Alternative NF-κB Regulation of Skeletal Muscle Oxidative Metabolism

DISSERTATION

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Abstract

Skeletal muscle facilitates movement through contraction, which requires substantial energy production. Therefore, proper metabolism is essential for muscle homeostasis. Interestingly, muscle fibers with more oxidative metabolism are more resistant to atrophy caused by chronic inflammation and cancer cachexia. Understanding how metabolism is regulated in muscle homeostasis may reveal mechanisms of disease pathogenesis and therapeutic intervention.

NF-κB signaling regulates a wide array of skeletal muscle processes. For example, aberrant classical NF-κB activation in skeletal muscle has been directly linked to atrophy, inflammation, and cancer progression. However, less is known about the role of alternative NF-κB in skeletal muscle homeostasis. Evidence suggests that alternative NF-κB directly stimulates transcription of PGC-1β, a master regulator of mitochondrial biogenesis, and thereby regulates skeletal muscle oxidative metabolism. Here, we sought to understand in vivo the role of alternative NF-κB in skeletal muscle physiology, skeletal muscle homeostasis, and overall metabolic health. We therefore generated a transgenic mouse expressing the alternative NF-κB mediator IKKα in skeletal muscle. We find that alternative NF-κB is sufficient to induce slow Type I myofiber specification, without causing muscle atrophy. Furthermore, IKKα transgenic mice were significantly more active than their wild type littermates, suggesting healthier, more fatigue resistant skeletal
muscles. This in vivo evidence reinforces the distinction between classical and alternative NF-κB signaling, suggests a role for alternative NF-κB in promoting slow Type I myofibers, and provides a potential tool to study the link between fiber type and atrophy resistance.

In addition to alternative NF-κB, we also studied muscle homeostasis in the context of the transcription factor MyoD. MyoD is a key regulator of skeletal myogenesis that directs contractile protein synthesis, but whether this transcription factor also regulates skeletal muscle metabolism, and particularly aerobic respiration, has not been explored. In a genome-wide bioinformatics analysis of skeletal muscle cells, we unexpectedly observed that MyoD directly binds to numerous metabolic genes, including those associated with mitochondrial biogenesis, fatty acid oxidation, and the electron transport chain. Results in cultured cells and adult skeletal muscle confirmed that MyoD regulates oxidative metabolism through multiple transcriptional targets, including PGC-1β. We find that PGC-1β gene expression is cooperatively regulated by MyoD and the alternative NF-κB signaling pathway. Bioinformatics evidence suggests that this cooperativity between MyoD and alternative NF-κB extends to other metabolic genes as well. Together, these data uncover a novel role for MyoD in regulating the metabolic oxidative capacity of mature skeletal muscle to ensure that a sufficient energy pool is available to support muscle contraction.

Collectively, this dissertation highlights novel regulatory mechanisms uniquely structured to support the fundamental metabolic needs of skeletal muscle.
Dedication

This document is dedicated to my family and friends.
Acknowledgments

First, I would like to thank my mentor, Dr. Denis Guttridge, for everything. I could not emphasize enough how much his mentoring, love for science, and unwavering patience with my development have impacted my life. I would also like to thank the members of my committee, Dr. Martha Belury, Dr. Jeffrey Parvin, and Dr. Qianben Wang for their time and support throughout this process. Additionally, I greatly appreciate the help I received from members of the Guttridge lab, whose friendships I will always cherish. Finally, I would like to thank my family for their love and support.
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CHAPTER 1

INTRODUCTION

1.1 Skeletal muscle

Movement is facilitated by the coordinated contraction and relaxation of skeletal muscles, which are each composed of multinucleated myofibers. During myofiber development there is a large proliferation of contractile myofilaments as well as energy producing mitochondria. Both of these components are essential for repeated cycles of muscle contraction.

Differentiation

Muscle differentiation, also known as myogenesis, occurs at the cellular level with muscle precursor cell proliferation, migration, and fusion into multinucleated myofibers. Each of these myofibers carries out sarcomere formation and mitochondrial biogenesis, which are critical to its ability to contract. Myogenesis occurs similarly in both embryonic and adult muscle. Embryonic muscle formation first involves the differentiation of myoblasts into primary and secondary myofibers. Subsequent fusion of muscle
progenitors into these fibers supports muscle growth\textsuperscript{4}. Similarly, injury to mature muscle stimulates a process of regeneration, where resident muscle stem cells become activated, migrate, differentiate, and fuse into existing myofibers\textsuperscript{9,10}.

Myogenesis is regulated at the transcriptional level by a network of highly conserved myogenic regulatory factors (MRFs). These factors include Myf5, MyoD, myogenin, and MRF4. MyoD was first identified by its ability to independently convert C2H10T1/2 embryonic fibroblasts into myoblasts\textsuperscript{11}. This was soon followed by the discovery of additional MRFs capable of inducing muscle characteristics in non-muscle lineages\textsuperscript{12-16}. Each MRF contains a basic domain which mediates binding to consensus E-box DNA sequences. Additionally, they each contain a helix-loop-helix domain required to form heterodimers with E proteins.

Throughout myogenesis, MRFs temporally cooperate to induce the expression of essential muscle genes. The somewhat redundant and temporal nature of the MRFs has been most clearly demonstrated through a series of gene knockout experiments. Despite the ability of Myf5 and MyoD to independently stimulate muscle, knockout mice of either gene were reported to develop normal skeletal muscle\textsuperscript{17,18}. This was in sharp contrast to Myf5\textsuperscript{−/−} MyoD\textsuperscript{−/−} double knockout mice, which were completely devoid of skeletal muscle formation and myogenin expression\textsuperscript{19}. These results suggested that these MRFs were able to functionally compensate for each other. Closer analysis revealed that, although the individual Myf5\textsuperscript{−/−} and MyoD\textsuperscript{−/−} mice were able to form skeletal muscle, Myf5\textsuperscript{−/−} mice exhibited delayed embryonic myogenesis while MyoD\textsuperscript{−/−} mice had prolonged Myf5
expression, presumably to compensate for the lack of MyoD\textsuperscript{17,18}. Together, these results suggest that Myf5 and MyoD are able to regulate some of the same essential genes required for myogenesis. A similar analysis of \textit{myogenin} null embryos revealed some myofiber formation, but also late-stage myoblasts that had not fully differentiated\textsuperscript{20}. They also had normal expression of MyoD and reduced MRF4. Together, these data suggest that Myf5 and MyoD are critical for muscle specification, while myogenin and MRF4 are more downstream effectors of myogenesis.

The role of MyoD in myogenesis is unique because it is important for early progression of muscle differentiation and is still expressed in mature myofibers\textsuperscript{21}. As myoblasts differentiate into myotubes, ChIP-seq studies reveal that MyoD binds to genes largely related to the cytoskeleton, muscle contraction, and the sarcomere\textsuperscript{22}. However, MyoD activation of genes is not as simple as a single on switch during differentiation. MyoD ChIP experiments revealed that it temporally binds to different genes throughout myogenesis to directly regulate various patterns of gene expression\textsuperscript{23}. In addition to its temporal binding, a subset of genes bound by MyoD are only expressed after subsequent recruitment of the histone acetyltransferase PCAF\textsuperscript{22,24}. An additional mechanism by which MyoD regulates gene expression is by modifying higher-order chromatin structure. MyoD directly interacts with the chromatin architectural protein CTCF to regulate expression of the cell cycle inhibitor p57Kip2\textsuperscript{25}. MyoD also directly associates with Brg1 during early stages of myogenesis to spatially cluster and temporally restrict expression of late-stage myogenic genes\textsuperscript{26}.
**Contraction**

Muscle contraction is mediated by myosin and actin filaments organized into sarcomeres that extend along the length of myofibers. At rest, the cross bridges between myosin and actin are sterically hindered through the binding of a multimeric complex of troponin proteins and a linear chain of tropomyosin. Upon muscle stimulation, Ca\(^{2+}\) is released from the sarcoplasmic reticulum which, when bound to the troponin complex, induces a conformation change in tropomyosin that allows binding of myosin to the exposed actin filament. The myosin and actin filaments are then free to pull along one another to produce force.

The ability of myosin to release from actin and reset its mechanical conformation is required for additional rounds of contraction. ATP is central to this process. Through direct binding to myosin, ATP allows myosin to release its grip on actin. This is followed by ATP hydrolysis and movement of myosin back into a conformation of high potential energy. At this stage, myosin is prepared for the next round of Ca\(^{2+}\) stimulated contraction. Contraction then proceeds when tropomyosin allows myosin to bind actin. Subsequently, ADP and inorganic phosphate are released by myosin, which coincides with muscle contraction.

Skeletal muscles throughout the body have different functional demands and are therefore composed of myofibers geared towards strength or endurance. These differences can be visually distinguished by the white color of fast Type II myofibers compared to the
distinct red color of slow Type I myofibers derived from their higher myoglobin content. Early studies associated this color difference with slow or fast contractile properties\textsuperscript{30}. More complex mechanical and biochemical classifications have led to the identification of four distinct myosin types: I, IIa, IIx, and IIb\textsuperscript{31}. One series of experiments examined succinate dehydrogenase (SDH) activity, reflective of oxidative metabolism, in fast twitch fibers\textsuperscript{32}. They found that some fast fibers had more SDH staining and were more resistant to fatigue, indicative of distinct IIa and IIb fiber types. The identification and characterization of myosin led to the discovery of an addition myosin heavy chain IIx, with unique transcript and fiber contractile properties intermediate of IIa and IIb\textsuperscript{31,33}.

The molecular and physiological cues regulating myosin fiber type specification are not completely understood. For example, altering motor neuron stimulation rates in myofibers can reprogram fast and slow fiber types\textsuperscript{34}, and muscle denervation leads to preferential fiber type atrophy\textsuperscript{35}. These results suggest that the motor neuron is a critical upstream regulator of determining muscle fiber type. On the other hand, motor neuron denervation does not affect early fiber type determination in neonatal rat hind limb muscles, suggesting that while the motor neuron can affect some physiological properties of myofibers, there are also intrinsic signals that regulate muscle fiber type identity\textsuperscript{36}.

Some of the muscle intrinsic factors proposed to regulate myofiber specification include SOX6, SIX1, SIX4, and the transcriptional coactivators PGC-1\(\alpha\) and PGC-1\(\beta\). The transcription factors SOX6, SIX1, and SIX4 promote a fast muscle phenotype and suppress slow fiber identity\textsuperscript{37}. In contrast, PGC-1\(\alpha\) and PGC-1\(\beta\) over expression promotes a more
oxidative muscle phenotype, along with Type I or Type IIx myofibers, respectively\textsuperscript{38,39}. The relationship between PGC-1 coactivator expression and myofiber specification is, however, somewhat ambiguous since the patterns of myofiber changes in \textit{PGC-1\(\alpha\)} and \textit{PGC-1\(\beta\)} knockout mice do not correspond with the over expression models\textsuperscript{40,41}. Together, these data reveal that there are likely multiple muscle intrinsic factors involved in myofiber type specification\textsuperscript{42-44}.

1.2 Metabolism

ATP production and hydrolysis have a central role in every cycle of muscle contraction\textsuperscript{45}. The metabolic profile of a myofiber therefore often corresponds with its contractile properties and energetic needs. As such, mechanisms regulating metabolism have a key role in maintaining muscle homeostasis.

Glycolysis

Glycolysis is the process of metabolizing glucose to pyruvate\textsuperscript{46}. In doing so, ATP and NADH are synthesized. ATP can directly be used to fuel energy-dependent processes in the cell, while pyruvate is enzymatically broken down to Acetyl-CoA and fed into the citric acid cycle. As an electron donor, NADH can shuttle into the mitochondria and support the electron transport chain\textsuperscript{47,48}. While the products of glycolysis help to fuel oxidative phosphorylation, glycolysis itself is a relatively less efficient producer of ATP.
compared to aerobic respiration. It is, however, particularly suited for producing energy quickly and under anaerobic conditions.

During short periods of intense exercise, glycolysis becomes a major contributor of ATP in skeletal muscle. This shift is not due to limited oxygen being available for oxidative phosphorylation. Rather, evidence suggests that high glycolytic flux is directly responsible for restricting oxidative phosphorylation, and both of these processes remain in balance to sustain optimal ATP concentrations for fueling muscle contraction. The contribution of glycolysis versus oxidative phosphorylation is complemented by myosin heavy chain isoforms that consume ATP at different rates, with fast Type II myosin consuming ATP faster than slow Type I myosin.

**Oxidative phosphorylation**

The process of oxidative phosphorylation is centered around the electron transport chain and its production of ATP. It does this by taking electrons from donors such as NADH and passing them through multiple intermediate complexes to oxygen, an electron acceptor. The energy released by this passing of electrons is used to transport protons across the inner mitochondrial membrane and form a gradient of potential energy. As the proton gradient is released through an ATP synthase enzyme, ADP is phosphorylated to form ATP.

Glycolysis provides substrates for oxidative phosphorylation in part through the citric acid cycle. In addition to the NADH produced during glycolysis that directly supports
the electron transport chain, pyruvate metabolized into Acetyl-CoA enters the citric acid cycle to produce GTP and additional NADH. Large amounts of Acetyl-CoA can also be derived from fatty acid β-oxidation. The process of one glucose molecule producing four Acetyl-CoA molecules, which then each produces three NADH molecules through the citric acid cycle drives a complex and highly efficient process of obtaining energy.

**Mitochondrial biogenesis**

Stimulation of skeletal muscle through exercise can increase mitochondrial volume upwards of five-fold\(^53,54\). Naturally, greater mitochondrial volume corresponds with a greater capacity for oxidative phosphorylation\(^55\). Mitochondrial biogenesis and homeostasis are dynamic processes involving a carefully regulated balance of fusion and fission\(^56,57\). Specific proteins such as OPA1\(^58\), Mfn1\(^59\), and Mfn2 are essential for fusion of the mitochondrial inner and outer membranes, and defects in these can lead to mitochondrial DNA (mtDNA) mutations and aberrant mtDNA distribution. Similarly, mutations in the mitochondrial fission protein Drp1 also affect mitochondrial morphology, distribution, and function\(^60\).

In addition to regulated fusion and fission, mitochondrial biogenesis requires a coordinated proliferation of structural and enzymatic proteins, ribosomes, and mtDNA. This includes the expression of both mitochondrial and nuclear encoded genes\(^61\). Some of the transcription factors implicated in coordinating expression of these genes include NRF1, NRF2, PPAR\(\alpha\), and ERR\(\alpha\). NRF1 is particularly important for directly stimulating
expression of mitochondrial transcription factor A (TFAM), which is essential for mtDNA replication and commonly correlates with mitochondrial genome copy number \(^{62-64}\). The mitochondrial genome encodes vital subunits of respiratory complexes I, III, IV, and V \(^{65}\). Other genes required for mtDNA replication include POLRMT, TFB2M, and MTERF1 \(^{66,67}\). By comparison, NRF2 stimulates transcription of cytochrome c oxidase subunit IV (COXIV) \(^{68}\). NRF1 and NRF2 also enhance the mitochondrial transport machinery. In addition, PPAR\(\alpha\) and ERR\(\alpha\) enhance expression of enzymes involved in fatty acid oxidation, the citric acid cycle, and the electron transport chain.

**PGC-1 coactivators**

The coordination of multiple transcription factors targeting various components of mitochondrial structure and dynamics was simplified by the discovery of the overarching transcriptional coactivator PGC-1\(\alpha\) and its family members PGC-1\(\beta\) and PRC. Each of these coactivators contains an amino-terminal activation domain important for recruitment of histone acetyltransferases SRC-1 and CBP/p300. Also present near the amino-terminal are LXXLL motifs for interaction with nuclear receptors. Commonalities near the carboxyl-terminus include an RNA recognition motif, arginine-serine rich domains, and other specific sites identified to interact with transcription factors.

The PGC-1 family was first identified by the interaction of PGC-1\(\alpha\) with the nuclear receptor PPAR\(\gamma\) \(^{69}\). Since then, PGC-1\(\alpha\) and PGC-1\(\beta\) have been shown to directly interact with NRF1, NRF2, ERR\(\alpha\), PPAR\(\alpha\), and PPAR\(\beta\) \(^{70-73}\). Through these interactions,
PGC-1α and PGC-1β expression enhances the mitochondrial biogenesis program. This has perhaps been most clearly demonstrated in vivo where transgenic over expression of either coactivator specifically in skeletal muscle increases mitochondrial biogenesis and oxidative phosphorylation\(^{38,39}\).

Despite interactions with many of the same transcription factors to effectively enhance mitochondrial biogenesis, functional and regulatory differences between PGC-1α and PGC-1β have been revealed. In skeletal muscle, exercise induces PGC-1α expression. However, PGC-1β does not respond to this same stimulus, suggesting that PGC-1α supports muscle adaptations to metabolic demands, while PGC-1β may distinctly support basal oxidative phosphorylation. Another stimulus that was found to induce PGC-1α and not PGC-1β expression was cold exposure, again supporting the role of PGC-1α in mature muscle adaptation.

**1.3 NF-κB signaling**

NF-κB signaling has been implicated in cell differentiation\(^ {1,74,75}\), survival\(^ {76}\), migration\(^ {4}\), proliferation\(^ {77}\), and metabolism\(^ {1,78,79}\). Intense focus has gone into defining the relevant signaling components and target genes involved in regulating these and other processes. The delineation of classical and alternative NF-κB signaling has been instrumental in our understanding of skeletal muscle development and disease pathogenesis.
Overview of NF-κB and IκB subunits

The NF-κB family of transcription factors is composed of five subunits – p65, RelB, c-Rel, p50, and p52 – that bind DNA in the form of homo- and heterodimers. Each subunit contains a Rel homology domain at its amino terminus, which is required for dimerization, DNA binding, and interaction with Inhibitor of κB (IκB) proteins. However, only p65, RelB, and c-Rel contain a transactivation domain (TAD) on their carboxyl terminus, suggesting that dimers not containing these particular subunits function as transcriptional repressors. In addition to the Rel homology domain and TAD, the NF-κB subunits contain a nuclear localization signal (NLS) that aids import of the dimers into the nucleus.

One notable layer of NF-κB regulation is the IκB family. The IκB proteins IκBα, IκBβ, IκBε, IκBζ, BCL-3, IκBNS, p100, and p105 each contain ankyrin repeat domains which mediate protein-protein interactions. By interacting with NF-κB subunits and sterically covering the NLS, IκB proteins inhibit nuclear translocation and thus restrict NF-κB-mediated transcription. Degradation of IκB proteins occurs by phosphorylation and subsequent K48-linked polyubiquitination. Alternatively, K48 polyubiquitination of p100 and p105 induces partial proteolysis to the p52 and p50 subunits of NF-κB.

Once NF-κB dimers are activated and translocated into the nucleus, they bind to consensus NF-κB binding sites composed of characteristic GGGRNNYYCC sequences. Following binding, there are several ways that NF-κB can then regulate transcription. The
most recognized mechanism involves recruitment of the transcription Mediator complex\textsuperscript{81}. The most common NF-kB dimer, p65-p50, is also known to recruit histone acetyltransferases (HATs), such as PCAF, CBP, and p300\textsuperscript{82}. On the other hand, NF-κB can also act as a transcriptional repressor through direct binding of p50-p50, p50-p52, or p52-p52 dimers to NF-κB sites, recruitment of histone deacetylases HDAC1 or HDAC2, or recruitment of the DNA methyltransferase DNMT1\textsuperscript{83,84}.

**Classical NF-κB signaling**

The most ubiquitously expressed NF-κB heterodimer, p50-p65, is sequestered in the cytoplasm by IκBα and IκBβ and becomes activated via the classical NF-κB signaling pathway. This pathway is regulated by an IκB kinase (IKK) complex composed of catalytic subunits IKKα and IKKβ, and a regulatory subunit NF-κB Essential Modulator (NEMO or IKKγ).

Together, the IKK complex phosphorylates IκBα at Ser32 and Ser36, leading to its ubiquitination and proteasomal degradation\textsuperscript{85}. The degradation of IκBα allows the translocation of p50-p65 to the nucleus where the heterodimer then binds to a specific DNA consensus sequence and subsequently stimulates the initiation of gene expression. Transcriptional activity of the NF-κB subunit p65 is enhanced by post-translational modifications such as phosphorylation, which is in part controlled by components of the
IKK complex. Most extracellular inducers of NF-κB operate via activation of the IKK complex, which serves as an essential signaling hub\textsuperscript{86}.

The basic components operating upstream of IKK depend in part on extracellular signaling, but common components include members of the TNF receptor-associated factor (TRAF) family\textsuperscript{86}. TRAFs are E3 ubiquitin ligases, which are recruited to the cytoplasmic portion of cell surface receptors by adaptor proteins such as MyD88, TRADD, and FADD. Stimulation in response to inflammatory mediators IL-1 and LPS results in the recruitment of MyD88, IRAK, and TRAF6, which in turn activates IKK, degrades IκB, and induces NF-κB nuclear translocation and gene transcription. In contrast, TNFα stimulation leads to receptor recruitment of TRAF2 and additional E3 ubiquitin ligases cIAP1 and cIAP2, leading to activation of the kinase RIP1. Polyubiquitination and activation of RIP1 is required for the recruitment of NEMO and subsequent activation of the IKK complex\textsuperscript{85}.

**Alternative NF-κB signaling**

Extracellular stimuli such as CD40L, BAFF, and lymphotoxin-β activate an alternative, or non-canonical, NF-κB signaling pathway, which is distinct from classical NF-κB both in its upstream mechanisms of activation and downstream transcriptional signals\textsuperscript{87}. In contrast to the classical NF-κB signaling effectors IKKβ and NEMO, alternative NF-κB is dependent upon NF-κB inducing kinase (NIK) and IKKα. Under basal conditions, NIK concentrations are repressed by constitutive TRAF3-dependent
ubiquitination and degradation. Upon stimulation of alternative NF-κB, NIK accumulates following degradation of TRAF3. NIK then phosphorylates IKKα and simultaneously functions as a docking site for the phosphorylated IKKα and p100. This facilitates IKKα phosphorylation of p100. This event induces p100 ubiquitination, which is followed by p100 partial proteolysis to form the mature NF-κB subunit, p52. Analogous to the p50-p65 heterodimer, p52-RelB translocates to the nucleus to regulates target gene expression.

**Classical NF-κB signaling in skeletal muscle**

The classical pathway of NF-κB is basally activated in myoblasts, and evidence indicates that this suppresses their differentiation. Supporting studies have utilized myoblast cell lines in vitro, as well as neonatal muscle development as an in vivo model of myogenesis. During neonatal development, when myoblasts are differentiating and fusing into existing myofibers, classical NF-κB activity declines, consistent with its role as an inhibitor of myogenesis. Furthermore, genetic mouse models lacking the p65 subunit of NF-κB have increased myofiber numbers and greater expression of genes such as myosin and troponin during neonatal development.

Classical NF-κB suppresses myogenesis through multiple mechanisms. MyoD, a critical regulator of muscle differentiation, can be transcriptionally or post-transcriptionally repressed by NF-κB. In addition, NF-κB directly induces transcription of Yin Yang 1, which cooperates with the PRC2 polycomb complex to epigenetically repress expression.
of myofibrillar genes$^{97}$. NF-κB also enhances transcription and stability of cyclin D1, a known suppressor of muscle differentiation$^{77,98}$. The current model suggests that as myoblasts receive the proper cues to initiate differentiation, activation of classical NF-κB declines, thus allowing cells to proceed through their terminal differentiation program$^{94,99}$.

In contrast to the role of classical NF-κB in early myogenesis, activation of classical NF-κB in mature muscle is generally associated with disease pathogenesis. The one exception has been a described relationship between classical NF-κB activity and neuromuscular junction formation$^{100}$. However, in diseases such as muscular dystrophy$^{101}$, inflammatory myopathies$^{101,102}$, sarcopenia$^{103}$, cancer cachexia$^{104,105}$, and disuse atrophy$^{106,107}$, aberrant classical NF-κB activity is detrimental to muscle health. The pathological role of classical NF-κB was most directly demonstrated in a mouse model where constitutively active IKKβ was over expressed in skeletal muscle fibers, resulting in severe muscle atrophy and reduced force production$^{105}$. In addition, the ability of classical NF-κB to inhibit myogenesis can impair muscle regeneration, which has been demonstrated to exacerbate cancer cachexia pathogenesis$^{108}$.

**Alternative NF-κB signaling in skeletal muscle**

As myoblasts differentiate into mature muscle, classical NF-κB declines while alternative NF-κB becomes activated$^1$. This has been observed during neonatal muscle development when processing of p100 processing declines over time$^{94}$. Although the
upstream signals regulating this switch are yet unknown, the downstream role of alternative NF-κB in muscle has been explored. Unlike classical NF-κB, alternative NF-κB does not regulate muscle differentiation or cause muscle atrophy\(^1\). It instead enhances mitochondrial biogenesis in mature muscle. In vivo, over expression of IKKα in muscle stimulates mitochondrial biogenesis and preferential formation of oxidative fiber types. Alternatively, muscles from RelB\(^{-/-}\) mice are less oxidative than those of wild type littermates. These effects were demonstrated to result, at least in part, through direct regulation of PGC-1β transcription by RelB\(^{78}\).
CHAPTER 2

ALTERNATIVE NF-kB REGULATION OF SKELETAL MUSCLE

OXIDATIVE METABOLISM IN VIVO

2.1 Introduction

Skeletal muscle comprises approximately 40% of human body mass, and its health is critical for voluntary skeletal locomotion. It is capable of performing a range of tasks ranging from quick feats of strength to extended bouts of endurance. Additionally, skeletal muscle is directly involved in maintaining glucose homeostasis and can communicate with other tissues such as adipose\textsuperscript{109}, brain\textsuperscript{110}, and pancreas\textsuperscript{111} through secreted signaling molecules.

The ability of skeletal muscle to accommodate a range of functional demands is due to both its heterogeneity and plasticity. The myosin filaments responsible for mediating these contrasting functions are functionally specialized and categorized into Type I, IIa, IIx, and IIb fibers, with slow Type I fibers ideal for low intensity, sustained muscle activity. Myosin types are complemented by a combination of glycolysis and oxidative metabolism suited to produce a continual energy supply.
Through physical activity, muscles can adapt to functional demands by tuning their structural and metabolic characteristics. Comparatively, conditions that restrict muscle use, such as having a cast or being bedridden, can quickly cause muscle atrophy. Additionally, mitochondrial mutations can feed back to muscle health and cause various myopathies. Understanding the signaling pathways regulating muscle health, disease pathogenesis, and metabolism could provide new avenues for therapeutic intervention.

The NF-κB pathway has been implicated in multiple aspects of skeletal muscle health and development, including differentiation, oxidative metabolism, and atrophy. Classical and alternative NF-κB signaling have distinct functions in muscle. Classical NF-κB signals through the kinase IKKβ to induce activation of the p65-p50 heterodimer and expression of classical NF-κB target genes. Its aberrant activation has been associated with inflammation, cancer, and muscle atrophy.

On the other hand, alternative NF-κB signaling is regulated by an IKKα homodimer complex, which leads to partial proteolysis of p100 to p52, and subsequent translocation of a RelB-p52 transcription factor complex to the nucleus. RelB transcriptional activity has been associated with mitochondrial biogenesis and oxidative metabolism. In particular, it directly activates transcription of PGC-1β, a master regulator of mitochondrial biogenesis. Along with in vitro experiments, the link between alternative NF-κB and oxidative metabolism was consistent in muscles of IKKα−/− and RelB−/− mice. Additionally, adeno-associated virus (AAV) expressing IKKα was injected directly into tibialis anterior (TA) muscles of neonatal mice. IKKα expression was driven by a skeletal
actin promoter to restrict transgene expression to skeletal muscle fibers. After four months, injected muscles had more mitochondrial content and PGC-1β gene expression.

Although alternative NF-κB has been demonstrated in cultured cells and individual TA muscles to regulate oxidative metabolism, we sought to understand how manipulation of alternative NF-κB signaling on a broader scale could impact overall animal health. In addition, a transgenic mouse system targeting all skeletal muscles could potentially identify alternative NF-κB mediated crosstalk with non-muscle tissues or mechanisms of disease pathogenesis. We therefore generated a transgenic mouse expressing HA-tagged IKKα driven by a muscle-specific skeletal actin promoter. Analysis of these transgenic mice revealed a range of predicted and surprising effects on both skeletal muscle and overall transgenic mouse health. Our study demonstrates a clear distinction between classical and alternative NF-κB signaling in vivo. Data also indicate that alternative NF-κB signaling is more complicated than simply being a substitute for exercise and may have a more defined role in skeletal muscle development and basal energy metabolism.

2.2 Materials and Methods

Materials. Antibodies for slow MyHC (M8421) and α-tubulin (T5168) were purchased from Sigma-Aldrich, HA (MMS-101P) from Covance, and IKKα (IMG-136A) from Imgenex. Antibodies against Complex I subunit NDUFS3 (459130), and Complex II subunit Fp (459200) were obtained from Invitrogen.
Mice. All animals were housed, maintained, and used according to protocols approved by the Institutional Animal Care and Use Committee at The Ohio State University. IKKα transgenic mice were generated in the Genetically Engineered Mouse Modeling Core of The Ohio State University and maintained in an FVB/N background.

Gene expression analysis. Tissue RNA was homogenized in TRIZOL (Life Technologies, 15596081), followed by cDNA synthesis (Invitrogen, 28025). Real time PCR was performed using SYBR Green (Roche, 04913914001). Samples were normalized to β-actin.

Mitochondrial DNA quantitation. Total DNA from tissue was extracted using a solution of 10 mM Tris HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 0.2 M NaCl, and 20 mg/mL proteinase K incubated overnight at 55°C. Purification of DNA was then performed by isopropanol precipitation and ethanol washes. DNA was dissolved in TE buffer. Mitochondrial DNA was measured by real time PCR of the mitochondrial cytochrome C oxidase subunit 1 gene. These values were normalized to nuclear DNA as quantitated by real time PCR of glyceraldehyde 3-phosphate dehydrogenase.

Histology and immunohistochemistry. Tissues were sectioned at 8 μm on a cryostat (Leica, CM 3050S). Sections were subsequently stained with hematoxylin and eosin or stained by immunohistochemistry for slow MyHC at a 1:500 dilution factor.
**Fiber size.** MicroSuite Pathology software was used to measure fiber sizes from H&E sections. All fibers were measured on each of 5 sections per TA muscle. Sections were acquired across the length of the muscle.

**Grip strength.** Force measurements were made using an Ametek DFE II Series force gauge. An average of 10 consecutive grip measurements was made for each 6-week old mouse and normalized to body weight.

**Comprehensive Lab Animal Monitoring System.** Mice were allowed to acclimate for 12 hours prior to measurements. They were housed individually at thermoneutrality (28°C) with 12-hour light-dark cycles. Mice were given ad libitum access to food and water. Measurements for O₂ consumption, CO₂ release, and activity were measured in 8 minute intervals over three days. Ambulation was recorded in the x, y, and z planes and pooled to determine total activity.

**Glucose tolerance test.** Mice were fasted overnight and weighed prior to the experiment. 1 mg glucose per gram body weight was injected into the tail vein using a 10% glucose solution. Blood glucose was measured from the ear at 0, 10, 30, 60, and 120 minutes using a MediSense Precision PCx glucose monitoring system.
**Statistical analysis.** Quantitative data are presented as mean ± SEM. Comparisons between two different conditions were assessed using two-tailed Student’s t-test. Statistical significance was evaluated as $p < 0.05$.

### 2.3 Results

**Specific expression of transgenic IKKα in skeletal muscle**

Alternative NF-κB signaling has been implicated in the regulation of skeletal muscle metabolism. This is mediated through IKKα, the transcription factor RelB, and its direct transcriptional target gene $PGC-1\beta^\alpha$. These mechanisms were carefully dissected using genetic manipulation of muscle cells in culture, IKKα−/− and RelB−/− mouse models, and over expression of IKKα in mouse TA muscles with an adeno-associated virus (AAV). However, studies have yet to determine the long-term *in vivo* impact of alternative NF-κB on muscle and overall metabolic health.

To better understand the role of skeletal muscle alternative NF-κB signaling *in vivo*, we generated a transgenic mouse model that would express IKKα specifically in mature skeletal muscle. The DNA construct integrated into the transgenic model utilized a human skeletal actin (HSA) promoter to drive HA-tagged IKKα expression in skeletal muscle fibers (Figure 1A). Expression was verified in culture using C2C12 myoblasts and myotubes, and HeLa non-muscle cells transfected with either an empty DNA vector or the HSA-IKKα expression plasmid (Figure 1B). As we predicted, expression of the HA-tagged
IKKα was specific to the C2C12 cell line and dramatically increased upon C2C12 differentiation to myotubes, consistent with a promoter that drives expression in skeletal muscle fibers.

To translate this into an *in vivo* system, we generated transgenic mice by integrating the IKKα construct into the genomes of FVB/N mice. Lines of transgenic mice derived from independent zygote injections were then genotyped for integration of the expression plasmid and tested for expression of the HA-tagged IKKα transgene. Transgene expression was specific to the quadriceps (Quad), TA, and gastrocnemius (Gastroc) skeletal muscles, while absent from non-skeletal muscle tissues (Figure 1C). When we compared independent transgenic lines for IKKα expression, we found a wide range of expression levels, enabling us to analyze relatively low and high expressing transgenic mice (Figure 1D). The following data was generated with females of the relatively high expressing Line #6, which was selected in order to amplify phenotypic differences with wild type littermates and because we observed no detrimental effects of excessive IKKα expression on muscle or overall mouse health. Together, these data demonstrate that we generated multiple transgenic mouse lines, each expressing IKKα specifically in skeletal muscle.

**IKKα does not induce muscle atrophy**

Whereas alternative NF-κB signaling has been identified as an enhancer of mitochondrial biogenesis, classical NF-κB signaling has been implicated in muscle atrophy\(^1,10^5\). Since these two pathways have crosstalk mechanisms as well as some shared
signaling components, including IKKα, we wanted to determine whether IKKα over expression *in vivo* causes muscle atrophy. We therefore stained TA cross sections of 12-week old littermates with hematoxylin and eosin (H&E) and measured fiber cross sectional area (Figure 2A). On average, there was no difference between the fiber sizes of wild type and IKKα transgenic mice (Figure 2B). In contrast to our transgenic IKKα mouse model, we also utilized a tamoxifen inducible, constitutively active IKKβ expressed specifically in skeletal muscle fibers. As we would expect from this positive control, we observed substantial muscle atrophy (Figure 2C, 2D). Significantly, the IKKα transgenic mice also had a normal distribution of fiber sizes, consistent with healthy muscle (Figure 2E). There was also no significant difference in the overall body weight of wild type and IKKα transgenic mice, which would be expected if there was systemic muscle atrophy (Figure 2F). In addition to the anatomical features of muscle atrophy, we also measured grip strength of the transgenic mice and found no functional decline in their skeletal muscle (Figure 2G). These results suggest that over expression of IKKα, unlike IKKβ, does not cause muscle atrophy, and muscle atrophy would not be an extraneous factor in our other analyses of the IKKα transgenic mice.

**Transgenic IKKα selectively induces a slow myofiber phenotype**

We then wanted to determine whether transgenic IKKα expression *in vivo* is sufficient to induce a slow, oxidative myofiber phenotype. Gene expression results from TA muscles of 5-day old transgenic pups compared to wild type littermates revealed higher
mRNA levels of mitochondria related genes, including *PGC-1α, PGC-1β, COX5a*, and *cytochrome C* (Figure 3A). However, western blot analysis of respiratory chain proteins NDUFS3 and SDHA, associated with mitochondria respiratory chain complexes I and II, respectively, indicated no difference between wild type and transgenic mouse littermates at postnatal day 5 (Figure 3B). We also examined older transgenic mice, expecting to find a more pronounced effect of transgenic IKKα on myofiber phenotype over time. However, similar analyses of mRNA and protein expression at 12-weeks of age revealed no significant differences between skeletal muscles of wild type and transgenic mice (Figure 3C, 3D).

Given the gene expression patterns, we were surprised to find that 12-week old transgenic mice had elevated concentrations of mtDNA compared to wild type littermates (Figure 3E). This would suggest that, although there are limited differences in the expression of genes involved in respiratory chain activity, IKKα expression positively affects mitochondrial biogenesis, albeit in a limited capacity. Since increased mitochondrial biogenesis is associated with a slow muscle fiber phenotype, we explored whether there was a change in myofiber composition. When we used immunohistochemistry to stain TA cross-sections for slow muscle fibers, we found an increase in the number of slow fibers (Figure 3F, 3G). These results complement the observed increase in mtDNA content and suggest that IKKα has some capacity to affect muscle oxidative metabolism.
Transgenic IKKα in skeletal muscle affects metabolic health

In addition to examining the role of IKKα expression in individual skeletal muscles, we wanted to explore the overall metabolic health of the transgenic mice. We therefore utilized a Comprehensive Lab Animal Monitoring System (CLAMS). Since we had observed differences in mtDNA and fiber type, but not respiratory chain gene expression, we were initially surprised to find that the transgenic mice consumed significantly more oxygen than their wild type littermates (Figure 4A). However, when CO₂ production was factored in, we found no significant impact of IKKα transgene expression on the respiratory exchange ratio (Figure 4B). Rather than an overall shift from glycolysis towards oxidative phosphorylation, the increased oxygen consumption by the transgenic mice was due to higher ambulatory activity (Figure 4C).

We also examined the ability of IKKα transgenic mice to maintain glucose homeostasis, since diabetes has been associated with perturbed muscle metabolism. Glucose tolerance tests indicated that the transgenic mice did not have a significantly improved or impaired ability to resolve excess glucose from the bloodstream (Figure 4D). Together, our results suggest that skeletal muscle IKKα promotes some aspects of oxidative metabolism and may more broadly improve overall health by promoting activity.

2.4 Discussion

Disease conditions such as cancer cachexia, sarcopenia, and chronic heart failure cause skeletal muscle atrophy. This occurs preferentially in fast glycolytic fibers, while
more oxidative Type I fibers remain relatively healthy. Evidence implicating alternative NF-κB signaling in the regulation of skeletal muscle oxidative metabolism suggested this pathway could serve a protective role in these disease conditions. Developing an \textit{in vivo} model of alternative NF-κB activation in skeletal muscle would allow us to understand the role of this pathway in general health and homeostasis, and whether alternative NF-κB could be a useful therapeutic target for certain types of muscle atrophy.

One of our first observations of the IKKα transgenic mice was their healthy physical appearance and normal behavioral patterns. More rigorous analysis of their skeletal muscles compared to those of wild type littermates revealed nearly identical fiber sizes and no functional decline in grip strength, indicating that substantial over expression of IKKα does not induce muscle atrophy. Conflicting studies in recent years had suggested that IKKα and IKKβ are each sufficient to cause muscle atrophy through redundant roles in the IKK complex\textsuperscript{112}. One particular study electroporated a plasmid expressing constitutively active IKKα in soleus muscle and found that it was significant muscle atrophy\textsuperscript{112}. However, our data suggest this is not the case. In addition to our transgenic IKKα mouse model, we generated a tamoxifen inducible, constitutively active IKKα mouse. When constitutively active IKKα was expressed in skeletal muscle, we did not observe any atrophy (data not shown). Together, these data enrich our understanding of NF-κB signaling in skeletal muscle by identifying a link between IKKβ and muscle atrophy.
that is distinct from IKKα. The signaling distinction between IKKα and IKKβ is also significant for other studies examining the NF-κB pathway.

When analyzing skeletal muscles of the IKKα transgenic mice, we were surprised to find that IKKα affected only select aspects of muscle metabolism. Published experiments utilizing AAV to over express IKKα in skeletal muscle at postnatal day 2 showed significant increases in mtDNA content, slow Type I fibers, and mitochondrial gene expression, including PGC-1β and proteins of the respiratory chain. In contrast, our IKKα transgenic mice, which begin expressing IKKα as early as embryonic day 9.5, had limited mitochondrial gene expression increases on the mRNA level and no difference in respiratory chain protein expression. The IKKα transgenic mice did, however, develop elevated amounts of mtDNA and Type I fibers. Since an increase in Type I fibers typically corresponds with more oxidative metabolism, it was interesting to observe an apparent disconnect between these characteristics. If indeed there is compromised energy metabolism in the IKKα transgenic muscles, it would seem that the mice would be more susceptible to fatigue and therefore not exhibit increased ambulation. Further analysis of the contractile properties and mitochondrial functional capacity of these muscles would enhance our understanding of how these changes enrich or impair muscle function.

The switch towards Type I fibers without increased respiratory chain proteins suggests these phenotypic characteristics are not necessarily coupled, and IKKα regulates a subset of signals important for determining muscle fiber type. The downstream targets of IKKα or its signaling crosstalk could elucidate mechanisms of fiber type specification.
Some of the signals associated with fiber type specification include AMPK\textsuperscript{114}, PGC-1α\textsuperscript{38}, Baf60c\textsuperscript{115}, Fnip1\textsuperscript{116}, and Tbx15\textsuperscript{117}, though these determinations are often made without a mechanistic understanding of how they specify fiber type. Therefore, the signaling pathway and genetic targets of IKKα strengthen our limited understanding of how fiber types are determined.

As the IKKα transgenic mice matured from postnatal day 5 neonates to adults, the enhanced mitochondrial gene expression seen at the RNA level dissipated. One gene whose expression appeared lower in the IKKα transgenic adults compared to wild type was PGC-1α, a master regulator of mitochondrial biogenesis recognized for its role in adapting oxidative metabolism in response to stimulatory conditions such as exercise and cold. This result suggests that reduced PGC-1α expression is modulating respiratory chain gene expression in the adult IKKα transgenic mice, and regulators of PGC-1α transcription are signaling through PGC-1α to adjust respiratory chain gene expression to physiologically wild type levels. In addition, even though PGC-1β is a downstream target of alternative NF-κB signaling, PGC-1β expression is not elevated in adult IKKα transgenic mice, suggesting that a similar transcriptional repression mechanism may be targeting both PGC-1α and PGC-1β. Significantly, the IKKα transgenic mice still developed slow Type I muscle fibers, suggesting that IKKα, perhaps through RelB, has additional gene targets beyond PGC-1β that stimulate an oxidative muscle phenotype.
Figure 1: Specific expression of transgenic IKKα in skeletal muscle

A. Diagram of the DNA construct used to generate transgenic mice, including the human skeletal actin promoter (HSA), HA tag, and IKKα gene. 

B. C2C12 or HeLa cells were transfected with empty pcDNA3 plasmid or plasmid containing the HA-tagged IKKα transgene. C2C12 cells were then maintained in growth media (GM) or cultured in differentiation media (DM) to form myotubes.

C. Tissues from a transgenic mouse were harvested and examined by western blot for HA expression. 

D. TA muscle tissue from 12-week old wild type and independent lines of transgenic mice were homogenized and probed for IKKα expression.
Figure 2: Transgenic IKKα does not cause muscle atrophy

Continued
Figure 2 continued

A. Sections of TA muscle from wild type and transgenic littermates were stained with H&E. B. Average fiber sizes from 12-week old wild type and transgenic mice were calculated from TA sections stained by H&E (n=4). C. Sections of TA muscle from control and constitutively activated IKKβ muscles were stained by H&E (n=4). C. Sections of TA muscles from mice containing a control or muscle specific, inducible, constitutively active IKKβ were stained by H&E. D. Average fiber sizes from control and constitutive IKKβ muscles were measured (n=4). E. Fiber size measurements comparing wild type and IKKα transgenic mice are displayed as a percent of the total fiber population (n=4). F. Body weight of wild type and transgenic 12-week old littermates was measured (n=8). G. Grip strength of 12-week old mice was measured (n=6). *, p < 0.05.
Figure 3: IKKα selectively induces a slow myofiber phenotype

A. RNA was harvested from TA muscles of 5-day old wild type and transgenic littermate pups, followed by quantitation by real time qRT-PCR (n=4). B. Protein from 5 day old pups was analyzed by western blot. C. RNA was harvested from TA muscles of 12-week old wild type and transgenic littermates, followed by quantitation by real time qRT-PCR (n=4). D. Protein from 12-week old mice was analyzed by western blot. E. Total DNA was isolated from TA muscles of wild type and transgenic littermates. Mitochondrial DNA and nuclear DNA were quantitated by real time PCR. Mitochondrial DNA is normalized to nuclear DNA (n=4). F. Cross sections of TA muscles were stained by immunohistochemistry for slow myosin heavy chain. G. Fibers positive for slow myosin heavy chain staining were quantified from stained TA muscle sections. Positive fibers per TA section were calculated for wild type and transgenic littermates (n=5). *, p < 0.05.
Figure 4: Transgenic IKKα in skeletal muscle increases ambulation

A. Wild type and transgenic littermates were housed in a CLAMS and O₂ consumption was measured (n=8). B. Respiratory exchange ratio were calculated from CLAMS (n=8). C. Ambulation was measured from wild type and transgenic mice in the CLAMS (n=8). D. A Glucose tolerance tests were performed on 12-week old wild type and transgenic mice (n=8). *, p < 0.05.
CHAPTER 3

MYOD REGULATES SKELETAL MUSCLE OXIDATIVE METABOLISM
COOPERATIVELY WITH ALTERNATIVE NF-κB

3.1 Introduction

Skeletal myogenesis involves the fusion of proliferating myoblasts into multinucleated, contractile myotubes. At the molecular level, myogenic differentiation is controlled through a highly conserved family of skeletal muscle specific transcription factors including MyoD, Myf5, myogenin, and MRF4. These factors function as heterodimers with E protein subunits that bind to E box consensus sites within promoters and enhancers of genes involved in proliferation, migration, fusion, and contraction. MyoD in particular is important for postnatal myoblasts to progress through differentiation to form adult skeletal muscle.

As myoblasts differentiate, MyoD binding increases on a multitude of genes, including those that form the contractile apparatus. Studies performed on the myosin heavy chain, skeletal α-actin, and troponin promoters demonstrated that MyoD can mediate transcriptional activation of these myofibrillar genes through its recruitment of
chromatin remodeling factors\textsuperscript{24,123,124}. Expression of these genes is vital to the development and maintenance of myofiber contractile function. Consistent with this requirement, deletion of MyoD in mice weakens muscle contraction\textsuperscript{125}.

In addition to the development of contractile structures, myogenesis is associated with a shift in metabolism from glycolysis to oxidative phosphorylation, including a corresponding increase in mitochondrial biogenesis\textsuperscript{126}. This metabolic shift is presumably required to synthesize the large pool of ATP needed to sustain the energy demands of contracting muscle. Members of the PPAR\(\gamma\) coactivator-1 (PGC-1) family, mainly PGC-1\(\alpha\) and PGC-1\(\beta\), interact with and stimulate the transactivation function of NRF-1, NRF-2, PPAR\(\alpha\), PPAR\(\delta\), and ERR\(\alpha\) to enhance mitochondrial content and the oxidative capacity of muscle\textsuperscript{127}. Consequently, transgenic expression of either PGC-1\(\alpha\) or PGC-1\(\beta\) in myofibers increases mitochondrial biogenesis and oxidative phosphorylation\textsuperscript{38,39}.

Our laboratory recently implicated the alternative NF-\(\kappa\)B signaling pathway in the regulation of PGC-1\(\beta\)\textsuperscript{78}. In contrast to canonical NF-\(\kappa\)B signaling, the alternative pathway is regulated by an I\(\kappa\)B kinase \(\alpha\) (IKK\(\alpha\)) homodimer complex, which phosphorylates the p100 precursor protein, resulting in its partial proteolysis and formation of the mature p52 subunit of NF-\(\kappa\)B\textsuperscript{128}. The p52 subunit forms a heterodimer with RelB, which then translocates to the nucleus to bind NF-\(\kappa\)B consensus binding sites and stimulate transcription. We found that RelB binds within the first intron of the PGC-1\(\beta\) gene, which is sufficient to activate PGC-1\(\beta\) transcription and promote mitochondrial biogenesis and oxidative phosphorylation in skeletal muscle.
During our exploration into the metabolic regulation of skeletal muscle, we were intrigued to discover through chromatin immunoprecipitation sequencing (ChIP-seq) that MyoD binds to numerous metabolic genes, including PGC-1β. Through genetic manipulation of cultured muscle cells and in vivo skeletal muscle, we have uncovered that MyoD is a regulator of oxidative muscle metabolism. Furthermore, we reveal that MyoD regulation of metabolic genes such as PGC-1β depends on cooperative activity with the alternative NF-κB transcription factor RelB via chromatin remodeling. These data thereby establish a novel regulatory link between MyoD, alternative NF-κB, and mitochondrial oxidative metabolism.

3.2 Materials and Methods

Materials. Antibodies against MyoD (sc-304), RelB (sc-226), and PGC-1β (sc-67286), were obtained from Santa Cruz Biotechnology, Inc., PGC-1α (ab54481) from Abcam, IKKα (IMG-136A) from Imgenex, phosho Ser2 Polymerase II (MMS-129R) from BioLegend, Trimethyl-Histone H3 Lys4 (17-614) from EMD Millipore, and α-tubulin (T5168) from Sigma-Aldrich. Antibodies against Complex I subunit NDUFS3 (459130), Complex II subunit Fp (459200), and Complex III subunit Core 1 (459140) were obtained from Invitrogen. siRNAs for IKKα, RelB, MyoD, and PGC-1β were obtained from Thermo Scientific. Basic human FGF (G5071) was purchased from Promega, Insulin (I0516), gelatin (G1393), doxycycline (D9891), FCCP (C2920), and hyaluronidase (H4272) from Sigma-Aldrich, and collagenase P (11249002001) and dispase
(04942078001) from Roche. pGIPZ and pTRIPZ plasmids were obtained from Thermo Scientific. pLenti-CMV-rtTA3-Blast (plasmid #w756-1) and pX330-U6-Chimeric_BB-CBh-hSpCas9 (plasmid #42230) were obtained through Addgene.

**Cell culture.** C2C12 murine myoblasts were cultured in high-glucose DMEM containing 10% fetal bovine serum and antibiotics as previously described\(^\text{104}\). Cells were transfected with Lipofectamine 2000 (Invitrogen, 11668-019) following the manufacturer’s instructions. C2C12 myoblasts were differentiated in high-glucose DMEM supplemented with 2% horse serum, 100 ng/mL insulin, and antibiotics. Primary murine myoblasts were isolated from embryonic day 2 neonates as previously described\(^\text{129}\) and differentiated in DMEM supplemented with 10% FBS. Human primary skeletal muscle myoblasts were purchased from Lonza (CC-2580) and cultured according to their recommended protocols.

**Bioinformatics and statistical analysis.** hMyoD (Accession: GSM1218850) raw data were downloaded from a public database and aligned to hg19 using Bowtie 2. Only uniquely alignable reads were kept and all duplicates removed. HOMER was used for peak calling, annotation, and visualization. HOMER assigns peaks to the gene with the nearest transcriptional start site. For ChIP-seq visualization, BED files were uploaded to the UCSC database\(^\text{130}\). hRelB (Accession: GSM1329661) peak files were provided by Dr. Benjamin E. Gewurz\(^\text{80}\). HOMER was used to annotate peaks and create a visualization file. We then identified overlapping genes between these ChIP-seq datasets and a previously published
microarray dataset comparing wild type and $RelB^{-/}$ soleus muscle. Functional categories for these genes were identified using Toppgene. HOMER was used for annotation and gene ontology analysis of MyoD ChIP-seq data derived from C2C12 myotubes.

Quantitative data are presented as mean ± SEM. Comparisons between two different conditions were assessed using two-tailed Student’s t-test and comparisons between 4 different conditions were analyzed using a two-way ANOVA. Statistical significance was evaluated as $p < 0.05$.

**Chromatin immunoprecipitation.** ChIP analysis of C2C12 cells and tissues was performed as previously described with 2 μg antibody per sample. 8-week old mice were used for tissue ChIPs. Each ChIP assay included an IgG control experiment in parallel. Results expressed as Fold Enrichment represent pulldown of the experimental antibody relative to the pulldown of the IgG control.

**Electroporation.** Six-week old mice were first injected in the TA muscle with 15 μL of 2 mg/mL hyaluronidase, which improves transfection efficiency. After 50 minutes, mice were then injected with 30 μg plasmid DNA or 5 μg siRNA. Electroporation was performed using a BTX Electro Square Porator ECM 830 at 100 V/cm. Mice were anesthetized with isoflurane throughout the procedure.
**Gene expression analysis.** Total RNA was isolated using TRIZOL (Life Technologies, 15596081), followed by cDNA synthesis with M-MLV reverse transcriptase (Invitrogen, 28025). Real-time RT-PCR was performed using SYBR green (Roche, 04913914001). Samples were normalized using β-actin as a housekeeping gene.

**Analysis of mitochondria and respiration.** Seahorse XF®24 analysis of C2C12 cells to measure basal and maximal OCR was performed as suggested by the manufacturer. Comparisons between C2C12 myoblasts and myotubes were normalized to total myonuclei number. Cellular ATP concentration was quantified in C2C12 cells using the CellTiter-Glo Luminescent Cell Viability kit (Promega, G7570).

OCR of muscle tissue was measured using the Seahorse XF24 Islet Capture Microplates (Seahorse Bioscience, 101122-100). Tissues were dissected into 1.5 mm cross-sections and incubated for 1 hour in DMEM supplemented with 10 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate. Basal OCR measurements were obtained immediately prior to addition of FCCP. Following OCR measurements, tissues were dried at 60°C for 48 hours. All tissue OCR measurements were then normalized to dry tissue weight.

Citrate synthase activity was measured from TA muscles 4 weeks after electroporation with pGIPZ control or pGIPZ-shMyoD plasmid following the protocol reported by Spinazzi et al. Briefly, muscle homogenate was incubated with Acetyl CoA,
oxaloacetic acid, and DTNB, followed by spectrophotometry measurements at 412 nm for 3 minutes.

**Metabolic flux analysis.** Fatty acid β-oxidation flux was determined by measuring the detritiation of [9,10-\(^3\)H]-palmitic acid\(^{133,134}\). C2C12 dox-treated TRIPZ-shMyoD or TRIPZ-shControl myotubes were suspended in 0.5 mL fresh media. In parallel, a duplicate set of cells were pre-incubated with 100 μM etomoxir (Sigma, E1905), an inhibitor of carnitine palmitoyltransferase I (CPT1), for one hour. The experiment was initiated by adding 3 μci [9,10-\(^3\)H]-palmitic acid complexed to 5% BSA (lipids free; Sigma) and, 2 hours later, media was transferred to a 1.5 mL microcentrifuge tube containing 50 μL 5N HCl. The tubes were then placed in 20 mL scintillation vials containing 0.5 mL water, with the vials capped and sealed. \(^3\)H\(_2\)O was separated from non-metabolized [9,10-\(^3\)H]-palmitic acid by evaporation diffusion for 24 hours at room temperature. Mitochondria-dependent β-oxidation rate was determined by the difference between the oxidation rate in the absence or in the presence of CPT1 inhibitor.

TCA cycle flux was determined by the rate of \(^{14}\)CO\(_2\) released from [2-\(^{14}\)C]-pyruvate\(^{135}\). C2C12 dox-treated TRIPZ-shMyoD myotubes or TRIPZ-shControl myotubes were suspended in 1 mL fresh media. To facilitate the collection of \(^{14}\)CO\(_2\), cells were dispensed into 7 mL glass vials (Thermo, TS-13028) each containing a 0.5 mL PCR tube with 50 μL 0.2M KOH glued to the sidewall. After adding 0.5 μci [2-\(^{14}\)C]-pyruvate, the vials were capped using a screw cap with rubber septum (Thermo, TS-12713). The assay
was stopped 2 hours later by injection of 100 μL 5N HCl and the vials were kept at room temperate overnight to trap the \(^{14}\)CO\(_2\). The 50 μL KOH in the PCR tubes were then transferred to scintillation vials containing 10 mL scintillation solution for counting. A cell-free sample containing 0.5 μci \([2-^{14}\text{C}]\text{-pyruvate}\) was included as background control.

**CRISPR design and screening.** Guide RNA sequences were identified using a publicly available resource developed by Feng Zhang’s lab at Broad Institute of MIT and Harvard (http://crispr.mit.edu/). Sequences 72 bp apart were identified on either side of the NF-κB site located approximately 40 kb downstream from the transcriptional start site. Similarly, sequences 150 bp apart were identified on either side of MyoD site 9 of the \(PGC-1\beta\) gene. Sequences were then cloned into the pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid and transfected into C2C12 cells. Individual cell clones were grown and screened by PCR for the specific deletion.

**Mice.** Animals were housed in the animal facility at The Ohio State University Comprehensive Cancer Center, fed a standard diet, and kept at constant temperature and humidity. Treatment of mice was in accordance with the institutional guidelines of the Institutional Animal Care and Use Committee. \(MyoD^{+/}\) mice (stock #002523) were obtained from The Jackson Laboratory.
Nuclease Accessibility Assay. A restriction enzyme accessibility assay utilizing ligation-mediated PCR was performed as previously described with the following modifications. Briefly, cells were collected and incubated on ice for 5 minutes in CE buffer (10 mM HEPES pH 7.6, 60 mM KCl, 1 mM EDTA) supplemented with 0.25% NP40. Isolated nuclei were then washed with CE buffer and digested in NEB Buffer 3.1 and 0.2 U/µg PvuII for 8 minutes at 37°C. DNA from the reaction was purified with a Qiagen DNeasy purification kit. DNA was subsequently ligated and quantitated as previously described.

3.3 Results

MyoD binds to a network of metabolic genes

MyoD is a master regulator of muscle differentiation with several important functions attributed to its target genes. Most notably, MyoD regulates expression of myofibrillar genes that form the sarcomere and facilitate muscle contraction. We were interested in what other cellular processes could be regulated by MyoD. To explore this, we analyzed MyoD ChIP-seq data from murine C2C12 myotubes. As expected, MyoD binding was strongly associated with muscle contraction and differentiation (Figure 5A).

However, the top biological processes associated with MyoD binding were unexpectedly related to metabolism, a surprising finding since the regulation of muscle metabolism has not been directly linked to MyoD (Figure 6A). Gene ontology (GO) categories related to oxidative metabolism, including mitochondrial biogenesis, fatty acid metabolism, and the TCA cycle, were prevalent throughout the analysis (Figure 6B). The
A wide range of functions found by GO analysis was supported by our finding that MyoD binds to specific target genes implicated in mitochondrial biogenesis, fatty acid oxidation, mitochondrial fission, electron transport, and mitochondrial protein translation (Figure 6C). As a reference and positive control, MyoD was also found to bind to the myogenin gene (Figure 5B). These results imply that MyoD directly regulates a broad collection of metabolic genes involved in multiple aspects of mitochondrial respiration.

**MyoD regulates skeletal muscle oxidative metabolism**

To examine whether MyoD is a bona fide regulator of oxidative metabolism, we first generated a C2C12 myoblast cell line containing a doxycycline (dox)-inducible shRNA targeting MyoD (TRIPZ-shMyoD). When C2C12 TRIPZ-shMyoD cells were differentiated into mature myotubes and subsequently treated with dox, MyoD levels declined over time. In contrast, no change in MyoD expression was observed in a separately generated C2C12 cell line expressing a dox-inducible scrambled shRNA (TRIPZ-shControl) (Figure 7A). To assess if MyoD knockdown had a functional consequence in these myotubes, we measured their oxygen consumption rate (OCR) using the Seahorse Bioscience XF24 (as illustrated in Figure 7B). We found that C2C12 TRIPZ-shMyoD myotubes treated with dox had a significantly lower OCR than dox-treated TRIPZ-shControl myotubes (Figure 7C). Furthermore, treatment of these myotubes with the mitochondrial uncoupling agent carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), which produces maximal oxygen consumption, revealed an
even greater difference in OCR between control and shMyoD myotubes (Figure 7C). These results suggested that MyoD is required to maintain mitochondrial respiratory capacity. Significantly, the reduced respiration of TRIPZ-shMyoD myotubes was associated with reduced ATP content, indicating the functional importance of this decreased respiration (Figure 8A).

To better understand the role of MyoD in mitochondrial metabolism, we measured TCA cycle activity and fatty acid β-oxidation in dox-treated C2C12 TRIPZ-shControl and TRIPZ-shMyoD myotubes by using radiolabeled substrates [2-14C] pyruvate and [9,10-3H] palmitic acid (Illustrated in Figure 7D and Figure 7E). Compared to control myotubes, silencing MyoD reduced both TCA cycle activity and fatty acid β-oxidation (Figure 7D and Figure 7E). These results, along with the OCR analyses, further support that MyoD is required to maintain normal mitochondrial metabolism in skeletal muscle.

To assess the metabolic significance of MyoD in vivo, we adapted the Seahorse XF24 to monitor metabolism of intact, non-permeabilized muscle tissue (Figure 7B). To test the validity of using this platform in measuring muscle tissue respiration, we first measured basal OCR from tibialis anterior (TA) muscles. Following the addition of titrating concentrations of FCCP, we observed stimulated OCR measurements (Figure 8B), as would be expected from a mitochondrial uncoupling agent. To then confirm that the Seahorse platform could effectively contrast OCR between intact muscle tissues with known differences in oxidative capacity, we tested this assay on slow twitch soleus muscles in comparison to fast twitch extensor digitorum longus (EDL) muscles. As anticipated,
OCR values were higher in the more oxidative soleus muscle (Figure 8C). In addition, FCCP treatment effectively stimulated the OCR of both soleus and EDL muscles (Figure 8C). These OCR measurements from tissues were performed on a multi-well plate, which allowed for simultaneous measurements from 3 EDL muscles and 3 soleus muscles divided into 10 total tissue sections per muscle group. These results demonstrate that the Seahorse XF24 platform could reliably be used to measure and contrast the respiration of intact skeletal muscle tissue.

In an effort to identify if MyoD has a functional significance in adult muscle, we examined TA muscles of MyoD−/− mice and found that the lack of MyoD was associated with reduced basal OCR compared to wild type littermates (Figure 7F). Furthermore, stimulation of these tissues with FCCP revealed an accentuated difference in maximal OCR, indicating reduced mitochondrial respiration capacity in MyoD−/− muscles. To circumvent potential metabolic differences due to the absence of MyoD during embryogenesis and postnatal muscle development in MyoD−/− mice, we electroporated adult wild type murine TA muscle with either a pGIPZ plasmid constitutively expressing an shRNA targeting MyoD (pGIPZ-shMyoD) or a pGIPZ control plasmid. After a four-week post-transfection period, we were able to achieve a fiber transfection efficiency of nearly 60% (Figure 8D). Western blot analysis showed that efficient knockdown of MyoD corresponded with significantly lower basal and maximal OCR (Figure 7G). As an additional functional endpoint, we assessed the enzymatic activity of citrate synthase, a critical component of the TCA cycle. Consistent with the TCA cycle metabolic activity
measured in C2C12 myotubes silenced for MyoD (Figure 7D), analysis of electroporated tissues depleted of MyoD revealed significantly less citrate synthase activity (Figure 7H). Furthermore, electroporation of pGIPZ-shMyoD in TA muscle led to a pronounced reduction in the mRNA or protein expression of mitochondrial genes, including ATP synthase lipid-binding protein (ATP5γ), cytochrome c oxidase subunit Va (COX5a), cytochrome c (Cyto C), mitochondrially encoded cytochrome c oxidase I (MTCO1), NADH dehydrogenase-ubiquinone 30 kDa subunit (NDUFS3), succinate dehydrogenase complex subunit A flavoprotein (SDHA), and ubiquinol-cytochrome c reductase core protein I (UQCRC1) (Figure 7I and Figure 8E). Such differences in mitochondrial gene products strongly support that MyoD functions in skeletal muscle as a regulator of mitochondrial biogenesis and oxidative metabolism.

**MyoD binds to the PGCG-1β gene**

During muscle differentiation, respiration increases, along with PGCG-1β gene expression and to a lesser extent PGCG-1α (Figure 9A, 9B). To gain insight into the mechanism by which MyoD directly regulates respiration, we examined ChIP-seq data of C2C12 cells at the PGCG-1α and PGCG-1β gene loci, since these genes are established master regulators of mitochondrial biogenesis. In C2C12 myoblasts, MyoD binds multiple sites along the PGCG-1β gene with additional peaks bound in differentiated myotubes (Figure 9C). This was in sharp contrast to the PGCG-1α gene, which surprisingly showed little to no evidence of MyoD binding in both myoblasts and myotubes (Figure 10).
Further ChIP-seq analysis of the *PGC-1α* gene locus revealed that RNA polymerase II (Pol II) binding was absent in myoblasts and myotubes, suggesting that differentiation is not sufficient to induce *PGC-1α* transcription (Figure 10). Further inquiry revealed the combined presence of histone marks H3K4me3 and H3K27me3 in myotubes, indicative of a poised, yet transcriptionally suppressed bivalent gene (Figure 10). Interestingly, H3K27me3 is not present at the *PGC-1β* locus in myoblasts, suggesting this bivalent chromatin structure uniquely regulates the induction of *PGC-1α* expression in differentiated muscle. Consistent with this, a repressive H3K27me3 mark was also found on the *PGC-1α* gene of myotubes when an independent ChIP-seq data set was used (Accession: GSM818949). In sharp contrast, the *PGC-1β* gene had substantially more Pol II binding across its gene locus, H3K4me3 at its promoter, and an absence of H3K27me3, all indicative of a transcriptionally active gene in myotubes (Figure 11A). These results highlight distinct differences in the transcriptional regulation of these co-activators during skeletal muscle differentiation.

Since MyoD binding increases at the *PGC-1β* locus during muscle differentiation, coincident with increased *PGC-1β* expression, we sought to determine whether MyoD directly regulates *PGC-1β* transcription. We began by validating the MyoD ChIP-seq data with a series of ChIP reactions to assess MyoD binding during C2C12 differentiation. Using the myogenin promoter as a positive control for MyoD binding, results confirmed that MyoD occupies multiple sites along the first two introns of the *PGC-1β* gene (Figure 9D). While MyoD was bound to some of these sites with varying intensities in myoblasts,
the binding at sites 2, 9, and 12 significantly increased upon differentiation, coincident with increased PGC-1β transcription. These results were confirmed in primary muscle cells where, similar to C2C12 cells, MyoD binding increased during myogenesis (Figure 11B). MyoD binding at these 3 sites was then confirmed in vivo. ChIP assays in adult TA muscle tissue demonstrated that MyoD was significantly bound at sites 2, 9, and 12 of the PGC-1β gene locus in mature muscles (Figure 9E). The binding of MyoD to the myogenin promoter was used as a positive control for primary myotubes as well as TA muscles. Together, these results demonstrate that MyoD binding to the PGC-1β gene increases during myogenesis at three particular likely enhancer sites and remains bound in adult skeletal muscle.

**MyoD is a direct regulator of PGC-1β transcription**

To understand whether MyoD is important for promoting PGC-1β expression, we measured PGC-1β expression in MyoD-/- mice. Compared to wild type littermates, both PGC-1β mRNA and protein were reduced in MyoD-/- TA muscles (Figure 12A, 12B). This difference was not a developmental characteristic of the MyoD-/- mice since a similar reduction of PGC-1β expression was observed following the knockdown of MyoD in adult wild type TA muscles (Figure 12C, 12D).

We then wanted to determine whether the positive correlation between MyoD and PGC-1β expression is due specifically to MyoD binding on the PGC-1β gene. We utilized a CRISPR/Cas9 genome editing strategy to remove a 150 bp region of the PGC-1β gene
containing MyoD site 9 (ΔMyoD9\textsuperscript{CRISPR}) in C2C12 myoblasts (Figure 12E). ChIP analysis confirmed that ΔMyoD9\textsuperscript{CRISPR} led to the elimination of MyoD binding at the site 9 locus, but binding at sites 2 and 12 were unaffected (Figure 12F). Significantly, MyoD binding at the myogenin promoter was also not affected in C2C12 ΔMyoD9\textsuperscript{CRISPR} myotubes (Figure 13A), indicating that this genomic deletion did not impair MyoD binding at a known MyoD target gene besides PGC-1β. Compared to control C2C12 myotubes, ΔMyoD9\textsuperscript{CRISPR} myotubes expressed significantly less PGC-1β mRNA and protein (Figure 12G, 12H). Furthermore, ChIP experiments detected significantly less Pol II phospho Ser2 (P-Pol II) on the PGC-1β gene locus of ΔMyoD9\textsuperscript{CRISPR} myotubes (Figure 12I). Collectively, these data suggest that MyoD regulates PGC-1β transcription through direct DNA binding.

**MyoD and alternative NF-κB signaling cooperatively regulate PGC-1β**

Our previous findings demonstrated that PGC-1β is transcriptionally regulated by the alternative signaling pathway of NF-κB\textsuperscript{78}. Specifically, this occurs through two RelB binding sites located in intron 1 of PGC-1β approximately 40 and 65 kb downstream from the transcriptional start site (Figure 14A). Consistent with this binding and the role of PGC-1β in the regulation of oxidative phosphorylation, TA muscles from adult RelB\textsuperscript{-/-} mice consumed significantly less oxygen than TA muscles from wild type littermates (Figure 14B). Combined with our current results with MyoD, these findings suggested that the regulation of muscle oxidative metabolic capacity by RelB and MyoD might be linked
through their shared regulation of PGC-1β. To gain insight into this regulation, we utilized CRISPR/Cas9 to specifically delete a small 72 bp region at the first RelB binding site, 40 kb downstream of the PGC-1β transcriptional start site (ΔNF-κB\textsuperscript{CRISPR}) (Figure 14C). Deletion of this region in C2C12 cells eliminated RelB binding on the PGC-1β gene (Figure 14D). Consistent with our previous findings, C2C12 ΔNF-κB\textsuperscript{CRISPR} myotubes expressed markedly less PGC-1β than control myotubes (Figure 14E). Significantly, knockdown of MyoD in C2C12 ΔNF-κB\textsuperscript{CRISPR} myotubes led to a further reduction of PGC-1β expression (Figure 14E). Similar results with respect to PGC-1β were obtained when alternative NF-κB signaling was decreased through depletion of RelB or its upstream regulator IKKα by siRNA knockdown in combination with MyoD knockdown (Figure 15A, 15B). These data support the notion that MyoD and alternative NF-κB signaling exhibit additive activities in the regulation of PGC-1β.

Given that MyoD and RelB bind to multiple sites on the PGC-1β locus and have complementary functions, we then asked if these transcription factors have a reciprocal effect on their binding affinities to the PGC-1β gene. We began by determining whether alternative NF-κB signaling affects the ability of MyoD to bind the PGC-1β gene. Indeed, knockdown of either RelB or IKKα by siRNA restricted MyoD binding to multiple sites on the PGC-1β gene (Figure 15C, 15D). MyoD binding at the PGC-1β locus was also lower in primary myotubes as well as TA muscle tissues from RelB\textsuperscript{−/−} compared to RelB\textsuperscript{+/+} mice (Figure 15E and Figure 14F). Together, these data indicate that the alternative
signaling pathway of NF-κB promotes MyoD binding to the *PGC-1β* gene. To determine whether the ability of RelB to impact MyoD binding was directly due to its own binding on the *PGC-1β* gene, we performed MyoD ChIP experiments on C2C12 ΔNF-κB<sup>CRISPR</sup> myotubes. Impressively, MyoD binding to *PGC-1β* was profoundly reduced by the selective removal of the local RelB binding site (Figure 14G).

We then used C2C12 ΔMyoD9<sup>CRISPR</sup> myotubes to examine whether the specific removal of the MyoD enhancer binding site 9 of the *PGC-1β* gene could reciprocally affect RelB binding. Results showed that C2C12 ΔMyoD9<sup>CRISPR</sup> myotubes had dramatically less RelB binding on the *PGC-1β* gene compared to control myotubes (Figure 14H). Therefore, regulation of *PGC-1β* transcription is dependent on the mutual binding of MyoD and RelB to enhancer elements within the *PGC-1β* gene.

Since MyoD and RelB binding sites on the *PGC-1β* gene locus are not located in close proximity to one another (Figure 14A), we asked whether their ability to influence each other’s binding activity involves chromatin remodeling. To test this notion, nuclease accessibility assays were performed. Data revealed that the PvuII restriction site adjacent to MyoD binding site 9 on the *PGC-1β* gene became more susceptible to digestion upon myoblast differentiation (Figure 14I), consistent with a more transcriptionally active gene. However, deletion of MyoD binding at site 9 of *PGC-1β* resulted in less PvuII digestion, suggesting more restricted and condensed chromatin compared to control myotubes (Figure 14J). Likewise, a similar reduction in chromatin accessibility was observed in
C2C12 ΔNF-κB\textsuperscript{CRISPR} myotubes (Figure 14K). Together, our results show that \textit{PGC-1β} transcription is regulated by the cooperative binding of MyoD and RelB to the \textit{PGC-1β} gene.

**MyoD regulates oxidative metabolism through multiple gene targets**

ChIP-seq results from Figure 6 showed that MyoD binds to multiple metabolic genes, suggesting that its regulation of adult skeletal muscle oxidative metabolism extends beyond \textit{PGC-1β}. We tested this notion \textit{in vivo} by knocking down \textit{PGC-1β} alone or in combination with MyoD in TA muscles (Figure 16A). As expected, \textit{PGC-1β} knockdown alone was sufficient to reduce muscle OCR and expression of several mitochondrial genes (Figure 16B, 16C). Significantly, additional knockdown of MyoD with PGC-1β had an even more dramatic effect. These results support our bioinformatics analysis showing that MyoD regulates mitochondrial biogenesis and muscle respiration through multiple transcriptional targets including, but not limited to, \textit{PGC-1β}.

**MyoD and RelB potentially cooperate to regulate numerous mitochondrial genes**

Our findings that MyoD regulates metabolism through numerous gene targets and cooperates with RelB to regulate \textit{PGC-1β} led us to hypothesize that a similar cooperative mechanism exists between these two transcription factors on multiple metabolic genes. We therefore analyzed publicly available MyoD and RelB ChIP-seq datasets to identify genes containing binding sites for both transcription factors\textsuperscript{138}. Since RelB ChIP-seq data in
skeletal muscle was not available, we utilized human RelB ChIP-seq data from lymphoblastoid B cells and pared down annotated peaks with microarray data identifying RelB regulated genes in mouse soleus muscle\textsuperscript{78,80}. This analysis revealed a total of 206 genes containing both human MyoD and RelB binding sites (hMyoD, hRelB) whose functions are tightly linked to mitochondria. A sampling of these genes included Nuclear Respiratory Factor 1 (NRF1), Optic Atrophy 3 (OPA3), Peroxisome Proliferator-Activated Receptor delta (PPAR\(\delta\)), and Sirtuin 4 (SIRT4) (Figure 17A). Through ChIP assays for MyoD and RelB on differentiated human primary myotubes, we confirmed that MyoD and RelB co-occupy NRF1, OPA3, PPAR\(\delta\), and SIRT4 genes in skeletal muscle (Figure 17B). When we categorized the full collection of MyoD and RelB co-occupied genes into functional classes using ToppGene, we identified numerous biological processes and cellular components strongly associated with mitochondrial biogenesis and oxidative metabolism (Figure 17C). In sharp contrast, this was not true for categories of muscle differentiation and muscle contraction, suggesting that the role for MyoD in regulating sarcomeric genes and genes involved in myogenesis, does not involve cooperation with alternative NF-\(\kappa\)B signaling (Figure 17C). The cooperation of MyoD and alternative NF-\(\kappa\)B is instead required for global regulation of skeletal muscle oxidative metabolism.

3.3 Discussion

MyoD is considered a master switch transcription factor with critical roles in embryonic skeletal muscle development and the injury repair process of postnatal
In contrast, the role of MyoD in maintaining the homeostasis of adult skeletal muscle has been less appreciated. Presumably, the low basal expression of MyoD by myofiber nuclei is sufficient to synthesize contractile genes that form the sarcomere to generate force. Despite the integral role of energy metabolism in muscle function, myogenic transcription factors such as MyoD have not been directly identified to regulate mitochondrial biogenesis, although previous work describing the regulation of MyoD by circadian rhythm genes did indirectly reveal a connection to mitochondrial function. However, the fundamental mechanisms by which MyoD directly targets metabolic genes to maintain mitochondrial homeostasis have not been established. Our current findings highlight a direct role for MyoD in maintaining the oxidative metabolic capacity of homeostatic adult skeletal muscle. Our data support that in adult muscle, a basal level of MyoD expression is sufficient to transcriptionally regulate a large cohort of genes involved in mitochondrial biogenesis and aerobic respiration. Thus, we propose that MyoD is not only responsible for the generation of the sarcomeric genes involved in contractile function, but it also ensures that a sufficient pool of ATP is available to support skeletal muscle contraction.

Our data additionally suggest that MyoD regulation of oxidative metabolism occurs through the cooperation of the alternative NF-κB signaling pathway. For PGC-1β, this cooperativity directly regulates transcription, and our bioinformatics analysis revealed numerous other metabolic genes that appear to be regulated by a similar mechanism. Of
particular interest among these genes is \textit{PPAR}\(\delta\), whose function in regulating oxidative metabolism in adult skeletal muscle is well established\textsuperscript{141}. ChIP-seq analysis revealed that the \textit{PPAR}\(\delta\) gene contains multiple MyoD binding peaks, which are juxtaposed to RelB binding sites, similar to what we identified in the \textit{PGC-1}\(\beta\) gene locus. Another interesting gene was \textit{SIRT4}, which contained prominent overlapping MyoD and RelB binding peaks. From the perspective of how MyoD and RelB binding sites are positioned relative to each other on the different metabolic genes that we identified, it will be interesting to determine in future studies whether the cooperative functions between these transcription factors are manifested through distinct regulatory mechanisms.

One surprising result from the MyoD ChIP-seq data set came when we compared the \textit{PGC-1}\(\alpha\) and \textit{PGC-1}\(\beta\) loci. Although both are considered major regulators of mitochondrial biogenesis, ChIP-seq data at the \textit{PGC-1}\(\alpha\) locus showed no evidence of MyoD binding in either myoblasts or myotubes. In addition, ChIP-seq data in myotubes revealed the absence of Pol II, along with repressive H3K27me3 marks, which suggests that the induction in \textit{PGC-1}\(\alpha\) transcription during muscle differentiation might be quite modest. \textit{PGC-1}\(\beta\) expression instead appears uniquely associated with skeletal muscle differentiation\textsuperscript{142,143}, and our data demonstrating a much greater expression of \textit{PGC-1}\(\beta\) compared to \textit{PGC-1}\(\alpha\) in differentiated myotubes is consistent with these results. This regulatory distinction is significant because physiological stimuli such as cold exposure and exercise induce \textit{PGC-1}\(\alpha\) expression, but not \textit{PGC-1}\(\beta\)\textsuperscript{142}. These conclusions are consistent with our data demonstrating that knockdown of MyoD in adult skeletal muscle
decreases PGC-1β protein (Figure 12B). Interestingly, we observed that under similar conditions PGC-1α protein expression was also decreased (Figure 13B). Since MyoD binding sites were not identified in the PGC-1α gene locus, it suggests that MyoD regulates PGC-1α either though an indirect mechanism, or through other transcriptional regulatory domains that lie outside the regions we explored. Another possibility is that MyoD might impact the stability of the PGC-1α mRNA or protein. Although additional studies will be required to better define the regulation of PGC-1α and PGC-1β co-activators during skeletal myogenesis and in adult muscle, our results nevertheless add to the current understanding of the regulatory mechanisms distinguishing these co-activators, and further defines a role for PGC-1β in supporting the fundamental metabolic requirements of skeletal muscle during homeostasis.

In addition to muscle development and homeostasis, our findings may also be applicable to our understanding of rhabdomyosarcoma, which is the most common soft tissue cancer in children. Rhabdomyosarcoma tumor cells express MyoD, and the most prevalent theory regarding the development and persistence of this cancer is that tumor cells originating from skeletal muscle are unable to properly differentiate. This disparity in part results from the inability of MyoD to properly bind to and activate its target myogenic genes. Interestingly, a study examining the metabolic properties of human rhabdomyosarcoma tumors revealed a dependence on glycolysis reminiscent of the Warburg effect. Given our current results, it is intriguing to posit that loss of MyoD function not only compromises the differentiation status of rhabdomyosarcoma tumor
cells, but in addition contributes to the metabolic shift through the decline of PGC-1β and potentially other regulators of oxidative metabolism.
Figure 5: MyoD binds to genes related to muscle differentiation and contraction

A. MyoD ChIP-seq from C2C12 myotubes was annotated, followed by gene ontology enrichment analysis. B. MyoD binding was visualized by ChIP-seq at the myogenin gene.
Figure 6: MyoD binds to a network of metabolic genes

Continued
A. MyoD ChIP-seq using C2C12 myotubes was annotated followed by gene ontology analysis using HOMER. The top 10 biological processes are listed, with categories related to metabolism listed in dark blue. B. Selected biological processes and cellular components related to mitochondrial biogenesis and function are listed. C. MyoD ChIP-seq peaks are visualized on selected genes with functions related to oxidative metabolism.
Figure 7: MyoD regulates skeletal muscle oxidative metabolism

Continued
Figure 7 continued

F

Continued
Figure 7 continued

A. C2C12 cells containing a stable, dox-inducible shRNA targeting MyoD or dox-inducible scrambled shRNA were differentiated for 4 days and subsequently treated with 2 µg/mL dox for 3 days. MyoD expression was assessed by western blot. B. Diagram of Seahorse analysis for C2C12 myotubes or muscle tissue. C. C2C12 TRIPZ-shControl and TRIPZ-shMyoD myotubes were treated with dox for 3 days. Basal OCR measurements were immediately followed by addition of FCCP and measurement of maximal OCR. (n=10 per cell line) D. C2C12 TRIPZ myotubes treated with dox were provided with [2-14C] pyruvate and the resulting 14CO2 was measured as an indicator of TCA cycle activity. (n=3 per cell line) E. Similarly, TRIPZ myotubes treated with dox were provided with [9,10-3H] palmitic acid and the resulting metabolized 3H2O was measured as an indicator of fatty acid β-oxidation. (n=3 per cell line) F. Basal and maximal OCR was measured from TA muscles of 12-week old MyoD−/− mice and wild type littermates using the Seahorse XF24. Values are normalized to dry tissue weight. (n=9 samples per condition from 6 total tissues) G. Basal and maximal OCR were measured on a single multi-well plate from 3 TA muscles electroporated with pGIPZ-shMyoD and 3 TA muscles electroporated with control pGIPZ. 10 tissue sections were analyzed per experimental condition. Knockdown was assessed by western blot. H. TA muscles electroporated with pGIPZ-shMyoD or control pGIPZ were homogenized and lysate was used to measure citrate synthase activity. (n=3 muscles per condition) I. TA muscles electroporated with pGIPZ-shMyoD or control pGIPZ were assessed by western blot. *, p<0.05 relative to Control. #, p<0.05 relative to Basal OCR. Error bars indicate mean ± SEM.
Figure 8: MyoD regulates muscle metabolism in vitro and in vivo

Continued
Figure 8 continued

A. C2C12 TRIPZ-shControl and TRIPZ-shMyoD myotubes were treated with dox, followed by ATP measurement normalized to protein. (n=5 per condition) B. OCR from wild type TA muscles was measured before and after treatment with the indicated concentration of FCCP. C. Basal OCR from wild type EDL and soleus muscles was measured and normalized to tissue dry weight. FCCP was added to induce maximal OCR response from EDL and soleus. (n=9 samples per condition from 6 total tissues) D. Wild type mouse TA muscles were electroporated with pGIPZ. Four weeks later, GFP⁺ and total fibers were quantitated from muscle cross-sections. (n=6 sections from 2 tissues) E. Wild type mouse TA muscles were electroporated with control pGIPZ in one leg and shMyoD pGIPZ plasmid in its other leg. Expression of mitochondrial genes was evaluated by qRT-PCR. For each mouse, gene expression in the shMyoD leg was compared to the control leg of the same mouse. Each paired control leg was set to a relative value of 1. (n=3 per condition) *, p<0.05 relative to control or EDL. #, p<0.05 relative to Basal OCR. Error bars indicate mean ± SEM.
Figure 9: MyoD binds to the PGC-1β gene
A. OCR was measured in C2C12 myoblasts and 4-day differentiated myotubes. Values are normalized to myonuclei number. (n=10 per condition) B. mRNA was extracted from C2C12 myoblasts and myotubes and quantitated by qRT-PCR. (n=3 per condition) C. Schematic representation of the PGC-1β gene locus showing exon-intron borders and the size of intron 1 compared to the entire gene. MyoD and Pol II ChIP-seq data from C2C12 myoblasts and myotubes are aligned to the gene schematic. D. MyoD ChIP assays on C2C12 myoblasts and myotubes were performed at the myogenin promoter as a positive control and the indicated sites along the PGC-1β gene. (n=3 per condition) E. MyoD ChIP assays were performed on 6-week old murine TA muscles, with liver as a negative control. (n=3 per condition) *, p<0.05. Error bars indicate mean ± SEM.
Figure 10: The PGC-1α gene is bivalently marked and not directly induced by MyoD

Schematic representation of the PGC-1α gene locus showing exon-intron borders and the size of intron 1 compared to the entire gene. ChIP-seq visualization of MyoD, Pol II, H3K4me3, and H3K27me3.
Figure 11: The PGC-1β gene is not repressed by H3K27me3 in C2C12 cells and is bound by MyoD in primary myoblasts and myotubes

A. H3K4me3 and H3K27me3 ChIP-seq analysis on C2C12 myoblasts and myotubes. B. Primary myoblasts were isolated from mice at postnatal day 4 and cultured for 4 days. Myotubes were differentiated for 2 days prior to MyoD ChIP. (n=3 per condition) *, p<0.05. Error bars indicate mean ± SEM.
Figure 12: MyoD is a direct regulator of PGC-1β transcription

Continued
Figure 12 continued

A. mRNA was extracted and quantitated from TA muscles of \textit{MyoD}^{+/+} and \textit{MyoD}^{-/-} mice. (n=3 per condition) B. Protein extracts from TA muscles of \textit{MyoD}^{+/+} and \textit{MyoD}^{-/-} mice were analyzed by western blot. C. TA muscles from 8 week-old wild type mice were electroporated with pGIPZ-shMyoD in one leg and pGIPZ control in the other leg. RNA was extracted after 4 weeks and analyzed by qRT-PCR. Data are paired for each mouse, with expression from knockdown in each pGIPZ-shMyoD treated TA muscle compared relative to the control of the same mouse. (n=3 muscles per condition) D. Protein extracts from TA muscles electroporated with control pGIPZ or pGIPZ-shMyoD were assessed by western blot. E. PCR of C2C12 wild type and \textit{ΔMyoD}^{9\text{CRISPR}} cells demonstrating the deletion of 150 bp at the MyoD binding site 9 locus on the \textit{PGC-1β} gene. F. MyoD ChIP at the indicated sites on the \textit{PGC-1β} gene comparing C2C12 control and \textit{ΔMyoD}^{9\text{CRISPR}} myotubes. (n=3 per condition) G. RNA was extracted from C2C12 control and \textit{ΔMyoD}^{9\text{CRISPR}} myotubes and analyzed by qRT-PCR. (n=3 per condition) H. Protein from C2C12 control and \textit{ΔMyoD}^{9\text{CRISPR}} myotubes was analyzed by western blot. I. P-Pol II ChIP was performed on C2C12 control and \textit{ΔMyoD}^{9\text{CRISPR}} myotubes. (n=3 per condition) *, p<0.05. Error bars indicate mean ± SEM.
Figure 13: Removal of MyoD site 9 by CRISPR does not disrupt MyoD binding at the myogenin gene promoter

A. MyoD ChIP at the myogenin promoter was performed on 4-day differentiated C2C12 Control and ΔMyoD9CRISPR myotubes. (n=3 per condition) B. Wild type TA muscle was electroporated with control pGIPZ or pGIPZ-shMyoD. After 4 weeks, western blot was performed. The PGC-1α blot was obtained from the same western blot depicted in Figure 71, hence the same corresponding α-Tubulin blot is shown here.
Figure 14: MyoD and RelB cooperatively regulate PGC-1β transcription

Continued
Figure 14 continued
A. Gene schematic of the \( PGC-1\beta \) gene visualized with MyoD ChIP-seq data. RelB binding sites are depicted within the first intron of the gene schematic. The RelB site removed by CRISPR is indicated with an asterisk. 

B. OCR was measured from TA muscles of \( RelB^{+/+} \) and \( RelB^{-/-} \) littermates. (n=9 samples per condition from 6 total tissues) 

C. PCR comparing control and \( \Delta NF-\kappa B^{CRISPR} \) myotubes demonstrating the 72 bp deletion at the RelB binding site. 

D. ChIP for RelB was performed on C2C12 control and \( \Delta NF-\kappa B^{CRISPR} \) myotubes. (n=3 per condition) 

E. \( PGC-1\beta \) mRNA expression was compared between C2C12 control myotubes, \( \Delta NF-\kappa B^{CRISPR} \) myotubes, and \( \Delta NF-\kappa B^{CRISPR} \) myotubes transfected with MyoD siRNA. (n=3 per condition) 

F. MyoD ChIP was performed on TA muscles of 6-week old \( RelB^{+/+} \) and \( RelB^{-/-} \) littermates. (n=3 per condition) 

G. MyoD ChIP was performed on C2C12 control and \( \Delta NF-\kappa B^{CRISPR} \) myotubes at the indicated sites along the \( PGC-1\beta \) gene. (n=3 per condition) 

H. RelB ChIP on the \( PGC-1\beta \) gene comparing C2C12 control and \( \Delta MyoD9^{CRISPR} \) myotubes. (n=3 per condition) 

I. PvuII nuclease accessibility was assessed in C2C12 myoblasts and myotubes at MyoD site 9. (n=3 per condition) 

J. C2C12 control and \( \Delta MyoD9^{CRISPR} \) myotubes were compared using a PvuII nuclease accessibility assay. (n=3 per condition) 

K. Control and \( \Delta NF-\kappa B^{CRISPR} \) myotubes were also assessed by PvuII nuclease accessibility at the \( PGC-1\beta \) gene. (n=3 per condition) 

*, p<0.05 relative to Control. #, p<0.05 relative to \( \Delta NF-\kappa B^{CRISPR} \) control myotubes. Error bars indicate mean ± SEM.
Figure 15: MyoD and RelB cooperatively regulate PGCMβ transcription
A. C2C12 cells were transfected with the indicated siRNA and differentiated for 4 days. Knockdown was assessed by western blot and mRNA was quantitated by qRT-PCR. (n=3 per condition) B. C2C12 cells were transfected with the indicated siRNA, differentiated, and assessed by western blot and qRT-PCR. (n=3 per condition) C. C2C12 cells were transfected with control or RelB siRNA, differentiated for 4 days, followed by MyoD ChIP. (n=3 per condition) D. Similarly, C2C12 cells were transfected by control or IKKα siRNA followed by differentiation and MyoD ChIP. (n=3 per condition) E. Primary myoblasts were isolated from RelB+/+ and RelB–/– littermates and differentiated for 2 days prior to MyoD ChIP. (n=3 per condition) *, p<0.05 relative to Control. #, p<0.05 relative to single siRNA knockdown. Error bars indicate mean ± SEM.
Figure 16: MyoD regulates oxidative metabolism through multiple gene targets

A. TA muscles of 6-week old mice were electroporated with the either pGIPZ control or pGIPZ-shMyoD, and control or MyoD siRNA. Knockdown was assessed by western blot.

B. Basal OCR from electroporated TA muscles described in A were determined using the Seahorse XF24. (n=6 samples per condition from 9 total tissues)

C. mRNA from TA described in A was analyzed by qRT-PCR. (n=3 per condition) *, p<0.05 relative to Control. #, p<0.05 relative to PGC-1β siRNA. Error bars indicate mean ± SEM.
Figure 17: MyoD and RelB may cooperatively regulate numerous mitochondrial genes

Continued
A. ChIP-seq peaks for human MyoD and human RelB are visualized with gene diagrams depicted above peaks for \textit{NRF1}, \textit{OPA3}, \textit{PPAR\delta}, and \textit{SIRT4} genes. B. Differentiated human skeletal muscle cells were analyzed by ChIP for MyoD and RelB binding at the genes indicated. An asterisk indicates the peak in A that was chosen to confirm binding by ChIP. (n=3 per condition) C. Gene ontology analysis by ToppGene was performed on genes common to hMyoD ChIP-seq, hRelB ChIP-seq, and microarray results from human myotube cultures, human lymphoblastoid B cells, and murine wild type versus \textit{RelB}^{-/-} soleus muscle, respectively. Selected gene ontology categories are presented with p-values, with accompanying non-significant categories listed with n.s.
CHAPTER 4

CONCLUSION

This dissertation advances our understanding of the molecular signals regulating skeletal muscle oxidative metabolism. Mitochondrial biogenesis and oxidative phosphorylation have important roles in fueling skeletal muscle contraction. The regulation of these processes is often studied through the lens of exercise and other conditions of physiological stress. However, there is significantly less understanding of whether there are specific regulators of basal energy metabolism that support skeletal muscle homeostasis. Here, we identify a mechanism by which oxidative metabolism is coupled directly to muscle differentiation and homeostasis. This occurs in part through MyoD and RelB co-regulation of PGC-1β, a master regulator of mitochondrial biogenesis.

Regulation of PGC-1β transcription by MyoD

Despite PGC-1β being sufficient to induce mitochondrial biogenesis in multiple tissue types, there is very little known about what regulates its transcription. The identification of MyoD as one such factor suggests a physiological role for PGC-1β in
skeletal muscle homeostasis. Further dissecting the regulatory mechanisms utilized by MyoD and other signaling pathways will inform our understanding of skeletal muscle metabolic homeostasis and potential avenues of disease pathogenesis.

From our MyoD ChIP-seq data, we identified twelve distinct MyoD binding sites along the *PGC-1β* gene locus in C2C12 myotubes. This included eight sites where MyoD binding increased during differentiation and four sites where MyoD binding did not differ between myoblasts and myotubes. We had focused our initial study on the sites where enhanced MyoD binding corresponded with differentiation and enhanced *PGC-1β* transcription. However, the sites with fixed MyoD binding affinity could indicate that a second mechanism of MyoD regulation is targeting the same gene. The activity of MyoD has been shown to convert between myoblasts and myotubes through interactions with the protein KAP1, which functions as a scaffold for coactivators p300 and LSD1 and repressors G9a and HDAC1. Differentiation induces phosphorylation of KAP1, which then selectively releases G9a and HDAC1, leading to transcriptional activation. KAP1 presence on a subset of the identified MyoD binding sites at the *PGC-1β* gene would suggest MyoD is regulating transcription through multiple mechanisms. Further determination of whether KAP1 and its binding partners undergo a functional switch during differentiation at the static MyoD binding sites would further support this mechanism.

In contrast to the sites of static MyoD binding, MyoD recruitment to the *PGC-1β* gene during differentiation may enhance transcription by altering chromatin structure.
Mechanisms involving chromatin looping and MyoD binding have been demonstrated to activate or repress transcription\textsuperscript{25,26}. One of these mechanisms involves CTCF-mediated chromatin looping, which prevents premature gene expression in the myoblast prior to muscle differentiation\textsuperscript{25}. MyoD directly interacts with CTCF and, although this does not cause CTCF to be displaced, it disrupts CTCF-mediated chromatin dynamics to promote transcription. The loss of looping coincides with displacement of the cohesion protein Rad21, which is involved in CTCF interactions. If a similar mechanism occurs at the PGC-1\(\beta\) gene, we would expect to observe chromatin looping in C2C12 myoblasts and loss of this structure in myotubes. This determination could be made with a chromatin capture conformation experiment. Interestingly, preliminary analysis of publicly available CTCF ChIP-seq data in C2C12 cells reveals CTCF binding on the PGC-1\(\beta\) gene. Following this same line of thinking, we would expect by ChIP to detect MyoD recruitment to the CTCF site upon C2C12 differentiation. Additionally, Rad21 and CTCF Re-ChIP in myoblasts, and loss of this signal in myotubes, would be significant evidence that this particular CTCF site is regulating chromatin architecture at the PGC-1\(\beta\) gene.

**Regulation of PGC-1\(\beta\) transcription by NF-\(\kappa\)B**

Alternatively, chromatin structure dynamics are mediated by NF-\(\kappa\)B in macrophages to induce transcription of the iNOS and osteopontin genes\textsuperscript{148,149}. Although these published regulatory mechanisms occur through the NF-\(\kappa\)B subunit p65, it is plausible that RelB can mediate similar interactions. In macrophages, LPS stimulation
induces DNA binding of p65 and AP-1, which interact and subsequently recruit p300 to create local DNA looping. The looping allows p300 to activate transcription of the nearby gene. One straightforward way to test whether DNA looping on the PGC-1β gene occurs via RelB binding would be to compare C2C12 wild type and ΔNF-κB<sup>CRISPR</sup> myotubes by chromatin conformation capture. Additionally, p300 ChIP experiments on these myotubes could identify whether RelB is recruiting this histone acetyltransferase to induce transcription.

The program rVista2.0 identified three putative NF-κB binding sites along the PGC-1β gene that are conserved across species. We have demonstrated that RelB binds to two of these sites in C2C12 myotubes and mature muscle. During muscle differentiation, classical NF-κB activity is elevated in myoblasts and declines<sup>94</sup>. Additionally, the classical NF-κB subunit p65 has been associated with multiple mechanisms inhibiting muscle differentiation<sup>77,97,98,104</sup>. We examined the possibility that p65 is binding to consensus NF-κB binding sites on the PGC-1β gene to prevent premature transcription. We found that indeed p65 binds to two of these sites in myoblasts and disappears in myotubes (data not shown). Given the transcriptional profile of PGC-1β during differentiation, these results suggest that p65 directly represses PGC-1β transcription in myoblasts and a differentiation switch to alternative NF-κB activates PGC-1β transcription in myotubes.

Mechanisms of p65-mediated transcriptional repression have been described in non-muscle systems that could be applicable to PGC-1β regulation and muscle metabolism. One relatively simple model includes p65 recruitment of HDAC1, HDAC2,
or DNMT1\textsuperscript{83,84}. The presence, indicated by ChIP, of HDAC1, HDAC2, or DNMT1 in myoblasts would be expected to decline as myoblasts differentiate to myotubes. Use of the HDAC inhibitor trichostatin A could suggest HDAC1 or HDAC2 are actively involved in transcriptional repression if myoblast treatment increases \textit{PGC-1}\textsubscript{β} gene acetylation and increases its transcription. Additionally, ChIP experiments in p65\textsuperscript{-/-} myoblasts and ΔNF-κB\textsubscript{CRISPR} myoblasts could demonstrate that the recruitment of one or more of HDAC1, HDAC2, or DNMT1 is p65-dependent.

\textbf{Regulation of oxidative metabolism by classical NF-κB}

The data presented in this dissertation center around models of muscle differentiation and mature muscle homeostasis. While this certainly provides us with important insights into how metabolism is regulated in normal muscle, it may also inform our understanding of disease pathogenesis. Chronic obstructive pulmonary disease, chronic heart failure, and diabetes are associated with low-grade systemic inflammation and impaired skeletal muscle metabolism\textsuperscript{150-152}. However, there is a limited understanding as to whether these characteristics are directly and mechanistically related. Our findings suggest possible mechanisms for how chronic inflammation, through classical NF-κB activation in skeletal muscle, could impair skeletal muscle oxidative metabolism.

While our focus has been on how the alternative NF-κB signaling pathway enhances oxidative metabolism, the vast majority of disease mechanisms implicating NF-κB are in reference to classical NF-κB. One way to reconcile this potential conflict is by
recognizing that binding sites for classical and alternative NF-κB are not mutually exclusive\textsuperscript{80}. We find direct evidence of this on the \textit{PGC-1β} gene where one of its NF-κB binding sites is initially bound by p65 in myoblasts and is subsequently bound by RelB in myotubes (data not shown). Under conditions of chronic inflammation, p65 may directly repress \textit{PGC-1β} transcription, either through recruitment of transcriptional repressors or by sterically disrupting RelB binding. Furthermore, since RelB and MyoD cooperatively bind a wide array of metabolic genes in skeletal muscle, disruption of RelB binding could have genome-wide effects on metabolic gene expression.

Classical NF-κB activation in disease conditions could also potentially affect skeletal muscle metabolism through inhibition of alternative NF-κB signaling and RelB binding. Evidence in peripheral blood mononuclear cells suggests transcriptional targets of classical NF-κB inhibit alternative NF-κB activity through degradation of its upstream mediator NIK\textsuperscript{153}. Although this study was not performed in skeletal muscle, we could use mouse models of skeletal muscle-specific constitutively active classical NF-κB or chronic inflammation to investigate whether similar crosstalk occurs in skeletal muscle. NF-κB signaling crosstalk in skeletal muscle could immediately expand the scope of our findings. Since we demonstrated that RelB is important for maintaining metabolic homeostasis, classical NF-κB–induced loss of RelB binding on \textit{PGC-1β} and other metabolic genes would clearly elucidate why skeletal muscle metabolism is impaired in conditions of chronic inflammation. Combining a mouse model of chronic inflammation or
constitutively active classical NF-κB in skeletal muscle with a RelB ChIP-seq in skeletal muscle would be a key piece of evidence linking these mechanisms.

Rhabdomyosarcoma is a pediatric cancer thought to arise from the skeletal muscle lineage. In many cancers, classical NF-κB is aberrantly activated and associated with cancer proliferation, inflammation, transformation, metastasis, and survival. Evidence suggests that classical NF-κB is activated in rhabdomyosarcoma and maintains the cancerous phenotype in part by inhibiting differentiation. Interestingly, rhabdomyosarcoma cells are particularly dependent on glycolysis rather than oxidative phosphorylation. Whether this metabolic characteristic is directly related to NF-κB signaling has not been established. Through the direct binding of p65 to oxidative metabolism genes or signaling crosstalk with alternative NF-κB, the glycolytic reliance of rhabdomyosarcoma cells could be driven by a classical NF-κB signaling mechanism.

The partially differentiated phenotype of rhabdomyosarcoma cells has been associated with the inability of MyoD to properly bind its target genes. The findings presented in this dissertation demonstrate that, at least on the PGC-1β gene, MyoD binding occurs cooperatively with RelB. Conversely, if we knock down RelB expression or eliminate its binding site, MyoD binding on the PGC-1β gene is impaired. Therefore, in much the same way classical NF-κB could be affecting oxidative metabolism through its effects on RelB binding, inhibition of alternative NF-κB signaling could affect MyoD binding at metabolic genes. One small example of this was demonstrated in the rhabdomyosarcoma RD cell line where forced expression of MEF2C could rescue MyoD
binding at some genes important for differentiation such as *TNNT3*, but not at the *COX6A2* gene\textsuperscript{138}. This result suggests that proper MyoD binding in rhabdomyosarcoma may require an additional cooperative binding factor such as RelB to target oxidative metabolism genes. Performing a RelB ChIP-seq in rhabdomyosarcoma cells could tell us how alternative NF-κB and specifically RelB binding to metabolic genes is affected compared to normal muscle. Further comparison of these results to the MEF2C over expression data in RD cells might also suggest MyoD and RelB cooperative binding is an important mechanism regulating rhabdomyosarcoma metabolism.

Muscle atrophy resulting from diabetes, cancer cachexia, sarcopenia, and sepsis does not homogenously affect skeletal muscle. In each of these conditions slow Type I myofibers are preferentially spared, suggesting a link between oxidative metabolism and resistance to atrophy\textsuperscript{158-161}. Consistent with these observations, skeletal muscle specific transgenic expression of PGC-1α in a mouse model of aging protects against atrophy\textsuperscript{162}. Similar studies using PGC-1α or PGC-1β over expression in skeletal muscle suggest that these coactivators may protect against atrophy by inhibiting classical NF-κB signaling\textsuperscript{163,164}. Although the specific mechanism of NF-κB inhibition was not determined, some of the data from Eisele et al suggested PGC-1α and PGC-1β are able to directly bind and stabilize the NF-κB inhibitor IκBα\textsuperscript{164}. Understanding whether there is a direct link between PGC-1α, PGC-1β, and classical NF-κB signaling would help us understand how certain muscle fibers avoid atrophy, with possible therapeutic applications. Since we know that alternative NF-κB induces *PGC-1β* transcription, this would also be a unique crosstalk
mechanism through which alternative NF-κB inhibits classical NF-κB. Understanding the intertwined signaling between classical NF-κB, alternative NF-κB, and the PGC-1 coactivators in skeletal muscle homeostasis supports the framework necessary to efficaciously treat multiple scenarios of skeletal muscle atrophy.
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