The relationship between metabolic circumstance and epigenetic acetylation in myoblast fate and function

Sean Lim

A thesis submitted in partial fulfillment of the requirements for the Master's degree in Interdisciplinary Health Science

Supervisors:
Dr. Keir Menzies
Dr. Mathieu Lavallée-Adam

Interdisciplinary School of Health Science
Faculty of Health Science
University of Ottawa, Ottawa, ON.

Abstract

Muscle tissue is grown and maintained by muscle stem cells termed satellite cells. Activated satellite cells become myoblasts, which must proliferate then differentiate into functional muscle. This process, known as myogenesis, is controlled by a cascade of epigenetic regulatory events. One facet of this regulation is histone acetylation, which can be influenced by the availability of metabolites within a cell. In this study, the ability of glucose, pyruvate, or glutamine to change histone acetylation levels in cultured myoblasts was investigated. Changing concentrations of glucose or pyruvate had no effect but decreasing the availability of glutamine in cell culture from 2mM to 0.2mM resulted in proliferating myoblasts accruing a hyperacetylated histone phenotype. However, when the same concentration of glutamine was used on differentiating myoblasts the hyperacetylated phenotype was lost and no change to differentiation was observed. This study demonstrates the potentials and limitations of altering epigenetic acetylation with metabolic circumstance.

Le développement du tissu musculaire est soutenu par les cellules souches musculaires, communément appelées cellules satellites. Les cellules satellites activées se transforment en myoblastes qui doivent ensuite proliférer et se différencier en muscle fonctionnel. Ce processus, connu comme myogenèse, est contrôlé par une cascade de régulation épigénétique. Un aspect de ce processus est l'acétylation d'histones, qui peut être influencée par la disponibilité de métabolites dans la cellule. Dans cette étude de cas, la capacité du glucose, pyruvate, ou glutamine à changer les niveaux d'acétylation d'histones a été examinée. Le changement des concentrations de glucose ou de pyruvate n'a généré aucun effet, mais la

diminution de la disponibilité de la glutamine dans la culture cellulaire de 2mM à 0.2mM a eu pour résultat une prolifération de myoblastes présentant un phénotype d'histones hyper-acétylées. Pourtant, quand la même concentration de glutamine a été utilisée pour différencier les myoblastes, le phénotype hyper-acétylé n'a pas été observé et aucun changement de différenciation n'a pu être détecté. Cette étude démontre le potentiel et les limites des modifications de l'acétylation épigénétique selon les circonstances métaboliques.

Acknowledgements

So often we are loudly told that our accomplishments should fill us with pride. It is only in the quiet moments of our lives that we allow our accomplishments to fill us with gratitude as well.

Thank you, Dr. Keir Menzies, for affording me the opportunity to join your lab. The experience and support that were made available to me helped with every step forward. Your passion for exploration will make scientific adventurers of us all.

Thank you, Dr. Mathieu Lavallée-Adam for your mentorship and guidance. Through your lab and your work, I have been able to approach problems from a different point of view that I might have otherwise overlooked or been too intimidated to bother understanding. By your words of wisdom, I will forever criticize my colleagues by saying they have too many words on their slideshow presentations.

I would like to thank my thesis advisory committee, Dr. Mireille Khacho, and Dr. Yan Burelle for their ever-helpful instruction and critique. I believe every word I shared with either of you over the entirety of my Master's degree was invaluable and contributed to my success.

The members of my lab all deserve praise for their support and comradery. A special thanks to Dr. Gregory Addicks for inducting me into the world of laboratory biology and stem cell work. Your expertise always gave me something to aspire to. Thank you, Dr. Alex Green, for the direction and encouragement you gave me. The questions you asked, and encouraged me to ask, about my work always led to large leaps forward. To Mahdi Ahsan, thank you very much for keeping me sane with good conversation when the cells grew slow, and the gels ran long.

I would also like to extend a warm thank you to the MATRIX program for my funding support and to Dr. David Patten and Colleen Paquette for helping organize such an enriching environment.

Finally, thank you to my friends and family for their ongoing support in my quest to be a mad scientist. Thank you, Mummsy, for doing everything a mother does while also finding time to argue with me about nutrients, glucose tolerance, and the biological role of fats. Devon Miles, thank you for discussing my project with me when I needed you to and not discussing my project with me when I needed you not to. A big thanks to Alex Pajot for your provision of support and entertainment during this experience.

Table of Contents

List of Figures

List of Tables

List of Illustrations

List of Abbreviations

ACL	ATP Citrate Lyase
ADP	Adenosine Diphosphate
aKG	Alpha Ketoglutarate
AMP	Adenosine Monophosphate
AMPK	AMP-Activated Protein Kinase
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
bp	Base Pairs
ChIP-seq	Chromatin Immunoprecipitation Sequencing
CoA	Coenzyme A
DIPG	Diffuse Intrinsic Pontine Gliomas
DM	Differentiation Media
DMEM	Dulbeccos's Modified Eagle Medium
ECE	Extracellular Environment
ECM	Extracellular Matrix
ETC	Electron Transport Chain
FAD	Flavin Adenine Dinucleotide
FGF	Fibroblast Growth Factor
GM	Growth Media
H3	Histone 3
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
JAK	Janus Kinase
JNK	c-Jun N-terminal Kinase
KAT	Lysine Acetyltransferase
KDAC	Lysine Deacetylase
MAPK	Mitogen-activated Protein Kinase
MRF	Myogenic Regulatory Factor
MyHC	Myosin Heavy Chain
NaB	Sodium Butyrate
NAD	Nicotinamide Adenine Dinucleotide
NF-?B	Nuclear Factor kappa-light-chain-enhancer of Activated B Cells
OAA	Oxaloacetic acid
OBCF	Ottawa Bioinformatics Core Facility
OXPHOS	Oxidative Phosphorylation
PBS	Phosphate Buffered Saline
PDC	Pyruvate Dehydrogenase Complex
PTM	Post-Translational Modification
PVDF	Polyvinylidene Fluoride
Pyr	Pyruvate
ROS	Reactive Oxygen Species

SAM	A-Adenosyl Methionine
SC	Satellite Cell
SITA	Stable Isotope Tracing Analysis
STAT	Signal Transducer and Activator of Transcription
TBS	Tris Buffered Saline
TBS-T	TBS with Triton-X
TCA	Tricarboxylic Acid
TNFα	Tumour Necrosis Factor alpha

Chapter 1 - Theoretical Foundation

Skeletal muscle is one of the most abundant tissues, making up roughly 40% of total body mass (Proctor et al., 1999). The adaptability of muscle tissue is unparalleled in the body with simple training inducing muscle hypertrophy and increasing muscle strength. Muscle is also susceptible to insults and diseases that affect this dynamic such as sarcopenia or cachexia. Along with their plasticity, skeletal muscle also possesses a large capacity for regeneration after injury. Adult muscle stem cells, termed satellite cells (SCs), are responsible for the adaptability and regeneration of skeletal muscle tissue (Dumont, Bentzinger, et al., 2015). The functionality of the satellite cell population in skeletal muscle tissue is paramount for its continued health, and SCs require a precise system of intrinsic and extrinsic cues to function properly (Francetic & Li, 2011; Hernandez-Hernandez et al., 2017; Nederveen et al., 2019). Below is a brief overview of the role of SCs in muscle and key mechanisms and pathways that regulate their cell fate decisions.

1.1 – Skeletal Muscle Stem Cells

1.1.1 - Satellite Cells and Skeletal Muscle Homeostasis

Tissue homeostasis is the fundamental basis for life in all complex organisms, and in most cases is facilitated at the cellular level. Reduced function of cells associated with these processes will result in tissue deterioration leading to potentially fatal pathological conditions. Skeletal muscle tissue homeostasis is no exception, and the tissue's decline is a hallmark of aging and part of the terminal route for patients suffering from cancer, chronic obstructive

pulmonary disease, or heart failure (von Haehling & Anker, 2014). Skeletal muscle is mainly composed of long cylindrical fibers joined by a structural extracellular matrix (ECM). When these fibers are damaged, the main avenue by which they are regenerated is by muscle stem cells, termed satellite cells (Dumont, Bentzinger, et al., 2015). These SCs are sporadically dispersed along the muscle fibers between the plasmalemma of mature myofibers and the basal membrane (Dumont, Bentzinger, et al., 2015; Dumont, Wang, et al., 2015). This forms their stem cell niche in which they exist in a quiescent state, constitutively expressing the paired box protein Pax7, and waiting for an activation signal upon which they are recruited to regenerate existing myofibers or initiate myogenesis to form new muscle. Maintaining this quiescent state is an integral facet of the long-term stability of the SC population and its regenerative capacity (Mourikis et al., 2012). Quiescence is different from other forms of cell cycle exit in that it is reversible and the SC can enter into cell division when needed. Two forms of cell division, symmetrical and asymmetrical, can occur to maintain the SC population through multiple regenerative episodes (Dumont, Bentzinger, et al., 2015). Symmetrical division involves a SC dividing into two daughter cells that have equivalent cell fate characteristics with the original SC. Both maintain Pax7 expression (Dumont, Bentzinger, et al., 2015). This mode of division favours replenishing the SC population, also known as SC self-renewal. Asymmetrical division occurs when a SC division results in one daughter cell with equivalent cell fate to the original SC, and one daughter cell that is committed to becoming a myogenic progenitor. Myogenic progenitors produced this way will express the myogenic regulatory factors (MRFs) myf5 and MyoD accompanied by an attenuation of Pax7 expression implicating their loss of stemness (Motohashi & Asakura, 2012). More information on MRFs can be found below. Both

modes of cell division theoretically maintain the integrity of the SC population, but disease and aging are taxing on the size of the SC population along with the cells' overall myogenic potential and ability to make cell fate decisions (Barani et al., 2003; Marsh et al., 1997). Cell fate decisions are mediated by a variety of factors, most notably Pax7 which is necessary for the establishment and maintenance of a SC population (Dumont, Bentzinger, et al., 2015). Additionally, SCs exist as a heterogenous population and those that do not express Myf5, suggestive of less commitment to the myogenic process, have a greater capacity of self-renewal (Dumont, Bentzinger, et al., 2015).

1.1.2 - Myogenesis

Myogenesis is the term used to describe the process of SCs becoming muscle fibers. To ensure tissue homeostasis, SCs must progress through the steps of myogenesis in which they enter the cell cycle to proliferate and exit the cell cycle to differentiate into functional muscle tissue (Dumont, Bentzinger, et al., 2015). The process begins when quiescent SCs first receive signals that tell the cells to grow or repair muscles. These signals can come in many forms including changes to their stem cell niche or specific signals sent through their microenvironment. For example, the ECM surrounding SCs can trap growth factors (i.e. FGF2, HGF) that, upon injury, are released and activate SCs (DiMario et al., 1989; Gresham et al., 2021; Tatsumi et al., 1998). This prompts the SCs to ready themselves from the quiescent, or G_0 state, and enter the G_{alert} state (Siegel et al., 2011). This intermediate alert state is necessary to prime the SC for their first cell cycle of division as the initial cycle post-quiescence takes longer

than subsequent ones (Siegel et al., 2011). Cells proliferating from the G_0 state do so at a slower rate than those beginning in the G_{alert} state (Rodgers et al., 2014). Further activation signals induce pathways that alter the SC epigenome and transcriptome to facilitate the cell transforming into a myoblast that proliferates rapidly. The ability of myoblasts to proliferate and grow their number is required for the regenerative process to be successful (Pietsch, 1961). While likely a heterogenous population, myoblasts at this stage express Pax7, and/or Myf5, and/or MyoD (Dumont, Bentzinger, et al., 2015). Myoblasts may downregulate MyoD and return to a non-proliferating state similar to a quiescent SC or they may further commit, decreasing Pax7 expression (Dumont, Bentzinger, et al., 2015; Zammit et al., 2004). These myoblasts then commit further, becoming myocytes, which can fuse to existing myofibers to repair them or form new myotubes to grow muscle. The various steps of myogenesis are accompanied by the expression of complementary MRFs (**Figure 1.1**), a family of helix-loop-helix transcription factors that control myogenesis through a regulatory cascade (Asfour et al., 2018). MRF expression and activity must be tightly regulated to ensure proper myogenesis. Expression of differentiation-related genes during the proliferative step of myogenesis will be deleterious to the overall process. Thus, while myoblasts enter the cell cycle, they must repress the premature expression of MRFs that would cause early differentiation. An example of this selective repression is the mitogen-activated protein kinase (MAPK) p38y, which phosphorylates MyoD during the early steps of myogenesis (Gillespie et al., 2009). This leads to a transcriptional complex that occupies and represses the myogenin promoter, helping prevent premature differentiation (Gillespie et al., 2009). The SC is said to be in a transcriptionally poised state in which it is ready to express muscle-specific genes but has repressive

epigenomics marks that keep these genes dormant (Dumont, Bentzinger, et al., 2015; Khilji et al., 2018). In this case, the binding of MyoD to the *myogenin* promoter prepares the cellular machinery for transcription, but extensive repressive methylation of histone H3K9 near the promoter prevents premature expression(Gillespie et al., 2009). After proliferation however, these repressive methylation marks are removed from the muscle-specific genes and begin to accumulate on genes related to cell division (Gillespie et al., 2009; Liu et al., 2013). With the cell exiting the cell cycle and muscle-specific genes beginning to be expressed the cell enters terminal differentiation.

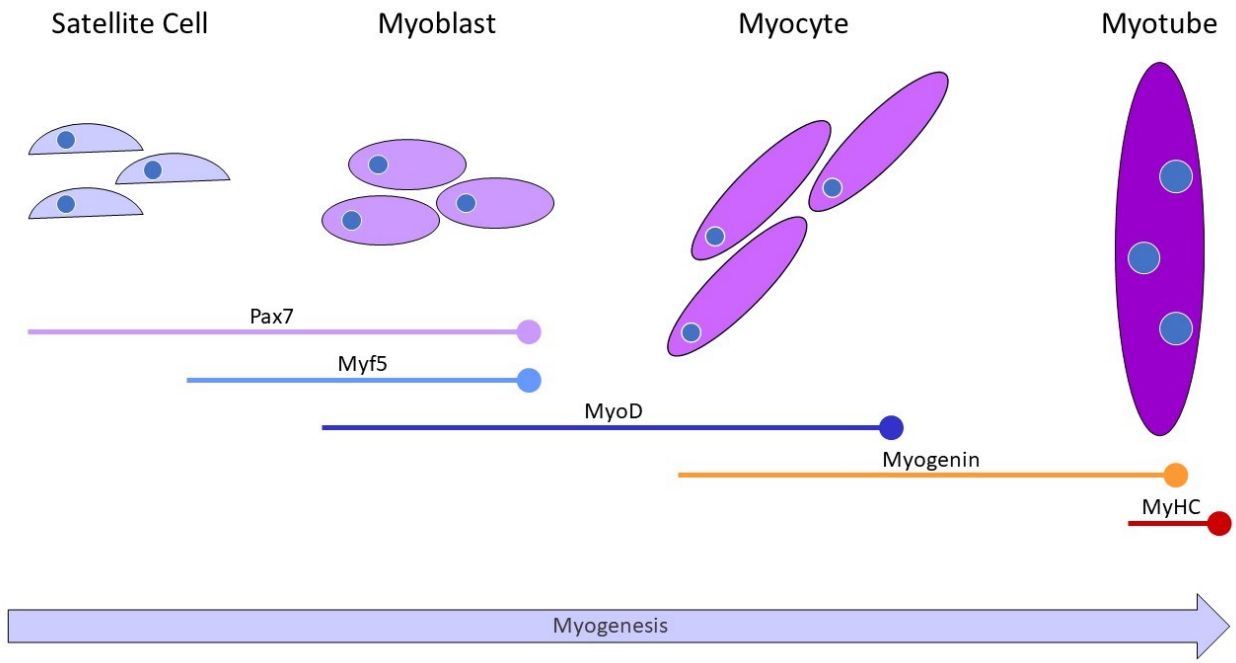

Figure 1.1: Changing protein expression as satellite cells progress through the muscle regeneration pathway. Adapted from (Dumont, Bentzinger, et al., 2015).

1.1.3 - Regulatory Pathways of Proliferation and Differentiation

Once myogenesis is initiated, myoblasts must balance their role between proliferation and differentiation as both cannot be accomplished at the same time. If proliferation goes on indefinitely, then the regeneration of the muscle tissue will be delayed as no cells will be differentiating to repair the myotubes. But as noted above, if differentiation occurs too early, then there will not be enough cells to contribute to an effective regenerative process. These cell fate decisions are orchestrated by specific pathways within the cell. What follows is a brief overview of some of the most relevant pathways which are illustrated in **Figure 1.2**.

After injury or insult, the skeletal muscle tissue environment is in a proinflammatory state. This causes an elevation in cytokines such as tumour necrosis factor alpha (TNFα). In myoblasts, TNFα promotes proliferation by repressing differentiation through activation of the c-Jun N-terminal kinase (JNK) pathway (Alter et al., 2008). This mode of induction of a pro-proliferative state is also seen in the action of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which promotes the expression of Cyclin D1 to inhibit differentiation, thereby increasing the propensity for proliferation (Guttridge et al., 1999). Similarly, p38γ acts through MyoD to produce a repressive transcriptional complex on the pro-differentiation myogenin promoter site, which sustains proliferation (Gillespie et al., 2009). A pattern that emerges through a review of the various signaling pathways of regulating myogenic lineage progression is that proliferation is promoted by inhibition of differentiation. Myoblasts, once activated, have the prerogative to proliferate. The cells will continue to proliferate until programmed otherwise, thus preventing the transmission of new instructions to differentiate is the main way the cell avoids premature differentiation. By tightly regulating proliferation and

differentiation, the cells have a method to repopulate the SC population and to regenerate muscle.

Calcium has been implicated as an important component for differentiation since 1969 (Shainberg et al., 1969). Calcineurin, a calcium-dependant serine/threonine phosphatase, indirectly activates MyoD to promote myogenin expression and entry into the differentiation process (Friday et al., 2003).

The Janus kinases (JAK) are a family of non-receptor tyrosine kinases that consist of JAK1, JAK2, JAK3, and TYK2 in mammals (Jang & Baik, 2013; Moresi et al., 2019). The main role of these proteins is to transduce signals by phosphorylating associated members of the signal transducer and activator of transcription (STAT) protein family. The STAT family is comprised of STAT1-4, STAT5A, STAT5B, and STAT6. The general mechanism of action of the JAK/STAT pathways is that JAKs receive an activation signal, phosphorylate their respective STATs, then the STATs translocate to the nucleus and act as transcription factors (Jang & Baik, 2013; Moresi et al., 2019). JAK1-STAT1-STAT3 and JAK3-STAT1-STAT3 pathways promote proliferation through suppression of precocious differentiation (Jang & Baik, 2013; Sun et al., 2007). This is achieved by reducing the transcriptional activity of MyoD and MEF2 (Jang & Baik, 2013). On the other hand, the JAK2-STAT2-STAT3 pathway promotes differentiation via MyoD and MEF2, and is important for terminal differentiation (Wang et al., 2008). The role of TYK2 in differentiation, if it exists, has yet to be delineated.

A shift in signalling from Notch- to WNT-mediated pathways during the myogenic process is also required for both proliferation and differentiation to be successful (von Maltzahn et al., 2012). Notch signaling is important during proliferation to maintain early

lineage fate (Conboy et al., 2003). The Notch ligand Delta-1 promotes proliferation and decreases the expression of Numb, a Notch inhibitor (Conboy et al., 2003). The functionality of Notch signaling then declines due to increased Numb expression, which degrades Notch through a ubiquitin-mediated reaction and prevents Notch transcription as the myoblast progress towards, and eventually into, differentiation (Brack et al., 2008; Flores et al., 2014). This decline attenuates Notch's inhibition of ß-catenin allowing for canonical, or classical, WNT cascade activity, which results in differentiation (Sethi & Vidal-Puig, 2010; von Maltzahn et al., 2012). As WNT signaling takes over, the degradation complex associated with ß-catenin is inactivated and ß-catenin is allowed to enter the nucleus, recruit transcriptions factors, and facilitate transcription of differentiation genes (von Maltzahn et al., 2012). The crosstalk between Notch and WNT signaling seems to involve GSK3ß, a kinase at the intersection of multiple signaling pathways including G protein-coupled receptor activation and cell-cycle regulation (Doble & Woodgett, 2003), but the exact mechanism remains elusive (Brack et al., 2008). Both of these signaling pathways are also related to metabolism. Notch signaling has recently become recognized as a key player in metabolism and has implications in glucose utilization and insulin sensitivity in muscle (Bi & Kuang, 2015). WNT signaling is well characterized to have the ability to coordinate metabolic pathways in murine adipose tissue under different conditions (e.g. nutritional surplus, fasting) (Sethi & Vidal-Puig, 2010). WNT's participation in regulating myogenesis may provide another avenue for metabolism to influence the process. The transient nature of Notch and WNT signalling pathways during myogenic lineage progressions forces us to ask questions about how the metabolic landscape of the cell changes with them.

Beyond WNT and Notch, many of the myogenesis regulatory pathways discussed above are also related to metabolism. JNK activation, for example, is associated with high levels of reactive oxygen species (ROS) produced by metabolism within the mitochondria (Dougherty et al., 2004; Xie et al., 2018). This form of a receptor-independent stimulus of the JNK pathways is most applicable to circumstances of hypoxia followed by reoxygenation. Nonetheless, it is also suggestive of a link between metabolism and the fate of myoblasts between proliferation versus differentiation.

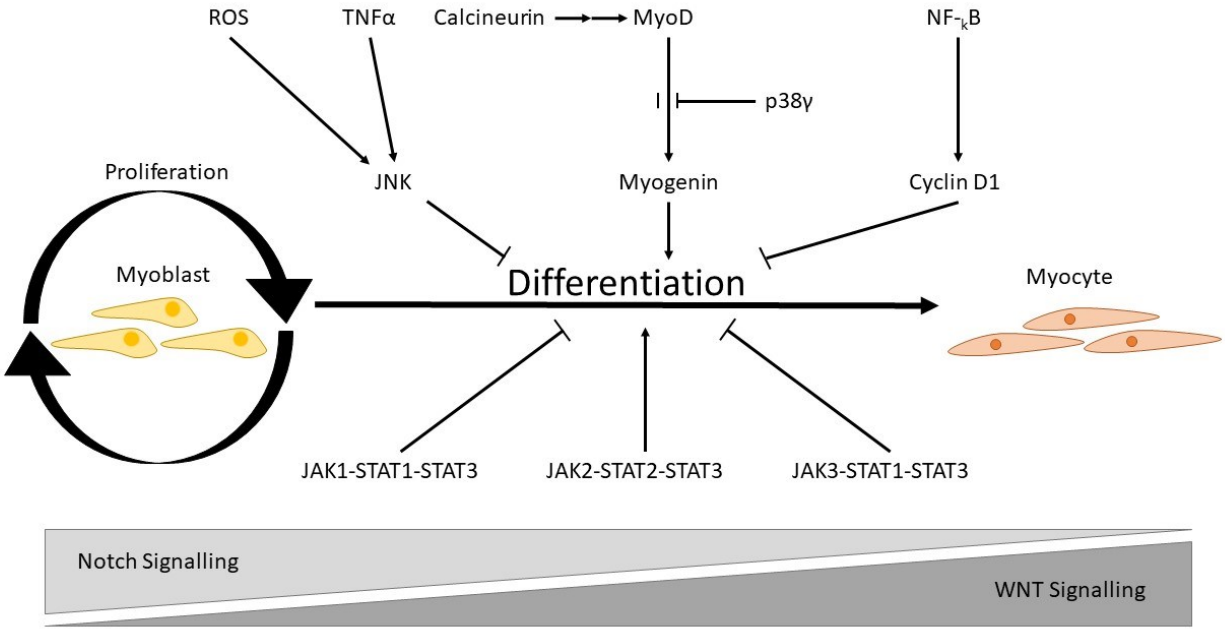

Figure 1.2: Regulatory pathways for proliferation and differentiation.

1.2 - Metabolism of Proliferating Cells

Proliferation is an important step of the myogenic process and the cell's metabolic state during proliferation needs to be understood in this context. Proliferation metabolism is a balancing act of supplying enough energy for cellular processes and enough substrates for

macromolecules synthesis (Song et al., 2019). Glucose is one of the keystone molecules for energy and anabolic substrate provision within the cell. Glycolysis is the process in which glucose is broken down into pyruvate and provides adenosine triphosphate (ATP) and reducing equivalents along the way. The process requires the reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH to move forward, therefore coupling glycolysis with a pathway that regenerates NAD^+ is paramount to the continual metabolism of glucose. The cell possesses two distinct ways of regenerating NAD^+ using the products of glycolysis. Pyruvate derived from glycolysis can be transported to the mitochondria where it is decarboxylated to acetyl-coenzyme A (acetyl-CoA), which then enters the tricarboxylic acid (TCA) cycle. This produces an abundance of ATP through the use of the electron transport chain (ETC), which relies on the oxidation of NADH in the TCA cycle, a process termed oxidative phosphorylation (OXPHOS). Alternatively, pyruvate can be converted to lactate in the cytosol and used to oxidize NADH back into NAD^+ for future use in glycolysis in a process referred to as fermentation. Each of these methods of NAD^+ regeneration has its pros and cons. Regenerating NAD^+ in the TCA cycle produces ample amounts of ATP to fuel the cell, however, this process is also relatively long and complex, involving the transport of substrates to the mitochondria and the use of a broad range of proteins and pathways. This encumbers the ability of this process to adapt quickly to rapid energy need changes in the cell and requires an ample supply of oxygen for the ETC. Conversion of pyruvate to lactate does not produce ATP but it does replenish NAD^+ rapidly. The pyruvate is also not necessarily lost as lactate can be converted back into glucose in the liver (Pilkis et al., 1979). The simplicity of this pathway allows it to adapt quickly to meet energy needs and can be completed under hypoxic conditions (i.e. anaerobic glycolysis). The choice of NAD^+ regeneration

method is of course not a binary option as both pathways for the regeneration of NAD^+ can be present and active at the same time. It is assumed though that OXPHOS, when there is enough oxygen present to facilitate the respiration needed to convert pyruvate to CO2, is preferential as it produces much more ATP per molecule of glucose. In practice, however, proliferating myoblasts rely more heavily on glycolysis than OXPHOS for energy production regardless of oxygen abundance, and only preferentially use OXPHOS once they slow proliferation and differentiate (Kraft et al., 2006; Leary et al., 1998; Lyons et al., 2004). The preference for glycolysis in the presence of ample oxygen is referred to as aerobic glycolysis, or the Warburg effect, and may be explainable when other facets of the cell's metabolic needs other than just energy production are considered.

The Warburg effect was first described in the 1920s in the context of cancer cells (Warburg, 1925). Since then, it has been extensively interrogated to help describe why cells with functional mitochondria and sufficient oxygen supply would choose to forgo OXPHOS for the comparatively inefficient energy production of aerobic glycolysis. One possible explanation is that ATP production requirements can be satisfied regardless of the method used under the conditions of proliferating cells (Vander Heiden et al., 2009). *In vivo*, proliferating cells are supplied consistently with nutrient-rich blood. Similarly, cell culture models of proliferating cells are surrounded by nutrient-rich media that is usually formulated with excess nutrients and replaced on a schedule such that nutrients are never depleted. Under these circumstances, the efficiency of ATP synthesis per unit of nutrient (e.g., glucose) becomes a moot point as the supply of nutrients would not be rate-limiting. That being said, there are limitations to the applicability of the Warburg model to describe proliferative metabolism. Stoichiometrically, the

conversion of 1 molecule of glucose to 2 molecules of lactate results in no loss or gain of NAD^+, NADH, or carbon. This leaves no room for biomass synthesis in the equation and the carbon incorporated into lactate is effectively lost by the muscle cell if it is not regenerated by the liver then transported back (Liberti & Locasale, 2016). Furthermore, the mitochondria and metabolic pathways contained therein seem integral to providing substrates for the biosynthetic process (Wellen & Thompson, 2012). This suggests that the mitochondria are still being used for some aspect of metabolism in proliferating cells.

As previously stated, the balancing act of how to utilize the products and intermediate metabolites of metabolism is an important consideration for proliferating cells. A prime example of this is acetyl-CoA, which can be involved in energy production, biosynthesis, or cell fate decisions depending on the context. A select overview of the interconnected metabolic pathways with regards to cell fate decisions and biosynthesis is illustrated in **Figure 1.2**. Citrate, an intermediate of the TCA cycle, is required for macromolecule synthesis and is the main mode of transport for acetyl-groups between the mitochondria and the cytosol for lipid synthesis (Vander Heiden et al., 2009). Glutamine is an important anaplerotic precursor used to bolster TCA cycle intermediates during metabolic activity (Bowtell & Bruce, 2002). Glutamine can elevate citrate production by being converted into the precursor of citrate, oxaloacetate, in the mitochondria (DeBerardinis et al., 2007). A lack of glutamine in human cancer cells results in depletion of TCA intermediates and eventual cell death (Yuneva et al., 2007). This dependency is independent of ATP production from glutamine and is instead related to the production of anabolic substrates and reducing equivalents (i.e. NADH, NADPH). Though this work was done in cancer it may be suggestive of broader generalizations that can be made about all rapidly

proliferating cells (Palm & Thompson, 2017), as all these cell types will require abundant substrates for macromolecule synthesis in some way, shape, or form.

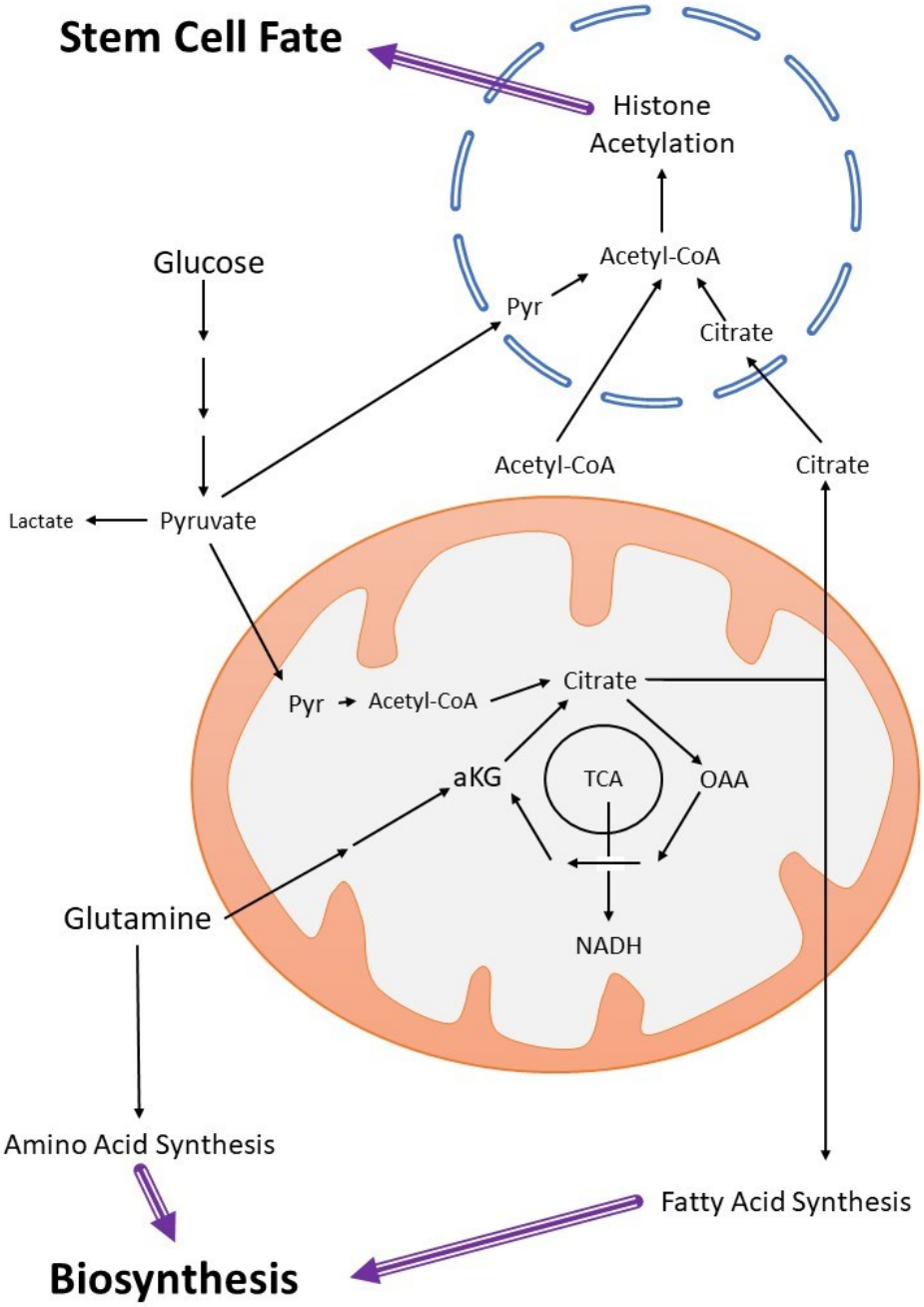

Figure 1.3: Subcellular metabolite pathways map. Metabolic products and intermediate metabolites are utilized for regulatory events that direct cell fate decisions and contribute to the biosynthesis of new material in proliferating cells. Pyr, Pyruvate; aKG, alpha ketogluturate; OAA, Oxaloacetic acid

1.3 – Metabolism and Epigenetics

1.3.1 - Epigenetics Modifications and Metabolic Cofactors

The term “epigenetics” seems to have a context dependant definition that has resulted in it being ill-defined (Deans & Maggert, 2015). For the purpose of this work, however, epigenetics is the modification of gene expression within a cell that is not dependant on changes to that cell’s DNA sequence. This can be achieved by the covalent modification of DNA, binding of chromatin remodelling proteins to covalently modified DNA, post-translational modification of DNA associated proteins like histones, action of ATP-dependant remodelling complexes, and action of regulatory RNA structures (Li, 2002; Wosczyna et al., 2021). Epigenetic modifications like the trimethylation of H3K4 in quiescent stem cells primes genes for expression upon SC activation (Liu et al., 2013). The general collection of these modifications is known as the epigenome and is a complex language of chromatin structure and post-transcriptional regulation (Segales et al., 2015). Myoblasts must make deliberate cell fate decisions based on the availability of metabolites for energy and biomass production. Informed decision making is achieved through coordinated epigenetic-regulation of gene expression that is heavily influenced by factors exogenous and endogenous to the cell (Boonen et al., 2009; Dumont, Bentzinger, et al., 2015). It is well established that signal-transduction can inform cellular decisions related to metabolism. Classically, these signals originate from hormones and growth factors that result in transcriptional or regulatory changes in the cell that then go on to inform metabolism. What has been proposed in recent years is the idea that metabolism itself

can also inform transcriptional and regulatory events to change cellular processes (Wellen & Thompson, 2012). What facilitates this is the ability of metabolites to directly impact the rate of regulatory events. This paradigm is perfectly encapsulated by AMP-activated protein kinase (AMPK) whose activity is based on the ratio of the metabolites AMP and ATP that reflects, in part, the energetic state of the cell. Metabolism, which changes the AMP/ATP ratio will directly alter the regulation of proteins by AMPK. The reoccurring motif across these types of metabolism driven mechanisms is the ability of certain metabolites to directly alter the activity of signaling proteins, metabolic enzymes, and transcriptional factors through their use as cosubstrates in regulatory events (Brunet & Rando, 2017; Khacho & Slack, 2017; Ren et al., 2017)**.** One group of this metabolically driven regulatory machinery are chromatin modifying enzymes and their respective metabolic cosubstrates.

1.3.2 - Metabolic Influence on the Histone Code

The nucleosome is the fundamental unit of chromatin and is made up of an octamer consisting of two of each of the core histones H2A, H2B, H3 and H4. These histones are subject to post-translational modifications (PTMs), known as the histone code, that alter their interaction with associated DNA and other proteins.

Methylation of DNA and DNA-associated histones can cause alterations to the structure of a cell's chromatin that will influence gene expression. Methylation of DNA generally decreases gene expression but can also increase expression if the methylation inactivates a negative regulatory element (Jones & Takai, 2001). Methylation of histones can result in active

or repressive chromatin structures (Serefidou et al., 2019). The methylation state of the epigenome is mediated by the abundance of S-adenosyl methionine (SAM) and associated one-carbon metabolic pathways (Serefidou et al., 2019)**.** SAM acts as a carrier for the one-carbon methyl group that will be used as an epigenetic modification, and the source of that methyl group can be derived from numerous different metabolites and pathways. Historically, methylation has been considered an irreversible process but recent discoveries of a flavin adenine dinucleotide (FAD) dependant and a Fe^{2+}/alpha ketoglutarate dependant mechanism have proven otherwise (Rotili & Mai, 2011).

Similar to histone methylation, the acetylation of histones is a form of epigenetic control that is also influenced by metabolic activity (Canto et al., 2009; Cohen et al., 2004; Fulco et al., 2008; Fulco et al., 2003; Ryall et al., 2015). Acetylated lysine on the tails of histones, most commonly histones H3 and H4, can act as binding sites for bromodomain containing proteins, alter chromatin localization and structure, and often weakens the interaction between the nucleosome and associated DNA resulting in increased transcriptional permissiveness (Clayton et al., 2006; Jacobson et al., 2000; Li et al., 2007; Moresi et al., 2019; Schneider & Grosschedl, 2007). Some histone acetylation sites are also associated with gene repression. Specifically, acetylation of histone H4K20 was discovered to be co-localized with known transcription repressors (Kaimori et al., 2016). Residues near these acetylation sites along with the sites themselves can be acted upon by multiple proteins resulting in a complex network of competing modifications (Brower-Toland et al., 2005; Wang et al., 2018).

Acetylation requires acetyl-CoA, which can best be thought of as an “activated acetyl group”. The association of the acetyl group with coenzyme A puts it into an energetic state that

makes the acetylation of other proteins and metabolites more favourable (Lipmann & Kaplan, 1946). Acetyl groups are added to histones by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs) at lysine residues on the exposed histone tails. It is the equilibrium established by these two enzyme families that dictates the histone acetylation state of a cell. These same enzymes can modify other proteins and are also referred to as lysine acetyltransferases and acetylases (KATs and KDACs).

Epigenetic factors are dynamically changing within SCs during myogenesis. Before an activation signal is received, the SC epigenome is in a poised state with both positive (e.g., H3K4me3, H3K9ac, K4K8ac) and negative (e.g., H3K27me3, H4K20ac) histone modifications present (Dumont, Bentzinger, et al., 2015; Kaimori et al., 2016; Khilji et al., 2018). This bivalent state keeps developmental genes silenced but primed for transcription once the negative regulatory modifications are removed. Once activation has occurred, SCs enter a proliferative state in which genes specific to muscle cells continue to be repressed, while genes required for cell division enter a transcriptionally permissive state. When SCs begin differentiation, genes related to proliferation begin to accumulate repressive modifications and take on a stable, dormant state (Liu et al., 2013). The cell's attention is then focused on producing proteins required for muscle functioning and incorporation into myofibers. All throughout this process, metabolic cosubstrates are used to add and remove epigenetic modifications, which indicate that metabolism might be able to alter the rate of these changes.

1.3.3 - Histone Acetylation Metabolism

Histones, as part of the cellular machinery that packages DNA into chromatin, are involved in the regulation of gene expression, and their acetylation depends on the metabolic state of the cell. In SCs there is still much to be understood about how acetylation is targeted towards specific histones associated with particular genes via interactions with transcription factors, and when histone acetylation occurs through an undirected mechanism of general histone acetylation events (Peleg et al., 2016). There is evidence to support loci-specific histone acetylation occurring during certain stages of the myogenic process. This is achieved by the coordination of the KAT p300 with MyoD during early differentiation (Di Padova et al., 2007). It seems that while the activity of KATs can be unspecific, they can also be recruited to specific genes by relevant transcriptional factors, which result in targeted acetylation. This specification is not absolute as demonstrated by the fact that the colocalization of p300 with MyoD during periods of proliferation and differentiation in myoblasts is only about 35% (Khilji et al., 2018). Nonetheless, this is sufficient to elicit hyperacetylation at genomic sites containing motifs that are known to be bound by MRFs during the early stages of differentiation in myoblasts (Bergstrom et al., 2002).

Deacetylation is the other side of the equilibrium that establishes the acetyl state of histones. Deacetylation occurs in either an NAD^+-dependant or -independent manner (Menzies et al., 2016). Deacetylation by class III KDACs, termed sirtuins, requires NAD^+ as a cosubstrate and can therefore act as a nutrient-sensing mechanism for the cell. Through this reaction, NAD^+ is catalyzed into NAM and the acetyl group from the target histone is removed and used to form O-acetly-ADP-ribose. Sirtuins have strong connections to the modulation of lifespan in many model organisms and imply a relationship between nutrient sensing and longevity (Sauve

& Youn, 2012). Class I, II, and IV KDACs work independently of NAD^+ and use a catalytic zinc containing domain to hydrolyze acetyl-groups from histones to produce free acetate. The rate of deacetylation on histones varies between different residues, which adds an additional layer of complexity to the authorship of the histone code (Takahashi et al., 2006).

What makes histone acetylation so interesting is its close link with energy metabolism. The main facets of energy production in myoblasts are the combination of glycolysis, the TCA, and OXPHOS. These processes are linked together by the cosubstrate NAD^+ because of its ability to be reversibly oxidized and reduced throughout the different metabolic pathways (Donohoe & Bultman, 2012), which is an important factor for Sirtuin-directed histone deacetylation. Energy metabolism is also connected by acetyl-CoA, which is an integral part of many metabolic pathways, such as the TCA cycle and fatty acid metabolism, where it functions as an acetyl-group carrier in both anabolic and catabolic reactions. It is also a necessary substrate for histone acetylation. Generally, increased histone acetylation levels correspond to an excess of energy in the cell as abundant acetyl-CoA levels increase KAT activity (Donohoe & Bultman, 2012). Deacetylation is the other side of the equilibrium and, generally, occurs at a greater frequency when energy levels are low in the cell through the induction of NAD^+-driven sirtuin activity (Li & Kazgan, 2011). As the cell breaks down excess nutrients through glycolysis and/or the TCA cycle, it drives the reduction of NAD^+ to NADH. NAD^+ is then regenerated from NADH during aerobic respiration or fermentation, supplying NAD^+ to continue participating in cellular reactions. The fluctuation of the NAD^+/NADH ratio will however affect the availability of NAD^+ as a substrate for Sirtuin deacetylation activity, thereby changing acetylation levels. Unlike class I, II and IV KDACs, which use zinc containing domains to hydrolyze the bond that connects

acetyls groups to their proteins, sirtuin activity depends on NAD^+ as a cofactor to deacetylate proteins. Sirtuins, therefore, act as a bridge connecting energy metabolism and histone acetylation. Our group previously demonstrated that the NAD^+/NADH ratio is dynamic through the process of SC differentiation, boosting NAD^+ improves SC function during muscle regeneration experiments, and NAD^+ elevates muscle SC numbers in aged animals (Zhang et al., 2016).

The physiological foundation that instructs the addition and removal of acetyl groups from histones has not been completely delineated. The cosubstrates of KATs and sirtuins are by default integrated into the cell's metabolism and are, therefore, important regulators of both metabolic processes and histone acetylation. Epigenetic regulation of SCs by histone acetylation should therefore not be viewed as a stand-alone pathway but rather as a point of integration for multiple environmental changes driven by muscle damage or metabolism. Changes to overarching histone acetylation patterns have a strong correlation with functional decline in cells (i.e. ability to respond to activation signals) and must therefore play some sort of role in cell fate and function (Benayoun et al., 2015).

1.4 – Cell Fate and Metabolism

1.4.1 - Myoblast Fate and Factors improving it

Although SCs are the point of conception for muscle tissue regeneration, myoblasts are the beginning of the active phase of the process. Myoblasts are the first non-quiescent cell type

that progresses towards muscle repair. Their contribution to myogenesis can be split into two domains, proliferation and differentiation, which are easily recapitulated in cell culture. Activated SCs initially proliferate as myoblasts so that more cells are available to contribute to the regenerative process, given that a single cell's contribution is not sufficient for effective muscle repair and that SCs are generally not abundant in healthy muscle (Pietsch, 1961). Factors that improve myoblast proliferation will do so either by directly increasing progression through the cell cycle or by helping repress premature differentiation. Both of these mechanisms are integral to the continued health of muscle tissue and their dysfunction leads to decline (Conboy et al., 2003).

MyoD conducts the switch from proliferative to differential activity by inducing p21 and p57 to inhibit cell cycle progression, and by promoting the expression of myogenin (Halevy et al., 1995; Hollenberg et al., 1993). Myogenin will then go on to induce the expression of muscle-specific genes such as myosin heavy chain, α-actin, and voltage-dependant sodium channels (Davie et al., 2007). Myoblast differentiation will therefore be improved by factors that help the cells express these muscle-specific genes either by increasing transcription or ensuring the provision of substrates for producing these new macromolecules.

1.4.2 - Cell Fate Influence of Histone Acetylation and the Acetyl-CoA metabolome

The extent of histone acetylation and the acetyl-CoA metabolome is of special interest in myoblasts because the cell's focus is on the production of new proteins and biomass for eventual incorporation into a myofiber. Following muscle damage, myoblasts undergo a period

of rapid proliferation. This occurs to expand the pool of available cells for subsequent repair events and to contribute additional cells to those destined for differentiation and tissue regeneration (Dumont, Bentzinger, et al., 2015). Myoblast proliferation, which will directly affect the final efficacy of the repair process, is influenced by the environment around the cell and available metabolites (Boonen et al., 2009; Ryu et al., 2016). All DNA and cellular machinery must be reproduced for the cell to divide. Histone acetylation plays an important role in the replication of DNA as poly-acetylation of histones H3 and H4 is required for efficient replication activation during S-Phase (Unnikrishnan et al., 2010). Also, increasing the pool of available acetyl-CoA will be beneficial to producing new cellular machinery as this substrate is required for membrane lipid synthesis along with other macromolecules. The importance of acetylation and acetyl-CoA on the proliferation of cells is exemplified by the oscillation of yeast. When acetyl-CoA levels are elevated, the yeast genome becomes hyperacetylated with modifications often occurring around genes related to cell division and expansion of the yeast population (Kaelin & McKnight, 2013). Impairment of the production of acetyl-CoA attenuates the proliferation of yeast under these conditions and prevents their genome from becoming hyperacetylated. Though this work was performed in yeast, parallels to proliferating cells in other organisms can be drawn given the similar requirement for acetyl-CoA as an anabolic substrate and for acetylation events. Unfortunately, the transduction of metabolic factors into cell phenotype has not yet been delineated in higher organisms.

During differentiation, cells with increased histone acetylation should have more active transcription rates that could result in increased expression of genes associated with muscle function and therefore a more effective myogenic process. Similar to the circumstance of

proliferation, increased acetyl-CoA may also benefit the production of novel muscle-specific macromolecules further enhancing the myogenic process. Thus, environmental conditions that alter the acetyl-CoA metabolome, acetylation levels, or deacetylation through the NAD metabolome, may be responsible for tuning myogenesis and could be potential avenues to develop therapies for pathological conditions in skeletal muscle.

1.4.3 - Metabolic Modulation of Satellite cell fate

The physiological niche in which SCs reside has not yet been fully characterized due to limitations of metabolomic technology and the difficulties of isolating the cells without disturbing the niche. As speculated by Ryall (Ryall, 2013), the metabolic milieu of the SC niche may be different than that of the greater extracellular environment (ECE) of muscle fibers. When damage occurs to this tissue, disrupting the connective tissue used to differentiate the SC niche from the ECE, the SC will be exposed to an altered metabolic landscape (Ryall, 2013). This plays an important role in activating the SC and allowing it to successfully perform myogenesis but may also provide an altered metabolic landscape that may alter SC fate and function (DiMario et al., 1989; Tatsumi et al., 1998). Quiescent SCs depend on fatty acid oxidation to fulfill their energy requirements while activated SC and proliferating myoblasts heavily depend on glycolysis (Purohit & Dhawan, 2019). It is therefore important to understand how changes to the metabolic environment might alter the efficacy of myogenesis. Furthermore, mitochondrial function is able to control stem cell function during aging and may very well be a potential

driver of cell fate decisions that go beyond harvesting energy for cellular processes (Zhang et al., 2012).

One possible mechanism for energy metabolism to act as a driving force of epigenetic regulation is through histone acetylation. Nuclear acetyl-CoA synthesis is rate-limiting for histone acetylation (Takahashi et al., 2006) so metabolic circumstances that increase this synthesis will be able to alter cell fate through histone acetylation. Metabolic changes can also change transcription and protein PTMs that accompany a change in cell phenotype. In general, metabolic circumstances that result in a reduction of energy stores will switch on cellular pathways that reduce energy consumption, while increasing the production of energy carriers such as ATP. This is, in part, achieved through the deacetylating activity of sirtuin SIRT1, which reprograms the transcriptome of cells to best cope with the altered energetic state (Canto et al., 2009). The multitude of downstream effects produced by these transcription changes can alter DNA repair, apoptosis propensities, and cell growth (Cohen et al., 2004).

This work aims to identify a general epigenetic governing mechanism that regulates myoblast fate and function, and is linked to cellular metabolism. The complexity of this endeavour necessitates that the first step will be to understand larger cellular patterns within the myoblast such as global histone acetylation under different phases of cell fate including proliferation and differentiation. This work will also examine how certain environmental conditions that alter cellular metabolism affect global histone acetylation and how this alters myoblast fate and function in skeletal muscle.

1.5 – Research Question

The process of myogenesis has a well-documented connection to epigenetic cues. These epigenetic cues may be susceptible to influence by metabolism in some capacity. The relationship between metabolism and its ability to alter myogenesis through epigenetics has not been investigated. To address this gap in knowledge, my research question is as follows: Can changes to histone acetylation caused by metabolism impact cell fate during myogenesis? Three objectives will be addressed in this work:

1. Are cultured primary myoblasts a useful cell culture model of histone acetylation?
2. Can histone acetylation levels be changed by altering metabolite availability?
3. Do changes to histone acetylation levels correlate with differences in protein expression related to myogenic cell fate?

1.6 – Hypothesis

In myoblasts, histone acetylation is susceptible to change caused by the availability of metabolites in the cell's local microenvironment. These changes to acetylation alter cell fate decisions about proliferation and expression of proteins associated with differentiation.

Chapter 2: Cultured primary myoblasts are a consistent and robust model for histone acetylation.

2.1 – Introduction

In their stem cell niche within the body, SCs and their progenitors are exposed to a very specific local environment. Almost everything about their environment from the temperature, availability of oxygen, supply of nutrients, presence of other cells, and even the elasticity (Hu et al., 2011) of the surface they reside on can impact their function (Bentzinger et al., 2013). The complexity of this environment also makes it difficult to study these cells while they are still in their niche. Reliably monitoring and altering the SC niche environment for the purpose of an experiment is challenging. There are no guaranteed ways to influence the environment or monitor the influence of an intervention, that would not disturb the niche. This had led to studies being carried out using cell cultures, which have contributed valuable knowledge and given us insight into how muscle regeneration works and ways to improve it. However, this is based on the understanding that once the cells are removed from their niche and cultured in a laboratory setting, they cease to be their canonical selves. Once removed from their niche, the SCs are no longer quiescent and differentiate to myoblasts, which then proliferate in cell culture. This current work does not seek to investigate the activation of satellite cells so this difference is permissible, but the cell culture model is essential to study histone acetylation in a controlled environment and must be validated.

Additionally, there is accumulating publicly available datasets that examine chromosomal histone acetylation in myoblasts using chromatin immunoprecipitation sequencing (ChIP-seq) (Asp et al., 2011; Consortium, 2012; Hamed et al., 2017; Kelly et al., 2018; Moris et al., 2018; Pope et al., 2014; Yucel et al., 2019; Yue et al., 2014). In order to

leverage these existing databases to complement our research, we would need a method to standardise the datasets to allow statistically sound comparison. We sought to validate a recently published bioinformatics tool titled ChIPseqSpikeInFree that claims to provide a method for estimating scaling factors used to standardise reported values without the need for a spike-in standard which is missing from most published datasets (Jin et al., 2020). The development of this algorithm was prompted by the assumption made during ChIP-seq experiments that the yield from the workup of a sample is identical between treatment groups, which has recently been understood to be a flawed assumption (Chen et al., 2015; Jin et al., 2020). In these cases, a spike-in standard can be used wherein an exogenous analyte is introduced into the samples and used as an absolute measurement to normalize all other measurements (Chen et al., 2015). However, the experiments that have been used to generate most of the relevant online datasets have not included this standard. This strategy would therefore allow the leveraging of non-standardized online databases to interrogate the role of global histone acetylation across different treatment groups and potentially between datasets.

The work in the following section is to establish the cell culture model of primary myoblasts as a useful model to explore histone acetylation during myogenesis and to validate the R package ChIPseqSpikeInFree. To this end, the myoblasts were tested for the consistency of their histone acetylation state under baseline conditions, along with the protocols used to measure proliferation of the cells and isolate histones from cultured cells. The results from the original ChIPseqSpikeInFree paper were replicated then the algorithm was applied to a dataset that included a spike-in standard for comparison.

2.2 – Materials and Methods

Primary Myoblast Isolation

Primary myoblasts were isolated from the hindlimbs of NTacC57BL/6 mice. Briefly, whole hindlimb muscles are dissected from a single mouse euthanized by cervical dislocation. Both hindlimb muscles are washed in phosphate-buffered saline (PBS, Wisent inc.) then minced using a sterilized razor blade in a 30mm petri dish with a 1% collagenase, 0.4% dispase solution (Sigma-Aldrich) and left to digest for 15 minutes at 37°C in an incubator. The digest is passed through a 5 ml pipette repeatedly until smooth then incubated for an additional 15 minutes at 37°C. The digest is then passed through a 100-micron strainer, collected, and pelleted in a centrifuge at 500 rcf. The supernatant is aspirated off and the pellet is resuspended in Ham's F-10 growth medium (Wisent inc.) supplemented with 20% v/v bovine calf serum (Sigma Life Sciences), 1% v/v penicillin/streptomycin (Sigma Life Sciences), and 0.005% FGF (Abcam). The whole suspension is pre-plated on an uncoated 100mm petri dish and incubated for 2 hours, as a pre-plating procedure to discard fibroblasts. After incubation, the media from the plate and any cells suspended within are re-plated to a collagen-coated 100mm petri dish and incubated overnight. Collagen-coated dishes are prepared at least one day in advance by pipetting 10 ml of 0.01% collagen into each petri dish, followed by the complete aspiration of the solution off of the dish. Wet collagen-coated petri dishes are then left to dry aseptically. The plate with the transferred cells will undergo another 2-hour pre-plating on plain petri dishes then re-plating onto collagen-coated petri dishes after they grow to 80% confluency in order to further enrich for myoblasts. The time it takes to reach 80% confluency depends on the number of myoblasts

successfully isolated but will usually take 2 – 4 days. Once sufficiently enriched, myoblasts are trypsinized off plates using 0.05% trypsin (Wisent inc.), pelleted, and resuspended in the same HAM's F-10 media with an addition of 10% v/v DMSO (Fisher BioReagents). 1 ml of the 10% DMSO media is used per plate of trypsinized cells which is then stored at -80°C for future use.

Primary Myoblast Cell Culture

Primary myoblast samples from the isolation protocol are defrosted, resuspended in HAM's F- 10 growth media, and pelleted. The supernatant is then aspirated and replaced with fresh media to resuspend the cells. Generally, one ml of frozen cells will be plated onto two dishes. The cells are divided onto 100mm collagen-coated dishes such that each plate is 50% confluent. This level of confluency is used so that the cells will continue to proliferate rapidly but do not overcrowd. Cells at extremely low confluency will proliferate very slowly and cells at too high of confluency with prematurely differentiate. During the initial plating of defrosted cells, they are fed with a total of 8ml of media. Cells are grown and re-plated before they reach 80% confluency, as above this confluency they are at risk of differentiating. Subsequent media replacements after the initial plating during defrosting or re-plating are done with 6ml of media. The initial use of 8ml of media is to facilitate easy distribution of the cells uniformly across the plate. After the initial distribution of cells, 6ml of media is sufficient to satisfy the cells' metabolic requirements for the two days between media changes. When cells are split between plates to reduce confluency, all the cells from each of the plates of the same cell line are mixed together so that the final plates will not be affected by potential plate-to-plate

disparities that arose during the culture process. Cells are grown at 37°C with a supply of 5% CO_2.

Sodium Butyrate Treatment for Positive Acetylation Control

Cultured myoblasts at approximately 60% confluency had their media replaced with new growth media containing 5mM sodium butyrate (NaB). Sodium butyrate is an HDAC inhibitor that inhibits most classes of HDACs thereby causing hyperacetylation in cell culture (Davie, 2003). The cells were grown in this media for 24 hours before they were sampled and frozen for later analysis.

Whole-Cell Lysis Histone Extraction

Petri dishes containing the primary myoblasts had their growth media aspirated. The dishes were then washed with PBS, which was again aspirated off. The dishes were chilled and 500uL of ice-cold RIPA lysis buffer (**Table 2.1**) with protease inhibitors and sodium butyrate was added to each plate. The cells on the dishes were scraped off and placed into centrifuge tubes on ice. The cells within the buffer were sonicated using a probe sonicator (Fisher Scientific inc.) at 30% power 3 times. Each sonication step lasted 3 seconds and then the cells were placed on ice for 10 seconds to prevent degradation of the sample caused by heat generated by the sonication process. Sonicated samples were centrifuged at 4 °C for 10 minutes at 16000 rcf. The supernatant was then reserved and stored at –80 °C for later analysis. The pellet was discarded.

Table 2. 1: Description of RIPA lysis buffer ingredients and quantities.

RIPA Lysis Buffer	
NaCl	150mM
DOC	0.5% w/v
SDS	0.1% w/v
Triton X-100	1% v/v
Tris pH 8.0	50mM
ddH20	prn
COmplete Protease Inhibitor (Roche)	1 tablet per 10ml, added just before use
Sodium Butyrate	10mM, added just before use

Histone Isolation with Acid Extraction

As has been previously described (Shechter et al., 2007), cells were collected from plates and washed with 5mM sodium butyrate (Alfa Aesar) in PBS (Wisent inc.). The cells were pelleted and resuspended in extraction buffer made from PBS containing 5mM sodium butyrate (Alfa Aesar), 0.5% Triton X-100 (Fisher Bioreagents), 2mM phenylmethylsulphonyl fluoride (Thermo Scientific), and 0.02% NaN (Fisher Chemical). The solution is stirred by inversion at 4°C for 10 minutes to lyse the cells. The solution was pelleted, and the supernatant was discarded. The solution was resuspended in half the original volume of extraction buffer then pelleted again. The pellet was resuspended in 0.2M HCl and the histones were acid extracted overnight at 4° C with constant inversion mixing. Finally, the solution was pelleted at 4 °C for 10 minutes at 16000 rcf and the supernatant, a mixture of acid soluble nuclear proteins including histones, was removed. A 2M NaOH solution was used to approximately neutralize the solution by adding one tenth the volume of 0.2M HCL that was previously used before it is stored at -20° C for later analysis.

Cell Sample Homogenization

Some samples were subject to an additional step using a 0.5 ml disposable pellet pestle and microtube (Fisher Scientific, Canada) to ensure cell membrane lysis (**Figure 2.1**). These samples were placed in these specially designed pellet tubes and pulverized for 30 seconds on ice using the supplied pestle.

Western Blotting Electrophoresis and Transfer

Samples were separated by electrophoresis on a 15% acrylamide stain-free SDS-page gel (Bio-Rad). The gels were run at 35mA for 1.5 hours or until the dye front ran off. The gel was then photographed in a Bio-Rad Chemidoc using the stain-free gel setting with 45 seconds of activation and automatic exposure time, as a method for quantifying protein loading in each lane. After imaging, the gel was equilibrated in transfer buffer containing 20% methanol for 15 minutes. The proteins in the gel were then transferred onto an Immun-Blot polyvinylidene fluoride (PVDF) membrane (Bio-Rad) at 100V for 1 hour. After the transfer is completed, the membranes are again imaged on the Bio-Rad Chemidoc using the stain-free blot setting for estimating the relative total protein content. Ponceau staining of the PVDF membrane was also used to measure total protein content when stain-free gels were unavailable.

Immunoblotting

The PVDF membranes with the transferred proteins were blocked with 10% powdered milk in tris-buffered saline (TBS). Blocking is done for 1 hour at room temperature with constant stirring. The membranes are then incubated with a primary antibody at a 1:1000 dilution (Anti-acetyl-Histone H3, EMD Millipore; Histone H3 D1H2 XP Rabbit mAb, Cell Signalling; Acetylated Lysine Antibody, Cell Signalling; Anti-myosin Heavy chain MF20, DSHB). After the primary incubation, the membranes are quickly rinsed twice with TBS containing 0.01% triton-X (TBS-T) then washed for 15 minutes in TBS-T at room temperature. The secondary incubation then begins with fresh 10% powdered milk in TBS and an appropriate HRP-conjugated secondary antibody at a dilution of 1:5000. The secondary incubations were done for 1 hour at room temperature or overnight at 4°C. After, the membrane is once again rinsed quickly twice then

washed for 15 minutes in TBS-T. When the washing is completed, the membrane is covered in 1 ml of imaging solution (Clarity Western ECL Substrate, Bio-Rad) and imaged in a Chemidoc using the chemiluminescence setting. An initial exposure time is automatically calculated by the instrument and used for a photo, then exposure times above and below the automatically calculated one are manually inputted and additional images are taken. The range of exposure times is to ensure that all lanes in the photos are adequately imaged while not being overexposed.

Membrane Stripping

Membranes requiring a second round of immunoblotting were stripped after the first round of imaging. To first determine the minimum amount of stripping required to remove the first round's signal, the membranes were washed in stripping buffer (Restore PLUS Wester Blot Stripping Buffer, Thermo Scientific) for 30 minutes then reblotted with a secondary antibody and imaged. This was repeated until no signal was seen. Stripping for 1 hour at room temperature was chosen as the optimal method and used in all protocols moving forward.

Microscopy Cell Counting for Proliferation Rate

Cell Imaging

Previously isolated and frozen cells were plated at low confluency on 0.01% collagen-coated 6-well dishes. One day after platting, the dishes were then placed in a Zeiss Cell Discoverer 7 and Zen Blue software was used to randomly assign 5 non-overlapping frames to

each well. The position of these frames is saved by the program and used on subsequent days to take pictures of the same places on each plate every day. This allows for the same local population of cells to be photographed and counted throughout the experiment. Each plate was photographed daily, immediately after the replacement of the growth media to limit the number of floating cells in the images.

Cell Counting

The number of cells in each image was counted using an automated algorithm set up in Zen Blue. Since the location of each image is randomly assigned, there were occasionally images that had no cells on the first day. These images were identified by the automatic counting algorithm and then manually reviewed to ensure that the problem was with the image and not the algorithm. When an image did not contain cells, it was censored from the analysis. The selection of 5 random frames provided enough images to allow for some censoring while maintaining a minimum of 3 images for analysis.

Statistical Methodology

An unpaired two-tailed student's *t*-test was used to compare western blotted samples in the NaB positive control experiment and the acetylation between passage number experiment. These experiments were between two groups with at least three measurements per group. A one-way analysis of variance (ANOVA) was used to compare the histone acetylation levels from

cell lines derived from three different mice. The standard error of the mean was used as positive and negative error bars on all graphs.

For calculation of proliferation rate, the number of cells was plotted vs. time, and the resulting graph was used to identify the exponential phase of growth. This exponential phase was then log-transformed, and a linear regression was done to calculate a slope for the now linear graph. The average slope and standard deviation of the four cell lines were then used to report the proliferation rate.

ChIPseqSpikeInFree Validation

The validation of the algorithm was divided into two phases: replication and application. In the first phase of validation, the algorithm was used to replicate a selection of scaling factors used to normalize ChIPseq data from the source publication's benchmark analysis. This was performed on the source publication's "Dataset 3" which is from a 2019 analysis of histone methylation changes in primary gliomas tumour lines (Harutyunyan et al., 2019; Jin et al., 2020).

In the second phase, the algorithm was tested against published ChIPseq data that included a spike-in-determined scaling factor that was not previously included in the original publication of the algorithm (Maganti et al., 2018).

2.3 – Results

Histone Isolation and protocol Validation

To ensure that histones can be successfully extracted for future experiments, we tested 7 different protocols for their ability to produce an isolate containing histone 3 (H3, **Figure 2.1**). The protocols were compared based on the amount of H3 in their resulting isolate per amount of starting material (i.e. cells) used to generate said isolate. Myoblasts grown to 80% confluency were sampled and their histone extracted in several different ways, based on a previously published acid extraction of histones protocol (Shechter et al., 2007), to determine the optimal method. The cells were scraped off the plate in either PBS or hypotonic lysis buffer containing detergents (**Figure 2.1**). The cells were then either allowed to be passively lysed by the buffer during inversion mixing or underwent mechanical homogenization using a pestle. After the lysis, cytosolic debris were removed from the sample through centrifugation and the pelleted nuclei were kept. These nuclei were either suspended in acid so that the acid-soluble histones would diffuse out into solution or the nuclei were sonicated to disrupt the nuclear membrane and release the proteins. The last method used was to take the cells directly from cell culture, scrape them off the plate with RIPA buffer (see **Section 2.2**) and sonicate them to disrupt both the cellular membrane and nuclear membrane.

Western blot loading was done such that each lane contained extract from approximately the same number of cells. All isolation methods produced a signal at a reasonable molecular weight (**Figure 2.1**) for the target protein when blotted with an anti-H3 antibody, suggesting that all methods extracted histones from the cells at varying efficiencies. The whole-cell lysis method produced the strongest signal comparatively and was used to move forward in experiments.

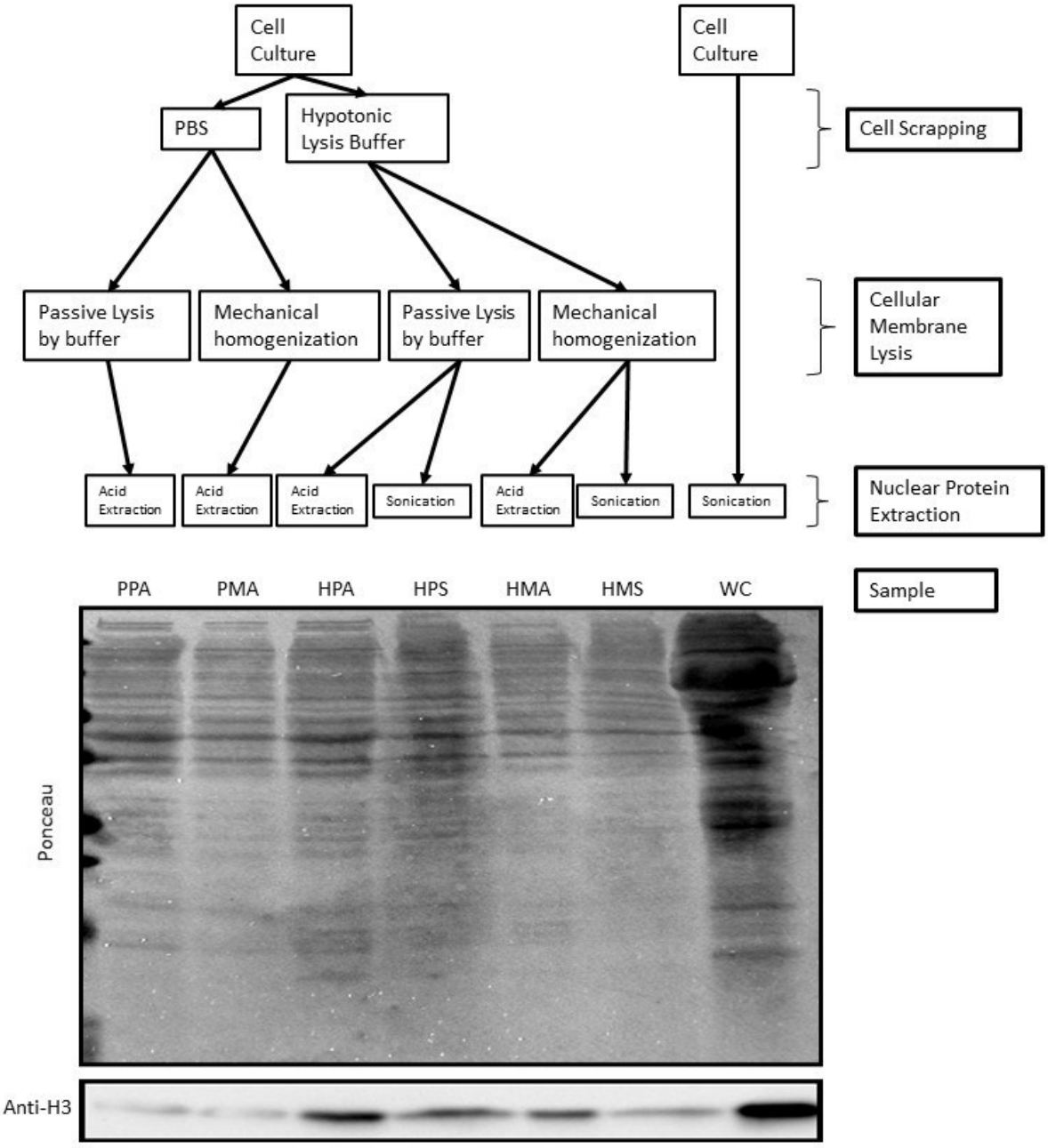

Figure 2. 1: ***Histone isolation and protocol validation.*** *Histones were isolated from cultured myoblasts using seven different methods. The resulting isolate from each method was then immunoblotted for total histone H3 content and ponceau stained for total protein content. Samples are coded using three letters. The first letter indicates cell scraping in either* PBS *or* Hypotonic *lysis buffer. The second letter indicates cellular membrane lysis using either* Passive lysis by the buffer *or* Mechanical *homogenization. The third letter indicates the method of extracting histone from the nucleus either by* Acid extraction *or* Sonication. *WC indicated whole-cell lysis in which the sample was not prepared, only sonicated. (n=1).*

Positive Control Validation

Cells treated with 5mM of NaB for 24h had a significantly higher level of acetylation (**Figure 2.2**, $p<0.05$, two-tailed student's *t*-test). These samples were used as a positive control for acetylation in western blots moving forward.

Stripping Validation

Stripping was performed in 30-minute intervals followed by immunoblotting with a secondary HRP-conjugated antibody and imaged (**Figure 2.3**). It was determined that after 1 hour of stripping at room temperature there was no signal at exposure times under 2 minutes from the initial immunoblotting done with an anti-acetyl antibody. As 2 minutes is more than the exposure time used for most blots it was decided that a 1-hour stripping at room temperature was sufficient to remove previous antibodies from the membrane.

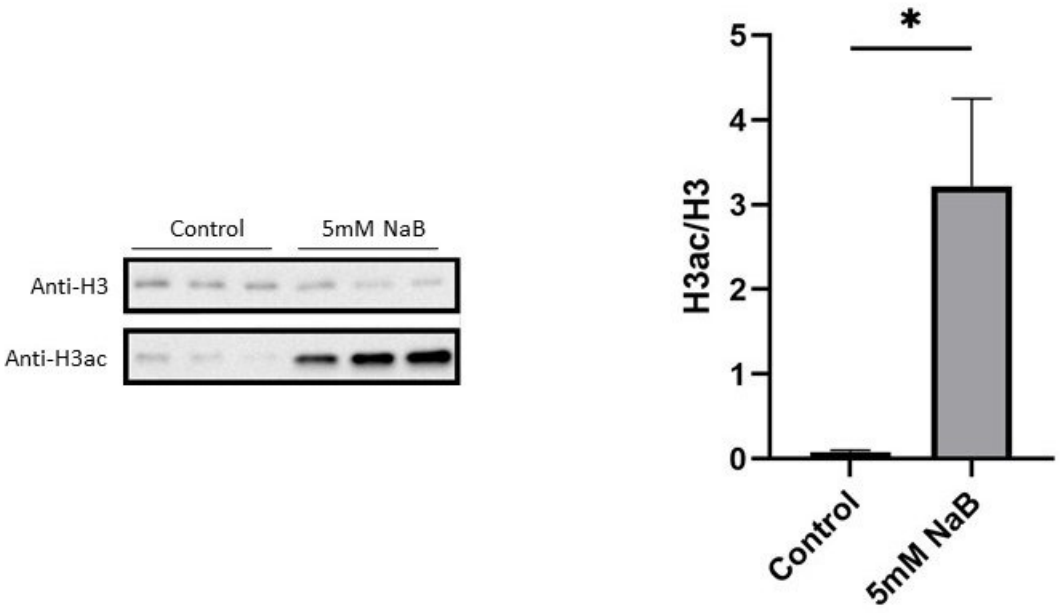

Figure 2. 2: ***Sodium butyrate (NaB) validation as positive acetylation control****. Cultured myoblasts were grown in growth media containing 5mM of the HDAC inhibitor sodium butyrate for 24h. These cells were then scrapped and sonicated. The resulting lysate was then immunoblotted for histone H3 content and acetylated histone H3 content. * p-value <0.05 (two-tailed unpaired t-test, n=3)*

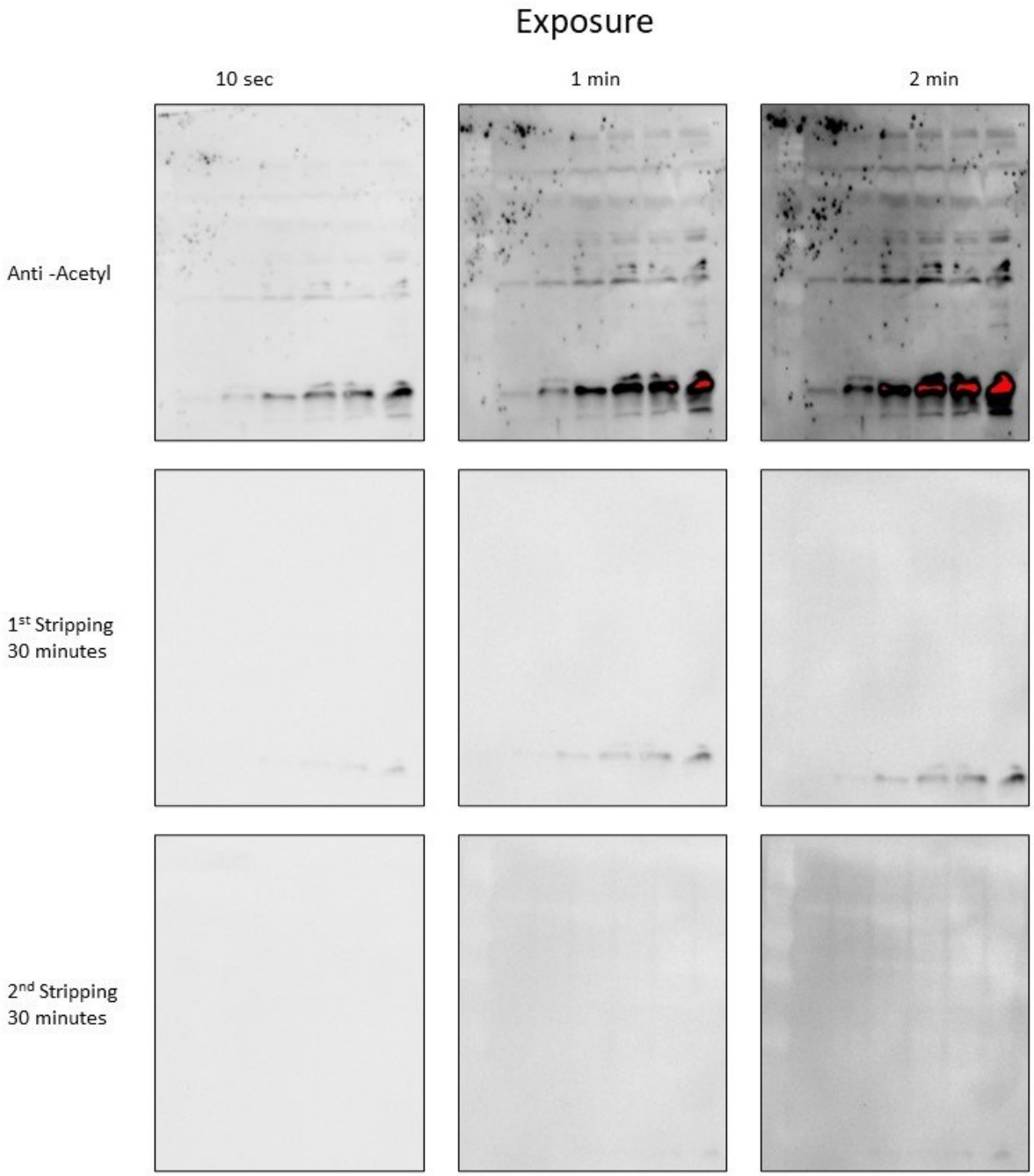

Figure 2. 3: ***Stripping validation****. A western blot was performed using isolate from cultured myoblasts loaded at progressively larger protein content. The sample was then immunoblotted for acetylated protein and imaged. After imaging, the blot was stripped using stripping buffer for 30 minutes then reimaged. Stripping was performed an additional time until almost no signal was seen except as high exposures.*

Histone Acetylation Between Different Mice

For cultured primary myoblasts to be a useful model of general histone acetylation, the variability of histone acetylation under baseline conditions should be minimal. To evaluate the variability between cells derived from different animals, cultured primary myoblasts from three mice had their histones extracted and analyzed by western blotting for changes in general acetylation using an anti-acetyl antibody (**Figure 2.4**). No significance was found between the general histone acetylation levels of the three mice (*p*-value=0.998, One-way ANOVA). Mouse 1, 2, and 3 had acetylation levels of 0.98±0.09, 0.98±0.04, and 1.04±0.18 (average ± standard deviation) respectively.

General Histone and H3 Acetylation Across Passage Numbers

Primary myoblasts are cultured through successive passages to facilitate the expansion of the cell population for experiments. If general histone acetylation significantly changes depending on passage number then this effect will need to be accounted for when experiments aimed at changing histone acetylation are conducted. To investigate if passage number affects general histone acetylation, primary myoblasts cultured from different passage numbers had their histones extracted and analyzed by western blotting using an anti-acetyl antibody. For myoblasts derived from a single mouse (**Figure 2.5 A**), the average acetylation over passages 1-7 was 1.45±0.03 (Average ± standard deviation). A second experiment was performed with myoblasts from 3 different mice comparing acetylation between passage 1 and passage 5 using

an anti-acetyl-H3 antibody (**Figure 2.5 B**). This antibody is a polyclonal antibody targeted at all H3 acetylation sites. The average acetylation of passages 1 and 5 across all three primary myoblast cell lines was 0.82±0.06 and 0.84±0.06, respectively. There was no significant difference in acetylation between passages 1 and 5 (*p*-value=0.78, two-tailed *t*-test).

Proliferation Rate

The proliferation rate of primary myoblasts is important to know because the confluency of primary myoblasts in cell culture affects their functions. High confluency myoblasts will begin to spontaneously elongate and differentiate. By understanding the rate of proliferation, we can better prevent high confluency conditions. The accurate determination of proliferation rate may also be one avenue to determine how experimental conditions affect the fate and function of myoblasts.

A protocol was investigating for determining the proliferation rate of myoblasts using a Zeiss Cell Discoverer 7, which takes an image of the same location on a cell culture plate across multiple days. The imaging method tracked the same local cell population across multiple days and gave results expected of proliferating cells. A representative picture is shown in **Figure 2.6** along with an example image of the automatic cell count quantification. When cells are plated at a low confluency (<50%) and allowed to proliferate without interference, cultured primary myoblasts displayed a lag, exponential, and plateau phase of proliferation as is expected. The average proliferation rate within the exponential phase was 0.43±0.05 during the exponential phase of proliferation.

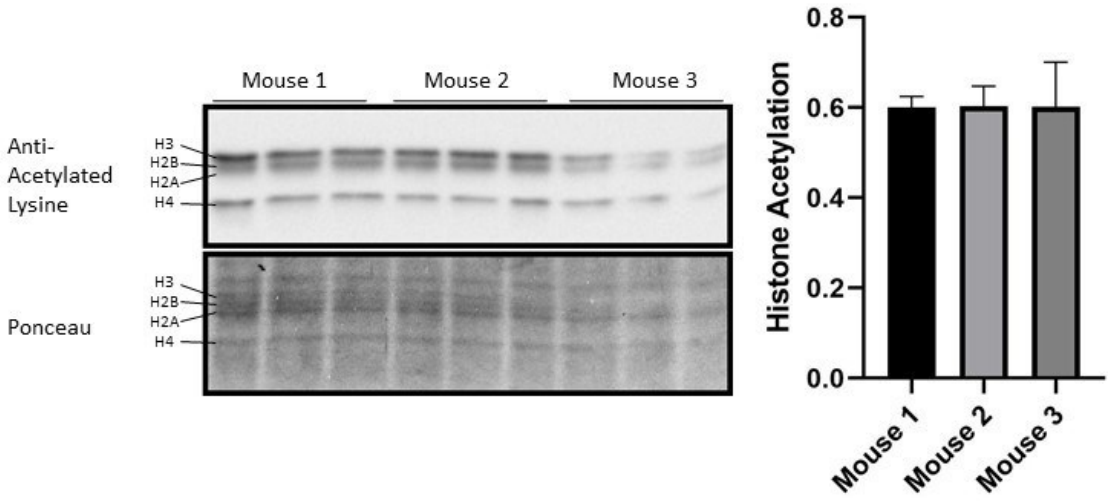

Figure 2. 4: ***General histone acetylation between mice****. Histones were isolated from three different mice and immunoblotted for acetylated histone proteins and total isolated histone protein content. Molecular weight and characteristic band pattern were used to identify the bands which correspond to histones. p-value=0.999 (One-way ANOVA, right panel, n=3).*

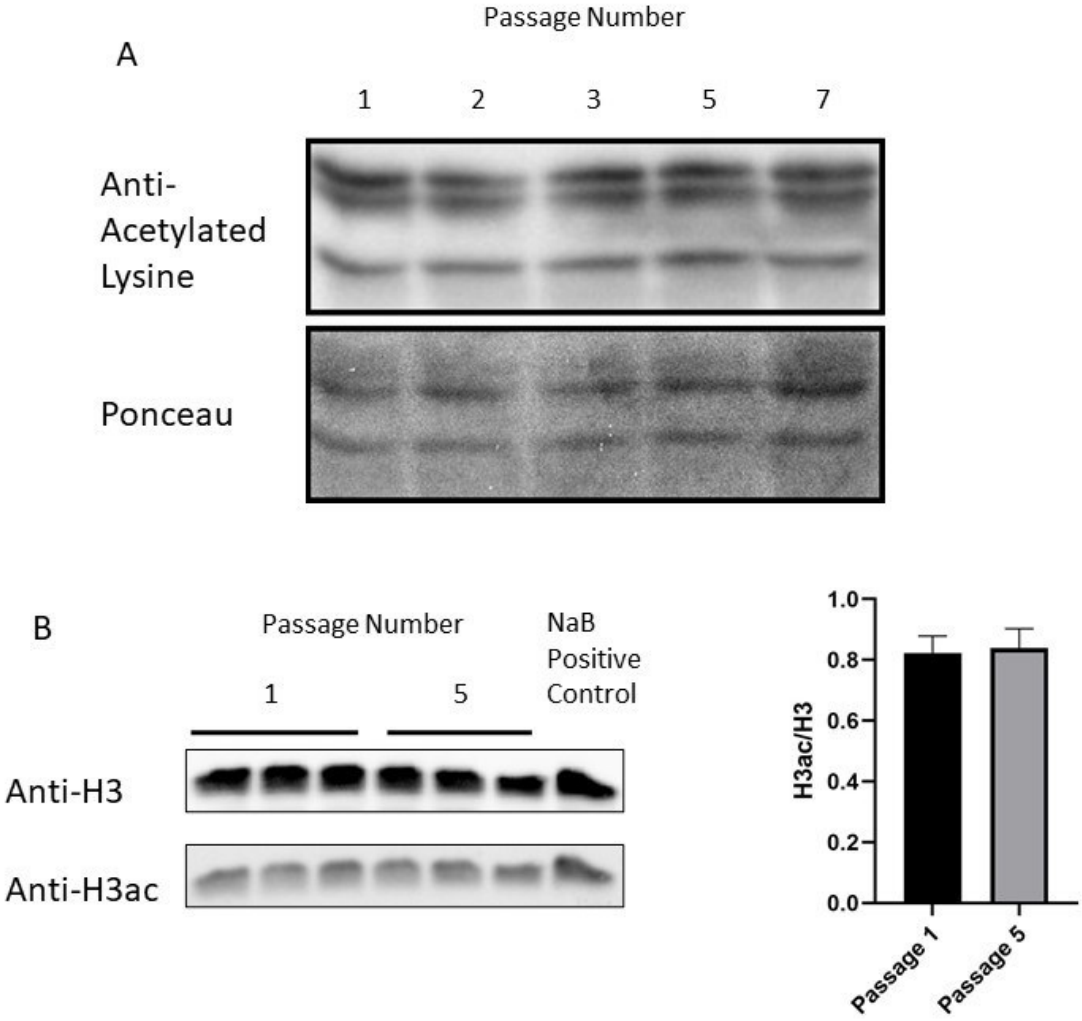

Figure 2. 5: ***Acetylation between culture passages****. Histones from cultures myoblasts derived from a single mouse were collected after consecutive passages.* ***A)*** *Isolated histones from passaged 1-7 were isolated from these samples and immunoblotted for general acetylated protein and total histone protein content. Molecular weight and band pattern were used to identify histones (n=1).* ***B)*** *Histones were isolated from passages 1 and 5. These samples were immunoblotted for histone H3 and acetylated histone H3 with the inclusion of a NaB positive control for acetylation. p-value=0.767 (two-tailed unpaired t-test, n=3, bottom right panel).*

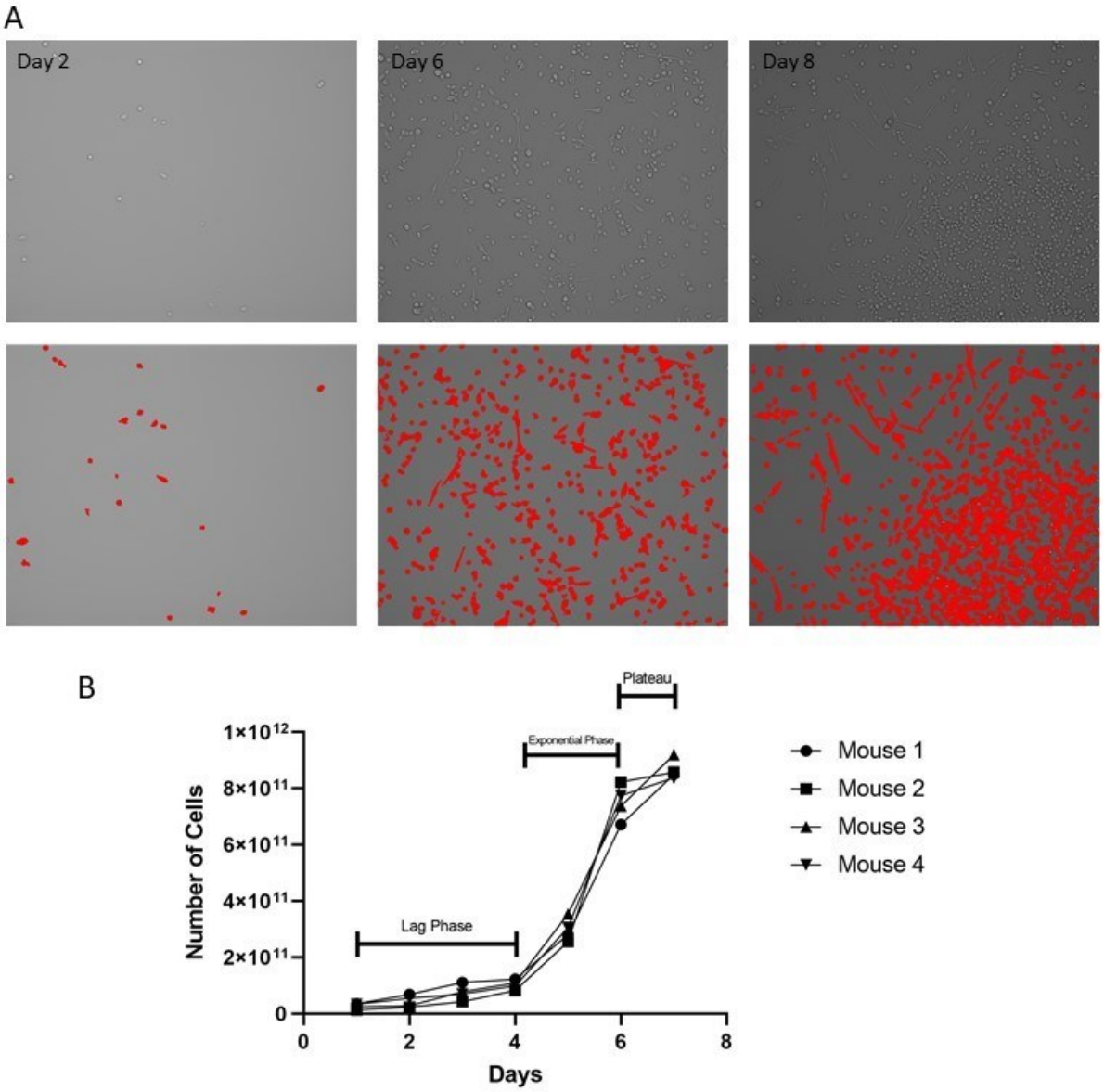

Figure 2. 6: ***Proliferation rate of cultured primary myoblasts****. Cells were platted at a low confluency (<40%) on 6-well collagen coated plates. Three wells were used per animal. 24h after the initial plating, five observational frames were randomly assigned to each well, which indicate the area that would be photographed on subsequent days. The same area of each plate was photographed every day to track the proliferation of myoblasts. The number of cells was then counted using an automatic algorithm in Zen Blue (Zeiss) and plotted against time. Lag, exponential, and plateau indicate the characteristic phases of growth expected for cultured proliferating cells.*

ChIPseqSpikeInFree Validation

The algorithm ChIPseqSpikeInFree was made to facilitate the calculation of scaling factors for ChIPseq data between groups without the need for a spike-in standard to have been incorporated in the original experiment (Jin et al., 2020). If this algorithm is accurate, it would allow researchers to utilize and reinterpret publicly available ChIPseq data. To validate the ChIPseqSpikeinFree algorithm, the results from the source publication's benchmark analysis were replicated using the paper's "Dataset 3" (Jin et al., 2020). The data was filtered and processed using their documented method. The resulting scaling factors for the ChIPseq data were compared to the published values. We were able to replicate some results, but others differed significantly, notably the diffuse intrinsic pontine gliomas (DIPG) samples DIPGVI and DIPGXIII. Correspondence with the paper's author revealed that the problem could be solved by manually setting a corrected parameter (cutoff_1stTurn) based on visual inspection of a diagnostic plot. This correction allowed us to replicate most of the results, indicating that we were using the software correctly.

We then applied that ChIPseqSpikeInFree algorithm against published ChIPseq data, which included a spike-in-determined scaling factor (Maganti et al., 2018). This allows for comparison between factors attained using the algorithm and those attained using an actual spike-in standard. In this evaluation, the scaling factors generated by the algorithm were poorly correlated with the spike-in-determined factors (**Figure 2.7**, simple linear regression, r=0.002). These scaling factors differed by close to a factor of 10 in some cases (H3K27me3-R4_S6, H3K27me3-Sh3-1_S4).

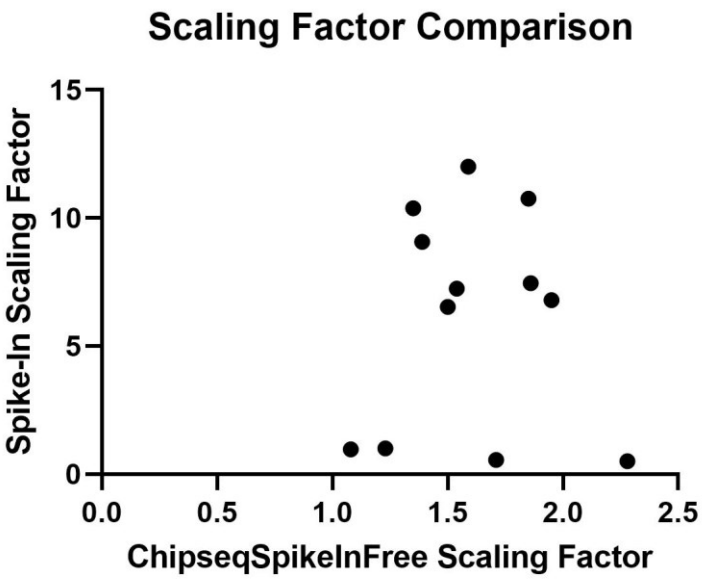

Figure 2. 7: ***Comparison of scaling factors derived from either the algorithm ChIPseqSpikeInFree or from a spike-in standard****. The same published ChIPseq data was used to produce both sets of scaling factors (Maganti et al., 2018). No correlation was found between the scaling factors produced by the different methods.*

2.4 – Discussion

Histones are nuclear proteins that are closely associated with DNA. These histones can be modified to form what is known as the histone code, a multifaceted program to control transcription and gene expression (Hansen et al., 1998; Jaskelioff & Peterson, 2003). Many of the PTMs that form this histone code, such as histone acetylation, rely on cosubstrates that are derived from cellular metabolism (Reid et al., 2017). Exactly how histone acetylation is affected by changes to metabolic circumstance and how it may affect myoblast function is not known. The establishment and validation of a cell culture model for studying histones and specifically histone acetylation is an important first step towards answering some of these questions.

The initial step required to study histones is to lyse the cells that contain them and remove them into a sample that can be analyzed. Seven different methods of histone isolation were investigated and assessed based on their ability to produce a consistent and histone-rich sample (**Figure 2.1**). These methods were built upon previously published protocol for isolating histones for PTM analysis (Shechter et al., 2007; Sidoli et al., 2016). The main contention during the investigation was whether or not isolating histones from a cell requires a stepwise process of first isolating the nuclei then extracting the histones, or if the histones can be directly extracted from whole cells by using sonication. The latter whole-cell extraction method was originally incorporated into the experiment to give a point of comparison that would allow us to judge if histones were being lost from the other samples during the extraction process. However, sonicating whole cells turned out to be the best option for our needs. All histone

isolation methods that were assessed produced a sample that gave an H3 signal when immunoblotted. The whole-cell lysate gave the strongest signal normalized to the initial cell count, suggesting that the other methods either failed to liberate some of the histones from the nucleus or lost the histones at some point in the isolation process. If the acetylation state of the histone is heterogeneous across the histone population and the procedure chosen to isolate them does not extract them all, it is possible that the procedure may select for certain histones with specific acetylation levels. For example, histones that are acetylated to a greater extent will have weaker interactions with DNA and may be extracted more easily than less acetylated histones. This would bias results towards acetylated histones. As such, the whole cell lysate procedure was chosen since its high H3 signal was indicative that it retrieved the most histones from the cells, and the small number of steps required for the procedure limits opportunities for histones to be lost or degraded during the workup. Using a whole-cell lysate will therefore result in more dependable results that are not affected by differences in isolation efficiency or be as prone to bias.

To have a positive control for histone acetylation in future experiments we cultured and treated cells with sodium butyrate, a class I and II HDAC inhibitor, then isolated their histones. Treatment of myoblasts with 5mM NaB for 24h produced a clear increase in histone acetylation when compared to untreated cells grown in normal growth media (**Figure 2.2**). The use of this treatment to investigate the cellular phenotype produced by histone hyperacetylation is limited, however. Treating myoblasts with 5mM of NaB caused them to elongate and resulted in a large proportion of cells that detached from the cell culture dish after 24h. These two

changes to the cell culture were taken as an indication that the treatment had some deleterious effect on the health of the myoblasts and so it was not used as a potential treatment to investigate the role of histone acetylation on cell fate. Nonetheless, extract from cells treated with 5mM NaB are a robust positive control for histone acetylation.

To determine the acetylation state of histones by immunoblotting, it is ideal to examine both histone protein levels and their acetylation status using the same immunoblot. Using the same blot for both measurements minimizes errors that may be introduced by differences in western blot loading. To do this we validated the use of a stripping procedure by interrogating the ability to completely remove the initial signal from the blot before re-probing with the next antibody (**Figure 2.3**). After each 30 min stripping step the blots were once again washed, blocked, then incubated with secondary antibody and imaged. After a total of one hour of stripping at room temperature, all traces of the signal from the first round of antibody blotting were successfully removed. This led us to the conclusion that a one-hour stripping procedure will successfully remove the antibodies from the blot in our future standardized experiments.

The last step in validating our cell culture model of histone acetylation was to investigate the variability of histone acetylation under baseline conditions. The histone isolation with the acid extraction protocol (**Figure 2.1**) successfully extracts histones from the nuclei of myoblasts and was used for some initial experiments, but since it increased the yield of histones per sample later experiments used the whole-cell isolation protocol. Following western blotting there exists a banding pattern near the 15kDa range which represents the

proteins of histones H3, H2B, H2A, and H4 (highest to lowest) which is unique to histones. This band pattern can be taken as evidence for the presence of histones (Shechter et al., 2007). The presence of histones at this location of the blot can also be corroborated by other blots (**Figure 2.5**) that use an anti-H3 antibody that gives a band at the same height as the H3 band from the previous pattern's signal. The switch to using antibodies directed at H3 acetylation versus general acetyl-lysine antibodies was done because assays run with the general acetyl-lysine antibodies utilized ponceau staining for protein content quantification. Ponceau is a non-specific protein stain and since we were moving towards using a whole-cell lysate instead of isolated nuclei from which histones were then acid extracted, we became less confident in the reproducibility and robustness of this non-specific method. Alternatively, focusing on the acetylation state of H3 then using this information to suggest changes to global histone acetylation is an accepted practice (Galdieri & Vancura, 2012; Pavlidou et al., 2019; Vogelauer et al., 2000; Yucel et al., 2019), though it does have some limitations in this context. Specifically, if the acetylation state of histone H2A, H2B, or H4 is affected by the metabolic conditions of an experiment to a different extent as H3, then our assays will not be able to detect this difference. Future work may need to further interrogate the other core histones for different metabolic responsiveness.

Histone acetylation in primary cultured myoblasts does not exhibit significant mouse to mouse variability nor does it vary between cells analyzed at different low passage numbers. The latter point may not hold true at late passage numbers when the myoblasts may begin to progress towards senescence, so the work herein will be done within these low passage

numbers. The combined protocol for isolating and culturing myoblasts, then extracting histones from them is a reliable way to investigate changes to histone acetylation in cell culture.

The proliferation rate of myoblasts isolated from different mice is very similar and clearly demonstrates the three phases, lag, exponential, and plateau, expected of rapidly dividing cells (**Figure 2.6**). If cells from different mice are plated at similar confluency, they can be expected to grow at a similar rate. This is extremely important for experiments where counting the number of cells every day to make sure that they are similar across treatment groups may not be feasible. By knowing that they will proliferate at a similar rate we can be much more confident that we are not comparing cells of grossly differently confluency, which may alter their epigenetic landscape or protein expression.

In an effort to corroborate our future analysis of general histone acetylation levels in cell culture experiments, we also explored the idea of examining general histone acetylation using existing myoblast chip-seq datasets. To facilitate this endeavour we attempted to validate the scaling factor algorithm ChIPseqSpikeInFree (Jin et al., 2020) by first replicating the results of the publication then by applying the algorithm to a dataset that utilized a spike-in standard for comparison. During the replication phase in which we attempted to reproduce the results from the source paper's benchmark analysis, we were initially unable to reproduce all of the reported scaling factors. After contacting the paper's author we were informed that the problem could be due to poor enrichment, broad peaks, or small library size of the ChIPseq dataset. However, these problems could be corrected by manually setting a correction

parameter “cutoff_1stTurn”. This per-sample parameter setting, which must be set to achieve the non-replicated results, was not documented in association with the source paper’s published results. This raised some replicability concerns for us, but the ability to replicate most of the results indicated that we were using the software correctly and gave us confidence in moving on to the application phase of validation.

To validate the algorithm's ability to produce accurate scaling factors we applied it against a published ChIPseq dataset that included spike-in-determined scaling factors (Maganti et al., 2018). The original data analysis for this publication was generated by the Ottawa Bioinformatics Core Facility (OBCF). Contact with the OBCF gave us confidence in the results and that the samples do not have poor enrichment, broad peaks, or a small library size because these factors may impede the application of the ChIPseqSpikeInFree algorithm. Unfortunately, the scaling factors produced by the algorithm had no correlation with those produced using a spike-in standard. Though it is expected that there would be some differences between the scaling factors produced by the different methods, the algorithm completely failed to reflect the much higher scaling factors calculated using the spike-in standard. Thus, we were not able to move forward with further applications of the algorithm.

2.5 – Conclusion

Cultured myoblasts isolated from the hindlimbs of NTacC57BL/6 mice are a reasonable model for cell culture studies of histone acetylation. Extraction of histone by sonication results in a whole-cell lysis that is rich in histones and minimizes histone loss compared to more complicated isolation procedures. Histone acetylation in cultured primary myoblasts is

consistent between different mice and over multiple early passages. These cells display the canonical lag, exponential, and plateau phases of proliferation. This was determined through automated cell counting using a Zeiss Cell Discoverer 7, which is an efficient and accurate way to measure proliferation rates in cultured myoblasts. Our evaluation of the ChIPseqSpikeInFree algorithm found it inaccurate in estimating scaling factors compared to those generated using a spike-in standard.

The work in this section is not making any research claims and therefore does not have any limitations *per se*. The conclusions drawn do not seek to make statements about anything outside of this particular cell culture model, and therefore have no assumptions that need to be stated about broader implications. Future work may be needed to establish the same baseline consistency (i.e., consistent histone acetylation between mice and low passage numbers) when using other methods of analysis such as ChIP-seq or proteomics. (Brockers & Schneider, 2019)

Chapter 3: Histone acetylation in primary cultured myoblasts is sensitive to the availability of exogenous glutamine but not pyruvate or glucose.

3.1 – Introduction

Myogenesis requires the effective differentiation of myoblasts into functional muscle tissue. This entails an orchestrated cascade of cell fate decisions that facilitates the repair of damaged tissue or growth of muscle. Activated myoblasts initially proliferate before undergoing myogenic differentiation directed by a cascade of cellular signals (Alter et al., 2008; Conboy et al., 2003; Flores et al., 2014; Gillespie et al., 2009; Guttridge et al., 1999; Jang & Baik, 2013). A balance must be reached between proliferation, to create enough cells for the myogenic process, and differentiation, to contribute mature proteins to the myogenic process. The differentiation process is regulated by genetic and epigenetic factors that determine the expression patterns of myogenic genes. Part of the epigenetic regulation includes the enrichment of histone acetylation after myoblast activation, following the expression of MyoD (Khilji et al., 2018).

The most basic unit of chromatin is the nucleosome, which is composed of 146 base pairs (bp) of DNA wrapped around a histone octamer. This octamer consists of two of each of the core histones H2A, H2B, H3, and H4 (Brockers & Schneider, 2019). Acetylation can occur on the tails of any of the core histones and is a fundamental epigenetic modification that can help regulate gene transcription (Hansen et al., 1998). This modification is regulated by the equilibrium established by lysine acetyl transferases (KATs) and lysine deacetylases (KDACs) which add or remove acetyl groups, respectively. Histone acetylation is an important regulatory component of epigenetics that requires histone acetyltransferases (HATs), a subset of KATs, and the cosubstrate acetyl-CoA. Histone acetylation can provide a binding recognition site for

bromodomain-containing proteins, alter chromatin subnuclear structure, and weaken the interaction between histones and associated DNA (Brown et al., 2008; Clayton et al., 2006; Li et al., 2007; Schneider & Grosschedl, 2007). Histone acetylation is a major part of a larger transcriptional regulatory mechanism known as the histone code which encompasses all posttranslational modifications (PTMs) to histone protein primarily focused around their unstructured ends knows as histone tails. Many of these PTMs, including histone methylation and acetylation, require metabolite cosubstrates that are derived from cellular metabolism and, in some cases, can be limited in quantity. This allows for the possibility that changes to the availability of these metabolite cosubstrates might change the extent of epigenetic PTMs. If this is true, then it provides a mechanism for cellular metabolism to directly drive epigenetic changes in cell fate and function. A mechanism that is theoretically likely but has yet to be fully delineated.

The acetylation of histones requires the presence of an acetyl group containing substrate, in the form of acetyl-CoA, that will be transferred onto the histone by a HAT. Acetyl-CoA is an acetyl group that has been primed for reaction through association with coenzyme A. The association puts the acetyl group into an energy state that makes the acetylation of other proteins favourable (Lipmann & Kaplan, 1946). The physiological concentration of acetyl-CoA is rate-limiting to HAT activity, thus fluctuations in acetyl-CoA levels can alter the rate of HAT activity (Reid et al., 2017). The sensitivity of HATs to the concentration of acetyl-CoA has been investigated with yeast models within a nutrient-limited setting. When acetyl-CoA levels are elevated, the yeast genome becomes hyper-acetylated with many of these modifications affecting genes related to cell division and expansion of the yeast population (Kaelin &

McKnight, 2013). The importance of acetyl-CoA and acetylation has also been investigated to some extent in cell culture models of stem cells. Pluripotent stem cells have a 2-fold higher concentration of acetyl-CoA compared to differentiating stem cells, and this seems to contribute to the epigenetic regulation of their stemness (Moussaieff et al., 2015). We, therefore, hypothesize that modulating acetyl-CoA pools in primary myoblasts, a mitotic cell with differentiation capacity, may change histone acetylation levels and subsequently influence their fate and function. This work can help us understand the link between acetyl-CoA substrate availability and myoblast function, which could lead to novel therapies for deteriorating muscle in myopathies or aging.

The link between histone acetylation and the metabolic landscape of cultured, proliferating primary myoblasts requires some consideration. Proliferating cells must balance their use of acetyl-containing metabolites between energy production, macromolecule synthesis, and regulatory events such as histone acetylation (Song et al., 2019). The concentration of acetyl-CoA is an amalgamation of the availability of different precursor metabolites, such as glutamine or pyruvate, for its synthesis. Different cellular compartments, however, have different acetyl-CoA pools. The nuclear and cytosolic acetyl-CoA pools have a limited capacity for exchange making them functionally separate (Bulusu et al., 2017). This is because acetyl-CoA's polarity and complicated chemical structure prevent it from readily diffusing across biological membranes such as the nuclear envelope (Kaelin & McKnight, 2013). Since only nuclear acetyl-CoA can contribute to histone acetylation we must consider what metabolites, and sub-cellular pathways, might be able to influence the nuclear pool.

Glutamine can be used for energy production by the mitochondria and can regenerate pools of metabolites used for macromolecule synthesis or regulatory events through anaplerotic reactions (Yang et al., 2014; Yang et al., 2009). This includes the biosynthesis of nucleotides, glutathione synthesis for redox regulation, amino acid synthesis, histone and DNA methylation, and the generation of alpha-ketoglutarate for the TCA cycle (Nguyen & Durán, 2018). Glutamine is a prominent source of carbon and nitrogen in proliferating cells (Daye & Wellen, 2012) that is generally considered essential for rapidly proliferating cells, similar to myoblasts, to grow in culture (Rubin, 2019). Glutamine deprivation has been examined in cancer cells as it can result in cell-cycle arrest and the activation of apoptosis (Chen & Cui, 2015). By contributing carbon to the tricarboxylic acid (TCA) cycle, glutamine frees up other sources of carbon, such as glucose, for use in producing acetyl-CoA (DeBerardinis et al., 2007). This may be part of the mechanism by which glutamine can increase histone acetylation. As another potential mechanism for influencing histone acetylation, glutamine can be transformed into citrate by the TCA cycle in the mitochondria and then sent to the nucleus where it can add to the acetyl-CoA pool. In the nucleus, citrate is converted to acetyl-CoA by ATP citrate lyase (ACL) and is important for maintaining histone acetylation since silencing of ACL in mammalian cell lines results in decreased histone acetylation (Wellen et al., 2009). Modulating glutamine concentrations under different cellular states such as proliferation or differentiation could contribute to changes in histone acetylation in myoblasts.

Another well-known synthesis pathway of acetyl-CoA is through the decarboxylation of pyruvate. Pyruvate is the end product of glycolysis and can be transported into the nucleus, rather than the mitochondria, where it is directly converted into Acetyl-CoA by the pyruvate

dehydrogenase complex (PDC) and used for histone acetylation (Sutendra et al., 2014). Therefore, pyruvate may be able to alter nuclear acetyl-CoA levels and subsequently histone acetylation (Sutendra et al., 2014). Similarly, glucose availability may also alter histone acetylation because of its important role in energy production in the cell and its ability to be metabolized via glycolysis to pyruvate and subsequently acetyl-CoA. Promoting glycolysis can increase the expression of HATs such as GCN5, lending further credence to the ability of these metabolites to influence epigenetic events (Sajjanar et al., 2019). The evidence is growing to support that metabolites such as pyruvate and glucose play a role in stem cell regulation through metabolic modulation (Song et al., 2019). The importance of these metabolites in cell culture is obvious, however, the potential for manipulation of their concentrations to alter histone acetylation and cell fate or function is not. By investigating how metabolite concentrations influence histone acetylation, it may be possible to change or improve myoblast function during important periods of myogenesis.

Since the activity of HATs is sensitive to the availability of acetyl-CoA (Reid et al., 2017), and acetyl-CoA participates in intermediary metabolism (i.e., compounds that are produced and consumed throughout metabolic pathways but are not end products) (Kaelin & McKnight, 2013), it may be possible to change histone acetylation by altering metabolism and the metabolites that feed into acetyl-CoA pools. If cell fate decisions can be controlled this way then this may constitute an avenue to address disease phenotypes. The work presented in this section investigates how metabolism can affect global H3 acetylation by subjecting primary myoblasts to different concentrations of glucose, pyruvate, and glutamine.

3.2 – Materials and Methods

Metabolite Treatment

Primary myoblasts isolated from mice, as described in **Section 2.2**, were grown in growth media on plates coated with 0.01% collagen. Three separate primary myoblast cell lines, derived from three mice, were grown to approximately 60% confluency under normal conditions. At this point, the growth media was replaced by Ham's F10 media lacking either glucose, pyruvate, or glutamine (Wisent inc.). The missing metabolite for each growth media was then supplemented back at varying concentrations to create 5 different media preparations for each missing metabolite that ranged from low to high when compared to the standard media. The concentrations of glucose, pyruvate, and glutamine in standard media are 25mM, 1mM, and 2mM respectively. In total, 15 treatments were tested for their ability to change histone acetylation. These treatments are outlined in **Table 3.1**. The cells were grown in the treated media for 48 hours before they were scrapped and frozen in RIPA at -80°C for later analysis. All media used are supplemented with 20% v/v bovine calf serum (Sigma Life Sciences), 1% v/v penicillin/streptomycin (Sigma Life Sciences), and 0.005% FGF (Abcam).

Table 3. 1: Metabolite concentrations used for each treatment type.

	Metabolite Treatments														
Metabolites (mM)	Glucose Treatment					Pyruvate Treatment					Glutamine Treatment				
Glucose	2.5	12.5	25	50	125	25	25	25	25	25	25	25	25	25	25
Pyruvate	1	1	1	1	1	0.1	0.5	1	5	10	1	1	1	1	1
Glutamine	2	2	2	2	2	2	2	2	2	2	0.2	1	2	10	20

Proliferation Rate with Metabolite Treatment

Cultured myoblasts from three mice were plated onto 6-well collagen-coated plates at low confluency. The same media preparations used for the pyruvate and glutamine treatment experiments, as seen in **Table 3.1**, were used in this experiment. The cells were first counted 24 hours after the initial plating. This was done to allow the cells time to adhere to the 6-well plates. Every subsequent 24 hours, the media in each well was replaced with new media containing the same concentration of metabolites as before. Immediately after media replacement, the cells on the plate were counted. This was done to reduce the amount of floating material in the media so that it would not interfere with cell counting. The cells were counted using a Cell Discoverer 7 (Zeiss) as was previously described above (See **Section 2.2** for details).

The cells were allowed to proliferate and were counted until they reached the plateau phase of cell growth. This was determined by having a similar cell count for subsequent days after the plates reached confluency >100%. Once counting was concluded the proliferation rate for each cell line under each treatment was calculated for the exponential phase. This was then plotted against the concentration of pyruvate or glutamine that was used to grow the cells. Separately, the proliferation rate was plotted against the corresponding histone acetylation level of the cells that were subject to the same pyruvate or glutamine treatment.

Statistical Methodology

An unpaired, two-tailed student's *t*-test was used to analyze histone acetylation between concentrations of metabolites. Statistical significance was assessed based on a *p*-value

< 0.05. Linear regressions were performed using a 95% confidence interval to determine relations between variables.

3.3 - Results

Acetylation with Metabolite Treatment

To determine if changing the concentration of acetyl-CoA generating metabolites in growth media can alter a cell's histone acetylation state, three primary myoblasts cell lines were treated with varying levels of either glucose, pyruvate, or glutamine. The level of histone acetylation in mouse primary myoblasts was measured after they were grown in media containing either 2.5mM, 12.5 mM, 25mM, 50mM, or 125mM glucose for 48 hours (**Figure 3.1A**). The normal HAM's F-10 growth media used in cell culture has a glucose concentration of 25mM. No significant difference in histone acetylation was found between cells grown in 2.5mM glucose and 25mM glucose treatment groups (*p*-value=0.75, two-tailed *t*-test), or 125mM glucose and 25mM glucose treatment groups (*p*-value=0.36, two-tailed *t*-test). A linear regression of the data collected from three animals returned an R^2-value of 0.07. Thus, no correlation or significant changes were observed in myoblast histone acetylation, despite an almost fifty-fold difference in the amount of glucose available to the cells.

To investigate whether histone acetylation can be modulated through changing the availability of exogenous pyruvate, cells were grown in media containing either 0.1mM, 0.5mM, 1mM, 5mM, or 10mM pyruvate for 48 hours (**Figure 3.1B**). No significance was found between the histone acetylation of the 10mM treatment group and the 1mM treatment group (*p*-

value=0.66, two-tailed t-test), which is the standard concentration of pyruvate in normal HAM's F-10 growth media. Treatment of cells with 0.5mM pyruvate resulted in the lowest average for histone acetylation, however, no significance was found between the 0.5mM pyruvate and 1mM pyruvate treatment groups (*p*-value=0.46, two-tailed *t*-test).

Glutamine is an anaplerotic metabolite required for proliferating cells (Brockers & Schneider, 2019). To investigate how glutamine availability effect histone acetylation, cells were grown in media containing either 0.2mM, 1mM, 2mM, 10mM or 20mM glutamine for 48 hours. In normal growth media, the concentration of glutamine is 2mM. Interestingly, when cells were treated with different concentrations of glutamine, the cells exposed to lower concentrations of glutamine had higher levels of histone acetylation. Cells treated with glutamine concentration less than 2mM showed increased histone acetylation, whereas higher concentration seemingly had no effect (**Figure 3.1C**). Cells treated with 0.2mM glutamine had significantly higher histone acetylation compared to the 2mM glutamine treatment group (*p*-value=0.042, two-tailed *t*-test). This is contradictory to the assumption that increasing glutamine availability will increase acetyl-CoA levels, thereby increasing histone acetylation.

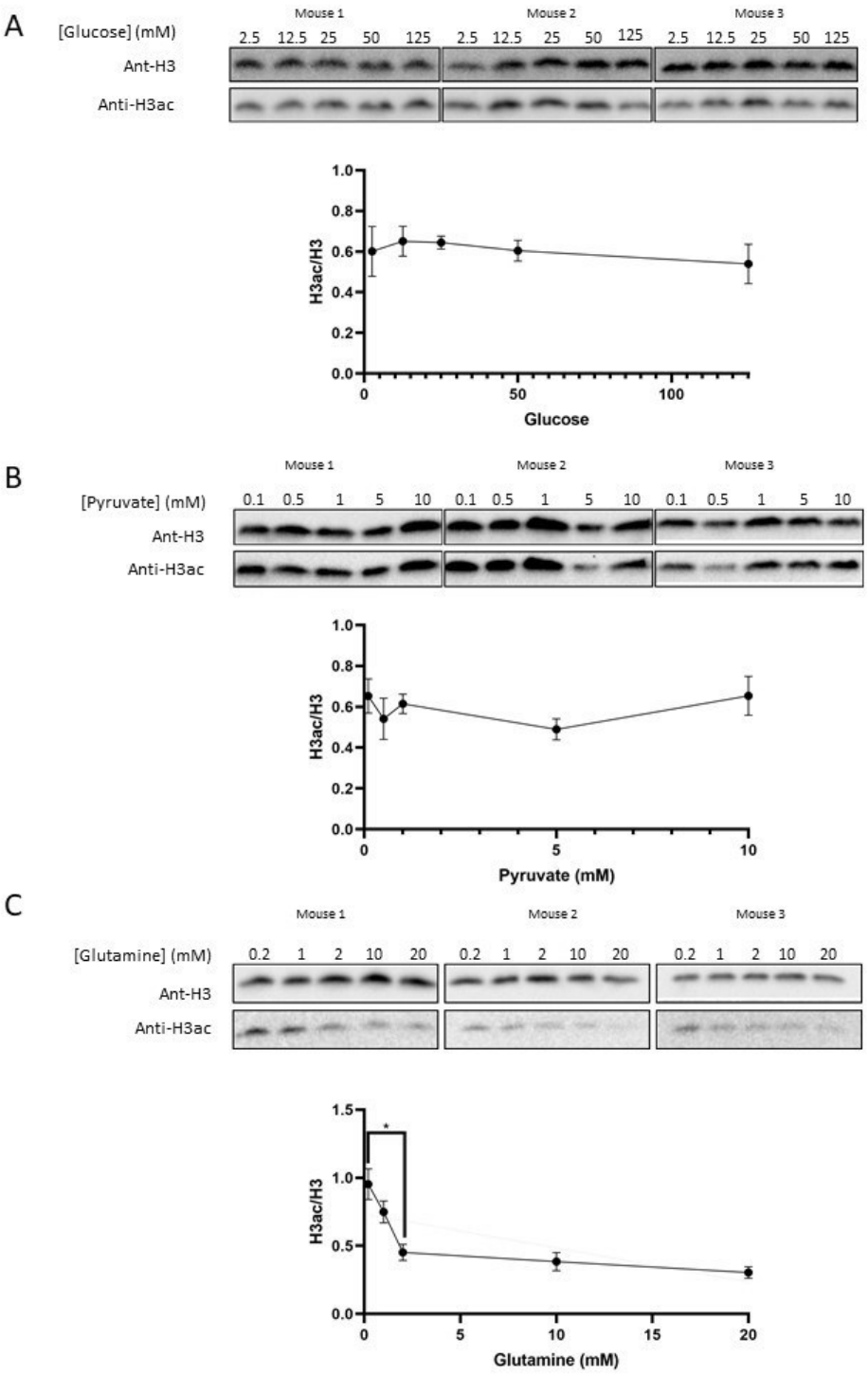

*Figure 3. 1**: Histone Acetylation with metabolite treatment**. Cultured myoblasts were treated with different concentrations of either glucose, pyruvate, or glutamine to investigate how this would affect the acetylation state of their histones. Data is presented at mean ± SEM (n=3). **A)** Cells were treated with 2.5, 12.5, 25, 50, or 125mM glucose for 48h. 25mM glucose is the standard concentration of glucose in the growth media. **B)** Cells were treated with 0.1, 0.5, 1, 5, or 10mM of pyruvate for 48h. 1mM pyruvate in the standard concentration of pyruvate in growth media. C) Cells were treated with 0.2, 1, 2, 10, or 20mM glutamine for 48h. 2mM glutamine is the standard concentration of glutamine in growth media. * p-value<0.05 (two-tailed unpaired t-test, n=3)*

Proliferation Rate with Metabolite Treatment

To investigate how the availability of pyruvate and glutamine might impact myoblast proliferation, primary myoblasts were cultured with different concentrations of each metabolite. The proliferation rate of myoblasts is not correlated with the concentration of pyruvate in the growth media (**Figure 3.2B**, two-tailed *t*-test, *p*-value=0.54). The slope of the linear regression for these parameters was 0.01 suggesting a very small effect of pyruvate on proliferation rate if any exists at all within these concentrations. Furthermore, the small slope increases the likelihood that small variations in the measurement of proliferation rate, and not a biological response from the cells to the different concentrations of pyruvate, are causing the trends seen in the data. In standard growth media the concentration of glutamine is 2mM. This experiment showed that if the concentration of glutamine exceeds 1mM the proliferation rate of myoblasts declines (**Figure 3.2A**). The proliferation rate of primary myoblasts in cell culture is negatively correlated (two-tailed *t*-test, *p*-value=0.009) between glutamine concentration and proliferation rate. The trend above 2mM glutamine shows an obvious decline with increasing glutamine concentrations and is likely responsible for the negative correlation of the linear regression. Between 0.5-2 mM glutamine, the correlation between metabolite concentration and proliferation is less obvious. All cell lines have a higher relative proliferation at 1mM glutamine compared to 0.5mM or 2mM, and this may represent an optimal glutamine concentration for proliferating primary mouse myoblasts. This may be due to some deleterious effect of glutamine on proliferation at higher concentrations, and a negative effect on proliferation because of a lack of nitrogen-containing substrates at the lowest concentration.

Increased histone acetylation has previously been shown to correlate with the expression of genes associated with population expansion in yeast (Kaelin & McKnight, 2013). To investigate if this correlation remains true in the context of myoblast proliferation, the proliferation rate of myoblasts grown in different concentrations of pyruvate or glutamine was plotted against histone acetylation data for each of the corresponding treatment concentrations. Cells with higher levels of histone acetylation under a specific metabolite treatment had larger proliferation rates under the same metabolite treatment. Histone acetylation and proliferation rate has a significant positive correlation (two-tailed *t*-test, n=29, *p*-value=0.0367, **Figure 3.3C**). This analysis agrees with the previous findings on the effect of glutamine and pyruvate on the proliferation rate. The glutamine treatment of cells altered histone acetylation levels and also altered proliferation rate. However, pyruvate treatment did not alter either.

Whether acetylation increases proliferation, proliferation increases acetylation, or both occurring together is a coincidence is not addressed by this experiment and requires further investigation.

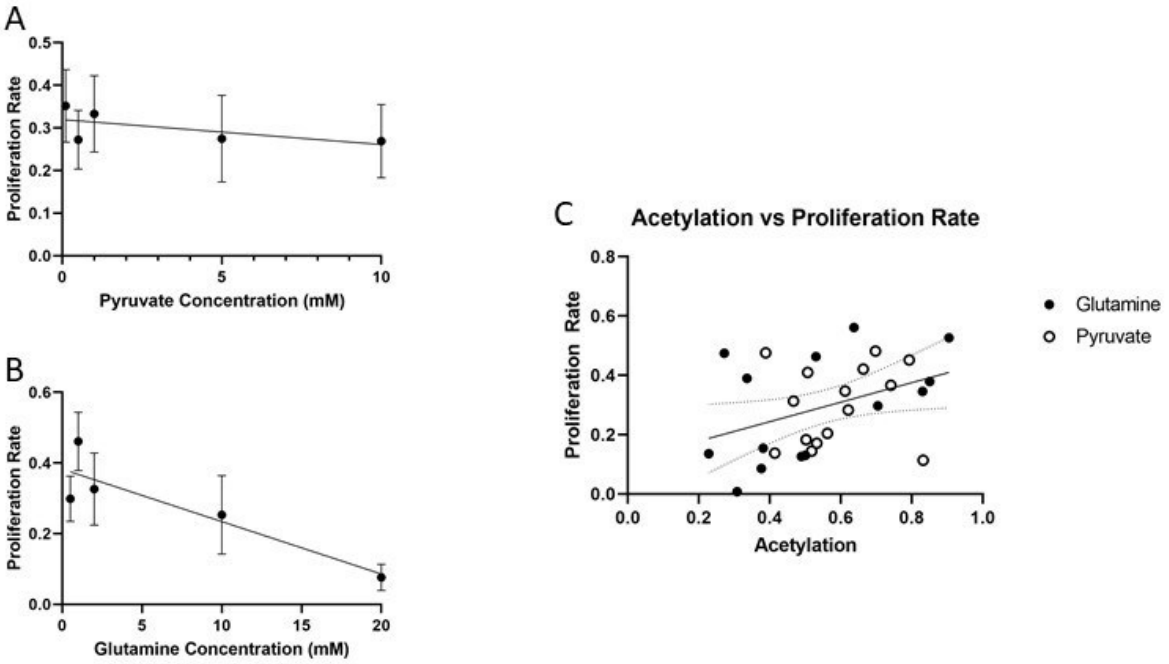

Figure 3. 2: ***Proliferation with metabolite treatment****. Different concentrations of glutamine and pyruvate were used to culture myoblasts. Their proliferation was monitored using the same protocol as in Chapter 2. The exponential phase of their growth curves was used to measure their proliferation rate and was plotted against glutamine concentration. A) Cultured myoblasts were grown in 0.1, 0.5, 1, 5, 10mM pyruvate. Linear regression determined the relationship between pyruvate and proliferation rate to be non-significant (two-tailed t-test, p-value=0.54, n=3). Data represented as mean ± SEM. B) Cultured myoblasts were grown in 0.2, 1, 2, 10, and 20mM glutamine. The relationship between glutamine and proliferation rate was significant based on linear regression (two-tailed t-test, p-value=0.0092, n=5). Data represented as mean ± SEM. C) Data was pooled from both proliferation rate experiments with glutamine and pyruvate along with the corresponding histone acetylation experiments (Fig. 3.1) to investigate the relationship between proliferation and histone acetylation. Linear regression was performed to estimate a line of best fit (solid) and 95% interval (dotted) for the data. There was a significant relationship between acetylation and proliferation rate (two-tailed t-test, p-value=0.0367, n=29).*

3.4 – Discussion

The original motivation for treating myoblasts with various metabolites was to increase the availability of acetyl-CoA for histone acetylation events. Histone acetylation is correlated with the progression of myoblasts through commitment and differentiation (Khilji et al.), so providing ample substrates for acetylation may facilitate differentiation and improve the myogenic process. Mechanistically, changes to metabolite concentration can change the availability of metabolite cofactors required for histone acetylation and this should change the acetylation state of histones (Reid et al., 2017). To investigate this, myoblasts from three mice were isolated and grown in media containing different concentrations of either glucose, pyruvate, or glutamine to assess their effect on H3 acetylation (**Figure 3.1**). H3 acetylation is a common measurement used to investigate histone acetylation (Galdieri & Vancura, 2012; Pavlidou et al., 2019; Vogelauer et al., 2000; Yucel et al., 2019). The normal concentrations for each of these metabolites were based on the concentrations of each that are normally found in HAM's F-10 growth media. It is assumed that lowering the concentrations of these metabolites does not represent a caloric restriction to the cell as there are other nutrients in excess within the growth media for the cell to use. The metabolite treatment should represent a mild change to the cellular environment and not a stressor that might elicit some sort of stress-response or caloric restriction response from the cell. The goal of the experiments is to investigate the cell's reaction to an altered metabolite landscape, not to understand the cell's ability to cope with adverse conditions nor to simulate the fasting versus fed states of a cell.

Metabolite treatment experiments with glucose and pyruvate did not show any obvious correlation between metabolite concentration and H3 acetylation. None of the treatment concentrations of glucose or pyruvate reached statistical significance when compared to the normal concentration (**Figure 3.1**)**.** The normal concentrations for glucose and pyruvate are 25mM and 1mM respectively. Since histone acetylation events catalyzed by HATs are sensitive to changes in concentration of their cosubstrate acetyl-CoA this is an interesting result (Brunet & Rando, 2017; Reid et al., 2017). The presence of high concentrations of glucose or pyruvate was hypothesized to increase the available carbon pool for acetyl-CoA production and thereby increase the rate of histone acetylation. There are four possible explanations for the lack of increased histone acetylation for these treatments: 1) Acetyl-CoA derived from glycolysis is not a rate-limiting factor for histone acetylation. 2) The extra substrates are metabolized to chemical species other than acetyl-CoA, or potentially fully oxidized to CO_2. 3) Acetyl-CoA is formed in the cytosol and consumed by cytosolic protein acetylation, steroid synthesis, and fatty acid synthesis before it can increase nuclear acetyl-CoA concentrations (Pietrocola et al., 2015).-4) the substrates increase acetyl-CoA levels and raise nuclear acetyl-CoA concentration, but histone deacetylation activity increases in a compensatory mechanism. Previous evidence in hyperglycemic conditions (50mM glucose), similar to our high glucose treatment group, suggests that hyperglycemia can induce the expression of acetyltransferase p300, which would increase histone acetylation level (Pirola et al., 2010). This work was done in human umbilical vein endothelial cells, so generalizations to mouse myoblasts cannot be made. Since we did not observe elevated H3 acetylation, it could be that the cells were not incubated long enough to elicit a similar hyperglycemic reaction. Previous work cultured cells in hyperglycemic conditions

for 22 days, however, a minimum time required for the effect was not investigated (Pirola et al., 2010; Roy et al., 1990). This hyperglycemic response would likely represent a stress response more so than the ability of metabolites to directly affect epigenetic regulation through cosubstrate availability. Alternatively, the extra glucose could be metabolized rapidly, as suggested above, in primary myoblasts. As a product of glycolysis, a similar explanation may be true for pyruvate. The cell may simply have other uses for the extra glycolytic metabolites that prevent them from contributing to histone acetylation or a longer treatment time is needed for the effects on histone acetylation to become apparent.

Glutamine is the most abundant free amino acid in the human body, and glutamine starvation is associated with cell stress and apoptosis (Dominique, 2017). Despite its abundance, glutamine is consumed rapidly by cells because it is involved in a wide array of metabolic functions (Dominique, 2017; Labow & Souba, 2000). Generally, glutamine's roles in the cell can be categorized into either nitrogen transport, redox regulation, use as a metabolic intermediate, or use as an energy source (Labow & Souba, 2000). Glutamine can also act as an anaplerotic metabolite that can be used to replenish TCA cycle metabolites when they are depleted by the cell shifting metabolic emphasis towards biomass production. Given that proliferating myoblast would require TCA metabolites to build new biomass, higher levels of glutamine would replenish these lost metabolites. If this were the case, then acetylation would be predicted to either decrease at lower glutamine concentrations (0.2mM and 1mM in **Figure 3.1C**) or stay the same, as the cell would not have excess acetyl-CoA for acetylation events. However, our results show that this is not the case as acetylation increases in myoblasts with reduced glutamine media concentrations. A possible explanation for these results is that higher

concentrations of glutamine (4mM) has been previously shown to inhibit pyruvate dehydrogenase complex (PDC) activity by inducing sirt4 to hydrolyze lipoamide cofactors from PDC, causing a decrease in acetyl-CoA levels (Kankotia & Stacpoole, 2014; Mathias et al., 2014). This would explain the relatively high histone acetylation levels at low glutamine concentrations when presumably the inhibition of PDC is lowest. This mechanism has been established in cancer models but has not been investigated in mouse myoblasts. Our result suggests that there is a baseline inhibition of PDC by glutamine at normal media concentrations of 2mM glutamine, which could be modulated to alter acetyl-CoA production from pyruvate. Thus, at glutamine concentrations between 2mM and 20mM, the mechanism inhibiting the flux of pyruvate through the PDC may limit the production of glucose and pyruvate derived acetyl-CoA. Additionally, 5mM glutamine concentrations have been shown to increase mitochondria uncoupling in cell culture models of human myoblasts, possibly contributing to lower histone acetylation, as substrates are fully oxidized in the mitochondria to compensate (Krajcova et al., 2015). If this is the case, the glutamine in the 2mM-20mM treatments would be consumed by anaplerotic reactions as the TCA cycle works to make up for the uncoupling of the mitochondria leaving little left to contribute to acetyl-CoA production. Thus, given that higher concentrations of glutamine can both inhibit the PDC and uncouple mitochondria, it is understandable that low glutamine concentrations would potentially favour the creation of acetyl-CoA, contributing to H3 acetylation.

The elevated acetylation levels at lower concentrations of glutamine also support the assumption that the cells have sufficient substrates under all metabolite treatments for regular energy production and acetylation. If this were not the case, then the decrease in available

metabolites caused by the lower glutamine concentration should have been detrimental to the cell, but instead, there were increases in H3 acetylation and proliferation.

Since proliferation is an important aspect of myoblast function, we sought to investigate how our metabolite treatments affected it. This would also confirm if there is a correlation between acetylation and proliferation as has been demonstrated in other models of histone acetylation (Kaelin & McKnight, 2013). The proliferation rate of primary myoblasts was measured under various concentrations of pyruvate and glutamine. The total number of cells in each treatment group was counted on subsequent days during the exponential growth phase and used to establish proliferation rates. These rates were then plotted against metabolite treatment concentration (**Figure 3.2A&B**) and H3 acetylation levels analyzed previously (**Figure 3.2C**). Glutamine has a greater influence over proliferation than pyruvate, but the mechanism for this remains unclear. It is counter intuitive that a decrease in available metabolites results in an increase in proliferation. Caloric restriction decreases the proliferation rate of most cell types in mice (Hsieh et al., 2005). However, caloric restriction is likely not applicable in this circumstance because the growth media used in these experiments is formulated to impose no metabolic limits on the cell. It is unlikely that the effect of the glutamine treatments on proliferation is the result of some change to caloric availability. As previously mentioned, higher glutamine concentrations promote mitochondrial uncoupling in human myoblasts (Krajcova et al., 2015). What was also noted in these studies is that mild hypoglutaminemia (0.3mM) provided optimal conditions for proliferation (Krajcova et al., 2015). On the other hand, murine derived C2C12 myoblasts exhibit enhanced proliferation when treated with 3mM alanyl-glutamine which breaks down into free alanine and glutamine in the cell (Liu et al., 2018).

However, this phenotype in C2C12 cells may be due to a synergistic effect between alanine and glutamine as supplementation with only one of the amino acids did not elicit the same result. The relationship between glutamine availability and proliferation remains poorly understood, but lower concentrations of glutamine between 0.2mM and 1mM may represent an optimal range for proliferation in cell culture.

There is correlation between acetylation and proliferation, but it is very weak. The data was not segregated by metabolites (i.e., whether the cells were treated with glutamine or pyruvate was not labelled) during the statistical analysis. Therefore, the correlation does not consider the potential effects of either metabolites, only the effects of acetylation. However, the experiment cannot draw any conclusion about causation between proliferation and acetylation.

3.5 – Limitations

Acetylation with Metabolite Treatment

The methods used herein to investigate the acetylation of histones under metabolite treatment measure global H3 acetylation. While measuring the acetylation of H3 has been used many times as an indicator of the general global acetylation state of histones (Galdieri & Vancura, 2012; Pavlidou et al., 2019; Vogelauer et al., 2000; Yucel et al., 2019), the western blotting technique is unable to differentiate which genes these histones are associated with. The treatments of cells with different concentrations of glucose, glutamine and pyruvate might have also altered the H3 acetylation status at specific genes without having affected total H3

acetylation. It is also possible that the sensitivity of our assay to measure H3 acetylation status was not great enough to measure more subtle fluctuations for each of the treatments. However, more sensitive measurements would have to be validated to ensure that any changes seen were not due to natural fluctuations in histone acetylation level. This would likely require a complete repeat of the validation of this histone acetylation model (see **Chapter 2**) using any new technique.

Pyruvate is produced from glucose during glycolysis. In the experiments that used varying concentrations of pyruvate, there may have been compensatory metabolic activity that allowed the cell to either increase or decrease glycolysis in order to regulate cellular pyruvate levels. The same may be true of the experiments that used different concentrations of glucose, wherein the cells could have used the normal levels of pyruvate in the media to compensate for low glucose concentrations. This however does not explain why elevated concentrations of the metabolites did not have our predicted effects on histone acetylation or any effect on proliferation. Future works should look into more specific mechanisms for preventing carbon derived from glycolysis from contributing to acetyl-CoA.

3.6 – Future Directions

Future work should focus on tracking changes to the flux of acetyl groups through metabolic pathways during the various treatments, possibly using metabolomics methods such as stable isotope tracing analysis (SITA) (Tumanov et al., 2015). This will allow a clearer understanding of how certain metabolites contribute to the pool of acetyl-CoA available for

histone acetylation and if these pools can be altered meaningfully by metabolite modulation. For example, SITA using labelled glucose could be performed in medias with different concentrations of glutamine to determine if glutamine changes acetylation by affecting the utilization of glycolysis derived acetyl-CoA for histone acetylation. Metabolomics may also reveal other unexpected contributions to the metabolism of the cultured cells from the growth media used. It could very well be that the cell uses alternative fuel sources in the growth media when glutamine levels are low, and this alternative fuel source causes the increased histone acetylation. Along the same lines, further delving into the details of which histones had their acetylation state changed by the metabolite treatment may yield interesting results regarding metabolism's ability to alter the epigenome of myoblasts. ChIP-seq experiments with spike-in standards would be a great way to interrogate this question further and would also address if gene specific changes to histone acetylation are occurring. Under these circumstances, spike-in standards are required to normalize between treatment groups since the change in total histone acetylation renders standard normalization protocols inaccurate (Chen et al., 2015).

3.7 – Conclusion

Metabolism plays an important role in the regulation of histone acetylation through the availability of metabolite cofactors. Cell culture experiments on primary myoblasts isolated from mice showed that changes to glucose and pyruvate concentrations in growth media does not affect the global acetylation state of H3. The concentration of glutamine, however, does impact histone acetylation with lower concentrations of 0.2mM glutamine increasing histone

acetylation compared to 2mM glutamine. Further investigation is required to elucidate the mechanism for this increase in histone acetylation and if higher concentrations of glutamine somehow inhibit acetylation. Acetylation and proliferation appear to be positively correlated in primary mouse myoblasts.

Chapter 4: Myoblast histone hyperacetylation induced by glutamine depletion is lost during differentiation and does not affect the expression of contractile proteins.

4.1 – Introduction

Differentiation is paramount for both the myogenic process during development and for muscle regeneration following damage (Dumont, Bentzinger, et al., 2015). Myoblasts, cells derived from activated muscle stem cells (SCs), must differentiate to produce the proteins and cellular structures required to form mature myotubes. Multiple histone modifications control gene expression during differentiation that can be altered by muscle regulatory factors (MRFs) (Asp et al., 2011). These histone modifications contribute to the global changes of the epigenome that occur during differentiation (Asp et al., 2011). For example, MyoD positively regulates muscle gene expression during differentiation and MyoD binding coincides with histone acetylation (Cao et al., 2010). Specific histone acetylation influences transcriptional rates of genes during myogenesis as histones associated with muscle specific genes are known to be hyperacetylated during differentiation (Khilji et al., 2018). This specificity is achieved by myogenic transcription factors, such as p300, directing histone acetyltransferase (HAT) activity (Bergstrom et al., 2002; Khilji et al., 2018). All these processes are influenced by histone acetylation which, as we have previously demonstrated, can be altered by decreasing the glutamine content of the growth media from 2mM to 0.2mM (**Chapter 3**).

Increased histone acetylation correlated positively with proliferation rate as well. Therefore, modulating histone acetylation using low glutamine media may promote proliferation and potentially inhibit differentiation, which is a common element of pro-proliferative mechanisms (Alter et al., 2008; Gillespie et al., 2009). Alternatively, increased histone acetylation may not drive proliferation so much as it facilitates the process. Histone

acetylation may be a context specific driver of cell fate. If this is the case, then increased histone acetylation may also facilitate and improve differentiation. To investigate this, we differentiated myoblasts under various circumstances with low glutamine media. By increasing general acetylation, it may be possible to alter muscle specific gene expression and influence the myogenic process.

The change of individual histone marks during differentiation has been previously examined (Asp et al., 2011; Cao et al., 2010). However, we sought to determine how global histone acetylation changed during differentiation. Additional investigations were performed to determine if global histone acetylation could be modulated with glutamine treatment during differentiation and if this altered the capacity or progression of differentiation in some manner.

4.2 – Materials and Methods

Induction of Differentiation Using HAM's F-10 Media

Myoblasts, isolated using the protocol previously mentioned (See **Section 2.2**), were cultured to 80% confluency in growth media. They were then switched into media containing 5% horse serum (Wisent inc.). This media is made from the same HAMs F-10 media used to make the growth media. HAMs F-10 media was supplemented with 1% v/v penicillin/streptomycin (Sigma Life Sciences), but unlike the growth media no fibroblast growth factor (fgf) or bovine calf serum was added. Instead, 5% v/v horse serum was added. Myoblasts were kept in this media for the duration of differentiation required for each experiment.

Differentiation Validation

To validate whether myoblasts could be successfully differentiated in HAMs F-10 media with 5% horse serum, three groups of myoblasts were prepared in triplicate from cells previously isolated from three different mice. Cells were grown to 80% confluency then placed in differentiating media. On day 0, one group of cells were scrapped off their plates and stored at -80 °C for later analysis. This group represents an undifferentiated control for the experiment. On day 2 the media was replaced, and another group of cells were scrapped off the plate and stored. This group represents an early point in the differentiation process. Differentiation media was replenished on day 4. On day 5, the final group of cells were scrapped off the plate and stored for later analysis. This last group represents cells near the endpoint of differentiation when mature muscle proteins are being expressed in full and fusion of cells into multinucleated myotubes has progressed to an appreciable degree.

The cells collected from this experiment were then lysed and prepared for western blotting at the same time to minimize possible differences introduced by the workup process.

Early Timepoints of Differentiation

Initial differentiation experiments were performed in which the cells were placed in differentiation media for >2 days. Crucial regulatory events occur early on in the differentiation process (Li & Schwartz, 2001), including localization of HAT's to loci related to myogenesis (Khilji et al., 2018), that may be attenuated at later timepoints. To investigate the expression of myosin heavy chain (MyHC) at earlier timepoint, cells were differentiated for 0.5 – 12 hours. Five groups of myoblasts were prepared in triplicate from 3 different mice. The cells were

switched into differentiation media and one group of cells from each mouse were scrapped as an undifferentiated control. The other groups were then scrapped at their respective timepoint. All samples were kept at -80°C until the end of the experiment at which point, they were all lysed and prepared for western blotting together.

The specific process used of switching all the samples to differentiating media at the same time is intended to limit any differences between the confluency of cells during differentiation. The cells are plated and grown to the same confluency. The switch to differentiating media ostensibly halts any further proliferation since the cells stop receiving signals from growth factors to proliferate. Therefore, each plate in each sample group should have approximately the same amount of cells on them. This experiment then assumes that any differences in confluency, and any effects on differentiation that confluency might have, are negligible between the sample groups.

Rate of Change of Acetylation during Glutamine Treatment

Media containing 0.2mM glutamine caused a hyperacetylated histone phenotype in primary myoblasts, but the rate at which acetylation is accrued is not known. To assess the rate at which media containing 0.2mM glutamine changes histone acetylation, samples were prepared that were exposed to the low glutamine media for various time-points between 30 minutes to 48 hours. Eight plates were prepared per mouse for 3 different mice. Myoblasts were plated and grown to 60% confluency. 48 hours before the cell scrapping, one set of myoblasts was changed to low glutamine media. This was repeated with each time point (i.e., each time point *t* was switched to low glutamine media *t* hours before all the cells were

scrapped). This way, all the myoblasts could be scrapped at the same time and all the myoblasts would be scrapped at relatively similar confluency. Samples were then stored at -80°C for later analysis.

Differentiation with Glutamine Treatment

Cells from 3 mice, with 6 plates of cells prepared per mouse, were cultured. Two plates from each mouse were switched to 0.2mM, 1mM and 2mM glutamine growth media, respectively, when they reached 60% confluency. 48h after the initial media switch the cells were at 80% confluency. Half the cells, one from each group receiving different concentration of glutamine media, were scrapped and stored at -80°C. The other half of the cells were placed into differentiation media containing the same concentration of glutamine as the growth media they were just in. The remaining cells were differentiated for 48 hours in this media before they were scrapped and stored for later analysis.

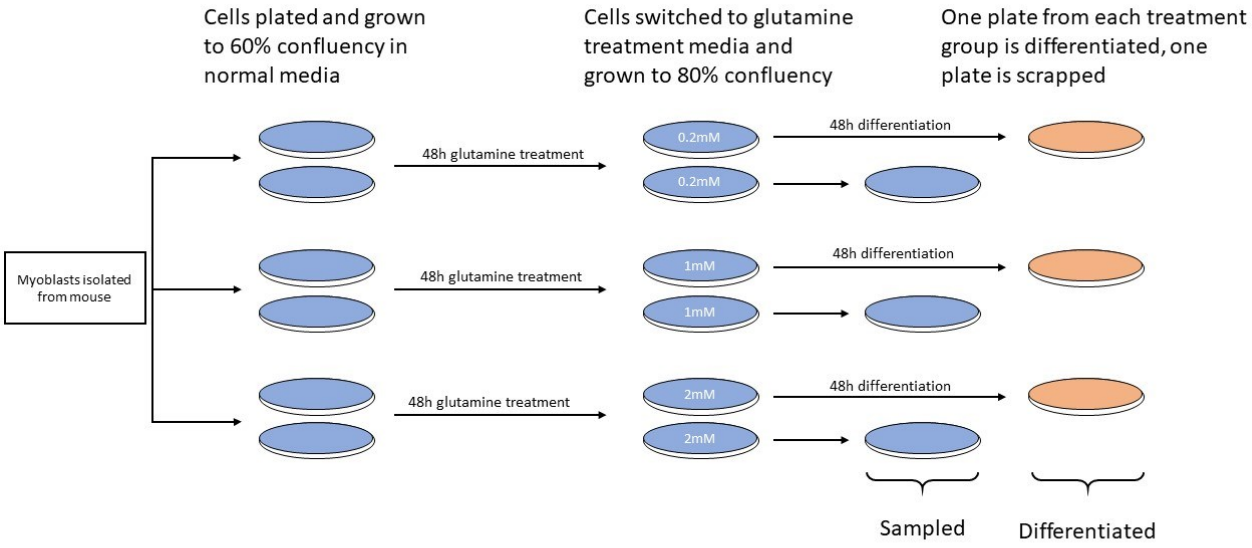

Figure 4. 1: Workflow for Differentiation with Glutamine Treatment experiment.

Western Blotting

Analysis of samples by western blotting was done for total protein content, myosin heavy chain, histone H3, and acetylated histone H3 (Anti-myosin Heavy chain MF20, DSHB; Histone H3 D1H2 XP Rabbit mAb, Cell Signalling; Anti-acetyl-Histone H3, EMD Millipore) was performed as previously described (see **Section 2.2**).

Statistical Methodology

A paired, two-tailed student's *t*-test was used to analyze histone acetylation and MyHC expression between different timepoints of differentiation or treatment. Statistical significance was assessed based on a p-value < 0.1.

4.3 – Results

Histone Acetylation during Myoblast Differentiation in HAM's F-10 Media

Cell cultures of primary, murine myoblasts are generally differentiated in Dulbecco's modified eagle medium (DMEM) containing 2-5% horse serum (Hindi et al., 2017). Since the goal of the experiments in this chapter are to look at the effects of metabolites on differentiation, it was prudent to keep as much metabolic consistency as possible between the proliferation and differentiation medias. To this end, the ability of HAM's F-10 media to differentiate myoblasts was investigated since this is the same media that the myoblasts are proliferated in. Cells differentiated in HAM's F-10 media containing 5% horse serum differentiated successfully displaying similar characteristics to those that were previously differentiated in DMEM. The MyHC expression of cells increased significantly (p-value=0.0083, paired two-tailed t-test) after 5 days of differentiation (**Figure 4.2C**). The presence of MyHC at the day 0 timepoint is not unexpected as within the heterogenous cell culture population it is possible that some cells prematurely differentiated enough to express MyHC.

This experiment also defined the natural state of histone acetylation during differentiation when there is no change to the metabolic profile of the differentiation media. General histone acetylation during differentiation remained relatively constant between the undifferentiated cells and the cells differentiated for 2 and 5 days, respectively (**Figure 4.2B**). As with previous work (See **Section 2.4**), H3 has been used to suggest trends in global histone acetylation.

Histone acetylation is thought to be dynamic during differentiation (Hamed et al., 2013; Saraiva et al., 2010), however, our results indicated the contrary. It is possible that the extent of differentiation at later time points may hide some of the nuanced changes to histone acetylation experienced by the cell. To investigate this possibility, myoblasts at earlier timepoints of differentiation were sampled and their histone acetylation state was measured. Cells were placed in differentiation media for 0.5 – 12 hours (**Figure 4.3**). The expression of MyHC was significantly increased after only 0.5 h (*p*-value=0.003, paired two-tailed *t*-test) suggesting that differentiation had indeed begun and had begun rapidly. MyHC expression continued to rise as differentiation was allowed to progress for longer periods of time. Once again, histone acetylation remained relatively constant throughout with no significance found between timepoints (**Figure 4.3**).

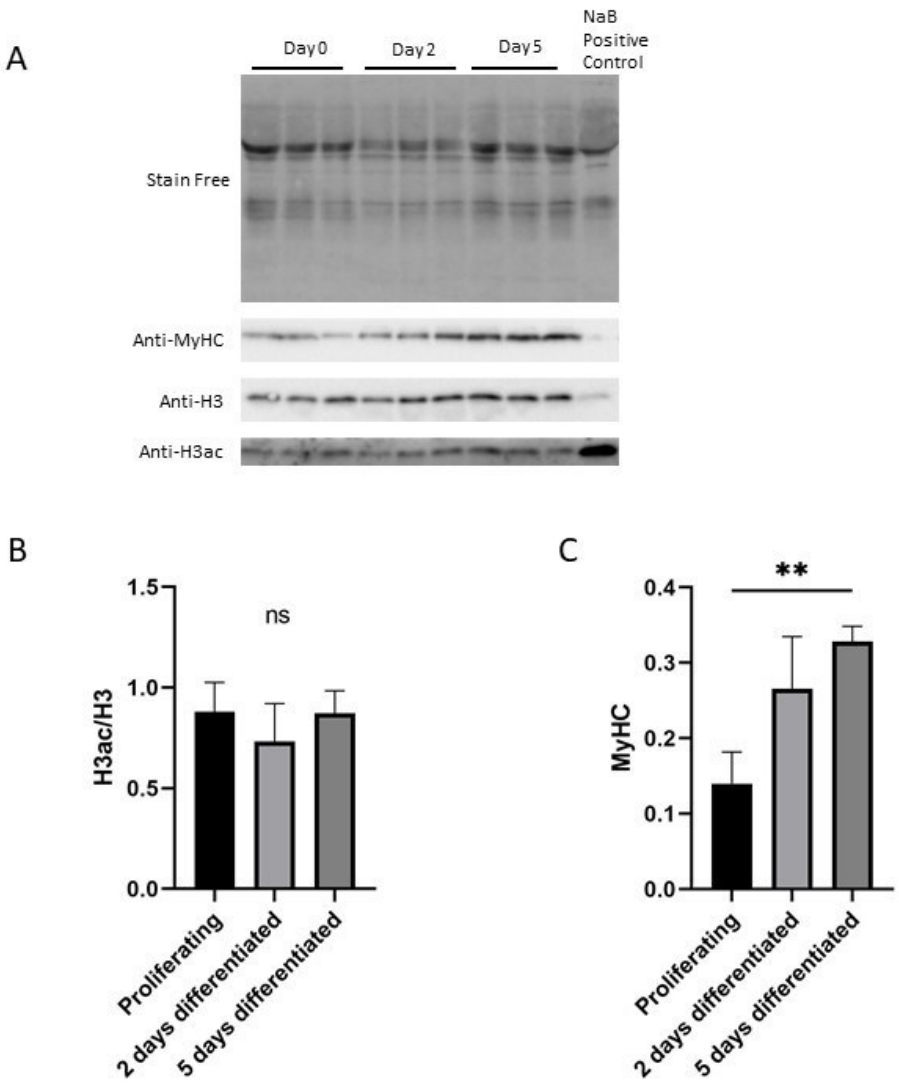

Figure 4. 2: ***Acetylation and Myosin heavy chain (MyHC) expression during differentiation****. Cultured myoblasts were differentiation for 2 and 5 days. A) Cells were immunoblotted for MyHC, histone H3, and acetylated histone H3. Total protein content was assessed using a stain free gel. B) Quantification of histone acetylation of the myoblast before they were differentiated (Proliferating) and when they were differentiated for 2 or 5 days. C) Quantification of the expression of MyHC for the same myoblast. p-value= 0.0083 (paired two-tailed t-test, n=3).*

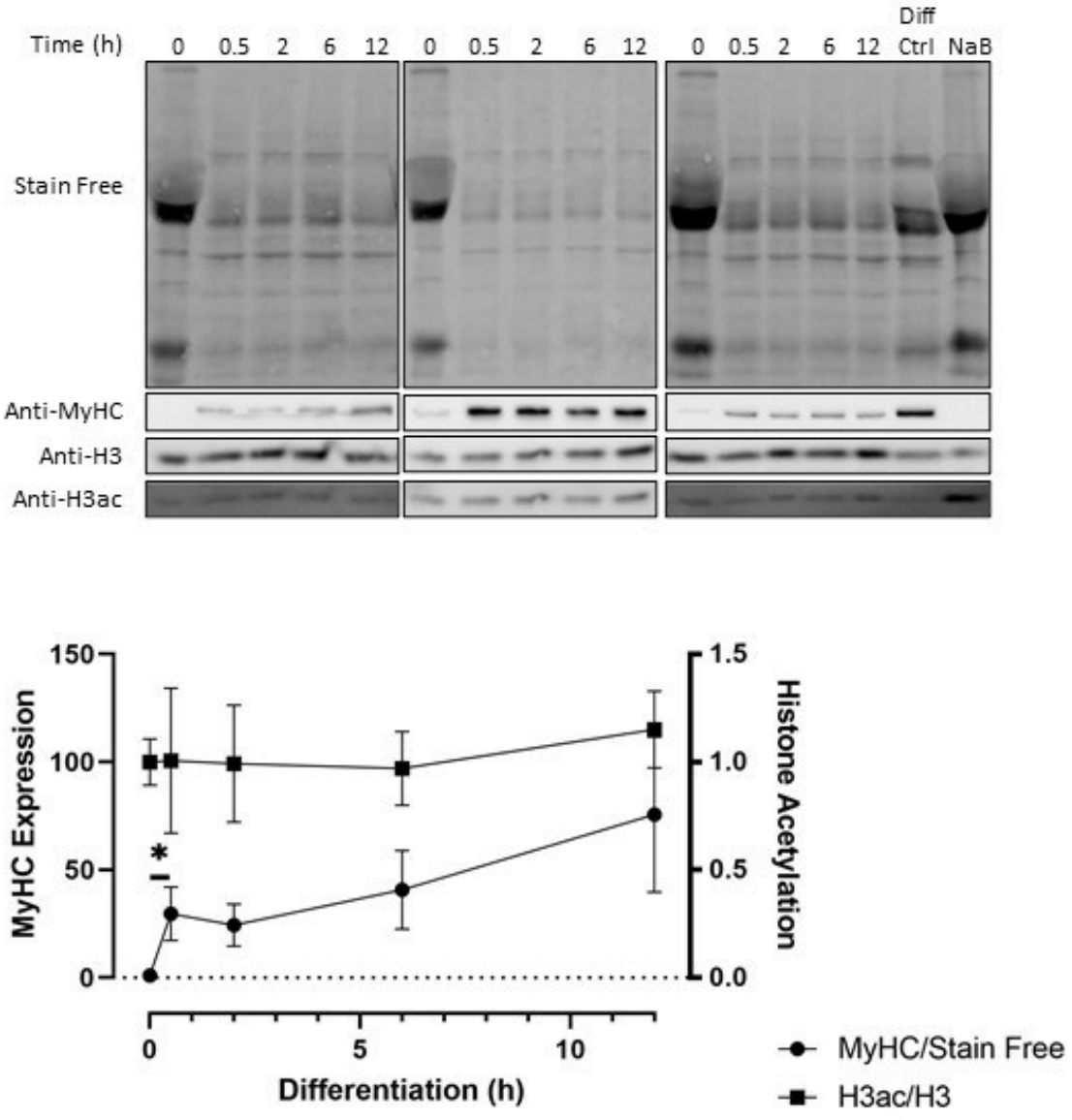

Figure 4. 3: ***Early timepoints of differentiation****. To investigate what happens to histone acetylation and myosin heavy chain (MyHC) expression at the beginning of differentiation, cultured myoblasts were differentiated for 0.5, 2, 6, and 12h. A differentiation control was included that consisted of cell extract for myoblasts that had been differentiated previously for 5 days. Data presented as mean ± SEM. p-value=0.003 (paired two-tailed t-test, n=3)*

Rate of Change of Acetylation during Glutamine Treatment in Proliferating Myoblasts

Primary myoblasts cultured in Ham's F-10 media containing 0.2mM glutamine have a hyperacetylated histone phenotype, as previously described. However, this phenotype was only evaluated at a timepoint 48 hours after media was switched to the 0.2mM glutamine media. If histone acetylation is to be modulated this way to alter differentiation, the rate at which this change occurs should be evaluated as this may impact its ability to alter the capacity or progression of differentiation. Since myoblasts begin to express MyHC within the first 30 minutes of being placed into differentiation media, a similar if not faster development of the hyperacetylted phenotype could aid glutamine's ability to exert an effect. Histone acetylation began changing very rapidly upon switching to a growth media containing 0.2mM glutamine (**Figure 4.4**). A peak of acetylation was seen at the 24h timepoint, after which the histone acetylation of all cell lines decreased slightly at the 48h timepoint. Acetylation significantly increased by > 200% at 24h compared to the 0h timepoint (*p*-value=0.094, paired two-tailed t-test). However, the difference in acetylation between the 24h and 48h timepoint was not significant (*p*-value=0.81).

Differentiation with Glutamine Treatment

As the concentration of glutamine in growth media is decreased from 2mM to 0.2mM, histone acetylation increases in proliferating myoblasts in a dose-response relationship (**Section 3.3**). The increases to histone acetylation correlates positively with increased myoblasts proliferation (**Section 3.3**). If histone acetylation promotes proliferation it may inhibit differentiation, resulting in a similar, albeit inverse, correlation. Histone acetylation may

alternatively not promote or inhibit any particular cell fate decision, but rather facilitate and improve which ever cell fate decision has already been chosen by the myoblasts. Declining glutamine concentrations would then be expected to increase gene expression associated with differentiation. To investigate this, cells were grown and differentiated in media containing 0.2mM, 1mM, or 2mM glutamine. The expression of MyHC was used as an indicator of differentiation as it has been in previous studies (Jang & Baik, 2013; Pavlidou et al., 2019).

Myoblasts grown in growth media containing 0.2mM, 1mM or 2mM glutamine showed decreased histone acetylation as the concentration of glutamine increased (**Figure 4.5**). Myoblasts grown in 2mM glutamine had significantly less histone acetylation than those treated with 0.2mM glutamine (*p*-value=0.096, two-tailed t-test). This was the same result as previous experiments that also investigated histone acetylation with glutamine treatment in undifferentiated cells (**Figure 3.1**). However, when the myoblasts were placed into differentiation media with the same concentrations of glutamine, the hyperacetylated phenotype was lost in the 0.2mM glutamine group. The histone acetylation between the differentiated cells did not display any of the dose-response relationship with glutamine concentration that the undifferentiated cells showed.

MyHC expression did not differ significantly between differentiated groups based on the concentration of glutamine that the cells were exposed to. The only significant difference to MyHC was between the undifferentiated and differentiated groups suggesting the competency of the differentiation process in all treatment.

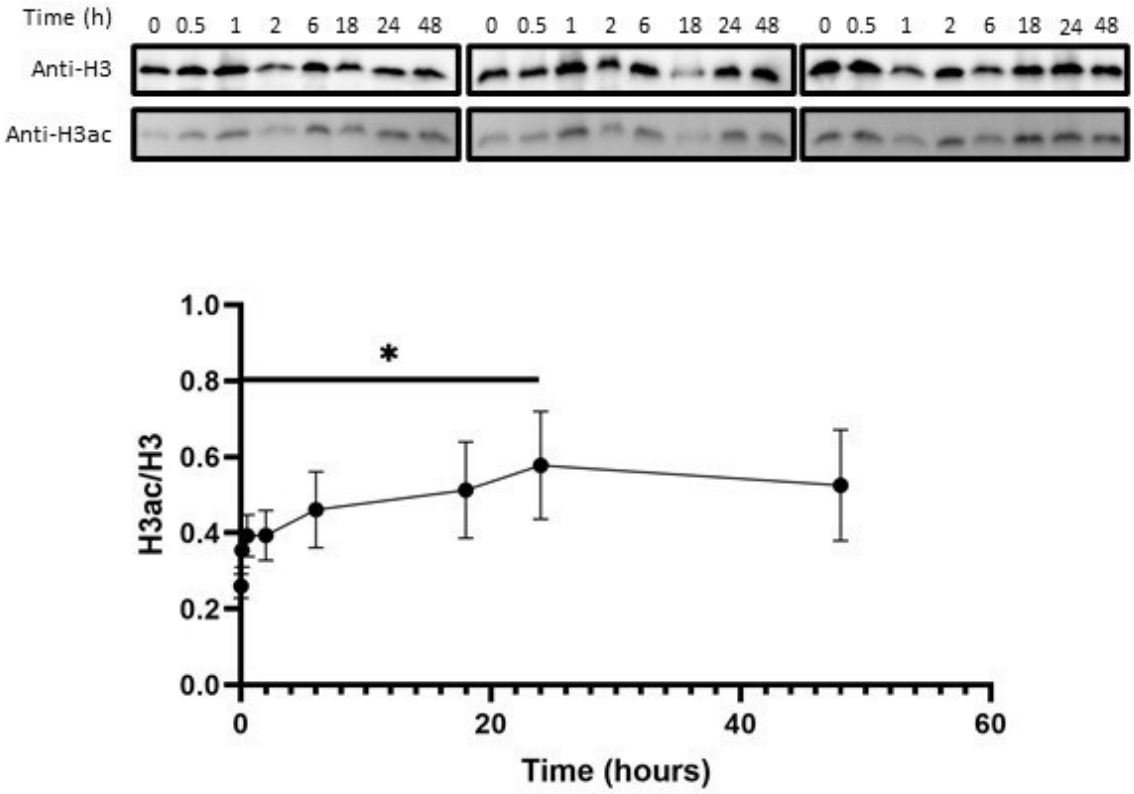

*Figure 4. 4:**Timecourse of acetylation change with glutamine treatment.** Cultured myoblasts were grown in growth media containing 0.5mM glutamine for 0, 0.5, 1, 2, 6, 18, 24, or 48 hours. They were then sampled and immunoblotted for histone H3 and acetylated histone H3. P-value=0.094 (paired two-tailed t-test, n=3). Acetylation data presented as the average of three replicates with error bars as SEM.*

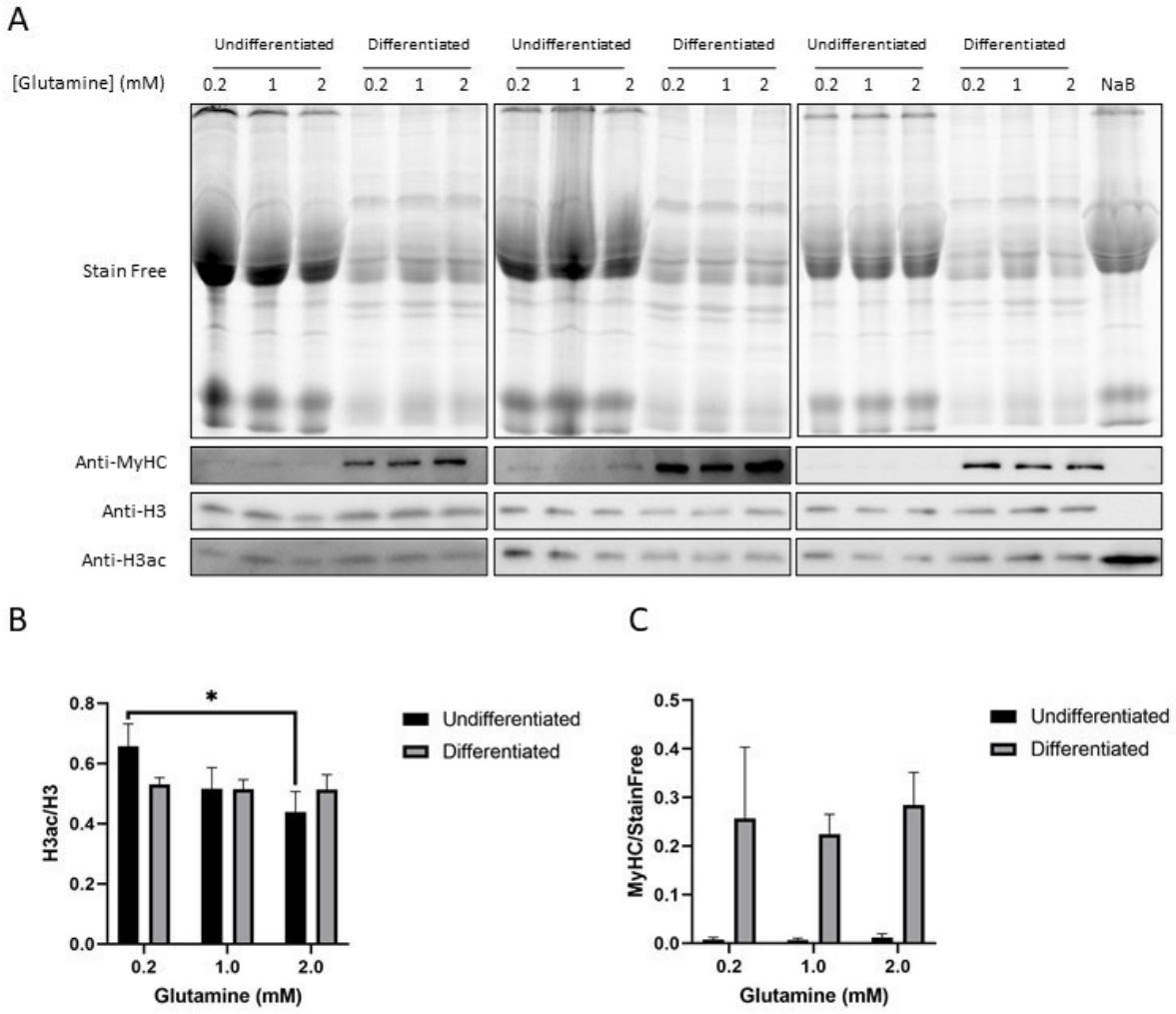

*Figure 4. 5:**Differentiation of myoblast with glutamine treatment.** Myoblasts were differentiated with different concentrations of glutamine. Cultured myoblasts were differentiated in media containing either 0.2, 1.0, or 2.0mM glutamine. The 2.0mM glutamine group represents a control for the normal glutamine content of cell culture media. A) The cells were then sampled and immunoblotted for myosin heavy chain (MyHC), histone H3, and acetylated histone H3. Total protein was quantified using a stain free gel. B) Quantification of H3 acetylation between treatment groups (P-value=0.096, unpaired two-tailed t-test, n=3). C) Quantification of MyHC between treatment groups. Quantitative data presented at mean ± SEM.*

4.4 - Discussion

The goal of this work was to examine the relationship between histone acetylation and myoblast differentiation along with how it might be manipulated through modulating the availability of glutamine.

When myoblasts are differentiated in cell culture, they are switched into a media that encourages differentiation, lacking the growth factors that promote proliferation and supress differentiation. Validation experiments were performed to ensure that the myoblasts differentiate in Ham's F-10 containing 5% horse serum, and that this media sufficiently activated cell differentiation by monitoring the expression of MyHC, a marker of myoblasts differentiation (Jang & Baik, 2013; Pavlidou et al., 2019). This allows the metabolic profile of the proliferating and differentiating media to be more consistent since they will both be based on Ham's F-10. The effects of changing the concentration of glutamine can then be investigated. Cultured primary myoblasts differentiate competently when exposed to Ham's F-10 media containing 5% horse serum (**Figure 4.2**). The expression of MyHC has been used in other works as a measurement of myogenic competency in muscle stem cells (Jang & Baik, 2013; Pavlidou et al., 2019). In this experiment, the expression of the contractile protein MyHC was significantly higher ($p<0.1$, two-tailed t-test) at day 2 compared to day 0 and continued increasing into day 5 (**Figure 4.2C**). This method seems to be a reasonable protocol for myogenic differentiation in cell culture, similar to other experiments that differentiate myoblasts in DMEM (Hindi et al., 2017). The cell culture media Ham's F-10 is therefore a viable choice for proliferating and differentiating primary myoblasts.

In the absence of any alterations to the concentration of glutamine in the media, histone acetylation remains consistent after 2 days and 5 days of differentiation compared to histone acetylation in proliferating myoblasts (**Figure 4.2B**). Histone acetylation during earlier points of differentiation (<12h) are also fairly consistent (**Figure 4.3**). Though there should be a large change in the epigenetic landscape of the cells as they switch from proliferation to differentiation (Asp et al., 2011), the net amount of histone acetylation does not change drastically. This does not mean that histones associated with specific genes might not have meaningful changes occur to their acetylation state, rather that across the whole genome there is no sharp increase or decrease to acetylation. This result contrasts previous reports of the dynamic nature of histone acetylation during differentiation (Hamed et al., 2013; Saraiva et al., 2010). However, these previous works have either investigated histone acetylation at a specific gene locus or in stem cell models other than primary myoblasts.

MyHC expression is the metric that has been used to reflect differentiation throughout these experiments. After being changed to differentiating media, the cells began to express MyHC at a significantly higher level after only 30 minutes (**Figure 4.3**). The quick onset of MyHC expression in the early timepoint experiments suggests that the cells rapidly begin making the contractile protein. Therefore, the window for altering the expression of this protein occurs very early on in the differentiation process and may continue for up to 5 days (**Figure 4.2**). However, the timeframe in which MyHC expression increases may be expanded by the heterogeneity of the cell culture population and not reflect the timeframe of differentiation for a single cell.

Glutamine is a non-essential amino acid that is a prominent source of carbon and nitrogen in proliferating cells (Daye & Wellen, 2012). Glutamine can be used for energy production and to regenerate pools of metabolites through anaplerotic reactions (Yang et al., 2014; Yang et al., 2009). When cultured myoblasts are exposed to glutamine concentrations < 2mM histone acetylation increases. The transition to this hyperacetylated histone state is not instantaneous. The cells must recognize the change to their environment and respond, which takes time. We investigated the rate at which histone acetylation changes when the cells are exposed to low concentrations of glutamine. To delineate this timeframe, we looked at the acetylation of histones at early time point of incubating myoblasts with low glutamine media. Once cells are switched into growth media containing 0.2mM glutamine, acetylation begins to change in the first 30 minutes (**Figure 4.4**). When myoblasts are cultured in media containing 0.2mM glutamine, histone acetylation steadily increases until 24 hour at which point it hits a peak. After 48 hours of treatment with 0.2mM glutamine the histone acetylation state of the cell may stabilize and plateau slightly below the 24h peak, but more data point past the 48 hour mark are required to support this assertion.

The rapid response to the new metabolic landscape and subsequent increase in histone acetylation is promising since it aligns well with when the cells begin to express MyHC during differentiation. Since the cells respond to the treatment in a timeframe similar to when they respond to differentiation cues it is possible for glutamine treatment to affect differentiation if the cells are grown in normal media then switched to differentiation media that contains 0.2mM glutamine. In a theoretical *in vivo* setting, these results suggest that local glutamine

levels could be lowered acutely to coincide with myoblasts activation and influence histone acetylation in these cells.

The quick response to low glutamine concentrations also speaks to the plasticity of the histone acetylation state in the cell. Within minutes the cells begin responding to the switch in media. If higher or lower levels of acetylation are identified as beneficial to specific stages of myogenesis or any other cellular process, it may be possible to adjust the levels of histone acetylation in real-time to best compliment specific stages in the process. This, however, would require further work to establish a causal link between histone acetylation and process efficacy.

The final test performed was to determine whether the increase in acetylation caused by low glutamine treatment has any effect on MyHC expression and if the elevated acetylation persists through differentiation. Cultured myoblasts grown in media containing 0.2mM glutamine have increased histone acetylation. As the concentration of glutamine in the media is raised, the hyperacetylated phenotype is attenuated. This attenuation continues until a concentration of 2mM glutamine is reached. In previous experiments (**Figure 3.2**), concentrations of 2mM to 20mM glutamine result in a plateau and did not continue to decrease histone acetylation. When cells were differentiated with concentrations of glutamine in the differentiation media of either 0.2mM, 1mM, or 2mM glutamine, no significant difference between MyHC expression was seen. The undifferentiated cells treated with the different concentrations of glutamine display the same negative correlation between acetylation and glutamine concentration that were previously seen, but this pattern does not hold when the cells are differentiated. Once again, acetylation during differentiation remained consistent between groups. It may be the mechanism that resulted in hyperacetylation is no

longer present or relevant in myoblasts once they begin to differentiate. Alternatively, it could be that whatever mechanisms govern the acetylation state of the cell during differentiation (i.e., coordination of acetylation events by MRFs) overrides the modulation to histone acetylation induced by a low glutamine growth media. Either way, this data suggests that low glutamine media is not an effective mode of altering histone acetylation levels during differentiation, and that low glutamine media does not change the expression of MyHC in differentiating myoblasts.

4.5 – Limitations

The goal of using and validating the HAM's F-10 media for differentiating was to keep the metabolite profile of the media as close as possible between proliferating and differentiating culture. The difference of 10% bovine calf serum and 5% horse serum introduces variability between the two media which cannot be avoided. Elucidating the complete metabolic profile of each media would be helpful to account for any variability, but lot-to-lot differences in the serums used would likely require repeated testing for each new media preparation. Thus, care should be taken when comparing cells grown in the proliferating media and those in the differentiating media.

4.6 – Future Directions

The work presented here looks at the sum of the acetylation state of all histones. A higher resolution investigation into which histones are being acetylated and which genes they are associated with may result in interesting findings. This has been done previously, but not under circumstances of low glutamine growth media (Asp et al., 2011; Cao et al., 2010). The global increase in acetylation occurring in undifferentiated cells may not be occurring on genes related to muscle, thereby making the increased acetylation with low glutamine treatment less relevant in the context of myogenesis. On the other hand, the loss of the hyperacetylated phenotype during differentiation may be occurring globally but not on histones associated with muscle-specific genes. Being able to compare acetylation between genes and not just between treatment groups may illuminate more about the relationship.

When differentiation is induced in cultured myoblasts there is always a population of myoblasts that fail to differentiate and become, essentially, quiescent. These cells are referred to as the reserve cell population (Laumonier et al., 2017). This population is likely similar to the population of myoblasts *in vivo* that would resist differentiation and return to the stem cell niche as quiescent satellite cells in order to maintain the satellite cell population. The presence of this reserve population is likely due to the action of the tumour suppressor p53 (Flamini et al., 2018) and the metabolite treatments used herein could also be investigated for their ability to maintain or increase this reserve population. With work already being done relating histone acetylation and muscle health (Ryall et al., 2015), this may provide another path of potential mechanisms for its action.

4.7 – Conclusion

In order to maintain some semblance of metabolite consistency across cell culture experiments, proliferating and differentiating myoblasts in HAM's F-10 media is a viable alternative to proliferating in HAM's F-10 and differentiating in DMEM. Myoblasts differentiated in HAM's F-10 have a significantly higher expression of MyHC after 2 days of differentiation. The expression of MyHC, however, begins as early as 30 minutes into the differentiation process. Histone acetylation seems relatively constant throughout all time points of differentiation explored in this work.

When myoblasts are placed in media containing 0.2mM glutamine, histone acetylation quickly begins to increase until it reaches a peak at 24h. When myoblasts are differentiated in media containing glutamine concentrations that increase histone acetylation in proliferating

cells, the hyperacetylated phenotype is lost. Though differentiation continues successfully in media containing 0.2mM, 1mM, or 2mM glutamine, no difference in MyHC expression was seen between the different concentrations of glutamine. While low glutamine treatment remains a viable way to increase histone acetylation in proliferating myoblasts, it does not increase acetylation in differentiating myoblasts nor does it change MyHC expression.

Chapter 5: Conclusion

The process of myoblast proliferation and differentiation defines myogenesis and is responsible for muscle tissue upkeep. While the influence of specific epigenetic modifications on this process has been interrogated repeatedly (Alter et al., 2008; Gillespie et al., 2009; Guttridge et al., 1999; Wang et al., 2008), there is a lack of understanding of global histone acetylation in this context. Various studies have identified changes to histone acetylation related to specific genes (Canto et al., 2009; Cohen et al., 2004; Fulco et al., 2008; Kaimori et al., 2016; Ryall et al., 2015), but none have investigated the relationship between global histone acetylation and myogenesis. This study addressed whether global histone acetylation in myoblasts could be changed by altering the metabolic profile of cell culture conditions and whether these changes to acetylation altered proliferation or differentiation. The results indicate that glutamine plays an important role in determining the histone acetylation state of proliferating myoblasts, with low glutamine conditions resulting in a hyperacetylated phenotype and increased proliferation. Although the causal link between global histone acetylation and proliferation cannot be established by this work, it may suggest a role for metabolic regulation of stem cell numbers *in vivo*. However, upon commitment to differentiation, myoblasts in low glutamine conditions lose this hyperacetylated phenotype and differentiate the same as they would under normal metabolic conditions. These findings demonstrate the importance of the metabolic environment in epigenetic regulation requiring cosubstrates and the need for further research into how metabolism interacts with cell fate decisions.

In their natural environment, muscle stem cells exist in a segregated niche with unique metabolite availability (Chakrabarty & Chandel, 2021; Dumont et al., 2015), and a cell's

metabolism contributes to cell fate decisions (Bahat & Gross, 2019; Somasundaram et al., 2020). The metabolic environment these cells are exposed to, when they exit their niche to contribute to myogenesis, may be very different and could therefore play a role in deciding their fate and function (Ryall, 2013). If this is the case, then modulating a stem cell's metabolic profile may provide an avenue for influencing cell fate and function in beneficial ways. Ultimately, this work demonstrated that exposing proliferating myoblasts to increasing concentrations of glutamine, glucose, or pyruvate, thereby increasing the availability of carbon sources for acetyl-CoA production, does not increase histone acetylation. This is in light of the fact that histone acetylation is rate limited by nuclear acetyl-CoA production, which is sensitive to substrate availability (Takahashi et al., 2006). There is some disconnect between supplying cells with extra metabolites to generate epigenetic cosubstrates and the actual utilization of these metabolites. Future works should examine metabolite utilization by myoblasts, using techniques such as stable isotope-resolved metabolomics, and if there is a strategy to bolster the metabolome of cells in a way beneficial to epigenetic regulation.

Bibliography:

Alter, J., Rozentzweig, D., & Bengal, E. (2008, Aug 22). Inhibition of myoblast differentiation by tumor necrosis factor alpha is mediated by c-Jun N-terminal kinase 1 and leukemia inhibitory factor. *J Biol Chem, 283*(34), 23224-23234. https://doi.org/10.1074/jbc.M801379200

Asfour, H. A., Allouh, M. Z., & Said, R. S. (2018, Jan). Myogenic regulatory factors: The orchestrators of myogenesis after 30 years of discovery. *Exp Biol Med (Maywood), 243*(2), 118-128. https://doi.org/10.1177/1535370217749494

Asp, P., Blum, R., Vethantham, V., Parisi, F., Micsinai, M., Cheng, J., Bowman, C., Kluger, Y., & Dynlacht, B. D. (2011, May 31). Genome-wide remodeling of the epigenetic landscape during myogenic differentiation. *Proc Natl Acad Sci U S A, 108*(22), E149-158. https://doi.org/10.1073/pnas.1102223108

Barani, A. E., Durieux, A. C., Sabido, O., & Freyssenet, D. (2003, Nov). Age-related changes in the mitotic and metabolic characteristics of muscle-derived cells. *J Appl Physiol (1985), 95*(5), 2089-2098. https://doi.org/10.1152/japplphysiol.00437.2003

Benayoun, B. A., Pollina, E. A., & Brunet, A. (2015, Oct). Epigenetic regulation of ageing: linking environmental inputs to genomic stability. *Nat Rev Mol Cell Biol, 16*(10), 593-610. https://doi.org/10.1038/nrm4048

Bentzinger, C. F., Wang, Y. X., Dumont, N. A., & Rudnicki, M. A. (2013, Dec). Cellular dynamics in the muscle satellite cell niche. *EMBO Rep, 14*(12), 1062-1072. https://doi.org/10.1038/embor.2013.182

Bergstrom, D. A., Penn, B. H., Strand, A., Perry, R. L. S., Rudnicki, M. A., & Tapscott, S. J. (2002). Promoter-Specific Regulation of MyoD Binding and Signal Transduction Cooperate to Pattern Gene Expression. *Molecular Cell, 9*(3), 587-600. https://doi.org/10.1016/s1097-2765(02)00481-1

Bi, P., & Kuang, S. (2015, May). Notch signaling as a novel regulator of metabolism. *Trends Endocrinol Metab, 26*(5), 248-255. https://doi.org/10.1016/j.tem.2015.02.006

Boonen, K. J., Rosaria-Chak, K. Y., Baaijens, F. P., van der Schaft, D. W., & Post, M. J. (2009, Jun). Essential environmental cues from the satellite cell niche: optimizing proliferation and differentiation. *Am J Physiol Cell Physiol, 296*(6), C1338-1345. https://doi.org/10.1152/ajpcell.00015.2009

Bowtell, J. L., & Bruce, M. (2002). Glutamine: an anaplerotic precursor. *Nutrition, 18*(3), 222-224. https://doi.org/10.1016/s0899-9007(01)00795-x

Brack, A. S., Conboy, I. M., Conboy, M. J., Shen, J., & Rando, T. A. (2008, Jan 10). A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. *Cell Stem Cell, 2*(1), 50-59. https://doi.org/10.1016/j.stem.2007.10.006

Brockers, K., & Schneider, R. (2019, Feb). Histone H1, the forgotten histone. *Epigenomics, 11*(4), 363-366. https://doi.org/10.2217/epi-2019-0018

Brower-Toland, B., Wacker, D. A., Fulbright, R. M., Lis, J. T., Kraus, W. L., & Wang, M. D. (2005, Feb 11). Specific contributions of histone tails and their acetylation to the mechanical stability of nucleosomes. *J Mol Biol, 346*(1), 135-146. https://doi.org/10.1016/j.jmb.2004.11.056

Brown, C. R., Kennedy, C. J., Delmar, V. A., Forbes, D. J., & Silver, P. A. (2008, Mar 1). Global histone acetylation induces functional genomic reorganization at mammalian nuclear pore complexes. *Genes Dev, 22*(5), 627-639. https://doi.org/10.1101/gad.1632708

Brunet, A., & Rando, T. A. (2017, Apr). Interaction between epigenetic and metabolism in aging stem cells. *Curr Opin Cell Biol, 45*, 1-7. https://doi.org/10.1016/j.ceb.2016.12.009

Bulusu, V., Tumanov, S., Michalopoulou, E., van den Broek, N. J., MacKay, G., Nixon, C., Dhayade, S., Schug, Z. T., Vande Voorde, J., Blyth, K., Gottlieb, E., Vazquez, A., & Kamphorst, J. J. (2017, Jan 17). Acetate Recapturing by Nuclear Acetyl-CoA Synthetase 2 Prevents Loss of Histone Acetylation during Oxygen and Serum Limitation. *Cell Rep, 18*(3), 647-658. https://doi.org/10.1016/j.celrep.2016.12.055

Canto, C., Gerhart-Hines, Z., Feige, J. N., Lagouge, M., Noriega, L., Milne, J. C., Elliott, P. J., Puigserver, P., & Auwerx, J. (2009, Apr 23). AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature, 458*(7241), 1056-1060. https://doi.org/10.1038/nature07813

Cao, Y., Yao, Z., Sarkar, D., Lawrence, M., Sanchez, G. J., Parker, M. H., MacQuarrie, K. L., Davison, J., Morgan, M. T., Ruzzo, W. L., Gentleman, R. C., & Tapscott, S. J. (2010, Apr 20). Genome-wide MyoD binding in skeletal muscle cells: a potential for broad cellular reprogramming. *Dev Cell, 18*(4), 662-674. https://doi.org/10.1016/j.devcel.2010.02.014

Chen, K., Hu, Z., Xia, Z., Zhao, D., Li, W., & Tyler, J. K. (2015, Dec 28). The Overlooked Fact: Fundamental Need for Spike-In Control for Virtually All Genome-Wide Analyses. *Mol Cell Biol, 36*(5), 662-667. https://doi.org/10.1128/MCB.00970-14

Chen, L., & Cui, H. (2015, Sep 22). Targeting Glutamine Induces Apoptosis: A Cancer Therapy Approach. *Int J Mol Sci, 16*(9), 22830-22855. https://doi.org/10.3390/ijms160922830

Clayton, A. L., Hazzalin, C. A., & Mahadevan, L. C. (2006, Aug 4). Enhanced histone acetylation and transcription: a dynamic perspective. *Mol Cell, 23*(3), 289-296. https://doi.org/10.1016/j.molcel.2006.06.017

Cohen, H. Y., Miller, C., Bitterman, K. J., Wall, N. R., Hekking, B., Kessler, B., Howitz, K. T., Gorospe, M., de Cabo, R., & Sinclair, D. A. (2004, Jul 16). Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science, 305*(5682), 390-392. https://doi.org/10.1126/science.1099196

Conboy, I. M., Conboy, M. J., Smythe, G. M., & Rando, T. A. (2003, Nov 28). Notch-mediated restoration of regenerative potential to aged muscle. *Science, 302*(5650), 1575-1577. https://doi.org/10.1126/science.1087573

Consortium, E. P. (2012, Sep 6). An integrated encyclopedia of DNA elements in the human genome. *Nature, 489*(7414), 57-74. https://doi.org/10.1038/nature11247

Davie, J. K., Cho, J. H., Meadows, E., Flynn, J. M., Knapp, J. R., & Klein, W. H. (2007, Nov 15). Target gene selectivity of the myogenic basic helix-loop-helix transcription factor myogenin in embryonic muscle. *Dev Biol, 311*(2), 650-664. https://doi.org/10.1016/j.ydbio.2007.08.014

Davie, J. R. (2003, Jul). Inhibition of histone deacetylase activity by butyrate. *J Nutr, 133*(7 Suppl), 2485S-2493S. https://doi.org/10.1093/jn/133.7.2485S

Daye, D., & Wellen, K. E. (2012, Jun). Metabolic reprogramming in cancer: unraveling the role of glutamine in tumorigenesis. *Semin Cell Dev Biol, 23*(4), 362-369. https://doi.org/10.1016/j.semcdb.2012.02.002

Deans, C., & Maggert, K. A. (2015, Apr). What do you mean, "epigenetic"? *Genetics, 199*(4), 887-896. https://doi.org/10.1534/genetics.114.173492

DeBerardinis, R. J., Mancuso, A., Daikhin, E., Nissim, I., Yudkoff, M., Wehrli, S., & Thompson, C. B. (2007, Dec 4). Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci U S A, 104*(49), 19345-19350. https://doi.org/10.1073/pnas.0709747104

Di Padova, M., Caretti, G., Zhao, P., Hoffman, E. P., & Sartorelli, V. (2007, Dec 28). MyoD acetylation influences temporal patterns of skeletal muscle gene expression. *J Biol Chem, 282*(52), 37650-37659. https://doi.org/10.1074/jbc.M707309200

DiMario, J., Buffinger, N., Yamada, S., & Strohman, R. C. (1989, May 12). Fibroblast growth factor in the extracellular matrix of dystrophic (mdx) mouse muscle. *Science, 244*(4905), 688-690. https://doi.org/10.1126/science.2717945

Doble, B. W., & Woodgett, J. R. (2003, Apr 1). GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci, 116*(Pt 7), 1175-1186. https://doi.org/10.1242/jcs.00384

Dominique, M.-D. (2017). *Glutamine*. https://doi.org/10.1201/9781315373164

Donohoe, D. R., & Bultman, S. J. (2012, Sep). Metaboloepigenetics: interrelationships between energy metabolism and epigenetic control of gene expression. *J Cell Physiol, 227*(9), 3169-3177. https://doi.org/10.1002/jcp.24054

Dougherty, C. J., Kubasiak, L. A., Frazier, D. P., Li, H., Xiong, W. C., Bishopric, N. H., & Webster, K. A. (2004, Jul). Mitochondrial signals initiate the activation of c-Jun N-terminal kinase (JNK) by hypoxia-reoxygenation. *FASEB J, 18*(10), 1060-1070. https://doi.org/10.1096/fj.04-1505com

Dumont, N. A., Bentzinger, C. F., Sincennes, M. C., & Rudnicki, M. A. (2015, Jul 1). Satellite Cells and Skeletal Muscle Regeneration. *Compr Physiol, 5*(3), 1027-1059. https://doi.org/10.1002/cphy.c140068

Dumont, N. A., Wang, Y. X., & Rudnicki, M. A. (2015, May 1). Intrinsic and extrinsic mechanisms regulating satellite cell function. *Development, 142*(9), 1572-1581. https://doi.org/10.1242/dev.114223

Flamini, V., Ghadiali, R. S., Antczak, P., Rothwell, A., Turnbull, J. E., & Pisconti, A. (2018, Mar 13). The Satellite Cell Niche Regulates the Balance between Myoblast Differentiation and Self-Renewal via p53. *Stem Cell Reports, 10*(3), 970-983. https://doi.org/10.1016/j.stemcr.2018.01.007

Flores, A. N., McDermott, N., Meunier, A., & Marignol, L. (2014, Sep). NUMB inhibition of NOTCH signalling as a therapeutic target in prostate cancer. *Nat Rev Urol, 11*(9), 499-507. https://doi.org/10.1038/nrurol.2014.195

Francetic, T., & Li, Q. (2011, May). Skeletal myogenesis and Myf5 activation. *Transcription, 2*(3), 109-114. https://doi.org/10.4161/trns.2.3.15829

Friday, B. B., Mitchell, P. O., Kegley, K. M., & Pavlath, G. K. (2003, Apr). Calcineurin initiates skeletal muscle differentiation by activating MEF2 and MyoD. *Differentiation, 71*(3), 217-227. https://doi.org/10.1046/j.1432-0436.2003.710303.x

Fulco, M., Cen, Y., Zhao, P., Hoffman, E. P., McBurney, M. W., Sauve, A. A., & Sartorelli, V. (2008, May). Glucose restriction inhibits skeletal myoblast differentiation by activating SIRT1 through AMPK-mediated regulation of Nampt. *Dev Cell, 14*(5), 661-673. https://doi.org/10.1016/j.devcel.2008.02.004

Fulco, M., Schiltz, R. L., Iezzi, S., King, M. T., Zhao, P., Kashiwaya, Y., Hoffman, E., Veech, R. L., & Sartorelli, V. (2003). Sir2 Regulates Skeletal Muscle Differentiation as a Potential Sensor of the Redox State. *Molecular Cell, 12*(1), 51-62. https://doi.org/10.1016/s1097-2765(03)00226-0

Galdieri, L., & Vancura, A. (2012, Jul 6). Acetyl-CoA carboxylase regulates global histone acetylation. *J Biol Chem, 287*(28), 23865-23876. https://doi.org/10.1074/jbc.M112.380519

Gillespie, M. A., Le Grand, F., Scime, A., Kuang, S., von Maltzahn, J., Seale, V., Cuenda, A., Ranish, J. A., & Rudnicki, M. A. (2009, Dec 28). p38-{gamma}-dependent gene silencing restricts entry into the myogenic differentiation program. *J Cell Biol, 187*(7), 991-1005. https://doi.org/10.1083/jcb.200907037

Gresham, R. C. H., Bahney, C. S., & Leach, J. K. (2021, Jul). Growth factor delivery using extracellular matrix-mimicking substrates for musculoskeletal tissue engineering and repair. *Bioact Mater, 6*(7), 1945-1956. https://doi.org/10.1016/j.bioactmat.2020.12.012

Guttridge, D. C., Albanese, C., Reuther, J. Y., Pestell, R. G., & Baldwin, A. S., Jr. (1999, Aug). NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. *Mol Cell Biol, 19*(8), 5785-5799. https://doi.org/10.1128/mcb.19.8.5785

Halevy, O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D., & Lassar, A. B. (1995, Feb 17). Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science, 267*(5200), 1018-1021. https://doi.org/10.1126/science.7863327

Hamed, M., Khilji, S., Chen, J., & Li, Q. (2013). Stepwise acetyltransferase association and histone acetylation at the Myod1 locus during myogenic differentiation. *Sci Rep, 3*, 2390. https://doi.org/10.1038/srep02390

Hamed, M., Khilji, S., Dixon, K., Blais, A., Ioshikhes, I., Chen, J., & Li, Q. (2017, Nov 2). Insights into interplay between rexinoid signaling and myogenic regulatory factor-associated chromatin state in myogenic differentiation. *Nucleic Acids Res, 45*(19), 11236-11248. https://doi.org/10.1093/nar/gkx800

Hansen, J. C., Tse, C., & Wolffe, A. P. (1998, Dec 22). Structure and function of the core histone N-termini: more than meets the eye. *Biochemistry, 37*(51), 17637-17641. https://doi.org/10.1021/bi982409v

Harutyunyan, A. S., Krug, B., Chen, H., Papillon-Cavanagh, S., Zeinieh, M., De Jay, N., Deshmukh, S., Chen, C. C. L., Belle, J., Mikael, L. G., Marchione, D. M., Li, R., Nikbakht, H., Hu, B., Cagnone, G., Cheung, W. A., Mohammadnia, A., Bechet, D., Faury, D., McConechy, M. K., Pathania, M., Jain, S. U., Ellezam, B., Weil, A. G., Montpetit, A., Salomoni, P., Pastinen, T.,

Lu, C., Lewis, P. W., Garcia, B. A., Kleinman, C. L., Jabado, N., & Majewski, J. (2019, Mar 19). H3K27M induces defective chromatin spread of PRC2-mediated repressive H3K27me2/me3 and is essential for glioma tumorigenesis. *Nat Commun, 10*(1), 1262. https://doi.org/10.1038/s41467-019-09140-x

Hernandez-Hernandez, J. M., Garcia-Gonzalez, E. G., Brun, C. E., & Rudnicki, M. A. (2017, Dec). The myogenic regulatory factors, determinants of muscle development, cell identity and regeneration. *Semin Cell Dev Biol, 72*, 10-18. https://doi.org/10.1016/j.semcdb.2017.11.010

Hindi, L., McMillan, J. D., Afroze, D., Hindi, S. M., & Kumar, A. (2017, May 5). Isolation, Culturing, and Differentiation of Primary Myoblasts from Skeletal Muscle of Adult Mice. *Bio Protoc, 7*(9). https://doi.org/10.21769/BioProtoc.2248

Hollenberg, S. M., Cheng, P. F., & Weintraub, H. (1993, Sep 1). Use of a conditional MyoD transcription factor in studies of MyoD trans-activation and muscle determination. *Proc Natl Acad Sci U S A, 90*(17), 8028-8032. https://doi.org/10.1073/pnas.90.17.8028

Hsieh, E. A., Chai, C. M., & Hellerstein, M. K. (2005, May). Effects of caloric restriction on cell proliferation in several tissues in mice: role of intermittent feeding. *Am J Physiol Endocrinol Metab, 288*(5), E965-972. https://doi.org/10.1152/ajpendo.00368.2004

Hu, X., Park, S. H., Gil, E. S., Xia, X. X., Weiss, A. S., & Kaplan, D. L. (2011, Dec). The influence of elasticity and surface roughness on myogenic and osteogenic-differentiation of cells on silk-elastin biomaterials. *Biomaterials, 32*(34), 8979-8989. https://doi.org/10.1016/j.biomaterials.2011.08.037

Jacobson, R. H., Ladurner, A. G., King, D. S., & Tjian, R. (2000, May 26). Structure and function of a human TAFII250 double bromodomain module. *Science, 288*(5470), 1422-1425. https://doi.org/10.1126/science.288.5470.1422

Jang, Y. N., & Baik, E. J. (2013, Apr 1). JAK-STAT pathway and myogenic differentiation. *JAKSTAT, 2*(2), e23282. https://doi.org/10.4161/jkst.23282

Jaskelioff, M., & Peterson, C. L. (2003, May). Chromatin and transcription: histones continue to make their marks. *Nat Cell Biol, 5*(5), 395-399. https://doi.org/10.1038/ncb0503-395

Jin, H., Kasper, L. H., Larson, J. D., Wu, G., Baker, S. J., Zhang, J., & Fan, Y. (2020, Feb 15). ChIPseqSpikeInFree: a ChIP-seq normalization approach to reveal global changes in histone modifications without spike-in. *Bioinformatics, 36*(4), 1270-1272. https://doi.org/10.1093/bioinformatics/btz720

Jones, P. A., & Takai, D. (2001, Aug 10). The role of DNA methylation in mammalian epigenetics. *Science, 293*(5532), 1068-1070. https://doi.org/10.1126/science.1063852

Kaelin, W. G., Jr., & McKnight, S. L. (2013, Mar 28). Influence of metabolism on epigenetics and disease. *Cell, 153*(1), 56-69. https://doi.org/10.1016/j.cell.2013.03.004

Kaimori, J. Y., Maehara, K., Hayashi-Takanaka, Y., Harada, A., Fukuda, M., Yamamoto, S., Ichimaru, N., Umehara, T., Yokoyama, S., Matsuda, R., Ikura, T., Nagao, K., Obuse, C., Nozaki, N., Takahara, S., Takao, T., Ohkawa, Y., Kimura, H., & Isaka, Y. (2016, Apr 11). Histone H4 lysine 20 acetylation is associated with gene repression in human cells. *Sci Rep, 6*, 24318. https://doi.org/10.1038/srep24318

Kankotia, S., & Stacpoole, P. W. (2014, Dec). Dichloroacetate and cancer: new home for an orphan drug? *Biochim Biophys Acta, 1846*(2), 617-629. https://doi.org/10.1016/j.bbcan.2014.08.005

Kelly, R. D. W., Chandru, A., Watson, P. J., Song, Y., Blades, M., Robertson, N. S., Jamieson, A. G., Schwabe, J. W. R., & Cowley, S. M. (2018, Oct 2). Histone deacetylase (HDAC) 1 and 2 complexes regulate both histone acetylation and crotonylation in vivo. *Sci Rep, 8*(1), 14690. https://doi.org/10.1038/s41598-018-32927-9

Khacho, M., & Slack, R. S. (2017, Dec). Mitochondrial activity in the regulation of stem cell self-renewal and differentiation. *Curr Opin Cell Biol, 49*, 1-8. https://doi.org/10.1016/j.ceb.2017.11.003

Khilji, S., Hamed, M., Chen, J., & Li, Q. (2018). Loci-specific histone acetylation profiles associated with transcriptional coactivator p300 during early myoblast differentiation. *Epigenetics, 13*(6), 642-654. https://doi.org/10.1080/15592294.2018.1489659

Kraft, C. S., LeMoine, C. M., Lyons, C. N., Michaud, D., Mueller, C. R., & Moyes, C. D. (2006, Apr). Control of mitochondrial biogenesis during myogenesis. *Am J Physiol Cell Physiol, 290*(4), C1119-1127. https://doi.org/10.1152/ajpcell.00463.2005

Krajcova, A., Ziak, J., Jiroutkova, K., Patkova, J., Elkalaf, M., Dzupa, V., Trnka, J., & Duska, F. (2015, Feb). Normalizing glutamine concentration causes mitochondrial uncoupling in an in vitro model of human skeletal muscle. *JPEN J Parenter Enteral Nutr, 39*(2), 180-189. https://doi.org/10.1177/0148607113513801

Labow, B. I., & Souba, W. W. (2000, Dec). Glutamine. *World J Surg, 24*(12), 1503-1513. https://doi.org/10.1007/s002680010269

Laumonier, T., Bermont, F., Hoffmeyer, P., Kindler, V., & Menetrey, J. (2017, Jun 14). Human myogenic reserve cells are quiescent stem cells that contribute to muscle regeneration after intramuscular transplantation in immunodeficient mice. *Sci Rep, 7*(1), 3462. https://doi.org/10.1038/s41598-017-03703-y

Leary, S. C., Battersby, B. J., Hansford, R. G., & Moyes, C. D. (1998). Interactions between bioenergetics and mitochondrial biogenesis. *Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1365*(3), 522-530. https://doi.org/10.1016/s0005-2728(98)00105-4

Li, B., Carey, M., & Workman, J. L. (2007, Feb 23). The role of chromatin during transcription. *Cell, 128*(4), 707-719. https://doi.org/10.1016/j.cell.2007.01.015

Li, E. (2002, Sep). Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet, 3*(9), 662-673. https://doi.org/10.1038/nrg887

Li, X., & Kazgan, N. (2011). Mammalian sirtuins and energy metabolism. *Int J Biol Sci, 7*(5), 575-587. https://doi.org/10.7150/ijbs.7.575

Li, Y. P., & Schwartz, R. J. (2001, Jun). TNF-alpha regulates early differentiation of C2C12 myoblasts in an autocrine fashion. *FASEB J, 15*(8), 1413-1415. https://doi.org/10.1096/fj.00-0632fje

Liberti, M. V., & Locasale, J. W. (2016, Mar). The Warburg Effect: How Does it Benefit Cancer Cells? *Trends Biochem Sci, 41*(3), 211-218. https://doi.org/10.1016/j.tibs.2015.12.001

Lipmann, F., & Kaplan, B. (1946). A common factor in the enymatic acetylation of sulfanilamide and of choline. *J. Biol. Chem., 162*, 743-744.

Liu, L., Cheung, T. H., Charville, G. W., Hurgo, B. M., Leavitt, T., Shih, J., Brunet, A., & Rando, T. A. (2013, Jul 11). Chromatin modifications as determinants of muscle stem cell quiescence and chronological aging. *Cell Rep, 4*(1), 189-204. https://doi.org/10.1016/j.celrep.2013.05.043

Liu, Z., Huang, C., Liu, Y., Lin, D., & Zhao, Y. (2018). NMR-based metabolomic analysis of the effects of alanyl-glutamine supplementation on C2C12 myoblasts injured by energy deprivation. *RSC Advances, 8*(29), 16114-16125. https://doi.org/10.1039/c8ra00819a

Lyons, C. N., Leary, S. C., & Moyes, C. D. (2004, Jun). Bioenergetic remodeling during cellular differentiation: changes in cytochrome c oxidase regulation do not affect the metabolic phenotype. *Biochem Cell Biol, 82*(3), 391-399. https://doi.org/10.1139/o04-040

Maganti, H. B., Jrade, H., Cafariello, C., Manias Rothberg, J. L., Porter, C. J., Yockell-Lelievre, J., Battaion, H. L., Khan, S. T., Howard, J. P., Li, Y., Grzybowski, A. T., Sabri, E., Ruthenburg, A. J., Dilworth, F. J., Perkins, T. J., Sabloff, M., Ito, C. Y., & Stanford, W. L. (2018, Nov). Targeting the MTF2-MDM2 Axis Sensitizes Refractory Acute Myeloid Leukemia to Chemotherapy. *Cancer Discov, 8*(11), 1376-1389. https://doi.org/10.1158/2159-8290.CD-17-0841

Marsh, D. R., Criswell, D. S., Carson, J. A., & Booth, F. W. (1997, Oct). Myogenic regulatory factors during regeneration of skeletal muscle in young, adult, and old rats. *J Appl Physiol (1985), 83*(4), 1270-1275. https://doi.org/10.1152/jappl.1997.83.4.1270

Mathias, R. A., Greco, T. M., Oberstein, A., Budayeva, H. G., Chakrabarti, R., Rowland, E. A., Kang, Y., Shenk, T., & Cristea, I. M. (2014, Dec 18). Sirtuin 4 is a lipoamidase regulating pyruvate dehydrogenase complex activity. *Cell, 159*(7), 1615-1625. https://doi.org/10.1016/j.cell.2014.11.046

Menzies, K. J., Zhang, H., Katsyuba, E., & Auwerx, J. (2016, Jan). Protein acetylation in metabolism - metabolites and cofactors. *Nat Rev Endocrinol, 12*(1), 43-60. https://doi.org/10.1038/nrendo.2015.181

Moresi, V., Adamo, S., & Berghella, L. (2019). The JAK/STAT Pathway in Skeletal Muscle Pathophysiology. *Front Physiol, 10*, 500. https://doi.org/10.3389/fphys.2019.00500

Moris, N., Edri, S., Seyres, D., Kulkarni, R., Domingues, A. F., Balayo, T., Frontini, M., & Pina, C. (2018, Dec). Histone Acetyltransferase KAT2A Stabilizes Pluripotency with Control of Transcriptional Heterogeneity. *Stem Cells, 36*(12), 1828-1838. https://doi.org/10.1002/stem.2919

Motohashi, N., & Asakura, A. (2012, Nov). Molecular Regulation of Muscle Satellite Cell Self-Renewal. *J Stem Cell Res Ther, Suppl 11*. https://doi.org/10.4172/2157-7633.S11-e002

Mourikis, P., Gopalakrishnan, S., Sambasivan, R., & Tajbakhsh, S. (2012, Dec). Cell-autonomous Notch activity maintains the temporal specification potential of skeletal muscle stem cells. *Development, 139*(24), 4536-4548. https://doi.org/10.1242/dev.084756

Moussaieff, A., Rouleau, M., Kitsberg, D., Cohen, M., Levy, G., Barasch, D., Nemirovski, A., Shen-Orr, S., Laevsky, I., Amit, M., Bomze, D., Elena-Herrmann, B., Scherf, T., Nissim-Rafinia, M., Kempa, S., Itskovitz-Eldor, J., Meshorer, E., Aberdam, D., & Nahmias, Y. (2015, Mar 3). Glycolysis-mediated changes in acetyl-CoA and histone acetylation control the early differentiation of embryonic stem cells. *Cell Metab, 21*(3), 392-402. https://doi.org/10.1016/j.cmet.2015.02.002

Nederveen, J. P., Fortino, S. A., Baker, J. M., Snijders, T., Joanisse, S., McGlory, C., McKay, B. R., Kumbhare, D., & Parise, G. (2019, Nov 1). Consistent expression pattern of myogenic regulatory factors in whole muscle and isolated human muscle satellite cells after eccentric contractions in humans. *J Appl Physiol (1985), 127*(5), 1419-1426. https://doi.org/10.1152/japplphysiol.01123.2018

Nguyen, T.-L., & Durán, R. V. (2018). Glutamine metabolism in cancer therapy. *Cancer Drug Resistance*. https://doi.org/10.20517/cdr.2018.08

Palm, W., & Thompson, C. B. (2017, Jun 7). Nutrient acquisition strategies of mammalian cells. *Nature, 546*(7657), 234-242. https://doi.org/10.1038/nature22379

Pavlidou, T., Marinkovic, M., Rosina, M., Fuoco, C., Vumbaca, S., Gargioli, C., Castagnoli, L., & Cesareni, G. (2019). Metformin Delays Satellite Cell Activation and Maintains Quiescence. *Stem Cells Int, 2019*, 5980465. https://doi.org/10.1155/2019/5980465

Peleg, S., Feller, C., Ladurner, A. G., & Imhof, A. (2016, Aug). The Metabolic Impact on Histone Acetylation and Transcription in Ageing. *Trends Biochem Sci, 41*(8), 700-711. https://doi.org/10.1016/j.tibs.2016.05.008

Pietrocola, F., Galluzzi, L., Bravo-San Pedro, J. M., Madeo, F., & Kroemer, G. (2015, Jun 2). Acetyl coenzyme A: a central metabolite and second messenger. *Cell Metab, 21*(6), 805-821. https://doi.org/10.1016/j.cmet.2015.05.014

Pietsch, P. (1961). Differentiation in regeneration I. The development of muscle and cartilage following deplantation of regenerating limb blastemata of Amblystoma larvae. *Developmental Biology, 3*(3), 255-264. https://doi.org/10.1016/0012-1606(61)90046-x

Pilkis, S. J., Park, C. R., & Claus, T. H. (1979). Hormonal Control of Hepatic Gluconeogenesis. In (pp. 383-460). https://doi.org/10.1016/s0083-6729(08)60988-7

Pirola, L., Balcerczyk, A., Okabe, J., & El-Osta, A. (2010, Dec). Epigenetic phenomena linked to diabetic complications. *Nat Rev Endocrinol, 6*(12), 665-675. https://doi.org/10.1038/nrendo.2010.188

Pope, B. D., Ryba, T., Dileep, V., Yue, F., Wu, W., Denas, O., Vera, D. L., Wang, Y., Hansen, R. S., Canfield, T. K., Thurman, R. E., Cheng, Y., Gulsoy, G., Dennis, J. H., Snyder, M. P., Stamatoyannopoulos, J. A., Taylor, J., Hardison, R. C., Kahveci, T., Ren, B., & Gilbert, D. M. (2014, Nov 20). Topologically associating domains are stable units of replication-timing regulation. *Nature, 515*(7527), 402-405. https://doi.org/10.1038/nature13986

Proctor, D. N., O'Brien, P. C., Atkinson, E. J., & Nair, K. S. (1999, Sep). Comparison of techniques to estimate total body skeletal muscle mass in people of different age groups. *Am J Physiol, 277*(3), E489-495. https://doi.org/10.1152/ajpendo.1999.277.3.E489

Purohit, G., & Dhawan, J. (2019). Adult Muscle Stem Cells: Exploring the Links Between Systemic and Cellular Metabolism. *Front Cell Dev Biol, 7*, 312. https://doi.org/10.3389/fcell.2019.00312

Reid, M. A., Dai, Z., & Locasale, J. W. (2017, Nov). The impact of cellular metabolism on chromatin dynamics and epigenetics. *Nat Cell Biol, 19*(11), 1298-1306. https://doi.org/10.1038/ncb3629

Ren, R., Ocampo, A., Liu, G. H., & Izpisua Belmonte, J. C. (2017, Sep 5). Regulation of Stem Cell Aging by Metabolism and Epigenetics. *Cell Metab, 26*(3), 460-474. https://doi.org/10.1016/j.cmet.2017.07.019

Rodgers, J. T., King, K. Y., Brett, J. O., Cromie, M. J., Charville, G. W., Maguire, K. K., Brunson, C., Mastey, N., Liu, L., Tsai, C. R., Goodell, M. A., & Rando, T. A. (2014, Jun 19). mTORC1 controls the adaptive transition of quiescent stem cells from G0 to G(Alert). *Nature, 510*(7505), 393-396. https://doi.org/10.1038/nature13255

Rotili, D., & Mai, A. (2011, Jun). Targeting Histone Demethylases: A New Avenue for the Fight against Cancer. *Genes Cancer, 2*(6), 663-679. https://doi.org/10.1177/1947601911417976

Roy, S., Sala, R., Cagliero, E., & Lorenzi, M. (1990, Jan). Overexpression of fibronectin induced by diabetes or high glucose: phenomenon with a memory. *Proc Natl Acad Sci U S A, 87*(1), 404-408. https://doi.org/10.1073/pnas.87.1.404

Rubin, H. (2019, Apr 2). Deprivation of glutamine in cell culture reveals its potential for treating cancer. *Proc Natl Acad Sci U S A, 116*(14), 6964-6968. https://doi.org/10.1073/pnas.1815968116

Ryall, J. G. (2013, Sep). Metabolic reprogramming as a novel regulator of skeletal muscle development and regeneration. *FEBS J, 280*(17), 4004-4013. https://doi.org/10.1111/febs.12189

Ryall, J. G., Dell'Orso, S., Derfoul, A., Juan, A., Zare, H., Feng, X., Clermont, D., Koulnis, M., Gutierrez-Cruz, G., Fulco, M., & Sartorelli, V. (2015, Feb 5). The NAD(+)-dependent SIRT1 deacetylase translates a metabolic switch into regulatory epigenetics in skeletal muscle stem cells. *Cell Stem Cell, 16*(2), 171-183. https://doi.org/10.1016/j.stem.2014.12.004

Ryu, D., Zhang, H., Ropelle, E. R., Sorrentino, V., Mazala, D. A., Mouchiroud, L., Marshall, P. L., Campbell, M. D., Ali, A. S., Knowels, G. M., Bellemin, S., Iyer, S. R., Wang, X., Gariani, K., Sauve, A. A., Canto, C., Conley, K. E., Walter, L., Lovering, R. M., Chin, E. R., Jasmin, B. J., Marcinek, D. J., Menzies, K. J., & Auwerx, J. (2016, Oct 19). NAD+ repletion improves muscle function in muscular dystrophy and counters global PARylation. *Sci Transl Med, 8*(361), 361ra139. https://doi.org/10.1126/scitranslmed.aaf5504

Sajjanar, B., Siengdee, P., Trakooljul, N., Liu, X., Kalbe, C., Wimmers, K., & Ponsuksili, S. (2019). Cross-talk between energy metabolism and epigenetics during temperature stress response in C2C12 myoblasts. *Int J Hyperthermia, 36*(1), 776-784. https://doi.org/10.1080/02656736.2019.1639834

Saraiva, N. Z., Oliveira, C. S., & Garcia, J. M. (2010, Dec 26). Histone acetylation and its role in embryonic stem cell differentiation. *World J Stem Cells, 2*(6), 121-126. https://doi.org/10.4252/wjsc.v2.i6.121

Sauve, A. A., & Youn, D. Y. (2012, Dec). Sirtuins: NAD(+)-dependent deacetylase mechanism and regulation. *Curr Opin Chem Biol, 16*(5-6), 535-543. https://doi.org/10.1016/j.cbpa.2012.10.003

Schneider, R., & Grosschedl, R. (2007, Dec 1). Dynamics and interplay of nuclear architecture, genome organization, and gene expression. *Genes Dev, 21*(23), 3027-3043. https://doi.org/10.1101/gad.1604607

Segales, J., Perdiguero, E., & Munoz-Canoves, P. (2015, May). Epigenetic control of adult skeletal muscle stem cell functions. *FEBS J, 282*(9), 1571-1588. https://doi.org/10.1111/febs.13065

Serefidou, M., Venkatasubramani, A. V., & Imhof, A. (2019). The Impact of One Carbon Metabolism on Histone Methylation. *Front Genet, 10*, 764. https://doi.org/10.3389/fgene.2019.00764

Sethi, J. K., & Vidal-Puig, A. (2010, Mar 15). Wnt signalling and the control of cellular metabolism. *Biochem J, 427*(1), 1-17. https://doi.org/10.1042/BJ20091866

Shainberg, A., Yagil, G., & Yaffe, D. (1969). Control of myogenesis in vitro by Ca2+ concentration in nutrittonal medium. *Experimental Cell Research, 58*(1), 163-167. https://doi.org/10.1016/0014-4827(69)90127-x

Shechter, D., Dormann, H. L., Allis, C. D., & Hake, S. B. (2007). Extraction, purification and analysis of histones. *Nat Protoc, 2*(6), 1445-1457. https://doi.org/10.1038/nprot.2007.202

Sidoli, S., Bhanu, N. V., Karch, K. R., Wang, X., & Garcia, B. A. (2016, May 17). Complete Workflow for Analysis of Histone Post-translational Modifications Using Bottom-up Mass Spectrometry: From Histone Extraction to Data Analysis. *J Vis Exp*(111). https://doi.org/10.3791/54112

Siegel, A. L., Kuhlmann, P. K., & Cornelison, D. D. (2011, Feb 2). Muscle satellite cell proliferation and association: new insights from myofiber time-lapse imaging. *Skelet Muscle, 1*(1), 7. https://doi.org/10.1186/2044-5040-1-7

Song, C., Xu, F., Ren, Z., Zhang, Y., Meng, Y., Yang, Y., Lingadahalli, S., Cheung, E., Li, G., Liu, W., Wan, J., Zhao, Y., & Chen, G. (2019, Aug 13). Elevated Exogenous Pyruvate Potentiates Mesodermal Differentiation through Metabolic Modulation and AMPK/mTOR Pathway

in Human Embryonic Stem Cells. *Stem Cell Reports, 13*(2), 338-351. https://doi.org/10.1016/j.stemcr.2019.06.003

Sun, L., Ma, K., Wang, H., Xiao, F., Gao, Y., Zhang, W., Wang, K., Gao, X., Ip, N., & Wu, Z. (2007, Oct 8). JAK1-STAT1-STAT3, a key pathway promoting proliferation and preventing premature differentiation of myoblasts. *J Cell Biol, 179*(1), 129-138. https://doi.org/10.1083/jcb.200703184

Sutendra, G., Kinnaird, A., Dromparis, P., Paulin, R., Stenson, T. H., Haromy, A., Hashimoto, K., Zhang, N., Flaim, E., & Michelakis, E. D. (2014, Jul 3). A nuclear pyruvate dehydrogenase complex is important for the generation of acetyl-CoA and histone acetylation. *Cell, 158*(1), 84-97. https://doi.org/10.1016/j.cell.2014.04.046

Takahashi, H., McCaffery, J. M., Irizarry, R. A., & Boeke, J. D. (2006, Jul 21). Nucleocytosolic acetyl-coenzyme a synthetase is required for histone acetylation and global transcription. *Mol Cell, 23*(2), 207-217. https://doi.org/10.1016/j.molcel.2006.05.040

Tatsumi, R., Anderson, J. E., Nevoret, C. J., Halevy, O., & Allen, R. E. (1998, Feb 1). HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. *Dev Biol, 194*(1), 114-128. https://doi.org/10.1006/dbio.1997.8803

Tumanov, S., Bulusu, V., & Kamphorst, J. J. (2015). Analysis of Fatty Acid Metabolism Using Stable Isotope Tracers and Mass Spectrometry. *Methods Enzymol, 561*, 197-217. https://doi.org/10.1016/bs.mie.2015.05.017

Unnikrishnan, A., Gafken, P. R., & Tsukiyama, T. (2010, Apr). Dynamic changes in histone acetylation regulate origins of DNA replication. *Nat Struct Mol Biol, 17*(4), 430-437. https://doi.org/10.1038/nsmb.1780

Vander Heiden, M. G., Cantley, L. C., & Thompson, C. B. (2009, May 22). Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science, 324*(5930), 1029-1033. https://doi.org/10.1126/science.1160809

Vogelauer, M., Wu, J., Suka, N., & Grunstein, M. (2000, Nov 23). Global histone acetylation and deacetylation in yeast. *Nature, 408*(6811), 495-498. https://doi.org/10.1038/35044127

von Haehling, S., & Anker, S. D. (2014, Dec). Prevalence, incidence and clinical impact of cachexia: facts and numbers-update 2014. *J Cachexia Sarcopenia Muscle, 5*(4), 261-263. https://doi.org/10.1007/s13539-014-0164-8

von Maltzahn, J., Chang, N. C., Bentzinger, C. F., & Rudnicki, M. A. (2012, Nov). Wnt signaling in myogenesis. *Trends Cell Biol, 22*(11), 602-609. https://doi.org/10.1016/j.tcb.2012.07.008

Wang, K., Wang, C., Xiao, F., Wang, H., & Wu, Z. (2008, Dec 5). JAK2/STAT2/STAT3 are required for myogenic differentiation. *J Biol Chem, 283*(49), 34029-34036. https://doi.org/10.1074/jbc.M803012200

Wang, Z., Long, H., Chang, C., Zhao, M., & Lu, Q. (2018, Sep). Crosstalk between metabolism and epigenetic modifications in autoimmune diseases: a comprehensive overview. *Cell Mol Life Sci, 75*(18), 3353-3369. https://doi.org/10.1007/s00018-018-2864-2

Warburg, O. (1925). The Metabolism of Carcinoma Cells. *The Journal of Cancer Research, 9*(1), 148-163. https://doi.org/10.1158/jcr.1925.148

Wellen, K. E., Hatzivassiliou, G., Sachdeva, U. M., Bui, T. V., Cross, J. R., & Thompson, C. B. (2009). ATP-Citrate Lyase Links Cellular Metabolism to Histone Acetylation. *Science, 324*(5930), 1076-1080. https://doi.org/10.1126/science.1164097

Wellen, K. E., & Thompson, C. B. (2012, Mar 7). A two-way street: reciprocal regulation of metabolism and signalling. *Nat Rev Mol Cell Biol, 13*(4), 270-276. https://doi.org/10.1038/nrm3305

Wosczyna, M. N., Perez Carbajal, E. E., Wagner, M. W., Paredes, S., Konishi, C. T., Liu, L., Wang, T. T., Walsh, R. A., Gan, Q., Morrissey, C. S., & Rando, T. A. (2021, Jul 1). Targeting microRNA-mediated gene repression limits adipogenic conversion of skeletal muscle mesenchymal stromal cells. *Cell Stem Cell, 28*(7), 1323-1334 e1328. https://doi.org/10.1016/j.stem.2021.04.008

Xie, S. J., Li, J. H., Chen, H. F., Tan, Y. Y., Liu, S. R., Zhang, Y., Xu, H., Yang, J. H., Liu, S., Zheng, L. L., Huang, M. B., Guo, Y. H., Zhang, Q., Zhou, H., & Qu, L. H. (2018, Sep). Inhibition of the JNK/MAPK signaling pathway by myogenesis-associated miRNAs is required for skeletal muscle development. *Cell Death Differ, 25*(9), 1581-1597. https://doi.org/10.1038/s41418-018-0063-1

Yang, C., Ko, B., Hensley, C. T., Jiang, L., Wasti, A. T., Kim, J., Sudderth, J., Calvaruso, M. A., Lumata, L., Mitsche, M., Rutter, J., Merritt, M. E., & DeBerardinis, R. J. (2014, Nov 6). Glutamine oxidation maintains the TCA cycle and cell survival during impaired mitochondrial pyruvate transport. *Mol Cell, 56*(3), 414-424. https://doi.org/10.1016/j.molcel.2014.09.025

Yang, C., Sudderth, J., Dang, T., Bachoo, R. M., McDonald, J. G., & DeBerardinis, R. J. (2009, Oct 15). Glioblastoma cells require glutamate dehydrogenase to survive impairments of glucose metabolism or Akt signaling. *Cancer Res, 69*(20), 7986-7993. https://doi.org/10.1158/0008-5472.CAN-09-2266

Yucel, N., Wang, Y. X., Mai, T., Porpiglia, E., Lund, P. J., Markov, G., Garcia, B. A., Bendall, S. C., Angelo, M., & Blau, H. M. (2019, Jun 25). Glucose Metabolism Drives Histone Acetylation

Landscape Transitions that Dictate Muscle Stem Cell Function. *Cell Rep, 27*(13), 3939-3955 e3936. https://doi.org/10.1016/j.celrep.2019.05.092

Yue, F., Cheng, Y., Breschi, A., Vierstra, J., Wu, W., Ryba, T., Sandstrom, R., Ma, Z., Davis, C., Pope, B. D., Shen, Y., Pervouchine, D. D., Djebali, S., Thurman, R. E., Kaul, R., Rynes, E., Kirilusha, A., Marinov, G. K., Williams, B. A., Trout, D., Amrhein, H., Fisher-Aylor, K., Antoshechkin, I., DeSalvo, G., See, L. H., Fastuca, M., Drenkow, J., Zaleski, C., Dobin, A., Prieto, P., Lagarde, J., Bussotti, G., Tanzer, A., Denas, O., Li, K., Bender, M. A., Zhang, M., Byron, R., Groudine, M. T., McCleary, D., Pham, L., Ye, Z., Kuan, S., Edsall, L., Wu, Y. C., Rasmussen, M. D., Bansal, M. S., Kellis, M., Keller, C. A., Morrissey, C. S., Mishra, T., Jain, D., Dogan, N., Harris, R. S., Cayting, P., Kawli, T., Boyle, A. P., Euskirchen, G., Kundaje, A., Lin, S., Lin, Y., Jansen, C., Malladi, V. S., Cline, M. S., Erickson, D. T., Kirkup, V. M., Learned, K., Sloan, C. A., Rosenbloom, K. R., Lacerda de Sousa, B., Beal, K., Pignatelli, M., Flicek, P., Lian, J., Kahveci, T., Lee, D., Kent, W. J., Ramalho Santos, M., Herrero, J., Notredame, C., Johnson, A., Vong, S., Lee, K., Bates, D., Neri, F., Diegel, M., Canfield, T., Sabo, P. J., Wilken, M. S., Reh, T. A., Giste, E., Shafer, A., Kutyavin, T., Haugen, E., Dunn, D., Reynolds, A. P., Neph, S., Humbert, R., Hansen, R. S., De Bruijn, M., Selleri, L., Rudensky, A., Josefowicz, S., Samstein, R., Eichler, E. E., Orkin, S. H., Levasseur, D., Papayannopoulou, T., Chang, K. H., Skoultchi, A., Gosh, S., Disteche, C., Treuting, P., Wang, Y., Weiss, M. J., Blobel, G. A., Cao, X., Zhong, S., Wang, T., Good, P. J., Lowdon, R. F., Adams, L. B., Zhou, X. Q., Pazin, M. J., Feingold, E. A., Wold, B., Taylor, J., Mortazavi, A., Weissman, S. M., Stamatoyannopoulos, J. A., Snyder, M. P., Guigo, R., Gingeras, T. R., Gilbert, D. M., Hardison, R. C., Beer, M. A., Ren, B., & Mouse, E. C. (2014, Nov 20). A comparative encyclopedia of DNA elements in the mouse genome. *Nature, 515*(7527), 355-364. https://doi.org/10.1038/nature13992

Yuneva, M., Zamboni, N., Oefner, P., Sachidanandam, R., & Lazebnik, Y. (2007, Jul 2). Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells. *J Cell Biol, 178*(1), 93-105. https://doi.org/10.1083/jcb.200703099

Zammit, P. S., Golding, J. P., Nagata, Y., Hudon, V., Partridge, T. A., & Beauchamp, J. R. (2004, Aug 2). Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? *J Cell Biol, 166*(3), 347-357. https://doi.org/10.1083/jcb.200312007

Zhang, H., Ryu, D., Wu, Y., Gariani, K., Wang, X., Luan, P., D'Amico, D., Ropelle, E. R., Lutolf, M. P., Aebersold, R., Schoonjans, K., Menzies, K. J., & Auwerx, J. (2016, Jun 17). NAD(+) repletion improves mitochondrial and stem cell function and enhances life span in mice. *Science, 352*(6292), 1436-1443. https://doi.org/10.1126/science.aaf2693

Zhang, J., Nuebel, E., Daley, G. Q., Koehler, C. M., & Teitell, M. A. (2012, Nov 2). Metabolic regulation in pluripotent stem cells during reprogramming and self-renewal. *Cell Stem Cell, 11*(5), 589-595. https://doi.org/10.1016/j.stem.2012.10.005