Branched-Chain Keto Acid Dehydrogenase Kinase Depletion Affects Branched-Chain Alpha-Keto Acid Dehydrogenase Activity and Muscle Cell Differentiation

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Abstract

Branched-chain amino acids are indispensible amino acids that are known for having anabolic effects in skeletal muscle. These effects BCAAs elicit in existing skeletal muscle are well documented in literature. However, much less is known about how they affect the process of myogensis and muscle regeneration. Data generated in our lab have shown that the intact BCAA catabolic pathway is required for proper skeletal muscle cell differentiation. In the current study, the main enzymes involved in the catabolism of BCAAs were assessed, in addition to the activity of the rate-limiting enzyme (Branched-chain α-ketoacid dehydrogenase - BCKD) in the pathway. Increasing the flux of BCAAs through this pathway via siRNA mediated knockdown of a negative regulator of the pathway augmented differentiation. It is evident that BCAA catabolism is intricately involved in the process of muscle cell differentiation. Further study into the regulation of BCAA catabolism during differentiation is warranted; to aid in the development of nutritional based interventions for populations suffering from muscle wasting condition.

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Abbreviations

4E-BP1 eIF4e-binding protein-1
AKT *also known as* protein kinase B (PKB)
AMPK AMP-activated protein kinase
BCAA branched-chain amino acids
BCAT2/BCATm branched-chain amino acid transferase-2 (mitochondrial isoform)
BCKA branched-chain α-keto acid
BCKD/BCKDH branched-chain α-keto-dehydrogenase complex
BCKDE1α branched-chain α-keto-dehydrogenase E1 α-polypeptide
BDK branched-chain α-keto-dehydrogenase kinase
bHLH basic helix-loop-helix
CDK cyclin-dependent kinase
DEPTOR DEP domain-containing mTOR-interacting protein
eIF4E eurkaryotic translation initiation factor-4E
eIF4F eurkaryotic translation initiation factor-4F
eIF4G eurkaryotic translation initiation factor-4G
ERK extracellular signal-regulated kinase 2
FKBP12 12-kDa FK506-binding protein
FoxO forkhead box-O
GAP Guanine activating protein
GTP/GDP guanine triphosphate/guanine diphosphate
GATOR GAP activity towards Rags
HMB β-hydroxy-β-methylbutyrate
IGF-1 insulin-like growth factor-1
IGF-2 insulin-like growth factor-2
IMP inosine monophosphate
IMP2 IGF-2 mRNA-binding protein
IRS1 insulin receptor substrate-1
JNK *jun* N-terminal kinase
KIC α-ketoisocaproate
KIV α-ketoisovalerate
KMV α-keto-β-methylvalerate
MAPK mitogen activated protein kinase
MEF2 myocyte enhancement factor-2
MHC-1 myosin heavy chain-1
MLST8 mammalian lethal with SEC13
MPD muscle protein degradation
MPS muscle protein synthesis
MRF myogenic regulatory factor
mTORC1 mammalian target of rapamycin complex-1
mTORC2 mammalian target of rapamycin complex-2
MuRF-1 muscle ring finger protein-1
Myf5 myogenic factor-5
Myf4 myogenic factor-4 (myogenin)
Pax7 paired box-7

PDK phosphoinositide-dependent kinase
PGC-1α PPAR γ _coactivator of 1α
PHB2/MEA prohibitin2 /repressor of estrogen activity
PI3K phosphoinositide 3-kinase
PNC purine nucleotide cycle
PP2CM protein phosphatase 1K
PRAS40 proline-rich AKT substrate of 40 kDa
Rb retinoblastoma protein
Rag RAS-related GTP-binding protein
RAPTOR regulatory associated protein of mTOR
Rbp-j Recombining binding protein suppressor of hairless
Rheb ras homolog enriched in brain
RICTOR rapamycin-insensitive companion of mTOR
ROS reactive oxygen species
SDHA succinate dehydrogenase
S6/rpS6 ribosomal protein S6
S6K1 p70 ribosomal protein S6 kinase 1
siRNA small interfering RNA
shRNA small hairpin RNA
TGF-β transforming growth factor-β
TCST two-component signal transduction
TSC tuberous sclerosis complex
UPS ubiquitin proteasome system

Introduction

Branched-chain amino acids (BCAA) (isoleucine, leucine and valine) are found in high-quality protein sources, and are well known components of a healthy diet. Not only do BCAAs comprise a large percentage of the amino acids (AA) incorporated into body proteins, they additionally exhibit cell signalling characteristics. BCAA's signalling characteristics have been studied extensively in mature skeletal muscle, although little attention has been given to their role in muscle recovery/development. Research in our lab and others shows that the BCAA catabolic pathway must be tightly regulated to ensure proper physiological functioning. BCAA metabolism is altered in many diseased states including obesity and type 2 diabetes (Lynch & Adams, 2014a; Mccormack et al., 2013; She et al., 2009), heart disease (Wang et al., 2016), and genetic disorders like maple syrup urine disease (MSUD)(Indo et al., 1987). Many of these diseases associated with a disrupted BCAA catabolic pathway include some form of skeletal muscle impairment. However, little is known whether the catabolic pathway of BCAAs plays a distinct role in muscle cell differentiation. Two mitochondrial-localized enzymes regulate the first two steps in BCAA catabolism. Branched-chain amino acid transferase-2 (BCAT2), the first enzyme in the pathway, catalyzes the transamination of all three BCAAs into their keto-acid equivalents. The second enzyme in the pathway, Branched-chain α-keto-acid dehydrogenase (BCKD), oversees the irreversible decarboxylation of all three BCAA derived keto-acids into acyl-CoA derivatives. The activity of BCKD in skeletal muscle is low under basal conditions, however, interventions that increase its activity, solely in skeletal muscle, have dramatic effects on whole body BCAA levels (Ishikawa et al., 2017). Therefore, investigating BCKD's

activity, in addition to its abundance, may yield greater insight into its role in muscle cell differentiation. The tightly controlled BCAA catabolic pathway is highly dependent upon BCKD activity. Two enzymes, a kinase and a phosphatase, antagonistically regulate BCKD activity. Altering the expression of the BCKD regulating enzymes could help elucidate the relevance of BCKD activity during muscle cell differentiation.

Evidence has shown BCAA metabolites possess anabolic-signalling characteristics, although the specific role they play in muscle cell differentiation remains to be determined. The regulatory properties of these metabolites could have positive effects on skeletal muscle mass, which, for a number of diseases, is an important indicator of both disease progression, and general quality of life. Thus, understanding the mechanisms underlying BCAA mediated muscle recovery/development may aid in uncovering potential therapeutic targets for the purpose of maintaining muscle mass.

Literature Review

Skeletal muscle's contribution to health

Skeletal muscle (SM) is the largest metabolic contributor to whole-body protein and energy-metabolism. Many metabolic disorders have their principle effects stemming from, or exerted on SM. Functions of SM include the regulation of glucose homeostasis in addition to being the body's principle reservoir for amino acids. SM mass and strength are highly associated with a number of metabolic diseases and overall risk of mortality (Atherton et al., 2016). Thus, attenuation of SM losses in both strength and mass has attracted vast research interests.

Factors regulating skeletal muscle mass

To address the topic of disease-mediated muscle atrophy/loss, one must first understand the consequences of muscle disuse atrophy. Muscle-disuse atrophy can lead to problems on its own, including decreases in physical-working capacity, decreases in quality of life, metabolic disorders, and increases in all-cause mortality (Atherton, 2016). A consequence of many diseased states involves some periods of immobilization. Immobilization itself is an atrophic-inducing condition. Wall et al., (2013) conducted a study that involved short-term immobilization lasting a mere 14-days. They showed 8% reductions in cross-sectional area (CSA) and 23% reductions in strength of quadriceps fibers (Wall et al., 2013). Additionally, muscle unloading has been shown to suppress the post-absorptive (fasted-state) and postprandial responses (fed-state) to muscle protein synthesis (MPS), while increasing muscle protein degradation (MPD) in animal models

(Atherton, 2016). Immobilization-induced losses in muscle mass are in part mediated through the up-regulation of the ubiquitin proteasome and autophagy-lysosomal pathways (Jackman & Kandarian, 2004).

The Effects of Nutrition and Exercise on Skeletal Muscle Mass

Two antagonistic processes determine protein balance within skeletal muscle: 1) muscle protein degradation (MPD), and 2) muscle protein synthesis (MPS). These two processes together are referred to as protein turnover. When protein balance leans towards a positive balance, muscle anabolism occurs, while muscle catabolism occurs when there is a negative balance. Protein turnover is highly responsive to two main stimuli: exercise and dietary protein ingestion. Many studies have sought out to assess how to optimally increase muscle protein balance via manipulation of these two factors.

In young healthy adults, exercise has been shown to stimulate muscle protein synthesis, leading to muscle hypertrophy (Adegoke et al., 2012; Marcotte et al., 2015; Phillips et al., 1999; Wilkinson et al., 2008). Provision of dietary protein at the cessation of exercise has been shown to augment MPS rates, while having moderate effects on MPD (Moore, Prior, et al., 2009; Moore, Tarnopolsky, et al., 2009; Pendergast et al., 2011; S. Phillips et al., 2016; S. M. Phillips et al., 1997; Tipton et al., 1999). Simply put, muscle mass is a function of exercise training, and an adequate intake of dietary proteins. Resistance exercise (RE) is the preferred method to accumulate muscle mass, compared to endurance exercise. A single bout of RE has been shown to stimulate MPS rates by 40-150% above basal levels. (Burd et al., 2008). The increase in MPD is thought to replenish intracellular

amino acid pools, a consequence of MPS drawing upon those pools for the incorporation into body proteins (Burd, 2008).

Current recommendations state that dietary protein intake should be equal to 0.8g of protein, per kg BW/day (S. Phillips, 2016). This blanket approach to dietary protein recommendations has been challenged due to the complexities and deviations from "normal" that many populations experience ie. age, disease, and athletes. Growing evidence supports an increase in dietary protein recommendations (S. Phillips, 2016). For example, aging individuals are subject to age-related losses in skeletal muscle mass, known as sarcopenia. Increasing dietary protein ingestion above the current recommendations in these individuals has been shown to slow the effects of sarcopenia (Bradlee et al., 2017; Morley & Phillips, 2013; S. Phillips, 2016; Volpi et al., 2013; Wolfe, 2012).

Myogenic Differentiation/Muscle Regeneration

mTOR function and regulation

Before delving into the complicated signalling pathways involved in myogenic differentiation, it is important to introduce the central hub to which all these signalling pathways converge upon.

Perhaps the most important protein complex involved in protein metabolism and whole cell function is the mechanistic (mammalian) target of rapamycin (mTOR). This section will explore the function and relevance of this far-reaching metabolic integration centre, which is integral to all cell functions.

The discovery of mTOR was preceded by two main events. The first event was the discovery of what would be the eventual inhibitor of the mTORC1 complex. This inhibitory chemical is produced by the bacterium *Streptomyces hygroscopicus*, found in the soils of Easter Island in 1975 by Suren Sehgal et al., which they named rapamycin, paying homage to the native name for the island, Rapa Nui (Sehgal et al., 1975). The second event would not be until 1991, where Heitman et al. described rapamycin's effects in arresting cell cycle progression in the G1 phase in yeast cells (Heitman, 2015). Building upon the genes identified via generation of rapamycin-resistant yeast mutants by Heitman et al., (TOR1, TOR2, and FKBP12), Sabatini et al. discovered the mammalian orthologs of the TOR1 and TOR2 complexes (Sabatini et al., 1994). Similarly to yeast TOR, mTOR exists as two distinct complexes in mammalian cells, mTORC1 and mTORC2. Rapamycin sensitive mTOR, or mTORC1, represents a central component to the translational efficiency and translational capacity of a cell (McGlory et al., 2016).

mTORC1 is a multi-subunit protein complex consisting of five subunits: mTOR, regulatory associated protein of mTOR (RAPTOR), proline-rich AKT substrate of 40 kDa (PRAS40), mammalian lethal with SEC13 protein 8 (mLST8), and DEP domain-containing mTOR-interacting protein (DEPTOR). RAPTOR facilitates rapamycin's inhibition of mTORC1 activity, through its ability to bind the FK506-binding protein of 12 kDa (FKBP12). RAPTOR is also thought to regulate the complexes assembly upon activation (Laplante & Sabatini, 2009). While MLST8's function remains undefined, PRAS40 and DEPTOR function as negative regulators of the complex (Peterson et al., 2009).

mTORC1 has been described as a signal integration centre that regulates cellular growth and proliferation. Upon the presence of adequate growth factors, amino acids,

oxygen and cellular energy status, mTORC1 is activated (Laplante, 2009). p70 ribosomal protein S6 kinase (S6K1) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) are the best-characterized downstream substrates of mTORC1 (depicted in **fig 1-1**). Activation of these two substrates following the upstream activation of mTORC1, triggers a cascade of events culminating in an increased translational efficiency and capacity of the cell. S6K1 and 4E-BP1 phosphorylation stimulates ribosomal biogenesis (the protein making machinery of the cell), in addition to promoting ribosomal binding to mRNA (enhances translational efficiency) (McGlory, 2016).

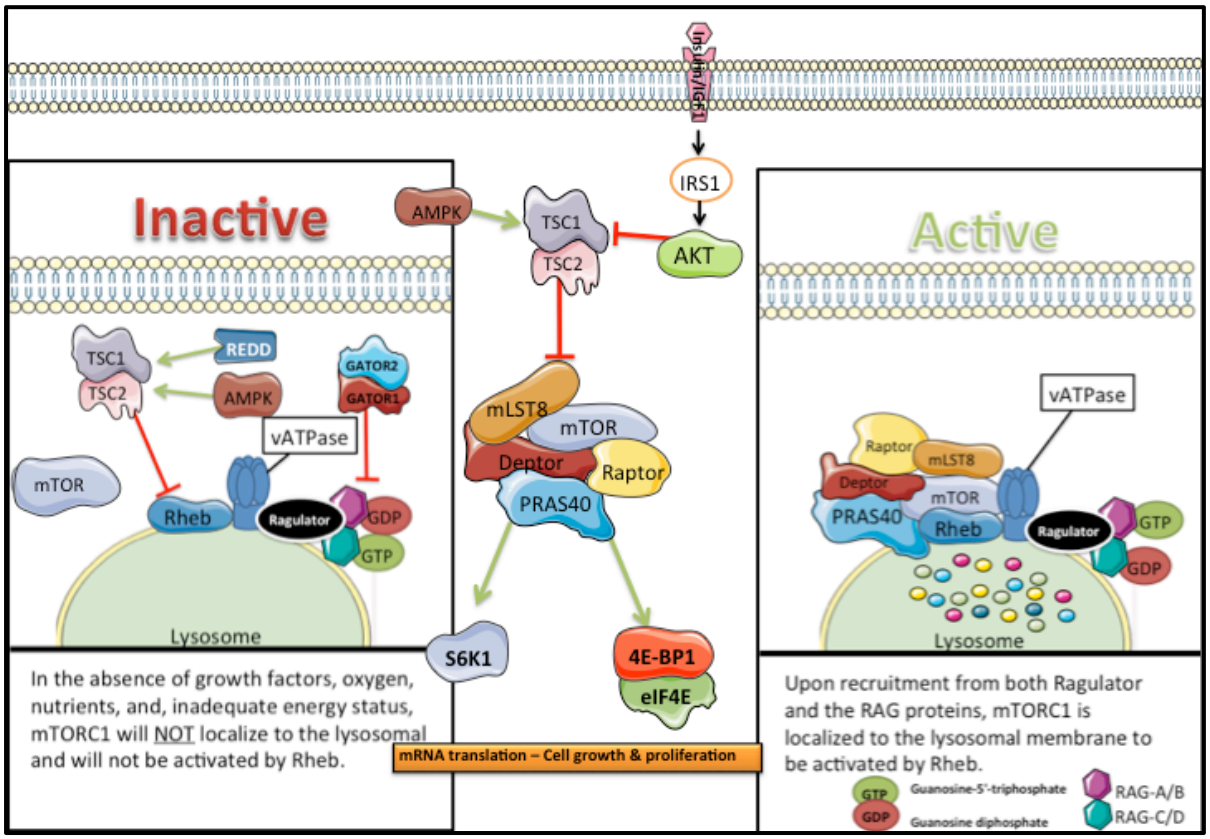

Figure 1-1. mTORC1 signalling pathway schematic, displaying both the inactive (left panel) and active (right panel) complex organizations. The central figure depicts the simplified signalling pathway containing the most well defined mTORC1 substrates. Mechanistic target of rapamycin complex 1 (mTORC1), regulatory-associated protein of mTOR (RAPTOR), mammalian lethal with SEC13 protein 8 (mLST8), proline-rich AKT1 substrate 1 (PRAS40), domain-containing mTOR-interacting protein (DEPTOR).

mTORC1 activity is determined by four factors as previously mentioned, the most actively studied of these factors include amino acids and growth factors. The binding of a growth factor such as insulin-like growth factor-1 (IGF-1) to its membrane localized receptors triggers the downstream activation of protein kinase B/AKT, ultimately leading to the phosphorylation of tuberous sclerosis complex (TSC). TSC is a heterodimeric complex that directly inhibits Ras homolog enriched in brain (Rheb) via its GAP activity, keeping Rheb in an inactive GDP-loaded state (Laplante, 2009). The phosphorylation of TSC complex by AKT results in its dissociation, allowing GTP loading of Rheb and the direct activation of mTORC1 through a poorly defined mechanism (Laplante, 2009).

The amino acid sensing mechanisms of the cell are extremely complex with much research indicating that the main site of AA-induced mTORC1 activation lies at the lysosomal membrane (depicted in the right side panel of **Fig 1-1**). Emerging evidence suggests that AA-induced mTORC1 activation can occur at other cellular organelles such as the Golgi apparatus, although more research needs to be conducted (Zheng et al., 2016). Currently, three defined signalling molecules exist that link AA sensing to mTORC1 activation. Firstly, the heterodimeric Rag GTPases (Rags), (RagA/B – RagC/D) represent the machinery necessary for mTORC1 translocation to the lysosomal membrane to become fully activated (Zheng, 2016). There are four Rag GTPases and current research indicates a redundancy between RagA/B, and for RagC/D. Upon AA stimulation Rag A/B becomes GTP loaded, and RagC/D GDP loaded, representing their active forms. Upon arrival of the Rags and mTORC1 to the lysosomal membrane, the second signalling molecule, Ragulator, tethers the Rags to the membrane when in the presence of amino acids. Ragulator is thought to operate as a guanine nucleotide exchange factor (GEF) towards RagA/B, which

leads to their GTP-loaded active state, consequently upregulating mTORC1 activity (Bar-Peled et al., 2012; Zheng, 2016). The third component, vacuolar H+ ATPase (v-ATPase) is not well characterized in its functioning. v-ATPase is located in the lysosomal membrane, and is thought to sense amino acids in the lysosomal lumen by an inside-out mechanism (Zoncu et al., 2011). Sabatini and colleagues believe it functions as a mediator of nucleotide loading of the Rags, and their subsequent activation, indicating it is a necessary component in the amino acid sensing activation of mTOR1 (Zoncu, 2011).

The regulation of muscle mass is regulated by both nutrition and the tension loading of skeletal muscle (Dickinson et al., 2011). Since mTORC1 is well defined as a nutrient sensor it is logical to assume that it mediates the responses of anabolic signalling. This is because AAs act not only as substrates for endogenous proteins, but can also activate mTORC1.

It is well accepted that resistance exercise (RE) increases MPS, a key component of the hypertrophic response in skeletal muscle (Adegoke, 2012). Many studies in rodents have shown impairments of adaptive hypertrophy following the administration of rapamycin, suggesting mTORC1 is a critical regulator of muscle fibre size (Bodine et al., 2001). Additional studies have shown acute bouts of RE stimulate muscle protein synthesis (MPS), hypertrophy, and ribosomal biogenesis (Figueiredo et al., 2015; West et al., 2016; Wilkinson, 2008). These effects are all attenuated by rapamycin administration confirming a definitive role for mTORC1 in these RE-induced responses (Marcotte, 2015; Ogasawara et al., 2016). Prolonged stimulation of mTORC1 signalling caused by concurrent training (endurance exercise (EE) and RE together) in the past has been shown to diminish some of the positive effects of RE (Hickson, 1980; Leveritt et al., 1999). Studies have shown

that chronic RE results in a reduction in the phosphorylation of S6K1, although it is reversible given a recovery period (Ogasawara et al., 2013). A recent study has found that EE training does not attenuate increases in muscle strength and fibre hypertrophy resultant from successive RE training bouts (Kazior et al., 2016). Although some studies have displayed contrary data supporting mTORC1 signalling in RE, the bulk of available data suggests mTORC1 is essential for increasing MPS, muscle hypertrophy and ribosomal biogenesis. Future research into the mechanisms regulating mTORC1's roles in RE and EE training needs to be completed to clarify the extent to which mTORC1 regulates these processes.

Regulation of Myogenic Differentiation/Muscle Regeneration

Myogenesis is the complex process of muscle formation. It is through this process in which muscle is developed during embryonic development, in mature skeletal muscle this process is referred to as either secondary myogenesis or muscle regeneration. Myogenesis begins during embryogenesis. Originating from the paraxial mesoderm, primary muscle cells will set the groundwork for later, or secondary-myogenesis to build upon.

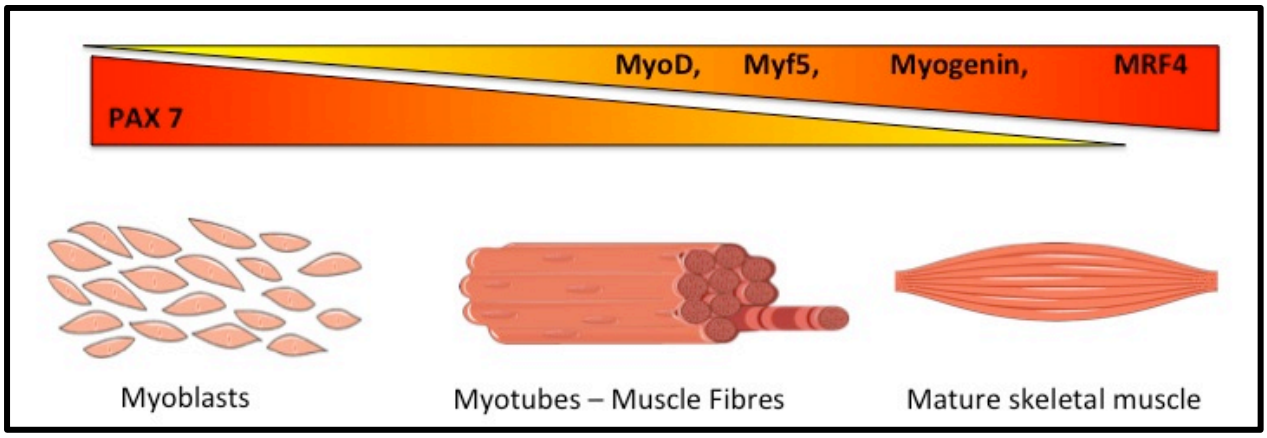

Figure 1-2. Schematic of myogenesis displaying the temporal rise and fall of myogenic regulatory factors during differentiation from satellite cells, to mature skeletal muscle. Paired box 7 (Pax7), Myogenic differentiation 1, or myogenic factor 3 (MyoD), Myogenic factor 5 (Myf5), Myogenic regulatory factor 4, or herculin (Mrf4) Image modified from (Hettmer & Wagers, 2010)

Myogenic regulatory factors

The myogenic regulatory factors (MRFs) belong to the superfamily of basic-loop-helix (bHLH) transcription factors. The HLH domain triggers a dimerization of the MRFs with E-proteins. Upon heterodimerization the affinity for the E-box promoter via a consensus DNA sequence (CANNTG) on many muscle specific genes increases, thereby enhancing the transcription of those muscle-specific genes (Lassar et al., 1989; Murre et al., 1989). There are four main MRFs that will be the focus of discussion in this section. The four MRFs include: Myogenic factor 5 (Myf5), Myogenic differentiation 1 or myogenic factor 3 (MyoD), myogenin, and Myogenic regulatory factor 4 or herculin (Mrf4). These MRFs appear throughout myogenesis in a temporal manner committing myogenic progenitors towards terminal differentiation as depicted in **Fig 1-2**. Gene inactivation studies provided much of the groundwork of understanding for this relatively new

research field. Studies investigating MyoD and Myf5 gene inactivation revealed redundant functioning between these two early regulators of myogenesis (Braun et al., 1992; Rudnicki et al., 1992). Verifying this redundancy became clear when double homozygous knockouts in mice for MyoD and Myf5 were completed, showing no signs of muscle formation (Rudnicki et al., 1993). However, individual knockouts for either yielded limited deficiencies in muscle formation (Braun, 1992; Rudnicki, 1992). Clearly, MyoD and Myf5 are required for muscle cell differentiation, particularly in the early stages.

These early studies have determined that MyoD and Myf5 are essential to the determination towards myogenic lineage and that their expression precedes that of myogenin and MRF4. Myogenin is essential to muscle differentiation. Its expression commences at the onset of differentiation in myoblasts, immediately following myoblast proliferation. Mice depleted of myogenin die at birth, due to severe skeletal muscle deficiencies. Although the embryonic lethality of the myogenin-null mice results from the failure of muscle fibre formation, myoblast number in this mice remains normal, which suggests that myogenin may regulate myoblast fusion (Nabeshima et al., 1993; Venuti et al., 1995). Myogenin has been shown to regulate proteins involved in the cell cycle and, through a p38 MAPK-dependent manner, it promotes cell cycle arrest, an essential step for differentiation to occur. One mechanism through which myogenin suppresses the cell cycle is by its direct regulation of miR-20a, a micro RNA that is known to deplete levels of E2F1 and E2F3 transcription factors (Liu et al., 2012). E2F transcriptional activity is essential for cell cycle progression, thereby, suggesting myogenin regulates the cell cycle by down regulating E2F transcriptional activity. Myogenin is required for terminal differentiation. Inactivation of myogenin causes cleavage of fully matured myotubes into mononucleated

cells (Mastroyiannopoulos et al., 2012). These mononucleated cells are then able to re-enter the cell cycle, although this mechanism is not thought to be a purely myogenin dependent process. Lastly, the fourth MRF, MRF4, has much less known about its role in muscle differentiation. MRF4 is expressed in a biphasic manner, where it is initially expressed during embryonic myogenesis, and once again during muscle regulation. MRF4 is expressed in mature skeletal muscle in response to muscle damage, although its levels are not detectable during satellite cell proliferation or early differentiation (Hinterberger et al., 1991; Pavlath et al., 2003; Z. Zhou & Bornemann, 2001). MRF4 appears to share some redundancy with myogenin, when mice were depleted of MRF4 the authors found myogenin mRNA increased four-fold, which they concluded as the compensatory factor leading to a relatively normally muscle phenotype (Zhang et al., 1995)

Taken together, these gene inactivation studies indicate that MyoD and Myf5 are important for determination to myogenic lineage, while myogenin and MRF4 are necessary for terminal differentiation of adult muscle fibres.

Satellite cell's role in myogenesis

Myogenesis in a post-natal context is largely dependent upon a sub-population of quiescent cells that contain myogenic potential termed satellite cells. Post-natal satellite cell populations are maintained by the expression of a transcription factor, paired box 7 (Pax7). These cells remain in a dormant phase until signaled by stress or trauma (Seale et al., 2000; von Maltzahn et al., 2013). Pax7 null mice have the same number of satellite cells at birth as WT controls, only to be lost in a temporal manner (Philippos et al., 2012). Therefore, Pax7 expressing satellite cells are essential to maintaining satellite cell

populations. It has been shown that Pax7 levels decrease upon myoblasts expansion, with a corresponding rise in MyoD levels, followed by an increase in myogenin expression (Halevy et al., 2004). During this process MyoD regulates the differentiation potential of myoblasts, while Myf5 controls their proliferation potential (Gayraud-Morel et al., 2007). The rise in the number of cells co-expressing MyoD and myogenin determines the intermediate stage of adult myogenesis, where the expanded myoblast population begins to fuse and form multi-nucleated myotubes (Myer et al., 2001). Therefore, tightly regulated, temporal expression of these myogenic regulatory factors orchestrates the proliferation of satellite cells and ultimately their differentiation and fusion onto existing muscle fibres.

Cell cycle exit

Following satellite cell expansion, cell cycle arrest is essential to committing myoblasts towards the differentiation pathway and successful muscle regeneration. There are two primary cell cycle inhibitors intricately involved in myogenesis, they are p21 and retinoblastoma (Rb). p21 is essential to terminal differentiation and permanent cell cycle exit following satellite cell expansion. The rise in p21 transcription can be partially attributed to the increase MyoD expression, through a p53-independent mechanism (canonical regulation of p21 is controlled by p53) (Halevy et al., 1995; Walsh & Perlman, 1997). Once p21 expression has been stimulated, subsequently facilitating cell cycle withdrawal, its levels remain constitutively elevated, even upon re-stimulation with mitogens (Walsh, 1997). In a low-serum (<2% fetal bovine serum or horse serum) environment, myoblasts begin to express myogenin, followed by the increase in the transcription of p21, demonstrating a commitment towards differentiation (Walsh, 1997).

Simultaneously, the tumour suppressor protein Rb, is essential for terminal differentiation and suppressing apoptotic signalling in myotubes. The cyclin-dependent kinases phosphorylate Rb, inactivating it, allowing E2F transcription factors to be freely expressed. The E2F transcription factors then drive the cell cycle through the G1 phase, allowing for progression into S-phase (Bartek et al., 1996). In the event where Rb is in a hypophosphorylated state (active), S-phase entry is blocked, thereby promoting cell cycle arrest, a step required for satellite cells to differentiate, fuse, and form multi-nucleated myotubes.

Nutrition's effects on Myogenic Differentiation/Muscle Regeneration

Muscle regeneration is an energy-dependent process, involving the accretion of myofibrillar proteins. In this section the nutritional effects of dietary carbohydrates and protein on muscle regeneration will be assessed.

Because muscle regeneration is a costly metabolic process, adequate energy must be provided to the system. Carbohydrates are one of the body's primary sources of fuel, thus, direct provision of carbohydrates may effect muscle regeneration. One study using C2C12 myoblasts saw a significant reduction in the myoblast fusion index and myotube formation in a 5mM glucose supplemented medium, compared to a 25mM medium concentration (Fulco et al., 2008). It is interesting to note that upon retroviral induction of a dominant negative AMPK that differentiation was rescued in its entirety, suggesting a definitive role for AMPK signalling in cell energy status regulation and subsequent control over a cells capacity to differentiate (Fulco, 2008). Currently only a few comparative

studies exists *in vivo* looking at the effects of carbohydrates on skeletal muscle development. One such study showed that in fish a diet comprised of 15-25% carbohydrates optimally stimulated myogenic regulatory factors such as MyoD, Myf5, myogenin, and the Pax7:MyoD ratio (an indicator of satellite cell myogenesis in vertebrates) (Chapalamadugu et al., 2009). Another study in fish sought out to determine the effects of carbohydrate restriction on protein turnover, a proxy measure of muscle regeneration. There were two experimental conditions, one being the control diet, the second being a diet completely void of carbohydrates. Although this experimental design seemed unnecessarily extreme, the data regarding protein turnover rates were informative. The fish in the carbohydrate depleted diet group saw no differences in protein synthesis rates, yet protein turnover was drastically upregulated. This effect was due to the near 8-fold increase in muscle protein degradation rates, proving that carbohydrate provision limits the breakdown of muscle proteins for the purpose of meeting energy requirements, via amino acid oxidation, while not limiting the rates of protein synthesis (Peragon et al., 1999). Taken together, carbohydrates appear to be required to sustain energy levels so that muscle regeneration can occur, principally by limiting the rates of protein degradation. However, these effects are likely confined to energy provision, thereby, lipids may regulate these effects to a similar degree.

Once we can establish that the energy demands of a cell are met, the next question seeks to answer how protein metabolism effects muscle regeneration. Dietary protein ingestion provides the amino acids necessary for protein synthesis, a process required for muscle regeneration. Not only do these amino acids act as substrate for translation, but also are essential to the signalling cascades that lead to protein synthesis, or in their

absence, protein breakdown. The first challenge was identifying the amino acid sensing mechanisms of a cell. As alluded to in the mTOR section of this review, the answer lied within mTORC1's ability to sense intracellular amino acids concentrations. Many studies have investigated the potential of individual amino acids to stimulate muscle protein synthesis and the subsequent muscle hypertrophy. However, not many studies have investigated how single amino acids regulate myogenic differentiation/muscle regeneration.

One study investigated the effect leucine plays in proliferation and differentiation of primary satellite cells excised from preterm-rats (Dai et al., 2015). Dai et al., (2015) found that leucine promoted both the proliferation and differentiation of these satellite cells, and that MyoD and myogenin protein expression was positively correlated with leucine dosage. When these cells were co-incubated with leucine (2mM – highest experimental dose) and rapamycin (50nM) the expression of MyoD protein was more than halved, while myogenin expression remained elevated (Dai, 2015). One issue with the Dai et al., (2015) study was that the leucine + rapamycin experiments only lasted for a mere 8-hours of differentiation. This short time course did not allow, or account for, the complex milieu of myogenic regulators that changes over the full 72-hours of differentiation, as they themselves depicted in their control studies. Therefore, the claim that leucine's action on MyoD is sustained throughout the whole process of differentiation may be erroneous.

Furthermore, another group has shown using C2C12 myoblasts and primary satellite cells from mice that cells deprived of leucine fail to differentiate. Additionally, the expression of MyoD is also suppressed under such conditions, while Myf5 mRNA is elevated (Averous et al., 2012). Unlike the study by Dai et al., (2015) the authors also

showed that leucine's effect on MyoD expression was apparent from day 0 all the way to day 5 of differentiation. In this experiment leucine deprivation was able to suppress MyoD expression significantly from 8-hours to the end of their differentiation protocol on day 5. Most interestingly, the authors suggest leucine's main role in early differentiation may not necessarily be promoting MyoD expression, but this lack of MyoD may be explained by the mere absence of differentiation. The authors also note that Myf5's role in differentiation is not well defined, although the rise in Myf5 mRNA may support the notion that Myf5 regulates a cells ability to differentiate in the absence of MyoD, or causes cells to enter a quiescent state (Young & Wagers, 2010), which is also seen in a portion of cells when leucine is deprived.

Pereira et al (2014) devised a model to test leucine's effect on muscle regeneration in mice. The authors subjected 2-month old mice to cryolesion of the soleus muscle while being supplemented with leucine, beginning 3-days prior to muscle injury, outwards to day 10 post-injury. It was found that leucine supplementation increased myofiber size, in addition to lowering collagen type III accumulation on day 10 post-injury (indicative of poorer injury recovery). Leucine supplementation was also shown to decrease the activation of autophagy mediators, such as FOXO3a, as well as reducing the accumulation of ubiquitinated proteins (Pereira et al., 2014). Not only were these leucine-derived effects seen at a molecular level, they were also witnessed at the level of muscle function. Leucine supplemented mice experienced a reduction in strength losses following muscle-injury, while fatigue resistance of the soleus was also elevated when compared to controls. The authors concluded that the positive effects of leucine on muscle regeneration in this case, was not being facilitated through the PI3K – AKT – mTOR axis, but its role was more

pertinent to the inhibition of proteolysis via reductions in protein ubiquitination and FOXO3a activity. Although this may come as a surprise, the authors make note that prolonged leucine supplementation loses its ability to stimulate both the PI3K – AKT – mTOR pathway and protein synthesis (Macotela et al., 2011; Suryawan et al., 2011). Taken together, the lack of mTOR activity and the downregulation of FOXO3a and ubiquitinated proteins in the face of leucine supplementation, suggests leucine positively regulates muscle regeneration via its ability to suppress proteolysis.

Factors and signalling pathways regulating myogenesis

mTor and myogenesis

The first instances of mTOR's regulatory control of myogenesis became evident when many studies reported impaired differentiation in various muscle cell lines in the presence of rapamycin (Conejo et al., 2001; Coolican et al., 1997; Erbay & Chen, 2001). Rapamycin, as previously discussed is an inhibitor of mTORC1. Interestingly, rapamycin-resistant mTOR mutants rescued C2C12 differentiation in the presence of rapamycin (Erbay, 2001). This suggests that mTOR-mediated differentiation functions through both rapamycin-dependent and independent mechanisms. Cells expressing kinase-inactive mTOR mutants were also able to differentiate in the presence of rapamycin, implying that the rapamycin-independent function of mTOR might also be independent of its kinase activity (Ge et al., 2009).

Much of the research involving mTOR's role in differentiation is studied by examining the role of mTORC1 subunit, RAPTOR. Experiments knocking out or

overexpressing RAPTOR had positive and negative effects on differentiation, respectively (Ge, Yoon, et al., 2011). It is postulated that RAPTOR's negative influence over differentiation may be acting through the uncoupling of the PI3K/Akt signalling pathway, via serine phosphorylation of insulin-like substrate-1 (IRS1) (Ge, 2011).

A major discovery of mTOR's role in differentiation is its regulatory control over insulin-like growth factor-2 (IGF-2). A simple, yet elegant experiment was conducted by Erbay et al. (2003) where they took two groups of C2C12 myoblasts, one being the control or "donor" group, the other to be incubated with rapamycin, the experimental or "recipient" group. The donor group was induced to differentiate 24 hours prior to the recipient group. At the 24-hour mark the now "conditioned-medium" from the donor group was transferred to the recipient group and supplemented with 100nM concentration of rapamycin. This medium exchange occurred two more times, until the cells had differentiated for 72 hours. Differentiation in the recipient group was completely rescued by the conditioned medium, despite the presence of rapamycin. Erbay et al. (2003) knew this supported the notion that a secreted factor was mediating differentiation, which was confirmed to be IGF-2. It turns out that mTOR mediates the transcription of IGF-2 independently of its kinase activity through a muscle specific enhancer (Erbay et al., 2003). One proposed mechanism of this effect is through mTOR's suppression of mir-125b, a microRNA that targets IGF-2, triggering its suppression (Ge, Sun, et al., 2011). Another mechanism regulating IGF-2 expression is mTOR's direct kinase activity towards IGF-2 mRNA-binding protein (IMP2), which enhances IGF-2 translation (Dai et al., 2011). Taken together, IGF-2's regulatory control over differentiation appears to be primarily regulated by both kinase dependent and independent functions of mTOR.

AKT and Myogenesis

Protein Kinase B or AKT is a serine/threonine kinase, which acts as part of the PI3K/AKT/mTOR signalling pathway. There are three known isoforms of AKT (1-3). AKT1 is involved mainly with cell growth and proliferation (Li et al., 2013) and muscle hypertrophy (Bodine, 2001). AKT2 has been linked to glucose homeostasis thus, is understandably expressed in insulin-sensitive tissues (Cho et al., 2001). Lastly, AKT3 is expressed primarily in lung, kidney and brain (Brodbeck et al., 1999). For our purposes the focus is on AKT1 and AKT2. Depletion of AKT1 in 10T1/2 mouse embryonic fibroblasts (MEFs) impaired myotube formation by 80% and myocyte fusion index by 90% (Wilson & Rotwein, 2007), adding to the body of evidence that AKT1 serves an important role in muscle regeneration.

Insulin and insulin-like growth factor (IGF) activate AKT through their interactions with the insulin-like growth factor receptor (IGFR). IGFR is a tyrosine kinase, which activates insulin-receptor substrate (IRS) via phosphorylation (Virkamäki et al., 1999). The phosphorylation of IRS triggers the translocation of phosphatidylinositol 3-kinase (PI3K) to the inner border of the cell membrane. PI3K contains catalytic subunits that produces the membrane bound phosphatidylinositol (3,4)-bisphosphate and eventually phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 then causes the localization of all pleckstrin homology domain containing proteins to the cell membrane, one being AKT and its direct activator phosphoinositide-dependent kinase 1 (PDK1) (Virkamäki, 1999).

Surprisingly, fairly few studies have investigated the role AKT serves in skeletal muscle differentiation. One study shows that AKT1 expression is required for the activity of the transcription factor MyoD, although its actions are independent of MyoD levels (Wilson, 2007). The authors also found that AKT1 depletion had no effect on cell viability or proliferation, which contradicts another study that stated AKT1 was required for cell proliferation (Heron-Milhavet et al., 2006). Heron-Milhavet at al. (2006) saw reductions in cyclin A expression, and failure to enter S-phase of the cell cycle in response to interference RNA targeted at AKT1. AKT2 too has been implicated in the regulation of myogenic differentiation. Two studies so far have implicated its role in muscle cell differentiation. The first proposed mechanism of AKT2's effect on cell cycle exit described by Heron-Milhavet et al. (2006) states that AKT2 binding to p21, a cell cycle inhibitor, leads to its nuclear accumulation, triggering cell cycle exit. The second proposed role of AKT2-mediated myogenic differentiation is via its inhibitory action towards Prohibitin2 (PHB2)/Repressor of estrogen activity (REA), a negative regulator of differentiation. PHB2/REA has been found to bind and inhibit MyoD, in addition to reducing MEF2 transcriptional activity, thereby impairing differentiation (Sun et al., 2004). Early experiments by Sun et al. (2004) had shown that AKT2 and PHB2/REA are inversely expressed at the onset of myogenic differentiation, and that during unperturbed differentiation AKT2 levels rise while PHB2/REA levels fall (Héron-Milhavet et al., 2008). Additional experiments showed that co-expression of AKT and PHB2 resulted in a decline of MyoD-PHB2 binding, rescuing muscle cell differentiation (Sun, 2004). Loss of function experiments that were conducted on AKT2 resulted in the failure of cells to exit the cell cycle, alongside a rise in the expression of PHB2/REA, subsequently impairing

differentiation (Héron-Milhavet, 2008). Taken together the current literature proves that AKT and at least two of its different isoforms are essential to cellular proliferation, cell cycle arrest, differentiation, and hypertrophy.

MAPK Signalling and Myogenesis

Multiple signalling cascades converge to properly regulate myogenesis, one of them being mitogen-activated protein kinases (MAPKs). MAPKs, as the name suggests, are responsive to mitogenic signals. These mitogenic signals regulate many cell functions including proliferation, differentiation (Madhala-Levy et al., 2012) and apoptosis (Keren et al., 2006). The MAPK superfamily is highly conserved in mammals. It is principally comprised of the extracellular signal regulated kinases (ERKs), p38 MAPKs, Jun amino-terminal kinases (JNKs) and Stress-activated protein kinases (SAPK) (Segalés, Perdiguero, et al., 2016). This literature review will focus on p38 MAPKs due to their established role in various stages of myogenesis. In mammals there exists four p38 MAPK family members: p38α, p38β, p38Y, and p38δ. Specific inhibitors to p38α/p38β have aided in identifying many physiological functions carried out by these kinases. One study found that myoblast fusion, and eventual myotube formation is ablated in the presence of p38α/p38β inhibitors. ((Lluís et al., 2006). However, when the master p38 kinase, MKK6, is overexpressed, muscle cells are able to overcome the inhibitors, expressing myogenic markers and the formation of myotubes (Lluís, 2006). This suggests the activation/phosphorylation of p38α/p38β is required for myotube formation. A more recent study in C2C12 myoblasts using genome-wide based analyses of p38α found that during muscle cell differentiation p38α was bound to a large number of active and inactive

promoters, further providing evidence of p38α's role in the transcriptional regulation of myogenic differentiation (Segalés, Islam, et al., 2016).

Furthermore, p38α/p38β has also been shown to be necessary for satellite cell activation and the transcription of MyoD (Jones et al., 2005). Once MyoD transcription has been initiated, p38α/p38β has been found to promote its association with the E-box protein, E47, via direct phosphorylation (Lluís et al., 2005). The heterodimerization of MyoD and E47 is essential for muscle-specific gene transcription (Lluís, 2005). Lastly, at the onset of myoblast differentiation cell cycle arrest needs to occur. One of the mechanisms regulating this is p38α. p38α-induced cell cycle arrest is ultimately achieved via the downregulation of cyclin D1 expression, in a JNK pathway dependent manner (Perdiguero et al., 2007). Even later in the differentiation process p38α coordinates myoblast fusion via the upregulation of the tetraspanin, CD53, a prominent mediator of myoblast fusion (Liu, 2012). In conclusion, p38 MAPK signalling is a large and essential contributor to muscle cell differentiation.

Notch Signalling and Myogenesis

Notch signaling is a highly complex and evolutionarily conserved pathway overseeing many roles in embryogenesis (Artavanis-Tsakonas et al., 1999), tumorigenesis (Evans & Calvi, 2015), metabolism (Pajvani et al., 2011), stem cell population maintenance (Androutsellis-Theotokis et al., 2006), and myogenesis (Conboy & Rando, 2002). The components of the Notch signalling pathway are comprised of four-mammalian receptors (NOTCH1-4), five-ligands, and both an extracellular (NECD) and intracellular (NICD) domains. Ligand binding occurs with the larger NECD, and conveys the signal to the smaller

NICD. Following ligand binding, a cleavage event separates the NICD from the intracellular-side of the cell membrane, allowing it to translocate to the nucleus where it binds Rbp-j and transcribes notch target genes (Kopan & Ilagan, 2009).

The onset of muscle regeneration in adult tissues begins with an early muscle progenitor-type cells, satellite cells. In response to stress or injury, satellite cells awaken from their quiescent state and enter the cell cycle so that they can provide the necessary nuclei to support the repair of existing muscle fibers. Notch signalling is required for satellite cell proliferation and population maintenance, in addition to its effects in blocking differentiation (Conboy, 2002; Shan et al., 2017). Notch signalling's role in satellite cell population maintenance is achieved by positively expressing the transcription factor Pax7, without which would result in dedifferentiation, away from a myogenic lineage (Shan et al., 2016). Experiments in which the Notch target Rbp-j is inactivated during development, satellite cell population levels decline, leading to the impairment of muscle regeneration (Vasyutina et al., 2007). In mature skeletal muscle that same inactivation of Rbp-j results in a decline of satellite cell population, which is accompanied by their immediate myogenic differentiation. This causes the prevention of S-phase entry, resulting in the failure of satellite cell expansion, an essential step in muscle regeneration (Bjornson et al., 2012). Studies overexpressing a constitutively active NICD found that Notch signalling's induction of Pax7 was directly regulated via its interaction with Rbp-j, which colocalizes with the NICD to the Pax7 promoter, promoting its activation (**Figure 1-3**) (Wen et al., 2012). Further experiments by Wen et al. (2012) used a lentivirus-mediated knockdown of Rbp-jκ, which completely removed any NICD association with the Pax7 promoter region, thereby preventing Pax7 expression. This resulted in premature satellite cell cell-cycle exit, failure

of satellite cell expansion and the subsequent impairment of differentiation. Notch signalling is also involved in the negative regulation of MyoD and myogenin, which is believed to be distinct from the NICD-Rbp-jκ interaction, and instead be regulated through canonical notch signalling (depicted on the right side of **Figure 1-3**) (Olguin et al., 2007; Wen, 2012). All together, the growing literature around Notch signalling confirms its importance in regulating satellite cell population, cell fate determination, and its requirement for muscle regeneration.

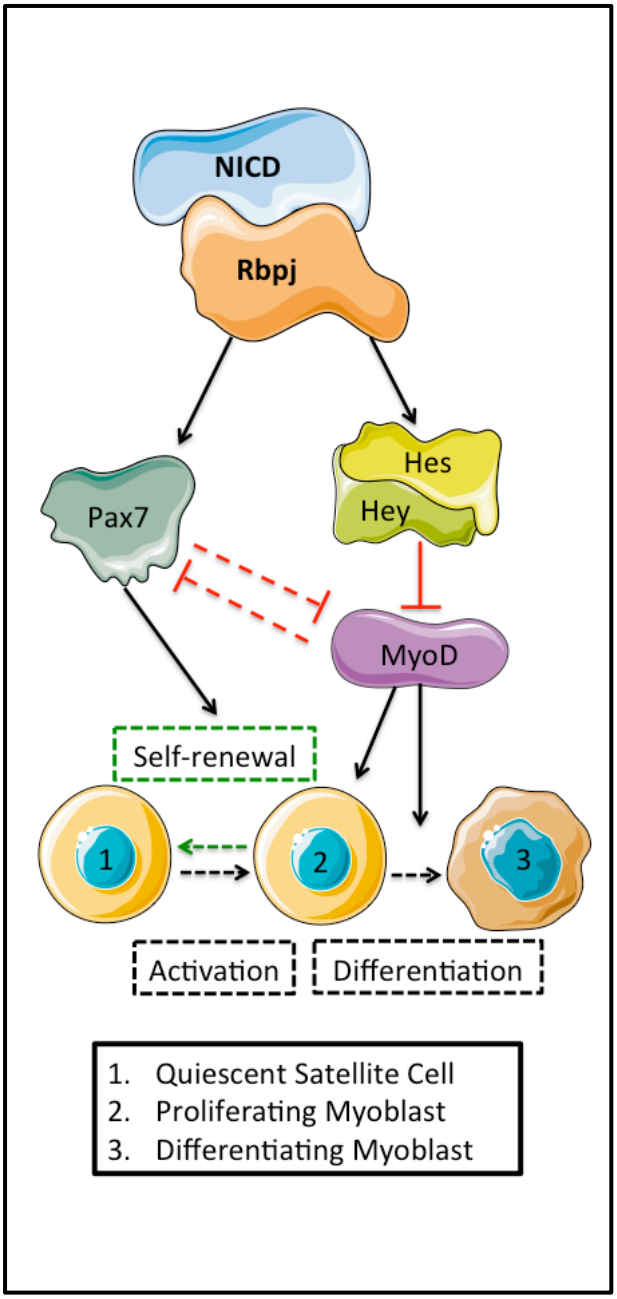

Figure 1-3. Notch signalling effects on satellite cell activation and early differentiation. Quiescent satellite cells predominantly express Pax7. Upon activation the satellite cells start to upregulate their expression of MyoD while Pax7 expression falls proportionately. Following the rise of MyoD and fall of Pax7 expression the cells exit the cell cycle and begin to differentiate. Notch signalling promotes the binding of the NICD with Rbp-j κ which upregulates the transcription of Pax7. The canonical targets of Notch signalling, Hes and Hey family proteins, are also activated to suppress MyoD expression. Overall, Notch signalling decreases as the myoblasts begin to differentiate. Image modified from (Wen, 2012). Recombining binding protein suppressor of hairless (Rbpj), Notch intracellular domain (NICD), Hairy and enhancer-of-split (HES), Hes-related repressor (HEY).

Mitochondrial Regulation of Myogenesis

The mitochondrion has many functions within the cell. Recently, mounting evidence suggests that they may also be regulating cell differentiation, particularly muscle cell differentiation. *In vitro* studies investigating the mitochondrial changes that occur during myoblast differentiation have shown a dramatic increase in mitochondrial enzyme activity (Barbieri et al., 2011; Moyes et al., 1997; Remels et al., 2010). Additionally, mitochondrial biogenesis has been found to increase during myoblast differentiation and muscle regeneration (Barbieri, 2011; Duguez et al., 2002; Remels, 2010; Wagatsuma et al., 2011). These factors alone are convincing enough that mitochondrial function is required for myoblast differentiation, leading to the question, "Do mitochondria regulate myoblast differentiation through an energy-mediated mechanism, or through other mitochondrial functions?"

A study examining mitochondrial function and its importance in differentiation was conducted using human primary muscle cultures. Ethidium bromide was used to inhibit mitochondrion DNA replication and transcription, which impaired myotube formation (Herzberg et al., 1993). Another study using C2C12 muscle cells found that inhibition of mitochondrial protein synthesis via tetracycline treatment also impaired terminal differentiation, while showing that these effects were independent of cell energy status (Hamai et al., 1997). Rochard et al. (2000) showed that the impairment of mitochondrial translation by chloramphenicol treatment negatively affected myoblast differentiation. This treatment perturbs proper assembly of 4 out the 5 respiratory complexes, rendering the organelle inert, yet this did not affect cell viability (Rochard et al., 2000). They also showed that increasing mitochondrial activity via p43 expression enhanced myoblast

differentiation (Rochard, 2000). p43 overexpression has been shown to stimulate mitochondrial gene transcription, ultimately leading to a rise in mitochondrial protein synthesis (Casas et al., 1999). Impressively, this enhancement in myoblast differentiation was accomplished in 10% serum conditions, meaning the QM7 myoblasts were able to overcome the cell-cycle promoting effects of a high-serum condition (Rochard, 2000). These results were replicated using two additional mitochondrial function inhibitors, oligomyosin and carbonyl cyanide p- (trifluoromethoxy) phenylhydrazone (FCCP). Lastly, Rochard et al. (2000) showed that disruption of mitochondrial activity negatively affected myogenin expression, in addition to drastically diminishing the expression of the acetylcholine receptor subunit alpha (AchRα), another marker of differentiation (Rochard, 2000). A follow-up study was completed by the same laboratory investigating the role that c-myc plays in mitochondrial-mediated myogenesis. Their main findings indicate that mitochondrial activity negatively regulates c-myc expression, which leads to permanent cell cycle withdrawal for myoblasts. Using FACS analysis, Seyer et al. (2006) found that overexpression of p43 increased the number of cells in the G0/G1 phase, conversely, c-myc overexpression stimulated cell cycle progression into S-phase, which also mirrored the response to chloramphenicol treatment. A possible explanation is that c-myc is a known suppressor of p21 expression, which is a major component of the cellular machinery that induces cell cycle arrest (Seyer et al., 2006). In conclusion, mitochondrial function independent of energy production is required for proper myoblast differentiation. Further study to discern the exact mechanisms involved are warranted.

BCAA metabolism

BCAA catabolism is often dysfunctional during diseased states including obesity & type 2 diabetes mellitus (Lynch, 2014a; Mccormack, 2013; She, 2009), heart disease (Wang, 2016), and maple syrup urine disease (MSUD) (Indo, 1987). Specifically, MSUD, a condition involving a loss-of-function mutation in BCKD, displays signs of skeletal muscle impairment (Ferriere et al., 1984). Dysregulation of BCAA metabolism in those diseased states may just be a symptom, or they could represent a primary driver in muscle impairment.

Impaired BCAA metabolism and disease

Many studies have begun looking into whole body BCAA metabolism due to their metabolic dysregulation in many diseased states including types 1 and 2 diabetes, renal failure, and heart disease. She et al. (2007) conducted a study utilizing a transgenic mouse depleted of global BCATm. Interestingly, a plethora of metabolic changes were present in these mice. Phenotypic changes included reductions in body weight (~10-15%) and adiposity, while also displaying resistance to high-fat diet induced obesity, despite increases in food intake (She et al., 2007). The starkest metabolic change was the improvement in insulin sensitivity, observed by basal plasma insulin levels falling from 0.75 ng/ml to 0.26 ng/ml (She, 2007). The authors also noted a significant rise in protein turnover, evident from the increase in protein synthesis rates in all tissues measured (heart, gastrocnemius, kidney, epididymal fat, and liver), while also noting a rise in urine 3-methylhistidine levels, indicative of protein degradation (She, 2007). It was hypothesized that the high leucine concentrations resultant from the removal of its primary catabolic

enzyme (BCATm) was causing an upregulation in protein translation, due to leucine's ability to stimulate mTORC1 activity. This rise in protein turnover may explain the increase in energy expenditure observed in the BCATm knockout mice.

In a follow-up study She et al., (2010) sought out to assess the effects of BCATm depletion on energy metabolism and exercise performance. The authors hypothesized that the drastic rise in plasma BCAA levels from the previous study could impact exercise performance, based upon past literature investigating BCAA supplementation and exercise performance. BCATm depleted mice were subjected to a treadmill test starting with a short endurance-training period (3-days) followed by the incremental-intensity test until exhaustion. Following training the transgenic mice ran for a reduced time (32% reduction) and distance (24% reduction) compared to littermate controls (She et al., 2010). The BCATm depleted mice displayed significant exercise capacity impairment, evident by a rise in plasma ammonia and lactate levels. Additionally, intramuscular levels of the lactate to pyruvate ratio increased similarly to plasma levels. Overall, the BCATm depleted mice appear to be in energy crisis based upon the significant reductions of gastrocnemius ATP concentrations, the accumulation of plasma ammonia and lactate, and the seven-fold rise in muscle inosine monophosphate (IMP) levels, indicative of an upregulated purine nucleotide cycle (PNC). These changes may represent a compensatory mechanism to replenish TCA cycle intermediates that are lacking in the knockout mice. A likely explanation for the rise in PNC activity could be from the reduction in glutamate synthesis, a product of BCAA transamination that is catalyzed by BCATm (She, 2010). The authors argue that this reduction in glutamate synthesis causes a decrease in the malate-aspartate shuttle system activity, which leads to a rise in cytosolic NADH and is associated with

lactate accumulation. Recently, a metabolic analysis study was completed comparing type 2 diabetic (T2D) patients against BMI-matched controls. Remember that T2D patients are one of the populations that have altered BCAA metabolisms, particularly noted by increased plasma BCAA levels. The authors found during baseline measurements that the T2D group had significantly reduced activities of malate-aspartate shuttle enzymes (cytosolic: aspartate aminotransferase - 25%, mitochondrial: aspartate aminotransferase - 30%, and malate dehydrogenase - 45%) (Hussey et al., 2013). The reductions in the malate-aspartate shuttle system enzymes, as alluded to by She et al., (2010) could be caused by an impairment of glutamate synthesis, which the BCATm enzyme regulates. These findings suggest that BCAA metabolic dysfunction in T2DM may be due to the impairment of BCATm functioning, providing a link between BCATm dysregulation and T2D.

There exist 3 studies to date, to my knowledge, that assess the effects of BDK knockout in mice. BDK is the enzyme that negatively regulates BCKD activity, and thus the activity of the entire BCAA catabolic pathway. The first study conducted in 2006 was a whole-body knockout, which saw 15% reductions in body weight, alongside reductions in brain, muscle, and adipose tissue, while liver and kidney weights increased (Joshi et al., 2006). Most notably these mice suffered neurological abnormalities involving epileptic seizures. The authors believed this might have been due to a hyper-phosphorylation of eIF2-alpha in brain tissue that would result in a decrease in protein synthesis. However, body weight and general appearance of the BDK knockout mice was rescued by a high-protein diet (50% kcal from protein). But the authors did not mention what affect this had on the severity or frequency of the seizures. This study improves our understanding of how essential a properly regulated BCAA catabolic pathway is to health.

The second study was completed in 2017. It sought out to assess how a muscle-specific knockout of BDK would affect protein metabolism. Plasma BCAAs were significantly reduced in the muscle-specific BDK knockout mice, similarly to that of the whole body BDK knockout mice. The degree at which the muscle-specific BDK knockout effects plasma BCAA concentrations proves the central role skeletal muscle plays in global BCAA metabolism. On the other hand, this fails to explain the absence of any tissue or body weight changes, in addition to the absence of any neurological abnormalities witnessed in the whole body BDK knockout mice (Ishikawa, 2017). The main findings included an increased sensitivity of mTORC1 to high-protein diet feedings, or BCAA supplementation. The authors speculated that the increase in mTORC1 sensitivity may be due to the chronic reduction in BCAA levels, leading to a hyper-sensitization to BCAAs. Additional experiments showed a 50% reduction in mTORC1 activity when animals were placed on a low-protein diet (8% of kcals from protein) and was accompanied by significant reductions in myofibrillar proteins (Ishikawa, 2017). Upon BCAA supplementation these losses were attenuated and mTORC1 activity was fully restored/enhanced.

In a follow-up study, which used the same muscle-specific BDK knockout model, the authors investigated its effects on endurance exercise and energy metabolism. Interestingly, there were no basal differences in exercise performance, only post-endurance exercise training saw a difference in performance (~12% reduction in BDK knockout performance) (Xu et al., 2017). The authors also noted reductions in muscle glycogen in the knockout mice post training, in addition to a rise in the acyl-carnitine production, which they claim may impair energy metabolism in muscle (Xu, 2017).

These three studies show the importance and impact that BDK has over BCAA metabolism and the subsequent effects that an accelerated BCAA catabolic pathway has on global energy and protein metabolism.

The BCAA catabolic pathway

Two mitochondrial enzymes regulate the initial two steps in the BCAA catabolic pathway, branched-chain amino acid transferase-2 (BCAT2/BCATm) and Branched-chain alpha keto-acid dehydrogenase (BCKD). BCAT2 oversees the transamination of BCAAs into their respective keto-acids, which are then further catabolized by BCKD. BCKD is a mitochondrial inner-membrane localized protein complex that shares structural and functional similarities to both pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Indo, 1987). It orchestrates the decarboxylation of BCAA derived keto-acids, representing the first irreversible and rate-limiting step in BCAA catabolism. BCKD is comprised of three component parts: the heterotetrameric branched-chain α-keto-acid decarboxylase component (E1: 2α-subunits & 2β-subunits), dihydrolipoyl transacylase component (E2), and the dihydrolipoyl dehydrogenase component (E3) (Matsumoto et al., 2010). BCKD is under tight regulation between a phosphatase and kinase that positively and negatively regulate its activity, respectively. The phosphatase termed protein phosphatase 2Cm (PP2Cm) is a mitochondrial matrix phosphatase. This enzyme has been linked to cell survival, embryonic development and cardiac function (Lu et al., 2007). PP2Cm positively regulates BCKD activity via the removal of a phosphorylation on S293 of BCKD's E1α

subunit (**Fig. 1-4**). The inhibitory phosphorylation is catalyzed by branched-chain α-keto-acid dehydrogenase kinase (BDK). Regulation of these two proteins has garnered some attention because of their potential to modulate BCAA homeostasis.

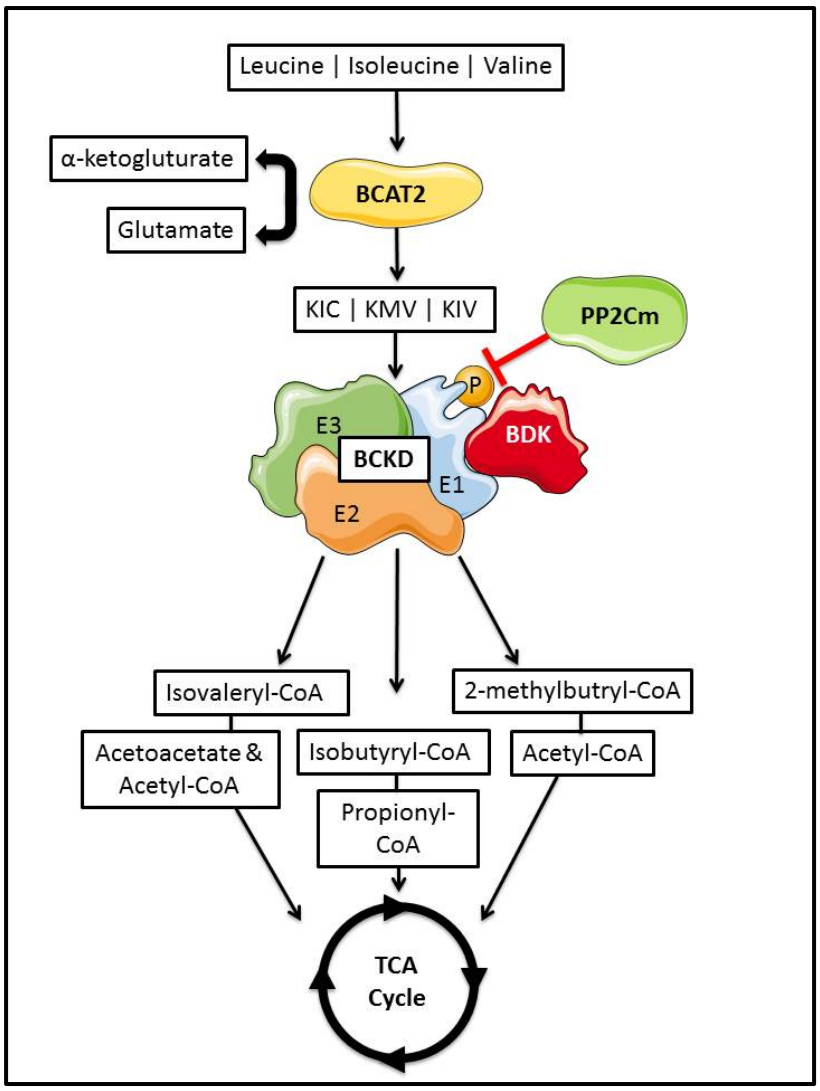

Figure 1-4. Branched-chain amino acid catabolic pathway. Schematic of the BCAA catabolic pathway, depicting its two main catabolic enzymes, BCATm and BCKD, respectively. BCATm controls the reversible transamination step that converts all three BCAAs into their keto-acid derivatives, while simultaneously converting α-ketoglutarate to glutamate. The second step is the irreversible decarboxylation of the BCAA derived keto-acids, forming their acyl-CoA derivatives, which can be siphoned off at any time for free fatty acid synthesis, ketone production and other cellular functions. Abbreviations: α-Ketoglutarate (αKG), Branched-chain amino acid (BCAA), mitochondrial branched-chain amino acid transferase (BCATm), Branched-chain alpha keto-acid dehydrogenase (BCKD). Figure modified from (She et al., 2013).

Protein Phosphatase 2Cm (PP2Cm)

Although the inhibitory kinase (BDK) of BCKD has been known for more than three decades, the characterization and cloning of its activating phosphatase has remained elusive up until recently (Y Shimomura et al., 1990).

Encoded by the PPM1K gene, protein phosphatase 2Cm (PP2Cm) is a mitochondrial Ser/Thr phosphatase localized to the matrix, is a member of the protein phosphatase 2C family, and is highly conserved among vertebrates (Lu, 2007). PP2Cm has been shown to be essential in the following cell functions: development, cell survival, apoptosis and metabolism. However, this phosphatase is not ubiquitously expressed, the highest levels have been found in brain, heart, kidney and hepatic tissues, while being lowly expressed in skeletal muscle (M. Zhou et al., 2012). Additionally, a loss of function mutation of PP2Cm results in a mild phenotype of MSUD, typically witnessed in cases where BCKD is mutated (Oyarzabal et al., 2013).

Using X-ray crystallography PP2Cm structure has been identified by Wynn et al., (2012), its notable feature, a 10-Ångstrom wide cleft, was determined to be the phosphatase's active site. They further cloned PP2Cm and found that its activity requires the binding of two Mn^{2+} ions, disruption of these binding sites results in enzyme inactivity (Wynn et al., 2012). Arg-104 within PP2Cm's active site is essential to substrate recognition. When the Arg-104 is mutated to alanine, PP2Cm's K*m* for the phosphorylated BCKD catalytic moiety increases 60-fold (Wynn, 2012).

Under conditions of reduced BCAA concentrations PP2Cm is transcriptionally downregulated (M. Zhou, 2012), whereas BDK translation would consequently increase (Doering et al., 2000; Harris et al., 2001). The antagonism between these two enzymes is dynamically regulated by BCKA concentrations, where high BCKA concentrations up-regulate PP2Cm activity towards BCKD, and down-regulate BDK activity, and vice-versa with respect to low BCKA concentrations (M. Zhou, 2012).

Impairments in BCAA catabolism are not limited to patients with MSUD related genetic defects. Impaired BCAA catabolism is also present in cases of ischemia and heart failure, resulting in high BCAA concentrations in myocardial tissue (Wang, 2016; M. Zhou, 2012). One of the first studies describing PP2Cm found that its levels decreases under myocardial stress (Lu, 2007). Zhou et al., (2012) suggested PP2Cm regulation may not be restricted to just myocardial stress, but by widespread oxidative stress, witnessed in obesity and type 2 diabetes. Zhou et al., (2012) also found that PP2Cm expression is linked to nutrient availability, similarly to its counterpart BDK. However, to date, there is no information regarding the mechanisms regulating PP2Cm's reduction following cardiac stress, albeit, research investigating PP2Cm's function is growing. A study using mouse embryonic stem cells displaying PP2Cm deficiencies showed marked increases in superoxide and ROS production, and ectopic expression of PP2Cm returned their levels to baseline. Clearly, PP2Cm serves a role in managing oxidative stress, although further study is warranted to determine whether this is through direct or indirect mechanisms. However, PP2Cm's role in skeletal muscle remains to be defined, as previously stated PP2Cm expression in skeletal muscle is relatively lower than in the myocardium. Therefore,

research investigating PP2Cm is required to elucidate its physiological relevance in skeletal muscle metabolism.

Branched chain alpha-keto dehydrogenase kinase (BDK)

BDK is a nuclear-encoded mitochondrial protein kinase (Muller & Danner, 2004). BDK's kinase activity, phosphorylating serine 293 and serine 303 on BCKD's E1-alpha subunit, show that it indeed functions as a serine/threonine kinase. However, its sequence and some structural characteristics show it is more similar to histidine kinases, which would be debated during the early characterization of BDK. Later studies would show that BDK could not function as a histidine kinase for two reasons. One, the phosphorylation event on BCKD's E1-alpha subunit was not a result of histidine-mediated phosphotransfer, as shown by Davies et al., (1995) and two, the conserved histidine residue is centralized to BDK's hydrophobic core, meaning it is inaccessible for histidine-mediated phosphotransfer in the first place (Attwood, 2013; Davie et al., 1995).

Rat BDK exists in a dimer-tetramer equilibrium, although characterization using X-ray crystallography yields only the dimer organization, while the relevance of BDK tetramerization is unknown (Machius et al., 2001). The two domains of BDK (K and B as denoted by Machius et al., (2001)) each contain unique features. The K domain shares structural homology to protein histidine kinases in its nucleotide-binding domain, while the B domain has a structure containing a four-helix bundle domain, which appear in two-component signal transduction (TCST) systems (Machius, 2001). A TCST system coordinates stimulus-response signals in a cell, typically in response to changing

environmental conditions, and is the principle signalling mechanism in bacteria (Stock et al., 2000).

BDK's kinase activity requires two cofactors magnesium and potassium (Machius, 2001). Additionally, in its inactive state BDK is ADP-loaded, which needs to be removed for its full activation. It is thought that the lipoyl-bearing domain of E2 (a subunit of BCKD) could facilitate its removal rendering it active (Machius, 2001).

Mitochondrial extracts from C57BL/6 mice displayed high levels of expression of BDK in skeletal muscle, and at lower levels in kidney, brain, and liver, respectively. BDK mRNA levels were not expressed in parallel with protein expression, suggesting that there are additional regulatory factors controlling translation (Muller, 2004). Muller et al., (2004) went on to define an upstream open reading frame within the 5'-untranslated region of BDK mRNA, which contains elements that negatively regulate BDK translation.

Once expressed BDK is localized to the mitochondrial matrix where its activity is regulated via allosteric inhibition. The branched-chain alpha keto-acids (BCKAs) in particular are the most potent endogenous BDK inhibitors. A study conducted by Paxton & Harris (1984) using a perfused rat heart model, established the K*m* values of the BCKAs towards BDK, their concentrations were as follows for α-ketoisocaproate (KIC), α-keto ß-methylvalerate (KMV), and ketoisovalerate (KIV), 70μM, 100μM, and 250μM, respectively (Paxton & Harris, 1984). Exogenous inhibitors of the BDK complex do exist, although KIC's affinity for BDK seems to be greater than most of them. Therefore, reducing the concentrations of BCKAs, as would be the case under protein restriction, should increase BDK activity, subsequently decreasing BCKD activity. Evidence supporting this assumption showed that rats on a low protein diet had increased BDK activity in liver extracts; this

increase is thought to be facilitated through the increased expression of BDK, and thereby, an increase in its association with BCKD (Popov et al., 1995). Conversely, high protein diets decrease the expression of BDK (Lynch, 2014a). Altogether, BDK expression is regulated translationally, in a tissue-dependent manner, while its activity is under tight control of the BCAA derived keto-acids.

Recent evidence has suggested that yet another layer of complexity exists in regulating the function of the BCAA catabolic pathway. BCATm and BCKD have been introduced in this literature review as two distinctly separate enzymes, each having a unique primary function. One study by Islam et al., (2007) challenges this assertion based on what they believe occurs between these two complexes. The term supramolecular complex, or metabolon, refers to the physical interaction between the two complexes. The significance of this supramolecular complex-interaction resides in the efficiency of substrate channeling from one complex to the other. The physical interaction occurs between the E1-alpha subunit of BCKD and the PLP-linked BCATm, PLP is the BCATm cofactor pyridoxal 5-phosphate (Islam et al., 2007), ultimately promoting the efficient transfer of BCKAs. Experiments completed by Islam et al., (2007) observed a 12-fold increase in the catalytic E1-alpha BCKD subunit activity, when in the presence of PLP-hBCATm. Two factors promote the dissociation of the metabolon, the first being a high phosphorylation status of BCKD, and second, the local rise in the NADH/NAD+ ratio (Islam, 2007). Both of these factors would be present under conditions that reduce intracellular BCAA levels, such as protein restriction or fasting. This pioneering study has improved our understanding of the complexities involved within the BCAA catabolic pathway and a better understanding of the kinetics of BCAA catabolism.

BCAA metabolites effects on skeletal muscle anabolism

Two metabolites of leucine metabolism are at the forefront of BCAA mediated anabolism research. These are β-hydroxy β-methylbutyrate (HMB) and α-ketoisocaproate (KIC). Similar to leucine, both KIC (Yoshizawa et al., 2004) and HMB (Pimentel et al., 2011) are able to activate mTORC1, based on the increased phosphorylation of mTORC1 substrates, p70S6K1 and 4E-BP1. KIC has been also shown to promote MPS in piglets (Escobar et al., 2010). However, the anabolic effects of HMB have received greater attention, showing that supplementation in rats and piglets has been found to stimulate muscle protein synthesis, reduce muscle protein breakdown (Gerlinger-Romero et al., 2017; Giron et al., 2016; Noh et al., 2014) and increase satellite cell proliferation (Kao et al., 2016). A controversy regarding KIC studies and its ability to stimulate muscle protein synthesis questions whether KIC is having this effect, or if it is merely the result of KIC being transaminated back into leucine (Moghei et al., 2016). However, one study using rats showed that KIC can increase protein synthesis without changing intracellular leucine concentrations (Chua et al., 1979).

Currently, the mechanism of HMB-mediated anabolism remains poorly understood. HMB has recently been shown to dampen the fasting induced rise in atrogenes, specifically, Bbox32 & Trim63, possibly explaining the anti-catabolic effects of HMB administration (Gerlinger-Romero, 2017). Furthermore, research has indicated that HMB reverses glucocorticoid-induced myopathies through the inhibition of FOXO1 nuclear translocation, ultimately decreasing ubiquitin proteasome system (UPS) responses, reversing losses in grip strength, muscle mass, and muscle damage (Noh, 2014). Not only does HMB stimulate global protein synthesis, its effects on muscle protein synthesis appear to be greater than in

visceral tissues (Kao, 2016). However, in this study HMB supplementation appeared to have no impact on the autophagy-lysosomal or ubiquitin-proteasome pathways; this could possibly be explained due to the short-time period of the study, lasting only 26.25 hours (Kao, 2016).

Clearly some contradictory evidence exists, whether or not KIC and HMB contributes to both sides of the protein balance equation (increase: muscle protein synthesis, decrease: muscle protein breakdown). Therefore, additional research needs to be completed, however, at this time the majority of research emphasizes their anabolic and anti-catabolic effects on SM.

BCAAs and their metabolites effects on skeletal muscle differentiation

There have been relatively few studies investigating the effects of BCAAs on skeletal muscle differentiation. Much of the research, as previously alluded to, has been investigated in existing muscle, centralized around the hypertrophic responses mediated by the BCAAs. One recent study has investigated the effects of varying BCAA ratios on C2C12 proliferation and differentiation. (Duan et al., 2017). The authors found that an optimal ratio of BCAAs (Leucine - 1; Valine - 0.25; Isoleucine - 0.25) stimulated multiple myogenic regulatory factors in both the proliferative and differentiation phases. The optimal BCAA ratio shifted more cells into S-phase, when compared to the two other experimental BCAA ratio groups (No BCAAs - decrease 20%, 1:1:1 BCAA ratio - decrease 10%) (Duan, 2017). Gene expression of mTOR related genes and of MRFs were generally upregulated during both proliferation and differentiation phases under the optimal BCAA ratio condition, where MuRF1 and MAFbx gene expression was generally downregulated

(Duan, 2017). This new data supporting the positive effects that an optimal ratio of BCAAs has on muscle cell proliferation and differentiation, provides novel insight into BCAAs capacity to improve muscle regeneration. Surprisingly, this is the only study to date, to our knowledge, which looks explicitly at muscle cell differentiation in response to BCAA manipulation.

Research investigating BCAA catabolism and its effects on muscle cell differentiation are still in its infancy. With that being said, the combination of recent research with older studies novel findings may be more pertinent now than they once were. Two studies investigating the effects of a hyperlipidemic correcting drug, chlorophenoxyisobutyrate, otherwise known as clofibrate, found cases where it was causing what they described as *muscular syndrome* (Paul & Adibi, 1979; Teräväinen et al., 1977) The condition exhibited signs and symptoms of muscle soreness, weakness, and tenderness, accompanied by an increase in serum creatine phosphokinase (indicative of muscle damage) and transaminase activity. Additionally, it was found that a 2-week clofibrate treatment in rats elicited multiple metabolic changes including: a reduction in gastrocnemius weight and protein content, a decrease (40%) in fasting insulin, an increase in liver mass and protein content, and finally, a reduction in glycogen stores in both liver and gastrocnemius (Paul, 1979). A few years later another group would define the endogenous inhibitor of the BCKD complex, BDK, which is now known to be directly inhibited by clofibrate (Harris et al., 1982). Similarly to the leucine metabolite, KIC, clofibrate inhibits the interaction between BCKD and BDK, which consequently upregulates the BCAA catabolic pathway (Yoshiharu Shimomura et al., 2006). Shimomura et al., (2006) postulates that the clofibrate induced myopathies may be a result of an accelerated BCAA catabolic pathway. The well-

established effects that BCAAs, particularly leucine, have on MPS, as well as leucine's insulinotropic effects, account well for the signs & symptoms of clofibrate induced - *muscular syndrome*.

Research Objectives

The process of skeletal muscle formation is immensely complex, and is consequently subject to a vast number of complications along its many checkpoints. Through the assessment of some of these checkpoints our study will explore the role the BCAA catabolic pathway serves in the process of muscle cell differentiation. It has been shown previously in our lab that the two main catabolic enzymes of the BCAA catabolic pathway are essential to the proper progression of differentiation. If either one of the enzymes are removed from muscle cells, myotube formation is absent. Therefore, this study will explore how the flux through this pathway changes during differentiation, and how altering this catabolic flux will affect differentiation.

My study's goal was to determine the regulatory role BCKD has during skeletal muscle differentiation. Much of the existing literature has investigated the anabolic responses elicited by the branched-chain amino acids in mature skeletal muscle. However, much less research has been done to elucidate how this subset of amino acids regulates skeletal muscle differentiation in vitro. My study aims to:

i. Measure the expression of enzymes responsible for regulating BCKD activity, and thus, the activity of entire BCAA catabolic pathway, during muscle cell differentiation.

ii. Determine how the activity of the rate-limiting enzyme of the BCAA catabolic pathway (BCKD) changes during muscle cell differentiation.

iii. Determine whether altering the activity of BCKD during muscle cell differentiation affects myotube formation, and which signalling mechanisms are regulating this change?

iv. Evaluate if supplementing BCAA-derived metabolic products, of which BCKD regulates endogenously, can rescue muscle cell differentiation under conditions of BCKD depletion?

Study Hypotheses

i. That the kinase and phosphatase responsible for inhibiting and promoting BCKD activity, respectively, will change during differentiation.

ii. That BCKD activity will increase similarly in parallel with the levels of the catalytic subunit of BCKD during differentiation.

iii. Depleting myoblasts of BDK will positively affect muscle cell differentiation.

iv. Supplementing BCKD metabolic products under conditions of BCKD depletion will rescue differentiation.

Manuscript

Branched-chain keto acid dehydrogenase kinase depletion effects branched-chain alpha-keto acid dehydrogenase activity and muscle cell differentiation

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Abstract

Branched-chain amino acids and their metabolites have been shown to possess anabolic-signalling characteristics in existing skeletal muscle. They have also displayed pro-myogenic characteristics in both *in vitro* and *in vivo*. In our lab we have shown, via siRNA mediated depletion studies, that the two main catabolic enzymes of the BCAA catabolic pathway are essential to successful L6 muscle cell differentiation. In this current study we have modified an assay that measures BCKD (the rate-limiting enzyme in the BCAA catabolic pathway) activity, and for the first time measured it during L6 muscle cell differentiation. It was found that BCKD activity decreases significantly following the onset of differentiation ($p < 0.01$). Additionally, we have shown that increasing the flux through the BCAA catabolic pathway via depletion of the negative regulator of BCKD activity, BDK, augmented differentiation ($p < 0.05$). BDK depletion has also caused a trend for AKT activation to increase at the onset of differentiation when compared to controls. These results suggest that an intact BCAA catabolic pathway is required for muscle cell differentiation, and further research is warranted into accessing the downstream metabolites of the pathway, and how they may regulate skeletal muscle protein and energy metabolism.

Introduction

Preventive medicine and therapy is becoming commonplace when discussing health care. One of the largest preventative and modifiable factors effecting health is dietary nutrition. Westernized countries especially, are experiencing an obesity epidemic leading to an increase in mortality and morbidities, adding to the ever-increasing economic burden

of an aging population (Dietz, 2015; Hruby & Hu, 2015; Trasande & Elbel, 2012). Lifestyle choices centered on physical activity and nutrition can modify the risk of developing chronic, non-communicable diseases such as (i) cardiovascular disease (Konstantinidou et al., 2014) , (ii) T2DM (Ley et al., 2014), (iii) and most cancers (Mayne et al., 2016). Many of the benefits derived from physical activity can be traced back to skeletal muscle health and function. The principal reasons skeletal muscle is so important to metabolic health are its roles in energy and protein metabolism. In fact, skeletal muscle is responsible for the majority (80%) of insulin-stimulate glucose uptake in the body (Olefsky, 1999) and is the body's main reservoir of amino acids (Argilés et al., 2016). Metabolic dysfunction is primarily associated with a reduction in insulin sensitivity and having greater muscle mass is inversely associated with insulin resistance and prediabetes (Srikanthan & Karlamangla, 2011). Therefore, nutritional interventions that aim to increase muscle mass are of great importance to metabolic health and essential to minimizing co-morbidities. Branched-chain amino acids are at the forefront of skeletal muscle metabolism research, because of their role in skeletal muscle anabolism, and because their catabolism is dysregulated in metabolic diseases like T2DM. The particular focus on skeletal muscle metabolism is due to the uniqueness of the BCAA catabolic pathway that is primarily localized to skeletal muscle. The metabolites of BCAA metabolism have also garnered attention due to their anabolic effects in existing skeletal muscle, but their ability to effect muscle regeneration remains much less well understood. Thus, determining the contribution of BCAA catabolism and its metabolites to the enhancement of muscle regeneration, and consequently the maintenance of muscle mass are of paramount importance to the prevention of metabolic diseases.

Materials and Methods

Cell culture

Using either L6 or C2C12 cell lines from *American Type Culture Collection*, cells were cultured in either 10cm, 6-well, or 12-well plates using either α–modified MEM (AMEM) for L6 cells, or Dulbecco's Modified Eagle's Medium (DMEM) for C2C12 cells. Growth medium (GM) was made by supplementing AMEM or DMEM with 10% FBS and 1% antibiotic/antimycotic agents: (FBS - Gibco #10082147: antibiotic/antimycotic - Wisent #450-115-EL). To ensure cell retained the ability to differentiate properly cells were passed every second day upon reaching 70% confluency.

mRNA isolation – *BDK and PP2Cm experiments*

L6 myoblasts were seeded in 10cm plates and differentiated for 5 days. Commencing on day 0 of differentiation RNA was isolated from cells using TRIzol Reagent and PureLink RNA Mini Kit (Life Technologies: #12183018A) following manufacturer's instructions. Following RNA isolation, RNA integrity (A260/280 – 1.7-2.0) and concentration was established using Smart Spec™ Plus (Bio-Rad Laboratories Ltd Life Science Group). RNA samples were then stored at -80°C. cDNA was synthesized using iScript Advanced cDNA Synthesis Kit (Bio-Rad Laboratories #172-5038) following manufacturer's instructions. Real-time (RT) Polymerase Chain Reaction (PCR) was completed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad #1725271) using the Real-Time PCR Detection System (Bio-Rad CFX96™). Data was then analyzed

using Bio-Rad - CFX Manager™ software using the 2-ΔΔCT method. HPRT [Forward: 5'-GCTTTCCTTGGTCAAGCAC-3' - Reverse: 5'-TCCAACAAAGTCTGGCCTGA-3']

Gene knockdown – *BDK and BCKD Knockdown Experiments*

Knockdown experiments utilized siRNA oligonucleotides for target genes, using the reverse transfection method. L6 or C2C12 myoblasts were seeded (2.5 x 10^5 cells/well) into 6-well culture plates. After adding cells to the wells they were incubated in either control transfection medium, or transfection medium containing siRNA targeted towards a specific gene of interest. Control medium contained scramble oligonucleotides (Sigma-Aldrich: #SIC001) (final concentration at 30nM/well), lipofectamine RNAiMAX (Life Technologies #13778150), Opti-MEM (Life Technologies #31985070), and 1ml of antibiotic/antimycotic free medium (10% FBS: AMEM/DMEM), at ratios according to manufacturers instructions. Cells were transfected with the above constituents at the same ratios, replacing scramble oligonucleotides with BDK oligonucleotides (Sigma: NM_019244 - SASIRn01_00080842 - CUAUGCAUGGCUUUGGCUU) (final concentration at 30nM/well) or BCKD oligonucleotides (Sigma: NM_012782 - SASI_Rn01_00064150 - CAGAUCGUGAUCUGUUACU) (final concentration at 30nM/well). Twenty-four hours post-transfection, 1ml of medium (10%FBS, 1% antibiotic/antimycotic: AMEM/DMEM) was added to each well. Following an additional 24 hours period, cells were either shifted into differentiation medium (2% horse serum, 1% antibiotic/antimycotic: AMEM/DMEM), or harvested using a lysis buffer, as previously described.

BCKD Activity Assay

The branched-chain alpha-keto dehydrogenase (BCKD) complex oversees the irreversible oxidative decarboxylation of the BCAA derived keto-acids into their respective Acyl-coA derivatives. Cells were seeded with either 10^5 x 1.5 – 2.5 L6 myoblasts, in 12, or 6-well cell culture plates, respectively. The resultant CO_2 given off via BCKD's decarboxylation activity towards the keto-acid derived from 1-^{14}C labelled valine (American Radiolabeled Chemicals #ARC 0277-50 µCi) is collected on [2M] NaOH soaked filter paper wicks. The now radiolabelled bicarbonate on the filter paper wicks is inserted into 20ml scintillation vials (Perkin-Elmer #6008118) containing 3.5ml of scintillation fluid (Ecolite+ - MP Biomedicals #01882475). The wick containing vials were then subjected to radiation counting (Tri-Carb Liquid Scintillation Counter), equated to protein content (Pierce BCA Protein Assay Kit - Thermo Scientific #23225), and expressed as relative values of counts-per-minute (CPM)/ µg of protein. The assay development went through three generations of testing, with the third being the final version. The second-generation assay was used for the BCKD activity during differentiation experiments **(Fig 2-4)**. The second-generation assay was also used for the first two BDK knockdown experiments measuring BCKD activity **(Fig 2-5a)**, while the third generation was used for the remaining BDK knockdown experiments **(Fig 2-5b)**. Details of the assay development can be found in the results section.

Harvesting Cells

Cells were harvested with 50µl or 100µl of lysis buffer, which was added to each well in experiments using 12 or 6-well plates, respectively. All experiments followed this harvesting method, with the exception of the mRNA experiments. Lysis Buffer formulation

(diluting in ddH2O)(Final Concentrations): 1mM ethylenediaminetetraacetic acid (EDTA) , 2% sodium dodecyl sulfate (SDS), 25 mM Tris-HCl pH 7.5, 10 µl/ml protease inhibitor cocktail (Sigma #P8340),10 µl/ml phosphatase inhibitor cocktail (Sigma #P5726), 1 mM dithiothreitol (DTT). Cells were then collected from the wells using a cell scraper. The lysate was then passed up and down to lyse the cell membranes using a 1-ml syringe fitted with a 26-guage needle. Cell Lysates were immediately put on ice and stored at -20°C until used for analysis.

Western blotting

Following sample collection, protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Scientific #23225). Samples were then mixed with 4x Laemmli loading buffer at a ratio of 3:1 (sample: loading buffer). Equal amounts of protein were boiled for 5 minutes, vortexed, and then loaded into 10% or 15% SDS-page gels, depending upon proteins to be blotted. Following electrophoresis, proteins were transferred to PVDF membranes overnight at 4°C. Following transfer, membranes were blocked in 5% non-fat milk for 1 hour at room temperature, and then rinsed 3x 5-minutes in TBST. Membranes were then incubated in primary antibody (diluted in 2.5%, non-sterile bovine serum albumin) overnight at 4°C. The next day membranes were rinsed 3 x 5-minute in TBST, then incubated with either HRP-conjugated anti-rabbit or mouse secondary antibody diluted in 5% non-fat milk for 3 hours at room temperature. Following an additional 3 x 5-minute washes in TBST, membranes were incubated in HRP chemiluminescent substrate (Millipore #WBKLS0500) for 5 minutes. Luminescence was then obtained using Bio-Rad ChemiDoc MP imager, and signals were analyzed using Bio-Rad: Image Lab 5.2 software.

BCKD knockdown supplemented with BCKD metabolic products

To assess the effects of reinstituting the downstream metabolites of BCKD under BCKD depleted conditions on muscle cell differentiation, L6 muscle cells (2.5×10^5) were seeded in 6-well culture plates the same day as transfection. The transfection medium contained lipofectamine RNAiMAX (Life Technologies 313778150) + Opti-MEM (Life Technologies #31985070) + 1ml /well of antibiotic free GM (AMEM medium + 10% FBS) and either scrambled oligonucleotides (Sigma-Aldrich: #SIC001), or BCKD oligonucleotides (Sigma: NM_012782 - SASI_Rn01_00064150 - CAGAUCGUGAUCUGUUACU). After 24 hours post-seeding an additional 1ml of GM was added to each well (AMEM + 10% FBS + 1% antibiotic/ antimycotic) and cells were allowed to proliferate for an additional 24 hours. Once the cells reached >90% confluency, they were rinsed in warm PBS and shifted into DM (AMEM + 2% horse serum + 1% antibiotic/ antimycotic). A third of the well conditions received both BCKD siRNA transfection medium supplemented with the following at [3μM]: isovaleryl-CoA – (Sigma Aldrich I9381), isobutyryl-CoA - (Sigma-Aldrich I0383), 2-methylbutyryl-L-carnitine - (Sigma Aldrich 50405), β-hydroxyisovaleric acid-d8 - (Toronto Research Chemicals H943607). Supplemented medium was refreshed every day with the other DM until day 5 of differentiation. Samples were harvested using 100μl of lysis buffer and stored at 20°C until used for analysis.

Primary antibodies: anti-mouse - myosin heavy chain-1 (1:500 dilution of a 27μg/ml stock concentration: Developmental Studies Hybridoma Bank #MF20), anti-mouse -tropomyosin (1:500 dilution of a 27μg/ml stock concentration), anti-rabbit - BCKDHA (1:1000 dilution: Abcam – ab90691), anti-rabbit - BCKDK (BDK) (1:2000 dilution: Thermo Fisher – PA5-

31455), anti-rabbit - PARP (1:1000 dilution: Cell Signaling Technologies (CST)#9532), anti-rabbit - caspase-7 (1:1000: CST#12728), anti-rabbit - phospho-Akt (Ser473)(1:1000 dilution – CST #4060), anti-mouse - gamma tubulin (1:10,000 dilution: Sigma #T6557). anti-rabbit - succinate dehydrogenase (SDHA) (1:1000 dilution: CST#11998).

Secondary Antibodies: HRP-conjugated anti-rabbit IgG (1: 10,000 dilution: CST #7074), Anti-mouse IgG-HRP-conjugated (1:10,000 dilution: CST #7076).

Statistics

Two-tailed, unpaired *t*-test was used to assess differences between two groups (denoted using "*" = $p < 0.05$, "**" = $p < 0.01$). Two-way ANOVA was used to assess differences between more than two groups using Tukey's multiple comparisons test (letters denote significant differences). In the evident that a group variance was equal to zero, that group would be removed from two-way ANOVA analysis and would later be subjected to unpaired T-test analysis. Results are expressed as means and SEM. Significance was established when $p < 0.05$. Graphpad Prism 6/7 software was used for data analysis and graph production. Figure illustrations were sourced from Servier Medical Art by Servier Powerpoint image bank (http://www.servier.com/Powerpoint-image-bank, https://creativecommons.org/licenses/by/3.0/).

Results

To understand how BCKD activity may be changing during differentiation its negative regulator, BDK, was measured during a five-day protocol of C2C12 and L6 muscle cell differentiation. In L6 muscle cells BDK mRNA expression was elevated significantly on day 1 of differentiation ($p < 0.05$) when compared to day 2 and 5 of differentiation **(Fig 2-**

1a). However, the same increase was not witnessed when measuring BDK protein expression **(Fig 2-1b)**. To test whether this was a cell specific response or antibody artifact, BDK protein was additionally measured in C2C12 muscle cells, which yielded the same pattern of expression **(Fig 2-1d)**. The positive regulator of BCKD activity, PP2Cm, had a trend for its mRNA to increase in a stepwise manner, reaching statistical significance by day 3 of differentiation **(Fig 2-1c)**. However, the high variability between experimental replicates is the likely cause of day 4 and 5 of differentiation not reaching a statistical significance increase. Taken together, BDK protein expression remaining unchanged during differentiation, PP2Cm mRNA expression increasing, and previous data from our lab showing BCKD-E1α protein expression rises during differentiation, it is likely BCKD activity will increase comparatively.

The BCAA catabolic enzymes have been shown to be required for muscle cell differentiation (Zameer N . Dhanani and Olasunkanmi J. Adegoke, 2016), therefore it is necessary to be able to measure the activity of the rate-limiting enzyme in the pathway, and see if it changes throughout the course of differentiation. A BCKD activity assay had been developed by Chuang et al., (2000) to be used with lymphocytes. However, there was no existing assay protocol available to measure BCKD activity in adherent muscle cells. The BCKD complex oversees the irreversible oxidative decarboxylation of the BCAA derived keto acids into their respective acyl-CoA derivatives. The resultant CO_2 given off (when radiolabelled) can be collected and measured, as an indicator of BCKD activity.

In the first test I used 12-well plates with homemade cell insert cups (modified microcentrifuge tubes). The cups were intended to suspend the NaOH soaked filter paper wicks above the cells and reaction mixture for the purpose of collecting radiolabelled CO_2.

Due to the complexity and work intensiveness of this process it was quickly abandoned. The main challenge of the first round of testing and its eventual failure was the inability to create the integral airtight seal, which was required to differentiate between control (GM + Buffer + Cells without 1-^{14}C labelled valine) and experimental (GM + Buffer + Cells + Reaction mix containing 1-^{14}C labelled valine) wells. The first successful assay generation alternatively used filter paper wicks suspended from the tape-covered ceiling. Radiation counts from the first generation assay depicted in **Fig 2-2a.** displays encouraging results. However the "Live" (GM + Buffer + Cells + Reaction mix containing 1-^{14}C labelled valine) conditions had too a large range and were too similar to control conditions. Because of this large range the protocol required further optimization. It should be noted that any CPM values that appear under control well conditions, where no radiation should have been given off, is due to air-leakage from adjacent "Live" wells. These air-leakages are due to the quality of adhesive on the clear tape used to form the well ceiling and human error.

The second generation of the modified assay involved suspending filter paper wicks imbedded into the clear tape used to seal the wells. The initial problem with this model was that the filter papers were soaked in a NaOH solution, rendering the adhesive on the tape useless. I created a system where I would add an extra 2-4mm of width to the filter paper wicks, which I would then bend at a ninety-degree angle, and attach to the clear tape **(Supplementary Fig 2)**. I then used a small piece of clear tape (just enough to cover the extension of the wick) to anchor the small tab in place. Using this method the bulk of the filter paper wick was exposed and projected perpendicularly from the clear tape roof of the well (towards the bottom of the well) and was free to get coated in the [2M] NaOH solution. The resultant changes in the second generation culminated in much smaller radiation

count ranges and larger differences between the control (GM + Buffer + Cells without 1-^{14}C labelled valine) and experimental (GM + Buffer + Cells + Reaction mix containing 1-^{14}C labelled valine) conditions **(Fig 2-2b)**.

Using the second generation of the assay, BCKD activity was measured in L6 muscle cells over the course of 5 days. The first round of testing produced reliable reads, with minimal intra-condition variability **(Fig 2-3)**. BCKD activity was then successfully measured over five experimental replicates. BCKD activity was significantly lowered by 36% from day 0 to day 1 ($p < 0.05$) **(Fig 2-4)**, and further significant reductions of~32% were witnessed on day 2 and remained lowered until day 5 ($p < 0.05$)**(Fig 2-4)**.

Since we have previously shown that BCKD knockdown impairs differentiation. Conversely, how would differentiation be affected if the activity of that same enzyme were to be increased? Increasing BCKD activity is achievable via knockdown of its negative regulator BDK. Under conditions of BDK depletion, using the second generation assay, BCKD activity levels doubled on day 0 of differentiation ($p < 0.05$) (**Fig 2-5a)**. Because research has shown that BDK depletion can drastically upregulate BCKD activity in muscle (~15-20-fold increase when BDK was knocked out) I hypothesized that the assay may be reaching a limit in its ability to detect further changes in BCKD activity (Ishikawa, 2017). This led to further optimization of the assay.

The third generation of the modified assay kept many aspects of the second generation, with the addition of proportional increases in: reaction mix, cell medium, and buffer volumes. Theses proportional changes refer to the doubling of cells and well-space when moving from the 12-well culture plate to the 6-well plate. These changes were accompanied by even greater increases in the filter paper wicks size (5.3-fold increase in

area) and NaOH volume (3-fold increase), with the function of raising the limit of detection, or saturation point, of the assay. Taken together the 6-well plate enabled us to work with higher radioactive counts, which yielded smaller percentage error, better myotube formation, more sample lysate for analysis, and most importantly, raising the maximum capacity of the assay to detect radiolabelled CO_2. BCKD activity was found to increase ~5-fold on day 0 of cells depleted of BDK using the third generation assay **(Fig 2-5b)**. However, western blot analysis showed BDK knockdown efficiency was reduced following the last two days of differentiation **(Fig 2-5c)**. The reduced BDK knockdown efficiency on day 3 and 4 of differentiation may be abolishing additional effects on the later stages of differentiation.

Now that we are aware that BCKD activity decreases during the normal course of differentiation, despite the concomitant rise in BCKD-E1α. We hypothesized whether increasing its activity would positively affect differentiation, working under the assumption that the BCKD metabolic products would have positive effects on differentiation. BDK depleted C2C12 myoblasts displayed significant increases in MHC-1 expression on day 3 and 4 of differentiation ($p < 0.05$**(Fig 2-6b)**. Additionally, tropomyosin displayed significant increases under BDK depleted conditions on day 1, 2, and 4 of differentiation ($p < 0.05$), yet day 3 variability was too high to reach significance **(Fig 2-6c)**. Clearly, the increased expression of these two myofibrillar proteins indicates differentiation is occurring earlier and to a greater extent in C2C12 cells depleted of BDK.

We next sought to determine whether increasing the flux through the BCAA catabolic pathway could be causing differentiation to occur at a faster rate. This was believed because BCAAs final metabolism feeds into the citric acid cycle, providing carbon

skeletons for anaplerosis, thereby affecting cell energy metabolism. BCAAs are known to be oxidized at a higher rate during instances of greater energy demand, such as exercise (Yoshiharu Shimomura, 2006). Therefore, by speeding up the BCAA catabolic pathway via BDK depletion, we hypothesized that this may be upregulating signalling mechanisms promoting greater oxidative capacity within the cell. Mitochondrial content increases under normal conditions of muscle cell differentiation, causing glycolytic myoblasts to shift towards a more oxidative metabolism in myotubes, thus we measured a marker of mitochondrial content and oxidative phosphorylation (OXPHOS) capacity, succinate dehydrogenase (SDHA) (Sin et al., 2016). SDHA levels remained stable during differentiation, no significant differences were found between the control and intervention groups **(Fig 2-6d)**. Up to this point, no proxy of protein synthesis had been measured, which could aide in explaining the increased accretion of myofibrillar proteins under BDK depleted conditions, so ph-(S473) AKT was measured. AKT phosphorylation had a trend to be greater on day 0, under BDK knockdown conditions ($p < 0.0948$) **(Fig 2-6e)**. This finding is in agreement with AKT's role in early differentiation, in addition to the likelihood of mTORC1 being activated, which is necessary for differentiation (Gardner et al., 2012; Heron-Milhavet, 2006; Héron-Milhavet, 2008). Thereby, BDK depletion may be facilitating an accelerated rate of differentiation through this pathway.

At the onset of differentiation, particularly on day 1, many cells unadhere from cell culture plates during the normal course of differentiation. However, the number of unadherent cells present in the BDK knockdown wells appeared to be greater. To test whether BDK depletion was affecting cell death, markers of apoptosis were measured. I found no significant treatment effects on the expression of unprocessed caspase-7 or poly

(ADP-ribose) polymerase (PARP) **(Fig 2-7a, b)**. Apoptosis has been reported to be necessary to support successful differentiation (Fernando et al., 2002; Nakanishi et al., 2005; Sandri & Carraro, 1999; Schöneich et al., 2014). Alternatively, measuring markers of another catalytic process, like autophagy, could yield additional understanding of the effect BDK depletion may be having on cell viability during the early phase of differentiation.

As previously stated knockdown of BCKD- E1α impaired differentiation of L6 muscle cells (Zameer N . Dhanani and Olasunkanmi J. Adegoke, 2016). Provision of the substrate products immediately downstream of BCKD may rescue differentiation. L6 cells were transfected with siRNA targeted against BCKD- E1α and were supplemented with three of the BCAA derived metabolites immediately downstream of BCKD, isovaleryl-CoA, isobutyryl-CoA, 2-methylbutyryl-CoA, and β-hydroxy β-methylbutyrate(an anti-proteolytic leucine metabolite). The BCKD metabolic products were unable to rescue differentiation, MHC-1 expression in the control group was significantly higher than both intervention groups, which both remained significantly lowered compared to the control ($p < 0.01$) **(Fig 2-8a)**. Additionally, to assess whether rates of protein synthesis were changing under BCKD knockdown conditions, ph(S473)AKT was measured. There were no differences between any of the groups for ph(S473)AKT **(Fig 2-8b)**.

Fig 2-1

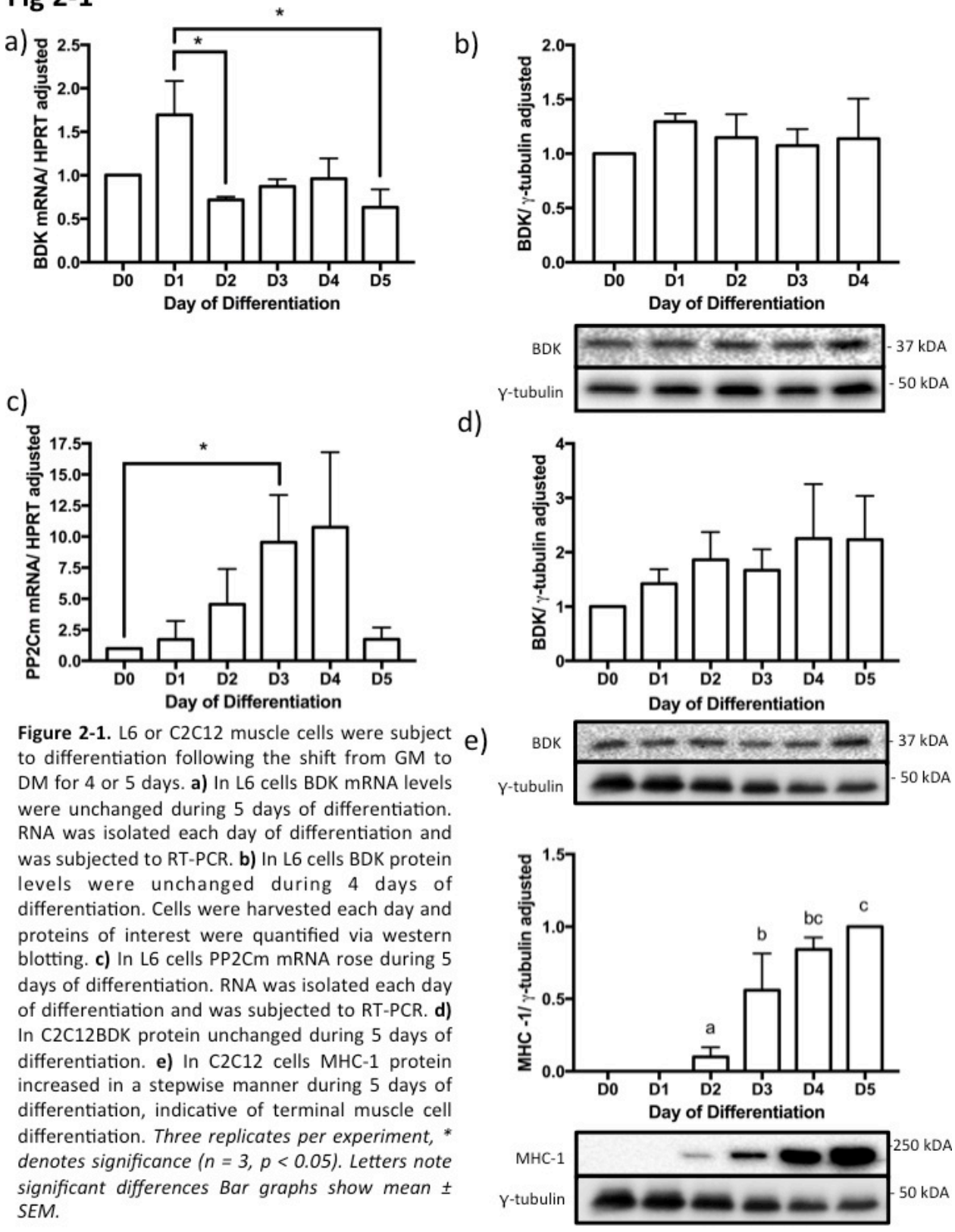

Figure 2-1. L6 or C2C12 muscle cells were subject to differentiation following the shift from GM to DM for 4 or 5 days. **a)** In L6 cells BDK mRNA levels were unchanged during 5 days of differentiation. RNA was isolated each day of differentiation and was subjected to RT-PCR. **b)** In L6 cells BDK protein levels were unchanged during 4 days of differentiation. Cells were harvested each day and proteins of interest were quantified via western blotting. **c)** In L6 cells PP2Cm mRNA rose during 5 days of differentiation. RNA was isolated each day of differentiation and was subjected to RT-PCR. **d)** In C2C12BDK protein unchanged during 5 days of differentiation. **e)** In C2C12 cells MHC-1 protein increased in a stepwise manner during 5 days of differentiation, indicative of terminal muscle cell differentiation. *Three replicates per experiment, * denotes significance (n = 3, p < 0.05). Letters note significant differences Bar graphs show mean ± SEM.*

Fig 2-2

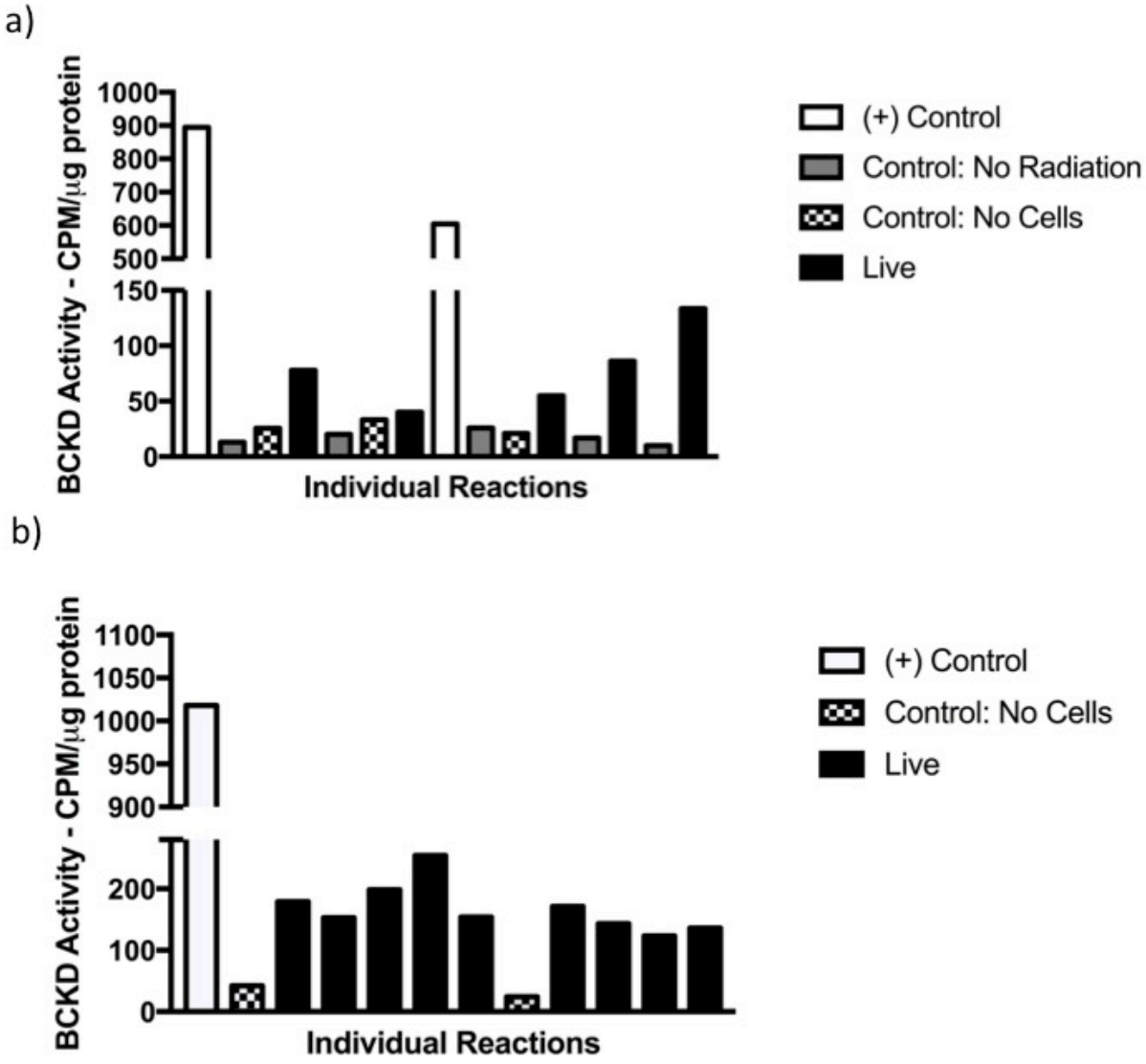

Figure 2-2. BCKD activity assay development of the first and second assay generations. **a)** L6 muscle cells were measured for BCKD activity on day 0 of differentiation using the 1st assay generation. Open-bars represent positive controls, solid-grey bars had L6 cells with no 1-^{14}C-valine, checkered bars had no L6 cells with 1-^{14}C-valine **b)** L6 muscle cells were measured for BCKD activity on day 0 of differentiation using the 2nd assay generation. *Both figures data were the result of single experiment tests to ensure assay efficacy.*

Fig 2-3

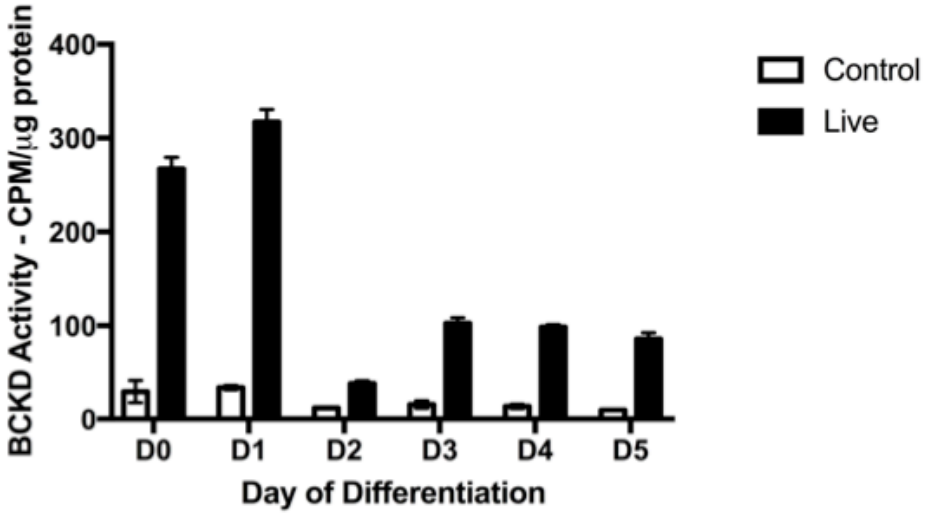

Figure 2-3. Single experiment of the second generation of BCKD activity test, during differentiation. Relative values of BCKD activity in counts-per-minute (CPM), expressed per µg of protein. Control = cells incubated in growth medium without 1-^{14}C-valine. Live = cells incubated with growth medium and 1-^{14}C-valine. Bar graph show means of technical replicates of a single experiment ± STD (n=1).

Fig 2-4

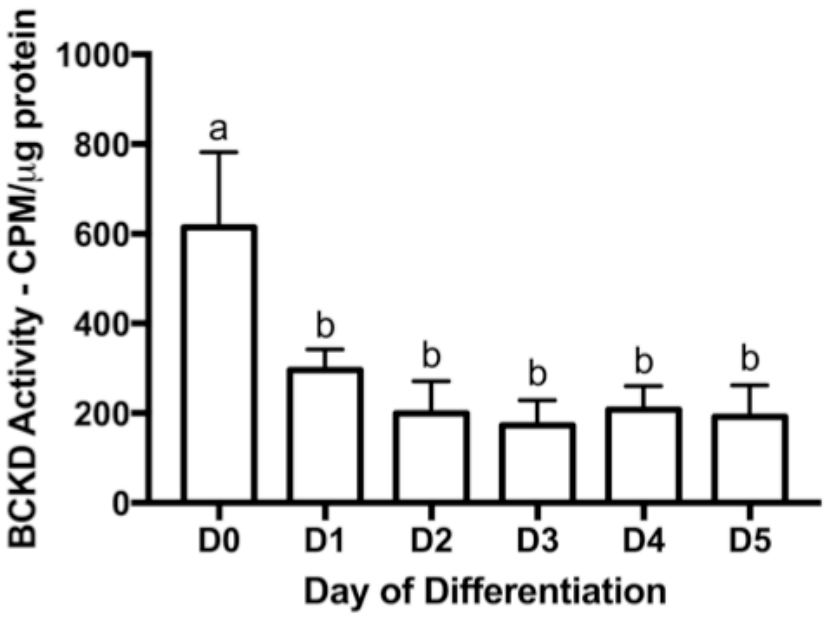

Figure 2-4. BCKD activity is reduced following the first 48 hours of differentiation and plateaus until the end of the experiment on day 5 of differentiation. BCKD activity assay (2nd generation) values adjusted per µg of protein during differentiation in L6 muscle cells. *Three replicates per experiment, letters denote significant difference (n = 5, p < 0.05). Bar graphs show mean ± SEM*

Fig 2-5

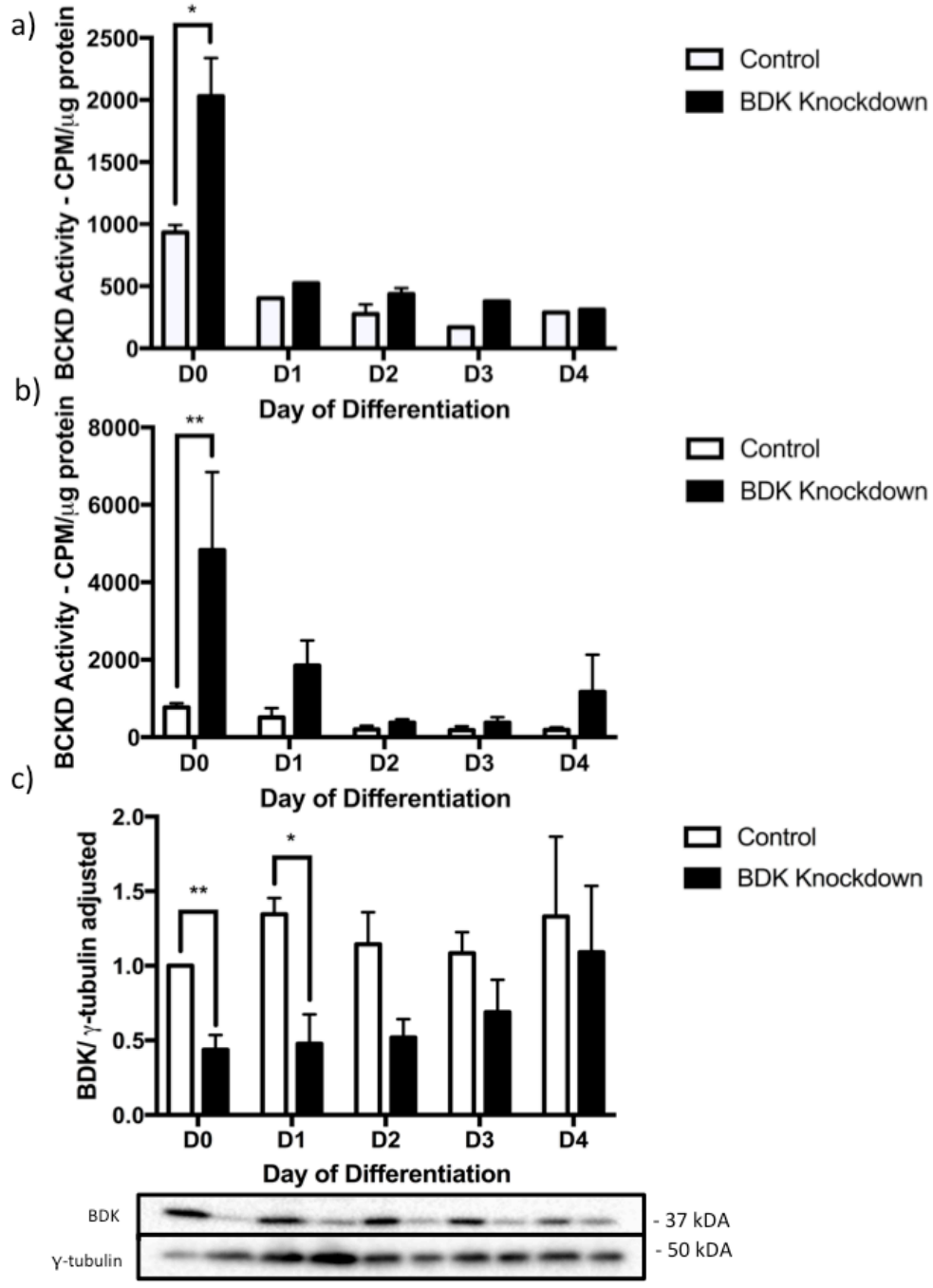

Figure 2-5. siRNA mediated BDK knockdown in L6 muscle cells significantly increases BCKD activity in L6 muscle cells. **a)** BCKD activity was measured using the second generation BCKD activity assay generation up to day 4 of differentiation (n = 2) **b)** BCKD activity was measured using the third generation BCKD activity assay generation up to day 4 of differentiation (n=3). **c)** Western blotting analysis of BDK protein from third generation BCKD activity assays (n=3). *Three replicates per experiment, (*= p < 0.05 - ** = p <0.01)). Bar graphs show mean ± SEM*.

Fig 2-6

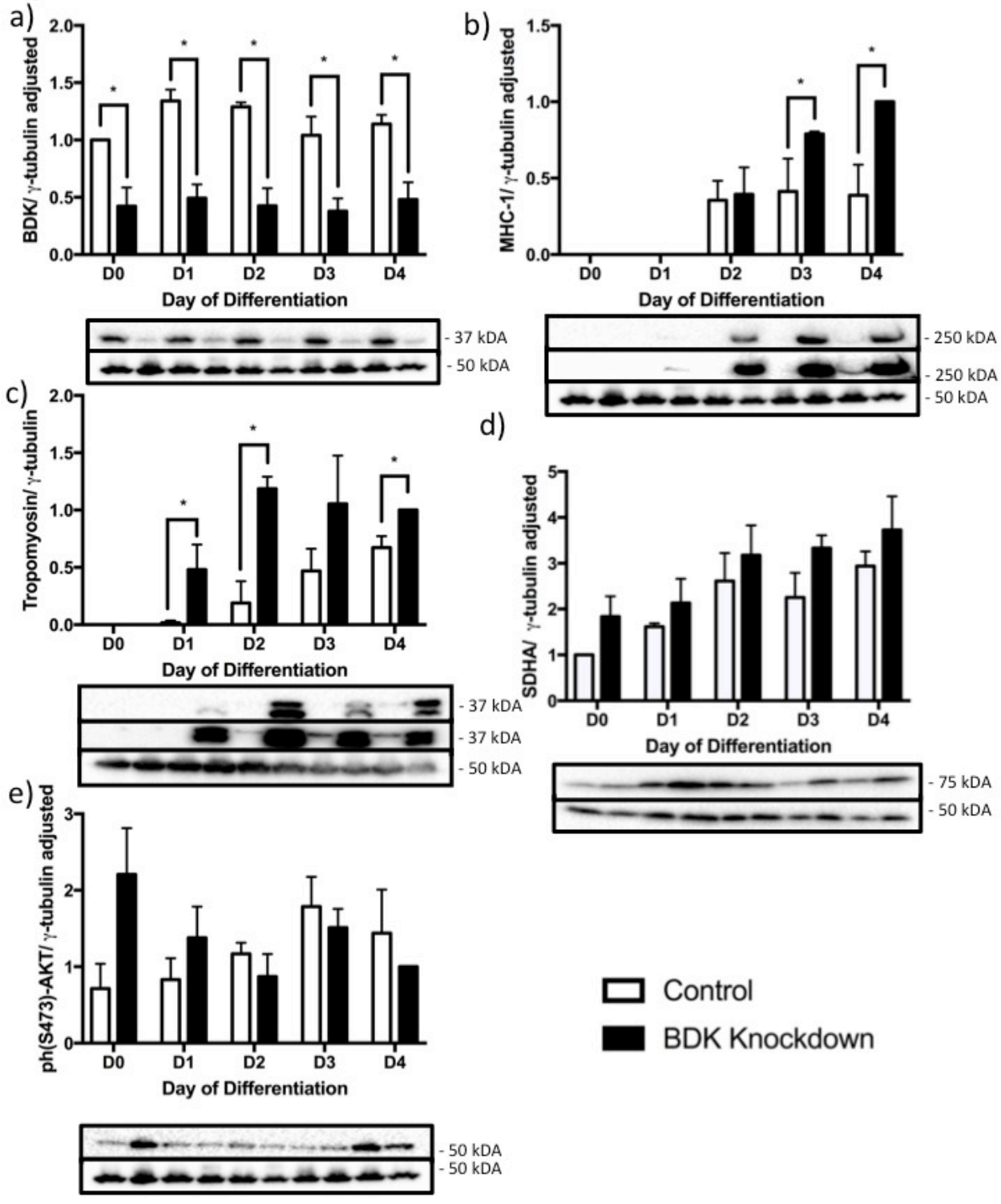
a)
BDK/ γ-tubulin adjusted
2.0
1.5
1.0
0.5
0.0
D0
D1
D2
D3
D4
Day of Differentiation
- 37 kDA
- 50 kDA
b)
MHC-1/ γ-tubulin adjusted
1.5
1.0
0.5
0.0
D0
D1
D2
D3
D4
Day of Differentiation
- 250 kDA
- 250 kDA
- 50 kDA
c)
Tropomyosin/ γ-tubulin
2.0
1.5
1.0
0.5
0.0
D0
D1
D2
D3
D4
Day of Differentiation
- 37 kDA
- 37 kDA
- 50 kDA
d)
SDHA/ γ-tubulin adjusted
5
4
3
2
1
0
D0
D1
D2
D3
D4
Day of Differentiation
- 75 kDA
- 50 kDA
e)
ph(S473)-AKT/ γ-tubulin adjusted
3
2
1
0
D0
D1
D2
D3
D4
Day of Differentiation
- 50 kDA
- 50 kDA
Control
BDK Knockdown

Figure 2-6. siRNA mediated BDK depletion in C2C12 muscle cells during 4 days of differentiation. **a)** Western blot analysis of BDK. **b)** Western blot analysis of MHC-1. Second MHC-1 panel is of a longer exposure time **c)** Western blot analysis of tropomyosin. Second tropomyosin panel is of a longer exposure time **d)** Western blot analysis of SDHA. **e)** Western blot analysis of Ph-(S473)-AKT. *Three replicates per experiment, * denotes significance (n = 3, p < 0.05). Bar graphs show mean ± SEM*. The top panels in each figure display the protein of interest. Lowest panel of blots in each figure is γ-tubulin.

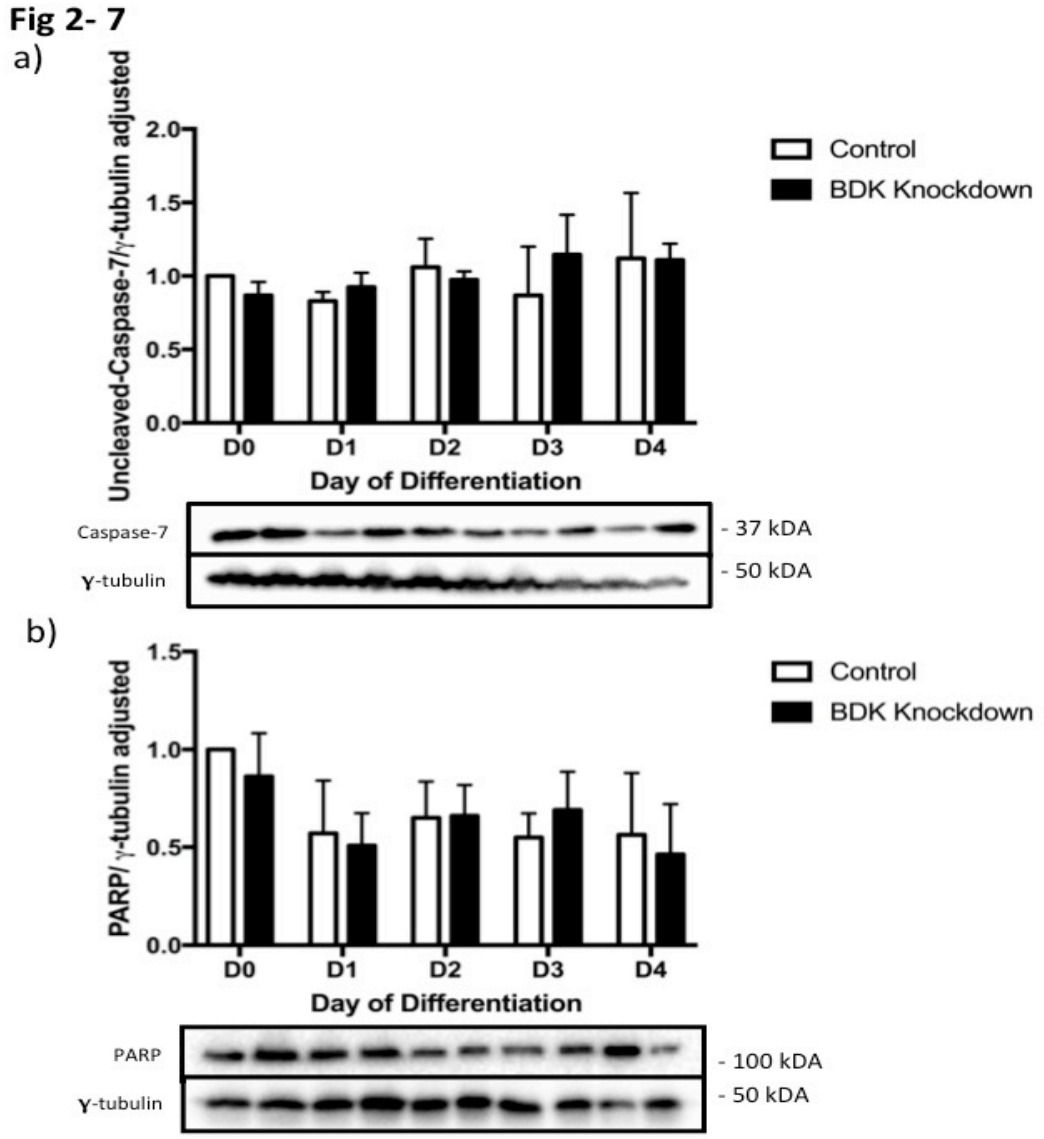

Fig 2-7. Markers of apoptosis do not change during 4 days of differentiation in L6 muscle cells under conditions of BDK depletion. **a)** Western blot analysis of uncleaved/unprocessed caspase-7. **b)** Western blot analysis of uncleaved/ unprocessed PARP. Two replicates per experiment. (n=2) Bar graphs show mean ± STD

Fig 2-8

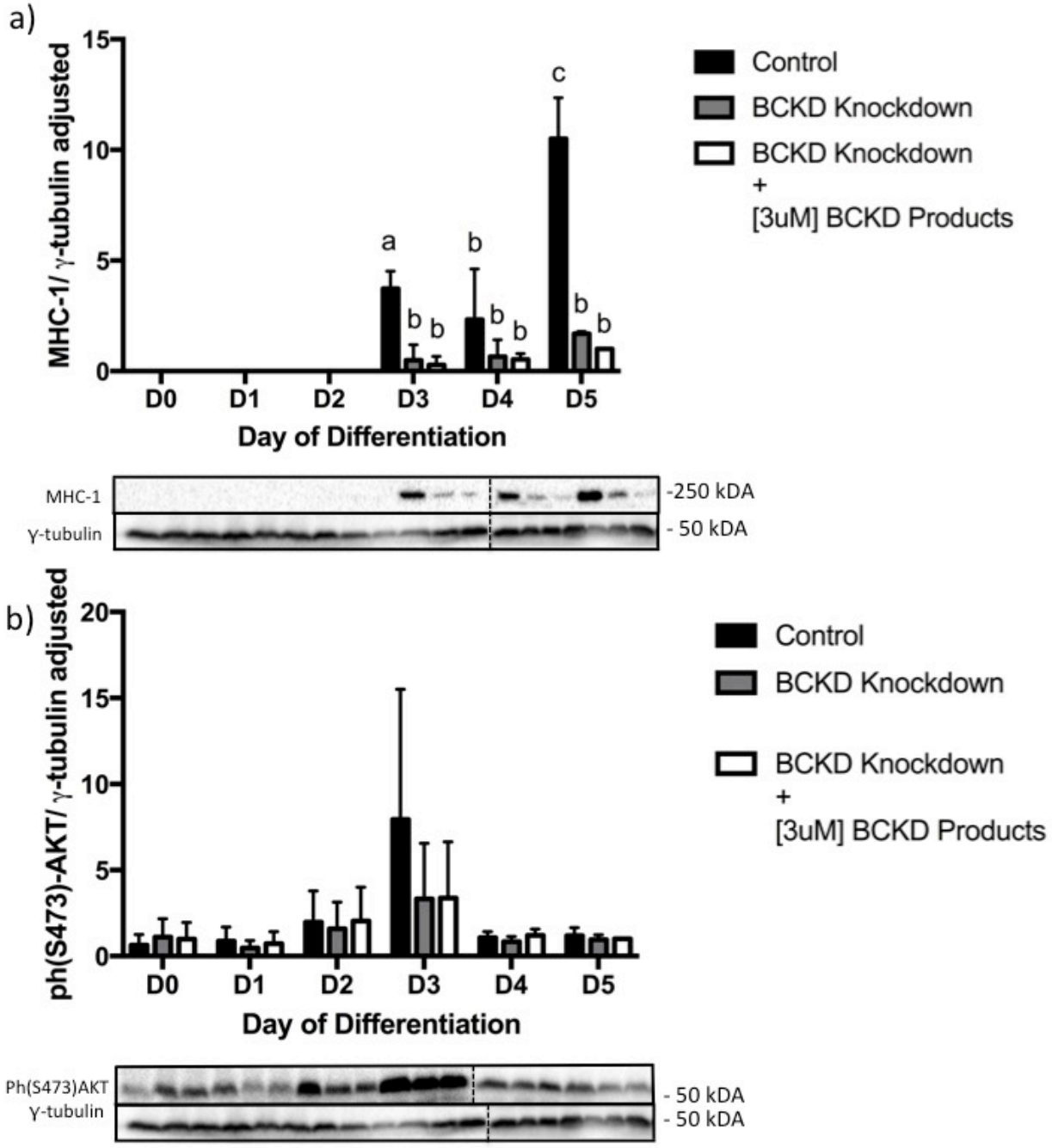

Fig 2-8. BCKD depletion impairs myotube formation and the accretion of the myofibrillar protein MHC-1. L6 muscle cells were subject to three conditions: 1) Control, 2) siRNA mediated BCKD knockdown, 3) siRNA mediated BCKD knockdown supplemented with the downstream metabolites of BCKD. The blots shown amalgamate two membrane images together. **a)** Western blot analysis of MHC-1 during differentiation **b)** Western blot analysis of ph-(S473)AKT during differentiation. *Three replicates per experiment, letters denote significance (n = 2, p < 0.05). Bar graphs show mean ± STD. Dotted line indicates merged blots.*

Fig 2-9

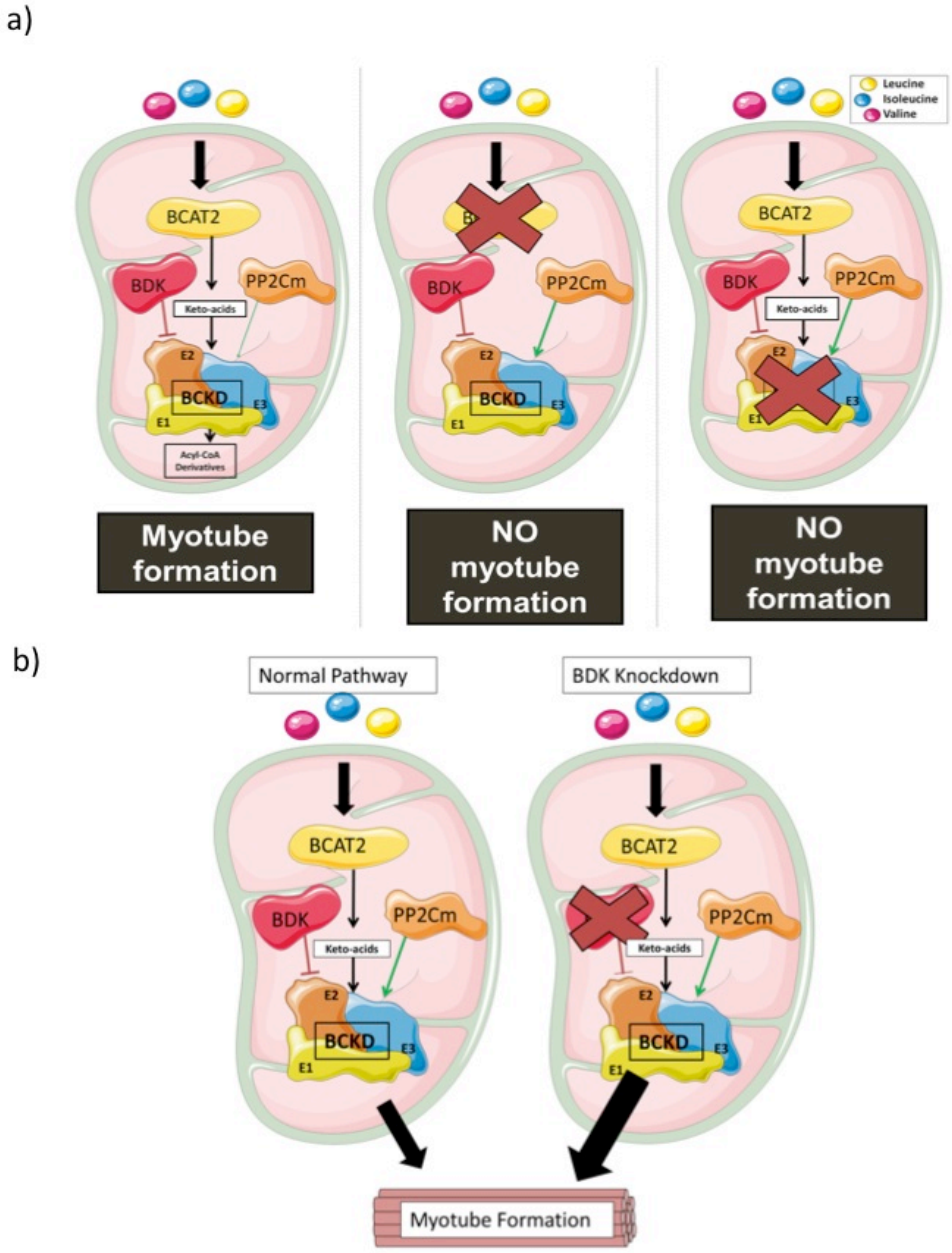

Figure 2-9. Overall schematic of BCAA catabolic pathway regulation and muscle cell differentiation**. a)** In vitro siRNA mediated gene knockdowns of the BCAA catabolic pathway inhibit muscle cell differentiation. Inhibiting either one of its two main catabolic enzymes resulted in the impairment of muscle cell differentiation. **b)** Schematic model of BCAA catabolic pathway with BDK depletion augments myofibrillar protein accretion indicative of myotube formation.

Discussion

For the first time in skeletal muscle cells we have shown that BCKD activity decreases throughout the process of differentiation. This finding was contrary to what I had hypothesized. Previous work from our laboratory had shown that the catalytic subunit of BCKD, E1α, increased throughout muscle cell differentiation. The increase in E1α protein expression led us to believe that the activity of the complex would increase concomitantly with differentiation.

I began by assessing the regulatory mechanisms of BCKD via measuring its two-antagonistic regulatory proteins. The expression of BDK protein, the negative regulator of BCKD, remained unchanged throughout a 5-day differentiation protocol. Previous reports have shown that BDK is regulated at the level of translation, through an element contained within BDK mRNA's 5' untranslated region (Muller, 2004). Yet BDK mRNA was assessed and its expression was elevated on day 1 of differentiation, significantly above that of day 2 and 5 ($p < 0.05$). BDK mRNA for day 0, 3 and 4 did not reach a significant difference from day 1 levels. BDK protein expression has been shown to be regulated by nutrient availability, the greater the concentration of BCKAs in the cell the lower BDK expression, and vice versa (Doering, 2000; Lynch & Adams, 2014b; Popov, 1995). My in vitro protocols call for medium replacement every 24 hours, meaning that the nutrient conditions should remain rather stable, thus BDK expression may as well **(Fig 2-1b, d)**. However, because BDK had never been studied in a cell model of differentiation, and the enzyme it regulates increases during differentiation, it was within reason to assume BDK may be subject to additional regulatory mechanisms.

One point that needs to be addressed in regard to the usage of both L6 and C2C12 muscle cells is due to antibody specificity. Commercially available antibodies against BDK had exclusive species cross-reactivity with mice (C2C12), instead of rats (L6). Side-by-side western blots were later run to access the antibodies validity in BDK detection in both the C2C12 and L6 cell lines. These side-by-side blots displayed similar results between C2C12 and L6 BDK expression, blot location, and was further emphasized in siRNA knockdown studies using L6 cells.

My next experiment found that the positive regulator of BCKD activity, PP2Cm, had a trend for its mRNA expression to increase during differentiation. PP2Cm mRNA levels on day 3 were significantly different than day 0 ($p < 0.05$)**(Fig 2-1c)**, although generation of protein data was not possible due to antibody unavailability. However, the physiological relevance of this rise in PP2Cm mRNA expression may be limited because its expression is dwarfed by that of BDK in skeletal muscle. This high expression of BDK in skeletal muscle explains the tissues relatively low basal BCKD activity, whereas PP2Cm is lowly expressed (M. Zhou, 2012). Further evidence supporting BDK's essential role in BCKD regulation in skeletal muscle has become evident since the generation of the muscle-specific BDK knockout mouse (Ishikawa, 2017). These mice display very similar BCAA plasma profiles (~40% reductions) to whole-body BDK knockout mice (~50% reductions), emphasizing the physiological relevance of skeletal muscle BDK.

Once we established how BCKD's regulatory factors were expressed during differentiation, it was time to investigate BCKD activity directly. Following the development of a modified BCKD activity assay, we were able to directly measure enzymatic activity of BCKD. This is achieved using 1-^{14}C labelled valine. Valine was the molecule of choice for

measuring BCKD activity because it, of all three BCAAs, is catabolized at the highest rate by BCKD (Sokatch et al., 1981; Yoshida et al., 1986). The radiolabeled valine molecule is not uniformly labeled, we purposefully chose this isotope because only carbon-1 is radioactive and it is this carbon that is removed via BCKD's decarboxylation action**.** The modifications of this assay were rather extensive and many rounds of testing were conducted to ensure efficacy. I was faced with two main challenges during the first few rounds of testing. The first challenge was ensuring that each experimental well was airtight, because the radiolabelled carbon from the 1-^{14}C-valine would be transferred to CO_2, following its decarboxylation by BCKD. On the first generation of the assay I tried out a variety of taping methods to address the prevention of air leakages, because the efficacy of the assay was dependent upon the collection of the aforementioned radiolabelled CO_2.

The second challenge involved the actual collection of the radiolabelled CO_2 itself. The purpose of the NaOH soaked filter paper wicks was to collect the radiolabelled CO_2 via the chemical reaction that occurs between CO_2 and NaOH, which would react forming bicarbonate, leaving the ^{14}C on the wicks. The wicks were then subjected to radiation counting. The original assay developed by Chuang et al., (2000) called for the production of specialized cell inserts to hold these wicks, but the inserts were custom made and I could not find any substitute that would serve my purposes. I had improvised a method using microcentrifuge tubes that were transversally cut through the midsection, inverted with the lid closed, and the cap was wrapped with Parafilm® to ensure it was water-tight. Although this method could have worked, I could not devise a way to suspend them above my monolayer of cells, Chuang et al., (2000) had designed the assay with the intention of using suspended cells. This meant I had to come up with an alternative means of keeping

the wicks contained within the closed environment, without making direct contact with the 1-^{14}C-valine containing medium. I finally solved the issue by suspending the filter paper wicks from the ceiling of each well, which happened to work with the taping method I had devised to overcome the first challenge. Using small pieces of tape, I anchored the wicks to the tape that would form the ceiling of each well. Finally, I was able to begin testing whether or not I would be able to produce reliable, reproducible results.

I began my testing protocols using four different conditions. All four conditions were in the presence of growth medium, the conditions were as shown in **(Fig 2-2a).** The condition termed, "Live" had all the final components: i) cells, ii) growth medium, iii) medium buffer, iv) 1-^{14}C-valine containing reaction mix, and iv) NaOH soaked filter paper wicks. The positive control was comprised of 20µl of the 1-^{14}C-valine containing growth medium, which was added to the scintillation fluid and was then subjected to radiation counting. The remaining two conditions were controls, missing either cells, or 1-^{14}C-valine. Both controls contained NaOH wicks, and at the end of each protocol, samples from these wells too were counted. The first generation assay in **Fig 2-2a** displays high variability among the counts-per-minute (CPM) of the "Live" (GM + Buffer + Cells + Reaction mix containing 1-^{14}C labelled valine) conditions. Additionally, the CPM reads of the "Live" conditions were too similar to that of the control conditions. The CPM generated from the control conditions is often the residual, or basal registers of the scintillation counter, or the result of an air leak from a "Live" condition. The two problems with the first generation assay were improved upon the second generation development. The variability between "Live" groups was lowered and their CPM reads were much greater than the control conditions **(Fig 2-2b)**. This was accomplished by improvement of the taping protocol.

Once optimized, I was able to measure BCKD activity during differentiation as shown in the first test round **(Fig 2-3)**.

BCKD activity declined following the first 24 hours of my differentiation protocol and remained lowered until the experiments cessation on day 4 **(Fig 2-4).** BCKD activity fell by ~36% by day 1 ($p < 0.05$) and remained significantly lowered by ~68% from day 2 to day 5 ($p < 0.01$), despite the concomitant rise in the abundance of BCKD's catalytic subunit, E1α**,** which had been shown in prior studies in our lab. It is likely that the relatively high levels of BCKD activity on day 0 and day 1 are a result of growth factors from the growth medium. Not only was there a ~36% drop in BCKD activity, which was consistent across all experiments from day 0 to day 1, but during testing rounds when myotubes were exposed to growth medium (as opposed to DM), BCKD activity rose to near day 0 levels (**Supplementary Fig 1)**. Nevertheless, the fall in BCKD activity during differentiation suggests a greater association between BDK and BCKD, which is indicative of a reduction in intracellular BCAAs, and thus BCKAs (She, 2013). However, past work in our lab has shown that BCAA levels remain constant in differentiating L6 cells, meaning the association between these two proteins should not change. Because BCKD activity has never been studied during muscle cell differentiation it is possible that it may be subject to regulation via an alternative mechanism that remains to be defined.

During the development of a new assay, it is essential that a few detection parameters be defined. The modified BCKD activity assay has, at this stage, proven able to detect a minimum signal enabling the discernment between BCKD decarboxylation and negative control conditions. However, there is a finite capacity of the assay to detect the main output product, the radiolabelled CO_2, because the available NaOH may become

saturated rendering it unable to bind additional CO_2. In this case the ceiling of detection, or the "saturation point" of the assay is controlled by four factors: i) filter paper size, ii) amount/concentration of NaOH added to the wicks, and iii) amount/concentration of 1-^{14}C labelled valine in the reaction wells, and most importantly the number of cells per reaction (Cell number is controlled via protein concentration).

The siRNA-mediated knockdown of BDK was capable of raising BCKD activity as hypothesized **(Fig 2-5a)**. However, as previously mentioned, muscle-specific BDK knockout mice displayed BCKD activity levels between 15-20-fold of controls, thus I believed the doubling effect witnessed in **Fig 2-5a** was not truly reflective of BCKD's activity. Using the third-generation assay for the remaining BDK depletion experiments it became evident that a saturation point had been reached using the second-generation assay **(Fig 2-5b)**. And thus, the highest BCKD activity witnessed on day 0 using the second generation assay did not reflect the true increase (2-fold increase from day 0 control to knockdown in BCKD activity). The third-generation assay clearly depicts a much larger increase in BCKD activity on day 0, under knockdown conditions (~5-fold increase from day 0 control to knockdown in BCKD activity)**(Fig 2-5b)**. Increasing the ceiling of detection, or saturation point, for the third generation was achieved by increasing the size and volume of the filter paper wick and NaOH, respectively, when moving from a 12-well plate to a 6-well plate format. Note that the increase in filter paper wick size (5.3-fold increase in area) and NaOH volume (3-fold increase) exceeded the doubling-increase of the reaction mix, cell medium, cell number, and buffer volumes.

The third generation BCKD activity assays with BDK depletion used another siRNA oligo against BDK, which depleted BDK expression to similar levels as the first oligo used in

the 2nd generation assay experiments. However, upon using the second oligo, the specific cell line, the oligo, or the combination of the two, resulted in the reduction of the transfected cells ability to proliferate. This led to a sub-optimal confluency (<90%) even after an additional 24-hour period to allow the cells to proliferate. Therefore, to bring the cells back up to the appropriate confluency required for successful differentiation I needed a method to increase the number of cells per well, while retaining efficient BDK knockdown. A previously used technique in our lab involved transfecting three times the number of cells (using 3x the number of wells) typically used per experimental conditions and condensing them into a single well (Ratio of 3:1). For example, all experiments conducted had three technical replicates per condition, and this experiment is repeated for five days. Thus, a single experimental replicate requires 15-wells per condition (Control and BDK knockdown). On the day of transfection, I transfected 15-wells worth of cells (2.5 x 10^5 /well) under the control condition and 45-wells worth for the BDK knockdown condition. I allowed all these cells to proliferate over the next 24-hours, allowing for sufficient time to pass for the transfection to have an effect. At the 24-hour mark I trypsinized all the control wells, centrifuged them, re-suspended them in 15 milliliters of medium, and re-seeded them into 15-wells (1ml of suspension/well). I completed the same procedure for the BDK knockdown wells, however when I re-suspended the cells in medium, instead of adding 45 milliliters I added only 15 milliliters of medium. I then re-seeded those cells into 15-wells, thereby, condensing three times the number of cells into a single well. This procedure was able to rescue cell confluency 24-hours later, and subsequently differentiation.

I next sought out how siRNA-mediated BDK depletion would affect muscle cell differentiation. Because the removal of the main BCAA catabolic enzymes impaired differentiation, perhaps increasing flux through the pathway would positively effect differentiation. These experiments were conducted in C2C12 muscle cells because the knockdown efficiency in these cells was greater and lasted throughout our entire protocol, compared to L6 muscle cells **(Fig 2-6a)**. BDK depleted cells displayed a premature increase in the accretion of two myofibrillar proteins, MHC and tropomyosin, when compared to control cells **(Fig 2-6b, c)**. There was also a trend for AKT(S473) phosphorylation ($p < 0.0948$) to be greater under BDK knockdown conditions on day 0 compared to the control **(Fig 2-6e)**.

Cells with high concentrations of BCAAs elicit robust mTORC1 activation, which has been shown to feedback onto the PI3K-AKT signalling axis, uncoupling the axis from the insulin receptor, via a phosphorylation event on insulin receptor substrate-1 (Carlson et al., 2004; Hançer et al., 2014; Smadja-Lamère et al., 2013). The increase in AKT(S473) phosphorylation on day 0 may be the result of greater signalling coupling between the insulin receptor and the PI3K signalling pathway **(Fig 2-6e)**. The greater coupling may be caused by the decrease in intracellular BCAA concentrations, typically observed under BDK depleted conditions (Ishikawa, 2017; Joshi, 2006). The increased activation of AKT could be one of the factors accelerating differentiation under BDK depleted conditions, based upon AKT's role in early differentiation (Heron-Milhavet, 2006; Héron-Milhavet, 2008; Sun, 2004; Wilson, 2007).

Another mediator involved in the early stages of differentiation is the mitochondrion. Mitochondrial biogenesis has been shown to occur during both muscle cell

differentiation in vitro (Sin, 2016), and muscle regeneration in vivo (Wagatsuma, 2011). Succinate dehydrogenase (SDHA) is the only enzyme involved in both the citric acid cycle and electron transport chain, thereby it is a good indicator of mitochondrial energy capacity. However, SDHA levels remained unchanged during differentiation and no differences were seen under conditions of BDK depletion **(Fig 2-6d)**. Further experiments will measure the activity of SDHA under BDK depleted conditions to gain a better understanding whether or not SDHA function is altered under BDK depletion. The increased rate of differentiation is evident due to the aforementioned increased accretion of myofibrillar proteins, MHC and tropomyosin **(Fig 2-6b, c)**. A possible explanation is that differentiation is simply occurring at a faster rate, thereby, a faster increase in mitochondrial content. I suspect the effects of chronically reducing intracellular BCAAs through the increased rate of BCAA catabolic flux is important to differentiation, based on the increased activation of AKT and the likely stimulatory effect it exerts on mTORC1. Taken together, the dynamic nature of these signalling pathways and their effects on differentiation under BDK depleted conditions require further investigation.

The enhancement of differentiation caused by BDK depletion may be mediated by an effect on cell viability, during the early stages of differentiation. I had noticed muscle cell differentiation is always accompanied by the accumulation of unadherent cells by day 1 of differentiation, especially under BDK knockdown conditions, which is indicative of non-viable cells. Because cell viability and subsequently, cell confluency, have been shown to effect differentiation, I measured two markers of apoptosis. There were no differences in apoptosis between the BDK depleted condition and control, based on the unprocessed caspase-7 and poly (ADP-ribose) polymerase (PARP) **(Fig 2-7a, b).** However, cells that

became detached from the plate are washed away following the normal harvesting protocol. Perhaps gathering those cells and combining them with the rest of the cell lysate would yield a better understanding of the role apoptosis may be having in BDK depleted cells. Another explanation of the mass of unadherent cells could be that only these cells received apoptotic signalling instruction, and the subset of cells that survived this phase never had a significant increase in apoptotic signalling. Evidence supporting this notion refers to C2C12 cells that can be separated into subgroupings. One of these subgroups do not undergo apoptosis, nor do they differentiate, Schöneich et al., refer to them as "Reserve" cells (Schöneich, 2014). These "Reserve" cells enter a quiescent state upon exposure to DM, and are believed to reflect satellite cell functioning in mature skeletal muscle. Additionally, it has been shown that the caspase-3 is required for differentiation to occur in C2C12 cells (Fernando, 2002; Larsen et al., 2010), however in mature myotubes anti-apoptotic signalling is elevated (Xiao et al., 2011). This compartmentalization of cells and their ability to serve different roles during differentiation should influence our assessments of muscle cell differentiation, and perhaps we should investigate interventional changes according to these subgroups once better defined. Taken together, assessment of other mechanisms that could be regulating cell viability during differentiation, like autophagy, warrant further study because of its established role in myogenic differentiation (McMillan & Quadrilatero, 2014; Pizon et al., 2013; Sin, 2016).

Prior research in our lab has shown that cells depleted of BCKD-E1α fail to differentiate. If the impairment in differentiation is due to the absence, or the reduction in BCKD derived metabolites, it is logical to assume if provided exogenously differentiation may be rescued. I then supplemented the BCKD depleted condition with each of the

immediate downstream metabolites of BCKD, derived from the BCAA keto-acids. Supplementation with BCKD products was unable to rescue differentiation under BCKD depleted conditions; evident by MHC-1 levels being suppressed to a similar degree as the knockdown alone **(Fig 2-8a)**. In addition, no effect of either intervention treatment was witnessed in ph(S473)AKT analysis. This may be a product of a reduction in cell viability witnessed in BCKD depleted muscle cells (Zameer N . Dhanani and Olasunkanmi J. Adegoke, 2016), and is possibly resultant from the accumulation of intracellular BCAAs and BCKAs.

Conclusions

We have presented novel findings observing BCKD activity in muscle cells. To our knowledge this is the first time BCKD activity has been measured during muscle cell differentiation in vitro. Because of the decrease in BCKD activity and the constant expression of its negative regulator, BDK, the association between the two must be increasing during differentiation. However, the mechanisms mediating this increased association remain to be elucidated. Furthermore, siRNA mediated depletion of BDK resulted in an increased and premature expression of myofibrillar proteins. This could possibly be explained by our observations of greater AKT activation during early differentiation, which may be stimulating mTORC1 activity, consequently upregulating cellular processes necessary for differentiation.

Taken together, we provide greater evidence supporting the role BCAA catabolism serves as a regulating factor involved in muscle cell differentiation **(Fig 2-9).**

Future Directions and Study Limitations

Our next objectives include:

1) Determine to what degree BDK and BCKD associate during differentiation via immunoprecipitation studies.
2) Determine the role of PP2Cm in muscle cell differentiation. If PP2Cm knockdown studies are completed will this yield the opposite result witnessed during BDK depletion?
3) Determine whether the past increases in BCAT2 and BCKD-E1α proteins observed during differentiation were not merely the result of the concomitant rise in mitochondrial content. Measuring mitochondrial content during differentiation, mainly via respiratory chain proteins, could be used as a standard for the rate increase of mitochondrial content. Then by comparing the changes in BCAA catabolic expression and mitochondrial content via regression analysis, it can be determined if these increases in BCAA catabolic enzymes are rising proportionately with mitochondrial content. (Isolating mitochondria may be favourable to eliminate any newly translated cytosolic BCAT2 or BCKD)
4) Investigate how the increase in BCKD flux affects energy metabolism during differentiation. Radiolabelling BCAAs or BCKAs represents one method for assessing the contribution of BCAA catabolic flux to the TCA cycle. The output measure of this experiment would assess the incorporation of those radiolabelled carbon skeletons into TCA cycle intermediates. Comparing the amount of radiolabelled TCA cycle intermediates between normal and BDK depleted conditions would give indication whether or not BCAA flux could disproportionately effect energy substrate provision.

5) Investigate how mTORC1 activity is altered under conditions of BDK depletion. The most well defined substrates of mTORC1 include, S6K1, S6, 4E-BP1 and ULK1. Measuring these downstream targets of mTORC1 will determine whether ribosomal biogenesis, translation efficiency, or autophagy, are alternatively regulated under conditions of BDK depletion.

The nature of *in vitro* studies is to determine specific mechanisms, which in turn, subjects them to operating under isolated conditions. Further study *in vivo* will elucidate how all of the mammalian body systems affect the results generated from this *in vitro* study.

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Appendices

Supplementary Figures

Fig 1

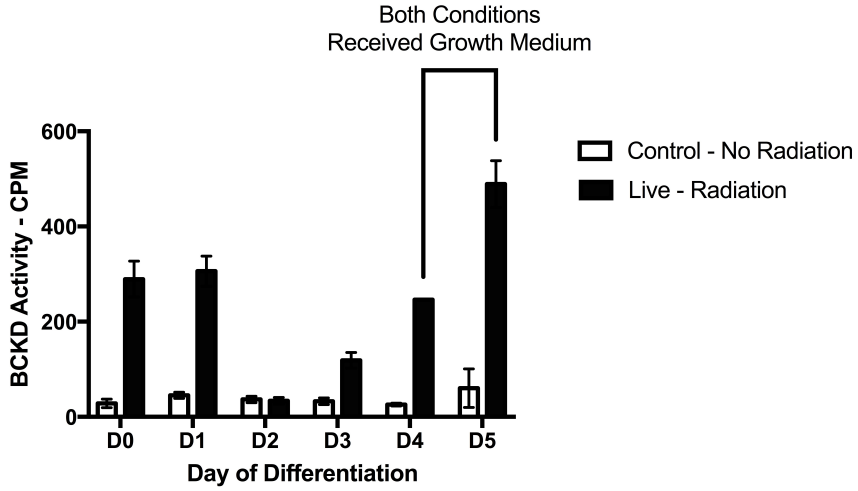

Fig 1. BCKD activity during differentiation of L6 muscle cells, first differentiation test. The second generation BCKD activity assay was used and the final two days L6 myotubes were exposed to GM (AMEM + 10% FBS + 1% Ab). The final two days that were exposed to GM were similar to initial days of differentiation. Thereby, growth factors may be mediating BCKD activity, as opposed to the processes driving differentiation. Under the "No Radiation" data CPM values are present to a small degree, due to leakage from adjacent wells that contained the radioisotope.

Fig 2

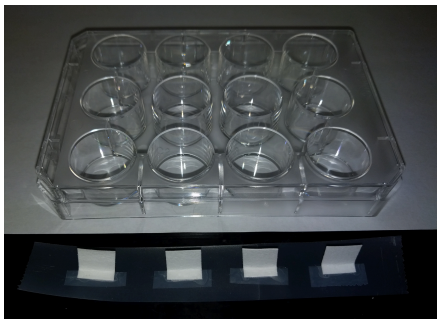

Fig 2. Setup example of second BCKD activity assay generation. A 12-well plate is shown with one of three pieces of clear tape with filter paper wicks anchored.

Fig 3

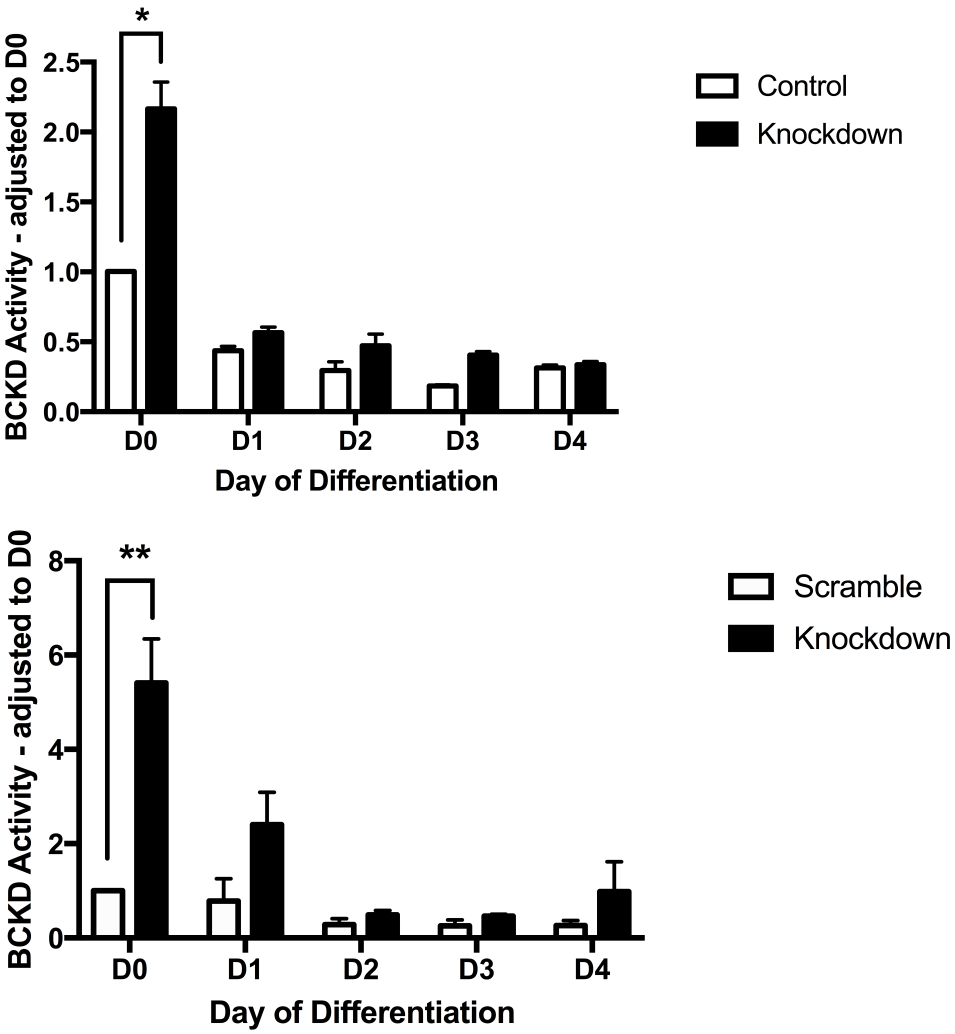

Figure 3. siRNA mediated BDK knockdown in L6 muscle cells significantly increases BCKD activity in L6 muscle cells. **a)** BCKD activity was measured using the second generation BCKD activity assay generation up to day 4 of differentiation (n = 2) **b)** BCKD activity was measured using the third generation BCKD activity assay generation up to day 4 of differentiation (n=3). *Three replicates per experiment, (*= p < 0.05 - ** = p <0.01)). Bar graphs show mean ± SEM*. All data in these figures are normalized to the first condition, unlike the CPM corrected values depicted in the body of this work.

Fig 4

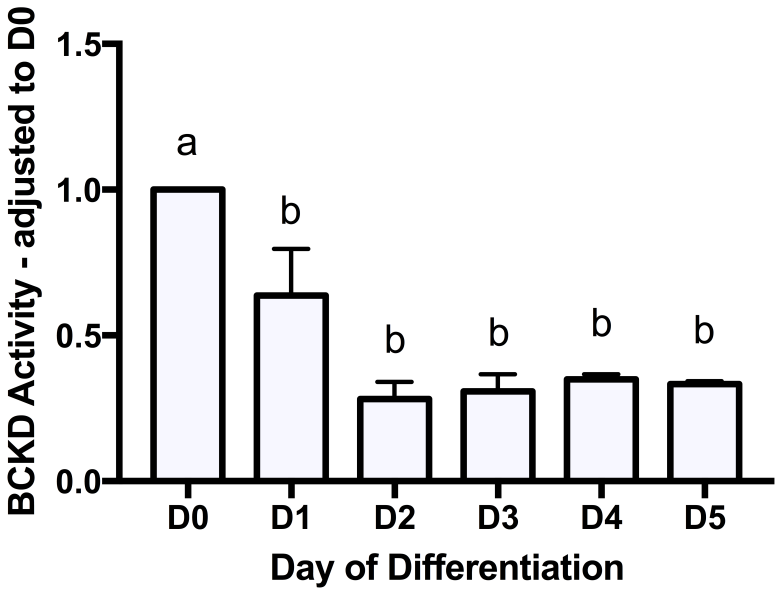

Figure 4. BCKD activity is reduced following the first 48 hours of differentiation and plateaus until the end of the experiment on day 5 of differentiation. BCKD activity assay (2nd generation) values normalized to day 0 of differentiation in L6 muscle cells. *Three replicates per experiment, letters denote significant difference (n = 5, p < 0.05). Bar graphs show mean ± SEM*

BCKD Activity Assay

Materials and Methods

- 6-well culture plate
- Clear tape
- Filter paper wicks (1.3cm x 2cm)
- 2 M NaOH
- Heat Incubator-Shaker
- AMEM Medium (when using L6 cells)
  - Growth Medium (GM)
  - Differentiation Medium (DM)
- Krebs Ringer Phosphate Buffer (KRB)
  - KRB formulation

0.018M	$NaHPO_4$
0.68% (w/v)	NaCl
0.045% (w/v)	KCl
0.03% (w/v)	$MgSO_4$

0.02% (w/v)	$CaCl_2$

  - 1mg/ reaction well - Thiamine hydrochloride
    - Working dilution: 1mg per 856µl of KRB = 14mg per 12ml of KRB
- Reaction Mixture → Typical working volume 1ml (936.65µl PBS, 43.35µl valine (50mg/ml stock), 20µl 1-^{14}C labelled valine)
  - Phosphate Buffer Saline
  - Valine 50mg/ml mixture
  - 1-^{14}C labelled valine
- 15% Trichloroacetic acid (TCA)
- Lysis Buffer

Steps

1) Inside the sterile hood aspirate medium from all wells in plate, replenish with 200ul/well of GM (day 0) or DM (day 1 – day 5).
2) Move to radioactive bench in lab, immediately add 856 ul/well of KRB.
   i. KRB buffer should be supplemented with thiamine hydrochloride. Each well should receive 1mg B_1 (1mg per 856ul KRB)
3) Add 61ul of Reaction Mixture/well – Equal to 0.12µCi
4) Administer 60ul of 2M NaOH to each filter paper wick, while they are anchored in place to the clear strip of tap that will run across the top of each well. (Once the wicks are secured on the mounting strip, place the mounting strip on its side. It is at this point you should administer the NaOH).
5) Place clear tape (mounted with filter paper wicks) across desired wells.
   i. Ex. When a single plate contains more than one experimental condition run the strips of scotch tape across one another (as shown in **Supp. Fig 1b**)
6) Cover all desired wells with the single strip of clear tape (make sure the wicks are centralized on all wells).
7) Make sure the initial strips of clear tape are well fastened and the paper wicks are secure on them (not hanging off at a single corner).
8) Start to apply additional strips of clear tape starting, covering the remaining gaps left by the initial strips of clear tape (**Supp. Fig 1c**).
9) Once all wells are well covered make sure to run your finger around the rim of each well, ensuring there are no bubbles or gaps compromising the seal of EACH well.
10) Place 6-well plate lid over top of the newly taped plate and then wrap a single piece of clear tape around the entire plate and lid. Some of the lids (company dependent) have well protrusions that help seal the wells (pressing the tape down uniformly around each well)(Single band of clear tape keeping the lid on, as seen in **Supp. Fig 2a**).
11) Place plate in heat incubator-shaker for 1.5 hours at 50 rpm and at 37 degrees Celsius.
12) At the 1.5 hour mark, remove plate from heat incubator-shaker and bring to radiation bench.

13) Using a 21-gauge needle add 50ul of 15% (w/v) TCA to each well, to stop the reaction.
14) Immediately following *step 12* cover holes with additional strips of clear tape, and place back in incubator for 30 minutes.
15) During this 30 minute waiting period I prepare three things:
    i. Label and fill 20ml liquid-scintillation vials with 3.5mls of scintillation fluid.
    ii. Label centrifuge tubes required for cell lysate.
    iii. Add 7ul (10ul/ml) of phosphatase inhibitor to lysis buffer (usually kept in 700ul volumes) and let thaw.
16) Remove plate from heat incubator-shaker and bring to radiation bench, using a small set of scissors, make an incision in the clear tape, just along the inside perimeter of the plate, and cut along the perimeter line along three sides (as seen in **Supp. Fig 2b**).
17) At this stage it is easy to peal back all of the clear tape as a single unit, you will find it looks like a basket weave to which all the filter paper wicks should still be secured (**Supp. Fig 2c**).
18) Using a cleaned (70% ethanol admin and wiped with Kim wipes) set of forceps remove the blank filter paper wicks (the two that were placed between the wells) and place into their respectively labelled scintillation vials.
19) Then remove each filter paper wick individually from the clear tape basket weave and place into their respective scintillation vials.
20) Aspirate the contents of each well, then rinse twice with ice cold PBS.
21) Following the second rinse with ice cold PBS, add 100ul/well of lysis buffer.
22) Harvest cells following typical cell harvesting protocol.

Figure 1.

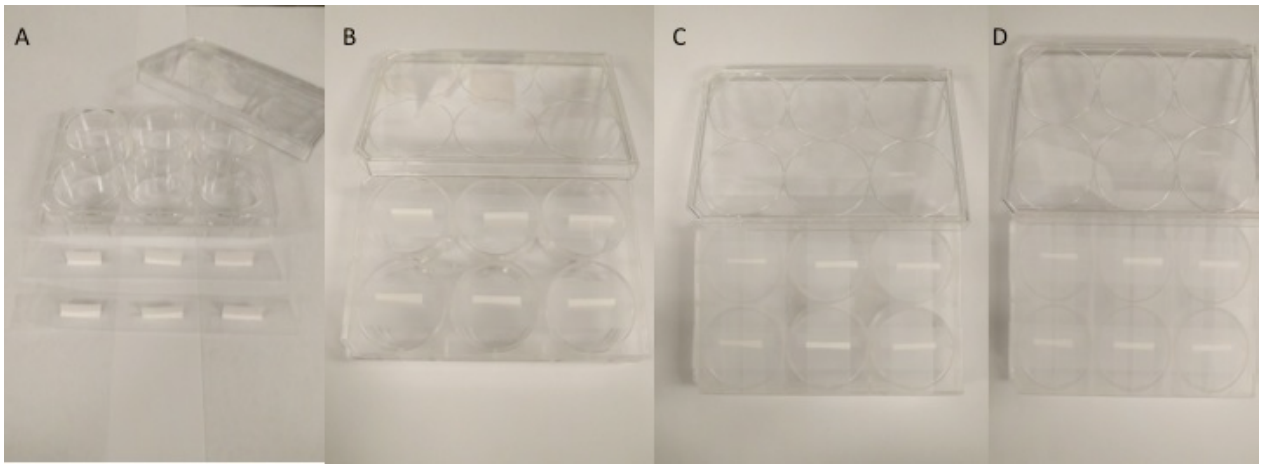

Figure 2.