blots of PGC-1α, MFN-1, DRP-1 and quantification of blots in Min Fasted and Re-Feed gastrocnemius muscle. * denotes different to Fasted state. Significance was set at $p<0.05$. Students pre-planned t-test.

CHAPTER 4

THE EFFECT OF VOLUNTARY WHEEL ACTIVITY ON AMPK SIGNALING IN RESPONSE TO PHYSIOLOGICAL FASTING DURING CANCER CACHEXIA

4.1 Abstract

The adenosine monophosphate protein kinase (AMPK) is a key cellular energy sensor and regulator of skeletal muscle metabolic processes such as protein turnover and mitochondrial quality control. AMPK is chronically elevated during energy stress conditions such as fasting and acute exercise. Cancer cachexia is a debilitating disease that is often accompanied by decreased physical activity and chronic energy stress. Increased physical activity has been demonstrated to improve indices of muscle protein turnover and mitochondrial quality control under basal conditions. However, far less is understood about physical activity and the regulation of AMPK signaling during fasting and diseased conditions. Therefore, the purpose of this study was to determine the effect of voluntary wheel activity on AMPK signaling in response to physiological fasting during cancer cachexia. Male C57BL/6 (B6, N=16) and ApcMin/+ (Min, N=18) were subjected to either a 4 weeks of voluntary wheel running followed by a 12 hour fast, or a12 hour fast. Following completion of each treatment, hindlimb muscles were harvested and the gastrocnemius homogenized for protein analysis. Fasted Min mice exhibited body weight loss and reduced gastrocnemius mass when compared to fasted B6 mice

however, voluntary wheel activity attenuated fasting induced bodyweight loss in Min mice when compared to fasting alone. The fasting induction of AMPK (T172) and ACC (S79) was not altered in B6 mice but was suppressed by voluntary wheel activity in Min mice. Wheel activity induced PGC-1α in Min mice only, MFN-1 and DRP-1 were induced in both B6 and Min mice when compared to fasted condition. The fasting induction of FOXO3a (S416), Atrogin-1, MuRF-1, ULK-1 (S555), LC3 (II/I) and P62 were suppressed by wheel activity in Min mice only. Wheel activity induced TSC2 (S1347) in B6 and Min mice when compared to the fasted condition alone. Our results demonstrate the 4 weeks of voluntary wheel activity is sufficient to improve cachectic muscle AMPK signaling in response to a physiological fast. Furthermore, our results suggest that wheel activity improves mitochondrial quality control and AMPK mediated protein degradation signaling.

4.2 Introduction

Cancer cachexia is a debilitating wasting syndrome that occurs in approximately 80% of all cancer patients and is responsible for approximately 30% of all cancer related deaths (Argiles et al., 2010; Baracos, 2006a, 2006b, 2011; Evans et al., 2008; K. Fearon et al., 2011; K. C. Fearon & Baracos, 2010). Cachexia is infrequently identified or diagnosed and rarely treated(Baracos, 2011, 2013; Blum et al., 2010; Bruggeman et al., 2016). Cachexia development is not associated with all cancers, but the progression of cachexia is directly associated with cancer patient morbidity and mortality (Baracos, 2006a; Sadeghi et al., 2018; Solheim et al., 2011; Solheim et al., 2018). The loss of muscle mass and strength can result in increased fatigue and lead to a decrease in physical activity which can potentially further exacerbate muscle mass loss

and contribute to metabolic dysfunction (Drescher et al., 2016). A more recent study of patients with cancer of the gastrointestinal tract also demonstrated a substantial loss of mass and strength regardless of daily nutritional intake, suggesting a chronic energy deficit (Drescher et al., 2016).

The adenosine monophosphate protein kinase (AMPK) serves as the cellular energy sensor of the cell and is also responsible for regulating metabolic homeostasis and muscle mass (Thomson, 2018; Thomson et al., 2008). In healthy skeletal muscle, AMPK activation will suppress the metabolically expensive mTORC1 signaling pathway and induce the activation of energy generating pathways through the activation of downstream targets PGC-1α, FOXO3a, and ULK-1 (Shaw, 2009; Thomson, 2018; Thomson et al., 2008; Viollet et al., 2009; Zhao & Klionsky, 2011; Zong et al., 2002). Activation of these proteins will induce muscle mitochondrial biogenesis/remodeling, protein degradation, and autophagy which serve to free up amino acids for energy metabolism and help maintain blood glucose levels during prolonged periods of fasting or stress (Sandri, 2016; Schwalm et al., 2015; Thomson, 2018; Toyama et al., 2016; Zhao & Klionsky, 2011). Short term fasting is a normal physiological process that often occurs during sleep and contributes to the diurnal variation of protein turnover (Chang, Yoshihara, Machida, & Naito, 2017; Harfmann, Schroder, & Esser, 2015). Research has demonstrated that short term fasting in normal healthy skeletal muscle does not activate the AMPK signaling pathway is well tolerated (Chang et al., 2017; Dethlefsen et al., 2018; Martinez-Lopez et al., 2017; K. Nakashima, Yakabe, Yamazaki, & Abe, 2006). Our laboratory has previously reported that despite normal food intake, AMPK is chronically activated in cachectic skeletal muscle (Puppa, Gao, et al., 2014; Puppa et al.,

2012; White, Puppa, Gao, et al., 2013). Furthermore, the chronic activation of AMPK also coincides with decreased physical activity, disrupted mitochondrial quality control, and protein turnover (Baltgalvis et al., 2010; Hardee et al., 2018; Puppa, Gao, et al., 2014; Puppa, Murphy, Fayad, Hand, & Carson, 2014; White, Baltgalvis, et al., 2011; White, Baynes, et al., 2011b; White, Puppa, Gao, et al., 2013). These studies suggest that cachexia induces a constant metabolic stress that results in energy deficiency and the perpetual activation of AMPK. However, since cachectic skeletal muscle already presents with elevated AMPK, whether short term fasting could exacerbate this signaling pathway requires further investigation.

The loss of body weight and skeletal muscle mass is often accompanied by decreased physical activity and exercise (Baltgalvis et al., 2010). Our laboratory has previously reported that the decrease of voluntary wheel running activity actually precedes the initiation of body weight loss in $Apc^{Min/+}$ mice (Baltgalvis et al., 2010). Coinciding with decreased wheel running activity, our laboratory has also reported elevated AMPK and suppressed oxidative metabolism occurring as early as 15 weeks (Baltgalvis et al., 2010; Carson et al., 2015; Puppa et al., 2012; White, Baltgalvis, et al., 2011; White et al., 2012). Interestingly, increased activity and exercise have been demonstrated to induce LKB1 rather than AMPK protein expression as well as mitochondrial biogenesis markers in rat skeletal muscle (Taylor et al., 2004; Taylor et al., 2005). Surprisingly, these changes occurred despite a decrease in AMPK kinase activity (Taylor et al., 2005). In human skeletal muscle increased activity and exercise training has been demonstrated to suppress AMPK activity following a prolonged bout of exercise suggesting that training improves the skeletal muscle capacity to handle metabolic stress,

possibly through improved mitochondrial function and quality control (McConell et al., 2005; Taylor et al., 2005). While it is well established that acute exercise activates AMPK our laboratory has established that exercise training was able to attenuate the cachexia induced chronic activation of this pathway and improve muscle mass loss (Hardee et al., 2016; Puppa et al., 2012). These results suggest that increased physical activity and exercise training may improve metabolic capacity and mitochondrial quality during cancer cachexia and alleviate the chronic deficit of energy (ATP generation) thus lowering AMPK's activity. Similar results were also demonstrated using high and low frequency electrical stimulation in cachectic muscle (Hardee et al., 2016; Puppa, Murphy, et al., 2014). Seven repeated bouts of high frequency stimulation were able to lower AMPK phosphorylation in cachectic $Apc^{Min/+}$ and stimulate muscle protein synthesis 3 hours post the final treatment (Hardee et al., 2016).

As mentioned previously, numerous models of cancer cachexia have reported both elevated AMPK and disrupted aberrant downstream signaling related to protein turnover and mitochondrial quality control (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017; Pigna et al., 2016a; White, Baltgalvis, et al., 2011). The use of exercise training to attenuate or improve AMPK phosphorylation during cancer cachexia has been demonstrated as an effective treatment however, whether this effect improves the protein synthesis, degradation, and mitochondrial quality control response to a short term fast remains to be determined. Therefore, the purpose of this study was to determine if 4 weeks of voluntary wheel running activity improves aberrant AMPK signaling in response to physiological fasting during in tumor bearing mice.

4.3 Methods

Animals

The *Apc*$^{Min/+}$ mouse is a genetic model of colorectal cancer and cachexia (Baltgalvis et al., 2008). These mice harbor a heterozygous mutation in the adenomas polyposis coli (APC) gene, which promotes the development of intestinal tumors beginning as early as 4 weeks of age (Moser et al., 1995). Mice develop an IL-6-dependent cancer cachexia phenotype between 3 and 6 months of age (Baltgalvis et al., 2008). Due to the slow onset and progression of body weight loss this model is advantageous as treatments can be started after the initiation of cancer cachexia. Male *Apc*$^{Min/+}$ mice on a C57BL/6 background were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred at the University of South Carolina's Animal Resource Facility. All mice used in the current study were obtained from the investigators breeding colony within the Center for Colon Cancer Research Mouse Core. At 3-wk of age mice were genotyped as previously described. Mice were individually housed, kept on a 12:12-h light-dark cycle, and had access to standard rodent chow (#8604 Rodent Diet; Harlan Teklad, Madison, WI) and water ad libitum. 12 hours prior to sacrifice all mice had food removed from cage. Body weight measurements were taken weekly and the percentage body weight loss from peak body weight (~10-15 weeks of age) was calculated. Mice lacking the *Apc*$^{Min/+}$ mutation (C57BL/6) served as controls for all experiments. The University of South Carolina's Institutional Animal Care and Use Committee approved all animal experimentation in this study.

Voluntary Wheel Running

Voluntary wheel running was used as a marker of volitional physical activity and was performed as previously described (Baltgalvis et al., 2010). At ~14-15 wks. of age, wild-type and *Apc*$^{Min/+}$ were housed individually in cages with 9.5-in.-diameter stainless steel activity wheels (MiniMitter, Bend, OR). Running activity was monitored daily from 14-15 weeks of age to 18-19 weeks of age. Bicycle computers (Specialized, Morgan Hill, CA) with magnetic sensors measured average speed, distance, time, and maximum speed, and the data were recorded daily.

Western Blotting

Western blot analysis was performed as previously described (Fix et al., 2018). Briefly, frozen gastrocnemius muscle was homogenized in ice-cold Mueller buffer and protein concentration was determined by the Bradford method. Crude muscle homogenates were fractionated on 6 – 15% SDS-polyacrylamide gels and transferred to PVDF membranes overnight. Membranes were stained with Ponceau red to verify equal loading and transfer for each gel. Membranes were blocked at room temperature for 1 – 2 hrs. in 5% Tris-buffered saline with 0.1% Tween-20 (TBST) milk. Primary antibodies for puromycin, p-FOXO3(S416), FOXO3, p-ULK-1 (S555), ULK-1, P62, LC3II/I, MuRF-1, Atrogin-1 (ECM Biosciences), p-AMPK (T172), AMPK, p-ACC (S79) , ACC, p-TSC2 (S1387), TSC2, p-raptor (S792), raptor, DRP-1, FIS-1, MFN-1 (Abcam), PGC-1α (Abcam), and GAPDH will be diluted 1:5000 – 1:2000 in 5% TBST-milk followed by a 4-8-hr incubation with membranes at 4 degrees. Anti-rabbit or anti-mouse IgG horseradish-peroxidase conjugated secondary antibody was incubated with the

membranes at 1:2000 dilutions for 1 hour in 5% TBST-milk at room temperature. Enhanced chemiluminescence (ECL) (GE Healthcare Life Sciences, Piscataway, NJ) was used to visualize the antibody-antigen interactions. Images were digitally scanned and quantified by densitometry using imaging software (Image J; NIH). All antibodies were from Cell Signaling unless otherwise stated.

Plasma IL-6 concentrations

Plasma IL-6 concentrations were determined as previously described (Hardee et al., 2016). A commercially available IL-6 enzyme-linked immunosorbent assay kit was obtained from BD Biosciences (San Diego, CA, USA) and the manufacturer's protocol was followed. Briefly, a Costar clear 96-well plate (Corning, NY, USA) was coated with IL-6 capture antibody and allowed to incubate overnight. The next morning, the plate was blocked with assay diluent buffer, washed, and IL-6 standards and plasma samples were added in duplicate to the plate. The plate was again washed and sAV-HRP reagent was added to each well. After several washes, TMB substrate was added and the reaction was developed for 20 minutes. The reaction was stopped with sulfuric acid and absorbance was read in a Bio-Rad iMark plate reader (Hercules, CA, USA) at 450 nm.

Cytochrome C Oxidase Enzyme Assay

Cytochrome-c oxidase (COX) activity was assessed in whole muscle homogenates from the gastrocnemius muscle. For whole muscle analysis, gastrocnemius tissues were homogenized in extraction buffer (0.1M KH2PO4/Na2HPO4 and 2 mM EDTA, pH 7.2). COX enzyme activity was determined by measuring the rate of oxidation of fully reduced cytochrome c at 550 nm as previously described (Fix et al., 2018).

Statistical Analysis

Results are reported as the means ± standard error. An unpaired student's pre-planned t-test. The accepted level of significance was set at $p<0.05$ for all analysis. Statistical analysis was performed using Prism GraphPad 7 (GraphPad Software Inc., La Jolla, CA).

4.4 Results

Animal Characteristics Fasting vs. Wheel+Fasting

We first examined animal characteristics of our mice, there was a significant difference in age between B6 fast and wheel+fast mice (Table 4.1). Interestingly, spleen weight was decreased in the wheel+fast group compared to fasting alone. Additionally, seminal vesicle weight was elevated in B6 wheel+fast mice compared to fasting. In Min mice, there was no difference in pre-fast bodyweight loss (Table 4.1). However, fasting (post-fast) bodyweight loss was reduced in Min wheel+fast mice when compared to fasted Min mice alone (Table 4.1). Wheel access prior to fasting attenuated the loss of gastrocnemius mass when normalized to bodyweight in Min mice. IL-6 values and total polyps were similar between fast and wheel+fast Min mice (Table 4.1). Interestingly, these changes occurred despite Min mice running significantly less kilometers per week and per day on the wheels than B6 mice (Figure 4.1B-C) and suggest that even minimal physical activity is sufficient to improve fasting induced changes during cancer cachexia.

AMPK Activation in Wheel+Fasting Mice

In order to examine the activation of AMPK following wheel+fasting we examined B6 and Min mice that had been subjected to a short term fast or allowed free access to a wheel for 4 weeks and then subjected to a short term fast. In B6 mice there was no significant alteration to AMPK or ACC with wheel+fast when compared to fasting alone (Figure 4.2A). Interestingly, wheel access prior to fasting drastically reduced AMPK and ACC phosphorylation in Min mice (Figure 4.2B). These results suggest that increased physical activity over the course of 4 weeks was able to inhibit the fasting induction of AMPK following a short term fast.

AMPK Immediate Downstream Targets in. Wheel+Fasting Mice

Due to the suppression of AMPK in Min mice and we next examined if the changes in AMPK activation upstream were reflected downstream by mTORC1 signaling (Figure 4.3A-B). We examined TSC2 and Raptor, two known immediate downstream of AMPK targets that regulate mTORC1. In B6 mice wheel access prior to fasting did not alter TSC2 and Raptor phosphorylation (Figure 4.3A). In Min mice Raptor also remained un-altered by wheel access when compared to fasting alone (Figure 4.3B). Interestingly, TSC2 phosphorylation was induced nearly 2 fold by wheel access when compared to fasting in Min mice. (Figure 4.3B). These results suggest that despite changes in AMPK phosphorylation up-stream mTORC1 signaling appears largely un-affected by wheel access prior to fasting. Furthermore, while AMPK phosphorylation may have been suppressed it is interesting to speculate if AMPK enzyme activity actually still elevated or if the beta subunits may be compensating for a lack of alpha. We next

examined the downstream signaling of AMPK pertaining to protein degradation related to the ubiquitin proteasome (FOXO3a) and autophagy ULK-1. FOXO3a phosphorylation was suppressed by wheel access prior to fasting in B6 and Min mice (Figure 4.3A-B). ULK-1 phosphorylation at Serine 555 was only suppressed in Min mice with wheel access when compared to fasting alone (Figure 4.3B).

AMPK Mediated Protein Turnover During Wheel+Fasting

Furthermore, downstream E3 ligases MuRF-1 and Atrogin-1 were suppressed in B6 and Min with wheel access prior to fasting (Figure 4.4A-B). Downstream targets LC3B (II/I) and P62 were both suppressed in wheel+fasting Min mice only. In line with previous literature, P62 and LC3B were actually induced in wheel+fasting B6 mice compared to fasting alone (Figure 4.4A). We next examined if wheel+ fasting had any effect on puromycin incorporation which is indicative of protein synthesis. Puromycin incorporation was only induced Min wheel+fast groups when compared to fasting alone (Figure 4.4B). Collectively these results suggest that the suppression of fasting induced AMPK by wheel access serves to inhibit the fasting induction of E3 ligases and indices of autophagy in Min mice and prevent the fasting induced suppression of protein synthesis in Min mice.

AMPK Mediated Mitochondrial Quality Control During Wheel+Fasting

AMPK has the unique ability to control and regulate mitochondrial quality control through peroxisome proliferator gamma co-activator 1 (PGC-1α) and dynamin related protein 1 (DRP-1). We next examined if wheel access prior to fasting regulated these processes. B6 mice demonstrated no alterations in PGC-1α protein expression despite an

increase in cytochrome C oxidase (COX) activity (Figure 4.5A). Mitochondrial dynamics proteins DRP-1 and MFN-1 were both induced suggesting that mitochondrial remodeling is occurring with wheel access prior to fasting (Figure 4.5A). Interestingly, despite a suppression of AMPK activation with wheel access, Min mice exhibited a robust induction of PGC-1α, DRP-1, and MFN-1 when compared to fasting alone (Figure 4.4B). These changes were also accompanied by an induction of COX activity which is indicative of increased mitochondrial content. COX enzyme activity was actually tightly correlated to distance run in Min mice (Figure 4.4B).

4.5 Discussion

AMPK is an established regulator of skeletal muscle protein turnover and metabolism (Kemp et al., 2007; Kjobsted et al., 2018; Koh et al., 2008; Sakamoto et al., 2004; Sakamoto et al., 2005; Sanchez, Csibi, et al., 2012; Thomson, 2018; Thomson et al., 2008; Zhao & Klionsky, 2011). During prolonged energy deficiency such as fasting or strenuous exercise, AMPK exerts regulatory control of both mTORC1 signaling and degradation through control of its downstream targets Raptor, TSC2, FOXO3a, and ULK-1 (Bujak et al., 2015; Canto & Auwerx, 2009; Z. P. Chen et al., 2000; Friedrichsen et al., 2013; Gwinn et al., 2008; Jager et al., 2007; J. Kim et al., 2011; Laker et al., 2017; McConell et al., 2005; Mihaylova & Shaw, 2011; Mounier et al., 2015; K. Nakashima & Yakabe, 2007; Sakamoto et al., 2004). Furthermore, AMPK evidence also suggest that AMPK is an essential regulator of skeletal muscle mitochondrial quality control through PGC-1α and DRP-1 (Z. Chen et al., 2019). In healthy skeletal muscle, AMPK will suppress the mTORC1 signaling pathway through TSC2/Raptor while activating degradation pathways. Simultaneously AMPK will stimulate PGC-1α to generate more

mitochondria to compensate for the chronic energy deficit (Canto & Auwerx, 2009). However, our laboratory and others have demonstrated that despite the chronic elevation of AMPK in cachectic skeletal muscle PGC-1α expression remains suppressed (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017; White, Baynes, et al., 2011b; White, Puppa, Gao, et al., 2013).

Our laboratory has also demonstrated that TSC2 and Raptor are induced while mTORC1 is suppressed in cachectic Min skeletal muscle (White, Puppa, Gao, et al., 2013). Furthermore, this suppression of mTORC1 through AMPK coincides with a robust induction of ubiquitin proteasome and autophagy lysosomal activity (Aversa, Pin, Lucia, Penna, Verzaro, Fazi, Colasante, Tirone, Rossi Fanelli, et al., 2016; Baracos, 2000; Penna, Costamagna, et al., 2013; Sandri, 2016; White, Baynes, et al., 2011a). Expression of E3 ligases Atrogin-1 and MuRF-1 are controlled by FOXO3a, whose activity is regulated by AMPK (K. Nakashima & Yakabe, 2007). Previous studies have demonstrated that AMPK activation both in-vitro and in-vivo FOXO3a and increases the expression of E3 ligases and autophagy markers (K. Nakashima & Yakabe, 2007; Puppa, Gao, et al., 2014; Sanchez, Csibi, et al., 2012; White, Puppa, Gao, et al., 2013). Additionally, AMPK is also a critical regulator of the autophagy lysosomal system through ULK-1 (J. Kim et al., 2011; Laker et al., 2017; Sanchez, Csibi, et al., 2012). During energetic stress such as acute exercise or fasting, ULK-1 is phosphorylated by AMPK and initiates the activation of autophagy through downstream targets (Kemp et al., 2007; J. Kim et al., 2011; Laker et al., 2017; Sanchez, Csibi, et al., 2012). Autophagy has recently become a target of cancer cachexia researchers and has been reported as elevated in human colorectal cancer patients and pre-clinical models (Aversa, Pin, Lucia,

Penna, Verzaro, Fazi, Colasante, Tirone, Fanelli, et al., 2016; Penna et al., 2014; Penna, Costamagna, et al., 2013; Pettersen et al., 2017). However, an exact mechanism of elevated autophagy in cachectic skeletal muscle has yet to be fully elucidated however, some researchers have suggested an association between cancer induced inflammation and AMPK (Pettersen et al., 2017). In the current study we report the novel findings that wheel running prior to fasting was sufficient to suppress the fasting induction of AMPK in Min mice and suppress downstream degradation targets FOXO3a and ULK-1 thus, alleviating the activation of E3 ligases and autophagy. Additionally, we report that wheel running was also sufficient to restore mitochondrial quality control proteins PGC-1α, MFN-1 and DRP-1 while also inducing COX enzyme activity in Min mice. Collectively these results demonstrate that 4 weeks of voluntary wheel running prior to fasting is sufficient to attenuate aberrant AMPK mediated degradation signaling. Furthermore, 4 weeks of wheel activity also restored indices of mitochondrial quality control and improves cachectic skeletal muscles response to a short term fast.

Increased physical activity as a treatment modality has become an important area of interest in cancer cachexia (Argiles et al., 2012; Carson et al., 2015; Grande et al., 2015; Solheim et al., 2018). Numerous studies have investigated the association between physical activity, developing cancer, and skeletal muscle protein turnover (Antoun & Raynard, 2018; Booth, Roberts, & Laye, 2012; Hardee, Counts, & Carson, 2019). Studies and clinical trials have also suggested that prognosis is improved in physically active cancer patients due to exercise performed after the cancer diagnosis (Alves, da Cunha, da Paixao, & Brum, 2015; Argiles et al., 2012; Belloum, Rannou-Bekono, & Favier, 2017; Gould et al., 2013; Grande et al., 2015). Additionally, similar results have

been reported in pre-clinical models of cancer cachexia as well, wheel running activity in C26 cancer mice blocked the induction of AMPK mediated degradation pathways in gastrocnemius muscle (Pigna et al., 2016b). Additionally, our laboratory and others have demonstrated the ability of muscle contraction to attenuate some of these markers of degradation in cachectic muscle, further establishing the regulatory power of activity and contraction (Hardee et al., 2016; Pigna et al., 2016b; Puppa, Murphy, et al., 2014). We add to these findings by demonstrating that FOXO3a phosphorylation by AMPK, Atrogin-1, and MuRF-1 were all suppressed with 4 weeks of wheel running prior to fasting in both B6 and Min mice. Interestingly, ULK-1 phosphorylation, P62, and LC3B (II/I) were actually induced by wheel running prior to fasting in B6 mice which corroborates previous studies by our laboratory and others in healthy adult skeletal muscle (Fix et al., 2018; Yan et al., 2012). However, wheel running activity prior to fasting suppressed ULK-1 phosphorylation and its downstream targets P62 and LC3B (II/I) in Min mice, demonstrating the therapeutic effect of physical activity in the caner environment. Despite the robust changes in protein degradation markers we report that wheel running prior to fasting had little to no effect on the phosphorylation of TSC2 and Raptor. Interestingly, TSC2 phosphorylation was actually further induced in Min mice while Raptor was actually suppressed in B6 mice when compared to fasting alone. Previous research in non-muscle cells has suggested that Raptor phosphorylation is actually required for the suppression of mTORC1 in response to fasting (Gwinn et al., 2008). Despite these slight changes in TSC2 and Raptor we report that 4 week of wheel running did not alter skeletal muscle puromycin incorporation in B6 and Min mice which is indicative of protein synthesis. Collectively, these results demonstrate that the

suppression of AMPK in Min mice was not reflected downstream in regard to mTORC1 signaling and skeletal muscle protein synthesis. However, these results suggest that the attenuation in muscle mass loss is most likely attributed to a suppression of degradation rather than protein synthesis.

In addition to regulating skeletal muscle mass, AMPK also exerts control over muscle metabolism and mitochondrial quality control (Canto et al., 2010; Laker et al., 2017; Mishra & Chan, 2016; Zong et al., 2002). In healthy human skeletal muscle, exercise training has been demonstrated to improve the response to fasting when compared to untrained muscle (Dethlefsen et al., 2018; McConell et al., 2005). Furthermore, AMPK has emerged as a critical regulator of muscle mitochondrial health via its regulation of mitochondrial biogenesis (PGC-1α) and fission (DRP-1) (Z. Chen et al., 2019; Jager et al., 2007). These processes are critical for the maintenance of skeletal muscle during periods of energy deficiency such as fasting and acute exercise. However, numerous disease models including cancer cachexia report that skeletal muscle AMPK is chronically activated suggesting a state of constant energy stress or deficiency (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017; Bujak et al., 2015; Hall et al., 2018; Puppa, Gao, et al., 2014; Rohm et al., 2016; White, Puppa, Gao, et al., 2013). Our laboratory has previously reported that AMPK is robustly activated in severe cancer cachexia in both the Lewis Lung Carcinoma (LLC) and $Apc^{Min/+}$ mouse models of cancer cachexia while PGC-1α is actually suppressed (Puppa, Gao, et al., 2014; Puppa et al., 2012; White, Puppa, Gao, et al., 2013). In the current study PGC-1α was only induced by wheel running in Min mice prior to fasting. Interestingly, this induction occurred despite a suppression of AMPK phosphorylation upstream. The disconnect of AMPK and PGC-

1α in cachexia has been previously reported by our laboratory (Puppa, Gao, et al., 2014; Puppa et al., 2012; White, Puppa, Gao, et al., 2013). Furthermore, results from the current study further corroborate the ability of increased physical activity and exercise to attenuate chronically activated AMPK in cachexia and restore the activation of PGC-1α. In addition to the increase in PGC-1α, wheel running prior to fasting also induced mitochondrial remodeling by inducing both MFN-1 and DRP-1 in both B6 and Min mice which are critical regulators of mitochondrial health. In Min mice the induction of these proteins coinciding with the restoration of PGC-1α is suggest of improved mitochondrial quality control and remodeling. It is interesting to speculate how exactly the activation of PGC-1α is occurring, previous studies have demonstrated that AMPK phosphorylation may not always accurately reflect kinase activity. The decrease in phosphorylation of AMPK in the current study may be occurring outside of kinase activity which may still serve to induce PGC-1α. Furthermore, these results may also suggest a role for the sirtuins, especially SIRT-1 which has been demonstrated to be a critical co-factor for PGC-1α activation and reflective of AMPK kinase activity. Collectively, these results suggest that 4 weeks of voluntary wheel running is sufficient to restore aberrant AMPK signaling and induce mitochondrial remodeling in fasted Min mice.

In conclusion we demonstrate a role for increased physical activity through wheel running to attenuate aberrant AMPK activation in fasted cachectic skeletal muscle. We provide evidence that the suppression of AMPK by wheel running prior to fasting attenuates protein degradation through suppressing E3 ligase and autophagy activation. These results demonstrate that the sparing of muscle mass is occurring due to changes in degradation rather than AMPK mediated mTORC1 signaling. Furthermore, the current

study demonstrates that wheel running activity is sufficient to restore skeletal muscle mitochondrial quality control through PGC-1α, MFN-1, and DRP-1. These results suggest that the improvements in mitochondrial quality control may improve the ability of cachectic skeletal muscle to handle a short term fast.

Table 4.1. Animal Characteristics of Fast and Wheel+Fast Mice

	WT	AMPK KO	Min	Min KO
Sex	Male	Male	Male	Male
Age (weeks)	16.1	15.9 ± 0.1	21.2 ± 1.0	19.0 ± 1.3
N	6	10	10	6
Peak BW (g)	23.5 ± 0.5	23.1 ± 0.7	23.9 ± 0.4	23.9 ± 0.6
BW (g)	23.3 ± 0.6	23.3 ± 0.6	23.2 ± 0.4	22.9 ± 0.6
Pre Fast BW (g)	23.2 ± 0.5	23.2 ± 0.5	23.1 ± 0.4	22.8 ± 0.6
BW @ Sac (g)	22.5 ± 0.5	22.3 ± 0.6	21.2 ± 0.8	22.2 ± 0.8
BW Change % (BW-Peak BW/Peak BW)	1.0 ± 1.0	2.0 ± 0.3	3.7 ± 0.01	4.0 ± 1.0
Fasting BW Change % (Pre Fast BW-BW @ Sac/Pre Fast BW)	3.0 ± 1.0	4.0 ± 0.1	9.8 ± 0.2	3.0 ± 1.0*
Sac Glucose (mg/dL)	140.7 ± 7.3	125.7 ± 7.1	112.7 ± 5.3	132.8 ± 10.7 *
Food (g/day)	3.4 ± 0.2	3.2 ± 0.1	2.9 ± 0.3	3.1 ± 0.2
IL-6 (pg/ML)	0.0 ± 0.0	0.0 ± 0.0	15.6 ± 4.6	17.7 ± 3.8
Total Polyps	0.0 ± 0.1	0.0 ± 0.1	36.3 ± 6.7	36.9 ± 10.2
Gastrocnemius (mg)	120.2 ± 3.0	118.8 ± 2.1	99.9 ± 6.9	119.8 ± 3.6 *
Stomach (mg)	250.7 ± 22.7	257.6 ± 9.8	423.9 ± 38.1	283.3 ± 21.7*
Liver (mg)	1007 ± 40.7	970.5 ± 26.9	1287.3 ± 56.7	1039.7 ± 104.9 *
Spleen (mg)	60.7 ± 2.2	83.9 ± 16.9	344 ± 39.2	150.7 ± 29.8 *
Sem Vesicles (mg)	176.4 ± 11.6	155.0 ± 14.4	131.3 ± 24.0	166.0 ± 18.5
Testes (mg)	165.8 ± 10.5	168.9± 1.9	168.7 ± 11.4	170.5 ± 3.1
Epdidymal Fat (mg	237.3 ± 30.3	237.0 ± 22.3	99.1 ± 39.3	202.3 ± 15.0 *
Tibia Length	16.9 ± 0.1	17.1 ± 0.1	16.8 ± 0.1	16.9 ± 0.1

B6 Fast and Min Fast groups are the same mice from specific aim 2. Values are means ± SEM. Age given in weeks (wks). Body weights given in grams BW Change is determined from BW-Peak BW/Peak BW, Fasting BW Change determined from Pre-Fast BW-BW @ Sac/Pre-Fast BW. All tissue weights expressed in milligrams (mg). IL-6 values expressed as picograms per milliliter (pg/ml). Fast denotes 12-hour fast during the 12-hour light cycle. Wheel+Fast denotes 4 weeks of wheel access prior to 12-hour fast during the light cycle. Min denotes $Apc^{Min/+}$ mice . B6 denotes C57BL/6 mice *Significantly different from Fed state within genotype. $p<0.05$ (students pre-planned t-test within genotype).

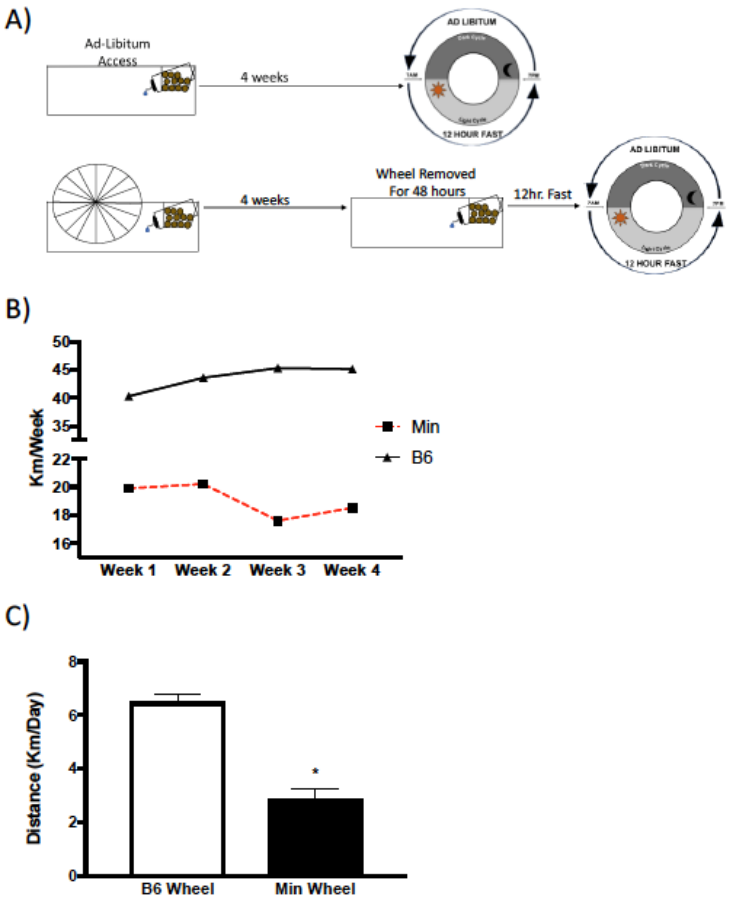

Figure 4.1 Experimental Design and Wheel Running Distance. A) Experimental design figure depicting 12 hour light cycle fasting protocol and wheel running protocol. B) Kilometers per week (Km/Week) in B6 and Min mice. C) Average distance (Km/Day) in B6 wheel and Min wheel mice. * denotes different to B6 wheel. Significance was set at $p<0.05$. Students pre-planned t-test.

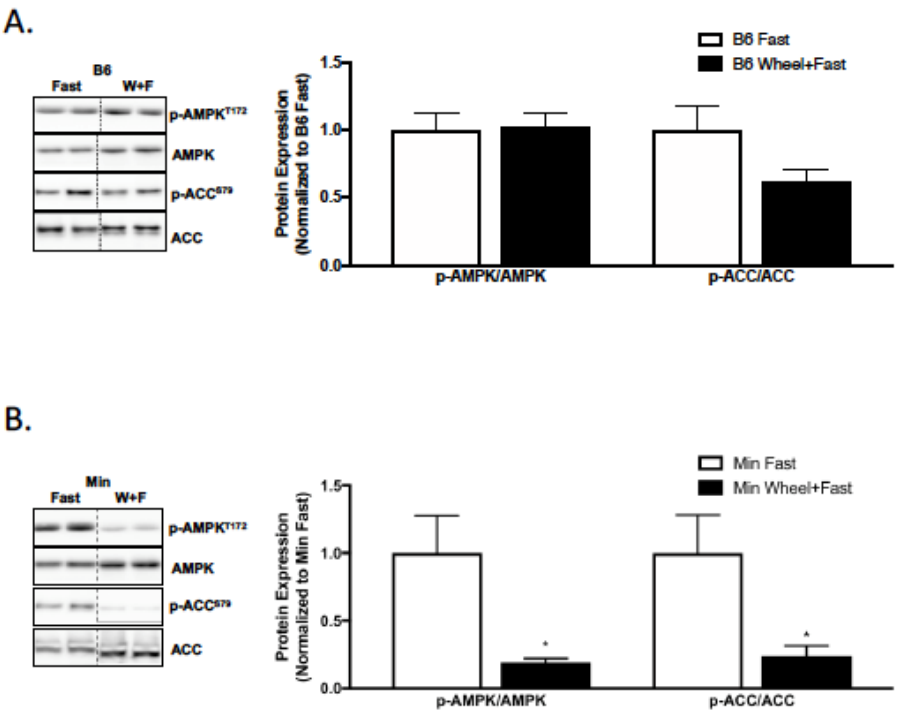

Figure 4.2 AMPK Activation in Fasted and Wheel+Fasted Mice. A) Representative western blots of p-AMPK (T172), p-ACC (S79), ACC, and quantification of blots in B6 Fasted and Wheel+Fasted gastrocnemius muscle. B) Representative western blots of p-AMPK (T172), p-ACC (S79), ACC, and quantification of blots in Min Fasted and Wheel+Fasted gastrocnemius muscle. * denotes different to Fed state. Significance was set at $p<0.05$. Students pre-planned t-test.

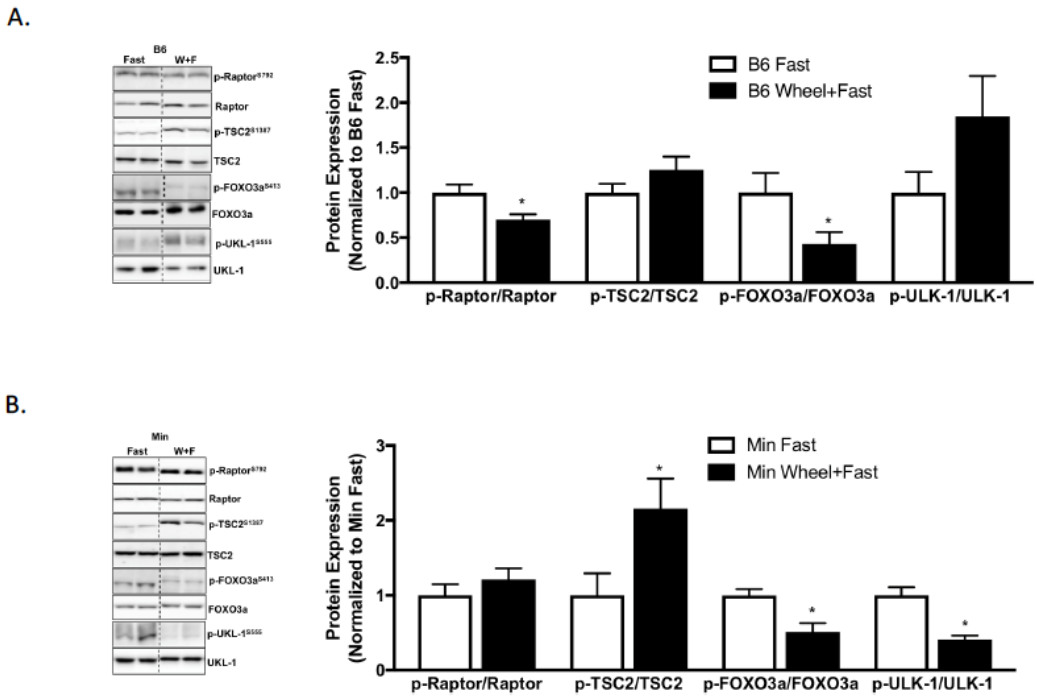

Figure 4.3. AMPK Downstream Signaling in Fasted and Wheel+Fasted Cachectic Skeletal Muscle. A) Representative western blots of p-Raptor (S792), Raptor, p-TSC2 (S1387), TSC2, p-FOXO3a (S413), FOXO3a, p-ULK-1 (S555), ULK-1, and quantification of blots in B6 Fasted and Wheel+Fasted gastrocnemius muscle. C) Representative western blots of p-Raptor (S792), Raptor, p-TSC2 (S1387), TSC2, p-FOXO3a (S413), FOXO3a, p-ULK-1 (S555), ULK-1, and quantification of blots in Min Fasted and Wheel+Fasted gastrocnemius muscle. * denotes different to Fed state. Significance was set at $p<0.05$. Students pre-planned t-test.

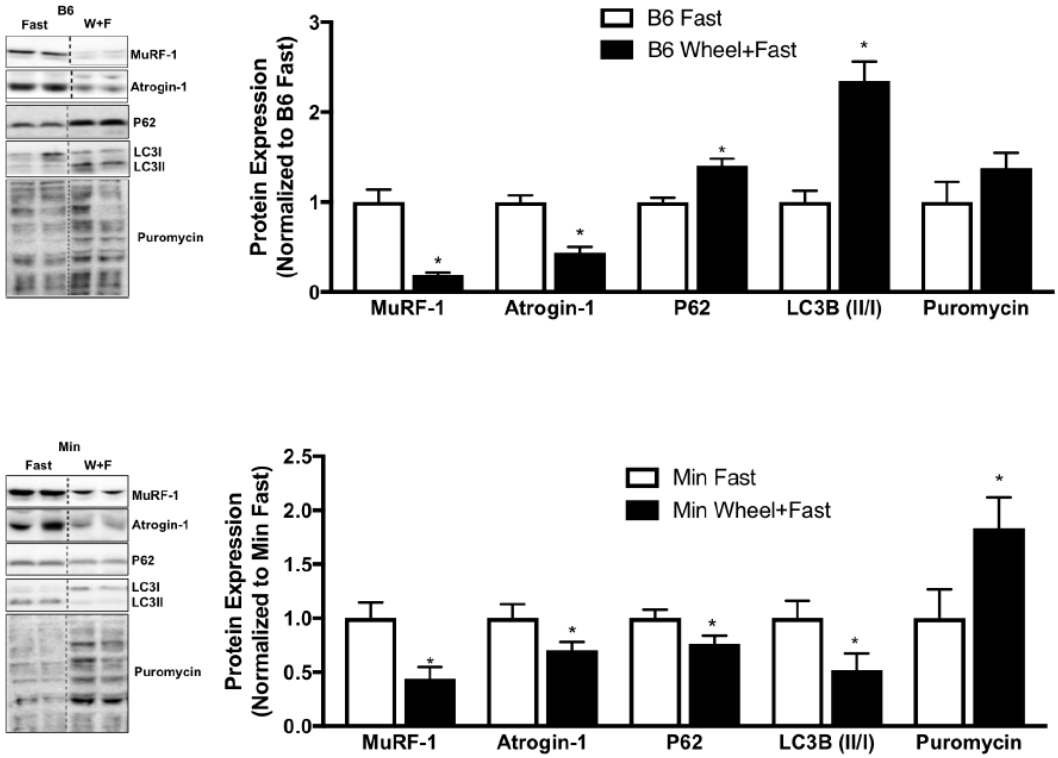

Figure 4.4. Protein Turnover in Fasted and Wheel+Fasted Cachectic Skeletal Muscle. A) Representative western blots of MuRF-1, Atrogin-1, P62, LC3 I/II, Puromycin, and quantification of blots in B6 Fasted and Wheel+Fasted gastrocnemius muscle. C) Representative western blots of MuRF-1, Atrogin-1, P62, LC3 I/II, Puromycin, and quantification of blots in Min Fasted and Wheel+Fasted gastrocnemius muscle. * denotes different to Fed state. Significance was set at $p<0.05$. Students pre-planned t-test.

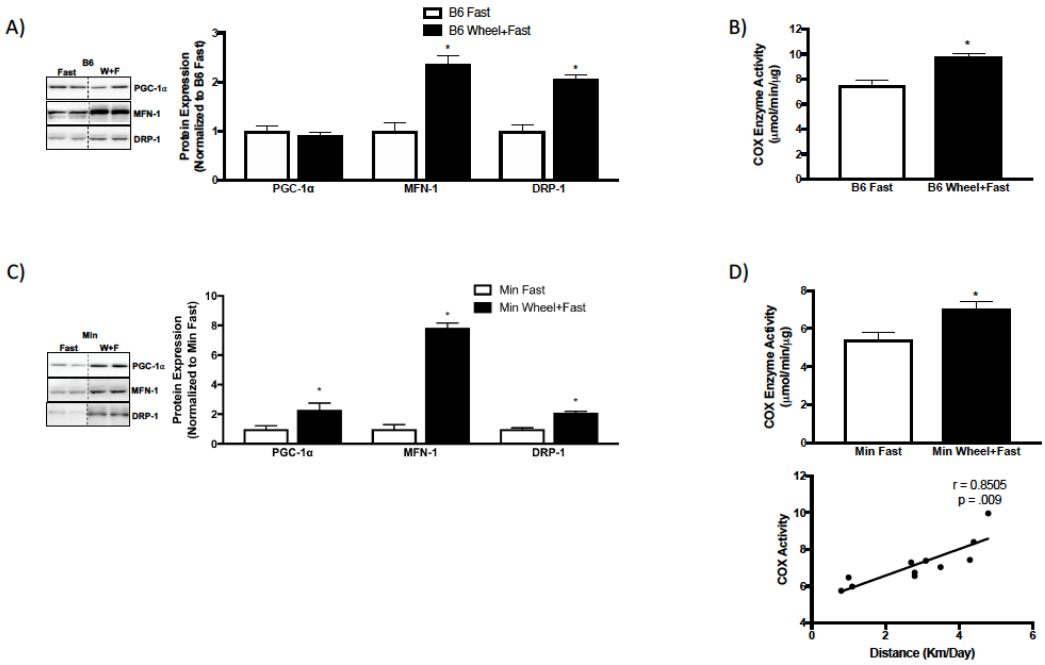

Figure 4.5. Mitochondrial Quality Control in Fasted and Wheel+Fasted Cachectic Skeletal Muscle. A) Representative western blots of PGC-1α, MFN-1, DRP-1, and quantification of blots in B6 Fasted and Wheel+Fasted gastrocnemius muscle. B) COX enzyme activity in B6 Fasted and Wheel+Fasted gastrocnemius muscle. C) Representative western blots of PGC-1α, MFN-1, DRP-1, and quantification of blots in Min Fasted and Wheel+Fasted gastrocnemius muscle. D) COX enzyme activity and correlation of COX activity to Distance (Km/Day). * denotes different to Fed state. Significance was set at $p<0.05$. Students pre-planned t-test. Pearson Correlation where appropriate.

CHAPTER 5

OVERALL DISCUSSION

The overall purpose of the current dissertation was to determine if cachexia associated aberrant AMPK signaling is responsive to fasting, feeding, and increased wheel running activity to regulate skeletal muscle proteostasis. Our central hypothesis was that cancer cachexia induces the disruption of AMPK signaling by fasting and feeding through dysfunctional mitochondrial quality control leading to muscle mass loss. We also further hypothesized that increased physical activity would improve mitochondrial quality control and thus improve the regulation of AMPK to fasting and feeding improving protein turnover and attenuating wasting. Utilizing manipulations of AMPK signaling in each aim we sought to test our question and hypothesis and determine the role of AMPK in skeletal muscle protein turnover. Key findings from this dissertation experiments demonstrated that (1) AMPK is required for the cachexia suppression of skeletal muscle protein synthesis through TSC2 and Raptor during a short term fast, however we also demonstrate that AMPK is required for the cachexia induction of degradation through E3 ligases and autophagy during fasting, and we identify that AMPK is required for the cachexia disruption of mitochondrial dynamics. (2) Cancer cachexia accelerates the skeletal muscle physiological fasting response through AMPK, a short term fast accelerates skeletal muscle protein degradation, suppresses protein

synthesis, and induces mitochondrial fission protein DRP-1. (3) Refeeding following a short term fast suppresses AMPK and downstream signaling suggesting that cachectic skeletal muscle is not anabolic resistant but rather hyper-sensitive to periods of fasting. (4) 4 weeks of voluntary wheel activity inhibits the fasting induction of AMPK, restores mitochondrial quality control, and attenuates muscle mass loss in cachectic skeletal muscle.

5.1 AMPK and Cachexia: Imbalanced Protein Turnover

A negative energy balance, resulting from both reduced production of ATP and increased energy expenditure is commonplace in cancer patients (Jager et al., 2007; Kemp et al., 2007; Kjobsted et al., 2018; Koh et al., 2008). The increase of energy expenditure in cachexia is prevalent in human and pre-clinical cachexia (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017; Sandri, 2016; Tsoli et al., 2014; White, Baynes, et al., 2011b). The energy sensing molecule adenosine monophosphate protein kinase AMPK regulates energy metabolism and homeostasis in response to metabolic stressors such as fasting and exercise (Bujak et al., 2015; Friedrichsen et al., 2013; Kjobsted et al., 2018). AMPK is known for facilitating a host of metabolic processes and couples cellular energy status to protein synthesis. AMPK activity is disrupted during the progression of cancer cachexia (Hardee et al., 2017; Sanchez, Csibi, et al., 2012; Sandri, 2016). Multiple pre-clinical models of cachexia demonstrate an elevation of AMPK activity during the later stages of cachexia (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017; Puppa et al., 2012; Rohm et al., 2016; White, Baynes, et al., 2011a). Often coinciding with the elevation of AMPK in cachectic muscle is the suppression of protein synthesis (mTORC1) and acceleration of degradation (E3 ligases and autophagy)

pathways (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017; Puppa et al., 2012; Rohm et al., 2016; White, Baynes, et al., 2011a). While this induction of AMPK activity has been established, very little has been done to mechanistically examine the role of AMPK in the regulation of protein turnover. Our laboratory has previously suggested that AMPK inhibition in cultured myotubes incubated with IL-6 and LLC media is capable of restoring mTORC1 and protein synthesis (White, Baynes, et al., 2011b; White, Puppa, Gao, et al., 2013). However, recent evidence has suggested that the timing of AMPK activation may contribute to cachexia induced muscle mass loss (Rohm et al., 2016). Activation of AMPK via AICAR immediately following C26 tumor implantation attenuated tumor growth and cachexia induce muscle mass loss (Rohm et al., 2016). Taken together these results although ambivalent, definitively demonstrate a role for AMPK in the regulation of muscle mass during cancer cachexia. The current dissertation is the first study to mechanistically examine the cellular processes AMPK regulates in response to fasting, feeding, and activity during cancer cachexia.

AMPK Regulation of TSC2 and Raptor: Fasting, Feeding, and Activity in Cachexia

An intriguing question among researchers is whether cachectic muscle retains anabolic plasticity (Hardee et al., 2017). This could prove beneficial for clinicians looking to provide treatment and improve muscle mass and function in cachectic cancer patients. While circadian fluctuations in protein turnover are the established norm in muscle mass regulation, it has become evident that protein synthesis is highly responsive to environmental stimuli (Chang et al., 2017; Lefta, Wolff, & Esser, 2011). Fasting, feeding, and activity are extremely potent regulators of skeletal muscle protein synthesis in basal healthy conditions; however, numerous wasting conditions have demonstrated

perturbed sensitivity to these anabolic signals (Argiles, Lopez-Soriano, et al., 2015; Bodine, 2013; Bowen et al., 2015). Short term fasting is a normal physiological process that often occurs during sleep and contributes to the diurnal variation of protein turnover (Lefta et al., 2011). Research has demonstrated that short term fasting in normal healthy skeletal muscle does not activate the AMPK signaling pathway is well tolerated (Bujak et al., 2015; Canto et al., 2010; Galic et al., 2018). However, our laboratory was one of the first to suggest that the chronic activation of AMPK may be responsible for the cachexia disruption of protein synthesis after an acute 5 hour fast (White, Baynes, et al., 2011a). The current studies demonstrate that the fasting induction TSC2 and Raptor require AMPK in cachectic skeletal muscle. Furthermore, we demonstrate that cancer cachexia accelerates the skeletal muscle physiological response to fasting by robustly inducing AMPK after a mere 12-hour fast during the light cycle. Min mice that were fasted for 12-hours during the light cycle exhibited a robust activation of both TSC2 and Raptor phosphorylation by AMPK which were not present in their fed counterparts. The AMPK phosphorylation of TSC2 and Raptor are believed to serve as a metabolic checkpoint that shuts off mTORC1 activity (Gwinn et al., 2008). However, recent evidence has suggested that the phosphorylation of Raptor and not TSC2 is critical in this process (Gwinn et al., 2008).

Since cancer cachexia cannot be reversed by conventional nutritional supplementation, it is also necessary to further our understanding of anabolic resistance (Bruggeman et al., 2016; Dillon et al., 2012; Hardee et al., 2017). Growing evidence is supporting the disruption of protein synthesis during cancer cachexia; however, very few studies have examined if nutrients or feeding can stimulate anabolic signaling (Evans et

al., 2008; A. M. H. Horstman et al., 2016; Kitagawa, Haji, & Amagai, 2017). We provide evidence in the current studies that 1-hour of refeeding following a 12-hour short term fast suppresses AMPK and its phosphorylation of downstream targets TSC2 and Raptor. Interestingly, this suppression of AMPK signaling was not reflected in downstream puromycin incorporation which is indicative of protein synthesis. The disparity between the two may be an issue of timing as the refeeding paradigm was only 1 hour. However, it is interesting to speculate how long the suppression of AMPK occurs for post feeding, the decrease in phosphorylation may only be an acute transient suppression. Furthermore, the composition of the food may contribute to protein synthesis as well, cancer patients are still capable of inducing protein synthesis in response to protein ingestion however, the degree of activation appears to be severely impaired (Dillon et al., 2012). A newer approach using specially formulated medical food that is high in protein and leucine has shown promise in cancer patients with involuntary weight loss and may be applicable to the current data presented (Deutz et al., 2011).

Physical activity and exercise have been deemed critical regulators of skeletal muscle AMPK (Canto & Auwerx, 2009; Z. P. Chen et al., 2000; Friedrichsen et al., 2013; Laker et al., 2017). Acute exercise has been extensively investigated to induce skeletal muscle AMPK to compensate for the energy stress (Friedrichsen et al., 2013; Jorgensen et al., 2005). Increased physical activity as a treatment modality has become an important area of interest in cancer cachexia (Alves et al., 2015; Argiles et al., 2012; Grande et al., 2014). Numerous studies have investigated the association between physical activity, developing cancer, and skeletal muscle protein turnover (Ali & Garcia,

2014; Alves et al., 2015; Argiles et al., 2012; Aversa et al., 2017; Grande et al., 2015; Hardee et al., 2016; Hardee et al., 2017; Khamoui et al., 2016). Studies and clinical trials have also suggested that prognosis is improved in physically active cancer patients due to exercise performed after the cancer diagnosis (Gould et al., 2013; Grande et al., 2015). Additionally, similar results have been reported in pre-clinical models of cancer cachexia as well, wheel running activity in C26 cancer mice blocked the induction of AMPK mediated degradation pathways in gastrocnemius muscle (Pigna et al., 2016b). In the current study we provide evidence that 4 weeks of voluntary wheel activity prior to fasting is sufficient to inhibit AMPK phosphorylation and activation of Raptor. These results also demonstrated that wheel activity blocked the fasting suppression of protein synthesis. Our laboratory has previously demonstrated that two models of contraction are capable of suppressing AMPK and inducing protein synthesis in cachectic skeletal muscle (Puppa, Gao, et al., 2014; White, Baynes, et al., 2011a). Seven bouts of high frequency electrical stimulation as well as an acute bout of low frequency stimulation were able to suppress AMPK, restore mitochondrial biogenesis, and induce protein synthesis in Min and LLC mice (Hardee et al., 2016; Puppa, Murphy, et al., 2014).

AMPK Mediated Protein Degradation in Cachexia

It remains mostly unclear how tumors initiate the signal to stimulate muscle protein degradation and cachexia. The often anatomically distant location between tumors and the wasting muscles are suggestive of tumor derived factors such as cytokines into circulation to initiate signaling processes that cause muscle wasting (Baracos, 2006a; Bowen et al., 2015; Johns et al., 2012). A hallmark feature of cancer cachexia is the net loss of skeletal muscle protein through selective degradation pathways (Penna et al.,

2014; Pettersen et al., 2017; Tisdale, 2009). A consistent finding that translates from pre-clinical models to the clinic is the elevation of muscle protein degradation while synthesis is suppressed (Penna, Ballaro, Beltra, De Lucia, & Costelli, 2018). While cancer cachexia may be a multifactorial condition, clear evidence suggest that the ubiquitin proteasome system and autophagy lysosomal system are major contributors to muscle mass regulation (Penna, Costamagna, et al., 2013; Sandri, 2016; Yuan et al., 2015).

AMPK is a critical regulator of both the ubiquitin proteasome system through FOXO3a and the autophagy lysosomal system through ULK-1 (Ju et al., 2016; K. Nakashima & Yakabe, 2007; Sandri, 2016). Phosphorylation of these two downstream targets induces both E3 ligases and autophagy indices (Ju et al., 2016; K. Nakashima & Yakabe, 2007; Sanchez, Csibi, et al., 2012; Sandri, 2010, 2016). Activation of these two degradation pathways has been studied extensively in catabolic conditions such as fasting and cachexia and are believed to be key mediators of muscle mass loss (Ju et al., 2016; K. Nakashima & Yakabe, 2007; Pettersen et al., 2017; Pigna et al., 2016b; Sandri, 2016). AMPK regulation of FOXO3a and ULK-1 has been demonstrated through loss and gain of function studies (Sandri et al., 2004). Treatment of mice with AICAR infusion has demonstrated a robust activation of FOXO3a and E3 ligases Atrogin-1 and MuRF-1 (Sanchez, Csibi, et al., 2012). Multiple pre-clinical models and human cancer patients have reported elevated E3 ligase expression (Bodine & Baehr, 2014; Bowen et al., 2017; Mueller et al., 2016b). In addition to E3 ligase expression, the process of autophagy has recently become a target of interest in cachexia (Penna et al., 2014). Both human cancer patients and pre-clinical models of cachexia have demonstrated inductions of the autophagy lysosomal system (Penna, Costamagna, et al., 2013). In the current

dissertation we demonstrate that a short term 12-hour fast induces FOXO3a and ULK-1 phosphorylation in Min mice and this induction requires AMPK. The phosphorylation of these proteins leads to the induction of Atrogin-1, MuRF-1, and LC3B (II/I). Interestingly, in line with our protein synthesis data described above, re-feeding following a 12-hour fast suppressed the induction of these proteolytic systems. The suppression of these signaling processes by re-feeding suggest that despite the failure to induce protein synthesis, lowering AMPK via re-feeding may alleviate the elevation of muscle protein degradation acutely. While the fasting induction of these proteins is not a new concept the current dissertation is the first to report these findings in cachexia and that acute re-feeding is capable of suppressing that fasting activation. AMPK mediated protein degradation during a fast in healthy skeletal muscle is typically not activated until 24-48 hours of fasting is achieved (Galic et al., 2018). We demonstrate that cachectic skeletal muscle induces these pathways after only 12-hours.

Many cancer patients suffer from chronic fatigue, either from the disease itself or its treatment, which is often a confounder that limits regular exercise practice (Grande et al., 2015). This lack of physical activity or exercise has been demonstrated to occur in both the clinic and pre-clinical models (Grande et al., 2015; Roberts et al., 2013). While the targeting of aberrant protein degradation processes has been suggested as a potential treatment for cachexia, it is entirely plausible that inhibiting protein degradation could prove detrimental to systemic metabolism and muscle function (Cohen, Nathan, & Goldberg, 2015). Furthermore, damaged proteins induced by exercise must be removed for successful regeneration and muscle function. Muscle protein degradation is stimulated by both acute endurance and resistance exercise (Vainshtein, Tryon, Pauly, & Hood,

2015; Wolfe, 2006). In contrast, exercise training can decrease indices of protein breakdown in muscle, which may be related to improved protein quality in previously cachectic muscle (Vainshtein et al., 2015). Indeed, voluntary wheel running decreased muscle E3 ligase mRNA expression and autophagy protein expression in tumor bearing mice (Pigna et al., 2016b) . In the current studies we demonstrate that 4 weeks of voluntary wheel activity suppressed the fasting induction of Atrogin-1,MuRF-1, P62 and LC3B. These results are in line with previous reports demonstrating treadmill exercise prior to tumor inoculation decreased muscle protein breakdown in rats (Salomao, Toneto, Silva, & Gomes-Marcondes, 2010). While exercise-induced improvements have not been observed in all preclinical studies, this could be related to variables such as tumor type or exercise intensity (Khamoui et al., 2016).

5.2 Mitochondrial Quality Control and AMPK

Mitochondrial respiration and ATP synthesis are intimately linked to cellular energy utilization (Wai & Langer, 2016). Protein synthesis is an energy demanding process and is responsible for approximately 20-30% of ATP consumption (Hardee et al., 2017). Many non-ribosomal energy-consuming enzymes and kinases are required for the synthesis, assembly, and maintenance of ribosomes (Kressler et al., 2010a). Collectively, the energetic cost of protein synthesis implicates mitochondrial function and quality are important in protein turnover (Carson et al., 2015; Hardee et al., 2017). AMPK tightly couples energy consumption to oxidative phosphorylation and protein synthesis (Kjobsted et al., 2018; Suliman & Piantadosi, 2016). The process of mitochondrial quality control involves the processes of biogenesis, fission and fusion (dynamics), and the selective turnover of mitochondria (mitophagy) (Kjobsted et al., 2018; Suliman &

Piantadosi, 2016; Vitorino et al., 2015; Wai & Langer, 2016). Mitochondrial quality control has recently become an intriguing therapeutic target of cancer cachexia (Vitorino et al., 2015). Recent evidence suggest a role for the disruption of these processes in the regulation of metabolic homeostasis.

Dysfunctional mitochondrial quality control and oxidative metabolism are found in many diseases and pathological conditions (Argiles, Lopez-Soriano, et al., 2015; Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017; Marzetti et al., 2017; Vitorino et al., 2015; White et al., 2012). Accelerated protein degradation and suppressed synthesis in wasting muscle has recently been linked to dysfunctional mitochondrial quality control and AMPK may be the link between these cellular processes (B. N. VanderVeen, Fix, et al., 2017). Increased activity and exercise have been well studied as potent stimulators of mitochondrial quality control and has been studied as a therapeutic for cancer (Alves et al., 2015; Argiles et al., 2012; Aversa et al., 2017). Endurance training is associated with increased mitochondrial density and metabolic enzymes in skeletal muscle (Egan & Zierath, 2013; Forbes et al., 2012; Hood et al., 2015). Exercise improves mitochondrial function and has the capacity to increase remodeling of the mitochondrial network through improving dynamics (Fix et al., 2018; Yan et al., 2012). We have previously reported that activity decreases before bodyweight loss suggesting that a lack of activity contributes to a disuse type loss of mitochondrial capacity and function in cachexia (Baltgalvis et al., 2010). These findings suggest a role for increased activity and exercise as a potential therapy or countermeasure to the cachexia induced muscle mass loss.

AMPK and PGC-1α in Cachexia: Disconnected?

AMPK is activated during energy stress and will induce mitochondrial biogenesis and remodeling to meet the demand for ATP (Hood, 2009; Hood et al., 2015; Jorgensen et al., 2005; Koh et al., 2008). Mitochondrial biogenesis is modulated by AMPK through its activation of PGC-1α and is actually required for the induction of the PGC-1α promoter (Penna et al., 2018). Most genes involved in mitochondrial metabolism seem to be under the control of PGC-1 family of transcription factors (Penna et al., 2018). Studies utilizing AMPK knockout models have demonstrated a blunted response to exercise and decreased mitochondrial content (Viollet et al., 2009). Despite the chronically elevated activation of AMPK in cachectic skeletal muscle PGC-1α is actually suppressed demonstrating a disconnect between these two signaling molecules (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017; Puppa, Gao, et al., 2014; Puppa et al., 2012; White, Baltgalvis, et al., 2011). Furthermore, our laboratory has previously reported that cachectic skeletal muscle has depleted mitochondrial number and citrate synthase activity (Puppa et al., 2012; White, Baltgalvis, et al., 2011). The loss of mitochondria and lack of biogenesis in cachectic skeletal muscle has recently been determined to occur prior to bodyweight loss in LLC tumor bearing mice (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017). The use of contraction models using electrical stimulation have been employed by our laboratory previously and have proven effective at inducing PGC-1α protein expression as well as cytochrome C oxidase (COX) activity (Hardee et al., 2016; Puppa, Murphy, et al., 2014). In the current dissertation we demonstrate that the

cachexia suppression of PGC-1α does not require AMPK. These results are not un-expected given that there appears to be a disconnect between AMPK activation and PGC-1α in cachectic skeletal muscle. However, we also demonstrate that 4 weeks of voluntary wheel running activity is able to restore AMPK phosphorylation to basal levels following a fast and induce PGC-1α protein expression and COX activity in Min mice. The induction of mitochondrial biogenesis and content in cachectic skeletal muscle may serve to alleviate the energy stress induced by fasting and alleviate AMPK activation restoring protein synthesis and attenuating degradation. Collectively these results in context with previous literature in the field suggest that increased wheel running activity improves mitochondrial quality control, thus alleviating the activation of AMPK during a short term fast and attenuating dysfunctional protein turnover in cachectic skeletal muscle.

AMPK and Mitochondrial Dynamics

Different mitochondrial morphologies have been proposed to modulate oxidative metabolism (Wai & Langer, 2016). Elongated mitochondria have been proposed to sustain ATP production during stress whereas fragmented mitochondria are believed to facilitate mitophagy and turnover of damaged mitochondria (Trewin, Berry, & Wojtovich, 2018). Recent evidence has come to light highlighting AMPK as a potential regulator of these processes (especially fission) (Z. Chen et al., 2019). Studies suggest that the process of mitochondrial fission induced atrophy is modulated through AMPK mediated FOXO3a signaling (Romanello et al., 2010). Myofibers transfected with a dominant negative AMPK inhibited the fasting induction of fission proteins DRP-1 and FIS-1 and spared muscle mass (Romanello et al., 2010). Coinciding with the activation

of AMPK in cachectic skeletal muscle is the induction of fission and suppression of fusion proteins (White et al., 2012). Our laboratory has previously demonstrated that suppressing the cachexia activation of AMPK through inhibiting IL-6 attenuated the induction of mitochondrial fission and restored fusion to basal levels in-vivo and in-vitro (White et al., 2012). While recent studies have shown a direct link to mitochondrial fission and AMPK in catabolic conditions such as fasting, disuse, and cachexia very little has actually examined mitochondrial fusion (Iqbal, Ostojic, Singh, Joseph, & Hood, 2013; Jheng et al., 2012; White et al., 2012). One recent study suggests a role for MFN-2 in the regulation of muscle mass in cachectic cancer patients and mice (Xi et al., 2016). There are conflicting reports of AMPK regulating mitochondrial fusion proteins, some suggest a potential link however, a phosphoproteomic study demonstrated that AMPK did not interact with any known mitochondrial fusion protein but was responsible for fission in fasting conditions (Z. Chen et al., 2019; Toyama et al., 2016). However, in non-muscle cells a recent study suggest that AMPK activation of fusion prevented drug induced mitochondrial damage (Kang et al., 2016). The activation of mitochondrial fission by AMPK under energy stress classically serves to induce autophagy/mitophagy processes and activate biogenesis to replace the damaged mitochondria (Z. Chen et al., 2019; Toyama et al., 2016). In cachectic skeletal muscle biogenesis is suppressed suggesting a net loss of mitochondria and a constant energetic stress that may explain why AMPK and fission are chronically elevated. In the current studies we provide evidence that the cachexia suppression of MFN-1 and induction of DRP-1 require AMPK following a short term physiological fast. Unexpectedly, we also report that MFN-1 is induced 8 fold by re-feeding following a short term fast in both B6 and Min mice. The

induction of MFN-1 in Min mice by re-feeding occurred simultaneously with a suppression of AMPK suggesting MFN-1 is a target of AMPK however, whether this is direct or in-direct requires further investigation. Interestingly, DRP-1 was not altered by re-feeding in Min mice despite the AMPK suppression. In accordance with previously reported literature we also demonstrate that 4 weeks of wheel activity prior to fasting induced mitochondrial fission and fusion in B6 and Min mice. These changes coincided with a suppression of AMPK activation when compared to fasted Min mice alone and suggest that the restoration of mitochondrial quality control proteins improves the cachectic skeletal muscles ability to cope with a short term fast and as mentioned previously above restore protein turnover.

5.3 Overall Summary

Cachexia is a multifactorial syndrome that is associated with multiple systemic disruptions such as chronic inflammation, anemia, and insulin resistance that can impact muscle mass and metabolism (Argiles, Busquets, Stemmler, & Lopez-Soriano, 2014; Baracos et al., 2018). AMPK is often deemed as a cellular fuel gauge that modulates metabolism and protein turnover (Koh et al., 2008). AMPK is supposed to stimulate ATP synthesis while concurrently suppressing ATP consumption (Kjobsted et al., 2018). Thus, AMPK has been implicated in the regulation of mTORC1, E3 ligases, and autophagy/mitophagy (Carson et al., 2015; Hardee et al., 2017). Dysfunctional AMPK signaling is a common hallmark of cancer cachexia and is indicative of energetic stress (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017; Puppa et al., 2012; Rohm et al., 2016; Sandri, 2016; White, Baynes, et al., 2011b). Often coinciding with the elevation of AMPK in cachexia is the suppression of muscle protein synthesis and induction of

degradation (White, Baynes, et al., 2011b; White, Puppa, Gao, et al., 2013). These processes make up muscle proteostasis and are important for the regulation of skeletal muscle mass (Sanchez, Candau, et al., 2012; Sandri, 2016). The purpose of the current dissertation was to investigate if cachexia associated aberrant AMPK signaling is responsive to fasting, feeding, and increased wheel running activity to regulate skeletal muscle proteostasis.

Healthy mitochondria are essential for metabolism and what is commonly referred to as substrate flexibility. The concept of substrate flexibility is the ability of a tissue to freely switch substrates in order to generate ATP and restore metabolic homeostasis. Failure to do this results in energy stress and deficiency that can lead to disrupted metabolism and the activation of proteolytic pathways. Catabolic conditions such as fasting have been long studied as a classical activator of AMPK and its induction of protein degradation however, very little has been done to investigate fasting in cachexia (Bagherniya et al., 2018; Canto et al., 2010). Classically, under metabolic stress such as exercise or fasting where carbohydrates are depleted, metabolism will switch to utilizing lipids as fuel to generate ATP. This process is controlled by AMPK and its activation of ACC which then signals to initiate lipolysis and subsequently beta oxidation in the mitochondria. It is reasonable to suggest that in tumor bearing mice where mitochondrial quality control is disrupted that an acute fast could exacerbate the lack of substrate flexibility and lead to metabolic disruption. In the current study we provide evidence that AMPK activation during an acute fast in Min mice may be due to a lack of substrate flexibility. The activation of AMPK in Min mice by an acute fast is coupled with disrupted fusion, fission, and biogenesis. Fasting has recently been suggested as a

method to improve the response to chemotherapeutics in non-cachectic cancer patients however, our current results demonstrate that cachectic skeletal is hyper-sensitive to periods of short fasting (Brandhorst & Longo, 2016). In the current series of studies, we identify that cancer accelerates the fasting activation of AMPK signaling and induces muscle protein degradation. Furthermore, we identify that AMPK is required for the fasting induction of protein degradation in tumor bearing mouse skeletal muscle.

An important question that has come to the forefront of cachexia is whether skeletal muscle retains its anabolic plasticity to respond to nutrients and activity/exercise. Studies in cachectic cancer patients have demonstrated that the response to nutrients and exercise are often blunted and require a potent stimulus to override the block of protein synthesis (A. M. H. Horstman et al., 2016; K. Nakashima et al., 2006; Rennie et al., 1982). Furthermore, the use of increased physical activity as a therapeutic to improve mitochondrial quality control and protein synthesis has recently been suggested (Hardee et al., 2017; B. N. VanderVeen, Hardee, et al., 2017). We provide evidence that cachectic skeletal muscle responds to re-feeding following a fast by suppressing AMPK mediated degradation but fails to induce protein synthesis. These results suggest that an acute fast sensitizes skeletal muscle AMPK signaling to re-feeding and the influx of nutrients. Additionally, we also provide evidence that increased activity via wheel running suppresses the fasting induction of AMPK in cachectic skeletal muscle restoring mitochondrial quality control, protein turnover, and attenuating muscle mass loss. These results suggest that modulating AMPK through increased physical activity alleviates the fasting disruption of mitochondrial quality control and muscle protein turnover and in cachectic skeletal muscle.

Taken collectively the current series of studies provides evidence that AMPK is a critical modulator of skeletal muscle protein turnover and metabolism in cachectic skeletal muscle. Previous reports from our laboratory have suggested the disruption of AMPK during cachexia however, the current dissertation mechanistically examines AMPK's regulation of protein turnover and mitochondrial quality. The use of our muscle specific knockout model identified targets of regulation by AMPK in cachexia and fasting and spared gastrocnemius muscle mass. Additionally, we provide evidence that cachectic skeletal muscle has altered sensitivity to fasting and feeding and their roles in regulating protein turnover through the AMPK signaling pathway. Finally, we demonstrate that increased physical activity in cachectic mice alleviates aberrant AMPK signaling and restores mitochondrial quality control. The restoration of mitochondrial quality control may serve to improve the physiological response to short term fasting and attenuate disrupted proteostasis attenuating muscle mass loss . Overall, we identify AMPK as a potential therapeutic target for cancer cachexia and the regulation of proteostasis. Modu lating AMPK activity through interventions such as increased physical activity and exercise training could prove beneficial in the preservation of muscle mass through restoring mitochondrial quality control.

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APPENDIX A

DETAILED PROTOCOLS

Tissue Homogenization

1. Materials and Reagents

    a. Mueller Buffer

Mueller Buffer	Stock Solution	Desired Concentration	Volume of stock needed(ul)
HEPES	500mM	50mM	600
Triton-X100	100%	0.10%	6
EGTA (pH 8.0)	500mM	4mM	48
EDTA (pH 8.0)	500mM	10mM	120
Na4P2O7	100mM	15mM	900
β-glycerophosphate	2M	100mM	300
NaF	500mM	25mM	300
NaVO4	1M	5mM	50
dH2O	-	-	3585
Protease Inhibitor	-	-	60

    b. Mueller Diluent:

Mueller Diluent	Stock Solution	Desired Concentration	Volume of stock needed (ul)
Glycerol	100%	50%	1500
Na4P2O7	100mM	50mM	1500
EGTA (pH 8.0)	500mM	2.5mM	15
β-mercaptoethanol	500mM	1mM	6
Protease Inhibitor	-	-	30

2. Procedure

    a. Weigh out the samples to be used and place weighted portion into an Eppendorf tube labeled with the sample and M.

    b. Add 10ul/mg tissue of Mueller buffer to the homogenization tube and add

sample.

c. Homogenize in glass on glass tissue homogenizer keeping the sample in ice while homogenizing. (homogenize ~30s check sample repeat if needed)

d. Wash glass tissue homogenizer 3 times by dH2O between two samples.

e. Transfer tissue homogenate back to Eppendorf tube.

f. Spin samples at 13,000rpm for 10min at 4℃.

g. Transfer supernatant to clean Eppendorf tube labeled with sample and D, discard the pellet.

h. Add 5ul/mg tissue of Diluent buffer to the D tube and vortex.

i. Run protein assay (Bradford).

j. Dilute samples down to a working concentration in a new tube labeled with the sample and the working concentration. Keep both D tube and diluted samples in -80℃.

Protein Assay (Bradford)

1. Materials and Reagents
    a. Bradford Reagent (Bio-Rad, Catalog #: 5000006)
    b. Make a stock of 1mg/ml BSA in PBS, store aliquot in -20°C.
    c. Costar 96 well plate
2. Procedure
    a. In a clear flat bottom 96 well plate create a standard curve with the 1ug/ul BSA solution from 0-14ug in duplicate or triplicate

b. Dilute samples 1:5 in a new tube with water (5ul sample: 20ul dH20)
c. Add 5ul of the diluted samples to the wells of the plate being sure to run them in duplicate or triplicate
d. Make a 1:5 dilution of Bradford reagent. You will need enough for 300ul/well. Be sure to clean the glassware well before you use it with soap and water.
e. Add 300ul of diluted Bradford reagent to each well.
f. Let sit in dark drawer for 15 minutes
g. Read in plate reader at 595nm
h. Calculate protein concentration based on standard curve
i. Create curve being sure to subtract out the zero value from both curve and samples.
j. Calculate protein concentration using y=mx+b equation (sample = con*slope + intercept -> con=(sample-intercept)/slope)

SDS-PAGE/Western Blot

1. Materials and Reagents
    a. Acrylamide Solution (38.5% Acrylamide + 1% Bis-Acrylamide) 1.5M Tris-HCL, pH 8.8
    b. 1.0M Tris-HCL, pH 6.8
    c. 10% SDS
    d. 10% APS
    e. TEMED

f. 4×SDS Loading Buffer: 200 mM Tris-Cl (pH 6.8), 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol

g. SDS Running Buffer: 25mM Tris, 200mM Glycine, 0.1% SDS Protein Ladder (Bio-Rad, Catalog #: 1610373)

h. Transfer Buffer: 25mM Tris, 200mM Glycine, 20% Methanol 1×TBST: 50 mM Tris-Cl, 150 mM NaCl, 0.1% Tween-20 5% milk (solved in 1×TBST)

i. ECL plus (Thermo Scientific, Catalog #: 1610373)

2. Procedure – SDS-PAGE

    a. Make gel. Base the % off of what molecular weight the protein of interest is.

    b. Prepare samples

    c. Pipette desired amount of protein into Eppendorf tube

    d. Add correct volume of 4 × SDS loading dye

    e. Vortex and do a quick spin

    f. Heat in heat block at 100°C or in boiling water for 5 minutes

    g. Quick spin

    h. Load 4ul of protein ladder to the 1st lane in the gel

    i. Load all of sample on gel

    j. Run gel at 200V for ~1h or until samples have run through the gel

3. Procedure – Transfer

    a. Prepare 750ml of transfer buffer

b. Prepare membrane by placing in a small amount of methanol for 1 minute
c. Pour methanol into the transfer buffer and wash membranes with the transfer buffer
d. Set up the transfer with the black side of the holder facing down, sponge, blotting paper, gel, membrane (be sure there are absolutely no bubbles between the gel and the membrane), blotting paper, sponge, white/clear side.
e. Carefully close the sandwich and place in the transfer box black side to black side and clear side facing the red side of the transfer box.
f. Place ice pack in the box and pour remaining transfer buffer into the box.
g. Transfer either overnight at 70mA or for 200min at 200mA making sure to pack well with ice.

4. Procedure – Probing
    a. Prepare 5% milk in 1×TBST solution
    b. Remove membrane from transfer and place in ponceau solution for 3-5 minute
    c. Rinse off excess ponceau with dH2O and place membrane in plastic sheet and scan into computer
    d. Wash off ponceau with 1×TBST
    e. Block the membrane for 1h in 5% TBST milk solution (made in step 13)
    f. Incubate in primary antibody in milk solution
    g. Wash membranes 3× with 1×TBST for 5 minutes each
    h. Incubate in secondary antibody in milk solution for 1 hour

i. Wash membranes 3× with 1×TBST for 5 minutes each

j. Develop with ECL or ECL quantum.

Cytochrome C Oxidase Activity

1. Materials and Reagents

    a. 20 mM KCN; MW= 65.12, 13.02 mg/10 ml dH_2O

    b. 100 mM KPO_4 Buffer (pH 7)

        1. 0.1 M KH_2PO_4 (pH 5)

        2. 0.1 M $K_2HPO_4.3H_2O$ (pH 8)

    c. 10 mM K-Phosphate Buffer

    d. Extraction Buffer (100 mM Na-K-Phosphate, 2 mM EDTA; pH 7.2)

        1. 500 ml 0.1 M Na_2HPO_4. $2H_2O$;

            1. 8.9 g sodium phosphate with 0.372 g EDTA up to 500 ml

        2. 200 ml 0.1 M KH_2PO_4

            1. 2.7 g potassium phosphate with 0.149 g EDTA up to 200 ml.

        3. combine both solutions and pH to 7.2

    e. Test Solution (reduced cytochrome c, 2 mg/ml), for 10 ml (enough for 36 microplate wells);

        1. 20 mg of horse heart cytochrome c (Sigma, C-2506) in a scintillation vial

        2. add 1 ml of 10 mM KPO_4 buffer and dissolve cytochrome c

3. make up a small volume of 10 mg/ml sodium dithionite-10 mM KPO_4 stock solution (make fresh each experiment and use within twenty minutes)
4. add 40 μl of the dithionite stock solution to the test solution and observe red-orange colour change
5. add 8 ml of ddH_2O
6. add 1 ml of 100 mM KPO_4 buffer.

2. Procedure

a. Place powdered muscle samples in liquid N_2.
b. Add 50 μl of extraction Buffer to 1.5 ml Eppendorf tubes in the aluminium block on ice. (One Eppendorf per sample).
c. Add 5-7.5 mg tissue to each tube, recording exact tissue mass. Mix by tapping.
d. Add the volume of Extraction Buffer required to obtain a 20-fold dilution.
e. Add a stir bar and mix for 15 min. Make up Test Solution during this time and wrap in foil.
f. Sonicate each tube 3 x 3 seconds, cleaning the probe between samples.
g. Pipette some of 20-fold sample extract into new Eppendorf tube and add volume of Extraction Buffer required to obtain an 80-fold dilution. (eg. 50 μl of 20-fold extract + 150 μl Ext. Buffer = 200 μl of 80-fold sample extract). Keep 80-fold sample extract tube on ice for duration of experiment
h. Add 270 μl of Test Solution into 4-8 wells of 96-well microplate and

incubate at 30°C for 10 minutes to stabilize the temperature and absorbance.

i. Open KC4 plate reader program (on Triton). Select CONTROL icon, then PRE-HEATING tab, enter 30°C and select ON. (Do not run assay until KC4 temperature has reached 30°C.)

j. Select WIZARD icon, then READING PARAMETERS icon.

    1. Select Kinetic for Reading Type.
    2. Select Absorbance for Reader and 550 nm for wavelength (drop-down menu).
    3. Select Sweep for Read Mode.
    4. Select 96 Well Plate (default) for Plate Type.
    5. Enter first and last well to be read (eg. A1 and A4 if reading 4 samples simultaneously).
    6. Select Yes and Pre-heating and enter 30 for Temperature Control.
    7. For Shaking enter 0 for both intensity and duration (shaking is not necessary, and it will delay the first reading).
    8. Do not select either of the two options for Pre-reading.
    9. Click on the KINETIC… rectangular tile to open the Kinetic window.
    10. Enter run time (1 minute is recommended) and select MINIMUM for Interval time (under these conditions the minimum Interval time should be 3 seconds).

11. Select Allow Well Zoom During Read to see data in real time (optional).
12. Under Scales, checkmarks should appear for both Auto check boxes. Do not select Individual Well Auto Scaling.
13. Press OK to return to Reading Parameters window. Press OK to return to Wizard window. Press OK. Do not save the protocols

k. Set the multipipette to 250 μl and secure 4-8 yellow tips on the white projections (make sure they are on tight and all at the same height).

l. In a second, clean 96 well plate, pipette samples into 4-8 empty wells (start with A1). Recommended volumes: 30 μl of 80-fold extract for Mixed Gastroc, 10 μl for Heart. Adjust volumes according to oxidative capacity of the tissue. (eg. 25 μl for Red Gastrocnemius and 35 μl for White Gastrocnemius).

m. Remove microplate with Test Solution in 4-8 wells from the incubator (as long as it has been incubating for 10 minutes). Place this plate beside the plate with the sample extracts in it.

n. On KC4 program, select the READ icon and press the START READING icon, then press the READ PLATE button. A box will appear that says, “Insert plate and start reading”. Do not press OK yet but move the mouse so that the cursor hovers over the OK button.

o. Using the multipipette (set to 250 μl) carefully draw up the Test Solution. Make sure the volume is equal in all the pipette tips, and that no significant air bubbles have entered any of the tips.

p. Pipette the Test Solution into the wells with the sample extracts (the second plate). As soon as all the Test Solution has been expelled from the tips (do not wait for the second push from the multipipette), place the plate onto the tray of the plate reader and with the other hand on the mouse, press the OK button. (Speed at this point is paramount, as there is an unavoidable latency period between the time of pressing the OK button and the time of the first reading.)

q. If desired, add 5 μl KCN to one of the wells to measure any absorbance changes in the presence of the CYTOX inhibitor.

r. Once reading is complete, hold the CTRL key on the keyboard, and use the mouse to click once on each of the squares corresponding to a well that had sample in it. Once all the desired wells have been highlighted by a black square (up to a maximum of 8 wells), let go of the CTRL key and a large graph will appear with lines on it representing each sample.

s. To obtain the rate of change of absorbance over different time periods, select Options and enter the amount of time for which you would like a rate of change of absorbance to be calculated. The graph, along with one rate (at whichever time interval is selected) for each sample can be printed on a single sheet of paper, and the results can be saved.

t. The delta absorbance will appear in units of mOD/min and the number given will be negative. Convert this to OD/min by dividing by 1000 and omit the negative sign in the calculation. (eg. if Mean V: -394.8 mOD/mn, then use 0.395 OD/min

APPENDIX B

PROPOSAL

2.1 Specific Aims

Cancer Cachexia

Cancer-induced cachexia is a wasting syndrome that occurs in approximately 80% of all cancer patients and results in 30% of deaths (Argiles et al., 2010; Narsale & Carson, 2014; Puppa et al., 2012). Cancer-induced cachexia is a debilitating disease that diminishes quality of life due to severe loss of skeletal muscle mass and function (Baracos, 2011, 2013; Baracos et al., 2018). There are currently no approved therapeutic approaches for cancer-induced cachexia, therefore it is necessary to elucidate key underlying factors and processes as potential therapeutic targets. Skeletal muscle wasting during cancer cachexia occurs through altered protein turnover (suppressed anabolic signaling/increased degradation) (Aversa, Pin, Lucia, Penna, Verzaro, Fazi, Colasante, Tirone, Fanelli, et al., 2016; Baracos, 2000). Our laboratory and others have demonstrated suppressed anabolic signaling through mechanistic target of rapamycin (mTORC1) and the induction of muscle protein degradation in pre-clinical models of cancer cachexia (White, Puppa, Gao, et al., 2013). Coinciding with the suppression of mTORC1 signaling is the chronic activation of the adenosine monophosphate protein kinase (AMPK) (White, Puppa, Gao, et al., 2013). AMPK has been deemed a critical regulator of skeletal muscle degradation through the regulation E3 ligases and the process of autophagy, defined as the removal of organelles and protein aggregates through the lysosomal proteasome system (Lira et al., 2013). While altered protein turnover has been well characterized in the cachectic skeletal muscle of tumor bearing mice, less is understood about the role of AMPK in this process.

AMPK – The Checkpoint of Protein Metabolism

Skeletal muscle proteostasis fluctuates diurnally depending on different fasting and feeding durations as well as in response to activity and exercise stimuli. Adequate nutrient availability is key in the net loss and gain of skeletal muscle protein. AMPK is a nutrient sensitive energy sensor and is essential for skeletal muscle metabolic homeostasis (Romanello et al., 2010; Romanello & Sandri, 2015). AMPK may be capable of being activated by many different stimuli as well as diseases such as cancer cachexia. During severe cancer cachexia, skeletal muscle AMPK is dysregulated and chronically activated which could have dire consequences regarding muscle proteostasis. The most widely investigated activators of skeletal muscle AMPK is the process of prolonged energy deficiency (fasting). While fasting has been proposed to have beneficial effects in regard to aging and some cancers, less is understood about its role in cancer cachexia and the regulation of muscle proteostasis. Exercise or increased physical activity has been demonstrated to induce AMPK in a dose/intensity dependent manner and stimulate mitochondrial biogenesis and glucose uptake by skeletal muscle. However, less is understood about the role of exercise training or chronic increased activity in the regulation of AMPK, especially in diseased states such as cancer cachexia. Our laboratory has previously demonstrated that both treadmill exercise training and electrical stimulation (contraction) actually attenuate the chronic cachexia activation of AMPK and improve indices of mitochondrial quality control.

AMPK has long been established as a critical regulator of both protein synthesis and protein degradation. AMPK exerts its regulation the process of muscle protein synthesis through two inhibitory phosphorylation events. When activated, AMPK will

phosphorylate tuberous sclerosis protein 2 (TSC2) and raptor which inhibit mTORC1 activity. Inhibition of mTORC1 activates 4EBP1 and inhibits P70S6K, thus inhibiting the initiation of translation and ribosomal proteins. In addition to suppressing protein synthesis AMPK also controls degradation through the activation of its direct downstream targets ULK-1 and FOXO3a. Activation of these targets through phosphorylation induces both the ATP-independent autophagy lysosomal proteasome and the ATP-dependent ubiquitin proteasome respectively (FOXO3a is capable of regulating both degradation systems). The degradation of proteins through the ubiquitin proteasome is a highly coordinated process and in skeletal muscle utilizes the muscle specific E3 ligases Atrogin-1 and MuRF-1 which are controlled upstream by FOXO3a and were discovered initially through the studies examining starvation and fasting conditions. These E3 ligases allow for targeted proteins to be recognized by the 26S proteasome and ensure specificity of the UPS and additional adaptor proteins(Murton et al., 2008). The process of autophagy is induced through the AMPK activation of ULK-1 and has been extensively investigated with numerous catabolic conditions including fasting (Yan et al., 2012). Autophagy is a highly conserved cellular degradation pathway that can be initiated selectively and non-selectively by sequestering cytosolic substrates within a phagophore which is then conjugated with ubiquitination-linked proteins and degraded by a lysosome (Sanchez, Csibi, et al., 2012; Vainshtein & Hood, 2015).

Conversely, a common suppressor of AMPK is nutrient stimulation through the consumption of glucose, a mixed meal (feeding), or amino acids (Koh et al., 2008; Long & Zierath, 2006). Feeding has been widely investigated as a regulator of mTORC1 and AMPK. The consumption of glucose or a mixed meal will induce a glucose and insulin

response activating the IGF-1/AKT signaling cascade(Sengupta et al., 2010). AKT will then phosphorylate TSC2 releasing its inhibition of mTORC1(Sengupta et al., 2010). Both AMPK and mTORC1 are energy sensing kinases and regulate one another through pathway cross talk. AMPK and mTORC1 negatively regulate one another, for example during energy demand AMPK is activated thereby suppressing energy consuming pathways (mTORC1) (Koh et al., 2008; Long & Zierath, 2006; Sanchez, Csibi, et al., 2012). Muscle mTORC1 signaling is activated with both exercise and feeding and will phosphorylate ULK-1 at Ser 757 and inhibit the ULK-1 activation of downstream autophagy signaling (Laker et al., 2017; Sanchez, Csibi, et al., 2012). To date, our lab and others have demonstrated that the cachectic environment elevates skeletal muscle AMPK following a 5 hour fast, showing that a very short period of fasting is capable of inducing AMPK in the cancer environment, suggestive of metabolic dysregulation (Penna et al., 2010; White, Baynes, et al., 2011a; White, Puppa, Gao, et al., 2013). Concomitant with the induction of AMPK, our laboratory has demonstrated mTORC1 suppression during cancer cachexia, signifying a role for AMPK in the suppression of anabolic signaling (Hardee et al., 2018; Hardee et al., 2016; White, Puppa, Gao, et al., 2013). Furthermore, preliminary data from our laboratory suggests that the cachexia-related metabolic dysfunction sensitizes skeletal muscle response to fasting. While the chronic activation of AMPK and its potential role in anabolic suppression has been examined in detail, the role of this signaling axis in the regulation of protein turnover (synthesis and degradation through UPS and autophagy) is poorly understood in the skeletal muscle of cachectic mice. Moreover, given the sensitivity of this pathway to nutrient availability, further

investigation is warranted to elucidate if the feeding regulation of autophagy is disrupted in the cancer environment.

Purpose and Hypothesis

The **long-term goal** of this research proposal is to establish AMPK's role in the regulation of skeletal muscle mass during cancer cachexia. The **purpose of the current proposal** is to determine if cachexia associated aberrant AMPK signaling is responsive to fasting, feeding, and increased wheel running activity to regulate skeletal muscle proteostasis. **Our central hypothesis** is that the cachexia activation of AMPK alters the skeletal muscle response to fasting, feeding, and voluntary wheel activity in a phenotype specific way through the acceleration protein degradation while suppressing synthesis.

Specific Aims

Using preclinical models of cancer cachexia and manipulations of the AMPK signaling pathway, we plant to test our hypothesis using the following 3 specific aims:

Specific Aim 1: Determine if downstream AMPK signaling is necessary for muscle protein turnover in cachectic skeletal muscle.

Specific Aim 2: Determine if cancer cachexia disrupts the physiological regulation of AMPK signaling by fasting/feeding in skeletal muscle.

Specific Aim 3: Determine if 4 weeks of voluntary wheel running activity regulates aberrant AMPK signaling in response to physiological fasting during cancer cachexia.

While the suppression of anabolic signaling and the activation of AMPK has been established in cachectic skeletal muscle, less is known about how AMPK mechanistically regulates total protein turnover during cancer cachexia and if these effects are dependent on muscle phenotype. Significant gaps remain in our understanding of the cachexia

induced activation of AMPK, whether AMPK is necessary for the attenuation of muscle mass loss remains to be elucidated. The results from these studies will provide mechanistic insight into the AMPK regulation of muscle during cancer cachexia and provide new potential mechanisms and therapeutic targets to further the cachexia field.

Specific Aim 1: Determine if downstream AMPK signaling is necessary for muscle protein turnover in oxidative and glycolytic cachectic skeletal muscle.

Aim 1.1: Determine the role of AMPK in the cancer cachexia regulation of TSC2 and raptor mediated skeletal muscle protein synthesis.

Aim1.2: Determine the role of AMPK in the cancer cachexia regulation of ULK-1 and FOXO3 mediated skeletal muscle degradation through autophagy and E3 ligase expression.

Aim 1.3: Determine the role AMPK in the cancer cachexia regulation of skeletal muscle mitochondrial quality control processes of biogenesis, fission, and fusion.

Specific Aim 2: Determine if cancer cachexia disrupts the physiological regulation of AMPK signaling by fasting/feeding in oxidative and glycolytic skeletal muscle.

Aim 2.1: Determine the effect of fasting and re-feeding on the cachexia regulation of protein synthesis via AMPK signaling and downstream targets TSC2 and raptor.

Aim 2.2: Determine the effect of fasting and re-feeding on the cachexia regulation of AMPK degradation targets ULK-1 and FOXO3 and their downstream processes of autophagy and E3 ligase expression.

Aim 2.3: Determine the effect of fasting and re-feeding on the cachexia regulation of AMPK mediated mitochondrial quality control processes biogenesis, fission, and fusion.

Specific Aim 3: Determine if in oxidative and glycolytic cachectic skeletal muscle, 4 weeks of voluntary wheel running activity regulates aberrant AMPK signaling in response to physiological fasting.

Aim 3.1: Determine if 4 weeks of voluntary wheel running activity regulates the cachexia disruption of AMPK signaling and downstream targets TSC2, raptor, and muscle protein synthesis in response to a physiological fast.

Aim 3.3: Determine if 4 weeks of voluntary wheel running activity regulates the cachexia disruption of AMPK downstream degradation targets ULK-1, FOXO3 in response to a physiological fast.

Aim 3.4: Determine if 4 weeks of voluntary wheel running activity regulates the cachexia disruption of AMPK mediated mitochondrial quality control processes biogenesis, fission, and fusion in response to a physiological fast

Proposed Working Model

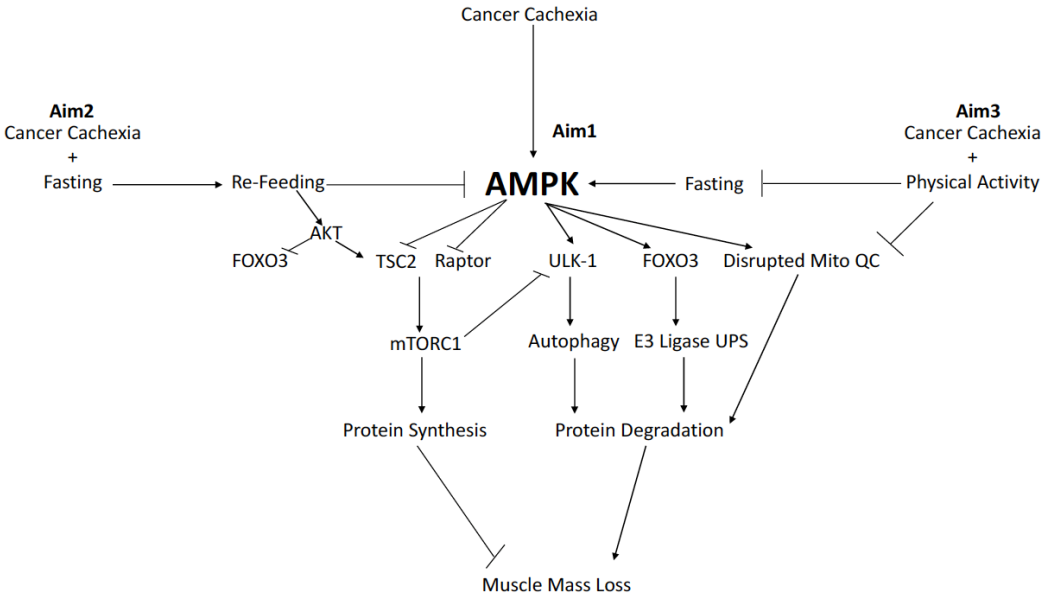

Figure B.1. Proposed working model of AMPK regulation of proteostasis during cancer cachexia**.** The overall purpose of the study is to determine the role of AMPK in the disruption of protein turnover during cancer cachexia. **Our central hypothesis** is that the cachexia activation of AMPK alters the skeletal muscle response to fasting, feeding, and voluntary wheel activity in a phenotype specific way through the acceleration protein degradation while suppressing synthesis. Specific aim 1 will determine if downstream AMPK signaling is necessary for muscle protein turnover in oxidative and glycolytic cachectic skeletal muscle. Specific aim 2 will determine if cancer cachexia disrupts the physiological regulation of AMPK signaling by fasting/feeding in oxidative and glycolytic skeletal muscle.. Specific aim 3 will determine if in oxidative and glycolytic cachectic skeletal muscle, 4 weeks of voluntary wheel running activity regulates aberrant AMPK signaling in response to physiological fasting..

2.2 Preliminary Data

AMPK Regulation of Anabolic Signaling in $Apc^{Min/+}$ Mice

Our laboratory has published extensively utilizing the $Apc^{Min/+}$ mouse model of cancer cachexia(Puppa et al., 2012; White, Baynes, et al., 2011a). The cachexia induced activation of AMPK has been demonstrated by our laboratory to occur during chronic inflammation and the progression of cancer cachexia. While our laboratories initial studies demonstrated a role for AMPK in the regulation muscle protein turnover, there has not been any further mechanistic examination of this signaling process. The activation of AMPK during cachexia suppresses basal muscle protein synthesis through inhibition of mTORC1 signaling. Our research group has demonstrated that this chronic activation of AMPK can be attenuated utilizing an IL-6 receptor antibody and an acute injection of glucose (nutrients) (White, Baynes, et al., 2011a). However, these studies did not detect a change in total muscle protein synthesis. The cancer cachexia field has continually measured AMPK in many pre-clinical models but has yet to establish its true regulatory role in skeletal muscle protein turnover.

In many models that have impaired anabolic signaling such as cancer cachexia, there is often an induction of AMPK activation (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017; Puppa, Gao, et al., 2014; White, Puppa, Gao, et al., 2013). These results suggest that mTORC1 is an essential part of normal ATP production and its disruption induces energetic stress and AMPK activity (Brown, Rosa-Caldwell, Lee, Blackwell, et al., 2017). The suppression of AMPK in P70S6 kinase deficient myotubes has been demonstrated to rescue myotube size and protein synthesis signaling further demonstrating a role for AMPK in the regulation of anabolic dependent signaling

processes (White, Puppa, Gao, et al., 2013). Studies have also demonstrated that the constant infusion of AICAR suppresses P70S6 kinase activation leading to suppressed synthesis rates (Suwa et al., 2003). This suppression is directly correlated to the chronic activation of AMPK (Suwa et al., 2003). Conversely, activation of mTORC1 in AMPK deficient myotubes has been demonstrated to robustly induce synthesis rate and lead to a 1.5 fold increase in myotube size (White, Puppa, Gao, et al., 2013). Recent cachexia research suggests that stimulating AMPK using AICAR is sufficient to attenuate inflammation during cancer cachexia and improve loss of mass (Hall et al., 2018). However, our key preliminary data suggest that the loss of AMPK during cancer cachexia is sufficient to induce skeletal muscle anabolic signaling proteins ribosomal protein S6 (rpS6) and 4-eukaryotic binding protein 1 (4EBP-1) when compared to control $Apc^{Min/+}$ mice (Figures B.2A, 2B, and 2C).

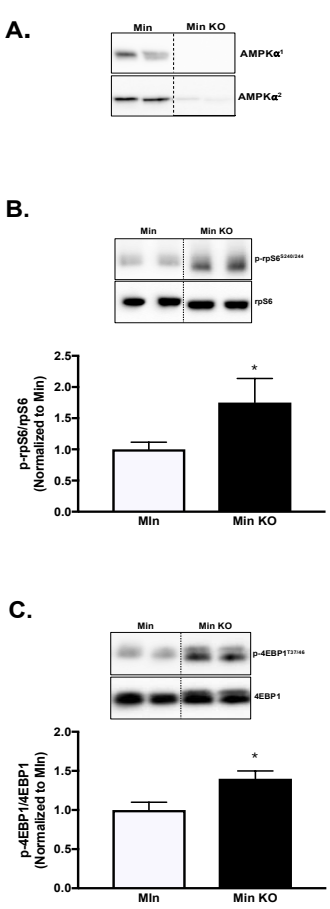

Figure B.2. AMPK Regulation of Skeletal Muscle Anabolic Signaling During Cancer Cachexia. A) Total protein expression of AMPK$^{\alpha 1}$ and AMPK$^{\alpha 2}$ in the gastrocnemius of *Apc*$^{Min/+}$ (Min) and AMPK$^{\alpha 1\alpha 2}$ *Apc*$^{Min/+}$ (Min KO) mice. B) *Upper:* Immunoblot of p-rpS6$^{S240/244}$ and rpS6 in the gastrocnemius muscle of Min and Min KO mice. *Lower:* Quantification of above immunoblot. C) *Upper:* Immunoblot of p-4EBP-1$^{T37/46}$ and 4EBP-1 in the gastrocnemius muscle of Min and Min KO mice. *Lower:* Quantification of above immunoblot. N=6-10 mice per treatment group. Data presented as means ± standard error. Students pre-planned t-test. Statistical significance was set at $p<0.05$. All values normalized to Min. * denotes statistically significant from Min.

Cachexia, AMPK, Fasting, and Autophagy

AMPK has been very well characterized for its roles in exercise and metabolism (K. Nakashima & Yakabe, 2007; Sanchez, Csibi, et al., 2012) . However, only a few researchers have examined AMPK's role in cancer induced muscle wasting. These studies have provided putative mechanisms of action and provided the initial evidence that place AMPK at the center of muscle wasting. Few studies have mechanistically tried to investigate the role it plays in regulating autophagy and protein synthesis during the progression of the disease (Hall et al., 2018; Rohm et al., 2016; White, Puppa, Gao, et al., 2013). AMPK as described above is a master energy sensor and regulator of skeletal muscle metabolism and is capable of being activated by numerous stimuli. Downstream it regulates a host of signaling processes related to lipid metabolism, glucose metabolism, protein synthesis, degradation, autophagy, and mitochondrial biogenesis which are all disrupted in cancer cachexia (Argiles, Busquets, et al., 2015; Carson et al., 2016; Sandri, 2016).

To date, our lab and others have demonstrated that the cachectic environment elevates skeletal muscle AMPK and indices of autophagy following a 5 hour fast, showing that a very short period of fasting is capable of inducing AMPK in the cancer environment, suggestive of metabolic dysregulation (Penna et al., 2010; White, Baynes, et al., 2011a; White, Puppa, Gao, et al., 2013). However, we now have key preliminary data demonstrating that AMPK signaling is induced during the fed (ad libitum access to food) state during cancer cachexia when compared to B6 mice (Figure B.3A, B, and C).

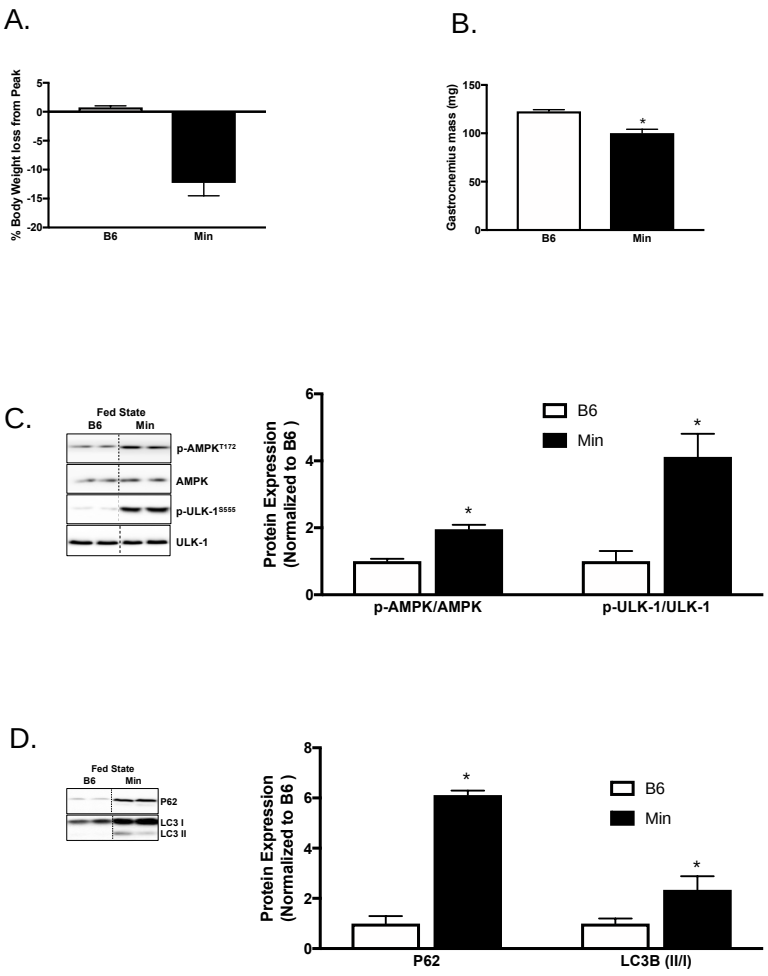

Figure B.3. AMPK Signaling in the Fed State During Cancer Cachexia. A) % bodyweight loss from peak in B6 and Min mice. B) Gastrocnemius mass in B6 and Min mice. C) *Left:* Representative immunoblots of p-AMPK^{T172}, AMPK, p-ULK-1^{S555}, and ULK-1 in the gastrocnemius of Fed B6 and Min mice. *Right:* Quantification of immunoblots. D) *Left:* Representative immunoblots and quantification of P62 and LC3B II/I in the gastrocnemius of Fed B6 and Min mice. Values are means ± SEM. Significance was set at $p<0.05$. * Signifies different from B6. N=8-9 per group. Students pre-planned t-test used for all analysis.

AMPK has long been known to be a key regulator of autophagy (Kjobsted et al., 2018). While some initial studies reported conflicting results on AMPK's role in autophagy, it was later determined that these results were dependent on cell type and conditions used. Under normal basal conditions both mTORC1 and AMPK are associated with ULK-1(J. Kim et al., 2011). Classically, a fast or nutrient deprivation will lead to AMPK activation and suppress mTORC1 which promotes autophagy through the direct phosphorylation of ULK-1(Bagherniya et al., 2018). Cancer cachexia and skeletal muscle autophagy has become a very intriguing area of investigation (Penna et al., 2014). Numerous mouse models of cancer cachexia have demonstrated elevations in LC3 as well as p62 (Penna, Costamagna, et al., 2013; Tessitore et al., 1994; Tessitore et al., 1993). The gastrocnemius muscle of C26 and LLC tumor bearing mice exhibited robust inductions of Beclin-1, LC3 II, and p62 at 11 and 14 days which coincided with a reduction in cathepsin activity(Pettersen et al., 2017; Talbert et al., 2014; White, Baltgalvis, et al., 2011). Our laboratory has also demonstrated that LC3 II and Beclin-1 are also induced in the Min mouse model of cancer cachexia (White, Baynes, et al., 2011a).

Interestingly, some researchers argue that the elevation in LC3 and p62 is indicative of dysfunctional clearance of the autophagosome by the lysosome. The induction of skeletal muscle autophagy in tumor bearing animals is typically demonstrated by increased LC3 II levels, which are further induced in the presence of a lysosomal inhibitor such as colchicine (a recently employed technique to determine true autophagy flux(Ju et al., 2010). The common link in pre-clinical models of cachexia appears to be LC3 II and p62, which are often reported as accumulated in cachectic mice

(Pigna et al., 2016b). Moreover, preliminary data from our laboratory suggests that the cachectic environment sensitizes skeletal muscle to a 12-hour fast through the hyper-activation of AMPK and induction of autophagy when compared to B6 mice (Figures B.4A, B, C, D, and E). We also have preliminary data demonstrating that AMPK is necessary for the 12-fast induction of autophagy in *Apc*$^{Min/+}$ Mice (Figures B.5 A and B).

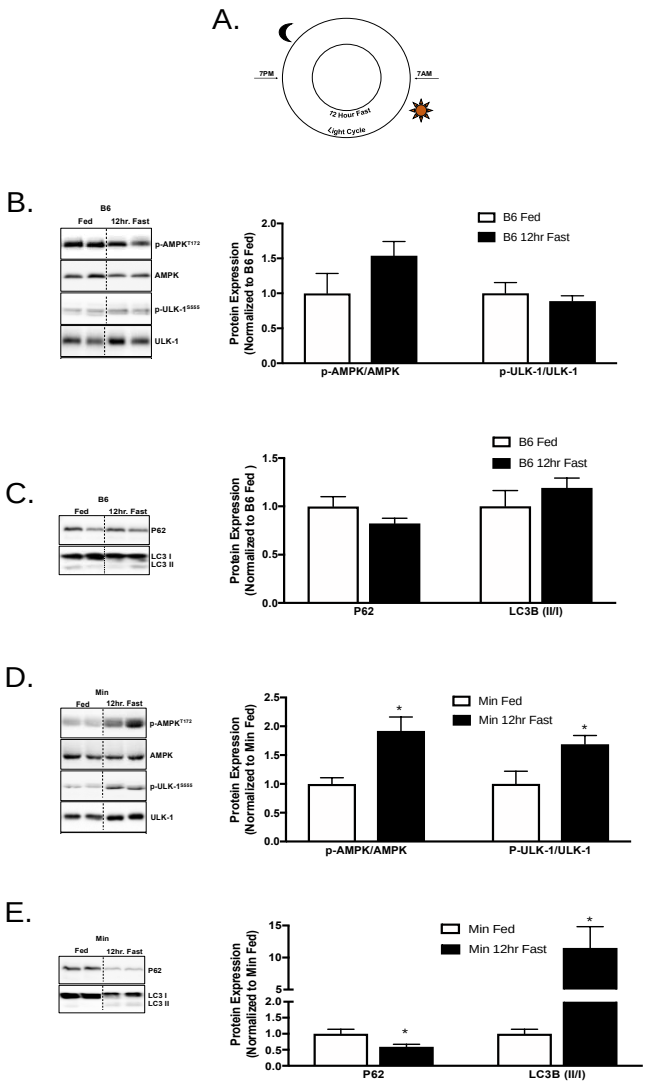

Figure B.4. Skeletal Muscle AMPK and Autophagy Response to a 12-hour Fast. A) Experimental design figure depicting when 12-hour fast and ad-libitum access occurred. B) *Left:* Representative immunoblots of p-AMPK (T172), AMPK, p-ULK-1(S555), and ULK-1 protein expression in gastrocnemius of B6 fed and 12-hour fasted mice. *Right:* Quantified results of immunoblots. C) *Left:* Representative immunoblots of P62 and LC3 II/I protein expression in gastrocnemius of B6 fed and 12-hour fasted mice. *Right:* Quantified results of immunoblots D) *Left:* Immunoblot of p-AMPK^{T172}, AMPK, p-ULK-1^{S555}, and ULK-1 protein expression in gastrocnemius of Min fed and 12-hour fasted mice. *Right:* Quantified results of immunoblots. E) *Left:* Immunoblot of P62 and LC3 II/I protein expression in gastrocnemius of Min fed and 12-hour fasted mice. *Right:* Quantified results of immunoblots. Values are means ± SEM. Significance was set at $p<0.05$. * Signifies different from Fed state. N=8-9 per group. Students pre-planned t-test used for all analysis.

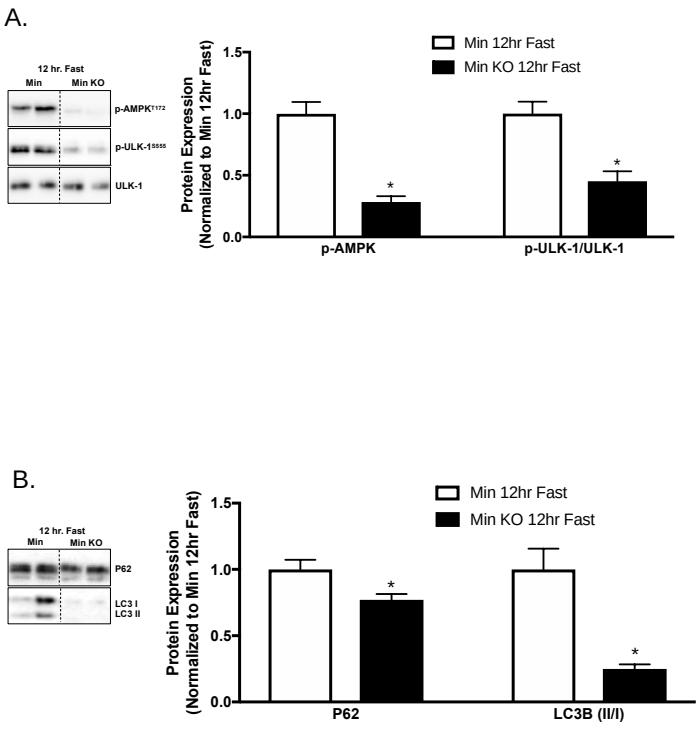

Figure B.5. AMPK Regulation of Fasting Induced Autophagy in *Apc*$^{Min/+}$ Mice. A) *Left:* Representative immunoblots of p-AMPK^{T172}, AMPK, p-ULK-1^{S555}, and ULK-1 in the gastrocnemius of Min and Min KO mice. *Right:* Quantification of immunoblots. D) *Left:* Representative immunoblots of P62 and LC3B II/I in the gastrocnemius of Fed B6 and Min mice. *Right:* Quantification of immunoblots. Values are means ± SEM. Significance was set at $p<0.05$. * Signifies different from Min. N=7-10 per group. Students pre-planned t-test used for all analysis.

Cachexia and the Response to Feeding

Of recent interest in cancer cachexia and protein synthesis is the new concept of anabolic resistance (A. M. H. Horstman et al., 2016). Many cancer patients experience weight loss at the time of diagnosis and as mentioned previously, muscle loss is indicative of poor prognosis in many cancers(Baracos, 2006a, 2011; Baracos et al., 2018; Romanello & Sandri, 2015). An intriguing question among researchers is whether cachectic muscle retains anabolic plasticity. This could prove beneficial for clinicians looking to provide treatment and improve muscle mass and function. While circadian fluctuations in protein turnover are the established norm in muscle mass regulation, it has become evident that protein synthesis is highly responsive to environmental stimuli. Nutrients or feeding and exercise are extremely potent stimulators of skeletal muscle protein synthesis in basal healthy conditions; however, numerous wasting conditions have demonstrated reduced sensitivity to these anabolic signals(Argiles, Lopez-Soriano, et al., 2015; Bodine, 2013; Bowen et al., 2015). Even the smallest decrement in daily protein synthesis could significantly impact muscle maintenance in wasting conditions, it is logical to suggest that anabolic resistance could also contribute to muscle loss during cancer cachexia (Cuthbertson et al., 2005). Surprisingly, the cancer cachexia field is actually lacking in the study of pre-clinical model studies examining anabolic resistance. However, our laboratory has demonstrated blunted mTORC1 activation in response to glucose administration in cachectic skeletal muscle (White, Puppa, Gao, et al., 2013). We have key preliminary data demonstrating that free ad-libitum access to a food pellet following a 12-hour fast is sufficient to induce blood glucose and phosphorylation of AKT (T308) in $Apc^{Min/+}$ mice (Figure B.6A and B).

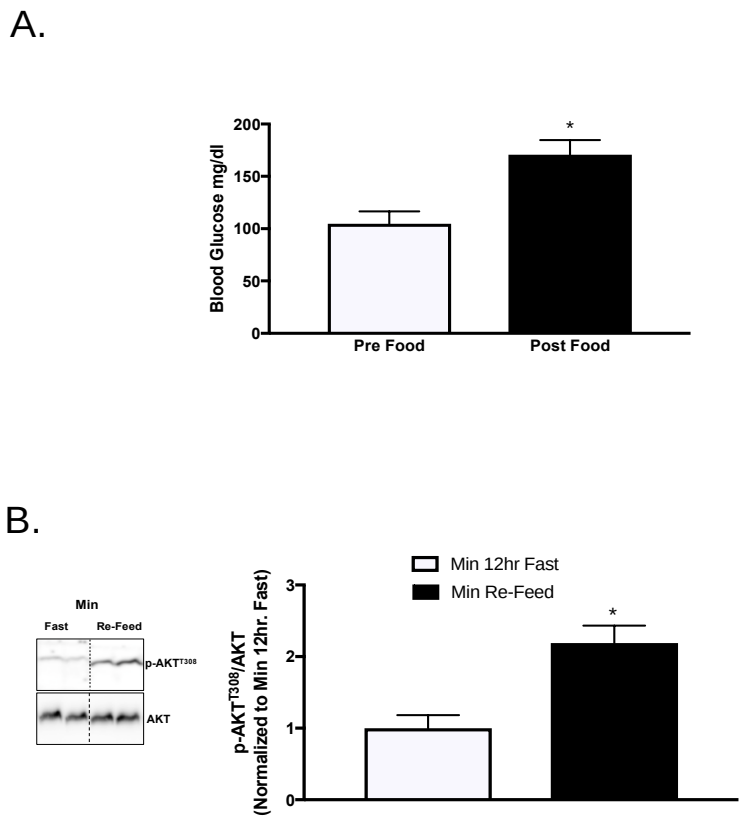

Figure B.6. 1-hour Re-Feed Induces Blood Glucose and AKT. A) Blood glucose of 1-hour Re-Feed mice pre and post food access. B) *Left:* Representative immunoblots of p-AKT^{T308} and AKT in the gastrocnemius of 12-hour fast and 1-hour Re-Feed and Min mice. *Right:* Quantification of immunoblot. Values are means ± SEM. Significance was set at $p<0.05$. * Signifies different from Min 12-hour fast. N=8-10 per group. Students pre-planned t-test used for all analysis.

2.3 Research Design and Methods

*Specific Aim 1: Determine if downstream AMPK signaling is necessary for muscle protein turnover in oxidative and glycolytic cachectic skeletal muscle***.**

Aim 1.1: Determine the role of AMPK in the cancer cachexia regulation of TSC2 and raptor mediated skeletal muscle protein synthesis.

Aim1.2: Determine the role of AMPK in the cancer cachexia regulation of ULK-1 and FOXO3 mediated skeletal muscle degradation through autophagy and E3 ligase expression.

Aim 1.3: Determine the role AMPK in the cancer cachexia regulation of skeletal muscle mitochondrial quality control processes of biogenesis, fission, and fusion.

Premise

The loss of muscle mass during cancer cachexia is detrimental to quality of life and is indicative to poor prognosis (Baracos, 2000). Additionally, muscle mass loss is often an indicator of poor response to cancer treatment and therapeutics such as chemotherapy (Barreto et al., 2016). The induction of muscle mass loss during cancer cachexia has often coincided with the induction inflammation and AMPK. AMPK heterotrimeric complex expression has also been previously reported to be regulated by muscle phenotype. However, the consequences and mechanisms of this differential expression has not yet been determined. As previously mentioned, AMPK is capable of suppressing anabolic signaling through mTORC1 and inhibiting protein synthesis (Koh et al., 2008). AMPK has also been established as a key modulator of mitochondrial quality

control, having direct regulation of mitochondrial biogenesis and fission. Furthermore, AMPK is capable of regulating multiple downstream signaling pathways contributing to skeletal muscle degradation such as the ubiquitin proteasome and autophagy lysosome system (Kjobsted et al., 2018).

Aim 1.1 Experimental Design

The purpose of Aim 1.1 is to determine the role of AMPK in the cancer cachexia regulation of TSC2 and raptor mediated skeletal muscle protein synthesis. We hypothesize that loss of skeletal muscle AMPK will alleviate the cachexia-induced inhibitory AMPK phosphorylation of TSC2 at (Ser1387) and raptor at (Ser792). We also hypothesize that the loss of AMPK will increase protein synthesis in glycolytic but not oxidative muscle. We will measure phosphorylated TSC2 Ser1387 and raptor Ser792 using western blotting. We will measure skeletal muscle protein synthesis using puromycin incorporation (SUnSET technique). Briefly, at approximately 12 weeks of age both control floxed $Apc^{Min/+}$ (non HSA containing) and Min KO mice received 5 daily injections of tamoxifen to induce HSA Cre recombinase deletion of $AMPK^{\alpha 1}$ and $AMPK^{\alpha 2}$ in skeletal muscle. A one week washout period following tamoxifen treatment will be completed to avoid any acute effects of the treatment. At 14 weeks of age mice initiated the study and will be tracked weekly for bodyweight and activity until sacrifice at ~20 weeks of age. At sacrifice tissues will be dissected, weighed and snap frozen in liquid nitrogen. The gastrocnemius and soleus muscle will be utilized in all analysis for Aim 1.

Aim 1.1 Primary Outcomes

Indices of Cachexia:

The progression of cachexia in the male *Apc*$^{Min/+}$ mouse has been well described (Baltgalvis et al., 2010). Body weight will be measured weekly throughout the early and mid-stages of adulthood of the mice to determine the relative loss of body weight in individual mice. The muscle and tissue weights of each mouse will be determined at sacrifice. Intestines will be collected and analyzed for total tumor burden. Blood will be collected immediately prior to sacrifice and plasma IL-6 levels will be examined using an ELISA.

Analysis of Skeletal Muscle AMPK, TSC2, raptor, and Puromycin:

Phosphorylated AMPK (T172) and total AMPK, will be measured using will be measured in the gastrocnemius and soleus using western blotting techniques (Fix et al., 2018). To truly assess AMPK activation phosphorylated ACC (S79) and total ACC will also be measured. Phosphorylated and total TSC1/2, and raptor will be measured using will be measured in the gastrocnemius and soleus using western blotting techniques (Fix et al., 2018). Protein synthesis will be determined using the SUnSET technique as previously described (Hardee et al., 2018). Briefly, 30 minutes prior to sacrifice mice will receive an intraperitoneal injection of puromycin at a concentration of 0.040 μmol/g body weight in warm sterile PBS. Protein homogenates are then fractionated on gels using western blotting technique as previously described. Blots are incubated in puromycin antibody at a dilution of 1:2000 for a duration of 4-6 hours. Total puromycin incorporation will be expressed relative to Min. The phosphorylated and total ratios of

these proteins will be quantified using Image J software and graphed statistically using Prism GraphPad 7.

Aim 1.1 Secondary Outcomes

Skeletal Muscle AKT:

Phosphorylated and total protein expression of AKT T308 and total AKT will be measured in the gastrocnemius using western blotting techniques(Fix et al., 2018). The phosphorylated and total ratio of this protein will be quantified using Image J software and graphed statistically using Prism GraphPad 7.

Aim 1.1 Statistical Analysis

Determining Statistical Significance:

The purpose of this experiment is to determine the role of AMPK on the regulation of skeletal muscle anabolic signaling during cancer cachexia. Mice will be divided into two groups consisting of Min and Min KO. A pre-planned students t-test will be employed to conduct analysis and significance set at $p<.05$. All statistical analysis will be performed using GraphPad Prism 7 software. Values will be presented as means ± the standard error of the mean.

Power analysis:

A power analysis was performed using a similar experimental design to determine the sample size needed to detect a significant difference. Power (1-β) was set to 0.8 and error of probability (α) was set at 0.05. Based on previous cancer cachexia analysis of anabolic signaling proteins and preliminary data from our laboratory, to achieve significance for

an estimated 20 ± 5% difference between groups, a minimum sample size of 6 is needed for each group.

Aim 1.2 Experimental Design

The purpose of Aim 1.2 is to determine the role of AMPK in the cancer cachexia regulation of ULK-1 and FOXO3 mediated skeletal muscle degradation through autophagy and E3 ligase expression. We hypothesize that loss of skeletal muscle AMPK will inhibit the cachexia activation of ULK-1 and FOXO3 in both oxidative and glycolytic skeletal muscle. The same experimental groups as Aim 1.1 will be used in Aim 1.2. At approximately 12 weeks of age both control floxed mins (non HSA containing) and Min KO mice will receive 5 daily injections of tamoxifen to induce HSA Cre recombinase deletion of $AMPK^{\alpha 1}$ and $AMPK^{\alpha 2}$ in skeletal muscle. A one week washout period following tamoxifen treatment will be completed to avoid any acute effects of the treatment. At 14 weeks of age mice will initiate the study and will be tracked weekly for bodyweight and activity until sacrifice at ~20 weeks of age. Similar muscle preparation of protein homogenates (gastrocnemius and soleus) will be performed as in aim 1.1, however protein expression for skeletal muscle degradation markers will be analyzed.

Aim 1.2 Primary Outcome

ULK-1and FOXO3a:

Phosphorylated ULK-1 S555 and total ULK-1 Phosphorylated and total protein expression will be measured by Western blot as previously described in both the gastrocnemius and soleus muscle (Fix et al., 2018). FOXO3a activation will be

determined by S253 phosphorylation. Proteins will be expressed relative to Min or Control.

E3 Ligases Atrogin-1 and MuRF-1:

Total protein expression will be measured by Western blot as previously described (Fix et al., 2018) for E3 ligases Atrogin-1 and MuRF-1. Proteins will be expressed relative to Min or Control.

Autophagy Signaling, P62, and LC3II/I:

total P62, and LC3II/I will be examined via western blot as previously described. Proteins will be expressed relative to Min or Control.

Aim 1.2 Secondary Outcome

Protein ubiquitination:

Total ubiquitination of muscle proteins will be determined by Western blot as previously described (Fix et al., 2018) . Total ubiquitination will be expressed relative to Min or Control.

Aim 1.2 Statistical Analysis

Determining Statistical Significance: See Aim 1.1

Power Analysis: See Aim 1.1

Aim 1.3 Experimental Design

The purpose of Aim 1.3 is to determine the role AMPK in the cancer cachexia regulation of skeletal muscle mitochondrial quality control processes of biogenesis, fission, and fusion. We hypothesize that loss of muscle AMPK during cachexia suppresses mitochondrial biogenesis and fission while inducing fusion. The same experimental groups as Aim 1.1 will be used in Aim 1.2. At approximately 12 weeks of age both control floxed mins (non HSA containing) and Min KO mice will receive 5 daily injections of tamoxifen to induce HSA Cre recombinase deletion of $AMPK^{\alpha 1}$ and $AMPK^{\alpha 2}$ in skeletal muscle. A one week washout period following tamoxifen treatment will be completed to avoid any acute effects of the treatment. At 14 weeks of age mice will initiate the study and will be tracked weekly for bodyweight and activity until sacrifice at ~20 weeks of age. Similar muscle preparation of protein homogenates will be performed as in aim 1.1, however protein expression for mitochondrial quality control will be measured and analyzed.

Aim 1.3 Primary Outcomes

Mitochondrial Quality Control: To assess AMPK regulation of mitochondrial quality control during cancer cachexia. Total protein expression for Pgc-1α, MFN-1, and DRP-1 will analyzed via western blot as previously described.

Aim 1.3 Statistical Analysis

Determining Statistical Significance:

See Aim 1.1

Power Analysis See Aim 1.1

Aim 1 Overall Interpretation and Potential Pitfalls

This set of experiments will establish the role of AMPK in the regulation of skeletal muscle protein turnover during cancer cachexia. Aim 1 will establish of AMPK is necessary for muscle protein turnover in oxidative and glycolytic cachectic skeletal muscle . I have strong preliminary data demonstrating that the loss of skeletal muscle $AMPK^{\alpha1\alpha2}$ increases indices of the mTORC1 pathway that control anabolic signaling and protein synthesis in the gastrocnemius (glycolytic) of $Apc^{Min/+}$ mice. Furthermore, this aim will also establish if cachexia-induced changes in mitochondrial quality occur through AMPK. Additionally, this aim will establish if cachexia mediated changes in muscle mass are related to increased skeletal muscle protein synthesis or a suppression degradation through utilization of the muscle specific AMPK knockout. Valuable information can be gained if any of my hypothesis are rejected, and loss of skeletal muscle AMPK in $Apc^{Min/+}$mice does not improve any of these processes.

Aim 1 Methods

Animals

The $Apc^{Min/+}$ mouse is a genetic model of colorectal cancer and cachexia (Baltgalvis et al., 2010). These mice harbor a heterozygous mutation in the adenomas polyposis coli (APC) gene, which promotes the development of intestinal tumors beginning as early as 4 weeks of age (Shoemaker, Luongo, Moser, Marton, & Dove, 1997). Mice develop an IL-6-dependent cancer cachexia phenotype between 3 and 6 months of age (Baltgalvis et al., 2010). Due to the slow onset and progression of body weight loss this model is advantageous as treatments can be started after the initiation of

cancer cachexia. Male *Apc*$^{Min/+}$ mice on a C57BL/6 background were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred at the University of South Carolina's Animal Resource Facility. All mice used in the current study were obtained from the investigators breeding colony within the Center for Colon Cancer Research Mouse Core. At 3-wk of age mice were genotyped as previously described (Baltgalvis et al., 2009). Mice were individually housed, kept on a 12:12-h light-dark cycle, and had access to standard rodent chow (#8604 Rodent Diet; Harlan Teklad, Madison, WI) and water ad libitum. 12 hours prior to sacrifice all mice had food removed from cage. Body weight measurements were taken weekly and the percentage body weight loss from peak body weight (~12-20 weeks of age) was calculated. Mice lacking the *Apc*$^{Min/+}$ mutation (C57BL/6) served as controls for all experiments. The University of South Carolina's Institutional Animal Care and Use Committee approved all animal experimentation in this study.

Skeletal Muscle Deletion of AMPK$^{\alpha 1\alpha 2}$

To generate a skeletal muscle specific knockout of AMPK$^{\alpha 1\alpha 2}$, mice that contained individually floxed alleles for AMPK$^{\alpha 1}$ and AMPK$^{\alpha 2}$ were a kind gift from Dr. Hoh-Jin Koh at the University of South Carolina. These mice were then crossed together to generate a double floxed AMPK$^{\alpha 1\alpha 2}$ mouse. The AMPK$^{\alpha 1\alpha 2}$ was then crossed with *Apc*$^{Min/+}$ mice to generate an AMPK$^{\alpha 1\alpha 2}$ *Apc*$^{Min/+}$ mouse. Tamoxifen inducible Mer Cre Mer driven by a Human Skeletal Actin promoter (HSA) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA)(McCarthy, Srikuea, Kirby, Peterson, & Esser, 2012). These mice were then crossed with our AMPK$^{\alpha 1\alpha 2}$ to generate AMPK$^{\alpha 1\alpha 2}$

HSA Cre mice. Finally, the AMPK$^{\alpha1\alpha2}$ HSA Cre mice were crossed with AMPK$^{\alpha1\alpha2}$ *Apc*$^{Min/+}$ mice giving a 50% chance of the offspring producing an AMPK$^{\alpha1\alpha2}$ *Apc*$^{Min/+}$ HSA Cre mouse. Tamoxifen injection via intraperitoneal cavity deletes skeletal muscle AMPK$^{\alpha1\alpha2}$ in the *Apc*$^{Min/+}$ mouse (Min KO).

Tissue Collection

Mice were anesthetized via a subcutaneous injection of ketamine/xylazine/acepromazine cocktail (1.4 ml/kg body weight) prior to sacrifice. Hind limb muscles were rapidly excised, cleared of excess connective tissue, rinsed in PBS, weighed, and snap frozen in liquid nitrogen. The gastrocnemius muscle was cut at the mid-belly, and the proximal portion was split laterally and medially for protein. The left and right soleus muscles were weighed and processed for protein analysis. Blood was collected prior to muscle collection via retro-orbital eye bleed with heparinized capillary tubes, placed on ice, and centrifuged (10,000 x g for 10 min at 4°C). The supernatant was removed and stored for plasma IL-6 analysis. Plasma and tissue samples were stored at -80°C until further analysis.

Western Blotting

Western blot analysis was performed as previously described (Fix et al., 2018). Frozen gastrocnemius muscle was homogenized in ice-cold Mueller buffer and protein concentration was determined by the Bradford method. Crude muscle homogenates were fractionated on 6 – 15% SDS-polyacrylamide gels and transferred to PVDF membranes overnight. Membranes were stained with Ponceau red to verify equal loading and transfer for each gel. Membranes were blocked at room temperature for 1 – 2 hrs. in 5% Tris-

buffered saline with 0.1% Tween-20 (TBST) milk. Primary antibodies for p-AKT (T308), AKT, puromycin, p-FOXO3(S253), FOXO3, p-ULK-1 (S555), ULK-1, P62, LC3II/I, MuRF-1, Atrogin-1 (ECM Biosciences), p-AMPK (T172), AMPK, p-ACC (S79) , ACC, p-TSC2 (S1387), TSC2, p-raptor (S792), raptor, DRP-1, FIS-1, MFN-1 (Abcam), PGC-1α (Abcam), and GAPDH will be diluted 1:5000 – 1:2000 in 5% TBST-milk followed by a 4-8-hr incubation with membranes at 4 degrees. Anti-rabbit or anti-mouse IgG horseradish-peroxidase conjugated secondary antibody was incubated with the membranes at 1:2000 dilutions for 1 hour in 5% TBST-milk at room temperature. Enhanced chemiluminescence (ECL) (GE Healthcare Life Sciences, Piscataway, NJ) was used to visualize the antibody-antigen interactions. Images were digitally scanned and quantified by densitometry using imaging software (Image J; NIH). All antibodies were from Cell Signaling unless otherwise stated.

Plasma IL-6 concentrations

Plasma IL-6 concentrations were determined as previously described (B. N. VanderVeen, Hardee, Fix, & Carson, 2018). A commercially available IL-6 enzyme-linked immunosorbent assay kit was obtained from BD Biosciences (San Diego, CA, USA) and the manufacturer's protocol was followed. Briefly, a Costar clear 96-well plate (Corning, NY, USA) was coated with IL-6 capture antibody and allowed to incubate overnight. The next morning, the plate was blocked with assay diluent buffer, washed, and IL-6 standards and plasma samples were added in duplicate to the plate. The plate was again washed and sAV-HRP reagent was added to each well. After several washes, TMB substrate was added and the reaction was developed for 20 minutes. The reaction

was stopped with sulfuric acid and absorbance was read in a BioRad iMark plate reader (Hercules, CA, USA) at 450 nm.

Statistical Analysis

Results are reported as the means ± standard error. An unpaired student's pre-planned t-test or One-way ANOVA was employed when appropriate. Post-hoc analyses were performed with Student-Newman-Keuls methods when appropriate. The accepted level of significance was set at $p<0.05$ for all analysis. Statistical analysis was performed using Prism GraphPad 7 (GraphPad Software Inc., La Jolla, CA).

*Specific Aim 2: Determine if cancer cachexia disrupts the physiological regulation of AMPK signaling by fasting/re-feeding in oxidative and glycolytic skeletal muscle***.**

Aim 2.1: Determine the effect of fasting and re-feeding on the cachexia regulation of protein synthesis via AMPK signaling and downstream targets TSC2 and raptor.

Aim 2.2: Determine the effect of fasting and re-feeding on the cachexia regulation of AMPK degradation targets ULK-1 and FOXO3 and their downstream processes of autophagy and E3 ligase expression.

Aim 2.3: Determine the effect of fasting and re-feeding on the cachexia regulation of AMPK mediated mitochondrial quality control processes biogenesis, fission, and fusion.

Premise

Nutrient availability or energy status is a critical regulator of AMPK. Skeletal muscle proteostasis fluctuates diurnally depending on different fasting and feeding durations as well as in response to activity and exercise stimuli. Adequate nutrient

availability is key in the net loss and gain of skeletal muscle protein. AMPK is a nutrient sensitive energy sensor and is essential for skeletal muscle metabolic homeostasis (Romanello et al., 2010; Romanello & Sandri, 2015). A lack of available energy (ATP) results in the activation of AMPK in order to activate energy generating pathways (degradation) while suppressing energy consuming pathways (protein synthesis). AMPK is capable of being activated by many different stimuli as well as diseases such as cancer cachexia. During severe cancer cachexia, skeletal muscle AMPK is dysregulated and chronically activated which could have dire consequences regarding muscle proteostasis. The most widely investigated activators of skeletal muscle AMPK is the process of prolonged energy deficiency (fasting). Our laboratory has previously reported elevated AMPK following an acute 5 hour fast (Cannavino, Brocca, Sandri, Bottinelli, & Pellegrino, 2014). This activation is not present in non-tumor bearing mice and suggest that cachectic skeletal muscle may be hyper-sensitive to fasting conditions exacerbating the AMPK response. While fasting has been proposed to have beneficial effects in regard to aging and some cancers, less is understood about its role in cancer cachexia and the regulation of muscle proteostasis.

Conversely the abundance of nutrients following the ingestion of glucose or a mixed meal (feeding) has been previously demonstrated to suppress AMPK. Feeding induces the IGF-1/AKT signaling pathway which will control skeletal muscle protein synthesis (K. Nakashima et al., 2006). The capacity cellular energy status or feeding to induce mTORC1 and suppress AMPK has been characterized extensively in normal physiological conditions (Bagherniya et al., 2018; Galic et al., 2018; K. Nakashima et al., 2006; Russo et al., 2018). Some even argue that mTORC1 may actually be a homeostatic

ATP energy sensor. Nutrient stimulation as well as insulin and certain cytokines will induce the AKT phosphorylation of TSC2 releasing the block and allowing rheb to induce mTORC1 and its activation of downstream targets. We have previously demonstrated an acute glucose injection was capable of attenuating AMPK and inducing mTORC1 but was unable to induce protein synthesis. In light of this, it is worth mentioning the newly defined concept of anabolic resistance or the inability to respond to nutrients has recently become an interesting and widely debated topic in cancer cachexia. While circadian fluctuations in protein turnover are the established norm in muscle mass regulation, it has become evident that protein synthesis is highly responsive to environmental stimuli. Nutrients or feeding and exercise are extremely potent stimulators of skeletal muscle protein synthesis in basal healthy conditions; however, numerous wasting conditions have demonstrated reduced sensitivity to these anabolic signals (Argiles, Lopez-Soriano, et al., 2015; Bodine, 2013; Bowen et al., 2015).

While the concept of fasting has been suggested to be therapeutic in some diseases and even cancer less is understood about its role in cachexia. Therefore, we hypothesize that the fasting induced changes in energy status will exacerbate the AMPK mediated downstream process of degradation and suppress muscle protein synthesis. Furthermore, we hypothesize that re-feeding following a fast will attenuate the fasting induced activation of AMPK signaling. Additionally, we also believe that these events will be differentially regulated by muscle phenotype.

Aim 2.1 Experimental Design

The purpose of Aim 2.1 is to determine the effect of fasting and re-feeding on the cachexia regulation of protein synthesis via AMPK signaling and downstream targets TSC2 and raptor. We hypothesize that AMPK and downstream targets TSC2 and raptor will be robustly activated in the glycolytic but not oxidative muscles of fasted *Apc*$^{Min/+}$ (Min) mice when compared to ad libitum fed Mins and C57BL/6 (B6). We also hypothesize that a 1 hour re-feed following a fast will attenuate the AMPK phosphorylation of TSC2 and raptor which will induce protein synthesis in both glycolytic and oxidative cachectic muscle. To test this hypothesis male B6 and Min mice ~18-20 weeks of age will be stratified into either fed (ad-libitum food access during the 12 hour dark cycle) fasted (food removed from cages during the 12 hour light cycle) or re-feed (fasted during the 12 hour light cycle and allowed access to a food pellet for 1 hour). Following completion of each groups fed or fasted treatment mice will be sacrificed, and tissues collected for analysis similar to Aim 1.

Aim 2.1 Primary Outcomes

Indices of Cachexia: See Aim 1.1

Analysis of Skeletal Muscle AMPK, TSC2, raptor, and Puromycin: See Aim 1.1

Aim 2.1 Secondary Outcomes

Skeletal Muscle AKT: See Secondary Outcomes Aim 1.1

Aim 2.1 Statistical Analysis

Determining Statistical Significance: See Aim 1.1

Power Analysis:

A power analysis was performed to determine the sample size needed to observe statistical significance using a two-way ANOVA. Power (1-β) was set to 0.8 and error of probability (α) was set at 0.05. Based on previously published results and our preliminary data, to achieve significance difference between groups for an estimated 20 ± 5% difference between groups, a minimum sample size of 7 is needed for each group.

Aim 2.2 Experimental Design

The purpose of Aim 2.2 is to determine the effect of fasting and re-feeding on the cachexia regulation of AMPK degradation targets ULK-1 and FOXO3 and their downstream processes of autophagy and E3 ligase expression. We hypothesize that fasting exacerbates the activation of ULK-1 and FOXO3 in cachectic glycolytic but not oxidative skeletal muscle. We also hypothesize that a 1 hour refeed will suppress the induction of these proteins. Mice from Aim 2.1 will be utilized in this experiment.

Aim 2.2 Primary Outcomes

FOXO3a: See Aim 1.2

Atrogin-1 and MuRF-1: See Aim 1.2

Autophagy Signaling, P62, and LC3II/I: See Aim 1.2

Aim 2.2 Secondary Outcomes

Protein Ubiquitination: See Aim 1.2

Aim 2.2 Statistical Analysis

Determining Statistical Significance: See Aim 2.1

Power: See Aim 2.1

Aim 2.3 Experimental Design

The purpose of Aim 2.3 is to determine the effect of fasting and re-feeding on the cachexia regulation of AMPK mediated mitochondrial quality control processes biogenesis, fission, and fusion. We hypothesize that fasting will exacerbate the chronic cachexia activation of AMPK, suppressing mitochondrial biogenesis and fusion while inducing fission in glycolytic but not oxidative muscle. We also hypothesize that 1 hour of refeeding will be sufficient to suppress these signaling events.

Aim 2.3 Primary Outcomes

Mitochondrial Quality Control: See Aim 1.3

Aim 2.3 Statistical Analysis

Determining Statistical Significance: See Aim 1.1

Power: See Aim 2.1

Aim 2 Overall Interpretation and Potential Pitfalls:

Aim 2 will determine if cancer cachexia disrupts the physiological regulation of AMPK signaling by fasting/re-feeding in oxidative and glycolytic skeletal muscle . Furthermore, Aim 2 will elucidate if nutrients ingestion through re-feeding is sufficient to suppress AMPK during cancer cachexia. Aim 2 will also demonstrate if these processes are differentially regulated in oxidative and glycolytic muscle. We will examine immediate downstream AMPK targets that regulate muscle protein synthesis,

mitochondrial quality control, and degradation through E3 ligases and the lysosomal autophagy system. I have strong preliminary data that AMPK mediated autophagy is induced in Min mice during the fed state compared to B6 mice. Additionally, a fasting exacerbates the activation of AMPK and downstream ULK-1 autophagy signaling in Min mice when compared to the fed state. While there is strong rationale for the hyper-activation of AMPK and autophagy during fasting in cachectic skeletal muscle, less is understood about the role of feeding AMPK and mTORC1 in this process. However, I have strong preliminary data demonstrating that a 1-hour re-feed is capable of stimulating mTORC1 in cachectic skeletal muscle. This should provide valuable information related to the role of AMPK in the fasting and feeding regulation of skeletal muscle autophagy during cancer cachexia.

Specific Aim 2 Methods

Animals

Male C57BL/6 (B6) and *Apc*$^{Min/+}$ (Min) mice will be bred, housed, and monitored as described in Aim 1. For experiment 2 B6 and Min mice will be allowed access to ad libitum access to food, fasted 12-hours prior to sacrifice, or fasted 12-hours and allowed ad libitum access to a food pellet for 1-hour. The University of South Carolina's Institutional Animal Care and Use Committee approved all animal experimentation outline in this proposal.

Western Blotting

Western blot analysis was performed as previously described (Fix et al., 2018). Frozen gastrocnemius muscle was homogenized in ice-cold Mueller buffer and protein

concentration was determined by the Bradford method. Crude muscle homogenates were fractionated on 6 – 15% SDS-polyacrylamide gels and transferred to PVDF membranes overnight. Membranes were stained with Ponceau red to verify equal loading and transfer for each gel. Membranes were blocked at room temperature for 1 – 2 hrs. in 5% Tris-buffered saline with 0.1% Tween-20 (TBST) milk. Primary antibodies for p-AKT (T308), AKT, puromycin, p-FOXO3(S253), FOXO3, p-ULK-1 (S555), ULK-1, P62, LC3II/I, MuRF-1, Atrogin-1 (ECM Biosciences), p-AMPK (T172), AMPK, p-ACC (S79) , ACC, p-TSC2 (S1387), TSC2, p-raptor (S792), raptor, DRP-1, FIS-1, MFN-1 (Abcam), PGC-1α (Abcam), and GAPDH will be diluted 1:5000 – 1:2000 in 5% TBST-milk followed by a 4-8-hr incubation with membranes at 4 degrees. Anti-rabbit or anti-mouse IgG horseradish-peroxidase conjugated secondary antibody was incubated with the membranes at 1:2000 dilutions for 1 hour in 5% TBST-milk at room temperature. Enhanced chemiluminescence (ECL) (GE Healthcare Life Sciences, Piscataway, NJ) was used to visualize the antibody-antigen interactions. Images were digitally scanned and quantified by densitometry using imaging software (Image J; NIH). All antibodies were from Cell Signaling unless otherwise stated.

Plasma IL-6 concentrations

Plasma IL-6 concentrations were determined as previously described (Hardee et al., 2016). A commercially available IL-6 enzyme-linked immunosorbent assay kit was obtained from BD Biosciences (San Diego, CA, USA) and the manufacturer's protocol was followed. Briefly, a Costar clear 96-well plate (Corning, NY, USA) was coated with IL-6 capture antibody and allowed to incubate overnight. The next morning, the plate was blocked with assay diluent buffer, washed, and IL-6 standards and plasma samples were

added in duplicate to the plate. The plate was again washed and sAV-HRP reagent was added to each well. After several washes, TMB substrate was added and the reaction was developed for 20 minutes. The reaction was stopped with sulfuric acid and absorbance was read in a BioRad iMark plate reader (Hercules, CA, USA) at 450 nm.

Statistical Analysis

Results are reported as the means ± standard error. An unpaired student's pre-planned t-test or One-way ANOVA was employed when appropriate. Post-hoc analyses were performed with Student-Newman-Keuls methods when appropriate. The accepted level of significance was set at $p<0.05$ for all analysis. Statistical analysis was performed using Prism GraphPad 7 (GraphPad Software Inc., La Jolla, CA).

Specific Aim 3: Determine if in oxidative and glycolytic cachectic skeletal muscle, 4 weeks of voluntary wheel running activity regulates aberrant AMPK signaling in response to physiological fasting.

Aim 3.1: Determine if 4 weeks of voluntary wheel running activity regulates the cachexia disruption of AMPK signaling and downstream targets TSC2, raptor, and muscle protein synthesis in response to a physiological fast

Aim 3.2: Determine if 4 weeks of voluntary wheel running activity regulates the cachexia disruption of AMPK degradation targets ULK-1, FOXO3, and protein degradation in response to a physiological fast.

Aim 3.3: Determine if 4 weeks of voluntary wheel running activity regulates the cachexia disruption of AMPK mediated mitochondrial quality control processes of biogenesis, fission, and fusion in response to a physiological fast.

Premise

While it is well established that acute exercise activates AMPK our laboratory demonstrated that increased cage activity via voluntary wheel running was able to attenuate the cachexia induced chronic activation of this pathway and improve muscle mass loss (Hardee et al., 2016; Puppa et al., 2012). These results suggest that increased activity may improve mitochondrial quality control during cancer cachexia and alleviate the chronic energy deficit (ATP generation) thus lowering AMPK's activity. Similar results were also demonstrated using high frequency electrical stimulation in cachectic muscle(Hardee et al., 2016). Seven repeated bouts of high frequency stimulation were able to lower AMPK phosphorylation in cachectic $Apc^{Min/+}$ and stimulate muscle protein synthesis 3 hours post the final treatment(Hardee et al., 2016). These results demonstrate that exercise training in the cachectic state has the ability to improve skeletal muscle AMPK signaling and alleviate the AMPK mediated inhibition of mTORC1.

While cytokine treatments and anti-inflammatory therapies have been demonstrated to improve AMPK signaling during cancer cachexia, less is understood about the role exercise training in the regulation of fasting and autophagy. As mentioned previously, numerous models of cancer cachexia have reported both elevated AMPK and indices of autophagy (Pigna et al., 2016a; White, Baltgalvis, et al., 2011). The use of exercise training to attenuate or improve AMPK signaling during cancer cachexia has been utilized by our laboratory previously, however, whether this effect improves the protein synthesis, degradation, and downstream autophagy response to a 12-hour fast remains to be determined.

Aim 3.1 Experimental Design

The purpose of Aim 3.1 is to determine if 4 weeks of voluntary wheel running activity regulates the cachexia disruption of AMPK signaling and downstream targets TSC2, raptor, and muscle protein synthesis in response to a physiological fast. We hypothesize that 4 weeks of voluntary wheel running will attenuate the fasting activation of AMPK, TSC2, and raptor in $Apc^{Min/+}$ mice when compared to non-activity fasted $Apc^{Min/+}$ mice. Furthermore, we also believe that wheel running activity will improve these signaling processes more in glycolytic than oxidative muscle. To test this hypothesis C57BL/6 mice and $Apc^{Min/+}$ mice will be given access to a standard mouse wheel beginning at 14-15 weeks of age and monitored until sacrifice at 18-19 weeks of age. Mice will have the wheel removed 48 hours prior to sacrifice to avoid any acute effects of exercise and will be fasted for 12-hours (the same fasting paradigm as in Aim 2). Tissues will be collected as described in Aim 1 and will be compared to 12-hour fasted untrained mice from Aim 2. All analysis will be expressed relative to 12-hour fast.

Aim 3.1 Primary Outcomes

Voluntary Wheel Activity:

Voluntary wheel running will be used as our exercise training stimulus. At 14-15 weeks of age, all mice will be single housed and placed in cages with 9.5-in.-diameter stainless steel activity wheels (MiniMitter, Bend, OR). Running activity will be monitored daily from 14-15 to 18-19 weeks of age. Bicycle computers (Specialized, Morgan Hill, CA) with magnetic sensors are able to measure average speed, distance, time, and maximum speed, which will be recorded daily, however expressed as means within a week.

Indices of Cachexia: See Aim 1.1

Analysis of Skeletal Muscle AMPK, TSC2, raptor, and Puromycin: See Aim 1.1

Aim 3.1 Secondary Outcome

Skeletal Muscle AKT: See Secondary Outcomes Aim 1.1

Aim 3.1 Statistical Analysis

Determining Statistical Significance:

An unpaired pre-planned students t-test will be employed to conduct analysis between 12-hour fasted and exercise trained 12-hour fasted within each genotype and significance set at $p<.05$. All statistical analysis will be performed using GraphPad Prism 7 software. Values will be presented as means ± the standard error of the mean.

Power Analysis:

A power analysis was performed to determine the sample size needed to observe statistical

significance using a one-way ANOVA. Power (1-β) was set to 0.8 and error of probability (α) was

set at 0.05. Based on previously published results utilizing wheels, to achieve significance for an estimated 20 ± 5% difference between groups, a minimum sample size of 8 is needed for each group.

Aim 3.2 Experimental Design

The purpose of Aim 3.2 is to determine if 4 weeks of voluntary wheel running activity regulates the cachexia disruption of AMPK degradation targets ULK-1, FOXO3, and protein degradation in response to a physiological fast. We hypothesize that 4 weeks of voluntary wheel running will attenuate the fasting activation of ULK-1 and FOXO3 signaling in glycolytic and oxidative skeletal muscle of $Apc^{Min/+}$ mice. C57BL/6 and $Apc^{Min/+}$ Mice from Aim 3.1 will be utilized for this experiment.

Aim 3.2 Primary Outcomes

FOXO3a: See Aim 1.2

Atrogin-1 and MuRF-1: See Aim 1.2

Autophagy Signaling, P62, and LC3II/I: See Aim 1.2

Aim 3.2 Secondary Outcomes

Protein ubiquitination: See Aim 1.2

Aim 3.2 Statistical Analysis

Determining Statistical Significance: See Aim 3.1

Power Analysis: See Aim 3.1

Aim 3.3 Experimental Design

The purpose of Aim 3.3 is to determine if 4 weeks of voluntary wheel running activity regulates the cachexia disruption of AMPK mediated mitochondrial quality control processes of biogenesis, fission, and fusion in response to a physiological fast.

We hypothesize that 4 weeks of voluntary wheel running will attenuate the cachexia disruption of AMPK mediated mitochondrial quality control following a fast. We also hypothesize that wheel running activity will improve mitochondrial quality control more in glycolytic than in oxidative muscle. To test this hypothesis C57BL/6 and *Apc*$^{Min/+}$ Mice from Aim 3.1 will be utilized for this experiment.

Aim 3.3 Primary Outcomes

Mitochondrial Quality Control: See Aim 1.3

Aim 3.3 Secondary Outcomes

COX Enzyme Activity: Skeletal muscle cytochrome c oxidase activity (COX) will be measured using methods as previously described (Fix et al., 2018). Briefly, a 5-7mg piece of muscle will be homogenized in extraction buffer and analyzed spectrophotometrically using a kit from Sigma Aldrich (CYTOCOX1). The absorption at 550nm over a 1 minute period will determine COX activity.

Aim 3.3 Statistical Analysis

Determining Statistical Significance: See Aim 3.1

Power Analysis: See Aim 3.1

Aim 3 Overall Interpretation and Potential Pitfalls:

This set of experiments will establish the therapeutic potential of exercise training to improve cachectic the skeletal muscle autophagy response following a 12-hour fast. Furthermore, data from these experiments will further elucidate if exercise training can

attenuate the fasting induction of AMPK in cachectic skeletal muscle and improve muscle protein synthesis. We will also explore if improved mitochondrial quality control in response to exercise training coincides with the potential AMPK suppression. This Aim will give valuable information that can provide rationale to further explore exercise training and the regulation of AMPK mediated autophagy and protein synthesis. Furthermore, data from these experiments can provide intriguing rationale to explore if exercise training is capable of sensitizing skeletal muscle to feeding following a 12-hour fast.

Specific Aim 3 Methods

Animals

The *Apc*$^{Min/+}$ mouse is a genetic model of colorectal cancer and cachexia (Baltgalvis, Berger, Pena, Davis, Muga, et al., 2008; Mehl et al., 2005). These mice harbor a heterozygous mutation in the adenomas polyposis coli (APC) gene, which promotes the development of intestinal tumors beginning as early as 4 weeks of age (Moser et al., 1990). Mice develop an IL-6-dependent cancer cachexia phenotype between 3 and 6 months of age (Baltgalvis, Berger, Pena, Davis, Muga, et al., 2008; Mehl et al., 2005). Due to the slow onset and progression of body weight loss this model is advantageous as treatments can be started after the initiation of cancer cachexia. Male *Apc*$^{Min/+}$ mice on a C57BL/6 background were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred at the University of South Carolina's Animal Resource Facility. All mice used in the current study were obtained from the investigators breeding colony within the Center for Colon Cancer Research Mouse Core.

At 3-wk of age mice were genotyped as previously described (Baltgalvis, Berger, Pena, Davis, Muga, et al., 2008). Mice were individually housed, kept on a 12:12-h light-dark cycle, and had access to standard rodent chow (#8604 Rodent Diet; Harlan Teklad, Madison, WI) and water ad libitum. 12 hours prior to sacrifice all mice had food removed from cage. Body weight measurements were taken weekly and the percentage body weight loss from peak body weight (~10-15 weeks of age) was calculated. Mice lacking the $Apc^{Min/+}$ mutation (C57BL/6) served as controls for all experiments. The University of South Carolina's Institutional Animal Care and Use Committee approved all animal experimentation in this study.

Voluntary Wheel Running

Voluntary wheel running was used as a marker of volitional physical activity and was performed as previously described (26). At 5 wks. of age, wild-type and ApcMin/+ were housed individually in cages with 9.5-in.-diameter stainless steel activity wheels (MiniMitter, Bend, OR). Running activity was monitored daily from 14-15 weeks of age to 18-20 weeks of age. Bicycle computers (Specialized, Morgan Hill, CA) with magnetic sensors measured average speed, distance, time, and maximum speed, and the data were recorded daily.

Western Blotting

Western blot analysis was performed as previously described (Fix et al., 2018). Frozen gastrocnemius muscle was homogenized in ice-cold Mueller buffer and protein concentration was determined by the Bradford method. Crude muscle homogenates were fractionated on 6 – 15% SDS-polyacrylamide gels and transferred to PVDF membranes

overnight. Membranes were stained with Ponceau red to verify equal loading and transfer for each gel. Membranes were blocked at room temperature for 1 – 2 hrs. in 5% Tris-buffered saline with 0.1% Tween-20 (TBST) milk. Primary antibodies for p-AKT (T308), AKT, puromycin, p-FOXO3(S253), FOXO3, p-ULK-1 (S555), ULK-1, P62, LC3II/I, MuRF-1, Atrogin-1 (ECM Biosciences), p-AMPK (T172), AMPK, p-ACC (S79) , ACC, p-TSC2 (S1387), TSC2, p-raptor (S792), raptor, DRP-1, FIS-1, MFN-1 (Abcam), PGC-1α (Abcam), and GAPDH will be diluted 1:5000 – 1:2000 in 5% TBST-milk followed by a 4-8-hr incubation with membranes at 4 degrees. Anti-rabbit or anti-mouse IgG horseradish-peroxidase conjugated secondary antibody was incubated with the membranes at 1:2000 dilutions for 1 hour in 5% TBST-milk at room temperature. Enhanced chemiluminescence (ECL) (GE Healthcare Life Sciences, Piscataway, NJ) was used to visualize the antibody-antigen interactions. Images were digitally scanned and quantified by densitometry using imaging software (Image J; NIH). All antibodies were from Cell Signaling unless otherwise stated.

Plasma IL-6 concentrations

Plasma IL-6 concentrations were determined as previously described (Hardee et al., 2016). A commercially available IL-6 enzyme-linked immunosorbent assay kit was obtained from BD Biosciences (San Diego, CA, USA) and the manufacturer's protocol was followed. Briefly, a Costar clear 96-well plate (Corning, NY, USA) was coated with IL-6 capture antibody and allowed to incubate overnight. The next morning, the plate was blocked with assay diluent buffer, washed, and IL-6 standards and plasma samples were added in duplicate to the plate. The plate was again washed and sAV-HRP reagent was added to each well. After several washes, TMB substrate was added and the reaction was

developed for 20 minutes. The reaction was stopped with sulfuric acid and absorbance was read in a BioRad iMark plate reader (Hercules, CA, USA) at 450 nm.

Cytochrome C Oxidase Enzyme Assay

Cytochrome-c oxidase (COX) activity was assessed in isolated mitochondria and whole muscle homogenates from the gastrocnemius muscle. For whole muscle analysis, gastrocnemius tissues were homogenized in extraction buffer (0.1M KH2PO4/Na2HPO4 and 2 mM EDTA, pH 7.2). COX enzyme activity was determined by measuring the rate of oxidation of fully reduced cytochrome c at 550 nm as previously described(Fix et al., 2018).

Statistical Analysis

Results are reported as the means ± standard error. An unpaired student's pre-planned t-test. The accepted level of significance was set at $p<0.05$ for all analysis. Statistical analysis was performed using Prism GraphPad 7 (GraphPad Software Inc., La Jolla, CA).